Adult and Embryonic Stem Cell Sources for Use in a Canine Model of *In Utero* Transplantation

by

Andrea Kathleen Vaags

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Sciences
University of Toronto

© Copyright by Andrea Kathleen Vaags 2010
Adult and Embryonic Stem Cell Sources for Use in a Canine Model of *In Utero* Transplantation

Andrea Kathleen Vaags
Doctor of Philosophy
Institute of Medical Science
University of Toronto
2010

Abstract

Dogs are useful preclinical models for the translation of cell transplantation therapies from the bench to the bedside. In order for canine models to be utilized for stem cell transplantation research, it is necessary to advance discoveries in the fields of canine stem cell biology and transplantation. The use of side population hematopoietic stem cells (HSCs) has garnered much interest for the purification of mouse HSCs and has been translated to several other species, including human. In order to assess if this method of purification of HSCs could be useful for stem cell therapies in humans, safety and efficacy studies in a large animal model, such as the dog would be required. With this objective in mind, we isolated canine bone marrow-derived side population (SP) stem cells and assessed their multilineage differentiation *in vitro* and engraftment potential *in vivo*. Utilizing a pregating strategy to enrich for small, agranular SP cells we were able to enrich for blast cells, expressing the ABCG2 transmembrane pump known to be associated with murine and human SP cells. Canine SP cells were also enriched for C-KIT positive cells and lacked expression of CD34 as identified in other species. The small, agranular SP fraction had high CFU potential after long-term culture with canine bone marrow stromal cells and cytokine supplementation. Yet, canine SP cells
demonstrated low-level engraftment within the NOD/SCID-β2m<sup>−</sup> xenotransplantation model as compared to unfractionated canine bone marrow, which was indicative of suboptimal activation of quiescent canine SP cells within the murine bone marrow niche. A second source of transplantable canine stem cells was examined through the derivation of canine embryonic stem cells (cESCs). The cESC lines described herein were determined to have similar pluripotent stem cell characteristics to human embryonic stem cells, in that they were maintained in an undifferentiated state upon extended passaging as determined by their expression of the human stem cell markers, OCT3/4, NANOG, SOX2, SSEA3, SSEA4, TRA1-60, TRA1-81 and alkaline phosphatase. In addition, cESCs could be induced to differentiate to cells of the three germ layers within <i>in vitro</i> embryoid body cultures and adherent differentiation cultures. Importantly, these cESC lines were the first reported to differentiate <i>in vivo</i> within teratomas. One method of transplanting stem cells to canine recipients involves the delivery of donor cells to the yolk sacs of developing fetuses <i>in utero</i>. Utilizing cells labeled with supraparamagnetic particles conjugated to a Dragon Green fluorophore and the intracellular fluorescent dye, CMTMR, donor cells were tracked from the yolk sac injection site to fetal tissues after transplantation in early (day-25) and mid (day-35) gestation canine fetuses. Labeled cells were localized primarily to the fetal liver and developing bone marrow cavities when examined at gestational day 32, and had been redistributed to not only the fetal liver and bone marrow by day 42, but also to nonhematopoietic tissues, including the lungs and hearts. No labeled cells were detected within the yolk sacs of transplanted fetuses at either time point. These studies demonstrated the efficacy of yolk sac <i>in utero</i> transplantation for the delivery of donor cells to fetal tissues. Collectively, these results
indicate that canine stem cells with characteristics similar to human can be isolated and their engraftment, proliferation and differentiation may be assessed in future studies utilizing the canine \textit{in utero} transplantation model employing yolk sac delivery.
Acknowledgements

Throughout my post-secondary education I have been buoyed up by the support of my family. My mother has seen me through every step of the way; without her love and encouragement, much of what I have accomplished would not have been possible. I have also received on-going guidance and encouragement from my siblings, Christina, Lisa, Janet, Darren, Morgan and Owen, my stepfather, Steve, my father, Bob, stepmother, Shelley, my grandparents and the rest of our very large family.

The last five years have been brightened by the addition of my new family, including my beloved husband, Danny, his parents, Millie and Branko and all of their extended family and friends. I have been fortunate to have Danny’s support through the highs and lows of completing a PhD and he has always helped me to keep the big picture in sight.

Toronto has become like a second home to me during my PhD and this is due in large part to the amazing people that I have had the privilege to meet. Special thanks to my dear friends, Xin and Hong, for many science and life-related discussion as well as to all of the past and current Hough lab members, colleagues at Sunnybrook Research Institute and collaborators at the Ontario Veterinary College.

My academic development was supported by so many people who were willing to share their knowledge and passion for science, but I would particularly like to thank my mentors Drs. John Dick, Norman Iscove, Cathy Gartley, Stephen Kruth, and Mira Puri. It has been a privilege to learn from your combined expertise in stem cell biology, hematology, developmental biology, veterinary medicine and theriogenology.
Of course, none of this would have been achieved without the guidance and support of my supervisor and mentor, Dr. Margaret Hough. Margaret has given me the opportunity to grow as a scientist while instilling in me the importance of scientific rigor and integrity. Her ability to find ways of staying excited with the work, even when difficulties arise, has been a valuable lesson and made coming to the lab every day a pleasure. For the many, many hours of help with lab work, editing, discussion and listening, I am forever indebted.
Table of Contents

Abstract..............................................................................................................ii
Acknowledgements..........................................................................................v
Table of Contents.............................................................................................vii
List of Tables.....................................................................................................xii
List of Figures....................................................................................................xiii
List of Abbreviations........................................................................................xvi

Chapter 1. General Introduction.................................................................1
1.1. Preface.......................................................................................................2
1.2. Canine Models for Preclinical Research.................................................2
1.3. Canine Embryonic and Fetal Development.............................................5
1.4. Hematopoiesis.........................................................................................9
1.5. Canine Stem Cell Sources for Transplantation.......................................13
  1.5.1. Canine Adult Stem Cells.................................................................13
      1.5.1.1. Hematopoietic Stem Cells.......................................................14
      1.5.1.2. Side Population Stem Cells....................................................17
          1.5.1.2.1. Optimization of Hoechst 33342 staining.........................17
          1.5.1.2.2. Transmembrane pimps efflux Hoechst 33342..............19
          1.5.1.2.3. SP cell surface marker expression.................................21
          1.5.1.2.4. SP cells self-renew and proliferation in vitro
                        and in vivo...........................................................................22
      1.5.1.2.5. Canine SP cells...............................................................23
2.5.4. Canine SP cells do not give rise to colonies when plated directly into methylcellulose ..........................................................85

2.5.5. Canine SP cells form colonies after long-term culture on canine stroma...90

2.5.6. Engraftment of canine SP cells in NOD/SCID xenotransplantation model........................................................................91

2.6. Discussion.................................................................................................98

2.7. References..................................................................................................104

Chapter 3. Derivation and Characterization of Canine Embryonic Stem Cell Lines with In Vitro and In Vivo Differentiation Potential.............111

3.1. Attribution of Data.........................................................................................112

3.2. Abstract........................................................................................................113

3.3. Introduction.....................................................................................................114

3.4. Materials and Methods................................................................................117

  3.4.1. Mating and Embryo Collection...............................................................117

  3.4.2. In Vitro Culture of Canine Embryos and Derivation of cESC lines........117

  3.4.3. Establishment and Maintenance of cESC Lines......................................118

  3.4.4. RT-PCR....................................................................................................118

  3.4.5. Immunohistochemistry of cESC Colonies..............................................119

  3.4.6. Analysis of Alkaline Phosphatase Activity............................................121

  3.4.7. Karyotyping of ESCs..............................................................................121

  3.4.8. Teratoma Formation................................................................................121

  3.4.9. In Vitro Formation of Embryoid Bodies (EBs).........................................122

  3.4.10. In Vitro Differentiation of cESCs............................................................122
# 3.5. Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5.1. Embryo Collection and <em>In Vitro</em> Maturation</td>
<td>125</td>
</tr>
<tr>
<td>3.5.2. Establishment of cESC Lines</td>
<td>131</td>
</tr>
<tr>
<td>3.5.3. Expression of Pluripotency Markers by cESCs</td>
<td>132</td>
</tr>
<tr>
<td>3.5.4. Requirement of hLIF for Maintenance of Pluripotency</td>
<td>140</td>
</tr>
<tr>
<td>3.5.5. Karyotype Analysis</td>
<td>143</td>
</tr>
<tr>
<td>3.5.6. <em>In Vivo</em> Differentiation and Teratoma Formation</td>
<td>143</td>
</tr>
<tr>
<td>3.5.7. <em>In Vitro</em> Differentiation of cESCs</td>
<td>150</td>
</tr>
</tbody>
</table>

# 3.6. Discussion

158

# 3.7. References

164

---

## Chapter 4. Migration of Cells from the Yolk Sac to Hematopoietic Tissues after *In Utero* Transplantation in Early and Mid Gestation Canine Fetuses

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1. Attribution of Data</td>
<td>174</td>
</tr>
<tr>
<td>4.2. Abstract</td>
<td>175</td>
</tr>
<tr>
<td>4.3. Introduction</td>
<td>176</td>
</tr>
<tr>
<td>4.4. Materials and Methods</td>
<td>177</td>
</tr>
<tr>
<td>4.4.1. Animal husbandry</td>
<td>180</td>
</tr>
<tr>
<td>4.4.2. Canine bone marrow harvest and processing of bone marrow mononuclear cells (BMMCs)</td>
<td>180</td>
</tr>
<tr>
<td>4.4.3. Mesenchymal Stromal Cell (MSC) Cultivation and Characterization</td>
<td>181</td>
</tr>
<tr>
<td>4.4.4. BMMC and MSC labeling</td>
<td>182</td>
</tr>
<tr>
<td>4.4.5. <em>In utero</em> transplantation of labeled BMMC and MSC</td>
<td>182</td>
</tr>
<tr>
<td>4.4.6. Spay and fetal retrieval</td>
<td>183</td>
</tr>
<tr>
<td>4.4.7. <em>Ex vivo</em> whole body fluorescence imaging</td>
<td>184</td>
</tr>
</tbody>
</table>
4.4.8. Preparation of samples for histological analysis.................................184
4.4.9. Microscopy.........................................................................................185
4.4.10. Canine Y chromosome analysis......................................................185

4.5. Results..................................................................................................187

4.5.1. MSC characterization........................................................................187
4.5.2. Cell labeling with SPIO and CMTMR..............................................187
4.5.3. In utero cell transplantation..............................................................190
4.5.4. Ex vivo whole body fluorescence imaging......................................192
4.5.5. Histological analysis of fetal tissues.................................................197
4.5.6. Molecular analysis of fetal tissues.....................................................203

4.6. Discussion............................................................................................205

4.7. References............................................................................................209

Chapter 5. General Discussion and Future Directions........213

5.1. Canine Side Population (SP) Cells....................................................214
5.2. Canine Embryonic Stem Cells (cESCs)..............................................218
5.3. Canine In Utero Transplantation.......................................................221
5.4. Summary..............................................................................................224
5.5. References............................................................................................226
List of Tables

Table 1.1. Comparison of the findings of five groups establishing canine embryonic stem cell lines……………………………………………………………….28

Table 2.1. Antibodies utilized for characterization of canine SP cells…………………..68

Table 2.2. RT-PCR primers utilized to characterize the genes expressed by SP cells….70

Table 2.3. Percentages of canine bone marrow-derived SP cells falling within the small agranular and large, granular gated regions…………………………………...79

Table 2.4. Response rate of individual methylcellulose cultures and calculated CFU-C frequency for SP and non-SP cell fractions cultured for 6 weeks in LTC conditions prior to transfer to methylcellulose……………………………...92

Table 3.1: RT-PCR Primers Sets for Canine Genes………………………………………120

Table 3.2: PCR Primer Set for Amplification of Canine and Murine Beta-2-
Microglobulin from Genomic DNA………………………………………………123

Table 3.3: Optimal Stage of Development for In Vitro Embryonic Expansion of Inner Cell Mass…………………………………………………………….130

Table 4.1. Cell transplants completed in seven canine pregnancies…………………..191

Table 4.2. Assessment of canine Y chromosome amplification from donor cells transplanted to female fetuses………………………………………………204
List of Figures

Figure 1.1. Timeline of embryonic and fetal development milestones in canine pregnancies from gestational day 0-30.................................................7

Figure 1.2. The ontogeny of blood cell production from hematopoietic stem cell (HSC) to mature blood cells.................................................................10-11

Figure 1.3. Side population cell flow cytometry profile after Hoechst 33342 staining of canine bone marrow cells.................................................................18

Figure 2.1. Detection of the side population fraction in canine, mouse and human samples and inhibition of dye efflux with verapamil and fumitremorgin.....................................................74

Figure 2.2. Flow cytometric analysis of SP cell size and granularity for the total SP, large, granular SP (LG-SP) and small, agranular SP (SA-SP) subpopulations.................................................................76

Figure 2.3. Wright-stained cytospins of canine bone marrow stained with Hoechst 33342 gated into small, agranular (SA) and large, granular (LG) cells and differential counts........................................................................78

Figure 2.4. RT-PCR detection of transporter pump and lineage markers expressed in each SP and non-SP subfraction.................................................................81

Figure 2.5. Flow cytometric analysis of cell surface markers expressed by cells within each SP and nonSP fraction.................................................................83-84

Figure 2.6. Flow cytometric analysis of canine CD34 expression in relation to the SP fraction.................................................................86

Figure 2.7. CFU-Cs derived from unfractionated canine bone marrow, SP and nonSP fractions plated directly into human cytokine-supplemented methylcellulose medium.................................................................88-89

Figure 2.8. DX5+NKp46+ natural killer cells in the peripheral blood are diminished by anti-CD122 treatment of NOD/SCID-β2-microglobulin−/− mice........................................93

Figure 2.9. Average level of engraftment of canine CD45+ cells in anti-CD122-treated NOD/SCID-β2m−/− recipient mice transplanted intrafemorally with SP and non-SP fractions of canine bone marrow.................................................................95
List of Figures, continued

Figure 2.10. Distribution of engraftment levels of canine CD45+ cells in anti-CD122-treated NOD/SCID-β2m-/ recipient mice transplanted intrafemorally with SP and non-SP fractions of canine bone marrow…………………………97

Figure 3.1. Morphology of canine embryos and cESC outgrowths………………126-127

Figure 3.2. *In vitro* maturation of canine embryos and generation of cESC lines SK-1 and K-6………………………………………………………………………128

Figure 3.3. Expression of pluripotency markers by cESCs…………………………133-135

Figure 3.4. Expression of pluripotency markers was specific to undifferentiated regions of cESC colonies…………………………………………………136-137

Figure 3.5. Analysis of OCT3/4, SOX2 and NANOG expression in cESCs by RT-PCR………………………………………………………………………138-139

Figure 3.6. Spontaneous differentiation of cESCs after removal of hLIF from the culture medium……………………………………………………………141-142

Figure 3.7. Canine ESC derived from male and female embryos maintained a normal karyotype after multiple passages……………………………………144

Figure 3.8. *In vivo* differentiation of cESCs to tissues of the three embryonic germ layers……………………………………………………………………145-147

Figure 3.9. Teratoma tissues, derived after injection of cESC under the testis capsule of NOD/SCID and NOD/SCID-β2 mice, contained canine specific DNA……………………………………………………………………149

Figure 3.10. *In vitro* differentiation of cESCs to EBs containing cells of ectodermal, endodermal and mesodermal origin……………………………151-152

Figure 3.11. *In vitro* differentiation of cESCs to mesoderm, ectoderm and endoderm………………………………………………………………………154-156

Figure 4.1. Flow cytometric analysis of cell surface markers…………………………188

Figure 4.2. Efficiency of SPIO labeling of BMMCs and MSCs used for *in utero* transplantation……………………………………………………………………189
List of Figures, continued

**Figure 4.3.** Whole body fluorescence imaging of recipient dogs seven days after *in utero* transplantation………………………………………………193-194

**Figure 4.4.** Whole organ fluorescence imaging of day 35 recipient dogs seven days after *in utero* transplantation………………………………………………196

**Figure 4.5.** Detection of SPIO-labeled cells within fetal livers of fetuses transplanted at 35 days of gestation and retrieved at 42 days…………………..198-199

**Figure 4.6.** Detection of SPIO-labeled cells within fetuses transplanted at 25 days of gestation and retrieved at 32 days……………………………………200-201
List of Abbreviations

%                percent
°C                degrees Celsius
5-FU              5-flurouracil

ACK
AFP              alpha-fetoprotein
APC              allo-phycocyanine
ATP              adenosine tri-phosphate

BM                bone marrow
BMMC              bone marrow mononuclear cells
bp                base pairs
β2m               beta-2 microglobulin

cCD34             canine cluster of differentiation 34
cCD45             canine cluster of differentiation 45
cDNA              complementary deoxyribonucleic acid
cESCs             canine embryonic stem cells
CFCs              colony forming cells
CFU(s)            colony forming unit(s)
CFU-E             colony forming unit-erythrocyte
CFU-G             colony forming unit-granulocyte
CFU-GEMM          colony forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte
CFU-GM            colony forming unit-granulocyte, monocyte/macrophage
CFU-M             colony forming unit-monocyte/macrophage
cG-CSF            canine granulocyte-colony stimulating factor
cGM-CSF           canine granulocyte macrophage-colony stimulating factor
CLAD              canine leukocyte adhesion disorder
cm                centimetre
CNBD              could not be determined
cSCF              canine stem cell factor
CTL                control

DAPI              4',6-diamidino-2-phenylindole
DMEM              Dulbecco’s Modified Eagle’s Medium
DNA               deoxyribonucleic acid

EBs               embryoid bodies
EDTA              ethylenediaminetetraacetic acid
ESCs              embryonic stem cells
et al.            et alia

FACS              fluorescence activated cell sorting
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluoro-iso-thio-cyanide</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered saline solution</td>
</tr>
<tr>
<td>hESCs</td>
<td>human embryonic stem cells</td>
</tr>
<tr>
<td>HF</td>
<td>Hank’s buffered saline solution containing 2% fetal bovine serum</td>
</tr>
<tr>
<td>hFlt3L</td>
<td>human Flt3 ligand</td>
</tr>
<tr>
<td>hIL3</td>
<td>human interleukin 3</td>
</tr>
<tr>
<td>hIL7</td>
<td>human interleukin 7</td>
</tr>
<tr>
<td>HSCs</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>IMEM</td>
<td>Iscove’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>IUT</td>
<td><em>in utero</em> transplantation</td>
</tr>
<tr>
<td>IUSCT</td>
<td><em>in utero</em> stem cell transplantation</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KO SR</td>
<td>Knockout serum replacement</td>
</tr>
<tr>
<td>KSL</td>
<td>cKIT⁺Sca1⁺Lin⁻ cell</td>
</tr>
<tr>
<td>LG</td>
<td>large granular</td>
</tr>
<tr>
<td>LG-nonSP</td>
<td>large granular non-side population cell</td>
</tr>
<tr>
<td>LG-SP</td>
<td>large granular side population cell</td>
</tr>
<tr>
<td>LH</td>
<td>leutinizing hormone</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>Lin⁻</td>
<td>lineage negative cells</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>long-term culture-initiating cells</td>
</tr>
<tr>
<td>MEFs</td>
<td>murine embryonic fibroblasts</td>
</tr>
<tr>
<td>mESCs</td>
<td>murine embryonic stem cells</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MHz</td>
<td>megahertz</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MPS I</td>
<td>mucopolysaccharidosis type I</td>
</tr>
<tr>
<td>MSCs</td>
<td>multipotent mesenchymal stromal cells</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>N</td>
<td>number</td>
</tr>
<tr>
<td>ND</td>
<td>not done</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>no.</td>
<td>number</td>
</tr>
<tr>
<td>NOD</td>
<td>nonobese diabetic</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>nonobese diabetic/severe combined immune deficiency</td>
</tr>
<tr>
<td>NOD/SCID-β2m^-/-</td>
<td>nonobese diabetic/severe combined immune deficiency - β2-microglobulin null</td>
</tr>
<tr>
<td>nonSP</td>
<td>non-side population</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>all-trans retinoic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SA</td>
<td>small agranular</td>
</tr>
<tr>
<td>SA-nonSP</td>
<td>small agranular non-side population cell</td>
</tr>
<tr>
<td>SA-SP</td>
<td>small agranular side population cell</td>
</tr>
<tr>
<td>SSEA</td>
<td>stage-specific embryonic antigen</td>
</tr>
<tr>
<td>SNL</td>
<td>murine STO-derived feeder cells</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>SPIO</td>
<td>small paramagnetic iron oxide particles</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>StdDev</td>
<td>standard deviation</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>USD</td>
<td>United States of America Dollar</td>
</tr>
<tr>
<td>U.S.P.</td>
<td>United States Pharmacopoeia</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
CHAPTER 1

General Introduction and Literature Review
1.1 Preface

As human stem cell therapies move closer to the clinic, safety and efficacy testing in large animal models is warranted, particularly in the case of embryonic stem cell-derived therapies. The dog has a long-standing history of utility in translating research from the bench to the bedside. This thesis will focus on the dog as a useful preclinical model, with a specific focus on in utero stem cell transplantation, as well as the isolation and characterization of both adult and embryonic stem cells as potential cell sources for transplantation in the canine model. As such, I will limit discussions to canine-specific research and examples, except when noted. This introduction will cover the advantages of utilizing dogs for preclinical studies, followed by a brief overview of canine embryonic and fetal development to give relevant background information on canine embryogenesis as it relates to the development of canine embryonic stem cell lines, as well as midgestation fetal development as it relates to canine in utero transplantation. The state-of-the-art in adult and embryonic canine stem cell biology will also be introduced, with a summary of the transplantation models that can be used to assess the engraftment potential of canine stem cells.

1.2 Canine Models for Preclinical Research

The dog (Canis familiaris) has often been utilized as a preclinical model due to the similarities between human and dog physiology, disease presentation and clinical response (Starkey et al., 2005). Yet, not only are studies in dogs useful for their predictive outcomes of the safety and efficacy of human therapies, they are also useful for the treatment of dogs who have become one of man’s closest companions. Second
only to humans, health care expenditures for dogs in the United States are in excess of $20 billion USD (AVMA, 2007) and many pet owners are interested in new research to treat diseases in their companion animals. Thus, canine health care research is a very active field in its own right, with the benefit of many findings being directly translated to human medicine.

The advantages of utilizing dogs in preclinical studies are many fold. Ethical and cost considerations are major factors in large animal studies and as such many studies have elected to use canine models rather than nonhuman primates. In comparison to nonhuman primates, dogs are easy to care for and handle (Trobridge et al., 2005) and are less expensive to acquire and maintain. Dogs also acquire many of the same diseases that humans do; over 360 genetic disorders in human have an analogue in dogs. In most cases, the genetic mutation found in humans, maps to the same gene in dogs or a canine analogue of the gene (Patterson, 2000). Some of the diseases that afflict both dogs and humans include cardiomyopathies (Dixon and Spinale, 2009), blindness (Armstrong et al., 1982; Narfstrom et al., 1989; Acland et al., 2001; Aguirre et al., 2007; Vilboux et al., 2008), deafness (Hood et al., 2002; Rak et al., 2003), epilepsy (Hegreberg and Padgett, 1976; Loscher et al., 1985; Famula et al., 1997), lysosomal storage disorders (Kelly et al., 1983; Walvoort et al., 1985; Alroy et al., 1992; Menon et al., 1992), and cancers (Vail and MacEwen, 2000; Breen and Modiano, 2008).

Development of many biomedical tools for the treatment and study of disease in dogs have also driven canine models to the forefront of preclinical research. A number of canine-specific antibodies are now available for immunocytochemistry, flow cytometry, and Western blotting (Schuberth et al., 1998; Weiss, 2001), while the production of
canine chromosome paints has allowed for the determination of genetic aberrations responsible for many canine diseases (Courtay-Cahen et al., 2007; Thomas et al., 2007). In addition, the dog genome project has revealed that nucleotide conservation is high between the dog and other mammalian species. Despite the fact that the dog was the first to diverge from the human-dog-mouse common ancestor (Madsen et al., 2001; Murphy et al., 2001; Kirkness et al., 2003) it shares greater sequence identity with the human than the mouse does. More than 650 million base pairs (>25%) of the dog genome align uniquely to the human genome, including fragments of putative orthologs for 18,473 of 24,567 annotated human genes (Kirkness et al., 2003).

Similar to the human population, there are a variety of genetic backgrounds that can influence physiology and metabolism in dogs. Within the over 400 breeds recognized worldwide (Wilcox and Walkowicz, 1993) and the 162 recognized by the American Kennel Club (2010), there are known physiologic and metabolic idiosyncrasies that can influence the propensity for certain diseases within breeds as well as effect drug pharmacokinetics and responses to xenobiotics (Fleischer et al., 2008). Thus, studies in multiple breeds can simulate treatment outcomes within divergent human patient populations and reveal variable outcomes to therapies. Within-breed polymorphisms also contribute to an increased complexity of genetic backgrounds within dogs that lead to different responses to drug treatments and other therapies (Fleischer et al., 2008). Thus, out-bred canine subjects may be more predictive of complex human populations than commonly used inbred mouse populations.

Perhaps most importantly, the long lifespan of the dog makes it more suitable for preclinical studies than the mouse, as the long-term effects of treatments can be assessed.
Side effects of many drug, gene therapy and cell therapy treatments will only be apparent after an extended period of time, in excess of the normal life span of murine recipients. For example, transplantation of gene-modified stem cells in canine recipients has pointed to safety concerns that were not detected in murine models. The over expression of HOXB4 in CD34+ cells resulted in myeloid leukemia in canine recipients two years after transplantation (Zhang et al., 2007, 2008), while the same effects were not noted in murine recipients (Antonchuk et al., 2001).

Despite these benefits, there are limitations in the use of canine models. Due to our closer evolutionary relationship with nonhuman primates, studies utilizing nonhuman primates can be even more predictive of human therapy outcomes. Particularly in the case of gene-modified cell transplantation, nonhuman primates whose genetic material has greater conservation with our own can be more predictive of insertional mutagenesis sites within the DNA of modified cells (Trobridge et al., 2005). For example, there is evidence of recurrent insertion near Evi1 in rhesus macaques that received gammaretroviral vector gene modified CD34+ cells (Calmels et al., 2005), a finding which raises concerns about the safety of gene-modified cell transplantation in human patients and highlights the importance of preclinical testing in large animal models.

1.3 Canine Embryonic and Fetal Development

Despite the many similarities between humans and dogs in regards to organ physiology, the reproductive biology of dogs is quite different from that of humans. Bitches have an estrous cycle twice per year, at which time multiple ova are ovulated from both ovaries. The unfertilized eggs enter the oviducts, where fertilization takes
Fertilized ova will transition from the oviducts into the two horns of the uterus, which will become the site of implantation. The canine pregnancy is 63-65 days in length and during this two-month period, development occurs at a very rapid rate to produce fully formed puppies. The important developmental milestones in the canine pregnancy are summarized in Figure 1.1 and further detailed herein.

The progression of the canine pregnancy is often staged from the time of the luteinizing hormone (LH) surge, which is designated as day 0. The canine ovum is ovulated at day 1-2, undergoes oocyte maturation at day 4-5 and remains viable and receptive to fertilization within the uterine tubes for several days (Concannon, 2000). Unlike many species, canine oocytes are ovulated as primary oocytes and do not undergo meiosis to secondary oocytes until 2.5-3 days after ovulation. Canine sperm is also long-lived within the uterine tract and thus insemination from several days prior to or after ovulation can result in fertilization from day 1-8 (Concannon, 2000) (Tsutsui et al., 2009). In the event, that fertilization occurs prior to oocyte maturation, the male pronucleus must wait for the development of the female pronucleus before a new one-cell embryo is formed. In this way, the length of canine pregnancies is consistently 63-65 days from the LH surge, despite the large window of time in which fertilization can take place.

If fertilization occurs, the ovum divides to the 4-cell stage at day 8, 8-cell at day 9, 16-cell at day 10 and 32-cell at day 11 (Concannon, 2000). Between day 11-13, the
**Figure 1.1.** Timeline of embryonic and fetal development milestones in canine pregnancies from Day 0-30 after the luteinizing hormone (LH) surge. Data is compiled from references included in text of Introduction, p 5-9.
fertilized ova divide to the morulae or even early blastocyst stage when they transit into the uterine horns (Abe et al., 2008) and become evenly distributed within the horns from day 12-17 (Pretzer, 2008). Development continues with progression to the blastocyst stage at day 14 (Abe et al., 2008), followed by gastrulation being initiated during day 18-19 (Concannon, 2000). At this time, the three germ layers are established and the endoderm migrates to line the trophoblast becoming a bilaminar yolk sac. As the mesoderm migrates between the endoderm and trophoblast, the trilaminar yolk sac is formed. The trophoblast-derived amniotic folds fuse around the embryo to form the amniotic sac, while the endoderm-derived allantois gradually expands into the extraembryonic coelom. At this stage of development the blood islands form within the yolk sac mesoderm allowing primitive hematopoiesis and vasculogenesis to begin with the development of circulation between the yolk sac and the developing fetus (McGeady et al., 2006). By day 20-21, the embryo has developed to greater than 2 mm in diameter, the zona pellucida is absent, and the embryo touches the uterine wall and cannot be flushed from the uterine tract.

Implantation occurs at day 21-23 (Thatcher et al., 1994; Concannon, 2000); (Pretzer, 2008), at which time opposition of the maternal uterine wall and the yolk sac and trophoblast-derived choriovitelline membrane, forms the choriovitelline placenta, which can be visualized by ultrasound at day 25 (Kim and Son, 2007). This is followed by fusion of the allantois with the trophoblast, forming the chorioallantoic membrane, which in conjunction with the maternal tissues creates the chorioallanotic placenta. The choriovitelline and chorioallantoic placentae co-exist until the fourth week of gestation, when the choriovitelline placenta ceases to function in respiration and nutrient exchange.
Despite this, the canine yolk sac persists throughout development and remains an important site of erythropoiesis, with remnants of the yolk sac still present at birth (McGeady et al., 2006). The yolk sac vessels circulate directly through the region of the developing fetal liver, supplying oxygenated blood from the placenta. The fetal liver will become the site of the earliest definitive hematopoiesis, which is sustained by stem cells seeded from the aorta-gonad-mesonephros region. Thus, with anastomoses of the vitelline veins from the yolk sac with the endocardial tubes of the developing heart, circulation between the yolk sac and the fetus proper is fully established. It should be noted that while extraembryonic hematopoiesis is progressing in the yolk sac blood islands and circulation between the yolk sac and fetus proper are developing, the embryo has already undergone sufficient development that the cardiac tube is formed and a heart beat can be detected by ultrasound as early as day 23-24 (Kim and Son, 2007). By day 30, all organ systems are established and the second half of canine pregnancy is marked by rapid growth and functional maturation of each organ system.

