MITOCHONDRIAL AND APOPTOTIC DYNAMICS IN UNDIFFERENTIATED AND
DIFFERENTIATING PLURIPOTENT STEM CELLS

A dissertation presented by

Judith A. Newmark

to
The Department of Biology

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

in the field of
Biology

Northeastern University
Boston, Massachusetts
October 2009
Abstract

Embryonic stem (ES) cells are pluripotent and therefore they have the capacity to form any tissue type in the body. ES cells are also capable of unlimited self-renewal. Mouse ES cells are isolated and grown from blastocyst stage preimplantation embryos on a Primary Mouse Embryonic Fibroblast (PMEF) feeder layer of cells in the presence of Leukemia Inhibitory Factor (LIF). Pluripotent stem cells may also be derived from adult cells by using retroviral vectors to introduce the appropriate pluripotency genes. The resulting cells are termed induced pluripotent stem (iPS) cells, and they are thought to be equivalent to their ES cell counterparts. The potential for stem cell therapy using pluripotent stem cells relies on the ability to selectively direct the growth of particular types of cells. Pluripotent stem cells provide a unique model system for studying early development and tissue differentiation, creating new disease models, and identifying novel drug targets for therapeutic application.

Mitochondria are responsible for generating the energy for the cell, and they are also master regulators of apoptosis (programmed cell death). They have a mitochondrial membrane potential associated with them which reflects their level of activity. Active mitochondria localize to regions which require larger amounts of energy. Loss of mitochondrial membrane potential in cells is indicative of apoptosis. The published literature pertaining to mitochondria and apoptosis in pluripotent and differentiating stem cells is scarce and often conflicting. The overall goal of this research was to study the role of mitochondrial activity and apoptosis in pluripotent stem cell growth and differentiation. To accomplish this goal we defined four specific aims: 1) create and characterize an ES cell line from mtGFP-tg mice that have their mitochondria...
endogenously labeled with GFP, and then use the mtGFP-tg ES cell line to evaluate mitochondrial localization; 2) compare cell division, pluripotency markers, and differentiation markers in undifferentiated and differentiating mouse pluripotent stem cells by comparing the mtGFP-tg ES cell line, a conventional C57BL/6 ES cell line, and an iPS cell line; 3) analyze mitochondrial activity and localization in undifferentiated and differentiating mouse pluripotent stem cells by comparing the mtGFP-tg ES cell line, a conventional C57BL/6 ES cell line, and an iPS cell line; 4) analyze early, intermediate, and late stage apoptosis markers in undifferentiated and differentiating mouse pluripotent stem cells by comparing a conventional C57BL/6 ES cell line and an iPS cell line.

We successfully created an ES cell line from mtGFP-tg mice. The mtGFP-tg ES cells had a normal karyotype, and they demonstrated characteristics of pluripotency and differentiation capabilities comparable to conventional ES cells. Their mitochondrial GFP fluorescence was extremely bright and stable, allowing us to image, for the first time, mitochondria in undifferentiated and differentiating cells without the use of externally applied mitochondrial stains. Our imaging data represent the first 3D sectioning through an EB, made possible by using endogenous mitochondrial fluorescence.

Using our mtGFP-tg ES cell line in conjunction with a C57BL/6 ES and an iPS cell line, we confirmed that all three pluripotent stem cell lines had high levels of pluripotency markers in their undifferentiated state. Upon differentiation, the pluripotency markers decreased whereas markers for the three developing germ layers increased throughout differentiation. These data confirm the known characteristics of robust pluripotent stem cells.
We analyzed mitochondrial activity in all three pluripotent stem cell lines by using JC-1 and TMRE staining. We found that pluripotent stem cells had high levels of mitochondrial activity in their undifferentiated state. Mitochondrial activity was confined to the perimeters of undifferentiated pluripotent stem cell colonies, an observation which has not been published previously. Upon differentiation, mitochondrial activity significantly decreased. Early, intermediate, and late apoptosis were assessed in C57BL/6 ES and iPS cells. The two pluripotent stem cell lines showed low levels of apoptosis in their undifferentiated state. Upon differentiation, apoptosis significantly increased. These data represent the first full analysis of mitochondrial activity and apoptosis throughout the differentiation process. Taken together, these results support a role for mitochondrial activity and apoptosis in pluripotent stem cell growth and differentiation. In particular, high mitochondrial activity is associated with pluripotency whereas apoptosis is associated with loss of pluripotency.

To the best of our knowledge, these data provide the first analyses of mitochondrial activity and apoptosis in iPS cells. iPS cells were equivalent to conventional C57BL/6 ES cells in terms of pluripotency, mitochondrial activity, and apoptosis in their undifferentiated state. However, upon differentiation or long-term culture we found several significant differences in pluripotency, mitochondrial activity, and apoptosis thus bringing into question the complete equivalency of iPS cells to conventional ES cells.

All together, we conclude from our analyses that there are significant differences in pluripotency, mitochondrial activity, and apoptosis during differentiation of pluripotent stem cells and that there are several significant differences among the mtGFP-tg,
C57BL/6, and iPS pluripotent stem cell lines. These data increase our knowledge of the pluripotency and differentiation capabilities of pluripotent stem cells, which may impact the development of stem cell therapy for the treatment of human diseases.
Dedication

I would like to dedicate this dissertation to my daughter, Kira, who has inspired me to give my best as a wife, mother, and a scientist. Thank you baby goose!
Acknowledgements

I would like to thank Dr. Carol Warner for being such a wonderful and supportive research advisor and mentor. I am very grateful for her encouragement and support through my many happy years in her laboratory.

Additionally, the many alumni from the Warner Lab were pivotal to my successes. I am thankful for having met such wonderful friends. In particular I would like to thank Mike Byrne, Bob Crooker, Martina Comiskey, Sally DeFazio, Matt Devine, Carmit Goldstein, Paula Lampton, and Michele Mammolenti.

Thanks go to the many people involved in Gordon-CenSSIS, the Bernard M. Gordon Center for Subsurface Sensing and Imaging Systems, and particularly the laboratory of Charles A. DiMarzio. It was a pleasure to work with so many wonderful engineers who were also interested in Biology.

I would also like to thank my committee members for their time and efforts towards my research. Thank you to Dr. Erin Cram, Dr. Max Diem, Dr. Ann Kiessling, and Dr. Badrinath Roysam.

Finally, I would like to thank my family and friends for their support during this long process. They were always there for me when I needed a little extra push to keep going. This dissertation would not be possible without the support of all of these people.
# Table of Contents

Abstract..............................................................................................................4  
Dedication..........................................................................................................8  
Acknowledgements...........................................................................................9  
Table of Contents.............................................................................................10  
List of Abbreviations........................................................................................13  
List of Figures..................................................................................................15  
List of Tables....................................................................................................20  
List of Appendices............................................................................................21  
Introduction......................................................................................................22  
  Embryonic Stem Cells....................................................................................22  
  Induced Pluripotent Stem Cells....................................................................28  
  Differentiation of Stem Cells.......................................................................30  
  Long-term Stem Cell Culture.........................................................................33  
  Mitochondria..................................................................................................33  
  Apoptosis........................................................................................................38  
  Specific Aims....................................................................................................41  
Materials and Methods...................................................................................65  
  Cell Culture.....................................................................................................65  
  mtGFP-tg Embryonic Stem Cells..................................................................66  
  Validation of Pluripotent Stem Cell Lines.....................................................68  
  Mitochondrial Assays....................................................................................71  
  Apoptosis Assays............................................................................................72
References..................................................................................................................185

Appendix......................................................................................................................201

  Appendix 1: Neviparine Treatment of Embryonic Stem Cells.........................201

  Appendix 2: Multicolor Imaging of Embryonic Stem Cells.........................207

  Appendix 3: Multimodal Imaging of Embryonic Stem Cells.......................212
List of Abbreviations

- AFP-Alpha Fetoprotein
- ALS-Amyotrophic Lateral Sclerosis
- Anova-Analysis of Variance
- AP-alkaline phosphatase
- Bry-Brachyury
- BSA-Bovine Serum Albumin
- Caspase-Cysteine-Aspartic Acid Protease
- CCCP-carbonyl cyanide m-chlorophenylhydrazone
- CLSM-Confocal Laser Scanning Microscopy
- DIC-Differential Interference Contrast
- DMEM-Dulbecco’s Modified Eagle’s Medium
- EB-Embryoid Body
- ER-Endoplasmic Reticulum
- ES Cell-Embryonic Stem Cell
- FACS-Fluorescence Activated Cell Sorting
- FACScan-Fluorescence Activated Cell Scanning
- FADH2-Flavin Adenine Dinucleotide plus Hydrogen
- FBS-Fetal Bovine Serum
- Flk1-Fetal Liver Kinase 1
- FSC-Forward Scatter
- GAPDH-Glyceraldehyde 3-phosphate dehydrogenase
- GFP-Green Fluorescent Protein
- hES Cell-Human Embryonic Stem Cell
- IAP-Inhibitor of Apoptosis
- ICM-Inner Cell Mass
- iPS Cell-induced Pluripotent Stem Cell
- JC-1-5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
- Klf4-Kruppel-like Factor 4
- KSOM- Potassium Simplex Optimized Medium
- LIF-Leukemia Inhibitory Factor
- mES Cell-Mouse Embryonic Stem Cell
- mtGFP-tg-mitochondrial GFP transgenic
- MTT-3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
- N.A.-Not Applicable
- NADH-Nicotinamide Adenine Dinucleotide plus Hydrogen
- NEAA-Non-Essential Amino Acids
- N.S.-Not Significant
- Oct-4-Octomer 4, POU class 5 homeobox 1
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>OQM</td>
<td>Optical Quadrature Microscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBSAZ</td>
<td>Phosphate Buffered Saline with BSA and Sodium Azide</td>
</tr>
<tr>
<td>P#</td>
<td>Passage Number</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pEB</td>
<td>plated Embryoid Body</td>
</tr>
<tr>
<td>PGD</td>
<td>Preimplantation Genetic Diagnosis</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMEF</td>
<td>Primary Mouse Embryonic Fibroblasts</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
</tr>
<tr>
<td>Smac/Diablo</td>
<td>Second mitochondrial-derived activator of caspase/direct inhibitor of apoptosis protein-binding protein with low pI</td>
</tr>
<tr>
<td>Sox2</td>
<td>(Sex Determining Region Y)-box2</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>SSEA1</td>
<td>Stage Specific Embryonic Antigen-1</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TMRE</td>
<td>tetramethylrhodamine, ethyl ester, perchlorate</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethyl Rhodamine Methyl Ester</td>
</tr>
<tr>
<td>TPLSM</td>
<td>Two-Photon Laser Scanning Microscopy</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
</tbody>
</table>
List of Figures

Introduction

Figure 1: Preimplantation Mouse Embryo Development and Stem Cells..........43
Figure 2: Isolation of Mouse Embryonic Stem Cells.................................44
Figure 3: Primary Mouse Embryonic Fibroblasts and Embryonic Stem Cells.....45

Movie 1: Timelapse DIC Imaging of Mouse Embryonic Stem Cell

Growth.................................................................46

Movie 2: Timelapse TPLSM Imaging of Mouse Embryonic Stem Cell

Growth.................................................................47

Figure 4: Pluripotency Markers and a Teratoma........................................48

Movie 3: Beating Cells Grown from Mouse Embryonic Stem Cells..........49

Figure 5: Embryonic Stem Cell Therapy..................................................50
Figure 6: Methods for Deriving Pluripotent Stem Cells-1...........................51
Figure 7: Methods for Deriving Pluripotent Stem Cells-2...........................52
Figure 8: Deriving Induced Pluripotent Stem Cells....................................53
Figure 9: Mouse Embryoid Body Formation.............................................54

Movie 4: Timelapse DIC Imaging of Mouse iPS Cell Growth......................55

Figure 10: Phase Contrast Images of Plated Mouse Embryoid Bodies..........56
Figure 11: Directed Differentiation.......................................................57
Figure 12: Anatomy of a Mitochondrion..................................................58
Figure 13: Mitochondria in Stem Cells...................................................59
Figure 14: Apoptosis.........................................................................60
Figure 15: Apoptosis Signal Cascade.....................................................61
Figure 16: Apoptosis in Stem Cells.................................................................62

Materials and Methods

Figure 17: Relevant Markers of Apoptosis..................................................82
Figure 18: Flow Cytometry Instrumentation..............................................83
Figure 19: Example of a Flow Cytometry Histogram.................................84
Figure 20: Example of a Flow Cytometry Quadrant Analysis.........................85
Figure 21: The Keck 3D Fusion Microscope...............................................86
Figure 22: Optical Quadrature Microscopy...............................................87

Results

Figure 23: mtGFP-tg Mouse Tissues.............................................................116
Figure 24: Real-Time PCR Amplification of the GFP Transgene from the
  mtGFP-tg Mouse Colony.................................................................117
Figure 25: Ear Punch Phenotyping of mtGFP-tg Mice.................................118
Figure 26: Creation of Mouse mtGFP-tg Embryonic Stem Cells..................119
Figure 27: Mycoplasma Testing of mtGFP-tg ES Cells.................................120
Figure 28: Validation of Mouse mtGFP-tg Embryonic Stem Cells...............121
Figure 29: Differentiation of Mouse mtGFP-tg Embryonic Stem Cells..........122
  Movie 5: Beating Mouse mtGFP-tg Cells..............................................123
Figure 30: GFP Expression in Mouse mtGFP-tg Embryonic Stem Cells, an
  Embryoid Body, and a Plated Embryoid Body.....................................124
  Movie 6: Timelapse TPLSM of Mouse mtGFP-tg Cells Growth..............125
Figure 31: GFP Expression from Mouse Embryonic Stem Cells through 14 Days
  of Development..............................................................................126
Figure 32: GFP Imaging in Mouse mtGFP-tg Embryonic Stem Cells…………127

**Movie 7**: CLSM Stack of Mouse mtGFP-tg ES Cells…………………128

Figure 33: GFP Imaging in Mouse mtGFP-tg D7 Embryoid Bodies………………129

**Movie 8a**: CLSM Stack of a Mouse mtGFP-tg EB…………………………130

**Movie 8b**: TPLSM Stack of a Mouse mtGFP-tg EB…………………………130

Figure 34: mtGFP-tg Imaging in Mouse mtGFP-tg pD7 Plated EBs…………………131

Figure 35: MTT Assay on Mouse Pluripotent Stem Cell and Embryoid Body

Growth……………………………………………………………………132

Figure 36: Alkaline Phosphatase Activity in Mouse Pluripotent Stem Cells,

Embryoid Bodies, and Plated Embryoid Bodies…………………………….133

Figure 37: Alkaline Phosphatase Activity from Mouse Pluripotent Stem Cells

through 14 Days of Development………………………………………134

Figure 38: Oct-4 Expression from Mouse Pluripotent Stem Cells through 14 Days

of Development……………………………………………………………135

Figure 39: Germ Layer Expression from Mouse Pluripotent Stem Cells through

14 Days of Development………………………………………………….136

Figure 40: Mitochondrial GFP and Germ Layer Expression in Plated Embryoid

Bodies……………………………………………………………………….137

Figure 41: JC-1 in Mouse Pluripotent Stem Cells……………………………..138

Figure 42: JC-1 Imaging of Mouse Pluripotent Stem Cells…………………..139

**Movie 9**: Epifluorescence Stack of JC-1 and Hoechst Stained C57BL/6

Mouse ES Cells……………………………………………………………140

**Movie 10a**: TPLSM Stack of JC-1 Stained C57BL/6 Mouse ES Cells..141
Movie 10b: CLSM Stack of JC-1 Stained C57BL/6 Mouse ES Cells…141

Figure 43: TMRE in Mouse Pluripotent Stem Cells..............................142

Figure 44: GFP Expression versus TMRE Staining in Mouse mtGFP-tg Embryonic Stem Cells.................................143

Figure 45: JC-1 Staining of Mouse Pluripotent Stem Cells through 14 Days of Development..................................................144

Figure 46: JC-1 Imaging of Plated Mouse Embryoid Bodies.....................145

Figure 47: TMRE Staining of Mouse Pluripotent Stem Cells through 14 Days of Development..................................................146

Figure 48: Annexin V Staining in Mouse Pluripotent Stem Cells...............147

Figure 49: Caspase 3 Expression in Mouse Pluripotent Stem Cells ..........148

Figure 50: Annexin V and PI Staining of Mouse Pluripotent Stem Cells through 14 Days of Development..................................................149

Figure 51: Caspase 3 Expression from Mouse Pluripotent Stem Cells through 14 Days of Development..................................................150

Figure 52: TUNEL Assay from Mouse Pluripotent Stem Cells, Embryoid Bodies, and Plated Embryoid Bodies.................................151

Figure 53: Caspase 3 Imaging of Mouse Pluripotent Stem Cells and Plated Embryoid Bodies..................................................152

Figure 54: Phase Contrast Imaging of P100 Mouse Pluripotent Stem Cells......153

Figure 55: Alkaline Phosphatase Activity in Early versus Late Passage Mouse Pluripotent Stem Cells..................................................154

Figure 56: Oct4 Expression in Early versus Late Passage Mouse Pluripotent
Stem Cells........................................................................................................155

Figure 57: GFP Expression through 100 Passages of Mouse Pluripotent Stem
Cells..............................................................................................................156

Figure 58: JC-1 Staining through 100 Passages of Mouse Pluripotent Stem Cells
......................................................................................................................157

Figure 59: TMRE Staining through 100 Passages of Mouse Pluripotent Stem
Cells..............................................................................................................158

Figure 60: Annexin V and PI Staining through 100 Passages of Mouse Pluripotent
Stem Cells....................................................................................................159

Figure 61: Caspase 3 Expression through 100 Passages of Mouse Pluripotent
Stem Cells....................................................................................................160

Appendices

Figure 62A: Control Mouse Embryoid Bodies..............................................202
Figure 62B: Control Mouse Embryoid Bodies..............................................203
Figure 63A: Neviparine Treated Mouse Embryoid Bodies.........................204
Figure 63B: Neviparine Treated Mouse Embryoid Bodies.........................205
Figure 64: Multicolor Images of Mouse Oocytes.......................................209
Figure 65: Multicolor Images of Mouse 2-Cell Embryos..............................210
Figure 66: Multicolor Images of a Fibroblast and Mouse ES Cells.........211
Figure 67: Multimodal Imaging of Mouse Embryonic Stem Cells-1.........214
Figure 68: Multimodal Imaging of Mouse Embryonic Stem Cells-2.........215
Figure 69: OQM Imaging of Mouse ES Cells.............................................216
List of Tables

Introduction

Table I: Significant Pluripotent Stem Cell Developments..........................63
Table II: Induced Pluripotent versus Conventional Embryonic Stem Cell Lines
..................................................................................................................64

Materials and Methods

Table III: Pluripotent Stem Cell Lines.......................................................88
Table IV: Assays and Markers.................................................................89
Table V: Imaging and Detection Modalities..............................................90
Table VI: Statistical Analyses.................................................................91

Results

Table VII: Statistics on all Three Undifferentiated Mouse Pluripotent Stem Cell
Lines........................................................................................................161
Table VIII: Statistics on all Three Differentiating Mouse Pluripotent Stem Cell
Lines........................................................................................................162
Table IX: Statistics on Differentiating mtGFP-tg vs. C57BL/6 ES Cell Lines...163
Table X: Statistics on Differentiating mtGFP-tg ES vs. iPS Cell Lines.........164
Table XI: Statistics on Differentiating C57BL/6 ES vs. iPS Cell Lines.........165
Table XII: Statistics on Long-term Culture of Mouse Pluripotent Stem Cells...166

Appendices

Table XIII: Area and Number of Cells/Region in Control versus Neviparine
Treated Mouse EBs.................................................................................206
List of Appendices

Appendix 1: Neviparine Treatment of Embryonic Stem Cells.................................201

Appendix 2: Multicolor Imaging of Embryonic Stem Cells.................................207

Appendix 3: Multimodal Imaging of Embryonic Stem Cells.................................212
INTRODUCTION

Embryonic Stem Cells

Embryonic stem (ES) cells have two characteristics that make them unique: pluripotency and unlimited self-renewal. If they are maintained properly they can replicate indefinitely, and when the factors that maintain pluripotency are removed the cells will naturally form tissues from all three germ layers. Mouse ES cells were first derived in the laboratory in 1981 (Evans and Kaufman, 1981), and it took nearly 20 years for the research to mature enough to allow the first human ES cell line to be derived (Thomson et al., 1998). Since then the ES cell field has skyrocketed, and researchers produce thousands of new ES cell papers every year.

ES cells are derived from preimplantation embryos, which occur in the earliest phases of development. Figure 1A shows the typical preimplantation embryo development pathway for mice. An oocyte and sperm join to produce a fertilized 1-cell embryo (zygote). The embryo then undergoes a series of cleavage divisions to produce the later stage preimplantation embryos. Throughout the preimplantation period of embryonic development, the embryos are free-floating within the reproductive tract. They are surrounded by a protective zona pellucida (glycoprotein) shell. Around day 3 of development, embryos undergo compaction to a morula (~16-cell) stage. The cells lose their boundaries and begin to change. On the fourth day, the embryo forms a fluid filled cavity (blastocoel) within the embryo. The blastocoel forces some cells to the outside of the embryo (trophectoderm, TE) and leaves a clump of cells within and to the side of the embryo (inner cell mass, ICM). During normal development, the blastocyst will expand until it undergoes hatching, whereby the blastocyst forces its way out of the zona
pellucida. It will then implant into the uterus and continue through the fetal development phase, as shown in Figure 1B. During normal fetal development, the outer trophectodermal cells become the placenta, and the ICM cells become the embryo proper (fetus). It is possible to harvest fetal stem cells from a fetus, which are potentially useful for stem cell therapy despite having a more limited pluripotency than ES cells. Once a fetus is born and grows into a mature adult, specific tissues may be harvested to create an adult stem cell line. Adult stem cells are notoriously difficult to maintain in culture, due to having limited self-renewal, and they have limited pluripotency. Alternatively, a blastocyst may be placed into tissue culture in order to harvest ES cells that have the capability of producing tissues from all three germ layers: endoderm, mesoderm, and ectoderm.

In order to derive embryonic stem cells, the ICM is harvested from the blastocyst stage embryo. Figure 2 shows a simplified diagram of ES cell derivation. There are many potential versions of this technique, but in the simplest version blastocysts are collected and then plated onto primary mouse embryonic fibroblast (PMEF) feeder layers in a medium containing leukemia inhibitory factor (LIF). The blastocysts will hatch in the dish and attach to the feeder layer. Over the next few days, the TE cells will fall away and leave the isolated ICM to grow. Upon passaging of the ICM onto fresh feeder layers, the individual cells will grow into ES cell colonies that can be expanded and continued in culture indefinitely.

ES cell lines have now been derived from a variety of species including mouse, several non-human primate species, and humans. Table I lists several of the significant pluripotent stem cell developments over the years, many of which will be discussed in
Through the years of research on mouse ES (mES) and human ES (hES) cells, many similarities have been discovered. However, there have also been a few noticeable differences. Morphologically, mES grow in rounded colonies with indistinct cell boundaries whereas hES grow in flattened monolayers with more distinct cell boundaries (Verfaillie et al., 2002). Additionally, hES have much slower generation times compared to mES (Ohtsuka and Dalton, 2008). In terms of SSEA pluripotency markers, hES express SSEA3 and SSEA4 and then begin to express SSEA1 upon differentiation, whereas mES express SSEA1 in the pluripotent state (Draper and Fox, 2003). A wonderful review by Koestenbauer et al. (2006) discusses the differences in several pluripotency markers in detail. Another important difference between mouse and human ES cells is in the LIF requirement. Mouse ES cells require LIF to maintain pluripotency whereas hES are not dependent on LIF. Instead hES are grown with basic fibroblast growth factor (bFGF) or FGF-2 (Amit et al., 2000).

