TOWARD AN UNDERSTANDING OF HOW HYPERCAPNIA AFFECTS APOPTOSIS IN HUMAN PROMYELOBLASTS IN 3D SUSPENSION CULTURE SYSTEMS

BY

MOUNA HAMAD

A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

AT

THE SCHOOL OF CHEMICAL AND BIOMOLECULAR ENGINEERING
THE UNIVERSITY OF SYDNEY
2017
Declaration Statement

I declare that the work presented in this dissertation is original and the product of my own work except as acknowledged throughout the text.

This work has not been submitted for a degree at any other university.

This thesis contains materials published in (1), in which I am leading author, and is distributed throughout the thesis.

Mouna Hamad
February, 2017
Acknowledgments

First and foremost, I would like to thank God almighty, the Most Gracious, the Most Merciful, for granting me the knowledge and the ability to complete my PhD studies.

I would like to express my sincere gratitude and appreciation to my supervisor A/Prof Ali Abbas for his ongoing support and encouragement towards my research. Thank you Ali for your patience and understanding in all circumstances, thank you for your continuous enthusiasm and motivation, thank you for pushing me and allowing me to grow as a research scientist. I mostly appreciate you persuading me to present my work at numerous conferences/seminars/competitions and to meet new people, my experience has truly been valuable and much more than I ever imagined. I am also very grateful for your advice on both my research as well as on my future career, your immense knowledge and guidance was priceless. Finally, thank you for your assistance towards the publishing of this thesis document; it would not have been possible otherwise.

Huge thanks to my co-supervisor Dr Mohammad R. Irhimeh for his immense help in my integration into the cell world. Thank you Mohammad for teaching me all I need to know about cells and cell culturing and also for providing us with the cell line used throughout this project, it would not have been possible without your time and efforts.

I would also like to thank the School of Chemical and Biomolecular Engineering at the University of Sydney; it has been a pleasure being part of such a wonderful and warm community. Special thanks to the beautiful ladies in the office, Annette Karydis, Agnes Constanti, and Lynne, for always being welcoming and ready to help with a smile. Thank you to the beautiful Elizabeth Dobrinsky for our chats and giggles. Thank you to Sergio Londono and Bogumil Eichstaedt for all their help in the lab, I greatly appreciate your time and assistance.

Thanks to all my colleagues that I have met over the years for your help in one way or another: Harry Manoli, Muhamad Hafiz Abd Rahim, Hari Jeyaseelan, Eleonora Costamagna, Edoardo Bartolini, and all the PhD students in our postgraduate office. It was a pleasure being part of the team, and congratulations to all those who graduated.
Thank you to everyone within the Bosch facilities who made this project possible, Donna Lai, Sheng Hua, Angeles Sanchez-Perez, and Xiaosuo Wang. Special thanks to Angeles for the great amount of help and time she dedicated towards mentoring me in the flow cytometry section of this project. Thanks to Adrian Smith and Caroline Royle from the Centenary Institute for their assistance also in the flow cytometry.

I must also acknowledge the School of Chemistry at the University of Sydney, thank you for being my second family over the last several years and hopefully for many more to come. Thank you to Dr Ron Clarke, Dr Robert Baker, and Jeanette Hurst for the opportunity to be part of your community. Thanks to all the demo’s for being wonderful family, especially Peter Luke for the wonderful morning whistles! Huge thanks to the beautiful Sophie Patsalides and Marjan Ashna for your support and kindness especially during my pregnancy.

Very special thanks to my one and only friend Mengyu Wang. Mengyu, thank you for being the most wonderful, kind-hearted, amazing friend and office-neighbour anyone can ask for. Thank you for listening to my complaints and always making me feel better. Thank you for your very wise words and suggestions in all the times I needed to hear them. Thank you for our coffee-getaways which always made my days brighter. Thanks for your delicious drawer which was always full of yummy-ness. I cannot thank you enough for being a friend I will love and cherish forever, you made my PhD journey much more valuable. Congratulations on also finishing your PhD, cannot wait to graduate together!!

To my beloved family... Thank you to my dad who always believed in me and encouraged me, thanks for always showing me how proud you are of what I have achieved. I appreciate all your dedications towards helping reach this goal. Thank you to my brother Ahmed for all your support and for being my wonderful lab buddy during all holidays, I appreciate all the time you spent by my side, I would not have done it without you! Thank you to my brother Ibrahim for being a brilliant role model, I admire what you have achieved in your life thus far and wish you all the best for all to come.

To my dear husband Ahmad, I cannot thank you enough! Thank you for being there for me all throughout my candidature. Thanks for putting up with me in all my moods and for always giving me a shoulder to lean on during this rollercoaster. Thanks for your ongoing support,
your encouragement, and most importantly, your love. You are an amazing friend and husband, and now an even more wonderful father. I could not have become who I am today without you!

Last, but certainly not least, I would like to express my deepest sense of gratitude to my beloved mother. Mum, I am more than thankful for you. If it weren’t for you, I would not have even applied to continue my postgraduate studies. Thank you for always encouraging and motivating me to do better. Thank you for your patience and for your infinite help throughout all my years. Thank you for being the most remarkable role model both as a mother and as a teacher. Thank you for being my best friend and for making me the woman I am today. I continue to learn from you how to be a better person and a better mother every day. Thank you for simply being there for me, none of this would be possible without you!

To my baby Hussain, thank you for coming into this world and making my life so much more cheerful and meaningful. It was a tough ride for the two of us, but your infectious smile made every day a lot more bearable and brighter. My heart is an ocean and it is full of love for you habibi, this is all for you!
Preface

Basic biology teaches us that humans and all human cells breathe in oxygen and discard carbon dioxide. We are also taught that excessive amounts of carbon dioxide are detrimental to all human life forms. Hence the vast majority, in fact almost all, of human cell-related researches focus on carbon dioxide as the ‘bad guy’, the byproduct, the contaminant, or the toxin that must be eliminated from the cell culture system to prevent unfavourable effects upon the cells in focus. However, it must be highlighted that an immeasurable count of studies have and are currently being conducted on human stem cells. Stem cells are born within the human bone marrow and they exist in this environment for quite some time before differentiating and committing to a specific lineage and leaving for their destined site in the body. Nowhere in the literature is it stated that in the bone marrow these early stem cells exclusively breathe in oxygen, nor is it stated that these stem cells utterly rely on oxygen for their optimal proliferation and/or pluripotency. Nowhere in the literature is it demonstrated that carbon dioxide is a ‘contaminant’ or ‘toxin’ in the bone marrow which hinders stem cell proliferation capacity and must be eliminated. It seems, to the best of my knowledge, that researchers do not have any clear idea of the precise gas composition, specifically carbon dioxide, within the bone marrow niches which ensures an optimum microenvironment for stem cell proliferation and development.

Oxygen concentrations, on the other hand, have been researched much more than carbon dioxide to optimise in vitro culture conditions of bone marrow progenitor cells. It is now well known that a hypoxic environment is maintained within the bone marrow where oxygen tension ranges from 1 to 6% (2, 3). Human blood contains dissolved carbon dioxide at a concentration close to 5% (40 mmHg), but this is very different from the environment surrounding progenitor stem cells such as hematopoietic stem cells in vivo within bone marrow niches (3, 4). Very little evidence is present to provide a rationale for choosing this concentration (5%) of carbon dioxide for culturing progenitor cells of the bone marrow in vitro. Despite current literature not accurately defining the distribution of carbon dioxide in the bone marrow niches, it is nevertheless well established that the 5% carbon dioxide concentration used in standard cell culturing is lower than that surrounding the bone marrow-hematopoietic stem cells (3, 5). Only a few studies have been conducted on the effect of carbon dioxide on
mammalian stem cells and results are contradictory with some cell types favouring hypercapnia and others hypocapnia (high and low concentrations of carbon dioxide, respectively).

So we questioned... Why are stem cells in the literature always cultured at 5% carbon dioxide? Why is carbon dioxide completely overlooked in stem cell research altogether? Why is oxygen the primary gas examined in terms of stem cell proliferation? What if carbon dioxide played a critical role within the bone marrow microenvironment to ensure stem cell aptness? What if carbon dioxide is not the ‘bad guy’ after all?

Our hypothesis is that, similarly to oxygen, carbon dioxide is also involved in key role(s) in the proliferation and/or development of human stem cells. Whether progenitor stem cells favour carbon dioxide concentrations higher or lower than the commonly renowned 5% is the main broad topic of this thesis. From the above perspective, the thesis herein will examine the effects of hypo- and hypercapnia on human promyeloblasts, specifically focusing on the apoptotic capacity of these cells in this regard. Concurrently, the effect of the culture environment itself will also be scrutinized (two dimensional flasks versus a three dimensional stirred suspension bioreactor).

This study analyses the effects of hypo- and hypercapnia using the cell line KG-1a to model human hematopoietic stem cells. This is a reasonable assumption to make, given that both KG-1a and human hematopoietic stem cells express the CD34+ antigen and have analogous properties. To the best of our knowledge, this work is novel since no other research has described the optimization of KG-1a cell expansion under varying concentrations of dissolved carbon dioxide, nor has the apoptotic profile of these cells, or any other mammalian cell/cell line, been focused on in this manner. We firmly believe this work will significantly contribute towards innovative stem cell culturing methods and towards a better understanding of the in vivo microenvironment of hematopoietic stem cells.

This dissertation presents 6 chapters. Chapter 1 will introduce the reader to stem cells and their current use in research/therapy with specific focus on hematopoietic stem cells and their role towards the treatment of leukaemia. This chapter will also emphasise the importance of carbon dioxide in mammalian cell culture, particularly in the apoptosis cascade. Chapter 2 will review
the role of carbon dioxide in the hematopoietic stem cell microenvironment and the expansion of mammalian stem cells in hypo- and/or hypercapnic conditions. It will also review in detail conventional 2D culture methods versus 3D bioreactor culture systems and related configurations and parameters with specific focus on the various bioreactor systems used in the literature for the expansion of hematopoietic stem cells. Chapter 3 will discuss our experimental materials and methods in detail. The experimental work conducted will then be portrayed and results discussed in Chapters 4 and 5, respectively, then in Chapter 6 relevant conclusions and future recommendations will be made. This thesis will also provide a concise literature review on the culturing of hematopoietic stem cells in model-based controlled 3D culture systems. Since this review is slightly beyond the main scope of our work, it will be presented at the end of the dissertation for the reader’s expediency. The purpose of the latter review is to provide the reader with an indication of where the literature currently stands with respect to the expansion of hematopoietic stem cells in controlled 3D systems, which will, in turn, give insight into the future potential(s) of our own work presented herein.
Table of Contents

1. Chapter 1: An Introduction to Stem Cells..............................................................................7
   1.1 Background.......................................................................................................................... 7
   1.2 Ethics, Policies and Limitations.......................................................................................... 9
   1.3 Hematopoietic Stem Cells and Hematopoiesis ................................................................. 9
   1.4 Case Study: Hematopoietic Stem Cells and Leukaemia ..................................................... 13
   1.5 The Role of Carbon Dioxide in Cell Culture and in Apoptosis ........................................ 15
   1.6 Hypotheses and Objectives ................................................................................................. 18
   1.7 Thesis Outline ................................................................................................................... 18

2. Chapter 2: Literature Review ................................................................................................ 20
   2.1 The role of CO₂ in the HSC 3D Microenvironment ............................................................ 21
   2.2 The Expansion of Mammalian Stem Cells in Hypo- or Hypercapnic Conditions ............ 22
   2.3 Three Dimensional Culture Systems – Bioreactors ........................................................... 26
   2.4 Bioreactor Configuration and Parameters ........................................................................ 30
       2.4.1 Oxygen......................................................................................................................... 30
       2.4.2 Carbon Dioxide and pH ......................................................................................... 32
       2.4.3 Metabolic Byproducts .............................................................................................. 32
       2.4.4 Sheer Stress.............................................................................................................. 32
   2.5 Bioreactor Configurations and Operations ....................................................................... 33
       2.5.1 Brief Introduction and Scope of Review ................................................................. 33
   2.6 Uncontrolled HSC proliferation .......................................................................................... 34
       2.6.1 Stirred suspension .................................................................................................... 36
       2.6.2 Perfused Systems ..................................................................................................... 38
       2.6.3 Packed Bed .............................................................................................................. 39
       2.6.4 Hollow Fibre ........................................................................................................... 39
       2.6.5 Spinner Flasks ......................................................................................................... 40
       2.6.6 Rotating Wall Vessel Bioreactor (RWVB) ............................................................... 40
       2.6.7 Other ....................................................................................................................... 41
   2.7 Traditional Control (Feedback Control) of HSC proliferation .......................................... 43
       2.7.1 Perfused Systems .................................................................................................... 44
       2.7.2 Stirred Suspension ................................................................................................... 46
       2.7.3 Packed Bed ............................................................................................................ 47
       2.7.4 RWVB .................................................................................................................... 48
       2.7.5 Hollow Fibre .......................................................................................................... 48
       2.7.6 Other ....................................................................................................................... 49
   2.8 Commercially Available HSC Expansion Systems and Patents ........................................ 50
       2.8.1 Cytomatrix .............................................................................................................. 50
       2.8.2 Rotary Cell Culture Systems (RCCS) – Synthecon .................................................. 51

VIII
5.2 2D versus 3D .............................................................................................................. 93

6. Chapter 6: Conclusions and Future Directions .............................................................. 95

6.1 Toward an Understanding of How Hypercapnia Affects Apoptosis ............................. 95

6.1.1 Future Work ........................................................................................................ 96

6.2 Towards Autologous Transplantation – A future multi-scale systems approach in stem cell bioreactor operation ....................................................................................... 98

6.3 Population balance equations (PBE) ........................................................................ 100

6.4 Growth Models ....................................................................................................... 102

6.5 Model-Based Control and Optimization of HSC Proliferation ................................ 103

6.6 Conclusions and Future Remarks ............................................................................ 105

7. References .................................................................................................................. 107

Appendix I – Literature Review Summary Table .................................................................. 129

Appendix II - Research to Date .......................................................................................... 136
List of Tables

Table 1.1: Cytokines utilised in HSC culture ex vivo (22).................................................................11
Table 1.2: Summary of human HSC markers (22). ..........................................................................12
Table 1.3: Timeline of events for BMT (43). ....................................................................................14
Table 2.1: Summary of the literature on hypo/hypercapnia with respect to mammalian cells........24
Table 2.2: Comparison between stationary and stirred cell culture methods (92).........................27
Table 2.3: Critical parameters that manipulate stem cell expansion and/or differentiation in bioreactors (63). ................................................................................................................28
Table 2.4: The main characteristics of different bioreactors used for stem cell culture (63, 92). .................................................................................................................................29
Table 2.5: List of selected patents related to HSC proliferation in 3D bioreactors (2000 – 2015). ..........................................................................................................................51
Table 3.1: Summary of experiments conducted. ................................................................................59
Table 3.2: Properties of KG-1a cells.................................................................................................60
Table 3.3: Bioreactor System Configurations..................................................................................71
Table 4.1: Average cell size of KG-1a cells at varying CO\textsubscript{2} concentrations with time........75
Table 4.2: Average population doubling time (PDT) and viability of KG-1a cells grown at (1, 5, and 15) % CO\textsubscript{2} in air at three different starting cell concentrations. ...............................76
Table 4.3: Specific apoptosis (SA) of hypo- and hypercapnic populations (calculated in comparison to 5% CO\textsubscript{2} control populations) at three different starting cell concentrations. ................................................................................................................79
Table 4.4: Average population doubling time (PDT) and viability of KG-1a cells grown at (0.5, 5, and 15) % CO\textsubscript{2} in air ........................................................................................................83
Table 4.5: Specific apoptosis (SA) of hypo- and hypercapnic populations (calculated in comparison to 5 % CO\textsubscript{2} control populations). ........................................................................86
Table 5.1: Summary of identical experiments conducted at 15% CO\textsubscript{2} in air in 2D and 3D systems, respectively........................................................................................................93
Table 8.1: Literature summary of Sections 2.6 and 2.7. This table summarises studies which examined the expansion of blood or blood-derived (human or murine) cell expansion in an either controlled or uncontrolled bioreactor system. ................................. 129

List of Figures

Figure 1.1: Embryonic Stem Cell Differentiation into multiple cell lineages (11). ESC: embryonic stem cell .................................................................................................................. 7

Figure 1.2: Development of the blastocyst from the zygote; PB: Polar Bodies (14) ....................... 8

Figure 1.3: Stem Cell Division and Pluripotency. Stem cells are pluripotent and can divide or differentiate into progenitor cells which are multipotent. The latter can also divide or differentiate into cells of the same tissue. Differentiated cells can divide but are incapable of differentiating into other cell types (16). ................................. 8

Figure 1.4: Diagram of the anatomy of the bone (24). .................................................................. 10

Figure 1.5: Illustrative examples of blood samples from a healthy person versus leukaemia patient (24). .................................................................................................................. 13

Figure 2.1: New-type bioreactor developed by Zhou et al. which combines elements of both static and stirred culture systems (146). 1: grooved culture chamber; 2: medium inlet; 3: medium outlet; 4: cell inoculation port; 5: surface protuberance; 6: complementary flask; 7: gas mixture appliance. ............................................................. 42

Figure 2.2: Schematic diagram of the minibioreactor and data acquisition and control system used for the expansion of human hematopoietic cells (109). ......................................................... 47

Figure 2.3: The novel bioreactors for the cultivation of hematopoietic cells (115). ......................... 49

Figure 2.4: Commercially available bioreactor, Cytomatrix (175) .................................................. 51

Figure 3.1: First sketch of the bioreactor design ............................................................................. 68

Figure 3.2: Small scale sample bioreactor. ..................................................................................... 68

Figure 3.3: Final bioreactor manufactured and used in experimental. ............................................. 69

Figure 3.4: Schematic diagram of the stirred suspension bioreactor system ................................. 70

Figure 4.1: ProRes C5 images of KG-1a cells using Olympus CKX31/41 inverted microscope (40 times magnification) at 0, 72, 96, and 168 hours. ................................................. 74
Figure 4.2: KG-1a cells’ size in μm (y-axis) that were cultured at 1% (A), 5% (B) and 15% (C) CO₂ over time in hours (x-axis) at three different starting cell concentrations (→2.00 x 10⁵ cells/ml, →-3.00 x 10⁵ cells/ml, →- 4.00 x 10⁵ cells/ml). At time = 240 hours, cells cultured at (1 and 5) % CO₂ were dead and hence cell size is zero. Each time point represents average of three independent experiments ± standard deviation.

Figure 4.3: Proliferation curves of KG-1a cells cultured at CO₂ concentrations of 1% (A), 5% (B) and 15% (C) CO₂ over time, where y-axis and x-axis show cell concentration (cells/ml) and time (hours), respectively, each at three different starting cell concentrations (→2.00 x 10⁵ cells/ml, →-3.00 x 10⁵ cells/ml, →- 4.00 x 10⁵ cells/ml). Each time point represents three independent experiments ± standard deviation.

Figure 4.4: Flow cytometry analysis of FITC Annexin V (x-axis) and propidium iodide (PI) (y-axis) staining showing high CO₂ concentration preserves KG-1a cells from early apoptosis and delays cell death. KG-1a cells were cultured at three CO₂ levels [1% (top row), 5% (middle row) and 15% (bottom row)]. Two representative time points (96 hours (left) and 192 hours (right)) were selected to show the pattern. The quadrants in each blot show percentages of KG-1a that are alive (Q4), early apoptotic (Q3), late apoptotic (Q2) or necrotic cells (Q1).

Figure 4.5: Error bar plots showing the effect of (1, 5, and 15) % CO₂ concentrations on KG-1a cell count. The x-axis for all plots shows % CO₂, while the y-axis shows in (A) % live KG-1a cell fraction, in (B) % early apoptotic KG-1a cell fraction, in (C) % late apoptotic KG-1a cell fraction, and in (D) necrotic KG-1a cell fraction. Two representative time points [96 hours (○) and 192 hours (□)] were selected to show the pattern. Each time point represents three numbers of independent experiments ± standard deviation.

Figure 4.6: KG-1a cells’ size in μm (y-axis) that were cultured at 0.5 % (→), 5 % (→-), and 15 % (→- C) CO₂ over time in hours (x-axis). Each time point represents average of three independent experiments ± standard deviation.

Figure 4.7: Flow cytometry analysis of KG-1a cell surface antigen integrity at time of maximum cell concentration reached. KG-1a cells were cultured at three CO₂ levels [0.5 % (left), 5 % (middle) and 15 % (right)]. The second quadrant (Q2) in each blot shows the percentage of KG-1a cells that are CD34⁺ positive.

Figure 4.8: Proliferation curves of KG-1a cells cultured at CO₂ concentrations of 0.5 % (→), 5 % (→-) and 15 % (→- C) CO₂ over time, where y-axis and x-axis show cell concentration (cells/ml) and time (hours), respectively. Each time point represents three independent experiments ± standard deviation.
Figure 4.9: Flow cytometry analysis of FITC Annexin V (x-axis) and propidium iodide (PI) (y-axis) staining showing high CO\textsubscript{2} concentration preserves KG-1a cells from early apoptosis and delays cell death. KG-1a cells were cultured at three CO\textsubscript{2} levels [0.5 % (top row), 5 % (middle row) and 15 % (bottom row)]. Three representative time points (46 hours (left), 125 hours (middle), and 190 hours (right)) were selected to show the pattern. The quadrants in each blot show percentages of KG-1a that are alive (Q4), early apoptotic (Q3), late apoptotic (Q2) or necrotic cells (Q1).

Figure 4.10: Glucose depletion curves of KG-1a cells cultured at CO\textsubscript{2} concentrations of 0.5 % (---), 5 % (- - -) and 15 % (--- C) CO\textsubscript{2} over time, where y-axis and x-axis show glucose concentration (g/ml) and time (hours), respectively. Each time point represents three independent experiments ± standard deviation.

Figure 4.11: pH curves of KG-1a cells cultured at CO\textsubscript{2} concentrations of 0.5 % (---), 5 % (-----) and 15 % (------ C) CO\textsubscript{2} over time, where y-axis and x-axis show pH and time (hours), respectively. pH was continuously measured during the experiment and recorded every 17 minutes.
Abstract

Stem cells have more recently attracted interest in scientific research and their use is increasingly developing in many therapeutic fields and applications (6, 7), such as cell transplantation therapy (8), cardiac cell therapy (6), genetic manipulation studies (7), tissue differentiation/regeneration (9), and many more. Culturing stem cells, however, is a complex challenge particularly at the clinical scale where there are critical shortages. In this regard, the establishment of an \textit{in vitro} environment near-identical to that of the \textit{in vivo} bone marrow one is of significant interest, particularly if such system can also be readily controlled and/or optimised. The body has numerous types of cells, all growing in varying conditions of gas distribution, nutrients, blood flow, growth surface(s), pH, and temperature. For instance, hematopoietic stem cells are found in both the blood and the bone marrow; nevertheless, the microenvironment differs greatly between the two sites. Human blood has a maintained dissolved carbon dioxide concentration close to 5% (40 mmHg), which is very different from the environment surrounding hematopoietic stem cells \textit{in vivo} within niches.

A great deal of cell-based work, as evidenced in the literature, is conducted at 5% carbon dioxide concentration and is predominantly cultured on flat two-dimensional surfaces (e.g. T-flasks, well plates). These protocols and methods are straightforward, simple to handle, and of low economic costs. They however do not necessarily replicate the niche microenvironment and are limited in terms of gas exchange which is only permissible at the medium surface, and uncontrolled concentration gradients are very likely to occur in the medium itself, while experimental replications are not guaranteed. It is therefore a major challenge to replicate the real microenvironment \textit{in vitro} with new systems that control parameters such as, carbon dioxide/oxygen concentrations, nutrient levels, pH and others. Three dimensional apparatus on the other hand allow increased volume space for the cells to grow in and more adequately resemble the \textit{in vivo} environment of cells. In order for stem cells to be considered for successful therapeutic use, the \textit{in vitro} expansion of these cells must be satisfactory, and this will require 3D mimicking of the stem cell niche microenvironments, including the replication and control of carbon dioxide concentration.

The stem cell niche microenvironment variables (oxygen/carbon dioxide tension, nutrients, proteins, pH, temperature...) directly manipulate stem cell proliferation, differentiation, and
apoptotic capacity. Besides oxygen, dissolved carbon dioxide in mammalian cells is equally significant, but has been rather disregarded throughout the literature. Humidified cell incubators are generally maintained at 5% carbon dioxide in air in the vast majority of hematopoietic stem cell-related proliferation studies. Evidence to provide justification for selecting this specific concentration of carbon dioxide in hematopoietic progenitor cell proliferation studies is lacking. The literature does not currently define the precise distribution of carbon dioxide within niches, it is however established that the 5% used for typical cell proliferation is lower than that actually encircling hematopoietic stem cells in human tissue. In mammalian cells, carbon dioxide is essential but the precise role of carbon dioxide in cell culture is intricate because it can exist in many forms and can be regarded as both a nutrient and a potentially inhibitory byproduct. Hence, both build-up and excessive removal are unfavourable.

**Hypotheses**

This thesis pursues to prove the following hypotheses:

i. Hypercapnia decelerates proliferation rate but increases overall expansion of hematopoietic progenitor stem cells (KG-1a) without disturbing cell surface integrity.

ii. Apoptosis of KG-1a cells is delayed under the effect of hypercapnia in both 2D and 3D culture systems.

iii. KG-1a cells can successfully proliferate within a 3D bioreactor culture system to yield higher cell concentrations than 2D systems.

To examine the above hypotheses, the following steps were undertaken:

i. Assess the proliferation, morphology, apoptotic capacity of KG-1a cells in 2D culture systems.

ii. Research, design, and manufacture of a suitable bioreactor to carry out 3D studies in.

iii. Set up 3D culture system comprised of: online monitoring and control circuit and feeding circuit.
iv. Assess the proliferation, morphology, apoptotic capacity, cell surface antigen integrity, pH, and metabolism of KG-1a cells in 3D culture system.

**Results**

Experiment 1 revealed that both hypo- and hypercapnia had no substantial effect on hematopoietic stem cell-morphology in terms of cell shape and size. Also hypercapnia decreased proliferation rate but extended survival, where apoptosis was delayed. Cells also reached higher cell concentrations in hypercapnic conditions (15% carbon dioxide in air) in comparison to the 5% carbon dioxide control and 1% hypocapnic cells. The hypocapnic conditions (1% carbon dioxide in air), although increased the cell population doubling time, decreased the maximum cell concentration reached over time in comparison to both the control and the hypercapnic cells. Hence, having cells which cycle at a slower rate would be favourable for sustaining survival and for mimicking the *in vivo* environment where it is established that cells proliferate at a gradual pace.

