Fundamental Differentiation and Growth Characterization of Murine Embryonic Stem Cells in Varied Culture Conditions

by

Yasunori Hashimura

B.S. Chemical Engineering
Massachusetts Institute of Technology, 2002

Submitted to the Biological Engineering Division
In Partial Fulfillment of the Requirements for the
Degree of

Master of Engineering in Biological Engineering

at the
Massachusetts Institute of Technology
June 2004

Signature of Author _____
Biological Engineering Division
March 25, 2004

Certified by _____
Jean-Francois Hamel
Research Engineer and Lecturer of Chemical Engineering
Associate Industry Liaison
Thesis Supervisor

Accepted by _____
Roger Kamm
Professor of Mechanical and Biological Engineering
MEBE Program Director

Accepted by _____
Alan Grodzinsky
Professor of Electrical, Mechanical, and Biological Engineering
BE Graduate Program Chair

Fundamental Differentiation and Growth Characterization of Murine Embryonic Stem Cells in Varied Culture Conditions

by

Yasunori Hashimura

Submitted to the Biological Engineering Division
On March 25, 2004 in Partial Fulfillment of the
Requirements for the Degree of
Master of Engineering in Biological Engineering

ABSTRACT

Although embryonic stem (ES) cells and their pluripotent capability have been elucidated for decades, little study has been done on obtaining the pluripotency profile of ES cells in the incipient stages of differentiation. In this research, an ES cell line with transfected green fluorescent protein (GFP) co-expressed by an Oct-4 promoter was analyzed by fluorescence-activated cell sorter (FACS) to obtain such profile. As Oct-4 is an ES cell differentiation marker whose expression varies with pluripotency, GFP expression could simply be measured in these cells to determine how pluripotent they are as a population. The differentiation characterization of ES cells was also conducted with different culture conditions of reduced serum and glucose concentrations both in the presence and absence of leukemia inhibitory factor (LIF) which prevents spontaneous differentiation, as well as at varied LIF concentrations and seeding densities. In addition, fundamental growth kinetic and metabolic profiles were obtained to get a more complete picture of how ES cells behave under these varied culturing conditions.

The doubling time (t_d) of R1 Oct4-GFP cell line was found to be 13 hours in LIF^+ culture and 8 hours in culture with LIF addition after 7 days of LIF withdrawal, implying that cell proliferation rate is higher for cells receiving a sudden upregulation of genes controlling cell division through LIF addition. Although the upregulation of the genes is rapid, the downregulation of these genes through LIF withdrawal was found to take 6-7 days, while 3-4 days were required to downregulate the *pou5f1* gene (which controls Oct4 expression). Higher concentration of LIF resulted in higher ES cell proliferation rate, but GFP^+ expression was unaffected by LIF concentration. Higher seeding density resulted in greater improvement in GFP^+ expression for LIF^+ culture but lower non significant reduction in GFP^+ expression in LIF^- culture. Low level of glucose in medium led to reduction in the rate of ES cellular mechanisms and lower $Y_{lac/gluc}$ (8-49 % versus 40-60 % in high glucose), but metabolic rates were consistent with cells grown in high glucose medium, implying more efficient glucose metabolism through oxidative phosphorylation. The level of serum in medium had no effect on GFP^+ expression or cell proliferation rate in LIF^+ cultures, but reduction in GFP^+ expression level was higher and t_d was longer in low-serum culture (71 ± 33 hours versus 35 ± 9 hours) in the absence of LIF.

Thesis Supervisor: Jean-Francois Hamel
Title: Research Engineer and Lecturer of Chemical Engineering
Associate Industry Liaison

Acknowledgements

After two years of endeavoring in a relatively new field of embryonic stem cells, I have finally begun to appreciate the intense hard work and effort that scientists and researchers around the globe put in every day, especially those pioneers who venture into the unprecedented unknown. I quickly learned that the information and data that can be looked up in the form of journal articles and reports are important, yet miniscule compared to the knowledge one can gain from the expertise of professors, researchers, and fellow colleagues who devote their lives to improving and furthering science in whatever field they have chosen to explore. With that said, I would like to thank the following people for their efforts, in helping me through the past two years in all research aspects, as well as in helping me grow and mature:

Professor Engelward for her expertise regarding embryonic stem cells and her assistance in every technical matter involving these cells, including their culturing, plating and maintenance techniques;

Professor Zandstra of the University of Toronto for granting me access to his transfected cell line R1 Oct4-GFP and for his publications that provided the backbone for this research, as well as Ali Khademhosseini and Judy Yeh of the Langer Lab and for their assistance in matters involving FACS and HPLC;

Daryl St. Laurent (MIT '02), Jiovani Visaya (Amherst '05) and members of the Hamel Lab for their support and assistance and in data acquisition and other aspects of laboratory work;

And finally, my primary thesis advisor Jean-Francois Hamel for his continuing guidance, patience, and understanding of the various experimental and personal obstacles that I encountered over the course of these years and his assistance in guiding me through my research and keeping me on track to graduate.

Lastly, this research was supported in part by the Engineering Research Centers Program of the National Science Foundation under NSF Award EEC 9843342, and I would like to thank the foundation for their financial assistance, without which this research would have encountered even greater challenges.

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 What are embryonic stem cells?

Embryonic stem (ES) cells have drawn much attention from researchers in the last few decades ever since the first successful culturing and expansion of ES cells in vitro by Evans and Kaufman in 1981, given their pluripotent nature and their ability to be cultured and expanded relatively easily with the addition of certain transcription factors to prevent differentiation. Although pluripotent cells had been studied at least since the mid-1970s on embryonic carcinoma (EC) cells derived from a type of tumor called teratocarcinoma, these cells did not seem ideal for use in therapy because they had come from tumors[1] and they often had karyotype abnormalities[2]. Another type of pluripotent cells called embryonic germ (EG) cells were first isolated in 1998 by Gearhart et al. from the primordial germ cells of the gonadal ridge of a 5-10-week fetus, but these cells could only remain pluripotent for about 20 passages[3] and showed evidence that they may be incapable of undergoing normal development and differentiation due to epigenetic modifications[4].

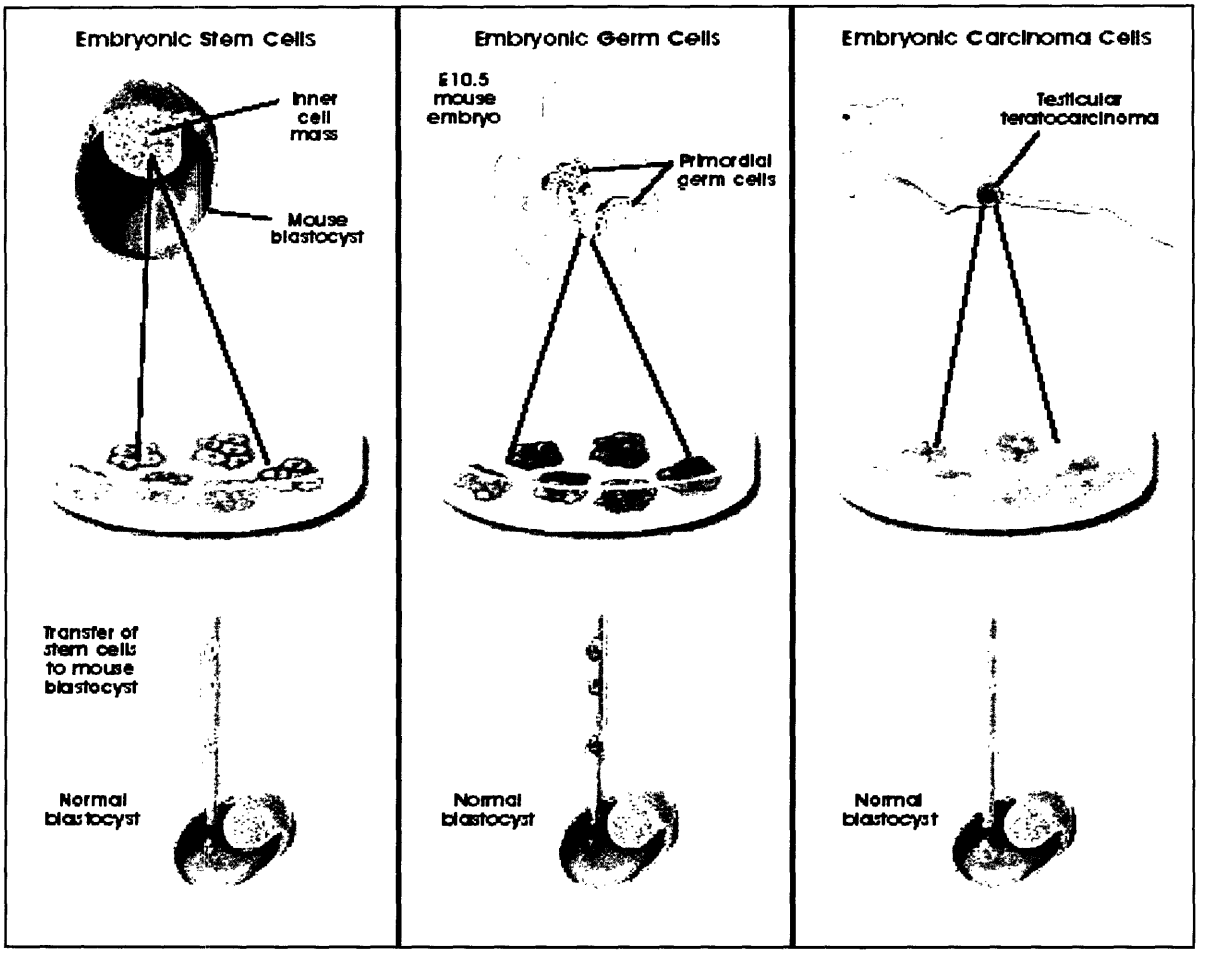


Figure 1: Origins of mouse pluripotent stem cells

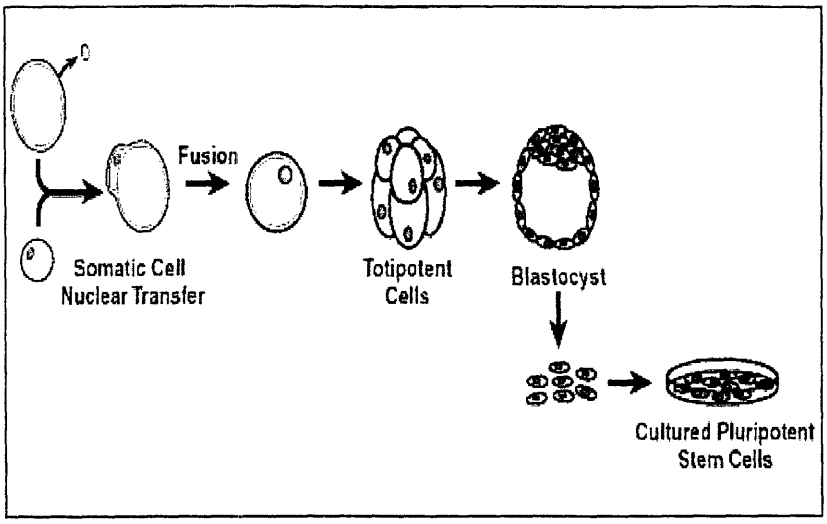


Figure 2: Simplified process of somatic nuclear transfer

ES cells, on the other hand, have been shown to develop normally into an entire organism through reproductive cloning[5] without having being derived from tumor and have therefore been identified as having the best developmental potential out of the three types of pluripotent cells to date (Figure 1)[6]. The ultimate goal of ES cell research is, however, that these cells be used in the near future to cure genetic diseases, to end problems frequently encountered in tissue and organ rejections, and even to perform genetic screening, by means of therapeutic cloning and not reproductive cloning. In cloning, an enucleated embryo is given a nucleus from a somatic (adult) cell from a patient in what is called somatic cell nuclear transfer, and with the addition of electric stimulation, the embryo can be induced to divide normally (Figure 2)[7]. However, instead of inserting this embryo into a surrogate mother as in reproductive cloning, ES cells in therapeutic cloning are withdrawn from the inner cell mass of the embryo and plated on tissue culture dish to undergo differentiation *in vitro*, thereby avoiding the ethical issue of sacrificing the life of the clone to obtain organs and tissues of the same genetic makeup as that of the patient from whom the nucleus of the somatic cell was derived. Since ES cells have pluripotent capability to divide into essentially any type of cells in a human body, the ultimate goal is to arrive at a complete differentiation protocol for deriving each tissue type and organ from the cells that serve as the origin of essentially all cell types: ES cells.

1.2 Embryonic stem cells

Embryonic stem cells are derived from the inner cell mass (ICM) of a 4 to 5-day old embryo called the blastocyst. Once removed, these cells from the ICM can be cultured into ES cells, which can undergo hundreds of replications and more than 2 years of self-renewal and still maintain a normal karyotype [8,9]. This can occur as long as pluripotency is maintained by adding different transcription factors to the culture, which depends on the mammal from which the cells were derived. For instance, leukemia inhibitory factor (LIF) can be used to maintain pluripotency in mouse ES cells, but basic fibroblast growth factor (bFGF) from the mitotically inactivated mouse embryonic fibroblasts (EF) must be used as feeder layer to prevent differentiation in human ES cells.

By far the most important quality of ES cells is their pluripotency, which means that they have the capability of differentiating into essentially any cell types within an organism (Figure 3)[10], with the exception of extraembryonic membranes, such as parietal endoderm, yolk sac visceral endoderm, and trophoblast of the placenta [11,12,13]. More importantly, this differentiation

process can occur both *in vivo* as well as *in vitro*, which allows the ethical issue of reproductive cloning in human beings to be avoided. The pluripotency, or a cell's ability to give rise to virtually all of the different types of cells in an organism, is a characteristic that gives these cells potential for great scientific applications in the field of medicine, specifically gene therapy and gene targeting technology [14].

In mice, ES cell pluripotency can be maintained either by adding a transcription factor called LIF or growing these cells on mouse embryonic fibroblast (mEF) feeder layer that expresses LIF. The signal transduction of LIF involves the

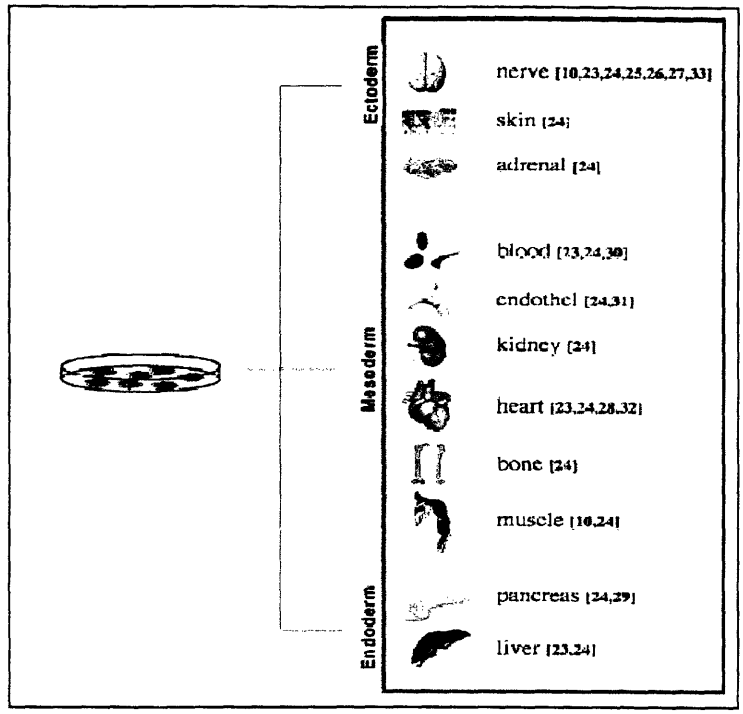


Figure 3: Developmental potential of human ES cells *in vitro*

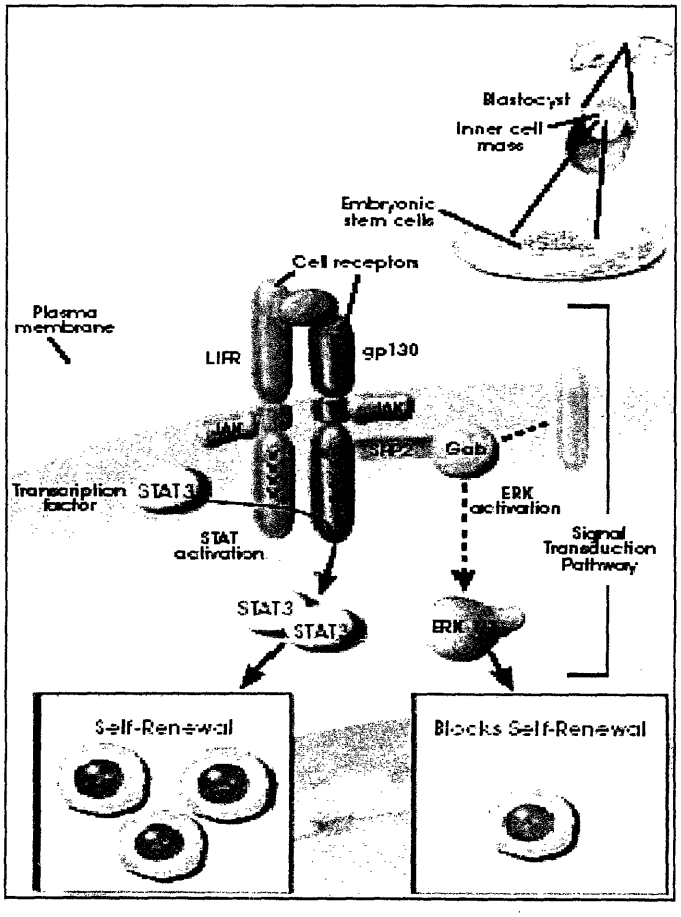


Figure 4: Signaling pathways in ESC self-renewal

binding of LIF-R and gp130-R to form a heterogeneous receptor complex, which triggers the activation of the latent transcription factor STAT3 through the JAK/STAT pathway, a necessary event *in vitro* for the proliferation of mouse ES cells[15,16,17]. STAT3 and a different transcription factor called Oct-4 then act in concert with telomere-synthesizing enzyme telomerase to maintain ES cells in an undifferentiated state, indicating that telomere length is invariably linked to cell differentiation[17]. In addition to this pathway, there appears to be a separate signaling pathway involving ERK that blocks self-renewal, as depicted in Figure 4[6].

Another important aspect of ES cells is their nearly unlimited self-renewal capability. ES cells can undergo hundreds of passages over years of culturing without differentiating, by growing them under standard culturing conditions with

certain transcription factors. It has been recently shown, however, that presence of differentiating cells in culture can induce the differentiation of other cells even under optimal growing conditions when the appropriate transcription factors are added[18], a phenomenon especially true in human ES cells[9,19]. Isolating a pure, undifferentiated population of ES cells before starting on a research is therefore essential to obtaining and maintaining pluripotency in ES cell culture.

This can, however, be performed using flow cytometry technology, since there are plenty of known transcription factors such as Oct4 and stage-specific embryonic antigens (SSEAs) that are expressed in high levels only by ES cells and are expressed in increasingly lower levels as they differentiate. By conjugating these transcription factors with antibodies that are tagged with a fluorochome, one could easily sort out the cells that express antigens in high amounts, that is, those that are undifferentiated.

Finally, ES cells can be cultured very easily as long as sterile conditions exist, with culturing conditions resembling those of ordinary mammalian cells. They also grow according to symmetric growth kinetics, which allows these cells to be expanded readily, and this quality greatly facilitates the way in which research can be conducted on ES cells, something that is uncommon in adult stem cells.

One of the challenges facing ES cell research, however, is that most of the available experimental data have come from mice[20] and there are obvious differences between mouse and human ES cells which make simple correlation between the species impossible. First, human ES cells do not respond to LIF[9,19] as mouse ES cells do, and they are also much more difficult to propagate because of their long doubling time (approximately 23 hours compared to 12 hours for mouse ES cells). Also, they differ in the expression of certain markers and consequently probably some of the signaling pathways as well[21]. Passaging also results in low viability of human ES cells, and spontaneous differentiation and difficulty faced in achieving clonal growth of human ES cells have hindered isolation of pure, pluripotent cell population for clinical use[20]. And the greatest challenge of all is the fact that ES cells, as the origin of essentially all cells, must undergo numerous stages of differentiation to get to the adult cells that are needed for therapeutic purposes, and the differentiation protocols for only the major categories of adult cells have been discovered, namely, for hematopoietic[22], endothelial[23], cardiac[24,25], and neuronal tissues[26,27].

1.3 Adult stem cells

Adult stem cells, also known as somatic stem cells, are similar to ES cells in their long-term self renewal and differentiation capabilities. Typically, somatic stem cells generate an intermediate cell type(s) called progenitor cells before they achieve a fully differentiated state[28], and they can also replicate just as ES cells do. Somatic stem cells and ES cells, however, differ significantly in where they can be found as well as in their characteristics. First, there are numerous different types of somatic stem cells, and each is derived from a different tissue type within a body. For instance, hematopoietic stem cells (HSCs) are derived from bone marrow, and neural stem cells (NSCs) are found in nerves. Where they are derived influences which type of cells they can differentiate into, since their primary functions are to maintain homeostasis, while replacing cells that die as a result of injury or disease[29,30]. They are also much closer in the steps of differentiation required to reach a particular type of adult cell since they are only

multipotent, rather than pluripotent like ES cells. This allows for much easier therapeutic solutions to be devised, and some well-studied somatic stem cells such as HSCs and mesenchymal stem cells of the bone marrow are already in clinical use for leukemia and skeletal regeneration therapy, respectively[31]. As an added bonus, there is also no ethical issue with these cells unlike ES cells, since they can be derived directly from the patients themselves.

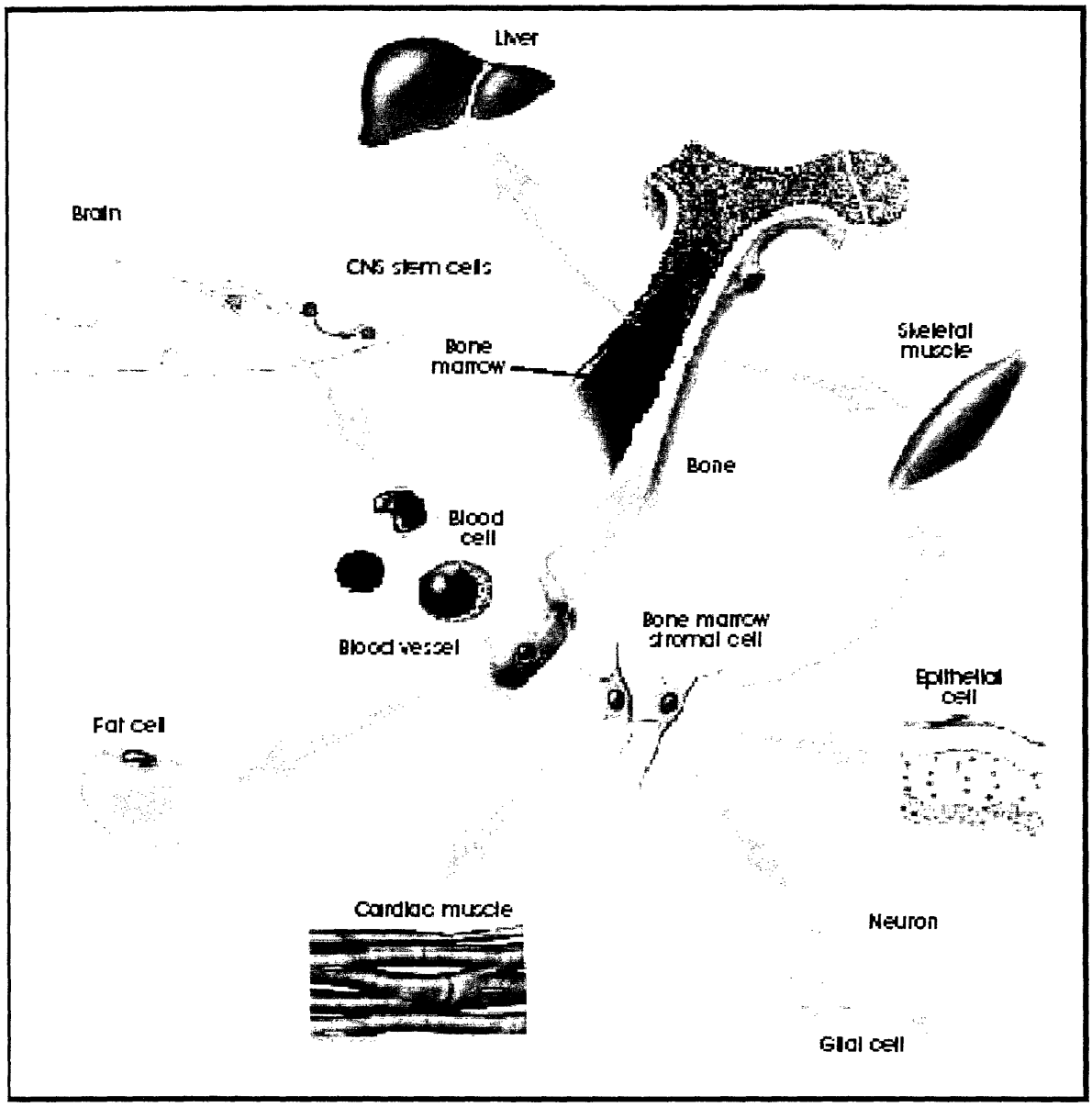


Figure 5: Example of plasticity of adult stem cells

In the recent years, there has been even higher interest in somatic stem cell research, with studies indicating that somatic stem cells also have a great degree of plasticity, or ability to generate cell types other than those from which the stem cells were derived (Figure 5)[28]. For instance, brain-derived stem cells can regenerate the entire hematopoietic system in lethally irradiated mice[32], as well as myocytes[33,34]. Stem cells from skin tissue can differentiate into neurons *in vitro*[35], and those cells derived from bone marrow can differentiate into chondrocytes, osteoblasts, and adipocytes[36,37,38], skeletal muscle cells[38,39] , liver cells[40], and endothelial cells[41].

The idea is that particular types of somatic stem cells that show high level of plasticity may be used to replace ES cell research.

As promising as these results are, however, somatic stem cells can never possess the developmental capability of ES cells since they are derived from the soma, not germ cells. Adult stem cells are also very rare within a human body, with only an estimated 1 in every 10,000 to 15,000 cells in bone marrow being HSC[42], and some stem cells are inaccessible due to where they are located in a human body. Furthermore, there are problems with the growth kinetics of adult stem cells, since they appear to follow asymmetric growth kinetics, whereby a stem cell divides to give rise to an identical stem cell and a transit cell destined to produce a differentiated cell lineage[43,44]. With such mode of replication, simple culturing techniques for expanding cells would inevitably dilute out the stem cell population, and hence, achieving a pure population of somatic stem cells in any significant number is difficult if not extremely challenging. As a result, there is no definitive argument that can be made for either ES cells or adult stem cells as to which is "better," and one must merely take into account all of the advantages and disadvantages of each and choose which system is preferred based on the objective of the research.

2 Research Objective and Experimental Design

2.1 Objective of research

This master's thesis research was designed and conducted because of lack of basic information available on ES cells such as cell growth kinetics and metabolic data, especially those acquired under varied culturing conditions. The biology of ES cells and how they can be used for gene therapy are an immensely complex and important field of research, but the underlying characteristics of ES cells and how they behave under simple culturing conditions are simple yet powerful information that could potentially be useful to researchers in the developmental stage of therapy, and especially once the *in vitro* differentiation protocols for all tissues and organs from ES cells have been established. In this growing field, there is always a need to facilitate and speed up the expansion of ES cells, and knowing the growth kinetic and metabolic profiles of ES cells and how they are affected under certain culturing conditions could make a world of difference. In addition, these changes in growth kinetics and metabolic profile could be used to make correlation to the level of differentiation occurring in cells, which, if strong, could be useful in detecting unexpected differentiation in cells.

While simple in nature, this type of characterization is nonetheless only useful if there is assurance that the cells do not undergo any significant level of differentiation while growing. While adding LIF gives some level of assurance that differentiation does not occur, studies have shown that presence of LIF does not guarantee maintenance of undifferentiated ES cells[18]. Performing primary and secondary antibody conjugations to a desired differentiation marker would eliminate this uncertainty, but this procedure would quickly become tedious if performed on a daily basis over a span of 10 to 14 days. For this characterization to be possible, a simpler method of detecting differentiation therefore became necessary.

For this reason, it was convenient in this research to use a cell line that expresses a reporter gene under the control of a promoter belonging to a transcription factor specific to ES cells so that pluripotency could be readily measured without antibody conjugation. The R1 Oct4-GFP cell line that was procured was indeed a standard mouse R1 stem cell line with the reporter GFP gene driven by an Oct-4 promoter, a construct developed and stably transfected into the ES cell line by Professor Peter Zandstra of University of Toronto, who recently demonstrated a linear relationship between GFP expression and Oct-4 expression in these cells[45]. As mentioned before, Oct-4 is a transcription factor expressed from the *pou5f1* gene and is necessary to establish[46] and maintain[47] the undifferentiated state of the ICM. In fact, Oct-4 expression is limited to pluripotent stem cells *in vivo* and *in vitro*, and pluripotent cells not expressing Oct-4 apparently do not exist[48]. By using this unique cell line that expresses GFP along with Oct-4, one could obtain pluripotency information from the cells as frequently as necessary, noting how the growth kinetics, metabolic profile, and differentiation state change in ES cells as culturing conditions are varied.

