Characterisation of Placental Mesenchymal Stromal Cells and their Role in Cord Blood Transplantation.

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ABSTRACT

Cord blood transplantation (CBT) is an alternative to unrelated bone marrow transplantation in pediatric patients, while in adult patients the limited cell dose in cord blood (CB) unit results in delayed engraftment. To circumvent cell dose limitation, various methods have been investigated. Ex-vivo expansion of hematopoietic stem cells (HSC) is feasible but does not enhance engraftment due to HSC exhaustion. Use of double cord blood transplantation (DCBT) shows improved engraftment when compared to single unit transplantation, with median neutrophil engraftment at day 23 for recipients of DCBT, compared to 26-27 days for recipients of single cord blood transplantation (SCBT). However, engraftment is ultimately derived from single CB unit with reducing rates of chimerism seen up to day 100. The HSC share an intimate relationship with the BM microenvironment. Myeloablative conditioning using chemotherapy and radiotherapy may damage the microenvironment, which may contribute to delayed engraftment especially when the cell dose is limited. On the other hand mesenchymal stromal cells (MSC) could be used to restore this microenvironment. MSC are non-cycling cells having fibroblastic morphology, which express mesenchymal markers (CD73, CD105, CD90, CD29, and CD44), lack hematopoietic markers (CD45 and CD34) and differentiate into mesodermal lineages. MSC have been isolated from different tissues and show comparable characteristics to BM MSC. Recently, the placenta has been identified as a potential source of MSC and may have advantages to BM MSC due to a higher expansion potential and stronger immunosuppressive properties. This study has characterized cells obtained from the foetal aspect of the placenta and evaluated whether co-transplantation of placental MSC would enhance engraftment. Plastic adherent cells isolated from the placenta demonstrated typical characteristics of MSC. In 6 individual experiments, 4 cohorts of 24 NOD/SCID mice were evaluated. Cohort 1 received 5×10^4 $CD34^+$ cells from unit (U) 1 (SCBT); cohort 2 received 5×10^4 $CD34^+$ cells from U1+ 4×10^4

MSC (SCBT+MSC); cohort 3 received 2.5 $\times$ 10^4 $CD34^+$ cells from U1+ 2.5 $\times$ 10^4 $CD34^+$ cells from U2 (DCBT); cohort 4 received 2.5 $\times$ 10^4 $CD34^+$ cells from U1+ 2.5$\times$ 10^4 $CD34^+$ cells from U2+ 4 $\times$ 10^4 MSC (DCBT+MSC). Haemopoietic engraftment evaluated after 6-8 weeks was similar in recipients of SCBT and DCBT. MSC co-transplantation demonstrated enhanced engraftment in DCBT (51.8 ± 6.8% vs. 14.9 ± 6.5%; p=0.04) with an increased trend in SCBT (48.7 ± 7.7% vs. 17.5 ± 6.1%; p=0.07). In DCBT, co-transplantation of placental MSC reduced single CB unit dominance. Self-renewal capacity of engrafted HSC was assessed by serial transplantation in secondary recipients. Secondary recipients were infused with engrafted human cells from primary mice transplanted with or without MSC. In secondary transplantation of 17 evaluable mice, 13 engrafted at levels of 1-6.5%. Despite enhanced engraftment in primary mice, long-term engraftment capacity was unaltered with MSC co-transplantation. Furthermore, to study the potential mechanisms behind enhanced engraftment, eGFP transduced placental MSC and PKH-26 red labelled CB $CD34^+$ cells were traced in live mice. Imaging studies showed MSC migrated to the pelvic region and improved CB $CD34^+$ homing. Co-transplantation of placental MSC enhanced CB engraftment and may act by improving homing of $CD34^+$ cells.

PREFACE

Cord blood transplantation (CBT) in adults is restricted due to the limiting cell dose. Co-transplantation of bone marrow (BM) mesenchymal stromal cells (MSC) has been shown to enhance engraftment of CBT. Although BM MSC are well characterised are a rare population and their numbers decrease with age. Recently the placenta has been explored as a potential source of MSC. However there is limited literature available regarding its use in CBT. This study characterises the adherent cell population obtained from the placenta and investigates the role of these cells in CBT in non-obese diabetic/severely immuno-deficient (NOD/SCID) mice.

Chapter I

The literature review and the basis for setting the hypothesis of the study are discussed in detail. It also states the objectives to be achieved.

Chapter II

This chapter deals with the procedures describing the tissue selection, dissociation procedures and isolation of cells from the placenta. This is followed by investigating the phenotype of the tissue isolated primary cells and culture expanded adherent cells. The differentiation potential of these adherent cells has been demonstrated. The cell morphology, proliferation, and karyotype are also described. This chapter concludes that the adherent cells obtained from the foetal aspect of the placenta are non–haemopoietic progenitors, capable of self-renewal, differentiating into at least three mesenchymal lineages (bone, cartilage, fat) and expressing common MSC markers while lacking HSC markers. These cells also possess fibroblastic morphology demonstrating similar characteristics to BM MSC.

Chapter III

This chapter describes primary and serial transplantation studies in the NOD/SCID mice model. Here the method of isolating $CD34^+$ haemopoietic progenitor cells from CB unit and

cryopreservation is described, along with detailed explanations of the experiments conducted on NOD/SCID mice. This chapter deals with results from 6 individual primary transplantation experiments, in which 4 cohorts were evaluated. Cohort 1 received 5×10^4 $CD34^+$ cells from unit (U) 1 (SCBT); cohort 2 received 5×10^4 $CD34^+$ cells from U1+ 4×10^4 MSC (SCBT+MSC); cohort 3 received 2.5×10^4 $CD34^+$ cells from U1+ 2.5×10^4 $CD34^+$ cells from U2 (DCBT); cohort 4 received 2.5×10^4 $CD34^+$ cells from U1+ 2.5×10^4 $CD34^+$ cells from U2+ 4×10^4 MSC (DCBT+MSC). Co-transplantation of MSC from the placenta demonstrated enhanced engraftment in DCBT with an increased trend in SCBT. Moreover, in DCBT, co-transplantation of placental MSC reduced single CB unit dominance. This chapter also describes the serial transplantation experiments in secondary recipients. It demonstrated that despite enhanced engraftment in primary mice, long-term engraftment capacity was unaltered with co-transplantation of the placental MSC.

Chapter IV

There is limited published literature addressing the homing of the MSC.This chapter describes a live imaging assay to study the migration of placental MSC to delineate the mechanism of HSC supportive role. This chapter explains in detail the gene manipulation of the placental MSC to transduce green fluorescent protein which was imaged into the mice after IV injections at various intrvals. Co-transplantation of placental MSC enhances haemopoietic engraftment by increasing homing and retention of the CB $CD34^+$ cells to the haemopoietic site. This chapter also shows that the preincubation of MSC with anti-CXCR4 antibody, neither inhibited its migration to the pelvic region nor altered the engraftment of CB $CD34^+$.

Chapter V

This chapter summarizes the observations and findings conducted during the research project. Placental MSC demonstrate similar morphological, immunophenotypical and differentiation characteristics to BM MSC. This study has demonstrated that at equivalent cell

dose single and DCBT leads to similar engraftment. There was improved engraftment in mice that received placental MSC in both settings. The co-transplantation of MSC leads to reduced dominance of single CB unit in DCBT. How these results fit in with the work of other researchers is also discussed in detail. This chapter also summarises the attempts to understand the mechanism of MSC homing to BM by the blocking of CXCR4 by T140 peptide. Furthermore, limitations of the study are stated, and the direction for future work described.

ABBREVIATIONS

ANCs	absolute neutrophil count
AHSCT	allogeneic haemopoietic stem cell transplantation
Bp	base pairs
BFU-E	burst forming unit erythroid
BM	bone marrow
BMP	bone morphogenetic protein
BMT	bone marrow transplantation
BSA	bovine serum albumin
BSC	bio-safety cabinet
CB	cord blood
CBT	cord blood transplantation
CD	cluster of differentiation
CFU	colony forming unit
CFU-F	colony forming unit –fibroblast
CFU-GM	colony forming unit granulocytes and macrophage
CFU-GEMM	colony forming unit granulocyte, erythroid, macrophages and megakaryocyte
CPD	cumulative population doubling
DCBT	double cord blood transplantation
DMEM	Dulbecco's minimum essential media
DMSO	dimethyl sulfoxide
DNA	deoxy ribonucleic acid.
ECM	extra cellular matrix
EDTA	ethylenediamine tetra acetic acid
EPO	erythropoietin

ESC	embryonic stem cells
FACS	fluorescence activated cells sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte –macrophage-colony stimulating factor
GVHD	graft versus host disease
Gy	Gray
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney
HGF	haemopoietic growth factors
HLA	human leucocyte antigen
HSC	haemopoietic stem cells
HSCT	haemopoietic stem cell transplantation
Ig	immunoglobulin
IL-1	interleukin-1
IMDM	Iscove's minimum defined media
IMVS	Institute of Medical and Veterinary Sciences
ITS	insulin transferring selenous
ISCT	International Society for Cellular Therapy
IVIS	in-vivo imaging system
LB	Luria broth
MACS	magnetic activated cell sorting

MNCs	mononuclear cells
MoAB	monoclonal antibody
MPP	multi potent progenitors
MRI	magnetic resonance imaging
MSC	mesenchymal stromal cells
MSCV	murine stem cell virus
MUD	matched unrelated donor
NOD-SCID	Non obese diabetic-severe combined immune deficient
O_2	oxygen
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PC5	phyco erythrin-cyanine 5
PD	population doubling
PDT	population doubling time
PE	phyco erythrin
PDGF	placental-derived growth factor
REC	research ethics committee
RO	reverse osmosis
ROI	region of interest
SC	sideward scatter
SCBT	single cord blood transplantation
SCT	stem cell transplantation
SCF	stem cell factor
SDF-1	stromal derived factor-1

SCS	sodium chloride /sodium citrate
STR	short tandem repeats
TEM	transendothelial migration
TNC	total nucleated cells
TRM	transplantation related morbidity
TPO	thromboprotein
UBMT	unrelated bone marrow transplantation
UCB	umbilical cord blood
URD	unrelated donor
URDT	unrelated donor transplantation
VCAM	vascular adhesion molecule
VLA-4	very late antigen -4
VLA-5	very late antigen-5

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PUBLICATION

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CONFERENCE PRESENTATIONS

International conference

An oral presentation at the European Bone Marrow Transplantation (EBMT) 2008 Congress held in Florence, Italy, 30 March - 2 April, 2008. Co-transplantation of placental derived mesenchymal stromal cells produces superior engraftment of umbilical cord blood compared to double unit umbilical cord blood transplantation. Smita. Hiwase, Pamela. Dyson, S. Young, L.B. To, Ian. Lewis* IMVS (Adelaide, AU)

National conferences

An oral presentation "Placental Derived Mesenchymal Stromal Cells Enhances Umbilical Cord Blood Engraftment" at the conference HAA-2007 (HSANZ/ANZSBT/ASTH-2007 ASM) held 14-17 October 2007- at Gold Coast Convention & Exhibition Centre, Surfers Paradise, Queensland.

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Poster presentation "Determination of the optimal technique to increase stem cell dose for cord blood transplantation" held at the National Wine Centre during 2008 Postgraduate Research Expo conducted by the Faculty of health Sciences.

Division of Haematology Seminar Presentation

An oral presentation “Determination of the optimal technique to increase stem cell dose for cord blood transplantation” held at IMVS August 2007.

Chapter 1 - Literature Review

1.1 Haemopoiesis

Haemopoiesis refers to the continuous lifetime process of blood cell formation in which the haemopoietic stem cells (HSC) give rise to all the blood components in living organisms. The mature blood cells are terminally differentiated cells with a limited life span, and are constantly replaced by less differentiated precursors that are descended from primitive progenitors. In haemopoiesis the most primitive cells are the HSC which possess the ability to self renew, proliferate and to undergo multilineage differentiation into mature blood cells (1). Additionally a single HSC can restore the complete haemopoietic system of a myeloablated host (2).

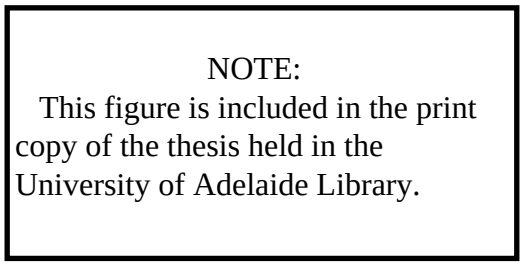
According to the functional properties, HSC can be classified into long-term self-renewing HSC, short-term self-renewing HSC and the multi-potent progenitors (MPP) (3). The long-term HSC give rise to short-term HSC which descend to MPP (4). The mitotic stimulation of MPP leads to formation of mature blood cells including erythrocytes, neutrophils, eosinophils, monocytes, mast cells, platelets, and lymphocytes, while its self-renewal capacity is compromised (Figure 1).

The HSC and the bone marrow (BM) microenvironment share an intimate bond in haemopoiesis. The environment where stem cells reside is termed niche. It is a three dimensional structure comprising different stromal cells, blood vessels, and extra cellular matrix. The supporting stromal cell population is derived from the distinct progenitors known as mesenchymal stromal cells (MSC).

MSC can differentiate into adipocytes, fibroblasts, osteoblasts, and chondrocytes. MSC and its differentiated descendants, secrete cytokines such as interleukins, macrophage colony stimulating factor, stem cell factor (SCF) granulocyte colony stimulating factor (G-CSF) and

Figure 1. Haemopoietic stem cell (HSC) hierarchy: HSC differentiate into common myeloid progenitor (CMP) and lymphoid progenitor (CLP). CMP have potential to form colony forming unit (CFU)-granulocyte, erythroblasts, macrophages, eosinophils and megakaryocytes (CFU-GEMM). CFU-GEMM can differentiates into CFU-granulocyte and macrophage (CFU-GM); and erythroid and megakaryocyte progenitors (MEP). MEP differentiate into megakaryocyte and erythroid progenitor, which subsequently forms erythrocytes and platelets. The CFU-GM gives rise to neutrophils, eosinophils, basophils and monocytes. The common lymphoid progenitor (CLP) give rise to T and B progenitor cells that go through several stages and produce T, B and NK cells.

Figure 1



Adapted from http://daley.med.harvard.edu/assets/Willy/willy.htm

granulocyte-macrophage colony stimulating factor (GM-CSF) (5). These cytokines bind to the specific receptors on the HSC and activate downstream signalling pathways necessary for cell survival, self-renewal, proliferation and differentiation (6-9).The osteoblasts maintain stem cell quiescence through the interaction of specific signaling pathways (10, 11). Thus the MSC and the HSC are intrinsically linked.

The long-term self-renewal capacity of the HSC, along with proliferation and terminal differentiation to distinct cell lineages is vital for the lifelong reconstitution of the blood cells. The HSC undergo differentiation by asymmetric cell division (12) which is dependent on the specific signalling pathways regulated by cytokines and haemopoietic growth factors. The acquired defects in this complex process give rise to a range of stem cell disorders such as leukaemia and myeloproiferative disorders. Haemopoietic stem cell transplantation (HSCT) is a therapeutic strategy utilised in the treatment of these disorders.

1.2 Haemopoietic stem cell transplantation (HSCT)

Intravenous infusion of HSC to recipients is known as HSCT. Prior to HSCT, recipients are treated with high dose chemotherapy and/or radiotherapy to eradicate tumour cells and immunosuppress the recipients to avoid graft rejection. The first reported case study was published in 1939 (13)where a patient with aplastic anaemia received an intravenous bone marrow transplantation from his brother. Since then BM has been used as the traditional source of HSC and has been termed as the bone marrow transplantation (BMT). Significant progress in BMT occurred only after the 1950s, when it was observed that murine marrow ablated by radiation could be restored by BM infusion from normal mice (14, 15). Since then BMT has been used to restore the HSC that have been destroyed by radiation or chemotherapies in haematological disorders or to correct immunological disorders.

In BMT, intial attempts are made to destroy the abnormal/ leukaemic HSC and the stroma by radiation and chemotherapy. Subsequently, normal BM function is restored by

transplanting HSC derived from the patient (autologous transplantation) or from the donor (allogeneic transplantation). In autologous transplantation, the patient's own stem cells from bone marrow (BM) or peripheral blood (PB) are infused after high dose myeloablative therapy. In allogeneic haemopoietic stem cell transplantation (AHSCT) the donor's BM or PB is used as the source. The donor can be syngeneic, a HLA-matched sibling, a haploidentical family donor a HLA matched unrelated donor (URD) or cord blood (CB) unit.

In BMT, the BM is harvested by repeated aspiration from the posterior iliac crest under general anaesthesia. This is associated with the risks of general anaesthesia and discomfort to the patient/donor. Stem cells have also been collected from the peripheral blood of donors, where donors are treated with G-CSF which causes proliferation of neutrophils and releases proteases that detaches $CD34^+$ cells from bone marrow and subsequently release them to circulation. These circulating $CD34^+$ cells are then collected by leukapheresis.

The advantage of autologous HSCT is the lack of graft rejection and no requirement of immunosuppressive drugs. However this technique is not useful for immunological disorders and some malignancies. The lack of a graft-versus-tumour effect and the possible contamination of the graft with malignant cells may moreover result in recurrence of the underlying disease.

1.2.1 Allogeneic haemopoietic stem cell transplantation (AHSCT)

AHSCT refers to the use of HSC from a donor. The donor can be related, unrelated or a CB unit. Thomas *et al* performed the first syngeneic stem cell transplantation in a patient with end stage leukaemia (16). AHSCT became feasible in the early 1960s, following identification and typing of the human leukocyte antigens (HLA), the major histocompatibility complex which recognises self and unself. Subsequently in the 1970s, this resulted in improved outcomes when Thomas and colleagues treated end stage leukemic patients with total body irradiation (TBI) and cyclophosphamide followed by infusion of BM from their HLA-identical siblings (17, 18).The recognition of the graft-versus-leukaemia/tumour effect, whereby the donor lymphocytes can

eradicate tumour cells that survive preparative regimens, has further substantiated the role of AHSCT for treating various haematological malignancies.

In AHSCT the degree of HLA matching is the most important component of success. HLA molecules are present on the cell surface. They are involved in the intercellular recognition and discrimination between self and non–self cells. The major HLA antigens are HLA-A, B, C, DP, DQ, and DR. The degree of HLA-mismatch in AHSCT can result in severe acute and chronic graft-versus-host disease (GVHD). Better understanding of the immune mechanism, discovery of the superior chemotherapeutic and antibiotic agents, the establishment of BM registries and blood banks further led to development of AHSCT.

Since the expansion of donor registries AHSCT has emerged as a potentially curative treatment for patients with haematological malignant and non-malignant disorders. However it is limited by several factors. Only 25 to 30% of eligible patients have HLA matched sibling donors (19). The patients without well-matched sibling donors may be eligible for unrelated donor transplantation (URDT). Although 70 to 80% of caucasian patients have a suitable matched unrelated donor, less than 20% of referred patients proceed to the transplantation. There is also a delay of almost 4-5 months from initiation of the search to transplantation. AHSCT is also limited by toxicity associated with GVHD, an immunological reaction by the donor lymphocytes against the recipient, causing dermatitis, enteritis and hepatitis.

The immune response is accentuated and possibly stimulated by injury resulting from the preparative regimen used before transplantation. GVHD and its treatment with immunosuppressive agents are associated with increased transplantation related mortality (TRM) and morbidity. The improvement in donor selection, supportive care and management of GVHD has improved outcomes of transplantation. However, limitations persist including the toxicity of treatment and lack of donors. Therefore alternative sources of HSC are being explored, of which CB is the most promising.

1.3 Cord Blood Transplantation

In 1983 Edward Boyse suggested the possibility of transplanting stem cells derived from CB(20). Gluckman *et al* performed the first successful HLA matched sibling CBT in a child with Fanconi anemia (21). This patient remains alive and well at 17 years of age (22). The success of CBT in a small number of patients and single institute studies increased the interest in this field, and over a period of time it formed the foundation of CBT (23). The preliminary data demonstrates the progression of CBT in treating various haematological disorders (23-25). CBT became a valid alternative to unrelated bone marrow transplantation (UBMT) in paediatric patients with haematological malignancies. As a result CB banks were established worldwide, giving fast and easy access to CB units. To date over 20,000 CBT have been performed worldwide, with the majority in the unrelated setting (26). Therefore one could say that CB offers an alternative source of HSC for transplantation in recipients who do not have an identified donor.

1.3.1 Limitations of Cord Blood Transplantation

As part of the banking process, CB units are routinely typed for HLA antigens and ABO blood groups. Prior to cryo-preservation CB units are tested for infectious agents. For this reason the delay between the start of search and transplantation is less compared to that for unrelated match donor (URD) identified from BM registries (19). The median time required to obtain a suitable URD was 49 days (range 32-293) while a suitable CB unit was only 13.5 days (2-387) (27) This reduction in search time is critical in many patients. The transplantation can be scheduled as per the patient's need rather than the availability of the donor, and also this rapid availability of the CB unit could be valuable in patients with high risk disease. In addition the CB banks can become a source of HSC for specific ethnic groups who are under-represented on the BM donor registries.

Studies have shown that CB contains a significantly higher number of committed progenitor cells compared to the PB or BM (28). In addition, CB progenitor cells possess both a higher proliferative potential and a greater capacity for self-renewal and long-term culture growth than progenitors from BM (29). This may mean that lesser cell doses may be adequate (29). Moreover, CB grafts have greater engraftment potential than the adult HSC (30).

The other important benefit of CBT is the reduced incidence and severity of GVHD compared to unrelated donor (URD) transplantation (31). Despite HLA mismatching, CBT is associated with low GVHD risk compared to URD transplantation. Davies *et al* previously reported a probability of grade III-IV acute GVHD of 32% and 49% in recipients of BM mismatched at 0 and 1 HLA antigens respectively (32) while Wagner *et al* reported grade III-IV GVHD in 11% recipients of UCB disparate in 0, 1, or 2 HLA antigens. The reduced incidence of GVHD in recipients of CB is due to the absence of cytotoxic T lymphocytes in CB (33) and reduced immuno reactivity of CB monocytes and lymphocytes compared to PB (34). This allows CBT to be performed with greater degrees of HLA disparity than would normally be accepted for URDT (27, 35). Thus increasing the donor pool. Moreover increased awareness about the valuable source of HSC in CB, has led to the development of CB registries, resulting in the establishment of CB banks worldwide. This continued expansion of extensive libraries of CB units allows rapid identification and availability.

Generally the collection of BM from an URD involves anxiety, hospitalisation, anaesthesia and postoperative pain. In addition, there is URD attrition due to ageing, relocation, loss of interest, and medical complications, whereas there is no donor attrition for CBT. The easy and prompt access to CB without any inconvenience to the donor makes it an appealing source of HSC.

Despite these advantages the main limitation of CBT is the finite cell dose available from a single CB unit. Although CB contains more primitive HSC per unit volume than BM (36) it

is not sufficient to overcome the limited cell dose. The limited volume from each CB unit results in a cell dose that is 10 fold lower than the BM cell dose (37, 38). There are roughly one tenth as many nucleated and $CD34^+$ cells in CB grafts as in allogeneic BM grafts, thereby limiting its use to smaller patients predominantly.

The recommended cell dose for successful CBT is 1.7×10^5 CB $CD34^+$ cells/kg body weight or 2.5×10^7 TNC/kg body weight. Wagner *et al* reported delayed neutrophil engraftment in patients who received a lower CB TNC dose. Median time to neutrophil (ANC$\geq 0.5 \times 10^9$/L) engraftment was 15, 21, 23 and 34 days in patients who received $>5.4 \times 10^5$/kg, 2.8 to 5.4×10^5/kg, 1.7 to 2.7×10^5/kg and $<1.7 \times 10^5$/kg $CD34^+$ cells respectively (39). In children weighing greater than 40 kg and in adults it is difficult to acquire the required number of cells from a single CB unit.

The early studies of CBT in adults reported a high rate of graft failure: 160/562 patients failed to achieve myeloid engraftment and 102 patients died prior to achieving absolute neutrophil count of 0.5×10^9/L (40, 41). The limited number of cells derived from a single CB unit are unable to provide durable engraftment in heavier children and adults. This has been confirmed by studies indicating that the cell dose is the most important predictor of the outcome of the CBT (40, 42, 43).

Despite CBT being associated with delayed neutrophil and platelet engraftment compared to URD BMT, studies have shown comparable rates of TRM (44, 45) and disease free survival (39, 40). The findings from prior studies confirm that in patients where matched URD is not available, CB could be used as a potential source of HSC.

However, 90% of patients who undergo a CB unit search are withdrawn from CBT due to the limited cell dose available from a CB unit (46). It is therefore important to develop strategies to circumvent the cell dose limitation observed with a single CB unit in CBT. These strategies include more efficient collection of CB units, minimising the loss of cells during

cryopreservation and thawing procedures, ex-vivo expansion of HSC, transplantation of two or more CB units, and co-transplantation of MSC.

However, to date the optimal approach to CBT that provides the increased cell dose required to improve engraftment and survival has not been defined.

1.3.2 Strategies to improve CBT

To circumvent the limited HSC number from a single unit various strategies have been explored including ex-vivo expansion, multi CB unit transplantation and co-transplantation of MSC.

1.3.2.1 Ex-vivo expansion of cord blood

To expand the cell numbers in a graft, ex-vivo culture systems have been used (47). Over the past decade researchers have tried to gain insight into the ex-vivo expansion of HSC. Understanding the process that regulates the expansion and proliferation of HSC has led to suggestions that the ex-vivo expansion of CB HSC may result in the provision of a graft with an adequate number of HSC. However, the initial ex-vivo expansion of stem cells has achieved limited success.

The ex-vivo expansion system can be categorised as either stroma free cytokine cocktail driven expansion systems, which may or may not contain serum, or the systems which use stromal feeder layers with or without cytokines. In cytokine driven expansion, 50 fold expansion of CB $CD34^+$ cells was accomplished by a cytokine cocktail of interleukin-1 (IL-1), IL-3, IL-6, GM-CSF, and SCF (48). Other cytokines which also play a role in the HSC expansions include Flt-3 ligand (FL) and thrombopoietin (49-51).

In the majority of studies, short-term culture (less than 6 days) in stroma free, serum free or serum supplemented systems with cytokines led to increased CFC, CD34 cells and total

cells (52-54). However, ex-vivo expansion compromises self-renewing cell (SRC) frequency and long-term engraftment potentials (49, 55). Similar findings that the ex-vivo expansion results in rapid engraftment in primary recipients but compromises the long-term engraftment in the secondary and tertiary recipients have been reported (55). These results are consistent with the notion that cytokine driven expansion results in exhaustion of primitive repopulating HSC. However, other features may also contribute to impaired engraftment. This includes interference of the signalling pathways of self-renewal (56) or altered expression of VLA-4 and VLA-5 that leads to a homing defect resulting in poor engraftment (57). The short-term ex-vivo expansion maintains the homing related characteristics of the CB HSC (58) while the increased caspase activation in the ex-vivo expanded cells leads to induction of apoptosis (59).

The role of stromal support for the ex-vivo expansion of HSC is contentious and the engraftment capacity of the ex-vivo expanded HSC also depends on the type of stroma used. Gan *et al* has also shown that cultures of progenitors on allogeneic human stromal feeders resulted in loss of engraftment capacity of BM and CB $CD34^+$ cells (60). However culturing of $CB34^+$ cells on AC6.21 stromal cell line (derived from murine BM) maintained SRC cells (61). Maintenance of the long-term engraftment potentials in primary, secondary and possibly tertiary recipients with ex-vivo expanded CB cells in AFT024 (murine stromal cells derived from foetal liver) contact and non-contact culture systems has been demonstrated (62). Likewise, Nolta *et al* reported that long-term engraftment of HSC which was expanded using the AFT024 cell line was superior compared to HSC expanded on human stromal cells (63). In the clinical settings where transplantation of ex-vivo expanded CB HSC was performed in high risk chronic myeloid leukaemia (CML) patients (n=2) successful engraftment was achieved using ex-vivo expanded CB HSC with the AastromReplicell system (ex-vivo cell production system) along with unmanipulated CB cells. The graft had exclusive donor origin cells (64). However in another clinical trial of 28 patients, ex vivo expansion using AstromReplicell system increased the cell

numbers but failed to hasten the myeloid engraftment (65). In an autologous transplantation trial, 18/19 breast cancer patients who received ex-vivo expanded BM cells from the small aliquots achieved successful engraftment (66). However, in the autologous setting it is not clear if engraftment was the result of infused ex-vivo expanded cells or could be attributed to endogenous recovery. Shpall *et al* demonstrated early neutrophil and platelet engraftment with 37% survival rate at 30 months when transplantation was performed with the combination of ex-vivo expanded and un-manipulated HSC cells in patients with haematological malignancies (n=34) and breast cancer (n=3) (67). However the clinical efficacy of expanded cells contributing to long-term engraftment has not been demonstrated. In summary, ex-vivo expansion is able to expand total cell numbers but it compromises the long-term engraftment capacity of the HSC.

Furthermore, for ex-vivo expansion of HSC for clinical applications, HSC need to be cultured in serum free media, and recombinant growth factors and cytokines used should be of clinical grade. The focus of future efforts should be on refining culture methods, optimising the combination and concentration of specific cytokines, along with assessing the effect in vivo of the ex-vivo manipulation of progenitors for long-term engraftment. This might allow the expansion of primitive HSC without compromising their self-renewal capacity.

1.3.2.2 Multi-unit cord blood transplantation

Mathe *et al* in 1969 was first to report the combined transplantation of BM from different donors as a method of increasing graft cell dose (68). Similarly Chen *et al* demonstrated that combining two different T cell depleted, HLA mismatched BM resulted in enhanced myeloid engraftment in a murine model (69). As cell dose is a major limiting factor in CBT and furthermore CB unit cells have reduced immuno reactivity compared to adult source of the HSC (37), combining two or more partially matched CB units could increase the cell dose without significant increase in GVHD. Zanjani *et al.* demonstrated the enhanced engraftment following multiple unit CBT in a sheep model. Nevertheless the long-term engraftment was achieved from

only one of the transplanted CB units (70). Recently, another murine study demonstrated that double CBT (DCBT) leads to an increase in engraftment (71). On the contrary Kim *et al.* reported similar engraftment in murine model, following single cord blood transplantation (SCBT) and DCBT, despite double the dose of the MNC being used in DCBT (72)

The improved early engraftment following DCBT in animal studies led to clinical studies. Early studies were mainly case reports and small case series. Shen *et al* reported four patients with advanced solid tumours who received high-dose chemotherapy and HLA-mismatched, unrelated multi-CBT. Of these patients, three achieved complete remission and one achieved a partial remission. Little or no GVHD was reported. Weinreb *et al* reported a patient who received high-dose chemotherapy followed by transfusion of 12 randomly selected units of CB. Similarly De Lima *et al* reported an early engraftment following DCBT (73).These early results demonstrated that HLA-mismatched and unrelated multi-CBT may engraft with little or no GVHD and may hasten recovery from marrow suppression. These early encouraging murine and clinical results increased awareness of DCBT. Barker *et al* reported that despite single unit predominance following DCBT, all patients engrafted. During DCBT, median TNC cell dose infused was 3.5×10^7/kg and the median time to neutrophil engraftment was 23 (range 15-41) days (74), which was shorter than that following SCBT (37, 44). Other clinical studies also have demonstrated the safety, efficacy, feasibility and early neutrophil engraftment following DCBT in adults (75, 76). Brunstein *et al* reported that DCBT is safe and feasible following non-myeloablative conditioning chemotherapy (n=110). In contrast to the Barker study, the median TNC dose infused in SCBT (n=17) and DCBT (n=93) was similar (3.7×10^7/kg vs. 3.3 x 10^7/kg) and resulted in similar myeloid engraftment kinetics. In all these clinical and murine studies of DCBT only one unit dominated the engraftment and ultimately contributed to long-term haemopoiesis. The predominance of single donor unit engraftment is not determined by nucleated cell dose, $CD34^+$ cell dose and degree of HLA-disparity, ABO blood group, sex match

or relative viability of the unit (74-77). Single unit dominance could be due to a graft-versus-graft reaction in DCBT, resulting in rejection of the non-dominant unit. Moreover Barker *et al* suggested that the unit with the higher CD3 count dominated engraftment (75). This is supported by the murine model where Kim *et al* demonstrated that both CB units engrafted when committed cells were depleted from the graft (72) suggesting that committed cells contributed to graft to graft rejection. However, in another study the dominance of the single unit was not predicted by $CD3^+$ count of the unit (78). At present the mechanism of single unit engraftment is not clear. Does the second CB unit provide the extra cells compared to SCBT or, as suggested by Barker's group, does the non-dominant unit facilitate the engraftment of the dominant unit (74). Single unit dominance could be due to a graft-versus-graft reaction in DCBT, rejecting the non-dominant unit. In summary, DCBT increases the cell dose and may improve early engraftment, however only one unit engrafts for reasons not clearly understood.

