Analysis of Stem and Progenitor Cell Responses to Variations in Bioprocess Culture Variables

by

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Abstract

The realization of many potential cell therapies will depend on the development of culture protocols that consistently expand stem and progenitor cell numbers while retaining their developmental properties. Hematopoietic cell lines were used to develop predictive mathematical models of cytokine-dependent proliferation. First, Hill-type empirical models provided a quantitative understanding of how cytokines (IL-3 and GM-CSF) individually influenced the growth rate. Then a competitive model of cytokine interactions was developed that effectively predicted the growth rates for TF-1 and MO7e cells exposed to combinations of IL-3 and GM-CSF, over wide concentration ranges.

Mouse embryonic stem cells (mESC) were used to study the influence of various culture variables on their proliferation and embryoid body (EB) formation potential. The medium pH, osmolality, basal medium composition, as well as serum and serum replacement (SR) concentrations were found to modulate mESC responses. For most of these variables, conventional cultivation methods did not expose the cells to levels that significantly decreased culture output. However, for feeder-containing cultures, the osmolality increased to over 400 mOsm/kg and, when ESC were cultured in such a medium, the EB yield was decreased by more than 50% compared to media at 300 and 335 mOsm/kg. Likewise, pH dose response experiments revealed that, within 48 h, compared to a medium initially at pH 7.3, the EB yield decreased by approximately 50% when R1 cells were cultured at either pH 7.15 or 7.45, with even lower yields at more extreme pH conditions. The mESC responses were also found to be serum concentration-dependent with maximal results at 5% serum in DMEM compared to the commonly used 15%. In DMEM:F12, a reduced dependency on serum was observed. When SR was used, both the proliferation and EB formation potential were maximal with 5% SR in DMEM. In DMEM:F12, 1.7% SR was sufficient to maintain the EB yield of mESC lines with a similar growth rate. Taken together, these results demonstrate that multiple critical culture variables can profoundly influence the responses of mESC and that these variables need to be kept within defined ranges to maintain the mESC in culture without compromising their developmental potential.

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List of Abbreviations and Symbols

bFGF	Basic fibroblast growth factor (also known as FGF-2)
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CFC	Colony forming assay
CRU	Competitive repopulation unit
DMEM	Dulbecco's modified Eagle's medium
EB	Embryoid Bodies
EC	Embryonal carcinoma
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
Epo	Erythropoietin (a cytokine)
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FCS	Fetal calf serum
Flt-3	Flt-3 ligand (a cytokine)
G-CSF	Granulocyte-colony stimulating factor (a cytokine)
GM-CSF	Granulocyte Macrophage-colony stimulating factor (a cytokine)
GM-CSFR	Granulocyte Macrophage-colony stimulating factor receptor
GPC	Glycerophosphorylcholine
hESC	Human embryonic stem cells
HIL-6	Hyper-Interleukin-6 (a cytokine)
HSC	Hematopoietic stem cells
ICM	Inner cell mass
IL-11	Interleukin-11 (a cytokine)
IL-2	Interleukin-2 (a cytokine)

IL-3	Interleukin-3 (a cytokine)
IL-5	Interleukin-5 (a cytokine)
IL-6	Interleukin-6 (a cytokine)
IMDM	Iscove's modified Dulbecco medium
JAK	Janus Kinase
LIF	Leukemia inhibitory factor (a cytokine)
LIFR	Leukemia inhibitory factor receptor
LTC-IC	Long term culture-initiating cell
M-CSF	Macrophage-colony stimulating factor (a cytokine)
MDCK	Madin-Darby canine kidney
MEF	Mouse embryonic fibroblasts
mESC	Mouse embryonic stem cells
MTG	Monothioglycerol
PB	Peripheral blood
pH_e	Extracellular pH
pH_i	Intracellular pH
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
SF	Stem Cell or Steel Factor (a cytokine)
SSEA	Stage specific embryonic antigen
STAT	Signal transducer and activation of transcription
TRA	Tumor recognition antigen
VEGF	Vascular endothelial growth factor
Wnt	Wingless

Symbols

$\overline{\mu}$	Average specific growth rate (h^{-1})
μ_{sat}	Average saturation specific growth rate (h^{-1})
$\mu_{sat\,IL-3}$	IL-3 average saturation specific growth rate (h^{-1})
$\mu_{sat\,GM}$	GM-CSF average saturation specific growth rate (h^{-1})

α_1	Ratio of IL-3 half-saturation constant to GM-CSF half-saturation constant
α_2	Ratio of GM-CSF half-saturation constant to IL-3 half-saturation constant
HCO_3^-	Sodium bicarbonate concentration (mM)
Gln	Glutamine concentration (pM)
Glu	Glucose concentration (pM)
K_{GM}	GM-CSF half saturation constant (ng/mL)
K_{IL3}	IL-3 half saturation constant (ng/mL)
K_L	Cytokine half saturation constant (ng/mL)
L	Cytokine concentration (ng/mL or pg/mL)
Lac	Lactate concentration (pM)
n	Hill's exponent (-)
pCO_2	Partial pressure of carbon dioxide (mm Hg)
q_L	Cell specific cytokine uptake rate (pg/h-10^6 cells)
$sGlnUR$	Cell specific glutamine uptake rate (pmol/h-10^6 cells)
$sGUR$	Cell specific glucose uptake rate (pmol/h-10^6 cells)
$sLPR$	Cell specific lactate production rate (pmol/h-10^6 cells)
t	Time (h)
X_o	Initial cell concentration (cells/mL)
X_v	Viable cell concentration (cells/mL)

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Dedication

Have they never considered the functioning of the heavens and the earth, and have they never observed closely anything that Allah (God) has created? And has it never occurred to them that their life term might already have drawn near to its end? Then after this warning of the Messenger what else can there be in which they will believe? Whomever Allah deprives of guidance, there is no guidance for him, for Allah leaves such people wandering about blindly in their contumacy.

The Quran (Chapter 7 (Al-Araf), verses 185-186)

To

My Teachers, Parents, Mona, Hassan, Safwan, Suha and Rayyan

1 Introduction and Literature Review

Stem cell biology has come of age. Unequivocal proof that stem cells exist in the hematopoietic system has given way to the prospective isolation of many tissue-specific stem and progenitor cells, the initial delineation of their properties and expressed genetic programs, and much work developing their utility in regenerative medicine. By definition, stem cells have the ability to both perpetuate themselves through self-renewal divisions and generate mature cells through differentiation. Although it seems reasonable to propose that each tissue arises from a tissue-specific stem cell, the rigorous identification and isolation of the somatic stem cells has been accomplished only in a few instances. For example, hematopoietic stem cells (HSC) have been isolated from mice and humans, and have been shown to be responsible for generation and regeneration of the blood forming and immune systems. HSC have already been used extensively in therapeutic settings[1]. The recent isolation and growth of human embryonic stem cells (hESC) in culture has caused much excitement and has raised hopes that in future it may be possible to generate, *in vitro*, new tissues and organs to replace those damaged by age and disease. The ability of stem cells to undergo stable genetic transformations and produce altered gene products for the lifetime of the host also makes them especially useful targets for gene therapy. Considerable progress has already been made in bioengineering relatively simple tissues such as skin, cartilage, and bone. To grow complex organs composed of many different cell types will likely require manipulating stem cells, which have a greater developmental potential.

A prerequisite for the clinical use of stem cells is a thorough understanding of *in vitro* conditions that help determine stem cell *self-renewal* (i.e., the ability of stem cells to renew themselves by dividing into the same non-specialized cell type), *differentiation* (i.e., the process whereby the stem cells acquire the features of specialized cells such as liver, heart or muscle cell), *apoptosis* (i.e., programmed cell death) or lineage commitment decisions that must be identified, defined and optimized in order to realize the potentials of regenerative medicine. Strategies and techniques that favor the selection, isolation and enrichment of specific cell populations must also be developed.

In the absence of such detailed understanding, the clinical potential of stem cells will likely remain unexploited.

A body of evidence suggests the possibility to expand *in vitro* the desired functional cells for implantation into humans by manipulating specific environmental signals that control the process of tissue formation. Soluble or membrane-bound cytokines control cell proliferation, differentiation and survival by interacting with cells via specific trans-membrane receptors and trigger signal cascades within the cells that alter gene expression and determine or modify cell function. Also, the 3-dimensional extracellular matrix conveys information that can control cell growth and development.

The orchestration of stem cell self-renewal, proliferation, differentiation and apoptosis *in vitro* will require not only the identification of key molecular interactions but also the spatial and temporal aspects of these interactions. This represents one challenge faced by the emerging field of stem cell bioengineering. Another equally important challenge is to be able to expand stem cells to large numbers in culture while maintaining their developmental potential. For example, as many as 3×10^{11} tumor infiltrating lymphocytes were needed to mediate beneficial effects in clinical trials[2], while 2×10^{11} myeloid cells would be necessary for a treatment to prevent neutropenia following chemotherapy[3]. It is therefore reasonable to assume that for clinical realization of stem cell based therapies, it will be necessary to grow and maintain relatively large numbers of undifferentiated stem cells. Human embryonic stem cells (hESC) have a doubling time of ~ 40 h[4] and extensive cell expansion (in large-scale bioreactor cultures) would be needed to produce clinically relevant numbers of cells. Expanding the hESC numbers with maintenance of developmental potential in a large-scale bioreactor would be challenging as the pH, osmolality and composition of the culture medium (serum containing vs. serum-free, for example), shear stress, dissolved oxygen tension, nutrient consumption and inhibitory metabolite accumulation, mode of reactor operation (for example batch or fed-batch vs. perfusion), etc., all become important variables to be optimized for a given system to realize the success of the culture. This requires that stem cell responses to variations in these culture variables be first thoroughly understood in

small scale cultures with the hope that results from these studies would then guide large scale culture development.

1.1 Hematopoiesis

Hematopoiesis is a complex, tightly-regulated process by which a variety of specialized blood cell types are generated from a relatively small population of pluripotent hematopoietic stem cells (HSC). Mature blood cells have diverse functions ranging from oxygen transport and blood clotting to infection fighting. Because the terminal blood cells have limited life spans and are unable to replicate, hematopoiesis is necessary to sustain adult life, even in the absence of disease. The production of mature blood cells from their precursors involves a series of differentiation events that take place over a large number of cell generations. This results in a recognizable hierarchy of hematopoietic progenitor cells of decreasing proliferative and differentiative potentials (Figure 1.1).

The HSC are defined functionally by their ability to generate and sustain multi-lineage hematopoiesis after transplantation into a hematologically-compromised host. To maintain the stem cell population and generate more mature cells at the same time, a stem cell has to preserve a balance between self-renewal on the one hand, and lineage commitment followed by terminal differentiation into erythroid, myeloid or lymphoid cells on the other. During the latter process, the progenitors maintain the capacity to undergo cell division, but their differentiation potential becomes more and more restricted. An intricate network of cytokines and cell-cell interactions constantly ensures that the appropriate quantities of the different cell types are produced. Failure of these control mechanisms can result in various malignancies. Although phenotypic and functional properties have been extensively characterized (reviewed in references[5-7]), the fundamental question of how HSC self-renewal is regulated remains unanswered. In most cases, combinations of growth factors that can induce potent proliferation cannot prevent the differentiation of HSC in long-term cultures. Although some progress has been made in identifying culture conditions that maintain HSC activity in culture (for

example, see references[8-10]), it has proved exceedingly difficult to identify combinations of defined growth factors that cause a significant expansion in culture in the number of progenitors with transplantable HSC activity.

1.1.1 Hematopoietic Cytokines

Cytokines are secreted or membrane-bound protein molecules that promote intercellular communication and mediate a number of biological functions such as cell growth and differentiation as well as immunity and inflammatory responses (Table 1.1).

The most studied cytokines can be categorized into three general families based on their receptors: the tyrosine kinase receptor family, hematopoietic growth factor receptor family and the gp130 receptor family. Flt-3 Ligand (Flt-3) and Steel Factor (SF) are examples of cytokines binding to receptors with tyrosine kinase activity. The receptors for cytokines belonging to the hematopoietic growth factor family do not possess an intrinsic tyrosine kinase domain but bind to cytoplasmic kinases. Interleukin-3 (IL-3), granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are examples of this family. The gp130 family includes interleukin-6 (IL-6), interleukin-11 (IL-11) and leukemia inhibitory factor (LIF) among others. Receptors for these cytokines share the common receptor chain, gp130.

One characteristic that many cytokines share is an ability to maintain the viability of their respective target cells by suppressing apoptosis *in vitro*[11]. A second function shared by cytokines is an ability to simulate the proliferation of the appropriate progenitor cell populations, i.e., to act as mitogens. The effect of the cytokines on the survival of hematopoietic cells can be uncoupled from proliferation. For example, exposure to low concentrations of macrophage-colony stimulating factor (M-CSF) promotes survival (but not proliferation) of macrophages while higher concentrations promote both survival and proliferation[12].

Another characteristic of cytokines is their ability to provide the conditions whereby multipotent and lineage-restricted progenitors undergo differentiation and development to produce mature cells[13]. Some cytokines play a crucial role in the production of specific lineages such that, for example, antibodies against erythropoietin (Epo) cause anemia when injected into animals. Other factors such as IL-3 and GM-CSF exert actions on a variety of lineages. Table (1.2) lists some of the important cytokines and their actions. A number of these cytokines are known to be produced by fibroblasts and endothelial cells as well as macrophages and lymphocytes, all of which help form the unique and heterogeneous microenvironment of the bone marrow.

It is clear from Table (1.2) that more than one cytokine can act on a particular lineage. There are several reasons for this redundancy:

1) Sequential Action: Cytokines may act at specific phases in the differentiation sequence of specific lineages. For example, Epo acts on more mature erythroid precursors whereas IL-3 and SF act on early progenitors.
2) Recruitment: Adult stem cells appear to require a combination of two or more growth factors for proliferation, e.g., SF + Flt-3 + IL-11. SF alone maintains the survival of stem cells but induces little proliferation unless an additional cytokine is present.
3) Synergy: Enhancement of proliferation by combinations of growth factors such as SF + G-CSF or GM-CSF results in a significant increase in the number of cells per colony compared to either growth factor alone.

The overall picture of cytokine regulation of hematopoiesis that has emerged from extensive research in this field is that most of the primitive hematopoietic cells in the adult are in a dormant non-cycling state. Their recruitment into a cycling state is influenced by early acting cytokines (e.g., IL-6, G-CSF, and SF). The proliferation of these cycling multipotent cells is controlled by intermediate growth factors (e.g., IL-3, GM-CSF). The late-acting lineage specific cytokines (e.g., Epo, IL-5, M-CSF) control the survival and maturation of committed lineage specific progenitors[14].

Receptors for some of the cytokines, such as those of IL-3, IL-6, LIF and GM-CSF, are comprised of two membrane-spanning subunits; a specific α chain that binds the ligand with low affinity and a common β (or gp130) chain that is shared by these cytokines and is required for high-affinity binding and signal transduction. Ligand binding results in dimerization of the two subunits, followed by rapid and reversible tyrosine phosphorylation of a number of cellular proteins including the β subunit of the receptor. Janus kinases (JAKs) that are constitutively associated with the α chain mediate this phosphorylation. Upon activation, JAKs phosphorylate several substrates including the signal transducer and activator of transcription (STAT) proteins and tyrosine residues of the β_c chain that serve as docking sites for various signaling proteins. Further details about the receptor structure and activation mechanisms can be found in reviews[15-19].

1.2 Origin and Derivation of Embryonic Stem Cells

Several discoveries in the areas of cell and developmental biology during the past 30 years proved to be pivotal events that have profoundly influenced our view of life: i) the establishment of embryonic stem cells (ESC) derived from mouse[20, 21] and human embryos[22], ii) the creation of genetic mouse models of disease through homologous recombination in ESC, iii) the reprogramming of somatic cells after nuclear transfer into enucleated eggs[23], and iv) the demonstration of germ-line development of ESC *in vitro*[24-26]. Because of these breakthroughs, cell therapies based on an unlimited, renewable source of cells have become an attractive concept in regenerative medicine.

Many of these advances are based on developmental studies of mouse embryogenesis. The fertilized egg has the ability to generate an entire organism. This capacity, defined as *totipotency*, is retained by the early progeny of the zygote up to the eight-cell stage of the morula (Figure 1.2).

Subsequently, cell differentiation results in the formation of a blastocyst, composed of outer trophoblast cells and the inner cell mass (ICM). Cells of the ICM are *pluripotent*, i.e., they retain the ability to develop into all the cell types that comprise the embryo

proper. *In vivo*, the cells of the ICM give rise to *multipotent* progenitors of the three germ layers, i.e., the ectoderm, mesoderm and endoderm. Embryonic stem cell lines are derived from the blastocyst state embryo. These cells can be maintained undifferentiated *in vitro* for prolonged periods of time and, upon exposure to appropriate culture conditions, can be induced to differentiate to produce the cells of the three germ layers.

Embryonic stem cell research dates back to the early 1970s, when a striking observation was reported: early mouse embryos grafted into adult mice produced *teratocarcinomas* - malignant multi-differentiated tumors containing a significant population of undifferentiated cells[27,28]. Previously, studies of spontaneously occurring terato-carcinomas had established that the undifferentiated component, *embryonal carcinoma* (EC), could be propagated in culture. This also proved to be true of embryo-derived teratocarcinomas, and EC cells were established as cell lines[29, 30]. After transplantation to extrauterine sites of appropriate mouse strains, EC cells produced either benign teratomas or malignant teratocarcinomas[28, 31]. Clonally isolated EC cells retained their capacity for differentiation and could produce derivatives of all three primary germ layers. EC cells, however, showed chromosomal aberrations[32], lost their ability to differentiate[33] or differentiated *in vitro* only under the influence of chemical inducers[34]. Maintenance of the undifferentiated state relied on cultivation with feeder cells[35] and, after transfer into early blastocysts, EC cells only sporadically colonized the germ line[36]. These data suggested that the EC cells did not retain the pluripotent capacities of early embryonic cells and had undergone cellular changes during the transient tumorigenic state *in vivo* (see review by Andrews[37]).

The direct *in vitro* propagation of mouse embryonic cells was the logical next step and, in 1981, two groups succeeded in cultivating pluripotent cell lines from mouse blastocysts using either a feeder layer of mouse embryonic fibroblasts (MEF)[20] or an embryonic carcinoma cell-conditioned medium[21]. These cell lines, later termed mouse embryonic stem cells (mESC), originate from the ICM of the blastocyst (Figure 1.2).

The protocols for mESC derivation are relatively simple and remain unchanged to the present day. Embryos at the expanded blastocyst stage are plated, either intact or following immunosurgical isolation of the ICM, onto a feeder layer. After several days of culture, the cell mass is disaggregated and replated onto fresh feeders. Various types of differentiated colonies arise along with colonies of a characteristic undifferentiated morphology. The latter are individually dissociated and replated. If secondary colonies of undifferentiated cells arise, these can generally be expanded further and continuous ES cell lines established. These cells proliferate rapidly in culture and clonal populations can be readily initiated from single cells.

The pluripotency of mESC was demonstrated *in vivo* by their introduction into blastocysts. The resulting mouse chimeras demonstrated that mESC could contribute to all cell lineages including the germ line[38, 39]. *In vitro*, mESC showed the capacity to reproduce the various somatic cell types[20, 40, 41] and only recently were found to develop into cells of the germ line[24-26]. The establishment of human ES cell lines (hESC) from *in vitro* fertilized embryos[22] and the demonstration of their *in vitro* developmental potential[42-46] have evoked widespread discussions concerning not only future applications of hESC in regenerative medicine but also about their ethical and legal implications.

1.3 Maintenance of Undifferentiated State of mESC

The efficiency of mESC derivation proved strain dependent, and inbred mice, like the 129 strain, demonstrated the highest rates of success for the generation of ES cells[47]. Once established as permanent lines, mESC displayed an almost unlimited proliferation capacity *in vitro*[48] and retained the ability to contribute to all cell lineages. *In vitro*, the mESC maintained a relatively normal and stable karyotype and were characterized by a relatively short generation time of ~ 12 - 15 h with a short G1 cell-cycle phase[49-51]. However, maintenance of the undifferentiated stem cell phenotype is not cell-autonomous. Media containing all necessary metabolites and nutrients are not sufficient to support either derivation or maintenance of mESC. Co-culture with a feeder layer was originally considered essential. Subsequently, it was discovered that the feeders could be

substituted by conditioned media preparations[52]. This suggested that the critical requirement is to provide trophic stimulation, without which the ESC differentiate. This interpretation was confirmed by the subsequent finding that a single cytokine, leukemia inhibitory factor (LIF), could sustain mESC self-renewal in the absence of feeders[53, 54]. LIF is produced by feeder cells, and its expression is stimulated by the presence of ESC[55]. Furthermore, feeders lacking a functional LIF gene do not support ESC propagation effectively[56]. In mESC, LIF predominantly activates STAT3. On withdrawal of LIF or feeders, or the inactivation of STAT3, proliferation continues but differentiation is induced, and stem cells do not persist beyond a few days[57].

Studies on hematopoietic stem cell expansion had suggested that the number of ligand-receptor complexes on the cell surface could influence the stem cell fate decision[58]. According to this model, when a relevant ligand-receptor interaction falls below a certain threshold, the probability of differentiation is increased; otherwise, self-renewal is favored. Examination of ESC over a range of LIF concentrations demonstrated that LIF supplementation had little effect on growth rates, but it significantly altered the probability of cells undergoing self-renewal versus differentiation[59]. To further address this question, a designer cytokine (a fusion protein of sIL-6/sIL-6R linked to a flexible peptide chain) called Hyper-IL-6 (HIL-6)[60] together with LIF were employed to experimentally and computationally test their capacity to sustain ESC self-renewal. Quantitative measurements of ES cell phenotypic markers, functional assays (embryoid body (EB) formation), and transcription factor (Oct-3/4) expression over a range of LIF and HIL-6 concentrations demonstrated a superior ability of LIF to maintain ES cell pluripotentiality at higher concentrations (500 pM)[61]. These results supported a ligand/receptor signaling threshold model of ESC fate modulation that requires appropriate types and levels of cytokine stimulation to maintain self-renewal[58].

1.4 Characterization of mESC Responses

mESC are identified by a number of *in vitro* phenotypic, functional and gene expression assays. mESC exhibit a high nuclear to cytoplasm ratio, grow in compact colonies and have a relatively short G_1 cell cycle phase[50]. It is now well established that undifferentiated mESC express a specific cell surface antigen, stage specific embryonic antigen-1 (SSEA-1), which is first expressed on cells of the preimplantation embryo at around the 8-cell stage and is present on most of the ICM cells[62, 63]. Expression of SSEA-1 increases as the ESC form primitive ectoderm cells, but diminishes upon further differentiation[62, 63]. The precise function of SSEA-1 is not known; a suggested role includes the mediation of mouse embryo compaction[64]. Although SSEA-1 has not been identified as a marker for any particular cell lineage, it is also found in the adult mouse brain, kidney and sperm[63]. Another phenotypic marker that is differentially expressed as mESC undergo differentiation is E-cadherin which is a Ca^{2+}-dependent cell adhesion molecule that mediates cell-cell interactions in the formation of the trophectoderm and compaction of preimplantation mouse embryos[65-68]. Pluripotent cells of the ICM and the primitive ectoderm express E-cadherin, but expression is downregulated during mesoderm formation at gastrulation[69, 70]. In addition to LIFR and gp130, mESC also express the receptor for stem cell factor, c-kit[71, 72], and possess high activities for the enzymes alkaline phosphatase (AP) and telomerase[73]. Differentiation is accompanied by a fall in alkaline phosphatase activity[74].

In vivo functional assays for mESC include their abilities, on ectopic transplantation, to give rise to teratocarcinomas containing a wide range of differentiated cells and to contribute to normal embryonic development, resulting in germline competent chimeric mice, when injected into mouse embryos[38]. One *in vitro* functional assay used to measure mESC pluripotentiality is the assessment of their ability to form multiple lineages in EB formation cultures under appropriate conditions. In addition, undifferentiated mESC have higher EB formation efficiencies than differentiated cells. As the ability to form EBs correlates with other ESC markers such as SSEA-1 expression and AP activity[59], this assay can be used to assess mESC developmental potential. mESC

differentiation may also be analyzed by assaying the germline specific Oct-3/4 protein, an octamer-binding transcription factor[75-77] that acts on multiple target genes and regulates early developmental events[78]. Oct-3/4 is expressed at high levels in undifferentiated cells and is downregulated upon differentiation both *in vivo* and *in vitro*[79]. Another homeobox-containing gene, ehox, has recently been identified as having differential expression during mESC differentiation. In contrast to Oct-3/4, ehox expression is upregulated upon ESC differentiation[80].

1.5 Molecular Mechanisms Underlying Self-Renewal of mESC

In vivo, zygotic expression of the transcription factor, Oct-3/4, is essential for the initial development of pluripotentiality in the ICM as mutant null embryos develop only to the blastocyst stage and do not form ICM[77, 81]. In mESC, continuous function of Oct-3/4 is necessary to maintain pluripotency[82]. A less than two-fold increase in expression causes differentiation into primitive endoderm and mesoderm, whereas downregulation of Oct-3/4 induces the formation of trophectoderm concomitant with a loss of pluripotency (see Figure 1.3(B))[82]. Maintenance of Oct-3/4 expression is not in itself sufficient to sustain the pluripotent phenotype. Propagation of the pluripotent mESC phenotype requires both the intrinsic activity of Oct-3/4 and the cytokine-induced action of STAT3.

Recently, two groups identified the homeodomain protein Nanog as another key regulator of pluripotentiality[83, 84]. In preimplantation embryos, its expression is restricted to and required in ICM cells from which ES cells can be derived. The dosage of Nanog is a critical determinant of cytokine-independent colony formation, and forced expression of this protein confers constitutive self-renewal in ES cells without gp130 stimulation. Nanog may therefore act to restrict the differentiation-inducing potential of Oct-3/4. LIF, when applied to serum-free ES cell cultures, is insufficient to maintain pluripotency or block (neural) differentiation. In combination with bone morphogenetic protein (BMP), LIF sustains self-renewal, multilineage differentiation, chimera colonization, and germ-line transmission properties. The critical contribution of BMP is to induce expression of Id (inhibitor of differentiation) genes via the SMAD pathway (Figure 1.3 (A)). Forced

expression of Id genes liberates ES cells from BMP or serum dependence and allows self-renewal in LIF alone. Blockade of lineage-specific transcription factors by Id proteins enables the self-renewal response to LIF/STAT3 signaling[85]. Somewhat surprisingly, activation of the ERK mitogen-activated protein kinase (MAPK) pathway, seems not to be required for ES cell self-renewal[86, 87]. ERK activation is normally a key signal for cell-cycle progression through the cyclin D/CDK checkpoint in G_1[88]. In fact, inhibition of the ERK-activating enzyme MEK actually enhances self-renewal, implying that there is a pro-differentiative effect of ERK activation (Figure 1.3(A)). This may relate to growth factors and other inductive stimuli that signal through the Ras-Raf-MEK-ERK cascade and also to integrin-mediated ERK activation[71].

Overall, the self-renewal of ES cells appears to depend on a balance between conflicting intracellular signals. The dual requirements of achieving and maintaining a high level of STAT3 activation and a low level of ERK activity may underlie some of the difficulties experienced in ES cell derivation, where the alternative outcome of differentiation is generally favored. In this context, application of the MEK inhibitor PD05809 appears to increase the efficiency of ES cell establishment by promoting expansion of primary stem cell colonies[89]. However, it remains unclear how this pathway interacts with Nanog, Oct-3/4, and LIF signaling to regulate pluripotentiality (see Figure 1.3).

Finally, a recent study has implicated Wnt-signaling pathways in the maintenance of ESC pluripotency. Wnt pathway activation by a specific pharmacological inhibitor (BIO; 6-bromoindirubin-3'-oxime) of glycogen synthase kinase-3β (GSK-3 β) maintains the undifferentiated phenotype in both mouse and human ES cells and sustains expression of the pluripotent stage-specific transcription factors Oct-3/4 and Nanog[90]. Whether or not Wnt signaling has an effect on hESC self-renewal over longer periods through multiple passages remains to be determined. The reversibility of the BIO-mediated Wnt-activation in hESC also suggests a practical application of GSK-3β-specific inhibitors to regulate early steps of differentiation, which may prove valuable for the derivation of cells suitable for regenerative medicine.

The mESC property of self-renewal therefore depends on a stoichiometric balance among various signaling molecules, and an imbalance in any one can cause the identity of the mESC to be lost. Other molecular markers potentially defining pluripotentiality include Rex1[91], Sox2[92], Genesis[93], GBX2[94], UTF1[95, 96], and Pem[97, 98]. All of these have been shown to be expressed in the ICM of blastocysts and are downregulated upon differentiation; however, they are not exclusively expressed by pluripotent embryonic stem cells and can be found in other cell types in the soma. Their potential role in maintaining pluripotentiality or self-renewal remains to be determined

1.6 Human ESC Lines

The techniques used to isolate and culture mESC proved critical to the generation of hESC lines from preimplantation "spare" embryos produced by *in vitro* fertilization[22,46,99] and after *in vitro* culture of blastocysts[100]. The resulting hESC shared some fundamental characteristics of murine lines, such as Oct-3/4 expression, telomerase activity, and the formation of teratomas containing derivatives of all three primary germ layers in immunodeficient mice[22, 46]. Similar to mESC, hESC maintained proliferative potential for prolonged periods of culture and retained a normal karyotype[101]. In contrast to mESC, hESC formed mainly cystic EBs[102] and displayed proteoglycans (TRA-1–60, TRA-1–81, GCTM-2) and different subtypes of stage-specific antigens (SSEA-3, SSEA-4), which were absent from mouse ES cell lines.

Several potentially important differences exist between mouse and human ESC. hESC have a longer average population doubling time than mESC (30–35 h vs. 12–15 h[101]). With murine cells, it is possible to substitute the feeder layer of embryonic fibroblasts with recombinant LIF. In contrast, LIF is insufficient to inhibit the differentiation of hESC[22, 46] which continue to be routinely cultured on feeder layers of MEF or feeder cells from human tissues. The identity of the essential self-renewal signals provided to hESC by MEF feeder cells remain ill defined. The cultivation of hESC on extracellular matrix proteins, such as Matrigel (a complex mixture of ECM proteins isolated from Engelbreth-Holm-Swarm tumor) or laminin with MEF-conditioned media[103] causes hESC to express

high levels of SSEA-3 along with α_6- and β_1-integrins, which are involved in cell interactions with laminin[103]. These results show that application of extracellular matrix-associated factors can be employed to improve the culture and maintenance of pluripotent hESC.

hESC lines have now been cultivated both on human feeders to avoid xenogenic contamination[104, 105] and in the absence of feeder cells under serum-free conditions[105]. These technological advances suggest that new hESC lines free from potential retroviral infections will be prepared and that these cells, unlike most of those currently available, might be suitable for eventual therapeutic applications.

Although the principal techniques necessary to culture (up to 80 and more passages) and manipulate hESC have been established (cell cloning[101], cryo-preservation[106], transfection[107], and gene targeting by homologous recombination[108]), other methods (single cell dissociation and proliferation) are still not yet optimal. Because of the variabilities among hESC lines (growth characteristics, differentiation potential, and culturing techniques), it will be important to define a reliable set of molecular and cellular markers that characterize the undifferentiated pluripotent (stemness) or differentiated state of hESC. Recent attempts to define molecular markers of undifferentiated cells, however, indicate similar pattern of marker expression among four hESC lines maintained in a feeder-free culture system[109] and examined after long-term culture[4]. It is evident that the present data, although limited, does allow an unambiguous molecular definition of pluripotent stem cell properties. The application of transcriptome profiling with proteomic analyses to ES cell lines may prove useful to define which lines and growth conditions are optimal for human ES cells *in vitro*.

Formulation of optimal culture conditions for hESC will depend on whether the cell line will be used as a cell source for therapeutics or as a model to study early human development. For the former application, the cell line will need to have enough proliferative capacity to provide a sufficient quantity of cells, reliably differentiate into an appropriate cell population and remain karyotypically stable during expansion,

differentiation as well as after transplantation. Xenogenic-free culture conditions would need to be developed for these cells. In addition, therapeutic applications will require that the cell line be compatible with FDA-type regulations and hence it is likely that there will be a change to the production of hESC under current Good Manufacturing Practices (cGMP) regulations. All methods will need to be well defined and subjected to quality control procedures. Bulk culture systems for hESC are still in their infancy and much bioprocess R&D is needed to improve these systems for optimal production of hESC and their derivatives.

If hESC are to be used as a model to study early human development, the most desirable characteristics will be appropriate proliferation and cell cycle regulation, gene expression and ability to appropriately respond to developmental cues. Pluripotency is a key feature as these cells will be used to study cell fate choices.

For both of the above goals, generating and maintaining the cells in defined culture conditions will allow the establishment of more reproducible cultures that can be consistently maintained in multiple laboratories. These conditions will include a defined matrix and medium supplemented with recombinant proteins, and passaging which allows cell seeding at a consistent cell density. Moreover, the culture conditions should maintain cells phenotypically and karyotypically stable for the required time period and allow them to retain the capacity for appropriate and reproducible differentiation.

The molecular regulation of hESC self-renewal is less well understood than for mESC. With current protocols, hESC can be maintained either on feeder cells in a serum-free medium supplemented with basic fibroblast growth factor (bFGF)[110] or on matrigel or laminin-coated plates in the presence of bFGF and MEF conditioned medium[103]. Cells grown under these conditions for > 100 population doublings retained normal karyotypes and stem cell characteristics, including their *in vitro* and *in vivo* differentiation potential. hESC express both Oct-4 and Nanog[90, 111] suggesting that this aspect of their regulation may be similar to that observed in mESC. A requirement for Nanog in the direction of self-renewal of hESC seems probable but remains to be established[112]. Oct-3/4 appears

to be required to suppress extra-embryonic differentiation in hESC[113, 114]. Extrinsic signals that support hESC self-renewal have yet to be definitively identified but may be different from those effective on mESC. Although hESC can self-renew in the absence of exogenously added LIF, this does not necessarily mean that STAT3 activation is not important for the self renewal of hESC. It is possible that autocrine signaling through gp130 occurs in these cultures and that the extent of this is sufficient for human but not for mouse ESC. Alternatively, hESC express a higher effective level of Nanog than mESC and hence may not require a cooperative interaction between STAT3 and endogenous Nanog in order to self-renew effectively[112]. A trivial explanation for the reported lack of effect of LIF on hESC is that most studies used recombinant mouse LIF which does not bind to the human LIF receptor[115]. Further studies are therefore needed to define the molecules needed for the maintenance of hESC and uncover the molecular mechanisms that regulate their self-renewal.

1.7 Embryoid Body Formation Based Differentiation of mESC

In 1985, an *in vitro* model of mouse embryogenesis based on differentiating mESC was presented for the first time[40]. When grown in the absence of feeder cells and LIF, in suspension, in bacterial dishes, in spinner flasks, in a semisolid methylcellulose medium or as hanging drops, mESC are able to differentiate spontaneously[40, 116, 117]. Because of the ability of the differentiated cells to form spheroid aggregates mimicking postimplantation embryonic tissues, they were termed embryoid bodies (EBs). Following aggregation, the outer layer of the EBs is specified as extra-embryonic primitive endoderm and it further differentiates into visceral endoderm[40]. Extracellular matrix, which is secreted from the primitive endoderm layer, then forms a thick basement membrane, separating the primitive endoderm from the inner mass of the EBs. Along the inside of the basement membrane, a primitive ectoderm layer is formed and cavitation occurs in the core of the EBs. This series of processes is considered to be an *in vitro* model recapitulating early embryonic development from the blastocyst stage to the egg cylinder stage (E3.5-E6.5)[118]. In contrast to tumor cell derived spheroids, oxygenation in EBs was shown to be very efficient[119]. Cells within developing EBs differentiate to more

advanced stages of embryogenesis, resulting in various committed cell types including hematopoietic precursors[120, 121], cardiomyocytes[117, 122], skeletal muscle cells[123] and many others.

Establishment of EBs has facilitated the investigation of several aspects of mouse development *in vitro*. First, EBs are a powerful tool for characterizing the function of precursor cells, which is very difficult to perform *in vivo* as it is nearly impossible to isolate early stage cells from developing embryos. Second, EBs derived from mESC in which both alleles of a specific gene have been disrupted by targeted mutagenesis often represents a fast alternative for investigating the impact of a given null mutation. The same holds true if the deletion of a given gene induces early embryo death.

It is well known that mESC derivation is strain dependent with 38% of the explanted blastocysts from the 129Sv strain giving rise to mESC while only 1% of the C57Bl/6J ICM strain generally develops into a mESC line *in vitro*[124]. It has proved impossible so far to isolate a single mESC line from FVB blastocysts. These results suggest that the genetic background and perhaps even minor epigenetic differences between strains and perhaps profound differences among species might predetermine whether the ICM cells can survive on feeder cells in the presence of LIF or not.

It is also well known that self-renewal capacity and differentiation control are highly variable among even isogenic mESC lines generated in different laboratories. EB development is therefore influenced not only by the genetic background of the mESC line used but also by the complex conditions under which that mESC line had been generated. In addition, both the pattern and the efficiency of differentiation are affected by parameters like mESC density, medium components (high glucose concentration, i.e., at least 4.5 g glucose/L is required) and amino acids, growth factors and extracellular matrix (ECM) proteins, pH and osmolality, and the quality of the fetal calf serum (FCS)[125]. Because the differentiation efficiency depends on the presence of FCS, and even the "batch" of serum used, many efforts have been taken to avoid the resulting uncertainties, e.g., by using chemically defined media[126, 127] or, more recently, by substitution of FCS

with BSA fraction V[128]. Furthermore, different ES cell lines display unique developmental properties *in vitro*[125, 129].

Additional parameters that influence mESC potential and EB differentiation include the developmental stage of the embryo at the time of mESC generation, the time required to reach confluency *in vitro*, the type of feeder cells, the source and batch of serum and, last but not least, the handling of the cells by the investigator. Furthermore, the self-renewal capacity strongly influences EB development[130]. The initial proliferation of mESC, for example, determines the number of ESC which aggregate to form EBs, and the final number of cells in the compact EB aggregate strongly influences cardiomyogenesis and hematopoiesis[131, 132]. Thus, mESC proliferation and maintenance of pluripotency significantly contribute to the subsequent differentiation potential in EBs. It is generally accepted that mESC are reprogrammed to delete all epigenetic modifications[133, 134], but examples of epigenetic inheritance exist[135, 136] and may not only influence the differentiation potential but additionally contribute to the divergence of the differentiation potential observed in EB development.