In Experiment 2, results showed that both hypo- and hypercapnia had no considerable effect on cell morphology in terms of cell size. However, flow cytometry analysis revealed that hypocapnia decreased the expression of the CD34+ surface antigen, the latter being unaffected by hypercapnia with respect to the control populations. Once again, hypercapnia also yielded a higher maximum cell concentration, but this was reached later in time compared to the other populations. Proliferation was slowest in hypocapnic populations (highest population doubling time) despite these cell populations reaching maximum cell concentration in the shortest time. According to apoptosis, hypercapnia clearly delayed the progress of apoptosis in the KG-1a cells, and vice versa for the hypocapnic cells, confirming results from Experiment 1. This is also parallel to the glucose depletion studies, where the cells that cycled slower (hypercapnic) metabolized glucose at a respectively slower rate and cells that cycled faster (hypocapnic) metabolized glucose at a much faster rate. Therefore, cells which cycle at a slower rate are favourable for sustaining survival and delaying apoptosis. This also aligns with *in vivo* environment where it is well established that cells expand at gradual paces.

In comparing the hypercapnic component of Experiments 1 and 2, it can be seen that 15% carbon dioxide in air had an analogous effect on KG-1a cells regardless of the culture system (2D or
Cell size in both cultures is almost equivalent and maximum cell number at approximately the same time in both 2D and 3D cultures (168 hours and 166 hours, respectively) is comparable: $5.09 \times 10^6 \pm 3.13 \times 10^5$ and $4.18 \times 10^6 \pm 3.51 \times 10^4$, respectively. The % specific apoptosis is however lower in the 3D culture system (-0.68 vs. -0.06), accompanied by a lower population doubling time (43.3 vs. 44.5) despite the identical experimental conditions, hence a better quality of cells in the 3D populations. In other words, the lower the population doubling time, the lower the % specific apoptosis, the slower the cells proliferate, the more guarded they are against DNA damage, the less the % apoptotic capacity. This proves our hypotheses that both hypercapnia and a 3D culture system combined are more effective in producing greater cell numbers in the same amount of time in contrast to hypercapnia alone in a traditional 2D culture system.

**Conclusions**

Researchers in the stem cell culturing field have broadly overlooked carbon dioxide and focused more on the effect of oxygen. Almost all cell proliferation systems in the literature include the culturing of stem cells at 5% carbon dioxide. Despite human blood containing an analogous 5% dissolved carbon dioxide, stem cells in the bone marrow exist in a microenvironment with a higher carbon dioxide concentration. This thesis aimed at analysing and investigating the effects of hypo- and hypercapnia on the proliferation of hematopoietic progenitor stem cells (KG-1a cell line). In addition, these cells’ apoptotic capacity, morphology, surface antigen integrity, and metabolism were also examined under varying carbon dioxide conditions. This work also highlighted the significance of culturing stem cells in 3D bioreactor vessels compared to 2D ones. Once again, the 3D system is higher in similarity to the actual in vivo microenvironment of these cells.

This work analysed the effects of hypo- and hypercapnia using the cell line KG-1a to model real human hematopoietic stem cells. This is a reasonable assumption to make, given that both KG-1a and human hematopoietic stem cells express the CD34$^+$ antigen and have other analogous properties (detailed in Section 3.1). This work was successful in proving that in both 2D and 3D culture systems, hypercapnia (15% carbon dioxide in air) decelerates proliferation rate but increases overall expansion of hematopoietic progenitor stem cells (KG-1a) without disturbing cell surface integrity in comparison to hypocapnic (0.5-1% carbon dioxide in air) and control (5% carbon dioxide in air) populations. Also, we established that the apoptotic capacity of these
KG-1a cells is delayed under the effect of hypercapnia in both 2D and 3D culture systems. And finally, this work has demonstrated that KG-1a cells can successfully proliferate within a 3D culture system (bioreactor) to yield comparable cell concentrations to traditional 2D systems (culture flasks).

Since human stem cells are difficult to obtain with respect to strict ethics and policies governing their availability and use, growing and culturing stem cells in scalable quantities is therefore a huge advantage for both research and therapy. This study has succeeded in adding to the current body of knowledge that exists on hematopoietic stem cell expansion and it may potentially provide the foundation to enhancing proliferation systems of hematopoietic stem cells and therefore contribute to resolving numerous diseases.
Abbreviations

Antifoam C (AFC)  Henry’s law gas constant ($K_H$)
Bicarbonate (HCO$_3^-$)  Human leukocyte antigen (HLA)
Bone marrow (BM)  Interleukin (IL)
Bone marrow mononuclear cells (BM MNCs)  Long-term bone marrow cultivation (LTBC)
Bone marrow transplantation (BMT)  Long-term culture-initiating cells (LTC-IC)
Carbon dioxide (CO$_2$)  Low-density mononuclear cells (LDMC)
Carbonate (CO$_3^{2-}$)  Mononuclear cell (MNC)
Carbonic acid (H$_2$CO$_3$)  Nitrogen N$_2$
Chinese hamster ovary (CHO)  Oxygen (O$_2$)
Colony-forming cell (CFC)  Peripheral blood (PB)
Colony-forming unit-granulocyte macrophage (CFU-GM)  Platelet-derived growth factor (PDGF)
Colony-stimulating factor (CSF)  Population balance equations (PBE)
Cord blood (CB)  Population balance model (PBM)
Dissolved oxygen (DO)  Pressure of CO$_2$ ($P_{CO_2}$)
Embryonic stem cell (ESC)  Reactive oxygen species (ROS)
Flow cytometry (FCM)  Red blood cell (RBC)
Foetal bovine serum (FBS)  Rotating wall vessel bioreactor (RWVB)
Graft-versus-host-disease (GVHD)  Specific apoptosis (SA)
Hematopoietic stem and progenitor cell (HSPC)  Tumour infiltrating lymphocytes (TIL)
Hematopoietic stem cell (HSC)  White blood cell (WBC)
Chapter 1: An Introduction to Stem Cells

1.1 Background

The human body is an intricate system compiled of hundreds of cell types, all organized into specialized tissues, that constitute specific organs which are crucial for regular functioning of the body. Stem cells are a distinctive class of cells in that they are unspecialized, can self-renew, (10) and can differentiate to give rise to any foetal or adult cell type (muscle cells, nerve cells, fat cells and skin cells) (Figure 1.1).

Figure 1.1: Embryonic Stem Cell Differentiation into multiple cell lineages (11). ESC: embryonic stem cell
Embryonic stem cells (ESCs) are extracted from the inner cell mass of the developing mammalian blastocyst (Figure 1.2) (12, 13). These cells are also characterized by their infinite capability to self-renew while maintaining their aptitude to differentiate into all three primary germ layer cell lineages (Figure 1.3) (7, 12).

Figure 1.2: Development of the blastocyst from the zygote; PB: Polar Bodies (14).

A stem cell’s proliferative capability begins to decrease as it commits to a specific lineage through its development; at this stage, it is identified as a progenitor cell (7). These progenitor stem cells, also known as adult stem cells or tissue-specific cells, are multipotent and have a lower proliferative capability in contrast to premature stem cells; nevertheless, they are still capable of dividing into multiple cell types (7). For example, hematopoietic stem cells (HSCs) are the progenitor stem cells of all blood cells and many cells of the adult immune system (15).

Figure 1.3: Stem Cell Division and Pluripotency. Stem cells are pluripotent and can divide or differentiate into progenitor cells which are multipotent. The latter can also divide or differentiate into cells of the same tissue. Differentiated cells can divide but are incapable of differentiating into other cell types (16).
Stem cells distinctively divide asymmetrically, i.e. the parent cell divides to produce two daughter cells with separate fates: a new stem cell and a progenitor cell destined for terminal differentiation (17). Asymmetric differentiation maintains homeostasis within regenerative tissue such as the bone marrow (BM), in other words, it ensures a balanced number of stem cells is kept while differentiated progenitor stem cells are produced in parallel to meet the requirements of regular cell turnover (17).

Stem cells have more recently attracted interest in scientific research and their use is increasingly developing in many therapeutic fields and applications (6, 7). The latter include cell transplantation therapy (8), cardiac cell therapy (6), genetic manipulation studies (7), tissue differentiation/regeneration (9), and many more.

1.2 Ethics, Policies and Limitations

The employment of ESC, especially from human sources, in scientific research is currently an elevated topic in ethical and political guidelines in many countries (18). On one hand, human ESCs demonstrate great potential benefits in the research and treatment of disease; however, their exploitation remains controversial since they are initially obtained from embryos of aborted foetuses (18, 19). In Australia, using human ESCs in reproductive cloning is prohibited as stated by the Prohibition of Human Cloning Act 2002 (19). Nevertheless, The Research Involving Human Embryos Act 2002, which came into effect in June 2007, allows the use of excess human ESCs for research purposes only, given that the researcher/supervisor has obtained a license from the National Health and Medical Research Council Licensing committee (19).

Since human stem cells are difficult to obtain with respect to strict ethics and policies governing their availability and use, growing and culturing stem cells in scalable quantities is therefore a huge advantage for both research and therapy. This leads to the broad aim of this work which is to discover means of enhancing stem cell proliferation.

1.3 Hematopoietic Stem Cells and Hematopoiesis

BM is the flexible, spongy tissue found within large bones in the hollow spaces (Figure 1.4). This material is rich in blood vessels and fibres that generate white blood cells (WBCs), red
blood cells (RBCs), and platelets (20). WBCs are the principal components of the immune system, RBCs are responsible for the transport of carbon dioxide (CO₂) and oxygen (O₂) to and from the lungs to all the body’s cells, while platelets aid in preventing bleeding (20).

BM was suggested the dominant site for early hematopoiesis in adult humans over a century ago (21) and HSCs are the cells accountable for blood renewal. These cells are primarily found within the BM at very low concentrations (0.01-0.05%) and in the peripheral blood (PB) at even lower concentrations (0.001%) (22). Nonetheless, the turnover of cells in the hematopoietic system in a man weighing 70kg is close to \(10^{12}\) cells/day (23).

The HSC is possibly the most thoroughly investigated and characterised somatic stem cell and the most commonly used in research and therapeutical practices. Even so, \textit{in vitro} HSC-expansion remains somewhat a challenge which hinders their therapeutic potential.

![Bone Anatomy](image)

\textbf{Figure 1.4:} Diagram of the anatomy of the bone (24).

It was only a few decades ago that the concept of “niches” was proposed for HSCs within the complex three-dimensional BM structure (25). A niche is a specialized anatomical site, or microenvironment, fundamental for maintaining normal stem cell functions (self-renewal, differentiation, quiescence, and migration) (26). BM niches produce signals and cell adhesion
molecules that together give rise to the HSC-niche synapse (17). These signals regulate HSC dormancy, proliferation, and differentiation (17). At least two HSC BM niches have been described: endosteal niche (supports steady-state hematopoiesis and maintains HSC quiescence) and perivascular niche (allows self-renewing HSCs to access the vasculature) (27-29). Each niche provides a specialised microenvironment in which the HSCs’ pluripotency is maintained (cells self-renew) but differentiation is inhibited (17).

Table 1.1: Cytokines utilised in HSC culture ex vivo (22).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flt-3L, FL</td>
<td>Survival of HSCs</td>
<td>(30, 31)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Mobilization of HSCs to PB</td>
<td>(32)</td>
</tr>
<tr>
<td>IL-3</td>
<td>Proliferation of HSCs (with IL-6)</td>
<td>(33)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Proliferation of HSCs (with IL-3)</td>
<td>(34)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Proliferation of HSCs</td>
<td>(35)</td>
</tr>
<tr>
<td>IL-11</td>
<td>Shortens the G0 period of the cell cycle of HSCs</td>
<td>(36)</td>
</tr>
<tr>
<td>Jagged-1</td>
<td>HSC self-renewal</td>
<td>(37)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Mitogen for connective tissue cells</td>
<td>(31)</td>
</tr>
<tr>
<td>Stem cell factor</td>
<td>Growth factor for HSC progenitor cells</td>
<td>(33)</td>
</tr>
<tr>
<td>Thrombopoietin</td>
<td>Stimulator of megakaryocytopoiesis</td>
<td>(31, 36)</td>
</tr>
</tbody>
</table>

CSF: colony-stimulating factor; IL: interleukin; PDGF: platelet-derived growth factor.

Hematopoiesis within these niches is relatively complex and involves several molecular and cellular interactions. HSCs secrete glycoproteins, also known as cytokines, which induce/inhibit stem cell differentiation, apoptosis, and expansion depending on the concentrations in which they are present. It is possible to supplement cell cultures with cytokines to provide essential physical and physiological support and increase cell culture quality. Table 1.1 above summarizes a few examples of cytokines used in the ex vivo culturing of HSCs.

HSCs are also characterized by specific surface markers. Identifying and separating HSCs is solely dependent on precise surface markers which are summarized in Table 1.2 Since these
markers are not however entirely exclusive to HSCs, and in order to avoid yielding heterogeneous populations during any selection, combinations of positive and negative markers are used instead (22).

Despite the mass literature present on HSCs, nevertheless, current literature still falls short of accurately describing the composition of the HSC BM microenvironment, especially in terms of CO₂ gas distribution. Yet, in order to successfully develop rational methods for the expansion of HSCs, an understanding of this microenvironment is crucial alongside other factors discussed above (cytokines, surface markers, and others). This thesis will pursue to comprehend the significance of CO₂ in the HSC early life cycle and also the role of CO₂ in the BM on HSC proliferation/expansion, apoptosis, morphology, metabolism, and CD34⁺ integrity. This initiative is novel and has not been attempted before in the literature since CO₂ has been almost completely overlooked in this regards.

Table 1.2: Summary of human HSC markers (22).

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34⁺ or -</td>
<td>Cell–cell adhesion</td>
</tr>
<tr>
<td>Thy 1⁻ (CD90)</td>
<td>Cell–cell interaction</td>
</tr>
<tr>
<td>CD38⁻</td>
<td>Synthesizes cyclic ADP-ribose; indicates differentiation to both erythroid and myeloid progenitors</td>
</tr>
<tr>
<td>c-Kit⁺ (CD117)</td>
<td>Tyrosine kinase receptor for stem cell factor (SCF)</td>
</tr>
<tr>
<td>AC133⁻ (CD133)</td>
<td>Unknown function</td>
</tr>
<tr>
<td>lin⁻ (lineage)</td>
<td>Generic designation for several markers of blood cell lineages</td>
</tr>
</tbody>
</table>
1.4 Case Study: Hematopoietic Stem Cells and Leukaemia

HSCs are, at present, one of the main stem cell classes used clinically. They are used for the treatment of immunodeficiency and hematopoietic-related disorders, such as leukaemia (15, 38). Leukaemia is often described as a ‘liquid cancer’. This means the disease develops from cells within the BM, the bloodstream, and/or the lymphatic system (39, 40). Immature WBCs proliferate uncontrollably inside the body of a leukaemia patient and out crowd the BM and enter the bloodstream and lymph system (Figure 1.5) (39, 41). These abnormal WBCs do not carry out the tasks of normal WBCs, they grow faster than all other cells, and they do not die when they should (i.e. irregular apoptosis) (39, 40). A person with leukaemia may develop several adverse affects, some of which include infection, anaemia, bleeding, bruising, decreased levels of immunity, and in severe cases death (40, 41).

In the mid 1860s, arsenicals were first utilised for the treatment of leukaemia up until the 1940s when work begun on the development of folate and its antagonists. Between these two periods, no progress was made for acute leukaemia and only little expectation for cure was given to patients. Over the past 20 years, and despite the fact that no significant new chemotherapy agents have been introduced, 75-80% overall cure rates are now reported (42). The biotechnology revolution between the ‘70s and the ‘90s opened many doors for bone marrow transplantation (BMT) practitioners in health care by the end of the 20th century (Table 1.3).
Table 1.3: Timeline of events for BMT (43).

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1892</td>
<td>Marrow is given as an extract for the treatment of anaemia and tuberculosis</td>
</tr>
<tr>
<td>1900</td>
<td>Successful red blood cell transfusion</td>
</tr>
<tr>
<td>1930</td>
<td>Discovery of human blood groups</td>
</tr>
<tr>
<td>1940</td>
<td>Intravenous-administration of BM</td>
</tr>
<tr>
<td>1945-1965</td>
<td>ABO blood grouping, understanding of B and T cells and antigens</td>
</tr>
<tr>
<td>1950</td>
<td>Successful cryopreservation of BM</td>
</tr>
<tr>
<td>1959</td>
<td>First reports of BMT for solid tumours</td>
</tr>
<tr>
<td>1965</td>
<td>Importance of HLA discovered</td>
</tr>
<tr>
<td>1968</td>
<td>First reports of BMT in children</td>
</tr>
<tr>
<td>1980</td>
<td>Advances in marrow purging, GVHD management, peripheral stem cell harvesting</td>
</tr>
<tr>
<td>1990s</td>
<td>Better understanding of HLA matching, peripheral stem cell transplants</td>
</tr>
</tbody>
</table>

HLA: human leukocyte antigen; GVHD: graft versus host disease

The use of blood and BM HSC for treatment of disease dates back to the early 1920s; thus, progress in research over the years has led to the innovation of clinical blood and BMT (44). BMT is an imperative therapy for thousands of people who would otherwise die of their disease, and leukaemia patients are very often directed towards this option (43). It can be split into three categories based on the nature of the donor which can be the patient him/herself, an identical twin, or another person (autologous, sygeneic, and allogeneic, respectively). The most critical problem today in curing leukaemia is the enormous shortage in the number of donors. Only 30-40% of patients have an HLA-matched sibling/parent/relative, and the odds of acquiring HLA-compatible marrow from an unrelated donor are no doubt very slim (20).

Therefore, the aim of this project was to work towards resolving the major obstacle that leukaemia patient still face and that is the difficulty in obtaining a healthy and well-matched BM
sample for transplantation after chemotherapy and/or radiation. The proposed idea is to find ways to expand an initial small number of the patient’s very own healthy cells into maximum numbers, in minimum time for autologous transplantation.

To do so, we must start off by better understanding the HSC-niche microenvironments and discover successful methods of expansion that will meet therapeutic requirements. This is the broad aim of this thesis where hematopoietic progenitor stem cells are being cultured under varying CO₂ conditions and in 3D bioreactor environments in aim of mimicking their actual in vivo microenvironments and maximizing their proliferation. This also demands continuous evolution of bioreactor technology for efficient and rapid expansion of cell lines utilising the patient’s own cells as starter cultures. We have chosen to focus on CO₂ since it has been somewhat disregarded throughout the literature and we firmly believe that this gas plays an integral role in the HSC niche. The next section will provide the reader with an introduction to CO₂ in mammalian cell systems and the gas’ role in the apoptotic cascade, while the influence of varying CO₂ concentrations on the proliferation of these cells in the literature will be presented in Sections 2.1 and 2.2. The background to bioreactor technology and its associated operational parameters in stem cell culturing are reviewed and discussed in Sections 2.4 and 2.5.

1.5 The Role of Carbon Dioxide in Cell Culture and in Apoptosis

In mammalian cells, CO₂ is essential for the synthesis of pyrimidines, purines, and fatty acids (45). It is also the end product of aerobic metabolism in cells from which it then diffuses into the tissue spaces and then into the capillaries. All living cells hence require CO₂ for proliferation. If the partial pressure of CO₂ (Pco₂) increases over a certain limit, the metabolic activity of cells will be suppressed. Numerous suggestions have been put forward to explain this proliferation inhibitory effect of CO₂: (a) CO₂ initiates a decrease in the intracellular pH, (b) enzymatically catalysed reactions and enzyme synthesis are inhibited or stimulated, (c) the effect that CO₂ may have on the cell membrane may indirectly affect proliferation inhibition (46). Nevertheless, it is most likely that two major factors are involved in this phenomenon: (i) the modification of the cell membrane and/or the function of membrane proteins due to both aqueous CO₂ and HCO₃⁻, and (ii) alteration of cytoplasmic enzyme behaviour (46).
The precise role of \( \text{CO}_2 \) in cellular niches is intricate because it can exist in many forms \{\text{CO}_2, \text{bicarbonate} (\text{HCO}_3^-), \text{carbonic acid} (\text{H}_2\text{CO}_3), \text{or carbonate} (\text{CO}_3^{2-})\} \) and can be regarded as both a nutrient and a potentially inhibitory byproduct. Because \( \text{CO}_2 \) is a non-polar molecule, it can easily diffuse across cellular membranes and reduce intracellular pH. It has thus been shown that elevated \( P_{\text{CO}_2} \) levels are detrimental to mammalian cells and can inhibit many key processes, such as proliferation, nutrient utilization, recombinant protein production, and protein glycosylation (47-55). Hence, both build-up and excessive removal are unfavourable (56).

Humidified cell incubators are typically controlled at 5\% \( \text{CO}_2 \) levels in air. This concentration of \( \text{CO}_2 \) ensures that the buffer within the cell medium works as intended to maintain a somewhat neutral pH. This is a close mimic of the actual \textit{in vivo} system; our blood contains dissolved \( \text{CO}_2 \) at a concentration close to 5\%, the majority in the form of \( \text{HCO}_3^- \) which acts as a pH buffer to permit gas, nutrient and metabolite fluctuations without causing significant pH variations. However, the concentration of 5\% dissolved \( \text{CO}_2 \) in cell cultures will alter accordingly based on the cellular release of \( \text{CO}_2 \), lactate, and other byproducts during proliferation/metabolism. The result is excess \( \text{CO}_2 \) forced into or out of the atmosphere to maintain a balance between the two systems via what is known as Henry’s Law.

Henry’s law (Equation 1) states that “at a \textit{constant} temperature, the amount of a given gas that dissolves in a given type and volume of liquid is directly proportional to the partial pressure of that gas in equilibrium with that liquid”; in other words, the solubility of a gas in a liquid is directly proportional to the partial pressure of that gas above the liquid.

\[
p = K_H c \tag{1} \quad \text{Equation 1: Henry’s Law}
\]

Where \( p \) is partial pressure of the solute in the gas above the liquid, \( c \) is concentration of the solute, and \( K_H \) is Henry’s law gas constant. \( K_H \) for \( \text{CO}_2 \) = 29.41 L.atm/mol.

In closed static cell culture systems, the cells proliferate and simultaneously produce \( \text{CO}_2 \) which builds up in the headspace. According to Henry’s law, this prevents the gas from diffusing out of the medium, resulting in poorly dissociated \( \text{HCO}_3^- \) which generates an excess of hydrogen ions, in turn, decreasing overall pH. The solution to this is a very large headspace (at least tenfold greater than the medium volume) which is easily achievable in small scale cultures, but becomes near impossible as experiments are scaled up. Another solution is a system with continuous air
flow in and out of the culture vessel via filters which is not very effective for static 2D flasks. The more practical solution is for the cells to be cultured in stirred suspension in 3D bioreactors with continuous flow of air in and out of the vessel, accompanied by monitoring and control of dissolved gas concentrations within the cell medium.

The stem cell niche (O2/CO2 tension, pH, temperature...) directly manipulates stem cell proliferation, differentiation, and apoptotic capacity (57). Apoptosis was first described over 40 years ago as genetically programmed cell death. When a mammalian cell undergoes necrosis, it dies inadvertently due to injury and deteriorates uncontrollably by swelling and bursting coupled with the initiation of an inflammatory response in the surrounding tissue (58). Unlike necrosis, apoptosis is induced by specific signals (e.g. heat shocks, drugs, infection, toxins) activating the cell’s genetic control machinery via cell surface death receptors (58). This is then followed by a cascade of intracellular responses which remain only partially understood: signal transduction $\rightarrow$ transcription factor activation $\rightarrow$ stimulation of apoptosis-associated genes (58). The process then terminates in cell destruction and DNA degradation.

The mitochondrial membrane permeability is deemed accountable for the activation of the apoptosis cascade; the stability of this membrane prevents it (59). In cell cultures, elevated CO2 concentrations (i.e. higher than 5% in air) acidify cell medium, and acidic pH values stabilise the mitochondrial membrane (59, 60); hence, it can be hypothesised that increased CO2 levels may deter apoptosis in this way. Moreover, reactive oxygen species (ROS) are reported to be the main stimulants of apoptosis (61). Increased levels of CO2 decrease cellular consumption of O2 which consequently reduces ROS production independently of medium pH (59, 62), once again delaying apoptosis (59).

Therefore, CO2 can be regarded as both an essential gas and toxin to stem cells. Wider investigations need to be undertaken in this field in order to acquire a clearer picture of the level of the necessity/toxicity of CO2 to stem cells, especially within the BM. Throughout this project, the extent of the influence of CO2, both hypo- and hypercapnia (decreased and increased levels of CO2, respectively), on hematopoietic progenitor stem cell apoptosis was explored. It was hypothesised that hypercapnia delays apoptosis while hypocapnia accelerates it.
1.6 Hypotheses and Objectives

In Summary, this dissertation explores the following hypotheses:

i. Hypercapnia decelerates proliferation rate but increases overall expansion of hematopoietic progenitor stem cells (KG-1a) without disturbing cell surface integrity.

ii. Apoptosis of KG-1a cells is delayed under the effect of hypercapnia in both 2D and 3D culture systems.

iii. KG-1a cells can successfully proliferate within a 3D culture system (bioreactor) to yield higher cell concentrations than 2D systems.

To examine the above hypotheses, the following steps were undertaken:

i. Assess the proliferation, morphology, apoptotic capacity of KG-1a cells in 2D culture systems.

ii. Research, design, and manufacture of a suitable bioreactor to carry out 3D studies in.

iii. Set up 3D culture system comprised of: online monitoring and control circuit and feeding circuit.

iv. Assess the proliferation, morphology, apoptotic capacity, cell surface antigen integrity, pH, and metabolism of KG-1a cells in 3D culture system.