1.3.2.3 Co-transplantation of MSC in CBT

As described previously haemopoiesis is responsible for the formation of blood cells in the living organism which predominantly takes place in the BM after birth. Abnormalities in haemopoiesis lead to different haematological diseases. HSCT following high dose chemotherapy and/or radiotherapy is commonly used for treating patients with high risk acute leukaemia and relapsed or refractory lymphomas. The recipients of unmanipulated allogeneic BMT contain only host-type MSC in their BM (78,79) although damaged by chemotherapy (80-82).The effect on stromal cells and microenvironment damage during the haemopoietic transplantation is unclear, and during CBT where limited cell dose is an issue, any damage to the normal stroma caused during the preparatory regimen could contribute to delayed engraftment. MSC are the integral part of BM niche where HSC reside and proliferate. An average BM graft contains 2-5 MSC per 1×10^6 MNC, and hence on average 400-1000 MSC /kg will be present in graft containing 2×10^8/kg cells (83). Moreover, MSC have not been

detected in G-CSF and/or chemotherapy mobilized PB graft or CB(79). Thus the limited or negligible supply of MSC may hinder haemopoietic engraftment, and as a result this has led to the concept that co-transplanting MSC along with HSCT may enhance engraftment.

The presence of MSC in the BM stroma was first reported by Friedenstein *et al* (84). Due to the lack of a specific marker, it is difficult to identify MSC in vivo, potentially restricting the use of expanded MSC. The high proliferative potential of MSC allows rapid in vitro expansion of MSC (85) making it an appropriate cell population to investigate. Furthermore, Dexter *et. al.* has demonstrated that stromal cells are able to sustain long-term haemopoiesis in vitro (86). The MSC also possess immunosuppressive properties (87) which may allow easier acceptance of the new graft. Replenishing the microenvironment by co-transplanting MSC during HSCT has been evaluated in murine and clinical studies.

Murine studies have demonstrated that co-transplantation of MSC is safe, feasible and enhances the engraftment of HSC (72, 88-93). Different sources of HSC (PB or BM) and MSC were used for HSCT in the above studies; however, the cell dose is a major limiting factor for CBT rather than for PB or BM SCT. Only a few studies have used CB HSC (72, 89, 94) Similarly, the source of MSC also varied between studies. Foetal lung derived MSC (94) have also been used. The clinical utility of such a source of MSC is limited because of understandable ethical issues. While BM and adipose MSC have been used in other studies, no comparative data is available regarding the optimal source of MSC. As well as differing sources of MSC the immunophenotype of the cells also varied between studies. In most of the studies MSC lacked expression for haemopoietic markers such as CD34 and CD45, however Noort *et al* used foetal lung MSC (94) that expressed HSC markers, raising the possibility of a haemopoietic origin.

Furthermore, to exclude homing interference (getting trapped in various tissues), delivering the cells directly at the site has been evaluated. Some studies have used direct intra

bone marrow (IBM) injection of HSC while others have used infusion of cells through peripheral veins. IBM injections were found to be advantageous for homing and lodgement of HSC (95-99). When CB $CD34^+/CD38^-$ cells were transplanted in NOD/SCID mice by IBM injection, 15 fold higher SCID repopulating cells was achieved compared to intravenous injection(95). Similarly Zhang *et al* demonstrated that IBM injection of human BMC leads to more rapid haemopoietic engraftment than IV injection (93). However Muguruma *et al* demonstrated similar engraftment of CB $CD34^+$ cells following IV and IBM injections. Moreover, following IBM injection, MSC did not integrate in the BM of the opposite tibia that did not receive injections. On the side that received IBM injections, the injected human MSC were able to self renew and generate cells exhibiting the phenotypic and functional characteristics of human BM in mice (100) indicating that the IBM injections only improve injected MSC localization at the site of injection rather than the engraftment of all haemopoietic sites. The ex-vivo expansion of MSC does not impair their effect on HSC engraftment. In't Anker *et al* demonstrated that both culture expanded and non-expanded MSC enhances the engraftment of CBT in NOD/SCID mice and following in-vitro expansion MSC did not lose their haemopoietic supporting ability (89).

The Stro-1 antigen potentially defines a MSC subset. Bensidhoum *et al* demonstrated higher haemopoietic engraftment when $Stro\text{-}1^-$ cells were co-infused with $CD34^+$ cells than when $Stro\text{-}1^+$ derived cells were used (101). Stro-1 is a marker for primitive MSC progenitors, while progression of these precursors towards maturity leads to an osteoblastic phenotype, losing STRO-1 expression (102, 103). Similarly, culture-expanded BM MSC begin to undergo partial osteogenic differentiation (104). It is known that the osteoblast produces essential factors for haemopoiesis (105) which may contribute to enhanced engraftment.

Another important issue which needs addressing is the MSC cell dose. It may influence haemopoietic engraftment. Kim *et al* co-transplanted 1×10^5 to 1.6×10^6 MSC along with 1×10^5 $CD34^+$ cells in NOD/SCID mice and demonstrated that co-transplantation of MSC enhances

HSC engraftment in a dose dependent manner (106). Similarly, other researchers have also demonstrated that co-transplantation of MSC enhances human $CD34^+$ cells engraftment in a dose dependent fashion in NOD/SCID mice (92, 107). This was more obvious when low doses of CB CD34+ cells were given (107).

There is limited published literature regarding the role of the MSC in DCBT. Kim *et al* demonstrated that the co-transplantation of the BM MSC enhances the engraftment of DCBT and allowed engraftment of both the cords. This murine study also demonstrated that lineage depletion of MNC of the CB alleviates single donor predominance. This suggests that graft-versus-graft reaction with immunological competition between two grafts may be responsible for the single donor predominance. On the co-transplantation of MSC, the single cord dominance was reduced leading to enhanced engraftment (72).

Limited clinical experience has demonstrated the feasibility and safety of co-transplantation of in-vitro expanded autologous and allogeneic MSC in patients with haematological and non-haematological disorders. The first clinical trial using 1-2 × 10^6/Kg MSC resulted in rapid haemopoietic recovery (ANC $\geq$0.5 × 10^9/L in 8 days and platelet $\geq$20 ×10^9/L in 8.5 days)(6). In this study a high dose of $CD34^+$ cells (median dose 13.9 × 10^6/kg) was used. There was no control group and hence the role of MSC in enhancing engraftment could not be ascertained. Nevertheless co-infusion of culture expanded MSC seems safe, feasible and without any major adverse effects (6).

Subsequently Lazarus *et al* reported a multicentre trial of co-transplantation of culture-expanded MSC with HLA-identical sibling HSCT (n=46). The median times to neutrophil (ANC $\geq$ 0.5 x 10^9/L) and platelet (platelet count $\geq$ 20 x 10^9/L) engraftment were 14 days and 20 days respectively (108). No acute or long-term MSC associated adverse effects were seen, thus confirming the safety (108). In this study the HSC source was PB and BM, in which cell dose is not a major limiting factor, in contrast to CBT.

Macmillan *et al* reported a phase I-II clinical trial in which ex-vivo culture-expanded MSC from haplo-identical parental donors were infused at the time of CBT (n=8). No serious adverse events were observed with allogeneic MSC infusion. All eight evaluable patients achieved neutrophil engraftment at a median of 19 days. The probability of platelet engraftment was 75%, at a median of 53 days. This study demonstrated that infusion of ex-vivo culture-expanded haplo-identical MSC into unrelated paediatric CBT recipients could be performed safely (109). Likewise, a study conducted in 14 patients who received co-transplantation of donor MSC along with haplo-identical HSC, achieved haemopoietic engraftment and there was no graft failure, in contrast to 15% graft failure in the historical control group. This was most probably due to their potent immunosuppressive effect on alloreactive host T lymphocytes escaping the preparative regimen (110). Thus these clinical trials have demonstrated the feasibility and safety of MSC infusion. However Wang *et al* reported that the expansion capacity of MSC in patients who receive an HSCT is damaged, and no donor MSC engraftment was demonstrated (111). As previously mentioned for establishing lifelong haemopoiesis, durable long-term engraftment of the HSC is essential. For this the HSC must home to the BM, survive within the BM, proliferate and differentiate. The healthy stromal cells in the BM compartment contribute to haemopoiesis. The animal and clinical studies have demonstrated the safety, feasibility of co-transplantation of MSC along with HSC. However there are limited studies addressing the influence of the MSC in DCBT.

Currently BM represents the main source for MSC. The BM MSC is a rare population, approximately 0.001- 0.01% in the adult BM (112) and it decreases with age (113). Therefore it may not be always feasible to use BM-MSC, thus necessitating the search for an alternative source. Including BM, MSC have been isolated from different sources including adipose tissue (114), foetus (94, 115) umbilical cord (116, 117), amniotic fluid (118), placenta (119-123) compact bone and dental pulp (124).

1.3.3 Placenta as source of MSC

The placenta is a hemochorial organ, resulting from rapid proliferation and differentiation of trophectoderm and mesenchymal cells of embryonic origin. The intimate relation of the placenta and CB may indicate a role in foetal haemopoiesis, and the placenta produces haemopoietic growth factors (125). The placental stroma forms a large number of cell types including trophoblasts, fibroblasts, immune cells, and vascular endothelium. The placenta has cells derived from the mesenchyme of foetal origin tissue, and has been recently explored as a potential source of MSC (119-123, 126). The MSC obtained from the placenta has higher expansion potential than the BM MSC (121, 127) and the immunosuppressive properties of placental MSC are significantly higher than those of BM MSC (128). The morphological, immunophenotype and differentiation characteristics of adherent cells derived from the placenta are similar to those of BM MSC (127). Rodrigo *et al* demonstrated that the gene expression profiles of BM MSC and the umbilical cord vein are comparable (129). The similarities between cultured BM derived MSC and placenta derived MSC (127), UC vein (129) implies that one could utilise the placenta as a potential, more accessible source for obtaining allogeneic MSC.

The procurement of the placenta is non-invasive, and it also has the advantage of no ethical concerns. Placenta derived MSC are at less risk of viral infection when compared to BM MSC. Placenta derived MSC could be readily available and obtained at short notice without delaying treatment or causing any discomfort after BM harvesting. As a result the placenta could be more effective allogeneic source of MSC than the BM or the cells obtained from embryonic tissue.

The preliminary studies have revealed variation in the isolation procedure of the MSC. Some studies have isolated cells from explants (cultures initiated from pieces of tissue), while few studies have isolated MSC by positive selection using a combination of STRO-1, a putative marker for BM MSC and vascular cell adhesion molecule-1 (VCAM-1), or by selecting the most

commonly occurring MSC markers ecto-5'nucleotidase known as CD73 and endoglin (CD105). Moreover, there are discrepancies in expansion and culturing methods (described in detail in Chapter 2).

Despite an increase in the use of MSC along with the HSC in both preclinical and clinical settings, little is known about what happens to the systematically injected MSC cells in the live subject. After systemic injection where do these cells go? Do they remain at the injection site or migrate, and do they enhance the homing of the co-transplanted cells? Does the co-transplantation of MSC in DCBT influence the graft composition? The answers to these questions would help to understand the possible role of the MSC in haemopoietic engraftment. Further studies should be conducted addressing these questions, and to understand the role of MSC in facilitating the engraftment of HSC.

Currently, in the majority of studies BM is the main source of MSC. There are limited studies on the role of placental MSC in haemopoiesis and its effect on the graft contribution in DCBT.

1.4 Rationale of the Study

CB is an important alternative for unrelated allogeneic BM/PB transplantation. A potential advantage includes reduced incidence of GVHD. CBT is used with more HLA disparity, thus increasing the donor pool. However cell dose is the major limiting factor of CBT in adults. To achieve the cell dose for adult patients various strategies are being explored such as ex vivo expansion or by combining two CB units. Current literature reveals that ex vivo expansion of stem cells leads to an increase in their proliferative capacity, but compromises self-renewal capacity thus limiting long-term engraftment capacity. As the HSC share an intimate relationship with MSC present in the bone marrow, co transplanting MSC with CB CD34 could enhance engraftment of the HSC, thus overcoming the cell dose limitation in CBT.

1.4.1 Hypothesis

1. Co-transplantation of placental derived MSC enhances engraftment of single and double cord blood units.
2. Placental MSC co-transplantation does not compromise HSC function.
3. Placental MSC migrate to the haemopoietic sites and enhance homing of the HSC.

1.4.2 Aims

1. To isolate, expand and characterise the adherent cells derived from the placenta.
2. To assess the effect of placental MSC on haemopoietic engraftment following SCBT and DCBT in NOD/SCID mice.
3. To study the effect of the placental MSC on the maintenance of the long-term engraftment potentials of the $CD34^+$ human cells.
4. To study the effect of co-transplanted placental MSC on CB $CD34^+$ homing, and possibly gain insight into the mechanism behind enhanced engraftment

Chapter 2 - Characterisation of adherent cells from the placenta

2.1 Introduction

The pluripotent HSC have capacity to self-renew, prolifrate and differentiat into mature haemopoietic cell type. The most mature haemopoietic cells live for only a few hours (granulocytes) or weeks (erythrocytes). This means that every day, about 10^{13} new myeloid cells must be produced to replenish the supply (130). This huge number of mature cells can be replaced by the small HSC population, 0.01% to 0.05% of the total marrow cells, demonstrating the proliferative and differentiating capacity of stem cells (130). The sites of haemopoiesis change during the life cycle. Embryonic HSC develop from mesenchymal cells in the yolk sac. After the 12th week, foetal liver is the main haemopoietic organ, along with spleen. From the 20th week BM becomes increasingly important, and by the time of birth, bone marrow is the main haemopoietic organ (131). For the first 2-3 years of life, active BM is found in all the bones. Later, there is gradual replacement of active (red) marrow by inactive (fatty tissue) marrow towards adulthood; while the active haemopoiesis is restricted to the epiphysis of long bones, and the axial skeleton. Localization of the HSC within adult BM is highly organized, with hierarchical, lineage-specific spatial positioning. The stem cell localization studies suggest that there are anatomically and physiologically distinct niches for the HSC.

The haemopoietic niche is a three dimensional structure composed of MSC, osteoblasts, fat cells, endothelial cells, vessels, and extra cellular matrix (consisting of collagen,

fibronectin, and proteoglycan constituents). Two haemopoietic niches have been described: the osteoblast niche and the vascular niche. Undifferentiated HSC reside in the osteoblast niche which maintain HSC quiescence and self-renewal, while progenitors that express lineage-commitment demonstrate selectivity to the vascular niche (132-134). Interaction of the committed progenitors with sinusoidal endothelium at the vascular niche is thought to induce haemopoietic expansion. The osteoblast niche is formed by osteoblasts and the other mesenchymal derived stromal cells, such as reticular cells, fibroblasts and adipocytes. As mentioned earlier, undifferentiated HSC localize to the osteoblast niche and the stem cell-niche interaction mediates the balance between quiescence, self-renewal and differentiation of HSC. Osteoblasts of MSC origin mediate HSC adherence to a niche within the endosteal region. The molecular mechanism by which the osteoblastic niche regulates the HSC cycling is unclear. It is known that the osteopontin is expressed on the endosteal surface, provides anchorage to α and β integrins on the HSC, and contributes towards the HSC homing and trans-marrow migration. Angiopoietin-1 is a signalling adhesion molecule expressed on osteoblasts, binds to its receptor Tie2 on HSC, and might induce adhesion and quiescence within the niche. Other factors secreted by osteoblasts include ligands for Notch receptors and N-cadherin which also contributes to the interaction of HSC and osteoblasts in the niche (135).

Besides producing mature, specialised cells such as osteoblasts, reticular and other stromal cells which interact with the HSC, MSC are also directly involved with regulation of the haemopoietic process. The molecular pathways of interaction between MSC and HSC are being actively studied. MSC secretes multiple chemokines including CXCL1, CXCL2, CXCL5, CXCL8, CXCL10, CXCL11, CXCL12, CX3CL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL20 and CCL6(136). They also express various chemokine receptors such as CCR1, CCR2, CCR3, CCR4, CCR5, CCR7, CCR9, CX3CR1, CXCR3, CXCR4, CXCR5 and CXCR6(136). In vitro Honczarenko *et al* has shown that CXC and CC chemokines induced directional migration of

BM MSC. It has also been reported that BM MSC play an important role in promoting migration, homing and localization of the HSC to the BM (137, 138). In vitro, more primitive haemopoietic progenitors possess a higher affinity to MSC than the committed progenitors. This interaction between HPC and HSC is mostly mediated through fibronectin-1, VCAM-1, cadherin 11, CX43, TGFβI, and integrin β-like1. Following injection of human MSC into the BM of immunodeficient mice, human MSC have been shown to differentiate into stromal cells, pericytes, myofibroblast, bone lining osteoblast, endothelial cells, osteocytes and reconstituting the BM microenvironment(100) emphasising the important role of MSC in haemopoiesis.

Stem cell transplantation is the potential curative treatment for patients with advanced haematological and non-haematological malignancies. During stem cell transplantation patients are treated with high dose chemotherapy and radiotherapy to eradicate the malignant cells, immunosuppress the recipient, and provide a space or niche for the infused stem cells. The myeloablative conditioning chemotherapy and radiotherapy may damage the microenvironment which could contribute to delayed engraftment especially when the HSC dose is limited. Potentially, MSC could be used to restore this microenvironment (139, 140). Moreover, culture expanded MSC have unique immunomodulatory properties which makes them suitable for therapeutic use. They express HLA class I molecules and lack any expression for HLA class II and other co-stimulatory molecules like CD40 and CD80, making them undetectable by the immune system. MSC also possess immunosuppressive properties(87) which may allow easier acceptance of the new graft. Therefore, replenishing the microenvironment by MSC during HSCT could be a rational strategy to improve engraftment. However, in a BM, CB or PB graft there are very few to negligible MSC which may be inadequate to replace the damaged BM microenvironment, thus co-transplantation of MSC has been studied in murine and clinical studies. Murine studies have demonstrated that co-transplantation of MSC enhances the engraftment of HSC (89-95). Similarly, clinical studies have also demonstrated that infusion of

ex-vivo expanded autologous, donor origin and third party MSC along with autologous, allogeneic and CBT is safe and feasible (6, 108, 109, 141) and possibly improves haemopoietic engraftment (6).

Currently BM represents the main source for MSC. However, BM MSC is a rare population, approximately 0.001- 0.01% in the adult BM (112) and it decreases with age (113). Harvesting MSC from BM is an invasive procedure and may necessitate the need for general anaesthesia. Therefore it may not always be feasible to use BM-MSC, thus necessitating the search for an alternative source. Although MSC can be isolated from different sources the optimal source for enhancing HSC engraftment is yet to be identified.

Recently placenta has been explored as a potential source of MSC (121-125, 130). Placental derived MSC have a number of advantages including easy non-invasive procurement, higher expansion potentials than BM MSC (121) and lower risk of viral infection. Moreover, the immunosuppressive properties of placental MSC are significantly stronger than that of the BM MSC (128). It has been demonstrated in murine studies that the placenta serves as a source and a functional niche for HSC (142, 143). Moreover the intimate relation of placenta and CB may indicate a role in foetal haemopoiesis, and it is known that the placenta produces haemopoietic growth factors (125) including stem cell factor (SCF), Flt 3 ligand, interleukin-6 (IL-6), and macrophage stimulating factors which support expansion of the HSC (144). All these features make placenta an appealing tissue source of MSC.

MSC are a rare cell population that occur at low frequency in-vivo and lack specific markers to identify them in-vivo. The current data obtained on MSC is based on studies performed on in-vitro expanded cells. The therapeutic use also requires high cell numbers, necessitating in-vitro expansion of MSC. Currently there is inconsistency in methods to isolate and expand the initial cell population. This chapter addresses the isolation, characterisation, differentiation potential, proliferation capacity and genomic stability of placental MSC. After

optimising the isolation procedure and the placental source, adherent cells isolated from the foetal aspect of the placenta were enriched by sequential passaging. Subsequently, these placental adherent cells were characterised by their morphological features, surface antigen expression and differentiation potential. The placental MSC were culture expanded to use for co-transplantation studies, hence their genomic stability was assessed by karyotyping at various passages.

MSC cell biology is evolving. There is no clear definition of MSC, however the International Society for Cellular Therapy (ISCT) has definied MSC based on its morphology, differentiation potentials and surface antigen expression. MSC are non–haemopoietic progenitor cells, isolated by adherence to plastic substrate. They have self-renewal potential; differentiate into at least three mesenchymal lineages (bone, cartilage, fat); express common MSC markers while lack HSC markers; and possess fibroblastic morphology (145). The results of the various experiments has shown that adequate cells can be consistently obtained from the foetal aspect of the placenta. Thus the placenta can be a source of allogeneic MSC which satisfy the requirements set forth by ISCT.

2.2 Materials and Methods

2.2.1 Sample collection

The placenta samples and CB units were collected after informed consent from donors from the maternity unit at The Women's and Children's Hospital, Adelaide, SA, Australia, with the protocol approved by the Research Ethics Committee (REC 1916/2/2010). All the procedures performed during the experiments, from collection of samples to processing, isolation of cells, expansion and transplanting in the mice were performed in a bio safety class II (BSC II) cabinet with "biohazard" laminar flow hoods in a sterile environment to exclude contamination by any micro-organisms.

2.2.2 Selection of tissue

Initially, three different anatomical sites of the placenta were selected. The proximal region, the foetal aspect of the placenta (comprising the villous chorion), was selected by making an incision just below the cord insertion about 2 cm in length. The outer-most amnion was dissected and discarded, while the deflected part of the foetal membrane was finely minced. From the distal region, the maternal aspect of the placenta (decidua basalis), a similar incision was made at the surface adjacent to the endometrium and for the third source, the cord matrix obtained from the two veins in the umbilical cord was excised and cut into 2 cm pieces. Approximately 10 g of tissue from each source was processed. Figure 2 shows the anatomical sites of the three sources of the placental tissue processed.

2.2.3 General media and solutions used for the dissociation of the tissue and cell isolation and culture

Refer to Table 1

2.2.4 Enzymes for dissociation of the tissue

Collagenase type 1 [Gibco/Invitrogen, Melbourne, Australia] was diluted, 1% in sterile PBS (10 g/L) and Dispase [Gibco/Invitrogen] was diluted to 0.1 % in sterile PBS (1g/L) according to the manufacturer's instruction. DNase [Sigma-Aldrich, Sydney Australia] was prepared as 1 mL aliquots of 100 μg/mL stock solution in sterile PBS [Sigma-Aldrich]. Trypsin

Figure 2. Cross section of the placenta demonstrating maternal and foetal aspect of placenta: Mesenchymal stromal cells were isolated from three different anatomical location of placenta

i. Proximal placenta (foetal aspect of placenta)

ii. Distal placenta (maternal aspect of placenta)

iii. Cord matrix (cord vessels)

Adapted from - the image at: www.siumed.ed/~dking2/erg/placenta.htm

Table 1: General media and solutions used for tissue digestion, cell isolation and cell culture

Media	**Source**
Dulbecco's modified eagle medium	SAFC, Biosciences, Australia
Hanks' Balanced Salt Solution	SAFC Biosciences, Australia
HEPES buffered saline	SAFC Biosciences, Australia
Phosphate buffered saline (PBS)-	Sigma-Aldrich, Australia
Fungizon	Sigma-Aldrich, Australia
Penicillin/ Streptomycin	Sigma-Aldrich, Australia
L-glutamine	Sigma- Aldrich, Australia
Foetal Calf Serum	JRH Biosciences, Australia
Trypsin	Gibco/Invitrogen, Australia
Collagenase I	Gibco/Invitrogen, Australia
DNase	Sigma-Aldrich, Australia
Dispase	Gibco/Invitrogen, Australia

1× [Gibco/Invitrogen] was distributed in 5 mL aliquots. The aliquots were stored at -20^{0}C to -80^{0}C as required.

2.2.5 Tissue processing for enzymatic dissociation

Approximately 10 g of the tissue was washed three times with HBSS supplemented with 1% penicillin/streptomycin and 0.1% fungizon. After washing, it was finely cut and digested by the combination of enzymes (refer to Table 2) with constant gentle agitation of varying duration at 37^{0}C.

On achieving single cell suspension, the dissociation was ceased by adding 5 mL of MSC medium consisting of DMEM medium low glucose, supplemented by 10% FCS, 1% penicillin 100 U/mL /streptomycin (100 μg /mL), and 1% L-glutamine. The cell suspension was filtered through a 70 μm pore strainer [BD, Falcon Biosciences, CA] and the MNCs were isolated by using lymphoprep [Axis-Shield PoC AS, Oslo-Norway] density gradient centrifugation. For isolating the desired cell population, plastic adherence to substrate method demonstrated in Figure 3 was used.

2.2.6 Cell isolation and culture establishment

Primarily the $6.7 \pm 0.6 \times 10^7$ of MNCs obtained after tissue dissociation were plated in a T175 cm^2 tissue culture flask [CellStar, Greiner Bio-one GmbH/ Frickenhausen Germany] in DMEM low glucose medium [SAFC Biosciences] supplemented with 10% FCS, 1% penicillin/streptomycin and 1% glutamine and incubated at 37^{0}C in atmosphere containing 5% CO_2 and 97% humidity and left undisturbed for 7 days. During this time the adherent cells attached to the plastic substrate while the non-adherent cells floated. On the 8th day the non-adherent cells were discarded and the adherent cells were left undisturbed and were fed by fresh media. Media change was performed every 3 - 4 days to replenish the nutrients and also to avoid build-up of metabolic by-products and dead cells. The adherent cell density and

Table 2: Optimisation of enzyme digestion method to achieve maximum single, live cells from placental tissue

Combination of enzymes	**Duration of incubation at 37^0C**
Collagenase I + Dispase	4 hours
Collagenase I + DNase	4 hours
Collagenase I + DNase+ Dispase	16 hours
Trypsin+ DNase	4 hours
Collagenase I + DNase	16 hours
Collagenase I + Dispase	16 hours

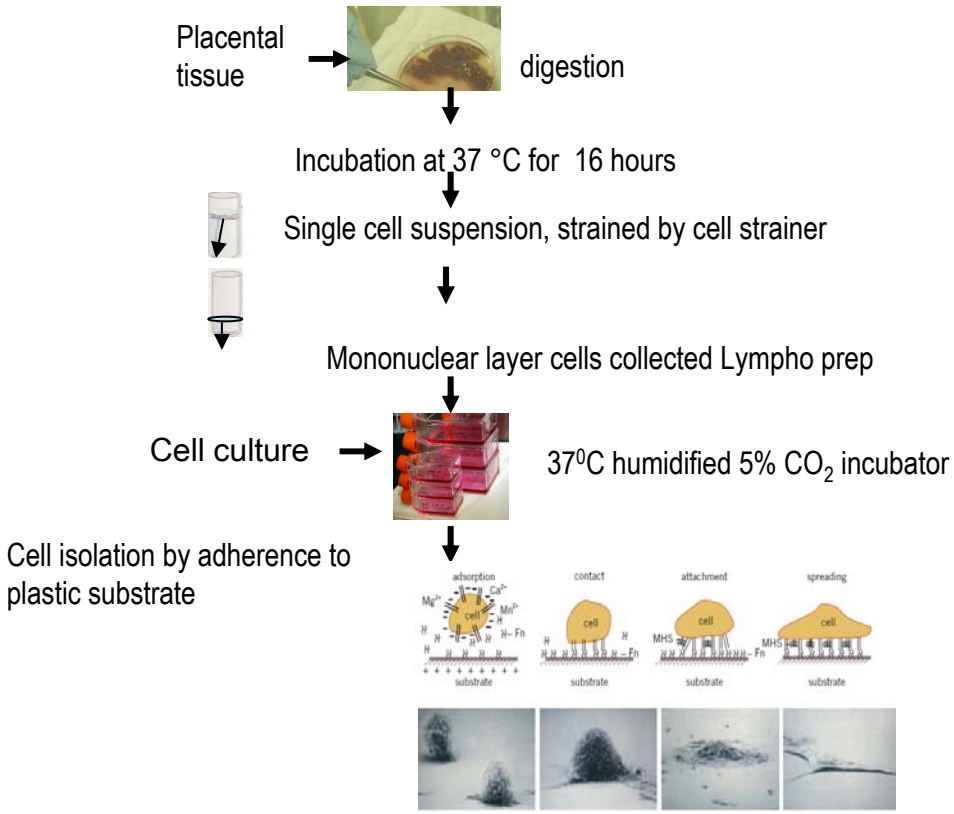


Figure 3. Schematic representation of adherent cell isolation: The MNC obtained from enzymatic dissociation of placental tissue were plated in a tissue culture flask. The plastic substrate adherent cells were trypsinised and replated for enriching the adherent cell population.

morphology were observed under an inverted microscope. On achieving 85 - 90% confluency, the adherent cells were isolated by trypsin and subcultured at a plating density 0.3 $\times 10^6$ in a fresh tissue culture flask T175 cm^2.

2.2.7 Trypan blue assay

Cell viability was measured using the trypan blue exclusion assay. Twenty μL of the MNC suspension was mixed with 20 μL of trypan blue dye and incubated for 2 minutes. Subsequently 10 μL of the mix was added to the Neubauer's chamber (haematocytometer) and the viable cells were counted by excluding the blue coloured dead cells.

2.2.8 Adherent cell harvesting

After plating the MNC cell suspension in appropriate culture medium, the MNC cytoskeletal structure binds to the surface of the tissue culture flask and spreads as a monolayer of adherent cells. After reaching 85 - 90 % confluency, these cells were harvested by trypsinization. Briefly the culture medium was discarded and the flask content was washed twice with HBSS to eliminate traces of FCS, as it inactivates the trypsin. To detach the adherent cells from the substrate the cells were incubated with 5 mL trypsin (0.25%) for 2 - 3 minutes at room temperature. Following trypsin treatment adherent cells detached and became round rather than spindle shaped. However some cells remained attached to the surface of the flask. These were dislodged by mechanically tapping the flask bottom thus breaking the fibres. The residual trypsin was inactivated by 10 mL media supplemented with 10% FCS. The cell suspension was washed twice with HBSS and resuspended in 5 mL of media and a viability check was performed by trypan blue dye exclusion assay (2.2.7).

2.2.9 Adherent cell expansion in-vitro

To enrich the adherent cell population 3×10^5 were replated in T175 cm flasks using 25 mL culture medium consisting of DMEM medium low glucose, supplemented by 10% FCS, 1% penicillin 100 U/mL /streptomycin (100 μg /mL), and 1% L-glutamine. Within an hour the rounded cells started attaching to the flask surface and regained their fibroblastic shape. The adherent cell population was harvested on achieving 85% confluency by trypsinization and further enriched by in vitro expansion.

2.2.10 Cryopreservation of cells

Cells were cryopreserved in cryoprotectant consisting of 20% dimethylsulfoxide (DMSO) [Sigma-Aldrich] in DMEM supplemnted by 20% FCS. A fresh batch of cryoprotectant at 4^0C was added drop wise to the cell suspension with gentle mixing in 1:1 ratio. This suspension was then dispensed into 2 mL labelled cryovials which were transferred to isopropyl alcohol freezing chambers and left in a - 80^0 C freezer for 24 hours, for controlled rate freezing. The following day the vials were transferred to the liquid nitrogen storage tank and stored at -196^0 C.

