

The Regulation of Stem Cell Engraftment



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Abstract

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The engraftment of haemopoietic stem/progenitor cells (HSPCs) from umbilical cord blood (UCB) into adult recipients, although advantageous in terms of sourcing units, the decreased need to match donor and recipient and reduced risk of graft versus host disease (GvHD), is delayed compared to grafts using HSPCs from mobilised peripheral blood (MPB) or bone marrow (BM). One reason for this is the limited number of HSPCs (CD34⁺/CD133⁺ cells) in a unit of UCB compared to MPB or BM. The CXCR4-CXCL12 axis is widely recognised as a key player in the bone marrow homing, retention, and engraftment of HSPCs. The aim of this thesis was to investigate whether the engraftment of HSPCs from UCB into the bone marrow could be improved. Firstly, a novel *in vitro* 3D time-lapse chemotaxis assay to assess the homing capacity of human UCB CD133⁺ HSPCs, towards the chemokine CXCL12 was developed. One advantage of this assay was that it distinguished cell chemotaxis from chemokinesis and allowed these parameters to be quantified. Human UCB CD133⁺ HSPC chemotaxis towards CXCL12 was inhibited by the CXCR4 antagonist, AMD3100. Importantly, the presence of CXCL12 or AMD3100 had no effect on cell chemokinesis. To complement the *in vitro* chemotaxis assay, a short term *in vivo* homing assay in NSG mice was successfully established. The effect of siRNA silencing of the CXCR4 co-receptor, CD164, which is also expressed on CD133⁺ HSPCs, on cell migratory and homing ability was investigated. CD164 knock-down using siRNA in human UCB CD133⁺ HSPCs did not demonstrate an effect on homing to NSG bone marrow *in vivo* or chemotaxis to CXCL12 *in vitro*. However, homing to NSG mouse spleen was significantly reduced in cells silenced for CD164. Following this, an 8 day HSPC expansion system using nanofibre scaffolds (Nanex) and differing cytokines was investigated. These serum and feeder free conditions yielded a significant expansion of cells that retained CD133⁺CD34⁺ expression and their *in vitro* chemotactic ability to CXCL12. Time constraints did not permit the engrafting ability of these cells to be analysed in an *in vivo* HSC reconstitution assay that was initiated. However these studies will provide the basis to support future related research in this laboratory.

Publications

Publications

- Zhou B, Tsaknakis G, Coldwell KE, Khoo CP, Roubelakis MG, Chang CH, Pepperell E, Watt SM. A novel function for the haemopoietic supportive murine bone marrow MS-5 mesenchymal stromal cell line in promoting human vasculogenesis and angiogenesis. Br J Haematol 2012 May; 157(3):299-311

Manuscripts in preparation

- Pepperell EE and Watt SM. CXCL12 modulates chemotaxis but not chemokinesis of individual hematopoietic CD133⁺ stem/progenitor cells *in vitro*

Published meeting abstracts

- Pepperell EE and Watt SM (2012). CXCL12-induced trafficking of individual haematopoietic CD133⁺ stem/progenitor cells *in vitro*. NHS Blood and Transplant UK R&D conference, Oxford University, Oxford, UK.
- Pepperell EE and Watt SM (2012). A novel method to track human CD133⁺ haemopoietic stem/progenitor cell chemotaxis towards CXCL12. Chemotactic cytokines Gordon Research Conference, Barga, Italy.
- Pepperell EE and Watt SM (2012). A time-lapse microscopy method to visualize and quantify human haemopoietic CD133⁺ cell chemotaxis to CXCL12. Chemotactic cytokines Gordon Research Conference, Barga, Italy
- Pepperell EE, Woll P, Forde SP, Watt SM (2011). CD164 modulates the homing of human umbilical cord blood CD133⁺ cells to the bone marrow *in vivo*. NHS Blood and Transplant UK R&D conference, Cambridge University, Cambridge, UK.

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Acknowledgments and Declaration

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Common abbreviations

Abbreviation	Full name
AGM	Aorta, gonads and mesonephros
Angptl5	Angiopoietin-like 5
APC	Allophycocyanin
BM	Bone marrow
BMSC	Bone marrow stromal cell
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation antigen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CXCL12	CXC-chemokine ligand 12
DAPI	4',6-diamidino-2-phenylindole
dmPGE2	16, 16-dimethyl prostaglandin E2
ECFC	Endothelial colony forming cell
FACS	Fluorescence activated cell sorter
FGF-1	Fibroblast growth factor-1
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FLT-3	FMS-related tyrosine kinase 3
G-CSF	Granulocyte colony stimulating factor
GMP	Granulocyte-monocyte progenitor
GPCR	G-protein coupled receptor
GvHD	Graft versus host disease
eGFP	Enhanced green fluorescent protein
HCELL	Haemopoietic cell E-L-selectin ligand
HLA	Human leucocyte antigen
HSC	Haemopoietic stem cell
HSPC	Haemopoietic stem/progenitor cell
HUVECs	Human umbilical vein endothelial cells
IGFBP2	Insulin-like growth factor-binding protein 2
IL	Interleukin
LDL	Acetylated low density lipoprotein
LMPP	Lymphoid primed multi-potent progenitor
LSK cells	Lin ⁻ Sca-1 ⁺ Kit ⁺ cells
Mab	Monoclonal antibody
MEP	Megakaryocyte-erythrocyte progenitor
MLP	Multi-lymphoid progenitor
MMP	Matrix metalloproteinase
MNCs	Mononuclear cells
MPB	Mobilised peripheral blood
MPP	Multi-potent progenitor
MSC	Mesenchymal stem cell
MS-5	Murine stromal cell line 5
NOD-scid	Non-obese severe combined immune-deficient
NSG	NOD scid IL2 receptor gamma chain knockout

PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PKC	Protein kinase C
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
SCF	Stem cell factor
SLAM	Signalling lymphocyte activation molecules
SNS	Sympathetic nervous system
siRNA	Small interfering RNA (ribonucleic acid)
shRNA	Short hairpin RNA
SRC	SCID repopulating cell
TNC	Total nucleated cells
TPO	Thrombopoietin
UCB unit	Umbilical cord blood unit
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
YFP	Yellow fluorescent protein

Chapter 1. Introduction

1.1 Literature review

1.1.1 Stem cells

Regenerating systems, composed of specialised, mature cells, such as the intestine, skin and blood, require continuous replenishment. These mature cells are not capable of further growth and proliferation, and so the task falls on primitive cell populations capable of re-populating tissues, known as stem cells. Stem cells have self-renewing potential, or at least the potential for extensive proliferation and the ability to develop into functional differentiated cells over an individual's lifetime (reviewed in (Hsu and Fuchs, 2012; Lo and Scadden, 2011; Nakada et al., 2011; Watt, 2011)). Although most cells in the adult human are differentiated, self-renewing stem cells may also be present, although this varies amongst different tissues. Adult stem cells are required to maintain and provide function in a number of tissues. The tissues where stem cells are particularly prevalent are those that require the replenishment of differentiated cells in environments of continuous renewal such as the blood, skin and gastrointestinal tract (reviewed in (Arwert et al., 2012; Doulatov et al., 2012; Hsu and Fuchs, 2012; Nakada et al., 2011)).

Stem cells not only self-renew, but also often have multilineage differentiation potential, giving rise to progeny comprising different cell types (Luis et al., 2012). There is evidence that this is achieved by asymmetric cell division to produce self-renewing and differentiated progeny, rather than individual stem cells either self-renewing or producing progeny which differentiate (Knoblich, 2008). This process

has been extensively studied in *Drosophila* and *Caenorhabditis elegans* and less so in mammals. However, these studies suggest that the asymmetrical division is either achieved by unevenly distributing mRNA and proteins in the cytoplasm before mitosis or by exposure to different environmental signals post-division (reviewed by (Hsu and Fuchs, 2012; Knoblich, 2008; Wilson and Trumpp, 2006)). Studies in haemopoietic stem cells (HSCs) suggested that the environmental influence is provided by the stem cell niche and that a pro-differentiation environment would induce asymmetric division to produce differentiated progeny, whereas in a pro-renewal environment, symmetric division would retain the HSC pool (reviewed by (Luis et al., 2012; Wilson and Trumpp, 2006)).

1.1.1.1 Haemopoietic stem cells

The development of the haemopoietic system in vertebrates is characterised firstly by a wave of primitive haemopoiesis initiated in the yolk sac, and later, by a definitive wave of haemopoiesis (Medvinsky et al., 2011). During early embryonic haemopoietic development, the haemangioblast, derived from one of the three germ layers (the mesoderm), contributes to the formation of blood islands containing primitive red blood cells and vasculature (Dzierzak and Speck, 2008). In the mouse, the production of nucleated erythrocytes occurs in the yolk sac between day 7-8 of gestation (reviewed by (Cumano and Godin, 2007; Luis et al., 2012)). After this, haemopoietic progenitors originate from the aorta, gonads and mesonephros (AGM) region of the embryo proper during mouse development and haemopoietic cells have been visualised budding from the dorsal aorta, suggesting the existence of haemogenic endothelium (Tavian et al., 2005). Definitive haemopoiesis, defined as the process whereby HSC generate all blood lineages throughout adult life, occurs in the mouse

embryo from day 10.5-11.5 of gestation (reviewed by (Luis et al., 2012; Medvinsky et al., 2011)). The close association between endothelial and haemopoietic cells during primitive and definitive haemopoiesis is widely accepted. However, controversy remains regarding the relationship between the haemangioblast and haemogenic endothelium and whether the haemogenic endothelium is the origin of definitive HSCs (reviewed by (Jaffredo et al., 2005)). HSCs are thought to then migrate to the fetal liver, which becomes the major site of haemopoiesis until birth. In the latter stages of gestation, cells migrate to the bone marrow (reviewed by (Luis et al., 2012)). The HSCs continue to proliferate before transiting to quiescent adult HSCs within the first few weeks of birth (Bowie et al., 2006). The location of definitive haemopoiesis throughout gestation to birth is illustrated in Figure 1.1.

The mobility seen in HSCs during gestation can extend into adulthood. During homeostasis, HSCs reside principally in the bone marrow, but have the potential to move within the niche. In response to stress, HSCs and their progeny may enter the blood stream before trafficking to other organs such as the liver or spleen (at least in the mouse) (Hsu and Fuchs, 2012). Most self-renewing adult HSCs are thought to be quiescent under homeostatic conditions. This is supported by the resistance of the adult stem cell pool to cycling cell targeting chemotherapies, whereas their progeny are actively cycling and contributing to mature blood cell production (reviewed by (Luis et al., 2012)).

It has been suggested that the long term repopulating HSCs (LT-HSC) might be held in reserve as a quiescent storage population which can be reversibly reactivated and mobilised in response to stress, before returning to dormancy once homeostasis has been re-established (Foudi et al., 2009; Kiel et al., 2007a).

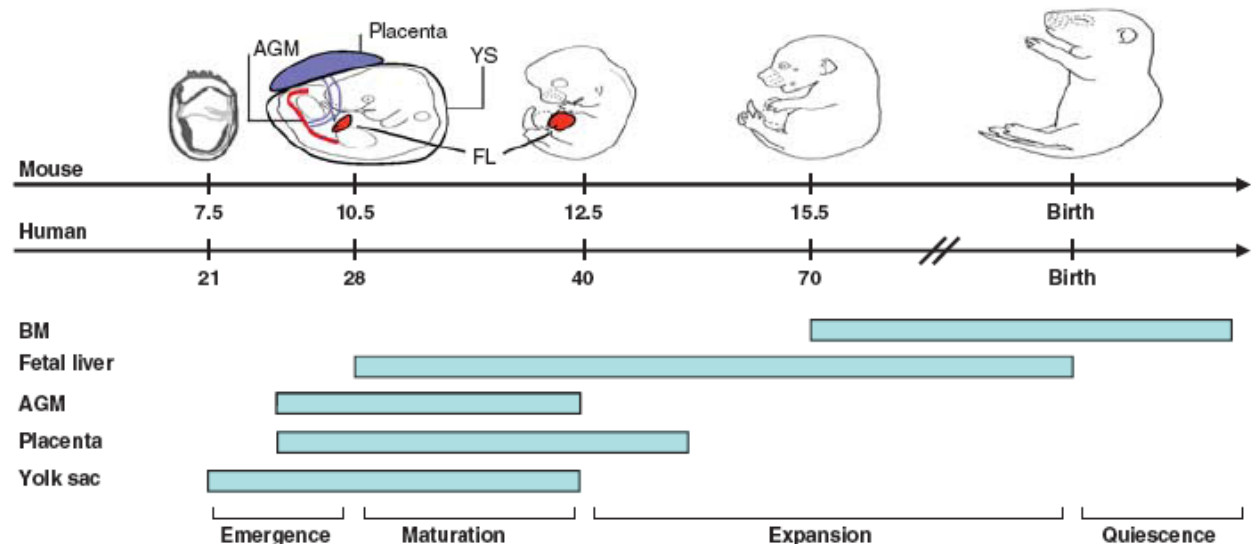


Figure 1.1. The progression of definitive haemopoiesis in mouse and human. The sites of active haemopoiesis are shown at the relevant stage in development by the bars below the timeline. Image reproduced with permission from Luis *et al.* Leukaemia 2012;26,86-90 (Luis et al., 2012).

The short-term re-populating HSCs (ST-HSCs), which are derived from LT-HSCs, are thought to promote initial or short-term reconstitution of haemopoietic lineages (reviewed by (Hsu and Fuchs, 2012; Luis et al., 2012; Ratajczak, 2008)). The progeny of HSCs form an ordered hierarchy which is thought to initially include a multi-potential progenitor (MPP) with lymphomyeloid potential (Goardon et al., 2011). Historically, it has been postulated that differentiation into common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) then occurs, and that these precursors further differentiate independently to form cells of the myeloid lineage (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes and myeloid dendritic cells) or lymphoid lineage (natural killer (NK) cells, T, B cells and lymphoid dendritic cells) respectively (Ratajczak, 2008). This traditional hierarchy is illustrated in Figure 1.2, with an updated understanding of the hierarchy shown in Figure 1.13. This updated system includes a lymphoid primed multi-potent progenitor (LMPP) with granulocyte-macrophage but not

megakaryocyte-erythroid potential that has been described for the mouse (Doulatov et al., 2010). As the equivalent progenitor was not fully characterised in humans, a multi-lymphoid progenitor (MLP) capable of producing monocytes, macrophages and dendritic cells was suggested for human haemopoiesis (Doulatov et al., 2010). A diagram detailing the suggested hierarchy and phenotype of the LMPP/MLP is shown later in Figure 1.13. More recently, Goardon *et al.* suggested that, in the human, there was an undefined intermediate population immediately downstream of the MPP which precedes the more committed progenitors (Goardon et al., 2011). This population most closely resembled the LMPP in studies dissecting the normal and leukaemic human stem cell hierarchy (Goardon et al., 2011).

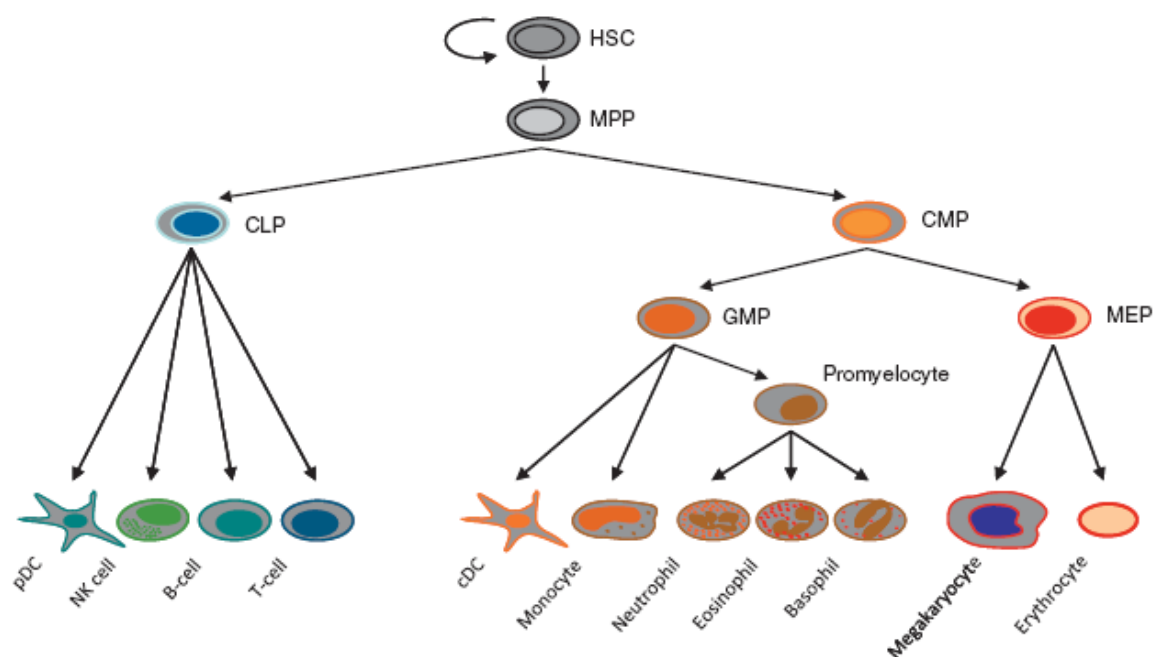


Figure 1.2. The hierarchical development of the haemopoietic system. Self renewing HSCs generate multipotent progenitors (MPP) through asymmetric division. The common lymphoid and common myeloid progenitors (CLP and CMP) give rise to lymphoid and myeloid progeny (including the granulocyte-monocyte progenitor (GMP) and megakaryocyte-erythrocyte progenitor (MEP)). Image reproduced with permission from Luis *et al.* Leukaemia 2012;26,86-90 (Luis et al., 2012).

1.1.1.2 Mesenchymal stem cells

Bone marrow mesenchymal stem/stromal cells (BMSCs/MSCs) are a non-haemopoietic population of cells found in the bone marrow, that have the ability to differentiate *in vivo* into chondrocytes, adipocytes and osteoblasts (Dazzi et al., 2012). The dynamic role of these 'skeletal stem cells' in regulating haemopoiesis, bone formation, and in their own regeneration in the bone marrow is being increasingly appreciated (reviewed by (Bianco, 2011b; Scadden, 2012)). However, primitive MSCs are difficult to distinguish from their undifferentiated progeny and fibroblasts but are reported to express CD105, CD73, CD90, CD44, CD71, Stro-1, CD106 (VCAM-1) and CD29 in the absence of the common leucocyte marker CD45 (Dazzi et al., 2012). In mice, these Nestin⁺ cells, capable of self renewing, or at least proliferation while maintaining multipotency, highly express HSC maintenance genes and also control HSC homing to and egress from the bone marrow (Mendez-Ferrer et al., 2010b). Schajnovitz *et al.* have recently reported that equivalent reticular MSC cells isolated from human bone marrow express CD146, CD106 and Nestin (Schajnovitz et al., 2011). However, particularly within the bone marrow, the term MSC is thought to cover a heterogeneous population (Park et al., 2012). Most recently, Park *et al.* have shown bone marrow derived MSCs, which meet the criteria detailed above *in vitro*, although capable of self-renewal and serial transplantation, are restricted to the osteolineage *in vivo* (Park et al., 2012). Human MSCs have also been shown to support blood vessel formation from human endothelial cells (Watt et al., 2010), have immunomodulatory properties (reviewed by (Dazzi et al., 2012)) and be capable of interstitial and intravascular translocation to injury sites (Park et al., 2012).

1.1.1.3 Endothelial cells

Endothelial stem/progenitor cells generate blood vessels in the embryo, allowing organs to develop by providing oxygen and the trafficking of cells and nutrients. These cells are thought to be derived either as committed endothelial precursors from the mesoderm, from a haemangioblastic intermediate which gives rise to endothelial cells and haemopoietic cells, or possibly via the haemogenic endothelium (reviewed by (Cumano and Godin, 2007; Gordon-Keylock and Medvinsky, 2011)). It is logical that an integral relationship should exist between the blood cells and the cells that constitute the blood vessels. During the proposed transition from endothelial precursor to HSC, CD34 and VE-cadherin (CD144) are expressed (Medvinsky et al., 2011) (Figure 1.3), and these markers also continue to be expressed by adult endothelial cells (Watt et al., 2010). Endothelial progenitor cells, termed endothelial colony forming cells or ECFCs, persist in the adult and have been isolated from peripheral blood, tissues and bone marrow (Mund et al., 2012)(reviewed by (Watt et al., 2010)). Much work has been done to identify the phenotype of ECFCs and these cells share markers with haemopoietic stem/progenitor cells (HSPCs) but lack the common leucocyte marker CD45 (reviewed by (Timmermans et al., 2009; Yoder, 2012)). Their function in the adult, to maintain the vascular system, requires constant regeneration which can occur in three ways. Firstly, by angiogenesis (the formation of new vessels by sprouting from existing vessels), secondly, by vasculogenesis (the differentiation of mature endothelial cells from endothelial progenitors) and finally, by arteriogenesis (remodelling the arterioles to increase lumen size) (Watt et al., 2010). The early evidence that ECFCs enter the blood stream from the bone marrow challenged previous ideas that, in the adult, only angiogenesis and not vasculogenesis could

provide regeneration (reviewed by (Timmermans et al., 2009)). Studies investigating the importance of vasculogenesis in the bone marrow have shown that haemopoietic reconstitution in the adult mouse is dependent on VEGFR2-mediated endothelial regeneration following myeloablation (Hooper *et al.* 2009).

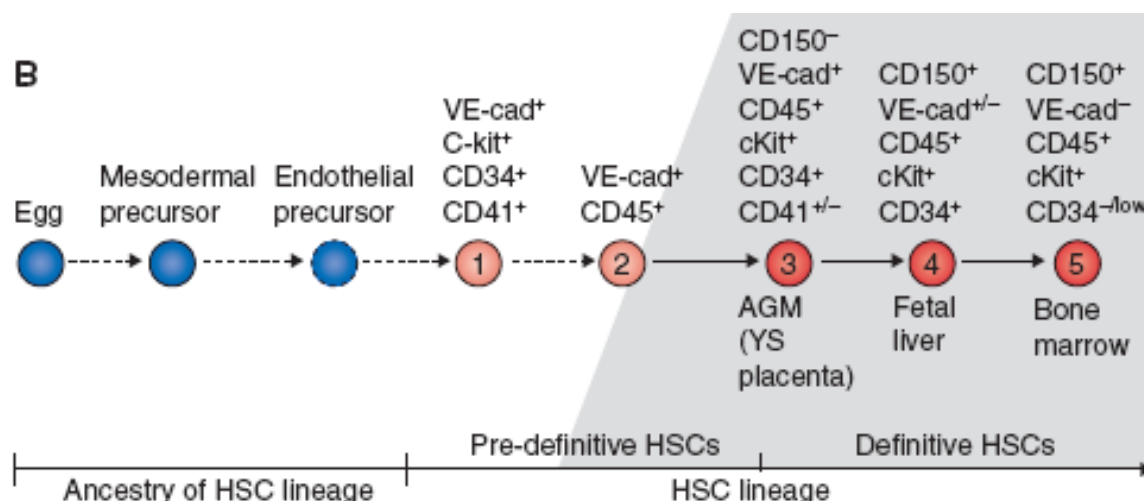


Figure 1.3. HSC development from the mesodermal and endothelial precursors. The surface expression of markers on transitional stages of proposed HSC development from endothelium in the mouse. The dotted arrows represent presumptive transitions, and the solid arrows, defined populations on definitive HSCs. Image reproduced with permission from Medvinsky *et al.* Development 2011;138,1017-1031 (Medvinsky et al., 2011).

1.1.2 Bone marrow development

The bone marrow is the main site for haemopoiesis in adults (Watt and Forde, 2008) and produces myeloid and B lymphoid cells, whereas T cells mature in the thymus. The development of the bone marrow was shown in early studies using ectopically implanted bone marrow, which was shown to have complete haemopoietic regeneration potential (Meck et al., 1973; Tavassoli and Crosby, 1968). Bone develops as a result of proliferating mesenchymal cells forming an aggregation of chondroblasts which promote collagenous fibre formation, allowing chondrocytes to develop within the fibrous matrix. Once the chondrocytes become hypertrophic they

secrete a collagen and fibronectin matrix. Formed cartilage is calcified leaving matrices on which osteoid bone can form. Distinctive sinusoidal endothelium develops and the bone is repopulated with HSCs. Expanding haemopoiesis is associated with bone resorption by osteoclasts, creating a central space which is filled with red bone marrow (haemopoietic and stromal cells) and yellow/white bone marrow (adipose) (Tavassoli, 1984). Bone forming osteoblasts deposit bone on the cancellous bone and once this metabolic process is over, they form either osteocytes in the mineralised bone or form bone lining cells (reviewed by (Bianco, 2011a)). These are not the osteogenic cells that form a single layer on the surface of bones (endosteum) and have been shown to regulate HSCs and differentiation (Calvi et al., 2003; Wu et al., 2008; Zhang et al., 2003; Zhu et al., 2007), which have been termed osteoblasts in some studies. However, osteoblastic-like cells expressing osteopontin have been shown to negatively regulate the HSC pool size, whereas stroma from osteopontin knock-out mice increased the HSC pool (Nilsson et al., 2005; Stier et al., 2005).

Sinusoidal endothelial cells, unique to the haemopoietic tissues including the bone marrow and distinct from other vascular endothelial cells, are also required for haemopoiesis (Bianco, 2011a; Hooper et al., 2009). These specialised cells constitutively express factors that are important for HSC mobilisation, homing and engraftment, such as the cytokine CXC-chemokine ligand 12 (CXCL12), endothelial cell selectin (E-selectin) and vascular cell adhesion molecule 1 (VCAM-1) (Frenette et al., 1998; Mazo et al., 1998; Mazo et al., 2011). The arterioles which open into the sinusoids provide a slow blood flow from the nutrient artery, which enters midway along the bone marrow and extends to both ends of the bone, allowing HSPCs from

the circulation to adhere to and extravasate through the endothelium and lodge extra-vascularly (Ellis et al., 2011; Mazo et al., 1998; Sipkins et al., 2005). Although these sinusoidal endothelial cells are covered with a discontinuous layer of perivascular/mesenchymal stromal cells, there is controversy about whether they provide a separate environment to the endosteum for supporting HSPCs (Kiel et al., 2005; Sipkins et al., 2005). More recently, *in vivo* imaging studies in mice have identified single HSPCs crossing the bone marrow endothelium between 15 minutes-15 hours post infusion, by which point few remained in the sinusoids (Ellis et al., 2011). However, as Figure 1.4 illustrates, endosteal and endothelial cells are found throughout the bone and have not been confirmed as providing separate HSC microenvironments. Indeed, Ellis *et al.* calculated that trabeculae were approximately 192 μ m apart on average and that sinusoids were approximately 45 μ m apart. Therefore, assuming an HSPC to be 8 μ m in diameter, they concluded that all HSPCs in the bone marrow were <12 cell diameters from bone and <3 cell diameters from vasculature (Ellis et al., 2011).

1.1.2.1 The bone marrow niche

The niche, or specialised stem cell microenvironment, was first described by Schofield in 1978 (Schofield, 1978). Schofield proposed that the cell-cell contact between HSCs and the bone was responsible for maintaining HSCs in an immature state with unlimited proliferative abilities (Schofield, 1978). Lord *et al.* had previously suggested that the bone marrow (BM) conformed to a well-defined arrangement, and that HSPC populations reside at the endosteum or in the bone marrow cavity depending on their relative state of differentiation (Lord et al., 1975).

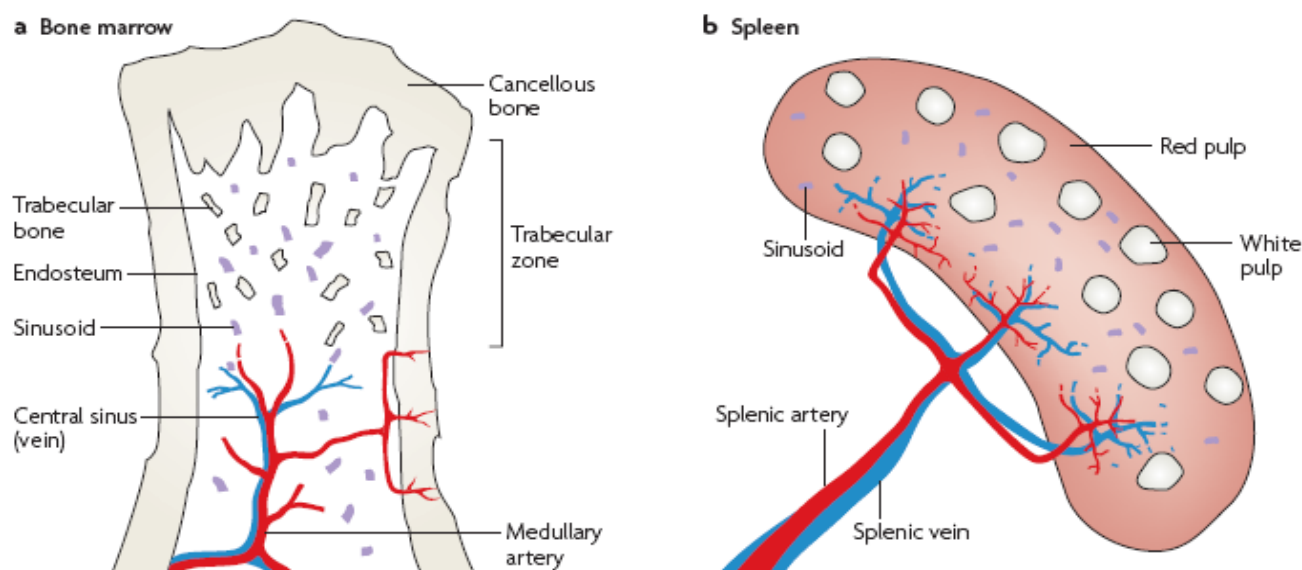


Figure 1.4. Diagrams of the adult haemopoietic organs. The bone marrow (a) and spleen (b). The bone marrow contains stromal cells that support haemopoiesis. This occurs within the cavity, in association with the fenestrated sinusoids, trabecular bone and endosteum lining the vascularised and innervated cancellous bone. The spleen also contains low levels of HSCs, in the red pulp, and can support extramedullary haemopoiesis when the bone marrow is impaired. Image reproduced with permission from Kiel and Morrison Nat Rev Immunol 2008;8,290-301 (Kiel and Morrison, 2008).

Subsequent *in vitro* studies demonstrated that endosteal cells support the maintenance of HSCs and showed selective lodgement of HSC enriched populations specifically at the endosteal region of the bone marrow following transplantation (Nilsson et al., 2001; Taichman et al., 1996). Although later studies also supported the endosteal niche as a key component regulating HSC quiescence, the size of the HSC pool (Nilsson et al., 2005; Stier et al., 2005) and a primary site for HSC residence (Calvi et al., 2003; Zhang et al., 2003), evidence of an additional supportive niche that did not require bone was emerging (Kiel and Morrison, 2006; Kiel et al., 2007b; Kiel et al., 2005). However, the identification of a perivascular mesenchymal progenitor that could re-store the niche (Sacchetti et al., 2007) and CXCL12-expressing adventitial reticular (CAR) cells that were located adjacent to HSCs at endosteal and

perivascular sites, suggested a role for cell types other than bone and vasculature (Sugiyama et al., 2006). This led to the realisation that the bone marrow niche is likely to be composed of different types of bone, vascular, mesenchymal/stromal, adipocytic and perivascular cells (reviewed by (Kiel and Morrison, 2008)) and most recently osteomacs (bone marrow macrophages) and T cells (reviewed by (Lo Celso and Scadden, 2011)) (Figure 1.5).

The production of adipocytes from bone marrow MSCs is thought to regulate the amount of space within the bone marrow that can generate haemopoiesis (Bianco et al., 1988; Naveiras et al., 2009). Although cellular constituents of the niche are unlikely to have completely independent roles, the notion that the endosteum or osteoblastic niche maintains the dormant cells, or those with a higher haemopoietic potential (Haylock and Nilsson, 2007), whilst the perivascular cells can promote HSPC cycling and differentiation next to the vasculature is widely supported (Malhotra and Kincade, 2009; Trumpp et al., 2010).

More recently, Nestin⁺ perivascular stromal cells innervated by the sympathetic nervous system (SNS) have been identified adjacent to bone, and are required for HSC homing and retention (Mendez-Ferrer et al., 2010b). These MSCs highly express HSC maintenance genes (such as CXCL12, SCF, IL-7, VCAM-1 and Angpt-1) but also downregulate osteoblastic differentiation during HSC mobilisation and were shown to differentiate into osteoblasts when stimulated (Mendez-Ferrer et al., 2010b), similar to the adipo-osteogenic progenitors near sinusoids described by Omatsu *et al.* (Omatsu et al., 2010).

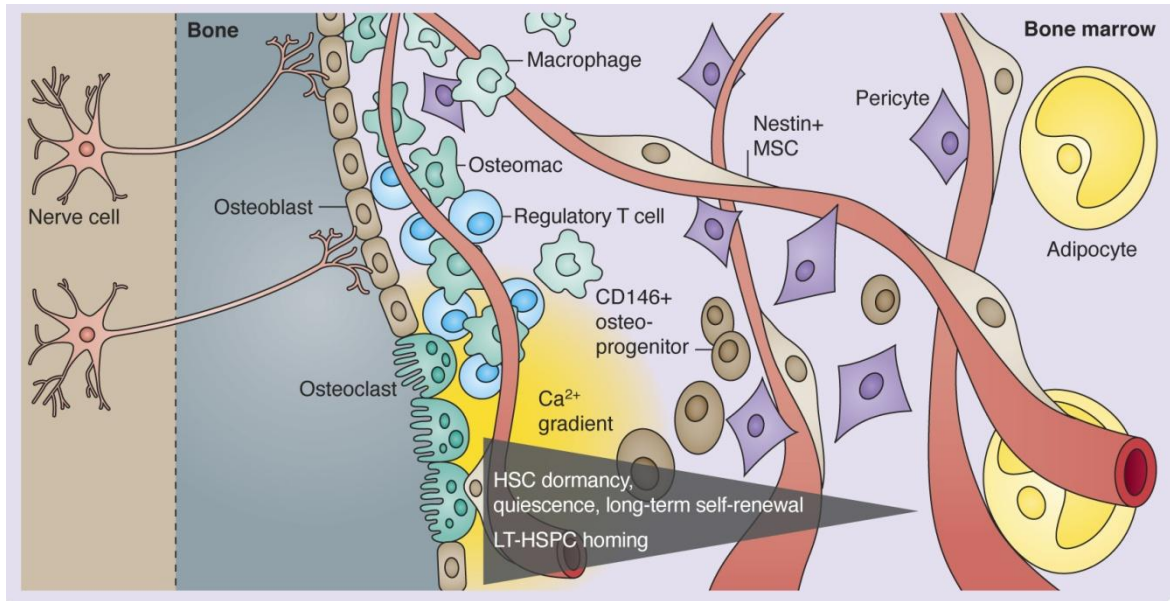


Figure 1.5. The proposed HSC bone marrow microenvironment. The current model of the bone marrow niche containing MSCs and pericytes lining sinusoids and the endosteal cells including bone destroying osteoclasts and osteoblastic cells. The authors note the current controversy surrounding the term osteoblast and use this term to refer to the entire lineage of osteoblastic-like cells rather than those with metabolic activity. The role of calcium in the stem cell niche (Adams et al., 2006) is also shown. The recently identified osteo-macrophages (different from osteoclasts) are also shown along with regulatory T cells. Image reproduced with permission from Lo Celso and Scadden *J Cell Sci* 2011;124:3529-3535 (Lo Celso and Scadden, 2011).

The SNS innervations to these stromal/reticular cells, associated with blood vessels, have been shown to regulate HSC movement controlled by circadian oscillations in CXCL12 expression (Mendez-Ferrer et al., 2008; Mendez-Ferrer et al., 2010a). More recently, other molecules, including tetraspanins, such as CD82 and CD9 have been shown to play a role in homing and engraftment (Larochelle et al., 2012; Leung et al., 2011). Other factors, cytokines and pathways involved in regulating HSC mobilisation, homing and engraftment are illustrated in Figure 1.6. In addition to these, the importance of extracellular matrix components such as hyaluronic acid has been described (reviewed by (Sackstein, 2011)). HSPCs express CD44, a receptor for hyaluronic acid, which is a glycosaminoglycan and component of the HSC niche

providing a physical scaffold and retention platform for HSPCs (Haylock and Nilsson, 2006). A specialised glycoform of CD44, termed HCELL (haemopoietic cell E-L-selectin ligand), the most potent E- and L- selectin ligand which is expressed by human (and not mouse) HSPCs, can also regulate HSPC migration and extravascular lodgement (Sackstein, 2011).

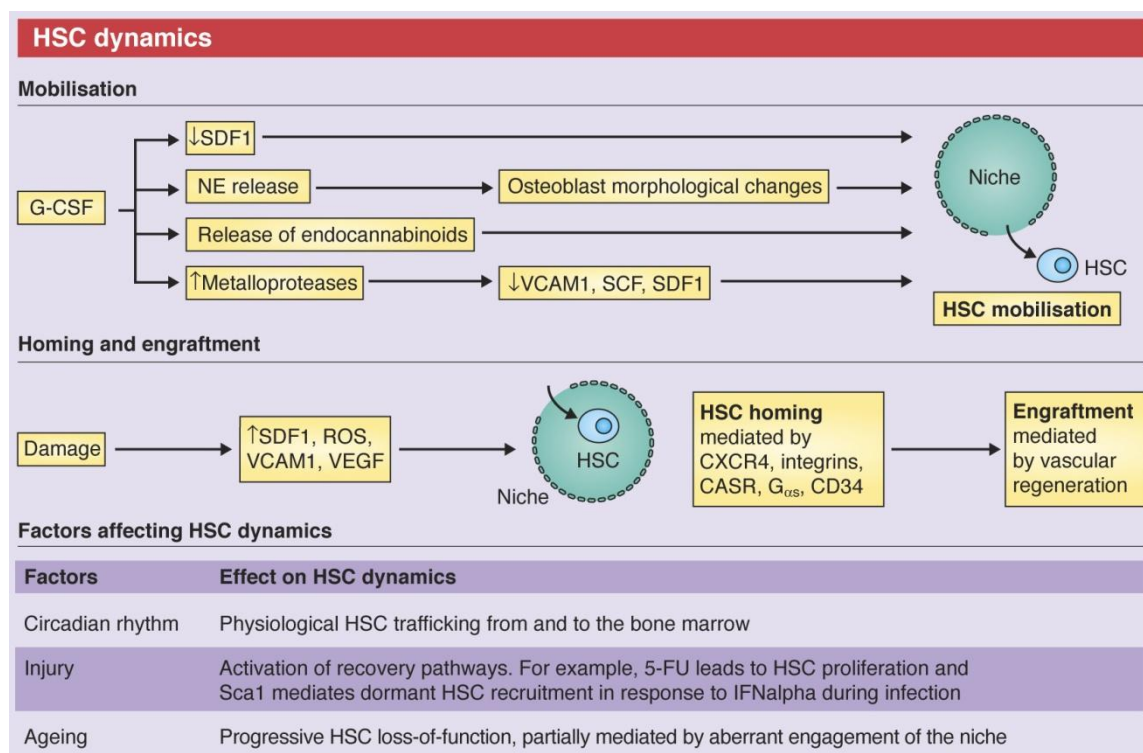


Figure 1.6. The dynamics controlling HSC mobilisation from and homing and engraftment to the bone marrow. HSC movement to and from the bone marrow is controlled by local factors such as cytokines, ligands, proteases and osteoblastic cells, the sympathetic nervous system and the requirement to regenerate. HSC dynamics are affected by physiological factors such as circadian rhythms, injury and ageing. Image reproduced with permission Lo Celso and Scadden J Cell Sci 2011;124:3529-3535 (Lo Celso and Scadden, 2011).

Although the preference for long-term repopulating HSCs to localise in close proximity to the endosteum has been demonstrated using Hoescht 33342 perfusion (Lo Celso et al., 2009; Winkler et al., 2010), fluorescent cell labelling (Ellis et al., 2011) and *ex vivo* and live imaging (Bengtsson et al., 2011; Lo Celso et al., 2009; Xie et al.,

2009) in mice, distinct niches have not been attributed to this. Rather, a joint periendosteal-perivascular niche has been suggested (Lo Celso et al., 2009) where the contribution of locally secreted niche-factors and the amount of blood perfusion enriches for stem cell function (Winkler et al., 2010). A recent review by Bianco has suggested that the mesenchymal/osteoprogenitors require their own niche in order to self-renew which is offered by the sinusoids, and as such, a symbiotic relationship exists between the niche cells (both skeletal and haemopoietic) and the sinusoids (Figure 1.7) (Bianco, 2011b).

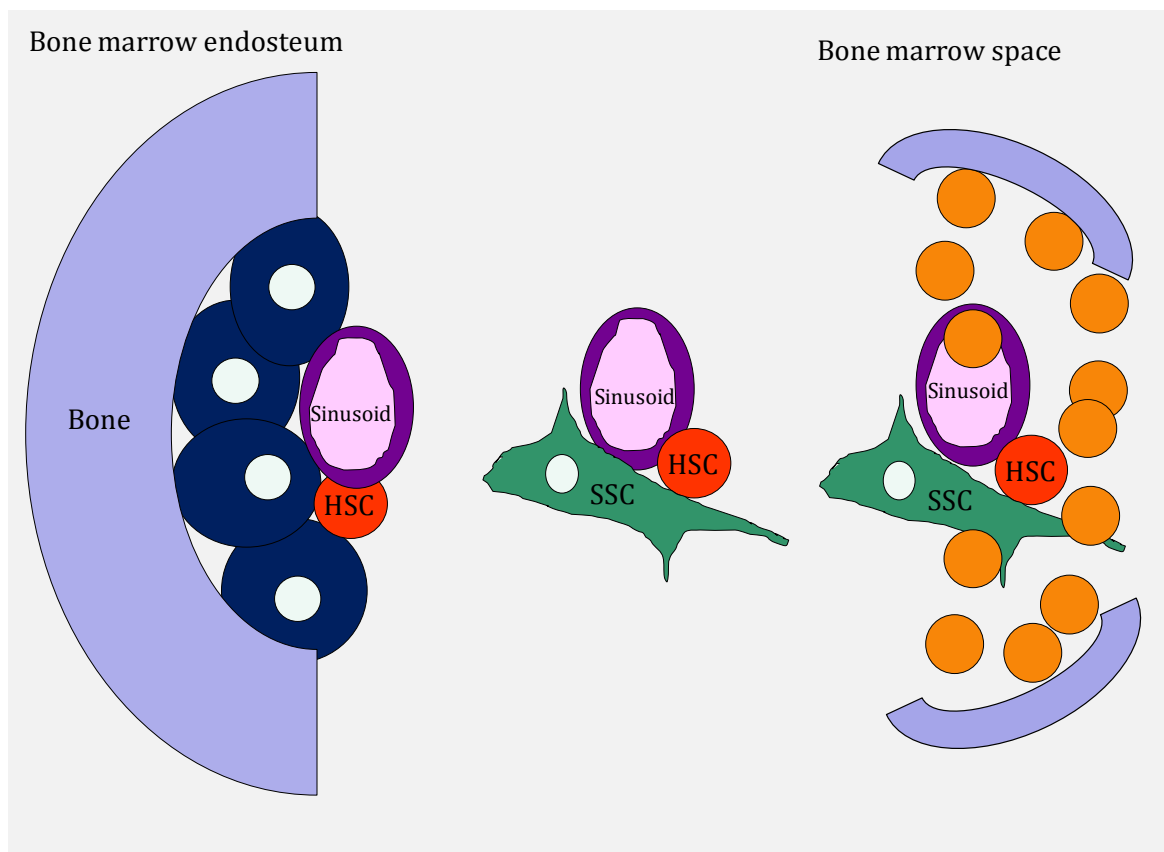


Figure 1.7. The dual niche within the bone marrow. One of the recent views of this combined niche is that mesenchymal/skeletal stem cells (SSC) and HSCs share a sinusoidal niche. For the dormant HSCs, this is next to the bone (endosteum). For HSC expansion, this occurs in the bone marrow space along with expansion of skeletal stem cell progeny. HSC proliferation/quiescence and bone formation/resorption occur as a balancing act so that haemopoiesis subtracts bone and fills marrow space whereas SSC differentiation into adipocytes and bone limits haemopoiesis by subtracting available space. Image adapted from Bianco P *Endocrinology* 2011;152:2957-2962 (Bianco, 2011b).

1.1.2.2 The spleen as a site for haemopoiesis

The spleen is a site for extra-medullary haemopoiesis in mice, although its existence is not essential for haemopoiesis (reviewed in (O'Neill et al., 2011)). Following intravenous injection of murine HSPCs into mice, the cells are rapidly cleared from the blood stream and home to other sites of extramedullary haemopoiesis (liver and spleen) (Wright et al., 2001). Cycling HSPCs (from mobilised peripheral blood (MPB)) have been seen in the spleen and liver within 1 minute of i.v. infusion into mice (1-2 circulations) (Wright et al., 2001), whereas HSPCs have been visualised in the bone marrow of mice within 14-20 minutes of injection (Ellis et al., 2011; Lo Celso et al., 2009). However, it is only the bone marrow and spleen that retain HSPCs beyond 48 hours (Papayannopoulou, 2003) and most of the cells eventually reside in the bone marrow for long-term repopulation in mice (Kahn et al., 2004; Kollet et al., 2001; Peled et al., 1999a).

However, small numbers of LTR-HSCs have been shown to reside in the murine spleen, sufficient to provide lymphoid, myeloid and dendritic repopulation (Wolber et al., 2002; Tan and O'Neill, 2010). Therefore, as well as being a secondary lymphoid organ (reviewed by (Brendolan et al., 2007)), the murine spleen can also support the development of myeloid cells (reviewed by (Tan and O'Neill, 2010)). Although the reconstitution potential between murine BM and spleen derived HSCs has been shown not to differ, spleen HSCs cycled twice as often as BM HSCs (Morita et al., 2011). Indeed, early studies showed that mice engrafted with murine spleen cells produced circulating leucocytes faster than BM recovered cells (Szilvassy et al., 1999). These spleen-derived HSPCs are thought to be more committed myeloerythroid progenitors which can produce novel dendritic-like cells (reviewed

in (O'Neill et al., 2011)), and which have been shown to provide the radio-protective platelets required to rescue lethally irradiated mice through the critical period of bone marrow failure (Na Nakorn et al., 2002). Less is known, however, regarding the role of the human spleen in extramedullary haemopoiesis, but HSCs have been detected in human spleen (Dor et al., 2006).

1.1.3 Chemokines and receptors important in homing/engraftment

Chemokines are low molecular weight chemotactic cytokines that mediate the induction of directional cell migration through binding to type 1 G-protein coupled chemokine signalling receptors (Dar et al., 2006). This is true for the typical receptors only as the atypical chemokine receptors do not engage in G-protein coupled receptor (GPCR) mediated signalling when bound to chemokines. These chemokine receptors are sub-grouped into 4 classes based on which type of chemokines they bind (CC, CXC, CX₃C, XC) (Broxmeyer, 2008). The known relationships between chemokines and receptors are shown in Table 1.1 (Schall and Proudfoot, 2011).

1.1.3.1 CXCR4 and its cognate ligand CXCL12

In the haemopoietic system, much work has focused on CXCL12 (stromal cell-derived factor 1) which was cloned in its α form (most predominantly expressed) and β form in 1993 (Tashiro et al., 1993). CXCL12, which is expressed on bone marrow endothelial cells (Imai et al., 1999; Peled et al., 1999a), at high levels on reticular cells in the bone marrow (Sugiyama et al., 2006) and osteoblasts (reviewed by (Sharma et al., 2011)), exclusively interacts with CXCR4 and CXCR7 (Table 1.1). CXCL12 has been shown to be upregulated in the bone marrow by stressors including hypoxia, DNA-

damaging chemotherapy agents and G-CSF, to restore haemopoietic homeostasis [Ceradini et al., 2004; Demaison et al., 2002; Dar et al., 2005; Xie et al., 2009].

Table 1.1. Chemokine and chemokine receptor interactions.

Chemokine ligand (alternative name)	Chemokine receptor												Atypical		
	CC-family														
	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	CCR12	DARC	D6	CCX CKR	
CC-family															
CCL1 (I-309)								●							
CCL2 (MCP1)		●	●									●	●		
CCL3 (MIP1α)	●				●								●		
CCL3L1 (LD78)	●				●										
CCL4 (MIP1β)	●				●								●		
CCL5 (RANTES)	●		●		●							●	●		
Mouse CCL6 (C10)	◆												●		
CCL7 (MCP3)	●	●	●		●							●	●		
CCL8 (MCP2)		●	●									●	●		
Mouse CCL9 (MIP1γ)	◆														
CCL11 (Eotaxin)		●	●		●							●	●		
Mouse CCL12 (MCP5)		●													
CCL13 (MCP4)		●	●		●							●	●		
CCL14 (HCC1)	◆														
CCL15 (HCC2)	◆		●									●			
CCL16 (HCC4)	◆														
CCL17 (TARC)				●								●	●		
CCL18 (PARC)			●									●			
CCL19 (MIP3β)							●							●	
CCL20 (MIP3α)						●									
CCL21 (SLC)							●							●	
CCL22 (MDC)				●								●	●		
CCL23 (MPIF1)	◆									◆					
CCL24 (Eotaxin 2)			●												
CCL25 (TECK)									●					●	
CCL26 (Eotaxin 3)			●												
CCL27 (CTACK)										●					
CCL28 (MEC)			●							●					
CXC-family															
CXCL11 (I-TAC)			●												

Table 1.1. Chemokine and chemokine receptor interactions (continued).

Chemokine ligand (alternative name)	Chemokine receptor									
	CXC-family							XC-family XCR1	CX ₃ C-family CX ₃ CR1	Atypical DARC
	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CXCR7			
CXC-family										
CXCL1 (GRO α)		●								●
CXCL2 (GRO β)		●								●
CXCL3 (GRO γ)		●								●
CXCL4 (PF4)										
CXCL5 (ENA78)		●								●
CXCL6 (GCP2)	●	●								●
CXCL7 (NAP2)		●								●
CXCL8 (IL-8)	●	●								●
CXCL9 (MIG)			●							
CXCL10 (IP10)			●							
CXCL11 (I-TAC)			●				●			●
CXCL12 (SDF1)				●			●			
CXCL13 (BCA1)					●					
CXCL14 (BRAK)										
Mouse CXCL15 (Lungkine)										
CXCL16						●				
CC-family										
CCL11 (Eotaxin)			●							
XC-family										
XCL1 (Lymphotactin)								●		
XCL2 (SCM1 α)								●		
CX ₃ C-family										
CX ₃ CL1 (Fractalkine)									●	

- | | |
|-------------------------|---|
| ● High affinity agonist | ● Antagonist |
| ● Low affinity agonist | ◆ High affinity agonist activated from low affinity precursor |

Known relationships between the listed chemokines and chemokine receptors. Atypical receptors bind chemokines but do not engage G-proteins. Image reproduced from Proudfoot and Schall, Nat Rev Immunol 2011;11,355-363 (Schall and Proudfoot, 2011).

CXCL12 was initially identified as having a chemotactic effect on human peripheral blood lymphocytes and monocytes *in vitro*, and mononuclear cells *in vivo* (Bleul et al., 1996) and HSPCs (Aiuti et al., 1997). CXCL12 was also shown to be the most potent chemokine for inducing actin polymerisation in lymphocytes. Bleul *et al.* also demonstrated using pertussis toxin that the receptor required for chemotaxis to CXCL12 was a G_o-protein coupled receptor (CXCR4) (Bleul et al., 1996). CXCR4 is expressed on the cell surface of most leucocyte populations, including HSPCs (Deichmann et al., 1997). It is also found on bone marrow endothelium and mesenchymal stromal/reticular cells where it has a maintenance/binding function and can internalise and re-secrete functional CXCL12 (Dar et al., 2005; Kortessidis et al., 2005; Sugiyama et al., 2006). Early *in vivo* studies showed that CXCL12 deficient mice had a deficit of B-cell progenitors and were defective for B lymphopoiesis and myelopoiesis in the bone marrow, whilst CXCR4^{-/-} mice have a similar phenotype (Nagasawa et al., 1996; Zou et al., 1998).

The structure of CXCL12 and CXCR4 are highly conserved amongst species, with murine and human CXCL12 being cross reactive (Dar et al., 2006). CXCL12 has been shown to be a survival factor for mouse and human HSPCs (Broxmeyer et al., 1999; Lataillade et al., 2000) and was shown to enhance engraftment of scid repopulating cells (SRCs) (Broxmeyer et al., 2003a; Broxmeyer et al., 2003b). CXCL12 binding to CXCR4 activates the coupled G protein, triggering G_i which signals for motility, polarisation, adhesion and possibly proliferation (Hartmann et al., 2008) (reviewed by (Sharma et al., 2011)) (Figure 1.8). CXCR4 has been shown to regulate migration and survival of HSPCs (reviewed by (Sharma et al., 2011)). CXCR4 is harboured intracellularly and its expression at the surface of HSPCs is dynamic and has been

shown to fluctuate in response to culture conditions and CXCL12 presentation (Kollet et al., 2002). The formation of a CXCL12 chemokine concentration gradient drives CXCR4-mediated cell migration and has been shown *in vivo* (Jung et al., 2006).

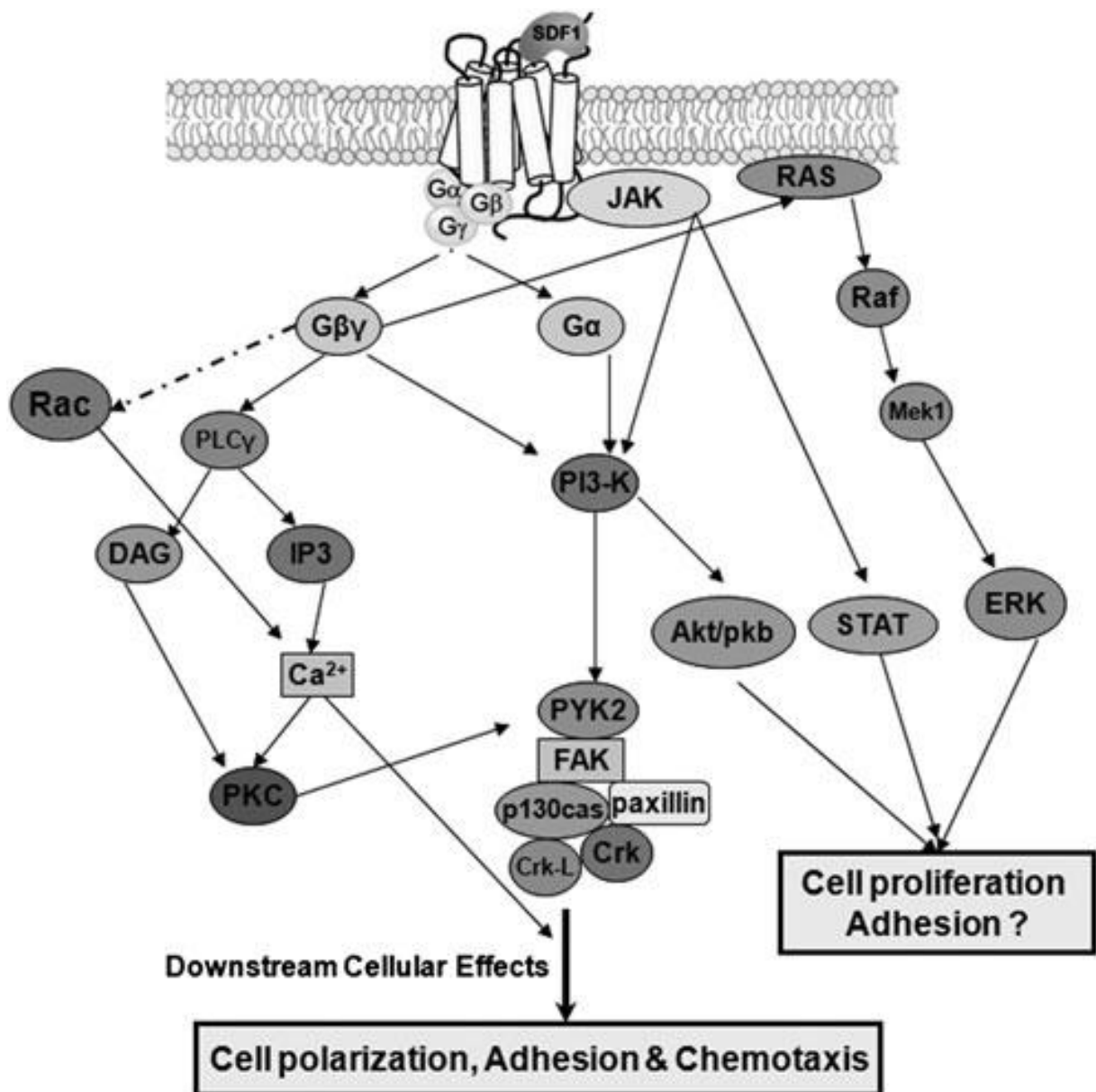


Figure 1.8. Signalling cascade downstream from CXCL12 binding the G-protein coupled receptor (GPCR) CXCR4. CXCL12 binding to CXCR4 triggers Gi protein mediated activation of PI3K/Akt, PKA, PKC, RAS/MAPK and Gi-independent events (JAK/STAT). The downstream cellular effects for HSPCs are adhesion, polarisation and chemotaxis and controversially, proliferation. Image reproduced with permission from Sharma *et al.* Stem cells and Development 2011;20,6 (Sharma et al., 2011).

The bone marrow stromal cell populations produce extracellular matrix components such as fibronectin and heparan sulphate (Balduino et al., 2005). The binding of CXCL12 to this matrix provides a platform for CXCL12 gradient formation *in vitro*, allowing presentation to its chemokine receptor on HSPCs (Forde et al., 2007).

AMD3100, a CXCR4 antagonist, was shown to exclusively inhibit CXCL12-stimulated CXCR4-mediated intracellular signalling and chemotaxis, and not other chemokine receptors tested (Hatse et al., 2002). The specificity of AMD3100 for CXCR4 alone is due to high affinity interactions between an aspartate residue at each end of the ligand binding crevice of CXCR4 (Figure 1.9), which is also highly conserved amongst species (Hatse et al., 2002).

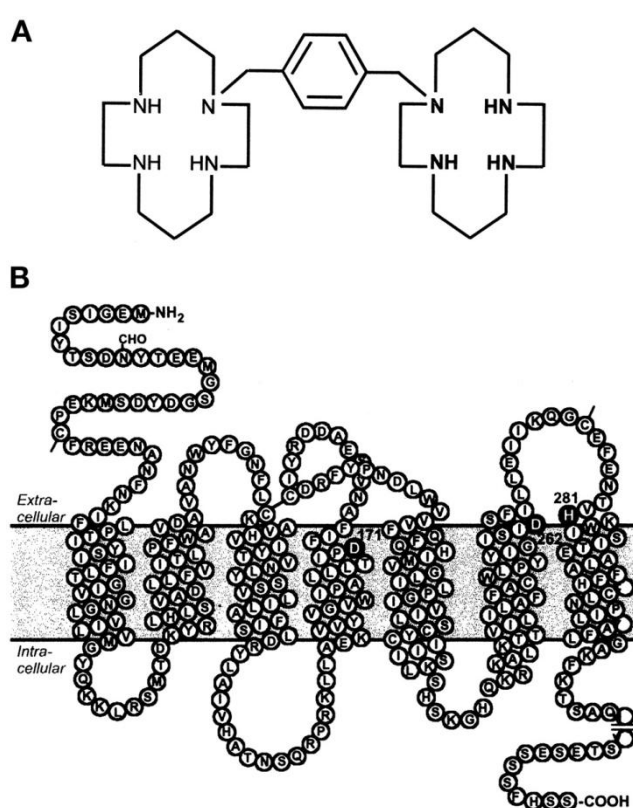


Figure 1.9. Structure of CXCR4 and its antagonist AMD3100. A) Chemical structure of AMD3100, 1,1'-[1,4-phenylenebis(methylene)]-bis-1,4,8,11-azatetradecane. B) Amino acid sequence of the seven transmembrane receptor CXCR4. Figure reproduced with permission from Hatse *et al.* FEBS Letters 2002;527,255-262 (Hatse et al., 2002).

1.1.3.2 CXCR7 may affect the efficiency of CXCR4-CXCL12 interaction

CXCR7 has been identified as a receptor for CXCL12 as well as for CXCL11, albeit with lower affinity (Balabanian et al., 2005; Burns et al., 2006). Ligand binding to CXCR7 does not stimulate GTP γ S binding to G proteins and therefore CXCR7 is now accepted by the field as an atypical chemokine transmembrane receptor (reviewed in (Graham et al., 2012)). Hartmann *et al.* reported that although CXCL12 interaction with CXCR7 alone did not trigger motility in CD34⁺ cells, blocking CXCR7 did reduce the efficiency of the response (Hartmann et al., 2008). This is because CXCR7 does internalise its cognate ligands and targets them for degradation, therefore acting as a scavenger to regulate the CXCR4-CXCL12 interaction (Naumann et al., 2010). CXCL11 binding to CXCR7 can be out competed when CXCL12 that is not bound to CXCR4 requires internalisation to mediate the CXCR4-CXCL12 axis (Berahovich et al., 2010). CXCR7 knock-out mice show lethal cardiac defects (Sierro et al., 2007) and its expression has been found on activated HUVECs (Burns et al., 2006) and B cells (Sierro et al., 2007). However, the expression of CXCR7 on HSPCs has not been confirmed (Berahovich et al., 2010). Indeed, Tarnowski *et al.* found minimal CXCR7 expression on normal human CD34⁺ cells from umbilical cord blood (UCB), MPB or bone marrow, but high expression on several human myeloid cell lines (Tarnowski et al., 2010). Additionally, CXCR7 binding to CXCL12 cannot be inhibited by AMD3100, the CXCR4 antagonist (Naumann et al., 2010; Berahovich et al., 2010).

1.1.3.3 The role of CXCR4 and CXCL12 in HSPC homing/engraftment

CXCL12 is required to retain HSCs in the niche by interaction with CXCR4, and equally can be reversed to maintain haemopoietic homeostasis (Hartmann et al., 2008; Sugiyama et al., 2006). A review of recent evidence suggests that CXCL12 is required

to maintain LTR-HSCs at the endosteum (Itkin and Lapidot, 2011). CXCR4-CXCL12 binding has been shown to be required for HSC quiescence (Nie et al., 2008) as CXCL12 loss in adult mice increases the number of cycling HSCs (Tzeng et al., 2011). Schajnovitz *et al.* have recently shown that CXCL12 secretion from reticular bone marrow MSCs, which is dependent on cell-cell contact and communication via connexion gap junctions, was required for HSPC contact with BMSCs and HSC pool maintenance (Schajnovitz et al., 2011). Petit *et al.* provided earlier evidence for this retention interaction between CXCR4 and CXCL12 as the mobilising agent G-CSF decreased bone marrow CXCL12 and increased CXCR4 (Petit et al., 2002). The requirement for CXCR4-CXCL12 in HSPC engraftment to the BM of NOD-scid mice was also shown by early studies blocking CXCL12 and/or CXCR4 (Kollet et al., 2001; Peled et al., 2000; Peled et al., 1999b). CXCR4 may require additional co-receptors, such as the transmembrane receptor Robo4, amongst other receptors. Robo4 has been shown to be required for HSC retention and therefore long term repopulating ability in the bone marrow (Smith-Berdan et al., 2011).

Although the role of CXCR4 in localisation of HSCs in the bone marrow is widely accepted (Lo Celso and Scadden, 2011), the role of CXCR4-CXCL12 in immediate (less than 16 hour) homing to the murine bone marrow may not be as necessary as its role in homing to the spleen in mice (Kahn et al., 2004). Indeed, early studies found that bone marrow homing of mouse HSPCs was not blocked by pertussis toxin, which blocks G α i-signalling (Wiesmann and Spangrude, 1999). However, in human HSPCs, blocking CXCR4 with neutralising antibodies and pertussis toxin was later shown to reduce homing to murine bone marrow and spleen (Kollet et al., 2001).

1.1.3.4 Integrins, factors and downstream signalling also involved in HSPC homing and engraftment

HSPC homing, engraftment and retention of HSPCs to bone marrow also requires co-operation from other molecules. These include the integrins, selectins and sialomucin families, and also growth factors such as stem cell factor (SCF) (reviewed in (Ratajczak et al., 2012)). The integrins are composed of α and β heterodimer subunits (Humphries et al., 2006). Human UCB CD34⁺ HSPCs have been reported to express $\alpha_4\beta_1$ (VLA-4), $\alpha_5\beta_1$ (VLA-5), α_6 , α_7 , α_9 and β_2 integrin subunits. They also express CD11a (LFA-1), CD54 (ICAM-1), ICAM-3, CD58 (LFA-3) and CD44, which binds hyaluronic acid (Lee-Thedieck et al., 2012; Schreiber et al., 2009; Watt and Forde, 2008; Zheng et al., 2003). The HCELL (haemopoietic cell E-/L-selectin ligand) glycoform of CD44 has been shown to activate VLA-4 G-protein dependent signalling, triggering chemokine independent HSPC migration and extravascular lodgement (reviewed by (Sackstein, 2011)). Other glycoproteins including the pan-leucocyte marker CD45, a phosphatase, have been shown to play a key role in HSPC homing and repopulation of the murine bone marrow (Shivtiel et al., 2011).

CXCR4-CXCL12 signalling also activates the integrins VLA-4, VLA-5 and LFA-1 (reviewed by (Sharma et al., 2011)). The effect of CXCL12 stimulation has been shown to induce the transition of VLA-4 from a low to high affinity state for adhesion to VCAM-1 (Foguenne et al., 2009). Early studies showed that the integrins which bind to the bone marrow endothelial/mesenchymal stromal cell expressed ligands ICAM-1 (LFA-1), VCAM-1 (VLA-4) (Imai et al., 1999; Peled et al., 1999a) and VLA-5 (which binds fibronectin along with VLA-4) (Craddock et al., 1997) were shown to be crucial for the homing and engraftment of CD34⁺ cells in NOD-scid mice (Peled et al.,

2000; Kollet et al., 2001). Later, a role for SCF was described, as 24 hour culture of MPB HSPCs was shown to increase surface expression of VLA-4 and VLA-5 and increase engraftment in NOD-scid mice (Hart et al., 2004). In 2007, Forde *et al.* showed that CXCR4, VLA-4 and VLA-5 co-immunoprecipitated with the sialomucin CD164 in response to CXCL12 presented on fibronectin, demonstrating that these pathways co-operate in human UCB CD133⁺ cells (Forde et al., 2007). This was shown to activate PKC- ζ and Akt signalling (Forde et al., 2007). The downstream signalling pathways from these molecules are important for HSPC function but are not clearly defined. Protein kinase C (PKC) inhibition has been shown to block CD34⁺ cell homing (Kollet et al., 2001) and more recently, protein kinase B (PKB) inhibition was shown to increase human CD34⁺ cell migration, homing and 6 week engraftment (Buitenhuis et al., 2010).

Proteases, including matrix-metalloproteinases (MMPs) and the CXCL12 protease CD26, complement proteins and extracellular matrix components such as hyaluronic acid, also play a role in the homing (and mobilisation) process to and from the bone marrow (reviewed by (Marquez-Curtis et al., 2011)).

1.1.4 The CXCR4-CXCL12 axis and the sialomucin CD164

The sialomucins are a family of transmembrane glycoproteins, the function of which depends on their state of O-glycosylation. The sialomucins, which include CD34, PSGL-1, podocalyxin and CD164, are expressed on HSPCs and vasculature, amongst other tissues, and have a role in haemopoiesis (Furness and McNagny, 2006). For example, CD34 has been suggested to block cell adhesion and enhance migration in HSPC trafficking, whilst podocalyxin correlates with migration and haemopoietic

tissue seeding (Nielsen and McNagny, 2009b; Nielsen and McNagny, 2009a). CD164, a homodimeric type I integral transmembrane sialomucin adhesion receptor that is expressed intracellularly and at the cell surface (Bae et al., 2008; Zannettino et al., 1998; Chan et al., 2001), has also been shown to play a role in human UCB CD133⁺ cell migration to CXCL12 *in vitro* (Forde et al., 2007).