1.7 Thesis Outline

This dissertation presents 6 chapters. Chapter 1 will introduce the reader to stem cells and their current use in research/therapy with specific focus on hematopoietic stem cells and their role towards the treatment of leukaemia. This chapter will also emphasise the importance of carbon dioxide in mammalian cell culture, particularly in the apoptosis cascade. Chapter 2 will review the role of carbon dioxide in the hematopoietic stem cell microenvironment and the expansion of mammalian stem cells in hypo- and/or hypercapnic conditions. It will also review in detail conventional 2D culture methods versus 3D bioreactor culture systems and related configurations and parameters, with specific concentration on various bioreactor systems used in
the literature for the expansion of hematopoietic stem cells. Chapter 3 will discuss experimental materials and methods in detail. The experimental work conducted will be portrayed and results discussed in Chapters 4 and 5, respectively, then in Chapter 6 relevant conclusions and future recommendations will be made. This thesis will also provide a concise literature review on the culturing of hematopoietic stem cells in model-based controlled 3D culture systems. Since this review is slightly beyond the main scope of our work, it will be presented at the end of the dissertation for the reader’s expediency. The purpose of the latter review is to provide the reader with an indication of where the literature currently stands with respect to the expansion of hematopoietic stem cells in controlled 3D systems, which will, in turn, give insight into the future potential(s) of our own work presented herein.
Chapter 2: Literature Review

Culturing stem cells is a complex challenge with regards to establishing an *in vitro* environment near-identical to that of the *in vivo* one of the cells of interest in terms of culture parameters. The body has several types of cells, all growing in varying conditions in terms of gas distribution, nutrients, blood flow, growth surface(s), pH, and temperature. For instance, HSCs/RBCs are found in both the blood and the BM; nevertheless, the microenvironment differs greatly between the two sites. Human blood has a maintained dissolved CO₂ concentration close to 5% (40 mmHg), which is very different from the environment surrounding blood progenitor stem cells (HSCs) *in vivo* within BM niches (3, 4). In order for stem cells to be considered for successful therapeutic use, the *in vitro* expansion of these cells must be satisfactory, and this will require 3D mimicking of the stem cell-niche microenvironments.

Of these numerous physio- and biochemical parameters which manipulate and/or regulate cell proliferation, dissolved O₂ (DO) is regarded the most significant of the cellular microenvironment (63-65) and is thus the most examined throughout. The literature broadly agrees that since a hypoxic microenvironment is maintained within human BM with O₂ tension lying between 1 and 6%, hypoxic conditions (O₂ concentrations of approximately 2-5%) are thus optimal in order to maintain cell pluripotency, whereas atmospheric O₂ levels (approximately 20%) enhance proliferation and division (66).

Besides O₂, dissolved CO₂ in mammalian cells is equally significant, but has been rather disregarded throughout the literature. Humidified cell incubators are generally maintained at 5%
CO₂ in air in the vast majority of HSC-related proliferation studies. Evidence to provide justification for selecting this specific concentration of CO₂ in hematopoietic progenitor cell proliferation studies is lacking. The literature does not currently define the precise distribution of CO₂ within BM niches, it is however established that the 5% used for typical cell proliferation is lower than that actually encircling BM HSCs in human tissue (3, 5).

2.1 The role of CO₂ in the HSC 3D Microenvironment

Each cell class exists within a unique environment which provides the cell with optimal conditions for optimal cell proliferation and expansion, thus all cells respond and behave differently to different surroundings. Therefore, in order to successfully optimise the expansion of HSCs for these reasons discussed, it is first essential to comprehend the actual in vivo environment of these cells. The design of this defined cell expansion protocol must take into account several parameters, such as media composition, sheer stress, pressure, temperature, and dissolved gas composition. Herein, this review will be specifically focusing on the role of CO₂ in the HSC-microenvironment.

As suggested over a century ago, the BM is the chief site of hematopoiesis in human adults (21); however, more recently, it was suggested that the BM itself is a more complex 3D structure comprising of subordinate sections known as “niches” (25). These niches are distinct subdivisions that are crucial for the maintenance of normal HSC-operations, such as self-renewal, differentiation, quiescence, and migration (26). Two examples of niches identified within the BM include: (i) the endosteal niche, which maintains hematopoiesis and quiescence, and (ii) the perivascular niche, which permits HSC-migration to the vasculature (27-29).

The vast majority of HSC-related studies report culturing these cells in 5% CO₂ concentrations in air. This particular concentration of CO₂ sustains the media by maintaining the buffer within a rather neutral pH range. Nevertheless, limited verification exists to justify this CO₂ concentration for culturing BM progenitor cells. Physiologically dissolved CO₂ is typically equivalent to a $P_{\text{CO}_2}$ of 50-70 mmHg (5% dissolved) in human blood at 37°C, but this value differs significantly to that of HSC-niches within the BM. Current literature only vaguely describes CO₂ distribution in
the BM niches, it is however well established that the 5% CO\textsubscript{2} depended in standard cell expansion studies is \textit{lower} than that surrounding blood progenitor stem cells in BM niches (3, 5).

In summary, cell proliferation is dependent upon numerous factors, more critically O\textsubscript{2} and CO\textsubscript{2}. Varying these parameters generates variations within the overall cellular system. These variations can then be consequently used to optimise cell proliferation \textit{in vitro} (63-65). Ideal values of CO\textsubscript{2} are essential in order to minimize process and inconsistency and maximize efficiency. This strategy must consist of precise monitoring and accurate control of the overall system to achieve process standardization.

2.2 The Expansion of Mammalian Stem Cells in Hypo- or Hypercapnic Conditions

The literature has witnessed the optimization of stem cell expansion in terms of suitable DO concentrations on a much wider scale than that of dissolved CO\textsubscript{2}. A hypoxic environment is maintained within the BM where DO concentrations lie between 1 and 6% (2, 3). Generally, hypoxic environments (DO concentrations of 2-5%) have been shown to retain stem cell pluripotency, while normoxic conditions (DO concentration of about 20%) encourage mature cell proliferation and proliferation (66). Cell cultivation in normoxic/hyperoxic conditions would hence prompt cells to differentiate, i.e. to lose pluripotency, and cell viability would decrease simultaneously due to oxidative stress (63). Hence, decreased O\textsubscript{2} levels are favoured not only for the maintenance of cell integrity, but also because this is a closer mimic of the almost anaerobic \textit{in vivo} environment surrounding BM progenitor cells.

Carbon dioxide is itself a toxic gas and has been shown in the literature to have inhibitory effects on cell cultures. At higher concentrations of dissolved CO\textsubscript{2}, combined with higher partial pressures, and lower temperatures, CO\textsubscript{2} equilibrium is shifted towards the aqueous phase (67). As mentioned, pH within cell culture medium is directly related to the concentrations of dissolved CO\textsubscript{2} and lactate. Both CO\textsubscript{2} and lactate, which are produced via cellular respiration, are present in equilibrium with water in the form of carbonic and lactic acids. Elevated pH (greater than 7.0-7.4) levels were found to induce megakaryocytic and erythroid differentiation. On the other hand, decreased pH levels have been illustrated to favour granulocyte proliferation and
differentiation (68, 69). Parolini et al. revealed that pH levels lower than 6.4 cause a sharp decrease in cell survival (70).

Dissolved CO$_2$ in mammalian cells is crucial for steady cell proliferation. It is essential for pyrimidine, purine, and fatty acid synthesis (45). CO$_2$ is also the end product of cellular aerobic metabolism from which it then diffuses into the tissue and finally into the capillaries. However, the exact purpose that intracellular CO$_2$ serves is much more complex since it can exist in several forms: CO$_2$, HCO$_3^-$, H$_2$CO$_3$, or CO$_3^{2-}$; it can either be considered a nutrient, or a potentially inhibitory byproduct. Elevated $P_{\text{co}_2}$ levels can hinder key cellular performances, such as proliferation and expansion, nutrient consumption, recombinant protein production, and protein glycosylation (47-55). The inhibition of cell proliferation due to excessive CO$_2$ has been hypothesized to be a result of: (a) a decrease in intracellular pH, (b) inhibition or stimulation of enzymatically catalysed reactions, or (c) modification(s) in the cell membrane and/or in membrane proteins via CO$_2$ and HCO$_3^-$ (46). It can thus be concluded that both the build-up as well as excessive removal of intracellular CO$_2$ are unfavourable (56).

ROS are the main stimulators of apoptosis and are suppressed by elevated levels of CO$_2$ (61). In other words, an increase in $P_{\text{co}_2}$ will bring about apoptosis inhibition. The mitochondria play a major role in this cascade of events (59, 71); changes in the mitochondrial membrane permeability could potentially trigger apoptosis, while the stabilization of the latter could hinder it (59). Cell medium has been shown to acidify in excessive CO$_2$ (59), simultaneously, the mitochondrial membrane has been shown to stabilize in acidic conditions (60), hence it can be speculated that elevated CO$_2$ levels slow down apoptosis in this way. The literature has demonstrated that CO$_2$ acts as a weak acid to maintain intracellular pH at relatively low levels. CO$_2$ diffuses across the cell membrane, combines with intracellular water to create carbonic acid, which then dissociates into HCO$_3^-$ and hydrogen ion and consequently regulates the intracellular pH (59, 72-74).

The affinity between O$_2$ and haemoglobin is significantly influenced by CO$_2$, consequently manipulating the oxyhemoglobin dissociation curve (75). Increases in $P_{\text{co}_2}$ bring about a right-hand shift of the curve and vice versa. The latter is known as the Bohr effect and is principally the outcome of subsequent pH alterations (75). So, increases in $P_{\text{co}_2}$ lead to a decrease in the affinity between O$_2$ and haemoglobin and a decrease in pH.
Throughout the literature, humidified cell incubators are typically controlled at 5% CO\(_2\) levels in air in the vast majority of cell-culture studies. This concentration of CO\(_2\) ensures that the buffer within the medium works as intended to maintain a somewhat neutral pH. This is also a close mimic of the actual in vivo system, where human blood contains dissolved CO\(_2\) at a concentration close to 5% (40 mmHg), but is very different from the environment surrounding progenitor stem cells such as HSCs in vivo within BM niches (3, 4). Very little evidence is present to provide a rationale for choosing this concentration of CO\(_2\) for culturing progenitor cells of the BM. Oxygen concentration, on the other hand, has been researched much more than CO\(_2\) to optimise in vitro culture conditions of BM progenitor cells. It is now well known that a hypoxic environment is maintained within the BM where O\(_2\) tension ranges from 1 to 6% (2, 3). Despite current literature not accurately defining the distribution of CO\(_2\) in the BM niches, it is however well established that the CO\(_2\) concentration (5%) used in standard cell culturing practices is lower than that surrounding the BM-HSCs in the tissues (3, 5).

From Table 2.1, it is evident that a wide gap exists in the literature with respect to the proliferation of cells in hypo-/hypercapnic conditions, and an even wider gap in terms of HSC proliferation in such conditions. This area has only been narrowly investigated with only very few findings reported. It also appears that conflicting evidence is present amongst these articles, where few suggested the advantages of hypercapnia and others the disadvantages, similarly in terms of hypocapnia.

Table 2.1: Summary of the literature on hypo/hypercapnia with respect to mammalian cells.

<table>
<thead>
<tr>
<th>CO(_2)</th>
<th>Result</th>
<th>Reference/Year</th>
<th>Agrees/Disagrees with thesis hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% CO(_2)</td>
<td>Lowered pH (6.0-6.4)</td>
<td>(76)</td>
<td>Agrees</td>
</tr>
<tr>
<td></td>
<td>Erythropoiesis stimulation</td>
<td>1967</td>
<td></td>
</tr>
<tr>
<td>10% CO(_2)</td>
<td>Enhanced hamster embryo development</td>
<td>(73)</td>
<td>Agrees</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1987</td>
<td></td>
</tr>
<tr>
<td>35 mmHg pCO(_2)</td>
<td>Maximum productivity</td>
<td>(77)</td>
<td>Agrees</td>
</tr>
<tr>
<td>CO₂ pressure</td>
<td>Effect</td>
<td>Survival/Activity</td>
<td>Year</td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>-------------------</td>
<td>------</td>
</tr>
<tr>
<td>103 mmHg pCO₂</td>
<td>Productivity reduction</td>
<td>72% viability</td>
<td>1996</td>
</tr>
<tr>
<td>148 mmHg pCO₂</td>
<td>CHO cell proliferation inhibition</td>
<td>58% viability</td>
<td></td>
</tr>
<tr>
<td>70 mmHg pCO₂</td>
<td>Regulated excitatory synaptic interactions of brainstem neurons</td>
<td></td>
<td>(78) 2008</td>
</tr>
<tr>
<td>6% CO₂ in 3% O₂</td>
<td>Prevents cell damage and preserves a high number of functional HSCs and progenitors</td>
<td></td>
<td>(59) 2009</td>
</tr>
<tr>
<td>120 mmHg pCO₂</td>
<td>Inhibited epithelial cell proliferation</td>
<td></td>
<td>(79, 80) 2009/2011</td>
</tr>
<tr>
<td>CO₂-Free</td>
<td>17.25 ± 3.65-fold expansion was attained in human HSCs from umbilical CB</td>
<td></td>
<td>(81) 2011</td>
</tr>
<tr>
<td>120 mmHg pCO₂</td>
<td>Inhibited fibroblast proliferation</td>
<td></td>
<td>(80) 2011</td>
</tr>
<tr>
<td>Atmospheric CO₂ – (0.04%)</td>
<td>Reduced cell density of MSCs compared with standard 5% CO₂ cultures</td>
<td></td>
<td>(57)</td>
</tr>
<tr>
<td>1.5% CO₂</td>
<td>Enhanced expression of pluripotency and differentiation genes in mouse ESCs</td>
<td></td>
<td>(82) 2014</td>
</tr>
<tr>
<td>0.05% CO₂ in 20% O₂</td>
<td>Stem cell activity significantly decreased</td>
<td></td>
<td>(83)</td>
</tr>
<tr>
<td>9% CO₂ in 5% O₂</td>
<td>Stem cell activity maintained</td>
<td>Survival of CD34⁺ cells doubled</td>
<td>2014</td>
</tr>
</tbody>
</table>
Survival of CFCs cells doubled
Decreased apoptosis

CHO: Chinese hamster ovary; CB: cord blood; CFC: colony-forming cell

In this concise review, we have illustrated where the literature currently stands with respect to cell proliferation in varying CO₂ environments. The latter has been very thoroughly investigated in terms of O₂, but for some reason, CO₂ has been dismissed, despite being a crucial factor for cell proliferation and expansion. In future work, we expect to see more studies focusing on this topic with the aim of improving cell (specifically HSC) proliferation so as to mimic their actual *in vivo* environment.

### 2.3 Three Dimensional Culture Systems – Bioreactors

Successful stem cell engineering greatly depends on appropriate cell cultivation techniques, the essence of which is 3D culture systems, or bioreactors. Bioreactor design, management, and process have been well established over the years in engineering fields, but they are rather innovative in biotechnology and medicine. The term ‘bioreactor’ generally refers to a closed culture system that permits the control and monitoring of physiochemical and biochemical parameters that influence biological systems (84). In line with the requirement of diverse cell/tissue types, bioreactor engineering has witnessed the development of more detailed and intricate systems that meet the needs of the specific cell type and/or mimic the cell type’s distinct organ to enhance proliferation. Specifications and designs of the varying bioreactor types have been illustrated in the literature in great detail (63). Bioreactors are used extensively for the production of many practical products such as pharmaceuticals, various DNA vaccines (85), monoclonal antibodies (86), Insulin (87), antibiotics (88), ethanol (89), and citric acid (90). This is most commonly achieved via the use of biological agents, including microorganisms (bacteria, fungi, yeast), enzymes, plant and mammalian cells (91).

A great deal of cell-based work in the literature encompasses cell proliferation on flat two dimensional surfaces (e.g. T-flasks, well plates, blood bags), where the cells adhere and grow in cell clusters known as embryoid bodies (EB) in static conditions (12, 63). These methods are straightforward, simple to handle, and of low economical costs. Their drawbacks however are
that gas exchange is only permissible at the medium surface, and concentration gradients are very likely to occur in the medium itself (63). Therefore, it is almost impossible to control parameters such as O₂ levels and pH (63). Three dimensional apparatus hence allow for an increased surface area for the cells to grow in and more strongly resemble the *in vivo* environment of cells (Table 2.2) (63).

Table 2.2: Comparison between stationary and stirred cell culture methods (92).

<table>
<thead>
<tr>
<th>Parameter(s)</th>
<th>Static Culture</th>
<th>Dynamic Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient availability</td>
<td>Concentration gradients exist</td>
<td>Homogenous distribution</td>
</tr>
<tr>
<td></td>
<td>No control; gas exchange mainly occurs at medium surface</td>
<td>Homogenous distribution via sparging and agitation</td>
</tr>
<tr>
<td>O₂</td>
<td>No online monitoring system; pH gradients exist</td>
<td>Online monitoring possible; uniform pH throughout medium</td>
</tr>
<tr>
<td>pH</td>
<td>Must handle the culture; contamination</td>
<td>Sampling of the media possible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>without handling the culture directly; decreased contamination</td>
</tr>
<tr>
<td>Sampling</td>
<td>Culture must be handled during cell passage</td>
<td>Handling only occurs during inoculation and harvesting</td>
</tr>
</tbody>
</table>

Stem cell culturing in 3D agitated bioreactors is overall advantageous over standard methods of static two-dimensional (2D) culturing and have several key advantages. These include achievable high cell densities, ease of scale-up and control, homogeneity, particularly with respect to O₂ distribution, and other benefits (93). Through the stirred suspension technique, the cells are exposed to elevated and enhanced amounts of nutrients and gases. Moreover, the various physiochemical (pH, temperature, osmolarity, DO, shear stress) and biochemical (nutrients, metabolic waste products, growth factors, cytokines) parameters can also be controlled much more easily and analysed for improved results (Table 2.3) (63, 65).
Table 2.3: Critical parameters that manipulate stem cell expansion and/or differentiation in bioreactors (63).

<table>
<thead>
<tr>
<th>Variable Category</th>
<th>Examples of critical parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiochemical</td>
<td>• pH values</td>
</tr>
<tr>
<td></td>
<td>• Temperature</td>
</tr>
<tr>
<td></td>
<td>• Osmolarity (measurement of the osmotic pressure of a given growth medium) (94)</td>
</tr>
<tr>
<td></td>
<td>• DO tension</td>
</tr>
<tr>
<td></td>
<td>• Hydrodynamic shear stress</td>
</tr>
<tr>
<td>Biochemical</td>
<td>• Nutrients</td>
</tr>
<tr>
<td></td>
<td>• Metabolic byproducts (lactate and ammonia)</td>
</tr>
<tr>
<td></td>
<td>• Growth factors and Cytokines</td>
</tr>
</tbody>
</table>

A study by Cameron et al. which investigated the development of human ESC-derived EBs reported a 15-fold increase in the total number of EB-derived cells in a stirred suspension culture in contrast to just a 4-fold increase in stationary 2D cultures (65). King and Miller (93) reported expansion of mouse neural stem cells for 44 days in stirred bioreactors, and more recently Amit et al. (95) described a system supporting a suspension culture of human pluripotent stem cells where they reported stable karyotype and pluripotency even after 20 passages, and increases in cell numbers by 25-fold in just ten days (95).

One of these more advanced bioprocesses is culturing ESCs in three-dimensional (3D) stirred suspension culture, i.e. a medium-perfused bioreactor system. Stirred suspension bioreactors are capable of producing high quantities of desired mammalian cells, and when coupled with computerised online monitoring and control, the operator has increased control over several parameters. Various other bioreactor systems alongside stirred suspension are available for stem cell culturing and they are listed in the table below with their main characteristics (Table 2.4).
Table 2.4: The main characteristics of different bioreactors used for stem cell culture (63, 92).

<table>
<thead>
<tr>
<th>Bioreactor Configuration</th>
<th>Advantages and Disadvantages</th>
</tr>
</thead>
</table>
| Stirred suspension Bioreactor (96) | • Economical and easy to control  
• Homogeneous system can be achieved; minimal concentration gradients  
• Allows both suspension and adherent growth (when microcarriers are used)  
• Online monitoring and control is possible  
• Hydrodynamic shear stress due to agitation can be detrimental to cells |
| Roller bottles (97) | • Only allows anchorage-dependent cell culture  
• Low-cost solution  
• Concentration gradients are minimized, but still exist within medium |
| Wave bioreactor | • Monitoring and control solutions are widely available  
• Disposable system and easily scalable  
• Sampling, monitoring and control are not as straightforward as with other systems |
| Rotating wall vessel bioreactor | • Better stem cell expansion I shorter time period  
• Low-shear stress  
• Efficient gas transfer  
• Complex system  
• Not easily scalable  
• Build-up of toxic metabolic byproducts is minimized |
| Hollow-fibre bioreactor | • Low shear stress environment  
• Concentration gradients are formed within the system |
### Bioreactor Configuration and Parameters

Selecting optimal culture parameters for *ex vivo* expansion is a significant challenge. Generally, it involves numerous parameters that require both close examination and management to ensure optimal and accurate results of cell proliferation. Alongside suitable temperature and pH values, the proliferation of cells is dependent not only on the supply, distribution, and uptake of O\(_2\) and nutrients (glutamine, glucose, and others), but also on the production of growth modulating chemical substances. Altering these parameters creates variations within the bioreactor system that can be used to direct cell behaviour *in vitro* (63-65). Hence, strategies are essential such that process and inconsistency are minimized whereas efficiency is maximized. These strategies consist of precise monitoring and accurate control of the overall system to achieve process standardization.

The stem cell culture niche is overall very intricate and mimicking it *in vitro* involves of precise monitoring and accurate control of the overall system to achieve process standardization and to enhance stem cell proliferation. Altering these parameters creates variations within the bioreactor system that can be used to direct stem cell behaviour *in vitro* (63-65). This is discussed in more detail in the next few sections.

#### 2.4.1 Oxygen

Of the many parameters that regulate and influence ESC proliferation and/or differentiation in bioreactors, O\(_2\) tension is probably the most critical component of this microenvironment (63-65). In cell culture, O\(_2\) tension directly affects stem cell proliferation via altering the production of growth factors, surface markers, transcription factors and by also interfering in the cellular respiratory pathways (68). The literature broadly illustrates that hypoxic conditions (low O\(_2\))
concentrations of about 2-5%) are favoured in order to retain ESC pluripotency, while normoxic condition (atmospheric O₂ concentrations of about 20%) enhance the proliferation and expansion of mature cells (66). ESCs can only be obtained in relatively low numbers and hence need to be cultured and grown into large numbers in vitro; if this cultivation were to be undertaken at atmospheric O₂ levels, not only would the cells be triggered to differentiate (thus loss of pluripotency), but cell viability would also decrease as a result of oxidative stress (63). Cell proliferation is more likely maintained by decreased O₂ levels (2-5%) so as to mimic cellular in vivo environment within the BM which is almost anaerobic. Nevertheless, significantly low O₂ concentrations (0-1%) have been reported to induce a state of dormancy in cell cultures and thus halt cell expansion (66).

Optimal culture conditions that favour an increase in viable cell proliferation were investigated by dos Santos et al., who focused on the effect of low O₂ levels (2%) on human mesenchymal stem cells (MSC) proliferation kinetics and metabolism (63, 98). Their results illustrated two important points; first, that MSC expansion was more efficient at 2% O₂ levels when compared to those at normal O₂ levels (98), and that mouse neural ESCs showed higher levels of proliferation at 2-5% O₂ levels also in contrast to cells at normoxic conditions (63, 98). Stem cell proliferation is not only enhanced in hypoxic conditions, but apoptosis is also reduced (99).

In several studies on murine ESCs, O₂ tension has also been shown to have considerable effects on the differentiation of these cells (63, 99). Low O₂ levels could lead to spontaneous differentiation if it is not properly controlled (63). Bauwens and co-workers revealed that hypoxic conditions increased the yields of cardiomyocytes (12), while Dang and colleagues demonstrated that these conditions increased the yields of hematopoietic cells (63).

Oxygen partial pressure was also demonstrated in the literature to significantly affect the expansion of pluripotent cells. Serra et al. conducted an experiment whereby two bioreactors were monitored and controlled for O₂ partial pressure in parallel (100). The group controlled pO₂ at 5% and 30% air saturation in each bioreactor respectively. They found that with higher air saturation (30%), cell expansion was enhanced by day 11 to almost three times that of the bioreactor with lower air saturation (100).
According to cell differentiation with respect to O₂ levels, some debate exists in the literature. Ezashi et al. reported that hypoxic conditions of approximately 5% O₂ caused a reduction in cell differentiation in comparison to that of normoxic condition (21% O₂). However, Chen and co-workers illustrate that both concentrations of O₂ have no significant effect on the cells in terms of differentiation (101, 102).

2.4.2 Carbon Dioxide and pH
Discussed in Sections 1.5 and 2.1.

2.4.3 Metabolic Byproducts
Cell proliferation and respiration are fuelled by carbon and nitrogen sources. Glucose and glutamine are the two key sources of these substrates in cell culture (68). Nutrient and metabolite concentrations can also have unfavourable effects on the stem cell fate in the culture in terms of cell proliferation, differentiation, and/or death. ESCs require an energy source for proliferation; however, these metabolic byproducts are also produced during the culture and need to be monitored. High levels of the latter generate an acidic environment where pH decreases throughout the cell culture process (63, 68). Consequently, these unfavourable conditions could initiate cell death or a hindrance in cell proliferation (63). From this, in order to generate stem cells in decent numbers while sustaining their pluripotency, close monitoring and control of nutrient and metabolite levels is essential. Alongside monitoring, the cell culture can also be maintained by replacing, or partially replacing, the cell medium with fresh medium so as to dilute the concentration of the toxic metabolites and provide additional nutrients for the cells.

2.4.4 Sheer Stress
Another factor that is prone to have a detrimental effect on the in vitro stem cell culture is hydrodynamic sheer stress (impeller speed, diameter, geometry/position) (63). The stem cells will be grown in stirred suspension where agitation gives rise to an energy transfer from the stirring bar to the medium (63). This generates areas of strong disorder and instability within the bioreactor which, in turn, initiates localized sheer on regions such as the surface of cell aggregates, cells attached to microcarriers, or single cells in suspension, which results in cell damage/death (63). Moreover, shear stress can also be a stimulus for the differentiation of the stem cells (63). Agitation is however necessary within the cell system to allow for uniform
distribution of medium and nutrients amongst the cells and consequently a homogeneous environment (103). With an increase in revolution per minute (rpm) and impeller size, there is an increase in hydrodynamic shear stress (93); it has been reported that stem cell proliferation is impeded at rpm values of 120 and over where the cells enter necrosis (104). HSCs are relatively sensitive to shear; stirring at 60rpm can be harmful (69). A challenge we face here is finding the optimal levels of agitation and the most favourable stirring conditions within the bioreactor that the cells will need in order to grow to a maximum number while maintaining their survival and proliferative capability.

Simultaneously, the methods of pumping gases (O\textsubscript{2} or CO\textsubscript{2}) into the bioreactor must be taken into consideration; this is because forcing gas bubbles into the medium and/or vigorous stirring may also be a reason of stem cell damage/death (63). Rapid gas sparging creates turbulence with the medium itself. This not only results in hydrodynamic shear stress at the cellular membranes, but also in the physiological damage of the cell and thus the cease of its ability to function (63). Nevertheless, it has also been accounted that the insertion of probes/sensors into the medium also increases shear stress since the latter disrupt flow patterns (105).