Maintenance of ES cells traditionally requires the cells to remain on a PMEF feeder layer. This layer is typically inactivated by mitomycin c or irradiation. Additionally, the ES cells require LIF (for mouse) or bFGF (for human) and serum. New formulations have led to serum free and feeder layer free culture systems, including the advent of a fully chemically defined medium (Ludwig et al., 2006). Figure 3 shows sample images by Phase Contrast and DIC microscopy of PMEF feeder layers and mouse C57BL/6 ES on feeder layers. Fibroblasts spread out on the bottom of the dish to form a lawn of cells upon which the ES cell colonies grow. Mouse ES cells form distinguished colonies on the surface of the fibroblasts (Figure 3), whereas human ES cell colonies are more flattened with indistinct boundaries. ES cells grow quickly and therefore they must
be passaged every couple of days to fresh feeder layers. Movie 1 shows C57BL/6 ES cell growth as imaged by timelapse DIC Microscopy. The cells are very active and merge in and out of ES cell colonies during growth. Movie 2 shows C57BL/6 ES cells, containing Hoechst stained nuclei, as imaged by timelapse Two Photon Laser Scanning Microscopy (TPLSM). In Movie 2, the cells move and divide as the colony grows.

ES cells are identified by several hallmarks of pluripotency. There are a series of pluripotency markers associated with stemness. Examples include SSEA1 (mouse ES), SSEA 3 and 4 (human ES), high telomerase activity, alkaline phosphatase expression, and the transcription factors Oct-4, Nanog, and Stat 3. Figure 4A shows a model ES cell with some of these important pluripotency markers on the cell surface and in the cell nucleus. Markers such as SSEA1, SSEA4, and TRA 1-60 are cell surface markers that can be easily labeled with antibodies for detection. Some other markers in Figure 4A, such as Oct-3/4 and Nanog, are transcription factors that reside in the cell nucleus. Another hallmark of ES cells is their ability to differentiate into tissues representing all three germ layers: endoderm, mesoderm, and ectoderm. One method of testing this is to differentiate the ES cells in vitro and identify developing cells from all three germ layers. Features such as beating cells, which represent cardiomyocyte differentiation, may often be identified. Movie 3 shows an example of Phase Contrast Microscopy of beating cells, in real time, formed from the in vitro differentiation of C57BL/6 ES cells. One in vivo mechanism for testing the pluripotency of ES cells is to inject them into nude mice and allow them to form a teratoma, a cancerous mass containing tissues from multiple lineages. An example of a teratoma is shown in Figure 4B. This teratoma is a mature cystic teratoma containing structures from all three germ layers. Hair is visible on the
right side of the teratoma. A final method for ensuring the pluripotency of ES cells is to inject them into mouse blastocysts to form chimeras. If the ES cells are capable of developing into a full grown mouse with ES cell representation in the germ line, the ES cell line is considered pluripotent.

Pluripotency is the key to the potential of stem cell therapy. As is suggested in Figure 5, ES cells provide a unique tool for assaying for new drug targets or testing toxicity, studying development and cell differentiation, understanding birth defects, modeling diseases, and even for creating tissues for stem cell transplantation therapy. Stem cells provide a good model system for identifying novel drug targets and testing drug toxicity because, unlike most conventional cell lines derived from cancerous cells, stem cells are naturally self-renewing. ES cell lines created from abnormal embryos model birth defects and provide an unlimited source of cells for performing research towards the understanding and treatment of these birth defects. Finally, much can be learned about the mechanisms of differentiation by studying stem cells as they differentiate into various tissues. The question-mark in Figure 5 reflects that ongoing research may lead to as yet undiscovered uses for stem cells. Research has produced a vast amount of data towards properly differentiating and using stem cells to treat diseases such as diabetes, spinal cord injury, and cardiac malfunctions (Gaetani et al., 2009; Guo and Hebrok, 2009; Kim and de Vellis, 2009). The first clinical trial using hES cells, for spinal cord injury, was approved by the FDA this year (http://www.geron.com/media/pressview.aspx?id=1148), although it is currently on hold pending further review by the FDA. Additionally, researchers have hypothesized the role of a cancer stem cell in several types of cancer (Rosen and Jordan, 2009), and research
towards understanding the link between stem cells and cancer is leading to the development of new treatment methodologies.

Ongoing ethical debate concerning the use of embryos in order to derive human ES cells has led to the derivation of pluripotent stem cells using alternative methods. Figures 6 and 7 diagram several of these alternative methods. Traditional derivation of ES cells (method A in Figure 6) has been described previously. Embryonic germ cell embryos (method B in Figure 6) are possible in animal models, but their pluripotency and self-renewal may be somewhat limited. Methods such as “dead” embryos (method C in Figure 6) and altered nuclear transfer embryos (method I in Figure 7) avoid the ethical implications of depriving a potential being of life, but the resulting cell lines may be impaired due to a deficiency in the embryo. Parthenogenetic ES cells (method F in Figure 6) also avoid depriving a potential being of life, as parthenotes are unable to naturally develop to term, but it is extremely difficult to find a readily available source of oocytes. ES cells produced from genetically abnormal embryos (method D in Figure 6) and fusion of skin cells with ES cells (method J in Figure 6) produce abnormal ES cells that are useful for research but are not currently viable for therapy. ES cells from a single-cell biopsy of an embryo (method E in Figure 6) are controversial as it is unknown whether the surviving embryo may sustain any lasting effects from the biopsy procedure. Methods G and H in Figure 7 both are forms of cloning, and they are currently banned from human embryo research in many parts of the world. In the USA they are ineligible for federal funding. Currently, the most favored alternative method is induced pluripotent stem (iPS) cells (method K in Figure 7), which do not require oocytes or
involve the destruction of embryos. This method will be described in detail in the following section.

**Induced Pluripotent Stem Cells**

The first derivation of iPS cells came from the laboratory of Shinya Yamanaka in 2006 and used just four factors (Oct3/4, Sox2, c-Myc, and Klf4) to reprogram mouse fibroblasts to a pluripotent state (Takahashi and Yamanaka, 2006). These results were quickly confirmed by another group (Wernig et al., 2007). Yamanaka swiftly shifted his research towards replicating this work in the human system, and he and another group accomplished this goal within a year (Takahashi et al., 2007; Yu et al., 2007). The Thomson group was able to substitute Nanog and Lin28 for Klf4 and c-Myc. While the research seemed very robust, the recent publications announcing that adult mice could be generated from iPS cells solidified the pluripotent capabilities of iPS cells (Boland et al., 2009; Zhao et al., 2009). The methods involved in deriving iPS cells are outlined in Figure 8, and Maherali and Hochedlinger (2008) have published an outstanding review of the techniques. In Figure 8 adult cells, such as skin fibroblasts, are treated with retroviruses carrying the four transcription factors. After a lengthy culture, cells expressing pluripotency markers and exhibiting self-renewal are selected for further testing. Cell lines which pass these tests are considered good iPS cell lines. As shown in Figure 8, iPS cells can undergo genetic repair to fix a genetic abnormality before differentiation, or they may be directly differentiated. These differentiated cells may be used in screening for novel drug targets or they may be used for transplantation therapy.
Since the early derivations of iPS cells there have been many advances. Mouse iPS cells have been used to treat sickle cell anemia in mice (Hanna et al., 2007), and an iPS model for ALS was created using human cells (Dimos et al., 2008). One of the potential problems with iPS cells is that the inclusion of c-Myc leads to high rates of tumor formation. Two groups found that although c-Myc enhanced iPS derivation success, it was not required and therefore could be eliminated from the reprogramming cocktail (Nakagawa et al., 2008; Wernig et al., 2008b). Additionally, other factors may be eliminated from the reprogramming mix by using an adult cell that inherently possesses high levels of one or more of the required transcription factors (Kim et al., 2008). One other notable concern with the creation of iPS cells is in the use of retroviruses to transmit the transcription factors. Several types of systems have been created to either change the type of virus used or to eliminate viral vectors altogether (Huangfu et al., 2008; Okita et al., 2008; Stadtfeld et al., 2008; Wernig et al., 2008a; Kaji et al., 2009; Kim et al., 2009; Woltjen et al., 2009). One final barrier to the successful use of iPS cells has been the very low reprogramming efficiency. Several groups recently reported that suppression of the p53 pathway was able to significantly increase reprogramming efficiency (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). iPS research has been moving forward extremely quickly, and it has caused such a shift in the pluripotent stem cell field that Shinya Yamanaka was awarded the Albert Lasker Basic Medical Research Award in 2009. Winners of this award frequently also win the Nobel Prize for Medicine.

The majority of evidence thus far supports the claim that iPS cells are fully equivalent to conventional ES cells. Table II compares the two types of pluripotent stem
cell lines, and shows that there are a few notable differences. The different source and derivation protocols for each have already been discussed. One notable difference is in the efficiency of derivation. iPS cells still lag far behind conventional ES cells in efficiency (0.01-0.1% efficiency for iPS cells as compared to 22-75% efficiency for ES cells), however the source material for iPS cells is more plentiful. iPS and conventional ES cells have similar culture conditions, morphology, proliferation, and pluripotency. However, another notable difference is in gene expression. An analysis by Chin et al. (2009) showed that thousands of genes had different expression levels in iPS cells compared to conventional ES cells. Many of these genes were pluripotency genes that were insufficiently activated and genes from the originating adult cell (fibroblast) that were insufficiently suppressed. One final notable difference is that iPS cells can easily be created to match a patient, whereas with conventional ES cells the source embryo is destroyed. Patient matched ES cells would need to be created using a technique such as somatic cell nuclear transfer or fusion of a patient’s cell with an already established ES cell line.

**Differentiation**

The potential of pluripotent stem cells is in their ability to differentiate into a variety of different tissue types. In the laboratory, pluripotent cells are differentiated through a two stage process. In the first stage Embryoid Bodies (EBs) are created. Figure 9 diagrams EB differentiation and shows example images of growing EBs. Although there are two parallel methods for forming EBs, the common theme is that pluripotent cells are removed from the PMEF feeder layer and transferred to a culture
medium without LIF (or bFGF). In the first method, pluripotent stem cells are counted upon removal from the feeder layer and placed into hanging drops of medium, without LIF, with an equal number of cells per drop (Figure 9A, Forming EBs by hanging drop). This method produces EBs with consistent size and shape. Once the EBs form they are transferred to a new plate to continue their growth. The simplest method of forming EBs is to remove the cells from the feeder layer and place them in a culture dish containing medium without LIF (Figure 9A, EBs in suspension). This method produces EBs with a variety of sizes and shapes. Movie 4 shows iPS cells forming EBs as imaged by timelapse DIC Microscopy. The movie begins with small clumps of cells with distinct boundaries. The cells compact into a ball of cells with indistinct boundaries, although the EB itself has a distinct boundary. The EB then grows in size.

In the second stage of differentiation, EBs are plated onto gelatin coated plates (Figure 9A, Plated EB colonies on gelatin) for continued growth. Before plating, EBs seem to be in a suspended state of development. They lose their pluripotency, but they do not truly begin to undergo specific tissue formation. Figure 9B shows images of EBs on day 3, day 4, day 6, day 7, and day 1 after plating (pD1). During EB growth, the EBs become larger and they develop more defined edges. Plated EBs (Figure 9B pD1) attach to the gelatin coated plate and cells begin to grow away from the colony. During the plated EB stage of differentiation, a number of different tissues begin to develop spontaneously. Figure 10 shows Phase Contrast Microscopy images of several potential tissues that developed spontaneously in plated C57BL/6 cells. The examples include: neurons, which develop far away from the originating EB and have long projections protruding from the cell body; epithelial cells, which are cuboidal and form large patches
of cells; fibroblasts, which are numerous and reside just at the edge of the originating EB; epithelium, which forms at the edge of colonies as tight layers of long cells that wrap around the colony; and pigmentation, which develops in random patches that sometimes has channels in the middle. There are a few tissue types that form better tissues when seeded upon a scaffold rather than by undergoing this classical two step process of differentiation. For example, bone engineering requires a scaffold for proper growth (Jukes et al., 2008).

Differentiation can either be spontaneous or directed. Removal of pluripotent cells from PMEFs and LIF releases cells from pluripotency and it allows them to undergo spontaneous differentiation. However, differentiation to a desired tissue type can be directed. Figure 11 shows an example of directed differentiation to several tissue types. In Figure 11, hES cells are directed to neural crest cells by using Fluorescence Activated Cell Sorting (FACS) to isolate cells that are p75+, HNK1+, and AP2+. These cells can be cultured in serum free medium with the appropriate factors to make neurons or Schwann cells. Alternatively, the cells can be cultured with serum and then sorted by FACS for the CD73+ mesenchymal cell population. These mesenchymal cells can then be directed, using the appropriate factors, to create adipogenic cells, chondrogenic cells, osteogenic cells, or smooth muscle cells. Directed differentiation requires knowledge of specific developmental pathways and the necessary requirements for those cells to develop. When the proper factors are added at the proper time, reasonably high levels of purity for a desired type of cell can be achieved. For example, a protocol has been developed for generating high purity oligodendroglial cells to aid in repairing spinal cord injuries (Hatch et al., 2009).
Long-term Cell Culture

One of the qualities commonly reported for new pluripotent stem cell lines is their stability during long-term culture. Although some groups find no differences, their assessment is often cursory and simply an observation that the cells continue to grow and appear morphologically unchanged. Stability of pluripotent stem cells during long-term culture has been challenged by reports of aneuploidy and other chromosomal abnormalities, and differentiation (Greenlee et al., 2004; Inzunza et al., 2004; Maitra et al., 2005; Mikkola et al., 2006; Li et al., 2007; Rebuzzini et al., 2008). However, there is evidence that the effects of long-term culture are not reflective of intrinsic cell instability, but are in fact due to lack of appropriate culture techniques (Brimble et al., 2004; Yao et al., 2006). Brimble et al. (2004) showed that using the typical method of trypsinizing cells induces karyotype instability, whereas ES cells that were mechanically passaged remained stable. As culture techniques advance perhaps scientists will be able to better maintain pluripotent stem cells for extended culture without inducing changes. However, it remains good advice to periodically assay pluripotent stem cell cultures to ensure phenotypic and genotypic stability.

Mitochondria

Mitochondria generate the energy for the cell in the form of ATP and they also participate in apoptosis, or programmed cell death, in stressed cells (Figure 12). The main function of mitochondria is energy production through respiration. Energy production begins with the breakdown of glucose to pyruvate in the cytosol by glycolysis.
Pyruvate, and fatty acids, are imported into mitochondria and enter the Krebs (citric acid) cycle where they are oxidized to produce NADH, FADH$_2$, and CO$_2$ waste. NADH and FADH$_2$ carry electrons to the electron transport chain (respiratory chain). Here, the electrons are carried by cytochrome c, transferred to oxygen, and the protons are released into the intermembrane space via proton pumps such as cytochrome c oxidase, thus creating an electrochemical proton gradient within the mitochondrion. This gradient drives the synthesis of ATP, which is then used as energy for the cell. The role of mitochondria in energy production is outside the realm of this dissertation.

The role of mitochondria in apoptosis is central to this dissertation. Mitochondria have several pro-apoptotic and anti-apoptotic proteins associated with them, including many Bcl-2 family members. The balance of these proteins is at least partially responsible for preventing or promoting apoptosis in cells. During apoptosis, mitochondria receive signals which block the anti-apoptotic proteins. In response, mitochondrial membranes become permeable and cause cytochrome c to be released into the cytoplasm thus disrupting the mitochondrial membrane potential gradient. Cytochrome c and other released proteins are then free to activate caspases, which are the effector molecules of apoptosis. The next section will discuss apoptotic cell death in greater detail.

The structure of mitochondria is relevant to their function. Mitochondria have their own DNA (mtDNA), although they also rely on proteins encoded in the nucleus and imported into the mitochondria. Mitochondria often have multiple copies of mtDNA. The number of mitochondria/cell and DNA molecules/mitochondria varies widely among types of cells (Robin and Wong, 1988). Cells also vary in the number and morphology of
mitochondria in relation to cellular function. Some types of cells have complex tubular networks of mitochondria (Westermann, 2002; Twig et al., 2006), whereas other types of cells contain very few and singular mitochondria. Figure 12 shows a model of a cross-section of a single mitochondrion, which is approximately 0.5 x 1 µm in size. The mitochondrion consists of an outer membrane, an inner mitochondrial membrane that is folded into cristae to provide more surface area for respiration, and a matrix within the inner membrane. As mentioned previously, in the process of generating energy mitochondria pump protons across the inner membrane to the intermembrane space thus leaving the interior matrix negatively charged. As a result of this, they have a mitochondrial membrane potential associated with them. Mitochondria have two states pertaining to their mitochondrial membrane potential. High mitochondrial membrane potential mitochondria are active whereas low mitochondrial membrane potential mitochondria are inactive.

Due to the variety of live cell dyes available, the study of mitochondria is particularly amenable to live cell imaging, which can be done microscopically to provide information on localization and mitochondrial activity, or by Fluorescence Activated Cell Scan (FACScan) analysis to provide information on protein expression and mitochondrial activity. In particular, MitoTracker dyes (from Invitrogen, Carlsbad, CA) label all mitochondria, and dyes such as JC-1 and TMRE label mitochondria differentially on the basis of their mitochondrial membrane potential (activity). However, we have found that many of the mitochondrial dyes are not very bright or they photobleach easily. Additionally, the dyes show difficulty in penetrating thick specimens, such as EBs, and in providing reliable data during optical sectioning. In contrast, green fluorescent protein
(GFP) conjugates are extremely bright and photostable (Stepanenko et al., 2008). Thus, we undertook the generation of a new mitochondrial GFP ES cell line in order to develop a reliable means of analyzing mitochondria in ES cells and the differentiating tissues arising from them.

Mitochondrial function and localization have been associated with viability in oocytes, embryos, and adult cells (Van Blerkom et al., 2000; Van Blerkom et al., 2002; Warner et al., 2004; Levitt et al., 2006). Very little is known about mitochondria in stem cells and their differentiating progeny, and the published data are often conflicting. A few studies, using Transmission Electron Microscopy (TEM), found that there are few mitochondria in ES cells and that they localize in small perinuclear groups (St. John et al., 2005; Cho et al., 2006). Also, the mitochondria have a singular morphology, rather than a network, and are spherical to oval in shape with few cristae (Baharvand and Matthaei, 2003; Oh et al., 2005). Figure 13A shows a TEM image of a hES cell with a large nucleus and a few, singular mitochondria clustered next to the nucleus (St. John et al., 2005).

Three groups have assessed mitochondrial activity in ES cells. St. John et al. (2005) stained hES with JC-1 and found that undifferentiated cells had low mitochondrial activity (no image was published of undifferentiated cells). In contrast, Chung et al. (2007) saw many highly polarized mitochondria in mES cells (Figure 13B). The mES cells in Figure 13B were stained with JC-1 dye to differentiate between active (red) and inactive (green) mitochondria so that overlapping active and inactive mitochondria are yellow. The cells have a large nucleus with mitochondria squeezed in the remaining space around the nucleus. There are numerous active mitochondria visible. Lonergan et
al. (2008; 2009) stained mouse and monkey ES cells with MitoTracker Red and also saw numerous, active mitochondria in undifferentiated cells. Additionally, they found that Rhesus monkey ES cells had mitochondria that formed a cap on the nucleus and speculated that this may be a feature of pluripotency. It is important to note that the Lonergan et al. (2008; 2009) studies are published abstracts rather than peer-reviewed publications; thus there are actually only two peer-reviewed published reports of mitochondrial activity in ES cells. As these data are somewhat conflicting, and certainly sparse, more research concerning mitochondrial activity in ES cells became a major thrust of the research reported in this dissertation.

The published data concerning mitochondrial localization and activity during differentiation is equally conflicting. A few groups have detected changes in mitochondrial localization and activity with differentiation, although one group saw no change in hES mitochondrial activity by JC-1 staining (Saretzki et al., 2008). Cho et al. (2006) observed that the mitochondria in hES moved from their small clusters and dispersed through the cytoplasm during differentiation. St. John et al. (2005) studied cardiomyocyte differentiation in hES using JC-1 and found that mitochondrial activity increased and mitochondria polarized to one side of the cell before forming a bipolar arrangement. In contrast, Chung et al. (2007) saw decreases in mitochondrial activity with cardiomyocyte differentiation in mES cells. Schieke et al. (2008) performed a more complicated analysis of mES mitochondrial activity. They sorted undifferentiated mouse ES cells into high and low mitochondrial membrane potential populations and then differentiated them. The two populations of cells did not differ in morphology or pluripotency markers. However, only high mitochondrial membrane potential cells were
able to form teratomas, whereas low mitochondrial membrane potential cells were very good at making mesoderm. Interestingly, all of the published data seems to agree that the number of mitochondria increases with differentiation (von Wangenheim and Peterson, 1998; St. John et al., 2005; Cho et al., 2006; Facucho-Oliveira et al., 2007; Saretzki et al., 2008). However, more work in this field is necessary in order to determine whether undifferentiated ES cells have high mitochondrial activity and how this activity changes during differentiation.

To the best of our knowledge, there are no published reports concerning the distribution or activity of mitochondria in aging pluripotent stem cells. However, there is a single study that has shown mitochondrial aggregation in aging adult stem cells (Lonergan et al., 2006; Lonergan et al., 2007). Additionally, data on mitochondrial localization or activity in iPS seem to be nonexistent (Parker et al., 2009). Given the paucity of data and the conflicting reports, the analysis of mitochondrial activity and localization in pluripotent stem cells and their differentiating progeny is ripe for study.

**Apoptosis**

Apoptosis, or programmed cell death, refers to the cell’s ability to detect flaws within it or to receive signals from outside the cell that indicate that cell death would benefit the whole organism. This is in contrast to necrosis, where cells die as a result of induced damage to the cell such as by physical means. Necrosis leads to localized tissue damage due to the violence of cell death, whereas apoptosis provides a means for the cell to disassemble itself without damage to surrounding cells and tissues. Figure 14 shows a simplified diagram of the apoptotic process. A normal cell first detects the signal from
the intrinsic or extrinsic apoptotic pathway that something is wrong with the cell. It then undergoes cell shrinkage and chromatin condensation. The DNA becomes fragmented, and finally the cell breaks apart into apoptotic bodies that are lysed and phagocytosed by other cells.

There are two intricately linked pathways leading to apoptosis that are represented in Figure 15. Both pathways involve a series of caspases, which are cysteine-aspartic acid proteases. They reside in the cell in a procaspase (precursor) form that is cleaved by other molecules to the produce the activated form. Figure 15 shows that in healthy cells, the inhibitor of apoptosis (IAP) family of anti-apoptotic proteins block apoptosis. The intrinsic apoptotic pathway detects changes within the cell that indicate stress or DNA damage. Mitochondria then receive apoptotic signals that lead to their depolarization and release of cytochrome c and second mitochondrial-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein-binding protein with low pI (DIABLO). In turn, cytochrome c works with APAF-1 in the apoptosome to activate caspase 9 while the Smac/DIABLO protein blocks the IAP and other anti-apoptotic proteins. The extrinsic pathway detects binding of extracellular signals (death ligands) to cell surface receptors. Caspase 8 is then activated, followed by caspase 7. Both the intrinsic and extrinsic pathways converge on caspase 3, which cleaves substrates within the nucleus that ultimately mediate DNA fragmentation and the breaking apart and phagocytosis of the dying cell.

There are several hallmarks of apoptosis that allow apoptotic cells to be identified. By light microscopy the characteristic blebbing of apoptotic cells can be seen. The Annexin V reagent binds to phosphatidylserine, which flips over in an apoptotic cell.
DNA staining allows the visualization of condensed chromatin, which is indicative of apoptosis. Common assays to detect DNA fragmentation include the TUNEL assay. Dyes that differentially stain mitochondria detect the loss of mitochondrial membrane potential that accompanies apoptosis. And there are numerous other assays for caspase activity and the individual molecules in the apoptotic signal cascade. By combining these assays not only can cells that are undergoing apoptosis be detected, but also what stage of apoptosis they are in can be ascertained.

Researchers have used several of these methods to study apoptosis during development. Jurisicova and Acton (2004) provide a thorough review of the role of apoptosis in human preimplantation development. Pluripotent stem cells also undergo apoptosis. Qin et al. (2007) notes that hES cells, unlike mES cells, have high rates of spontaneous apoptosis. The loss of viability after freeze thawing cells is due to apoptosis, rather than necrosis, and can be suppressed by using a caspase inhibitor (Heng et al., 2006; Heng et al., 2007). As hES colonies grow their centers compact and TUNEL positive particles appear (Johkura et al., 2004). Additionally, localized areas of apoptosis and caspase 3 activity are seen in differentiating EBs (Murray and Edgar, 2004).

