XENOTRANSPLANTATION OF HUMAN UMBILICAL CORD
PERIVASCULAR CELLS IN A FEMORAL DEFECT

by

Rano Matta

A thesis submitted in conformity with the requirements
for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

Copyright © 2009 by Rano Matta
Abstract

Xenotransplantation of Human Umbilical Cord Perivascular Cells in a Femoral Defect

Rano Matta

Master of Applied Science

Institute of Biomaterials and Biomedical Engineering

University of Toronto

2009

This work examines the osteogenic potential and immune-privileged properties of human umbilical cord perivascular cells (HUCPVCs) in normal Wistar rats and athymic rnu/rnu rats for up to 60 days. HUCPVCs demonstrated a mesenchymal stromal cell phenotype, assayed through flow cytometry, and RT-PCR analysis detected their expression of osteogenic genes. A bone tissue engineering construct was developed through centrifugal seeding of HUCPVCs onto calcium phosphate-coated PLGA scaffolds. These cell-scaffold constructs were transplanted into bilateral femoral defects. HUCPVCs did not induce any systemic biological response in normal rats; however, they did not engraft and impaired bone healing up to 60 days. When transplanted into athymic rats, HUCPVCs were detected up to 30 days in the femoral defects, improved bone regeneration at 15 and 30 days, as measured by micro computed tomography, and expressed osteogeneic proteins. These findings demonstrate that HUCPVCs are suitable for bone tissue engineering studies in larger animals.
Acknowledgements

I wish to express my sincerest gratitude to Dr. John E. Davies, AKA JED, for giving me the opportunity to become a Bonehead. With your unique teaching style and discerning scientific perspective, the lesson’s I have learned in this short time have been invaluable, and time I have spent in the Bone interface lab has been very memorable and enjoyable. JED’s enthusiasm to teach, his humility, his patience with me, and his commitment to each and every student’s success, are examples I will carry with me throughout my career. JED you’re one of a kind!

I am grateful to Dr. Vanessa Mendes, Susan Carter, and Dr. Yuko Ujiie for their technical assistance with animal surgeries and help surrounding many aspects of my work. I am also grateful to Vanessa for her friendship and for filling in as my ‘lab mom’. I am also grateful to Dr. Michel Araujo for teaching me to use the MicroCT, for his patience with my never-ending requests, and for wearing his sunglasses indoors. Thanks to Patralika for her help with the histology and especially for her kind words and reassurance during those long hours at the microtome. I am grateful to Limin Guan for always providing Osteoscaf on short notice. I wish to also express my appreciation to Elaine Cheng for her help with flow cytometry and for her friendship. A sincere thanks to Alejandro Gomez for his assistance with flow cytometry, PCR, and all things HUCPVC, and most importantly for the sushi lunches, heated debates, and memorable times in the lab. Also, a sincere thanks to Catalina Estrada for her help with PCR and for cheering me up with her infectious sense of humour. Thanks to all the members of the Bone interface group that I have worked with: Rahul, Dave Lickorish, Nazlee, Hamideh, Reynaldo, Ferryal, Val, Lorraine, Dave Grant, Elena, and James; your friendship and support will not be forgotten.

To my boys Akram, Amir, Jack, John, Mike, Rami, Sam, Sherif, and Tamer, thanks for your support, friendship, and the copious amounts of chicken wings throughout this process. To Donna, the past two years would not have been the same without you, thank...
you for your care, for putting up with me, and for brightening up my days.

Most importantly, I wish to thank my parents and my brother for their unbelievable love and support. You have enabled me to reach higher each day, and your encouragement is why I continue to work and succeed. This one’s for you!
Contents

1 Introduction .................................................. 1
  1.1 Osteogenesis and Fracture Healing ....................... 1
    1.1.1 Early blood interactions in the fracture site .......... 2
    1.1.2 Coagulation, Provisional Matrix Formation, and Inflammation 2
    1.1.3 Granulation Tissue and Angiogenesis .................. 3
    1.1.4 Osteogenesis ........................................... 3
    1.1.5 Clinical problem and motivation ....................... 4
  1.2 Regenerative medicine and tissue engineering for bone repair 6
    1.2.1 Bone biomaterials ...................................... 7
    1.2.2 Cell-based therapies ................................... 10
  1.3 Multipotent mesenchymal stromal cells .................... 12
    1.3.1 Bone marrow stromal cells as a therapeutic cell source 14
    1.3.2 Bone marrow stromal cells for bone tissue engineering  15
    1.3.3 Immunological properties of mesenchymal progenitor cells 16
  1.4 Human umbilical cord perivascular cells ................... 18
    1.4.1 Human umbilical cord perivascular cells as an osteogenic cell source 20
  1.5 Rationale .................................................. 22
  1.6 Hypothesis .................................................. 23
  1.7 Objectives .................................................. 23
# 2 Materials and Methods

2.1 Cell isolation and culture ................................. 25
2.2 Cell phenotyping and gene expression .................... 26
  2.2.1 Flow cytometry ........................................ 26
  2.2.2 RT-PCR .............................................. 28
2.3 Tissue engineering of a cell-scaffold construct ............ 28
  2.3.1 Preparation of a three-phase resorbable scaffold ....... 28
  2.3.2 Cell seeding .......................................... 30
  2.3.3 Assessment of cellular attachment and viability ....... 31
  2.3.4 Visualizing cell attachment on cell-scaffold constructs .... 32
2.4 In vivo transplantation ...................................... 33
  2.4.1 Cell-scaffold construct preparation ................... 33
  2.4.2 Animal subjects ...................................... 33
  2.4.3 Femoral implantation .................................. 33
  2.4.4 Blood sampling ...................................... 36
  2.4.5 Morphometric analysis of bone healing ................. 36
  2.4.6 RT-PCR tracking of HUCPVCs implanted in rat femora .... 38
  2.4.7 Histology and Immunohistochemistry .................. 38
  2.4.8 Statistics ........................................... 40

# 3 Results

3.1 Cell Immunophenotyping and gene expression ................. 41
  3.1.1 Flow Cytometry ...................................... 41
  3.1.2 RT-PCR .............................................. 45
3.2 Scaffold seeding .......................................... 46
3.3 Animal Studies ............................................ 50
  3.3.1 Xenotransplantation in Wistar rats .................... 50
  3.3.2 Xenotransplantation in athymic rats ................... 65
4 Discussion

4.1 Phenotyping of human umbilical cord perivascular cells .......................... 79
  4.1.1 Human umbilical cord perivascular cells have a mesenchymal stromal cell phenotype ................................................................. 80
  4.1.2 Human umbilical cord perivascular cells express several osteogenic genes ................................................................. 85

4.2 Development of a tissue engineered construct suitable for orthotopic implantation ......................................................................................... 86
  4.2.1 Centrifugal seeding enables efficient seeding of calcium phosphate coated scaffolds ................................................................. 86

4.3 Transplantation of human umbilical cord perivascular cells in immunocompetent rats ................................................................. 90

4.4 Xenotransplantation of HUCPVCs does not elicit a systemic immune response ......................................................................................... 91
  4.4.1 HUCPVCs cause a local immune response and do not engraft in immunocompetent rats ................................................................. 93

4.5 Transplantation of human umbilical cord perivascular cells in athymic rats ................................................................. 97
  4.5.1 HUCPVCs enhance bone regeneration in athymic rats ............... 98
  4.5.2 HUCPVCs engraft and contribute to bone regeneration ........... 99

5 Conclusions .................................................................................................................. 104

Bibliography .................................................................................................................. 106
# List of Tables

2.1 Primary antibodies for flow cytometry analysis .......................... 27  
2.2 Distribution of primary antibodies per tube of cells for flow cytometry analysis. ................................................................. 27  
2.3 Primers for amplification of osteogenic markers used in RT-PCR for analysis of RNA isolates from HUCPVCs and excised human bone. ........... 29  
2.4 Distribution of animals and time points for evaluation. ................... 34  
3.1 p-Values in blood count between the experimental group and control rats that received only the CP-PLGA scaffold without HUCPVCs. ............ 52
List of Figures

1.1 Timeline for normal fracture healing events. . . . . . . . . . . . . . . . . . 2

2.1 Schematic of the centrifugation seeding method. . . . . . . . . . . . . . . . . 31
2.2 Photographs of the sequence of surgical steps. . . . . . . . . . . . . . . . . 35
2.3 Mineral volume measurements in the femoral defect using Micro computed
tomography. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 37

3.1 Flow cytometry data of surface markers comparing HUCPVCs with hBM-
SCs. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 42
3.2 Representative flow cytometry histograms. . . . . . . . . . . . . . . . . . 44
3.3 RT-PCR data comparing cDNA from HUCPVCs grown in normal growth
medium to cDNA from human bone with regard to osteogenic markers. . 45
3.4 Scanning electron microscopy images of HUCPVCs cultured on scaffold
for five days after centrifugal seeding. . . . . . . . . . . . . . . . . . . . 47
3.5 Optimization of scaffold seeding. . . . . . . . . . . . . . . . . . . . . . . . 48
3.6 Mean blood count parameter time-course values with standard deviations. 53
3.7 Post-operative weights of animals receiving HUCPVCs+scaffold, and con-
trol animals receiving only scaffold. . . . . . . . . . . . . . . . . . . . . 54
3.8 Healing of femoral defect in Wistar rats transplanted with HUCPVCs. . 55
3.9 Progression of fibrous tissue formation and scaffold resorption in a femoral
defect in Wistar rats transplanted with HUCPVCs. . . . . . . . . . . . . . 57
3.10 Identification of fibrous tissue in a femoral defect in Wistar rats transplanted with HUCPVCs at 10 days. ............................. 59
3.11 Unusual trabecular outgrowth observed in experimental and control femurs after 30 and 60 days. ........................................ 60
3.12 Human specific staining of HUCPVCs in femoral defects in Wistar rats. ............................ 61
3.13 Cellular infiltration in HUCPVC-treated defects in Wistar rats. .............................. 62
3.14 CD68 staining in HUCPVC-treated defects in Wistar rats. .............................. 63
3.15 Healing of femoral defect in athymic rats transplanted with HUCPVCs. .................. 66
3.16 MicroCT analysis of bone healing in athymic rats transplanted with HUCPVCs. 68
3.17 HUCPVCs in culture stained with DiD and DiR. ........................................ 69
3.18 Resin sections observed by phase contrast microscope under DiR optical filter. ............................................................... 71
3.19 HUCPVCs survive in an athymic rat after seven days. ...................................... 72
3.20 HUCPVCs survive in an athymic rat after 10 days. ...................................... 74
3.21 HUCPVCs contribute to new bone in an athymic rat after 15 days. ........ 76
3.22 HUCPVCs persist in new bone until 30 days and express osteogenic proteins. 77
Chapter 1

Introduction

1.1 Osteogenesis and Fracture Healing

Bone is a dynamic tissue that is constantly being remodeled through the processes of osteogenesis and resorption\(^1\). Osteogenesis, the laying down of new bone matrix, is carried out by osteoblasts, the cell responsible for bone formation. The synthetic activity of osteoblasts is counteracted by osteoclasts, multi-nuclear cells responsible for bone resorption.

Fracture healing is a complex process in which damaged bone restores its original architecture through a cascade of molecular and cellular events\(^2\). The process of fracture healing is unique, in that it does not form a poorly organized replacement matrix, otherwise known as scar tissue, but rather regenerates the original matrix and retains its mechanical properties\(^3\). Normal fracture and bone repair follows a known sequence of events described below: haematoma formation, inflammation, angiogenesis, osteogenesis, and bone remodeling\(^4\) (Figure 1.1).
1.1.1 Early blood interactions in the fracture site

In the event of a fracture, blood from ruptured and destroyed tissue invades the fracture site\(^4\). This is followed by haematoma formation and platelet degranulation, releasing mostly TGF\(\beta\) and PDGF\(^5,6\), which are important in regulating migration, proliferation, and differentiation of committed mesenchymal progenitor cells\(^7\). At the same time, the complement system is activated releasing inflammatory products\(^8\). Inflammatory cells invade the site and release IL-1 and IL-6, which may be important in regulating the early events of the fracture healing process\(^6\).

1.1.2 Coagulation, Provisional Matrix Formation, and Inflammation

Platelet activation also results in the formation of a clot. The process of clot formation by platelets occurs in concert with the coagulation pathway. Activated platelets catalyze the production of thrombin, which then stabilizes the growing thrombus. This is done through the production of fibrin from fibrinogen. The end result is a three dimensional provisional matrix composed of fibrin\(^9\). Many signaling molecules are released in the clot, primarily by platelets. A chemotactic gradient of cytokines, growth factors and chemoattractants causes migration of inflammatory cells (neutrophils and monocytes), as well as mesenchymal cells and osteoprogenitors into the defect site. Initially neutrophils invade the defect site, lasting for the first 24-48 hours, however monocytes, upon differentiating into macrophages, and fibroblasts eventually overtake them\(^9\). Inflammatory products
released by the neutrophils and macrophages act as mediators of the inflammatory response (IL-1, IL-6, IL-8, tumor necrosis factor-α, and macrophage colony stimulating factor)\textsuperscript{10}.

### 1.1.3 Granulation Tissue and Angiogenesis

The proliferation of monocytes and macrophages, and later fibroblasts and vascular endothelial cells at the defect site leads to the formation of granulation tissue to replace the clot. Granulation tissue is seen as early as three days after fracture\textsuperscript{4}. Angiogenesis accounts for almost 60% of the granulation tissue and is observed in the wound site as a result of endothelial cells migrating from existing venules and forming capillary tubes\textsuperscript{11}. The other major activity within granulation tissue is synthesis of a connective tissue matrix by fibroblasts, consisting of collagen and proteoglycans. As wound healing progresses, the clot becomes rich in collagen, specifically collagen III\textsuperscript{9}. Some fibroblasts differentiate to myofibroblasts and they are responsible for wound contraction during this stage.

### 1.1.4 Osteogenesis

Following wound contraction in a fracture, when the fracture is not adequately stabilized, there is often formation of a callous and cartilage, which is then calcified, forming bone\textsuperscript{2}. It is important to note that most fractures show some degree of motion\textsuperscript{12}. This results in healing which includes both endochondral and intramembranous ossification, also known as ”primary” and ”secondary fracture healing”, respectively. Though, when there is significant stability in the fracture to prevent cartilage formation, a primary callus is formed and bone formation occurs by intramembranous ossification on both faces of the fracture site\textsuperscript{2}.
Woven Bone formation

During the formation of granulation tissue, pericytes from the newly formed vessels and osteoprogenitor cells from the marrow and the periosteum infiltrate the fracture site. Migration of mesenchymal and osteoprogenitor cells is mediated by platelet-derived signals. Migrating osteogenic cells attach to cell adhesion molecules such as bone sialoprotein (BSP), osteocalcin (OC), and osteopontin (OP) within the provisional matrix. Upon attachment, they begin to express bone morphogenetic proteins (BMPs). BMPs induce the differentiation of osteoprogenitors to mature secretory osteoblasts; BMPs 2, 6 and 9 are primarily involved in the differentiation of pluripotent mesenchymal stem cells into osteoprogenitor cells, and BMPs 2, 4, 7 and 9 further differentiate them to become osteoblasts. Immature secretory osteoblasts first produce an afibrillar matrix on the host bone. This matrix, typically 0.2-0.5 μm thick, is electron dense and consists of non-collagenous proteins, such as bone sialoprotein and osteopontin and proteoglycans from the plasma. These proteins provide nucleation sites for calcium mineralization, resulting in the formation of a non-collagenous, calcified layer called the cement line. After this, fully differentiated osteoblasts deposit a collagenous matrix, consisting of primarily type I collagen onto the afibrillar zone. Osteoblasts continue laying down collagen, which becomes mineralized, eventually becoming enveloped in their own matrix and forming osteocyte lacunae. This primary mineral matrix has an architecture known as woven bone. Woven bone is rapidly forming and allows for some mechanical stability in the fracture site until it is further remodeled into more mature lamellar bone.

1.1.5 Clinical problem and motivation

A bone defect may not heal because of local factors (infection, mechanical instability, inadequate vascularity, poor bone contact, magnitude of injury) and systemic conditions (malnutrition, smoking, diabetes, metabolic bone disorders). When the normal endoge-
nous mechanisms are not able to restore the lost bone, such as in non-union fractures, removal of benign bone tumors, or large-scale traumatic bone injury, surgical intervention is necessary.

Current therapeutic approaches to repair large bone defects can be divided into two groups: (1) without graft transplant (metallic implants, Ilizarov technique, and distraction osteogenesis), and (2) with graft transplant (autologous, allogenic, or xenogenic bone grafts, or the use of different graft substitutes). The use of metallic implants for rigid internal fixation, using either plates or intramedullary rods, is useful in restoring shape, mechanical function, and load bearing in the bone. However, use of these implants does not allow complete bone repair and remodeling since the metal plates and rods are not resorbable and cannot be remodeled by the body. As well, one must consider the implant surface and geometry, and the anatomical site into which the implant is placed, to ensure adequate osseointegration and mechanical support for the implant. One method that does not make use of internal fixation, The Ilizarov technique, consists of an osteotomy followed by bone distraction and avoids complications associated with poor implant osseointegration and graft integration. However, it is often a difficult procedure both for the surgeon and the patient. Alternatively, there are bone grafts and bone graft substitutes, which provide an osteoconductive matrix in which endogenous bone can grow, and often osteoinductive growth factors.

**Autograft**

There are approximately 2.2 million cases of skeletal defects world wide per year that require bone graft. Autograft, cancellous bone harvested from one anatomical site in the patient, often the iliac crest, and implanted in the defect, is the current gold standard for bone graft procedures. It provides osteogenic cells and bone marrow, osteoinductive proteins and factors, as well as an osteoconductive collagen matrix. However, autograft requires a second operation at the tissue harvest site, and this can create complications
including increased postoperative pain, bleeding, haematoma, infection and donor-site morbidity\textsuperscript{21,22}. Moreover, there is a limited supply of bone that can be harvested, specifically in the pediatric and geriatric patient population, and this makes it ineffective for treating large volume defects. While producing the most clinically predictable results, reports show that autograft still has a failure rate of 13-30\%\textsuperscript{23}.

**Allograft**

In reconstruction of large cortical and cancellous bone defects, a unique biological alternative to autograft is allograft, which is taken from a cadaver. While commonly used, allografts present the potential for disease transmission from donor to recipient, or immune responses to the mismatched antigens present in donor bone\textsuperscript{24–26}. Moreover, allograft bone has been shown to decrease in strength over time, with failure rates after massive allograft reconstructions reported around 50\% after 10 years in vivo\textsuperscript{27}.

Given the shortcomings with current graft and non-graft procedures, researchers have turned their attention to other therapeutic strategies. Tissue engineering strategies, combining porous scaffold with adult human stem cells and growth factors as the osteogenic agents are a less intrusive alternative that can supplement current bone repair techniques and address the need for large quantities of viable bone.

