Effects of Oxygen on Embryonic Stem Proliferation, Energetics, and Differentiation into Cardiomyocytes

by

Daryl E. Powers

Bachelor of Science in Chemical Engineering
Northeastern University, 2001

Submitted to the Department of Chemical Engineering in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Chemical Engineering

at the

Massachusetts Institute of Technology

May 2007

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Author..................................................

Department of Chemical Engineering
May 22, 2007

Certified by...........................................

Clark K. Colton
Professor of Chemical Engineering
Thesis Supervisor

Accepted by...........................................

William Deen
Professor of Chemical Engineering
Chairman, Committee for Graduate Students
Acknowledgements

This work would not have been possible without the help and support of many people. I am grateful for the guidance of my thesis advisor, Clark Colton, who provided me freedom to explore during my research and advice to keep me focused. Clark gave me valuable lessons and instruction on the preparation of display materials, research reports, and presentations, and the quality of my work has greatly benefited from having worked with him.

I am also grateful for the support of the rest of the laboratory, particularly Anna Pisania and Michael Rappel. They have been invaluable colleagues as well as friends and the 5 years of research have much more enjoyable and productive because I have had the pleasure of working with them. Although I will not miss spending entire nights working with them on grant proposals, I will miss our daily interactions. I would also like to thank Ryan Huang and Joao Paulo Mattos Almeida, who worked with me to perform experiments and assisted with obtaining some of the data shown in this thesis. I also am grateful to have a capable successor, Jeffrey Millman, who will carry on with this research and answer some of the scientific questions that I was unable to address. Finally, I would like to thank Kevin Brower, Amy Lewis, Michelle Miller, and Jin Zhou for the general support that they provided during the course of research.

I would also like to acknowledge the assistance of my thesis committee. Susan Bonner-Weir and Gordon Weir provided helpful advice and a biological perspective to keep me on track. Kenneth Smith and Linda Griffith provided me valuable suggestions during thesis committee meetings and helped me focus on the most important problems. Klearchos Papas played an important role training me and helping me to get started working in the lab.

Last of all, I want to acknowledge the other people in my life who have provided support. I am grateful for all of the great friends that I have made at MIT including Ramin Haghgoie, Brad Cicciarelli, Chad Augustine, Keith Tyo, Patrick Underhill, Jason Fuller, Sam Ngai, Andre Ditsch, Luwi Oluwole, Michelle Wu, Yuhua Hu, Hairong Tang, Josh Tam, Joanna Yu, and all of the guys who have played various IM sport with me. Finally, and most importantly, I want to acknowledge my family including my mother (Vivian), father (Richard), two brothers (Craig and Brent), and more recently my girlfriend (Yina). They have provided me the support that I needed to complete this project. No matter how poorly research has gone, I have always been confident in their love. This has been my most valued asset of all.
dedicated to my mother and father, for teaching me the value of hard work
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Abstract

Most embryonic stem (ES) cell research has been performed using a gas-phase oxygen partial pressure (pO$_{2\text{gas}}$) of 142 mmHg, whereas embryonic cells in early development are exposed to cellular pO$_2$ (pO$_{2\text{cell}}$) values of about 0-30 mmHg. Murine ES (mES) cells were used as a model system to study the effects of oxygen on ES cell proliferation, phenotype maintenance, cellular energetics, and differentiation into cardiomyocytes. It was found that undifferentiated mES cells are capable of surviving and proliferating at pO$_2$ conditions in the range of 0-285 mmHg, with only moderately decreased growth at the extremes in pO$_2$ over this range. Oxygen levels had no effect on the maintenance of the undifferentiated phenotype during culture with the differentiation-suppressing cytokine leukemia inhibitory factor (LIF) in the culture medium, and low oxygen had, at most, a small differentiating-promoting effect during culture without LIF. Aerobic metabolism was used to generate approximately 60% of the energy required by undifferentiated mES cells at high pO$_2$, but substantially smaller fractions when cells were oxygen starved. This shift from aerobic to anaerobic respiration occurred within 48 hr with minimal cell death. Oxygen was found to substantially affect the differentiation of mES cells into cardiomyocytes. Reduced pO$_{2\text{cell}}$ conditions strongly promoted cardiomyocyte development during the first 6 days of differentiation, after which oxygen primarily influenced cell proliferation. Using silicone rubber membrane-based dishes to improve oxygenation and an optimized cardiomyocyte differentiation protocol, it was possible to reproducibly obtain 60 cardiomyocytes per input ES cells and a cell population that was 30% cardiomyocytes following 11 days of differentiation. These results, obtained using a pO$_{2\text{gas}}$ of 7 mmHg during the first 6 days of differentiation, represent a 3-fold increase relative to those obtained with a pO$_{2\text{gas}}$ of 142 mmHg throughout differentiation. This work has shown that undifferentiated ES cells are able to adapt to their environmental pO$_2$ and are relatively insensitive to its variations, whereas during differentiation oxygen affects cell fate decisions. Oxygen control can be used to improve directed ES cell differentiation into cardiomyocytes and oxygen may play a more important role in early embryonic development than heretofore appreciated.

Thesis Supervisor: Clark K. Colton
Title: Professor of Chemical Engineering
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Chapter 1. Background and Overview of Results
Background

Embryonic stem (ES) cells have the potential to generate any type of cell found and provide material for cell and tissue for transplantation to treat diseases such as diabetes, leukemia, and Parkinson's disease (Bonner-Weir et al. 2000; Kaufman et al. 2001; Kim et al. 2002; Soria 2001). For the promise of ES cell technology to be realized, development of efficient techniques for expansion of undifferentiated cells and subsequent directed differentiation of these cells must be developed, and this requires improvement of current practices requires an understanding of how ES cells interact with their microenvironment and development of methods to control these interactions. The oxygen partial pressure (pO₂) at the cell surface to which the cell is exposed (pO₂cell) is potentially one important environmental factor that can be manipulated for the improvement of ES cell culture protocols.

Oxygen levels during in-vivo development and in-vitro cell culture

Mammalian tissues, especially those in the developing embryo, are usually exposed to pO₂ values far below atmospheric levels. Values as low as 25 mmHg have been reported for the rat microvasculature (Intaglietta et al. 1996). Venous human fetal blood in the second and third trimester ranges from 25 to 30 mmHg (Siggaard-Andersen and Huch 1995; Soothill et al. 1986). Even lower pO₂ values are found in gestational sac during the first trimester (Jauniaux et al. 2003a). Current thinking is that the gestational sac limits fetal exposure to oxygen, providing a low pO₂ environment. In addition, the sac provides an environment containing antioxidant molecules, which provide protection from oxidative damage (Jauniaux et al. 2003b). pO₂ values within early embryos are likely to be lower because circulation does occur until a well-formed vascular network is present (Maltepe and Simon 1998; Palis et al. 2001). Even after a well-developed vascular network exists, there are still hypoxic regions within an embryo (Land 2004; Lee et al. 2001).

Currently, most in vitro stem cell work is performed at poorly controlled pO₂ levels that are usually much higher than that of the embryo in vivo. Typically, cells are incubated in a humidified atmosphere consisting of 95% air/5% CO₂, resulting in a gas phase pO₂ (pO₂gas) of 142 mmHg. Most cell culture is done on dishes made of polystyrene, a polymer with very low oxygen permeability, leading to a cell surface pO₂ (pO₂cell) that is different from the gas phase and usually unknown (Metzen et al. 1995; Tokuda et al. 2000; Yarmush et al. 1992). The pO₂cell depends on several factors, including depth of medium, density of cells, cellular respiration, and pO₂gas, which are usually not taken into account (Csete 2005; Tokuda et al. 2000). The failure of oxygen-impermeable culture dishes to adequately control pO₂cell was first recognized using theoretical models of oxygen transport, (McLimans et al. 1968; Stevens 1965) and has been subsequently verified using microelectrodes (Metzen et al. 1995; Pettersen et al. 2005; Tokuda et al. 2000; Wolff et al. 1993).
To improve \( pO_{2_{cell}} \) control, researchers have either cultured cells on gas permeable dishes or used convective oxygen transport in mechanically mixed or perfused vessels. For our work, we elected to use gas-permeable dishes, since these can be used with standard static culture protocols with minimal modification. The ability of such dishes to control \( pO_{2_{cell}} \) to a value near \( pO_{2_{gas}} \) was shown theoretically (Jensen et al. 1976) and then experimentally (Wolff et al. 1993) with fluoroethylene-propylene copolymer (FEP-teflon) membrane-based dishes. Compared to FEP-teflon, silicone rubber has about a 100 fold higher oxygen permeability (Avgoustiniatos 2002; DuPont 1996), and is therefore the preferred membrane material for oxygen delivery in membrane aerated bioreactors (Henzler and Kauling 1993; Rishell et al. 2004a; Rishell et al. 2004b; Yoshino et al. 1996) and in devices for high density culture of hybridoma cells (Falkenberg 1998). These commercially available devices are too large for stem cell research, and we manufactured our own silicone rubber membrane-based dishes.

**Effects of oxygen on growth, cellular energetics, and stem cell differentiation**

Cell survival and proliferation can be adversely affected by \( pO_{2} \) conditions that are sub-optimal. Culture of cells at a \( pO_{2} \) higher than needed exposes them to higher concentrations of reactive oxygen species (ROS), which damage lipids, proteins, and nucleic acids (Lee and Wei 2005; Saretzki et al. 2004) and can lead to senescence and cell death (Parrinello et al. 2003). For this reason, and perhaps others, numerous cell types have a higher growth rate at \( pO_{2_{gas}} \) conditions less than 142 mmHg. These cell types include fibroblasts (Bradley 1978; Parrinello et al. 2003), hematopoietic cells (Bradley 1978; Hevehan et al. 2000; Mostafa et al. 2000), neural progenitors (Milosevic et al. 2005; Morrison et al. 2000; Studer et al. 2000), muscle satellite cells (Chakravarthy et al. 2001; Csete et al. 2001), CHO and hybridoma cells (Goetz 1975; Miller et al. 1987), as well as early embryos of both humans and mice (Dumoulin et al. 1999; Orsi and Leese 2001; Quinn and Harlow 1978). Providing cells with too little oxygen can also be harmful and lead to apoptotic or necrotic cell death (Brunelle and Chandel 2002).

Cellular energetics are affected by the culture \( pO_{2_{cell}} \) conditions (Miller et al. 1987), and oxygen starvation requires cells to rely on anaerobic glycolysis for their energy supply. Mouse, bovine, and human blastocysts have a high glycolytic capacity and can readily convert glucose into lactic acid (Devreker and Englert 2000; Harvey et al. 2002; Hewitson and Leese 1993; Houghton et al. 1996). Despite these high levels of anaerobic glycolysis, 75 to 85% of the ATP production is reported to occurs through oxidative phosphorylation at high \( pO_{2_{gas}} \) (Houghton 2006; Houghton et al. 1996; Thompson et al. 1996). Under normal oxygen culture conditions, ES cells also have relatively high levels of glycolytic enzymes during culture with at a \( pO_{2_{gas}} \) of 142 mmHg that are further increased at 7 mmHg reduced \( pO_{2_{gas}} \) conditions through the action of HIF-1\( \alpha \) (Iyer et al. 1998; Wenger 2002). Quantitative oxygen consumption and lactate production measurements have not been done with ES cells have not been reported, and it is unknown how these parameters are affected by the culture \( pO_{2_{cell}} \), so the relative contribution of oxidative phosphorylation and anaerobic glycolysis is unknown.
The pO\textsubscript{2}cenI used for culture can affect whether stem cells remain undifferentiated. Culture of hematopoietic progenitor cells at reduced pO\textsubscript{2}gas favors self-renewal, while increased pO\textsubscript{2}gas favors differentiation and maturation (Cipolleschi et al. 1997; Hevehan et al. 2000; Mostafa et al. 2000; Ramirez-Bergeron et al. 2004). Similar behavior is observed in CNS progenitor and neural crest stem cells (Morrison et al. 2000; Studer et al. 2000), and the notch pathway has been implicated the oxygen dependent phenotype maintenance of both neural stem and muscle satellite cells (Gustafsson et al. 2005). Studies with ES cells show no consensus about whether oxygen affects the undifferentiated phenotype. Depending on the study, low oxygen promotes differentiation (Jeong et al. 2007), prevents differentiation (Ezashi et al. 2005), or has no effect on maintenance of the ES cell phenotype (Forsyth et al. 2006; Ludwig et al. 2006; Peura et al. 2005).

In addition to potentially influencing ES cells to enter into a pathway that leads to differentiation, it has been shown that the culture pO\textsubscript{2} affects the types of differentiated cells that are formed when stem cells differentiate. Low oxygen has also been attributed to an increase in osteogenic markers in differentiating rat mesenchymal stem cells (Lennon et al. 2001) and to an enhancement in the differentiation of neural stem and precursor cells into dopamine producing neurons and dopamine producing sympathoadrenal lineage cells (Morrison et al. 2000; Studer et al. 2000). During ES cell differentiation, reduced pO\textsubscript{2} culture leads to an increase in the differentiation of ES cells into various cells of the hematopoietic lineage (Adelman et al. 1999; Potocnik et al. 1994), early chondrocytes (Chen et al. 2006a; Chen et al. 2006b), and cardiomyocytes (Bauwens et al. 2005).

Culture pO\textsubscript{2} affects cells through the alteration of the cellular redox state (Zupke et al. 1995), and through the regulation of the transcription factor subunit hypoxia inducible factor 1\alpha (HIF-1\alpha) (Lee et al. 2004). The HIF-1\alpha gene is constitutively transcribed over all oxygen concentrations (Semenza et al. 1999), but the protein concentration of HIF-1\alpha increases exponentially as oxygen concentration decreases (Iyer et al. 1998; Jiang et al. 1996). In HeLa cells, HIF-1\alpha protein reaches half its maximum expression at 1.5-2\% oxygen and maximum expression at 0.5\% oxygen (Jiang et al. 1996). HIF-1\alpha protein concentrations are also increased during low oxygen culture in mouse ES cells (Iyer et al. 1998). Dozens of genes are known to be up-regulated due to the expression of the transcription factor HIF-1, notably glucose transporters, glycolytic enzymes, erythropoietin (EPO), and vascular endothelial growth factor (VEGF) (Iyer et al. 1998). HIF-1 is necessary for the proper development of embryos. In HIF-1\alpha null mutant mouse embryos, embryonic lethality occurs by day E11 (Iyer et al. 1998).

**Differentiation of ES cells into cardiomyocytes**

Cardiomyocytes derived from differentiated embryonic stem (ES) cells hold promise as a treatment for heart disease (Klug et al. 1996), but obtaining sufficient number and purity of functional cells is challenging (Xu et al. 2006). The use of a various molecules to direct cardiomyocyte differentiation has been studied extensively, and positive results have been reported with retinoic acid, nitric oxide, TGF\beta-1, FGF, erythropoietin, BMP-2, BMP-4, ascorbic
acid, retinoic acid, DMSO, noggin, 5-azacytidine, and VEGF (Chen et al. 2006c; Fukuda and Yuasa 2006; Yoon et al. 2006).

Little attention has been given to the possible effects that oxygen might have on cardiomyocyte differentiation, and there is a only a single study exploring how oxygen affects this process (Bauwens et al. 2005). This work showed only a very small benefit of low oxygen culture that could only be observed after substantial genetic selection to purify cardiomyocytes. However, the differentiation methods used in this study produced poor cardiomyocyte cell fractions which potentially obscured a more significant effect of oxygen on ES cell differentiation into cardiomyocytes.

Overview of Results

The work described in this thesis focused on quantitatively studying the effects of dissolved oxygen during (1) culture of undifferentiated ES cells and (2) differentiation of these cells into cardiomyocytes. In all of the studies performed consideration was given to the pO2 experienced by the cells, and when required, methods were developed to minimize differences between the pO2gas and the pO2cell. All studies were performed using murine ES (mES) cells as a model system.

Effects of oxygen on ES cell growth and energetics

To study the implications of performing ES cell culture using pO2 values that are much higher than those during in-vivo development and to gain a better understanding of ES cell physiology we measured (1) the cellular growth rate and undifferentiated cell fraction in differentiation-suppressing and -permissive conditions at pO2gas conditions in the range of 0-285 mmHg, (2) the temporal change in cell viability, maximal oxygen consumption rate (OCR), and lactate dehydrogenase (LDH) activity after a step change in the pO2gas from 142 to 0 mmHg, and (3) the OCR and lactate production rate (LPR) after prolonged culture at pO2gas conditions in the range of 0-285 mmHg. Growth of undifferentiated mES cells was exponential at all pO2 conditions with a specific growth rate of 1.2 day⁻¹ at pO2gas in the range of 36 - 142 mmHg, decreasing to about 0.9, 1.0, and 0.6 day⁻¹ at 285, 7, and 0 mmHg, respectively. Culture pO2gas had no effect on the maintenance of mES cells in their undifferentiated state in differentiation-suppressing conditions, and low pO2gas had, at most, a small differentiation promoting effect in differentiation-permissive conditions. Undifferentiated mES cells were able to survive oxygen starvation with negligible cell death by increasing anaerobic metabolism, as indicated by a two-fold increase in intracellular LDH activity that occurred within 48 hr of exposure to anoxia. OCR and LPR were about 29 and 110 amol/cell sec, respectively, for cells maintained at a pO2gas of 142 mmHg or greater. Decreasing pO2gas to 36 mmHg or lower decreased OCR and increased LPR. The fraction of ATP generated aerobically was 60% at 285 and 142 mmHg and decreased to 50, 30, and 0% at pO2 of 36, 7, and 0 mmHg, respectively, but the total ATP production rate was about 280 amol/cell sec at all pO2. In conclusion, undifferentiated ES cells adapt their
energy metabolism to survive and proliferate at all pO₂ between 0 and 285 mmHg. Oxygen has minimal effects on undifferentiated cell growth and phenotype when differentiation is exogenously suppressed, but may exert some influence on ES cell phenotype maintenance under differentiating conditions.

Silicone rubber membranes for control of cellular oxygen

To identify methods that could be used for the culture of differentiating ES cells at well-defined cellular oxygen levels we studied (1) the oxygen supply to monolayers of cells and cellular aggregates growing on polystyrene dishes, FEP-teflon membranes, and silicone rubber membranes in static culture, (2) methods that could be used to promote attachment of cells to silicone rubber, and (3) cardiomyocytic differentiation of mES cells on silicone rubber membranes. Oxygen transport was significantly improved using by culturing cells on membrane-based dishes, especially those made with silicone rubber. During monolayer culture, the difference between the gas phase and the cellular pO₂ (ΔpO₂) was less than 1 mmHg on silicone rubber, independent of cell density and medium depth, whereas ΔpO₂ could be 15 and 150 mmHg or higher on FEP-teflon membranes and polystyrene dishes, respectively. Use of silicone rubber membrane-based dishes also greatly improved oxygenation of cultured aggregates, however there were still oxygen gradients within the tissue. Undifferentiated mES cells did not adhere to native silicone rubber, but treatment with either a polyelectrolyte multilayer or adsorption of fibronectin prior to cell addition allowed for robust attachment. Using the fibronectin adsorption technique, differentiating ES cells could be cultured on silicone rubber membrane-based plates for at least 11 days. Significantly more cells were present on the silicone rubber membrane-based dishes than parallel polystyrene dishes, presumably due to the increased oxygen availability on silicone rubber. In conclusion, there is a strong theoretical justification for the use of silicone rubber membrane-based culture surfaces for the study of the effects of dissolved oxygen on cell function, and it possible to modify the silicone surface so it can be used for the culture of adherent cells.

Methods for differentiation of ES cells into cardiomyocytes

To show that oxygen might affect differentiation of ES cells into cardiomyocytes and find methods to further improve the robustness of the process we studied differentiation at several pO₂ conditions using (1) a neural differentiation protocol with embryoid bodies (EBs) formed in suspension or in hanging drops, (2) supplemental ascorbic acid to promote cardiomyogenesis, (3) a range of initial cell numbers for EB formation and time in hanging drop EB culture, and (4) a change to serum-free medium at different times during differentiation. After 24 days of neuronal differentiation, more cells were cardiomyocytes at a pO₂gas of 7 relative to 142 mmHg. The fraction of cardiomyocytes reached its maximal value within about 10 days of differentiation, and supplemental ascorbic acid could be used to promote cardiomyocyte differentiation at all oxygen levels. The highest fraction and total number of cardiomyocytes were obtained when mES cells were differentiated as EBs in hanging drops with
500 cells per drop, EBs were cultured for 2 days in hanging drops before being transferred to adherent culture on silicone rubber membrane-based dishes, and the medium was changed from serum-containing to serum-free medium after 5 days. Such conditions produced 29, 16, and 7% cardiomyocytes, after 11 days at a pO$_{2\text{gas}}$ of 7, 36, and 142 mmHg, respectively, in a preliminary experiment. In conclusion, oxygen control could be used to improve the differentiation of mES cells into cardiomyocytes, and a protocol was developed to improve mES cell differentiation into cardiomyocytes. This protocol was used in subsequent work described below to definitively demonstrate that oxygen affects cardiomyocyte differentiation.

**Effects of oxygen on ES cell differentiation into cardiomyocytes**

To show that oxygen affects cardiomyocyte differentiation and better understand the time and specific oxygen levels over which an effect is present we (1) quantitatively studied cardiomyocyte differentiation at different pO$_2$ conditions in 7 independent experiments, (2) studied the effect of temporal variation in pO$_2$ on the total cell number and fraction and total number of cardiomyocytes after 10 days of differentiation, and (3) estimated the pO$_{2\text{cell}}$ during differentiation using theoretical models. Oxygen reproducibly affected mES cell differentiation into cardiomyocytes. After 10 or 11 days of differentiation, the highest number fraction of cardiomyocytes was 31 ± 6% with a pO$_{2\text{gas}}$ of 7 mmHg, compared to 23 ± 9% and 9 ± 4% at 36 and 142 mmHg, respectively. Culture at 7 mmHg resulted in a substantial decrease in total cell number relative to that at 36 or 142 mmHg, both of which had comparable cell numbers after 11 days. As a result, the total number of cardiomyocytes was similar at 7 and 142 mmHg but significantly higher at 36 mmHg. Low oxygen (7 mmHg) exerted its strongest cardiomyocyte differentiation-promoting effect during the first 6 days of differentiation, and subsequent increases in pO$_{2\text{gas}}$ to 142 mmHg could be used to maximize the total cardiomyocyte number without a decrease in purity. Theoretical models of oxygen transport showed that cells differentiated at high pO$_{2\text{gas}}$ were well-oxygenated during early stages of culture, and some cardiomyocytes were apparently able to form at high pO$_{2\text{cell}}$ conditions. Oxygen starvation was predicted to occur in some tissue clumps after 11 days of differentiation at all pO$_{2\text{gas}}$ due to the increasing size of cell aggregates with increasing time in culture and pO$_{2\text{gas}}$. In conclusion, oxygen substantially affects the differentiation of mES cells into cardiomyocytes, and pO$_2$ control can be used to increase the fraction and total number of cardiomyocytes obtained from the differentiation of ES cells.
Chapter 2. Effects of Oxygen on ES Cell Growth and Energetics
To study the implications of performing ES cell culture using pO2 values that are much higher than those during in-vivo development and to gain a better understanding of ES cell physiology we measured (1) the cellular growth rate and undifferentiated cell fraction in differentiation-suppressing and -permissive conditions at pO2gas conditions in the range of 0-285 mmHg, (2) the temporal change in cell viability, maximal oxygen consumption rate (OCR), and lactate dehydrogenase (LDH) activity after a step change in the pO2gas from 142 to 0 mmHg, and (3) the OCR and lactate production rate (LPR) after prolonged culture at pO2gas conditions in the range of 0-285 mmHg. Growth of undifferentiated mES cells was exponential at all pO2 conditions with a specific growth rate of 1.2 day⁻¹ at pO2gas in the range of 36 – 142 mmHg, decreasing to about 0.9, 1.0, and 0.6 day⁻¹ at 285, 7, and 0 mmHg, respectively. Culture pO2gas had no effect on the maintenance of mES cells in their undifferentiated state in differentiation-suppressing conditions, and low pO2gas had, at most, a small differentiation promoting effect in differentiation-permissive conditions. Undifferentiated mES cells were able to survive oxygen starvation with negligible cell death by increasing anaerobic metabolism, as indicated by a two-fold increase in intracellular LDH activity that occurred within 48 hr of exposure to anoxia. OCR and LPR were about 29 and 110 amol/cell sec, respectively, for cells maintained at a pO2gas of 142 mmHg or greater. Decreasing pO2gas to 36 mmHg or lower decreased OCR and increased LPR. The fraction of ATP generated aerobically was 60% at 285 and 142 mmHg and decreased to 50, 30, and 0% at pO2 of 36, 7, and 0 mmHg, respectively, but the total ATP production rate was about 280 amol/cell sec at all pO2. In conclusion, undifferentiated ES cells adapt their energy metabolism to survive and proliferate at all pO2 between 0 and 285 mmHg. Oxygen has minimal effects on undifferentiated cell growth and phenotype when differentiation is exogenously suppressed, but may exert some influence on ES cell phenotype maintenance under differentiating conditions.