CD164 has two mucin domains (mucin I and II) in its extracellular region (Figure 1.10) (Chan et al., 2001). The class I (105.A5) and class II (103B2) monoclonal antibodies recognise different glycosylated epitopes which have restricted expression on different cell types (Zannettino et al., 1998; Jorgensen-Tye et al., 2005). The class II epitope, 103B2, is highly expressed on the long term re-populating Lin⁻CD133⁺CD34⁺CD38^{lo/-} and Lin⁻CD133⁺CD34⁻CD38^{lo/-} cells from fetal liver, adult bone marrow and UCB cells, erythromyeloid and associated endothelial and stromal cells, monocytes and osteoblasts (Watt et al., 1998; Watt et al., 2000; Zannettino et al., 1998). The class III monoclonal antibody, N6B6, can be used to identify all isoforms of CD164 as it recognises a ubiquitously expressed epitope on the molecule backbone (Watt et al., 1998).

Initial functional studies by Zannettino *et al.* demonstrated that blocking CD164 with the 103B2 antibody reduced the adhesion of human CD34⁺ HSPCs cells to bone marrow MSC *in vitro* and blocked the entry of these cells into cycle (Zannettino et al., 1998). CD164 has been shown to interact with CXCR4 on CD133⁺ HSPCs cells from UCB following exposure to CXCL12 presented on fibronectin *in vitro* (Forde et al., 2007). Forde *et al.* demonstrated that transient blockade of CD164, using either the monoclonal antibody 103B2 or siRNA, reduced the migration of CD133⁺ HSPCs to CXCL12 presented on fibronectin *in vitro* and reduced the activation of CXCR4

downstream signalling pathways (Forde et al., 2007). Further evidence for the co-operation of CD164 in migration and adhesion of HSPCs is provided by Wagner *et al.* (Wagner et al., 2007). Here, adhesion assays using gravitational force demonstrated that CD34⁺ cells that adhered to a layer of human MSC had upregulated expression of genes including CD164, compared to the non-adherent fraction (Wagner et al., 2007). More recently, CD164 knockdown was shown to reduce proliferation and metastasis *in vitro* in the colon cancer cell line, HCT116 (Tang et al., 2012).

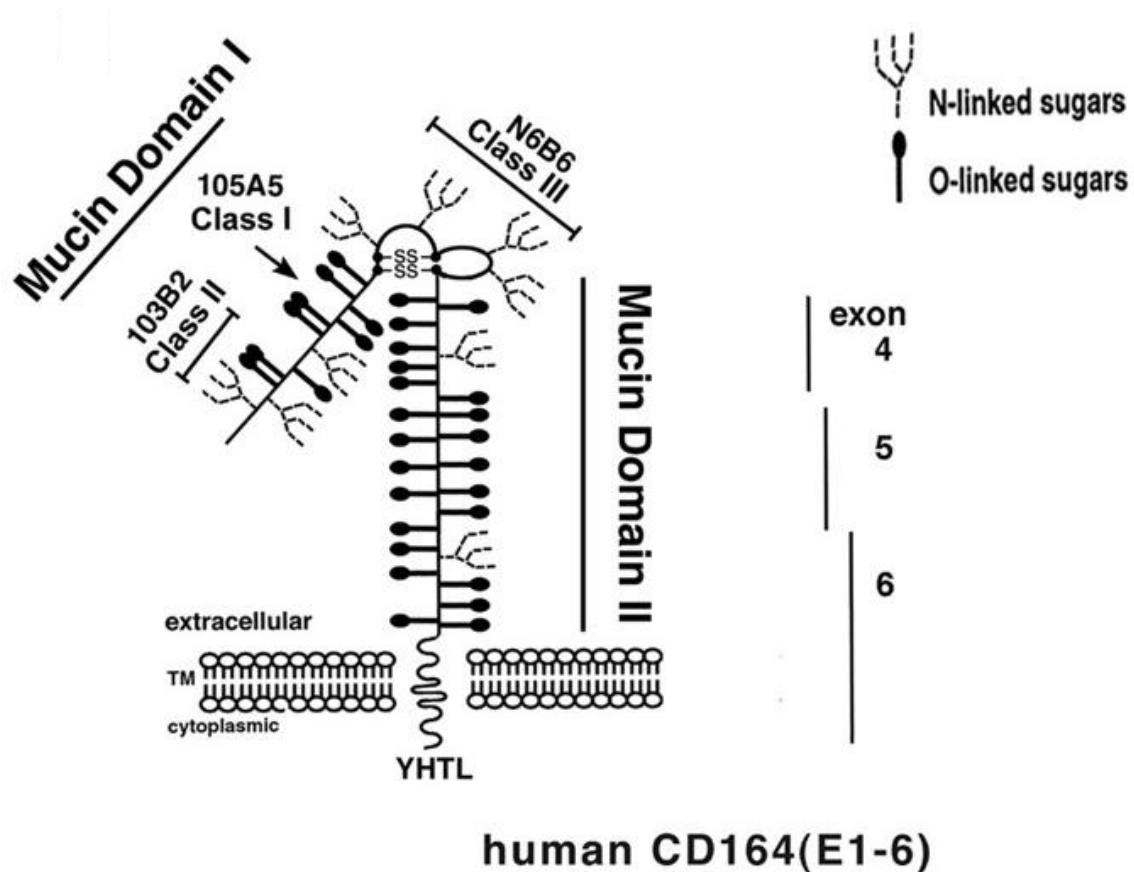


Figure 1.10 Structure of human CD164. Structure showing exons 1-6 of CD164. The glycosylation pattern and antibody class I-III binding sites are also shown. The transmembrane molecule has a cytoplasmic tail containing a YHTL motif. Image reproduced with permission from Chan *et al.* J Biol Chem 2001;276,2139-2152 (Chan et al., 2001).

1.1.5 Cell migration and chemotaxis

Signal transduction and chemotaxis in eukaryotic organisms has been widely studied in *Dictyostelium discoideum*, especially towards cAMP (Shaffer, 1975; Ueda et al., 2001; Shaffer, 1975). Chemotaxis towards a cAMP gradient is G-protein mediated. PIP₂ is phosphorylated to PIP₃ by PI3K at the leading edge of the migrating cell and degraded to PIP₂ by PTEN (phosphatase and tensin homologue deleted on chromosome 10) at the rear (Figure 1.11). For chemotaxis to be demonstrated, a gradient of the stimuli must be present, otherwise the response measured is chemokinesis (random cell movement).

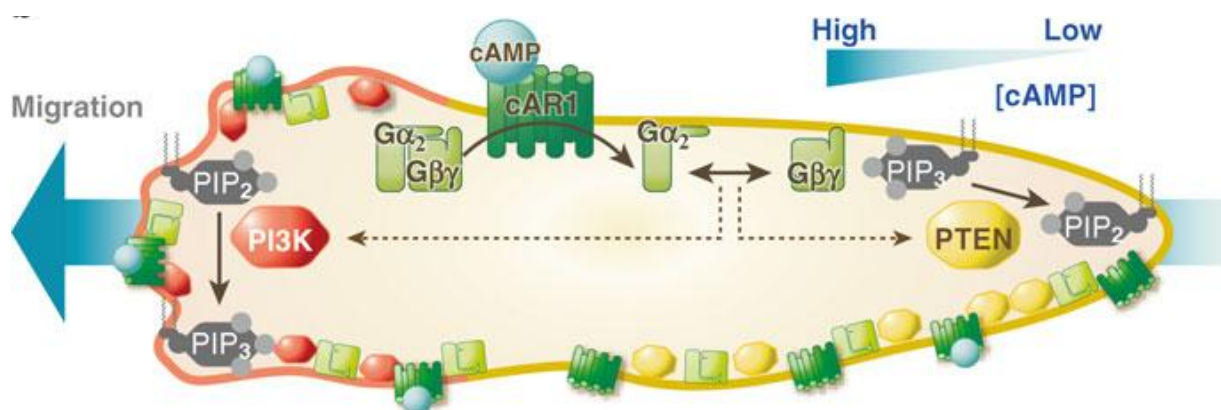


Figure 1.11. Chemotactic signalling of *Dictyostelium discoideum* towards cAMP. In response to a cAMP gradient, PIP₂ phosphorylation at the leading edge and PIP₃ degradation at the rear of the cell enables chemotactic migration in *Dictyostelium discoideum*. Image reproduced with permission from Miyana, Y. and Ueda, M. (2011) from “Single-Molecule Kinetic Analysis of Stochastic Signal Transduction Mediated by G-Protein Coupled Chemoattractant Receptors” in Cell Signaling Reactions p35-37 (Miyana and Ueda, 2011).

1.1.5.1 Conventional and evolving assays for studying migration in vitro

Although the migration of mammalian cells has been widely studied, the assays in use such as the transwell assay, do not easily distinguish chemotaxis and chemokinesis

(Entschladen et al., 2005), although checkerboard transwell assays have been used (Kim and Broxmeyer, 1998). Additionally, CD34⁺ cells have shown a maximal chemotactic response to CXCL12 in the transwell assay at 4 hours (Kim and Broxmeyer, 1998). However, *in vivo* imaging of infused HSPCs in mice has shown that cells appeared in the BM sinusoids 15 minutes post infusion, but that transendothelial migration into the extravascular space continued to the final 15 hour timepoint (Ellis et al., 2011). The need for assays that observe cell migration and enable the effect of inhibitors on these aspects of migration to be studied for example, as well as providing stable gradients for periods greater than 4 hours, has been recognised (Toetsch et al., 2009).

The evolution of chemotaxis assays from basic chambers to more physiologically relevant assays has been reviewed by Toetsch *et al.* (Toetsch et al., 2009), where they described the establishment of several assays utilising microscopy and tracking software rather than counting methods. The use of hydrogels as matrices, which more closely resemble the biological environment, and allow the diffusion of chemoattractant solutions, has also been advocated. The importance of assays that enable the migratory response to be studied in 3D environments that more closely resemble the extra-cellular components *in vivo* was demonstrated in CD34⁺ cells by Peled *et al.* (Peled et al., 2000) and in other cell types such as dendritic cells (Renkawitz et al., 2009). Peled *et al.* determined that CD34⁺ cell polarization and migration was shown to be dependent on VLA-4 and VLA-5 in 3D collagen-laminin-fibronectin gels, whereas adhesion to 2D fibronectin was mediated by VLA-5 but not VLA-4 (Peled et al., 2000). These integrins have been shown to have important, and distinct roles *in vivo*, and therefore the use of assays to represent different

extracellular environments are important when studying cell function *in vitro*. Toetsch *et al.* have also described microfluidic systems which have been developed to more closely resemble leucocyte migration using shear flow techniques (Toetsch *et al.*, 2009).

1.1.6 Transplantation

HSPCs can be sourced from UCB, MPB or bone marrow (Watt and Forde, 2008). As the bone marrow is the major site for HSC and hence blood cell production, severe malignant and non-malignant disorders of the haemopoietic system require replacement of the HSCs (Table 1.2) if the patient is to restore normal haemopoiesis (Li and Sykes, 2012; Liao *et al.*, 2011).

1.1.6.1 Umbilical cord blood as a source of HSPCs

Allogeneic UCB stem cell transplantation can be used for both unrelated and related transplants in patients with a variety of haematological malignancies and disorders (Delaney *et al.*, 2010b; Gluckman E, 2009; Li and Sykes, 2012; Liao *et al.*, 2011). In 1988, the transplantation of a single unit of UCB from an HLA-identical sibling resulted in complete long-term haematological and immunological reconstitution of the recipient with Fanconi's anaemia (Gluckman *et al.*, 1989). Since 1993, unrelated UCB transplants have been performed, and more recently, have been extended to adults, where an HLA-matched BM donor is not available and where the number of HSPCs is sufficient (Smith and Wagner, 2009; Gluckman *et al.*, 2011). Over 25, 000 UCB stem cell transplants have been performed worldwide (Kurtzberg, 2011).

The advantages of UCB HSC transplants over conventional adult sources of HSPCs (BM or MPB) are primarily the ease of procurement and availability of units, lack of attrition of donors, the reduced risk of graft versus host disease (GvHD) in recipients, and a greater tolerance for HLA mismatching between donor and recipient (Gluckman E, 2009; Gluckman, 2011; Gluckman et al., 2011; Li and Sykes, 2012; Liao et al., 2011; Gluckman E, 2009; Gluckman, 2011; Rocha et al., 2000).

Table 1.2. Indications for haemopoietic cell transplantation.

Clinical category	Clinical subcategory	Examples of disease or clinical setting	Type of haemopoietic stem cell transplantation (HCT)
Malignant tumours	Haematological malignancies	Leukaemias, lymphomas, multiple myeloma	Autologous or allogeneic HCT
	Solid tumours	Metastatic melanoma	Autologous HCT combined with adoptive cell therapy
		Metastatic renal cell carcinoma	Allogeneic HCT
Genetic disease	Immunodeficiency diseases	RAG2-deficient SCID	Allogeneic HCT
	Haemoglobinopathies	Sickle cell disease	Allogeneic HCT
Solid organ transplantation	Allogeneic solid organ transplantation	Kidney transplantation	Combined Allogeneic HCT and solid organ transplantation (experimental)
	Xenogeneic solid organ transplantation	Kidney transplantation	Combined xenogeneic HCT and solid organ transplantation (experimental)
Autoimmune disease	-		Autologous or allogeneic HCT

Clinical conditions which are treated by autologous (own cells) and allogeneic (donor cells) haemopoietic cell replacement are listed. Table adapted with permission from Li and Sykes Nat Rev Immunol 2012;12 (Li and Sykes, 2012).

Acute GvHD is the main source of morbidity and mortality in MPB transplanted patients and is caused by donor T cells, however T cell depletion is associated with

infections and relapse risk (Chaidos et al., 2012). Recently, higher doses of invariant natural killer cells in MPB grafts have been associated with acute GvHD protection (Chaidos et al., 2012). The reduction of GvHD with UCB is thought to be due to the immunological immaturity of UCB T and dendritic cells and the enrichment of T-reg cells and so unrelated and HLA-mismatched UCB transplants can be performed (Gluckman et al., 2011; Li and Sykes, 2012).

Leukaemia free survival has been shown to be similar for all three sources of stem cells (Gluckman E, 2009; Gluckman et al., 2011). However, early engraftment (granulocyte and platelet recovery) is slower with UCB grafts even when comparing HLA-matched UCB to HLA-matched BM sources, exposing the patient to infection and increasing the likelihood of graft failure (Gluckman et al., 2011; Li and Sykes, 2012). A number of studies have shown that the incidence of graft failure is highly negatively correlated with the total nucleated cell (TNC) count and CD34⁺ cell dose in UCB units (reviewed in (Delaney et al., 2010b)). Kurtzberg *et al.* have shown that $>5 \times 10^7$ TNC/kg improved the speed of neutrophil and platelet recovery whereas $<2.5 \times 10^7$ TNC/kg was associated with decreased survival (Kurtzberg et al., 2008). Barker *et al.* showed that similar survival could be achieved using $>5 \times 10^7$ TNC/kg where two HLA antigens were mismatched and 2.5×10^7 TNC/kg for one antigen mis-match (Barker et al., 2010). For >70 kg adults, less than 10% of single UCB units meet the requirement of 2.5×10^7 TNC/kg (Delaney et al., 2010b; Kurtzberg, 2011). Thus, research investigating UCB expansion, the use of 2 UCB units for transplantation, or both (Delaney et al., 2010a), have increased the use of UCB in adults. Double UCB unit transplantation has offered comparable results for heavier patients to that achieved with lighter recipients receiving one UCB unit (reviewed by (Petropoulou and Rocha,

2011)). However, only one unit will preferentially engraft the recipient (Kurtzberg, 2011).

1.1.7 Techniques to improve UCB engraftment

HSPC engraftment is measured short term by the time to neutrophil or platelet recovery, or longer term to haemopoietic reconstitution (Petropoulou and Rocha, 2011). In addition to double UCB unit transplants, techniques currently being studied to improve UCB engraftment include the use of reduced intensity conditioning and co-infusion with accessory cells such as haploidentical T cell depleted CD34⁺ cells or MSCs (reviewed by (Delaney et al., 2010b; Li and Sykes, 2012; Petropoulou and Rocha, 2011)). Early studies in mice showed that the co-injection of human endothelial cells, and co-transplantation of MSCs with human HSPCs improved engraftment (Chan et al., 2007; in 't Anker et al., 2003; Park et al., 2007; Salter et al., 2009).

Direct injection of cells into the bone has also been explored following studies showing greater engraftment in mice, but mixed results have been reported (reviewed by (Petropoulou and Rocha, 2011)). Frassoni *et al.* showed that intra-bone injection reduced delayed platelet recovery and the incidence and severity of GvHD seen with i.v. infusion (Frassoni et al., 2008). However, in a smaller study, intra-bone infusion of two UCB units did not offer an advantage over i.v. infusion of two UCB units (Brunstein et al., 2009). Finally, the *ex vivo* or *in vivo* modulation of UCB units by manipulation or expansion of the unit with drugs, factors or cytokines prior to transplantation has gained increasing interest (Chou et al., 2010; Delaney et al., 2010b; Petropoulou and Rocha, 2011).

Ex vivo modulation of UCB generally targets the CXCR4-CXCL12 axis. For example, inhibition of CD26 by Diprotin A can enhance HSPC engraftment due to inhibition of its proteolytic activity on CXCL12 (Campbell et al., 2007; Campbell and Broxmeyer, 2008; Christopherson et al., 2007). Other studies found that short term (<48 hour) culture with SCF was associated with increased CXCR4 expression (Peled et al., 1999b) and this improved the engraftment potential of UCB units into NOD-scid mice (Zheng et al., 2003; Zheng et al., 2005). Alternatively, post-transplant transient blockade of CXCR4 with AMD3100 has demonstrated increased overall survival and enhanced primary and secondary reconstitution in mice, although short term peripheral blood B cell counts were lower than non-treated mice (Kang et al., 2010).

Additionally, regulation with prostaglandin E2 has been investigated for enhancing HSC engraftment (reviewed by (Durand and Zon, 2010)). The regulation of haemopoietic homeostasis by prostaglandins was identified in 1983 (Estrov and Resnitzky, 1983). Recent studies have shown that a 2 hour incubation or pulsing with dmPGE2 increased CXCR4 gene and cell surface expression in CD34⁺ cells (Cutler et al., 2011; Goessling et al., 2011) and that this was associated with increased HSC survival (Hoggatt et al., 2009). Goessling *et al.* and Cutler *et al.* have also demonstrated that 1-2 hour *ex vivo* stimulation of human UCB CD133⁺ cells with 16, 16-dimethylprostaglandin E2 (FT1050) improved engraftment in a non-human primate model and in a Phase I clinical trial (Cutler et al., 2011; Goessling et al., 2011). This shows that the effects of manipulating UCB units on cell migration/homing/engraftment ability is a useful area for further investigation. These approaches to improve the capacity of the available cells to home to and engraft into the bone marrow offer an alternative that may negate the problems that

have been associated with the *ex vivo* expansion of repopulating cells (Larochelle et al., 2012).

Although culturing HSPCs *ex vivo* in cytokine cocktails stimulates proliferation of these cells, the LT-HSCs may be lost as the cells start cycling and differentiating, particularly as differentiated cells provide feedback (Csaszar et al., 2012; Kirouac et al., 2010). This effect impairs bone marrow *in vivo* repopulation ability in xenograft models (references in (Larochelle et al., 2012)) with as little as 24 hours *ex vivo* culture (Ahmed et al., 2004). Retroviral and lentiviral techniques to genetically manipulate HSPCs either require the cells to be cycling, or to be cultured in cytokines for efficient transduction respectively (references in (Larochelle et al., 2012)). Studies investigating the effect of 72 hour cytokine culture on HSPCs showed that homing and engraftment mechanics to murine bone marrow were affected in terms of decreasing the requirement for VLA-4 and CXCR4 and increasing the requirement for VLA-5 (Foguenne et al., 2009). The culture was suggested to increase VLA-4 affinity such that it was maximally expressed and did not respond to CXCL12, and therefore homing/engraftment was not specifically localised to CXCL12 expressing regions but to extramedullary sites expressing VCAM-1 (Foguenne et al., 2009). Recent studies have shown that disruption of a polarised membrane domain enriched with tetraspanins, CD133 and VLA-4, which is seen during increased cell cycling, could explain the loss of repopulating ability associated with *ex vivo* culture (Gillette et al., 2009; Larochelle et al., 2012). The disruption of the tetraspanin CD82 has been recently shown to decrease HSPC adhesion to stroma, homing and engraftment (Larochelle et al., 2012).

1.1.8 HSPC expansion to enhance engraftment

1.1.8.1 Defining human HSC repopulating ability in xenograft models

Various *in vivo* models have been established to estimate and improve either the number or function of long-term re-populating cells in the human graft. The gold standard for determining efficient HSPC homing to bone marrow niches is their subsequent haematological reconstitution following transplantation in mammals (humans, or as surrogates in immunodeficient mice or non-human primates) (Bhatia et al., 1997; Goessling et al., 2011; Larochelle et al., 1996; Larochelle et al., 2012). Humanised murine surrogate models are more often used due to costs, time and ethics (Doulatov et al., 2012). Historically, severe combined immune-deficient (scid) mice lacking T and B cells were found to support engraftment of human haemopoietic cells (reviewed by (Doulatov et al., 2012)). Later, this mouse strain was backcrossed with the nonobese diabetic mouse to generate NOD-scid mice with improved human engraftment but still with active NK cells (Figure 1.12) (reviewed by (Doulatov et al., 2012)). More recently, variations on this model have included the NOD/ShiLtSz-scid/IL2R γ null (NSG) mouse strain with deleted or truncated IL-2R common γ chain (NOD/ShiJic-scid/IL2R γ null) (NOG) or using anti-CD122 in NOD-scid mice which prevents signalling through IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 and therefore results in a complete loss of B, T and NK cells (McDermott et al., 2010; Doulatov et al., 2012). The NSG mice have shown improved (reported 1.5-5 fold) bone marrow engraftment of human cells compared to NOG mice or NOD-scid mice (McDermott et al., 2010; Doulatov et al., 2012). However, the majority of reconstitution seen with human cells is of the lymphoid rather than erythro-myeloid lineages, and as such is still sub-optimal for modelling successful bone marrow reconstitution (Figure 1.12).

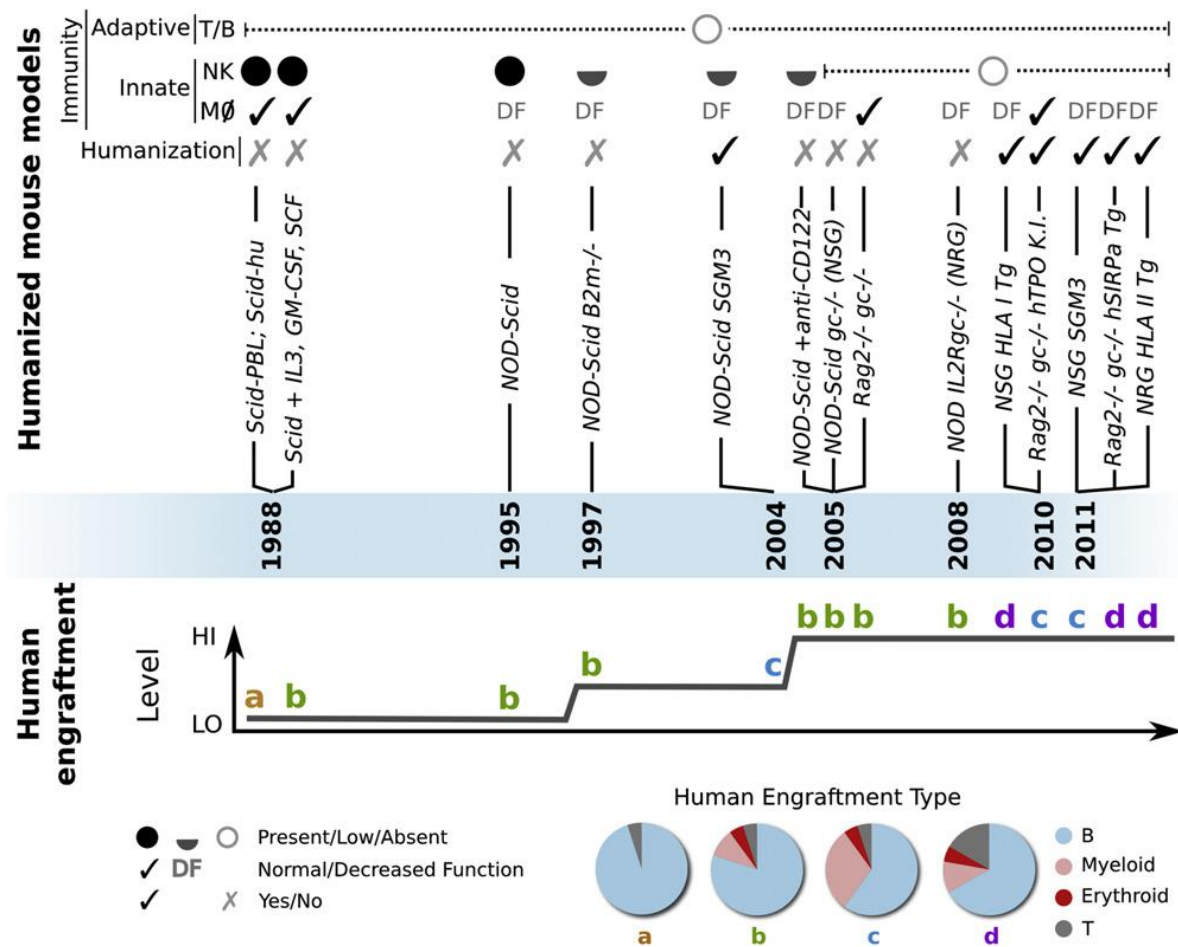


Figure 1.12. The development of immune-deficient and humanised mouse models. The development of immune-deficient mouse strains over time is shown along with the extent of humanisation and immunity within each strain. The type of engraftment seen with each strain is referenced to the pie charts underneath each strain. Image reproduced with permission from Doulatov *et al.* 2012 *Cell Stem Cell*;10,120-136 (Doulatov et al., 2012).

1.1.8.2 Identifying haemopoietic stem cells

There is no single marker with which to identify HSCs. Instead, HSCs can be identified, or at least enriched using a combination of markers (Ratajczak, 2008). The HSC-containing subset of bone marrow cells does not express lineage antigens (Lin⁻), which are expressed on the mature, differentiated cells, such as CD13 and CD33

(myeloid cells), CD19 (B cells), CD61 (megakaryocytes), CD3 (T lymphocytes) and CD56 (NK cells) and glycophorin A (erythroid cells) (reviewed in (Wilson and Trumpp, 2006)).

For defining murine HSPCs, the Lin⁻Sca-1⁺c-Kit⁺ (LSK) phenotype, which included CD34⁺ cells with the capacity to reconstitute mice, was used, but more recently, the signalling lymphocyte activation molecule (SLAM) panel CD150⁺CD48⁺CD41⁻ have been used in addition to the LSK phenotype (Kiel et al., 2005; Doulatov et al., 2012) (Figure 1.13). *In vivo*, LT-HSCs have been located based on BrdU retention (although this is controversial as a sensitive detector of HSCs (Kiel et al., 2007a)), and Hoechst 33342^{lo} uptake from the circulation (Winkler et al., 2010). The use of metabolic fluorochromes, such as Rhodamine 123, where low staining is expected due to the p-glycoprotein pump, or by measuring high aldehyde dehydrogenase (ALDH) activity have also been used (Ratajczak, 2008; Spangrude et al., 1991; Udomsakdi et al., 1991). However, these techniques are not as useful for defining human HSCs because in culture, the rhodamine low phenotype is lost, whilst SRC ability is maintained (Ito et al., 2010). Equally, CD38, which is not expressed on HSCs but is gained with increasing haemopoietic maturity (McGuckin et al., 2003), may also be lost on maturing HSPCs in culture (Ito et al., 2010). Hence, the CD34⁺CD38⁻Rho^{lo} phenotype does not reflect maintenance of SRCs in culture (Dorrell et al., 2000; Ito et al., 2010).

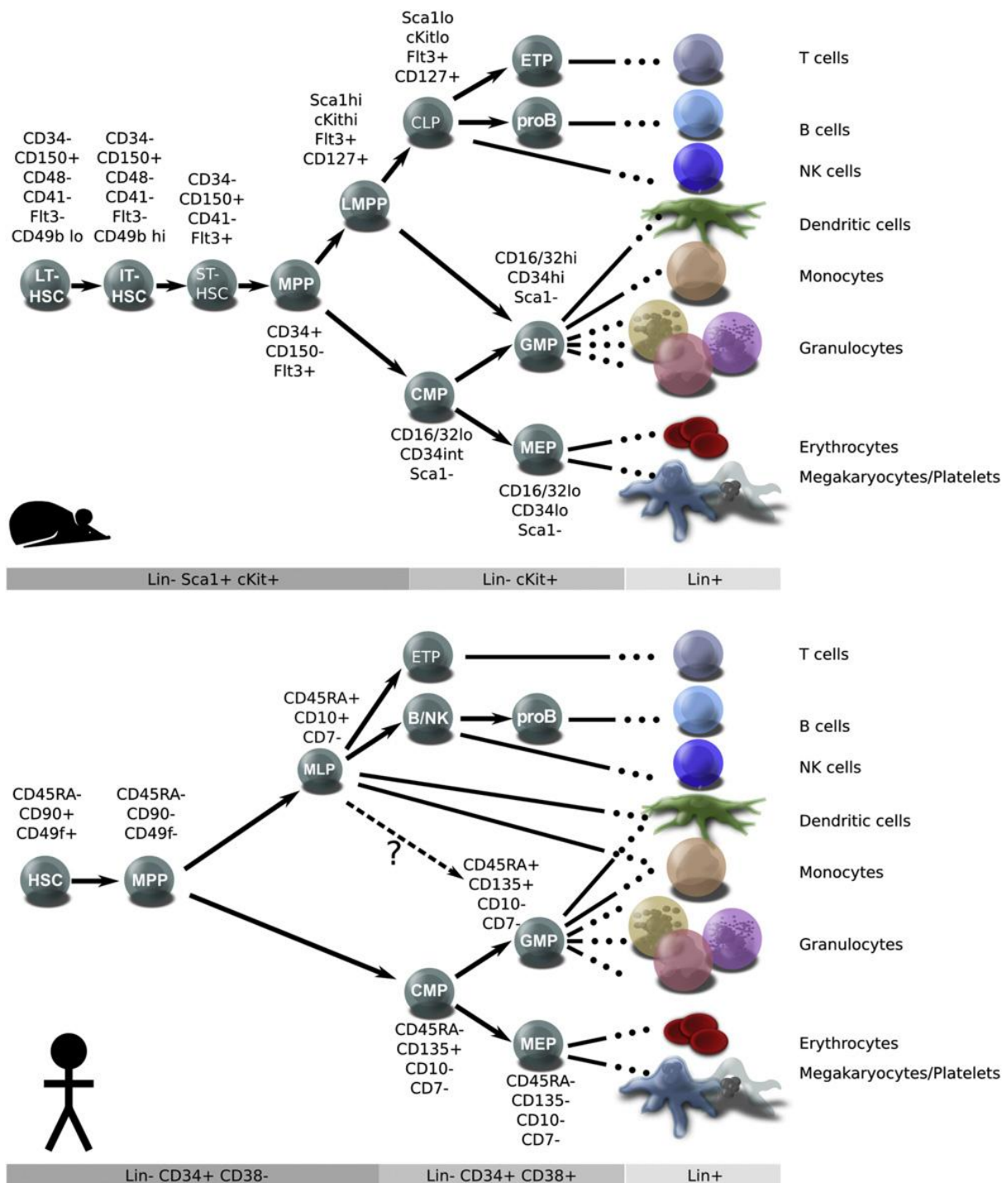


Figure 1.13. Lineage determination of mouse and adult HSCs and their progeny. Surface markers defining long term (LT), intermediate (IT) and short term (ST) mouse HSCs which show decreasing self renewing potential and give rise to multipotent progenitors (MPP). In humans, the heterogeneity of the HSC has not been clarified. The MPP differentiates into multi-lymphoid (LMPP/MLP) and myeloid biased progenitors (CMP) which differentiate into T, B, NK cells and dendritic lineages and myelo-erythroid progeny respectively. CD133 (not shown) is also expressed on human HSCs. Image reproduced with permission from Doulatov *et al.* 2012 Cell Stem Cell;10,120-136 (Doulatov et al., 2012).

Although human CD34^{low/-} HSCs are capable of long-term multilineage engraftment and reconstituting capability in mice (Lange et al., 2007), CD34 has been calculated to be expressed on >99% of HSCs (Doulatov et al., 2012). However, as CD34 is also expressed on more differentiated haemopoietic cells (and endothelial cells) it cannot define the HSC when used alone (Ratajczak, 2008; Doulatov et al., 2012). More recently, additional surface markers (Lin⁻CD34⁺CD38⁻CD90⁺CD49f⁺CD45RA⁻) have been shown to define the SRCs (Figure 1.13) (reviewed by (Doulatov et al., 2012)). Human cells with some, or all of this surface phenotype have also recently been shown to contain SRCs in culture expanded cells (Butler et al., 2012; Gori et al., 2012). The current phenotypes of the mouse and human HSC hierarchy are illustrated in Figure 1.13.

1.1.8.3 The use of CD133 to isolate HSPCs

Labelling mononuclear cells (MNCs) with magnetic beads conjugated to antibodies for CD133 (AC133) and separating them on a magnetic column provides a useful starting point of an immature population of HSPCs from UCB (Ratajczak, 2008), as employed in this thesis. In 1997, the production of AC133, a monoclonal antibody, that bound to a novel antigen on CD34⁺ HSCs was described (Miraglia et al., 1997; Yin et al., 1997). Early studies comparing populations isolated using CD34 and CD133 from the same UCB have suggested their expansion and multilineage capacity *in vitro* are similar (Kobari et al., 2001). However, CD133 has been shown to appear before CD34 at the surface of both UCB and BM CD117⁺CD38^{low} populations, suggesting it to be a useful tool for isolating more immature HSC populations that do not yet express cell surface CD34 (McGuckin et al., 2003). Ito *et al.* more recently demonstrated that CD133⁺ staining, could be used to enrich for SRCs in *ex vivo* cultures (Ito et al., 2010).

1.1.8.4 The use of cell monolayers for HSC maintenance/expansion

Cytokine cultures alone have been shown to impair regenerative function with increasing culture of human UCB CD34⁺ cells (Holmes et al., 2012). The niche provides support and *in vitro* studies have shown the stromal “feeder” cell layers to be vital for the maintenance of multipotent HSPCs (Wagner et al., 2007). BMSCs have been shown to support the *in vitro* expansion of HSPCs cultured in the presence of cytokines. Early studies showed that these cultures retain a population of long term culture initiating (LTC-IC) or cobblestone forming cells (CAFC) and SRC ability (Piacibello et al., 1999; Rappold et al., 1999; Sutherland et al., 1989). Human brain endothelial cells (with or without direct cell contact) were also shown not only to support the maintenance, but also increase the frequency of SRCs, in adult human bone marrow CD34⁺ cell cultures (Chute et al., 2002). In contrast to Chute *et al.*, HSPC expansion was shown to be dependent on HSPCs binding to Notch-ligand on E4ORF1 lentiviral transduced mouse endothelial serum-free cultures by Butler *et al.* (Butler et al., 2010). More recently, E4ORF1 lentiviral transduced human endothelial cells have been shown to support serum-free HSPC expansion when cultured with additional cytokines, beyond that seen without endothelial support (Butler et al., 2012). The endothelial cultures supported almost 7-fold expansion of SRCs compared to uncultured cells and 2-fold greater expansion of SRCs compared to cytokines alone, and showed that 90% of secondary transplanted recipients engrafted (Butler et al., 2012).

Although these cultures on cell monolayers offer angiocrine support to the HSPCs, these cultures still required the addition of cytokines such as SCF, Fms-like-tyrosine kinase 3 ligand (Flt-3 ligand), thrombopoietin (TPO) and in some cases interleukin

(IL-6). Ding *et al.* have recently demonstrated that HSPCs were depleted from the bone marrow upon deletion of SCF from endothelial cells or leptin⁺ stromal cells, but not upon its deletion from haemopoietic cells and osteoblastic cells (Ding *et al.*, 2012). In addition to demonstrating the importance of SCF *in vivo*, this also demonstrates the importance of both endothelial and stromal components for HSC function, which may not be replicated by *in vitro* culture models using feeder layers composed of one cell type.

Other approaches to expand HSPCs have included the use of transcription factors such as HOXB4 transduced directly using retroviral vectors into mouse bone marrow cells (Antonchuk *et al.*, 2002). HOXB4-GFP transductions have resulted in expansion and improved engraftment in an autologous non-human primate competitive repopulation model compared to YFP (yellow fluorescent protein) transduced cells, although 2 of 3 animals died within 3 months (Watts *et al.*, 2012). The use of MS-5 stromal cells engineered to produce HOXB4 and HOXC4 homeoproteins, has enabled passive expansion of HSCs (3-6 times) which show improved engraftment, without genetic alteration (Amsellem *et al.*, 2003; Auvray *et al.*, 2012). Although removing the need for retroviral vectors has improved the safety of gene transfer, the use of cellular co-cultures, particularly those using transformed and/or non-human cells are still a concern for the clinical applicability of such therapies.

1.1.8.5 HSPC expansion without cell feeder layers

Non-cellular, non-viral protocols, in the form of scaffolds, suspension cultures and additional cytokines or factors have provided an exciting array of potentially clinically applicable HSPC expansion techniques. Different nanofibre scaffolds were shown to provide different levels of expansion by Chua *et al.* (Chua *et al.*, 2007). The

scaffolds that showed improved 6 week engraftment from uncultured cells in NOD-scid mice (although this was <1% chimerism) supported 200-fold CD34⁺ cell expansion over 10 days (Chua et al., 2007). Ventura Ferreira *et al.* have demonstrated expansion of UCB CD34⁺ cells on a 2D fibrin scaffold over 7 days which retained multilineage engraftment capability in NSG mice (Ventura Ferreira et al., 2012b). More recently, a 3D fibrin scaffold seeded with MSCs and cultured in SCF, TPO, FGF-1, Angptl5 and IGFBP2 has shown a 15 fold improvement in human cell engraftment over non-cultured cells in NSG mice (Ventura Ferreira et al., 2012a).

A further approach taken has been to culture UCB HSPCs with conventional cytokines and additional growth factors or ligands without scaffold support (reviewed by (Dahlberg et al., 2011)). These include the growth factors Angptl5 and IGFBP2, which have been shown to enhance UCB HSPC 11 day expansion and *in vivo* reconstitution both separately but with additional improvement when combined, generating a 20 fold increase in SRCs (NOD-scid) in the best condition (Zhang et al., 2008). This was subsequently confirmed in NSG mice (Drake et al., 2011). However, more recently, Angptl4, Angptl5, IGFBP2 and combinations of these factors were shown to have variable engraftment potential, and more frequently acted to maintain SRCs similar to uncultured cells rather than improving engraftment after 8-11 day *ex vivo* culture (Blank et al., 2012).

The aryl hydrocarbon receptor antagonist StemRegenin1, which, over 3 weeks expanded CD34 cells 669 fold, has been shown to improve secondary transplant capacity compared to uncultured cells (Boitano et al., 2010). Notch-ligand mediated UCB CD34⁺ cell expansion of 200 fold was also shown to enhance short term and longer term SRC frequency (15 and 6 fold) respectively in mice. A phase I trial

showed that infusing a Notch-expanded unit (approximately 150 fold expansion of CD34⁺ cells) with an unmanipulated unit shortened the time to neutrophil recovery in patients, regardless of which unit contributed most to the myeloid donor cells (Delaney et al., 2010a). A similar effect has been observed with pleiotrophin which may activate Notch signalling pathways, but without the need for cell-cell contact (Himburg et al., 2010). Most recently, Csaszar *et al.* have overcome a fundamental problem which is associated with expanding HSPCs in culture due to the inevitable feedback inhibition by differentiated cells (Csaszar et al., 2012; Kirouac et al., 2010). An automated closed system bioreactor optimally fed UCB HSPCs using an algorithm designed to reduce endogeneously produced negative feedback signals in the culture, yielding an 11 fold increase in SRCs over 12 days (Csaszar et al., 2012).

The translation possibility to the clinic for these therapies has recently been questioned (Chou et al., 2010), in terms of the isolation of HSPC populations (e.g. CD34/CD133) prior to expansion and the feasibility of clinical grade manufacture. However, in the successful phase I trial, Delaney *et al.* showed far superior CD34 cell numbers in expanded units despite initial CD34 selection (Delaney et al., 2010a). Additionally, the use of closed system bioreactors, serum free-conditions and synthetic scaffolds will enhance the potential for clinical manufacture (Csaszar et al., 2012; Ventura Ferreira et al., 2012a).

1.2 Key objectives and aims of this research

The overall objective of the research was to investigate mechanisms which regulate the engrafting capacity of HSPCs derived from human UCB, and specifically to:

- 1) Establish an *in vitro* migration assay to measure individual human UCB CD133⁺ cell chemotactic and chemokinetic response to CXCL12, by:
 - Defining a 3D gel matrix through which the cells can migrate
 - Optimising migration in terms of CXCL12 concentration, gel matrix concentration and time course
 - Defining the effect of CXCL12 and AMD3100 on UCB CD133⁺ cell chemotaxis and chemokinesis
- 2) Determine the role of CD164 in regulating CD133⁺ HSPC migration *in vitro* and homing *in vivo*, by:
 - Establishing an *in vivo* homing assay
 - Optimising the silencing of CD164 in UCB CD133⁺ cells
 - Assessing the effect of CD164 silencing in UCB CD133⁺ cells on *in vivo* homing and *in vitro* chemotaxis
- 3) Investigate the effect of culture conditions on human UCB CD133⁺ cell expansion and function by:
 - Developing a scaffold from which to investigate HSPC expansion *in vitro*
 - Assessing the effect of culture conditions on expansion
 - Determining whether expanded HSPCs retain migratory function in the *in vitro* chemotaxis assay

Chapter 2. Materials and Methods

2.1 Reagents

Table 2.1. Reagents with company details

Reagents	Company
2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF-AM)	Invitrogen Ltd., Paisley, UK
Accutase in PBS with 0.5 mM EDTA	PAA Laboratories GmbH, Pasching, Austria
Acetylated low density lipoprotein (LDL)	Athens Research and Technology Inc., Athens, USA
Bovine Serum albumin (BSA)	Sigma Aldrich Ltd., Gillingham, UK
CD133 MicroBead Kit	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Collagenase from Clostridium histolyticum	Sigma Aldrich Ltd., St. Louis, Miss, USA
Collagen I bovine gel	Invitrogen Ltd., Paisley, UK
CXCL12 (stromal derived factor 1- α)	Peprtech, Rocky Hill, NJ, USA
DAPI (4',6-diamidino-2-phenylindole)	Invitrogen Ltd., Paisley, UK
DAB substrate kit	Vector Laboratories Inc., Burlingame, CA, USA
Dimethylsulphoxide (DMSO) Hybri-Max	Sigma Aldrich Ltd., Gillingham, UK
Dulbecco's Phosphate Buffered Saline (DPBS)	Lonza Biologics, Tewkesbury, UK or PAA Laboratories GmbH, Pasching, Austria
Dulbecco's Modified Eagle Medium	Lonza Biologics, Tewkesbury, UK
EDTA	Sigma Aldrich Ltd., Gillingham, UK
Endothelial Cell Basal Medium-2 (EBM-2)	Lonza Biologics, Tewkesbury, UK
Endothelial Growth Medium-2 (EGM-2)	Lonza Biologics, Tewkesbury, UK
Ethanol	VWR International Ltd., Lutterworth, UK
Falcon centrifuge tubes	BD Biosciences, Franklin Lakes, CA, USA
FcR Blocking Reagent	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Fetal Calf Serum (FCS)	Hyclone, Salt Lake City, USA or PAA Laboratories GmbH, Pasching, Austria

Fibronectin	Sigma Aldrich., Gillingham, UK
Fungizone	Sigma Aldrich., Gillingham, UK
Hanks' Balanced Salt Solution (HBSS)	PAA Laboratories GmbH, Pasching, Austria or Lonza Biologics, Tewkesbury, UK
Isopropanol	VWR International Ltd., Lutterworth, UK
Lipofectamine 2000	Invitrogen Ltd., Paisley, UK
Luria tablets	Sigma Aldrich., Gillingham, UK
LSM 1077 Lymphocyte Separation Medium	PAA Laboratories GmbH, Pasching, Austria
MEM- α medium (Gibco)	Invitrogen Ltd., Paisley, UK
Myelocult medium	Stem Cell Technologies, Grenoble, France
Nanex Cytokine Cocktail	Arteriocyte, Cleveland, Ohio, USA
Nanex HSC expansion medium	Arteriocyte, Cleveland, Ohio, USA
Nuclease free water	Invitrogen Ltd., Paisley, UK
OptiMEM I reduced serum media	Invitrogen Ltd., Paisley, UK
Paraformaldehyde	Sigma Aldrich., Gillingham, UK
Penicillin-Streptomycin (100x)	PAA Laboratories GmbH, Pasching, Austria
Plasma transfer set	Baxter Healthcare Ltd., Thetford, UK
Propidium Iodide (cat no. P4864)	Sigma Aldrich Ltd., Gillingham, UK
Recombinant human angiopoietin-like 5 (Angptl5)	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Recombinant human Fms-like tyrosine kinase 3 ligand (Flt-3 ligand)	R&D systems, Minneapolis, MN, USA
Recombinant human IGFBP2	R&D systems, Minneapolis, MN, USA
Recombinant human Interleukin-6 (IL-6)	R&D systems, Minneapolis, MN, USA
Recombinant human Stem Cell Factor (SCF)	R&D systems, Minneapolis, MN, USA
Recombinant human Thrombopoietin (TPO)	R&D systems, Minneapolis, MN, USA
SOC medium	Invitrogen Ltd., Paisley, UK
Stemspan serum-free expansion medium (stemspan)(Cat. No. 09650)	Stem Cell Technologies, Grenoble, France
To-pro-3	Invitrogen Ltd., Paisley, UK
Trisodium Citrate	VWR International Ltd., Lutterworth, UK
Trypan Blue stain 0.4% (w/v)	Invitrogen Ltd., Paisley, UK

Trypsin-EDTA (1x) 0.5mg/ml-0.22mg/ml in PBS without Ca ⁺⁺ and Mg ⁺⁺	PAA Laboratories GmbH, Pasching, Austria
Tween-20	Sigma Aldrich Ltd., Gillingham, UK
VECTASTAIN ABC kit	Vector Laboratories Inc., Burlingame, CA, USA

2.2 Cells

2.2.1 Cell lines

Human embryonic kidney cells (HEK293FT) and human dermal fibroblasts (hDF) were purchased from Lonza Biologics and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza Biologics) with 10% (v/v) fetal calf serum (FCS) (PAA Laboratories GmbH) unless otherwise stated. BMEC-60 cells, obtained from Prof. E van der Schoot, the Netherlands (Rood et al., 2000), were cultured in complete endothelial growth medium-2 (EGM-2) (made from endothelial cell basal medium-2 (EBM-2) (Lonza Biologics) supplemented with EGM-2 Single Quots (consisting of undisclosed concentrations of fetal calf serum (FCS), hydrocortisone, fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), long R insulin-like growth factor-1 (R³-IGF-1), ascorbic acid, epidermal growth factor (hEGF), gentamycin sulphate, amphotericin-B (GA-1000), and heparin in EBM-2, all from Lonza Biologics) unless otherwise stated.

2.2.2 Primary cells

2.2.2.1 Human bone marrow stromal cells (BMSC)

BMSC were purchased from Lonza Biologics at passage 1 and cultured in Myelocult medium (Stem Cell Technologies). They were expanded, trypsinised as described in

section 2.2.3 and frozen in aliquots at passage 2, and used upon thawing without further passage.

2.2.2.2 Endothelial cells from human umbilical vein (HUVEC) and umbilical cord blood (UCB)

Human umbilical cord blood (UCB) units were collected fresh from the umbilical cord vein by gravity feed or the umbilical cord was used to produce human umbilical vein endothelial cells (HUVECs). These were sourced from normal births from the John Radcliffe Hospital, Oxford with written informed pre-consent and ethical approval from the Oxford Radcliffe Research Ethical Committee for the Use of Umbilical Cord Blood following Delivery as a Source of Stem Cells for Study of Cell Differentiation in Normal and Disease Conditions (CO2.313). Cells isolated were used as anonymised linked donations either under an HTA licence or with ethical permission from the Berkshire Research Ethics Committee (10/H0505/34).

Endothelial cells from UCB (clones 301109 and 120110) were kindly provided by Dr Cheen Khoo, NHS Blood and Transplant, Oxford, UK at passage 2-3. The cells were cultured in complete EGM-2 medium with an additional 10% (v/v) FCS (Hyclone), unless otherwise stated.

HUVECs were isolated from human umbilical cord less than 3 hours old using sterile technique in a laminar flow cabinet as described by Jaffe *et al.* (Jaffe et al., 1973). The intact, undamaged section of cord was between 10-30cm in length. The cord was kept moist using HBSS (Hanks' balanced salt solution) with Ca^{++} and Mg^{++} (PAA Laboratories GmbH) throughout the procedure. After massaging the cord to dislodge any large blood clots, the umbilical vein was cannulated using a plasma transfer set with cannulator and luer adaptor (Baxter Healthcare Ltd., Thetford, UK) and secured

using two clamps. A 20ml syringe was attached to the kit and 50ml HBBS with Ca^{++} and Mg^{++} (PAA Laboratories GmbH) perfused through the cord, whilst massaging, until the fluid became clear. A 0.1% (w/v) collagenase solution was made using 50mg collagenase (Type 1-AS, Sigma Aldrich Ltd.) in a sterile vial and 50ml HBSS with Ca^{++} and Mg^{++} (PAA Laboratories GmbH). The dissolved collagenase was then perfused through the vein and the lower end clamped once the entire vein contained collagenase. The vein was further perfused with collagenase until it appeared inflated. The cord was then placed on a sterile tray and incubated at 37°C in a Nuaire DH autoflow incubator (5% CO_2) for approximately 20 minutes. To collect the endothelial cells, the cord was gently massaged above a 50ml sterile falcon tube and the clamp released. Fifty to 100ml HBSS with Ca^{++} and Mg^{++} (PAA Laboratories GmbH) was perfused through the cord and collected in Falcon tubes. The cell suspension was then centrifuged at 350 x g, acceleration 9, deceleration 4 for 10 minutes at 22°C in a Hettich Rotina 46R centrifuge (DJB Labcare, Newport Pagnell, UK). The supernatant was removed and the loose pellet re-suspended in 10ml HBSS with Ca^{++} and Mg^{++} (PAA Laboratories GmbH). The cell suspension was centrifuged at 350 x g, acceleration 9, deceleration 4 for 10 minutes at 22°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The supernatant was carefully removed and the loose pellet resuspended at room temperature in 20ml EGM-2 medium. The cells were seeded into a T75 flask (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands) and incubated for 24 hours in a Nuaire DH autoflow incubator at 37°C (5% CO_2). After 24 hours, the flask was rinsed twice with 5ml HBSS without Ca^{++} and Mg^{++} (PAA Laboratories GmbH) and the media replaced (20ml EGM-2 medium (Lonza Biologics), 1% (v/v) fungizone (Sigma Aldrich Ltd.), 1% (v/v) penicillin-streptomycin (PAA

Laboratories GmbH)). The media were changed every 3-4 days until cells reached 80-90% confluence (approximately 9 days after isolation).

Once the HUVECs had reached confluence, the cells were passaged (P1) by washing twice with 20ml HBSS and then incubated with 3ml trypsin-EDTA (PAA Laboratories GmbH) for 10-15 minutes in a Nuaire DH autoflow incubator at 37°C (5% CO₂). The cell number was counted using a haemocytometer and cell viability assessed using 0.4% (w/v) trypan blue stain (Invitrogen Ltd.). Master stocks of HUVECs at passage two (P2) were cryopreserved by re-suspending cells in HUVEC freezing mix (70% (v/v) EGM-2 medium (Lonza Biologics), 20% (v/v) FCS (Hyclone) and 10% (v/v) dimethylsulphoxide (DMSO) (Sigma-Aldrich Ltd.)) and storing as described in section 2.2.3. For routine culture, HUVECs were cultured in complete EGM-2 medium unless otherwise stated.

2.2.2.3 Human UCB CD133⁺ cells

2.2.2.3.1 Isolating the mononuclear cell fraction from UCB

Human UCB units were collected fresh from the umbilical vein of the placenta of normal births, with written informed pre-consent and ethical approval from the Oxford Radcliffe Research Ethical Committee for the Use of Umbilical Cord Blood following Delivery as a Source of Stem Cells for Study of Cell Differentiation in Normal and Disease Conditions (CO2.313). UCB units were either processed fresh or kept at 4°C overnight. Cells were used as anonymised linked donations either under an HTA licence or with ethical permission from the Berkshire Research Ethics Committee (10/H0505/34).

CD133⁺ cells were isolated from human UCB after obtaining the mononuclear cell (MNC) fraction. The entire procedure was performed in a laminar flow hood under aseptic conditions. UCB units were diluted 1:1 if the white blood cell (WBC) count was $< 7 \times 10^6$ cells/ml and 1:2 if the WBC count was $> 7 \times 10^6$ cells/ml in filter sterilised HBSS without Ca⁺⁺ and Mg⁺⁺ (PAA Laboratories GmbH) containing 3g/l trisodium citrate (VWR International Ltd.) at room temperature and mixed by inversion. Thirty ml of diluted blood was either pipetted into Accuspin tubes (Sigma Aldrich Ltd.) containing 17ml LSM 177 (density 1.077g/ml) (PAA Laboratories GmbH) or gently layered over 20ml LSM 177 (density 1.077g/ml) (PAA Laboratories GmbH), which had been brought to room temperature. Mononuclear cells were separated by centrifugation at 970 x g, acceleration 9, deceleration 1 for 10 minutes using Accuspin tubes or at 630 x g, acceleration 9, deceleration 0 or 1 for 30 minutes if layering at 22°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The mononuclear cell layer was collected and washed with 4°C 50ml HBSS without Ca⁺⁺ and Mg⁺⁺ (PAA Laboratories GmbH) containing 3g/l trisodium citrate (VWR International Ltd.) by centrifugation at 630 x g, acceleration 9, deceleration 9 for 10 minutes at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The cell pellet was then re-suspended in 50ml HBSS without Ca⁺⁺ and Mg⁺⁺ (PAA Laboratories GmbH) containing 3g/l trisodium citrate (VWR International Ltd.) and centrifuged at 350 x g, acceleration 9, deceleration 7 for 10 minutes at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The cell pellet was then re-suspended in 20ml 4°C MACS buffer (DPBS with 0.5% (w/v) BSA and 2mM EDTA, pH7.2) and filtered through a 40µm nylon cell strainer (BD Biosciences). A 140µl sample taken for determining the white blood cell number on a Sysmex haematology analyser (Sysmex Corporation, Hyogo, Japan) (using option 3). The remaining sample was centrifuged at 350 x g for 7 minutes, acceleration 9,

brake 7 at 4°C in a Hettich Rotina 46R centrifuge and either frozen as MNCs at 1×10^8 cells per ml as described in section 2.2.3 or processed further to isolate the CD133⁺ cell fraction (described in section 2.2.2.3.2).

2.2.2.3.2 Immuno-magnetic purification of CD133⁺ cells from MNCs

CD133 is a cell surface antigen that is expressed by an immature subset of human CD34⁺ haemopoietic stem/progenitor cells (HSPC) and a proportion of CD34⁻ cells (McGuckin et al., 2003). The MNC fraction, obtained as described in section 2.2.2.3, was re-suspended in 300µl 4°C MACS buffer per 1×10^8 cells. The CD133 microbead kit (Miltenyi Biotec GmbH) was used to purify the CD133⁺ cells from the MNCs, as illustrated in Figure 2.1. To prevent the antibody binding to Fc receptors, 100µl FcR Blocking Reagent (human IgG) per 1×10^8 cells and 100µl CD133/1 (mouse IgG1, clone AC133/1) per 1×10^8 cells, both from the CD133 MicroBead Kit (Miltenyi Biotec GmbH), were added to the re-suspended cells. The cells were incubated with the CD133/1 antibody, which was conjugated to magnetic microbeads, for 30 minutes in a 4°C refrigerator. The CD133/1 antibody recognises epitope 1 of the antigen, AC133, which is present on the most immature human HSPCs. The cells were then washed in 20ml 4°C MACS buffer and centrifuged at 350 x g for 10 minutes, acceleration 9, brake 7 at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The cells were then re-suspended in 5ml 4°C MACS buffer.

An LS column (Miltenyi Biotec GmbH) was equilibrated on a MidiMACS separation magnet (Miltenyi Biotec GmbH) with 3ml ice cold MACS buffer. The MACS buffer was kept on ice throughout the separation described below. The cell suspension was passed through a 30µm pre-separation filter (Miltenyi Biotec GmbH) and then through the equilibrated LS column (Miltenyi Biotec GmbH) attached to a MidiMACS

separation magnet (Miltenyi Biotec GmbH). The column was then washed three times with 4ml 4°C MACS buffer so that only magnetically labelled cells (CD133⁺) were retained in the column. The column was removed from the magnet and the labelled cells were then flushed out of the column with 5ml 4°C MACS buffer using a plunger from the LS column kit. To increase the purity of CD133⁺ cells the cells were passed through a second equilibrated LS column (Miltenyi Biotec GmbH) and the washing procedure repeated. The labelled cells were then flushed out of the column with 5ml MACS buffer using a plunger from the LS column kit and centrifuged at 350 x g for 5 minutes, acceleration 9, brake 7 at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The pellet was re-suspended in 1ml MACS buffer and a 10µl sample diluted 1:1 with 0.4% (w/v) trypan blue stain (Invitrogen Ltd.) to assess viability. Cells were counted using a haemocytometer (Neubauer) and 8x10⁴ cells taken for assessment of purity using flow cytometry as described in section 2.4.2.1.

The remaining CD133⁺ cell suspension was centrifuged in 5ml MACS buffer at 350 x g for 7-10 minutes, acceleration 9, brake 7 at 4°C in a Hettich Rotina 46R centrifuge. The cells were either cryopreserved as described in section 2.2.3 or used fresh for assays. Before use in assays, freshly harvested or cryopreserved and thawed CD133⁺ cells were cultured in stemspan medium (containing undisclosed quantities of bovine serum albumin, recombinant human insulin, human transferrin (iron saturated), 2-mercaptoethanol, Iscove's MDM and undisclosed supplements) (Stem Cell Technologies) with 100ng/ml stem cell factor (SCF), Fms-like-tryosine-kinase 3 ligand (Flt-3 ligand), interleukin-6 (IL-6) and 20ng/ml thrombopoietin (TPO) (all recombinant human from R&D Systems) at a concentration of 5x10⁵ cells per ml in

tissue culture plates (BD Biosciences) and incubated at 37°C (5% CO₂) in a Nuair DH autoflow incubator for 24 hours unless stated otherwise.

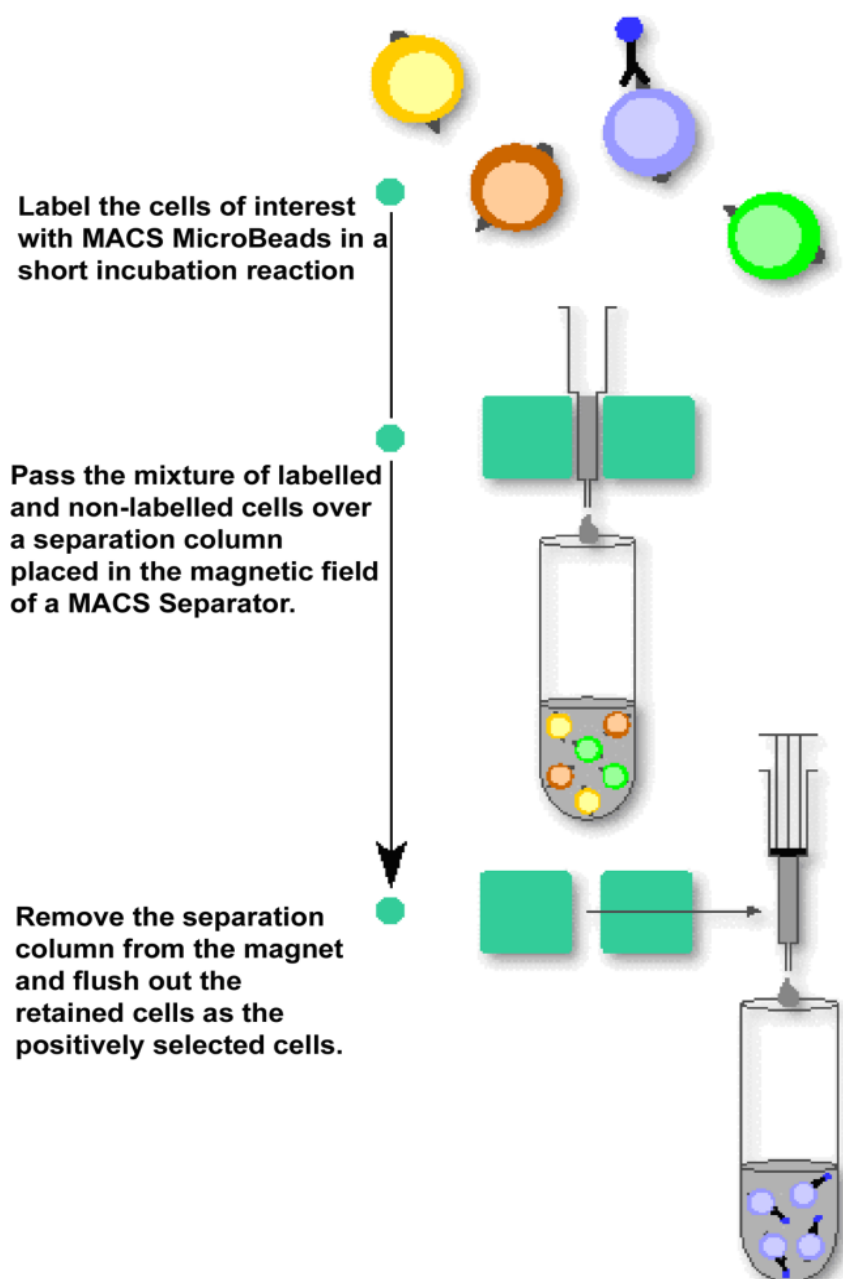


Figure 2.1. Labelling and separation of human UCB CD133⁺ cells. Following incubation of AC133/1 conjugated to magnetic microbeads, the cells were passed through a separation column attached to a magnet. The magnetically labelled cells captured in the column were then flushed out with MACS buffer, leaving a population of CD133⁺ cells. Image taken from Miltenyi Biotec GmbH (<http://www.miltenyibiotec.com>).

2.2.3 Cell passage, counting and freezing procedures

Where cells were passaged, this refers to the detachment of adherent cells from the culture flasks or plates (BD Biosciences). For this procedure, the cell medium was removed and cells were washed twice in 10-20ml PBS (PAA Laboratories GmbH) prior to incubating with 2-5ml trypsin-EDTA or accutase (PAA Laboratories GmbH) for 10-20 minutes at 37°C in a Nuair DH autoflow incubator in 5% CO₂ in air.

The enzyme solutions were neutralised using the standard cell culture medium as described for each type of cell in section 2.2. The cells were collected into a Falcon tube (BD Biosciences) and centrifuged at 350 x g, acceleration 9, deceleration 4 for 10 minutes at 22°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The cells were re-suspended in an appropriate volume for counting (approximately 1x10⁶ cells/ml) in their standard culture media and an 8µl sample taken. This was diluted 1:1 with trypan blue stain (Invitrogen Ltd.) in order to ensure the cells counted using a bright field microscope using a Nikon Eclipse TE300 (Nikon UK Ltd., London, England) were viable. Counting was performed by seeding the cell suspension containing trypan blue underneath a cover slide on a haemocytometer (Neubauer). Using the outer 4 corner chambers of the haemocytometer, approximately 100 cells were counted. To calculate the volume of cells/ml of solution the following formula was used:

(Number of cells counted/4) x 10⁴ (area of haemocytometer) x 2 (trypan blue dilution)

The required volume of cells was then taken for further culture, storage or experiments. For CD133⁺ suspension cells, after pipetting to mix the well, a sample was taken and counted using the same counting procedure as above.

For freezing, cells were re-suspended in freezing mix (90% (v/v) FCS (PAA Laboratories) and 10% (v/v) dimethylsulphoxide (DMSO) (Sigma-Aldrich Ltd.)) and frozen at 1×10^6 cells per ml per cryovial unless otherwise stated. Cryovials containing cells were immediately transferred to a Mr Frosty containing room temperature isopropanol (VWR International) and stored at -80°C for 24 hours before being transferred to gaseous phase liquid nitrogen ($<-150^{\circ}\text{C}$).

2.3 Antibodies

For flow cytometry mouse anti-human antibodies were used at test quantities unless stated otherwise: CD164 (N6B6) (BD Biosciences) and goat anti-mouse-APC secondary (Invitrogen Ltd.), CD164-PE (N6B6) or CD164-647 (N6B6), CXCR4-APC (12G5) or CXCR4-PerCP (12G5), CD45-PerCP (2D1), CD90-FITC (5E10), CD38-PeCy5 (HIT2), CD34-PerCP (8G12), CD45RA-PE (HI100) (BD Biosciences), CD34-APC (AC136), CD133/2-PE (293C3), CD133/2-APC (293C3) (Miltenyi Biotec GmbH) and the equivalent volumes of the isotype controls mIgG2a-APC, mIgG2b-APC, mIgG2a-PE, mIgG2b-PE (Miltenyi Biotec GmbH), mIgG2a-APC, mIgG2a-PE, mIgG1-PE, mIgG1-PerCP (BD Biosciences) and mIgG1-FITC (Dako, Glostrup, Denmark) were used in 100 μl total volumes made up with MACS buffer. For *in vivo* or set-up experiments the following antibodies were used at test quantities unless stated otherwise: mouse anti-human CD45 (HI30) (BD Biosciences), 1.25 $\mu\text{g}/\text{ml}$ rat anti-mouse CD45-FITC (30-F11) (Invitrogen Ltd.), 1 $\mu\text{g}/\text{ml}$ anti-mouse CD45-PerCP (30-F11), 0.125 $\mu\text{g}/\text{ml}$ anti-mouse Ter119 (TER119) (Biolegend, San Diego, CA, USA) or mIgG1-APC (BD Biosciences) or rat IgG2b-FITC (Invitrogen Ltd.) isotype controls were used per 100 μl of staining buffer (PBS (PAA Laboratories) with 5% BSA (Sigma Aldrich Ltd.)),

10% (v/v) human FcR block (Miltenyi Biotec GmbH) and 0.5% (v/v) of a titrated mouse FcR block kindly supplied by Dr. Petter Woll).

2.4 Flow Cytometry

2.4.1 *The principle*

Flow cytometry analyses the physical characteristics of fluorescently labelled individual cells. The cell suspension is injected into the column by placing the tube under the sample injection port (Figure 2.2A). The conical shape of the lower chamber and the under-pressure of the sheath fluid (hydrodynamic focusing) pressurises the flow stream so that the cells pass through the laser beam in single file. Different types of cells cause different forward and side scattering of light when passing through the laser beam. The scattered light is measured by a detectors set at angles of 13° for forward light scattering and 90° for side light scattering detection. The forward scattered light is roughly proportional to the size of the cell or inversely proportional to the refractive index of the cell, and the side scatter indicates the granularity and nuclear shape (the complexity of the cell) as the light is refracted from the inside of the cell outwards. An obscuration bar prevents direct interference by the laser alone on the detectors. Fluorophores conjugated to antibodies can be used to gather further information on the types of cells present in the sample. The fluorophore is excited by the relevant laser and absorbs the light and then re-emits the light at a different wavelength on return to ground state. The lasers in the BD LSR II (BD Biosciences) are UV (355nm), blue (488nm) or red (630nm). Filters at the fluorescent detectors allow the measurement of a particular wavelength of light. The filter configuration in the BD LSR II (BD Biosciences) for the 488nm laser is

illustrated in Figure 2.2B. The setup consists of long pass filters which block the passage of light below the stated wavelength and band pass filters. Band pass filters allow the passage of light of a wavelength below the stated centre wavelength plus the stated bandwidth, e.g. 530/30 allows the passage of 515-545nm wavelengths of light. The information can be displayed using FACS Diva software (BD Biosciences) as a voltage peak, which can be displayed as a light scatter histogram or dotplot. Multiparametric analysis combines the size and complexity information with the fluorescence to create an integrated phenotype for the cell of interest. The fluorophores used in this thesis are listed in Table 2.2 along with the filters used to detect emission and the excitation and emission wavelengths.