1.2 **Regenerative medicine and tissue engineering for bone repair**

When a multi-cellular organism sustains injury it restores homeostasis by one of two mechanisms. The first is the formation of a biological matrix to re-establish both physical and physiological continuity of the damaged area. The second is the process of regeneration, whereby developmental pathways are reactivated to restore the architecture and function of the original tissue. Regenerative medicine then seeks devise new therapies in
cases where the body’s own ability to restore the tissue is insufficient. Strategies developed to accomplish this fall into three general categories: (1) cell and growth-factor based therapy, (2) the use of biomaterials alone, or (3) the use of functional tissue engineered constructs from cells and biomaterials.

Tissue engineering is an interdisciplinary field that merges medicine, materials science and engineering in the design of functional artificial constructs for the purposes of "maintenance, regeneration, or replacement of malfunctioning tissues" \(^{28}\). To direct tissue repair, cells are seeded onto a resorbable scaffold, which provides them with mechanical and spatial cues that can direct their growth and differentiation in vitro, and allow delivery to the transplant site. Transplanted cells directly replace damaged endogenous cells or direct tissue replacement by the endogenous machinery through the release of soluble factors. This approach can theoretically be used to repair any tissue. However, to successfully regenerate the desired tissue one must consider each element of a tissue engineered construct.

### 1.2.1 Bone biomaterials

To differentiate between cellular therapy and tissue engineering\(^{29}\), the use of a biocompatible scaffold is essential to provide a three-dimensional framework in which cells can migrate, organize, and differentiate. For bone tissue engineering, scaffolds are designed to enhance specific attributes\(^{30}\): (1) biocompatibility - the ability to surpass an immunogenic and chronic inflammatory response, (2) osteoconductivity - a porous structure and high surface roughness which enables the infiltration of cells and neovasculature and the attachment, survival, and proliferation of osteogenic cells, (3) osteointegration - the formation of a direct structural and functional connection between the mineralized tissue and the surface of the scaffold, and (4) biodegradability - damaged tissue can be initially replaced with the scaffold, but is then able to regenerate and completely replace the synthetic matrix\(^{31}\).
Porosity is a decisive characteristic for scaffolds used as bone substitutes, as it has been shown to influence bone deposition\textsuperscript{32}. Porosity must be controlled to balance the ability to allow cellular infiltration and mechanical integrity. Pore shape and size must be suitable for osteoblast infiltration and complete interconnectivity is necessary to ensure adequate nutrient exchange and cell viability throughout the scaffold.

**Natural biomaterials**

Bone biomaterials are either naturally or synthetically derived. Natural bone substitutes include demineralized bone matrix (DBM) and collagen. Allogenic DBM has been used for several decades in humans for the treatment of non-unions and large defects, and is typically used as a bone graft "extender" or enhancer rather than a substitute\textsuperscript{32;33}. DBM is produced by decalcification of cortical bone, typically by hydrochloric acid, leaving a trabecular collagenous backbone that can serve as an osteoinductive and osteoconductive scaffold. The decalcification also decreases DBM’s antigenic stimulation and may enhance the release of bone morphogenic protein and other bone growth factors\textsuperscript{33}. Collagen is another naturally-derived bone biomaterial also typically used as a graft extender or carrier material for growth factors and stem cells\textsuperscript{34;35}. It is processed from purified collagen material (normally from animal sources) into aqueous solutions or suspensions at adequate pH. It can then be manufactured into porous collagen scaffolds or sponges, either through freeze-drying or exogenous cross-linking using a variety of chemical agents. As with any polymer, the degree of cross linking of collagenous materials directly affects its chemical, physical, and mechanical properties\textsuperscript{23}. Collagen sponges are currently the most effective delivery vehicle for recombinant bone morphogenetic proteins in bone tissue engineering applications\textsuperscript{23}. 
Synthetic biomaterials

Synthetic materials used for bone substitutes generally fall under three categories: ceramics, degradable polymers, or polymer-ceramic composites. Synthetic bone substitutes must be biocompatible and capable of osseointegration. They should also possess similar strength and elastic modulus to the bone being replaced, in order to prevent stress shielding and maintain adequate toughness for cyclic loading.\(^{20}\)

Ceramics are made from inorganic, non-metallic minerals that can possess a crystalline structure. Calcium phosphates, calcium sulfates, calcium carbonates, and bioactive glass are the most commonly used ceramics for bone substitutes\(^{20;32;36}\). Ceramic materials are favoured as bone substitutes for their high compressive strength. While ceramics do not exist naturally, and do not have intrinsic osteoconductive or osteoinductive capabilities, they have been used successfully for bone reconstruction both alone and when combined with stem cells and growth factors\(^{32;37;38}\). The commonly used tricalcium phosphate (TCP) has a stoichiometry similar to precursor bone, whereas hydroxyapatite (HA) is the mineral component of bone\(^{20}\). These ceramics work by preventing soft tissue formation and promoting the formation of an osteoid directly onto their surfaces. This osteoid then mineralises and the resulting new bone undergoes remodeling. While both TCP and HA are biocompatible, porous TCP is resorbable and is eventually replaced by the endogenous bone, while HA will not degrade and does not integrate well with host bone\(^{37}\). The large difference in mechanical strength of the HA implants as compared to the surrounding lamellar structure can result in stress shielding in the healing bone and fracture upon cyclic loading. Thus, HA is often combined with TCP or other materials for improved resorption and functionality\(^{36;39;40}\).

There is an increasing focus on the use of degradable polymers and polymer-ceramic composites for bone tissue engineering, owing to their “biocompatibility” and simple manufacturing\(^{31;41–43}\). Many different synthetic polymer matrices have been used for bone tissue engineering including polycaprolactones\(^{41;44}\), polylactide, polyglycolide\(^{45}\), and as-
Chapter 1. Introduction

sociated copolymers (polylactic-co-glycolic acid)\textsuperscript{31,42}. Although polymeric biomaterials are often "biocompatible", some materials may trigger acute and chronic inflammatory responses\textsuperscript{46}. Different polymer matrices have different mechanical properties, degradation times, and degradation mechanisms. For example, polylactic-co-glycolic acid will undergo bulk degradation and can elicit a chronic inflammatory response in vivo\textsuperscript{31}.

Polymer-ceramic composites can overcome some of the disadvantages of both materials, while maintaining their advantageous properties - namely, the array of different mechanical properties and degradation times of polymers and the mechanical strength and biocompatibility of ceramics. One example of a polymer-ceramic composite used for bone tissue engineering is a calcium phosphate (CP) coated CP-polylactic-co-glycolic acid (PLGA) composite scaffold developed by Lickorish et al.\textsuperscript{31}. This scaffold draws upon the ability of PLGA to be manufactured with a highly porous architecture, similar to trabecular bone, and combines this with embedded calcium phosphate particles, which enhance the stiffness of the polymeric matrix. The third phase, a biomimetic CP film, covers the external surface of the scaffold and minimizes the fibrous tissue encapsulation that occurs at the host/material interface.

1.2.2 Cell-based therapies

Cell-based therapies, as they apply to bone tissue engineering, focus on the development of engineered cells and tissues for the purposes of restoring function through transplantation. Cellular therapies have entered clinical practice, primarily through the widespread adoption of bone marrow transplantation. The development of techniques to expand cells ex vivo through advances in mesenchymal stem cell culture and bioreactor design for scaffold seeding has also led to the successful engineering of tissue constructs for bone repair. As a result products such as Trinity Matrix (Blackstone Medical, Springfield, MA) and Collagraft (NeuColl, Campbell, CA), allogenic cell-based implant constructs, have received regulatory approval and are currently being used clinically.
Chapter 1. Introduction

The source of cells used remains a primary issue in cell-based bone therapies. The use of a patient’s own cells (autologous transplantation), is appealing in that it does not require the use of immunosuppressive drugs and avoids the risk of immune rejection based on differences in histocompatibility antigens. However, there is often difficulty obtaining sufficient numbers of healthy cells for replacement, especially in elderly patients or patients with metabolic bone disorders, and thus additional time is required for ex vivo expansion before transplantation. As such, there is great interest in using allogenic or "universal" cell sources\textsuperscript{47-49}, from another patient, to create "off the shelf" therapies. This strategy however must consider the immunological complications that can arise with HLA mismatched donor and patient.

Traditional sources of cells for bone tissue engineering have included marrow stromal cells from humans\textsuperscript{36}, sheep\textsuperscript{50}, dogs\textsuperscript{39}, rats\textsuperscript{51}, pigs\textsuperscript{52}, and rabbits\textsuperscript{41;42}, and periosteum derived progenitor cells\textsuperscript{53;54}. While these are the most commonly used source, they have been criticized for requiring an invasive harvesting procedure and containing a low yield of progenitor cells available for regeneration\textsuperscript{55}. Embryonic stem cells (ESC) are an abundant source of pluripotent stem cells that have been used for bone tissue engineering in animal studies\textsuperscript{56}, however there are currently no practical techniques available to limit their pluripotency and control their differentiation in order to prevent teratoma formation. As well, their harvesting is fraught with ethical issues. However, with the current development of a reliable and safe method of producing induced pluripotent stem cells from skin fibroblasts or other sources, ESCs may become a feasible cell source for bone tissue engineering. To circumvent these problems, several groups are now looking into alternative sources of adult stem and osteoprogenitor cells, from adipose tissue, skeletal muscle, and umbilical cord tissue.
1.3 Multipotent mesenchymal stromal cells

Multipotent mesenchymal stromal cells (MSCs), also known as mesenchymal stem cells, are plastic-adherent cells isolated from the bone marrow and other tissues that can give rise to multiple connective tissues. More than fifty years ago, Till and McCulloch showed that cells from the bone marrow stroma were capable of developing colonies of cells\(^{57}\); these colonies are clones and the initiating cell is called a colony-forming unit-fibroblast (CFU-F). Friedenstein later showed the multipotential of these marrow derived MSC colonies in an ex vivo clonal assay, demonstrating their differentiation into various cell types, including osteoblasts\(^ {58} \).

Subsequent work revealed the plasticity of bone marrow stromal cells, demonstrating that these cells could be differentiated to osteoblasts, chondroblasts, and adipocytes simply by changing the culture environment. Based on these findings, Caplan defined the mesenchymal stem cell as a cell "whose progeny eventually give rise to skeletal tissues."\(^ {59} \) Moreover, he proposed the mesengenic process, outlining a process through which differentiation of the mesenchymal stem cell gives rise to the multiple mesenchymal lineages\(^ {60} \). However, MSCs derived from many sources represent a non-homogeneous population of cells and therefore, not all cells have the same potential for differentiation. More recently, investigators have suggested a hierarchal differentiation process, in contrast to Caplan’s mesengenic process. Muraglia et al. first hypothesized a tripotent stem cell based on human bone marrow stromal cell clones, whose default lineage is osteogenic\(^ {61} \). More recently, Sarugaser et al. have demonstrated a quintipotential stem cell whose default lineage is fibroblastic, based on single-cell seeded clonal self-renewal and multilineage differentiation assays with umbilical cord perivascular cells\(^ {62} \).

Although bone marrow has been the primary source for MSCs, reports have identified essentially identical cells from different sources, including umbilical cord blood\(^ {40} \) and Wharton’s Jelly\(^ {63} \), placenta\(^ {64} \), skeletal muscle\(^ {65} \), and adipose tissue\(^ {66} \), and virtually every other connective tissue. These different sources and their corresponding culture
systems have resulted in different denominations: Mesenchymal Stem Cells, Mesenchymal Progenitor Cells, Marrow Stromal cells, Skeletal Stem Cells. At the same time, authors have reported varied characteristics of these cells, depending on their source tissue, method of isolation and expansion, and their approach to characterizing the cells. In order to remedy this and facilitate the direct comparison of reported biologic properties and experimental outcomes in the field, the International Society for Cellular Therapy has proposed a minimum set of standards to define a multipotent mesenchymal stromal cell:  

1. **Adherence to plastic:** MSCs must be plastic adherent when cultured in standard tissue culture flasks under normal culture conditions. In contrast to this criteria, Wan et al. have claimed that their is an MSC population, specifically in bone marrow, that is non-adherent and only becomes adherent once differentiated.  

2. **Specific surface antigen expression:** $\geq 95\%$ of the cell population must express CD105, CD73 and CD90, as measured by flow cytometry. In addition, these cells must lack expression ($\leq 2\%$ positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Besides these standard markers, others have characterized MSCs using CD146, STRO-1 coupled with vascular cell adhesion molecule-1 (VCAM-1/CD106), neuron-glial Ag 2 clone 7.1 (NG2), and adhesion molecules CD29, CD44, CD49b, CD58 and CD166 and platelet-derived growth factor receptor.  

3. **Multipotential differentiation:** MSCs must be able to differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts. Others have also shown differentiation to smooth muscle cells, and tenocytes. More recently, some groups have shown that their plasticity extends to non-mesodermal-derived tissues, describing Multipotent Adult Progenitor Cells (MAPCs). These non hematopoietic (CD45-negative) cells are harvested from the bone marrow and cultured in low
serum medium containing specific growth factors. Reports have demonstrated that MAPCs can give rise to endothelial cells\cite{76} and, more surprisingly, to neuroectodermal (Schwann cells)\cite{77} and endodermal (hepatocytes)\cite{78,79} derivatives. There is still debate as to whether MAPCs are a subpopulation of MSCs, or a distinct adult stem cell population maintaining the differentiation potential of embryonic stem cells\cite{80}.

Regardless of the confusion in the multipotent stromal cell literature, there is a consensus that these cells possess significant therapeutic potential both in animal models and when used clinically in humans.

1.3.1 Bone marrow stromal cells as a therapeutic cell source

Adult human bone marrow contains a rare population (1:10,000 to 1:100,000) of multipotent mesenchymal stromal cells\cite{55}. Like MSCs isolated from other sources, the adherent cells from the bone marrow represent a heterogeneous population of multipotent cells whose true stem cell component is limited. Bone-marrow-derived MSCs (BMSCs) are functionally defined as nonhematopoietic, multipotential cells that support hematopoietic stem cell expansion in vitro and can differentiate into cells of different connective tissues. They are plastic adherent cells, that have a fibroblastic morphology in their undifferentiated state and can be extensively expanded in vitro. Human BMSCs have been harvested from the bone marrow of adults, children, and human fetuses\cite{44,81}.

Since Friedenstein demonstrated their osteogenic differentiation\cite{82}, BMSCs have been shown to differentiate into bone, adipose tissue, cartilage, muscle, and tendon in vitro. They have also been shown to have immunoprivileged and immunosuppressive properties in vitro and in vivo\cite{83,84}.

Because of their ability to differentiate into multiple tissues and be tolerated in allogenic transplantation, BMSCs have significant therapeutic potential. Adult BMSCs
transplanted into fetal sheep have been shown to engraft in numerous organs and differentiate along numerous tissue-specific lineages. In mice and sheep that received total body irradiation, BMSCs enhanced engraftment of donor hematopoietic cells after cotransplantation. In humans, Horwitz et al. demonstrated engraftment of functional allogenic BMSCs in children with osteogenesis imperfecta, resulting in increased bone marrow density. BMSCs have also been used to regenerate the marrow environment in advanced breast cancer patients after myeloablative therapy.

In addition to these therapeutic applications, BMSCs represent a valuable cell source for bone tissue engineering because of their demonstrated osteogenic potential, simple culture protocols, and immunoprivileged properties.

### 1.3.2 Bone marrow stromal cells for bone tissue engineering

In bone tissue engineering, human BMSCs are the most commonly used cell source. Since Goshima et. al first demonstrated new bone deposition in porous bioceramic scaffolds seeded with rat BMSCs, BMSCs have been combined with porous scaffolds to regenerate bone in experimentally induced osseous defects in both small and large animals.

Although these studies and others have provided promising results, the clinical applications of MSCs remains limited due to the relative difficulty of the harvesting procedure, and the quality of cells obtained when harvesting autologous cells from patients who may be elderly or sick. Studies have shown that the age of the donor affects the performance of the cells, as fetal MSCS have a greater expansion capacity in vitro, faster doubling time, and higher clonal frequency than adult MSCs. This can be attributed due to their having longer telomeres than adult MSCs. Also, as a result of their low clonal frequency, a significant expansion period ex vivo is required to produce a sufficient number of cells for transplantation. These current limitations highlight the need for a more robust donor cell population.
1.3.3 Immunological properties of mesenchymal progenitor cells

When considering the use of MSCs for cell-based therapies, one must consider their immunological properties. In this regard, MSCs have been reported to be uniquely immunoprivileged and immunosuppressive in vitro\textsuperscript{94–96} and in vivo\textsuperscript{47;97–99}. It is well established that undifferentiated MSCs constitutively express low surface densities of MHC class I molecules, responsible for presenting peptides derived from cytosolic proteins, and are negative for MHC class II molecules, responsible for antigen presentation to CD4\textsuperscript{+} T-cells. Moreover, they lack expression of costimulatory molecules CD40, CD80, and CD86, which could potentially induce T-cell anergy. Together, these properties may affect their ability to escape recognition by HLA-mismatched hosts and mitigate an immune response.

MSCs are capable of suppressing T-cell proliferation induced by alloantigens, mitogens, as well as activation by CD3 and CD28 antibodies in mixed lymphocyte reactions (MLR)\textsuperscript{96}. This suppression appears to have no immunological restriction, with similar suppressive effects observed with cells that were autologous, allogenic\textsuperscript{100;101}, or xenogenic\textsuperscript{95} to the responder cells. The mechanism of T-cell suppression is mainly driven by soluble factors, as demonstrated by continued suppression of T-cell proliferation when MSCs are separated from the responder cells (peripheral blood monocytes) by a semi-permeable membrane (transwell)\textsuperscript{94}. Although the in vitro capacity of MSCs to suppress T-cell proliferation has been convincingly demonstrated, some have hypothesized that the "immunosuppressive" effect of MSCs is in fact a non-specific antiproliferative effect. This hypothesis is supported by a recent study which showed that MSCs were capable of suppressing tumor cell apoptosis and proliferation\textsuperscript{102}. MSCs are also effective at suppressing the differentiation of monocytes and CD34\textsuperscript{+} progenitors into mature dendritic cells (DC), resulting in the formation of immature DCs with a decreased production of proinflammatory cytokines, and an increased production of anti-inflammatory cytokines\textsuperscript{96}. This suppressive effect of MSCs on dendritic cell differentiation is also mediated by soluble factors. Additionally, MSCs have also been shown to inhibit the proliferation of B cells.
Chapter 1. Introduction

and the proliferation, cytokine production, and cytotoxicity of natural killer cells. While these findings agree that MSCs can modulate the different immune cells in vitro, there is still debate about the immunological properties of MSCs in vivo. The immunoprivileged and immunomodulatory effects of MSCs have been explored in several animal models. Studies have shown that coinfusion of MSCs with allogenic skin grafts and bone marrow transplants in baboons reduces lymphocyte proliferation and prolongs graft survival. Systemic MSC infusion has been shown to be effective in treating graft-versus-host-disease (GVHD) following hematopoietic stem cells grafts in mice. This has generated a clinical interest in their use for treating GVHD and autoimmune disorders, with 17 clinical studies underway or recently completed to test the effectiveness of MSCs for treating GVHD after solid organ and stem cell transplants.