1.8 Culture Environment and Maintenance of Stem Cell Potential

Cellular homeostasis (maintenance of a stable internal environment) is an important regulator of many cell functions such as cell division, protein synthesis, differentiation, cell-to-cell communication and cytoskeleton dynamics, as well as metabolism[137]. Therefore, any disruptions that may occur in cellular homeostasis as a result of trauma or stress can have significant implications for normal growth and development. Stress can be induced by using inappropriate media formulations, oxygen or carbon dioxide levels, pH, osmolality or even the presence of visible light. Several studies using animal models have shown that preimplantation embryos are sensitive to environmental conditions that can affect future growth and developmental potential, both pre- and post-natally. These studies were initiated with the observation that cultured mouse embryos, after transfer to surrogate mothers, resulted in reduced fetal growth compared with that of their *in vivo*

counterparts[138, 139]. Further studies on the mouse have confirmed this effect and identified possible culture conditions which may act deleteriously[140-144]. These and other similar studies have given rise to the notion that immediate manifestations of culture-induced stress include altered homeostasis and perturbed metabolism, which lead to altered cell function and impaired energy production, respectively[145-147]. A downstream effect of culture-induced stress is altered gene function[148]. The potential influences of culture environment on the embryo's responses are summarized in Figure 1.4.

1.8.1 Serum

Most *ex vivo* expansions of hematopoietic cultures have been carried out in media containing 10-20% fetal calf serum or horse serum. A number of studies have also reported expansion in serum-free media (see Sandstrom et al.[149] for a review). Serum functions as a source of hormones and essential nutrients. It also can protect cells from environmental damage by providing enzymes, enzyme inhibitors and other molecules that react with or bind to toxins. Serum alters the physiological/physicochemical properties of the culture environment by coating surfaces with macromolecules and buffering or altering the pH, osmolarity, surface tension and viscosity. Addition of serum to culture medium, however, makes its composition undefined, adds uncontrolled variability and complicates the clinical use of cells. Serum-containing media are superior for expansion of the granulocyte and monocyte lineages[150, 151]. Serum-free media, on the other hand, promote greater expansion of erythroid and megakaryocytic lineages primarily because serum contains transforming growth factor β (TGFβ), a potent inhibitor to the expansion of these lineages[152]. Autologous serum or plasma, however, can partly alleviate this shortcoming of serum-free media[150]. Serum has also been reported to either increase or decrease the expression of various genes in several cell types. For instance, addition of serum to the culture medium of beta TC1 cells (a pancreatic cell line) increased the mRNA levels of *c-fos* and *c-jun*[153, 154]. Increasing the serum concentration from 2.5% to 10% in cultures of HL60 cells increased the CD13 receptor surface expression by 100%[155]. This increase in CD13 surface expression was correlated with a 30% increase in CD13 mRNA levels. Serum also protected HL60 cells

from hydrodynamic damage under conditions of high agitation[155]. In contrast, reducing the serum concentration from 5% to 0.5% increased the mRNA and protein levels of progesterone receptor in MCF-7 human breast cancer cells[156]. Similarly, decreasing the serum concentration from 10% to 0.5% increased the collagen mRNA levels in smooth muscle cells[157].

Mammalian embryos are not exposed to serum *in vivo*[148]. Studies using more defined embryo culture systems have shown that serum induces premature blastulation in domestic animal embryos, affects embryo morphology and leads to perturbations in the ultra-structure, energy metabolism and cryotolerance of blastocysts[158-161]. Extensive work in recent years has helped to elucidate possible mechanisms behind these observations. Specifically, fetal overgrowth has been associated with reduced fetal methylation and expression of IGF2R, indicating that there were epigenetic alterations in imprinted genes during the preimplantation period *in vitro* when embryos were exposed to serum[162].

1.8.2 Culture Osmolality

Most measured values reported in the literature are osmolality (osmoles per kilogram, Osm/kg) rather than osmolarity (osmoles per liter, OsM). However, in the relatively dilute solutions that are physiologically relevant, these two measures differ negligibly. Both osmolality and osmolarity are intrinsic properties of a solution and are distinguishable from tonicity which describes the osmotic effect of a solution on a particular entity such as a cell and is thus context specific.

Animal cells regulate their size precisely. This is true not only for somatic cells but also for the cells of eggs and early embryos. Oocytes grow to a precise diameter, characteristic of each species, and the egg maintains that diameter for an extended period of time, implying that mechanisms exist by which an egg can determine and control its size. Subsequently, as the embryo cleaves into successively smaller cells, each embryonic stage possesses blastomeres that are maintained at characteristic dimensions.

The membranes of animal cells are generally highly permeable to water and cannot tolerate substantial hydrostatic pressure gradients. Water movement across the membranes is in large part dictated by osmotic pressure gradients. Thus any imbalance of intracellular and extracellular osmolality is paralleled by respective water movement across the cell membrane and subsequent alteration of the cell volume.

Cells accumulate a number of substances such as proteins, amino acids and carbohydrate metabolites. The concentration of these substances is higher within the cells than in the extracellular fluid. The excess cellular concentrations of these organic substances are counterbalanced by lower intracellular ion concentrations. Most cells extrude Na^+ in exchange for K^+ by the Na^+ - K^+ - ATPase pump. The cell membrane is only slightly permeable to Na^+, and the exclusion of impermeable Na^+ outweighs the cellular osmolality created by the impermeant organic solutes [double Donnan hypothesis[163]]. On the other hand, the cell membrane is highly permeably to K^+. The exit of K^+ creates an outside-positive cell membrane potential that drives Cl^- out of the cell. The low intracellular Cl^- concentration compensates for the excess intracellular concentration of organic substances.

1.8.2.1 Regulation of Volume Control

Delicately balanced mechanisms are brought into play to maintain a normal volume after the cells are exposed either to hypotonic or hypertonic solutions. Three sequential responses occur when cells are exposed to a hypertonic extracellular fluid. An initial rapid response involves the movement of water out of the cell, causing it to shrink. A second slower response follows in which ions and/or organic osmolytes move from the environment into the cell such that cells approach the original volume (regulatory cell volume increase, RVI). If the volume is still not restored, a very slow third response occurs in which genes are activated to stimulate the synthesis of organic osmolytes intracellularly. Exposure to a hypotonic solution results in the same sequence of responses, each in reverse. It should be kept in mind, however, that exposure of cells to

anisotonic extracellular fluid does not only modify the cell volume but also the volume of intracellular organelles such as the mitochondria[164]. Furthermore, in parallel to cellular osmolality, the intracellular ionic strength is altered even if the extracellular ionic strength is kept constant. Thus the sequelae of osmotic alterations of cell volume are not necessarily identical to the consequences of isotonic alterations of cell volume[165]. During cell swelling, cells extrude ions thus accomplishing regulatory volume decrease (RVD), whereas during cell shrinkage, cells accumulate ions to achieve RVI. The activation of ion release during RVD is paralleled by inhibition of ion uptake mechanisms, and the ion uptake during RVI is accompanied by inhibition of ion release mechanisms. Thus, the simultaneous stimulation of ionic mechanisms for RVD and RVI is largely avoided[166].

1.8.2.2 Organic Osmolytes

The cellular accumulation of electrolytes after cell shrinkage is limited, as high ion concentrations interfere with the structure and function of macromolecules including proteins[167-170]. Furthermore, alterations of ion gradients across the cell membrane would affect the respective transporters. An increase of intracellular Na^+ activity, for instance, would reverse Na^+/Ca^+ exchange and thus increase intracellular Ca^+ activity, which would in turn affect a multitude of cellular functions[171, 172]. To circumvent the untoward effects of disturbed ion composition, cells rely on so-called osmolytes – molecules that correct osmolality without compromising other cellular functions[173, 174]. A large body of work convincingly demonstrates that these osmolytes are compatible with biochemical function even at high (molar) concentrations. Three groups of osmolytes are used in mammalian cells: (i) polyalcohols such as sorbitol, glycerophosphorylcholine (GPC) and inositol; (ii) methylamines such as betaine; and (iii) amino acids and their derivatives such as glycine, glutamine, glutamate aspartate and taurine. Beyond their function in cell volume regulation, osmolytes can be protective against the destructive effects of excessive temperature[175, 176] and desiccation[177, 178]. Furthermore, they have been found to ease cell membrane assembly[179].

Sorbitol and GPC are synthesized within the cell in which they are accumulated as organic osmolytes. However, most organic osmolytes are instead transported into the cell from the environment via specific transporters. Four such osmolyte transport systems have been identified in mammals: the betaine transporter, β-amino acid or the taurine transporter (system β), the Na^+/myo-inositol transporter and system A, which transports neutral amino acids. All of these transport systems respond to increased tonicity by increasing their rate of substrate transport[174, 180]. In each case, the increased rate of transport is due to an increase in the maximal transport velocity (V_{max})[181-183]. The rate of osmolyte transport increases slowly, reaching a maximal level only hours to days after the external osmolality is increased[184, 185].

Van Winkle et al.[186] first proposed that mammalian embryos utilize organic osmolytes and their presence protects embryos against increased osmolality. Evidence has since accumulated that early mammalian embryos use a range of organic compounds for osmoprotection. McKiernan et al.[187] examined the effect of each of the common amino acids and taurine, separately and in combination, on the development of hamster one-cell embryos to blastocysts. Three amino acids – glutamine, glycine and taurine – were found to greatly stimulate development and several others were found to stimulate development in the presence of glutamine. Glutamine has been found to be especially beneficial for the culture of preimplantation stage embryos of mouse, hamster, cow and human[188-192]. An extensive body of work has shown that, when added as a group, Eagle's nonessential amino acids (NEAA) plus glutamine and taurine are stimulatory to cleavage stage mouse, bovine and human embryo development[146, 193-196]. This group overlaps considerably with the stimulatory amino acids identified in the hamster[187].

Many of the amino acids that have been found to be protective of osmotic stress and beneficial to embryo development, especially glutamine, taurine, glycine and alanine, are established organic osmolytes in somatic cells as well[169, 197]. In Madin-Darby canine kidney (MDCK) cells, in which the effect of osmolality on intracellular amino acid content has been studied most systematically, taurine, alanine, glutamine, glycine, proline and serine were found to accumulate in response to hypertonicity and to act as organic

osmolytes[183, 198]. Comparing the amino acids accumulated in MDCK cells with those found beneficial to preimplantation embryo development reveals that the two groups are almost identical. This raises the possibility that one of the major functions of beneficial amino acids is to act as organic osmolytes.

1.8.2.3 Molecular Mechanism of Cellular Protection from Hyperosmolality

Hypertonicity stimulates a diverse array of signal transduction pathways. Cell swelling has been shown to stimulate protein kinase C[199], focal adhesion and phosphatidylinositol 3-kinases (PI3K)[200], as well as to trigger the mitogen activated protein kinase (MAPK) cascade leading to the activation of cJun-N-terminal kinase (JNK) or extracellular signal regulated kinases (ERK1 and ERK2)[201-204]. Similarly, osmotic cell shrinkage has been shown to activate protein kinase C[199], the MAPK cascade leading to ribosomal S6 protein kinase[205, 206], and either p38 MAPK or JNK[207, 208]. These signal transduction pathways eventually result in increase or decrease in expression of a wide variety of genes.

In somatic cells, both cell swelling and cell shrinkage markedly influence the expression of a wide variety of genes. Hypertonicity increases sorbitol concentration by raising the amount and activity of aldose reductase – the enzyme that catalyzes the synthesis of sorbitol from glucose[209-211]. The enzyme is upregulated by hypertonic extracellular addition of NaCl or raffinose but not of membrane permeable solutes such as urea or glycerol. It is presumably an increase in intracellular ionic strength that stimulates the aldose reductase transcription rate[210, 212]. The aldose reductase transcription rate peaks at a level approximately 18-fold higher than control after 24 h of hypertonicity; then, as sorbitol and other organic osmolytes accumulate, the transcription rate falls to a steady-state level approximately 5 times that of the control[213]. Cell shrinkage has been shown to stimulate the expression of heat shock proteins that serve to stabilize the proteins and thus to counteract the detrimental effects of increased salt concentrations[214, 215]. Increased intracellular ionic strength also stimulates transcription of genes for betaine and inositol

transporters[216]. Cell shrinkage stimulates Na^+-coupled transport of neutral amino acids[217, 218] and proteolysis[219, 220] and inhibits protein synthesis[221]. Cell shrinkage also stimulates the expression of genes with diverse functions not obviously related to RVI such as P-glycoprotein[222], cyclooxygenase-2[223], c-fos[215, 224], phosphoenolpyruvate carboxykinase[225] and tyrosine hydroxylase[226], to name a few. Cell shrinkage is frequently observed to alkalinize cells, at least partially due to activation of the volume regulatory Na^+/H^+ exchanger[227, 228]. Alkalanization after cell shrinkage should stimulate glycolysis[229, 230].

Conversely, cell swelling stimulates the expression of immediate early genes, *c-fos* and *c-jun*[231, 232], α-*actin*[233] and *tublin*[234], MAPK cascades in cardiac myocytes[231], and TNF-α in macrophages[235]. Cell swelling inhibits proteolysis and stimulates protein synthesis[221, 236] and leads to cytosolic acidification[237, 238], which has been explained by the exit of bicarbonate ion through anion channels[239]. Swelling also increases glycogen synthesis and inhibits glycolysis[240, 241].

Information on the mechanisms triggering altered gene expression remains scanty. Some evidence points to the involvement of the cytoskeleton[242]. The expression of aldose-reductase is regulated by a distinct osmolality-responsive element[209, 243]. The stimulation of *c-fos* expression by swelling of cardiomyocytes depends on tyrosine phosphorylation[232]. The *c-jun* transcription after swelling of hepatocytes is at least partially the result of MAPK activation followed by phosphorylation of c-jun[244, 245]. Hypertonicity has also been shown to alter karyotype[246].

Cell proliferation has been shown to correlate with increases of cell volume in fibroblasts[247, 248], lymphocytes[249, 250], HL-60 cells[251, 252], hybridoma cells[253] and HeLa cells[254]. In contrast to cell proliferation, cell differentiation is accompanied by cell shrinkage in erythroleukemia cells[255-257], HL-60 leukemia cells[258, 259] and EMT6/ro mouse mammary sarcoma cells[260].

In summary, cells are able to control their size precisely in the face of an osmolality challenge by taking up organic osmolytes from the medium as well as by inducing expression of a wide variety of genes. Increases or decreases in osmolality of medium also profoundly influence the proliferation of cells.

1.8.3 Culture pH

The ability to regulate ionic homeostasis is essential for normal cell function. Intracellular levels of ions such as calcium (Ca_i^{+2}), hydrogen (H_i^+ or pH_i), magnesium (Mg_i^{+2}) and phosphate (PO_{4i}^{-3}) regulate a multitude of cellular functions such as cell division, protein synthesis, cytoskeletal dynamics, metabolism and energy production. It is also known that the difference in ionic composition between the cell and its microenvironment is a universal source of cellular potential energy[261]. Transmembrane ionic gradients – particularly that of sodium – are the driving force used to accomplish both organ-specific cellular functions (e.g., action potential in nerves, reabsorption and secretion by kidney and cerebrospinal fluid formation), and general cellular functions essential for cell growth and division (e.g., entry of nutrients and amino acids, as well as regulation of pH_i and free calcium). Therefore, it is essential for normal cell development that ionic homeostasis be tightly regulated. Aberrations in cellular homeostasis result in perturbed cell function and loss of developmental competence. This has significant implications for ESC cultures where the target is to expand ESC numbers without loss of developmental potential. To ensure this, ESC culture must be performed under conditions where cellular homeostatic stress is not too great.

Developmental biologists generally divide the genome into two parts: one consisting of genes directly involved in determination, differentiation and morphogenesis and the other part consisting of genes that regulate metabolic functions. Genes involved in metabolic control are generally not considered important from a developmental point-of-view [262]. However, it is increasingly being recognized that ions and small molecules operating in complex networks are likely involved in the sequential control of developmentally important genes[263, 264]. Ionic signaling occurs when an ovum is activated at fertilization,

and ion transport systems become active at specific times to produce fluid-filled cavities in the embryo such as the blastocoel and the amnion[265]. Similarly, two specific physiological systems in which ions are involved in developmentally important phenomena in the early embryo are: i) regulation of pH_i which emphasizes the fact that some transport systems change with development according to the embryo's needs at each stage of the life cycle, and ii) regionalization of the Na^+/K^+ - ATPase in the embryo which results in the formation of the blastocyst through cavity formation, thereby establishing the earliest patterned structure in mammalian development[263].

In general, the intracellular concentrations of Na^+ and K^+ are held constant. However, they are maintained at concentrations very different from those found in the extracellular microenvironment. The intracellular concentration of Na^+ is low while its extracellular concentration is high. The reverse is true for K^+. Consequently, large electrochemical gradients due to these two ions exist across the cell membrane. Both of these ions are however, in a constant state of flux. At any given time, their flux is the net result of the rate at which the ion is lost by leaking down its electrochemical gradient and the rate at which it is retrieved by Na^+/K^+ pumps which expend chemical energy derived from the hydrolysis of ATP. The energy, in the form of these ion gradients (mainly of Na^+), is then used for other energy-requiring transports, such as co- and counter-transport of protons, chloride, phosphate, glucose and amino acids. Further discussion of these dynamic homeostatic systems can be found in standard references[261, 266].

The normal functions of cellular proteins depend on the degree of ionization of their component amino acids. The ionization in turn depends on the presence of a stable acid-base status in the cells. The distribution of H^+ ions across the plasma membrane of most cells is such that the internal pH (pH_i) is much higher than that predicted if H^+ were passively distributed. For an average membrane potential of –60 mV and for an external pH of 7.4, a pH_i value of 6.4 would be expected if the hydrogen ions are in electrochemical equilibrium across the membrane[267, 268]. Therefore, all cells have mechanisms for H^+ extrusion that maintain pH_i at a value which is well above equilibrium and is compatible with the necessities of cytoplasmic reactions. This fact has

also been demonstrated in mammalian embryos including the human, where it is observed that the pH of the external medium (pH_e) does not equate to pH_i. Numerous studies performed during the last two decades have demonstrated that changes in pH_i occur during metabolic and developmental transitions in a large variety of cells[269-271]. Acidic cytoplasmic conditions are usually associated with a quiescent or dormant cellular state while an increase in pH_i is often a signal for cellular activation[271, 272].

1.8.3.1 Effect of Culture Medium on Intracellular pH (pH_i)

Some components of the culture medium have been shown to have significant effects on pH_i regulation by somatic cells as well as embryos. For example, levels of both lactate and amino acids in the medium have been shown to alter pH_i. Low levels of lactate in the medium (5 mM D/L-lactate) significantly reduced the pH_i of mouse embryos by around 0.2 units[273, 274]. Although only L-lactate can be metabolized by cells, both D- and L-lactate affect pH_i[273, 274]. The other important components of the medium for pH_i regulation are the amino acids. Proportions of some of the NEAA such as taurine and glycine exist as zwitterions at physiological pH. Zwitterions can move readily across the membrane and bind protons and therefore buffer pH_i. Taurine, glycine and glutamine are present at high concentrations in the female reproductive tract[275] and quite likely these amino acids are able to regulate pH_i *in vivo*. The capacity of amino acids in the culture medium to buffer pH_i has been demonstrated in mouse pronuclear stage embryos. Incubation of embryos in the presence of the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), results in a significant decrease in pH_i. Addition of NEAA to a culture medium containing DMO reduced the resultant acidification and therefore increased the ability of embryos to buffer protons and maintain pH_i[276]. Furthermore, addition of amino acids to the culture medium prevents the efflux of endogenous amino acids and would therefore assist in maintaining the intrinsic buffering capacity of the cytoplasm[277]. As discussed in Section 1.7.3, NEAA can also protect cells against stress caused by hyperosmolality.

1.8.3.2 Regulation of pH_i

In order to maintain pH_i at a constant and steady level, the cell must be able to regulate and neutralize intracellular acids such as lactic acid – a by-product of metabolism. Cells have two mechanisms to regulate pH_i: i) short-term regulation and (ii) long-term regulation.

Short-term regulation of changes in pH_i is achieved by physiochemical buffering of the cytoplasm as well as buffering by the organelles themselves. The intrinsic buffering capacity can be measured and expressed as the millimoles of protons that can buffered by the cytoplasm of the cell. A higher value indicates an increased ability of the cytoplasm to buffer against a pH challenge. Typically, the intrinsic buffering capacity of cells is reported to range from 12 to 30 mM/ΔpH. Hamster oocytes, however, have been reported to have a much higher buffering capacity (51 mM/ΔpH)[278-280]. This increased buffering capacity of hamster oocytes may be a mechanism to compensate for the reduced capacity to restore pH due to lack of functional membrane transporters. The intrinsic buffering capacity of the cytoplasm can buffer pH_i in a matter of seconds.

For long-term stability and regulation of pH_i, it is necessary to either extrude the protons or buffer them by the import of bicarbonate ions. This is achieved by specific transporters in the cell membrane including proton pumps (H^+ ATPases)[281], proton channels[282] and ion transporters that drive H^+ or equivalent H^+ and HCO_3^- ions into and out of the cell (Figure 1.5). The latter include an Na^+/H^+ exchanger encoded by members of the sodium hydrogen exchanger (NHE) gene family (NHE1-9)[283, 284], Na^+ - dependent HCO_3^- co-transporters (NBC)[285], an Na^+-independent Cl^-/HCO_3^- transporter (AE)[286, 287], Na^+-dependent Cl^-/HCO_3^- transporter (NDCBE) and the recently described Cl^-/OH exchanger[288]. Other membrane transporters such as the lactate-proton co-transporter may also participate in intracellular pH regulation[289, 290] (Figure 1.5).

NHE is an electroneutral ion exchanger that pumps H^+ out of the cell when it is turned on by intracellular acidosis. The H^+ efflux is driven by the inwardly directed Na^+

electrochemical gradient. First discovered in kidney and intestine[283], the antiporter has now been found in most tissues of eukaryotic cells. So far, nine members of the NHE family sharing 30 - 60% homology have been cloned. The activity of the exchanger increases exponentially to restore pH_i as acidification increases[278]. The exchanger extrudes protons in exchange for Na^+ ions and regulates pH_i in the acid to neutral range. The amino-terminal domain is sufficient to transport the ions[291, 292]. The C-terminal region features an ATP and other protein binding sites in addition to many phosphorylation sites and is thus involved in neurohormonal regulation of the ion-exchange activity[293-295]. The NHE1 isoform is ubiquitously expressed in somatic cells and its activity has also been demonstrated in embryos from several strains of mice[274] as well as from hamster[278, 280], cow[296], and human[297].

The kinetic features of the NHE isoforms have been studied after stable transfection in antiporter-deficient fibroblast cell lines. NHE1-3 typically show similar Michaelis constants for external Na^+ (K_{mNa} = 4 – 18 mM). The kinetics for intracellular H^+ are also similar and fit a sigmoidal curve which suggests the presence of an intracellular H^+ modifier site in addition to the H^+ transport site[298]. The NHE1-3 exhibit half-maximal activity at intracellular pH values between 6.2 and 6.8. The pH sensitivity of NHE1 is mechanistically complex. It is regulated by an autoinhibitory domain (residues 636-656) that prevents protonation of the pH sensor[299-301] and another domain that is more directly responsible for the maintenance of pH sensitivity (residues 567 – 635)[291, 295]. Amino acids 515-595 in the C-terminal domain are particularly critical for the pH sensitivity of the antiporter[301].

All NHE isoforms show dependence on ATP, as a drop in intracellular ATP dramatically decreases their activity in several cell types[301, 302]. The ATP dependence of NHE1 cannot be explained entirely by the requirement of phosphorylation, as mutation of all known phosphorylation sites does not abolish the metabolic regulation of the antiporter[294]. The cytoskeleton is likely to participate in this process and a non-diffusible effector that binds ATP is probably required to activate the antiporter in the presence of ATP[303, 304].

The NHE is also regulated by cell volume[303, 304]. NHE1 is stimulated by hypertonicity and, under similar conditions, the activity of NHE2 is also increased. Under hypotonic conditions, the activity of both isoforms is inhibited. In contrast, NHE3 is markedly inhibited by hypertonic cell shrinkage but is unaffected by hypotonicity[227, 302, 305]. A phosphorylation-independent mechanism likely underlies the volume regulation of the antiporter and hence the "volume sensitive site" is different from the site(s) postulated to mediate the stimulatory effects of calcium and growth factors[306].

The HCO_3^-/Cl^- exchanger transports bicarbonate ions out of cytoplasm in exchange for Cl^- and regulates pH_i in the neutral to alkaline range. When the pH_i of the cell rises above the physiological set point, the HCO_3^-/Cl^- exchanger is activated and acidifies the cytoplasm until the pH_i is restored to its physiological level[307]. The HCO_3^-/Cl^- exchanger has been extensively studied in erythrocytes. HCO_3^-/Cl^- exchangers are members of the anion exchanger (AE) gene family ubiquitously expressed in vertebrate tissues and are expressed very early during embryogenesis. As early as the two-cell stage, embryos are able to recover from an alkaline load by switching on this exchanger[308, 309]. The HCO_3^-/Cl^- exchanger is utilized by mouse[310], hamster[279], cow[296] and human[297] embryos to regulate pH_i in the alkaline range. Both the NHE and HCO_3^-/Cl^- exchangers can take several minutes to restore pH_i to its physiological level.

1.8.3.3 Influence of pH on Cellular Functions and Development

It is well recognized that pH_i is an important epigenetic regulator of somatic cell functions[269, 270]. In mammalian cells, pH_e is easily disturbed as weak acids and bases from cell metabolism can accumulate significantly in the culture media of long-term and high cell density cultures. In somatic cells, pH_e has been shown to have many diverse effects, including those on proliferation rate[311, 312], differentiation[313-315], metabolism and Ca^{+2} homeostasis[272, 316-318], protein synthesis, degradation and glycosylation[319-322], cell motility, contractility and cell-cell coupling[323], cell adhesion[324], expression of various cell surface receptors[316], glucose as well as amino acid transport[325], binding of growth factors

to extracellular matrix proteins[326, 327], expression of matrix metalloproteinase-9[328] and cell death[329, 330]. Clinically, the acidic pH_e has been shown to modulate the sensitivity of solid tumors to radiation and chemotherapeutic agents[331, 332]. In addition, acidic pH_e increases the expression of i) platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cells[333], ii) vascular endothelial growth factor (VEGF) in glioma and gliobastoma[334, 335], and iii) IL-8 in human pancreatic adenocarcinoma[336-339] and ovarian carcinoma cells [340] through activation of transcription factors NF-κB and/or AP-1.

Given the extensive work that has been done on the influence of pH in somatic cells, we might expect that alterations in pH_i will have important consequences for mammalian embryos and that different medium formulations that influence pH_i might enhance or reduce embryo development. Exposure of preimplantation embryos to culture conditions that result in the cytoplasm becoming either weakly acidic or weakly basic causes significant reductions in the ability of the embryos to develop in culture[273, 278, 279, 341]. Furthermore, exposure of two-cell embryos to either acidic or basic conditions results in disruptions in mitochondrial distribution and microfilament organization which in turn are correlated with perturbed development in culture[341-343]. Embryonic stem cells could also be profoundly influenced by changes in external pH.

1.9 Mouse ESC as a Model System

Although yeasts, worms and flies are excellent models for studying the cell cycle and many developmental processes, mice are far better tools for probing the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share. Like humans and many other mammals, mice naturally develop diseases that affect these systems, including cancer, atherosclerosis, hypertension, diabetes, osteoporosis and glaucoma. In addition, certain diseases that afflict humans but normally do not strike mice, such as cystic fibrosis and Alzheimer's, can be induced by manipulating the mouse genome and environment. Adding to the mouse's appeal as a model for biomedical research is the animal's relatively low cost of maintenance and its

ability to quickly multiply, reproducing as often as every nine weeks. A substantial proportion of scientific breakthroughs has been as a result of experiments conducted on living mice, their organs or cells. Both the human[344] and the mouse[345, 346] genomes have been sequenced and comparative maps illustrate the high degree of homology between the two genomes. It is reported that, although the mouse genome is 14% smaller than the human genome, over 90% of the two genomes can be partitioned into regions of conserved synteny and both contain approximately thirty thousand genes[346].

The mouse is an ideal model organism for human disease. Not only are mice physiologically similar to humans, but a large genetic reservoir of potential models of human disease has been generated through the identification of >1000 spontaneous, radiation- or chemically induced mutant loci. In addition, a number of recent technological advances have dramatically increased our ability to create mouse models of human disease. These technological advances include the development of high resolution genetic and physical linkage maps of the mouse genome, which in turn are facilitating the identification and cloning of mouse disease loci. Furthermore, transgenic technologies that allow one to ectopically express or make germ-line mutations in virtually any gene in the mouse genome have been developed, as well as methods for analyzing complex genetic diseases. Further, to date, >100 mouse models of human disease where the homologous gene has been shown to be mutated in both human and mouse have been developed. In the vast majority of these models, the mouse mutant phenotype very closely resembles the human disease phenotype and these models therefore provide valuable resources to understand how the diseases develop and to test ways to prevent or treat these diseases. However, in certain cases, the knockout mice may reproduce only some of the human disease phenotype, may be more severely affected than human cases, or may have no clinical phenotype at all. Under these circumstances, the disease pathology can become more complex, causing the researcher to evaluate basic differences in mouse and human biology as well as questions of genetic background, alternate pathways, and possible gene interactions. Similarly, for many human infectious diseases (e.g., HIV) despite the use of immunodeficient severe combined immunodeficiency (SCID) mice, the mouse remains, at best, an imperfect model to study the disease

pathogenesis. Some would, therefore, argue that human variation and complex diseases can be studied and modeled only in our own species.

Certainly there are common human diseases that do not occur naturally in the mouse, such as mental illness, autism, and Alzheimer's. However, using technologies to genetically alter their genomes, researchers have created mice that recapitulate many, but not all, of the features of human Alzheimer's[347]. Using detailed behavioral phenotyping and standardized tests, investigators are defining traits in the mouse that may be equivalent to human mental illness and behavioral disorders[348]. An advantage of many of these mouse models is the ability to dissect out parts of a complex human disorder and study the contributions of individual components to the complete phenotype. The influence of environmental factors on a genetic trait can be studied under controlled conditions in the mouse. This characteristic is extremely important in cancer, where effects of diet and chemical exposures on an underlying genetic predisposition may be difficult to discern. Investigators have discussed how the genetic resources of inbred mouse strains with varying cancer susceptibilities, recombinant inbreds, consomics, and congenics can be applied to study human cancer or other complex traits[349]. Thus, it may be concluded that, mouse models are useful in many ways and similar to human diseases and this similarity allows the mouse to be a good model system for human biomedical research.

The field of stem cell research has benefited greatly by having access to all stages of the developing mouse embryo. A considerable amount of knowledge regarding human hematopoietic stem cells, for example, has been garnered from studying the fetal murine bone marrow and liver. Mice have also been used in gene therapy applications to prove that inserted genes are functioning properly, that they do not have a deleterious effect on the organism and that they correct the phenotypic defect[350]. Mouse models of other human diseases such as Parkinson's and Alzheimer's have also been developed. mESC are readily available and their responses can be characterized by both phenotypic and functional assays. Since mice have been extensively used in biomedical research as an acceptable model for humans and murine embryonic stem cells have been extensively

studied and characterized, they will be used in this study to develop an understanding of how culture variables influence the maintenance of stem cell potential.

1.10 Thesis Objectives

Extensive research is rapidly developing our understanding of the molecular control of proliferation and differentiation of stem and progenitor cells. These advances have given hope for the cellular therapy of various disorders for which no efficacious treatments currently exist. In sharp contrast, related bioengineering developments are still in their infancy. The available tissue culture technology for primary cells is limited and scale-up capability is little explored. Yet many of the promises of new treatments cannot be realized unless a transplantable number of cells can be generated and manipulated *ex vivo*. Recently bioengineers have started developing protocols for large-scale expansion and maintenance of neural[351-354] and mouse and human embryonic stem and derived cells[355-359]. However, growing cells in a large bioreactor is a complex task. Protocols would have to be developed to maintain the expansion of cells for an extended period of time. Ranges of different culture variables have to be determined within which such cultures could be performed. The dynamics of large-scale cultures are entirely different from those of the well plates or T-flasks that are commonly used to grow these cells in the laboratory. The ability to adequately provide oxygen to cells often becomes a key issue. Furthermore, the selection of an appropriate growth medium, concentration gradients of pH and metabolites, shear stress, growth factor requirements, etc., become important factors in the design and successful operation of these large-scale bioreactors. The bioreactor microenvironment may therefore be completely different than that found *in vivo*. The challenge is to increase the stem cell content without compromising their developmental potential.

From the information presented thus far, it is evident that both mouse and human ESC can be cultured in the lab while maintaining their developmental properties. It is also becoming clear that these cells may play a crucial therapeutic role in the treatment of various currently non-curable disorders in the near future. However in order to treat

potentially millions of patients, it will be necessary to develop methods to generate hESC in large quantities. To date, relatively few studies have been published highlighting the large-scale production of terminally differentiated cells from mouse ESC[355, 357, 360]. Only one study has so far reported on the maintenance of mouse ESC in a small-scale perfusion bioreactor[359]. The research presented in this thesis will examine the influence of environmental variables on the maintenance of stem cell potential and the ranges of these variables within which these cultures can be conducted in small- and potentially large-scale cultures. Specifically, this project is concerned with developing a quantitative understanding of cytokine dependent proliferation of cells using hematopoietic cell lines as a model system as well as developing an understanding of the impact such culture variables as basal medium composition, pH, osmolality, serum and serum replacement concentration can have on the maintenance of stem cell potential using mouse ESC as a model.

In order to be able to grow primary hematopoietic cells in large-scale bioreactors for transplantation and other applications, it is instructive to study how single and multiple cytokines and their concentrations will influence their proliferation. Primary hematopoietic cultures, in general, have various limitations: they are inherently heterogeneous in nature with a variety of different cell types differing in their differentiation and proliferative status present at any given time in the culture. These cultures are labor intensive and subject to donor-to-donor variability. Besides, they require three to four months to assess changes in their most primitive progenitor numbers (using the long term culture-initiating cell (LTC-IC) or competitive repopulation unit (CRU) assays). These issues posed major obstacles to the use of primary cells for initial experimental studies as well as mathematical model development. We therefore chose to use cytokine-dependent cell lines for initial studies since they are homogenous in nature, yield reproducible results, and their responses can be analyzed within days. The first specific objective of this project was to investigate the effect of two cytokines (IL-3 and GM-CSF) and their interactions on proliferation of hematopoietic cell lines TF-1 and MO7e as a function of cytokine concentration by means of dose-response experiments as well as to develop empirical mathematical models of cytokine-dependent growth of both

cell lines when cytokines are present alone and in combination. This work is described in Chapter 2.

The second specific objective of this project was to study the influence of various environmental variables on the proliferation (measured by the mean of growth rate) and maintenance of stem cell potential (using the EB formation assay) of the mESC lines, R1 and EFC. To this end, dose-response experiments were carried out to study the influence of the pH and osmolality of the medium (Chapter 3), as well as the basal medium composition and serum as well as serum replacement concentrations (Chapter 4) on the proliferation and maintenance of stem cell potential of mESC.

Table 1.1 Characteristic Features of Cytokines

- Cytokines are (glyco) proteins that function as signaling molecules with molecular weights in the range of 6 - 30 kDa. Many cytokines form higher molecular weight oligomers.
- Cytokines are potent and are active at nanomolar or even picomolar concentrations.
- Cytokines are not constitutively produced; various inducing stimuli regulate their production at the transcription or translational level.
- Cytokines exert their action via autocrine or paracrine mechanisms.
- Binding of cytokine to their cognate receptors is a high affinity process with a dissociation constant, K_d, in the range of $10^{-9} - 10^{-12}$ M.
- Cytokine actions may be classified as:

Pleiotropic:	The cytokine tends to have multiple target cells and multiple actions
Redundant:	The same effect may be produced by different cytokines
Synergistic:	Cellular response to two cytokines is greater than the sum of responses observed in the presence of each cytokine alone.