2.2 Experimental design

In order to accomplish these goals, several experiments were designed to give a fundamental understanding of how ES cells react to various medium conditions. Studies with other mammalian cells and stem cells have suggested that numerous physicochemical parameters such as pH, dissolved oxygen (DO), and glucose can significantly affect cell responses[49]. While pH and DO could also have been studied, glucose was selected as a parameter in this research, since the means for measuring metabolite concentrations was readily accessible in the lab. In addition to glucose, serum and LIF were also studied for a more comprehensive characterization of ES cells. Serum was selected because it is an important component for cell growth due to the presence of countless growth factors, and LIF was an obvious choice as it affects cell differentiation. Glucose, as the favored energy source in animals, was also a necessary parameter for a detailed characterization study.

The glucose and serum experiments were each conducted with 2 types of medium containing either high or low concentration of the component, to determine how cell growth and nutrient metabolism vary with glucose and serum concentrations. Standard ES medium was used as the high glucose or high serum medium in these experiments, with the other 2 types of medium containing less of one of those components. In addition, LIF was used as an additional parameter in these experiments, so that the effect of low glucose and low serum on cell differentiation could be determined for both LIF^+ and LIF^- cultures. The FACS data were also obtained for both 6-well seeded plates as well as 6-cm maintenance plates so that results could be analyzed for both types of TC plates.

The LIF concentration experiment included both the effect of various LIF concentrations on cell pluripotency, as well as the fate of ES cell pluripotency as they are cultured with extended LIF withdrawal. Since non-commercial LIF was estimated to be about 10 times as concentrated and potent as commercial LIF (ESGRO® ES1106 requires 1 μL LIF per mL of medium), the cell differentiation profiles were determined at LIF concentrations of 2 μL/mL (standard concentration used), 0.5 μL/mL, 0.125 μL/mL, and 0.03125 μL/mL. These seemingly random values were chosen to simplify the LIF dilution procedure, as each concentration is merely a 4:1 dilution of the previous vial. FACS data were obtained both for cells grown at various LIF concentrations as well as for cells with extended LIF withdrawal of over 2 weeks. Only non-commercial LIF was used in this research, and all of the LIF used was derived from one large batch of culture and purified as described in Appendix F: LIF Purification Protocol.

In addition to a LIF concentration experiment, a cell concentration (seeding density) experiment was also conducted to determine whether seeding density has any effect on how cells differentiate. The cell densities were varied at $2x10^6$, $1x10^6$, and $0.5x10^5$ cells in 6-well plate wells, and both LIF^+ and LIF^- cultures were used in this experiment. Only FACS analysis was performed in this experiment, however, since cell growth and metabolic data are invariably defined by the seeding density and therefore nonessential to this research.

Prior to these studies, an experiment was conducted with both the original R1 cell line and the transfected R1 Oct4-GFP cell line to confirm that the stable transfection of *gfp* gene into the genome did not alter the cell growth kinetics and metabolic profile of the cells. These values were also compared to the data obtained from J1 cell line, a different but also commonly-used murine ES cell line, to check for consistency in the values. Aside from these experiments, several studies were also conducted to determine the error in seeding cells onto 6-well plate

wells, as well as the rate of medium evaporation in an incubator and the rate at which medium components degrade over time after repeated cycle of heating and cooling in the water bath and the refrigerator, respectively. These studies were used to make fine adjustments in the data analyses, and the setup and results for each study are available in separate appendices at the end of the document.

In order to check for consistency of results, most of these experiments were conducted with two R1 Oct4-GFP cell populations: an ordinary, unsorted cell population expanded from a vial of cells acquired from the Langer Lab, as well as a Top 20 % GFP^+ cell population. This sorted cell population, as well as a third population that was only used in the preliminary differentiation characterization experiment (Bottom 20 % GFP^+), was sorted out by a FACS instrument at the MIT flow cytometry after an initial expansion of R1 Oct4-GFP cells. Only those cells with the top 20 % and bottom 20 % of positive GFP expression were collected from the sorting event, and these cell populations (T20 and B20), along with unsorted R1 Oct4-GFP and R1 cells, were expanded and frozen down according to the protocol as stated in Appendix D: Cell Maintenance Protocols at passage number of 12, 12, 4, and 1, respectively, with passage number 1 starting from the vials that were initially acquired from the Langer Lab. The difference in 11 passages was believed to have a non significant effect on the outcome of the research.

For FACS analysis involving percentage of GFP^+ expression, 10^2 on the FL1-H sorting channel was used as the threshold value, above which was considered to be GFP^+. The FACS settings, as described in greater detail in Appendix G: Becton Dickinson FACScan Settings and Operation, were also kept constant throughout this research for consistency. The Cedex cell counting instrument was set for "standard cells," which was optimized for seven parameters to exclude all non-cell particles or aggregates based on appearance. At this setting, only those cells with diameter between 8 and 40 μm could be accurately measured, but typical diameter of ES cells was well within this range (~16 μm). This instrument was primed every day before use and shut down after use, but it required no periodic calibration. The YSI 2700 SELECT™ instrument, which analyzes metabolites in culture by enzymatic reaction into hydrogen pyroxide, was calibrated before each day of use with 5.00 g/L glucose and lactate calibration solution and 5.00 mM glutamine calibration solution. Glucose and lactate membranes were replaced approximately every two weeks, and glutamine membrane was replaced every week.

3 Checking for Stable Profiles in R1 Oct4-GFP Cell Line

3.1 R1 cell line experiment

3.1.1 Experimental setup

This preliminary experiment was conducted to compare the cell growth kinetics and metabolic consumption or production rate of R1 and R1 Oct4-GFP cells under two different culturing conditions: one with continuous addition of LIF and the other with addition of LIF after 7 days of LIF withdrawal. The first condition tested the similarities in growth and metabolic profiles of these cell lines while in the undifferentiated state, while the second tested the similarities in these profiles upon a week of differentiation. Both were necessary to confirm that the transfection of the *gfp* gene had not significantly altered the growth kinetic and metabolic profiles of the R1 cell line so that results from this research would be applicable to ES cells in general and not just to this particular transgenic cell line.

One vial each of R1 and unsorted R1 Oct4-GFP was thawed down and plated with 0.2 % v/v LIF, added to facilitate cell pluripotency recovery as described in Appendix D: Cell Maintenance Protocols under "Thawing down stem cells." Each cell population was cultured on 6-cm plates, and LIF^- cultures were split from LIF^+ cultures at Day 5 upon LIF withdrawal. On every day prior to seeding, either the medium was changed or cells were passaged to ensure rapid recovery of cells after thawing. The LIF^+ cultures were seeded onto 6-well plates in triplicates at $3.5x10^5$ cells per well on Day 9, and time samples were taken three times a day at 8 am, 4 pm, and 12 am for a total of 11 time samples over a 4-day time span. The LIF^- cultures were cultured without LIF for 7 days before LIF was added again on Day 11, and these cultures were seeded onto 6-well plates in triplicates at $5x10^5$ cells per well on Day 17 with time samples taken three times a day for a total of 11 time samples, just as for the LIF^+ cultures.

For daily sampling, 2 mL of supernatant was first withdrawn from each well, with 1 mL frozen down for future metabolic analyses with YSI 2700 SELECT™. Cells were then trypsinized by adding 0.5 mL trypsin to each well, and additional amounts of PBS were added to allow for uniform mixing of cells using a 1000 µL pipette. 1 mL PBS was added for Cedex analysis (making the total volume 1.5 mL) since 1 mL is required for the instrument and some liquid will invariably be left on the surface of the well. This experiment lasted for 21 days in total.

3.1.2 Results

Assuming no cell death, cell growth follows a first order kinetics in viable cell density (VCD), with μ as the specific growth rate ($time^{-1}$):

$$\frac{dN}{dt} = \mu N$$

$$N = N_o e^{\mu t}$$

$$\mu = \frac{\ln N - \ln N_o}{t - t_o} = \frac{\ln \frac{N}{N_o}}{t - t_o},$$

where N is VCD in cells/mL of culture,
t is time in hr.

Since doubling time (t_d) is simply $\frac{\ln 2}{\mu}$ under the assumption of no death, VCD data from Cedex were used to calculate t_d by plotting the average VCD over time on a semi-log plot with a weighed linear regression applied to the data points (which reflect time points). The no-death assumption was valid in this experiment due to high constant viability maintained in all cultures. Only the first 4 time points for LIF^{-7} and 7 time points for LIF^{+} were used in determining the doubling time, since they were graphically shown to be in the exponential phase of growth. Since each data point was based on the average of 3 independent but identical samples, the standard deviation (SD) was calculated separately and applied as σ_i in the Taylor expansion formula for weighted linear regression as described in Appendix H: Error Propagation.

Table 1: Doubling time of R1 and R1 Oct4-GFP cells

	R1 (hours)	R1 Oct4-GFP (hours)
LIF^{+}	13.3 ± 0.3	13.6 ± 0.2
LIF^{-7}	7.6 ± 0.4	8.2 ± 0.3

The results, summarized in Table 1, indicate that R1 and R1 Oct4-GFP have statistically non significant difference in t_d at both LIF^{+} and LIF^{-7} conditions, with the significance of difference under those conditions at 45 % and 89 % confidence level, respectively, as determined by the Maple script for confidence level calculation for weighted linear regression. This consistency is indicative of the fact that these cell lines have similar cell growth kinetics even when significant change is induced by LIF withdrawal. It is also interesting to note that the difference in t_d between the two culturing conditions are statistically significant in both R1 and R1 Oct4-GFP cell lines (both at 99.9 % confidence level), with much more rapid cell growth observed in LIF^{-7} culture. For R1 and R1 Oct4-GFP cell lines, t_d is longer in LIF^{+} culture than in LIF^{-7} culture by 75 % and 66 %, respectively. As results from latter experiments will indicate, this rapid growth in LIF^{-7} cultures is characteristic of culture that has recently experienced LIF addition following a period of LIF-depletion, which may be the result of sudden upregulation of genes controlling cell division.

The metabolic data for glucose, lactate, and glutamine were analyzed for each 8-hour time point interval to obtain the metabolic consumption or production rate at each culturing condition, and VCD data were used to calculate the average VCD over each time interval so that

the metabolic rate could be determined on a cell number basis. The correction factor of 0.01 g/hr medium evaporation rate from Appendix A: Medium Evaporation Study was used to make fine adjustments to the metabolic rates, and SD was calculated using error propagation formulas.

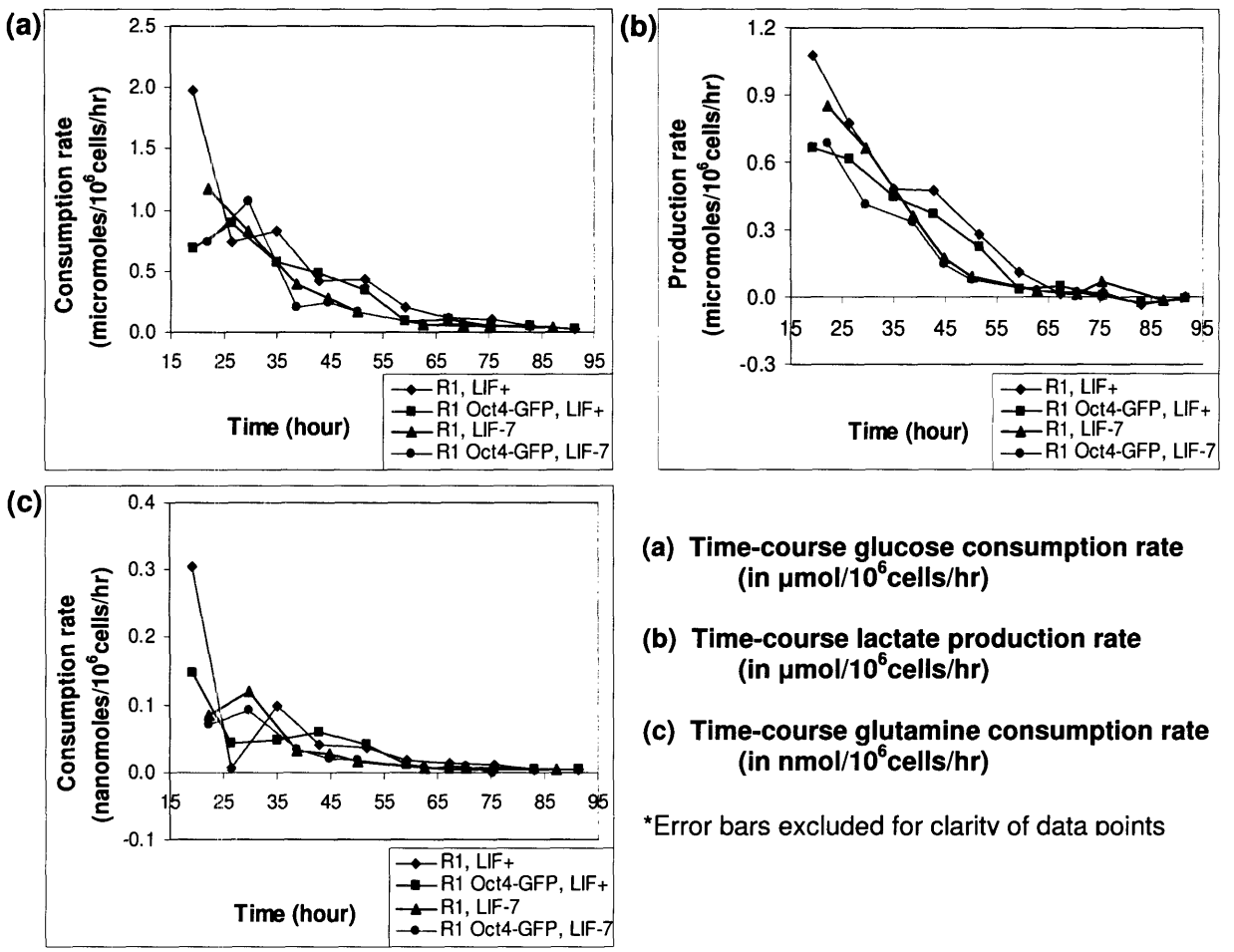


Figure 6: Time-course metabolic rates of both R1 cell lines under LIF^{+} and LIF^{-7} conditions

The metabolic rates for glucose (a), lactate (b), and glutamine (c) were analyzed at each time point interval and plotted over the course of the experiment. The four sets of data in each graph correspond to four unique conditions used in the experiment, arising from 2 different cell lines (R1 and R1 Oct4-GFP) and 2 LIF conditions (continuous LIF addition and LIF addition after 7 days of withdrawal).

As the graphs in Figure 6 indicate, both R1 and R1 Oct4-GFP cells have similar trend for metabolic rates under both LIF^{+} and LIF^{-7} conditions, with high metabolic rates at high glucose and glutamine concentrations and reduction in metabolism as the culture becomes increasingly acidic from pH 8.0 to 6.8 in linear fashion with time until it stabilizes at pH of 6.8 or 6.9. Although the concentration of nutrients have been shown to affect metabolic rates indicative of Monod-type saturation model[50], pH probably played a more significant role, given that the metabolic rates approached zero before the nutrients were close to being depleted (3.2 mM glucose and 0.2 mM glutamine still remained at the last time point). This phenomenon of increase in metabolic rates at high pH has been observed in many studies involving animal cells,

and it is hypothesized that this is due either to increase in glycolytic enzyme activity[51] or change in membrane potential which affects the glucose transport rate through the membrane[52]. Although the error bars are not shown in the graphs, SD averaged out to be 0.18 μmol/10^6cells/hr for glucose, 0.17 μmol/10^6cells/hr for lactate, and 0.02 nmol/10^6cells/hr for glutamine, which is enough to show that the metabolic rates in Figure 6 are at least consistent, if not in agreement, with one another.

The only major discrepancy between R1 and R1 Oct4-GFP is the high metabolism of glucose and glutamine initially observed within the first day of seeding, which can be attributed partly to the slightly lower initial seeding density of R1 cells that altered the apparent nutrient concentration seen by the cells and hence affected their metabolic rates. As a point of comparison, baby hamster kidney cells in other studies have been shown to have a maximum glucose uptake rate of 1.95 μmol/10^6cells/hr[53], which is consistent with the result obtained in this experiment. A yield calculation of lactate from glucose ($Y_{lac/glu}$) revealed consistent $Y_{lac/gluc}$ values, with their respective range at 44-94 % and 51-94 % for R1 LIF^+ and R1 Oct4-GFP LIF^+ cultures and 54-86 % and 54-84 % for R1 LIF^- and R1 Oct4-GFP LIF^- cultures.

3.1.3 Conclusion

The results from this experiment revealed consistent growth kinetics profiles of R1 and R1 Oct4-GFP cells at both LIF^+ and LIF^{-7} conditions, with t_d of approximately 13 hours for LIF^+ cultures and 8 hours for LIF^{-7} cultures, indicating statistically significant difference in doubling time between cells grown with continuous LIF addition and those with periodic LIF withdrawal. The t_d in LIF^+ culture was found to be 75 % and 68 % longer than the t_d in LIF^{-7} culture for R1 and R1 Oct4-GFP cells, respectively. The metabolic profiles of R1 and R1 Oct4-GFP were also consistent for glucose, lactate, and glutamine, giving a strong indication that *gfp* gene did not significantly affect the R1 cell line characteristics through its stable transfection. $Y_{lac/gluc}$ calculation revealed strong similarities between R1 and R1 Oct4-GFP cells at both LIF^+ and LIF^- conditions and consistency between LIF^+ and LIF^- conditions for both cell populations.

3.2 J1 cell line experiment

3.2.1 Experimental setup

In order to ensure that R1 cell line has similar growth kinetics and metabolic profiles compared to other ES cells, a different murine ES cell line called J1 was studied under LIF^+ condition. Similar methods of culturing were used in this experiment with the exception of counting cells on a hemacytometer and seeding cells on 6-cm plates. Results from this experiment were compared to those results obtained from the R1 and R1 Oct4-GFP experiment and checked for consistency.

For this experiment, one vial of J1 cells was thawed down and plated on a 6-cm plate with 0.2 % v/v non-commercial LIF as stated above. After medium exchange on Day 2, the cells were seeded onto five 6-cm plates on the following day at $3x10^5$ cells per plate as determined by manual counting using a hemacytometer, to be analyzed twice a day in 12-hour intervals starting on Day 4. LIF was added every day prior to seeding as well as on the day of seeding, and enough medium to make the total culture volume 4 mL was added to each plate.

For daily sampling, supernatant was first withdrawn from one of the plates, with 1 mL of it frozen down for future metabolic analyses with YSI 2700 SELECT™. Cells were then trypsinized using 1 mL trypsin, and 2.5 mL of fresh medium was added to the plate for a uniform mixing of cells using a 1000 µL pipette. A small volume (approximately 100 µL) of cells was diluted 2:1 with 0.2 % trypan blue, and the cells were counted on a hemacytometer. The metabolic analyses and cell counting were performed in triplicate from the same plate, and SD of those measurements was used in the error propagation analysis. This experiment lasted for 6 days.

3.2.2 Results

Viable cell density (VCD) data were analyzed as described in Section 3.1.2 to determine the doubling time (t_d) for J1 cells. Due to the low initial seeding density, all 5 time points in this experiment were in the exponential phase and were therefore used in t_d calculation. Based on these time points, t_d was found to be 10.7 ± 0.1 hours, which was statistically different from the R1 cell line at 86 % confidence level and R1 Oct4-GFP cells at 99.9 % confidence level. This result further confirms the consistency of the cell growth profile of the R1 cell lines, as the difference observed between J1 and R1 Oct4-GFP cell lines is not only an order of magnitude greater than the difference observed between R1 and R1 Oct4-GFP cell lines but also statistically significant. Even for the R1 and J1 cell lines where t_d are not statistically significant, the confidence level indicates that the difference in t_d is greater between J1 and R1 cells than between R1 and R1 Oct4-GFP cells (86 % versus 45 % confidence level).

The metabolic rates for glucose, lactate, and glutamine were also calculated from the metabolic data as described in Section 3.1.2. Since the decrease in metabolic rate over time for J1 cell line was much more gradual, only the maximum and minimum values are presented.

Table 2: Metabolic rates of J1 cell line under LIF^+ condition

	Max rate ($\mu mol/10^6 cells/hr$)	Min rate ($\mu mol/10^6 cells/hr$)
Glucose consumption	0.48	0.30
Lactate production	0.55	0.28
Glutamine consumption	0.15*	0.04*

* Glutamine consumption rate is in units of $nmol/10^6 cells/hr$

From error propagation, SD of the metabolic rates was found to be 0.74 $\mu mol/10^6 cells/hr$ for glucose, 0.48 $\mu mol/10^6 cells/hr$ for lactate, and 0.50 $nmol/10^6 cells/hr$ for glutamine. The comparison of metabolic rates from Table 2 and Figure 6 along with their SDs strongly suggest that J1 and R1 cells metabolize glucose and glutamine and produce lactate in a consistent manner and are therefore similar from a metabolic standpoint. Although the range of values for metabolic rates is somewhat smaller for the J1 cell line, this discrepancy can be attributed to the difference in cell lines, as well as to the different culturing condition and cell counting method used in this experiment. The $Y_{lac/gluc}$ ratio was also found to be between 47 % and 57 %, which is consistent with the results from the R1 cell line experiment.

3.2.3 Conclusion

Comparisons between R1 cell lines and J1 cell line revealed significant discrepancy in cell growth kinetics, with 21 % shorter t_d for J1 cell line compared to R1 cell lines, but consistency was observed in basic metabolic profiles, with $Y_{lac/gluc}$ values for J1 cells ranging between 47 % and 57 %. The upper limit of the range is significantly lower for J1 cells than for R1 cells, perhaps owing to the faster t_d of J1 cells that forces them to utilize energy more efficiently.

The discrepancy in cell growth kinetics could be attributed to the different culturing conditions (on 6-cm plates in a different incubator: Revco Ultima Series standard-size CO_2 incubator instead of ThermoForma Steri-Cycle^{TM} CO_2 incubator) and cell counting method (manual cell counting by hemacytometer) used in this experiment, which might have added systematic uncertainties to the experiment. Nonetheless, this experiment demonstrated the discrepancy in cell growth and metabolic characteristics that can be exhibited in different cell lines and the importance of performing a preliminary check on their characteristics before conducting multiple experiments. Furthermore, R1 Oct4-GFP cells were shown to be more similar to R1 cells than J1 cells in both proliferation rate and metabolism, which is further evidence that the *gfp* gene transfection did not significantly affect R1 cell characteristics.

4 Preliminary Analysis of R1 Oct4-GFP

4.1 Differentiation experiment

4.1.1 Experimental setup

In this preliminary experiment, the GFP^+ expression level of ES cells both seeded in 6-well plates and maintained on 6-cm plates was analyzed in LIF^+ and LIF^- cultures for purposes of obtaining a detailed differentiation profile of ES cells. All three cell populations as discussed in Section 2.2 were studied so that the differences among the populations, including the degree to which each loses pluripotency, could be quantified. For this reason, both the FACS histograms and the graphical representations of GFP^+ expression level are provided in this section.

One vial each of Top 20 % sorted (T20), Bottom 20 % sorted (B20), and unsorted R1 Oct4-GFP cells were thawed down and plated in standard ES medium with 0.2 % v/v non-commercial LIF, added to facilitate cell pluripotency recovery as described in the cell maintenance protocol under "Thawing down stem cells." Each cell population was initially plated in a 6-well plate well, and the cells were expanded and maintained on 6-cm plates and then on 10-cm plates as they grew. On every day either the medium was changed or cells were passaged to ensure rapid recovery of cells after thawing.

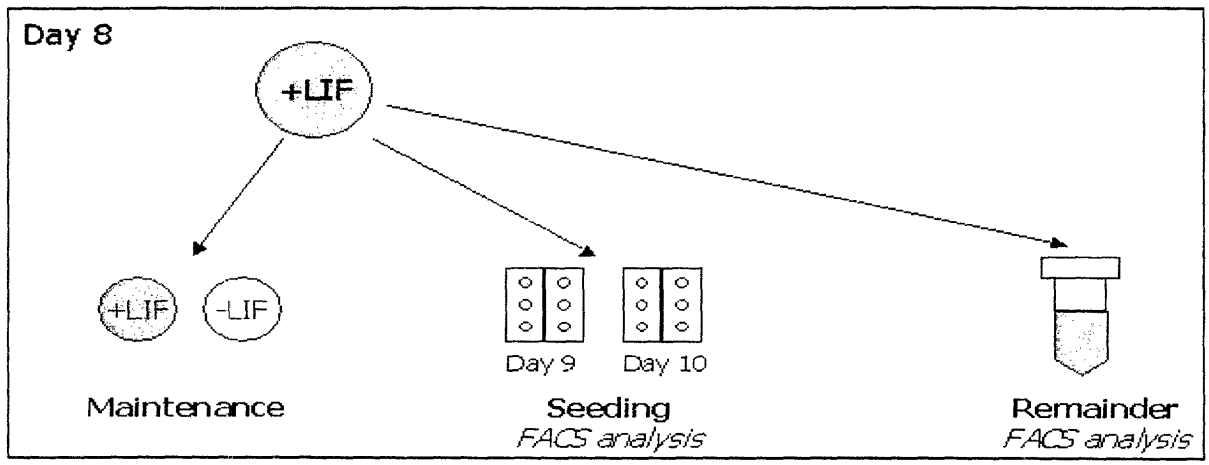


Figure 7: Experimental setup in differentiation experiment

On each seeding, LIF^+ cells from the maintenance plate were seeded onto 6-well plates for FACS analyses in the following two days, as well as passaged onto 6-cm plates for maintaining cells for subsequent passages. The remainder of cells from the maintenance plate was analyzed by FACS, and this procedure was repeated for all 3 cell populations. For Seeding #1, 10-cm plate was used as the maintenance plate, and LIF^- cultures were also started.

On Day 8, seeding and LIF withdrawal were initiated (Figure 7). Each of the three 10-cm plates (corresponding to the 3 cell populations) was seeded in four 6-well plate wells for two days of sampling after seeding at both LIF^+ and LIF^- conditions, as well as on two 6-cm plates used for maintaining cells for next seeding at both LIF^+ and LIF^- conditions. For LIF^+ culture,

0.2 % v/v LIF was added to the medium for each passage or medium exchange, while LIF^- culture did not receive LIF for the duration of the experiment. FACS analysis was performed on the remainder of the cells for pluripotency information on the maintenance plate. Seeding was performed every other day at a reasonable concentration to ensure exponential growth between time points, for a total of 4 seedings.

For daily sampling, supernatant was first withdrawn from each well, and cells were trypsinized by adding 0.25 mL trypsin to each well. Approximately 1mL of PBS was then added to allow for uniform mixing of cells using a 1000 µL pipette, and the mixture was then centrifuged in an IEC Centra-M microcentrifuge for 1 minute at 15,600 xg to remove the red-colored trypsin). These cells were then resuspended in cell density-dependent amount of PBS and left on ice until FACS analysis was performed. This experiment lasted for 16 days.

4.1.2 Results

The histogram plots of percent GFP expression were obtained from the FACScan instrument for both LIF^+ and LIF^- cultures, grown in both the 6-well plates used for seeding and the 6-cm plates used for maintaining cells. The following figures from Figure 8 to Figure 11, which are analyses of cells on the maintenance plates, visually show how levels of pluripotency in cells vary as population over time, both in cultures with LIF supplementation and LIF withdrawal. All of the histograms have "count" on the linear y-axis and "FL1-H" corresponding to GFP expression on the logarithmic x-axis.