Some authors have suggested that MSCs are hypo-immunoprivileged and represent an immune tolerant universal donor. Their findings suggest that MSC transplants do not induce immunoreactivity, even to xenografts in immunocompetent animals. There is some evidence that supports this notion; engraftment of human MSCs in fetal sheep after the development of the immune system; engraftment of human MSCs and improvement in osteogenic repair in normal rats; engraftment of MSCs and improvement of cardiac function from mouse to rat, porcine to rat, and human to rat. Based on these findings, Chiu has theorized that MSCs, when implanted do not express MHC antigens and co-stimulant molecules, avoiding recognition and activation of the host effector T-cells. Additionally, they are capable of downregulating T-cell proliferation and activation, as described in vitro. Despite the presence of “danger signals” as a result of surgical trauma at the implant site, the T cells are not activated, in keeping with the Two-signal theory for “immune synapsis”. Once the MSCs have differentiated and begin to express MHC antigens and costimulatory molecules, the effects and response to tissue injury would have subsided, and the cells would have effectively evaded recognition by the immune immune system. However, Grinnemo et al. have presented conflicting
findings, indicating that xenotransplantation of human MSCs into the infarcted hearts of immunocompetent rats induces an acute immune response, with macrophage infiltration at the transplant site, and graft rejection\textsuperscript{83}. When the transplantation was repeated in immune deficient rats receiving immunosuppression, hMSCs were found in large quantities and survived in vivo up to six weeks\textsuperscript{83,107}. Grinemmo et al. have postulated two reasons for the xenogenic rejection of MSCs: (1) The increased inflammatory signals in a newly infarcted adult myocardium lead to the expression of costimulatory factors, making this an unfavorable environment for xenotransplant engraftment. (2) hMSCs, although capable of modulating immune responses in vitro by suppressing the formation of CD4 and CD8 T-cells, may not be sufficiently robust in an inflammatory xenogenic setting where they encounter both acquired and innate immunity. Our group has made similar observations, showing that hMSCs from the umbilical cord perivasculature injected into a skeletal muscle defect elicit an macrophage infiltration and do not survive beyond seven days\textsuperscript{108}. These findings warrant further examination of xenotransplantation of MSCs in other tissues to better understand the general mechanisms at work.

### 1.4 Human umbilical cord perivascular cells

The umbilical cord is a rapidly developing tissue in placental mammals that connects the fetus with the placenta, providing the fetus with nutrients and oxygenated blood and carrying away deoxygenated, nutrient depleted blood. The human umbilical cord normally contains two arteries and one vein, which are surrounded by a gelatinous substance, called Wharton’s Jelly, that is comprised mainly of fibroblast-like cells and mast cells embedded in an extracellular matrix rich in proteoglycans. E.W. Parry was the first to describe the "unusual fibroblast cells" of the Wharton’s Jelly as "mesenchymal" through in situ analysis of cord sections using histology and electron microscopy\textsuperscript{109}. He suggested that these cells were responsible for producing the collagen and other components of the
Wharton’s Jelly matrix, and they were distinct from the adventitial muscle cells on the vascular musculature. These cells were largely ignored until McElreavey et al. were the first to isolate, culture, and attempt to characterize the cells in the Wharton’s Jelly. Soon after, Takechi et al. provided evidence to support Parry’s postulation that these cells were not smooth muscle cells, describing them as myofibroblasts after in situ labeling of vimentin, desmin, $\alpha$-actin, and myosin. Naughton et al. then demonstrated that these cells were capable of differentiation to “prechondrocytes” when treated with TGF$\beta$ in vitro. This generated a burst of interest in the stem cell potency of cells from the Wharton’s Jelly, with authors reporting their differentiation into neural-like cells, cardiomyocytes expressing N-cadherin and cardiac troponin I among other cardiomyocyte markers, osteoblasts, and adipocytes. Wang et al. also first identified that this stromal population expressed known MSC markers CD105 (SH2 or endoglin) and CD73 (SH3), and coupled with its multipotency, represented a mesenchymal progenitor population similar to BMSCs. A more recent study by Jo et al. however, claims that stromal cells from the umbilical cord are more primitive, or ”immature”, than marrow stromal cells, expressing embryonic pluripotency markers NANOG, OCT-4, SSEA-3, SSEA-4, Tra-160, and Tra-181 and showing a lack of senescence and consistent expression of telomerase activity. However, there are conflicting reports of the expression of these embryonic markers and this may be due to the different isolation and culture methods used, which target different areas of the cord.

With these findings in mind, Sarugaser et al. hypothesized that there must be a mesenchymal progenitor population in the umbilical cord which enables it to expand 30-50 cm in length during the 40 week gestation period and this population would reside near its oxygen and nutrient source. They isolated a non-hematopoietic cell population from the perivascular region (0.5-1 mm from center of the vessel) of the human umbilical cord by digesting the tissue surrounding the vessels. These cells have a fibroblastic morphology in vitro and have been shown to express CD73, CD105, CD90, and CD44.
In addition to these markers, HUCPVCs also express high levels of CD146, α-actin, desmin, vimentin, and 3G5, a known pericyte marker. Surprisingly however, these cells do not express STRO-1, which has been shown to select for a more homogeneous MSC population.

Human umbilical cord perivascular cells (HUCPVCs) as a candidate MSC population for cell-based therapies pose several advantages over BMSCs. They are capable of osteogenic, chondrogenic, adipogenic, and myogenic differentiation. Moreover, they have been shown to improve regeneration of damaged mouse femora and to improve angiogenesis and healing in full thickness skin defects in NOD/SCID mice (unpublished results, John E. Davies lab, University of Toronto). Sarugaser et al. have shown that HUCPVCs have a significantly greater clonal frequency (1:333) compared to BMSCs (1:10,000 to 1:100,000). Baksh et al. have shown that HUCPVCs demonstrate a higher proliferative potential than human BMSCs and osteogenic differentiation of these cells proceeds more rapidly than in BMSCs. HUCPVCs also express higher levels of CD146, a recognized MSC marker, relative to BMSCs. Most importantly for cell-based therapies, HUCPVCs, which are major histocompatibility complex class I and II negative, express both an immunoprivileged and immunomodulatory phenotype in vitro. Moreover, MHC class I expression levels can be manipulated, allowing the possibility of avoiding alloreactive T cells or a host immune response upon transplantation. All this suggests that HUCPVCs are a suitable candidate for cell-based therapies and tissue engineering.

1.4.1 Human umbilical cord perivascular cells as an osteogenic cell source

The osteogenic potential of MSCs is well established in vitro, and in small and large animals, as well as humans, as discussed previously. Similarly, the in vitro osteogenic potential of stromal cells from the umbilical cord and the umbilical cord blood has been demonstrated in several reports. However, owing to their relative novelty, the in vivo
osteogeneic potential of MSCs from the umbilical cord connective tissue is not as widely reported.

Diao et al. report on the in vivo implantation of a progenitor cell population isolated from the Wharton’s Jelly surrounding the blood vessel, on a nano-hydroxyapatite/collagen/poly-(lactic acid) composite scaffold in an ectopic site in Balb/c nude mice. They found that the cells grew into pores of the scaffold and formed bone-like matrix, and following 12 weeks of implantation, preosteoblasts, and osteoblasts were observed under transmission electron microscopy. They also showed that these cells stained positively for human osteocalcin. Osteogenesis was not observed in the control scaffolds, and so they postulated that the bone growth in the scaffold was most likely of human origin, although they did not confirm this through cell tracking or immunohistochemistry.

Sarugaser et al. were the first to demonstrate the in vivo osteogenic potential of HUCPVCs. HUCPVCs were transplanted through the knee into the intrafemoral space of NOD/SCID mice and were shown to survive up to six weeks. Furthermore, they showed that at 2 and 4 weeks there was significantly more healing of bone and cartilage in the HUCPVC-injected femurs than in contralateral sham-injected controls. The transplanted cells were shown to produce collagen II in the femoral growth plate and osteocalcin was present in the cytoplasm of cells as well as in the osteoid being produced. However, the majority of cells in the healing tissue did not label positively for human nuclei and so it was postulated that HUCPVCs were largely responsible for recruiting the mouse mesenchymal progenitors to repair the damaged tissue, most likely through soluble factors.

Zhang et al. found similar results in NOD/SCID mice using cells extracted from the human umbilical cord in a manner similar to HUCPVCs, and they were able to track the fate of the cells up to 2 months through staining for human nuclei (lamins A and C). They also compared the osteogenic potential of the umbilical cord cells with adult bone marrow and fetal bone marrow, and found that the bone marrow cells generated a
greater mineral volume and new bone volume in vitro and in vivo, respectively.

1.5 Rationale

Cells from the human umbilical cord vasculature have been shown to derive early osteo-
progenitors through culture condition manipulation\(^6\), providing an alternative source
over the commonly used human bone marrow mesenchymal stromal cells (BMSCs) for
bone-regenerative therapies. In a comparison of BMSC and human umbilical cord perivas-
cular cells (HUCPVCs), Baksh et al. have demonstrated that HUCPVCs proliferate faster
than BMSCs. This can translate into a shorter turnaround time to generate therapeu-
tic doses of cells for transplantation. Combined with the known osteogenic capabilities of
biocompatible calcium phosphate-coated scaffolds, HUCPVCs make an attractive candi-
date for bone tissue engineering therapies.

Previous work with HUCPVCs in immunodeficient mice has demonstrated their os-
teogenic potential in vivo\(^6\). Following injection of HUCPVCs into the intrafemoral space
of NOD/SCID mice, Sarugaser et al. observed significantly greater bone density at two
and four weeks as compared to the sham controls. Other authors have demonstrated the
osteogenic potential of Wharton’s Jelly cells, which are closely related to HUCPVCs, in
an ectopic site in Balb/c nude mice\(^1\) and in NOD/SCID mice. One common thread
among these in vivo experiments is the use of immune deficient animals. Due to the com-
plex interactions that occur during cell transplantation and bone healing, it is necessary
to observe the function of these cells in an immunocompetent animal model in order to
make clinically relevant conclusions.

HUCPVCs and other Wharton’s jelly-derived MSCs have been shown to be immuno-
privileged and immunomodulatory in vitro\(^9\). In vivo, studies have shown that al-
logenic and xenogenic adult MSCs can be tolerated in immunocompetent hosts in a
subcutaneous pocket, the brain tissue, cardiac tissue, and bone. However,
other studies have observed graft rejection in the same tissues using both allogenic\(^{124,125}\) and xenogenic\(^{83,107}\) transplants. Thus, there is a lack of consensus within the literature regarding the in vivo fate of transplanted MSCs.

The main purpose of this work is, therefore, develop a bone tissue engineering construct that will be used to assess the osteogenic potential of HUCPVCs in a larger animal model than those employed previously. It will be important to assess if HUCPVCs can avoid a host immune response, survive, and differentiate in an immunocompetent Wistar rat and whether they will be functionally active and contribute to bone regeneration.

### 1.6 Hypothesis

Human umbilical cord perivascular cells will avoid a host immune response, survive, and differentiate when transplanted into an immunocompetent host. Moreover, when combined with osteoconductive scaffold, they will enhance and contribute to bone regeneration when compared to an empty scaffold.

### 1.7 Objectives

The objectives of this work are to:

1. Immunophenotype HUCPVCs and compare them to BMSCs, and assess their osteogenic gene expression.

2. Assess the in vivo osteogenic potential of HUCPVCs.

3. Determine if HUCPVCs are immunopriveleged in vivo in a xenogenic model.

These objective will be accomplished by:
1. Comparing surface marker phenotype of HUCPVCs to hBMSCs through flow cytometry and examining their osteogenic gene expression through RT-PCR and comparing this to gene expression in human bone.

2. Developing a tissue engineering construct that can deliver HUCPVCs to an orthotopic defect. This will involve:
   
   - devising a cell seeding technique on porous scaffolds and assessing its suitability for attaching HUCPVCs through the use of an alamar blue cell metabolism assay.
   - determining the appropriate subculture routine to expand HUCPVCs on the porous scaffolds after seeding and assessing cell distribution and cell morphology through scanning electron microscopy and light microscopy.

3. Assessing whether there is a systemic immune response to xenotransplantation of HUCPVCs in an immunocompetent Wistar rat through a complete blood count analysis.

4. Quantifying, through micro computed tomography evaluation, the reparative bone formation of the HUCPVC-seeded constructs in rat femoral defects at several time points.

5. Tracking HUCPVC fate and contribution to bone regeneration in the femoral defects through fluorescence microscopy, immunohistochemistry, and standard histology.
Chapter 2

Materials and Methods

2.1 Cell isolation and culture

Frozen aliquots of human umbilical cord perivascular cells, either first or second passage after harvest, were kindly provided by Tissue Regeneration Therapeutics, Inc (Toronto, ON). HUCPVCs were harvested according to the protocol described by Sarugaser et al. \(^6^2\). The cells were thawed and plated in T-75 and T-150 (75 and 150 cm\(^2\), respectively) tissue culture polystyrene dishes (Falcon) in supplemented medium (SM) (80% α-MEM; Gibco, 10% antibiotic stock solution [0.1% Penicillin, Sigma-Aldrich, St. Louis, MO; 1% Gentamicin, Sigma-Aldrich, St. Louis, MO; and 0.3% Fungizone], 10% fetal bovine serum [FBS]; HyClone, Lot # KPJ22093), which was changed every 2 days. Cells were incubated at 37°C and 5% CO\(_2\). At day 4, adherent cells, judged 80% to 90% confluent by phase contrast microscopy, were passaged using 0.05% trypsin/0.02% EDTA solution (Gibco, Invitrogen, Carlsbad, CA). Cells used for seeding studies and transplantation were pooled from four different donors and three different passages (P3-P5, the maximal cultivation time was 3 weeks). Cells used for flow cytometry were obtained from four donors and two passages (P2 and P3). Cells used for PCR were obtained from a single donor population (n=1) after P5.
Chapter 2. Materials and Methods

2.2 Cell phenotyping and gene expression

2.2.1 Flow cytometry

HUCPVCs were analysed for cell surface markers, listed in table 2.1. Some samples were processed with the help of Tissue Regeneration Therapeutics, and flow cytometry data was collected with the help of Tissue Regeneration Therapeutics and Cytoquest Corp. (Toronto, ON). HUCPVCs were either thawed directly before analysis (referred to as 'thaw' or 'cryopreserved') or plated, trypsinized, and passaged before analysis (referred to as 'plated'). For staining, \( \leq 1 \times 10^5 \) cells were pelleted and resuspended in PBS.

To stain, non-specific binding was blocked 1% bovine serum albumin in PBS for 30 minutes on ice. The cells were then incubated with fluorescently conjugated primary antibodies, according to table 2.1, for 30 minutes on ice. For CD90, cells were incubated with a non-fluorescent conjugated primary followed by incubation with a FITC-conjugated secondary antibody for 30 minutes. Control cells were prepared by omitting the primary antibody and compensation controls were created according to table 2.2. Flow cytometry compensation was performed primarily by Elaine Cheng from Tissue Regeneration Therapeutics. In each case, the cells were gently pelleted and washed with PBS rinses between each incubation step. The same analysis was performed on cryopreserved human bone marrow stromal cells. Cryopreserved HUCPVCs were obtained from four different donors, (n=3 donor populations at P2 and n=1 donor population at P3), plated HUCPVCs were obtained from three different donors (n=2 donor populations at P1 and n=1 donor population at P2), and human bone marrow stromal cells (hBMSCs) from one donor (n=1 donor populations used at P1 and P2) were analyzed. Samples were analysed using a Beckman Coulter flow cytometer and data was processed using proprietary software. Results for each cell type were averaged across the donors and passages and the mean percent positive expression and mean fluorescence intensity (MFI) and standard deviations were reported.
### Table 2.1: Primary antibodies for flow cytometry analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Fluorescent Conjugate</th>
<th>Manufacturer</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD73</td>
<td>Mouse</td>
<td>PE</td>
<td>BD Biosciences</td>
<td>550257</td>
</tr>
<tr>
<td>CD90</td>
<td>Mouse</td>
<td>Alexa Fluor 647</td>
<td>BioLegend</td>
<td>328116</td>
</tr>
<tr>
<td>CD105</td>
<td>Mouse</td>
<td>APC</td>
<td>BioLegend</td>
<td>323208</td>
</tr>
<tr>
<td>CD146</td>
<td>Mouse</td>
<td>PE</td>
<td>BD Biosciences</td>
<td>550315</td>
</tr>
<tr>
<td>NG2</td>
<td>Mouse</td>
<td>FITC</td>
<td>R&amp;D Systems</td>
<td>FAB2585F</td>
</tr>
<tr>
<td>PDGF-RB</td>
<td>Mouse</td>
<td>PE</td>
<td>R&amp;D Systems</td>
<td>FAB1263P</td>
</tr>
<tr>
<td>MHC I</td>
<td>Mouse</td>
<td>PE</td>
<td>BD Pharmigen</td>
<td>555553</td>
</tr>
<tr>
<td>MHC II</td>
<td>Mouse</td>
<td>FITC</td>
<td>BD Pharmigen</td>
<td>555558</td>
</tr>
<tr>
<td>CD40</td>
<td>Mouse</td>
<td>PE-Cy5</td>
<td>BD Biosciences</td>
<td>555590</td>
</tr>
<tr>
<td>CD80</td>
<td>Mouse</td>
<td>FITC</td>
<td>BD Pharmingen</td>
<td>555683</td>
</tr>
<tr>
<td>CD86</td>
<td>Mouse</td>
<td>PE</td>
<td>BD Pharmingen</td>
<td>555658</td>
</tr>
</tbody>
</table>

### Table 2.2: Distribution of primary antibodies per tube of cells for flow cytometry analysis.

<table>
<thead>
<tr>
<th>Tube</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD80  FITC</td>
<td>CD146 PE</td>
<td>CD105 APC</td>
</tr>
<tr>
<td>2</td>
<td>MHC II FITC</td>
<td>MHC I PE</td>
<td>CD90 Alexa 647</td>
</tr>
<tr>
<td>3</td>
<td>PDGF-RB PE</td>
<td>CD40 PE-Cy5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NG2 FITC</td>
<td>CD73 PE</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CD86 PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td>Unstained</td>
<td></td>
</tr>
<tr>
<td>FL1 Positive</td>
<td>CD90 (Primary)</td>
<td>FITC IgG (Secondary)</td>
<td></td>
</tr>
<tr>
<td>FL2 Positive</td>
<td></td>
<td>CD73 PE</td>
<td></td>
</tr>
<tr>
<td>FL4 Positive</td>
<td></td>
<td>CD90 Alexa 647</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 RT-PCR

RNA was extracted from HUCPVCs (P5) in monolayer in normal SM using TRI Reagent (Applied Biosystems, Austin, TX) according to the manufacturer’s RNA Isolation procedure. cDNA was synthesized using 0.2-1 µg total RNA in the presence of gDNA Wipeout Buffer 7X, RNase-free water, reverse transcriptase, RT Buffer 5X, and RT Primer mix (All from the Quantitect Reverse Transcription Kit For cDNA Synthesis, Qiagen, Mississauga, ON) according to the manufacturer’s protocol. cDNA from human bone was kindly provided by Dr. Bernhard Ganss. The DNA sample was stored frozen until use. PCR was performed in a 10.5 µL reaction solution containing 1 µl 10x PCR Accuprime buffer, 0.3 µl of 50M MgCl2, 0.25 µl of 10 mM dNTP, 0.5 µl primer, 0.5 µl of undiluted cDNA and 0.04 µl of Platinum Taq polymerase (Sigma-Aldrich, St. Louis, MO). The PCR conditions were as follows: 5 min at 94°C followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s, and final extension for 10 min at 72°C. Primer sequences are provided in table 2.3.