Much of the apoptosis in pluripotent stem cells can be related to the culture conditions. One influence on the level of apoptosis in ES cells, as determined by Annexin V staining, is related to the dose of LIF (Viswanathan et al., 2003; Figure 16A). Figure 16A shows that by increasing the concentration of LIF the percentage of apoptotic cells, as assessed by Annexin V staining and FACScan analysis, decreases. Other groups have also shown that LIF has anti-apoptotic properties and that LIF starvation leads to apoptosis of ES cells (Furue et al., 2005; Duval et al., 2006). An elegant study recently
found a direct link between apoptosis and pluripotency. Fujita et al. (2008) discovered that induction of differentiation by retinoic acid (RA) led to a rapid increase in caspase 3 activity (Figure 16B1 and B2). Figure 16B1 shows a control set of cells (triangle) and a RA treated (differentiation induced) group of cells plotted vs. caspase 3 activity. RA induces differentiation, and so with differentiation there is a rise in the level of caspase 3 activity within a few hours of stimulation. Figure 16B2 shows a similar experiment with RA induction (time representing the time of treatment) and a caspase 3 sensor (Casp<sup>sensor</sup>) that translocated to the nucleus with caspase 3 activity. Again, within hours of RA induction the caspase sensor moves from the cytosol to the nucleus (see Pos. for the positive control comparison). Fujita et al. (2008) then linked caspase 3 to cleavage of the pluripotency marker Nanog, which released the ES cells from their pluripotent state. Thus, apoptosis and pluripotency are linked.

The importance of mitochondrial activity and apoptosis to cell viability led us to form the following specific aims for our research.

**Specific Aims**

1) Create and characterize an ES cell line from mtGFP-tg mice that have their mitochondria endogenously labeled with GFP. Use the mtGFP-tg ES cell line to evaluate mitochondrial localization.

2) Compare cell division, pluripotency markers, and differentiation markers in undifferentiated and differentiating mouse pluripotent stem cells by comparing the mtGFP-tg ES cell line, a conventional C57BL/6 ES cell line, and an iPS cell line.
3) Analyze mitochondrial activity and localization in undifferentiated and differentiating mouse pluripotent stem cells by comparing the mtGFP-tg ES cell line, a conventional C57BL/6 ES cell line, and an iPS cell line.

4) Analyze early, intermediate, and late stage apoptosis markers in undifferentiated and differentiating mouse pluripotent stem cells by comparing a conventional C57BL/6 ES cell line and an iPS cell line.

Each section of this dissertation has the Figures (first) and Tables (second) at the end of the section.
Figure 1: Preimplantation Mouse Embryo Development and Stem Cells. A) Oocytes are fertilized by sperm, and then they undergo cleavage divisions to the blastocyst stage of preimplantation embryo development (adapted from Molecular Biology of the Cell, ISE by Alberts et al., Copyright 2002 by Garland Science-Books. Reproduced with permission of Garland Science-Books in the format Dissertation via Copyright Clearance Center.). B) At this point the blastocyst can develop into a fetus and then a full grown adult. One can derive fetal stem cells from the fetus and adult stem cells from the adult (top scheme). Alternatively, the blastocyst can be used to produce ES cells that are pluripotent and can produce all three germ layers (bottom scheme). Scale bar = 50 µm; Newmark and Warner, unpublished data.
Figure 2: Isolation of Mouse Embryonic Stem Cells. To harvest ES cells from mice, blastocyst stage preimplantation embryos are collected. The blastocysts are plated onto fibroblast feeder layers in the presence of LIF. The outgrowths are expanded into a fully developed ES cell line. Reprinted with permission from www.openbiosystems.com/GeneTargeting/MEScells/.
Figure 3: Primary Mouse Embryonic Fibroblasts and Embryonic Stem Cells. Phase Contrast and DIC Microscopy Images of fibroblast feeder layers and C57BL/6 ES cell colonies growing on a fibroblast feeder layer. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Movie 1: Timelapse DIC Imaging of Mouse Embryonic Stem Cell Growth. C57BL/6 ES cells were grown on a PMEF feeder layer in a Bioptechs Delta T culture chamber on the Keck 3DFM and imaged every 15 min by DIC microscopy. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Movie 2: Timelapse TPLSM Imaging of Mouse Embryonic Stem Cell Growth. C57BL/6 ES cells were stained with Hoechst, grown on a PMEF feeder layer in a Bioptechs Delta T culture chamber on the Keck 3DFM, and imaged every 15 min by TPLSM microscopy. The corresponding DIC image was taken at the start of the culture. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Figure 4: Pluripotency Markers and a Teratoma. A) Diagram of a typical ES cell, which expresses many of the pluripotency markers shown in this figure. B) Example of a teratoma, which is a tumor composed of multidifferentiated tissue (KW project funded by the National Library of Medicine).
Movie 3: Beating Cells Grown from Mouse Embryonic Stem Cells. C57BL/6 ES cells were cultured into EBs and then plated onto gelatin for further culture. Beating cells were captured by live Phase Contrast Microscopy and presented in real time. The scale bar = 50 µm and the cells are approximately 5-10 µm in size; Newmark and Warner, unpublished data.
Figure 5: Embryonic Stem Cell Therapy. ES cells have the potential to be used in testing drug targets, toxicity testing, studying cell differentiation, and understanding birth defects among other potential uses.
Figure 6: Methods for Deriving Pluripotent Stem Cells-1. Many alternative methods to the traditional derivation of ES cells have been developed in order to minimize ethical and/or financial concerns.
Figure 7: Methods for Deriving Pluripotent Stem Cells-2. Many alternative methods to the traditional derivation of ES cells have been developed in order to minimize ethical and/or financial concerns.
Figure 8: Deriving Induced Pluripotent Stem Cells. iPS cells are created from adult cells by introducing pluripotency factors. iPS cells have the same potential as ES cells to be used in drug testing, transplantation therapy, or repairing genetic defects. Reprinted by permission from Macmillan Publishers Ltd: Nature, Passier et al. (2008) 453(7193): 322-329.
**Figure 9: Mouse Embryoid Body Formation.** A) EBs are formed by removing ES cells from their feeder layers and removing LIF from the culture medium. They may be formed by hanging drops or in suspension. They are then plated onto gelatin for further differentiation. B) Phase Contrast Microscopy of EBs during different days of growth. D=Day, pD=plated Day. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Movie 4: Timelapse DIC Imaging of Mouse iPS Cell Growth. iPS cells were placed in a Bioptechs Delta T culture chamber on the Keck 3DFM, in medium without LIF, and imaged by DIC microscopy every 15 min. The iPS cells formed EBs. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Figure 10: Phase Contrast Images of Plated Mouse Embryoid Bodies. Plated EBs spontaneously form a variety of tissue types in culture. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Figure 11: Directed Differentiation. Directed differentiation of ES cells to specific cell types requires knowledge of specific factors that identify the desired cell types. Reprinted by permission from Macmillan Publishers Ltd.: Nature Biotechnology, Lee et al. (2007) 25(12): 1468-1475.
Figure 12: Anatomy of a Mitochondrion. Mitochondria are approximately 0.5 x 1 µm in size. They have an outer membrane, folded inner membrane of cristae for energy production, and an inner matrix. Reprinted with permission from http://micro.magnet.fsu.edu.
Figure 13: Mitochondria in Stem Cells. A) Mitochondria (circled) in hES cells were imaged by TEM. Reprinted with permission from CLONING AND STEM CELLS 7/3, published by Mary Ann Liebert, Inc., New Rochelle, NY. Scale bar = 200 nm. B) Mitochondria in mES cells were stained with JC-1 and imaged by Confocal Laser Scanning Microscopy (CLSM). Green is inactive mitochondria, red is active mitochondria, and yellow is overlapping inactive and active mitochondria. Scale bar = 20 µm. Reprinted by permission from Macmillan Publishers Ltd.: Nature Clinical Practice Cardiovascular Medicine, Chung et al. (2007) 4 Suppl 1: S60-67.
Figure 14: Apoptosis. The process of apoptosis (programmed cell death) begins with a normal cell receiving an apoptotic signal from the intrinsic or extrinsic pathway. The cell undergoes shrinkage and chromatin condensation, nuclear collapse, apoptotic body formation, and finally lysis and phagocytosis of apoptotic bodies. Illustrated by Jen Philpott and published at www.scq.ubc.ca/apoptosis/. Reprinted using the license http://creativecommons.org/licenses/by-nc-sa/3.0/.
Figure 15: Apoptosis Signal Cascade. Apoptosis has an extrinsic pathway, which receives signals from outside of the cell and works through caspase 8, and an intrinsic pathway, which receives signals from within the cell and works through mitochondria and caspase 9. Both pathways converge at caspase 3 and lead to the final stages of apoptosis. Reprinted with permission from Imgenex Corp.
Figure 16: Apoptosis in Stem Cells. A) Higher concentrations of LIF are correlated with a decrease in apoptosis as detected by Annexin V staining. B1) Retinoic acid (RA) induction of differentiation is correlated with increasing caspase 3 activity. Reprinted with permission from Wiley. B2) Induction of differentiation, as indicated by the time on the DAPI nuclear stained images, is correlated with movement of a caspase 3 sensor to the nuclei thus indicating caspase 3 activity. Reprinted from Fujita et al. (2008) with permission from Elsevier.
## Table I: Significant Pluripotent Stem Cell Developments

<table>
<thead>
<tr>
<th>Year</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995</td>
<td>Non-human primate ES cells created</td>
<td>(Thomson et al., 1995)</td>
</tr>
<tr>
<td>1997</td>
<td>First cloned mammal (Dolly)-reprogrammed adult nucleus</td>
<td>(Wilmut et al., 1997)</td>
</tr>
<tr>
<td>1998</td>
<td>Human ES cells created</td>
<td>(Thomson et al., 1998)</td>
</tr>
<tr>
<td>2002</td>
<td>Primate parthenogenetic ES cells created</td>
<td>(Cibelli et al., 2002)</td>
</tr>
<tr>
<td>2004</td>
<td>ES cells from a human morula</td>
<td>(Strelchenko et al., 2004)</td>
</tr>
<tr>
<td>2005</td>
<td>First verifiable cloned human blastocyst</td>
<td>(Stojkovic et al., 2005)</td>
</tr>
<tr>
<td>2005</td>
<td>ES cell lines with specific genetic disorders</td>
<td>(Verlinsky et al., 2005)</td>
</tr>
<tr>
<td>2005</td>
<td>ES cells from single mouse blastomeres</td>
<td>(Chung et al., 2006)</td>
</tr>
<tr>
<td>2006</td>
<td>Successful altered nuclear transfer ES cells</td>
<td>(Meissner and Jaenisch, 2006)</td>
</tr>
<tr>
<td>2006</td>
<td>Human ES cells grown in chemically defined medium</td>
<td>(Ludwig et al., 2006)</td>
</tr>
<tr>
<td>2006</td>
<td>Mouse iPS cells created</td>
<td>(Takahashi and Yamanaka, 2006)</td>
</tr>
<tr>
<td>2007</td>
<td>Mouse eggs and sperm created from ES cells in same dish</td>
<td>(Kerkis et al., 2007)</td>
</tr>
<tr>
<td>2007</td>
<td>Human parthenogenetic ES cells created</td>
<td>(Kim et al., 2007)</td>
</tr>
<tr>
<td>2007</td>
<td>Human iPS cells created</td>
<td>(Takahashi et al., 2007; Yu et al., 2007)</td>
</tr>
<tr>
<td>2007</td>
<td>Mouse sickle cell anemia treated with iPS cells</td>
<td>(Hanna et al., 2007)</td>
</tr>
<tr>
<td>2008</td>
<td>Human iPS cells from ALS patient created</td>
<td>(Dimos et al., 2008)</td>
</tr>
<tr>
<td>2009</td>
<td>FDA approval for first hES therapy for spinal cord injury</td>
<td><a href="http://www.geron.com">www.geron.com</a></td>
</tr>
<tr>
<td>2009</td>
<td>Adult mice generated from iPS cells</td>
<td>(Boland et al., 2009; Zhao et al., 2009)</td>
</tr>
<tr>
<td>2009</td>
<td>Increased iPS generation success by silencing p53</td>
<td>(Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009)</td>
</tr>
</tbody>
</table>
Table II: Induced Pluripotent versus Conventional ES Cell Lines

<table>
<thead>
<tr>
<th>Source</th>
<th>iPS Cells</th>
<th>Conventional Embryonic Stem Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Adult cells</td>
<td>Blastocyst or embryonic cells</td>
</tr>
<tr>
<td>Derivation</td>
<td>Induction of Oct-4, Sox2, Klf4, and c-Myc (optional) using viruses, genes, or proteins</td>
<td>Plate with LIF on PMEFs (mouse) Plate with bFGF on PMEFs (human)</td>
</tr>
<tr>
<td>Efficiency of Derivation</td>
<td>0.01-0.1%</td>
<td>Mouse: strain dependent, but typically 50-75% (Bryja et al., 2006) Human (clinical grade): 22% (Crook et al., 2007)</td>
</tr>
<tr>
<td>Culture Conditions</td>
<td>Culture with LIF (mouse) or bFGF (human) on PMEFs</td>
<td>Similar</td>
</tr>
<tr>
<td>Morphology</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Similar</td>
<td></td>
</tr>
<tr>
<td>Pluripotency</td>
<td>Mouse cells: capable of producing adult Human cells: teratoma formation</td>
<td></td>
</tr>
<tr>
<td>Gene Expression Profile</td>
<td>3947/17620 genes significantly different (79% lower than ES) (Chin et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Patient Matched Cells</td>
<td>ALS, Diabetes, Huntington’s Disease, Muscular Dystrophy</td>
<td>ES cells with diseases created after PGD, but not patient matched</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Cell Culture

Three mouse pluripotent stem cell lines were used in this research (Table III). The first was a C57BL/6 ES cell line purchased from Open Biosystems (Huntsville, AL) and originally derived from a blastocyst in Dr. Rudolph Jaenisch’s lab. The second pluripotent stem cell line was an iPS cell line derived from PMEFs in Dr. Jaenisch’s lab (Wernig et al., 2007). This was a generous gift provided by Stemgent (Cambridge, MA). The third cell line was a mtGFP-tg ES cell line derived in our lab and described in further detail below.

All three pluripotent stem cell lines were maintained on a PMEF feeder layer (mitomycin-c treated CF-1, Millipore, Billerica, MA). They were cultured using ES cell medium, which consisted of Dulbecco’s Modified Eagle’s Medium (DMEM, Mediatech, Manassas, VA), 15% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1000 U/ml leukemia inhibitory factor (LIF, Millipore), 4 mM l-glutamine, 1x nonessential amino acids, 1x sodium pyruvate, 1x penicillin/streptomycin, and 0.1 mM β-mercaptoethanol (all from Invitrogen, Carlsbad, CA). The cells were trypsinized (0.25% Trypsin, Chemicon) and passaged onto fresh feeder layers every couple of days. On occasion, cells were frozen using ES cell freeze medium (Millipore) and stored in liquid nitrogen.

In order to induce cell differentiation, we followed the ATCC standard protocol for making EBs, which entailed trypsinizing and culturing the ES cells in MEF medium, which is equivalent to the ES cell medium without LIF, and removing the ES cells from the fibroblast feeder layer. EBs were cultured in low attachment dishes (Corning,
Corning, NY) for up to seven days. To induce further differentiation we plated the EBs onto 0.1% gelatin (Millipore) coated dishes in MEF medium. Plated EBs could be cultured for many days, but we typically ended the culture before 10 days.

**mtGFP-tg Embryonic Stem Cells**

In order to generate a mitochondrial GFP ES cell line, we obtained three male mitochondrial green fluorescent protein (mtGFP-tg) mice as a kind gift from the laboratory of Dr. Hiroshi Shitara (Department of Laboratory Animal Science, Metropolitan Institute of Medical Science, Tokyo, Japan) (Shitara et al., 2001; Nagai et al., 2004). These mice contain the sequences for both enhanced GFP and the signal sequence of cytochrome c oxidase subunit VIII on the background of a C57BL/6 mouse. Upon receiving the mice, we discovered that the mice were contaminated with helicobacter. After a three month treatment regimen with a four drug combo (Bio-Serv, Frenchtown, NJ), the mice were declared clean and released from quarantine. We then established a breeding and genotyping program within our own laboratory.

The three male mice were mated with C57BL/6 female mice, and the resulting F1 mice underwent genotyping/phenotyping in order to determine GFP zygosity. (Through analysis of the F1 litter results, we discovered that our original three male mice were heterozygous for GFP.) For GFP genotyping, an ear punch was collected from individual mice, and DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA). RNase A (4 µl of 100 mg/ml) was added after tissue digestion in order to eliminate RNA. The resulting DNA was quantitated and the concentrations of each sample were adjusted to 10-20 ng/µl for PCR. A GAPDH control PCR was run for each sample. The
reaction mix contained 20 ng DNA, 300 nM each of forward (5'-AGGTCGGTGTAACGGATTTG-3') and reverse (5'-TGTAGACCATGTAGTTGGT-3') primers, 1X SYBR green master mix, and nuclease free water. Samples were run on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) using the PCR protocol 50°C 2 min; 95°C 15 min; 40 cycles of 95°C 15 s, 60°C 30 s, 72°C 30s; 72°C 10 min, 4°C 2 min. The GFP PCR reaction mix contained 50 ng DNA, 300 nM each of forward (5'-ACCACCTACCTGAGCACCCAGTC-3') and reverse (5'-GTCCATGCCGAGAGTGATCC-3') primers, 1X SYBR green master mix, and nuclease free water. The PCR protocol used was 50°C 2 min; 95°C 15 min; 40 cycles of 95°C 15 s, 59°C 30 s, 72°C 30s; 4°C 2 min.

In order to study the pattern of mitochondrial GFP expression, several tissues were harvested from a sacrificed mouse and imaged by epifluorescence microscopy. It was determined that visual examination of ear punches would be sufficient for phenotyping. To maintain the mtGFP-tg colony, each mouse was ear punched upon weaning. The ear punches were examined by epifluorescence microscopy, and only the GFP positive mice were used in experiments or for breeding.

Embryonic stem cells were produced generally following the procedure of Bryja et al (2006). Briefly, one-cell embryos were collected from superovulated female mice and then placed into culture in KSOM (Millipore) until they reached the blastocyst stage. Fresh PMEFs were plated onto gelatin coated 12-well plates a day ahead of blastocyst formation. The blastocysts were placed one per well onto the fibroblasts in ES cell medium. They were cultured for several days until the trophectoderm cells fell away and the inner cell mass (ICM) cells began to grow out, at which time the ICMs were
transferred onto fresh fibroblasts. After a couple of more days of growth, the ICMs were trypsinized and passaged (P0). After two more days of growth, the cells were trypsinized and passaged again leading to the establishment of two cell lines. One cell line was chosen for continued characterization based on good morphology and growth characteristics. This cell line was named mtGFP-tg1 (mitochondrial green fluorescent protein-transgenic line 1).

Validation of Pluripotent Cell Lines

A summary of all assays and markers used in these experiments is presented in Table IV. The alkaline phosphatase, Oct-4, and germ layer assays were performed on all three pluripotent stem cell lines grown in duplicate, such that each time point is duplicated, with the results reported as the mean ± the standard deviation (SD). FACScan data were collected on 10,000 cells for each sample.

Mycoplasma: mtGFP-tg ES cells were examined for mycoplasma contamination by culturing without antibiotics/antimycotics for several passages and then staining the cells with 2 µg/ml Hoechst (Invitrogen). The cells were imaged using the 100X objective and epifluorescence imaging. Cytoplasmic or extracellular Hoechst staining are indicative of mycoplasma contamination.

MTT: The MTT assay measures cell proliferation as reflected by the number of mitochondria. The MTT assay was performed in replicate using ES cells on PMEFs with ES cell medium (n=18), ES cells in MEF medium (EB formation, n=12), individual EBs
(C57BL/6 and iPSC n=10, mtGFP-tg n=8), and plated EBs (C57BL/6 n=20, iPSC and
mtGFP-tg n=19). For the ES cell and the EB formation assays, the cells were counted
and an equal number were plated in each well of a flat-bottomed 96 well culture plate.
For the EB assay, individual EBs were cultured, using the same number of cells for each,
in hanging drops so that the resulting EBs were equivalent in size. The same number of
EBs was added to each well of a flat-bottomed 96-well culture plate. For the plated EB
assay, the same number of EBs was added to each well of a gelatin coated flat-bottomed
96-well culture plate. For all assays, the cells were grown in 200 µl cultures before
adding 20 µl of 20 mg/ml MTT (Sigma, St. Louis, MO) in PBS. After 24 h of further
culture, the plates were processed by adding 50 µl PBS to each well, centrifuging,
removing the supernatant, adding 150 µl acidified isopropanol, dissolving the purple
precipitate by vigorous pipetting, and then reading the absorbance at 565 nm using a
Synergy HT plate reader (Biotek, Winooski, VT). The results were reported ± the SD.

Alkaline phosphatase: alkaline phosphatase activity is found in pluripotent cells.
We performed an alkaline phosphatase (AP) analysis according to manufacturer’s
instructions using the Alkaline Phosphatase Detection Kit (Millipore), which uses Fast
Red Violet and Napthol AS-BI Phosphate. Positive colonies appear pink. We considered
a colony to be AP positive when >75% of the colony was positive. We performed an AP
assay, in duplicates for each cell line, on each day of development from pluripotent cell
through 14 days of development and counted the number of positive colonies using Phase
Contrast Microscopy.
Oct-4: Oct-4 is a marker of pluripotency. We stained cells for Oct-4 essentially as described in Mitalipov et al. (2003). Cells were trypsinized (for FACScan) and then fixed in 3.7% formaldehyde and permeabilized using 0.5% saponin/PBS before staining. The primary antibody used was 2 µg/ml anti-Oct-4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody was 7.5 µg/ml goat anti-mouse IgG Cy3 (Jackson Immunoresearch, West Grove, PA). An IgG2b isotype control (eBioscience, San Diego, CA) was also used. After staining, cells were washed in PBS and either imaged by Epifluorescence Microscopy or analyzed by FACscan. We performed an Oct-4 assay on alternate days of development from pluripotent cell through 14 days of development.

Karyotype: A karyotype analysis was performed on mtGFP-tg ES cells by Cell Line Genetics (Madison, WI). The C57BL/6 ES cells and iPS cells were previously karyotyped by their source companies. We prepared mtGFP-tg ES cells by passaging them to a T-25 culture flask and then sent them to Cell Line Genetics overnight.

Germ layers: Three germ layer markers were analyzed in order to study tissue differentiation in pluripotent and differentiating cells. The markers used were: alpha fetoprotein (AFP, endoderm), Brachyury (Bry, mesoderm), and Nestin (ectoderm) (Dubois et al., 2006; Kwon et al., 2006; Martin and Kimelman, 2008). In order to determine whether our pluripotent stem cells could produce tissues from all three germ layers, we trypsinized (for FACScan), fixed, permeabilized, and stained ES cells, EBs, and plated EBs using these typical markers for each germ layer and analyzed the results
by FACscan analysis or Epifluorescence Microscopy. Anti-alpha 1-fetoprotein antibody (AFP, Dako, Denmark) was used neat to stain for endoderm, 5 µm/ml anti-brachyury antibody (Bry, Abcam, Cambridge, MA) was used to stain for mesoderm, and 1:200 anti-nestin antibody (Abcam) was used to stain for ectoderm. A rabbit polyclonal isotype control (Abcam) was also used. Goat anti-rabbit IgG-R (Santa Cruz Biotechnology) was used at 4 µg/ml as a secondary antibody. We performed all three germ layer assays on alternate days of development from pluripotent cell through 14 days of development.

**Mitochondrial Assays**

Two main assays, included in Table IV, were used to detect mitochondria: JC-1 and TMRE (both from Invitrogen). Both dyes stain all mitochondria and are able to differentiate between active and inactive mitochondria in living cells by the mitochondrial membrane potential. All assays were performed on all three pluripotent stem cell lines grown in duplicate, such that each time point is duplicated, with the results reported ± the SD. FACScan data was collected on 10,000 cells for each sample.

**JC-1:** JC-1 staining was performed on trypsinized cells (for FACScan) or plated cells (for imaging). Cells were immersed in 2 µM JC-1 in medium and placed in the incubator for 15 min. For one ES cell experiment, 1 µl CCCP (Invitrogen) was added to the staining solution to inhibit mitochondrial activity by uncoupling the mitochondria. The cells were then washed in medium for fluorescence imaging or washed in PBS for FACScan analysis. Active mitochondria fluoresce red whereas inactive mitochondria fluoresce green. Thus double positive (red and green fluorescence) cells contain both
active and inactive mitochondria, green fluorescent cells contain inactive mitochondria, and red fluorescent cells contain only active mitochondria. Most cells contain a mixture of active and inactive mitochondria; therefore the analysis of mitochondrial activity is focused on the percentage of double positive cells. We performed a JC-1 assay on each day of development from pluripotent cell through 14 days of development.

