Improving Hematopoietic Stem Cell Transplantation by Developing New Tools to Reduce Donor Cell Rejection

by

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Abstract

Patients undergoing hematopoietic stem cell transplantation (HSCT) face hurdles such as conditioning-related mortality, graft rejection and insufficient donor cell dose that impede recovery. To address these issues, we investigated the role of cell surface protein transfer in influencing graft rejection and the use of blood reprogramming intermediates as a novel source of donor cells, respectively. We found that the transfer of host MHC class I proteins onto donor cells protects the donor cells from NK cell-mediated and macrophage-mediated rejection. These results suggest that mild conditioning regimens that spare host NK cells and macrophages can be used on patients without compromising graft tolerance. Next, we attempted but failed to generate blood reprogramming intermediates using the non-viral piggyBac system. Moving forward, we should investigate the use of other methods to generate blood reprogramming intermediates. Altogether, our study provided novel insights and possible solutions to the aforementioned complications faced by patients receiving HSCTs.
Acknowledgments

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List of Abbreviations

ALL  Acute lymphoid leukemia
AML  Acute myeloid leukemia
APC  Antigen presenting cell
ATG  Anti-thymocyte globulin
BMC  Bone marrow cell
Cl₂MDP  Dichloromethylene diphosphonate
CML  Chronic myeloid leukemia
DLI  Donor lymphocyte infusion
DOX  Doxycycline
ESC  Embryonic stem cell
FBS  Fetal bovine serum
Fgf4  Fibroblast growth factor 4
FISH  Fluorescence in situ hybridization
Foxp3  Forkhead box P3
G-CSF  Granulocyte colony-stimulating factor
GdCl₃  Gadolinium chloride
GFP  Green fluorescent protein
GM-CSF  Granulocyte-monocyte colony-stimulating factor
GvHD  Graft-versus-host disease
HLA  Human leukocyte antigen
HSCT  Hematopoietic stem cell transplantation
ICC  Immunocytochemistry
Ig  Immunoglobulin
IgG  Immunoglobulin G
IL-2  Interleukin-2
IL-2R  Interleukin-2 receptor
IL-2Rγ  Interleukin-2 receptor gamma chain
iPSC  Induced pluripotent stem cell
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibition motif
KIR  Killer Ig-like receptor
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Ly49</td>
<td>A set of receptors on NK cells that bind to MHC class I proteins</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>mESC</td>
<td>Mouse embryonic stem cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKG2D</td>
<td>An activating receptor on NK cells</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time-polymerase chain reaction</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immune deficiency</td>
</tr>
<tr>
<td>SHP-1</td>
<td>A tyrosine phosphatase</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical cord blood</td>
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Chapter 1
Introduction

1.1 Current state of hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is a medical procedure commonly used to treat patients with hematologic disorders and cancers. This procedure involves the replacement of the patient’s diseased hematopoietic system with HSCs from a healthy donor. Acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and acute lymphoid leukemia (ALL) are examples of hematologic cancers treated with HSCT. Hematologic disorders such as Fanconi’s anemia and sickle cell anemia are also treated with HSCT (Copelan 2006).

Prior to transplantation, patients undergo conditioning regimens that consist of immunosuppressive treatments and/or total body irradiation. Conditioning regimens can be categorized into myeloablative and non-myeloablative or reduced intensity. The aim of myeloablative regimens is to use intense conditioning to eradicate the malignancy and to make room in the bone marrow niche for the donor cells. For non-myeloablative/ reduced intensity regimens, the aim is not to eradicate but suppress the function of host immune cells to facilitate donor cell engraftment. To eliminate cancer cells, non-myeloablative/ reduced intensity regimens utilize the potential graft-versus-malignancy effect.

Each individual expresses a set of major histocompatibility complex (MHC) or human leukocyte antigen (HLA) molecules on the cell surface. The set of MHC molecules expressed by an individual is determined by two MHC haplotypes, one inherited from each parent. The MHC/HLA molecules give cells an identity and differences in MHC/HLA expression between
host and donor cells can lead to graft rejection or the development of graft-versus-host disease (GvHD) after HSCT. Donor cells are typed for HLA-A, HLA-B, HLA-C and HLA-DR. A good match between the host and donor cells at these loci is associated with better engraftment and patient survival outcome (Flomenberg, Baxter-Lowe et al. 2004).

HSCs for bone marrow reconstitution can be derived from HLA-matched (sharing both MHC haplotypes) siblings, HLA-matched unrelated donors or haploidentical (sharing one MHC haplotype) family members. Donor cell engraftment and patient survival is heavily dependent on the degree of HLA matching between the patient and donor. HLA-mismatch can lead to adverse effects such as graft failure or the development of GvHD. Hence, HLA-matched sibling donors are preferred over HLA-matched unrelated donors, and haploidentical donors are the least preferred. The chance of having a HLA-identical sibling is only 25% while the chance of finding a HLA-matched unrelated donor ranges from 10-90% depending on ethnicity (Beatty, Mori et al. 1995; Koh and Chao 2008; Pidala, Kim et al. 2012). Although the likelihood of finding a HLA-matched unrelated donor can be very high for some ethnicities, the process from identifying a donor to transplantation can take months. For patients without HLA-matched siblings requiring immediate transplants, waiting for HSCs from a HLA-matched unrelated donor is impractical. For these patients requiring urgent grafts, donation from haploidentical donor is more feasible because virtually all patients have a haploidentical family member who is readily available to provide a donation.

Historically, bone marrow cells (BMCs) aspirated from donors are used for transplants. The long term repopulating stem cell population, which represents about 1% of the BMCs (Korbling and Anderlini 2001), is characterized by the expression of CD34 (Civin, Strauss et al. 1984). Over
the years, it was recognized that circulating peripheral blood also contains HSCs (Bensinger, Weaver et al. 1995; Korbling, Przepiorka et al. 1995; Schmitz, Dreger et al. 1995). Since then, it has also been exploited as a source of donor cells for transplants. Furthermore, umbilical cord blood (UCB) also contains stem cells (CD34/CD133 cells), and is often used for transplants in smaller children (Broxmeyer, Douglas et al. 1989). The cell composition of each source differs, giving each source its unique advantages and disadvantages.

1.2 Pre-conditioning regimens

Prior to transplantation, patients undergo preconditioning regimens that consist of immunosuppressive drugs and/or total body irradiation. Conventional myeloablative conditioning regimens use intense doses of immunosuppressive drugs and total body irradiation to suppress host immune cells, clear the host bone marrow niche and eradicate the malignancy. Although relapse rates are lower when patients undergo harsh myeloablative conditioning regimens, this advantage is offset by an increase in treatment-related mortality (Clift, Buckner et al. 1990).

The toxicity of myeloablative conditioning regimens prompted the development of non-myeloablative or reduced intensity regimens. Instead of relying on high doses of total body irradiation to eradicate the malignancy, non-myeloablative/ reduced intensity regimens utilize the graft-versus-malignancy effect. In non-myeloablative/ reduced intensity regimens, low dose total body irradiation along with immunosuppressive drugs, such as fludarabine, are used to promote donor cell engraftment. In these regimens, donor lymphocyte infusions (DLI) are often used to enhance the graft-versus-malignancy effect. The graft-versus-malignancy effect arises from the eradication of host cancer cells by donor immune cells (Loren and Porter 2008). In addition to
milder toxicity, non-myeloablative/ reduced intensity regimens also have a lower incidence of infection compared to myeloablative regimens (Meyers, Flournoy et al. 1982). However, non-myeloablative/ reduced intensity regimens are associated with a higher risk of graft rejection.

1.3 Sources of donor cell

Traditionally, HSCs aspirated from the bone marrow have been utilized to reconstitute the immune system. Over time, peripheral blood and UCB were recognized as sources of HSC for transplants. Each source of HSCs has its advantages and disadvantages with regards to speed of immune reconstitution, availability to patients, graft rejection, risk of developing GvHD and graft-versus-malignancy effects.

1.3.1 Bone marrow

BMCs aspirated from iliac crests were the first source of donor cells used for bone marrow transplantation. The cells that repopulate the host bone marrow constitute about 1% of the donor marrow cells and express the surface marker CD34 (Korbling and Anderlini 2001). The first HSCT was performed with BMCs donated from genetically identical siblings (Thomas, Lochte et al. 1959). Soon after, bone marrow grafts from non-identical siblings were used (Buckner, Epstein et al. 1970). As our understanding of HLA and their impact on graft tolerance advanced, HSCT were then performed with marrow grafts from unrelated donors (Hansen, Clift et al. 1980).
1.3.2 Peripheral blood

HSCs are constantly released from the bone marrow niche and circulate in the peripheral blood system. Hence, peripheral blood can serve as a source of HSC. Only about 0.06% of the circulating nucleated cells are CD34 cells which is significantly less than the bone marrow, but the number of circulating HSC can be enhanced by treating donors with granulocyte colony-stimulating factor (G-CSF) or granulocyte-monocyte colony-stimulating factor (GM-CSF) that releases stem cells from the bone marrow niche.

Donor cells derived from peripheral blood are capable of reconstituting the immune system faster than BMCs (Talmadge, Reed et al. 1997). Peripheral blood contains more T cells than the bone marrow and this difference may explain the difference in reconstitution rate. Also because of the high T cell content, transplants performed with peripheral blood stem cells have a higher risk of developing acute and chronic GvHD (Cutler, Giri et al. 2001) and therefore, peripheral blood has been primarily used for autologous transplantations in which hosts do not develop GvHD.

1.3.3 Umbilical cord blood

UCB was identified as a source of HSC after Boyse observed that lethally irradiated mice can be rescued by infusion of syngeneic neonatal mouse blood (Broxmeyer, Douglas et al. 1989). In humans, typically, 80-100 ml of blood and an average of one billion cells can be collected from each umbilical cord (Rogers, Sutherland et al. 2001). Upon collection, cells are cryopreserved and stored in liquid nitrogen for future use. UCB is rich in stem/progenitor cells but the small volume of UCB limits the number of cells that can be retrieved for transplantation.
The first successful cord blood transplantation was performed on 5-year-old boy with Fanconi anemia over 20 years ago (Gluckman, Broxmeyer et al. 1989). Since then, twenty five thousand cord blood transplantations have been performed. UCB has several advantages over BMCs and peripheral blood cells. For instance, UCB cells are more readily available for transplant than BMCs or peripheral blood cells because they are banked. Moreover, UCB can be easily collected with no risk to the donor whereas BMC and peripheral blood collection can cause discomfort and tissue damage.

Despite the large number of registered donors, at least 10% of patients are unable to find a donor match with no more than one HLA disparity (Pidal, Kim et al. 2012). Transplants with UCB are less stringent and grafts with 1-2 HLA disparities between the host and donor are tolerated. Moreover, transplants with UCB have lower incidences of GvHD without compromising the graft-versus-malignancy effect (Wagner, Barker et al. 2002). The graft-versus-malignancy effect uses donor lymphocytes to eradicate host cancer cells and to enhance this effect hosts are often infused with donor lymphocytes post transplantation (Loren and Porter 2008). DLI is often not available for patients receiving UCB grafts because UCB can only be collected once from a donor. In addition, immune reconstitution after UCB transplantation is slower than BMC and peripheral blood cells, thus putting the patients at a higher risk for opportunistic infections (Copelan 2006).

1.4 Graft rejection

A complication faced by HSC recipients is graft rejection. The risk of graft rejection is enhanced when there is a greater HLA disparity between the host and donor, and in patients receiving non-myeloablative or reduced intensity conditioning regimens. Although a harsher myeloablative
conditioning regimen can reduce the risk of graft rejection, this advantage is offset by the increase in treatment-related mortality (Clift, Buckner et al. 1990). Therefore, there is a need to develop a firmer understanding of the interactions between donor and host cells in order to improve graft tolerance after non-myeloablative/ reduced intensity regimens.

1.4.1 The importance of MHC/HLA-matching

MHC molecules are surface proteins involved in antigen presentation. In humans, MHC molecules are also called HLA molecules. There are two classes of MHC proteins: class I and class II. In humans, class I molecules are called HLA-A, B, and C, while in mice, they are known as H-2K, D and L. Class II molecules are called HLA-DR, DP, and DQ in humans, and I-A and I-E in mice. MHC class I and II proteins differ in the types of antigens they present, their localization, and their function. MHC class I proteins are expressed on all nucleated cells, displaying antigens derived from the cytoplasm. On the other hand, MHC class II proteins are displayed on antigen presenting cells (APCs) such as dendritic cells and B cells, and present antigens derived from the extracellular space (Germain and Margulies 1993).

One function of MHC class I proteins is to help our body distinguish between normal and infected or transformed cells. In normal cells, the repertoire of peptides presented on MHC class I proteins are all self-derived. Under these conditions, naïve T cells remain unstimulated and no immune response is invoked. Upon infection, APCs present foreign peptides on MHC class I proteins, and this MHC class I/peptide complex can stimulate naïve T cells. This stimulation leads to production and proliferation of mature cytotoxic T cells that are specific to the presented antigen. Hereinafter, cells that are infected with the same pathogen are immediately lysed by the active T cells (Rock and Shen 2005). On the other hand, MHC class II proteins display proteins
derived from extracellular pathogens and bacteria and present to helper T cells. This interaction with helper T cells leads to recruitment of immune cells to the site of infection and subsequent removal of the pathogen (Neefjes, Jongsma et al. 2011).

In addition to identifying pathogens, the MHC molecules expressed by a cell has implications for graft tolerance. The spectrum of MHC molecules expressed on a given cell is determined by MHC haplotypes inherited from the parents. Transplantation outcomes are highly dependent on the degree of HLA-matching between the host and donor. Hosts and donors are typically typed for HLA-A, B, C and DRB1 and mismatching at these loci is associated with graft rejection, increased risk of developing GvHD and adverse patient outcomes (Sasazuki, Juji et al. 1998; Flomenberg, Baxter-Lowe et al. 2004).

1.4.2 T cells

T cells are members of the adaptive immune system. Conventionally, all T cells were thought to be MHC-restricted, meaning they will only respond to foreign antigens presented on self MHC molecules. However, there is a subpopulation of T cells that can detect allogeneic MHC molecules on non-self cells (Suchin, Langmuir et al. 2001). Differences between MHC and minor histocompatibility antigens on host and donor cells can lead to T-cell mediated graft rejection or GvHD in HSCT. T cells can recognize foreign cells by one of two allorecognition pathways: the direct and indirect pathway (Figure 1.1) (Lafferty, Prowse et al. 1983; Sherman and Chattopadhyay 1993). In the direct pathway, host T cells recognize donor antigens on donor MHC molecules and donor APCs. In the indirect pathway, host T cells recognize donor antigens internalized, processed and presented on host MHC molecules and host APCs.
1.4.3 NK cells

Natural killer (NK) cells belong to the innate immune system and like other immune cells their role is to find and eliminate foreign cells. NK cells express inhibitory receptors that bind to self MHC class I proteins, and stimulatory receptors that interact with various ligands including MHC class I homolog MICA (Bauer, Groh et al. 1999). While stimulatory receptors have immunoreceptor tyrosine-based activation motifs (ITAMs), inhibitory receptors have immunoreceptor tyrosine-based inhibition motifs (ITIMs) that recruit SHP-1 to initiate downstream inhibitory signaling (Lanier 2003). The NK cell’s decision to lyse cells depends on the balance of incoming inhibitory and activating signals. Typically, inhibitory signals can override the activating signals if both signals are present in equal strength. In mice, inhibitory receptors belong to the Ly49 receptor family (Yokoyama 1995) while in humans, inhibitory receptors are known as killer immunoglobulin (Ig)-like receptors (KIRs) (Lanier 2005).

Unlike T cells which are activated by the presence of allogeneic antigens, NK cells are activated when they detect the absence of ‘self’. The ‘missing self’ hypothesis was developed by Karre and Ljunggren (Karre, Ljunggren et al. 1986) to describe NK cell’s ability to detect the absence of self and the mechanism they use to differentiate between self and non-self cells (Figure 1.2). According to the ‘missing self’ hypothesis, NK cells will spare cells that express sufficient self MHC class I proteins. However, in an event where there is a loss of MHC class I or expression of the incorrect MHC class I proteins, these cells are immediately lysed.

The ‘missing self’ hypothesis was formulated to describe the phenomenon of hybrid resistance (Cudkowicz and Bennett 1971). Hybrid resistance refers to the rejection of parental (A or B) bone marrow grafts in F1 (A x B) hybrids, where A and B represent MHC haplotypes. The
The NK cells in F1 hybrids recognize cells that express both A and B MHC class I proteins as ‘self’. Grafts from either parent only express A or B MHC class I proteins, and thus, are considered as ‘non-self’ by F1 hybrids.