2.3 Tissue engineering of a cell-scaffold construct

2.3.1 Preparation of a three-phase resorbable scaffold

Three-phase resorbable calcium phosphate (CP) - coated poly(lactic-co-glycolic acid) (PLGA) scaffolds (Osteoscaf™, Tissue Regeneration Therapeutics, Inc. Toronto, ON) were kindly prepared with the help of Limin Guan at Tissue Regeneration Therapeutics, Inc. Scaffolds were prepared as previously described by Lickorish et al.31. Briefly, CP cement particles were prepared by mixing equimolar TTCP and DCPA with deionized distilled water (ddH2O) at 100% relative humidity for 24 h. These were ground and sieved through 45 µm. To prepare the scaffold, PLGA was dissolved in DMSO at a concentration of 11.5% (w/v). To this solution, CaP particles were added at a CaP/PLGA ratio of
Table 2.3: Primers for amplification of osteogenic markers used in RT-PCR for analysis of RNA isolates from HUCPVCs and excised human bone.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Product Size (kbp)</th>
</tr>
</thead>
</table>
| Osteocalcin | S - ATGAGAGCCCTCACA CACTCCTC  
                AS - GCCGTAGAAGCGCCGATAGGC | 294 |
| OPN         | S - GCCGAGGTGATAGTGTGGTT  
                AS - GTGGGTTTCAGCAGC ACTCTGGT | 242 |
| ALP         | S - CCTCCTCGGAAGACACTCTG  
                AS - AGACTGC GCCTGGTAGTTTG | 239 |
| BSP         | S - ATCATAGCCATCGTAGCCTTGT  
                AS - AATGAAAACGAAAGAAAGCGAAG | 450 |
| RUNX2       | S - AGATGATGACACTGCCCACCTCTG  
                AS - GGGATGAAATGCTTTGGGAAC TGC | 125 |
| rhGAPDH     | S - ACCACAGTCCATGCCCATCAC  
                AS - TCCACCACCCTGGTTGCTGTA | 452 |

S - sense sequence, AS - anti-sense sequence.
2:1 (w/w). Sugar crystals with size ranges of 0.85 to 1.18 mm were dispersed in the CaP/PLGA and the mixture was solidified at -18 C. The PLGA was precipitated and the sugar crystals leached out by soaking in three changes of ddH$_2$O. This was used to produce blocks of the polymer that were 10 cm×10 cm×2.5 cm. Scaffolds with a diameter of 2.3 mm diameter and 2.3-2.5 mm height were trephined out of the block. To deposit a thin surface coating of CaP onto and throughout the pores of the scaffold, prepared scaffolds were pre-wetted in 70% ethanol and immersed in 3×SBF (500 ml/g scaffold) for 24 hrs at 37°C. Coated scaffolds were washed in ddH$_2$O and air dried. Following coating, scaffolds were gamma-sterilized, followed by disinfection with 70% ethanol prior to seeding HUCPVCs.

2.3.2 Cell seeding

Cells were seeded on CP-PLGA scaffolds in one of two ways:

1. Static seeding: Approximately $7.2 \times 10^5$ HUCPVCs were detached from culture plates, suspended in 300 ml SM, and pipetted onto the scaffold in a six well polystyrene plate. The scaffold was incubated at 37°C and 5% CO$_2$ in the suspension for one hour after which it was resuspended in 1 ml of fresh SM, and placed on a rotating shaker at 37°C and 5% CO$_2$.

2. Centrifugation seeding: A 0.25, 0.5, or 1.0 $\times 10^6$ cells/ml suspension in 1 ml of SM was added to a 1.5 ml eppendorf tube containing a scaffold as pictured in figure 2.1. The solution was centrifuged at 30 g and 4°C for five-one minute intervals with mixing to resuspend the cell pellet between centrifugation intervals. The scaffolds were then incubated in the suspension at 37°C and 5% CO$_2$ on a rotating shaker for one hour after which the medium was replaced with fresh SM. This was changed every two days thereafter.
The effect of fluid flow during the incubation period on cell attachment to the scaffold was assessed by either laying the tube containing the cell suspension horizontally or standing it vertically on the rotating shaker during the one hour incubation period post-centrifugation. While standing on a shaker, the fluid flow inside the container and around the scaffold is reduced because of the small diameter of the 1.5 ml eppendorf tubes used. However, when the tube is laid horizontally, there is significantly greater fluid flow and movement of the scaffold along the length of the tube.

2.3.3 Assessment of cellular attachment and viability

To quantify the number of viable cells attached on the scaffold, an alamar blue (AB) cell viability assay (AbD Serotec, Raleigh, NC) was used. The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction.

A standard curve was prepared by plating HUCPVCs in a 96-well plate in densities ranging from 1,520 cells/well to 152,000 cells/well and allowing them to attach overnight.
in SM with 2% FBS at 37°C and 5% CO₂. After the attachment period, SM was replaced with fresh SM and AB solution was added to a final concentration of 10%. Cells were incubated in this solution for three hours at 37°C and 5% CO₂. As negative control (no reduction) AB solution was added to the medium without cells and the positive control (100% reduction) was AB solution added to medium and autoclaved for 15 minutes.

Cell-scaffold constructs were also incubated in 1 ml of the 10% AB solution under identical conditions and 100 µl was collected after three hours to measure the fluorescence.

The fluorescence of test and control wells was read at an excitation wavelength of 530 nm and emission wavelength of 590 nm with a BioTek Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Viable cell numbers were calculated using the following equation (F: Fluorescence):

\[
\frac{F_{\text{scaffold+cells}} - F_{\text{blank}}}{F_{100\% \text{reduction}} - F_{\text{blank}}} \times \text{slope}_{\text{stdcurve}}
\]

(2.1)

### 2.3.4 Visualizing cell attachment on cell-scaffold constructs

After five days of culture, scaffolds were fixed in 10% neutral buffered formalin for 20 minutes for toluidine blue staining or in Karnowsky’s fixative for two hours for scanning electron microscopy.

To visualize the overall distribution of cellular material on the scaffolds, cell-scaffold constructs were immersed in a 1% toluidine blue solution for 20 minutes, followed by washing in tap water overnight. Stained constructs were then observed and photographed under a dissecting microscope in combination with a computer-supported imaging system (Openlab 4.0.4, Improvison, Coventry, England).

To observe the morphology of HUCPVCs attached to the CaP-PLGA scaffolds, fixed cell-scaffold constructs were critical point dried and coated with gold using a Polaron sputter coater. Cell morphology was visualized by scanning electron microscopy (Hitachi S2500, Japan).
Chapter 2. Materials and Methods

2.4 In vivo transplantation

2.4.1 Cell-scaffold construct preparation

HUCPVCs at passage three or four were stained with Vybrant DiD, DiR, or not stained. Staining was performed by detaching HUCPVCs from culture flasks with 0.05\% trypsin/0.02\% EDTA and washing twice in PBS. Cells were then resuspended at $1 \times 10^6$/ml cells in SM and incubated for 5 minutes at 37\°C and 15 minutes at 4\°C in a solution of 5 $\mu$l DiD or DiR/ml of cell suspension. The labelled cells were then allowed to recover for 5 minutes in SM and then they were rinsed twice with PBS before proceeding to scaffold seeding.

Cell were then resuspended at $0.25 \times 10^6$ cells in 1000 $\mu$l in a 1.5 ml microcentrifuge tube. Scaffolds were soaked in SM at 37\°C and 5\% CO$_2$ for one hour then placed in the cell suspension and seeded by centrifugation as described in section 2.3.2. The constructs were cultured for five days prior to transplantation.

2.4.2 Animal subjects

Ethical approval was obtained from the University of Toronto Animal Care Committee (Protocol #20007165). 39 Wistar rats (weight 250-300 g, Charles River Laboratories, Wilmington, MA) and 36 rnu/rnu nude rats (Crl:NIH-Foxn1rnu, weight 200-250 g, Charles River Laboratories, Wilmington, MA) were kept in a controlled environment, (athymic rats were kept in a sterile facility) and given free access to food and water throughout the study period.

2.4.3 Femoral implantation

Animals were anesthetized with inhalation anesthesia (isofluorane in nitrous oxide and oxygen, 900 ml total flow rate; 4\% induction and 2\% maintenance), using a nose cone and sterile conditions. Analgesia (0.01 to 0.05mg/kg Buprenorphine) was administered
subcutaneously both pre-operatively and post-operatively. Each femur was exposed by a lateral incision, and a bilateral defect, 2.3mm diameter, was created in the distal metaphysis using a 2.3mm dental burr under constant saline irrigation. The drilled hole was made only through a single cortex. Figure 2.2 depicts the steps of the surgical procedure. Scaffolds were implanted in the defect and left for up to eight weeks. Cell-scaffold constructs were implanted contra-lateral to an empty scaffold in each animal. Three rats acted as controls for blood sampling, receiving empty scaffolds in both femurs. Table 2.4 gives an overview of the distribution of different groups and time points.

<table>
<thead>
<tr>
<th>Time Points</th>
<th>Experimental</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>5+2 for RNA</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>5+1 for RNA</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td>4+1 for RNA</td>
<td>5 + 5*</td>
</tr>
<tr>
<td>30</td>
<td>5+1 for RNA</td>
<td>5 + 5*</td>
</tr>
<tr>
<td>60</td>
<td>5+1 for RNA</td>
<td>4**</td>
</tr>
</tbody>
</table>

*Animals received scaffolds seeded with $7.5 \times 10^5$ HUCPVCs instead of $2.5 \times 10^5$.

**Sacrificed at 45 days. ***Controls not receiving any HUCPVCs, only empty scaffold.
Figure 2.2: Sequence of surgical steps. (a) The femur is exposed. (b) A 2.3 mm defect is drilled in the distal aspect of the femur with constant saline irrigation. (c) The defect is exposed and cleaned of any debris. (d) The cell-scaffold construct is loaded into the defect. (Not pictured) The defect is then secured by suturing the overlaying muscle prior to wound closure with surgical staples.
2.4.4 Blood sampling

Under anesthesia, blood samples of 200-500 µl venous blood and the weight of the Wistar rats, were taken every five days, starting with the day of surgery, throughout the study period. The blood was analysed using a Coulter Ac·T diffTM blood counter (Beckman Coulter, Fullerton, CA). White (WBC) and red blood count (RBC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDV), mean platelet volume (MPV), and differential blood count (lymphocytes, monocytes, granulocytes) served as parameters to detect any systemic influences after xenotransplantation.

2.4.5 Morphometric analysis of bone healing

Animals were euthanised by cervical dislocation after CO₂ exposure at each of the time points indicated above. Femurs were harvested, trimmed leaving the epiphyses and some of the distal metaphysis. They were then fixed in 10% neutral buffered formalin or zinc formalin (Sigma-Aldrich, St. Louis, MO) for a minimum of 48h. Defects were observed macroscopically under a dissecting microscope.

To evaluate bone healing, micro computed tomography of the harvested femurs was performed after fixation using a microtomography system (MicroCT40, Scanco Medical, Basserdorf, Switzerland). All trimmed femur samples were placed in a poly-methyl-metacrylate (PMMA) holder with tap water or fixative and scanned at 70kVp and 114 µA. The specimens were scanned in high-resolution mode with an X, Y, and Z resolution of 6 µm, and acquisition files were obtained at 1000-1300 projections with 2,048 samples each (per 180 of rotation), 0 angle increment, 300ms of integration time, and 1 frame averaging. The scanning time for each specimen was approximately 2.1-2.3 hours.

Total mineral volume in the defect site was determined using custom software (Scanco Medical, Basserdorf, Switzerland). Briefly, the marrow cavity in the defect site was con-
Chapter 2. Materials and Methods

Figure 2.3: Mineral volume measurements in the femoral defect using Micro computed tomography. (a) The bone in the marrow cavity is contoured throughout the entire volume of the defect to determine the mineral volume (MV). (b) The total volume of the defect is determined by calculating the volume of a cylinder of the same height as the one measured here.

toured throughout the entire defect as pictured in figure 2.3a. The software determined the mineral volume (MV) in the marrow cavity based on a threshold determined by the user within the defect volume. The measurements spanned the defect site (2.3 mm depth). Three measurements within the center of the defect site were taken to determine the height of the defect. This height was measured from the supposed edge of the damaged cortex to the endosteal surface of the opposing cortex, as seen in figure 2.3b. The average of these measurements was used as the height of a hypothetical cylinder in the defect (2.3 mm diameter) and a volume was calculated (TV). A ratio of MV:TV was reported for each femur to assess the extent of bone regeneration.
2.4.6 RT-PCR tracking of HUCPVCs implanted in rat femora

One femur from each time point was used for RNA extraction. The femur was harvested after sacrifice and snap frozen in liquid nitrogen. The excess bone was trimmed using a sterile drill bit, leaving only the defect and some surrounding cortical bone. The trimmed piece was then snap frozen in liquid nitrogen, placed in Trizol reagent, and crushed using a cooled mortar and pestel. The trizol and tissue fragments were then collected and the RNA from this was purified as described above. RNA was converted to cDNA, as described above, and RT-PCR was performed using human specific primers, shown in table 2.3, and conditions described above.

2.4.7 Histology and Immunohistochemistry

Harvest and trimmed femurs were either processed for paraffin embedding or undecalcified resin embedding. For paraffin embedding, femurs were decalcified in a formic acid (22.5%)/sodium citrate (10%) solution, dehydrated in grades of ethanol, and embedded in paraffin. Sections were prepared at 6 µm on a microtome and stained with hematoxylin and eosin, Goldner's trichrome, Maximow's stain for bone marrow, and Masson's trichrome (see Appendix for protocols).

For immunohistochemistry, femur sections were incubated in a 1 mg/ml trypsin solution containing 4 mM CaCl$_2$, 200 mM Tris, pH 7.7 (Sigma-Aldrich, St. Louis, MO) in a 37°C water bath for 30 minutes to unmask the antigens. Endogenous peroxidase in the tissue was quenched by incubating the sections with 3% hydrogen peroxide in methanol for 10 minutes. To identify human cells in the defect, femur sections were incubated with a monoclonal mouse anti-human nuclei antibody (1:50, Millipore Chemicon, Billerica, MA) or monoclonal mouse anti-human mitochondria antibody (1:20, Millipore Chemion, Billerica, MA) in 0.1% PBS-Triton overnight at 4°C. After rinsing in 0.05% PBS-Tween, specimens were incubated for 30 minutes with a biotinylated anti-mouse
IgG (H+L) adsorbed against rat (1:200, Vector Laboratories, Burlingame, CA). Formation of an avidin-biotin complex was performed on the sections using a standard Vector ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer protocol. Vector Novared (Vector Laboratories, Burlingame, CA) was used as a chromogen to visualize the reaction product. Sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO). The stained sections were then dehydrated and sealed with Entellan mounting medium (EMD Chemicals, San Diego, CA).

The expression of human osteogenic proteins was identified using monoclonal mouse anti-human osteopontin and osteocalcin antibodies (1:20, R&D systems, Minneapolis, MN). Activated macrophages in the defect site were identified using monoclonal mouse anti-rat CD68 antibody (1:50, AbD Serotec, Raleigh, NC). All sections were observed and photographed with a light microscope (Leitz Aristoplan, Leica Microsystems, Wetzlar, Germany) in combination with a computer-supported imaging system (Openlab 4.0.4, Improvison, Coventry, England).

Undecalcified resin histology

A modified version of the Osteo-Bed Bone Embedding Kit protocol for small samples was used for undecalcified resin embedding (Donath Technique - Exakt Products Histology, US).

Briefly, femurs were harvested, trimmed, and fixed in 3% paraformaldehyde for 24-48 hours. They were then scanned by microCT as described above. After scanning, they were placed in specimen glass vials and washed in dH$_2$O for 1 hour. Next, the samples were dehydrated in grades of acetone: 50% for two days, 1 change after 8 hours; 75% for two days, 1 change after 8 hours; 100% for two days, 1 change after 8 hours; and a 50:50 solution of acetone and Osteobed monomer for 24 hours. Then, the samples were infiltrated with the monomer Osteo-Bed I for 4 days (1 change after 2 days) under vacuum at room temperature. After this, the samples were embedded in the Osteobed
polymer solution (1.4g benzoyl peroxide/100mL monomer) for 4 days (1 change after 2 days) under vacuum at 4°C before being placed in the oven at progressively higher temperatures (25°C for 3 days, 50°C for 3 days, and 60°C for overnight) to allow the resin to set.

After setting, the glass vials were broken and the resin blocks were ground and polished and glued to backup slides. From the backup slides sections were cut, ground, and polished to a final thickness of 30-40 µm. These sections were then analysed under a phase contrast microscope equipped with a fluorescent lamp and optical filters for DiD (Omega XF47, Omega Optical, Brattleboro, VT) and DiR (Omega XF112).

2.4.8 Statistics

Statistical analysis on blood count parameter data was analysed by the method of generalized estimation mating equations (GEE) using Matlab R2007a software (The Mathworks, Natick, MA) and the GEEQBOX toolbox (University of Pennsylvania, Philadelphia, PA). The blood count data was first imported into Matlab and transformed using the ‘box-cox’ function, which transforms non-normally distributed data to a set of data that has an approximately normal distribution. The transformed data was then input into the GEEQBOX function and processed as a normal distribution with an AR(1) correlation structure. From the output, the p-value for the covariate parameter estimates based on the robust covariance matrix were reported for each parameter. P-values lower than 0.05 were considered significant and this indicated that the experimental and control data were statistically different.