Table 2.2. Fluorochrome emission and excitation

Fluorochrome (channel)	Excitation wavelength (nm)	Emission wavelength (nm)	Filter
DAPI	355	455	440/40
BCECF-AM	488	530	530/30
GFP	488	509	530/30
FITC	488	520	530/30
PE	488	578	575/26
PerCP	488	678	670/14
Pe-Cy5	488	667	660/20
Pe-Cy7	488	785	780/60
Propidium Iodide	488	617	575/26
APC	633	660	660/20
Alexa Fluor 647	633	668	660/20
To-pro-3	633	661	660/20

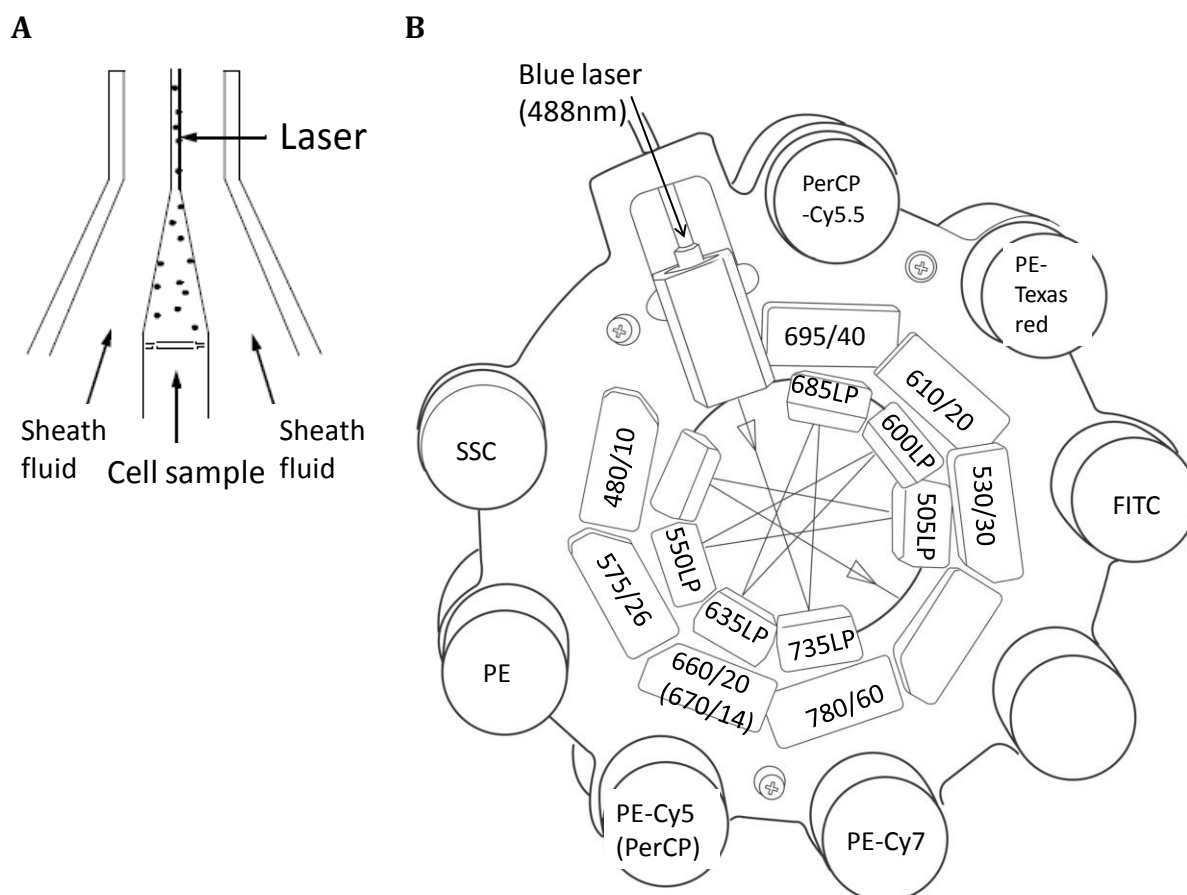


Figure 2.2. The workings of a flow cytometer. A) The sample injection port injects the cell sample into the column for detection by lasers. B) The filter configuration allowing the detection of fluorophores excited by the 488nm laser is demonstrated. Diagrams adapted from the BD LSR II User's guide (BD Biosciences).

2.4.2 Flow cytometry method

For all staining for flow cytometry, unless otherwise stated, 5×10^4 cells were re-suspended with the stated antibody or isotype control in 100 μ l MACS buffer. After 30 minutes incubation in darkness, on ice, cells were washed in 3ml MACS buffer at 425 x g for 5 minutes in a Heraeus Megafuge 1.0S centrifuge (DJB Labcare). The supernatant was poured off and excess buffer blotted onto tissue paper and the cell pellet re-suspended in 350 μ l ice cold MACS buffer. Immediately before each sample was run on the flow cytometer, samples were vortexed gently and 1nM to-pro-3

(1:100) or 0.1µg/ml DAPI (1:50,000) (Invitrogen Ltd.) viability stain was added. Where necessary, compensation controls were prepared and used to correct for spectral overlap, as described in section 2.4.2.3. The cells were analysed on a BD LSR II flow cytometer (BD Biosciences) using the BD FACSDiva 6 software program (BD Biosciences), which was set-up using cytometer setup and tracking (CST) beads (BD Biosciences) before every use, by collecting at least 10,000 viable cells per sample.

2.4.2.1 Assessing the purity of UCB CD133⁺ cells by flow cytometry

Human UCB CD133⁺ cells (8×10^4) were pelleted in 5ml MACS buffer at 350 x g for 7-10 minutes, acceleration 9, brake 9 at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The pellet was re-suspended in 40µl FcR blocking reagent (Miltenyi Biotec GmbH) and 160µl MACS buffer and kept on ice for 15 minutes. One hundred µl cells were aliquoted into FACS tubes containing test quantities of either CD133/2-PE (293C3) and human CD34-APC (AC136) antibody (Miltenyi Biotec GmbH) or mIgG2b-PE and mIgG2a-APC (Miltenyi Biotec GmbH) isotype controls. The cells were incubated on ice, in darkness, for 30 minutes and prepared as described in section 2.4.2. Representative flow cytometry histograms and dot plots show examples of a CD133 enriched population stained for CD133-PE and CD34-APC (Figure 2.3).

2.4.2.2 Adherent cell characterisation by flow cytometry

HUVECs, UCB endothelial cells, BMSCs and hDFs were phenotyped by staining for cluster of differentiation (CD) antigens as described by Zhang *et al.* (Zhang et al., 2009). The flask, containing confluent cells, was washed twice with 10ml HBSS without Ca⁺⁺ and Mg⁺⁺ (PAA Laboratories GmbH) and passaged using 1ml accutase (PAA Laboratories GmbH) for endothelial cells or 1x trypsin-EDTA (PAA Laboratories GmbH) for BMSC/hDF and incubated for 5 minutes at 37°C in a Nuair DH autoflow

incubator (5% CO₂). The cells were collected into a Falcon tube (BD Biosciences) and washed in their respective media containing serum. The supernatant was discarded and the wash repeated to remove accutase and phenol red from the medium using 10ml MACS buffer at 350 x g for 5 minutes at 22°C in a Hettich Rotina 46R centrifuge. The cells were re-suspended at 2x10⁶ cells per ml in FACS buffer. FcR blocking reagent (human IgG) (Miltenyi Biotec GmbH) was added (10% (v/v)) and incubated on ice for 15 minutes. The appropriate antibody or isotype control was added to 100µl aliquots at the following pre-titrated concentrations or volumes: 6.25ng/ml CD31-PE (WM59), 1µg/ml CD44-PE (515), 10µg/ml CD29-PE (MAR4), 1.25µg/ml CD73-PE (AD2), 0.625µg/ml CD14-Pe-Cy7 (M5E2), 1.25µg/ml CD45-PerCP (2D1), 50µg/ml CD90-FITC (5E10), 2.5µg/ml CD106-FITC (51-10C9) (BD Biosciences), 5µg/ml CD105-FITC (166707), 3.5µg/ml CD166-PE (105902) (R&D Systems), 1.1µg/ml CD34-APC (AC136), 3.3µg/ml CD133/2-PE (293C3), 0.825µg/ml CD133/2-APC (293C3) (Miltenyi Biotec GmbH), 8µg/ml CD146-FITC (P1H12) (EMD Millipore Corp., Billerica, MA, USA), 0.4µg/ml CD144-PE (16B1) (eBioscience Ltd., Hatfield, UK), or test quantities of mIgG1-PE or mIgG1-APC isotype control (R&D Systems), mIgG1-FITC isotype control (Dako), mIgG1-PE, mIgG1-PerCP, mIgG2a-PeCy7 isotype controls (BD Biosciences), mIgG2b-PE, mIgG2b-APC and mIgG2a-APC isotype controls (Miltenyi Biotec GmbH). Cells were kept on ice, in darkness for 30 minutes then washed and prepared for flow cytometry as described in section 2.4.2.

2.4.2.3 Multicolour flow cytometry

Where multiple fluorochrome-conjugated antibodies were used to stain the same sample of cells, compensation controls were required to correct for spectral overlap. This was performed using the BD CompBeads Plus kit (BD Biosciences).

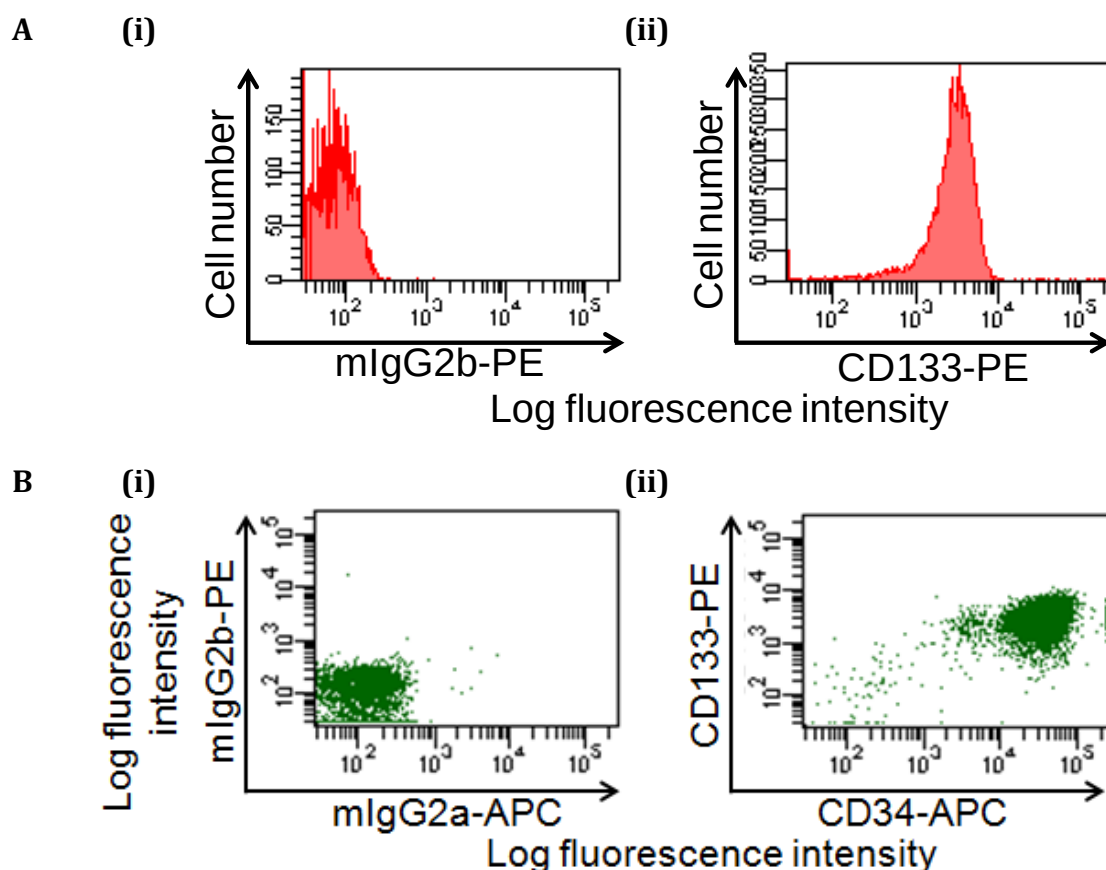


Figure 2.3. CD133 enriched populations express CD133 and CD34. CD133 enriched populations were incubated as described with A (i) mIgG2b-PE, (ii) CD133-PE, B (i) mIgG2a-APC and mIgG2b-PE and (ii) CD34-APC and CD133-PE and assessed by flow cytometry. Plots show histograms or scatter plots of the viable population as assessed on a BD LSR II flow cytometer.

One drop of vortexed anti-mouse Igk polystyrene beads (BD Biosciences), along with 100 μ l MACS buffer and the test volume of a single fluorochrome-conjugated antibody were incubated for 20 minutes at room temperature in the dark. This was repeated for each different fluorochrome used in the experiment. After washing at 425 x g for 5 minutes in a Heraeus Megafuge 1.0S centrifuge, a drop of vortexed Negative Control (BSA) beads (BD Biosciences) was added along with 350 μ l MACS buffer. Each tube was run on the BD LSR II (BD Biosciences) flow cytometer and 5,000 events collected. Compensation was calculated and applied using the BD FACS Diva software (BD Biosciences) (Figure 2.4) after gating as shown in Figure 2.5. A FSC/SSC gate was

applied to the bead population (P1) as demonstrated in Figure 2.5A(i) for PE and Figure 2.5A(ii) for PeCy5 respectively. From this gate the negative and positive populations were gated, as demonstrated for PE and PeCy5 (Figure 2.5B(i and ii) respectively). This determined the percentage of spectral overlap for each fluorochrome, as demonstrated by an example in Figure 2.4.

The difference in PE emission in the FITC channel before and after the compensation had been applied is demonstrated in Figure 2.5C(i) and (ii) respectively. The difference in the PE signal detected in the PE and FITC channel before and after the compensation had been applied is demonstrated in Figure 2.5D(i) and (ii) respectively. Before compensating, the PE signal was distorted due to spectral overlap into the FITC channel. Once the compensation had been applied, the PE signal detected minimal FITC fluorescence.

2.4.2.4 The use of DAPI as a viability stain for flow cytometry

DAPI can be used to exclude dead cells in non-fixed samples using the UV laser as described by Gordon *et al.* (Gordon et al., 2003). DAPI was titrated using concentrations ranging from 0.01µg/ml-5µg/ml (Figure 2.6). Staining of the populations with disrupted membranes, without non-specifically shifting the fluorescence of the live populations, was found to be optimal at a dilution of 1:50,000 (0.1µg/ml) (Figure 2.6B).

DAPI was validated as a dye for detecting non-viable cells by flow cytometry by comparing staining patterns on human UCB mononuclear cells, which had been frozen and thawed immediately prior to staining, to the viability dyes propidium iodide (PI) and to-pro-3 (Figure 2.7). The stained non-viable populations had the same forward and side scatter profile amongst all 3 dyes. The majority of the non-

viable cells (shown in purple for PI, blue for top-ro-3 and green for DAPI) had a low forward scatter (Figure 2.7A-C(i)). The use of DAPI enabled the use of a viability indicator with all of the fluorochromes used in this thesis and in multi-colour flow cytometry experiments. This is because DAPI is the only fluorophore used here to be excited by the UV laser and the emission wavelength (455nm) does not spill into any other channels.

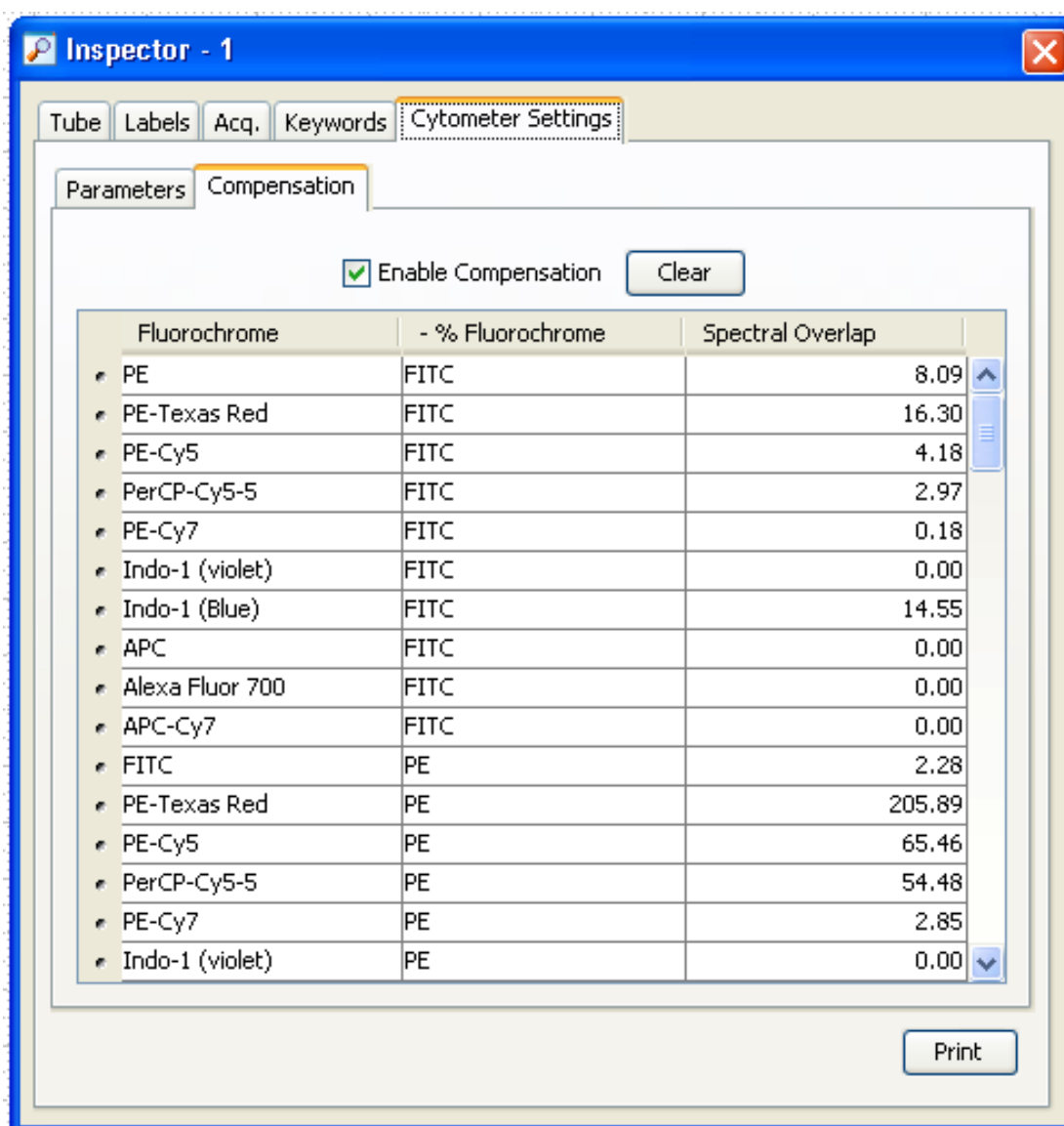


Figure 2.4. Calculating compensation using FACS Diva software. Once each compensation control had been run and the negative and positive bead populations gated (Figure 2.5), compensation was calculated and applied automatically with no manual adjustment.

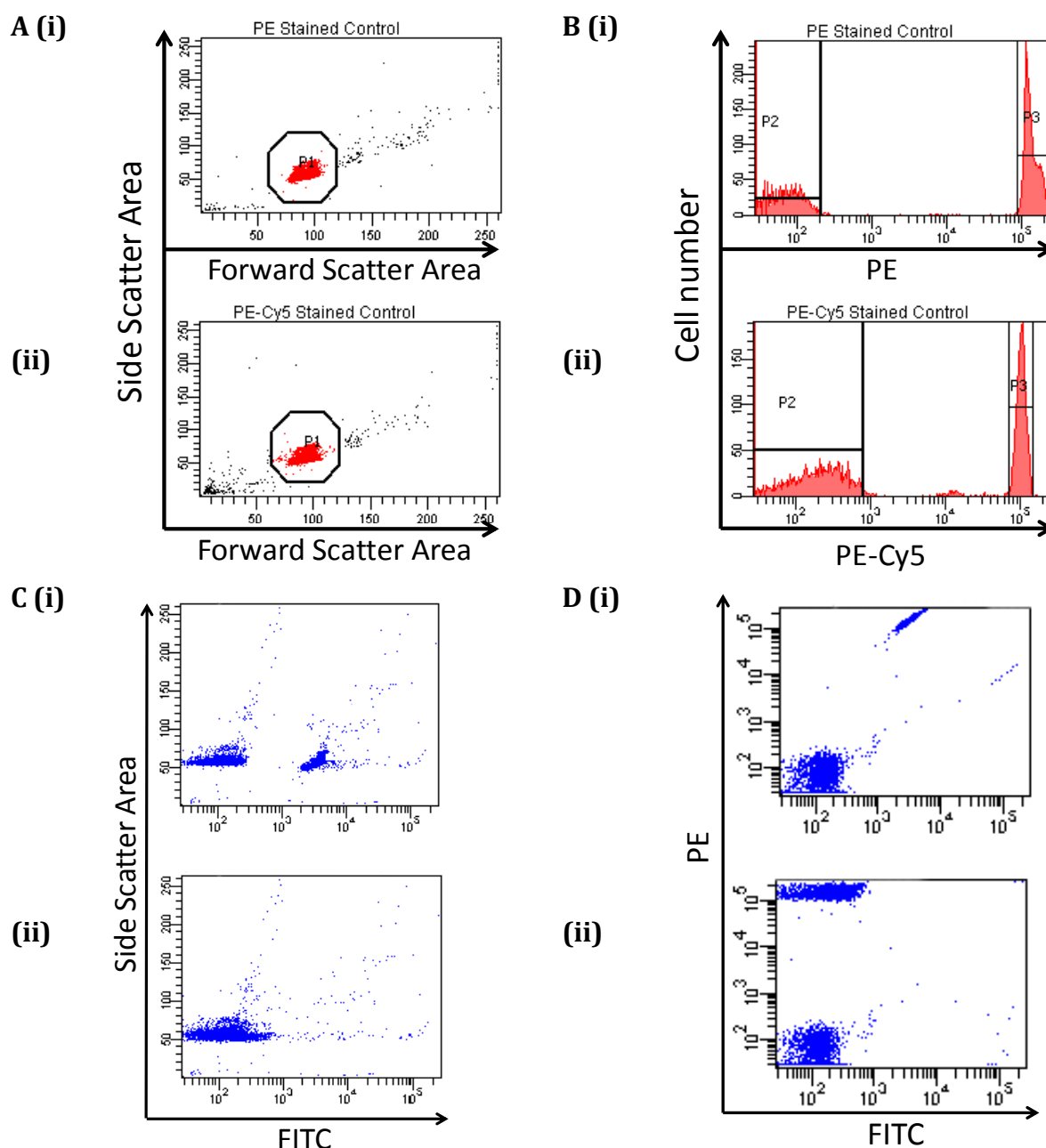


Figure 2.5. Representative compensation controls. Representative compensation set-up demonstrating A) forward scatter (area) vs. side scatter (area) gating on the beads (P1 gate) for (i) a PE stained compensation control and (ii) a PeCy5 stained compensation control. B) Histograms showing negative (P2 gate) and positive bead populations (P3 gate) for (i) PE and (ii) PeCy5 stained beads are shown. C) Side scatter (area) vs. FITC log fluorescence dot plots demonstrate the spectral overlap by PE (i) before and (ii) after compensation was calculated and applied using FACS diva software (Figure 2.4). D) PE vs. FITC log fluorescence dot plots demonstrate (i) the spectral overlap of PE emission into the FITC channel before compensation had been applied also affected the PE signal but this was corrected in (ii) after compensation was calculated and applied using FACS diva software.

PI and to-pro-3 use restricts the amount of compatible fluorochrome conjugated antibodies for single staining and would generally require compensation for multi-colour staining. Figure 2.7 compared the percentage of viable cells detected by the three dyes. Based on the negative gates (no dye control) (Figure 2.7A-C(ii)), it is clear that DAPI is the only dye that does not cause a fluorescence shift in the entire population (Figure 2.7C(iii)). This is most evident using to-pro-3 where the percentage of non-viable cells was 13.8% based on the non-stained control gating (Figure 2.7B(ii)). PI and DAPI gave non-viable percentages of 10.7% and 9.9% respectively (Figure 2.7A and C(iii) respectively).

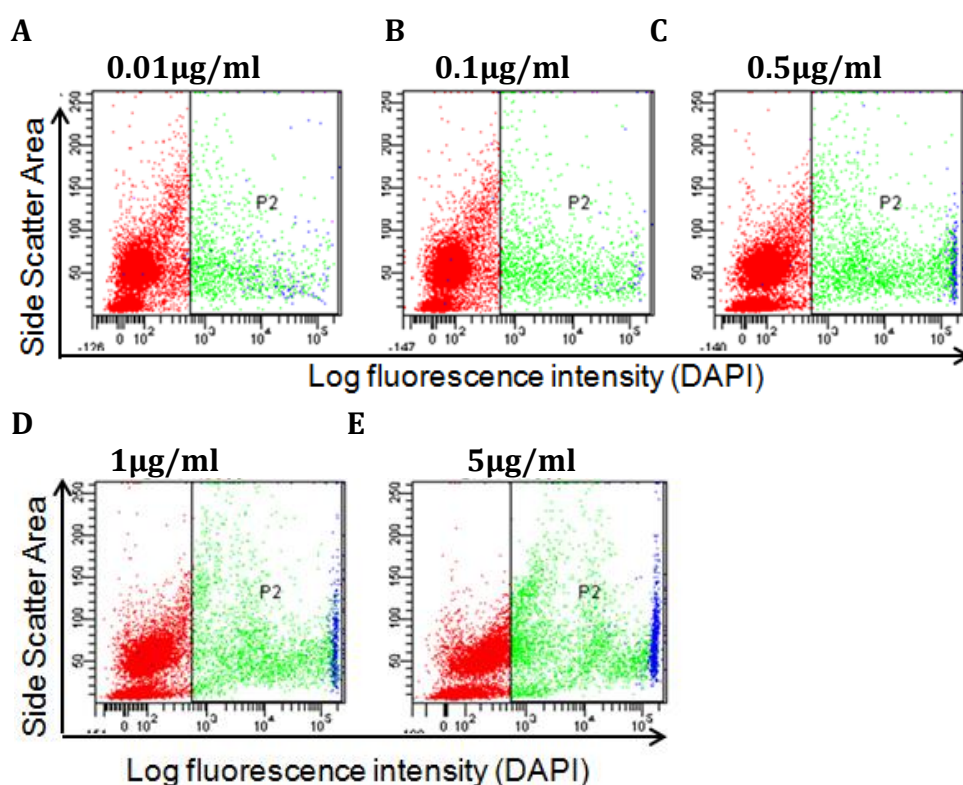


Figure 2.6. Titrating DAPI on mononuclear cells. Mononuclear cells from umbilical cord blood which had been thawed from frozen to ensure some non-viable cells were aliquoted into FACS tubes. Prior to running the samples on a BD LSR II flow cytometer, DAPI was added at concentrations of A) 0.01µg/ml, B) 0.1µg/ml, C) 0.5µg/ml, D) 1µg/ml, E) 5µg/ml. Non-viable cells were identified by emitting fluorescence after excitation of the DAPI bound to cellular double stranded DNA of cells with disrupted membranes using the UV (355nm) laser. As DAPI concentration was increased, the cell populations shifted to the right in the fluorescence channel.

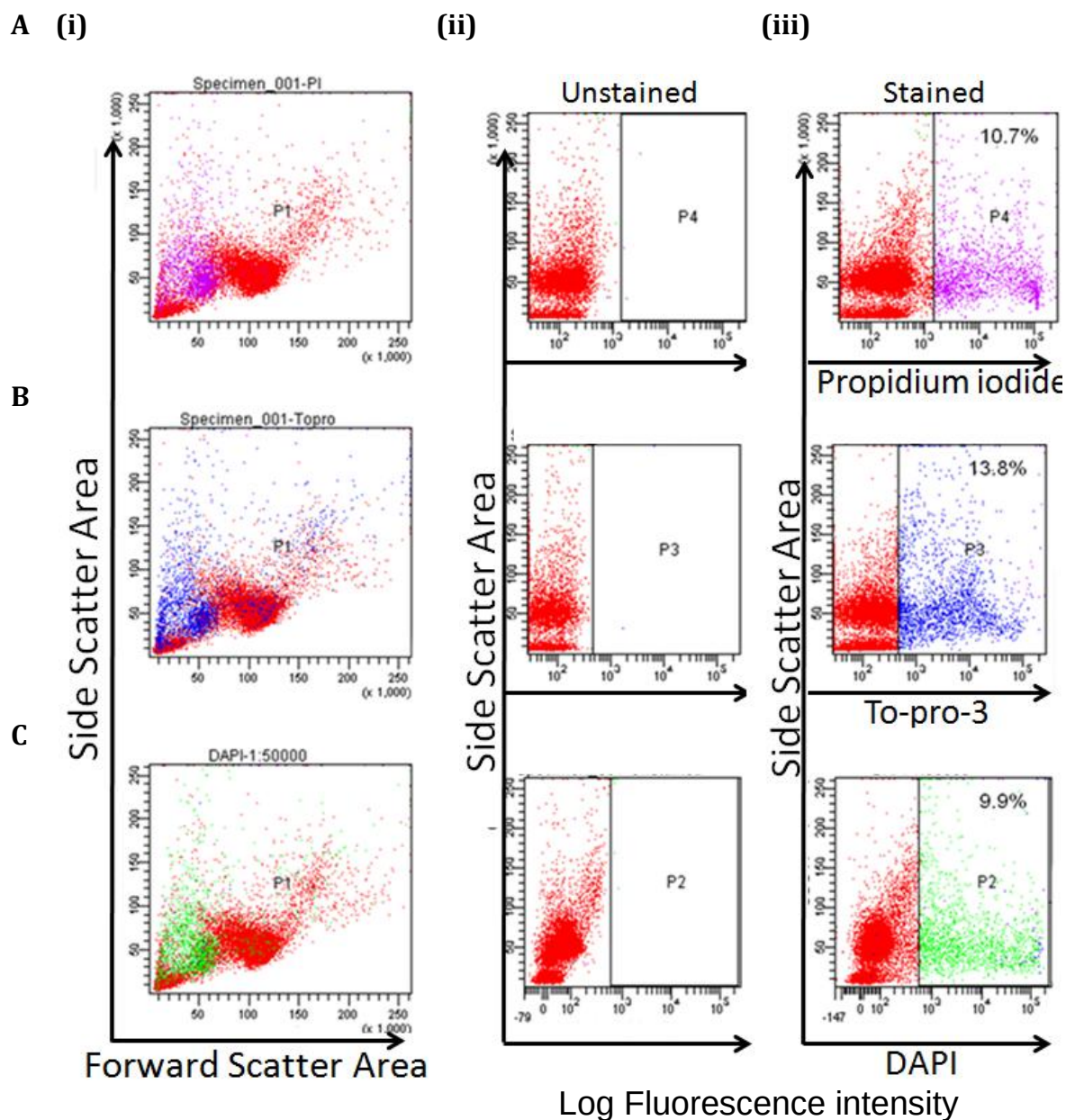


Figure 2.7. Comparing DAPI to PI and to-pro-3 viability dyes. Thawed human MNCs were used to compare non-viable cell staining to existing dyes using optimal concentrations of A) propidium iodide (1:100), B) to-pro-3 (10nM) and C) DAPI (0.1 μ g/ml) were added prior to running the sample. Scatter plots showing (i) FSC vs. SSC showing non-viable populations (purple, blue, green) determined by fluorescence minus one gating using the non-stained controls for each fluorochrome (ii) to provide the positive (non-viable) population in the presence of the viability stains (iii).

2.5 shRNA amplification and lentiviral vector production

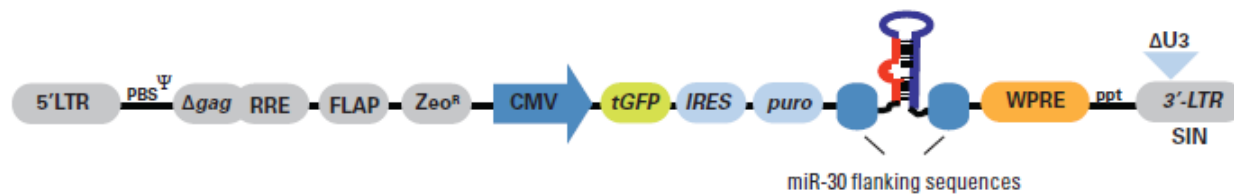
2.5.1 Plasmids

Two human pGIPZ shRNA vector clones targeting CD164 (A-B) and a non-silencing shRNA control were purchased as glycerol stocks from Open Biosystems (Thermo Fisher Scientific Inc., Lafayette, USA). The hairpin sequences and identification are detailed in Table 2.3. The pGIPZ construct is shown in Figure 2.8A and the vector elements described in Table 2.4. The inclusion of turboGFP (green fluorescent protein) gene allowed identification of transfected cells by fluorescence. The SINHIV-1eGFP glycerol stock, described by Demaison *et al.* was obtained from Professor Adrian Thrasher, UCL, London, along with the packaging plasmid Δ 8.91 and envelope plasmid VSVg. These 3 plasmids were co-transfected to generate lentiviral particles (describe in section 2.5.1.5). This second generation self inactivating (SIN) amphotrophic HIV-based lentiviral vector expressed enhanced GFP (eGFP) under control of the spleen focus forming viral (SFFV) promoter (Demaison et al., 2002), and was used as described by Zhang *et al.* (Zhang et al., 2009). The construct is shown in Figure 2.8B and the vector elements described in Table 2.4.

Table 2.3. pGIPZ shRNA mir oligo sequence details

Name	Oligo ID	Hairpin sequence (sense loop antisense)
CD164-A	V2LHS_71805	TGCTGTTGACAGTGAGCGAAGGCAGATACTGCTGTATTTATAG TGAAGCCACAGATGTATAAATACAGCAGTATCTGCCTGTGCCT ACTGCCTCGGA
CD164-B	V2LHS_71808	TGCTGTTGACAGTGAGCGCGCAGATGTTCTTCTATCAAATTAG TGAAGCCACAGATGTAAATTTGATAGAAGAACATCTGCATGCCT ACTGCCTCGGA

A



B



Figure 2.8. Vector genomes. Diagram illustrating A) the pGIPZ shRNA genome (image taken from www.openbiosystems.com) and B) eGFP vector genome (taken from Forde S, DPhil thesis, University of Oxford, UK).

Table 2.4. Vector element functions

Vector element	Function
CMV Promoter	RNA Polymerase II promoter
FLAP (containing a central polypurine tract (cPPT) and central termination site (CTS))	Required for efficient translocation into the nucleus of non-dividing cells and minus-strand DNA synthesis
Primer binding site (PBS)	Initiates reverse transcription
HIV-1 psi (Ψ)	Packaging signal including a 5' terminal fragment of the gag sequence
WPRE (woodchuck post-transcriptional regulatory element)	Enhances the stability and translation of transcripts
turbo (tGFP)/enhanced (eGFP)	Green fluorescent protein marker
IRES	Internal ribosome entry site
SFFV promoter (spleen focus forming viral)	Promoter which drives expression from the enhancer region (U3-LTR) of SFFV
cPPT (central polypurine tract)	Facilitates initiation of plus strand DNA synthesis
Puro ^r	Puromycin resistance (mammalian selectable marker)
AMP ^r	Ampicillin resistance (bacterial selectable marker)
pUC ori	High copy replication and maintenance in <i>e.coli</i>
5'LTR	5' long terminal repeat
3' LTR	3' long terminal repeat lacking enhancer elements in $\Delta U3$ to prevent native transcriptional activity
SIN (self-inactivating)	Self inactivating vector due to the deletion in the U3 region of the 3'LTR
RRE	Rev response element (env fragment)
ZEO ^r	Zeocin resistance (bacterial selectable marker)

2.5.1.1 Transforming vectors into JM109 competent cells

The packaging vector (pΔ8.91), envelope vector (pVSV-G) and the lentiviral eGFP vector were individually transformed into *E. coli* JM109 competent bacteria (Stratagene, Agilent Technologies UK Ltd., Wokingham, UK). This was performed by thawing the competent bacteria on ice, mixing, and then aliquoting 100μl bacteria and 0.8μl β-mercaptoethanol into each of four pre-chilled Falcon tubes (one for the pUC18 control and one for each vector). The bacteria were incubated on ice for 10 minutes and swirled every 2 minutes. The vector DNA was diluted to 20-30ng/μl in nuclease free water (Invitrogen Ltd.) and 1μl vector DNA (or 1μl pUC18 control) added to the relevant tubes. The tubes were swirled gently and incubated on ice for a minimum of 20 minutes. The tubes were then heat pulsed in a 42°C water bath for 45 seconds, then incubated on ice for 2 minutes. Pre-heated (37°C) SOC medium (0.9ml) (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate, 20mM glucose per litre) (Invitrogen Ltd.) was added and the tubes shaken at 225-250rpm for 1 hour at 37°C in an Innova 4200 incubator (New Brunswick Scientific, Enfield, CT, USA). Luria Bertani (LB) agar was made by dissolving 35g of LB agar powder (Sigma Aldrich Ltd.) in 1 litre of distilled water and heating to boiling point with regular swirling in a microwave. 100μl of each transformation mixture and 5μl of the pUC18 control transformation mixture were plated, under sterile conditions, onto LB agar plates containing 100μg/ml ampicillin. The plates were incubated overnight at 37°C in an incubator.

2.5.1.2 Amplifying DNA from a single colony or glycerol stocks

Bacteria containing plasmids from glycerol stocks (see section 2.5.1) or one bacterial colony from a transformation were inoculated into 2ml LB broth (made by dissolving a 1.1g tablet from Sigma Aldrich Ltd. (composed of 10 g/l enzymatic digest of casein, 5 g/l yeast extract (low sodium), 5 g/l sodium chloride, 2 g/l inert agents, and 22 g/l total solids) in 50ml water and autoclaving) containing 100µg/ml ampicillin in 50ml Falcon tubes (BD Biosciences). This mixture was shaken at 250rpm at 37°C for 4-8 hours before the mini-culture was inoculated into autoclaved LB broth containing ampicillin in 1l conical flasks (or 200ml for high copy plasmids e.g. pVSV-G and eGFP and 250ml Luria broth containing ampicillin for low copy plasmids e.g. Δ8.91 and pGIPZ plasmids). The flasks were shaken for 18-19 hours at 37°C. The bacterial pellet was then harvested by centrifugation at 4070 x g for 20 minutes at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). All supernatant was removed and the pellets were frozen in airtight conditions (parafilm sealed bucket centrifuge tubes) at -20°C.

2.5.1.3 Purifying lentiviral DNA

The plasmid DNA amplified in 2.5.1.2 was purified using an Endofree plasmid Giga or Maxi prep kits (Qiagen Ltd., Crawley, UK) as described in the manufacturer's protocol. To extract the DNA, the frozen pellet was brought to room temperature and re-suspended in lysis solution in the bucket centrifuge tubes. The lysis solution consisted of RNase A solution in buffer P1 solution to give a final concentration of 100µg/ml with 1:1000 LyseBlue reagent. The solution was vortexed and pipetted up and down until the bacteria were re-suspended completely. Buffer P2 (containing sodium hydroxide) was then added and the solution mixed thoroughly by inverting vigorously until the whole mixture had turned blue over a 5 minute period at room

temperature. The DNA was then precipitated using chilled buffer 3 (containing potassium dodecyl sulphate and acetic acid) and vigorously inverted until the mixture became colourless and non-viscous. The precipitated lysate was run non-agitated through a QIAfilter cartridge at room temperature over a 10 minute period before gently depressing the plunger to filter the lysate. Buffer ER was then added to the lysate, inverted 10 times and incubated on ice for 30 minutes. A Qiagen-tip 500 column was equilibrated with QBT buffer before applying the filtered lysate. The column was then washed twice with buffer QC. The DNA was then eluted from the column with buffer QN and precipitated with 0.7 volumes of room temperature isopropanol. This was immediately centrifuged at 4460 x g for 60 minutes at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare) and the supernatant decanted. The pellet was re-suspended in 70% (v/v) ethanol to remove precipitated salt and displace the isopropanol and the tubes centrifuged at 4460 x g for 5 minutes at 4°C in a Hettich Rotina 46R centrifuge (DJB Labcare). The supernatant was decanted and the remaining ethanol evaporated for 10 minutes at room temperature or in a low heat block until the pellet was dry. The DNA pellet was re-dissolved in nuclease free water (Invitrogen Ltd.) (300µl for high copy plasmids and 100µl for low copy plasmids) overnight at 4°C. The purified DNA was stored at -20°C.

2.5.1.4 Determining DNA concentration using the NanoDrop spectrophotometer

The concentration and purity of the DNA purified in 2.5.1.3 was measured using the NanoDrop Micro-Volume UV-Vis Spectrophotometer for Nucleic Acid and Protein Quantitation (Thermo Fisher Scientific). The NanoDrop spectrophotometer was zeroed using 1µl nuclease free water (Invitrogen Ltd.). DNA (1µl) was then applied to the NanoDrop spectrophotometer and absorbance of the sample at 260nm used to

determine DNA quantity. The purity of the sample was also determined by dividing the absorbance at 260nm by the absorbance at 280nm.

2.5.1.5 Co-transfection of plasmids

The lentiviral eGFP vector was produced by co-transfection of the three plasmids (50µg eGFP, 17.5µg VSV-G and 32.5µg Δ8.91) into HEK293FT cells. This was achieved using Lipofectamine 2000. Two T75 cell culture flasks containing 90% confluent HEK293FT cells pre-cultured in DMEM (Lonza Biologics) with 10% (v/v) FCS (PAA Laboratories GmbH) without geneticin were used to propagate the lentiviral vector. 3.75ml OptiMEM I medium (Invitrogen Ltd.) was added to two Falcon tubes and then 200µl lipofectamine 2000 (Invitrogen Ltd.) was added to tube one, whilst 50µg eGFP, 17.5µg pVSV-G (envelope protein) and 32.5µg pΔ8.91 (packaging plasmid) were added to tube two. The contents of tube one was added to tube two and mixed by inverting 5 times, followed by a 20 minute incubation at room temperature. The transfection mix was then divided between the two T75 flasks of HEK293FT cells, each containing 15ml serum free DMEM medium. The flask was incubated overnight in a Nuaire DH autoflow incubator at 37°C (5% CO₂) and the media replaced in the morning. The medium containing crude lentiviral vector particles was harvested 48 hours after transfection, centrifuged at 350 x g for 5 minutes in a Hettich Rotina 46R centrifuge at room temperature and frozen in 0.5-1ml aliquots at -80°C.

2.5.1.6 Lentiviral titrations

The supernatant containing lentiviral vector particles harvested in 2.5.1.5 was titred using serial dilutions of the vector to transduce HEK293FT cells previously cultured in standard conditions for 24 hours in 24 well plates (5x10⁴ cells seeded per well) (Corning). Dilutions were made as described in Table 2.5 in serum free DMEM (PAA

Laboratories GmbH) and applied to the 24 well plate as listed. After a 4-6 hour incubation in a Nuaire DH autoflow incubator at 37°C (5% CO₂), the transduction (lentiviral) solution was removed and replaced with 1ml of DMEM with 10% (v/v) FCS and 1% (v/v) 1x Pen-Strep (both from PAA Laboratories GmbH). The titre was deduced after culturing the cells for 48 hours in a Nuaire DH autoflow incubator at 37°C (5% CO₂) by counting green colonies under a Nikon Eclipse TE300 microscope (Nikon UK Ltd). The transducing units (TU)/ml were calculated using the formula:

Number of fluorescent colonies counted x dilution factor x 40 = TU/ml

The eGFP lentiviral titres achieved were between 1.5×10^6 – 3×10^7 TU/ml.

Table 2.5. Method for producing serial dilutions of the eGFP lentiviral vector

Preparing serial dilutions (96 well plate)			Applying viral vectors (24 well plate)			
Well	DMEM (µl)	Viral vector (µl)	Destination well	DMEM (µl)	Volume diluted viral vector (µl)	Dilution (fold)
A1	80	20 (neat)	A1	225	25	5
A2	80	20 (from A1)	A2	225	25	25
A3	80	20 (from A2)	A3	225	25	125
A4	80	20 (from A3)	A4	225	25	625
A5	80	20 (from A4)	A5	225	25	3125
A6	80	20 (from A5)	A6	225	25	15625
A7	80	20 (from A6)	B1	225	25	78125
A8	80	20 (from A7)	B2	225	25	390625

2.5.1.7 Transducing HUVECs with the eGFP lentiviral vector

The multiplicity of infection (MOI) for HUVEC transduction was optimised to between 4-7 using the eGFP lentiviral vector by Zhang *et al.* (Zhang et al., 2009). Three batches of isolated HUVECs (see 2.2.2.2) were transduced with the eGFP lentiviral vector at an MOI of 7. Neat lentiviral vector was applied to the required volume of serum free EGM-2 medium (Lonza Biologics) to obtain the desired MOI. After a 4-6 hour incubation in a Nuair DH autoflow incubator at 37°C (5% CO₂), the lentiviral particle containing media was removed and replaced with EGM-2 complete media (Lonza Biologics). Greater than 98.9% transduction efficiency was achieved in all three batches of HUVEC at 2 days post transduction (Figure 2.9A). eGFP expression was shown to persist after the HUVECs had been cultured under standard conditions for 3 weeks. The effect of eGFP lentiviral transduction on cell growth in all three batches transduced with eGFP lentiviral vector was assessed by counting the number of viable cells harvested at each passage following transduction. This was compared to the same batch of cells cultured without transduction. Although there was an initial reduction in cell number, this had recovered within two passages for all batches (Figure 2.10).

2.5.1.8 Transducing UCB endothelial cells and BMEC-60 cells with eGFP

BMEC-60 and endothelial cells from UCB were transduced when 70% confluent at an MOI of 5-10 using the eGFP lentiviral vector. Neat thawed lentiviral vector supernatant was applied to the required volume of serum free EGM-2 medium (Lonza Biologics) to obtain the desired MOI. After a 4-6 hour incubation in a Nuair DH autoflow incubator at 37°C (5% CO₂), the lentiviral particle containing medium was removed and replaced with EGM-2 complete medium (Lonza Biologics).

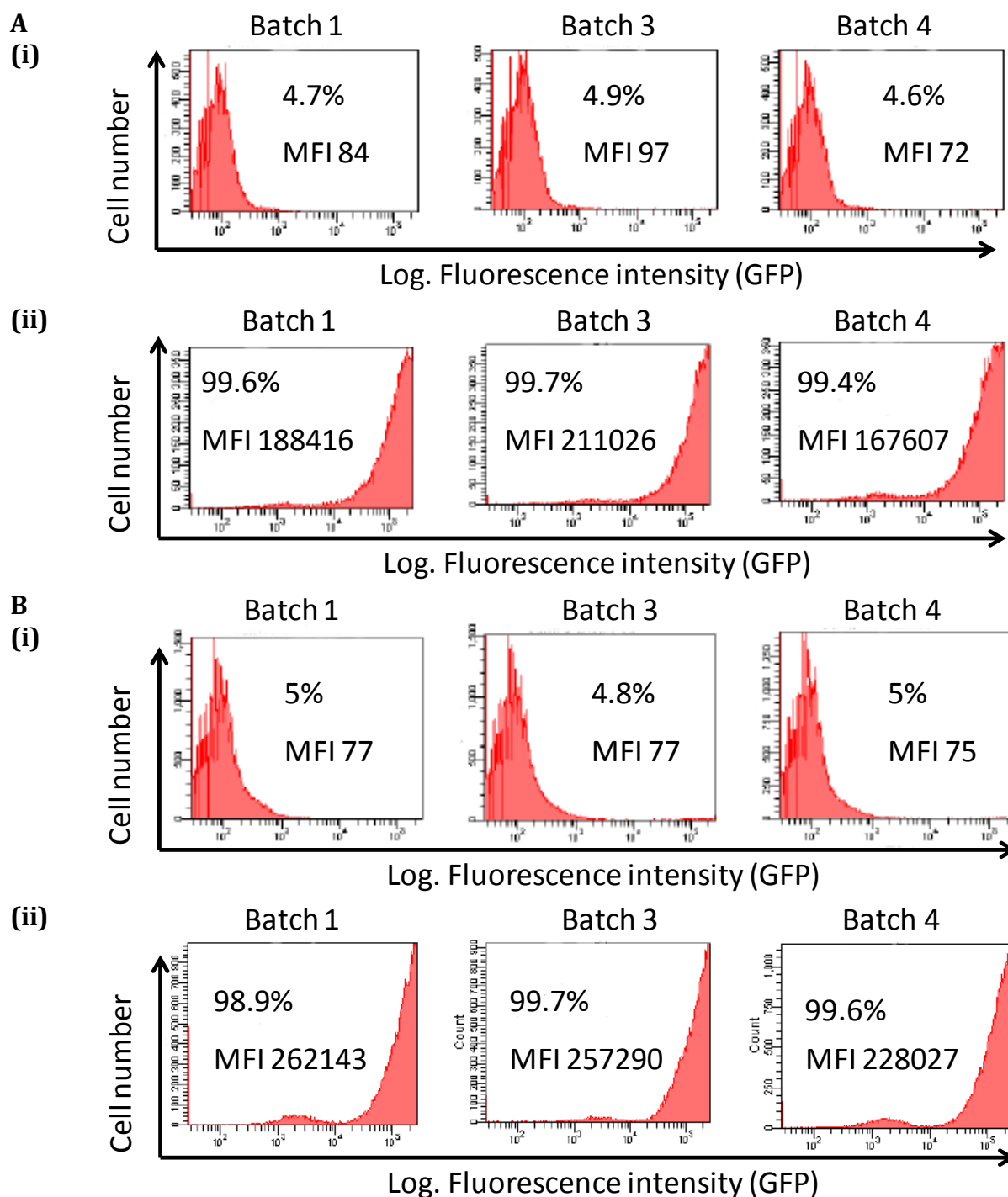
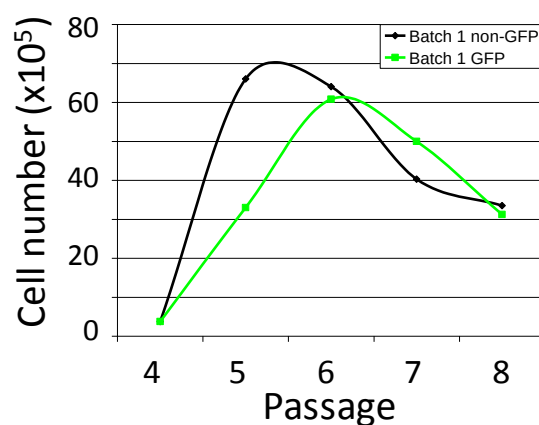
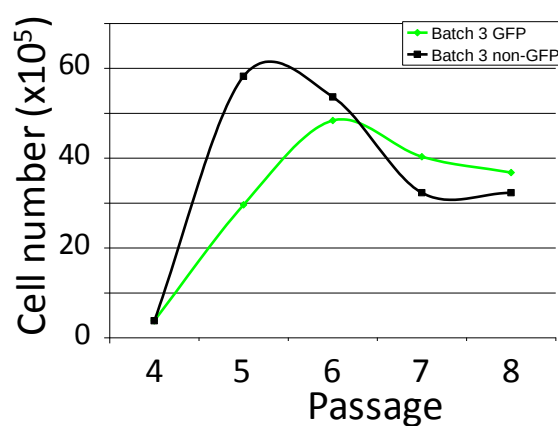


Figure 2.9. HUVECs transduced with eGFP retain fluorescence in culture. Three batches of HUVECs (P3) were transduced with the eGFP lentiviral vector at an MOI of 7. Representative histograms are shown and display median fluorescence intensity (MFI) and the % cells positive for eGFP. A) Transduction efficiency was assessed by flow cytometry two days post transduction (ii) for eGFP expression compared to (i) non transduced cells. B) eGFP expression was shown to persist in culture for at least 3 weeks (P8) as assessed by flow cytometry in (i) non transduced and (ii) eGFP transduced cells.

A



B



C

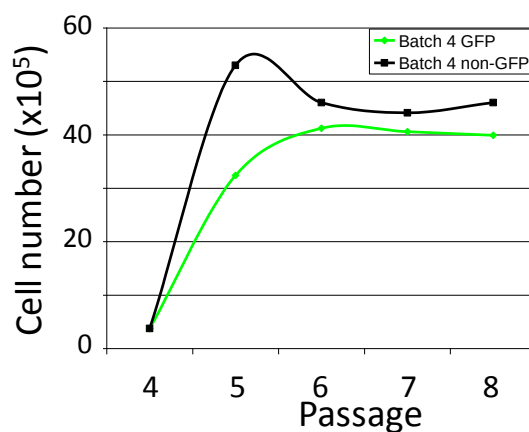


Figure 2.10. Assessing the effect of transduction with eGFP on HUVEC growth. Three batches of HUVECs (P3) were transduced with the eGFP lentiviral vector at an MOI of 7. The growth rate of A) batch 1, B) batch 3 and C) batch 4 eGFP transduced HUVECs (green line) was compared to non-transduced HUVECs (black line) by counting the number of cells harvested at each passage (n=1).

2.6 Nucleofection

2.6.1 siRNA oligo sequences

The CD164 siRNA targeting sequences CD164-1 and 2 described by Forde *et al.* (Forde et al., 2007) were custom synthesised by Sigma Aldrich Ltd. A pool of non-targeting control siRNAs (ON-TARGETplus) was also purchased (Dharmacon, Thermo Fisher Scientific Inc.). The sequences are specified in Table 2.6. The shRNA pGIPZ vector sequences (detailed in 2.5.1) produced as described in sections 2.5.1.2, 2.5.1.3 and 2.5.1.4 were also used. The location the sense RNA targets for the siRNA and shRNA on the CD164 structure and full sequence are shown using colour coding in Figure 2.11 and Figure 2.12 respectively.

Table 2.6. siRNA sequence details

siRNA ID	Sequence
CD164-1	5'-CCCGAACGUGACGACUUUA-3'
CD164-2	5'-GGACUGGUGAUUCAUUUGU-3'

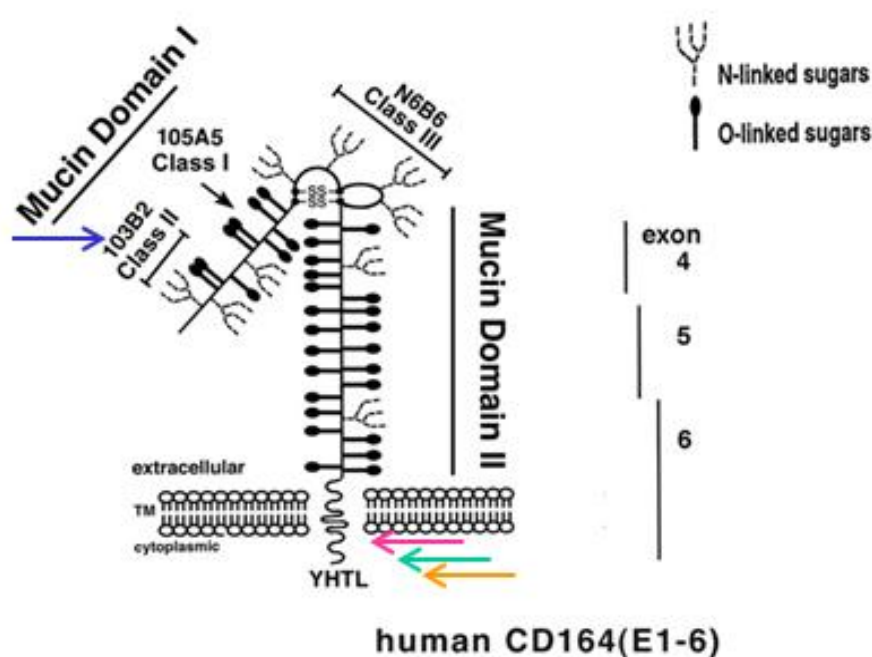


Figure 2.11. Diagrammatic protein structure of human CD164. The coloured arrows mark the respective siRNA and shRNA targets. Image taken from Chan J Y *et al.* J. Biol. Chem. 2001;276:2139-2152 (Chan et al., 2001).

Exon 1	
ATG TCG CGG CTC TCC CGC TCA CTG CTT TGG GCC GCC ACC TGC CTG GGC GTG CTC TGC GTG	60
M S R L S R S L L W A A T C L G V L C V	
CTG TCC GCG GAC AAG AAC ACG ACC CAG CAC <u>CCG AAC GTG ACG ACT TTA</u> GCG CCC ATC TCC	120
L S A D K N* T T Q H P N* V T# T# L A P I S#	
-->	
AAC GTA ACC TCG GCG CCG GTG ACG TCC CTC CCG CTG GTC ACC ACT CCG GCA CCA G/AA ACC	180
N* V T# S# A P V T# S# L P L V T# T# P A P E T	
TGT GAA GGT CGA AAC AGC TGC GTT TCC TGT TTT AAT GTT AGC GTT GTT AAT ACT ACC TGC	240
C\$ E G R N S C\$ V S C\$ F N* V S V V N* T T C\$	
Exon 3	
TTT TGG ATA GAA TGT AAA G/AT GAG AGC TAT TGT TCA CAT AAC TCA ACA GTT AGT GAT TGT	300
F W I E C\$ K D E S Y C\$ S H N* S T V S D C\$	
Exon 4	
CAA GTG GGG AAC ACG ACA GAC TTC TGT TCC G/TT TCC AC GGC ACT CCA GTG CCA ACA GCC	360
Q V G N* T T D F C\$ S V S# T# A T# P V P T# A	
Exon 5	
AAT TCT ACA G/CT AAA CCC ACA GTT CAG CCC TCC CCT TCT ACA ACT TCC AAG ACA GTT ACT	420
N* S# T# A K P T# V Q P S# P S# T# T# S# K T# V T#	
Exon 6	
ACA TCA G/GT ACA ACA AAT AAC ACT GTG ACT CCA ACC TCA CAA CCT GTG CGA AAG TCT ACC	480
T# S# G T# T# N* N T# V T# P T# S# Q P V R K S# T	
TTT GAT GCA GCC AGT TTC ATT GGA GGA ATT GTC CTG GTC TTG GG\T GTG CAG GCT GTA ATT	540
F D A A S F I G G I V L V L G V Q A V I	
TTC TTT CTT TAT AAA TTC TGC AAA TCT AAA GAA CGA AAT TAC CAC ACT CTG TAA	594
F F L Y K F C K S K E R N Y H T L STOP	
ACAGACCCATTGAATTAATAAGGACTGGTGAATTCATTTGTGTAACCTACTGAAGCCAAATACTACTCTTTTAAGATGTCCACATGGAAGACGCT	689
Signal	
ATTCCAGGATCTTTAAATTTCCATGGATGCATATAGGATGTTTGGGAGCATCATCCGTGAAGAAAAATCAATTAAATCATTTGTGTTCAACAGGA	784
PolyA-1	
ATATTTAAAAATATCTGCATG AATCCTGTGGCTGTCTTATTTTAAATAGCTGTCTGTGGGATTATATTTTTTTCCTTAACATGCCAAATAT	878
AACTTTCTGAAAGTGATGGAATGTTGTCTTGTGCAGACAACATCATGGCTCTTGGCAGTTTAAATTTAGTAATTTTAAATTTAGTGAACAGAAT	973
TGAGAAGAACGTGCCAAATGAGAATCAATTAGGTGGATTTTGGCTGTCAITTCAAAAGTGGAAATAAATTTATTAATTTAGTAGTACTAAATGGT	1068
ATCCTTAGATTAATAATTTGTGCTTGATAACAGCTGTTTTTCTACATTAG\AAATAAGATGCCACACAGGAATACATTCAGATTTAAAGAA	1162
E I R C H T R N Y I P D L Y Y	
A\TGAAAGGATACCATTTAGTGTGTATAACAGATTATTGTTTCATACTTGTAAGCATCTTATGTCTTGGAGTATAAAGAACAGTGCCTTAGAAG	1256
ACAGTGAAAGGTAAAGCTAGCTTAATGTCTATGATTGTTCTTTGACATTAAGGAAGTAAAGGATTGGTCAGAGGATGTAACCTGATGTGAGCA	1351
TAGTAAACCTGTTTATGATATCATACTGTTAATATTTTATTGAAATTTATTTTCAGAGCGGAGAACTTAAGCTAAAGTCTGTTATACAGAATT	1446
GAAAGCCTTCGTATCTTGAACCTCCCAACATTTTCTTATGGCTGTGAAAGTATAGAGCTAAATTTGATTTAATTACACTTTCCTTTTGACTTTT	1541
MGC-GP-F4	
AAAAAAAGTATGCTAGCACTATT/GTACCTTGAAAGGATTTCCACCAGAC/TGCTCTGAGTAGTGACTTCTTTGGTGAGGCAAGAAGGATATAC	1634
ATTATTTTAGAATCATTTACTATTAAATGAGACAATCATATTATTTAGAAATCATTTTAAATGAGACAATCATTTTAAAGTTTAAAGATAA	1729
CAGAAGTGACCAATGTAATTTTCAACACCTAAGGATTTTGGTTGATCAGGTTACTGTAGATTTTACTGATTGCTCGGATGAATAGACTGT	1824
GCCTTTCTTTTCTCTCCCTTCTTCTTCCATAGTATAAATAGCATGCATACCTTTAAGTCTATAGTTTCTCCTTTAGAGGGTCTGTC	1919
TTTCAAGTTTATAGAGGTTTACTTCTCCCTTGCCCTTGACTCATTGGACTAGTGACAGAGGCTTTAAGTAGTTTAAATGGGCTTTTGCTTTTCTAGGT	2014
MGC-GP-B4	
CATTACGTTTTTTTATTTAGTTTCTTTAGCC/AAATAGTGGCTGAGTTTCGCACTTG/ATTTTCAATATTTTATAGTAAGAAATGACAACTGCTT	2107
Signal	
GTGTTTCATTTTCAATAACAACTCTGCATTTAGATAACTATTAAAGGGTGTGTAAG ATGAAGATTTACTGTTTCTTTGTTACTCGTTGGTACAGCT	2201
GTTTGTTTTACTTGCACATTTGTACATATACTTAATGTTTCAAGTGCCTTAATGTTTAAATCTCTGGCTTCAAGTTTCTTGGGGAAGGTC	2296
GGTTTACCTCACATTTTGTTCATTAGTAATATTCTAGGTACCTCACAAATGTATTATGGTGCCATGGCTGTTAGTTTATTTAGTGAGTGCTG	2391
MGC-F7	
TAGGATTAATTCGAAATAGGCAGAA/TTCATTCTCCCAAGGTGG/CAAAATAGCTATACTGATGTAATTGTCATTACCTGGGTATGAAT	2484
TCCCTGACACACATTTATGTCAACATATGTAGCAAAATTTGTGAAACATAACAATTTGAAGCTTCTGTAATTTTGAGCACTGCTCTAACAAACA	2579
GCATAATATAAAATAGTTAGATTTTGCAAGTCTACAAATGAGCTCTTGCAACAGAACTCACAGCCTTTTACTTTTCCCTTAACCTTAGCAA	2674
TGTAGTATCTTGAGCCATTAATTTTGGGTTTTTTTAAATCCAGAAGGTATATAGAAACCTTTTCAGATTTTTCATCTGATTTGTTCTTGCAGA	2769
CD164-PolyA-3R	
TGTTCTTCTATCAAAATACCTTATTTTACCTTAC/AGATATTTGTTGCACAGGCAGA/TACTGCTGTATTTTACAGATTTCTATTTTCAGTTTCATTAA	2862
Signal	
AAACTGCAAAACCAATCTGTATCATGTACCAAACTGACTTAAATAAATTACATGTTTATTGAATT	2929
PolyA-3	

Figure 2.12. cDNA sequence of human CD164. cDNA sequence showing the location of the siRNA sequence by coloured underlining for CD164-1 (blue), CD164-2 (pink), and the shRNA sequences CD164-A (orange) and CD164-B (green). Sequence taken from Chan J Y *et al.* J. Biol. Chem. 2001;276:2139-2152 (Chan et al., 2001).

2.6.1.1 *The method*

The Amaxa human CD34 cell nucleofector kit (VPA-1003) (Lonza Biologics) was used to transfect the siRNA duplexes detailed in Table 2.6 or shRNA plasmid DNA detailed in Table 2.3 into human UCB CD133⁺ cells. CD133⁺ cells, isolated and frozen as described in section 2.2.2.3, were thawed and cultured in stemspan medium (Stem Cell Technologies) with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (R&D Systems) for 1-2 or 24 hours prior to nucleofection as indicated for each experiment. In the final optimised experiments, 5×10^5 - 1×10^6 cells were centrifuged at 350 x g for 10 minutes at 22°C in a Hettich Rotina 46R centrifuge (DJB Labcare) and all of the supernatant was removed. For each sample, 100µl nucleofector solution (Lonza Biologics) pre-warmed to room temperature was used to re-suspend the cell pellet. This was added to either 2-5µg pGIPZ plasmid shRNA or 30-1200nm siRNA (final concentration) as indicated for each experiment and immediately transferred into a cuvette (Lonza Biologics). The cuvettes were placed in the Amaxa Nucleofector II (Lonza Biologics) one at a time and the pre-defined programs U-001 or U-008 were applied. Immediately after, 500µl pre-warmed stemspan medium (Stem Cell Technologies) with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (R&D Systems) was added to the cuvette. The entire content of a cuvette was transferred to a well of a 12 well plate containing 1.5ml pre-warmed stemspan (Stem Cell Technologies) with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (R&D Systems). Cells were then returned to the incubator in the 12 well plates for 24 hours before checking cell viability, transfection efficiency and knock-down using flow cytometry. The results from the optimisation of the Amaxa nucleofector program and the concentration of siRNA/shRNA are described in section 4.4.1.3 and Appendix I.

2.7 Migration assays

2.7.1 2D μ -slide chemotaxis assays

The chemotaxis assay was initially developed for adherent cells using the 2D μ -slide chemotaxis product (Ibidi GmbH, Munich, Germany). Endothelial cells from UCB or HUVEC (section 2.2.2.2) were passaged using trypsin-EDTA (PAA Laboratories GmbH). Importantly, the EBM-2 medium with 0.5% FCS (v/v) (Lonza Biologics) and the μ -slides were pre-equilibrated at 37°C in a Nuaire DH autoflow incubator (5% CO₂) 24 hours prior to the experiment. Cells were re-suspended at 2-3x10⁶ cells/ml in the pre-equilibrated EBM-2 medium with 0.5% FCS (v/v) (Lonza Biologics). Ports 6 and 7 were closed with plugs (8, Figure 2.13A) and 6 μ l cell suspension seeded into the observation port (5, Figure 2.13A) of a slide using bevelled pipette tips (Grenier Bio-One, Frickenhausen, Germany). The same volume was then slowly withdrawn from the opposite port (4, Figure 2.13A) to fill the observation area (1, Figure 2.13A) with cells. The plugs were removed and all ports were covered with caps (9, Figure 2.13A), and incubated in a humidified incubator at 37°C (5% CO₂) for 4-6 hours until the cells had adhered. The caps were removed and the plugs replaced in ports 6 and 7 before cells were washed with EBM-2 medium with 0.5% (v/v) FCS (Lonza Biologics) by repeating the filling procedure to remove any remaining traces of the EGM-2 culture medium (Lonza Biologics) and dead cells. The reservoirs either side of the observation chamber were then filled slowly by moving the plug from port 7 to port 4 and injecting 45 μ l EBM-2 medium with 0.5% (v/v) FCS (Lonza Biologics) into port 5 (Figure 2.13A). The plug in port 6 was then transferred to port 7 and 45 μ l EBM-2 medium with 0.5% (v/v) FCS (Lonza Biologics) injected into port 5 (Figure 2.13A). The media containing chemo-attractant (10% (v/v) FCS (PAA Laboratories GmbH),

or the control media (no chemo-attractant) in a total volume of 18 μ l was added to port 7 or 6, and an equal volume of medium withdrawn from the opposite reservoir (port 7 or 6) in order to establish a stable gradient over the cells. The filling chambers were then covered with plugs and the slide secured in a 37°C heat chamber surrounding the microscope. Using a Nikon Eclipse TE300 microscope, a Prior proscan II controlled motorised stage (Nikon UK Ltd.) and a Hamamatsu ORCA-ER camera and SimplePCI automated image capture camera device software (Hamamatsu Photonics UK Ltd., Welwyn Garden City, UK), each chamber was photographed at x4 magnification at regular intervals (3-5 minutes) for 22 hours, generating time-lapse images.