1.4 Hematopoiesis

Hematopoiesis is the production of all of the mature cells of the blood system from a pool of hematopoietic stem cells (HSCs). HSCs have the ability to self-renew to produce additional HSCs, as well as to differentiate to produce all of the different mature blood cell types. In this way, the HSC pool is maintained throughout the life of the organism, while supporting the need to constantly produce new mature blood cells. HSCs generate cells of the mature lineages through a series of intermediate progenitors, each of which is increasingly restricted in its ability to self-renew and generate multiple lineages (Figure 1.2). The mature blood cells are divided into two main classes; the lymphoid and myeloid
Figure 1.2. The ontogeny of blood cell production from hematopoietic stem cell (HSC) to mature blood cells. Intermediate progenitors include the hematopoietic progenitor cell (HPC), common myeloid progenitor (CMP), lymphoid-primed
Figure 1.2. (continued) multipotent progenitors (LMPP), common lymphoid progenitor (CLP), colony forming unit – granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte (CFU-GEMM), and colony forming unit – granulocyte, monocyte/macrophage (CFU-GM). Adapted from M. William Lensch, retrieved May 3, 2010. http://daley.med.harvard.edu/assets/Willy/ hematopoiesis.jpg and (Luc et al., 2007)
cells. The lymphoid cells include the T cells, B cells, natural killer cells and lymphoid dendritic cells which constitute the adaptive immune system. The myeloid lineage is composed of the granulocytes, monocytes, macrophages, myeloid dendritic cells, megakaryocytes and platelets, which are important components of the innate immune system responsible for inflammation, phagocytosis and coagulation. In addition, the myeloid cells include erythroid cells, also known as the red blood cells, which are responsible for carrying oxygen throughout the organism.

During early fetal development, hematopoiesis is extra-embryonic and begins in the blood islands of the yolk sac with the production of immature red blood cells that develop in close proximity to the endothelial cells that form the vasculature (Ribatti et al., 2000). Early yolk sac hematopoiesis is restricted to the production of primitive erythrocytes that retain their nuclei and possess embryonic hemoglobin molecules (Palis and Yoder, 2001). A second wave of yolk sac hematopoiesis is thought to produce macrophage (Moore and Metcalf, 1970; Palis et al., 1999) and megakaryocyte (Xu et al., 2001) progenitors, as well as definitive erythroid progenitors (Wong et al., 1986a, b; Palis et al., 1999). While hematopoiesis continues within the yolk sac, HSCs arise independently in the intraembryonic aorta-gonad-mesonephros region within the fetus proper (Medvinsky et al., 1993; Muller et al., 1994; Medvinsky and Dzierzak, 1996). Soon after the development of organized structures in the fetal liver, it is colonized by HSCs and becomes the main site of definitive hematopoiesis for the production of all of the mature blood cell types. The fetal liver remains the main site of hematopoiesis throughout the perinatal period, but during mid to late gestation the bone marrow spaces develop and are populated with HSCs. In the adult, hematopoiesis is limited to the bone marrow, with the
maturation of lymphoid cells being supported by the thymus, spleen and lymph nodes (Medvinsky and Dzierzak, 1996).

1.5 Canine Stem Cell Sources for Transplantation

In order for stem cell therapies to be completed in canine models, the isolation and characterization of stem cell populations is required. The study of canine stem cells has grown out of many advances developed in murine and human stem cell research. The techniques for the isolation of canine stem cells have taken cues primarily from work done with human stem cells, as the characteristics and cell surface marker expression of canine cells is most similar to those of human stem cells.

1.5.1 Canine Adult Stem Cells

Adult stem cells are undifferentiated cells found throughout the body, that divide to replenish dying cells and regenerate damaged tissues. They have the ability to self-renew and generate some or all of the cells types of the organ from which they originated.

The study of adult stem cells in dogs has been primarily limited to bone marrow and peripheral blood-derived cells (McSweeney et al., 1996; Kadiyala et al., 1997). More recently, adult stem cells have also been isolated from canine adipose tissue (Neupane et al., 2008; Vieira et al., 2009), umbilical cord blood (Seo et al., 2009; Zucconi et al., 2010), liver (Arends et al., 2009), mammary tissue (Cocola et al., 2009), dental pulp (Iohara et al., 2009), and hair follicles (Kobayashi et al., 2009).

The phenotypical and functional assays employed in human stem cell biology translate well to canine adult stem cells and have provided evidence for the presence of
stem cell populations in dogs that are similar to human stem cells and can thus be useful for preclinical studies in canine models of stem cell transplantation.

1.5.1.1 Hematopoietic stem cells

The existence of a multipotential hematopoietic stem cell (HSC) was first postulated by Maximow in 1909 when he detected a cell with a “lymphocyte” appearance that was capable of migrating from the blood to other tissues, where it was able to proliferate and differentiate along lineage specific pathways (Gunsilius et al., 2001). This hypothesis gained further support in the 1950’s following several reports of successful recovery from irradiation damage by bone marrow transplantation (Lorenz et al., 1951; Main and Prehn, 1955; Barnes et al., 1956; Ford et al., 1956; Thomas et al., 1957). The work of Till and McCulloch utilizing bone marrow transplantation for repopulation experiments provided the most compelling evidence for a self-renewing HSC. Multilineage hematopoietic colonies of donor origin within the spleens of irradiated mice, called colony-forming unit-spleen, were formed following transplantation of small numbers of bone marrow-derived cells (Till and McCulloch, 1961). This indicated that a rare population of cells in the bone marrow was capable of producing colonies of new blood cells. They also showed that cells from these colonies gave rise to similar colonies upon transplantation into secondary recipients, indicating the capacity for self-renewal (Siminovitch et al., 1963).

Thus by definition, HSC have the potential to self-renew, differentiate into all of the mature cell types of the hematopoietic system and reconstitute the entire adult
hematopoietic cell repertoire upon transplantation into lethally irradiated recipients. The majority of HSCs reside in the adult bone marrow cavity, but HSCs also circulate via the peripheral blood and can thus be found in many other tissues at low levels (Wright et al., 2001).

Many HSC transplantations have been completed with unfractionated canine bone marrow as a source of HSCs, as this cell source contains not only HSCs required for long-term engraftment and self-renewal of donor cells, but also contains progenitor cells that support short-term reconstitution and more mature cells which may provide cytokines and growth factors to aid donor HSC survival, engraftment and proliferation. More recently, purified canine HSCs have been used for transplantation as this allows for delivery of a greater number of cells with stem and progenitor properties in a smaller total cell number. The durability of engraftment is often related to the abundance of stem cells present within a cell graft and transplantation of a defined number of stem cells allows for greater reproducibility between experiments. Thus, stem cell purification can increase the engraftment seen in recipients and allows researchers to determine the stem cell properties of defined subpopulations of cells. Enrichment of canine HSCs has been accomplished through a variety of methods including density-gradient centrifugation (Wijewardana et al., 2007), canine CD34 (cCD34) antibody selection (McSweeney et al., 1996, 1998; Bruno et al., 1999, 2001; Blakemore et al., 2004), wheat germ agglutinin binding (Wijewardana et al., 2007), Rhodamine expulsion (Niemeyer et al., 2001; Wijewardana et al., 2007) and more recently Hoechst 33342 expulsion (Arends et al., 2009; Iohara et al., 2009).
Since the development of the cCD34 antibody in 1998 (McSweeney et al., 1998), immunoselection of cCD34⁺ HSCs has become the most widely utilized method of HSC isolation in canine stem cell transplantation studies. With current protocols, it is possible to enrich the CD34⁺ fraction to greater than 95% purity (Suter et al., 2004, 2007) and the use of negative selection of lineage-restricted markers (Bruno et al., 2001) or supravital dye stained cells (Niemeyer et al., 2001) further increases the resulting cell purity.

The utility of cCD34⁺ cells for stem cell transplantation has been assessed through autologous or DLA-matched transplantation into myeloablated dogs, which resulted in long-term multilineage reconstitution of canine recipients (Bruno et al., 1999). If the purity of the cCD34⁺ cells was greater than 95%, full hematopoietic recovery occurred but was delayed, while transplantation of bone marrow enriched for cCD34⁺ cells (20-70% purity) but not depleted of cCD34⁻ cells resulted in more rapid reconstitution, which was attributed to short-term reconstitution by cCD34⁻ progenitor and mature hematopoietic cells, but may also be due to the effect of cytokines produced by cells in the CD34⁻ fraction which supported the engraftment and proliferation of the cCD34⁺ HSCs.

The characterization of CD34⁻ HSCs in the bone marrow of mouse and human has indicated that CD34⁺ selection may result in the exclusion of some primitive HSCs that also have long-term reconstitution potential in transplantation studies (Osawa et al., 1996; Morel et al., 1998; Sato et al., 1999; Morita et al., 2006). One method that enriches for CD34⁻ HSCs is based on the efflux of the supravital dye, Hoechst 33342, and identifies a fraction of cells termed the side population (SP) (Goodell et al., 1997).
Identification of CD34− HSCs from canine bone marrow has not been established and thus this thesis will address if CD34− SP HSCs can be isolated from canine bone marrow.

1.5.1.2 Side Population Stem Cells

A distinct population of HSCs can be isolated from bone marrow based on the efflux of Hoechst 33342 (Hoechst) dye when examined by UV flow cytometry at two emission wavelengths simultaneously (424 nm (Hoechst Blue) and 675 nm (Hoechst Red)). This population forms a “hook” distinct from the main cell population in a flow plot of Hoechst Blue versus Hoechst Red and as such has been named the side population (SP) cells (Figure 1.3). Hoechst dye rapidly diffuses into all cells, but is rapidly effluxed by HSCs. The SP cell is a rare subset of cells, approximately 0.05 – 0.1% of total bone marrow cells in mice (Goodell et al., 1996) while reported percentages for human, rhesus monkey and pig are 0.03-0.8% (Goodell et al., 1997; Asakura and Rudnicki, 2002), 0.01-0.07% (Goodell et al., 1997), and 0.05-0.09% (Goodell et al., 1997), respectively. Preliminary studies in our laboratory indicated that cells with an SP phenotype could be isolated and constituted 0.08% of the canine bone marrow (Grantab, 2004), but a functional analysis of these cells was not completed.

1.5.1.2.1 Optimization of Hoechst 33342 staining

Goodell and colleagues have determined that the concentration of Hoechst dye and length of staining time are critical variables in the dynamic process of staining SP cells. Thus, optimization of the staining protocol for each species and tissue type tested is required. Incubation with 5 µg/ml Hoechst 33342 for 90 minutes is required for isolation of murine bone marrow SP cells (Goodell et al., 1996), while 5 µg/ml and 120 minutes was
Figure 1.3. Side population cell flow cytometry profile after Hoechst 33342 staining of canine bone marrow cells. SP cells are detected as a “hook” population of cells to the left of the main cell population when excited with an ultraviolet laser and examined at 424 nm (Hoechst Blue) and 675 nm (Hoechst Red) emission wavelengths.
determined to be optimal for human, rhesus macaque and porcine bone marrow SP cells (Goodell et al., 1997; Goodell, 2005).

Ibrahim et al. (2007) have shown, with real-time flow cytometric assessment of murine bone marrow cells stained with Hoechst, that every cell passes through the SP hook as the intracellular concentration of Hoechst dye increases until eventually all cells become part of the main population of cells saturated with Hoechst dye. Only a small population of cells, namely the HSCs, that actively efflux the dye remains within the SP hook region. With extended incubation, the HSCs that are slow to accumulate Hoechst dye will also become part of the main population. As such, it is critical to determine the optimal staining time for each cell source and species, to determine the window of time during which the majority of the cells are within the main population and the HSCs are falling within the SP hook region.

1.5.1.2.2 Transmembrane pumps efflux Hoechst 33342

Early studies speculated that the low level of Hoechst fluorescence exhibited by SP cells was due to the active efflux of the Hoechst dye by the P-glycoprotein (Pgp), encoded by the multidrug resistance gene, Mdr1. Expression of Mdr1 had been identified in early hematopoietic progenitors (Chaudhary and Roninson, 1991) and thus the side population phenotype was largely attributed to Mdr1 (Bunting et al., 2000). Yet the Hoechst efflux activity of murine HSCs was only partially blocked by the Pgp inhibitor, verapamil (Zijlmans et al., 1995), which indicated that another transporter might be responsible for the Hoechst dye efflux of SP cells. Mice with targeted disruptions for the Mdr1a and Mdr1b genes showed no difference in the percentage of SP cells in mutant
versus wild-type mice, which further confirmed that Pgp is not required for the SP phenotype. Furthermore, rhodamine effluxing cells were absent in Mdr1a/1b-/- mice indicating the importance of Pgp for rhodamine efflux and the presence of another transporter for Hoechst efflux (Zhou et al., 2001). More recently, specific inhibition of another pump, the ABCG2 transporter, with fumitremorgin C resulted in complete inhibition of the SP phenotype (Zhou et al., 2001; Scharenberg et al., 2002). ABCG2 belongs to the adenosine triphosphate-binding cassette (ABC) family of transporters and has been shown to be essential for the SP phenotype in Bcrp1 knockout mice with a disrupted murine Abcg2 gene (Bcrp1) (Zhou et al., 2002). Bcrp1-/- mice had fewer SP cells, which lacked expression of c-Kit and Sca1 and were incapable of repopulation in lethally irradiated mouse recipients.

At the molecular level, Zhou et al. (Zhou et al., 2001) have shown that expression of Abcg2 is greatest in the primitive CD34+KSL compartment of mice in comparison to the CD34-KSL fraction, while Scharenberg et al. (Scharenberg et al., 2002) have further shown that ABCG2 is expressed in relatively high levels in human SP, CD34+/CD38- and CD34+/KDR+ populations and drops sharply in committed progenitors including CD34+/CD38+, CD34+/CD33+ (committed myeloid progenitors) and CD34+/CD10+ (committed lymphoid progenitors) populations. Interestingly, expression is low in most maturing hematopoietic cells but rises again in natural killer cells and erythroblasts (Zhou et al., 2001; Scharenberg et al., 2002).

Most strikingly, not only was the expression of ABCG2 associated with and responsible for the SP phenotype, but it has been shown to be a characteristic of other sources of multipotent stem cells. For example, inhibition of ABCG2 in embryonic stem
cells induced the loss of Nanog expression and differentiation of these cells towards the ectodermal lineage (Susanto et al., 2008). In addition, the ability of HSCs to efflux Hoechst may be dependent on the state of activation of the stem cells being purified. Uchida et al. (Uchida et al., 2004) have shown that juvenile mouse bone marrow-derived HSCs do not as effectively efflux Hoechst as do adult bone marrow derived HSCs and that activation of adult BM with 5-FU increases the proportion of SP cells. It has also been shown that as cells begin to differentiate, their ability to efflux Hoechst is rapidly lost (Wolf et al., 1993). Thus, expression of ABCG2 in stem cells may be a functional determinant of the multipotent state.

1.5.1.2.3  SP cell surface marker expression

SP cells can be further characterized by the presence and absence of various cell surface markers. In mouse, human and rhesus monkey, it has been found that bone marrow derived SP cells express CD45 and MHC Class I, while they expressed no lineage markers (Lin-) and low to no levels of CD34 which is a classical marker for HSCs. It has been postulated that this lack of CD34 expression is indicative of a more primitive phenotype of HSC within the SP fraction (Goodell et al., 1997). Interestingly, rhesus CD34−SP cells plated on bone marrow stroma acquire the expression of CD34 in order to become capable of differentiation into myelomonocytic, megakaryocytic and erythroid progeny (Goodell et al., 1997). In addition, the majority of HSCs in murine bone marrow are CD34lo/c but increase CD34 expression following cell activation (Sato et al., 1999) further corroborating the hypothesis that SP cells represent a quiescent, more primitive HSC within the bone marrow. Despite the lack of CD34 expression, murine bone marrow-derived SP cells do express the classical stem cells markers Sca-1 and c-Kit
(Goodell et al., 1997) and thus murine SP cells are classified as CD34<sup>−</sup>c-Kit<sup>−</sup>Sca-1<sup>−</sup>Lin<sup>−</sup>Hoechst<sup>lo</sup> cells.

### 1.5.1.2.4 SP cells self-renew and proliferate in vitro and in vivo

The murine bone marrow-derived SP population isolated with the procedure of Goodell et al., is highly enriched for hematopoietic colony forming units (CFUs), long-term culture-initiating cells (LTC-IC) and SCID repopulating cells (Goodell et al., 1997). SP cells with hematopoietic colony formation activity have also been isolated from other tissue types, including muscle (Jackson et al., 1999), kidney, lung and small intestine (Asakura and Rudnicki, 2002). In addition, cells with a SP phenotype and tissue-specific mature cell differentiation were isolated from neurospheres (Hulspas and Quesenberry, 2000; Kim and Morshead, 2003) and the mammary gland (Welm et al., 2002; Alvi et al., 2003).

SP cells in mice have been assayed for their myelomonocytic and erythroid CFU potential in methylcellulose medium, wherein they give rise to multilineage colonies of mature hematopoietic cells. The growth of rhesus monkey SP cells, on the other hand, could not be directly supported in methylcellulose medium and required a pre-incubation period of 25-35 days in long-term culture with bone marrow stroma and cytokines (Goodell et al., 1997). During this process, the CD34<sup>−</sup> SP cells acquired CD34 expression and were then capable of generating colonies in cytokine-supplemented methylcellulose cultures. Limiting dilution LTC-IC assays performed on rhesus unfractionated bone marrow and CD34<sup>−</sup>SP cells, indicated an enrichment for LTC-IC activity in the SP fraction with a frequency of 1 out of 7 SP cells compared to 1 of 6060 for unfractionated
bone marrow. A similar phenomenon has been discovered in human SP cells, which had no CFU potential in methylcellulose until they underwent 5-7 days of cytokine-supported suspension culture (Guo et al., 2003). This in vitro expansion of SP cells resulted in a 176-fold enrichment for LTC-ICs compared to non-SP cells.

Murine SP cells also demonstrate robust in vivo long-term reconstitution potential as seen by their ability to engraft at high levels in sublethally irradiated NOD/SCID recipients. As little as one murine SP cell is capable of multilineage reconstitution when transplanted with irradiated accessory cells (Uchida et al., 2003; Camargo et al., 2006), although the frequency of reconstitution varies from 20-96% of transplanted mice with different protocols to enrich for the most primitive stem cell fraction (Uchida et al., 2003; Matsuzaki et al., 2004; Camargo et al., 2006). To date, no data have been published on the potential of rhesus monkey or human SP cells for short or long-term reconstitution of the murine NOD/SCID xenotransplantation model, nor in autologous recipients.

1.5.1.2.5 Canine SP cells

Due to the difficulty in identifying cell surface markers exclusively expressed by all hematopoietic stem cells as well as limitations in the availability of canine-specific antibodies for cell purification, the property of Hoechst efflux from SP cells may facilitate enrichment of HSCs from canine bone marrow samples. Preliminary studies in our laboratory identified an SP profile for canine bone marrow (Grantab, 2004), though further characterization of the functional properties of these cells was required to determine if this population was enriched for HSC potential.
In addition, two recent reports of the utilization of the side population (SP) phenotype in canine tissues were published. Arends et al. reported the presence of a Hoechst 33342-effluxing cell population within the liver of adult dogs that is enriched for progenitors committed to the hepatic lineage. The liver SP fraction constituted approximately 3% of the non-parenchymal cell fraction and contained both cCD45 positive and negative cells. Both fractions appeared to contain progenitor cells based on cell morphology and expression of hepatic progenitor markers, keratin 7, FN14, KRT19, and KRT7. Sub-fractionation based on cCD45 expression revealed that the CD45+ SP fraction also expressed higher levels of ABCG2 and CD44, which were not detected in the CD45− SP fraction. No in vitro or in vivo characterization of these cells was reported.

Canine side population stem cells have also been isolated from the dental pulp of dogs’ teeth (Iohara et al., 2009). A subfraction of the SP cells that were CD31−CD146−, had the ability to form tube-like structures in vitro and regenerate the pulp tissue in an amputated pulp model, complete with capillaries and neuronal cells within 14 days in vivo. These studies indicate that SP purification of dental pulp stem cells may be useful for future transplantation studies aimed at regeneration of dental pulp and point to the usefulness of the SP phenotype, which can be utilized in dogs without the need for antibody-based isolation of stem cell populations.

1.5.1.3 Multipotent Stromal Cells

Multipotent stromal cells (MSCs), also known as marrow stromal cells or mesenchymal stem cells (Horwitz et al., 2005), are a stromal cell population found in various tissues with the ability to self-renew while maintaining their multipotency. The
ability to self-renew and differentiate is most often assessed with in vitro culture and differentiation assays that promote the formation of osteoblasts, adipocytes and chondrocytes. As these abilities are not attributable, to the same degree, to every cell within a population of MSCs, it is not yet clear if the variation in proliferation and differentiation is due to the number of “true” progenitors within the culture or variable capacities of individual progenitors (Seruya et al., 2004). Despite a consensus on the criteria for a cell to be called a MSC, they are a highly studied cell type for transplantation due to their ease of isolation and maintenance in culture, as well as for their noted ability to suppress T-cell proliferation and reduce inflammatory responses both in vitro and upon transplantation (Krampera et al., 2003; Le Blanc et al., 2003; Rasmusson et al., 2003; Tse et al., 2003). Initially, MSCs were identified in the bone marrow at a low frequency (0.001-0.01% of the bone marrow mononuclear fraction) and more recently have been isolated from adipose tissue, skeletal muscle, umbilical cord blood, peripheral blood and teeth. Characterization of MSCs was primarily based upon their adherence to tissue culture plastic, but has also been refined with the addition of immunophenotyping. As bone marrow-derived MSCs are derived from the non-hematopoietic fraction, a lack of hematopoietic markers, such as CD45, CD34, CD11b, CD14, and CD19, is required for their classification. An MSC-specific cell surface marker has yet to be identified, but positive expression of CD44, CD73, CD90, CD105, CD106, CD146 and CD166, which are also expressed on other cell types, has aided the characterization of MSCs (Buhring et al., 2009). In addition, the ability of MSCs to differentiate to multiple cell types of the mesenchymal lineage, including osteoblasts,
chondroblasts and adipocytes is useful to confirm the isolation of MSCs (Dominici et al., 2006).

MSCs have been isolated from a variety of canine tissues including bone marrow (Kadiyala et al., 1997; Jung et al., 2009), umbilical cord blood (Jang et al., 2008; Seo et al., 2009; Zucconi et al., 2010) and adipose tissue (Neupane et al., 2008; Vieira et al., 2009). In all cases, these cells have been isolated by their adherence to tissue culture dishes, possess a fibroblastic morphology upon passaging and can be maintained in culture for many passages. Canine bone marrow and umbilical cord blood MSCs have osteochondrogenic potential, both in vitro (Kadiyala et al., 1997) and in vivo (Jang et al., 2008; Jung et al., 2009), while neurogenic (Seo et al., 2009) and adipogenic (Zucconi et al., 2010) potential have also been characterized in umbilical cord blood derived MSCs. MSCs isolated from canine adipose have osteochondrogenic potential (Vieira et al., 2009) but also possess the ability to give rise to adipocytes and myocytes (Vieira et al., 2009).

Preclinical studies with induced spinal cord injury in beagles, has shown engraftment of canine MSCs into the injury site and improved recovery at 5 weeks post spinal cord injury (Jung et al., 2009). Interestingly, despite the supposed immune privilege of MSCs, this study noted that autologous MSCs had a greater effect and persisted at the injury site longer than allogeneic transplants. Thus canine MSCs isolated from various tissue sources are multipotential, have the ability to engraft in vivo and can be utilized in stem cell transplantation therapies.
1.5.2 Canine Embryonic Stem Cells

Embryonic stem cells (ESC), with the potential to differentiate into any cell type in the body, have garnered much interest as a potential source of cells for regenerative medicine. ESCs are characterized by the ability to (1) self-renew and expand in an undifferentiated state upon extended culture, (2) differentiate in vitro to cells of the three embryonic germ layers, (3) differentiate in vivo through formation of teratomas, and (4) contribute to the development of chimeric animals, including germ cell contribution. As one defining characteristic of ESCs is the ability to form teratomas upon transplantation, safety concerns have been raised about the use of ESC-derived cells for regenerative medicine in humans. In order for ESC-derived stem cell therapies to be moved into the clinic, it will be necessary to assess their safety and efficacy in a large animal model. With this in mind, several research groups, including our own, have been interested in the development of canine ESC (cESC) lines. A summary of the findings of each research group is given in Table 1.1.

From 2006 to 2008, three reports of the derivation of cESC-like cells lines were published. The first two reports, showed that cESCs could be derived from blastocyst stage embryos, expressed the classical ESC marker OCT3/4 as well as the mouse ESC marker SSEA1, and to varying degrees, differentiated to cell types of one or more of the embryonic germ layers (Hatoya et al., 2006; Schneider et al., 2007). The utility of these cESC lines was limited, as they could only be maintained in culture for eight passages before they were lost. The work of Hayes et al. (2008) improved on that of the first cESC lines, in that one line selected for in-depth characterization, could be cultured for greater than 32 passages, while maintaining the ability to differentiate in vitro to cell types of the
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeder layer</td>
<td>Mechanical</td>
<td>Mechanical</td>
<td>Mechanical</td>
<td>Not removed</td>
<td>Enzymatic immunodissection or Not removed</td>
</tr>
<tr>
<td>Expression of Alkaline Phosphatase</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Expression of OCT4/SOX2/NANOG</td>
<td>√(^1)</td>
<td>√(^2)</td>
<td>√(^3)</td>
<td>√(^4)</td>
<td>√(^5)</td>
</tr>
<tr>
<td>Expression of SSEA1</td>
<td>√</td>
<td>√</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Expression of SSEA3/ SSEA4</td>
<td>X(^6)</td>
<td>X</td>
<td>X</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Expression of TRA1-60/ TRA1-81</td>
<td>ND</td>
<td>ND</td>
<td>X</td>
<td>√</td>
<td>X(^7)</td>
</tr>
<tr>
<td>Form embryoid bodies</td>
<td>√</td>
<td>ND</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In vitro differentiation</th>
<th>Ecto, meso, endo</th>
<th>Ecto, meso, endo</th>
<th>Ecto, meso, endo</th>
<th>Ecto, meso, endo</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo teratoma</td>
<td>ND</td>
<td>ND</td>
<td>X</td>
<td>√</td>
</tr>
<tr>
<td>formation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vivo differentiation</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Ecto, meso, endo</td>
</tr>
<tr>
<td>confirmed</td>
<td></td>
<td></td>
<td></td>
<td>Meso</td>
</tr>
<tr>
<td>Derivation efficiency</td>
<td>2 of 80</td>
<td>1 of 8</td>
<td>4 of 22</td>
<td>12 of 25 (FBS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 of 24 (SRM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39 of 130</td>
</tr>
<tr>
<td>Maximum undifferentiated passages</td>
<td>8</td>
<td>8</td>
<td>34</td>
<td>30</td>
</tr>
</tbody>
</table>

MEFs: mouse embryonic fibroblasts; SNL: murine STO-derived feeders; √ characteristic is present in eESC line(s) derived; X characteristic is not present in eESC line(s) derived; ND not done; 1OCT4 protein, SOX2 and NANOG-ND; 2mRNA only; 3OCT4 protein, SOX2-ND, NANOG-mRNA only; 4mRNA and protein; 5OCT4 and SOX2-mRNA and protein, NANOG-mRNA only; 6SSEA3-ND; 7canine-derived support cells express TRA1-81; Ecto: ectoderm; Meso: mesoderm; Endo: endoderm
three germ layers (Hayes et al., 2008). Despite repeated attempts, these cell lines did not possess the ability to undergo *in vivo* differentiation, as assessed by the formation of teratomas, upon transplantation into immunocompromised mice.

The cESC lines established by our research group added to the field by demonstrating not only the expression of pluripotency genes, such as OCT3/4, NANOG, and SOX2, the ability to differentiate *in vitro* to cells of the three germ layers, but also the ability to differentiate *in vivo* with the formation of teratomas containing cells of endodermal, mesodermal and ectodermal origin (Vaags et al., 2009). The derivation and characterization of our cESC lines is detailed extensively in Chapter 4.

Recently, Wilcox et al. have also published the development of an additional 39 cESC-like lines (Wilcox et al., 2009). These cell lines possessed many defining characteristics of embryonic stem cells, including the ability to differentiate *in vitro* to cell types of the three germ layers, but upon transplantation into immunocompromised mice only small teratomas were formed. These masses contained immature cells that were only found to express smooth muscle actin, indicating differentiation towards the mesodermal lineage (Wilcox et al., 2009). The most stringent defining characteristic of ESCs is the ability to contribute cells to the three somatic germ layers as well as germ cells, through the formation of chimeric animals. This has not been required for the characterization of human ESC lines due to the ethical constraints of such an experiment. This bioethical limitation does not hold for canine research, but the technical aspects of canine embryo manipulation for injection of cESCs into blastocysts are not well developed. Work in somatic cell nuclear transfer for the development of cloned canids is ongoing (Lee et al., 2005; Hong et al., 2009; Kim et al., 2009; Oh et al., 2009), and many
of the advances in this field involving the \textit{in vitro} manipulation and maturation of embryos as well as control of the estrous cycle of surrogate bitches will be useful for future development of cESC chimeric animals. The development of cESC chimeric dogs would provide the gold-standard confirmation of the isolation of \textit{bona fide} canine ESCs.

1.6 Models to Assess Canine Stem Cell Engraftment

The potential of canine stem cells, either adult or embryonic, must be assessed with \textit{in vivo} models that support the engraftment and long-term proliferation of the cell types being studied. Much work has been done in the field of adult canine bone marrow transplantation, while a limited number of studies have also utilized the canine \textit{in utero} transplantation model. The usefulness of the NOD/SCID xenotransplantation model is well defined for human stem cells, but very little work has been done to determine its ability to assess the stem cell potential of canine stem cells.

1.6.1 Canine \textit{In Utero} Transplantation

\textit{In utero} transplantation (IUT) is the delivery of cell therapies to the developing fetus, in order to take advantage of (1) the small size of the recipient allowing for high donor to host cell ratios to be achieved with transplantation of relatively small numbers of cells, (2) the preimmune status of the fetus, and (3) early correction of disease phenotypes with the delivery of normal or gene-modified donor cells. In addition to work done in mice (Chen \textit{et al.}, 2009b; Panaroni \textit{et al.}, 2009; Tondelli \textit{et al.}, 2009), rats (Chen \textit{et al.}, 2009a), cats (Abkowitz \textit{et al.}, 2009) and sheep (Almeida-Porada \textit{et al.}, 2000; Mackenzie and Flake, 2001; Schoeberlein \textit{et al.}, 2005; Nagao \textit{et al.}, 2009), the dog model has also been utilized for \textit{in utero} transplantation studies.
Studies to determine the efficiency of donor cell engraftment in canine fetal recipients have demonstrated that low level (approx. 1%) chimerism can be achieved in the absence of *in utero* myeloablation. Work from our laboratory has employed yolk sac delivery of transplants to midgestation (day 32-45) fetuses to test the safety of delivery and persistence of gene-modified long-term marrow culture hematopoietic cells of canine (Lutzko *et al*., 1999) and human (Omori *et al*., 1999) origin. All transplanted puppies were chimeric with high levels of human cell engraftment at 10-12 weeks as assessed by CFU assays, which declined rapidly to less than 1% donor chimerism by 17-21 weeks. In similar studies, Blakemore *et al*. (2004) have assessed the engraftment of canine CD34\(^+\) hematopoietic stem cells transplanted to the peritoneal cavity. Again, only low level (1%) microchimerism was detected in various tissues, including the thymus, liver, skin, spleen and intestine.