Statistical analysis on all other data, including the cell seeding counts and morphometric data, was performed by a two-sample t-test assuming equal variances in Excel 2007 (Microsoft, Redmond, WA). P-values from the t-tests were reported and values lower than 0.05 were considered significant.
Chapter 3

Results

3.1 Cell Immunophenotyping and gene expression

3.1.1 Flow Cytometry

Cryopreserved HUCPVCs (n=3 at passage 2 and n=1 at passage 3) which were thawed and directly analysed, and plated HUCPVCs which were trypsinized and resuspended prior to analysis (n=2 at passage 1 and n=1 at 2) were characterized by flow cytometry. These were compared with cryopreserved hBMSCs (thawed and analysed) from passages 1 and 2 (n=1) (see figure 3.1; representative histograms of this data can be found in figure 3.2). ≥96.5% of cryopreserved HUCPVCs, as well as plated HUCPVCs and hBMSCs expressed CD73, CD90, CD105, PDGF-Rβ, and MHC I. However, surface antigen levels on hBMSCs of CD105 (hBMSCs: 36.1±8.1 MFI; HUCPVC thaw: 9.1±4.3 MFI; HUCPVC plate: 13.1±8.4) and PDGF-Rβ (hBMSC: 33.2±1.2 MFI; HUCPVC thaw: 6.7±2.3 MFI; HUCPVC plate: 12.1±5.8 MFI) were greater than those on HUCPVCs. As well, the surface antigen levels of MHC I appears to be highly variable in cryopreserved HUCPVCs (standard deviation: 25.0 MFI), however the total surface antigen levels are significantly greater in hBMSCs (67.2±3.2 MFI) as compared to plated HUCPVCs (33.1±9.5 MFI).

93.2±2.8% of cryopreserved HUCPVCs expressed CD146, and this number increased
slightly in plated cells (95.4±3.2%). Most interestingly, the percentage of cells expressing CD146 was significantly higher in HUCPVCs than in hBMSCs (65.5±1.3%). Additionally, the relative CD146 expression per cell was greater in HUCPVCs (24.97 MFI, plated; 15.29 MFI, thaw) than hBMSCs (5.91 MFI).

A subpopulation of cryopreserved HUCPVCs expressed NG2 (28.0±18.9%), which was similar to the percentage of hBMSCs (37.5±14.2%). This population was significantly increased in plated HUCPVCs (88.80±15.75%). Plated HUCPVCs also express increased surface antigen levels of NG2 (6.4±1.2 MFI) when compared to hBMSCs (1.8±0.4 MFI) and cryopreserved HUCPVCs (2.2±0.4).

A negligible population of cryopreserved HUCPVCs expresses CD40 (2.8±1.4%) and CD80 (0.8±0.4%), while a significantly greater population expresses CD86 (77.4±25.6%). This percentage is higher in hBMSCs (94.9±3.8%) and lower in plated HUCPVCs (69.2±19.1%), though not significantly. Regardless of this high percentage of CD86 expression in cryopreserved HUCPVCs, the relative surface antigen levels of CD86 per cell (2.8±0.9) is comparable to CD40 (7.4±9.3) and CD80 (2.4±0.9). A small fraction of all cells observed expressed MHC II (HUCPVC Thaw: 14.3±11.0%; HUCPVC Plate: 7.7±4.2%; hBMSC: 18.6±7.6%).

**Figure 3.1 (following page)**: Flow cytometry data of surface markers comparing HUCPVCs with hBMSCs. (a) Averaged percentage of cells expressing specific surface molecules. (b) Averaged mean fluorescence intensity from cells expressing specific surface molecules. Cryopreserved HUCPVC data (HUCPVC Thaw) represent the averaged results across samples from passage 2 (n=3 donors) and 3 (n=1 donor). Plated HUCPVC data (HUCPVC Plate) represent the averaged results from samples across passage 1 (n=2 donors) and 2 (n=1 donor). Cryopreserved hBMSC data (hBMSC) represents the averaged results from samples across passage 1 and 2 (n=1 donor). Representative histograms are provided in figure 3.2.
Figure 3.2: Representative flow cytometry histograms. Flow cytometry data for one cryopreserved cord sample at passage 2, one plated cord sample at passage 1, and one cryopreserved hBMSC sample at passage 2. The black histogram is the cell staining of the blank controls, the red histogram is the antibody-stained cells. The percentage of cells staining for each surface marker is provided in figure 3.1.
Figure 3.3: RT-PCR data comparing cDNA from undifferentiated HUCPVCs to cDNA from human bone with regard to osteogenic markers. Alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN), rat and human GAPDH (rhGAPDH).

3.1.2 RT-PCR

cDNA was translated from RNA that was extracted from excised human bone and HUCPVCs cultured in monolayer in normal supplemented medium at passage five. The gene expression profile of important bone matrix proteins was compared by RT-PCR. As shown in figure 3.3, expression of the alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), and RUNX2, the osteogenic master gene, was detected in the HUCPVCs. Expression of osteocalcin (OCN), which is a late marker of osteoblast differentiation, was not detected in HUCPVCs, but was detected in human bone.
3.2 Scaffold seeding

HUCPVCs were seeded onto calcium phosphate (CP)-polylactic-co-glycolic acid (PLGA) scaffolds by either centrifugal or static seeding. Static seeding was performed using a single density of cells \((7.17 \times 10^5\) cells) and centrifugal seeding was performed using both \(2.5 \times 10^5\) and \(5.0 \times 10^5\) cells. Scanning electron microscopy of fixed cell-scaffold constructs, as seen in figure 3.4, demonstrated that cells seeded by centrifugation maintained a fibroblastic morphology. Cells were able to expand into confluent monolayer and multilayer cultures on the scaffold after five days of culture and were visible inside the surface pores of the scaffold structure.

When comparing the efficiency of static to centrifugal seeding by an alamar blue assay of seeded scaffolds, centrifugal seeding enabled a greater number of cells to attach to the scaffolds with higher efficiency, as seen in figure 3.5a. Seeding efficiency for static seeding was \(3.69\pm0.5\%\) (n=3), for centrifugal seeding using an initial seeding density of 250,000 cells, it was \(13.6\pm1.7\%\) (n=3), and for an initial seeding density of 500,000 cells, it was \(12.9\pm0.2\%\) (n=3).

In choosing the optimal cell seeding density, it is necessary to balance between maximizing the number of viable cells that attach to the scaffold and preventing excessive bridging of the scaffold pores by cells throughout the expansion period. Figure 3.5b shows that the number of viable cells attached to the scaffold is positively correlated with the initial number of cells seeded by centrifugation. The seeding efficiency decreases from \(8.1\pm1.9\%\) for 250,000 cells (n=6), to \(6.1\pm1.1\%\) for 500,000 cells (n=6), \(5.4\pm0.9\%\) for 750,000 cells (n=15), and \(4.7\pm0.7\%\) for 1 million cells (n=6).

The optimal seeding density was found to be \(2.5 \times 10^5\) cells, based on the distribution of cells on the scaffold seen in figure 3.5e, as there was considerable pore-bridging when seeding an initial density of \(5.0 \times 10^5\) (figure 3.5f) and \(1.0 \times 10^6\) (figure 3.5g), as observed by a toluidine blue staining of the seeded scaffolds.

The effect of fluid flow on cell attachment to the scaffold was assessed by either verti-
Figure 3.4: Scanning electron microscopy images of HUCPVCs cultured on scaffold for five days after centrifugal seeding.
cally or horizontally positioning the microcentrifuge tube, containing the cell suspension, on the rotating shaker during the one hour incubation period post-centrifugation. When the tube is seated vertically on a shaker, the fluid flow inside the container and around the scaffold is reduced because of the small diameter base of the 1.5 ml eppendorf tubes. However, when the tube is laid horizontally, there is significantly greater fluid flow and movement of the scaffold along the length of the tube. An alamar blue cell viability assay, performed after the one hour incubation, demonstrated that the horizontal position of the tube, which corresponds to a 'high flow', reduces the number of viable attached cells on the scaffold (n=3, figure 3.5c).

\textbf{Figure 3.5 (following page):} Optimization of scaffold seeding. (a) Alamar blue cell viability assay demonstrating centrifugal seeding enables significantly greater attachment of viable cells with greater efficiency (n=3 scaffolds per density). Asterisks indicates statistically significant difference; *, p≤0.0009. (b) The number of viable cells attached to scaffold two days after seeding, increases with initial seeding density using centrifugation. Standard deviations were obtained and presented as standard error bars (n=6 scaffolds per density, n=15 for scaffolds seeded with 750,000 cells). (c) The effect of fluid flow on cell attachment and viability. When the scaffold is standing (low flow conditions), cell viability is greater in the suspension (0 days) and on the scaffold (5 days) than when sitting (high flow). Standard deviations were obtained and presented as standard error bars (n=3 scaffolds per variable); *, p≤0.001. (e) Scaffolds that were seeded with 250,000 cells stained with toluidine blue to visualize cellular material after growth for 5 days, show optimal coverage of the scaffold, without pore bridging, compared to scaffolds seeded with (f)500,000, (g) and 1,000,000 cells. (d) Blank (control) scaffold stained with toluidine blue.
Chapter 3. Results

(a) 

(b) 

(c) 

(d) 

(e) 

(f) 

(g)
3.3 Animal Studies

3.3.1 Xenotransplantation in Wistar rats

Blood count

A sample of blood was taken from 36 animals transplanted with HUCPVCs and three control rats who did not receive HUCPVCs, every five days post-transplantation. These samples were used to do a complete blood count and the cell counts from the experimental animals were compared with those from the control animals by the method of generalized estimation equations. For each blood count parameter, a correlation value was calculated to determine the correlation, if any, between the control and experimental values. A p-value was also determined for this correlation parameter based on the null hypothesis that there is no difference between the response variables. These p-values were reported for each blood count parameter in table 3.1. p \leq 0.05 was considered statistically significant, which indicates that the null hypothesis can be rejected. As well for each blood count parameter, the average values (X) and the standard deviation served as descriptive parameters and are shown as time-course plots in figure 3.6. The p-values were not significant (\geq 0.05) for the white blood cell count (WBC), lymphocyte count (LY\#), monocyte count (MON\#), platelets (PLT), and mean platelet volume (MPV). All the other parameters had p-values that were significant (\leq 0.05).

In terms of the time course mean values of the parameters that had a p \geq 0.05, the white blood cell count in both the control and experimental animals, increased within five days from the surgery, and remained nearly steady for the remainder of the study period. There was a decrease in the mean white blood cell count in the control animals on day 15 (X_{control} = 5.36 \pm 1.53 \times 10^3/\mu l vs. X_{HUCPVC} = 8.18 \pm 2.14 \times 10^3/\mu l), which occurred as a result of a larger drop in two of the three control animals. This decrease promptly returned to the pre-existing levels on day 20 and remained constant until the end of the study period. The experimental animals saw a small drop in mean WBC beginning
on day 30 ($X_{HUPVC} = 7.48 \pm 1.82 \times 10^3/\mu l$) and continuing on day 35 ($X_{HUPVC} = 7.25 \pm 1.66 \times 10^3/\mu l$).

The mean lymphocyte count followed a similar trend to the WBC, both for the experimental and control animals. This is expected as the lymphocytes made up on average 77.9% of the total WBC.

The mean monocyte count in the control and experimental groups saw several perturbations throughout the study period. The control group had the lowest mean monocyte count at 15 days ($X_{control} = 0.87 \pm 0.20 \times 10^3/\mu l$) and the highest mean monocyte count at 20 days ($X_{control} = 1.77 \pm 0.35 \times 10^3/\mu l$). For the experimental animals, the highest mean monocyte number was seen on day 10 ($X_{HUPVC} = 1.75 \pm 0.63 \times 10^3/\mu l$) and the lowest mean count was seen on day 30 ($X_{HUPVC} = 1.00 \pm 0.30 \times 10^3/\mu l$).

The mean granulocyte count in the control group increased beginning on day 5 from basal levels (Day 0: $X_{control} = 0.30 \pm 0.17 \times 10^3/\mu l$ vs. $X_{HUPVC} = 0.13 \pm 0.14 \times 10^3/\mu l$) and peaked at day 10 ($X_{control} = 1.43 \times 10^3/\mu l$ vs. $X_{HUPVC} = 0.50 \pm 0.28 \times 10^3/\mu l$). It returned close to initial levels by day 15 ($X_{control} = 1.10 \pm 0.15 \times 10^3/\mu l$) and this was followed by a gradual increase until 35 days which was not seen in the experimental animals.

The mean platelet count in the experimental animals decreased initially from 766 ± 172 to 658 ± 195. Conversely, in the control animals, it initially increased from 743 ± 182 to 1089 ± 303 on day 5. A similar increase was seen in the experimental animals however it occurred later at 10 days, during which time the platelet count in the control animals began to decrease, reaching its lowest point on day 15 ($X_{HUPVC} = 535 \pm 193$). Both control and experimental counts leveled off beyond day 20.

The mean plasma volume (MPV) also showed variability throughout the study period, with the control animals showing an increase around day 15 that remained until day 30. Contrastingly the experimental mean plasma volume level showed less fluctuation, with the highest value on day 30 ($X_{HUPVC} = 7.65 \pm 2.57$) and the lowest value on day 40.
(X_{HUCPVC} = 6.08 \pm 1.95).

The weights of the rats were also tracked over the course of 40 days, as seen in figure 3.7, and compared to control animals. No significant differences or perturbations in the weights were observed and the animals appeared healthy throughout the study.

**Table 3.1:** p-Values in blood count between the experimental group and control rats that received only the CP-PLGA scaffold without HUCPVCs. n=36, rats receiving HUCPVCs. n=3, control rats receiving only scaffold.

<table>
<thead>
<tr>
<th>p-value ≤</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>0.2949</td>
</tr>
<tr>
<td>RBC</td>
<td>1.02E-11</td>
</tr>
<tr>
<td>HGB</td>
<td>2.74E-08</td>
</tr>
<tr>
<td>HCT</td>
<td>3.67E-09</td>
</tr>
<tr>
<td>MCV</td>
<td>0.0175</td>
</tr>
<tr>
<td>MCH</td>
<td>5.34E-04</td>
</tr>
<tr>
<td>MCHC</td>
<td>9.65E-07</td>
</tr>
<tr>
<td>PLT</td>
<td>0.5251</td>
</tr>
<tr>
<td>LY#</td>
<td>0.9432</td>
</tr>
<tr>
<td>MON#</td>
<td>0.9385</td>
</tr>
<tr>
<td>GR#</td>
<td>1.26E-11</td>
</tr>
<tr>
<td>RDW</td>
<td>0.0323</td>
</tr>
<tr>
<td>MPV</td>
<td>0.8656</td>
</tr>
</tbody>
</table>

**Bone healing**

HUCPVC-seeded constructs were implanted in a femoral defect in the distal metaphysis of male wistar rats. The rate of new bone formation was decreased within the defect area
Figure 3.6: Mean blood count parameter time-course values with standard deviations. Experimental, n=36; control, n=3.
Figure 3.7: Post-operative weights of animals receiving HUCPVCs+scaffold, and control animals receiving only scaffold. Experimental, n=36; control, n=3.

after HUCPVC transplantation compared with controls, as shown by micro computed tomography analysis of the mineral content within the defect seen in figure 3.8a. The difference was statistically significant at seven days (p≤0.001) as determined by a student’s t-test. This was also apparent by visual inspection of the two-dimensional micro-CT images of femur cross sections, specifically at seven days in figures 3.8b-c. Macroscopic inspection of the femurs at 60 days post surgery (see figures 3.8d-e) revealed that some treated defects had still not completely healed, whereas, control defects showed complete closure.

In histological sections (see figure 3.9), a fibrous tissue encapsulation was observed surrounding the HUCPVC treated defects at seven days, and this fibrous tissue persisted surrounding scaffold remnants until 60 days, although it decreased in size throughout the study. This fibrous tissue is collagenous as evidenced by birefringence under polarizing filters (figure 3.10a) and by continuity with new bone when stained with Goldner’s trichrome (figure 3.10b).

Control defects consisted mainly of osteoid and bone appositional to the scaffold after seven days, with a fibrous tissue distributed throughout the defect. Very little fibrous
tissue remained in the defect by 30 days, with some remnants of scaffold remaining. Fibrous tissue was not observed at 60 days in the control defects. In both the control and the experimental animals, the scaffold was resorbed as the study proceeded, and by 60 days it was eventually engulfed in the new trabecular bone.

In addition to the impaired healing observed, massive trabecular bone outgrowths appeared towards the distal end of the metaphysis and the epiphyses in one HUCPVC treated femur at 30 days (figure 3.11a), and another at 60 days (figure 3.11c). They also appeared in a control femur at 60 days (figure 3.11b). These growths are unusual and were observed away from the defect site.

**Survival of human cells and immune cell infiltration**

Transplanted HUCPVCs were tracked using an anti-human nuclei antibody raised in mouse. In figure 3.12a cells were found near the scaffold in the hematopoietic cell infiltrate occurring in the defect at four days. Some positive staining material (not pictured) appeared diffuse, similar to destroyed nuclei. At seven days, there are very few cells remaining in the extracellular matrix of the defect, as pictured in figure 3.12c.

RT-PCR was attempted using the RNA extracted from the femoral defects to detect

---

**Figure 3.8 (following page):** Healing of femoral defect in Wistar rats transplanted with HUCPVCs. (a) MicroCT measurements of the mineral volume indicates that there is a greater mineral content in the control defects versus the experimental defects, most significantly at 7d. Standard deviations are presented as standard error bars (n=5, for 15d n=4). Asterisks indicates statistically significant difference; *, p≤0.001. (b) Cross sections of femurs taken by micro-CT show greater trabecular bone formation in the cortical space of a control defect (scaffold only) when compared to (c) a HUCPVC treated defect after 7d. (d) A macroscopic view of the femurs from 60d shows complete healing in the control femur as compared to (e) incomplete healing of the defect transplanted with HUCPVCs.
Chapter 3. Results

(a)

(b)

(c)

(d)

(e)
human specific genes outline in table 2.3. Results were inconsistent and the extracted RNA appeared to be of poor quality.

Femurs from seven days stained with Maximows stain (see figure 3.14b,a,c), used to identify hematopoietic cells in bone marrow, show a significant cell infiltrate in the experimental defect compared to controls. Within this infiltration, in figure 3.14c there are identifiable eosinophils, which suggest that there should also be other immune cells present. CD68 staining, for activated rat macrophages confirms this, showing a greater infiltration of macrophages in the presence of HUCPVCs in the defect site as compared to control scaffolds (see figure 3.14a).