Table 1.2: Examples of hematopoietic cytokines and their actions

Cytokine	Molecular Wt. (kDa)	Origin	Receptor	Action
Epo	18-32	Kidney, fetal liver	EpoR; 507 amino acid polypeptide	Stimulation of CFU-E, proliferation and differentiation of BFU-E
G-CSF	19-25	Monocyte, macrophage	G-CSFR, 130 kDa high affinity oligomer	Proliferation and differentiation of neutrophils; used for mobilization of stem cells in peripheral blood (PB)
GM-CSF	22	Macrophage or endothelial cells	Low affinity α subunit (85 kDa); high affinity β subunit common to IL-3 and IL-5	Growth and differentiation of CFU-GM, CFU-Mk; activation of neutrophils, eosinophils and endothelial cells
IL-3	28	T cells, mast cells	Low affinity α subunit (70 kDa); high affinity β subunit common to GM-CSF and IL-5	Stimulation of CFU-GM, BFU-E, CFU-Mk
IL-6	26	T cells, B cells, monocytes, endothelial cells	IL-6R; low affinity α chain (80 kDa); gp130 (β sub unit) common to other family members	Proliferation and differentiation of CFU-GM, BFU-M, CFU-Mk
SF	20-35	Marrow stromal, fibroblasts	c-Kit, 145 kDa, intrinsic tyrosine kinase	Stimulation of BFU-E, CFU-GM, pluripotent stem cells as well as leukemic cells

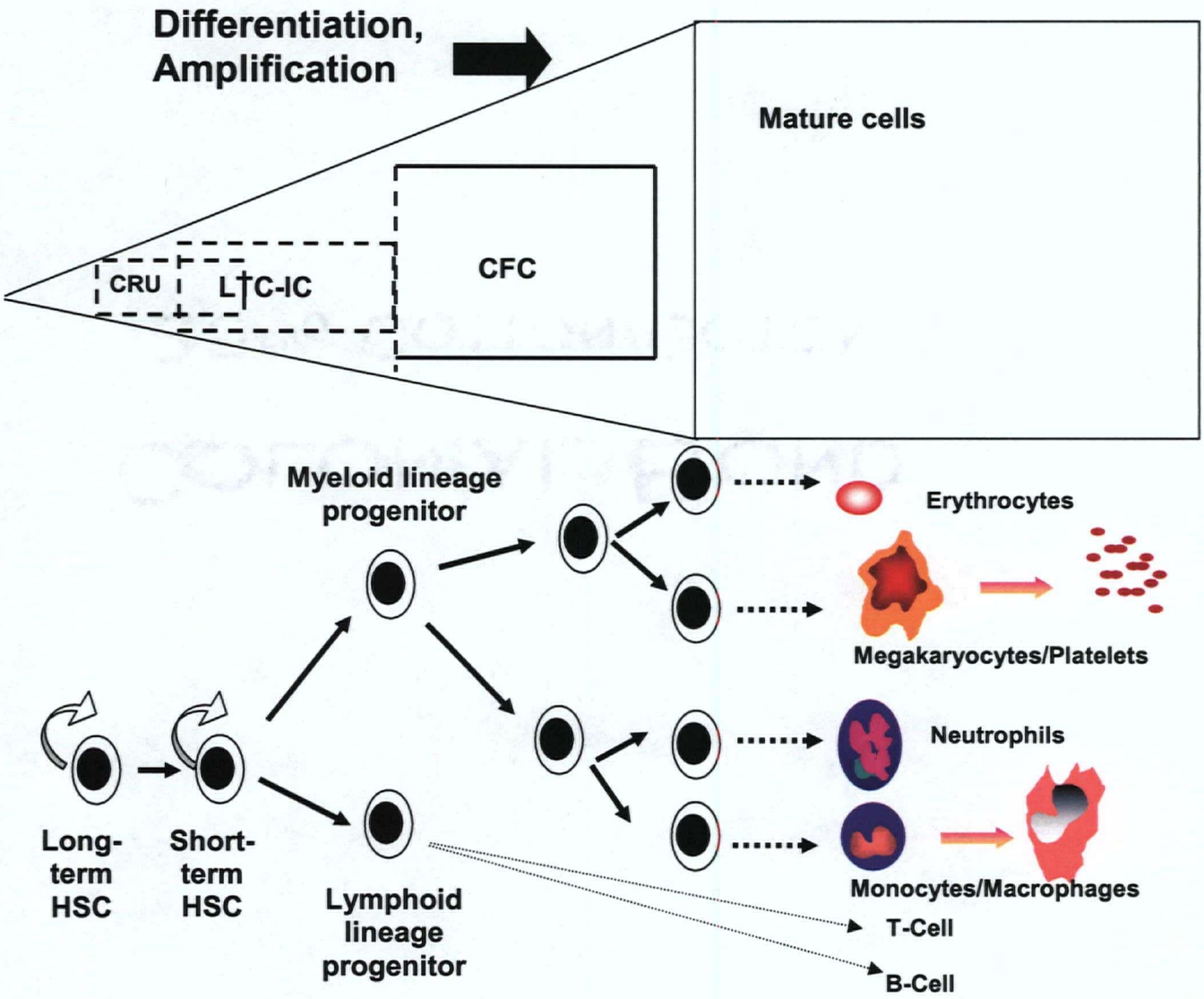

Figure 1.1 **Hematopoietic hierarchy.**

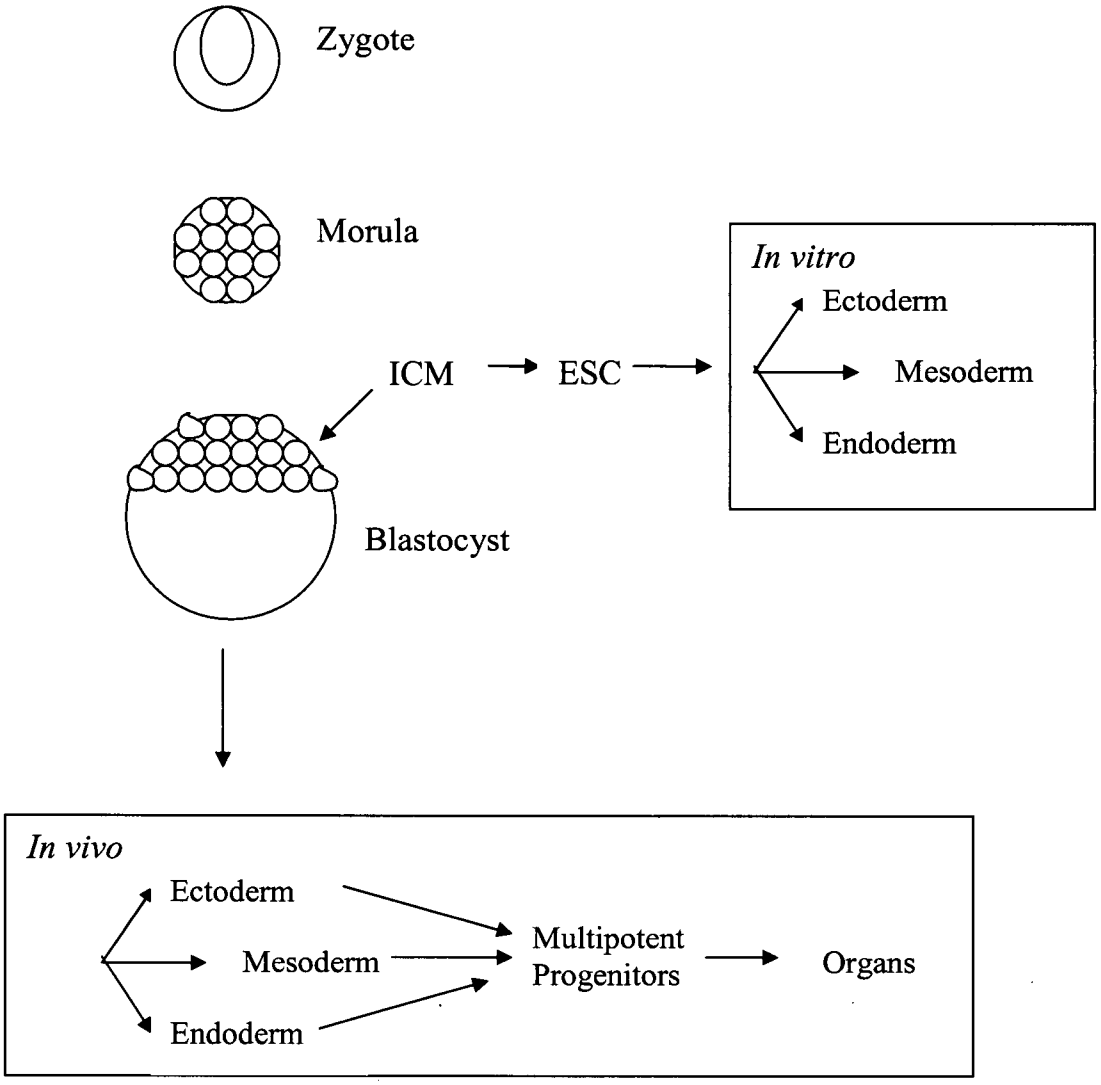

Figure 1.2 **Stem cell hierarchy. Adopted from Wobus et al. (2005) Physiol Rev 85, 635-678.**

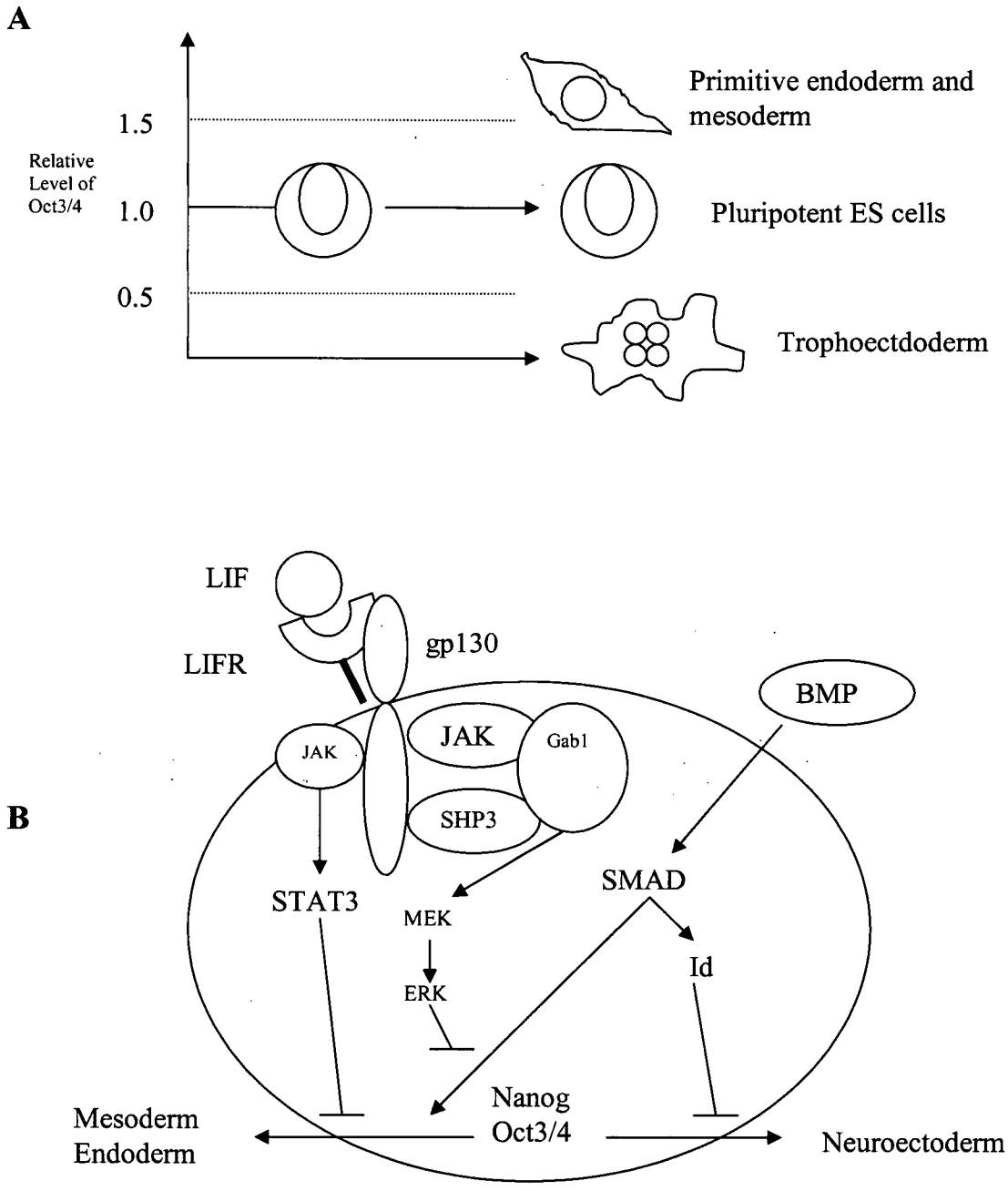

Figure 1.3 Regulation of self-renewal of mESC by Oct-3/4, Nanog, BMP-dependent SMAD and LIF dependent JAK/STAT3 signaling pathways. Adopted from Wobus et al. (2005) Physiol Rev 85, 635-678.

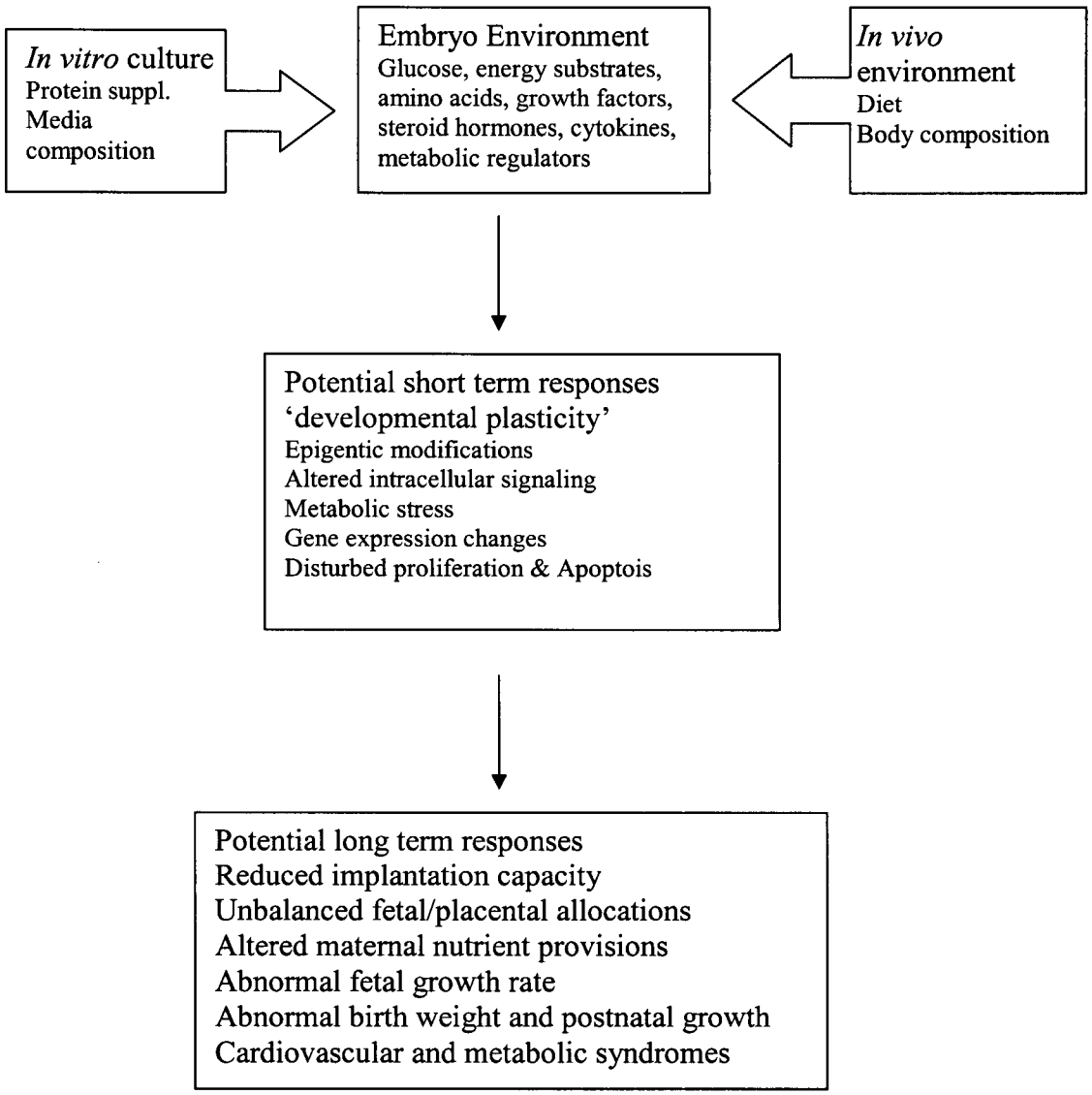

Figure 1.4 Schematic representing the potential interactions between the environment of the embryo, either *in vitro* or *in vivo*, the embryo's short-term responses, and their long-term consequences. Adopted from Fleming et al.(2004) Biol Reprod 71, 1046-1054..

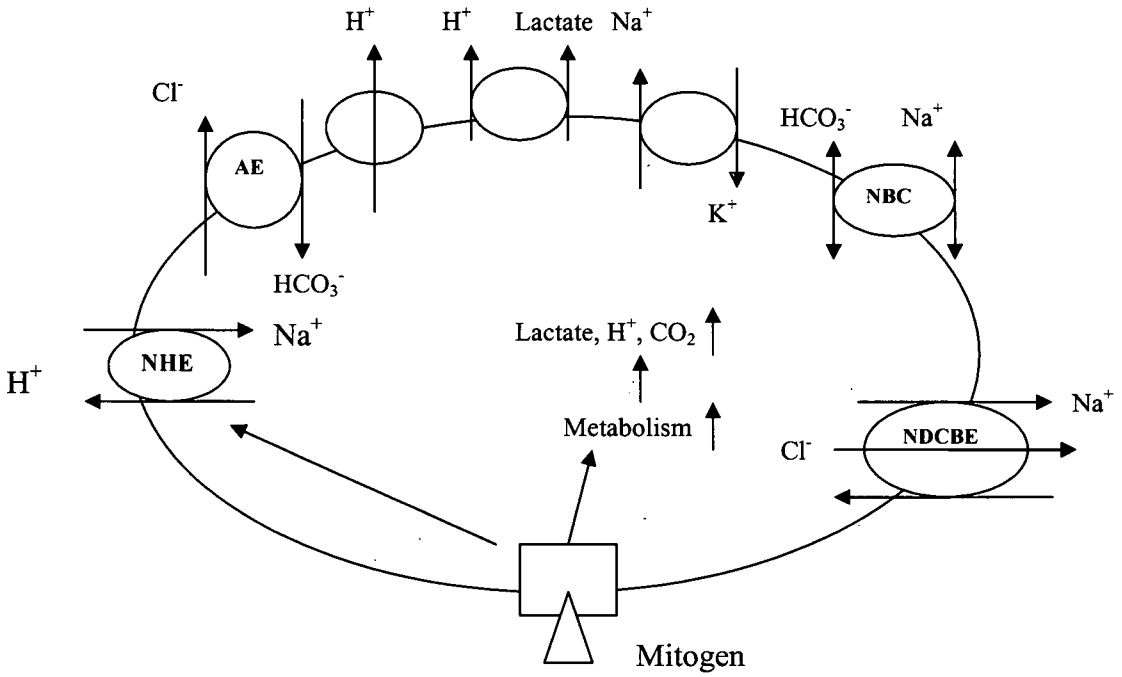

Figure 1.5 Summary of the main acid/base transporters of mammalian cells. Receptor activation by mitogens induces activation of the Na^+/H^+ exchanger (NHE) leading to alkalanization of the cytosol. The transporter H^+-ATPase, NHE, lactate$^-$-H^+ symporters, Na^+/HCO_3^- cotransporter (NBC), Cl^-/HCO_3^- exchanger (AE) and Na^+ dependent Cl^-/HCO_3^- exchanger (NDCBE) all are involved in regulation of pH_i. Adopted from Schreiber (2005) J Membr Biol 205, 129-137.

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2 Empirical Models of the Proliferative Response of Cytokine Dependent Hematopoietic Cell Lines[a]

2.1 Introduction

Identifying conditions that support the *ex vivo* expansion of human hematopoietic stem cells without compromising their developmental potential remains a challenging task. Recently, the use of engineering principles to analyze and optimize various culture parameters has made significant contributions towards achieving this goal[1-5]. However, many aspects of these processes are still poorly understood. A central role in this regulatory process is played by cytokines that are critical mediators of cellular proliferation, differentiation and survival. Many of the cytokines required by stem cells have now been cloned and purified to homogeneity. For therapeutic culture processing involving stem and progenitor cells, optimization of likely complex cytokine cocktails will be needed to derive the most consistent and effective use of these valuable materials. In order to optimize *ex vivo* expansion protocols, there is a need for more quantitative information about how changing cytokine concentrations alter primitive hematopoietic cell proliferation and differentiation responses.

Although substantial progress has been made in elucidating the molecular basis of cell growth regulation, there has been limited development of reliable mathematical models that predict the growth response of cells to mitogenic cytokines. Probably the simplest and most widely used approach in this regard has been to apply the 2-parameter empirical Monod model to describe the growth rate dependence of cells on the concentration of serum[6] or insulin[7] added to the medium.

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Other specific cytokine effects that have been modeled include the interleukin-2 (IL-2)-stimulated proliferation of T cells[8] and the epidermal growth factor (EGF)-stimulated proliferation of fibroblasts[9]. Cantrell and Smith[8] proposed a conceptual model for the cell cycle progression of T cells that accounted for the observed effects of changes in IL-2 concentration on the IL-2 receptor density and the duration of receptor activation. Their study suggested that a threshold number of ligand-receptor interactions must occur before quiescent T cells will initiate DNA synthesis. Knauer et al.[9] developed a mechanistic model that relates fibroblast proliferation in response to EGFR binding by EGF. Assuming a simple reversible bimolecular receptor-ligand interaction, a first-order internalization rate and negligible recycling, they derived an expression relating the total number of occupied receptors to EGF uptake. Again it was proposed that the mitogenic response depended on a minimum threshold of receptor occupancy followed by a simple linear increase in the probability of activating DNA synthesis according to the number of signaling complexes formed. When the maximum number of cells that could be stimulated to enter the S-phase was achieved, a ceiling was reached. Lauffenburger and coworkers[10-12] further extended this model by including the hypothesis that the mitogenic activity of EGF-EGFR binding on the cell surface will be attenuated by receptor down-regulation or depletion of the ligand from the medium by cellular internalization and degradation.

There are many complex mathematical models in the theoretical biology and biochemical engineering literature[13]. However, most practicing biologists and biochemical engineers have not found these to be very useful. A major practical obstacle has been to obtain the large number of parameters required for each new cell population and each new cell environment. Most often these parameters are determined by using pooled data from different cell types, and there have been only a few efforts to critically test the predictive capability of such complex models[12, 14].

Microbiologists as well as environmental engineers have extensively used unstructured models to describe the growth of microorganisms on a limiting substrate. Given the simplicity of these empirical models, it is a concern that they may not adequately capture

the complexity of biological responses. Recently, however, we have shown how systematic experimentation and empirical modeling can greatly simplify the analysis of multiple interacting cytokine effects on a mixed cell culture system[15]. A central composite experimental design was used to measure the concentration effects of three cytokines on murine hematopoietic stem, progenitor and total cell numbers generated by 14-day serum-free cultures. These data were used to derive both polynomial and modified Monod models that fit the results for all three populations. The common models for the different cell types greatly simplified the perceived complexity of the multiple interacting cytokines in mixed culture systems by revealing characteristic qualitative trends. The model parameters varied considerably between the cell types providing a coherent view of the quantitative differences between their responses.

IL-3 and GM-CSF are members of the type I cytokine superfamily and their receptors consist of a ligand-specific, but low-affinity α-chain, and a common β_c-chain to which IL-3 and GM-CSF bind competitively when complexed with their α–chains[16,17]. IL-6 is a member of a different cytokine family that utilizes gp130 as a common signaling element in the receptor complex. Thus, at the cell surface, interactions between IL-3 and GM-CSF receptors would be expected whereas interactions of either of these cytokines with IL-6 receptors would not be predicted.

Given recent progress in the field of *ex vivo* expansion of hematopoietic stem and progenitor cells, it has become increasingly important to develop predictive semi-empirical models that can assist with the analysis of such complex systems for clinical cell cultures. To investigate the interactions of different cytokines and their influence on hematopoietic cells, we have selected a relatively simple biological system provided by two immortal, but cytokine-dependent hematopoietic cell lines: MO7e and TF-1 cells. Because of their stability as relatively homogeneous populations, these cell lines are widely utilized for bioassays of various cytokines and as model systems to investigate mitogenic signal transduction pathways. The central objective of this work was to develop practical predictive models for the growth rates of these cells as a function of

cytokine doses and interactions. By not taking into account the complex details of ligand binding and intracellular signaling, much simpler models having only a limited number of parameters could be formulated. These relatively simple models, developed in terms of readily measurable variables, should provide a foundation for future empirical or mechanistic additions that may include cytokine dependent self-renewal, differentiation and apoptosis.

2.2 Materials and Methods

2.2.1 Cell Lines

MO7e cells were originally derived from a patient with acute megakaryocytic leukemia [18] while TF-1 cells were derived from a patient with severe pancytopenia[19]. Both lines were maintained in Iscove's medium (IMDM, Invitrogen Life Technologies, Burlington, ON) with 5% fetal calf serum (FCS) (Invitrogen) and 5 ng/mL of either human IL-3 or human GM-CSF (gifts from Novartis, Basel, Switzerland). Both cell lines were grown in the presence of various concentrations of IL-3 and GM-CSF alone as well as in combination such that the ratio [IL-3]/[GM-CSF] was kept constant at 2, 3.5 and 10.5 respectively to cover a relatively wide range of these cytokines. TF-1 cells were also grown in the presence of various concentrations of human IL-6 alone (gift from Novartis, Basel, Switzerland) as well as in combination with IL-3 or GM-CSF.

2.2.2 Cell Proliferation Assay

Exponentially growing MO7e or TF-1 cells were harvested from T-flasks and washed twice in IMDM + 5% FCS by centrifuging and resuspending in fresh medium to remove residual cytokines from the inoculum culture, and incubated overnight without any cytokine additions. Fifty μL of cells suspended in IMDM + 5% FCS (at a final concentration of 5×10^4 cells mL^{-1}) were then added to an equal volume of the same medium containing the desired concentrations of IL-3, GM-CSF and IL-6 (alone or in combination as described above) in triplicate individual wells of a 96-well microtitre plate. The cultures were incubated for 72 or 120 h at 37°C in a humidified atmosphere of 5% CO_2. At the end of the incubation period, 20 μL of MTS/PMS solution (Promega, Madison, WI) was added to all the wells and the plate was incubated for another hour prior to measuring the absorbance at 490 nm using a micro-plate reader (Molecular Devices, Sunnyvale, CA). The absorbance values were converted to viable cell concentrations using appropriate standard curves prepared for either the TF-1 or MO7e

exponentially growing cells. The average specific growth rate ($\overline{\mu}$) was calculated using the equation:

$$\overline{\mu} = \frac{\ln\left[X_v / X_o\right]}{\Delta t} \tag{2.1}$$

where X_v is the viable cell concentration (cells/mL) at the end of the growth period (72 or 120 h), X_o is the inoculum viable concentration (cells/mL) and Δt is the time interval (h).

2.2.3 Cytokine Depletion Analysis by ELISA

Human IL-3 and GM-CSF concentrations were determined using sandwich enzyme-linked immunosorbent assays (ELISA)[20] with capture and detection antibodies against human IL-3 and GM-CSF from BD Biosciences (Mississauga, ON).

2.2.4 Calculation of Cell Specific Cytokine Uptake Rate

The average cell specific cytokine uptake rate, q_L (pg/h-10^6 cells), was calculated from:

$$q_L = \frac{\Delta L}{\Delta t \left(\frac{X_v - X_o}{\ln\left(X_v / X_o\right)} \right)} \times 10^6 \tag{2.2}$$

where ΔL is the change in cytokine concentration (pg/mL), Δt is the time interval (h) and X_v and X_o are the final and initial cell concentrations (No. of cells/mL) respectively.

2.2.5 Mathematical Modeling of Cytokine Dependent Proliferation

2.2.5.1 Initial Single Cytokine Model

Both MO7e and TF-1 cells exhibit a saturation-type of population growth kinetics as a function of cytokine concentration. Hence, the following Monod-type equation was initially employed to model the average specific growth rate of these cell lines as a function of the cytokine concentration.

$$\bar{\mu} = \frac{\mu_{sat} L}{K_L + L} \tag{2.3}$$

where μ_{sat} (h^{-1}) is the average specific growth rate when the cytokine concentration approaches saturation; L is the initial concentration of cytokine (ng/mL) and K_L is the average apparent half-saturation constant (ng/mL). The statistical software package, R, (http://www.r-project.org/) was employed to determine the kinetic parameters, μ_{sat} and K_L, using a nonlinear least-squares method.

2.2.5.2 Improved Single Cytokine Model

The Monod-type model, Eq. (2.3), showed a consistent and systematic lack of fit to the growth rates measured at intermediate and high cytokine concentrations. The following Hill-type function was therefore tested next:

$$\bar{\mu} = \frac{\mu_{sat} L^n}{K_L^n + L^n} \tag{2.4}$$

where n is Hill's exponent. When initially developed, the Hill equation sought to describe the binding of oxygen to hemoglobin[21]. However, it is now widely regarded as an empirical description of complex binding phenomena. The Hill coefficient, n, describes the shape of the curve. When $n > 1$, the curve shows a sigmoidal behavior with $n > 5$ representing a step change in response. When $n < 1$, the curve exhibits a more gradual rise to the maximum value. Hill's function reduces to the Monod model Eq. (2.3) when $n = 1$. All of the parameters of Eq. (2.4) were obtained via nonlinear regression analysis as described above.

2.2.5.3 Cytokine Interaction Models

Few attempts to mathematically model cytokine interactions have been reported, although there is considerable experience with modeling drug interactions in the pharmaceutical field[22]. However, the methods used are diverse and can predict very different results when applied to the same set of data, such that a given drug combination may be synergistic according to one method and antagonistic according to another[22]. In the work reported here, we define a synergistic interaction as one where "the effect of the combination is greater than the sum of the effects of its individual constituents". It is important to note that, although numerous articles have reported synergistic effects of cytokines on hematopoietic cells in culture[23-25], many of the results obtained do not in fact satisfy this definition.

To model the growth rates observed in the presence of two cytokines, two independent approaches were adopted. First, the following mathematical relationship, developed from an adaptation of a pharmacodynamic model[26], was used to accommodate the possibility of synergistic cytokine interactions:

$$\bar{\mu} = \frac{\mu_{sat\,IL3}\, L_{IL3}^{n}}{K_{IL3}^{n} + L_{IL3}^{n}} + \frac{\mu_{sat\,GM}\, L_{GM}^{m}}{K_{GM}^{m} + L_{GM}^{m}} + \gamma \left(\frac{\mu_{sat\,IL3}\, L_{IL3}^{n}}{K_{IL3}^{n} + L_{IL3}^{n}} \frac{\mu_{sat\,GM}\, L_{GM}^{m}}{K_{GM}^{m} + L_{GM}^{m}} \right) \qquad (2.5)$$

where γ is an interaction parameter. $\gamma > 0$ indicates a synergistic interaction as the overall growth rate becomes greater than the sum of the growth rates obtained with single cytokines. $\gamma < 0$ implies an overlapping of the effects of the two cytokines. The value of γ was determined by minimizing the sum of squared differences between the experimental and predicted growth rates using the "Solver" function in Excel 2000 (Microsoft, Redmond, WA).

The second modeling approach was based on an analogy to the situation where the presence of one substrate inhibits the utilization of another[27]. Given that IL-3 and GM-CSF bind to a

common β_c chain and a competition between IL-3 and GM-CSF for β_c has been observed experimentally[28, 29], the following competitive model was developed:

$$\bar{\mu} = \frac{\mu_{sat\,IL3}\, L_{IL3}^{n}}{K_{IL3}^{n} + L_{IL3}^{n} + \alpha_2\, L_{GM}^{m}} + \frac{\mu_{sat\,GM}\, L_{GM}^{m}}{K_{GM}^{m} + L_{GM}^{m} + \alpha_1\, L_{IL3}^{n}} \tag{2.6}$$

where $\alpha_2 = K_{IL3}^{n} / K_{GM}^{m}$ *and* $\alpha_1 = 1/\alpha_2$.

Here, the third term in each denominator predicts the competitive influence of another cytokine based on the ratio of K_L values obtained from the single cytokine responses. This model is fully predictive in the sense that all of the parameters can be determined from independent measurements in single-cytokine experiments.

2.3 Results

2.3.1 Dose-Response Analysis of MO7e and TF-1 cells

Dose-response experiments were performed with MO7e and TF-1 cell lines in the presence of IL-3, GM-CSF and IL-6. Since the two cell lines exhibited similar responses, primarily the results for the TF-1 cells are shown.

Figure 2.1 compares the experimentally determined average growth rates of TF-1 cells with the results predicted by Eqs. (2.3) and (2.4) for various doses of IL-3, GM-CSF or IL-6. It can be seen that the Hill-type model, Eq. (2.4), provided a better fit to the measured growth rates for both the 72 h and 120 h experiments than the Monod-type model, Eq. (2.3). The Monod-type model overestimated the growth rates at intermediate cytokine concentrations, underestimated the growth rates at low and high cytokine concentrations and gave a 5 to 200 times higher χ^2/DOF goodness-of-fit statistics (Table 2.1). The improved fit of the Hill equation was due to the additional parameter, n. However, for TF-1 cells, the fit was not significantly altered when the parameter n was kept constant at a value of 0.5 (i.e., approximately the average n determined for TF-1 cells in response to IL-3, GM-CSF and IL-6 for both the 72 h and 120 h time points) (Figure 2.2 and Table 2.1). The constant value of n, however, varied depending on the cell line and for MO7e cells at 72 h, $n \sim 1.57$ for GM-CSF and $n \sim 1.35$ for IL-3 (Table 2.2). Both of these values of n decreased by approximately 60% for the 120 h data.

Dose-response analyses also revealed that for the 72 h TF-1 cultures, the apparent half-saturation constant of GM-CSF was about two times lower than that of IL-3 (Table 2.2). For MO7e cells, the apparent half-saturation constant of GM-CSF was also consistently lower than that of IL-3. This suggests that GM-CSF is a more potent stimulator than IL-3 for both of these cell lines as has also been reported[30] where it was found that the doubling time of TF-1 cells grown in GM-CSF was significantly lower than that of cells grown in IL-3.

The MO7e apparent half-saturation constant for both IL-3 and GM-CSF also decreased with duration of the culture by 73% and 16%, respectively for IL-3 and GM-CSF between 72 h and 120 h and for TF-1 cells, by 84% and 83% respectively during the same interval.

Figure 2.3 shows the cell concentration and the cell-specific GM-CSF uptake rate for TF-1 cells cultured at various cytokine concentrations for 72 h. It can be seen that the uptake rate increased with increasing GM-CSF concentration in contrast to the growth rate that saturated quickly as a function of the cytokine concentration. These results show that cytokine uptake rate and cell proliferation are uncoupled processes in these cells. To check that GM-CSF was not degraded when incubated at 37°C or lost by adhesion to the tissue culture plastic, GM-CSF was in incubated in culture media for 120 hours at 37°C and the level of GM-CSF was found not to decrease (data not shown).

2.3.2 Determination of Cytokine Interactions

Figure 2.4 shows the growth rates of MO7e cells over a 72 h period when IL-3 and GM-CSF were present alone or together. The overall growth rate of cells exposed to IL-3 and GM-CSF in combination was less than the sum of the growth rates achievable with IL-3 or GM-CSF separately, i.e., the mitogenic activity of IL-3 and GM-CSF on this cell line was not synergistic.

Two models were developed to describe the interactions of the cytokines on the growth rates of MO7e and TF-1 cells, as described in the mathematical modeling section. As shown in Figure 2.5, Eq. (2.5) provided only a rough fit of the experimental data for TF-1 cells when IL-3 and GM-CSF were added in ratios of 2, 3.5 and 10.5 at 72 h (see Table (2.3) for χ^2/DOF values). However, the agreement of Eq. (2.5) with experimental data at 120 hour is reasonable. The value of the interaction parameter, γ, estimated to be between –23.7 to –37.3 for both IL-3 and GM-CSF for the 72 and 120 h data (Table 2.4), indicates a non-synergistic but overlapping effect of these two cytokines.

The competitive model (Eq. 2.6) provided a good fit (Table 2.3) of the experimentally observed growth rates of both cell lines as illustrated in Figure 2.6 for TF-1 cells cultured for 72 and 120 h with IL-3 and GM-CSF concentrations at ratios of 2, 3.5 and 10.5.

To test the wider applicability of this model for a case where receptor subunits are not shared, the interactive effects on TF-1 cells of a third cytokine, IL-6, with either IL-3 or GM-CSF were investigated. As is evident from the results shown in Figure (2.7a) for IL-3+IL-6 and in Figure (2.7b) for IL-6+GM-CSF, the cell growth rates observed did not fit the competitive model, Eq. (2.6), consistent with the different receptor specificities of IL-6.

2.4 Discussion

Analyses of the cytokine dose-responses of both MO7e and TF-1 cells to IL-3 and GM-CSF revealed that the Monod-type model did not fit the results obtained at intermediate and high cytokine concentrations but that this could be corrected by using the more general Hill-type model. In order to correct for the more gradual approach to saturation exhibited by the TF-1 cells, the values of n required were consistently ~ 0.5. However, values of $n > 1$ were required to fit the model to the 72 h MO7e dose-response data and these decreased to $n<1$ for the 120 h data where cells reached the declining growth phase of culture. The value of n is therefore cell line and cytokine specific and may vary with the culture conditions. It is remarkable that the Hill-type model continued to fit better than the Monod-type model even as growth rates were reduced by non-cytokine factors.

The dose response data and relative K_L values in Table 2.2 show that GM-CSF stimulated a higher growth rate of TF-1 cells than did the same concentration of IL-3. This may be due to a greater number of GM-CSF receptors on the surface of these cells. Radiolabeled ligand-receptor binding assays have indicated that, for TF-1 cells, the number of low affinity GM-CSF receptors (α-chain receptors) to be ~9000 per cell[31, 32], while the number of low affinity IL-3 receptors has been reported to be ~340 per cell[32]. The equilibrium dissociation constant for GM-CSF is 189 pM while that of IL-3 is 244 pM[33]. GM-CSF also appears to have a greater ability to recruit the β_c chain than IL-3[28, 34]. GM-CSF therefore appears to be a more potent growth stimulator for TF-1 cells than IL-3.

As the cytokine concentration was lowered, there was a concomitant decrease in the rate at which the population expanded. This may be due to insufficient stimulation to initiate entry into the S phase of cell cycle and/or initiation of apoptotic mechanisms.

Comparison of the 72 and 120 h dose-response data also revealed that the growth rate of TF-1 and MO7e cells decreased with culture duration as the cells passed from the exponential to the declining growth phase. This may also be partially due to cytokine

depletion as was shown to occur when TF-1 cells were cultured in a GM-CSF-containing medium. These decreases were most likely caused by the accumulation of inhibitory metabolites like lactate and ammonium and a decrease in pH.

An important conclusion indicated by the present studies is that proliferation and cytokine uptake appeared to be uncoupled processes. For instance, while cellular proliferation was observed to saturate quickly as the cytokine concentration was increased, cytokine uptake rate continued to increase dramatically. Evidence in the literature suggests the existence of a soluble form of the GM-CSF receptor α-chain (solGMRα)[35, 36]. The solGMRα is secreted by a variety of cell types including myeloid cell lines[37, 38], bone marrow progenitors, monocytes, and macrophages[39] and its secretion is upregulated by GM-CSF in normal human monocytes in a dose-dependent manner[39]. The solGMRα binds to GM-CSF and is able to interact with the common β_c-chain such that high affinity binding of solGMRα with β_c is indistinguishable from that of transmembrane GM-CSF receptor α-chain (trmGMRα) with β_c[40]. The addition of GM-CSF to TF-1 cells starved of cytokines for 24 h elicited either a marked increase[41] or virtually no change[42] in the expression of β_c, whereas there was a marked down-modulation of trmGMRα. Therefore, solGMRα may also be involved in the uptake of GM-CSF from the extracellular medium in these studies and thereby contributes to the uptake of GM-CSF at concentrations that have already reached the levels required to maximize the number of cells stimulated to proliferate.