Since the ‘missing self’ hypothesis was formulated, numerous studies have provided strong evidence that support this hypothesis. It has long been observed that cells lacking or having reduced MHC or HLA class I protein expression are more prone to NK cell-mediated lysis than normal cells (Gidlund, Orn et al. 1981; Harel-Bellan, Quillet et al. 1986). The susceptibility to NK cell-mediated lysis of HLA-deficient cells can be reversed by the introduction of HLA class I genes (Storkus, Alexander et al. 1989). Introduction of unmatched murine MHC class I proteins into these same HLA-deficient cells did not confer protection from NK cells, indicating that NK cells are repressed through specific interactions with matched HLA proteins. Using murine cells, Ohlen and colleagues (Ohlen, Kling et al. 1989) forced the expression of H-2D<sup>d</sup> on allogeneic C57BL/6 (H-2D<sup>b</sup>) cells. The transgenic expression of H-2D<sup>d</sup> alone protected C57BL/6 cells from rejection in H-2D<sup>d</sup>-expressing mouse strains such as B10.D2. From a different perspective, Held and colleagues (Held, Cado et al. 1996) showed that expression of Ly49A inhibitory receptors on NK cells from H-2<sup>b</sup> mice can prevent lysis of H-2<sup>d</sup> target cells. The interaction of Ly49A receptors with H-2D<sup>d</sup> molecules on the H-2<sup>d</sup> target cells repressed the lytic activity of H-2<sup>b</sup> NK cells.

The role of NK cells in graft rejection is also evident from transplants in immunocompromised murine hosts. For instance, non-obese diabetic (NOD)/severe combined immune deficiency (SCID) mice are often used to measure human cell engraftment, but because they have residual
active NK cells, donor cell engraftment in these mice can be enhanced when they are treated with antibodies against NK cells (Shultz, Banuelos et al. 2003). Likewise, donor cell engraftment is greater in NK cell-deficient NOD/SCID/gamma (Shultz, Lyons et al. 2005) and NOD/SCID/B2m−/− (Shultz, Banuelos et al. 2003) mice than in NOD/SCID mice with residual NK cells. NOD/SCID/gamma and NOD/SCID/B2m−/− mice are deficient in NK cells because of null mutations to their IL-2Rγ chain and beta-2-microglobulin, respectively. Signaling through the IL-2R is essential for NK cell development and the beta-2-microglobulin mutation results in the absence of MHC class I proteins which interrupts NK cell development.

1.4.4 Macrophages

Like NK cells, macrophages also belong to the innate immune system. The role of macrophages in graft rejection is not as intensively studied as NK cells, but current studies show that macrophages have a prominent role in graft rejection. Terpstra and colleagues (Terpstra, Leenen et al. 1997) showed that eliminating macrophages by treating SCID mice with liposomes containing dichloromethylene diphosphonate (Cl2MDP) improved engraftment of AML cells and UCB cells. SCID mice are deficient in T and B cells but have functional NK cells and macrophages. The Cl2MDP liposomes are phagocytosed by macrophages and the subsequent release of clodronate kills the macrophages (Van Rooijen and Sanders 1994). Elimination of macrophages in SCID mice enabled a higher engraftment of donor cells.

Using a similar approach, Liu and colleagues (Liu, Xiao et al. 2012) showed that Rag−/−γ−/− mice treated with gadolinium chloride (GdCl3) can tolerate allogeneic BALB/c donor cells. Rag−/−γ−/− mice are deficient in mature T, B and NK cells, having only functional macrophages. Adsorption
of GdCl$_3$ induces apoptosis of macrophages (Hardonk, Dijkhuis et al. 1992), thereby preventing the rejection of allogeneic cells.

Moreover, Shultz and colleagues (Shultz, Banuelos et al. 2003) demonstrated that engraftment in NOD/SCID/B2m$^{-/-}$ mice can be significantly enhanced by treating them with anti-CD122 antibodies. NOD/SCID/B2m$^{-/-}$ mice are deficient in T, B and NK cells, having only macrophages. The interaction between IL-2 and its cognate receptor IL-2R found on NK cells and macrophages is crucial for growth signal transduction. Anti-CD122 antibodies targets β chain of the IL-2R and terminate the signaling between IL-2 and IL-2R. The inhibition of macrophages in NOD/SCID/B2m$^{-/-}$ mice enabled higher engraftment of allogeneic cells.

The mechanism of macrophage-mediated rejection is not well known. Like NK cells, macrophages express both stimulatory and inhibitory receptors. In mice, the stimulatory and inhibitory receptors are called paired Ig-like receptors A and B, respectively (Blery, Kubagawa et al. 1998). The human equivalent of these receptors is known as Ig-like transcripts, leukocyte Ig-like receptors or monocyte/macrophage Ig-like receptors (Colonna, Navarro et al. 1997; Cosman, Fanger et al. 1997). Like NK cell receptors, macrophage stimulatory and inhibitory receptors have ITAM and ITIM cytoplasmic domains, respectively. Moreover, inhibitory receptors on macrophages bind to MHC class I proteins and this interaction leads to macrophage repression. The structural and functional similarities between macrophage and NK cell inhibitory receptors suggest that macrophage can, like NK cells, detect the absence of ‘self’.
1.5 Immunocompromised mouse models

Immunocompromised mouse models have become invaluable in understanding the in vivo role of immune cells in HSC graft rejection. The SCID mutation was first described on the C.B-17 mouse strain. The SCID mutation results in a defect in the DNA repair system, rendering T and B cells incapable of gene rearrangement to produce functionally mature T and B cells. Since its discovery, the SCID mutation was then crossed onto the NOD background to produce NOD/SCID mice. The NOD/SCID mice have additional reductions in NK cells and macrophage function than C.B-17/SCID mice (Shultz, Schweitzer et al. 1995). Although NK cells and macrophages are reduced in NOD/SCID mice, they are still capable of graft rejection. This is exemplified by several groups who showed that human cell engraftment can be improved by treating NOD/SCID mice with anti-CD122 antibodies that target NK cell and macrophages (Shultz, Banuelos et al. 2003; McKenzie, Gan et al. 2005).

To further improve donor cell engraftment, especially human donor cells, NOD/SCID mice were crossed with mice bearing the IL-2Rγ null mutation to produce NOD/SCID/gamma mice (Shultz, Lyons et al. 2005). The IL-2Rγ chain is involved in various cytokine signaling pathways important in NK cell development. The NK cell deficiency in NOD/SCID/gamma mice supports higher engraftment of donor cells compared to NOD/SCID mice (Shultz, Lyons et al. 2005) and anti-CD122 treated NOD/SCID mice (McDermott, Eppert et al. 2010).

1.6 Intercellular protein transfer

Cell function and identity is often characterized by its surface protein expression. For example, MHC class II proteins are only found on APCs such as B cells, dendritic cells and macrophages.
Almost 40 years ago, there were studies describing the presence of surface proteins not typically associated with the particular cell type (Bona, Robineaux et al. 1973; Hudson, Sprent et al. 1974; Sharrow, Ozato et al. 1980). These results were puzzling and challenged the views on cell phenotype and function at that time. Years later, *in vivo* and *in vitro* models provided insights into the mechanism, types of molecules transferred and the purpose of protein transfer.

1.6.1 Mechanisms of protein transfer

Proteins can be transferred from one cell to another through several mechanisms (Davis 2007). Firstly, proteins can be cleaved from one cell and interact with receptors on another cell. For instance, major histocompatibility complex class I homologues MIC molecules cleaved and shed from tumour cells can interact with NKG2D receptors on T and NK cells (Groh, Wu et al. 2002). Proteins can also be transferred in exosomes, which are protein-bearing vesicles formed from multivesicular bodies. It has been shown that MHC molecules transferred via exosomes secreted from APCs are functional (Raposo, Nijman et al. 1996; Zitvogel, Regnault et al. 1998). In addition to proteolytic cleavage and exosome release, the observation of membrane nanotubes forming between two cells prompted the idea that proteins can be transferred through these tubes (Rustom, Saffrich et al. 2004). However, there is a lack of definitive proof that these membrane structures support protein transfer (Davis 2007).

Recently, a new form of protein transfer called trogocytosis was described. Trogocytosis, derived from the Greek word ‘trogo’ meaning to gnaw or nibble, is a term used to describe the transfer of plasma membrane and its associated proteins from one cell to another (Joly and Hudrisier 2003). Protein transfer by trogocytosis is rapid and can occur as fast as a few minutes after co-culturing cells (Carlin, Eleme et al. 2001; Vanherberghen, Andersson et al. 2004). Cell-cell contact is
necessary for trogocytosis to occur and proteins are transferred as a whole and in large quantities (Russo, Zhou et al. 2000; Carlin, Eleme et al. 2001; Vanherberghen, Andersson et al. 2004). The exact mode by which trogocytosis occurs is still unknown, but current work suggests that proteins are transferred on cell membrane fragments or lipid rafts. It has also been postulated that this transfer may involve receptor-ligand interactions and actin cytoskeleton remodeling.

1.6.2 Cells involved in protein transfer

Intercellular protein transfer has primarily been documented to occur between immune cells. For example, T, NK cells, and APCs have been documented to capture MHC class I and II proteins from neighbouring cells. B cells can also acquire foreign proteins from other cells but these proteins are often internalized, processed, and presented on their own MHC molecules (Davis 2007).

Although not as prominently studied as intercellular protein transfer between immune cells, protein transfer between cells outside of the immune system has also been observed. For example, during the development of the eye in Drosophila, the bride of sevenless (boss) protein is transferred between cells (Cagan, Kramer et al. 1992). Moreover, cleaved EphB-ephrinB complexes are transferred between cells during axon guidance and migration (Zimmer, Palmer et al. 2003). Also, the CCR5 receptor for HIV-1 can be transferred onto CCR5-negative cells. The transferred CCR5 receptors are functional and can make CCR5-negative cells susceptible to HIV-1 infection (Mack, Kleinschmidt et al. 2000).
1.6.3 Purpose of protein transfer

Several possible functions for protein transfer have been proposed. For example, the transfer of MHC class II proteins onto CD4 T cells has implications for establishing tolerance to self (Patel, Arnold et al. 1999). Once CD4 T cells acquire MHC class II proteins from APCs, they can be presented to other CD4 T cells. The number of transferred MHC class II proteins on CD4 T cells is less than the number of MHC class II proteins presented by professional APCs such as dendritic cells. This presentation of a reduced number of MHC class II proteins by CD4 T cells to other CD4 T cells can induce anergy, the state of immune unresponsiveness.

Also, the transfer of MHC class I proteins onto APCs has been suggested as a mechanism of cross-presentation or cross-priming (Russo, Zhou et al. 2000). Cross-presentation is a process used by professional APCs to present extracellular antigens on MHC class I molecules. Conventionally, peptides presented on MHC class I proteins are derived from the cytoplasm of APCs. If an APC is infected or transformed, foreign peptides are presented and recognized by T cells, leading to the stimulation and proliferation of T cells. However, in an event where an infection or transformation does not affect APCs, then T cells cannot be stimulated and the appropriate immune response cannot be initiated. To overcome this barrier, APCs internalize and present extracellular antigens through the cross-presentation pathway. The transfer of whole MHC class I/peptide complexes from neighbouring cells may be a rapid, alternative mechanism of cross-presentation.

Moreover, the acquisition of donor MHC molecules by host APCs may influence allore cognition after transplantation (Herrera, Golshayan et al. 2004). T cell-mediated graft rejection can be initiated by two pathways: direct and indirect. In direct allore cognition, host T cells respond to
donor MHC/peptide complexes presented on donor APCs. In the indirect pathway, host T cells respond to donor antigens presented on self MHC molecules and self APCs. The ability of dendritic cells to acquire MHC molecules led Herrera and colleagues (Herrera, Golshayan et al. 2004) to postulate a third mechanism of allore cognition, called the semidirect pathway. In the semidirect pathway, host T cells respond to donor MHC molecules transferred onto host APCs.

1.7 Donor cell dosage

A potential drawback in HSCT is a having insufficient donor cells, especially in UCB transplants. The use of UCB in transplants not only reduces the risk of developing GvHD in transplants but it also allows more patients to receive treatment because UCB grafts with 1-2 HLA disparities can be tolerated in patients. Over the years, UCB has mainly been used to treat children because the CD34 cell dose in UCB is lower than a BM graft. Typically, BM grafts contain $3 \times 10^6$ CD34 cells/kg host weight while UCB grafts contain about $2.5 \times 10^6$ CD34 cells/kg host weight but can contain less than $1.7 \times 10^5$ CD34 cells. Transplants with grafts containing less than $1.7 \times 10^5$ CD34 cells or $1.8 \times 10^7$ nucleated cells/kg host weight usually had unsatisfactory outcomes (Wagner, Barker et al. 2002). Problems with low cell dosage include slow engraftment rates and low engraftment success. To solve this problem of cell insufficiency, various groups have looked into infusing patients with multiple UCB units or combining cells from different sources, and HSC expansion.

1.7.1 Infusing patients with cells from multiple donors

In adult UCB recipients, CD34 cell dose is positively correlated with survival (Laughlin, Barker et al. 2001). Insufficient CD34 and nucleated cells in a single cord for an adult host is a major
problem impeding the use of UCB in adult patients. To increase the CD34 and nucleated cell
dose in UCB grafts, Barker and colleagues (Barker, Weisdorf et al. 2005) infused patients with
two partially HLA-matched UCB units. Although two cords are transplanted, only one
dominated and contributed to donor chimerism. Neither CD34 cell dose nor degree of HLA-
matching predicted which of the two cords would dominate. In fact, even low CD34 cell dose
UCB units (1.5x10^5 cells/ kg host weight) were able to engraft and dominate in double cord
transplants. However, it is interesting to note that the dominating cord always had a higher dose
of CD3 cells. This implies that T cell content in a cord influences engraftment success. Kim and
colleagues (Kim, Chung et al. 2004) found similar results when they performed double cord
transplants in NOD/SCID mice. Dominance of one cord was seen in mice transplanted with
cords containing total nucleated cells. On the other hand, engraftment of both cords was
sustained in mice transplanted with cords depleted of lineage-committed cells. Again, this
confirms that mature immune cells can influence engraftment success.

1.7.2 HSC expansion

To increase the number of HSC, several methods have been developed to expand HSC from
UCB. Efforts are focused on UCB expansion because expansion of HSC derived from BM and
peripheral blood has been unsuccessful. Altogether, current results demonstrate that HSC can be
expanded, though some methods are more efficient than others, and the expanded cells are safe
to use in patients. However, for precautionary reasons, expanded cells have always been
transplanted with an unmanipulated cord in preclinical and clinical trials. Whether
transplantation of the expanded cells alone can support long-term engraftment in humans is not
yet known.
1.7.2.1 Cytokine cocktails

To expand HSC, several groups have used different cytokine cocktails. Our group (Rogers, Yamanaka et al. 2008) has developed a serum- and feeder-free culturing system that supports the proliferation of long term repopulating cells over differentiated cells. In this system, HSCs from UCB were cultured in the presence of Fibroblast growth factor 4 (Fgf4), Stem cell factor (SCF) and Flt3-ligand to achieve a modest 3.5-fold increase in long term repopulating cells. Shpall and colleagues (Shpall, Quinones et al. 2002) cultured HSC in a different combination of cytokines (SCF, thrombopoietin and G-CSF) and also obtained a modest 4-fold increase in HSCs. In their preclinical studies, Shpall and colleagues showed that expanded HSCs are safe to use in patients. Although expanded HSCs using cytokine cocktails are safe to use in patients, the modest increase in HSCs from one cord may not be enough for a transplant, which requires >1.7x10^5 CD34 cells/ kg host weight.

1.7.2.2 Notch-ligand mediated expansion

Delaney and colleagues (Delaney, Varnum-Finney et al. 2005; Delaney, Heimfeld et al. 2010) used a different approach to expand HSC ex vivo. In their approach, they harnessed the molecular and signaling pathways involved in governing cell fate and renewal in HSC. One pathway is the Notch-ligand signaling pathway which is involved in the development of several tissues including the central nervous system. Similarly, CD34 cells express Notch receptors and signaling through these Notch receptors enhances stem/progenitor proliferation and impedes differentiation (Milner, Kopan et al. 1994; Varnum-Finney, Xu et al. 2000). In their approach, Delaney and colleagues cultured HSCs with immobilized Notch ligands and their system were able to expand CD34 cells 164-fold after 16 days of culture.
In a phase I clinical trial, patients were transplanted with one unmanipulated cord and one cord expanded using the Notch-ligand system. Neutrophil engraftment was significantly faster in patients infused with one unmanipulated and one Notch-expanded cord than patients receiving two unmanipulated cords. Like double cord transplants, one cord eventually dominated and contributed to long term engraftment. The unmanipulated cord dominated more often, presumably because of the lack of T cells in the expanded cord. Delaney and colleagues noted that the expanded cord contributes to the myeloid lineage and to B cells but does not produce T cells.