Due to the disease effects that can occur before birth, IUT is of interest for the treatment of diseases that can be diagnosed prenatally and corrected by postnatal bone marrow transplantation. Canine models of hematopoietic cell-based abnormalities have been utilized to test the ability of *in utero* transplanted cells to reverse disease phenotypes and extend the life of affected dogs. Our research group has been interested in using IUT for the treatment of dogs with the inherited disorder, Mucopolysaccharidosis I (MPS I), which results from a lack of alpha-L-iduronidase enzyme activity. IUT of genetically corrected canine hematopoietic progenitors to the yolk sacs of midgestation fetuses resulted in engraftment but not amelioration of disease (Lutzko *et al*., 1999). The levels of engraftment were as high as 12% within the bone marrow when assayed by CFU assays but there were only a small proportion of provirally-marked cells within the blood
(1%) and no iduronidase transcript or enzyme was detected. Dogs that were boosted with autologous iduronidase-transduced cells did not mount an immune response to iduronidase, indicating that tolerance towards the iduronidase enzyme had been achieved, but all MPS I affected dogs died at 8-11 months of age with complications of the MPS I disease. Thus the levels of engraftment and retroviral gene expression were concluded to be insufficient for reversal of the affects of the MPS I disease. In contrast, Flake et al. have successfully treated canine leukocyte adhesion deficiency (CLAD) dogs with IUT. In many cases, IUT yielded sufficient levels of engraftment to reverse the effects of CLAD, and chimerism could be enhanced with postnatal boosting with bone marrow from the same donor (Peranteau et al., 2009). It is interesting to note that the levels of donor chimerism were never above 2% in the absence of postnatal boosting, and yet were sufficient for the reversal of the disease phenotype. Postnatal boosting resulted in stable 35-45% mixed chimerism in 30-40% of the dogs receiving a same donor bone marrow transplant. Thus it was concluded that IUT resulting in ≥1% donor chimerism sufficiently tolerated recipients to subsequent donor cell transplantation.

1.6.2 NOD/SCID Xenotransplantation

In order to study the potential of canine stem cells in vivo, an appropriate transplantation model is required. Transplantation of canine SP cells into canine recipients would be the preferred system in which to assess this potential but due to the high cost and ethical considerations of utilizing canine recipients, the use of the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse xenotransplantation system was warranted prior to moving studies into canine recipients. The NOD/SIC model has been extensively utilized for the in vivo assessment of human stem cells
(Kollet et al., 2000; Ito et al., 2002; Mazurier et al., 2003; Yahata et al., 2003) and thus we speculated that it might also support the engraftment and proliferation of canine adult and embryonic stem cells.

The NOD/SCID mouse is useful for transplantation studies as these mice have an impaired immune system relative to NOD or SCID mice and thus do not reject xenotransplants. The NOD phenotype of insulin-dependent diabetes mellitus is polygenic and involves mutations to numerous insulin-dependent diabetes loci (Prochazka et al., 1987, 1989; Wicker et al., 1995) which result in the development of autoimmunity in NOD mice, along with defects in the complement pathway (Baxter and Cooke, 1993) and macrophage function (O'Brien et al., 2002, 2006). In contrast, the SCID phenotype is due to a mutation within the scid gene that results in deficient activity of the DNA repair enzyme, Prkdc. The lack of Prkdc results in deficient V(D)J recombination, preventing productive rearrangement of the immunoglobulin and T cell receptor genes, and thus the B and T cells of the immune system fail to mature (Bosma et al., 1983). Interestingly, NOD/SCID mice do not have autoimmune diabetes as found in NOD mice, because they lack B and T cells due to the scid mutation. NOD/SCID mice do still have NK cells, though their number is decreased (Shultz et al., 1995).

The work of several groups to enhance human engraftment in NOD/SCID mice through the use of gene-modified transgenic mice with compromised immune systems, intrafemoral injection to enhance homing to the bone marrow niche and treatment with an anti-CD122 antibody to further inhibit the recipient immune system have allowed for the detection of previously unidentified stem cell populations (Mazurier et al., 2003; Shultz
et al., 2003; McKenzie et al., 2005). Modifications to the NOD/SCID xenotransplantation protocol employed in this study are detailed herein.

The nonobese diabetic/severe combined immune deficiency (NOD/SCID) xenotransplantation model provides a powerful tool to characterize human HSCs as these mice are deficient in mature lymphocytes and serum Ig which allows for engraftment of non-autologous cells. Modification of the NOD/SCID assay through the use of genetic depletion of natural killer (NK) cells in NOD/SCID-β2-microglobulin-null (NOD/SCID-β2m-/-) mice has increased the efficiency of human short-term repopulating cell engraftment by 10-fold (Kollet et al., 2000, 2001). Due to their deficiency in β2-microglobulin, NOD/SCID-β2m-/- mice express almost no MHC class I molecules and thus CD8⁺-T-cells, NK cells and NK1⁺-T-cells are almost completely depleted (Christianson et al., 1997). This lack of T-cells and NK cells is beneficial in a xenotransplantation setting, as foreign donor cells are not attacked and cleared by the recipient’s innate immune system.

Injection of cells directly into the bone marrow cavity of the femur is known as intrafemoral transplantation and enables robust myeloerythroid repopulation 2 weeks after transplantation with engraftment at levels 30-fold greater in the bone marrow and 16-fold greater in the peripheral blood compared to mice receiving intravenous injection (Mazurier et al., 2003). However, levels of engraftment stabilize to those similar to intravenous injection at later time points. Not only does intrafemoral transplantation enhance detection of short-term repopulation but also improves long term repopulation as shown by serial transplant of CD34⁺/CD38⁻/Lin⁻ cord blood cells by Yahata et al. (2003).
In comparison to intravenous injection, intrafemoral transplantation resulted in a 15-fold higher frequency of detection of SCID repopulating cells (1 in 660 for intravenous versus 1 in 44 for intrafemoral).

Further enhancement of the NOD/SCID assay can be achieved through depletion of several mature hematopoietic cell populations including NK cells and macrophages by pre-treatment of NOD/SCID mice with an anti-CD122 antibody (TMβ1) (Tanaka et al., 1991), directed against the IL-2Rβ chain. IL2 acts as a growth factor for mature NK cells, which preferentially express IL-2Rβ (Biron et al., 1990) and it is through IL2 signaling that the proliferation and cytotoxic activity of NK cells is regulated (Phillips et al., 1989). Intraperitoneal injection of TMβ1 has been shown to selectively deplete splenic NK function in various mouse strains and lasts for at least 5 weeks (Tanaka et al., 1993). Even in NOD/SCID-β2m<sup>-/-</sup> mice, with no NK cell cytotoxic activity, anti-CD122 treatment results in a 2-fold increase in the level of short term repopulating cells, indicating that CD122-blockade may affect other factors, such as myeloid cells, which are important in the regulation of xenotransplant engraftment (Shultz et al., 2003).

Unlike human to mouse xenotransplant studies, the use of murine xenotransplantation models to study the stem cell potential of canine derived cells is not well established. One study employed the NOD/SCID xenotransplantation model with intravenous injection of 1 x 10<sup>7</sup> bone marrow mononuclear cells and achieved short-term repopulation of 6.8% within the bone marrow and 23.3% in the peripheral blood (Niemeyer et al., 2001) at 4-8 weeks post transplantation. Interestingly, transplantation of 1-6 x 10<sup>4</sup> Rho<sup>lo</sup> purified HSCs resulted in no engraftment in the bone marrow and only
2.5% in the peripheral blood. Through further modification of the NOD/SCID transplantation model used by Niemeyer et al. for canine transplantation, with the use of intrafemoral injection and treatment with anti-CD122 depleting antibodies in transgenic NOD/SCID-β2m<sup>-/-</sup> mice, we attempted to increase the engraftment read-out of canine SP cells. We also utilized intratesticular injection of NOD/SCI-β2m<sup>-/-</sup> mice to assess the proliferation and differentiation of cESCs.

1.7 Experimental Rationale and Hypotheses

The research detailed in this thesis was undertaken to more fully establish the utility of canine preclinical models for the translation of human stem cell therapies. As new stem cell sources are discovered in mice and humans with the potential to be used in regenerative medicine and tissue engineering, it is important that analogous cells be characterized in large animal models to allow for preclinical testing of therapies employing these cell sources.

The isolation of SP HSCs in mouse and human bone marrow, based on the efflux of Hoechst dye, has been employed for several years in the research setting (Goodell et al., 1997). As such, I undertook to determine if canine bone marrow contained a side population of cells with equivalent in vitro and in vivo properties to those isolated in mouse and human. The identification of a canine SP cell was particularly useful in that it allowed for the isolation of HSCs on the basis of a functional property of stem cells, namely the activity of the ABCG2 transmembrane pump, rather than relying on antibody based purification. The identification of a canine SP cell was also of interest as it allowed for the isolation of a CD34<sup>+</sup> HSC, which had not been previously identified in dog, to assess the multilineage differentiation and engraftment potential of an HSC population.
thought to represent a more quiescent, primitive stem cell than the classical CD34\(^+\) HSC (Goodell \textit{et al.}, 1997).

The use of differentiated human ESCs for regenerative medicine may provide an unlimited cell source for transplantation, but the production of transplantable material must be refined in order to obtain a highly specific cell population and to avoid the risk of ESC-derived teratomas. Due to the high risk of ESC-derived transplants containing undifferentiated cells, the safety of any differentiation protocols must be assessed in large animal models before commencing human treatments. Thus, development of canine embryonic stem cells with properties similar to those of human ESCs was required. I undertook to derive cESCs, as the lines that had been previously obtained by other groups did not have all of the hallmark characteristics of human ESCs; namely they were unable to form teratomas upon \textit{in vivo} transplantation in NOD/SCID mice (Hatoya \textit{et al.}, 2006; Schneider \textit{et al.}, 2007; Hayes \textit{et al.}, 2008). Teratoma formation of cESCs was essential if these cells are to be used to assess the safety of differentiation protocols for the creation of transplantable cells and to ensure that ESC-derived cells no longer retain the ability to form teratomas upon transplantation into recipients.

\textit{In utero} transplantation (IUT) holds the promise of the amelioration of disease prior to birth, but from a theoretical standpoint is also interesting in that it may allow for allogeneic transplantation in preimmune fetuses without the need for immune suppression as well as for the development of high donor chimerism through the transplantation of relatively large cell numbers into relatively small recipients. Yet, studies in the canine IUT model utilizing yolk sac delivery of cell transplants have resulted in very low-level chimerism (Lutzko \textit{et al.}, 1999; Omori \textit{et al.}, 1999). To determine if cells transplanted to
the yolk sac of midgestation fetuses migrated to fetal tissues, I undertook cell labeling and tracking studies. This work was important to establish the feasibility of utilizing the yolk sac delivery route for future IUT studies and to elucidate the distribution of donor cells within fetal organs.

To address these objectives, this thesis has been divided into three research chapters, which address the isolation and characterization of canine side population cells (Chapter 2), the derivation and characterization of canine embryonic stem cells (Chapter 3), and the tracking of cells transplanted to the yolk sacs of canine *in utero* transplantation recipients (Chapter 4). An overall discussion of the findings is presented in Chapter 5.
1.8 References


8. Club AK. Complete Breed List. 2010


41. Grantab R. Isolation and characterization of canine side population cells. Thesis (MSc)--University of Toronto, 2003 2004


78. McGeady TA, Quinn PJ, FitzPatrick ES et al. Veterinary Embryology. 2006


CHAPTER 2

Canine Bone Marrow-Derived Side Population Stem Cells as a Potential Cell Source for Transplantation
2.1. Attribution of Data

I performed all the work described in this chapter with the following exceptions: canine bone marrow harvests were performed by Dr. Stephen Kruth and Dr. Marcus Litman at the Ontario Veterinary College, University of Guelph; the initial testing of Hoechst 33342 staining on bone marrow samples from mouse and dog and human cord blood, as well as inhibition of transporter pumps by verapamil and fumitremorgin were performed by Rama Grantab, Suzana Rosic-Kablar and Susan Andras; operation of the BD Diva and Aria flow cytometers was performed by Gisele Knowles and Arian Khandani, Dr. Yanzhen Zheng and Dr. Anderson Goncalves assisted with design of RT-PCR primers.
2.2. Abstract

Techniques for the efficient isolation of hematopoietic stem cells (HSCs) from canine bone marrow are less developed than those for mouse or human samples. Although isolation with a canine-specific CD34 antibody can be performed, this strategy excludes the most primitive CD34\(^{-}\) stem cells. Hoechst 33342 staining has been used in several species, including humans, to isolate primitive CD34\(^{-}\) HSCs from a variety of tissues. In the studies reported herein, we assessed Hoechst 33342 staining as a strategy to isolate hematopoietic stem and progenitor cells from canine bone marrow. A side population (SP) of cells in canine bone marrow was detected after Hoechst staining, which could be consistently isolated following preincubation with cytokines. To reduce the heterogeneity of the SP fraction, cells were further fractionated by gating on small agranular (SA) or large granular (LG) cells. Cytocentrifugation and morphological examination of SP cells falling in the SA region (SA-SP) indicated that this fraction consisted of predominantly blast-like stem cells and erythroblasts. Erythroblasts and monocytes were the predominant cell types in the LG-SP fraction. Flow cytometric analysis confirmed that the SA-SP fraction was enriched for HSCs and progenitors while CD34\(^{+}\) cells were isolated in the LG- and SA-nonSP fractions. RT-PCR analysis indicated that the SA-SP cells, expressed high levels of the ABCG2 transporter, the transmembrane pump known in other species to be responsible for the SP phenotype, whereas cells of the LG-SP fraction expressed high levels of the MRP1 efflux pump. Long-term culture initiating cell (LTC-IC) and colony forming unit (CFU) assays revealed that the SA-SP was enriched in LTC-IC which, after culture on a feeder layer of canine stromal cells supplemented with cytokines gave rise to colonies in
methylcellulose. By comparison, CFUs were present in the nonSP fractions (SA-nonSP and LG-nonSP) and established colonies after direct plating in methylcellulose. SA-SP cells did not significantly contribute to long-term engraftment of NOD/SCID-β2m−/− mice, while limited engraftment was detected in mice receiving cells of the SA-nonSP fraction or unfractionated canine bone marrow. These studies demonstrated the SP fraction of canine bone marrow provided an enriched source of hematopoietic progenitor cells with long-term culture initiating potential that were not optimally supported by the stem cell niche within NOD/SCID-β2m−/− mice and may require further activation to allow readout of their stem cell potential in vivo.
2.3. Introduction

Hematopoietic stem cells (HSCs) have the capacity to self renew and to differentiate into all cells comprising the blood and immune system. HSCs with long-term multilineage reconstituting potential efflux fluorescent dyes including Rhodamine 123 and Hoechst 33342 allowing for the functional isolation of HSCs from a number of species (Spangrude and Johnson, 1990; Baum et al., 1992; Wolf et al., 1993; Goodell et al., 1996; Leemhuis et al., 1996). One procedure involving the staining of bone marrow mononuclear cells with Hoechst 33342 and dual-wavelength fluorescence activated cell sorting (FACS) identifies a distinct low fluorescing subpopulation termed the side population (SP) (Goodell et al., 1996, 1997).

The observation that the SP profile is diminished after loss of expression of multidrug transporters and after treatment of cells with verapamil (Goodell et al., 1996), a known inhibitor of ATP hydrolysis, or with fumitremorgin C (Scharenberg et al., 2002), a specific inhibitor of the breast cancer resistance protein, ABCG2 (Rabindran et al., 2000), suggests that members of the ATP-cassette transporter super-family are responsible for the SP phenotype (Bunting et al., 2000; Zhou et al., 2001; Scharenberg et al., 2002; Zhou et al., 2002). The enforced expression of ABCG2 in bone marrow cells results in reduced differentiation both in vitro and in vivo (Zhou et al., 2001), while ectopic ABCG2 expression in human embryonic stem cells enhances self-renewal in differentiation-inducing, basic fibroblast growth factor-free culture medium (Zeng et al., 2009). Thus, ABCG2 expression may be a property of many stem cell populations and can be used as a prospective functional marker for HSC enrichment.
In the mouse, the Hoechst-purified SP fraction comprises 0.07% of bone marrow mononuclear cells and is 1,000- to 3,000-fold enriched for HSC activity relative to unsorted bone marrow (Goodell et al., 1996). The presence of SP cells has also been confirmed in human, nonhuman primate, zebrafish and pig bone marrow in addition to human cord blood and fetal liver (Goodell et al., 1997; Uchida et al., 2001; Heinz et al., 2002; Dor et al., 2006; Tsinkalovsky et al., 2007). Bone marrow-derived SP cells do not express lineage antigens (Lin⁻) and lack the HSC antigen CD34. SP cells are highly enriched for colony forming units (CFUs) and long-term culture-initiating cells (LTC-IC) as well as cells that initiate multilineage hematopoietic reconstitution of mice (Goodell et al., 1996, 1997). Unlike murine SP cells, which demonstrate CFU potential upon direct plating in methylcellulose cultures, rhesus monkey (Goodell et al., 1997) and human (Guo et al., 2003) SP cells must first undergo long-term culture to induce maturation from a CD34⁻ to CD34⁺ phenotype before they can demonstrate CFU potential. These findings suggest that stem cells with an SP phenotype may be more primitive and quiescent than HSCs isolated with other methodologies. Although the SP fraction of bone marrow is heterogeneous with respect to reconstituting activity, long-term repopulating HSCs can be enriched by isolating cells with the strongest dye efflux activity (Tip-SP cells) that express c-kit. Transplantation of even a single cKit⁺Sca1⁻Lin⁻Tip-SP cell can repopulate irradiated NOD/SCID mice at a very high, though not absolute, frequency (Matsuzaki et al., 2004; Camargo et al., 2006).

Given the potential of bone marrow-derived SP cells to generate the entire hematopoietic repertoire these cells may provide an ideal source of stem cells for use in transplantation medicine. However, demonstration of the functional potential and
developmental capacity of SP cells has been largely limited to murine studies. Before SP cells can be used in the clinical setting, their utility must be demonstrated in large animal models.

Inbred dogs with genetic disorders homologous with human diseases have historically been used as large animal models in preclinical studies to develop novel strategies for bone marrow transplantation (Maris and Storb, 2002; Georges and Storb, 2003; Kolb et al., 2003) as well as gene and stem cell-based therapies (Abrams-Ogg et al., 1993; Bienzle et al., 1994; Shull et al., 1996; Lutzko et al., 1999, 1999; Moise, 1999; Lutzko et al., 2002; Meertens et al., 2002). Dogs share many biochemical and physiologic characteristics with humans and thus they often exhibit a greater resemblance to human diseases than do their rodent counterparts. Moreover, stem cell doses and clinical procedures used for dogs are similar to those used in human protocols and thus knowledge and technical expertise acquired in canine preclinical studies are often directly translatable to the human clinical setting.

The specific objectives of these studies were to isolate SP cells from canine bone marrow and determine if these cells were enriched for stem cell potential as had been reported in other species. Utilizing a modified SP isolation protocol, which included a preincubation step with stem cell-supporting cytokines and selection of small agranular cells, we reproducibly isolated canine bone marrow-derived SP cells. To demonstrate that the SP fraction of canine bone marrow provided an enriched source of blast cells with the ability to form multilineage colonies we employed a methylcellulose colony forming unit (CFU) assay as well as long-term culture with canine bone marrow stroma to assess LTC-IC potential. In addition, the ability of SP cells to contribute to hematopoiesis in the
NOD/SCID xenotransplantation model was utilized to assess the *in vivo* reconstitution capacity of canine SP cells.
2.4. Materials and Methods

2.4.1. Veterinary Procedures, Tissue Harvesting and Processing

All animal experimental protocols were approved by the Animal Care Committees of the University of Guelph and Sunnybrook Research Institute. Murine bone marrow, peripheral blood, and spleens were harvested using standard protocols and processed for single cell suspensions. Briefly, peripheral blood was harvested into heparinized saline (100 U.S.P units/ml Hepalean (Organon Canada Ltd., Toronto, ON, Canada) in 1X Hank’s Buffered Saline Solution (HBSS; Wisent, St. Bruno, QC, Canada) and red blood cells were lysed with an equal volume of ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA (Sigma, Oakville, ON, Canada) in HBSS) for 5 minutes at room temperature. Bone marrow was isolated from murine femurs and tibiae after crushing bones with a mortar and pestle and passing cells through a 40 µm mesh. Spleens were cut into small pieces and gently pressed through a 40 µm mesh.

Canine bone marrow was aspirated from the humeral bone marrow cavities into heparinized saline and shipped to Sunnybrook Research Institute for processing within 12 hours of collection. Bone marrow samples were diluted 1:1 with HBSS and 25 ml of sample was layered over 15 ml of Ficol Paque PLUS (GE Healthcare, Baie d’Urfe QC, Canada) in a 50 ml centrifuge tube. Samples were centrifuged at 1400 rpm for 30 minutes at room temperature with the brake off. Mononuclear cells were collected from the Ficol-HBSS interface and washed with HBSS. Residual red blood cells were lysed with ACK buffer. 2 x 10⁷ cells/ml were cryopreserved at -80°C in fetal bovine serum (FBS; Hyclone, Logan, UT, USA) supplemented with 10% DMSO (Sigma).
Canine stromal feeder layers were established by plating $2 \times 10^4$ cells/cm$^2$ bone marrow mononuclear cells in low-glucose $\alpha$MEM (Wisent) with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM Glutamax (Invitrogen, Mississauga, ON, Canada) as previously described for the establishment of canine mesenchymal stromal cells (Kadiyala et al., 1997). Stromal cells were irradiated at 60 Gy prior to use as feeders for long-term cultures or accessory cells for co-injection into immunocompromised mice.

2.4.2. **Hoechst 33342 staining for SP cells**

One to four hours prior to addition of Hoechst 33342, canine bone marrow was incubated at 37°C in $\alpha$MEM (Wisent) with 10% FBS (Hyclone), 2 mM Glutamax, 100 U and 100 $\mu$g/ml Penicillin/Streptomycin, 10 mM HEPES (Invitrogen), 0.1 mM $\beta$-mercaptoethanol (Sigma), and 5 ng/ml of each cytokine: canine stem cell factor (cSCF), hFlt3L and hIL7 (R&D Systems, Minneapolis, MN, USA). Cells were stained for 90 minutes with the addition of 5 ng/ml Hoechst 33342 (Sigma) for 90 minutes, according to published protocols (Goodell, 2005). Propidium iodide (PI; 2 $\mu$g/ml; Sigma) staining was used to exclude dead cells. SP cells were sorted using a FACS Digital Vantage (DiVa) cell sorter (BD Biosciences, Mississauga, ON, Canada) with published settings (Goodell, 2005).

2.4.3. **Antibody Staining**

Cells were resuspended at $1 \times 10^7$ cells/ml in HBSS with 2% FBS (HF) and stained on ice for 30 to 60 min. Antibodies and concentrations used are outlined in Table 2.1.
**Table 2.1.** Antibodies utilized for characterization of canine SP cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone or Product Number</th>
<th>Supplier</th>
<th>Isotype for secondary antibody</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>cCD4:FITC</td>
<td>MCA1038F</td>
<td>Serotec</td>
<td>-</td>
<td>1:100</td>
</tr>
<tr>
<td>cCD11b</td>
<td>MCA1777S</td>
<td>Serotec</td>
<td>mIgG</td>
<td>1:100</td>
</tr>
<tr>
<td>cCD11c</td>
<td>MCA1778S</td>
<td>Serotec</td>
<td>mIgG</td>
<td>1:100</td>
</tr>
<tr>
<td>cCD14</td>
<td>α-M-M9/ CAM36A</td>
<td>VMRD</td>
<td>mIgG</td>
<td>1:100</td>
</tr>
<tr>
<td>cCD34:biotin</td>
<td>2E9/550427</td>
<td>BD</td>
<td>-</td>
<td>1:100</td>
</tr>
<tr>
<td>cCD45:biotin</td>
<td>MCA1042B</td>
<td>Serotec</td>
<td>-</td>
<td>1:400</td>
</tr>
<tr>
<td>hCD117</td>
<td>555714</td>
<td>BD</td>
<td>mlg</td>
<td>1:100</td>
</tr>
<tr>
<td>cB-cell/CD21:PE</td>
<td>MCA1781PE</td>
<td>Serotec</td>
<td>-</td>
<td>1:50</td>
</tr>
<tr>
<td>cMHC Class I</td>
<td>H58A</td>
<td>VMRD</td>
<td>mIgG</td>
<td>1:50</td>
</tr>
<tr>
<td>cMHC Class II:FITC</td>
<td>MCA1044F</td>
<td>Serotec</td>
<td>-</td>
<td>1:50</td>
</tr>
<tr>
<td>cThy1/CD90</td>
<td>DH2A</td>
<td>VMRD</td>
<td>mlgM</td>
<td>1:100</td>
</tr>
<tr>
<td>Streptavidin:PE</td>
<td>016-110-084</td>
<td>Jackson</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Rat anti-mouse IgG:FITC</td>
<td>F-4018</td>
<td>Sigma</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Rat anti-mouse IgG + M:PE</td>
<td>115-116-068</td>
<td>Jackson</td>
<td>-</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Stained cells were resuspended in HF and 2µg/ml PI and viable cells were selected with gates set to exclude PI\(^+\) cells. Controls included unstained samples as well as samples stained with FITC-, PE- or APC-labeled isotype antibodies. Analyses were performed on a FACSCalibur or a LSRII flow cytometer (BD Biosciences) and FlowJo 8.7 software was used for data analyses (Treestar, Ashland, OR, USA).

2.4.4. **Analysis of Gene Expression**

Total RNA was isolated with a RNeasy Plus Mini kit (Qiagen, Mississauga, ON, Canada), following manufacturer’s instructions. Two rounds of linear RNA amplification were completed with a MessageAMP aRNA amplification kit (Ambion, Austin, TX, USA). Amplified RNA was primed with oligo dT and reverse transcribed to first strand complementary DNA (cDNA) using Superscriptase II reverse transcriptase (Invitrogen). PCR reactions were carried out with 25-30 cycles at a 60°C annealing temperature. Primer pairs are detailed in Table 2.2.

2.4.5. **CFU-C and LTC-IC Assays**

Total canine bone marrow mononuclear cells (1 x 10\(^5\) cells/30 mm dish), SA-SP or LG-SP (1 x 10\(^4\) cells/dish) and SA-nonSP or LG-nonSP (2 x 10\(^5\) cells/dish) were plated directly into human cytokine-supplemented methylcellulose (Methocult H4435 Enriched, StemCell Technologies, Vancouver, BC, Canada) to assess the frequency of colony forming units (CFUs) in each cell fraction. Cultures were incubated in a humidified chamber at 37.8°C for two to four weeks. Individual colonies were plucked, cytocentrifuged, and Wright stained to determine the cell composition of colonies.
Table 2.2. RT-PCR primers utilized to characterize the genes expressed by SP cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Name</th>
<th>Sequence (5’→3’)</th>
<th>Size amplified (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cABCG2</td>
<td>cABCG2-A1</td>
<td>GTGGTTGAGAAGAAGCTCTT</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>cABCG2-S1</td>
<td>CCACAACCTGGTTTCAATCCT</td>
<td>167</td>
</tr>
<tr>
<td>cMDR1</td>
<td>cMDR1-2799F</td>
<td>TTTGACTCGGGAGCAGAAGT</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>cMDR1-2970R</td>
<td>ATTTGCCACCAAGTAGGCAC</td>
<td>171</td>
</tr>
<tr>
<td>cMRP1</td>
<td>cMRP1-2753F</td>
<td>CAACTCCTCCTCCTACAGCG</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>cMRP1-2935R</td>
<td>AAGGAGATGAAAAAGCCCGAT</td>
<td>182</td>
</tr>
<tr>
<td>cC-KIT</td>
<td>cC-KIT-F</td>
<td>CCCATTTAACCGAAGGAGAA</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>cC-KIT-R</td>
<td>TCTCCGTGTGATCTTCCTGCTT</td>
<td>211</td>
</tr>
<tr>
<td>cFLK1</td>
<td>cFLK1-F</td>
<td>GGTATGGTCCTTGCTTGCTAGA</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>cFLK1-R</td>
<td>CAGTGGTATCCCCTGTCATCG</td>
<td>172</td>
</tr>
<tr>
<td>cCD4</td>
<td>cCD4-358F</td>
<td>CCTTCCTGGACTGTAGGAGC</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>cCD4-523R</td>
<td>AACACCAGCAATTCCCACCTC</td>
<td>165</td>
</tr>
<tr>
<td>cCD19</td>
<td>cCD19-663F</td>
<td>AAGGTACTGGCTGCTGGAGA</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>cCD19-806R</td>
<td>TCGGTCAATCGCTTCTCTCTT</td>
<td>143</td>
</tr>
<tr>
<td>cCD45</td>
<td>cCD45-2040F</td>
<td>TTTCTGAGGATGATCTGGG</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>cCD45-2155R</td>
<td>GTGTGCCCCTCCTCCATTGAT</td>
<td>115</td>
</tr>
<tr>
<td>cGAPDH</td>
<td>cGAPDH-713F</td>
<td>TATCAGTTGGATCCTGACCTG</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>cGAPDH-884R</td>
<td>GCGTCCAAGGTGGAAGAGT</td>
<td>171</td>
</tr>
</tbody>
</table>
Findings were confirmed by Dr. Alden Chesney, a hematopathologist at Sunnybrook Health Sciences Centre.

To determine the LTC-IC frequency of each bone marrow fraction, cells were plated in human Myelocult medium (H5100, StemCell Technologies) supplemented with 10 ng/ml of cSCF, hFIt3L, hIL3, hIL6, cGM-CSF and cG-CSF (R & D Systems) in 96-well flat bottom tissue culture plates (Nunc, Thermo Fisher Scientific, Rochester, NY) containing 60 Gy gamma-irradiated canine marrow stromal feeders. SP cells were plated over a range of concentrations (100, 300, 500, 1,000, or 5,000 cells per well) with 10 replicate wells for each cell concentration. The non-SP cell fractions were also plated at 1 x 10^5 – 1 x 10^6 cells per well. After 35 days, adherent and nonadherent cells were pooled and replated in Methocult and CFUs assayed after 8-12 days. Samples were scored positive when dishes contained one or more colonies consisting of 50 or more cells. The frequency of LTC-IC was calculated using L-Calc software (StemCell Technologies).