These studies also revealed that the apparent half-saturation constant constants (K_L) of both cytokines varied with duration of culture and could depend on culture conditions. A decrease in K_L values would mean that lower cytokine concentrations are required to maximize growth over longer batch culture times – an observation that might have bioprocessing implications for the expansion and maintenance of progenitor and differentiating cells. However, in stem cell cultures, it has been shown that higher cytokine concentrations can be required for the maintenance of self-renewal and suppression of differentiation[43, 44]. The variation of K_L values could be explained on the basis of the increasing cell density per well such that the metabolic activity of reducing

MTS per individual cell decreased as has been previously observed[45]. These findings also signify a need for further investigations to determine the impact that bioprocess variables have on effective cytokine utilization. For example, additional experiments could be carried out to study how changes in the extra-cellular pH as well as a build-up of inhibitory metabolites like lactate and ammonia alter cytokine activity or cytokine-receptor binding affinity, and how conditioned medium affects cytokine stability.

The results reported here indicate that there is an overlapping interaction between IL-3 and GM-CSF as they affected the growth rates of the cell lines employed in this study. The expression pattern of IL-3 and GM-CSF receptors on TF-1 may therefore explain these observations. Since the number of GM-CSFR α-chains is far greater than that of IL-3R, as discussed above, at high initial concentrations of GM-CSF it is likely that most of the β_c chains are complexed with GM-CSFRα molecules allowing them to out-compete IL-3Rα molecules for the β_c chains. However, when the initial GM-CSF concentration is low, it is rapidly depleted from the culture medium thereby making the IL-3 molecules more competitive. A report by Gesner et al.[46] that GM-CSF competition with IL-3 on KG-1 cells was concentration-dependent supports this view.

Multiple cytokines are often simultaneously administered to hematopoietic as well as tissue engineering cultures to stimulate cell survival, proliferation and differentiation responses. These cytokines can interact in synergistic, overlapping or antagonistic ways. The empirical modeling approach using Hill's function was extended to address the cytokine interaction and its influence on the growth rates of the cell lines used in the study. The additive interaction model developed agreed well with the experimental data obtained at three different [IL-3]/[GM-CSF] ratios for 120 h cultures of TF-1 cells. The competitive model developed has the advantage that it is based on parameters obtained from single cytokine measurements and hence can 'predict' the outcome when both cytokines are simultaneously present. The model was tested at various IL-3 to GM-CSF concentration ratios and found to predict the experimentally observed results. Also when parameters (α_1 and α_2) were obtained from a particular IL-3 to GM-CSF concentration ratio in the single cytokine experiments, they could be used to predict the results obtained at a different IL-3 to GM-

CSF concentration ratio (data not shown). As expected, the competitive model did not fit the growth response data obtained in the presence of IL-3 and IL-6 or GM-CSF and IL-6, where these cytokine pairs do not share receptor subunits.

These cytokine interaction models are not mechanistic and do not provide detailed information about ligand-receptor interactions or intracellular signaling processes. However, this approach does has the advantage that it depends only on extracellular parameters that can be relatively easily measured and does not necessitate the determination of an impractically large number of parameters, as is required by many mechanistic cellular models.

2.5 Conclusions

Models of hematopoietic cell growth rate dependence on medium cytokine additions have been developed. An empirical approach was adopted because it depended only on extracellular parameters that can be relatively easily measured, compared to much more complex mechanistic cellular models. Interestingly, a Monod-type model was poorly fit to IL-3, IL-6 or GM-CSF dose-response data. Instead, it was proposed that a Hill-type relationship provides a more appropriate model, although it requires one additional parameter. When IL-3 and GM-CSF were added together, it was shown that their effects on growth did not interact synergistically or even additively. A competitive model was developed by modifying the Hill-function to include the competition between IL-3 and GM-CSF for their common receptor (β_c) subunit. This model had no new parameters beyond those obtained from the single cytokine cultures and provided good prediction of the growth rates for both cell lines when they are exposed to combinations of IL-3 and GM-CSF, over a wide range of concentration. The competitive receptor system provided a clear rationale for the predictive model developed, suggesting that this modeling approach may be effective for a wide range of other shared receptor subunit systems. Although empirical, these Hill's and competitive mathematical models both could be used to quantitatively predict the proliferative responses of hematopoietic cells to a wide range of cytokine stimulation.

Table 2.1 χ^2/DOF statistics for goodness-of-fit test comparing Monod-type model Eq. (2.3), Hill-type function Eq. (2.4) with exponent '*n*' fixed (*n* = 0.5) as well as Hill function with variable exponents against experimental growth rates of TF-1 cells grown in the presence of IL-3, IL-6 or GM-CSF alone.

χ^2 / DOF			
Cytokine	**Monod Model**	**Hill function (n = variable)**	**Hill function (n = fixed at 0.5)**
		72 h	
IL-3	1.3×10^{-4}	2.0×10^{-6}	9.7×10^{-6}
IL-6	4.4×10^{-5}	4.1×10^{-6}	4.1×10^{-6}
GM-CSF	2.8×10^{-4}	2.6×10^{-6}	2.9×10^{-5}
		120 h	
IL-3	4.8×10^{-5}	9.7×10^{-7}	9.6×10^{-7}
IL-6	5.4×10^{-5}	2.9×10^{-6}	5.2×10^{-6}
GM-CSF	1.7×10^{-4}	7.9×10^{-7}	8.2×10^{-7}

Table 2.2 **Hill model kinetic parameters for cell lines, TF-1 and MO7e cells growing in IL-3, IL-6 and GM-CSF[a]**

	IL-3						GM-CSF					
	72 h			**120 h**			**72 h**			**120 h**		
Cell Line	μ_{sat} h^{-1}	K_L ng/mL	n (-)	μ_{sat} h^{-1}	K_L ng/mL	n (-)	μ_{sat} h^{-1}	K_L ng/mL	n (-)	μ_{sat} h^{-1}	K_L ng/mL	n (-)
TF-1	0.044 ± 0.003	0.040 ± 0.002	0.40 ± 0.06	0.028 ± 0.004	0.0062 ± 0.0007	0.48 ± 0.02	0.045 ± 0.003	0.017 ± 0.004	0.68 ± 0.05	0.030 ± 0.003	0.0031± 0.0004	0.49 ± 0.03
MO7e	0.039 ± 0.004	0.84 ± 0.02	1.35 ± 0.05	0.024 ± 0.003	0.23 ± 0.02	0.50 ± 0.04	0.042 ± 0.005	0.092 ± 0.003	1.57 ± 0.02	0.036 ± 0.003	0.077 ± 0.002	0.62 ± 0.07

	IL-6					
	72 h			**120 h**		
Cell Line	μ_{sat} h^{-1}	K_L ng/mL	n (-)	μ_{sat} h^{-1}	K_L ng/mL	n (-)
TF-1	0.026 ± 0.004	0.09 ± 0.005	0.49 ± 0.03	0.018 ± 0.004	0.364 ± 0.02	0.57 ± 0.06

[a] the values are mean ± std. dev of three independent experiments

Table 2.3 **χ^2/DOF statistics for goodness-of-fit test comparing the two cytokine interaction models against experimental growth rates of TF-1 cells grown in the presence of combination of IL-3, IL-6 and GM-CSF.**

Cytokine Interaction Model	Cytokine Conc. Ratio	χ^2 /DOF
	72 h	
Equation (2.5)	[IL-3]/[GM-CSF] = 2	4.98×10^{-4}
Equation (2.5)	[IL-3]/[GM-CSF] = 3.5	2.69×10^{-4}
Equation (2.5)	[IL-3]/[GM-CSF] = 10	3.03×10^{-4}
Equation (2.6)	[IL-3]/[GM-CSF] = 2	1.77×10^{-4}
Equation (2.6)	[IL-3]/[GM-CSF] = 3.5	5.4×10^{-5}
Equation (2.6)	[IL-3]/[GM-CSF] = 10	7.60×10^{-5}
Equation (2.6)	[IL-6]/[IL-3] = 8	5.30×10^{-3}
Equation (2.6)	[IL-6]/[GM-CSF] = 16	8.97×10^{-4}
	120 h	
Equation (2.5)	[IL-3]/[GM-CSF] = 2	2.37×10^{-4}
Equation (2.5)	[IL-3]/[GM-CSF] = 3.5	8.80×10^{-5}
Equation (2.5)	[IL-3]/[GM-CSF] = 10	1.03×10^{-4}
Equation (2.6)	[IL-3]/[GM-CSF] = 2	1.13×10^{-4}
Equation (2.6)	[IL-3]/[GM-CSF] = 3.5	2.83×10^{-5}
Equation (2.6)	[IL-3]/[GM-CSF] = 10	4.03×10^{-5}
Equation (2.6)	[IL-6]/[IL-3] = 8	6.63×10^{-4}
Equation (2.6)	[IL-6]/[GM-CSF] = 16	5.20×10^{-4}

Table 2.4 **The values of interaction parameter γ computed to compare the additive interaction model (Equation 2.5) against experimental growth rates of TF-1 cells grown in the presence of combination of IL-3 and GM-CSF.**

Cytokine Conc. Ratio	γ (h)
72 h	
[IL-3]/[GM-CSF] = 2	-23.70
[IL-3]/[GM-CSF] = 3.5	-25.65
[IL-3]/[GM-CSF] = 10	-24.79
120 h	
[IL-3]/[GM-CSF] = 2	-36.46
[IL-3]/[GM-CSF] = 3.5	-37.33
[IL-3]/[GM-CSF] = 10	-28.0

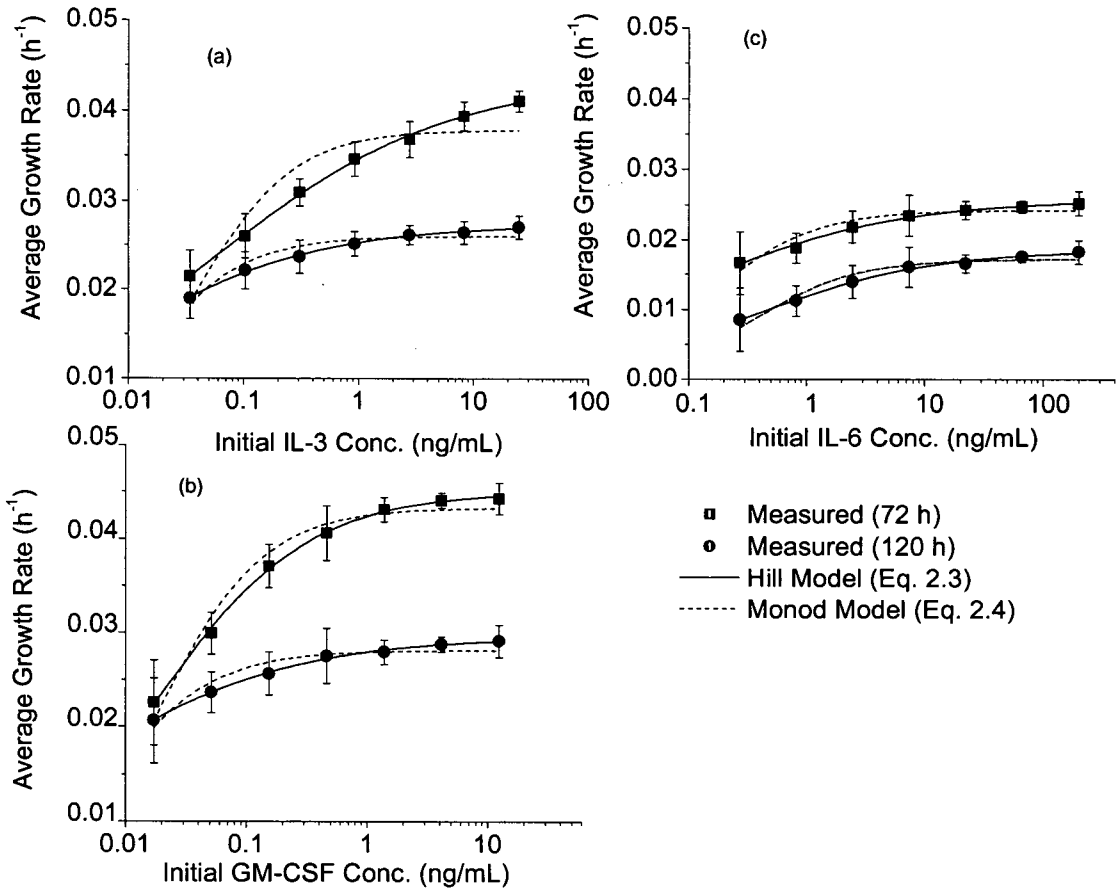

Figure 2.1 **Comparison of the growth rate of TF-1 cells cultured for either 72 or 120 h in the presence of (a) IL-3, (b) GM-CSF and (c) IL-6 with predictions of Monod- and Hill-type models. Data are mean ± standard deviation of three independent experiments. Legend applies to all three panels.**

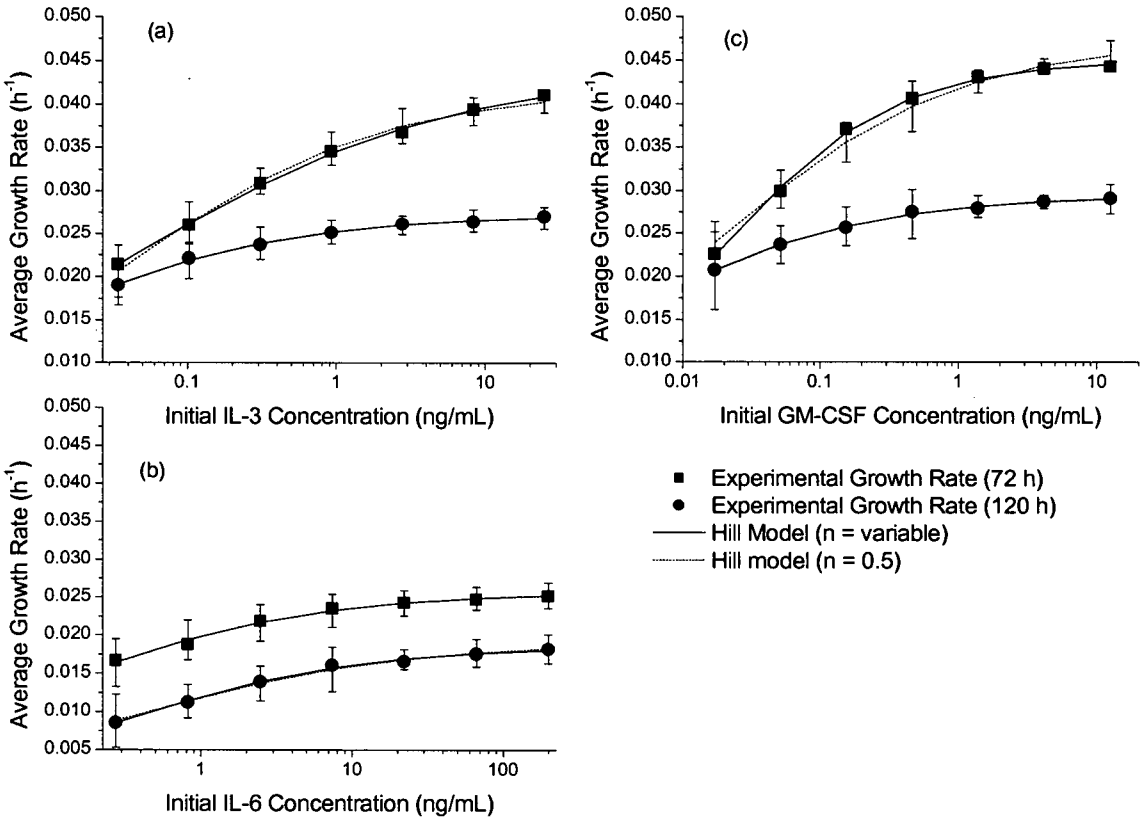

Figure 2.2 **Comparison of the growth rate of TF-1 cells cultured for either 72 or 120 h in the presence of (a) IL-3, (b) GM-CSF and (c) IL-6 with prediction of a Hill-type model with *n* fixed at 0.5 or varied according to the value obtained from non-linear regression of the data. Data are mean ± standard deviation of three independent experiments. The same legend applies to all three panels.**

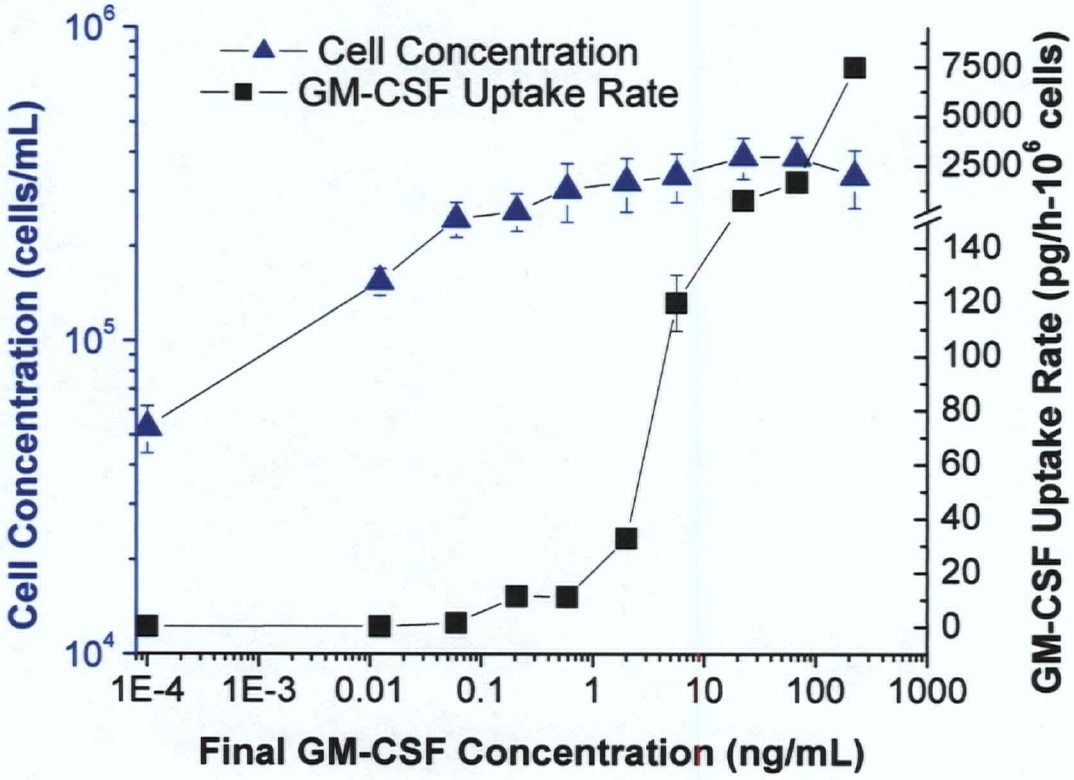

Figure 2.3 **Cell concentration and GM-CSF uptake rate as a function of final GM-CSF concentration for 72 h cultures of TF-1 cells.. The rate of GM-CSF uptake was calculated from the rate of GM-CSF depletion from the medium as determined by ELISA. Data are the mean ± standard deviation of two independent experiments.**

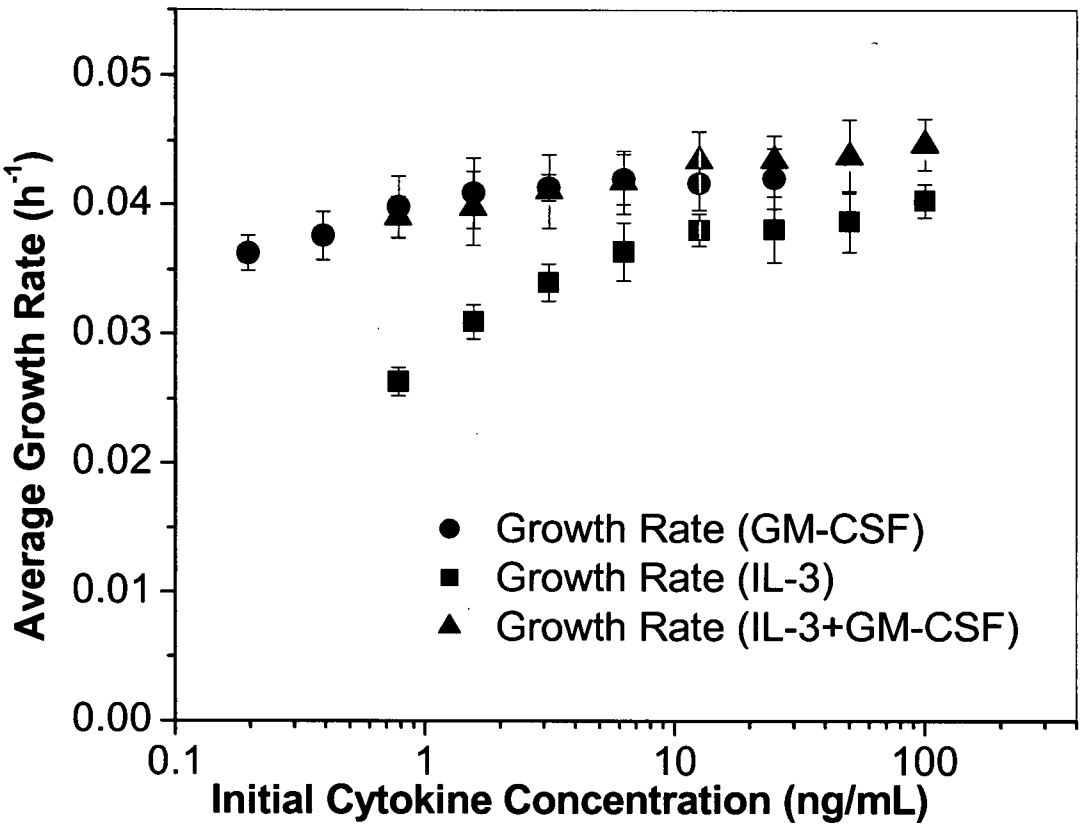

Figure 2.4 **Growth rates of MO7e cells cultured for 72 h in the presence of IL-3 alone, GM-CSF alone or IL-3 and GM-CSF together at a ratio of [IL-3]/[GM-CSF] = 2. Abscissa scale is initial IL-3 concentration when IL-3 and GM-CSF were used in combination. Data are mean ± standard deviation of three independent experiments.**

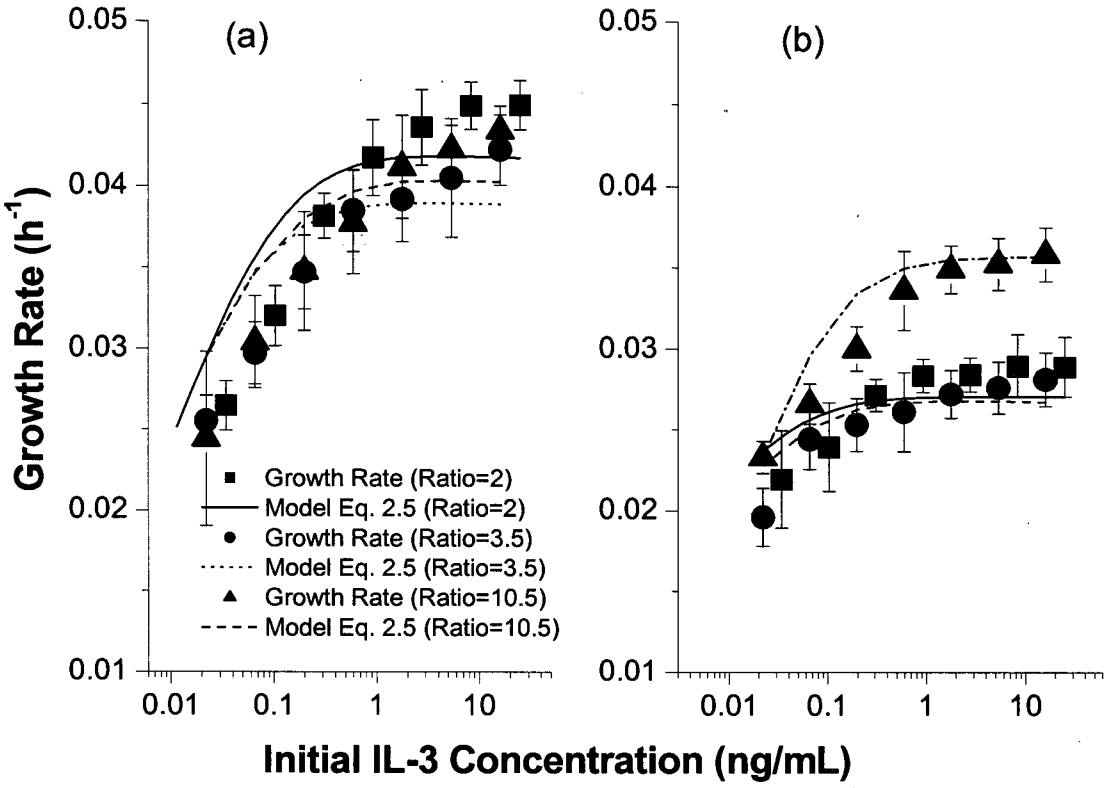

Figure 2.5 **Comparison of the growth rate of TF-1 cell cultured in the presence of both IL-3 and GM-CSF for (a) 72 and (b) 120 h with the predictions of an additive Hill-type model (Eq. 2.5). In each set of experiments, the [IL-3]/[GM-CSF] ratio remained constant at fixed values of 2, 3.5 and 10.5. Data are mean ± standard deviation of three independent experiments. The legend applies to both panels.**

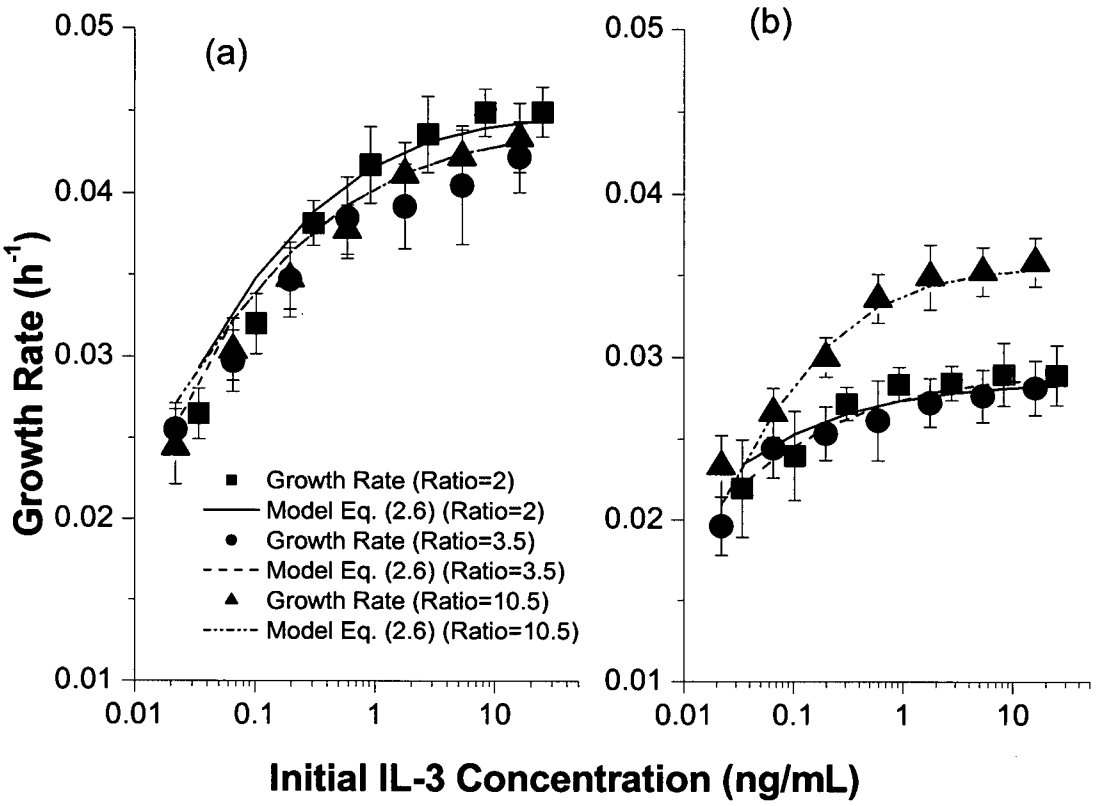

Figure 2.6 **Comparison of the growth rate of TF-1 cells cultured in the presence of both IL-3 and GM-CSF for (a) 72 and (b) 120 h with the predictions of a competitive Hill-type model (Eq. 2.6). In each set of experiments, the [IL-3]/[GM-CSF] ratio remained constant at fixed values of 2, 3.5 or 10. Data are mean ± standard deviation of three independent experiments. Figure legend applies to both panels.**

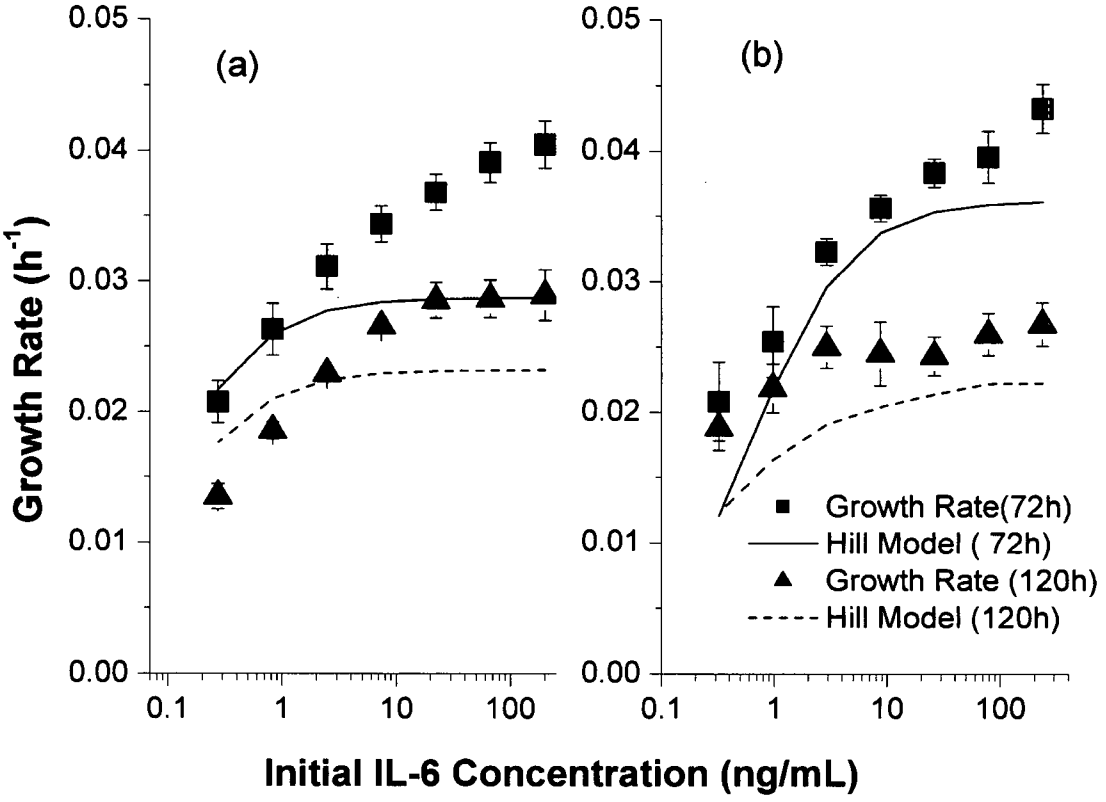

Figure 2.7 Comparison of the growth rate of TF-1 cells cultured in the presence of (a) IL-3 + IL-6 or (b) IL-6 + GM-CSF for 72 and 120 hours, with the predictions of a competitive Hill-type model (Eq. 2.6). In each set of experiments, the [IL-6]/[IL-3] and [IL-6]/[GM-CSF] ratios remained constant at fixed values of 8 and 16 respectively. Data are mean ± standard deviation of three independent experiments. Figure legend applies to both panels.

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3 Culture pH and Osmolality Modulate Proliferation and Embryoid Body Formation Potential of Murine Embryonic Stem Cells[b]

3.1 Introduction

Pluripotent embryonic stem cells (ESC) have been derived from the inner cell mass of blastocyst stage embryos of murine, primate as well as human sources[1-4]. These cells exhibit indefinite replicative potential and the ability to differentiate into derivatives of all three germ layers. The manipulation of stem cells is expected to provide new means to treat conditions that are currently incurable. Stem cells are subject to a wide variety of control mechanisms and hence progress in harnessing these cells to repair damaged tissues will rely upon identifying the critical genetic and culture factors that influence these regulatory mechanisms.

Whereas human embryonic stem cell-derived cells have tremendous potential in many experimental and therapeutic applications, they have a doubling time of the order of 35-40 h[5]. The realization of stem cell clinical therapies depends on the development of consistent, scalable protocols to expand their numbers without compromising their developmental potential. Maintaining a large pool of high purity stem cells in culture represents a great challenge as, although mouse or human ESC can be readily expanded in culture, many types of differentiated cells are also produced in these cultures. To maximize ESC numbers and minimize the fraction of diverse differentiated cells, culture conditions that favor ESC self-renewal need to be defined.

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Murine ESC are pluripotent cells derived from the early mouse embryo. When cultured in the presence of mouse embryonic fibroblasts (MEF) and/or leukemia inhibitory factor (LIF), these cells can proliferate while maintaining their capacity to differentiate into a wide variety of other cell types[1]. Removal of MEF or LIF causes ESC to spontaneously differentiate, following a reproducible temporal pattern of development that in many ways recapitulates early embryogenesis[6]. As ESC differentiate in culture, they typically form three-dimensional aggregates of cells called embryoid bodies (EBs). Over time, EBs increase in cell number and complexity as cells from the three germ layers are formed. EB formation has been shown to recapitulate the formation of a three-dimensional architecture wherein cell-cell and cell-matrix interactions are thought to support the development of the three embryonic germ layers and their derivatives[6,7]. Generation of a wide variety of cell types, e.g., cardiac myocytes, hematopoietic cells, neurons and pancreatic cells, from ESC have been reported [8-13].

Mouse and human ESC can be cultured in the lab while maintaining their developmental properties. It is also becoming clear that human ESC may play a crucial therapeutic role in the treatment of various currently non-curable disorders in the near future. However in order to treat potentially millions of patients, it will be necessary to develop methods to generate these cells in large quantities. To date, relatively few studies have been published highlighting the large-scale production of undifferentiated as well as terminally differentiated cells from mouse ESC[14-17]. To scale up these bioreactors, it will be necessary to determine the ranges of culture variables within which these cells can be maintained in an undifferentiated state. Murine embryonic stem cells (mESC) provide a practical model for stem cell culture process research as they can be readily obtained at relatively high purities and can be propagated indefinitely. However, stem cell bioengineering is still in its infancy and a thorough understanding of environmental tolerance ranges of either mouse or human ESC is still lacking. Because the mouse is widely used as a model for conducting *in vitro* and *in vivo* studies to gain insight into the developmental biology and regenerative medicine that will eventually benefit humans, it is important to develop an understanding of the influence of the culture environment on

the maintenance of the stem cell potential of mESC. The insight gained through mESC should also be beneficial towards developing methods for the expansion of human embryonic stem cells (hESC) in culture without loss of their developmental potential.

ESC are normally cultured in well-plates, dishes or T-flasks with daily replacement of medium such that they are subject to variations in the cellular microenvironment. The rapid exhaustion of cultivation area in these systems necessitates frequent passaging of cells to prevent the spontaneous differentiation that otherwise ensues. Most importantly, the batch-type approach does not provide steady-state operating conditions, resulting in temporal variation of the culture microenvironment that can potentially influence the cellular output of ESC cultures.

In the case of mammalian cells, weak acids from cell metabolism normally accumulate in longer-term and high density cultures. Culture extracellular pH has been shown to have diverse influences on cells, including effects on proliferation rate[18, 19], differentiation[20-22], metabolism[23-25], protein synthesis, degradation and glycosylation[26-28], glucose transport and glycolysis, branched chain amino acid oxidation[29] as well as expression of cell surface receptors[23] and amino acid transport[30]. At low extracellular pH, there may be nutrient uptake limitations and cells may not be able to maintain sufficient intracellular levels of nutrients for growth[31]. A stimulation of glucose transport in alkaline pH conditions has been reported for many cell systems, including rat adipocytes and liver cells[32]. Under alkaline pH, nutrient molecules may have the wrong ionic charges for uptake and cell surface proteins may assume the wrong configuration for their proper function[31].

Raised osmolality has also been shown to affect both cell growth and recombinant protein production by CHO[33, 34] as well as hybridoma cells[35, 36]. Osmotic stress affects cells by the mechanical consequences of changes in volume or turgor pressure and by the

effects on macromolecules of changes in ionic strength and molecular crowding. Adaptive responses to osmotic stress include the acute influx of salts and water (called regulatory volume increase, RVI), the chronic accumulation of organic osmolytes and the activation of the genes for heat shock proteins, aldose reductase and the transporters of betaine, inositol and taurine. The increased expression of aldose reductase occurs when the cell volume falls and the intracellular concentrations of Na^+ and K^+ salts rise. Apparently, it is the increased ion concentration, not the decreased volume that is the stimulus[37, 38]. By the same reasoning, the osmotic induction of heat shock genes may also depend on the intracellular inorganic ion concentration. Increased expression of HSP-70 mRNA in response to a high NaCl concentration is reduced when betaine and inositol are present in the medium (and the cells), compared to the much greater and more prolonged elevation when there is no betaine or inositol in the medium[39]. The development of a mouse zygote to a blastocyst is severely impaired when the concentration of NaCl exceeds 115 mM (~290 mOsm/kg). The development of the embryo is not affected if the NaCl concentration is as low as 80 mM (230 mOsm/kg)[40]. Two key enzymes of glycolysis, LDH and pyruvate kinase, become essentially inoperable in the presence of high salt concentrations[41]. Moreover, a decreased rate of protein synthesis and the disruption of gene expression with increasing salt concentration has also been reported in mammalian embryos[42,43]. Hypo- or hyper-osmotic conditions activate a variety of kinases, including stress- and mitogen-activated kinases[44-46] and Rho kinase[47] among others. Hyperosmotic stress has been shown to induce proteosome-mediated degradation of cyclin D1 in a lymphoma cell line[48]. Janus kinase 2 and calmodulin have been shown to activate the Na^+/H^+ exchanger in CHO-K1 cells[49].