2.2.11 Thawing of cryopreserved samples

The frozen aliquots were rapidly thawed by immersion in a water bath at 37^0 C. The cell suspension was pippetted and transferred into a 15 mL polypropylene tube; 3 mL thawing media (IMDM supplemented with 10% FCS, 5% ACD, 50 U/mL DNAse) was added drop-wise to ensure assimilation of the DMSO. The remaining tube was filled with media and centrifuged at 1400 rpm for 5 minutes at 22^0 C. The cell pellet was washed twice to ensure the removal of any residual DMSO prior to culture or transplantation. DNAse in thaw media reduced the cellular clumping caused by the DNA from dead cells.

2.2.12 Bone marrow MSC isolation and culture establishment

Normal BM was obtained after informed consent from human donors (Royal Adelaide Hospital). The MNCs were obtained by lymphoprep. Briefly, BM was diluted with HBBS and subsequently 12 mL lymphoprep (Axis-Shield PoCAS, Oslo-Norway) was under laid in each tube and centrifuged at 1400 rpm for 30 minutes at 22^0 C with no break. The interphase of MNCs layer was separated using a transfer pipette. The MNCs obtained were diluted with HBSS to eliminate traces of lymphoprep and were resuspended in MSC media and incubated at 37^0 C in atmosphere containing 5% CO_2 and 97% humidity. These cultures were left undisturbed for 7 days. On the 8^{th} day the non-adherent cells were discarded and the adherent cells were nourished by fresh media. Media change was performed every 3 - 4 days. Adherent cells were harvested and further enriched (as described in section 2.2.9).

2.2.13 Surface antigen expression assessment

A panel of antibodies was used to study the surface antigen expression of the cells obtained from primary tissue and the expanded cells at passage 2 (refer to Table 3). The antibodies used against MSC antigens were CD106, CD29, CD44 conjugated to fluorescein isothiocyanate (FITC), while CD105, CD73, CD90 were conjugated to phycoerythrin (PE). The CD45 antibody was conjugated to FITC, while CD34 and CD31 antibodies conjugated to PE were used. The antibodies against immunological markers HLA-MHC class I conjugated to FITC and HLA-MHC class II conjugated to PE [BD Biosciences, San Jose, Ca USA] were selected.

The respective isotype control was used as control for the background fluorescence. A Blocking buffer and the immuno fluorescence wash buffer were used for preparing samples for flow cytometry. The blocking buffer was made from single strength PBS supplemented with 2% v/v BSA [Sigma-Aldrich] and 2% v/v human serum [Sigma-Aldrich]. The immunofluorescence

Table 3: Panel of antibodies used for studying surface antigens expression on single cell suspension of placental tissue

Sample tube No.	Fluorescein isothiocynate (FITC)	Phycoerythrins (PE)
1	Isotype IgG_1	Isotype IgG_1
2	HLA-I	
3		CD73
4	CD14	CD105
5	CD45	CD34
6	HLA-I	HLA-II
7	CD44	CD90
8	CD29	CD166
9	CD31	CD80
10	CD106	CD73
11	IgM	
12	STRO-1	

wash buffer was prepared by adding 5% FCS to single strength PBS. The buffers were maintained at 4^0C.

2.2.13.1 Cell staining

Cells were washed twice with HBSS at 1400 rpm for 5 minutes at 22^0 C and resuspended at 3 - 4 × 10^6/ mL in 1 mL of blocking buffer. Subsequently the cells were incubated at 4^0C for 10 minutes to prevent non-specific antibody binding. Aliquots of 5 μL of respective antibodies were dispensed into the labelled tubes; two antibodies, one conjugated to FITC and the other to PE in each tube, were added in sequence (refer to Table 3). For tubes No.2 (FITC) and No.3 (PE) a single antibody (positive control) was used for setting compensation. 2 × 10^5 cells were dispensed per tube and incubated in the dark at 4^0C for 30 minutes. The cells were then washed twice with wash buffer. After the final wash supernatant was discarded and the cells were resuspended in 0.5 mL of FACS-fix buffer. Into tube No. 11, 50 μL IgM control for STRO-1 (donated by Professor Gronthos) and to tube No.12, 50 μL of STRO-1 antibody were added. The cells were resuspended in blocking buffer and incubated on ice for 30 minutes to inhibit non-specific binding. Subsequently the cells in tubes No.11 and No. 12 were incubated for 30 minutes in the dark at 4^0 C with the secondary antibody IgM FITC.

2.2.13.2 Sample acquisition

The stained cell suspension was evaluated by two colour flow cytometry using the Coulter Epics XL Flow cytometer (Coulter, Miami, FL). The list mode data was analysed by CXP software. Forward scatter and side scatter were used to detect the cell population, and the cell population of interest was gated excluding non-viable cells and debris. This was then displayed on single parameter histograms FITC (FL 1) or PE (FL 2) where the horizontal axis represents the parameter's signal value in channel numbers and the vertical axis represents the number of

events per channel number. Background fluorescence was determined by the use of appropriate isotype controls run for each sample analysed. The laser compensation was adjusted independently for fluorochrome, according to the level of fluorescence emitted by MoAb incubated with the corresponding opposing isotype control. Before running the test sample the flow cytometer was calibrated by using standard calibration fluorescent beads.

2.2.14 Differentiation study of adherent cells obtained from the placenta

The adherent cells obtained from the foetal aspect of the placenta along with the BM MSC were assayed at passage 3 (P3) in lineage specific condition for evaluating its differentiation potential to adipocytes, osteocytes and chondrocytes using well established methods(146).

2.2.14.1 Adipogeneic assay

1 $\times 10^5$ culture expanded cells from the foetal aspect of the proximal placenta were plated in six well plates in triplicate. On achieving 60% confluency, adipogeneic media containing 0.5 nM Isobutylmethylxanthine, 0.5 μM hydrocortisone, 60 μM Indomethacin, 10% FCS, 2 mM L-Glutamine [SAFC, Biosciences], 1 mM sodium pyruvate [Sigma-Aldrich], 100 μM L-ascorbate-2-phosphates, 50 μg/mL penicillin/gentamicin, made in alpha-MEM [SAFC, Biosciences] was added. A twice weekly media change was performed for two weeks. Adipogenesis was confirmed by staining with Oil Red O stain (146). The adipogeneic assay was ceased after two weeks as the cells started aggregating and forming clusters. These cell cultures were washed twice with PBS and fixed in 4% paraformaldehyde for 15 minutes followed by air drying. Subsequently the cell clusters were immersed into Oil Red O stain solution [Sigma-Aldrich] (0.5 g Oil Red O in 100 mL of isopropanol stock solution) prepared by mixing 5 mL of stock solution to 5 mL of RO water for 30 minutes, followed by three washes. Staining was observed under an Olympus SZ-PT light microscope and digitally photographed.

2.2.14.2 Mineralization assay

1 ×10^5 culture expanded cells were plated in a six well plate in triplicates. On reaching 70% confluency mineralization induction media containing 5% FCS, 50 μg/mL penicillin, 50 μg/mL gentamicin, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM L-ascorbate-2-phosphate, 10^{-7}M dexamethasone [Sigma-Aldrich], 10 mM HEPES [SAFC, Biosciences], 1.8 mM KH_2PO_4 [Sigma-Aldrich]in alpha-MEM was added. The media was replaced weekly for 4 weeks and the osteoblastic differentiation was evaluated by detection of calcium deposits by alizarin red dye (146). After 4 weeks of incubation in mineralization media the cells were washed with PBS and fixed with 10% formalin for an hour at room temperature. This was followed by three washes with distilled water and staining with 1% alizarin red stain [ProSci tech, Qld, Australia], prepared in 2% ethanol in water for 5 minutes at room temperature. Excess stain was removed by washing the cells five times with distilled water.

2.2.14.3 Chondrogenesis assay

0.5 ×10^6 culture expanded cells in 10 mL tube were incubated in chondrocyte stimulating media consisting of 100 × insulin transferring selenous (ITS) acid premix, 100 μM L-ascorbate-2-phosphate, 50 μg/mL penicillin, 50 μg/mL gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10^{-7} M dexamethasone, 10 ng/mL TGF β3, 200 μL of each added to 0.125% BSA in 20 mL of DMEM-high glucose. The media was replaced twice weekly for 4 weeks. The supernatant was aspirated and the clustered cells were washed with PBS twice and fixed in 500 μL of 4% paraformaldehyde. Chondrogenesis was detected by alcian blue staining [all reagents from Sigma-Aldrich] (146). The chondrocyte media was aspirated and the clustered ball of cells were washed with PBS twice and fixed in 500 μL of 4% paraformaldehyde. Following fixing, the

cluster of cells was placed in a mould for embedding in wax and washed in ethanol for 30 minutes, followed by xylene for 30 minutes. The clustered cells were then placed into liquid wax, followed by instant cooling which solidified the wax into blocks. The blocks were sectioned by using the rotary microtones and then stained by haematoxylin and eosin (H &E) and alcian blue stains (pH 0.2) and were then air dried. The sections were studied under an Olympus SZ-PT light microscope and digitally photographed.

2.2.15 Proliferation kinetics of the adherent cells obtained from the placenta-CFU-F assay

The cells obtained from the different anatomical locations of the placenta were assessed for colony forming unit-fibroblast (CFU-F) capacity. The placental cells at passage 2 were plated in 100 mm tissue culture plate at a plating density of 3×10^3 in triplicates. Media was replaced every 2 days. Non-adherent cells were discarded and the colonies were counted at day 2, 6 and 8. On the day of scoring the plates were washed three times with PBS, and were stained and fixed with toluidine blue [Sigma-Aldrich] prepared at 0.01% concentration in 2% formalin in PBS. The cells were incubated overnight with 5 - 8 drops of toluidine blue stain, and on the following day washed with distilled water to remove excess stain, and then air dried (147). The following day the cells were imaged using an Olympus digital camera.

2.2.15.1 Population doubling and population doubling time

On reaching 80 to 85% confluency at passage 2-3 the adherent cells were trypsinised and the viability count was performed. The cell population doubling was calculated as PD = (log harvested cells-log of plated cells) / 0.301. Population doubling time (PDT) is the period required

for performing one doubling and it was calculated using the following equation PDT = (log 2 × T) / (log harvested cells - log of plated cells) (148).

2.2.16 Karyotyping

The adherent cells isolated from the placenta have the ability to rapidly expand. It has been demonstrated that the in vitro expansion of cells might alter the karyotype (149-151), potentially affecting the safety and therapeutic efficacy of the culture expanded cells. Therefore, at various passages, cells were examined for their cytogenetic stability. Using three different established cell lines obtained from culture expanded cells of the foetal aspect of the proximal placenta (n=3), 5 $\times 10^5$ cells were plated at various passages (P2, P10 and P12). To arrest the cells in metaphase, the multiplying cells reached 60 - 70 %, confluency, colcemid [Gibco] was added at final concentration of 0.1µg/mL. This was followed by incubation in 5% CO_2 at 37^0C for 2 hours. The cells were then dislodged by trypsinization and fixed. The fixed cells were spread on to a wet methanol cleaned slides followed by overnight drying at 60^0C. The slides were immersed in 2 × Sodium chloride/sodium citrate (SSC) [GeneWorks, Hindmarsh, SA, Australia] at 60^0C for an hour followed by two washes in RO water. On drying slides were then banded using trypsin /Leishman's stain. The metaphases were karyotyped by G banding using the LUCIA chromosome automatic analysis system. For each sample a minimum 30 metaphase were analysed. The chromosomal abnormalities such as deletion, inversion, translocation, formation of ring chromosome and change in the number, were studied. If any of these abnormalities were detected in at least 3 cells in a sample, it was considered a clonal abnormality (152).

2.3 Observations and results

In this study 17 samples of placenta were used. Adherent cells with fibroblastic morphology were successfully isolated from 15 placentas, while 2 samples were discarded due to contamination in culture.

2.3.1 Tissue dissociation and cell isolation

While optimising the tissue dissociation methods different combinations were tried in parallel in triplicates and assessed. Initial observation showed that on dissociation of the placental tissue with combinations of collagenase 1 and dispase, collagenase 1 and DNase when incubated for 4 hours at 37^0 C, resulted in low yields $0.8 \pm 0.9 \times 10^6$ cells/g $1.5 \pm 1.4 \times 10^6$ cells/g of tissue respectively. Following this, the incubation period was increased to 16 hours which resulted in a higher cell number with an average of $7.5 \pm 4.3 \times 10^6$ cells/g when dissociated with the collagenase 1 and DNase enzyme only, while the combination of collagenase 1 + DNase + Dispase resulted in fewer viable cells, $2.4 \pm 0.02 \times 10^4$ MNCs/g of tissue. The tissue when incubated with the trypsin and DNase combination for 4 hours, yielded single viable cell suspension ranging from $2.5 \pm 0.2 \times 10^5$ cells/g single viable cells (refer to Table 4). Therefore collagenase 1 and DNase with 16 hours incubation time at 37^0 C seemed an optimal approach for isolation of MNCs from the placenta. Following these trials, a fixed amount of placental tissue was digested with a combination of collagenase 1 and DNase enzymes at 37^0 C for 16 hours, and on average $7.5 \pm 4.3 \times 10^6$ MNCs /g of tissue were obtained after each dissociation. The placental source, methods, reagents used and experimental conditions while processing each placental sample were consistent. The experiments were conducted in parallel. For the initial three experiments dissociation was conducted on tissues from three anatomical sites. After observing that the cells from cord matrix had lower proliferation rate compared to the cells obtained from foetal and maternal aspect of placenta, cells were isolated from the later two

Table 4: Single, live cell yield from placental tissue according to method of enzyme digestion

Combination of enzymes	**Incubation Duration at 37^0C**	**Observations**
Collagenase I + Dispase	4 hours	few viable clumped cells
Collagenase I + DNase	4 hours	few viable cells
Collagenase I + DNase+ Dispase	16 hours	tissue over digested, higher number of dead cells
Trypsin+ DNase	4 hours	viable single cells
Collagenase I + DNase	16 hours	viable single cells
Collagenase I + Dispase	16 hours	few viable cells

sources. Furthermore when no significant difference was noted between the two sources cells were isolated only from the foetal aspect of the placenta.

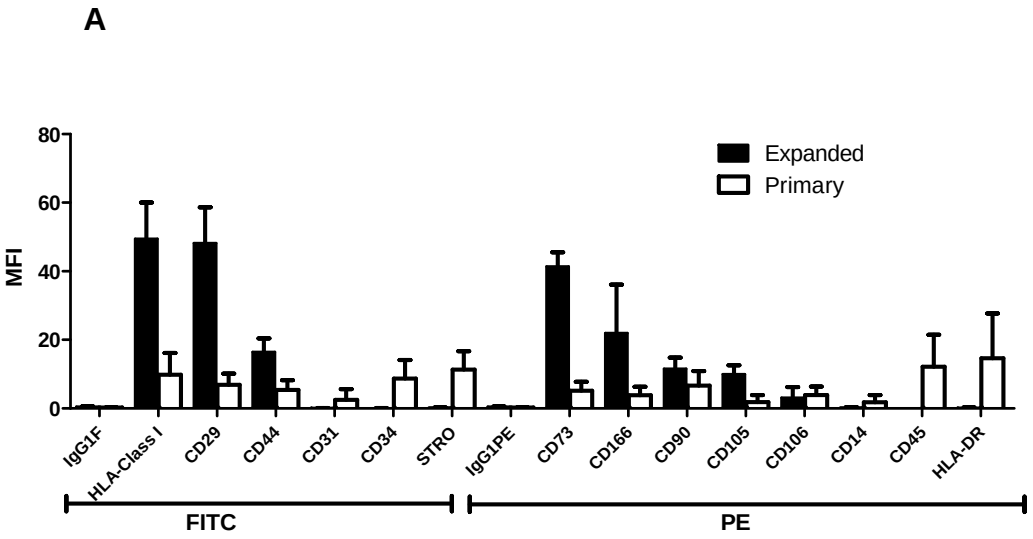
After tissue dissociation, MNCs were plated at $6.7 \pm 0.6 \times 10^7$ cells in a T175 cm^2 tissue culture flask. They adhered within 3 - 4 hours of plating. On days 8-10, patchy colonies of adherent cells on the surface of the tissue culture flask were noted with non-adherent floating cells. The plated cells initially formed patchy colonies that grew as a monolayer of adherent cells having a spindle shape and showing fibroblastic morphology (Figure 4A). These colonies grew and formed a confluent monolayer of cells within 14 - 28 days. These cells were sub-cultured to enrich the adherent population. On average $3.47 \pm 0.4 \times 10^6$ adherent cells were harvested from the first passage achieved within 14 - 28 days of initial plating.

2.3.2 Surface antigen expression

The surface antigen expression was studied on primary cells obtained from the placental tissue and the cells were isolated by plastic adherence to the substrate. Cells were counted marker positive if the mean fluorescence level was at least 0.5 log units above the isotype control cells. Results show a change in mean florescence intensity (MFI) of positive cells relative to background control. The primary cell isolated from the placental tissue was a population expressing heterogeneous markers including CD34, CD45, HLA-DR, CD14, STRO-1, HLA-ABC, CD31, CD73, CD105, CD90, CD44, CD106, and CD166.

The surface antigen expression of culture expanded cells differed from the primary cells (Figure 5A). The passage 2 cells expressed common MSC markers and lacked HSC, macrophage (CD14) and endothelial (CD31) markers (Figure 5B). The surface expression of the placenta and the BM MSC at passage 2 was assessed under parallel condition and was comparable (Figure 5C). STRO-1 is a putative marker for BM MSC (153). Hence its expression was studied and compared on the cells obtained from the different dissociation protocols. The mean fluorescence intensity on the primary cells for STRO-1 expression obtained from the tissue

Figure 4 A. Morphological features of placental adherent cells and colony forming unit-fibroblast (CFU-F): (A) The adherent placental cells obtained after plating MNC exhibit fibroblastic morphology and resemble like bone marrow-mesenchymal stromal cells when observed under inverted microscope (magnification 10 x).



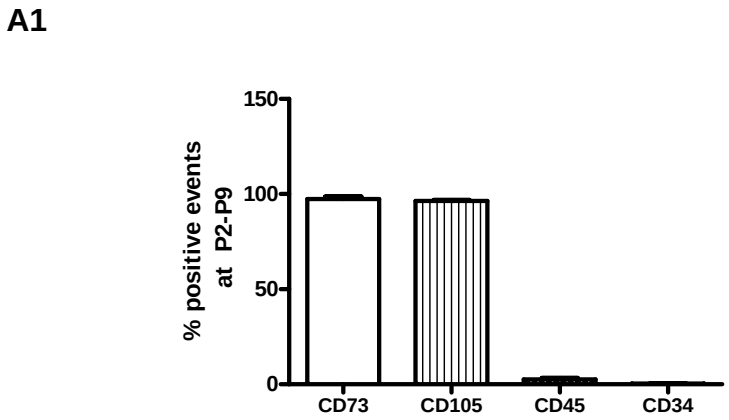


Figure 5 A-A1. Comparison of surface antigen expression on primary and culture expanded adherent placental cells: (A) Primary cells obtained from placenta are heterogeneous and express haemopoietic, endothelial, macrophage and mesenchymal markers. However, following expansion (after passage 2) adherent cells express only MSC markers and lack haemopoietic, endothelial and macrophage markers. Data is presented as MFI of the various markers as compared to isotype control.

(A1) 97% of the adherent placental cells express CD73 and CD105 MSC markers

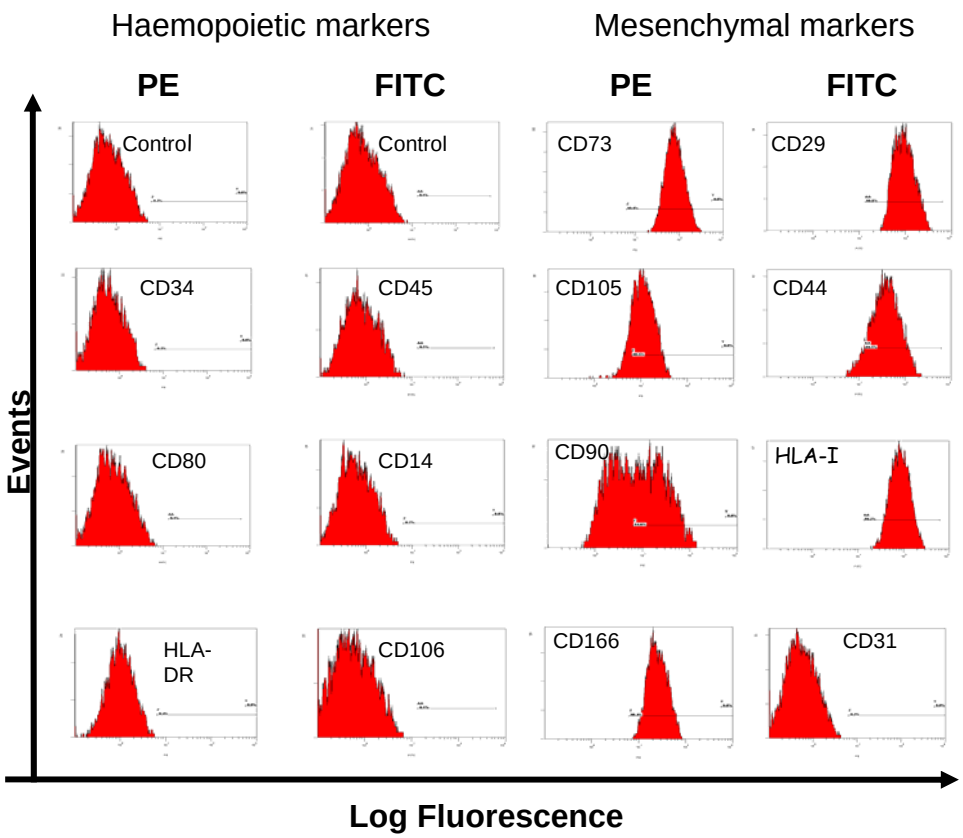


Figure 5B. Immunophenotype of adherent cells isolated from the placenta : Adherent placental cells at passage 2 exhibit common MSC markers (CD73,CD29,CD105, CD44, CD90, HLA-I, CD166) and lack haemopoietic markers (CD45, CD14, CD34 and HLA-DR).

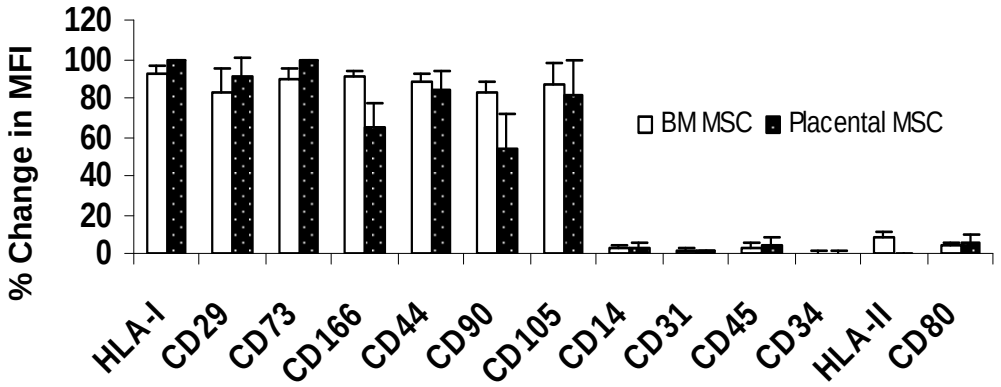


Figure 5C. Surface antigen expression of the adherent placental cells obtained from foetal aspect of placenta and BM MSC: Cells from both sources were cultured in identical conditions and harvested at 85% confluence (passage 2). No difference in surface antigen expression was identified.

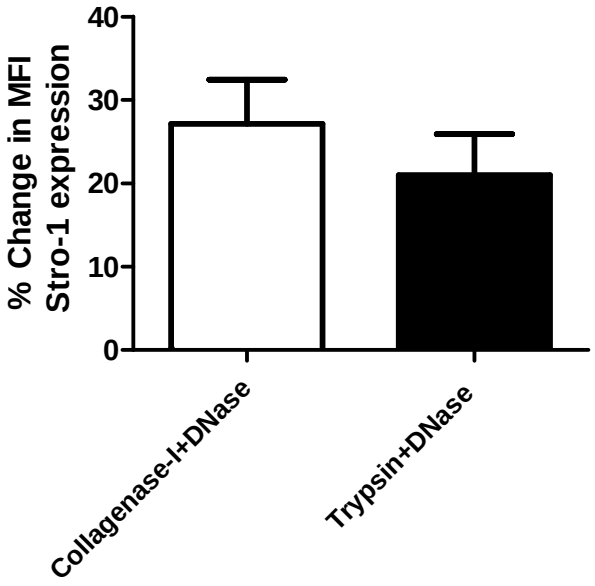


Figure 5D. STRO-1 expression according to method of placental tissue digestion: Placental tissue obtained from the foetal aspect of placental was digested with either collagenase 1 + DNAse or with trypsin + DNAse combination and STRO-1 expression was assessed. The STRO-1 expression was higher (27 ± 5.3) on placental cells digested with collagenase-1 + DNAse combination compared to cells digested with the trypsin + DNAse combination (22 ± 4.9)(n=3).

digested by the collagenase-1 + DNase for 16 hours was 27 ± 5.3% higher than the expression on the cells obtained by trypsin + DNase dissociation for 4 hours, 21.0 ± 4.9% (Figure 5D). Subsequently on enriching the adherent cell population the expression for STRO-1 was completely lost (Figure 5A) indicating maturation of the progenitors. Similarly, BM MSC gradually loses STRO-1 expression (153).

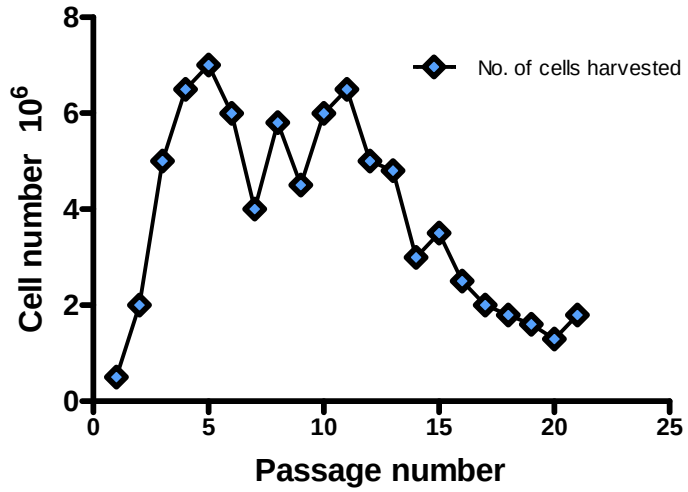
2.3.3 Proliferation potential of adherent cells obtained from the placenta

The adherent cells, after reaching 85% confluency were harvested by trypsinization. The interval required by a cell population to double is the population doubling (PD) and each passage number indicates the number of harvest or the trypsinization steps performed during the culture period. In this study from passage 2 the cells had a steadily increasing growth rate, with an average 3 - 5 PD (Figure 6A). The MSC are a rare population and are obtained after ex-vivo expansion, thus monitoring the proliferation potentials of MSC is critically important. The PD is a more accurate measure to assess the cell growth. At the 6^{th} passage 16 cumulative population doublings (CPD) were observed, followed with 27 CPD at 9^{th} passage. As the passage number increased the proliferation potentials of the cells decreased, and an increase in the population doubling time (PDT) was noted (Figure 6B). Adherent cells reached 80 to 90% confluency and weekly trypsinization yielded 4.5 to 6 $\times 10^6$ cells every harvest. As the passage number (P14-P18) increased it took a longer time to achieve the same confluency and the harvested cell number decreased ranging from 3 - 3.5 $\times 10^6$ (10 fold expansion). Further decrease was noted in the number of harvested cells ranging from 1 - 1.5 $\times 10^6$, and longer PDT was needed to reach the same confluency. The cells reached senescence at P22 - P26.

2.3.4 CFU-F assay

The proliferation potentials of the cells obtained from the foetal aspect of the placenta were higher compared to the cells obtained from the matrix of the vessel of the umbilical cord,

A



B

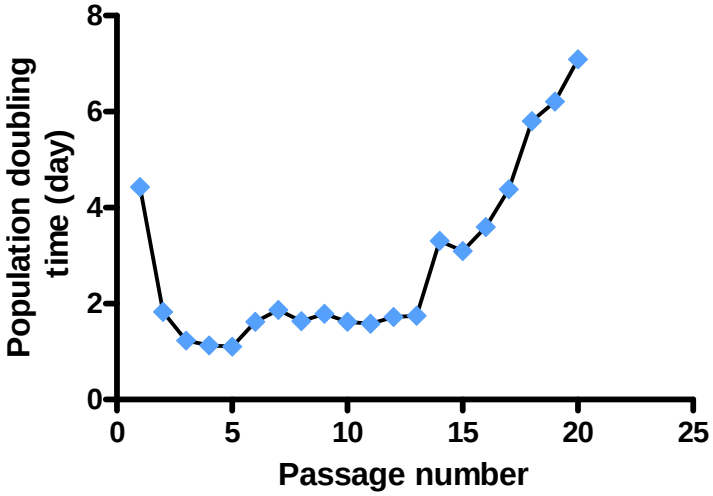


Figure 6A-B. Proliferation potential of adherent placental cells reduces with higher passage number: (A) 3 x 10^5 of adherent cells from the foetal aspect of the placenta at different passages were plated and the number of cells harvested were plotted against the passage number. The proliferation potential reduces at higher passages.

(B) The population doubling time (PDT) was assessed after plating a fixed number of cells at different passages. The PDT was calculated using the following formula PDT = (log 2 × T) / (log harvested cells - log of plated cells). [T=time].

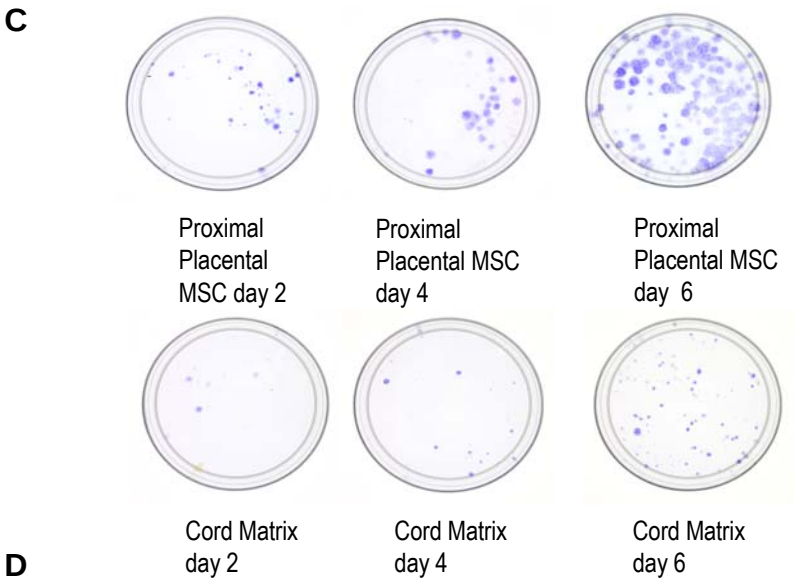


Figure 6C-D. Proliferation potential of adherent cells varies according to anatomical location of placental tissue: (C) At passage 2, adherent placental cells (3×10^3) from three different anatomical locations of the placenta were plated and CFU-F were assessed at day 2, 4 and 6. Adherent cells isolated from the proximal placental tissue have higher CFU-F quantity compared to the adherent cells obtained from cord matrix.

(D) Cells 3 x 10^5 from proximal and distal aspect of placenta were plated in a T75 cm^2 flask and on day 6 confluency was observed under inverted microscope (4 x). The proliferation potential of proximal and distal aspect the placenta was comparable while cord matrix was lower.