2.4.6. In Vivo NOD/SCID Mouse Repopulation Assays

Twenty-four hours prior to cell transplantation, 8-10 week old female NOD/SCID-β2-microglobulin−/− mice (The Jackson Laboratory, Bar Harbor, MN, USA) were irradiated with 3.4 Gy then injected intraperitoneally with 200 µg of purified anti-CD122 antibody. Depletion of CD122+ cells was assessed by flow cytometric analysis of peripheral blood cells stained with NK-specific markers, DX5 (pan NK cells, CD49b, #13-5971-85) and NKp46 (clone 29A1.4, CD335, #12-3351-82 (eBioscience, San Diego, CA, USA). Mice were co-injected intrafemorally with either canine male SP cells (3 x 10^3, 7 x 10^3, 1 x 10^4 or 4 x 10^4 cells per mouse) or non-SP cells (1 x 10^4, 1 x 10^5, 1 x 10^6).
in combination with accessory cells (5 x 10^4 irradiated female canine stromal cells) in a volume of 20-30 µl. Control mice received 2 x 10^6 total canine bone marrow mononuclear cells or 5 x 10^4 irradiated canine stromal cells. Engraftment of recipients was assessed 12 weeks post injection by flow cytometric analysis of mouse bone marrow samples stained with a canine CD45 antibody (1:400 dilution, AbD Serotec, Raleigh, NC, USA). Mice containing more than 0.1% cCD45^+ cells were determined to be positive for engraftment with canine cells. Prior to analyzing samples for donor canine cell engraftment, the cCD45 antibody was tested for canine hematopoietic-specific binding by assessing antibody reactivity with canine and murine bone marrow samples. To assess the sensitivity of the assay to detect canine cells in recipient mice, mixtures of canine bone marrow (0%, 1%, 5%, 10% and 100% canine bone marrow) with murine bone marrow were prepared. Cell mixtures (1 x 10^6) were blocked with mouse Fc receptor block (clone 2.4G2, Sunnybrook Antibody Facility, Toronto, ON, Canada) for 15 minutes, followed by primary antibody staining with a canine-specific CD45-biotin antibody (1:400) for 30 minutes. Secondary antibody staining was completed with a 1:500 dilution of Streptavidin-PE (Jackson ImmunoResearch, West Grove, PA, USA) for 15 minutes. After washing, cells were resuspended in HF supplemented with 2 µg/ml PI. Live-cell gates were set to exclude nonviable PI^+ cells. Unstained, secondary only and isotype controls were used to set thresholds for positively stained samples.
2.5. Results

2.5.1. Detection of SP Cells in Canine Bone Marrow

To determine whether a Hoechst 33342-effluxing SP of cells were present in adult canine bone marrow, samples derived from 7 individual adult canine donors were stained with Hoechst 33342 and analyzed by dual wavelength, ultraviolet flow cytometry. We also included bone marrow from a Mucopolysaccharidosis type I (MPS I)-affected dog, mouse bone marrow and human cord blood (representative plots, Fig 2.1 A). All samples showed the presence of SP cells, with the mean percentage of canine SP cells being 0.08 ± 0.08%. The percentage of SP cells among dog samples was highly variable (0-0.23%), with no SP cells being detected in approximately 25% of samples analyzed. Similar to other species, the SP fraction in canine bone marrow was partially blocked with increasing concentrations of verapamil, the broad spectrum inhibitor of multidrug resistance pumps (Fig 2.1 B). Total depletion of the SP fraction was only achieved using 50 µM fumitremorgin C, a specific inhibitor of the ABCG2 transporter pump (Figure 2.1 C). The high level of variability among canine samples in both the presence and percentage of the SP population within samples from the same donor dog was a concern. Furthermore, detection of SP cells from cryopreserved marrow samples obtained from a given dog showed significant variability among experiments. To diminish the variability in the proportion of SP cells among samples, bone marrow mononuclear cells were prestimulated for one to four hours in a recovery medium supplemented with canine stem
Figure 2.1. Detection of the side population fraction in canine, mouse and human samples and inhibition of dye efflux with verapamil and fumitremorgin. (A) Flow cytometric plots of UV (red) versus UV (blue) from bone marrow of a normal and a mucopolysaccharidosis (MPS I) affected dog. Mouse bone marrow and human cord blood served as positive controls for the procedure. Gates indicate percentage of SP cells in each sample. (B) The effect of the pump inhibitor, verapamil, at increasing concentrations on dye efflux of Hoechst by canine SP cells. (C) The effect of the ABCG2-specific pump inhibitor, fumitremorgin, at increasing concentrations on dye efflux of canine SP cells.
cell factor (cSCF), human Flt3 ligand (hFlt3L) and human IL7 (hIL7) (Fahlman et al., 1994; Jacobsen et al., 1994; McKenna et al., 1995; Rusten et al., 1996; Shah et al., 1996). After a one-hour pretreatment, the average percentage of SP cells detected was 0.94 ± 0.10% of the total viable cells and 2.33 ± 1.69% after a four-hour incubation. An increase in the proportion of PI<sup>lo</sup> cells was noted after the four-hour pretreatment suggesting that the prolonged incubation time was associated with increased cell death. Based on this, all SP cell isolations were performed using a one-hour preincubation in recovery medium prior to Hoechst staining.

Flow cytometric analysis of SP cell forward scatter (FSC) versus side scatter (SSC) indicated that the SP fraction was heterogeneous with respect to cell size and granularity (Figure 2.2 A). Since hematopoietic stem and progenitor cells are typically small, agranular cells, we further enriched the SP fraction by separating the small, agranular (SA) fraction from the large, granular (LG) fraction as shown in Figure 2.2 B. Thus, four populations of cells from the total bone marrow were derived: the small, agranular side population (SA-SP); the small, agranular, non-side population (SA-nonSP); the large, granular side population (LG-SP); and the large, granular non-side population (LG-nonSP) fractions (Figure 2.3 A). The proportion of SA-SP cells was 1.18 ± 0.14 % (range 0.88 – 1.39%) of the total SA cells or 0.13 ± 0.02 % of the total viable cells, while the majority of the SP hook was composed of LG-SP cells, presenting an average percentage of 0.89 ± 0.11 % (range 0.65-1.08%) of the total LG cell fraction or 0.69 ± 0.09 % of the total viable cells (Table 2.3).
Figure 2.2. Flow cytometric analysis of SP cell size and granularity for the total SP, large, granular SP (LG-SP) and small, agranular SP (SA-SP) subpopulations. (A) The total viable bone marrow cells were plotted by Hoechst 33342 Red versus Blue to show the
**Figure 2.2.** (continued) total SP fraction, which was gated and plotted by forward scatter (FSC) versus side scatter (SSC) to indicate the heterogeneity of SP cell size and granularity. (B) The total viable bone marrow cells were gated into two populations of large, granular cells (LG) and small, agranular cells (SA). Gating on the SP population, the size and granularity of the LG-SP and SA-SP subfraction was detected on FSC versus SSC plots.
Figure 2.3. Wright-stained cytospins of canine bone marrow stained with Hoechst 33342 gated into small, agranular (SA) and large, granular (LG) cells and differential counts. The gating strategy in (A) results in four sub-populations that were cytocentrifuged and Wright stained. (A i) The LG-SP fraction shows lighter staining of the uncondensed chromatin of blast cells. (B) Differential counts of cell types present in each sub-fraction.
Table 2.3. Percentages of canine bone marrow-derived SP cells falling within the small agranular and large, granular gated regions.

<table>
<thead>
<tr>
<th>Viable % of total</th>
<th>Total SP cell % of viable</th>
<th>SA % of viable</th>
<th>SA SP % of viable</th>
<th>SA SP % of viable</th>
<th>LG % of viable</th>
<th>LG SP % of viable</th>
<th>LG SP % of viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>52.6</td>
<td>0.94</td>
<td>11.4</td>
<td>1.18</td>
<td>0.13</td>
<td>77.0</td>
<td>0.89</td>
</tr>
<tr>
<td>StdDev</td>
<td>1.36</td>
<td>0.10</td>
<td>1.29</td>
<td>0.14</td>
<td>0.02</td>
<td>1.26</td>
<td>0.11</td>
</tr>
</tbody>
</table>

N = 14 samples
To assess the cellular composition of the SP and nonSP fractions, cytospins were prepared and differential hematopoietic cell counts determined by a hematopathologist, Dr. Alden Chesney. The majority of stem cell-like blast cells were detected in the SA-SP fraction (Figure 2.3 A, SA-SP panel). The SA-SP fraction also contained a high proportion of erythroblasts (42% of SA-SP), while the LG-SP fraction consisted primarily of erythroblasts and monocytes, which combined, made up 76% of the LG-SP fraction (Figure 2.3 B). Thus, gating on the small agranular cells resulted in enrichment of stem cell-like blasts and erythroblasts and excluded a subpopulation of differentiated myeloid cells present in the LG-SP fraction.

2.5.2. SA-SP Cells Express High Levels of the ABCG2 Transporter

To determine whether the SP and nonSP fractions isolated from the small, agranular and large, granular regions showed differential expression of transmembrane efflux pumps, RT-PCR was performed. Analyses revealed that the ABCG2 transporter was expressed in the SA-SP and SA-nonSP fractions while low levels of expression were noted in the LG-SP and LG-nonSP fractions (Figure 2.4 A). By contrast, the multidrug resistance pump, MDR1, was only expressed in the SA-nonSP fraction (Figure 2.4 B), while MRP1 was highly expressed in all fractions except for the SA-SP fraction (Figure 2.4 C). Thus the transporter primarily responsible for Hoechst dye efflux by SA-SP cells was ABCG2, while both the ABCG2 and MRP1 transporters likely contributed to the dye efflux from LG-SP cells. Analysis of the expression of stem cell antigens, C-KIT and FLK1, revealed differential expression, with the majority of C-KIT+ cells falling into the SA-SP gate and the majority of FLK1 cells falling into the SA-nonSP gate. Controls to
Figure 2.4. RT-PCR detection of transporter pump and lineage markers expressed in each SP and non-SP subfraction. Primers specific for transporter pumps (A) ABCG2, (B) MDR1, (C) MRP1; stem cell markers (D) C-KIT, (E) FLK1; and lineage restricted (F) CD4; and B lymphoid cells (G) CD19 positive cells were employed. To control for the amount of template, (H) canine CD45 was amplified. Samples are LG-SP (Lane 2), LG-non-SP (Lane 3), SA-SP (Lane 4), LG-SP (Lane 5), total bone marrow mononuclear cells (Lane 6) and no template control (Lane 7). A 100 bp ladder was run for each sample (Lane 1).
expressing B lymphocytes in both the SA-SP and SA-nonSP fractions (Figure 2.4 E). In dog, CD4 is highly expressed by T cells and neutrophilic granulocytes (Moore et al., 1992), as well as on monocytes, macrophages, and dendritic cells (Rabanal et al., 1995). The diminished levels of CD4 expression by SA-SP cells confirmed that this fraction was largely devoid of lineage-restricted cells (Figure 2.4 D).

2.5.3. Expression of lineage markers by SP cells

Since SP cells from other species offer an enriched source of stem cells, we characterized canine SP cells for expression of lineage-restricted antigens. Antibody staining revealed that the nongated SP cell fraction contained cells expressing the mature myeloid antigens, CD11b, CD11c, and CD14 as well as very low levels of the lymphoid antigens, CD90/Thy1.1 and B-cell marker (Figure 2.5 B).

Gating separately on the LG and SA fractions revealed that the majority of lineage-restricted cells could be separated from the stem cells. For example, CD14+ cells fell within the LG-SP gate and thus were removed from the SA-SP fraction. These studies supported our earlier conclusion that the SP fraction was heterogeneous, consisting of both lineage-committed cells in addition to stem and progenitor cells.

Although pregating diminished the proportion of lineage committed cells in the SA-SP fraction, flow cytometric analysis indicated that this fraction still contained a low proportion of cells expressing the B cell marker, CD4, CD11b, CD11c and CD90/Thy1 (Figure 2.5 F). It should be noted that in dogs, the canine CD4 antibody reacts not only with an antigen expressed on T cells, but it also recognizes an antigen expressed on myeloid cells. Thus these data are consistent with the differential hematopoietic cell
Figure 2.5. Flow cytometric analysis of cell surface markers expressed by cells within each SP and nonSP fraction. Cells were gated on total viable cells and then sub-gated on (A) total non-SP cells, and (B) total SP cells. From the total viable cells, gates were also placed for the LG fraction, which was sub-gated on (C) LG-non-SP, and (D) LG-SP. The SA fraction was sub-gated on (E) SA-non-SP, and (F) SA SP.
Figure 2.5. Flow cytometric analysis of cell surface markers expressed by cells within each SP and nonSP fraction. Cells were gated on total viable cells and then sub-gated on (A) total non-SP cells, and (B) total SP cells. From the total viable cells, gates were also placed for the LG fraction, which was sub-gated on (C) LG-non-SP, and (D) LG-SP. The SA fraction was sub-gated on (E) SA-non-SP, and (F) SA SP.
counts, showing that the SA-SP fractions consisted primarily of stem cell-like blasts and erythroblasts, with a small proportion of monocytes and lymphocytes.

As expected, a large proportion of the cells contained within the LG-nonSP fraction (Figure 2.5 C) expressed the mature myeloid cells markers CD14 and CD11c. The SA-nonSP fraction (Figure 2.5 E) contained mature lymphoid cells expressing the B cell marker and CD4 in addition to myeloid cells expressing CD11c\(^+\) and CD14\(^+\).

Of note, the SP fractions, like other species studied to date, contained very few cells expressing the hematopoietic stem cell antigen, CD34. The majority of CD34\(^+\) cells were found within the nonSP gates, in both the LG-nonSP (Figure 2.5 C) and SA-nonSP (Figure 2.5 E) fractions. Further assessment of the distribution of the CD34\(^+\) cells, indicated that they showed a distinct localization pattern from SP cells (Figure 2.6 A-D) with the majority of CD34\(^+\) SP cells being localized to the LG-SP fraction (Figure 2.6 D).

2.5.4. **Canine SP cells do not give rise to colonies when plated directly into methylcellulose**

Previous studies established that the mouse SP fractions provide an enriched source of colony forming cells (CFCs). To determine whether canine SP cells were enriched in CFCs, we first determined whether human-cytokine supplemented methylcellulose could support colony formation of canine CFCs by plating \(1 \times 10^5\) unfractionated canine bone marrow cells directly into methylcellulose. Within 2 weeks, the canine bone marrow cultures produced 29.5 ± 3.4 colonies (Figure 2.7 A; n=6, two dogs assayed in triplicate). Multi-lineage differentiation was present within the bone marrow of each dog tested as seen by the presence of CFU-GM, CFU-G, CFU-M and
Figure 2.6. Flow cytometric analysis of canine CD34 expression in relation to the SP fraction. (A) Cells were gated on total viable cells (grey) and then sub-gated on the SA (green) and LG (blue) cells. The distribution of the total SP cells (red) is indicated in relation to the total CD34+ cells (black). (B) The CD34+ cells (black) are primarily in the nonSP region, with a smaller number of CD34+ cells in the total SP fraction. (C) Within the SA fraction, the majority of CD34+ (black) cells are in the nonSP region. (D) Within the LG fraction the majority of CD34+ cells (black) are in the nonSP region, and the CD34+ cells detected in the SP fraction of the total viable cell plot (B) are present in the LG-SP fraction.
CFU-E colonies (Figure 2.7 B). CFU-GEMM colonies were not detected which suggested that the human cytokine-supplemented methylcellulose may not show complete cross-species reactivity with canine hematopoietic stem cells or that the frequency of GEMM progenitors was sufficiently low in canine bone marrow that plating of $1 \times 10^5$ cells was insufficient to detect CFU-GEMMs. Ash et al. (1981) found a frequency of $10.8 \pm 1.3$ human bone marrow-derived CFU-GEMM colonies per $1 \times 10^6$ bone marrow mononuclear cells plated, yet found a modification of the methylcellulose medium from αMEM to Iscove’s modified Dulbecco’s medium (IMDM) and the addition of β-mercaptoethanol supported CFU-GEMM colony formation at a frequency of $146\pm58$ per $1 \times 10^6$ cells plated. This data indicated the importance of optimizing the growth medium to assess the colony-forming potential of a cell source of interest. The methylcellulose medium utilized in these studies contained IMDM and β-mercaptoethanol supplemented with human cytokines, but a similar analysis of the frequency of CFU-GEMMs present in canine bone marrow has yet to be reported in this medium. Analysis of the potential of canine SP cells to give rise to colonies after direct plating in human cytokine-containing methylcellulose indicated that canine SP cells, similar to rhesus monkey SP cells, did not form colonies despite plating as many as $1 \times 10^4$ cells SA-SP and LG-SP cells and extended incubation for up to four weeks (Figure 2.7 C).

By comparison, the LG-nonSP and SA-nonSP fractions were also plated directly into methylcellulose and gave rise to colonies within 2 weeks of culture initiation. The LG-nonSP fraction gave rise to $11.7 \pm 2.0$ colonies, while the SA-nonSP produced $4.0 \pm$
Figure 2.7. CFU-Cs derived from unfractionated canine bone marrow, SP and nonSP fractions plated directly into human cytokine-supplemented methylcellulose medium. (A)
Figure 2.7. (continued) The proportion of CFU-C colony types from $1 \times 10^5$ total canine bone marrow cells of two individual dogs. Each sample was assayed in triplicate. (B) Wright staining of individual colonies confirmed the presence of each colony type. (C) The average number of CFU-Cs within each subfraction of Hoechst stained canine bone marrow. Each sample was assayed in triplicate.
0.6 colonies after plating 2 x 10^5 cells (Figure 2.7 C). Thus CFCs were present in nonSP fractions.

### 2.5.5. Canine SP cells form colonies after long-term culture on canine stroma

Since colonies formed in methylcellulose after plating cells from either the unfractionated canine bone marrow or nonSP fractions, it was unlikely that the absence of CFUs in the SA-SP and LG-SP fractions was due to the inability of canine CFCs to respond to human cytokines. Alternatively, it was likely that the SP fractions contained more primitive cells that were unable to differentiate in methylcellulose but could be transitioned to a CFC after culture on a feeder layer of canine stroma, as had been shown to be the case with rhesus monkey SP cells (Goodell et al., 1997). To address this possibility, we used the long-term culture-initiating cell (LTC-IC) assay to analyze bone marrow fractions for multipotent progenitors and stem cells.

Long term cultures were established by plating 10-5000 SP or 1 x 10^3-1 x 10^5 nonSP cells on an irradiated layer of canine bone marrow-derived stromal cells in human Myelocult medium supplemented with canine- or human-specific cytokines (10 ng/ml of cSCF, hFlt3L, hIL3, hIL6, cGM-CSF, and cG-CSF). Within one to two weeks after culture initiation, cobblestone areas were visible in a proportion of cultures seeded with SA-SP, LG-SP and LG-nonSP cultures, while no cobblestone areas were detected in the SA-nonSP cultures.

Five weeks after initiation, cultures were harvested and the contents of the entire well were plated in methylcellulose. Methylcellulose cultures were scored positive when dishes contained one or more colonies of fifty or more cells. Poisson statistics were
utilized to determine the frequency of LTC-ICs for each SP and nonSP cell fraction. Limiting dilution analysis revealed that an average of 1 in 300 SA-SP cells and 1 in 1892 LG-SP cells were LTC-ICs (Table 2.4). By contrast, only 1 in 22802 SA-nonSP cells and 1 in 80622 LG-nonSP cells gave rise to colonies. Cytospin preparations of all colonies demonstrated multilineage differentiation of cells in the SA-SP and SA-nonSP fractions, while only CFU-M colonies developed from cells originating from the LG-SP and LG-nonSP fractions.

2.5.6. Engraftment of canine SP cells in NOD/SCID xenotransplantation model

The NOD/SCID xenotransplantation model allows for the assessment of the self-renewal and differentiation potential of various stem cell sources. Although NOD/SCID-β2m−/− mice have a deficiency in CD8+ and natural killer T cells, which allows for more robust engraftment than that seen in NOD/SCID mice, further immuno-depletion using anti-CD122 treatment can increase engraftment of xenotransplants (McKenzie et al., 2005). Irradiated NOD/SCID-β2m−/− mice treated with 200 µg of anti-CD122 antibody (clone TM-β1, kind gift of Tanaka) demonstrated a reduction of recipient NK cells within the peripheral blood (PB) as shown by the loss of the small, low granularity population (6% of untreated versus 1% of CD122-treated PB; Figure 2.8 A and C). Cytotoxic NK cell depletion was confirmed by a reduction of PB cells expressing the murine pan-NK marker, DX5, and the NK cell specific marker, NKp46 (Figure 2.8 B and D). Twenty-four hours after treatment with the anti-CD122 antibody, the percentage of DX5 positive cells (16% in untreated) had decreased to 10%, while the percentage of NKp46 positive
Table 2.4. Response rate of individual methylcellulose cultures and calculated CFU-C frequency for SP and non-SP cell fractions cultured for 6 weeks in LTC conditions prior to transfer to methylcellulose.

<table>
<thead>
<tr>
<th>Cell dose</th>
<th>Nellie 1</th>
<th>Nellie 2</th>
<th>Wassy 1</th>
<th>Wassy 2</th>
<th>Female 1</th>
<th>Female 2</th>
<th>Male 1</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA SP</td>
<td>10</td>
<td>ND</td>
<td>1 in 298</td>
<td>ND</td>
<td>ND</td>
<td>CNBD‡</td>
<td>0/3</td>
<td>1 in 1567</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>ND</td>
<td>(479-186)</td>
<td>2/3</td>
<td>(174-45)</td>
<td>ND</td>
<td>0/3</td>
<td>(422-581)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>3/4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1317</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>4/4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4/4</td>
<td>ND</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>4/4</td>
<td>3/3</td>
<td>0/1</td>
<td>4/4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>4/4</td>
<td>ND</td>
<td>2/2</td>
<td>2/2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SA non-SP</td>
<td>1e3</td>
<td>ND</td>
<td>CNBD‡</td>
<td>ND</td>
<td>CNBD‡</td>
<td>ND</td>
<td>0/3</td>
<td>1 in 10735</td>
</tr>
<tr>
<td></td>
<td>1e4</td>
<td>0/4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/3</td>
<td>2/3</td>
<td>2/3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LG SP</td>
<td>100</td>
<td>ND</td>
<td>CNBD‡</td>
<td>ND</td>
<td>ND</td>
<td>1 in 940</td>
<td>1/3</td>
<td>1 in 1267</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0/4</td>
<td>1/3</td>
<td>(1504-76)</td>
<td>1/3</td>
<td>(2039-150)</td>
<td>0/3</td>
<td>CNBD‡</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0/4</td>
<td>2/2</td>
<td>588</td>
<td>2/3</td>
<td>787</td>
<td>3/4</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>0/4</td>
<td>2/3</td>
<td>2/3</td>
<td>3/4</td>
<td>ND</td>
<td>3/3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>0/4</td>
<td>ND</td>
<td>3/3</td>
<td>4/4</td>
<td>ND</td>
<td>3/3</td>
<td>ND</td>
</tr>
<tr>
<td>LG non-SP</td>
<td>1e4</td>
<td>0/3</td>
<td>CNBD‡</td>
<td>1/3</td>
<td>(135006-39075)</td>
<td>1/3</td>
<td>1 in 24513</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>2e4</td>
<td>0/4</td>
<td>ND</td>
<td>1/3</td>
<td>(135006-39075)</td>
<td>3/4</td>
<td>(39075-15378)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5e4</td>
<td>2/3</td>
<td>18022</td>
<td>3/4</td>
<td>15378</td>
<td>ND</td>
<td>102189</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>1e5</td>
<td>0/2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

ND: Not done
CNBD: Could not be determined, data with no responses cannot be analyzed by L-CALC software
CNBD‡: Could not be determined, data with 100% responses cannot be analyzed by L-CALC software
Figure 2.8. DX5$^+$NKp46$^+$ natural killer cells in the peripheral blood are diminished by anti-CD122 treatment of NOD/SCID-$\beta$2-microglobulin$^{-/-}$ mice. The proportion of small, low granularity natural killer cells in untreated mice (A) is greater than mice treated with the anti-CD122 antibody (C). The proportion of DX5$^+$NKp46$^+$ natural killer cells (B) is also decreased in treated mice (D).
cells had been decreased from 10% to 4%. This finding indicated that cytotoxic NK cells, which are positive for both DX5 and NKp46, had been reduced in the NOD/SCID-β2m−/− mice with anti-CD122 treatment.

Once we had determined that NOD/SCID-β2m−/− mice were further immuno-depleted with anti-CD122 treatment, we assessed which SP or nonSP fractions contained the long-term SCID repopulating cells. 2 x 10⁶ – 1 x 10⁷ canine total bone marrow, 1 x 10³ – 1 x 10⁵ SP, and 1 x 10⁴ – 1 x 10⁶ non-SP cells were transplanted intrafemorally and engraftment of canine cells was assessed at 12 weeks post-transplantation by flow cytometric detection of canine CD45 (cCD45) positive cells. This time point was selected as engraftment of donor cells after 12 weeks is thought to be indicative of long-term engraftment for human transplants into the NOD/SCID model (Hogan et al., 2002). To ensure that cCD45 staining was specific to canine cells and did not cross react with host mouse cells, the antibody was tested on mouse and canine bone marrow, as well as mixtures of the two samples. With mouse Fc block pretreatment, the cCD45 antibody had very low background binding to mouse cells (≤ 0.05%, data not shown). The sensitivity of this flow cytometric assay was determined to be reliable with as few as 0.1% canine cells in a background of 99.9% mouse cells. As such, the cut off for a positive engraftment result was set at 0.1% cCD45+ cells in samples taken from transplanted mice.

Engraftment of canine cells was detected at low levels in mice that received SA-SP, SA-nonSP and LG-SP cells (Figure 2.9), while engraftment was below the limit of detection in mice that received LG-nonSP cells or irradiated canine stromal cells. As the percentage of cCD45+ cells in the injected femur and contralateral femur and tibiae for
Figure 2.9. Average level of engraftment of canine CD45+ cells in anti-CD122-treated NOD/SCID-β2m-/- recipient mice transplanted intrafemorally with SP and non-SP fractions of canine bone marrow. Marrow stromal cells (MSC) were transplanted alone to determine background level of engraftment for accessory cells. Total bone marrow mononuclear cells (BMMC) were transplanted to determine the lower limit of cells required for engraftment in this xenotransplantation model. The bone marrow harvested from the injected femur (grey bars) and the contralateral femur and tibiae (white bars) were assayed separately.
each cell type and dose was near the limit of detection, a highly variable distribution of
engraftment levels was noted (Figure 2.10), though engraftment was most robust in mice
transplanted with cells from the SA-nonSP fraction with a mean total activity of $0.54 \pm 
0.22\%$ in the injected femur and $1.26 \pm 0.55\%$ in the contralateral femur and tibiae with
transplantation of $5 \times 10^5$ SA-nonSP cells. For comparison, unfractionated canine total
bone marrow cells were also transplanted to anti-CD122-treated NOD/SCID-β2m−/− mice,
and showed engraftment ranging from 0 - 11.1% after 12 weeks in vivo. The greatest
levels of engraftment were seen with transplantation of $5 \times 10^6$ or $1 \times 10^7$ total bone
marrow cells, which resulted in a mean total activity of $1.80 \pm 0.80\%$ cCD45+ cells in the
injected femur and $3.85 \pm 1.85\%$ in the contralateral femur and tibiae with
transplantation of $1 \times 10^7$ total bone marrow cells.
Figure 2.10. Distribution of engraftment levels of canine CD45+ cells in anti-CD122-treated NOD/SCID-β2m/- recipient mice transplanted intrafemorally with SP and non-SP fractions of canine bone marrow. Marrow stromal cells (MSC) were transplanted alone to determine background level of engraftment for accessory cells. Total bone marrow mononuclear cells (BMMC) were transplanted to determine the lower limit of cells required for engraftment in this xenotransplantation model. The bone marrow harvested from the injected femur (●) and the contralateral femur and tibiae (▲) were assayed separately.
2.6. Discussion

We undertook these studies to determine if hematopoietic stem cells could be purified from canine bone marrow based on exclusion of the vital dye Hoechst 33342 as had been demonstrated in other species (Goodell et al., 1997). Our studies showed for the first time that, like other species, canine bone marrow-derived HSCs had an innate ability to efflux Hoechst dye, while the majority of mature cells within the bone marrow did not possess this activity.

We found that the Hoechst staining procedure resulted in variability among experiments in both the presence and proportion of SP cells. To reduce this variability, bone marrow samples were recovered in medium supplemented with the stem cell supporting cytokines, cSCF, hFlt3L and hIL7. Analysis of the size of cells within the SP fraction indicated that there was significant heterogeneity within this population. Furthermore, flow cytometry indicated that the SP fraction contained mature cells expressing lineage-restricted markers including the monocyte marker, CD14. Since HSCs are small in size with low granularity, we subfractioned the bone marrow by establishing gates on cells with a low FSC and SSC profile to enrich for the stem cell fraction. This strategy allowed us to identify a SP fraction, which we termed the SA-SP that was enriched for stem cell-like blast cells with a high nucleocytoplasmic ratio. Depletion of the large, granular SP cells from the total SP fraction diminished the contamination of SP cells with CD14+ myeloid cells. Using this strategy, the proportion of SA-SP cells in canine bone marrow samples was 0.13% with the standard error among samples decreasing from ± 0.08% to 0.02%.
Verapamil, an inhibitor of MRP1 and MDR1, was able to reduce the number of cells within the total SP fraction but did not completely eliminate the SP phenotype suggesting that the MRP1 and MDR1 pumps were responsible for Hoechst efflux in a proportion of cells. The Hoechst<sup>lo</sup> phenotype of canine CD14<sup>+</sup> myeloid lineage cells may be attributed to slower kinetics of Hoechst dye accumulation within this population of cells, or to the presence of additional transmembrane pumps effluxing the Hoechst dye. RT-PCR analysis for expression of ABCG2, MDR1 and MRP1 efflux pumps revealed that the LG-SP fraction expressed predominantly MRP1 whereas the primary pump responsible for dye efflux from SA-SP cells was the ABCG2 pump. Given the minimal expression of the ABCG2 transporter by LG-SP cells, in conjunction with high expression of MRP1, it is likely that the MRP1 transmembrane pump is responsible for Hoechst dye expulsion in CD14<sup>+</sup> cells. This conclusion is further supported by studies with human cells demonstrating that CD14<sup>+</sup> monocytes expressed MRP1 (Laupeze <i>et al.</i>, 2001) and MDR1 (Drach <i>et al.</i>, 1992) and both pumps were capable of transporting Hoechst (Neyfakh, 1988). Thus, exclusion of large, granular cells enabled depletion of CD14<sup>+</sup> cells with the ability to efflux Hoechst dye, thereby enriching the SA-SP fraction with a greater proportion of blast cells. The enrichment of blast cells was also indicated by the high level of C-KIT expression within the SA-SP fraction and low-level of FLK1 expression. Work by Haruta <i>et al.</i> (2001) supports this finding, as murine bone marrow-derived SP cells were found to express c-kit but lacked expression of flk1.

As seen in other species, canine SA-SP cells expressed low to undetectable levels of the hematopoietic stem cell antigen, CD34, and were primarily negative for antigens whose expression is limited to lineage-restricted hematopoietic cells. A proportion of
cells within the SA-SP fraction reacted with a canine B cell antibody, the myeloid lineage markers, cCD11b and cCD11c, and the T-cell antigen, CD4, also expressed on monocytes, granulocytes and dendritic cells (Moore et al., 1992). Microscopic analysis of sorted SA-SP cells corroborated the cell surface staining, as within this fraction 10% lymphocytes and 6% monocytes. An additional proportion of cells (42%) were identified as erythroblasts by histopathology. The presence of these cells was not confirmed by flow cytometry as no erythroblast antibodies are available for use in dogs. It is interesting to note that several studies have reported expression of ABCG2 on Ter119+ murine erythrocytes and erythroblasts (Zhou et al., 2001; Tadjali et al., 2006) and therefore, it is likely that similar to mouse, canine erythroblasts express the ABCG2 pump resulting in their colocalization with HSCs within the SA-SP fraction.