1.8 Reprogramming blood cells

In 2006, Takahashi and Yamanaka (Takahashi and Yamanaka 2006) demonstrated that ectopic expression of just four transcription factors (c-Myc, Klf4, Oct4 and Sox2) in lineage-committed cells can reprogram these cells to a pluripotent state. These reprogrammed cells were termed induced pluripotent stem cells (iPSCs). Fibroblasts have been traditionally used for reprogramming because of their high reprogramming efficiency. To collect fibroblasts from patients, a skin biopsy is performed but this procedure can cause discomfort to the patient. On the other hand, blood cells can be easily collected from patients and hence, have been targeted for reprogramming. Recent studies show that stem/progenitor and terminally differentiated blood cells can also be reprogrammed (Brown, Rondon et al. 2010; Loh, Hartung et al. 2010; Seki, Yuasa et al. 2010). All blood-iPSCs show characteristics of pluripotent stem cells, including correct localization of pluripotency markers (such as Oct4, Nanog, and Tra1-60) as determined by immunocytochemistry, and the ability to form embryoid bodies and/or teratomas that contain all three germ layers (endoderm, ectoderm and mesoderm).
1.8.1 Methods of reprogramming

To successfully reprogram cells, transgene containing the Yamanaka or reprogramming factors must be introduced into the cell. There are several methods of reprogramming cells and they can be broadly categorized into genomic integrative or non-integrative methods. For non-viral methods, reprogramming factors can be introduced into the cells using liposomes or by electroporation. To reduce the number of integration sites, there is a trend towards replacing single factor vectors with vectors containing all four Yamanaka factors in tandem. Each reprogramming method has its advantages and disadvantages in terms of transfection and reprogramming efficiencies and whether it will cause genomic alterations that will affect the use of the iPSCs in clinical applications.

1.8.1.1 Methods involving genomic integration

Viral systems, such as retrovirus (Takahashi and Yamanaka 2006) and lentivirus (Yu, Vodyanik et al. 2007), are popular reprogramming methods because of their high infection and reprogramming efficiencies. Retroviruses are suitable for infecting dividing cells while lentiviruses are able to infect both dividing and non-dividing cells. Retrovirus and lentivirus with constitutively active promoters are uncontrollably silenced by the host cells after about two weeks post infection. The introduction of a drug-inducible promoter on lentiviruses gives researchers temporal control over transgene expression (Brambrink, Foreman et al. 2008).

Despite the high reprogramming efficiency with viral systems, iPSC lines produced from retrovirus and lentivirus are not recommended for use in a clinical setting because the transgenes cannot be removed from the host genome. Transgenes can be hazardous because they can be
integrated into vital genomic regions and disrupt normal cell function. Also, there is a risk that the transgenes will spontaneously turn ‘on’ as time progresses and generate teratomas in patients. In order to make transgene-free iPSC lines, viral vectors have been modified to include loxP terminal sequences (Hanna, Wernig et al. 2007). Transgenes can then be removed via the transient introduction of Cre recombinase.

The piggyBac transposon system is an alternative reprogramming method involving genomic integration (Woltjen, Michael et al. 2009). This system uses a transposase to ‘cut and paste’ transposons containing the Yamanaka factors into the host genome. Cells are transfected with a transposon containing the reprogramming factors and a non-integrating plasmid coding for the transposase because the transposase is only required transiently for transposon integration. The transgenes are preferentially integrated into site containing TTAA sequences (Cary, Goebel et al. 1989). The expression of transgene can be temporally controlled by having a drug-inducible promoter such as a tetracycline-inducible promoter. After a stable iPSC line is produced, transgenes can be excised by re-introducing the piggyBac transposase. Unlike the cre/loxP recombination system that leaves a loxP sequence behind after transgene removal, the piggyBac transposon system allows for footprint-free removal of transgene. The ability to remove without a footprint is beneficial because the host genome is left unchanged (conserved), there is no risk of transgene reactivation and iPSC differentiation is not hindered by possible basal expression of transgene.

1.8.1.2 Methods involving no genomic integration

Adenovirus, Sendai virus, episomal vectors and mRNA/protein are examples of non-integrating reprogramming methods (Gonzalez, Boue et al. 2011). The non-integrating reprogramming
factors are lost with cell division and often require multiple transfections. For instance, reprogramming using recombinant reprogramming proteins require multiple rounds of transfection, with the number of transfection rounds positively correlating with iPSC colony formation (Kim, Kim et al. 2009). Reprogramming with synthetic mRNA requires even more rounds of transfection (daily transfections for up to 17 days) to produce iPSCs (Warren, Manos et al. 2010).

Although there is a safety appeal with these non-integrating methods, they typically have low transfection and reprogramming efficiencies. For example, the transfection efficiency of episomal vectors is two orders of magnitude less than lentiviral transfection, resulting in significantly fewer reprogrammed cells (Yu, Vodyanik et al. 2007; Yu, Hu et al. 2009). Likewise, the efficiency of generating iPSCs from human (Zhou and Freed 2009) and mouse fibroblasts (Stadtfeld, Nagaya et al. 2008) using adenoviruses is <0.0002%, which is over two orders of magnitude less efficient than using integrative viral systems (Takahashi and Yamanaka 2006).

### 1.8.2 Successfully reprogrammed blood cell types

Some studies show that the differentiation stage of a hematopoietic cell influences reprogramming efficiency. For instance, Eminli and colleagues (Eminli, Foudi et al. 2009) demonstrated using a secondary reprogramming system that stem/progenitor cells reprogram 300 times more efficiently than mature T and B cells. Similarly, Hanna and colleagues (Hanna, Markoulaki et al. 2008), also using a secondary system, showed that immature B cells reprogram more efficiently than terminally differentiated B cells. However, there has not yet been a study that compares the reprogramming efficiency of stem/progenitor cells, T cells and B cells using a
primary system. Primary iPSCs can be produced from various blood cell types, including stem/progenitor cells, T cells, and B cells, using both integrative and non-integrative reprogramming methods.

1.8.2.1 Stem and progenitor cells

Stem/progenitor cells can be reprogrammed using both genomic integrative and non-integrative methods. Loh and colleagues (Loh, Agarwal et al. 2009) provided the first evidence that human blood cells can be reprogrammed to generate iPSCs. Using retroviruses encompassing the four Yamanaka factors, peripheral blood CD34 cells can be reprogrammed at a frequency of about 0.01%. Other reports using integrative viral systems to reprogram stem/progenitor cells from cord blood (Giorgetti, Montserrat et al. 2009; Takenaka, Nishishita et al. 2010) and peripheral blood (Loh, Hartung et al. 2010) showed similar reprogramming efficiency. Reprogramming cord blood stem/progenitor cells using Sendai viruses showed greater success with a reprogramming efficiency of >0.1% (Ban, Nishishita et al. 2011).

1.8.2.2 T cells

Like stem/progenitor cells, groups have reprogrammed T cells using genomic integrative and non-integrative methods. Several groups have shown that mature, lineage-committed cells are more difficult to reprogram than stem/progenitor cells (Hanna, Markoulaki et al. 2008; Eminli, Foudi et al. 2009) presumably because stem/progenitor innately express numerous pluripotent, proliferative and self-renewal genes. Loh and colleagues (Loh, Hartung et al. 2010) used multiple rounds of lentivirus transfections to reprogram peripheral T cells. T cell-iPSCs were produced at a frequency of about 0.0008%-0.001%. Seki and colleagues (Seki, Yuasa et al. 2010;
Seki, Yuasa et al. 2012) showed that T cells can be reprogrammed using non-integrating Sendai virus at a rate of 0.1%.

1.8.2.3 B cells

Similar to stem/progenitor and T cells, viral system are not effective or efficient at reprogramming B cells. Wada and colleagues (Wada, Kojo et al. 2011) generated B cell-iPSCs using retroviral transduction at a frequency ranging from 0.0003%-0.0025%. However, Rajesh and colleagues (Rajesh, Dickerson et al. 2011) showed that B cell can be reprogrammed at a higher efficiency using episomal vectors.

1.8.3 Epigenetics and partially reprogrammed cells

As a cell differentiates and becomes lineage-committed, its genome acquires an epigenetic signature specific to mature cell types. These epigenetic changes include DNA methylation and histone modifications (Reik 2007). Upon transcription factor-mediated reprogramming, whether the epigenetics fingerprint reverts back to an embryonic state is debatable and still under investigation. Current work shows strong evidence that reprogrammed cells retain the epigenetic memory of their cell of origin. For instance, Kim and colleagues (Kim, Doi et al. 2010) showed that iPSCs derived from blood cells exhibited a DNA methylation pattern more similar to blood cells and had a higher propensity to form blood colonies than iPSC derived from dermal fibroblast. Moreover, Bar-Nur and colleagues (Bar-Nur, Russ et al. 2011) demonstrated that iPSC derived from pancreatic beta cells could differentiate into insulin-secreting cells more easily than embryonic stem cells or iPSC derived from non-beta pancreatic cells.
Epigenetic memory represents a barrier to reprogramming cells. To fully reprogram a cell, the complete set of pluripotent, self-renewal and proliferative genes must be reactivated. Incomplete reactivation of the full set of pluripotent, self-renewal and proliferative genes can lead to the generation of partially reprogrammed cells or reprogramming intermediates. Several groups (Mikkelsen, Hanna et al. 2008; Chan, Ratanasirintrawoot et al. 2009) showed that only a rare number of colonies become true iPSCs while the remaining colonies represents reprogramming intermediates. Although the morphology of these reprogramming intermediates is similar to true iPSCs, gene expression and epigenetic signature differ. In particular, some lineage-specific genes were downregulated while self-renewal and proliferative genes but not pluripotent genes were reactivated. This intermediate profile gives these partially reprogrammed cells the ability to proliferate but also retain the potential to differentiate into the cell of origin. The balance between retaining the original somatic cell characteristics while acquiring the proliferative ability of stem cells makes reprogramming intermediates ideal for cell therapy. Partially reprogramming CD34 HSCs using a drug-inducible promoter for the Yamanaka factors should result in a controllable system for proliferating HSCs that are usable in HSCT.

1.9 Goals of the study

Conditioning-related mortality and insufficient donor cell dose are barriers to successful HSCTs. To overcome these obstacles, non-myeloablative or reduced intensity conditioning regimens and HSC expansion methods have been developed. However, there are flaws associated with these current solutions. For instance, the milder non-myeloablative conditioning regimens have a greater risk of graft rejection and current HSC expansion methods either only produce moderate increases in HSC number or the differentiation of the expanded HSCs is biased towards the myeloid lineage. Therefore, there is a need for new solutions and improvements to these current
problems. In this study, we want to explore alternative new solutions to the problem of graft rejection and insufficient donor cell number. Over the years, the phenomenon of intercellular surface protein transfer has attracted attention. Molecules such as MHC class I proteins have been documented to transfer between cells and although the precise role of these transferred proteins remains elusive, current studies suggest that they have immunomodulatory roles. To shed some light onto this topic, we want to investigate the role of intercellular transfer in HSCT and its influence on graft rejection. Specifically, we want to investigate whether transferred MHC class I proteins have immunosuppressive roles. In addition, we want to explore the idea of partially reprogramming HSCs as a novel method of cell expansion to solve the ongoing problem of insufficient donor cell dose, particularly with UCB transplants. We reason that partially reprogrammed HSCs are an ideal source of donor cells because they should have iPSC-like proliferative properties and also retain blood properties because of epigenetic memory. Therefore, they should preserve the full differentiation potential of HSCs. The new knowledge on these issues from our study will advance the field of HSCT and also change the lives of patients on their journey to recovery.
1.10 Chapter 1 figures

**Figure 1.1: T cell allorecognition.** Host T cells can recognize foreign donor antigens via the a) direct and b) indirect pathway. In the direct pathway, host T cells recognize donor antigens presented on donor MHC molecules and donor APCs. In the indirect pathway, host T cells recognize donor antigens presented on host MHC molecules and host APCs. Figure adapted from (Archbold, Ely et al. 2008).
Figure 1.2: The ‘missing self’ hypothesis. NK cells express inhibitory receptors and stimulatory receptors. NK cell’s decision to lyse a target cell depends on the incoming inhibitory and stimulatory signals. In the presence of both signals, inhibitory signal is able to override the stimulatory signal. a) In the presence of self MHC molecules, NK cell’s lytic activity is repressed. b) The loss of self MHC molecules or the expression of the incorrect MHC molecules can trigger NK cell-mediated lysis. Figure adapted from (Kumar and McNerney 2005).
Chapter 2
Investigating the role of trogocytosis in HSCT

2.1 Introduction

HSCT is a medical procedure commonly used to treat patients with blood disorders or cancers. Cells used for transplantation can be derived from the bone marrow (Thomas and Storb 1970), peripheral blood (Bensinger, Weaver et al. 1995; Korbling, Przepiorka et al. 1995; Schmitz, Dreger et al. 1995) or UCB (Broxmeyer, Douglas et al. 1989). Conventionally, full myeloablative conditioning regimens that use high doses of immunosuppressive drugs and total body irradiation are used to pre-condition patients for transplants. Now, there is a growing trend towards using milder non-myeloablative or reduced intensity conditioning regimens in place of the conventional, highly toxic myeloablative regimens, especially in high risk patients such as older patients or patients with co-morbidities (Diaconescu, Flowers et al. 2004; Sorror, Maris et al. 2004). Despite the reduction in treatment-related mortality, these milder regimens are associated with lower rates of donor cell engraftment (Mattsson, Ringden et al. 2008).

By having a better understanding of the mechanism of donor cell rejection, we can develop new methods to improve donor cell engraftment after non-myeloablative or reduced intensity regimens. In our previous study (Yamanaka, Wong et al. 2009), we transplanted human UCB (hUCB) into NOD/SCID mice and assessed engraftment at various time points post transplantation. Flow cytometry analysis revealed that all surviving donor cells were positive for both donor and host MHC class I proteins. Detailed analysis showed that all the double MHC class I-positive cells were of donor origin, and host MHC class I proteins were transferred onto donor cells by trogocytosis. This was an unusual finding because MHC class I proteins identify
cells as self and non-self, and the presence of both host and donor MHC class I proteins on donor cells after transplantation suggests that they have a dual identity.

Considering that NOD/SCID mice retain residual NK cells and macrophage activity (Shultz, Schweitzer et al. 1995), we hypothesize that the transfer of host MHC class I proteins to the donor cells disguised them as ‘self’ and hence, protected them from NK cell-mediated and macrophage-mediated rejection in the NOD/SCID mice. Furthermore, we argue that in the NOD/SCID mouse model all engrafting cells are a result of the survival and expansion of a subset of transplanted cells that have undergone trogocytosis immediately after transplantation and are thus protected from residual NK cell and macrophage activity.

NK cells express inhibitory receptors that recognize self MHC class I proteins and they function following the ‘missing self’ hypothesis (Karre, Ljunggren et al. 1986). According to the ‘missing self’ hypothesis, down-regulation or the absence of self MHC class I proteins on donor cells can make these cells targets for NK cells. There are numerous studies validating the ‘missing self’ hypothesis, and demonstrating the importance of inhibitory receptor-self MHC class I protein interaction in repressing NK cell-mediated lysis (Ohlen, Kling et al. 1989; Held, Cado et al. 1996). A common theme in these studies is that as long as the MHC class I proteins and the inhibitory receptors are matched, cell lysis is prevented. In regards to our study, the transferred host MHC class I proteins on transplanted donor cells could inactivate host NK cells and macrophages through interactions with their inhibitory receptors.

In addition to NK cells, macrophages also express inhibitory receptors for self MHC class I proteins. The role of these macrophage inhibitory receptors is still unclear but their structural
similarity to NK cell’s inhibitory receptors suggests that they may share a similar function (Colonna, Navarro et al. 1997; Cosman, Fanger et al. 1997). Also like NK cells, macrophages have shown to play a substantial role in graft rejection (Terpstra, Leenen et al. 1997; Liu, Xiao et al. 2012).

Unlike NK cells and macrophages, which are activated by the absence of self MHC class I proteins, T cells are activated by the detection of non-self MHC class I proteins through the direct or indirect allorecognition pathway (Fleischhauer, Kernan et al. 1990). Hence, the acquired host MHC class I proteins do not protect donor cells from T cell-mediated rejection. In our present study, we want to understand the contribution of NK cells and macrophages to graft rejection in HSCT by modeling human HSCT using intraspecific (mouse-to-mouse) and xenotransplantations (human-to-mouse).

We hypothesize that donor cell survival and successful engraftment is dependent on acquisition of host MHC class I proteins after non-myeloablative or reduced intensity conditioning regimens. The transferred host MHC class I proteins disguise donor cells as ‘self’, and protect them from host immune cells that express inhibitory receptors for self MHC class I proteins, such as NK cells and macrophages. Confirming that NK cell-mediated and macrophage-mediated lysis is naturally blocked through trogocytosis will allow for the development of conditioning regimens that can take advantage of trogocytosis and may lead to improved engraftment rates and decreased treatment-related mortality.
2.2 Materials and Methods

2.2.1 Ethics statement

Hospital personnel collected patient consent and UCB samples. Samples collection was approved by the University of Toronto and Mount Sinai Hospital Research Ethics Boards. All animal work was approved by the Animal Care Committee at the Toronto Centre for Phenogenomics.

2.2.2 hUCB collection, processing and cryopreservation

hUCB samples were collected at the time of delivery by trained hospital personnel following protocols approved by the Mount Sinai Hospital and University of Toronto Human Ethics Committee. Only samples tested negative for HIV I/II, HTLV-I/II, Hepatitis B (HBs Ag), Hepatitis C (anti HVC), CMV and VDRL were used in our study.

When hUCB samples were received, samples were processed by removing RBCs using Pentaspan (Bristol-Myers Squibb). Pentaspan was added to the UCB at a blood:Pentaspan ratio of 1:5 and centrifuged at 50 g, 10 °C for 10 min to pellet RBCs. The supernatant was collected and centrifuged at 400 g, 10 °C for 10 min. The cell pellet was then resuspended in freezing buffer consisting of 45% DMEM/ 45% serum/ 10% dimethyl sulphoxide (Edwards Lifesciences). After processing, samples were cryopreserved and stored in liquid nitrogen until use.