**TMRE:** TMRE staining was performed on trypsinized cells for FACScan. We found that although cells stained very brightly for fluorescence imaging, they photobleached very quickly making imaging difficult. Cells were immersed in 100 nM TMRE in medium and placed in the incubator for 10 min. The cells were then washed in PBS and analyzed by FACScan. Active mitochondria fluoresce more brightly than do inactive mitochondria. Therefore TMRE captures the full gradient of mitochondrial activity. We performed a TMRE assay on each day of development from pluripotent cell through 14 days of development.

**Apoptosis Assays**

Several assays were used to detect apoptosis and are summarized in Table IV. As apoptosis is a process rather than a single step, we chose to analyze indicators from early through late stages of apoptosis. We chose one marker of early apoptosis ( Annexin V), one marker of intermediate apoptosis (caspase 3), and one marker of late apoptosis (TUNEL). Additionally, the JC-1 and TMRE assays for mitochondrial activity reflect early to intermediate apoptosis by measuring a loss of mitochondrial activity. These four markers are circled in Figure 17, which is adapted from Figure 15 in the Introduction. It
is worthwhile to note that, with the exception of the assays for mitochondrial activity, our apoptosis assays do not differentiate between the intrinsic and extrinsic apoptotic pathways. A loss of mitochondrial activity would indicate that the intrinsic apoptotic pathway is activated. Additionally, López-Hernández et al. (2006) present evidence that phosphatidlyserine externalization is more closely associated with the extrinsic apoptotic pathway than with the intrinsic apoptotic pathway. All assays were performed on cell lines grown in duplicate, such that each time point is duplicated, with the results reported as the mean ± the SD. FACScan data was collected on 10,000 cells for each sample.

Annexin V: Annexin V detects phosphatidylserine, which flips over in the cell membrane during the early stages of apoptosis (Figure 17A). Cells that are positive for Annexin V have begun the process of apoptosis, but are still able to be rescued from cell death. Annexin V staining (Invitrogen kit) was performed on trypsinized (FACScan) or plated cells (for imaging). Cells were resuspended in 1X Annexin binding buffer. C57BL/6 and iPS cells received 5 µl Annexin V 488 and 1 µg/ml PI as a counterstain to detect necrotic cells. The cells were incubated at room temperature for 15 min. Cells then received 400 µl Annexin binding buffer and they were kept on ice until analysis. We performed an Annexin V assay on each day of development from pluripotent cell through 14 days of development. The mtGFP-tg ES cells were not tested for Annexin V due to the interference of the GFP fluorescence with the detecting antibody.

Mitochondrial assays: JC-1 and TMRE both detect apoptosis as a loss in mitochondrial membrane potential. Loss of mitochondrial membrane potential occurs in
early to intermediate apoptosis as mitochondria become leaky and release cytochrome c and Smac/DIABLO (Figure 17B). For JC-1, apoptotic cells lose active (red fluorescent) mitochondria and become greener. Thus, the population of double positive (red and green fluorescent) cells decreases and the population of green fluorescent cells increases. For TMRE, apoptotic cells lose intensity and become fainter red. As stated above, we performed the JC-1 and TMRE assays on each day of development from pluripotent cell through 14 days of development.

Caspase 3: Caspase 3 is an intermediate apoptosis marker. Both the extrinsic and intrinsic apoptosis pathways converge on caspase 3 (Figure 17B). Caspase 3 staining (Image iT LIVE Caspase 3, Invitrogen kit) was performed on trypsinized cells (for FACScan) or plated cells (for imaging). We performed a caspase 3 assay on each day of development from pluripotent cell through 14 days of development. The mtGFP-tg ES cells were not tested for caspase 3 due to the interference of the GFP fluorescence with the detection reagent.

TUNEL: The TUNEL assay measures the DNA fragmentation that occurs in late stage (irreversible) apoptosis (Figure 17B). We used the APO-BrdU TUNEL Assay Kit (Invitrogen) to detect DNA fragmentation in the C57BL/6 and iPSC pluripotent stem cell lines. Cells were trypsinized and fixed in 1% formaldehyde on ice for 15 min. They were then centrifuged, resuspended in ice cold 70% ethanol, and kept in the freezer for a couple of days until processing. Fragmented DNA was labeled, including using the kit’s positive and negative control cells (human lymphoma cell line), following the kit’s
instructions and then the samples were analyzed by FACScan. We performed a TUNEL assay on pluripotent cells, D7 EBs, and pD7 EBs. The mtGFP-tg ES cells were not assessed by using the TUNEL assay due to the interference of the GFP fluorescence with the detecting antibody.

**Flow Cytometry**

Flow cytometry instruments use a flow cell to pass a sample through a beam of light one cell at a time. The advantage is that the instrument is extremely fast, making it possible to analyze 10,000 cells in a minute. Therefore it is easy to acquire data on an entire population of cells quickly. The disadvantage is that although we can amass a large quantity of population data, the data from each single cell were not available using our instrument. Some new flow cytometers do have the capability of acquiring an image of each cell as it flows through the cytometer. However, there are no morphological data or localization data available from conventional flow cytometry, which is why flow cytometry needs to be complemented by microscopic imaging, as described in the next section.

Our flow cytometer is a FACScan (Becton Dickinson (BD), Franklin Lakes, NJ), which is shown in Figure 18A. Figure 18B shows the typical laser path through a FACScan and the resulting parameters that may be collected. In Figure 18B, the laser travels to the cell, and then forward scatter (FSC) light is collected on the opposite side. Side scatter (SSC) and FL1-FL4 fluorescence are measured from the side of the cell. Mirrors deflect the light to the appropriate detectors. Examples of some of the common dyes (fluorochromes) used in flow cytometry are listed by the respective fluorescent
channels (FL1-FL4). Typically, we measure forward scatter (FSC, reflective of cell size), side scatter (SSC, reflective of cell granularity), FL1 fluorescence, FL2 fluorescence, and FL3 fluorescence. Our instrument does not have a FL4 fluorescence channel.

To prepare samples for flow cytometry, they are dissociated into a single cell suspension, stained for a particular marker (if desired), and placed into a sample tube with a small amount of sheath fluid (BD). Preliminary experiments are performed in order to set the appropriate conditions for each channel and to determine whether a fluorescence marker bleeds into another channel. Compensation may be applied to correct for any bleedthrough.

Once the data are collected, one can view these data using the appropriate software tools. There are two major types of graphs used to analyze the data: histogram, and dot plot. An example of a flow cytometry histogram is shown in Figure 19. In the histogram shown, the fluorescence intensity is plotted (x-axis) vs. a count of the number of cells at each given intensity (y-axis). Typically this will look similar to a normal curve, and a mean fluorescence intensity can be calculated. It is common to include an unstained autofluorescence control sample (purple histogram) along with the positively labeled sample (green histogram). In a dot plot, two channels are plotted against one another (Figure 20). One type of dot plot is to plot FSC vs. SSC in order to find a specific cell population within a mix of cells. One can then draw a gate around this population to exclude other cell types. Additionally, as shown in Figure 20, one may plot one fluorescence channel against another. In this case, a cross is drawn on the graph to define quadrants. Thus the percentages of the cell population that falls into each quadrant can be calculated. Each assay will fit more readily into either histogram or quadrant
analysis depending on whether you are looking at one parameter (histogram) or two (quadrant).

Keck 3D Fluorescence Microscope Imaging

The Keck 3DFM was built using a grant from the W.M. Keck Foundation with the purpose of creating a multimodal microscope to image samples in the same place and at the same time (Townsend et al., 2005; Warger et al., 2007). Figure 21A shows the diagram of the original Keck 3DFM concept, which consists of a fixed specimen stage on a microscope with fluorescence confocal, reflectance confocal, 2-photon, OQM, and DIC microscopy modalities. These modalities will be described in further detail below. Figure 21B shows images of the actual instrument. In contrast to flow cytometry, the advantages of imaging are that a wealth of morphological and localization information can be collected from samples, albeit at the cost of time. Microscopy is particularly appropriate to use for imaging of cells within the context of their natural tissues.

The Keck 3DFM has a number of modalities that are useful alone or in conjunction with the other modalities. Table V lists a variety of the modalities used in this research, and they will all be discussed in further detail below. Phase Contrast Microscopy is a potential capability of the Keck 3DFM, if the appropriate objectives were purchased; however because of the lack of Phase Contrast lenses for the Keck 3DFM we used the Nikon Diaphot in our laboratory for Phase Contrast Microscopy. We used Differential Interference Contrast (DIC) Microscopy on the Keck 3DFM. Both imaging modalities employ the use of specialized light modulators to enhance the
contrast in thin or low contrast specimens, and the images are collected by a SPOT camera (Diagnostic Instruments, Sterling Heights, MI).

The Keck 3DFM has a variety of modalities for fluorescence imaging. Epifluorescence imaging uses widefield excitation from a mercury lamp and widefield emission and image collection by a SPOT camera to collect the fluorescence throughout an entire sample. Thick specimens may appear blurry due to the stacking of fluorescence from cells above and below the focal plane. To eliminate fluorescence from outside of the focal plane, Confocal Laser Scanning Microscopy (CLSM) employs the use of a pinhole in the emission path. Therefore, although the excitation and emission are widefield (through the entire sample), the light that is collected by the photomultiplier tube (PMT) detector is only from the focal plane. The CLSM can be adjusted so that reflected light, rather than fluorescence, is collected in a Reflectance Confocal Microscopy (RCM) mode. In a further advancement, Two-photon Laser Scanning Microscopy (TPLSM) was developed to provide a minimally invasive method of imaging fluorescence from a sample. TPLSM employs a specialized laser to pump extremely fast femtosecond pulses of long wavelength (infrared) photons onto the sample. This technique relies on the ability of those photons to hit a fluorophore simultaneously to cause fluorescence excitation. This will only happen in the focal plane, thereby eliminating out of focus fluorescence. A PMT is also used to collect the emissions from the sample. An additional benefit to TPLSM is that the long wavelength light is far less damaging to cells than the typical shorter wavelengths used in other fluorescence microscopy methods. It is important to note that the PMTs have a 56% smaller field of view than the SPOT camera. Although the long-wavelength of the TPLSM penetrates the
sample well, thick specimens show a loss of fluorescence signal approximately 200 µm into the sample.

Optical Quadrature Microscopy (OQM), formerly called the Quadrature Tomographic Microscope (QTM), was invented in Dr. Charles DiMarzio’s laboratory at Northeastern University (Hogenboom et al., 1998; Glina et al., 1999; DiMarzio et al., 2001; Stott et al., 2001; Townsend et al., 2003). OQM uses a Helium Neon 633 nm laser. A diagram of the OQM is shown in Figure 22A. Light from a 633 nm laser is split into a signal pathway, which passes through the sample and objective lens, and a reference pathway, which is shifted by a quarter wave plate and does not pass through the sample. The two pathways are recombined and analyzed from four views in order to mathematically produce the OQM image. The OQM is reflective of the absorbance throughout a sample and has been used to accurately count the number of cells in preimplantation embryos (Warger II et al., 2005; Newmark et al., 2007). Sample DIC and OQM images of mouse preimplantation stage embryos are shown in Figure 22B. The color bar indicates the change in phase of light through the sample. This modality was used in conjunction with the other Keck 3DFM modalities for multimodal imaging of ES cells (Appendix 3, Figures 67 and 68).

The Keck 3DFM also has a Bioptechs Delta T Culture Chamber (Butler, PA) for long-term imaging of live samples. For our research, pluripotent stem cells were placed in a 2 ml volume of culture medium in the Bioptechs Delta T dish. An oil overlay was added to help prevent evaporation. The dish was placed on the stage, which provides heat, and a heated lid was placed on top to seal the chamber. A regulated line of humidified, mixed gas (5% CO₂, 5% O₂, and 90% N₂) was connected to the heated lid
and the chamber was turned on. Timelapse DIC, CLSM, or TPLSM imaging was performed on pluripotent stem cells in the chamber for up to 24 h.

The images collected for this research were analyzed using Metamorph software (Universal Imaging Systems, Downingtown, PA). Metamorph was used to collate stacks of image slices from 3D sectioning experiments or timelapse images. The brightness and contrast of some images was adjusted, and some images were pseudocolored for effect. Additionally, fluorescence images were overlayed such that multiple colors from multiple indicators could be viewed simultaneously.

**Long-term Culture of Pluripotent Stem Cells**

Pluripotent stem cells were cultured for a long-term period in order to determine the stability of their phenotypes. All three pluripotent stem cell lines were cultured for 100 passages and their mitochondrial and apoptotic profiles were assessed every five passages. The mitochondrial and apoptosis assays included JC-1, TMRE, Annexin V, and caspase 3. Alkaline phosphatase expression was analyzed at P54, a midpoint. Additionally, Oct-4 and SSEA1 expression were assessed at P100 and compared to early passage data.

**Statistical Analyses**

We calculated means and standard deviations from the data. Then, a series of statistical analyses were performed. These analyses are summarized in Table VI. All three pluripotent stem cell lines were compared using the single factor Analysis of Variance (Anova), and then the individual pluripotent stem cell lines were compared in
pairs by using the t-test. In order to analyze the 14 day differentiation data, a two factor Anova with replication was used to analyze all three pluripotent stem cell lines and then the two factor Anova with replication was used again to compare pairs of pluripotent stem cell lines. We also separately analyzed D1-D7 and pD1-pD7 as compared to the overall 14 day differentiation. The long-term cell culture data were analyzed using the two factor Anova without replication statistical test first on all three pluripotent stem cell lines and second using pairs of pluripotent stem cell lines. The MTT assay required the use of the single factor Anova to compare the three pluripotent stem cell lines and t-tests to compare pairs of pluripotent stem cell lines. For the MTT assay, each stage of differentiation was analyzed separately because the nature of the assay made it inappropriate to use a two factor Anova to analyze the three pluripotent stem cell lines across the four separate stages of differentiation that were analyzed. For all tests, statistically significant results were considered to be $P < 0.05$. 
Figure 17: Relevant Markers of Apoptosis. A) Annexin V binds to phosphatidlyserine that has flipped in the cell membrane of an apoptotic cell (early marker of apoptosis). Reprinted by permission from Macmillan Publishers Ltd.: Nature Reviews Molecular Cell Biology, Orrenius et al. (2003) 4: 552-565. B) Mitochondrial membrane leakage (early to intermediate apoptosis) releases cytochrome c. Caspase 3 (intermediate marker of apoptosis) is a place of convergence of the intrinsic and extrinsic apoptotic pathways. DNA fragmentation, which can be detected by the TUNEL assay (late marker of apoptosis), is a final step in apoptosis. Reprinted with permission from Imgenex Corp.
Figure 18: Flow Cytometry Instrumentation. A) Our Becton Dickinson FACScan. B) Diagram depicting the laser path and readout channels for a FACScan. Our instrument does not have a FL 4 channel. Reprinted with permission from www.facs.scripps.edu.
Figure 19: Example of a Flow Cytometry Histogram. The x-axis marks the fluorescence intensity on a log scale while the y-axis marks the number of cells (counts) on a linear scale. Typically a histogram includes an unstained autofluorescence control (the purple histogram) and a positive sample (the green histogram). Newmark and Warner, unpublished data.
Figure 20: Example of a Flow Cytometry Quadrant Analysis.
Cells are plotted with the log scale of fluorescence 1 (green fluorescence) on the x-axis and log scale fluorescence 2 (red fluorescence) on the y-axis. When we draw a cross we create quadrants and can measure the percentages of cells in each quadrant. Newmark and Warner, unpublished data.
Figure 21: The Keck 3D Fusion Microscope.  A) Diagram depicting the multi-modality of the Keck 3DFM for "same place-same time" imaging (Warner et al., 2004).  B) Images of the Keck 3DFM; Newmark and Warner, unpublished.
Figure 22: Optical Quadrature Microscopy.  A) Diagram of OQM laser path. The laser splits into a reference path (REF) and a signal path (SIG) going through the sample, and then the signals are recombined before image collection by the four cameras (Warger II et al., 2005).  B) Preimplantation stage mouse embryos imaged by DIC (grayscale) and OQM (color).  The color bar indicates the change in phase of light through the sample.  The red end of the spectrum is associated with background while the blue end of the spectrum is associated with a greater phase change (Warner et al., 2004).
Table III: Pluripotent Stem Cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>C57BL/6</th>
<th>iPS</th>
<th>mtGFP-tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Mouse</td>
</tr>
<tr>
<td>Tissue Source</td>
<td>Blastocyst</td>
<td>MEF</td>
<td>Blastocyst</td>
</tr>
<tr>
<td>Vendor</td>
<td>Open Biosystems</td>
<td>Stemgent</td>
<td>Derived in our lab</td>
</tr>
<tr>
<td>Category</td>
<td>Assay/Marker</td>
<td>Purpose</td>
<td>Source</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-----------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>Mycoplasma (Hoechst)</td>
<td>Hoechst stains cell nuclei; mycoplasma would show cytoplasmic or extracellular staining</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>MTT</td>
<td>Measures cell proliferation</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>alkaline phosphatase (AP)</td>
<td>Enzymatic assay; AP activity indicates pluripotency</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>Oct-4</td>
<td>Antibody to detect presence of Oct-4 (pluripotency)</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>Karyotype</td>
<td>Assay for chromosome number and normality</td>
<td>Cell Line Genetics</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>AFP</td>
<td>Antibody to detect endoderm</td>
<td>Dako</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>Bry</td>
<td>Antibody to detect mesoderm</td>
<td>Abcam</td>
</tr>
<tr>
<td>Validation of Pluripotent Stem Cell Lines</td>
<td>Nestin</td>
<td>Antibody to detect ectoderm</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mitochondrial Assays</td>
<td>JC-1</td>
<td>Stains mitochondria differently based on mitochondrial membrane potential</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Mitochondrial Assays</td>
<td>TMRE</td>
<td>Stains mitochondria so that fluorescence intensity correlates with mitochondrial membrane potential</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Apoptosis Assays</td>
<td>Annexin V</td>
<td>Stains phosphatidlyserine molecules on the outside of the membrane of apoptotic cells; early apoptosis marker</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Apoptosis Assays</td>
<td>JC-1</td>
<td>Stains mitochondria and detects loss of mitochondrial membrane potential; intermediate apoptosis marker</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Apoptosis Assays</td>
<td>TMRE</td>
<td>Stains mitochondria and detects loss of mitochondrial membrane potential; intermediate apoptosis marker</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Apoptosis Assays</td>
<td>caspase 3</td>
<td>Stains caspase 3; intermediate apoptosis marker</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Apoptosis Assays</td>
<td>TUNEL</td>
<td>Stains DNA strand breaks; late apoptosis marker</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
Table V: Imaging and Detection Modalities

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase Contrast microscopy</td>
<td>Nikon Diaphot microscope in our lab</td>
<td>Enhances contrast by changing the phase difference between the object and the background</td>
</tr>
<tr>
<td>DIC microscopy</td>
<td>Keck 3DFM</td>
<td>Enhances contrast by shearing polarized light such that the gradient of the phase is reflected in the image</td>
</tr>
<tr>
<td>Epifluorescence microscopy</td>
<td>Keck 3DFM, Nikon microscope in our lab</td>
<td>Illuminates and detects fluorescence in a wide-field image</td>
</tr>
<tr>
<td>Confocal microscopy</td>
<td>Keck 3DFM</td>
<td>Uses a pinhole to detect fluorescence from a single focal plane</td>
</tr>
<tr>
<td>Two-photon microscopy</td>
<td>Keck 3DFM</td>
<td>Uses the interaction of 2 low-intensity photons to excite fluorescence in a single focal plane</td>
</tr>
<tr>
<td>FACScan</td>
<td>Our lab</td>
<td>Detects fluorescence one cell at a time in a population of cells streaming through a flow cytometer</td>
</tr>
</tbody>
</table>
**Table VI: Statistical Analyses**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Statistical Test</th>
<th>Data Analyzed</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single factor Anova</td>
<td>MTT</td>
<td>AP</td>
<td>Oct-4</td>
<td>AFP</td>
<td>Bry</td>
</tr>
<tr>
<td>All three pluripotent cell lines: Undiff.</td>
<td>t-test</td>
<td>AP</td>
<td>Oct-4</td>
<td>Oct-4</td>
<td>AFP</td>
<td>Bry</td>
</tr>
<tr>
<td>Pairs of stem cell lines: Undiff.</td>
<td>Two factor Anova</td>
<td>JC-1 Green</td>
<td>JC-1 Green</td>
<td>JC-1 Green</td>
<td>JC-1 Green</td>
<td>JC-1 Green</td>
</tr>
<tr>
<td>All three pluripotent cell lines: 14 Days Diff.</td>
<td>Two factor Anova with replication</td>
<td>JC-1 double positive</td>
<td>JC-1 double positive</td>
<td>JC-1 double positive</td>
<td>JC-1 double positive</td>
<td>JC-1 double positive</td>
</tr>
<tr>
<td>Long-term cell culture</td>
<td>Two factor Anova without replication</td>
<td>MTT assay</td>
<td>GFP expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTT assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* mtGFP-tg not included
RESULTS

mtGFP-tg Mice and Embryonic Stem Cells

Mice

At the beginning of this research, we considered several methods for visualizing mitochondria in ES cells. Mitochondrial specific dyes were used, but they were transient (could not be used for timelapse imaging) and were unable to penetrate the thick EBs. We also considered using a kit such as Invitrogen’s Organelle Lights Mito-GFP kit, but this would also provide only transient labeling. Therefore, we decided that developing an ES cell line from a mouse with GFP labeled mitochondria would provide the best opportunity for visualizing mitochondria in ES cells and their progeny. We imported three mtGFP-tg male mice from Japan (Shitara et al., 2001; Nagai et al., 2004) for the purpose of creating a mtGFP-tg ES cell line. These mice were bred to C57BL/6 females and the F1 progeny were used to confirm GFP expression in the mtGFP-tg mice. The ratio of F1 progeny expressing GFP to those not expressing GFP was approximately 1:1 for all three founder males, which means that the founder males were all heterozygous. We analyzed several tissues, including oocytes, for GFP expression. Figure 23 shows fluorescent images from a selection of these tissues. Figure 23A shows a DIC image of a mtGFP-tg mouse oocyte along with the corresponding pseudocolored TPLSM and CLSM images of mitochondrial GFP fluorescence. The mitochondria were more heavily concentrated in the center of the oocyte, but they were also spread diffusely throughout much of the oocyte. Figure 23B shows mitochondrial GFP, by Epifluorescence Microscopy, in an oviduct and uterus, liver, and bronchi. The oviduct and uterus were uniformly fluorescent, the liver had spots of high intensity, and the bronchi were
particularly fluorescent at their edges. Overall, the fluorescence was extremely bright and stable, and it was expressed in all of the tissues sampled.

Our next step was to develop a genotyping protocol, using ear punches as a tissue source, in order to analyze zygosity in the progeny of the original mice. A real-time PCR protocol successfully amplified GFP. Figure 24 shows the real-time PCR results from one experiment on eight mice. The cycle number is plotted vs. the fluorescence intensity of the SYBR green DNA labeling. Samples A-D show increasing fluorescence beginning around cycle 25 and they cross the green horizontal threshold by cycle 40. Thus samples A-D are positive for the GFP transgene. Samples A and B cross the threshold several cycles before samples C and D cross the threshold, indicating that samples A and B likely have more copies of the GFP transgene than samples C and D. The other four samples show random background fluorescence that does not cross the threshold and therefore, we concluded that they were negative for the GFP transgene. Without having a known homozygous tissue sample to analyze in comparison with our unknown samples, the genotyping protocol could not be used to determine zygosity with certainty. Since our main requirement was the expression of GFP, we decided that homozygosity vs. heterozygosity was not important when choosing mice to breed to create embryos for ES cell development. Therefore, instead of using PCR we decided that analyzing the ear punches using Epifluorescence microscopy would be sufficient for simply showing GFP expression (phenotyping). Figure 25 shows an example of a GFP negative ear punch and a GFP positive ear punch from mice in our mtGFP-tg mouse colony. The GFP negative mouse has autofluorescent hair shafts, but there is no fluorescence visible in the tissue. The GFP positive mouse has autofluorescent hair shafts and GFP fluorescence in the
tissue. The bright fluorescent spots at the base of the hair shaft are approximately 40 µm in size and most likely are hair follicles. Thus, the mouse colony was maintained as a mtGFP positive colony without knowledge of zygosity.