In this project, we have chosen to specifically focus on CO\textsubscript{2}; yet, it is not sufficient to merely focus on this parameter alone, for the culture environment itself is just as significant in achieving maximum cell densities. Hence, a detailed literature review on the culturing of HSCs in bioreactors was conducted and is presented in the following sections. This was in order for us to draw a clear picture of the optimal bioreactor system for our cells in. From this literature review, an individual bioreactor was designed and manufactured to not only meet literature recommendations/achievements but to also comply with our requirements in order to confirm the potential of cell proliferation in 3D systems compared to 2D ones with the above parameters taken into consideration.

### 2.5 Bioreactor Configurations and Operations

#### 2.5.1 Brief Introduction and Scope of Review

From the literature, it can be noted that the optimization of HSC cultivation in a bioreactor has only been narrowly investigated (91). The \textit{in vitro} propagation of HSCs in bioreactors is still in
its infancy and remains largely under-developed. The development of such stable systems will potentially provide valuable means for the investigation of signal transduction mechanisms that influence hematopoiesis and hematopoietic malignancies, such as leukaemia (106). Moreover, uniform ex vivo systems will aid in the comprehension of the dynamics and mechanisms of cell differentiation and hematopoietic cell-development (106).

Growing and expanding HSCs in bioreactors was initially put forward to overcome traditional shortfalls in the standard cultivation methods using TCP-flasks. Various bioreactor designs have been conceptualised and engineered for HSC-cultivation since the 1990s (107, 108). The latter include stirred tank (109), fixed bed (110, 111), airlift (112), membrane (112), and perfusion bioreactor (113, 114). Reviews on bioreactor applications for HSC cultivation and stem cell cultivation in bioreactors can be found in the literature (23, 63, 107, 115).

The following sections provide a broad literature review on studies that have investigated the proliferation/expansion of HSCs in 3D bioreactor systems, under either uncontrolled or controlled (traditional or model-based control) conditions. Please refer to Appendix for a summary of Sections 2.6 and 2.7.

2.6 Uncontrolled HSC proliferation

The cell type required for BM transplantation differs depending on the type of application. The cells that are suggested for short-term rapid recovery of the hematopoietic system are the mature progenitors: colony-forming unit-granulocyte macrophage (CFU-GM). However, patients undertaking ablative chemotherapy and radiotherapy treatment critically require long-term reconstitution; in this case, patients depend mostly on primitive stem cells: long-term culture-initiating cells (LTC-IC) (116).

Retrospective assays are employed to quantitatively measure the adequacy of hematopoietic cells during their earlier stages of differentiation to produce certain quantities and forms of adult blood cells under maximally stimulatory conditions (117). Through these assays, several CFC types alongside more primal LTC-IC are distinguished in vitro (117, 118). LTC-IC support both human and murine hematopoietic cell differentiation, or hematopoiesis, and are deemed HSCs (119) which is why most HSC bioreactor cultivation and/or optimization studies mainly
concentrate on improving CFC and LTC-IC yields as an indication of cell expansion (118). CD34\(^+\) analysis by means of flow cytometry (FCM) is another method used to evaluate the competence of HSC, especially prior to a BM transplant (69, 118). Although these \textit{in vitro} assays may correlate with the stem cell content within a given HSC sample, they are not however competent enough to give an absolute measure of HSC content over a broad range of conditions (69).

As discussed in Chapter 1, the \textit{in vitro} culture of human hematopoietic cells is of high clinical importance and has more recently started to receive significant attention. The more popular methods utilised for the proliferation and expansion of these cells include static 2D culture methods with only fewer reports that discuss the use of 3D culture systems (109). 3D culture systems can be both static and dynamic. Bioreactor cell cultivation allows for the monitoring, control, and optimisation of the process. From the literature, it can be noted that the optimisation of HSC cultivation in a bioreactor has only been narrowly investigated (91). Furthermore, even fewer reports exist on the use of bioreactors coupled with strict control and optimisation of variables. This review will now discuss papers that have explored non-controlled and non-optimised HSC expansion in 3D bioreactor systems.

The earliest documentation of an \textit{in vitro} liquid-culture system for the cultivation of progenitor hematopoietic cells was in the very early 1970’s by Dexter \textit{et al.} (120, 121) in 2D dishes. It was not until 15 years later that an 8-12-fold expansion of murine pluripotent hematopoietic cells \textit{in vitro} was reported in four days of culture, also in 2D dish, by Iscove \textit{et al.} (122). In 1990, Naughton \textit{et al.} suggested the culturing of HSCs in a 3D culture system (suspended nylon mesh system). The group developed this intricate 3D microenvironment to support hematopoiesis over a 12-week experimental period. They found that after analysis, both human and rat BM cultures revealed multi-lineage hematologic expression and active proliferation of immature cells (123). Also in 1990, Knazek \textit{et al.} showed that human tumour infiltrating lymphocytes (TIL) could be expanded efficiently in a hollow fibre bioreactor system (Cellmax) reaching 124-1170-fold over a 14-32 culture day period with an average viability of 91%. The cell characteristics were analysed and shown to be similar to TIL grown in gas-permeable culture bags. The group estimated that this protocol would help decrease technical laboratory time expended by 80% and would also decrease incubator space required (124).
2.6.1 Stirred suspension

Stirred suspension bioreactors were first used for HSC cultivation by Sardonini and Wu (1993) (112) who analysed and compared the expansion and differentiation of low-density mononuclear cells (LDMC) obtained from human BM in static culture (T-flasks) and in various bioreactors (suspension, microcarrier, airlift, and hollow fibre). Cell cultures in all cases were carried out via the use of added cytokines. It was reported that the total cell expansion in static culture ranged between 4.4- and 32-fold. Using a normalized value of 1 for static cultures, LDMC expansion in the suspension bioreactor reached a value of 1.6 under the same conditions, which was the highest of all bioreactor types (112).

Zandstra et al. also developed a successful system for the production of human hematopoietic progenitors in stirred suspension bioreactors. The group investigated the potential of stirred suspension bioreactors to support hematopoiesis from a starting inoculum of human BM cells with growth factor supplements. Their results showed that after four weeks of stirred culture, the number of total viable cells and CFC increased by 16- and 22-fold, respectively (117). Zandstra et al. then conducted a series of experiments to investigate the parameters that may hinder the cytokine-mediated expansion of primitive HSCs in stirred suspension cultures of normal human marrow cells. First, cells were exposed to specific cytokine concentrations and the group achieved a 45-fold expansion in CFC numbers within two weeks along with a 2.5-fold expansion of LTC-IC (125). Afterwards, the cells were exposed to a 5-fold higher level of these cytokines and expansion in two weeks was enhanced to 66-fold and 9-fold of CFC and LTC-IC, respectively (125). Similarly, Kwon et al. reported a 5-fold and 4-fold increase in hematopoietic primitive progenitor cells (stem-cell-antigen-1 positive cells) and LTC-ICs, respectively, when murine HSCs were cultured in 50 ml stirred bioreactors for 14 days (119).

Collins et al. evaluated glucose and lactate metabolic rates for hematopoietic cultures of CB mononuclear cell (MNC), PB MNC, and PB CD34+ cells in spinner flasks and in T-flasks using both serum-containing and serum-free media (model-based control). It was reported that the time of maximum CFC counts in each culture directly coincided with the time of maximum glucose uptake and lactate production for a broad range of seeding densities and cytokine combinations. A two-population model was developed to illustrate lactate production for cell cultivation in both T-flasks and spinner flasks. The group concluded that this model could potentially be employed
to predict harvest time which directly correlates to maximum CFC percentage in culture (126).
After this, Collins et al. then analysed the same hematopoietic cell cultures for their proliferation and expansion in stirred bioreactors in both serum-containing and serum-free medium (traditional control). This study is the first to analyse the proliferation of hematopoietic cells in serum-free stirred bioreactors. It was reported that all cell cultures proliferated well in the spinner flasks in comparison to that in T-flasks in both medium types. The group also stated that proliferation of these hematopoietic cells in the spinner flasks was dependant on agitator design, rpm, and also seeding density, where serum-free cultures required a higher inoculum density. Finally, it was noted that serum-free spinner flask culture proliferation was comparable to that observed in serum-containing medium (127).

A study by Kim et al. developed a large-scale suspension bioreactor system for the production of clinically relevant numbers of CFU-GM from an initial small inoculum of BM aspirate (for short-term rapid recovery of hematopoiesis). The group first conducted factorial experiments with three variables to investigate the effects of three variables (initial cell density, medium exchange rate, and supplement of stroma-conditioned medium) on the expansions of total cells and CFU-GM. Highest levels of total cells and CFU-GM were observed in cultures with a high initial cell density (10^6 cells/ml), a high medium exchange rate (20-40% (v/v) per day), and supplemented with stroma-conditioned medium (116). Large-scale suspension cultures were then performed with high initial cell density and medium supplemented with growth factors. The numbers of total cells and CFU-GM expanded to an average 8- and 5-fold respectively over a 14-day period. Subsequent runs were performed with different growth factors and supplemented with stroma-conditioned medium where total cells and CFU-GM numbers increased to 3- and 17-fold over a 14-day period (116).

Xiong et al. evaluated the effects of expanding and maintaining multipotency of human umbilical CB hematopoietic progenitor cells in a novel 3D system, CultiSpher G, in stirred suspension mode in the presence of cytokines and stromal cells. At day 12, CFU-C and CD34^+ cells were 23.3-, and 9.6-fold in the 3D system, respectively, in comparison to 19.2-, and 3.4-fold in the 2D system, respectively (128).
Jing et al. examined the effects of agitation speed on the ex vivo expansion of CB HSCs in stirred flasks. The group noted significantly higher CD34+ percentages at 30 rpm in contrast to 60 and 80 rpm, 3.01 ± 0.26 versus 2.13 ± 0.24 and 1.39 ± 0.19, respectively (129).

2.6.2 Perfused Systems

Wang et al. reported the development of a novel continuous perfusion bioreactor for long-term BM cultivation (LTBC) in 1992 (130). The group first investigated the ability of the bioreactor to support LTBC at incubation temperatures of 33°C and 37°C. Highly porous collagen microspheres were introduced to the bioreactor to provide a natural extracellular matrix and an increased surface area for cell attachment, interaction, and proliferation. The bioreactor was found to successfully support hematopoiesis in vitro for at least four months with continuous output of viable hematopoietic cells and at both temperatures (130).

Koller et al. describe methods for the large-scale expansion of human hematopoietic LTC-IC and progenitor cells from BM MNCs also in a continuously perfused bioreactor system. The cells were cultured for 14 days and an overall 10- and 7.5-fold expansion was obtained for total cell number and LTC-IC, respectively (131). The group reported that overall, three billion cells were produced from the equivalent of a 10-15 ml BM aspirate (131).

Oh et al. designed experiments to identify factors that reduce the ex vivo single batch expansion of BM cells in perfusion-based bioreactor systems long-term. It was reported that harvesting half the cell population periodically can lead to extended culture periods by means of increasing the size of the proliferation surface area required (132). Based on cell expansion that could potentially be obtained from the harvested cell population, the group was able to achieve a calculated cell expansion exceeding 100-fold, and a CFU-GM expansion exceeding 30-fold over a 27-day culture period (132).

In more recent work, Schmelzer et al. developed and compared multicompartment hollow-fibre membrane-based 3D perfusion bioreactor for the long-term cultivation of human BM MNCs with and without hydroxyapatite scaffolds mimicking the in vivo bone matrix (133). The cells were analysed for gene expression, surface markers, metabolic activity, hematopoietic potential, viability, and attachment. The group found that the 3D perfusion bioreactor culture enabled long-term maintenance of primary human BM cells with the hydroxyapatite scaffolds (133).
2.6.3 Packed Bed

Wang et al. developed a LTBMC system composed of a packed-bed bioreactor and initiated with murine BM with packed, highly porous bovine collagen microspheres as the matrix support for BM cell proliferation. When compared to cultures grown in conventional 2D flasks, it was found that cultures grown in the bioreactor grew in a 3D configuration comparable to that in vivo. This group also reported that cell output from the 3D culture system at 37°C was practically equivalent to that at 33°C; also, multilineal differentiation of the HSCs was achieved through this system in the absence of growth factors (excluding those in the serum) (134). However, a drawback to the use of these collagen spheres is that the porous lattice structure which is an arrangement of pores (200-300 μm) is closer to providing a 2D local microenvironment for the cells as opposed to the 3D space assumed in the BM (135).

Mantalaris et al. developed a 3D BM culture system in which marrow cells were also cultivated in a bioreactor packed with porous microspheres. The overall system was configured to mimic the in vivo BM for the ex vivo erythropoiesis of human cells. The group reported erythroid cell production that lasted for more than 5 weeks with 60% erythroid cells in the non-adherent cell population in contrast to erythropoiesis in standard 2D flask cultures. The group also reported that microscopic examination of a thin section of the spheres showed the marrow cells populating the pores in a 3D manner with cell-cell contact, resembling interactions within the BM tissue in vivo (136).

2.6.4 Hollow Fibre

Malone et al. characterized human TILs expanded in hollow-fibre bioreactors, as an alternative to semi-permeable bags and roller bottle systems, for therapeutic use in cancer patients. The group reported that the mean number of days required to reach successful initiation for all tumour types was 29 ± 16 days, while therapeutic doses of TIL required an average of 88 ± 23 days. The overall success rate and characteristics of TIL expanded in the hollow-fibre bioreactors were proven similar to those of TIL expanded in semi-permeable plastic bags (137).

Recently, Xue et al. developed an ex vivo 3D bio-mimetic co-culture platform using a hollow fibre bioreactor for HSC expansion. The efficiency of the overall system was determined via comparison to identical data from cultures in 2D T-flasks. The group revealed that both systems
showed comparable results in terms of supporting total cell and CD34\(^+\) progenitor cell expansion, as well as cell performance; however, cells collected from the 3D systems had higher clonogenic ability (138).

### 2.6.5 Spinner Flasks

Levee et al. reported a 12- to 24-fold multilineage expansion of adult human BM cells in 16 to 19 days via the use of alginate-poly-L-lysine microencapsulation systems combined with rapid media exchange in glass spinner flasks (139). The group stated that visually identifiable colonies within the capsules were accountable for the cell expansion; further suggesting that microencapsulation of human HSCs permits the outgrowth of progenitor cells and could provide a novel culture system for the examination of HSC- proliferation and differentiation (139).

A comparative gene-expression analysis of CD34\(^+\) hematopoietic stem and progenitor cells (HSPC) grown in static (well plates) and 3D stirred (spinner flasks) culture systems was conducted by Li et al. The gene expression profiling of CD34\(^+\) HSPCs of both systems was compared using SMART-PCR and cDNA arrays in order to understand the genetic differences provoked by culture environments. Apart from acquiring higher CD34\(^+\) and CFC counts in the stirred cultures in comparison to the static ones, the group’s analyses also showed that 103 and 99 genes were significantly expressed in CD34+ cells from static and stirred systems, respectively. Similar levels of expression were found in 91 of those genes, while 12 showed differential transcription levels (140).

### 2.6.6 Rotating Wall Vessel Bioreactor (RWVB)

Kedong et al. showed that UCB-HSCs and UCB-MSCs can be successfully expanded and harvested simultaneously in a rotating wall vessel bioreactor (RWVB) in the presence of low doses of cell factors when using microencapsulated feeder cells and glass-coated styrene colpolymer microcarriers. The co-culture was also performed within well plates and spinner flasks for comparison. The fold expansion of total cell numbers in the RWVB, CFU-C, CD34\(^+\)/CD45\(^+\)/CD105\(^-\) (HSCs), CD34\(^-\)/CD45\(^-\)/CD105\(^+\) (MSCs) and after 12 days of culture were \((3.7 \pm 0.3)\)-, \((5.1 \pm 1.2)\)-, \((5.2 \pm 0.4)\)-, and \((13.9 \pm 1.2)\)-fold, respectively. The group demonstrated that these values were significantly superior to those obtained from well plates and spinner flasks (141). Kedong et al. then demonstrated that the expansion of UCB-HSCs can be
achieved at a large scale with the support of microencapsulated stromal cells using a RWVB (with traditional feedback control) (142). The microencapsulated MSCs support the expansion of HSCs through soluble growth factors, and the RWVB provides an environment that is favoured for dynamic co-cultures. The group reported the expansion of total cells, CD34+ cells and CFU-Cs at day 7 of culture to be $(107.05 \pm 6.08)$-, $(26.52 \pm 1.5)$-, and $(19.2 \pm 3.18)$-fold expansion, respectively, which were significantly higher than that observed in 2D tissue-culture plates.

Yuan et al. encapsulated human CB MNCs in alginate 3D static and RWVB systems. The cell number amplification, CD34+ expansion, and the colony forming capacity of these systems was compared to those of the standard 2D system. The group reported that the 3D alginate culture systems displayed higher efficacy in CB HSC proliferation for extended periods without loss of pluripotency (143).

2.6.7 Other

Banu et al. developed a LTBM bioreactor culture system and analysed the maintenance, preservation, and colony activity of CD34+ cells cultured for three to six weeks with various lower concentrations of cytokines than those more commonly used. Cells were grown in a novel, 3D, porous cell-growth matrix (Cellfoam™) (144). Lower cytokine concentrations were chosen in order to mimic the steady state in vivo hematopoietic environment and were found to expand certain components of the heterogeneous CD34+ hematopoietic progenitor cell population. Expansion was also noticeably higher in the 3D systems in comparison to that in the 2D plastic dishes (144).

A modern commercially available bioreactor (Dideco ‘Pluricell System’) was used by Astori et al. in recent preclinical and murine studies in which the group demonstrated an MNC and CD34+ fold expansion of $230.4 \pm 91.5$ and $21.0 \pm 11.9$, respectively, after 12 days of culture. This bioreactor is a closed system that contains an expansion chamber and uses serum-free medium and was showed improved engraftment in sublethally irradiated severe combined immunodeficient and non-obese diabetic mice models (145).
Zhou et al. developed a new-type bioreactor (Figure 2.1) which combined elements of both static and stirred culture systems and then compared HSC/Progenitor cells \textit{ex vivo} expansion in both. Proportions and fold expansion of CD34$^+$ cells in both static and cyclic systems were close. However, static displayed favourable results in total expansion of cells, while cyclic had a mean proportion of CD34$^+$CD38$^-$ four times of that in static culture. Through these results, the group demonstrated that the static culture environment more closely encouraged HSCs to differentiate into progenitor cells, while the cyclic culture system was more efficient in the expansion of primitive HSCs (146).

Higuchi et al. reported the development of a direct \textit{ex vivo} HSC expansion method which involves faster filtration of umbilical CB through polyurethane foaming membranes as opposed to conventional methods of HSC-purification (147). The group then used 3D bioreactor systems for HSC expansion and achieved increased numbers of total cells (6.6- to 45.7-fold) (147).

A simple eccentric stirred tank mini-bioreactor was characterized in terms of mixing performance and culture adequacy by Bulnes-Abundis et al. (148). The group used laser induced fluorescence experiments and computational fluid dynamics computations to determine optimal conditions required for adequate mixing (eccentricity of impeller shaft, round bottom tank,
inclination of impeller disc). Using this system, human HSCs were expanded tenfold in suspension with no pH and DO monitoring nor control (148).

2.7 Traditional Control (Feedback Control) of HSC proliferation

As outlined above, HSC have extensive potential applications in both research and medicine. The chief aspect these applications have in common is the requirement of a large population of HSCs. In order to meet this prerequisite, the following conditions must be met: (a) maximized cell expansion in minimum time, (b) straightforward cell harvest protocols, (c) controlled cell culture system, and (d) clinical/therapeutical validity.

(a) In the case of leukaemia patients, for example, patients will require the largest number of cells possible for successful stem cell transplantation. These cells must not only be patient-specific, but must also be cultured during the chemotherapy procedure (in a maximum of two weeks). Hence, cells will be required ‘on demand’ and in adequate numbers per patient (149). The cultivation vessel (bioreactor) must also accommodate for two important points: (i) a small amount of cells in the early stage of culture, (ii) a considerable increase in cell number and volume during the cultivation without disturbances to the conditions and control of the overall system (149).

(b) A facile method of harvest must be prepared when the cells are finally required for transplantation. This method must minimize both cell loss and damage in order to maximize the value of the transplant and minimize any chances of contamination. Moreover, this method must be both time and economically efficient.

(c) As we illustrated above in great detail, control and optimization of HSC-culture systems is integral bearing in mind the many parameters that directly influence the proliferation and expansion of these cells. Minor alterations in any of these parameters can lead to considerable variations in the proliferation and/or differentiation of HSCs proliferation patterns.

(d) The vast majority of HSC culture systems use either cell lines of animal origin, or animal serum. These must be taken into careful consideration when contemplating human clinical use.
This review will now summarize and discuss papers that have explored the traditional control of mammalian HSC expansion in 3D bioreactor systems.

2.7.1 Perfused Systems

Early studies conducted by Koller et al. examined the effects of reduced O₂ tension and perfusion in long-term hematopoietic cultures (150). The group revealed that reduced O₂ tension increased total cell numbers by 5-fold in CB suspension cultures. Moreover, the low O₂ levels, which more closely approximate the in vivo environment, not only favoured increases in both number and frequency of CFCs, but also enhanced the proliferation and maintenance of human stromal and progenitor cells in vitro. Finally, the group revealed that continuous medium perfusion alongside the low O₂ tension increased total cell production when compared to static cultures under ambient O₂ (150).

Palsson et al. explored the dependence of human HSC proliferation on gas phase O₂ concentration, seeding density, and time of cell harvest. The cells were cultivated in continuous perfusion bioreactor systems under specific operating condition; the group was able to achieve a population of human mononuclear BM cells which expanded up to 20- to 25-fold over a 14-day period (151).

Van Zant et al. compared the efficacy of the ex vivo expansion of UCB and mobilised PB in continuous perfusion bioreactors to produce clinically relevant numbers of stem/progenitor cells. Under ideal conditions (inoculum density, growth factor combinations, presence/absence of allogeneic stroma) total cell density, CFU-GM and LTC-IC populations in mobilised PB were expanded by approximately 50-, 80-, and 20-fold, respectively, over 14 days. After evaluating UCB in the same manner, a 40- to 60-fold expansion of CFU-GM was reported which was equivalent to a 40-fold expansion in comparison to the inoculum (152).

Sandstrom et al. explored the ex vivo expansion of PB MNCs cultured both directly and after CD34⁺ selection in both static and continuously perfused cultures. Cultures were inoculated with either MNCs or CD34⁺ cells. The group revealed that similar average total cell expansion was observed in both static and perfused cultures for both MNC and CD34⁺ cell cultures (153). However, perfusion cultures were shown to maintain higher concentrations of LTC-IC for both MNC and CD34⁺ cell cultures (153).
Meißner et al. developed and compared two continuously perfused systems for the cultivation and expansion of human hematopoietic progenitor cells: fixed bed and fluidized bed bioreactors. It was reported that the fixed bed bioreactor system was the favoured model for cultivation and expansion of these cells, where the group reported a permanent production of progenitor cells and mature blood cells and the expansion of very early progenitor cells and later progenitor cells up to 4.2-fold, 7-fold, and 1.8-fold, respectively (111). However, this system was found to be unsuccessful due to its insufficient capability of expansion of MNCs and the difficulty associated with harvesting the cells from the scaffold matrix (135).

Bachier et al. evaluated the use of ex vivo cultured progenitor cells for hematopoietic restructure following high-dose chemotherapy in breast cancer patients. Controlled perfusion bioreactors were used for the ex vivo expansion over a 12-day culture period. The inoculum of BM aspirate consisted of a total of $675–1125 \times 10^6$ MNCs; Median fold expansion of nucleated cells, CFU-GM, CD34+lin−, and LTC-IC was 4.9, 9.5, 0.42, and 0.32, respectively (154). It was shown that ex vivo expansion of progenitor cells from an initial small volume of BM aspirate in perfusion bioreactor systems permits hematopoietic reconstitution after high-dose chemotherapy (154). In a similar study, Chabannon et al. also reported a clinical pilot study whereby the feasibility and safety of producing hematopoietic progenitor cells from a small marrow sample for clinical use after high-dose chemotherapy in breast cancer patients was investigated (155). A small aspirate of BM was obtained and used to inoculate two bioreactors over a 12-day culture period. Fold expansion of total cell number and CFU-GM ranged from 3.32 to 4.94, and 0.52 to 26.6, respectively (155).

An automated continuous perfusion culture vessel (AastromReplicell) for HSC expansion was developed by Aastrom Biosciences and was used by Stiff et al. for the expansion of BM MNCs. Nineteen breast cancer patients then underwent autologous transplantation exclusively using the ex vivo expanded small aliquot of BM cells after chemotherapy. Hematopoiesis was maintained at 24 months in 18/19 of the patients, and no tumour cells were observed in any of the patients after the expansion and transplantation. Hence, the group demonstrated that is feasible to perform autotransplants solely using BM cells grown in ex vivo perfusion bioreactors starting from only a small aliquot (156).
The AastromReplicell was also used by Jaroscak et al. for the *ex vivo* expansion of UCB. The group then performed phase 1 trial augmenting conventional UCB transplants with the *ex vivo*-expanded cells. Results revealed that UCB cells could be expanded in 12 days where CFU-GM counts were maximal, but CD34⁺lin⁺ counts were poor. The expansion system was proven safe since the patients did not experience any adverse effects from the infusion of the expanded cells (157).

### 2.7.2 Stirred Suspension

Collins et al. illustrated hematopoietic cell expansion with controlled pH and DO in a stirred bioreactor. The group reported that, and through the use of a cell-dilution feeding protocol, the maximum specific O₂ consumption rate for each culture closely matched the maximum percentage of CFCs within that culture. Glycolysis was also monitored within this research via the examination of the ratio of lactate production to O₂ consumption and it was concluded that post-progenitor cells of the granulomonocytic lineage obtain most of their energy from glycolysis than do CFCs; thus monitoring metabolic parameters will allow the estimation of the percentages of both CFCs and more mature cells in culture (158).

McDowell and Papoutsakis investigated the effect of increases in agitation intensity on receptor surface content and mRNA levels in CD13 cells, and on the metabolism of HL60 cells in stirred tank bioreactors. It was concluded that agitation had a dose-dependent effect on the proliferation of HL60 cells cultured in 5% foetal bovine serum (FBS) and that HL60 metabolism was dose dependent upon the agitation rate (159). This group then went on to examine the effect of serum concentrations in cell media on the CD13 receptor surface content and mRNA levels of HL60 cultured in agitated bioreactors. Increased serum levels were found to increase CD13 receptor expression, reduce the transduction of fluid-mechanical forces, and reduced glucose consumption/lactate production rates (160). McDowell and Papoutsakis then assessed the effect of decreasing extracellular pH on the CD13 receptor content and HL60 metabolism. It was reported that reduced pH levels lead to increases in CD13 surface receptor content, decreases in HL60 average size, and lower glucose consumption/lactate production rates (161).