---

**Figure 3.9 (following page):** Progression of fibrous tissue formation and scaffold resorption in a femoral defect in Wistar rats transplanted with HUCPVCs. (top) H&E cross sections from rat femora transplanted with HUCPVCs+scaffold, (bottom) and control CaP-PLGA scaffolds at 7d, 30d (cross sections) and 60d (longitudinal sections). Faster osteogenesis and scaffold resorption is observed within the femora implanted with empty scaffold. Greater fibrous tissue formation is observed appositional to the scaffolds in the HUCPVC group, persisting until 60d. s - scaffold, example is encircled in yellow; f - fibrous tissue, example is encircled in black, t - trabecular bone; FW = 4.3 mm.
Figure 3.10: Identification of fibrous tissue in a femoral defect in Wistar rats transplanted with HUCPVCs at 10 days. (a) Paired bright field (left) and polarized light images (right) from 10d post-operation defect sections. Each show considerable connective tissue matrix which is birefringent, identified by red arrows (thus collagen containing). FW = 858 µm. (b) Goldner’s trichrome stain of another 10d section confirms that the extracellular matrix is collagen containing (green, identified by red arrow) and continuous with newly formed bone. Right image is a magnification of inset box; r - FW = 858 µm, l - FW = 343 µm.
Figure 3.11: Unusual trabecular outgrowth observed in experimental and control femurs after 30 and 60 days. (a) At 30d, trabecular growth is observed mainly within the medulla in femur 23L, transplanted with HUCPVCs, and confined to a small outgrowth near the epiphysis. (b) At 60d, femur 25R, a control that received only scaffold, reveals a massive outgrowth in the epiphysis, away from the original defect site. (d) Longitudinal section of the same femur shows the outgrowth mainly within the epiphysis, happening through the growth plate. (c) At 60d, femur 26R, a femur that received HUCPVCs, reveals an outgrowth in the epiphysis, away from the original defect site. (e) Longitudinal sections of the same femur at different depths reveal the trabecular growth in the epiphysis of the femur.
Figure 3.12: Human specific staining of HUCPVCs in femoral defects in Wistar rats. (a) Human nuclear staining (red) at 4 days (c) and 7 days. At 4d positive staining cells are near the scaffold, in the cellular infiltration (black arrows). Few HuNu+ cells remain within the extracellular matrix surrounding the scaffold at 7d (one is identified by a black arrow). (b), (d) Negative controls, (primary antibody omitted). (a), (c) - FW=343 μm; insets FW=214 μm; (b), (d) - FW=2.14 mm.
Figure 3.13: Cellular infiltration in HUCPVC-treated defects in Wistar rats. (a) Staining with Maximow’s stain for bone marrow reveals a greater hematopoietic cellular infiltration in the presence of HUCPVCs at 7 days post-operatively, compared to (b) empty scaffold control; FW=858 µm. (c) A magnification of (a). Eosinophils indicated by black arrows; FW=136 µm.
Figure 3.14 (following page): CD68 staining in HUCPVC-treated defects in Wistar rats. (a) A greater activated macrophage (brown cells) infiltration is seen in the presence of HUCPVCs at 7 days post-operatively, compared to (c) empty scaffold control; FW=858 μm. (b) A magnification of (a). ED1+ cells indicated by black arrows; FW=343 μm. (d) A magnification of (c) shows fewer ED1+ cells within the defect; FW=343 μm. (e) Negative control (primary antibody omitted); FW=858 μm.
3.3.2 Xenotransplantation in athymic rats

Tissue engineered constructs, using pooled HUCPVCs from five different donors were cultured as previously described and implanted into a femoral defect in athymic rats (strain: Crl:NIH-Foxn1nu). This animal model, as compared to the previously used Wistar rats, has a normal B immune system, while functional T lymphocytes are absent. This is similar to the more common NOD/SCID mouse model, which has an impaired ability to make T or B lymphocytes, or activate the complement system, and cannot efficiently fight infections nor reject tumors or transplants.

Bone healing

The rate of new mineral volume formation in athymic rats was increased within the defect area, as compared to controls, after HUCPVC transplantation. This was demonstrated through morphometry and histological analysis (figure 3.15). Fibrous tissue formation was observed within the HUCPVC defects at four days, which was eventually replaced by new bone after 45 days. The majority of the scaffold was also resorbed by that point (see figure 3.15). HUCPVC-treated defects contain a greater mineral deposition at 15 and 30 days based on micro-CT morphometry (figure 3.16a). When the initial number of seeded cells was increased from 250,000 to 750,000, this enabled an increase in mineral volume when comparing HUCPVC-treated defects to control defects to become statistically significant at 15 days (figure 3.16b). There was no significant difference observed however at 30 days (figure 3.16c) when the initial seeding density was increased to 750,000 cells.

Tracking human cells using a fluorescent carbocyanine dye in undecalcified resin sections

HUCPVCs were stained with DiR and DiD, fluorescent carbocyanine cell membrane dyes, prior to transplantation. After harvesting the femurs, they were embedded in
methylmethylacrylate resin, cut, ground, and polished into fine sections. The sections were analyzed by a phase contrast microscope equipped with a fluorescent lamp and optical filters for DiR.

In culture plates, fixed cells stained with DiR or DiD and DAPI were detectable as seen in figure 3.17. Cell membranes were fluorescent, however fluorescence was not detected uniformly throughout the culture as seen in figures 3.17a and 3.17c. Cells stained with DiR were cultured and analysed by flow cytometry in between passaging throughout a 3 week culture period. It was found that the mean fluorescence intensity of the cells decreased almost linearly with increasing population doublings as seen in figure 3.17. In resin sections from animals transplanted with DiR stained cells, fluorescent cells/signals were not detected in the femoral defects after 15 days as seen in figure 3.18. In sections from animals transplanted with DiD stained cells, fluorescent cells were also not detected in the femurs due to autofluorescence from the scaffold (not shown).

**Survival and engraftment of human cells and expression of human osteogenic proteins**

Paraffin embedded sections of the femurs transplanted with HUCPVCs were stained with anti-human nuclear, anti-human mitochondria, and anti-human osteopontin and osteocalcin. HUCPVCs appeared in a loose connective tissue matrix at seven days (figure 3.19a), which was distinct from the matrix formed by the endogenous rat cells. Some

---

**Figure 3.15 (following page):** Healing of femoral defect in athymic rats transplanted with HUCPVCs. A femoral defect treated with HUCPVCs at 4d shows infiltration of hematopoietic cells and some fibrous tissue formation. At 7d, fibrous tissue and some regenerated trabecular bone is observed in the HUCPVC-treated defect. At 15d and 45d, fibrous tissue is replaced by trabecular bone, which is eventually resorbed along with the remnants of scaffold; FW = 4.3 mm.
Figure 3.16: MicroCT analysis of bone healing in athymic rats transplanted with HUCPVCs. (a) MicroCT analysis of femoral defects indicates a greater mineral content in the defect at 15 and 30 days. (b) When the initial cell seeding density was increased from 250,000 to 750,000, the increase in mineral volume at 15 days post-operative is statistically significant, as determined by student’s t-test, when compared to a control femur. ** indicates statistical significance ($p \leq 0.029$, n=5). (b) At 30 days post-operative, however, the difference is no longer statistically significant ($p=0.23$, n=5).
positively staining cells were also observed in newly forming osteoid at the edge of the defect (3.19c), and this HuNu staining was confirmed by detecting positive anti-human mitochondrial stain in the same region (3.19d).

At 10 days, the Hunu\textsuperscript{+} cells took on a more rounded morphology and appeared embedded in the new bone (see figure 3.20a). In serial sections, cells in the vicinity of the Hunu\textsuperscript{+} also stained positively for human osteopontin (figure 3.20b). The HuNu staining was also confirmed by observing positive anti-human mitochondrial stain in the same region (figure 3.20c). In serial sections, cells in the vicinity of the human mitochondria\textsuperscript{+} cells also stain for human osteocalcin (figure 3.20e,d).

At 15 days, Hunu\textsuperscript{+} cells appeared embedded in trabecular bone near the scaffold remnants. Very few Hunu\textsuperscript{+} cells were detected in the trabecular bone in the defect, relative to the amount of bone that had formed.

At 30 days, some osteocytes within the defect stain positively for human nuclear antigen and cells in their vicinity in serial sections, express cytoplasmic human osteocalcin and osteopontin (figure 3.22). Again very few Hunu\textsuperscript{+} cells were detected relative to the size of the defect. At 45 days, Hunu\textsuperscript{+} cells were not found in the bone or extracellular matrix in the defect (not shown).

---

**Figure 3.17 (following page):** HUCPVCs in culture stained with DiD and DiR. (a) Fluorescence image taken with DiD filter of HUCPVCs in culture stained with DiD. Signal is not uniform throughout. A pseudocolor has been applied to the fluorescence signal using ImageJ software to contrast the dapi staining, FW\approx 2\,\text{mm}. (b) Higher magnification of (a) taken with DiD filter, FW\approx 120\,\mu\text{m}. (c) Fluorescence image taken with DiR filter of HUCPVCs in culture stained with DiR, FW\approx 2\,\text{mm}. (d) Higher magnification of (c) taken with DiR filter, FW\approx 120\,\mu\text{m}. (e) A plot of the mean fluorescence intensity, as measured by flow cytometry (FL5 filter) of HUCPVCs stained with DiR at variation population doublings after staining. The mean fluorescence intensity decreases in a linear fashion with increased doublings.
Figure 3.18: Resin sections observed by phase contrast microscope under DiR optical filter. 
(a) Trans image showing scaffold (black) embedded in connective tissue and trabecular bone 
in defect (b) Corresponding fluorescence image taken with DiR filter shows little signal, FW = 2.144 mm. 
(c) Higher magnification trans image of the same section. 
(d) Corresponding higher magnification fluorescence image, FW = 858 µm.
Figure 3.19 (following page): HUCPVCs survive in an athymic rat after seven days. (a) Human cells are identified by localization within a loose connective matrix (encircled by black dashed line), distinct from the more dense surrounding endogenous matrix at seven days; FW=858 µm. (c) Hunu$^+$ cells are also seen in the newly forming osteoid at the edge of the defect (black arrows) and this is confirmed by (d) positive staining of human mitochondria (black arrows); FW=429 µm. (b) Negative control for (a) (IgG isotype antibody); FW=858 µm. (e) Negative control for (c),(d) (IgG isotype antibody); FW=429 µm.
Figure 3.20 \textit{(following page)}: HUCPVCs survive in an athymic rat after 10 days. (a) Hunu$^+$ cells appear embedded in the forming bone at 10 days (black arrows). (b) In serial sections, cells are positive for cytoplasmic osteopontin (black arrows); FW=343 $\mu$m. (c), staining for human mitochondria in forming bone (black arrows); (d) In serial sections, cells are positive for cytoplasmic osteocalcin (black arrows); FW=429 $\mu$m. (e) Another section revealing positive staining of human mitochondria (black arrows); FW=429 $\mu$m, (f) high magnification of (e); FW=172 $\mu$m. (g) Negative control for (a),(b) (IgG isotype antibody); FW=429 $\mu$m. (h) Negative control for (c),(e),(f),(d) (IgG isotype antibody); FW=429 $\mu$m.
Chapter 3. Results

(a)  

(b)  

(c)  

(d)  

(e)  

(f)  

(g)  

(h)
Figure 3.21: HUCPVCs contribute to new bone in an athymic rat after 15 days. (a) Hunu⁺ cells appear embedded in the forming bone at 15 days (black arrows); FW=429 µm, (b) Magnification of (a); FW=172 µm. (c) Negative control for (a) (IgG isotype antibody); FW=429 µm.
Figure 3.22 (following page): HUCPVCs persist in new bone until 30 days. (a) Hunu$^+$ osteocytes (identified by black arrows) embedded in trabecular bone, appositional to remnants of scaffold; FW=343 µm. (b) Positive staining of human mitochondria at 30d (black arrows); FW=172 µm. (c) Hunu$^+$ and (e) h−Osteopontin$^+$ positive cells in serial sections at 30d (black arrows); FW=343 µm. (d) Hunu$^+$ positive and (f) h−Osteocalcin$^+$ positive cells in serial sections at 30d (black arrows); FW=343 µm. (g) Negative control for (a),(b)(IgG isotype antibody); FW=429 µm. (h) Negative control for (c),(e),(d),(f) (IgG isotype antibody); FW=429 µm.
Chapter 4

Discussion

4.1 Phenotyping of human umbilical cord perivascular cells

This work aimed to analyse the surface marker expression of human umbilical cord perivascular cells, after plating and detachment and directly from thaw, and comparing these to human bone marrow stromal cells from thaw. Previous work by Sarugaser et al. has demonstrated that HUCPVCs are multipotent, capable of differentiating to bone, cartilage, muscle, fat, and fibroblast lineages. In addition to this, Baksh et al. have demonstrated that HUCPVCs express mesenchymal stromal cell surface markers and are capable of osteogenic differentiation. The cell phenotyping described in this work was then conducted to confirm the already established mesenchymal stromal cell phenotype of HUCPVCs. In addition to the surface marker assay, the expression of osteogenic genes in HUCPVCs grown in normal supplemented medium was compared to the expression in human bone, so as to gauge the osteogenic potential of non-differentiated HUCPVCs. These assays were necessary in order to determine the nature of the cells before using them as a cell source for bone regeneration in vivo.
4.1.1 Human umbilical cord perivascular cells have a mesenchymal stromal cell phenotype

In this study, surface markers assayed by flow cytometry were chosen based on different lines of reasoning described below.

CD105, CD73, and CD90 have been defined by the International Society for Cellular Therapy as the minimal surface markers that should be expressed by MSCs. In addition, MSCs must lack expression (≤ 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and MHC class II. This work did not assay CD45, CD34, CD14 or CD11b, CD79 or CD19, as some of these markers, particularly CD45 and CD34, had already been assayed in this cell population (results not shown) and were not expressed. The flow cytometry experiments presented in this work were gated on the cell population that had been shown in previous experiments to be CD45− and CD34−. All cell populations analysed had ≥96.5% positive expression of CD73, CD90, and CD105. The positive expression of these markers, along with plastic adherence and multilineage potential shown by Sarugaser et al. demonstrates that HUCPVCs meet the minimal criteria for a multipotent mesenchymal stromal cell. One unusual finding in this work appears in the surface antigen levels of CD105, with hBMSCs expressing greater amounts than both thawed and plated HUCPVCs. CD105 is closely associated with tumor angiogenesis, as studies have shown that CD105 expression is up-regulated in the endothelium of various tumor tissues compared with that in normal tissues. In mesenchymal stromal cells, CD105 expression has been linked to increased plasticity along the endothelial lineage in cells derived from AML patients. CD105 expression in BMSCs is also associated with higher clonal recovery and increased expression of pluripotency markers OCT4 and NANOG when compared to CD105− subpopulations of BM mononuclear cells. These findings suggest that CD105 expression is associated with a more immature fraction of MSCs. This, however, does not correspond with the present increased surface antigen
levels of CD105 in hBMSC compared to HUCPVCs. The hBMSCs used in this study come from a more mature source (adult human bone marrow vs. post-natal umbilical cord) and they are not as closely associated with the vasculature and endothelium as the umbilical cord perivascular cells. In fact, as discussed below, an increased percentage of cells expressing CD146 in HUCPVCs when compared to hBMSCs, may suggest that HUCPVCs are a more primitive MSC population. Therefore, the functional significance of CD105 surface antigen levels in hBMSCs and HUCPVCs is not clear and warrants further examination.

The combination of CD146, NG2, and PDGF-Rβ was selected for flow cytometry analysis as it has been used to identify pericytes/perivascular cells that can differentiate into multiple mesenchymal lineages. These markers have linked MSCs to pericytes/perivascular cells, and this has led some authors to suggest that the perivascular region is the anatomical origin of MSCs. This would explain the phenotypic and functional similarities that have been previously demonstrated between HUCPVCs and hBMSCs.

HUCPVCs have previously been shown to express CD146 at levels higher than bone marrow stromal cells, and this was confirmed in this work. A significantly greater percentage of HUCPVCs assayed compared to hBMSCs expressed CD146, regardless of whether they were plated or cryopreserved. CD146 (S-endo1, Mel-CAM, Muc18, or gicerin) is an adhesion molecule present on endothelial cells and is also expressed on circulating endothelial cells, widely considered to be mature endothelial cells detached from injured vessels. It is also present on smooth muscle and myofibroblasts in situ, and it is present on pericytes, stromal, and perivascular mesenchymal stem cells. Shi et al. found that STRO-1+/CD146+ sorted hBMSCs did not express CBFA1 and osteocalcin, markers of early and late osteogenic populations, respectively, and they concluded that hBMSCs exhibit a preosteogenic phenotype in human bone marrow aspirates. Covas et al. propose that CD146 could be used as a marker for more primitive mesenchymal
stem cells, primarily based on its expression on pericytes and its signaling pathway, which comprises proteins involved in osteogenic differentiation\textsuperscript{132}. Supporting this notion, Sacchetti et al. have identified osteogenic progenitors in CD146\textsuperscript{+} subendothelial cells that are also capable of forming the stromal cells that support hematopoiesis\textsuperscript{134}. As discussed above, Baksh et al. have demonstrated that HUCPVCs, which contain a higher percentage of CD146\textsuperscript{+} cells compared to hBMSCs, have a higher rate of proliferation and osteogenic differentiation\textsuperscript{55}. The higher percentage of CD146\textsuperscript{+} cells and the higher surface antigen levels of CD146 on HUCPVCs suggests that these cells are a more primitive MSC population than BMSCs.

A significantly greater proportion of HUCPVCs that were plated and trypsinized prior to analysis are NG2\textsuperscript{+} as compared to HUCPVCs and hBMSCs taken directly from thaw. This might suggest that cryopreservation quenches NG2 surface marker expression, which is restored in culture, or that culture conditions select for an NG2\textsuperscript{+} subpopulation of cells. The latter possibility however is less likely as both populations of HUCPVCs, from thaw and from culture, had been passaged in culture at least to P1 at some point. NG2 is a chondroitin sulfate proteoglycan, that is primarily expressed on the surface of polydendrocytes\textsuperscript{135}. These are a fourth major glial population in the central nervous system, which are distinct from astrocytes, mature oligodendrocytes and microglia, and they are widely accepted as oligodendrocyte precursor cells\textsuperscript{136}. In normal development, NG2 is found on pericytes in large vasculogenic vessels such as the aorta at low frequencies\textsuperscript{137}. NG2 is also found on pericytes in the neovasculature associated with pathological conditions\textsuperscript{138}. In BMSCs, it is expressed by cells that are also positive for CD73, CD105 and CD166 in all passages of BM samples\textsuperscript{70}. Ozerdem et al. have shown that NG2, along with PDGF-R\textsubscript{β}, is consistently expressed by the mural cell component of developing vasculature regardless of the mechanism of vascularization (vasculogenesis or angiogenesis) and the nature of the vasculature (macro or micro)\textsuperscript{139}. Along with NG2, in this work, all the cell types assayed were PDGF-R\textsubscript{β}\textsuperscript{+}. Hirshci et al. have tried to mimic blood vessel as-


assembly by coculturing endothelial cells (EC) with a multipotent mesenchymal cell line. In their work, they have provided strong evidence for PDGF-β as a paracrine effector in EC-directed mesenchymal recruitment, proliferation and through direct contact with ECs, differentiation toward an SMC/pericyte fate. Together then, these findings point to NG2 and PDGF-Rβ as markers primarily expressed by pericytes/perivascular cells in neovasculature and playing an important role in directing vascularization.

In addition to the idea that NG2+ PDGF− Rβ+ cells play a role in vascular development, Crisan et al. show that single cultured perivascular cells, (identified as CD146+ NG2+ PDGF− Rβ+ ALP+ and CD34− CD45− vWF− CD144−), contain a high frequency clonal population. In their findings, all clones yielded osteocytes, chondrocytes, and adipocytes when grown in the appropriate differentiation conditions. Taking this into account, and the findings of Baksh et al. discussed above with regard to CD146, these findings may suggest a novel physiological role for NG2+ CD146+ PDGF− Rβ+ MSCs as circulating cells which play a prominent role in vascular development and are capable of contributing to mesenchymal tissues. Most importantly in this work, these findings point to the potential use of HUCPVCs as a therapeutic cell source in damaged tissue.