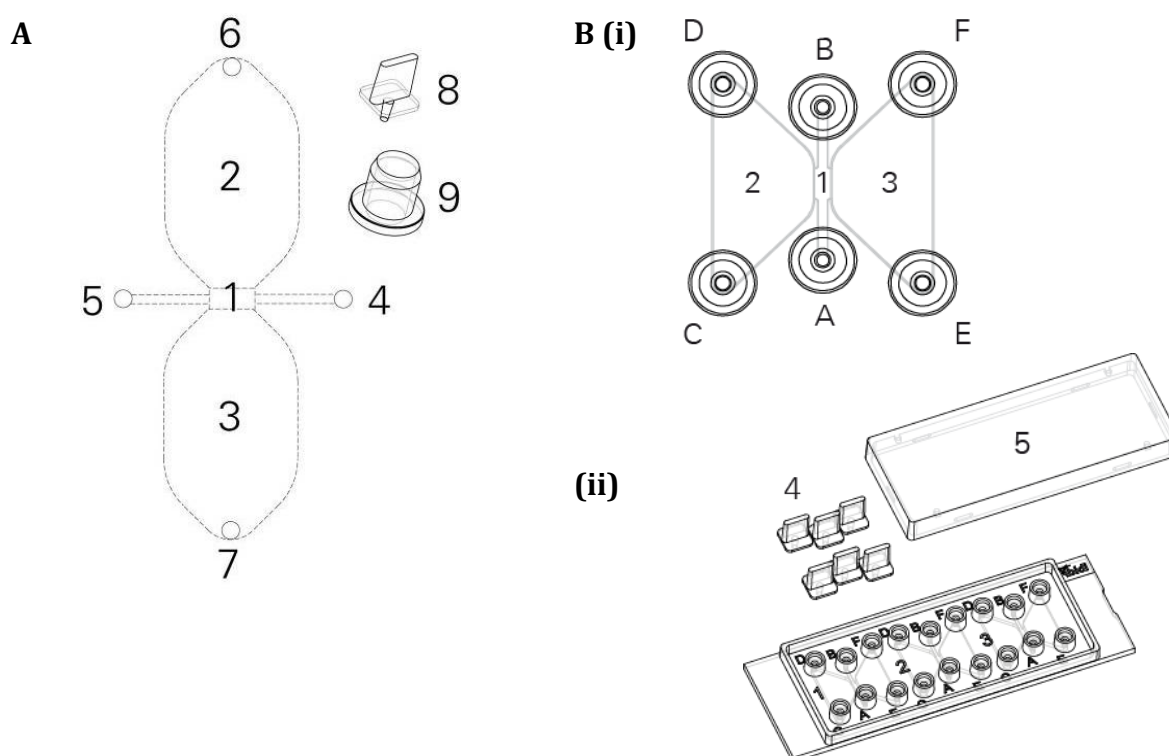


Figure 2.13. Diagrams of 2D and 3D μ -slide chemotaxis slides. Diagrams detailing A) a 2D μ -slide chemotaxis slide chamber (one of three), plugs (8) and caps (9) and B) (i) a 3D μ -slide chemotaxis slide chamber (one of three), plugs (4) and cover (5) and (ii) demonstrating the three chambers contained within each μ -slide. Image taken with kind permission from Ibidi GmbH (www.ibidi.com).

2.7.2 3D μ -slide chemotaxis assays

Human UCB CD133⁺ cells were isolated as described in section 2.2.2.3 and used fresh or from frozen following a 24 hour incubation with stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL6 or 20ng/ml TPO unless otherwise stated. The CD133⁺ cells were washed in stemspan medium alone and re-suspended at 3×10^6 cells/ml in a cold 1mg/ml collagen I solution (Invitrogen Ltd.) unless otherwise stated, consisting of 20% (v/v) collagen I bovine gel (5mg/ml), 10% (v/v) 10x MEM- α medium (Invitrogen Ltd.), 5% (v/v) NaHCO₃ (7.5%), 0.5% (v/v) NaOH (1M) and 64.5% (v/v) distilled deionised H₂O. 3D μ -slide chemotaxis slides and stemspan medium were pre-equilibrated for 24 hours in a Nuaire DH autoflow incubator 37°C (5% CO₂) incubator before use. As illustrated in Figure 2.14. To seed the cells, 6 μ l of the cell-gel suspension was immediately and quickly seeded into port B and the same volume withdrawn from port A using bevelled pipette tips (Grenier Bio-one) so that the cells filled the observation area (1, Figure 2.13B). Each slide was then covered with a lid (5, Figure 2.13B), and incubated in a humidified incubator at 37°C (5% CO₂) upside-down for 1 hour until the gel had set. The reservoirs either side of the observation chamber were then filled slowly through ports D or C and F or E with 60-70 μ l stemspan medium alone (control) or stemspan medium containing 0.2-2 μ g/ml CXCL12 (Peprotech) (chemo-attractant media) (as optimised in Chapter 3) in order to establish a stable CXCL12 gradient over the cells.

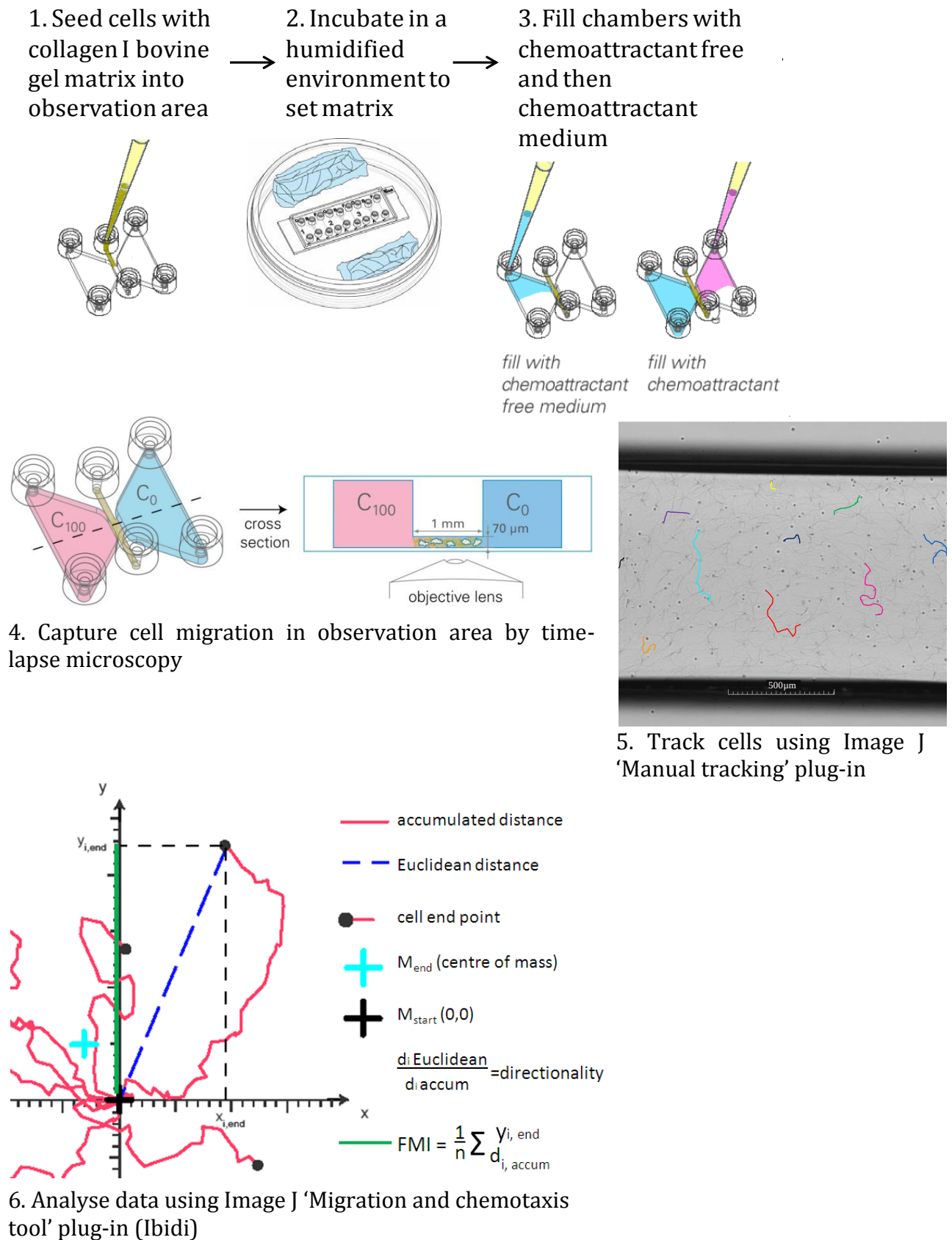


Figure 2.14. Method for the 3D μ -slide chemotaxis assay. Stepwise illustration of the method described in 2.7.2. The slide and analysis images were obtained with kind permission from Ibidi GmbH.

This was repeated for multiple conditions (3 per slide) (Figure 2.13B(ii)). The filling chambers were then closed with plugs (4, Figure 2.13B) to prevent evaporation and the slide secured in a 37°C humidified heat chamber surrounding the microscope. Using a Nikon Eclipse TE300 microscope, a Prior proscan II controlled motorised stage (Nikon UK Ltd.) and a Hamamatsu ORCA-ER camera and SimplePCI automated image capture camera device software (Hamamatsu Photonics UK Ltd.), each chamber was photographed at x4 magnification at regular intervals (3-5 minutes) for 18-22 hours, generating time-lapse images.

2.7.2.1 Chemotaxis data analysis

The images were exported as multipage TIFF files to either enable cell tracking using the Image J (rsbweb.nih.gov/ij/) 'Manual Tracking' plug-in ($n \geq 30$ cells tracked per chamber) or for uploading for automated tracking by www.wimasis.com (Wimasis GmbH, Munich, Germany). The Image J 'Chemotaxis and Migration Tool' plug-in (Ibidi GmbH) enabled the data to be quantified in terms of six chemotaxis and chemokinesis parameters and represented graphically. The parameters were the displacement of the centre of mass (average position of all tracked cell end positions), forward migration index (the ratio between the net distance on the relevant axis and the total accumulated distance), accumulated distance (total cell path), Euclidean distance (straight distance between cell start and end point), directionality (the ratio between Euclidean and accumulated distance) and cell velocity. The tracking and analyses are shown diagrammatically in Figure 2.14. The Rayleigh test provided a measure of statistically inhomogeneously distributed data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The calculations used to generate these parameters are described by Zengel *et al.* (Zengel *et al.*, 2011).

2.7.3 Transwell migration assay

Specialised transwell plates and membrane inserts (BD Biosciences) were coated with 10µg/ml fibronectin (Sigma Aldrich Ltd.) in PBS (PAA Laboratories GmbH) overnight at 4°C. The fibronectin solution was removed and the plate air dried in a sterile biosafety cabinet. Human UCB CD133⁺ cells isolated as described in section 2.2.2.3 were cultured for 24 hours in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. The cultured cells were washed in stemspan medium in a Hettich Rotina 46R centrifuge at 350 x g for 5 minutes (DJB Labcare). Human CXCL12 (Peprotech) was applied at the desired concentration in the lower wells in 400µl stemspan medium (Stem Cell Technologies) or stemspan medium alone for control wells. Pelleted CD133⁺ cells were re-suspended in stemspan medium alone at a concentration of 1x10⁶-2x10⁶ cells/ml and 1-2x10⁵ (100µl) CD133⁺ cells applied above the transwell (see Figure 2.15A). The equivalent volume and quantity, or part thereof, of cells added above the transwell filter were kept in the incubator for labelling with BCECF-AM. 30 minutes before the end of the assay the cells were labelled by re-suspending 1x10⁶ cells/ml in HBSS with Ca⁺⁺ and Mg⁺⁺ (PAA Laboratories GmbH) and adding 25nmol/l 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF-AM) (Invitrogen Ltd.) fluorescent indicator. The cells were incubated for 30 minutes in the dark in a Nuaire DH autoflow incubator at 37°C (5% CO₂) before washing with PBS (PAA Laboratories) in a Hettich Rotina 46R centrifuge at 350 x g for 5 minutes (DJB Labcare). The supernatant was removed and the cells re-suspended in the same volume of MACS buffer as the volume of media removed prior to labelling. The transwell plates were incubated for 4-5 hours at 37°C (5% CO₂) in a Nuaire DH autoflow incubator before the cells in the lower chamber and on the lower side of the membrane were harvested. For quantification of

migration, the input number of BCECF-AM labelled CD133⁺ cells (determined by equivalent volume) was added to the harvested cells for each respective well. Each sample was analysed by flow cytometry to detect fluorescent and non-fluorescent populations. Migration was calculated by gating the non labelled cells (P4) in the total population to give a percentage of input (BCECF-AM fluorescent cells) (P3) (Figure 2.15B). As this was a known number, this enabled the number of migrating cells to be derived. This was then converted to a percentage by dividing by the number of input cells and multiplying this by 100. Control conditions where either no CXCL12 was added, CXCL12 was added above and below the filter or above the filter with the cells, were included for every experiment (demonstrated in Figure 2.15B(i)). Based on the transwell migration results obtained by others within the laboratory, two CXCL12 (Peprotech) concentrations were tested in the transwell migration assays using CD133⁺ cells. Figure 2.15C shows that there was a linear migration response to the increasing concentrations of CXCL12 with 200ng/ml and 350ng/ml.

2.8 Co-culture vascular tubule assay

The original method was described by Zhang *et al.* using hDF and endothelial cells from umbilical cord blood (Zhang et al., 2009). In this thesis, hDF or BMSCs (stromal cells) (see sections 2.2.1 and 2.2.2) were seeded into 48 well plates pre-coated with rat tail collagen type I (BD Biosciences) in complete EGM-2 medium (Lonza Biologics) and allowed to adhere for several hours in a Nuaire DH autoflow incubator (5% CO₂) at 37°C. The number of stromal cells seeded ranged from 1x10⁴–4x10⁴ per well.

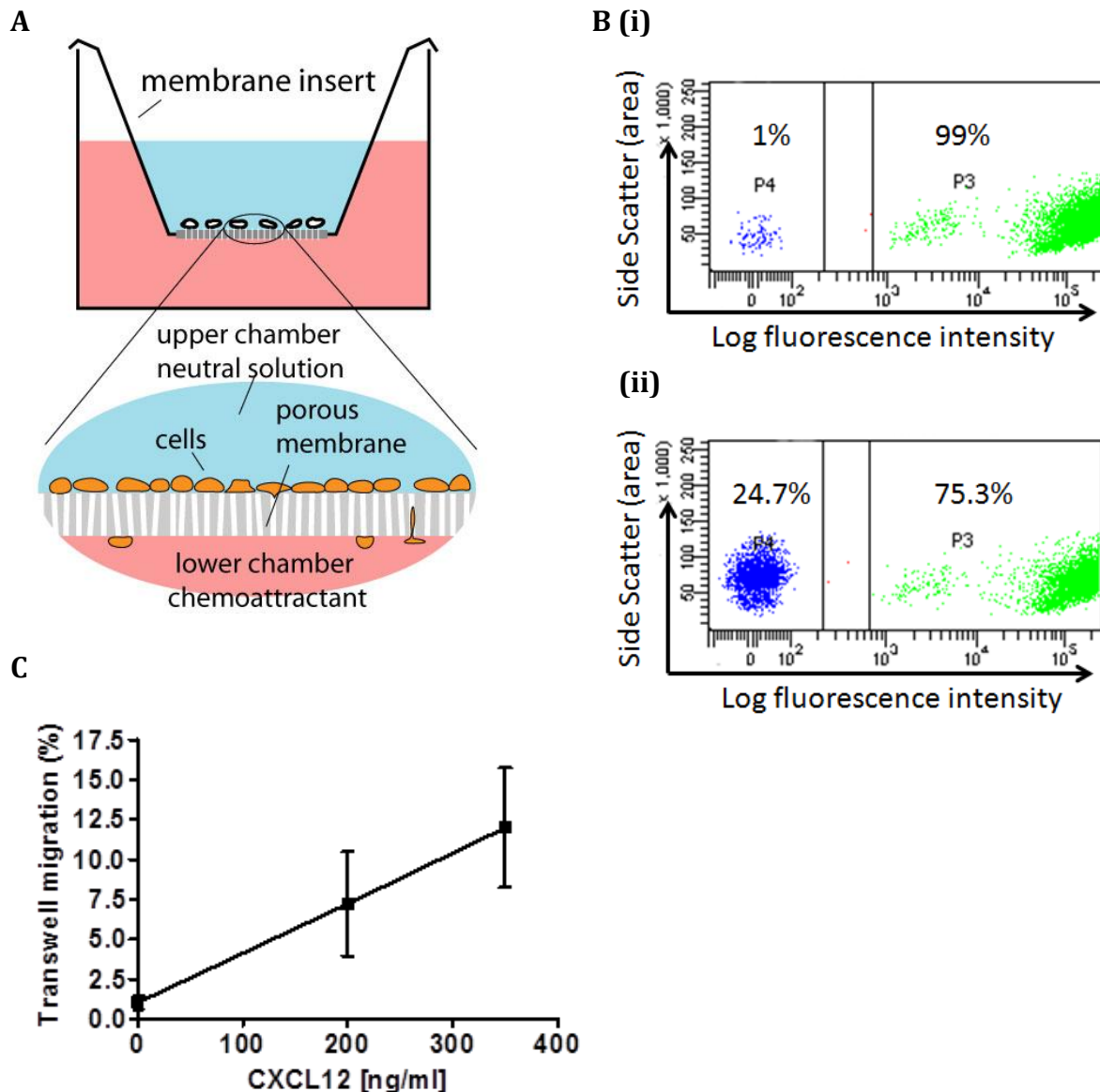


Figure 2.15. Method for transwell migration assay. The transwell migration experiments were conducted as described in section 2.7.3 using a transwell porous filter as demonstrated in (A). B) Migrated CD133⁺ cells (gate P4, blue), which had been cultured in stemspan medium with 100ng/ml SCF, Flt3 ligand, IL-6 and 20ng/ml TPO for 24 hours prior to the experiments, were collected and pooled with BCECF-AM labelled CD133⁺ cells (gate P3, green) used for quantification as described in section 2.7.3 and analysed by flow cytometry. Representative flow cytometry dot plots demonstrate CD133⁺ cells which had migrated across the membrane where (i) no CXCL12 was applied (control) and (ii) where 350ng/ml CXCL12 (Peprotech) was applied below the transwell filter. C) 200ng/ml and 350ng/ml CXCL12 (Peprotech) in duplicate or triplicate were used to select a concentration for optimal CD133⁺ cell transwell migration. Data are shown as mean $\pm$ SEM (n=3 independent experiments).

The endothelial cells (from UCB, HUVEC or BMEC-60) were then added in EGM-2 medium at the desired ratio (range 2×10^3 – 6×10^3) based on the results described by Zhang *et al.* (Zhang et al., 2009). The plates were incubated in a Nuaire DH autoflow incubator (5% CO₂) at 37°C and EGM-2 complete media was replaced every 2 days. After 14-17 days in culture, cells were fixed in 70% (v/v) ethanol (VWR International Ltd.) and refrigerated until the vessels were stained using the method described by Zhang *et al.* (Zhang et al., 2009). The plates were blocked using PBS with 3% (w/v) BSA for one hour at room temperature. The mouse anti-human CD31 (clone WM59) antibody (Serotec Ltd., Kidlington, UK) was made up at a dilution of 1:2000 in PBS with 3% (w/v) BSA and 200µl per well added and incubated for 18 hours at 4°C.

The plate was brought to room temperature before washing 3 times with PBS (PAA Laboratories GmbH). A goat anti-mouse IgG biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) diluted to 1:200 in PBS with 2% BSA was added at a volume of 200µl per well for one hour at room temperature. In the meantime, the VECTASTAIN ABC kit (Vector Laboratories Inc.) immunoperoxidase solution was prepared by adding 2 drops of Reagent A (Avidin DH) and 2 drops of Reagent B (Biotinylated Horseradish Peroxidase H) per 10ml PBS (PAA Laboratories GmbH). After washing the plate 3 times with PBS (PAA Laboratories GmbH), 200µl VECTSTAIN ABC immunoperoxidase solution was added per well and incubated at room temperature for 40 minutes. The Avidin DH has a high affinity for biotin and so binds to both the anti-mouse IgG biotinylated secondary antibody and the Biotinylated Horseradish Peroxidase H. The plate was then washed 3 times in PBS (PAA Laboratories GmbH). Immediately before use, the DAB (3, 3'-diaminobenzidine) substrate kit (Vector Laboratories Inc.) was prepared to allow visualisation of the

tubules. This comprised 2 drops of Buffer Stock Solution, 4 drops DAB stock solution, 2 drops hydrogen peroxide and 2 drops nickel solution (Vector Laboratories Inc.) per 5ml distilled water. Once 200µl of the DAB solution had been added per well, the development of the staining was monitored at room temperature using a Nikon Eclipse TE300 microscope (Nikon UK Ltd.) for 5-10 minutes before washing for 5 minutes in water. The wells were then air dried before storing at room temperature. Four photographs per well were taken at x4 magnification using the bright field channel of a Nikon Eclipse TE300 microscope and a Hamamatsu ORCA-ER camera (Hamamatsu Photonics UK Ltd.).

As an alternative, eGFP lentiviral vector labelled endothelial cells (2.5.1.7 and 2.5.1.8) were used so that vessels could be seen during the assay by excitation using a 488nm blue light in a Nikon Eclipse TE300 fluorescent microscope. At the end of the assay, the plates were fixed with 200µl 4% (w/v) paraformaldehyde (PFA) in PBS per well at room temperature for 10 minutes to retain the eGFP within the cell. The PFA was removed and wells were washed with distilled water for 5 minutes. PBS (PAA Laboratories GmbH) was added to the wells and the plates refrigerated at 4°C after sealing with parafilm. Adobe Photoshop CS (Adobe Systems Inc., San Jose, USA) was used to process the images by inverting the images and increasing the contrast between the stained vessels and the background to allow optimal resolution for quantification using Angiosys software (TCS Cellworks, Buckingham, UK).

2.9 Nanex expansion assay

Nanex Nanofibre (aminated electrospun polyethersulphone) pre-coated plates (Arteriocyte Inc., Cleveland, Ohio, USA) were used with different combinations of

culture medium. The conditions suggested by Arteriocyte (Nanex HSC serum free expansion media with the Nanex cytokine cocktail (1:1000) and 40µg/ml acetylated LDL (Athens Research & Technology Inc., Athens, GA, USA)) were defined as standard Nanex conditions. Other conditions used were Nanex HSC expansion media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (defined as Nanex with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO), and Nanex HSC expansion media with 100ng/ml IGFBP2 (R&D Systems) and Angptl5 (Miltenyi Biotec) as well as 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (defined as Nanex with IGFBP2 and Angptl5). CD133⁺ cells that had been isolated from UCB, as described in section 2.2.2.3 were used fresh or cultured for 4 hours after thawing before seeding onto the Nanex scaffold at a concentration of 1000 cells/ml in 3ml per well of a 6 well Nanex plate (Arteriocyte). The surface expression of CD133, CD34 and CD45 was assessed by flow cytometry using the antibodies and flow cytometry protocol described in sections 2.3 and 2.4.2 respectively. After 8 days in culture, without any agitation or feeding, the cells were collected. This was performed by pipetting the media off and rinsing the wells with PBS (PAA Laboratories GmbH). 500µl trypsin-EDTA (PAA Laboratories GmbH) was then added per well for 1 minute before collecting using PBS and pooling with the media already collected from all wells. The cells were centrifuged immediately at 350 x g for 5 minutes at 22°C in a Hettich Rotina 46R centrifuge (DJB Labcare), re-suspended in 1ml Nanex HSC expansion medium and counted on a haemocytometer in order to determine the total nucleated cell (TNC) count. Cells were analysed for the surface expression of markers such as CD45, CD34, CD133, and on some occasions CXCR4, CD45RA, CD38 and CD90 (all as detailed in section 2.3), by flow cytometry as described in section 2.4.2. On some occasions, a sample of the entire harvest was taken for CD133 re-purification using immuno-magnetic beads

(described in section 2.2.2.3.2). The re-purified cells were assessed for migratory ability in the 3D μ -slide chemotaxis assay described in section 2.7.2.

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2.10.1.1 Titrating anti-mouse CD45-PerCP

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The text and figures originally presented above has been redacted as it cannot be made freely available via ORA because of the sensitive nature of the work.

2.11 Statistical analysis

Where differences between conditions were assessed for significance, data were analysed using non-parametric statistical tests such as the Mann Whitney test. This was because the data sets were too small and the variances between groups too different to assume the data were normally distributed, and the use of non-parametric statistical tests reduced the likelihood of finding false significance. Where differences in expression of CD164 were compared to control expression standardised to 100%, the Wilcoxon Signed Rank test was used in order to compare the mean value to a hypothetical 100. Where the effect of NT and CD164-2 siRNA on homing and chemotaxis were compared for significance, a two-way ANOVA test was used in order to analyse the significance of the differences both between treatment groups, and within repeat experiments as the recoveries varied. In all cases, raw data and/or median, means and standard error of the mean (SEM) when averaging independent experiments, or standard deviation when averaging within experiments, are shown as indicated for each figure. When data parameters were correlated, using paired data from independent experiments, the line was fitted using linear regression and the Spearman's rank test was used to determine the correlation using Prism (Prism, GraphPad Software Inc., La Jolla, CA, USA). The R value displayed the goodness of fit, while the p value determined whether the slope was significantly different from 0. All of the above statistical tests were performed using Prism (www.graphpad.com/prism). For the chemotaxis assay, the Rayleigh test was used to assess if the data were significantly inhomogeneously distributed in any direction. For all of these tests, increasingly significant findings were determined by p values of * <0.05 , ** <0.01 , *** <0.001 .

Chapter 3.

Developing a novel *in vitro* chemotaxis assay for haemopoietic stem/progenitor cells

3.1 Aims and objectives

To develop a method for using a novel chemotaxis assay to study the directed migration of haemopoietic stem/progenitor cells (HSPC) towards CXCL12 *in vitro*.

The specific aims were to:

- 1) Optimise the method including;
 - a. Type of microscope slide
 - b. Gel matrix requirement
 - c. CXCL12 titration
 - d. Migration timecourse
 - e. Automated vs. manual analysis of data
- 2) Investigate the effect of CXCL12 gradients on human umbilical cord blood (UCB) CD133⁺ cell chemotaxis and chemokinesis
- 3) Investigate the effect of AMD3100, an antagonist of the CXCL12 receptor, CXCR4, on human UCB CD133⁺ cell chemotaxis and chemokinesis to CXCL12.

3.2 Introduction

Directed cell migration (chemotaxis) towards a stimulus is a well defined function of many mammalian and non-mammalian cells (Bonner and Savage, 1947). The ability of cells to migrate towards the appropriate signals is vital from the embryonic stages of development through to adult life (Dormann and Weijer, 2006). Examples in the adult include the need for immune cells to migrate to respond to infection, and for endothelial cells to migrate to sites of injury to repair damaged vessels. Indeed, endothelial cell migration has been demonstrated in the adherent cell version of the assay described here (Rothmeier et al., 2009; Zengel et al., 2011). However, to the author's knowledge, no such assay has been described for human suspension cells.

Directed cell migration is also of therapeutic importance in the field of haemopoietic transplantation. Graft failure occurs if insufficient HSPCs engraft into the host, which could be caused either by cells not engrafting upon reaching the bone marrow, or simply not migrating to the bone marrow. Delayed grafts, as occurs with umbilical cord blood (UCB) HSPCs, also pose significant risks of infection to patients. As engraftment is associated with the numbers of CD34⁺ HSPCs in the UCB graft (Kurtzberg, 2011), one approach to improve the success of UCB grafts is to improve the capacity of the injected HSPCs to reach the bone marrow and restore haemopoiesis (Mazo et al., 2011).

The engraftment process itself is complex and multistage. Migration of the HSPCs from the circulation to the bone marrow is a key part of the process, but must be followed by lodgement in an appropriate environment to enable subsequent engraftment (Papayannopoulou, 2003). The interaction between CXCR4, a receptor expressed on HSPCs, and its cognate chemokine CXCL12, has been widely

documented as a key factor in HSPC migration (Aiuti et al., 1997; Deichmann et al., 1997; Hatse et al., 2002; Imai et al., 1999) (reviewed by (Sharma et al., 2011)). The production of a CXCL12 gradient *in vivo* was demonstrated by Jung *et al.*, where anabolic parathyroid hormone increased CXCL12 levels in the long bones and correspondingly decreased these levels in the serum (Jung et al., 2006). Migration assays, such as the use of the Boyden chamber (Boyden, 1962), where cells migrate through a membrane, have been used to attempt to replicate this *in vitro* (Bleul et al., 1996; Hatse et al., 2002). However, in these transwell assays, although HSPC migration to CXCL12 is seen, the gradient is step-like and unstable (an equilibrium between upper and lower compartments is quickly formed), and perhaps not the best assay to mimic *in vivo* homing, as reviewed by Entschladen *et al.* (Entschladen et al., 2005).

The gold standard for determining efficient homing of HSPCs to bone marrow niches and their subsequent haematological reconstitution following transplantation in model systems is to use surrogate animal models (Goessling et al., 2011; Larochelle et al., 2012). However, these are time consuming and costly. An initial *ex vivo* migration assay, which reduces animal usage and allows refinement of conditions for pre-transplant manipulation of cells so that their migration abilities can be assessed and optimised prior to transplant, would make screening of expansion/stimulation protocols more efficient. Peled *et al.* demonstrated that there was a strong link between cells migrating to CXCL12 *in vitro* and cells that could engraft NOD-scid mice *in vivo*, by injecting migrating and non-migrating fractions of human CD34⁺CD38⁻ cells (Peled et al., 1999b). Therefore, this chapter aimed to develop a migration assay to

overcome the limitations of existing end-point assays in order to manipulate CD133⁺ cell chemotaxis to CXCL12 in subsequent chapters.

The use of matrices, such as collagen I, to provide a 3D migration environment for cells has been documented. Weidt *et al.* established a microscope based assay using collagen gel and timelapse microscopy to visualise human CD133⁺ cell movement in the presence of CXCL12 (Weidt *et al.*, 2004). Although this assay provided an indication of cell response to chemokines (e.g. CXCL12), the lack of a gradient eliminated the ability to investigate chemotaxis (Weidt *et al.*, 2004).

The application of methods enabling HSPC migration to be studied extensively and on a single cell basis by timelapse microscopy has been described as elementary (Weidt *et al.*, 2004). However, the importance of maintaining a stable gradient in these microscopy assays has also been recognised (Toetsch *et al.*, 2009). The sensitive nature of HSPCs combined with their weak adhesion to extracellular matrix (ECM) coated surfaces excludes the possibility for a suitable 2D assay of this format. Therefore, this chapter describes the development of a time-lapse assay combining all of these challenging factors, including the additional advantage of a stable chemokine gradient (for up to 48 hours), enabling both the chemotaxis and chemokinesis of human UCB derived CD133⁺ HSPCs to be observed and quantified.

3.3 Results

3.3.1 Establishing the 2D chemotaxis assay using adherent cells

Initially, only 2D chemotaxis μ -slides, recommended for adherent cells, were available from Ibidi GmbH (Figure 2.13A). Endothelial cells derived from human umbilical vein cord (HUVEC) or UCB, as described in section 2.2.2.2, were used to establish the 2D μ -slide chemotaxis assay as described in section 2.7.1. The endothelial cells were trypsinised and seeded in the central observation chamber in EBM-2 medium with 0.5% (v/v) fetal calf serum (FCS). After allowing the cells to adhere, the reservoirs were filled with EBM-2 with 10% (v/v) FCS for the control (so that there was no gradient in either direction) (Figure 3.1A(i)), or, EBM-2 with 0% FCS in one reservoir and EBM-2 with 10%(v/v) FCS in the other, to create a gradient (Figure 3.1A(ii)). Cell migration was captured by time-lapse microscopy over a 22 hour period. Cell tracking, trajectory plot and data generation was performed using Image J software and manual tracking and chemotaxis and migration tool plug-ins, as described in section 2.7.2.1.

Endothelial cells showed significant chemotaxis to a 0-10% (v/v) FCS gradient in EBM-2 media (Figure 3.1B(i)), determined by the Rayleigh p values which tested for significant inhomogeneous distribution when $p < 0.05$. In all experiments, in the presence of a 0-10% FCS (v/v) gradient, $**p \leq 0.005$ compared to $p \geq 0.05$ in the control conditions (EBM-2 with 10% (v/v) FCS in both reservoirs) ($**p < 0.01$, Rayleigh test, $n=3$) (Figure 3.1B(i)). The mean $\pm$ SEM of the Rayleigh values for migration in a 0-10% FCS gradient was 0.002 ± 0.002 compared to 0.403 ± 0.218 for the 10-10% (v/v) FCS control.

In the control condition there was also a minimal forward migration index or displacement of the centre of mass seen from the zero starting position (mean $\pm$ SEM 0.01 $\pm$ 0.02 and 18.0 μ m $\pm$ 14.4 respectively) (Figure 3.1B(ii and iii)). However, when a gradient was established, using 0-10% (v/v) FCS, an increase in the forward migration index and displacement of centre of mass in the direction of the gradient was seen (mean $\pm$ SEM 0.07 $\pm$ 0.01 and 71.2 μ m $\pm$ 15.8 respectively) (Figure 3.1B(ii and iii)). A small increase was observed in the mean $\pm$ SEM directionality when the 0-10% (v/v) FCS gradient was present (0.15 $\pm$ 0.02), compared to control (10-10% (v/v) FCS) (0.12 $\pm$ 0.01) (Figure 3.1B(iv)). No difference was seen in the mean $\pm$ SEM accumulated distance (903 μ m $\pm$ 231 and 929 μ m $\pm$ 223) and mean $\pm$ SEM velocity (0.75 μ m/min $\pm$ 0.08 and 0.81 μ m/min $\pm$ 0.03) between the control and test conditions respectively (Figure 3.1B(v and vi)). However, this was to be expected as FCS was present in both conditions.

3.3.2 CXCR4 surface expression on CD133 enriched populations

Once the 2D μ -slide assay had been established, the assay was tested for human UCB CD133⁺ suspension cells but in the presence of the chemokine CXCL12. The expression of the CXCL12 cognate receptor CXCR4 was first examined on the surface of the UCB CD133⁺ cells prior to performing the migration assay. Cells enriched using immunomagnetic bead selection from UCB and cultured overnight with SCF, Flt-3 ligand, IL-6 and TPO (described in section 2.2.2.3) were 94.0% $\pm$ 2.0 positive for CD133, as assessed by flow cytometry (section 2.4.2.1) (Figure 3.2A and C(i)). The level of CXCR4 on the surface of these cells varied (Figure 3.2B(ii)).

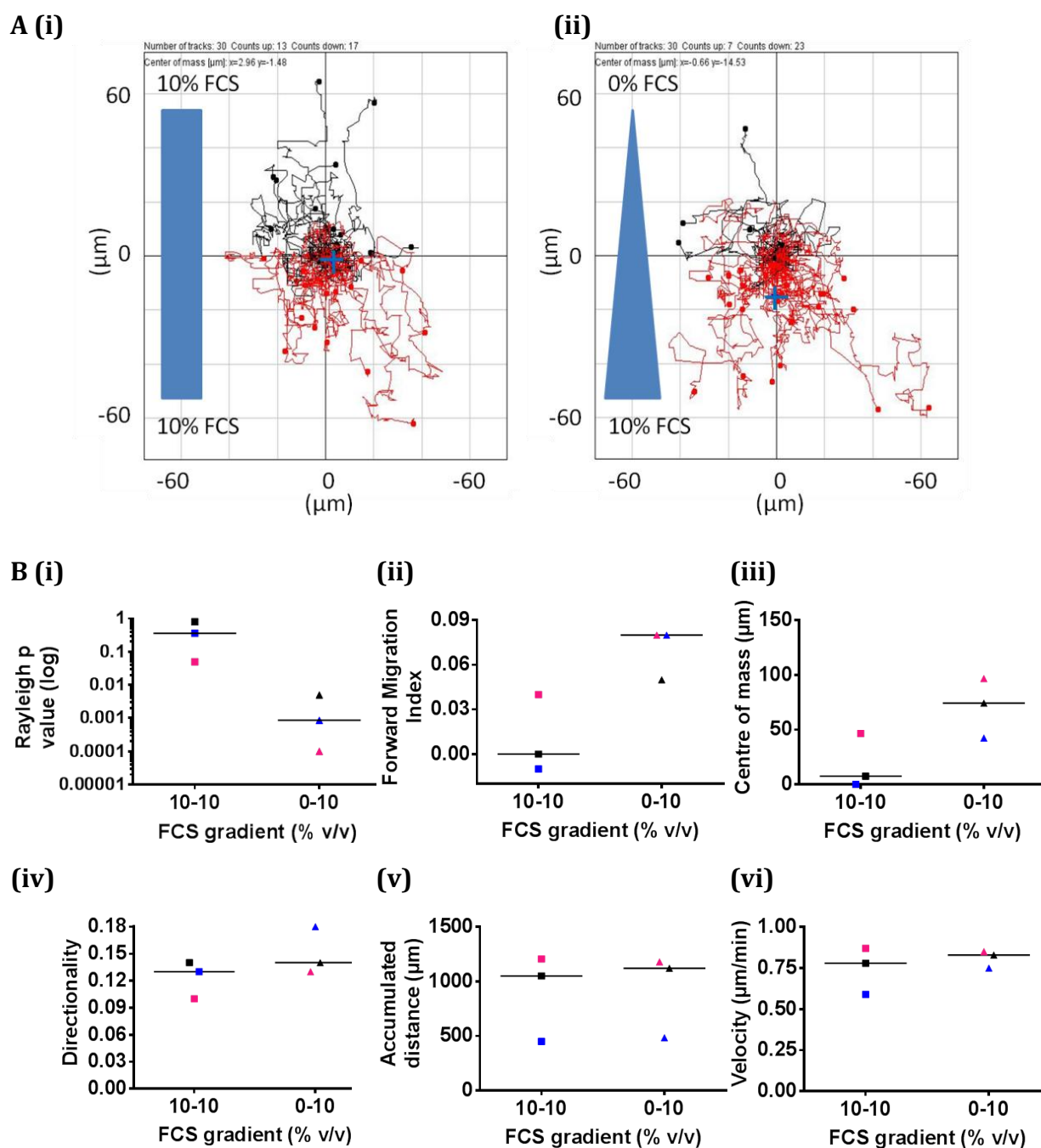


Figure 3.1. Establishing migration using 2D μ -chemotaxis slides and human endothelial cells derived from human UCB or umbilical vein. The cells were seeded in the observation chamber of the μ -slide, as described in section 2.7.1, and allowed to adhere. After washing, the reservoirs were filled with EBM-2 and a gradient of either 10-10% (v/v) FCS (control) or 0-10% (v/v) FCS was established. A) Representative plots demonstrate cell movement over 22 hours (n $\geq$ 30 cells tracked per condition). B) Graphs display the data generated as described in section 2.7.2.1 from individual experiments (colour coded) and median (black line) for (i) Rayleigh p value, (ii) forward migration index, (iii) displacement of centre of mass, iv) directionality, (v) accumulated distance and (vi) velocity (n=3 independent experiments).

The mean percentage of CD133⁺ cells expressing CXCR4 above the arbitrary gate set for the isotype control was 74.9%±4.7 (Figure 3.2C(i)). The median fluorescence intensity (mean±SEM 726.4±153.3) demonstrated a clear shift from isotype control (38±8.48) suggesting that all the CD133⁺ cells express CXCR4 but to varying extents (Figure 3.2C(ii)).

3.3.3 Attempting to use 2D slides for non-adherent cells

Prior to the manufacture of 3D μ -slide chemotaxis slides for non-adherent cells (Figure 2.13B), the 2D slides (Figure 2.13A) were tested using human UCB CD133⁺ cells. Grassinger *et al.* reported HSPC chemotaxis to CXCL12 or osteopontin using these 2D μ -slides by counting the number of cells in the reservoirs (Grassinger et al., 2009). When attempted in house, this method, which involved filling the reservoirs first and then adding cells into the observation chamber, the cells leaked straight into the reservoirs upon their addition. In further experiments, the 2D slides were coated with fibronectin and collagen in an attempt to promote CD133⁺ cell adhesion to the observation area, and to provide a platform for migration. However, cells in the observation area were always flushed into the reservoirs, as a result of the flow of liquid over the loosely adherent/non-adherent cells. Subsequently, the 3D μ -slide chemotaxis slide prototypes were provided by Ibidi GmbH (Figure 2.13).

A method suitable for assessing HSPC chemotaxis in these 3D μ -slides was developed in this chapter. In order for the cells to be retained in the observation area between the reservoirs, a gel matrix compatible with HSPCs and the 3D μ -slides needed to be established first.

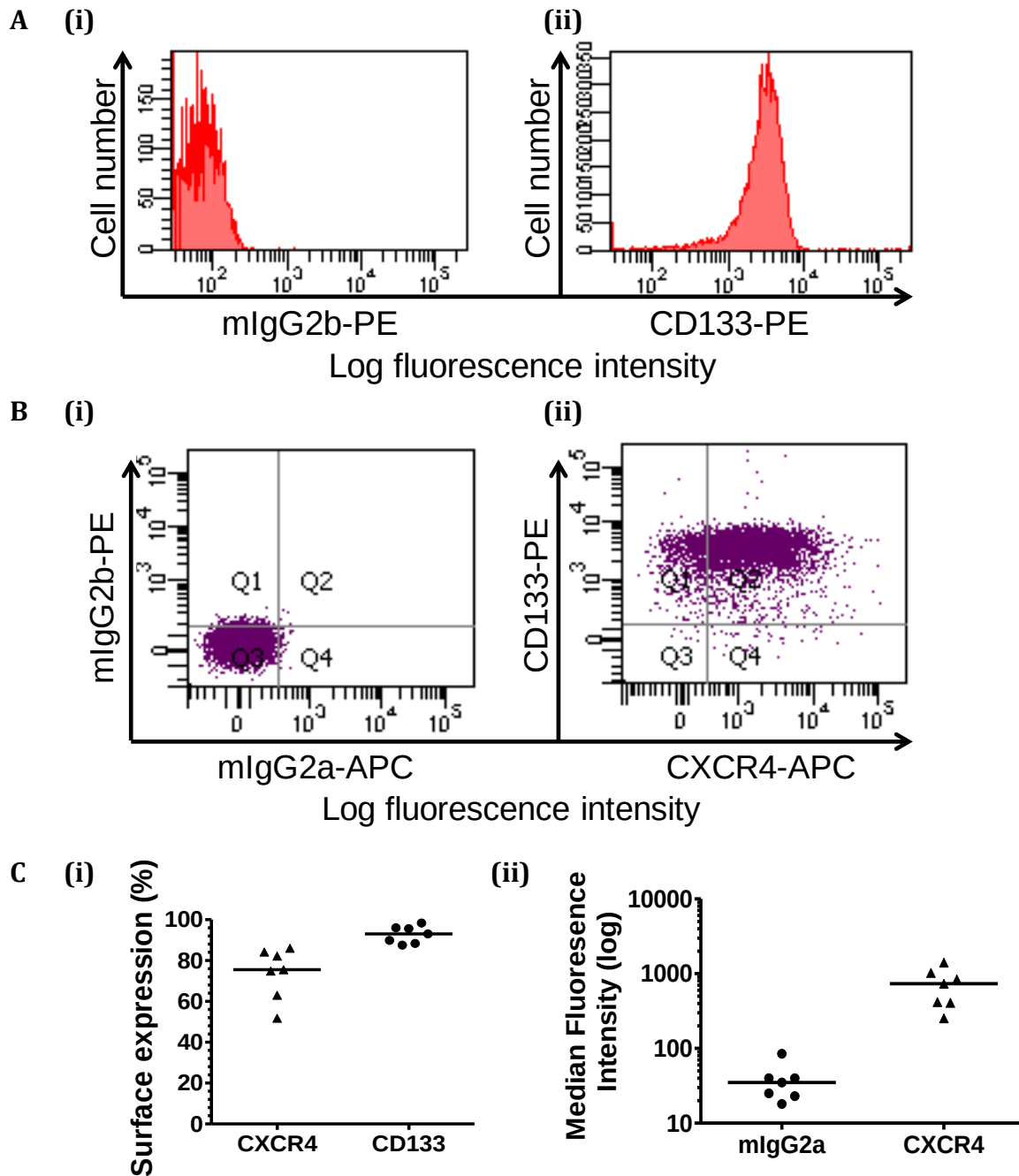


Figure 3.2. CD133 and CXCR4 expression on human UCB CD133 enriched populations. Purified UCB CD133⁺ cells cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were analysed by flow cytometry for CD133 and CXCR4 surface expression as described in section 2.4.2. Representative histograms or dot plots are shown for A) (i) mlgG2b-PE isotype control and (ii) CD133-PE, B) co-stains of (i) mlgG2a-APC vs. mlgG2b-PE and (ii) CXCR4-APC vs. CD133-PE. C) (i) The percentage of cells expressing CXCR4 or CD133 on their surface above the gate set for the respective isotype controls and (ii) the median fluorescence intensities (MFI) for the mlgG2a-APC isotype control and the CXCR4-APC stained CD133 enriched cells. Both graphs display individual data points and the median values of these points (line) (n=7).

Due to the nature of the assay, which required the μ -slide and media to be equilibrated at 37°C in an incubator for 24 hours in advance, the gel matrix had to be compatible with being seeded at this temperature through narrow channels, in order to prevent bubble formation which would distort cell migration.

3.3.4 Selecting 3D matrices for chemotaxis slides using non-adherent cells

In a preliminary experiment, three different gel matrices were tested in the μ -slide chambers. The suitability for use was assessed in terms of whether the viscosity, aeration and speed of gel setting enabled its use in this assay. The ability for the UCB CD133⁺ cells to move through the gels was also observed by time-lapse microscopy. A 50% (v/v) matrigel (a gelatinous ECM-like protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells) (Figure 3.3A) was tested as it is widely used as a substrate for specialised cell culture or as a 3D gel matrix for cell invasion assays.

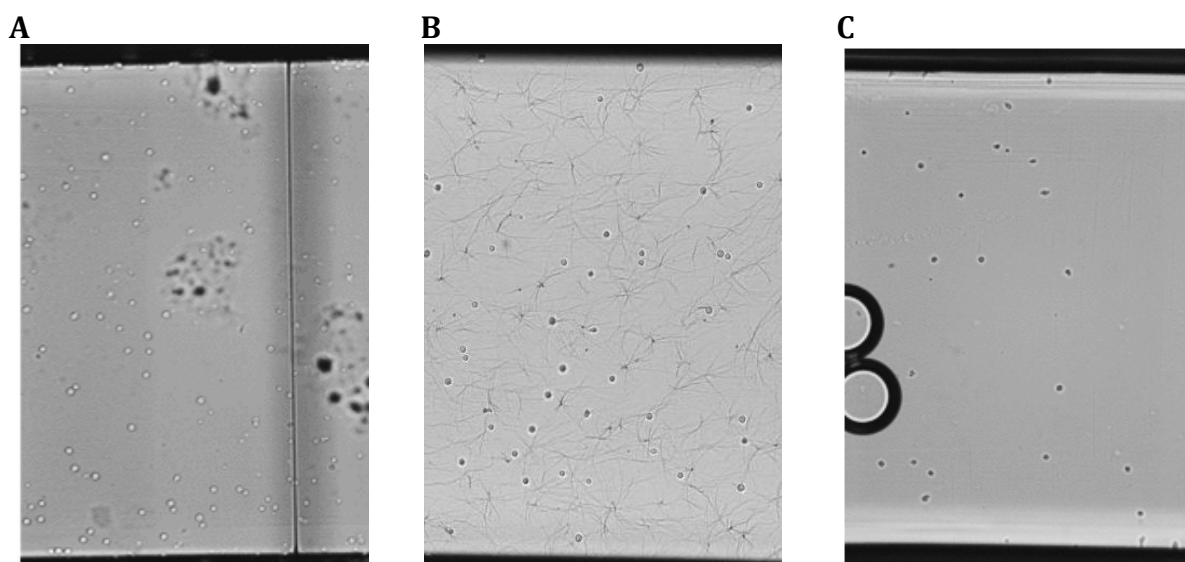


Figure 3.3. Selecting 3D matrices for UCB CD133⁺ cell migration. Human UCB CD133⁺ cells cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan alone and seeded in a 3D μ -slide chemotaxis chamber (Ibidi GmbH) using A) a 50% (v/v) matrigel, B) a 1mg/ml collagen I gel, or C) 100% (v/v) methylcellulose. Cell retention in the gel, and ability to migrate was assessed using timelapse microscopy (x4 magnification images shown).

Although this concentration of matrigel did retain the cells and some cell movement was observed, the use of collagen I gel to provide a 3D migration environment for dendritic cells had been documented using the 3D μ -slide chemotaxis chambers (http://www.ibidi.com/service/application_notes) and this proved successful for HSPCs (Figure 3.3B). Methylcellulose did retain some cells and allow cell movement, but its viscosity and aeration made it difficult to use for this application due to bubble formation (Figure 3.3C). Consequently, future experiments used collagen I gel.

3.3.5 Titrating CXCL12 in the 3D chemotaxis assay

Ibidi GmbH demonstrated directional migration of dendritic cells towards 1.25 μ g/ml CCL19 using 3D μ -slide chemotaxis chambers (http://www.ibidi.com/service/application_notes). To optimise the chemokine used for human UCB CD133⁺ cell migration in this thesis, different concentrations of CXCL12, the key homing chemokine for HSPCs were tested. The human UCB CD133⁺ cells, which had been freshly isolated (described in section 2.2.2.3) and cultured for 24 hours in stemspan media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, were washed in stemspan alone. The cells were re-suspended in a 1mg/ml collagen I gel at 3x10⁶ cells/ml and 6 μ l seeded per chamber of a 3D μ -slide chemotaxis slide as described in section 2.7.2. Different concentrations of CXCL12 in stemspan media were used to create a gradient over the cells. These ranged from 0.2 μ g/ml to 2 μ g/ml CXCL12 and included a control where no CXCL12 was added. The optimal concentration for human UCB CD133⁺ cell chemotaxis using Peprotech CXCL12 was 1 μ g/ml, as indicated by the peak displacement of centre of mass (mean $\pm$ SEM 257.84 μ m $\pm$ 112.9 compared to control 48.8 μ m $\pm$ 8.9) (Figure 3.4A and C and Figure 3.5A).

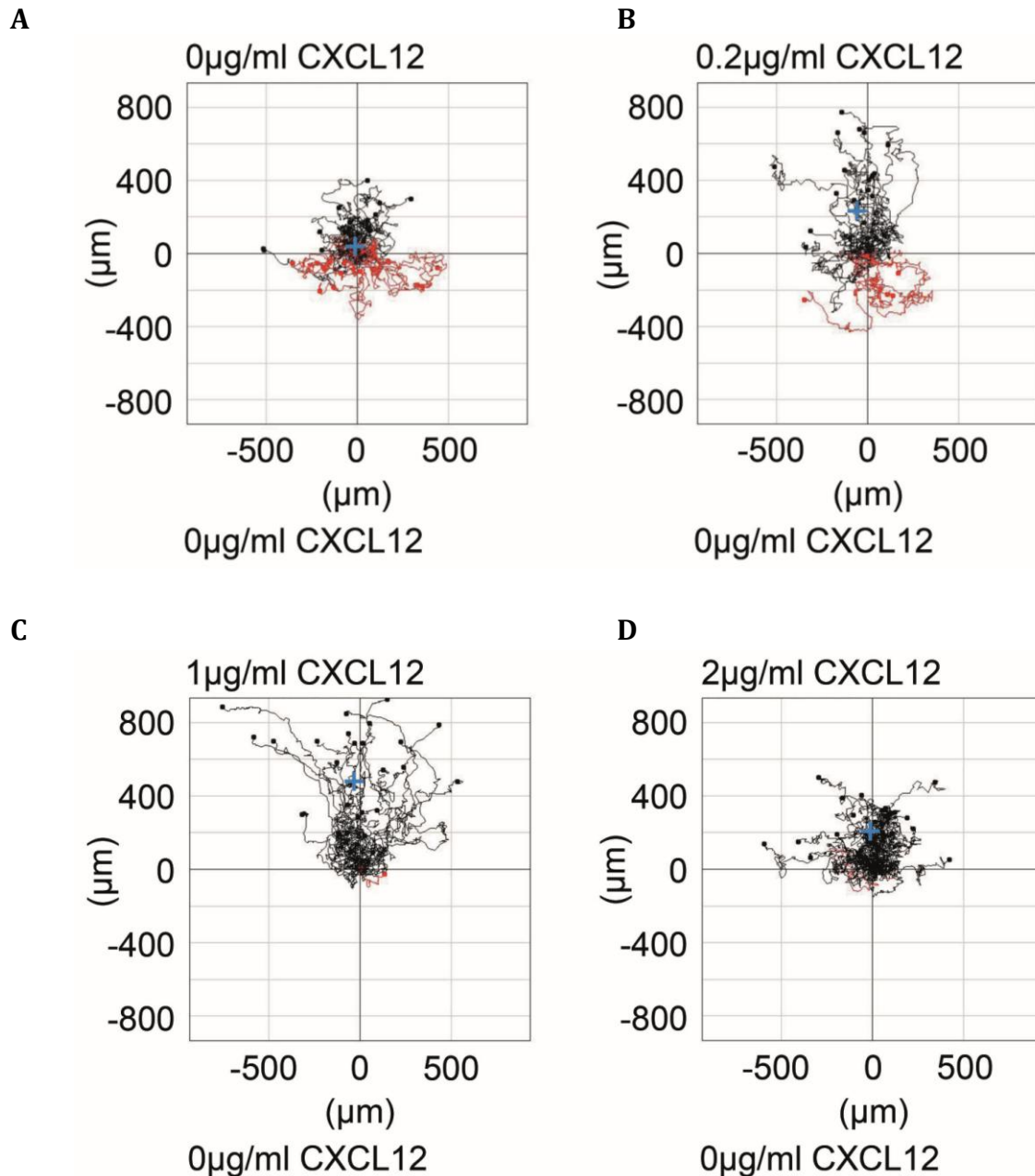


Figure 3.4. CD133⁺ cells migrate optimally to 1 μg/ml CXCL12. Human UCB CD133⁺ cells that had been cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium alone and re-suspended in a 1mg/ml collagen I gel before seeding into the 3D μ-slide chemotaxis chamber (Ibidi GmbH). With the exception of the control (A) (0 μg/ml CXCL12), a CXCL12 gradient was established using (B) 0.2 μg/ml, (C) 1 μg/ml and (D) 2 μg/ml CXCL12. The cells were captured over a 22 hour period by timelapse microscopy in a heated chamber. Image J was used to manually track 30 cells per group. The blue cross represents the displacement of the centre of mass. Representative trajectory plots of tracked human UCB CD133⁺ cell migration towards different CXCL12 concentrations are shown (n=3 independent experiments). The chemotaxis and migration tool (Ibidi GmbH) was used to analyse and quantify the migration (Figure 3.5).

A bell shaped response was also observed in response to CXCL12 in the forward migration index, peaking at 1µg/ml CXCL12 (mean±SEM 0.18±0.05 compared to control 0.02±0.00) (Figure 3.5B). The cell directionality was increased with all concentrations of CXCL12 tested compared to 0µg/ml CXCL12 indicating that the cell path was straighter (Figure 3.5C). Interestingly, there was no difference in cell velocity or accumulated cell distance with increasing concentrations of a CXCL12 gradient, or indeed in the absence of CXCL12, suggesting that human UCB CD133⁺ cell chemokinesis is not influenced by the presence of CXCL12 (Figure 3.5D and E).

3.3.6 Establishing an optimal concentration of collagen I gel

Different concentrations of collagen I gel were tested for human UCB CD133⁺ cell chemotaxis and chemokinesis, ranging from 0.5-3mg/ml. The human UCB CD133⁺ cells, which had been freshly isolated or thawed from frozen (described in section 2.2.2.3) and cultured for 24 hours in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, were washed in stemspan alone. The cells were re-suspended in a 0.5, 1 or 3mg/ml collagen I gel at 3x10⁶ cells/ml and seeded into a 3D µ-slide chemotaxis slide. Cell migration towards 1µg/ml CXCL12 (optimised in section 3.3.5) was tracked over 22 hours as described in section 2.7.2. As demonstrated by Figure 3.6A, cells were not retained within the 0.5mg/ml gel as well as in higher gel concentrations. The increase in the formation of collagen fibres with increasing concentrations of collagen gel can be seen in Figure 3.6A-C. At a concentration of 3mg/ml, although the cells were generally retained very well within the observation chamber, a lack of uniformity in the collagen fibre formation was seen (Figure 3.6C).

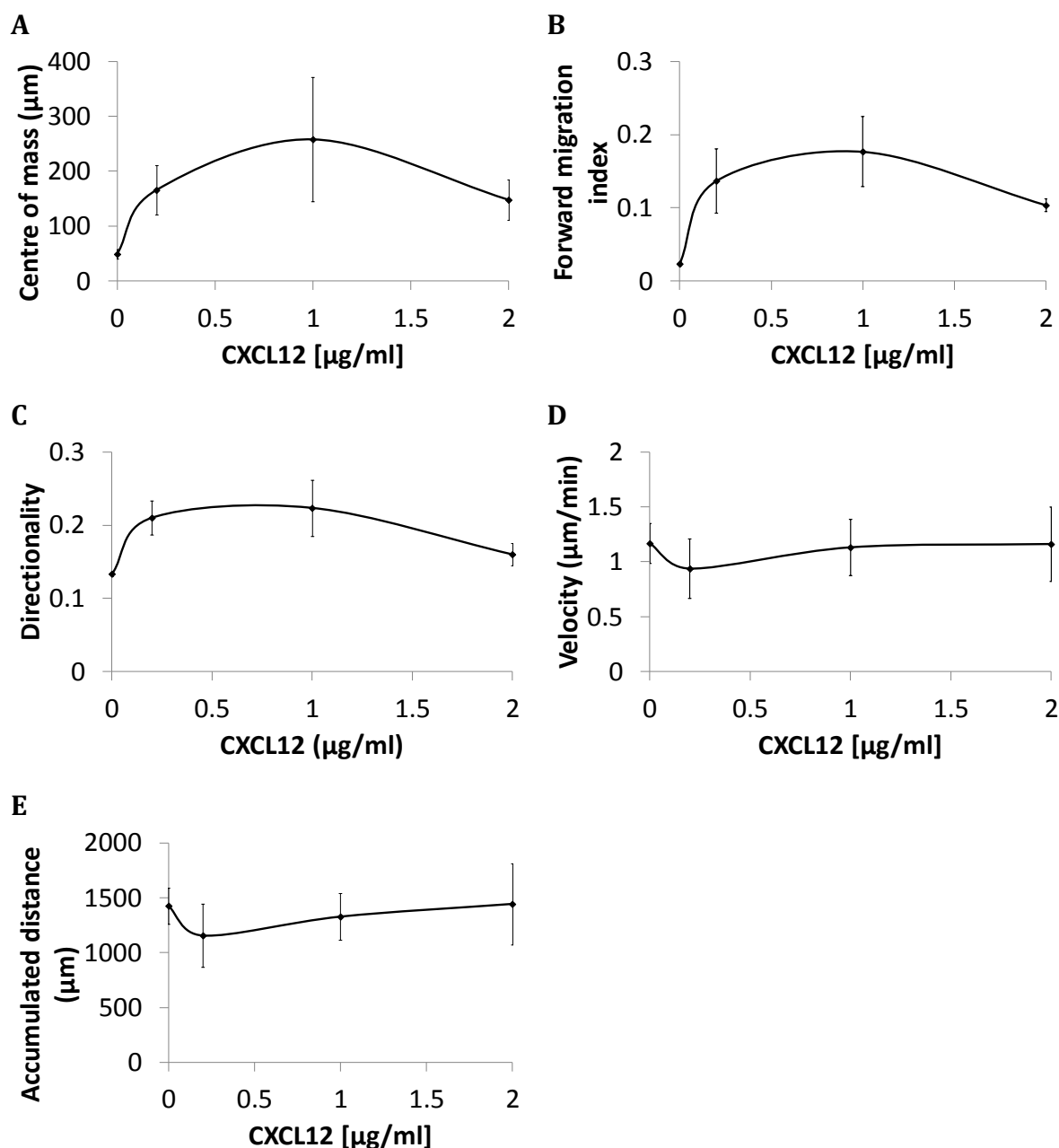


Figure 3.5. CD133⁺ cell chemotaxis, but not chemokinesis, is influenced by CXCL12. Human UCB CD133⁺ cells that had been cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium alone and re-suspended in a 1mg/ml collagen I gel before seeding into the 3D μ -slide chemotaxis chamber (Ibidi GmbH). With the exception of the control (0 $\mu\text{g/ml}$ CXCL12), a CXCL12 gradient was established using 0.2 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ or 2 $\mu\text{g/ml}$ CXCL12. The cells were captured over a 22 hour period by timelapse microscopy in a heated chamber. Image J was used to manually track the migration paths of 30 cells per group (representative trajectory plots shown in (Figure 3.4)) and the chemotaxis and migration plug-in (Ibidi GmbH) (described in section 2.7.2.1) was used to generate the following data (A) displacement of the centre of mass, (B) forward migration index, (C) directionality, (D) velocity and (E) accumulated distance shown as mean $\pm$ SEM (n=3 independent experiments).

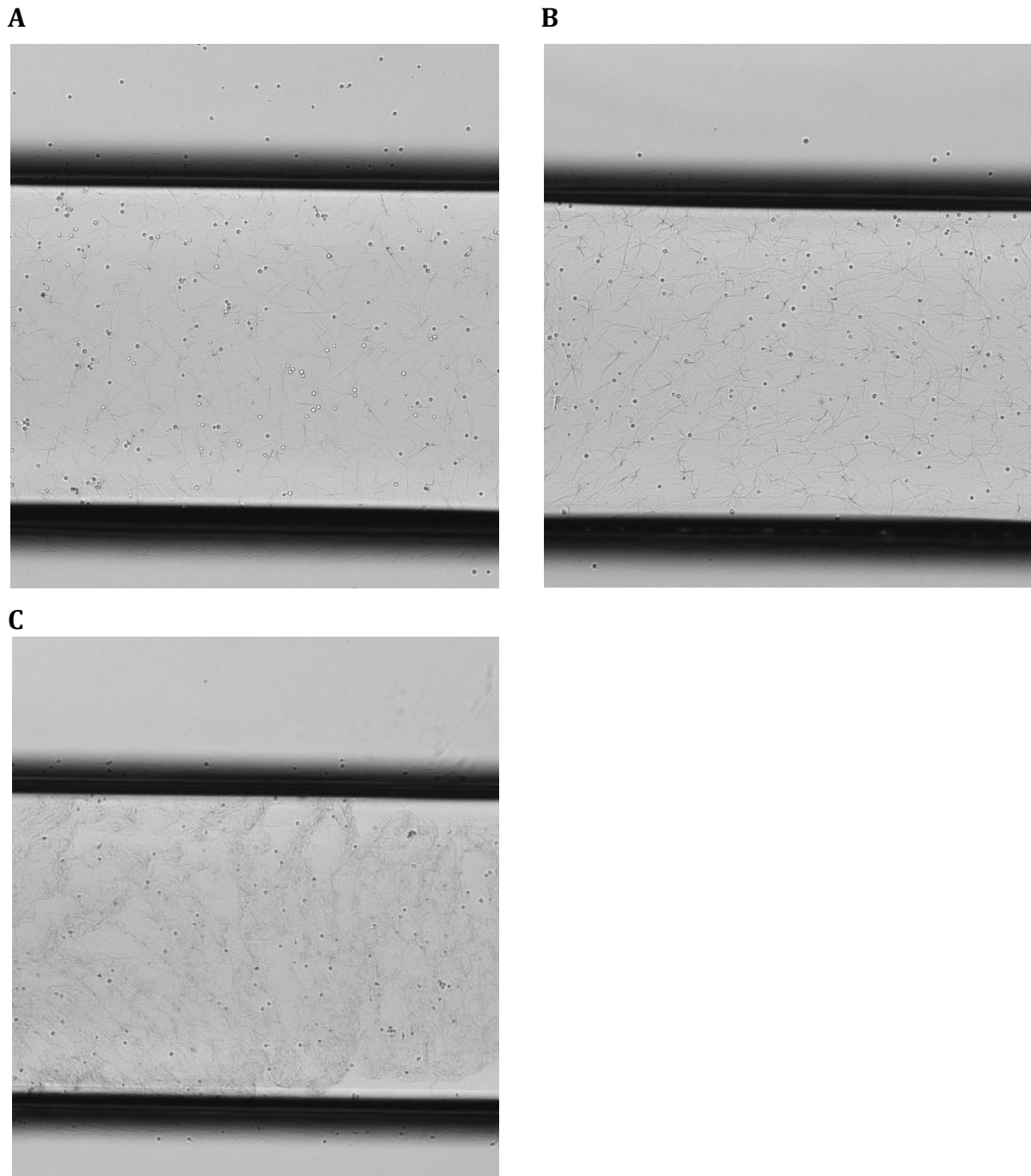


Figure 3.6. CD133+ cells seeded in 0.5-3mg/ml collagen I gel. Human UCB CD133+ cells that had been cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium alone, re-suspended in either (A) 0.5mg/ml, (B) 1mg/ml and (C) 3mg/ml collagen I gel and seeded into the observation chamber of a 3D μ -slide chemotaxis slide (Ibidi GmbH). A CXCL12 gradient was established using 1 μ g/ml CXCL12. Representative images show the cells embedded in the gels at time point 0 (x4 magnification). The cells were captured over a 22 hour period by timelapse microscopy in a heated chamber. Image J was used to manually track and analyse 30 cells per group as described in 2.7.2.1 (data analysed in Figure 3.7).

The data generated from tracking the migrating cells was analysed and displayed in Figure 3.7. Significant chemotaxis was observed in all experiments for all gel concentrations ($p < 0.05$, Rayleigh test). The mean $\pm$ SEM of the Rayleigh p values for CD133⁺ cell migration in the presence of CXCL12 were 0.002 ± 0.001 , 0.003 ± 0.002 and 0.011 ± 0.011 in 0.5mg/ml, 1mg/ml and 3mg/ml collagen I gels respectively. The displacement of the centre of mass and forward migration index, was on average increased with the 3mg/ml collagen I gel. However, when the 3mg/ml collagen I gel was compared with the 0.5 or 1mg/ml collagen I gels, there was a greater degree of variability in the displacement of the centre of mass amongst independent experiments (mean $\pm$ SEM $153.6\mu\text{m} \pm 49.0$ compared to $94.6\mu\text{m} \pm 19.0$ and $113.7\mu\text{m} \pm 1.1$ respectively, $n \geq 3$) (Figure 3.7A). This could be explained by the inconsistency in collagen fibre formation at high concentrations due to the increased speed at which the gel set. If the gel was setting when the cells were seeded and the fibres were disrupted, considerably reduced cell movement would occur. This is further exemplified when comparing the ranges in the forward migration index (0.10 to 0.11 vs. 0.06 to 0.22), displacement of the centre of mass ($111.8\mu\text{m}$ to $115.4\mu\text{m}$ vs. $26.9\mu\text{m}$ to $256.2\mu\text{m}$), accumulated distance ($1012\mu\text{m}$ to $1451\mu\text{m}$ vs. $350\mu\text{m}$ to $1467\mu\text{m}$) and velocity ($0.77\mu\text{m}/\text{min}$ to $1.16\mu\text{m}/\text{min}$ vs. $0.27\mu\text{m}/\text{min}$ to $1.18\mu\text{m}/\text{min}$) for 1mg/ml and 3mg/ml collagen I gels respectively (Figure 3.7A-D).

On average, human UCB CD133⁺ cell movement in a 1mg/ml collagen I gel, in terms of velocity was $0.92 \pm 0.12\mu\text{m}/\text{min}$ and accumulated distance was $1159\mu\text{m} \pm 146$ (mean $\pm$ SEM, $n=3$). The 1mg/ml collagen gel also showed the least variability in forward migration index and displacement of the centre of mass between independent experiments (Figure 3.7A-B). This was because the gel formed

consistently in each experiment but was concentrated enough to retain the cells whilst allowing the least restrictive cell movement. Therefore, 1mg/ml collagen gels were used in the remaining experiments.