Introduction

Embryonic stem (ES) cells have the potential to generate any type of cell for transplantation to treat diseases such as diabetes, leukemia, and Parkinson’s disease (Bonner-Weir et al. 2000; Kaufman et al. 2001; Kim et al. 2002; Soria 2001). Development of efficient techniques for expansion of undifferentiated cells and subsequent directed differentiation requires an understanding of how ES cells interact with their microenvironment and methods to control these interactions. The oxygen partial pressure (pO2) to which the cell is exposed (pO2cell) is potentially one important environmental parameter.

Mammalian tissues are usually exposed to pO2 values far below atmospheric levels, such as 25 mmHg in the rat microvasculature (Intaglietta et al. 1996), 15 to 45 mm Hg in the reproductive tract of rhesus monkeys (Fischer and Bavister 1993), and 25 to 30 mmHg in venous human fetal blood in the second and third trimester (Sigggaard-Andersen and Huch 1995; Soothill et al. 1986). Lower values occur in the gestational sac during the first trimester (Jauniaux et al. 2003b), which also provides an environment containing antioxidant molecules to further reduce...
oxidative damage (Jauniaux et al. 2003a). pO2 values within early developing embryos are likely to be even lower because circulation does not occur until a well-formed vascular network is present (Maltepe and Simon 1998; Palis et al. 2001), and there are hypoxic regions even after a well-developed vascular network exists (Land 2004; Lee et al. 2001).

Stem cell culture is usually performed at pO2 levels much higher than that of the embryo in vivo, typically in a humidified atmosphere consisting of 95% air/5% CO2 that results in a gas phase pO2 (pO2gas) of 142 mmHg. Most experiments with ES cells are carried out in polystyrene culture dishes, which have very low oxygen permeability, and oxygen is supplied by diffusion through the medium. Oxygen concentration gradients exist in the medium that cause pO2cell to be different from pO2gas (Metzen et al. 1995; Tokuda et al. 2000; Yarmush et al. 1992). The value of pO2cell depends on several factors, including medium depth, cell density, cellular oxygen consumption rate, and pO2gas, which are usually not taken into account (Csete 2005; Metzen et al. 1995). As a consequence, the value of pO2cell during ES cell culture is usually not controlled to be in the range experienced by the early embryo. The implications of this poor control are not well understood. Published data obtained with ES and other cell types suggest that cell survival and proliferation, maintenance of the stem cell phenotype, and cellular energetics are three important parameters that can be significantly affected by the magnitude of pO2cell.

Cell proliferation can be adversely affected by too high or too low a pO2. Culture at a pO2 higher than needed exposes cells to higher concentrations of reactive oxygen species (ROS), which damage lipids, proteins, and nucleic acids (Lee and Wei 2005; Saretzki et al. 2004) and can lead to senescence and cell death (Parrinello et al. 2003). Many cell types have a higher growth rate at pO2gas conditions less than 142 mmHg, including fibroblasts (Bradley 1978; Parrinello et al. 2003), hematopoietic cells (Bradley 1978; Hevehan et al. 2000; Mostafa et al. 2000), neural progenitors (Milosevic et al. 2005; Morrison et al. 2000; Studer et al. 2000), muscle satellite cells (Chakravarthy et al. 2001; Csete et al. 2001), CHO and hybridoma cells (Goetz 1975; Miller et al. 1987), as well as early embryos of both humans and mice (Dumoulin et al. 1999; Orsi and Leese 2001; Quinn and Harlow 1978). Providing too little oxygen can also be harmful and lead to apoptotic or necrotic cell death (Brunelle and Chandel 2002).

In addition to growth, pO2gas can affect whether stem cells remain undifferentiated. Culture of hematopoietic progenitor (Cipolleschi et al. 1997; Hevehan et al. 2000; Mostafa et al. 2000; Ramirez-Bergeron et al. 2004), CNS progenitor (Studer et al. 2000), and neural crest stem cells (Morrison et al. 2000) at reduced pO2gas favors self-renewal, while increased pO2gas favors differentiation and maturation. Other studies suggest that reduced pO2gas increases the growth rate of ES cells and might be beneficial for maintaining the undifferentiated phenotype, but only two or three values of pO2gas were examined (Carmeliet et al. 1998; Ezashi et al. 2005; Forsyth et al. 2006; Gibbons et al. 2006; Hopfl et al. 2002; Iyer et al. 1998; Kurosawa et al. 2006; Ludwig et al. 2006; Ording et al. 2005; Peura et al. 2005; Wang et al. 2006).

Cellular energetics are affected by pO2cell (Miller et al. 1987), and survival at low oxygen requires anaerobic glycolysis for ATP generation. Mouse, bovine, and human blastocysts have high glycolytic capacity and can readily convert glucose into lactic acid (Devreker and Englert
2000; Harvey et al. 2002; Hewitson and Leese 1993; Houghton et al. 1996). Nonetheless, 75 to 85% of the ATP production in embryos occurs through oxidative phosphorylation at high pO$_2$gas (Houghton 2006; Houghton et al. 1996; Thompson et al. 1996). The high levels of glycolytic enzymes in ES cells during culture at a pO$_2$gas of 142 mmHg are further increased at reduced pO$_2$gas conditions through the action of HIF-1$\alpha$ (Iyer et al. 1998; Wenger 2002). Quantitative oxygen consumption and lactate production measurements with ES cells have not been reported, and it is unknown how these parameters are affected by the culture pO$_2$cell.

In this study we performed a systematic quantitative investigation of the effects of pO$_2$ on growth rate, stem cell phenotype, and energetics of murine ES (mES) cells. Varying pO$_2$gas in the range from 0 to 285 mmHg had a limited effect on the growth rate of undifferentiated mES cells, both with and without inclusion of the differentiation-suppressing cytokine leukemia inhibitory factor (LIF) in the culture medium. Changing pO$_2$gas had no effect on the maintenance of the undifferentiated phenotype during culture with LIF, but high pO$_2$gas favored continued expression of Oct 3/4 during culture without LIF. Aerobic metabolism generated approximately 60% of the energy required by mES cells at high oxygen conditions, but substantially smaller fractions if cells were oxygen starved. This shift from aerobic to anaerobic respiration occurred rapidly with minimal cell death.

Materials and Methods

Cells and media

Two mES cell lines were used, passage 22 CCE (Keller et al. 1993; Robertson et al. 1986) from Stem Cell Technologies (Vancouver, BC) and unknown passage number D3 (Doetschman et al. 1985) from ATCC (CRL-1934, Manassas, VA). Undifferentiated cells were grown in high glucose DMEM (30-2002, ATCC) supplemented to 10% (v/v) ES cell qualified fetal bovine serum (FBS) (06905, Stem Cell Technologies, or SCRR 30-2020, ATTC). The medium was further supplemented to a final concentration of 1000 U/ml leukemia inhibitory factor (LIF, ESG1106, Chemicon, Temecula, CA) and 0.1 mM 2-mercaptoethanol (M-7522, Sigma-Aldrich, St. Louis, MO). Differentiation was carried out in an otherwise identical medium without LIF.

Cell culture

Unless otherwise stated, cells were grown with 4 ml of medium in 25 cm$^2$ cell culture flasks (353109, Becton Dickinson, Franklin Lakes, NJ) that were treated for 30 min with a sterile 0.1% (w/v) solution of gelatin (G-2500, Sigma-Aldrich) in tissue culture water (25-055-CM, Mediatech, Herndon, VA). Cells were plated at a density of about 12,000 cells/cm$^2$. Medium was exchanged daily, and cells were detached with 0.25% trypsin (30-2101, ATCC) every two days. Split fractions were chosen so that cells were always plated at 12,000 cells/cm$^2$. Medium was incubated in the appropriate pO$_2$gas condition for 24 hr prior to use.
Gas phase pO$_2$ control

Cell culture vessels were placed inside polystyrene chambers (MIC-101, Billups-Rothenburg, Del Mar, CA) contained within a standard incubator (OWJ272A0, Queue Systems, Parkersburg, WV) maintained at 37 °C. An open dish of deionized water in each chamber provided humidification. The desired pO$_2$gas was attained using premixed gas containing 5% CO$_2$ and 40%, 5%, 1%, or 0% O$_2$ (certified medical gas from Airgas, Hingham, MA). The flow rate of this gas to the chambers was 2 l/min for 15 min for an initial purge following closure of the chamber (after cell medium exchange or passage) and was 30 ml/min at all other times.

Cell enumeration, membrane integrity, and apoptosis assessment

After detachment with trypsin, cell number and membrane integrity were evaluated by staining cells using a Guava Viacount assay kit (Guava Technologies, Hayward, CA) and acquiring data with a Guava PCA flow cytometer. Apoptotic cell fraction was determined using an Annexin V assay kit (Guava Technologies).

Oxygen consumption rate measurement

Cells were detached with trypsin and resuspended at a density of between 6 and 12 x 10$^6$ viable cells/ml in fresh culture medium equilibrated to 37 °C and ambient oxygen. The oxygen consumption rate (OCR) of this suspension was measured with a Micro Oxygen Monitoring System (FO/SYS2-T250, Instech Labs, Plymouth Meeting, PA) as described previously (Papas et al. 2007). Briefly, a 200 µl aliquot of the cell suspension was sealed within the titanium chamber stirred with a glass-coated magnetic stirring bar. The time-dependent pO$_2$ within the chamber was recorded with a fluorescence-based oxygen sensor, and the data at pO$_2$ values greater than 30 mmHg were fit to a straight line by linear regression analysis. The oxygen consumption rate (OCR) was evaluated from

$$\text{OCR} = V_{ch} \alpha \frac{\Delta pO_2}{\Delta t}$$

where $V_{ch}$ is the chamber volume (200 µl), $\alpha = 1.19 \times 10^{-9}$ mol/cm$^3$ mmHg is the Bunsen solubility coefficient for oxygen in medium (Avgoustiniatos 2002), and $\Delta pO_2/\Delta t$ is the slope of the fitted line. OCR/cell was determined by dividing the total OCR by the total number of viable cells that were loaded into the chamber. All measurements were completed within 1 hr of trypsin detachment of the cells.

Lactate concentration measurement

Assay reagent, prepared fresh for each test, consisted of 1 U/ml lactate oxidase (L-0638, Sigma-Aldrich), 0.025 U/ml peroxidase (P-6782, Sigma-Aldrich), and 0.1 mg/ml of o-dianisidine (F-5803, Sigma-Aldrich) in DPBS. Samples of supernatant medium from cell cultures were collected, and lactate concentration was measured by adding 100 µl of assay
reagent to 50 μl of diluted sample (final concentration 0.1 – 0.3 mM lactate) in wells of a 96-well plate. The plate was incubated for 30 min at 37 °C, 100 μl of 12 N sulfuric acid was added to stop the reaction, absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA), and lactate concentration was determined with a standard curve prepared for each test using lactic acid (L-1750, Sigma-Aldrich). Dulbecco’s phosphate buffered saline (DPBS) (21-030-CM, Mediatech) was used as a diluent throughout.

**Lactate production rate calculation**

We assumed that the lactate production rate (LPR) is proportional to the number of cells (N) at any instant of time (t), which can be represented by

\[ \text{LPR} = V_m \frac{dC}{dt} = \lambda N \]  

(2.2)

where \( V_m \) is the medium volume, \( C \) is the lactate concentration in the medium, and the coefficient \( \lambda \) is the LPR per cell, which is assumed to be constant during an experiment. Rearranging and integrating yields

\[ V_m(C_f - C_0) = \lambda \int_0^{t_f} N \, dt \]  

(2.3)

where subscripts 0 and f represent the initial and final conditions, and \( t_f \) is the duration of the experiment. When cells are in exponential growth, which applied in all of our experiments, we can write

\[ N = N_0 \exp(\mu t) \]  

(2.4)

where \( N_0 \) is the initial number of cells, and \( \mu \) is the specific growth rate. Substituting Eqn. (2.4) into Eqn. (2.3) and integrating leads to

\[ \lambda = \frac{V_m(C_f - C_0)}{N_0 \left[ \exp(\mu t_f) - 1 \right]} \]  

(2.5)

from which \( \lambda \) is evaluated, since all quantities in Eqn. (2.5) were known. In our experiments, we measured final cell number \( N_f \) at \( t = t_f \), which was used to estimate \( N_0 \) with Eqn. (2.4).

**LDH activity measurement**

A 100-μl aliquot containing \( 10^5 \) cells was added to a 1.5 ml tube containing 1 ml of DPBS and centrifuged for 3 min at 300xg to obtain a cell pellet. To lyse the cells, the
supernatant was removed, and 1 ml of 1% (v/v) Triton X-100 (T-9284, Sigma-Aldrich) in DPBS was added to each sample. The tubes were then briefly mixed using a vortex mixer and kept at room temperature for 1 hr. LDH activity in the samples was measured with an Ektachem Vitros DT 60 II (Johnson and Johnson Clinical Diagnostics, Rochester, NY), which monitored the kinetics of the change in reflection density on a test slide as pyruvate and NADH were enzymatically converted into lactate and NAD by LDH in the test sample.

**Immunocytochemistry for ES cell-specific markers**

Samples with 10⁵ cells were centrifuged for 3 min at 300xg, and the supernatant was removed and replaced with 750 μl of DPBS. After briefly mixing to resuspend the cells, 250 μl of 4% (w/v) paraformaldehyde (Alfa Aesar, Ward Hill, MA) in DPBS was added to each sample. The cells were fixed for 20 min, washed with 1 ml DPBS, and resuspended in 100 μl of DPBS. For Oct 3/4 immunostaining, 100 μl of 1% (w/v) saponin (S-4521, Sigma-Aldrich) in DPBS was added to the sample, the cells were incubated for 10 min for permeabilization, then washed and resuspended in 50 μl of 1% (v/v) FBS in PBS. Samples for SSEA-1 immunostaining were centrifuged and resuspended in 50 μl of 1% FBS in PBS without saponin treatment. All samples were incubated in the 1% FBS solution for 30 min, then 5 μl of a 1:10 dilution of either anti-Oct 3/4 (611202, BD Transduction Laboratories, Franklin Lakes, NJ) or anti-SSEA-1 (MC-480 ascites, Developmental Studies Hybridoma Bank, Iowa City, IA) was added to each tube and the samples were incubated for 1 hr. To each tube 1 ml of 1% FBS solution was added and the samples were centrifuged. The supernatant was discarded and the cells were resuspended in 50 μl of an appropriate PE conjugated secondary antibody (115-116-075 or 115-116-146, Jackson ImmunoResearch) diluted 1:500. The samples were incubated 30 min in the dark, then washed 3 times with 1 ml of PBS, and fluorescence intensity data were acquired with a flow cytometer (Guava Technologies) using the Express software module. All steps were performed at room temperature.

**Estimation of pO₂cell**

pO₂cell was estimated by equating the rate of diffusion of oxygen through the stagnant medium to the rate of oxygen consumption by

$$\frac{D\alpha}{L} [pO_2gas - pO_2cell] = \rho \left[ \frac{V_{max} \cdot pO_2cell}{K_m + pO_2cell} \right]$$

(2.6)

where D and α are the diffusivity and solubility of oxygen in cell culture medium, respectively, L is the medium depth, pO₂gas and pO₂cell are the gas phase and cell surface pO₂ values, respectively, ρ is the viable cell density on the surface, Kₘ is the Michaelis constant for oxygen consumption, and Vₘₐₓ is the experimentally measured OCR/cell. The presence of a small pO₂ drop across the layer of cultured cells was ignored. For our calculations we used D = 2.97 x 10⁻⁵ cm² sec⁻¹, α = 1.19 x 10⁻⁹ mol cm⁻³ mmHg⁻¹, L = 0.15 cm, and Kₘ = 0.44 mmHg
Eqn. (6) was solved for \( pO_{2}\text{cell} \) by iterative solution (Excel solver).

**Estimation of specific growth rate**

The cell specific growth rate, \( \mu \), was approximately constant during culture of undifferentiated cells. The specific growth rate was determined for these cultures using linear regression of the cumulative cell number versus time plotted on a semi-logarithmic scale (as in Fig. 2.1A). The specific growth rate varied with time in culture when ES cells were differentiating (as in Fig. 2.3). The instantaneous specific growth rate at a given time \( t \) was determined by numerically differentiating the data using the average of the forward, backward, and central differences, according to

\[
\mu = \frac{\ln \left( \frac{N}{N_0} \right)}{3(t - t_0)} + \frac{\ln \left( \frac{N_1}{N} \right)}{3(t_1 - t)} + \frac{\ln \left( \frac{N_1}{N_0} \right)}{3(t_1 - t_0)}
\]

(2.7)

where \( N_0, N, \) and \( N_1 \) are the cumulative cell numbers at successive times \( t_0, t, \) and \( t_1, \) respectively.

**Assessment of oxidative DNA damage**

A single cell gel electrophoresis (comet) assay (Tice et al. 2000) was used to detect DNA damage. This assay was done with and without the use of formamidopyrimidine DNA glycosylase (FPG, F-3174, Sigma Aldrich), which creates strand breaks at sites of 8-oxoguanine (Collins and Dusinska 2002), a well-documented product of oxidative DNA damage (Helbock et al. 1999). Undifferentiated mES cells were cultured at specified \( pO_2 \text{gas} \) for 72 hr, then detached with trypsin and resuspended at a concentration of 200 cell/\( \mu l \) in 37 °C, 1% (w/v) low melting point agarose (A-6560, Sigma-Aldrich) in DPBS. Methods for alkaline single-cell gel electrophoresis were subsequently followed (Collins and Dusinska 2002). Comets were stained with 50 nM Sytox Orange dye (S11368, Molecular Probes, Eugene, OR), images were acquired with a 10x objective on a Zeiss epifluorescence microscope, and image analysis was performed using NIH Image (Helma and Uhl 2000). Samples with oxidative DNA damage (positive controls) were prepared by treating cells with 25, 85, and 150 \( \mu M \) hydrogen peroxide for 30 min immediately before embedding them in low melting point agarose.

**Statistics**

Statistical tests were done using Microsoft Excel. Results were deemed significant if the two-tailed \( P \) value was 0.05 or less.
Results

Cellular pO2

Estimates of pO2cell, calculated from Eqn. (2.6), for a range of cell densities, 0.1 to 2.0 x 10^5 cell/cm^2, corresponding to the lowest and highest cell density present before and after passaging cells, respectively, are tabulated in Table 2.1. Although the magnitude of the pO2 drop across the culture medium was small in all cases, the difference between pO2gas and pO2cell was large relative to pO2gas at very low oxygen concentrations, e.g., cells cultured using a pO2gas of 7 mmHg were often exposed to pO2cell values that approached 0.

Cell growth

Undifferentiated CCE and D3 mES cells at pO2gas of 0, 7, 36, 142, and 285 mmHg grew exponentially for up to 25 days (Fig. 2.1A). There were no temporal changes that suggested senescence, differentiation, or long-term conditioning to the culture conditions. The fraction of cells with intact cell membranes was between 94 and 98%, which further suggested that undifferentiated mES cells survived and proliferated in all conditions tested. Both CCE and D3 cells had roughly the same specific growth rate (Fig. 2.1B) and were tolerant of a wide range of oxygen conditions. Growth was significantly suppressed under anoxia, but the cells continued to grow exponentially with a doubling time between 24 and 27 hr. Elevated pO2gas of 285 mmHg also caused a decrease in growth rate in both cell lines relative to that at 142 mm Hg. D3 cells consistently grew faster at a pO2gas of 36 mm Hg compared to 142 mmHg. The specific growth rate μ in four experiments was 1.05 ± 0.12 day^{-1} at 142 mm Hg and 1.15 ± 0.10 day^{-1} at 36 mm Hg. This small, but statistically significant difference (P < 0.01, paired t-test) would lead to a three-fold difference in cell number after 10 days of culture. No difference in growth at these same two pO2gas conditions was observed with CCE cells.

Oxidative DNA damage

Comet assays run with and without FPG using cells cultured for 72 hr at each pO2gas condition showed nearly identical means of tail length and moment, both measures of the relative amount of DNA damage, at all pO2gas. Cells treated with hydrogen peroxide displayed dose-dependent increases in mean tail length and moment, and a further dose-dependent increase occurred when the assay was performed with FPG. These results suggest that pO2gas did not significantly affect the amount of oxidative DNA damage, a result consistent with the growth rate data.

Undifferentiated stem cell phenotype at different pO2gas

When using culture medium containing LIF, which suppresses mES cell differentiation (Viswanathan et al. 2002), pO2gas did not affect the fraction of cells with an undifferentiated stem cell phenotype, as assessed by the fraction of cells expressing SSEA-1 and Oct 3/4. Both of these markers were present on 85 – 95% of all cells over a 20 day period at all pO2gas (Fig. 2.2).
Thus, increasing or decreasing the oxygen concentration did not induce differentiation in mES cells when it was exogenously suppressed with LIF and cells were passaged every 2 days.

Without LIF in the culture medium, CCE cells readily differentiated and the specific growth rate (Fig. 2.3) decreased with increasing time after LIF withdrawal. After a time lag of about two days, the specific growth rate, on average, decreased at a similar rate for pO$_2$gas conditions ranging from 7 to 142 mmHg, and reached a minimum after about 10 days (Fig. 2.3F). Changing pO$_2$gas in this range did not significantly delay or accelerate the onset or rate of differentiation. Individual experiments were reproducible at 285, 36, and 7 mmHg, less so at other conditions. At the extremes of 0 and 285 mmHg the decrease in specific growth rate was larger and began earlier. The specific growth rate at 285 mmHg was suppressed to a greater extent, relative to that at other values of pO$_2$gas, than was observed in the presence of LIF. A small number of experiments with D3 cells suggested similar trends (data not shown), which occurred more slowly due to a decreased rate of spontaneous differentiation.