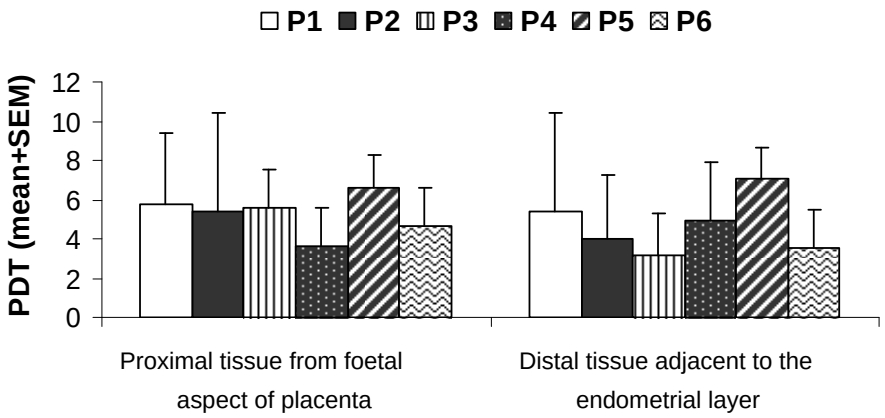


Figure 6E. Population doubling time (PDT) is comparable in adherent cells obtained from the proximal (foetal) and distal (maternal) aspect of placenta: Adherent cells (3×10^5) isolated from proximal (foetal) and distal (maternal) aspect of the placenta (n=6) were plated and PDT was studied. Average PDT of adherent cells obtained from foetal and maternal aspect of the placenta was comparable. (P1 = placenta 1, P2 = placenta 2, P3 = placenta 3, P4 = placenta 4, P5 = placenta 5 and P6 = placenta 6). Data represented as mean PDT+ SEM.

as more colonies of cells were formed in the same duration when plated at similar plating density (Figure 6C). Growth kinetics between the proximal and distal portion of placenta was also compared and no significant difference was noted (Figure 6D-E). There was no significant difference between the sources and hence for subsequent experiments only one source, foetal aspect of placenta was used.

2.3.5 Differentiation potentials of adherent cells obtained from the placenta

The adherent cells obtained from the foetal aspect of the proximal placenta were assessed and compared to BM MSC. The placental MSC at passage 2 were stimulated for 4 weeks by chondrogeneic media. After 24 hours of incubation in chondrogeneic media cells clumped and aggregated forming a spherical structure. Alcian blue staining demonstrated the presence of chondroitin sulphate, an important structural component of cartilage.

The adipogeneic assay conducted for 12 days demonstrated formation of bright red droplets of fat indicated by staining with oil red O, demonstrating adipogenesis. Subsequently, osteogenesis was not quantified but was assessed by the presence of calcium deposits detected by the alizarin red stain after incubating the cells in osteogeneic media for 4 weeks. The BM MSC was used as the positive control and the differentiation of the culture expanded cells obtained from the proximal placenta cells were comparable (Figure 7). The differentiated cells were not quantitated.

2.3.6 Karyotyping of the culture expanded cells

The cytogenetic analysis of cultured placental MSC (n = 9) was tested until passage 12. At P12, cultured cells had undergone 32 CPD and demonstrated normal chromosomal numbers. The structural integrity and lack of any clonal abnormalities indicates that the culture expanded

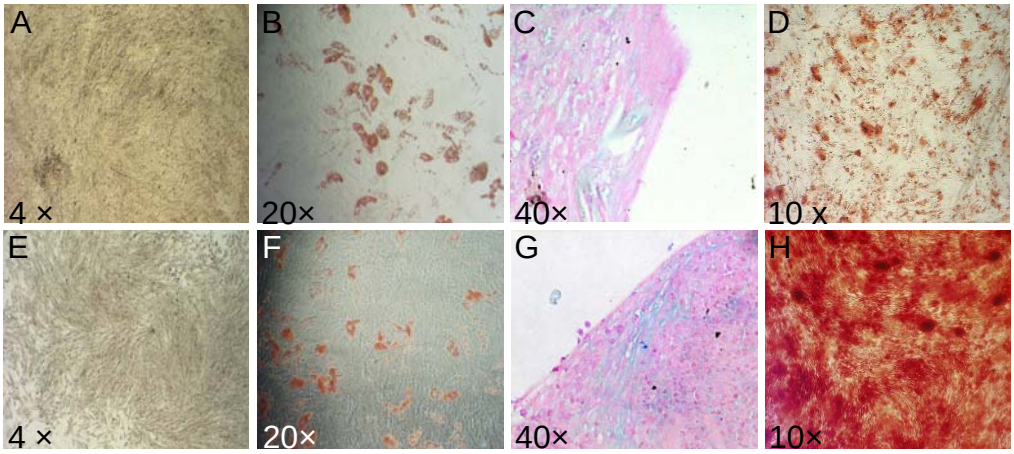


Figure 7. Placental MSC demonstrate multi-lineage differentiation properties similar to bone marrow MSC:

i. Control cells: BM MSC (A), adherent cells obtained from placenta (E).

ii. Oil Red O staining dem onstrates adipogenesis of BM (B) and placental MSC (F).

iii. Alcian blue staining demonstrates chondrogenic differentiation of BM (C) and placental MSC (G).

iv. Alizarin red staining dem onstrate os teogenic differentiation of BM (D) and placental MSC (H).

placental MSC maintain genetic stability. These cells displayed a progressive decrease in proliferation after passage 14. A sample of karyotype at passage 12 is demonstrated (Figure 8).

2.4 Discussion

The MSC is a rare population obtained primarily from BM, but other sources including adipose tissue, dental pulp, amnionotic fluid, kidney, liver, placenta, cord and foetal lung have also been identified. The MSC population is relatively fewer in adult PB and in term CB. Foetal organs such as liver, kidney, lung and BM contain MSC; however their utility as a source of MSC involves ethical issues and difficulty in obtaining the samples. Comparatively easy procurement of the placenta without invasive procedures makes it a valuable source of MSC. Furthermore, the cells obtained from the placenta have higher expansion potentials than the BM MSC (123), possess stronger immunosuppressive properties and are at less risk of acquired viral infection.

The placenta is a fetomaternal organ which develops from the blastocyst (6 - 7 days after fertilization). The foetal portion is the villous chorion and the decidua basalis is the maternal portion (154). Full term placenta can be divided into different anatomical regions: amnion, chorionic plate, decidua basalis and parietalis. The chorion is composed of mesenchyme, a region of extravillous proliferating trophoblasts and chorionic villi. Chorionic villi originate from the chorionic plate and anchor the placenta through the trophoblasts of the basal plate and maternal endometrium. MSC have been isolated from different anatomical sites of the placenta, including amniotic fluid (118), amnion and chorion (128), decidua basalis, decidua parietalis (121) and chorionic villi (122).

The success of isolating MSC and the origin of MSC (foetal or maternal) depends upon the anatomical site of the placenta used to isolate the MSC. MSC were isolated from only 6/10 and 4/10 samples of decidua parietalis and decidua basalis respectively, and all were of maternal origin (121). Second trimester amniotic fluid is also a rich source of foetal MSC but

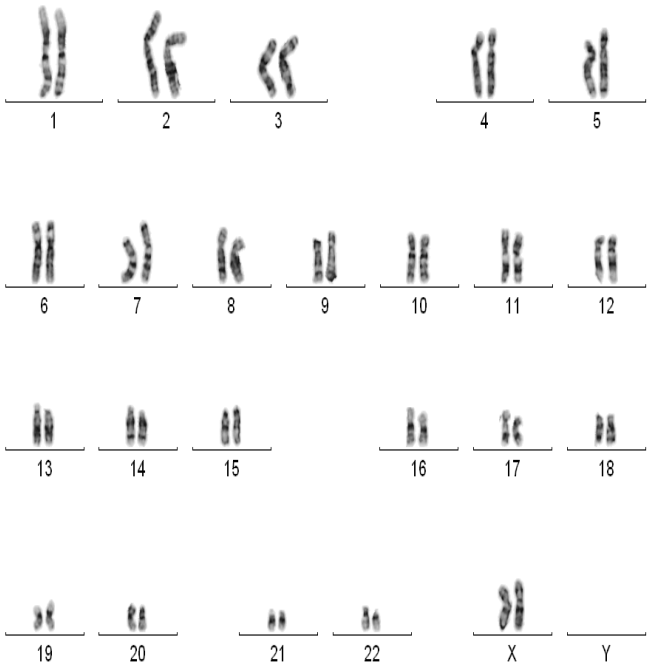


Figure 8. Placental MSC exhibit normal karyotype: Above is a representative karyotype at passage 12. Adherent cells from the foetal aspect of the placenta exhibit normal karyotype 46XX. Karyotype analysis was performed on three different placenta at three time points P2, P10 and P12. Abnormalities were not noted in the assessed 9 karyotypes.

harvesting it involves amniocentesis with its attendant risks. However full term (third trimester) amniotic fluid is not a good source of MSC: only 2/10 samples resulted in foetal MSC (121). MSC could also be isolated from 8/10 and 7/10 samples from amnion of second and third trimester placenta respectively. Moreover, third trimester chorion MSC showed significantly more cells positive for MSC markers than amnion epithelia cells (146). In the current study MSC were isolated from cord vessel matrix, chorionic villous (foetal aspect), decidua basalis, and decidua parietalis along with chorionic tissue (maternal aspect of placenta). From the 17 placental samples processed MSC were successfully isolated from 15, while two samples were discarded due to contamination.

Cord vessel matrix-MSC had lower proliferation potential compared to the MSC obtained from both placental tissues (foetal and maternal aspect). The proliferation capacity of MSC obtained from both sources of placenta was similar. However, the MSC obtained from decidua basalis and parietalis are of maternal origin (121). Hence, to avoid the contamination of maternal tissue, the foetal aspect of the placenta was used to isolate the MSC for further experiments. Other groups have also isolated MSC from all samples of chorionic villi and >95% of MSC were of foetal origin (120, 122). This suggests that chorionic tissue is a rich source of the foetal MSC.

MSC have been isolated and culture expanded since the 1970's. However there is inconsistency in the isolation and culture techniques used to expand MSC. Techniques, have included isolating placental MSC through explant culture, in which the cells were outgrown from the pieces of tissue attached to culture plates (122, 155, 156). Other methods include mechanical and enzymatic dissociation of placental tissue. There is variation between studies in the dissociation enzymes and procedures used for example trypsin (122, 129), collagenase(146), collagenase and DNAse (156, 157). In the current study chorionic tissue was minced and different enzymatic dissociation methods were compared to optimise viable, single

cell yields. Tissue dissociation with collagenase I plus dispase or collagenase plus DNase for 4 hours resulted in a high rate of cell death and excessive cell clumping. Similarly, tissue dissociation with collagenase, DNase plus Dispase or Collagenase I plus Dispase for 16 hours resulted in a high rate of cell death. Collagenase I plus DNase for 16 hours or trypsin plus DNase for 4 hours resulted in high numbers of single, viable cells. Cells obtained from trypsin plus DNase resulted in lower expression of STRO-1 compared to tissue dissociation with collagenase I plus DNase. This could be attributed to the difference in the dissociation potency of the enzymes used; also the collagenase used might have stronger dissociation strength. Other research groups have also used collagenase and DNase either in combination or as a single enzyme. In the current study, addition of DNase to collagenase reduced cell clumping and made it easier to get single cell suspensions.

MSC occur in a very low percentage in their innate state but a vast number of MSC are used in both preclinical and clinical settings, up to 10^9 cells (158), thus in-vitro expansion is necessary. Most studies relied on the plastic adherent characteristic of MSC and expanded the adherent population while discarding non-adherent populations. Nevertheless, immunophenotype markers to negatively or positively select MSC have been used. The STRO-1 marker has been used by Gronthos and colleagues to select the MSC progenitors and expand the selected cells. Isolated MSC by these markers, although low in number (1.4%), had a highly proliferative capacity (153). CD133 is another marker used to isolate MSC ($CD133^+$) from CB and peripheral blood, but it also resulted in isolation of heterogeneous populations along with isolated cells expressing Oct4 (159). Sorrentino *et al* have used $CD146^+$ to isolate BM MSC and demonstrated that these positively selected cells have mesenchymal differentiation potential and support HSC growth (160). Fluorescence-activated cell sorting of human bone marrow MNC based on expression of CD73, CD130, CD146, CD200, and $\alpha V/\beta 5$ integrin allowed for 100, 256, 278, 333 and 1750 fold enrichment of recovered CFU-Fs respectively (161). In the

current study MSC were expanded by using their plastic adherent properties, a well-established method (84, 112). The frequency of MSC found in various tissues differs. MSC isolated from the first trimester liver and BM were 11.3 ± 2.0/10^6 nucleated cells and 12.3 ± 3.6/10^6 nucleated cells (162); and Caplan *et al* reported that new-born BM has 1 MSC per 10,000 nucleated cells (163). In the current study, the MSC obtained after the first passage were 5.7 - 7% of MNC compared to 1.4% from the BM MNCs (153) and 0.01% of MNCs (163). This variation in MSC could be due to tissue source, dissociation and isolation method.

The variation in techniques of isolating the initial cell population and the lack of a specific marker to define MSC has held back the characterisation of MSC. In an attempt to unify the definition of MSC, the International Society for Cellular Therapy (ISCT) has proposed criteria that include a combination of positive and negative immunophenotype markers along with plastic adherence and morphological features of MSC. The Mesenchymal and Tissue Stem Cell Committee of the ISCT defines the immunophenotype of MSC: the cells must be positive for CD105, CD73, and CD90 and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR (145). In the current study, primary non-expanded MNCs obtained from the placenta exhibited both HSC and common MSC markers, thus demonstrating a mixture of haemopoietic and non haemopoietic cells. In 't Anker *et al* also observed this feature(89). However culture expanded cells consistently expressed CD73 (SH3/SH4), CD105 (SH2), CD90 (Thy-1), CD44, CD29 and HLA-class I and lacked expression of haemopoietic markers including CD34 and CD45, the endothelial marker CD31, and the macrophage marker CD14. They also did not express co-stimulatory molecules CD80, or CD31, the platelet endothelial adhesion molecule, and HLA-DR/MHC – classII(164-166). Taken together the immuno phenotype of the placental adherent cells it is comparable with common MSC immunophenotype. For therapeutic uses, the release criteria of MSC mandate >85% of cells should express CD73 and CD105. In the current study, cells were consistently positive for these markers during all passages (Figure 5 A1).

BM MSC can differentiate into a range of mesodermal lineages including adipose tissue, bone, cartilage, ligaments, nerve cells and tendons (167-169). In the current study, using an appropriate stimulus, the adherent cells obtained from the foetal aspect of the placenta differentiated into adipogeneic, chondrogeneic and osteogeneic lineages similar to the in-vivo lineage differentiation of BM MSC. These findings are in agreement with known literature.

Preclinical and clinical studies have used the culture expanded MSC without any short-term or long-term adverse effects. Recently, however concern was raised that the long-term in-vitro expansion of MSC may lead to malignant transformation (149-151, 170). These studies have highlighted the critical significance of assessing genetic stability of the long-term culture expanded cells. In the current study, despite high proliferation capacity, the placental MSC karyotype was structurally and numerically stable as tested over 32 cumulative population doublings (CPD) (achieved after 4 months of in-vitro expansion) in 12 passages. Similarly, others have also reported the karyotypic stability of ex-vivo expanded MSC over P1 to P30 (171, 172). The mRNA expression of tumour suppressor genes such as p53, p16, retinoblastoma genes and H-Ras oncogene was not altered in culture expanded MSC (172). Moreover the telomerase activity found in 85% of human cancers (173) was not detected in early and late passages of MSC (172).The process of malignant transformation requires an initial step of immortalization and subsequent acquisition of full malignant phenotype. In the current study, the population doubling progressively decreased after passage 14, and reached senescence at passage 22. At passage 21 and passage 22 cells did not expand in the appropriate culture conditions, demonstrating that these cells were not immortalized. Other groups also reported that after multiple passages MSC proliferation reduces until reaching senescence (171). In the current study, the mean CPD of placental MSC was 50 ± 0.66 PD, similar to the study of Campagnolin nucleated cells (162). Moreover in the current study IV tail injection of MSC from passage 2 - 6 (shown in chapter 3) did not form any tumours, although mice were sacrificed at 6

- 8 weeks after transplantation at the age of 16 - 18 weeks old. These observations emphasise the critical importance of karyotypic analysis of in-vitro culture expanded cells before its administration to patients. Also raised is the issue as to what passage number MSC should be ideally used for clinical studies. At passage 1 some expression of HSC markers is seen and by passage 2 the required cell number might not be available. Placental MSC have high expansion potential and large cell numbers are achievable at early passages.

This study has efficiently isolated MSC from the foetal aspect of the placenta. The obtained cell population is non–haemopoietic progenitor isolated by adherence to plastic substrate; capable of self-renewal; differentiates into at least three mesenchymal lineages (bone, cartilage, fat); expresses common MSC markers while lacking HSC markers, and possesses fibroblastic morphology.

2.4.1 Summary

This chapter has dealt with establishing the protocol for successful dissociation of the placental tissue to obtain single cell suspension, followed by isolation and expansion of the MSC. It also deals in detail with the materials and methods used for characterisation of these cells. The results of the cell phenotype and the differentiation potentials are demonstrated and discussed.

Briefly, the foetal aspect of placental tissue was digested with collagenase-1 plus DNase-1 enzymes. Plastic adherent cells demonstrated fibroblastic morphology and expressed the common MSC markers (CD73, CD105, CD90, CD44, and CD29. HLA-ABC/MHC-I) and lacked haemopoietic, endothelial and macrophage markers. Using an appropriate stimulus these adherent cells differentiated into adipogeneic, chondrogeneic and osteogenic lineages similar to the BM MSC. The cytogenetic study revealed that these cells have a normal diploid karyotype after ex-vivo expansion. This demonstrates that the placenta is a rich source of MSC, and could be used for cell-based therapies.

The importance of BM MSC in providing support for growth and differentiation of HSC has been previously reported (94, 141, 174, 175). As the in-vitro characteristics of BM MSC and the placental MSC are similar, placental MSC have been selected to determine whether they have any in-vivo haemopoietic supporting characteristics. This is discussed in detail in Chapters 3 and 4.

Chapter 3 - Cord Blood Transplantation

3.1 Introduction

Pre-clinical data demonstrates the presence of HSC in CB (28, 176-178). Compared to the adult source of HSC the cellular component of CB has advantages. These include a higher potential to form colonies in culture and a higher proliferative rate than other sources (31, 32). The CB $CD34^+/CD38^{low}$ cells have longer telomeres than stem cells from the BM (179) which might confer proliferative advantages. The lack of cytotoxic T lymphocytes in CB (33) and reduced immuno reactivity of CB (37) reduces the incidence of GVHD, thus allowing CBT with a greater degree of HLA-mismatch.

However in larger children and adults the limited cell dose from a single CB unit results in delayed engraftment and increased rates of graft failure. Prior studies have demonstrated the adverse impact of low cell dose on engraftment, transplant related mortality (TRM) and survival (77, 180-182). Patients who received a CB $CD34^+$ cell dose $<1.7 \times 10^5$/kg had 70% TRM (41). Hence, Eurocord has recommended that units containing less than 3×10^7/kg nucleated cells should not be transplanted (182, 183). As a result up to 90% of patients do not receive a CBT due to the limited cell dose from a single unit limiting the majority of adult patients from undergoing CBT.

To circumvent the cell dose limitation, various methods have been investigated in both laboratory and clinical settings. These include ex-vivo expansion of CB $CD34^+$ cells, DCBT and co-transplantation of MSC along with CBT. The ex-vivo expansion of HSC increases the CFCs, $CD34^+$ and total cell numbers (52-54). However, it compromises self-renewal capacity resulting in exhaustion of HSC leading to long-term engraftment failure (49, 55, 183). In clinical settings,

transplantation of ex-vivo expanded CB HSC has achieved limited success. Other strategies to increase cell dose include combining two or more CB units (described in detail in chapter 1) or co-transplantation with MSC. It is well-established that the HSC shares an intimate relationship with the BM microenvironment in haemopoiesis. The environment where stem cells reside is termed the niche; it is a three-dimensional structure comprising different stromal cells, blood vessels, and extra cellular matrix. The majority of supporting stroma is derived from MSC. MSC are non-cycling cells having fibroblastic morphology, expressing common MSC markers, lacking HSC markers and able to differentiate into at least three mesodermal lineages (145). The myeloablative conditioning with chemotherapy and radiotherapy may damage the microenvironment which may contribute to delayed engraftment, especially when the cell dose is limited. Therefore MSC could be used to restore this microenvironment (139, 140). MSC also possess immunosuppressive properties (87) which may allow easier acceptance of the new graft. Hence replenishing the microenvironment by MSC during HSCT could be a rational strategy. However, in a BM, CB and PB graft there are very few to negligible MSC which may not be adequate to replace the damaged BM microenvironment, thus co-transplantation of MSC has been studied in murine and clinical studies.

Murine studies have demonstrated that co-transplantation of MSC enhances the engraftment of HSC (72, 88-92). In these studies the source of HSC and MSC varied, and only few studies have used CB HSC while others have used BM and PB HSC. Similarly the MSC cell dose used also varied between studies. Kim *et al* co-transplanted 1×10^5 to 1.6×10^6 MSC along with 1×10^5 $CD34^+$ cells in NOD/SCID mice and demonstrated that co-transplantation of MSC enhances HSC engraftment in a dose-dependent manner (106). Likewise other researchers also demonstrated that co-transplantation of MSC enhances human $CD34^+$ cells engraftment in a dose-dependent fashion in NOD/SCID mice (92, 107). This was more obvious when low doses of CB $CD34^+$ cells were given (107). There is limited published literature

regarding the role of MSC in DCBT. Kim *et al* demonstrated that the co-transplantation of BM MSC enhances the engraftment of DCBT, allowes engraftment of both CB units, and reduces single CB unit dominance (72).

Clinical studies have shown MSC transplantation is feasible. Koc *et al* reported that co-transplantation of autologous MSC along with autologous $CD34^+$ cells in breast cancer patients was feasible, safe and with no acute or long-term adverse effects. Although this study demonstrated rapid neutrophil and platelet engraftment compared to historical control, the $CD34^+$ cell dose used was very high and there was no control arm (6). Similarly Lazarus *et al* reported a trial of co-transplantation of culture-expanded MSC along with HLA-identical sibling HSCT (n=46). The median times to neutrophil (ANC $\geq 0.5 \times 10^9$/L) and platelet (platelet count $\geq$ 20 x 10^9/L) engraftment were 14 days and 20 days respectively (108). No acute or long-term MSC associated adverse effects were seen, thus confirming the safety (108). In another trial patients received co-transplantation of donor MSC along with haplo-identical HSC. MSC infusion was well tolerated and there was no graft failure, in contrast to 15% graft failure in the historical control group (141). Macmillan *et al* reported a phase I-II clinical trial in which ex-vivo culture-expanded MSC from haplo-identical parental donors were infused at the time of CBT (n=8). There were no acute MSC related toxicities and engraftment was possibly improved (109). All of these clinical studies have demonstrated that infusion of ex-vivo expanded MSC along with autologous, allogeneic and CBT is safe and feasible, and there is a suggestion that engraftment may be enhanced, albeit only slightly (6, 108, 109, 141). Larger randomised studies will be required to study its influence on engraftment, graft failure, GVHD, relapse, disease free survival and overall survival.

Presently BM represents the main source for MSC, while this study has characterised the cells obtained from the foetal aspect of the placenta (Chapter 2). The role of the placental MSC in CBT has been investigated. The CB $CD34^+$ from single or double CB units were co-

transplanted with placental MSC in the NOD/SCID mouse model. Also the role of placental MSC in the graft contribution in DCBT has been explored.

The NOD/SCID mice model selected for this study recapitulates features of human haemopoiesis including the anatomical distribution of infused cells. This model supports the multi-lineage differentiation of HSC without the influence of exogenously supplied human growth factors. Murine experiments were performed with approved protocol from the Animal Ethics Committee, IMVS (project number 36/06) and The University of Adelaide Ethics Committee (AEC Project No. M-20-2006).

For long-term engraftment, the sustained self-renewal of the donor CB $CD34^+$ cells is critical. Because of limited lifespan of NOD/SCID mice, short-term engraftment and proliferative capacity of CB stem cells can be studied easily; however it is difficult to study long-term engraftment of CBT. To assess the long-term engraftment ability of CB $CD34^+$ in-vivo, serial transplantation was performed in secondary hosts. For secondary transplantation whole BM cells harvested from the engrafted primary mice (co-transplanted with or without placental MSC) were injected into the secondary host. Additionally the in-vitro colony forming unit (CFU) potential of the CB $CD34^+$ cells obtained by sorting the whole BM of the primary engrafted mice was assessed.

This chapter deals with the experiments conducted, materials and methods used, results, discussion of the results achieved, followed by the conclusion.

3.2 Hypothesis

1. Co-transplantation of placental derived MSC enhances engraftment of single and double cord blood units.
2. Placental MSC co-transplantation does not compromise HSC function.

3.3 Aims

1. To determine the optimal strategy to enhance engraftment of CB using the NOD/SCID mouse model.
2. To verify that at equivalent cell dose the engraftment achieved is similar in SCBT and DCBT respectively.
3. To study the effect of the placental MSC on the maintenance of the long-term engraftment potentials of the human $CD34^+$ cells.

3.4 Materials and methods

3.4.1 Animal model for the transplantation study

NOD/SCID mice were obtained from the animal house of the Institute of Medical and Veterinary Science (IMVS) Veterinary Services Division, Hanson Building, Adelaide, South Australia. The mice were housed in filter top isolator cages, in ultraclean pathogen free conditions. Mice were maintained on autoclaved food and water, with supplemented Septrin (Sigma Pharmaceuticals, Australia) 5 mL / L of water as a prophylactic measure.

3.4.2 Collection and processing of cord blood

CB was collected after informed consent from donors at the maternity unit of The Women's and Children's Hospital, Adelaide, South Australia. CB was drained into Baxter CPDA-1 single blood packs (450 mL, FBR 7110/ FGR 8104). CB samples were processed within 8 to 48 hours of collection.

On average 30 to 45 mL CB volume was collected. An aliquot of 7 mL of CB was diluted with 28 mL of HBSS in 50 mL tubes. The dilution of blood with HBSS prevented RBC clotting

and subsequently 12 mL lymphoprep [Axis-Shield PoCAS, Oslo-Norway] was unde laid in each tube. Samples were centrifuged at 1400 rpm for 30 minutes at 22^0C with no break. The interphase of the MNCs layer was separated using a transfer pipette. The MNCs obtained were diluted with HBSS to eliminate the traces of the lymphoprep and the anti coagulants. For 10 mL MNCs 40 mL of HBSS was added. An average of 1.5 -3.5 × 10^8 MNCs were isolated from a CB unit. The cell pellet was washed twice with MACS buffer and spun down at 1200 rpm for 5 minutes at 22^0C. MNCs were quantitated with the trypan blue dye exclusion method (described in Chapter 2). Subsequently in a 5 mL round bottom sterile tube the final volume of the MNCs in 300 µl was aliquoted. The $CD34^+$ cells from the MNC were isolated by magnetic assisted cell separation the Mini MACS IM technology [MiniMACS, Miltenyi Biotech, Germany].

3.4.2.1 Cord blood CD34+ cell immunomagnetic isolation

The $CD34^+$ cells were positively selected from the MNCs suspension by immunomagnetic cell isolation. Under sterile conditions freshly collected MNCs 10^8 per 300 µl in MACS buffer were suspended in 5 mL round bottom sterile tubes and 100 µl per 10^8 cells of FcR blocking reagent [Miltenyi Biotech] was added followed by 100 µl of MACS $CD34^+$ micro beads. This mix was incubated for 30 minutes at 4^0C in the dark. Labelled MNC were then washed with 5 mL MACS buffer and cell pellet collected by centrifuging at 1400 rpm for 5 minutes at 22^0C.

The MiniMACS magnet was attached to the MACS multi stand and the separation column was applied to the MiniMACS magnet. To prime the column 1 mL of MACS buffer was allowed to run through. The pre-labelled MNCs were passed through a separation filter [Steri-Dual TM filter] to remove any clumps and then applied to the column, allowing the MNCs to pass through the column with 3 × 1 mL MACS buffer. Subsequently the column was removed from the magnet separator, placed on a suitable sterile collection tube and the attached $CD34^+$

cells were flushed out with 1 mL MACS buffer. To enhance the purity of $CD34^+$ cell population the magnetic separation step was repeated with the eluted cells. The obtained $CD34^+$ cells were washed twice with MACS buffer and spun at 1400 rpm for 5 minutes at 22^0C.

3.4.2.2 Purity check of CB CD34+ cells

The purity was determined by flow cytometry. Purified $CD34^+$ cells were stained with phycoerythrin (PE) conjugated antibodies against human CD34. Cells were incubated on ice for 30 minutes followed by washing twice in flow buffer. The cell suspension was analysed by flow cytometry using the Coulter Epics XL. These $CD34^+$ cells were cryopreserved for future use.

3.4.2.3 Cryo-preservation of CB CD34+

CB $CD34^+$ cells were resuspended into 700 µL MACS buffer and added to 700 µL of cold cryo protectant made of 20% dimethyl sulfoxide [DMSO, Sigma] and 20% FCS in DMEM. This suspension was then dispensed into 2 mL labelled cryovials which were slow frozen in isopropyl alcohol freezing chambers. These vials were then transferred into a -70^0C freezer for the next 24 hours and the following day transferred to liquid nitrogen.

3.4.3 Radiation dose

NOD/SCID mice were sub-lethally irradiated with 325 cGy by Linear accelerator machine in the Department of Radiotherapy, Royal Adelaide Hospital, South Australia on the day prior to transplantation. The mice were transported in a perspex container with individual chambers reducing the movements of the mice; each mouse received an equivalent radiation dose.

3.4.4 Cohort's distribution and cell dose for transplantation study.

In the current study the $CD34^+$ dose was fixed at 5×10^4 in all the cohorts irrespective of SCBT or DCBT. This dose was based on our laboratory's previous experience demonstrating

mean engraftment of approximately 30%, thereby allowing the effect of MSC co-transplantation to be determined. This MSC dose is clinically achievable and has been earlier tested (6). All the experiments were conducted in parallel using 4 cohorts.

Cohort 1 received 5×10^4 CB $CD34^+$ cells from unit U1 denoted as single cord blood transplantation (SCBT); Cohort 2 received 5×10^4 $CD34^+$ cells from U1 + 4×10^4 placental MSC denoted as SCBT+ MSC; Cohort 3 received 2.5×10^4 CB $CD34^+$ cells from U1 +2.5×10^4 CB $CD34^+$ cells from U2 denoted as double cord blood transplantation denoted as (DCBT); Cohort 4 received 2.5×10^4 CB $CD34^+$ cells from U1 +2.5×10^4 $CD34^+$ cells from U2 + 4×10^4 placental MSC denoted as DCBT+ MSC (Figure 9).

3.4.5 Placental MSC preparation for injection

Previously cryopreserved placental MSC at P2 were thawed and plated in a T175 cm^2 flask, then harvested on achieving 80-85% confluency (as described in Chapter 2), A single cell suspension was prepared. The viability was assessed by the trypan blue exclusion assay and the required amount was mixed with the CB $CD34^+$ cells. The mix was then aspirated into ultra fine 29 gauge needle 500 µL syringe [BD] and 200 µLof cell suspension was injected.

3.4.6 CB CD34+ preparation for mice injection

Previously cryopreserved CB $CD34^+$ cells were thawed and viability assessed by trypan blue exclusion assay. On day 0 (injection day) CB $CD34^+$ cells with or without placental MSC were inoculated via tail vein injections. Mice were distributed in four treatment cohorts.