These studies indicated that canine SA-SP cells display several properties associated with primitive hematopoietic stem and progenitor cells, as have been seen in SP cells isolated from other species. Although, canine SA-SP cells did not form colonies when plated directly in human cytokine-supplemented methylcellulose, they formed multipotent colonies representing the myelomonocytic and erythroid lineages after long-term culture on a feeder layer of canine bone marrow stroma. It is important to note that the canine SA-SP fraction was enriched in LTC-ICs relative to the LG-nonSP or SA-nonSP fractions. Moreover, the majority of the CD34+ cells, which are the cell type utilized for most canine HSC transplants, localized within the LG-nonSP and SA-nonSP fractions. The ability of the nonSP cells to form colonies upon direct culture in methylcellulose, while demonstrating limited colony formation after long-term culture, suggested long-term culture initiating cells were found in the CD34+ SA-SP fraction while
CD34+ cells with short-term CFU potential were located in the LG- and SA-nonSP fractions.

Assessment of the in vivo hematopoietic repopulation potential of canine SA-SP cells showed a surprisingly limited level of engraftment in the NOD/SCID-β2m–/– xenotransplantation model. Even more striking was the 4-fold higher engraftment achieved by the SA-nonSP fraction. The higher than expected engraftment from the SA-nonSP fraction may be due to the presence of stem cells with greater SCID-repopulating cell (SRC) potential than those of the SA-SP fraction. Based on lineage marker flow cytometric analysis of the SA-SP and SA-nonSP fractions, we speculate that the engraftment seen in mice transplanted with the SA-nonSP fraction is due to the presence of CD34+ stem cells, which were enriched within this cell fraction. The comparatively lower engraftment of cells of the SA-SP fraction may be due to the more primitive, quiescent nature of cells in this fraction. In support of this hypothesis, studies in the baboon have shown that SCID-repopulation by baboon CD34+ cells is representative of the engraftment of short-term repopulating cells rather than cells with long-term repopulation potential in baboon recipients (Horn et al., 2003). Thus, the NOD/SCID xenotransplantation model was found to primarily detect more committed progenitors than those capable of long-term engraftment after autologous transplantation in a large animal model. Alternatively, the lack of NOD/SCID engraftment for the SA-SP fraction may indicate that in the dog, the majority of SRCs have a non-SP phenotype.

These results, taken together with the in vitro potential of the canine SA-SP and SA-nonSP fractions, suggest that one of the limiting factors of these studies may be the suitability of the immunocompromised mouse xenotransplantation model for the
assessment of long-term engraftment potential of canine HSCs. Although human cytokines can be used in the absence of commercially available canine cytokines to support canine hematopoiesis in vitro, the support provided is incomplete as evidenced by the lack of detection of CFU-GEMMs after plating canine bone marrow in human cytokine-supplemented methylcellulose. In addition, the cross-reactivity of many murine cytokines with canine HSCs has not been thoroughly established. Thus, the low levels of engraftment detected using the xenotransplantation model may point to incomplete in vivo stimulation of canine cells by murine cytokines provided by the NOD/SCID transplantation model. Furthermore, engraftment of recipient mice injected with unfractionated canine bone marrow, which included mature hematopoietic and stromal cells along with HSCs, suggested that the cytokines produced by support cells were essential for repopulation of the murine hematopoietic system with canine cells. The identification of canine support cells for cotransplantation that secrete the cytokines necessary to support robust canine HSC engraftment and proliferation is one avenue of future study. Cotransplantation of irradiated unfractionated canine bone marrow or peripheral blood cells with HSCs may increase chimerism in conjunction with post-transplant boosting with additional cells or purified canine cytokines to support the long-term engraftment of canine cells. To demonstrate the SRC potential of canine SA-SP cells, it may be important to activate quiescent canine SA-SP cells by a short period of in vitro culture prior to transplantation to induce their maturation to the CD34+ stage of development. This view is supported by our LTC-IC assays in which canine SP cells required maturation under long-term culture conditions in order to demonstrate their potential to give rise to colonies in methylcellulose. Thus, a similar strategy may
facilitate SP cell engraftment and differentiation within the xenotransplantation model. It is also interesting to note that since the isolation of human and rhesus monkey SP cells in 1997 (Goodell et al., 1997), no reports of the in vivo engraftment potential of these cells in a NOD/SCID xenotransplantation model have been published. Thus, it may not be surprising that this HSC surrogate assay had limited ability to support the proliferation of canine HSCs and resulted in low-level chimerism. Alternatively, these studies cannot rule out the possibility that canine SP cells lack in vivo HSC activity. Future studies employing transplantation into canine recipients would provide a better environment in which to assess this possibility.

Most efforts to develop transplantation protocols for canine hematopoietic stem cells have focused on CD34\(^+\) cells, based on the well-accepted idea that at least a subset of these cells are responsible for durable engraftment in bone marrow transplant recipients (Bruno et al., 1999, 2001). Our studies demonstrated that a large proportion of stem cell-like blast cells are localized within the SA-SP fraction and do not express CD34. These blast cells could be induced to give rise to colonies in methylcellulose after long-term culture with canine stromal cells and cytokines, yet, were unable to engraft NOD/SCID recipients. These findings indicated that further stimulation of this blast cell population might be required to determine their in vivo repopulation potential. Thus, novel methods of stem cell isolation that combine the CD34\(^+\) cell fraction with the more quiescent CD34\(^-\) fraction may provide a larger cell source for transplantation with robust repopulation potential in canine recipients.


2.7. References

1. Abrams-Ogg AC, Kruth SA, Carter RF et al. Clinical and pathological findings in
dogs following supralethal total body irradiation with and without infusion of autologous

2. Ash RC, Detrick DA, Zanjani ED. Studies of human pluripotential hemopoietic stem


cells: long-term maintenance of in vitro activated progenitors without marrow ablation.

transduced with retroviral vectors give rise to long-term multi-lineage hematopoiesis.

6. Bruno B, Nash RA, Wallace PM et al. CD34+ selected bone marrow grafts are
radioprotective and establish mixed chimerism in dogs given high dose total body

bone marrow cells results in expansion of side population stem cells in vitro and


49. Zhou S, Morris JJ, Barnes Y et al. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to

CHAPTER 3

Derivation and Characterization of Canine Embryonic Stem Cell Lines with *In Vitro* and *In Vivo* Differentiation Potential

This chapter is a modified version of the following publication:

3.1 Attribution of Data

I performed all the work described in this chapter with the following exceptions: the derivation of the canine embryonic stem cell lines SK-1 and SK-4 was completed by Suzana Rosic-Kablar, and their alkaline phosphatase activity (Figure 4.3 D) and in vitro differentiation potential (Figure 4.12) were confirmed by Suzana. PCR primers used throughout this project were designed by Yan-zhen Zheng (Table 4.1. and 4.2.). Animal breeding and embryo collection was completed by Cathy J. Gartley. G-banding and canine chromosome identification was done by Daniel A.F. Villagomez (Figure 4.7 A-C). Cell types present within teratomas were confirmed by Alden Chesney.
3.2 Abstract

Embryonic stem cells (ESCs) represent permanent cell lines that can be maintained in an undifferentiated state. In an environment that induces differentiation, they form derivatives of the three embryonic germ layers: mesoderm, ectoderm and endoderm. These characteristics give ESCs great potential for both basic research and clinical applications in the areas of regenerative medicine and tissue engineering. The establishment of ESCs from large animals that model human diseases is of significant importance. We describe the derivation of permanent canine cell lines from preimplantation stage embryos. Similar to human ESCs, canine ESCs expressed OCT3/4, NANOG, SOX2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and alkaline phosphatase while they expressed very low levels of SSEA-1. They maintained a normal karyotype and morphology typical of undifferentiated ESCs after multiple in vitro passages and rounds of cryopreservation. Plating cells in the absence of a feeder layer, either in attachment or suspension culture, resulted in the formation of embryoid bodies and their differentiation to multiple cell types. In vivo, canine ESCs gave rise to teratomas comprising cell types of all three embryonic germ layers. These cells represent the first pluripotent canine ESC lines with both in vitro and in vivo differentiation potential and offer the exciting possibility of testing the efficacy and safety of ESCs based therapies in large animal models of human disease.
3.3 Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) of preimplantation stage embryos. They are capable of differentiating to the three embryonic germ layers and of unlimited in vitro self-renewal and proliferation while maintaining their pluripotent phenotype. Murine ESC (mESC) lines were first established by culturing blastocysts on a feeder layer of mouse embryonic fibroblasts (MEFs) (Evans and Kaufman, 1981; Martin, 1981). Cells from established mESC lines have been used to study mechanisms of cell differentiation during embryogenesis and have also demonstrated the potential of in vitro differentiation into a wide spectrum of cell types including hematopoietic cells, cardiomyocytes, neuronal cells, islet cells and hepatocytes (Keller, 2005; Murry and Keller, 2008).

Human ESC (hESC) lines have also been established (Thomson et al., 1998; Reubinoff et al., 2000) which, like mESCs, differentiate into diverse cell types. These findings have fuelled optimism that ESCs may hold tremendous potential as unlimited sources of cells for transplantation and tissue regeneration in the treatment of many diseases including disorders of the hematopoietic system, cardiomyopathies, neural and muscular degenerative disorders and autoimmune diseases. Although hESCs could solve problems of donor shortages for transplantation therapies and provide treatment for debilitating diseases, thorough assessment of the efficacy and safety of ESCs using appropriate large animal models that accurately predict therapeutic outcomes of these cells in transplantation and tissue repair is required before their clinical application. Thus an important challenge for the future application of ESCs is the derivation of ESC lines from biomedically relevant large animal research models.
Murine models of human diseases have contributed significantly to the development of novel therapies, although the physiological differences existing between mice and humans limit the direct translation of knowledge gained to the clinical setting. Although nonhuman primate ESC lines derived from rhesus monkey (Thomson et al., 1995), common marmoset (Thomson et al., 1996) and cynomolgus monkey (Suemori et al., 2001) are available, preclinical studies using these models are restricted to research centres with the appropriate animal care capabilities. Furthermore, well-characterized nonhuman primate models of human diseases are not available. Inbred dogs with genetic disorders that recapitulate human diseases are widely available and offer alternative models that have been successfully used to advance novel therapies into the clinic (Tsai et al., 2007; Wayne and Ostrander, 2007). Dogs share many biochemical and physiological characteristics with humans and thus diseases in dogs more accurately mimic human diseases than do their rodent counterparts. In addition, knowledge and technical expertise acquired in canine studies are often directly translatable to the human clinical setting. Dogs have been instrumental in the development of human bone marrow transplantation and gene therapy protocols (Baron and Storb, 2006; Casal and Haskins, 2006; Lillicrap et al., 2006; Ponder, 2006; Malech and Hickstein, 2007) and have also made valuable contributions to the development of therapies for diabetes (Banting, 1937), cardiovascular (Moise, 1999) and orthopaedic diseases (Egermann et al., 2005; Pearce et al., 2007). Furthermore, the hazards of novel therapeutics may be better revealed in canine (Zhang et al., 2007, 2008) than murine models (Sauvageau et al., 1995; Antonchuk et al., 2002) and thus safety and efficacy studies in dogs should be considered prior to implementation of clinical trials in humans.
The availability of canine ESCs (cESCs) would profoundly facilitate the development of ESC-based therapies for the treatment of inherited and acquired human diseases. Herein we report the establishment of cESC lines derived from canine blastocysts. Cells from these lines were maintained in culture for many months while retaining the defining features of ESCs. CESC more closely resemble human than mESC in their expression of cell surface antigens, growth rates and passage requirements.
3.4. Materials and Methods

3.4.1. Mating and Embryo Collection

Animal protocols were approved by the University of Guelph Animal Care Committee. Sixteen bitches were used: thirteen mixed breeds ranging in size from 15-33 kg and three beagles. Bitches were artificially inseminated with semen from large mixed breed dogs. Routine surgical ovariohysterectomy under general anaesthesia was performed 10-16 days after the surge of leutinizing hormone (LH) or 6-12 days after first mating. Uterine horns were flushed with modified Dulbecco’s Phosphate Buffered Saline (PBS) (Invitrogen, Burlington, Canada). Embryos were assessed for stage of development and quality as described (Seidel and Stringfellow, 1998) and then transferred to CO₂ independent medium (Invitrogen) for transport to Sunnybrook Health Sciences Centre.

3.4.2. In Vitro Culture of Canine Embryos and Derivation of cESC lines

Embryos were plated on γ-irradiated (35Gy) MEFs and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% ESC-qualified fetal bovine serum (FBS) (Sigma, Oakville, ON, Canada). Complete ESC medium consisted of Knockout (KO) DMEM or DMEM/F12 medium supplemented with 0.1 mM β-mercaptoethanol, 5 µM thymidine, 15 µM cytidine, 15 µM guanosine, 15 µM adenosine and 15 µM uridine nucleosides (Sigma), 0.2 mM GlutaMax, 0.1 mM non-essential amino acids, penicillin (100 IU/ml), streptomycin (50 µg/ml) (Invitrogen), human LIF (10 ng/ml hLIF) (Chemicon, Temecula, CA, USA), human basic fibroblast growth factor (4 ng/ml hbFGF) (R&D Systems, Minneapolis, MN, USA), and 15% FBS
or KO Serum Replacement (KO SR) (Invitrogen). Embryos were incubated at 37.5°C and 5% CO₂.

### 3.4.3. Establishment and Maintenance of cESC Lines

Embryos were cultured in complete DMEM/F12 supplemented with 15% FBS or KO SR and allowed to hatch spontaneously or by cutting through the zona pellucida with a fine scalpel blade. Five to seven days after hatching, inner cell mass (ICM) outgrowths were disaggregated into small pieces by mechanical cutting using a glass capillary microdispenser (Drummond Scientific, Broomall, PA, USA). Clusters of ES-like cells were transferred at high density to fresh, irradiated MEFs and resulting colonies were further sub-cultured every 2-4 days by mechanical manipulation. Once established (passage 6-10), ESCs were passaged every 5-7 days by exposure to 0.125% dispase (Stem Cell Technology, Vancouver, BC, Canada) in Hanks’ Balanced Salt Solution (HBSS) supplemented with 2% FBS for 45 min followed by exposure to 0.02% EDTA (Sigma) for 1-2 min at RT. Cultures were maintained at 37.5°C in 5% CO₂, with half medium changes completed on alternate days. Cell lines were cryopreserved in 90% FBS and 10% DMSO under slow cooling conditions to –80°C. Cell lines derived under SRM conditions were then transferred to liquid nitrogen for long-term storage; while cell lines derived under FBS conditions were only stored at -80°C and long-term viability was compromised.

### 3.4.4. RT-PCR

Total RNA was prepared using an RNeasy kit following the manufacturer’s instructions (Qiagen, Mississauga, ON, Canada). 100 ng of mRNA was primed with oligo dT and reverse transcribed (RT) to cDNA using Superscriptase II reverse transcriptase
(Invitrogen). Primers are listed in Table 3.1. RT-PCR reactions consisted of a 3-min denaturation at 94°C, followed by 40 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. A final extension for 7 min was completed at 72°C. OCT3/4 RT-PCR reactions were modified to 45 cycles, with primer annealing at 50°C. RT-PCR-specific amplification of target genes was confirmed by DNA sequencing.

3.4.5. Immunohistochemistry of cESC Colonies

CESC colonies were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. Cells were incubated for 1 h in 10% goat serum, 3% BSA, 0.05% Triton X-100 (Sigma) in PBS then with anti-human stage-specific embryonic antigen (SSEA)-1 (MAB4301), SSEA-3 (MAB4303), SSEA-4 (MAB4304); keratin sulphate-associated antigens TRA-1-60 (MAB4360) or TRA-1-81 (MAB4381; Chemicon), OCT3/4 (SC9081; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NANOG (500-P236; Peprotech, Rocky Hill, NJ, USA or H00079923-M08; Abnova, Taipei City, Taiwan) or SOX2 (#01438; Stem Cell Technologies) in PBS at 4°C overnight. Appropriately matched anti-mouse-IgG or IgM, anti-rat-IgM or anti-rabbit-IgG-Cy5 labelled secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) staining for 1 h in PBS, was followed by a 0.1 μM Sytox Green (Invitrogen) nuclear stain for 15 min. Secondary antibody controls were completed for all samples. Slides mounted with DAKO fluorescent mount (Dako, Mississauga, ON, Canada) were viewed with an Axiovert 200M epi-fluorescence microscope using Axiovision Rel. 4.5 software or a LSM 510 confocal imaging system on an Axiovert 100 microscope (Carl Zeiss, Toronto, ON, Canada).
Table 3.1: RT-PCR Primers Sets for Canine Genes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine OCT3/4</td>
<td>Dog-POU5F1-S1</td>
<td>TGACGACAACAAAAAATCT</td>
</tr>
<tr>
<td></td>
<td>Dog-POU5F1-A1</td>
<td>CAGGCATGTGTTCTCCAG</td>
</tr>
<tr>
<td>Canine NANO2</td>
<td>cNANO2100F</td>
<td>CCTGCATCCTTGCCAAATGTC</td>
</tr>
<tr>
<td></td>
<td>cNANO2197R</td>
<td>TCCGGGCTGTCTGAGTAAG</td>
</tr>
<tr>
<td>Canine SOX2</td>
<td>cSOX2aL</td>
<td>AACCCCAAGTGATGCAAACTC</td>
</tr>
<tr>
<td></td>
<td>cSOX2aR</td>
<td>CGGGGCCCCGTATTTTATAATC</td>
</tr>
<tr>
<td>Canine GBX2</td>
<td>cGBx2-482F</td>
<td>TGCAGGCGTCGCTCGTAG</td>
</tr>
<tr>
<td></td>
<td>cGBx2-602R</td>
<td>TCCGAGCTGTAGTCCAGATCA</td>
</tr>
<tr>
<td>Canine REX1</td>
<td>cRex1-561F</td>
<td>GAGAAAGCATCTCCTCGTCCCA</td>
</tr>
<tr>
<td></td>
<td>cRex1-711R</td>
<td>GCGTTTCACATCTCTCCAA</td>
</tr>
<tr>
<td>Canine AFP</td>
<td>cAFP-F</td>
<td>TGCCAGGCTCAGGGTGTAG</td>
</tr>
<tr>
<td></td>
<td>cAFP-R</td>
<td>TAAACTCCCAAAGCAGCACGA</td>
</tr>
<tr>
<td>Canine Nkx2.5</td>
<td>cNkx2.5-F</td>
<td>CCAAGGACCCTCGAGCTGA</td>
</tr>
<tr>
<td></td>
<td>cNkx2.5-R</td>
<td>CGACAGATACCGCTGCTGCT</td>
</tr>
<tr>
<td>Canine myosin light chain-1 (MLC-1)</td>
<td>cMLC-1F</td>
<td>CCACTCTGGGTGAGGGGCTA</td>
</tr>
<tr>
<td></td>
<td>cMLC-1R</td>
<td>GGGCTGCCGTTAGGATTTC</td>
</tr>
<tr>
<td>Canine GATA4</td>
<td>cGATA4-F</td>
<td>AACGGAAGCCCAAGAACCTT</td>
</tr>
<tr>
<td></td>
<td>cGATA4-R</td>
<td>GCCACATTGGCTGGAGTTGCT</td>
</tr>
<tr>
<td>Canine GATA6</td>
<td>cGATA6-F</td>
<td>CATTTGGAGGAAACCGTGAA</td>
</tr>
<tr>
<td></td>
<td>cGATA6-R</td>
<td>CCAGCAGATTGAGGACTCCCTT</td>
</tr>
<tr>
<td>Canine Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>cGAPDH-F</td>
<td>TATCAGTTGGATCTGACCTG</td>
</tr>
<tr>
<td></td>
<td>cGAPDH-R</td>
<td>GCGTCGAAGGTTGAAGAGT</td>
</tr>
</tbody>
</table>
3.4.6. Analysis of Alkaline Phosphatase Activity

Cells were treated with BM Purple AP substrate (Roche, Mississauga, ON, Canada) according to the manufacturer’s instructions. MESC and their differentiated progeny were used as positive and negative controls, respectively.

3.4.7. Karyotyping of ESCs

High resolution metaphase chromosome preparations from cultured cESCs at passages 6, 10, 15 and 23 were prepared using standard techniques (Barnes and Maltby, 1986) and GTG banding (Seabright, 1971). Chromosomes were counted and analyzed following the partially standardized G-banded canine karyotype (Switonski et al., 1996).

3.4.8. Teratoma Formation

CESCs were injected bilaterally under the testis capsules of NOD/SCID-β2M or NOD/SCID mice (Jackson Laboratories, Bar Harbor, ME, USA) (~5 x 10^5 cells per site). Mice were sacrificed 7-12 weeks later, teratomas were excised and fixed overnight in 10% neutral-buffered formalin (Fisher Scientific) or with the AMeX protocol (100% acetone (Sigma), followed by clearing in 100% methyl benzoate (Sigma) and 100% xylene (Sigma) (Sato et al., 1986) prior to paraffin embedding. Von Kossa, Masson-Trichrome and Neuron Specific Enolase (NSE) staining was performed as described (Sheehan and Hrapchak, 1981) (Department of Pathology, Sunnybrook Health Science Centre). Immunofluorescence staining with anti-human smooth muscle actin (F3777), beta-tubulin (F2043), GFAP (G3893; Sigma), nestin (N17220; BD Biosciences, Mississauga, ON, Canada), SOX17 (AF1924; R&D Systems) and anti-mouse MHC Class I (CL9013F; Cedarlane, Burlington, ON, Canada) was completed as described above.
DNA was extracted from 50-80 µm testis tissue sections using the RecoverAll Total Nucleic Acid Isolation kit (Ambion, Austin, TX, USA). PCR, using primers listed in Table 3.2 for canine and murine beta-2-microglobulin, was performed using conditions outlined above.

3.4.9. In Vitro Formation of Embryoid Bodies (EBs)

Hanging drop EBs were formed from cESCs dissociated with either 0.125% dispase or TrypLE (Invitrogen) in complete DMEM/F12 supplemented with 15% FBS without hLIF or hbFGF as described (Wobus et al., 1991; Metzger et al., 1994). After 4 days, EBs were transferred to nonadherent culture dishes until harvest. EBs were fixed in 4% paraformaldehyde for 15 min, transferred to 30% sucrose and embedded in OCT medium for cryosectioning. Sections were incubated with antibodies against human OCT3/4, NANOG, SOX2, GFAP, AFP (A8452; Sigma), NCAM (AB5032; Chemicon), Beta-III-tubulin (CBL412; Cymbus Biotechnology, Hampshire, UK), CD201 (#557950) and murine VEGFR2 (#555308; BD Biosciences), at 4°C overnight. Secondary antibody staining was performed with anti-mouse-Cy5 or anti-rabbit-Cy3 antibodies for 1 h at RT and nuclei were stained with DAPI.

3.4.10. In Vitro Differentiation of cESCs

**Endothelial Cells:** CESC or day 2-4 EBs were plated on Collagen IV (Sigma) treated tissue culture plates. Cells were cultured in alpha-MEM medium supplemented with 10% FBS and 0.1 mM β-mercaptoethanol. The medium was exchanged every 2-3 days and endothelial cells were identified after 10-14 days using an anti-porcine-CD31-RPE antibody (MCA1746PE; Serotec, Raleigh, NC, USA).
**Table 3.2:** PCR Primer Set for Amplification of Canine and Murine Beta-2-Microglobulin from Genomic DNA.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine beta-2-microglobulin</td>
<td>cgB2M-3765F</td>
<td>CATTTGTCTTTCCCTCATGTCC</td>
</tr>
<tr>
<td></td>
<td>cgB2M-3927R</td>
<td>GCTTTTCATCTCCTTTCCGTTC</td>
</tr>
<tr>
<td>Murine beta-2-microglobulin</td>
<td>mgB2M-4807F</td>
<td>GCCCAGACAAGCAGTTACCA</td>
</tr>
<tr>
<td></td>
<td>mgB2M-4950R</td>
<td>GGAAGGGAGGGAGAGAGAGGA</td>
</tr>
</tbody>
</table>
**Cardiac Cells:** EBs were formed in hanging-drop cultures in complete DMEM/F12/FBS with or without 100 ng/ml hCripto-1 (R&D Systems, Minneapolis, USA). At day 4, EBs were plated onto tissue culture plates and maintained for up to 30 days. Cultures were assessed by RT-PCR for expression of canine Nkx2.5, MLC-1, GATA4, GATA6 and GAPDH. Primers are listed in Table 3.1.

**Hematopoietic Cells:** ESCs were plated on OP9 cells (Cho et al., 1999) in medium supplemented with 50% conditioned medium from canine long-term bone marrow cultures (Meertens et al., 2002), 10 ng/ml each of hFlt3-L and cSCF (R&D Systems). Differentiation to hematopoietic cells was confirmed by flow cytometry of cells stained with the canine specific CD45-FITC (MCA1042F; Serotec).

**Neurons and Glial Cells:** ESCs were plated onto nontissue culture grade plates in DMEM/F12 without hLIF and supplemented with 15% FBS and 1 µM all-trans retinoic acid (RA) (Sigma). Four days after plating, aggregates were replated on tissue culture slides in medium lacking RA. Neuronal cell and astrocyte differentiation were confirmed by morphological appearance and immunohistochemistry with anti-human NCAM (Chemicon) and GFAP (Sigma).

**Endoderm:** CESC or day 2-4 EBs were plated onto tissue culture plates in DMEM/F12 supplemented with 15% KO SR without hLIF. The cultures were maintained for up to 30 days and assessed for the expression of canine alpha-fetoprotein (AFP) by RT-PCR with primers listed in Table 3.1.
3.5. Results

3.5.1. Embryo Collection and In Vitro Maturation

Progression of canine pregnancies is measured from the time of the preovulatory LH surge (day 0) until parturition at day 63-65. Ovulation of multiple immature germinal vesicle stage oocytes occurs two days after the LH surge with oocytes completing meiosis during their tubal transport. Unlike most other mammalian species, canine oocytes remain viable within the oviduct for four to five days. Furthermore, spermatozoa typically survive four to six days after mating. Following fertilization of oocytes within the oviducts, morulae to early blastocyst stage embryos enter the lumen of the uterine horns between days seven to 14 and are evenly distributed via transuterine migration. Loss of the zona pellucidae and embryo implantation into the endometrium occurs between days 16-22 (Concannon et al., 2001; Reynaud et al., 2006; Pretzer, 2008). Based on the above information, we initiated experiments to identify the optimum time after the LH surge to harvest blastocyst stage embryos. Ten to 16 days after the LH surge or six to 12 days after artificial insemination, bitches underwent routine surgical ovariohysterectomy and embryos were recovered by flushing the uterine horns. One hundred and twenty-two embryos were harvested from 16 bitches, each yielding four to 14 embryos with a mean of eight. Harvested embryos ranged from 16-cell stage to hatched embryos (Figure 3.1 A-G). To identify in vitro culture conditions that supported canine embryos, day 14 embryos were plated on fresh MEFs in DMEM/F12 or KO DMEM medium supplemented with hLIF, bFGF and either 15% FBS or 15% KO SR. Cultures were assessed for their ability to support embryo attachment to feeders, in vitro maturation and hatching (Figure 3.2 A-E, I). Embryos cultured in DMEM/F12
Figure 3.1. Morphology of canine embryos and cESC outgrowths.
Figure 3.1. Morphology of canine embryos and cESC outgrowths. Morulae-stage embryos harvested on day-12 (A) and blastocyst-stage embryos harvested on day-14 (B) after the LH surge. (C) Early stage morula harvested on day-12 post LH surge. (D) In vivo hatched embryo harvested on day-15 post LH surge showing empty zona pellucida. (E, F, G) Freshly harvested embryos initially collapsed when placed in culture (E) but rapidly regained their typical morphology after 2-4 h in culture (F, G). (H, I) After hatching, embryos were left unmanipulated for 5-9 days during which time robust proliferation of ICM cells occurred resulting in the expansion of ICM outgrowths as seen over a 24 h period. (J) Outgrowths were mechanically cut and replated onto fresh MEF feeder layers. (K, L) Small groups of cells gave rise to many phenotypically distinct colonies. Scale bar, 100 μm.
Figure 3.2. *In vitro* maturation of canine embryos and generation of cESC lines SK-1 and K-6. (A-D) Phase contrast images showing developmental stages of canine embryos collected 12 days post LH surge and cultured for 6 days on MEFs. (A) Morula stage embryo. (B) Blastocyst stage embryo. (C) Expanded blastocyst-stage embryo. (D) Hatching embryo. ICM outgrowth eight days after mechanical cutting (E) of the zona pellucida and seven days after spontaneous hatching (I). Outgrowths were mechanically cut and transferred to a new MEF feeder layer (F). Continued mechanical passaging resulted in colonies with both 3-D (G) and flattened (G, H) morphologies. cESCs after adaptation to dispase dissociation maintain colonies with either a 3-D (J) or flattened (K, L) morphology. Scale bar, 100 μm.
supplemented with FBS attached to MEFs and developed to expanded blastocysts that hatched spontaneously. KO DMEM media supplemented with FBS also supported in vitro maturation of embryos to the expanded blastocyst stage, but fewer embryos hatched. Supplementation of either medium with KO SR limited embryo attachment, but addition of 20% FBS during initial overnight culture facilitated embryo attachment after which time blastocyst expansion and hatching occurred in KO SR supplemented medium. All subsequent embryos were cultured in DMEM/F12 media supplemented with either FBS or KO SR.

To identify the optimal time for embryo harvest, 25 embryos, were classified based on the day of LH surge and developmental stage at the time of collection (Table 3.3). Eleven days after the LH surge, embryos were at the 16-cell stage of development (Figure 3.1 C) whereas embryos harvested 12 days after the surge were primarily at the compact morulae or early blastocyst stages (Figure 3.1 A). Embryos harvested 14 or 15 days post LH surge were expanded blastocysts or hatching embryos, respectively (Figure 3.1 B, D-G). Morula and blastocyst stage embryos attached to MEFs within 24 to 48 h of plating (Figure 3.2 A-B). This was followed by significant embryo expansion and increased numbers of cells of the ICM (Figure 3.2 C, D). Blastocysts continued their in vitro maturation to expanded blastocysts with many undergoing spontaneous hatching six to eight days after plating (Figure 3.2 I). The zona pellucidae of expanded blastocysts that did not spontaneously hatch were mechanically cut to facilitate release and outgrowth of the ICM (Figure 3.2 E). Within three to five days of hatching, large outgrowths of embryo-derived ICM were detected in both KO SR (Figure 3.2 E) and FBS (Figure 3.2 I) supplemented cultures. Hatching blastocysts or 16-cell stage embryos did not produce...
Table 3.3: Optimal Stage of Development for In Vitro Embryonic Expansion of Inner Cell Mass.

<table>
<thead>
<tr>
<th>Days after Leutinizing Hormone Surge</th>
<th>Embryonic Developmental Stage</th>
<th>Collected Embryos</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Attached</td>
<td>Expanded</td>
<td>Hatched</td>
</tr>
<tr>
<td>11</td>
<td>16-Cell Embryo</td>
<td>4</td>
<td>1/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>11-13</td>
<td>Morula/Early Blastocysts</td>
<td>9</td>
<td>7/9*</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>14-16</td>
<td>Hatching Blastocysts</td>
<td>8</td>
<td>3/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>

*Two embryos from this group did not attach. While in suspension, the ICM expanded and one embryo hatched spontaneously while the zona pellucida was removed mechanically from the other. Cells from these embryos did not remain viable for more than four weeks.
ICM outgrowths. These studies indicated that the optimal time of embryo harvest for maximum recovery and in vitro maturation under the culture conditions described above was between days 12 and 14 after the LH surge. Furthermore, these culture conditions facilitated the in vitro maturation of compact morulae and early stage blastocysts to expanded blastocysts with subsequent hatching.