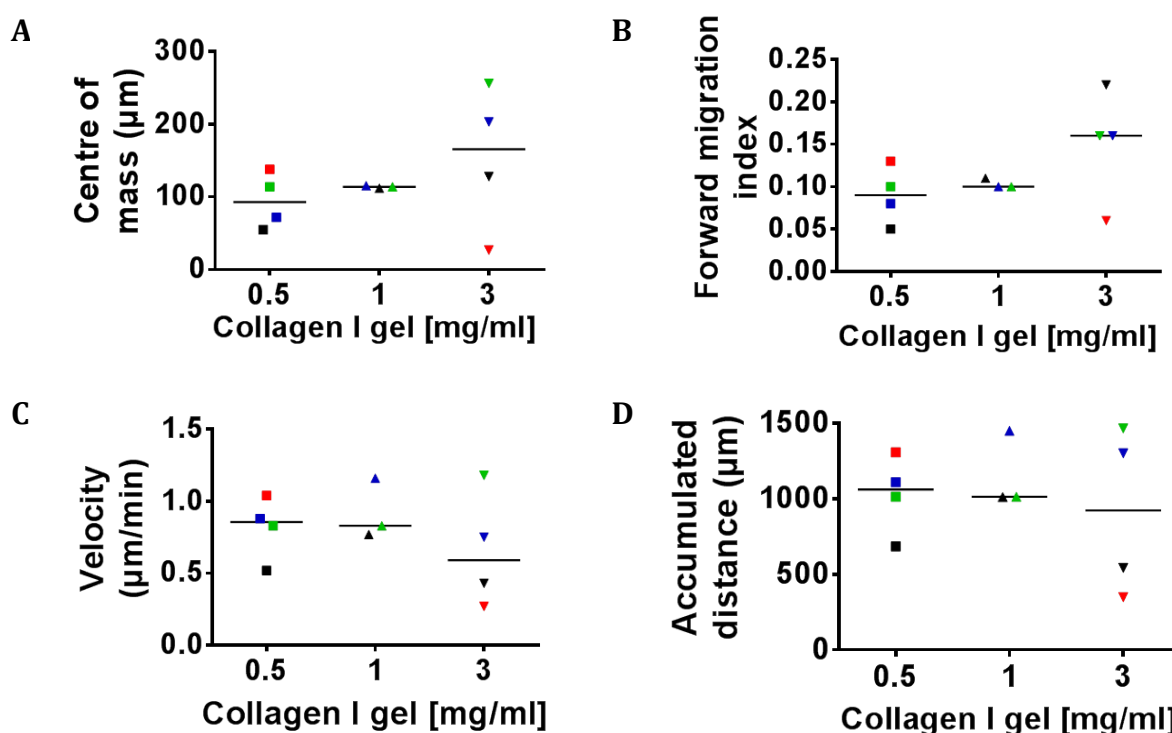


Figure 3.7. CD133⁺ cells migrate through collagen I gel. Human UCB CD133⁺ cells that had been cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium alone, re-suspended in either 0.5mg/ml, 1mg/ml or 3mg/ml collagen I gel and seeded into the observation chamber of 3D μ -slide chemotaxis slide (Ibidi GmbH). A 1 $\mu\text{g}/\text{ml}$ CXCL12 gradient was established. The cells were captured over a 22 hour period by timelapse microscopy in a heated chamber. Image J was used to manually track the migration paths of 30 cells per group. The chemotaxis and migration plug-in (Ibidi GmbH) was used to generate A) displacement of the centre of mass, B) forward migration index, C) velocity, and D) accumulated distance. Data are shown as individual experiments (colour coded) and median (line) ($n \geq 3$ independent experiments).

3.3.7 Establishing an optimal time point to track chemotaxis

The effect of time on migration was investigated between 4 and 22 hours. The human UCB CD133⁺ cells, which had been freshly isolated (described in section 2.2.2.3) and cultured for 24 hours in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, were washed in stemspan alone. The cells were re-suspended in a 1mg/ml collagen I gel at 3×10^6 cells/ml and 6 μ l seeded per chamber of a 3D μ -slide chemotaxis slide. Cell migration towards 1 μ g/ml CXCL12 (optimised in section 3.3.5) was tracked over 22 hours as described in 2.7.2. Data was taken at 4, 10, 18 and 22 hour timepoints and migration was displayed as trajectory plots (Figure 3.8A-D). The data were analysed as described in section 2.7.2.1 to determine the optimal timepoint for cell migration in this assay (Figure 3.9A(i)-D(i)). Migration over time without CXCL12 being present was also assessed at these time points (Figure 3.9A(ii)-D(ii)).

The displacement of the centre of mass increased over a 4-22 hour period in a linear fashion in the presence of a 1 μ g/ml CXCL12 gradient, but not without CXCL12 (Figure 3.9A(i and ii)). CD133⁺ cells in the presence of a 1 μ g/ml CXCL12 gradient displayed a plateau of forward migration between 4-10 hours, compared to those without CXCL12 (Figure 3.9B(i and ii)). Accumulated distance also increased linearly, however, this occurred both in the presence and absence of a 1 μ g/ml CXCL12 gradient (Figure 3.9C(i and ii)). Cell velocity increased steadily over time, in the presence or absence of a CXCL12 gradient, with the largest increase seen during the first 4 hours (mean+SEM 0 to 0.71 μ m/min $\pm$ 0.14, n=3) (Figure 3.9D(i and ii)). Subsequent experiments recorded chemotaxis over 22 hours.

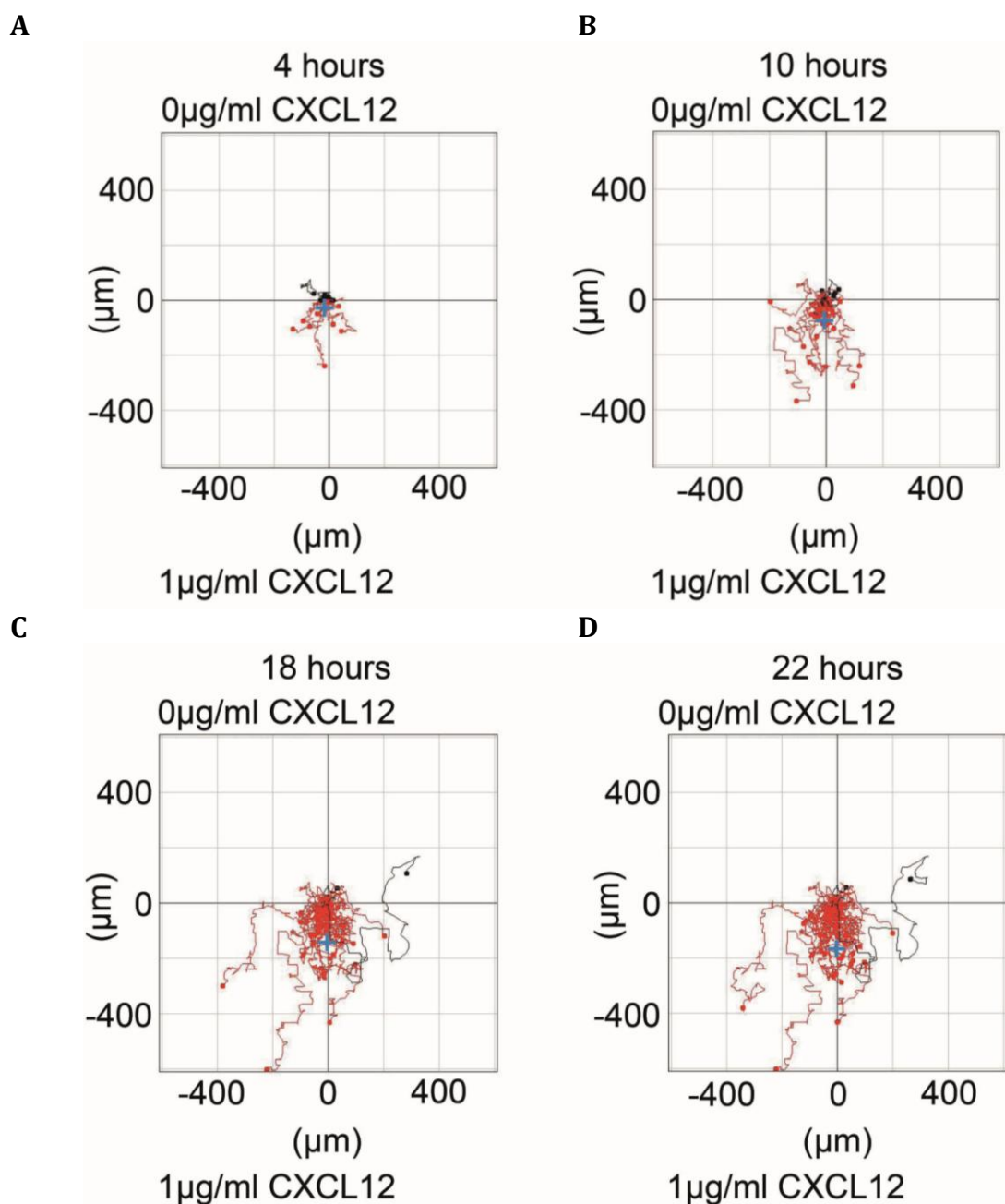


Figure 3.8. CD133⁺ cell migration increases up to 22 hours in the presence of CXCL12. Human UCB CD133⁺ cells that had been cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium alone, re-suspended in 1mg/ml collagen I gel and seeded into the observation chamber of a 3D μ -slide chemotaxis slide (Ibidi GmbH). A CXCL12 gradient was established using 1 $\mu\text{g}/\text{ml}$ CXCL12. The cells were captured over a 22 hour period by timelapse microscopy in a heated chamber. Image J was used to manually track the migration paths of 30 cells per group. Representative trajectory plots show the migration paths and centre of mass (blue cross) at (A) 4, (B) 10, (C) 18 and (D) 22 hours into the assay. The chemotaxis and migration plug-in (Ibidi GmbH) was used to analyse the data as described in section 2.7.2.1 (Figure 3.9) (n=3 independent experiments).

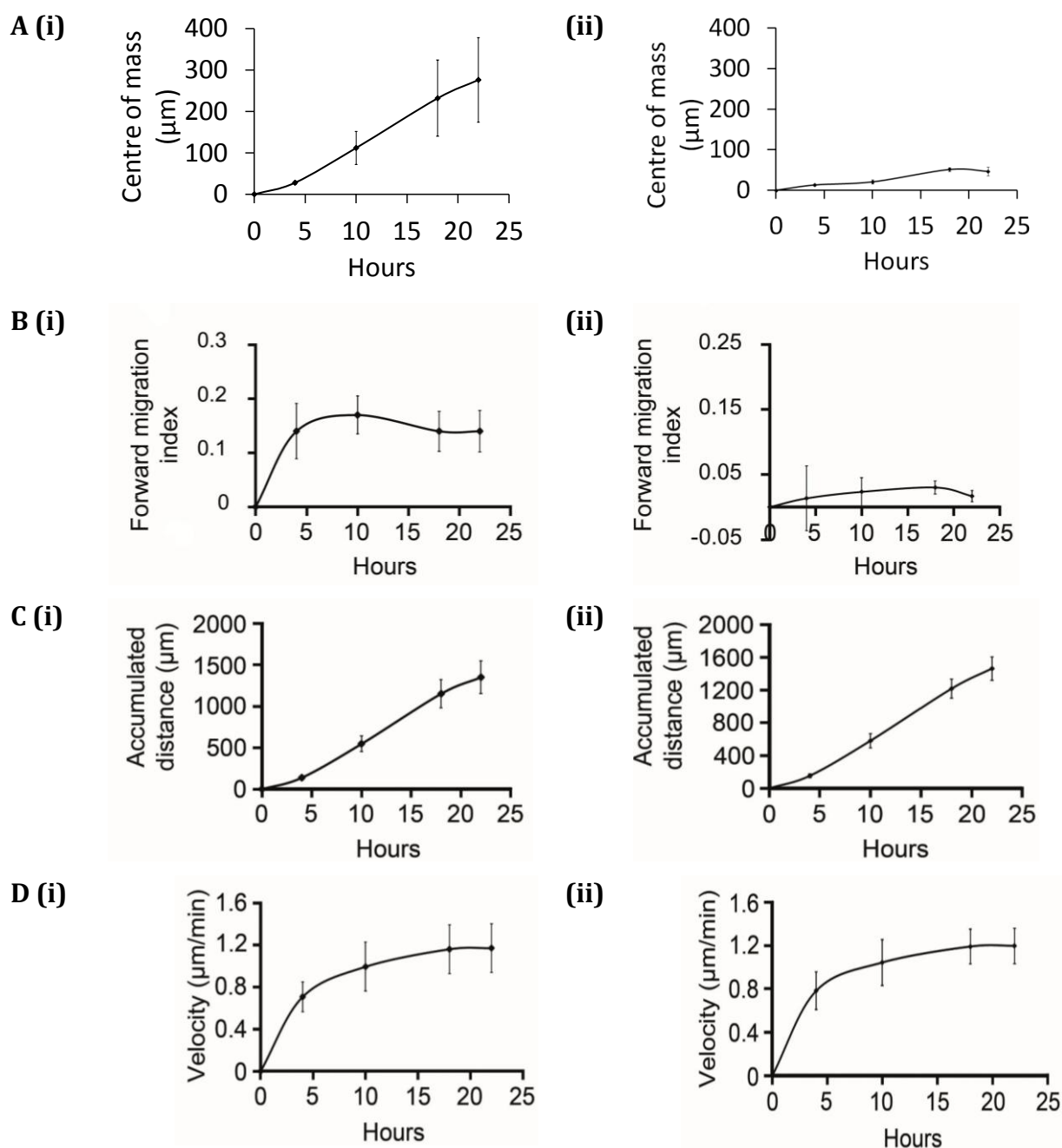


Figure 3.9. CD133+ cell chemotaxis increases over time in the presence of CXCL12. Human UCB CD133+ cells that had been cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium alone, re-suspended in a 1mg/ml collagen I gel and seeded into the observation chamber of 3D μ -slide chemotaxis slide (Ibidi GmbH). A CXCL12 gradient was established using 1 $\mu\text{g}/\text{ml}$ CXCL12 (i), or no gradient (ii). Image J was used to manually track the migration paths of 30 cells per group over a 22 hour period. Representative trajectory plots show the migration paths at 4, 10, 18 and 22 hours into the assay (Figure 3.8). The chemotaxis and migration plug-in (Ibidi GmbH) (described in section 2.7.2.1) was used to generate A) displacement of centre of mass, B) forward migration index, C) accumulated distance, and D) velocity with 1 $\mu\text{g}/\text{ml}$ CXCL12 (i) and without CXCL12 (ii) for all measures of migration. Values are mean $\pm$ SEM (n=3 independent experiments).

3.3.8 The final optimised method confirmed CD133⁺ cell migration to CXCL12

Using the optimised conditions described in sections 3.3.5 to 3.3.7, the chemotactic and chemokinetic response of human UCB CD133⁺ cells to 1µg/ml CXCL12 was determined. The human UCB CD133⁺ cells, which had been thawed from frozen or freshly isolated (described in 2.2.2.3) and cultured for 24 hours in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, were washed in stemspan medium alone. The cells were re-suspended in a 1mg/ml collagen I gel at 3x10⁵ cells/ml and 6µl seeded per chamber of a 3D µ-slide chemotaxis slide. Cell migration towards 1µg/ml CXCL12, or no CXCL12 was tracked over 22 hours, and data derived using the Image J plug-ins, as described in sections 2.7.2 and 2.7.2.1.

In the presence of a 1µg/ml CXCL12 gradient, human UCB CD133⁺ cells seeded in a 1mg/ml collagen I gel and tracked over 22 hours displayed chemotaxis (Figure 3.10A). This was determined by the Rayleigh p values in the presence of a CXCL12 gradient (**p<0.01 for all experiments) compared to no CXCL12 (p>0.05). The individual Rayleigh p values for each experiment are listed in Table 3.1. The mean±SEM Rayleigh values were ***p=0.001±0.001 with a CXCL12 gradient and p=0.336±0.073 with no CXCL12. The migration towards CXCL12 could also be seen in the forward migration index (0.126±0.024) and displacement of centre of mass (189.0µm±44.3) compared to without CXCL12 (0.015±0.008 and 10.8µm±11.3 respectively) (n=8 independent experiments) (Figure 3.10B(i-iii)). Increased directionality was also seen in the presence of CXCL12 (0.205±0.015) compared to no CXCL12 (0.128±0.006) (n=8 independent experiments) (Figure 3.10B(iv)). There was no increase in cell velocity (mean±SEM 1.0µm/min±0.1 and 1.1µm/min±0.1) or accumulated distance (mean±SEM 1258µm±94 and 1389µm±91) in the presence or

absence of a CXCL12 gradient respectively (n=8 independent experiments). This confirmed the earlier findings (Figure 3.5 and Figure 3.9) that human UCB CD133⁺ cell chemokinesis was not influenced by the presence of CXCL12 (Figure 3.10B(v-vi)).

Table 3.1. Summary of Rayleigh p values.

Experiment	Rayleigh p value	
	0µg/ml CXCL12	1µg/ml CXCL12
1	0.69	***2.0x10 ⁻⁸
2	0.17	***6.0x10 ⁻⁸
3	0.57	***8x10 ⁻⁵
4	0.18	**4.0x10 ⁻³
5	0.16	***3.0x10 ⁻¹⁰
6	0.26	**6.0x10 ⁻³
7	0.45	***5.0x10 ⁻⁵
8	0.21	***6.0x10 ⁻⁹

Rayleigh p values from human UCB CD133⁺ cell migration in the absence or presence of a 1µg/ml CXCL12 gradient (**p<0.01, ***p<0.001, n=8 independent experiments).

3.3.9 Validating the measures of migration in the 3D µ-slide chemotaxis assay

The measures of chemotaxis (displacement of the centre of mass, forward migration index and directionality) and chemokinesis (random cell movement) (accumulated distance and velocity) were compared to each other. This was in order to ensure the measures used were valid and accurately represented the movement of the cells. A Spearman's rank correlation analysis was performed using data from 15 independent experiments using human UCB CD133⁺ cells. To maximise the amount of data for the correlation analysis, these experiments included those where cells had been nucleofected with a non-targeting siRNA sequence (described in section 2.6.1.1) as well as the non treated cells used throughout this chapter (prepared as described in section 2.7.2).

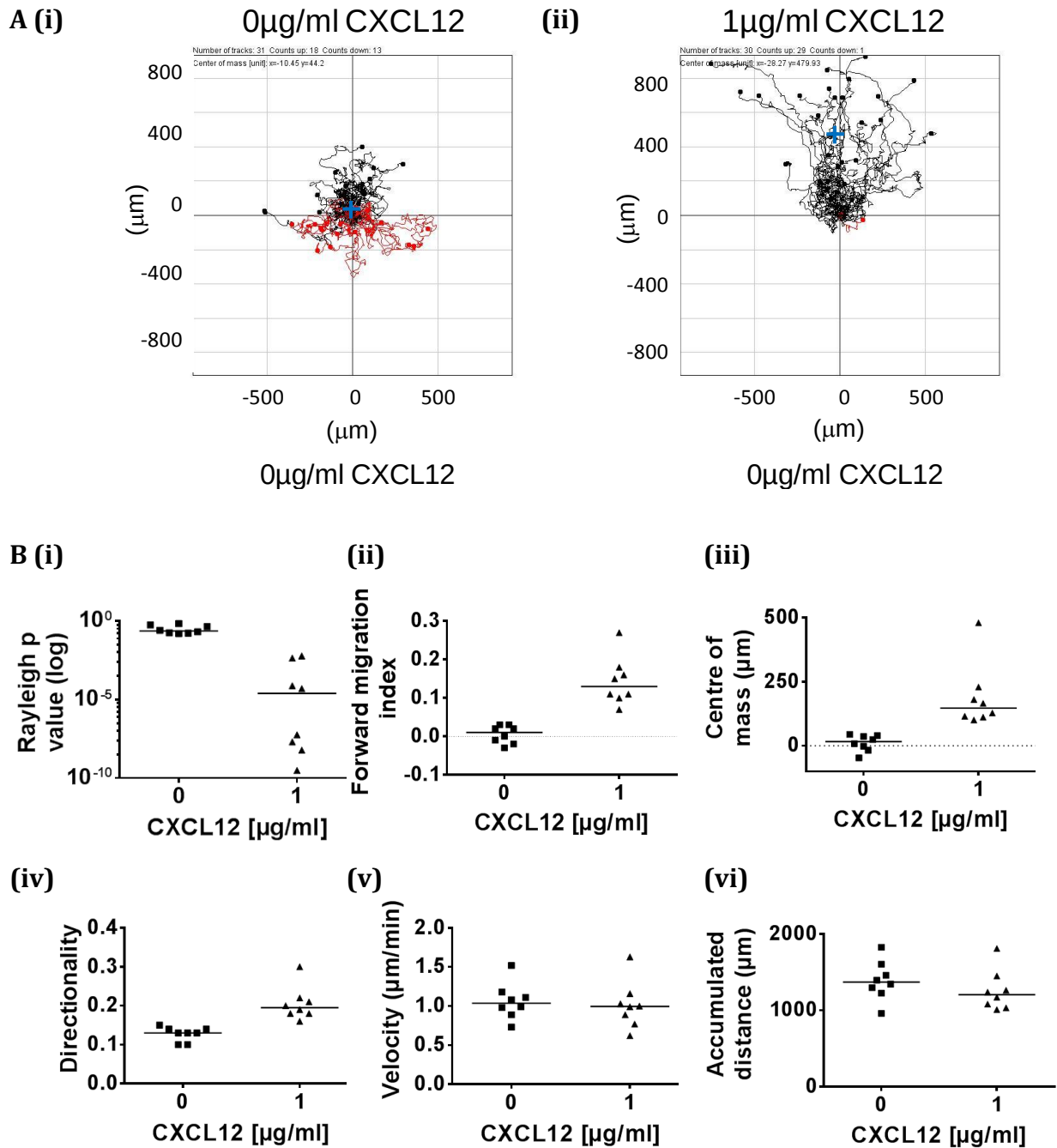


Figure 3.10. CD133⁺ cells migrate to 1 μ g/ml CXCL12. Human UCB CD133⁺ cells cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium and re-suspended in a 1mg/ml collagen I. The cells were seeded in 3D μ -slide chemotaxis chambers (Ibidi GmbH) for 20-22 hours. Cell migration was tracked using Image J plug-ins. A) Representative trajectory plots show cell movement in the presence of (i) no gradient and (ii) a 1 μ g/ml CXCL12 gradient (the blue cross represents the displacement of the centre of mass). B) Quantified migration data is displayed as independent experiments and median (line) for (i) the Rayleigh test p values (testing for inhomogeneous distribution where $p < 0.05$ was significant), (ii) forward migration index, (iii) displacement of the centre of mass, (iv) directionality, (v) cell velocity and (vi) accumulated distance ($n = 8$ independent experiments).

Forward migration index and the displacement of the centre of mass were highly correlated within experiments ($R=0.951$, $***p<0.0001$) (Figure 3.11A). Unsurprisingly, the directionality and forward migration index were also highly significantly correlated ($R=0.881$, $***p<0.0001$) as the forward migration index is a measure of overall chemotactic efficiency, and directionality the straightness of the cell path (Figure 3.11B). Similarly, the displacement of the centre of mass also correlated with directionality ($R=0.801$, $***p<0.0003$) (Figure 3.11C). Velocity and accumulated distance were highly significantly correlated ($R=0.870$, $***p<0.0001$) as the further the faster the cells move, the further they can travel in the given time (Figure 3.11D). A negative, highly significant correlation was observed between the Rayleigh p values (significantly inhomogeneously distributed data) and both the displacement of centre of mass and forward migration index ($R=-0.918$, $***p<0.0001$ and $R=-0.906$, $***p<0.0001$ respectively) (Figure 3.11E-F). This confirmed that the Rayleigh test was a useful tool for defining chemotaxis, as the greater the displacement of centre of mass or forward migration index, the more significant the p value.

No significant correlations were seen between the displacement of the centre of mass and velocity or accumulated distance (Table 3.2). Neither was there any correlation between Rayleigh p values and velocity or accumulated distance (Table 3.2). This supported the earlier findings (Figure 3.5, Figure 3.9 and Figure 3.10) that chemotaxis is not dependent on a change in velocity or accumulated distance (chemokinesis).

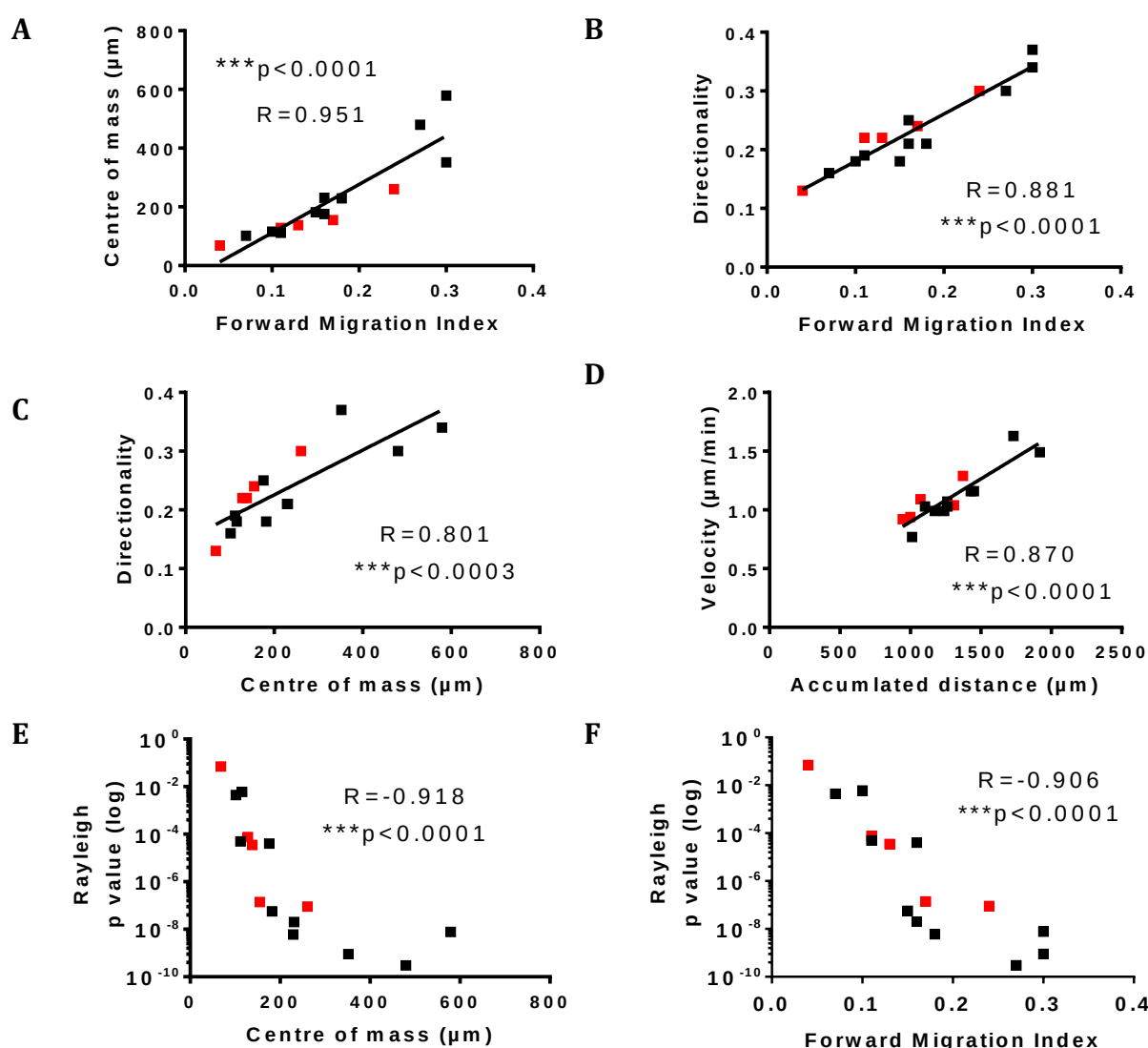


Figure 3.11. Validating the μ -slide chemotaxis assay measures of migration. Human UCB CD133⁺ cells cultured in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6, and 20ng/ml TPO for 24 hours (including some nucleofected with a non-targeting siRNA described in section 2.6.1.1, coloured red) were washed in stemspan medium alone. The cells were re-suspended in 0.5 or 1mg/ml collagen I gels and seeded into 3D μ -slide chemotaxis chambers (Ibidi GmbH) for 17 hours if nucleofected or 22 hours otherwise. Image J was used to manually track at least 30 randomly selected cells per condition to generate quantitative data. The measures of migration were correlated with each other using Spearman's rank correlation to determine the R value and p value for A) displacement of centre of mass and forward migration index, B) directionality and forward migration index, C) directionality and displacement of the centre of mass, D) velocity and accumulated distance, E) displacement of the centre of mass and Rayleigh p value (log), and F) forward migration index and Rayleigh p value (log). The line was fitted to the individual data points using linear regression (where data were not displayed on a logarithmic scale) (Graphpad Prism) (n=15 independent experiments $**p<0.01$, $***p<0.001$).

Table 3.2. Summary of correlation between measures of chemotaxis.

Correlation	R value	P value
Accumulated distance vs. COM	0.361	0.187
Velocity vs. COM	0.459	0.086
Accumulated distance vs. directionality	0.029	0.912
Velocity vs. Accumulated distance	0.870	***<0.0001
COM vs. directionality	0.801	***0.0003
FMI vs. directionality	0.881	***<0.0001
FMI vs. Rayleigh	-0.906	***<0.0001
COM vs. Rayleigh	-0.918	***<0.0001
Velocity vs. Rayleigh	-0.278	0.317
Accumulated distance vs. Rayleigh	-0.311	0.260

The analysis between the measures of migration listed was performed by Spearman's rank correlation (n=15, **p<0.01, ***p<0.001). Abbreviations are displacement of the centre of mass (COM), forward migration index (FMI).

3.3.10 AMD3100 blocks CD133⁺ cell chemotaxis but not chemokinesis

To demonstrate that human UCB CD133⁺ cell migration to CXCL12 could be blocked, the CXCR4 receptor antagonist AMD3100 was used. This has been used previously in the laboratory to block CXCR4-CXCL12 mediated wound repair in the scratch assay (Zhou et al., 2012). The human UCB CD133⁺ cells, which had been thawed from frozen or freshly isolated (described in section 2.2.2.3) and cultured for 24 hours in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, were washed in stemspan medium alone. The cells were re-suspended in a 1mg/ml collagen I gel at 3x10⁶ cells/ml and 6µl seeded per chamber of a 3D chemotaxis slide. Cell migration towards no CXCL12 (control) (Figure 3.12A) or 1µg/ml CXCL12 without or with 1µM or 10µM AMD3100, was tracked over 22 hours (Figure 3.12B-D respectively). Trajectory plots and quantitative data were derived using Image J plugins, as described in section 2.7.2.1. With 1µM and 10µM AMD3100 (Figure 3.12C-D),

CD133⁺ cell chemotaxis to CXCL12 was reduced to migration patterns similar to those seen with no CXCL12 present (Figure 3.12A).

With the CXCL12 gradient and no AMD3100, the Rayleigh p values were $**p < 0.01$ for all experiments, however with 1 μ M and 10 μ M AMD3100, the Rayleigh p values were $p > 0.05$ for all experiments. Therefore the cell distribution towards CXCL12 was not significantly inhomogeneously distributed in the presence of AMD3100 and this was comparable to control distribution (Rayleigh p value mean $\pm$ SEM 0.253 ± 0.108 , $n=4$). With no AMD3100, the mean $\pm$ SEM p values for human UCB CD133⁺ cell chemotaxis to CXCL12 were 0.001 ± 0.001 ($n=4$), and with 1 μ M AMD3100 0.650 ± 0.170 ($n=3$) and 10 μ M AMD3100 0.481 ± 0.146 ($n=4$) (Figure 3.13A(i)). A decrease in forward migration index from 0 μ M AMD3100 was observed when 1 μ M and 10 μ M AMD3100 were added (mean $\pm$ SEM values were 0.11 ± 0.02 with no AMD3100 and 0.01 ± 0.01 for 1 μ M ($p=0.06$, $n=3$) and 0.01 ± 0.01 for 10 μ M AMD3100 ($*p=0.03$, $n=4$) (Mann Whitney test)) (Figure 3.13A(ii)). A decrease was also seen for the displacement of the centre of mass (mean $\pm$ SEM values were $126.5 \mu\text{m} \pm 21.1$ for no AMD3100 to $24.7 \mu\text{m} \pm 18.1$ for 1 μ M ($p=0.06$, $n=3$) and $7.5 \mu\text{m} \pm 10.6$ for 10 μ M AMD3100 ($*p=0.03$, $n=4$) (Mann Whitney test)) (Figure 3.13A(ii)).

Interestingly, there was no detrimental effect on human UCB CD133⁺ cell chemokinesis with a CXCL12 gradient, since neither the accumulated distance nor the velocity were affected by the absence (mean $\pm$ SEM $1167 \mu\text{m} \pm 79$ and $1.0 \mu\text{m}/\text{min} \pm 0.0$ respectively, $n=4$) or presence of 1 μ M (mean $\pm$ SEM $1200 \mu\text{m} \pm 179$ and $1.0 \mu\text{m}/\text{min} \pm 0.1$ respectively, $n=3$) or 10 μ M AMD3100 (mean $\pm$ SEM $1255 \mu\text{m} \pm 100$ and $1.0 \mu\text{m}/\text{min} \pm 0.1$ respectively, $n=4$) (Figure 3.13A(iv and v)).

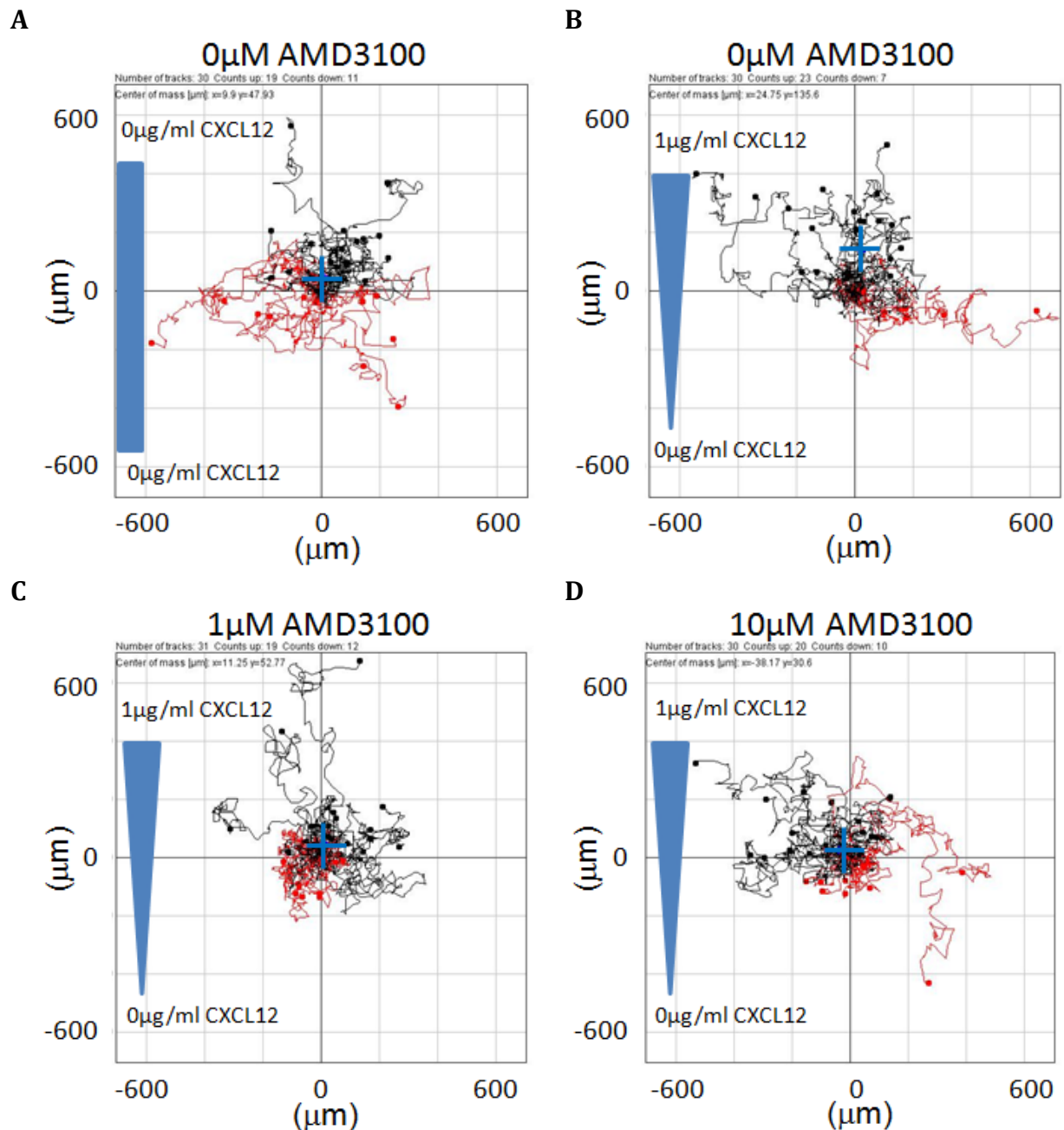


Figure 3.12. AMD3100 blocks CD133+ cell chemotaxis in the 3D μ -slide assay. Human UCB CD133+ cells cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium and re-suspended in a 1mg/ml collagen I. The cells were seeded in 3D μ -slide chemotaxis chambers (Ibidi GmbH). The movement of at least 30 cells were tracked for 22 hours using Image J plug-ins. Representative trajectory plots show migration (A) without a CXCL12 gradient or in the presence of a 1 μ g/ml CXCL12 gradient with (B) 0 μ M AMD3100, (C) 1 μ M AMD3100 and (D) 10 μ M AMD3100. The data were analysed using the Image J Chemotaxis and migration plug-in and is shown in Figure 3.13 ($n \geq 3$ independent experiments).

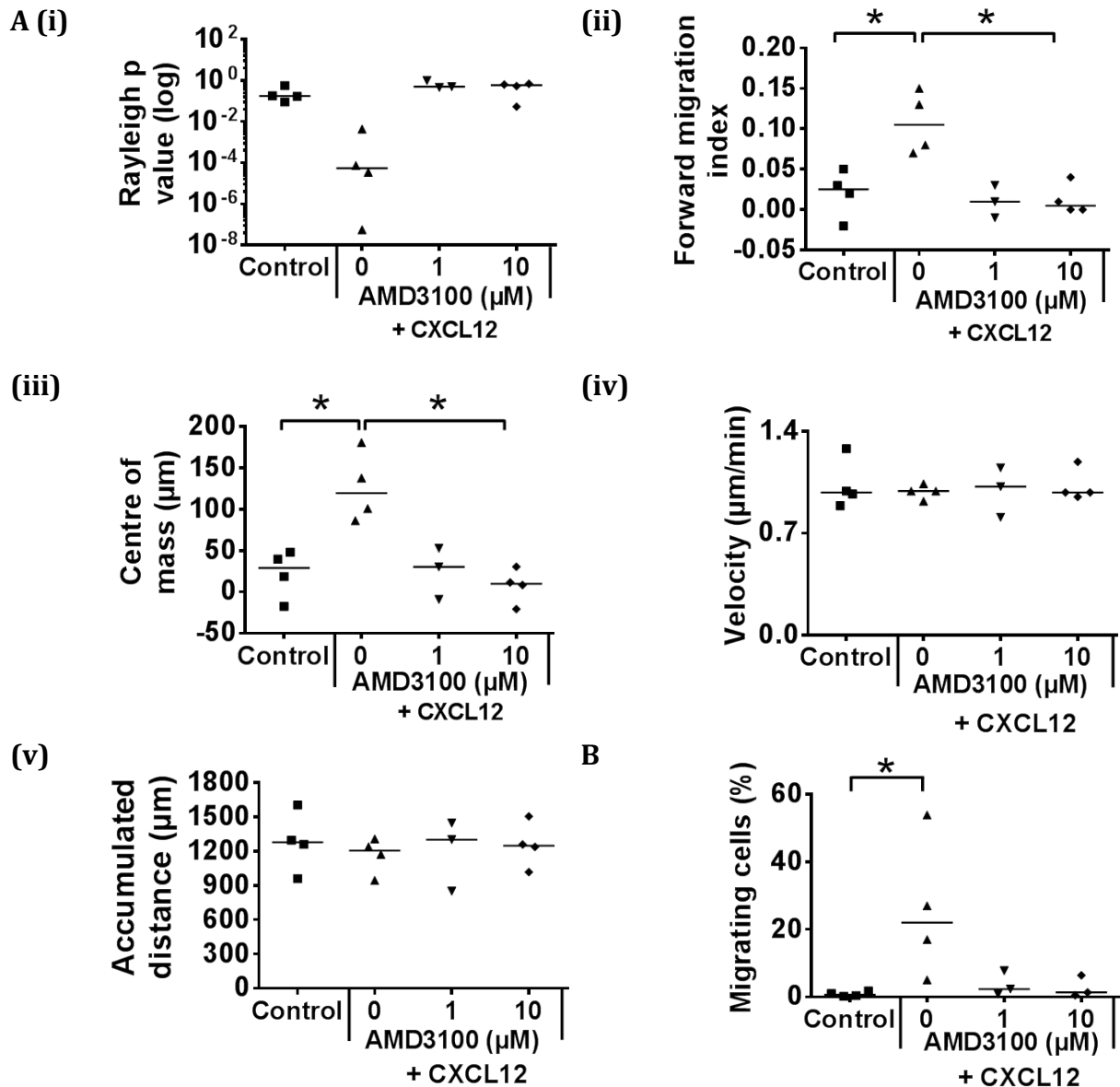


Figure 3.13. AMD3100 blocks CD133⁺ cell chemotaxis but not chemokinesis.

Human UCB CD133⁺ cells cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed in stemspan medium and either seeded into A) 3D μ -slide chemotaxis chambers (Ibidi GmbH) in a 1mg/ml collagen I gel, or B) 2×10^5 CD133⁺ cells were seeded onto a transwell fibronectin coated porous filter (Corning) for 4 hours. In A) the movement of at least 30 cells towards no CXCL12 (control) or 1 μ g/ml CXCL12 with or without AMD3100 as indicated, were tracked for 22 hours using Image J plug-ins to generate (i) Rayleigh p values (significant where $p < 0.05$), (ii) forward migration index, (iii) the displacement of the centre of mass, (iv) velocity and (v) accumulated distance, and in B) cell migration through the transwell towards no CXCL12 (control) or 0.35 μ g/ml CXCL12 with or without AMD3100 as indicated, was calculated by determining the % cells migrating compared to input by flow cytometry. Graphs display individual data and median (line) ($n \geq 3$ independent experiments). Statistics between conditions for A)(ii-v) and B) were calculated using the Mann Whitney test (* $p < 0.05$).

This also confirmed that the cells remained viable and able to function in the presence of AMD3100 as they would in control conditions (mean $\pm$ SEM 1281 μ m $\pm$ 132 and 1.0 μ m/min $\pm$ 0.1 respectively, n=4). These data further validate the use of 3D μ -slide chemotaxis assay for investigating chemotaxis and chemokinesis.

In order to provide a comparison to an existing migration assay, the standard transwell migration assay with and without AMD3100 was performed in parallel to the 3D μ -slide chemotaxis assay. The experiment was performed as described in section 2.7.3. The human UCB CD133⁺ cells were prepared as for the 3D μ -slide chemotaxis assay, but seeded onto fibronectin coated transwell inserts. Migration towards 350ng/ml CXCL12 (CXCL12 titration shown in section 2.7.3) was assessed by determining the percentage of cells that had migrated compared to the input number of cells (fluorescently labelled) by flow cytometry (detection method described in section 2.7.3). With the addition of AMD3100, the percentage of cells migrating was reduced from where no AMD3100 was added (mean $\pm$ SEM 25.8% $\pm$ 10.4, n=4) to 3.7% $\pm$ 2.1 (n=3) with 1 μ M AMD3100 and 2.8% $\pm$ 1.8 with 10 μ M AMD3100, but this was not statistically significant (p=0.11 when comparing 0 μ M AMD3100 to either 1 μ M or 10 μ M AMD3100) (Figure 3.13B). Therefore, the cell migration through the transwell was not reduced to the extent of control with no CXCL12 (mean $\pm$ SEM 0.9% $\pm$ 0.4), as this was significant from migration to CXCL12 (*p=0.03, n=4, Mann Whitney test) (Figure 3.13B).

3.3.11 Pooling CD133⁺ cells from different donors does not affect migratory capacity but batch variation is seen

In some experiments CD133⁺ cells from two or more UCB units were pooled where cell numbers from a single unit were insufficient for experimental use. The effect of pooling units on migration was compared to single unit migration to ensure this did not account for any differences in the data presented above. The average values were similar between pooled and non-pooled units for forward migration index, directionality and velocity, but slightly increased in non-pooled units for the displacement of the centre of mass and the accumulated distance (Figure 3.14A(i-v)). These differences were not significant ($p > 0.05$, Mann Whitney test). The mean $\pm$ SEM values for pooled ($n=8$) and non-pooled ($n=7$) CD133⁺ cells were $166\mu\text{m}\pm 34$ and $212\mu\text{m}\pm 94$ for the displacement of the centre of mass, 0.16 ± 0.03 and 0.16 ± 0.02 for forward migration index, 0.24 ± 0.03 and 0.22 ± 0.02 for directionality, $1160\mu\text{m}\pm 64$ and $1285\mu\text{m}\pm 94$ for accumulated distance and $1.1\mu\text{m}/\text{min}\pm 0.0$ $1.1\mu\text{m}/\text{min}\pm 0.1$ for velocity respectively.

Variability in the migratory response of human UCB CD133⁺ cells from single UCB units to CXCL12 was also observed (Figure 3.14A(i-v) non-pooled). The ranges were as follows: displacement of the centre of mass (pooled $68\mu\text{m}$ to $352\mu\text{m}$ and non-pooled $77\mu\text{m}$ to $480\mu\text{m}$), forward migration index (pooled 0.04 to 0.30 and non-pooled 0.07 to 0.27), directionality (pooled 0.13 to 0.37 and non-pooled 0.15 to 0.30), accumulated distance (pooled $946\mu\text{m}$ to $1451\mu\text{m}$ and non pooled $1012\mu\text{m}$ to $1731\mu\text{m}$) and velocity (pooled $0.92\mu\text{m}/\text{min}$ to $1.29\mu\text{m}/\text{min}$ and non-pooled $0.77\mu\text{m}/\text{min}$ to $1.63\mu\text{m}/\text{min}$).

This varied UCB CD133⁺ cell migration to CXCL12 was also observed in the transwell migration assay (pooled range 12.4% to 53.7% and non pooled 4.7% to 59.5%) (Figure 3.14B). There were no differences in transwell migration between the mean $\pm$ SEM for pooled (26.7% $\pm$ 7.4, n=5) and non-pooled CD133⁺ cells (26.4% $\pm$ 16.7, n=3). The variability observed between experiments suggested that there were individual differences in the ability of UCB units from different donors to migrate in both the *in vitro* transwell and μ -slide chemotaxis assays.

The migration seen in the transwell migration and μ -slide chemotaxis assays was also compared directly. For the 3D μ -slide chemotaxis assay, the % of cells migrating was calculated by determining the percentage of migrating cells (moving greater than one cell diameter) displaying chemotaxis towards CXCL12 (defined as the number of cells out of the total tracked whose paths ended in the direction of the gradient on the relevant axis). In four experiments, CD133⁺ cells were split in half and put into each assay in parallel. Similar responses were seen in both assays, however the percentage of cells displaying chemotaxis towards CXCL12 seen in the μ -slide chemotaxis assay was much greater than the transwell assay (mean $\pm$ SEM of 82.5% $\pm$ 6.6 and 26.5% $\pm$ 11.8 respectively). This could be because the 3D μ -slide chemotaxis assay is more sensitive at detecting moving cells than the transwell assay.

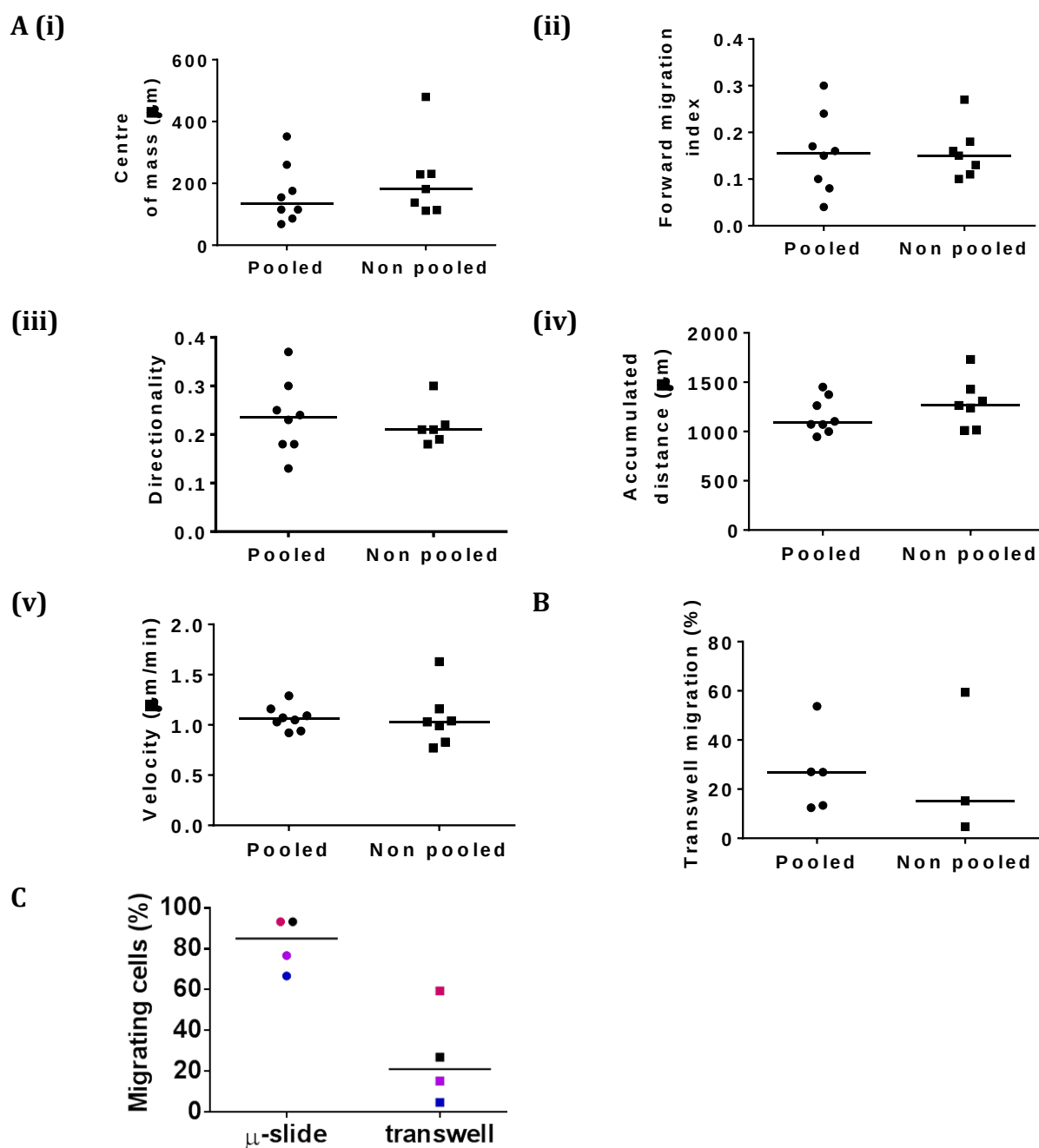


Figure 3.14. Comparing the migration ability of pooled and non-pooled CD133⁺ cells in the $\mu\text{-slide}$ chemotaxis assay and the transwell migration assay. Pooled or non pooled human UCB CD133⁺ cells cultured in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO for 24 hours were washed and either A) seeded into the 3D $\mu\text{-slide}$ chemotaxis assay (Ibidi GmbH) in a 1mg/ml collagen I gel and tracked for 18-22 hours in the presence of a CXCL12 gradient (described in section 2.7.2.1) to generate (i) displacement of the centre of mass, (ii) forward migration index, (iii) directionality, (iv) accumulated distance, (v) velocity ($n=8$ pooled and $n=7$ non pooled), or B) seeded into the transwell assay for 4 hours in the presence of a CXCL12 gradient (as described in section 2.7.3) ($n=5$ pooled and $n=3$ non pooled). C) Cells (pooled and non-pooled) were compared in both assays in parallel (matched experiments are colour coded) ($n=4$). Data from individual experiments and median values (horizontal line) are displayed.

3.4 Discussion

CXCL12, a chemokine expressed by bone marrow mesenchymal stem/stromal cells, osteoblasts and endothelial cells, has been widely documented as a key chemo-attractant for HSPCs expressing CXCR4 (Aiuti et al., 1997; Dar et al., 2006; Imai et al., 1999; Peled et al., 1999b; Yun and Jo, 2003). CXCL12 can also bind to CXCR7, where it is internalised, but significant expression of CXCR7 on HSPCs has not been demonstrated (Berahovich et al., 2010; Graham et al., 2012; Tarnowski et al., 2010).

In this chapter, a novel and reproducible μ -slide chemotaxis assay has been developed using 3D μ -slide chemotaxis chambers from Ibidi GmbH (Munich, Germany). In this assay, the cells migrate through a collagen I matrix in which they are embedded towards a soluble gradient of CXCL12. Although previous end-point assays, such as the transwell assay, have shown cell migration towards CXCL12, these simply give a percentage of cells migrating across a membrane. Distinguishing between chemotactic and chemokinetic effects of stimuli such as CXCL12 have been shown in this system (Kim and Broxmeyer, 1998), however demonstrating the mechanisms affected by inhibitors of migration, such as AMD3100 have proven more difficult. The transwell assay is also dependent on the experimenter recovering all of the cells in the lower well, which may have adhered to the fibronectin, or been lost during pipetting and washing whilst preparing for flow cytometric or counting analysis. Using the 3D μ -slide chemotaxis assay, time-lapse microscopy and Image J plug-ins allows cell tracking which generates quantitative, visual and statistical analysis on both cell chemotaxis and chemokinesis.

Nevertheless, the transwell assay offers a useful and popular system to determine whether cells are willing to actively crawl through transwell filters in the presence of

a stimulus, as the pore dimensions are smaller than the cells. Although the 3D μ -slide chemotaxis assay provided a stable gradient for at least the 22 hours measured, the transwell assay was conducted for the standard 4 hours. This was because Kim and Broxmeyer showed no additional CD34⁺ transwell migration to CXCL12 beyond this time point, and showed an increase in cell proliferation and apoptosis (Kim and Broxmeyer, 1998), which is a confounding variable when relying on input cell number to determine final migration percentages. Using the 3D μ -slide chemotaxis assay, we demonstrated increasing cell migration for 22 hours, indicating that these cells do not stop responding to CXCL12 after 4 hours, as the transwell assay would suggest (Kim and Broxmeyer, 1998).

When developing the assay, we firstly established that methylcellulose did not prove practical for this application. Matrigel, which is commonly used in invasion assays (Havens et al., 2006), was also tested. Although this proved practical and retained the cells, matrigel was not fully validated as collagen I was deemed more similar to the extra-cellular matrix found in the bone marrow niche and had been shown to function in this assay by Ibidi GmbH. Collagen I gels (0.5 to 3mg/ml) supported CD133⁺ cell movement in terms of accumulated distance and velocity. Chemotactic migration towards CXCL12 was on average slightly, although not significantly ($p>0.05$), higher with the 3mg/ml collagen I gel, but variability amongst independent experiments was increased when compared with 0.5 or 1mg/ml collagen I gels. This variability resulted from a lack of uniformity in the collagen fibre formation at 3mg/ml concentrations due to the speed at which the gel set when less diluted. Therefore 1mg/ml collagen I gel was chosen for the experiments.

We noted that human UCB CD133⁺ cells displayed increasing chemotaxis up to 1µg/ml CXCL12 (sourced from Peprotech) in the 3D µ-slide chemotaxis assay. This was higher than the frequently used concentration of 0.2µg/ml in the transwell assay (Forde et al., 2007). However, Aiuti *et al.* have shown that CD34⁺ cells from UCB required a higher concentration of CXCL12 than those from MPB for peak migration in the transwell assay (approx 0.3µg/ml CXCL12) (Aiuti et al., 1997). In house titration experiments (section 2.7.3) also found improved UCB CD133⁺ cell transwell migration to higher than previously used concentrations of CXCL12.

Additionally, Bleul *et al.* demonstrated that 1µg/ml CXCL12 provided the peak of lymphocyte migration in the transwell assay (Bleul et al., 1996). In further support of the use of this concentration in the 3D µ-slide chemotaxis assay, Weidt *et al.* used 1µg/ml CXCL12 in a 3D collagen based assay looking at the effect of CXCL12 stimulation on CD133⁺ movement (Weidt et al., 2004). Seemingly contrary to the findings of this thesis, where no effect on chemokinesis was observed, Weidt *et al.* described an overall increase in accumulated distance migrated but a decrease in cell velocity in the presence of 1µg/ml CXCL12 compared to no CXCL12 (Weidt et al., 2004). However, it should be noted that the experiments conducted by Weidt *et al.*, took place over a 90 minute period and in the presence of surrounding CXCL12 rather than a stable gradient as used here. Weidt *et al.* also reported no effect on cell chemokinesis at the single cell level under these conditions, and put their findings down to an overall increase in the number of cells displaying motility (Weidt et al., 2004). This was a measure not obtained in the 3D µ-slide chemotaxis experiments described here.

In the final validated procedure, there were significant differences in chemotaxis but not chemokinesis between the cells in the presence of a CXCL12 gradient and those with no CXCL12 gradient. The mean $\pm$ SEM Rayleigh test p values were ***0.001 $\pm$ 0.001 and 0.336 $\pm$ 0.073 respectively, confirming that the cells were distributed in a significantly inhomogeneous fashion in the presence of the CXCL12 gradient but not without (***p<0.001, n=8). Differences were also seen in the displacement of centre of mass in the presence of CXCL12 compared to no CXCL12 (189.0 μ m $\pm$ 44.3 compared to 10.8 μ m $\pm$ 11.3, n=8) and forward migration index (0.126 $\pm$ 0.024 compared to 0.015 $\pm$ 0.008, n=8). Increased directionality was also seen in the presence of CXCL12 compared to no CXCL12 (0.205 $\pm$ 0.015 and 0.128 $\pm$ 0.006 respectively).

To validate the 3D μ -slide chemotaxis assay, we confirmed that chemotaxis was inhibited with AMD3100. This CXCR4 antagonist prevents CXCL12 binding, and thus preventing CXCL12-dependent migration and confirming the key role of CXCR4 in CD133⁺ cell chemotaxis (Hatse et al., 2002; Kawaguchi et al., 2009). Interestingly, the variance in CXCR4 surface expression (Figure 3.2C) did not correlate with chemotaxis (the displacement of centre of mass or forward migration index) when a Spearman's Rank Correlation was performed (data not shown). However, the surface expression was measured on a sample of cells prior to performing the chemotaxis assay and CXCR4 expression is known to be dynamic and is harboured intracellularly (Kollet et al., 2002), therefore these results are difficult to interpret.

Additionally, we noted that there was no effect of AMD3100 on human UCB CD133⁺ cell chemokinesis, in terms of the accumulated distance and cell velocity. Therefore, CXCR4 receptor blockade by AMD3100 affected CD133⁺ cell CXCL12 sensing, but not random cell motility. This, in addition to the finding that CXCL12 only acted on

chemotaxis with no effect on chemokinesis, demonstrated the advantages of this system. CXCL12 was shown to have activity in transwell chemotaxis assays and induce actin polymerisation in human peripheral blood lymphocytes by Bleul *et al.* in 1996 (Bleul et al., 1996). Voermans *et al.* also demonstrated that transwell migratory capacity of human UCB HSPCs to CXCL12 was determined by their capacity to efficiently polymerise F-actin (Voermans et al., 2001). Therefore, it was suggested that actin polymerisation, which has been shown to occur faster in cells over-expressing CXCR4 in response to CXCL12 (Kahn et al., 2004), and subsequent cell motility, required a stimulus such as CXCL12.

Although it has been suggested that chemotaxis does not occur without increasing chemokinesis (Entschladen et al., 2005), we have shown that these measures are not always indicative of one another. The studies here demonstrated that human UCB CD133⁺ cells suspended in stemspan medium without cytokines in a collagen I gel displayed random migration (chemokinesis) with no additional stimulation required. This was independent of CXCR4-CXCL12 interactions, as chemokinesis was not affected by the presence or absence of CXCL12 or AMD3100. Ibidi GmbH have demonstrated that HT-1080 cell chemokinesis (velocity) can be doubled in the presence of FCS, compared to no FCS, demonstrating that the 2D μ -slide version of this assay can identify changes to chemokinetic measures (Zengel et al., 2011).

The number of human UCB CD133⁺ cells migrating in the transwell assay differed from those migrating in the 3D μ -slide chemotaxis assay. The transwell migration results described here are consistent with those described in the literature for HSPCs (approximately 20-30%) (Aiuti et al., 1997; Kollet et al., 2002). The difference in the percentage of cells migrating towards CXCL12 between the transwell and 3D μ -slide

chemotaxis assays can be partly explained by the sensitivity of detection of migrating cells, but the mechanics of the two assays and the different ECM components used in each assay should also be taken into consideration. Studies have demonstrated that the migration mechanisms of dendritic cells differ dramatically when a soluble gradient is presented in a 3D environment compared to when presented on a 2D substrate (Renkawitz et al., 2009). It has also been shown that in CD34⁺ cells, polarisation and migration was dependent on VLA-4 and VLA-5 in 3D collagen-laminin-fibronectin gels, whereas adhesion to 2D fibronectin was mediated by VLA-5 but not VLA-4 (Peled et al., 2000).

The active migration in the transwell assay, which was dependent on CXCL12, was performed through 2D fibronectin. Migration through the 3D μ -slide chemotaxis assay required the cells to move through a 3D collagen I gel matrix, where chemotaxis, but not motility was dependent on CXCL12. Collagen I and fibronectin are extracellular matrices, which are linked to the cytoskeleton of cells by specific integrins, which mediate cell adhesion, motility and spreading (Humphries et al., 2006). While HSPCs are known to bind to fibronectin through $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5), they are not generally reported to express significant levels of the collagen receptors, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ (Humphries et al., 2006). However, human UCB CD34⁺ cells have been shown to secrete matrix metalloproteinases (MMPs) including MMP-9 (which degrades CXCL12) and MMP-2 (which degrades collagen I and CXCL12) (Wysoczynski et al., 2005). This MMP secretion was shown to enhance cell migration in the transwell assay through matrigel and ECM components such as hyaluronic acid (Wysoczynski et al., 2005). Human MMPs constitute a family of 24 endopeptidases which degrade extracellular matrices, including gelatinases and

collagenases (Zitka et al., 2010). They also interact with other substrates which include chemokines, cell surface receptors, growth factors, cytokines, adhesion molecules, MMPs, proteinases and proteinase inhibitors (reviewed by (Shirvaikar et al., 2012)).

We noted that human UCB CD133⁺ cell velocity increased over time in the presence and absence of a CXCL12 gradient, and that the greatest increase was during the first 4 hours of the assay (0 to 0.71 ± 0.14 $\mu\text{m}/\text{min}$; mean $\pm$ SEM, n=3). We have not examined protease secretion by our cells which may enhance UCB CD133⁺ cell motility or chemotaxis in our 3D μ -slide chemotaxis assay. However, it has been reported that MMPs, including proMMP-9 and proMMP-2, which is activated by MMP-2, are able to modulate human CD34⁺ cell migration to CXCL12 and engraftment (Shirvaikar et al., 2008; Shirvaikar et al., 2011). Furthermore, chemokines and growth factors such as SCF and CXCL12 have been shown to induce secretion of MMP-2 and MMP-9 by human UCB CD34⁺ cells and improve cell migration towards CXCL12, enhance homing *in vivo* (Marquez-Curtis et al., 2011; Zheng et al., 2003; Zheng et al., 2005) and enable bone marrow egress (Heissig et al., 2002). Thus, it is possible to speculate that prior treatment of UCB CD133⁺ cells in our system with SCF and/or subsequent exposure to CXCL12 could induce MMP-2 secretion by these cells, resulting in the degradation of collagen I and an initial increase in cell motility.

The μ -slide chemotaxis assay generates substantial data (quantitative measures of chemokinesis (random cell movement) including cell velocity, distance travelled as well as measures of chemotaxis and directionality). In the studies presented here, the data was produced by manual tracking. Wimas GmbH semi-automated tracking was tested alongside manual tracking in an effort to automate the cell tracking. In

preliminary experiments, the Wimasis algorithm was unable to follow cells for the duration of the experiment and to distinguish between cells it had already followed earlier in the time course. Although the Wimasis automated tracking provided data that were comparable to manual tracking in terms of the direction of the centre of mass, the difference was in the distance of the displacement of centre of mass. The sensitivity in following an entire cell's path by time-lapse rather than real time imaging, especially with non-adherent fast moving cells, was lost in the automated algorithm. For the analysis of differences in chemotactic parameters, manual tracking has therefore proven preferable. However, for experiments where chemotaxis is expected to be either present or not present in easy to follow cells, for example migrating adherent endothelial cells towards a 0:10% FCS gradient, or no gradient, the Wimasis software can provide data that resembles manual tracking.

The advantages of timelapse microscopy, enabling assessment of chemotaxis by individual cell tracking has been recognised, along with the importance of maintaining a stable gradient in physiologically relevant assays using hydrogels such as collagen (Toetsch et al., 2009). The stability of the gradient over the cells in the observation chamber has been demonstrated in the 2D μ -slide using dye (Zengel et al., 2011). As the dimensions of the observation area in the 2D and 3D μ -slide chemotaxis slides are the same, the same principal should apply. Therefore, the 3D μ -slide chemotaxis assay meets these demands and can be extended by using multi-fluorescence microscopy to provide detailed information on heterogeneous populations. Randomly selecting cells to track provides an accurate representation of migration. The assay allows the user to observe not only the chemotactic and chemokinetic effects of a substance on cell migration, but also the strength of

chemotactic activity and the cell morphology, viability and cell interactions during the migratory process through a defined ECM. The described findings demonstrate the advantages of this system as the effect of different conditions on the ability of low numbers of primary cells to undergo both chemotaxis and chemokinesis can be thoroughly investigated.

In conclusion, this 3D μ -slide chemotaxis assay is a useful tool enabling the user to observe the exact cellular response to chemotactic or chemokinetic stimulants, whilst using fewer cells than transwell migration assays. This is particularly important when considering rare populations, e.g. patient samples or CD133⁺/CD34⁺ cells derived from UCB. Future work could examine if there is a correlation between activity in the chemotaxis assay and homing or engraftment to the bone marrow *in vivo*. This would then provide a screen for conditions which may improve or affect the ability of these cells to reach the bone marrow.

Chapter 4. The role of CD164 in haemopoietic stem/progenitor cell

chemotaxis

4.1 Aims and objectives

- 2) Modulating CD164 expression in CD133⁺ cells derived from human umbilical cord blood (UCB)

- 4) Assessing the effect of CD164 modulation on human UCB CD133⁺ cell chemotaxis and chemokinesis *in vitro*

Parts of the text and figures originally presented above and in this Chapter have been redacted as it cannot be made freely available via ORA because of the sensitive nature of the work.

4.2 Introduction

Although the term “homing” has proved controversial in its definition, it has been suggested that this is the process by which haemopoietic cells successfully lodge in a supportive environment i.e. the bone marrow niche prior to their engraftment (the onset of their proliferation to reconstitute the host) (Papayannopoulou, 2003). For the purpose of this thesis, the term “homing” has been used to describe the migration of the cells to the bone marrow and spleen [REDACTED]. Although it is known that HSPCs also transiently lodge non-selectively in non-haemopoietic tissues merely following haemodynamic flow shortly after intravenous infusion, within 48 hours longer term and functional lodgement is restricted to the spleen and bone marrow (Papayannopoulou, 2003).

In order to further understand the mechanisms by which HSPC home to the bone marrow and spleen, it is important to consider the key homing molecule CXCL12, and its receptor CXCR4. CXCL12 knock-out has been shown to be perinatally lethal in mice and required for B cell lymphopoiesis (Nagasawa et al., 1996). CXCR4, which is expressed on HSPCs, has been shown to be essential for their engraftment in the spleen and bone marrow (Kollet et al., 2001; Peled et al., 1999b). Although CXCR4⁺CD34⁺CD38⁻ cells have been shown to engraft NOD-scid mice, CXCR4 is known to be harboured intracellularly and can be rapidly induced at the cell surface where it becomes functional (Kollet et al., 2002; Rosu-Myles et al., 2000). There is evidence to suggest that increasing CXCR4 expression in HSPC can improve migration *in vitro* and engraftment *in vivo* (Peled et al., 1999b). More recently, a possible association between increasing CXCR4 expression with dimethylprostaglandin E2 (dmPGE2) and improved UCB engraftment in non-human primates has been suggested (Goessling et

al., 2011). As upregulation of CXCR4 can improve engraftment, other surface molecules expressed on HSPC subsets and which act as CXCR4 co-receptors, such as CD164 (Zannettino et al., 1998; Forde et al., 2007), could potentially play a role in maintaining CXCR4 on the cell surface, or, promoting its interaction with the relevant integrins, such as VLA-4 (Forde et al., 2007).