On average, the fraction of CCE cells expressing Oct 3/4 also decreased with time without LIF in the culture medium, except for pO$_2$gas of 285 mmHg and some experiments at 142 mmHg, at which the fraction decreased and then rebounded (Fig. 2.3A and 2.3B). Individual experiments were reproducible at 7 and 285 mmHg but less so at 142 and 36 mmHg, similar results were observed at 0 and 7 mmHg. The averaged data (Fig. 2.3F) demonstrate a decrease in the fraction of Oct 3/4 positive cells with decreasing pO$_2$gas. Because of the scatter in the data, this trend was only significant (P < 0.04) when 142 and 7 mmHg were compared at day 6, and 285 and 7, 36, or 0 mmHg were compared on day 16. After about 16 days, the average fraction of Oct 3/4 positive cells converged to about 30% at 0, 7, and 36 mmHg and was about 55% and 70% at 142 and 285 mmHg, respectively.

**Culture without passaging**

In experiments described to this point, medium was exchanged daily, and cells were passaged every 2 or 3 days. Fresh medium was preequilibrated to the desired pO$_2$ so that there was minimal disturbance associated with medium exchange; however, during passaging the mES cells were exposed to ambient oxygen for about 1 hr and to the stress of trypsin detachment, which may have selectively harmed either differentiated or undifferentiated cells. To study effects of pO$_2$gas on mES cell growth and differentiation without these potential artifacts, D3 and CCE cells were plated at a density of 100 cells/well in 6-well plates containing 3 ml of culture medium (three wells for each condition) both with and without supplemental LIF. The plates were maintained for 8 days without passaging the cells or changing the medium. Plates were kept at a pO$_2$gas of 142 or 7 mmHg continuously (24 hr/day), or at a pO$_2$gas of 7 mmHg for 23 hr/day and 142 mmHg for 1 hr/day to mimic the temporary exposure to elevated pO$_2$ that occurs during normal passaging.

Growth and spontaneous differentiation data from these experiments are summarized in Table 2.2. With and without LIF, exposure of D3 and CCE ES cells to a pO$_2$gas of 7 mmHg for 23 hr/day and a pO$_2$gas of 142 mmHg for 1 hr/day did not significantly affect specific growth rate
or the fraction of cells expressing SSEA-1 and Oct 3/4, relative to culture at 7 mmHg continuously. For CCE cells cultured without LIF, pO$_{2\text{gas}}$ did not affect the spontaneous loss of the undifferentiated, Oct 3/4 positive phenotype, but a significantly higher fraction of cells were positive for SSEA-1 at a pO$_{2\text{gas}}$ of 142 mmHg relative to 7 mmHg (P = 0.02), suggesting that the pO$_{2\text{gas}}$ was affecting the phenotype (SSEA-1 expression) of the differentiated cells that formed. For D3 cells with LIF in the culture medium, there was a significantly smaller fraction of Oct 3/4 positive D3 cells at 7 mmHg, relative to 142 mmHg (P = 0.01). Without LIF, D3 cells had a small loss of SSEA-1 and Oct 3/4 expression relative to culture with LIF. No significant differences between the pO$_{2\text{gas}}$ conditions were observed for D3 cells cultured in medium without LIF.

Temporal response to anoxia

The ability of mES cells to grow in anoxia with a doubling time of about 24 hr indicated that mES cells are well suited to, or can readily adapt to, low oxygen conditions. To further explore this behavior, undifferentiated mES cells were subjected to a change in pO$_{2\text{gas}}$ from 142 to 0 mm Hg. The fraction of cells with intact membranes, the fraction of apoptotic cells, OCR/cell immediately after reoxygenation, and LDH activity were measured at various times up to 50 hr after the transition to anoxia (Fig. 2.5).

Exposure to anoxia decreased the fraction of cells with intact membranes by about 2-5%. Similarly, there was a very small increase in the fraction of cells that were apoptotic, which never exceeded 9% under anoxia compared to about 4% in cells exposed to 142 mmHg. Thus, exposure to anoxia caused only a small fraction of cells to become apoptotic and die, and more than 90% remained alive. Oxygen deprivation caused mES cells to rapidly down-regulate aerobic metabolic pathways as indicated by a decrease in OCR that began immediately after the initiation of anoxia and was nearly complete within 1 day. There was little change in the intracellular LDH activity for the first 10 hr of exposure to anoxia, after which it increased to more than twice that found in cells cultured at 142 mm Hg.

Cellular energy metabolism

The data in Fig. 2.5 provide evidence that mES cells increased anaerobic metabolism when exposed to hypoxia or anoxia. To quantify this further, we estimated the rate of ATP produced aerobically and anaerobically under each of the pO$_2$ conditions tested. OCR/cell was used as a measure of oxidative phosphorylation and LPR/cell as a measure of anaerobic glycolysis. ATP production rate was estimated by assuming production of 6 mol of ATP per mol of oxygen consumed and 1 mol of ATP per mol of lactate formed (Lindqvist et al. 2002; Miller et al. 1987).

A relatively high level of anaerobic metabolism occurred in the mES cells, even at high pO$_{2\text{gas}}$ at which there was a plentiful supply of oxygen to the cells (Fig. 2.6). Nearly 40% of the ATP production was provided by anaerobic metabolism of glucose into lactate at 142 mmHg or greater. When pO$_{2\text{gas}}$ was reduced to 36 mmHg, a small but significant decrease in OCR and
increase in LPR was observed. This shift to increased anaerobic metabolism became more pronounced as the pO$_2$gas decreased to 0 mm Hg, a condition in which no significant aerobic ATP production could occur. Overall, the total estimated ATP production rate remained nearly constant as long as oxygen was present, and decreased about 10% at 0 mmHg.

**Discussion**

We measured the growth rate and Oct 3/4 expression of undifferentiated mES cells cultured with and without LIF at pO$_2$gas in the range of 0 – 285 mmHg in order to determine whether oxygen level affected proliferation and undifferentiated phenotype maintenance of in differentiation-suppressing and -permissive conditions. We also studied mES cell energetics over the same range of pO$_2$gas to gain further insight into the extent to which mES cells adapt to different environmental oxygen conditions.

Growth of undifferentiated mES cells was moderately sensitive to pO$_2$ (Fig. 2.1), which is consistent with most previous studies. When compared to culture at a pO$_2$gas of 142 mmHg, the specific growth rate (Fig. 2.1B) was similar at 36 mmHg, moderately reduced at 7 and 285 mmHg as others have found (Ezashi et al. 2005; Iyer et al. 1998; Ludwig et al. 2006; Saretzki et al. 2004), and further reduced at 0 mmHg. D3 cells had a slightly higher growth rate at a pO$_2$gas of 36 relative to 142 mmHg, while growth rates for CCE cells were the same. When LIF was present in the culture medium, the cells remained essentially undifferentiated, and changes in pO$_2$gas did not affect the spontaneous loss of the undifferentiated ES cell phenotype (Fig. 2.2), although a small effect was observed with D3 cells at 7 mmHg if they were cultured for 8 days without passaging (Table 2.2).

Our data suggest neither harm nor benefit to culturing undifferentiated mES cells at pO$_2$gas lower than 142 mmHg, except for a modest increase in growth rate of D3 cells. There may be situations where ES cell growth and survival are enhanced at low pO$_2$ conditions, such as during the establishment of new ES cell lines (Gibbons et al. 2006; Wang et al. 2006) and during culture at very low cell densities or sub-optimal culture conditions (Forsyth et al. 2006; Ording et al. 2005). Reduced pO$_2$gas conditions may also reduce the occurrence of chromosomal abnormalities in hES cells (Forsyth et al. 2006), but similar results have not been observed with mES cells (Wang et al. 2006). We found no affect of culture pO$_2$ in the range of 0 – 285 mmHg on the amount of oxidative DNA damage in undifferentiated mES cells.

When LIF was removed, the cells differentiated. The decrease in growth rate with time was not affected by pO$_2$gas from 7-142 mmHg (Fig. 2.3), but there were differences at the extremes of 0 and 285 mmHg as there were for growth in the undifferentiated state. Differentiation occurred much faster in the CCE cell line than it did in the D3 cell line. Such variations between ES cell lines have been observed by others in many different aspects of culture (Ward et al. 2004). The rate at which CCE cells lost Oct 3/4 expression increased with decreasing pO$_2$gas in the range of 0-285 mmHg (Fig. 2.4); the effect was statistically significant after 6 and 16 days, but not at other times because of scatter in the data. In contrast to this
apparent trend, no significant oxygen-dependent differences in Oct 3/4 positive cell fraction were observed in a separate experiment during which no passages were performed over a period of 8 days of continuous culture without LIF (Table 2.2). In this experiment, the fraction of Oct 3/4 positive cells at 142 mmHg was only 29%. Although within the range observed at 142 mmHg in experiments with frequent passages, it is possible that this low value left little room for a significant oxygen effect at 7 mmHg to occur. It is also possible that the difference in differentiation behavior in continuous experiments and those with multiple passages reflects, in part, differences in the relative ability of differentiated and undifferentiated cells to survive and grow with regular passages at different pO$_{2\text{gas}}$.

Rigorous studies of the effects of pO$_2$ on spontaneous differentiation have not been reported, but some experiments of this type have been carried out. A reduction in pO$_{2\text{gas}}$ to 36 mmHg decreased the rate of spontaneous differentiation or produced no marked change in 4 published studies using hES cells (Ezashi et al. 2005; Forsyth et al. 2006; Ludwig et al. 2006; Peura et al. 2005). Recently it has been reported that HIF-1α signaling present at low oxygen can inhibit self-renewal of mES cells by regulation of the LIF-Stat3 pathway (Jeong et al. 2007). Although we saw some evidence of a decrease in self-renewal at low pO$_2$, this effect was much less pronounced than that previously reported. Nonetheless, the involvement of the LIF-Stat3 pathway may explain the differences observed with mES and hES cells, since this signaling pathway is important for the maintenance of mES but not hES cells in an undifferentiated state (Daheron et al. 2004).

The limited effects of culture pO$_2$ on ES cell proliferation, particularly under anoxia, led us to further investigate ES cellular energetics. We measured the rates of lactate production and oxygen consumption (Fig. 2.6A) as indicators of the contributions of aerobic and anaerobic metabolism to the total cellular ATP production rate at all pO$_{2\text{gas}}$ conditions (Fig. 2.6B). With excess oxygen, 40% of the ATP demand of ES cells was satisfied through anaerobic glycolysis, which is higher than the 15-25% reported for early embryos (Houghton 2006; Houghton et al. 1996; Thompson et al. 1996). The reliance on anaerobic metabolism increased further with oxygen depletion, and all of the ATP was produced anaerobically under anoxia. The total ATP production rate remained nearly constant during this change, and ES cells could complete this adaptation in less than 2 days without significant loss of viability (Fig 2.5).

Use of anaerobic metabolism of glucose and the ability of ES cells to adapt to reduced pO$_2$ conditions including anoxia is logical from a developmental viewpoint, since fetal development occurs in a very low pO$_2$ environment. The pO$_2$ in the maternal endometrium supporting an early embryo is about 40 mm Hg, which in turn produces an oxygen concentration of about 0.05 mM in the surrounding tissue (Rodesch et al. 1992). Compared to oxygen, glucose is in substantial excess, with a fasting plasma concentration of approximately 3-4 mM (Weijers et al. 2002). An early embryo can therefore maximize its energy production rate by using both aerobic and anaerobic metabolism of glucose. The fact that mES cells utilize anaerobic metabolism to a substantial extent even at high pO$_2$ may also partially explain their ability to survive hyperoxia, since such a metabolic profile reduces the generation of harmful ROS
(Andreyev et al. 2005). This profile is also observed in tumor cells (Gatenby and Gawlinski 2003), and it has been shown that the glycolytic and the cancer phenotypes are very closely linked to one another (Kondoh et al. 2005; Ramanathan et al. 2005).

Many other cells increase anaerobic metabolism when confronted with hypoxia, which was first observed by Pasteur during yeast fermentation (Berg et al. 2002). The Pasteur effect has been particularly well documented in CHO and hybridoma cultures (Lin and Miller 1992; Miller et al. 1987). Although the Pasteur effect is commonly observed, not all cells can adapt equally well to survive hypoxic/anoxic insult. As ES cells differentiate into mature cells, a shift to aerobic metabolism will occur for many lineages (Sharma et al. 2006), and some of the resulting cells will become sensitive to oxygen deprivation. Study of the energetics of differentiated cells obtained from ES cells could potentially be used as an evaluative tool for determination of cell maturation.

Our work shows that for bioprocess development with cells having the properties of undifferentiated mES cells, pO₂ control is not likely to be a critical parameter. The ideal pO₂cell is about 40 mmHg, which is likely to maximize growth without leading to excessive lactate formation and might also provide some protection from DNA damage. However, both lower and higher pO₂cell conditions can be tolerated without excessive cell death or undesired changes in cell phenotype. We have observed a large effect of reduced pO₂ during the directed differentiation of ES cells to cardiomyocytes (Chapter 5). Under these conditions pO₂ control is much more important and is an area that warrants further study.
### Tables and Figures

#### Table 2.1. Oxygen conditions used for culture

<table>
<thead>
<tr>
<th>% Oxygen</th>
<th>pO$_2$gas (mmHg)</th>
<th>V$_{max}$ (amol/sec cell)</th>
<th>pO$_2$cell (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>285</td>
<td>28</td>
<td>262 – 284</td>
</tr>
<tr>
<td>20</td>
<td>142</td>
<td>29</td>
<td>118 – 141</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>23</td>
<td>18 – 35</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>17</td>
<td>0.5 – 6.5</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

- **Gas Phase**
  - The oxygen content in the pre-mixed gas cylinders (containing 5% CO$_2$) was specified, and the pO$_2$gas was determined from pO$_2$gas = (% oxygen)(P$_{atm}$ – P$_{H_2O}$), where P$_{atm}$ is the atmospheric pressure and P$_{H_2O}$ = 47 mmHg is the vapor pressure of water at 37 °C.

- **Maximal OCR**
  - OCR measured experimentally (Fig. 2.6).

- **Cellular pO$_2$**
  - pO$_2$cell calculated with Eqn (2.6) for extreme low and high densities of 0.1 and 2.0 x 10$^5$ cell/cm$^2$ (about 2 – 50% confluence), respectively.
Table 2.2. Growth and spontaneous differentiation of mES cells cultured without passaging

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>pO₂_gas Exposure (mmHg)</th>
<th>With LIF</th>
<th>Without LIF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Growth Rate (day⁻¹)</td>
<td>SSEA-1 Positive (%)</td>
<td>Oct 3/4 Positive (%)</td>
</tr>
<tr>
<td>CCE</td>
<td>142 (24 hr/day)</td>
<td>1.08 ± 0.02</td>
<td>83 ± 6</td>
</tr>
<tr>
<td></td>
<td>7 (24 hr/day)</td>
<td>1.07 ± 0.02</td>
<td>88 ± 3</td>
</tr>
<tr>
<td></td>
<td>7/142 (23/1 hr/day)</td>
<td>1.06 ± 0.02</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>D3</td>
<td>142 (24 hr/day)</td>
<td>1.17 ± 0.01</td>
<td>94 ± 2</td>
</tr>
<tr>
<td></td>
<td>7 (24 hr/day)</td>
<td>1.08 ± 0.01</td>
<td>95 ± 1</td>
</tr>
<tr>
<td></td>
<td>7/142 (23/1 hr/day)</td>
<td>1.06 ± 0.03</td>
<td>93 ± 1</td>
</tr>
</tbody>
</table>

Cells were plated at a density of 11 cell/cm² and grown for 8 days without passaging. In two groups, pO₂_gas was maintained at 142 or 7 mmHg continuously. A third group was cultured for 23 hr/day at 7 mmHg and 1 hr/day at 142 mmHg. Data are from a single experiment and are reported as mean ± SD for three different wells at each condition.
Figure 2.1. Effects of oxygen on undifferentiated ES cell growth

(A) Cumulative cell number versus time for undifferentiated mES cells (CCE) maintained at different $p_{O_2_{gas}}$ conditions. Data are from an experiment that is representative of results obtained with both CCE and D3 cells. (B) Specific growth rates of the two cell lines as determined from an exponential fit of data, such as that plotted in panel A. Cell densities ranged from 0.1 to $2.0 \times 10^5$ cell/cm$^2$ in these experiments.
Figure 2.2. ES Cell phenotype during culture with LIF

(A) Growth rate and (B) Oct 3/4 expression of ES cells cultured in medium containing supplemental LIF at different pO$_{2gas}$. Results are from a single experiment with CCE ES cells.
Figure 2.3. Effects of oxygen on differentiating ES cell growth

Specific growth rate of CCE ES cells grown in culture medium without LIF beginning on day 0. Results from up to four independent experiments at different pO$_{2\text{gas}}$ conditions are shown in panels A-E. Data obtained from comparable times for each pO$_{2\text{gas}}$ were averaged, and the result is plotted in panel F.
Figure 2.4. Effects of oxygen on Oct 3/4 expression during differentiation

Fraction of Oct 3/4 positive CCE cells during culture in medium without LIF beginning on day 0. Results from up to three independent experiments at different pO$_2$gas conditions are shown in panels A-E. Data obtained from comparable times for each pO$_2$gas were averaged, and the result is plotted in panel F.
Figure 2.5. Temporal response of ES cells to changes in oxygen

(A) The fraction of membrane intact and apoptotic cells (Annexin V positive) and (B) cellular oxygen consumption rate (measured under normoxic conditions) and intracellular LDH activity plotted as a function of the time spent in anoxia. Undifferentiated CCE cells were initially grown at pO$_2$gas of 142 mm Hg, before being cultured under anoxia for periods of time shown. Each point represents a single measurement except for the OCR measurements, which represent the mean ± SD of at least three replicates. Each symbol represents data taken with the same initial batch of cells.
Figure 2.6. Effects of oxygen on ES cell energetics

(A) Oxygen consumption rate per cell and lactate production rate per cell and (B) the corresponding ATP production rates for undifferentiated mES cells cultured at different pO$_{2_{gas}}$ conditions. Each data point is the mean ± SD of at least three independent experiments. No differences between CCE and D3 cells were observed, and the plotted data were obtained from separate experiments with both cell lines (one cell line per experiment). All cells were cultured at least 50 hr at the indicated pO$_2$ prior to assessment.
Chapter 3. Silicone Rubber Membranes for Control of Cellular Oxygen
To identify methods that could be used for the culture of differentiating ES cells at well-defined cellular oxygen levels we studied (1) the oxygen supply to monolayers of cells and cellular aggregates growing on polystyrene dishes, FEP-teflon membranes, and silicone rubber membranes in static culture, (2) methods that could be used to promote attachment of cells to silicone rubber, and (3) cardiomyocytic differentiation of mES cells on silicone rubber membranes. Oxygen transport was significantly improved using by culturing cells on membrane-based dishes, especially those made with silicone rubber. During monolayer culture, the difference between the gas phase and the cellular pO2 (ΔpO2) was less than 1 mmHg on silicone rubber, independent of cell density and medium depth, whereas ΔpO2 could be 15 and 150 mmHg or higher on FEP-teflon membranes and polystyrene dishes, respectively. Use of silicone rubber membrane-based dishes also greatly improved oxygenation of cultured aggregates, however there were still oxygen gradients within the tissue. Undifferentiated mES cells did not adhere to native silicone rubber, but treatment with either a polyelectrolyte multilayer or adsorption of fibronectin prior to cell addition allowed for robust attachment. Using the fibronectin adsorption technique, differentiating ES cells could be cultured on silicone rubber membrane-based plates for at least 11 days. Significantly more cells were present on the silicone rubber membrane-based dishes than parallel polystyrene dishes, presumably due to the increased oxygen availability on silicone rubber. In conclusion, there is a strong theoretical justification for the use of silicone rubber membrane-based culture surfaces for the study of the effects of dissolved oxygen on cell function, and it possible to modify the silicone surface so it can be used for the culture of adherent cells.

**Introduction**

With few exceptions, small-scale mammalian cell culture experiments in academic labs are performed in glass or polystyrene dishes that kept stationary within an incubator. The partial pressure of oxygen (pO2) in the culture dish is determined by the incubator gas, which is usually a humidified atmosphere consisting of 95% air/5% CO2 that results in a gas phase pO2 (pO2gas) of 142 mmHg. However, since oxygen concentration gradients exist in the medium, the pO2 at the cell surface (pO2cell) can be lower than pO2gas and is usually unknown.

The failure of oxygen-impermeable glass and polystyrene culture dishes to adequately control pO2cell was first recognized using theoretical models of oxygen transport, which showed that significant differences between pO2,gas and pO2cell would exist at high cellular respiration rates (McLimans et al. 1968; Stevens 1965). Subsequent experiments with microelectrodes have verified the presence of large pO2 gradients in the medium (Metzen et al. 1995; Pettersen et al. 2005; Tokuda et al. 2000; Wolff et al. 1993). These gradients have been exploited in static culture systems designed to measure oxygen consumption rate (OCR) of cells in monolayer culture and in embryoid bodies (Guarino et al. 2005; Lopes et al. 2005). In addition to affecting steady state oxygen concentration profiles, researchers have also found that traditional static culture systems make it nearly impossible to study effects of intermittent hypoxia because of the
long time scales associated with oxygen diffusion through the static medium layer (Baumgardner and Otto 2003).

To achieve $p_{O_2\text{cell}}$ control, cells can be cultured on gas-permeable dishes or in dishes that are mechanically mixed or perfused so as to induce convective oxygen transport. Mechanical mixing is the preferred method for oxygenation of large-scale bioreactors. However this method introduces additional equipment requirements that become cumbersome for small-scale research laboratories. Additionally, mixing in culture vessels induces shear stresses on cells, which may affect their function (Garin and Berk 2006). Carefully designed perfusion systems can avoid this problem, however they are known to be difficult to work with and susceptible to contamination. In contrast, culture on gas-permeable membranes requires no additional equipment and no changes in the culture environment other than the surface on which the cells are growing.

The ability of an oxygen permeable bottom membrane to control $p_{O_2\text{cell}}$ to a value near $p_{O_2\text{gas}}$ was shown theoretically (Jensen et al. 1976) and then experimentally (Wolff et al. 1993) with fluoroethylene-propylene copolymer (FEP-teflon) membrane-based dishes that significantly decreased the difference between $p_{O_2\text{gas}}$ and $p_{O_2\text{cell}}$. Selection of the FEP-teflon membrane was made because of its optical clarity and strength, not because it is the optimal material for oxygen transfer. Specialty dishes made with this membrane on the bottom are commercially available (Lumox dishes, Greiner Bio-One, Munich) with either a hydrophobic or plasma gas-treated hydrophilic surface and are occasionally described in the literature for mammalian cell culture.

The oxygen permeabilities of FEP-teflon and silicone rubber are $0.2-0.4 \times 10^{-14}$ and $26 \times 10^{-14}$ mol/cm mmHg sec, respectively (Avgoustiniatos 2002; DuPont 1996; Koros et al. 1981). Silicone rubber is therefore the preferred membrane material for oxygen delivery and is used widely in membrane aerated bioreactors (Henzler and Kauling 1993; Rishell et al. 2004a; Rishell et al. 2004b; Yoshino et al. 1996) and in devices for high density culture of hybridoma cells (Falkenberg 1998) or pancreatic islets of Langerhans (Papas et al. 2005). These commercially available devices are too large for stem cell research, which requires multiple conditions with expensive cells and reagents. Small-scale dishes incorporating these membranes are not commercially available.