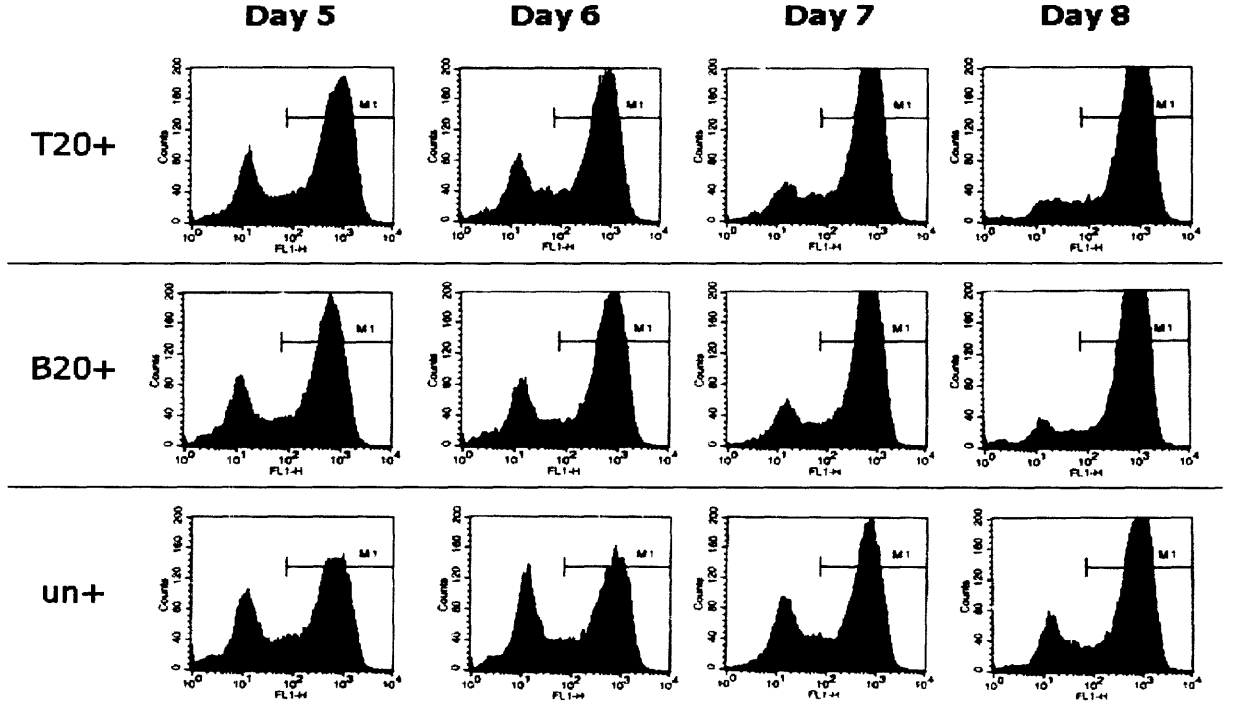


Figure 8: FACS histograms of LIF^+ cultures with 1-day passages

These histograms represent FACS analyses that were conducted daily over 4 days leading up to Seeding #1. On these days, passages were performed daily with LIF addition for all cell populations. The GFP expression level for each histogram is based on approximately 50,000 cells.

Between Day 5 and Day 8, where LIF was added to all cultures at 2 % v/v and passages were performed daily, there is an obvious increase in peak height for high GFP expression (~10^3 on FL1-H) and decrease in peak height for low GFP expression (~10^1 on FL1-H) for all three cell populations over time. These peak heights can be readily correlated to fractions of cells at each GFP expression level, since all the graphs are based on the same number of cells at approximately 50,000 cells. This implies that there are increasingly higher percentage of cells that are GFP^+ and increasingly lower percentage of cells that are GFP^- in the presence of LIF, which is expected as LIF not only prevents spontaneous differentiation but also increases the proliferation rate of undifferentiated cells in a more dramatic manner than partially differentiated cells [54]. This result also visually proves the effectiveness of the non-commercial LIF used in this research, as well as the reliability of the *pou5f1-gfp* gene construct in allowing the correlation of ES cell pluripotency to be made through level of GFP expression.

The difference among the cell populations also exists, but the difference only appears to lie in the fractions of cells that fall under the 2 peaks, with T20 and B20 showing greater similarity to each other than to the unsorted cell population. This is supported by the fact that T20 and B20 were both sorted for GFP^+-expressing cells and therefore have greater fraction of cells expressing high level of GFP than unsorted cells. It is also interesting to note the drastic improvement in GFP^+ expression level for the unsorted cell population, resembling on Day 8 the distribution on Day 6 of T20 and B20 population. This explains why T20 and B20 are also very similar to each other in GFP expression, as any differences that existed between these populations must have been compensated by the improvement in GFP^+ expression in B20 cells as LIF was added in the expansion of cells, either prior to making a freezing stock or after the vials were thawed down in preparation for this experiment.

It is also interesting to note that there are 2 distinct peaks where the majority of the cells in a population lies instead of a smooth, Gaussian distribution over a broad range of GFP expression. This perhaps is indicative of the bistability of the *gfp* gene, with the gene either turned on after upregulation or off upon downregulation. From this perspective, the cells should naturally be distributed in this way, as there are 2 possible states for a gene to be in, with each state assuming a Gaussian distribution in a probabilistic manner. The small fraction of cells that fall between the peaks probably corresponds to those cells that are in the process of being upregulated or downregulated.

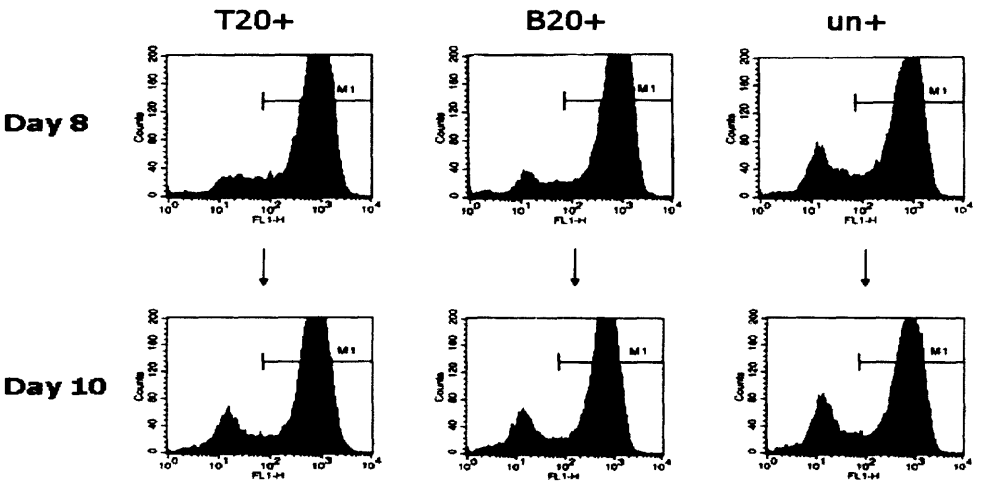


Figure 9: FACS histograms for transition between 1-day and 2-day passages

For each cell population, the pair of histograms represents a shift in GFP expression observed between 1-day passaging and 2-day passaging transition in the maintenance plates. 2-day passaging was initiated for the first time on the maintenance plates on Day 8, the effect of which was seen for the first time on Day 10.

The histograms in Figure 9 reveal a negative trend for cell population pluripotency when daily passaging is changed to 2-day passaging for all 3 cell populations even when LIF is added, as evidenced by the increase in the fraction of cells with low GFP-expression. This result confirms the notion that cells stay healthier if passaged more frequently, which can be an indication for the existence either of an optimal seeding density or passaging frequency for cells.

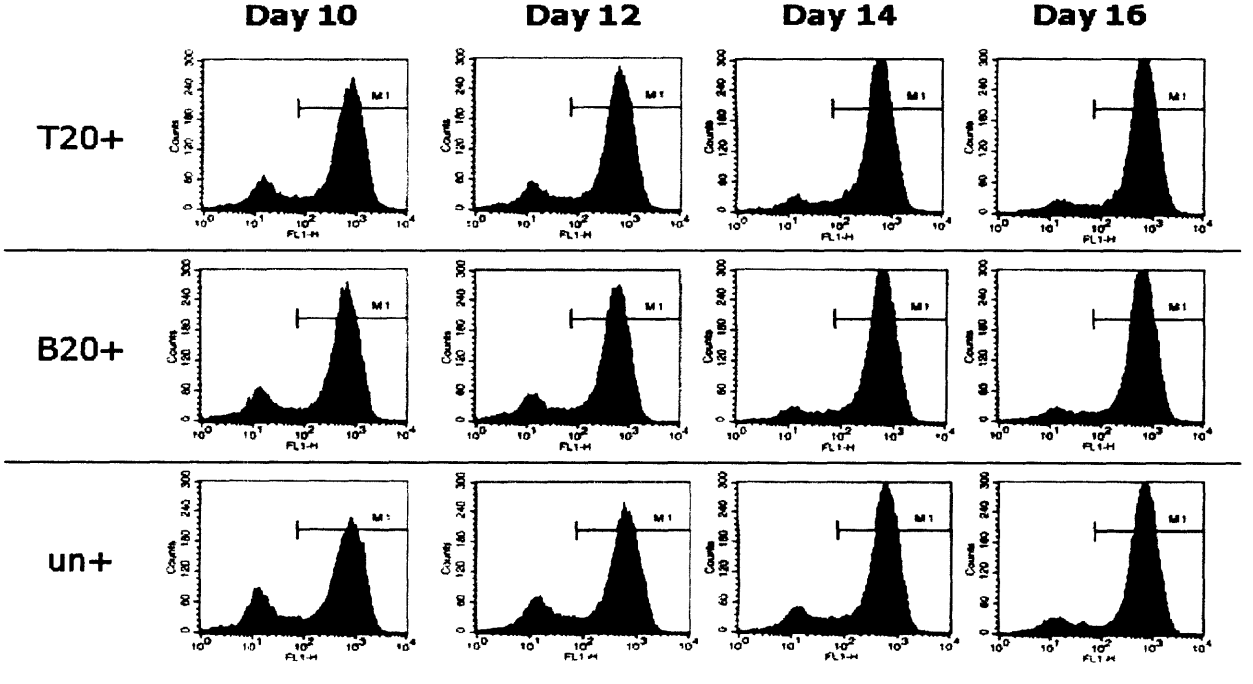


Figure 10: FACS histograms of LIF$^+$ cultures with 2-day passages

These histograms represent FACS analyses that were conducted on LIF$^+$ cells every two days after Seeding #1 on days when cells were passaged. The GFP expression level for each histogram is based on approximately 50,000 cells.

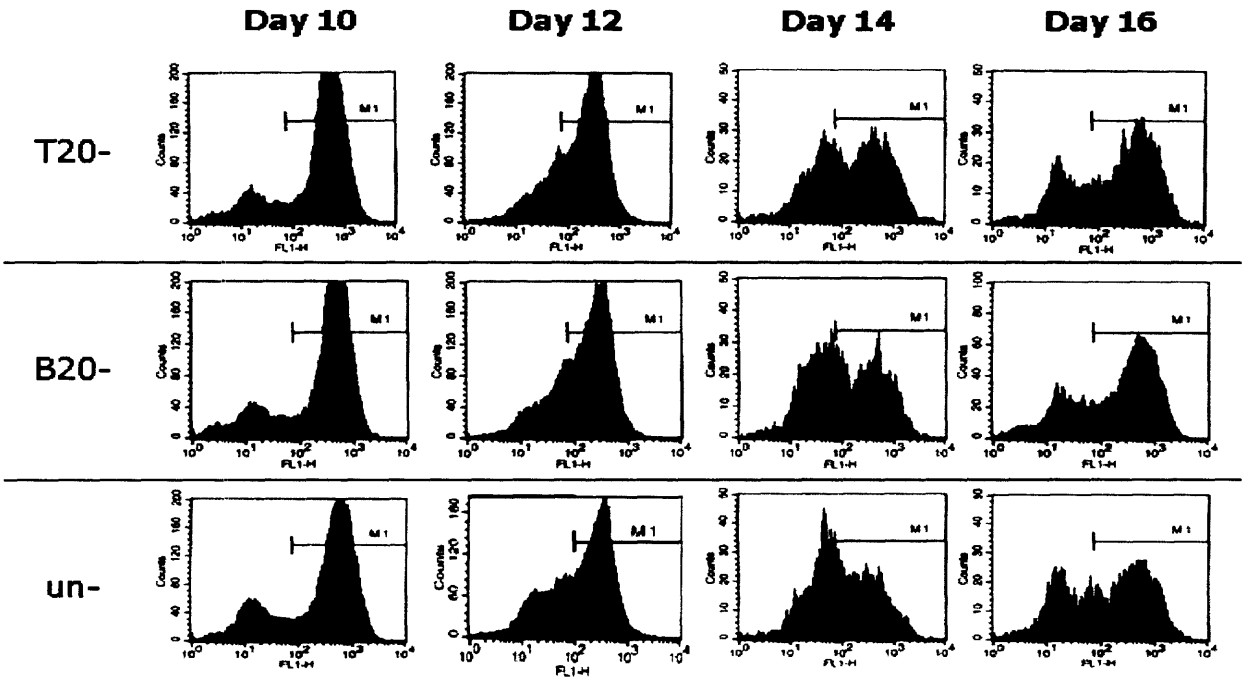


Figure 11: FACS histograms of LIF⁻ cultures with 2-day passages

These histograms represent FACS analyses that were conducted on LIF⁻ cells every two days after Seeding #1 on days when cells were passaged. The GFP expression level for each histogram is based on approximately 50,000 cells for Days 10 and 12 and 10,000 cells for Days 14 and 16, with the exception of B20 cells on Day 16, which was based on 20,000 cells.

In Figure 10 and Figure 11, the FACS histograms of LIF^+ and LIF⁻ cultures between Day 10 and Day 16 are shown, respectively. In the LIF^+ cultures, further improvement in GFP^+ expression is seen, with the gradual elimination of the GFP⁻ expression peak, as expected from the continual addition of LIF. In the LIF⁻ cultures, the decline in GFP expression is characterized by merging of the 2 peaks at mid-expression level (~10^2 on FL1-H), followed by the formation of distinct peaks with larger fraction of cells expressing low level of GFP on Day 14 (see row with unsorted LIF⁻). This further supports the idea that the *gfp* gene acts like a switch, since the merging of the peaks is simply the movement of the high GFP-expression peak caused by LIF withdrawal.

The apparent improvement in GFP^+ expression peak observed on Day 16 was an anomaly that can be attributed to the high adherence of partially differentiated cells to the plates. This was a qualitative judgment made during passaging by observing the untrypsinized cells on the LIF⁻ plates and noting the similarity in morphology to the flattened, jagged-edged cells in the extended LIF⁻ culture in the LIF concentration experiment (Figure 27e). If a significant portion of the partially differentiated cells remained attached to the plates, a disproportionately higher percentage of undifferentiated cells would have been analyzed by FACS, thereby distorting the distribution of GFP expression in the histograms.

To get a better idea of how GFP^+ expression of cells in maintenance plates varies over time, FACS data were compiled and analyzed for the percentage of cells in each population with GFP^+ expression (as determined to be the percentage over the threshold value of 10^2 in FL1-H using the FACS settings as described in Appendix G: Becton Dickinson FACScan Settings and

Operation). The error in FACS measurements from the seeding variation study (2 SD of 4.06 % and 4.32 % GFP^+ expression for T20/B20 and unsorted cell populations, respectively) was used as error bars, and a vertical dotted line was used to indicate the day on which 2-day passages were commenced. These results are summarized in the figure below.

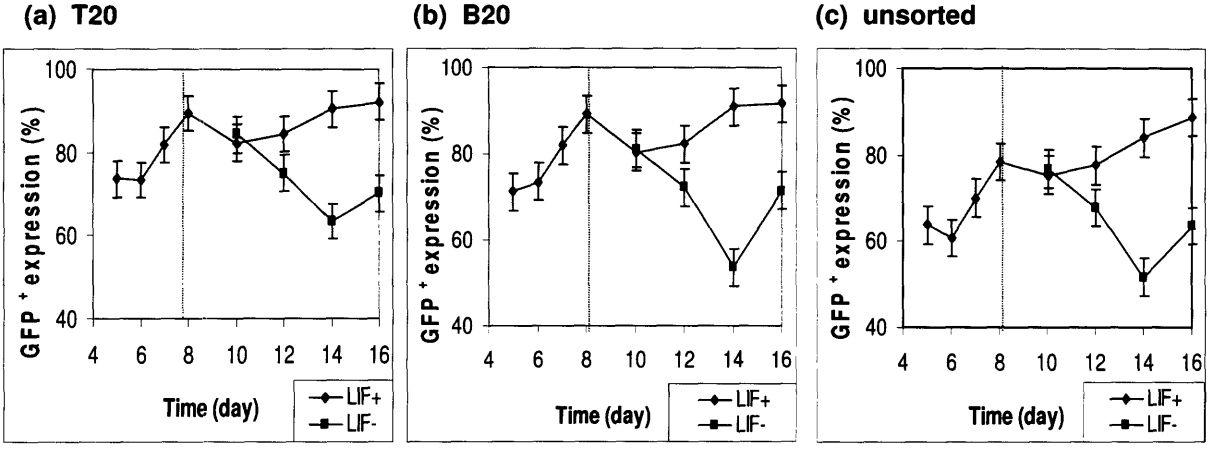


Figure 12: Percent GFP^+ expression over time on 6-cm plates

For each cell population, the percentage of cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ culture was compared to the percentage of GFP^+ cells in LIF^- culture. The error bars reflect 2 SD of 4.06 % for T20 and B20 cells and 4.32 % for unsorted cells, as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures.

The sharp, upward trend in the values is apparent in the initial phase of the experiment for all three cell populations, followed by a more gradual upward trend for the LIF^+ cultures after the start of the 2-day passages. This overall upward trend agrees with the histograms, indicating improvement in pluripotency with LIF addition. As expected, the LIF^- cultures drop in GFP^+ expression steadily for the next few passages, resembling the trend observed in the histograms. The anomaly of improvement in GFP^+ expression observed between Day 14 and Day 16 in the LIF^- cultures is also apparent in these graphs.

It is interesting to note that the improvement in GFP^+ expression by LIF addition between Day 8 and Day 14 is less drastic than the decrease in GFP^+ expression through LIF withdrawal. This stems from the fact that cells at this stage (at 80 % GFP^+ expression) are closer to the theoretical maximum of 100 % GFP^+ expression and therefore only have 20 % of cells that can be affected by LIF addition, whereas the drop in GFP^+ expression is more drastic since 80 % of cells can be affected by LIF withdrawal. LIF withdrawal also appears to have a more rapid effect on the *pou5f1* promoter than on genes affecting cell division, with significant decline in GFP^+ expression observed just 3 to 4 days after LIF withdrawal. The effect of LIF on cell proliferation will be discussed later in the LIF concentration experiment.

For a more in-depth analysis of the differentiation profile of ES cells, the results from the seeded plates were analyzed. It was first necessary, however, to compare the FACS results for cells grown on maintenance plates and those grown on seeded plates to ensure that these data were comparable.

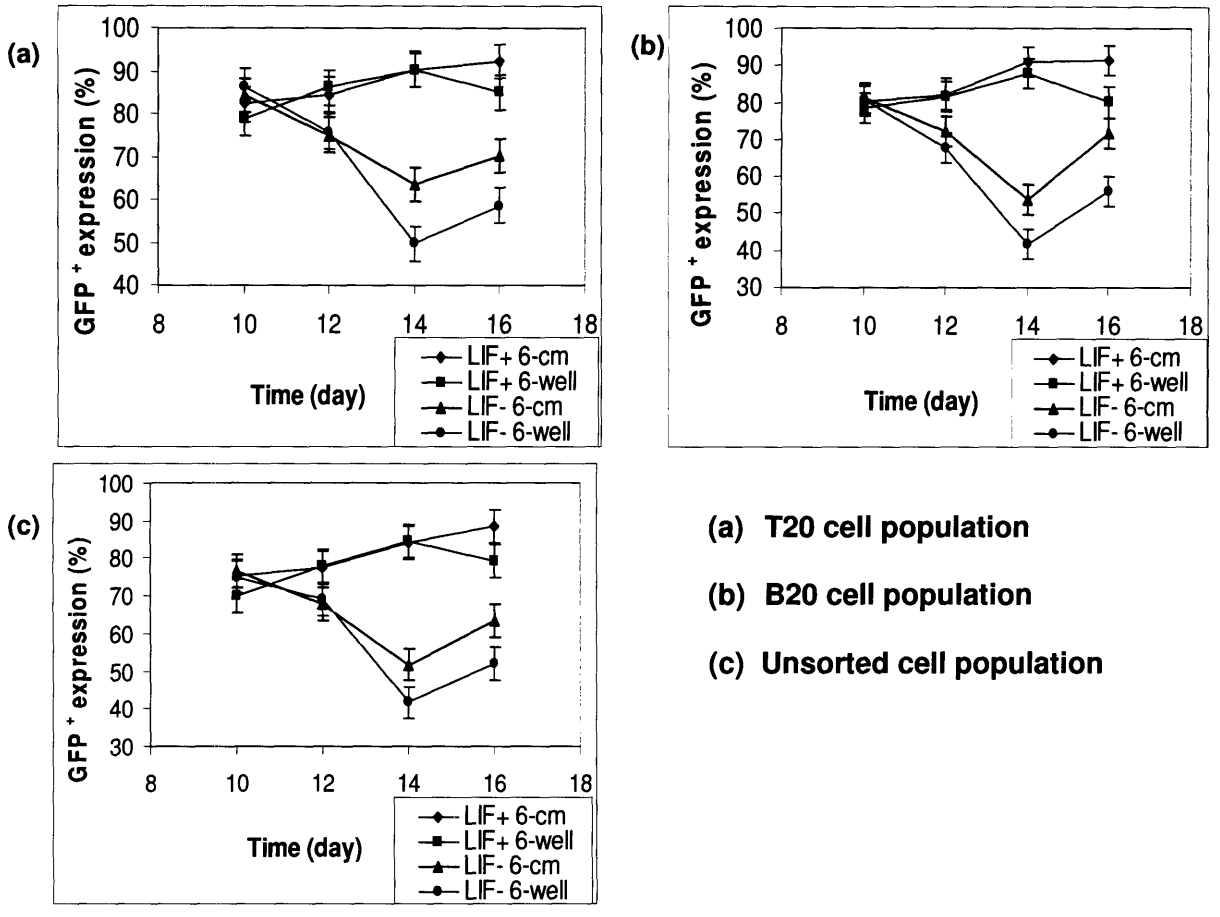


Figure 13: Comparison of FACS results for 6-cm and 6-well plates

For each cell population, the percentage of cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ and LIF^- cultures on 6-cm maintenance plates was compared to the percentage of GFP^+ cells on 6-well seeded plates. The error bars reflect 2 SD of 4.06 % for T20 and B20 cells and 4.32 % for unsorted cells, as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures, as well as for 6-cm and 6-well plates.

According to the figure above, which compares such data for Days 10, 12, 14, and 16, there is non significant difference in GFP^+ expression between cells grown in 6-cm plates and those grown in 6-well plate wells for LIF^+ cultures, which is to be expected. LIF^- cultures seem to follow a similar trend initially, but there is significant difference in the last 2 passages (Seedings #3 and #4) for all 3 populations, which could have resulted from seeding these plates too low and thereby disrupting cell-cell signaling crucial for maintaining cell pluripotency. This low seeding density is apparent in the histograms from Day 14 and Day 16 for LIF^- cultures (Figure 11), which show jagged distribution of cells for all cell populations, with the y-axis scale corresponding to frequency adjusted from "200" to "50" to reflect the reduced number of cells analyzed in FACS from 50,000 to 10,000. This hypothesis of low seeding density affecting GFP^+ expression in LIF^- cultures was later tested in the seeding density experiment (Section 5.4).

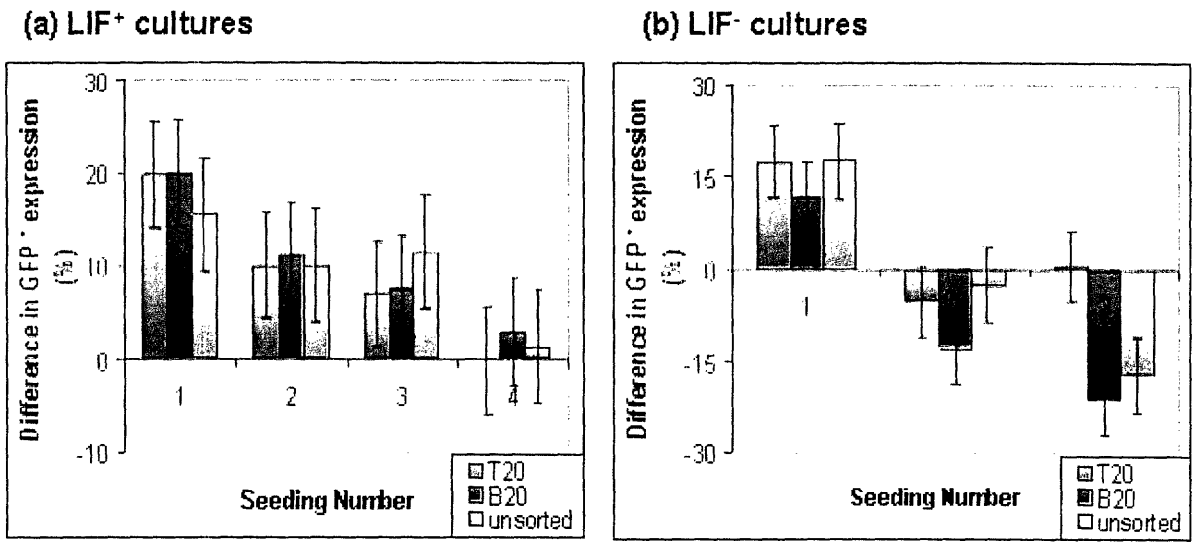


Figure 14: Change in GFP$^+$ expression level from Day 1 to Day 2 for each seeding

Each graph shows the change in GFP$^+$ expression from Day 1 to Day 2 at each seeding for the 3 cell populations cultured in 6-well plates, shown in blue (T20), maroon (B20), and yellow (unsorted) bars. The error bars (5.74 % for T20 and B20 cells and 6.11 % for unsorted cells) reflect 2 SD values from Appendix B: Seeding Variation Study that underwent error propagation.

The FACS data for seeded plates were analyzed for change in percentage of cells with GFP$^+$ expression to determine how cell pluripotency differs from Day 1 to Day 2 in a 2-day passage, since only results corresponding to Day 2 of the seeded plates could be obtained for maintenance plates. According to Figure 14a, the LIF$^+$ cultures initially experience the greatest boost in percentage of cells with GFP+ expression from Day 1 to Day 2 of each seeding, and this increase gradually tapers off with each seeding until the difference becomes insignificant. This shows that GFP$^+$ expression improves with time within a passage and that passaging can have a detrimental effect on the cells, since the increase in GFP$^+$ expression between passages observed in is less than the increase between days within a passage in Figure 14a. This detrimental effect is most likely due to the time of trypsinization during passaging, but the results show that the cells can recover from it over the course of two days and even improve cell pluripotency by the next passage. The reduction in GFP$^+$ expression boost over seedings is probably due to its proximity to the theoretical maximum, as presented before.

The results for LIF$^-$ cultures reveal a similar increase in GFP$^+$ expression from Day 1 to Day 2 initially in Seeding #1, but a gradual decrease with subsequent seedings (Seeding #4 is not shown due to the anomaly in data as discussed before). This increase is presumed to be the result of residual LIF remaining in the LIF$^-$ cultures or the time delay involved in turning off the *pou5f1* promoter upon LIF withdrawal. As for the decreases observed in subsequent seedings, they are presumably due to further degradation of residual LIF or the *pou5f1* gene finally being turned off. The drastic drop in GFP$^+$ expression for LIF$^-$ cultures compared to the slight increase for LIF$^+$ cultures observed in the maintenance plates could be partially attributed to the fraction of cells that are affected by LIF in each culture as presented before, but it is probably more sensible that this is due to the combined negative effect of LIF withdrawal and trypsinization for the LIF$^-$ cultures.

4.1.3 Conclusion

For all ES cell populations with LIF addition to the cultures, there was improvement in the percentage of cells with GFP^+ expression over time, with less improvement seen in 2-day passages compared to 1-day passages, which could be attributed to the possible dependence of improvement in GFP^+ expression on the fraction of cells that are affected by LIF addition at any given time. It is also possible that there is an optimal seeding density that allows cells to maintain high GFP expression, since the actual act of passaging was shown to be detrimental to cell pluripotency (and therefore simply increasing the passaging frequency should not be beneficial for the cell pluripotency).

As expected, LIF withdrawal led to drastic reduction in percentage of cells with GFP^+ expression, which is probably due either to the high fraction of cells in culture that are affected by LIF withdrawal or that passaging and LIF withdrawal have an additive negative effect on GFP^+ expression. LIF withdrawal also appears to have a rapid effect on downregulating the *pou5f1* gene with reduction in GFP^+ expression observed within 3 to 4 days of LIF withdrawal, which is faster than its effect on cell proliferation rate, as discussed in latter experiments. It is unclear, however, whether the effect is due to reduction of LIF within the culture to below its effective concentration for maintaining cell pluripotency or to the time delay that is involved with its signal transduction on a cellular level.

There was also non significant difference in the pluripotency of cells grown in 6-cm plates compared to the pluripotency of cells grown on 6-well plate wells for all cell populations, which was as expected. The difference in GFP^+ expression between the unsorted cell population and T20/B20 cell populations was greater than the difference between T20 and B20 cell populations, which is expected since T20 and B20 cells were both sorted for GFP^+ expression. In this experiment, GFP^+ expression was also shown to improve with LIF addition such that different cell populations can end up having similar distribution of GFP expression in cells, as seen in the histograms.

The anomaly of increase in GFP^+ expression observed in the last seeding of LIF^- culture was attributed to the high level of adherence exhibited by partially differentiated cells, which resulted in a disproportionately higher ratio of undifferentiated to differentiated cells to be analyzed by FACS. In the future, this can be prevented by slightly increasing the trypsinization time for cells, although this may end up damaging healthier, undifferentiated cells.