Following transplantation, animals were inspected daily. Animals demonstrating evidence of distress, weight loss above 10%, ruffled fur, or lethargy were checked three times

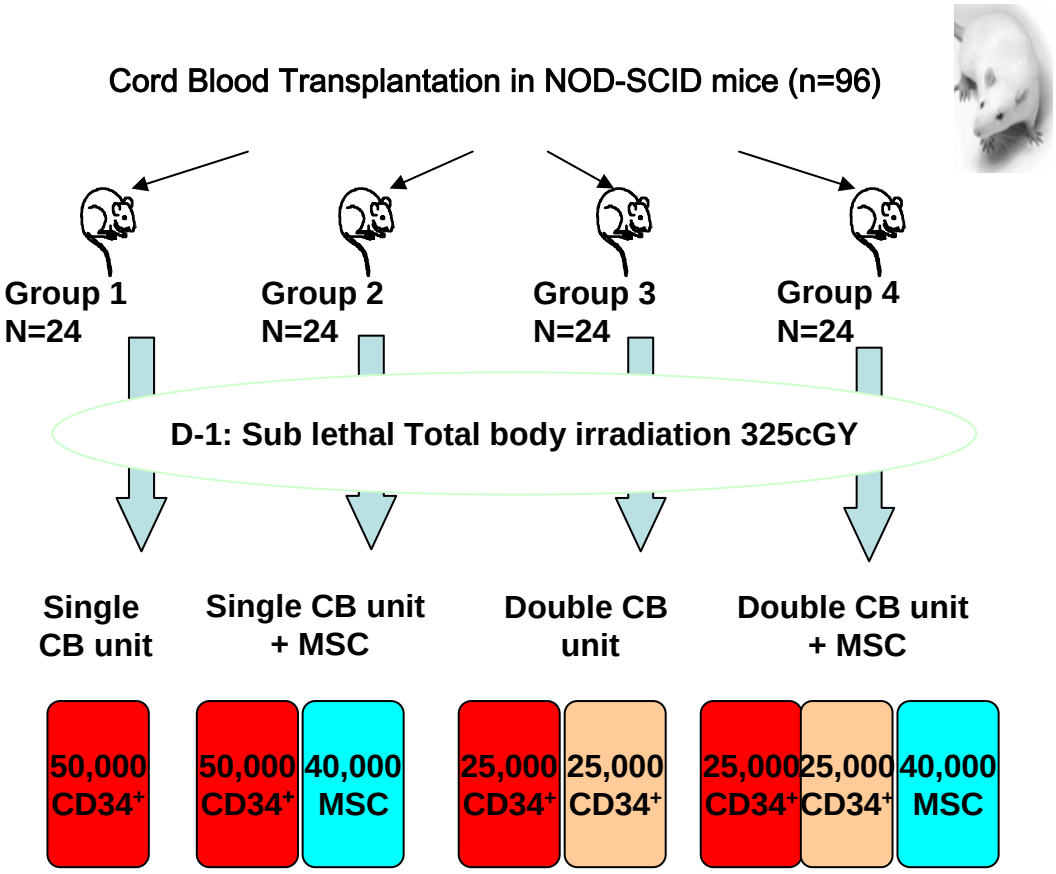


Figure 9. Schematic representation of transplant study: CB $CD34^+$ cells along with or without placenta derived MSC were injected in 96 NOD/SCID mice via tail vein. Six independent experiments were performed with four mice in each cohort and all experiments were conducted in parallel conditions. Engraftment of the human CD45 cells in murine BM was studied after 6-8 weeks.

per day and if continuing to deteriorate were euthanized immediately. Mice were sacrificed 6-8 weeks post-transplantation and engraftment evaluated.

3.4.7 Mice euthanization and dissection procedure

Mice were sacrificed by carbon dioxide ($C0_2$) inhalation. Subsequently the dead mouse was pinned to the dissection board with the abdomen facing up. The skin of the mouse was lifted up with forceps, holding anterior to the urethral opening, and the upper skin layer was cut open along the ventral midline from the groin to the chin, taking precautions to only cut the skin. The tissue around the pelvic area near the head of the femur was cut, all the ligaments and the tendons were separated and the femur was dislocated from the pelvis. The muscle tissue attached to the bone was teased until the bone was completely exposed and the femurs were decapitated.

3.4.8 Murine bone marrow harvesting

The murine bones were then placed in test tubes containing PBS supplemented with 2% FCS. BM was aspirated 2-3 times along with PBS until all the cells were liberated from the bone. The cells were filtered through a 70 μm filter [Steri-DualTM filter BD] and were quantitated by flow cytometry.

3.4.9 Cell staining for flow cytometry

The percentage engraftment of human cells was assessed using 3 colour immuno phenotyping with human specific CD45- Phycoerythrin–Cy5 (PC5) [Beckman and Coulter, Marseille Cedex 9, France] murine specific CD45-FITC [BD Biosciences Pharmingen, San Jose, CA] and human markers for myeloid and lymphoid cells CD33- Phyoerthrin (PE), CD19-PE, CD34-PE and CD 3-PE [BD Pharmingen] were used.

The collected BM cells were washed twice with HBSS at 1400 rpm for 5 minutes at 22^0C followed by suspending the cells in blocking buffer in 1 mL at 4^0C for 15 minutes. Aliquots of 50 μL (1×10^6) whole BM cells were dispensed into round bottom flow tubes and the respective antibodies were added for staining for 30 minutes at 37°C in the dark at 4^0C. The cells thus obtained were processed for staining with a panel of antibodies (Table 5) and appropriate isotype controls were used for background staining.

The red cells were lysed by incubating in 1 mL of 8.3 % NH_4Cl lysing buffer Pharm Lyse [BD Biosciences Pharmingen] for 15 minutes at room temperature followed by washing twice with HBSS supplemented with 2% human serum and 2% bovine serum albumin (BSA) and suspended in 350 μL of 2% para formaldehyde in PBS.

3.4.9.1 Sample acquisition and criteria for assessment of engraftment

The stained cell suspension was displayed as scatter plots. Forward scatter and side scatter were used to detect the cell population. The cell population of interest was gated to exclude non-viable cells and debris. A dot plot was used to provide a two-parameter display of data. An example of the flow chart is shown (Figure 10). Initially, the isotype control determined where the quadrant markers were placed. This quadrant marker divides two-parameter plots into four sections to distinguish the populations that were considered negative, single positive, or double positive.

A template was developed to detect the populations based on the locations of these cell populations. Multi-lineage engraftment was defined by the presence of human $CD45^+$ $CD\ 33^+$, $CD45^+$ $CD34^+$, $CD45^+$ $CD19^+$ and $CD45^+$ $CD3^+$. A minimum of 30,000 events were analysed using EPICS-PROFILE II XL, flow cytometer [Coulter]. The list mode data was analysed using CXP software.

Table 5: Panel of antibodies used for studying the surface antigen expression on the cells obtained from the transplanted murine BM

Sample No.	Phycoerythrin–Cy5 (PC5)	Fluorescein isothiocynate (FITC)	Phyoerthrin (PE)
1	IgG_1	IgG_2	IgG_1
2	Human CD45	IgG_2	IgG_1
3	IgG_1	murine CD45	IgG_1
4	IgG_1-	IgG_2	Human CD19
5	Human CD45	murine CD45	Human CD33
6	Human CD45	murine CD45	Human CD3
7	Human CD45	murine CD45	Human CD34
8	Human CD45	murine CD45	Human CD19

Figure 10. Immunophenotype profile of the human cells regenerated in murine BM: Three colour flow cytometry was used to measure the human cell in murine BM after 6-8 weeks of transplant. BM cells expressing the different human lineages is depicted as the percentage positive cells compared to the isotype control.

A: Cell population gated.

B,C & D: Controls set for FITC, PCY-5 and PE.

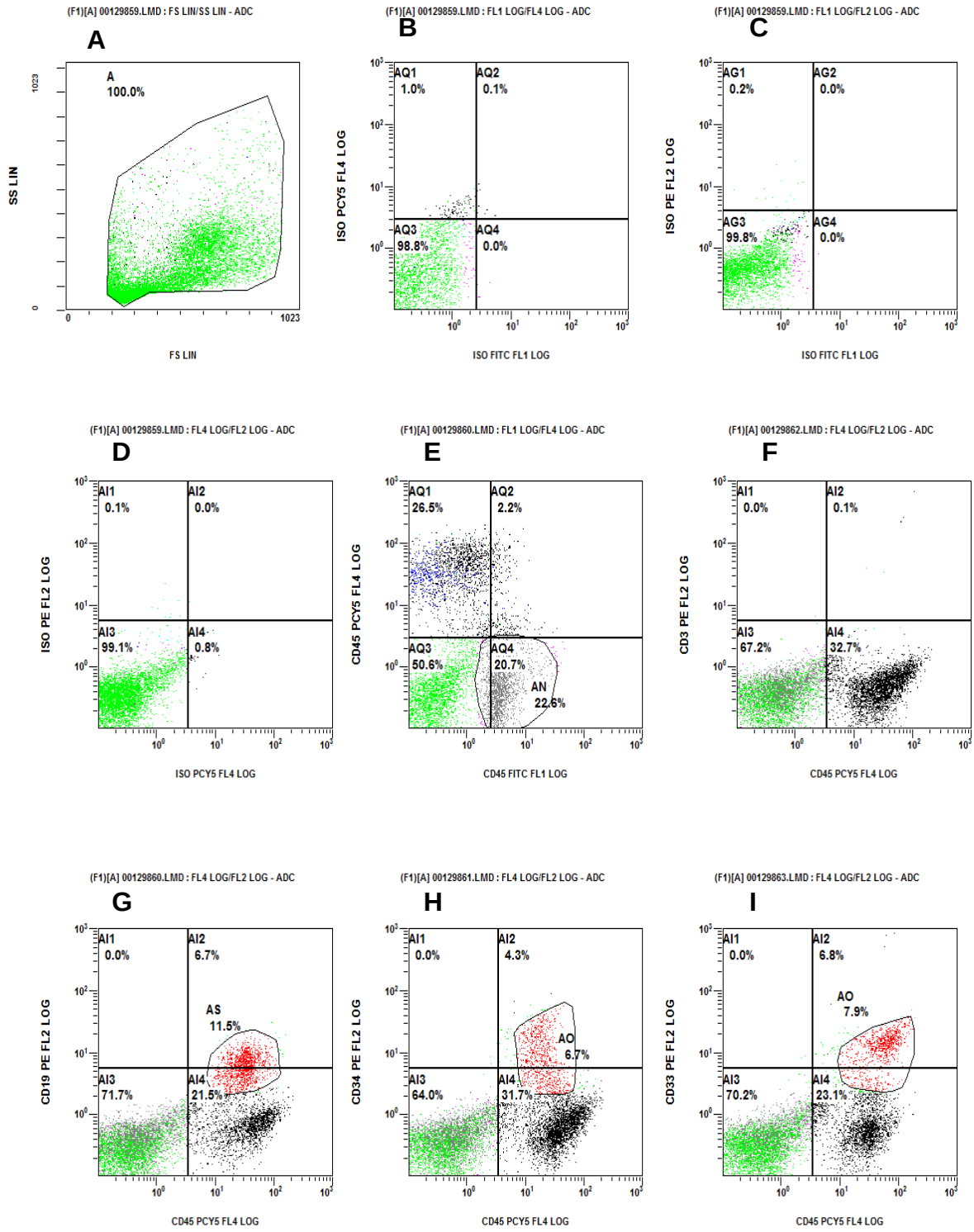
E: Murine haemopoietic cells represented by CD45 FITC.

F: Expression of human CD45 population shown by PCY-5 while expression of the human CD3 lineage is not detectable.

G: Expression of human CD19 lineage on human CD45 cells.

H: Expression of human CD34 lineage on human CD45 cells.

I: Expression of human CD33 lineage on human CD45 cells.


(F1)[A] 00129859.LMD : FS LIN/SS LIN - ADC
A
A
100.0%
SS LIN
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0
1023
(F1)[A] 00129859.LMD : FL1 LOG/FL4 LOG - ADC
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AQ1
1.0%
AQ2
0.1%
AQ3
98.8%
AQ4
0.0%
ISO PCY5 FL4 LOG
ISO FITC FL1 LOG
(F1)[A] 00129859.LMD : FL1 LOG/FL2 LOG - ADC
C
AG1
0.2%
AG2
0.0%
AG3
99.8%
AG4
0.0%
ISO PE FL2 LOG
ISO FITC FL1 LOG
(F1)[A] 00129859.LMD : FL4 LOG/FL2 LOG - ADC
D
AI1
0.1%
AI2
0.0%
AI3
99.1%
AI4
0.8%
ISO PE FL2 LOG
ISO PCY5 FL4 LOG
(F1)[A] 00129860.LMD : FL1 LOG/FL4 LOG - ADC
E
AQ1
26.5%
AQ2
2.2%
AQ3
50.6%
AQ4
20.7%
AN
22.6%
CD45 PCY5 FL4 LOG
CD45 FITC FL1 LOG
(F1)[A] 00129862.LMD : FL4 LOG/FL2 LOG - ADC
F
AI1
0.0%
AI2
0.1%
AI3
67.2%
AI4
32.7%
CD3 PE FL2 LOG
CD45 PCY5 FL4 LOG
(F1)[A] 00129860.LMD : FL4 LOG/FL2 LOG - ADC
G
AI1
0.0%
AI2
6.7%
AS
11.5%
AI3
71.7%
AI4
21.5%
CD19 PE FL2 LOG
CD45 PCY5 FL4 LOG
(F1)[A] 00129861.LMD : FL4 LOG/FL2 LOG - ADC
H
AI1
0.0%
AI2
4.3%
AO
6.7%
AI3
64.0%
AI4
31.7%
CD34 PE FL2 LOG
CD45 PCY5 FL4 LOG
(F1)[A] 00129863.LMD : FL4 LOG/FL2 LOG - ADC
I
AI1
0.0%
AI2
6.8%
AO
7.9%
AI3
70.2%
AI4
23.1%
CD33 PE FL2 LOG
CD45 PCY5 FL4 LOG

Engraftment was expressed as the percentage of cells expressing human specific CD45 marker. The mouse was considered engrafted when greater than 0.5 % human CD45 cells were detected in their BM(63).

3.4.10 Serial Transplantation

The extended engraftment potential of the human $CD34^+$ cells in the BM from the primary engrafted recipient was assessed in secondary recipients (n=21).The BM cells from the primary recipients were collected (Figure 11) as described previously. The engrafted mice were identified by flow cytometry.

For the secondary transplantation the mice were divided into two cohorts (n=22). Each cohort had 11 mice (one died on the day of injection). One cohort received cells from the primary recipient mice that had initially been transplanted with CB $CD34^+$ cells with placental MSC irrespective of single or DCBT. The second cohort received cells from the mice that had received only CB $CD34^+$ cells irrespective of single or DCBT. The secondary recipients in both the cohorts received cells from the primary engrafted mice BM ranging from 0.5×10^5 – 13×10^5 along with 4×10^4 placental MSC (Figure 11).

3.4.11 Colony forming unit assay

To determine if engrafted cells from the primary recipient mice were able to produce haemopoietic colonies the CFU assay was performed. The human $CD34^+$ population obtained from the BM of engrafted primary mice were labelled with appropriate antibody (as described earlier in this section) and $CD34^+$ cells were sorted.

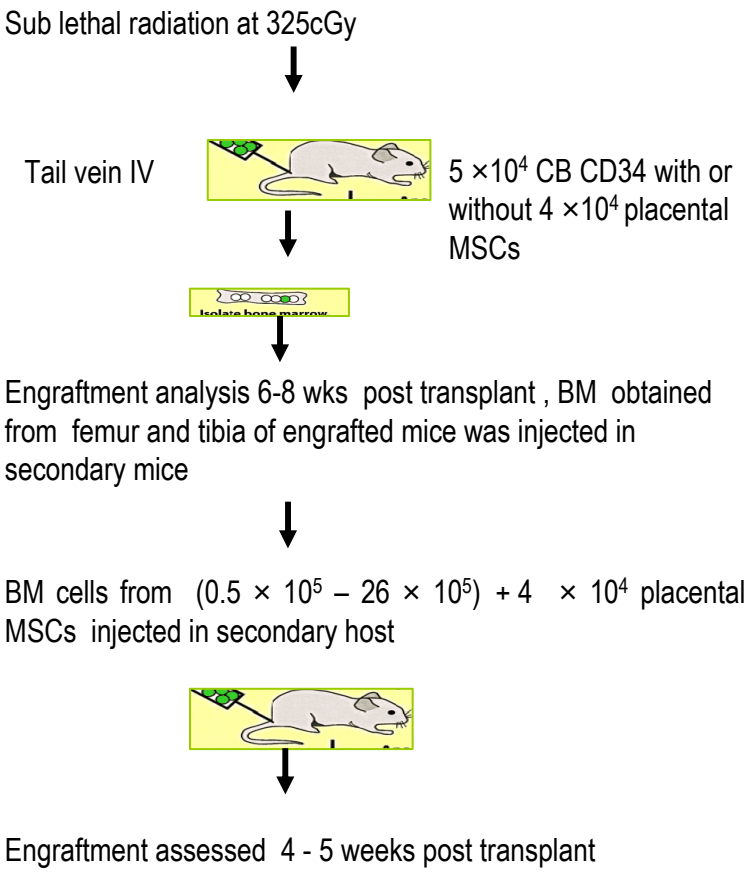


Figure 11. Schematic diagram of serial transplant experiment: Role of the placental MSC on the long term engraftment potentials of the CB $CD34^+$ in the secondary mice (n=22) was assessed. BM obtained from the primary engrafted mice that were co transplanted with or without MSC was injected into two cohorts of secondary mice and the human cell engraftment was assessed after 4 - 5 weeks of transplantation.

3.4.11.1 $CD34^+$ cells sorting by Fluorescence-Activated Cell sorting (FACS)

The cryopreserved cells from BM aspirates of the primary engrafted mice were thawed. The mice BM cells were incubated with the appropriate amount of human specific PE conjugated to anti CD34, human specific to PCy-5 conjugated to anti CD45, and mice specific FITC conjugated to anti CD45 for 30 minutes in the dark at 4^0C, followed by washing twice with the sterile MACS buffer. Appropriate isotype controls were used for background staining. The $CD34^+$ cells were sorted by fluorescence activated cell sorting [Coulter ALTRA] and plated.

3.4.11.2 Methyl cellulose preparation

The sorted cells were assayed on semi-solid medium prepared from methyl-cellulose. For preparing the methyl-cellulose, 8.1 g powder was added to a 500 mL bottle with magnetic stirrer and autoclaved for 45 minutes at 125^0C. After cooling, 270 mL of sterile IMDM was added to the powder and stirred overnight. The following day, 180 mL of FCS along with 60 mL of 10% BSA was added and on complete dissolving of the powder, 3.5 mL of methyl cellulose was aliquoted into 10 mL of conical tubes and stored at 20^0C.

3.4.11.3 Cytokine combination for CFU assay

The cytokine mix was prepared at 10 × concentration of 200 ng / mL SCF, 200 ng / mL G-SCF, 200 ng / mL GM-CSF, 200 ng / mL of IL-3, 200 ng / mL of IL-6, 30U/mL EPO with human growth factors(63).

3.4.11.4 Cell plating for CFU assay

Methyl cellulose was thawed by keeping it at 4^0C a day prior to starting the assay (as thawing in water bath leads to formation of clumps). The enriched human $CD34^+$ cells were prepared in IMDM at a final concentration 1000, 3000 and 5000 cells per mL and were added to the 3.5 mL methyl cellulose tubes along with 100 µL of the 10 × cytokine mix. This was

thoroughly mixed by vortexing for 30 seconds and by gently aspirating with syringe (taking care to avoid any air bubble formation). 1.2 mL of the suspension was added to each 35 mm tissue culture plate in triplicates.

These plates were placed in a sterilised cake box containing 100 mL of sterile water and were incubated in a humidified incubator for 14 days at 37^0C with 5% CO_2. The functional capacity of the cells was assessed after 14 days by assaying the haemopoietic progenitors for forming the different lineage colonies.

3.4.12 Chimerism analysis for contribution of donor cord blood units

In DCBT the donor origin in the engrafted mice was confirmed by STR PCR using the AMPFLSTR Identifiler Kit (AB Applied Biosystems, Warrington, UK). DNA was extracted from the mice marrow by the QIA ampDNA extraction kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The markers provided in Identifiler plus PCR Amplification Kit were D8S1179, D21S11, D7S820, CSD1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, FGA, Vwa, TPOX, D18S51, D5S818, FGA and Amelogenin.

3.4.12.1 DNA extraction from the human cells obtained from engrafted mice

The engrafted human cells from the BM of the NOD/SCID mice were harvested and processed by using a QIA ampDNA extraction kit according to the manufacturer's instructions. Briefly, 20 μL of proteinase K was dispensed into a 1.5 mL microcentrifuge tube and 5×10^6 cells in 200 μL were added to it. This was followed by adding 200 μL of Buffer AL and 15 seconds vortexing. The above mix was incubated for 10 minutes at 56^0C and centrifuged briefly. Subsequently 200 μL 100% ethanol was added and briefly vortexed for 15 seconds to remove drops from the inside of the lid. The mixture was then carefully applied to the spin column inserted in a 2 mL collection tube and centrifuged at 8000 rpm for 1 minute. Following this the

column was placed on a clean 2 mL collection tube and 500 µL of buffer AW1 was added and spun at 8000 rpm for 1 minute. Subsequently the spun columns were placed on clean 2 mL collection tube and 500 µL of buffer AW2 was added. Further these columns were centrifuged at 1400 rpm for 3 minutes and placed them on clean 1.5 mL microcentrifuge tube. The elution of DNA was performed by adding 200 µL buffer AE, followed by incubation for 1 minute at room temperature and spinning down at 8000 rpm for 1 minute.

The eluted DNA was quantitated by using the nano-drop spectrophotometer. Purity was estimated by measuring the absorbance at 260 nm and 280 nm. Working concentration of 3 ng /µL was used.

Master Mix was prepared by mixing of 4.8 µL of the reaction mixture (nucleotides dATP, dGTP, dCTP, dTTP, bovine serum, 0.05 % azide in buffer and salt, water), 2.5 µL of primer set, 0.23 µl Ampli Taq gold polymerase. 1 µL of the DNA sample (3 ng /µL) and 7.5 µL of Master Mix was added to a 200 µL PCR reaction tube. The PCR machine was set for heating and cooling at precise temperature. The initial incubation step involved 11 minutes at 95^0C to activate the Taq gold polymerase followed by 28 cycles in the following sequence, denaturation cycle at 94^0C for 1 minute, annealing at 59^0C for 1 minute, and extension at 72^0C for a minute. This was followed by an extension step at 60^0C for 60 minutes to ensure copying of any remaining single stranded DNA. The PCR product was stored at 4^0C.

3.4.12.2 DNA electrophoresis

The PCR product was identified by its size using the ABI 3730 DNA Analyser by capillary electrophoresis. Each fluorescence tagged fragment was detected by a laser beam that identifies the STR product. Each dye emits light at a different wavelength when excited by a laser light; the four dyes were distinguished in a single lane. The laser beam detected only one strand for each amplified DNA fragment, as only one primer of each pair was labelled with the 5-FAM, Joe or NED. These NHS-ester dyes were detected as blue, green and yellow respectively

on the ABI 3730 DNA Analysers. A mixture of 10 μL HiDi formamide and 0.4 μL ROX 500 size standard samples was prepared; 10 μL to each well was added followed by adding 1.5μL of sample product to the appropriate well. Then 2 μL of Identifiler allelic ladder was added to the appropriate well plate.

The samples were then placed in a thermal cycle and denatured at 95^0C for 3-5 minutes, followed by immediate chilling for at least 3 minutes in an ice-water bath. After loading the specimen into the capillary, voltage was applied causing the fragments to electrophorese through the capillary which separated according to size. When fragments reach the read region, light from the laser excites the fluorescence dyes. The emitted light is focused onto a spectrograph and separated chromatically. The digital output from the analyser was read and interpreted by ABI Genemapper ID software.

3.4.13 Quantitation of donor chimerism in DCBT

Corresponding fluorescence curves were printed for each sample. The donor samples CB unit U1, U2 and MSC were printed on a page and aligned in the size modus to obtain rapid facts on informatives. Water was used as a negative control. The amplified PCR product from DNA of the engrafted NOD/SCID mice BM cells was compared with the informatives.

The informative peaks were analysed as those unique to the donors, identified according to their size. These peaks were then matched to the post-transplantation samples, obtained from the engrafted mice. The particular loci give information pertaining to the origin of the engrafted cells, thus determining the donor. Subsequently the contribution of each donor was calculated by using the informative peak area. The area of CB donor 1 (U1) peak and the CB donor 2 (U2) peak were added and the percentage of the donor contribution in the graft was calculated (184).

3.4.14 Statistical analysis

All the results are expressed as the mean, median and standard error of human cells engrafted in each group. The differences between the quantitative measurements between the transplanted mice from the different cohorts were compared and analysed using the student paired t test. The level of significance was assessed by P value ≤ 0.05

3.5 Results

In six independent transplantation experiments, 96 NOD/SCID mice were injected with CB $CD34^+$ with or without placental MSC. HSC engraftment was assessed after 6-8 weeks of CBT by evaluation of human CD45 cells present in murine BM. The primary transplantation experiments were conducted in a total of 96 mice; out of these 18 (19%) mice died within 2 to 3 weeks of transplantation and were not evaluable. After 6-8 weeks post transplantation, 78 (81%) mice were evaluable for haemopoietic engraftment. Out of 78 mice, 69 (88%) mice engrafted while 9(11%) mice failed to engraft with human cells (Figure 12). Engraftment varied from 0.5-89%. The engrafted human $CD34^+$ cells underwent myeloid and lymphoid differentiation.

3.5.1 At equivalent cell doses SCBT and DCBT produce similar engraftment

Equivalent $CD34^+$ cells were injected in the SCBT and DCBT cohort and there was no significant difference in the haemopoietic engraftment. The mean $CD45^+$ engraftment in SCT and DCBT cohorts was 17.5 ± 6.1% and 14.9 ± 6.5% respectively (p=0.4; Figure13A). The transplanted CB $CD34^+$ cells were able to differentiate into both myeloid and lymphoid lineage in both the cohorts. CD19 (60.7 ± 4.2 vs. 56.8 ± 5.6, p=0.2), CD33 (28.1 ± 4.3 vs. 18 ± 4.2, p=0.19) CD34 (14.3 ± 1.8 vs. 17 ± 1.6, p=0.17) engraftment was similar between SCBT and DCBT cohorts (Figure 13A-B).

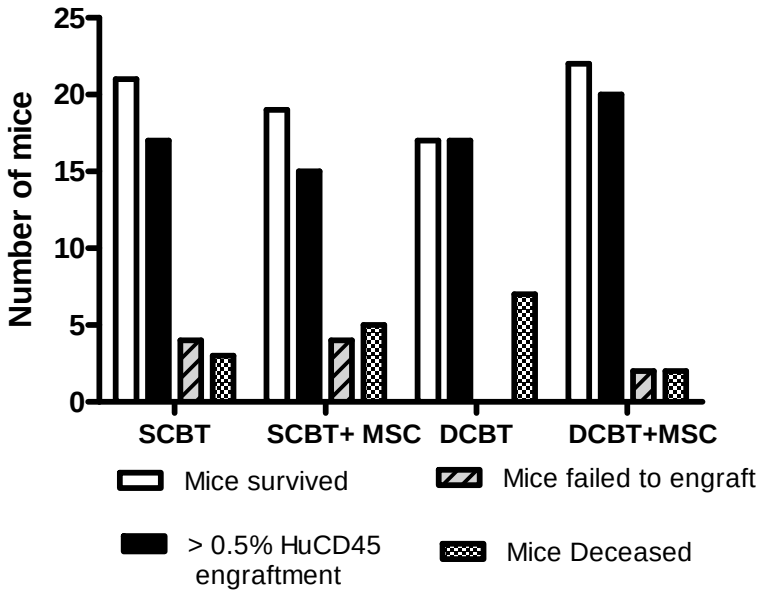


Figure 12. Haemopoietic engraftment and survival following cord blood transplantation: Primary transplantation was performed in NOD/SCID mice (n=96) with CB $CD34^+$ cells with or without placental MSC. The human cell engraftment was analysed 6 weeks after CBT. The mice which died within 6 weeks of transplantation were not evaluated for engraftment.

Figure 13 A-C. Co-transplantation of placental MSC significantly enhances engraftment of double cord blood units and maintains multi-lineage differentiation: (A) CB $CD34^+$ cells with or without placental MSC were injected via tail vein injection into mice. The engraftment was assessed by detection of human cells in murine BM by flow cytometry, analysed 6 weeks after CBT. Cohorts co-transplanted with MSC show enhanced haemopoietic engraftment of SCBT (48.7 $\pm$ 7 vs. 17.5 $\pm$ 6.1 %, p=0.07) and DCBT (51.8 $\pm$ 6.8% vs. 14.9$\pm$ 6.5, p=0.04)

SCBT = single cord blood transplant

DCBT = double cord blood transplant

(B) Co- transplantation of MSC maintains the multi lineage differentiation in the graft. This was assessed by three colour flow cytometry. Results represented are expression of lineage specific antigen on human CD45 cells

(C) Distribution of engraftment in mice co-transplanted with or without MSC. Data represents three ranges of engraftment:

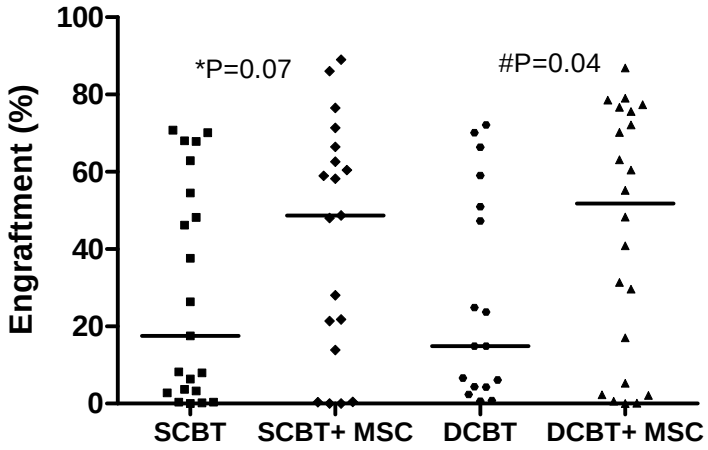
i. 0 - 30%

ii. 31 - 60%

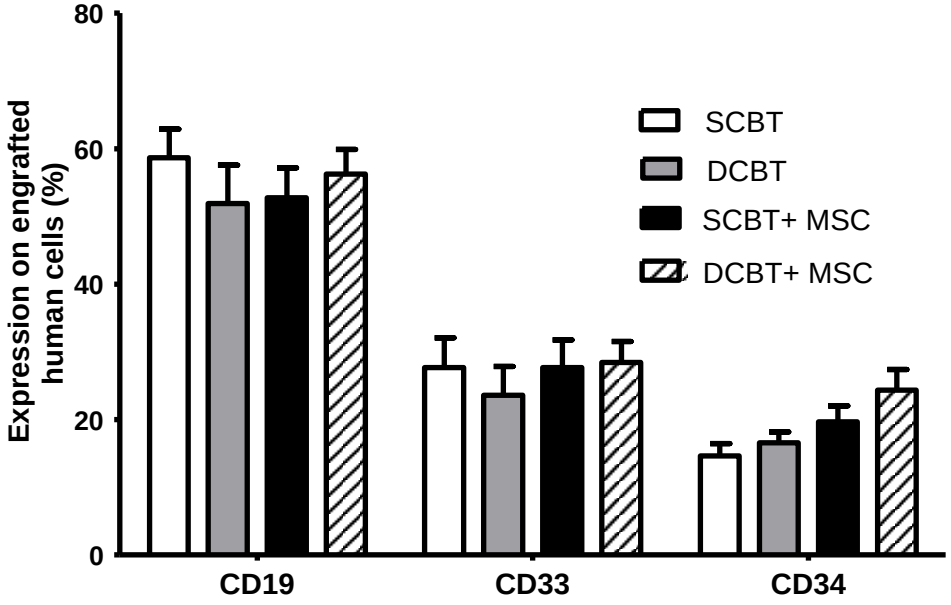
iii. 61 - 90%

Mice co-transplanted with MSC showed higher rates of engraftment compared to mice not receiving MSC.