Silicone rubber surfaces must be modified prior to use for the culture of adherent cells (Hsiue et al. 1993). Treatment with plasmas of various compositions are sometimes used for this purpose (Hsiue et al. 1993; Monge et al. 2003), but surfaces modified in this way are not stable for extended periods of time due to migration of the functional groups from the surface into the bulk of the silicone material (Evaer et al. 1996; Rangel et al. 2004; Williams et al. 2004). Modifications in which functional coatings are grafted onto the silicone rubber are more stable, but infrequently used (Ai et al. 2002; Okada and Ikada 1995). Recently, there has also been interest in the use of polyelectrolyte multilayers for the surface modification of silicone rubber because this method is inexpensive, easy to apply, and has very flexible chemistry (Ai et al. 2003; Berg et al. 2004; Elbert et al. 1999; Mendelsohn et al. 2003; Yang et al. 2003).

Here we show theoretical justification for the use of silicone rubber as a membrane material for the precise control of the $p_{O_2\text{cell}}$ for both cell monolayers and aggregates, using mES
cells as our model system. We also show that a simple physical adsorption of fibronectin to silicone rubber is sufficient for robust cell adhesion and we show that these materials can be used to study the effects of oxygen on the differentiation of mES cells into cardiomyocytes using an 11-day differentiation protocol, the last 9 days of which occur with the cells attached to silicone rubber membrane-based dishes.

**Materials and Methods**

**Estimation of \( pO_2 \text{cell} \) for cell monolayers**

The \( \Delta pO_2 \) was estimated by equating the rate of one-dimensional diffusion of oxygen through the stagnant medium and the culture dish bottom to the rate of oxygen consumption by cells according to the following equation

\[
\Delta pO_2 = \rho V_{\text{max}} \left[ \frac{D_m \alpha_m}{L_m} + \frac{D_b \alpha_b}{L_b} \right]^{-1}
\]  

(3.1)

where \( V_{\text{max}} \) is the experimentally measured OCR/cell, \( \rho \) is the cell surface density, \( D \) and \( \alpha \) are the diffusivity and solubility of oxygen, respectively, \( L \) is the depth, and subscripts \( m \) and \( b \) refer to the medium and dish bottom, respectively. For our calculations we used 2.97, 2.21, 0.028, and 0.011 x 10\(^{-5}\) cm\(^2\)/sec as the values for the diffusivity of oxygen in culture medium, silicone rubber, FEP-teflon, and polystyrene, respectively (Avgoustiniatos 2002; Hodge et al. 2001; Koros et al. 1981), and 1.19, 11.9, 5.06, and 8.6 x 10\(^{-9}\) mol/cm\(^3\) mmHg as values for the solubility of oxygen in culture medium, silicone rubber, FEP-teflon, and polystyrene, respectively (Avgoustiniatos 2002; Boersma et al. 2003; DuPont 1996). We have previously measured the cellular oxygen consumption rate (OCR) of fully-oxygenated mES cells to be 29 amol/cell sec (Chapter 2), and this value was used for \( V_{\text{max}} \).

**Estimation of transient change in \( pO_2 \text{cell} \) after changing \( pO_2 \text{gas} \)**

The transient \( pO_2 \) at the culture dish-medium interface was estimated numerically using the finite element package Comsol Multiphysics. Simulations were done in the absence of cells and results are reported as fractional approach to the final steady-state \( pO_2 \) versus the time after a step change in \( pO_2 \text{gas} \).

**Estimation of \( pO_2 \) for cell aggregates**

The steady-state \( pO_2 \) profile in dishes containing cellular aggregates was estimated numerically using the finite element package Comsol Multiphysics. Simulations of oxygen reaction and diffusion were performed for a uniform square array (1mm center-to-center spacing) of hemispherical aggregates attached to the bottom of an infinite plate covered with 5 mm of medium. It was assumed that the cell volume was 9 x 10\(^{-10}\) cm\(^3\), the oxygen permeability of the
tissue was $1.19 \times 10^{-14}$ mol/cm sec mmHg (Avgoustiniatos 2002), and oxygen consumption followed Michaelis-Menten kinetics with a $K_m$ of 0.44 mmHg (Wilson et al. 1979). All other parameters were as described in the previous sections.

**Silicone rubber membranes**

Silicone rubber sheeting was purchased from Specialty Manufacturing (Saginaw, MI). The sheet was optically clear (gloss finish) and was 0.005 inch (127 μm) thick. Prior to use, the membrane material was cut to the desired shape with scissors and sterilized by autoclaving for 30 min. at 121 °C.

**Machined silicone rubber membrane holder**

A custom-made holder for the membrane was fabricated at the MIT central machine shop. This holder consisted of a polysulfone cylinder 6 cm in diameter with 4, 1 cm diameter holes bored through its length. The main body of the cylinder was 4.5 cm long, and an identical piece 1.8 cm long which served as the membrane support was attached to it with 4, 1 cm screws. The silicone membrane was sealed to the main body with a very thin layer of silicone adhesive (59530, Henkel Loctite Corp., Rocky Hill, CT), and then the membrane support was fastened to the main body to secure the membrane. The device was autoclaved for 30 min prior to use, and the top of the wells was covered with the cover from a sterile 60 mm polystyrene dish.

**Custom-made silicone rubber membrane-based plate**

Most of the bottom surface of the 8 central wells of 24-well tissue culture plates (353047, Becton Dickinson) was removed using a 3/8 x 3 inch fixed handle nutdriver (12, Cooper Hand Tools, Apex, NC) heated in a Bunsen burner to melt a hole in the plastic. A sterile scalpel was used to trim the edges of the holes. A very thin layer of silicone adhesive (59530, Henkel Loctite Corp., Rocky Hill, CT) was spread around each of the holes. A rectangular 8.5 x 4.5 cm piece of silicone rubber membrane (non-reinforced vulcanized gloss/gloss 0.005 inch, Specialty Manufacturing, Saginaw, MI), previously sterilized by autoclaving, was placed over the holes and manually pressed and stretched so that the silicone sheet was flat (no wrinkles) and sealed onto the plate bottom. After allowing the adhesive to cure for 24 hr, the plates were completely filled with a 70% ethanol solution for 1 hr and dried overnight under a germicidal UV lamp in a biological safety cabinet. Plates were discarded after a single use.

**Layer-by-layer film growth**

Polyelectrolyte multilayers (PEMs) were prepared by adapting previously published methods (Ai et al. 2003). Solutions in Dulbecco's phosphate buffered saline (DPBS) (21-030-CM, Mediatech, Herndon, VA) were prepared containing 3 mg/ml poly-sodium 4-styrene-sulfonate (PSS) (561967, Sigma-Aldrich, St. Louis, MO), 2 mg/ml poly-ethyleneimine (PEI) (P3143, Sigma-Aldrich), 0.5 mg/ml poly-D-lysine hydrobromide (PDL) (P1149, Sigma Aldrich), 1 mg/ml type A gelatin (G2500, Sigma-Aldrich), 1mg/ml type B gelatin (G9391, Sigma-Aldrich),
and 50 μg/ml fibronectin (F1141, Sigma-Aldrich). All coating steps were carried out at room temperature using 1 ml of solution per well of a 24 well silicone rubber membrane-based plate.

PSS solution (polyanion) was added to an untreated plate and incubated for 1 hr, followed by a 10 min wash with DPBS, a 1 hr incubation with PEI (polycation), and a 10 min wash with DPBS. Two additional incubations with both PSS and PEI (30 min each) with intervening washes (10 min) were done to build a (PSS-PEI)_3 PEM. To this PEM an additional 3 layers each of type B gelatin (polyanion) and PDL (polycation) were formed, using 30 min incubations with the polyelectrolyte solution, and 10 min washes with DPBS. The final PEM structure was thus (PSS-PEI)_3-(yype B gelatin–PDL)_3. After assembly of the PEM an additional 20 hr incubation with type A gelatin, type B gelatin, or fibronectin was performed. The plates were then washed and incubated for 24 hr with DPBS prior to emptying the wells and adding cell culture medium and cells.

**Physical adsorption of proteins**

Untreated plates were incubated in either a 0.1% (w/v) solution of gelatin (G-2500, Sigma-Aldrich) in tissue culture water (25-055-CM, Mediatech, Herndon, VA) or a 2 μg/ml solution of fibronectin (F1141, Sigma-Aldrich) in DPBS. Immediately before plating cells, the solution was removed and replaced with pre-warmed ES cell medium.

**Cardiomyocyte differentiation**

ES cells were differentiated into cardiomyocytes following protocols described elsewhere (Chapter 4). Briefly, mES cells were differentiated in hanging drops containing 500 cells using DMEM with 10% FBS. After 2 days the cell aggregates were transferred into a culture plate previously coated with fibronectin, onto which the cells attached and grew. Three days later, the medium was changed to a serum-free ITS medium. Media were exchanged daily after plating of cells. At the end of differentiation cells were removed from the dish by treatment with trypsin, counted, fixed with 4% paraformaldehyde, and immunostained with an antibody to sarcomeric myosin heavy chain (MF-20) for analysis using a flow cytometer to determine cardiomyocyte fraction.

**Nuclei enumeration**

Supernatant medium from a well (1ml) was mixed with an equal volume of a lysis solution containing 1% Triton X-100 (T9284, Sigma Aldrich) and 0.1 M citric acid (C1909, Sigma Aldrich) in DI water and stored at 4 °C for up to 1 day prior to analysis. Attached nuclei were counted by adding 0.5 ml of lysis solution to the well and incubating for 10 min. The solution was then removed, and 0.5 ml of DPBS was added to the well, pipetted up and down to remove any remaining cells, and saved for analysis. Nuclei were stained using a Guava Viacount assay kit (Guava Technologies, Hayward, CA) and data were acquired with a Guava PCA flow cytometer. 

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Results

Theoretical comparison of dish materials for monolayer culture

The difference between $pO_{2,\text{gas}}$ and $pO_{2,\text{cell}}$ ($\Delta pO_2$) determined for monolayers of cells cultured on polystyrene, FEP-teflon, and silicone rubber using steady state simulations is shown in Fig. 3.1A. The $\Delta pO_2$ is highly dependent on the cell surface density (OCR density) and the medium height during culture on a polystyrene dish. For high cell densities and medium depths the magnitude of $\Delta pO_2$ can become quite large and can exceed 142 mmHg, which is the usual $pO_{2,\text{gas}}$ used for culture, causing cells to become oxygen starved. The overall magnitude of the $\Delta pO_2$ and its dependence on the medium height and cell surface density is significantly reduced using an FEP-teflon membrane, and reduced to almost 0 using a silicone rubber membrane-based culture dish. For conditions commonly encountered for undifferentiated ES cell culture, 1-2 x $10^5$ cell/cm$^2$ and a medium height of 2 mm, the absolute $\Delta pO_2$ is 15–35, 3-6, and 0.1-0.3 mmHg on polystyrene, FEP-teflon, and silicone rubber, respectively. Higher cell densities can be encountered during differentiation, and the $\Delta pO_2$ will be greater in such circumstances.

Theoretical comparison of effect of dish material on transient response

The time for the $pO_{2,\text{cell}}$ to equilibrate after a step change in $pO_{2,\text{gas}}$ was determined for polystyrene, FEP-teflon, and silicone rubber dishes using finite element simulations (Fig. 3.1B). The equilibration time was quite long on a polystyrene dish and was highly dependent on the medium volume. Approximately 1000, 4000, and 20000 sec were required to reach 90% of the steady state $pO_{2,\text{cell}}$ at medium depths of 1, 2, and 5 mm, respectively, in a polystyrene dish. The time to reach 90% of the equilibrium $pO_{2,\text{cell}}$ was about 100 and 500 sec at depths of 1 and 5 mm, respectively, on an FEP-teflon membrane, and 10 seconds in a silicone rubber dish with any medium height.

Theoretical comparison of dish materials for aggregate culture

The local $pO_2$ profile within dishes containing 100 $\mu$m hemispherical cell aggregates spaced 1 mm apart (2.3 x $10^5$ cell/cm$^2$) was determined for 3 different $pO_{2,\text{gas}}$, using numerical simulations (Fig. 3.2). In this case there was no single $pO_{2,\text{cell}}$ value, due to the presence of oxygen concentration gradients within the cell clump. At a $pO_{2,\text{gas}}$ of 142 mmHg (upper panels) there was a 55 mmHg drop in $pO_2$ from the gas to the surface of the aggregates ($\Delta pO_{2,ag}$) in polystyrene dishes, which was slightly larger than the $\Delta pO_2$ for a cell monolayer at the same density (40 mmHg). Membrane-based dishes reduced $\Delta pO_{2,ag}$ to 24 and 4 mmHg on FEP-teflon and silicone rubber, respectively. There was an additional 45 mmHg $pO_2$ decrease within aggregates on polystyrene, compared 30 and 27 mmHg on FEP-teflon and silicone rubber, respectively. The total $pO_2$ drop from the gas to the region of the aggregate with the lowest local $pO_2$ was 100, 54, and 31 mmHg on polystyrene, FEP-teflon membranes, and silicone rubber membranes, respectively. When the $pO_{2,\text{gas}}$ was decreased to 36 or 7 mmHg, areas of oxygen
starvation began to appear in the aggregate center. These areas were minimized by culturing on a silicone rubber membrane-based dish, but could not be eliminated because they were caused primarily by mass transfer resistance within the aggregate itself.

Cell attachment to silicone rubber

Undifferentiated mES cells did not attach to native silicone rubber or silicone rubber that was pretreated with gelatin prior to cell addition (Fig. 3.3). When mES cells were placed into such wells, they formed freely floating embryoid bodies (EBs) after 24 hr in culture. Exposure of the membrane to 2 μg/ml fibronectin in DPBS for a day prior to addition of the cells was sufficient for attachment of 99% of the cells. However, there was a small but significant decrease in the total cell number on the silicone rubber treated with fibronectin relative to the tissue culture plastic. The cause for this is not known. A 2 μg/ml fibronectin solution effectively coated the silicone rubber and promoted cell attachment if it was prepared in DPBS (as described above) or a serum-free ITS medium, but cells would not attach to the silicone rubber surface when the 2 μg/ml fibronectin solution was made in DMEM with 10% FBS (data not shown). This was presumably a result of competitive adsorption of serum proteins onto the silicone surface, which limited fibronectin adsorption.

Tissue culture polystyrene did not require pretreatment with any protein layer prior to cell addition. In all polystyrene conditions tested, greater than 99% of the cells were attached after a day in culture. Pretreatment of polystyrene with fibronectin or to a lesser extent gelatin, caused the cells to have a more widely spread morphology but did not affect cell proliferation (Fig. 3.3).

Deposition of a (PSS-PEI)₄-(PDL-gelatin)₃ PEM onto silicone rubber prior to cell addition could be used to promote cell adhesion (Table 3.1). Comparison of Table 3.1 with Fig. 3.3 shows that cells adhered better to silicone rubber with a PEM than they did to unmodified silicone. However, attachment to these materials still was not as robust as it was to any of the tissue culture polystyrene surfaces tested. Also, the PEM–gelatin structures did not support attachment as well as the single layer of fibronectin adsorbed onto silicone rubber. Other modifications of the PEM method examining the use of fewer layers were also tested with results comparable to those shown in Table 3.1 (data not shown).

It was observed that cells did not readily adhere to the entire surface of the silicone rubber membranes when they were placed in the machined membrane holder. The cells preferentially collected at the outer edges of each well, where they attached and grew. This behavior was seen using both physical adsorption of fibronectin and PEMs to promote cell attachment. Such non-uniform attachment was never observed in the custom-made silicone rubber-based polystyrene plates, and therefore such dishes were used in all subsequent experiments.

Cardiomyocyte differentiation on polystyrene dishes and silicone rubber membranes

Fibronectin-coated silicone rubber membranes could be used for adherent culture of ES cells differentiating into cardiomyocytes. The fraction of cardiomyocytes, total cell number, and
total cardiomyocyte number after 11 days of differentiation are shown in Fig. 3.4 for experiments carried out on silicone rubber membranes and polystyrene dishes. There were significantly fewer cells in the polystyrene relative to the silicone rubber membrane-based plates. Comparison of the cell density on the polystyrene dish \(5\times 10^5\) cell/cm\(^2\) and medium depth (5 mm) with Fig. 3.1 suggested that there was significant oxygen depletion in these dishes, even at a \(pO_{2\text{gas}}\) of 142 mmHg. Use of an oxygen sensitive dye on the bottom of polystyrene and silicone rubber membrane-based wells verified that significant oxygen depletion was present in the polystyrene dishes, but not in wells with a silicone rubber membrane bottom (data not shown).

A higher fraction of cells were cardiomyocytes on the polystyrene dish relative to the silicone rubber membrane at a \(pO_{2\text{gas}}\) of 36 and 142 mmHg, while at a \(pO_{2\text{gas}}\) of 7 mmHg there were a lower fraction of cardiomyocytes on polystyrene dishes relative to silicone rubber membranes. The cells also adopted a much more widely spread morphology on the polystyrene dishes compared to the silicone rubber membranes (not shown). No spontaneous contraction was observed in any \(pO_{2\text{gas}}\) condition on the polystyrene dishes, whereas on silicone rubber membranes an excellent correlation was found between immunostaining for MF-20 and the area covered with spontaneously contracting cells (Chapter 4). Similar results were seen in a subsequent experiment, but there is no apparent explanation for this result.

**Effect of fibronectin adsorption conditions on cardiomyocyte differentiation**

As was noted above, undifferentiated ES cells did not adhere to silicone rubber if it was not pre-incubated with fibronectin or if it was pre-incubated with fibronectin in DMEM with 10% FBS. Table 3.2 shows the fraction of cardiomyocytes, total cell number, and total cardiomyocyte number after 11 days of cardiomyocyte differentiation (protocol described in Chapter 5) on silicone rubber surfaces treated with fibronectin in PBS, with fibronectin in DMEM with 10% FBS, and without fibronectin pre-treatment. Pretreatment with fibronectin did not dramatically affect the total cell number at either \(pO_{2\text{gas}}\) condition. However, adsorption of fibronectin from a solution in PBS led to a significant increase in the fraction of cardiomyocytes, relative to the other two conditions at a \(pO_{2\text{gas}}\) of 7 mmHg. In contrast, the fraction and total number of cardiomyocytes at 142 mmHg was higher for the silicone rubber surfaces not pre-treated with fibronectin, or pretreated with fibronectin in DMEM with 10% FBS.

Embryoid bodies (EBs) adhered to and spread well on surfaces treated with fibronectin in PBS at \(pO_{2\text{gas}}\) of 7 and 142 mmHg, whereas EBs cultured at a \(pO_{2\text{gas}}\) of 142 mmHg did not readily adhere to the other silicone rubber surfaces. In these wells, multiple EBs combined to form large aggregates before some weak attachment occurred after several days. In contrast, EBs cultured at a \(pO_{2\text{gas}}\) of 7 mmHg were able to attach within 1 day to the surfaces without fibronectin pre-treatment or those treated with fibronectin in DMEM with 10% FBS, although the cells did not spread as much after the initial attachment of the EB, relative to the silicone rubber surface treated with fibronectin in PBS (data not shown). These differences in adhesion
and aggregation are the likely source for the relative differences observed in the cardiomyocyte
differentiation at the two pO$_{2gas}$ values on the un-treated silicone rubber surfaces.

**Discussion**

We studied whether silicone rubber membrane based dishes were an improvement on
existing polystyrene and FEP-teflon membrane based dishes for the precise control of the pO$_{2cell}$
during static culture with cell monolayers and aggregates. We also investigated possible surface
modifications that could be used to promote cell attachment to the silicone rubber surface. These
surface modified dishes were then used for the study of the effect that oxygen has on the
differentiation into cardiomyocytes.

Our theoretical simulations show that membrane based culture dishes improve the
oxygenation of cells in static culture and are consistent with the available literature
(Avgoustiniatos 2002; Jensen et al. 1976; Wolff et al. 1993). Previously published data are not
available to compare FEP-teflon membranes to silicone rubber, nor are there to our knowledge
published experiments showing that membrane-based culture dishes can be used to reduce the
time for equilibration of the pO$_{2cell}$ after a change in pO$_{2gas}$. Our results in both of these aspects
are logical and are a simple extension on previous modeling efforts.

The differences between silicone rubber and FEP-teflon are of practical significance
because the only widely commercially available membrane-based dish is constructed of FEP-
teflon. Our results show that this dish is a significant improvement over polystyrene, but it is not
nearly as effective as silicone rubber for the control of pO$_{2cell}$. FEP-teflon membranes have a
ΔpO$_2$ that is about 15 mmHg at high cell densities (Fig. 3.1), which is adequate for culture of
cells at a pO$_{2gas}$ of 142 mmHg. A ΔpO$_2$ of 15 mmHg is relatively large if it occurs during studies
at physiological pO$_2$ conditions in the range of 0-40 mmHg. Such experiments require a silicone
rubber membrane or convective mixing for precise pO$_{2cell}$ control.

Precise pO$_{2cell}$ control is not possible during culture of cell aggregates. Membrane-based
dishes improve oxygenation of aggregates in culture relative to polystyrene dishes, however
there are still oxygen gradients within the tissue that can be significant, as has been previously
predicted (Avgoustiniatos 2002). These gradients can result in tissue being oxygen starved,
especially at reduced pO$_{2gas}$ conditions. Quantitative studies of pO$_2$ effects on cell aggregates
must therefore consider aggregate sizes to estimate the range of pO$_{2cell}$ values that are present.

The transfer of a cell culture protocol from polystyrene dishes to FEP-teflon or silicone
rubber will introduce changes in the cell-substrate interactions in addition to changes in cell
oxygenation. A method described previously using a PEM to functionalize the silicone rubber
was used to promote cell attachment (Ai et al. 2003). Although the PEM improved cell
attachment compared to native silicone rubber, the best attachment was found using simple
physical adsorption of fibronectin onto the silicone rubber. The use of physical adsorption was
also much easier to accomplish and required only an incubation of a single solution, whereas
forming a full PEM required 14 different incubation steps (with a wash between each step). It is
not clear why others found physical adsorption of fibronectin to silicone rubber to be inefficient (Cunningham et al. 2002), but it may be partially due to the solutions that were used. We found that incubation of the silicone rubber with fibronectin solution in PBS promoted very good cell adhesion, whereas if the fibronectin solution contained 10% serum the subsequent cell adhesion was poor.

Fibronectin-coated silicone rubber membrane-based dishes could be used to study the effect that changing pO$_{2cell}$ has on the differentiation of ES cells into cardiomyocytes. These experiments were performed by forming EBs in hanging drops for 2 days, then transferring them to an adherent dish for 9 additional days. On silicone rubber membrane-based dishes, we observed the maximal cardiomyocyte fraction occurred at the lowest pO$_{2gas}$ condition (7 mmHg) with decreasing fractions at 36 and 142 mmHg, and the most total cardiomyocytes were obtained at a pO$_{2gas}$ of 36 mmHg. Use of a polystyrene dish resulted in significantly fewer cells presumably due to oxygen limitations, and showed that the maximum cardiomyocyte fraction was obtained at a pO$_{2gas}$ of 36 mmHg. There was also no increase in the total number of cardiomyocytes in pO$_{2gas}$ conditions less than 142 mmHg using a polystyrene dish. This experimental system appears to be one in which the use of a gas impermeable dish to study oxygen effects gives presumably misleading results due to differences between the pO$_{2gas}$ and pO$_{2cell}$.