Unlike hematopoietic stem cells, for which quantitative functional assays of stem cell potential have been defined and validated, such assays for mouse or human ESC are less well developed. Self-renewal is generally measured by the ability of mouse ESC to continuously proliferate in culture while maintaining undifferentiated colony morphology. The most rigorous *in vivo* assay to establish the functionality of cultured mouse ESC is blastocyst injection and measurement of their ability to give rise to

chimeric mice, since an ESC contribution is required for all adult tissue, including germ cells[50]. Two *in vitro* assays have been used extensively as surrogates for chimera formation when testing culture reagents or examining the consequences of genetic manipulation. The colony-forming cell (CFC) assay is used to determine the plating efficiency of ESC populations under various conditions and thus may be considered indicative of their self-renewal potential. Formation of EBs can be performed at a clonal level *in vitro* and reflects multilineage differentiation potential[51]. Assessment of pluripotency of mouse ESC has also relied on the expression of selected molecular markers. These have included alkaline phosphatase, the POU transcription factor Oct-4, and stage-specific embryonic antigen 1 (SSEA-1). We have recently demonstrated the superiority of the embryoid body formation assay over phenotypic characterization by Oct-4 and SSEA-1 staining to monitor the loss of mouse ESC potential[52].

To successfully grow the number of cells required for clinical applications, it is essential to know how the pluripotentiality of hESC is affected by changes in various bioprocessing variables such as oxygen tension, pH, osmolality, shear, glucose and glutamine concentrations, etc. In addition, the ranges of these bioprocess variables within which such cultures could be performed without compromising the hESC developmental potential need to be determined. Once the acceptable ranges of these various culture variables have been found, optimization of culture conditions to maximize yields can be carried out. To this end, we have used mESC as a model system as they are readily available in high numbers and purities and are more readily maintained and manipulated in culture compared to hESC. For example, mESC are passaged as single cells while hESC, at present, require passaging as small aggregates such that even cell numbers are difficult to enumerate and control.

Physiochemical and nutritional culture parameters are increasingly being recognized as important determinants of culture performance for recombinant protein production as well as for hematopoietic cultures in cellular therapy applications. Culture pH is an important parameter whose effect on embryonic stem cell culture performance remains

largely unexplored. In the present study, the mESC lines, R1 and EFC, have been used as model systems to study the effect of two important culture variables, medium pH and osmolality, on proliferation and EB formation potential. Most mESC differentiation protocols consider EB formation as the first step. Hence, it is important to define those culture conditions that would maximize the EB yield (total number of EBs obtained per initial mESC inoculated) from differentiating mESC. We have examined the changes in pH and other culture variables in mESC cultures following the conventional passaging protocol and show that proliferation and EB formation potential are greatly influenced both by pH as well as the previous growth history of inoculum cells, i.e., whether they were grown on irradiated feeders or in gelatinized dishes. Dose-response experiments then show that both pH and osmolality influence the growth rate and EB forming potential of both R1 and EFC cells. In addition, both metabolism and cell size exhibit a strong correlation with pH. Flow cytometric analysis is then used to enumerate the Oct-4 and Annexin-V positive cells under various pH conditions.

3.2 Materials and Methods

All reagents were obtained from StemCell Technologies Inc. [STI], Vancouver, BC, unless otherwise indicated.

3.2.1 Growth of Mouse Embryonic Fibroblasts (MEF)

Day 12.5 MEF from a CD-1 mouse strain were thawed onto a gelatinized dish and maintained at 37°C in humidified air with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μM monothioglycerol (MTG) (Sigma-Aldrich, Oakville, ON). Cells to be irradiated were trypsinized, resuspended in 4 mL of medium, and exposed to 60 Gy from an x-ray source before replating for use as feeder cells.

3.2.2 Growth of Mouse Embryonic Stem Cells (mESC)

The R1[53], and EFC[54] murine ESC were routinely maintained in 37°C humidified air with 5% CO_2 on a layer of irradiated MEF and fed daily with a complete change of ESC maintenance medium. The maintenance medium consisted of high-glucose DMEM supplemented with 15% ESC-qualified fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 2 mM glutamine, 1,000 U/mL leukemia inhibitory factor (LIF), 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μM MTG. R1 cells were from passage 17 while EFC cells were from passage 11 and had been frozen at 10^6 cells per vial. Both cell lines were used within 5-8 passages of initial thawing. Cells were passaged every second day in maintenance cultures. To passage the cells, a single-cell suspension was generated by treatment with 0.25% trypsin and 1 mM EDTA (T/E) (Invitrogen Life Technologies, Burlington, ON) for 5 min until the cells detached from the culture vessel surface. T/E activity was then quenched with DMEM supplemented with 10% FBS. The cells were centrifuged at 1,200 rpm for 7 min and resuspended in ESC maintenance

medium. Viable cells were plated at ~2 × 10^4 cells/cm^2 in either 10 cm irradiated MEF dish (10 mL of maintenance medium) or a 6 cm gelatinized dish (4 mL maintenance medium) and cultured for a further 48 h at 37°C, 5% CO_2 before harvesting for the pH or osmolality experiments.

3.2.3 Determination of Growth Rate

The viable cell concentration was determined by either counting cells on a hemocytometer or by using an automated cell counter, Cedex (Innovative Directions, Pinole, CA) which, in addition to providing viable cell numbers and viability values, also computed the distribution of cell diameters. The average specific growth rate ($\overline{\mu}$) of mESC under various pH and osmolality conditions was calculated using the equation:

$$\overline{\mu} = \frac{\ln\left[X_v / X_o\right]}{\Delta t} \qquad (3.1)$$

where X_v (cells/mL) is the viable cell concentration at the end of the culture period (48 h), X_o (cells/mL) is the inoculum viable cell concentration and Δt (h) is the time interval.

3.2.4 Preparation of Medium at Desired pH or Osmolality

The pH of the ESC maintenance medium was adjusted to different values ranging from 6.7 to 7.9 by adding 5N HCl or 5N NaOH and then incubating the medium overnight to equilibrate in a 5% CO_2 atmosphere. In some experiments, a powdered DMEM formulation without glucose, glutamine, sodium bicarbonate, sodium pyruvate and phenol red (Sigma-Aldrich, Oakville, ON) was used to prepare the maintenance medium and sodium bicarbonate was added to obtain the desired pH since the equilibrium bicarbonate concentration ($[HCO_3^-]$, mM) at 37°C is related to the medium pH and the partial pressure of CO_2 (pCO_2, mmHg) in the gas phase via the following equation[55]:

$$\log[HCO_3^-] = pH + \log[pCO_2] - 7.543. \qquad (3.2)$$

A custom-made DMEM formulation (JRH Biosciences, Lenexa, KS) with a low NaCl content (500 mg/L vs. standard 6400 mg/L) was used to prepare the ESC maintenance medium and then appropriate volumes of 1 M NaCl (prepared in DMEM) were added to adjust the osmolality of the medium to between 250 and 500 mOsm/kg. Both the pH and osmolality experiments were carried out on gelatinized 60 mm tissue culture dishes (Sarstedt, Montreal, PQ).

3.2.5 Measurement of pH or Osmolality

All medium samples were measured after equilibration for several hours in an incubator containing 5% CO_2. Samples of equilibrated media were aseptically removed as quickly as feasible from the culture dishes into 1.7 mL Eppendorf tubes, which were filled completely and capped. The pH measurements were conducted using a Bayer Rapidlab 348 gas analyzer. Prior to each use, the electrodes were calibrated using pH 6.8 and 7.3 buffer solutions. Visual inspection of the culture plates was also done at the start of the experiment, before medium exchange at 24 or 72 h as well as before culture harvesting at 48 or 96 h. The medium contained phenol red which changes color from red (neutral pH) to yellow (acidic pH) or magenta (basic pH), allowing an approximate confirmation that the pH was in the correct range.

Osmolality is a measure of the total solute concentration in a solution. The medium osmolality was measured by freezing point depression using an Advanced Osmometer 3D3 (Advanced Instruments, Norwood, MA). The osmometer was cooled using a mixture of ethylene glycol and several other unspecified chemicals. It was calibrated using 100, 290 and 1500 mOsm/kg standard solutions (Advanced Instruments). The instrument was operated using a sample volume of 250 μL. All osmolality values were calculated as the average measurement from duplicate samples.

3.2.6 Measurement of Glucose, Lactate, Glutamine and Ammonium Concentrations

Glucose and glutamine are the primary carbon and energy sources in mammalian cell cultures. Glucose, lactate, glutamine and ammonium concentrations were determined using an YSI 7100MBS analyzer (Yellow Springs Instruments, Yellow Springs, OH). The instrument was operated according to the instructions provided by the manufacturer. The system solutions included a reagent pack with all the necessary standards and buffers.

The average cell specific glucose uptake rate was calculated from:

$$sGUR = -\frac{1}{\log\text{mean}\, X_v}\frac{\Delta Glu}{\Delta t}\times 10^6 \tag{3.3}$$

where $sGUR$ is the average cell specific glucose uptake rate (pmol/10^6 cell-h), ΔGlu is the change in glucose concentration (pM), log mean X_v is the logarithmic mean average viable cell concentration (cells/mL) and Δt is time interval (h).

Similarly, the average cell specific lactate production rate, $sLPR$ (pmol/10^6 cell-h) was determined from:

$$sLPR = \frac{1}{\log\text{mean}\, X_v}\frac{\Delta Lac}{\Delta t}\times 10^6 \tag{3.4}$$

where ΔLac is the change in lactate concentration.

The average cell specific glutamine uptake rate, $sGlnUR$ (pmol/10^6 cell-h) was determined using:

$$sGlnUR = -\frac{1}{\log\text{mean}\, X_v}\frac{\Delta Gln}{\Delta t}\times 10^6 \tag{3.5}$$

where ΔGln is the change in glutamine concentration. Since measurements were made on samples collected every 24 h, spontaneous glutamine degradation in culture was assumed to be negligible (since the first-order degradation constant, $k = 0.004675/h$ [56]).

The yield of lactate from glucose ($Y_{Lac/Glu}$) was calculated from the equation:

$$Y_{Lac/Glu} = -\frac{\Delta Lac}{\Delta Glu} \tag{3.6}$$

3.2.7 Preparation of mESC for pH or Osmolality Experiments

The mouse ESC were thawed and cultured for 2 passages on irradiated feeders as described above. To prepare the cells for exposure to various pH or osmolality solutions, they were harvested by trypsinization as described above, resuspended in ESC maintenance medium, and preplated on tissue culture plates for 15-20 minutes at 37°C, 5% CO_2, in order to deplete contaminating MEF. At the end of this pre-plating step, the non-adherent and loosely adherent ESC were collected by gently washing the surface of the tissue culture plate. The cells were then centrifuged, resuspended in ESC maintenance medium and viable cell numbers were counted on a hemocytometer with trypan blue dye or on an automated cell counter (Cedex, Innovative Directions, Pinole, CA) to distinguish live from dead cells. The frequency of contaminating MEF in the undifferentiated (day 0) ESC samples was estimated to be less than 0.2% based on cell size during counting (MEF cells are easily distinguishable from mESC as they are bigger in size (generally $\geq$16 μm in diameter) and appear to have irregular morphology). All cultures for the various pH and osmolality experiments initially contained ~2×10^4 cells/cm^2 in a 6-cm dish (4 mL medium). The conditioned medium from each of these cultures was collected after 24 hours and replaced with fresh medium at the same pH or osmolality. The following day, the cultures were harvested by trypsinization and the cell concentrations were determined as described above.

3.2.8 Embryoid Body Formation Assay

To determine the effect of maintenance medium pH or osmolality on the ability of mouse ESC to form EBs, the latter were differentiated in a semi-solid medium consisting of 0.9% methylcellulose, 15% FBS, 2 mM glutamine, 150 μM MTG and the remainder IMDM. Single-cell suspensions were collected on day 0, or prepared after harvesting the cells from the pH and osmolality experiments. Defined numbers of cells (1000 for EFC cells, 2500 for R1 cells) were plated in 35 mm low adherence Greiner dishes in the ESC differentiation methyl-cellulose medium described above to determine the efficiency of EB formation. EB numbers were determined microscopically after 5–6 days of culture. It is important to note that the pH of the methylcellulose medium was not adjusted. Figure (3.1) shows a representative picture of 3-dimensional cell aggregates that were considered as EBs and counted as such, while Figure (3.2) shows the cell aggregates that were not considered to be EBs. Morphologically, only those aggregates were considered as EBs that had a 3-dimensional (more or less spherical) shape, fairly smooth margins and a minimum diameter of around 130-150 μm. Considerable variation in the sizes of counted EBs was observed and the mean diameter was 230 ± 73 μm. The largest EBs counted were ~350 μm in diameter. The EB output as a function of initial input number of cells, in the range of 500 – 5000 cells/mL, remained more or less linear between. At higher initial cell densities, both the EB output and the morphology of the resulting EBs varied considerably (data not shown). The percent EB formation efficiency was calculated by dividing the total number of EBs observed by the number of cells plated, and multiplying by 100. Typically % EB formation efficiencies varied between 0.5 – 10%. The EB yield per initial cell for each pH or osmolality culture was determined by calculating the number of EBs that would have formed if all the cells harvested had been inoculated in EB cultures and dividing that number by the total viable cell density at the start of the culture.

3.2.9 Flow Cytometry

The antibody used for apoptosis analysis was purified fluorescein isothiocyanate–conjugated Annexin-V (BD Pharmingen). Single-cell suspensions collected on day 0 or prepared from R1 cells exposed to various pH and osmolality conditions were washed twice in cold PBS and then resuspended in 1X binding buffer at a concentration of 10^6 cells/mL. Five μL Annexin-V were added to a 100 μL aliquot of cells. The cells were gently mixed and incubated for 15 minutes at room temperature in the dark. Four hundred μL of binding buffer were added to each tube and the samples were analyzed by flow cytometry using a FACSCalibur flow cytometer and CELL Quest software (BD Pharmingen, Mississauga, ON). The forward- versus side-scatter profile was used to gate on viable cells, and an unstained sample (treated with the test cells under otherwise identical conditions) was employed to determine the appropriate gating for quantification of expression.

Cells to be stained for Oct-4 were resuspended in 100 μL of Hanks' buffered saline solution plus 2% FBS (HF) and fixed with 100 μL of Intra Prep Permeabilization Reagent 1 (Immunotech, Westbrook, ME) for 15 minutes at room temperature. The cells were then washed with HF and permeabilized with IntraPrep Permeabilization Reagent 2 for 5 minutes before incubation with a 1:100 dilution of mouse anti-mouse Oct3/4 monoclonal antibody (Transduction Laboratories, Lexington, KY) for 15 minutes at room temperature. The cells were washed with HF before staining with allophycocyanin-labeled anti-mouse immunoglobulin G1 (BD Pharmingen). A sample stained with secondary antibody only was used to determine the appropriate gating for quantification of expression. The samples were analyzed by flow cytometry as described below.

Flow cytometry analyses were conducted using a Becton Dickinson FACSCalibur instrument (BD Biosciences, San Jose, CA). This device is a non-sorting analytical flow cytometer that uses an air-cooled argon ion laser to deliver 488 nm light at approximately 15 mW. The flow cytometer was calibrated using Calibrite beads (BD Pharmingen). A

2 mL single-cell sample was placed in a 5 mL plastic tube and air pressure was used to transport the cells into the flow chamber. From the chamber, the cells were injected into a flow cell while suspended in a sheath fluid. The device was set to analyze 10^4 viable cells gated appropriately.

3.2.10 Statistics

Data are reported as mean ± standard error of the mean (SEM), unless otherwise noted. Statistical comparisons were performed using a two-tailed paired Student's t-test. An asterisk (*) was used to denote statistical significance ($p < 0.05$).

3.3 Results

3.3.1 Culture Variables during Routine Passaging of mESC

This study examined the influence of culture variables on the growth rate and EB formation potential of mESC. First R1 mESC-conditioned media were analyzed to determine the temporal variations over the course of conventional cultures, inoculated at 2.5×10^4 cells/cm^2 on irradiated feeder cells with daily feeding of maintenance medium. During the first 24 h, glucose, glutamine, ammonium, pH and osmolality (Figure 3.3) showed relatively modest changes that were increased 2- to 3-fold in the next 24 h, consistent with the increased cell concentration before passaging at 48 h.

Gelatinized surfaces can replace feeder cells though the ESC colonies appear increasingly differentiated after two successive passages on gelatin. Nonetheless, this is a useful experimental system for assessing culture variable impacts on ESC for 1 or 2 passages in the absence of any confounding effects of the variables on the feeder cells. To compare these cultures, R1 mESC were grown on gelatinized dishes and it can be seen (Figure 3.4) that the measured culture variables consistently changed less than in the feeder-containing cultures. Irradiated feeders alone decrease the pH only by 0.05 units and consume less than 3 mM glucose over a 48 h period (data not shown). In most cases, these reductions were smaller than those seen in Figure (3.3), consistent with the absence of the feeder metabolism and their stimulatory effects on ESC. The one exception was that the osmolality increased from 330 to over 400 mOsm/kg in the feeder cultures compared with only up to 365 mOsm/kg in the gelatinized cultures. This is probably due to cell lysis and hydrolysis of the irradiated feeder cell components. The greatest changes (Figure 3.4) in culture variables occurred when the cell concentrations were highest during the final 24 h (24 – 48 h) of culture prior to passaging.

Dose-response experiments were carried out with glucose, glutamine and ammonium as variables. The growth rate of the R1 mESC was independent of the glucose concentration except at its lowest initial value of 1.25 mM where the glucose decreased to ~ 0 mM while the growth rate decreased by only ~12% (Figure 3.5a). Compared to control cultures containing 20 mM glucose, the EB formation potential was also significantly decreased ($p < 0.05$) when the initial glucose concentrations was 2.5 mM or lower. There was a more gradual increase in EB yield between 2.5 and 20 mM such that 5 mM or more glucose would appear to maximize the EB yield. A similar dependence on glutamine was observed (Figure 3.5b) with EB yields significantly decreased ($p < 0.05$) in cultures initially at 1 mM glutamine and the maximum yields were obtained from 2 mM glutamine or more present in the medium. Both the growth rate and the EB forming ability were significantly lower ($p < 0.01$) when glutamine was not added to the initial medium compared to the control culture with 4 mM glutamine.

Ammonium dose-response experiments, depicted in Figure (3.5c), show that the R1 mESC growth rates were significantly decreased ($p < 0.05$) when ammonium was present at a concentration of 4.5 mM or higher compared to the control culture containing 1.5 mM ammonium. The EB yield was significantly decreased ($p < 0.05$) only when ammonium was present at a concentration of 6 mM or higher. Due to the presence of ammonium in the serum (15% of medium), it was not possible to test ammonium at 0 mM. The data shown in Figure (3.4) clearly illustrated the fact that, following the conventional passaging protocol, the glucose and glutamine concentrations never fell to the threshold levels described above while the ammonium build-up was never more than 3 mM (data not shown). Based on these observations, these variables were considered not to influence the proliferation and EB formation potential of mESC lines and hence were not further explored.

3.3.2 Osmolality Dose-Response Experiments

The influence of the osmolality of the medium on the growth rate and EB yield of R1 cells was determined by means of dose-response experiments carried out in the range of

200 – 500 mOsm/kg at an initial pH of 7.3. The R1 cells grew relatively slowly at the low and high ends of this range compared with the conventional osmolality of 300 mOsm/kg (Figure 3.6). The EB yield per initial cell increased linearly when the osmolality was raised from 200 to 300 mOsm/kg and then declined sharply between 335 and 500 mOsm/kg. This was due to a decline in the number of cells capable of forming EBs at low and high osmolalities with the result that there was almost a 5-fold decline in EB yield at 200 and 500 mOsm/kg compared to the culture with an osmolality of 300 mOsm/kg. The EB yield of R1 cells showed a plateau in the range of 300 – 335 mOsm/kg. Since the feeder containing cultures had over 400 mOsm/kg levels, these results favored the used of feeder-free gelatinized dish cultures to study pH effects without influences from high osmolality.

3.3.3 pH Dose-Response Experiments

The gelatinized dish based pH dose-response experiments determined the effect of initial culture pH on the growth rate and EB yield of R1 cells exposed to a range of pH values for 48 h (Figure 3.7). The initial and final average pH values as well as the corresponding logarithmic average pH values to which cells were exposed during the final 24 h in culture are shown in Table (3.1). The R1 mESC growth rate as well as the EB yield was strongly influenced at both the low and high initial pH values compared to an initial pH 7.3. There was almost a 3-fold decline in the EB yield at pH 6.7 and 7.7 compared to that at pH 7.3. An initial pH of 7.3 maximized the growth rate and EB yield of R1 cells, except that the growth rate of these cells did not significantly decrease at pH 7.6. The growth rate declined rapidly in cultures exposed to pH 7.75 and higher with virtually no viable cells remaining in cultures with a pH of 7.9. The reduced EB yield from low and high pH cultures appeared to be due to both the reduced growth rate as well as the reduced fraction of cells capable of forming EBs, i.e., lower frequency (% EBs) of cells capable of forming EBs (Figure 3.8).

The pH dose-response was refined to include more pH values as well as the culture time extended to 96 h, to assess the influence of prolonged exposure of R1 cells to different

pH conditions. As depicted in Figure (3.9), data with trends similar to the 48 h cultures were obtained. Again, a pH 7.3 environment maximized the EB yield. Even though the growth rate of R1 cells was not influenced by pH in the range of 7.15 to 7.6, the EB yield was significantly higher at pH 7.3 compared to 7.15 or 7.45. These results demonstrate that pH is an important culture parameter that strongly influences the performance of mESC cultures within the ranges of standard maintenance cultures conditions (Figure 3.4).

An additional pH dose-response analysis was performed with the EFC cells, a different mESC line. The growth rate and mean EB yield of EFC cells was significantly higher at all pH values compared to R1 cells. The EB yield of EFC cells increased almost linearly with increasing pH up to 7.3 and then declined at pH 7.6 (Figure 3.10). As was the case with the R1 cells, pH 7.3 produced the maximum EB yield and the EB output was significantly less for cultures exposed to either pH 7.0 or 7.6. Like R1 cells, the growth rate of EFC cells was not significantly different between pH 7.3 and 7.6.

A systematic comparison of the pH dose-response of R1 cells in which the inoculum cells were taken from cultures that were maintained either on feeders or on gelatinized dishes was also performed. As shown in Figure (3.11), over the range of pH values tested (6.7 – 7.7), the growth rate as well as the EB formation potential of R1 cultures inoculated with cells grown on feeders is significantly higher than those cultures that were initiated with cells taken from gelatinized dishes. Table (3.2) summarizes the results of 2-way ANOVA analysis showing that both pH and surface used for inoculum growth significantly influenced the growth rate as well as EB formation potential (EBs/initial cell) of R1 cells. No interaction was, however, detected between the pH and the growth surface (gelatin or feeders) on either the EB formation potential or the growth rate of R1 cells. These results further strengthen the observations stated above regarding the metabolic data (Figures 3.3 and 3.4) that mESC are better maintained on feeders than gelatin.

3.3.4 Influence of pH on Metabolism of mESC Line, R1

Glucose, lactate and glutamine concentrations were measured and the corresponding metabolic rates were determined during a 48 h culture of R1 cells. Like growth rate and EB yield, the cellular metabolism of mESC was strongly influenced by variations in pH. As shown in Figure (3.12a), the average *sGUR* and *sLPR* both increased monotonically with increasing pH. When the initial culture pH was raised from 6.7 to 7.6, *sGUR* increased more than two-fold from 53 pmol/10^6 cell-h to 124 pmol/10^6 cell-h. The corresponding increase in *sLPR* was amost five-fold from 41 pmol/10^6 cell-h to 193 pmol/10^6 cell-h. Thus, Figure (3.12b) shows that the lactate to glucose yield coefficient, $Y_{Lac/Glu}$, increased from 0.84 ± 0.2 at pH 6.7 to 1.62 ± 0.05 at pH 7.3. The glutamine metabolism was also influenced by pH. The cell specific glutamine uptake rate, *sGlnUR*, was highest at pH 6.7 (38 ± 5 pmol/10^6 cell-h) and lowest at pH 7.3 (27 ± 2 pmol/10^6 cell-h).

3.3.5 Variation of Cell Size as a Function of pH

Changes in cell size provide another indication of how the cells respond to a particular culture environment. Figure (3.13) shows the variation of average cell diameter as a function of pH for R1 cells exposed to different pH environments for 48 or 96 h. A dose-dependent increase in average viable cell diameter with pH was observed. Cells exposed to pH 6.7 for 48 and 96 h had mean diameters of 13.27 ± 0.17 and 13.28 ± 0.15 μm, respectively, while at the other extreme, pH 7.75, the mean diameters were 15.03 ± 0.16 and 15.15 ± 0.21 μm, respectively. For both the 48 and 96 h cultures, R1 cells exposed to the pH range 6.7 – 7.15 had similar average diameters that were significantly lower than those of cells at pH 7.3. Similarly, the cells exposed to media at pH 7.45 – 7.75 had average diameters that were significantly larger than at pH 7.3.

3.3.6 Variation of Oct-4 Positive and Annexin-V Positive Cells with pH

Flow cytometric analysis was carried out to determine whether exposure to extremes of pH causes a down-regulation of Oct-4 – a transcription factor that is highly expressed by ESC but not by their differentiated progeny. In addition, cells were stained with Annexin-V – a marker of early apoptotic cells – to determine whether exposure to extreme pH values induced cell death. Figure (3.14a) shows that there were no statistically significant differences in the percentage of Oct-4+ cells after 48 h of exposure to acidic (6.7) pH compared to the control at pH 7.3. Exposure to alkaline pH (7.75), however, decreased the percentage of Oct-4 positive cells after 48 h of exposure. After 96 h of exposure to both pH 6.7 and 7.75, however, there were significant declines in Oct-4+ cells compared to cells exposed to pH 7.3. Figure (3.14b) demonstrates that exposure of R1 cells to either acidic (6.7) or alkaline (7.75) media during the first 48 h induced cell death, as the percentage of Annexin-V positive cells at these conditions was significantly higher than at pH 7.3. However, there is an apparent adaptation to both acidic and alkaline pH conditions, as the number of Annexin-V positive cells at 96 h was not significantly different across the range of pH values tested.

3.3.7 Reversibility of pH Effects

The other aspect of pH influence on the proliferation and EB forming potential of mESC addressed in these studies was to determine whether the observed pH effects are permanent or if cellular responses would be restored back to their original values once the medium pH was returned to 7.3. R1 cells were seeded in 60 mm gelatinized dishes in triplicate and exposed to pH values of 6.7, 7.3 and 7.75. The initial pH variation during this time course experiment is shown in Figure (3.15). For the R1 cells with 24 h exposure to pH 6.7 and 7.75, the medium was removed at 24 h and replaced with a medium at pH 7.3. For cells exposed for 48 h, their medium was exchanged at 24 h at their respective pH values. At the end of 48 h, all cells were harvested, counted and EB assays were initiated. All the cultures were then passaged twice in pH 7.3 medium, i.e., cultured for 96 h using the conventional maintenance protocol. The normalized growth

rate and % EBs formed, with respect to values obtained at pH 7.3, are shown in Figures (3.16) and (3.17), respectively. At 48 h, as expected, cells exposed to pH 7.3 had the highest growth rate as well as % EBs. The 24 h or 48 h exposures to pH 6.7 significantly ($p < 0.05$) decreased the growth rate as well as the frequency of stem cells in those cultures while the 24 h exposure to pH 7.75 had a relatively less detrimental effect on either stem cell frequency or growth rate compared to the 48 h exposure where both the growth rate and % EB formed were significantly ($p < 0.05$) decreased. The influence of pH on mESC responses is not permanent as cells begin to recover their original state soon after their return to pH 7.3 conditions. All the cultures (previously exposed to different pH conditions) that had been allowed to grow in pH 7.3 medium for another 96 h had either restored or started to recover their growth rate and stem cell frequency (% EBs) compared to the pH 7.3 control condition, thereby showing that the influence of pH on mESC is not permanent.

3.4 Discussion

R1 cells growing on feeders typically show a 10-fold expansion in cell concentration over a 48 h culture period. Consequently, due to this large increase in cell numbers, metabolic and physiochemical variables also indicate large variations. It is important to note that the larger variations in culture variables seen when R1 cells were grown on feeders is due to the stimulation provided by feeders to the R1 cells and is not due to the metabolism of the feeders. In addition to feeders, mESC can also be maintained on gelatinized surfaces where the pH, osmolality and metabolic profiles are relatively less changed compared to when these cells are grown on feeders. R1 cells can be maintained on gelatin, but at a lower growth rate and with decreasing EB formation potential. This is likely due to the lack of feeder secreted soluble factors[57, 58] in addition to extracellular matrix proteins deposited by feeders that can also play an important role in cell-cell signaling and maintenance of pluripotency of mouse ESC[59]. Based on the variation with time of pH and osmolality seen in these experiments, it is suggested that, although not very convenient, a medium exchange at around 36 h after culture initiation would help minimize the variation in culture variables and subsequent detrimental effects on the proliferation of mouse ESC and their ability to form EBs.

This study has shown that EB yields from mESC cultures were very sensitive to the medium pH. The 96 h experiments showed that the EB yield was significantly lower even at 0.15 pH units higher or lower than 7.3. The mESC growth rates were maintained over a relatively broader range of pH but more extreme pH values reduced growth rates. The trends were confirmed with another mESC line, EFC, derived from a 129J strain of mice on gelatinized surfaces[54] (R1 cells were derived on feeders from a 129/Sv strain). Due to their derivation on gelatin in the presence of LIF, EFC cells can be propagated on gelatin for multiple passages without an appreciable decline in either their growth rate or EB forming potential. R1 cells, on the other hand, retain a higher EB formation potential when grown on feeders and show a decline both in growth rate and EB forming potential when transferred from feeders to gelatinized surface. Since most experiments in this

study were conducted on gelatinized dishes, EFC cells therefore had much higher growth rates and EB yields compared to R1 cells. From a bioprocessing point of view, a feedback control of pH would probably improve the culture's EB yield and maintain the growth rates.

Like pH, the results obtained from osmolality experiments (Figure 3.6) also show that the growth rate and EB yield of mESC cultured on gelatinized surfaces is influenced by extremes of osmolality. For R1 mESC, initial medium osmolality values of 300 and 335 mOsm/kg resulted in both growth rate and EB yields being maintained. Although experiments were not conducted to determine the intracellular pH (pH_i) of cells when exposed to various osmolality or extracellular pH (pH_e) conditions, both of these variables can theoretically influence pH_i. When pH_e is decreased, the elimination of protons by the Na^+/H^+ exchanger can become less efficient (as indicated by an increased counter-gradient of protons across the cell membrane[60]), thereby leading to a lower pH_i. A reverse situation might be expected in the case of an increased medium osmolality. The increased extracellular concentration of Na^+ ions can facilitate the exchange of protons by the exchanger, as a consequence of the increased Na^+ gradient across the cell membrane. This might induce a cytoplasmic alkalization leading to an increase in pH_i[60].

The increase in glucose and lactate metabolism at high pH values, observed for mESC lines (Figure 3.12), has also been reported for transformed cells[61, 62]. One possible explanation lies in the increase of the activity of glycolytic enzymes under alkaline conditions. It is known, for example, that hexokinase has an optimal pH of 8[63]. An increase in pH_i stimulates the activity of phosphofructokinase, PFK[64]. The activity of PFK isolated from mouse embryos can be significantly altered by changes in pH of around 0.2 units[65]. Another explanation is that increasing the pH alters the membrane potential, changing the glucose transport rate through the membrane[66]. The lactate production rate increased because of a higher glycolytic activity under alkaline pH conditions. A third explanation is that lactate transport out of the cell is mediated via a lactate/H^+ co-transporter which is only slightly sensitive to pH_i but is strongly dependent on pH_e. It has been shown that alkaline pH enhances[67] and acidic pH inhibits[67, 68] the rate

of lactate exit from the cell. The depression of lactate release results in an inhibition of the glycolytic enzymes through a feedback mechanism which increases the concentration of glycolytic intermediates and hence lowers the overall lactate production rate. The yield coefficient of lactate from glucose increased at elevated pH values. This may suggest a self-regulation to keep the internal pH constant. The greater amount of lactate formed allows the cell to survive under alkaline conditions. Similarly, less lactate is produced at low pH as has been observed for other cell lines[25]. The glutamine consumption rate had a minimum at pH 7.3. The glutamine uptake rate was higher at low pH where glucose utilization was minimal. Glutamine could be used preferentially as an energy substrate at low pH. The glutamine uptake rate increased at high pH values possibly due to the increased glutaminase activity which is known to have a pH optimum of around 8.

The mean viable cell diameter (and hence cell volume) was found to change as a function of extracellular pH (Figure 3.13). The volume of cells exposed to pH 7.75 was 32% greater than those of cells exposed to pH 7.3 which in turn were 13% greater in volume compared to cells exposed to pH 6.7. It is therefore possible that variation in glycolysis rates reported in Figure (3.12) is partly due to this change in cell volume. An increase in cell diameter with pH has also been reported for hematopoietic cells[23].

Analysis of cells by flow cytometry for detection of early apoptotic cells (Annexin-V+) revealed that, during the first 48 h, exposure to acidic or alkaline pH induced apoptosis in cells (Figure 3.14). There was no apparent increase in the number of floating (dead) cells or cellular debris found in the supernatant collected before cells were harvested for flow cytometry. However, there was an apparent adaptation with time as there was no significant difference in Annexin-V positive cells exposed to these different pH conditions for another 48 h. It is also plausible that under extreme pH conditions, cells that acquire tolerance to low or high pH were being selected. A phenotypic analysis of mouse ESC was also done to determine the expression of Oct-4, a key transcription factor highly expressed by these cells. There was a decline in number of Oct-4 positive cells at pH 6.7 but only after 96 h of exposure to this condition. This result confirmed our

previously reported observation that Oct-4 is a lagging indicator of loss of pluripotency of mouse ESC[52].

The influence of pH on mESC responses is not permanent and once the cells exposed to extreme pH conditions are returned to normal conditions, they gradually recover their growth rate as well as EB formation potential. This demonstration of reversibility of pH effects on mESC responses implies that perhaps cells capable of forming EBs are not permanently lost under extreme pH conditions. However, even the short term influence of pH on mESC responses is still significant from the subsequent differentiation point of view as it is quite possible that cells exposed to adverse pH conditions may not generate fully functional differentiated progeny. The short term pH effects are also important from the point of view of expansion of mESC in culture, as adverse pH conditions both reduce the growth rate as well as final cell number generated in those cultures.

3.5 Conclusions

The conventional mESC maintenance protocol of daily medium exchange and passaging cells every other day resulted in glucose and glutamine concentrations that remained within ranges that should have little influence on the growth rate or EB yields. Similarly, ammonium was not found in mESC cultures at the elevated concentrations that would influence the mESC responses. However, the pH and osmolality did vary considerably during the conventional passaging protocol. Based on the dose-response experiments, it was found that mESC growth rates did not significantly decrease for relatively broad pH and osmolality ranges while the EB yield was more sensitive to pH and osmolality variations. Hence to maximize EB formation from mESC lines, the initial medium pH should be maintained close to 7.3 and the osmolality within the range of 300 – 335 mOsm/kg. Maximizing EB yield is important as it is generally the first step in differentiation protocols. The influence of pH on mESC responses does not appear to be permanent and, once cells exposed to extreme pH conditions are returned to pH 7.3, they gradually restore their growth rate as well as EB forming ability. These studies indicate that, for culturing hESC, particular attention also be paid to determining the optimal pH and osmolality conditions.

Table 3.1 Average initial, final and logarithmic averaged pH values that R1 cells were exposed to during the final 24 h (24 – 48 h) period in culture during pH dose-response experiments reported in Figure (3.9). Values shown are drawn from a subset of five experiments.

Average Initial pH ± SD	Average Final pH ± SD (at 48 h)	Logarithmic Average pH during 24-48 h period
6.72 ± 0.02	6.80 ± 0.1	6.72
6.86 ± 0.03	6.80 ± 0.07	6.83
7.01 ± 0.02	6.84 ± 0.04	6.92
7.15 ± 0.05	6.97 ± 0.06	7.06
7.30 ± 0.04	7.05 ± 0.03	7.17
7.45 ± 0.03	7.24 ± 0.04	7.34
7.62 ± 0.04	7.39 ± 0.07	7.5
7.75 ± 0.02	7.75 ± 0.06	7.75

Table 3.2 Summary Table of 2-way ANOVA to determine the influence of pH and inoculum growth history on EB yield and growth rate of R1 cells.