**ES Cells**

In order to create our mtGFP-tg ES cell line, GFP phenotypically positive mice were crossed to produce one-cell embryos, which were harvested and analyzed for GFP expression using Epifluorescence microscopy. Figure 26A shows an Epifluorescence Microscopy image of one of four GFP positive mtGFP-tg 1-cell embryos that were harvested for our first attempt to make mtGFP-tg ES cells. The mtGFP-tg positive embryos were plated onto PMEFs, and they successfully attached. Figure 26B shows Phase Contrast Microscopy and Epifluorescence Microscopy images of the ICM outgrowth, on a PMEF feeder layer, which resulted in the mtGFP-tg ES cell line shown in Figure 26C. The ICM outgrowths were passaged, and one outgrowth from our first attempt and one outgrowth from our second attempt were able to continue growing in culture (mtGFP-tg1 and mtGFP-tg2). Phase Contrast Microscopy and Epifluorescence Microscopy images of mtGFP-tg1 at passage 1 (P1) on a PMEF feeder layer are shown in Figure 26C. Two ES cell colonies are visible. The overall morphology of both mtGFP-tg ES cell lines appeared normal in comparison to a C57BL/6 ES cell line. However, the mtGFP-tg2 ES cell line had a slower rate of proliferation than C57BL/6 ES cells and mtGFP-tg1 ES cells and was not further characterized.

The mtGFP-tg1 ES cell line was further characterized and is referred to as mtGFP-tg (without the “1”) throughout this dissertation. First, the mycoplasma assay detected no evidence of mycoplasma in the mtGFP-tg ES cell line. Figure 27 shows DIC
and Epifluorescence Microscopy images of a Hoechst stained mtGFP-tg ES cell colony. Negativity for mycoplasma was concluded because there was no Hoechst staining in the cytoplasm or outside of the cells. Next, mtGFP-tg ES cells were shown to express alkaline phosphatase (AP) activity and Oct-4 pluripotency markers. Figure 28A shows a Phase Contrast Microscopy image of the AP assay on mtGFP-tg ES cells. Pink, AP positive, colonies are visible on the PMEF feeder layer. The vast majority of colonies were AP positive. Figure 28B shows an Epifluorescence Microscopy image of Oct-4 (red) staining in mtGFP-tg ES cells. The ES cell colonies were positive for Oct-4 staining whereas the PMEF feeder layer did not have Oct-4 staining. We sent a sample of mtGFP-tg ES cells to a company, Cell Line Genetics, and they determined that the mtGFP-tg ES cells had a normal, male karyotype (Figure 28C). Additionally, we were able to maintain the cell line for more than 100 passages demonstrating long-term stability.

Upon applying our differentiation protocol, mtGFP-tg ES cells readily formed EBs, and they differentiated into a wide variety of tissues after plating the cells onto gelatin. The differentiated tissues included the formation of beating cells. Figure 29A shows Phase Contrast Microscopy images from the EB and pEB stages of growth. The third image shows a pEB that has a region of cells that were beating (circled). Movie 5 shows, by live TPLSM imaging, pD7 mtGFP-tg cells that are beating. Many of the cells were GFP negative for reasons that will be discussed later. The mtGFP-tg cell line was analyzed in duplicate from ES cell through 14 days of differentiation (seven days of EB growth and seven days of pEB growth) for AP activity (by microscopy) and germ layer expression (by FACScan) in order to quantitate differentiation. The results are expressed
as mean percent positive ± SD for AP and mean fluorescence ± SD for each germ layer. Figure 29B graphs the 14 day differentiation vs. the percentage of AP positive colonies. The percentage of AP positive colonies dropped dramatically throughout development to reach almost 0% positive. Figure 29C graphs the 14 day differentiation vs. the relative mean fluorescence of the AFP (endoderm), Bry (mesoderm), and Nestin (ectoderm) germ layer markers. All three germ layer markers were lower in the early EB stages compared to ES cells. However, the markers increased so that they were higher than ES cell expression by the end of differentiation. Bry expression was consistently lower than expression of the other two germ layer markers, but comparisons among the three markers should not be interpreted strictly as the three different antibodies used most likely have different binding capabilities. Statistical analyses of AP activity, Oct-4 expression, and germ layer expression are presented together with the data from the other two pluripotent stem cell lines in the next section (C57BL/6 ES and iPS). All together, these results provide evidence that the mtGFP-tg cell line that we produced is a healthy, pluripotent ES cell line.

Next, we analyzed GFP expression in mtGFP-tg cells by fluorescence microscopy of ES cells, EBs, and plated EBs. The GFP fluorescence was extremely bright and stable, allowing us to perform 3D sectioning of the samples using CLSM or TPLSM. Figure 30 shows examples of DIC, Epifluorescence, and TPLSM imaging from ES cells (A), an EB (B), and a pEB (C). The mtGFP-tg ES cell colony in Figure 30A has bright, concentrated spots of fluorescence at the edges of the nuclei. Epifluorescence Microscopy of the mtGFP-tg EB in Figure 30B is blurry due to the thickness of the sample. However, the TPLSM image shows rings of mitochondrial GFP fluorescence surrounding the cell.
nuclei. The mitochondrial distribution pattern in the pEB in Figure 30C is somewhat variable, most likely due to the various cell types present in the sample. To image differentiating mtGFP-tg ES cells, we placed the cells into a Bioptechs Delta T culture chamber on the Keck 3DFM and imaged the formation of EBs by TPLSM once every 15 min. Movie 6 shows a small clump of cells that aggregate together, and then the cluster of cells grows in size. The mitochondrial distribution changes rapidly between the frames, and intensity across the EB is variable. It would be extremely informative to repeat this experiment by imaging once a minute (instead of once every 15 min) and to collect the corresponding DIC images as well. Using the Keck 3DFM, this experiment would have to be performed using manual image collection, rather than automated image collection, and it would be difficult to switch between the TPLSM and DIC modes within one minute. Thus the experiment would be difficult, although not impossible.

Interestingly, we noticed during data collection that the GFP fluorescence expression FACScan histogram shifted so that the mean fluorescence intensity was decreasing during the 14 day differentiation of the mtGFP-tg ES cells. Although there were still cells expressing high levels of GFP fluorescence, the range of fluorescence intensity values broadened so that many cells were also expressing lower levels of GFP fluorescence. We assessed GFP expression in mtGFP-tg ES cells in duplicate by FACScan analysis throughout the 14 day differentiation period and the results are expressed as mean fluorescence ± SD in Figure 31A. We analyzed the data by two-factor Anova without replication and found that the GFP mean fluorescence intensity for the population of cells did in fact decrease significantly throughout differentiation (P < 0.001). We attempted to identify the source of the decrease in GFP mean fluorescence
intensity, which could be due to a loss of mitochondria or a loss of GFP expression in cells, by Epifluorescence imaging of pEBs. Although we found no consistent pattern of GFP negative cells, there was a trend towards patches of cuboidal epithelial type cells that were negative. Figure 31B shows DIC and Epifluorescence Microscopy images of two examples of patches of GFP negative cells (circled). These cells have a morphology typically associated with epithelial cells. A few potential explanations for the observed loss of GFP expression in differentiating mtGFP-tg ES cells are presented in the Discussion section.

We took advantage of the brightness and stability of GFP in the mtGFP-tg ES cell line to do more imaging of mitochondria throughout the differentiation process. Four examples of mtGFP-tg ES cells were imaged by DIC, Epifluorescence, and CLSM and they are shown in Figure 32. The first two examples were imaged using low (20x) magnification whereas the second two examples were imaged using high (60x) magnification. The pattern of mitochondrial distribution is variable, although in the higher magnification images there are distinct clusters of mtGFP fluorescence next to the cell nuclei. Movie 7 presents a z-sectioned stack of CLSM images taken every 2 µm through a mtGFP-tg ES cell colony. Again, although mtGFP fluorescence is apparent throughout the cytoplasm, there are distinct clusters of fluorescence next to the cell nuclei.

Figure 33 shows three examples of D7 mtGFP-tg EBs imaged by DIC, Epifluorescence, CLSM, and TPLSM. The Epifluorescence images are completely blurred due to the thickness of the EBs. CLSM and TPLSM are better able to resolve the mitochondrial distribution in individual cells. mtGFP fluorescence is spread through
more of the cytoplasm than the mtGFP fluorescence in ES cells (Figure 32). However, there are still distinct clusters of mtGFP fluorescence. Movies 8A and 8B show z-sectioned stacks, taken every 5 µm, of CLSM and TPLSM images from a mtGFP-tg D7 EB. The TPLSM images have better resolution than the CLSM images, but they both show variability in mitochondrial distribution albeit with distinct clusters. To the best of our knowledge, these movies represent the first imaging of mitochondria throughout an EB.

Finally, Figure 34 shows DIC and Epifluorescence Microscopy images of pD7 EBs. Inconsistency of the GFP expression by pD7 is seen as evidenced by fluorescently negative regions of cells in pD7 EBs and will be discussed more thoroughly in the Discussion section. The pattern of mitochondrial distribution in pD7 EBs is extremely variable. There are regions of diffuse and clustered mitochondria. Throughout all of the mtGFP-tg images, we did not find a single pattern of mitochondrial distribution. The mitochondria appeared perinuclear, diffuse, or clustered. The mitochondrial localization in ES cells, EBs, and pEBs perhaps depends on the stage of the cell cycle and the health of the individual cell and will be addressed in the Discussion. In summary, the creation of mtGFP-tg ES cells has allowed us to image, for the first time, mitochondria during the growth and differentiation of ES cells.

**Validation of Rate of Proliferation and Pluripotency in all Stem Cell Lines**

**Rate of Proliferation**

We began our comparison of the mtGFP-tg pluripotent stem cell line to a C57BL/6 ES cell line and an iPS cell line by analyzing the rate of proliferation of all
three pluripotent stem cell lines by using the MTT assay and measuring absorbance. We assessed all three cell lines in replicate at four different time points during differentiation: pluripotent (ES) cell growth (n=18), EB formation from ES cells (n=12), EB growth (C57BL/6 and iPS n=10, mtGFP-tg n=8), and pEB growth (C57BL/6 n=20, iPS and mtGFP-tg n=19). The graphs of mean absorbance ± SD are shown in Figure 35, and we analyzed the significance using a single factor Anova and t-tests. ES cells had low absorbance values, and there were no significant differences among the three cell lines. During EB formation the absorbance readings were much higher, and all three cell lines were significantly different from one another by a single factor Anova (P < 0.001). The iPS cell line had a higher proliferation than the C57BL/6 cell line, and the mtGFP-tg cell line proliferation was even higher than the iPS cell line. t-tests also showed that all cell lines were significantly different from one another (P < 0.001), except the C57BL/6 cell line, which was significantly lower than the iPS cell line by P < 0.05. During EB growth, there were no significant differences between the cell lines. However, during pEB growth the three cells lines were significantly different from one another (P < 0.001 by single factor Anova and t-tests) with C57BL/6 cells having the lowest proliferation and the iPS cells having the highest proliferation (Tables VIII-XI).

**Pluripotency**

First, AP activity was assessed, by imaging, for all three cell lines in duplicate during 14 days of differentiation. Figure 36 shows Phase Contrast Microscopy images of the three cell lines at pluripotency (undifferentiated), at D3 EB, and at D7 pEB. Pluripotent cells had high AP activity in most colonies whereas the activity was almost completely eliminated at pD7. Figure 37 shows a graph of the 14 day differentiation vs.
AP activity as mean percent positive colonies ± SD. AP activity in undifferentiated cells was analyzed by using a single factor Anova among the three cell lines or by t-tests between pairs of cell lines (Table VII). The results for differentiation were analyzed by using a two factor Anova with replication (Tables VIII-XI). The results show that AP activity in all three cells lines was the same in undifferentiated pluripotent stem cells. The AP activity in the differentiated cells significantly decreased throughout the 14 day differentiation to almost 0% positive colonies. These data included the significant decreases in AP activity that occurred during both D1-D7 and pD1-pD7. Additionally, there were significant differences among the cell lines (Tables VIII-XI). The C57BL/6 and mtGFP-tg ES cell lines, which were not significantly different from one another, generally had higher AP activity than the iPS cell line.

We tested a second pluripotency marker by using the Oct-4 marker. Oct-4 was measured for all three pluripotent stem cell lines, in duplicate by FACScan analysis, in undifferentiated stem cells and on alternate days during the 14 day differentiation process. The results are presented as mean fluorescence ± SD in Figure 38. Oct-4 expression in undifferentiated cells was analyzed by using a single factor Anova among the three cell lines or by t-tests between pairs of cell lines (Table VII). The results for differentiation were analyzed by using a two factor Anova with replication (Tables VIII-XI). Undifferentiated pluripotent stem cells had high levels of Oct-4 that were not significantly different among the cell lines. Oct-4 showed significantly decreased expression with overall differentiation, as expected. Notably, Oct-4 decreased significantly during D1-D7, but generally had no significant change in expression during pD1-pD7. Oct-4 had a transient increase in expression on D7 that may be due to day to
day variation. There were generally no significant differences among the cell lines during any stage of differentiation. The one exception to these observations occurred when comparing the mtGFP-tg ES cell line with the iPS cell line during pD1-pD7 (Table X). The results showed a significant decrease with differentiation, most likely due to the lower Oct-4 expression values at pD4 compared to the C57BL/6 ES cell line. Additionally, the mtGFP-tg ES cell line had significantly lower Oct-4 expression than the iPS cell line.

We analyzed AFP (endoderm), Bry (mesoderm), and Nestin (ectoderm) germ layer expression for all three pluripotent stem cell lines, in duplicate by FACScan analysis, in undifferentiated cells and on alternate days during the 14 day differentiation process. The results are expressed as mean fluorescence relative to undifferentiated cells ± SD in Figure 39. Germ layer expression in undifferentiated cells was analyzed by using a single factor Anova among the three cell lines or by t-tests between pairs of cell lines (Table VII). The results for differentiation were analyzed by using a two factor Anova with replication (Tables VIII-XI). The results for undifferentiated pluripotent stem cells show no significant differences in germ layer expression among the three cell lines. We expected loss of pluripotency during differentiation, as evidenced by diminishing AP activity (Figure 37) and Oct-4 expression (Figure 38), to correlate with an increase in germ layer marker expression. However, all three germ layer markers actually decreased during the pluripotent stem cell to EB transition. The germ layer markers then increased significantly throughout overall differentiation. There were several individual differences among the germ layer markers during the D1-D7 and pD1-pD7 stages of differentiation. Additionally, there were a few instances during the D1-D7
and pD1-pD7 stages of differentiation where the cell lines showed significant differences from one another. However, there were no significant differences among the cell lines, for any germ layer marker, during overall differentiation.

We attempted to correlate the expression of each germ layer marker with mitochondrial localization by immunostaining mtGFP-tg pEBs for each marker and then imaging them by Epifluorescence Microscopy. The staining results are presented in Figure 40. The three germ layer markers (red) tended to be expressed mostly at the edges of the pEB colonies, adjacent to bright mtGFP staining (green). Areas with high intensity staining are marked in Figure 40 with white arrows, and areas of overlapping germ layer and mtGFP expression are yellow. Although there are several regions of overlapping germ layer and mtGFP expression, there are also areas where the two markers are separated. mtGFP should be expressed in every cell but, as discussed previously, GFP negative cells appear with differentiation. As expected with a mix of tissue types, each germ layer marker appeared in patches rather than throughout the entire sample. Although we could not correlate AFP expression with a pattern of mitochondrial localization, Bry was associated with cells containing clustered mitochondrial fluorescence and Nestin was associated with cells containing perinuclear mitochondrial fluorescence.

**Mitochondrial Activity and Localization in Pluripotent Stem Cell Lines**

We analyzed mitochondrial activity and distribution in pluripotent stem cells and through 14 days of differentiation using the dyes JC-1 and TMRE, and the endogenous fluorescence of mtGFP-tg ES cells. A summary of the statistical analyses of all the data
is included in Tables VII-XI. In cells stained with JC-1, active mitochondria fluoresce red whereas inactive mitochondria fluoresce green. Thus double positive (red and green fluorescence) cells contain both active and inactive mitochondria, green fluorescent cells contain only inactive mitochondria, and red fluorescent cells contain only active mitochondria. Most cells contain a mixture of active and inactive mitochondria; therefore our analysis of mitochondrial activity was focused on the percentage of double positive cells. Figure 41 presents the results from one experiment (from duplicates) of JC-1 staining analyzed by FACScan quadrant analysis. One set of cells was also treated with the mitochondrial inhibitor CCCP in order to test the effectiveness of JC-1 staining. The results of JC-1 staining in pluripotent stem cells were analyzed by single factor Anova and t-tests (Table VII). In the quadrant analyses presented in Figure 41 the upper left quadrant contains JC-1 red single positive cells (active mitochondria only), the upper right quadrant contains JC-1 double positive cells (active and inactive mitochondria), and the lower right quadrant contains JC-1 green single positive cells (inactive mitochondria only). The lower left quadrant contains poorly stained or compromised cells. JC-1 staining of pluripotent stem cells revealed mitochondrial activity to be quite high with 81-97% double positive (active and inactive mitochondria) cells (Figure 41). The remaining cells were primarily green single positive (inactive mitochondria only) cells (3-18%). Treatment of pluripotent stem cells with the mitochondrial inhibitor CCCP brought the mitochondrial activity (double positive cells) down to less than 20%. There were no significant differences among the cell lines (Table VII).

Figure 42 shows images of the three pluripotent stem cell lines stained with Hoechst and JC-1 and then imaged using DIC and Epifluorescence microscopy. The
The majority of the active mitochondria were located on the outside of the stem cell colonies (red staining) whereas inactive mitochondria (green staining) were found throughout the colonies. Three-dimensional sectioning of these stem cell colonies by Epifluorescence or TPLSM and CLSM confirmed that the red (active mitochondria) fluorescence was on the outside of the colonies (Movies 9, 10A, 10B). Movie 9 shows z-sectioning, by Epifluorescence Microscopy, every 5 µm through a C57BL/6 ES cell colony stained with Hoechst (blue) and JC-1 (active mitochondria in red and inactive mitochondria in green). The active mitochondria were located at the edges of the ES cell colony whereas the inactive mitochondria were found throughout the colony. Movies 10A and 10B show z-sectioning, by TPLSM (Movie 10A, inactive mitochondria) and CLSM (Movie 10B, active mitochondria), every 5 µm through a C57BL/6 ES cell colony stained with JC-1. Again, the active mitochondria were located at the edges of the ES cell colony whereas the inactive mitochondria were found throughout the colony. These results cannot be attributed to a dye penetration issue as evidenced by the JC-1 green (inactive mitochondria) fluorescence within the colonies. Additionally, we stained single cell suspensions from each cell line during passaging and then plated them to ensure dye penetration. The results also showed mitochondrial activity to be limited to the edge of the colonies (data not shown). Furthermore, JC-1 staining of ES cells performed in another lab produced similar results (personal communication, Celine Filippi, University of Edinburgh, ISSCR Annual Conference 2008).

TMRE was also used to stain pluripotent stem cells for mitochondrial activity. Figure 43 shows the fluorescence histogram results of one experiment (from duplicates) analyzed by FACScan. The results of TMRE staining (thick lines) in pluripotent stem
cells were analyzed by single factor Anova and t-tests (Table VII). The results confirmed the high mitochondrial activity that we saw by using JC-1 staining (Figure 41). There were no significant differences among the cell lines (Tables VII).

We questioned whether GFP fluorescence in the mtGFP-tg ES cells would reflect mitochondrial activity, given that the GFP label was conjugated to the cytochrome c oxidase subunit VIII which is actively involved in respiration, or just simply serve as a marker of mitochondrial presence. A comparison of GFP fluorescence with TMRE fluorescence by FACScan dot plot analysis did not produce a diagonal slope (Figure 44). Instead, the data produced an almost straight line correlating to a wide range of GFP fluorescent values with only a small range of TMRE fluorescent values. Therefore, mtGFP fluorescence may be used as a marker for mitochondrial localization, but it does not correlate well with mitochondrial activity as assessed by TMRE staining.

JC-1 staining of all three pluripotent stem cell lines was assessed, in duplicate by FACScan analysis, through 14 days of differentiation and the results are expressed as mean percent positive ± SD in Figure 45. The results were analyzed by using a two factor Anova with replication (Tables VIII-XI). Figure 45 presents the results of JC-1 staining with JC-1 double positive cells (active and inactive mitochondria), JC-1 green single positive cells (inactive mitochondria only), and JC-1 red single positive cells (active mitochondria only) separated into three graphs. All three pluripotent stem cell lines demonstrated a significant decrease in double positive cells (mitochondrial activity) throughout differentiation. Concurrently, the percentage of JC-1 green (inactive mitochondria only) cells increased significantly throughout differentiation whereas the percentage of JC-1 red (active mitochondria only) cells remained near zero percent.
Therefore, the decrease in double positive cells (active and inactive mitochondria) was linked directly to the increase in cells with only inactive mitochondria. The JC-1 red (active mitochondria only) cells did show a slight, but significant, increase in the percentage of positive cells throughout differentiation. Despite the decrease in double positive cells, mitochondrial activity remained fairly high as approximately 70% of cells were still double positive with JC-1 staining at the end of the time course.

There were several statistical differences in JC-1 staining among the cell lines throughout differentiation. There was a tendency for the iPS cell line to have a higher percentage of JC-1 double positive cells and a lower percentage of JC-1 green single positive cells than the other two cell lines. The mtGFP-tg ES cell line tended to have a lower percentage of double positive cells and a higher percentage of JC-1 green single positive cells than the other two cell lines. Thus, the iPS cells had the highest mitochondrial activity and the mtGFP-tg ES cells had the lowest mitochondrial activity.

We stained pD7 EBs from all three pluripotent stem cell lines with Hoechst and JC-1 and then imaged them by Epifluorescence Microscopy. The results are presented in Figure 46. Hoechst stained nuclei are blue, JC-1 stained inactive mitochondria are green, and JC-1 stained active mitochondria are red. The C57BL/6 and iPS cell lines have regions containing high mitochondrial activity (red), but there are also several cells without active mitochondria. The mtGFP-tg cells are almost uniformly positive for mitochondrial activity (red staining). In other areas of the mtGFP-tg pEB there was more variability in mitochondrial activity (data not shown), however the images were not as clear as the one shown in Figure 46. Overall, the imaging data generally match the
FACScan analyses that determined that approximately 70% of the cells in pD7 EBs were positive for mitochondrial activity.

TMRE staining of all three pluripotent stem cell lines was assessed, in duplicate by FACScan analysis, through 14 days of differentiation and the results are expressed as mean fluorescence ± SD in Figure 47. The results were analyzed by using a two factor Anova with replication (Tables VIII-XI). TMRE staining, as presented in Figure 47, also confirmed a significant decrease in mitochondrial activity throughout the 14 days of differentiation. All three pluripotent stem cell lines displayed similar losses of mitochondrial activity, although there were statistically significant differences among the cell lines. The mtGFP-tg cell line generally had significantly higher mitochondrial activity compared to the other two cell lines. Interestingly, the C57BL/6 cell line had significantly higher mitochondrial activity than the iPS cell line during D1-D7, but then the iPS cell line had significantly higher mitochondrial activity than the C57BL/6 cell line during pD1-pD7. For the entire 14 day differentiation period the difference between the C57BL/6 ES cell line and the iPS cell line was not significant.

**Apoptosis in Pluripotent Stem Cell Lines**

We analyzed three markers of apoptosis, Annexin V, caspase 3, and TUNEL in the C57BL/6 ES cell line and the iPS cell line. We did not perform these experiments on the mtGFP-tg ES cell line because of the interference of the endogenous green fluorescence with the assays. A summary of the statistical analyses of all the data are included in Tables VII-XI. First, Annexin V staining was assessed, in duplicate by FACScan analysis, to detect early apoptosis and PI staining was used to detect necrosis.
Figure 48 shows Annexin V (FL1) and PI (FL2) staining data from one experiment (from duplicates). In the quadrant analyses the upper two quadrants contain cells that are positive for PI (necrosis), the lower left quadrant contains healthy cells, and the lower right quadrant contains Annexin V positive (apoptotic) cells. The results of Annexin V and PI staining were analyzed by t-tests (Table VII). Annexin V stained 26-34% of the pluripotent stem cells while the levels of necrosis (PI staining) were very low in both stem cell lines (3-5%). The C57BL/6 ES cell line was not significantly different from the iPS cell line for either assay (Table VII).