4.2 Kinetic and metabolic experiment

4.2.1 Experimental setup

In this experiment, preliminary cell growth and metabolic profiles of R1 Oct4-GFP cells were obtained for purposes of determining whether the unsorted population varies in cell growth and metabolic profiles with the Top 20 % sorted (T20) population. While Bottom 20 % sorted (B20) population was initially considered for part of the study, it was omitted after the results from the preliminary differentiation study revealed the similarity between T20 and B20 populations.

Two vials each of T20 and unsorted R1 Oct4-GFP cells were thawed down and plated onto 6-well plate wells, one with 0.2 % v/v non-commercial LIF, added to facilitate cell pluripotency recovery as described in the cell maintenance protocol under "Thawing down stem cells," and the other without LIF. Cells were replated onto larger plates when they became confluent, and medium was changed every day. On Day 6, both cell populations were seeded onto 6-well plates for differentiation analysis, and T20 and unsorted cell populations were seeded in triplicate for cell growth and metabolic analyses. These analyses were conducted twice a day for Cedex and YSI, for a total of 5 consecutive time points over 3 days.

For each sampling, 2 mL of supernatant was first withdrawn from each well, with 1 mL frozen down for future metabolic analyses with YSI 2700 SELECT^{TM}. Cells were then trypsinized by adding 0.25 mL trypsin to each well, and additional amounts of PBS were added to allow for uniform mixing of cells using a 1000 µL pipette. 1 mL PBS was added for Cedex analysis (making the total volume 1.25 mL) since 1 mL is required for the instrument and some liquid will invariably be left on the surface of the well. This experiment lasted for 8 days in total.

4.2.2 Results

Viable cell density (VCD) results from Cedex were analyzed by performing doubling time (t_d) calculation as explained in Section 3.1.2. The first 7 time points were used for calculating t_d in LIF^+ cultures, while only 4 time points could be used for determining t_d in LIF^- cultures due to higher initial seeding densities in the LIF^- cultures. The values for t_d along with their standard deviation as determined by the Taylor expansion formula for weighted linear regression as described in Appendix H: Error Propagation are shown in the table below.

Table 3: Doubling time of T20 and unsorted R1 Oct4-GFP

	T20 (hours)	Unsorted (hours)
LIF^+	11.9 ± 0.2	12.5 ± 0.2
LIF^-	11.6 ± 0.3	12.5 ± 0.3

The results seem to indicate that T20 and unsorted R1 Oct4-GFP cells have seemingly significant difference in t_d at both LIF^+ and LIF^- conditions, but that very little difference in t_d exists between LIF^+ and LIF^- cultures for both cell populations. Due to the scatter in data and the discrepancy in the number of data points used between LIF^+ and LIF^- conditions, however, the differences were all found to be equally non significant. The significance of the discrepancy between T20 and unsorted cells at LIF^+ and LIF^- conditions was found to be at 46 % and 64 % confidence level, respectively, and the difference between LIF^+ and LIF^- conditions for T20 and unsorted cells was at 49 % and 46 % confidence level, respectively. The non significant difference between T20 and unsorted cells can be explained by the fact that these cell populations are derived from the same cell line.

Nonetheless, it is interesting to note how similar the t_d of T20 and unsorted cell populations are under different culturing conditions of LIF^+ and LIF^-, implying that perhaps LIF withdrawal has a non significant effect on overall ES cell growth. This result is validated by a study by Zandstra, et al. which has shown that cell proliferation is independent of LIF concentration tested over a broad range of concentrations (0.5 pM to 500 pM) including a condition where α-LIF monoclonal antibody was added to nullify the effects of low levels of LIF in the serum-containing medium. This study showed that the difference in maximum doubling time for ES cell cultures grown either with 500 pM LIF or α-LIF was non significant (13.6 ± 0.4 hours) [55]. A recent study by Viswanathan, et al., however, contradicts this claim by showing that the overall proliferation of ES cells, especially the undifferentiated population, improves with LIF concentration, also tested between no LIF and 500 pM LIF (Table 4) [54].

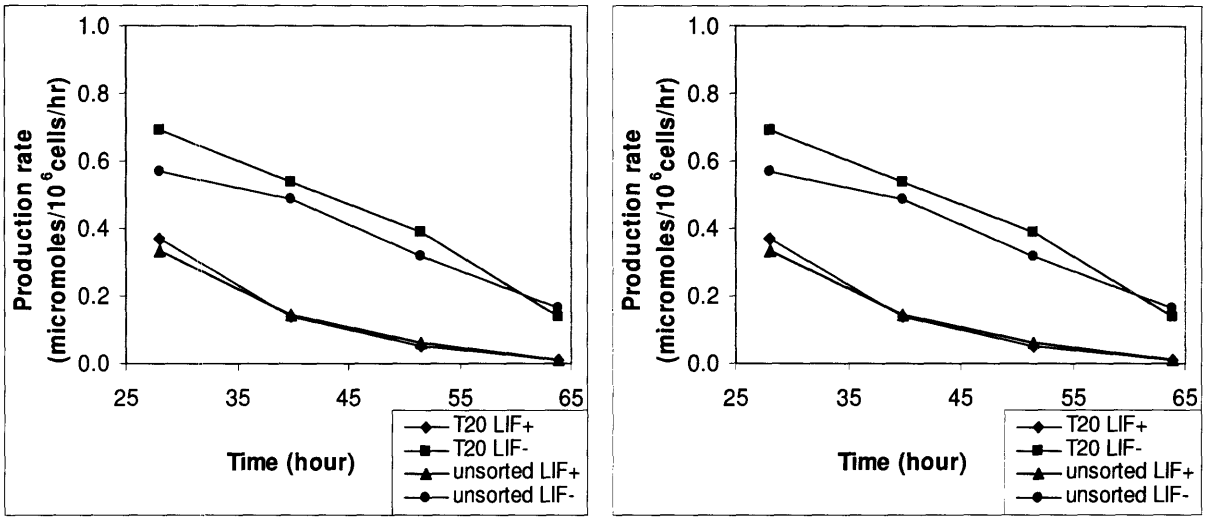
Table 4: Specific growth rate at various LIF concentrations (from Viswanathan, et al)

	Specific growth rate ($hour^{-1}$)	
	GFP^+	GFP^-
No LIF	0.008 ± 0.002	0.008 ± 0.001
0.5 pM LIF	0.01 ± 0.002	0.006 ± 0.001
5 pM LIF	0.04 ± 0.005	0.01 ± 0.006
50 pM LIF	0.06 ± 0.01	0.01 ± 0.005
500 pM LIF	0.08 ± 0.008	0.008 ± 0.001

This apparent contradiction could have stemmed from the differences in the way LIF was added to the cultures in these studies, just as the difference in t_d between LIF^+ and LIF^- cultures was significant for R1 cell line experiment (for which LIF^- culture experienced temporary LIF withdrawal followed by LIF addition) but non significant for this experiment (for which LIF was never added to the LIF^- culture). As suggested in Section 3.1.2, it is conceivable that the addition of any amount of LIF after periodic withdrawal has a boosting effect, perhaps in a concentration-dependent manner, on undifferentiated cell growth by upregulating their genes controlling cell division. Alternatively, if the downregulation of these genes through LIF withdrawal takes a few days to take effect, then adding LIF to the pre-seeding culture could potentially affect the outcome of the experiment where LIF concentration is the parameter.

The metabolic data for glucose and lactate were analyzed with the correction factor of 0.01 g/hr for evaporation at each 12-hour time point interval to obtain the metabolic consumption or production rate at both LIF^+ and LIF^- conditions for T20 and unsorted cell populations. The

average VCD over the time interval was used as before to calculate the metabolic rate on a cell number basis, and SD was calculated as before, using error propagation formulas.



(a) Time-course glucose consumption rate (in μmol/10^6cells/hr)

(b) Time-course lactate production rate (in μmol/10^6cells/hr)

* Error bars excluded for clarity of data points

Figure 15: Time-course metabolic rates of T20 and unsorted cells for LIF^+ and LIF^- conditions

The metabolic rates for glucose (a) and lactate (b) were analyzed at each half-day interval and plotted over the course of the experiment. The four sets of data in each graph correspond to four unique conditions used in the experiment, arising from 2 different cell populations (T20 and unsorted cells) and 2 LIF conditions (LIF addition and LIF withdrawal).

As the graphs in Figure 15 indicate, the glucose consumption rates ($q_{glucose}$) and lactate production rates ($q_{lactate}$) are both higher under LIF^+ condition than under LIF^- condition, even though the difference is not statistically significant due to the large SD at each data point (The error in $q_{glucose}$ for T20 and unsorted cells was found to be 0.26 and 0.40 μmol/10^6cells/hr, respectively, while the error in $q_{lactate}$ for T20 and unsorted cells was 0.50 and 0.37 μmol/10^6cells/hr). The trend of the graph also differs between cultures, with LIF^+ cultures behaving in a manner resembling exponential decay as in the R1 cell line experiment and LIF^- cultures outlining a linear decline in metabolic rate. These non significant differences in both the absolute value and the trend of the metabolic rates, however, can be attributed to higher pH (average pH of 7.3 on the last day), higher glucose concentration (7.17 mM), and lower lactate concentration (12.3 mM) found in the LIF^- cultures, since these cultures were seeded with cells at a much lower concentration by accident. Although the actual seeding concentration cannot be determined, VCD for unsorted and T20 LIF^- cultures on the day after seeding were $2.4x10^5$ and $2.3x10^5$ cells, while VCD for corresponding unsorted and T20 LIF^+ cultures were $9.9x10^5$ and $9.8x10^5$ cells, respectively. Incidentally, the LIF^+ cultures on the last day of the experiment had an average pH of 6.9, glucose concentration of 3.98 mM, and lactate concentration of 13.8 mM.

The difference between T20 and unsorted cell populations was also less pronounced than the difference between R1 and R1 Oct4-GFP (unsorted) cells in the R1 cell line experiment, which can be attributed to the fact that both T20 and unsorted cell populations are derived from the same cell line (R1 Oct4-GFP). Therefore, these cell populations should naturally have similar metabolic profiles compared to R1 and R1 Oct4-GFP cells, as they differ only in the fraction of undifferentiated cells in the population.

4.2.3 Conclusion

The results from this experiment revealed consistent growth kinetic profiles of T20 and unsorted R1 Oct4-GFP cell populations at both LIF^+ and LIF^{-7} conditions, with t_d of 11.8 hours for T20 cell cultures and 12.5 hours for unsorted cell cultures. These values were determined by confidence level calculation to have non significant difference. The difference in t_d between LIF^+ and LIF^- cultures were less pronounced and less statistically significant for both T20 and unsorted cells, indicating that either LIF plays little role in cell proliferation or that downregulation of genes governing cell division by LIF withdrawal takes a while to take effect. The metabolic profiles of T20 and unsorted cells were also consistent for glucose and lactate, although the data followed different trends. This was suggestive of difference in the mechanism governing glucose metabolism, which was perhaps caused by the low seeding density of cells in LIF^- cultures that led to low metabolism of glucose into lactate and consequently little acidification of the cultures.

5 Variation in Culturing Conditions

5.1 Low glucose experiment

5.1.1 Experimental setup

In this experiment, the glucose concentration in medium was varied in order to determine whether low glucose medium has any effect on ES cell growth, metabolism, and pluripotency. Since glucose is a main source of nutrient in any animal cells, it was hypothesized that low glucose medium would have a significant effect on cell growth and metabolic profiles. However, it was difficult to ascertain to what extent these profiles would be affected or whether this lowered cell growth and metabolism would have any effect on ES cell pluripotency.

Two vials each of Top 20 % sorted (T20) and unsorted R1 Oct4-GFP cells were thawed down and plated with 0.2 % v/v non-commercial LIF, added to facilitate cell pluripotency recovery as described in the cell maintenance protocol under "Thawing down stem cells." Since the cell density of unsorted cell vials ($2x10^6$ cells/vial) was twice that of T20 cell vials ($1x10^6$ cells/vial), the unsorted cells were initially plated onto 6-cm plates while T20 cells were cultured in 6-well plate wells. On Day 2, each cell population was split into 2 plates of different glucose concentrations, one at standard concentration of 4500 mg/L (25 mM) and the other at reduced concentration of 1500 mg/L (8.33 mM). As cells grew, they were expanded and maintained in 6-cm plates. On every day either the medium was changed or cells were passaged to ensure rapid recovery of cells after thawing. On Day 5, each plate was split into 2 separate plates, one with LIF^+ and the other with LIF^-. For LIF^+ culture, LIF was added to the medium for each passage or medium exchange, while LIF^- culture did not receive LIF for the duration of the experiment.

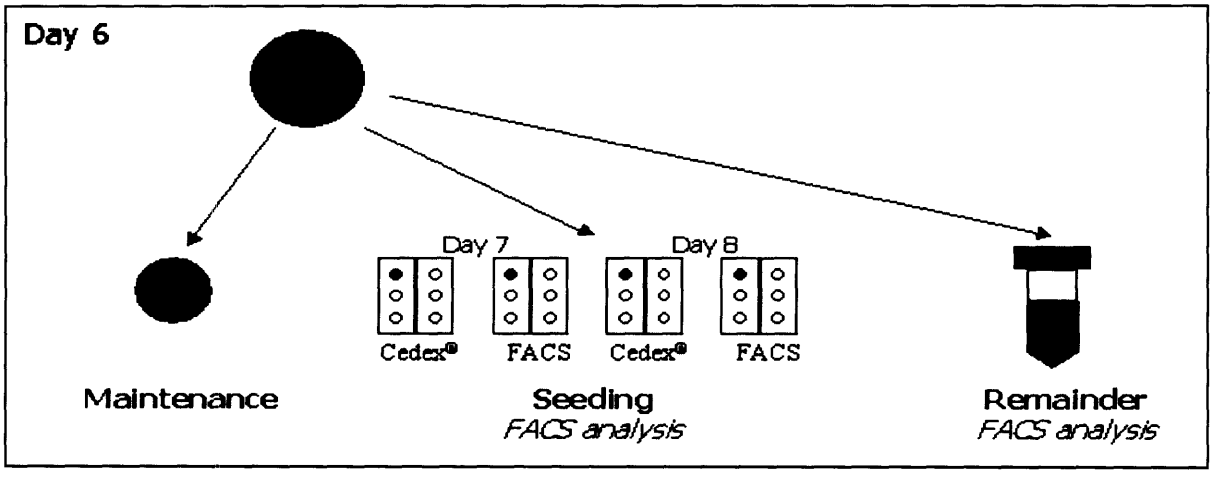


Figure 16: Experimental setup in low glucose experiment

For each seeding, cells from the maintenance plate were seeded onto 6-well plates for FACS and Cedex analyses in the following two days, as well as passaged onto 6-cm plates for maintaining cells for subsequent passages. The remainder of cells from the maintenance plate was analyzed by FACS, and this procedure was repeated for all 3 cell populations as well as for LIF^- cultures. Only on Seeding #1 was the 10-cm plate used as the maintenance plate.

On Day 6, seeding was initiated (Figure 16). Cells from each of the 8 plates (2 conditions for 3 variables: T20 and unsorted; standard ES and low-glucose ES medium; LIF^+ and LIF^-) were seeded in four 6-well plate wells for two days of sampling after seeding (each requiring 1 plate for FACS analysis and 1 plate for Cedex analysis), as well as onto one 6-cm plate used for maintaining cells for next seeding. FACS analysis was performed on the remainder of the cells for pluripotency information on the maintenance plate. Seeding was performed every other day at a reasonable concentration to ensure exponential growth between time points. Three seedings were performed in this experiment, which lasted 12 days.

For daily sampling, supernatant was first withdrawn from each well and frozen down for future metabolic analyses with YSI 2700 SELECT™. Cells were then trypsinized by adding 0.25 mL trypsin to each well, and additional amounts of PBS were added to allow for uniform mixing of cells using a 1000 µL pipette. 1 mL PBS was added for Cedex analysis (making the total volume 1.25 mL) since 1 mL is required for the instrument and some liquid will invariably be left on the surface of the well. Similar amount of PBS was added for FACS analysis so that the cells could be mixed and centrifuged (to remove the red-colored trypsin) in an IEC Centra-M microcentrifuge for 1 minute at 15,600 xg. These cells were resuspended in cell density-dependent amount of PBS and left on ice until FACS analysis was performed.

5.1.2 Results

The histogram plots of percent GFP^+ expression (as determined to be the percentage over the threshold value of 10^2 in FL1-H using the FACS settings as described in Appendix G: Becton Dickinson FACScan Settings and Operation) were obtained from FACS for both LIF^+ and LIF^- cultures for both cell populations, in both the 6-well plates used for seeding and the 6-cm maintenance plates. The results for the maintenance plates are summarized in the figures below.

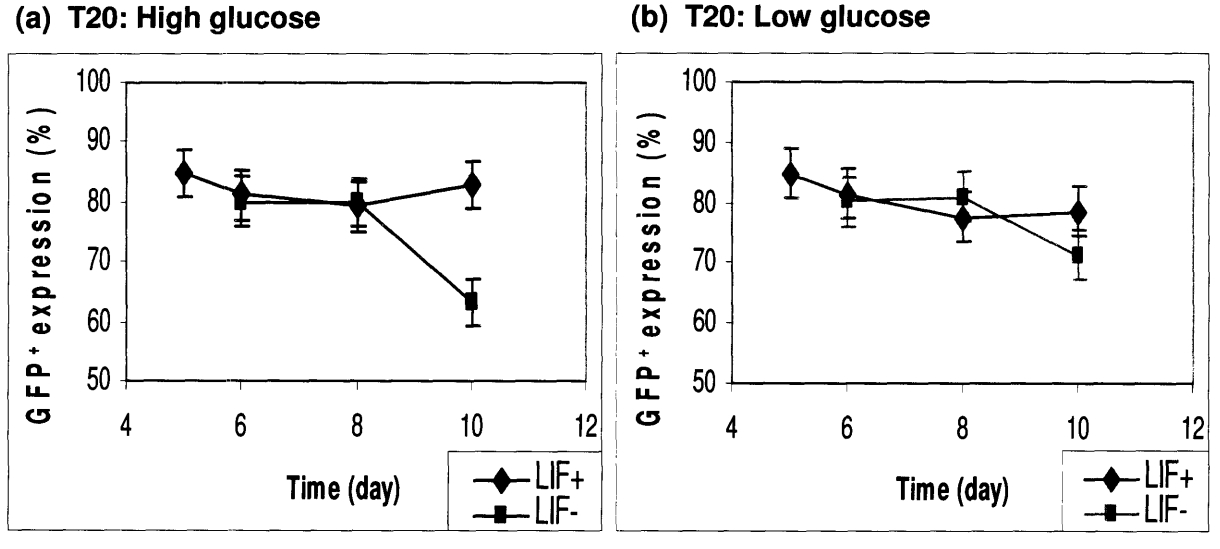


Figure 17: FACS results for T20 cells on 6-cm plates with glucose concentration variation

The percentage of T20 cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ culture was compared to that of GFP^+ cells in LIF^- culture in 6-cm plates for both high glucose and low glucose conditions. The error bars reflect 2 SD of 4.06 % as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures.

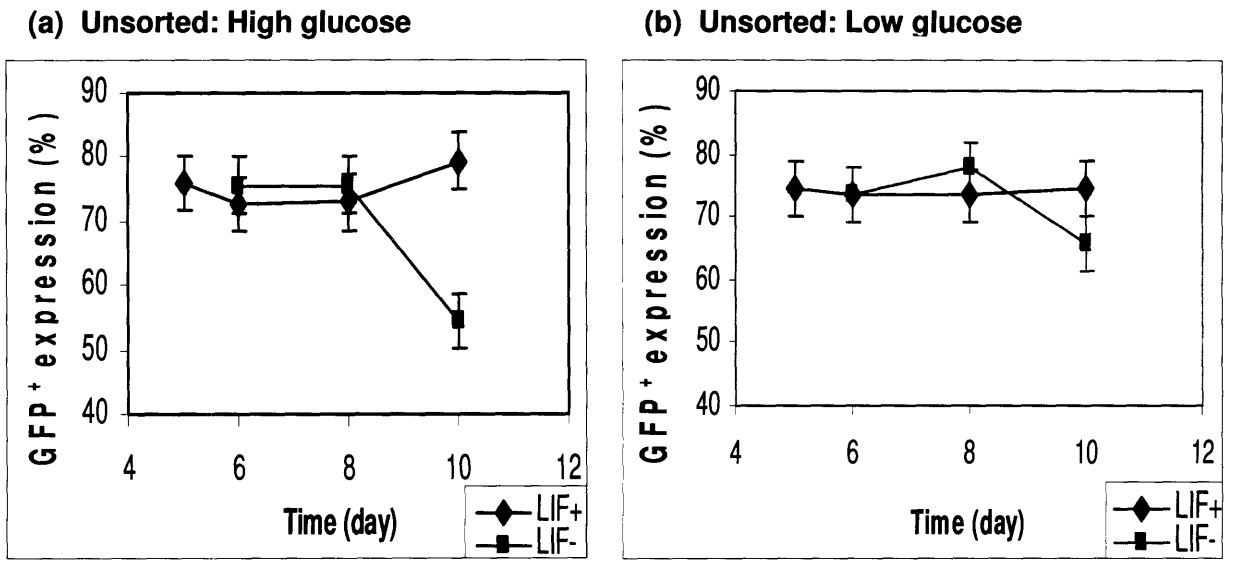
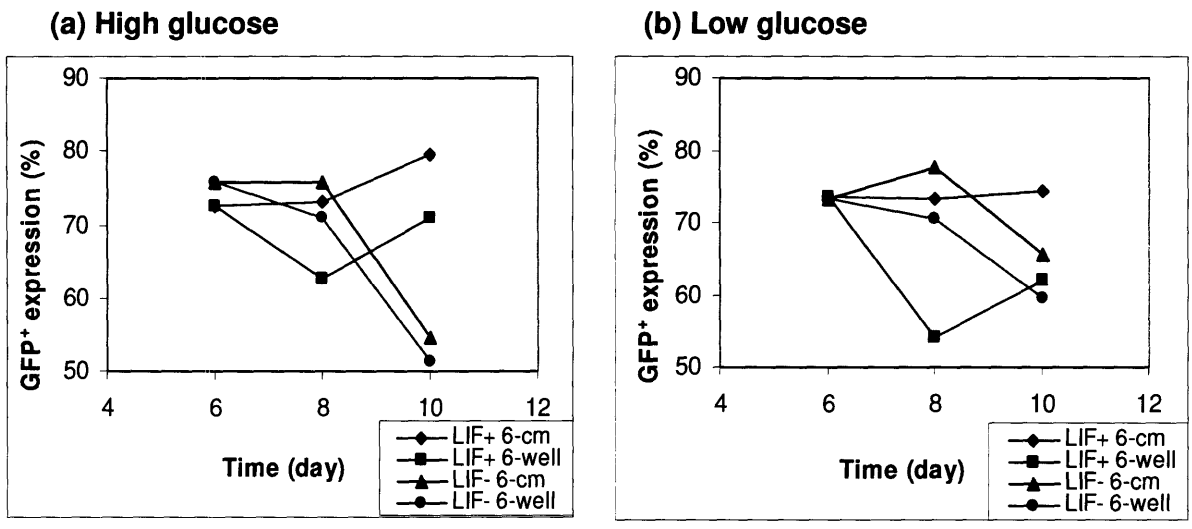


Figure 18: FACS results for unsorted cells on 6-cm plates with glucose concentration variation

The percentage of unsorted cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ culture was compared to that of GFP^+ cells in LIF^- culture in 6-cm plates for both high glucose and low glucose conditions. The error bars reflect 2 SD of 4.32 % as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures.

Seeding was initiated on Day 6, but results from Day 5 are also included in these figures because LIF withdrawal was initiated on this day. For at least 3 days after LIF withdrawal and 2 days after 2-day passaging is initiated, there is no statistically significant difference observed in percentage of cells with GFP^+ expression level either between LIF^+ and LIF^- cultures or between high glucose and low glucose cultures, for both cell populations. Small dips in GFP^+ expression are also seen in this experiment right after the transition in passaging frequency. The significant difference is first observed between LIF^+ and LIF^- cultures 4 to 5 days after LIF withdrawal and 3 to 4 days after 2-day passaging is initiated for cells grown in high glucose, which agrees with the results from the preliminary differentiation experiment. However, the difference in GFP^+ expression between LIF^+ and LIF^- cultures for cells grown in low glucose culture is not significant, which can be explained by the low metabolism of glucose in these cultures, possibly leading to lowered level of cellular mechanisms and hence delayed responses to extracellular stimuli.



* Error bars excluded for clarity of data points

Figure 19: Comparison of FACS results for unsorted cells in 6-cm and 6-well plates

The percentage of unsorted cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ and LIF^- cultures on 6-cm maintenance plates was compared to the percentage of GFP^+ cells on 6-well seeded plates. The error bars reflect 2 SD of 4.32 % for unsorted cells, as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures, as well as for 6-cm and 6-well plates.

Although percent GFP^+ expression data for seeded plates were also obtained from FACS, they were not analyzed as in the preliminary differentiation experiment due to inconsistency in results observed between seeded plates and maintenance plates. As Figure 19 shows, the consistency in the data trend still holds for the high-glucose cultures (which corroborates the results from the preliminary differentiation experiment), but large fluctuations are visible in the low-glucose LIF^+ cultures for reasons that are unclear.

Nonetheless, there are likely physical differences between 6-cm and 6-well plates that affect the GFP expression level in ES cells, especially since GFP^+ expression was higher in 6-cm plates than in 6-well plates under all culturing conditions in all cell populations for all seedings. For instance, the reason could be that gas exchange occurs poorly in 6-well plates due to significantly higher plate-depth per plate-diameter ratio (0.46 versus 0.25 in 6-cm plates) as well as the low-evaporation lid on the Becton-Dickinson 6-well plates that reduces gas exchange between the headspace of the plate and the ambient air in the incubator chamber. As a result, culturing conditions such as lower pH induced by the buildup of CO_2 and lowered cellular respiration due to depletion of O_2 might have been affected to cause noticeable differences in GFP^+ expression level, which were perhaps magnified when cultured in lower level of glucose. Further study with O_2 and CO_2 concentration variation is necessary to determine whether these gas concentrations can in fact affect the level of pluripotency in ES cells.

To determine whether the lowered level of cellular mechanisms caused by low glucose affects cell proliferation rate, comparisons in cell growth kinetics were made using an estimate in doubling time (t_d) calculated from the viable cell density (VCD) data of seeded plates. Since each seeding was performed over 2 days, t_d could simply be determined by using the sets of cell density measurements from each seeding. The doubling time comparisons over 3 seedings for T20 cell populations are provided in the figure below. The checkered column with a dotted

outline indicates t_d calculation which may not be reliable due to low cell density (less than $1x10^5$ cells/well) and therefore not appropriate for comparison.

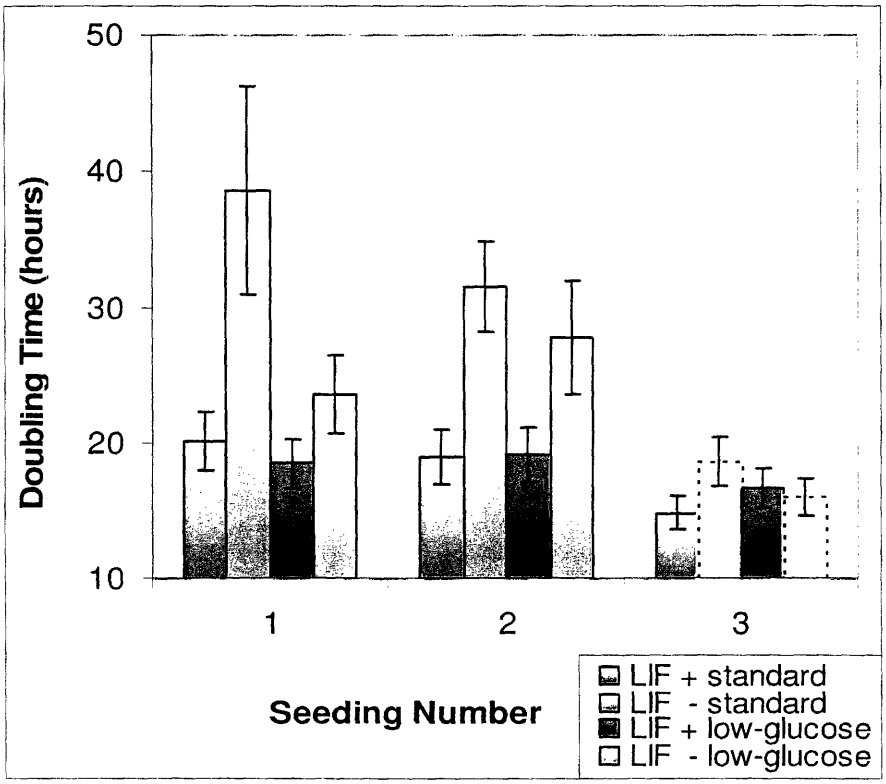


Figure 20: Doubling time of T20 cells in standard and low-glucose cultures

Doubling time was calculated from the 2 time points at each seeding, and the error bars were determined through error propagation of 1 SD for VCD data of 6 % from Appendix B: Seeding Variation Study. The checkered columns represent possibly unreliable t_d values due to low cell density.