The use of a fibronectin coating is clearly beneficial for cardiomyocyte differentiation at a pO$_{2gas}$ of 7 mmHg. However, the robust attachment and spreading that this treatment provides is detrimental to cardiomyocyte differentiation at a pO$_{2gas}$ of 142 mmHg. This is most likely due to the fact that large aggregates rapidly form after transferring the EBs to a non-adherent dish on day 2. Such aggregates will generate a low local pO$_2$ (Chapter 5) that likely promotes cardiomyocyte differentiation, even at high pO$_{2gas}$. Further study is required to verify that this phenomenon can explain the results in Table 3.2.

Our work shows that silicone rubber membrane-based dishes offer substantial improvements in tissue oxygenation over polystyrene and FEP-teflon membrane-based culture vessels, and that physical adsorption of fibronectin to silicone rubber surfaces can be used to promote prolonged ES cell attachment. Based on these findings, fibronectin-coated silicone rubber membrane-based dishes were used in subsequent studies of the effects that oxygen has on the differentiation of ES cells into cardiomyocytes (Chapter 4 and 5).
Tables and Figures

Table 3.1. Cell attachment to polystyrene and silicone rubber functionalized with a PEM

<table>
<thead>
<tr>
<th>Surface</th>
<th>Cell Number (10^4 cell)</th>
<th>Total</th>
<th>Attached</th>
<th>Suspension</th>
<th>% Attached</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC Polystyrene unmodified</td>
<td>59 ± 4</td>
<td>58 ± 4</td>
<td>1.0 ± 0.3</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>TC Polystyrene Gelatin A</td>
<td>56 ± 3</td>
<td>56 ± 3</td>
<td>0.52 ± 0.04</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>TC Polystyrene Gelatin B</td>
<td>58 ± 1</td>
<td>57 ± 1</td>
<td>0.69 ± 0.07</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Silicone Rubber PEM – Gelatin A</td>
<td>52 ± 8</td>
<td>50 ± 8</td>
<td>1.9 ± 0.2</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Silicone Rubber PEM – Gelatin B</td>
<td>47 ± 3</td>
<td>45 ± 3</td>
<td>2.0 ± 0.1</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Silicone Rubber PEM – Fibronectin</td>
<td>38 ± 3</td>
<td>36 ± 3</td>
<td>1.2 ± 0.3</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>

26 x 10^4 viable cells were placed into each well of a 24-well plate and cultured for 18 hr prior to assessing cell attachment. Results are from a single experiment and are reported as the mean ± SD for triplicate wells for each condition.
Table 3.2. Effect of surface treatment on cardiomyocyte differentiation on silicone rubber

<table>
<thead>
<tr>
<th>Surface</th>
<th>pO$_{2\text{gas}}$ (mmHg)</th>
<th>Fraction MF-20$^+$ (%)</th>
<th>Cell Number ($10^5$ cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>No treatment</td>
<td></td>
<td>9 ± 1</td>
<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>Fibronectin in PBS</td>
<td>7</td>
<td>21 ± 1</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>Fibronectin in</td>
<td></td>
<td>7 ± 2</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>DMEM w/ 10% FBS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td></td>
<td>12 ± 1</td>
<td>19 ± 10</td>
</tr>
<tr>
<td>Fibronectin in PBS</td>
<td>142</td>
<td>3 ± 2</td>
<td>26 ± 9</td>
</tr>
<tr>
<td>Fibronectin in</td>
<td></td>
<td>8 ± 8</td>
<td>13 ± 9</td>
</tr>
<tr>
<td>DMEM w/ 10% FBS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fraction of cells that were cardiomyocytes, total number of cells, and total number of cardiomyocytes for cells cultured at constant pO$_{2\text{gas}}$ conditions of 7 or 142 mmHg for 11 days in silicone rubber membrane based dishes without pre-treatment, treated with fibronectin in PBS, and treated with fibronectin in DMEM with 10%FBS. The number fraction of cardiomyocytes was determined by flow cytometry of MF-20-immunostained, trypsin-dispersed cell samples. Data are shown as mean ± SD for triplicate wells in a single experiment with R1 ES cells. Similar results were observed in a comparable experiment with J1 ES cells. All experiments were started with 30 EBs/well (0.15 x $10^5$ cells/well).
Figure 3.1. Theoretical predictions for $pO_{2\text{cell}}$ during monolayer culture

The predicted $pO_{2\text{cell}}$ for ES cell monolayers as a function of cell density and medium height on polystyrene, FEP-teflon membrane, and silicone rubber membrane surfaces. The OCR/cell used was 29 amol/cell sec, and the $pO_{2\text{cell}}$ was determined using Eqn.1. (B) The fractional approach of the $pO_{2\text{cell}}$ to steady state after a step change in $pO_{2\text{gas}}$ as estimated using numerical simulations in a cell-free system.
Figure 3.2. Theoretical predictions for pO$_2$cell during aggregate culture

Simulations of the pO$_2$ profile within dishes containing 100 μm radius hemispherical aggregates spaced 1 mm apart ($2.3 \times 10^5$ cell/cm$^2$) on the bottom surface of polystyrene, FEP-teflon membrane, and silicone rubber membrane-based culture dishes for pO$_2$$_{gas}$ of 142, 36, and 7 mmHg. The pO$_2$ profile shown is on a line passing through the center of an aggregate and perpendicular to the bottom of the culture dish. The dashed lines on the plots indicate the locations of the aggregate upper and lower surfaces. The upper left panel shows the entire distance from the top of the medium to the bottom of the culture vessel, whereas the other three panels are zoomed in to show the regions within 100 μm of the aggregate top and bottom surfaces. The distances greater than 2 mm represent areas within the culture dish and the plotted lines showing the local pO$_2$ profiles terminate at the lower surface of the culture vessel, the location of which is different for each of the three different dish materials.
Figure 3.3. Adhesion of ES cells to polystyrene and silicone rubber

Adhesion of mES cells to polystyrene and silicone rubber in their native states or with 0.1% gelatin in water or 2 μg/ml fibronectin in PBS adsorbed onto the surface for 24 hr prior to cell addition. Below each pair of micrographs is quantitative data for the total, attached, and suspension cell numbers. Results shown are mean ± SD for three wells in a 24-well plate seeded with $2 \times 10^5$ cell 26 hr prior to performing the cell counts.
Figure 3.4. Differentiation on silicone rubber membranes and polystyrene dishes

Fraction of cells that were cardiomyocytes (top), total number of cells (middle), and total number of cardiomyocytes (bottom) for cells cultured at constant $pO_2$gas conditions of 7, 36, or 142 mmHg for 11 days in silicone rubber membrane based dishes (white bars) and polystyrene dishes (black bars). The number fraction of cardiomyocytes was determined by flow cytometry of MF-20-immunostained, trypsin-dispersed cell samples. Data for silicone rubber membrane-based dishes are shown as mean $\pm$ SD for 7 independent experiments (data presented in more detail in Chapter 5), while data for polystyrene dishes are shown as mean $\pm$ SD for 3 replicate wells in a single experiment. All experiments were started with 30 EBs/well ($0.15 \times 10^5$ cells/well).
Chapter 4. Methods for Differentiation of ES Cells into Cardiomyocytes
To show that oxygen might affect differentiation of ES cells into cardiomyocytes and find methods to further improve the robustness of the process we studied differentiation at several pO₂ conditions using (1) a neural differentiation protocol with embryoid bodies (EBs) formed in suspension or in hanging drops, (2) supplemental ascorbic acid to promote cardiomyogenesis, (3) a range of initial cell numbers for EB formation and time in hanging drop EB culture, and (4) a change to serum-free medium at different times during differentiation. After 24 days of neuronal differentiation, more cells were cardiomyocytes at a pO₂ of 7 relative to 142 mmHg. The fraction of cardiomyocytes reached its maximal value within about 10 days of differentiation, and supplemental ascorbic acid could be used to promote cardiomyocyte differentiation at all oxygen levels. The highest fraction and total number of cardiomyocytes were obtained when mES cells were differentiated as EBs in hanging drops with 500 cells per drop, EBs were cultured for 2 days in hanging drops before being transferred to adherent culture on silicone rubber membrane-based dishes, and the medium was changed from serum-containing to serum-free medium after 5 days. Such conditions produced 29, 16, and 7% cardiomyocytes, after 11 days at a pO₂ of 7, 36, and 142 mmHg, respectively, in a preliminary experiment. In conclusion, oxygen control could be used to improve the differentiation of mES cells into cardiomyocytes, and a protocol was developed to improve mES cell differentiation into cardiomyocytes. This protocol was used in subsequent work described in Chapter 5 to definitively demonstrate that oxygen affects cardiomyocyte differentiation.

**Introduction**

Most embryonic stem (ES) cell research is performed in incubators with a humidified 95% air/5% CO₂ gas mixture, resulting in a gas-phase oxygen partial pressure (pO₂) of 142 mmHg. Embryonic cells in early development are exposed to pO₂ values of about 0-30 mmHg (Intaglietta et al. 1996; Jauniaux et al. 2003b; Siggaard-Andersen and Huch 1995), and the effects of such conditions on differentiating ES cells are poorly understood.

Low pO₂ conditions affect cells cultured in vitro by decreasing the exposure to reactive species generated by cellular respiration (Andreyev et al. 2005) and by regulating the stability of the oxygen-responsive transcription factor HIF-1α (Lee et al. 2004), but other mechanisms may also be involved (Yun et al. 2005). Some cells are known to preferentially grow at low pO₂ conditions rather than the standard 142 mmHg, including fibroblasts (Bradley 1978), muscle satellite (Chakravarthy et al. 2001), hematopoietic (Hevehan et al. 2000), mesenchymal (Robins et al. 2005), and neural stem cells (Morrison et al. 2000). Previous studies with undifferentiated ES cells have shown some improvement in survival and cloning efficiency using reduced pO₂ conditions, but most of the observed effects have been relatively minor (Forsyth et al. 2006; Ludwig et al. 2006; Wang et al. 2006).

ES cell differentiation studies that show strong effects of low pO₂ conditions have been limited to hematopoietic (Adelman et al. 1999; Potocnik et al. 1994; Ramirez-Bergeron et al. 2004) and chondrocytic differentiation (Chen et al. 2006a; Chen et al. 2006b). Oxygen has been
shown to affect both the maintenance of the stem cell phenotype and the cell types obtained following differentiation of a more diverse set of adult stem cell types, including mesenchymal (Lennon et al. 2001), hematopoietic (Cipolleschi et al. 1997; Hevehan et al. 2000; Mostafa et al. 2000; Ramirez-Bergeron et al. 2004), and neural stem cells (Morrison et al. 2000; Studer et al. 2000).

ES cell differentiation generally favors the formation of ectodermal lineages (neurons) (Tropepe et al. 2001) and the early stages of the first widely used neural differentiation protocols were designed with no specific neural inductive agents (Lee et al. 2000). These methods initially differentiate the cells in aggregates called embryoid bodies (EBs) using basal medium with serum. Following the initial differentiation in the presence of serum, a simple chemically defined medium (ITS medium) is used. This method for differentiation is among the least complex, but is not generally used for cardiomyocyte differentiation.

Cardiomyocytes are usually generated from ES cells through the formation of EBs in inverted drops (hanging drops) of basal medium with serum containing 500 – 700 cells (Guan et al. 2001). After 3 days, EBs are transferred to bacteriological dishes on which they grow for an additional 3-5 days in suspension before being placed into a gelatin-coated dish to which the EBs attach and grow (Boheler et al. 2002). Numerous variations of this basic protocol exist, and other completely different methods have been studied including formation of EBs in non-adherent dishes instead of hanging drops (Kattman et al. 2006), culturing the EBs and cardiomyocytes exclusively in suspension in a bioreactor (Bauwens et al. 2005), or performing all steps of differentiation attached to a flask, without formation of EBs (Yamashita et al. 2005).

In addition to changes in the culturing techniques, various molecules have been shown to direct cardiomyocyte differentiation, including retinoic acid, nitric oxide, TGFβ-1, FGF, erythropoietin, BMP-2, BMP-4, ascorbic acid, retinoic acid, DMSO, noggin, 5-azacytidine, and VEGF (Chen et al. 2006c; Fukuda and Yuasa 2006; Yoon et al. 2006). Of these factors, erythropoietin, VEGF, and nitric oxide (through iNOS) are all known to be positively regulated under hypoxia through the activity of HIF-1α (Kasuno et al. 2004; Semenza 2000). Nonetheless, the single previous study of reduced oxygen culture using a stirred system showed only a very small positive effect of reduced oxygen culture. The overall differentiation into cardiomyocytes was so poor that extensive genetic selection for cardiomyocytes had to be performed to even quantify the increased cardiomyogenesis that occurred at reduced oxygen conditions (Bauwens et al. 2005).

In the present study we found that in a static, attached culture system, reduced oxygen caused a significant increase in differentiation of mES cells into cardiomyocytes using a simple neural differentiation protocol with EB formation. Cardiomyocytes first appeared on day 6 of differentiation, and the maximal fraction of cells that were cardiomyocytes was reached between days 10 and 14. The cell number per EB and time spent in EB culture were both found to affect the differentiation of mES cells into cardiomyocytes independent of the pO2gas used, and changing these parameters and the medium formulation could increase ES cell differentiation into cardiomyocytes.

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Materials and Methods

Undifferentiated ES cell culture

J1, R1, and D3 ES cells were obtained from ATCC (SCRC 1010, SCRC 1036, CRL 1934, Manassas, VA), and CCE ES cells (Keller et al. 1993; Robertson et al. 1986) were obtained from Stem Cell Technologies (Vancouver, BC). Undifferentiated cells were grown in high glucose DMEM (30-2002, ATCC) supplemented to 10% (v/v) ES cell qualified fetal bovine serum (FBS) (06905, Stem Cell Technologies, or SCRR 30-2020, ATCC). The medium was further supplemented to a final concentration of 1000 U/ml leukemia inhibitory factor (LIF, ESG1106, Chemicon, Temecula, CA) and 0.1 mM 2-mercaptoethanol (M-7522, Sigma-Aldrich, St. Louis, MO). Medium was exchanged daily, and cells were detached with 0.25% trypsin (30-2101, ATCC) every two days. Split fractions were chosen so that cells were plated at approximately 12,000 cells/cm².

Neural differentiation protocol

A slightly modified version of a 5 stage ES cell differentiation protocol was used (Lee et al. 2000). Cell suspensions obtained after trypsin detachment of undifferentiated ES cells were centrifuged at 300 xg for 3 min, supernatant medium was removed, and cells were resuspended in ES cell differentiation medium that did not contain supplemental LIF (Table 4.4). The membrane-intact cell concentration was determined using trypan blue cell counts, and 7 x 10⁵ cells were placed into 60 mm hydrophobic FEP-teflon membrane-based dish (96077305, Greiner Bio-One, Monroe, NC) containing 2 ml of medium. A partial medium exchange was performed after 2 and 3 days by aspirating 2 ml of medium from the top of the dish and replacing it with fresh differentiation medium.

After 4 days in culture, the cells and cell aggregates were removed from the dish, and any adherent EBs were removed by pipetting the medium up and down. The EBs were placed a tube, allowed to settle, all but 1 ml was removed, and 3 ml fresh differentiation medium was added. The EBs were then aliquotted equally to 4 wells of a 12-well silicone rubber membrane-based plate (described in Chapter 3), that had been pre-incubated overnight with an ITS medium (Table 4.5) containing 5 μg/ml fibronectin (F1141, Sigma-Aldrich). Medium was exchanged with fresh ITS medium with fibronectin the day after plating the cells, and every two days thereafter.

After 7 days in attached culture, cells were detached and dispersed using trypsin and the viable cell number was determined with trypan blue staining and cell counting using a hemacytometer. Cells were then plated at a density of 10⁵ viable cell/cm² in a 24-well fibronectin-coated silicone rubber membrane-based plate. At this time the medium was changed to an ITS medium with N2 supplement, 0.2 mM ascorbic acid, and bFGF (Table 4.6). Medium was exchanged daily, and after 4 days, bFGF was no longer included in the medium. This protocol was used to generate the data in Table 4.1.
Shortened differentiation protocol

The neural differentiation protocol described previously was followed, except cells were not detached with trypsin after 11 days of culture. The medium for days 4 onward was ITS (Table 4.5) with 5 μg/ml fibronectin. This protocol was used to generate the data shown in Fig. 4.1. All subsequent experiments were performed with ITS medium without supplemental fibronectin.

Hanging drop embryoid body formation

Cell suspensions obtained after trypsin detachment of undifferentiated ES cells were centrifuged at 300 xg for 3 min, supernatant medium was removed, and cells were resuspended in ES cell differentiation medium that did not contain supplemental LIF. The membrane-intact cell concentration was determined using trypan blue cell counts, and the cell concentration was adjusted to obtain the desired number of cells in an EB. 20-μl drops of this cell suspension were aliquotted onto the inside surface of the lids of 10 x 10 cm Petri dishes (351112, Becton Dickinson) using an 8-channel pipette. The lids were inverted and placed onto the dish bottoms, which were filled with liquid to provide humidification.

Embryoid body transfer

After 2-5 days in hanging drops, the resulting embryoid bodies (EBs) were manually pipetted off of the Petri dish lid using wide-orifice pipette tips (3532, Molecular BioProducts, San Diego, CA) and placed into the wells of custom made fibronectin-coated silicone rubber membrane-based dishes. 30 and 10 EBs were placed into each well of 24 and 48 well plates, respectively, and the medium was mixed by pipetting to distribute the EBs uniformly across the plate bottom.

Culture of attached embryoid bodies

The EBs attached and spread within one day of transfer to the silicone rubber membrane-based dish. After the EBs had attached and grown for a day, medium was exchanged daily. The medium used depended on the experiment, and is clearly stated in the figure and table captions.

Humidification liquid for EB culture

For early experiments (Table 4.2 and Fig. 4.2) DPBS was used to provide humidification of hanging drop EBs. It was found that using 15 ml of a pre-warmed (37 °C) solution containing 75% DPBS (21-030-CM, Mediatech), 25% water, and 0.002% (w/v) gelatin better maintained the drop volume at 20 μl, so this was used in subsequent experiments (Fig. 4.3). Experiments were also performed using tissue culture deionized (DI) water and DI water with 32.8 g/L sodium chloride (4X PBS) to provide humidification for hanging drops (Table 4.3).
Optimizer cardiomyocyte differentiation protocol

On conclusion of the experiments described in this chapter, the following protocol was found to be robust, and was used extensively in future work (Chapter 5) and for the data presented in Fig. 4.3.

Cell suspensions obtained after trypsin-detachment of undifferentiated ES cells were centrifuged at 300 xg for 3 min, supernatant medium was removed, and cells were resuspended in ES cell differentiation medium that did not contain supplemental LIF (Table 4.7). The membrane-intact cell concentration was determined using trypan blue cell counts, and cells were diluted to a concentration of 25,000 membrane intact cells/ml. 20-μl drops of this cell suspension were aliquotted onto the inside surface of the lids of 10 x 10cm Petri dishes (351122, Becton Dickinson) using an 8-channel pipette. The lids were inverted and placed onto dish bottoms that were filled with 15 ml of a pre-warmed (37 °C) solution containing 75% DPBS (21-030-CM, Mediatech), 25% water, and 0.002% (w/v) gelatin.

After 2 days in hanging drops, the resulting embryoid bodies (EBs) were manually pipetted off of the Petri dish lid using wide-orifice pipette tips (3532, Molecular BioProducts, San Diego, CA) and placed into the wells of custom made fibronectin-coated silicone rubber membrane-based 24-well culture dishes. 30 EBs were placed into each well, and the medium was mixed by pipetting to distribute the EBs uniformly across the plate bottom.

The EBs attached and spread within one day of transfer to the silicone rubber membrane-based dish. The medium was removed from all of the wells 48 hours after EB transfer, and was replaced with 1ml of fresh ES cell differentiation medium. The following day, the medium was removed and replaced with 1 ml serum-free differentiation medium described in Table 4.8. Medium continued to be exchanged daily with the serum-free differentiation medium until the end of the experiment.

Cell Isolation for nuclei counts and flow cytometry

The medium was removed at the end of the experiment from the wells of the 24-well silicone rubber membrane-based culture dish, the cells were washed with DPBS, and 200 μl of trypsin solution was added to each well. After a 5 min incubation at 37 °C, 800 μl of ES cell differentiation medium was added to each well. The contents of the well were vigorously pipetted up and down using a 1 ml pipette to dislodge and disperse the cells, and were then transferred to a 1 ml tube. The contents were allowed to settle for 2 min, then the bottom 50 μl that contained any large clumps of cells and extra-cellular matrix were removed and discarded. Experiments showed that about 30% of the nuclei present in the well at all pO2gas were in this undissociated cell fraction that was discarded. The cell sample was then mixed briefly with a vortex mixer and a 50 μl aliquot was removed, added to 450 μl of lysis solution containing 1% Triton X-100 (T9284, Sigma Aldrich) and 0.1 M citric acid (C1909, Sigma Aldrich) in DI water, and saved for later analysis. The remaining 900 μl of cell sample was centrifuged at 300 xg for 3 min and the supernatant medium was discarded. Cells were resuspended in 750 μl DPBS, 250 μl of 4% (w/v) paraformaldehyde (Alfa Aesar, Ward Hill, MA) in DPBS was added, and
each sample was incubated for 20 min at room temperature. The samples were then centrifuged at 300 xg, the supernatant removed, and 1 ml of DPBS was added. Nuclei and fixed cell samples were stored at 4 °C prior to analysis.

Gas phase pO2 control

Cell culture vessels were placed inside sealed polystyrene chambers (MIC-101, Billups-Rothenburg, Del Mar, CA) inside a standard incubator (OWJ2720A, Queue Systems, Parkersburg, WV) maintained at 37 °C. The desired pO2gas was established and maintained using premixed gas containing 5% CO2 and 20%, 5%, 1%, or 0% O2 (certified medical gas from Airgas, Hingham, MA). The flow rate of this gas to the chambers was set to 2 l/min for 15 min for an initial purge following closure of the chamber (after cell medium exchange), and was set at 30 ml/min all other times. Gas was bubbled through a sealed bottle of water (in the incubator) and an open dish of deionized water in each chamber provided additional humidification.

Nuclei enumeration

Samples were stained using a Guava Viacount assay kit (Guava Technologies, Hayward, CA) and data were acquired with a Guava PCA flow cytometer.

Flow cytometry

Samples with 3 x 10^5 fixed cells were removed the fixed cell sample tubes and added to an equal volume of 1% (w/v) saponin (S-4521, Sigma-Aldrich) and incubated for 10 min to allow for cell permeabilization. Subsequently, the cells were washed and resuspended in 50 µl of 2% (v/v) FBS in PBS. Samples were incubated in the 2% FBS solution for 30 min, and then 5 µl of diluted primary antibody was added. Primary antibodies were anti-sarcomeric myosin heavy chain (MF-20, MF-20 supernatant, Developmental Studies Hybridoma Bank, Iowa City, IA) without dilution (final dilution 1:10), anti-neuron-specific class III β-tubulin (TUJ1, MAB1637, Chemicon) diluted 1:10 (final dilution 1:100), and anti-nebulin (N9891, Sigma Aldrich) diluted 1:10 (final dilution 1:100). Samples were incubated with the primary antibody for 1 hr, then 0.5 ml of 2% FBS solution was added to each tube and the samples were centrifuged. The supernatant was discarded and the cells were resuspended in 50 µl of goat anti-mouse PE-conjugated secondary antibody (115-116-146, Jackson ImmunoResearch, West Grove, PA) diluted 1:250 in 2% FBS. The samples were incubated 30 min in the dark, washed twice with 0.5 ml of PBS, and fluorescence intensity data were acquired using a flow cytometer (Guava Technologies) using the Express software module. All steps were performed at room temperature. Samples immunostained with anti-nebulin, a antibody specific for skeletal muscle, were used as negative controls.
Quantification of spontaneously contracting area

The entire surface 48-well plate was examined for spontaneously beating areas. The area covered by each spontaneously contracting cell cluster was estimated using a calibrated reticule. Areas that were moving but in which no active contraction was observed were not included in the analysis. The total contracting area was determined from a sum of the individual areas that were measured.