We analyzed intermediate apoptosis by assessing caspase 3 staining, by FACScan analysis, in the C57BL/6 and iPS cell lines (Figure 49). Figure 49 shows one experiment (from duplicates) and graphs the mean fluorescence of caspase 3 for each cell line. Caspase 3 expression was low, and a t-test showed that there was no significant difference between the two cell lines (Table VII). All together, these data on apoptosis in C57BL/6 ES and iPS cells show that although some pluripotent stem cells have evidence of the beginning stages of apoptosis (Figure 48), there are very few apoptotic cells that continue down the apoptotic pathway (low caspase 3 expression in Figure 49).

We continued our analysis of apoptosis in the C57BL/6 ES cells and iPS cells through 14 days of differentiation. Figure 50 shows Annexin V (apoptosis) and PI (necrosis) staining expressed as mean percent positive ± SD. The results were analyzed by using a two factor Anova with replication (Table XI). The data from D5-D7 were lost due to a technical error. Early apoptosis (Annexin V staining) increased rapidly during the pluripotent cell to EB transition and then remained high throughout the rest of the differentiation process. There was no significant difference in Annexin V staining for
D1-D7. However, there was a significant decrease in Annexin V during pD1-pD7. There were no significant differences in Annexin V between the C57BL/6 and iPS cell lines at any point. Necrosis (PI staining) remained relatively low throughout differentiation for the C57BL/6 and iPS cell lines. Necrosis significantly decreased during D1-D7, increased between D7 and pD1, and then significantly decreased again through pD1-pD7. The C57BL/6 and iPS cell lines were not significantly different for D1-D7, but they were significantly different for pD1-pD7 and for overall differentiation, most likely due to the higher PI staining in iPS cells at the pD1 timepoint.

To test intermediate apoptosis in differentiating C57BL/6 ES and iPS cells we measured caspase 3 expression through 14 days of differentiation. Figure 51 shows caspase 3 staining expressed as mean fluorescence ± SD. The results were analyzed by using a two factor Anova with replication (Table XI). Caspase 3 expression increased during the pluripotent cell to EB transition, but then caspase 3 expression did not change significantly during D1-D7. However, caspase 3 activity significantly decreased during pD1-pD7. Overall, the changes in caspase 3 expression with differentiation were significant. Additionally, the iPS cell line had significantly higher levels of caspase 3 expression than the C57BL/6 ES cell line.

Late stage apoptosis was measured in duplicate by using the TUNEL assay and FACScan analysis on pluripotent (ES) cells, D7 EBs, and pD7 EBs. Figure 52A shows the negative and positive control cells (human lymphoma cells) in a quadrant analysis from one experiment. FL-1 shows staining of DNA fragmentation and FL-2 shows PI staining. Since the cells are fixed for analysis, all cells should be PI positive. The lower two quadrants show cells that were not PI positive, while the upper left quadrant shows
cells that were PI positive but were not TUNEL positive, and the upper right quadrant shows TUNEL positive cells. The negative control cells were 4% positive for the TUNEL assay while the positive control cells were 28% positive for the TUNEL assay.

Figure 52B shows the results of the TUNEL assay on the C57BL/6 and iPS pluripotent stem cell lines expressed as mean percent positive ± SD. The results were analyzed by using a two factor Anova with replication (Table XI). The percentage of TUNEL positive cells significantly increased throughout differentiation. There were differences between the cell lines with the iPS cells having a higher percentage of TUNEL positive cells at D7 and the C57BL/6 cells having a higher percentage of TUNEL positive cells at pD7. Our TUNEL results confirm that there is a general increase in apoptosis throughout the differentiation process.

We attempted to analyze the localization of apoptosis by performing caspase 3 staining on pluripotent and differentiated stem cells. Figure 53 presents the results of caspase 3 and Hoechst staining in the C57BL/6 and iPS pluripotent stem cell lines as imaged by DIC and Epifluorescence Microscopy in undifferentiated (ES) cells and pD7 EBs. Caspase 3 imaging was difficult due to the low fluorescent signal. However, caspase 3 staining in Figure 53 showed a few localized spots of caspase 3 activity in ES cell colonies (white arrows) with more caspase activity visible in pD7 EBs (white boxes). The relatively long exposure time required to view a signal (several seconds) implies low caspase 3 activity. Overall, the results from microscopic imaging confirmed an increase in apoptosis and necrosis during differentiation that was observed with FACScan analysis.
**Long-term Cell Culture of Pluripotent Stem Cells**

C57Bl/6, iPS, and mtGFP-tg pluripotent stem cell lines were cultured for 100 passages and analyzed for pluripotency, mitochondrial activity, and apoptosis in order to assess the stability of their phenotypes. A summary of the statistical analyses of all the data is shown in Table XII. During the later passages of cell culture, the pluripotent cells seemed to increase their rate of proliferation, as evidenced by the necessity to split the cells at higher ratios in order to maintain the cultures for the days between the passages. Phase Contrast Microscopy images of the cells taken at P100 showed normal pluripotent stem cell morphology, although the colonies seemed slightly more rounded in shape (Figure 54). All three cell lines were morphologically similar to one another.

Alkaline phosphatase activity was analyzed in all three pluripotent stem cell lines. Early (P15) and intermediate (P54) passages (Figure 55) were compared by using a two factor Anova without replication (Table XII). The results showed a significant decrease in AP activity by P54 when analyzing all three pluripotent stem cell lines. There were no significant differences among the cell lines. Oct-4 expression was analyzed by FACScan in all three pluripotent stem cell lines. Early (P8) and late (P100) passage pluripotent stem cells (Figure 56) were compared by using a two factor Anova without replication (Table XII). There were no significant differences in Oct-4 expression between the passages or among the cell lines.

The mitochondria in the mtGFP-tg ES cell line were analyzed by GFP expression every five passages from P5 to P100 with the results shown in Figure 57. Interestingly, GFP expression increased significantly throughout much of the long-term culture period before dropping precipitously in the last ten passages (P < 0.001 by two factor Anova).
without replication). The increase in GFP fluorescence could be due to an increase in the number of mitochondria per cell. The drop in GFP fluorescence towards the end of the long-term culture could be due to a loss in the number of mitochondria per cell or due to an increase in differentiated cells, which were shown earlier to express decreased levels of GFP. These results are discussed further in the Discussion section.

Next, JC-1 staining for mitochondrial activity, for all three pluripotent stem cell lines through 100 passages, was analyzed by FACScan and expressed as the percentage of positive cells (Figure 58). The results were analyzed by using a two factor Anova without replication (Table XII). Mitochondrial activity proved to be somewhat variable during long-term culture, although overall there was a significant increase in mitochondrial activity. The percentage of JC-1 double positive cells (active and inactive mitochondria) increased in the later passages of cells (increased mitochondrial activity) whereas the percentage of JC-1 green cells (inactive mitochondria only) decreased significantly. The percentage of JC-1 red cells (active mitochondria only) increased significantly throughout the long-term culture despite remaining near zero percent. JC-1 double positive and JC-1 green cells showed significant differences among the cell lines, however the JC-1 red results were generally not significant for differences among the cell lines. Throughout the long-term culture, the C57BL/6 ES cell line had the highest levels of mitochondrial activity (high double positive cells and low green single positive cells) and the mtGFP-tg ES cell line had the lowest levels of mitochondrial activity with the iPS cell line having intermediate levels of mitochondrial activity.

TMRE staining for mitochondrial activity, in all three pluripotent stem cell lines through 100 passages, was analyzed by FACScan and expressed as mean fluorescence
Early apoptosis was analyzed in the C57BL/6 ES cells and iPS cells, through 100 passages, by Annexin V staining and FACScan analysis, and necrosis was analyzed by PI staining and FACScan analysis. The results are presented in Figure 60 as the percentage of positive cells, and the data were analyzed by using a two factor Anova without replication (Table XII). Annexin V staining was somewhat variable throughout the long-term culture. For much of the culture period, the percentage of Annexin V positive cells was diminished and then it increased during the last 15 passages. Overall, these changes were significant. The percentage of PI positive cells (necrosis) increased significantly, with a peak at passage 55, and then it decreased significantly. There were no significant differences between the cell lines for Annexin V staining, but the C57BL/6 ES cells were significantly different from the iPS cells for PI staining. The C57BL/6 ES cell line generally had higher levels of necrosis (PI) than the iPS cell line.

Intermediate apoptosis was assessed in the C57BL/6 ES cells and iPS cells, through 100 passages, by caspase 3 staining and FACScan analysis. Figure 61 presents the data graphed as mean fluorescence over 100 passages, and the results were analyzed by using a two factor Anova without replication (Table XII). The data from P20 were
lost due to a technical error. Caspase 3 expression remained nearly the same until P75 when expression levels increased significantly. The C57BL/6 ES cell line had levels of caspase 3 that were significantly greater than the levels in iPS cells. Overall, there seemed to be a trend towards increased apoptosis during the late passages of long-term culture of the C57BL/6 ES cells and iPS cells.
Figure 23: mtGFP-tg Mouse Tissues. A) DIC, TPLSM, and CLSM images of mtGFP-tg oocytes. The green images are pseudocolored. Scale bar = 50 µm. B) Epifluorescence images of mtGFP-tg mouse tissues. Bright fluorescence is visible in all tissues. Scale bar = 300 µm.
Amplification of GFP genomic DNA

Figure 24: Real-Time PCR Amplification of the GFP Transgene from the mtGFP-tg Mouse Colony. Samples from eight mice were analyzed for GFP gene presence by quantitative real-time PCR and plotted as cycle number versus fluorescence. The fluorescence signal indicates the presence of the GFP gene, and the cycle number where the fluorescence crosses the threshold (horizontal green line) indicates the number of copies of GFP. Thus samples A-D were positive for the GFP transgene, and samples A and B likely have more copies of the GFP transgene than samples C and D. The four negative samples have random, low fluorescence that does not cross the threshold.
Figure 25: Ear Punch Phenotyping of mtGFP-tg Mice. Epifluorescence Microscopy images of ear punches from mtGFP-tg mice. Autofluorescent hair shafts are visible in the GFP negative mouse whereas GFP fluorescence appears in the tissues of the GFP positive mouse. Scale bar = 50 µm.
Figure 26: Creation of Mouse mtGFP-tg Embryonic Stem Cells. Phase Contrast (grayscale) and Epifluorescence Microscopy (green) images of a A) a 1-cell embryo, B) an isolated inner cell mass (ICM), and C) the resulting mtGFP-tg ES cell colonies (at passage 1) that were derived from the 1-cell embryo and isolated ICM shown here. GFP fluorescence is visible in embryonic tissues but not in the fibroblast feeder layer under the ES cell colonies. Scale bar = 50 µm.
Figure 27: Mycoplasma Testing of mtGFP-tg ES Cells. DIC and Hoechst Epifluorescence Microscopy images were taken, using the 100X objective, of mtGFP-tg ES cells. The ES cell colonies are outlined in white. Hoechst nuclei are visible, but we did not see cytoplasmic or extracellular staining that would be indicative of mycoplasma contamination. Scale bar = 50 µm.
Figure 28: Validation of Mouse mtGFP-tg Embryonic Stem Cells.
Figure 29: Differentiation of Mouse mtGFP-tg Embryonic Stem Cells. A) DIC images of a mtGFP-tg EB, plated EB, and a plated EB with beating cells (circled). The scale bar = 50 μm and the cells are approximately 5-10 μm in size. B) Alkaline Phosphatase activity was assessed in duplicate on mtGFP-tg ES cells through 14 days of differentiation and reported as mean percent positive colonies ± SD. C) AFP (endoderm), Bry (mesoderm), and Nestin (ectoderm) expression was assessed by FACSscan analysis in duplicate from mtGFP-tg ES cells through 14 days of differentiation and expressed as relative mean fluorescence ± SD compared to ES/pluripotent cells.
Movie 5: Beating Mouse mtGFP-tg Cells. mtGFP-tg ES cells were cultured to EBs and then plated onto gelatin for further growth. The resulting beating cells were imaged by TPLSM in real time. Due to differentiation induced loss of GFP only some of the cells are GFP positive. The corresponding DIC image is shown. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 30: GFP Expression in Mouse mtGFP-tg Embryonic Stem Cells, an Embryoid Body, and a Plated Embryoid Body. A) DIC, Epifluorescence, and TPLSM images of a mtGFP-tg ES cell colony, B) DIC, Epifluorescence, and TPLSM images of a mtGFP-tg EB, and C) DIC and Epifluorescence images of a mtGFP-tg plated EB. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Movie 6: Timelapse TPLSM of Mouse mtGFP-tg Cell Growth. mtGFP-tg ES cells were placed in a Bioptechs Delta T culture chamber on the Keck 3DFM and imaged by TPLSM every 15 min. The mtGFP-tg cells formed EBs. The corresponding DIC image taken at the start of culture is shown. Scale bar = 50 µm.
Figure 31: GFP Expression from Mouse Embryonic Stem Cells through Day 14 of Development. A) GFP expression in pluripotent stem cells was analyzed in duplicate by FACScan analysis through 14 days of development and the mean fluorescence ± SD is graphed. Statistical analysis was by two factor Anova without replication; P < 0.001 for a decrease with differentiation. B) DIC and Epifluorescence Microscopy images of GFP negative cells on pD7. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 32: GFP Imaging in Mouse mtGFP-tg Embryonic Stem Cells.
Four examples of mtGFP-tg ES cell colonies are shown with Epifluorescence and CLSM imaging of their mitochondrial GFP expression. Scale bar = 50 µm.
Movie 7: CLSM Stack of Mouse mtGFP-tg ES Cells.
mtGFP-tg ES cells were imaged, by z-sectioning, every 2 µm by DIC and CLSM. The images were pseudocolored so that the mitochondrial GFP is green. Scale bar = 50 µm.
Figure 33: GFP Imaging in Mouse mtGFP-tg D7 Embryoid Bodies. Three examples of D7 EBs are shown with Epifluorescence, CLSM, and TPLSM imaging of mitochondrial GFP expression. Epifluorescence Microscopy produces blurred images of GFP due to the thickness of the samples. Scale bar = 50 µm.
Movies 8A and B: CLSM and TPLSM Stacks of a Mouse mtGFP-tg EB. mtGFP-tg EBs were imaged, by z-sectioning, every 5 µm by DIC, CLSM (10A), and TPLSM (10B). The images were pseudocolored so that the mitochondrial GFP is green. Scale bar = 50 µm.
Figure 34: GFP Imaging in Mouse mtGFP-tg pD7 Embryoid Bodies. Three examples of plated D7 EBs are shown with Epifluorescence images of mitochondrial GFP Expression. Expression is varied across different tissues within the field. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 35: MTT Assay on Mouse Pluripotent Stem Cell and Embryoid Body Growth. Pluripotent cells from all three cell lines were analyzed, by measuring absorbance, at four timepoints during differentiation: ES/pluripotent cells (n=18), EB formation (n=12), EB growth (C57BL/6 and iPS n=10, mtGFP-tg n=8), and plated EB (pEB) growth (C57BL/6 n=20, iPS and mtGFP-tg n=19). The results are graphed as mean absorbance ± SD. Each timepoint was subjected to a single factor Anova and t-tests, and significant differences were found for EB formation (P < 0.001) and for pEB growth (P < 0.001). All cell lines at EB formation were significantly different from one another by t-tests (P < 0.001), except the C57BL/6 ES cell line which was significantly different from the iPS cell line by P < 0.05. All cell lines at the pEB stage were significantly different from one another by t-tests (P < 0.001).
Figure 36: Alkaline Phosphatase Activity in Mouse Pluripotent Stem Cells, Embryoid Bodies, and Plated Embryoid Bodies. Phase Contrast Microscopy images of Alkaline Phosphatase activity (pink) in all three pluripotent cell lines. Alkaline Phosphatase activity is minimal by D7 of pEB development. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 37: Alkaline Phosphatase Activity from Mouse Pluripotent Stem Cells through 14 Days of Development. All three pluripotent stem cell lines were analyzed in duplicate for Alkaline Phosphatase activity throughout 14 days of differentiation, and the mean percent positive ± SD is graphed. Statistical analyses for undifferentiated cells were by single factor Anova among the three cell lines or by t-tests between pairs of cell lines, and the results are shown in Table VII. Statistical analyses for differentiation were by two factor Anova with replication and the results are shown in Tables VIII-XI.
Figure 38: Oct-4 Expression from Mouse Pluripotent Stem Cells through 14 Days of Development. All three pluripotent stem cell lines were analyzed in duplicate by FACScan analysis for Oct-4 expression throughout 14 days of differentiation, and the mean fluorescence ± SD is graphed. Statistical analyses for undifferentiated cells were by single factor Anova among the three cell lines or by t-tests between pairs of cell lines, and the results are shown in Table VII. Statistical analyses for differentiation were by two factor Anova with replication and the results are shown in Tables VIII-XI.
Figure 39: Germ Layer Expression from Mouse Pluripotent Stem Cells through 14 Days of Development. All three pluripotent stem cell lines were analyzed in duplicate by FACScan analysis for germ layer expression throughout 14 days of differentiation, and the mean expression relative to undifferentiated (ES) cells ± SD is graphed. Statistical analyses for undifferentiated cells were by single factor Anova among the three cell lines or by t-tests between pairs of cell lines, and the results are shown in Table VII. Statistical analyses for differentiation were by two factor Anova with replication and the results are shown in Tables VIII-XI.
Figure 40: Mitochondrial GFP and Germ Layer Expression in Plated Embryoid Bodies. DIC and Epifluorescence Microscopy images were collected of mitochondrial GFP (green) and each germ layer marker (red). Arrows mark bright germ layer staining. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 41: JC-1 in Mouse Pluripotent Stem Cells. Undifferentiated stem cells were stained with JC-1 to assess mitochondrial activity, and the cells were analyzed by FACScan quadrant analysis. One experiment (from duplicates) is shown. FL-1 green fluorescence represents inactive mitochondria and FL-2 red fluorescence represents active mitochondria. Thus JC-1 double positive cells have both active and inactive mitochondria, JC-1 green cells have only inactive mitochondria, and JC-1 red cells have only active mitochondria. The mitochondrial inhibitor CCCP was added to one set of cells and caused a decrease in mitochondrial activity (decreased FL-2 fluorescence). Statistical analyses were by single factor Anova among the three cell lines or by t-tests between pairs of cell lines. The results are shown in Table VII.
Figure 42: JC-1 Imaging of Mouse Pluripotent Stem Cells. DIC and Epifluorescence Microscopy images of pluripotent stem cells where Hoechst stained nuclei are blue, JC-1-stained inactive mitochondria are green, and JC-1 stained active mitochondria are red. Scale bar = 50 µm.
**Movie 9: Epifluorescence Stack of JC-1 and Hoechst Stained Mouse C57BL/6 ES Cells.** C57BL/6 ES cells were stained with Hoechst and JC-1 and imaged, by z-sectioning, every 5 µm by DIC and Epifluorescence microscopy. The images were pseudocolored and overlayed so that the Hoechst stained nuclei are blue, JC-1 stained inactive mitochondria are green, and JC-1 stained active mitochondria are red. Scale bar = 50 µm.
Movies 10A and B: TPLSM and CLSM stacks of JC-1 Stained Mouse C57BL/6 ES Cells. C57BL/6 ES cells were stained with JC-1 and imaged, by z-sectioning, every 5 µm by DIC microscopy, TPLSM (8A), and CLSM (8B). The images were pseudocolored so that the JC-1 stained inactive mitochondria are green and JC-1 stained active mitochondria are red. Scale bar = 50 µm.
Figure 43: TMRE in Mouse Pluripotent Stem Cells. Mitochondrial activity in undifferentiated stem cells was assessed by TMRE staining and was analyzed by FACScan analysis. One experiment (from duplicates) is shown. The resulting histograms show unstained autofluorescence controls (thin lines) and TMRE stained cells (thick lines). Statistical analyses were by single factor Anova among the three cell lines or by t-tests between pairs of cell lines. The results are shown in Table VII.
Figure 44: GFP Expression versus TMRE Staining in Mouse mtGFP-tg Embryonic Stem Cells. mtGFP-tg ES cells were stained with TMRE and analyzed by FACScan to assess mitochondrial activity. GFP was plotted versus TMRE in a dot plot analysis. GFP fluorescence shows little correlation with TMRE mitochondrial activity.
Figure 45: JC-1 Staining of Mouse Pluripotent Stem Cells through 14 Days of Development. Mitochondrial activity from pluripotent stem cells through 14 days of differentiation was assessed in duplicate by JC-1 staining followed by FACScan analysis, and the mean percent positive ± SD is graphed. Statistical analyses were by two factor ANOVA with replication and the results are shown in Tables VIII-XI.
Figure 46: JC-1 Imaging of Plated Mouse Embryoid Bodies. DIC and Epifluorescence Microscopy images of plated EBs stained so that Hoechst stained nuclei are blue, JC-1 stained inactive mitochondria are green, and JC-1 stained active mitochondria are red. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 47: TMRE Staining of Mouse Pluripotent Stem Cells through 14 Days of Development. Mitochondrial activity by TMRE staining was analyzed in duplicate by FACScan analysis from pluripotent stem cells through 14 days of development and the mean fluorescence ± SD is graphed. Statistical analyses were by two factor Anova with replication and the results are shown in Tables VIII-XI.
Figure 48: Annexin V Staining in Mouse Pluripotent Stem Cells. The C57BL/6 ES cells and iPS cells were tested for apoptosis by Annexin V staining (FL-1) and for necrosis by PI staining (FL-2). The stained cells were analyzed by FACSscan. One experiment (from duplicates) is shown. The results of the quadrant analyses show the percentage of apoptotic versus necrotic and healthy cells. Statistical analyses were by t-tests and the results are shown in Table VII.
Figure 49: Caspase 3 Expression in Mouse Pluripotent Stem Cells.
Caspase 3 expression in undifferentiated stem cells was analyzed by FACScan. One experiment (from duplicates) is shown. The histograms show unstained autofluorescence controls (thin lines) and caspase 3 stained cells (thick lines). Statistical analysis was by t-test and the results are shown in Table VII.
Figure 50: Annexin V and PI Staining of Mouse Pluripotent Stem Cells through 14 Days of Development. Apoptosis by Annexin V and necrosis by PI staining were analyzed in duplicate by FACScan analysis from pluripotent stem cells through 14 days of development and the mean percent positive ± SD is graphed. The Annexin V data from D5-D7 were lost due to a technical error. Statistical analyses were by two factor Anova with replication and the results are shown in Table XI.
**Figure 51: Caspase 3 Expression from Mouse Pluripotent Stem Cells through 14 Days of Development.** Apoptosis by Caspase 3 staining was analyzed in duplicate by FACScan analysis from pluripotent stem cells through 14 days of development and the mean fluorescence ± SD is graphed. Statistical analysis was by two factor Anova with replication and the results are shown in Table XI.
Figure 52: TUNEL Assay of Mouse Pluripotent Stem Cells, Embryoid Bodies, and Plated Embryoid Bodies. The TUNEL assay measures the DNA fragmentation that occurs during late stage apoptosis. A) Positive and negative control samples (human lymphoma cells) for the TUNEL assay were analyzed by FACScan. FL1 is staining of DNA fragmentation and FL2 is PI staining. TUNEL positive cells are in the upper right quadrant B) Apoptosis by the using the TUNEL assay was analyzed in duplicate by FACScan analysis in pluripotent stem cells, D7 EBs, and pD7 plated EBs and the mean fluorescence ± SD is graphed. Statistical analyses were by t-test for undifferentiated cells and by two factor Anova with replication for differentiating cells and the results are shown in Tables VII and XI.
Figure 53: Caspase 3 Imaging of Mouse Pluripotent Stem Cells and Plated Embryoid Bodies. DIC and epifluorescence Microscopy images of Hoechst stained nuclei (blue) and caspase 3 staining (green) in pluripotent stem cells and plated embryoid bodies. White arrows/boxes mark caspase positive cells. The scale bar = 50 µm and the cells are approximately 5-10 µm in size.
Figure 54: Phase Contrast Imaging of P100 Mouse Pluripotent Stem Cells. P100 pluripotent stem cells show similar morphology compared to low passage pluripotent stem cells, although they are slightly more crowded due to an increased rate of proliferation. Scale bar = 50 µm.
Figure 55: Alkaline Phosphatase Activity in Early versus Late Passage Mouse Pluripotent Stem Cells. Alkaline Phosphatase activity was analyzed in P15 and P54 pluripotent stem cells and the percentage of positive colonies is graphed. Statistical analysis was by two factor Anova without replication and the results are shown in Table XII.
Figure 56: Oct-4 Expression in Early versus Late Passage Mouse Pluripotent Stem Cells. Oct-4 expression was analyzed by FACScan in P8 and P100 pluripotent stem cells and the mean fluorescence is graphed. Statistical analyses were by two factor Anova without replication and the results are shown in Table XII.
Figure 57: GFP Expression through 100 Passages of Mouse Pluripotent Stem Cells. GFP expression was analyzed by FACScan throughout 100 passages of cell culture and the mean fluorescence intensity is graphed. GFP expression increased through P85 and then decreased through P100. These changes in GFP expression were both statistically significant (P < 0.001) by two factor Anova without replication.
Figure 58: JC-1 Staining through 100 Passages of Mouse Pluripotent Stem Cells. Mitochondrial activity in pluripotent stem cells was assessed by JC-1 staining and FACScan analysis through 100 passages of cell culture. Statistical analyses were by two factor Anova without replication and the results are shown in Table XII.
Figure 59: TMRE Staining through 100 Passages of Mouse Pluripotent Stem Cells. Mitochondrial activity in pluripotent stem cells was assessed by TMRE staining and FACScan analysis through 100 passages of cell culture. Statistical analyses were by two factor Anova without replication and the results are shown in Table XII.
Figure 60: Annexin V and PI Staining through 100 Passages of Mouse Pluripotent Stem Cells. Apoptosis was assessed by Annexin V staining and FACScan analysis, and necrosis was assessed by PI staining and FACScan analysis in pluripotent stem cells through 100 passages of cell culture. Statistical analyses were by two factor Anova without replication and the results are shown in Table XII.
Figure 61: Caspase 3 Expression through 100 Passages of Mouse Pluripotent Stem Cells. Apoptosis was assessed in pluripotent stem cells by caspase 3 staining and FACScan analysis through 100 passages of cell culture. The data from P20 were lost due to a technical error. Statistical analysis was by two factor Anova without replication and the results are shown in Table XII.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of Data: Figure #</th>
<th>All Three Cell Lines</th>
<th>mtGFP-tg vs. C57BL/6 ES</th>
<th>mtGFP-tg ES vs. iPS</th>
<th>C57BL/6 ES vs. iPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P value</td>
<td>Significance</td>
<td>P value</td>
<td>Significance</td>
</tr>
<tr>
<td>AP</td>
<td>37</td>
<td>0.73</td>
<td>N.S.</td>
<td>0.57</td>
<td>N.S.</td>
</tr>
<tr>
<td>Oct-4</td>
<td>38</td>
<td>0.57</td>
<td>N.S.</td>
<td>0.46</td>
<td>N.S.</td>
</tr>
<tr>
<td>AFP</td>
<td>39</td>
<td>0.48</td>
<td>N.S.</td>
<td>0.37</td>
<td>N.S.</td>
</tr>
<tr>
<td>Bry</td>
<td>39</td>
<td>0.46</td>
<td>N.S.</td>
<td>0.15</td>
<td>N.S.</td>
</tr>
<tr>
<td>Nestin</td>
<td>39</td>
<td>0.67</td>
<td>N.S.</td>
<td>0.41</td>
<td>N.S.</td>
</tr>
<tr>
<td>JC-1 double positive</td>
<td>41, 45</td>
<td>0.31</td>
<td>N.S.</td>
<td>0.19</td>
<td>N.S.</td>
</tr>
<tr>
<td>JC-1 Green</td>
<td>41, 45</td>
<td>0.33</td>
<td>N.S.</td>
<td>0.21</td>
<td>N.S.</td>
</tr>
<tr>
<td>JC-1 Red</td>
<td>41, 45</td>
<td>0.94</td>
<td>N.S.</td>
<td>0.81</td>
<td>N.S.</td>
</tr>
<tr>
<td>TMRE</td>
<td>43, 47</td>
<td>0.50</td>
<td>N.S.</td>
<td>0.97</td>
<td>N.S.</td>
</tr>
<tr>
<td>Annexin V</td>
<td>48, 50</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>PI</td>
<td>48, 50</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>caspase 3</td>
<td>49, 51</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>TUNEL</td>
<td>52</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