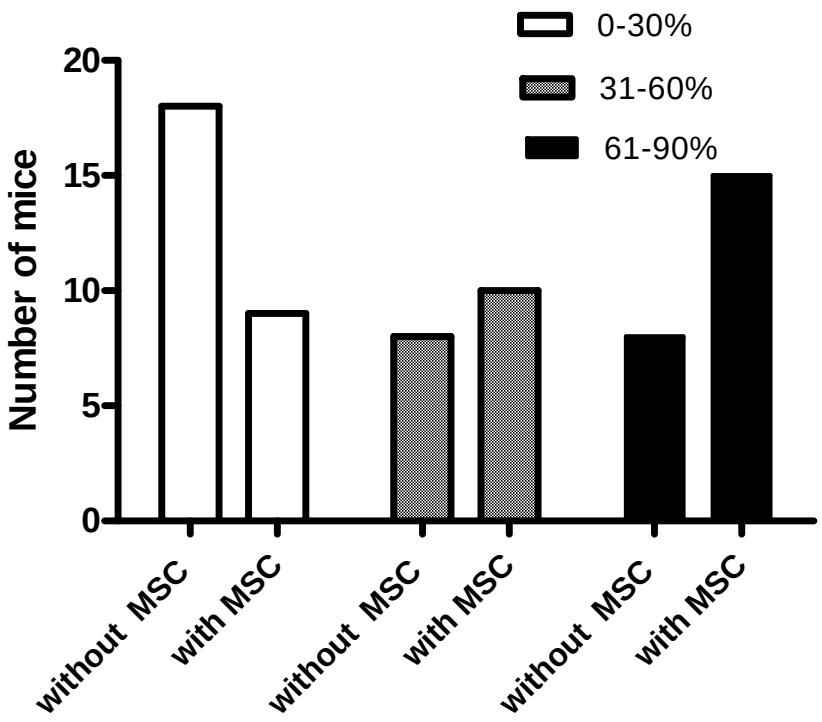
A


Engraftment (%)
100
80
60
40
20
0
*P=0.07
#P=0.04
SCBT
SCBT+ MSC
DCBT
DCBT+ MSC

B


Expression on engrafted human cells (%)
80
60
40
20
0
SCBT
DCBT
SCBT+ MSC
DCBT+ MSC
CD19
CD33
CD34

C


0-30%
31-60%
61-90%
Number of mice
20
15
10
5
0
without MSC
with MSC
without MSC
with MSC
without MSC
with MSC

3.5.2 Co-transplantation of placental MSC enhances engraftment of SCBT and DCBT

Co-transplantation of placental MSC along with SCBT (n=19) resulted in higher engraftment (48.7 ± 7.6%) compared to the cohort (n=21) that received only SCBT (17.5 ± 6.1%; p=0.07). Similarly, co-transplantation of MSC with DCBT (n=22) resulted in significantly higher engraftment (51.8 ± 6.8% vs. 14.9 ± 6.5%; p=0.04) compared to DCBT only (n=17; Figure 13A).

Nevertheless no significant difference in lineage differentiation capacity of engrafted human cells was demonstrated CD19 (60.7 ± 4.2% vs. 49.4 ± 4.4%), CD33 (28.1 ± 4.3% vs. 27.7 ± 4%), and CD34 (14.3 ± 1.8% vs. 16.9 ± 2.4%) engraftment was not significantly different between SCBT and SCBT + MSC. Similarly, the CD19 (56.8 ± 5.6% vs. 58.6 ± 3.6%), CD33 (18.0 ± 4.2% vs. 27.0 ± 3%), and CD34 (16.5 ± 2% vs. 24.3 ± 1.7%) engraftment was not significantly different between DCBT and DCBT + MSC (Figure 13B).

The engrafted mice were further subdivided into three cohorts based on engraftment. In this study the least engraftment accomplished was 0.5 to 30%, while the medium engraftment accomplished was 31 to 60% and the highest engraftment accomplished was > 60%. The highest engraftment levels were seen in 44% of mice, those that were co-transplanted along with MSC. When compared to this the least engraftment ranging from 0.5 to 30% was seen in 52% of mice in the cohort that had received only CB $CD34^+$ cells (Figure 13C).

3.5.3 Engrafted human cells maintain self-renewal *capacity in secondary hosts*

To determine self-renewal capacity of the engrafted human cells serial transplantation experiments were performed in 22 mice.

There were 4 cohorts of primary recipients and all the experiments were conducted in parallel. The $CD34^+$ dose was fixed at 5×10^4 in all the cohorts irrespective of SCBT or DCBT

the difference was with or without MSC. Post 6-8 weeks of transplantation the BM was harvested and subsequently assessed for human cell engraftment. The BM cells of the engrafted mice were then injected in secondary mice along with placental MSC.

One mouse died on the day of injection. Of the remaining mice 4/21 were euthanized due to weight loss after two weeks of transplantation and were not evaluable for engraftment. Thirteen of 17 evaluable mice engrafted and 4/17 mice failed to achieve more than 0.5% of human cell engraftment.

The human $CD45^+$ engraftment in secondary mice varied from 1 - 6.5%. Equivalent engraftment of secondary recipients was seen independent of whether the primary recipient had received placental MSC or not (Figure 14A). The in-vivo expansion of the infused CB $CD34^+$ cells was calculated by dividing total human haemopoietic cells obtained from individual mice by CB $CD34^+$ cells injected. Total human cells were derived from human cells obtained from the two femurs and two tibias which contain 25% of the total marrow content of a mouse (185). Pooled results showed initial primary in-vivo expansion of 190 ± 20 fold and 159 ± 50 fold in cohorts transplanted with and without MSC respectively (Figure 14B).

Multi-lineage differentiation capacity was also maintained in secondary recipients (Figure 14C). Serial transplantation of engrafted cells obtained from primary mice that had received only CB $CD34^+$ cells and CB $CD34^+$ cells along with placental MSC resulted in similar lineage and $CD34^+$ engraftment in secondary mice. There was no significant difference in CD19 (43.8 ± 9.7% vs. 55 ± 12.5%), CD33 (59.7 ± 5.3% vs. 66.2 ± 22.9%), and CD34 (30.7 ± 82% vs. 26.5 ± 9.8%) engraftment in both cohorts of secondary recipients.

3.5.4 Colony forming unit (CFU) assay

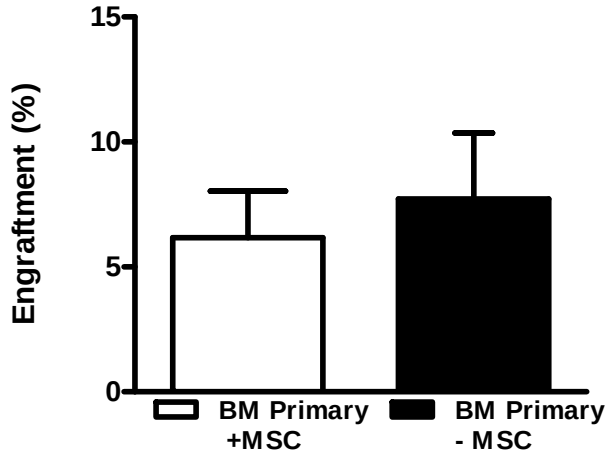
Human $CD34^+$ cell population was sorted from the BM of primary engrafted mice transplanted with CB $CD34^+$ and CB $CD34^+$ along with placental MSC. Sorted, live $CD34^+$ cells were plated for CFU-GM assay in methyl cellulose base along with growth factors. After 2

Figure 14 A-C. Serial transplantation demonstrates preservation of self renewal and multilineage differentiation capacity maintained in the secondary recipients : (A) Human cells obtained from murine BM was assessed for long term engraftment by serial transplantation. There was no significant difference (p=0.49) in engraftment in the secondary recipient that received BM from primary engrafted mice that were transplanted with MSC (7 ± 2.5) or without MSC (6.8 ± 2.2)along with CB $CD34^+$ cells.

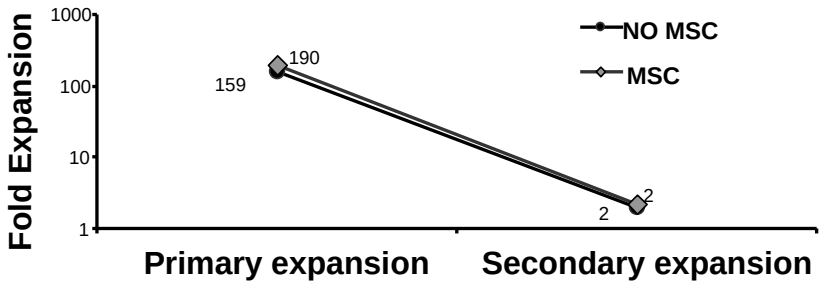
(B) The human $CD45^+$ population obtained from the BM of engrafted primary mice were sorted for $CD34^+$ cells obtained from the murine marrow. The CB CD34 cells retain self renewal capacity in the secondary mice in spite of massive expansion in the primary mice. The in-vivo expansion of the infused CB $CD34^+$ cells was calculated by dividing total human haemopoietic cells obtained from individual mice by CB $CD34^+$ cells injected.

(C) In the serial transplantation experiments, the secondary mice received engrafted cells from the primary mice that had received CB $CD34^+$ cells with or without MSC. This resulted in similar lineage engraftment in both the cohorts.

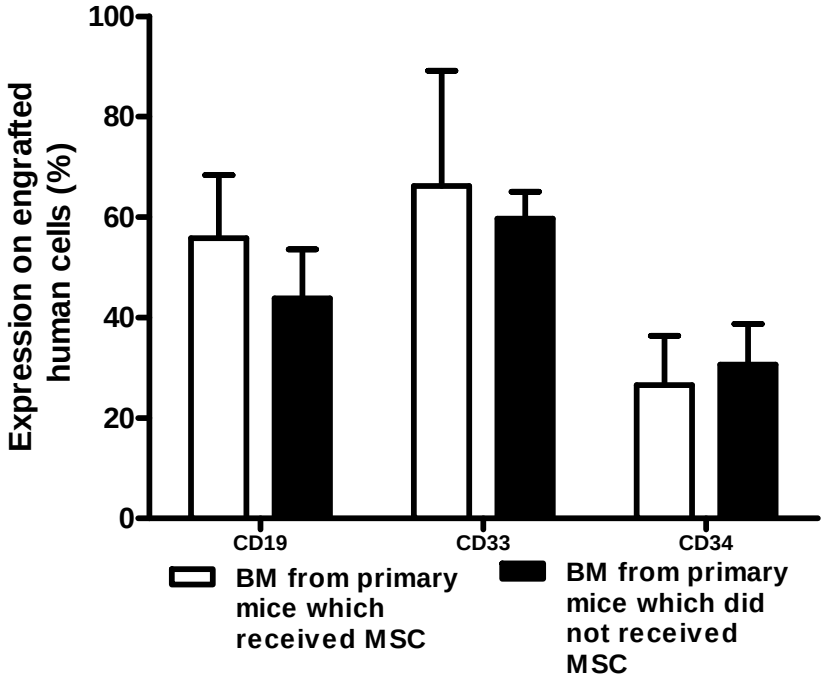
A


15
10
5
0
Engraftment (%)
BM Primary +MSC
BM Primary - MSC

B


1000
100
10
1
Fold Expansion
NO MSC
MSC
190
159
2
2
Primary expansion
Secondary expansion

C


Expression on engrafted human cells (%)
100
80
60
40
20
0
CD19
CD33
CD34
BM from primary mice which received MSC
BM from primary mice which did not received MSC

weeks of plating, colonies were identified as CFU-GM, CFU-GEM, and BFU-E depending on morphological features.

The colonies with star burst patterns of granulocytes and scattered large macrophages, with cells accumulated densely at the centre of the colony, were identified as CFU-GM colony and scored. When dense colonies with multiple clusters of reddish brown coloured erythrocytes were seen, they were identified as BFU-E colony and scored. The dense reddish brown erythroid colonies in close association with smaller densely packed granulocytes and round bright macrophages resembled both the myeloid and erythroid lineages and were dentified as CFU-GEMM colony and were counted (Figure 15A).

The total of 45 $\pm$ 6.2 CFU colonies/ 1000 CB $CD34^+$ cells were formed from the sorted CB $CD34^+$ cells obtained from the engrafted primary mice BM transplanted with CB $CD34^+$ cells along with placental MSC, while 44.8 $\pm$ 6.1 CFU colonies / 1000 CB $CD34^+$ cells were formed from the sorted CB $CD34^+$ cells obtained from the engrafted primary mice BM initially transplanted with CB $CD34^+$ cells only. There was no difference in the CFU capacity of human $CD34^+$ cells obtained from engrafted primary mice who had received CB $CD34^+$ along with placental MSC and only CB $CD34^+$.

The total of 25 $\pm$ 4.7 CFU-G colonies, 17.11 $\pm$ 4.9 BFU-E colonies, CFU-GEMM 3.6 $\pm$ 0.8 / 1000 CB $CD34^+$ cells were produced (Figure 15B) from the sorted CB $CD34^+$ cells obtained from the engrafted primary mice BM had been primarily transplanted with CB $CD34^+$ cells along with placental MSC. While 24.6 $\pm$ 5.4 CFU-G colonies, 16.7 $\pm$ 3.8 BFU-E colonies, CFU-GEMM 3.6 $\pm$ 1.8 / 1000 CB $CD34^+$ cells were formed from the sorted CB $CD34^+$ cells obtained from the engrafted primary mice BM who had been primarily transplanted with only CB $CD34^+$ cells.

In summary, both the cohorts showed a maximum of 25 - 28 CFU-G colonies/ 1000 sorted CB $CD34^+$, 16 - 17 BFU-E colonies and the least were 3 - 4 CFU-GEMM colonies per

Figure15 A-B. Human CFU produced from engrafted human cells. CFU assay was conducted on the engrafted human CB CD 34^+ cells obtained from the murine BM. Harvested BM from the primary mice was sorted for human CB $CD34^+$ cells. These generated the different lineages colonies. Examples of different colonies identified,

a. colony forming unit-granulocyte (CFU-G).

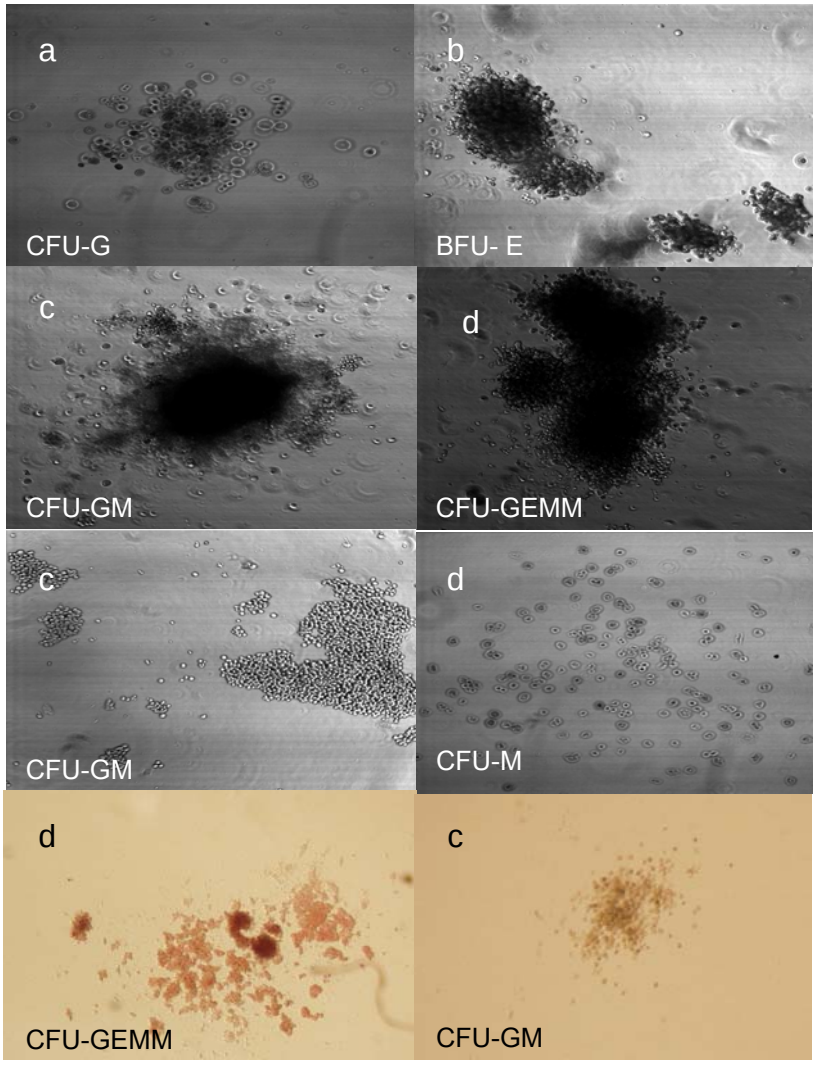
b. burst forming unit-erythroid (BFU-E).

c. colony forming unit-granulocyte macrophage (CFU-GM).

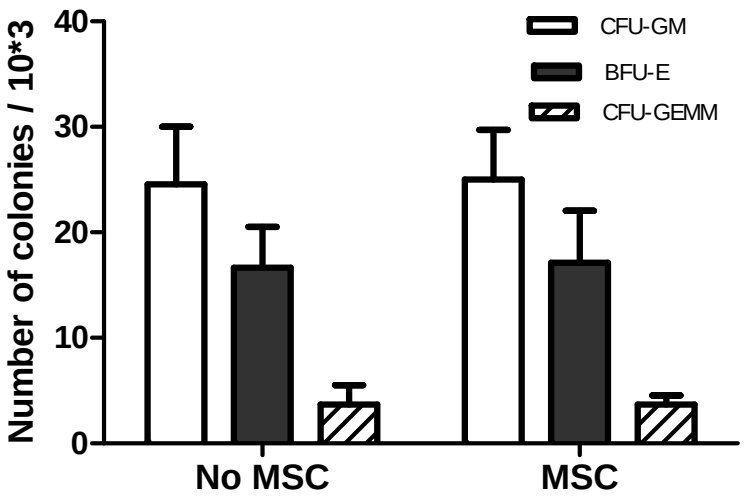
d. colony formi ng unit-granulocytes eryt hroid megakaryocytic macrophage (CFU-GEMM).

(B) The engrafted CB CD34 maint ains multi-potent haemopoietic progenitors. No significant difference in the colony fo rmation potentials was demonstrated from the primary host irrespective of co-transplantation of MSC.

A



B


Number of colonies / 10*3
40
30
20
10
0
CFU-GM
BFU-E
CFU-GEMM
No MSC
MSC

1000 CB $CD34^+$ with no significant difference noted in the colony formation capacity of the sorted human $CD34^+$ cells obtained from primary mice that had received CB $CD34^+$ cells with or without MSC.

3.5.5 MSC co-transplantation ameliorates single donor predominance

In DCBT, irrespective of co-transplantation of MSC, mixed chimerism was detected. In DCBT, the ratio of dominant to non-dominant unit was 3.6:1. Co-transplantation of MSC significantly reduced the ratio of dominant to non-dominants unit to 1.6:1. To calculate the donor contribution, the donor DNA profile was identified. An example of the donor DNA profile is shown (Figure 16A). Here the informative loci were D81179 and D7S820 for CB U1 and CB U2. In DCBT, irrespective of co-transplantation of MSC, mixed chimerism was detected while in the units that received only DCBT, single CB unit dominance was seen in the graft.

The allele peak area is proportional to its relative DNA mass. For quantitating the contribution of each donor CB units in DCBT the ratio of the contribution of the dominant CB unit and the non-dominant CB unit was calculated.

In DCBT, the ratio of dominant to non-dominant CB units was 3.6:1. Co-transplantation of placental MSC significantly reduced the ratio of dominant to non-dominant units to 1.6:1 (Figure. 16B). This suggests reduction of single CB unit dominance, and explains the enhanced engraftment in the cohort that received DCBT along with MSC.

3.6 Discussion

Studies evaluating the outcome of CBT have demonstrated the adverse impact of low cell dose on engraftment and survival (39, 77, 180, 181). Wagner *et al* reported TRM of 15%, 29% and 68% at 1 year in patients who received CBT with CD34+ cell dose of $>2.7 \times 10^5$/kg, 1.7 to 2.7×10^5/kg and $<1.7 \times 10^5$/kg respectively (39). Eurocord has recommended that units containing less than 3×10^7nucleated cells / kg should not be transplanted (180). However this

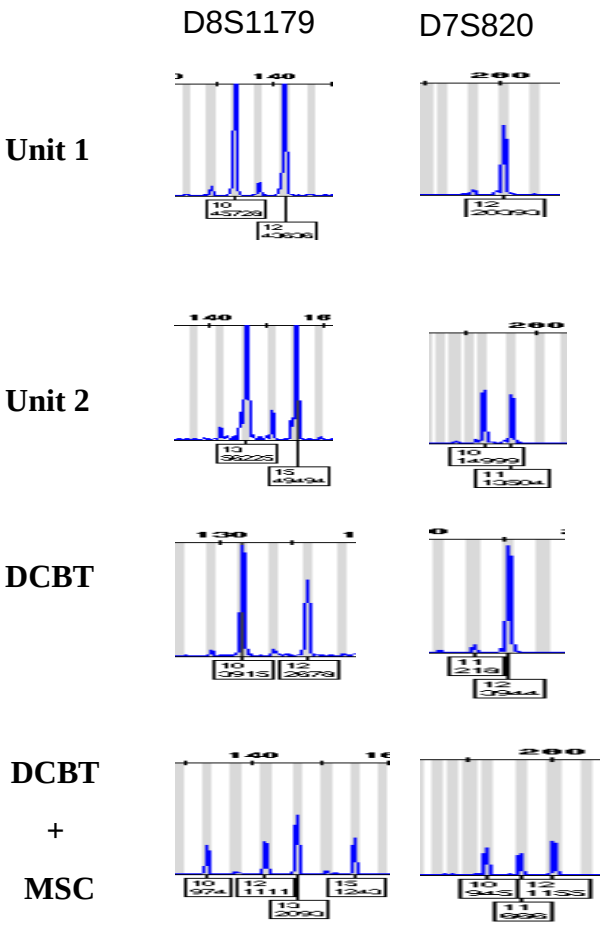


Figure 16A. Donor contribution in DCBT with or without MSC: Following DCBT, chimerism studies were conducted on engrafted cells using informative DNA alleles. The informative allele of both CB units were identified as unit 1 and unit 2 and these were matched with the alleles present in the engrafted cells from the murine BM. Above is an example of a mouse that received DCBT + MSC which shows presence of both the CB units (alleles 10,12 of unit 1 and 13,15 of unit 2) while the mouse that received DCBT only has single cord blood unit 1 engraftment only (allele 10,12 of single unit 1 are present).

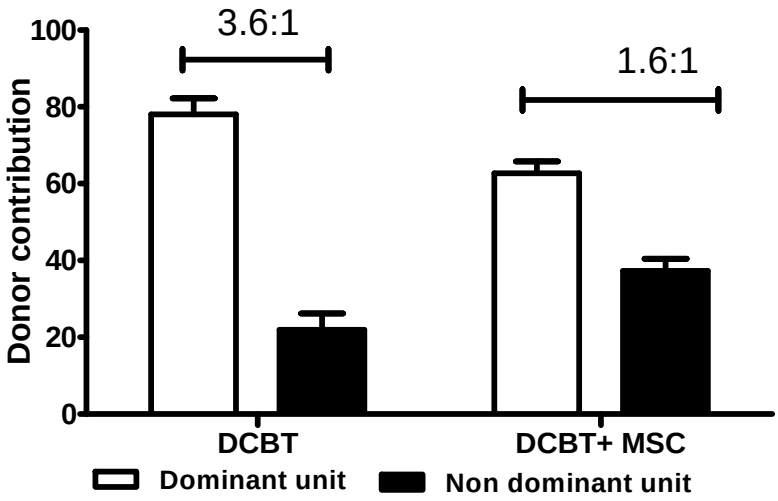


Figure 16B. Co-transplantation of placental MSC reduces single unit dominance in the DCBT**:** Sublethally irradiated NOD/SCID mice were injected with double cord blood (DCBT) with or without MSC and the contribution of each CB unit to engraftment was determined by assessing the expression of unique DNA markers. To quantitate the contribution of the specific donor, the allele peak area was measured. It is proportional to its relative DNA and the ratio of the contribution of the dominant CB unit and the non-dominant CB unit was calculated. Co-transplantation of the placental MSC reduces the ratio of dominant to non-dominant unit in DCBT (3.6:1 vs. 1.6:1; p=0.04).

limits the number of adult patients that can undergo CBT. Up to 90% of patients do not receive a CBT due to the limited cell dose from a unit (46). The potential strategies to increase cell dose include ex vivo expansion of CB $CD34^+$ cells and multiple cord blood transplantation.

Ex-vivo expansion of haemopoietic cells, while demonstrating capacity to increase total cell numbers, leads to exhaustion of the critical engrafting HSC (55). Following DCBT, improved engraftment and disease free survival of 75% and 54% at 1 and 3 years respectively in patients with high risk or advanced haematological malignancies has been reported (77). This has resulted in the use of CBT in adult patients. However, limitations remain for some patients where two suitably matched CB units of sufficient cell dose may not be identified. Murine studies of DCBT have given contrasting results. A study conducted by Nauta *et al* in NOD/SCID mice, showed that the DCBT resulted in a six fold increase in engraftment compared to SCBT, when double cell dose was infused in the DCBT compared to the SCBT cohorts (71). However, Kim *et al* could not demonstrate increased HSC engraftment in spite of the increased cell dose infused in DCBT cohort compared to SCBT cohort. This might be due to the difference in the cell populations infused. Also, MNCs might have varied widely in their CD34 content or there may have been a graft-versus-graft reaction reducing the engraftment of the CB units (72).

In this study mice received a fixed $CD34^+$ cell dose in both cohorts irrespective of SCBT or DCBT and achieved equivalent engraftment. These results this study are consistent with the notion of the clinical studies that the improved engraftment in DCBT is due to an increased cell dose effect. This is also in agreement with the double cell dose effect (72). In addition it has been reported that in DCBT, single CB unit predominance was seen in the majority of patients by day 100 (78). In murine and clinical studies of DCBT, despite of two CB units being transplanted, single units dominated engraftment (74, 75, 77, 186).

The exact mechanism of single unit dominance is not yet known but it is not related to the cell dose or HLA matching with recipient, ABO blood group, sex, or cell count. Barker *et al*

suggested that the dominance of the unit might be related to CD3 content of the unit which was supported by the murine study. However in another clinical study, dominance of the unit was not predicted by the CD3 content of the unit. Preclinical studies demonstrated that culture expanded MSC from the foetal organs, placenta and BM is feasible and safe (92, 94, 119, 187) and allows engraftment of both CB units in DCBT (72). Co-transplantation of MSC might suppress the graft versus graft effect and allow both CB units to engraft, thus improving engraftment (72).

In the current study, co-transplantation of placental MSC along with CB $CD34^+$ cells significantly improves engraftment following SCBT and DCBT. No lethal effect was observed in the cohorts that received placental MSC. Furthermore the data demonstrates that MSC supports both myeloid and lymphoid engraftment. The findings of this study are consistent with previously published literature. Noort *et al* and Kim *et al* reported the improved engraftment following co-transplantation of MSC along with SCBT (72, 94). The current study also demonstrates that co-transplantation of placental MSC reduces the single CB unit dominance and allows engraftment of both the CB units and thus enhances engraftment (3.6:1 to 1.6:1). Similarly Kim *et al* also reported contribution of both CB units in the engraftment following co-transplantation of BM MSC along with DCBT (2.8:1 to 1.8:1). They also noticed that, when committed cells were depleted from the DCBT, both the CB units engrafted. Together these findings may suggest that graft-versus-graft reaction might be rejecting one unit, and removing immunoreactive cells might allow both CB units to engraft.

Similarly, the immunosuppressive effect of MSC might be suppressing graft-versus-graft rejection and thus allows both CB units to contribute to the graft. However it must be emphasised that this is seen in an immunosuppressed murine model and may not reflect clinical results.

The differences in the current study and the published literature are the source of $CD34^+$ and MSC and the MSC cell dose used. Some of the studies have co-transplanted MSC

along with BM and PB $CD34^+$; however limited cell dose is the major limitation of CB. Hence the CB $CD34^+$ cells have been used for this study.

The sources of MSC used in other studies were foetal lung, BM, adipose tissue and placenta. Currently BM represents the main source for MSC. The BM MSC is a rare population, approximately 0.001- 0.01% in the adult BM (112) and it decreases with age (113). So it may not be always feasible to use BM-MSC. Noort *et al* have used the foetal lung MSC. The utility of such sources for further studies is limited because of the ethical issues involved with such a procedure.

The intimate relation of the placenta and CB may indicate a role in foetal haemopoiesis, and it is known that the placenta produces haemopoietic growth factors (125) and cytokines such as stem cell factor (SCF), Flt 3 ligand, interleukin-6 (IL-6) and macrophage stimulating factors which support expansion of the HSC (188). Additionally placental MSC are at less risk of viral infection making placenta an appealing tissue source of MSC. Hence the placenta was selected as a source of MSC.

Another difference between this study and published literature is the MSC cell dose used. In published studies, the MSC dose varied from $1\text{-}5 \times 10^6$ cells/kg. In this study the MSC dose used was lower and importantly is equivalent to a dose of 1×10^6 cells/kg, which is clinically achievable (6).

There are limited published clinical studies addressing the effect of MSC on HSC engraftment. All demonstrate that infusion of ex-vivo expanded autologous, donor origin and third party MSC along with autologous, allogeneic and CB cells is safe and feasible (6, 110, 111, 148) The marginal improvement in engraftment was reported when autologous MSC were infused along with autologous HSC in breast cancer patients (6). However Koc *et al* reported using higher $CD34^+$ cell dose and there was no control arm. Other clinical studies did not demonstrate similar benefit.

Any strategy that enhances engraftment must maintain the population of engrafting HSC. Ex-vivo expansion of CB $CD34^+$ with cytokines significantly increases the total cell number (53-55). However the ex-vivo expansion compromises SRC frequency and the long-term engraftment potential (49, 183).

In this study, serial transplantation experiments show engrafted CB HSC from both cohorts of the primary recipients (co-transplanted with or without MSC), were able to engraft in secondary recipients. Despite significantly higher expansion and enhanced short-term engraftment (6-8 weeks) of CB $CD34^+$ cells in primary recipients, co-transplantation of MSC did not exhaust the long-term engraftment capacity. Thus placental MSC co-transplantation maintains the engrafting human stem cells indicating that the co-transplantation of the placental MSC with the CB $CD34^+$ improves engraftment without exhausting the HSC. Another possible explanation is that the MSC provide additional cells that can be targeted by the reticuloendothelial system for removal, leaving the HSC to engraft.

Furthermore, the multi-lineage differentiation in the secondary host was also maintained. It was similar to the primary host, and was irrespective of co-transplantation of placental MSC in the primary host, thus demonstrating that placental MSC maintains multi-lineage differentiation.

The exact mechanism of the MSC supporting the haemopoietic activity is not yet completely understood. MSC secrete various cytokines which include interleukins, macrophage colony stimulating factor, SCF (189), granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (5, 6). These cytokines have the capacity to maintain and expand lineages and form specific colonies from the injected CB $CD34^+$cells. With the help of these cytokines, MSC might be providing the essential microenvironment needed for engraftment (108).

Moreover, the surface receptors present on the MSC might be facilitating homing of injected CB $CD34^+$ cells. This is discussed in Chapter 4.

3.6.1 Conclusion

The major limitation of CBT is cell dose. Strategies used to increase cell dose include ex-vivo expansion of CB $CD34^+$cells, DCBT and co-transplantation of MSC along with CBT. Although ex-vivo expansion increases cell count it reduces the long-term engraftment capacity.

Clinical and murine studies demonstrated that DCBT can improve short-term engraftment but only one unit engraft. The other strategy which needs exploring is co-transplantation of MSC along with CB $CD34^+$.

MSC forms the part of the BM niche where HSC live and proliferate. Stem cell derived factor-1 (SDF-1) secreted by MSC may regulate the proliferation, differentiation, attraction and retention of the HSC (150-152). High dose chemotherapy prior to HSCT damages this microenvironment and thus co-transplantation of MSC along with CB $CD34^+$ is a rational approach.