Previously published work from this laboratory showed that CD164 co-localised with CXCR4 and with the integrins VLA-4 and VLA-5 at the site of interaction with CXCL12 presented on fibronectin (Forde et al., 2007). Forde *et al.* also demonstrated that human UCB CD133⁺ HSPCs with transient CD164 blockade displayed reduced passage through fibronectin coated transwell filters towards CXCL12 *in vitro* (Forde et al., 2007).

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4.3 Previous research

Zannettino *et al.* and Forde *et al.* (unpublished data) previously used the 103B2 monoclonal antibody (mab) against the functional epitope of CD164 to block CD164 function in adhesion assays (Zannettino et al., 1998). Forde *et al.* also showed that the

CD164-2 siRNA sequence used in this thesis could knock-down CD164 at the protein level (Figure 4.1A(i)) and reduce human UCB CD133⁺ cell adhesion to BMSC, BMEC-60 and HUVEC *in vitro* (Figure 4.1A(ii) and B(i and ii)). This evidence formed the basis for these studies investigating a role for CD164 in bone marrow homing *in vivo*.

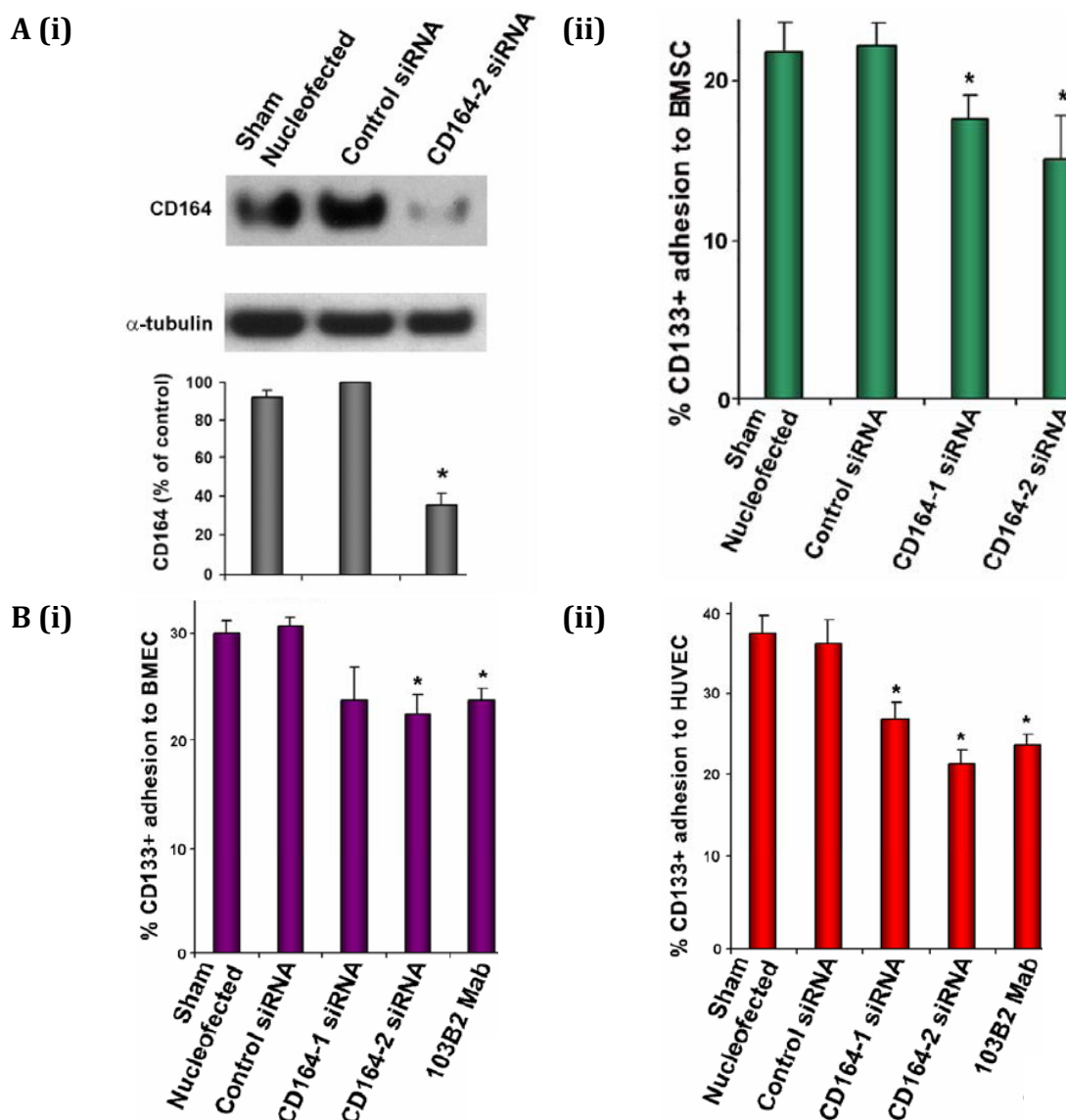


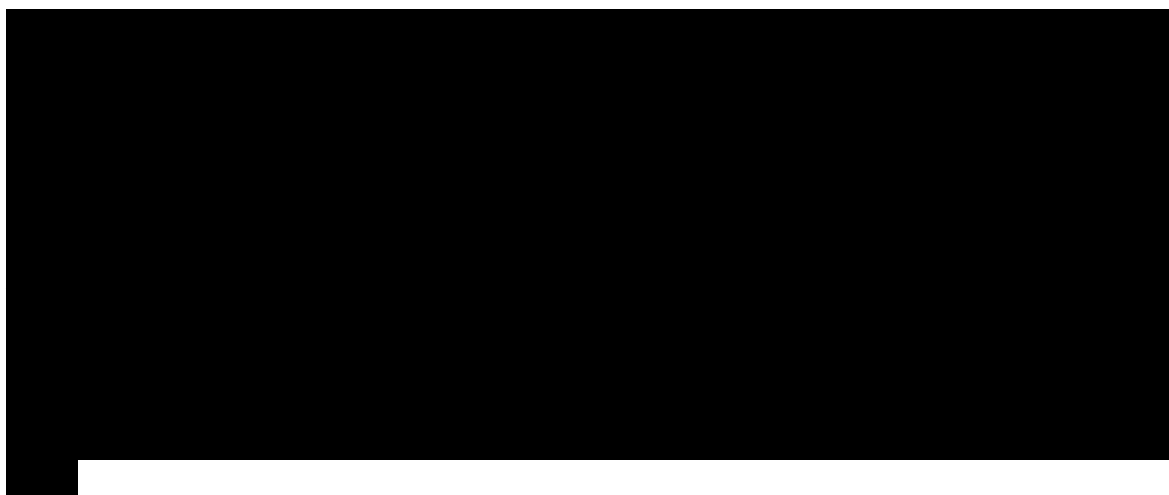
Figure 4.1. CD164 modulation and effect on adhesion to cell layers. A) CD164 was knocked down by nucleofecting human UCB CD133⁺ cells with sham (no siRNA), control siRNA, or CD164-1 or CD164-2 siRNAs, (i) protein level was assessed by Western blotting 48 hours post nucleofection and quantified by densitometry, (ii) adhesion of these nucleofected cells to BMSC layers was assessed. B) The adhesion of these nucleofected cells or human UCB CD133⁺ cells treated with the CD164 103B2 mab, to (i) BMEC-60 or (ii) HUVEC was assessed. Data are presented as means±SEM (n=3), *p<0.05 (Student's *t* test, where the siRNA nucleofected cells were compared to the sham nucleofected cells). The experiments and analysis shown here were performed by Forde *et al.* (unpublished data, 2004-2008).

4.4 Results

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4.4.1.1 Defining the final flow cytometry gating strategy for detecting human cells in harvested mouse bone marrow



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4.4.1.2 Determining conditions for optimal homing of human UCB CD133⁺ cells from murine bone marrow 22-44 hours after i.v. injection into NSG mouse

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4.4.1.3 Optimising human CD45⁺ cell recovery from mouse bone marrow

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4.4.1.4 The effect of mock nucleofection on human UCB CD133⁺ cell short term

homing in vivo

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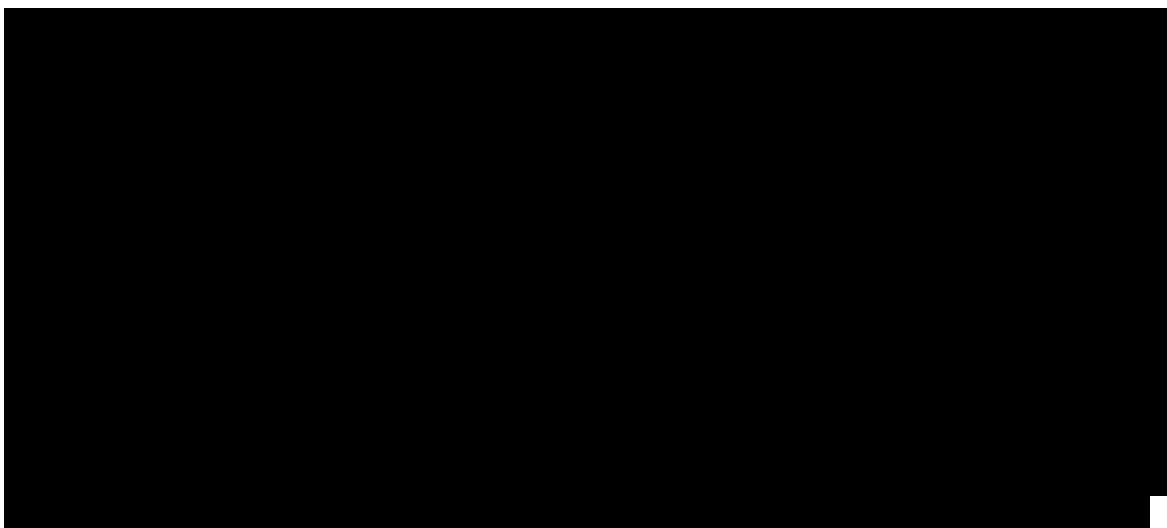
4.4.2.1 Assessing CD164 shRNA knock-down over time

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4.4.2.2 Using the CD164-B shRNA to investigate the effect on human UCB CD133⁺

cell homing to the bone marrow and spleen in NSG mice

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[REDACTED] Human CD133⁺ cells, isolated as described in section 2.2.2.3, were thawed (8x10⁶ in total) and cultured for 24 hours in stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO.

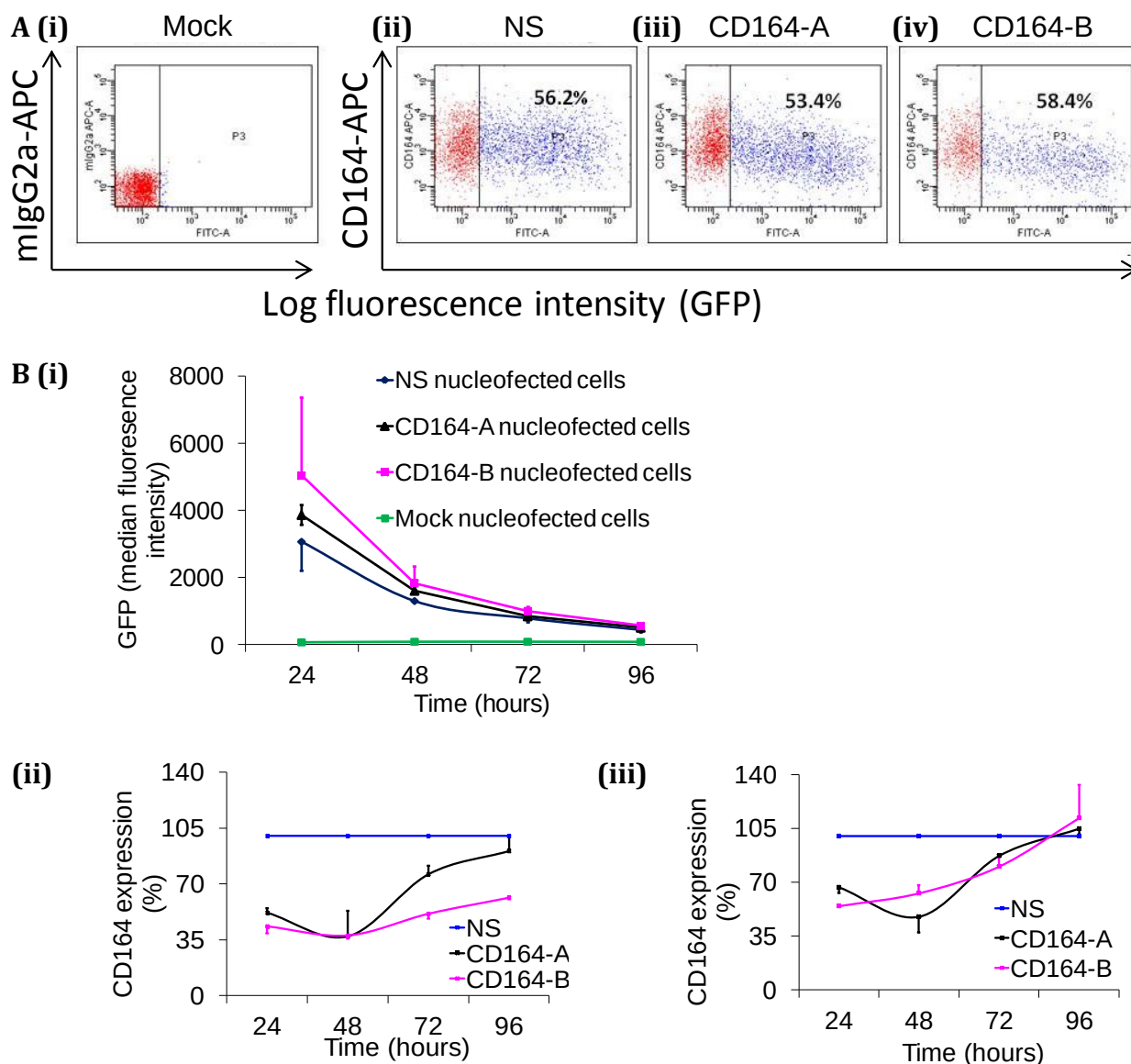
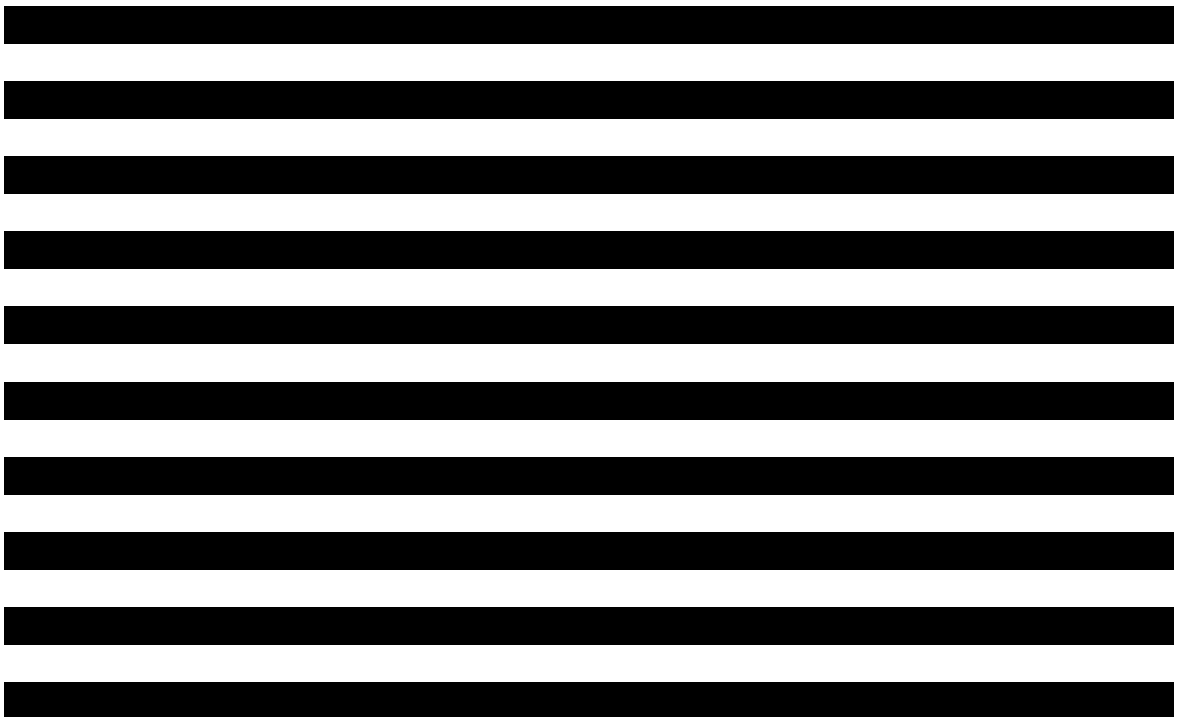


Figure 4.7. Nucleofecting human UCB CD133⁺ cells with CD164 shRNA. Human UCB CD133⁺ cells were either mock nucleofected, or nucleofected with 5 μ g non silencing (NS), CD164-A or CD164-B shRNA. A) Nucleofection efficiencies (GFP positivity) were assessed 24 hours post nucleofection by flow cytometry. Representative FACS dotplots are shown displaying the percentage GFP positive cells for (i) mlgG2a-APC isotype control vs. GFP in mock nucleofected cells, (ii) CD164-APC vs. GFP in NS shRNA nucleofected cells, (iii) CD164-APC vs. GFP in CD164-A shRNA nucleofected cells, and (iv) CD164-APC vs. GFP in CD164-B shRNA nucleofected cells. B) Expression of CD164 and GFP was assessed by median fluorescence intensity using flow cytometry over 96 hours post nucleofection, and is shown as mean $\pm$ SEM (n=2 independent experiments) for (i) GFP MFI in the nucleofected populations compared to mock nucleofected cells, (ii) CD164 surface expression in the GFP positive population of the CD133⁺ cells nucleofected with CD164-A or B shRNA, compared to those nucleofected with NS shRNA (standardised to 100%) and (iii) CD164 surface expression in the total population of the CD133⁺ cells nucleofected with CD164-A or B shRNA, compared to NS shRNA (standardised to 100%).

Counting the cells using a haemocytometer (described in section 2.2.3) revealed that the cell recovery from thawing was 50% (4×10^6 cells). 1×10^6 cells were nucleofected per cuvette with 5 μ g non-silencing (NS) or CD164-B plasmid shRNA (2 cuvettes each) using the program U-008 as described in 2.6.1.1. The nucleofection efficiency for these experiments (assessed by GFP fluorescence by flow cytometry) was 31.5% and 34.4% for NS nucleofected and CD164-B nucleofected cells respectively 24 hours post nucleofection (Figure 4.9A and B). The CD164 surface expression, determined by MFI, was reduced in the CD164-B nucleofected cells (GFP⁺) by 62.6% compared to NS control nucleofected cells 24 hours post nucleofection (Figure 4.9B(ii) and A(ii) respectively). However, CD164 MFI in the entire cell population was reduced by only 14.4% with CD164-B shRNA compared to NS shRNA nucleofected cell CD164 MFI.



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4.4.3.1 Optimising transient CD164 knockdown in CD133⁺ cells using siRNA

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[REDACTED] The siRNAs were used at a concentration of 1200nM, as this had been shown to give maximal CD164 knock-down in human UCB CD133⁺ cells by Western blotting (Forde et al., 2007), and in a preliminary titration experiment (Appendix I.II).

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Flow cytometry confirmed that 1200nM CD164-1 and CD164-2 siRNA provided the most efficient knock-down in CD164 expression at the cell surface of human UCB CD133⁺ cells (Appendix I.II). The control pool of non-targeting (NT) siRNA had no effect on the level of total surface CD164 expression compared to 30nM or 300nM NT siRNA (Appendix I.II).

Subsequently, a timecourse was carried out to assess the knock-down with 1200nM CD164-1 and CD164-2 siRNA compared to 1200nM NT control siRNA [REDACTED]. Human UCB CD133⁺ cells that had been isolated as described in section 2.2.2.3 and frozen, were thawed into stemspan medium without cytokines for 1-2 hours prior to nucleofection with program U-008 and NT, CD164-1 and CD164-2 siRNAs. Following nucleofection, the cells were cultured in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. CD164 surface expression was measured by taking a sample of the nucleofected cells and analysing these by flow cytometry using the CD164 mab N6B6 at 24, 48, 72 and 96 hours post nucleofection (Figure 4.11B). CD164-2 siRNA produced greater and more persistent knockdown than CD164-1 siRNA throughout the time course. CD164 median fluorescence intensity (MFI) was most effectively reduced by 1200nM CD164-2 siRNA between 24 to 48 hours post nucleofection (69.5%±15.8 and 72.6%±10.0 of the NT control MFI (standardised to 100%)) (n=2) (Figure 4.11B).

4.4.3.2 Using siRNA targeting CD164 to investigate the effect on CD133⁺ cell

homing to NSG mouse bone marrow

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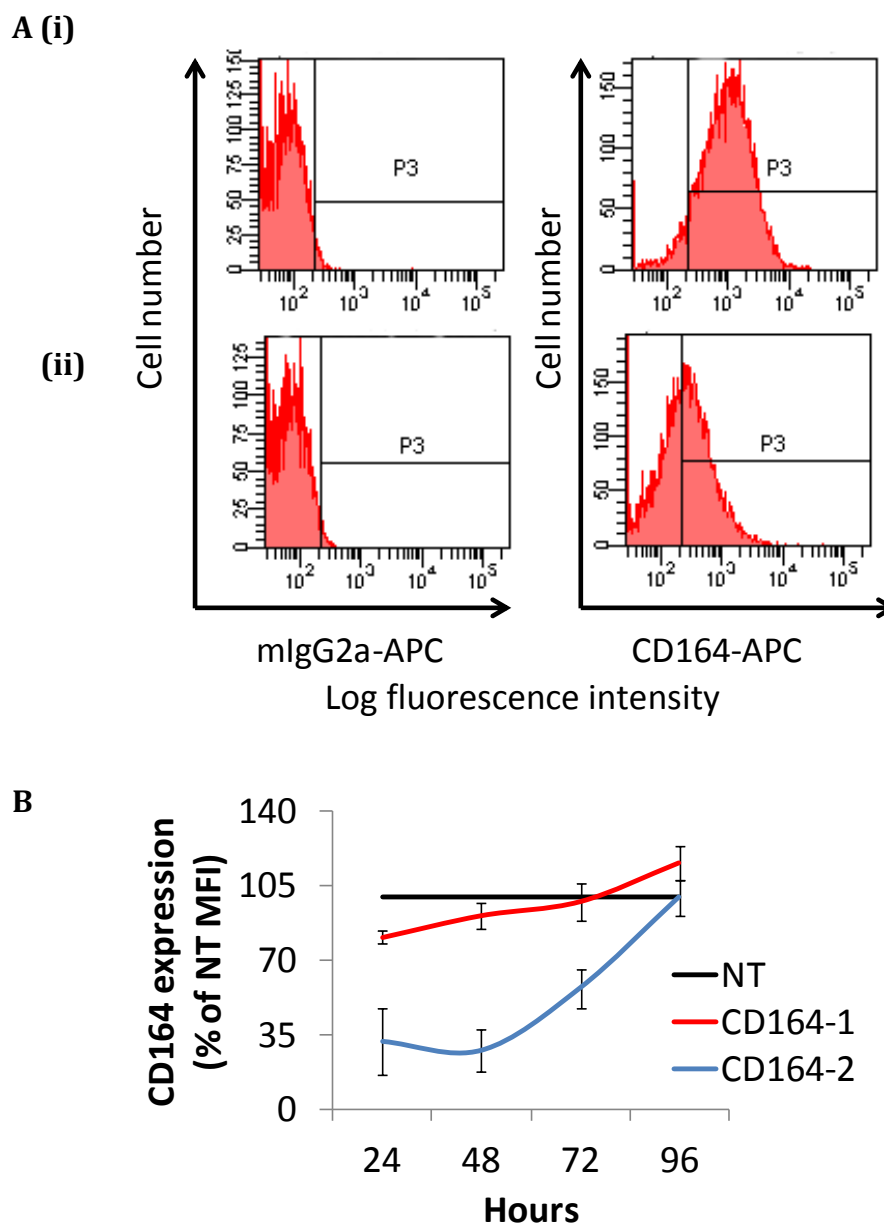


Figure 4.11. Silencing CD164 expression with siRNA. Human UCB CD133⁺ cells were nucleofected with 1200nM non-targeting (NT), CD164-1 or CD164-2 siRNA using the Amaxa nucleofection program U-008 with the CD34 nucleofection kit. CD164 surface expression was assessed at 24 hour intervals for 96 hours post nucleofection by flow cytometry. A) Representative flow cytometry histograms show (i) NT siRNA and (ii) CD164-2 siRNA nucleofected cells stained with mIgG2a-APC and CD164-APC (N6B6) 24 hours post nucleofection as indicated. B) The complete data are expressed as the % of CD164 median fluorescence intensity (MFI) for CD164-1 and CD164-2 siRNA nucleofected cells, where CD164 expression for the NT control siRNA nucleofected cells was standardised to 100%. Graph shows mean $\pm$ SEM (n=2 independent experiments).

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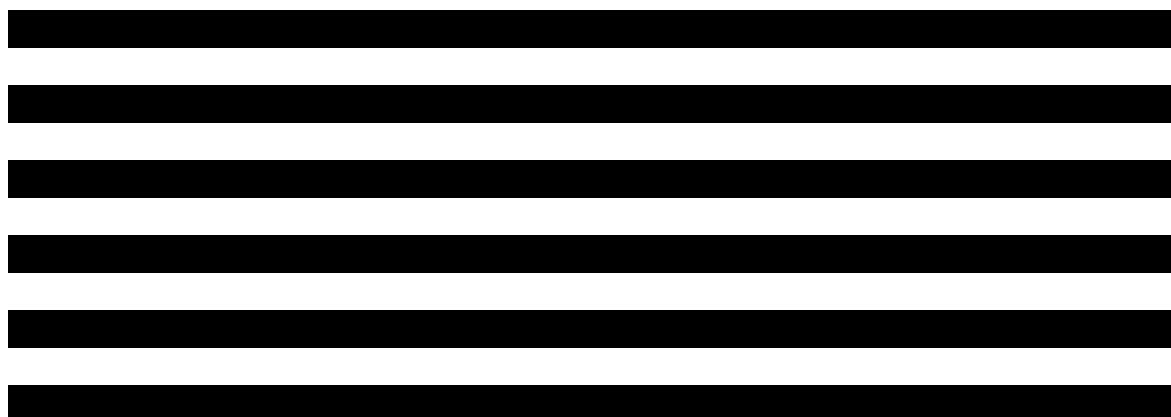


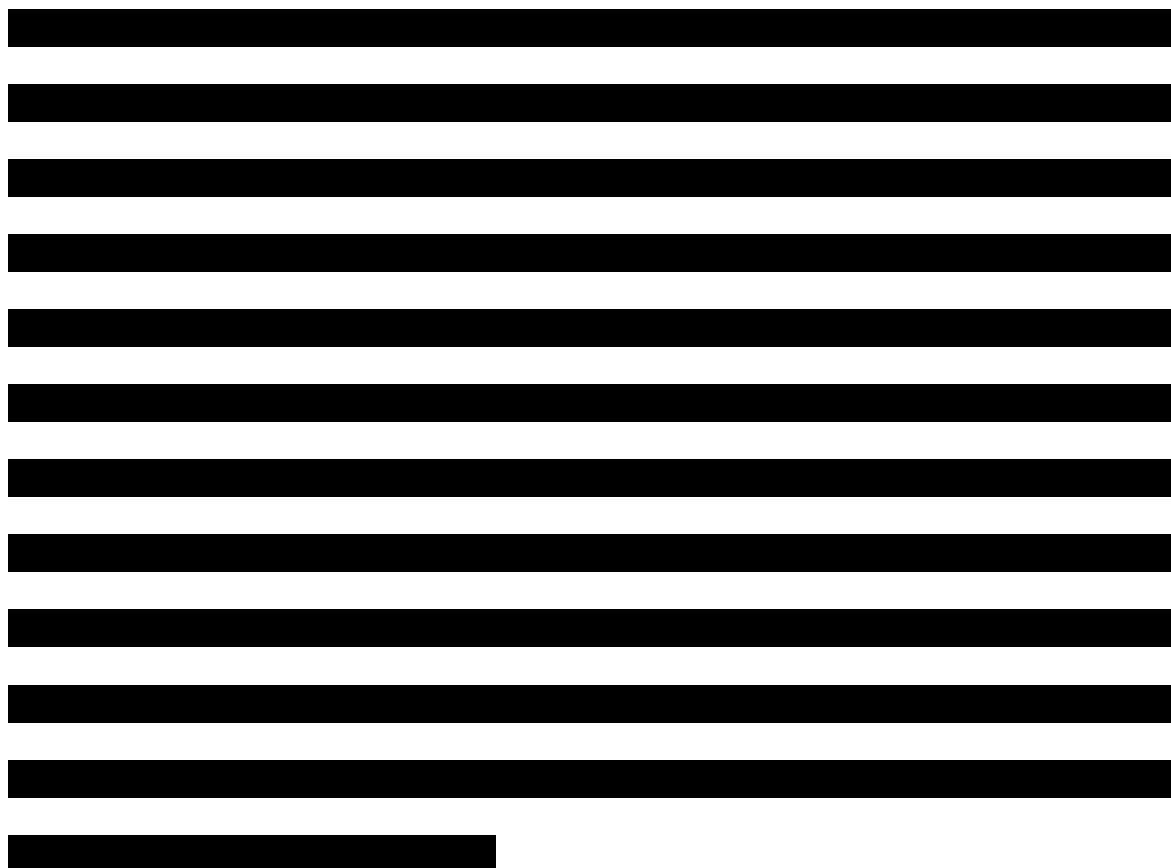
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4.4.3.3 CD164 silencing with siRNA reduces CD133⁺ cell homing to the spleen of

NSG mice

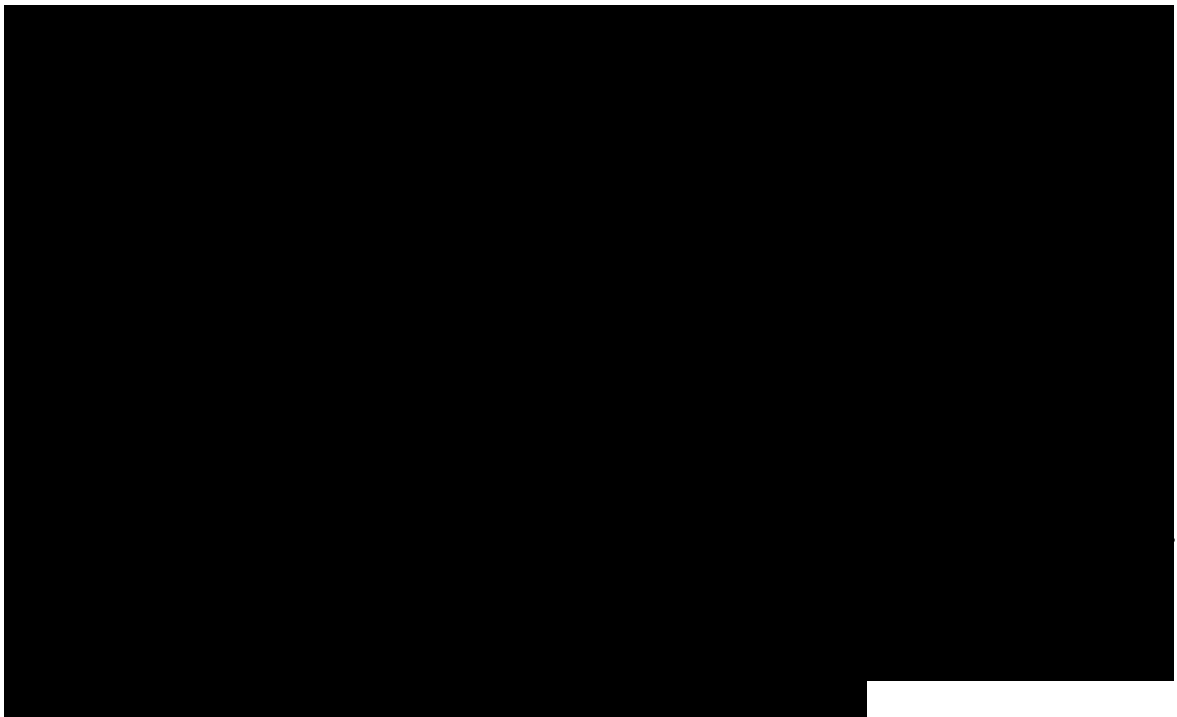




4.4.4 The effect of CD164 knock-down with CD164-2 siRNA on human UCB CD133+ cell migration in vitro

Previous experiments had demonstrated reduced transwell migration of human UCB CD133⁺ cells treated with CD164 siRNA and the 103B2 blocking antibody *in vitro* (Forde et al., 2007). In parallel with the *in vivo* homing experiments, where there were sufficient cells (identified where the colour coding is matched to the homing data), the human UCB CD133⁺ cells nucleofected with NT siRNA and CD164-2 siRNA were assessed for chemotaxis *in vitro* towards CXCL12

These experiments were performed using the 3D μ -slide chemotaxis assay developed in Chapter 3.



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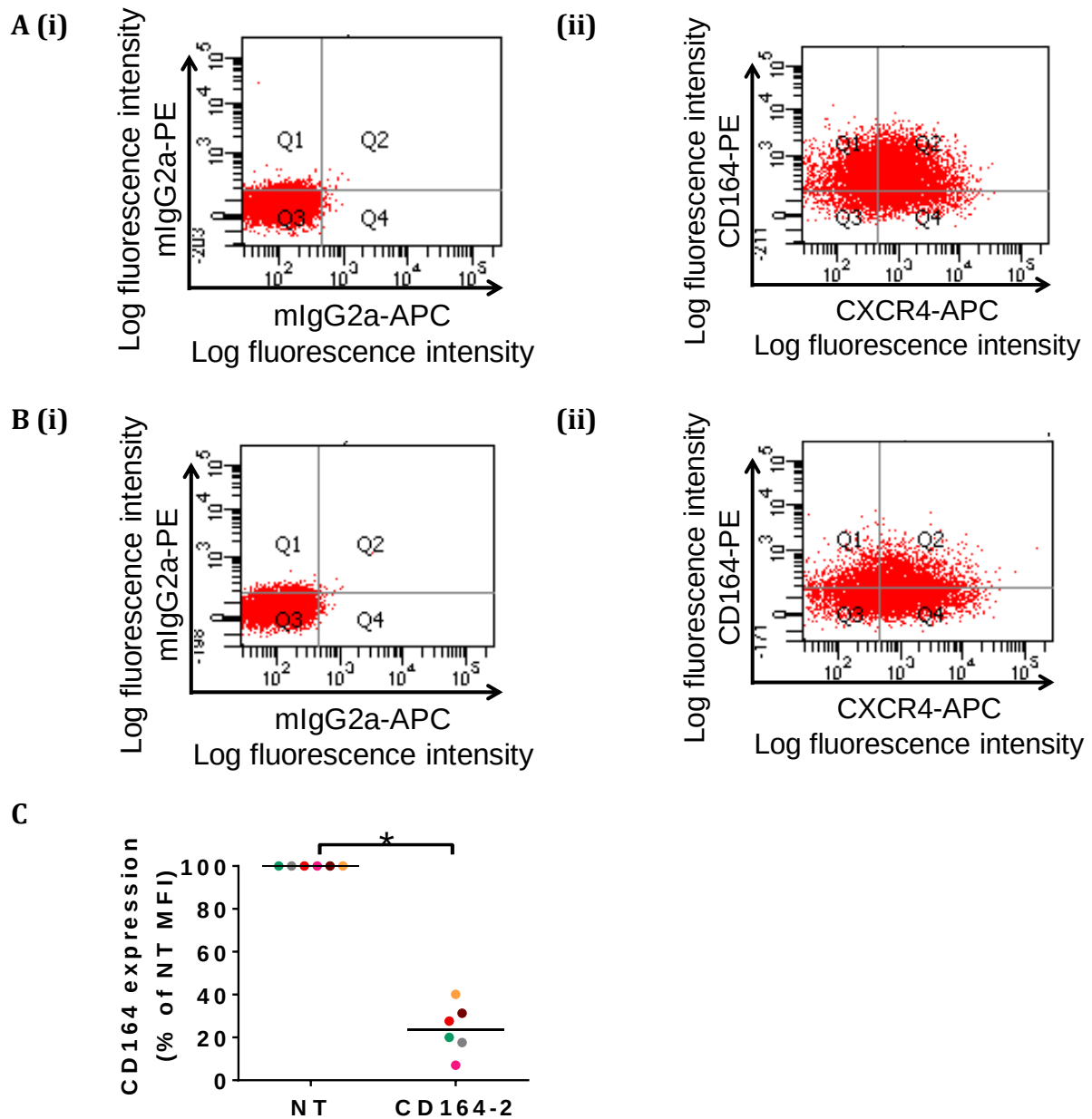


Figure 4.16. CD164 knock-down with siRNA in UCB CD133⁺ cells used in the 3D μ -slide chemotaxis assay. CD133⁺ cells were cultured in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO for 24 hours prior to nucleofection with 1200nM non-targeting (NT) siRNA or CD164-2 siRNA. After a further 30 hour culture period in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, a sample of the cells were analysed by flow cytometry. FACS dot plots, displaying one of the less efficient nucleofections show A) NT siRNA nucleofected cells and B) CD164-2 siRNA nucleofected cells stained for (i) mlgG2a-PE vs. mlgG2a-APC and (ii) CD164-PE (N6B6) vs. CXCR4 (12G5). The CD164 MFI on the CD164-2 siRNA nucleofected cells was calculated as a percentage of NT siRNA nucleofected cells, where the MFI was standardised to 100%. C) Individual data points () are shown along with median (horizontal line). * $p < 0.05$ (Wilcoxon signed rank test) (n=6 samples assessed by flow cytometry).

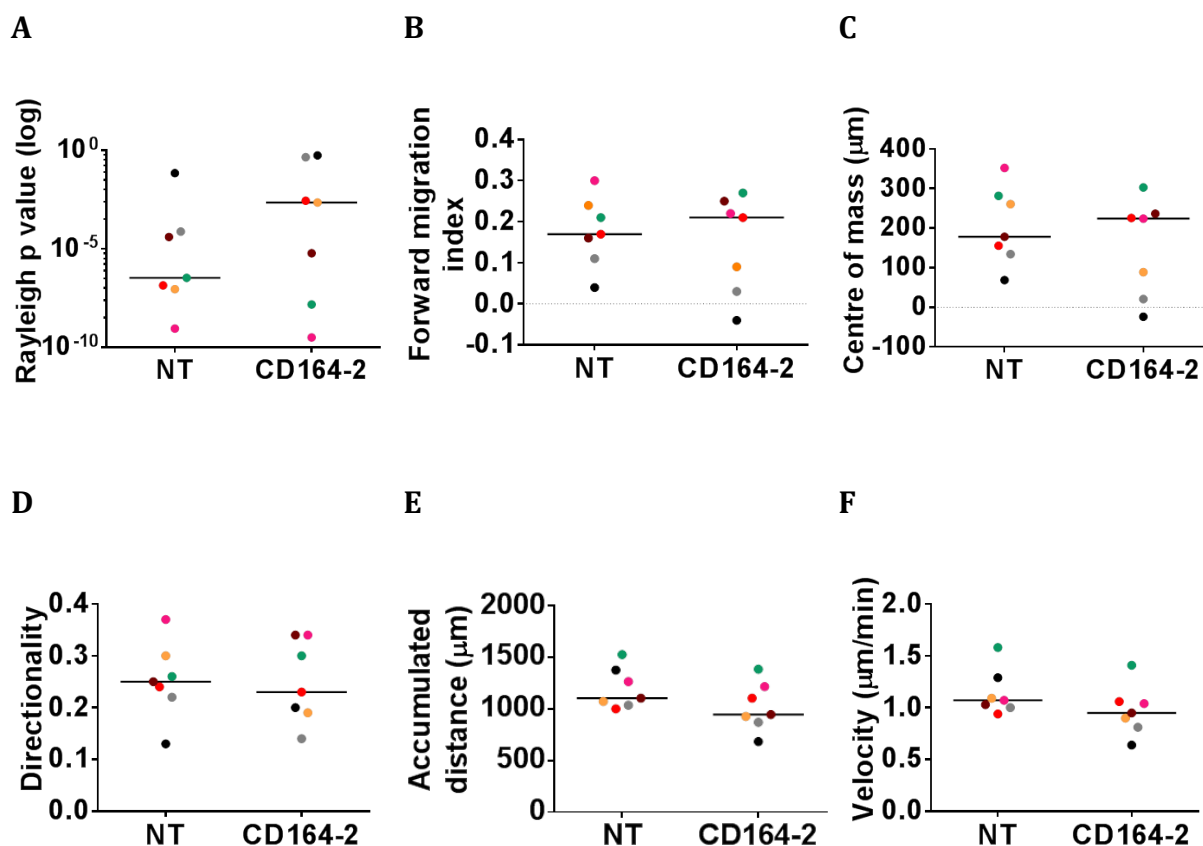


Figure 4.17. The effect of CD164 knock-down with siRNA on CD133+ cell chemotaxis to CXCL12. Human UCB CD133+ cells were cultured in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO for 24 hours prior to nucleofection with 1200nM non-targeting (NT) siRNA or CD164-2 siRNA. After a further 30 hours of culture, CD164 knock-down was assessed by flow cytometry (Figure 4.16). The cells were washed in stemspan alone, re-suspended in a 1mg/ml collagen I gel and seeded in a 3D μ -slide chemotaxis chamber (Ibidi GmbH). The migration of 30 cells per group towards 1 μ g/ml CXCL12 was tracked using Image J plug-ins over an 18 hour period. The chemotaxis and migration tool (Ibidi GmbH) was used to determine A) significantly inhomogeneous cell distribution towards CXCL12 (Rayleigh p value), B) forward migration index, C) displacement of the centre of mass, D) directionality, E) accumulated distance and F) velocity. Individual data points (paired as colours) and median (horizontal line) are shown (n=7 independent experiments).

4.5 Discussion

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The second aim was to knock-down CD164 in human UCB CD133⁺ cells [REDACTED]

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[REDACTED] Because the 103B2 mab used by Forde *et al.* (Forde et al., 2007) was unavailable, shRNA and siRNA oligos that were available commercially were used.

Firstly, the CD164 shRNAs with a GFP tag in the pGIPZ vector were tested. Theoretically, the cells expressing CD164 shRNA could be identified [REDACTED] by their GFP fluorescence. When the NS, CD164-A or CD164-B shRNAs were

nucleofected into human UCB CD133⁺ cells, the mean nucleofection efficiencies, assessed using GFP fluorescence by flow cytometry 24 hours post nucleofection were 50.5%±4.2, 63.5%±11.5 and 46.9%±8.3 respectively and thus could not provide complete knock-down, although the GFP should have enabled detection of nucleofected cells. During validation of the shRNA constructs, GFP expression and CD164 silencing were maintained for 24-48 hours post nucleofection, as determined by flow cytometry. [REDACTED]

[REDACTED] CD164 knock-down in GFP expressing populations was 62.9%±16.1 compared to respective non-silencing (NS) control populations 48 hours post nucleofection. [REDACTED]

[REDACTED] Because of these non-specific effects of the NS shRNA [REDACTED], an alternative method of CD164 knock-down was used.

Previously, Forde *et al.* demonstrated that CD164 blockade with the monoclonal antibody (mab) 103B2 or knock-down using CD164-2 siRNA could reduce CD133⁺ cell migration in transwell assays (Forde et al., 2007), and cell adhesion capacity (Figure 4.1). In the initial timecourse experiments, the CD164 knock-down obtained by CD164-2 siRNA in the whole population was much greater than was seen for the CD164-B shRNA (72.6%±14.1 compared to 37.2%±7.4 respectively 48 hours post

nucleofection). As this knock-down was more efficient and consistent, the effect of CD164-2 siRNA on CD133⁺ cell [REDACTED] migration was assessed. Unfortunately, the CD164-1 sequence did not show knock-down at the cell surface (Figure 4.11C) and therefore could not be used to validate the effects seen with the CD164-2 sequence in functional assays.

The CD164-2 siRNA produced reliable and consistent surface knock-down of CD164, as assessed by flow cytometry (between $76.1\% \pm 4.7$ (* $p=0.03$, $n=6$) [REDACTED] [REDACTED] for cells used in the chemotaxis [REDACTED] assays [REDACTED]), compared to NT siRNA nucleofected cells (standardised to 100%). Forde *et al.* reported a $56.6\% \pm 6.2$ reduction in total protein (assessed by Western blotting) 48 hours after nucleofecting the same CD164-2 siRNA into UCB CD133⁺ cells (Forde *et al.*, 2007). Due to the large cell numbers required for Western blotting, protein knock-down in CD133⁺ cells following CD164-2 siRNA nucleofection was not re-assessed in this thesis. Although the surface expression was deemed to be most important for these studies, Western blotting or intra-cellular staining would have demonstrated the protein knock-down for the timecourse of the homing assays. CD164 protein levels were shown to be reduced ($72.0\% \pm 3.8$) by Western blotting within 24 hours of CD164-2 nucleofection in HUVECs (Forde *et al.*, unpublished data) and thus suggesting that 24 hours is sufficient for protein knock-down. Future work should repeat these experiments in CD133⁺ cells to confirm protein knock-down for the time course of the [REDACTED] migration experiments.

[REDACTED]

[REDACTED]

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Results in Chapter 3 demonstrated a total blockade of chemotaxis in the 3D μ -slide assay using the specific CXCR4 receptor antagonist AMD3100. This confirmed that when the CXCL12-CXCR4 migratory axis was disrupted, significantly reduced chemotaxis could be detected in these assays. The Rayleigh test p values suggested that CD164 knock-down altered CD133⁺ cell chemotaxis in some experiments, however, considerable variability was seen amongst individual experiments.

Therefore, over the 7 experiments, no effect of CD164-2 siRNA nucleofection on CD133⁺ cell chemotaxis to CXCL12 was seen. Interestingly, the chemokinetic responses were less variable. Although a consistent trend towards decreased chemokinesis was seen, there was no significant effect on accumulated distance or velocity for cells which had been subjected to nucleofection with the NT and CD164-2 siRNAs ($p > 0.05$, determined by two-way ANOVA).

Previous research on CD164 has shown that CD164 and CXCR4 co-localised when presented with CXCL12 on fibronectin. Furthermore, significantly reduced transwell migration using the 103B2 antibody and CD164-2 siRNA was seen in CD133⁺ cells in the presence of fibronectin and CXCL12 and not without fibronectin (Forde et al.,

2007). Since CD164 was initially identified as having a role in adhesion (Zannettino *et al.*, 1998), and since Forde *et al.* also demonstrated co-immunoprecipitation of CD164, VLA-4 and VLA-5 with CXCR4 after stimulation with CXCL12 when presented on fibronectin, it is plausible that the CD164 blockade described previously was not only modulating CXCR4 but also interactions with fibronectin. Other molecules which regulate integrin binding to adhesion molecules have been found to co-localise with CXCR4 and have a function in human HSPC homing. Recently, the tetraspanin CD82 has been shown to polarise with CXCR4 and promote human HSPC migration and *in vivo* homing to CXCL12, and it is known that CD82 regulates the interaction of VLA-4 integrin with VCAM-1 on endothelium (Larochelle *et al.*, 2012). The interaction of CD164 with extra-cellular matrices (ECM) has also been shown by Havens *et al.* who found that basal and CXCL12 induced prostate cancer cell invasion through matrigel could be inhibited by the blocking CD164 (N6B6) (Havens *et al.*, 2006). This might suggest that CD164 regulates the speed of migration through such matrices. The 3D μ -slide chemotaxis assay showed a small decrease in chemokinesis through collagen I gel with CD164 siRNA nucleofected cells in all but one experiment. However, the effect of the N6B6 antibody on human UCB CD133⁺ cell chemotaxis was not tested.

The migration, adhesion and homing process has been demonstrated to be complex and involve multiple molecules other than CD82. Leung *et al.* demonstrated that blocking surface CD9 with a monoclonal antibody (ALB6) decreased CD34⁺ cell homing to bone marrow but not spleen, decreased adhesion to fibronectin and HUVECs and increased transendothelial migration towards CXCL12 (Leung *et al.*, 2011). Furthermore, the inhibition of CD26, which cleaves CXCL12, has been shown

to improve CXCR4 mediated engraftment using Diprotin A (Campbell et al., 2007; Campbell and Broxmeyer, 2008; Christopherson et al., 2007).

In the publications cited above, inhibition of the molecules of interest was achieved using monoclonal antibodies, inhibitors, or selection of fractions of cells with differential expression of the molecule of interest. However, the effects of gene silencing in human HSPCs on engraftment have been shown. For example, PTEN siRNA treated CD34⁺ cells showed enhanced engraftment *in vivo* (Kim et al., 2012). This was a gain of function change in a phosphatase which regulates CXCR4 cell signalling pathways involved in homing (Kim et al., 2012). In the absence of complete inhibitors of CD164 or if blocking mabs to CD164 remain unavailable, lentiviral vectors, which can provide efficient and stable modulation, may be required to further explore a role for this molecule *in vivo*. Alternatively, future experiments promoting increased CD164 surface expression (using pLOC lentivectors for example) could be used to assess the effect on cell function, providing that such over-expression does not adversely affect the survival of the targeted cells.

However, a role for CD164 in UCB CD133⁺ cell *in vitro* chemotaxis to CXCL12 has not been shown.

The siRNA silencing of CD164 was incomplete (~70-80%) and therefore may have been insufficient to consistently target the small percentage of cells that were tracked *in vitro*

However, if CD164 was vital for cell migration to CXCL12 it is likely that this would have been detected using the techniques presented here. In order to be sure that this level of knock-down using siRNA could affect cell migration, a control sequence such as a CXCR4 siRNA should also have been used in these studies.

The evidence so far from the literature suggests that CD164 plays a role in HSPC binding to bone marrow niche cells and preventing HSPC cycling when bound (Zannettino et al., 1998). CD164 also modulates the response of CXCR4 to CXCL12 by co-operating with CXCR4, VLA-4 and VLA-5 and could enhance cell movement through extracellular matrices (Havens et al., 2006; Zannettino et al., 1998; Forde et al., 2007). Other experiments where the function of cell surface molecules has been assessed in migration have used blocking antibodies (Campbell et al., 2007; Campbell and Broxmeyer, 2008; Christopherson et al., 2007; Larochelle et al., 2012). Therefore, future studies using the 103B2 monoclonal antibody (which was not available during these experiments) to block the functional epitope of surface CD164, may be used to repeat the work presented here and confirm whether there is a role for CD164 in human UCB CD133⁺ cell homing

Chapter 5. Expanding human umbilical cord blood CD133+ haemopoietic stem/progenitor cells, while maintaining their homing ability *in vitro*

5.1 Aims and objectives

The objective of this research was to investigate culture conditions that might expand human UCB CD133⁺ cells, whilst maintaining chemotactic ability *in vitro*.

The specific aims were:

- 1) To establish a 2D cell based co-culture system using endothelial cells and stromal cells to model the vascular niche *in vitro*, and if robust to investigate the ability of these co-cultures to expand human UCB CD133⁺ HSPCs
- 2) To use a nanofibre scaffold (specifically the Nanex scaffold) to expand human UCB CD133⁺ cells in the presence of different cytokines and growth factors
- 3) To assess the ability of these cells to retain homing ability using the *in vitro* 3D μ -slide chemotaxis assay described in Chapter 3.

5.2 Introduction

The literature describes several different approaches that have been taken to expand human HSPCs using supporting cells and/or growth factors or stimulators. It is well known that bone marrow mesenchymal stem/stromal cells (BMSCs), which support haemopoiesis in the bone marrow *in vivo*, enhance human HSPC expansion in the presence of added cytokines and growth factors *in vitro* (Piacibello et al., 1999; Rappold et al., 1999; Sutherland et al., 1989). However, the endothelium are also a vital component of the bone marrow niche. Hooper *et al.* demonstrated that VEGFR2-mediated regeneration of endothelial vessels in the bone marrow was required not only for bone marrow re-vascularisation in lethally irradiated mice, but also for their haemopoietic reconstitution (Hooper et al., 2009). To investigate the endothelium *in vitro*, Butler *et al.* demonstrated that E4ORF1 lentiviral vector transduced mouse bone marrow endothelial cells could support mouse HSPC expansion in the presence of soluble kit ligand (mouse stem cell factor) (Butler et al., 2010). These cells had increased colony forming units (CFU) after a 28 day culture compared to the point of seeding. More recently, Butler *et al.* have confirmed this using human umbilical vein endothelial cells (HUVECs) (Butler et al., 2012). HUVECs transduced with the E4ORF1 lentiviral vector supported the expansion of human UCB HSPCs in serum-free conditions with SCF, Flt-3 ligand and TPO, beyond the expansion seen without the HUVECs (Butler et al., 2012).

The usefulness of generating *in vitro* models which may mimic the bone marrow microenvironment has been recognised (Di et al., 2011). For this to be achieved, more than one cell constituent of the stroma should be represented. Recently, work towards modelling the niche in what has been proposed to be more representative

3D environments has been developed using scaffold-based culture systems (Di et al., 2011; Ventura Ferreira et al., 2012a). These have included perfused porous scaffolds which have been shown to support stromal, endothelial and HSPCs to the extent that they can network when implanted *in vivo* and create functional ectopic niches capable of chimaerism with their host (Güven et al., 2011; Song et al., 2010). Synthetic 2D polymer surfaces, including nanofibre scaffolds have also been used to expand HSPCs which retained engraftment potential in mice (Chou et al., 2010; Ventura Ferreira et al., 2012b). Very recently, a fibrin 3D scaffold seeded with UCB-derived MSCs has reported a 3×10^8 fold TNC expansion from UCB CD34⁺ cells after 14 days in culture with cytokines and factors (SCF, TPO, FGF-1, Angptl5 and IGFBP2). This MSC seeded fibrin scaffold provided the highest levels of engraftment in NSG mice above uncultured CD34⁺ cells and other scaffolds (including collagen) (Ventura Ferreira et al., 2012a).

Other approaches have expanded human HSPCs under serum free conditions with combinations of a standard panel of cytokines, namely SCF, Flt-3 ligand, IL-6 and TPO, as described by Butler *et al.* (Butler et al., 2012) or with additional factors. The latter have recently been reviewed by Delaney *et al.*, and have included the use of factors such as HOXB4, Notch ligands, Angiopoietin-like-5 and Stemregenin-1 (Boitano et al., 2010; Delaney et al., 2010b; Delaney et al., 2010a; Drake et al., 2011; Zhang et al., 2008). A commercially produced Nanex™ nanofibre scaffold (Arteriocyte) has recently been developed to expand UCB CD34⁺ cells for up to 12 days (Figure 5.1A and B). In these studies, fresh and frozen human UCB CD133⁺ cells expanded around 50 fold by days 7-8 and around 150-200 fold by day 12 of culture (Figure 5.1C and D).

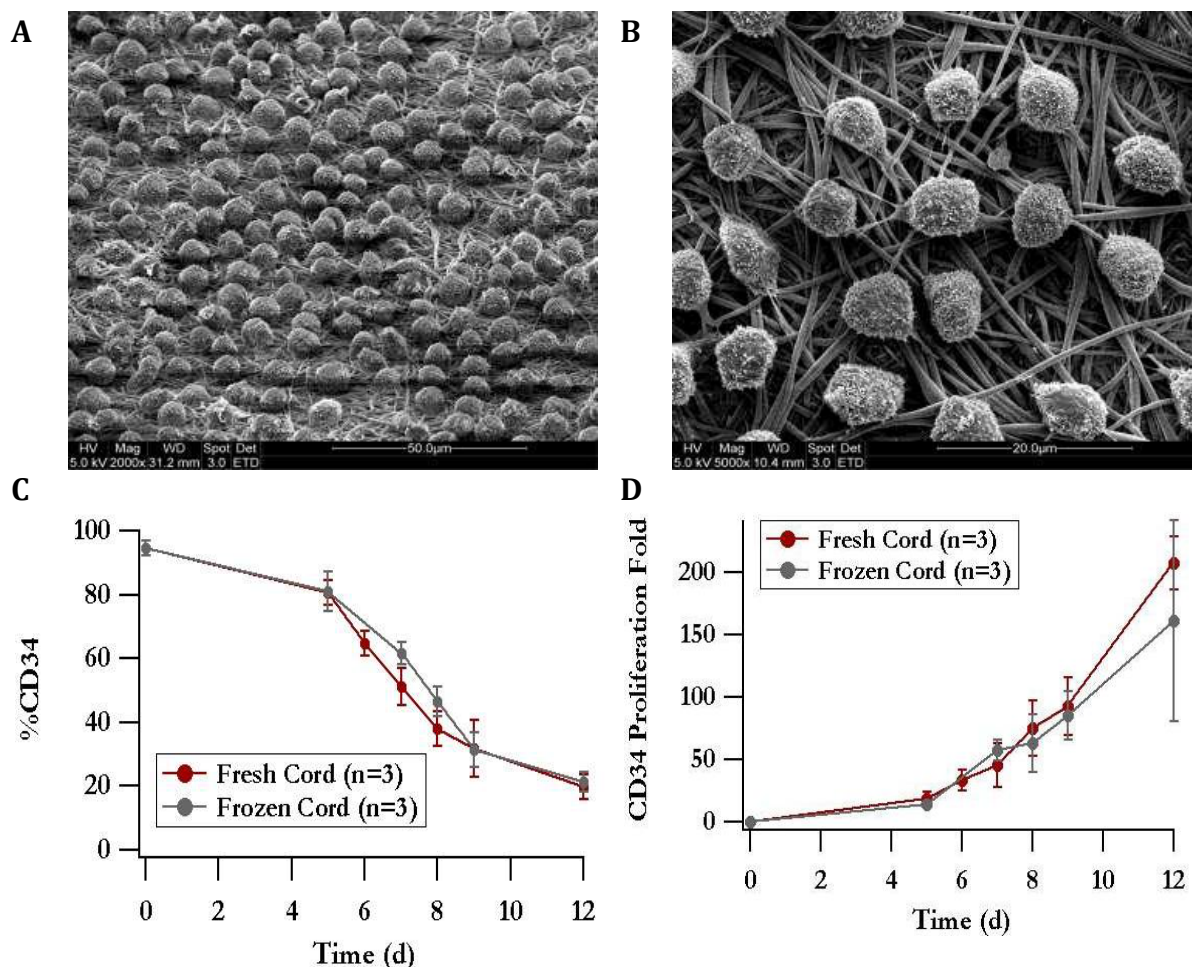


Figure 5.1. Human UCB CD34⁺ cell expansion on Nanex scaffolds. Fresh or frozen CD34⁺ cells from human UCB were cultured undisturbed for up to 12 days in Nanex HSC expansion medium complete with the Nanex cytokine cocktail and LDL. Scanning electron micrographs show CD34⁺ HSPCs growing on top of the Nanex surface at A) x5,000 magnification and B) x10,000 magnification. C) The percentage of CD34 cells present in the culture and D) the fold proliferation was assessed every 2 days. The figures and data were produced by and reproduced with kind permission from Dr Yukang Zhao, Arteriocyte.

Expanding human UCB HSPCs is important for providing higher numbers of HSPCs for transplantation of UCB in adults, while differentiating these cells into more mature progenitors can provide more mature myeloid cells to enhance short term engraftment of neutrophils and platelets. This is particularly pertinent for UCB where cell numbers and HSPC content is limited, where there is delayed engraftment and haematological reconstitution compared to bone marrow or mobilised peripheral

blood, where one UCB unit will engraft in preference to another in double unit transplants, and where *ex vivo* expansion prior to transplant is a strategy used to reduce delayed engraftment (Aljitawi, 2012; Dahlberg et al., 2011; Holmes et al., 2012; Larochelle et al., 2012). However, prior manipulation or expansion of HSPCs after *ex vivo* culture in cytokine cocktails for as little as 24 hours can compromise cell homing or migratory capacities and this can affect transplant outcomes (Ahmed et al., 2004).

5.3 Results

5.3.1 Establishing a vascular niche model in vitro

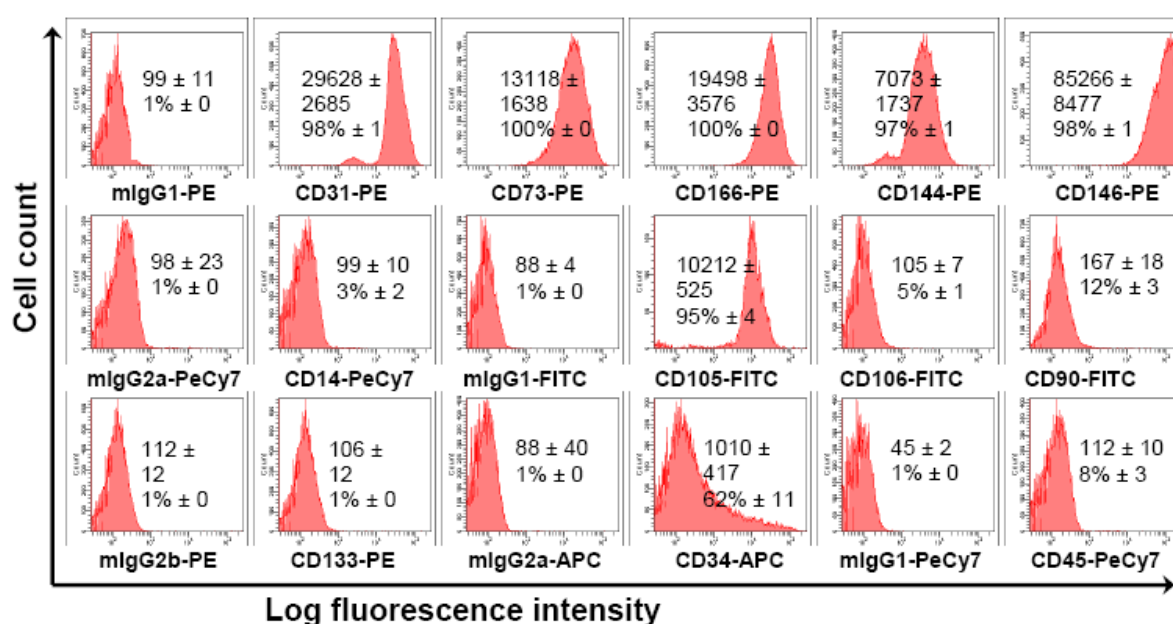
In these studies, human endothelial cells and bone marrow stromal cells (BMSC) were used to create a model of the vascular niche. The endothelial cells were sourced from human umbilical vein (HUVECs) or human UCB, which contains endothelial colony forming cells (Zhang et al., 2009; Zhou et al., 2012), or an immortalised human bone marrow endothelial cell line, BMEC-60. The primary BMSCs and endothelial cells from all three sources were first characterised and compared for cell surface markers (Table 5.1).

5.3.1.1 Phenotyping cellular components for the niche model

5.3.1.1.1 Characterising HUVECs

Three batches of HUVECs were isolated from umbilical cords (as detailed in section 2.2.2.2) and the phenotype confirmed by flow cytometry (passage 3-5) (Figure 5.2A). HUVECs were stained with the antibodies shown in Figure 5.2A as described in section 2.4.2.2 following culture in EGM-2 medium. The cells were positive for the surface markers CD31, CD73, CD144, CD146, CD166 and CD105, and partially positive for the haemopoietic and endothelial progenitor marker CD34. HUVECs were negative for the stromal marker CD90, the haemopoietic stem/progenitor marker CD133, the leucocyte common antigen CD45 and the monocyte marker CD14. The HUVECs displayed a typical endothelial cobblestone appearance under light microscopy (Figure 5.2B). Thus, the morphology and phenotype confirmed the endothelial cell nature of the HUVECs.

A



B

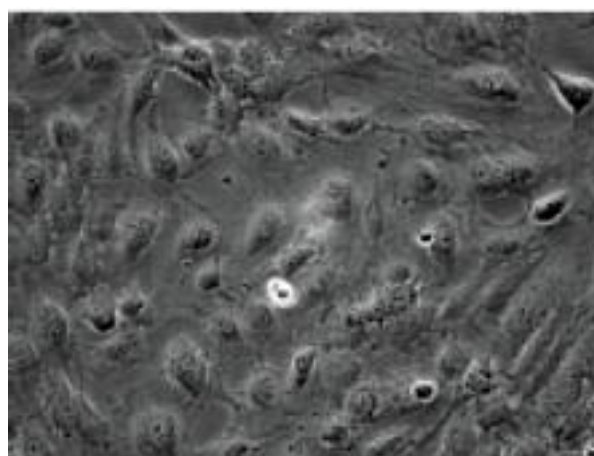
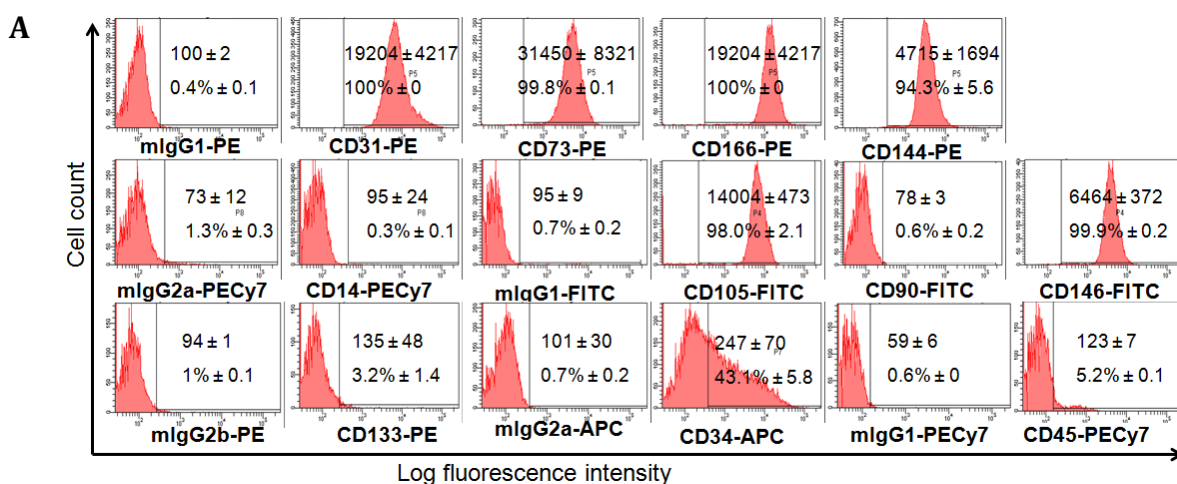


Figure 5.2. Phenotype and morphology of HUVEC. A) HUVECs were characterised by flow cytometry using directly conjugated antibodies to the cell surface antigens shown on the figure. Flow cytometry plots show representative histograms displaying median fluorescence intensity (MFI) ±SEM and the % of cells positive for each marker±SEM above an arbitrary gate set on the matched isotype control (arbitrary gate demonstrated in Figure 5.3) (n=3 independent batches at passage 3-5). B) HUVECs were photographed under phase contrast microscopy when confluent at x20 magnification and demonstrated a typical morphology.

5.3.1.1.2 Characterisation of endothelial cells from umbilical cord blood

Two batches of endothelial cells isolated from UCB were obtained from and had been phenotyped by Dr Cheen Khoo (passage 3-4) (Figure 5.3B). The cells were stained

with the antibodies shown in Figure 5.3 as described in 2.4.2.2 following culture in EGM-2 medium. The cells were positive for the surface antigens CD31, CD73, CD144, CD146, CD166, CD105, partially positive for CD34 and negative for CD90, CD133, CD45 and CD14. These markers confirmed the endothelial phenotype of these cells.