2.2.3 Mice

NOD/SCID and NOD/SCID/gamma mice were used as hosts, and C57BL/6 and FVB/NJ mice were used as donors. Mice were housed under pathogen-free conditions at the Toronto Centre for
Phenogenomics. All mice, aged 7-9 weeks, were purchased from the Toronto Centre for Phenogenomics or the Jackson Laboratory (USA).

NOD/SCID and NOD/SCID/gamma mice were irradiated 320 cGy and 200 cGy, respectively, using a GammaCell40 irradiator four hours prior to transplantation. For GdCl$_3$-treated mice, GdCl$_3$ (Sigma 439770-5G) was dissolved in sterile saline and administered intravenously at a dose of 20 mg/ kg host weight 24h prior to irradiation. Pre-conditioned hosts were transplanted 3.5 million (mouse BMC) or 10 million (hUCB) donor cells intravenously. All intravenous injections were done by Jennifer Whiteley. Mice were euthanized at different time points using CO$_2$.

2.2.4 Flow cytometry

Murine hosts were sacrificed from one week to eight weeks post transplantation. Peripheral blood was collected by heart puncture and femurs and tibias were excised and flushed for BMCs using 1 ml of PBS/ 2% FBS. Retrieved cells were centrifuged at 400 g, 10 °C for 5 min. Cell pellet was resuspended in 1 ml RBC Lysis Buffer to remove RBCs and centrifuged at 400 g, 10 °C for 5 min. RBC Lysis Buffer was removed after centrifugation and cell pellet was resuspended in PBS/ 2% FBS. Before staining, cells were incubated with anti-CD16/32 antibody (BD Pharmingen, USA) for 10 min in ice to block unspecific binding of antibodies to Fc (CD16/CD32) receptors. 7-AAD and antibodies against H-2K$^d$, H-2K$^b$, H-2K$^q$, HLA-ABC and CD45 were purchased from BD Pharmingen. All antibodies were titrated and cells were stained using the optimal concentrations for 30 min on ice. The optimal dose was determined by staining cells with varying concentrations of antibodies to obtain a concentration that gave the highest positive to negative signal ratio. The optimal concentration for antibodies against H-2K$^d$, H-2K$^b$,
H-2K\textsuperscript{d} and CD45 is 0.5 µg/ 10\textsuperscript{6} cells and the optimal concentration for the antibody against HLA-ABC is 0.025 µg/ 10\textsuperscript{6} cells. Corresponding isotype controls were used at the same concentration to determine unspecific and background signals and to discriminate between a positive and a negative signal. Isotype controls were used for every mouse in every experiment and isotype control graphs are shown as supplementary figures in Section 2.6. After incubation, cells were washed with 1 ml PBS/ 2% FBS and centrifuged at 400 g, 10 °C for 5 min. Cells were resuspended in 200 µl PBS/ 0.2% FBS and analyzed immediately using a Gallios Flow Cytometer (Beckman Coulter).

2.2.5 Trogocytosis-positive donor cell culture

Double MHC class I-positive cells were sorted for using a BD FACSArray and collected in DMEM/ 50% FBS by Annie Bang (Mount Sinai Hospital, Canada). Cells were centrifuged and cultured in DMEM/ 10% FBS/ antibiotics at 37 °C, 5% CO\textsubscript{2}. After culture, cells were collected and centrifuged at 400 g, 10 °C for 5 min to pellet cells. Cells were stained in the same procedure as described above and analyzed using a Gallios Flow Cytometer (Beckman Coulter).

2.2.6 Stromal and blood cell culture

Stromal cells were isolated from C57BL/6 mice following an established protocol (Zhu, Guo et al. 2010). Briefly, femurs and tibias from a C57BL/6 mouse were flushed with 3 ml PBS three times to remove the blood cells. The bone segments were then cut into small bone chips and washed with antibiotics. After the wash, the bone chips were cultured in DMEM/ 10% FBS/ 1 mg collagenase per 1 ml of media for one hour at room temperature. The media was discarded and the bone chips were then cultured in DMEM/ 10% FBS/ antibiotics for three days without changing media. When stromal cells started to appear on the third day of culture, media was
changed every other day. Bone chips were removed when stromal cells were about 30% confluent.

Blood cells were isolated from Balb/c mice following the protocol described in Section 2.2.4. Mononuclear blood cells were isolated using Ficoll (Sigma) and added to the stromal cell culture. The ratio of stromal: blood cells was approximately 1:1.5. Blood cells were cultured with stromal cells for three days before they were stained for MHC class I proteins and analyzed using flow cytometry.

2.2.7 Statistics

To determine if donor cell engraftment was different depending on the mouse strain used for donor cells, a two-way ANOVA followed by a Bonferroni post test were used to compare the two treatment groups. Significance was accepted as *p < 0.05. To determine if there was a significant loss of trogocytosis-positive cells after culture, a Student’s t-test was used. The same test was used to compare the difference in trogocytosis-positive cells between irradiated and non-irradiated, and GdCl$_3$-treated and non-treated NOD/SCID/gamma mice. All tests were completed using GraphPad-Prism software (La Jolla, California, USA).

2.3 Results

2.3.1 Antibodies against H-2K$^d$, H-2K$^b$, H-2K$^q$ and HLA-ABC do not cross react

To ensure that antibodies against H-2K$^d$, H-2K$^b$, H-2K$^q$ and HLA-ABC were specific to NOD/SCID and NOD/SCID/gamma cells, C57BL/6 cells, FVB/NJ cells, and human cells,
respectively, cross-reactivity tests were performed (Figure 2.1-2.6). IgG isotype controls (a, c, e) were used to gate and distinguish between positive and negative cell populations. All antibodies were specific and only stained for their respective cell types.

2.3.2 Trogocytosis protects donor cells from rejection in NOD/SCID mice

In order to prove that trogocytosis is not a product of xenotransplantation, a mouse intraspecific transplantation model was established. 3.5x10⁶ allogeneic C57BL/6 BMCs (H-2Kᵇ) were transplanted intravenously into sublethally irradiated (320 cGy) NOD/SCID mice (H-2Kᵈ). Animals were sacrificed weekly for up to five weeks post transplantation and the BMCs were isolated and subjected to flow cytometry analysis. At one week post transplantation, approximately 55% of the BMCs were of donor origin (H-2Kᵇ+) with an average of 91% (range: 88-97%) of them expressing both host and donor MHC class I protein (H-2Kᵈ+/H-2Kᵇ+), also referred to as trogocytosis-positive cells (Figure 2.7A). These results were similar to what was seen with our previous xenotransplantations where the majority of the human donor cells (95%+) were trogocytosis-positive (Yamanaka, Wong et al. 2009). However, unlike the hUCB cells used in the xenotransplantation study, allogeneic mouse donor cells can mature and become functional in NOD/SCID hosts. Full donor chimerism was achieved at two weeks post transplantation which suggests that stem and progenitor blood cells had undergone trogocytosis. At two weeks post transplantation, about 71% (range: 65-75%) of the donor cells were trogocytosis-positive (Figure 2.7B). As the host BMCs were eliminated by donor cells during the establishment of full donor chimerism, the single H-2Kᵇ+ donor cells that have not undergone trogocytosis were no longer targeted for destruction, which explains the increase in single positive donor cell population (Figure 2.7B-E). By five weeks post transplantation, only about 17% of the donor cells were trogocytosis-positive while the remaining donor cells were single H-2Kᵇ+ (Figure
2.7E). The negative relationship between the percentage of trogocytosis-positive cells and total donor cells (H-2K\textsuperscript{b+}) as donor chimerism is established is depicted in Figure 2.8. This result confirms that cells that have undergone trogocytosis are protected from host NK cell-mediated and macrophage-mediated rejection. Once the host immune cells are replaced during engraftment by the transplanted donor cells, there is no longer a survival advantage for these trogocytosis-positive donor cells and trogocytosis-negative or single H-2K\textsuperscript{b+} donor cells are able to survive and proliferate.

### 2.3.3 The transfer of host MHC class I proteins is not strain specific

To confirm that the transfer of host MHC class I proteins is not limited to donor cells from one mouse strain, 3.5x10\textsuperscript{6} allogeneic FVB/NJ BMCs (H-2K\textsuperscript{q}) were transplanted intravenously into sublethally irradiated (320 cGy) NOD/SCID mice. At one week post transplantation, 85\% (range: 84-86\%) of the donor cells were trogocytosis-positive (Figure 2.9A). After donor chimerism was established at around two weeks post transplantation, the ratio of trogocytosis-positive to trogocytosis-negative donor cell population decreased (Figure 2.9B). We compared these results with those from the transplants with C57BL/6 donor cells (Figure 2.7) using a two-way ANOVA followed by a Bonferroni post test and we found that there was no significant difference (p = 0.9) between the percentage of trogocytosis-positive C57BL/6 and FVB/NJ donor cells at any given time points (Figure 2.10). These results confirm that trogocytosis is not limited to donor cells from one mouse strain and they also strengthen our hypothesis on the role of trogocytosis in protecting donor cells from rejection during the early stages of engraftment.
2.3.4 Trogocytosis-positive cells are of donor origin and result from protein transfer

In our previous xenotransplantation study (Yamanaka, Wong et al. 2009), we used a combination of fluorescence in situ hybridization (FISH) and colony forming unit (CFU) cultures to show that trogocytosis-positive cells arise from the intercellular transfer of host MHC class I proteins onto donor cells and not from cell fusion or the exchange of genomic material. To confirm that trogocytosis-positive cells from intraspecific transplantations also arise from intercellular protein transfer, we used flow cytometry to sort for trogocytosis-positive cells, cultured them overnight and analyzed the MHC expression after culture. If the trogocytosis-positive cells in our intraspecific transplantation resulted from cell fusion or from the transfer of the MHC gene, then the donor cells should be able to maintain the expression of both host and donor MHC class I proteins when cultured *ex vivo*.

Trogocytosis-positive cells were sorted from NOD/SCID/gamma mice transplanted with C57BL/6 donor cells. These cells were then cultured overnight, collected the next day and re-stained for H-2K<sup>d</sup> and H-2K<sup>b</sup>. A post-sort analysis showed that about 86% of the sorted cells were trogocytosis-positive. After 18 hours in culture, we used a Student’s t-test to determine that there was a significant decrease in trogocytosis-positive cell (p < 0.01). Specifically, only 36% of the sorted cells remained double positive while the rest of the cells were single H-2K<sup>b</sup>+. (Figure 2.11). This confirms what we observed with the previous xenotransplantation study (Yamanaka, Wong et al. 2009). The inability to maintain expression of host MHC class I protein expression after culture confirms that trogocytosis-positive cells arise from the transfer of host MHC class I proteins to donor cells and not a result of cell fusion.
The flow cytometry sort was not ideal with only about 86% of the collected cells being trogocytosis-positive. The culture of a mixture of trogocytosis-positive and trogocytosis-negative donor cells raises the question of whether the increase in the percentage of trogocytosis-negative cells after culture is due to the selective loss of trogocytosis-positive cells. Although we cannot exclude this possibility, this is unlikely because if trogocytosis-positive donor cells are selectively eliminated then donor chimerism would not be able to establish in our transplant models where >90% of the donor cells are trogocytosis-positive cells at one week post transplantation. Hence, these results along with those obtained in our previous study (Yamanaka, Wong et al. 2009) demonstrate that trogocytosis arise from the transfer of host MHC class I proteins onto donor cells.

2.3.5 Stromal cells can donate MHC class I proteins to blood cells but stromal cells do not acquire non-self MHC class I proteins

Blood cells are in close contact and constant communication with stromal cells in the bone marrow niche. The intimate communication between blood cells and stromal cells led us to reason that donor blood cells acquire most of the host MHC class I proteins from host stromal cells. To investigate whether there is bidirectional transfer of MHC class I proteins between blood cells and stromal cells, we assessed MHC expression on CD45+ (blood) cells and CD45- (stromal) cells. In this experiment, the BMCs were harvested before full donor chimerism was established. This allowed us to determine if host and donor stromal cells undergo trogocytosis. Cells were first sorted for CD45+ and CD45- populations (Figure 2.12A). Each population was then interrogated for host and donor MHC class I proteins (Figure 2.12B and C). Host (H-2K<sup>d</sup>) stromal cells made up 77% (range: 76-78%) of the CD45- cells while donor stromal cells (H-2K<sup>b</sup>) comprised 18% (17-19%) of the population (Figure 2.12B). None of the stromal cells
(CD45-) were double positive. In contrast, 59% (58-60%) of donor blood cells (CD45+) were double-positive for host and donor MHC class I proteins confirming that trogocytosis had occurred (Figure 2.12C). These results demonstrate that stromal cells do not acquire non-self MHC class I proteins. There could be an intrinsic property of stromal cells that prevents them from acquiring MHC class I proteins but the biological reason for this is unknown.

We used an in vivo model to show that stromal cells do not acquire non-self MHC class I proteins while blood cells readily acquire host MHC class I proteins. However, it is unclear whether host stromal cells can act as a source of host MHC class I proteins for trogocytosis. To investigate whether host stromal cells can donate MHC class I proteins to donor blood cells, we cultured stromal cells from C57BL/6 mice (H-2K^b) with mononuclear blood cells from Balb/c mice (H-2K^d). In confirmation with the above experiment, stromal cells did not acquire non-self MHC class I proteins (Figure 2.13A). Although stromal cells did not acquire non-self MHC class I proteins, they are able to donate their MHC class I proteins to blood cells (Figure 2.13B). After three days of culture, about 27.5% of the blood cells were trogocytosis-positive. These results are in line with the in vivo results and confirm that trogocytosis involves the unidirectional transfer of MHC class I protein from stromal cells to blood cells.

2.3.6 Trogocytosis also protects donor cells from rejection in irradiated NOD/SCID/gamma mice

Studies with NOD/SCID mice as hosts demonstrated that NK cells and macrophages are prevented from lysing donor cells that have undergone trogocytosis. In order to separate the role of NK cells from that of macrophages we used NOD/SCID/gamma mice as hosts. NOD/SCID/gamma mice are similar to NOD/SCID mice in that they lack T and B cells but the
additional null mutation renders them deficient in NK cells. These mice enabled us to study the role of trogocytosis in transplantation and engraftment in the absence of T, B and NK cells, and allowed us to focus on the impact of macrophages.

3.5x10^6 allogeneic C57BL/6 donor BMCs were transplanted intravenously into sublethally irradiated (200 cGy) NOD/SCID/gamma mice (H-2K^d). Hosts were sacrificed weekly for up to four weeks post transplantation. At one week post transplantation, about 76.5% (range: 73-80%) of the donor cells were trogocytosis-positive (Figure 2.14A). Full donor chimerism was achieved at two weeks post transplantation. From two weeks post transplantation and onwards, we saw a gradual decrease in trogocytosis-positive donor cells and an increase in trogocytosis-negative donor cells (Figure 2.14B-D). By four weeks post transplantation, the majority of donor cells were trogocytosis-negative (Figure 2.14D). These results were similar to those with NOD/SCID mice transplanted with C57BL/6 BMCs (Figure 2.7). Also similar to NOD/SCID mice transplanted with C57BL/6 BMCs, there was a negative relationship between percentage trogocytosis-positive cells and total donor cells (Figure 2.15). This experiment clearly demonstrates that macrophages, like NK cells, can discriminate between cells expressing self and non-self MHC class I proteins and therefore do not attack donor cells that have undergone trogocytosis for MHC class I proteins. These results are in line with past studies that show that macrophages alone are sufficient at rejecting allogeneic donor cells (Shultz, Banuelos et al. 2003; McKenzie, Gan et al. 2005).
2.3.7 Host irradiation influences donor cell survival in NOD/SCID/gamma mice through macrophage activation

Macrophages are activated following exposure to ionizing radiation (Lorimore, Coates et al. 2001; Pandey, Shankar et al. 2005), and coupled with the fact that engraftment of donor cells has been reported in non-irradiated NOD/SCID/gamma mice, provides us with a system to test the influence of radiation-dependent activation on macrophages in donor cell rejection. We hypothesize that the pre-conditioning radiation used in our experiments activated host macrophages. Hence without irradiation, macrophages in NOD/SCID/gamma mice are not activated and are unable to attack donor cells. Therefore the population of donor cells that has not undergone trogocytosis will survive and proliferate, resulting in an increase in trogocytosis-negative donor cells at one week post transplantation.

As in the previous experiments, $3.5 \times 10^6$ allogeneic C57BL/6 BMCs were transplanted intravenously into non-irradiated NOD/SCID/gamma mice. As shown in Figure 2.16, the donor cell engraftment pattern differs between irradiated (Figure 2.14) and non-irradiated NOD/SCID/gamma mice. In non-irradiated mice, only about 43.5% (range: 42-45%) of the donor cells were trogocytosis-positive at two weeks post transplantation (Figure 2.16A). Compared to irradiated NOD/SCID/gamma mice at one week post transplantation (Figure 2.14), non-irradiated NOD/SCID/gamma at two weeks post transplantation had significantly less trogocytosis-positive donor cells ($p = 0.01$), despite the presence of host immune cells. The trogocytosis-positive donor cell population persisted for up to eight weeks post transplantation and contributed to about 38% (range: 37-39%) of the donor cells (Figure 2.16B). Throughout the eight weeks post transplantation, the single positive donor cells were not selected for lysis and removal despite the substantial presence of host immune cells because the macrophages in non-
irradiated NOD/SCID/gamma mice were not activated. In the absence of activated macrophages, both trogocytosis-positive and trogocytosis-negative donor cells were able to survive. Moreover, these results reinforce that trogocytosis is a natural event that occurs in our body and not a by-product of irradiation.