1 The data from the assays on all three pluripotent stem cell lines were compared by using a single factor Anova. The data from comparison of two cell lines were analyzed using the t-test.

2 N.S. = Not Significant

3 N.A. = Not Applicable
Table VIII: Statistics on all Three Differentiating Mouse Pluripotent Stem Cell Lines

<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of Data: Figure #</th>
<th>D1-D7 Significance</th>
<th>pD1-pD7 Significance</th>
<th>14 Day (Overall) Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Differentiation</td>
<td>Cell Line</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Pluripotency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>37</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Oct-4</td>
<td>38</td>
<td>P &lt; 0.001</td>
<td>N.S.²</td>
<td>N.S.</td>
</tr>
<tr>
<td>AFP</td>
<td>39</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
<td>N.S.</td>
</tr>
<tr>
<td>Bry</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Nestin</td>
<td>39</td>
<td>P &lt; 0.05</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mitochondrial Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JC-1 double positive</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Green</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Red</td>
<td>45</td>
<td>P &lt; 0.01</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>TMRE</td>
<td>47</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td>N.A.³</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>PI</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>TUNEL</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

¹The data from all three pluripotent stem cell assays during differentiation were compared by using a two factor Anova with replication.
²N.S. = Not Significant
³N.A. = Not Applicable
Table IX: Statistics on Differentiating mtGFP-tg vs. C57BL/6 ES Cell Lines

<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of Data: Figure #</th>
<th>D1-D7 Significance</th>
<th>pD1-pD7 Significance</th>
<th>14 Day (Overall) Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Differentiation</td>
<td>Cell Line</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Pluripotency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>37</td>
<td>P &lt; 0.001</td>
<td>N.S.²</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Oct-4</td>
<td>38</td>
<td>P &lt; 0.01</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>AFP</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Brg</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Nestin</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mitochondrial Activity</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 double positive</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Green</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Red</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>TMRE</td>
<td>47</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td>N.A.³</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>PI</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>TUNEL</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

¹The data from mtGFP-tg and C57BL/6 ES cell assays during differentiation were compared by using a two factor Anova with replication.
²N.S. = Not Significant
³N.A. = Not Applicable
### Table X: Statistics on Differentiating mtGFP-tg ES vs. iPS Cell Lines

<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of Data: Figure #</th>
<th>D1-D7 Significance</th>
<th>pD1-pD7 Significance</th>
<th>14 Day (Overall) Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Differentiation Cell Line</td>
<td>Differentiation Cell Line</td>
<td>Differentiation Cell Line</td>
<td></td>
</tr>
<tr>
<td>Pluripotency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>37</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Oct-4</td>
<td>38</td>
<td>P &lt; 0.01</td>
<td>N.S. (^2)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>AFP</td>
<td>39</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>N.S.</td>
</tr>
<tr>
<td>Bry</td>
<td>39</td>
<td>P &lt; 0.05</td>
<td>N.S.</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Nestin</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mitochondrial Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JC-1 double positive</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Green</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Red</td>
<td>45</td>
<td>N.S.</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>TMRE</td>
<td>47</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td>N.A. (^3)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>PI</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>TUNEL</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

1. The data from mtGFP-tg ES and iPS cell assays during differentiation were compared by using a two factor Anova with replication.
2. N.S. = Not Significant
3. N.A. = Not Applicable
Table XI: Statistics on Differentiating C57BL/6 ES vs. iPS Cell Lines

<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of Data: Figure #</th>
<th>D1-D7 Significance</th>
<th>pD1-pD7 Significance</th>
<th>14 Day (Overall) Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Differentiation</td>
<td>Cell Line</td>
<td>Differentiation</td>
</tr>
<tr>
<td>AP</td>
<td>37</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Oct-4</td>
<td>38</td>
<td>P &lt; 0.001</td>
<td>N.S. 2</td>
<td>N.S.</td>
</tr>
<tr>
<td>AFP</td>
<td>39</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>N.S.</td>
</tr>
<tr>
<td>Bry</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Nestin</td>
<td>39</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>JC-1 double positive</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Green</td>
<td>45</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Red</td>
<td>45</td>
<td>P &lt; 0.01</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>TMRE</td>
<td>47</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Annexin V</td>
<td>50</td>
<td>N.S.</td>
<td>N.S.</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>PI</td>
<td>50</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>51</td>
<td>N.S.</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>TUNEL</td>
<td>52</td>
<td>N.A. 3</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

1 The data from C57BL/6 ES and iPS cell assays during differentiation were compared by using a two factor Anova with replication.
2 N.S. = Not Significant
3 N.A. = Not Applicable
Table XII: Statistics on Long-term Culture of Mouse Pluripotent Stem Cells

<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of Data: Figure #</th>
<th>Across all Passage #s</th>
<th>Cell Line</th>
<th>Across all Passage #s</th>
<th>Cell Line</th>
<th>Across all Passage #s</th>
<th>Cell Line</th>
<th>Across all Passage #s</th>
<th>Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pluripotency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>55</td>
<td>P &lt; 0.05</td>
<td>N.S. 2</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Oct-4</td>
<td>56</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mitochondrial Activity</td>
<td>JC-1 double positive</td>
<td>58</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Green</td>
<td>58</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>JC-1 Red</td>
<td>58</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
<td>P &lt; 0.01</td>
<td>N.S.</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
</tr>
<tr>
<td>TMRE</td>
<td>59</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td>60</td>
<td>N.A. 3</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>P &lt; 0.001</td>
<td>N.S.</td>
</tr>
<tr>
<td>PI</td>
<td>60</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>caspase 3</td>
<td>61</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

1The data from all three pluripotent stem cell lines, through 100 passages of cell culture, were compared by using a two factor Anova without replication.
2N.S. = Not Significant; 3N.A. = Not Applicable
DISCUSSION

mtGFP-tg ES Cells

The first major accomplishment achieved during this research was the successful creation of a mtGFP-tg ES cell line. The impetus for the creation of this line came from our experiences using a variety of mitochondrial dyes. Many mitochondrial dyes, such as the MitoTracker series from Invitrogen, were not very bright. We also found that the dyes could not fully penetrate EBs, most likely due to a relatively impermeable outer surface (Sachlos and Auguste, 2008). We considered using an \textit{in vitro} cell labeling system such as the Invitrogen Organelle Lights Mito-GFP kit. However, this GFP labeling would also be transient and only last for a few passages. Therefore, our new mtGFP-tg1 ES cell line provides the opportunity to visualize mitochondria noninvasively in ES cells and EBs and to obtain a bright and stable signal that may be collected during long-term imaging of ES cell growth and differentiation.

We subjected the mtGFP-tg ES cells to a number of tests to validate the pluripotency and differentiation capabilities of the newly created line. The cells had a normal male karyotype as well as a normal morphology and proliferation rate. The cell line was determined to be mycoplasma free. Importantly, the cells had AP activity and expressed the Oct-4 pluripotency marker at levels equivalent to a standard C57BL/6 ES cell line. Upon differentiation, mtGFP-tg ES cells readily formed EBs, and pEBs developed into a wide variety of tissues including beating cells. Concurrently, the expression of pluripotency markers decreased and the three germ layer markers analyzed (AFP, Bry, Nestin) increased. All together, mtGFP-tg ES cells proved to be a healthy and robust ES cell line.
Interestingly, GFP expression was not completely stable, but instead it was found to be significantly different during differentiation and long-term cell culture. mtGFP-tg differentiating cells progressively decreased in their GFP fluorescence (Figure 31A, \( P < 0.001 \)). One potential explanation is that differentiating cells decrease their number of mitochondria per cell, but several studies have shown that pluripotent cells have few mitochondria and that the number increases with differentiation (von Wangenheim and Peterson, 1998; St. John et al., 2005; Cho et al., 2006; Facucho-Oliveira et al., 2007; Saretzki et al., 2008). Therefore, a decrease in the number of mitochondria per cell seems unlikely as an explanation of decreased GFP expression with differentiation.

Another potential explanation for the decrease in GFP expression during differentiation, confirmed by imaging in Figure 31B, is that selective cells are losing their GFP fluorescence. There are regions of negative cells in pEBs from mtGFP-tg ES cells. One potential cause of the appearance of GFP negative cells relates to the concurrent increases in apoptosis also seen during differentiation. GFP has been found to act as a biosensor, and loss of GFP fluorescence has been linked to cell death and apoptosis, although the mechanism for this is unclear (Geddes, 2006). Another possible cause for the loss of GFP in some cells is that the mtGFP-tg ES cell line may be heterozygous for GFP. Although we were not able to test this, future work could address this issue. The appearance of GFP negative cells in differentiation of the mtGFP-tg cell line could reflect the loss of the GFP transgene or the loss of the entire chromosome upon which the GFP transgene resides.

In contrast to the decrease in GFP expression during differentiation, GFP expression increased significantly throughout long-term culture, until dropping
precipitously in the last 10 passages (Figure 57, $P < 0.001$). The drop in fluorescence at
the end of the long-term culture period is correlated with a sudden increase in apoptosis,
and therefore one possible explanation is again that apoptosis induces loss of GFP
fluorescence. Alternatively, as discussed above, GFP transgene or chromosome loss
could be the explanation. But how can we explain the increase in GFP fluorescence
during the first 90 passages? One explanation is that there could be an increasing number
of mitochondria per cell. As stated previously, pluripotent cells have few mitochondria,
but as cells differentiate the number of mitochondria increases. Our data, which show a
significant decrease in AP in a later passage of pluripotent cells, only lends a small
amount of support to the idea that long-term culture cells have more differentiation.
Alternatively, the increase in GFP fluorescence could be due to the increase in the rate of
proliferation, and therefore an increase in the number of mitochondria per cell, that we
witnessed. The number of mitochondria per cell could be assessed in future work by
analysis of the amount of mtDNA per cell as described in Facucho-Oliveira et al. (2007).

We successfully used the mtGFP-tg ES cell line to image mitochondria at points
throughout the differentiation process. The fluorescence was bright and stable enough to
collect 3D stacks of images and to perform timelapse imaging. Our data represents the
first 3D sectioning through an EB with mitochondrial fluorescence. High resolution
timelapse microscopy of ES cells undergoing differentiation would provide a wealth of
information regarding the localization and morphology of mitochondria as it relates to
differentiation. Unfortunately, our culture chamber is only capable of maintaining a
stable environment for about 24 h, and so we were unable to achieve this long-term goal.
One unforeseen problem relating to the creation of our mtGFP-tg ES cell line is where to deposit the cells for safekeeping and potential distribution. Although there are a number of human pluripotent stem cell banks, there is no main repository for mouse pluripotent stem cells. The ATCC selectively chooses a few potential cell lines, but there are no guarantees of acceptance, and it certainly does not represent an official mouse pluripotent stem cell bank. The Riken BioResource Center Cell Bank in Japan seems to be the type of repository that would be desirable in the United States. Perhaps our mtGFP-tg ES cells will end up back in Japan!

**Mitochondria in Pluripotent Stem Cell Lines**

We analyzed the mitochondria in our three pluripotent stem cell lines by using endogenous mtGFP fluorescence and staining with JC-1 and TMRE. Although mtGFP expression was not correlated with mitochondrial activity, the JC-1 and TMRE dyes both were able to detect levels of mitochondrial activity. Throughout all of our experiments, the TMRE and JC-1 staining correlated well with one another.

We analyzed mitochondrial activity in pluripotent stem cells as well as throughout a 14 day differentiation protocol. Our results showed that all three pluripotent stem cell lines had a high level of mitochondrial activity, and that with differentiation this activity significantly decreased (Tables VII-XI). There have only been five groups that previously examined mitochondrial activity in embryonic stem cells (St. John et al., 2005; Chung et al., 2007; Lonergan et al., 2008; Saretzki et al., 2008; Schieke et al., 2008; Lonergan et al., 2009), and to our knowledge data on mitochondria in iPS cells are nonexistent (Parker et al., 2009). Saretzki et al. (2008) did not see any change in
mitochondrial activity during differentiation, but it seems that the initial mitochondrial activity levels in undifferentiated hES cells were reasonably high. St. John et al. (2005) used JC-1 to study mitochondrial activity in differentiating hES cells as they formed cardiomyocytes. It is unclear what their data on undifferentiated ES cells showed as the earliest presentation of JC-1 data is on already differentiating cells. However, St. John et al. (2005) found that the population of high membrane potential cells increased as cells differentiated from the migratory cell phase to cardiomyocyte. In contrast, Chung et al. (2007) found, by JC-1 staining, that mES had high levels of mitochondrial activity that decreased upon differentiation to cardiomyocytes thus supporting our observations. It is possible that these conflicting studies are species-specific differences for mitochondrial activity levels in pluripotent stem cells. Schieke et al. (2008) performed a more complex analysis by sorting their population of mES cells into low and high mitochondrial membrane potential (activity) populations as detected by TMRM, which is similar to TMRE. They found that the populations were equivalent in morphology and in the expression of SSEA1 and Flk1 pluripotency markers. Low mitochondrial membrane potential cells differentiated readily into mesoderm in vitro but they were unable to make teratomas, whereas high mitochondrial membrane potential cells did not readily form mesoderm in vitro but they were able to make teratomas containing all three germ layers. Thus Schieke at al. (2008) linked mitochondrial membrane potential with cell fate. It is difficult to compare our data to theirs because they did not measure mitochondrial activity beyond their original sorting. However, we speculate that high mitochondrial activity is a hallmark of pluripotency. Our data is most similar to that of Lonergan et al. (2008; 2009), who found that both mouse and monkey embryonic stem cell lines
possessed a high number of active mitochondria. None of these studies followed mitochondrial activity from the entire population of pluripotent stem cells throughout the daily differentiation process, and our findings are certainly the first to analyze mitochondrial activity in iPS undifferentiated and differentiating stem cells. Thus, the majority of data supports a role for high mitochondrial activity in pluripotent stem cells that decreases upon differentiation.

We also analyzed mitochondrial localization by imaging of mtGFP and JC-1 stained pluripotent stem cells and pEBs. By mtGFP imaging we were unable to determine a single pattern of mitochondrial distribution at any point during development. At times the mitochondria appeared diffuse, whereas we also observed perinuclear and clustered arrangements. Our data most closely resembles that of St. John et al. (2005) and Cho et al. (2006) who found, by TEM, that mitochondria in human ES cells formed small clusters. The only other mitochondrial distribution study in ES cells found that mitochondria in monkey ES cells formed a capped arrangement around the nucleus (Lonergan et al., 2009). It is possible that the variable arrangements of mitochondria are related to the stage of the cell cycle or are species specific. When we stained pluripotent stem cells with JC-1, we observed that active mitochondria (red fluorescence in Figure 42) were confined to the outside of pluripotent stem cell colonies. We speculate that the inside of the stem cell colonies may be anaerobic, and therefore the cells may not be using oxidative phosphorylation in order to generate energy. No group has previously looked at mitochondrial activity in terms of localization. It is interesting to note that although mitochondrial activity was very high by FACScan analysis, the activity did not seem as high by imaging. This most likely reflects that the cells for FACScan analysis
were trypsinized to a single cell suspension. A previous study on ICM outgrowths demonstrated that mitochondrial activity was affected by cell density, and that fewer cell contacts increased mitochondrial activity (Van Blerkom, 2008). Although this is noteworthy, this does not discredit our FACScan data since it is the relative activity throughout differentiation that is important. Our data confirm that mitochondrial activity is related to the differentiation of pluripotent stem cells.

**Apoptosis in Pluripotent Stem Cell Lines**

We investigated the levels of apoptosis throughout differentiation using assays for all stages of apoptosis in C57BL/6 ES cells and iPS cells. Annexin V staining was used to detect early apoptosis, caspase 3 expression was used to detect intermediate apoptosis, and the TUNEL assay was used to detect late (irreversible) apoptosis. By using this combination of assays we hoped to be able to go beyond identifying whether cells were apoptotic and to pinpoint where in the apoptosis process the cells were since much of early apoptosis is actually reversible (Geske et al., 2001).

We found that, in contrast to mitochondrial activity, undifferentiated pluripotent stem cells had lower levels of apoptosis and necrosis. Roughly 26-34% of cells showed signs of early apoptosis according to Annexin V staining (Figure 48). The percentage of necrotic (PI positive) cells was very low (Figure 48). The later stages of apoptosis were also low as assessed by caspase 3 staining (Figure 49) and the TUNEL assay (Figure 52). Imaging results showed a few localized spots of apoptosis. Much of the literature pertaining to pluripotent stem cells and apoptosis involves using the cells as a model to understand the relationships between apoptosis molecules or to understand the effects of
culture components. However, results have generally shown that mouse embryonic stem cells have low rates of spontaneous apoptosis, whereas human embryonic stem cells have higher rates of apoptosis (Qin et al., 2007). Additionally, Johkura et al. (2004) found that as human ES colonies grew, their centers compacted and TUNEL positive particles appeared. In contrast, we did not see more apoptosis in the center of our stem cell colonies. Interestingly, it has been found that LIF acts in an anti-apoptotic manner in ES cell culture (Furue et al., 2005), and LIF starvation leads to apoptosis (Duval et al., 2006).

During differentiation we saw the initial levels of apoptosis increase during the pluripotent stem cell to EB transition, most likely representing increases due to both the intrinsic and extrinsic apoptotic pathways. Specifically, Annexin V staining increased (Figure 50), caspase 3 expression increased (Figure 51), and TUNEL staining increased (Figure 52). Caspase 3 activity has previously been linked with apoptosis in differentiating cells. Murray and Edgar (2004) found that a large proportion of cell death in EBs was due to apoptosis rather than necrosis, and they found localized areas of apoptosis in EBs. Additionally, caspase 3 was activated in EBs and cytochrome c was released, implying decreased mitochondrial membrane polarization (activity).

The significance of increased caspase 3 during ES cell differentiation has been studied by Fujita et al. (2008). Through a series of studies, they first found that caspase 3 was elevated quickly during the earliest stages of differentiation and hypothesized that this was related to the inhibition of self-renewal in ES cells. However, other signs of apoptosis were not evident. They then found that caspase 3 in fact has a novel role outside of apoptosis whereby it releases cells from their pluripotent/self-renewal state by inducing cleavage of the transcription factor Nanog. This correlates well with other
studies which have shown a link between the apoptosis machinery and self-renewal (pluripotency) and differentiation. For example, Yamane et al. (2005) found that elevated levels of Bcl-2, an anti-apoptotic protein, inhibited differentiation of hES cells. Additionally, a series of five recent papers have presented data showing that suppression of the p53 pathway dramatically increased the success of reprogramming cells to iPS cells (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). Thus, the apoptotic and self-renewal pathways are intricately linked. Our data strongly supports the concept that increasing apoptosis is related to loss of pluripotency.

**Long-term Culture of Pluripotent Stem Cells**

We cultured our three pluripotent stem cell lines for 100 passages in order to analyze their long-term stability. There were no significant differences in morphology, but the cells seemed to have an increased rate of proliferation. An MTT assay comparing early passage and late passage pluripotent stem cells would give a definitive answer. Pluripotency, as analyzed by AP activity, was significantly lower for the three cell lines (Table XII), although this was not statistically significant when analyzing pairs of pluripotent stem cell lines. Although Oct-4 expression was also lower, this was not statistically significant. One study in human ES cell cells cultured for more than 200 population doublings found that AP, Oct-4, and SSEA markers remained high (Krylova et al., 2005). A more comprehensive analysis is needed to determine whether or not pluripotency is affected by long-term culture.
In contrast, mitochondrial activity for all three cell lines was significantly increased during long-term culture (Table XII; JC-1 double positive, JC-1 red single positive, and TMRE). The population of cells with inactive mitochondria concurrently decreased significantly. This trend occurred in all three cell lines, although there were also significant differences between the cells lines (Table XII). The increase in mitochondrial activity could potentially be linked to the observed increase in the rate of proliferation. The trend for apoptosis was much more difficult to discern as the pattern was extremely variable. However, there did seem to be a significant rise in Annexin V and caspase 3 in the later passages. This increase could be related to loss of pluripotency, increased cell death, or both.