De León et al. designed, characterized and applied a mini-bioreactor for the culture of human hematopoietic cells from umbilical CB. The system described is composed of mini-bioreactor coupled to a data acquisition and control system which continuously monitored pH, DO and
redox potential (Figure XX). A 10-, 9.2-, and 14-fold expansion of hematopoietic progenitors CFU-GM, total CFU, and MNC, respectively, was achieved in cultures controlled at 20% DO levels. Characteristic variations of O₂ uptake rate and culture redox potential were also determined for human hematopoietic cells in batch cultures with constant and predetermined pH and DO values (109).

Figure 2.2: Schematic diagram of the minibioreactor and data acquisition and control system used for the expansion of human hematopoietic cells (109).

Ratcliffe et al. demonstrated the potential of an automated replicate suspension bioreactor system with online monitoring and control to develop a HSC proliferation and differentiation process for erythroid committed cells (162). The group reported that cell proliferation was directly related to seeding density and O₂ levels. Cells reached up to 6 population doublings over a 10-day culture period from a starting cell density of 4 × 10⁴ cells/ml, with 7.5-15% DO. The data reported demonstrated that HSCs can be cultured at a 10 ml suspension scale, which was the lowest reported to date.

2.7.3 Packed Bed

The in vitro expansion of murine BM cells in airlift packed bed bioreactor systems was investigated by Highfill et al. In this paper, the optimal inoculation density of stromal cells was first determined; these cells were then subsequently seeded with fresh BM to initiate hematopoiesis. The metabolic activity in the immobilized bioreactor was analysed by measuring
concentrations of glucose and ammonia. It was reported that the metabolic activity and cell nature in systems with non-conditioned medium, conditioned medium from non-active cultures, and conditioned medium from active cultures varied significantly (163). The group then investigated the cultivation of BM cells in a large-scale airlift packed bioreactor system with pH and DO control. The culture was monitored for HSC proliferation, glucose uptake, and lactate production. The 500-ml perfusion culture system yielded $3.6 \times 10^8$ suspended BM cells over the course of 11 weeks (164).

### 2.7.4 RWVB

Liu *et al.* showed that it is more efficient to expand UCB-HSCs and progenitors using a RWVB in contrast to standard T-flasks. Cells were cultured for eight days and media was monitored for pH and osmolality every 24 hours. At the end of the culture time, the total cell number, CD34$^+$ count, and CFU-GM count increased ($435.5 \pm 87.6$)-, ($32.7 \pm 15.6$)-, and ($21.7 \pm 4.9$)-fold, respectively, as opposed to very mild changes in counts for cells grown in the T-flasks. This group also proposed that in order for an adult weighing 80 kg to receive an UCB transplant, two feeding-protocols and four RWVBs (500 ml each) would be required to provide a sufficient number of cells in six days (165).

### 2.7.5 Hollow Fibre

Whole blood (WB) is widely used in therapy but its storage time is very limited due to the rapid deterioration of hematopoietic progenitors during liquid storage; WB can be stored refrigerated between 48-72 hours. Hence, de Kreuk *et al.* proposed a method for the preservation of whole blood in an automated closed hollow-fibre bioreactor system. The group developed an infusible-grade solution for liquid storage of WB for up to 7 days (166, 167) and was successful in maintaining unprocessed WB in a plasma filtration system without significant cell loss (168).

Housler *et al.* demonstrated a 100- and 440-fold expansion of total cells and differentiation towards the RBC lineage after seven days of culture in a small-scale (2 ml) and larger scale (8 ml) compartment bioreactor (hollow-fibre system), respectively (169). The group then used a semicontinuous production process to produce a total cell expansion of approximately 15,000-fold over a culture period of 19 days (169).
2.7.6 Other

Jelinek et al. developed four novel bioreactors for the cultivation of hematopoietic cells: two for suspension cultures (a stirred system for stroma-free suspension culture, and a small-scale spinner flask), and two for immobilized co-culture (a fixed bed bioreactor for immobilized co-culture of stromal and hematopoietic cells on porous microcarriers, and a miniaturized loop reactor) (Figure XX) (115). Both production systems portrayed in this study encompass a process control and cultivation was initiated with an inoculum of low cell density. The screening systems however did not have parameter control. The group utilised these reactors for the cultivation of cell lines and primary cells; they were successful in achieving an 18-fold expansion of MNCs and an 8-fold expansion of CFCs over a 7-day cultivation in suspension cultures. In the fixed-bed reactor, CB cells were cultivated in co-culture with a murine cell line for 14 days with supplementation of cytokines. In this system, the group attained a 100-fold expansion of MNCs and a 114-fold expansion of CFU-GM (progenitor cells) (115).

![Diagram of bioreactors](image)

**Figure 2.3:** The novel bioreactors for the cultivation of hematopoietic cells (115).

Luni et al. developed an array of bioreactors at the microlitre scale (controlled buoyancy-driven thermoconvection stirring inside wells of standard 96-well plates) for the cultivation of hHSCs. Human UC-BD CD34+ cells were cultured for 7 days at five varying stirring conditions and were then characterized in terms of proliferation, viability, and CD34+ expression to verify the biocompatibility of the system. Numerical simulations and a 3D model were developed to investigate the temperature and the buoyancy-driven thermoconvection flow in the wells (170). Luni et al. then went on to develop a stirred multi-well bioreactor for the expansion of CD34+
UCB cells in hypoxic conditions. The group investigated the dependency of the expression of stem cell markers in CD34+ cells on O_2 levels. They found that hypoxic conditions allowed the CD34+ cells to maintain higher expression of the stem cell marker c-kit (171).

Timmins et al. achieved an expansion of approximately 10^7-fold over a culture period of 21 days using a controlled wave agitated bioreactor system. The group was capable of producing over 500 units of RBCs per UCB donation using fully defined culture medium (172).

In more recent work, Csaszar et al. developed a microbead-based process control system for the monitoring and control of endogenously produced signalling factors (173). The latter was incorporated into a fed-batch bioreactor and enhanced progenitor cell outputs from human CB stem cell cultures were facilitated. The group revealed enhanced *ex vivo* proliferation of hematopoietic progenitor cells at higher input cell densities and over longer periods of culture (173).

### 2.8 Commercially Available HSC Expansion Systems and Patents

It is beyond the scope of this review to cover all commercially available bioreactor systems and impractical to cover all patents describing 3D HSC expansion in bioreactor systems. Hence, only a few commercially available systems and selected patents which have specifically focused on HSC-proliferation within the time period of 2000 to 2015 will be presented. Table 2.5 briefly describes the relevant patents. Expansion bioreactor systems have been reviewed in the literature elsewhere (174).

#### 2.8.1 Cytomatrix

Cytomatrix very recently developed the commercial-scale manufacture of HSCs. Their technology includes a bioreactor solution within a closed bioreactor system, a 3D scaffold, short nanofibres technology, and niche factors including Ficolin and Delta-1 ligand (Figure XX). The theory behind the Cytomatrix is to use blood left in the umbilical cord and placenta after birth (which is abundant in HSCs) for BMT. The novelty of this system is that it produces more stem cells, in contrast to conventional laboratory techniques which produce mature blood cells (175). In order to expand HSCs in higher stem cells counts, the HSC-niche, which naturally occurs in
the human body, must be replicated. The Cytomatrix replicates this niche for successful HSC-expansion via the bioreactor, 3D scaffold, short nanofibres and factors (175).

Figure 2.4: Commercially available bioreactor, Cytomatrix (175).

2.8.2 Rotary Cell Culture Systems (RCCS) – Synthecon

The RCCS is a 3D culture system for culturing suspension and anchorage-dependant cells. Its unique features include its ability to co-culture cells while concurrently maintaining low shear force and high mass transfer of nutrients. This system can be used for stem cell maintenance, expansion, differentiation, tissue engineering, and more (176).

2.8.3 Micro-Matrix – Applikon

The micro-Matrix combines traditional stirred tank bioreactors and typical plate/flask systems. It is an array of fully-controlled and stirred bioreactors (24 bioreactors) at the millilitre scale. This down-scaled system offers control of several parameters (pH, temperature, dO₂, gas/medium feeding) (177).

Table 2.5: List of selected patents related to HSC proliferation in 3D bioreactors (2000 – 2015).

<table>
<thead>
<tr>
<th>Title of Patent and Brief Description</th>
<th>Patent Number/ Publication Year</th>
<th>Reference</th>
</tr>
</thead>
</table>

51
Bioreactor for mammalian cell growth and maintenance

- Bioreactor for the *ex vivo* expansion of normal mammalian cells, especially the maintenance and selective growth of human stem and/or hematopoietic cells.

- Bioreactor includes a disposable, self-contained cell cassette, a system manager, an incubator unit and a processor unit.

- The cell cassette includes a substantially circular cell growth chamber defined between a substantially planar cell bed and a gas permeable, liquid impervious membrane.

![Diagram of bioreactor components]

**Bioreactor**

- Bioreactor is comprised of three carrier plates, two partition wall elements with membranes configured permeable to nutrient medium and impermeable organic material being cultivated

- Of particular interest in this is the multiplication of hematopoietic parent cells

---

US6048721A (178)

2000

US6844187B1 (179)

2005
Continuous filling automatic cell culture system

- Different cell types are simultaneously cultured while the nutrient solution and growth factor levels remain within an appropriate range, and unwanted metabolites are removed.

- The system allows variable perfusion rates and encompasses breathable, waterproof, semi-permeable membranes.

Automatic cell culturing system

- Fully closed bioreactor system with automated: perfusion, feeding, metabolite removal, and cell harvest.
Automatic candidate stem cell culturing system

- Cell culture system especially designed for *in vitro* expansion of human stem cells and/or human HSCs

- Nutrient composition is automatically controlled and adjusted within the culture media to suit the cell requirements

- The system also has an auto-excretion structure for metabolized products

Devices and methods for growing human cells

- A bioreactor comprised of a reaction chamber, cell inoculation inlet, ports for: gas exchange, filter, medium
Method and device for perfusion and culture of hematopoietic cell

- A stirred bioreactor and culture chamber linked by a pipeline
- Cells are first grown to high densities in the culture chamber and then transferred to the stirred suspension vessel

Bioreactor System for Culturing Hematopoiesis Cell in Vitro

- Bioreactor system for external culture of the candidate cell
- Includes a material make-up bottle, a gas mixture device, and a tubular reactor with rectangular sectional area, wherein the lower place of a side of the tubular reactor is equipped with the liquid inlet, the upper place of the other side is equipped with the liquid outlet, the upper bottom surface of a side near the liquid inlet is equipped with inoculating inlet, the lower surface in the reactor is equipped with rib, the gas mixture device is connected with the material make-up bottle by the pipe, the liquid inlet is communicated with the liquid outlet by the pipe and the

CN1297659C
2007
(184)

CN101130745A/B
2008/2012
(185)
Novel biology reactor

- A bioreactor comprised of a controlling box, a container and a magnetic stirrer; inlets for feeding, DO, pH and a discharge outlet.

- HSCs can be increased by more than 50 times in expansion mode within two weeks.
2.9 Literature Conclusions

The utmost challenge we as researchers are still facing in biomolecular science is accurately defining the microenvironment within BM niches in terms of: gas composition (specifically CO$_2$), 3D structure, pressure, temperature, nutrient availability, and so on. Above all, an evident gap in the literature exists in terms of the precise role CO$_2$ plays on the cellular level. Clearly, optimal cellular functions are not possible in the absence of CO$_2$, i.e. it is just as indispensable as O$_2$; nevertheless we are yet to pinpoint its exact purpose. The literature only vaguely describes a correlation between CO$_2$ and the apoptosis cascade, and no mention is given to this gas’ direct role in the proliferation of any cell type (stem cells, adult stem cells, mature cells). Hence, the next section of this thesis will describe our work towards this goal. Our aim was to better understand the role and the significance of CO$_2$ on the proliferation and death of human HSCs in vivo within the BM from our in vitro studies. We investigated the effects of hypo- and hypercapnia on human promyeloblasts in both 2D and 3D culture systems. In the above literature review, we have analysed in great detail the current standing of the literature on uncontrolled and controlled HSC-proliferation in 3D bioreactor culture systems. Considerable progress has been made on developing a successful bioreactor system for the ex vivo expansion of HSCs, but it is still in its infancy, and papers discussing the model-based control and optimization of such systems are very scarce. In this project, and based on the literature findings presented above, we have designed an individual 3D stirred suspension bioreactor. All aspects of our design are presented in Chapter 3.10.

The majority of attempts made to expand HSCs ex vivo for in vivo engraftment in patients have been unsuccessful due to generation of inadequate cell counts or improper differentiation of the starting cell population (187). HSC cultivation is a rapidly developing area in both research and medicine in regards to the numerous applications this technology can potentially offer. However, the development of many potential therapeutic applications is hindered again by the limited availability of HSCs, and also by our narrow understanding of the BM microenvironment, particularly regarding CO$_2$. Alongside the possible clinical applications of ex vivo HSC-expansion systems, other potential applications also exist. For example, these model-systems could be used as platforms for pharmaceutical drug screenings and tissue engineering, or as models for fundamental hematopoiesis research (188). With time, hematopoiesis is becoming
more and more understood, particularly over the past decade, thus allowing researchers to build and develop systems that are close reproductions of the *in vivo* microenvironment.
Chapter 3: Materials and Methods

In view of the literature reviewed above, we intended to explore the effect of CO\textsubscript{2}, hypo- and hypercapnia (decreased and increased levels of CO\textsubscript{2}, respectively) on human promyeloblasts, while concurrently investigating the significance of the nature of the culture system itself (2D versus 3D). Broadly, two experiments were conducted and are summarised below in Table 3.1.

Table 3.1: Summary of experiments conducted.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell line</strong></td>
<td>KG-1a</td>
<td>KG-1a</td>
</tr>
<tr>
<td><strong>Hypocapnia/Hypercapnia</strong></td>
<td>1% CO\textsubscript{2}/15% CO\textsubscript{2} in air</td>
<td>15% CO\textsubscript{2}, 21% O\textsubscript{2}, 64% N\textsubscript{2}</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>5% CO\textsubscript{2} in air</td>
<td>5% CO\textsubscript{2}, 21% O\textsubscript{2}, 74% N\textsubscript{2}</td>
</tr>
<tr>
<td><strong>Culture System</strong></td>
<td>2D flasks</td>
<td>3D stirred suspension bioreactor</td>
</tr>
<tr>
<td><strong>Gases Controlled</strong></td>
<td>CO\textsubscript{2}</td>
<td>CO\textsubscript{2}, O\textsubscript{2}, N\textsubscript{2}</td>
</tr>
<tr>
<td><strong>Average Starting Cell Concentration</strong></td>
<td>$2.00 \times 10^5$ cells/ml, $3.00 \times 10^5$ cells/ml, $4.00 \times 10^5$ cells/ml</td>
<td>$4.98 \times 10^5 \pm 3.24 \times 10^4$</td>
</tr>
<tr>
<td><strong>Cell Count</strong></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Morphology (size)</strong></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Morphology (surface)</strong></td>
<td>¥</td>
<td>✓</td>
</tr>
</tbody>
</table>
antigens)

<table>
<thead>
<tr>
<th>Apoptotic Profile</th>
<th>✓</th>
<th>✓</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>pH</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>PDT</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>% SA</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

*In Experiment 1, the effect of the starting cell concentration was also examined.

PDT: Population doubling time; % SA: % Specific apoptosis.

Materials, methods, and apparatus used in both experiments will be presented in the following sections in detail.

### 3.1 Cell Line – Why KG-1a?

Table 3.2: Properties of KG-1a cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37 ± 0.5 °C</td>
<td>(189-191)</td>
</tr>
<tr>
<td>Media*</td>
<td>IMDM with 10% FBS or RPMI-1640</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>(192)</td>
</tr>
<tr>
<td>pCO₂</td>
<td>0.3 mmHg</td>
<td>(192)</td>
</tr>
<tr>
<td>pO₂</td>
<td>159 mmHg</td>
<td></td>
</tr>
<tr>
<td>O₂ specific uptake</td>
<td>4.0x10⁻¹⁷ mole/cell/hour</td>
<td>(189, 190)</td>
</tr>
<tr>
<td>Doubling time</td>
<td>30 hours</td>
<td>(190, 191)</td>
</tr>
<tr>
<td>Cell Diameter</td>
<td>6-14 μm</td>
<td>(190)</td>
</tr>
</tbody>
</table>

*IMDM supplemented with 10% FBS was used in this work
KG-1a cells belong to the human acute myelogenous leukaemia cell line that was blocked at a certain stage during their maturation. Thus they are very young myeloblasts (promyeloblasts). They are completely resistant to teleocidins and to tumour-promoting phorbol diesters (193). Furthermore, this cell line is immortal and only requires straightforward standard tissue culture techniques. KG-1a cells grow in suspension and thus it has been well established that their culturing does not involve cell removal from TCP-flask-surfaces (190, 191); this is also advantageous for their culturing in the stirred suspension bioreactor.

The KG-1a cells used in this experiment were donated by the Save Sight Institute of Sydney Eye Hospital. Optimal KG-1a culturing is achieved under the following conditions (Table 3.2):

Also, the KG-1a cell-doubling time is about 30 hours, which is relatively stable and short. In suspension, the KG-1a cells appear spherical in shape, with diameters ranging from 6-14 μm.

The KG-1a cell line was chosen as a model for human HSCs in this project. The reasons being:

- Both KG-1a cells and human HSCs express the CD34\(^+\) antigen which is a characteristic feature of hematopoietic stem and progenitor cells
- Both are non-adherent and spherical in shape
- Both KG-1a cells and human HSCs have relatively low doubling times (30 and 48 hours, respectively) (194)
- Both can be maintained in IMDM with 10% FBS
- Both are cultured at 37 ± 0.5 °C
- Most importantly, in the literature, the vast majority of work describes the proliferation of both these cells using 5% CO\(_2\) in air, hence the novelty of our work which uses CO\(_2\) concentrations higher and lower than this 5%.

Hence, in view of these above analogous properties between KG-1a cells and human HSCs, we firmly believe our chosen cell line provides a decent model for our system’s future application to leukaemia.
3.1.1 Significance of CD34⁺ as a Cell Viability Marker
Over the years, numerous studies concerning the culturing, proliferation, and/or the differentiation of HSCs involve the detection/analysis of the cell surface marker – the CD34⁺ antigen. This antigen is a distinctive mark of presence and viability of human hematopoietic stem and progenitor cells (195). CD34⁺ is a transmembrane glycoprotein and is classified as a sialomucin (adhesion molecule) (195). One of the main roles of the CD34⁺ antigen is the regulation of the adhesion of HSCs to the stroma (196).

Since both KG-1a cells and human HSCs express the CD34⁺ antigen, we have chosen to analyse the presence and integrity of the CD34⁺ antigen on the surface of the KG-1a cells using flow cytometry (details below in Section 3.9.3).

3.2 Cell Handling
During cell culture/passage and cultivation, a number of potential hazards are present. The system itself may become contaminated or the culture can pose the risk of becoming a significant biological hazard (depending on the cultured species). Hence, all culture components were sterile and free of microorganisms (bacteria and fungi). This was achieved through the sterilization of all equipment via autoclaving. During cell culture, the flask/vessel must was tightly sealed with appropriate gas inlets and outlets (0.3µm filters were used for the bioreactor inlet/outlet). All cell handling/transfer was carried out within a laminar flow hood.

3.3 Cell Culture and Cryopreservation
KG-1a cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 4mM stable L-alanyl-glutamine (GlutaMAX™, Gibco), 10% FBS (Lonza) and 5 ml Pen-Strep (100 U/ml penicillin and 100 µg/ml streptomycin) (Sigma Aldrich), to constitute the complete medium. Cells were cryopreserved with complete medium supplemented with 10% DMSO (Sigma Aldrich) and were rapidly thawed, washed twice with fresh media, and plated in standard tissue culture flasks (Sigma Aldrich). The KG-1a cells were regularly maintained and passaged twice weekly before the initiation of the experiment. In experiment 1 (2D analysis) the
utilised KG-1a cells were between passage 11 and 12, and in experiment 2 (3D analysis) they were between passages 13 and 15.

### 3.4 Culture Conditions

#### 3.4.1 Two-dimensional Assay

To establish the hypo- and hypercapnia environments (1% and 15%, respectively) and the 5% standard environment in Experiment 1, the incubators’ CO$_2$ levels were reprogrammed 24 hours prior to the commencement of the experiment [(i) Binder, CB series; (ii) Sanyo, 18AIC; (iii) Sanyo, 17AIC]. The cells were then maintained for 4 passages at the designated CO$_2$ levels, where the 5% CO$_2$ was the control. Upon beginning the experimentation, cells were incubated in each incubator in triplicate at three different starting cell concentrations (2-$ \times $, 3-$ \times $, and 4-$ \times $ $10^5$ cells/ml) in a total volume of 8 ml. The media was not changed throughout the culture period. Equal samples were taken from all flasks daily for 10 days (0, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 hours) and cells were analysed for live cell count, cell size, morphological appearance, counts of cell number and viability using a cell counter (TC10™, Bio-Rad), and cell apoptosis using FCM (FACSCalibur™, BD Biosciences).

#### 3.4.2 Three-dimensional Assay

To establish the hypo- and hypercapnia environments (0.5% and 15%, respectively) and the 5% standard environment in Experiment 2, the bioreactor CO$_2$ levels were adjusted 24 hours prior to the commencement of the experiment. Antifoam C (AFC) has virtually no effect on cell proliferation and differentiation (197). In these experiments, an AFC content of 0.0125% was used and resulted in a foam-free process in the bioreactor system during the sparging of air. To allow maximized homogenous mixing, with minimal shear stress, a floating stirrer bar was used. Sparging of air is necessary to ensure adequate concentrations of all gases for cell expansion. This was done by means of a custom made dispersion ring. Pumping of air was regulated by means of gas flow controllers and regulators.

Upon beginning the experimentation, cells were incubated in the bioreactor at an average starting cell concentration of 4.98 $\times $ $10^5 \pm $ 3.24 $\times $ $10^4$ cells/ml in a total volume of 250 ml. The media was not changed throughout the culture period. Equal samples were taken from the bioreactor in
triplicate daily for 11 days (at 0, 24, 46, 70, 94, 125, 144, 166, 190, 214, 238 hours); bioreactor medium level was not controlled since only a minute sample was withdrawn daily. The cells were then analysed for: viability and cell size using a cell counter (TC10™, Bio-Rad), cell apoptosis using FCM (FACSCalibur™, BD Biosciences), and cell surface antigens using FCM (FACSDivam LSRFortessa X-20, BD Biosciences). DO, CO₂, pH, and temperature were also recorded throughout the experiment every 17 minutes.

### 3.5 Cell Morphology, Proliferation and Population Doubling Time

After exposure to varying CO₂ levels, the morphological modifications in the KG-1a stem cells were examined after 0, 72, 96, and 168 hours using Olympus CKX31/41 Fluorescence Inverted Microscope with ProRes C5 Camera. Cells were analysed for cell count and cell size using TC10™ (BioRad). A 10 µl sample of cells was mixed with 10 µl trypan blue (BioRad, 145-0021); mixture was loaded onto a slide and automatically analysed. In order to compare rates of cell proliferation at the varying CO₂ levels (1, 5, and 15) %, the population doubling time (PDT) was calculated using the following equation:

\[
PDT = \frac{t}{\log(N/No) \times 3.31}
\]

Equation 2: Population doubling time

Where \(t\) is time, \(N\) is cell concentration at time \(t\), and \(N_0\) is cell concentration at \(t = 0\) (82).

When cells reached their maximum confluency, cell concentration was validated and compared to initial seeding concentration at time zero.

### 3.6 Glucose Depletion

Cell medium was analysed for glucose content (g/l) using a bioanalyser (YSI Bioanalyzer 2700, YSI Life Sciences). Samples were prepared as per instrument instruction manual and analysed after system calibration using glucose standard.
3.7 pH

pH was continuously measured (online) during the 3D experiment using a pH sensor (Applikon) and recorded every 17 minutes using LabView software.

3.8 Statistical Analysis

Results are expressed as the mean of three independent experiments (2D) or three independent samples from the culture medium (3D). Error bars in all figures represent the standard deviation. Statistical significance was determined by the Student’s t-test for two-tailed distributions, assuming equal variances for both samples using Excel software (Microsoft Corporation). Probability values less than 0.05 were considered statistically significant.

3.9 Flow Cytometry Analysis

Many techniques are crucial for the counting and/or characterization of stem cells (e.g. grid cytometry, microscope imaging, FCM). However, it is beyond the scope of this document to provide a detailed description and review of each technique. This project will focus on cell analysis via the use of FCM and TC10 automated cell counter.

Flow cytometers are now not only utilised for research in laboratories, but they are also an essential technique in the clinical field; they have become indispensable tools in both clinical and biomedical fields (198, 199). They have numerous applications; these include all forms of cell biology, immunology, genetics, cross-examination of cell membranes, cytoplasmic and nuclear antigens, as well as the interrogation of cells, either as a whole or on the scale of cellular constituents (organelles, nuclei, DNA/RNA, chromosomes, hormones, proteins, etc…) using appropriate probes (198-200).

Also, FCMs are used nowadays for cell and chromosome sorting, single cell molecular characterization, propagation of HSCs, and purification of cells and cell fragments (Table 9) (199). In terms of cells biology, viable cells are functional cells and are capable of reproducing (199). FCMs also determine viability based on membrane integrity – certain dyes only stain dead cells, thus unstained cells are deemed viable (199).
In this study, FCM was used to determine the apoptotic capacity of the KG-1a cells and also their cell-surface antigen (CD34\(^+\)) profile.

### 3.9.1 Apoptosis

Cells were analysed for apoptotic capacity using FCM (FACSCalibur\textsuperscript{™}, BD Biosciences). Cells were prepared prior to analysis using a FITC Annexin V Detection Kit with PI (BioLegend) as per manufacturer’s instructions. Briefly, cell samples were washed twice with Cell Staining Buffer and then resuspended in Annexin V Binding Buffer before being treated with FITC Annexin V and PI solutions. Cells were then gently vortexed and incubated for 15 minutes at room temperature in the dark before FCM analysis. FlowJoX 10.0.7r2 software was then used for data analysis. The gating strategy used was as follows: all events were first displayed using Annexin V versus PI. Then a region around the double negative fraction was drawn, which was then displayed using FSC versus SSC plot. Then a tight region around the subset of cells with low FSC was created (debris). The debris gate was then inverted creating a non-debris gate, which was used on the original/total population. Finally a new plot using Annexin V versus PI was used to display ‘all cells without debris’.