2.3.8 Macrophage-depleted NOD/SCID/gamma mice permit the survival of both trogocytosis-positive and trogocytosis-negative donor cells

To confirm that macrophages are responsible for the rejection of trogocytosis-negative donor cells in irradiated NOD/SCID/gamma mice, NOD/SCID/gamma mice were pre-treated with GdCl$_3$ before transplantation. GdCl$_3$ inhibits phagocytosis and eliminates macrophages (Hordonk, Dijkhuis et al. 1992). 3.5x10$^6$ allogeneic C57BL/6 BMCs were transplanted intravenously into sublethally irradiated (200 cGy) NOD/SCID/gamma hosts treated with GdCl$_3$ (20 mg/ kg host weight). NOD/SCID/gamma hosts treated with GdCl$_3$ had a different donor cell composition than non-treated hosts (Figure 2.14A: non-treated vs. Figure 2.17: treated). Treated hosts were more tolerant of trogocytosis-negative donor cells with an average of 42% (range: 34-50%) of the donor cells being trogocytosis-negative at one week post transplantation while the non-treated hosts only had an average of 24% (range: 20-27%). Compared to irradiated but non-treated NOD/SCID/gamma mice at one week post transplantation (Figure 2.14), irradiated and macrophage-depleted NOD/SCID/gamma mice at one week post transplantation had significantly less trogocytosis-positive donor cells (p = 0.04). This confirms that activated host macrophages target trogocytosis-negative donor cells and spare trogocytosis-positive donor cells.
2.3.9 Engrafted human donor cells are also trogocytosis-positive in NOD/SCID/gamma mice

Investigation of intraspecific transplantations with mice was important to understand the fundamentals of HSCT and graft rejection in a system that allows for the eventual establishment of a fully functional donor cell-derived immune system, but the ultimate question is whether the same phenomenon occurs in human HSCT. Xenotransplantations with immunocompromised mice are an invaluable tool to evaluate how human donor cells will behave in the human transplant setting. We have previously demonstrated that hUCB and hBMCs undergo trogocytosis and are protected from rejection in NOD/SCID mice (Yamanaka, Wong et al. 2009). Here, we are extending our xenograft studies to NOD/SCID/gamma mice to determine if macrophage-mediated rejection of human donor cells occurs.

1x10^7 hUCB (HLA-ABC) cells were transplanted intravenously into sublethally irradiated (200 cGy) NOD/SCID/gamma mice. As demonstrated in Figure 2.18, trogocytosis-positive cells were protected from lysis during early engraftment stages. Human cells colonized the host mice at a slower rate than mouse donor cells do, but we observed at one week post transplantation that donor cells contributed ~3% (range: 2.5-3.7%) of the total cells and 88% (range: 81-93%) of these cells were trogocytosis-positive (Figure 2.18A). By the second week, donor cell contribution increased to about 12% (range: 1.6-20%) and trogocytosis-positive donor cells made up about 77% (range: 67-84%) of these cells (Figure 2.18B). We observed that by three weeks post transplantation the donor cell contribution increased to 23% which is similar to the xenografts using NOD/SCID hosts but trogocytosis was observed in only 34% of the donor cells (range: 21-47%). This result was different from what we previously observed in NOD/SCID mice transplanted with hUCB, where trogocytosis-positive donor cells comprised >95% of all
donor cells (Yamanaka, Wong et al. 2009). We can conclude that the increase in donor cell contribution with NOD/SCID/gamma hosts was not as dependent on trogocytosis as it was with NOD/SCID hosts. The rejection of trogocytosis-negative donor cells was more apparent with NOD/SCID hosts because they have both NK cells and macrophages. It is also possible that after the initial radiation of the hosts and subsequent activation of the macrophages any newly produced host macrophages that arise from the bone marrow are not activated and are unable to remove trogocytosis-negative donor cells. We do not observe this when mouse cells are used as donor cells because they engraft and achieve donor chimerism rapidly.

2.4 Discussion

Graft rejection has always been a complication faced by patients receiving non-myeloablative or reduced intensity conditioning regimens, but because of their milder toxicity than myeloablative regimens, they are the preferred regimens amongst patients and healthcare providers. In order to improve graft tolerance after non-myeloablative or reduced intensity conditioning regimens, a firm understanding of host-donor cell interactions and the criteria that make donor cells resistant to attack is required. Xenotransplantation models provide clinically relevant information, but there are many unknown xenograft-related transplantation barriers that hinder the understanding of basic donor-host cell interactions. Our study presented here focused on mouse intraspecific transplantations to develop a fundamental understanding of donor-host cell interactions in transplantation.

The development of immunocompromised mouse models has greatly advanced our understanding of immune cell function in transplantation by allowing us to study the influence of immune cell subsets in isolation. A DNA repair defect in NOD/SCID mice makes them deficient
in mature T and B cells, which enabled us to study the influence of NK cells and macrophages on graft rejection (Shultz, Schweitzer et al. 1995). NOD/SCID mice have reduced NK cell and macrophage activity, but the residual cells that are present are sufficient at rejecting transplanted cells (Shultz, Banuelos et al. 2003; McKenzie, Gan et al. 2005). Despite the presence of active NK cells and macrophages, donor cells are still able to engraft. This has always been a conundrum but here we demonstrate that a subset of donor cells is able to engraft and proliferate in NOD/SCID mice because of trogocytosis.

This study explored the role of trogocytosis in HSCT and revealed the importance of host MHC class I protein acquisition in donor cell survival and engraftment. Early post transplantation, almost all of the donor cells in the host bone marrow niche were double positive for both the host and donor MHC class I proteins. The presence of host MHC class I proteins disguised the donor cells and allowed them to escape immunological surveillance in NOD/SCID and NOD/SCID/gamma mice. However, when full donor chimerism was achieved, or when host immune cells were inactive or depleted, having host MHC class I proteins was no longer a requirement for donor cell survival and we saw a decrease in the ratio of trogocytosis-positive to trogocytosis-negative donor cells.

We also showed that trogocytosis–positive cells lose expression of host MHC class I proteins when cultured ex vivo and coupled with the FISH results in our previous study (Yamanaka, Wong et al. 2009) demonstrate that all trogocytosis-positive cells are of donor origin and result from the transfer of host MHC class I proteins onto donor cells. Although cell fusion on a few cells may have occurred and was undetected in our tests, such rare events are unlikely responsible for our observations. Several mechanisms of protein transfer have been described
including proteolytic cleavage, membrane nanotubes, exosome release and trogocytosis (Davis 2007). Looking at the extent and frequency of MHC class I protein transfer, trogocytosis is most likely the mechanism of transfer in our transplantation system. Our assumption that the transferred MHC class I proteins are intact because they were able to suppress NK cells and macrophages excludes proteolytic cleavage as the protein transfer mechanism. Next, membrane nanotubes are rare (Rustom, Saffrich et al. 2004) and hence cannot account for the large percentage of trogocytosis-positive cells. Exosomes containing MHC molecules are primarily released from APCs (Zitvogel, Regnault et al. 1998; Utsugi-Kobukai, Fujimaki et al. 2003; van Niel, Porto-Carreiro et al. 2006). We showed that stromal cells are a major source of MHC molecules and stromal cells have not been documented to secrete exosomes. Thus, it is unlikely that exosome release is the mechanism of transfer in our transplantation study. The most likely mode of protein transfer in our study is trogocytosis because a large quantity of intact MHC class I proteins can be transferred within a short period of time (Vanherberghen, Andersson et al. 2004). In a setting where the donor cells are under constant immune surveillance, the immediate transfer of protective host MHC class I proteins onto donor cells is crucial.

Although we showed that donor cells acquire a majority of the host MHC class I proteins from stromal cells in the bone marrow niche, evidence obtained from our previous (Yamanaka, Wong et al. 2009) and current study suggest that donor cells can acquire MHC class I proteins from other sources. In our previous xenograft study (Yamanaka, Wong et al. 2009), we showed that donor cells in the bone marrow and peripheral blood maintain expression of host MHC class I proteins for up to seven months post transplantation. Likewise, we found in this intraspecific HSCT study that host MHC class I protein expression is maintained on circulating donor cells and when donor chimerism is established. Coupled with the fact that MHC class I proteins are
constitutively internalized (Mahmutefendic, Blagojevic et al. 2007), there must be a constant supply of host MHC class I proteins from both stromal and non-stromal cells. Indeed, Herrera and colleagues (Herrera, Golshayan et al. 2004) demonstrated that endothelial cells can donate MHC class I proteins to immune cells.

The role of intercellular protein transfer via trogocytosis in determining donor cell survival and engraftment in HSCT has not been described before. Trogocytosis, derived from the Greek word ‘trogo’ meaning to gnaw or to nibble, is the transfer of cell surface proteins believed to occur through the transfer of large cell membrane sections or lipid rafts that contain both the lipid bilayer and intact functional proteins (Joly and Hudrisier 2003). The study of intercellular protein transfer has focused primarily on the transfer between immune cells (Bona, Robineaux et al. 1973; Hudson, Sprent et al. 1974; Hudson and Sprent 1976; Arnold, Davidian et al. 1997; Huang, Yang et al. 1999) but transfer between non-immune cells or between blood and non-blood tissues can also occur (Cagan, Kramer et al. 1992; Anderson, Yu et al. 1996; Brezinschek, Oppenheimer-Marks et al. 1999; Mack, Kleinschmidt et al. 2000; Zimmer, Palmer et al. 2003; Yamanaka, Wong et al. 2009).

The purpose of protein transfer between cells is still unknown, but current studies suggest that immune responses can be modulated by protein transfer. In particular, the transfer of MHC molecules has immunomodulatory roles. For instance, the transfer of MHC class II proteins onto CD4 T cells have implications for self-tolerance as presentation of these acquired MHC class II proteins to other CD4 T cells can induce anergy (Patel, Arnold et al. 1999). Moreover, the transfer of whole MHC class I/peptide complexes onto professional APCs has been suggested as a mechanism of cross-presentation (Russo, Zhou et al. 2000) and a novel pathway of T cell
allorecognition (Herrera, Golshayan et al. 2004). Our study supports the idea that transferred MHC proteins can modulate immune responses. Specifically, transferred host MHC I proteins can inhibit NK cell-mediated and macrophage-mediated donor cell destruction.

In our previous trogocytosis study (Yamanaka, Wong et al. 2009), we demonstrated using aggregation chimeras that about 10-15% of blood cells undergo trogocytosis in a non-transplanted host. The chimeras were kept in a pathogen-free facility so the trogocytosis was not due to an immune reaction or antigen presentation triggered by an exogenous pathogen, instead it represents the normal level of trogocytosis that occurs in our bodies. In the HSCT setting, it is likely that only about 10-15% of the transplanted donor cells undergo trogocytosis, but we observe close to 100% of the donor cells as trogocytosis-positive early post transplantation because these 10-15% donor cells are the only cells that escape host immune surveillance, engraft and repopulate the bone marrow.

Our intraspecies HSCT study demonstrates that even when only a small subset of donor cells is able to evade immune surveillance, they can engraft and full donor chimerism can be established. Xenotransplants using NOD/SCID mice results in an equilibrium being established between trogocytosis-positive donor cells and host NK cells and macrophages. The human donor cells are never able to establish full donor chimerism due to limitations of the mouse bone marrow niche, thus they are constantly attacked but are able to maintain long term engraftment at moderate levels due to the ability of the donor cells to maintain trogocytosis. We predict that although the speed of donor cell engraftment is reduced in the continued presence of host NK cells and macrophages, in the clinical setting full donor chimerism is achievable in an acceptable time
frame as a human HSCT would demonstrate similar engraftment kinetics as observed with our intraspecies model and not the xenograft model.