In the figure above, gradual reduction in t_d over seedings is seen for standard medium cultures as cells become accustomed to the different culturing environment of 2-day passaging and 6-well plates, but no such reduction is observed in the low-glucose culture. There is no noticeable trend in cell proliferation observed over time, which could be another indication that cellular changes do not happen as rapidly for these cells due to limited nutrient availability. However, it is apparent that cell proliferation is only mildly affected by low glucose, as no significant difference between standard medium and low-glucose medium cultures is evident for the LIF^+ condition. Further study with longer experimental time is necessary to determine whether culturing under low glucose can actually lower cell proliferation significantly in the long run.

Using the viable cell density (VCD) data, the metabolic rates of ES cells grown under low-glucose and standard medium situations were also analyzed, with the glucose consumption rate ($q_{glucose}$) and lactate production rate ($q_{lactate}$) calculated for each parameter studied at each seeding using the method described in Section 3.1.2. The results are provided with minimum and maximum $q_{glucose}$ and $q_{lactate}$ values out of 3 seedings instead of their averages due to the

dependence of metabolic rates on the nutrient concentration and pH of the culture, both of which vary with cell density, and the average SD calculated from error propagation also helps give an idea of how significant the differences are.

Table 5: Metabolic rates of high-glucose cultures in LIF^+ and LIF^- conditions

	$q_{glucose}$ ($\mu mol/10^6 cells/hr$)			$q_{lactate}$ ($\mu mol/10^6 cells/hr$)		
	Min	Max	SD	Min	Max	SD
un LIF^+	**0.049**	**0.203**	0.016	**0.102**	**0.172**	0.019
un LIF^-	**0.047**	**0.532**	0.049	**0.145**	**0.706**	0.064
T20 LIF^+	**0.130**	**0.218**	0.014	**0.111**	**0.164**	0.011
T20 LIF^-	**0.128**	**0.753**	0.063	**0.131**	**0.812**	0.056

It is clear from Table 5 that there is no statistically significant difference between LIF^+ and LIF^- cultures in the standard medium for each cell population, which agrees with results from the previous experiments. The difference in also non significant when comparing the metabolic rates of unsorted and T20 cell populations. The minimum $q_{glucose}$ values show less deviation between LIF^+ and LIF^- cultures than between cell populations due to proper adjustments made to the seeding densities for each cell population in Seeding #1, but the maximum $q_{glucose}$ values show greater deviation between LIF^+ and LIF^- cultures since the LIF^- cultures were accidentally seeded at low concentrations in Seeding #3, resulting in high $q_{glucose}$ values. Although the actual seeding concentrations could not be determined, the average cell density on Day 1 of Seeding #3 for LIF^- cultures was 1.02×10^5 cells/well, compared to 13.78×10^5 cells/well for LIF^+ cultures. The data for $q_{lactate}$ are also in agreement with one another.

It is interesting to note that a yield calculation of lactate from glucose ($Y_{lac/gluc}$) from the maximum $q_{glucose}$ and $q_{lactate}$ values indicates that approximately 60 % of glucose is metabolized into lactate (40 % by oxidative phosphorylation) in low VCD, LIF^- cultures, while only 40 % is metabolized into lactate (60 % by oxidative phosphorylation) in high VCD, LIF^+ cultures. Since this result was convoluted with 2 different parameters, further comparison of $Y_{lac/gluc}$ values determined from comparable $q_{glucose}$ and $q_{lactate}$ (and therefore comparable VCD values) chosen from among the 3 seedings was made, which revealed no correlation between $Y_{lac/gluc}$ and LIF. This implies that more glucose must be metabolized through aerobic respiration in the citric acid cycle when cell density is high, resulting in the orange-yellow color of these cultures indicative of low pH. This agrees with results obtained in other studies for mammalian cells which suggest that the rate of glycolysis increases with elevated pH[56]. Even though this information is useful as an estimate of $Y_{lac/gluc}$, the actual percentages must be used with caution due to presence of 1 mM of pyruvate in the medium which is not included in the calculation. The actual $Y_{lac/gluc}$ would therefore be lower than those reported above.

Table 6: Metabolic rates of low-glucose cultures in LIF^+ and LIF^- conditions

	$q_{glucose}$ ($\mu mol/10^6 cells/hr$)			$q_{lactate}$ ($\mu mol/10^6 cells/hr$)		
	Min	Max	SD	Min	Max	SD
un LIF^+	**0.000**	**0.012**	0.002	**-0.015**	**0.005**	0.002
un LIF^-	**0.004**	**0.719**	0.029	**-0.025**	**0.702**	0.024
T20 LIF^+	**0.002**	**0.025**	0.003	**-0.020**	**-0.002**	0.009
T20 LIF^-	**0.002**	**0.772**	0.028	**-0.017**	**0.768**	0.024

The minimum $q_{glucose}$ for both cell populations in the low-glucose cultures was found to be essentially zero, which is expected, as only 0.3 mM of glucose remained by Day 1 of Seeding #1. The energy required for cell proliferation was probably supplied for the most part from sodium pyruvate in the culture, as well as from amino acids and possibly even lactate, as evident from the negative values for $q_{lactate}$ in Table 6 which implies lactate consumption. This is not observed in the situation resulting in maximum $q_{glucose}$, where low VCD allowed enough glucose to remain for substantial glucose consumption to occur between Day 1 and Day 2 of Seeding #3, especially for LIF^- cultures.

$Y_{lac/gluc}$ in the low-glucose cultures, as determined from maximum $q_{glucose}$ and $q_{lactate}$ values, was approximately 92 % in high VCD cultures and 51 % in low VCD cultures. This difference can be attributed to higher pH in these cultures (as determined by the red-orange color of in the cultures) due to low level of lactate production in these cultures.

5.1.3 Conclusion

Compared to high-glucose culture, cells grown in low-glucose culture were characterized by non significant difference in the percentage of cells with GFP^+ expression between LIF^+ and LIF^- cultures, perhaps owing to the reduction in cellular mechanisms including signal transduction capabilities as a result of limited nutrient availability. Cell growth data revealed a non-improvement in cell proliferation for low-glucose cultures over multiple seedings compared to high-glucose cultures that revealed gradual improvement, which also supports the previous conclusion drawn regarding slowed response to extracellular stimuli.

The metabolic rates of glucose and lactate were obviously lower on a per cell basis under low-glucose condition for both cell populations under both LIF^+ and LIF^- conditions, which is expected, as pH would be higher in these cultures as a result of lower level of accumulated lactate. This lowered metabolic rate is probably the cause of slower response of the low-glucose culture to LIF withdrawal as well as to changes in culturing environment such as seeding conditions. Glucose metabolism through oxidative phosphorylation was also shown to be higher in low-glucose condition (92 % and 51 % for high VCD and low VCD, respectively) than in high-glucose condition (60 % and 40 %), which implies that low level of glucose forces cells to harness energy more efficiently, while high level of glucose allows cells to metabolize glucose through a faster pathway at the expense of producing the toxic product lactate. Higher VCD, which reflects lower pH, also consistently resulted in higher level of glucose metabolism through the citric acid cycle for both high-glucose and low-glucose cultures.

For a more in-depth analysis of how ES cellular metabolism is affected by low level of glucose, a more complex system with a pH controller would be necessary so that constant pH can be maintained in culture, since pH appears to have a significant impact on glucose metabolic rate. In addition to CO_2 and air tanks and an incubator, such system would require a pH probe to keep track of pH in the culture, as well as a feedback system connected to the gas tanks so that the concentration of CO_2 in the CO_2-air gas mixture entering the headspace of the culture can be reduced as pH drops. Even the simplest system would require a pH probe and alkaline buffer injection system with feedback capability, in addition to the gas tanks and an incubator. By maintaining the pH in the ES cell culture at desired set points, it would be possible to determine whether glucose concentration in culture can indeed affect cellular metabolism over a range of pH values.

5.2 Low serum experiment

5.2.1 Experimental setup

In this experiment, the serum concentration in medium was varied in order to determine whether low serum medium has any effect on ES cell growth, metabolism, and pluripotency. Since serum plays an important role in many of the cellular mechanisms, it was hypothesized that low serum medium would have a significant effect on cell growth, metabolic, and even differentiation profiles, especially for ES cells due to their heightened metabolism and growth compared to adult cells. However, it remained to be seen to what extent these profiles would be affected.

Two vials of Top 20 % sorted (T20) and one vial of unsorted R1 Oct4-GFP cells were thawed and plated with 0.2 % v/v non-commercial LIF, added to facilitate cell pluripotency recovery as described in the cell maintenance protocol under "Thawing down stem cells." Each cell population was initially cultured in standard serum concentration of 15 % v/v to help improve pluripotency and growth recovery of cells, in 6-well plate wells. On Day 3, each cell population was split into 2 plates of different serum concentrations, one at standard concentration of 15 % v/v and the other at reduced concentration of 5 % v/v. As cells grew, they were expanded and maintained in 6-cm plates. On every day either the medium was changed or cells were passaged to ensure rapid recovery of cells after thawing.

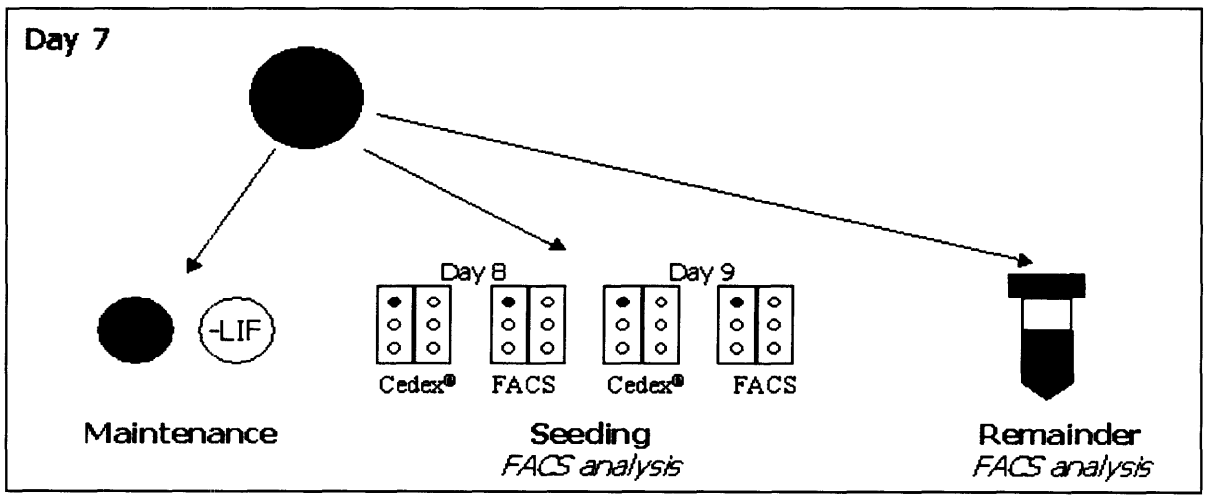


Figure 21: Experimental setup in low serum experiment

For each seeding, cells from the maintenance plate were seeded onto 6-well plates for FACS and Cedex analyses in the following two days, as well as passaged onto 6-cm plates for maintaining cells for subsequent passages. The remainder of cells from the maintenance plate was analyzed by FACS, and this procedure was repeated for all 3 cell populations as well as for LIF cultures. Only on Seeding #1 was the 10-cm plate used as the maintenance plate.

On Day 7, seeding and LIF withdrawal were initiated (Figure 21). Cells from each of the 4 plates (2 conditions for 2 variables: T20 and unsorted; standard ES and low-serum ES medium) were seeded in eight 6-well plate wells for two days of sampling after seeding (each

day requiring 2 wells for FACS analyses of LIF^+ and LIF^- and 2 wells for Cedex analyses of LIF^+ and LIF^-), as well as onto two 6-cm plate used for maintaining cells for next seeding, at both LIF^+ and LIF^- conditions. FACS analysis was performed on the remainder of the cells for pluripotency information on the maintenance plate. Unlike in the low glucose experiment, seeding density adjustments based on the cell density information obtained from the Cedex data was added to the procedure to ensure reasonable seeding conditions throughout the experiment. Three seedings were performed in this experiment.

For daily sampling, supernatant was first withdrawn from each well and frozen down for future metabolic analyses with YSI 2700 SELECT™ for measuring glucose, lactate, glutamine, and glutamate concentrations. Cells were then trypsinized by adding 0.25 mL trypsin to each well, and additional amounts of PBS were added to allow for uniform mixing of cells using a 1000 µL pipette. 1 mL PBS was added for Cedex analysis (making the total volume 1.25 mL) since 1 mL is required for the instrument and some liquid will invariably be left on the surface of the well. Similar amount of PBS was added for FACS analysis so that the cells could be mixed and centrifuged (to remove the red-colored trypsin) in an IEC Centra-M microcentrifuge for 1 minute at 15,600 xg. These cells were resuspended in cell density-dependent amount of PBS and left on ice until FACS analysis was performed. This experiment lasted for 13 days in total.

5.2.2 Results

The histogram plots of percent GFP^+ expression (as determined to be the percentage over the threshold value of 10^2 in FL1-H using the FACS settings as described in Appendix G: Becton Dickinson FACScan Settings and Operation) were obtained from FACS for both LIF^+ and LIF^- cultures for both cell populations, in both the 6-well plates used for seeding and the 6-cm maintenance plates. The results for the maintenance plates are summarized in the figures below.

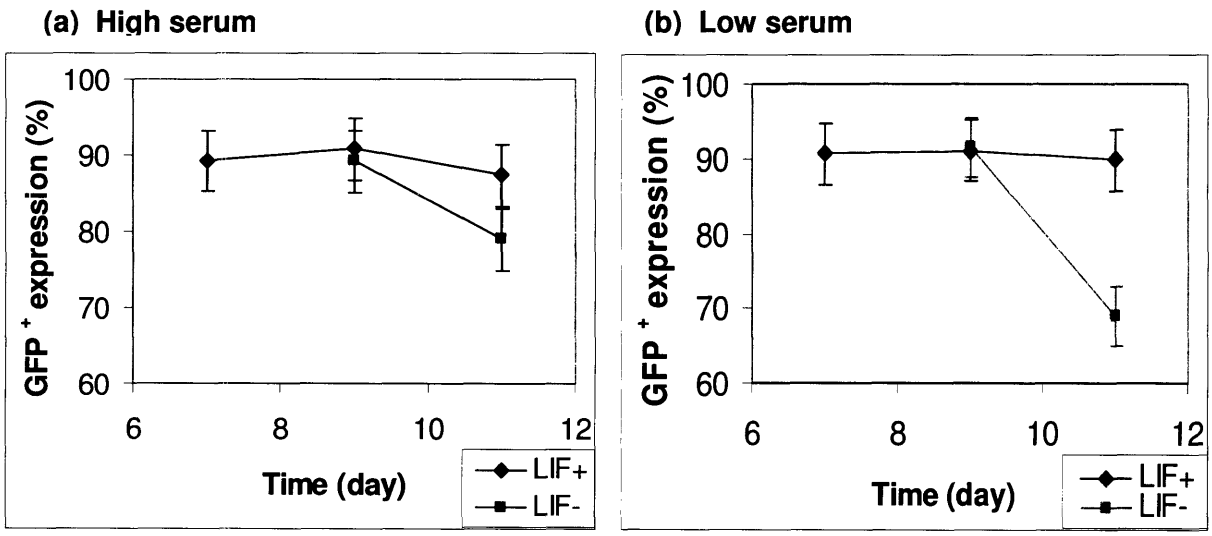


Figure 22: FACS results for T20 cells on 6-cm plates with serum concentration variation

The percentage of T20 cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ culture was compared to that of GFP^+ cells in LIF^- culture in 6-cm plates for both high serum and low serum conditions. The error bars reflect 2 SD of 4.06 % as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures.

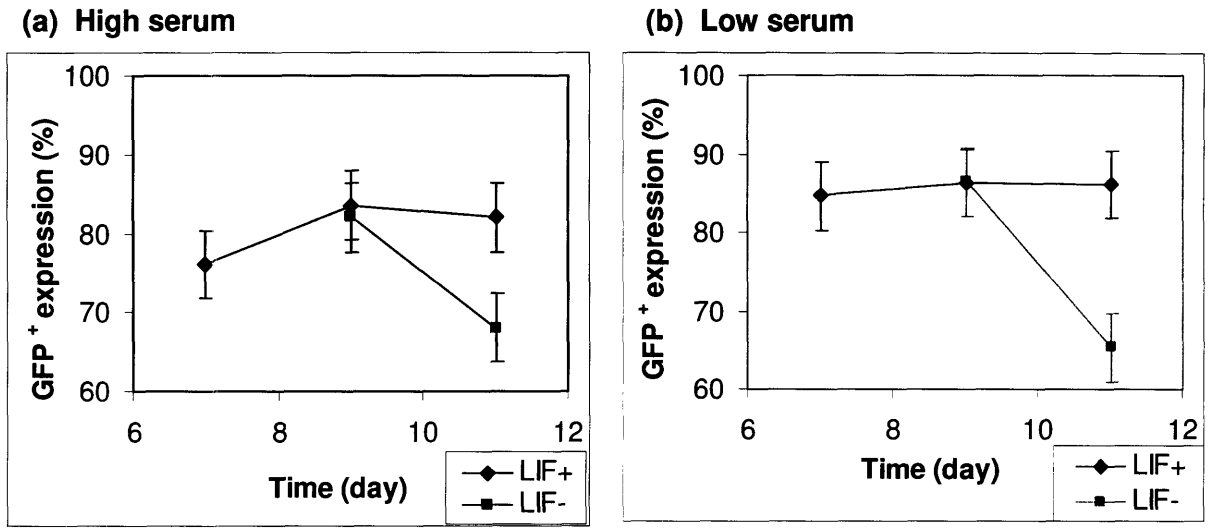


Figure 23: FACS results for unsorted cells on 6-cm plates with serum concentration variation

The percentage of unsorted cells expressing GFP^+ (FL1-H greater than 10^2) in LIF^+ culture was compared to that of GFP^+ cells in LIF^- culture in 6-cm plates for both high serum and low serum conditions. The error bars reflect 2 SD of 4.32 % as determined in Appendix B: Seeding Variation Study. The same values for error bars were used for LIF^+ and LIF^- cultures.

In this experiment, LIF withdrawal and seedings were both initiated on Day 7. Noticeable difference in the GFP^+ cell fraction between LIF^+ and LIF^- cultures is not apparent until 4 days after LIF withdrawal and 2-day passaging are initiated, but for both unsorted and T20 cell populations, there appears to be a more significant difference between LIF^+ and LIF^- cultures for cells grown in low-serum medium than high-serum medium. Furthermore, the combination of low serum and LIF^- appears to result in the lowest fraction of cells with GFP^+ expression. It is also evident from this experiment that ES cell pluripotency can be maintained in either high serum or low serum culture, as long as appropriate amount of LIF is provided. This shows that there is no advantage to using high serum medium in ES cell culture in the presence of LIF, but loss in pluripotency is reduced with high-serum medium in the absence of LIF. The mechanism by which low-serum medium affects ES cells cannot be determined from this experiment due to the presence of numerous different factors in serum, many of which has not been characterized. It suffices to say, however, that reduced level of serum combined with LIF withdrawal can have a greater negative effect on maintaining ES cell pluripotency than using standard level of serum and/or adding LIF.

Although FACS results for the 6-well seeded plates were obtained to allow for a more in-depth analysis of the behavior of ES cells, they were also left out of the analysis in this experiment due to improper mixing of samples in a majority of the seedings which led to poor data acquisition. The comparison between the maintenance plates and seeded plates is not provided for this experiment, but the variation between these plates was more obvious for both high-serum and low-serum cultures than in the low-glucose experiment.

Nonetheless, comparisons in cell growth kinetics were made by using an estimate of doubling time (t_d) calculated from viable cell density (VCD) data of seeded plates using the method described in Section 3.1.2. Since each seeding was performed over 2 days, t_d could simply be determined by using the sets of cell density measurements from each seeding. The doubling time comparisons over 3 seedings for T20 cell populations are provided in the figure

below. The checkered column with dotted outline indicates data point which may not be reliable based on irregularities seen in metabolic data and therefore not appropriate for comparison.

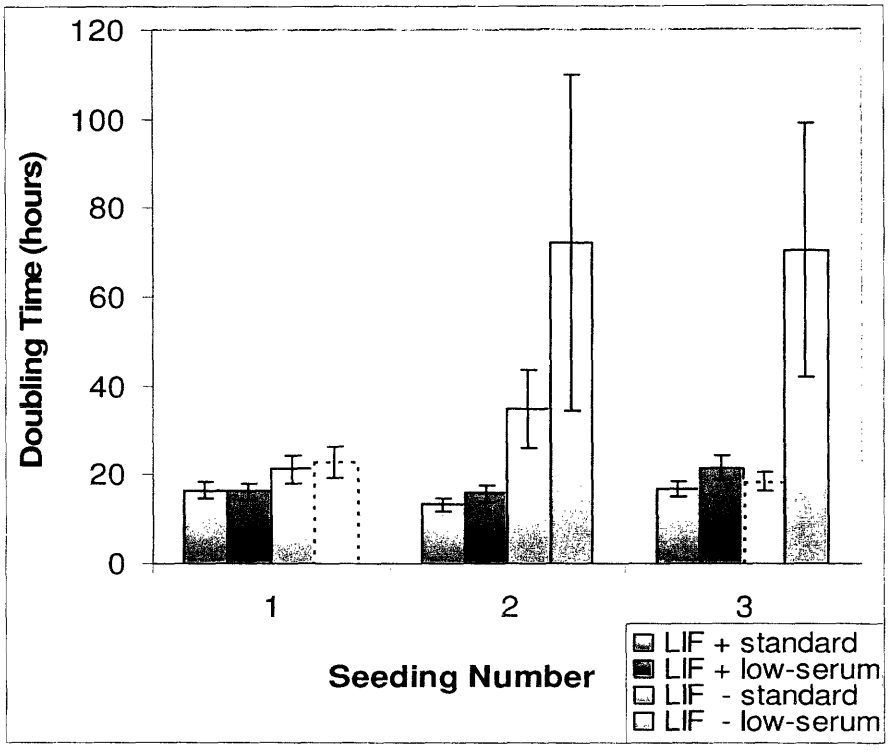


Figure 24: Doubling time of unsorted cells in standard and low-serum cultures

Doubling time was calculated from the 2 time points at each seeding, and the error bars were determined through error propagation of 1 SD for VCD data of 8 % from Appendix B: Seeding Variation Study. The checkered columns represent possibly unreliable t_d values based on irregularities observed in the corresponding metabolic rates.

In the figure, it is apparent that there is no significant difference between cells grown with standard medium and those grown under low-serum medium in the presence of LIF except for a slight increase in t_d observed over multiple seedings for the low-serum culture. The average doubling time of approximately 16 ± 2 hours for these cultures is slightly higher than 12 to 13 hours that were recorded on 6-cm maintenance plates in other experiments, but this discrepancy is within experimental uncertainties.

Compared to the LIF^+ cultures, the cultures without LIF have significantly higher t_d, with ES cells grown in standard medium recording t_d of 35 ± 9 hours at Seeding #2 and those grown in low-serum medium having an average t_d over Seedings #2 and #3 of 71 ± 33 hours. This trend is similar to the trend seen in the % GFP^+ expression in maintenance-plated cells, and cell proliferation rate can be correlated to % GFP^+ expression. These data confirm the results obtained in a study by Viswanathan, et al. which showed that LIF has the capability to increase pluripotency of ES cells by giving selective proliferation advantage to undifferentiated cells (Table 4)[54], but the results also show that low-serum medium adversely affects cell growth and pluripotency more so than standard medium only in the absence of LIF.

Using the VCD data, the metabolic rates of ES cells grown under low-glucose and standard medium conditions were also analyzed, with the glucose consumption rate ($q_{glucose}$) and lactate production rate ($q_{lactate}$) calculated for each parameter studied at each seeding using the method described in Section 3.1.2. The results are provided with minimum and maximum $q_{glucose}$ and $q_{lactate}$ values out of 3 seedings instead of their averages due to the dependence of metabolic rates on the nutrient concentration and pH of the culture, both of which vary with cell density, and the average SD calculated from error propagation also helps give an idea of how significant the differences are.

Table 7: Metabolic rates of cells grown with LIF in high-serum or low-serum medium

	$q_{glucose}$ (μmol/10^6cells/hr)			$q_{lactate}$ (μmol/10^6cells/hr)		
	Min	Max	SD	Min	Max	SD
un high	**0.161**	**0.221**	0.019	**0.111**	**0.182**	0.015
T20 high	**0.159**	**0.240**	0.017	**0.112**	**0.184**	0.013
un low	**0.164**	**0.220**	0.013	**0.123**	**0.168**	0.016
T20 low	**0.184**	**0.185**	0.017	**0.127**	**0.159**	0.014

Based on the table above, there appears to be no significant difference in $q_{glucose}$ or $q_{lactate}$ among the various cultures with different cell populations grown under 2 different serum concentrations in the presence of LIF, which is as expected based on the consistency in data observed for cell pluripotency as well as cell growth kinetics of LIF^+ cultures. $Y_{lac/gluc}$ was determined as an estimate to be 38 % (62 % by oxidative phosphorylation) for both unsorted cells grown in high serum and in low serum and 37 % and 39 % for T20 cells grown in high serum and low serum, respectively.

Table 8: Metabolic rates of cells grown without LIF in high-serum or low-serum medium

	$q_{glucose}$ (μmol/10^6cells/hr)			$q_{lactate}$ (μmol/10^6cells/hr)		
	Min	Max	SD	Min	Max	SD
un high	**0.172**	**0.359**	0.050	**0.105**	**0.283**	0.038
T20 high	**0.172**	**0.398**	0.031	**0.127**	**0.322**	0.024
un low	***0.064**	**0.482**	0.118	***0.175**	**0.431**	0.098
T20 low	**0.185**	**0.364**	0.041	**0.141**	**0.141**	0.034

* Unreliable data point for glucose consumption rate

As with LIF^+ cultures, the metabolic rates for different cultures grown under 2 different serum concentrations in the absence of LIF do not vary significantly with one another. $Y_{lac/gluc}$ was found to be 35 % and 45 % for unsorted cells grown in high-serum medium and low-serum medium, respectively, while T20 cells grown in high-serum medium and low-serum medium resulted in $Y_{lac/gluc}$ of approximately 39 % and 40 %, respectively.

5.2.3 Conclusion

High-serum medium offered no significant advantage to low-serum medium in maintaining ES cell pluripotency or increasing cell growth kinetics except in the LIF^- condition, where low serum led to higher reduction in percentage of cells with GFP^+ expression and much longer t_d (71 ± 33 hours) than the high-serum counterpart (35 ± 9 hours). The analysis of glucose and lactate metabolic rates revealed a high level of consistency, implying that cells still metabolize glucose at the same rate and in the same way regardless of the amount of serum present in the medium. The only effect of using low concentration of serum that was elucidated in this experiment was the longer t_d and higher reduction of GFP^+-expressing cell population fraction in the absence of LIF.

The yield of lactate from glucose was consistent between high-serum and low-serum cultures, as well as between unsorted and T20 cell populations and LIF^+ and LIF^- conditions. All $Y_{lac/gluc}$ values were calculated to be within the range of 35 to 40 %, indicating that level of serum in culture does not affect the metabolism of ES cells, even in the presence or absence of LIF. These values also agree with $Y_{lac/gluc}$ for high glucose in the previous experiment.

5.3 LIF concentration experiment

5.3.1 Experimental setup

In this experiment, the concentration of LIF added to R1 Oct4-GFP cell culture was varied in order to determine whether the amount of LIF affects cell growth and differentiation profiles of ES cells. This experiment was also conducted to ensure that the standard amount of LIF used in all of the experiments (2 % v/v non-commercial LIF) did not have detrimental effects on the cells, as excessive addition of LIF has been reported in other labs to have negative effects, at least on the appearance of cells. Descriptions on the procedure used to isolate LIF and where it was derived are provided in Appendix F: LIF Purification Protocol.

Top 20 % sorted (T20) cells from the seeding density experiment were plated onto 4 different 6-cm TC dishes with various concentrations of non-commercial LIF diluted in appropriate amounts of PBS. Using 4:1 serial dilution, 3 batches of LIF with dilutions of 4x, 16x, and 64x were made. Along with LIF with no dilution (1x), these diluted LIF were also added to 6-cm plates at 2 μL/mL for consistency, each seeded with $2x10^6$ cells in 4 mL of medium. Every other day, the cells were passaged onto fresh plates at the same concentration of $2x10^6$ cells with respective amounts of LIF, and the remaining cells were analyzed by FACS to obtain the differentiation profile of ES cells under different LIF concentrations. The Cedex data taken from the plates were also used to get approximate values for the cell growth kinetics of the cells at different LIF concentrations. There were a total of 4 passages in this experiment. In addition to the FACS and Cedex data, the pictures of the cells were taken from the microscope on Day 8 to confirm the reports of negative appearance that excessive LIF addition can cause.