3.5.2. Establishment of cESC Lines

Outgrowths were left unmanipulated for five to nine days after hatching (Figure 3.1 H, I) and then mechanically cut and replated onto fresh MEFs. Small groups of cells gave rise to compact colonies with an ESC-like morphology (Figure 3.1 J – L; Figure 3.2 F) consisting of cells with high nucleo-cytoplasmic ratios. After six to ten passages by mechanical cutting, cESC colonies were adapted to enzymatic passage by dissociation to small clumps with 0.125% dispase/EDTA (Figure 3.2 G, H, K, L). Exposure of embryo-derived outgrowths to 0.05-0.25% trypsin, 1% dispase or 0.1% collagenase type IV increased cell death and induced cESC differentiation. Established cell lines consisted of two phenotypically distinct cESC colonies with some colonies having distinct borders and a flattened appearance (Figure 3.2 H, K, L) whereas others were three-dimensional, round, tightly packed colonies, with distinct borders (Figure 3.2 G, J).

After two months of continuous culture with an undifferentiated phenotype, ten ESC-like lines were cryopreserved. Four lines were selected for detailed characterization. Two lines, SK-1 and SK-4, derived using FBS supplemented media, were cultured in vitro for more than 30 passages. These lines were recovered from cryopreservation for two years after their derivation, but with continued storage at -80ºC, lost viability. Lines WA-4 and K-6, derived using KO SR supplemented media, have been maintained for
more than 25 passages with an undifferentiated phenotype. These cESC lines were stored at both -80°C and -196°C and have retained viability after recovery from cryopreservation for more than one year.

3.5.3. Expression of Pluripotency Markers by cESCs

The expression of classical pluripotency markers by cESC lines SK-1, SK-4, K-6 and WA-4 was demonstrated with expression of the transcription factors OCT3/4, NANOG, and SOX2 (Figure 3.3 A-C (Line K-6) and data not shown). Immunofluorescence analysis indicated that both flattened and three-dimensional colonies expressed these proteins (Figure 3.4 A-D). SOX2 was detected in the nucleus of cells, while OCT3/4 and NANOG were localized to the nucleus of cells of flattened colonies and both the nucleus and cytoplasm of cells of three-dimensional colonies, which may be due to entrapment of excess antibody at higher cell densities. Two separate NANOG antibodies were employed and both gave the same staining pattern. RT-PCR analyses using primers specific for canine OCT3/4, NANOG, and SOX2 confirmed expression of these pluripotency markers (Figure 3.5 A, B) and indicated that although NANOG expression was downregulated after in vitro differentiation, OCT3/4 and SOX2 expression remained elevated in differentiating cESCs cultured in the absence of MEFs. CESCs, similar to ESCs derived from other species expressed alkaline phosphatase (Figure 3.3 Di, ii) (Benham et al., 1981; Andrews et al., 1984; Thomson et al., 1995, 1996; Thomson and Marshall, 1998; Park and Han, 2000; Suemori et al., 2001; Saito et al., 2002; Li et al., 2004). Furthermore, cESCs expressed the ICM specific makers, REX1 and GBX2 (Figure 3.3 E), although GBX2 expression was noted in terminally differentiated MEFs.
Figure 3.3. Expression of pluripotency markers by cESCs.
Figure 3.3. Expression of pluripotency markers by cESCs.
Figure 3.3. Expression of pluripotency markers by cESCs. Immunofluorescence analysis for expression (red fluorescence) of OCT3/4 (A), SOX2 (B), NANOG (C), SSEA-1 (F), SSEA-3 (G), SSEA-4(H), TRA-1-60 (I) and TRA-1-81 (J) by cESC colonies. Nuclei were counterstained with SYTOX Green (blue pseudo-coloured). Merged images demonstrated that expression of pluripotency markers was localized to the nucleus for OCT3/4 (A), SOX2 (B), NANOG (C) and the cell surface and matrix around individual cESCs for SSEA-3 (G), SSEA-4(H), TRA-1-60 (I) and TRA-1-81 (J). Analysis of alkaline phosphatase (D) expression by canine (i) and mouse (ii) ESC colonies. RT-PCR expression analysis of the ICM markers, GBX2 and REXI (E). Lane 1: Undifferentiated cESCs, line K-6, passage 9, Lane 2: Differentiated cESC-derived cells, line K-6, passage 12, Lane 3: Undifferentiated cESCs, line SK-1, passage 1, Lane 4: Undifferentiated cESCs, line I-2, passage 5, Lane 5: Undifferentiated cESCs, line WA-3, passage 4, Lane 6: MEFs, Lanes 7 and 8 show RT-PCR reactions in the absence of reverse transcriptase. Lane 9 shows an RT-PCR reaction in the absence of template. Lane 10, 100 bp DNA ladder. Scale bar, 50 μm.
Figure 3.4. Expression of pluripotency markers was specific to undifferentiated regions of cESC colonies.
Figure 3.4. Expression of pluripotency markers was specific to undifferentiated regions of cESC colonies. Immunofluorescence analysis for expression (green pseudo-coloured Cy5 fluorescence) of **NANOG** (A,C), **SOX2** (B) and **OCT3/4** (D) double stained for expression (Texas Red fluorescence) of **SSEA-3** (A), **SSEA-4**(B), **TRA-1-60** (C) and **TRA-1-81** (D) by cESC colonies. Nuclei were counterstained with SYTOX Green (blue pseudo-coloured). Merged images demonstrated that expression of pluripotency markers was limited to undifferentiated cells of cESC colonies. CESCs at the periphery of colonies undergoing spontaneous differentiation and MEFs were negative for expression of these markers. Scale bar, 100 μm.
Figure 3.5. Analysis of OCT3/4, SOX2 and NANOG expression in cESCs by RT-PCR. (A) Expression of OCT3/4 in cESCs of line SK-1 was maintained over multiple passages. OCT3/4 RT-PCR of cESC, line SK-1 at passage 1 (Lane 1 and 2) and passage 10 (Lane 3 and 4). Lanes 5 and 6 show OCT3/4 RT-PCR reactions in the absence of reverse transcriptase using RNA isolated from ESCs at passage 1 and passage 10, respectively. Lane 7, 100 bp DNA ladder. (B) Expression of OCT3/4, SOX2, NANOG and canine GAPDH in undifferentiated cESCs, line K-6 (Lane 1), differentiated cESCs, line K-6 (Lane 2), undifferentiated cESCs, line WA-4 (Lane 3), differentiated cESCs, line WA-4 (Lane 4), undifferentiated cESCs, line SK-I (Lane 5),
Figure 3.5. (continued) undifferentiated cESCs, line I-2 (Lane 6), EBs derived using cells from cESC line WA-4 (lane 7), and MEFs (lane 8). Lanes 9, 10, and 11 show RT-PCR reactions in the absence of reverse transcriptase. Lane 12 shows an RT-PCR reaction in the absence of template. RT-PCR-specific amplification of target genes was confirmed by DNA sequencing. Lane 13, 100 bp DNA ladder.
Since expression of the TRA-1 and the stage specific embryonic antigens (SSEA) are developmentally regulated during early embryogenesis and their expression is widely used to assess the differentiation state of ESCs (Draper et al., 2002; Henderson et al., 2002), we assessed expression of these antigens by cESC lines SK-1, SK-4, K-6 and WA-4. Similar to hESCs, cESCs expressed SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 3.3 G-J) with expression of these markers being downregulated upon ESC differentiation as shown by decreased staining of differentiating cells at the periphery of colonies (Figure 3.4 A-D). These markers were localized to the cell surface and spaces between individual cESCs as expected for detection of the high molecular weight glycolipid and proteoglycan antigens detected by these antibodies. In contrast to mESCs, cESCs expressed very low to undetectable levels of SSEA-1 (Figure 3.3 F). All staining was specific to cESC colonies as neither MEFs nor differentiating cells were stained (Figure 3.4 A-D) and secondary only controls were void of nonspecific staining (Figure 3.4 E).

3.5.4. Requirement of hLIF for Maintenance of Pluripotency

CESC, similar to mESC, required LIF for in vitro propagation in an undifferentiated state. When plated on MEFs in the absence of hLIF, cESC underwent spontaneous differentiation as indicated by changes in colony morphology and diminished OCT3/4, NANOG and SSEA4 expression (Figure 3.6 A-F). Although SOX2 expression remained high in differentiated cells, its localization shifted from nuclear to both cytoplasmic and nuclear. Since SOX2, in addition to being a pluripotency marker, is
Figure 3.6. Spontaneous differentiation of cESCs after removal of hLIF from the culture medium.
Figure 3.6. Spontaneous differentiation of cESCs after removal of hLIF from the culture medium. CESC colonies grown from the K-6 cell line in the presence (A, C, E) and absence (B, D, F) of hLIF were stained with antibodies against human SOX2, OCT3/4 and SSEA4. (A, C, E) CESC colonies cultured in the presence of LIF maintained expression of (A) SOX2 and (C) OCT3/4 as well as (E) SSEA4. In contrast, four days after removal of hLIF, (B, D, F) cESCs underwent spontaneous differentiation as indicated by the appearance of less tightly packed colonies with spindle shaped cells visible within the colonies. (B) SOX2 expression could still be detected at low levels within the nuclei of some cells, but was also highly expressed within the cytoplasm of spindle-shaped, differentiating cells present at the colony edges. (D) OCT3/4 expression was decreased and (F) SSEA4 expression was completely abrogated.
a marker of neural stem cells, detection of SOX2 in spindle-shaped cells suggested that cESCs had undergone spontaneous neuroectodermal differentiation.

### 3.5.5. Karyotype Analysis

Karyotype analysis of metaphase chromosomes prepared from lines WA-4 and K-6 at passages 10 and 15 indicated these lines were derived from male blastocysts (Figure 3.7 B, C). Chromosome analysis of metaphase spreads prepared from cESC line SK-1, at passage 6 and line SK-4 at passage 15 and 23 revealed both had normal female karyotypes (Figure 3.7 A). Although the limit of resolution precluded the unequivocal identification of normal chromosomes, no gross structural rearrangements were identified. Y-chromosome specific PCR confirmed the derivation of cESC lines from both male and female blastocysts (Figure 3.7 D).

### 3.5.6. In Vivo Differentiation and Teratoma Formation

To demonstrate the pluripotency of cESCs, colonies from cESC lines SK-1, SK-4, WA-4, and K-6 were injected under the testis capsule of NOD/SCID-β2M or NOD/SCID mice. Solid, noncystic testicular lesions, detectable as early as seven weeks after transplantation, developed in ~75% of injected testis. (Figure 3.8 A-D). Histological analyses revealed the presence of immature teratomas consisting of derivative cells of the three embryonic germ layers (Figure 3.8 E-H). Tissue types of mesodermal origin included bone (Figure 3.8 F, J), cartilage (Figure 3.8 J), connective tissue (Figure 3.8 K, blue stain), and dentine (Figure 3.8 N), while ectodermal derivatives included neural (Figure 3.8 M) and glial cells (Figure 3.8 I). In vivo differentiation of cESC-derived
Figure 3.7. Canine ESC derived from male and female embryos maintained a normal karyotype after multiple passages. Metaphase spreads (A, B) and karyogram (C) of cESC lines WA-4 (A, C) and SK-1 (B) showed normal XY (A, C) and XX (B) karyotypes consisting of 78 chromosomes. (D) Y chromosome specific PCR confirmed the XX and XY genotypes of seven cESC lines.
Figure 3.8. *In vivo* differentiation of cESCs to tissues of the three embryonic germ layers. (A-D) Teratomas derived after injection of cells from cESC lines SK-1, SK-4, WA-4 and K-6 under the testicular capsule of immunocompromised mice.
Figure 3.8. (continued) Tumours were detected at 12 weeks post-transplantation in three of four testis injected with cells from cESC line SK1, six of eight testis injected with cESC line SK4, five of six testis injected with cESC line K6, and four of six testis injected from cells from cESC line WA4. Black arrowheads point to teratomas that developed under the testis capsule. (E-H) H & E stained histological sections of SK1- (E), SK-4- (F), WA-4- (G) and K-6- (H) derived teratomas. Shown are (I) neural rosettes, (J) bone (arrow head) and connective tissue (arrow), (K) Masson-Trichrome (blue) staining confirmed the presence of connective tissue, (L) Von Kossa (dark brown) stained for the presence of bone, (M) Neuron Specific Enolase (NSE) (brown) confirmed neurons and glial cells and (N) gut-like (arrowhead) and dentine (arrow) structures. (O-T) Immunohistochemical staining of teratomas with ectodermal (O, P) endodermal (Q) and mesodermal (R) markers. (O) Immunofluorescence image of teratoma stained with anti-human nestin (red) and beta-tubulin (green) antibodies. The merged images indicated colocalized expression of the two neuron-specific markers to individual cells. Immunohistochemical staining of teratomas with antibodies specific for (P) GFAP (pink) (Q) SOX17 (red) and (R) smooth muscle actin (green). Staining of the teratoma tissue and adjacent murine testicular tissue with antibodies specific for (S) smooth muscle actin (green), and (T) MHC class I (pink). The solid white bar delineated the boundary between the cESC derived teratoma and NOD/SCID-β2 (N/S-β2) testicular tissue. Nuclei are stained with DAPI (blue). Representative special and
Figure 3.8. (continued) Immunohistochemical stains are shown for teratomas created from cESC lines SK-1 (I, M), SK-4 (J, N), WA-4 (K, L, O, Q, R), and K-6 (P, S, T).

Scale bar, 100 μm.
endoderm included gut-like structures (Figure 3.8 N). These findings were supported by staining teratoma sections with Von Kossa to confirm bone (Figure 3.8 L), with Masson’s trichrome to confirm connective tissue (Figure 3.8 K) and with NSE to confirm neurons and glial cells (Figure 3.8 M). Immunohistochemical staining using antibodies specific for neuroectodermal derivatives revealed colocalized expression of nestin and beta-tubulin (Figure 3.8 O) and expression of GFAP to elongated, spindle-shaped cells (Figure 3.8 P). To detect derivative cells of the endodermal and mesodermal germ layers, teratomas were stained with SOX17 (Figure 3.8 Q) and smooth muscle actin (Figure 3.8 R, S), respectively.

Given that immunocompromised mice are prone to the development of malignancies, studies were initiated to demonstrate that the teratomas were not of recipient origin. As all cells of NOD/SCID-β2M mice lack expression of beta-2 microglobulin (β2M), they do not express MHC class I at their cell surface and thus testicular tissues biopsied from these mice did not react with an anti-MHC-I antibody. However, teratomas reacted with the anti-MHC-I antibody demonstrating that teratomas were not of recipient origin (Figure 3.8 T). Furthermore, molecular confirmation of the presence of canine cells within the teratoma was demonstrated by PCR amplification of canine specific β2M (Figure 3.9). Murine-specific β2M was used to confirm the presence of amplifiable template. One large teratoma dissected from murine testicle tissue showed amplification of canine-specific β2M whereas canine-specific β2M was not detected in the adjacent testicle tissue (Figure 3.9, Lanes 2 and 3). Therefore, the teratomas developing in NOD/SCID-β2M mice were derived from cESCs.
**Figure 3.9.** Teratoma tissues, derived after injection of cESC under the testis capsule of NOD/SCID and NOD/SCID-β2 mice, contained canine specific DNA. (A) Canine and murine specific beta-2-microglobulin PCR of genomic DNA extracted from testis of NOD/SCID mice bearing a visible teratoma after injection with WA-4 cESCs (Lane 1-6) or K-6 cESCs (Lanes 7-10). The sample in Lane 2 was amplified from a testicle which was dissected away from the teratoma. The dissected teratoma from this testicle was amplified independently in Lane 3. Murine (Lane 11 and 12) and canine (Lane 13) bone marrow (BM) were used as negative and positive controls, respectively. A PCR reaction in the absence of template was completed as a negative control (Lane 14).
3.5.7. In Vitro Differentiation of cESCs

To further demonstrate the pluripotency of cESCs, in vitro differentiation studies were initiated. The ability of cESCs to form embryoid bodies (EBs) and undergo spontaneous differentiation was demonstrated after reaggregation of cESCs in hanging drop cultures (Figure 3.10 A, B). Differentiation, as indicated by loss of expression of NANOG, was noted although a proportion of cells retained expression of SOX2 and OCT3/4 (Figure 3.10 D-F).

The presence of differentiated cells was confirmed by immunohistochemical analysis of individual EBs stained with antibodies specific for antigens expressed on derivative cells of the three germ layers. Staining of EBs with antibodies specific for the glial cell marker, GFAP (Figure 3.10 G), the neuronal antigen, NCAM (Figure 3.10 H), and beta-III-tubulin (Figure 3.10 I) demonstrated differentiation of cESCs to ectodermal cell derivatives. Furthermore, double staining of EBs with the neural cell markers, GFAP and SOX2 (Figure 3.10 M), revealed colocalization of staining, further confirming specification of cESCs to derivative cell types of the ectodermal germ layer. ESCs also differentiated to endoderm as indicated by expression of AFP (Figure 3.10 J).

Differentiation of cESCs to derivative cell types of the mesodermal germ layer was demonstrated by expression of VEGFR2 (Figure 3.10 K) and CD201 (Figure 3.10 L). Furthermore, double staining of EBs with VEGFR2 and NCAM revealed non-overlapping expression of proteins to isolated regions within a single EB, indicating independent mesodermal and ectodermal specification (Figure 3.10 N). Limited antibody
Figure 3.10. *In vitro* differentiation of cESCs to EBs containing cells of ectodermal, endodermal and mesodermal origin.
Figure 3.10. *In vitro* differentiation of cESCs to EBs containing cells of ectodermal, endodermal and mesodermal origin. (A) Formation of EBs from line WA-4 after dissociation of cESCs into small clumps by mechanical cutting and reaggregation in hanging drop cultures. (B) Formation of EBs after dissociation of cESCs into a single cell suspension with trypsin and reaggregation in hanging drop cultures. EBs that formed in hanging drops were transferred to uncoated culture dishes where they enlarged and developed cystic formations within 7 to 14 days. (C-F) Immunohistochemical staining of cryosectioned EBs generated after reaggregation of small clumps of cESCs in hanging drop cultures, and analyzed for expression of *OCT3/4* (D), *SOX2* (E) and *NANOG* (F). (C) Rabbit secondary antibody-only staining control. (G-O) Immunohistochemical staining of cryosectioned EBs formed after reaggregation of single cell suspensions of cESCs in hanging drop cultures and stained with the ectodermal markers, GFAP (G), NCAM (H) and β-III-tubulin (I), the endodermal marker, AFP (J) and the mesodermal markers VEGFR2 (K) and CD201 (L). (M) Colocalization of staining (yellow) of the ectodermal markers GFAP (green) and the early neuroectodermal marker SOX2 (red) to the cystic body of an EB. (N) Double staining of an EB with the ectodermal marker, NCAM (green) and the mesodermal marker, VEGFR2 (red). (O) Rabbit and mouse secondary antibody-only staining control. Scale bar, 100 μm.
availability precluded triple staining to demonstrate the presence of derivative cell types of the three embryonic germ layers within individual EBs.

Studies were also initiated to demonstrate directed in vitro differentiation of cESCs to multiple cell types. Plating of EBs or small clumps of ESCs on Collagen IV coated plates resulted in ESC differentiation to endothelial cells as confirmed by the expression of PECAM-1 (CD31) (Figure 3.11 A). Within two weeks of culture, endothelial cells formed vascular-like structures while maintaining PECAM-1 expression (Figure 3.11 B, C). EBs cultured in ESC medium in the absence of LIF, initiated cardiomyocyte differentiation, which could be enhanced by supplementation of cultures with hCripto-1, as indicated by elevated MLC-1 and GATA4 expression (Figure 3.11 D). Cripto is a growth factor that acts early to determine the cardiac fate of differentiating ESCs and prevents differentiation to a neural fate (Xu et al., 1998; Parisi et al., 2003).

Differentiation of cESCs to hematopoietic cells was observed after coculture on a feeder layer of OP9 cells in medium supplemented with hFlt3-L, cSCF and canine long-term bone marrow culture conditioned media. Under these conditions, CESC developed large mesoderm-like colonies consisting of round hematopoietic-like cells (Figure 3.11 E). Flow cytometric analysis of an isolated population of cells based on forward and side scatter profiles indicated that approximately 25% of the in vitro differentiated cells expressed the canine hematopoietic specific antigen CD45. OP9 cells did not react with the canine specific CD45 antibody (Figure 3.12 F-I).

Directed differentiation of cESCs to neurons and glial cells was initiated by the formation of EBs in the presence of retinoic acid, as this compound has been shown to initiate neuronal differentiation from embryonal carcinoma cells
Figure 3.11. *In vitro* differentiation of cESCs to mesoderm, ectoderm and endoderm.
Figure 3.11. In vitro differentiation of cESCs to mesoderm, ectoderm and endoderm. (A-C) In vitro differentiation of cESCs to mesodermal derivative cell types. In vitro differentiation of EBs to endothelial cells after culture on Collagen IV coated tissue culture plates. Immunofluorescence microscopy of (A) an endothelial cell colony and (B) vascular-like formations stained directly with an anti-CD31-PE specific antibody. (C) Phase contrast image of vascular-like formations. (D) In vitro differentiation of cESCs (passage 17) to cardiac cells. RT-PCR analysis for expression of cardiac markers, NKX2.5, Myosin light chain-1 (MLC-1), GATA4 and GATA6. GAPDH served as a positive control for the presence of amplifiable template. Lane 1: EBs differentiated for 22 days in complete ESC media in the presence of hCripto-1, Lane 2: EBs differentiated for 30 days in the absence of Cripto-1, Lane 3: canine neonatal heart. (E-I) In vitro differentiation of cESCs (passage 15) to hematopoietic cells after coculture on an OP9 feeder layer. (E) Phase contrast microscopy of OP9 stromal cells cocultured for ten days with cESCs. (F) Flow cytometric analysis of unstained OP9 cells, (G) OP9 cells stained with the canine specific CD45 antibody, (H) OP9/cESC co-culture stained with isotype control and (I) OP9/cESC co-culture stained with the canine specific hematopoietic antigen, CD45. (J-Q) In vitro differentiation of cESCs to neuronal cells derived after culture of cells in media supplemented with retinoic acid. Phase contrast (J, L, N, P) and immunofluorescence (K, M, O, Q) images of in vitro differentiated cells expressing the neuron specific
Figure 3.11. (continued) marker NCAM (K) and the glial cell specific marker GFAP (M, O, Q). (R) In vitro differentiation of cESCs to cells of endoderm. RT-PCR analysis of expression of AFP and GAPDH in undifferentiated and differentiated ESCs. Lane 1: undifferentiated cESCs at P11 (no reverse transcriptase), Lane 2: undifferentiated cESCs at P11, Lane 3: Day-14 EBs, Lane 4: ESCs differentiated for 9 days in the absence of MEFs, Lane 5: Day 30 EBs, Lane 6: neonatal liver (no reverse transcriptase), Lane 7: neonatal liver and Lane 8: 100 bp ladder. Arrow indicates the expected 217 bp AFP amplified fragment. Scale bar, 100 μm.
After transfer of EBs to dishes in the absence of retinoic acid, large numbers of neurons expressing NCAM and glial cells expressing GFAP were observed (Figure 3.11 J-Q). To demonstrate the potential of cESCs to differentiate to endodermal cell types, we assessed expression of the endodermal marker, AFP. AFP is secreted by cells of the extra-embryonic yolk sac, fetal liver and intestines. AFP expression, as revealed by RT-PCR analysis, of cESCs induced to undergo spontaneous differentiation or day-4 EBs plated in medium supplemented with KO SR confirmed the presence of endodermal derivative cell types (Figure 3.11 R).
3.6. Discussion

The cESC lines reported herein have similar properties to ESC lines derived from other species including their ability to proliferate in vitro for multiple passages in an undifferentiated state and to give rise to derivative cell types of the three embryonic germ layers. Furthermore, cESCs, like their counterparts in other species expressed the pluripotency markers OCT3/4, SOX2, NANOG and alkaline phosphatase. CESCs expressed REX1 and GBX2, genes whose expression is found in cells of the ICM of the developing blastocyst (Pelton et al., 2002). Since expression of REX1 and GBX2 is downregulated in epiblast-stage embryos, these findings indicated that although ex vivo culture facilitated maturation of canine morulae and blastocysts, canine ICM cells did not progress to the epiblast stage. The morphology of cESC colonies was heterogeneous in that some colonies had a 3-D appearance more typical of mESCs while others had a flattened morphology more typical of hESCs. Similar to hESCs, cESCs expressed SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and did not express SSEA-1. However, cESC were similar to mESCs in that they were dependent on hLIF for maintenance of the undifferentiated state. Our finding that cESCs responded to human LIF is supported by studies reporting that Madin-Darby canine kidney cells expressed the LIF receptor and responded to hLIF by activation of JAK/STAT pathway (Buk et al., 2004).

Of particular importance, cESCs gave rise, in vivo, to teratomas after injection under the testis capsule of immunodeficient mice and, in vitro, to a number of different cell types including neuronal, hematopoietic, cardiac and endothelial cells when placed in a differentiating environment. Based on their ability to form teratomas and their phenotypic similarity to hESCs, the cESC lines reported herein represent the first bona
fida cESC lines and offer the exciting possibility of developing and assessing ESCs-based therapies in large animal models of human diseases.

Prior to the publication of our work, there were three published reports of the development of cESC lines (Hatoya et al., 2006; Schneider et al., 2007; Hayes et al., 2008). In the first two reports (Hatoya et al., 2006; Schneider et al., 2007), the cells described expressed alkaline phosphatase, OCT3/4 and, similar to mESCs, SSEA-1. The cells formed EBs in suspension culture that could be induced to differentiate to adherent cells with different morphologies (Hatoya et al., 2006) and to hematopoietic cells (Schneider et al., 2007). However, these cells showed limited self renewing potential with lines being lost after eight to ten passages in culture. In a third report (Hayes et al., 2008), the cESC lines described were phenotypically most similar to mink ESCs. These cells expressed alkaline phosphatase, telomerase, OCT3/4 and NANOG but lacked expression of the SSEA and TRA-1 antigens. The cells possessed a normal karyotype after multiple passages, showed the potential to differentiate in vitro to cells of the three germ layers but were unable to form teratomas after injection into immunocompromised mice.

Since the publication of our paper, a fifth manuscript describing the derivation of an additional 39 canine ESC-like cell lines was published (Wilcox et al., 2009). These lines were established using two different methodologies involving either immunodissection of the zona pellucidae or direct explanting of unmanipulated embryos. The lines derived using with the immunodissection method were more similar to those derived by Hayes et al. (Hayes et al., 2008) but were unique in that only a central “button” of cells within each colony displayed ESC characteristics. Cells surrounding the central button appeared, phenotypically, to resemble hypoblast cells (cHFs). The cells of
the central button of ESCs were incapable of independent growth and were lost when passed in the absence of cHFs. By contrast, the direct explant method produced cESC lines that could be mechanically passaged as small clumps for multiple passages and, similar to the lines derived by our group, expressed OCT3/4, NANOG, SOX2, SSEA3 and SSEA4 and lacked expression of SSEA1. Furthermore, the direct explant lines derived by Wilcox et al. could be induced to differentiate in vitro to embryoid bodies comprised of cells derived from the three embryonic germ layers. Intratesticular injection of these cells in NOD/SCID-β2-microglobulin−/− mice resulted in the development of small teratoma-like growths consisting of poorly differentiated cells (Wilcox et al., 2009).

A comparison of the methods employed for the derivation of cESCs revealed that the resulting cell morphologies are dramatically affected by both the removal of the zona pellucida prior to plating the embryos and ex vivo maturation of intact blastocysts. The cESC lines established by mechanical dissection of the zona pellucidae consisted of flat colonies with large, highly granular cells (Hatoya et al., 2006; Hayes et al., 2008), while the immunodissected lines established by Wilcox et al. had a central button of ESC-like cells surrounded by cHFs with endodermal characteristics (Wilcox et al., 2009). By contrast, the direct explant method employed by our group and Wilcox et al. resulted in tightly packed colonies of very small cells with a high nucleocytoplasmic ratio and low granularity. These cells displayed a phenotype very similar to mouse, nonhuman primate and human ESCs.

Immuno- or mechanical dissection of the zona pellucida results in the destruction of the fluid-filled blastocoel, containing many factors that support the development of both the ICM and trophectoderm cells (Dardik and Schultz, 1991a, b; Dardik et al.,
Thus, proteins such as OCT3/4, NANOG and the Kruppel-like factors (Ema et al., 2008; Nandan and Yang, 2009; Silva et al., 2009) that support pluripotency and suppress differentiation were kept in close proximity to the ICM cells when unmanipulated canine embryos were directly explanted and allowed to develop ex vivo. Studies to develop ESC lines from mouse strains which have proved recalcitrant to ESC derivation, such as C57BL/6 and CBA, have shown that epiblast expansion during the primary explant phase of ESC derivation, is essential for efficient establishment of mESC lines (Batlle-Morera et al., 2008). During the several days of ex vivo culture of canine embryos we have observed dramatic expansion of the ICM cells which may directly relate to the ability to create cESC lines with classical mouse and human ESC phenotypes. Thus the cell lines developed by the direct explant method may gain intrinsic support from growth factors expressed within the blastocyst until the cytokine supplements of hLIF and hbFGF as well as the growth factors expressed by MEFs can function to maintain the ICM-derived cESCs in an undifferentiated, self-renewing state.

While ESCs have demonstrated exciting potential for their projected use in the treatment of human diseases, difficulties and limitations must be overcome before establishing their use in humans. For example, although the NOD/SCID xenotransplantation model has been invaluable for assessment of the engraftment potential of hESC-derived cells, much of the knowledge gained in these studies is not directly translatable to humans. Furthermore, a defining characteristic of ESCs is their ability to give rise to teratomas and thus grafts contaminated with undifferentiated ESCs may produce teratomas in vivo (Laflamme and Murry, 2005). Due to the short lifespan of mice, longitudinal studies to assess teratoma formation and other safety issues are
limited. Furthermore, since microenvironment affects donor cell engraftment and teratoma development, these models may not accurately predict the safety and therapeutic outcomes of hESC-derived cells in human clinical trials. It also remains to be determined whether it is possible to obtain sufficiently high yields of ESC-derived cells to ameliorate diseases treatable by transplantation.

Nonhuman primate ESC lines have been generated (Thomson et al., 1995, 1996; Suemori et al., 2001) and several groups have used these cells to develop ESC-based therapies. Although these studies will undoubtedly provide powerful data, they are limited by the absence of naturally occurring nonhuman primate disease models for the assessment of therapeutic benefits. Therefore ESC lines derived from large animals that not only closely approximate humans in size and genetic background heterogeneity but also accurately recapitulate human diseases are required to demonstrate that the therapeutic benefits reported in rodents can be safely achieved in large animals.