This study provides evidence that upon transplantation, a subset of donor cells acquire ‘natural’ protection from NK cells and macrophages through the acquisition of host MHC class I proteins. This protected subset of donor cells, presumably about 10-15% of the transplanted cells, is sufficient to form a fully functional immune system (full donor chimerism). However, these trogocytosis-positive donor cells are not protected from host T cells because T cells are activated by the presence of non-self MHC class I proteins and there are no counter signals from inhibitory receptors. Altogether, our results suggest that preconditioning procedures and immunosuppressive drugs against NK cells and macrophages are not necessary for donor cell survival and successful engraftment and a milder regimen that only targets T cells can be employed.
Figure 2.1: NOD/SCID/gamma cells only stain positive for FITC H-2K\textsuperscript{d} and not PE H-2K\textsuperscript{b}. 
Figure 2.1: NOD/SCID/gamma cells only stain positive for FITC H-2K<sup>d</sup> and not PE H-2K<sup>b</sup>. NOD/SCID/gamma BMCs were singly stained for a) FITC IgG, b) FITC H-2K<sup>d</sup>, c) PE IgG, and d) PE H-2K<sup>b</sup>, and double stained for e) FITC and PE IgG and f) FITC H-2K<sup>d</sup> and PE H-2K<sup>b</sup>. The FITC and PE fluorescence is represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. When singly stained, NOD/SCID/gamma cells only stained positive for FITC H-2K<sup>d</sup>. Even in the presence of PE H-2K<sup>b</sup> (double staining) NOD/SCID/gamma cells only stained positive for FITC H-2K<sup>d</sup>. This confirms that FITC H-2K<sup>d</sup> specifically binds to NOD/SCID/gamma cells and PE H-2K<sup>b</sup> does not bind to NOD/SCID/gamma cells.
Figure 2.2: NOD/SCID/gamma cells only stain positive for PE H-2K$^d$ and not FITC H-2K$^q$. 
Figure 2.2: NOD/SCID/gamma cells only stain positive for PE H-2K<sup>d</sup> and not FITC H-2K<sup>a</sup>. NOD/SCID/gamma BMCs were singly stained for a) PE IgG, b) PE H-2K<sup>d</sup>, c) FITC IgG, and d) and FITC H-2K<sup>a</sup>, and double stained for e) PE and FITC IgG, and f) PE H-2K<sup>d</sup> and FITC H-2K<sup>a</sup>. The PE and FITC fluorescence is represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. When singly stained, NOD/SCID/gamma cells only stained positive for PE H-2K<sup>d</sup>. Even in the presence of FITC H-2K<sup>a</sup> (double staining) NOD/SCID/gamma cells only stained positive for PE H-2K<sup>d</sup>. This confirms that PE H-2K<sup>d</sup> specifically binds to NOD/SCID/gamma cells and FITC H-2K<sup>a</sup> does not bind to NOD/SCID/gamma cells.
Figure 2.3: NOD/SCID/gamma cells only stain positive for PE H-2K^d and not FITC HLA-ABC.
Figure 2.3: NOD/SCID/gamma cells only stain positive for PE H-2K^d and not FITC HLA-ABC. NOD/SCID/gamma cells were singly stained for a) PE IgG, b) PE H-2K^d, c) FITC IgG and d) FITC HLA-ABC, and double stained for e) PE and FITC IgG, and f) PE H-2K^d and FITC HLA-ABC. The PE and FITC fluorescence is represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. When singly stained, NOD/SCID/gamma cells only stained positive for PE H-2K^d. Even in the presence of FITC HLA-ABC (double staining) NOD/SCID/gamma cells only stained positive for PE H-2K^d. This confirms that PE H-2K^d specifically binds to NOD/SCID/gamma cells and FITC HLA-ABC does not bind to NOD/SCID/gamma cells.
Figure 2.4: C57BL/6 cells only stain positive for PE H-2K$^b$ and not FITC H-2K$^d$. 
Figure 2.4: C57BL/6 cells only stain positive for PE H-2K<sup>b</sup> and not FITC H-2K<sup>d</sup>. C57BL/6 BMCs were singly stained for a) PE IgG, b) PE H-2K<sup>b</sup>, c) FITC IgG and d) FITC H-2K<sup>d</sup>, and double stained for e) PE and FITC IgG, and f) PE H-2K<sup>b</sup> and FITC H-2K<sup>d</sup>. The FITC and PE fluorescence is represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. When singly stained, C57BL/6 cells only stained positive for PE H-2K<sup>b</sup>. Even in the presence of FITC H-2K<sup>d</sup> (double staining) C57BL/6 cells only stained positive for PE H-2K<sup>b</sup>. This confirms that PE H-2K<sup>b</sup> specifically binds to C57BL/6 cells and FITC H-2K<sup>d</sup> does not bind to C57BL/6 cells.
Figure 2.5: FVB/NJ cells only stain positive for FITC H-2Kq and not PE H-2Kd.
Figure 2.5: FVB/NJ cells only stain positive for FITC H-2K^q and not PE H-2K^d. FVB/NJ BMCs were singly stained for a) FITC IgG, b) FITC H-2K^q, c) PE IgG and d) PE H-2K^d, and double stained for e) FITC and PE IgG, and f) FITC H-2K^q and PE H-2K^d. The PE and FITC fluorescence is represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. When singly stained, FVB/NJ cells only stained positive for FITC H-2K^q. Even in the presence of PE H-2K^d (double staining) FVB/NJ cells only stained positive for FITC H-2K^q. This confirms that FITC H-2K^q specifically binds to FVB/NJ cells and PE H-2K^d does not bind to FVB/NJ cells.
Figure 2.6: hUCB cells only stain positive for FITC HLA-ABC and not PE H-2K$^d$. 
Figure 2.6: hUCB cells only stain positive for FITC HLA-ABC and not PE H-2K^d. hUCB cells were singly stained for a) FITC IgG, b) FITC HLA-ABC, c) PE IgG and d) PE H-2K^d, and double stained for e) FITC and PE IgG, and f) FITC HLA-ABC and PE H-2K^d. The PE and FITC fluorescence is represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. When singly stained, hUCB cells only stained positive for FITC HLA-ABC. Even in the presence of PE H-2K^d (double staining) hUCB cells only stained positive for FITC HLA-ABC. This confirms that FITC HLA-ABC specifically binds to hUCB cells and PE H-2K^d does not bind to hUCB cells.
Figure 2.7: Trogocytosis protects donor cells from rejection during early post-transplantation in NOD/SCID mice.
Figure 2.7: Trogocytosis protects donor cells from rejection during early post transplantation in NOD/SCID mice. Sublethally irradiated (320 cGy) NOD/SCID mice were transplanted with $3.5 \times 10^6$ C57BL/6 BMCs and sacrificed at a) one week (n = 2), b) two weeks (n = 3), c) three weeks (n = 3), d) four weeks (n = 5), and e) five weeks (n = 2) post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2K$^d$ and donor H-2K$^b$. Host H-2K$^d$ and donor H-2K$^b$ are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2K$^d$+/H-2K$^b$+) to trogocytosis-negative (H-2K$^b$+) donor cells is written in green. At one week post transplantation, about 91% of the donor cells were trogocytosis-positive. This confirms that trogocytosis-positive cells are protected from NK cell-mediated and macrophage-mediated rejection in NOD/SCID mice. When donor chimerism was established at two weeks post transplantation, the percentage of trogocytosis-positive donor cells decreased. This indicates that once donor chimerism is achieved, trogocytosis is no longer required for donor cell survival.
Figure 2.8: Percentage of trogocytosis-positive cells decreases as donor chimerism is established in NOD/SCID mice. Sublethally irradiated (320 cGy) NOD/SCID mice were transplanted with $3.5 \times 10^6$ C57BL/6 BMCs and sacrificed at a) one week ($n = 2$), b) two weeks ($n = 3$), c) three weeks ($n = 3$), d) four weeks ($n = 5$), and e) five weeks ($n = 2$) post transplantation. Engraftment of donor cells occurred rapidly with most mice reaching full donor chimerism between 2-3 weeks post transplantation. As the donor cells replace the host immune system, donor cell survival is no longer dependent on trogocytosis and the percentage of trogocytosis-positive cells decreases. Error bars represent standard deviation.
Figure 2.9: Host MHC class I protein transfers onto donor cells derived from different mouse strains. Sublethally irradiated (320 cGy) NOD/SCID mice were transplanted with 3.5x10^6 FVB/NJ BMCs and sacrificed at a) one week (n= 2), and b) two – three weeks (n= 4) post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2K^d and donor H-2K^q. Host H-2K^d and donor H-2K^q are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2K^d+/H-2K^q+) to trogocytosis-negative (H-2K^q+) donor cells is written in green. Similar to NOD/SCID mice transplanted with C57BL/6, a majority of the donor cells were trogocytosis-positive cells at one week post transplantation, and the percentage of trogocytosis-positive donor cells was reduced at two weeks post transplantation.
Figure 2.10: Comparing percentage trogocytosis-positive cells in NOD/SCID mice transplanted with C57BL/6 and FVB/NJ donor cells. There was no significant difference in the percentage of trogocytosis-positive cells over time when NOD/SCID mice were transplanted with C57BL/6 or FVB/NJ donor cells (p = 0.9). For both strains, over 80% of the donor cells were trogocytosis-positive and as donor chimerism is achieved, the percentage of trogocytosis-positive cells decreased at the same rate.
Figure 2.11: The loss of host MHC class I proteins after culture reveals the true identity of trogocytosis-positive cells. Sublethally irradiated (200 cGy) NOD/SCID/gamma mice were transplanted with 3.5x10^6 C57BL/6 BMCs and sacrificed at one week (n = 2) post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2K^d and donor H-2K^b. a) H-2K^d+/H-2K^b+ cells were sorted for by Annie Bang and cultured overnight in DMEM/10%FBS. b) Cultured cells were collected and re-stained for host H-2K^d and donor H-2K^b. Host H-2K^d and donor H-2K^b are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2K^d+/H-2K^b+) to trogocytosis-negative (H-2K^b+) donor cells is written in green. A post-sort analysis showed that about 86% of the sorted cells were trogocytosis-positive and after 18 h in culture, only 36% of the sorted cells remained trogocytosis-positive. This confirms that trogocytosis-positive cells arise from the transfer of MHC class I proteins and that trogocytosis-positive cells are of donor origin.
Figure 2.12: Host and donor stromal cells do not acquire foreign MHC class I proteins.

GdCl$_3$-treated and sublethally irradiated (200 cGy) NOD/SCID/gamma mice were transplanted with 3.5x10$^6$ C57BL/6 BMCs and sacrificed at one week post transplantation (n= 2). Host BMCs were flushed from the femurs and tibias and stained for CD45, host H-2K$^d$ and donor H-2K$^b$. a) BMCs were gated based on the presence and absence of CD45 expression. b) MHC expression on CD45- (stromal) cells. c) MHC expression on CD45+ (blood) cells. Host H-2K$^d$ and donor H-2K$^b$ are represented on the Y-axis and X-axis respectively. Although trogocytosis had occurred on CD45+ (blood) cells, CD45- (stromal) cells remained single positive for their respective MHC class I protein. This shows that stromal cells do not acquire non-self MHC class I proteins.
Figure 2.13: Stromal cells donate MHC class I proteins to blood cells. Stromal cells were isolated from C57BL/6 mice (H-2K^b) and passage two stromal cells were co-cultured with mononuclear bone marrow cells from Balb/c mice (H-2K^d) for three days in DMEM/ 10% FBS/antibiotics. After three days in culture, all cells were collected, stained for FITC H-2K^d and PE H-2K^b, and analyzed using flow cytometry. The expression of CD45 was used to distinguish between stromal (CD45-) and blood (CD45+) cells. a) Stromal cells do not acquire H-2K^d from blood cells. b) Blood cells acquire H-2K^b from stromal cells. This shows that stromal cells are a source of MHC class I proteins for trogocytosis on blood cells.
Figure 2.14: Trogocytosis protects donor cell from rejection during early post transplantation in NOD/SCID/gamma mice. Sublethally irradiated (200 cGy) NOD/SCID/gamma mice were transplanted with 3.5x10^6 C57BL/6 BMCs and sacrificed at a) one week (n = 2), b) two weeks (n = 3), c) three weeks (n = 3), and d) four weeks (n = 4) post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2K^d and donor H-2K^b. Host H-2K^d and donor H-2K^b are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2K^d+/H-2K^b+) to trogocytosis-negative (H-2K^d+/H-2K^b+) donor cells is written in green. These results confirm that trogocytosis-positive cells are protected from macrophage-mediated rejection in NOD/SCID/gamma mice.
Figure 2.15: Percentage of trogocytosis-positive cells decreases as donor chimerism is established in NOD/SCID/gamma mice. Sublethally irradiated (200 cGy) NOD/SCID/gamma mice were transplanted with $3.5 \times 10^6$ C57BL/6 BMCs and sacrificed at a) one week ($n = 2$), b) two weeks ($n = 3$), c) three weeks ($n = 3$), and d) four weeks ($n = 4$) post transplantation. Similar to NOD/SCID mice, donor chimerism was established approximately 2-3 weeks post transplantation. Once donor chimerism was established, the percentage of trogocytosis-positive cells decreased because donor cell survival was no longer dependent on the acquisition of host MHC class I proteins. Error bars represent standard deviation.
Figure 2.16: Donor cell survival is not dependent on acquisition of host MHC class I proteins in non-irradiated NOD/SCID/gamma mice. Non-irradiated NOD/SCID/gamma mice were transplanted with $3.5 \times 10^6$ C57BL/6 BMCs and sacrificed at a) two weeks ($n = 2$), and b) eight weeks ($n = 2$) post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2K$^d$ and donor H-2K$^b$. Host H-2K$^d$ and donor H-2K$^b$ are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2K$^d$+/H-2K$^b$+) to trogocytosis-negative (H-2K$^b$+) donor cells is written in green. Without radiation, macrophages in NOD/SCID/gamma mice were not activated and failed to reject trogocytosis-negative donor cells. This suggests that macrophages in irradiated NOD/SCID/gamma mice mediate rejection of trogocytosis-negative donor cells.
Figure 2.17: Donor cell survival is not dependent on acquisition of host MHC class I proteins in macrophage-depleted NOD/SCID/gamma mice. GdCl₃-treated (20 mg/ kg recipient weight) (n = 4) and sublethally irradiated (200 cGy) NOD/SCID/gamma mice were transplanted with 3.5x10⁶ C57BL/6 BMCs and sacrificed at one week post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2Kᵈ and donor H-2Kᵇ. Host H-2Kᵈ and donor H-2Kᵇ are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2Kᵈ+/H-2Kᵇ+) to trogocytosis-negative (H-2Kᵇ+) donor cells is written in green. Trogocytosis-negative donor cells were not targeted for destruction in macrophage-depleted NOD/SCID/gamma mice. Together with results from the radiation experiment in Figure 2.16, it is confirmed that macrophages are responsible for rejecting trogocytosis-negative donor cells in irradiated NOD/SCID/gamma mice.
Figure 2.18: The transfer of host MHC class I proteins also protects hUCB cells from rejection in NOD/SCID/gamma mice. Sublethally irradiated (200 cGy) NOD/SCID/gamma mice were transplanted with $1 \times 10^7$ UCB cells and sacrificed at a) one week ($n = 3$), and b) two weeks ($n = 3$) post transplantation. Host BMCs were flushed from the femurs and tibias and stained for host H-2K$^d$ and donor HLA-ABC. Host H-2K$^d$ and donor HLA-ABC are represented on the Y-axis and X-axis respectively. The ratio of trogocytosis-positive (H-2K$^d$/HLA-ABC+) to trogocytosis-negative (HLA-ABC+) donor cells is written in green. Similar to our intraspecific transplants, hUCB donor cell survival in irradiated NOD/SCID/gamma mice during the early stages of engraftment is dependent on the acquisition of host MHC class I proteins.
2.6 Chapter 2 supplementary figures

Figure S2.1: Corresponding IgG isotype controls for Figure 2.7.
**Figure S2.1: Corresponding IgG isotype controls for Figure 2.7.** IgG isotype controls for BMCs flushed at a) one week, b) two weeks, c) three weeks, d) four weeks, and e) five weeks post transplantation from sublethally irradiated NOD/SCID mice transplanted with C57BL/6 BMCs. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
**Figure S2.2: Corresponding IgG isotype controls for Figure 2.9.** IgG isotype controls for BMCs flushed at a) one week and b) two weeks post transplantation from sublethally irradiated NOD/SCID mice transplanted with FVB/NJ BMCs. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
Figure S2.3: Corresponding IgG isotype controls for Figure 2.11. IgG isotype controls for a) BMCs flushed at one week post transplantation from sublethally irradiated NOD/SCID/gamma mice transplanted with C57BL/6 BMCs, and b) the same BMCs after 18 h in culture. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
Figure S2.4: Corresponding IgG isotype controls for Figure 2.12. a) APC IgG isotype control and b) FITC and PE IgG isotype control. For a), the graph is presented as a histogram. Any signal to the left of the gate is negative. For b), Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. For both graphs, gates were drawn so that the IgG isotype control is negative for both its respective fluorescence.
Figure S2.5: Corresponding IgG isotype controls for Figure 2.13. Stromal IgG and blood IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
**Figure S2.6: Corresponding IgG isotype controls for Figure 2.14.** IgG isotype controls for BMCs flushed at a) one week, b) two weeks, c) three weeks, and d) four weeks post transplantation from sublethally irradiated NOD/SCID/gamma mice transplanted with C57BL/6 BMCs. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
Figure S2.7: Corresponding IgG isotype controls for Figure 2.16. IgG isotype controls for BMCs flushed at a) one week and b) eight weeks post transplantation from non-irradiated NOD/SCID/gamma mice transplanted with C57BL/6 BMCs. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
Figure S2.8: Corresponding IgG isotype controls for Figure 2.17. IgG isotype controls for BMCs flushed at one week post transplantation from GdCl$_3$-treated and sublethally irradiated NOD/SCID/gamma mice transplanted with C57BL/6 BMCs. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
**Figure S2.9: Corresponding IgG isotype controls for Figure 2.18.** IgG isotype controls for BMCs flushed at a) one week and b) two weeks post transplantation from sublethally irradiated NOD/SCID/gamma mice transplanted with hUCB cells. Host IgG and donor IgG are represented on the Y-axis and X-axis, respectively. Any signal above and to the right of the horizontal and vertical gate, respectively, is positive. The distribution of cells in each quadrant is represented by the percentages. Gates were drawn so that the IgG isotype control is negative for both FITC and PE fluorescence.
Chapter 3
Partially reprogrammed blood cells as a source of donor cells for HSCT

3.1 Introduction

Allogeneic HSCTs are commonly used as a part of the therapy to cure malignant and non-malignant blood diseases. Donor cells can be derived from bone marrow, peripheral blood, or UCB. Compared to bone marrow and peripheral blood, UCB has the benefits of immediate availability, low risk to donor, lower risk of developing GvHD, and patient tolerability of 1-2 HLA disparities (Wagner, Barker et al. 2002).

A potential drawback of UCB is its limited cell dose. A single UCB unit often has inadequate cells for larger adults. Wagner and colleagues (Wagner, Barker et al. 2002) showed that patients receiving less than \(1.7 \times 10^5\) CD34 cells/ kg patient weight or \(1.8 \times 10^7\) nucleated cells/ kg patient weight have unfavourable survival outcomes. To overcome this issue of insufficient cell dose in a single UCB unit, Barker and colleagues (Barker, Weisdorf et al. 2005) transplanted two closely HLA-matched cords into adult patients. In addition to double cord transplants, efforts are put into expanding HSC to augment cell dose. We (Rogers, Yamanaka et al. 2008) and other groups including Shpall and colleagues (Shpall, Quinones et al. 2002) have expanded UCB using different cytokine cocktails, but only a moderate increase in HSC number was achieved. Harnessing the Notch-ligand signaling pathway, Delaney and colleagues were able to achieve clinically significant expansion of UCB (Delaney, Varnum-Finney et al. 2005; Delaney, Heimfeld et al. 2010).
However, it’s important to note that there were no mature T cells after expansion, and the expanded graft contributes exclusively to myeloid formation when transplanted into patients. Also, like double cord transplants, when one unmanipulated cord and one expanded cord were co-transplanted, the unmanipulated cord always prevailed. As Delaney and colleagues suggested, this may be due to the lack of T cells in the expanded cord (Delaney, Heimfeld et al. 2010). This lack of mature T cells after expansion raises the question of whether expanded cords alone can successfully engraft in patients, especially after undergoing milder non-myeloablative or reduced intensity conditioning regimens. This weakness calls for new expansion methods that preserve the differentiation potential of all blood lineages.

In recent years, blood cells have been targeted as an accessible source of cells for reprogramming. Loh and colleagues (Loh, Agarwal et al. 2009) were the first group to show that human cord blood cells can be reprogrammed using a viral system. Following their study, several groups have shown that blood cells can be reprogrammed using genomic integrative and non-integrative systems (Loh, Hartung et al. 2010; Seki, Yuasa et al. 2010; Ban, Nishishita et al. 2011). The question of whether reprogrammed cells retain the epigenetic memory of their cell of origin is highly debatable, but current studies strongly suggest that they do (Kim, Doi et al. 2010; Bar-Nur, Russ et al. 2011).

Realistically, only a rare number of colonies become true iPSCs while the remaining colonies are only partially reprogrammed (Mikkelsen, Hanna et al. 2008; Chan, Ratanasirintragewoot et al. 2009). These reprogramming intermediates can be distinguished from fully reprogrammed cells by their morphology, gene expression and epigenetic signature (Chan, Ratanasirintragewoot et al. 2009). We are interested in stable reprogramming intermediates that express, at minimum, self-
renewal and proliferative genes and blood cell markers. We reason that stable partially reprogrammed UCB CD34 cells can serve as an unlimited pool of donor cells for HSCT because they should have the ability to proliferate like iPSCs but also retain HSC properties.