For every passage, the supernatant was first withdrawn from each plate, and the cells were trypsinized using 0.5 mL trypsin. 3.5 mL of warm, standard ES cell medium was then added to each plate, and upon mixing, 1 mL was removed from each plate for cell count using Cedex. Appropriate volume of cell culture was added to a new 6-cm plate balanced with fresh ES medium for a total of 4 mL, and 2 μL/mL of LIF from the respective dilution was added to each plate. The remainder of cell culture was spun down in an IEC Centra-M microcentrifuge for 1 minute at 15,600 xg to remove the red color of medium and trypsin, and the cells were resuspended in a cell-density dependent volume of PBS for FACS analysis. This experiment lasted 9 days.

5.3.2 Results

Viable cell density (VCD) data from Cedex were analyzed to determine the doubling time (t_d) of T20 cells cultured under different LIF concentrations using the same method as described in Section 3.1.2. Since each value for t_d was based only on 2 time points prior to passaging on consecutive passages, significance of difference in t_d could not be quantified on the basis of confidence level. Instead, 2 standard error of 12 % (as determined in Appendix B: Seeding Variation Study for T20 cell population) and 0.25 hour were used as error for VCD and time, respectively. Error in t_d, which was derived using error propagation formulas, was used as

error bar in Figure 25. In the graph, open circles correspond to possibly inaccurate t_d values as a result of improper mixing in the Cedex instrument that led to blank images to be included as part of VCD data, and dotted lines represent the corresponding portions of the trend in the data that were affected. The blank images for LIF^+ 64x dilution were eliminated from VCD calculation since they were few in number, but similar images were included in the calculation for LIF^+ 1x dilution, as they consisted of a major fraction of the 20 images taken in total, a standard number for a Cedex measurement.

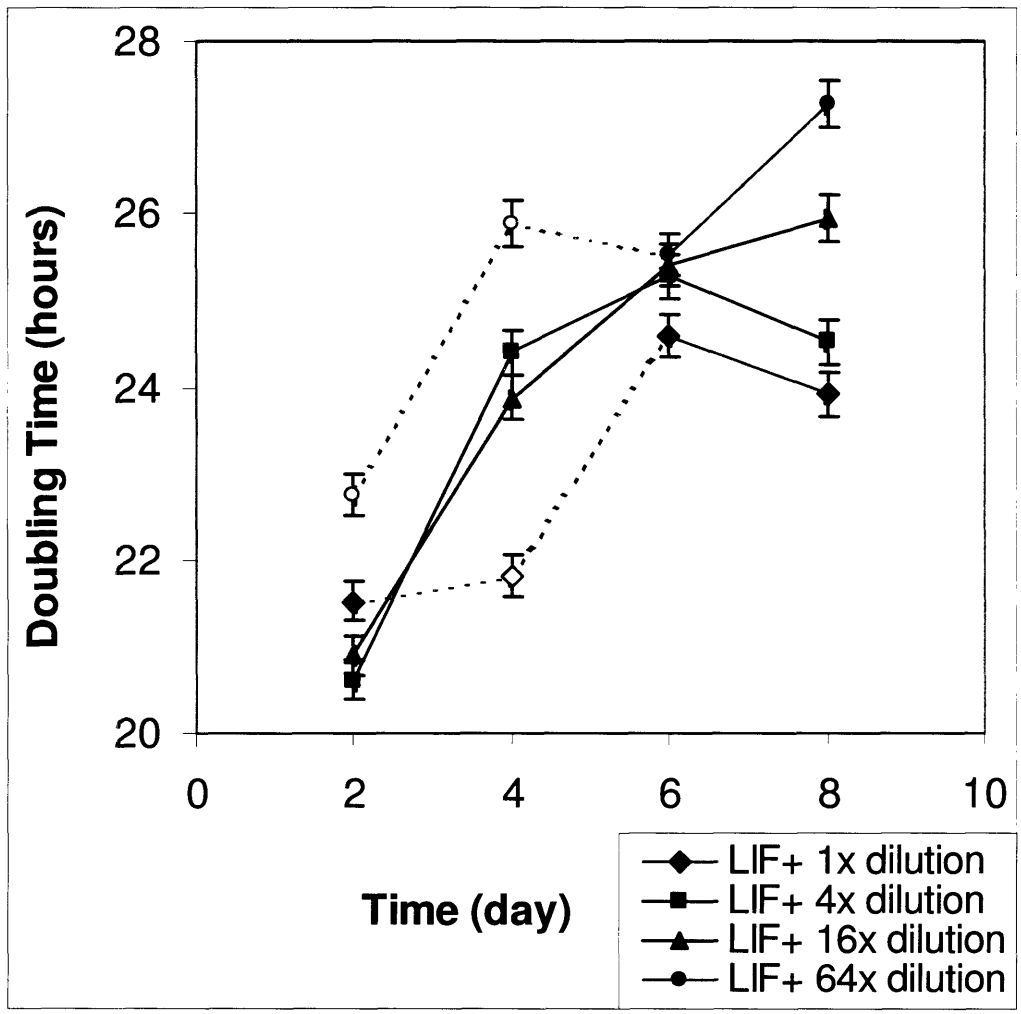


Figure 25: Doubling time at various LIF concentrations

Doubling time for T20 cells at each LIF concentration was calculated from 2 VCD counts: a known seeding concentration of $2x10^6$ cells at each passage and a Cedex cell count performed prior to the next passage after 2 days of culturing. The error bars were determined through error propagation of 1 SD for VCD data of 6 % from Appendix B: Seeding Variation Study. The open circles and dotted lines represent possibly unreliable t_d values and trend based on improper mising in the Cedex instrument.

If such data points are neglected from analysis, the general trend of the cells in the first 4 days of culturing appears to follow a gradual decrease in proliferation rate (increase in t_d), which was perhaps induced by the longer trypsinization time and time out of the incubator that the cells

experienced, as cell counting was necessary during each passage to ensure the seeding density of each plate at $2x10^6$ cells. This longer exposure to trypsin and other adverse culturing conditions perhaps incurred damage to the cells, which also explains why t_d was longer than was observed in other experiments. Another reason why t_d was significantly longer in this experiment stems from the fact that t_d in this experiment is measured from the point cells are seeded until the next passage and cells require time to adhere to the bottom of the plate and recognize the existence of other cells around them through cell-cell signaling before they can start proliferating. Therefore, this graph is helpful only to understand the difference in proliferation rates among the cultures with different LIF concentrations but not for obtaining useful values for t_d under these growing conditions. As a point of reference, the culture with two weeks of LIF withdrawal recorded an average t_d of 32.5 ± 0.3 hours before the cells became extremely difficult to trypsinize on the final day, which is significantly longer than t_d of cultures for which LIF was added.

Nonetheless, there are significant differences that become apparent starting on Day 6, with lower t_d for the culture with no dilution (1x). On Day 8, the cultures with 1x and 4x dilutions appear to level off in proliferation rates, while the cultures with 16x dilution and 64x dilution continue towards higher t_d. Based on these data, cell proliferation rate seems to be affected by LIF concentration, and a threshold value for maintaining stable t_d appears to be between 4x and 16x dilution of the non-commercial LIF. Based on personal communication with Dr. Vidya Jonnalagadda of the Engelward Lab, her data from a simple characterization study conducted between 1/9/04 and 1/19/04 suggested that this batch of non-commercial LIF was most effective at maintaining ES cells between 0.1 and 0.2 % v/v concentration (10 to 20x dilution equivalent), which agrees with our results.

As suggested in Sections 3.12 and 4.2.2, it is possible that LIF does in fact affect proliferation rate of ES cells in a dose-dependent manner but that the effects of LIF withdrawal in fact take 5-6 days to take effect due to slow downregulation of the genes controlling cell division. In this experiment, all 4 LIF^+ plates were derived from the same T20 cell culture with 2 % v/v non-commercial LIF added for over a week prior to the start of the experiment, and the reason why similar cell proliferation profile was observed in all 4 cell cultures for the first 4 days could be that the cells were all still growing as if 2 % v/v LIF were present in the cultures. Only after 6 days is there any difference in t_d observed (for the culture with no LIF dilution), and the apparent difference becomes more obvious after 8 days, after downregulation of the genes is complete.

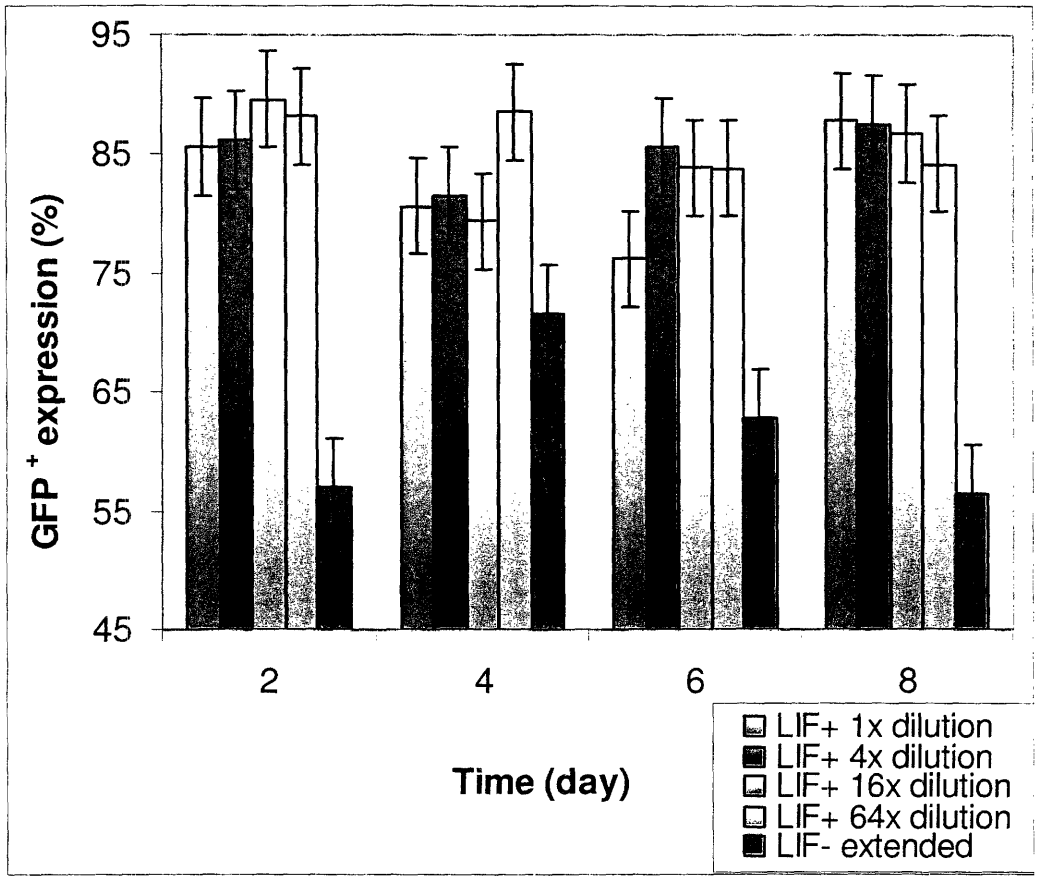


Figure 26: Percent GFP$^+$ expression at various LIF concentrations

The percentage of ES cells expressing GFP$^+$ (FL1-H greater than 10^2) at each LIF concentration was obtained every two days on the day of passaging and plotted next to one another for purposes of comparison. The error bars reflect 2 SD of 4.06 % for T20 cells, as determined in Appendix B: Seeding Variation Study. The same values for error bar were used for all LIF$^+$ cultures, as well as for extended LIF$^-$ culture.

The FACS results were analyzed for percentage of GFP$^+$ expression, and the average of triplicate measurements was presented in Figure 26. The error in FACS measurements from the seeding variation study (2 SD of 4.06 % GFP expression) was used as error bars.

Based on the figure, there is non significant difference in the fraction of undifferentiated cells over 4 passages across the different LIF concentrations, which shows that LIF concentration does not further improve cell pluripotency above a certain threshold level. Percent GPF$^+$ expression also never appreciably decreases over time for the range of concentrations studied, which is an indication that the non-commercial LIF used was still potent enough to prevent differentiation at 64x dilution. In comparison, LIF$^-$ culture decreases in pluripotency over time, although the reduction is not so obvious, as partially differentiated cells tend to remain tightly adhered to the bottom of the plate and avoid FACS analysis.

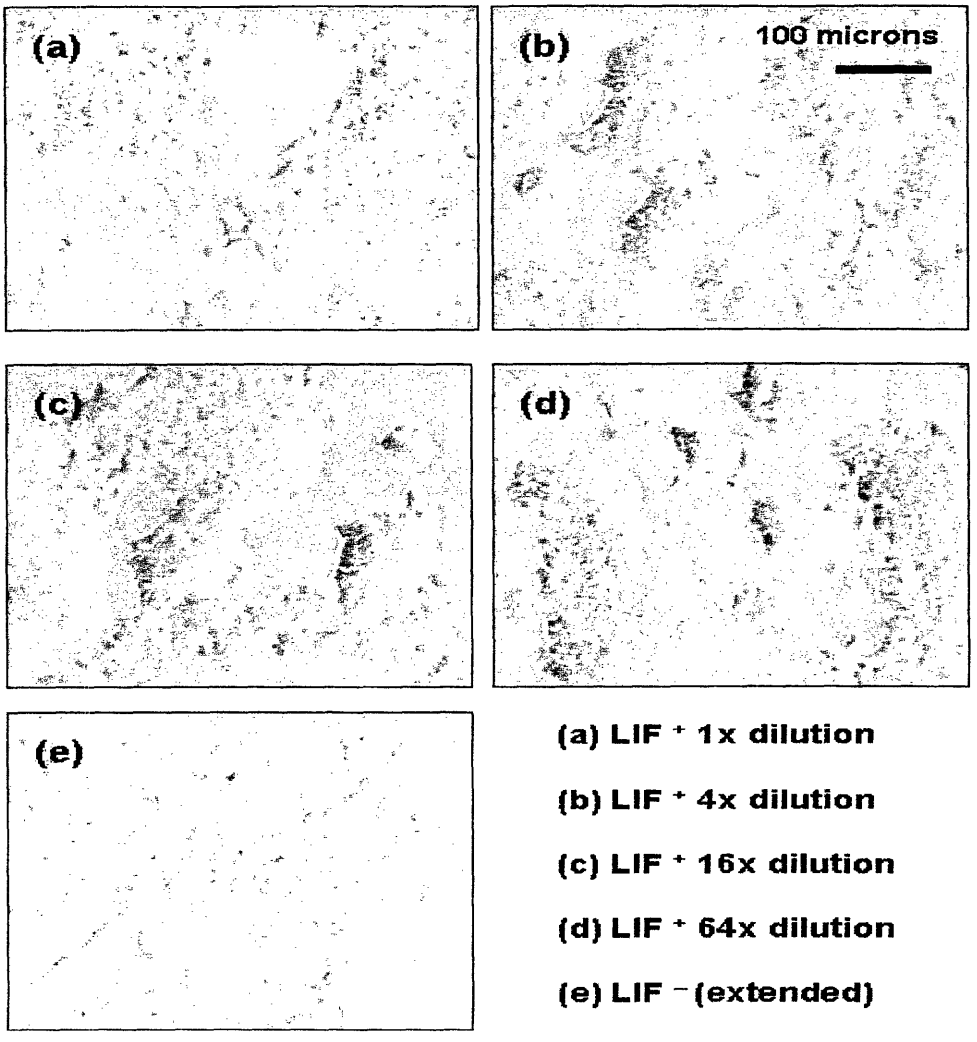


Figure 27: Pictures of cultures at various LIF concentrations

Each image, corresponding to a culture with different concentration of LIF, was taken separately by a digital camera and edited by a photo editing program. The brightness and contrast have been adjusted for highest clarity of each image, but the scale remains uniform for all cultures, with the black line in (b) corresponding to approximately 100 microns.

The digital pictures of the cultures in Figure 27, which were taken on Day 8, reveal similarities in the appearance of LIF^+ cells with rounded islands of cells with smooth edges that resemble oily droplets (Compare to LIF^- culture that has flat colonies with triangular, jagged edges that typify fibroblasts). Although the difference is not readily noticeable, there appears to be slight abnormality in the LIF^+ culture with no LIF dilution compared to the other 3 LIF^+ cultures, with less defined boundaries that indicate looser colony formation. In terms of characterization by appearance, there are no significant differences among the LIF^+ cultures studied, but perhaps there is a slight advantage to using LIF that has been diluted which gives a healthier appearance to ES cells.

5.3.3 Conclusion

Based on the results from this experiment, LIF concentration appears to have no concentration-dependent positive effect on ES cell pluripotency and appearance, as long as the amount of LIF added is above the threshold value necessary to maintain ES cells from differentiating. The experiment also revealed that 64x dilution of the non-commercial LIF is above this threshold value and therefore sufficient to maintain ES cell pluripotency. However, cell growth kinetic profiles showed a dose-dependent increase in cell proliferation rate with higher LIF concentration, which validated the use of 2 % v/v of LIF in all experiments in this research. Although the appearance of ES cells has been shown to be affected slightly by high LIF concentration, it remains to be seen whether excessive use of LIF adversely affects other aspects of ES cells, such as metabolic profiles.

5.4 Seeding density experiment

5.4.1 Experimental setup

In this experiment, the seeding density of R1 Oct4-GFP cells was varied in both LIF^+ and LIF^- cultures to determine whether the level of cell confluency has any effect on the differentiation profile of ES cells. Even though cell concentration can be considered to be a parameter in culturing conditions and therefore important for this research on characterization, the other major objective of this experiment was to confirm the hypothesis derived from the preliminary differentiation experiment that low seeding density can have added negative effect on cell pluripotency for LIF^- culture. Due to redundancy of information, cell growth data and consequently metabolic data were not obtained in this experiment.

Top 20 % sorted (T20) R1 Oct4-GFP cells maintained on 10-cm TC plates from the end of the Seeding Variation Study were split into LIF^+ and LIF^- cultures on 6-cm TC plates, with 2 % v/v non-commercial LIF added to LIF^+ cultures. On Day 2, cell density was checked using Cedex, and each of the 2 plates was passaged onto a 6-cm TC plate at a concentration of $2x10^6$ cells and kept as maintenance plate for the next seeding. T20 LIF^+ and LIF^- cultures were also seeded onto 6-well plate wells at 3 different concentrations of $0.5x10^6$, $1x10^6$, and $2x10^6$ cells/well to determine if cell density affects cell differentiation. A seeded plate was analyzed by FACS every day, and passaging and seeding for two days were conducted on every other day. The maintenance plates and the seeded wells were maintained at the same cell concentration throughout the experiment. Three seedings were performed in succession in this experiment.

For daily sampling, the supernatant was first withdrawn from the 6-well plate wells, and cells were trypsinized by adding 0.25 mL trypsin to each well. About 1 mL of PBS was later added to allow for uniform mixing of cells using a 1000 µL pipette, and the mixture was centrifuged for 1 minute using an IEC Centra-M Microcentrifuge at 15,600 xg to remove the red color of trypsin before a cell-density dependent amount of PBS was added to resuspend the cells prior to FACS analysis. This experiment lasted for 7 days.

5.4.2 Results

The FACS results were analyzed for percentage of GFP^+ expression (as determined to be the percentage over the threshold value of 10^2 in FL1-H using the FACS settings as described in Appendix G: Becton Dickinson FACScan Settings and Operation), and the average of triplicate measurements is presented in Figure 28. The error in FACS measurements from the seeding variation study (2 SD of 4.06 % GFP expression) was used as error bars. The three sets of seedings for both LIF^+ and LIF^- cultures are shown in the figures below.

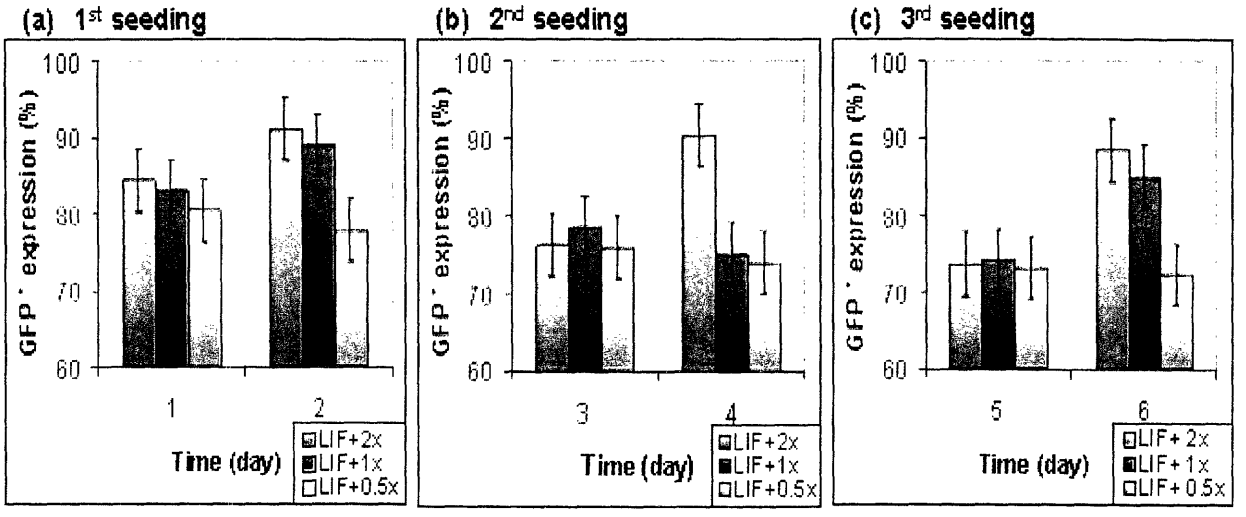


Figure 28: FACS results for LIF$^+$ cultures at various seeding densities

The percentage of T20 cells expressing GFP$^+$ (FL1-H greater than 10^2) in LIF$^+$ culture at each seeding density was obtained daily from seeded 6-well plates and plotted next to one another for comparison. Each graph corresponds to a seeding number. The error bars reflect 2 SD of 4.06 % as determined in Appendix B: Seeding Variation Study.

Based on Figure 28, seeding density appears to have no effect on pluripotency in LIF$^+$ cultures on the 1st day after seeding, but higher seeding density has a significant advantage to lower seeding density in improving percent of GFP$^+$ expression on the 2nd day after seeding in a concentration-dependent manner. The significance is especially significant for high seeding density of $2x10^6$ cells per plate, especially when compared to the lowest seeding density of $0.5x10^6$ cells per plate. One of the possible explanations for this phenomenon is that cells perhaps communicate better through cell-cell signaling at a higher seeding density since there is a higher concentration of cells around them, enabling them to upregulate important cellular processes for cellular growth and maintaining pluripotency. Another explanation is that the higher concentration of LIF per cell for cultures with lower seeding densities (since the same 2 % v/v LIF was added to each well) had a possible detrimental effect on the pluripotency of cells from adding too much LIF to the culture.

While the culture with initial seeding density of $2x10^6$ cells consistently improved in GFP$^+$ expression from Day 1 to Day 2 in each seeding, the culture with $0.5x10^6$ cells per plate experienced a non significant decrease in GFP$^+$ expression with time. The only inconsistency from the LIF$^+$ study was the improvement in GFP$^+$ expression from Day 1 to Day 2 observed in 1st and 3rd seedings but a decline observed in 2nd seeding for the culture with $1x10^6$ initial cell count, which could have resulted from inconsistent seeding between seedings that might have put the initial cell density closer to either $2x10^6$ cells or $0.5x10^6$ cells. The error bars do not reflect this error, as they were derived from SD of the seeding variation study and not from the SD of replicate measurements from this study.

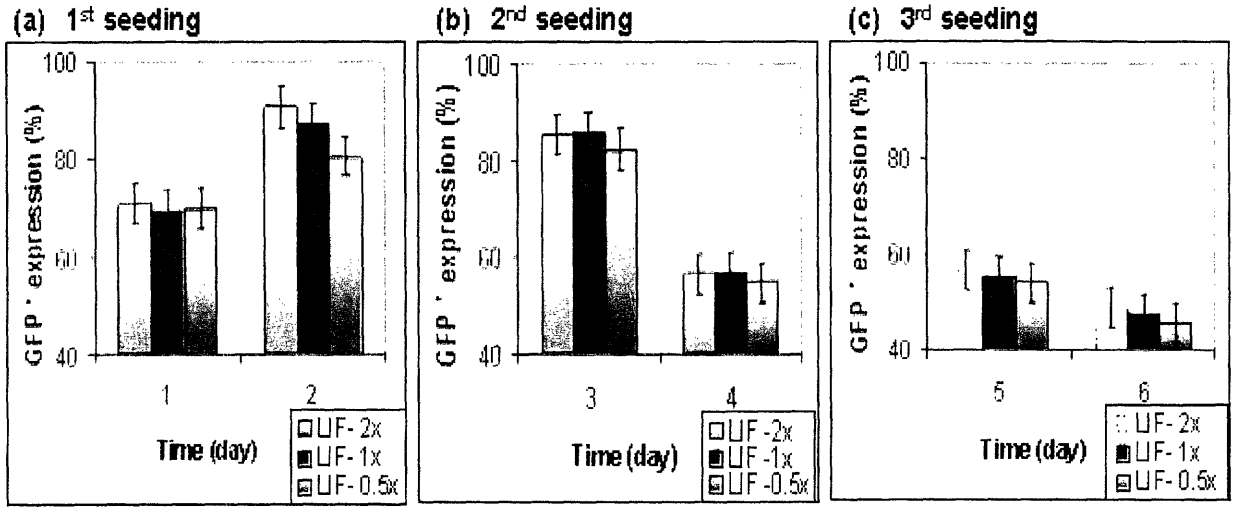


Figure 29: FACS results for LIF⁻ cultures at various seeding densities

The percentage of T20 cells expressing GFP⁺ (FL1-H greater than 10^2) in LIF⁻ culture at each seeding density was obtained daily from seeded 6-well plates and plotted next to one another for comparison. Each graph corresponds to a seeding number. The error bars reflect 2 SD of 4.06 % as determined in Appendix B: Seeding Variation Study.

The results for LIF⁻ cultures, summarized in Figure 29 show an increase in percent GFP⁺ expression from Day 1 to Day 2 for the 1st seeding but a significant drop in GFP⁺ expression for the 2nd seeding, followed by a non significant decrease in the 3rd seeding for all three seeding densities. The increase in GFP⁺ expression for the 1st seeding is probably just the result of withdrawing LIF on the day prior to seeding, which means that either residual LIF still remains in the culture to be effective or the reduction in GFP expression by the Oct4 promoter has not yet been activated by LIF withdrawal. The significant drop in GFP⁺ expression in the 2nd seeding is an indication that reduction in pluripotency by LIF withdrawal is finally taking effect, and the non significant decrease in the 3rd seeding indicates either that the rate of reduction in pluripotency decreases over passages or that percent GFP⁺ expression can only dip to a certain level. Although there was some indication that perhaps low seeding density leads to reduction in pluripotency, no statistically significant difference was observed among the various seeding densities for all 3 seedings in the LIF⁻ culture, in spite of the lack of data for $2x10^6$ initial cell count in the 3rd seeding due to low cell growth on the LIF⁻ maintenance plate.

5.4.3 Conclusion

Based on the results from this experiment, seeding density appears to have no effect on the pluripotency of cells cultured without LIF but significant effect on improving the pluripotency of cells cultured with LIF, with the highest seeding density ($2x10^6$ cells) resulting in

a significant increase in GFP^+ expression from Day 1 to Day 2 of each seeding, while the lowest seeding density ($0.5x10^6$ cells) resulted in no change from Day1 to Day 2 at each seeding. This can be attributed either to 1) the detrimental effect that excessive amounts of LIF can have on cells as LIF was added to culture on the basis of culture volume and not cell density, or 2) the upregulation of important cellular processes such as cell growth and maintaining pluripotency that occurs from cell-cell signaling at higher cell densities. Although no statistically significant difference in ES cell pluripotency was observed with seeding density for LIF^- culture, the hypothesis from the preliminary differentiation experiment that lower seeding density (at least by a factor of 5) leads to reduction in pluripotency for LIF^- culture was supported in this experiment, as data from the LIF^- cultures showed some correlation between seeding density and reduction in GFP^+ expression tested over seeding density of $0.5x10^5$ cells/plate and $2x10^5$ cells/plate. Although seeding densities lower than $0.5x10^5$ cells or higher than $2x10^5$ cells were not studied, it would be interesting to determine whether similar trend is observed at those concentrations.