	Degrees of freedom	Sum of Squares	Mean Sq	F value	Pr (>F)
Number of EBs/Initial Cell inoculated					
pH	4	0.394	0.0986	3.590	0.01*
Surface (PEF or Gelatin)	1	0.168	0.168	6.125	0.0158*
pH: Surface	4	0.078	0.019	0.712	0.587
Residuals	69	1.895	0.0275		
Growth Rate					
pH	4	0.0058	0.00145	8.574	1.122e-05 *
Surface (PEF or Gelatin)	1	0.0016	0.0016	9.45	0.0030 *
pH: Surface	4	0.00015	0.000037	0.2161	0.93
Residuals	69	0.012	0.00017		

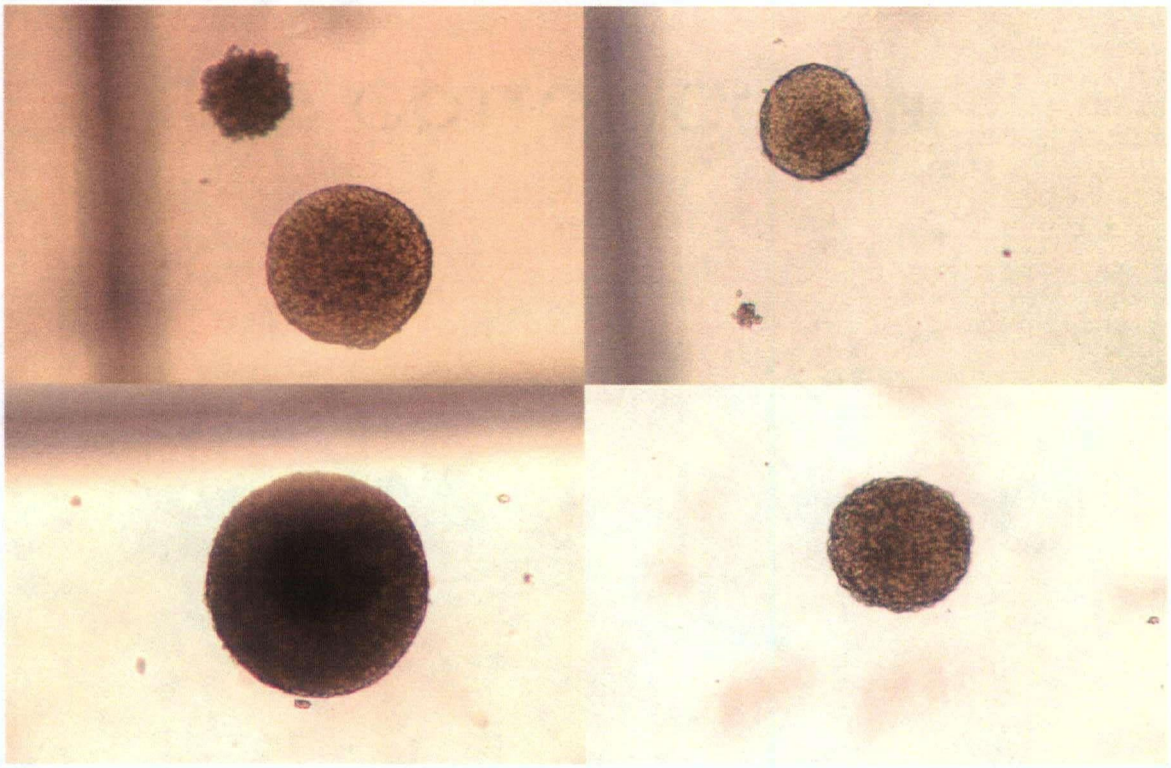

Figure 3.1 **Pictures of 3-dimensional cellular aggregates that were considered and counted as EBs.**

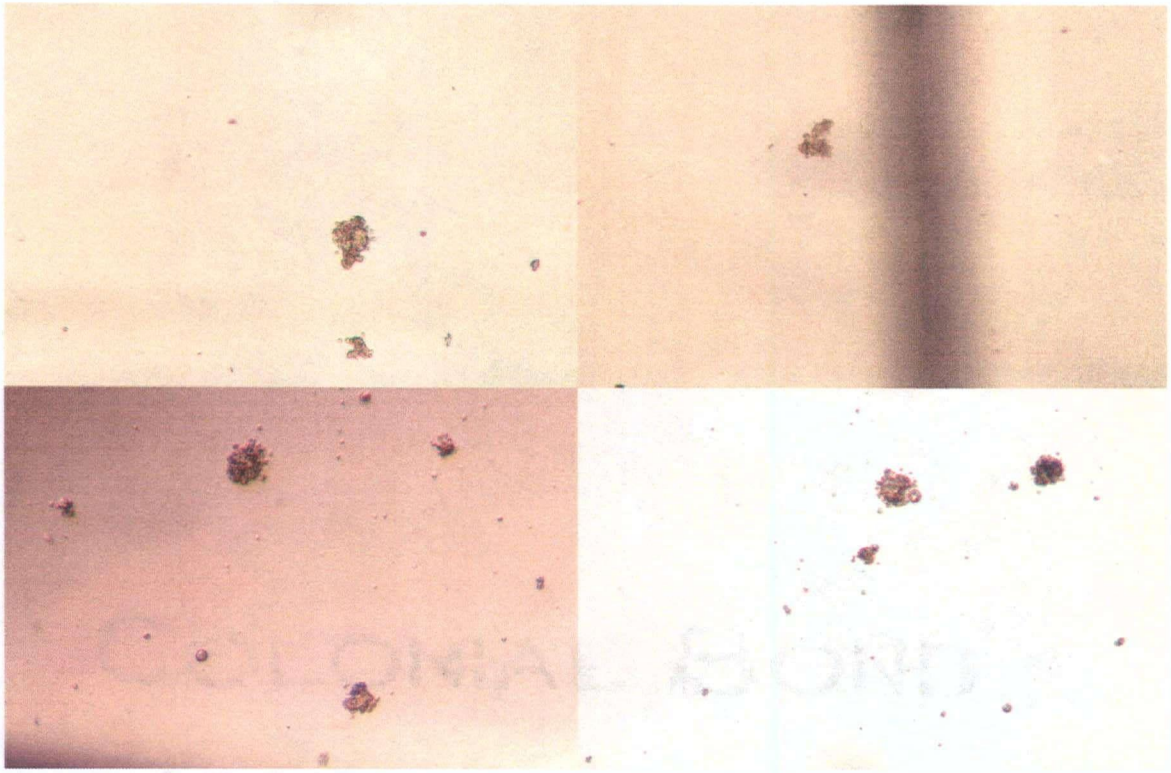

Figure 3.2 **Pictures of cellular aggregates that were not considered as EBs.**

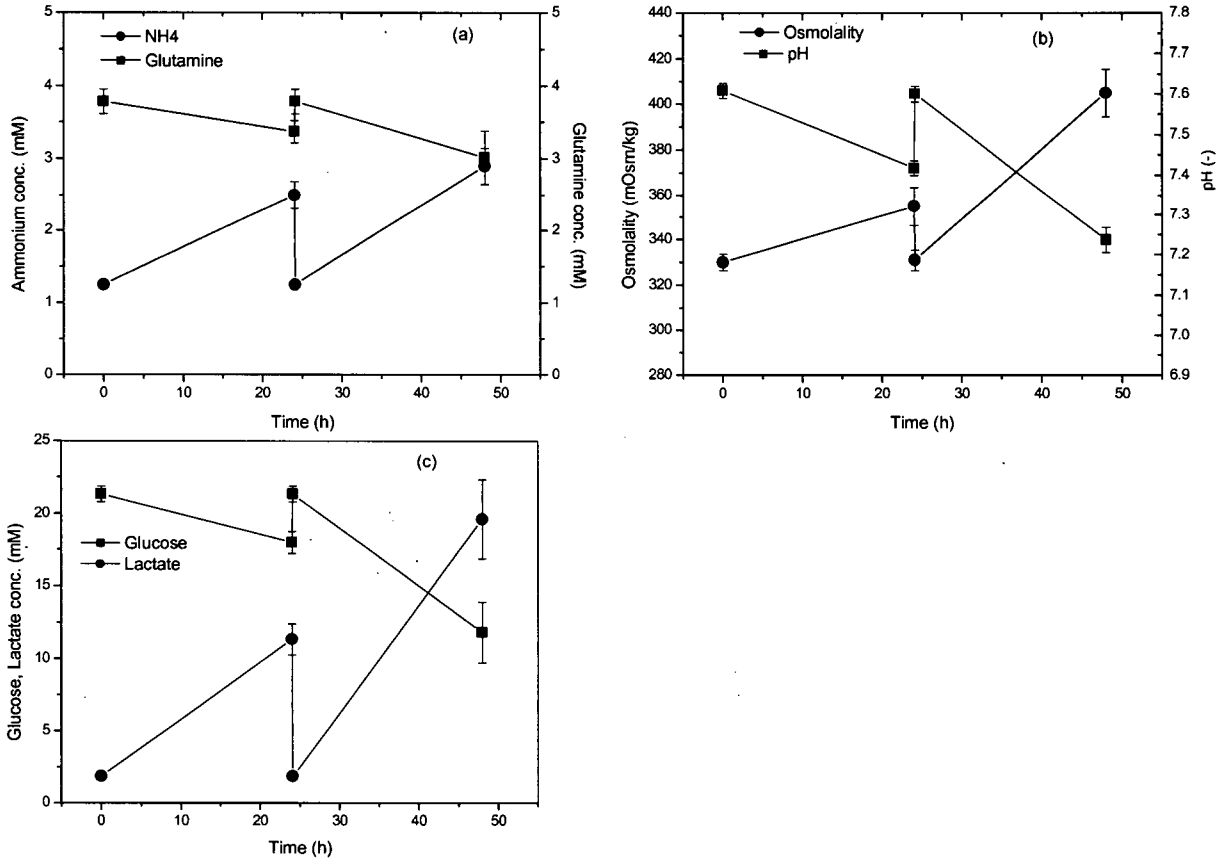

Figure 3.3 **Metabolic, pH and osmolality profiles of R1 mouse ESC grown on irradiated feeders following the conventional protocol of exchanging medium at 24 h and harvesting the cells at 48 h after inoculation in a maintenance medium of pH 7.6. Panel (a) shows glutamine and ammonium profiles, panel (b) depicts pH and osmolality profiles, and, panel (c) shows the glucose and lactate profiles. Values shown are mean ± SEM of three independent experiments.**

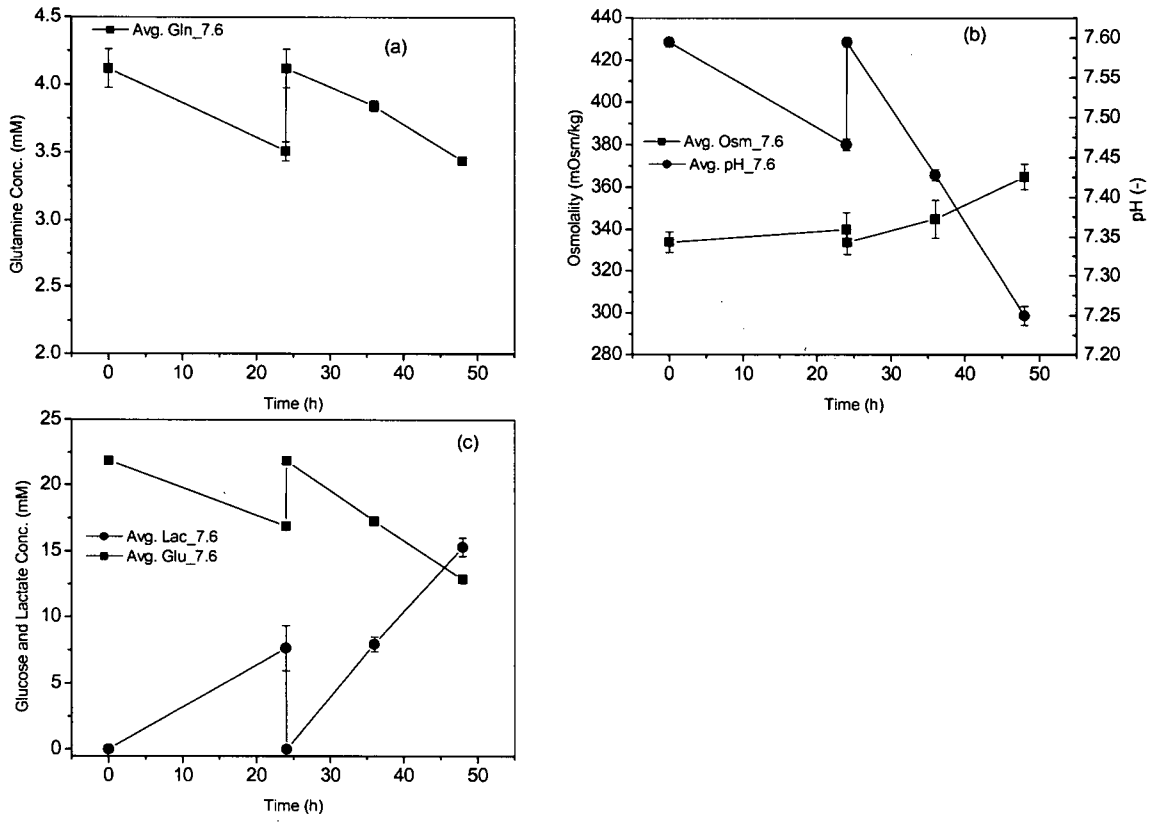

Figure 3.4 Metabolic, pH and osmolality profiles of R1 mouse ESC grown on 0.1% gelatin coated dishes following the conventional protocol of exchanging medium at 24 h and harvesting the cells at 48 h after inoculation in maintenance medium of pH 7.6. Panel (a) shows glutamine, panel (b) depicts pH and osmolality profiles and panel (c) shows the glucose and lactate profiles. Values shown are mean ± SEM of three independent experiments.

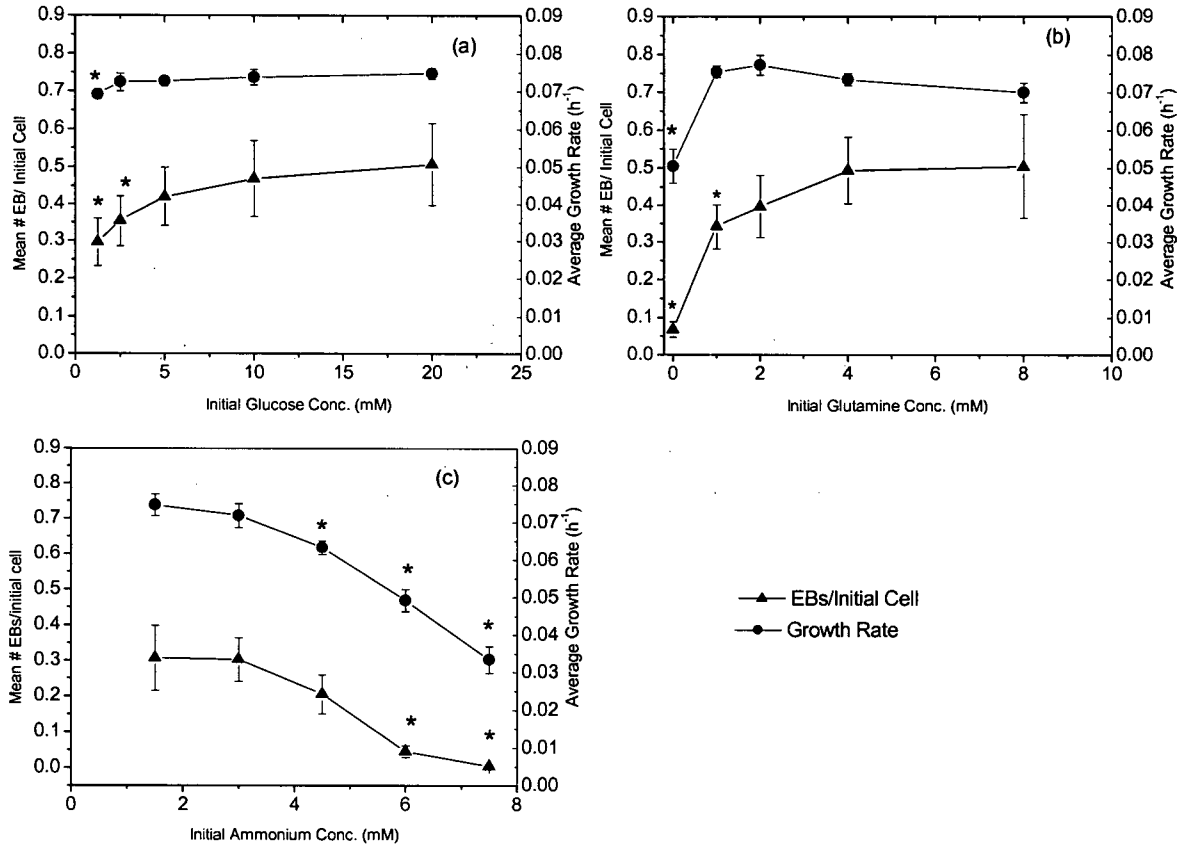

Figure 3.5 **Growth rate and mean EBs/initial cell inoculated of mouse ESC line R1 as a function of (a) initial glucose concentration (b) initial glutamine concentration and (c) initial ammonium concentration following conventional protocol of exchanging medium 24 h following inoculation and harvesting the cells 48 h after inoculation. The standard maintenance medium contains 20-22 mM glucose, 4 mM glutamine and generally around 1.5 mM ammonium. Values shown are mean ± SEM of seven experiments (* $p < 0.05$ compared to (a) 20 mM glucose (b) 4 mM glutamine and (c) 1.5 mM ammonium by paired t-test). Figure legend applies to all panels.**

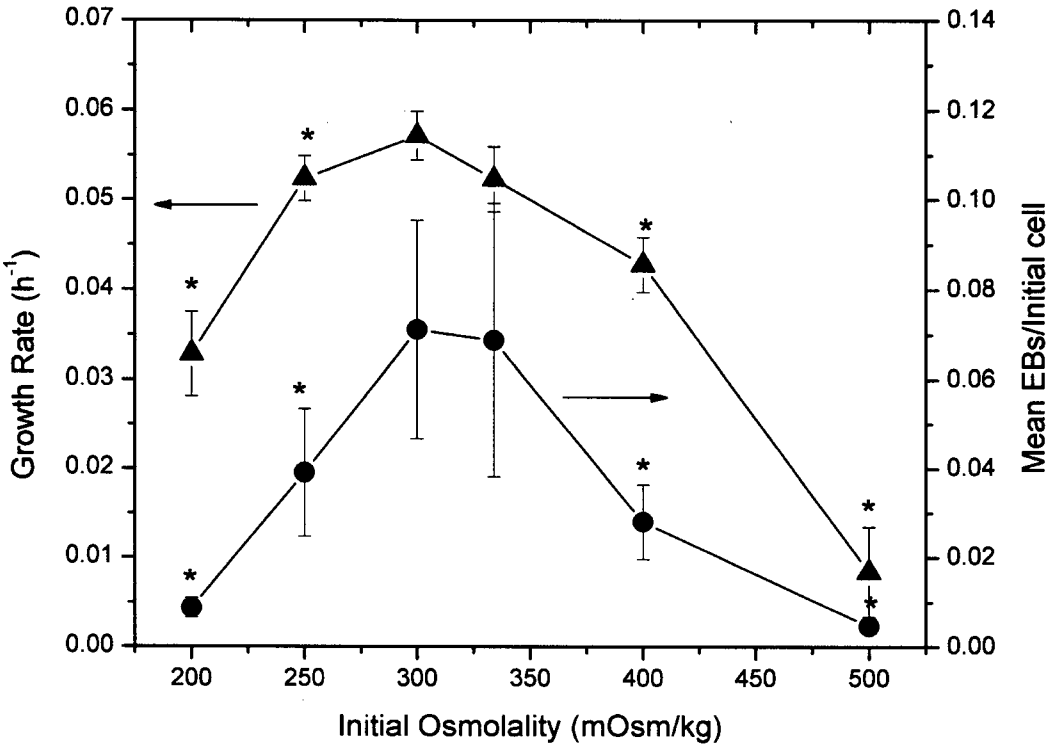

Figure 3.6 **Growth rate and mean EBs/initial cell inoculated of mESC line R1 as functions of medium osmolality following conventional protocol of exchanging medium 24 h following inoculation and harvesting the cells 48 h after inoculation. Values shown are mean ± SEM of fifteen experiments (* $p < 0.05$ compared to osmolality of 300 mOsm/kg by paired t-test).**

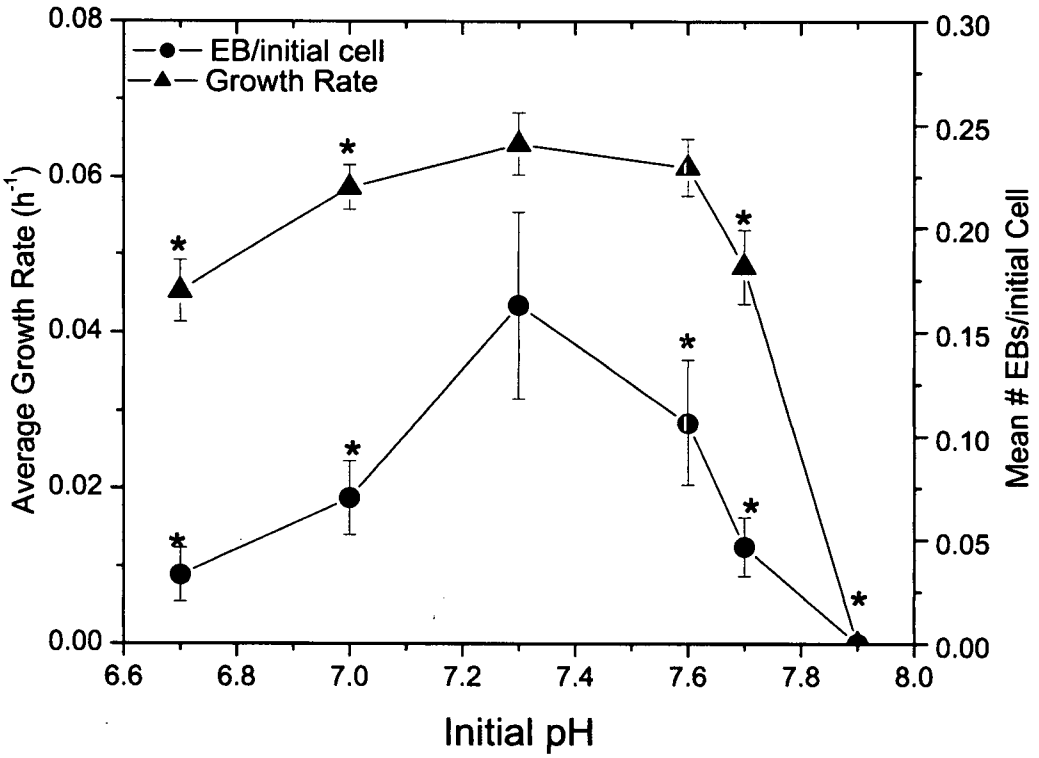

Figure 3.7 **Growth rate and mean EBs/initial cell inoculated of R1 mouse ESC as functions of initial pH following conventional protocol of exchanging medium, pre-equilibrated at the same pH, 24 h following inoculation and harvesting the cells 48 h after inoculation. Values shown are mean ± SEM of thirteen experiments (* $p < 0.05$ compared to pH 7.3 by paired t-test).**

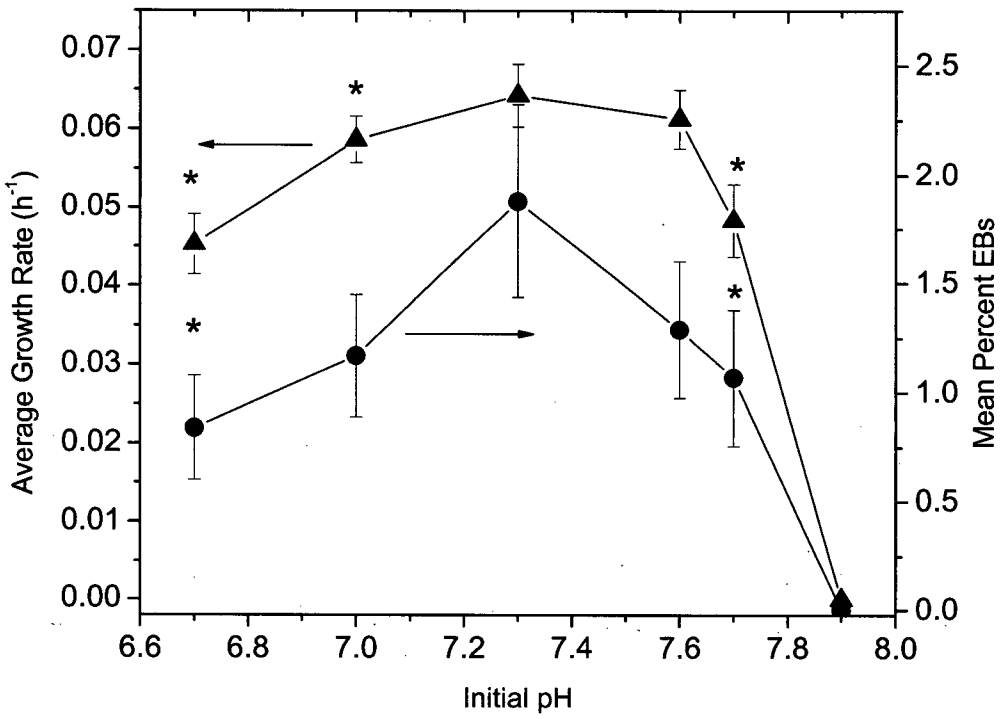

Figure 3.8 **Growth rate and mean % EBs of R1 mouse ESC as functions of initial pH following conventional protocol of exchanging medium, pre-equilibrated at the same pH, 24 h following inoculation and harvesting the cells 48 h after inoculation. Values shown are mean ± SEM of thirteen experiments (* $p < 0.05$ compared to pH 7.3 by paired t-test).**

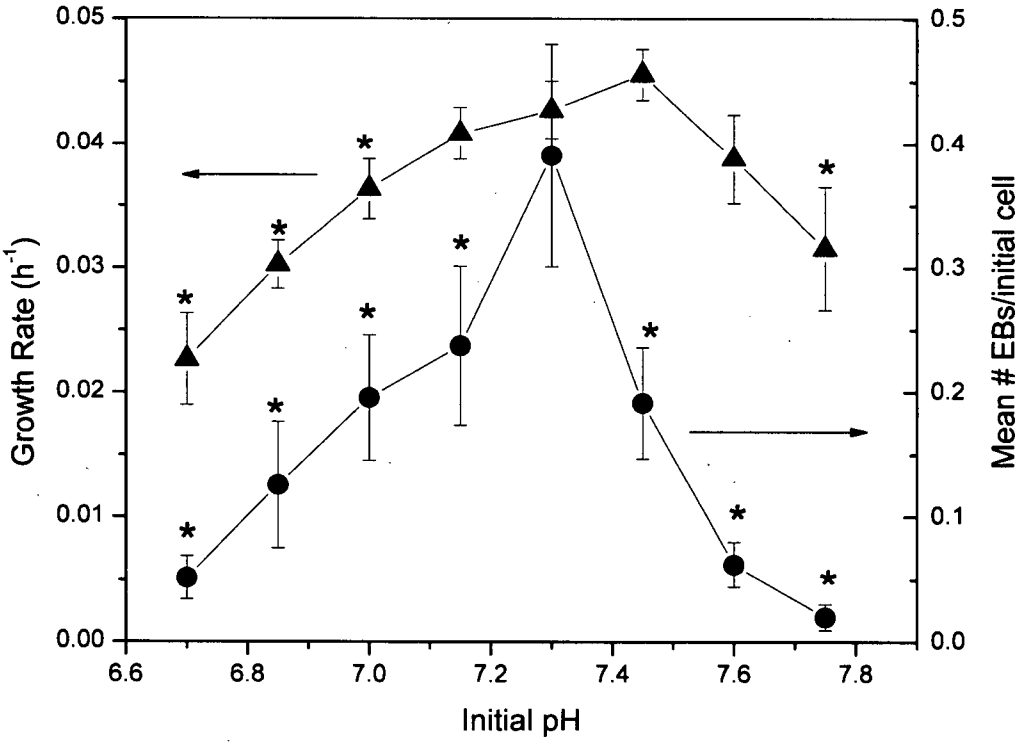

Figure 3.9 **Dose response analysis of growth rate and mean EBs/initial cell inoculated of mESC line R1 as functions of initial pH for two passages (96 h total exposure to various pH) following conventional protocol of exchanging medium, pre-equilibrated at the same pH, at 24 and 72 h following inoculation and harvesting the cells 48 and 96 h after inoculation. Values shown are mean ± SEM of seven experiments (* $p < 0.05$ compared to pH 7.3 by paired t-test).**

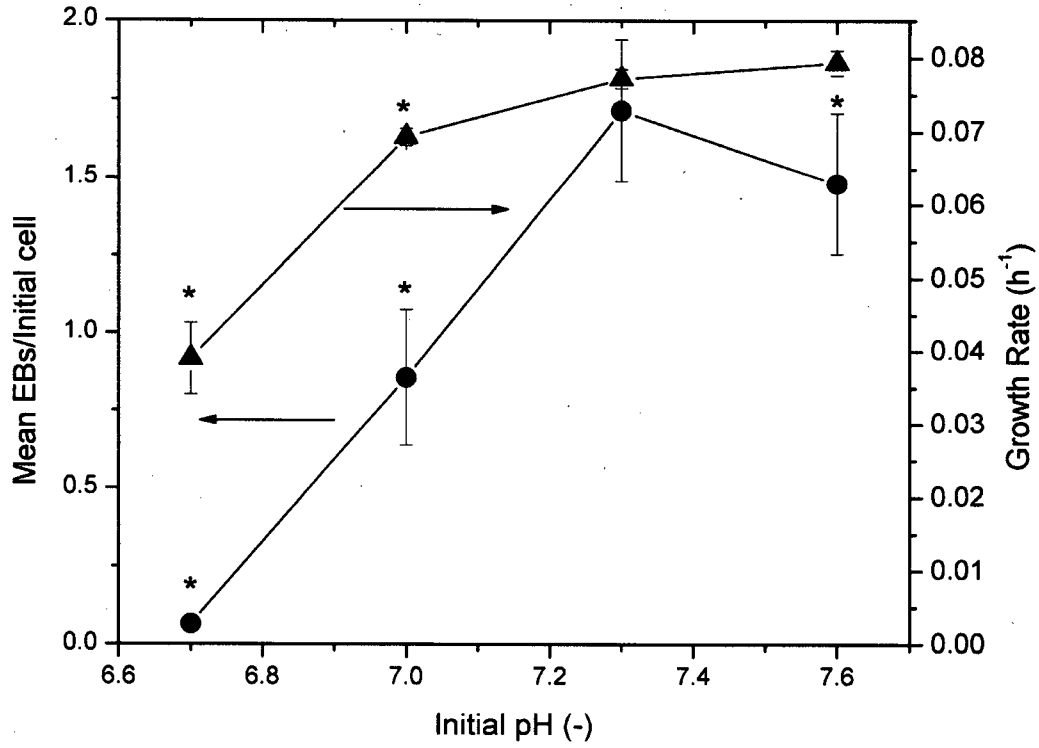

Figure 3.10 **Growth rate and mean EBs/initial cell inoculated of mESC line EFC as functions of initial pH following conventional protocol of exchanging medium, pre-equilibrated at the same pH, 24 h following inoculation and harvesting the cells 48 h after inoculation. Values shown are mean ± SEM of five experiments (* $p < 0.05$ compared to pH 7.3 by paired t-test).**

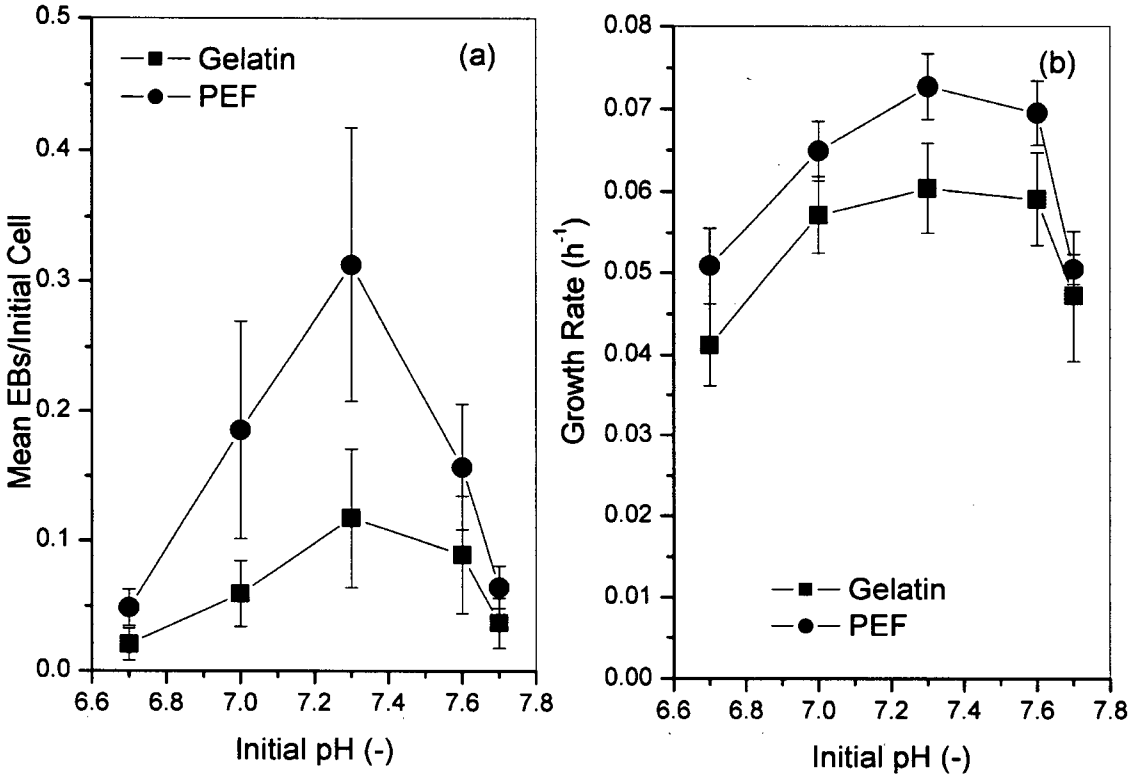

Figure 3.11 **(a) Mean number of EBs obtained per initial cell inoculated as a function of initial pH when the inoculum R1 mouse ESC were previously grown on 0.1% gelatin-coated dishes or on irradiated feeders. Two-way ANOVA analyses found significant differences both in growth rate and in mean EB/initial cell as a function of pH as well as previous growth history. (b) Growth rate as a function of initial pH when the inoculum R1 mouse ESC were previously grown on 0.1% gelatin-coated dishes or on irradiated feeders. All values shown are mean ± SEM of at least five independent experiments.**

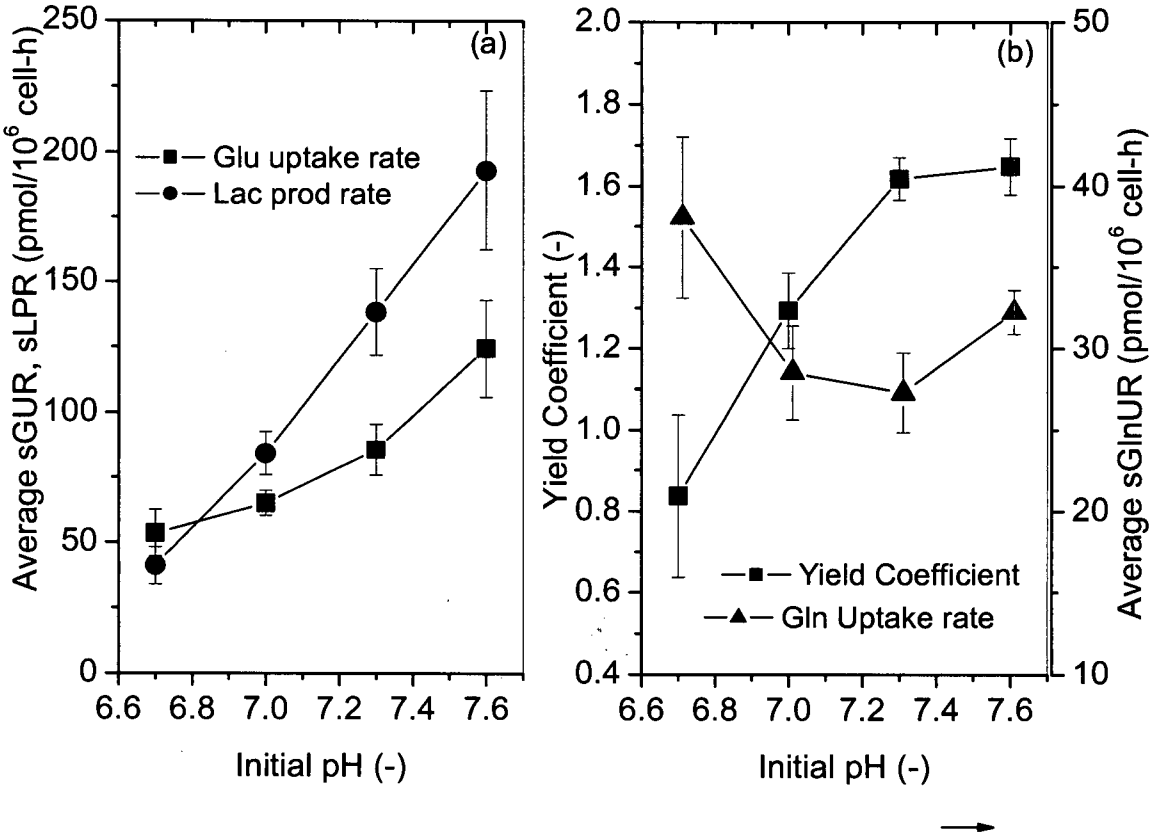

Figure 3.12 **(a) Cell specific average glucose uptake rate (sGUR) and lactate production rate (sLPR) as functions of initial pH of mESC line R1 used in the pH dose-response analyses depicted in Figure (3.5). (b) Lactate to glucose yield coefficient ($Y_{Lac/Glu}$) and average glutamine uptake rate (sGlnUR) as functions of initial pH of R1 mouse ESC used in the pH dose-response analyses depicted in Figure (3.5) Values shown are mean ± SEM of a subset of seven experiments.**