Young UCB cells are the ideal cells to reprogram because they possess fewer somatic mutations and should reprogram more efficiently than adult cells (Eminli, Foudi et al. 2009). In our study, we used the piggyBac transposon system to reprogram cells. Our piggyBac transposon system involves the genomic integration of tetracycline-inducible reprogramming factors, giving us temporal control of transgene expression. The advantage of using the piggyBac system is that it can produce safe, transgene- and footprint-free cell lines through the transient re-introduction of the transposase (Woltjen, Michael et al. 2009; Yusa, Rad et al. 2009). For use in patients, it is crucial to use footprint-free cell lines to avoid any genomic alterations and possible reactivation of the transgenes.

3.2 Materials and Methods

3.2.1 Derivation of Oct4-GFP mice

Oct4-green fluorescent protein (GFP) mice were gifts from Dr. Andras Nagy’s group (Mount Sinai Hospital, Toronto). Detailed development of these mice is described elsewhere (Viswanathan, Benatar et al. 2003). All cells from these mice express GFP under the control of the endogenous Oct4 promoter. The Oct4 gene is a reliable pluripotency marker and is expressed in embryonic stem cells and iPSCs.
3.2.2 Mouse embryonic fibroblast (MEF) isolation

MEFs were isolated from day 12.5 Oct4-GFP embryos following published protocols (Woltjen, Michael et al. 2009). Briefly, embryos were decapitated, internal organs were removed and fibroblast cells were dissociated with 0.25% trypsin/ 0.1% EDTA. Fibroblasts were then cultured in DMEM/ 10% FBS/ antibiotics/ glutamax, expanded, and frozen down until use.

3.2.3 Tail tip fibroblast isolation

The adult mouse tail was cut to remove 1 cm from the tip and the skin of the tip was discarded. The tail tip was then dissected into 2-4 mm sections and plated in a 3 cm culturing dish with DMEM/ 10% FBS/ antibiotics. Half of the medium was changed 72 h later and fibroblast colony formation was monitored for the next 72 h. When single adherent cells or small colonies of 3-5 cells were observed, the remaining tail tip tissue was removed. Culture medium was changed every other day. When fibroblasts were 80% confluent, they were trypsinized and replated at 1:3 (passage two). Fibroblasts were either used immediately or frozen down and stored in liquid nitrogen for future use.

3.2.4 Mouse BMC isolation

BMCs were isolated from adult Oct4-GFP mice. Femurs and tibias were retrieved from the mice and BMCs were flushed from each segment using 1 ml of PBS/ 2% FBS. Flushed cells were centrifuged at 400 g, 10 °C for 5 min. Cell pellet was resuspended in 1 ml RBC Lysis Buffer and centrifuged again at 400 g, 10 °C for 5 min. The cell pellet was then washed with antibiotics and cultured in DMEM/ 10% FBS/ antibiotics at 37 °C, 5% CO₂ or cryopreserved for future use.
3.2.5 hUCB

For details, see Section 2.2.2

3.2.6 Plasmid constructs

The piggyBac transposase, reverse tetracycline transactivator, and reprogramming factors plasmids were gifts from Dr. Andras Nagy’s group (Mount Sinai Hospital, Toronto). The piggyBac transposase plasmid is a non-dividing and non-integrating plasmid that is expected to be lost with cell division after the reverse tetracycline transactivator and reprogramming factors plasmids are integrated into the cell’s genome. The plasmid encoding the reverse tetracycline transactivator is enclosed by piggyBac terminal repeats and has a constitutively active promoter. Two different configurations of the plasmid encoding the reprogramming factors were used. One plasmid consists of the four reprogramming factors in the following order: c-Myc, Klf4, Oct4, Sox2 (MKOS). The MKOS plasmid is linked to a β-geo reporter gene. The second plasmid consists of a different order of the four reprogramming factors: Oct4, Sox2, Klf4 and c-Myc (OSKM). The OSKM transgene is linked to a fluorescent mCherry marker reporter gene. Both plasmid constructs have a tetracycline-on promoter. The tetracycline-on promoter is activated when both reverse tetracycline transactivator and DOX are present. In our system, reverse tetracycline transactivator is expressed in the transfected cells and DOX is added to the culture.

3.2.7 Transfection and cell culture

Prior to transfection, MEFs, mouse BMCs and human UCB were cultured in DMEM/ 10% FBS/ antibiotics. Cells were collected and centrifuged at 400 g, 10 °C for 5 min. The cell pellet was resuspended in Resuspension Buffer R (Invitrogen). Plasmids were then added to the cell
mixture. Cells were electroporated using the Neon Transfection System (Invitrogen) or the Amaxa Nucleofector (Lonza) following the manufacturer’s protocol. After transfection, cells were cultured in mouse embryonic stem cell (mESC) media with (for mouse BMCs and human UCB) or without (for fibroblast) a feeder layer. Two days after transfection, DOX (1500 ng/ml) was added to the culture to induce the transcription of the reprogramming factors. Media was changed every other day and cells were cultured with DOX until iPSC colonies were established. For secondary reprogramming, DOX was added when tail tip fibroblasts were established (passage 2) and when BMCs were isolated.

3.2.8 Immunocytochemistry

Cells were grown on 12-well plates and fixed with 10% neutral buffered formalin (NBF). After fixing, cells were washed once with PBS before blocking buffer (PBS/ 10% FBS/ 0.1% triton X-100) was applied for 30 min at room temperature. Primary antibodies against Oct3/4 (SC-8628 diluted 1/500 and SC-5279 diluted 1/100), Nanog (REC-RCAB0002PF diluted 1/200 and REC-RCAB0004PF diluted 1/100), SSEA-1 (MAB4301 diluted 1/100) and Sox2 (MAB2018 diluted 1/100) were applied and incubated with the cells overnight at 4 °C. After the overnight incubation, cells were washed with PBS and secondary antibodies conjugated with either Alexa Fluor 488 or 594 (diluted 1/500) were applied to visualize the primary antibodies. DAPI staining was used to visualize the nucleus of each individual cell.

3.2.9 Teratoma formation

To form teratomas, 1x10^6 iPSCs in 100 µl solution was injected intramuscularly into the legs of immunocompromised NOD/SCID mice. Mice were monitored for teratoma formation and were
immediately euthanized when tumour growth was observed on day 21. Teratomas were fixed in 10% NBF, dehydrated and embedded in paraffin blocks. 5 μm sections were subjected to hematoxylin and eosin (H&E) staining. Hibret Adissu (Toronto Centre for Phenogenomics, Toronto) analyzed for and identified endoderm, mesoderm and ectoderm tissues in these teratoma sections.

3.2.10 Karyotyping

OGMKOS iPSCs were sent to Cytogenomic Services at the Hospital for Sick Children for karyotyping.

3.2.11 Microscopy

All microscopic images were taken on the Leica DM IL microscope using a Hamamatsu ORCA-03G digital camera.

3.2.12 Chimeric mice

Chimeric mice were made by aggregating MEF-iPSCs with C57BL/6 embryos, as described before (Tanaka, Hadjantonakis et al. 2001). MEF-iPSCs contribution was scored based on coat colour.
3.3 Results

3.3.1 Primary fibroblast iPSCs can be derived from Oct4-GFP MEFs using the piggyBac system

A review of the literature and experience from our own lab has determined that mouse cells are easier to reprogram than human cells. Although our goal is to reprogram hUCB cells, we first evaluated whether mouse blood cells were amenable to reprogramming using the piggyBac system. Cells derived from Oct4-GFP mice were used for reprogramming because having the expression of GFP under the control of pluripotency marker Oct4 promoter can help us identify early stage iPSCs. This is advantageous when the reprogramming frequency is very low.

To confirm that cells derived from Oct4-GFP mice are amenable to reprogramming, we started with reprogramming MEFs, the easiest cell type to reprogram (Figure 3.1). Plasmids were introduced into MEFs using two different electroporation programs on the Neon Transfection System: program 17: 850 V, 30 ms, 2 pulses and program 20: 1150 V, 30 ms, 2 pulses. The following plasmids were introduced into the MEFs: piggyBac transposase, reverse tetracycline transactivator and MKOS transgene. MKOS is under the control of the tetracycline promoter and the promoter is inducible using reverse tetracycline transactivator and DOX. The MKOS vector was used at a concentration of 2 µg plasmids per 100,000 cells. After transfection, cells were given two days to recover in mESC media. On the third day, DOX (1500 ng/ml) was added to the culture to induce transcription of the reprogramming factors.

Only MEFs transfected using program 20 gave rise to colonies. After eight days in culture, only one colony expressed GFP. On day 20, the GFP+ colony was picked, replated on feeders and
cultured in mESC media with DOX. On day 23, the replated colonies were dissociated and reseeded on fresh feeders in mESC media without DOX. Fully reprogrammed cells will continue to exhibit iPSC characteristics in the absence of DOX. Indeed, the colonies retained iPSC morphology and continued to express GFP in the absence of DOX, confirming that they were fully reprogrammed.

The established MEF-iPSC line (OGMKOS) was tested for the expression of pluripotency-associated markers (Oct4, Nanog and SSEA1) using immunocytochemistry (ICC) (Figure 3.2). Day 24 colonies exhibited subcellular localization of all pluripotency markers. The established colonies were also able to form teratomas exhibiting all three germ layers in NOD/SCID mice (Figure 3.3). Southern blot showed that the OGMKOS cell line only had one integrated transgene copy (data not shown). Also, OGMKOS iPSCs carry trisomy 8 (Figure 3.4). Trisomy 8 is commonly seen in embryonic stem cells and iPSCs because this genomic alteration gives them a growth advantage which allows them to take over in culture (Liu, Wu et al. 1997).

3.3.2 Primary blood iPSCs cannot be derived from Oct4-GFP BMCs using the piggyBac transposon system

Having successfully established iPSCs from Oct4-GFP MEFs, we attempted to establish iPSCs from Oct4-GFP BMCs. After numerous attempts, we were unsuccessful in deriving iPSCs from Oct4-GFP BMCs (Figure 3.5). We first used the Neon Transfection System to introduce the piggyBac transposase, reverse tetracycline transactivator and MKOS plasmids (1-2 µg/100,000 cells) into the cells. This system has worked well for other cell types such as fibroblasts and kidney cells. We tried different electroporation settings (850-1500 V, 10-30 ms, and 2-3 pulses) to optimize transfection and cell survival. By transfecting GFP plasmids, we determined that the
transfection efficiency was about 1/50000 viable cells for program 20 which was the best efficiency we could get. Using 1 μg of piggyBac transposase, reverse tetracycline transactivator and MKOS plasmids per 100,000 cells, we were able to get a few Oct-GFP+ single cells and ~3-4 Oct4-GFP+ colonies (Figure 3.6). However, these colonies did not proliferate.

We subsequently used the Amaxa Nucleofector (programs X-001 and Z-001) to transfect BMCs with piggyBac transposase, reverse tetracycline transactivator and OSKM plasmids. The OSKM configuration carries a fluorescent mCherry reporter gene which allowed us to track transfection rates in live cells. The transfection efficiency was about 1/25000 cells. Up to 14 μg plasmids per 100,000 cells were used in transfections. Again, we were only able to get a few GFP+/mCherry+ single cells and a couple of GFP+/mCherry+ colonies (Figure 3.7). Like the colonies produced from the Neon Transfection System, these colonies did not proliferate. It is possible that the low transfection rates of blood cells prevented critical amounts of vector DNA to accumulate in these cells. Although we achieved an iPSC line from fibroblasts that contained only one copy of the MKOS vector, it is possible that blood cells require multiple copies of the MKOS vector and this was not achievable due to low transfection rates.

### 3.3.3 Primary blood iPSCs cannot be derived from hUCB cells using the piggyBac system

Following the unsuccessful attempts at producing iPSC from mouse BMCs, we then tried to reprogram hUCB. Like mouse BMCs, we used both the Neon Transfection (programs 17 and 20) and the Amaxa Nucleofector system (programs U-014, U-015 and T-023) to introduce MKOS and OSKM plasmid configurations into total nucleated cord blood and CD34-enriched cord blood cells. We had hypothesized that CD34 cells, which are enriched for HSCs and have been
reported to express Oct4, might be easier to reprogram. The mCherry reporter gene on OSKM plasmid allowed us to track transfected cells. mCherry+ cells were evident but they failed to form colonies, even after eight weeks of culture (data not shown).

3.3.4 Secondary fibroblast iPSCs can be derived from cells containing as few as one copy of the reprogramming factors

Both mouse BMCs and hUCB cells could be transfected and the expression of GFP suggested that endogenous pluripotency gene Oct4 was activated but no colonies could be sustained. This led us to hypothesize that the blood cells did not receive enough transgene copies and hence, insufficient reprogramming factor levels, to push them towards pluripotency. To verify this, we turned to the secondary reprogramming system. In the secondary system, cells from chimeric mice derived from DOX-inducible iPSCs are reprogrammed. We specifically used tissues from three chimeras generated from three independent iPSC lines (OGMKOS, and two lines from the Nagy lab (Woltjen, Michael et al. 2009)). While the OGMKOS iPSC line had only one copy of the Yamanaka factors, the two iPSC lines had ~9 copies of the reprogramming factors. Based on coat colour, all three chimeras were ~50% chimeric so we estimate that half of the isolated blood cells were of iPSC origin (‘secondary’ cell). Secondary cells arise from the iPSCs and already contain the transgenes thereby alleviating the need for transfection. These transgenes are under the control of a tetracycline-inducible promoter and can be reactivated by the addition of DOX. The secondary reprogramming system allowed us to circumvent the low transfection efficiency and enabled us to determine the ability to reprogram cells.

Tail tip fibroblasts were retrieved from three chimeras made from three different MEF-iPSC lines. All three iPSC lines were generated using the piggyBac transposon system and contain
various copies of DOX-inducible MKOS. Introduction of DOX reactivates the expression of the reprogramming factors, allowing us to generate secondary iPSCs. Fibroblasts from all three chimeras gave rise to secondary iPSCs. Fibroblasts with multiple copies of the transgenes formed iPSC-like colonies as early as eight days post DOX introduction. On the other hand, the fibroblasts with only one copy of the transgene formed colonies starting at 21 days post induction. DOX-independent colonies can be formed as early as 14 days for fibroblasts with multiple transgene copies and 28 days for fibroblasts with a single transgene copy. These secondary iPSCs expressed pluripotency-associated genes (Oct4, Nanog and SSEA-1) as determined by ICC (Figure 3.8).

3.3.5 Secondary blood iPSCs can only be derived from cells containing multiple copies of the reprogramming factors

To clarify, this experiment was done with Dr. Ian Rogers. BMCs were collected from the same three chimeras the tail tip fibroblasts were retrieved from. Unlike fibroblasts, we were only able to derive secondary blood iPSCs from the two chimeras produced from iPSCs containing multiple transgene copies. iPSC-like colonies started forming around three weeks post induction. However, some of these early colonies were DOX-dependent. These colonies expressed pluripotency markers Oct4, Nanog and Sox2 only in the presence of DOX (Figure 3.9). Once DOX was removed, expression of the pluripotency markers terminated and cells re-expressed blood-specific marker CD45 (Figure 3.9, Clone 1). It is interesting that some early iPSC clones retain the expression of CD45 in the presence of DOX (Figure 3.9, Clone 2). These are the kinds of colonies we are targeting because we think that they will most likely become stable reprogramming intermediates that have properties of their cell of origin and also have some stem cell properties such as the ability to proliferate. DOX-independent colonies can be established by
5-6 weeks post DOX introduction. These established DOX-independent colonies express pluripotency marker but do not express CD45 in the absence of DOX (Figure 3.9, Clone 3).

3.4 Discussion

Allogeneic HSCT is an indispensable treatment for patients with hematological disorders and cancers. The numerous advantages associated with using UCB as donor cells make it an ideal source of cells. However, UCB is not commonly used for larger children or adults because a single UCB unit typically contains insufficient cells for larger patients. To overcome this cell dose obstacle, double cord transplants have been performed and HSC expansion methods have been developed. Here in this study, we explored the feasibility of producing partially reprogrammed blood cells using the piggyBac transposon system as a source of donor cells for HSCT.

Partially reprogrammed cells represent a population of reprogramming cells with a distinct gene expression and epigenetic pattern that is different from the original cells they were derived from and different from established iPSCs (Mikkelsen, Hanna et al. 2008; Chan, Ratanasirintraewoot et al. 2009). Self-renewal and proliferative genes, but not pluripotency genes, are reactivated in these reprogramming intermediates, giving them the unlimited ability to expand.

We first attempted to reprogram BMCs from Oct4-GFP transgenic mice because murine cells are easier to reprogram than human cells. Using two electroporation systems and different combinations and amounts of reprogramming factors, we were unable to generate primary iPSCs from mouse BMCs. At most, we were only able to get 3-4 colonies that expressed GFP and/or mCherry. However, these colonies failed to proliferate. MEFs from the same mice were
reprogrammable hence the problem lies with the cell type and not the mouse strain. Next, we attempted to reprogram whole UCB and CD34-enriched cord blood using the same systems as mouse BMCs. Similar to mouse BMCs, we were unable to generate iPSC from cord blood cells. We were only able to get a few mCherry+ single cells indicating that transfection worked but there was no colony formation.