6 Discussion

The R1 cell line experiment was conducted as a preliminary check to test whether the stable transfection of *gfp* gene into the R1 cell line significantly altered the growth and metabolic characteristics of the cell line. In this experiment, the growth kinetics profiles of R1 and R1 Oct4-GFP cells were shown to be consistent at both LIF^+ and LIF^{-7} conditions, with t_d of approximately 13 hours for LIF^+ cultures and 8 hours for LIF^{-7} cultures, indicating statistically significant difference in doubling time between cells grown with continuous LIF addition and those grown with periodic LIF withdrawal. The doubling time was longer in LIF^+ than in LIF^{-7} cultures by 75 % and 66 % for R1 and R1 Oct4-GFP cell lines, respectively. The metabolic rates of these cell lines were also consistent between each other for glucose, lactate, and glutamine. $Y_{lac/gluc}$ calculation also revealed strong similarities between R1 and R1 Oct4-GFP cells at both LIF^+ and LIF^{-7} conditions as well as consistency between LIF^+ and LIF^- conditions for both cell populations, each ranging between 44 % and 94 % yield of lactate from glucose.

Comparisons between R1 cell lines and J1 cell line revealed significant discrepancy in cell growth kinetics, with 21 % shorter t_d for J1 cell line compared to R1 cell lines under a similar LIF condition. This discrepancy in cell growth kinetics could be attributed, however, to the different culturing conditions (on 6-cm plates and in a different incubator) and different cell counting method (manual cell counting by hemacytometer) used in this experiment, which might have added systematic uncertainties to the experiment. Consistency in the metabolic data was nonetheless observed with $Y_{lac/gluc}$ ranging between 47 % and 57 %, which showed that the J1 cell line not only exhibits similar metabolic profile as R1 cell lines but also utilizes energy from glucose more efficiently, possibly owing to its higher cell proliferation rate.

The preliminary characterization of R1 Oct4-GFP cell line involved two separate experiments: one for differentiation analysis and the other for cell growth and metabolic analyses. The histograms from the differentiation experiment visually showed the improvement and reduction in the percentage of GFP^+-expressing cells that arise from LIF addition and withdrawal, respectively. The drastic reduction in GFP^+ expression that was observed with LIF withdrawal could be attributed to the presence of high fraction of cells that can be affected by LIF withdrawal, as evidenced by GFP^+ cells accounting for 90 % of cells in these cultures. It is also possible that this reduction is caused by the added negative effect that LIF withdrawal and passaging (trypsinization and removing cells from optimal culturing environment) have on GFP^+ expression, which also helps explain why the improvement in GFP^+ expression in LIF^+ cultures is not as drastic.

The bistability of the *gfp* gene was suggested as an explanation for the shift in the distribution of the cells in the histograms over time in both LIF^+ and LIF^- cultures, but the time scale and the threshold concentration at which LIF affects the *pou5f1* gene and genes controlling cell division could not be fully elucidated from these experiments. Nonetheless, LIF withdrawal appears to have a rapid effect on downregulating the *pou5f1* gene, with reduction in GFP^+ expression observed within 3 to 4 days of LIF withdrawal, while the effect of LIF withdrawal on cell proliferation rate seems to occur at a slower rate.

This slow effect on cell proliferation was hypothesized from the preliminary kinetic and metabolic experiment, where non significant difference in t_d was found between LIF^+ and LIF^- cultures for both T20 and unsorted cell populations. This finding, which was validated by a

study by Zandstra, et al. but contradicted by another study by Viswanathan, et al., could be explained by the hypothesis that the effect of LIF withdrawal on cell proliferation is slow, such that it takes approximately a week for the effect to be noticeable. This hypothesis was later supported in the LIF concentration experiment, where t_d was found to vary with LIF concentration only after 6 days of culturing, which was presumably the time required to degrade or remove through endocytosis 2 % v/v LIF that was added to the cultures for a week prior to the start of the experiment. Further studies with variations in the period of LIF withdrawal and in LIF concentration are necessary to determine whether this delayed effect on cell proliferation is due to slow signal transduction for turning off the *pou5f1* promoter or to residual LIF remaining on the cell surface even after LIF is withdrawn. This latter situation could arise if endocytosis of LIF or degradation of LIF in culture is slow.

Although the effect of LIF on cell proliferation rate was not observed in the preliminary kinetic experiment, the results from the LIF concentration experiment revealed a dose-dependent increase in cell proliferation with higher LIF concentration, which confirmed the study by Viswanathan, et al. that LIF offers selective growth advantage to pluripotent cells [54]. Higher LIF concentration, however, was also found to offer no concentration-dependent advantage to ES cell pluripotency or cell morphology above a certain threshold level, implying that separate mechanisms involving LIF are used for maintaining cell pluripotency and increasing cell growth rate of undifferentiated cells.

Another hypothesis from the preliminary differentiation experiment, that low seeding density can have an added negative effect on cell pluripotency in LIF^- culture, was tested in the seeding density experiment where plates were seeded at 3 different seeding densities of $0.5x10^6$, $1x10^6$, and $2x10^6$ cells per plate. The results from this experiment revealed a non significant reduction in GFP^+ expression with lower seeding density in LIF^- cultures, but significant improvement in GFP^+ expression with higher seeding density in LIF^+ cultures. Although the results did not confirm the hypothesis, the positive effect of high seeding density on GFP^+ expression level in LIF^+ culture was elucidated over a small range of seeding densities studied. Further study is necessary to determine the optimal seeding density for maintaining ES cell pluripotency in culture at a given passage frequency in the presence of LIF.

In the low glucose experiment, the effect of low level of glucose on ES cells was found to be reduction in the rate of cellular mechanisms, as no improvement in t_d over multiple seedings and non significant difference in GFP^+ expression level between LIF^+ and LIF^- cultures were observed. The metabolic rates of glucose and lactate were also lower under the low-glucose condition, presumably due to low level of lactate present in these cultures resulting in higher pH. Glucose metabolism through oxidative phosphorylation was also found to be higher in low-glucose culture (51 % to 92 %) than in high-glucose culture (40 % to 60 %), implying that low level of glucose forces cells to harness energy more efficiently through a longer pathway, while high level of glucose allows cells to metabolize glucose through a faster pathway at the expense of producing lactate. In other words, ES cells metabolize glucose at a comparable rate in both high-glucose or low-glucose conditions, but reduced rate of cellular mechanisms is observed under low level of glucose, presumably since these cells must take a longer metabolic path to harness energy more efficiently.

In the low serum experiment, high-serum culture was found to offer no significant advantage to low-serum culture in maintaining ES cell pluripotency or increasing cell proliferation rate in the LIF^+ condition. In the LIF^- condition, however, low serum led to higher reduction in GFP^+ expression level and much longer t_d (71 ± 33 hours) than the high-serum

counterpart (35 ± 9 hours). Both the analysis of glucose and lactate metabolic rates and $Y_{lac/gluc}$ calculation revealed high level of consistency between high-serum and low-serum cultures, implying that ES cells still metabolize glucose at a similar rate and by similar mechanisms regardless of the amount of serum in the medium.

Appendix A: Medium Evaporation Study

(data procured by Jiovani Visaya, Amherst '05)

Three types of tissue culture (TC) dishes typically used in experiments involving adherent cells (10-cm, 6-cm, and 6-well plates) were placed in a ThermoForma Steri-Cycle™ CO_2 incubator (interior width x height x depth: 54.1 cm x 68.1 cm x 50.8 cm) with appropriate volume of medium typically added to each type of TC dish for culturing, and mass lost through medium evaporation was determined by periodic measurement on a Mettler-Toledo AE163 scale with 8 measurements taken over the course of 6 days. The approximate volume of medium initially placed in 10-cm, 6-cm, and 6-well plates was proportional to the growth surface area of each plate, at 10 mL, 4 mL, and 12 mL (2 mL per well), respectively. The same incubator that was used for all of the experiments involved in the thesis research was used in this study for consistency of results, and the same operating conditions of 37 °C temperature and 5 % CO_2 in humidified air were also maintained. Temperature was held constant in the incubator by direct chamber heating through heated walls, and the gas mixture entering the chamber was filtered by HEPA filter and circulated through the chamber by directed upward air flow. Since temperature sensors were located around the inner walls of the incubator, small variations in temperature were expected in the chamber, mostly between inner- and outer-edge positions.

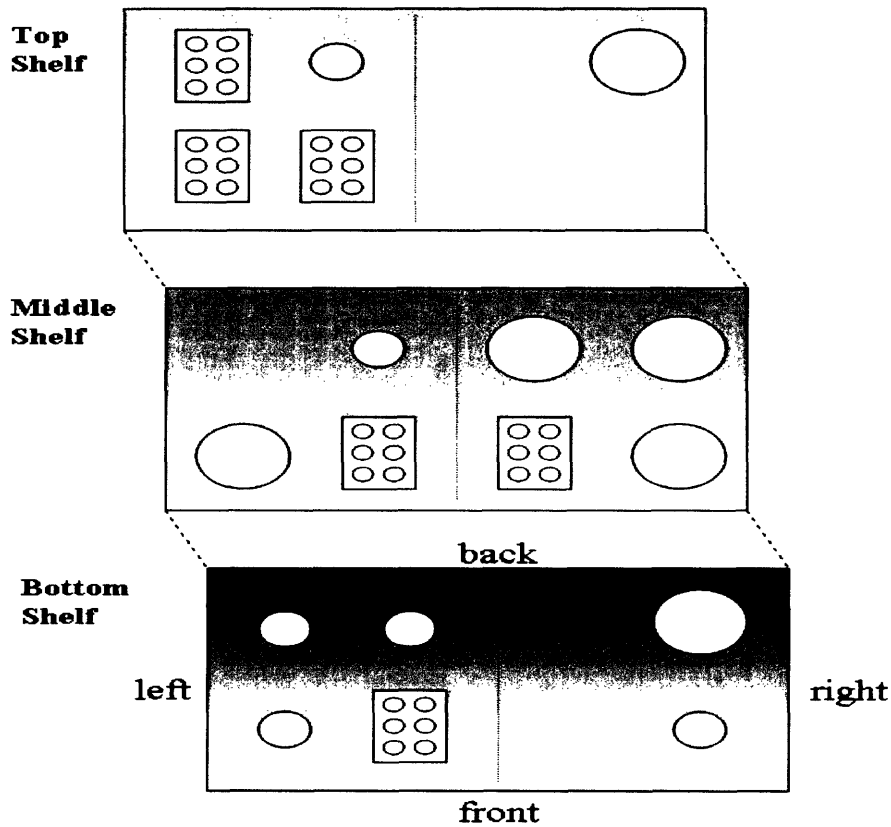


Figure 30: Arrangement of various TC plates in the incubator

Three types of plates were arranged in the incubator such that comparisons could be made for medium evaporation rates of plates placed on inner- and outer-edge positions; front and back positions; left and right positions; and top, middle, and bottom shelves.

Six plates of each type were arranged in the incubator as depicted in Figure 30, and each plate was returned to its respective location in the incubator every day upon weighing for consistency. The placement of the plates was devised so that, for each plate type, comparisons in the medium evaporation rate could be made between the inner- and outer-edge positions; left and right sides; top, middle, and bottom shelves; and front and back positions in the incubator. As a point of interest, the incubator door swings open to the right.

Each plate was weighed only once at each time point, but all plates were weighed 3 times prior to medium addition to determine an accurate mass of the plates as well as the standard deviation (SD) in weighing samples (ranging from 0.17 mg and 0.74 mg for all dish types). The other probable source of error, the error of the scale, was listed as 0.1 mg, which was negligible compared to this standard deviation of weighing the samples. Since measurements were taken approximately twice per day and medium evaporation typically exceeded 0.1 g (100 mg), the known errors were for the most part negligible.

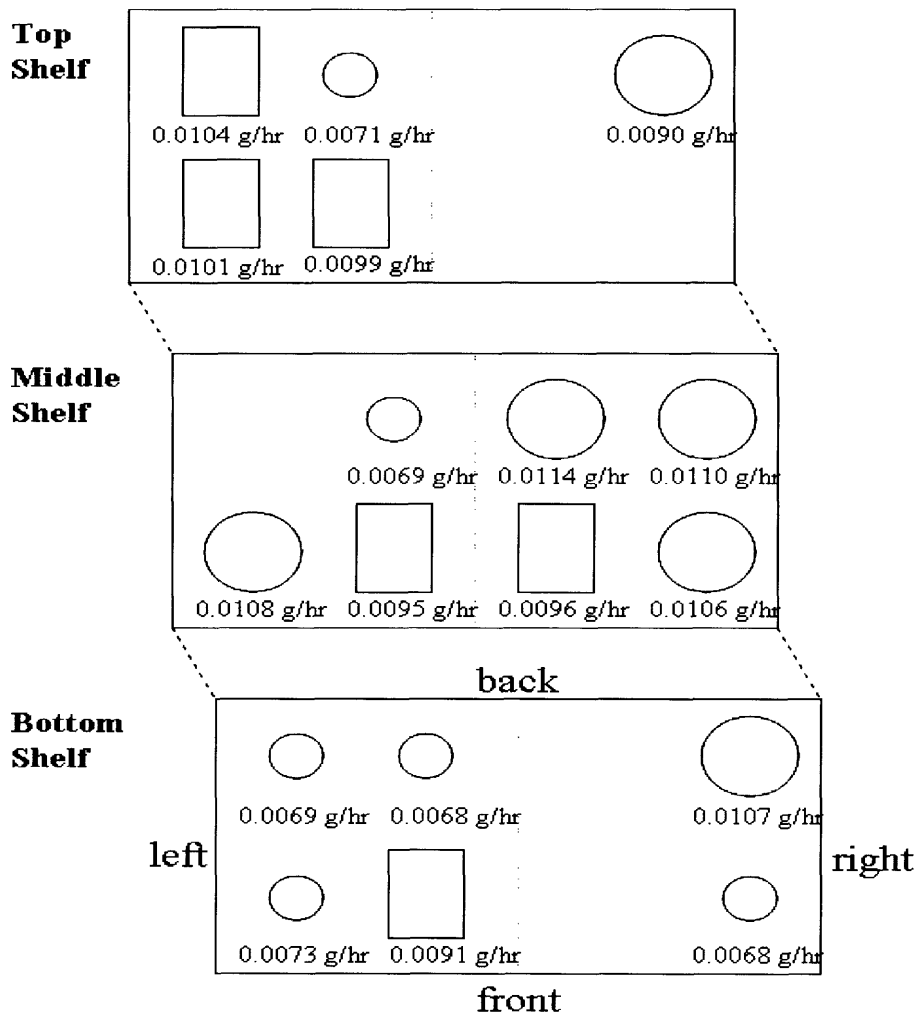


Figure 31: Rate of medium evaporation for each plate at each position in the incubator

The medium evaporation rate was determined for each type of plate at each position through a linear fit of 8 independent weighings performed over 6 days. The deviation in the values arising from the different positions in the incubator was determined to be statistically non significant.

The results from this study indicated that the evaporation rates of 10-cm, 6-cm, and 6-well plates were approximately 0.011 g/hr, 0.007 g/hr, and 0.010 g/hr, respectively (Figure 31). The deviations in evaporation rate seen among the 6 plates of same type could be attributed partially to differences in the gas flow rate around the plates as well as minor variations in the plates and the lids between which gas is allowed to enter and exit. The evaporation rate deviations were much less for 6-cm plates (1 SD of 0.2 mg/hr) and 6-well plates (0.5 mg/hr) compared to 10-cm plates (0.8 mg/hr), which was fortunate since all experimental results were based on data collected from 6-cm and 6-well plates. The evaporation rate for 6-well plates was comparable to that of 10-cm plates, but its low evaporation rate per plate area could be attributed to the low-evaporation lid on the Becton-Dickinson 6-well plates

These medium evaporation rates were used as correction factor for adjusting the apparent concentration of glucose, lactate, and glutamine for use in metabolic rate calculations. As metabolite mass was conserved over time, the actual metabolite concentration could be expressed as the following:

$$m_{k,i} = m_{k,i,apparent}$$

$$C_{k,i} \times V_i = C_{k,i,apparent} \times V_{i,apparent}$$

$$C_{k,i} = C_{k,i,apparent} \times \frac{V_{i,apparent}}{V_i}$$

where V is mass of the culture in mL ,
C is concentration of the metabolite in culture in g/L,
k is the type of metabolite, and
i is the specific time point.

Since rate of evaporation was constant over time, $V_{i,apparent}$ could be simplified as follows:

$$C_{k,i} = C_{k,i,apparent} \times \frac{V_i - [R \times (t_i - t_o)]}{V_i}$$

$$C_{k,i} = C_{k,i,apparent} \times \{1 - [\frac{R}{V_i} \times (t_i - t_o)]\}$$

where R is the evaporation rate in mL/hr assuming $d_{culture}$ of 1 mg/mL,
t is time in hr, with t_o as the initial time of seeding.

This last equation was used to calculate the actual glucose, lactate, and glutamine concentrations based on the values obtained from YSI 2700 SELECT™.

Table 9: Evaporation rate and TC parameters

	Evaporation rate (10-cm plate basis)	Circumference (10-cm plate basis)	Surface area (10-cm plate basis)
10-cm	1	1	1
6-cm	**0.66**	**0.58**	0.36

This study also showed that evaporation rate of round-surface plates was more a function of the circumference of the plate than the surface area of the plate when the lid was placed on it (Table 9), which is sensible given that the limiting factor in gas exchange is probably the small opening between the lid and the plate and not the interface between the liquid phase of the medium and the headspace under the lid.

A similar study was conducted in a Revco Ultima Series standard-size CO_2 incubator (interior width x height x depth: 50.8 cm x 71.1 cm x 45.7 cm) maintained at the same 37 °C and 5 % CO_2 conditions over 4 days. Medium was added to six TC plates, three 10-cm plates and three 6-cm plates, and the plates were placed on the middle shelf of the incubator. Each plate was weighed on a different Mettler-Toledo AE163 scale independently, and 9 measurements in total were taken over the course of 5 days. The amount of medium initially added to the plates was varied, with 10 mL, 8 mL, or 5 mL added to each of the 10-cm plates and 4 mL, 3 mL, or 2 mL added to each of the 6-cm plates to confirm that medium evaporation rate does not depend on the amount of medium in the plate.

Table 10: Evaporation rate of 10-cm and 6-cm TC plates with various amounts of medium

	Volume of medium (mL)	Evaporation rate (g/hr)
10-cm plate	10	0.0307
	8	0.0306
	5	0.0291
6-cm plate	4	0.0194
	3	0.0201
	2	0.0201

Table 11: Evaporation rate and TC parameters from a separate study

	Evaporation rate (10-cm plate basis)	Circumference (10-cm plate basis)	Surface area (10-cm plate basis)
10-cm	1	1	1
6-cm	**0.66**	**0.60**	0.36

As Table 10 shows, the amount of medium contained in a plate does not appear to affect the evaporation rate as long as the entire surface area is covered, a result which is also sensible since mass transfer of medium through polystyrene of the plate is essentially zero. The SD of evaporation rates of 3 plates for each type of TC plate was 0.40 mg/hr and 0.90 mg/hr for 6-cm

and 10-cm plates, respectively. These values were comparable to the range of SD in weighing samples, as determined in the previous study to be 0.17 mg/hr and 0.74 mg/hr. Even though evaporation data from this study were not consistent with data from the previous evaporation study discussed above, only data from the previous experiment were used in making adjustments to metabolic data, since the same CO_2 incubator was used in that study as in all other experiments of this research.

The results from that study also confirmed that the evaporation rate of medium has greater dependence on circumference of the plate than on surface area of the plate (Table 11). This comparison was made by taking the average evaporation rate of 3 plates for each plate type and then using the 10-cm plate as basis to draw comparisons, as in Table 9.

Appendix B: Seeding Variation Study

2 cell populations, Top 20 % sorted (T20) and unsorted R1 Oct4-GFP cells, were grown in the presence of LIF and expanded on four 6-cm plates, each for seeding onto three 6-well plate wells for determining the homogeneity of the culture after mixing when seeding and the error involved in seeding. At each seeding, 3 wells were used for cell counting with Cedex and the other 3 wells for FACS analysis, and the same procedure was used for the 2 cell populations. The supernatant from the wells used for Cedex counts was frozen down and was used later for calculating the error in metabolic measurements. In addition, a maintenance plate was also created for each cell population to allow multiple rounds of seeding to take place on subsequent days for a more precise determination of error. Only LIF^+ cultures were used in this study to simplify the data acquisition procedure, and error determined from this study was also applied to the experimental data obtained for LIF^- cultures.

4 seedings were performed in succession, but initial seeding density was maintained at approximately $1x10^6$ cells/well for all seedings since the error in seeding for cell densities would eventually be expressed as standard error (ε_x/x). By determining the standard deviation of 3 independent samples by Cedex, YSI, and FACS analyses, a more accurate value for the error bars could be derived for all data for which standard deviation from replicate measurements cannot be readily obtained. The seedings and analyses of seeded wells were performed every other day, with the Cedex analysis conducted before seeding so that the cell density could be predetermined for both cell populations, ensuring that the seeding density would not be too low or too high. The YSI analysis was performed at the same time after all the frozen samples were thawed down.

Table 12: Standard deviation in seeding for % GFP^+ expression

Seeding #	Unsorted (in %)	T20 (in %)
1	1.32	2.52
2	5.88*	2.94
3	2.31	0.62
4	2.86	7.61♣
Average	**2.16**	**2.03**

* Disregarded since only 2 of 3 samples were available for sampling

♣ Disregarded since some cells coagulated and precipitated out upon vortexing

For FACS analysis, standard deviation (SD) turned out to be approximately 2 % GFP^+ expression for both cell populations (Table 12). Since these numbers were obtained over a wide range of GFP^+ expression values (52 to 75 %) and no trend could be determined between GFP^+ expression and SD, average of SDs was calculated for each population, and 2.16 % and 2.03 % were used as the error in seeding for GFP^+ expression percentage for unsorted and sorted T20 R1 Oct4-GFP cells, respectively, in all data analyses involving fewer than 3 samples. For analyses involving 3 or more samples, the standard deviation of the samples was used. Since the scatter observed in the data is independent of the absolute value of the data in FACS analysis, standard deviation was used instead of standard error.

Table 13: Standard deviation in seeding for cell densities

Seeding #	Unsorted ($x10^5$ cells)	T20 ($x10^5$ cells)
1	2.86	2.16
2	5.06	3.49
3	0.99	2.28
4	1.83	0.67
Average	**2.98**	**2.65**

For cell density analysis, SD was calculated to be approximately $3x10^5$ cells for approximately $40x10^5$ cells (Table 13). Since these numbers all stemmed from error that occurred at the time of seeding, the error was ultimately expressed in the form of standard error based on the average cell density for each cell population, after the average of SDs was calculated for each cell population. Based on the average cell density of $38x10^5$ for the unsorted population and $42x10^5$ for the T20 population, the standard error in seeding for cell density was determined as 8 % and 6 %, respectively. These values were used as the standard error in analyses with fewer than 3 samples; otherwise, standard error was derived directly from the samples.

Table 14: Standard deviation in seeding for metabolic analysis

*Seeding #	Unsorted (in g/L)		T20 (in g/L)	
	Glucose	Lactate	Glucose	Lactate
2	0.025	0.074	0.031	0.082
3	0.015	0.108	0.019	0.126
4	0.030	0.099	0.017	0.127
Average	**0.023**	**0.094**	**0.022**	**0.112**

* Seeding #1 was not included in the analysis due to improper sample storage and preparation

For metabolic analysis, the frozen samples were first thawed overnight in a 4 °C refrigerator and vortexed immediately prior to taking measurements. Since the samples from the first round of seeding were initially not properly placed in the freezer, these samples had large deviations in the data set and were therefore discarded. For the other 3 rounds of seeding, measurements were taken in triplicates for each sample, and all 9 measurements (samples from 3 seeded wells measured in triplicates) were cumulatively treated as independent data points in determining the standard deviation, since that method most closely resembles the randomness involved in the actual culturing and accounts for both error in seeding and error in sampling. SD was calculated to be approximately 0.02 g/L for glucose and 0.10 g/L for lactate (Table 14).

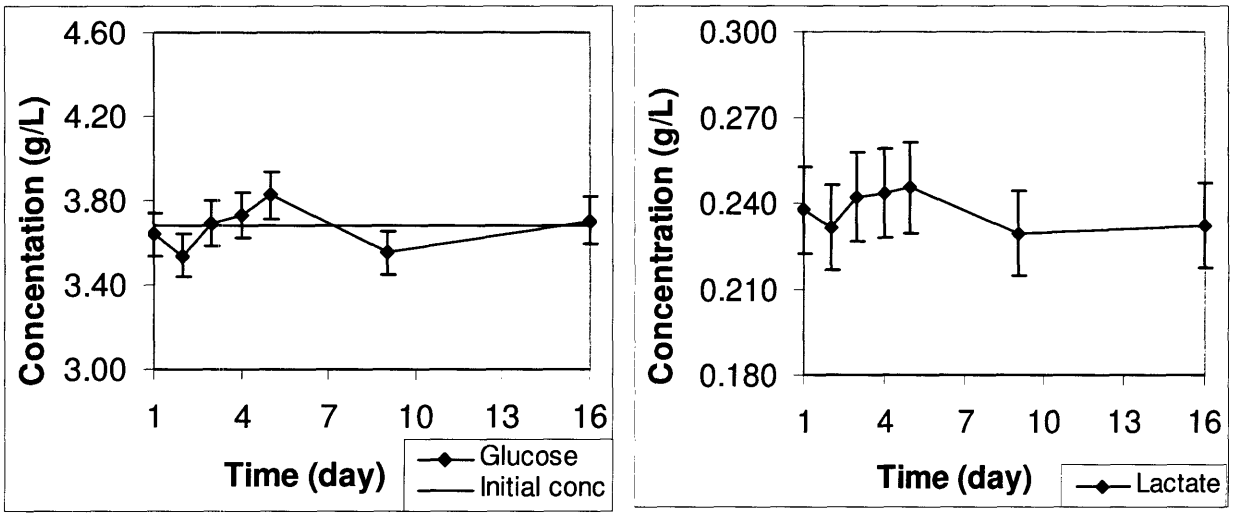
Since these deviations are not absolute and vary with concentrations of glucose and lactate, the standard error was calculated for each seeding for each cell population based on the SD and the average of glucose or lactate concentrations within each group, as these metabolic values were affected by cell density and hence varied slightly from seeding to seeding. The average of the standard errors was found to be 1.5 % for glucose in both populations and 4.0 % and 4.4 % for lactate in unsorted and sorted T20 R1 Oct4-GFP, respectively. These values were used as standard error for analyses involving fewer than 3 samples; otherwise, standard error was derived directly from the samples.

Appendix C: Medium Stability Study

(data acquired by Jiovani Visaya, Amherst '06)

Standard ES medium was checked for 2 nutrient concentrations (glucose and lactate) approximately every other day for 16 days to determine the stability of medium components in the 4 °C refrigerator over time. YSI 2700 SELECT™ was calibrated daily before data on media were taken to eliminate the error in instrumentation. Further, medium was initially aliquoted into a 50 mL tube, but the tube was warmed up in the incubator every day prior to sampling to simulate the actual cycle of warming up and cooling down that the medium experiences on a daily basis.

Metabolic concentrations were obtained three times for every day that the sample was taken. These multiple measurements were averaged and plotted with their standard deviation (SD) as determined in Appendix B: Seeding Variation Study to observe whether there is a systematic drift, due either to degradation of medium components in the tube or enzymatic membrane failure in the instrument. The results with the error bars are shown in the figures on the left, with the error bars representing 2 SD in magnitude for 95 % confidence level.



(a) Time-course glucose concentration (g/L) **(b) Time-course lactate concentration (g/L)**

Figure 32: Time-course glucose and lactate concentration in medium

The glucose and lactate concentrations in standard ES cell medium were measured daily or every few days over a time span of 16 days to determine whether medium component degradation occurs. The error bars reflect 2 SD as determined in Appendix B: Seeding Variation Study.

Both graphs show non-systematic fluctuations in the concentration of metabolic components, although glucose concentration, owing to its small relative error, has 3 out of 7 data points that are outside 2 SD. However, there is an upward trend for both components between Day 2 and Day 5, which indicates that degradation of medium components does not occur or have a significant effect on the overall fluctuations in the medium. Further, the initial glucose concentration at the time medium was made (shown as the pink horizontal line in Figure 32a at

3.68 g/L), is approximately the glucose concentration on Day 1 and Day 3 as well as on Day 16, indicating that the same amount of glucose is present on the first day and the last day of the experiment. Lactate data also confirm this result.

One interesting point to note is how similar the fluctuations in the graphs are. This could very well be attributed to the randomness in the YSI instrument since the same instrument measures both metabolic components simultaneously. However, since the detection of these components occurs with 2 separate membranes, it is more likely that the tube of medium was not mixed prior to sampling, leading to fluctuations in the glucose and lactate concentrations that were larger than the errors determined in the Seeding Variation Study, depending on the height of the tube from which the medium was withdrawn. This study nonetheless shows that the metabolic components do not degrade significantly enough to affect the data over a span of 16 days and that even without stirring the medium prior to sampling, the fluctuations are well within 3 SD of the expected value (initial concentration).