B

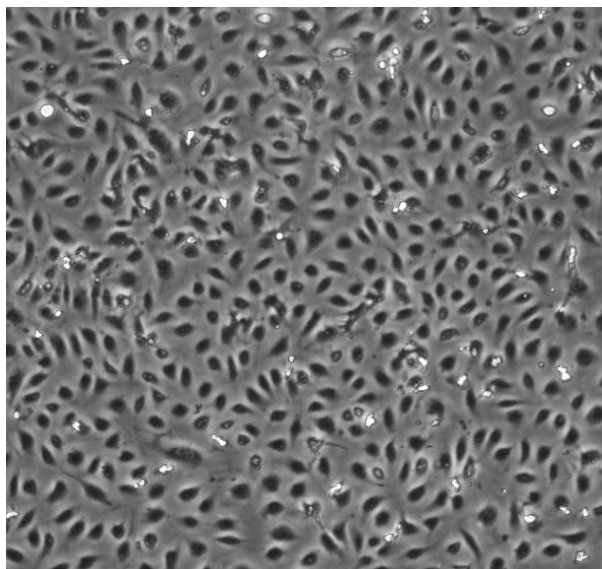


Figure 5.3. Phenotype and morphology of endothelial cells from UCB. A) UCB derived endothelial cells were kindly supplied and phenotyped by Dr Cheen Khoo. The characterisation was performed by flow cytometry using directly conjugated antibodies to cell surface antigens shown on the figure. Flow cytometry plots show representative histograms displaying median fluorescence intensity (MFI) ± SEM and the % of cells positive for each marker ± SEM above an arbitrary gate set on the matched isotype control (n=2 independent batches at passage 3-4). B) The cells were photographed under phase contrast microscopy when confluent at x10 magnification and demonstrated a typical morphology.

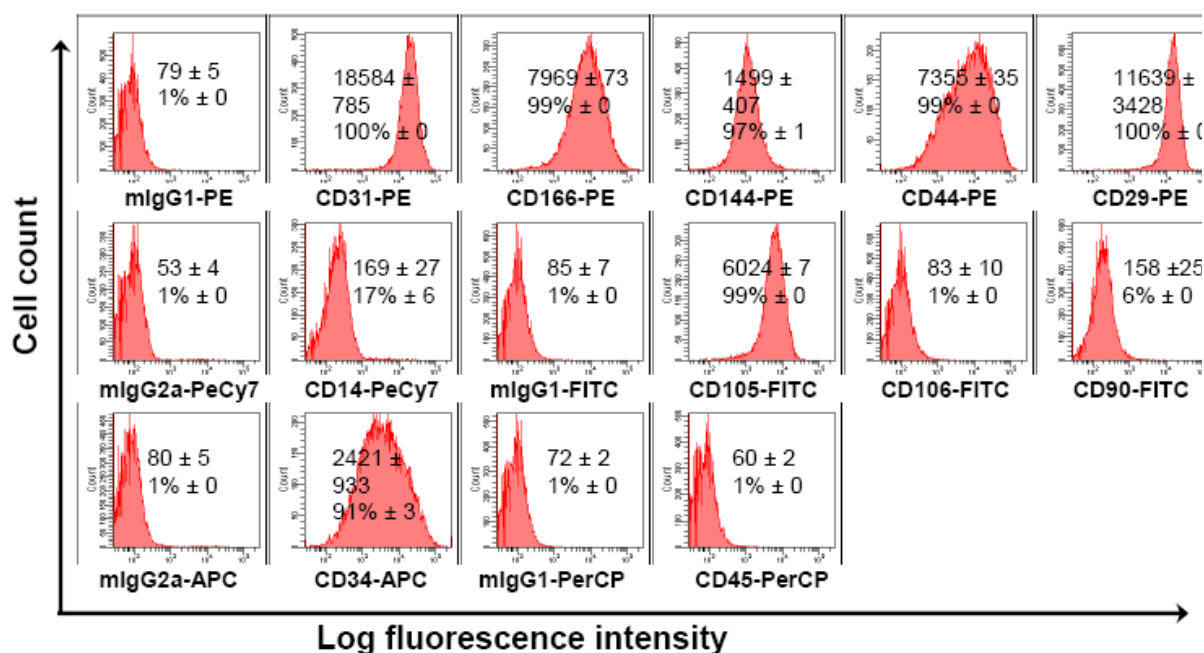
5.3.1.1.3 Characterisation of BMEC-60 cells

BMEC-60 cells (kindly supplied by Professor E van der Schoot, University of Amsterdam, The Netherlands) were originally derived from human bone marrow endothelial cells (BMEC-60) immortalised with a retroviral construct containing the HPV-16 E6/E7 genes (Rood et al., 2000). These cells were shown to be similar to primary human bone marrow endothelial cells in terms of phenotype (with the exception of E-selectin and VCAM-1 which were inducible by IL-1 β) and supporting adhesion of human HSPCs (Rood et al., 2000). Flow cytometry and morphology of BMEC-60 cells (described in Chapter 2) also confirmed an endothelial phenotype following culture in EGM-2 medium (Figure 5.4). BMEC-60 cells were positive for CD34, CD31, CD73, CD144, CD166, CD105 and the adhesion molecules CD44 and CD29. BMEC-60 cells were negative for CD90, CD45, CD14 and CD106 (VCAM-1). These markers confirmed the endothelial phenotype of the cells.

5.3.1.1.4 Characterisation of BMSC

BMSC were sourced from Lonza Biologics as cells which have long term culture initiating cell (LTC-IC) supportive ability. Batches 237, 562 and 396 were tested. The culture conditions for these cells are described in 2.2.2.1. Flow cytometric analysis confirmed that BMSCs were positive for CD90, CD105, CD166, CD44 and CD29 and negative for CD31, CD34, CD14 and CD45 (Figure 5.5A). Microscopy revealed the cells had an adherent fibroblastic morphology typical of stromal cells (Figure 5.5B).

A



B

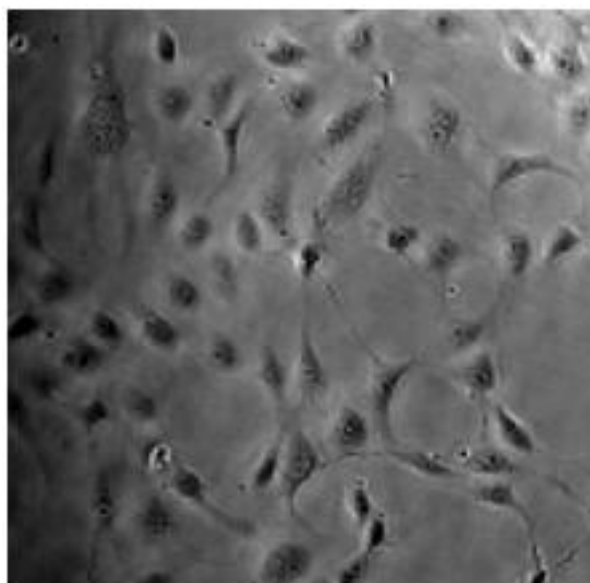
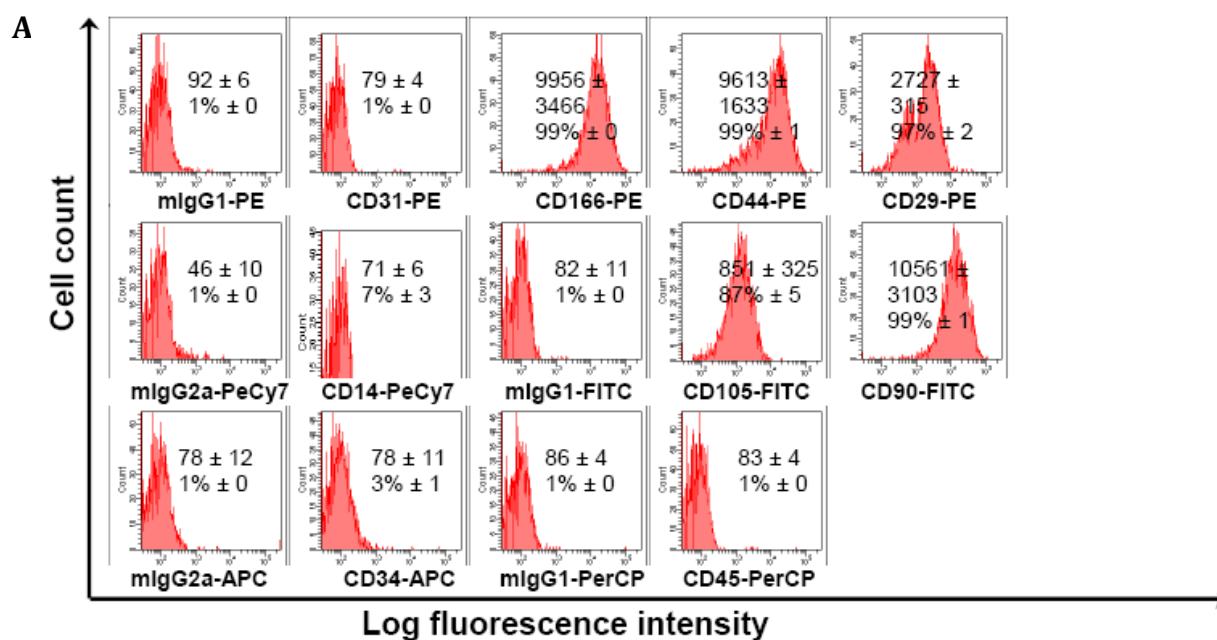


Figure 5.4. Phenotype and morphology of BMEC-60. A) BMEC-60 cells were characterised by flow cytometry using directly conjugated antibodies to the cell surface antigens shown on the figure. Flow cytometry plots show representative histograms displaying median fluorescence intensity (MFI) $\pm$ SEM and the % of cells positive for each marker $\pm$ SEM above an arbitrary gate set for the matched isotype control (n=2 repeats). B) BMEC-60 cells were photographed under phase contrast microscopy when confluent at x20 magnification and demonstrated a typical endothelial morphology.



B

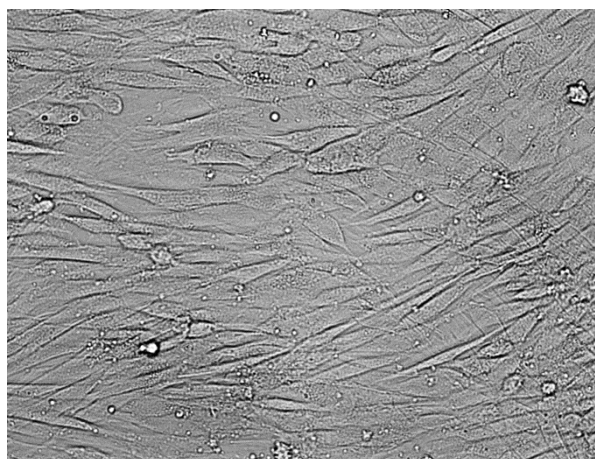


Figure 5.5. Phenotype and morphology of BMSC. A) BMSC were characterised by flow cytometry using directly conjugated antibodies to the cell surface antigens shown on the figure. Flow cytometry plots show representative histograms displaying median fluorescence intensity (MFI)±SEM and the % of cells positive for each marker±SEM (n=3 independent batches). B) BMSCs were photographed under phase contrast morphology when confluent at x20 magnification and demonstrated a typical BMSC morphology, even after culture in EGM-2 (as used for the co-culture assay).

Table 5.1. Summary of the phenotype of endothelial and stromal cells

	CD31	CD73	CD144	CD146	CD166	CD105	CD106	CD90	CD34	CD45	CD14	CD29	CD44
HUVEC	++	++	++	++	++	++	-	-	+	-	-	n/a	n/a
Endothelial cells from UCB	++	++	++	++	++	++	-	-	+	-	-	n/a	n/a
BMEC-60	++	++	++	n/a	++	++	-	-	++	-	-	++	++
BMSC	-	n/a	n/a	n/a	++	++	n/a	++	-	-	-	++	++

n/a = not applicable/not tested

5.3.1.2 Establishing the co-culture method

5.3.1.2.1 Setting up co-cultures using previously validated cell types

Studies from this laboratory have shown that HUVEC will form vascular tubule networks in the presence of hDFs and the murine MS-5 stromal cell line (Zhang et al., 2009; Zhou et al., 2012). In preliminary experiments aimed at establishing the BMEC-60/BMSC co-culture assay, the three batches of HUVECs (named 1, 3 and 4) (phenotyped in section 5.3.1.1.1) were co-cultured with hDFs. The batch of hDF that produced the best tubule formation from previously validated UCB endothelial cells transduced with the eGFP lentiviral vector (as described by Zhang *et al.* (Zhang et al., 2009)) was batch 1 (Figure 5.6). Therefore batch 1 hDF were used to assess the tubule forming ability of the three batches of characterised HUVECs which had been transduced with eGFP as described in section 2.5.1.7. The hDFs were trypsinised first and plated in 48 well collagen coated plates in complete EGM-2 medium at $2-4 \times 10^4$ cells per well.

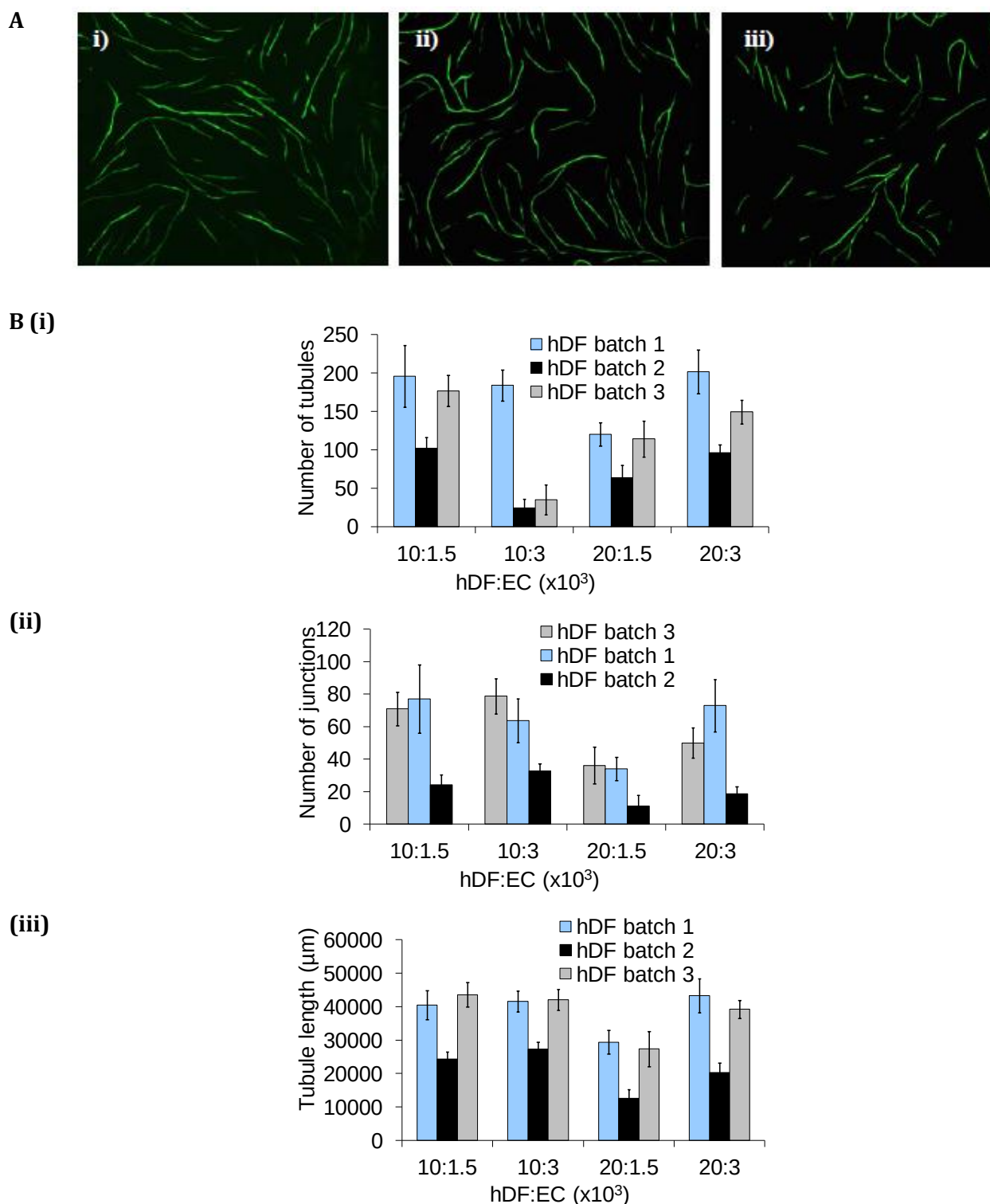


Figure 5.6 Analysis of vascular tubules generated by co-culture of UCB endothelial cells with hDFs. A)(i)-(iii) Representative photographs of hDF (i) batch 3, (ii) batch 1 and (iii) batch 2 when co-cultured with eGFP transduced endothelial cells (EC) from UCB at a ratio of 20x10³ hDF:3x10³ UCB EC. Photographs were taken using a fluorescent microscope on day 14 of the assay at x4 magnification. B) Angiosys software quantified (i) the number of tubules, (ii) the number of junctions and (iii) the tubule length, at different ratios of hDF:EC using images taken at day 14. Bars represent mean±standard deviation (n=3 replicates per ratio tested).

After several hours, the HUVECs were trypsinised and seeded into the hDF containing wells at $1.5\text{--}3 \times 10^3$ cells per well in a total volume of 500 μl complete EGM-2 medium. After 14 days of thrice weekly complete media changes, the cells were fixed in 4% (w/v) paraformaldehyde and photographed as described in section 2.8. All batches of HUVECs successfully produced visible tubules at all ratios tested (Figure 5.7A). The difference in tubule length and junctions between the ratios was quantitated using Angiosys software and the results are shown in Figure 5.7B. As this co-culture was successful, the experiment was repeated using BMSC and BMEC-60 cells in order to create a more representative model of the bone marrow niche.

5.3.1.2.2 Determining if bone marrow stromal cells could support endothelial cell vascular tubule formation

BMEC-60 cells were transduced with the eGFP lentiviral vector (as detailed in section 2.5.1.8) to enable the progression of tubule formation to be visualised during the 14 day culture period. Both eGFP and non eGFP BMEC-60 were co-cultured on three separate batches of BMSC from Lonza (named batch 237, 562, 396) to look for any differences in their ability to form tubules (Figure 5.8A). The cells were seeded in complete EGM-2 medium as described in section 5.3.1.2.1 and 2.8 at four different ratios (BMSC 4×10^4 or 7×10^4 and BMEC-60 4×10^3 or 6×10^3). These ratios were selected based on preliminary experiments conducted within the laboratory. After 14 days, all of the cells were fixed in 70% (v/v) ethanol for consistency and stained for CD31 (black stain) as described in section 2.8. Figure 5.8A showed that no differences were seen in tubule forming ability between BMEC-60 and eGFP transduced BMEC-60, however the tubule forming ability was poor compared to HUVECs on hDF.

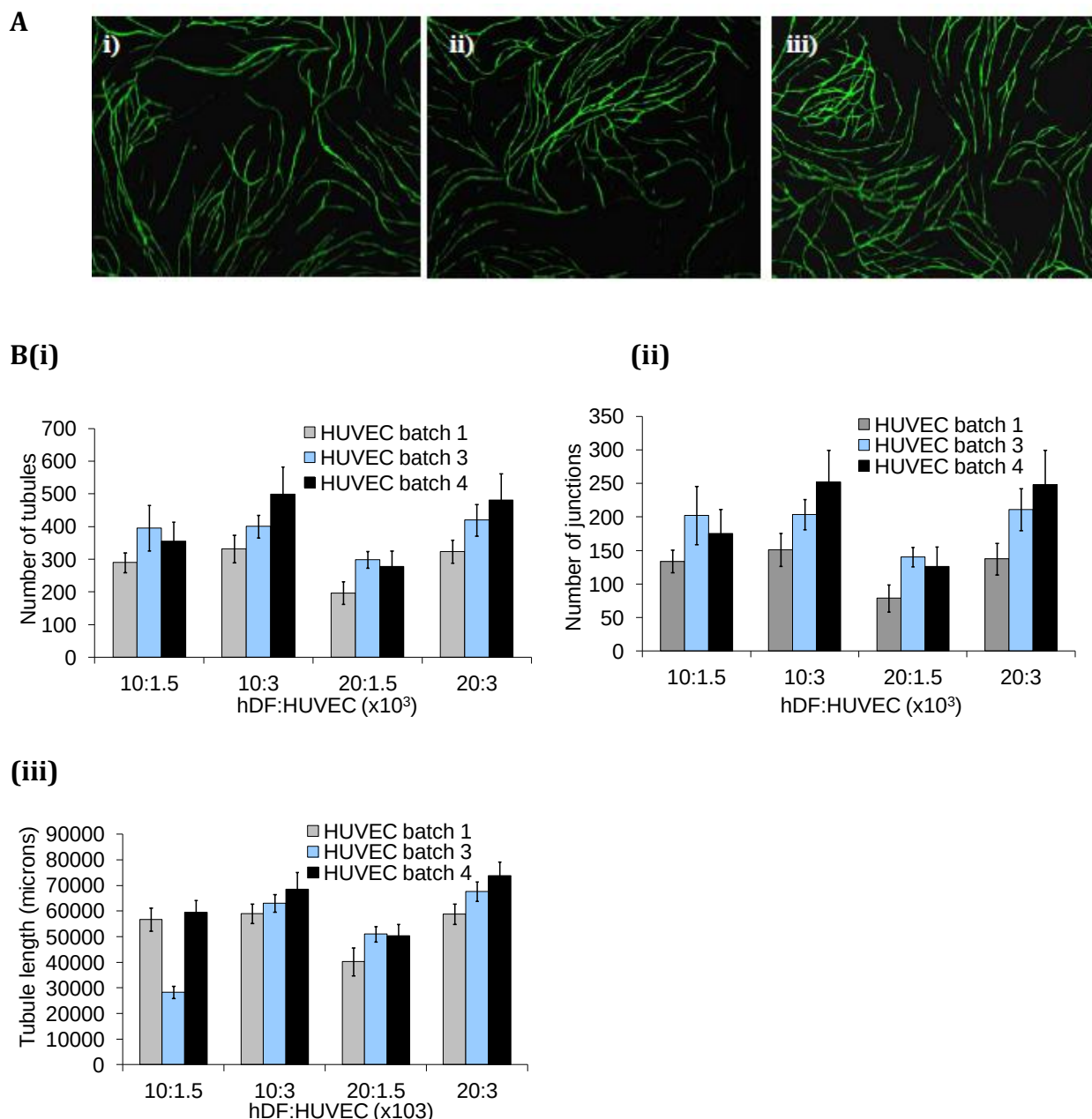


Figure 5.7. Analysis of tubules generated by co-culture of HUVECs with hDFs. A) Representative photomicrographs of eGFP transduced HUVEC batch (i) 1, (ii) 3 and (iii) 4 when co-cultured with hDF batch 1 at a ratio of 3×10^3 HUVEC: 10×10^3 hDF. Photographs were taken using a fluorescent microscope on day 14 of the assay at x 4 magnification. B) Angiosys software quantified (i) the number of tubules, (ii) the number of junctions and (iii) the total tubule length at the ratios of hDF:HUVEC indicated after 14 days of culture. Bars represent mean $\pm$ SEM (n=3 replicates per ratio tested).

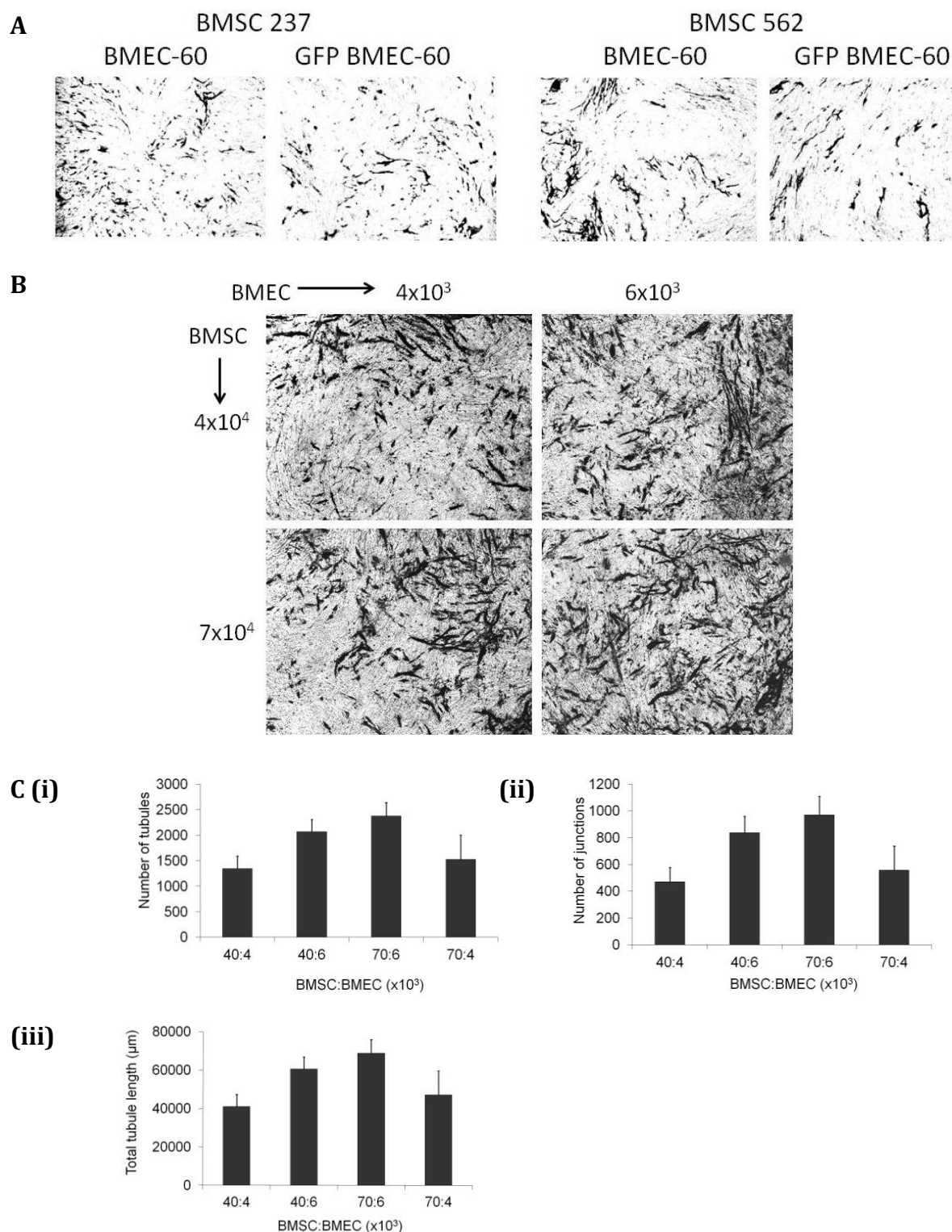


Figure 5.8. Co-culturing BMEC-60 with BMSC. A) Representative photographs taken at x4 magnification showing BMSC and BMEC-60 (either GFP transduced or non-transduced as stated) co-cultures with BMSC batch 237 and 562 after 17 days. B) Representative photographs taken at x4 magnification showing BMSC batch 396 and eGFP BMEC-60 after 17 days in culture. CD31 positive cells are stained black. C) Angiosys software quantified (i) the number of tubules, (ii) number of junctions and (iii) total tubule length using images taken at day 17. Bars represent mean $\pm$ standard deviation (n=3 replicates per ratio tested).

Figure 5.8B shows that BMSC batch 396 best supported BMEC-60 tubule formation compared to the other batches. The number of tubules, tubule length and number of junctions for each ratio tested from batch 396 were quantified using Angiosys (Figure 5.8C) (as described in section 2.8). The vascular tubules produced by BMEC-60 cells on BMSCs were short and stunted and consequently the quantitation proved difficult. However, quantitation suggested that as approximately twice the number of tubules were generated by BMEC-60 cells, with four times the number of junctions compared to that seen with HUVECs on hDFs (Figure 5.8C(i and ii)), the total tubule length overall was similar to that produced by HUVECs (Figure 5.7B(iii)). Figure 5.8C shows that the cell ratio producing the greatest number of BMEC-60 tubules with the most junctions from batch 396 was 70×10^3 BMSC: 6×10^3 BMEC-60.

As the tubules formed by BMEC-60 cells on BMSC were clearly different to those formed by HUVECs on hDFs, different stromal cell sources were investigated to see if the BMEC-60 networks could be improved or if this was an inherent property of the BMEC-60 cells. Experiments conducted by others within this laboratory had shown BMEC-60 did not produce tubules when cultured on hDF and hence the human stromal cell line, HS-5, was tested in order to eliminate the variability caused by using human primary BMSC at passage 2. HS-5 cells were tested in co-culture with BMEC-60 at the ratios listed in Figure 5.9. HS-5 supported the proliferation of BMEC-60 cells in rafts, but did not support vascular tubule formation (Figure 5.9).

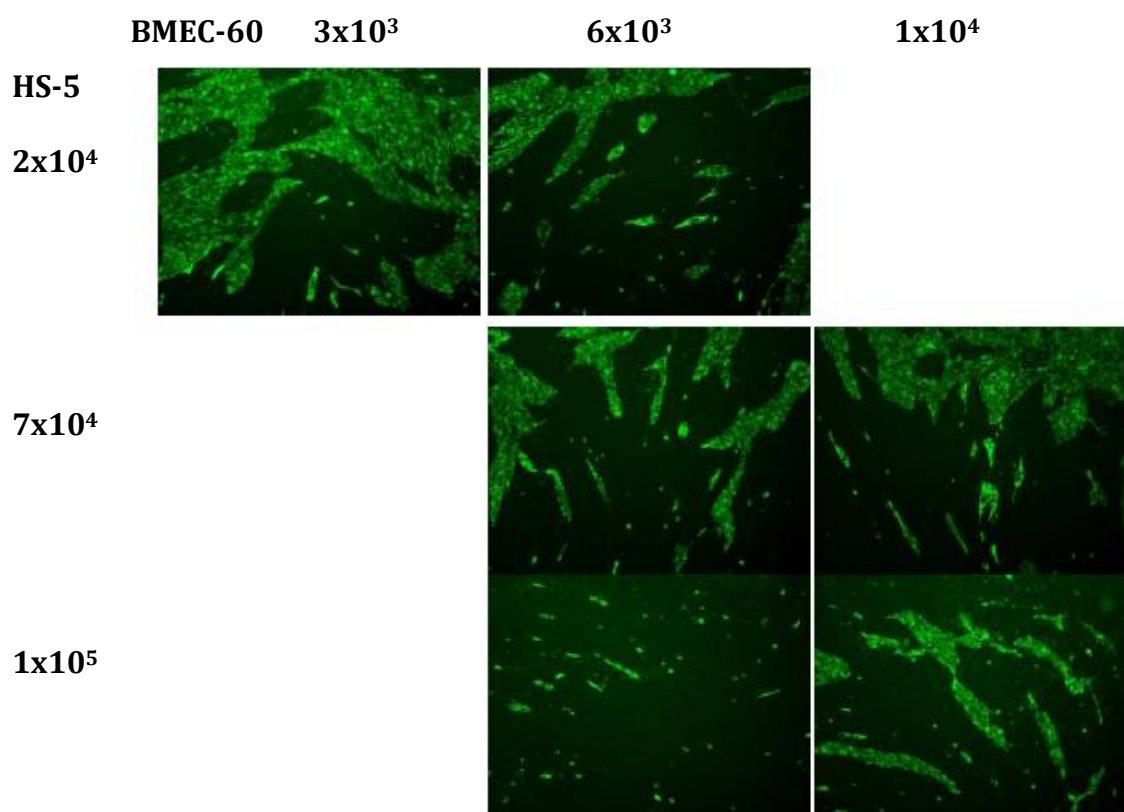


Figure 5.9. HS-5 human bone marrow stromal cells do not support vascular tubule formation by BMEC-60 cells. HS-5 and eGFP BMEC-60 cells were co-cultured for 17 days in EGM-2 medium at different ratio's shown and photomicrographs taken using a fluorescent microscope at x4 magnification.

Colleagues within the laboratory also using the BMEC-60 cell line confirmed that BMEC-60 co-cultured with BMSC did not consistently form robust vascular networks. Subsequently, endothelial cells from UCB (kindly provided and phenotyped by Dr Cheen Khoo, section 5.3.1.1.2) were tested with different batches of BMSC to determine if these would produce vascular networks with which to investigate human UCB CD133⁺ cell expansion. UCB endothelial cells were chosen with future studies in mind, as endothelial cells from UCB are contained within the UCB graft infused into patients undergoing bone marrow transplantation.

The co-culture experiment was performed as described in section 2.8 and section 5.3.1.2.2. Two different batches of UCB endothelial cells (unlabelled batch 301109

and an eGFP lentiviral vector transduced batch 120110) were tested. After 14 days, the cells were fixed in 70% (v/v) ethanol and stained for anti-CD31 (black stain) or 4% (v/v) paraformaldehyde in order to preserve the eGFP vasculature as described in section 2.8. Both UCB endothelial cell batches produced tubules on BMSC batches 237, 562 and 396 from Lonza Biologics that had been used to test the BMEC-60 cell tubule formation in section 5.3.1.2.2 (Figure 5.10A-C). The endothelial cells from UCB were also co-cultured with hDF batch 1 in the same experiments in order to provide a positive control (Figure 5.10D), as these had shown tubule support previously (Figure 5.6).

As these BMSCs supported UCB endothelial cell tubule formation and more so than hDF, it seemed unlikely that the inability of BMEC-60 to consistently form tubules was due to BMSC batch variation. The vascular tubule formation by UCB endothelial cells on different batches of BMSC was quantified using different ratios. Varying the cell numbers from $2-4 \times 10^3$ UCB endothelial cells in culture on 6×10^3 BMSC did not significantly affect observable tubule formation after 14 days in culture in complete EGM-2 medium (Figure 5.11A). The number of junctions, the number of tubules and the total tubule length were quantified by Angiosys (Figure 5.11B). BMSC batch 237 supported the formation of tubule networks that were more comparable to those supported by the hDF in terms of the number of junctions, tubule length and the length of tubules (Figure 5.11B(i-iii)). In contrast, BMSC batch 562 produced tubules which were more similar to BMEC-60 networks on BMSC, in terms of the overall tubule length, number of junctions and number of tubules formed (Figure 5.11B(i-iii)). Although these batches of BMSC produced vascular networks, there were batches of BMSC from Lonza Biologics that did not support UCB endothelial cell

tubule formation at all (414 and 746). Thus, there was considerable variability in the abilities of different BMSC batches from Lonza Biologics to support vascular tubule formation.

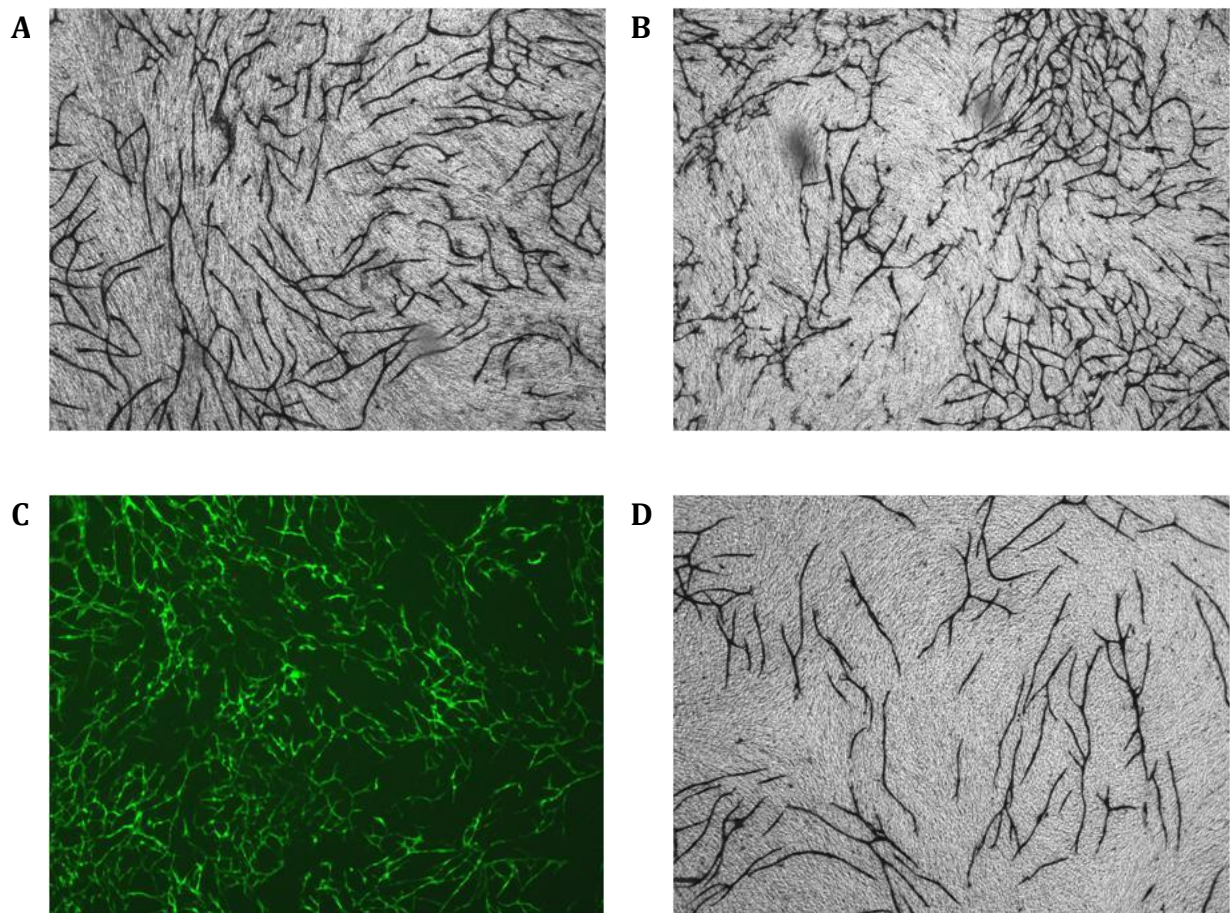


Figure 5.10. Human UCB endothelial cells form tubules when co-cultured with BMSC. Representative photomicrographs of UCB endothelial cells producing vascular tubules when cultured on different BMSC batches taken at x4 magnification after 14 days in culture show. UCB endothelial cell batch 301109 were cultured with A) BMSC batch 237, B) BMSC batch 562. C) The eGFP lentiviral vector transduced batch of UCB endothelial cells 120110 produced vascular tubules when cultured on BMSC batch 396. D) hDF batch 1 were used as a control with UCB endothelial cell batch 301109.

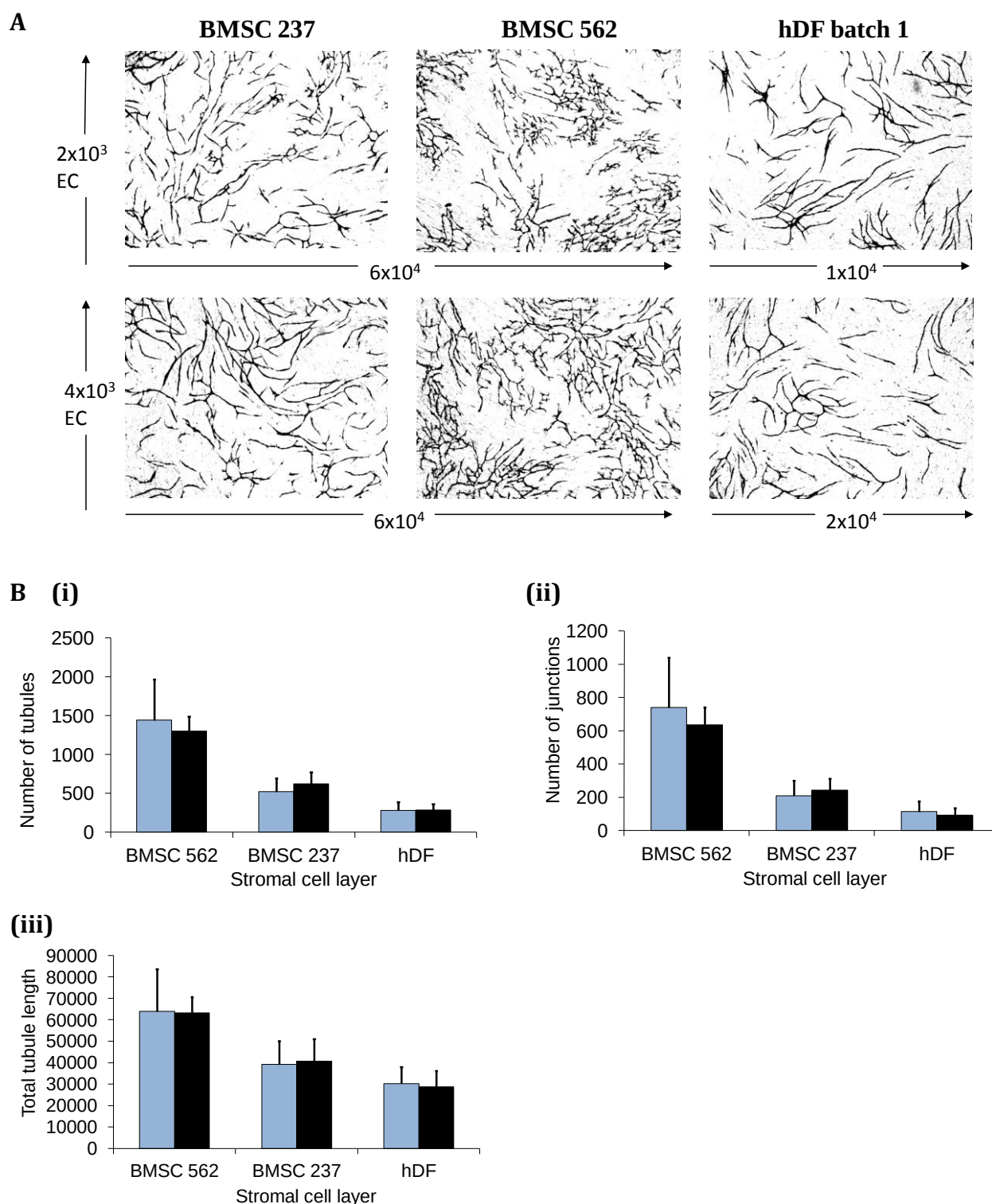


Figure 5.11. Comparing UCB endothelial cell tubule formation on different batches of BMSCs. A) UCB endothelial cells (EC) were cultured at 2x10³:6x10⁴ with BMSCs or 4x10³:6x10⁴ with BMSCs or at 2x10³:1x10⁴ with hDF or 4x10³:2x10⁴ with hDF for 14 days. B) The tubule formation was quantified using Angiosys and shown for the lower cell ratios (blue bars) and higher cell ratios (black bars) as (i) number of tubules, (ii) number of junctions and (iii) total tubule length. Mean±standard deviation is shown (n=3 replicates tested per ratio).

Preliminary experiments using the co-culture of UCB endothelial cells with BMSC to expand UCB CD133⁺ cells, which were conducted in collaboration with Dr Sarah Hale, showed that the co-cultures did not give a comparable expansion of the UCB CD133⁺ cells to that seen using standard CD133⁺ cell culture conditions (stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO) without co-cultures. As this co-culture system using endothelial cells with BMSCs to support vascular tubule formation did not appear to provide the most suitable or robust model from which to expand UCB CD133⁺ cells, this was abandoned in preference to the use of a commercially available Nanex nanofibre scaffold from Arteriocyte. In the next section, the use of different cytokines without or with this scaffold was tested as an alternative model system for human UCB CD133⁺ cell expansion *in vitro*.

5.3.2 Assessing the ability of non-cellular niche models to support haemopoiesis

5.3.2.1 Culturing human UCB CD133⁺ cells with the nanofibre scaffold

Nanex nanofibre scaffold plates (see Figure 5.1) for the expansion of HSPCs were provided by or purchased from Cambridge Bioscience. To examine different cytokine cocktails with this scaffold, the conditions suggested by the manufacturer (Nanex HSC Expansion Serum Free Media with the Nanex Cytokine Cocktail (1:1000), advised as containing undisclosed concentrations of SCF, Flt-3 ligand, IL-6, IL-3 and TPO, plus 40µg/ml acetylated low density lipoprotein (LDL)) (termed standard Nanex conditions), were compared to Nanex HSC Expansion Serum Free Media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (termed Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO). The assay was performed as described in section 2.9 using CD133⁺ cells isolated and purified from umbilical cord

blood, as described in section 2.2.2.3. The cells were freshly isolated and seeded immediately following purification. At the point of seeding the cells onto the Nanex scaffold at a concentration of 1000 cells/ml in 3ml per well of a 6 well plate in either the standard Nanex conditions or Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, the expression of CD133, CD34 and CD45 was assessed by flow cytometry as described in section 2.4.2. The mean $\pm$ SEM CD133 cell purity was 89.1% $\pm$ 0.8% (n=2). Of this population, 98.9% $\pm$ 0.5% of cells expressed CD45 and 89.7% $\pm$ 1.1% expressed CD34 as demonstrated in Figure 5.12.

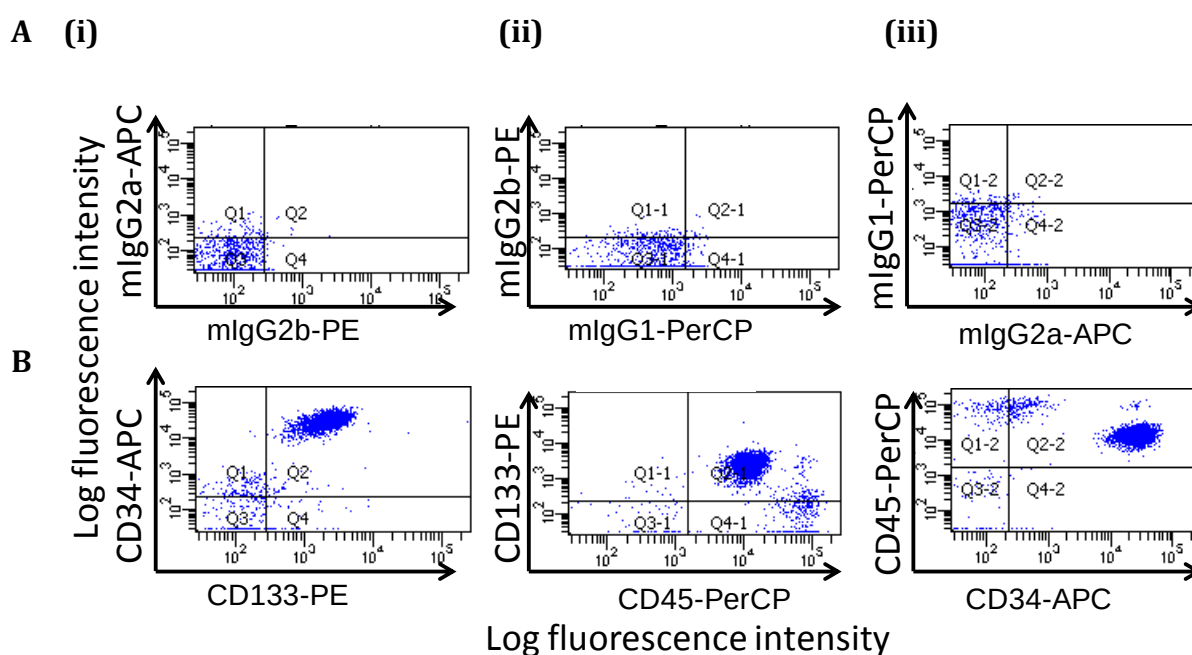


Figure 5.12. Expression of CD45, CD34, CD133 on CD133 enriched human UCB cells prior to culture. Human UCB CD133⁺ cells isolated and purified as described in section 2.2.2.3 were analysed by flow cytometry prior to seeding on the Nanex scaffold. Cells were stained using A) (i) mIgG2a-APC vs. mIgG2b-PE, (ii) mIgG2b-PE vs. mIgG1-PerCP, (iii) mIgG1-PerCP vs. mIgG2a-APC or B) (i) CD34-APC vs. CD133-PE, (ii) CD133-PE vs. CD45-PerCP, (iii) CD45-PerCP vs. CD34-APC as described in Chapter 2. Representative dot plots are shown.

After 8 days in culture, without any agitation or feeding, the cells were collected by pipette and viable cells counted on a haemocytometer using trypan blue as described in section 2.2.3. Cells were pooled from all wells for counting and further analysis to define TNC expansion per experiment. This was achieved by repeating the flow cytometry analysis performed initially (CD45, CD34 and CD133 expression).

Figure 5.13 demonstrates that the majority of the expanded cells retained CD45 expression and a smaller population retained CD133 and CD34 expression. The quantified analysis is shown in Figure 5.13A-C. The mean viability, assessed by negative staining for DAPI was $92.3\% \pm 2.8$ and $89.3\% \pm 0.3$ using the standard Nanex conditions and Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO respectively.

Figure 5.14A demonstrated that the TNC count and CD34⁺ cell count obtained from the Nanex standard conditions was greater than when using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. In 2 independent experiments, there was a 293.8 ± 19.6 fold expansion of TNC over 8 days of culture using standard Nanex conditions compared to a 114.6 ± 12.1 fold expansion of TNC using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (Figure 5.14C). A 246.0 ± 34.5 fold expansion of CD34⁺ cells was obtained after 8 days using the standard Nanex conditions compared to a 93.8 ± 14.2 fold expansion of CD34⁺ cells using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (Figure 5.14C). This suggested that the addition of IL-3 and/or acetylated LDL enhanced the TNC and CD34⁺ cell expansion 2-3 fold compared to 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO.

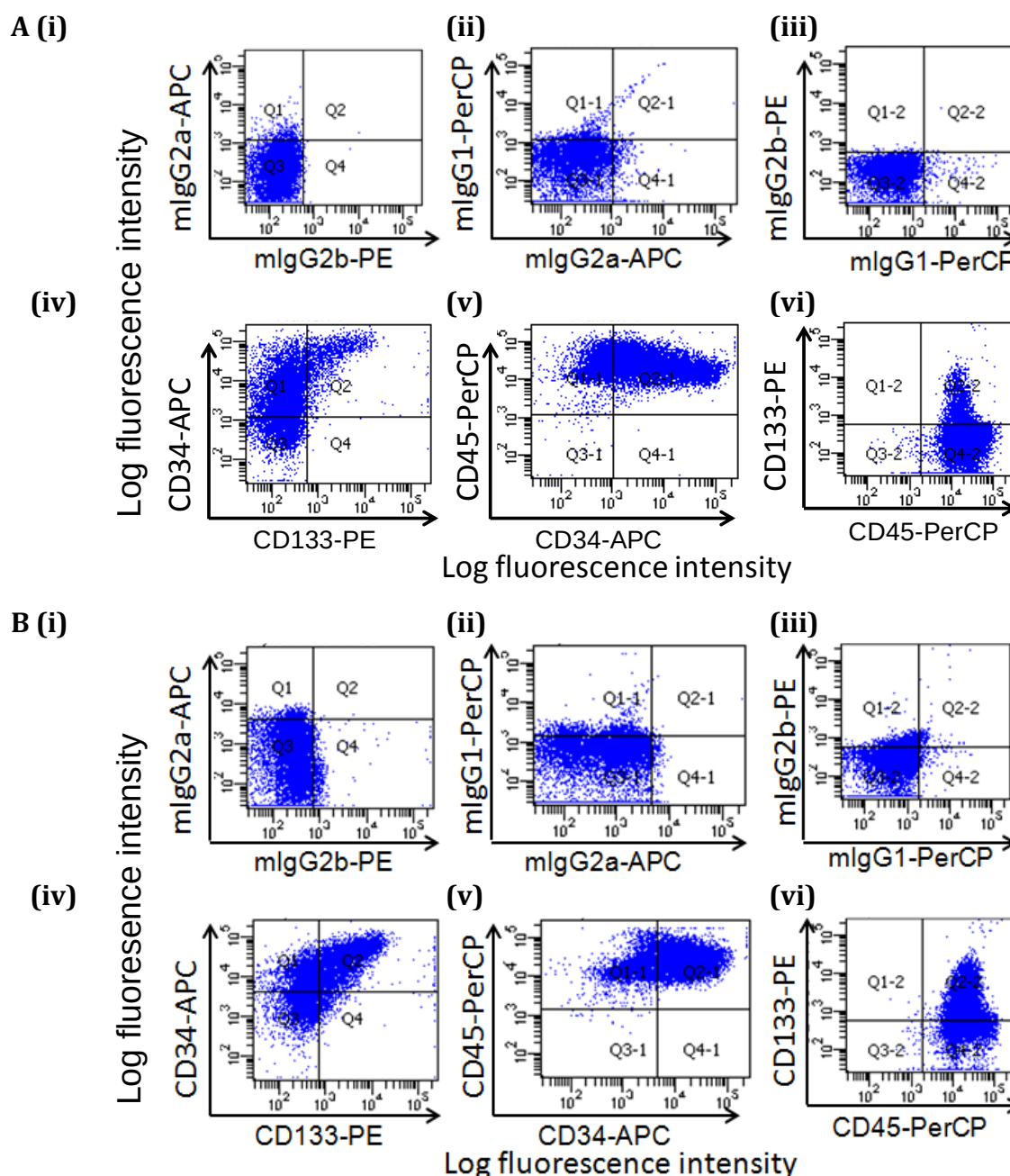


Figure 5.13. Nanex expanded cells retain a population of CD34⁺CD133⁺ cells. Human UCB CD133⁺ cells characterised by flow cytometry in Figure 5.12 were seeded at a concentration of 1000 cells/ml in 3ml per well of a 6 well Nanex plate (in triplicate). The cells were cultured in either A) standard Nanex conditions or B) Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. After 8 days in culture, the cells were harvested by pipette, pooled within conditions and counted using a haemocytometer. A sample of the entire population was then analysed by flow cytometry for (i) mlgG2a-APC vs. mlgG2b-PE, (ii) mlgG2a-APC vs. mlgG1-PerCP, (iii) mlgG1-PerCP vs. mlgG2b-PE (iv) CD34-APC vs. CD133-PE, (v) CD34-APC vs. CD45-PerCP, (vi) CD45-PerCP vs. CD133-PE as described in section 2.4.2. Representative dot plots are shown (n=2 independent experiments).

However, the absolute number of expanded CD133⁺ cells was slightly higher using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO compared to the standard Nanex conditions (Figure 5.14A). CD133⁺ cell expansion using the standard Nanex conditions was 56.5 ± 4.6 fold compared to CD133⁺ cell expansion in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO of 65.0 ± 3.5 fold expansion (Figure 5.14C).

The percentage of cells expressing CD45 after expansion was $97.9\% \pm 1.5\%$ for standard Nanex culture and $99.4\% \pm 0.2\%$ for Nanex culture with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. For cells expanded in standard Nanex conditions, the percentage of cells positive for CD34 was $74.0\% \pm 6.2\%$, and for Nanex with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO, $75.1\% \pm 3.6\%$ (mean $\pm$ SEM (n=2)). When looking at the percentage of CD133⁺ cells in the recovered population, it was evident that more than double the population of cells were CD133⁺ in the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO condition ($50.4\% \pm 2.1$), compared to the standard Nanex culture ($17.0\% \pm 0.4$) (n=2) (Figure 5.14B). Additionally, $15.7\% \pm 1.3$ and $46.4\% \pm 0.3$ of the TNCs were CD133⁺CD34⁺ using the standard Nanex conditions and Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO respectively. This suggested that the addition of IL-3 and/or acetylated LDL in the standard Nanex conditions may enhance CD133⁺ cell differentiation (Figure 5.14B).

As there appeared to be enhanced differentiation of the CD34⁺CD133⁺ HSPCS into CD34⁺CD133⁻ cells in the standard Nanex condition compared to the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO cultured cells (Figure 5.13A(iv) and B(iv)), the addition of other cytokines to the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO was tested in further experiments.

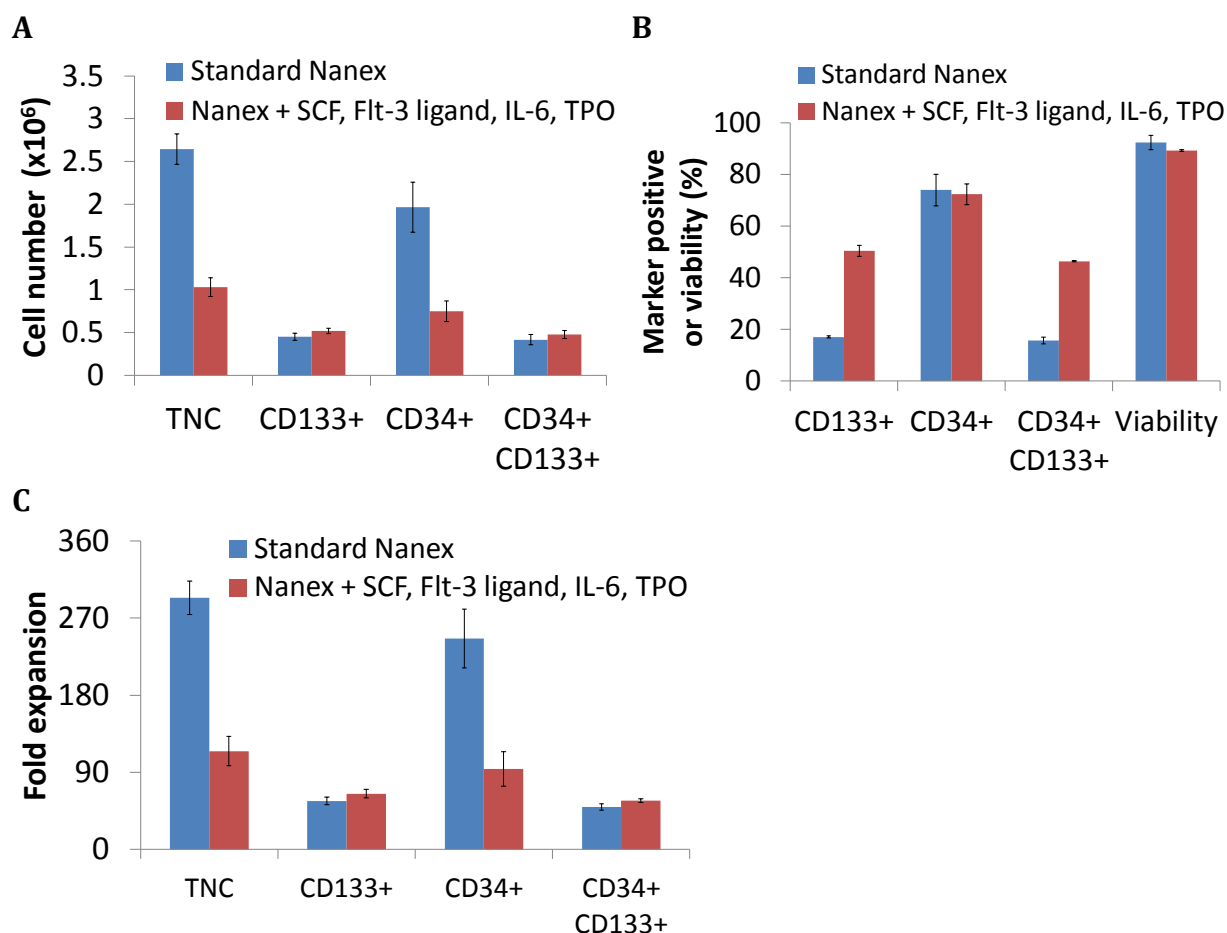


Figure 5.14. CD133⁺ cell expansion after 8 days in culture on Nanex plates. Human UCB CD133⁺ cells, at a concentration of 1000 cells/ml in 3ml, were seeded per well of a 6 well Nanex scaffold (in triplicate). The cells were cultured in either standard Nanex conditions (blue) or Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (red). After 8 days in culture, the cells were harvested by pipette and counted using a haemocytometer (A). A sample of the entire population was then analysed by flow cytometry for CD133, CD34 and viability (by DAPI negativity). B) The percentage of cells expressing each marker enabled calculation of (C) fold expansion from starting cell number (9000 cells). Data are presented as the mean±SEM (n=2 independent experiments).

5.3.2.2 Testing additional growth factors in the Nanex scaffold culture system

The Nanex scaffold expansion protocol was repeated using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO without or with the addition of 100ng/ml IGFBP2 and 100ng/ml Angptl5. Human UCB CD133⁺ cells were purified and frozen as described in section 2.2.2.3. Four hours prior to adding these cells to

the Nanex scaffold, they were thawed and cultured in Nanex HSC expansion media alone in tissue culture treated plates. Using trypan blue and a haemocytometer to count the cells, 3000 viable cells were then seeded per well of a Nanex scaffold plate as described in section 5.3.2.1. A sample of the seeded cells were analysed by flow cytometry for CD133 (86.7%±3.7), CD45 (95.1%±4.6) and CD34 (95.3%±3.7) expression (mean±SEM, n=3) (Figure 5.15).

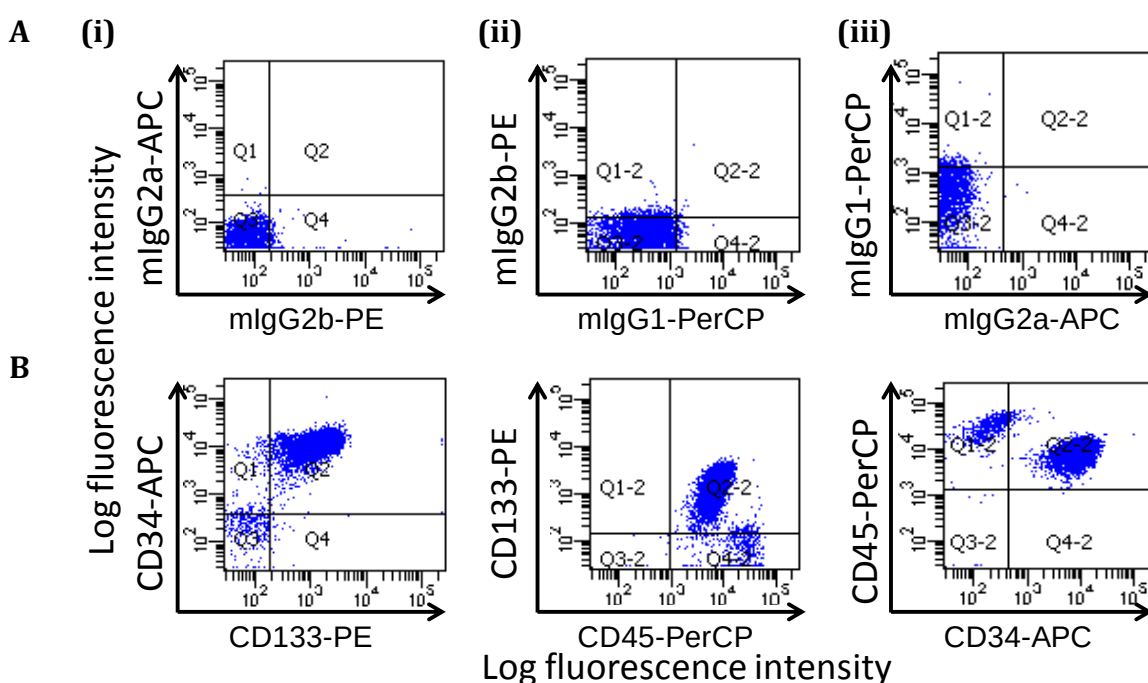


Figure 5.15. CD133 purified cells thawed and phenotyped at the start of the Nanex assay. Human UCB CD133⁺ cells isolated and purified as described in section 2.2.2.3 were analysed by flow cytometry prior to seeding in the Nanex culture system. Cells were stained using A) (i) mlgG2a-APC vs. mlgG2b-PE, (ii) mlgG2b-PE vs. mlgG1-PerCP, (iii) mlgG1-PerCP vs. mlgG2a-APC or B) (i) CD34-APC vs. CD133-PE, (ii) CD133-PE vs. CD45-PerCP, (iii) CD45-PerCP vs. CD34-APC as described in section 2.4.2. Representative dot plots are shown.

After 8 days in culture, the cells were harvested and counted (using a haemocytometer and trypan blue). A sample of the expanded cells were analysed by

flow cytometry for viability using DAPI and for CD133, CD45 and CD34 expression (Figure 5.16). In 3 independent experiments, the expanded cells retained CD45 expression ($96.8\% \pm 3.0$ and $94.9\% \pm 4.8$ using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and with the addition of 100ng/ml IGFBP2 and Angptl5 respectively ($n=3$)). A sub-population was both CD34 and CD133 positive ($38.7\% \pm 10.2$ and $37.7\% \pm 9.7$ using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and with the addition of 100ng/ml IGFBP2 and Angptl5 respectively ($n=3$)). The percentage of cells negative for DAPI was similar between conditions and to that seen for previous experiments ($85.0\% \pm 4.3$ and $86.6\% \pm 3.0$ using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and with the addition of 100ng/ml IGFBP2 and Angptl5 respectively ($n=3$)).

TNC expansion was 148.0 ± 48.6 fold for the cells cultured in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 209.0 ± 64.4 fold for the cells cultured with the additional growth factors (IGFBP2 and Angptl5) (Figure 5.17C). There was variability between the expansion of cells in these three experiments despite all samples being frozen and then thawed before use. The UCB units were derived from different donors which could account for the variability in expansion between samples (Figure 5.17C). Between the individual experiments, there was also variability seen in the number of CD34 cells expanded. However, the percentage of TNCs CD34⁺ remained very similar between the individual experiments ($78.7\% \pm 1.0$ in the cells cultured in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and $78.0\% \pm 1.0$ in the cells with the additional growth factors (IGFBP2 and Angptl5)) (Figure 5.17B).

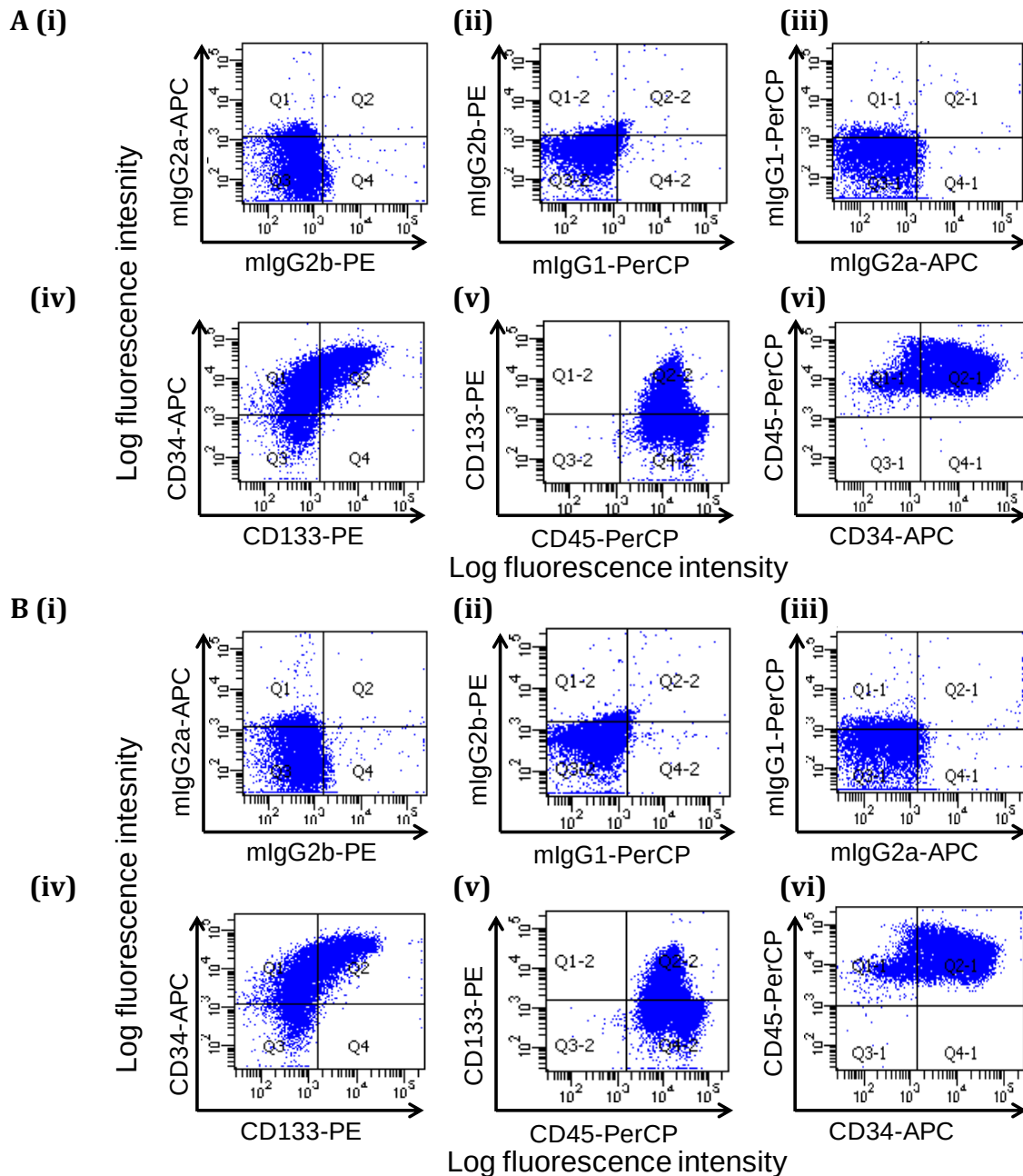


Figure 5.16. Nanex expanded cells retain CD45 expression and a population of CD133⁺CD34⁺ cells. Human UCB CD133⁺ cells were seeded at a concentration of 1000 cells/ml in 3ml per well of a 6 well Nanex scaffold. The cells were cultured in either A) Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (representative flow cytometry plots shown) or B) with the addition of 100ng/ml IGFBP2 and Angptl5. After 8 days in culture, the cells were harvested by pipette, pooled within conditions and counted using a haemocytometer. A sample of the entire population was then analysed by flow cytometry for (i) mlgG2a-APC vs. mlgG2b-PE, (ii) mlgG2b-PE vs. mlgG1-PerCP, (iii) mlgG1-PerCP vs. mlgG2a-APC (iv) CD34-APC vs. CD133-PE, (v) CD133-PE vs. CD45-PerCP, (vi) CD45-PerCP vs. CD34-APC as described in section 2.4.2. Representative dot plots are shown (n=3 independent experiments).