### 3.9.2 Specific Apoptosis

Specific Apoptosis (SA) was calculated as previously described (201) using the following formula:

\[
\text{%SA} = \frac{100 \times (Ae - Ac)}{100 - Ac}
\]

Equation 3: \textit{Specific Apoptosis}

Where \(Ae\) is percentage of apoptotic cells in the experimental population and \(Ac\) is percentage of apoptotic cells in the control population.

The lower the \% SA of the experimental cells, the lower the apoptotic capacity with respect to the control population.

### 3.9.3 Cell-Surface Antigen Profile

Cells were also analysed for surface antigen integrity using FCM (FACSDivam LSRFortessa X-20, BD Biosciences). Cells were prepared prior to analysis using a cocktail of four fluorochrome-conjugated primary antibodies and corresponding isotype controls (Human Hematopoietic
Progenitor Cell Multi-Colour Flow Kit, R&D Systems). Briefly, cell samples were first suspended in staining buffer and then incubated with fluorochrome-conjugated antibodies or isotype controls for 30 minutes. The cells were then washed and resuspended in staining buffer before being analysed by FCM. FlowJoX 10.0.7r2 software was then used for data analysis.

### 3.10 Bioreactor System

The 3D culture system is composed of three distinct subunits which are a) bioreactor, b) monitoring and control circuit, c) feeding circuit.

#### 3.10.1 Bioreactor

Following the extensive literature review on bioreactors and bioreactor applications to HSCs (Sections 2.3 and 2.4), the stirred suspension-type bioreactor was chosen due to its many advantages over others [62]. This bioreactor provides a homogeneous environment for the cells to grow in which means equal distribution of gases, nutrients, and pH for optimal cell proliferation.

The bioreactor used in this research was specially designed and custom made by Custom-Blown Glassware Pty Ltd (Sydney, Australia) to suit the needs of our project. The related review above was also used to design all aspects of our vessel. The stirred suspension bioreactor is appropriate for our cell line which grows in suspension, not on surfaces. For warming the cell culture, a water jacket was incorporated into the design. For ease of monitoring, several ports were included into the vessel lid for sensor probes. Also, the size was large enough to obtain a satisfactory amount of cells and to allow for the immersion of both a gas dispersion ring and floating magnetic stirrer bar, but also small enough to be performed on the bench top without posing any significant hazards. Most importantly, and in light of our elaborate literature review on CO₂ above, the bioreactor was manufactured to support the influx of gases (O₂, CO₂, and N₂) via the gas dispersion ring.

Initially, the rough sketch below (Figure 3.1) was proposed.
Figure 3.1: First sketch of the bioreactor design.

This was followed by the manufacture of a sample bioreactor at a smaller scale (Figure 3.2):

Figure 3.2: Small scale sample bioreactor.

The final product is shown in the following figure (Figure 3.3):
Figure 3.3: Final bioreactor manufactured and used in experimental.

3.10.2 Monitoring and Control Circuit

This was composed of:

- DO sensor
- CO₂ sensors (dissolved and headspace)
- Temperature sensor
- pH sensor
- Data acquisition: Software program (LabView)
3.10.3 Feeding Circuit

This was composed of:

- Gas flow controllers (1 × 20 sccm (CO₂), 1 × 100 sccm (O₂), 1 × 200 sccm (N₂))
- Gas cylinders and regulators with suitable stainless steel piping
- Water chiller (set to 37 °C)
- Floating magnetic stirrer
- Gas sparger (specially designed and manufactured – stainless steel ring with 1 mm pores)

3.11 Experimental Setup

A schematic diagram of the experimental system is shown in the figure below (Figure 3.4), while table 3.3 summarises the system configurations.

Figure 3.4: Schematic diagram of the stirred suspension bioreactor system
### Table 3.3: Bioreactor System Configurations.

<table>
<thead>
<tr>
<th>Component</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel Height</td>
<td>145 mm</td>
</tr>
<tr>
<td>Vessel Diameter</td>
<td>13 mm</td>
</tr>
<tr>
<td>Total Volume</td>
<td>750 ml</td>
</tr>
<tr>
<td>Working Volume</td>
<td>250 ml</td>
</tr>
<tr>
<td>Liquid Height</td>
<td>80 mm</td>
</tr>
<tr>
<td>Ports</td>
<td></td>
</tr>
<tr>
<td>3 × diameter</td>
<td>12 mm</td>
</tr>
<tr>
<td>1 × diameter</td>
<td>8 mm</td>
</tr>
<tr>
<td>3 × diameter</td>
<td>6 mm</td>
</tr>
<tr>
<td>Stirrer Bar</td>
<td>Floating magnetic stirrer</td>
</tr>
<tr>
<td></td>
<td>54 × 28 mm</td>
</tr>
<tr>
<td>Sparger (stainless steel ring)</td>
<td></td>
</tr>
<tr>
<td>Ring Diameter</td>
<td>100 mm</td>
</tr>
<tr>
<td>Orifice Diameter</td>
<td>1 mm</td>
</tr>
<tr>
<td>Sensors</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>Applikon</td>
</tr>
<tr>
<td>DO</td>
<td>Real Time Engineering</td>
</tr>
<tr>
<td>dCO₂</td>
<td>Real Time Engineering</td>
</tr>
<tr>
<td>CO₂</td>
<td>PASPORT (PS-2110)</td>
</tr>
<tr>
<td>Flow Controllers/Software</td>
<td>Gas</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>O2 MC-200SCCM-D</td>
<td></td>
</tr>
<tr>
<td>CO2 MC-20SCCM-D</td>
<td></td>
</tr>
<tr>
<td>N2 MC-100SCCM-D</td>
<td></td>
</tr>
<tr>
<td>Software 1 LabView</td>
<td></td>
</tr>
<tr>
<td>Software 2 Flow Vision SC Software</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4: Experimental Results

4.1 Introduction

Growing and culturing HSCs and other progenitor cells in scalable quantities is potentially a huge advantage for both research and therapy. However, researchers must first determine ideal parameters that offer these cells an optimal culture environment \textit{in vitro} prior to any clinical use. A typical HSC-culture system must encompass stable and well-adjusted physiological and chemical properties, such as: pH, temperature, osmolality, proteins, growth factors, and O$_2$ and CO$_2$ concentrations so as to overall mimic the physiological \textit{in vivo} environment of the cells. This would hence ensure optimal proliferation and maintenance of the cell population \textit{in vitro}.

In view of the literature presented throughout this thesis thus far, we intended to explore the influence of varying CO$_2$ concentrations (1, 5, and 15\%) in air on promyeloblast KG-1a cells using standard 2D cell-culture techniques. The CO$_2$ concentrations exploited herein were arbitrarily chosen and not optimised experimentally, yet derived from a physiological logic. Our hypothesis is that, similarly to O$_2$, CO$_2$ also participates in key role(s) within the BM progenitor cells niches. Is it possible that either hypo- or hypercapnia is favoured for optimal progenitor cells’ (such as HSCs) proliferation? Does CO$_2$ directly affect the expansion of KG-1a cells? We established that lowered CO$_2$ levels increase proliferation rate, but reduce overall cell expansion, while also and accelerating apoptosis; on the contrary, elevated CO$_2$ levels decrease proliferation rate, and increase overall cell expansion while delaying cell apoptosis, in comparison to the standard 5\% CO$_2$ control.
4.2 Experiment 1 - Hypercapnia Slows Down Proliferation and Apoptosis of Human BM Promyeloblasts

4.2.1 Cell Morphology

The cell morphology of KG-1a cells was examined with respect to shape and size. The shape of the cells was inspected by means of an inverted microscope at 0, 72, 96, and 168 hours, respectively. The latter revealed only slight differences between the varying CO\textsubscript{2} culture conditions, but the cells retained their myeloblast appearance (Figure 4.1) and only decreased slightly in size with an increase in confluency. This was then validated by cell size quantification (Figure 4.2) where all cultures showed very similar patterns in cell size. At the initiation of the experiment, cell size was 10.56 ± 0.59 μm across all populations. Then at 96 and 192 hours, it was 8.56 ± 0.09 μm and 8.33 ± 0.16 μm, respectively (Table 4.1). This trend was also observed in the FCM analysis.

![ProRes C5 images of KG-1a cells using Olympus CKX31/41 inverted microscope (40 times magnification) at 0, 72, 96, and 168 hours.](image)

Figure 4.1: ProRes C5 images of KG-1a cells using Olympus CKX31/41 inverted microscope (40 times magnification) at 0, 72, 96, and 168 hours.
Figure 4.2: KG-1a cells’ size in μm (y-axis) that were cultured at 1% (A), 5% (B) and 15% (C) CO₂ over time in hours (x-axis) at three different starting cell concentrations (2.00 × 10⁵ cells/ml, 3.00 × 10⁵ cells/ml, 4.00 × 10⁵ cells/ml). At time = 240 hours, cells cultured at (1 and 5) % CO₂ were dead and hence cell size is zero. Each time point represents average of three independent experiments ± standard deviation.

Table 4.1: Average cell size of KG-1a cells at varying CO₂ concentrations with time.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Cell Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% CO₂</td>
</tr>
<tr>
<td>0</td>
<td>10.44 ± 1.26</td>
</tr>
<tr>
<td>96</td>
<td>8.78 ± 0.19</td>
</tr>
<tr>
<td>192</td>
<td>8.56 ± 0.19</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean of three independent experiments ± standard deviation.

4.2.2 Average Cell Population Doubling Time (PDT) and Cell Proliferation

Table 4.2 below displays the average PDT and viability of KG-1a cells grown at (1, 5, and 15) % CO₂ in air at three different starting cell concentrations. Analysis of these values showed that
KG-1a cells cultured at 5% and 15% had an average PDT of $1.22 \pm 0.11$ and $1.51 \pm 0.17$ times higher than that of cells cultured at 1% CO$_2$, respectively (Table 4.2). This indicated that cells cultured at 1% CO$_2$ proliferated at a faster rate. Average cell PDT also increased with an increase in initial cell concentration (Table 4.2).

Table 4.2: Average population doubling time (PDT) and viability of KG-1a cells grown at (1, 5, and 15) % CO$_2$ in air at three different starting cell concentrations.

<table>
<thead>
<tr>
<th>Average Starting Cell Concentration (cells/ml)</th>
<th>CO$_2$ (%)</th>
<th>Average PDT (hours)</th>
<th>Average Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2.06E+05) ± (9.23E+04)</td>
<td>1</td>
<td>21.36</td>
<td>95 ± 0.03</td>
</tr>
<tr>
<td>(1.99E+05) ± (1.44E+04)</td>
<td>5</td>
<td>28.61</td>
<td>97 ± 0.01</td>
</tr>
<tr>
<td>(2.02E+05) ± (1.38E+04)</td>
<td>15</td>
<td>33.61</td>
<td>97 ± 0.02</td>
</tr>
<tr>
<td>(3.22E+05) ± (2.02E+05)</td>
<td>1</td>
<td>25.67</td>
<td>95 ± 0.02</td>
</tr>
<tr>
<td>(3.24E+05) ± (6.12E+04)</td>
<td>5</td>
<td>28.64</td>
<td>96 ± 0.02</td>
</tr>
<tr>
<td>(3.01E+05) ± (8.21E+04)</td>
<td>15</td>
<td>33.85</td>
<td>96 ± 0.02</td>
</tr>
<tr>
<td>(4.05E+05) ± (4.04E+04)</td>
<td>1</td>
<td>27.02</td>
<td>95 ± 0.01</td>
</tr>
<tr>
<td>(3.93E+05) ± (5.51E+03)</td>
<td>5</td>
<td>32.41</td>
<td>97 ± 0.02</td>
</tr>
<tr>
<td>(4.13E+05) ± (1.50E+04)</td>
<td>15</td>
<td>44.45</td>
<td>97 ± 0.01</td>
</tr>
</tbody>
</table>

*Starting cell concentration and viability are an average of three independent experiments ± standard deviation

In exploring the effect of hypo- and hypercapnia on KG-1a cell proliferation, it can be seen that cells grown in hypercapnia (15% CO$_2$) reached maximum cell concentrations $1.39 \pm 0.22$ and $1.06 \pm 0.08$ times higher than cells cultured at 1% and 5% CO$_2$, respectively (Figure 6.3). Also, and in parallel to the average PDT data, cells cultured in hypercapnia proliferated slower and reached their maximum cell concentration after 168 hours, in contrast to the cells cultured at 1%
and 5% which reached their maximum cell numbers prior to 168 hours. In summary, cells grown in hypocapnia proliferated faster and but also underwent apoptosis faster, whereas cells grown in hypercapnia proliferated and reached higher cell numbers slower with time, but also underwent apoptosis slower. Moreover, and analogous to the average PDT figures, it can be seen that proliferation in each culture increases with an increase in initial cell concentration (Figure 4.3). This trend can also be seen in the microscope images (Figure 4.1) where cells cultured at 1 and 5% CO₂ reached much lower levels of confluency in contrast to those cultured at 15% CO₂.

![Graph showing proliferation curves of KG-1a cells cultured at CO₂ concentrations of 1% (A), 5% (B) and 15% (C) CO₂ over time.](image)

**Figure 4.3:** Proliferation curves of KG-1a cells cultured at CO₂ concentrations of 1% (A), 5% (B) and 15% (C) CO₂ over time, where y-axis and x-axis show cell concentration (cells/ml) and time (hours), respectively, each at three different starting cell concentrations (%.00 × 10⁵ cells/ml, 3.00 × 10⁵ cells/ml, 4.00 × 10⁵ cells/ml). Each time point represents three independent experiments ± standard deviation.

**4.2.3 Cell Apoptotic Profile**

FCM data of the KG-1a cells at 96 and 192 hours confirmed the proliferation studies (Figure 4.4) in that cells grown in hypercapnia undergo apoptosis much later during the batch-culture period in contrast to cells grown in hypcapnia and control conditions. The percentage of live cells (Figure 4.4 A) is significantly (0.0252) higher for cells grown in hypercapnia at 192 hours. Also, cells grown in hypcapnia are more apoptotic at 96 hours in comparison to cells grown at 5%
and 15% CO₂ (Figure 4.4 B). Once again, with an increase in initial cell concentration within each culture group, there is a decrease in PDT, and hence a decrease in the rate of undergoing apoptosis. Also, the % SA of the hypocapnic cells is higher than that of the hypercapnic cells at both 96 and 192 hours (Table 4.3).

Figure 4.4: Flow cytometry analysis of FITC Annexin V (x-axis) and propidium iodide (PI) (y-axis) staining showing high CO₂ concentration preserves KG-1a cells from early apoptosis and delays cell death. KG-1a cells were cultured at three CO₂ levels [1% (top row), 5% (middle row) and 15% (bottom row)]. Two representative time points (96 hours (left) and 192 hours (right)) were selected to show the pattern. The quadrants in each blot show percentages of KG-1a that are alive (Q4), early apoptotic (Q3), late apoptotic (Q2) or necrotic cells (Q1).
Table 4.3: Specific apoptosis (SA) of hypo- and hypercapnic populations (calculated in comparison to 5% CO$_2$ control populations) at three different starting cell concentrations.

<table>
<thead>
<tr>
<th>Average Starting Cell Concentration (cells/ml)</th>
<th>CO$_2$ (%)</th>
<th>SA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>96 hours</td>
</tr>
<tr>
<td>(2.06E+05) ± (9.23E+04)</td>
<td>1</td>
<td>2.27</td>
</tr>
<tr>
<td>(2.02E+05) ± (1.38E+04)</td>
<td>15</td>
<td>0.87</td>
</tr>
<tr>
<td>(3.22E+05) ± (2.02E+05)</td>
<td>1</td>
<td>-0.16</td>
</tr>
<tr>
<td>(3.01E+05) ± (8.21E+04)</td>
<td>15</td>
<td>-0.05</td>
</tr>
<tr>
<td>(4.05E+05) ± (4.04E+04)</td>
<td>1</td>
<td>0.36</td>
</tr>
<tr>
<td>(4.13E+05) ± (1.50E+04)</td>
<td>15</td>
<td>-0.76</td>
</tr>
</tbody>
</table>

*Starting cell concentration is an average of three independent experiments ± standard deviation.
Figure 4.5: Error bar plots showing the effect of (1, 5, and 15) % CO₂ concentrations on KG-1a cell count. The x-axis for all plots shows % CO₂, while the y-axis shows in (A) % live KG-1a cell fraction, in (B) % early apoptotic KG-1a cell fraction, in (C) % late apoptotic KG-1a cell fraction, and in (D) necrotic KG-1a cell fraction. Two representative time points [96 hours (○) and 192 hours (□)] were selected to show the pattern. Each time point represents three numbers of independent experiments ± standard deviation.
4.3 Experiment 2 - Hypercapnia Slows Down Proliferation and Apoptosis of Human BM Promyeloblasts in 3D Suspension Bioreactor

In Section 4.2, we reported that increased dissolved CO₂ concentrations of 15% (hypercapnia) in standard 2D tissue culture flasks brought about enhanced cell proliferation with respect to the 5% control, as opposed to considerably decreased proliferation in hypocapnic populations (1% CO₂) (1). We also described that hypercapnia distinctly delayed apoptosis; similarly, hypocapnia was shown to accelerate cell apoptosis. Herein, we intend to investigate the effect of varying CO₂ concentrations (0.5, 5, and 15) % in air on promyeloblast KG-1a cells in a 3D stirred suspension bioreactor. The CO₂ concentrations exploited herein were arbitrarily chosen and not optimised experimentally, yet derived from a physiological logic. Our hypothesis is that hypercapnia slows down the proliferation and apoptosis of human BM promyeloblasts and 3D culture systems are favoured over conventional 2D methods. Will hypercapnia be optimal for progenitor cell proliferation (such as HSCs)? Will CO₂ have any direct effects on KG-1a cell-surface antigens? How will the 3D culture system compare overall to the 2D one? Our results confirmed the outcomes from the previous experiment in that KG-1a cell-apoptosis was delayed in hypercapnic conditions; this was accompanied by an increase in overall cell concentration and a decrease in cell proliferation rate with respect to both the hypocapnic and control populations.

4.3.1 Cell Morphology (Size)

KG-1a cell morphology was inspected in terms of cell size by means of TC10 daily. All cultures showed similar size development patterns (Figure 4.6). At the initiation of the experiments, cell size was 13.00 ± 0.00 µm across all populations, and then at 70 and 190 hours it was 10.00 ± 0.00 µm and 8.00 ± 0.00 µm, respectively.
Figure 4.6: KG-1a cells’ size in μm (y-axis) that were cultured at 0.5 % (→), 5 % (↔) and 15 % (←) CO₂ over time in hours (x-axis). Each time point represents average of three independent experiments ± standard deviation.

### 4.3.2 Cell Morphology (Cell-Surface Antigen Integrity)

Cells were analysed for cell surface antigen integrity when the maximum cell concentration was reached: 0.5% CO₂, 5.0% CO₂, and 15.0% CO₂ at 125, 144, and 166 hours, respectively. From the FCM data of the KG-1a cells (Figure 4.7), it can be seen that both the hypercapnic and control populations revealed comparable CD34⁺ positive staining (96.6 and 96.7%, respectively). On the other hand, the hypocapnic cells were only 87.9% positive for this surface antigen.

Figure 4.7: Flow cytometry analysis of KG-1a cell surface antigen integrity at time of maximum cell concentration reached. KG-1a cells were cultured at three CO₂ levels [0.5 % (left), 5 % (middle), 15 % (right)].
and 15% (right)]. The second quadrant (Q2) in each blot shows the percentage of KG-1a cells that are CD34+ positive.

4.3.3 Average Cell Population Doubling Time (PDT) and Cell Proliferation

KG-1a cell cultures at 0.5% CO₂ had an average PDT higher than that of both 5% and 15% cultures (46.11, 42.20, and 43.28, respectively) (Table 4.4). This indicates that cells cultured in hypocapnia proliferated at a much slower rate, whereas cells cultured in control conditions (5% CO₂) expanded the fastest.

According to cell proliferation, it can be seen from Figure 4.8 that cells cultured in hypercapnia reached maximum cell concentrations 2.54 ± 0.16 and 1.03 ± 0.26 times higher than cells cultured at 0.5% and 5%, respectively. However, the three different cultures reached maximum cell concentration at varying times: 0.5% CO₂ at 125 hours, 5% CO₂ at 144 hours, and 15% CO₂ at 166 hours.

Table 4.4: Average population doubling time (PDT) and viability of KG-1a cells grown at (0.5, 5, and 15) % CO₂ in air.

<table>
<thead>
<tr>
<th>Average Starting Cell Concentration (cells/ml)</th>
<th>CO₂ (%)</th>
<th>Average PDT (hours)</th>
<th>Average Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2.06E+05) ± (9.23E+04)</td>
<td>0.5</td>
<td>46.11</td>
<td>94.67 ± 2.89</td>
</tr>
<tr>
<td>(1.99E+05) ± (1.44E+04)</td>
<td>5</td>
<td>42.20</td>
<td>93.67 ± 0.58</td>
</tr>
<tr>
<td>(2.02E+05) ± (1.38E+04)</td>
<td>15</td>
<td>43.28</td>
<td>93.33 ± 1.53</td>
</tr>
</tbody>
</table>

*Starting cell concentration and viability are an average of three independent experiments ± standard deviation.*
Figure 4.8: Proliferation curves of KG-1a cells cultured at CO₂ concentrations of 0.5 % (→), 5 % (←→) and 15 % (→ C) CO₂ over time, where y-axis and x-axis show cell concentration (cells/ml) and time (hours), respectively. Each time point represents three independent experiments ± standard deviation.

4.3.4 Cell Apoptotic Profile

FCM data of the KG-1a populations at 46, 125, and 190 hours (Figure 4.9) was analogous to the proliferation studies in that cells grown in hypocapnic conditions underwent apoptosis much earlier than the control and hypercapnic cells. The percentage of live cells in the hypercapnic cultures was higher than the control and hypocapnic cells at all three time points. Similarly, the percentage of apoptotic cells in the hypocapnic cultures was higher than the control and hypercapnic cultures at all three time points. Also, the % SA of the hypocapnic cells is higher than that of the hypercapnic cells at all three time points (Table 4.5). In summary, cells grown in hypocapnic conditions expanded much slower and reached apoptosis much faster than other populations, whereas hypercapnic cells proliferated at a medium rate and reached their maximum cell number slower with time, but also underwent apoptosis slower than all other populations.
Figure 4.9: Flow cytometry analysis of FITC Annexin V (x-axis) and propidium iodide (PI) (y-axis) staining showing high CO₂ concentration preserves KG-1a cells from early apoptosis and delays cell death. KG-1a cells were cultured at three CO₂ levels [0.5 % (top row), 5 % (middle row) and 15 % (bottom row)]. Three representative time points (46 hours (left), 125 hours (middle), and 190 hours (right)) were selected to show the pattern. The quadrants in each blot show percentages of KG-1a that are alive (Q4), early apoptotic (Q3), late apoptotic (Q2) or necrotic cells (Q1).
Table 4.5: Specific apoptosis (SA) of hypo- and hypercapnic populations (calculated in comparison to 5 % CO₂ control populations).

<table>
<thead>
<tr>
<th>Average Starting Cell Concentration (cells/ml)</th>
<th>SA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ (%)</td>
<td>46 hours</td>
</tr>
<tr>
<td>0.5</td>
<td>3.36</td>
</tr>
<tr>
<td>4.98 × 10⁵ ± 3.24 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.16</td>
</tr>
</tbody>
</table>

4.3.5 Cell Metabolism and pH

Glucose depletion studies revealed that in hypocapnia, glucose was removed from the media at a faster rate than in hypercapnia and control conditions. Similarly, hypercapnic populations metabolized glucose at a much slower rate throughout the whole experiment (Figure 4.10).

Figure 4.10: Glucose depletion curves of KG-1a cells cultured at CO₂ concentrations of 0.5 % (---), 5 % (---) and 15 % (--- C) CO₂ over time, where y-axis and x-axis show glucose concentration (g/ml) and time (hours), respectively. Each time point represents three independent experiments ± standard deviation.

Figure 4.11 confirmed that hypercapnia acidified our cell media, whereas both the control and hypocapnic populations had comparable pH throughout the experiment at levels somewhat neutral.
Figure 4.11: pH curves of KG-1a cells cultured at CO\(_2\) concentrations of 0.5 % (---), 5 % (—) and 15 % (----- C) CO\(_2\) over time, where y-axis and x-axis show pH and time (hours), respectively. pH was continuously measured during the experiment and recorded every 17 minutes.
Chapter 5: Discussions – Hypercapnia Delays the Apoptosis Cascade

5.1 Discussions

The exploitation of *in vitro* expanded cells for tissue-based engineering research and therapy is a growing treatment modality. Each class of cells in the human body grows in a specific environment which provides the cell with optimal proliferation, hence all cells behave differently. Thus, for each cell type, the most favourable *in vitro* cell expansion protocol must be well-defined. This protocol encompasses not only culture medium composition, but also includes parameters such as gas concentrations, sheer force, pressure, temperature, and system nature (2D versus 3D) (3). The specific use of HSCs for the treatment of hematopoietic-related disorders, such as leukaemia (15, 38), is a continually rising field of interest. HSCs require specific culture conditions and parameters in order to provide them with a suitable environment to consequently obtain optimal proliferation. Herein, we have worked towards defining the most favourable *in vitro* cell expansion protocol for the optimal proliferation of KG-1a cells on a larger scale within a 3D culture system. The KG-1a cell line is a human acute myelogenous leukaemia cell line that was blocked during maturation. These cells are promyeloblasts and resistant to teleocidins (193). KG-1a cells are spherical in shape and grow in suspension which makes their culturing practical in 3D suspension bioreactors (190, 191). Most significantly, KG-1a cells express the CD34⁺
surface antigen similar to human HSCs, hence facilitating their use as a useful model for research which can be applied to human HSCs in the future.

Of the many parameters that regulate and influence stem cell proliferation and/or differentiation, O₂ tension is regarded the most critical and is subsequently the most explored throughout the literature. In cell culture, O₂ tension directly affects stem cell proliferation via altering the production of growth factors, surface markers, transcription factors and by also interfering in the cellular respiratory pathways (68). The literature broadly illustrates that hypoxic conditions (low O₂ concentrations of about 2-5%) are favoured in order to retain ESC pluripotency, while normoxic conditions (atmospheric O₂ concentrations of about 20%) enhance the proliferation and expansion of mature cells (66). Stem cells can only be obtained in relatively low numbers and hence need to be cultured and grown into large numbers in vitro; if this cultivation were to be undertaken at normoxic levels, not only would the cells be triggered to differentiate (thus loss of pluripotency), but cell viability would also decrease as a result of oxidative stress (63). Cell proliferation is more likely maintained by decreased O₂ levels (2-5%) so as to mimic cellular in vivo environment within the BM which is almost anaerobic. Nevertheless, significantly low O₂ concentrations (0-1%) have been reported to induce a state of dormancy in cell cultures and thus halt cell expansion (66).