As discussed previously, undifferentiated MSCs constitutively express low surface densities of MHC class I molecules and are negative for MHC class II molecules. Therefore to confirm a similar phenotype in HUCPVCs, MHC Class I and II were assayed by flow cytometry. MHC class I molecules are found on every nucleated cell of the body and they are responsible for presenting peptides derived from cytosolic proteins to T-cells. MHC class II molecules are found on professional antigen presenting cells (APCs) - dendritic cells, activated B cells, and macrophage - and they are responsible for antigen presentation of extracellular proteins to CD4+ T-cells. All cell populations assayed in this work had 100% positive expression of MHC class I molecules and a low percentage of cells were MHC class II+, as expected for a mesenchymal progenitor population. Duan
et al. have demonstrated that genetically modified donor tissue lacking MHC molecules could prevent neural xenograft rejection that was observed after wild-type mouse tissue transplantation\textsuperscript{143}. This suggests that HUCPVCs, owing to their lack of MHC class II expression, may be able to surmount a host immune response when transplanted allogenically or xenogeneically.

MSCs have also been shown to lack expression of costimulatory molecules CD40, CD80, and CD86\textsuperscript{95}. It is well accepted that T cells require costimulatory signals for optimal activation\textsuperscript{144}. CD80 (B7.1) and CD86 (B7.2) are intramembrane proteins expressed as monomers on activated APCs that both costimulate T cells through the ligation of CD28 (B7 receptor)\textsuperscript{145}, and initiate T-cell activation in the early phases of an immune response\textsuperscript{146}. Competitive inhibition of CD28 binding to CD80 and CD86, by a recombinant protein, CTLA4Ig, is effective at inducing indefinite islet xenograft (human to mouse) and cardiac allograft (rat) survival\textsuperscript{144}. CD40, which binds to its ligand CD154 (gp39), is also expressed by antigen presenting cells among other cell types\textsuperscript{147}. CD40-CD154 binding primarily mediates the expansion and differentiation of resting B cells, and is critical for the development of humoral immunity\textsuperscript{148}. However it is also implicated in T-cell priming\textsuperscript{147}. In vivo, Parker et al. have shown that anti-CD154 antibodies could indefinitely prevent alloislet graft rejection in 40-50% in mice that had their islets chemically destroyed\textsuperscript{149}. With these findings in mind, CD40, CD80, and CD86 expression are then important to consider when transplanting cells as they can give some early indication of a potential immune response. All cell populations assayed in this work were negative for CD40 and CD80, two of the three costimulatory molecules. However, in this work, hBMSCs had $94.9 \pm 3.8\%$ positive expression of CD86, and HUCPVCs had $77.4 \pm 25.6\%$ positive expression after thaw and $69.2 \pm 19.1\%$ positive expression after plating. This result is unexpected as others have reported that bone marrow stromal cells\textsuperscript{96} and specifically umbilical cord matrix cells do not express CD80 and CD86 costimulatory molecules\textsuperscript{95,121}. In this present work, the high percentage of HUCPVCs expressing CD86
may be negligible as all the cells analysed expressed low levels of CD40, CD80, and CD86 surface antigen as demonstrated by their mean fluorescence intensity. Without further experimental evidence in this work, and based on previous work showing that HUCPVCs suppress lymphocyte proliferation in coculture\textsuperscript{94}, it appears that the low surface expression of CD86 by HUCPVCs is of little consequence in terms of their immunosuppressive function.

4.1.2 Human umbilical cord perivascular cells express several osteogenic genes

RT-PCR analysis from HUCPVCs grown in normal supplemented medium demonstrates that HUCPVCs in non-differentiation conditions can express detectable levels of osteogenic genes alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), and the osteogenic master gene, RUNX2. HUCPVCs from passage 5 were used for this analysis as this was the latest passage of cells used for in vivo transplantation studies, and therefore this 'late' passage could provide information on the genotype of HUCPVCs used specifically for the animal experiments in this work. Cells from a single donor and passage (n=1) were assayed here, therefore these findings only provide a snapshot of HUCPVC osteogenic gene expression, and may not necessarily be applicable to other HUCPVC populations from different donors and passages.

The gene expression from HUCPVCs was compared to that from excised human bone samples, in order to confirm the correct gene product sizes. Osteocalcin (OCN) was the only gene that was not detected in HUCPVCs and this is somewhat expected, as this is a late marker of osteogenesis, and these cells were not grown in differentiating medium.

Typically, MSCs are differentiated in vitro before transplantation, as differentiated cells demonstrate a greater regeneration capacity in vivo when compared to undifferentiated MSCs\textsuperscript{72}. Differentiation is commonly performed using osteogenic induction medium, which contains growth factors and cytokines that direct the cells to differentiate into os-
teoblasts and produce a mineralized matrix and bone nodules in vitro. This approach however requires significant time in culture (up to 21 days), which may hinder its use in clinical applications. Thus, this work aims to understand the osteogenic potential of HUCPVCs grown in non-differentiating medium. Byrne et al. have shown that MSCs grown on porous collagen scaffold in non-osteogenic medium express higher gene levels of known bone markers collagen I (COL1), osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN)\textsuperscript{35}. Based on this work we might expect that the low levels of osteogenic genes expressed by HUCPVCs in normal supplemented medium could be up-regulated during subculture on porous osteoconductive scaffolds, however this was not performed in this study.

\section*{4.2 Development of a tissue engineered construct suitable for orthotopic implantation}

Bone tissue engineering involves the use of three-dimensional porous scaffolds that are seeded with an osteogenic cell population in vitro prior to implantation. Consequently, it is necessary to establish a rapid and effective method for seeding cells onto a three-dimensional scaffold in order to minimize the time necessary to prepare a suitable tissue-engineered construct. The present work aimed to develop an effective seeding protocol that could allow for the rapid seeding and culturing of a cell-scaffold construct for orthotopic implantation.

\subsection*{4.2.1 Centrifugal seeding enables efficient seeding of calcium phosphate coated scaffolds}

Several methods are currently used to seed cells onto scaffolds. The most common method is the static seeding approach, wherein cells are dispensed onto the scaffold, and allowed
to sit with the intention of cellular attachment and migration into the scaffold pores. This method, while relatively simple, usually leads to high seeding efficiency but poor uniformity, especially lacking of cells integrating in the central and bottom facing sections of scaffold.\textsuperscript{150}

A more advanced technique, which seeks to optimize the three dimensional distribution and penetration of cells into the pores of the scaffold uses a spinner flask where scaffolds are suspended within a stirred suspension of cells.\textsuperscript{151} This technique however has two potential shortcomings, requiring long seeding periods (typically 24h\textsuperscript{45}) and low efficiency when using low cell concentrations or small scaffold constructs.\textsuperscript{151} Moreover, in other seeding methods, cell viability has been shown to suffer from the constant collisions that occur between the scaffold and the walls of the flask,\textsuperscript{152} which would most likely occur in a spinner flask bioreactor. Thus, the present work looks at the suitability of using of centrifugal seeding to seed low cell densities ($\leq$1,000,000 cells/ml) onto small scaffold constructs (3 mm in height x 2.3 mm in diameter). This method was chosen based on the work of Godbey et al. who demonstrated centrifugal seeding of foreskin fibroblasts on PLGA scaffolds.\textsuperscript{45} In their work, they determined that the ideal rotor speed for cell viability equated to centrifugal forces of 35.0 and 52.5g at the inner and outer surfaces of the constructs. They also found that a centrifugation time of 5 to 10 minutes, broken into 1-minute segments, resulted in the highest number of seeded cells. Most importantly, they showed that centrifugal seeding was highly efficient for low cell densities, obtaining a 33% seeding efficiency when using 200,000 cells per construct. Roh et al seeded BMSCs onto poly(L-lactide-co-ε-caprolactone) scaffolds using the same parameters as Godbey et al., and they maintained 35% seeding efficiency at a higher cell density (1,000,000 cells/scaffold). In this present work, similar parameters were used in seeding the scaffolds, using a centrifugal force of 30.0 g, and a centrifugation time of 5 minutes, broken into 1-minute intervals. Centrifugal seeding of HUCPVCs was shown to be more effective, allowing greater cellular attachment (centrifugal: $64,315 \pm 10,014$
cells; static: 26,441±4018 cells) with a lower initial cell density (centrifugal: 500,000 cells; static: 717,000 cells). This translated to a seeding efficiency of approximately 12.9% for centrifugal seeding and 3.69% for static seeding. It was noted that the seeding efficiencies obtained in this study were lower for centrifugal seeding than those demonstrated by Godbey and Roh. Godbey et al. incubated the scaffold-cell constructs in a spinner flask for 24h post-centrifugation, whereas in this work the constructs were incubated in the centrifuged cell suspension on a rotating shaker for one hour. This was based on the work of Baksh, who demonstrated that the minimal time for cell attachment of BMSCs onto calcium phosphate scaffolds after dynamic seeding is one hour. This difference in incubation protocols may explain the lower seeding efficiencies obtained in this work.

It was also noted that the number of viable cells attached to the scaffolds when using an initial seeding density of 500,000 cells was nearly double the values determined in the seeding density analysis. A closer examination of this experiment reveals that the viable cells calculated for the 250,000 cell seeding density was also higher than in other experiments. Cell viability was determined by an alamar blue assay, which is a colorimetric assay based on cell metabolism. The disparity in the day-to-day results suggests that the alamar blue solution used for this experiment may have been more dilute than in other cases, and thus the metabolism reaction would have occurred faster, skewing the metabolic rate from the one calculated in the original standard curves used to calculate cell numbers. However, since the same solution was used for both the static and centrifugal seeding, it is then acceptable to assume that the relative cell counts amongst the two methods, and the conclusions to be drawn from them in terms of the optimal seeding protocol, are accurate.

This work also examined the effect of the initial seeding density on the number of viable attached cells by alamar blue cell viability assay and their distribution throughout the scaffold by toluidine blue staining. Control scaffolds, incubated in blank medium were used as controls for both assays. A density of 250,000 cells/scaffold was chosen as
the optimal initial cell seeding density since it provided the highest cell seeding efficiency (8.1%), and also because it resulted in a uniform coverage of the scaffold without significant scaffold pore bridging or aggregation of the cells. At higher cell densities, toluidine blue staining revealed that cells were more highly concentrated towards the edges of the scaffold, similar to the observations made by Weinand et al. when seeding chondrocytes by centrifugation onto porous PLGA mesh scaffolds\textsuperscript{155}. For bone tissue engineering applications, pore bridging poses a problem as it prevents osteogenic cell migration into the scaffold once implanted into a patient. Moreover, occluded pores prevent nutrients and vasculature from invading the interstices of the scaffold and this results in decreased cell viability towards the center of the implanted construct\textsuperscript{156}. Although scaffolds seeded with higher initial cell seeding densities contained a greater number of attached viable cells, they demonstrated pore bridging and cell aggregation making them unsuitable for in vivo implantation.

The effect of fluid flow on cell attachment during the incubation period, after centrifugation, was also investigated. Two levels of fluid flow were analysed and this was based on the position of the culture vessel, a microcentrifuge tube, on rotating shaker. When the tube was placed in a vertical, standing position, there was minimal fluid flow inside the small round bottom of the tube, where the scaffold rested vertically. When the tube was placed in a horizontal position this allowed for greater fluid flow along the length of the tube and this resulted in the scaffold shifting and colliding with the tube walls. Based on an alamar blue cell viability assay conducted after this incubation period, the lower fluid flow position of the tube allowed for increased viable cell attachment. This assay however cannot quantify non-viable cells, or cellular debris, that would have attached to the scaffold. However, it appears that the design of the high flow incubation chamber was rather poor, as it allowed the scaffold to shift and collide with the walls, and this has been shown to contribute to poor cell attachment in other seeding methods\textsuperscript{152}. After the incubation period, the scaffold constructs remained in the microcentrifuge tube in
a standing position on the same rotating shaker, which rotates in three dimensions, for the remainder of the culture period. This somewhat dynamic culture system was chosen since Baksh et al. have demonstrated that a dynamic culture system results in a greater quantity of deposited bone matrix by BMSCs on calcium phosphate substrates. In this work, the culture system configuration allowed for a continuous distribution of the culture medium throughout the interstices of the scaffold and it prevented excessive movement or collisions of the scaffold inside the tube.

4.3 Transplantation of human umbilical cord perivascular cells in immunocompetent rats

In this present work, HUCPVCs were seeded onto porous calcium phosphate-PLGA scaffolds and implanted into a femoral defect in immunocompetent Wistar rats.

The scientific rationale for xenotransplantation evolved from previous reports demonstrating the immunosuppressive and immunomodulatory characteristics of HUCPVCs and human MSC across species. The chosen xenogenic approach represents the maximum immunological incompatibility and thus if the grafts were not rejected one could predict that it would be safe to pursue allogenic transplantation with HUCPVCs, as some already have with hBMSCs. Several previous reports have indicated that hMSCs from different sources including bone marrow, adipose, Wharton’s jelly, and umbilical cord vasculature are immunosuppressive and immunomodulatory in vitro. More pertinent to this study however, some authors have indicated that MSCs are also immunopriveleged in vivo, capable of surmounting a host response when transplanted allogenically and xenogenically in immunocompetent animals. Authors have shown that pig, mouse, and human MSCs from marrow are capable of surviving in acutely infarcted myocardium in normal Lewis rats, and differentiating into a mature cardiomyocytic phenotype. Recently, Niemeyer et al. have shown that hMSCs can survive in...
a subcutaneous site in immunocompetent mice\textsuperscript{122} and can avoid immune detection and improve healing in a critical sized tibial defect in sheep\textsuperscript{97}. Others have reported the survival and expansion of MSCs from pigs Wharton’s jelly in 6-hydroxydopamine lesions of the striatal-nigral tract\textsuperscript{123,157} and human cells in the brains of hemiparkinsonian rats that were not immune-suppressed\textsuperscript{63}. It must be mentioned however that rejection reactions against MSCs have been reported in both allogenic and xenogenic animal models. Coyne et al. demonstrated that allogenic rat BMSCs transplanted into the adult hippocampus or striatum elicited an immediate inflammatory response and complete graft rejection by 14 days\textsuperscript{124}. Poncelet et al. have also observed rejection of allogenic swine MSCs injected subcutaneously and in a cardiac infarcted area, and they attribute the rejection to a donor-specific cellular and humoral response\textsuperscript{125}. Other reports have also demonstrated that xenograft MSCs cannot surmount a host immune response in cardiac\textsuperscript{83,107} and muscle tissue\textsuperscript{108}. Aside from the Niemeyer study, however, the conclusions of these studies cannot easily be related to the present work without restrictions since none of these studies looked at the fate of MSCs in an orthotopic defect.

4.4 Xenotransplantation of HUCPVCs does not elicit a systemic immune response

Because xenogenic transplantation of HUCPVCs without immunosuppression could lead to a generalized immune reaction in immunocompetent rats, in the present work 500 \(\mu\)L of blood were drawn every five days from the tail vein of the experimental animals recieving HUCPVCs and from control animals, and the samples were analyzed for complete blood counts. The weights of the animals were also tracked over this time period. The counts from treated animals were compared to counts from control rats that were not transplanted with HUCPVCs using the method of generalized estimation equations (GEE). Jager et al. have performed a similar blood count analysis on athymic rats that
were transplanted with cord blood stem cells into a critical size defect in the femur\(^{40}\). They observed no significant differences between the experimental and control animals, however they performed their analysis using a students t-test for independent statistical groups and the F-test. These tests assume that each of the observations within a data set are independent of all of the others. However, this approach is inappropriate since repeated observations were taken within subjects, and those tend to be correlated with one another\(^{158}\). In this present work, GEE is used to analyze the time-course blood count data. The analysis in this work then is more suited than a t-test and F-test, as it is meant to analyze longitudinal repeated measurements and other correlated response data\(^{159}\).

In this work the measured blood count parameters over the period of 40 days were considered the response data. A correlation parameter was calculated between the response data from the experimental and control animals, and a p-value was computed based on the null hypothesis that there is no difference between the experimental and control response variables. The null hypothesis was rejected if \(p \leq 0.05\), which indicated that there was a difference between the control and experimental groups. From the biological perspective, a parameter which obtained \(p \leq 0.05\) was affected by the HUCPVC treatment. In this work, it was found that the parameters which related to the immune system of the animal, namely the white blood cell (WBC), lymphocyte (LY\#), monocyte (MON\#), and granulocyte (GR\#) count, were not statistically different between the control and experimental animals, whereas all the other parameters had a \(p \leq 0.05\). This result seems unusual, however a closer look at the time-course plots of the data reveals that the parameters which were not significant showed large standard deviations. Additionally, the \(\alpha\) values calculated for these parameters, which are a measure of the power of the statistical parameters, were above 0.5, suggesting that the sample size was not large enough to make firm conclusions. In fact this is most clearly seen in the WBC counts for the control animals at 15 days. We observe a sudden drop in the mean WBC count and this occurs because of a large drop in two of the three control animals. A larger
sample size would most likely mask this large drop. Those parameters which obtained a \( p \leq 0.05 \) actually show little difference between the experimental and control groups. The significant p-values then may be a result of the small standard deviations within these measurements, which would allow any small difference between the experimental and control groups to become statistically significant.

To our knowledge, the only study which looks at the systemic immune response in immunocompetent animals during human MSC transplantation looked at the xenotransplantation of hMSCs into critical sized defects in sheep tibia and focused on the leukocyte and lymphocyte numbers in blood samples to assess the systemic immune response\(^97\). In that work, they report no significant differences between control and experimental groups, however as mentioned before, they incorrectly use a student’s t-test like Jager et al.\(^40\) to analyze what should be interpreted as longitudinal data. Still, their findings are not surprising as the massive trauma induced in the animals during the creation of a critical-size defect was likely to cause systemic changes that could potentially mask any additional changes as a result of xenotransplantation. In this work, the GEE analysis suggests that a larger control sample size is required (\( n \geq 3 \)). Without a larger sample size, which would improve the power of the statistical parameters, we cannot draw any strong conclusions from the statistical analysis. However, based on a visual inspection of the time-course blood count plots, the animal weights, and qualitative observations of the animals’ health post-operatively, it appears that there was no significant biological differences between controls and experimental animals to suggest a systemic immune response to xenotransplantation of HUCPVCs in immunocompetent rats.

### 4.4.1 HUCPVCs cause a local immune response and do not engraft in immunocompetent rats

HUCPVC-seeded constructs were implanted contralateral to empty scaffolds in femoral defects in Wistar rats. Mineral volume within the defect was quantified by microCT and
a ratio of mineral volume:total volume (the hypothetical volume occupied by the scaffold) was determined for each femur. Compared with controls, the rate of new bone formation was decreased within the defect area after HUCPVC transplantation. A fibrous tissue encapsulation was observed surrounding the HUCPVC-treated defects, which persisted until 60 days, whereas control defects consisted mainly of osteoid and bone appositional to the scaffold beginning from 7 days. A Goldner’s trichrome stain revealed that this fibrous tissue was collagenous, and this was confirmed by birefringence under polarizing filters. At later time points, the scaffold was resorbed and the fibrous tissue was replaced by osteoid and trabecular bone. Based on this progression, it appears that healing was impaired in the HUCPVC-treated defects compared to the controls.