The dog has historically been a powerful model used for the development of transplantation therapies (Shull et al., 1996; Storb et al., 1997; Georges and Storb, 2003; Kiem et al., 2004; Bauer et al., 2006; Sampaolesi et al., 2006; Malech and Hickstein, 2007) and cell tracking and imaging methodologies (Kraitchman et al., 1998; Croisille et al., 1999; Yung et al., 2003; Gallegos et al., 2004; Cheng et al., 2008; McCommis et al., 2008). Compared with mice, the large size of dogs is amenable to serial blood and marrow sampling, continuous intravenous infusions and scaling up therapies. Moreover, dosing and conditioning regimes as well as technical expertise developed in dogs are often directly translatable to the human clinical setting (Bauer et al., 2006; Graves et al., 2007; Parker et al., 2008; Sorror et al., 2008; Bauer et al., 2009). Work merging the use
of canine models and stem cell therapies has begun to show promise as a predictive approach for the evaluation of novel therapies as exemplified by the increased risk of leukemia revealed in long-term follow-up studies in dogs treated with HOXB4 gene modified cells (Sauvageau et al., 1995; Antonchuk et al., 2002; Zhang et al., 2007, 2008). In addition, over 360 genetic disease models have been described in dogs with more than 50% being true orthologues of their human genetic counterparts (Sargan, 2004). This availability of disease models that accurately recapitulate the disease phenotype in humans makes the dog a powerful model to assess the therapeutic benefits of novel treatment modalities. The completion of the sequencing of the canine genome (Lindblad-Toh et al., 2005) and the availability of canine BAC clone libraries (Li et al., 1999), genome-wide CGH microarrays (Thomas et al., 2005) and sets of whole chromosome-specific FISH painting probes (Breen et al., 2001)(Thomas et al., 2003) greatly facilitate experimental research in dogs (Shearin and Ostrander, 2010). There is also a large spectrum of commercially available canine tissue-specific antibodies and cytokines in addition to human and veterinarian reagents that function equally well in dogs. Thus, the established record of the canine model suggests that it will be instrumental in the translation of ESC-based therapies for tissue repair in humans.
3.7. References


CHAPTER 4

Migration of Cells from the Yolk Sac to Hematopoietic Tissues
after *In Utero* Transplantation in Early and Mid Gestation

Canine Fetuses
4.1 Attribution of Data

I performed all the work described in this chapter with the following exceptions: all live-animal procedures were completed at the Ontario Veterinary College, University of Guelph with assistance from the following veterinarians: canine bone marrow harvests were performed by Dr. Stephen Kruth and Dr. Marcus Litman; theriogenological procedures were performed by Dr. Cathy Gartley; surgical procedures were performed by Dr. Krista Halling; ultrasonography was performed by Dr. Howard Dobson; assistance in design of RT-PCR primers was by Dr. Yanzhen Zheng at Sunnybrook Research Institute.
4.2 Abstract

_In utero_ stem cell transplantation (IUSCT) offers a means of early intervention by cell therapy for the treatment of diseases prior to birth. Delivery of cells to the yolk sac is a noninvasive approach, which does not involve manipulation of developing fetal organs. Previous studies have resulted in low levels of engraftment at yolk sac injection, which drew into question the ability of cells transplanted by this approach to migrate into the fetus. To address this question, mesenchymal stromal cells (MSCs) or bone marrow mononuclear cells (BMMCs) labeled with superparamagnetic iron oxide particles (SPIO) and fluorescent markers were transplanted to the yolk sacs of day 25 or day 35 fetuses under ultrasound guidance. _Ex vivo_ whole body fluorescence imaging to assess levels of green (SPIO) and red (CMTMR) fluorescence in injected and noninjected control fetuses sacrificed at day 32 and 42 verified SPIO-labeled cell migration from the yolk sac injection site to fetal tissues. The signal was predominantly localized to the thoracic and abdominal regions, with no apparent fluorescence visible in the yolk sac. Histological analysis of fetal tissues by fluorescence microscopy to detect fluorophore co-localization with iron-reactive Prussian blue staining demonstrated that labeled cells migrated from the yolk sac to engraft predominantly in the fetal liver of recipients receiving either MSC or BMMC. Our studies demonstrated that injection of cells into the yolk sac during early fetal development is an effective approach for _in utero_ delivery of cells to fetal tissues.
4.3 Introduction

*In utero* stem cell transplantation (IUSCT) of fetal recipients is a therapeutic approach for the treatment of many congenital deficiencies. Diseases that can be corrected with postnatal bone marrow transplantation may be more effectively treated *in utero*. By taking advantage of the pre-immune status of the developing fetus allogeneic cells can be transplanted without immune suppression (Ashizuka *et al.*, 2006; Durkin *et al.*, 2008). The small size of the fetus may also allow for greater donor to host cell ratios to be achieved resulting in higher levels of mixed chimerism. In addition, for many severe congenital disorders, organ damage may occur in the fetus or newborn before postnatal bone marrow transplantation can be performed, making *in utero* treatment a necessity for preemptive therapy.

Although, IUSCT has been attempted in many species, including humans (Touraine *et al.*, 2004; Le Blanc *et al.*, 2005), non-human primates (Shields *et al.*, 2003)(Zanjani *et al.*, 1991), dogs (Lutzko *et al.*, 1999, 1999; Omori *et al.*, 1999; Lutzko *et al.*, 2002; Peranteau *et al.*, 2009), sheep (Zanjani *et al.*, 1992, 1997; Schoeberlein *et al.*, 2004; Ersek *et al.*, 2010), pigs (Fujiki *et al.*, 2003; Lee *et al.*, 2005) and mice (Chen *et al.*, 2009; Panaroni *et al.*, 2009), the engraftment levels achieved have been very low (< 1%). In most studies, cells were delivered to the intraperitoneal cavity, while in a few cases cells were injected into the extraembryonic yolk sac (Lutzko *et al.*, 1999, 1999; Omori *et al.*, 1999; Lutzko *et al.*, 2002). Yolk sac delivery is a preferred site for injection as this tissue is accessory to the developing fetus and can be more easily manipulated without harming the fetus. In addition, cells transplanted to the yolk sac may have access to fetal organs directly via the yolk stalk connecting the yolk sac to the fetal gut and associated organs.
such as the fetal liver, or by entering the vitelline circulation of the highly vascularized yolk sac for delivery to the fetal circulation via the vitelline veins which contribute to the sinusoids of the fetal liver. Despite the potential advantages of utilizing yolk sac delivery for cell transplantation, studies have not addressed whether cells transplanted into the yolk sac migrate from this site into the fetus and engraft recipient tissues.

In utero transplantation may also prove to be an effective strategy for the induction of immune tolerance to allogeneic cells for patients requiring postnatal cell therapy. Hematologic disorders, such as many of the lysosomal storage disorders, can be treated with stem cell transplantation, but this therapeutic option may be limited if a matched donor is not available. Furthermore, in order for stem cell therapies to be effective, a sufficient number of donor cells must engraft and persist. Use of conditioning regimes, including chemical and radiation therapy to ablate host hematopoietic cells and create space for donor cell engraftment have met with success, though continued immune suppression to prevent donor cell rejection may be required (Sorror et al., 2008). In utero transplantation could overcome this limitation by immunotolerizing the fetus to donor cells before the adult immune system is fully developed. Once immune tolerance has been achieved, low, non-therapeutic levels of engraftment could be enhanced after birth with same-donor stem cell transplantation (Peranteau et al., 2002; Ashizuka et al., 2006; Peranteau et al., 2009).

In practice, IUSCT has seen limited therapeutic success due to low levels of chimerism. The optimal gestational age of recipients, as well as the optimal cell type, dose and delivery site have yet to be determined. Our group has used the canine model to assess the utility of in utero transplantation for the treatment of Mucopolysaccharidosis
type I (MPSI). Previous studies have shown that injection of cells into the yolk sac of day 32-38 fetuses is safe although the levels of donor cell engraftment were too low to ameliorate disease symptoms (Lutzko et al., 1999). The aim of this study was to determine whether adult BMMCs or MSCs injected into the yolk sacs of early gestation, day-25 or -35 fetuses migrated from their site of injection into the fetus for engraftment in recipient tissues.
4.4 Materials and Methods

4.4.1 Animal husbandry

Mixed bred dogs were obtained from the Central Animal Facility at the University of Guelph and were maintained at the Ontario Veterinary College teaching hospital until they recovered from all procedures. The leutinizing hormone (LH) levels of bitches in estrous were monitored daily in order to accurately stage each pregnancy. Transcervical artificial insemination was conducted on 3 consecutive days following the LH surge. All animal use protocols were approved by committees at the University of Guelph and Sunnybrook Research Institute.

4.4.2 Canine bone marrow harvest and processing of bone marrow mononuclear cells (BMMCs)

Bone marrow was harvested using established procedures as previously described (Carter et al., 1992). One hundred to four hundred milliliters (ml) of adult canine bone marrow was aspirated from the proximal humeral bones in preservative-free heparin and shipped to Sunnybrook Research Institute on wet ice for processing within 6-12 hours of collection. To obtain BMMCs, bone marrow was diluted 1:1 with Hank’s buffered saline solution (HBSS; Wisent, St. Bruno, QC, Canada) and layered over Ficoll Paque Plus (GE Healthcare, Baie d’Urfe QC, Canada). Gradients were centrifuged at 1400 rpm at room temperature for 30 minutes with the brake off. The mononuclear layer was aspirated and washed with twice with HBSS. Red blood cell lysis was performed by adding an equal volume of ACK lysis buffer and incubating for 5 minutes at room temperature (150 mM...
NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA (Sigma, Oakville, ON, Canada) in HBSS). The BMMCs were cryopreserved in fetal bovine serum (FBS; Invitrogen, Mississauga, ON, Canada) containing 10% DMSO (Sigma, Oakville, ON, Canada) at –80°C until required.

4.4.3 Mesenchymal Stromal Cell (MSC) Cultivation and Characterization

Frozen stocks of BMMCs were washed to remove DMSO and 2.5 x 10⁴ cells/cm² were plated in 100 mm tissue culture dishes in 10 ml of complete medium (10% FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate and 2 mM Glutamax (Invitrogen) in low glucose αMEM (Wisent)). Cells were incubated at 37°C in a humidified 5% CO₂ environment. On day 4 of culture, the nonadherent cells were removed and fresh culture medium added. The cultures were fed every four days and passaged between days 10-13 by releasing cells with 0.025% trypsin (Invitrogen) exposure for 5 min. Cells were replated at 2 x 10³ cells/cm² for all subsequent passages. At passages 0 through 6, floating and adherent cells were assessed by flow cytometry using canine-specific CD11b, CD14, CD44 and CD45-biotin antibodies (AbD Serotec, Raleigh, NC, USA). Primary antibodies were diluted 1/100 in HBSS + 2% FBS and MSCS (1 x 10⁷ cells/ml) were stained for 20 minutes. Secondary antibodies used were anti-mouse IgG1-FITC (1/500 dilution, Sigma) and anti-rat IgG2a-FITC (1/500 dilution, Sigma) or Streptavidin-FITC (1/400 dilution, Jackson ImmunoResearch, West Grove PA, USA). Flow cytometry was performed on a FACSCalibur with CellQuest data acquisition software (BD Biosciences, Mississauga, ON, Canada). Data analyses were completed with FlowJo software (Treestar, Ashland, OR, USA).
4.4.4 BMMC and MSC labeling

Dragon Green™-labeled small, paramagnetic, iron oxide particles (SPIO; 10 μl/ml; Bang’s Laboratories, Fishers, IN, USA) were incorporated into BMMCs or MSCs (passage 3-6) by incubating cells in complete medium at 37°C, 5% CO2 humidity for 16 hours. For the final 30 minutes of SPIO labeling, double labeling with 5 μM Cell Tracker Orange (CMTMR, Molecular Probes, Invitrogen) was completed. SPIO and CMTMR-labeled BMMCs were washed two times with HBSS and resuspended at 1 x 10^8 cells/ml in unsupplemented IMEM (Invitrogen). Labeled MSC cultures were washed three times with HBSS and adherent cells were harvested by exposure to 0.025% trypsin for 5 min. MSCs were resuspended at 1 x 10^7 cells/ml in unsupplemented IMEM prior to injections.

4.4.5 In utero transplantation of labeled BMMC and MSC

Prior to transplantation, pregnant females were examined by transabdominal ultrasonography to confirm pregnancy and determine the number of fetuses present. On the day of transplantation, dogs were given butorphanol tartrate (Ayerst Laboratories, Saint-Laurent, PQ) 0.05 mg/kg intramuscularly, and anesthesia was induced with propofol (Mallinc-krodt Veterinary Inc., Ajax, ON) 4–6 mg/kg intravenously. Dogs were intubated and anesthesia was maintained with isoflurane (Ohmeda Pharmaceutical Products, Mississauga, ON). Ventral mid-line laparotomies were performed on gestational day 25 or 35 pregnant bitches to externalize the gravid uterus. The uterus was packed with saline-soaked laparotomy pads and periodically irrigated with sterile saline during the procedure.
An Ultramark 8 Ultrasound System (Advanced Technology Laboratories Canada Inc., Markham, ON) with a 7.5-MHz transducer was used to image the fetuses. The transducer cord was placed in a sterile cord cover and the transducer was placed in a sterile latex sheath (Ultracover, Parker Laboratories Inc., Orange, NJ). Sterile lubricant (Muko Lubricating Jelly, Ingram and Bell Medical, Don Mills, ON) was used for the interface. A dedicated instrument guide (Advanced Technology Laboratories Canada Inc.) and corresponding electronic markers on the image monitor were used to guide insertion of a 26-gauge, 3.5-inch spinal needle (Becton Dickinson Canada Inc., Mississauga, ON) into the yolk sac of each fetus. The stylet was removed and cells were injected into the yolk sac. Visualization of an air bubble on the sonograph confirmed appropriate targeting.

A total of $1 \times 10^6$ MSCs or $1-2.5 \times 10^7$ BMMCs were delivered to yolk sacs in a maximum volume of 100 µl IMEM. Based on the size of the fetus, cell injections ranged from $4 \times 10^8 - 1.8 \times 10^{10}$ MSCs/kg and $5.2 \times 10^8 - 2.2 \times 10^{11}$ BMMCs/kg fetal weight. At least one fetus of every pregnancy served as a mock-injected control, receiving 100 µl of IMEM alone. Following injection the uterus was returned to the abdomen and the incision sutured. Anesthesia time from induction to recovery was approximately two hours.

### 4.4.6 Spay and fetal retrieval

Seven or seventeen days post injection, pregnant bitches underwent routine spay to allow for fetal retrieval. Fetuses were removed from individual uterine sacs to allow for collection of fetuses with associated yolk sac structures. Fetuses were stored in $4\%$...
paraformaldehyde (Sigma) for up to 24 hours to preserve the tissues during imaging procedures and prior to dissection and histological tissue preparation.

4.4.7 *Ex vivo* whole body fluorescence imaging

Fetuses and fetal organs were imaged on a flat panel fluorescence imager (Kodak Imaging Station 2000MM) with FITC filters (excitation 485 nm; emission 535 nm). Images were obtained using KODAK 1D Image Analysis software (Eastman Kodak Company, Rochester, NY, USA).

4.4.8 Preparation of samples for histological analysis

Day 32 fetuses or day 42 fetal organs were fixed in 4% paraformaldehyde for 12-24 hours at 4°C, then processed in 30% sucrose for 24 hours at 4°C prior to embedding in Tissue-Tek OCT freezing compound (Sakura Finetek, Torrance, CA, USA). Tissues were placed in OCT in vinyl molds (VWR, Mississauga, ON, Canada) and flash frozen by floating in a bath of dry ice and ethanol (Sigma). OCT-embedded tissue biopsies or whole fetuses were cryosectioned at 4-10 um with a Leica CM3050 Cryotome (Leica Microsystems, Richmond Hill, ON, Canada) onto Superfrost Plus slides (Fisher Scientific Company, Ottawa, ON, Canada) and cryopreserved at -80°C to allow for future staining. Sections were stained with Prussian blue (10% HCl, 5% potassium ferrocyanide; Sigma) for 20 min followed by counterstaining with Nuclear Fast Red (Sigma) and mounting in Entellin (EMD Chemicals, Gibbstown, NJ, USA). To detect the presence of macrophage, selected sections were stained with anti-human MAC387 antibody (1/100 dilution; CBL-260, Chemicon, Temecula, CA, USA) overnight at 4°C, followed by staining with an anti
x-Cy5 antibody (1/500 dilution; Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour. All sections for fluorescence microscopy were stained with 0.1 µg/ml DAPI (Sigma) for 15 min followed by mounting with Molecular Probes anti-fade mountant (Invitrogen).

### 4.4.9 Microscopy

Light microscopy was conducted on a Leica DMLS microscope fitted with a DFC 300 camera and Fire Cam 1.2.0 imaging software (Leica). Epi-fluorescence microscopy was conducted on an Axiovert 200M microscope (Carl Zeiss Canada, Toronto, ON, Canada) fitted with an Orca ER camera (Hamamatsu Corporation, Bridgewater, NJ, USA) and AxioVision 4.1 imaging software (Carl Zeiss Canada).

### 4.4.10 Canine Y chromosome analysis

Several OCT sections were collected and DNA extracted with DNAzol (Invitrogen). DNA extracted from female fetuses (day 32) or fetal tissues (day 42) was used as a template (500 ng) for a canine Y chromosome specific PCR. Mixtures of male and female DNA extracted from canine bone marrow were amplified to determine the detection limit for this assay, with samples containing 10⁻⁶ to 100% male DNA. The canine Y chromosome-specific primers SRYF2 (5’-CTGCTGCAACAGGAGTACCA-3’) and SRYR1 (5’-AGGACGTTTTCGTTAGCCAGA-3’) detected the presence of male DNA in female recipients. The primers cGAPDH-603F (5’-CCAGAACATCATCCCTGCTT-3’) and cGAPDH-736R (5’-GGCAGGTCAGATCCACAACT-3’) were used in controls for detection of amplifiable
DNA. PCR amplification was conducted with 1X Buffer J (Epicentre Biotechnologies) and 1 U Taq polymerase. Cycling was done for 30s at 94°C, 30s at 58°C and 30s at 74°C for 35 cycles. Nested PCR reactions were completed with 500 ng of template DNA and the canine Y chromosome-specific primers SRYF2 and SRYR2 (5’-agcaagtttcaacgctatt-3’), followed by a second round of PCR amplification using primers SRYF2 and SRYR1. PCR products were electrophoresed through a 2% agarose gel containing 0.1 µg/ml ethidium bromide and imaged using a Gel Doc 2000 (Bio-Rad Laboratories, Mississauga, ON, Canada).
4.5 Results

4.5.1 MSC characterization

MSC cultures at passages 0-2 were examined by flow cytometry with canine-specific antibodies against CD45, CD44, CD14 and CD11b to confirm the MSC characterization initially based on plastic-adherence (Figure 4.1). The adherent cell fraction at passage 0 contained a small proportion of hematopoietic cells (1.79% CD45+) that decreased to 0.31% by passage 2. Between passages 0 to 2, CD14+ cells decreased from 3.28% to 0.42%, while CD11b+ cells decreased from 2.46% to 0.42% (Figure 4.1 C-E). In comparison, non-adherent cells collected at passage 0 showed an hematopoietic profile with the presence of 77.6% CD45+, 88.9% CD14+ and 13.3% CD11b+ cells (Figure 4.1 B). By passage 3, the adherent fraction possessed a typical MSC profile of CD44+CD45−CD14−CD11b−. All in utero transplants of MSCs were performed with passage 3-6 adherent cultures.

4.5.2 Cell labeling with SPIO and CMTMR

Tracking of injected cell populations was facilitated by labeling donor cells with SPIO particles (Figure 4.2 A). These particles are superparamagnetic beads with an attached Dragon Green™ fluorophore, which enable both magnetic resonance imaging and Prussian blue histological analysis as well as fluorescence imaging. In order to confirm the incorporation of SPIO labels into both BMMCs and MSC cultures, samples of labeled cells were examined by microscopy (Figure 1 A) and flow cytometry (Figure 4.2 B-C).
**Figure 4.1.** Flow cytometric analysis of cell surface markers expressed by (A) non-cultured canine BMMCs, (B) the non-adherent cells from MSC cultures at passage 0, and the adherent MSCs at (C) passage 0, (D) passage 1, and (E) passage 2. Antibody stains used were (from left to right): isotype control, cCD44, cCD45-biotin, cCD14, cCD11b, with FITC-labeled secondary antibodies; αmIgG2a for CD44, Streptavidin for CD45-biotin, and αmIgG1 for CD14 and CD11b. Isotype controls were used to set the limits for positive gates.
Figure 4.2. Efficiency of SPIO labeling of BMMCs and MSCs used for in utero transplantation. (A) Light micrograph of MSCs with incorporated SPIO. Flow cytometric analysis of (B) BMMCs and (C) MSCs prior to (B-C, left panels) and after SPIO-labeling (B-C, right panels showing green fluorescence of Dragon Green SPIO particles).
Due to the labeling of the SPIO particles with a Dragon Green™ fluorophore, labeled cells were detected as green fluorescence positive. Viable cells from BMMC and MSC cultures efficiently incorporated the SPIO label and contained 98% and 96% FITC positive cells, respectively (Figure 4.2 B-C).

An additional label, CMTMR, which diffuses freely through the membranes of live cells and reacts with intracellular components to label viable cells, can be detected by fluorescence imaging in the red fluorescence channel. Cells transplanted in all litters, except no.1 and 2, were co-labeled with CMTMR to further confirm that SPIO-labeled cells detected within recipients were of donor origin, rather than host cells that had taken up free SPIO particles released by donor cells. CMTMR labeled cells were detected by flow cytometry in the red fluorescence channel. Co-labeling of CMTMR was detected in all Dragon Green-SPIO positive cells.

4.5.3 In utero cell transplantation

A total of 58 fetuses from seven pregnancies were injected as summarized in Table 4.1. One day prior to fetal retrieval, all pregnant bitches were examined by ultrasound to assess the number and viability of fetuses present within the uterus. Normal development was confirmed by the presence of intact fetuses within each uterine sac and the visualization of regular heart rhythms.

Previous canine IUSCT demonstrated that injection of 0.5-1.5 x 10^7 cells into gestational day 32-38 fetuses was relatively safe with > 75% of fetuses being live born (Lutzko et al., 1999; Omori et al., 1999). Thus in our first series of injections, we used the yolk sac delivery route to deliver 1 x 10^6 MSCs or 1 x 10^7 BMMCs to day-35 fetuses. Recipients had an average fetal mortality of 7% by day 42 (litter no. 1 and 2).
<table>
<thead>
<tr>
<th>Litter no., Name of mother (abbreviation)</th>
<th>Day of gestation at transplant</th>
<th>Day of gestation at fetal retrieval</th>
<th>Crown rump length (cm)a</th>
<th>Mass (g)b</th>
<th>Total cell number injected and (estimated cells/kg)</th>
<th>Recipients/Total Fetuses</th>
<th>Recipients retrieved/Total Transplanted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, Clairol (C)</td>
<td>35</td>
<td>42</td>
<td>3.0</td>
<td>2.5</td>
<td>$1 \times 10^6$ b ($4 \times 10^6$) MSC</td>
<td>2/7</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1 \times 10^7$ b ($4 \times 10^6$) BMNC</td>
<td>3/7</td>
<td>3/2</td>
</tr>
<tr>
<td>2, Kiva (K)</td>
<td>35</td>
<td>42</td>
<td>3.0</td>
<td>1.9</td>
<td>$1 \times 10^6$ b ($4 \times 10^6$) MSC</td>
<td>1/7</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1 \times 10^6$ b ($4 \times 10^6$) BMNC</td>
<td>1/7</td>
<td>1/1</td>
</tr>
<tr>
<td>3, Misty (M)</td>
<td>25</td>
<td>32</td>
<td>0.9</td>
<td>0.11</td>
<td>$1 \times 10^6$ b ($9 \times 10^5$) MSC</td>
<td>2/7</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1 \times 10^7$ b ($9 \times 10^5$) BMNC</td>
<td>2/7</td>
<td>2/2</td>
</tr>
<tr>
<td>4, Pequena (P)</td>
<td>25</td>
<td>NAc</td>
<td>0.9</td>
<td>0.11</td>
<td>NA</td>
<td>4/9</td>
<td>4/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1 \times 10^7$ b ($9 \times 10^5$) BMNC</td>
<td>4/9</td>
<td>4/9</td>
</tr>
<tr>
<td>5, Fancy (F)</td>
<td>25</td>
<td>42</td>
<td>0.9</td>
<td>0.11</td>
<td>$1 \times 10^6$ b ($9 \times 10^5$) MSC</td>
<td>3/13</td>
<td>3/13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1 \times 10^7$ b ($9 \times 10^5$) BMNC</td>
<td>3/13</td>
<td>3/13</td>
</tr>
<tr>
<td>6, Fay (FY)</td>
<td>25</td>
<td>59d</td>
<td>0.9</td>
<td>0.11</td>
<td>$1.5 \times 10^6$ b ($1.4 \times 10^5$) MSC</td>
<td>4/11</td>
<td>4/11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$2.5 \times 10^7$ b ($2.2 \times 10^5$) BMNC</td>
<td>5/11</td>
<td>5/11</td>
</tr>
<tr>
<td>7, Playa (PY)</td>
<td>25</td>
<td>61d</td>
<td>0.9</td>
<td>0.11</td>
<td>$2 \times 10^6$ b ($1.8 \times 10^5$) MSC</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$7.5 \times 10^5$ b ($6.8 \times 10^5$) BMNC</td>
<td>5/11</td>
<td>5/11</td>
</tr>
</tbody>
</table>

a The average crown rump length was estimated from ultrasound images taken on the day of transplantation
b $L = 21W^{0.38}$ (Andersen and Goldman, 1970)

c Pregnancy 3 (P) was terminated at day 30 due to pyometra; no fetuses were retrieved
d Liveborn pups were delivered by elective Caesarean section; e One dog died at postnatal day 1; f One dog died at postnatal day 3

NA, Not applicable
To determine whether cells could be delivered at an earlier time point, we conducted transplants at gestational day 25 and retrieved fetuses seven (day 32) or seventeen (day 42) days later. Fetuses transplanted at gestational day 25 had a fetal mortality rate of 11% at day 32 (litter no. 3) and 0% at day 42 (litter no. 4). Of the two fetuses that died, both had received a transplant of $1 \times 10^7$ BMMCs, which was equivalent to $4 \times 10^9$ cells in day-35 recipients and $9 \times 10^{10}$ cells in day-25 recipients. This result raised the possibility that injection of high cell numbers may compromise fetal development. To address this possibility, day 25 fetuses were injected with increased cell doses of $2.2 \times 10^{11}$ BMMCs/kg or $1.8 \times 10^{10}$ MSCs/kg and recipients were allowed to develop to term. These studies indicated that fetuses injected with $9 \times 10^{10}$ BMMCs/kg or $9 \times 10^9$ MSCs/kg were lost during the prenatal period. Within the same pregnancies, fetuses injected with lower cells doses of $6.8 \times 10^9$ BMMCs/kg or $1.4 \times 10^{10}$ MSCs/kg resulted in viable fetuses, although 2 of 5 MSC-transplanted fetuses died in the early postnatal period. Pathological analysis of pups that died within the first week of life, demonstrated no evidence of infection or graft-versus-host disease on tissue examination.

4.5.4 *Ex vivo* whole body fluorescence imaging

The presence of Dragon Green/SPIO-labeled cells within transplanted fetuses was visualized by ex vivo whole body fluorescence imaging on a flat panel fluorescence imager. The green fluorescence intensity was examined for fetuses transplanted at day 35 and retrieved at day 42 (Figure 4.3 A-B) as well as for fetuses transplanted at day 25 and retrieved at day 32 (Figure 4.3 C-E). At the time of fetal retrieval, the day 42 fetuses had a crown-rump length of 5-7 cm, while the day 32 fetuses were 1.5-2 cm in length. The
**Figure 4.3.** Whole body fluorescence imaging of recipient dogs seven days after in utero transplantation retrieved at (A-B) 42 days and (C-E) 32 days of gestation.

Fluorescence images were taken with a Kodak 200M flat panel fluorescence imager with a 485 nm excitation and 535 nm emission filter set. (A,B,D) Stronger intensity of
Figure 4.3. (continued) black indicates greater FITC fluorescence in monochrome images. (A-B) Fetuses from litter no. 1 (Clairol) that were injected with either 1 x 10^6 MSCs (CR1 and CR2) or 1 x 10^7 BMMCs (CR3 and CR4) have greater green fluorescence than those that received a medium-only injection (CR5 and CL2). (C) Photograph of a fetus from the day 32 pregnancy (litter no. 2 - Millie) that were imaged in panel D and E. (D) Monochromatic imaging of FITC fluorescence of fetuses transplanted at day 25 and retrieved at day 32. Fetuses MR1, MR2, MR3, and MR4 received 1 x 10^6 MSCs; fetuses ML1, ML2 and ML3 received 1 x 10^7 BMMCs; and fetus ML5 received an injection of medium. (E) Pseudo-coloured image of FITC fluorescence showing relative fluorescence intensities.
green fluorescence shown as an increased intensity of black on the monochromatic images, indicated the presence of transplanted cells. Background levels of fluorescence were established using control-injected fetuses from the same litter.

In fetuses transplanted at day 35 and retrieved at day 42, the green fluorescence was primarily localized to the thorax and forelimbs, while the abdomen showed intermediate levels of fluorescence and no fluorescence was detected in the yolk sac (Figure 4.3 A-B). Green fluorescence was visible in pups that received either BMMCs or MSCs.

In fetuses transplanted at day 25 and retrieved at day 32, the highest levels of green fluorescence were localized to the fetal liver (Figure 4.3 D). Relative fluorescence intensities were determined and displayed in a pseudo-coloured image (Figure 4.3 E), which indicated that all fetuses that received a cell transplant had increased Dragon Green fluorescence over the background levels as determined by comparing fluorescence levels in the medium-only injected control fetus (fetus ML5). The majority of Dragon Green fluorescence was detected in the lower abdominal cavity and specifically, within the fetal liver. Two dogs that received $9 \times 10^9$ MSCs/kg (fetuses MR3 and MR4) showed markedly elevated fluorescence. Lower intensity fluorescence was also noted extending from the fetal liver along the spinal region and into the head of some fetuses. No fluorescence was detected in the yolk sacs of injected or noninjected fetuses.

Organs from day 42 fetuses were dissected and reimaged to characterize the localization of green fluorescence in greater detail (Figure 4.4). High levels of green fluorescence were detected within the livers (Figure 4.4 A i), hearts (Figure 4.4 A ii), forelimbs (Figure 4.4 A iii) and lungs (Figure 4.4 A iv) of fetuses transplanted with either
Figure 4.4. Whole organ fluorescence imaging of day 35 recipient dogs seven days after *in utero* transplantation. Fluorescence images were taken with a Kodak 200M flat panel fluorescence imager with a 485 nm excitation and 535 nm emission filter set. Stronger intensity of black indicates greater FITC fluorescence in monochrome images. (A) Tissue samples from fetuses that received $1 \times 10^6$ MSCs (CR1 and CR2) or $1 \times 10^7$ BMMCs (CR3 and CR4) have greater green fluorescence than those that received a medium-only injection (CR5 and CL2). Tissues examined were (i) liver, (ii) heart, (iii) forelimbs and (iv) lungs.
MSCs (fetus CR1) or BMMCs (fetuses CR3 and CR4). Background levels of fluorescence were noted in one fetus transplanted with MSCs (CR2) and two control fetuses (CR5 and CL2). The distribution of fluorescence in recipients injected with MSCs was greatest in the heart, forelimbs and lung, while the majority of the fluorescence in recipients injected with BMMCs was localized to the liver and lung.