Alongside O₂, CO₂ in mammalian cells is also imperative but disregarded throughout the literature. Physiologically dissolved CO₂ is generally equivalent to a Pco₂ of 50-70 mmHg in mammalian cells at 37°C (202) and altering this CO₂ level has been shown to have various effects on in vitro cultures. It was shown that high CO₂ concentrations stimulated erythropoiesis (76), increased blastocyst development (73), regulated excitatory synaptic interactions of brainstem neurons (78), decreased Chinese hamster ovary cell proliferation (77), decreased metabolism (50), inhibited epithelial cell (79, 80) and fibroblast proliferation (80). There is conflicting evidence in the literature about the effect of low CO₂ concentrations. It was shown that hypocapnia enhanced the expression of pluripotency and differentiation genes in mouse ESCs (82), but suppressed cell proliferation through NF-Kβ signalling pathway (203, 204). One study evaluated the proliferation of human HSCs from umbilical CB in roller bottles in a CO₂-free environment and their results showed an 18-fold expansion of total CFU in L-15 medium (81).
Within the stem cell niche, there exists a correlation between O$_2$ consumption and CO$_2$ production which, in turn, may allow cells to sense changes in CO$_2$ concentrations in parallel with O$_2$. Morton (1967) hypothesized that higher levels of CO$_2$ promote the flow of calcium into primitive cells of rat BM which, in turn, stimulates the cells to divide and proliferate at higher rates compared to cells cultured at lower CO$_2$ levels (76). Jyoti et al. have proposed that it is possible that a protein exists within cells that recognizes modifications in CO$_2$ concentrations (75). The up-regulation of this protein may potentially stimulate/inhibit various enzymatic pathways, metabolic activities, and/or membrane excitability. Since it is generally accepted in the literature that hypoxic conditions are favoured for HSC proliferation to mimic the cellular in vivo environment, could a hypo- or hypercapnic environment also be favoured for HSC proliferation over the typical 5%? To investigate this hypothesis, a comparative study was conducted to study the effect of hypocapnic (0.5-1% CO$_2$ in air) and hypercapnic (15% CO$_2$ in air) conditions on human KG-1a cells as compared to the commonly used 5% CO$_2$ control. The cells were examined for their expansion rate, proliferation, apoptotic profile, metabolism, and morphology.

Experiment 1 evaluated the KG-1a cell expansion in static 2D flasks at 1%, 5%, and 15% CO$_2$ in air. Concurrently, at each CO$_2$ concentration, cells were seeded at three different starting cell concentrations. This experiment firstly revealed that both hypo- and hypercapnia had no substantial effect on HSC-morphology in terms of cell shape and size as examined through an inverted microscope. Furthermore, hypercapnia decreased KG-1a cell proliferation rate but extended survival by delaying the apoptosis cascade. Cells also reached higher maximum concentrations in hypercapnic conditions in comparison to the 5% control and 1% hypocapnia. The hypocapnic populations, although had increased cell PDT, revealed decreased maximum cell concentration reached over time in comparison to both the control and the hypercapnic cells, as well as increased apoptosis rates. Hence, having cells which cycle at a slower rate would be favourable for sustaining survival and for mimicking the in vivo environment where it is established that cells proliferate at a gradual pace. According to initial cell concentration, it was understandably established that greater cell counts were produced with a greater starting cell volume.
Experiment 2 was an extension of our first where the same cells were seeded at elevated cell counts in hypo- and hypercapnic environments but in a 3D culture system. Once again, our experimental showed that both hypo- and hypercapnia had no considerable effect on cell morphology in terms of cell size. However, FCM analysis revealed that hypocapnia significantly decreased the expression of the CD34+ surface antigen, the latter being unaffected by hypercapnia with respect to the control populations. Hypercapnia also yielded a higher maximum cell concentration and lower PDT, and the contrary in the hypocapnic cell populations. According to apoptosis, hypercapnia clearly delayed the progress of apoptosis in the KG-1a cells once more and vice versa for the hypocapnic cells, confirming our results from Experiment 1. This is also parallel to the glucose depletion studies, where the cells that cycled slower (hypercapnic) metabolised glucose at a respectively slower rate and cells that cycled faster (hypocapnic) depleted glucose at a much faster rate from the medium.

To justify our novel outcomes, several hypotheses were put forward based on present literature. Firstly, it was hypothesised that hypercapnia relatively slows down proliferation and metabolism, and in turn, slows down cellular stress, and thus reduces the number of gene mutations that would potentially occur during cell proliferation/division, hence reducing the apoptosis rate, in turn, yielding higher cell concentrations per millilitre. Similarly, cells cultivated in hypocapnia proliferated at a much faster rate, and were thus subjected to more stress, hence causing more irregular gene mutations, rendering a higher rate of apoptosis.

Moreover, ROS are the main stimulators of apoptosis and are suppressed by higher levels of CO₂ (59, 61). So, our second hypothesis was that the ROS inhibition is accountable for the lower % SA observed in the hypercapnic populations. The acidified cell medium in the hypercapnic populations lead us to our third hypothesis: The apoptosis cascade is also greatly influenced by alterations in the mitochondrial membrane permeability (59, 71), where stability of the latter hinders apoptosis (59). The mitochondrial membrane has been shown to stabilise at acidic pH values (60), hence it can also be hypothesized that the increased CO₂ levels used in this experiment hindered apoptosis in this way. The latter was also illustrated by Carney et al. who revealed that CO₂ acts as a weak acid to maintain intracellular pH at relatively low levels by readily diffusing across the cell membrane and combining with intracellular water to form carbonic acid, which subsequently dissociates into HCO₃⁻ and hydrogen ion and regulates the
intracellular pH (73). This reasoning appears genuine since similar trends have also been demonstrated elsewhere in the literature (59, 72).

Moreover, CO₂ is the most significant of factors that manipulate the affinity between O₂ and haemoglobin and consequently modifying the oxyhemoglobin dissociation curve (75). This curve evidently shifts to the right with a subsequent increase in the PCO₂ and vice versa is also true. This shift in the oxyhemoglobin dissociation curve, brought about by modifications in PCO₂, is identified as the Bohr effect and is largely attributable to subsequent alterations in pH (PCO₂). Hence, with an increase in PCO₂, the affinity between O₂ and haemoglobin is decreased. Another hypothesis that can describe this study’s trend could be the relationship between O₂, CO₂, and haemoglobin. As mentioned above, with an increase in PCO₂ there is a subsequent decrease in the affinity between O₂ and haemoglobin and a decrease in pH. Indeed CO₂ is approximately 20 times more soluble in water than O₂ and has a solubility coefficient (kₚ) of 0.06 ml.dl⁻¹.mmHg⁻¹. This implies that about 5% of blood CO₂ exists as physiologically dissolved CO₂; hence humidified cell incubators in cell-culture studies are typically controlled at 5% CO₂ levels. CO₂ effortlessly diffuses across red blood cell membrane into the cell interior and is transported in the blood in three forms: 5% as dissolved CO₂, 5% carbaminohemoglobin, and 90% as HCO₃⁻ (205). The total CO₂ in the plasma is proportional to the partial pressure of the gas and its solubility coefficient (0.03 ml/100 ml plasma for each 1mmHg PCO₂) (45).

The formation of H₂CO₃ is catalysed by the enzyme carbonic anhydrase which is present at higher concentrations within RBCs. Thus, H₂CO₃ is generated in the RBC more than 10,000 times faster. Consequently, the concentration of HCO₃⁻ in RBCs rises in parallel. The RBC membrane itself is very permeable to HCO₃⁻, allowing the latter to diffuse rapidly down the concentration gradient from the RBC to the plasma, depolarizing the RBC membrane, which, in turn, draws anions into the RBC from the plasma (75); the latter being mostly chloride ions (Cl⁻). The substitution of HCO₃⁻ for Cl⁻ within the RBCs is labelled as the chloride shift (75). Increases in PCO₂ enhance the production of HCO₃⁻ without constraint, i.e. the CO₂ dissociation curve does not have a plateau resembling the oxyhemoglobin dissociation curve. It is worthy to note that stem cells possess a superior level of antioxidant defence (206) which could also be the justification of their survival at the varying CO₂ concentrations, where HSC are inclined to resist different micro-environmental conditions (59).
5.2 2D versus 3D

The majority of cell-culture studies in the literature describe cell proliferation in flat 2D flasks with the notion of ‘bioreactors’ only recently emerging into the cell-culture field. In Experiment 1, we showed that hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts in 2D flasks. In Experiment 2, we described the 3D culturing of promyeloblasts in a stirred suspension bioreactor. No previous study, to the best of our knowledge, has described the 3D culturing of such cells under varying CO₂ concentrations. This study is therefore novel in this respect. Moreover, no other study has examined the effect of CO₂ on human promyeloblast apoptosis, cell surface antigens, nor glucose depletion.

Table 5.1: Summary of identical experiments conducted at 15% CO₂ in air in 2D and 3D systems, respectively

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2D 15% CO₂</td>
<td>3D 15% CO₂</td>
</tr>
<tr>
<td>Average Starting Cell Concentration (cells/ml)</td>
<td>$4.13 \times 10^5 \pm 1.50 \times 10^4$</td>
<td>$4.98 \times 10^5 \pm 3.24 \times 10^4$</td>
</tr>
<tr>
<td>Maximum Cell Concentration (cells/ml)</td>
<td>$5.09 \times 10^6 \pm 3.13 \times 10^5$</td>
<td>$4.18 \times 10^6 \pm 3.51 \times 10^4$</td>
</tr>
<tr>
<td>At Time (hours)</td>
<td>168</td>
<td>166</td>
</tr>
<tr>
<td>Total Volume (ml)</td>
<td>8 ml</td>
<td>250 ml</td>
</tr>
<tr>
<td>Total Cell Count (cells)</td>
<td>$40.75 \times 10^6$</td>
<td>$10.46 \times 10^8$</td>
</tr>
<tr>
<td>PDT (hours)</td>
<td>44.5</td>
<td>43.3</td>
</tr>
<tr>
<td>% SA at time</td>
<td>-0.06 at 192 hours</td>
<td>-0.68 at 190 hours</td>
</tr>
</tbody>
</table>

In comparing Experiments 1 and 2, it can be seen that cell size in both cultures is comparable (Figures 4.2 and 4.6). Table 5.1 summarises the main aspects of both experiments (15% CO₂ are
compared only). Maximum cell number at approximately the same time (168 hours and 166 hours for 2D and 3D, respectively) is higher in the 2D culture, yet the overall cell count is much greater in the 3D system. This is due to the upscale in the bioreactor system with a total volume of 250 ml in contrast to the 8 ml of the 2D flasks. The 3D bioreactor systems are therefore more useful, under appropriately controlled conditions, for producing greater cell numbers in the same amount of time. The right bioreactor size is however expected to exist at a ‘sweet’ spot where differentiation does not occur. This question of bioreactor size, as well as the mode of bioreactor operation (eg. batch vs. continuous), are beyond the scope of this current work.

The % SA is lower in the 3D culture system (-0.68 vs. -0.06), accompanied by a lower PDT (43.3 vs. 44.5). These results once again prove our hypotheses: (i) 3D bioreactor culture systems produce cells of greater quality (less apoptotic cells compared to the control), and (ii) the slower the cells double, the more guarded they are against DNA damage, the less the apoptotic capacity. It seems logic to conclude that our 3D culture system was successful in producing much greater numbers of high quality cells in the same amount of time as our 2D systems.
Chapter 6: Conclusions and Future Directions

6.1 Toward an Understanding of How Hypercapnia Affects Apoptosis

Researchers in the stem cell culturing field have almost completely overlooked CO₂ in terms of mammalian stem cell proliferation and focused much more on the influences of O₂ alone. Despite human blood containing an analogous 5% dissolved CO₂, stem cells of the BM exist in a microenvironment of higher CO₂ concentration (3-5). Hence, it seems illogic to assume this 5% CO₂ for the proliferation of stem cells of the BM, but in fact, almost all cell proliferation systems in the literature do so. For this reason, our study’s aim was to analyse and investigate the effects of both hypo- and hypercapnia on the proliferation of hematopoietic progenitor stem cells (KG-1a cell line). In addition, these cells’ apoptotic capacity, morphology, surface antigen integrity, and metabolism were also examined under these varying CO₂ conditions. Furthermore, our work also highlighted the significance of culturing stem cells in 3D bioreactor vessels, contrary to conventional 2D ones. Once again, the 3D system provides greater similarity to the actual in vivo microenvironment of these cells.

In more detail, we deduced from our study that KG-1a cells favour an environment of higher $P_{\text{CO}_2}$ and slightly lower pH where they proliferate slower (decreased PDT) and undergo apoptosis at a decreased rate (lower % SA) in contrast to the hypcapnic and control populations. These results were true in both 2D and 3D culture systems. The cell morphology (shape and size) was not affected by neither hypocapnia nor hypercapnia, following analysis by inverted microscope; however, after FCM analysis, it appeared that hypercapnic and control cells had...
comparable cell surface antigen (CD34+) integrity in contrast to the hypocapnic ones which appeared to be impaired. Although the maximum cell concentration was greater in the 2D experiments compared to the 3D ones, it must be emphasized that the overall cell count is much greater in the 3D system since the total volume is over 30 times larger. Moreover, in our 3D populations, the increases in CO₂ brought about decreases in the medium pH, which, as justified above, may be accountable for the apoptosis delay also seen in these populations. Finally, KG-1a cell metabolism rate (glucose depletion) decreased with an increase in %CO₂; this agrees with the lower PDT seen for these hypercapnic populations. The overall conclusion is that although hematopoietic progenitor stem cells can be successfully maintained under atmospheric CO₂ pressures (5% in air); hypercapnic conditions seem to be advantageous for increased propagation and survival of hematopoietic progenitor stem cells.

At this point, the cellular mechanism(s) by which hypercapnia delays apoptosis and increases cell proliferation is yet to be determined, we have only hypothesised what we think is reasonable relative to the available current literature: (i) the suppression of the production of ROS, which are the main stimulators of apoptosis, is accountable for the hindered apoptosis observed in the hypercapnic cells, (ii) the mitochondrial membrane stability/permeability in the acidified medium hinders apoptosis.

This thesis analysed the effects of hypo- and hypercapnia using the cell line KG-1a as a model for human HSCs. Our study has illustrated that maintaining human KG-1a cells at elevated levels of CO₂ brought about prolonged survival rates and increased overall proliferation, accompanied by decreased proliferation rates. Likewise, reduced levels of CO₂ caused an increase in proliferation rates, but an overall decrease in KG-1a cell proliferation and an increase in apoptosis in comparison to the 5% CO₂ control. HSCs are at present one of the predominant stem cell classes seen in modern, wide-scale clinical applications. Thus, growing HSCs into scalable quantities is potentially a huge advantage for both research and therapy.

6.1.1 Future Work
Due to time constraints, our experiments looked at only one hypercapnic condition (15% CO₂), yet we were looking forward to completing a cell culture run at over 15%. It is worth saying that it is important to titrate the hypercapnic CO₂ concentrations (lower and higher than 15%) in order to optimise the culture conditions, especially for the expansion of human hematopoietic
progenitor stem cells in vitro. Also, in order to validate these results further, it is important to evaluate what effects optimum hypercapnic conditions have on HSC phenotype, gene expression, and on animal models. Future studies will require a clearer understanding of the genes expressed in both hypo- and hypercapnic conditions, proteins involved in both conditions, and perhaps signalling pathways implicated. In addition, to further mimic the BM microenvironment of HSCs, the cell medium must also be modified accordingly through the supplementation of cytokines and/or other growth factors, for example. Finally, it would also be worthy to examine the hypo- and hypercapnic effects on other cell lines and then on actual human HSCs to further validate our results.

Even after this research demonstrates that clinically meaningful numbers of human hematopoietic progenitors can be produced from an initial small inoculum of BM aspirate, there will remain further challenges to be conquered; such as, the addition of animal and/or human sera to the medium which may contain pathogens (116) – hence, the development of a substitute is crucial. This can either be the development of serum-free media (207) or the use of autologous plasma (208, 209), this topic has been reviewed in the literature (210, 211).

Despite our mass efforts in pioneering the design and development of a 3D cell culture system, it is still however in its early stages. The bioreactor itself must be optimised in terms of parameters, such as: sheer stress, pressure, feed gas pressure within vessel, medium sampling, redox potential, etc. This is in order to better understand whether or not our observations were purely a result of hypercapnia or a combination of parameters.

The next, and most important, phase is the development of a population balance model (PBM) which explicitly describes HSC proliferation in terms of: growth rate, overall cell number, division rate, cell death/apoptosis, dissolved gases (O₂ and CO₂), substrates (glutamine, glucose), metabolites (lactate, ammonia), and more. In addition, this model must take into account the bioreactor itself in terms of sheer stress and other hydrodynamic forces, as well as feeding into the cell medium and removal of waste products (in a continuously fed reactor), sampling out for analysis, and other factors. The model then requires validation (via several experimental runs) and coding. The model itself will then potentially serve as a means of dynamic optimisation for future bioreactor culture systems. Such an initiative could open new doors in HSCs research on one level and in stem cell biology overall on another level.
Moreover, the environment within the bioreactor itself must be optimised to further replicate the cellular microenvironment in terms of engineering. In other words, the BM niches are not a typical cylinder filled with liquid where cells grow and expand; it is much more complicated than that. The BM consists of smaller sub-compartments, pockets, surface areas for the cells to grow on, and other elements. Hence, a standard bioreactor on its own is inadequate and requires surfaces such as microcarriers, or beads, or matrices for the cells to proliferate on.

We have begun working towards these objectives firstly by developing a PBM for the proliferation of HSCs in a 3D suspension bioreactor, and secondly by designing and creating (using 3D printing) a replica of the BM for cells to grow on within a bioreactor. In our group, Bartolini et al. recently developed a mathematical model for the 3D expansion of ESCs (212). The effects of varying substrate and gas concentration on total cell number were modelled. They found that with a high starting concentration of O$_2$ in a fed-batch system, total cell number can be maximised. This model will be optimised further to include (i) the relationship between the %CO$_2$ and the death kinetics (apoptosis rate) and (ii) the effects of multiple metabolites (glutamine, glucose, lactate, ammonia).

6.2 Towards Autologous Transplantation – A future multi-scale systems approach in stem cell bioreactor operation

Biological systems are composed of numerous complex scales that communicate information to and from strata above and below (213). The biological processes of these systems can be organised into a hierarchy of spatial scales: genes, to proteins, to individual cells, to tissues, to organs, and finally to the individual organism that interacts with its surrounding environment (214).

Systems biology has been thoroughly investigated and developed over the last two decades with the aim of understanding biological behaviour at a global scale. These biological functions are the sum consequence of many complex mechanisms that occur at numerous scales: from the molecular to the ecosystem (215). In the context of stem cells and developmental biology, understanding how these growth systems are transformed with time is a key question yet to be answered. Existing experimental methods and techniques are still not competent enough in interpreting the various elements of these systems (216).
Mathematical modelling and simulation are computational tools that are at present modern being exploited for the comprehension, prediction, and portrayal of these mechanisms (natural or engineered mechanisms) by quantitative and integrative means (215). Successful analysis of these mechanisms must entail a clear understanding of the sub-interactions amongst the fundamental components of cells, organs, and systems in both the presence and the absence of disease (215). The construction of in-silico models has been used to generate experimental knowledge via software engineering methods (216). In other words, multi-scale techniques that permit the combining and simulation of models that span numerous spatial and temporal scales significantly promote the understanding of biological functions (215). The key method here is to incorporate known isolated mechanisms within simplified postulations where the knowledge is limited in order to analyse the developmental process (216, 217).

Owing to the great complexity of biological systems, in-silico methods have proven promising in studying development and in guiding future experimental methods and techniques by predicting unexpected trends through the varying of the model’s design. Potentially, the investigation and the implementation of mathematical models could open many doors to scientists and researchers not only by summing up known and predicted results but also by highlighting innovative approachable predictions and ideas (216). Models and experimental data provide enormous information on the intra- and inter-scale interactions and behaviour within the system itself and as a whole with the environment (215).

Mathematical models are utilised in continuum-based modelling techniques where mathematical equations or distinct modelling methods that are based on discrete units to model the heterogeneous microscopic elements (particulates or cells) are used to describe system properties (215). Models at the different scales must be coupled together in order to construct integrated models across these multiple scales to, in turn, achieve the objectives of systems biology entirely (215). In-silico models show great potential as common future techniques in both research and therapy. Today, many diverse models have been developed for solving multi-scale difficulties in various scientific disciplines and have been reviewed in the literature (215, 216).

Multicellular organisms’ development, structure, and function are maintained by cell populations within developmental systems (218, 219). Development is controlled by the cells through two main procedures: (a) cellular proliferation in which the cell grows and divides to give rise to two
daughter cells, and (b) cellular differentiation through which an immature cell takes on a more specialized cell type/line (219). Knowledge of the precise cellular mechanisms and how these systems are regulated remain vague; even with all the state-of-the-art techniques, the picture that is drawn the experimental data continues to be somewhat deficient (216). Mathematical modelling has been increasingly applied to various biological systems (220); in fact, interest in this area is emerging and the various advances and challenges have been reviewed extensively (220-223). The majority of these studies are specific to a particular biological system (224-227); few others have attempted more generic methods (228, 229).

Numerous models have been developed towards understanding cell population dynamics; other models identified expression patterns in stem cell systems development at the gene- and protein-level (226, 230). Patterns of stem cell divisions and cell regulation in sub-systems were also simulated from mathematical models (231, 232). These works and the weakness they incorporate have been reviewed extensively by Setty et al. (216). One such weakness includes the limited ability of in-silico models to clearly represent the dynamics within the individual cells and to accurately predict spatial distributions of cells and complex phenotypes (216). The most common and the utmost limitation of the majority of previous modelling efforts is that development is only taken into account at the single-scale of the biological system and all other related scales are ignored. Hence, in order to achieve a rational understanding of the comprehensive development of cells in the tissue, there is a definite requirement for in-silico models that facilitate the incorporation of the regulation of sub-systems into a thorough/complete multi-scale dynamic mathematical model; such models have also been reviewed (216).

Today, more bridges are being built between science and engineering where research is focusing more on applying in-silico modelling to various biological systems such as, stem cells. The many challenges and achievements in this area of multidisciplinary modelling were recently reviewed in several publications (219, 222, 223), but are beyond the scope of this dissertation.

6.3 Population balance equations (PBE)

Culturing any mammalian cell type involves accurate monitoring and control of in vitro physiological parameters during the cell culture. As discussed above, efficiency can be enhanced
by using mathematical models that predict population dynamics via the assimilation of kinetic data (cell size, cell numbers, cell death rate, metabolic uptake rates, differentiation rates…) (63).

Population balance equations (PBEs) are a mathematical description of a population of elements derived from the interaction of a single element with its environment. They are employed in various fields of biomolecular engineering in order to optimise both growth and control of a particular cell line via the use of a PBM. The PBM can either be a single or an integration of several PBEs.

In the case of stem cells, and in order to optimise their proliferation while maintaining their pluripotency, a great number of experiments must be conducted with varying culture conditions. This is not only uneconomic considering the high cost of cell culture materials, but also impractical bearing in mind the difficulties in obtaining stem cells in large numbers owing to strict ethics and policies. A PBM can then be used to enhance culture conditions in order to maximize results and minimize both time and experimental labour.

A PBM represents a number balance based on the particle state. This particle state vector is the domain in which the particles expansion is continuous, but does not exceed the bounds of this domain. This state vector can be based on a different number of variables which is needed to describe the rate of change in the cells. Several state variables include cell age, cell mass, cell surface area etc. (233).

PBM can be classified as ‘unstructured’ or ‘structured’, and also as ‘segregated’ or ‘unsegregated’. Unstructured population balances are less intricate than structured ones and employ a single state variable to illustrate the biophase in its environment. Structured population balances, however, singularly describe all the chemical components of this biophase by means of variables integrated into the PBE. On the other hand, an unsegregated model is based on the postulation that the cell population can be regarded as one constantly mixed biophase with no variations. A segregated model is again more complex and takes into account the heterogeneous nature of the cell expansion environment within the bioreactor. PBM can also be defined as mass structured or age structured. The former have both mass and time amongst their variables and obey the law of conservation of mass, the latter employ the age of the individual cell as the variable in the population overall.
All PBMs illustrate the temporal dynamics of a population of particles with respect to the single particle, or single cell kinetics. A generalised form of the population balance of cells in a working 3D culture volume can be described by:

Rate of cell accumulation = rate of cell birth + growth flux in − growth flux out − rate of cell division − rate of cell death (212)

The cell kinetics include (refer to Bartolini et al. (212) for detailed equation formulations):

- Occurrence of cell division
- Probability at which the individual cells divide, differentiate, and/or die
- State of newborn cells
- Growth rate of individual cells

In cell culture, in order to illustrate the entire cell property distributions, differentiation between the various cell states is initially required; hence the PBM must be segregated.

### 6.4 Growth Models

There are several different types of biological kinetics that can be attributed to cell growth for their use in PBMs or in logistic equations. These growth models are estimations of the rate at which cells will absorb mass from a substrate, usually only including one limiting substrate, unless rate constants (k) are used to define a number of different metabolite cycles. Some examples of the kinetics that can be used are reaction rate kinetics such as in Luni et al. where the reaction rates between receptor and ligand-receptor complex activation and deactivation are used as the single cell process kinetics (234). Another more complex method that is used in Hatzis et al. is the transition rate function for inter-stage transitions (235, 236). However, the most common kinetics that are used, are the simple Michaelis-Menten/Monod kinetics or variations of these kinetics, where the limiting substrate such as O$_2$ is taken into account (237-239). However, it is not usually dependent on other factors such as cell mass or surface area. In other cases where pluripotency is not sought the same kinetics can be attributed to limiting growth factors, where required for modelling differentiation (240).
6.5 Model-Based Control and Optimization of HSC Proliferation

In order to better comprehend the physical, mechanical, and biochemical elements of stem cell culturing, analysis of both experiments and quantitative mathematical models are required. As mentioned above, a PBM should ideally be employed in such experiments to improve culture conditions in order to amplify results and reduce both time and experimental labour. This segment will now summarise and discuss works that have explored the model-based control and optimization of mammalian HSC expansion in 3D bioreactor systems.

A few recent studies have been conducted on the proliferation and expansion of HSCs in bioreactors with an integrated PBE model. A review by Didwania et al. summarizes recent progress in developing a controlled artificial HSC niche in which current technologies for bioscaffold materials, microfluidic HSC niches and mathematical simulations of bioreactors were investigated (241).