Measurement of hanging drop volume

Hanging drops were aspirated using a 20 μl pipette, and the volume liquid taken into the pipette was adjusted so that it exactly matched the volume of liquid in the hanging drop. The pipette setting at which this occurred was taken to be the hanging drop volume.

Cell resistant polyelectrolyte multilayer coating

A coating to reduce protein adsorption and cell attachment to silicone rubber was prepared as described previously (Berg et al. 2004). Briefly, solutions of 0.01 M polyacrylic acid (PAA, 523925, Sigma Aldrich) and polyacrylamide (PAAm, 434949, Sigma Aldrich) were prepared in DI water, and the pH was adjusted to 3.0 using hydrochloric acid and sodium hydroxide. DI water adjusted to pH = 3.0 was also prepared. 3 ml of PAA solution was added to each well of a silicone rubber membrane-based 6 well plate and incubated for 15 min at room temperature. The wells were then washed three times using DI water (pH = 3). The first wash was 2 min, and the second and third washes were each 1 min. 3 ml of PAAm solution were then added to the wells, and an identical incubation and washing procedure was followed. The procedure was then repeated first with PAA and then PAAm solutions. A total of three incubations was performed with each of the polyelectrolyte solutions to form a (PAA-PAAm)₃ polyelectrolyte multilayer.

The plates were then dried, placed in a desiccation jar, subjected to a vacuum, and placed in a 95 °C oven overnight. The following day the plates were removed, washed for 15 min with 70% ethanol, and dried under a UV lamp in a biosafety cabinet. Prior to cell addition some wells were incubated for 1 hr with 2% (w/v) Pluronic F68 (61-161-RM, Mediatech) in DI water, which was removed prior to cell addition.

Results

Appearance of cardiomyocytes using neural differentiation methods

A 24-day protocol commonly used for neuronal differentiation produced spontaneously beating cells (cardiomyocytes) that first appeared on day 9. These cells were found at pO₂gas of 142 and 7 mmHg, but more were present at 7 mmHg. The spontaneously beating cells persisted throughout differentiation at 7 mmHg, whereas they were not observed at 142 mmHg after trypsin detachment and replating of cells, which occurred after 11 days. When the pO₂gas was varied temporally during differentiation, qualitative analysis of the spontaneous contraction after
24 days showed a greater coverage of spontaneously contracting cells at a pO$_{2\text{gas}}$ of 7 relative to 142 mmHg (Table 4.1). Cultures continuously maintained at 7 mmHg had the highest area covered by beating cells, with decreasing coverage as the total time cultured at 142 mmHg increased.

A subsequent experiment with the same differentiation protocol and quantitative analysis of cardiomyocyte (MF-20) and neural (TUJI) cell fraction using flow cytometry showed that 11 and 1% of cells were neurons and cardiomyocytes, respectively, if the entire protocol was carried out at 142 mmHg, whereas 1 and 32% of cells were neurons and cardiomyocytes, respectively, if the entire protocol was performed at 7 mmHg. The differences between the two conditions were readily apparent after 12 days in culture, when the MF-20 positive cell fraction was 2 and 22% at pO$_{2\text{gas}}$ of 142 and 7 mmHg, respectively. A substantial amount of cell death, especially at 142 mmHg, was observed during the trypsin-detachment and replating process that occurred after 12 days in culture, and this part of the protocol was not used in subsequent experiments.

**Temporal change in cardiomyocyte and total cell number**

The change in fraction of cardiomyocytes, total cell number, and total cardiomyocyte number are shown versus time during differentiation (Fig. 4.1). Cardiomyocytes first appeared at day 6 in these experiments, and the time of appearance was slightly delayed by culture at 7 mmHg. A similar delay at very low oxygen was reproducibly observed in other experiments. The cell number increased between 0 and 4 days during EB culture, remained flat or decreased for the following 4 days after transfer of the EBs to serum free ITS medium, then subsequently increased further. The total cardiomyocyte number increased between days 4 and 14, with a significantly increased number of cardiomyocytes present at 7 relative to 142 mmHg.

Other experiments showed comparable trends, with the maximal cardiomyocyte fraction occurring at day 8-14, then remaining steady or decreasing slightly (data not shown). Likewise, the maximum cardiomyocyte number occurred at about the same time that the maximal cardiomyocyte fraction was observed. Subsequent experiments therefore were focused primarily on the first 12 days of differentiation.

**Premature EB attachment**

During the experiments described previously, we observed that EBs would begin to adhere to the hydrophobic FEP-teflon membrane-based dishes beginning on day 3, and the degree of EB attachment was affected by pO$_{2\text{gas}}$. After the EBs attached to the plate, they spread and adopted a more sheet-like morphology. Subsequent experiments were performed using silicone rubber membranes to improve the control of pO$_{2\text{cell}}$, but EBs were able to adhere to this material after 2 days in culture (data not shown).

Treatment of the dishes with polyelectrolyte multilayers (PEMs) designed to prevent protein adsorption and cell adhesion (Berg et al. 2004) did not prevent EB attachment, but adsorption of Pluronic F68 onto the PEM prior to cell addition was able to reduce but not
eliminate EB adhesion (data not shown). Even when EBs remained unattached, they tended to form very large aggregates due to the combination of multiple EBs into a single aggregate.

**Hanging drop differentiation and enhanced cardiomyogenesis using ascorbic acid**

A series of experiments was performed in which cells were differentiated in hanging drops, which prevented EB agglomeration and attachment to the dish. These drops initially consisted of 200 cells in 20 μl of medium. The cardiomyocyte fraction following 11 days of differentiation is shown in Table 4.2, for experiments with and without supplemental ascorbic acid, a known promoter of cardiomyocyte differentiation (Takahashi et al. 2003). The increase in the fraction of cardiomyocytes obtained at a pO2gas of 36 relative to 142 mmHg was highly significant in these studies both with (P = 0.03) and without (P = 0.005) supplemental ascorbic acid. There was also a marginally significant increase in the fraction of cardiomyocytes at 36 relative to 7 mmHg (P = 0.06), and at 7 relative to 142 mmHg (P = 0.08) during differentiation without supplemental ascorbic acid. A highly significant increase in the fraction of cardiomyocytes obtained when ascorbic acid was included in the culture medium was observed (P = 0.0005), and ascorbic acid was effective at increasing the cardiomyocyte fraction to a about the same extent at all pO2gas tested.

Samples from these experiments were immunostained with a skeletal muscle-specific antibody (nebulin) to determine whether any of the MF-20 positive cells were skeletal muscle. The percentage of cells that were immunostained with nebulin was similar to the background staining in samples not treated with a primarily antibody (negative controls). This verified that the MF-20 positive cells after 11 days of differentiation were most likely cardiomyocytes.

During hanging drop differentiation it was observed that the volume of the medium drop could change due to vapor-liquid equilibrium effects. Depending on the liquid that was placed in the dish below the hanging drops and the temperature of the liquid and the dish itself when the drops were formed, the volume of the 20-μl hanging drops could increase or decrease to be in the range of 12-25 μl after 2 days of differentiation. A mixture of 75% PBS and 25% deionized water with a small amount of gelatin (0.002%) to reduce the surface tension was found to maintain the drop volume at about 20 μl if it was prewarmed to 37 °C prior to EB formation. Subsequent experiments showed that variations in the hanging drop volume over a period of two days did not substantially affect differentiation into cardiomyocytes, and a decrease in drop volume due to evaporation may have been beneficial (Table 4.3). The highest cardiomyocyte fraction and number was observed for hanging drops that had shrunk from 20 to 13 μl over the course of two days, while the least cardiomyocyte differentiation occurred in drops whose volume increased from 20 to 22 μl.

**Optimization for EB size and time of serum change**

A systematic variation in the number of cells used for the formation of EBs and the time in which the EBs were cultured in hanging drops before being transferred to adherent dishes was performed (Fig. 4.2). The time of transfer from serum containing medium to serum-free ITS
medium was varied in some of the experiments (panel A), and kept constant in others (panel B). For all of the protocol variations that were studied, there was always a higher surface coverage of beating cells at pO$_{2\text{gas}}$ of 7 or 36 relative to 142 mmHg.

For all pO$_{2\text{gas}}$, the highest coverage of beating cells was present when 500 cells were used for EB formation, EBs were transferred to adherent dishes on day 2, and serum was removed on day 5. The high surface coverage of spontaneously contracting cells was not due to an increase in total cell number, but was instead due to an increase in the fraction of cardiomyocytes (data not shown). Further increases in cell number in the initial EBs, or shortening the time in EBs to 1 day showed no additional increase in the surface coverage of beating cells (data not shown), relative to the conditions in Fig 4.2B.

The transfer to a serum-free medium was required for the maximal differentiation of mES cells into cardiomyocytes (Fig. 4.3). Culture in DMEM with 10% serum throughout the experiment did not affect the total cell number, but resulted in a reduced fraction of cells that were cardiomyocytes (and hence fewer total cardiomyocytes) in all pO$_{2\text{gas}}$ conditions relative to cultures that were transferred to serum-free ITS medium on day 5. The change to ITS medium did not affect the fraction or total number of cardiomyocytes at the respective pO$_{2\text{gas}}$ conditions relative to one another. In both cases, the highest fraction and total number of cardiomyocytes were obtained at pO$_{2\text{gas}}$ conditions of 7 and 36 mmHg, respectively.

**Comparison of immunostaining with MF-20 to spontaneously beating area**

We observed that the total number of cardiomyocytes determined by counting trypsin-dissociated cells immunostained with MF-20 correlated with the total area covered by spontaneously contracting cells (Fig. 4.4). Using linear regression we found that the correlation coefficient was 0.8 and that the intercept of the best-fit line was 0.01. From this fit of the data, the beating area attributed to each cardiomyocyte was 13 $\mu$m$^2$, which corresponds to a cell diameter of 4 $\mu$m if the cells were spherical. Spontaneously beating areas were usually present in large cellular aggregates that were three-dimensional in nature, which probably explains the small beating area per cell that was observed.

**Discussion**

We used a neural differentiation protocol to study whether changes in culture pO$_{2\text{gas}}$ could affect the differentiation of mES cells into cardiomyocytes because it contained no supplemental agents for cell specific differentiation other than serum, and used a chemically defined medium after initial EB formation. After showing that such conditions were conducive for cardiomyocyte differentiation, the timing of cardiomyocyte formation was studied so that experiments could be focused on the times of most importance. Alternative methods for EB formation and culture were studied in order to get reproducible differentiation in non-adherent EBs, while retaining control of the pO$_{2\text{cell}}$. Finally, the size and time the cells were in EBs was systematically studied in order to maximize differentiation into cardiomyocytes, and determine
whether low \( \text{pO}_2 \text{gas} \) conditions could stimulate cardiomyogenesis with extensive protocol variations.

A single study (Bauwens et al. 2005) has previously shown that low oxygen conditions can promote cardiomyocyte differentiation. However, the cardiomyocyte differentiation in the previous work was poor and the effect of low oxygen could only be measured if extensive genetic selection was performed to purify the cardiomyocytes. In contrast, the increased cardiomyocyte differentiation at reduced \( \text{pO}_2 \) conditions was obvious in our experiments, even using a simple neuronal differentiation protocol. Further improvements could be made to enhance cardiomyocyte differentiation, and make the effects of oxygen more obvious.

The appearance of cardiomyocytes after 6-8 days in culture is consistent with what others find with differentiating ES cells, as is the determination that 500 cells/EB is optimal for generation of cardiomyocytes (Guan et al. 2001). The use of a substantial period of serum-free culture for cardiomyocyte differentiation has only recently been used by others, and the beneficial effects are most likely due to removal of BMP signaling (Honda et al. 2006; Passier et al. 2005). We observed similar results, and serum removal after 5 days clearly increased the fraction and number of cardiomyocytes that were obtained from differentiation. We also found that ascorbic acid could be added to the medium to promote cardiomyocyte differentiation as previously reported (Takahashi et al. 2003), and it is likely that other cardiomyocyte promoting factors exist that could further improve differentiation.

Forming of EBs in simple suspension culture is much easier than using hanging drops, and is therefore generally preferred. However, premature (early) attachment of EBs clearly affects ES cells differentiation into cardiomyocytes (Fig. 4.2). The degree of attachment and time at which it occurred was affected by \( \text{pO}_2 \text{gas} \) during suspension culture, so this secondary oxygen effect is likely present if EB attachment is not well controlled. Indeed, the higher cardiomyocyte differentiation present in early experiments (Fig. 4.1) relative to later experiments (Table 4.2) may be due to this phenomenon. Ultra-low adherent polystyrene dishes are often used to prevent EB adhesion, however such devices offer poor \( \text{pO}_2 \text{cell} \) control. A cell resistant PEM could be used to reduce EB attachment to silicone rubber, however the EBs still agglomerated as others have also observed (Dang et al. 2002). Hanging drops were therefore used to prevent both the premature attachment and EB agglomeration to improve likelihood of obtaining reproducible results. The proximity of the EB to the gas liquid interface also means that the \( \text{pO}_2 \) at the EB surface would be similar to \( \text{pO}_2 \text{gas} \).

We found that cardiomyocyte differentiation could be assessed by immunostaining trypsin-dispersed cells with MF-20, and that results of this assay correlated with the total area covered by spontaneously contracting cells. The correlation of a functional assay (spontaneous contraction) with immunostaining verifies that we did indeed obtain an increase in functional cardiomyocytes using a \( \text{pO}_2 \text{gas} \) of 7 or 36 mmHg, relative to 142 mmHg. The quantification of spontaneously beating area offers a method for rapid screening of many different conditions, as was done in our studies of the optimal number of cells to use in EB formation (Fig. 4.2).
This work provides an overview of the development of a set of differentiation methods that could be used for the generation of cardiomyocytes from ES cells. These methods were developed to be (1) readily reproducible, (2) capable of generating high cardiomyocyte fractions, and (3) able to control the pO$_{2\text{cell}}$ to values near the pO$_{2\text{gas}}$. The resulting protocol meets these three goals, and during its development it was clear that low pO$_2$ culture increased ES cell differentiation into cardiomyocytes. Subsequent experiments performed with the protocol developed in this work are described in Chapter 5, and provide more insight into the effects of oxygen on mES cell differentiation.
Tables and Figures

Table 4.1. Spontaneously contracting cells after 24 days of neuronal differentiation

<table>
<thead>
<tr>
<th>Days</th>
<th>0-3</th>
<th>4-11</th>
<th>12-15</th>
<th>16-24</th>
<th>Relative Beating Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
<td>142</td>
<td>142</td>
<td>142</td>
<td>142</td>
<td>0</td>
</tr>
<tr>
<td>142</td>
<td>142</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>++</td>
</tr>
<tr>
<td>142</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>++++</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>142</td>
<td>++++</td>
</tr>
<tr>
<td>7</td>
<td>142</td>
<td>142</td>
<td>142</td>
<td>142</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>142</td>
<td>142</td>
<td>142</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

EBs were formed in suspension culture in DMEM with 10% FBS for 4 days (0-3), before being transferred to a serum-free ITS medium with fibronectin for an additional 7 days (4-11). Cells were then dispersed with trypsin, and replated with an ITS medium containing N2 supplement and bFGF for 4 days (12-15), and the same medium without bFGF was used for 9 additional days (16-24). The PO2gas is shown for each stage of differentiation and the conditions are ranked according to the number and relative size of the spontaneously beating areas that were observed in one well at the end of a single experiment.
Table 4.2. Differentiation into cardiomyocytes after 11 days using hanging drop culture (200 cell/EB) for 4 days

Fraction of trypsin-dispersed cells that were determined to be cardiomyocytes by immunostaining with MF-20 after 11 days of differentiation (%).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>142 ± 0.2</th>
<th>36</th>
<th>7</th>
<th>142</th>
<th>36</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1</td>
<td>0.5 ± 0.2</td>
<td>-</td>
<td>5 ± 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R1</td>
<td>0.8 ± 0.2</td>
<td>4 ± 2</td>
<td>3.3 ± 0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J1</td>
<td>0.4 ± 0.2</td>
<td>3.5 ± 1.9</td>
<td>6 ± 3</td>
<td>0</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>J1</td>
<td>1.0 ± 1.0</td>
<td>-</td>
<td>6.1 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J1</td>
<td>0.5</td>
<td>7</td>
<td>2</td>
<td>0.5</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>R1</td>
<td>-</td>
<td>12</td>
<td>2</td>
<td>-</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>J1</td>
<td>4.6</td>
<td>12</td>
<td>2</td>
<td>13</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>J1</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>J1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>16</td>
<td>24</td>
</tr>
</tbody>
</table>

Cells were differentiated in hanging drop EBs (200 cells/EB) in DMEM with 10% FBS for 4 days, then transferred to fibronectin-coated silicone rubber membrane based dishes with serum-free ITS medium for the remaining 7 days. Results shown are for independent experiments and, where shown, ± SD for triplicate samples from the same experiment.
Table 4.3. Effect of evaporation and condensation of water from hanging drops on cardiomyocyte differentiation

<table>
<thead>
<tr>
<th>Liquid for Humidification</th>
<th>Temperature prior to EB Formation (°C)</th>
<th>Drop Volume after 2 Days (µl)</th>
<th>Fraction of MF-20⁺ Cells (%)</th>
<th>Total Cell Number (x 10⁵)</th>
<th>MF-20⁺ Cells (x 10⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X PBS</td>
<td>37</td>
<td>13</td>
<td>42</td>
<td>23</td>
<td>9.4</td>
</tr>
<tr>
<td>PBS</td>
<td>25</td>
<td>18</td>
<td>36</td>
<td>10</td>
<td>3.6</td>
</tr>
<tr>
<td>PBS</td>
<td>37</td>
<td>19</td>
<td>33</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>75/25 PBS/DI Water</td>
<td>37</td>
<td>19</td>
<td>34</td>
<td>11</td>
<td>3.7</td>
</tr>
<tr>
<td>DI Water</td>
<td>37</td>
<td>22</td>
<td>25</td>
<td>10</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Hanging drops were formed using 20 µl of medium with different liquids, at different initial temperatures, placed in the bottom of each dish to provide humidification. The drop volume changed after two days due to evaporation or condensation of water. The fraction of cardiomyocytes, total cells, and total cardiomyocytes are shown after 11 days of differentiation performed in a single well using EBs from the indicated hanging drops. Differentiation was carried out using 500 J1 ES cell/EB for 2 days in DMEM with 10% FBS. EBs were then plated onto a fibronectin-coated silicone rubber membrane-based 24 well plate (30 EB/well), and the medium was switched to serum-free ITS on day 5. Both media contained 0.2 mM ascorbic acid.
Table 4.4. Formulation for undifferentiated ES cell maintenance medium and ES cell differentiation medium (without ascorbic acid)

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer and Catalogue #</th>
<th>Volume Added per Liter (ml)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified Eagles medium (DMEM)</td>
<td>ATCC SCRR 2010</td>
<td>868</td>
<td>ES cell qualified</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>ATCC SCRR 30-2020</td>
<td>100</td>
<td>ES cell qualified</td>
</tr>
<tr>
<td>L-alanyl-L-glutamine</td>
<td>ATCC SCRR 30-2115</td>
<td>10</td>
<td>200 mM stock solution</td>
</tr>
<tr>
<td>MEM non-essential amino acid solution</td>
<td>ATCC 30-2116</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2-mercaptoethanol stock solution</td>
<td>Custom made with Sigma Aldrich M7522</td>
<td>10</td>
<td>10 mM stock solution prepared by adding 28 μl of M7522 to 40 ml DMEM and sterile filtering</td>
</tr>
<tr>
<td>Leukemia inhibitory factor</td>
<td>Chemicon ESG 1106</td>
<td>1</td>
<td>10⁶ unit/ml stock solution</td>
</tr>
</tbody>
</table>

1 Leukemia inhibitory factor was only added to the undifferentiated ES cell maintenance medium
Table 4.5. Formulation for serum-free ITS medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer and Catalogue #</th>
<th>Volume Added per Liter (ml)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified Eagles medium (DMEM)</td>
<td>Mediatech 90-113-PB</td>
<td>485</td>
<td>4.08 g of 90-113-PB were dissolved in 485 ml WFI (Mediatech 25-055-CM) and sterile filtered</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>Mediatech 25-035-C1</td>
<td>10</td>
<td>7.5% (w/v) solution</td>
</tr>
<tr>
<td>F12 nutrient mixture</td>
<td>Invitrogen 31765-035</td>
<td>496</td>
<td></td>
</tr>
<tr>
<td>25M glucose solution</td>
<td>Sigma Aldrich G8769</td>
<td>1.6</td>
<td>Final glucose concentration of medium is 9 mM</td>
</tr>
<tr>
<td>Human insulin solution</td>
<td>Sigma Aldrich 19278</td>
<td>0.5</td>
<td>10 mg/ml solution</td>
</tr>
<tr>
<td>Holo transferrin solution</td>
<td>Custom made with Sigma Aldrich T1283</td>
<td>5</td>
<td>10 mg/ml stock solution prepared by dissolving 100 mg in 10 ml DPBS and sterile filtering</td>
</tr>
<tr>
<td>Sodium selenite solution</td>
<td>Custom made with Sigma Aldrich S9133</td>
<td>0.26</td>
<td>0.12 mM solution prepared by adding 50 ml DPBS to S9133</td>
</tr>
<tr>
<td>Component</td>
<td>Manufacturer and Catalogue #</td>
<td>Volume Added per Liter (ml)</td>
<td>Notes</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------------------</td>
<td>-----------------------------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dulbecco’s modified Eagles medium (DMEM)</td>
<td>Mediatech 90-113-PB</td>
<td>481</td>
<td>4.08 g of 90-113-PB were dissolved in 485 ml WFI (Mediatech 25-055-CM) and sterile filtered</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>Mediatech 25-035-CI</td>
<td>10</td>
<td>7.5% (w/v) solution</td>
</tr>
<tr>
<td>F12 nutrient mixture</td>
<td>Invitrogen 31765-035</td>
<td>491</td>
<td></td>
</tr>
<tr>
<td>25M glucose solution</td>
<td>Sigma Aldrich G8769</td>
<td>1.6</td>
<td>Final glucose concentration of medium is 9 mM</td>
</tr>
<tr>
<td>Human insulin solution</td>
<td>Sigma Aldrich I9278</td>
<td>2.5</td>
<td>10 mg/ml solution</td>
</tr>
<tr>
<td>Holo transferrin solution</td>
<td>Custom made with Sigma Aldrich T1283</td>
<td>10</td>
<td>10 mg/ml stock solution prepared by dissolving 100 mg in 10 ml WFI and sterile filtering</td>
</tr>
<tr>
<td>Sodium selenite solution</td>
<td>Custom made with Sigma Aldrich S9133</td>
<td>0.26</td>
<td>0.12 mM solution prepared by adding 50 ml DPBS to S9133</td>
</tr>
<tr>
<td>Putrescine solution</td>
<td>Custom made with Sigma Aldrich P5780</td>
<td>1</td>
<td>0.1 M solution prepared by adding 0.161 g P5790 to 10 ml DPBS and sterile filtering</td>
</tr>
<tr>
<td>Progesterone solution</td>
<td>Custom made with Sigma Aldrich P6149</td>
<td>0.1</td>
<td>0.2 mM solution prepared by adding 15.9 ml of DPBS to 1mg P6149</td>
</tr>
<tr>
<td>L-ascorbic acid stock solution</td>
<td>Custom made with Sigma Aldrich A4034</td>
<td>1</td>
<td>200 mM stock solution prepared by dissolving 396 mg of A4034 in 10 ml DPBS and sterile filtering</td>
</tr>
<tr>
<td>bFGF$^1$</td>
<td>R&amp;D Systems 3139-FB-025</td>
<td>1</td>
<td>10 µg/ml solution prepared by reconstituting bFGF in 2.5 ml WFI</td>
</tr>
</tbody>
</table>