Several groups have analyzed the effects of long term culture on mouse and human ES cells. A few groups have found ES cells to be relatively stable during long term culture, particularly with a chemically defined medium (Amit et al., 2000; Rosler et al., 2004; Krylova et al., 2005; Yao et al., 2006). However, there have been many reports of chromosomal instability, aneuploidy, and impaired differentiation as a result of long term culture (Greenlee et al., 2004; Inzunza et al., 2004; Maitra et al., 2005; Mikkola et al., 2006; Li et al., 2007; Rebuzzini et al., 2008). Interestingly, Chin et al. (2009) studied early passage vs. late passage human iPS cells and found that late passage iPS cells resembled human ES more than early passage iPS cells in that they had more appropriate levels of pluripotency markers and better suppression of fibroblast (the originating cell) markers. The only study to address apoptosis in long term cultured cells reported a decrease in caspase 3 expression (Greenlee et al., 2004).
Since iPS cells are relatively new and untested for stability in long-term culture, we took a closer look at the C57BL/6 vs iPS pluripotent stem cell lines (Table XII). Although C57BL/6 ES cells had higher levels of the pluripotency marker AP, these data were not statistically significant. C57BL/6 ES cells had significantly higher mitochondrial activity (JC-1 double positive and TMRE), apoptosis (caspase 3), and necrosis (PI) than iPS cells. It was perplexing to see increasing mitochondrial activity with decreasing pluripotency, an inverse relationship, whereas the differentiation data showed a direct relationship between the two. However, before any explanations are in order, these data need to be repeated. We decided against repeating the long-term culture experiments ourselves because a repetition of these data would be extremely time-consuming and costly. In order to determine whether the pluripotent stem cells become aneuploid during long-term culture, a karyotype analysis should be performed on the late passage pluripotent stem cells. Repetition of the long-term culture experiments would also afford an opportunity to quantitate the copy numbers of the GFP transgene in the mtGFP-tg ES cells to determine the zygosity of early (all cells GFP positive) vs. late (some cells GFP negative) passage cells. Thus we could answer whether the appearance of GFP negative cells within the mtGFP-tg population is due to loss of the GFP transgene.

**mtGFP-tg ES cells versus Conventional ES and iPS Cells**

We compared the mtGFP-tg ES cells to conventional C57BL/6 ES and iPS cells in terms of pluripotency and mitochondrial activity. We analyzed these characteristics throughout differentiation and long-term culture. The mtGFP-tg ES cells were generally
equivalent to the other two cell lines in growth and morphology, although the mtGFP-tg ES cells had increased levels of growth as compared to the C57BL/6 ES cells during EB formation and plated EB growth (Figure 35). In the undifferentiated state, mtGFP-tg ES cells were fully equivalent to C57BL/6 ES and iPS cells for pluripotency and mitochondrial activity (Table VII).

Analysis of these characteristics during a 14 day differentiation protocol resulted in some statistically significant differences between the mtGFP-tg and the other two pluripotent stem cell lines (Tables IX-X). mtGFP-tg ES cells were equivalent to C57BL/6 ES cells in terms of pluripotency (Table IX). However, mtGFP-tg ES cells had significantly higher AP activity than iPS cells throughout differentiation and significantly lower AFP expression during D1-D7 (Table X). In contrast, they had significantly lower Oct-4 expression than iPS cells during pD1-pD7. In terms of mitochondrial activity, the mtGFP-tg ES cell line was significantly different from the C57BL/6 ES and iPS cell lines throughout most of differentiation. Conflicting results showed the mtGFP-tg ES cell line to have significantly lower mitochondrial activity than the other two cell lines by JC-1 staining, but their mitochondrial activity was significantly higher than the other two cell lines by TMRE staining. This discrepancy is likely due to the brightness of GFP, which could falsely inflate the percentage of JC-1 green positive cells in the quadrant analyses of JC-1 staining. Since the percentages of green and red fluorescent cells are analyzed simultaneously in the quadrant analysis, a falsely inflated percentage of JC-1 green positive cells would automatically cause a falsely diminished percentage of JC-1 double positive or red positive cells. The same conflicting results appear in the analysis of mitochondrial activity among the cell lines during long-term culture (Table XII). Since
the TMRE assay is not dependent on the simultaneous analysis of green versus red fluorescence, it may provide a more reliable measure of mitochondrial activity in the mtGFP-tg ES cell line. Overall, there were no significant differences between the mtGFP-tg ES cells and the other two pluripotent stem cell lines that would indicate that the mtGFP-tg ES cell line is compromised in any way.

**iPS versus Conventional ES Cells**

One really interesting outcome of our work is the analysis of whether iPS cells are equivalent to conventional C57BL/6 ES cells in terms of pluripotency, mitochondrial activity, and apoptosis. We analyzed these characteristics throughout differentiation and long-term culture. iPS growth and morphology appeared identical to C57BL/6 ES cells throughout all experiments. In the undifferentiated state, iPS cells were equivalent to C57BL/6 ES cells in terms of growth (MTT), pluripotency, mitochondrial activity, and apoptosis (Table VII). These data support the idea that iPS cells are equivalent to classical ES cells.

The analysis became more complicated when we compared iPS cells to C57BL/6 cells in a 14 day differentiation protocol. Table XI takes a closer look at the the differences between iPS and C57BL/6 cells during differentiation. The MTT assay showed that iPS cells had a significantly higher rate of growth, as compared to the C57BL/6 ES cells, during EB formation and plated EB growth (Figure 35). iPS cells consistently expressed lower levels of pluripotency and differentiation markers than the C57BL/6 ES cells, although the only significant difference overall was in AP activity. iPS cells also had significantly lower AFP during D1-D7 and significantly lower Nestin
during pD1-pD7. With respect to mitochondrial activity, iPS cells were generally equivalent to C57BL/6 cells despite a consistent trend of higher mitochondrial activity. iPS did have significantly higher mitochondrial activity at two timepoints (D1-D7 JC-1 double positive and pD1-pD7 TMRE). The one exception to higher mitochondrial activity in iPS cells was by TMRE staining during D1-D7 where C57BL/6 cells had higher mitochondrial activity. The trend for apoptosis was more definitive. iPS cells had significantly higher levels of caspase 3 staining throughout the entire 14 day differentiation, and the iPS cell line also had significantly more TUNEL positive cells in the undifferentiated state and at D7 (Table XI). iPS cells may be inherently more prone to losing pluripotency as evidenced by lower expression of pluripotency markers such as AP activity and higher levels of apoptosis, caspase 3 in particular.

Our results warrant a word of caution regarding the equivalency of iPS cells to conventional ES cells. Although the majority of iPS studies have found them to be equivalent to conventional ES cells, there is still much that we do not know. A recent study found 3947 (out of 17,620) gene expression differences between iPS and ES cells (Chin et al., 2009). And an extremely harsh review of iPS cells questions the validity of many iPS cell claims (Liu, 2008). Our conclusions are not so harsh, but are merely a call for more research. Although in the undifferentiated state we found iPS cells to be equivalent to C57BL/6 ES cells, there were several notable differences in pluripotency, mitochondrial activity, and apoptosis during differentiation and long-term culture. The differences we found between ES cells and iPS cells make it very important to continue future research on both types of cells.
CONCLUSIONS AND FUTURE DIRECTIONS

A major accomplishment from our work was the generation of an ES cell line from mtGFP-tg mice. mtGFP-tg ES cells demonstrated many characteristics of pluripotency and they were able to differentiate into a variety of tissue types. Additionally, the endogenous GFP fluorescence was extremely bright and stable allowing us to perform timelapse imaging of ES cells and 3D sectioning through EBs. A mtGFP-tg ES cell line gives us the potential to view mitochondrial localization from the undifferentiated state all the way through terminal differentiation of cells to a specific tissue, thus affording us a new window into the dynamics of tissue development. It would be extremely interesting to use the mtGFP-tg ES cell line with a specific differentiation protocol, such as cardiomyocyte differentiation, and to perform timelapse imaging of mitochondrial GFP of the differentiation process.

Our research allowed us to conclude that pluripotent stem cells have high levels of mitochondrial activity and low levels of apoptosis in their undifferentiated state. We found that upon differentiation pluripotency decreased, germ layer expression increased, mitochondrial activity decreased, and apoptosis increased. All of these changes were significant, thus supporting a role for mitochondrial activity and apoptosis in normal pluripotent cell growth and differentiation. It remains to be seen whether the decrease in mitochondrial activity is universal across all differentiating cells, or whether certain subsets of cells have an increase in mitochondrial activity. Assessing mitochondrial activity in conjunction with a specific lineage marker during differentiation would help to answer this question. It would be particularly interesting to perform these assessments using a FACScan capable of collecting images of cells, such as the Amnis Imagestream,
so that mitochondrial activity and localization could both be correlated with cellular
differentiation. Additionally, it would be interesting to repeat the work on apoptosis
using markers that would distinguish between the intrinsic and extrinsic apoptotic
pathways. For example assays for caspase 9 (intrinsic) and caspase 8 (extrinsic) would
distinguish which apoptotic pathway is more prevalent in pluripotent stem cells and their
differentiating progeny.

Finally, we analyzed the differences between mtGFP-tg ES cells, iPS cells, and
conventional C57BL/6 ES cells. The cell lines were equivalent in every way in their
undifferentiated state. However, we found significant differences in pluripotency,
mitochondrial activity, and apoptosis between the cell lines during differentiation and
during long-term culture. In particular, we found several significant differences between
iPS and C57BL/6 ES cells thus bringing into question the complete equivalency of iPS
cells and conventional ES cells. This work addresses a major, ongoing question in the
field of stem cell research: are iPS cells truly equivalent to ES cells? Additionally, are
any discovered differences genuine differences between iPS cells and conventional ES
cells or are the differences due to variations among cell lines? It is important to
determine whether any discovered differences will affect the therapeutic potential of iPS
cells. Thus iPS cell research must continue with an open mind.

It is clear that mitochondrial activity and apoptosis are important factors in
pluripotent stem cell growth and differentiation. Future studies should be able to further
elucidate their roles. Pluripotent stem cells are heralded for their potential to provide
therapies, and even cures, for diseases. However, the field is still young and there is
much that we do not yet understand about pluripotent stem cells. Carol W. Greider,
recipient of the 2009 Nobel prize for Medicine, has stated that “curiosity-driven science is really important” and that disease oriented research isn’t the only way to reach the answer, but “both together are synergistic” (http://news.yahoo.com/s/ap/20091005/ap_on_re_eu/eu_nobel_medicine). Hence, the combination of basic and medical research on pluripotent stem cells provides the best path for developing stem cell therapy.
RESEARCH ACKNOWLEDGEMENTS

We sincerely thank Dr. Hiroshi Shitara (Department of Laboratory Animal Science, Metropolitan Institute of Medical Science, Tokyo, Japan) for providing us with the mtGFP-tg mice. Robert Crooker was instrumental in establishing the mtGFP-tg mouse colony and for his work in developing the genotyping and phenotyping protocols. We also thank Brad Hamilton from Stemgent for providing us with the mouse iPS cell line before it was ready to be released commercially.

Paula Lampton and I developed many of the ES cell procedures and protocols together. Assistance with ES cell culture and differentiation was provided by Robert Crooker. Matthew Devine performed several assays related to the differentiation experiments, and he also performed many of the long-term culture assays. William Warger III collected the multimodal images of ES cells, and critical help with imaging on the Keck 3DFM was provided by Gary Laevsky and Joseph Kerimo. I particularly want to thank Joseph Kerimo for establishing the culture chamber on the Keck 3DFM.

Funding for this research was provided by the Bernard M. Gordon Center for Subsurface Sensing and Imaging System (CenSSIS, NSF grant EEC-9986821).


Furue, M, Okamoto, T, Hayashi, Y, Okochi, H, Fujimoto, M, Myoishi, Y, Abe, T, 


Ludwig, TE, Levenstein, ME, Jones, JM, Berggren, WT, Mitchen, ER, Frane, JL, Crandall, LJ, Daigh, CA, Conard, KR, Piekarczyk, MS, Llanas, RA, Thomson,


Nagai, S, Mabuchi, T, Hirata, S, Shoda, T, Kasai, T, Yokota, S, Shitara, H, Yonekawa, H,
Hum Cell 17(4): 195-201.

Nakagawa, M, Koyanagi, M, Tanabe, K, Takahashi, K, Ichisaka, T, Aoi, T, Okita, K,
pluripotent stem cells without myc from mouse and human fibroblasts. Nat

Newmark, JA, Warger, WC, 2nd, Chang, C, Herrera, GE, Brooks, DH, DiMarzio, CA,
Warner, CM. (2007) Determination of the number of cells in preimplantation
embryos by using noninvasive optical quadrature microscopy in conjunction with

Oh, SK, Kim, HS, Ahn, HJ, Seol, HW, Kim, YY, Park, YB, Yoon, CJ, Kim, DW, Kim,
SH, Moon, SY. (2005) Derivation and characterization of new human embryonic


mouse induced pluripotent stem cells without viral vectors. Science 322(5903):
949-953.

Orrenius, S, Zhivotovsky, B, Nicotera, P. (2003) Regulation of cell death: The calcium-


APPENDIX 1: Neviparine Treatment of Mouse Embryonic Stem Cells

Nevirapine is an antiretroviral drug that is used to treat people infected with Human Immunodeficiency Virus (HIV). We conducted a study to determine the potential effects of Neviparine on the growth of ES cells. Mouse D8 EBs were cultured in Dr. Ann Kiessling’s lab (Bedford Stem Cell Research Foundation, Somerville, MA) by Lisa Fitzgerald. We received 10 control EBs and 10 Neviparine treated EBs for analysis. We stained all EBs with 1 µg/ml Hoechst and 200 nM MitoTracker Green FM and imaged them by Brightfield and Epifluorescence Microscopy using the Keck 3DFM. We sectioned through the EBs every 10 µm using a 4x, 6x, and 10x objective. Figures 62A and B show the 6x results for the control EBs, and Figures 63A and B show the 6x results for the Neviparine treated EBs. These Figures show the EBs as imaged by Brightfield Microscopy. Structural details are difficult to see given the large size of the samples that prevent the penetration of light through them. Adjacent to the Brightfield images are the corresponding Epifluorescence overlay images that show MitoTracker Green FM labeling and Hoechst stained nuclei. The control EBs are larger than the Neviparine treated EBs by a few hundred µm in both the x and y directions. The images were analyzed by Metamorph software. We measured the total area by colony tracing using the Brightfield images. Then we measured the number of cells, using the 10x images, in a circular region that was placed evenly within a dense area of the colony. The results are presented in Table XIII. Neviparine treated EBs were significantly smaller (P < 0.001) than the control EBs (area), and their cell density (# cells/region) was significantly lower (P < 0.05) than the control EBs. Thus Neviparine significantly inhibits ES cell growth.
**Figure 62A: Control Mouse Embryoid Bodies.** Brightfield and Epifluorescence Microscopy images of MitoTracker Green labeled mitochondria and Hoechst stained nuclei in control EBs. Scale bar = 200 μm; Kiessling, Fitzgerald, Newmark, and Warner, unpublished data.
Figure 62B: Control Mouse Embryoid Bodies. Brightfield and Epifluorescence Microscopy images of MitoTracker Green labeled mitochondria and Hoechst stained nuclei in control EBs. Scale bar = 200 µm; Kiessling, Fitzgerald, Newmark, and Warner, unpublished data.
Figure 63A: Neviparine Treated Mouse Embryoid Bodies. Brightfield and Epifluorescence Microscopy images of MitoTracker Green labeled mitochondria and Hoechst stained nuclei in Neviparine treated EBs. Scale bar = 200 µm; Kiessling, Fitzgerald, Newmark, and Warner, unpublished data.
Figure 63B: Neviparine Treated Mouse Embryoid Bodies. Brightfield and Epifluorescence Microscopy images of MitoTracker Green labeled mitochondria and Hoechst stained nuclei in Neviparine treated EBs. Scale bar = 200 µm; Kiessling, Fitzgerald, Newmark, and Warner, unpublished data.
Table XIII: Area and Number of Cells/Region in Control versus Neviparine Treated Mouse EBs.

<table>
<thead>
<tr>
<th>EB Number</th>
<th>Control EBs</th>
<th>Neviparine treated EBs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area (µm²)</td>
<td># Cells in region</td>
</tr>
<tr>
<td>1</td>
<td>482887</td>
<td>67</td>
</tr>
<tr>
<td>2</td>
<td>432237</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>635593</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>443689</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>458003</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>496813</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td>599951</td>
<td>59</td>
</tr>
<tr>
<td>8</td>
<td>348767</td>
<td>54</td>
</tr>
<tr>
<td>9</td>
<td>662311</td>
<td>51</td>
</tr>
<tr>
<td>10</td>
<td>441903</td>
<td>54</td>
</tr>
<tr>
<td>Mean</td>
<td>500214</td>
<td>59.4</td>
</tr>
<tr>
<td>Stnd. Dev.</td>
<td>100442</td>
<td>7.8</td>
</tr>
<tr>
<td>Standard Error</td>
<td>316.9</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Significance\(^1\) | P < 0.001 | P < 0.05
\(^1\)P values are from a t-test comparing the control EBs and the Neviparine treated EBs. Neviparine significantly lowered the size (area) of the EBs and the number of cells in the EBs.
APPENDIX 2: Multicolor Imaging of Mouse Embryonic Stem Cells

We initiated a multicolor imaging project in order to visualize the variety of structures within mouse oocytes, 2-cell embryos, and ES cells. We speculated that this information might be useful in context with our mitochondrial imaging studies since several cellular structures are associated with the mitochondria and microtubules are involved in mitochondrial movement. We stained our samples according to the Invitrogen protocols for each dye. In a variety of combinations we used Hoechst (nuclei), ER Tracker blue-white (endoplasmic reticulum, ER), LysoTracker (lysosomes), Tubulin Tracker (tubulin), SYTO 17 (nuclei), FM 1-43 (cell membrane), and MitoTracker Deep Red (mitochondria). Images were collected by Epifluorescence Microscopy on the Keck 3DFM, and they were processed using Metamorph software to pseudocolor and overlay them. We were able to use intensity to separate the brightly stained nuclei from the lower intensity ER staining. Figure 64 shows the results from oocyte imaging. Oocytes consistently showed a metaphase plate containing the chromosomes (blue) and tubulin derived spindle apparatus (green). ER (cyan) and mitochondria (red) were dispersed through the cytoplasm, although they concentrated more heavily near the metaphase plate. Thus they inhabit the same intracellular space and may have an association with one another. The oocyte in the bottom panel also showed membrane staining by FM 1-43 (red). Figure 65 shows the results from 2-cell imaging. In 2-cell embryos we saw the ER (cyan) and mitochondria (red) concentrated around the two nuclei (blue). Again, this implies a potential association between the ER and mitochondria. Tubulin (green) was concentrated between the two cells, and the membrane was stained with FM 1-43 (red). Figure 66 shows the results from ES cell imaging. Fibroblasts had very large nuclei (with
prominent nucleoli (SYTO). The ER (blue) seemed dispersed, and the mitochondria (red) and tubulin (green) were found clustered around the nucleus. In ES cells it was difficult to distinguish the ER (blue). It appeared as a faint blur through the cells. The tubulin (green) was also difficult to distinguish, although at times it strongly stained the cell boundaries. Nuclei (blue) were large and prominent. Lysosomes (green, bottom panel of images) were prominent, and they were heavily concentrated near the center (as determined by z sectioning) of the ES cell colony. The mitochondria (red) produced a bright signal around the nuclei and filled the cytoplasm, leading us to believe that mitochondria are plentiful in ES cells and thus influencing our decision to continue our study of mitochondria in ES cells.
Figure 64: Multicolor Images of Mouse Oocytes. Mouse oocytes were labeled using a variety of organelle specific stains and images were collected using DIC and Epifluorescence Microscopy. Fluorescent images were pseudocolored and overlayed in order to view several cellular structures simultaneously. The stains used included Hoechst (nuclei, blue), ER Tracker (ER, blue/cyan), Tubulin Tracker (microtubules, green), FM 1-43 (membrane, red), and MitoTracker Red (mitochondria, red). The ER Tracker signal could be separated from Hoechst based on brightness. Scale bar = 50 μm; Newmark and Warner, unpublished data.
**Figure 65: Multicolor Images of Mouse 2-Cell Embryos.** Mouse 2-cell embryos were labeled using a variety of organelle specific stains and images were collected using DIC and Epifluorescence Microscopy. Fluorescent images were pseudocolored and overlayed in order to view several cellular structures simultaneously. The stains used included Hoechst (nuclei, blue), ER Tracker (ER, cyan), Tubulin Tracker (microtubules, green), FM 1-43 (membrane, red), and MitoTracker Red (mitochondria, red). The ER Tracker signal could be separated from Hoechst based on brightness. Scale bar = 50 µm; Newmark and Warner, unpublished data.
Figure 66: Multicolor Images of a Fibroblast and Mouse ES Cells. Mouse C57BL/6 ES cells on fibroblasts were labeled using a variety of organelle specific stains and images were collected using DIC and Epifluorescence Microscopy. Fluorescent images were pseudocolored and overlayed in order to view several cellular structures simultaneously. The stains used included Hoechst (nuclei, blue), ER Tracker (ER, cyan), Tubulin Tracker (microtubules, green), LysoTracker (lysosomes, green), and MitoTracker Red (mitochondria, red). Scale bar = 50 µm; Newmark and Warner, unpublished data.
APPENDIX 3: Multimodal Imaging of Mouse Embryonic Stem Cells

The OQM has been used in conjunction with DIC Microscopy to accurately count the number of cells in high cell number preimplantation mouse embryos (Newmark et al., 2007). OQM provides a noninvasive alternate to Hoechst staining and TPLSM 3D sectioning for counting cells. We tested the Keck 3DFM and OQM imaging capabilities on mouse ES cells. First, we stained ES cells with 1 µg/ml Hoechst and 200 nM MitoTracker Green FM. We imaged the cells by DIC, CLSM, TPLSM, and Confocal Reflectance Microscopy (CRM). The results are shown in Figures 67-68. Figure 67 shows one ES cell colony. The CRM detected a strong signal from the nuclei, however the vertical lines (caused by a reflection of the coverslip) interfered with the image. The Hoechst and MitoTracker Green FM staining was bright. The nuclei are visible in the Hoechst Epifluorescence and TPLSM images. The mitochondria, which form a ring around the nuclei, are visible in the MitoTracker Epifluorescence and CLSM images. Figure 68 shows a separate ES cell colony. The staining was not as bright, but the CLSM detected the MitoTracker stained mitochondria surrounding the nuclei, and the TPLSM detected the Hoechst stained nuclei. The CLSM and TPLSM overlay shows the mitochondria surrounding the nuclei. The RCM image again detected a strong signal from the nuclei albeit with the reflection of the coverslip blurring the middle portion of the image.

We also imaged ES cells by OQM (Figure 69). The OQM easily differentiated between cell structure and background structure as shown in the top panel of Figure 69. By implementing linescans, we were able to see the stacking of cells within the ES cell colonies. Linescan 1 showed two levels of peaks (Layer 1 and Layer 2) above the level
of the background whereas Linescan 2 showed three levels of peaks (Layers 1-3) above the level of the background. We infer from these data that the light blue region correlates with one cell layer, the yellow region correlates with two cell layers, and the red region correlates with three cell layers. In this manner we likely can estimate the thickness (number of cells) in ES cell colonies by OQM. This could be confirmed by acquiring TPLSM z-sectioned images of Hoechst stained ES cells. Refinements to this procedure could allow us to accurately count the number of cells in ES cell colonies by OQM.
Figure 67: Multimodal Imaging of Mouse Embryonic Stem Cells-1. An ES cell colony was stained with Hoechst (nuclei) and MitoTracker GreenFM and imaged by DIC Microscopy, Reflectance Confocal Microscopy (RCM), Epifluorescence Microscopy, TPLSM (2P), and CLSM (1P). Scale bar = 50 µm; Warger, Newmark, and Warner, unpublished data.
Figure 68: Multimodal Imaging of Mouse Embryonic Stem Cells-2. C57BL/6 ES cells were stained with MitoTracker Green (green) and Hoechst (nuclei, blue) and imaged by DIC, CLSM, TPLSM, and RCM. Scale bar = 50 µm; Warger, Newmark, and Warner, unpublished data.
Figure 69: OQM Imaging of Mouse ES Cells. Unstained C57BL/6 ES cells were imaged by OQM. Processing of OQM images was able to clearly distinguish between cell structure and background structure. Linescans (black line) through OQM images are reflective of cell stacking. Warger, Newmark, and Warner, unpublished data.