Peng and Palsson formulated a mathematical model of O$_2$ diffusion, based on O$_2$ uptake rate (OUR) data, which was subsequently used to investigate factors contributing to hematopoietic bioreactor design, such as initial seeding density, medium depth, reactor configuration, and O$_2$ partial pressure (242). Very high and very low levels of O$_2$ can be harmful to cells causing toxicity and anoxia, respectively. Hence, a successful hematopoietic culture requires an optimal O$_2$ concentration range. Based on this mathematical model and experimental data, the in situ OUR measurements matched predicted O$_2$ limitations. Oxygen consumption was found to increase with culture time. The perfusion system used in this study for the expansion of BM cells was a radial-flow parallel-plate-type bioreactor and achieved a doubling in the number of cells over a 14-day culture period with a viability of 85-95% (242).

Konstantinov et al. developed and experimentally tested a mathematical computer model of spheroid movement in commercially available rotating-vessel bioreactor system and a static bioreactor while considering all inertial effects caused by both the production module rotation movement and by the relative movement of the spheroid in the fluid. The group cultivated mouse hematopoietic BM cells and Hodgkin’s disease-derived malignant cells and tested both bioreactors’ efficacy to support long-term BM cell cultures. A nonlinear system of differential
equations was developed to describe dynamic behaviour and the growth was approximated using a limited linear function.

In addition, equations were suggested to describe the mechanical properties of cell spheroids which were approximated to elastic shear stress-sensitive bodies (106). The group reported a higher content of CFC-GM cells in the rotating-vessel system. The composed mathematical models of the physio-mechanical behaviour of spheroids facilitated the estimation of the revolution frequency increase schedule; the aim was to optimise the rotation frequency increase schedule for various cells to reduce shear stress, enhance productivity, and endure the expansion of large spheroids (106).

Similarly, Pathi et al. investigated the role of nutrient supply on cell expansion in bioreactor design for cultivation of HSC. The group developed a mathematical model that quantitatively describes O2 levels in varying space and time and also simulates the growth of granulocyte progenitor cells alongside O2 consumption in a 3D perfusion bioreactor. They were able to deduce that maximum growth rates can be achieved based on a specific flow rate; flow rates higher or lower than this paramount lead to proliferation inhibition. The model further demonstrates the dependence of cell growth on O2 concentrations. The design of the reactor was also investigated via the model and was found to directly affect O2 distribution and thus cell growth; hence, an optimal bioreactor design must essentially include a large O2 distribution for maximized reactor yield (243). Further investigations on this topic can be found in the following reviews (22, 23, 69, 92, 107).

Ma et al. developed a numerical model that examined the fluid flow, shear stress, and nutrient distribution with a purpose-built, porous scaffold placed within a 3D rotating wall perfused bioreactor. Although the group did not experimentally validate this work, the simulation results revealed that several factors are required to support optimal hematopoietic cell proliferation suitable for BMT applications; these factors include: fluid dynamics within the scaffold similar to those in the in vivo BM microenvironment, inoculum density, transport properties (scaffold), consumption rates, and spatial average O2 concentration within the vessel which ranged from 4% to 21% (244).
Kresnowati et. al. discussed the model-based analysis and optimization of bioreactor for HSC cultivation. The group explored the development of a mathematical model to describe the dynamics in nutrient distribution and cell concentration in a micro-channel perfusion fed-batch bioreactor. This model was then further utilised to optimise cell proliferation by proposing alternative feeding strategies. It is imperative to maintain equilibrium between feeding rate and cell requirement; overfeeding may result in nutrient and byproduct accumulation, whereas underfeeding will cause cell starvation and hence decreases expansion rates (91). The model was useful in illustrating alternative feeding strategies that improved cell yield.

Doran et al. described the use of a simplified hollow fibre bioreactor system to produce blood products from umbilical CB-derived CD34⁺ HSPCs. Results were compared to cells cultured in the same optimised commercial medium in traditional static culture systems. Their results showed that although cell proliferation in the conventional cultures performed best, the simplified hollow fibre bioreactor system’s outcomes were almost equivalent in regards to total cell expansion and maturation. However, the simplified system employs significantly less protein and achieves an approximate 100-fold greater cell density (245).

Glen et al. compared the production of blood cells from directly isolated CD34⁺ hematopoietic progenitor cells to equivalent cells that had been pre-expanded for a week with Delta1 Notch ligand. Cells were cultured under erythroid expansion and differentiation conditions in a micro-scale suspension bioreactor with varying culture parameters. Growth trends were analysed through the measurement of phenotype markers and metabolites. The group found that erythroid differentiation chronology is somewhat controlled by the heterogeneous CD34⁺ progenitor compartment and that Delta1 ligand-mediated progenitor culture can modify cellular differentiation patterns (246).

**6.6 Conclusions and Future Remarks**

As presented above, very few studies in the literature provide sufficient information that evaluates the control and optimization of all parameters within a cell-culture system. In the majority of cases, complex kinetics are either overlooked, or the interaction between all parameters is ignored. The engineering of overall controlled systems for optimised HSC
cultivation is still in its early developmental stages and the application of PBE models to 3D bioreactor HSC culture systems has been limited to date. This is most likely due to the great intricacy in creating an exact, or close to exact, mimic of the in vivo microenvironment of stem cells niches (103). Relevant design parameters and physiochemical/biochemical parameters must be appreciated and investigated. These kinds of projects are multidisciplinary and require close integration of chemical and biomolecular/bioengineering, computer science, and medicine.

The utmost goal in this field is to engineer systems that are not only very close replicas of the in vivo hematopoietic environment, especially in terms of gas composition, but also sufficient, simple, flexible, and of high economical efficacy. These systems can potentially be employed for HSC expansion at the bedside for cell therapy of leukaemia and other blood-related and non-blood-related diseases. One major challenge is to increase both the productivity and the reliability of these hematopoietic ex vivo expansion systems by minimizing the number of initial cells required. The aim is to provide unlimited supplies of highly specialized cells for the purpose of stem cell therapy and tissue engineering as well as medical applications at the bedside. We believe our study has contributed

For the future, we can expect to see the development of satisfactory, straightforward, highly productive and reliable HSC-culture systems. These systems will be tested in clinical studies and will eventually be used in transplantation therapies for leukaemia, but also for the production of many other types of highly specialized cells for the treatment of various other diseases.
References


27. Anagnostou D, Matutes E. Bone Marrow Lymphoid Infiltrates: Diagnosis and Clinical Impact: Springer; 2012.
Flt3-ligand, interleukin (IL)-6 and IL-11 in a serum-free and stroma-free culture system. Br J Haematol. 2001;112.


62. Kuebler JF, Kos M, Jesch NK, Metzelder ML, van der Zee DC, Bax KM, Vieten G, Ure BM. Carbon dioxide suppresses macrophage superoxide anion production independent of


102. Chen HF. A reduced oxygen tension (5%) is not beneficial for maintaining human embryonic stem cells in the undifferentiated state with short splitting intervals. Hum Reprod. 2009;24(1).


132. Oh DJ, Koller MR, Palsson BO. Frequent harvesting from perfused bone marrow cultures results in increased overall cell and progenitor expansion. Biotechnology and Bioengineering. 1994;44(5):609-16.


159. McDowell CL, Papoutsakis ET. Increased agitation intensity increases CD13 receptor surface content and mRNA levels, and alters the metabolism of HL60 cells cultured in stirred tank bioreactors. Biotechnology and Bioengineering. 1998;60(2):239-50.

160. McDowell CL, Papoutsakis ET. Serum increases the CD13 receptor expression, reduces the transduction of fluid-mechanical forces, and alters the metabolism of HL60 cells cultured in agitated bioreactors. Biotechnology and Bioengineering. 1998;60(2):259-68.


180. 高春平, inventor; 高春平, assignee. Continuous filling automatic cell culture system2006.


184. 谭文松, 迟占有, 蔡海波, inventors; 上海伯瑞生物科技发展有限公司, 华东理工大学 assignee. Method and device for perfusion and culture of hematopoietic cell 2007.

185. 华平, 周美琴, 蔡海波, 谭文松, inventors; 华东理工大学, assignee. Bioreactor system for culturing hematopoiesis cell in vitro2012.


216. Setty Y. In-silico models of stem cell and developmental systems. Theoretical Biology and Medical Modelling. 2014;11(1).


Appendix I – Literature Review Summary Table

Table A.1: Literature summary of Sections 2.6 and 2.7. This table summarises studies which examined the expansion of blood or blood-derived (human or murine) cell expansion in an either controlled or uncontrolled bioreactor system.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Culture Period</th>
<th>Cell type</th>
<th>Bioreactor</th>
<th>Control type (uncontrolled, traditional control, model-based control)</th>
<th>Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>(123)</td>
<td>12 weeks</td>
<td>Human and rat BM</td>
<td>Suspended nylon mesh system</td>
<td>Uncontrolled</td>
<td>-</td>
</tr>
<tr>
<td>(124)</td>
<td>14-32 days</td>
<td>TIL</td>
<td>Hollow fibre (Cellmax)</td>
<td>Uncontrolled</td>
<td>124-1170-fold</td>
</tr>
<tr>
<td>(247)</td>
<td>-</td>
<td>LDMCs from human BM</td>
<td>Stirred suspension Microcarrier Airlift Hollow Fibre</td>
<td>Uncontrolled</td>
<td>4.4-32-fold</td>
</tr>
<tr>
<td>(117)</td>
<td>4 weeks</td>
<td>Cadaveric human BM cells</td>
<td>Flat-bottomed stirred suspension spinner flask</td>
<td>Uncontrolled</td>
<td>Viable cells: 16-fold CFC: 22-fold</td>
</tr>
<tr>
<td>(125)</td>
<td>2 weeks</td>
<td>Cadaveric human BM cells</td>
<td>Flat-bottomed stirred suspension spinner flask</td>
<td>Uncontrolled</td>
<td>CFC: 45-66-fold LTC-IC: 2.5-9-fold</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>Source</td>
<td>Method</td>
<td>Condition</td>
<td>Result</td>
</tr>
<tr>
<td>---</td>
<td>-------</td>
<td>--------</td>
<td>-------------------------</td>
<td>-----------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>119</td>
<td>14 days</td>
<td>Mouse BM cells</td>
<td>Stirred suspension</td>
<td>Uncontrolled</td>
<td>hematopoietic primitive progenitor cells: 5-fold LTC-IC: 4-fold</td>
</tr>
<tr>
<td>126</td>
<td>-</td>
<td>PB MNC</td>
<td>Stirred suspension (spinner flasks)</td>
<td>Uncontrolled</td>
<td>-</td>
</tr>
<tr>
<td>127</td>
<td>7 days</td>
<td>PB MNC</td>
<td>Stirred suspension (spinner flasks)</td>
<td>Uncontrolled</td>
<td>Total cells: ~6-fold</td>
</tr>
<tr>
<td>116</td>
<td>14 days</td>
<td>Human BM cells</td>
<td>Stirred suspension (spinner flask)</td>
<td>Uncontrolled</td>
<td>Total cells: 3.28-12.12-fold CFU-GM: 2.81-9.25-fold</td>
</tr>
<tr>
<td>128</td>
<td>12 days</td>
<td>human umbilical CB HPC</td>
<td>Stirred suspension (CultiSpher G)</td>
<td>Uncontrolled</td>
<td>TVC: 7.7-fold CFU-C: 23.3-fold CD34+: 9.6-fold</td>
</tr>
<tr>
<td>129</td>
<td>6 days 9 days</td>
<td>CB HSCs</td>
<td>Stirred suspension (stirred flasks)</td>
<td>Uncontrolled</td>
<td>CFC: 195.2 ± 24.6 g/cm³ CD34+: 3.01 ± 0.26 g/cm³</td>
</tr>
<tr>
<td>130</td>
<td>4 months</td>
<td>Mouse BM cells</td>
<td>Continuous perfusion</td>
<td>Uncontrolled</td>
<td>-</td>
</tr>
<tr>
<td>131</td>
<td>14 days</td>
<td>Human BM MNCs</td>
<td>Perfusion</td>
<td>Uncontrolled</td>
<td>Total cells: 10-fold LTC-IC: 7.5-fold</td>
</tr>
<tr>
<td>132</td>
<td>27 days</td>
<td>Human BM cells</td>
<td>Perfusion</td>
<td>Uncontrolled</td>
<td>Total cells: 100-fold CFU-GM: 30-fold</td>
</tr>
<tr>
<td>133</td>
<td>7 days</td>
<td>Human BM MNCs</td>
<td>Perfusion</td>
<td>Uncontrolled</td>
<td>CFU-E: BFU-E: CFU-GM: CFU-GEMM:</td>
</tr>
<tr>
<td>134</td>
<td>8 weeks</td>
<td>Mouse BM cells</td>
<td>Packed bed</td>
<td>Uncontrolled</td>
<td>-</td>
</tr>
<tr>
<td>136</td>
<td>5 weeks</td>
<td>Human BM cells</td>
<td>Packed bed</td>
<td>Uncontrolled</td>
<td>-</td>
</tr>
<tr>
<td>137</td>
<td>88 ± 23 days</td>
<td>Human TILs</td>
<td>Hollow Fiber</td>
<td>Uncontrolled</td>
<td>3 × 10¹⁰ cells</td>
</tr>
<tr>
<td>138</td>
<td>8 days</td>
<td>Human BM stroma HS-5</td>
<td>Hollow Fiber</td>
<td>Uncontrolled</td>
<td>Total cells: 5.18 × 10⁷ Total CD34+: 5.34 × 10⁶</td>
</tr>
<tr>
<td>Reference</td>
<td>Days</td>
<td>Cell Source</td>
<td>Culture System</td>
<td>Control</td>
<td>Total Cells</td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>-------------</td>
<td>----------------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>(139)</td>
<td>16-19 days</td>
<td>Human BM cells</td>
<td>Spinner flask</td>
<td>Uncontrolled</td>
<td>12-24-fold</td>
</tr>
<tr>
<td>(140)</td>
<td>7 days</td>
<td>Human CB</td>
<td>Spinner flask</td>
<td>Uncontrolled</td>
<td></td>
</tr>
<tr>
<td>(141)</td>
<td>12 days</td>
<td>UCB-HSCs and UCB-MSCs</td>
<td>RWVB</td>
<td>Uncontrolled</td>
<td></td>
</tr>
<tr>
<td>(142)</td>
<td>7 days</td>
<td>UCB</td>
<td>RWVB</td>
<td>Uncontrolled</td>
<td></td>
</tr>
<tr>
<td>(143)</td>
<td>12 days</td>
<td>Human CB MNCs</td>
<td>RWVB</td>
<td>Uncontrolled</td>
<td></td>
</tr>
<tr>
<td>(144)</td>
<td>3-6 weeks</td>
<td>CD34+ hematopoietic progenitor cells from human BM</td>
<td>CellFoam™</td>
<td>Uncontrolled</td>
<td>-</td>
</tr>
<tr>
<td>(145)</td>
<td>12 days</td>
<td>UCB</td>
<td>Dideco ‘Pluricell System’</td>
<td>Uncontrolled</td>
<td>MNC: 230.4 ± 91.5 CD34+: 21.0 ± 11.9</td>
</tr>
<tr>
<td>(248)</td>
<td>18 days</td>
<td>CB cells</td>
<td>unspecified</td>
<td>Uncontrolled</td>
<td>CD34+: 1.95 × 10^5-fold</td>
</tr>
<tr>
<td>(146)</td>
<td>7 days</td>
<td>CB</td>
<td>Static + Cyclic Bioreactor</td>
<td>Uncontrolled</td>
<td>Total cells: (7.23 ± 2.67)-fold</td>
</tr>
<tr>
<td>Study</td>
<td>Duration</td>
<td>Source</td>
<td>Culture</td>
<td>Control</td>
<td>Result</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>(147)</td>
<td>10 days</td>
<td>UCB</td>
<td>Bioreactor (other)</td>
<td>Uncontrolled</td>
<td>Total cells: 6.6-45.7-fold</td>
</tr>
<tr>
<td>(148)</td>
<td>12 days</td>
<td>Human HSCs (CD34+)</td>
<td>Stirred tank</td>
<td>Uncontrolled</td>
<td>10-fold</td>
</tr>
<tr>
<td>(150)</td>
<td>3-6 weeks</td>
<td>Human and Murine BM cells</td>
<td>Perfusion</td>
<td>Traditional control</td>
<td>Total cells: 5-fold CFU-C: 10-fold Thy1.2\textsuperscript{b} F4/80\textsuperscript{-} MAC1\textsuperscript{-}: 24-fold</td>
</tr>
<tr>
<td>(151)</td>
<td>14 days</td>
<td>Human BM cells</td>
<td>Continuous perfusion</td>
<td>Traditional control</td>
<td>MNC: 20-25-fold</td>
</tr>
<tr>
<td>(152)</td>
<td>6 days</td>
<td>UCB</td>
<td>Continuous perfusion</td>
<td>Traditional control</td>
<td>CFU-GM: 40-60-fold</td>
</tr>
<tr>
<td>(152)</td>
<td>14 days</td>
<td>MPB</td>
<td>Continuous perfusion</td>
<td>Traditional control</td>
<td>Total cells: 50-fold CFU-GM: 80-fold LTC-IC: 20-fold</td>
</tr>
<tr>
<td>(153)</td>
<td>10 days</td>
<td>PB MNCs</td>
<td>Continuous perfusion</td>
<td>Traditional control</td>
<td>CFU-GM: 17-19-fold</td>
</tr>
<tr>
<td>(111)</td>
<td>8 days</td>
<td>Human UCB</td>
<td>Continuous perfusion: Fixed and fluidized bed</td>
<td>Traditional control</td>
<td>CFU-GEMM: 4.2-fold CFU-GM: 7-fold BFU-E: 1.8-fold</td>
</tr>
<tr>
<td>(154)</td>
<td>12 days</td>
<td>Human BM MNCs</td>
<td>Perfusion</td>
<td>Traditional control</td>
<td>Nucleated cells: 4.9-fold CFU-GM: 9.5-fold CD34\textsuperscript{-}lin\textsuperscript{-}: 0.42-fold LTC-IC: 0.32-fold</td>
</tr>
<tr>
<td>(155)</td>
<td>12 days</td>
<td>Human BM cells</td>
<td>Perfusion</td>
<td>Traditional control</td>
<td>Total cells: 3.32-4.94-fold CFU-GM: 0.52-26.6-fold</td>
</tr>
<tr>
<td>(156)</td>
<td>12 days</td>
<td>Human BM cells</td>
<td>Continuous perfusion</td>
<td>Traditional control (?)</td>
<td>MNC: 4.2-4.8-fold CFU-GM: 11.0-fold</td>
</tr>
</tbody>
</table>
| (157)  | 12 days | UCB       | Continuous perfusion | Traditional control | CFU-F: 43.1-fold  
CD34"lin": 0.9-fold  
LTC-IC: 1.2-fold  
Viable cells: 2.4-fold  
CFU-GM: 82.7-fold  
CD34+: 0.5-fold |
| (158)  | -       | PB MNC    | Stirred suspension   | Traditional control | - |
| (159)  | -       | HL60 cells| Stirred suspension   | Traditional control | - |
| (160)  | -       | HL60 cells| Stirred suspension   | Traditional control | - |
| (161)  | -       | HL60 cells| Stirred suspension   | Traditional control | - |
| (109)  | -       | UCB       | Stirred suspension   | Traditional control | CFU-GM: 10-fold  
CFU: 9.2-fold  
MNC: 14-fold |
| (162)  | 10 days | UCB cells | Stirred suspension   | Traditional control | Total cells: 6-fold |
| (163)  | -       | Murine BM cells | Packed bed | - | - |
| (164)  | 11 weeks | Stromal cells | Packed bed | Traditional control | BM cells: 3.6 x 10^8 cells |
| (165)  | 8 days  | UCB       | RWVB                 | Traditional control | Total cells: (435.5±87.6)-fold  
CD34+: (32.7±15.6)-fold  
CFU-GM: (21.7±4.9)-fold |
<p>| (166)  | 7 days  | Whole blood| Hollow fiber        | - | - |
| (167)  | 7 days  | Whole blood| Hollow fiber        | - | - |</p>
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Duration</th>
<th>Material</th>
<th>Bioreactor Type</th>
<th>Control</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(168)</td>
<td>7 days</td>
<td>Whole blood</td>
<td>Hollow fiber</td>
<td>Traditional control</td>
<td>-</td>
</tr>
<tr>
<td>(169)</td>
<td>7 days</td>
<td>Umbilical CB and placenta-derived stem cells</td>
<td>Hollow fiber (2 ml) (8 ml)</td>
<td>Traditional control</td>
<td>RBC: 105 ± 33-fold RBC: 440-fold RBC:14,288 - fold</td>
</tr>
<tr>
<td></td>
<td>19 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(115)</td>
<td>14 days</td>
<td>KG-1 Human T cells CD34+ from CB</td>
<td>Novel continuous perfused bioreactor</td>
<td>Traditional control</td>
<td>MNC: 18-fold CFC: 8-fold</td>
</tr>
<tr>
<td>(170)</td>
<td>7 days</td>
<td>UCB</td>
<td>Microliter bioreactor array</td>
<td>Traditional control</td>
<td>Total cells: (17.1 ± 2.6)-fold</td>
</tr>
<tr>
<td>(171)</td>
<td>6 days</td>
<td>UCB</td>
<td>Stirred Multiwell Bioreactor</td>
<td>Traditional control</td>
<td>CD34+: (96 ± 1)% CD38+: (86 ± 3)% CD117+: (77 ± 3)% CD41+: (99 ± 1)% CD61+: (4 ± 1)%</td>
</tr>
<tr>
<td>(172)</td>
<td>21 days</td>
<td>UCB</td>
<td>Wave agitated bioreactor (BIOSTAT CultiBag RM bioreactor)</td>
<td>Traditional control</td>
<td>1.73 × 10^6-fold 2.25 × 10^8-fold</td>
</tr>
<tr>
<td></td>
<td>33 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(173)</td>
<td>16 days</td>
<td>UCB</td>
<td>-</td>
<td>Traditional control</td>
<td>CD34+: ~130-fold</td>
</tr>
<tr>
<td>(242)</td>
<td>14 days</td>
<td>Human BM cells</td>
<td>Radial-flow parallel-plate-type perfusion bioreactor</td>
<td>Model-based control</td>
<td>~2-fold</td>
</tr>
<tr>
<td>(106)</td>
<td>-</td>
<td>Murine hematopoietic BM cells Hodgkin’s disease-derived malignant cells</td>
<td>rotating-vessel (miniPERM)</td>
<td>Model-based control</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>Perfusion</td>
<td>Model-based control</td>
<td>-</td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>-----</td>
<td>-----------</td>
<td>---------------------</td>
<td>-----</td>
</tr>
<tr>
<td>(243)</td>
<td>-</td>
<td>-</td>
<td>Rotating wall perfused</td>
<td>Model-based control</td>
<td>CFU-GM: 16-fold</td>
</tr>
<tr>
<td>(244)</td>
<td>14 days</td>
<td>-</td>
<td>micro-channel perfusion</td>
<td>Model-based control</td>
<td>-</td>
</tr>
<tr>
<td>(91)</td>
<td>-</td>
<td>-</td>
<td>UCB</td>
<td>Model-based control</td>
<td>Total cells: 30-fold</td>
</tr>
<tr>
<td>(245)</td>
<td>-</td>
<td>CD34+ umbilical cord-derived cells</td>
<td>Stirred suspension (ambr micro-scale bioreactor)</td>
<td>Model-based control</td>
<td>Total cells: 2.7 ( \times 10^3 )-fold</td>
</tr>
</tbody>
</table>

- Information not reported

*TIL: human tumour infiltrating lymphocytes
*LDMC: low density mononuclear cells
*CB: cord blood
* hematopoietic progenitor cells
*CFC: colony forming cell
* bone marrow mononuclear cells
Appendix II - Research to Date

Publications


iv. Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas, Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts in 3D suspension bioreactor. (Under internal review)

Awards and Achievements

i. “Stem Cell Expansion Studies For the Treatment of Leukemia” Mouna Hamad, Ali
ii. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; The NSW Joint Chemical Engineer Committee (JCEC) Annual Postgraduate Symposium 2013: Engineer Australia Chatswood, Sydney Australia, 30th August, 2013 – (Oral) **First Place**

iii. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; 5th Annual Student Conference for Research Students 2013: The University of Sydney, Sydney Australia, 1st October, 2013 – (Oral) **First Place**

iv. “Modelling of Embryonic Stem Cell (ESC) Culture in 3D Suspension Bioreactors” Mouna Hamad, Harry Manoli, Ali Abbas; Research Conversazione 2013: The University of Sydney, Sydney Australia, 1st November, 2013 (Oral & Poster) **First Place**

v. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; 6th Annual Student Conference for Research Students 2014: The University of Sydney, Sydney Australia, 1st October, 2013 (Oral and Poster) **First Place**


ix. Winner of ‘Lab Demonstrator of the Semester’, Semester 2, 2014; Faculty of Chemistry, the University of Sydney, Sydney Australia, November 2014.
x. “Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts”
Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas; Postgraduate Cancer Research Symposium: The University of Sydney, Sydney Australia, 2nd December, 2016 (Oral)
First Place

Conferences

i. “Development of Novel Sialyltransferase Inhibitor Prodrugs” Mouna Hamad, Danielle Skropeta; RACI Biomolecular Division Conference: Torquay, Victoria, 4th December, 2011 (Poster – Abstract #084)

ii. “Optimal Model-Based Stem Cell Expansion In 3D Suspension For Bedside Applications” Mouna Hamad, Harry Manoli, Mohammad Irhimeh, Fariba Dehghani, Ali Abbas; Research Conversazione 2012: The University of Sydney, Sydney Australia, 2nd November, 2012 (Poster)

iii. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; The NSW Joint Chemical Engineer Committee (JCEC) Annual Postgraduate Symposium 2013: Engineer Australia Chatswood, Sydney Australia, 30th August, 2013 – (Oral) First Place


v. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; 5th Annual Student Conference for Research Students 2013: The University of Sydney, Sydney Australia, 1st October, 2013 – (Oral) First Place

vi. “Modelling of Embryonic Stem Cell (ESC) Culture in 3D Suspension Bioreactors” Mouna Hamad, Harry Manoli, Ali Abbas; Research Conversazione 2013: The University of Sydney, Sydney Australia, 1st November, 2013 (Oral & Poster) First Place
vii. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Harry Manoli, Ali Abbas; 21st NSW Stem Cell Network Workshop – The University of Sydney, 23rd September 2014 (Poster)


x. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; 6th Annual Student Conference for Research Students 2014: The University of Sydney, Sydney Australia, 1st October, 2013 (Oral and Poster) First Place


xii. “Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts” Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas; Postgraduate Cancer Research Symposium: The University of Sydney, Sydney Australia, 2nd December, 2016 (Oral) First Place