This chapter has discussed in detail the procedure of processing the CB unit, isolating CB $CD34^+$ and transplanting it into the mice. It also deals with the different strategies used to enhance the engraftment. It describes in detail the methodology of the experiments conducted and the assessment criteria, results and their explanation. The results of this chapter are summarised as follows:

1. Placental MSC co-transplantation significantly enhances engraftment of SCBT and DCBT.
2. In DCBT, a single unit dominates engraftment and co-transplantation of MSC allows engraftment of both units and reduces the dominance of the single CB unit.
3. Despite undergoing massive expansion in primary mice, placental MSC co-transplantation maintains the long-term engraftment capacity of CB $CD34^+$ progenitors.

4. Placental MSC co-transplantation does not alter the differentiation potentials of the CB HSC in primary and secondary host.

This study has demonstrated that placental MSC enhances haemopoietic engraftment and may overcome the limitation of low cell dose in CBT. This data should be confirmed by studies in higher animal models.

The mechanism of MSC action in the enhancement of engraftment needs elucidation. Chapter 4 addresses the role of the placental MSC in the homing of CB $CD34^+$ cells and also studies the migration of placental MSC in live mice and to the various internal organs.

Chapter 4 - Placental MSC migrate to haemopoietic sites and enhance homing of CB CD34+ resulting in improved engraftment

4.1 INTRODUCTION

The outcome of stem cell transplantation (SCT) depends on multiple factors, including HSC dose infused, the immune status of recipients and BM microenvironment. To attain engraftment, injected HSC have to migrate through the systemic and pulmonary circulation and home to the BM niche, where they self renew, proliferate and differentiate. High dose conditioning chemotherapy and radiotherapy damages not only HSC and leukaemic cells but also the microenvironment, along with marrow stromal cells. Following HSCT frequencies of colony forming unit-fibroblasts (CFU-F), the precursor compartment for the mesenchymal lineage, was reduced by 60-90% and the numbers did not recover up to 12 years after transplantation (190). It has also been verified that the damaged microenvironment could be restored by infusing MSC (139, 140).

In the previous chapter, cohorts of mice co-transplanted with placental MSC along with CB $CD34^+$ cells had significantly enhanced engraftment when compared to cohorts that received only CB $CD34^+$ cells. The transplanted cells proliferated, underwent multi-lineage differentiation and self-renewal as confirmed by engraftment in secondary recipients. A possible explanation for the improved engraftment may be restoration or facilitated repair of the damaged microenvironment by transplanted placental MSC. Previous studies have demonstrated the importance of stromal function following stem cell transplantation. In the current study, this was

achieved effectively by co-transplantation of placental MSC with CB $CD34^+$. However the mechanism of action remains unclear. The result of the engraftment studies demonstrates the benefit of MSC co-transplantation with a possible mechanism of action being functional improvement of the microenvironment. For this to occur, the injected MSC need to home to the BM microenvironment, but data on the homing of MSC is limited.

The characteristics of the HSC engraftment have been extensively studied, but little is known about the fate of infused placental MSC. The physical evidence of MSC engraftment is not robust in conventional BMT, and it is thought that the culture expanded MSC are not detectable. Following sibling allogeneic HSCT of whole BM graft or stromal and T cell depleted graft, all patients showed haemopoietic engraftment with donor cells. However the stromal cells were of host origin irrespective of the composition of graft (191). Other research groups also reported that following HSCT, marrow stromal microenvironment remains of host origin and donor cells do not contribute to reconstitution of the marrow microenvironment (109, 190, 192-195).However other groups have demonstrated that following sex mismatched allogeneic stem cell transplantation donor origin MSC engraftment was seen in 28 to 34% of patients (196, 197). In all of these studies, the graft used was whole BM or a stromal cell depleted graft. MSC are rare in BM and hence the MSC dose may not be adequate to engraft. Like HSC, there might be a critical MSC cell dose required for successful engraftment; however this needs to be established.

MSC are rare in BM and hence MSC may not be in sufficient numbers to engraft. Moreover, as MSC are a rare population, in transplantation studies MSC used are predominantly ex-vivo expanded at least up to 3-4 passages. However homing and migration of ex-vivo expanded MSC is controversial. Romboust *et al* suggested that MSC lose in-vivo homing ability to BM after 48 hours of expansion (170). It appears that passenger MSC when infused with donor BM do not contribute to long-term mesenchymal cell production. Their role in

short-term support of haematopoiesis has not been established and needs to be investigated further.

MSC support HSC by a number of mechanisms including production of cytokines such as stem cell derived factor-1 (SDF-1) which regulates proliferation, differentiation, attraction and retention of the HSC (95, 198, 199). Furthermore, homing of HSC depends on the interactions between SDF-1 and CXCR-4. Prior studies have demonstrated co-administration of neutralising anti CXCR4 or anti SDF-1 antibody reduces HSC homing to BM suggesting SDF-1/CXCR4 interaction plays an important role in HSC homing (200). Despite identification of several aspects of haemopoiesis, the initial homing and seeding of HSC and MSC after co-transplantation have not been studied extensively. There is limited data tracking transplanted cells after IV systemic injections. Furthermore, the migration and homing of culture expanded MSC have not been explored comprehensively. A technique to detect these cells in live animals would help to demonstrate the fate of IV injected MSC.

To achieve this requires labelling cells with suitable markers and having appropriate methods to track them in live animals. There are predominantly two ways of labelling cells: either directly or indirectly. In the direct method, radioactive (201) or iron particles (202) are loaded into the cells. However, this is of limited usefulness because the injected cells undergo division leading to decrease in the signal. The signal also decreases with radioactive decay and it also images the dead cells (203) making it difficult to interpret the results. Indirect labeling techniques involve labeling different cell components with a fluorescent dye. The dyes for example PKH26 or CFSE, can either be directly applied to the cell or a fluorescent reporter gene can be transduced into the cell. The indirect labelling methods depend on imaging of fluorescent reporter genes transduced into the cells which enables the detection of live cells. Previous studies have shown that fluorescence allows quantitative evaluation in mice (204) and using whole body imaging demonstrates the distribution of the IV injected cells.

The PKH26 red fluorescent linker incorporates the aliphatic reporter molecule in the cell membrane; however the signal decreases with cell division. But these cells were only traced for up to 48 hours and it was known from prior studies that the dye provides the fluorescent labelling over an extended period (175-177).

This chapter describes the experimental procedures for genetic manipulation of the placental MSC, labelling of the CB $CD34^+$ cells, and imaging the live animal using the non-invasive in vivo imaging system (IVIS). The experiments were conducted to address two important issues.

1. Do systemically injected placental MSC home to the haemopoietic sites?
2. Does co-transplantation of the placental MSC with CB $CD34^+$ increase the homing of the CB $CD34^+$ to the haemopoietic sites?

To distinguish the two cell populations, the placental MSC were transduced with enhanced green fluorescent protein (eGFP) using retroviruses and CB $CD34^+$ cells were labelled with PKH26 red fluorescent membrane stain. The mice were imaged by exposing to the appropriate filters with minimal overlap and distinct cell populations were detected and quantified.

4.1.1 Aims

1. To transduce placental MSC with eGFP protein.
2. To label CB $CD34^+$ cells with PKH26 red fluorescent membrane stain.
3. To trace the distinct cell population in live mice.

4.2 Materials and methods

4.2.1 Labelling of CB CD 34 with PKH 26

Cryopreserved CB $CD34^+$ cells were thawed (as described in Chapter 2, *2.2.11*) and were washed twice in PBS to ensure removal of all serum. Prior to staining, PKH26 red fluorescent dye [Sigma-Aldrich] was prepared according to the manufacturer's instructions. Briefly, 4 μM solution of PKH26 (working solution) was made by adding 2 μL of 1 mM PKH26 (stock solution) to 498 μL of the Diluent C. An equal volume of CB $CD34^+$ cells and working solution of PKH26 was slowly mixed and incubated for 2 minutes at 25^0C. Following incubation, staining was stopped by adding 2 mL of FCS for 1 minute. Subsequently cells were washed three times with 15 mL MSC medium (consisting of DMEM supplemented with 10% FCS 1% PS and 1% glutamine). Following a thorough wash, cells were resuspended in PBS at concentration of 5×10^5/mL (5×10^4 cells in 100 μL). The toxic effect of the PKH26 red dye on the CB $CD34^+$ cells was determined by assessing cell viability by trypan blue exclusion assay (described in detail in Chapter 2, *2.2.7*).

4.2.2 Placental MSC manipulation for eGFP tagging

The ex-vivo genetic manipulation of placental MSC was carried out by retroviral transduction. The MSC were trypsinised and washed in PBS. For transducing the MSC, initially competent DH5α *Escheria coli* (E-coli) cultures were established and DNA was extracted. Initially the transfection assays of the HEK 293 T cells were set, followed by collection of viral supernatant and transduction of the placental MSC.

4.2.2.1 Escheria coli transformation and plasmid DNA extraction

A murine stem cell virus internal ribosome entry site with eGFP (MSCV-IRES-eGFP) plasmid DNA (10 ng) was added to 100 μL of DH5α escherichia coli and incubated on ice for 20

minutes. Following heat shock treatment at 42^{0}C for 1 minute, the cells were returned to ice for 2 minutes, and subsequently incubated with SOC medium (SOC medium consisting of 20g tryptone [OXOID, Basingstoke Hampshire England] 5g yeast, 0.5g NaCl, 5g MgSO4, H2O, 20 mM glucose) at 37^{0}C for 30 minutes. These cells were then plated with a sterile spreader on a agar plate, and incubated overnight at 37^{0}C. A single colony was picked up and inoculated in 50 mL of Luria broth (LB) supplemented with 100 μg/mL ampicillin [Sigma, Aldrich]. The culture was incubated at 37^{0}C on the automatic shaker for 16 hours and subsequently centrifuged at 4000 rpm for 15 minutes at 4^{0}C. The DNA extraction was carried using the QIAGEN High Speed Midi prep Kit [QIAGEN, Vic, Australia] according to the manufacturers instruction. Briefly, the bacterial pellet was resuspended in 6 mL Buffer P1 [QIAGEN] supplemented with RNAse and then 6 mL of Buffer P2 [QIAGEN] was added and the tubes were inverted 6 times followed by incubating at room temperature for 5 minutes. Subsequently 6 mL of the chilled Buffer P3 [QIAGEN] was added to the lysate and mixed thoroughly by inverting the tubes 6 times. This mixture was transferred into the QIA filter cartridge and incubated at room temperature for 10 minutes. During the incubation period, a HiSpeed Midi tip was equilibrated with 4 mL of Buffer QBT [QIAGEN] and the column was allowed to empty by gravity flow. The plunger was inserted into the QIA filter midi cartridge to filter the cell lysate into the previously equilibrated HiSpeed Tip. The HiSpeed tip was washed with 20 mL buffer QC [QIAGEN]. Subsequently DNA was eluted with 5 mL of Buffer QF [QIAGEN]. Eluted DNA was precipitated by adding 3.5 mL of isopropanol, mixing the solution and incubating at room temperature for 5 minutes. The DNA elute was filtered through the QIA precipitator by using constant pressure. Subsequently DNA was washed with 2 mL of 70% ethanol, and dried on a heating block at 37^{0}C and eluted in 100 μL Buffer TE [QIAGEN].

4.2.2.2 Transient transfection of HEK 293T cell to produce live retro viruses

Retroviral supernatant for transfection of placental MSC was produced by transiently transfecting human embryonic kidney 293T (HEK293T) cells. The viral construct and ecotropic packaging plasmid (pEQ-Eco) were co-transfected into the HEK293T cells. HEK293T (1×10^6) cells were cultured in a T25 cm^2 flask for 24 hours and transfection was performed using serum-free OptiMEM 2000 [Invitrogen, CA, USA] along with Lipofectamine 2000 [Invitrogen] according to the manufacturer's instructions. Briefly, DNA solution and master mix were prepared separately. DNA solution consisted of 500 μL OptiMEM, 4 μg of MSCV-IRES-eGFP retroviral plasmid and 4 μg of packaging vector. Master mix was prepared by mixing 500 μL OptiMEM and 20 μL Lipofectamine 2000 reagent. The DNA solution and master mix were left at room temperature for 15 minutes and then combined gently. This mixture was kept at room temperature for 90 minutes and was subsequently gently added to 70% confluent HEK293T cells. After 24 hours, old media was replaced by 5 mL of fresh MSC media (MSC media was used as the target was transduction of MSC; this provides the optimum condition) and the viral supernatant was collected after 48 and 72 hours post transfection. The retroviral supernatant was collected from the transfected HEK293T cells and was centrifuged, followed by filtering through a 0.45 μM filter [Millipore]. This supernatant was stored at -80^0C or either used to infect the placental MSC. Transfection efficiency of HEK293T cells was assessed for eGFP fluorescence using flow cytometry.

4.2.2.3 Transduction of Placental MSC

Placental MSC at passage 2 were plated in MSC media (DMEM supplemented with 10% FCS, 1% glutamine 2mM, 1% penicillin and streptomycin) in a T175 cm^2 flask. On reaching 30-40% confluency, MSC were incubated with 10 mL of viral supernatant supplemented with 4 μg/mL polybrene [Sigma, Aldrich] and fungizone (1:100 dilution) for 8 hours at 37^0C.

Subsequently 8 hours post-transduction, 10 mL of fresh MSC media was added. Later, after 24 hours of incubation, media was replaced with 30 mL of fresh MSC media. On reaching 80% confluency, MSC were harvested and the transduction efficiency assessed. Cells expressing eGFP were sorted by Coulter ALTRA sorter and were further expanded by in-vitro culture (Figure 17A-C).

4.2.3 Transplantation of PKH26 labelled CB CD34 cells and eGFP transduced placental MSC in live mice

On the previous day of transplantation mice were sub-lethally irradiated at 325 cGy (described in detail in Chapter *3.4.3*). The experiments were conducted to test the role of placental MSC in homing of the HSC. Available mice were limited thus distributed into three cohorts, first cohort received 250 μL of PBS; the second cohort received 5×10^4 PKH26 red labelled CB $CD34^+$ cells; and the third cohort received 5×10^4 PKH26 red labelled CB $CD34^+$ cells along with 2×10^5 eGFP placental MSC via tail vein injection. The control cohorts were used to assess the background fluorescence. After 24 and 48 hours of IV injection mice were live imaged.

4.2.4 In vivo imaging system

The Xenogen IVIS facility was provided by Adelaide Microscopy Services. The IVIS consists of a charge coupled device (CCD) camera mounted on a light-tight black chamber provided with a moveable heated stage that allows different fields of view to be obtained. The photons emitted by fluorescent cells are detectable using the photon detectors when exposed to appropriate filters. Prior to imaging mice were anaesthetised with 2.5% isofluorane plus 100% oxygen vapours and were placed on the heated stage. To prevent the mice from moving, continuous gas anaesthetic was given via nose cone during the course of imaging. Both the cell populations, placental MSC-eGFP and the CB $CD34^+$ were visualised

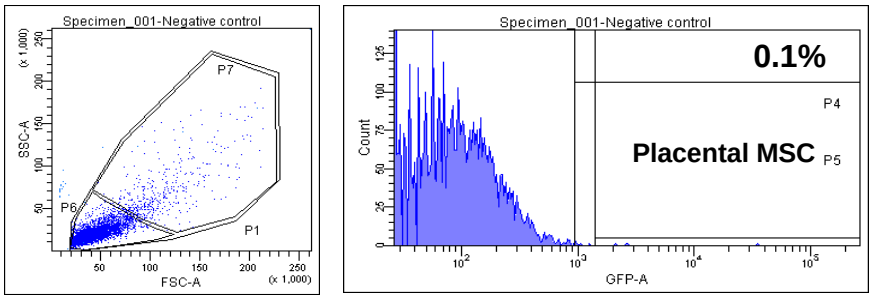
Figure 17A-C. Transduction efficiency of green fluorescence plasmid (eGFP) is high in placental MSC: Retroviral supernatant of HEK293T cells transfected with MSCV-IRES-eGFP retroviral plasmid and packaging vector were used to transfect placental MSC with eGFP. Transfection efficiency was assessed at passage 3.

(A) Shows the non induced placental MSC used as controls to subtract the background fluorescence.

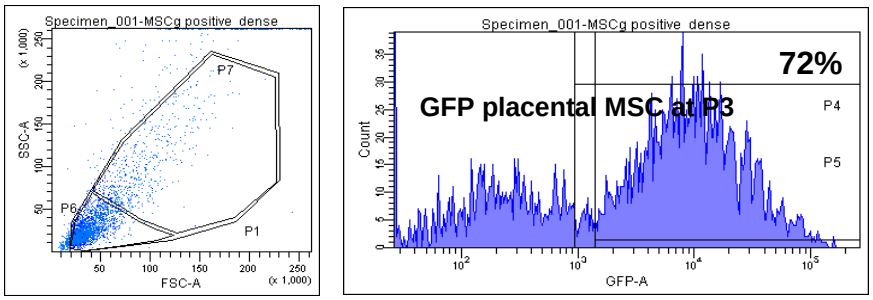
(B) Shows an example of transduction efficiency of the expanded placental MSC. At passage 3, 72% of transduced MSC were eGFP positive.

(C) On plating the FACS sorted eGFP positive placental MSC, 86-90% cells maintained the eGFP expression after expansion at passage 3-4 and these cells were used for in vivo cell tracing.

A


Specimen_001-Negative control
SSC-A
FSC-A
P7
P6
P1
Specimen_001-Negative control
Count
GFP-A
0.1%
P4
Placental MSC
P5

B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SSC-A
FSC-A
P7
P6
P1
Specimen_001-MSCg positive dense
Count
GFP-A
72%
GFP placental MSC at P3
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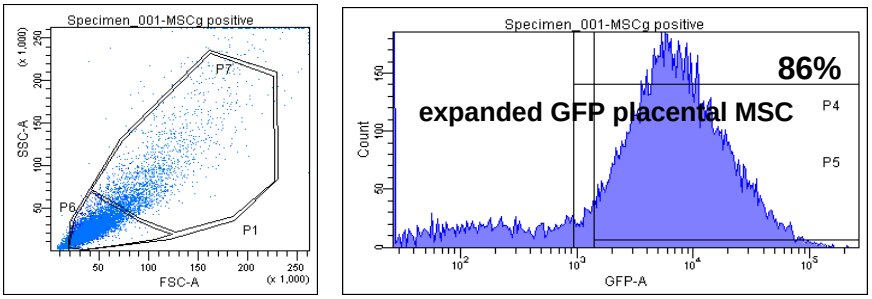
P5

C


Specimen_001-MSCg positive
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P1
Specimen_001-MSCg positive
Count
GFP-A
86%
expanded GFP placental MSC
P4
P5

sequentially as their excitation and emission frequencies were different with minimal overlap. The optical tracing of the fluorochrome labelled cells revealed consecutivlely the distribution of the distinct cell population in the live mice. First a photographic image of the mice was captured; subsequently the mice for 2 seconds were exposed to the GFP filter to detect fluorescence. Following this the mice were exposed for 1 second to the DsRed filter to detect PKH26 red fluorescence. This exposure time was selected taking care that it did not saturate the camera. The signal intensity was represented as a pseudo-colour image and was superimposed on the photographic image. The signal was graphically represented by an arbitrary pseudo-colour scale as photons emission / second (Figure 18).

Throughout all the experiments, imaging parameters and the pseudo-colour range scale were maintained. The magnitudes of the fluorescent signals originating from the injected cells were visualized and measured from the specific manually encircled regions of interest (ROI). The ROI were kept constant and the intensity was recorded as maximum photon counts within a ROI. Quantification of fluorescence was made by comparison with the appropriate controls. The mice were imaged post 24 hours of IV tail vein injections. Optical images were displayed using the Living Image ®2.50.1(Xenogen Corporation, Alameda, CA) and analysed using the IgorPro (Wavemetrics, Lake Oswego, OR) image analysis software. To further confirm the anatomical site of cell migration, mice were sacrificed after imaging and dissected. Organs including spleen, lung, heart, sternum and femur were isolated and placed on black paper (to avoid any fluorescence) and imaged ex-vivo. To determine the role of CXCR4 in facilitating homing, eGFP tagged MSC were pre-incubated with T140, an anti CXCR4 peptide. Placental MSC were incubated with T140 peptide (provided by Dr. Fuji, Department of Bio-organic Medicinal Chemistry Graduate School of Pharmaceutical Sciences, Kyoto University, Japan) for 30 minutes at room temperature and washed twice with wash buffer. Subsequently, placental MSC

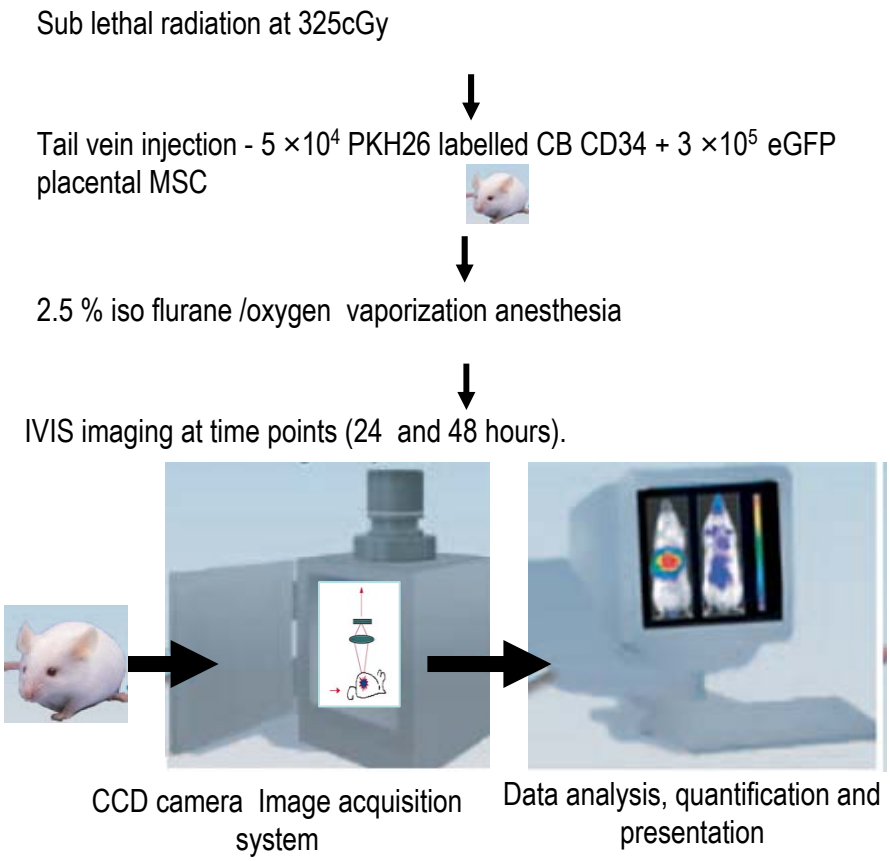


Figure 18. Tracing of labelled cells by in-vivo imaging: Schematic representation the of the in-vivo tracing of the placental eGFP-MSC and PKH26-red dye labelled CB CD34$^+$ cells. Following sub lethal irradiation eGFP-tagged MSC and PKH26 labelled CB CD34$^+$ cells were injected through tail vein. The mouse images were captured using IVIS imaging at 24 and 48 hours after injection. The mice were first exposed to GFP filter followed by the DsRed filter. The subsequent images were taken by charge couple camera (CCD) and analysed by the Living image software®2.50.1.

were injected into mice with or without PKH26 red fluorescent dye labelled CB $CD34^+$ cells as described previously, and were imaged by IVIS 24 hours post injections.

4.3 Results

The CB $CD34^+$ cells when observed under a fluorescent microscope showed uniform staining. The cellular viability was not compromised as assessed by trypan blue exclusion assay after labelling with 2 μM PKH26 red.

4.3.1 Expression of eGFP in Placental MSC

The positive eGFP cells were sorted and the transduction efficiency was 72%. The eGFP positive sorted cells were further enriched by culturing them for 8 -10 days before injecting in mice.

4.3.2 Tracking of fluorescent cells in live NOD/SCID mice

To assess the fate of the injected cells, images were acquired at 24 and 48 hours after IV tail vein injections. Quantitative analysis demonstrated the photon emission /second acquired 24 and 48 hours following IV tail injection of PKH26 red labelled CB $CD34^+$ cells and eGFP tagged placental MSC. For studying the cell migration and homing 15 mice received tail vein injections, of which 13 were evaluable. The mice numbers were small, and no significant difference was noted in 24 and 48 hours post injection for both the cell population, hence the data was pooled together and quantitated (Figure 19). The cohorts of mice injected with only PBS and only PKH26 red labelled CB $CD34^+$ cells were used to quantitate background fluorescence and compared to mice that were injected with both cell populations. The signal from the control mice (n=3), those that received only PBS, was $3.9 \pm 3\ 3 \times 10^{10}$ photon emissions/sec, applying GFP filter and similar photon emissions/sec was noted on applying DsRed filter. The eGFP MSC in the experimental mice (n=7) had signal intensity of $7.3 \pm 1.3 \times 10^{10}$ photon emissions/sec (Figure 19, 20). This demonstrates that placental MSC migrate to the

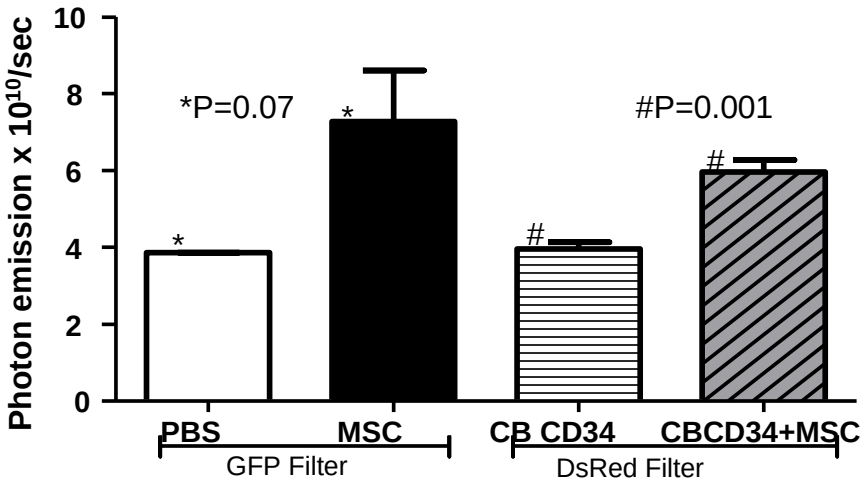
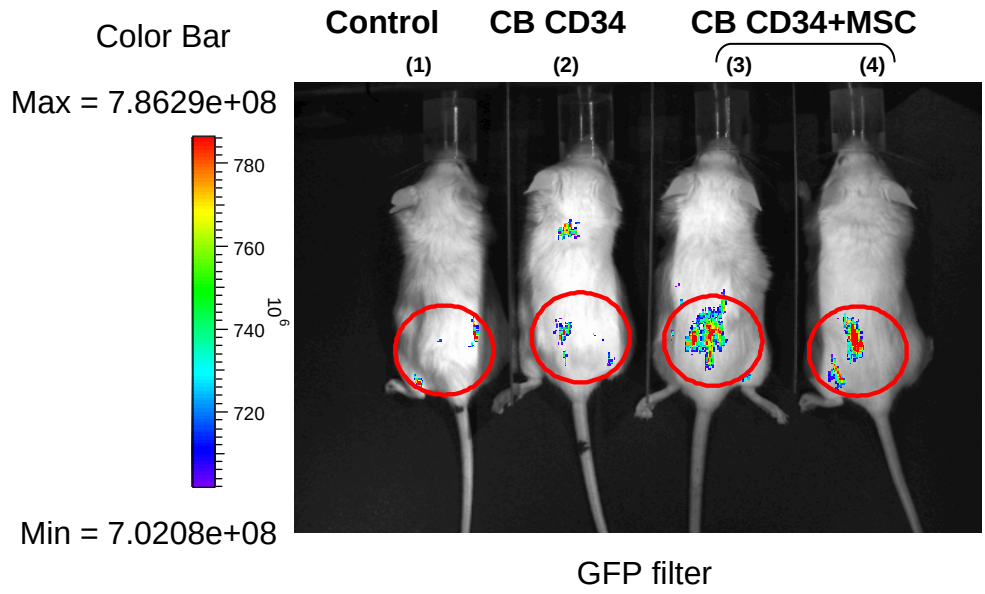


Figure 19. Co-transplantation of MSC enhances homing of CB-CD34$^+$ cells to the pelvic region: The PKH-26 red labelled CB CD34$^+$ and eGFP tagged placental MSC were traced 24 hours after the tail vein injection. Increased MSC in the pelvis (n = 7) were seen compared to the PBS control (p = 0.07). Co-transplantation of CB CD34$^+$ cells with MSC demonstrates enhanced homing of CB CD34$^+$ cells to the pelvic region compared to mice receiving CB CD34$^+$ cells alone (p = 0.001). The background fluorescence was assessed in mice injected with PBS It was similar between the DsRED and GFP filter.

Figure 20. Co-transplantation of eGFP-MSC results in higher homing of PKH26-red CB CD34$^+$ cells after 24 hours of IV tail vein injection: PKH26-red labelled CB CD34$^+$ cells were injected in the mice with or without eGFP tagged MSC. After 24 hours migration of MSC and CB CD34$^+$ cells was assessed. The placental MSC migrate to the pelvic region and enhance CB homing: Panel A (GFP filter) and Panel B (DsRed filter) showed higher signals in the mice injected with eGFP tagged MSC and PKH26 red labelled CD34$^+$ cells (3 and 4) compared to controls (1 and 2). There was co-localisation of CD34$^+$ and MSC signal at 24 hours. The control cohorts were used to assess the background fluorescence. The magnitudes of the fluorescent signals originating from the injected cells were visualized and measured from the specific manually encircled regions of interest (ROI).