$^1$ Was only added during first 4 days that N2 medium was used
Table 4.7. Formulation for cardiomyocyte differentiation medium (days 0-4)

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer and Catalogue #</th>
<th>Volume Added per Liter (ml)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified Eagles medium (DMEM)</td>
<td>ATCC SCRR 2010</td>
<td>868</td>
<td>ES cell qualified</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>ATCC SCRR 30-2020</td>
<td>100</td>
<td>ES cell qualified</td>
</tr>
<tr>
<td>L-alanyl-L-glutamine</td>
<td>ATCC SCRR 30-2115</td>
<td>10</td>
<td>200 mM stock solution</td>
</tr>
<tr>
<td>MEM non-essential amino acid solution</td>
<td>ATCC 30-2116</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2-mercaptoethanol stock solution</td>
<td>Custom made with Sigma Aldrich M7522</td>
<td>10</td>
<td>10 mM stock solution prepared by adding 28 μl of M7522 to 40 ml DMEM and sterile filtering</td>
</tr>
<tr>
<td>L-ascorbic acid stock solution</td>
<td>Custom made with Sigma Aldrich A4034</td>
<td>1</td>
<td>200 mM stock solution prepared by dissolving 396 mg of A4034 in 10 ml DPBS and sterile filtering</td>
</tr>
</tbody>
</table>
Table 4.8. Formulation for cardiomyocyte differentiation medium (day 5 onward)

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer and Catalogue #</th>
<th>Volume Added per Liter (ml)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified Eagles medium (DMEM)</td>
<td>Mediatech 90-113-PB</td>
<td>485</td>
<td>4.08 g of 90-113-PB were dissolved in 485 ml WFI (Mediatech 25-055-CM) and sterile filtered</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>Mediatech 25-035-CI</td>
<td>10</td>
<td>7.5% (w/v) solution</td>
</tr>
<tr>
<td>F12 nutrient mixture</td>
<td>Invitrogen 31765-035</td>
<td>496</td>
<td></td>
</tr>
<tr>
<td>25M glucose solution</td>
<td>Sigma Aldrich G8769</td>
<td>1.6</td>
<td>Final glucose concentration of medium is 9 mM</td>
</tr>
<tr>
<td>Human insulin solution</td>
<td>Sigma Aldrich 19278</td>
<td>0.5</td>
<td>10 mg/ml solution</td>
</tr>
<tr>
<td>Holo transferrin solution</td>
<td>Custom made with Sigma Aldrich T1283</td>
<td>5</td>
<td>10 mg/ml stock solution prepared by dissolving 100 mg in 10 ml DPBS and sterile filtering</td>
</tr>
<tr>
<td>Sodium selenite solution</td>
<td>Custom made with Sigma Aldrich S9133</td>
<td>0.26</td>
<td>0.12 mM solution prepared by adding 50 ml DPBS to S9133</td>
</tr>
<tr>
<td>L-ascorbic acid stock solution</td>
<td>Custom made with Sigma Aldrich A4034</td>
<td>1</td>
<td>200 mM stock solution prepared by dissolving 396 mg of A4034 in 10 ml DPBS and sterile filtering</td>
</tr>
</tbody>
</table>
Figure 4.1. Temporal appearance of cardiomyocytes during ES cell differentiation

Temporal changes in fraction of cells that were cardiomyocytes, total cell number, and total cardiomyocyte number during J1 ES cell differentiation. EBs were formed in suspension culture in DMEM with 10% FBS for 4 days, before being transferred to an adherent dish containing serum-free ITS medium with fibronectin for an additional 10 days of attached culture. Results are mean ± SD for 3 independent wells at each time point in a single experiment.
Figure 4.2. Effect of EB size and time in hanging drops on cardiomyocyte differentiation

The fraction of a 24-well plate that was covered with spontaneously contracting cells at different pO$_{2}$gas conditions after 11 days of differentiation is shown as a function of the number of cells present in the initial hanging drop and the time in hanging drop culture before transfer to a fibronectin-coated silicone rubber membrane-based 48 well plate (10 EB/well). The hanging drops were formed using DMEM with 10% FBS and were transferred to a serum free ITS medium 1 day after plating the EBs (A-C), or on day 5 (D-F). Results shown are the mean of two independent experiments performed with J1 ES cells.
Figure 4.3. Effect of serum removal on cardiomyocyte differentiation

Fraction of cells that were cardiomyocytes (top), total number of cells (middle), and total number of cardiomyocytes (bottom) for cells cultured at constant $pO_2_{gas}$ conditions of 7, 36, or 142 mmHg for 11 days. EBs were formed using 500 J1 ES cell/EB and were cultured for 2 days before being transferred to a fibronectin coated silicone rubber membrane-based 24 well plate (30 EB/well). Cells were maintained in serum containing DMEM for all 11 days (open bars) or were changed to a serum-free ITS medium after 5 days (solid bars). The number fraction of cardiomyocytes was determined by flow cytometry of MF-20-stained, trypsin-dispersed cell samples. Data shown as mean ± SD for 3 replicates in a single experiment.
Comparison between the total number of MF-20 positive cells counted using flow cytometry and the total surface area of the culture dish covered with spontaneously contracting cells estimated visually. The fraction of MF-20 positive cells ranged from 3 to 36% in these samples. The best-fit line was determined using linear regression, $R^2 = 0.80$. 

**Figure 4.4. Comparison of immunostaining with MF-20 to surface coverage of beating cells**
Chapter 5. Effects of Oxygen on ES Cell Differentiation into Cardiomyocytes
To show that oxygen affects cardiomyocyte differentiation and better understand the time and specific oxygen levels over which an effect is present we (1) quantitatively studied cardiomyocyte differentiation at different pO2 conditions in 7 independent experiments, (2) studied the effect of temporal variation in pO2 on the total cell number and fraction and total number of cardiomyocytes after 10 days of differentiation, and (3) estimated the pO2-cell during differentiation using theoretical models. Oxygen reproducibly affected mES cell differentiation into cardiomyocytes. After 10 or 11 days of differentiation, the highest number fraction of cardiomyocytes was 31 ± 6% with a pO2-gas of 7 mmHg, compared to 23 ± 9% and 9 ± 4% at 36 and 142 mmHg, respectively. Culture at 7 mmHg resulted in a substantial decrease in total cell number relative to that at 36 or 142 mmHg, both of which had comparable cell numbers after 11 days. As a result, the total number of cardiomyocytes was similar at 7 and 142 mmHg but significantly higher at 36 mmHg. Low oxygen (7 mmHg) exerted its strongest cardiomyocyte differentiation-promoting effect during the first 6 days of differentiation, and subsequent increases in pO2-gas to 142 mmHg could be used to maximize the total cardiomyocyte number without a decrease in purity. Theoretical models of oxygen transport showed that cells differentiated at high pO2-gas were well-oxygenated during early stages of culture, and some cardiomyocytes were apparently able to form at high pO2-cell conditions. Oxygen starvation was predicted to occur in some tissue clumps after 11 days of differentiation at all pO2-gas due to the increasing size of cell aggregates with increasing time in culture and pO2-gas. In conclusion, oxygen substantially affects the differentiation of mES cells into cardiomyocytes, and pO2 control can be used to increase the fraction and total number of cardiomyocytes obtained from the differentiation of ES cells.

Introduction

Cardiomyocytes derived from differentiated embryonic stem (ES) cells hold promise as a treatment for heart disease (Klug et al. 1996), but obtaining sufficient number and purity of functional cells is challenging (Xu et al. 2006). Most ES cell research has been performed in incubators with a humidified 95% air/5% CO2 gas mixture, resulting in a gas-phase oxygen partial pressure (pO2-gas) of 142 mmHg. Embryonic cells in early development are exposed to pO2-cell values of about 0-30 mmHg (Intaglietta et al. 1996; Jauniaux et al. 2003b; Siggaard-Andersen and Huch 1995), and the effects of such conditions on differentiating ES cells are poorly understood.

Previous research has investigated the use of a various molecules to direct cardiomyocyte differentiation. Positive results have been reported with retinoic acid, nitric oxide, TGFβ-1, FGF, erythropoietin, BMP-2, BMP-4, ascorbic acid, retinoic acid, DMSO, noggin, 5-azacytidine, and VEGF (Chen et al. 2006c; Fukuda and Yuasa 2006; Yoon et al. 2006). However, little attention has been given to the possible effects that oxygen might have on this process. The results of only a single study with reduced oxygen and cardiomyocyte differentiation showed a small
positive effect that was observed only after genetic selection for cardiomyocytes was performed (Bauwens et al. 2005).

In a static, attached culture system, we previously observed that reduced oxygen resulted in a significant increase in differentiation of mES cells into cardiomyocytes (Chapter 4), which could be further enhanced with supplemental ascorbic acid. In our studies, embryoid bodies (EBs) were formed in hanging drops for 2 days and then manually transferred to custom-made culture dishes having fibronectin-coated silicone rubber membranes at the bottom, to which the cells attached and grew. The silicone rubber membranes had very high permeability to oxygen that allowed use of static culture while retaining control of PO2cell at the membrane-cell interface to values similar to PO2gas (Chapter 3).

Here we describe the use the previously developed differentiation protocol to quantitatively study of the effect of oxygen on ES cell differentiation into cardiomyocytes. Control of the PO2cell to levels experienced by the developing embryo enhances differentiation of ES cells into cardiomyocytes. The fraction of cells that are cardiomyocytes is maximized by initial culture at PO2cell values less than 7 mmHg, whereas cardiomyocyte number is maximized by subsequent culture at high PO2cell. Under the best conditions examined, the fraction and number of cardiomyocytes increase 3-fold, compared to culture at high PO2 throughout differentiation.

Materials and Methods

Undifferentiated ES cell culture

J1 and R1 ES cells were obtained from ATCC (SCRC 1010, SCRC 1036, Manassas, VA), expanded on Mitomycin-C treated MEF cells, and frozen into vials following ATCC protocols. Vials were thawed into undifferentiated ES cell maintenance medium (described in Table 4.4) and plated at a density of about 50,000 cell/cm² on 25 cm² cell culture flasks (353109, Becton Dickinson, Franklin Lakes, NJ) that were treated for 30 min with a sterile 0.1% (w/v) solution of gelatin (G-2500, Sigma-Aldrich, St. Louis, MO) in tissue culture water (WFI, 25-055-CM, Mediatech, Herndon, VA). Medium was exchanged daily, and cells were detached with 0.25% trypsin (30-2101, ATCC) every two days. Split fractions were chosen so that cells were plated at approximately 12,000 cells/cm². Cells were used for differentiation experiments 4-6 days after thawing the cell vials.

Cardiomyocyte differentiation

Cardiomyocyte differentiation was performed using a previously developed protocol described in detail elsewhere (Chapter 4). Briefly, J1 mES cells were differentiated in hanging drops containing 500 cells using DMEM with 10% FBS. After 2 days the drops were transferred into a fibronectin-coated, silicone rubber membrane-bottomed, 24-well plate (30 EB/well). Three days later, the medium was changed to a serum-free ITS medium, which was used for the remainder of the experiment. Media was exchanged daily after plating of cells on day 2.
Cell Isolation for nuclei counts and flow cytometry

Cells were isolated as described previously (Chapter 4). Briefly, at the end of the experiments (8 or 9 days after plating EBs) the medium was removed from the wells of the 24-well silicone rubber membrane-based culture dish, and the cells were detached with trypsin. Samples were removed for nuclei counts, and the remaining cell suspension was fixed with paraformaldehyde and stored for subsequent immunocytochemistry.

Nuclei enumeration

Samples were stained using a Guava Viacount assay kit (Guava Technologies, Hayward, CA) and data were acquired with a Guava PCA flow cytometer.

Cell processing for histology

At the end of the experiments (8 or 9 days after plating EBs) the medium was removed from the wells of the 24-well silicone rubber membrane-based culture dish and the cells were washed with DPBS and incubated for 30 min at room temperature with 4% (w/v) paraformaldehyde solution. The cell sheet was then washed twice with 1 ml of DPBS, released from the dish bottom by stretching the silicone rubber membrane, and transferred into a 1.5 ml microtube. The tube was centrifuged at 300 xg and all but 100 µl of the DPBS was removed. 900 µl of 1% (w/v) agarose (5510UA, Life Technologies, Gaithersburg, MD) in DI water (solution at 95 °C) was added to the tubes, which were briefly mixed by vortexing. Samples were then centrifuged for 1 min at 20,000 xg and cooled for 1 hr at 4 °C. 500 µl of 4% (w/v) paraformaldehyde solution was added to the tubes, which were stored overnight. The agarose was removed from the tube the following day and trimmed with a razor blade so that only the pellet and a small additional amount of agarose remained. The pellets were then placed in histology cassettes and stored in DPBS prior to embedding. Histology specimens were embedded in paraffin and sectioned by the Joslin Diabetes Center histology core following standard protocols to yield 5-µm sections.

Silicone rubber membrane-based dishes

Custom-made silicone rubber membrane-based dishes were made as described previously (Chapter 3). Briefly, the bottom surface of the 8 central wells of 24-well tissue culture plates were removed and replaced with a 127-µm thick silicone rubber membrane. The plates were sterilized with 70% ethanol and dried under a UV lamp in a biological safety cabinet. One day prior to plating the cells, 1 ml of 2-µg/ml fibronectin (F1141, Sigma-Aldrich) in DPBS was added to each well and incubated overnight at 37 °C. Immediately before plating cells, the fibronectin solution was removed from all of the wells and replaced with 1ml pre-warmed ES cell differentiation medium.
Gas phase pO₂ control

The oxygen in the gas phase was controlled using premixed gas cylinders containing 5% CO₂ and 20%, 5%, or 1% O₂ as described previously (Chapter 4). Gas was bubbled through a sealed bottle of water (in the incubator) and an open dish of deionized water in each chamber provided additional humidification.

Flow cytometry

Flow cytometry was performed as described previously (Chapter 4). Primary antibodies were anti-sarcomeric myosin heavy chain (MF-20, MF-20 supernatant, Developmental Studies Hybridoma Bank, Iowa City, IA) with a final dilution of 1:10 and anti-cardiac troponin T (anti-cTnT, MS-295-P, Lab Vision Corp., Fremont, CA) with a final dilution of 1:100. A phycoerythrin-conjugated secondary antibody (115-116-146, Jackson ImmunoResearch, West Grove, PA) was used to detect primary antibody binding with a flow cytometer (Guava Technologies) using the Express software module.

Immunocytochemistry of sectioned tissue

Slides containing the 5-μm sections were deparaffinized and rehydrated by 7 min rinses with xylene (twice), 5 min rinses with 100% ethanol (twice), a 3 min rinse with 95% ethanol, a 10 min rinse with 70% ethanol, and 5 min rinses with deionized (DI) water (twice). Antigen retrieval was performed by boiling the sections for 10 min in a 10 mM sodium citrate (0754, Mallinckrodt, Paris, KY) solution adjusted to pH = 6. The slides were cooled to room temperature and endogenous peroxidase activity was quenched by incubating the slides for 10 min in a solution containing 0.3% hydrogen peroxide (386790, Calbiochem, La Jolla, CA) in DI water. Slides were then incubated for 1 hr in a solution containing 1% FBS in PBS, and subsequently incubated with diluted primary antibody overnight at 4 °C (same final dilution as was used for flow cytometry). After washing 3 times with 1% FBS in PBS, goat anti-mouse IgG (115-035-062, Jackson ImmunoResearch) diluted 1:50 in 1% FBS was added, and the slides incubated for 3 hr at room temperature. The slides were then washed 3 more times, a mouse PAP complex (223-005-024, Jackson ImmunoResearch) diluted 1:500 in 1% FBS was added, and an additional 1 hr incubation at room temperature was performed. After washing twice in PBS, antibody binding was visualized by incubating 2 min in a 2 mM solution of diaminobenzidine (D5637, Sigma Aldrich) containing 0.015% hydrogen peroxide. Slides were then counterstained by incubating for 15 sec in filtered hematoxylin (HHS16, Sigma Aldrich), followed by 3 subsequent washes in 30 mM sodium borate (B10267-34, EMD, Gibbstown, NJ). Slides were then dehydrated following the reverse procedure for rehydration and were preserved using Permount (SP15, Fisher Chemical, FairLawn, NJ). Slides were photographed and 1000 total cells in at least 20 different randomly selected areas (coordinates of photograph selected with a random number generator in Excel) of the section were examined for immunostaining (~50 cells per area), and positive and negative cells were counted.
Model for oxygen reaction and diffusion

A theoretical model of oxygen consumption and diffusion in the culture system was developed in order to describe the oxygen concentration in the tissue. Oxygen transport within the medium, silicone rubber membrane, and tissue was modelled using the oxygen conservation equation

\[ D\alpha V^2(pO_2) = R_v \]  

(5.1)

where \( D \) and \( \alpha \) are the effective diffusivity and solubility, respectively, of oxygen in the medium, silicone rubber membrane, or the tissue, and \( R_v \) is the volumetric oxygen consumption rate within the tissue and is equal to 0 in the medium and the silicone rubber. The boundary conditions for the model were \( pO_2 = pO_{2\text{gas}} \) at the gas/liquid and gas/silicone rubber interfaces and \( \nabla pO_2 = 0 \) at the edge of the unit well used for simulation. Continuity of flux and \( pO_2 \) were assumed at the silicone rubber/medium, silicone rubber/tissue, and medium/tissue interfaces. The product of the diffusivity and solubility of oxygen in culture medium, silicone rubber, and tissue was taken to be 3.53, 26.3, and \( 1.34 \times 10^{-5} \) mol/cm mmHg sec, respectively (Avgoustiniatos et al. 2007).

Determination of volumetric oxygen consumption rate

The volumetric oxygen consumption rate, \( R_v \) (amol/sec cm\(^3\)), in the tissue was assumed to follow Michaelis-Menten kinetics,

\[ R_v = V_{\text{max}} \frac{pO_2}{K_m + pO_2} \left( \frac{1}{V_{\text{cell}}} \right) \]  

(5.2)

where \( V_{\text{max}} \) is the maximal oxygen consumption rate (amol/cell sec), \( K_m \) is the Michaelis constant, which was taken to be 0.44 mmHg (Avgoustiniatos 2002; Avgoustiniatos et al. 2007; Wilson et al. 1979), and \( V_{\text{cell}} \) is the average volume of tissue associated with each cell, which includes the actual cell volume and associated interstitial volume.

\( V_{\text{max}} \) was measured with undifferentiated ES cells, whole EBs cultured for 3 days, and for cells that had been differentiated for 10 days according to the cardiomyocyte differentiation protocol. To measure \( V_{\text{max}} \), cells or tissue were put in fresh culture medium equilibrated to 37 °C and ambient oxygen and then placed into a Micro Oxygen Monitoring System (FO/SYS2-T250, Instech Labs, Plymouth Meeting, PA) as described previously (Papas et al. 2007). Undifferentiated cells were detached with trypsin and resuspended at a density of between 6 and \( 12 \times 10^6 \) viable cells/ml and placed in the chamber. With day 3 EBS, 100 EBs were removed from hanging drop culture after 3 days and pipetted into the OCR measurement chamber, allowed to settle for 5 min, and excess medium was removed. After 10 days of differentiation, the cell sheets and clumps were released from the dish bottom by stretching the silicone rubber
membrane, transferred into the OCR measurement chamber, allowed to settle 1 min, and excess medium was removed. After placing cells in the chamber and removing excess medium, the chamber was sealed and the measurement was performed. The time-dependent pO₂ within the chamber was recorded with a fluorescence-based oxygen sensor, and the data at pO₂ values greater than 30 mmHg were fit to a straight line by linear regression analysis. The oxygen consumption rate (OCR) was evaluated from the slope of this line and Vₘₐₓ was determined by dividing the total OCR by the total number of cells in the chamber. Total cell number was determined from cell counts from the undifferentiated ES cell suspension and from nuclei counts from the cells removed from the chamber following measurement with EBs or day 10 tissue. Measured values of Vₘₐₓ varied with pO₂ (Fig. 5.1). The data were fit to a function of the form

\[
V_{\text{max}} = \alpha + \beta \frac{pO_2}{\gamma + pO_2}
\]

yielding α and β = 10 and 20 amol/sec cell, respectively, and γ = 16 mmHg.

V₉₉ was determined with day 2 EBs by measuring the diameter using light microscopy and a calibrated reticule, then dissociating the EBs to obtain total nuclei counts, nₙ. V₉₉ was calculated from the ratio V₉₉/nₙ where V₉₉ is total volume of EBs analyzed, to obtain V₉₉ = 10.1 ± 1.5, 11.2 ± 1.4, and 12.7 ± 1.5 x 10⁻¹⁰ cm³/cell after 2 days at pO₂gas of 7, 36, and 142 mmHg, respectively. For cells after 11 days of differentiation, a field of view encompassing aggregated cells in a 5-μm tissue section was identified. The number of cells per unit volume of tissue was assumed to be equal to the number of nuclei having their centre within this volume, which was determined using light microscopy. It was assumed that the nuclei were spheres with a diameter, d, of 7-μm, and the number of nuclei counted in the section of thickness, t = 5 μm, was multiplied by the ratio of v/(v + d) to obtain the number of nuclei, nₙ, whose centre point was within the volume of tissue contained in the section. V₉₉ was calculated from the ratio V₉₉/nₙ, where V₉₉ = area of field of view • t is the volume of the section, to obtain V₉₉. Data for cells after 11 days of differentiation from 15 fields of view were averaged, yielding V₉₉ = 15 ± 4, 14 ± 6, and 14 ± 7 x 10⁻¹⁰ cm³ at pO₂gas of 7, 36, and 142 mmHg, respectively. The volume of tissue associated with each cell varied significantly within different regions of the tissue and this heterogeneity was not considered in the analysis.

**Geometric properties of cultured tissue**

To accurately model the oxygen consumption and diffusion, the geometric properties of the tissue were measured with light microscopy. The 2-day embryoid bodies were nearly perfect spheres with average radii of 99 ± 6, 115 ± 5, and 114 ± 8 μm for pO₂gas of 7, 36, and 142 mmHg, respectively. To characterize the tissue after 10 or 11 days in culture, 5-μm tissue sections taken from three independent experiments were analyzed by stereological point counting using at least 300 points from each of the 5-μm tissue sections (Weibel 1979). The tissue was present in the form of thin sheets or larger clumps of cells, and the volume fraction of tissue in each
morphology was determined. The fraction of tissue in clumps was 53 ± 6, 75 ± 9, and 90 ± 7 %
of the total tissue volume for cells cultured at P_{O_{2gas}} of 7, 36, and 142 mmHg, respectively. For
each clump of cells examined, the distance from the basal to apical surface (tissue thickness) and
the length of the clump was recorded (Table S3). Each clump was modeled as an oblate
hemispheroid with major and minor axis that corresponded to the measured height and half width,
respectively. The sheet-like tissue was modeled as an infinite thin slab with a thickness of 30, 30,
and 15 μm at P_{O_{2gas}} of 142, 36, and 7 mmHg based on measurements of representative cell
sheets; the model predictions were insensitive to variations in sheet thickness in the range of
0 – 30 μm.