Appendix D: Cell Maintenance Protocols

Freezing down stem cells

The cells were allowed to grow on 10-cm TC dishes until near confluent, using as many dishes as necessary for making a freezing stock. To ensure pluripotency of cells in the freezing stock, commercial LIF (ESGRO® ES1106 from Chemicon) or in-house LIF was always used for changing medium and for passaging, and the cells were never allowed to become overly confluent. Because in-house LIF was believed to be less potent than commercial LIF, twice the volume of LIF normally added to culture was used when using in-house LIF (at 2 μL/mL of medium). 10-cm dishes were used instead of 15-cm dishes to allow for easier and more uniform mixing of cells during resuspension.

Once the cells reached the desired concentration for freezing, spent medium was aspirated out of the dishes, and 2 mL trypsin-EDTA was added to each dish to detach the cells after washing each dish once using approximately 5 mL of sterile PBS to remove excess debris from the bottom of the dish. After 10 minutes in the incubator, 8 mL of warm fresh ES medium was used per dish to break up the cell clumps into individual cells. The cells from all the dishes were then transferred to large (15 mL or 50 mL) centrifuge tubes and mixed to ensure uniform cell concentration among the tubes. From one of these tubes, 1 mL of cell culture was transferred to an Eppendorf tube and put aside for manual or Cedex counting.

While the cells in the large tubes were centrifuged in an IEC Centra-8R centrifuge at 225 xg (1000 rpm) for 5 minutes, cell counting was performed to determine the approximate total number of cells available for freezing. After removing the supernatant from the centrifuged tubes, the cell pellet was resuspended using the amount of freezing medium (in mL) equal to the number of freezing vials that can be made from the pellet at a cell concentration of $1x10^6$ cells/vial (for plating on 6-cm TC dish after thawing), and 1 mL culture was aliquoted into each cryogenic vial and placed on ice for 10 to 15 minutes. LIF was usually added to the freezing medium before aliquoting to simplify the process.

The vials were then put in a labeled freezing box, which was placed thereafter in a styrofoam box insulated with paper towels and sealed using tape to slow down the freezing process as much as possible. Finally, the box was left in a –80 °C freezer overnight before being transferred to a –120 °C freezer in the Engelward lab for long-term storage.

Thawing down stem cells

For thawing down cells, a frozen vial of cells was transferred from the –120 °C freezer to the TC room via cryogenic vessel filled with dry ice or liquid N_2. To maintain high cell viability, the cells were partially thawed in the 37 °C water bath and then completely thawed by addition of warm fresh ES medium. The cells were then transferred to a 15 mL centrifuge tube and spun down at 225 xg for 5 minutes to remove the DMSO contained in the freezing medium. The supernatant was then aspirated out of the tube, and the cell pellet was resuspended in warm fresh ES medium before being plated onto a new gelatinized 6-cm TC dish with LIF and placed in the incubator. The cell concentration and viability were checked by visual inspection a few hours later to estimate the time for re-passaging and then reinspected every 12 hours to adjust the estimated time and to prevent the cells from becoming overly confluent.

Passaging stem cells

The cells were always passaged as the TC dish reached near confluency (about every 2 days), which was typically between $1x10^7$ and $2x10^7$ cells on a 10-cm plate. Having taken the dish out of the incubator, spent medium was first aspirated out of the dish, and 2 mL trypsin-EDTA was added to detach the cells after washing the bottom of the dish with approximately 5 mL PBS. After 10 minutes in the incubator, 8 mL of warm fresh ES medium was added, and cell concentration was either estimated by visual inspection before trypsinization (almost all of dish area covered by cells) or measured after trypsinization using manual or Cedex counting by transferring 1 mL of cell culture to an Eppendorf tube.

The volume of culture containing $2x10^6$ cells was then placed in a new gelatinized 10-cm TC dish, and fresh ES medium was added to make the total volume inside the dish 10 mL. LIF was finally added to the dish, the culture was thoroughly mixed, and the dish was placed back in the incubator.

For passaging of cells cultured in 6-well plates or 6-cm TC dishes, the cell number proportional to the growth area was used both for determining confluency and passaging, and total volumes of culture were made to be 2 mL and 4 mL, respectively.

Appendix E: Medium Formulation Protocols

Making standard ES medium

Standard ES medium consisted of the following components (per 500 mL):

409.1 mL Gibco® Dulbecco's Modified Eagle Medium (DMEM, Catalog # 11960-044)
75 mL Hyclone® fetal bovine serum (FBS, Lot #AMF15453)
5 mL Gibco® 10 mM non-essential amino acids (NEAA, Catalog # 11140-050)
5 mL Gibco® 100 mM sodium pyruvate (Catalog # 11360-070)
5 mL Sigma® 200 mM L-glutamine (Catalog # G7513)
0.9 mL Gibco® 55 mM (1000x) beta-mercaptoethanol (Catalog # 21985-023)

After thawing down L-glutamine and serum and warming up the rest of the ingredients in the 37 °C water bath, the components were mixed in the original DMEM container and filtered using a Zapcap® filter into a pre-autoclaved 500 mL glass bottle. As LIF has the tendency to degrade at high temperatures, it was never included in the medium formulation but was always added at 2 µL/mL of medium during each passaging, plating, and medium exchange.

Making low-glucose ES medium

Low-glucose ES medium consisted of the following components (per 500 mL):

409.1 mL Gibco® Low-Glucose DMEM with sodium pyruvate (Catalog # 10316-024)
75 mL Hyclone® FBS
5 mL Gibco® 10 mM NEAA
5 mL Sigma® 200 mM L-glutamine
5 mL Distilled autoclaved H_2O
0.9 mL Gibco® 55 mM (1000x) beta-mercaptoethanol

Low-glucose DMEM with sodium pyruvate contained 1000 mg/L of glucose as well as 1mM of sodium pyruvate, but with no phenol red (pH indicator). Distilled autoclaved H_2O was added in place of sodium pyruvate since concentrated 200 mM L-glutamine from Sigma® is shipped in H_2O instead of PBS. The same protocol as the one used for ES maintenance medium was used to make this medium.

Making low-serum ES medium

Low-serum ES medium consisted of the following components (per 500 mL):

409.1 mL Gibco® DMEM
25 mL Hyclone® FBS
50 mL Gibco® Phosphate Buffer Saline (PBS)
5 mL Gibco® 10 mM NEAA
5 mL Gibco® 100 mM sodium pyruvate
5 mL Sigma® 200 mM L-Glutamine
0.9 mL Gibco® 55 mM (1000x) beta-mercaptoethanol

Regular high glucose DMEM used for ES maintenance medium was also used to make this medium, but only 5 % FBS was added. The remaining volume was adjusted by adding PBS to the medium. As a result, the pH of this medium was slightly higher than the other formulations.

Making ES freezing medium

ES freezing medium consisted of the following components (per 10 mL):

8 mL ES maintenance medium (as prepared before)

1 mL Hyclone® FBS

1 mL Sigma® biotechnology grade DMSO (Catalog # D2438)

After thawing down serum and warming up the ES maintenance medium in the 37 °C water bath, these two components were first mixed together and then DMSO was slowly added to the mixture to prevent denaturing of proteins in the FBS. LIF was added to this freezing medium only before freezing down cells to prevent degradation of LIF. DMSO was added to freezing medium as freezing point modifier, and additional FBS served to keep the cells saturated with growth factors in the freezing and thawing process.

Appendix F: LIF Purification Protocol

Non-commercial LIF was made and purified using the protocol described below, obtained from Professor Bevin Engelward at MIT. All non-commercial LIF used in this research came from the batch that was made in the summer of 2002 with the help of Dr. Ryo Ohashi.

Recombinant LIF = 20 kDa
Thrombin = 34 kDa
Gex = 26 kDa

Day 1

1. Streak out JM109 (pGEX-LIF) onto LB agar + amp (final 50 μg/mL), incubate overnight (o/n) at 37 °C. Strain is stored at –80 °C.

Day 2

2. Select a colony of LB + amp plate and inoculate to 50 mL of LB broth + amp (final 50 μg/mL), incubate o/n at 37 °C with shaking (250 rpm). At same time, you can prepare four 2-L flasks with 500 mL of LB broth (autoclave and leave at room temperature).

Day 3

3. Inoculate 4 flasks of 500 mL of LB broth + amp (final 50 μg/mL) with 10 to 15 mL of overnight stationary culture, and incubate at 37 °C until OD get = 0.8 – 1.0 (will take approximately 4 hours). Flasks should be pre-warmed (if not, it will take longer).
4. Add IPTG (final 1 mM) and grow for another 3 hours. You could find 1 M stock in –20 °C.
5. Pellet the culture by centrifugation for 30 minutes at 3500 rpm (4 °C) with large capacity centrifuge. Decant and dispose of supernatant.
6. Freeze cell pellet at – 20 °C or continue to step 7 (but it will take super long).

Day 4

7. To each pellet (from 500 mL culture) add 5 mL of MTPBS. With the volume of the pellet this will be total about 10 mL.
8. Add PI cocktail and PMSF. Concentration of PI and PMSF is 1000x. Therefore 10 μl should be added to 10 mL of cell suspension.
9. Sonicate cell suspension. Sonicate 5 mL at a time and sample has to be kept on ice prior to sonication. Tip must be placed right below the surface of the sample. Duty cycle 50 %, output control 2, time 3 minutes with small probe.
10. Poll lysates and split into two equal volumes. Add Triton X-100 (10 %) to final concentration of 1 % and mix for a few times (invert). Leave on ice for 5 minutes.
11. Centrifuge for 30 minutes at 3500 rpm (4 °C).
12. Decant supernatant into a 50 mL. Centrifuge tube and keep on ice.
13. Take out 8 mL of glutathione Sepharose beads (for 2 L of culture) and spin down for 5 minutes at 2500 rpm (4 °C).
14. Wash with MTPBS (5 volumes of beads). Wash means remove supernatant and resuspend into MTPBS, and then centrifuge.

15. Resuspend in MTPBS as a 50 % v/v solution and store at 4 °C. For 1.33 mL of bead, add 1 mL of MTPBS (This will be a 50 % solution).
16. Add 5 mL of gex-lif supernatant to 2 mL of glutathione sepharose beads (in MTPBS).
17. Bind for 2 hours at 4 °C with agitation.
18. Centrifuge through 20 % sucrose (in MTPBS). Carefully layer beads suspension on top of equal volume of 20 % sucrose and centrifuge for 5 minutes at 2500 rpm (4°C).
19. Carefully take off supernatant and discard.
20. Wash beads with Wash buffer #1.
21. Wash beads with Wash buffer #2.
22. Resuspend beads with 10 mL of elution buffer.
23. Thrombin digestion. Add thrombin (human) to final 30 units /mL. Stock solution should be 100x, so 10 µl for 10 mL of beads suspension.
24. Digest o/n at room temperature.

Day 5

25. Spin down beads for 5 min at 3000 rpm (4 °C), save supernatant and wash pellet 5 times with 2 mL of elution buffer. Pool washes and spin a final time to get rid of residual beads. After that, glutathione sepharose beads have to be regenerated ASAP.
26. Perform Bradford assay.
27. Bring to 20 µg/mL. Reduce LIF concentration down to 20 µg/mL using 10 mg/mL BSA in elution buffer.
28. Sterile filter LIF and aliquot into 2 mL cryovials. Aliquot should be quick frozen in liquid N_2 and stored at –80 °C.

Bradford assay

1. Prepare BSA standard. 1, 2.5, 5, 7.5, 10 mg/mL of BSA will used as a standard.
2. Prepare 10x solution of each standard form 1 mg/mL BSA stock.
3. Take 100 mL of each of 10x standard and add to 700 mL of water and 200 mL of Biorad Dye to make up samples.
4. Form LIF extraction and make 10 to 100x dilution. Take 100 mL of each of 10x standard and add to 700 mL of water and 200 mL of Biorad Dye to make up samples.
5. Measure OD at 595 nm. Before measurement, wait 5 minutes to allow for warm up.

Regeneration of Glutathione Sepharose Beads

1. Pour sepharose beads into a Biorad empty column soon after use.
2. Wash column with 1 x bed volumes of 3 M NaCl in PBS.
3. Wash with 2 x 5 bed volumes of PBS.
4. Wash with 2 x 5 bed volumes of 20 % ethanol in PBS and store at 4 °C.

Appendix G: Becton Dickinson FACScan Settings and Operation

The BD FACScan is a fluorescence-activated cell scanning device, which has essentially the same capability as a FACSorter, in that cell analysis and fluorescence detection methods are the same in both instruments. The only difference is that a FACSorter is able to sort out cells into different chambers based on the gating set by the user and it can typically analyze samples faster than a FACScan. The cell analysis is based upon the principle that dyes emit light at a higher wavelength (lower energy) when they are excited by light at a lower wavelength (higher energy) due to dissipation of some energy to vibrational energy of the dye molecules Since the excitation wavelength and emission wavelength often vary depending on the type of dye used, some FACS instruments have multiple lasers that can excite dyes at various wavelengths, but many of the commonly-used dyes can be excited by blue light at 488 nm. FACScan is equipped with a 488 nm argon laser and can measure up to 3 different parameters determined by gating, as well as forward scatter (FSC), which determines the size of particle, and side scatter (SSC), which determines the shape of particle.

Table 15: FACScan instrument settings

Detector	Voltage	AmpGain	Mode
FSC	E00	1.00	Linear
SSC	269	1.00	Log
FL1	630	1.00	Log
FL2	405	1.00	Log
FL3	505	1.00	Log

The settings for BD FACScan were pre-adjusted and saved as a file by Ali Khademhosseini of the Langer Lab for optimal analysis of green light expressed by the R1 Oct4-GFP cell line, but these settings were checked to ensure that they were indeed optimized. The instrument settings are summarized in Table 15.

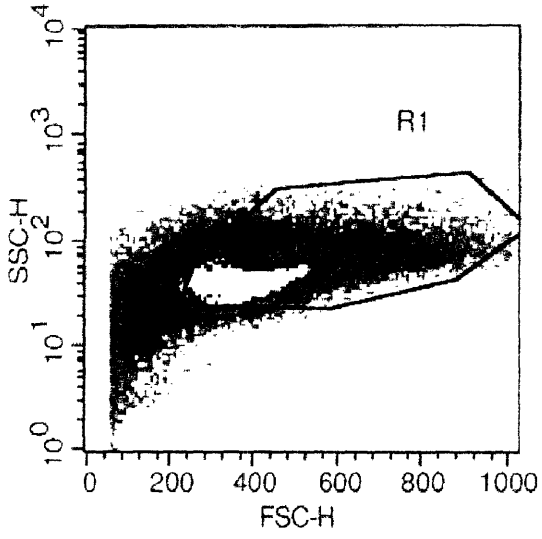


Figure 33: FSC and SSC gating

The BD FACScan instrument, located at the MIT flow cytometry facility, was connected to a Macintosh G4 computer with the software called CellQuest, which enabled data acquisition and analysis to be performed on the computer as the instrument measured the fluorescence intensity of the samples. The operational procedure as specified by the personnel at the MIT flow cytometry was used for acquisition and analysis of samples. FSC/SSC gating (see outlined area in Figure 33) was adjusted and set prior to any data acquisition to allow the greatest number of cells to be analyzed with the least number of debris. The GFP fluorescence results were plotted on dot histograms with the frequency on the linear y-axis and GFP expression level on the log x-axis.

Appendix H: Error Propagation

The uncertainty associated with any measurement can be summarized in the form of standard deviation (SD) with repeated measurements and in many cases further analyzed using the Student's t-test to determine whether the data are statistically different from each other to a defined confidence level. However, most data require some treatment and analysis before they can be presented in tables and figures, and data treatment can magnify the existing uncertainty in the pretreated data. Presented here are just three simple categories of error propagation and how the overall uncertainties for analyzed data from the experiments were derived. While simple calculations involved only one of these error propagation formulas, others such as determining the doubling time t_d (inverse of specific growth rate μ) from 2 time points involved all three to be performed simultaneously, but this procedure was greatly simplified by the use of Microsoft Excel®.

For any equation $x = f(u,v)$ where a and b are coefficients of variables u and v,

$$\sigma_x^{\,2} = \sigma_u^{\,2}\left(\frac{\partial x}{\partial u}\right)^2 + \sigma_v^{\,2}\left(\frac{\partial x}{\partial v}\right)^2$$ if one assumes u and v are uncorrelated[57].

- Category I: Addition and subtraction[57]

For $x = au \pm bv$, $\left(\frac{\partial x}{\partial u}\right) = a$ and $\left(\frac{\partial x}{\partial v}\right) = b$, so

$$\sigma_x^{\,2} = a^2\sigma_u^{\,2} + b^2\sigma_v^{\,2}, \text{ or}$$

$$\sigma_x = \sqrt{(a\sigma_u)^2 + (b\sigma_v)^2}$$

- Category II: Multiplication and division[57]

For $x = a\frac{u}{v}$, $\left(\frac{\partial x}{\partial u}\right) = \pm\frac{a}{v}$ and $\left(\frac{\partial x}{\partial v}\right) = \mp\frac{au}{v^2}$, so

$$\left(\frac{\sigma_x}{x}\right)^2 = \left(\frac{\sigma_u}{u}\right)^2 + \left(\frac{\sigma_v}{v}\right)^2, \text{ or}$$

$$\sigma_x = x\sqrt{\left(\frac{\sigma_u}{u}\right)^2 + \left(\frac{\sigma_v}{v}\right)^2}$$

- Category III: Natural logarithm[58]

  For $x = \ln(u)$, $\sigma_x \approx \frac{\sigma_u}{u}$, i.e. the error in the natural logarithm is approximately equal to the relative error of the number under the logarithm.

A more complicated error propagation calculation involving the error in doubling time t_d determined from 3 or more time points was performed using a Taylor series expansion formula for weighted linear regression, which is shown below.

For $x = f(u)$, the slope and error in slope of the weighted linear fit can be expressed as

$$Slope = \frac{\sum_{i=1}^{n} w_i \sum_{i=1}^{n} w_i u_i x_i - \sum_{i=1}^{n} w_i u_i \sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i \sum_{i=1}^{n} w_i u_i^2 - \left(\sum_{i=1}^{n} w_i u_i\right)^2}$$

and

$$\sigma_{Slope} = \sqrt{\frac{\sum_{i=1}^{n} w_i u_i^2}{\sum_{i=1}^{n} w_i \sum_{i=1}^{n} w_i u_i^2 - \left(\sum_{i=1}^{n} w_i u_i\right)^2}},$$

where $w_i = \frac{1}{\sigma_i^2}$

Appendix I: Confidence Level Calculation for Doubling Time (t_d)

Confidence level for significance of difference in weighted linear regression of paired data sets was determined using a Maple script written by Daryl St. Laurent (MIT '02). The script uses Student's t-test for paired data, which is based on the null hypothesis that makes an initial assumption that the paired data are not significantly different from each other. Based on the standard 95 % confidence level, the significance in doubling time (t_d) based on 3 or more data points was quantified. The script for determining the confidence level is given below:

```
Regress2 := proc(x1,y1,x2,y2)

  local X1, Y1, X2, Y2, n1, n2,
     SLOPE1, SLOPE2, t, p, Sx1, Sy1, Sxy1, Sx2, Sy2, Sxy2;

  X1 := Vector([flatten(x1)]); Y1 := Vector([flatten(y1)]);
  X2 := Vector([flatten(x2)]); Y2 := Vector([flatten(y2)]);

  n1 := nops([flatten(X1)]);
  n2 := nops([flatten(X2)]);

  Sx1 := sum('X1[i]^2',i = 1..n1)-(1/n1)*sum('X1[i]',i = 1..n1)^2;
  Sxy1 := sum('X1[i]*Y1[i]',i = 1..n1)-(1/n1)*sum('X1[i]',i = 1..n1)*sum('Y1[i]',i = 1..n1);
  Sy1 := sum('Y1[i]^2',i = 1..n1)-(1/n1)*sum('Y1[i]',i = 1..n1)^2;

  Sx2 := sum('X2[i]^2',i = 1..n2)-(1/n2)*sum('X2[i]',i = 1..n2)^2;
  Sxy2 := sum('X2[i]*Y2[i]',i = 1..n2)-(1/n2)*sum('X2[i]',i = 1..n2)*sum('Y2[i]',i = 1..n2);
  Sy2 := sum('Y2[i]^2',i = 1..n2)-(1/n2)*sum('Y2[i]',i = 1..n2)^2;

  SLOPE1 := Sxy1/Sx1;
  SLOPE2 := Sxy2/Sx2;

  t := abs((SLOPE1-SLOPE2)/sqrt(((Sy1-Sxy1^2/Sx1+Sy2-Sxy2^2/Sx2)/(n1+n2-
       4))*(1/Sx1+1/Sx2)));
  p := stats[statevalf,cdf,studentst[n1+n2-4]](t)-stats[statevalf,cdf,studentst[n1+n2-4]](-t);

  100*p;

  end proc:
```

References

[1] Lovell-Badge, Robin. (2001) "The future of stem cell research." *Nature.* 414: 88-91.

[2] Bradley, A. (1987) *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach.* IRL Press Limited, Oxford: 113-53.

[3] Chapter 3 of *Stem Cells: Scientific Progress and Future Research Directions.* (2001) Department of Health and Human Services. June 2001.

[4] Tada, M. et al. (1997) "Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells." *EMBO J.* 16(21): 6510-20.

[5] Wilmut, I. et al. (1997) "Viable offspring derived from fetal and adult mammalian cells." *Nature.* 385: 810-13.

[6] Appendix B of *Stem Cells: Scientific Progress and Future Research Directions.* (2001) Department of Health and Human Services. June 2001.

[7] http://www.futuresedge.org/Stem_Cells/index.html

[8] Smith, A.G. (2001) "Embryo-Derived Stem Cells: Of Mice and Men." *Annu. Rev. Cell. Dev. Biol.* 17: 435-62.

[9] Thomson, J.A. et al. (1998) "Embryonic stem cell lines derived from human blastocysts." *Science.* 282: 1145-47.

[10] Eiges, R., and Benvenisty, N. (2002) "A molecular view on pluripotent stem cells." *FEBS.* 529: 135-41.

[11] Nagy, A. et al. (1990) "Embryonic stem cells alone are able to support fetal development in the mouse." *Development.* 110: 815-21.

[12] Nagy, A. et al. (1993) "Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* 90: 8424-28.

[13] Tanaka, S. et al. (1998 "Promotion of trophoblast stem cell proliferation by FGF4." *Science.* 282: 2072-75.

[14] Thomas, K.R., and Capecchi, M.R. (1987) "Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells *Cell.* 51: 503-12.

[15] Burdon, T. et al. (1999) "Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells." *Cells Tissues Organs.* 165: 131-43.

[16] Matsuda, T. et al. (1999) "STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells." *EMBO J.* 18: 4261-69.

[17] Niwa, H. et al. (1998) "Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3." *Genes Dev.* 12: 2048-60.

[18] Mountford, P. et al. (1998) "Maintenance of pluripotential embryonic stem cells by stem cell selection." *Reprod. Fertil. Dev.* 10: 527-33.

[19] Reubinoff, B.E. et al. (2000) "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro." *Nat. Biotechnol.* 18: 399-404.

[20] Paul, G. et al. (2002) "Stem cells: hype or hope?" *DDT.* 7(5): 295-302.

[21] Odorico, J.S. et al. (2001) "Multilineage differentiation from human embryonic stem cell lines. *Stem Cells.* 19: 193-204.

[22] Palacios, R. et al. (1995) "In vitro generation of hematopoietic stem cells from an embryonic stem cell line." *Proc. Natl. Acad. Sci. USA.* 92: 7530-34.

[23] Wang, R. et al. (1992) "Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development." *Development.* 114: 303-16.

[24] Fleischmann, M. et al. (1998) "Cardiac specific expression of the green fluorescent protein during early murine embryonic development." *FEBS Lett.* 440: 370-76.

[25] Rohwedel, J. et al. (1994) "Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents." *Dev. Biol.* 164: 87-101.

[26] Tropepe, V. et al. (2001) "Direct neural fate specification from embryonic stem cells: a premature neural stem cell stage acquired through a default mechanism." *Neuron.* 30(1): 65-78.

[27] Bain, G. et al. (1995) "Embryonic stem cells express neuronal properties in vitro." *Dev. Biol.* 168: 342-57.

[28] Chapter 4 of *Stem Cells: Scientific Progress and Future Research Directions.* (2001) Department of Health and Human Services. June 2001.

[29] Holtzer, H. (1978). "Cell lineages, stem cells, and the 'quantal' cell cycle concept." *Stem cells and homeostasis.* Cambridge, New York: Cambridge University Press, 1-28.

[30] Leblond, C.P. (1964) "Classification of cell populations on the basis of their proliferative behavior." *National Cancer Institute.* 14: 119-50.

[31] Fuchs, E., and Segre, J.A. (2000) "Stem cells: a new lease on life." *Cell.* 100: 143-55.

[32] Bjornson, C.R. et al. (1999) "Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science.* 283: 534-37.

[33] Galli, R. et al. (2000) "Skeletal myogenic potential of human and mouse neural stem cells." *Nat. Neurosci.* 3: 986-91.

[34] Rietze, R.L. et al. (2001) "Purification of a pluripotent neural stem cell from the adult mouse brain." *Nature.* 412: 736-39.

[35] Toma, J.G. et al. (2001) "Isolation of multipotent adult stem cells from the dermis of mammalian skin." *Nat. Cell Biol.* 3: 778-84.

[36] Prockop, D.J. (1997) "Marrow stromal cells as stem cells for nonhematopoietic tissues." *Science.* 276: 71-734.

[37] Pittenger, M.F. et al. (1999) "Multilineage potential of adult human mesenchymal stem cells." *Science.* 284: 143-47.

[38] Reyes, M. et al. (2001) "Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood.* 98: 2613-23.

[39] Ferrari, G. et al. (1998) "Muscle regeneration by bone marrow-derived myogenic progenitors." *Science.* 279: 1528-30.

[40] Alison, M.R. et al. (2000) "Hepatocytes from non-hepatic, adult stem cells." *Nature*. 406: 257.

[41] Carmeliet, P., and Luttun, A. (2001) "The emerging role of the bone marrow-derived stem cells in (therapeutic) angiogenesis." *Thromb. Haemost.* 86: 289-97.

[42] Weissman, I.L. (2000) "Stem cells: units of development, units of regeneration, and units in evolution." *Cell.* 100: 157-68.

[43] Merok, J., and Sherley, J.L. (2001) "Breaching the kinetic barrier to in vitro somatic stem cell propagation." *J. Biomed. Biotech.* 1(1): 25-27.

[44] Sherley, J.L. (2002) "Asymmetric Cell Kinetics Genes: The Key to Expansion of Adult Stem Cells in Culture." *Stem Cells.* 20: 561-72.

[45] Viswanathan, S. et al. (2003) "Supplementation-Dependent Differences in Rates of Embryonic Stem Cell Self-Renewal , Differentiation, and Apoptosis." *Biotechnol. Bioeng.* 84(5): 505-517.

[46] Nichols, J. et al. (1998) "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4." *Cell.* 95: 379-91.

[47] Niwa, H. et al. (2000) "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells." *Nat. Genet.* 24: 372-76.

[48] Nichols, J. (2001) "Introducing embryonic stem cells." *Curr. Biol.* 11(13): R503-5.

[49] Zandstra, P.W. and Nagy, A. (2001) "Stem Cell Bioengineering." *Annu. Rev. Biomed. Eng.* 3: 275-305.

[50] Ozturk, S.S. and Palsson, B.O. (1991) "Growth, Metabolic, and Antibody Production Kinetics of Hybridoma Cell Culture: 2. Effects of Serum Concentration, Dissolved Oxygen Concentration, and Medium pH in a Batch Bioreactor." *Biotechnol. Prog.* 7:481-494.

[51] Eigenbrodt, E. et al. (1985) "New Perspectives on Carbohydrate Metabolism in Tumor Cells." *Regulation of Carbohydrate Metabolism.* Ed. R. Beitner. Boca Raton, FL: CRC Press, 1985. Vol. II, 141-79.

[52] Wilhelm, G. et al. (1971) "pH-Dependence of Aerobic Glycolysis in Ehrlich Ascites Tumour Cells." *FEBS Letters.* 17: 158-62.

[53] Cruz, H.J. et al. (1999) "Metabolic responses to different glucose and glutamine levels in baby hamster kidney cell culture." *Appl. Microbiol. Biotechnol.* 51: 579-585.

[54] Viswanathan, S. et al. (2003) "Supplementation-Dependent Differences in the Rates of Embryonic Stem Cell Self-Renewal, Differentiation, and Apoptosis." *Biotechnol. Bioeng.* 84(5): 505-17.

[55] Zandstra, P.W. et al. (2000) "LIF Concentration Modulates Embryonic Stem Cell Self-Renewal and Differentiation Independently of Proliferation." *Biotechnol. Bioeng.* 69(6): 607-17.

[56] Miller, W.M. et al. (1988) "Kinetic Analysis of Hybridoma Growth and Metabolism in Batch and Continuous Culture: Effect of Nutrient Concentrations, Dilution Rate, and pH." *Biotechnol. Bioeng.* 32: 947-65.

[57] http://www.mathworld.wolfram.com/ErrorPropagation.html.

[58] http://www.vims.edu/~david/MS505/property.pdf.