In the present work, femurs from seven days showed a significant cell infiltrate in the experimental defect when compared to controls. Within this infiltrate, eosinophils were identifiable. Moreover, CD68 (ED1) staining for activated macrophages revealed a massive infiltration of macrophages in the presence of HUCPVCs. Previously, others have reported that infiltration of macrophages and other immune cells into bony defects in response to foreign antigens impairs bone regeneration. Grundes et al. reported that the local activation of macrophages in an osteotomy induced an immature hypertrophic callus with reduced biomechanical characteristics. It has also been shown that macrophage depletion following anterior cruciate ligament reconstruction resulted in significant increases in osteoid formation and mineral apposition rates among experimental specimens. The mechanism through which macrophage activation impairs bone healing is most likely mediated by the release of cytoxic factors, which damage endothelium, fibroblasts, and parenchymal cells, and the release of large amounts of growth factors, such as TGF-β, that could promote fibrous tissue formation instead of bone tissue. In another hMSC transplant study, Neimeyer et al. observe a poor regeneration of bone when using xenograft MSCs as compared to autologous MSCs in sheep tibial defects. They assume that immunological rejection contributed "casually" to this poor healing,
however they do not explore this further. In this work, the slowed rate of healing and fibrous tissue formation observed, along with the immune cell infiltration into the defects in the presence of HUCPVCs, suggests that xenotransplantation of HUCPVCs causes an immune cell response which slows the regeneration of bone in the defect.

Such an infiltration of immune cells during xenotransplantation of MSCs is not unusual. Grinnemo et al. observe a similar round cell infiltrate at the site of injection of human bone marrow mesenchymal stromal cells in cardiac tissue in immunocompetent rats\textsuperscript{107}. These round cells were identified as macrophages through ED1 immunohistochemistry. Unpublished results from our own group have shown a similar macrophage infiltration at the site of injection of HUCPVCs and damage created in the anterior tibialis muscle of immunocompetent rats, and complete rejection of the cells by 7 days\textsuperscript{108}. Of note is a study conducted by Coyne et al.\textsuperscript{124} where they reported that allogenic BMSCs transplanted into the adult hippocampus elicited an immediate inflammatory response. As well, they observed a massive infiltration by ED1-positive microglia/macrophages and surrounded by a marked astrogliosis in the presence of MSCs. They showed complete donor rejection had occurred in the rat brain by 14 days. In contrast to this, Weiss et al. have reported the survival and expansion of pig and human MSCs in the brains of hemiparkinsonian rats that were not immune-suppressed\textsuperscript{63}. These contrasting findings suggest that transplantation studies in the brain are difficult to interpret and therefore cannot be easily related to the findings in this work. Nonetheless, several other studies, outlined previously in the introduction and above, highlight a growing body of evidence that suggests MSCs elicit an immune cell infiltration in immunocompetent hosts after xenotransplantation.

This present work also demonstrated that HUCPVCs could not be detected by immunohistochemical staining for human nuclear antigen beyond seven days. At four days, positively staining nuclei were detected, dispersed from the scaffold in the cell infiltration present in the defect. Few cells were detected at 7 days within the matrix that had formed
in the defect surrounding the remnants of scaffold. The rejection of xenogenic donor tissue, specifically organs, is normally mediated first by the complement system, then the adaptive or acquired immune system. In cellular transplants, it is primarily the adaptive immune system which is responsible. The adaptive responses usually commence later, often a week after transplantation. This time is required for antigen presentation, co-stimulation of T-cells, selection, and expansion of the responding T-cell population. Rejection occurs when cytotoxic T cells infiltrate the transplant site, recognize the donor antigens, and direct cell destruction. In the present work, the temporal and histological profile of HUCPVC rejection was not consistent with an adaptive immune response as graft rejection was nearly complete by seven days. This coupled with the macrophage and eosinophil infiltration in the femoral defect, and the likelihood of non-specific post-operative inflammation, suggests that the HUCPVCs were rejected by an inflammatory response. This is the same conclusion that Coyne et al. arrive at after making similar findings in the rat brain after MSC xenotransplantation. Coyne et al. also suggest that since MSCs secrete granulocyte/macrophage-colony stimulating factor, stromal-derived factor-1, and other cytokines which promote the differentiation and migration of macrophages and granulocytes, this would augment postoperative inflammation, and could lead to graft destruction. Grinnemo et al. however show significant proliferation in coculture experiments with rat lymphocytes that were re-exposed to hMSCs after xenotransplantation. This finding, coupled with the persistence of the hMSCs in athymic rats, which lack an adaptive immune response, led them to conclude that specific rejection of the hMSCs had occured rather than unspecific inflammation. Poncelet et al. make a similar conclusion after observing the rejection of allogenic swine MSCs injected subcutaneously and in a cardiac infarcted area. In this work, HUCPVCs were capable of surviving in athymic rats, as discussed later, however they did not survive in normal rats, similar to the findings by Grinemmo et al. mentioned above. Therefore, this finding may indicate the mechanism of cell rejection is by an adaptive immune re-
sponse. However, in this work, we cannot conclude whether the cells were rejected by a non-specific inflammatory response or an adaptive immune response. In order to confirm a non-specific mechanism of inflammation is responsible for HUCPVC destruction, it would be necessary confirm the lack of CD4$^+$ and CD8$^+$ cytotoxic T-cells present in the defect, which would exclude the possibility of an adaptive immune response.

It was noted in this study that massive trabecular bone outgrowths appeared towards the distal end of the metaphysis and the epiphyses in one HUCPVC treated femur at 30 days, and another at 60 days. This trabecular outgrowth also appeared most obviously in a control femur at 60 days, suggesting that this phenomenon was not related to HUCPVC transplantation. Upon closer inspection of these three cases, the growths appear unusual and most notable were observed away from the defect site and crossing the growth plate. We attribute this unusual trabecular bone formation to a dislocation of the femur around the growth plate during surgical manipulation, which would have resulted in abnormal mechanical loading which could stimulate abnormal trabecular bone growth in this area.

4.5 Transplantation of human umbilical cord perivascular cells in athymic rats

As a result of the graft rejection which occurred in Wistar rats, HUCPVC-seeded constructs were transplanted to femoral defects in athymic rats (strain: Crl:NIH-Foxn1nu). This animal model, as compared to the previously used Wistar rats, has a normal B immune system, while functional T lymphocytes are absent. This means that these animals are not capable of mounting an adaptive immune response to foreign antigens. This is similar to the more common NOD/SCID mouse model, which has an impaired ability to make T or B lymphocytes, or activate the complement system, and cannot efficiently fight infections nor reject tumors or transplants. The athymic rat has been shown to allow engraftement of hMSCs in the infarcted heart after the same cells were rejected in
Chapter 4. Discussion

Moreover, it has been demonstrated by Jager et al. to be a suitable host for bone tissue engineering studies using hMSCs.\textsuperscript{40}

4.5.1 HUCPVCs enhance bone regeneration in athymic rats

The rate of new bone formation in athymic rats, as assessed by microCT, was increased within the defect area after HUCPVC transplantation at 15 days and also at 30 days. A fibrous tissue was observed in the HUCPVC-treated defects beginning around seven days and this was eventually replaced by trabecular bone, most of which was resorbed along with the scaffold by 45 days post-transplantation. This work also looked at the in vivo effect of increasing the initial cell seeding density. Based on the findings discussed earlier, this would increase the viable number of cells attached to the scaffold. This was assessed at 15 and 30 days and it was demonstrated that the mineral volume within the defect increased further at 15 days, showing a statistically significant difference when compared to the control defect. There was however, no statistically significant difference observed in the mineral volume generated in the defect at 30 days. There was also no significant difference observed in the bone formation before 15 days and after 30 days. Others have reported similarly poor bone formation in animal models treated with hMSCs. Niemeyer et al. also report no significant bone formation, as compared to empty scaffolds, when hMSCs were transplanted into sheep tibial defects.\textsuperscript{97} Sidappa et al. show that goat MSCs are capable of producing significantly greater amounts of bone than human MSCs in an ectopic model in mice.\textsuperscript{72} The fact that the HUCPVCs could not induce a greater mineral volume in the defect around 7 and 10 days post-transplant may then be the result of the slower metabolic rate of human cells when compared to the endogenous rat cells.

In the present work, at 30 days post-transplantation, we do not see significantly greater mineral volume in the presence of HUCPVCs, even when the cell dosage was increased two-fold. Looking at the histological evidence, one might argue that even at
15 days, though the increase in mineral volume in the HUCPVC-treated defect may be statistically significant, this difference is still not biologically relevant. In contrast to these results, Sarugaser et al. found that HUCPVCs transplanted through the knee into the intrafemoral space of NOD/SCID mice induced significantly more healing of bone and cartilage in the HUCPVC-injected femurs than in contralateral sham-injected controls at 2, 4 and 6 weeks. This enhanced response to the HUCPVC injection in Sarugaser’s work as compared to the results in this work may be a result of the species difference. Viljanen et al. have shown that the activity per milligram of BMP is more than four-fold greater with allogenic compared to xenogenic BMP in a sheep skull defect model. To our knowledge, no such comparison has been performed with xenogenic and allogenic BMP on human MSCs, but perhaps HUCPVCs do not adequately respond to the local and systemic osteoinductive factors in rats as well as they do in mice. Another important difference between this present work and the Sarugaser study lies in the nature and extent of the orthotopic defect created. The far superior healing observed in the presence of HUCPVCs by Sarugaser may be a consequence of the significant damage that is caused to the epiphysis and distal metaphysis of the mouse femur. This damage, though capable of healing without intervention, requires a significant amount of time. Similarly, the drill-hole defect used in this present work is capable of healing without intervention as well, however this would occur within a presumably shorter time. Furthermore, the use of an osteoconductive scaffold in this present work, accelerates this healing, resulting in bridging of the defect by 30 days and complete cortical healing by 60 days. Therefore, the defect may not provide sufficient damage and the healing that occurs is too rapid to observe the osteoinductive ability of HUCPVCs.

4.5.2 HUCPVCs engraft and contribute to bone regeneration

HUCPVCs transplanted into the rat femurs were tracked in paraffin sections using antibodies against human nuclei and human mitochondria. Positively staining cells were
detected at 7 days in a loose connective tissue matrix which was distinct from the surrounding matrix. Though there was some non-specific staining observed, it was possible to differentiate the human cells from the endogenous rat cells based on nuclear specific-staining and matrix density. At 10 and 15 days, positive human cells were detected within the forming osteoid and in serial sections, stained positively for anti-human osteocalcin and anti-human osteopontin. It is important to note that although sections are 6 µm in thickness, it was still difficult to identify the same cells in serial sections. Much like Sarugaser et al. observed\textsuperscript{62}, HUCPVC-transplanted rat femurs displayed human-specific osteocalcin cytoplasmically and on the surface of newly forming bone. At 30 days positive osteocytes were detected embedded in the trabecular bone in the defect. Again in serial sections, human osteocalcin and osteopontin were detected in the osteocyte cytoplasm and the surrounding matrix. Positive human cells were not detected beyond 30 days, which appears to be sooner than in other studies. Previous in vivo studies conducted by Sarugaser et al. demonstrated that HUCPVCs survive in damaged mouse femurs up to six weeks post-transplantation. They observed the transplanted GFP\textsuperscript{+} HUCPVCs by flushing the marrow and recovering the adherent cells at six weeks post-transplantation. They also observed HuNu\textsuperscript{+} cells in parrafin sections at two, four, six weeks, and they observed human osteocalcin present in the cytoplasm of cells as well as on the contributed osteoid being produced on the surface of newly forming bone. Though the majority of cells did not label with the anti-human antibodies, as is the case in this work, they observed a significant therapeutic effect in conjunction with HUCPVC transplantation, and this was attributed to the HUCPVCs recruiting and directing the mouse mesenchymal progenitors. Goshima et al. have observed a similar phenomena, when they transplanted MSCs on ceramic cubes into mice and detected labelled osteocytes in newly formed bone on the surface of the ceramic\textsuperscript{165}. They could not detect labelled osteocytes within 56 days of implantation and they hypothesized that these were replaced by host-derived osteoblasts that continued the bone formation process\textsuperscript{129}. Thus, in this present work,
considering the low density of HUCPVCs that was transplanted, it is not surprising to find a low number of them engrafting in the rat femur and none detected in the newly formed bone or marrow beyond 30 days.

Despite extensive research in the optimization of bone tissue engineering methods, very few studies address the question of whether the implanted cells survive and are functional. In this present work, anti-human nuclear and mitochondrial antibodies were chosen to track implanted cells because of the relative simplicity of immunohistochemistry in terms of sample preparation, processing, and reliability. There were however other cell tracking techniques attempted in this work using fluorescent carbocyanine membrane dyes, however they posed several technical challenges. The use of fluorescent dyes, such as PKH-26 and CM-DiI, is currently the simplest and most popular method of tracking cells in vivo\textsuperscript{166,167}. These dyes have allowed more in-depth analysis of transplantation, specifically regarding cell fate, however they are limited by the number of cell divisions the transplanted cells can make before the signal disappears. Additionally, Kruyt et al. have demonstrated that CM-DiI dye transfer occurs both in vitro and in vivo between dead and living cells after 10 days, rendering the dye inaccurate for tracking beyond that point\textsuperscript{167}. In this present work, attempts were made to use DiD and DiR to track HUCPVCs in undecalcified sections of rat femurs. This carbocyanine membrane dyes have excitation and emission wavelengths that are outside the autofluorescent range of bone tissue (DiD - ex:648 em:670; DiR - ex:750nm em:782). However, it was found that the three-phase scaffold used as a component of the tissue-engineered construct was slightly autofluorescent in the DiD emission range, and this made it difficult to distinguish cellular signals from scaffold remnants in the tissue. Using DiR as the cell label, no signal was observed in undecalcified sections, and this is most likely because of signal depletion as a result of cell divisions that occurred during the culturing of the construct and in vivo. This was demonstrated in this work, by measuring the mean fluorescence intensity of cultured HUCPVCs stained with DiR for a three week period.
and observing that the fluorescence decreased almost linearly. Additionally, the femurs for this study were processed according to the protocol outlined by Ferrari et al. who employed it to analyse CM-DiI labeled MSCs in orthotopic defects. According to the manufacturer (Invitrogen), the chemistry of CM-DiI differs from DiR, making it more resistant to fixation by formaldehyde-based fixatives. Therefore, it is possible that the lack of DiR signal detected could be the result of denaturation of DiR during the sample processing in this work prior to embedding in methyl methacrylate. This was not tested prior to implantation, however it has been demonstrated by the manufacturer for DiI that formaldehyde fixation alone results in a quenching of the signal. In this work, this could potentially be tested by staining HUCPVCs with DiR in culture and fixing them with paraformaldehyde for different amounts of time after which their fluorescent signal could be analysed by flow cytometry.

Two of the the more stable and reliable techniques proposed to follow the fate of implanted cells in vivo are the transduction of detectable markers, such as β-galactosyl transferase (LacZ) and green fluorescent protein (GFP), and the use of in situ hybridization. GFP has been successfully used to track implanted cells by fluorescence in soft tissue, however its use for tracking cells in skeletal tissues is difficult primarily because of the natural background autofluorescence of bone, and also because it is difficult to preserve the GFP signal during tissue processing. However it is possible to track GFP-expressing cells in bone using an anti-GFP antibody. β-gal is more suitable for hard tissue, as it can be visualized as an intense blue intracellular precipitate in tissue sections without difficult processing techniques simply with the standard substrate X-Gal. In situ hybridization, a type of hybridization that uses a labeled complementary DNA or RNA strand to localize a specific DNA/RNA sequence in a tissue, is a valuable method for tracking transplanted cells in bone. Authors have devised methods of hybridizing the Y-chromosome using a fluorescent probe, making it possible to track male bone marrow cells in female recipients. More recently, others have used a digoxigenin-labeled probe,
visualized through a secondary antibody, for human-specific Alu repetitive sequences to track human cells in sheep tibia\textsuperscript{97}. These methods were not attempted in this present work, but they are worth employing in future in vivo studies using HUCPVCs to further understand their fate in orthotopic defects.
Chapter 5

Conclusions

From the work reported herein, it can be concluded that:

- HUCPVCs met the minimal criteria for multipotent mesenchymal stromal cells. Moreover, they expressed surface markers characteristic of pericytes/perivascular cells and they expressed low levels of MHC class I and did not express MHC class II or costimulatory molecules. Their surface marker phenotype was affected by plating and cryopreservation, specifically for NG2, and they differed from BMSCs in the percentage of positive cells expressing CD146, NG2, and CD86.

- Osteogenic gene expression was detected in undifferentiated HUCPVCs.

- Centrifugal seeding was a suitable method for seeding HUCPVCs onto porous calcium-phosphate-PLGA scaffolds. Moreover, HUCPVCs expanded to form semi-confluent monolayer and multilayer sheets and retained a normal fibroblastic morphology after five days of subculture on these porous scaffolds.

- HUCPVC xenotransplantation in normal Wistar rats did not cause any biological or observable systemic response, assessed through blood cell counts and animal weight, throughout a 40 day period.
• Undifferentiated HUCPVCs did not survive beyond seven days and impaired bone healing in a femoral defect in Wistar rats. An immune cell infiltration was observed in the presence of HUCPVCs in the defects at seven days which was greater than that observed in the defects.

• Undifferentiated HUCPVCs improved bone regeneration when implanted into a femoral defect in immune deficient athymic rats at 15 and 30 days post-op. This difference was statistically significant at 15 days when the dose of HUCPVCs was increased.

• Undifferentiated HUCPVCs survived, differentiated and contributed to new bone in the femoral defects in athymic rats and expressed human specific osteopontin and osteocalcin. However, the relative number of positive human cells detected in the femoral defects was low.


10. Gretzer, C et al. (2006) The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials. *J*
24. Lewandrowski, KU et al. (2001) Immune response to perforated and partially dem-


37. Papadimitropoulos, A et al. (2007) Kinetics of in vivo bone deposition by bone


48. Atoui, R et al. (2008) Myocardial regenerative therapy: Immunologic basis for the


61. Muraglia, A et al. (2000) Clonal mesenchymal progenitors from human bone marrow


83. Grinnemo, KH et al. (2004) Xenoreactivity and engraftment of human mesenchy-


115. Jo, CH et al. (2008) Fetal mesenchymal stem cells derived from human umbilical cord sustain primitive characteristics during extensive expansion. *Cell Tissue Res*


126. Duff, SE et al. (2003) Cd105 is important for angiogenesis: evidence and potential


137. Howson, KM et al. (2005) The postnatal rat aorta contains pericyte progenitor


146. Lanier, LL et al. (1995) Cd80 (b7) and cd86 (b70) provide similar costimulatory signals for t cell proliferation, cytokine production, and generation of ctl. *J Immunol* 154:97–105.