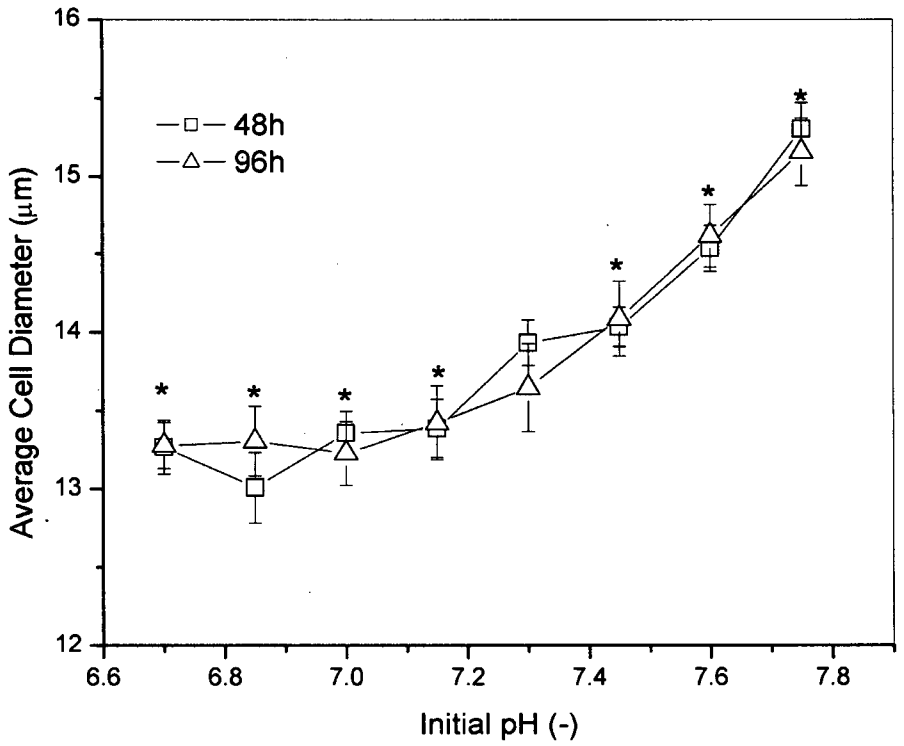

Figure 3.13 **Variation of average diameters of R1 mouse ESC as a function of initial pH (at initial osmolality of 300 mOsm/kg for cultures with initial pH of 6.7 – 7.3 and initial osmolality of 320 – 345 mOsm/kg for cultures with initial pH 7.45 – 7.75) used in the dose-response analyses depicted in Figures 3.5 and 3.6. Values shown are mean ± SEM of a subset of ten independent experiments. (* $p < 0.05$ compared to pH 7.3 by paired t-test).**

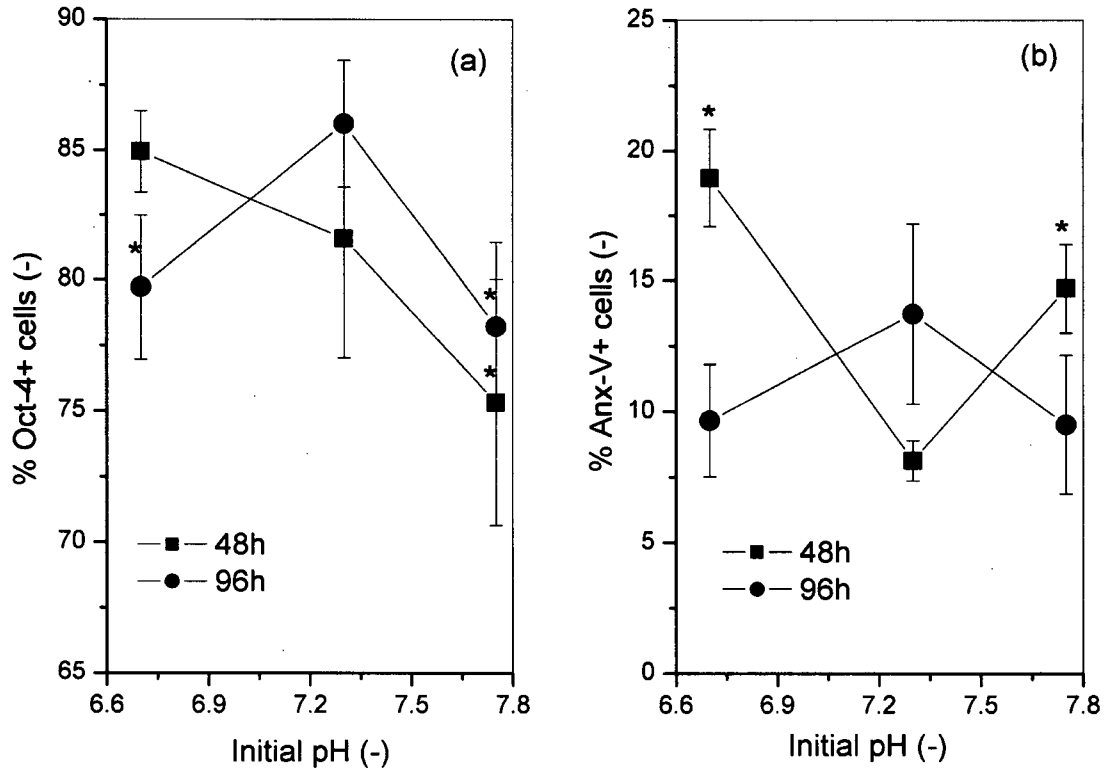

Figure 3.14 **(a) Variation of %Oct-4 positive cells from mESC line R1 as a function of initial pH during the dose-response experiments carried out for 48 and 96 h. (b) Variation of % Annexin-V positive cells from mESC line R1 as a function of initial pH during the dose-response experiments carried out for 48 and 96 h. Values shown are mean ± SEM of five experiments. (* $p < 0.05$ compared to pH 7.3 by paired t-test).**

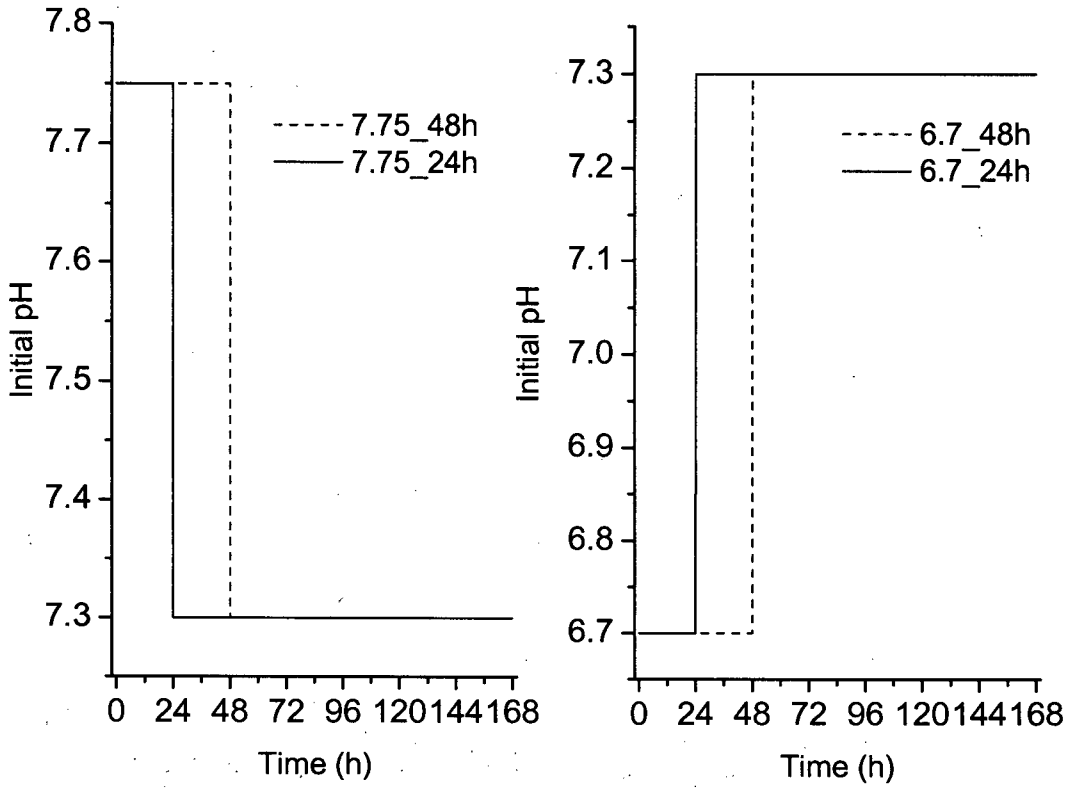

Figure 3.15 Reversibility of pH effect on responses of R1 cells. Variation of initial pH (6.7 and 7.75) as a function of time.

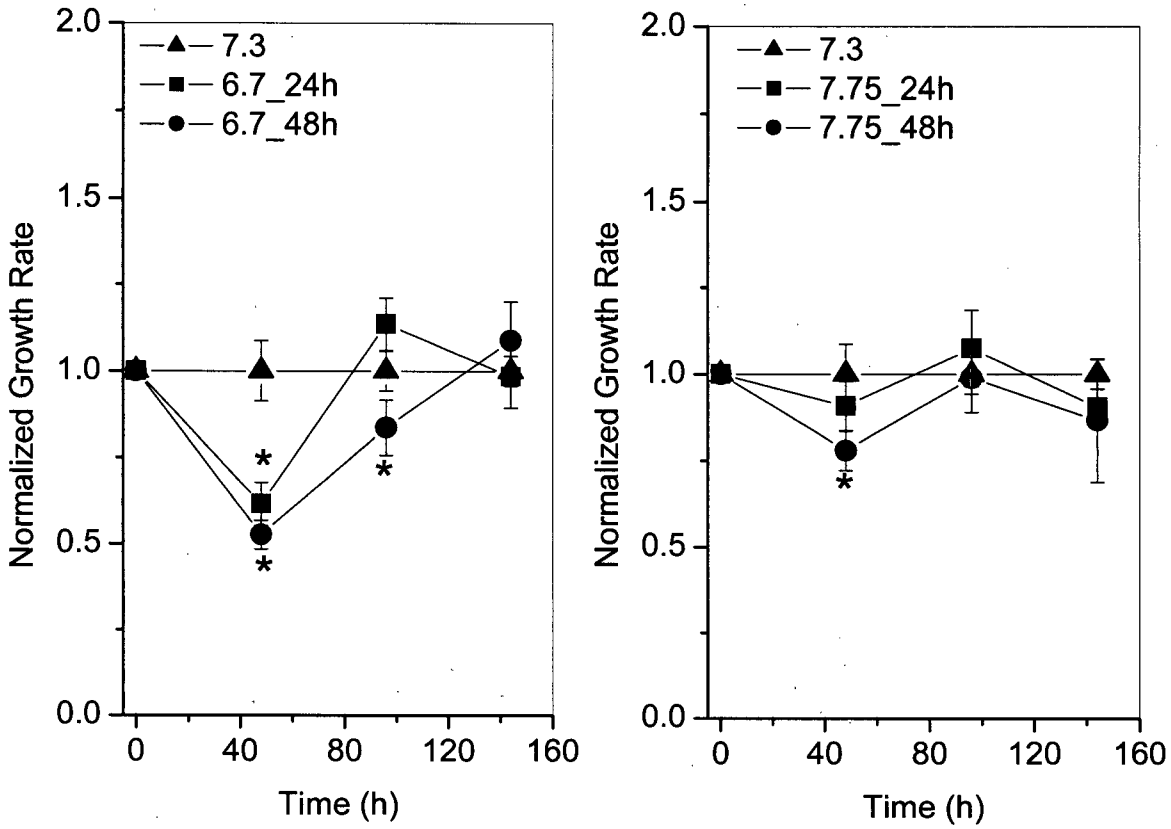

Figure 3.16 **Reversibility of pH effect on responses of R1 cells. Average normalized growth rates of R1 cells as functions of pH and duration of exposure. Cells were harvested after 48 h of setting up the experiment and cells were further grown for 48 or 96 h under pH 7.3 conditions. Values shown are mean ± SEM of an experiment carried out in triplicate. (* $p < 0.05$ compared to normali=zed pH 7.3 by paired t-test).**

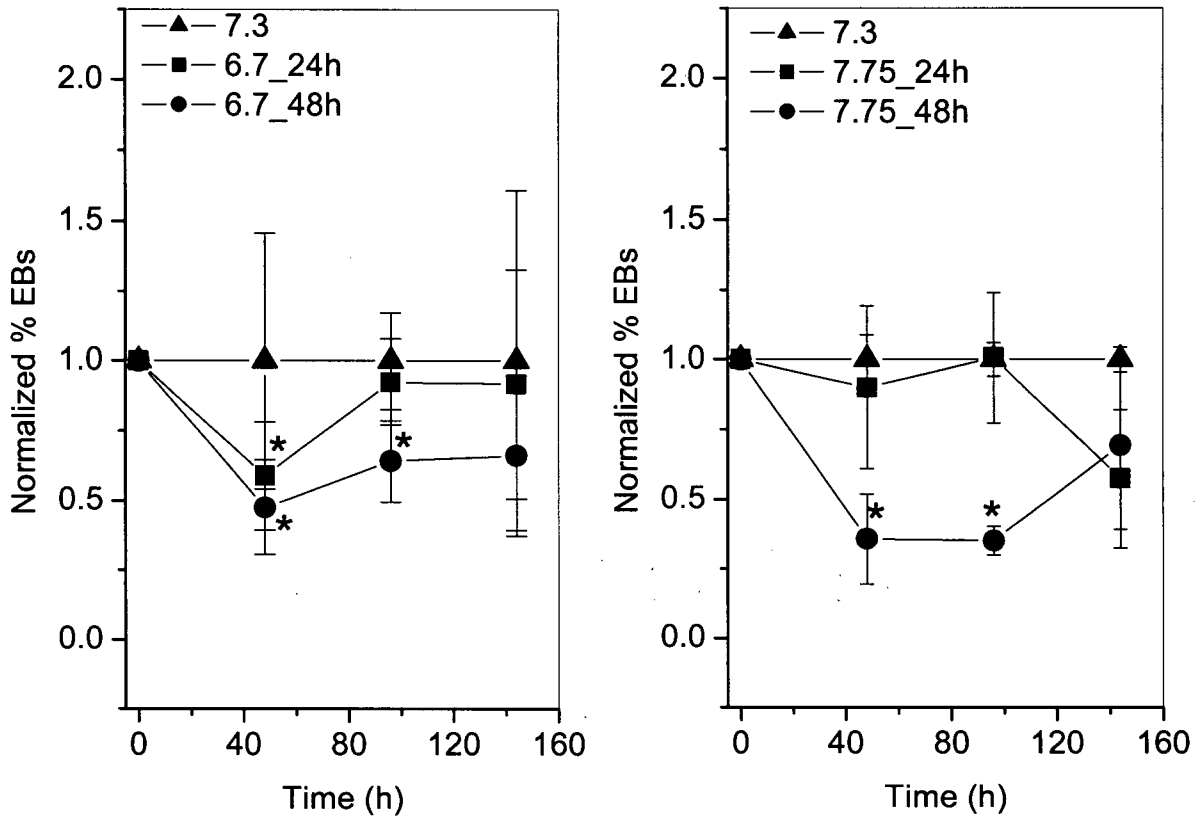

Figure 3.17 **Reversibility of pH effect on responses of R1 cells. Average normalized % EBs of R1 cells as functions of pH and duration of exposure. Cells were harvested after 48 h of setting up the experiment and cells were further grown for 48 or 96 h under pH 7.3 conditions. Values shown are mean ± SEM of an experiment carried out in triplicate. (* $p < 0.05$ compared to normalized pH 7.3 by paired t-test).**

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4 Basal Medium Composition and Serum or Serum Replacement Concentration Modulate Responses of Murine Embryonic Stem Cells[c]

4.1 Introduction

Stem cells are defined as cells that, at the single-cell level, are capable of both self-renewal and differentiation to specialized cell types[1]. Pluripotent embryonic stem cells (ESC) have been derived from the inner cell mass of blastocyst stage embryos of murine, porcine, primate as well as human sources. Human embryonic stem cells (hESC) exhibit indefinite replicative and developmental potential to differentiate into derivatives of all three germ layers even after prolonged culture. Whereas hESC-derived cells have tremendous potential in many experimental and therapeutic applications, they have a doubling time of the order of 35-40 h[2, 3] and extensive cell expansion will be required to meet the requirements for human therapies. Maintaining a large pool of uncommitted stem cells without compromising their developmental potential in culture represents a great bioprocessing challenge.

Murine ESC (mESC) can self-renew in vitro with leukemia inhibitory factor (LIF) in the presence of serum or mouse feeder layer cells, to obtain daughter cells that maintain their multilineage potential[4,5]. Similarly, derivation and propagation of hESC and establishment of permanent lines were first carried out in the presence of serum and mouse embryonic fibroblasts as feeder cells[6,7]. When mESC are maintained in feeder-free conditions, the fraction of undifferentiated cells begins to decline rapidly with consecutive passaging[8,9], and cells with a non-ES cell morphology quickly arise in culture, despite the presence of LIF. Thus, additional factors and/or the cell-cell contact provided by the feeders appear to be required to fully support the self-renewal of mESC.

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Removal of both LIF and feeders cause mESC to spontaneously differentiate, following a reproducible pattern of development that in many ways recapitulates early embryogenesis, including the formation of a complex three-dimensional architecture wherein cell-cell and cell-matrix interactions are thought to support the development of the three embryonic germ layers[10, 11]. As mESC differentiate in suspension or semi-solid culture, they typically form three-dimensional aggregates called embryoid bodies (EBs). Over time, these EBs increase in cell number and complexity as cells from the three germ layers are formed. Generation of a wide variety of apparent cell types – cardiac myocytes, hematopoietic cells, neurons, pancreatic islet cells, etc. – from mESC has been reported[12-15]. In an ideal scenario, differentiation of ESC could be directed to a pure population of the desired cell type. Culture conditions have been described that exclusively permit the formation of neural progenitor cells from mESC, albeit at very low cell frequencies[16]. In most cases, however, the knowledge to precisely control the mouse or human ESC fate decisions is still far from complete. Consequently, the most robust method for generating the most differentiated cell types is through the embryoid body formation step. Standard methods of EB formation include hanging drop, liquid suspension and methylcellulose based semi-solid cultures. These culture systems are thought to maintain a balance between the ESC aggregation necessary for EB formation and preventing EB agglomeration for efficient cell growth and differentiation[17].

The media commonly used in mammalian cell culture provide essential nutrients that are incorporated into dividing cells, such as amino acids, fatty acids, sugars, ions, trace elements, vitamins and co-factors, as well as molecules that are necessary for maintaining a proper chemical environment for the cells. Most media (e.g., minimal essential medium, Dulbecco's modified Eagle's medium (DMEM)) were developed specifically for use with serum supplementation and high-density growth of cells[18, 19]. In contrast, Ham's nutrient mixtures F12 and F10 were tailored towards growing specific cells (e.g., CHO, fibroblasts) at low density with a minimal amount of undefined protein added so that the effects of different nutrient components could be studied[20, 21]. The DMEM:F12 (1:1 vol/vol) medium was originally developed for growing cells in defined serum-free conditions[22] and can support high cell densities with serum or defined growth factor

supplements in serum-free media. These supplements, depending on the cell type and expected outcome of the culture, include cytokines, buffers, antioxidants, surfactants and shear protectants. The theoretical basis for medium development[23] as well as the protocols for developing serum-free media[21, 24-26] have been reviewed.

In addition to serum or serum substitutes, the basal medium formulation can also profoundly influence the expression of differentiated functions as has been demonstrated for the case of primary hepatocytes[27-32] and the human intestinal cell line Caco-2[33, 34]. It is well known that the conditions to which pre-implantation stage mammalian embryos are exposed in culture can have a profound effect on their subsequent development including physiology[35], metabolism[36,37] and viability[38,39]. Inappropriate media formulations, such as those lacking amino acids, have been shown to have profound negative effects on many aspects of embryo physiology. This was highlighted in a series of studies examining the rate of mRNA synthesis in pre-implantation embryos cultured in a medium at low (85 mM) or at high (125 mM) sodium chloride concentrations. It was determined that mRNA synthesis was reduced by 20% at the higher salt concentration and, furthermore, the resultant mRNA was less stable[40]. It was subsequently shown that the expression of nine genes in a medium containing amino acids was closer to that of *in vivo*-developed embryos than blastocysts developed in a simple medium lacking amino acids[41]. Micro-array technology has demonstrated that, out of a total of 22690 transcripts and variants assessed, on average only 39% were expressed by *in vivo*-developed mouse blastocysts. Whereas blastocysts cultured in a medium lacking amino acids had aberrant expression of 114 genes compared with *in vivo*-developed embryos, those blastocysts cultured in an amino acid-containing medium had only 29 genes misexpressed[42]. Similarly, the imprinting status of the H19 gene was shown to be aberrant in a medium lacking amino acids compared to an optimized medium containing amino acids[43].

As has been extensively reviewed[44, 45], serum functions as a source of hormones and essential nutrients, and alters the physiological/physiochemical properties of the medium. The use of serum to culture human cells for clinical applications is highly undesirable due to the risk of exposure to various pathogens. The general culture conditions for mESC

maintenance are well established. The most critical component of an ESC maintenance medium is serum. Serum can be highly variable from lot to lot, and can contain a variety of growth factors and other undefined components that can affect *in vitro* ESC plating efficiency, growth and can also promote differentiation of mESC. Hence only pre-screened batches of serum are used to maintain mESC in an undifferentiated state. Pre-screening serum, however, is both cost and labor intensive and considerably increases the overall cost of mESC culture. A commercial serum-free formulation, Knockout™ serum replacement (SR), has found widespread use both in mouse and human ESC culture as it directly replaces serum and maintains ESC in an undifferentiated state. The product is a better-defined (although the complete formulation is not disclosed) growth supplement and results in less ESC spontaneous differentiation. The manufacturer (Invitrogen, Carlsbad, CA) recommends it to be used for growth and maintenance of undifferentiated ESC at the same concentration as serum is used, generally 15%.

Compared to hematopoietic stem cells, for which quantitative functional assays of potential have been defined and validated, relatively less well developed assays exist for mouse or human ESC. Self-renewal can be measured by the ability of ESC to proliferate in culture while maintaining an undifferentiated colony morphology and teratoma formation capacity. The most rigorous *in vivo* assay to establish the functionality of cultured mESC is blastocyst injection followed by the measurement of their ability to give rise to chimeric mice. This assay provides proof that there has been ESC contributions to all adult tissue, including germ cells[46]. Two *in vitro* assays have been used extensively as surrogates for chimera formation when testing culture reagents or examining the consequences of genetic manipulation. The colony-forming cell (CFC) assay is used to determine the plating efficiency of ESC populations under various conditions and thus may be considered indicative of self-renewal potential. Formation of embryoid bodies (EBs) can be performed at a clonal level *in vitro* and reflects multilineage differentiation potential[47]. Assessment of the pluripotency of mouse ESC has also relied on the expression of selected molecular markers. These have included alkaline phosphatase, the POU transcription factor Oct-4, and the stage-specific embryonic antigen 1 (SSEA-1). We have recently demonstrated that the embryoid body

formation assay more rapidly detects decreasing mESC numbers than phenotypic characterization by Oct-4 and SSEA-1 under differentiating culture conditions[48].

In the present study, the murine ES cell lines, R1 and EFC, have been used as model systems to study the influence of basal medium composition and concentration of serum or SR on proliferation and maintenance of EB forming ability. Since EB formation is generally considered as the first step in most mESC differentiation protocols, it is important to study those culture variables that would influence the EB yield from differentiating mESC. In addition, defining the ranges of different variables that maximize the EB yield would accelerate bioprocess R&D in this field. We have examined the effect of basal medium composition along with serum or SR concentration in mESC cultures following the conventional passaging protocol and show that growth rate, EB formation potential and metabolism are greatly influenced by these variables. In addition, dose-response experiments were carried out to determine the optimum serum or SR concentration when two different basal media are present. These studies led to a series of time course experiments to show that the widely-used SR concentration of 15% is not optimal especially when an enriched basal medium such as DMEM:F12 is employed.

4.2 Materials and Methods

All reagents were obtained from StemCell Technologies Inc. [STI], Vancouver, BC, unless otherwise indicated.

4.2.1 Growth of Mouse Embryonic Fibroblasts (MEF)

MEF were cultured as described in Chapter 3 section 3.2.1.

4.2.2 Growth of Mouse Embryonic Stem Cells (mESC)

The mESC were thawed and cultured for 2 passages on irradiated feeders in the maintenance medium (DMEM + 15% serum). To prepare the cells for exposure to serum or SR concentrations, they were harvested by trypsinization, resuspended in ESC maintenance medium, and preplated on tissue culture plates for 15-20 minutes at 37°C, 5% CO_2, in order to deplete contaminating MEF. At the end of this pre-plating step, the non-adherent and loosely adherent ESC were collected by gently washing the surface of the tissue culture plate. The cells were then centrifuged, resuspended in ESC maintenance medium and viable cell numbers were counted on a hemocytometer with trypan blue dye or on an automated cell counter (Cedex, Innovative Directions, Pinole, CA) to distinguish live from dead cells. The frequency of contaminating MEF in the undifferentiated (day 0) ESC samples was estimated to be less than 0.2% based on cell size during counting (MEF cells are easily distinguishable from mESC as they are bigger in size (generally $\geq$16 μm in diameter) and appear to have irregular morphology). All cultures for the serum or SR experiments initially contained 2×10^4 cells/cm^2 in 4 mL of medium in a 60-mm gelatinized dish. The conditioned medium from each of these cultures was collected after 24 hours and replaced with fresh medium at the same serum or SR concentration. The following day, the cultures were harvested by trypsinization and the cell concentrations were determined as described above.

4.2.3 Determination of Growth Rate

The growth rate of both mESC lines was determined as described in Chapter 3 section 3.2.3. (Eq. 3.1)

4.2.4 Preparation of Media for Serum or Serum Replacement Dose-Response Experiments

The ES maintenance medium was prepared as described above using either DMEM or DMEM:F12 (Invitrogen Life Technologies, Burlington, ON) as the basal medium. Serum or SR was added at three different concentration levels: 1.67, 5 or 15% (v/v). To ensure that the pH values of both the DMEM- and DMEM:F12-based media are the same, a powdered DMEM formulation without glucose, glutamine, sodium bicarbonate, sodium pyruvate and phenol red (Sigma-Aldrich, Oakville, ON, Cat #D5030) was used. The sodium bicarbonate concentration was then adjusted to obtain the desired pH since the equilibrium bicarbonate concentration ($[HCO_3^-]$; mM) at 37°C can be related to the partial pressure of CO_2 (pCO2, mmHg) in the gas phase and the medium pH via the following equation[49]:

$$\log[HCO3^-] = pH + \log[pCO2] - 7.54. \quad (4.1)$$

Both the DMEM- and DMEM:F12-based media were adjusted to equilibrate at pH 7.3 in a 5% CO_2 atmosphere. When DMEM was used as the basal medium, the average osmolality of the ES maintenance medium containing 15% ES qualified serum was 334±5 mOsm/kg and, with 15% SR, it was 328±7 mOsm/kg. With DMEM:F12 as the basal medium, the 15% serum-containing medium had an average osmolality of 327±4 mOsm/kg while the medium containing 15% SR had an average osmolality of 322±5 mOsm/kg. On average, the changes in osmolality due to the lower concentrations of serum or SR used were within 10% of the average osmolality of the ES maintenance media with 15% serum or SR. Based on the osmolality dose–response experiments carried out separately (data shown in Chapter 3), such small changes in medium osmolality should not affect the proliferation and EB forming ability of either the R1 or

EFC cells. Both the serum and SR experiments were carried out on gelatinized 60-mm tissue culture dishes (Sarstedt, Montreal, PQ).

4.2.5 Measurement of Glucose, Lactate and Glutamine

These measurements were taken and metablic rates were determined as described in Chapter 3, section 3.2.5.

4.2.6 Preparation of mESC for Dose-Response Experiments

mESC lines were maintained and used for these experiments as described in Chapter 3, section 3.2.6.

4.2.7 Embryoid Body Formation Assay

The EB assays were initiated exactly as described in Chapter 3, section 3.2.7.

4.2.8 Statistics

Data are reported as mean ± standard error of the mean (SEM), unless otherwise noted. Statistical comparisons were performed using a two-tailed paired Student's t-test. An asterisk (*) was used to denote statistical significance ($p < 0.05$).

4.3 Results

The influence of serum, SR and basal medium (DMEM or DMEM:F12) on the 48 h proliferation response of both R1 and EFC mESC is shown in Figure (4.1). The EFC cells displayed a higher growth rate compared to the R1 cells under all conditions tested. In DMEM, both cell lines exhibited an increase in growth rate as a function of serum concentration that saturated at approximately the 5% level Figure (4.1a). The growth rate at the 1.67% serum level was significantly ($p < 0.05$) lower than at the recommended 15% level. A reduced dependence on serum concentration was observed when DMEM:F12 was used as the basal medium (Figure 4.1b). Even 1.67% serum was sufficient to maintain the growth rate of both cell lines within ~85% of the maximum observed with 15% serum. DMEM:F12 supported higher growth rates of both cell lines compared to DMEM at all concentrations of serum tested. In the dose-response experiments using DMEM with SR (Figure 4.1c), 5% SR again was sufficient for maximal growth, with even a significantly decreased ($p < 0.05$) growth rate at 15% SR for the EFC cells. The growth rate of both cell lines also decreased slightly at 15% SR with DMEM:F12 as the basal medium (Figure 4.1d).

The ability of both R1 and EFC mESC to form EBs (represented by the EB yield which is defined as the mean number of EBs/initial cell inoculated) was also tested for the two basal media (DMEM and DMEM:F12) and as a function of the serum or SR level. The EFC cells had much higher EB forming capacities than R1 cells under all conditions tested (Figure 4.2). In DMEM, the R1 cells exhibited a serum dose-dependent increase in EB yield while, for the EFC cells, the yield saturated at approximately a 5% serum concentration (Figure 4.2a). The response of both cell lines to SR dosages with DMEM as the basal medium is, however, quite different and exhibited an apparent high dose inhibition at a 15% SR concentration, e.g., the EB yield of the EFC cell line showed a decline at this level that was significantly lower ($p < 0.05$) than at either the 1.67 or 5% SR levels (Figure 4.2c). For both cell lines, 5% SR maximized the EB yield. The results of the serum dose-response experiments for both the R1 and EFC cells in DMEM:F12 are shown in Figure (4.2b). The EFC cells exhibited a dose-dependent increase in EB

formation potential while the response of the R1 cells was essentially independent of the serum concentration used. The EB yields of both cell lines were higher at 1.67% SR in DMEM:F12 compared to DMEM (Figures 4.2d vs. 4.2c). The EFC cells had a decreased ability to form EBs with increasing SR concentration with DMEM:F12, with a significantly ($p < 0.05$) lower EB yield at 15% SR compared to either 1.67 or 5% SR (Figure 4.2d). The 1.67% SR maintained the maximal EB forming capacity of EFC cells with higher concentrations of SR reducing the EB yield. The response of the R1 cells under these conditions, on the other hand, was essentially similar to that observed when these cells were exposed to serum with DMEM:F12. Again as was the case with growth rate, DMEM:F12, compared to DMEM, supported higher EB yields for both cell lines at all concentrations of serum tested.

The metabolism of both cell lines is influenced depending on whether serum or SR is used in conjunction with DMEM or DMEM:F12 as the basal medium. The changes in the cell specific glucose uptake rate (sGUR) of both cell lines when exposed to serum or SR with the two different basal media are shown in Figure (4.3). Contrary to the growth rate and EB formation data shown in Figures (4.1) and (4.2), R1 cells generally display a higher sGUR compared to EFC cells. Figure (4.3a) shows a dose-dependent increase in the sGUR of R1 cells with serum concentration in DMEM that saturates at the 5% serum level. The sGUR of EFC cells, on the other hand, is virtually independent of the serum concentration used. Both cell lines exhibit almost identical sGUR profiles when serum is used with DMEM:F12 (Figure 4.3b). Under these conditions, the glucose uptake rates are essentially independent of the serum concentration. When SR is present with DMEM, both cell lines show a dose-dependent increase in sGUR that appears to saturate at the 5% level as shown in Figure (4.3c). The response of both cell lines to SR with DMEM:F12 is similar to serum with DMEM:F12 – sGUR is independent of the SR concentration used (Figure 4.3d).

The cell-specific lactate production rates (sLPR) of both cell lines when exposed to various concentrations of serum or SR with DMEM or DMEM: F12 as the basal medium are shown in Figure (4.4). Since the R1 cells displayed higher sGUR under all conditions

tested as shown in Figure (4.3), they also exhibited higher sLPR values compared to the EFC cells. The R1 cells show an almost linear increase in sLPR as a function of serum concentration when DMEM is used as the basal medium, whereas the response of the EFC cells under these conditions is a decline in sLPR with increasing serum concentration (Figure 4.4a). Compared to Figure (4.3b) where both cell lines show almost identical sGUR responses, when serum is used in the presence of DMEM:F12, the sLPR response is quite different (Figure 4.4b). The R1 cells show a decline in sLPR as a function of serum concentration that appears to stabilize around 170 pmol/10^6 cell-h. The sLPR of the EFC cells, on the other hand, is unaffected by the serum concentration used and remains constant around 75 pmol/10^6 cell-h. In the presence of SR with DMEM as the basal medium, both cell lines display sLPR profiles that are essentially independent of the SR concentration (Figure 4.4c). When SR is used in conjunction with DMEM:F12, the sLPR of the R1 cells exhibited an apparent maximum at 5% SR, with lower sLPR values at both lower and higher SR concentrations. For the EFC cells, sLPR appeared to be almost independent of the SR concentration (Figure 4.4d).

Glutamine is used by most cell lines as a source of nitrogen and energy. The cell specific glutamine uptake rate (sGlnUR) of both the R1 and EFC cells is depicted in Figure (4.5). In the presence of serum with DMEM, the R1 cells showed an apparent linear increase in sGlnUR with serum concentration while the sGlnUR of the EFC cells was almost constant and independent of the serum concentration (Figure 4.5a). When DMEM:F12 is employed as the basal medium with serum, both cell lines exhibit sGlnUR values that are virtually independent of the serum concentration (Figure 4.5b). When SR is used, the sGlnUR of both cell lines is essentially independent of the SR concentration regardless of whether DMEM or DMEM:F12 is present as the basal medium. However, the magnitude of sGlnUR is quite different for the two basal media. For the R1 cells in DMEM, sGlnUR is around 12 pmol/10^6 cell-h while, for the EFC cells, it is around 6 pmol/10^6 cell-h (Figure 4.5c). However, in DMEM:F12, the sGlnUR of both cell lines is around 5 pmol/10^6 cell-h (Figure 4.5d).

The finding that a higher concentration (15%) of SR can be inhibitory for both the cell proliferation and EB forming ability of both the R1 and EFC cells was then further investigated. Three different SR lots (not previously used in our experiments) were acquired and the R1 cells were grown with 5% or 15% SR in either DMEM or DMEM:F12 for three consecutive passages to determine to what extent the observed responses might be sustained up to 144 h and in different SR lots. In DMEM, there was a gradual loss of EB forming ability as well as a decline in growth rate of R1 cells with consecutive passaging on gelatin, with all three SR lots behaving similarly (Figure 4.6a). Compared to the results obtained with 5% SR, there was a decline in both the growth rate and the EB yield of R1 cells when 15% SR was used (Figure 4.6b). On average, there was almost a 2-fold or more decline in the EB forming capacity of R1 cells exposed to 15% SR compared to 5% SR. The growth rate of R1 cells exposed to 15% SR was also lower by 22 – 25% (on average) compared to cells exposed to 5% SR. However, as was the case with 5% SR, the three different lots of SR behaved similarly at the 15% level. It was therefore clear that high concentrations of SR are inhibitory to both proliferation and EB formation in mESC lines. There was relatively little lot-to-lot variability.

In DMEM:F12 with 5% SR (Figure 4.6c), all three SR lots behaved similarly in terms of both the EB yield and growth rate of the R1 cells throughout the time course and there was a slow decline in both the EB yield and the growth rate of the R1 cells as a function of time due to consecutive passaging of these cells on gelatin. As shown in Figure (4.6d), when the R1 cells were exposed to 15% SR in DMEM:F12, there was on average a 2.5-fold or more decline in their EB forming capacity (compared to 5% SR) while the growth rate of these cells also declined by ~25%. However, the results obtained with all three SR lots were statistically indistinguishable at both the 5% and 15% SR levels. In DMEM:F12 with both 5% and 15% SR, the growth rate of the R1 cells declined significantly ($p < 0.05$) at 144 h compared to 48 h.

4.4 Discussion

Derivation and propagation of mESC have generally been carried out in the presence of fetal bovine serum (FBS) with mouse embryonic fibroblasts as feeder cells. Considerable attention is devoted to screening serum lots or purchasing pre-screened ESC-qualified serum prior to use because of lot-to-lot serum variability as well as its ability to maintain the mESC in an undifferentiated state. Serum is a very expensive component added to ESC maintenance media and, hence, a reduction in serum concentration while maintaining the pluripotent potential of mESC would be highly desirable. Recently, SR has increasingly been used in both mouse and human ESC cultures to maintain these stem cells in an undifferentiated state. It is recommended that the SR be added to the medium at the same concentration as serum is generally used, i.e., 15% for mESC.

The results of this study indicate that SR actually provides a stronger growth stimulatory effect at lower concentrations (1.67% and 5%) than serum at similar levels when DMEM is used as the basal medium. Similarly, EB yields are higher at lower (1.67% and 5%) SR concentrations compared to similar serum levels in DMEM. SR therefore appears to provide higher levels of factors (than serum) that stimulate higher growth rates and increase the EB yields of both the R1 and EFC cells. It would be interesting to perform cell cycle analysis of mESC grown in 5% SR and compare them with cells grown in either 5% or 15% serum to see if the stimulatory effect of SR actually reduces the overall cell cycle time by reducing the duration of the gap (G1 or G2) phases or if it affects the survival of the cells.

The results of this study also demonstrate the importance of basal medium selection for the maintenance of mESC in culture. While high glucose DMEM is the conventional basal medium used for mESC, the results presented here make a strong case for DMEM:F12 being a superior basal medium, in most cases increasing growth rate and EB yields. DMEM and Ham's F12 are often mixed to combine benefits from each formulation. For example, F12 provides small amounts of trace metals (copper and zinc) and some non-essential amino acids not present in DMEM. F12 also contains

hypoxanthine, linoleic acid, lipoic acid and thymidine that are absent from DMEM. DMEM has generally higher levels of vitamins. Hence, being an enriched medium, DMEM:F12 likely provides more of the components that cells require to grow and, hence, is able to reduce serum or SR dependence. Serum replacement, on the other hand, is a propritory supplement which consists of lipid-rich bovine serum albumin or albumin substitute (Albumax-I) and one or more ingredients selected from the following groups: amino acids, vitamins, antioxidants, insulin or insulin substitute, collagen precursors and trace elements. Since both DMEM:F12 and SR have many components in common, adding 15% SR to DMEM:F12 results in high-dose inhibition effect on both the proliferation and EB forming ability of mESC.