**Finite element model**

The tissue geometries described above were modeled as resting on the surface of a
127-μm thick silicone rubber membrane overlain with a 5 mm height of medium and placed in
the centre of a cylindrical well with a 2 mm radius, a size at which edge effects due to the well
walls were negligible. The oxygen conservation equation shown in Eqn. (5.1) with the
appropriate parameters and boundary conditions described above was solved for each individual
clump geometry using a numerical finite element method (Comsol Multiphysics).

**Tissue distribution of oxygen on day 11 tissue**

The calculations described above yielded the distribution of pO₂ throughout each of the
individual hemispheroidal and planar tissue geometries that was analyzed. For each geometry
simulated, surfaces of constant pO₂ were determined, and numerical integration was performed
to determine the volume of tissue between the surfaces of constant pO₂. The volume fraction of
tissue within specific limits of pO₂ in the entire dish, consisting of a sheet with a single average
radius and each of the individual clumps, was determined from

\[ \Phi_{ab} = \left( \phi_{sheet} \Phi_{ab,sheet} \right) + \phi_{clump} \frac{\sum_{j=1}^{n} V_{ab,j}}{V_{clump,n}} \tag{5.4} \]

where \( \Phi_{ab} \) is the total volume fraction with a pO₂ between a and b, \( \Phi_{ab,sheet} \) is the comparable
quantity for the tissue in the cell sheet, \( \phi_{sheet} \) and \( \phi_{clump} \) are the volume fractions of tissue in the
total preparation in the sheet and clump geometry, respectively, \( V_{ab,clump,n} \) is the volume of tissue
with a pO₂ between a and b in the n th clump of cells, and \( V_{clump,n} \) is the total volume of the n
clumps analyzed.

**Tissue distribution of oxygen within MF-20 + region**

Calculations similar to those described above were used to estimate the oxygenation of
the cells positively immunostained with MF-20. For these simulations the aggregate dimensions
were measured and MF-20$^+$ regions were identified in the 5-\(\mu\)m tissue sections. A 2D finite model for oxygen transport was used to predict the pO$_2$ profile within the entire aggregate. The area fraction of tissue in MF-20$^+$ regions within specific limits of pO$_2$ was determined using an equation analogous to Eqn (5.4) with \(\phi_{\text{sheet}} = 0\).

**Lactate production rate measurement**

After 10 days of differentiation, a complete medium exchange was performed, and cells were returned to their respective pO$_{2\text{gas}}$ conditions for an additional 5 hr of culture. After this time, the contents of each well were mixed, and samples were removed for lactate analysis, which was performed as described previously (Chapter 2).

**Results**

**Effect of oxygen on cardiomyocyte fraction and number**

Differentiating mES cells at different, constant pO$_{2\text{gas}}$ conditions for 10 or 11 days resulted in a large increase in the fraction of cells that were cardiomyocytes with decreasing values of pO$_{2\text{gas}}$ that was reproducibly observed in 7 independent experiments (Fig. 5.2). The highest number fraction of cardiomyocytes was 31 \(\pm\) 6\% with a pO$_{2\text{gas}}$ of 7 mmHg, compared to 23 \(\pm\) 9\% and 9 \(\pm\) 4\% at 36 and 142 mmHg, respectively. Culture at 7 mmHg resulted in a substantial decrease in total cell number relative to that at 36 or 142 mmHg, both of which had comparable cell numbers after 11 days. As a result, the total number of cardiomyocytes was similar at 7 and 142 mmHg but significantly higher at 36 mmHg.

**Histology after cardiomyocyte differentiation at different pO$_{2\text{gas}}$**

Histological examination of 5-\(\mu\)m tissue sections showed the cells cultured for 11 days at 7 mmHg preferentially formed thin cell sheets and smaller aggregates than were found at 36 or 142 mmHg (Fig 5.3). In all cases the MF-20 positive cells were found together in aggregates. Flow cytometry produced slightly higher estimates for number fraction of MF-20 positive cells than histological counting, but the increase in cardiomyocyte fraction with decreasing pO$_{2\text{gas}}$ was observed using both methods (Fig. 5.4). Immunostaining of serial sections with MF-20, and cTnT showed excellent co-localization of the two cardiomyocyte makers in all such sections examined (Fig. 5.5). Previous experiments also showed that there was a strong correlation between the total number of MF-20 positive cells and the area covered by spontaneously contracting cells (Chapter 4).

**Temporal variation of pO$_{2\text{gas}}$**

The effect of culturing cells at high and low pO$_{2\text{gas}}$ for different time periods in different order with the total culture period (10 days) held constant is shown in Fig. 5.6. After 10 days in culture, the fraction of cells that were cardiomyocytes was greater when the cells were initially started at 7 mmHg. The increased cardiomyocyte fraction was observed if days 0-6 were carried
out at 7 mmHg, and was not affected by increasing $pO_2_{gas}$ to 142 mmHg after day 6. Likewise, changing $pO_2_{gas}$ from 142 to 7 mmHg after day 2 had no positive effect on the cardiomyocyte fraction measured at day 10.

The total cell number was greatest for cultures in which the $pO_2_{gas}$ at days 6-10 was 142 mmHg, regardless of the $pO_2_{gas}$ used prior to day 6. Therefore, the total number of cardiomyocytes present at day 10 was highest if the $pO_2_{gas}$ was 7 mmHg during the first 6 days of culture, and then increased to 142 mmHg for the final 4 days. Using these $pO_2_{gas}$ conditions, 35% of cells were cardiomyocytes and 60 cardiomyocytes were generated for each initial ES cell, both of which represent a 3-fold increase relative to constant culture at 142 mmHg. The total cardiomyocyte number in this case was slightly higher than that obtained using constant culture at a $pO_2_{gas}$ of 36 mmHg in the same experiments, but the difference was not statistically significant (data not shown).

For cultures started at 7 mmHg, the increase in cardiomyocyte and total cell number that occurred during days 6-10 at 142 mmHg was accompanied by significant morphological changes. The cells that were in sheet-like and small aggregate structures on day 6 proliferated and migrated to form large aggregates similar in size to those present for cultures maintained at 142 mmHg for all 10 days. These aggregates contracted vigorously and contained numerous cardiomyocytes.

**Cellular energetics during and after cardiomyocyte differentiation**

The oxygen consumption and lactate production rate of mES cells that had been differentiated for 10 days was analyzed to obtain parameter estimates for finite element modeling and to determine the energetic profile of the cells (Table 5.1). The cellular oxygen consumption rate was relatively unchanged during differentiation, however there was a significant decrease in lactate production. The aerobic fraction of the total ATP synthesis was therefore significantly higher after 10 days of mES cell differentiation than it was in undifferentiated ES cells, as would be expected for maturing cells.

**$pO_2_{cell}$ during and after cardiomyocyte differentiation**

Silicone rubber membranes with high oxygen permeability were used as the bottom of the culture dishes in order to control the $pO_2_{cell}$ of cells attached to the bottom of the dish. Nonetheless, large gradients of $pO_2_{cell}$ existed within cellular aggregates. A theoretical model of oxygen consumption and diffusion was used to estimate the volumetric distribution of $pO_2_{cell}$ within the aggregates (Fig. 5.7). In day 2 embryoid bodies (EBs), about 60% of the tissue is at a $pO_2_{cell}$ less than 1 mmHg during culture at a $pO_2_{gas}$ of 7 mmHg, whereas more than 80% of tissue cultured at 36 mmHg is at a $pO_2_{cell}$ greater than 7 mmHg, and all of the tissue at 142 mmHg is at a $pO_2_{cell}$ greater than 94 mmHg.

After 2 days, the EBs attached, spread, and further proliferated on the silicone rubber membrane; the idealized spherical geometry was lost, and approximations of aggregate shapes were used in the model. Cells cultured at a high $pO_2_{gas}$ grow to form larger aggregates, with a
median height after 11 days in culture of 115, 195, and 270 μm at pO$_{2\text{gas}}$ of 7, 36, and 142 mmHg, respectively, based on measurements from sectioned tissue (Table 5.2). The increased length scale of the aggregates cultured at high pO$_{2\text{gas}}$ caused there to be oxygen starvation at the center of these cell clumps (Fig 5.8). About 21, 16, and 12% of the total volume of tissue was at a pO$_{2\text{cell}}$ of less than the $K_m$ for oxygen consumption (0.44 mmHg) at 7, 36, and 142 mmHg, respectively, after 11 days of culture.

Combining results such as those in Fig. 5.8 with immunostaining showing the precise location of the cardiomyocytes (Fig. 5.3), it was possible to predict the local pO$_{2\text{cell}}$ of the cardiomyocytes. A comparison of the volumetric distribution of pO$_{2\text{cell}}$ of all of the tissue during culture and that of cardiomyocytes at 142 mmHg is shown in Fig. 5.9. The overall pO$_{2\text{cell}}$ distribution shows no marked differences from that of the total tissue mass, suggesting that there is no correlation between local pO$_2$ and the location of the mature cardiomyocytes in day 11 tissue.

Discussion

We used a protocol previously optimized for the differentiation of mES cells into cardiomyocytes to study the effects that oxygen has on cardiomyocyte differentiation. Histological analysis was used to quantitatively verify the enhancement of cardiomyocyte differentiation at low pO$_2$ conditions, and to provide the approximate length scales of the tissue so that the actual pO$_{2\text{cell}}$ could be estimated with finite element simulations. Temporal variations in pO$_{2\text{gas}}$ were also performed to determine the time period over which a low pO$_{2\text{cell}}$ was required to promote cardiomyocyte differentiation, and to identify conditions that could be used to simultaneously maximize cardiomyocyte fraction and number.

Using the differentiation protocol developed previously, we were able to consistently obtain a cardiomyocyte purity of about 10% at 142 mmHg. This is comparable to results obtained by others using optimized differentiation protocols (Kanno et al. 2004; Kattman et al. 2006; Xu et al. 2006); our protocol was one such variation that used supplemental ascorbic acid and serum removal after day 5 to enhance cardiomyogenesis. The ability to obtain substantial cardiomyocyte differentiation at 142 mmHg allowed for the observation of the improvements with pO$_{2\text{cell}}$ control, that were not possible in the only other experiment that studied the use of oxygen control to improve ES cell differentiation into cardiomyocytes (Bauwens et al. 2005).

The demonstration of a 3-fold increase in cardiomyocyte fraction above levels typically obtained without purification is a significant advance. The positive effects of reduced pO$_{2\text{gas}}$ were found using a variety of differentiation protocols both without additional ascorbic acid and with removal of serum at different times, but reduced values of pO$_{2\text{gas}}$ did not result in high cardiomyocyte yields when combined with protocols that produced low yields at 142 mmHg. Nonetheless, the positive effect that low pO$_2$ culture has on ES cell differentiation into cardiomyocytes using a variety of conditions suggests that the benefits of using reduced oxygen
conditions are broadly applicable, and not simply the artifact of using one or two specific differentiation protocols.

The mechanism by which oxygen exerted its effects in our experiments is not known. The oxygen-sensitive transcription factor HIF-1 is active in ES cells cultured at 142 mmHg, and its activity is further enhanced during low pO2 culture (Iyer et al. 1998). In a HIF-1α knockout ES cell line, no cardiomyocyte differentiation is observed in-vitro during culture at 142 mmHg (Ateghang et al. 2006), and HIF-1 activity is seen in hypoxic regions of the developing chick heart (Wikenheiser et al. 2006). HIF-1 stabilization at low oxygen therefore seems a likely to be responsible for at least some of the effects observed in our experiments.

HIF-1 target genes include all of the enzymes necessary for glycolysis as well as VEGF and other proteins (Lee et al. 2004). VEGF enhances cardiomyocyte differentiation of ES cells (Chen et al. 2006c; Kattman et al. 2006), but it alone does not appear to have a sufficient effect on differentiation to explain our results. Signaling through ROS including nitric oxide affects ES cell differentiation into cardiomyocytes, and some of these pathways appear to affect HIF-1α expression as well (Ateghang et al. 2006; Kanno et al. 2004; Kasuno et al. 2004). As others have noted with the study of hematopoietic differentiation at low pO2 (Hevehan et al. 2000; Ramirez-Bergeron et al. 2004), the effects we observe are likely the result of a combination of factors, and extensive study will be required to understand the most oxygen-sensitive pathways and cells involved.

Oxygen exerted its strongest effects during the first 6 days of differentiation, the time period that immediately precedes the emergence of the first spontaneously-contracting and MF-20-positive cells, which both occur at approximately the sixth day of culture. Because, the oxygen effect is present before cardiomyocytes are present, we infer that oxygen affected differentiation into cardiomyocyte progenitor cells and/or induced an increase in proliferation of such progenitor cells relative to other cells types. The increase in total cardiomyocyte number suggests a direct positive effect of reduced oxygen on differentiation or proliferation along the cardiomyocyte lineage, but the exact mechanism and cell types through which this process is occurring is not yet known. After the appearance of cardiomyocytes, increased oxygen stimulated proliferation, presumably by minimizing the oxygen or other nutrient limitations as the cell number increased.

The cellular oxygen level can be predicted fairly well at early times in culture due to the idealized geometry, and these times are the most important for the cardiomyocyte promoting effect of low oxygen culture. It appears that during the first 6 days of culture at 142 mmHg most or all of the cells will remain well oxygenated, and therefore at least a modest level of cardiomyocyte differentiation is able to occur at high pO2cell conditions. However, culture at local pO2cell values ranging from less than 1 mmHg to several 10s of mmHg at early times substantially enhanced cardiomyocyte development relative to culture at high pO2.

The differentiating cells preferentially formed aggregates, which were limited in size by the availability of oxygen and other nutrients. Cells initially cultured at pO2gas of 7 mmHg would form large aggregates within 4 days of transfer to 142 mmHg. The cardiomyocytes were almost
exclusively present in aggregates, but their location within the aggregate could not be correlated with local pO$_{2\text{cell}}$ conditions (Fig. 5.9), and in many cases cardiomyocytes were observed to span the entire depth of a cell aggregate. A precise correlation of local pO$_{2\text{cell}}$ conditions to differentiation events would have to be done with tissue sections taken on day 6 or earlier, and would likely require identification of cardiac progenitor cells instead of mature cardiomyocytes. This is certainly an area that warrants further study.

Our finding that the pO$_2$ to which ES cells are exposed during culture can profoundly influence differentiation into cardiomyocytes has disparate ramifications. The markedly increased yield of cardiomyocytes may enhance prospects for their therapeutic use in heart disease. Control of dissolved oxygen is inexpensive and easily accomplished in biotechnological processes, making our results applicable to commercial development of stem cell technologies. Reduced oxygen culture with other proteins and small molecules may affect differentiation to other cell types. Because the cells in the early embryo are exposed to pO$_{2\text{cell}}$ conditions that lie within the range in which we observed large effects on differentiation, pO$_{2\text{cell}}$ may play a more important role in early embryonic development than heretofore appreciated.
Tables and Figures

Table 5.1. Oxygen consumption rate and lactate production of differentiating cells

<table>
<thead>
<tr>
<th></th>
<th>pO$_2$gas (mmHg)</th>
<th>OCR (amol/sec cell)</th>
<th>LPR (amol/sec cell)</th>
<th>Aerobic Fraction of Total ATP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated</td>
<td>142</td>
<td>29</td>
<td>110</td>
<td>60</td>
</tr>
<tr>
<td>ES Cells</td>
<td>36</td>
<td>23</td>
<td>160</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>17</td>
<td>210</td>
<td>32</td>
</tr>
<tr>
<td>Day 3 EBs</td>
<td>142</td>
<td>29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 10 Cardiomyocyte</td>
<td>142</td>
<td>27</td>
<td>29</td>
<td>85</td>
</tr>
<tr>
<td>Differentiation</td>
<td>36</td>
<td>17</td>
<td>48</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>18</td>
<td>96</td>
<td>55</td>
</tr>
</tbody>
</table>

Oxygen consumption rate (OCR) and lactate production rate (LPR) measured for undifferentiated mES cells, EBs after 3 days in hanging drop culture, and for cells that had been differentiated for 10 days using a cardiomyocyte differentiation protocol. Results for the undifferentiated cells were obtained from previous experiments (Chapter 2). EB and day 10 differentiated cells were measured in a single experiment, although similar results (slightly lower OCR and higher LPR) were obtained from experiments using other differentiation protocols. It was assumed that 6 ATP were made per mol of O$_2$ consumed, and 1 ATP was produced per mol lactate produced (Chapter 2) in order to calculate the aerobic fraction of total ATP.
Table 5.2. Data from sections used for finite element models

<table>
<thead>
<tr>
<th>pO₂gas (mmHg)</th>
<th>7</th>
<th>36</th>
<th>142</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>53</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>Number of</td>
<td>21</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>Clumps Analyzed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>115</td>
<td>195</td>
<td>270</td>
</tr>
<tr>
<td>Mean</td>
<td>120</td>
<td>190</td>
<td>280</td>
</tr>
<tr>
<td>Std Dev</td>
<td>60</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>Median</td>
<td>340</td>
<td>666</td>
<td>620</td>
</tr>
<tr>
<td>Mean</td>
<td>450</td>
<td>630</td>
<td>590</td>
</tr>
<tr>
<td>Std Dev</td>
<td>290</td>
<td>320</td>
<td>370</td>
</tr>
</tbody>
</table>

5-μm tissue sections from three experiments were analyzed to obtain morphology estimates that could be used in finite element models. Stereological point counting was performed to determine the volume fraction of tissue that was present in clumps (as opposed to cell sheets). The height of each clump was measured as the distance from its basal to apical surface using a calibrated reticule, and the length was measured in a similar manner. The basal surface was determined by the shape of the clump and the surface which was continuous with the cell sheet. Statistics for these measurements are given below.
Figure 5.1. Dependence of $V_{\text{max}}$ on $pO_{2\text{gas}}$ at which cells were cultured

The maximal normoxic OCR ($V_{\text{max}}$) of undifferentiated mES cells (closed circles), day 3 EBs (triangles), and day 10 tissue (open circles) is shown as a function of the culture $pO_{2\text{gas}}$. The fit of these data using Eqn. (5.3) is also shown. Data for $V_{\text{max}}$ with undifferentiated ES cells are reported as mean $\pm$ s.d. for at least 4 independent experiments, and data for differentiating cells are from single experiments.
Figure 5.2. Effects of oxygen on ES cell differentiation into cardiomyocytes

Fraction of cells that were cardiomyocytes (top), total number of cells (middle), and total number of cardiomyocytes (bottom) for cells cultured at constant pO₂gas conditions of 7, 36, or 142 mmHg for 11 days. The number fraction of cardiomyocytes was determined by flow cytometry of MF-20-stained, trypsin-dispersed cell samples. Data shown as mean ± SD for 7 independent experiments (at least 3 replicates per experiment), each started with 30 EBs/well (0.15 x 10^5 cells/well). P values were found using a two-tailed t-test for paired samples.
Figure 5.3. 5-μm tissue sections following 11 days of cardiomyocyte differentiation

Representative images of 5-μm tissue sections stained for MF-20 (brown) and counter stained with hematoxylin. Tissue was obtained after 11 days of differentiation from one of the experiments at each constant pO$_{2_{gas}}$ condition that contributed to the data shown in Fig. 5.1. The cells were removed and centrifuged into a pellet prior to sectioning, resulting in a random tissue orientation.
Figure 5.4. Cardiomyocyte quantification with flow cytometry and histological counting

Comparison of the number fraction of MF-20⁺ cells counted from stained 5-μm sections to that measured with flow cytometry of trypsin dispersed cells in three independent experiments. Open, gray filled, and black filled symbols represent samples taken from pO₂gas of 142, 36, and 7 mmHg, respectively, and each shape (circle, triangle, upside-down triangle) represents an independent experiment. Results for flow cytometry are the mean ± SD for samples from 3 independent wells in the same experiment, while the histological results are from the single section that was obtained in each experiment.
Figure 5.5. Co-localization of MF-20 and cTnT immunostaining

Consecutive 5-μm tissue sections stained with a primary antibody to sarcomeric myosin heavy chain (MF-20 - top) or cardiac troponin T (cTnT - middle), or no primary antibody (control - bottom). Images are representative of the co-localization of MF-20 and cTnT observed in day 11 tissue sections from all pO$_2$gas conditions.
Figure 5.6. Temporal modulation of pO$_{2}$gas affects differentiation

Fraction of cells that were cardiomyocytes, total number of cells, and total number of cardiomyocytes as determined by flow cytometry of trypsin-dispersed cell samples after 10 days of differentiation immunostained with MF-20. Cells were started at either 142 or 7 mmHg and then switched to the other condition on day 2, 4, 6, 8, or not at all. The 10 different experimental pO$_{2}$gas histories are shown at the bottom of the figure, and the day of change of the pO$_{2}$gas is noted. The number of independent experiments (n), each with three replicates, is given at the top of each column. Data are mean of the results of the independent experiments ± SD where n = 3, or ± range of the experiments where n = 2.
Figure 5.7. Volumetric distribution of $pO_{2\text{cell}}$ within differentiating cellular aggregates

A theoretical mathematical model for oxygen consumption and diffusion in the cellular aggregates was solved numerically to yield profiles of $pO_{2\text{cell}}$ within the tissue. Tissues were assumed to have uniform cell distributions and material properties. After 2 days culture using the hanging drop method, EBs were nearly perfect spheres and were transferred to fibronectin-coated silicone rubber surfaces, to which they attached and spread. The dimensions of the aggregates after 11 days in culture was measured, the median sized aggregate at each condition is drawn to scale above each panel. The line represents the cumulative volume fraction of tissue exposed to a $pO_2$ less than or equal to the indicated value. Each solid bar represents the fraction of the total volume of tissue within the aggregate that is exposed to a specific range of $pO_{2\text{cell}}$. These $pO_{2\text{cell}}$ ranges span values of 1-2, 2-5, or 5-10 within each decade on a logarithmic scale.
Figure 5.8. $pO_2$ profiles in day 11 aggregates

Lines of constant $pO_2$ are plotted for day 11 aggregates at $pO_{2\text{gas}}$ of 142, 36, and 7 mmHg. The numbers to the right of each shape correspond to the contour lines plotted. The aggregates shown have the mean dimensions from Table 5.2 and are representative of the profiles observed in aggregates of all sizes.
Figure 5.9. Volumetric distribution of pO$_2$ values in MF-20$^+$ regions

The line represents the cumulative volume fraction of tissue exposed to a pO$_2$ less than or equal to the indicated value. Each solid bar represents the fraction of the total volume of tissue within the aggregate that is exposed to a specific range of pO$_{2\text{cell}}$. The panel on the left shows the distribution in all of the tissue in the culture dish and the panel on the right shows the distribution in the regions that were positively immunostained with MF-20, which accounted for 11 ± 2% of the total tissue volume. Qualitatively similar results were observed in tissue cultured at 36 and 7 mmHg.
References


Helbock HJ, Beckman KB, Ames BN. 1999. 8-hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. Oxidants and Antioxidants, Pt B, p 156-166.