4.5.5 Histological analysis of fetal tissues

The livers of fetuses injected on day-35 and retrieved on day-42 showed high levels of green fluorescence (Figure 4.5 A). To confirm that Dragon Green/SPIO-labeled cells were responsible for the fluorescence seen, histological analysis for detection of the iron oxide core of the SPIO particles was achieved with iron-reactive Prussian blue staining of cryosectioned tissues. These studies revealed the presence of Prussian blue staining cells in the livers of fetuses receiving either MSCs (Figure 4.5 B i and iv) or BMMCs (Figure 4.5 B iii and vi). Control fetuses injected with only medium did not show the presence of blue stained cells (Figure 4.5 B ii and v). Fluorescence microscopy confirmed the presence of Dragon Green/SPIO and CMTMR triple-labeled cells within the fetal livers of recipients of MSC (Figure 4.5 C i and iv) and BMMC (Figure 4.5 C iii and vi) transplants, while fluorescence was not detected in medium-only injected controls.

Prussian blue staining of sagittal plane cryosections of whole fetuses injected on day-25 and retrieved on day-32, revealed positive cells were detected in primarily in the livers (Figure 4.6 A i). Prussian blue-staining positive cells were also detected in the medium-only injected controls (Figure 4.6 A iii). To further confirm the presence of
Figure 4.5. Detection of SPIO-labeled cells within fetal livers of fetuses transplanted at 35 days of gestation and retrieved at 42 days.
**Figure 4.5.** Detection of SPIO-labeled cells within fetal livers of fetuses transplanted at 35 days of gestation and retrieved at 42 days. (A) Whole organ fluorescence was detected with a flat panel imager prior to cryosecting livers into 10 μm slices and staining with (B) Prussian blue to detect the iron oxide core of the SPIO or (C) with DAPI for fluorescence microscopy to stain the cell nuclei and detect the Dragon Green fluorophore-labeled SPIO and CMTMR. The fetal livers of (B i and iv) MSC-injected and (A iii and vi) BMMC-injected fetuses were positive for Prussian blue staining while the (A ii and v) medium-injected fetuses had no staining for Prussian blue. The livers of (C i) MSC-injected and (C iii) BMMC-injected fetuses were positive for green fluorescence and CMTMR labeled-cells, while the (B ii) medium-injected control did not contain SPIO or CMTMR-labeled cells.
Figure 4.6. Detection of SPIO-labeled cells within fetuses transplanted at 25 days of gestation and retrieved at 32 days.
**Figure 4.6.** Detection of SPIO-labeled cells within fetuses transplanted at 25 days of gestation and retrieved at 32 days. (Ai) Fetuses were cryosectioned in 5 μm slices and stained with (A) Prussian blue to detect the iron oxide core of the SPIO or (B) with DAPI for fluorescence microscopy to the cell nuclei and detect the Dragon Green fluorophore-labeled SPIO. The fetal livers of (A ii) MSC-injected, (A iii) medium-injected, and (A iv) BMMC-injected fetuses were positive for Prussian blue staining, but only the livers of (B i) MSC-injected and (B iii) BMMC-injected fetuses were positive for green fluorescence, while the (B ii) medium-injected control did not have any green fluorescence. Prussian blue and Dragon Green-SPIO labeled cells were also found in the (C i-iii) hand plate and (D i-iii) humeri bone marrow cavities as shown by green fluorescence (C and D iii), iron reactive Prussian blue staining visualized by Nomarski imaging (C and D ii) and in the merged images with DAPI staining (C and D i). MAC387 positive cells (pseudocoloured pink) were found at the periphery of fetal livers (E i-iii) (representative fetus, Misty R4 shown). 10X magnification shows the peripheral location of MAC387+ cells (E i), while 100X magnification of the inset, show that the MAC387 staining does not co-localize with green fluorescence of the Dragon Green-SPIO labeled cells (E ii-iii).2
injected cells, fluorescence microscopy was undertaken for the detection of the Dragon Green fluorophore. Dragon Green fluorescence was detected within the livers of fetuses receiving MSCs (Figure 4.6 B i) and BMMC (Figure 4.6 iii) transplants while no fluorescence was detected in the liver of medium-injected control fetus (Figure 4.6 B ii). Thus, the presence of iron-reactive, Prussian blue staining cells within the livers of control fetuses, likely reflected the localization of fetal erythropoiesis to the liver at gestational day 32, which requires high levels of native iron for red blood cell development. Dragon Green-fluorescing cells were also detected in the developing limb bud (Figure 4.6 C i-iii) and humeral bone marrow cavities (Figure 4.6 D i-iii). No green fluorescence or Prussian blue staining was detected within the other fetal organs or within the extraembryonic yolk sac.

In order to further confirm that Prussian Blue-Dragon Green-CMTMR-positive cells were due to the presence of transplanted cells and not the uptake of free particles by host cells, macrophage-specific staining was completed on tissue sections, as host macrophages were speculated to be the cell type most likely to phagocytose free SPIO particles. The MAC387 antibody detected several macrophages at the periphery of the fetal livers of all dogs, as well as a small number of MAC387 positive cells within the liver tissue. The colocalization of the macrophage-specific stain with Prussian Blue-Dragon Green-positive cells was not detected in any injected or noninjected fetuses (Figure 4.6 E).
4.5.6 Molecular analysis of fetal tissues

Prior to the analysis of chimerism, all fetuses were sexed visually and by PCR amplification of a canine Y chromosome specific sequence from DNA extracted from a tail tip biopsy. Female fetuses were then assessed for microchimerism by semi-quantitative nested PCR detection of the canine specific Y chromosome sequence. The relative percentage of SRY positive cells was determined by amplification of serial dilutions of male DNA in a background of female DNA.

Following transplantation at day 35, day 42 fetuses (litter no. 1) exhibited microchimerism in the liver, lung, heart, and kidney. Engraftment levels were determined to be less than 0.001 – 0.01% by standard PCR and 0.01 – 0.1% by Q-PCR (Table 4.2). Litter no. 2 could not be analyzed, as all transplanted animals were male, except for the medium-injected control.

Fetuses transplanted at day 25 and retrieved at day 32 (litter no. 3) were assayed by sampling tissue in the liver region from sagittal cryosections. Tissue from the head region of each fetus was used as an internal negative control. The microchimerism detected was limited to samples taken from the liver and demonstrated low level amplification of the Y chromosome sequence in fetuses transplanted with MSCs (fetus MR4) and BMMCs (fetuses ML2 and ML3). No Y chromosome DNA could be amplified from the medium-only injected control fetus, ML5 (Table 4.2).
**Table 4.2.** Assessment of canine Y chromosome amplification from donor cells transplanted to female fetuses.

<table>
<thead>
<tr>
<th>Litter no., Mother</th>
<th>Fetus</th>
<th>Transplant</th>
<th>Sex of fetus</th>
<th>Standard SRY PCR, % positive cells</th>
<th>SRY Q-PCR, % positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, Clairol</td>
<td>CR1</td>
<td>MSC</td>
<td>F</td>
<td>0.0001-0.001</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td></td>
<td>CR2</td>
<td>MSC</td>
<td>F</td>
<td>0</td>
<td>0.001-0.01</td>
</tr>
<tr>
<td></td>
<td>CR3</td>
<td>BMMC</td>
<td>M</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CR4</td>
<td>BMMC</td>
<td>F</td>
<td>0.0001-0.001</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td></td>
<td>CR5</td>
<td>CTRL</td>
<td>F</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CL2</td>
<td>CTRL</td>
<td>F</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3, Misty</td>
<td>MR1</td>
<td>MSC</td>
<td>M</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>MR2</td>
<td>MSC</td>
<td>M</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>MR3</td>
<td>MSC</td>
<td>M</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>MR4</td>
<td>MSC</td>
<td>F</td>
<td>0.001-0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ML1</td>
<td>BMMC</td>
<td>M</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ML2</td>
<td>BMMC</td>
<td>F</td>
<td>0.001-0.01</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td></td>
<td>ML3</td>
<td>BMMC</td>
<td>F</td>
<td>0.01-0.1</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td></td>
<td>ML5</td>
<td>CTRL</td>
<td>F</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ND, not done
4.6 Discussion

Previous work has shown that low-level donor chimerism can be achieved in a canine in utero model of haploidentical adult bone marrow transplantation employing an intraperitoneal injection site (Blakemore et al., 2004). Previous work from our group utilized the yolk sac as a site for cell delivery, which also resulted in low-level chimerism (Lutzko et al., 1999; Omori et al., 1999). The aim of the study described herein was to address whether in utero transplantation to the extraembryonic yolk sac allows for efficient migration of cells from the yolk sac injection site and engraftment of donor cells within fetal tissues.

We found that in utero cell transplantation of 1 x 10^6 MSCs (4 x 10^8 cells/kg) or 1 x 10^7 BMMCs (4 x 10^9 cells/kg) in a canine model could be performed successfully at day-35 of gestation, resulting in a high percentage of transplant recipient survival (93%) seven days after injection. At the time of fetal retrieval, fetuses remained viable with steady heart rhythms and no visible gross abnormalities. We also showed that fetuses transplanted as early as 25 days of gestation remained viable for seven (retrieved at day 32) to seventeen (retrieved at day 42) additional days in utero with no apparent fetal abnormalities. Resorption rates were within a normal range (van der Beek et al., 1999).

By contrast, increased cell doses of 2 x 10^6 MSCs (1.8 x 10^10 cells/kg) or 2.5 x 10^7 BMMCs (2.2 x 10^11 cells/kg) injected into day-25 fetuses resulted in unacceptably high perinatal mortality of 100%. A two-fold increase in the total cell number transplanted to each fetus, resulted in high levels of in utero death in fetuses followed to term. High rates of mortality were also reported by Blakemore et al. (2004) when fetuses at gestational ages of day-31 or younger were injected intraperitoneally with up to 2.5 x 10^{10} CD34+
cells/kg (57% loss at day 31 and 86% at day 30). The authors speculated that the high mortality rate could be attributed to elevation of the diaphragm by an over-expanded peritoneal cavity due to the large volume of the cell transplant (40-70 µl). By contrast our studies, employing yolk sac injection at earlier time points in the canine pregnancy experienced low fetal mortality despite the larger volume of the cell transplants (100 µl). When cell doses were below $1.4 \times 10^{10}$ cells/kg ($1.5 \times 10^6$ cells/fetus) the fetal mortality rate was 0-25%, but a similarly large fetal loss of 75-100% was experienced if cell doses greater than $1.8 \times 10^{10}$ cells/kg were delivered. The lower mortality rate seen at an earlier gestational time point than that completed by Blakemore et al. (2004), after yolk sac injection is likely due to the accessory nature of the yolk sac, which allows for safe delivery of larger volumes of cells that gradually migrate from the yolk sac into the developing fetuses.

Tracking of labeled, transplanted cells was monitored by fluorescence imaging and histological analyses of whole fetuses and tissue sections. Fetuses transplanted with cells at 35 days of gestation and analyzed on a flat panel fluorescence imager seven days later, showed increased levels of green fluorescence within the thorax and forelimbs when examined, while the medium only-injected fetuses showed only background level fluorescence. Examination of the organs from day 35 fetuses harvested seven days later indicated that SPIO-labeled cells were primarily located within the liver, lung, heart and forelimbs. Histological analysis for co-localization of the green fluorescence of Dragon Green-labeled SPIO, red fluorescence of the CMTMR and iron oxide core of the SPIO as indicated by Prussian blue staining in triple-labeled cells confirmed the presence of donor cells.
Examination of fetuses transplanted at gestational day 25 and retrieved at day 32, showed high levels of green fluorescence within the fetal liver upon whole body imaging. Histological analysis confirmed the presence of SPIO-labeled cells within the liver tissue and also revealed a small number of labeled cells within the humeral bone marrow cavities and developing hand plates. This may indicate that by day 32, cells have migrated from the yolk sac to the liver, which is the primary site of fetal hematopoiesis, and are subsequently redistributed to the developing bone marrow cavities.

Taken together these data suggest that donor cells transplanted to the yolk sac follow the normal pattern of circulation of hematopoietic cells from the extraembryonic yolk sac to the fetal liver via the vitelline veins. Transplantation of cells into early in gestation (day 25) fetuses resulted in their localization primarily to the fetal liver. It was not until mid gestation (day 35) that the fetal circulation was sufficiently developed for transplanted cells to not only migrate from the yolk sac to the fetal liver, but than to be redistributed via the fetal circulation to various organs. Thus, labeled donor cells were detected in non-hematopoietic sites such as the lungs and heart only within fetuses retrieved at day 42, regardless of whether cells were transplanted at day 25 or 35.

Transplants of either BMMCs or MSCs resulted in microchimerism, but at levels of <1%. The ability of cells to migrate from the yolk sac to fetal tissues did not appear to be affected by the cell type used, as transplantation of BMMCs and MSCs resulted in tissue distribution to the same organ systems. Both cell types were found in the liver at day 32 and the liver, heart, lung and limbs at day 42. The overall distribution of cells to fetal organs at the time of fetal retrieval reflected the developmental stage of the recipient as those fetuses injected at day 25 and retrieved at day 32 showed localization of donor cells
to only the liver, whereas fetuses injected at day 35 and retrieved at day 42, showed localization of donor cells within a variety of tissues, including the liver, lung, heart and limbs. This may correspond to the migration of cells from the yolk sac to the fetal liver during early fetal development, followed by the redistribution of hematopoietic cells from the fetal liver to the other hematopoietic sites, such as the spleen and bone marrow, later in gestation. With continued development of the fetal circulation, donor cells may circulate to other nonhematopoietic organ systems and become entrapped within the lung and heart.
4.7 References


CHAPTER 5

Discussion and Future Directions
5 Discussion and Future Directions

As canine models gain recognition for their utility in the preclinical assessment of stem cell-based therapies for humans, it will be important for basic research in canine stem cell biology and transplantation models to keep pace with studies conducted in mice and humans. The research detailed in this thesis adds to the field of canine stem cell biology with the isolation and characterization of previously unidentified canine bone marrow-derived side population cells, as well as with the derivation and characterization of canine embryonic stem cell lines with both in vitro and in vivo differentiation potential. Finally, the assessment of the migration of cells transplanted into the yolk sacs of early and mid gestation canine fetuses adds to the field of in utero transplantation and suggested that this model could be utilized for future study of canine stem cell therapies.

5.1 Canine Side Population (SP) Cells

The isolation and characterization of canine side population cells was successfully demonstrated in Chapter 2. The presence of Hoechst dye-effluxing side population cells was confirmed in canine bone marrow and the reproducible purification of this population was enhanced with cytokine pretreatment to promote the recovery of bone marrow cells from freezing and activate the metabolic functioning of the transmembrane pumps responsible for Hoechst dye efflux. Enrichment for blast cells was achieved by excluding large granular cells from the SP fraction with the use of a pregating strategy. These modifications to the SP isolation protocol allowed for reproducible isolation of sufficient numbers of SP cells to assess their multilineage differentiation and proliferation potential both in vitro and in vivo.
Canine bone marrow-derived small agranular SP (SA-SP) cells were enriched for blast cells upon morphological examination of Wright-stained cytospins, but this cell fraction also contained a high proportion of erythroblast cells. The ability of erythroblasts to efflux Hoechst is supported by reports of high expression levels of both Abcg2 mRNA (Zhou et al., 2001; Scharenberg et al., 2002) and protein (Zhou et al., 2005)(Tadjali et al., 2006) in Ter119+ erythroblasts. Yet Yamamoto et al. (2007) have shown that although erythroblasts express large amounts of the ABCG2 protein, they did not show the SP phenotype. This is in contrast to our findings in canine bone marrow where the SP fraction contained a large proportion of erythroblasts. Thus in dog, the ABCG2 pump may be actively exporting Hoechst dye from erythroblasts, thereby facilitating the inclusion of a large number of these cells in the SA-SP fraction. In addition, a small proportion of cells isolated in the canine SA-SP fraction were also monocytic. Monocytes have been reported to express only low levels of ABCG2 (Zhou et al., 2001) and MDR1 (Drach et al., 1992) but high-level expression of the MRP1 transmembrane pump has been noted on CD14+ monocytes (Laupeze et al., 2001). Thus, the low level of Hoechst fluorescence in monocytes may be attributed to either a slow kinetic of dye accumulation or, more likely, to efflux of Hoechst dye with a pump other than ABCG2, such as MRP1. The co-purification of mature cell types after Hoechst staining has not been reported in other species, which points to canine-specific Hoechst-dye transport differences or a difference due to the staining protocol employed. Tadjali et al. (2006) have shown that increasing the Hoechst dye concentration increases the stringency of the SP protocol, resulting in fewer cells falling into the SP gate, and that a greater proportion of these cells expressed the ABCG2 transporter under these conditions. Tadjali et al. and others
(Goodell et al., 1996; Pearce and Bonnet, 2007) have employed pre-selection of Lin− cells prior to Hoechst staining, which resulted in the enrichment of HSCs with a KSL phenotype (Tadjali et al., 2006). Future canine SP cells studies will focus on the depletion of erythroblasts and monocytes from the canine bone marrow in order to further purify the SA-SP fraction to contain exclusively blast cells. Current protocols for lineage depletion of mature hematopoietic cells from dog bone marrow utilize antibodies against granulocytes, monocytes, B-cells and platelets (clone F72A, VMRD), neutrophils (α-neutrophil, VMRD) and CD8 (Serotec) positive cells, but do not specifically target erythroid lineage cells (Bruno et al., 2001; Niemeyer et al., 2001). To date, no canine-specific erythroid antibodies are commercially available, necessitating testing for cross-reactivity with available human erythrocyte/erythroblast antibodies.

The SA-SP fraction was enriched for long-term culture-initiating cell potential compared to large granular SP (LG-SP) cells or nonSP cells. This functional assay confirmed that canine bone marrow-derived SA-SP cells have multilineage differentiation potential as has been found for rhesus monkey and human SP cells. As in rhesus monkey (Goodell et al., 1997) and human (Guo et al., 2003) CFU assays, it was necessary to culture SP cells with feeder cells and/or cytokine stimulation to induce their ability to form colonies in methylcellulose cultures. Goodell et al. (1997) have previously shown that during long-term culture of PKH26-labeled CD34− rhesus monkey SP cells CD34 expression is upregulated after 14 days in cultures. The proportion of CD34+ cells increased to 51% of the PKH26-labeled cells by 35 days, at which time significant numbers of CFUs could be quantified in methylcellulose. CFUs were not detected from SP cells cultured for 0, 7, or 14 days, but could be detected as early as 25 days after
culture initiation. These finding suggests a possible mechanism whereby canine SP cells in long-term cultures may undergo activation to a CD34^+ phenotype in order to proliferate and produce colonies of the mature hematopoietic lineages. Future studies will assess the profile of CD34 expression on canine SP cells in long-term cultures.

Further characterization of the in vivo engraftment potential of canine SP cells was undertaken by intrafemoral transplantation in NOD/SCID-β2m^- mice and revealed that the murine stem cell niche may not efficiently support the in vivo proliferation and differentiation of canine SP cells. In fact, transplantation of cells from the SA-nonSP fraction, which contained a significant proportion of the CD34^+ cells, resulted in greater levels of engraftment. It is not surprising to see engraftment from CD34+ HSCs, as CD34^+ selection of canine bone marrow has been extensively used for gene-modified cell therapies which resulted in high level mixed chimerism and long-term multilineage hematopoiesis (Bruno et al., 1999, 2001; Hartnett et al., 2002; Kiem et al., 2004; Suter et al., 2007). The lack of in vivo engraftment for the SA-SP fraction, in conjunction with the necessity to activate canine SA-SP cells with long-term culture to assess their CFU potential, corroborated the hypothesis that SP cells are representative of a quiescent HSC that requires activation, which may occur concomitantly with the upregulation of CD34^+ expression, in order for proliferation and differentiation to be observed in the NOD/SCID xenotransplantation model.

Overall, these findings indicated that the NOD/SCID mouse is able to support canine long-term HSC engraftment, as significant engraftment was detected at 12 weeks upon transplantation of unfractionated canine bone marrow, but pointed to the need for further refinement of the NOD/SCID model to support engraftment of canine SP cells.
Future studies, utilizing short term *in vitro* maturation of canine SP cells may increase engraftment in NOD/SCID mice, while strategies to enhance *in vivo* SP maturation are another avenue of study. The cotransplantation of irradiated canine bone marrow or peripheral blood cells may provide the niche and cytokines required for robust canine SP engraftment, though boosting with subsequent injections of canine cells or purified cytokines may be necessary to induce extended proliferation and differentiation of engrafted SP cells. Future directions could include autologous SP cell transplantation into sublethally irradiated canine recipients, as this would remove the limitation of a lack of murine cytokine cross-reactivity with canine SP cells.

### 5.2 Canine Embryonic Stem Cells (cESCs)

The development of cESCs with the defining characteristics of human ESCs has been accomplished with the research detailed in Chapter 3. Human ESCs characterization has been defined by the maintenance of self-renewal in an undifferentiated state upon extended culturing, the expression of pluripotent stem cell markers, and the generation of cells derived from the three embryonic germ layers both *in vitro* and *in vivo*. The cell lines derived in these studies are an improvement on previously established cESC lines (Hatoya *et al.*, 2006; Schneider *et al.*, 2007; Hayes *et al.*, 2008) as they not only were maintained to high passage numbers, expressed classical human pluripotency markers and differentiated to cells of the three germ layers *in vitro*, but also possessed the ability to form teratomas *in vivo* which contained cells of each embryonic germ layer. This finding is novel as it represents the isolation of the first *bona fide* cESC lines based on the criteria utilized for the characterization of human embryonic stem cells and opens the possibility of producing cESC-derived cells for transplantation studies.
The morphology and expression of pluripotency antigens, including SSEA3, SSEA4, TRA1-60 and TRA1-81, while lacking the expression of SSEA1, indicated our cESCs are more similar to human ESCs than to mouse ESCs. Earlier reports of the derivation of cESCs indicated that resulting cell lines expressed the mouse-specific ESC marker, SSEA1 (Hatoya et al., 2006; Schneider et al., 2008), while expression of human-specific markers, SSEA3 and SSEA4 were either not tested or showed no staining. This difference in the expression profiles of ESC cell-surface carbohydrates may indicate that the cESCs derived by our group are derived from an embryonic cell more similar to those from which human ESCs are derived, as they expressed the human ESC markers, SSEA3, SSEA4, TRA1-60 and TRA1-81 and lacked expression of the mouse ESC marker, SSEA1.

By contrast, the dependence of cESCs on hLIF for their undifferentiated maintenance was a property more similar to mouse ESCs. Our cESC lines demonstrated rapid differentiation upon removal of hLIF from the cell culture medium, indicating that cESCs are responsive to hLIF and require it for their self-renewal. The ability of cESCs to respond to murine LIF was not assessed, but mouse and human LIF share 79% homology and show cross-species reactivity (Willson et al., 1992). Thus, the use of commercially available MEFs expressing murine LIF may be useful and more cost-effective for the maintenance of cESC lines in the future.

The ability of our established cESC lines to differentiate to cells of the three germ layers was confirmed both in vitro and in vivo. In vitro differentiation was primarily assessed through the formation of embryoid bodies and resulted in the development of aggregates containing cells derived from one or more of the embryonic germ layers. Due
to the lack of canine-reactive antibodies of different isotypes or from different species, it was not possible to confirm the presence of cells from each of the three germ layers within one embryoid body. Future experiments, to directly conjugate canine cross-reactive antibodies with fluorophores would facilitate such experiments.

We also attempted to differentiate cESC \textit{in vitro} with adherent cell culture techniques employing cytokines to induce their specific differentiation to endodermal, mesodermal and ectodermal derivatives. Further optimization of controlled \textit{in vitro} differentiation of cESCs to cell types of interest for transplantation will be an interesting area of future research. Many protocols for the directed differentiation of mouse and human ESCs to hematopoietic, cardiovascular and neural progenitors have been initiated (Keller, 2005; Murry and Keller, 2008). These studies indicated that pathways involved in directed differentiation of mouse and human ESCs are very similar, with only small modifications in the timing of the addition of cytokines being required to induce differentiation. Thus, it will be interesting to determine if these protocols can also be translated to cESCs, which would allow for subsequent safety and efficacy testing of transplants derived from differentiated ESCs in the canine large animal model. As developing large numbers of differentiated stem and progenitor cells from ESCs is a hurdle for transplantation studies (Keller, 2005; Murry and Keller, 2008), the use of the canine \textit{in utero} transplantation model may allow for the assessment of cell engraftment and safety with smaller numbers of cells than would be required for transplantation into adult recipients.

The teratomas formed by the transplantation of our cESCs under the testicular capsule of NOD/SCID-β2m−/− mice contained cell types derived from the three embryonic
germ layers, though the overall size of individual teratomas was modest in comparison to those routinely established with mouse ESCs. This may again suggest that the environment within NOD/SCID mice is suboptimal for the stimulation of canine stem cell proliferation. The use of more severely immunocompromised NOD/SCID-IL2R\(^{-/-}\) mice combined with anti-CD122 treatment may support more substantial teratoma formation from cESCs. Future studies, employing canine recipients for cESC transplants may also provide a better environment in which to assess the in vivo proliferation and differentiation potential of cESCs.

The gold standard to assess the self-renewal and multilineage contribution of ESCs is to create chimeric animals through the injection of ESCs into recipient blastocysts. This is a routine protocol for murine ESCs and is under development for monkey ESCs (Mitalipov et al., 2006). Similar protocols have not been established for canine blastocyst manipulation but when the technical aspects of in vitro maturation of canine embryos and transplantation into surrogate bitches have been elucidated, the development of cESC-derived chimeric dogs will definitively confirm the derivation of cESC lines with the same self-renewal capacity and multipotentiality of mouse ESCs.

### 5.3 Canine In Utero Transplantation

The migration of cells delivered by in utero transplantation to the yolk sacs of early and mid-gestation canine fetuses has been confirmed as shown in Chapter 4. As yolk sac, rather than the intraperitoneal, injection is technically less difficult and safer for fetal recipients, this finding confirms that this delivery route can be utilized to engraft cell transplants into canine fetal tissues.
Previous work from our group using yolk sac injection for cell transplantation resulted in low levels of engraftment detected after birth (Lutzko et al., 1999; Omori et al., 1999), which raised the possibility that cell were unable to migrate from the extraembryonic yolk sac to the developing fetus. The studies detailed in Chapter 4 of this thesis used transplantation of cells labeled with supraparamagnetic iron oxide particles conjugated to a Dragon Green fluorophore and an intracellular dye, CMTMR, to visualize the localization of transplanted cells in fetuses injected at both gestational day-25 and day-35. *Ex vivo* fluorescence imaging indicated that labeled cells were found exclusively within fetal tissues within seven to seventeen days of injection as no fluorescence was detected in the yolk sacs of transplanted or medium-only injected controls. Fetuses injected at day-25 and retrieved at day-32 showed Dragon Green fluorescence primarily within the region of the fetal liver, while those injected at either day-25 or day-35 and retrieved at day-42 demonstrated a more broad distribution of fluorescence within the livers, lungs, hearts and forelimbs.

Microscopic analyses of fetal tissues, confirmed the migration of labeled cells through visualization of triple-stained cells. Dragon Green and CMTMR fluorescence as well as iron-reactive Prussian blue staining colocalized within individual cells indicated that the fetal liver was the primary site of cell engraftment in early gestation (fetuses retrieved at day-32) with small numbers of cells in the developing bone marrow cavities. Later in gestation (fetuses retrieved at day 42) transplanted cells were still present within fetal livers, but had also become redistributed to nonhematopoietic organs such as the lungs and hearts. These findings suggested that cells transplanted to the yolk sac follow the normal migration of primitive hematopoietic cells via the vitelline vasculature to the
fetus proper by way of the fetal liver. Most of the fetal blood arriving via the yolk sac circulation is shunted through the liver to the caudal vena cava via the ductus venousus (McGeady et al., 2006), while a small volume passes into the hepatic sinusoids allowing transplanted cells access to the fetal liver to facilitate their engraftment. Seeding of the developing bone marrow cavities occurs with hematopoietic stem cells residing in the fetal liver (McGeady et al., 2006), and thus the localization of transplanted cells within the bone marrow cavities of fetuses transplanted at day-25 and retrieved at day-32 suggested that transplanted cells are also able to migrate along the same route as native fetal hematopoietic stem cells. As the fetal circulation becomes more highly developed, transplanted cells transit through other developing fetal organ systems and become entrapped in sites, such as the lungs and hearts.

Another finding of this study was the correlation between the cell number delivered to developing fetuses and fetal mortality. We found that cell doses in excess of approximately $1 \times 10^{10}$ cells/kg were lethal, with the highest doses resulting in death in utero and lower doses resulting in high mortality in neonates. Thus, in order to transplant large numbers of cells with long-term engraftment potential it will be beneficial to purify hematopoietic stem cells prior to in utero transplantation. The use of the SA-SP fraction of canine bone marrow may be one method of isolating an enriched source of cells for future transplantation into canine fetal recipients.

This work will aid future canine in utero transplantation studies, to assess the self-renewal and engraftment potential of canine SP cells in canine recipients. Due to the rarity of SP cells within the canine bone marrow, in utero transplantation may allow for the most robust engraftment with the smallest number of cells. Similarly, transplantation
studies with cord blood cells, which can only be isolated in small numbers, may be more effectively assessed in utero. Previous work has also shown that more ontogenetically-matched cell transplants may engraft better and provide more robust long-term chimerism (Flake et al., 1986; Zanjani et al., 1992, 1993, 1994; Shields et al., 1995; Golfier et al., 2000; Fujiki et al., 2003; Moustafa et al., 2004; Schoeberlein et al., 2004; Le Blanc et al., 2005; Ural et al., 2005), which would make canine cord blood cell transplantation an interesting avenue of future study.

5.4 Summary

The research I have completed adds to the field of stem cell transplantation by further establishing the canine preclinical model for the assessment of cell-based therapies for application to human regenerative medicine. The identification of a blast-enriched side population of cells within the canine bone marrow will be useful as a cell source for future hematopoietic stem cell transplantation studies, while the derivation of canine embryonic stem cells will allow future assessment of the efficacy and safety of ESC differentiation protocols in the development of transplantable cells. As cells produced from ESCs have the potential to elicit an immune response upon transplantation, the further development of the canine in utero transplantation model will provide an appropriate model for the assessment of allogeneic, ESC-derived cell transplantation. Due to the preimmune status of the developing canine fetuses, cESCs differentiated to cell types of interest for regenerative medicine may be assessed for their safety and engraftment in the canine in utero transplantation model without the need for DLA matching or myeloablation.
The canine preclinical model has been extensively utilized for bone marrow transplantation studies for the last 30 years, yet there remain many opportunities for novel research in the field of canine stem cell biology and regenerative medicine. The translation of preclinical findings in canine models to human medicine is well established and thus research employing specialized canine stem cell sources and transplantation models will be equally well recognized.
5.5 References


34. Ural SH, Sammel MD, Blakemore KJ. Determination of engraftment potential of human cord blood stem-progenitor cells as a function of donor cell dosage and