Like growth rate and EB forming capacity, the metabolism of both cell lines was influenced by the different basal media used with serum or SR. The cell specific glucose uptake (Figure 4.3), lactate production (Figure 4.4) and glutamine uptake (Figure 4.5) rates of both cell lines varied considerably depending on the basal medium present with serum or SR. Unlike the growth rate and EBs/initial cell, the R1 cells, under all conditions tested, had higher rates of all three of these metabolic rates compared to the EFC cells. Variations in sGUR as a function of serum concentration were similar to HeLa cells that have been reported to exhibit a decrease in sGUR as a function of serum concentration[50] while increasing concentrations of serum had no effect on glucose consumption for hybridoma cells[51]. Neither cell line exhibited a dependence of sGUR on SR concentration regardless of whether DMEM or DMEM:F12 was present as the basal medium. It is also interesting to note that in DMEM, the sGUR values of R1 cells were higher than those of EFC cells both in the presence of serum and SR. But, with DMEM:F12, both cell lines approach similar values of sGUR with either serum or SR. sLPR (Figure 4.4) and sGlnUR (Figure 4.5) show trends similar to sGUR for both cell lines.

Like serum, SR can have lot-to-lot variability problems. For instance, the osmolality of SR has been reported to influence the successful derivation of hESC lines (e.g. Melton lab hESC culture protocol, see footnote on final page in the HUES manual accessed at:

http://www.mcb.harvard.edu/Melton/hues/HUES_manual.pdf). In most cases, the recommended 15% SR levels resulted in sub-maximal growth rates and EB yields (especially for EFC in DMEM:F12). It may be that by optimizing the level of SR added this apparent high dose inhibition effect could be avoided and the actual variability of optimized SR additions reduced. With SR at levels such as 1.67 or 5%, instead of 15%, the expenses of these cultures would also be greatly decreased.

hESC are now routinely cultured in DMEM:F12 with 20% SR. It would be interesting to determine if lower SR concentrations could also sustain hESC in an undifferentiated state over multiple passages. However, the high dose inhibition effect of SR observed with mESC might not be observed with hESC as they are normally cultured on MEF feeders and the feeder "conditioning" of the medium could lower the concentration of components that caused inhibition to mESC.

In an ideal scenario, both mouse and human ESC should be cultured in completely defined serum-free conditions. In order to develop a serum-free medium, it would be advisable to perform experiments comparing new serum-free media to those supplemented with serum or SR at optimized levels (e.g. 5%) so as to adjust the concentrations of the serum-free components with a more appropriate positive control.

4.5 Conclusions

The results presented in this chapter highlight the fact that selection of the basal medium and the supplements can have a profound impact on the maintenance of the proliferation and EB formation potentials of mESC. The growth rate of both R1 and EFC cells show dependence on serum concentration when DMEM is used as a basal medium. However, this serum dependence can partially be removed by using an enriched basal medium such as DMEM:F12. SR at 5% levels seems to optimally maintain both proliferation and EB forming capacity of both cell lines and higher (15%) concentration appears to be inhibitory to both growth and EB yields of both cell lines. SR at 5% levels also maintains higher growth rate and EB yields than serum with DMEM thereby showing that it probably provides components of similar nature to cells that are also present in DMEM:F12. Hence a combination of high SR concentration and DMEM:F12 appear to cause a high dose inhibition to both growth rate and EB yields of both R1 and EFC cells. Metabolism of cells is also strongly influenced by the basal medium present along with serum or SR. There is apparently no lot-to-lot variability associated with SR and it is the high concentration being recommended to be used in mESC culture that causes problems.

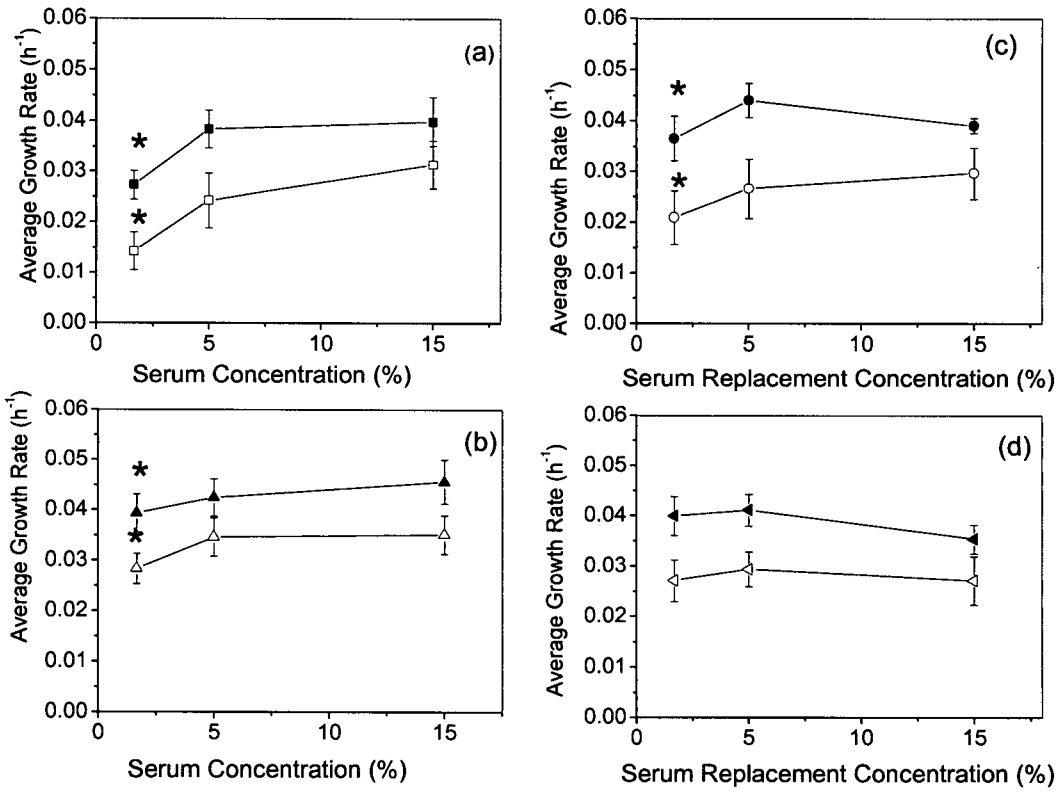

Figure 4.1 Average growth rate observed when R1 (open symbols) and EFC (closed symbols) cells were inoculated in the presence of (a) serum with DMEM as the basal medium, (b) serum with DMEM:F12 as the basal medium, (c) serum replacement with DMEM as the basal medium, and (d) serum replacement with DMEM:F12 as the basal medium. Values shown are mean ±SEM of six independent experiments. (* $p < 0.05$ compared to 15% serum or SR by paired t-test).

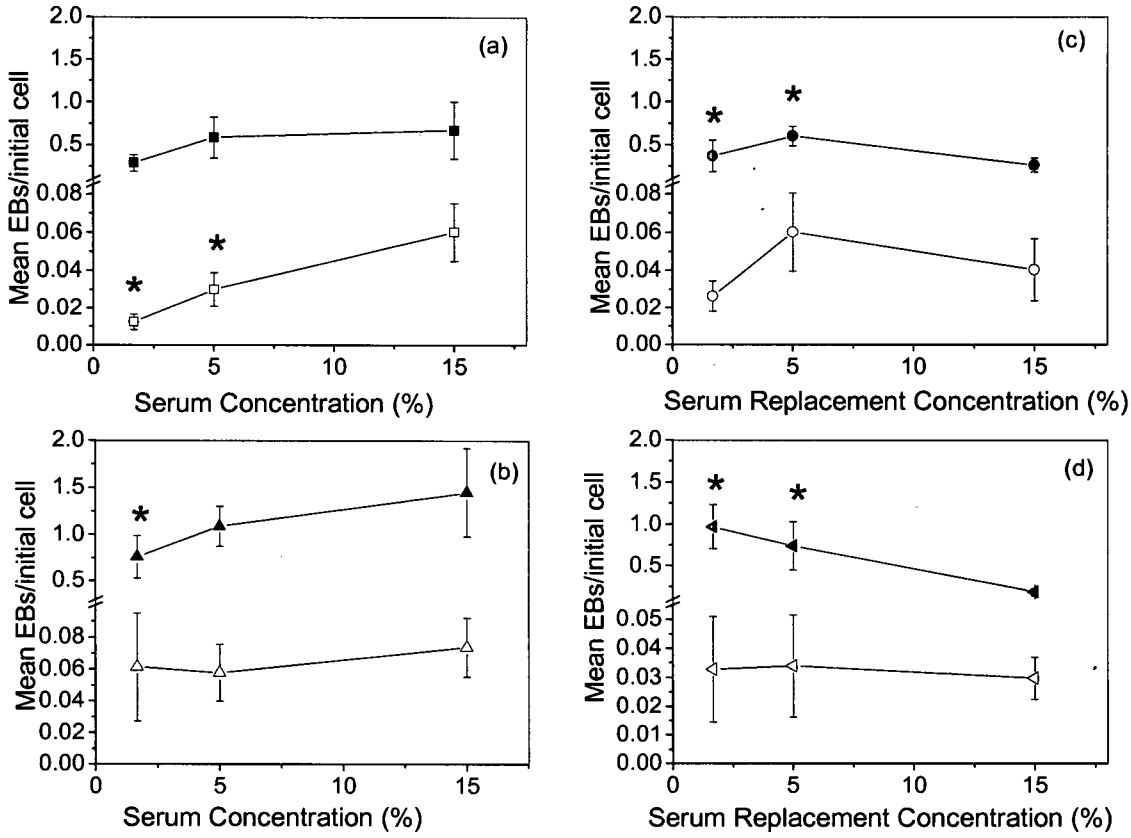

Figure 4.2 Mean number of EBs obtained per initial cell inoculated when R1 (open symbols) and EFC (closed symbols) cells were inoculated in the presence of (a) serum with DMEM as the basal medium, (b) serum with DMEM:F12 as basal the medium, (c) serum replacement with DMEM as the basal medium, and (d) serum replacement with DMEM:F12 as the basal medium. Values shown are mean ±SEM of six independent experiments. (* $p < 0.05$ compared to 15% serum or SR by paired t-test)

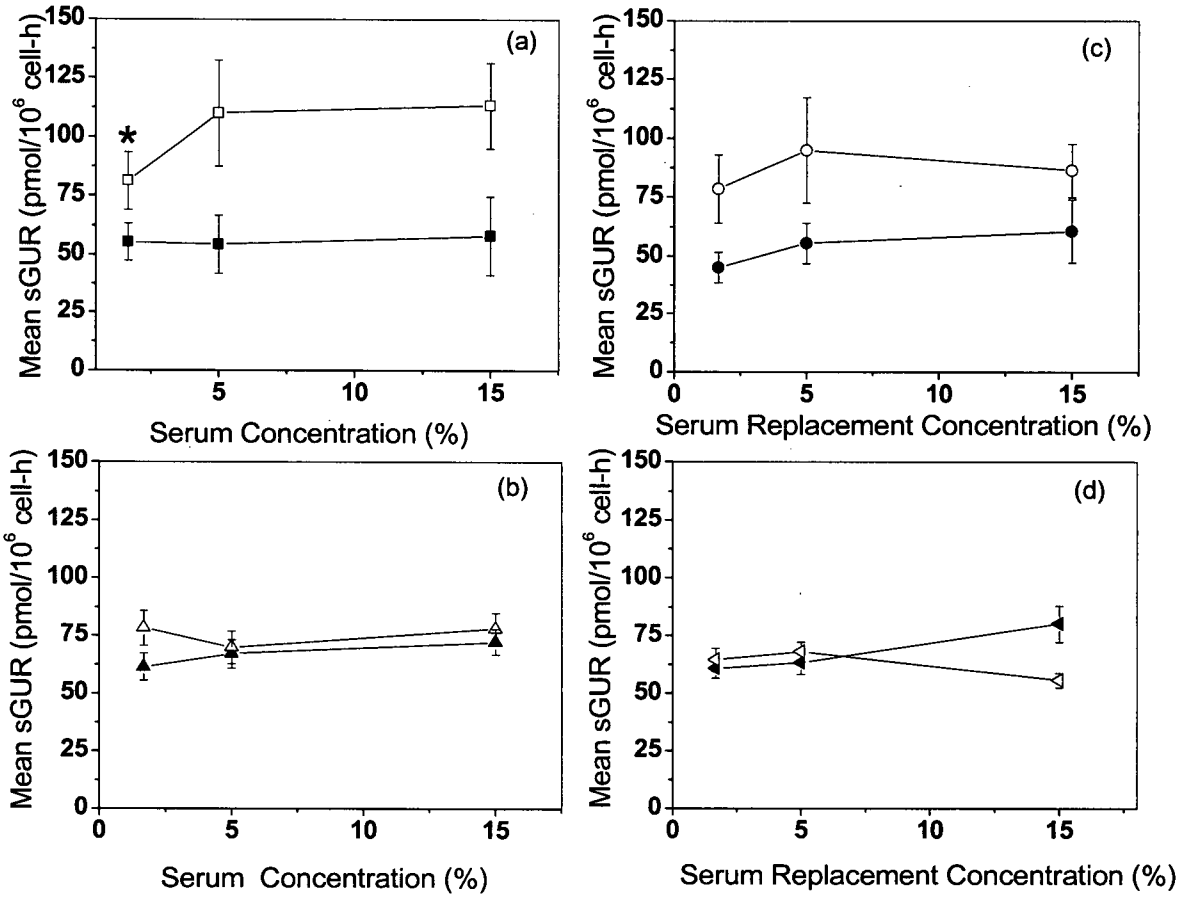

Figure 4.3 Cell specific glucose uptake rate (sGUR) obtained when R1 (open symbols) and EFC (closed symbols) cells were inoculated in the presence of (a) serum with DMEM as the basal medium, (b) serum with DMEM:F12 as the basal medium, (c) serum replacement with DMEM as the basal medium, and (d) serum replacement with DMEM:F12 as the basal medium. Values shown are mean ±SEM of six independent experiments. (* $p < 0.05$ compared to 15% serum or SR by paired t-test).

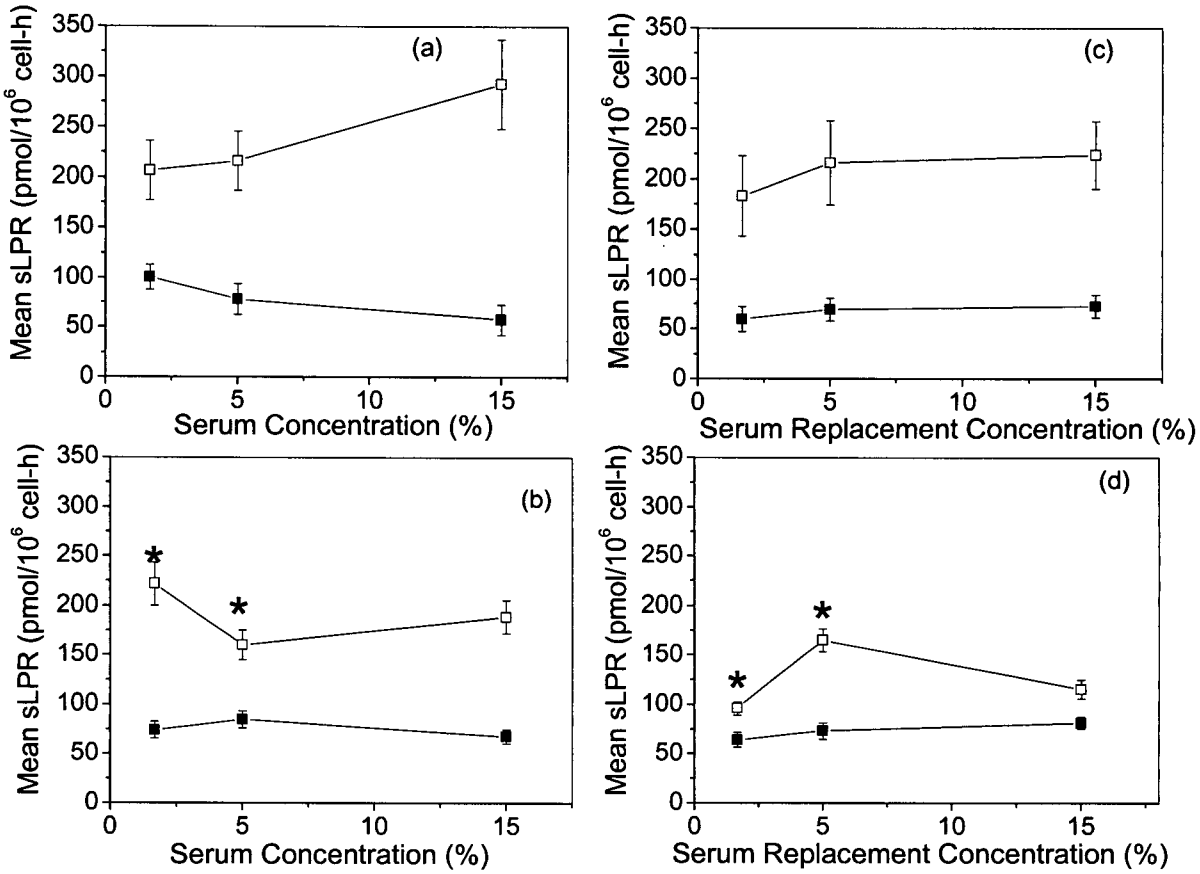

Figure 4.4 Cell specific lactate production rate (sLPR) obtained when R1 (open symbols) and EFC (closed symbols) cells were inoculated in the presence of (a) serum with DMEM as the basal medium, (b) serum with DMEM:F12 as the basal medium, (c) serum replacement with DMEM as the basal medium, and (d) serum replacement with DMEM:F12 as the basal medium. Values shown are mean ±SEM of six independent experiments. (* $p < 0.05$ compared to 15% serum or SR by paired t-test).

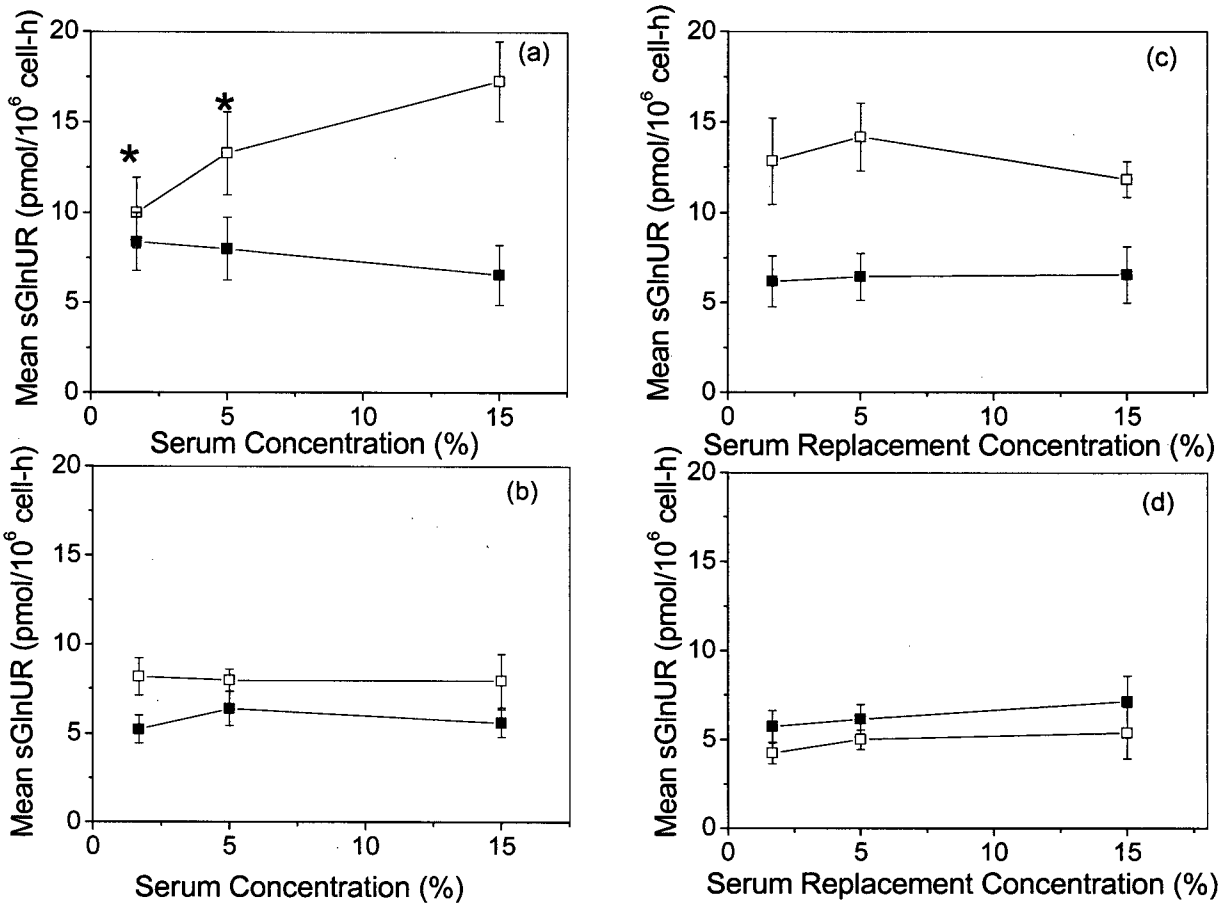

Figure 4.5 Cell specific glutamine uptake rate (sGlnUR) obtained when R1 (open symbols) and EFC (closed symbols) cells were inoculated in the presence of (a) serum with DMEM as the basal medium, (b) serum with DMEM:F12 as the basal medium, (c) serum replacement with DMEM as the basal medium, and (d) serum replacement with DMEM:F12 as the basal medium. Values shown are mean ±SEM of six independent experiments. (* $p < 0.05$ compared to 15% serum or SR by paired t-test).

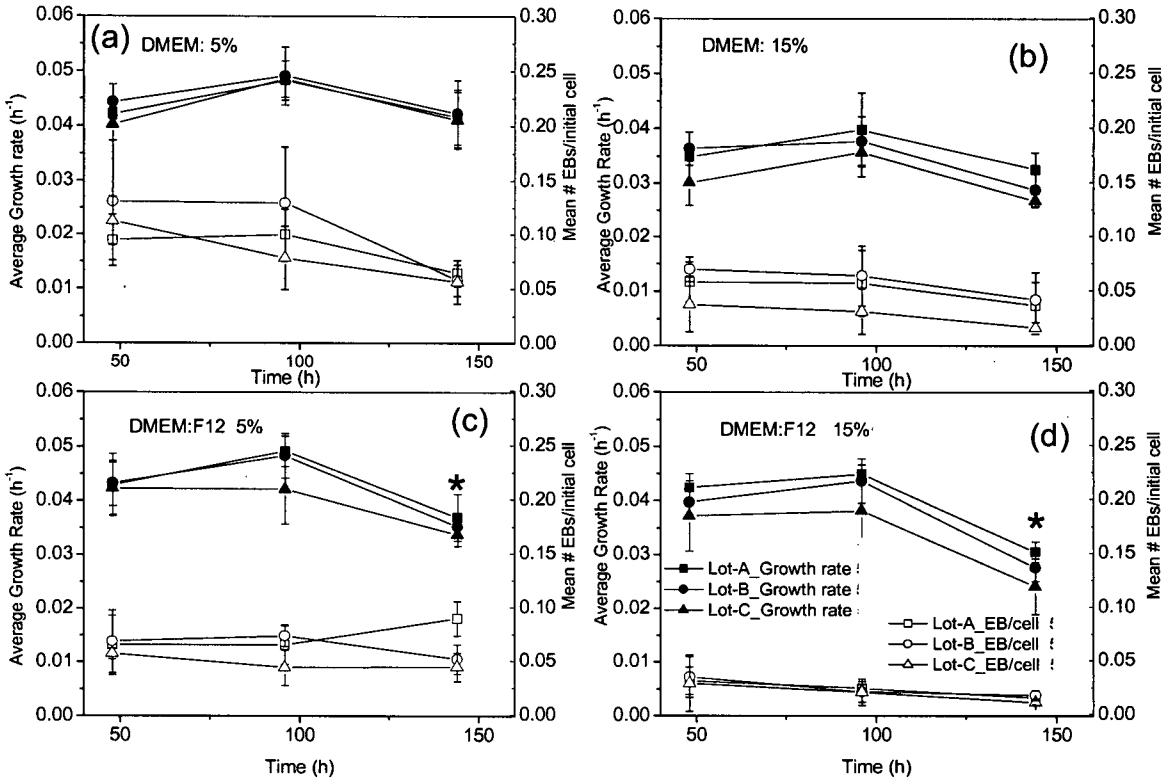

Figure 4.6 Mean number of EBs obtained per initial cell inoculated and growth rate of R1 cells when inoculated with three different serum replacement lots not used in previous experiments reported in Figures (4.1-4.5) (a) 5% SR with DMEM (b) 15% SR with DMEM (c) 5% SR with DMEM:F12 (d) 15% SR with DMEM:F12. Legend applies to all panels. Values shown are mean ±SEM of two independent duplicated experiments. (* $p < 0.05$ compared to 15% serum or SR by paired t-test).

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5 Conclusions and Future Directions

5.1 Conclusions

Many potential cellular therapies using adult or embryonic stem cells require that large numbers of stem cells be produced in culture. This in turn requires that the proliferation and the maintenance of the stem cell potential of these cells be carefully evaluated as a function of the culture environment to determine the range of variables within which such cultures should be carried out without compromising stem cell properties. For cellular therapy applications, both adult (such as hematopoietic) and embryonic stem cells could be used. As discussed in Chapter 1, the proliferation and stem cell potential of hematopoietic stem cells is strongly dependent on the kind and level of cytokines present in the medium. To expedite the bioprocessing research and development in this exciting area, there is a strong need to develop and validate mathematical models that can predict the influence of the culture environment and especially cytokine utilization and cytokine-dependent cell proliferation. The research presented in this thesis has therefore examined the influence of environmental variables on proliferation and maintenance of stem and progenitor cell potentials. Specifically, this project was concerned with developing a quantitative understanding of the cytokine-dependent proliferation of cells using hematopoietic cell lines as model systems. Due to various reasons, studies of the cytokine-dependent differentiation of hematopoietic cell lines are relatively difficult to perofrom and get any meaningful results. The mESC, on the other hand, were shown to differentiate readily under the influence of variety of different stimuli. The proliferation of mESC is however, cytokine independent and differentiation is generally carried out using non-cytokine agents such as retinoic acid (a vitamin A derivate) ascorbic acid (vitamin-C), and chemicals such as dimethylsulfoxide and 3-methoxybezene. Hence two mESC lines were chosen as model systems to develop an understanding of the impact of such environmental variables as basal medium composition, pH, osmolality, as well as serum and serum substitute concentration on the proliferation and maintenance of stem cell potential.

Mathematical models of the proliferation and differentiation responses of stem cells as a function of culture variables can greatly accelerate the bioprocess research and development needed before stem cells can safely be used in clinical settings. In the studies presented in this thesis, empirical mathematical models were developed to predict the proliferation response of two hematopoietic cell lines as a function of cytokine concentration when one or more cytokines are simultaneously present (Chapter 2). This modeling approach resulted in the development of a 'competitive model' to represent the competition reported in the literature between the two cytokines, IL-3 and GM-CSF, for the common receptor (β_c) subunit. This model had no new parameters beyond those obtained when the cells were cultured with single cytokines and provided good prediction of the growth rates for both cell lines exposed to combinations of IL-3 and GM-CSF, over a wide range of concentrations. The competitive receptor hypothesis provided a clear rationale for the predictive model developed, suggesting that this modeling approach may be effective for a wide range of other shared receptor subunit systems. The experiments reported here did not reveal a synergistic interaction between IL-3 and GM-CSF probably for two reasons: (i) at the signal transduction level, since IL-3 and GM-CSF share a common receptor subunit (β_c), common signal transduction molecules downstream of the receptors are likely employed by both cytokines, and (ii) growth rate is being used as the response variable. A synergistic increase in cell proliferation will probably occur only when two cytokines that employ completely different signal transduction mechanisms are employed. Also, since the calculation of growth rate involves a logarithmic transformation, it dampens the effect of any increase in cell concentration and hence a synergistic growth rate response may not be observed.

Hematopoietic stem cells are difficult to maintain and expand in culture and little expansion of HSC with a long term *in vivo* repopulating ability has so far been obtained. Consequently, mouse embryonic stem cells were employed as a model to further study the influence of culture environment on the proliferation and maintenance of stem cell potential. Biologists working with mESC agree to the notion that these cells require very careful handling and exposing them to inappropriate culture conditions results in loss of

the pluripotentiality of these cells. However there are no systematic reports in the literature about the responses of mESC to various culture conditions when varied over a wide range. In addition, the ranges of culture variables within which mESC cultures can be performed without adversely affecting the cells has not been established. The studies reported here demonstrate that both the pH and the osmolality of the medium are important modulators of mESC proliferation and EB formation potential. Results of dose-response experiments revealed that, within 48 h, the growth rate and yield of EBs decreased substantially when R1 or EFC cells were cultured under suboptimal pH or osmolality conditions. As has been reported for cell lines used in recombinant protein production processes, changes in the metabolism of both mESC lines also showed a strong correlation with pH. The average cell diameter was also found to be a strong function of pH. Extreme pH conditions induced apoptosis following 48 h of exposure, however, an apparent adaptation to these extreme pH values seemed to take place as, following 96 h of exposure to various pH conditions, there was no significant difference in the proportion of cells that were positive for Annexin-V. The pH of the mESC maintenance medium is conventionally around 7.3 at the beginning of each culture and the medium is exchanged the next day (i.e., after 24 h). Although not very convenient, another medium exchange could be performed after 36 h of culture since mESC start to grow exponentially after 24 h and their metabolism is most active during the last 24 h of the typically culture cycle where the cells are harvested and passaged after 48 h to avoid overgrowth. Such a procedure would reduce the pH decrease due to accumulation of lactic acid. However, the influence of pH on the proliferation and EB forming capacity of mESC is not permanent and these cellular responses were restored to their original values within 96 h of culture under optimal conditions. In contrast to pH, the osmolality of the medium could be varied over a relatively wide range (between 300 – 335 mOsm/kg) without significantly influencing the responses of mESC.

In addition to pH and osmolality, serum (or serum replacement) concentration and the basal medium used to prepare the mESC maintenance medium were found to profoundly influence the mESC responses. For both cell lines employed in these studies, the basal medium used turned out to be an important factor in determining the responses of cell

lines to different serum or serum replacement concentrations. For instance, a 5% serum concentration appears to be optimal in the presence of DMEM, while even 1.67% serum is sufficient to maintain both proliferation and EB formation potential with DMEM:F12 as the basal medium. Similar results were obtained when serum was replaced with serum replacement. Hence, using an enriched basal medium such as DMEM:F12 is beneficial to the maintenance of proliferation and stem cell potential of mESC. A striking observation of these studies was that the currently recommended level of SR to be used in preparing mESC maintenance media (15%) is actually inhibitory to both the proliferation and EB yield of mESC, and the effect becomes more pronounced when SR is added to DMEM:F12, where optimal responses were obtained at 1.67% SR. This was further confirmed by conducting independent experiments with R1 cells and three new lots of SR. The results of these experiments strongly argue that it is the high SR concentration used to culture mESC that lowers the growth rate and EB formation potential and that lot-to-lot variability is actually minimal. A similar high dose inhibition effect of SR has not been reported in hESC cultures as the 'conditioning' of SR containing hESC medium by MEFs eithers removes or lowers the concentration of those components that cause this effect. It is therefore quite possible that when mESC are cultured on MEFs, the high dose inhibition effect of SR would also be minimal or not detected. It is therefore recommended that to culture mESC on gelatinized surfaces, the maintenance medium be routinely prepared using DMEM:F12 as the basal medium supplemented either with serum or SR at the 5% level. The medium, after the addition of all the supplements, should be equilibrated to a pH of around 7.3 in order to optimize both the proliferation and EB forming potential of mESC. If SR is used instead of serum, this will help significantly in reducing the cost of ESC culture media and bioprocess development. Developing conditions that would minimize the spontaneous differentiation of mESC that ensue when they are plated on gelatin would also be very valuable. Different extracellular matrix (ECM) proteins such as laminin, fibronectin, collagen, etc., or their combinations, could replace gelatin and should be experimentally tested. As described in the literature review (Chapter 1), addition of MAP Kinase inhibitor PD05809 has been shown to enhance the derivation of mESC lines. It would be valuable to test this

compound's influence on the routine maintenance of different mESC lines and test whether mESC grown in its presence have a similar or altered differentiation potential.

5.2 Future Directions

The studies reported in this thesis have addressed the issue of the impact of culture variables on the mESC responses of proliferation and EB formation potential, but didn't investigate the mechanisms responsible for the observed responses. For example, whether pH influences only the metabolic enzymes or also the other signaling pathways that interact with these metabolic enzymes is currently unknown. Chronic exposure of cells to suboptimal culture conditions (such as low pH) would invariably invoke the cellular stress response. How this stress response interacts with the signaling pathways and the genes influencing the cellular fate decisions of self-renewal vs. differentiation is also currently unknown and experiments conducted to answer these questions could provide important insights about how the culture environment shapes these cellular responses.

Most studies reported in the literature on the influence of extracellular pH on intracellular pH and the resulting cellular responses were short term and did not consider the issue of chronic exposure of cells to suboptimal extracellular pH and its effect on intracellular pH as well as other cellular functions. Further, the pH experiments reported in this thesis did not address the mechanistic details of how various cell surface transporters or intracellular signaling molecules are being affected by different pH conditions. Most of the experiments reported in the literature on pH_i regulation in somatic cells as well as in embryos did not look at the chronic exposure of cells to low or high pH_e values and the resulting influence, for example, on the expression as well as function of various cell surface exchangers (like NHE1 or Cl^-/HCO_3^-). Furthermore, studies performed with cell lines used in recombinant protein production processes also reported an influence of pH on metabolism as both glucose uptake and lactate production rates changed as a function

of medium pH. None of these studies have reported any mechanistic details about how metabolism is affected by the changing pH conditions. It will be important to perform such mechanistic experiments, especially with hESC, as a detailed characterization of how these cells respond intracellularly to imposed chronic pH challenges will shed light on whether it is just the metabolism or also the signaling pathways that regulate cellular fate decisions that are being affected by variations in pH. Even if pH just influences the metabolism of ESC, it is quite possible that the metabolic enzymes interact with other signaling pathways operating in the cells and, hence, indirectly influence the proliferation and/or differentiation response. A recent study[2] that demonstrated the involvement of GSK3β, an enzyme with metabolic as well as other diverse functions, in the maintenance of the pluripotentiality of both human and mouse ESC supports this notion.

A related aspect is the formal demonstration of the expression and functional activation of one or more sodium-hydrogen exchanger (NHE) isoforms as well as other exchangers involved in pH_i regulation in hESC under both short- and long-term exposures to suboptimal pH conditions.

Our emphasis in the pH experiments reported in this thesis was to assess the mESC responses when they were exposed to various pH values under the standard conditions used to maintain these cells, i.e., medium exchange after 24 h of culture inoculation and cell harvesting after 48 h. We only investigated the influence of the variation of different culture variables on the EB yield of these cells as an indirect measure of their self-renewal ability, since a culture that contains a higher proportion of stem cells should give rise to more EBs. We didn't address the question of what might be the influence of the variation of such culture variables as pH, osmolality, as well as serum or serum replacement concentration on the cellular functions (e.g. differentiation, cell-cell adhesion or migration) of these cells. It would therefore be very informative, for example, to subject the mouse or human ESC (previously exposed to various pH or osmolality conditions for the duration of one passage – 48 h for mESC and 7 days for hESC) to differentiation by LIF (or bFGF) removal or addition of retinoic acid or DMSO

to the culture medium and determine whether the differentiation of these cells is affected compared to a control culture. It is conceivable that lineage commitment decision are influenced by prior exposure to different pH or osmolality conditions, resulting in enhanced or reduced production of one type of cells at the expense of others. The adhesion molecules, such as E-cadherin or gap junctions, expressed on the cell surface could be influenced by high or low pH conditions to which cells are exposed and hence could influence the plating efficiency of these cells in maintenance cultures as well as in EB and CFC assays. Similarly, the pH or osmolality of the methylcellulose-based differentiation medium was not altered in our experiments. Preparation of methylcellulose-based media adjusted to various pH or osmolality values could also influence the differentiation of cells and result in enhanced or reduced production of a particular type of cell. If this happens, it could be a relatively easy way to produce a desired cell type without going through the trouble of genetic alterations to enrich for that cell type.

During the course of this project, dose-response and factorial design experiments of glucose and glutamine concentrations were also used to study the influence of these variables on the proliferation and EB formation potential of mESC (Chapter 3). These experiments demonstrated that glutamine is far more important than glucose for the maintenance of the stem cell potential of mESC. The mESC proliferation as well as EB formation potential were significantly reduced when glutamine was absent from the medium compared to the control conditions of 4 mM glutamine even though the medium contained 20 mM of glucose under both conditions. On the other hand, the cellular responses were not as badly affected when glucose was present at low levels (1.25 mM) with 4 mM of glutamine in the medium. Hence, glutamine can apparently substitute for glucose deprivation, but the reverse is not true. Two-factor interactions between pH, glucose and glutamine were also significant when a 3×3 factorial experiment was performed (data not shown). It would therefore be important to thoroughly investigate these variables using both mouse and human ESC as models since a better understanding of their responses to systematic variations of pH, glucose and glutamine in a factorial

type experiment would help in developing an optimized medium for both of these cell types.

The production and maintenance of mESC is not of clinical significance. It will eventually be necessary to apply all that has been learned from mESC to the human system. The influence of pH, osmolality and other culture variables on the proliferation and differentiation potential of hESC has to be studied before these cells can be produced on a large scale under cGMP guidelines.

Although human ESC will be used in clinical settings, the use of mESC for further experimentation should not be abandoned. Rather, they should be used to investigate issues such as the development of large-scale bioreactor systems. They could also be used to study the influence of other culture variables, such as temperature, dissolved oxygen and shear rate that have not been addressed in this thesis. Not only are these culture variables important when studied alone, their potential interactions could also impact the responses of ESC. Hence, factorial design and response surface methodologies could be used to understand the impact of these interactions and to further optimize the levels of these variables.

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