CD34⁺ cell expansion was 107.0 ± 46.0 fold using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 146.7 ± 60.5 fold with the additional growth factors (IGFBP2 and Angptl5) (Figure 5.17C).

Similarly, the percentage of CD133⁺ TNCs was similar between the culture conditions ($38.1\% \pm 11.0$ for the cells cultured in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and $36.9\% \pm 10.7$ with the additional growth factors (IGFBP2 and Angptl5) (Figure 5.17B). The CD133⁺ cell fold increase was consistent between the individual experiments (52.3 ± 6.0 fold for the cells cultured in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 73.0 ± 6.3 fold with the additional growth factors (IGFBP2 and Angptl5)) (Figure 5.17C). The CD133⁺CD34⁺ cell expansion was 52.4 ± 4.0 fold for the cells cultured in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 73.3 ± 2.2 fold with the additional growth factors (IGFBP2 and Angptl5). These differences in cell population expansion between conditions were not statistically significant ($p > 0.05$, Mann Whitney test). Thus, the results suggested that the addition of IGFBP2 and Angptl5 may non-significantly enhance CD133⁺ and CD34⁺CD133⁺ cell numbers around 1.5 fold over 8 days.

The expression of additional markers was also tested on these expanded cells (Figure 5.18). The majority of CD34⁺ cells expressed CXCR4 with no differences between the two conditions ($75.1\% \pm 6.3$ and 76.5 ± 5.8 , compared to arbitrary gating on isotype controls, using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and with the addition of 100ng/ml IGFBP2 and Angptl5 respectively ($n=2$)) (Figure 5.18A(ii) and B(ii)).

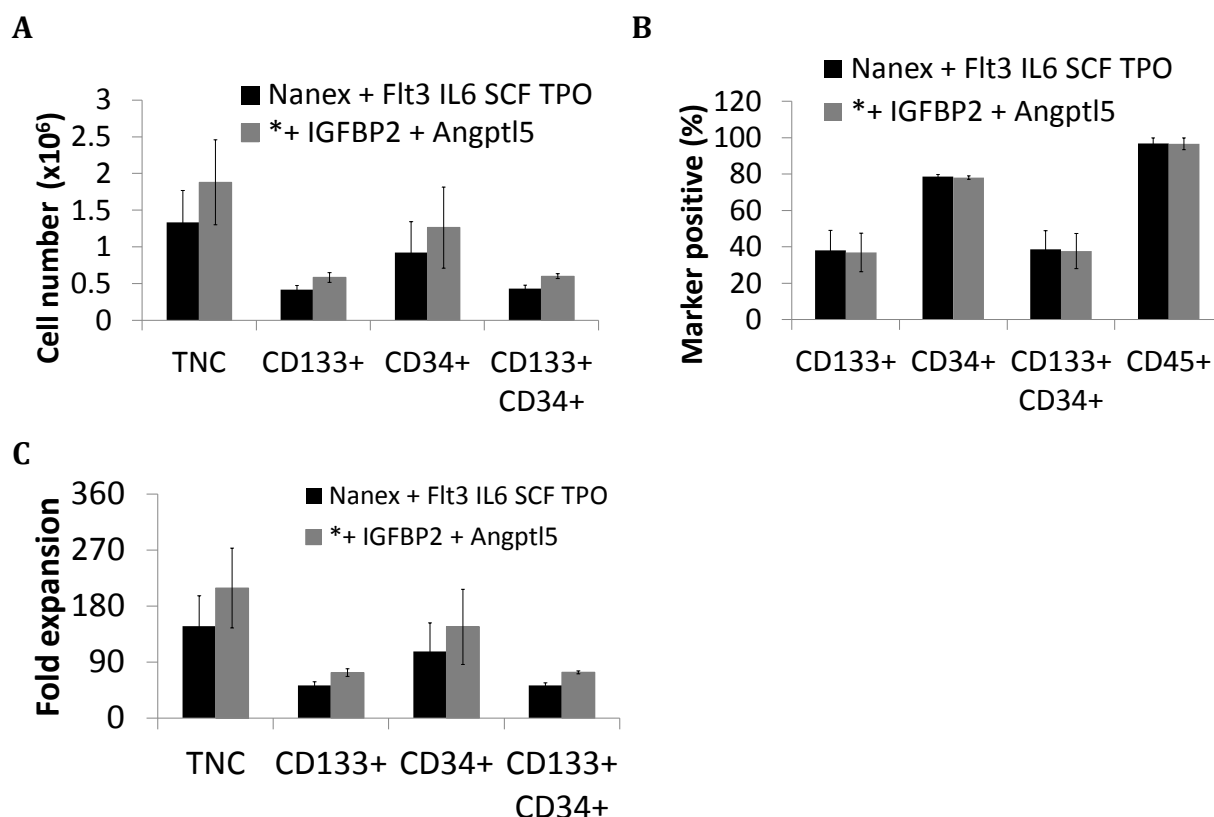


Figure 5.17. The addition of IGFBP2 and Angptl5 further improves CD34⁺CD133⁺ cell yield from Nanex expanded cells. CD133⁺ cells, at a concentration of 1000 cells/ml in 3ml were seeded per well of a 6 well Nanex scaffold (in 6 wells). The cells were cultured in either Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (black) or with the addition of 100ng/ml IGFBP2 and Angptl5 (grey). After 8 days in culture, the cells were harvested by pipette and counted by haemocytometer. A sample of the entire population was analysed by flow cytometry for the percentage of cells displaying CD45, CD133 and CD34 and DAPI (viability stain). A) The number of each expanded population, B) the percentage of cells positive for each marker or viability and C) the fold expansion from starting cell number (9000 cells) after 8 days in culture is shown as mean±SEM (n=3 independent experiments).

CD45RA staining showed a similar profile between the cells expanded using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and cells expanded with the addition of IGFBP2 and Angptl5 (Figure 5.18A(iv) and B(iv)). The percentage of cells that were CD133⁺CD45RA⁺ was 20.8% for cells expanded using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 21.1% with the addition of 100ng/ml IGFBP2 and Angptl5 (Figure 5.18A(iv) and B(iv)).

CD133⁺CD45RA⁻ populations could not be determined in the TNC population. The expression of CD38 and the HSPC/stromal marker CD90 was also measured and found to show negligible shifts in fluorescence from the isotype controls (Figure 5.18A and B(vi) and (viii)).

5.3.2.3 Testing the functional abilities of Nanex scaffold expanded cells using the 3D μ -slide chemotaxis assay

The human UCB CD133⁺ cells that had been cultured in the Nanex scaffolds for 8 days (section 5.3.2.2) were assessed for their ability to migrate to CXCL12 using the 3D μ -slide chemotaxis assay developed in Chapter 3. The CD34 population expanded in section 5.3.2.3 retained CXCR4 expression on the majority of TNCs (Figure 5.18A(ii) and B(ii)). A fraction of the total expanded population was re-selected for CD133 expression using microbeads (as described in section 2.2.2.3.2) immediately after harvesting and seeded into the chemotaxis assay as described in section 2.7.2. Unfortunately time constraints only permitted the completion of 2 chemotaxis experiments. The expression of CD133 and CXCR4 on CD133 purified cells before expansion is shown in Figure 5.19A(i-ii) and Figure 5.19A(iii-iv) respectively. This is also shown after expansion and re-purification of cells using CD133 microbeads for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO without and with 100ng/ml IGFBP2 and Angptl5 respectively (Figure 5.19B(i-iv) and C(i-iv) respectively). The starting CD133⁺ cell purity prior to expansion was 82.6% (MFI 1380) (Figure 5.19A(ii)).

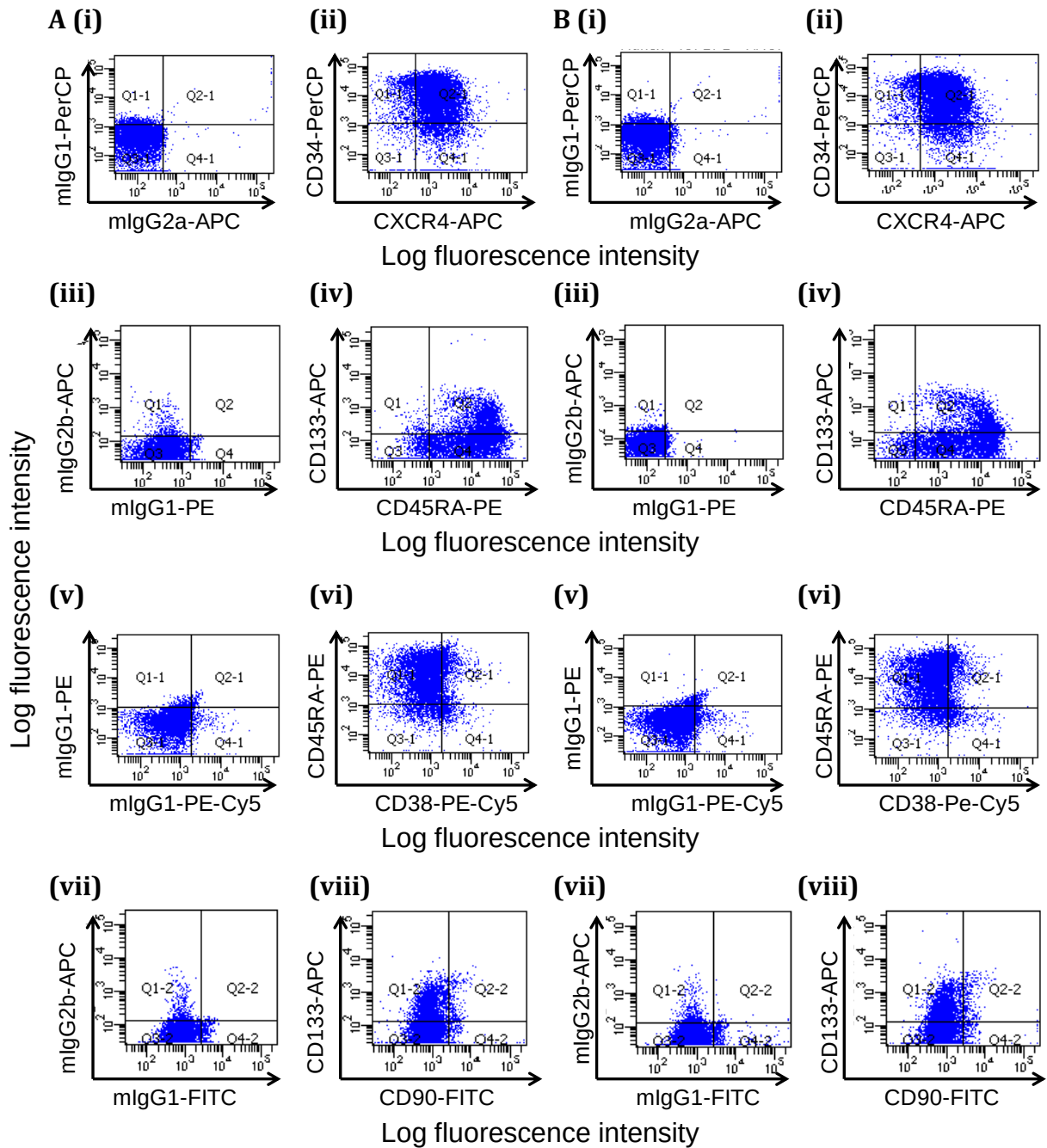


Figure 5.18. Additional characterisation of Nanex expanded cells. Human CD133⁺ cells, at a concentration of 1000 cells/ml in 3ml were seeded per well of a 6 well Nanex scaffold (in 6 wells). The cells were cultured in either A) Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO or B) with the addition of 100ng/ml IGFBP2 and Angptl5. After 8 days in culture, the cells were harvested and analysed by flow cytometry. Representative flow cytometry dot plots are shown for (i) mlgG2a-APC vs. mlgG1-PerCP, (ii) CXCR4-APC vs. CD34-PerCP, (iii) mlgG2b-APC vs. mlgG1-PE, (iv) CD133-APC vs. CD45RA-PE, (v) mlgG1-PE vs. mlgG1-PE-Cy5, (vi) CD45RA-PE vs. CD38-PE-Cy5, (vii) mlgG2b-APC vs. mlgG1-FITC, (viii) CD133-APC vs. CD90-FITC.

Re-selection of the expanded cells using CD133 magnetic beads yielded CD133⁺ cell purities of 96.1% (MFI 3816) for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (Figure 5.19B(ii)) and 96.3% (MFI 3878) for cells expanded with the addition of 100ng/ml IGFBP2 and Angptl5 (Figure 5.19C(ii)). CXCR4 expression was present on essentially all CD133⁺ cells, albeit ranging from low to higher levels of expression (MFI 277 and 276 to 295 for cells without and with expansion in SCF, IL-6, Flt-3 ligand and TPO without and with Angptl5 and IGFBP2 respectively). Although the profiles of expression exemplified by the FACS histograms altered before (Figure 5.19A(iv)) and after expansion (Figure 5.19B(iv) and C(iv)), the percentage of cells lying above the arbitrary gate set for the isotype controls ((Figure 5.19A-C(iii))) were similar for all cases (between 70.9%-73.7%).

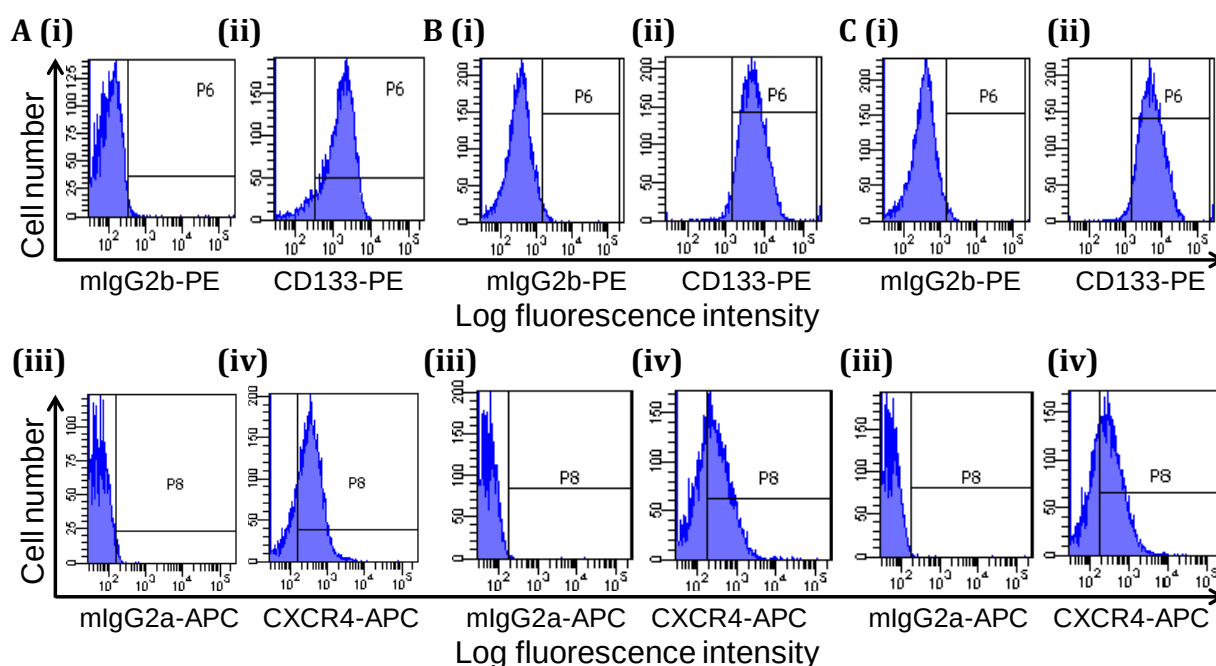


Figure 5.19. Characterising the expanded cells before the chemotaxis assay. Human UCB CD133⁺ cells were analysed by flow cytometry for (i) mlgG2b-PE, (ii) CD133-PE, (iii) mlgG2a-APC and (iv) CXCR4-APC A) prior to seeding the cells on the Nanex scaffold, or after being re-selected for CD133 surface expression using microbeads as described in section 2.2.2.3.2 after B) 8 day expansion in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and C) with the addition of 100ng/ml IGFBP2 and Angptl5. Representative histograms are shown.

The CD133 re-purification using magnetic beads recovered $51.8\% \pm 9.9$ and $43.8\% \pm 12.8$ of the expanded cell population expressing CD133 (based on the percentage of CD133 expression on the TNC by flow cytometry) for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and with the addition of 100ng/ml IGFBP2 and Angptl5 respectively (n=3). The migration of the re-purified CD133⁺ cells towards either no CXCL12 (control) or 1 μ g/ml CXCL12 was tracked over a 22 hour period as described in section 2.7.2 (Figure 5.20).

Cells from both expansion conditions in all experiments showed a displacement of the centre of mass and a forward migration index in the direction of CXCL12 compared to the control (no CXCL12). However, there was variability seen between the independent experiments, with the cells expanded in the second and third experiments showing more consistent chemotaxis to CXCL12. The displacement of the centre of mass towards CXCL12 for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO was $205.9\mu\text{m} \pm 60.8$ and with the addition of 100ng/ml IGFBP2 and Angptl5 was $242.1\mu\text{m} \pm 18.6$ (Figure 5.21B). The forward migration index towards CXCL12 for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO was 0.16 ± 0.03 and with the addition of 100ng/ml IGFBP2 and Angptl5 was 0.17 ± 0.06 (Figure 5.21C).

In the first experiment, when looking at whether the cell migration was distributed inhomogeneously in the direction of CXCL12 (Figure 5.21A), this was significant for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO ($**p=0.02 \times 10^{-1}$) ($**p<0.01$, Rayleigh test). This was not significant in cells expanded with the addition of 100ng/ml IGFBP2 and Angptl5 ($p=0.17$), despite a greater displacement of centre of mass than was seen for cells expanded in the other

condition. Therefore, cells expanded in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO showed a greater percentage of cells migrating to CXCL12, albeit with a lesser displacement of the centre of mass. Whereas, a lower percentage of cells expanded with the addition of Angptl5 and IGFBP2 migrated towards CXCL12, although, the cells that did, migrated much further, and hence the larger displacement of centre of mass. In the second and third experiment, the cells expanded in both conditions showed a highly significant inhomogeneous distribution towards CXCL12. The Rayleigh p values were $***p < 0.36 \times 10^{-4}$ and $***p < 0.84 \times 10^{-7}$ for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and with the addition of 100ng/ml IGFBP2 and Angptl5 respectively compared to the no CXCL12 control ($p > 0.13$) ($***p < 0.001$, Rayleigh test) (Figure 5.21A) (pink circles).

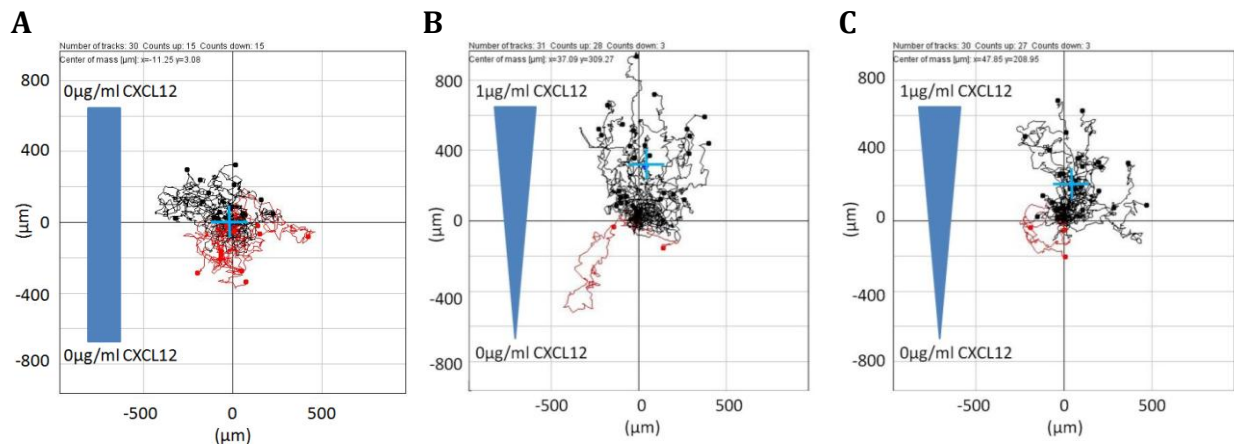


Figure 5.20. CD133⁺ cells expanded in the Nanex system retain migratory capacity to CXCL12. The CD133⁺ cells cultured for 8 days in Nanex scaffolds in section 5.3.2.2 and re-selected for CD133 surface expression using microbeads as described in Figure 5.19 were seeded into the 3D μ-slide chemotaxis assay as described in section 2.7.2. Migration in the presence of A) no CXCL12 (control) or towards 1 μg/ml CXCL12 was tracked over a 22 hour period as described in section 2.7.2 to generate trajectory plots showing migration of cells expanded in B) Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and C) Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 100ng/ml IGFBP2 and Angptl5. The plots shown were generated from experiment 2.

In terms of chemokinesis, Figure 5.21D and E shows that there were no overall differences in cell velocity or accumulated distance between cells in either expansion condition in the presence of CXCL12 or the control condition (no CXCL12). For cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO the velocity was $0.97\mu\text{m}/\text{min}\pm 0.1$ and $1.0\mu\text{m}/\text{min}\pm 0.1$ for cells expanded with the same cytokines but with the addition of 100ng/ml IGFBP2 and Angptl5 respectively (n=3). The accumulated distance was $1210\mu\text{m}\pm 186$ for cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and $1177\mu\text{m}\pm 143$ with the addition of 100ng/ml IGFBP2 and Angptl5 respectively (n=3). In the control, where no CXCL12 was present, the mean velocity and accumulated distance respectively were $0.97\mu\text{m}/\text{min}\pm 0.1$ and $1215\mu\text{m}\pm 158$ (n=3). Although the use of different cytokines did not affect cell chemokinesis between the expansion conditions, further studies are required to examine the variability seen in the chemotactic response to CXCL12 between expanded units and to compare the effect of stimulation with different cytokines on CD133⁺ cell chemotaxis.

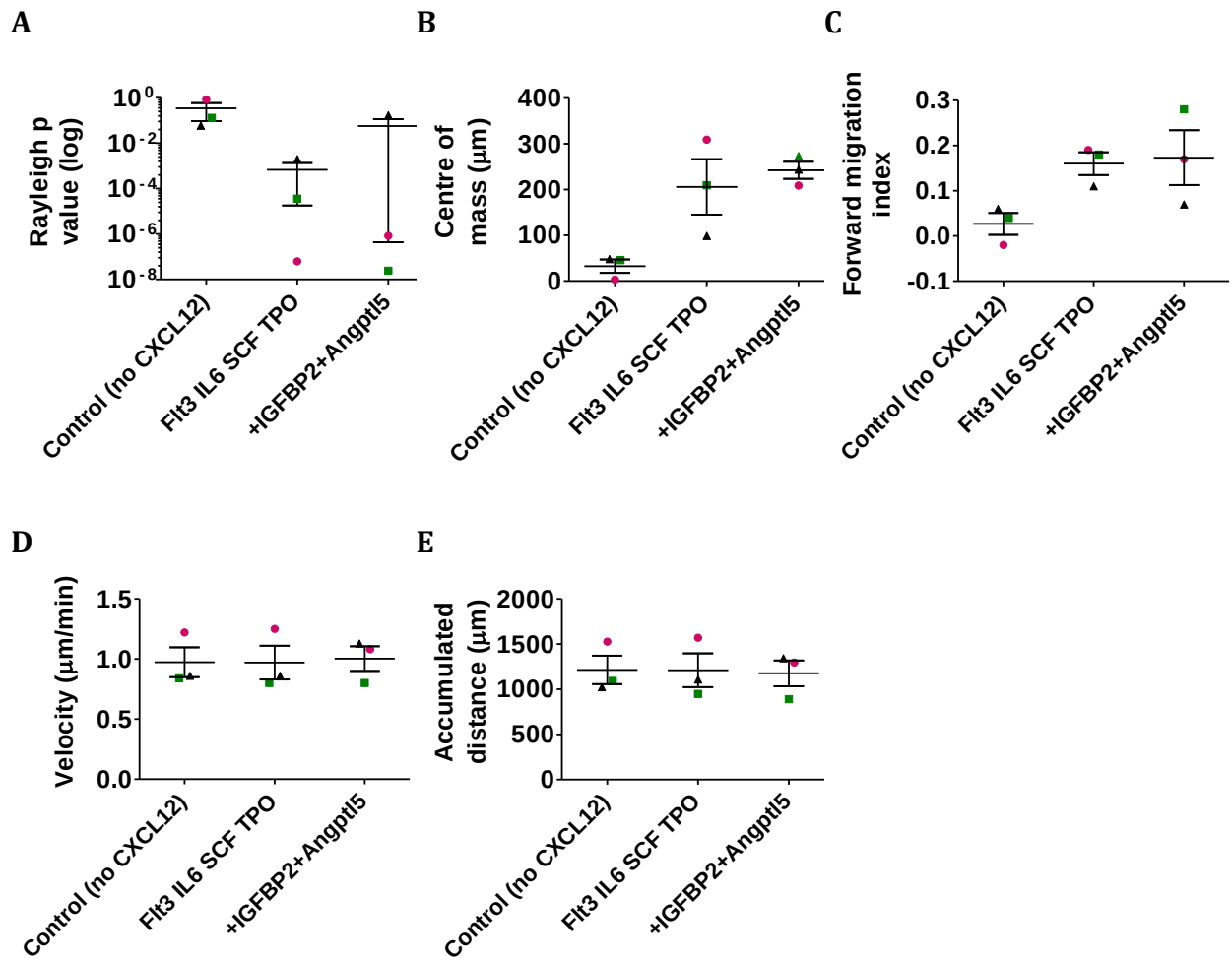


Figure 5.21. CD133⁺ cells expanded in the Nanex system display chemotaxis to CXCL12. The CD133⁺ cells were cultured for 8 days in Nanex scaffolds in section 5.3.2.2 using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (second data sets) or Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 100ng/ml IGFBP2 and Angptl5 (third data sets) as indicated on the X axis. The expanded cells were re-selected for CD133 surface expression using microbeads as described in Figure 5.19 and seeded into the 3D μ -slide chemotaxis assay as described in section 2.7.2. Migration in the presence of no CXCL12 (control) (first data set) or towards 1 μ g/ml CXCL12 (second and third data sets) was tracked over a 22 hour period as described in section 2.7.2 and analysed using the Chemotaxis and migration tool (Image J plug-in). Individual data and mean $\pm$ SEM from experiment 1 (black triangles), experiment 2 (pink circles) and experiment 3 (green squares) are shown for A) Rayleigh p values (significant at $p < 0.05$), B) displacement of the centre of mass, C) forward migration index, D) velocity and E) accumulated distance ($n=3$ independent experiments).

5.4 Discussion

In this chapter, different approaches were taken in an attempt to expand human UCB CD133⁺ cells *ex vivo*. In the first approach, attempts were made to recapitulate the vascular niche *in vitro* using a human bone marrow endothelial cell line (BMEC-60) and human bone marrow stromal cells (BMSCs). If this had been achieved, the ability of this niche model to support human UCB CD133⁺ cell expansion would have been examined. *In vivo* experiments have shown that endothelial cells are essential for reconstitution of the bone marrow following lethal irradiation (Hooper et al., 2009). The same researchers have demonstrated that human UCB HSPC expanded on transduced endothelial cells retained repopulation capacity *in vivo* (Butler et al., 2012). Although historically, BMSCs have also been shown to support human HSPC expansion in the presence of cytokines using long term culture initiating cell assays (LTC-IC) (Ploemacher et al., 1989; Rappold et al., 1999; Shih et al., 1999; Guven et al., 2011; Kranc et al., 2009), a combination of stromal and endothelial cells has not been used in an *in vitro* 2D culture system as a bone marrow niche model.

Studies within our laboratory had previously established a vascular network forming assay using hDFs and endothelial cells (Zhang et al., 2009). More recently, in studies not presented in this thesis, we demonstrated that the hDFs could be replaced with MS-5, but not NIH3T3, mouse stromal cell lines to support endothelial vascular network formation (Zhou et al., 2012). In an attempt to further emulate the human vascular niche, the HUVEC vs. hDF co-culture model was used as a control which we anticipated could be adapted to produce a more representative model of the bone marrow. Human BMSC which support LTC-IC, were substituted for hDF and two

sources of endothelial cells were tested. These were the human bone marrow endothelial cell line, BMEC-60, and endothelial cells derived from UCB.

BMEC-60 cells, which had been immortalised with a retroviral construct containing the HPV-16 E6/E7 genes and confirmed as being similar to primary bone marrow endothelial cells by Rood *et al.*, generated limited, stunted vascular tubules on BMSC. Although the tubules generated varied with the BMSC batch, these cells did not seem able to form integrated vascular networks on batches of BMSCs which were later shown to support the tubule formation of endothelial cells from UCB. Endothelial cells from UCB were chosen as these are contained within the UCB graft infused into patients undergoing bone marrow transplantation. Vascular network formation by endothelial cells varied depending on the batch of BMSC. In addition, preliminary experiments carried out in collaboration with Dr. Sarah Hale showed that expansion of UCB CD133⁺ derived cells was poor in comparison to our standard culture conditions on tissue culture treated plates (stemspan medium with SCF, Flt-3 ligand, IL-6 and TPO). Thus, this approach was abandoned in place of an artificial nanofibre scaffold (Nanex) developed by Arteriocyte.

This second approach assessed whether human UCB CD133⁺ cells could be expanded in different cytokine combinations in the presence of a Nanex nanofibre scaffold (sourced commercially from Arteriocyte). The Nanex nanofibre scaffold was reported by Arteriocyte to give a 75 fold expansion of fresh or frozen CD34⁺ cells from UCB over 8 days (Figure 5.1). Although Arteriocyte had also demonstrated that the cells expanded using the Nanex scaffold maintained and even increased CFU potential beyond that seen in fresh HSPCs, colony forming ability is not necessarily a determinant of *in vivo* HSC function. Peled *et al.* found that both the non-migrating

and migrating fraction of CD34⁺CD38⁻ cells in a Boyden assay both formed the same colonies but that the migrating cells showed significantly better engraftment *in vivo* (Peled et al., 1999b). Additionally, Voermans *et al.* found that *in vitro* migratory capacity of adult CD34⁺ cells was related to the clinical outcome following autologous transplantation in humans (Voermans et al., 2001). Further work using expanded CD34⁺ cells that did not successfully engraft mice showed impaired transwell migration towards CXCL12 (Foguenne et al., 2009). Therefore, our assays included a test of function in the form of the 3D μ -slide chemotaxis assay, developed in Chapter 3, to assess the cell response to CXCL12.

Our CD133 enriched cells from fresh UCB gave rise to a 246.0 ± 34.5 fold expansion of CD34⁺ cells and a 56.5 ± 4.6 fold expansion of CD133⁺ cells using the recommended culture conditions with the Nanex scaffolds for 8 days (standard Nanex conditions). Arterocyte advised that the cytokine cocktail contained SCF, Flt-3 ligand, IL-6, TPO and IL-3. If we replaced this cytokine cocktail with our panel of cytokines (SCF, Flt-3 ligand, IL-6 and TPO) and excluded LDL, CD34⁺ cell expansion was lower (93.8 ± 14.2 fold), while CD133⁺ cell expansion was on average higher (65.0 ± 3.5 fold). Additionally, a higher proportion of the TNC were CD133⁺ after 8 days in culture ($50.4\% \pm 2.1$), compared to standard Nanex conditions ($17.0\% \pm 0.4$). Thus, the standard Nanex expansion conditions appeared to promote HSPC differentiation.

In a preliminary flow cytometry experiment, more than half (approximately 60%) of the cells expanded in the standard Nanex condition expressed the myeloid marker CD33 compared to only 15% expression on the cells expanded in the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. Equal expression of the granulocytic marker CD66 was observed in both expansion conditions

(approximately 30%). There was negligible expression of CD3, CD19, CD56 or CD41a, suggesting that neither condition produced T cells, B cells, NK cells or megakaryocytes/platelets during the 8 day culture. Although CD34/CD33 co-staining was not performed, as $74.0\% \pm 6.2$ of the TNC expanded using the standard Nanex conditions expressed CD34, we can assume that some, if not all of the CD33 expressing cells were CD34 positive, and were therefore maturing into myeloid progenitors (CD34⁺CD33⁺) which have been shown to be incapable of forming colonies (Yamaguchi et al., 1993). This could be explained by the presence of IL-3 in the standard Nanex expansion conditions, as IL-3, in combination with SCF, Flt-3 ligand and IL-6 has been shown to support the differentiation of CD34⁺ cells into myeloid cells (Fadilah et al., 2007). However the other additive in the standard Nanex culture was acetylated LDL. The absence of lipoproteins has been shown to limit the differentiation of monocyte-derived dendritic cells in culture (Gogolak et al., 2007), and therefore the presence of acetylated LDL in the standard Nanex media may be driving differentiation towards the more mature myeloid lineage. Additionally, *ex vivo* expansion of UCB CD34⁺ cells with Flt-3 ligand, SCF and TPO with IL-3 was shown to result in a higher frequency of dendritic cell precursor surface markers compared to culture with Flt-3 ligand, SCF and TPO with IL-6 (Encabo et al., 2004). Therefore both IL-3 and acetylated LDL could be driving the increase in myeloid differentiation seen with the standard Nanex conditions.

These experiments investigating the expression of differentiation markers on the expanded cells could not be completed because of the time constraints of this thesis. Although further confirmation and a more detailed analysis of the cells generated is required, the initial results suggest that the system developed by Arterioocyte may be

beneficial for expanding one UCB unit for enhancing early neutrophil engraftment, perhaps when co-transplanted with an un-manipulated unit. It is well known that neutrophil engraftment is delayed with UCB compared to BM and MPB (Rocha et al., 2000; Rocha et al., 2001).

Although variability in expansion was seen between UCB units derived from different donors, the expansion of human UCB CD133⁺ cells did not appear to be detrimentally affected by freezing. Fresh cells expanded using the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO gave rise to a 93.8 ± 14.2 fold expansion of CD34⁺ cells compared to a 107.0 ± 46.0 fold expansion from thawed cells. However, more experimental data are required to confirm this.

In the second set of expansion experiments, human UCB CD133 enriched cells gave rise to a 107.0 ± 46.0 fold expansion of CD34⁺ cells over 8 days using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO. The addition of 100ng/ml IGFBP2 and Angptl5 increased this expansion almost 1.5 times to 146.7 ± 60.5 fold. A greater expansion of CD133⁺ cells was also seen after 8 days in culture with the addition of IGFBP2 and Angptl5 (73.0 ± 6.3 fold) compared to without (52.3 ± 6.0 fold). The same was seen with CD133⁺CD34⁺ cell expansion which was 52.4 ± 4.1 fold for the cells cultured in Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO and 73.0 ± 6.3 fold with the additional growth factors (IGFBP2 and Angptl5). Therefore, although further experiments need to be done, the results to date suggest that, although the TNC expansion was lower, the Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO improved CD34⁺CD133⁺ cell expansion compared to the standard Nanex conditions, while the addition of 100ng/ml IGFBP2 and Angptl5 to the SCF, Flt-3 ligand, IL-6 and TPO cocktail further increased the

expansion of CD34⁺CD133⁺ cells. When comparing these data to that published by Drake *et al.* using Angptl5 and IGFBP2 cultures, a 21 fold increase in CD133⁺CD34⁺ was observed after 10 days (Drake et al., 2011), suggesting that the Nanex scaffold used here could be enhancing expansion by greater than 3 fold. Although Drake *et al.* showed CD133⁺CD34⁺ cell number to correlate with SRC activity in NSG mice, we have yet to demonstrate this (Drake et al., 2011).

Further studies investigated the ability of the expanded, re-selected CD133⁺ cells to migrate to CXCL12 in the 3D μ -slide chemotaxis assay. With the exception of one experiment using cells expanded with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO with the addition of 100ng/ml IGFBP2 and Angptl5, all expanded cells showed chemotaxis. This was defined by the Rayleigh test for inhomogeneous distribution of data. Therefore, the CD133⁺ cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO showed definitive migration towards CXCL12 (**p<0.002). For the cells expanded with the addition of 100ng/ml IGFBP2 and Angptl5 however, although a similar displacement of the centre of mass was seen between the independent experiments, the mean Rayleigh p values achieved did not quite meet statistical significance (p=0.057). These experiments will need to be repeated with many more expanded UCB units before a conclusion can be drawn.

The average migratory ability of the cells expanded for 8 days using Nanex media with 100ng/ml SCF, Flt-3, IL-6 and 20ng/ml TPO (n=3) was compared to the average cell migration seen following 24 hour culture in stemspan with 100ng/ml SCF, Flt-3, IL-6 and 20ng/ml TPO (n=8, section 3.3.8). The displacement of the centre of mass towards CXCL12 in the cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO was comparable to the averages seen in CD133⁺ cells

used after a 24 hour culture period ($205.9\mu\text{m}\pm 60.8$ (n=3) compared to $189.0\mu\text{m}\pm 44.3$, (n=8) respectively). A similar finding was observed with the forward migration index when comparing the CD133⁺ cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO (0.16 ± 0.03 , n=3) to CD133⁺ cells cultured for a 24 hour period in stemspan medium with 100ng/ml SCF, Flt-3, IL-6 and 20ng/ml TPO (0.13 ± 0.02 , n=8). Directionality was similar (0.26 ± 0.03 , n=3 compared to 0.21 ± 0.02 , n=8), as were accumulated distance and velocity. Accumulated distance for CD133⁺ cells expanded using Nanex media with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO was $1210\mu\text{m}\pm 186$ (n=3), and velocity was $0.97\mu\text{m}/\text{min}\pm 0.10$ (n=3), compared to CD133⁺ cells cultured for a 24 hour period in stemspan medium with 100ng/ml SCF, Flt-3, IL-6 and 20ng/ml TPO ($1258\mu\text{m}\pm 4$, n=8 and $1.01\mu\text{m}/\text{min}\pm 0.11$, n=8 for accumulated distance and velocity respectively).

Unfortunately time constraints prevented this work from being repeated further, and from assessing the cells in an *in vivo* engraftment model, although the model was initiated in NOD-scid mice (as described in section 6.1). However, other laboratories have investigated the engraftment potential of expanded HSPCs. Holmes *et al.* have recently reported that human UCB CD34⁺ cells expanded for 6 days using IMDM with 10% (v/v) FCS and 100ng/ml Flt-3 ligand, SCF and TPO had impaired repopulating ability in NSG mice compared to non-expanded cells (Holmes et al., 2012). However, it is of note that neither the expanded or non-expanded cells from human UCB CD34⁺ cells shown by Holmes *et al.* expressed CXCR4 at the surface. Whether expanded or not expanded, our results demonstrate CXCR4 expression on the majority (>70%) of CD133⁺ cells from human UCB. Additionally, Holmes *et al.* reported that the majority of the expanded cells were CD38⁺CD90⁺ (Holmes et al., 2012). Our studies showed

that with the Nanex scaffold expansions, very few cells expressed CD38 or CD90 after 8 days' expansion. This suggests that the serum free Nanex expanded cells are phenotypically very different to cells expanded by Holmes *et al.* without a scaffold and in serum.

Earlier studies have consistently demonstrated a loss of CD38 expression during culture (Piacibello *et al.*, 1999; Rappold *et al.*, 1999) and this loss of CD38 does not correlate with cell engraftment ability (Ito *et al.*, 2010). Gori *et al.* most recently found that expanded cells with the CD34⁺CD38⁻CD90⁺CD49f⁺CD45RA⁻ phenotype were capable of long term engraftment (Gori *et al.*, 2012). Although we have seen limited evidence of CD45RA⁻ and CD90⁺ cells in the TNC expanded populations, the combination of these markers has not been tested on the CD133⁺CD34⁺ cells.

Zhang *et al.* reported (in the absence of a scaffold) that no additional effect on 10 day UCB CD34⁺ cell expansion above that seen with stemspan medium with recombinant human cytokines Flt-3 ligand, SCF, TPO was observed upon the addition of 100ng/ml IGFBP2 or 500ng/ml Angptl5 (Zhang *et al.*, 2008). However, the cells expanded with the addition of IGFBP2 or Angptl5 compared to cells expanded without repopulated NOD-scid mice significantly better (Zhang *et al.*, 2008). Additionally, they demonstrated that when both IGFBP2 and Angptl5 (500ng/ml) were added to 11 day human UCB CD34⁺ cell cultures with stemspan medium containing recombinant mouse SCF, TPO and FGF-1, significantly increased human chimerism in NOD-scid mice was seen compared to cells cultured without IGFBP2, without Angptl5, or without either (39.5%, 17.3%, 11.3% and 0.8% respectively) (Zhang *et al.*, 2008).

The Nanex scaffold demonstrated here achieved similar TNC expansion (239.7±98.1 fold) using Nanex medium with 100ng/ml SCF, Flt-3 ligand, IL-6 and 20ng/ml TPO

and 100ng/ml IGFBP2 and Angptl5 over 8 days as that described by Zhang *et al.* over 10-11 days (approximately 200 fold) (Zhang et al., 2008). Additionally, the CD133⁺ cells generated showed a chemotactic response to CXCL12. Therefore, future work should investigate whether the Nanex scaffold expanded HSPCs contain self-renewing stem cells and 1) are capable of engraftment, 2) whether this engraftment is improved compared to their non-expanded counterparts, and 3) whether the Nanex scaffold generates cells with additional repopulating ability compared to expansion without scaffolds (e.g. as shown by Zhang *et al.*) (Zhang et al., 2008). Unlike other published expansion procedures using alternative culture conditions above tissue culture plates (e.g. mouse cells or lentiviral transduced cells (Butler et al., 2012; Hooper et al., 2009)), the Nanex scaffold is animal, serum and viral free, and therefore has the potential for clinical application.

Chapter 6. General discussion

6.1 Review of the thesis

The aim of this thesis was to improve the homing or short term engrafting capacity of HSPCs derived from human UCB. The mechanisms by which this could be achieved were identified as 1) improving the homing capacity of individual HSPCs or 2) expanding these cells while maintaining their ability to home to and engraft in the bone marrow. As the identification of the human haemopoietic stem cell (HSC) is difficult and as these cells are rare, the experiments in this thesis concentrated on human UCB CD133⁺ HSPCs, which are thought to encompass both the HSC and less mature CD34⁺ cells (McGuckin et al., 2003). However, recent studies investigating HSPC homing and engraftment *in vivo* have used CD34 to enrich for SRCs and phenotyped these cells further using markers such as CD49f and CD45RA (Butler et al., 2012; Gori et al., 2012). Further characterisation of the CD133 enriched population used in this thesis would have enabled clarification as to the cell phenotypes contained in the 90-95% CD133⁺ population.

In order to investigate the potential homing capacity of individual cells in response to CXCR4-mediated signals, a 3D μ -slide chemotaxis assay was successfully developed first, as described in Chapter 3. The CXCL12-CXCR4 axis is widely recognised as playing a key role in HSPC homing to and retention and engraftment in the bone marrow. The assay measured individual human UCB CD133⁺ cell chemotaxis towards CXCL12 over time by time-lapse microscopy. This assay provided a tool which could track the movement of individual cells in a stable chemokine gradient over a 22 hour period by time-lapse microscopy. This provided advantages over existing end point

migration assays, such as the Boyden transwell assay, as information on chemokinetic as well as chemotactic parameters could be defined and quantified.

This chemotaxis assay required a 3D gel matrix through which the cells could migrate towards CXCL12 over a defined time period which corresponded to published *in vivo* homing assays (usually 16-20 hours) (Avigdor et al., 2004; Ellis et al., 2011; Foguene et al., 2009; Gori et al., 2012; Kollet et al., 2001; Larochelle et al., 2012; Leung et al., 2011). Human UCB CD133⁺ cells displayed optimal chemotaxis towards 1µg/ml CXCL12 through a 1mg/ml collagen I gel over 22 hours. The presence of CXCL12 did not affect cell chemokinesis. Additionally, CXCR4 chemotaxis was inhibited almost completely using the CXCR4 antagonist, AMD3100, which had no effect on cell chemokinesis. These results from this novel assay dispute the previous dogma that chemokines have dual effects on both chemotaxis and chemokinesis (Entschladen et al., 2005). This assay was subsequently used as a platform to measure the effects on human UCB CD133⁺ cell homing/migration after 1) modulating the expression of the sialomucin adhesion receptor CD164, which had previously been shown to act as a co-receptor for CXCR4 and 2) expanding human UCB CD133⁺ cells.

Chapter 4 set out to meet the first of these aims by knocking down CD164 in CD133⁺ cells from UCB. CD164 was previously shown to be expressed on long term repopulating human HSPCs (Buhring et al., 1999), as well as on CD34⁺CD133⁺ cells, and to play a role in their adhesion and migration to bone marrow stromal cells and CXCL12 (Forde et al., 2007; Zannettino et al., 1998). [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

In the absence of available antibodies to block the functional CD164 epitopes (103B2 and 105A5), transient silencing of human UCB CD133⁺ cells was attempted using the commercially available pGIPZ-GFP shRNAs targeting CD164. The pGIPZ construct contained a CMV promoter for the shRNA, which has been shown to be sub-optimal for HSPC transduction (Demaision et al., 2002), although the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which can improve transgene expression, was also contained in the pGIPZ vector (Ramezani et al., 2000). Although, the shRNA nucleofection efficiency was not complete (30-50%), in the initial experiments, the GFP⁺ cells showed a 62.9%±16.1 reduction in CD164 surface expression. Therefore, the vector had transfected the HSPCs and the promoter was driving shRNA expression. [REDACTED]

[REDACTED]

[REDACTED] This suggested that both the NS and CD164-B shRNA were non-specifically affecting or were toxic to human cell

function. [REDACTED]

[REDACTED]

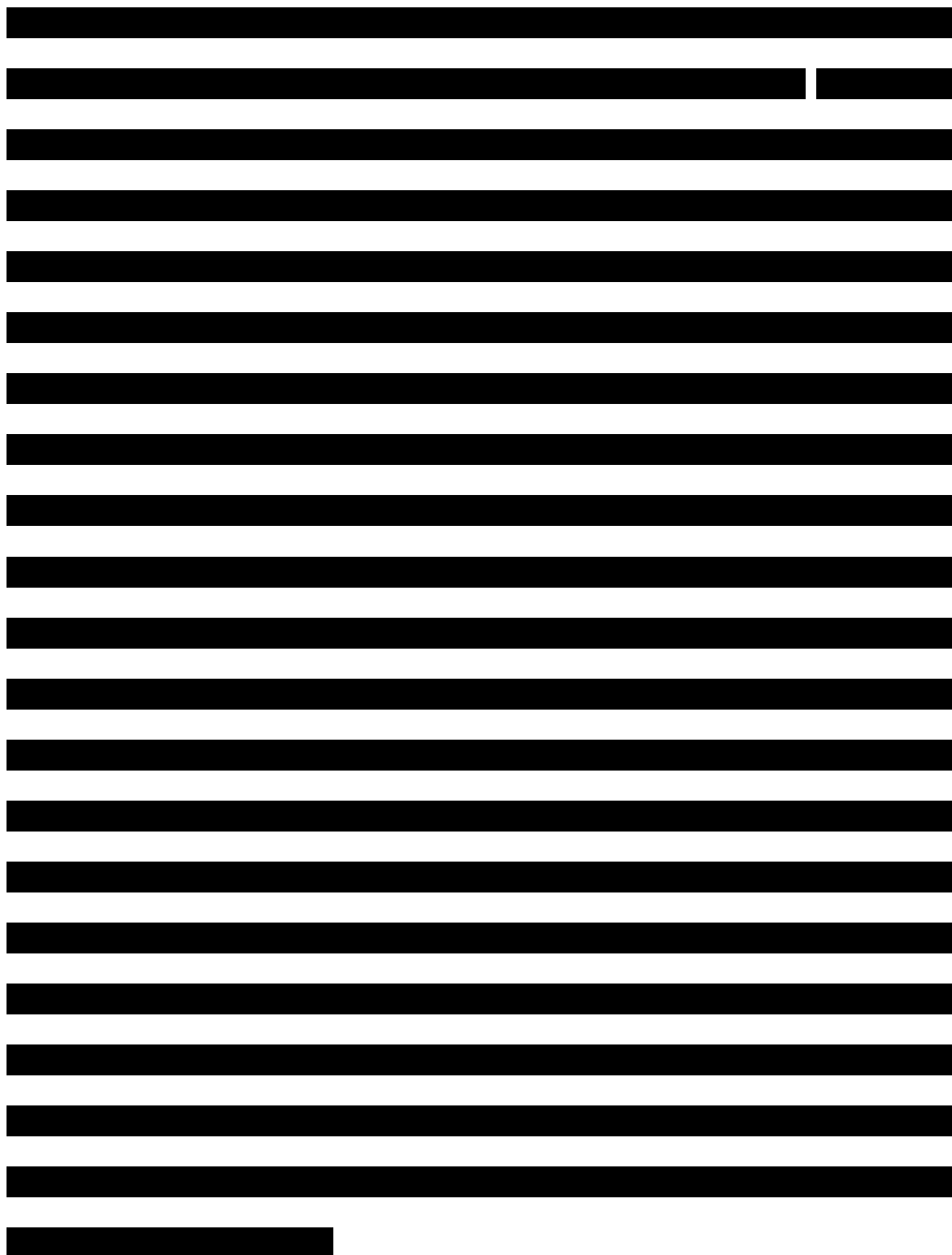
Subsequently, the siRNA sequences previously described by Forde *et al.*, (Forde et al., 2007) were tested. The CD164-2 sequence provided higher, although still not complete levels of CD164 surface knock-down in the total population of nucleofected human UCB CD133⁺ cells. Although this is a potential limitation to this research in terms of determining the effect of CD164 knock-down on the reconstituting sub-set of CD133⁺ cells from UCB, a more complete method of knock-down was not available. Additionally, a method to assess protein knock-down would have confirmed that intracellular levels of CD164 remained knocked-down throughout the time-course of these experiments and was therefore not available to be expressed at the cell surface under the conditions of the functional assays.

The effect of CD164-2 siRNA on [REDACTED] *in vitro* migration was tested over 7 independent experiments. The [REDACTED] chemotaxis of human UCB CD133⁺ cells nucleofected with NT or CD164-2 siRNA was variable. However, the mean difference between the [REDACTED] [REDACTED] chemotaxis of CD133⁺ cells nucleofected with NT or CD164-2 siRNA was not significantly different, suggesting CD164 does not play a vital role in the migration/homing of human HSPCs to the murine bone marrow. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED] A blockade of migration was demonstrated for CXCL12-dependent migration in the 3D chemotaxis assay using AMD3100.



Although the N6B6 antibody, which is commercially available, has been shown to block migration and invasion in cancer cells (Havens et al., 2006), this antibody did not block CXCL12 mediated migration in human UCB CD133⁺ cells (Forde et al.,

2007). In additional experiments not presented here, we noted that 24 hour culture in serum free medium with SCF, Flt-3 ligand, IL-6 and TPO increased the surface expression of CD164 on human UCB CD133⁺ cells. Whether or not this could affect homing compared to uncultured cells needs to be determined in future experiments.

Chapter 5 aimed to meet the final objective of improving the engraftment capability of UCB units by investigating the effect of culture conditions on CD133⁺ cell expansion and migratory capacity towards CXCL12. Initially, a cell based co-culture system designed to mimic the vascular niche was studied. The endothelial cells and stromal cells were chosen for a number of reasons. The ability of mouse bone marrow endothelial cells alone in supporting mouse HSPC proliferation had been shown (Butler et al., 2010) so we aimed to use human BMEC in the model. However, as BMECs did not generate vasculature on stromal cells, endothelial cells isolated from UCB, which are currently being investigated for their use in vascular regenerative medicine (Vanneaux et al., 2010; Watt et al., 2010), were tested with bone marrow stromal cells (BMSCs). Although the regeneration of bone marrow endothelium has been shown to be essential for HSPC engraftment, and therefore the restoration of haemopoiesis in myelosuppressed mice (Hooper et al., 2009), the importance of stromal cells in the bone marrow niche cannot be overlooked (Lo Celso et al., 2009). The existence of CXCL12⁺ stromal reticular cells and osteoblastic cells in the bone marrow, where HSCs have been visualised, supports the importance of non-endothelial cell types in haemopoiesis (Bengtsson et al., 2011; Ellis et al., 2011; Levesque and Winkler, 2011; Lo Celso et al., 2009; Winkler et al., 2010; Xie et al., 2009). For this reason, we wanted to establish an *in vitro* model to investigate the importance of stromal and endothelial cells in supporting haemopoiesis.

Our results demonstrated that neither the transformed BMEC-60 cell line, nor the batches of primary human BMSCs obtained from Lonza Biologics consistently produced robust vascular niche models with which to expand HSPCs. Butler *et al.* have recently shown that E4ORF1 transformed HUVECs can support human HSPC expansion of CD34⁺ cells by 150-fold and Lin-CD34⁺CD45RA-CD49f⁺ cells 23-fold in the presence of SCF, Flt-3 ligand and TPO and that these cells have secondary repopulation potential in mice (Butler et al., 2012). In preliminary experiments, our results demonstrated that neither BMSCs, BMEC-60 cells, primary endothelial cells from UCB, nor a combination of these, supported significant expansion of UCB CD133⁺ cells beyond that seen using standard serum free culture conditions without cells (SCF, Flt-3 ligand, IL-6 and TPO), as described by Rappold *et al.* (Rappold et al., 1999).

To take our studies forward, a commercially available nanofibre scaffold (Nanex), which had been demonstrated by Arteriocyte to expand cells beyond that seen in our standard scaffold free culture was acquired and tested. Using Nanex nanofibre scaffolds and serum free Nanex HSC expansion media and our standard panel of cytokines (100ng/ml Flt-3 ligand, SCF, IL-6 and 20ng/ml TPO), human UCB CD133⁺ cells were successfully expanded over 8 days. This assay demonstrated around 50 fold expansion of CD133⁺ cells on average, and an even greater expansion of CD34⁺ cells (approximately 100 fold) and TNCs. The addition of 100ng/ml IGFBP2 and Angptl5 factors further enhanced the number of CD133⁺ and CD34⁺ cells from UCB expanded over 8 days on the Nanex scaffolds to around 65 fold and 150 fold respectively. Both of these cultures yielded cells that not only retained CD133⁺CD34⁺ dual expression, but displayed migratory ability to CXCL12 in the 3D μ -slide

chemotaxis assay *in vitro* in preliminary experiments, although this was subject to variability and needs to be repeated further. Information on the expanded populations also needs to be collected in future experiments, in terms of the phenotype using markers of both SRCs and of haemopoietic lineage.

Had time not been constrained, additional steps would have been to investigate whether the cells that we had expanded retained engraftment capacity *in vivo*, as has been shown by other expansion conditions and factors (Boitano et al., 2010; Butler et al., 2012; Delaney et al., 2010a; Drake et al., 2011; Zhang et al., 2008). [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED].

6.2 Future work

The problems of increased graft failure and delays in engraftment exist with UCB compared to MPB or BM. Indeed, the numbers of viable nucleated cells and CD34⁺ cells collected from a single UCB unit and infused into patients is associated with the UCB graft success (Smith and Wagner, 2009). UCB cell numbers can be enhanced for transplantation by infusing multiple UCB units, however, studies demonstrate that

one UCB will 'win' or engraft in preference to the other unit (Kurtzberg, 2011). Although a greater number of CD34⁺ cells can be harvested from MPB donors, other factors, such as differential CXCR4 expression, could contribute to graft success (Smith and Wagner, 2009; Wagner et al., 2007). Cutler *et al.* recently demonstrated that human UCB CD34⁺ cells stimulated with dmPGE2 for 1-2 hours prior to injection into recipients showed improved engraftment capacity (Cutler et al., 2011). This was associated with upregulated CXCR4 gene and surface expression.

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Beyond this thesis, the 3D μ -slide chemotaxis assay provides an exciting tool for the investigation of the cellular response to chemokines, inhibitors and extra-cellular matrix components. Had time allowed, the 3D μ -slide chemotaxis assay would have been used as a tool for screening compounds that improve migration to CXCL12, and this could have included the use of dmPGE2 stimulated CD133⁺ cells. An *in vitro* tool such as this would reduce the use, time and expense of animal models when identifying such molecules. Future work should establish whether increased migratory ability in this assay correlates with improved *in vivo* function. Screening for stimulators of HSPC migration or donor sources of HSPCs which may have sub-optimal migration, could provide recipients with a better chance of successful engraftment.

This ties in with the Nanex scaffold expanded cells, which so far have only been tested for function in the chemotaxis assay. Future work should investigate whether the Nanex scaffolds, which provide serum and animal free expansion conditions, can expand human UCB CD133⁺ cells whilst maintaining their homing and longer term engraftment potential with additional cytokine combinations. [REDACTED]

[REDACTED] These assays could then be used to compare the engraftment potential of the expanded CD133⁺ cells to the equivalent dose of uncultured CD133⁺ cells, frozen at the time of isolation. Should this scaffold yield SRCs, these experiments could be scaled up and potentially produced under bioreactor conditions as described by Csaszar *et al.* to provide greater maintenance of expanded HSPCs (Csaszar et al., 2012).

Other future directions should consider whether expanding UCB cells with other factors such as StemRegenin, Notch ligands, or stimulators of other pathways, e.g. the Wnt pathway, can enhance the homing or shorten the time to haemopoietic reconstitution. Studies by Delaney *et al.*, where the Notch ligand delta1^{ext-IgG} was used with SCF, Flt-3 ligand, IL-6, IL-3 and TPO have expanded SRCs by 15 fold and in humans, shortened time to neutrophil engraftment from a median of 26 days to 16 days in a phase I trial, but have not shortened the time to platelet engraftment (Delaney et al., 2010a).

Finally, with regards to CD164 modulation, [REDACTED]

[REDACTED] the effect of CD164 blockade on CD133⁺ cell chemotaxis [REDACTED] using the monoclonal antibody 103B2 needs to be assessed. Such an approach has proved successful with other monoclonal antibodies, including those to CD9 and CD82 (Larochelle et al., 2012; Leung et al., 2011). Blockade of CD164 with a functional antibody could provide an answer as to whether CD164 has a role in controlling the cycling status of human CD133⁺ cells that home first to the spleen and then to the bone marrow of immune-deficient mice. Alternatively, if the effect of long term CD164 knockdown on HSPC bone marrow engraftment was to be studied, lentiviral vectors could be used. However, based on the experiments presented here with the pGIPZ shRNA, the use of other vectors would need to be considered.

Second generation self inactivating HIV-1 based lentiviral vectors pseudotyped with VSV-G have shown efficient transduction into UCB CD34⁺ cells (Guenechea et al., 2000; Liu et al., 2008; Uchida et al., 2011). Indeed, the eGFP lentiviral vector (from Professor Adrian Thrasher), used to successfully transduce HUVECs and endothelial

cells from UCB in Chapter 5, has been shown to transduce UCB CD34⁺ cells with a high efficiency (Demaision et al., 2002). Sub-cloning the shRNA targeting CD164 into this vector, as described by Montes *et al.* with a pRRL-GFP lentivector, for example (Montes et al., 2011), could provide a GFP tagged lentiviral vector targeting CD164 in the future. Alternatively, the pLKO.1 vectors encoding the CD164 shRNAs used in Chapter 4 have recently become available commercially (OpenBioSystems). The pLKO.1 vectors, although lacking a GFP reporter, may provide a better vector to investigate CD164 knock-down in HSPCs, as the shRNA are under the control of the human U6 promoter, rather than a cytomegalovirus (CMV) promoter as in the pGIPZ. The human U6 promoter has been shown to drive the expression of shRNAs with a 4 fold increase in knock-down efficiency compared to the murine U6 promoter in primary human CD34⁺ cells (Roelz et al., 2010). The U6 promoter has also been successfully used in the lentilox3.7 lentiviral vector, which also contained GFP, to transduce UCB CD34⁺ cells (Gupta et al., 2007). In the absence of obtaining this lentilox3.7 vector expressing GFP, eGFP could be excised from the Thrasher vector and cloned into the pLKO.1 lentiviral vector containing the CD164 shRNA as described by Wilber *et al.* (Wilber et al., 2011), or alternatively, by sub-cloning the CD164 shRNA into a pLKO.1 GFP vector, as successfully described for another shRNA into UCB CD34⁺ cells by Kranc *et al.* (Kranc et al., 2009).

6.3 Conclusion

To conclude, a novel chemotaxis assay has been developed which provides a method for analysing cell behaviour in the presence of any soluble chemokine, growth factor or inhibitor by enabling time-lapse tracking of individual cells over a stable gradient

for periods of time that correspond to *in vivo* homing assays. This assay could also be used to study preferable migration to conflicting gradients *in vitro*, as well the effects of HSPC expansion on migratory capacity. This was demonstrated here using CD133⁺ cells from UCB that were shown to expand over 8 days on Nanex scaffolds whilst retaining migratory function to CXCL12. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED] Further work needs to be performed to investigate the effects of HSPC manipulation by transfection and expansion on a nanofibre platform before these techniques could be considered for use in a clinical setting. Studies not presented here have shown that CD164 can be upregulated by standard 24 hour cytokine culture, and this could be a further area for investigation with regards *in vivo* homing and cell cycle status. The assays developed will therefore provide useful techniques that can be used in the laboratory for related or further studies.

Some of the text and figures originally presented in the above chapter has been redacted as it cannot be made freely available via ORA because of the sensitive nature of the work.

Appendix I. Titrating siRNA and shRNA oligos

The method described in section 2.6.1.1 was used to nucleofect the sequences into human UCB CD133⁺ cells using program U-008. The concentrations recommended by the manufacturers were tested in an initial experiment using shRNA (2-5 μ g). As 5 μ g CD164-B shRNA produced slightly more efficient nucleofection without adversely affecting viability (Figure I.I), this concentration was used for further experiments. Similarly, an initial experiment was performed using the siRNA using the manufacturers recommended concentration of siRNA (30-300nM) and that used previously by Forde *et al.* (Forde et al., 2007). The CD164-2 sequence was shown to effectively reduce CD164-2 expression at 1200nM and not below (Figure I.II).

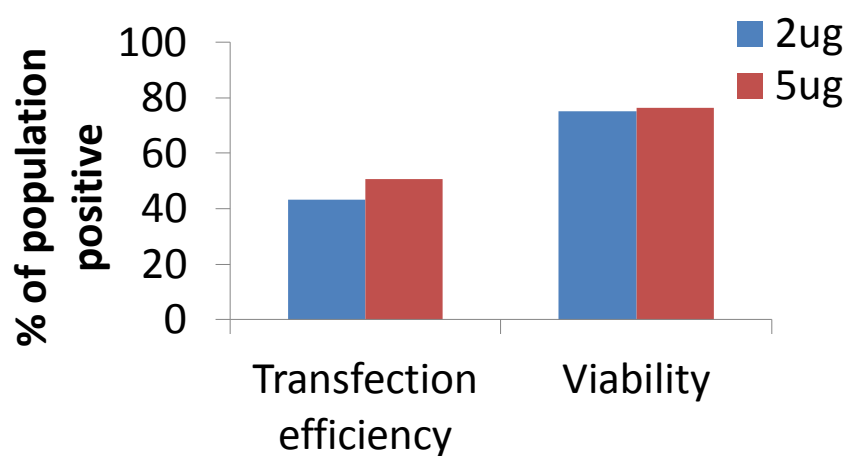


Figure I.I. Titration using CD164-B shRNA. Thawed and cultured CD133⁺ cells were nucleofected using program U-008 with either 2 μ g or 5 μ g non silencing or CD164-B shRNA (n=1). After 24 hours, cells were assessed by flow cytometry for nucleofection efficiency as determined by GFP expression and viability as assessed by DAPI or to-pro-3 negativity. The graphs represent CD164-B nucleofected cells only with the indicated amount of plasmid (n=1).

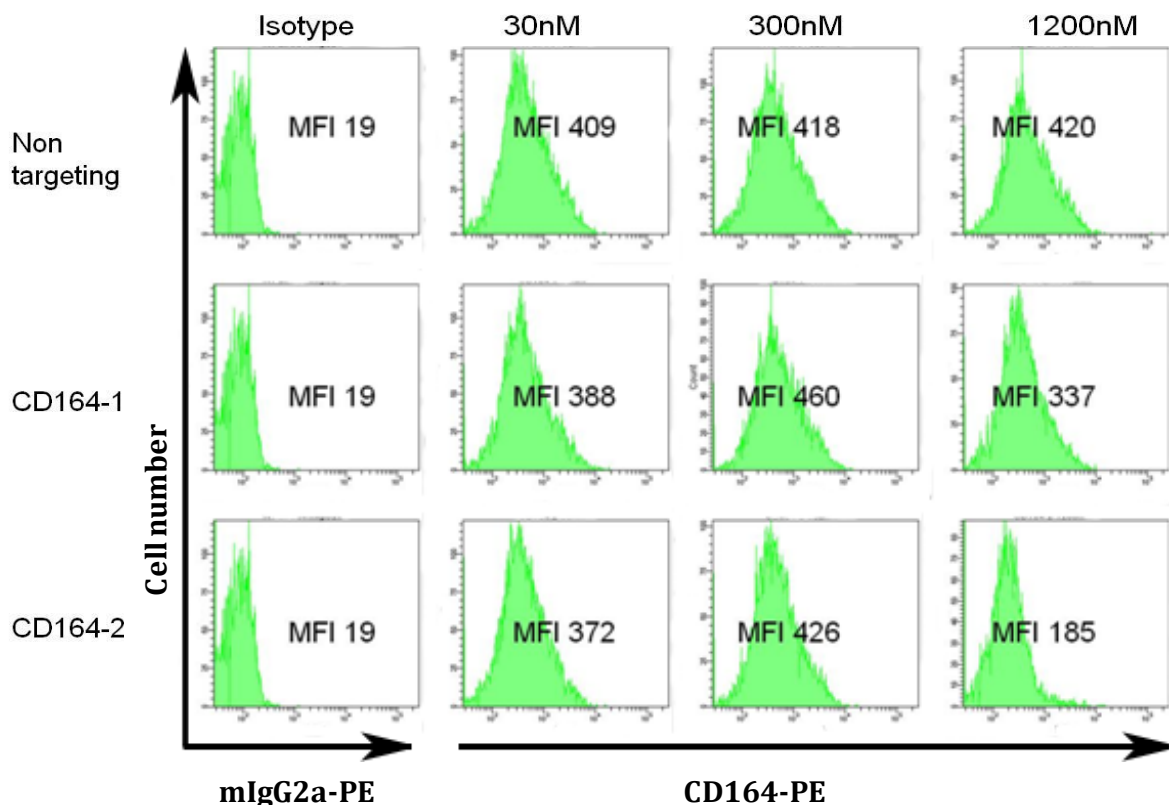


Figure I.II. Titration using siRNA oligo sequences. Concentrations ranging from 30nM-1200nM were tested for efficacy using the U-008 nucleofection program in human UCB CD133⁺ cells that had been isolated as described in section 2.2.2.3, thawed, and cultured for 1-2 hours in stemspan. Following nucleofection, the cells were cultured in stemspan medium with 100ng/ml SCF, Flt-3 ligand, IL6 and 20ng/ml TPO for 24 hours. CD164 surface expression was measured by taking a sample of the nucleofected cells and analysing by flow cytometry using the CD164 Mab N6B6. The most effective final concentration of siRNA was optimised and confirmed as 1200nM with the CD164- 2 sequence 24 hours post nucleofection. The CD164-1 sequence resulted in a lesser degree of knock-down to surface expression. Representative FACS histograms after the CD133⁺ cells were nucleofected with non-targeting siRNA (control), CD164-1 siRNA and CD164-2 siRNA at 30nM, 300nM and 1200nM final concentrations using program U-008 as indicated. mIgG2a-PE or CD164-PE expression is displayed as median fluorescence intensity (MFI) as measured by flow cytometry 24 hours post nucleofection (n=1).

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