We speculate that the blood cells failed to reprogram because not enough transgenes were integrated into the cells. This lack of transgene integration can be influenced by several factors including insufficient available transgenes for integration or by the cell’s DNA repair system. Up to 14 µg of plasmids per 100,000 cells were used for transfection hence the problem is not plasmid availability. Blood cells have a stringent DNA repair system and this may prohibit the integration of the Yamanaka factors (Bracker, Giebel et al. 2006). Indeed, the reprogramming efficiency of both mature and immature blood cells is higher with non-integrative methods than integrative methods (Giorgetti, Montserrat et al. 2009; Loh, Agarwal et al. 2009; Loh, Hartung et al. 2010; Seki, Yuasa et al. 2010; Ban, Nishishita et al. 2011; Chou, Mali et al. 2011).

To confirm our speculation, we turned to a secondary reprogramming system. Tail tip fibroblasts and BMCs were retrieved from chimeras made from three different MEF-iPSC lines. Two of the three lines contained multiple copies of the reprogramming factors (~9 copies) while one line only had one copy. Fibroblasts from all three chimeras were amenable to reprogramming whereas only BMCs from the two chimeras made from iPSCs with multiple transgene copies were amenable to reprogramming. In line with our speculation, these results showed that blood cells are amenable to reprogramming but require integration of multiple copies of the Yamanaka factors to produce high levels of the reprogramming factors. This abrogates the reason for using
the piggyBac system in the first place because it will become more difficult to cleanly remove all copies of the transgenes once the cells are partially reprogrammed.

Over the years, focus has shifted to using non-integrative methods to reprogram cells. These methods include Sendai viruses, episomal vectors, adenoviruses, and mRNA/protein delivery. Sendai virus and episomal vectors have been successfully used to reprogram human bone marrow, cord blood and peripheral T cells, and with high efficiency (>0.1%) (Seki, Yuasa et al. 2010; Ban, Nishishita et al. 2011; Chou, Mali et al. 2011). These non-integrative methods eliminate the need to remove transgenes after reprogramming or blood cell expansion. With such great reprogramming success using non-integrative methods, we should shift our focus towards using these methods to generate partially reprogrammed cells. Once we are successful in producing and expanding reprogramming intermediates, we will need to ensure that they are capable of giving rise to the whole range of hematopoietic cells and not just a subset as was observed with Delaney and colleagues’ study (Delaney, Heimfeld et al. 2010).
3.5 Chapter 3 figures

**Figure 3.1: Timeline of generating iPSCs from Oct4-GFP MEFs using the piggyBac transposon system.**
Figure 3.2: Primary MEF-iPSCs (OGMKOS line) generated from Oct4-GFP MEFs display pluripotency markers. MEF-iPSCs express Oct4 (top row), Nanog (middle row), and SSEA-1 (bottom row).
Figure 3.3: MEF-iPSCs (OGMKOS line) form teratomas consisting of tissues from all three germ layers. 1x10^6 iPSCs were injected into NOD/SCID mice and teratomas were retrieved from the mice three weeks post-injection. H&E staining of the sectioned tissues revealed the presence of endoderm (top), mesoderm (middle) and ectoderm tissues (bottom). Germ layers were identified and pictures were taken by Hibret Adissu.
Figure 3.4: MEF-iPSCs (OGMKOS line) carry trisomy 8.
Figure 3.5: Timeline of generating iPSCs from Oct4-GFP BMCs and hUCB cells using the piggyBac transposon system.
Figure 3.6: Oct4-GFP BMCs transfected with MKOS vector form colonies and express GFP but they do not proliferate. Oct4-GFP BMCs were transfected with 1 µg plasmid/100,000 cells and cultured for six weeks in mESC media and DOX (1500 ng/ml).
Figure 3.7: Oct4-GFP BMCs transfected with OSKM vector form colonies that display GFP and mCherry but they do not proliferate. Oct4-GFP BMCs were transfected with 5.7 µg plasmid/100,000 cells and cultured for two weeks in mESC media and DOX (1500 ng/ml).
Figure 3.8: Secondary fibroblast iPSCs display pluripotency markers. Chimeric mice were made from aggregating Oct4-GFP MEF-iPSCs with C57BL/6 embryos. Secondary fibroblast iPSCs express GFP (middle column) and pluripotency markers Oct4 (top row), Nanog (middle row), and SSEA-1 (bottom row).
Figure 3.9: Secondary blood iPSCs can only be generated from chimeric mice made from MEF-iPSCs containing multiple copies of the Yamanaka factors. iPSC-like colonies started to form around three weeks post DOX introduction but they were DOX-dependent (clone 1 and 2). DOX-independent colonies formed around six weeks post DOX introduction (clone 3).
Chapter 4
Summary, conclusions and future directions

Trogocytosis and its role in HSCT

HSCT is a potentially curative therapy for many patients with hematologic diseases. The procedure involves ablating the host’s diseased hematopoietic system and replacing it with donor’s healthy HSCs. Since this concept was conceived over 50 years ago, we have come a long way in terms of understanding and identifying factors that negatively influence engraftment outcomes and patient survival, and developing new methods to overcome these barriers. Despite this giant leap forward, we are still far from fully understanding all the issues associated with HSCT. For instance, issues such as high conditioning-related mortality, graft rejection and donor cell availability still need to be addressed. In this study, we propose novel perspectives to these issues that can lead to the development of alternative solutions.

Conventional myeloablative conditioning regimens are highly toxic because they uses high doses of cytotoxic drugs and total body irradiation to eradicate the malignancy and clear the patient’s bone marrow niche. Now, there is a growing trend towards using milder non-myeloablative and reduced intensity conditioning regimens. However, these milder alternatives are associated with a higher risk of graft rejection. The first aim of this study was to understand the interactions between host and donor cells after transplantation and the contribution of NK cells and macrophages to graft rejection. Using NOD/SCID and NOD/SCID/gamma hosts, our results showed that host MHC class I proteins transfer onto donor cells. During early post-transplantation, acquisition of host MHC class I proteins was crucial for donor cell survival and engraftment. We hypothesize that these acquired host MHC class I proteins disguise donor cells as ‘self’ and repress lysis by NK cell and macrophages through interactions with their inhibitory
receptors. When full donor chimerism is established, donor cell survival is no longer dependent on the presence of host MHC class I proteins.

In our previous study (Yamanaka, Wong et al. 2009), we showed that intercellular transfer of MHC class I proteins occurs naturally in our body and involves about 10-15% of the blood cells. As a result, a subset of the transplanted donor cells acquire host MHC class I proteins as it integrates into the host’s hematopoietic system. Through this intercellular protein transfer, donor cells also acquire ‘natural’ protection from host NK cells and macrophages. However, these transferred proteins do not confer protection from host T cells because T cells do not exhibit inhibitory receptors for self MHC class I proteins as do NK cells and macrophages. These observations prompted us to believe that we could harness this ‘natural’ protection from NK cells and macrophages and develop new conditioning regimens that only target T cells and hence, reduce the use of cytotoxic drugs and/or irradiation.

The concept of intercellular protein transfer has been described and studied intensively only recently. Moreover, the transfer of MHC class I proteins in the HSCT setting has not been previously described. Hence, a deeper understanding of this phenomenon needs to be established, and numerous questions regarding the feasibility and safety of this approach need to be answered before we can apply our ideas clinically.

Do HLA class I proteins transfer in human HSCTs?

Before we can apply our findings to human HSCTs, we need to confirm that HLA class I proteins also transfer in the human HSCT setting. To confirm this, we will enroll patients receiving grafts with HLA disparities. Blood samples are routinely retrieved from patients post
transplantation to evaluate chimerism, and neutrophil and platelet recovery. We could take a small aliquot of these blood samples, isolate and stain the nucleated cells for mismatched HLA proteins, and analyze using flow cytometry. If HLA proteins transfer occurs in the human HSCT setting then we would see a population of blood cells that are double positive for the host and donor mismatched HLA proteins. If HLA proteins do not transfer, then we would see no trogocytosis-positive populations. In our xenotransplantations, we showed that human cells indeed accept foreign MHC class I proteins. Hence, we expect HLA proteins to also transfer in human HSCTs.

Is there a clonal selection for donor cells that have acquired host MHC class I proteins?

A large portion of the donor cells at one week post transplantation in NOD/SCID and NOD/SCID/gamma mice had acquired host MHC class I proteins (trogocytosis-positive). This population can arise from one of two scenarios: 1) all donor cells undergo trogocytosis after transplantation, or 2) only a subset of donor cells undergoes trogocytosis but the survival of these cells are spared by host immune cells while trogocytosis-negative cells are eliminated. To verify which of the two scenarios it is, we will transplant a mixture of GFP+ and GFP- C57BL/6 BMC into sublethally irradiated NOD/SCID and NOD/SCID/gamma mice. If all donor cells undergo trogocytosis and have an equal chance of survival and engraftment, we expect the ratio of GFP+:GFP- donor cells to be the same as the initial donor cell ratio at the time of transplantation. However, if the transplanted donor cells are not all equal, then we expect the GFP+:GFP- ratio to differ from the transplanted donor cell ratio. We reason that the second scenario is more plausible than the first. Hence, we expect the ratio of GFP+:GFP- donor cells to
significantly differ from the initial transplanted ratio. In extreme cases, we expect to see hosts comprised of GFP+ cells only.

**Will depleting T cells alone in immunocompetent mice be sufficient to allow allogeneic donor cells to engraft and donor chimerism to establish?**

I have clearly demonstrated that trogocytosis protects donor cells from NK cell-mediated and macrophage-mediated rejection. On the other hand, T cell-mediated killing cannot be prevented by trogocytosis. This indicates that depletion of T cells alone is sufficient to allow donor cell engraftment. T cells can be broadly grouped into helper T cells expressing CD4 and effector or cytotoxic T cells expressing CD8. The cytotoxic T cells are responsible for the active elimination of incompatible grafts. Therefore, CD8 T cells should be targeted for depletion.

To assess whether depleting cytotoxic T cells alone is sufficient to allow engraftment, we will transplant allogeneic BMCs into immunocompetent mice depleted of cytotoxic T cells. These mice would then be sacrificed at different time points to evaluate trogocytosis and chimerism. There are currently several methods of depleting T cells and we will evaluate the effectiveness of each in promoting graft tolerance. For instance, anti-thymocyte globulins (ATGs) are commonly used in transplantations to deplete peripheral cytotoxic T cells and prevent T cell-mediated graft rejection (Beiras-Fernandez, Thein et al. 2003). Over the years, there has been an increasing interest in using regulatory T cells (Tregs) in modulating transplantation tolerance. Tregs are a subpopulation of T cells characterized by the expression of CD4, CD25 and Foxp3. The role of Tregs in immunosuppression and self-tolerance is evident from observations that mice deficient for Tregs develop autoimmune diseases (Asano, Toda et al. 1996) and that the human autoimmune disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked
syndrome) is caused by a mutation in the Foxp3 gene (Bennett, Christie et al. 2001). Numerous studies demonstrate that infusing Tregs along with donor cells can promote graft tolerance and engraftment (Joffre, Gorsse et al. 2004; Hanash and Levy 2005; Joffre, Santolaria et al. 2008). We will investigate the use of each method alone and in conjunction with each other to deplete T cells.

**Partially reprogramming blood cells as a method of increasing the donor pool**

Another prominent issue in HSCT is donor cell availability. Despite the large number of donors registered with bone marrow registries, a significant number of patients still cannot find a bone marrow donor match with no more than one HLA-mismatch. A greater HLA-mismatch is associated with adverse outcomes. On the other hand, UCB graft with 1-2 HLA disparities is tolerated. However, UCB has primarily been used for transplants in smaller children because of its low cell dose. Several groups have investigated different methods of expanding HSCs in UCB to augment the cell dose for transplant in larger patients. Expansion by culturing HSCs in the presence of several cytokine combinations led to a moderate increase in cell number but the size of these expansions was not clinically significant (Shpall, Quinones et al. 2002; Rogers, Yamanaka et al. 2008). By harnessing the Notch-ligand signaling pathway, Delaney and colleagues (Delaney, Varnum-Finney et al. 2005; Delaney, Heimfeld et al. 2010) were able to achieve clinically significant expansion of HSCs. However, these expanded cells do not give rise to T cell *in vitro* and *in vivo*. In our study, we investigated the feasibility of partially reprogramming blood cells as a method of HSC expansion.
In the reprogramming process, only a rare number of colonies will fully reprogram and become true iPSCs (Mikkelsen, Hanna et al. 2008; Chan, Ratanasirintrakul et al. 2009). The remaining colonies do not completely reprogram and remain as reprogramming intermediates. The gene expression and epigenetic state of these partially reprogrammed cells show remnants of the cell of origin but also exhibit reactivation of several self-renewal, proliferative and/or pluripotency genes. Hence, HSC reprogramming intermediates may represent a valuable source of cells for transplantation as they can proliferate like an ES-cell but also retain the potential to easily differentiate into blood cells.

In this study, we attempted to generate partially reprogrammed blood cells from mouse BMCs and hUCB cells using the piggyBac transposon system. We were unable to generate primary blood iPSCs but our results from secondary reprogramming suggest that blood cells require higher levels of Yamanaka factors than fibroblasts to reprogram. It would be counterproductive to reprogram blood cells with the piggyBac system if they require multiple copies of the Yamanaka factors because the transgenes will need to be excised afterwards. Recently, several groups have showed very efficient reprogramming of cord blood and peripheral T cells using genomic non-integrative methods (Seki, Yuasa et al. 2010; Ban, Nishishita et al. 2011; Chou, Mali et al. 2011). Non-integrative methods alleviate the need to remove transgenes after establishing a cell line. Moving forward, we will focus on generating partially reprogrammed cells from non-integrative methods such as using episomal vectors and Sendai viruses.

**How do we select for partially reprogrammed cells?**

The stable reprogramming intermediates we are targeting were ones that have reactivated, at minimum, self-renewal and proliferative genes but also express HSC-specific markers such as
CD34/CD133 and CD45. As Chan and colleagues (Chan, Ratanasirintrawoot et al. 2009) have indicated, partially reprogrammed iPSCs share similar morphology to true iPSCs. Hence, molecular techniques must be used in addition to morphology to identify these reprogramming intermediates. As colonies form, we will pick and expand individual colonies, and use ICC and RT-PCR to screen for the ideal partially reprogrammed cells. ICC and RT-PCR will be performed at different time points to ensure the colonies remain partially reprogrammed.

Do the partially reprogrammed cells differentiate into blood cells of all lineages?

After HSC expansion, it is essential that the expanded cells retain the ability to self-renew and differentiate into blood cells of all lineages. To evaluate the short and long term repopulating properties of these expanded cells, *in vitro* and *in vivo* methods can be employed. For *in vitro* methods, CFUs and long term culture-initiating cell (LTC-IC) assays can be used to evaluate the presence of progenitor and stem cells, respectively. We can also assess the repopulating properties of these expanded HSCs *in vivo* by transplanting them into lethally irradiated mice. To generate a microenvironment that supports human cells in the mice, human-specific growth factors can also be injected into the mice. To confirm that expanded HSCs are able to differentiate into blood cells of all lineages, differentiated blood cells can be analyzed for lineage-specific surface markers, such as CD3, CD14, CD19, and CD41, using flow cytometry.

Conclusions

New ideas are essential for moving the field of HSCT forward. Our study provided new perspectives and alternative solutions to common complications such as conditioning-related
mortality, graft rejection and insufficient donor cell dose faced by patients receiving HSCT. Using murine intraspecific transplantations, we showed strong evidence that transplanted donor cells can acquire host MHC class I proteins and these acquired proteins protect the donor cells from NK cell-mediated and macrophage-mediated rejection. Realistically, only a subset of the transplanted donor cells acquire host MHC class I proteins but this subset is sufficient to establish donor chimerism by 2-3 weeks post transplantation. Assuming that human transplants are similar to our mouse transplants, these results suggest that immunosuppressive drugs and radiation against NK cells and macrophages are not necessary when conditioning patients for transplantations. This will reduce the toxicity of conditioning regimens and therefore reduce conditioning-related mortality without compromising graft tolerance.

Next, we proposed that blood reprogramming intermediates can serve as a novel source of donor cells. All of our attempts at generating primary blood iPSCs from mouse BMCs and hUCB cells using the piggyBac system failed. Using a secondary reprogramming system, we showed that BMCs required more copies of the reprogramming factors than fibroblasts to reprogram. However, inserting multiple copies of the reprogramming factors is counterproductive when we want to produce safe cell lines for use in patients. Recently, groups have shown great success in generating blood iPSCs using genomic non-integrative methods (Seki, Yuasa et al. 2010; Ban, Nishishita et al. 2011; Chou, Mali et al. 2011; Seki, Yuasa et al. 2012). These non-integrative methods alleviate the need to remove the transgenes after reprogramming. Moving forward, we should focus on generating blood reprogramming intermediates using non-integrative methods. We are confident that partially reprogrammed HSCs would be great donor cell candidates for transplants because they should retain HSC properties while acquiring proliferative properties. HSC reprogramming intermediates should retain HSC properties because of incomplete
epigenetic remodeling and the acquired proliferative property should allow the HSCs to expand to clinically significant numbers.
References


the probability of finding an HLA-A, -B, -C and -DRB1 allele-matched unrelated donor and likelihood of subsequent transplant utilization." Bone marrow transplantation.