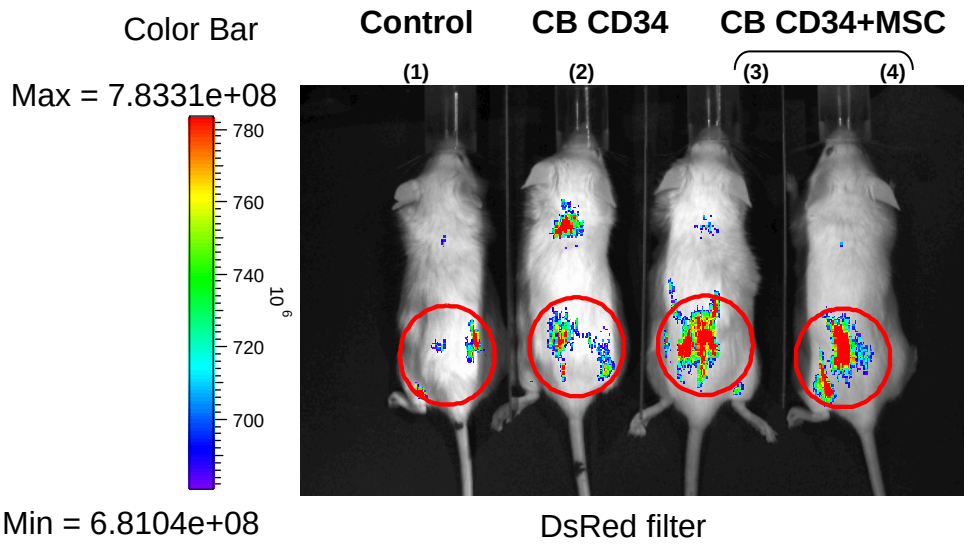
Figure 20

Panel A



GFP filter

Panel B



DsRed filter

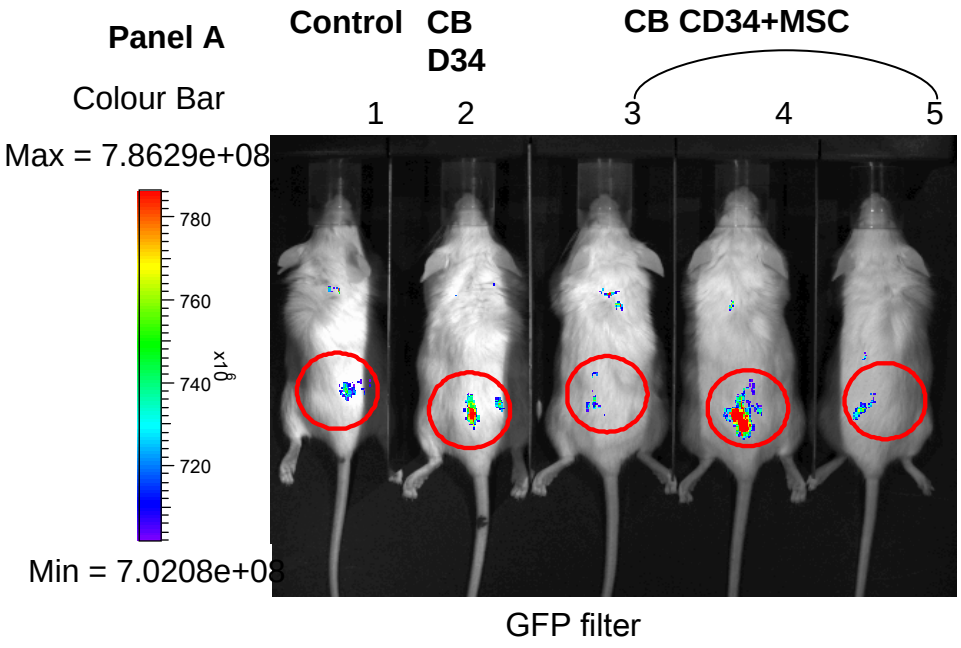
pelvic region (Figure 20, 21). Placental MSC were detected in the pelvic region of mice co-transplanted with PKH-26 red labelled CB $CD34^+$ cells and eGFP MSC ($7.3 \pm 1.3 \times 10^{10}$ photon emissions/sec). This is higher compared to the background photon emission level of 3.9 ± 3 in mice injected with PBS only ($p=0.07$) demonstrating that the MSC home to the pelvis. In the mice that were injected with only PKH26 red labelled CB $CD34^+$ cells, signal was detectable in the thoracic region, which may be due to cells in the lungs or the rib cage. Higher numbers of CB $CD34^+$ were detected in the pelvic region of mice co-transplanted with PKH-26 red labelled CB $CD34^+$ and eGFP MSC (6.0 ± 3.0; $p=0.001$) compared to control mice that received only PKH-26 red labelled CB $CD34^+$ cells (3.9 ± 1.7). This indicates that co-transplantation with MSC enhances homing of CB $CD34^+$ to the pelvic region.

After 48 hours of injection mice were sacrificed and major organs were imaged. Placental MSC predominantly migrated to the pelvic bone marrow, sternum, ribs, spleen, long bone ends, lungs and heart (Figure 22).

In summary, these results demonstrate that placental MSC migrate to murine BM and enhances the homing of CB $CD34^+$ cells. This study also determined how MSC migrate to BM of mice in limited number (n=7) consequently only minimum cohorts were used. The homing mechanism of HSC depends on the interactions between SDF-1 and CXCR4 (170). The role of SDF-1/CXCR4 axis in MSC homing has not been studied. To elucidate the role of SDF-1/CXCR4 axis, placental MSC were incubated with T140 peptide (CXCR4 antibody) and then injected into mice (n=3) with or without PKH26 red-labelled CB $CD34^+$ cells (n=3), and one mouse received PBS as described previously. Mice were imaged by IVIS 24 hours post injection. In the current study pre-incubation with CXCR4 antibody did not reduce homing of placental MSC to the pelvic region (Figure 23A), thus demonstrating that placental MSC homing is not dependent only on SDF-1/CXCR4 axis. These experiments also reaffirm earlier results

Figure 21. Co-transplantation of eGFP-MSC results in higher homing of PKH-red CB-CD34$^+$ cells after 48 hours of IV tail vein injection: PKH26-red labelled CB-CD34$^+$ cells were injected in mice with or without eGFP tagged MSC. After 48 hours of injections migration of MSC and CB-CD34$^+$ cells was assessed. At 48 hours placental MSC migrate to pelvic region and enhance CB homing: Panel A (GFP filter) and Panel B (DsRed filter) showed higher signals in mice injected with GFP tagged MSC and PKH26 red labelled CD34$^+$ cells (3, 4 and 5) compared to controls (1 and 2). There was co-localisation of CD34$^+$ and MSC signal.

Figure 21


Panel A
Control
CB D34
CB CD34+MSC
1
2
3
4
5
Colour Bar
Max = 7.8629e+08
780
760
740
720
x10
Min = 7.0208e+08
GFP filter

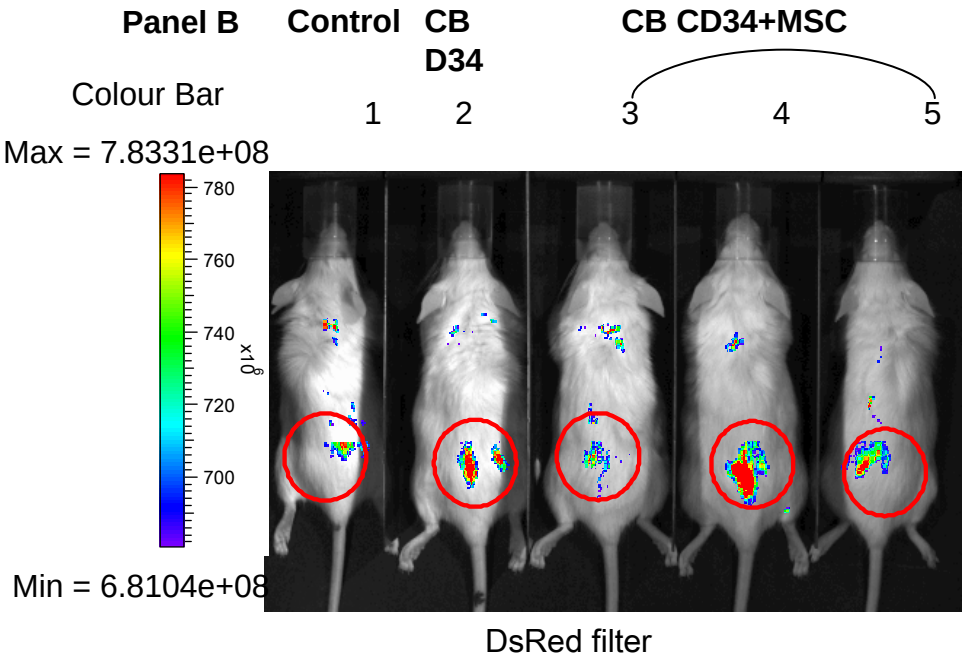
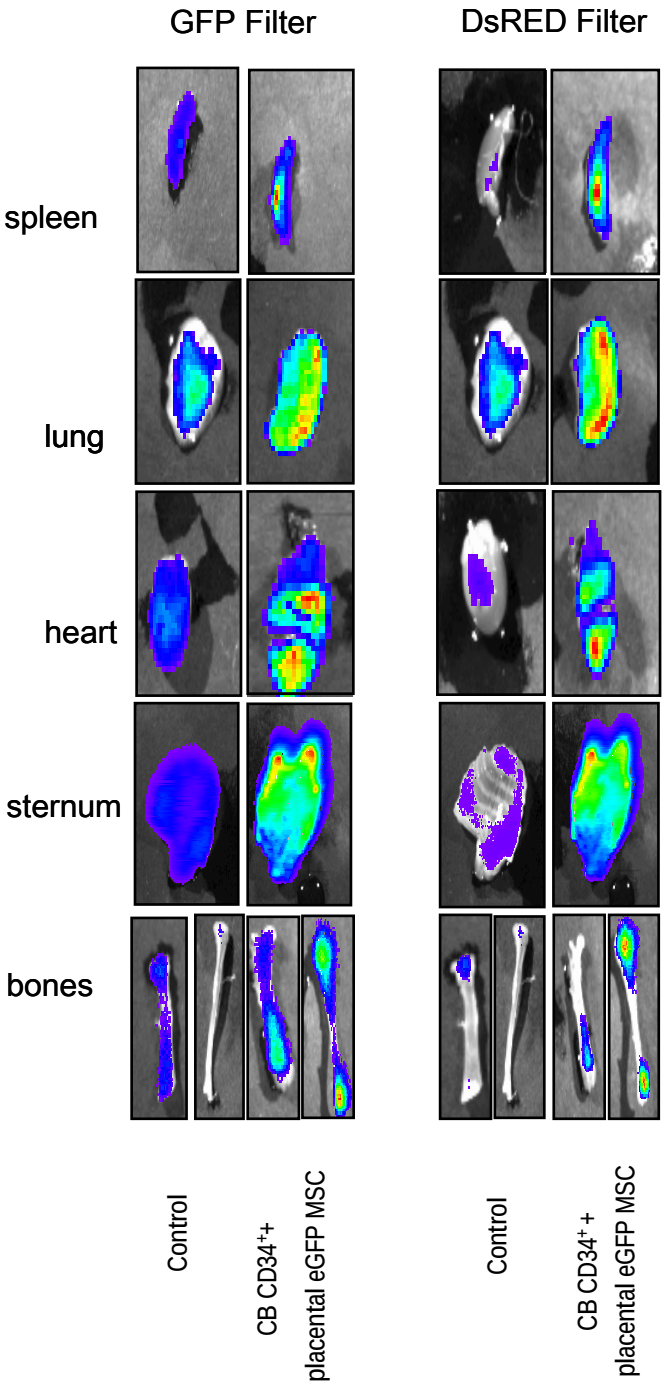

Panel B
Control
CB D34
CB CD34+MSC
1
2
3
4
5
Colour Bar
Max = 7.8331e+08
780
760
740
720
700
x10
Min = 6.8104e+08
DsRed filter

Figure 22. Tracing of GFP-MSC and PKH-CD34+ cells in internal organs of mice: PKH26- red labelled CB CD34$^+$ and eGFP tagged placental MSC were injected and fluorescence signals from the internal organs were studied after 48 hours. eGFP MSC and CB CD34$^+$ cells were traced by the GFP filter and DsRed filter respectively. The heat plot example shown, demonstrates higher signal emission from the organs obtained from the mouse that received eGFP transduced placental MSC and PKH26-red dye labelled CB CD34$^+$ compared to control.

Figure 22


GFP Filter
DsRED Filter
spleen
lung
heart
sternum
bones
Control
CB CD34++ placental eGFP MSC
Control
CB CD34+ + placental eGFP MSC

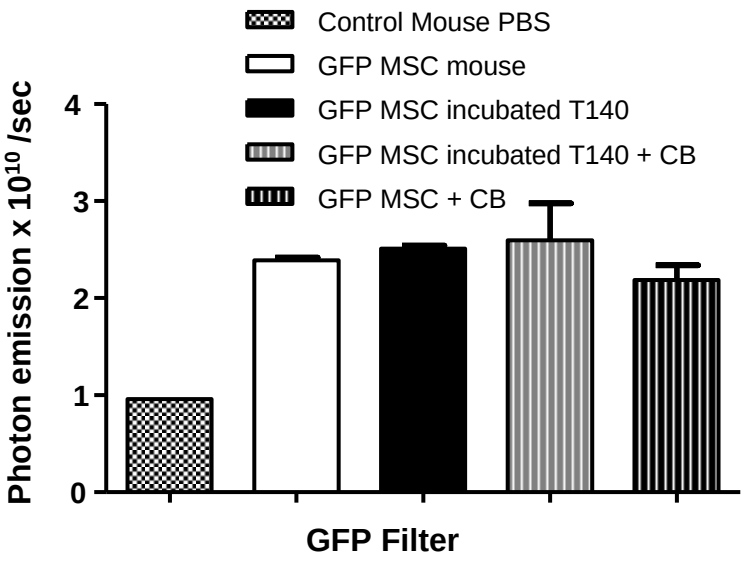


Figure 23A. Pre-incubation of placental MSC with T140 peptide (CXCR4 antibody) did not influence homing of MSC: The eGFP tagged MSC were pre-incubated with T140, an anti CXCR4 peptide for 30 minutes at room temperature and injected into the mice via tail vein injection with or without without CB $CD34^+$ cells. The preincubation with T140 did not reduce MSC homing to the pelvis.

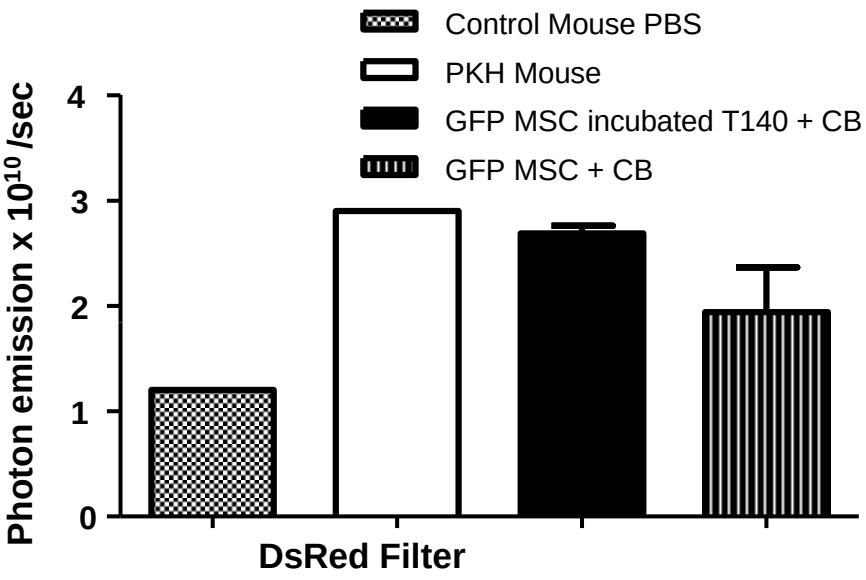


Figure 23B. Pre incubation of MSC's with T140 Ab did not influence CB $CD34^+$ cells homing: GFP-tagged placental MSC incubated with or without T140 (CXCR4 antibody) were co-transplanted with PKH labelled CB $CD34^+$ cells. Using live imaging technology, homing of CB $CD34^+$ cells was studied. The preincubation of placental MSC with T140 peptide did not influence homing of PKH26-red labelled CB $CD34^+$ cells to pelvis.

that co-transplantation of MSC along with CB $CD34^+$ enhances the homing of CB $CD34^+$ cells (Figure 23B).

4.4 Discussion

Despite the identification of several aspects of haemopoiesis little is known about the initial homing and seeding of HSC and MSC after co-transplantation. There are limited studies tracking the fate of placental MSC along with CB $CD34^+$ cells after IV systemic injections in mice.

In this study, the different cell types were labelled with eGFP or PKH26 red fluorescent dye. Tracing these cells provides information on their homing, expansion and engraftment. Previous studies have shown that fluorescence allows quantitative evaluation in mice (204) and using whole body imaging would help to demonstrate distribution of the IV injected cells. In this study the tagged CB $CD34^+$ and MSC were injected and in vivo migration was traced by IVIS.

In earlier animal and human studies of BMT, although HSC were of donor origin, BM stroma was predominantly of recipient origin. These studies set the dogma that replacing BM stroma is difficult, and following HSCT the BM stroma remains of recipient origin. However, infusion of donor MSC has repaired damaged tissue including myocardium, brain, lung, kidney and liver as demonstrated in different animal models (205-207). Horwitz *et al* reported osteoblast engraftment (1.5 to 2% donor cells) three months after allogeneic BMT for patients with osteogenesis imperfecta (208). Taken together, these results suggest that MSC can home to damaged organs and contribute to their repair.

During embryonic and foetal development MSC migrate in the bloodstream to seed new sites of haemopoiesis, as well as to various tissues (162, 209). MSC are also present in large numbers in foetal blood from at least 7 weeks of gestation and they persist until approximately 12 weeks of gestation. Hence, MSC have the capacity to migrate and home to various tissues during development. Recently, Muguruma *et al* have reported that following intra-bone marrow

injection, MSC engraft and integrate into the BM microenvironment of host mice (100). Bensidhoum *et al* also demonstrated that following intravenous injection, MSC engraft in murine BM, lungs, liver, spleen and muscles (103). In the current study, following systemic injection, it has been shown that the placental MSC predominantly migrated to the pelvic bone marrow, sternum, ribs, spleen, long bone ends, lungs and heart. Fouillard *et al* also reported that MSC help the regeneration of the BM stromal cells following infusion of allogenic MSC (210). Similarly, others have also reported that MSC migrate efficiently to BM, spleen (137, 211) lungs, brain, kidney, heart and eyes (205-207). Although most of the prior studies have shown that BM stromal cells or MSC migrate to different organs, this study has shown migration of MSC along with CB HSC to the haemopoietic sites and has traced them together in live mice. It has also shown co-transplantation of MSC increases homing of CB $CD34^+$ cells to BM.

Early studies which failed to demonstrate MSC engraftment following systemic infusion predominantly used BM or PB MSC. MSC are rare in BM and PB. It is estimated that human MSC are present at a frequency of 1 in 10^5 BM cells, and not detectable in peripheral blood stem cell graft (5, 80, 114). Therefore, a typical BM harvested graft may contain on average only 2-5 × 10^3 MSC/kg. As for $CD34^+$, the capacity of MSC to engraft in the BM may be related to the dose of MSC transplanted. The engraftment of donor-derived stromal cells capable of forming osteoblast in mice was detected only after injecting 120-180 × 10^6 of whole BM cells (134). In this study and other more recent studies higher doses of MSC were injected (≥50,000 MSC/mice). Another difference is the method of detection. This study has used live cell imaging for detection while studies which failed to demonstrate MSC homing to BM have used colony forming assay to detect. This may not be as sensitive in detecting MSC. The number of passages undergone during MSC expansion prior to injection also influences the engraftment

capacity of MSC (212). This variation between the studies might be the cause of discrepancy regarding the engraftment or homing potential of MSC.

There is limited published literature addressing the molecular mechanism of MSC homing. Sordi *et al* demonstrated that in vitro, migration of MSC in response to SDF-1 and CX3CL1 was abrogated by blocking CXCR4 and CX3CL1 with respective antibodies (211). Similarly, Wynn *et al* also demonstrated that the neutralizing anti-CXCR4 antibody inhibited MSC migration, indicating that MSC express the functionally active CXCR4 receptor, which contributes to MSC migration to BM (137). In the current study, preincubation of MSC with anti-CXCR4 antibody, neither inhibited its migration to the pelvic region nor altered the engraftment of CB $CD34^+$ cells Higher numbers of CB $CD34^+$ cells home to BM when co-transplanted with anti-CXCR4-preincubated-MSC compared to CB $CD34^+$ cells only. This may suggest that homing of all MSC may not be completely dependent on the CXCR4-SDF-1 axis, or it could be due to inadequate blocking of the CXCR4 receptor. It is also known that a subset of human BM MSC (2 to 25%) express chemokine receptors such as CXCR4, CX3CR1, CXCR6, CCR1, CCR7 and accordingly show chemotactic migration in response to the chemokines ligands such as CXCL12, CX3CL1, CXCL16, CCL3 and CCL19 respectively (211). Thus there is more than one migratory stimulus for BM-MSC(211). In MSC, CXCR4 mRNA levels are generally too low to be easily detectable by routine RT-PCR regardless of the passage number of the cells (137). Less than 1% of MSC express the cell membrane CXCR4 receptor(137). Brooke *et al* could not demonstrate the surface expression of CXCR4 on BM and placental MSC, although these cells were able to migrate in the direction of SDF-1, but intracellular CXCR4 receptor expression was found in 83 to 98% of cells. Another reason for the difference in CXCR4 expression could be the subset of MSC used for the studies. Lee *et al* reported that only rapidly self-renewing MSC (RS-MSC) express surface CXCR4. These cells have higher engraftment and rapid migration capacity compared to slowly renewing MSC (SR-MSC). CXCR4 levels were about 10 fold higher

in RS-MSC than SR-MSC (213). In this study placental MSC were used at 2 to 3 passages. During the logarithmic growth phase of MSC, RS MSC forms <10% of total MSC. Thus the current results could be explained by the low fraction of CXCR4 positive MSC, non-selected population, or the inadequate receptor blockade.

Co-transplantation of placental MSC along with CB $CD34^+$ demonstrated increased homing of $CD34^+$ cells in the pelvic region. Increased homing of CB $CD34^+$ to the BM could also be assessed by the CFU assay or CB $CD34^+$ cell quantitation in the harvested BM of transplanted mice. However post 16 hours of 2×10^5 $CD34^+$ cells injection only 0.02 to 0.12% of injected cells from the femur were detected (214) thus making it difficult to perform definitive CFU assays. Further-more there was a 1.6 to 2.7 fold increased in homing when MSC were co-transplanted with $CD34^+$ cells. There was significant variation in three mice (214). The current study could not document similar increase in homing using flow cytometry. This could be due to difference in events analysed (only 10,000 events were analysed in this study in contrast to 1×10^6 events analysed in the Chan *et al* study). Moreover the $CD34^+$ cell dose injected was low (5×10^4/mice) in the current study compared to 2×10^5 cells/mice injected in the Chan *et al* study. Also, the MSC dose injected was low (4×10^4 MSC/mouse) in the current study compared to a higher dose (1×10^6 MSC/mouse) used in the Chan *et al* study. The possible mechanism by which MSC increases HSC engraftment and homing may be SDF-1 secreted by MSC, which regulates the proliferation, differentiation and as well as attracts and retains the HSC(95, 198, 199).

Homing of HSC depends on the interactions between SDF-1 and CXCR-4. Peled *et al* demonstrated that interfering with the SDF-1/CXCR4 interaction by blocking with CXCR4 antibody significantly decreased HSC homing to BM(200). Muguruma *et al* demonstrated that primitive $CD34^+$ cells live in close contact with MSC in the endosteal region (100). Furthermore, the same study demonstrated that MSC integrate and differentiate into the haemopoietic

microenvironment (100). Thus MSC contribute to the formation of a “haemopoietic niche”. The IVIS data in the current study shows that enhanced homing of $CD34^+$ cells was facilitated by MSC, or alternatively increased retention of $CD34^+$ cells was induced by the MSC via cell to cell interaction or elaboration of haemopoietic cytokines. Further studies are needed to explain the molecular mechanism contributing to this effect. It is known that MSC produce various cytokines including IL-6, IL-11, GM-CSF, MCP-1, VCAM-1, ICAM-1(215), CXCL8, CXCL12, CCL2, CCL5, VEGF, and low amounts of CCL3 and angiopontin (211). In postnatal haemopoiesis, SDF-1 mediates selective BM homing and retention of HSC transplanted by intravenous infusion. In vitro, MSC supports the haemopoiesis and colony forming cell (CFC) production. In the presence of MSC progenitor output was 240.7 + 51.3 secondary CFC but no haemopoietic activity was detected after LTC in the absence of MSC(215). Moreover, neutralization of CXCR4 decreased the output of secondary progenitors by more than 50% compared to control cultures. Similarly, neutralization of SDF-1 induced a similar inhibitory effect. SDF-1 (726.3+140.9 pg/ml) was present in the supernatant of MSC but not in the conditioned medium of $CD34^+$ cells (215) demonstrating that SDF1 is secreted by MSC but not by $CD34^+$ cells. Thus SDF-1 is a critical factor in the regulation of haemopoiesis.

In the previous and current chapter it has been demonstrated that placental MSC enhances the engraftment of SCBT and DCBT. Bensidhoum *et al* also demonstrated that co-transplantation of MSC enhances the engraftment of CB $CD34^+$ cells (101). Placental MSC facilitates the homing of CB $CD34^+$ cells and may increase the retention of $CD34^+$ cells by cell-to-cell interaction or elevation of haemopoietic cytokines. The cells isolated from the placenta satisfied the criteria for defining MSC, It would be of interest to identify different cell populations within these placental MSC and compare their homing and engraftment potentials. However the focus of this study was to test whether placental MSC population enhances the homing and

engraftment of CB $CD34^+$ cells to the haemopoietic site. Results of the current study support the use of MSC for augmentation of engraftment following CBT.

4.5 Conclusions

This work has applied a live imaging assay to study the migration of MSC to delineate the mechanism of HSC supportive role. Placental MSC can be efficiently transduced with eGFP protein and can be traced to the haemopoietic sites following IV injection into live mice. Placental MSC migrate to BM and this is not completely dependent on CXCR4 function. Co-transplantation of placental MSC facilitates haemopoietic engraftment, perhaps by increasing homing and retention of the CB $CD34^+$ cells to the haemopoietic site.

Chapter 5 - Discussion

5.1 Final Discussion

During the last two decades there has been significant progress in the application of CBT (40, 216). CBT is an acceptable source of HSC for adult patients lacking a suitable matched unrelated donor (217). However cell dose remains the major limitation of CBT. Wagner *et al* have demonstrated the adverse effect of low cell dose on engraftment and survival (42). To overcome this limitation of CBT, various strategies have been used to increase CB cell dose. Some of the strategies include ex-vivo expansion of CB $CD34^+$ cells and transplantation of multiple CB units. Although ex-vivo expansion methods increase total cell numbers, this leads to exhaustion of the critical engrafting HSC (55) thus jeopardising engraftment capacity. DCBT increases the cell dose and provides an opportunity for patients who were otherwise disqualified on the basis of insufficient cells from a single CB unit (74). Studies have shown that DCBT has an additive effect in improving engraftment. However, only a single unit ultimately contributes to long-term haemopoiesis (74-77). Furthermore DCBT may be limited by the inability to find two suitably matched units.

Another aspect of SCT which potentially affects engraftment is damage to the microenvironment. Conditioning chemotherapy and/or radiotherapy not only damage the leukaemic cells and normal HSC, but also damage the marrow microenvironment which can compromise engraftment capacity (54). HSC shares an intimate relationship with the BM microenvironment and the majority of haemopoiesis supporting stromal cells are derived from MSC. Thus MSC could be used to restore the damaged microenvironment (139, 140) Furthermore, MSC also possess immunosuppressive properties (87), which may allow easier acceptance of the new graft. Kim *et al* reported that co-transplantation of BM MSC enhances engraftment of DCBT and reduces single CB unit dominance (72). Similarly, co-transplantation

of stromal cells in a murine model also enhanced HSC engraftment (72, 88, 89, 91, 92). In clinical studies infusion of ex-vivo expanded autologous, donor origin and third party MSC, along with autologous, allogeneic HSCT and CBT, has been demonstrated to be safe and feasible (6, 108, 109, 141)

Currently, BM represents the main source of MSC. Besides BM, MSC can also be isolated from various tissue sources such as adipose tissue (114), dental pulp (124), amniotic fluid (89, 118), placenta (123) cord (116) and the isolated cells have comparable characteristics to BM MSC. Nevertheless the optimal source of MSC for therapeutic application has not been defined. There is an intimate association between placenta and CB. The placenta plays a role in foetal haemopoiesis, as it produces haemopoietic growth factors (125) including SCF, Flt 3 ligand, IL-6, and macrophage stimulating factors (188). These properties make the placenta a promising source of MSC. Furthermore, cells obtained from the placental source are at less risk of viral infection, making the placenta an appealing source to consider.

The frequency of MSC varies between different sources. However, regardless the of source, the MSC population is rare. Therefore, for most therapeutic applications, including replacement of the damaged BM microenvironment, to obtain adequate MSC numbers requires in-vitro expansion. In this study 57 MSC per 1000 MNC were obtained from the foetal aspect of the placenta, compared to BM that has only 1 MSC per 10,000 nucleated cells (163). Similarly, Brooke *et al* reported higher number of placental MSC compared to BM MSC (123) thus confirming the placenta as a rich source of MSC. The current study has developed an optimal tissue dissociation protocol to isolate adherent cells from the foetal aspect of the placenta. The placental MSC were characterised and compared with BM MSC. The placental culture expanded cells consistently expressed CD73 (SH3/SH4), CD105 (SH2), CD90 (Thy-1), CD44, CD29 and HLA-class I, and lacked expression of haemopoietic markers including CD34 and CD45. This expression profile is comparable to BM MSC. Placental MSC are adherent cells with

fibroblast morphology and on an appropriate stimulus these adherent cells differentiated into adipogeneic, chondrogeneic and osteogeneic lineages similar to BM MSC. The cytogenetic study revealed that extensive in vitro expansion maintained the normal diploid karyotype. In summary, this study demonstrates that the placenta is a rich source of MSC, which could be a potential source for cell based therapies.

In this study, co-transplantation of placental MSC along with SCBT or DCBT enhanced engraftment. Placental MSC also reduced the dominance of the single unit in DCBT and allowed engraftment of both CBU. In the current study MSC were derived from the placenta, a readily accessible source of MSC. If these results are to be translated to a clinical setting, it is important that the dose of MSC used is achievable. A dose of 40,000 MSC per mouse, which is equivalent to 1×10^6/kg, was used in this study, and is a clinically achievable dose (6).

Any strategy that enhances engraftment must be able to maintain the long-term engraftment potential of HSC. In the current study serial transplantation demonstrated engrafted CB $CD34^+$ from both cohorts of the primary recipients (co-transplanted with or without MSC), were able to engraft in secondary recipients. Despite undergoing massive in-vivo expansion in the primary recipient, co-transplantation of placental MSC did not exhaust the long-term engraftment capacity of the HSC progenitors, indicating placental MSC improves engraftment without exhausting HSC progenitors. Furthermore, the multi-lineage differentiation in the secondary host was also maintained, irrespective of co-transplantation of placental MSC in the primary host, indicating placental MSC does not compromise the differentiation capacity of HSC.

Although several mechanisms have been proposed as to how MSC supports HSC engraftment, the precise mechanism remains unclear. In this study, migration and homing of CB HSC and placental MSC was studied. For this eGFP tagged MSC were co-transplanted along with PKH-26 red labelled CB $CD34^+$ cells. In vivo live mice imaging was performed by IVIS. Following infusion, eGFP tagged MSC migrate and home to lungs, spleen, ribs, pelvic bones

and long bone ends. CB $CD34^+$ cells were shown to home to pelvic BM and sternum. Co-transplantation of placental MSC enhanced the homing of CB $CD34^+$ cells to the pelvic bones compared to transplantation of CB $CD34^+$ cells only. Thus placental MSC increases the homing of CB $CD34^+$ cells to BM sites which may contribute to enhanced engraftment. This study also attempted to understand the mechanism of MSC homing to BM by the blocking of CXCR4 by T140 peptide. It did not reduce MSC homing to BM. Placental MSC express multiple cytokine and chemokine receptors which are involved in the homing and migration of MSC, and therefore the homing of MSC may not be dependent only on CXCR4-SDF1 interaction.

5.2 Conclusion

The major limitation of CBT is the limited cell dose. In this study the co-transplantation of placental MSC facilitated and enhanced engraftment. The findings of this study are as follows

1. The adherent cell population isolated from the placenta satisfy the criteria set forth by ISCT for defining MSC.
2. Placental MSC significantly enhances engraftment of SCBT and DCBT.
3. In DCBT, a single unit dominates the engraftment and co-transplantation of the placental MSC facilitates engraftment of both CB units.
4. Despite undergoing massive expansion in primary mice placental MSC maintains the long-term engraftment capacity of CB $CD34^+$ progenitors.
5. Placental MSC co-transplantation does not alter the differentiation potentials of the CB HSC.
6. Placental MSC co-transplantation increases the migration and homing of CB $CD34^+$ to the haemopoietic sites.

In summary this study has demonstrated that the co-transplantation of placental MSC may overcome the limitation of low cell dose in CBT.

5.3 Future Directions

For developing placental MSC for cellular therapies the in-vivo behaviour of the MSC after systemic administration needs to be understood. In the current study only the short-term fate of the injected MSC was studied. Whether MSC only help homing of the HSC to the haemopoietic sites or whether they engraft and differentiate into various lineages and produce the growth factors essential for HSC maintenance was not addressed in this study. The tracing of labelled placental MSC after 6 - 8 weeks may help to determine the long-term fate of injected MSC. Importantly, the experiments conducted in this study provide a method of enhancing engraftment, which should be confirmed in higher animal models. This could help us to better understand the role of MSC in reconstitution of the BM microenvironment, and also in enhancing HSC engraftment.

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