

Trafficking of mesenchymal stem cells

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Declaration of Originality.

The content of this thesis has not previously been submitted for a degree at Imperial College London, or any other University. To the best of my knowledge this thesis contains no content previously published or written by another person, except where due acknowledgement is made within the text.

Abstract.

In adult life mesenchymal stem cells (MSCs) reside primarily in the bone marrow and are defined according to their ability to self-renew and differentiate into tissues of mesodermal origin. Due to their immuno-modulatory properties and ability to form cartilage and bone, MSCs have clinical potential, for the treatment of autoimmune diseases and tissue repair.

This project determines the chemokine receptor profile on murine bone marrow MSCs at early and late passage and on human MSCs derived from a range of fetal tissues including fetal blood, bone marrow, amniotic fluid and placenta. The overwhelming result from this analysis is the consistency across species and tissue source with respect to chemokine receptor profiles. In addition it is clear that expression of specific chemokine receptors defines sub-populations of MSCs. Currently, clinical trials using MSCs have relied on continued *in vitro* culture in order to obtain sufficient numbers for treatment. Here, MSCs have been shown to lose external chemokine receptor expression and associated chemotactic ability, whilst growing in size upon continued culture. All cultured MSCs investigated in this thesis were shown to be a heterogeneous population of stem cells and progenitors that contained 'true' MSCs within its number.

This thesis investigates a pharmacological approach to mobilise endogenous MSCs from the bone marrow, increasing their numbers in the blood. It has previously been reported that administration of VEGF-A over 4 days followed by a single dose with a CXCR4 antagonist (AMD3100) causes selective mobilisation of

MSCs into blood. The VEGF biology of this response has been interrogated. MSCs were shown to express high levels of VEGFR-1 and lower levels of VEGFR-2 on the cell surface but do not express VEGFR-3. By blocking VEGFR-1 with mAbs during VEGF-A¹⁶⁵ treatment, a ten-fold increase in MSC mobilisation in response to AMD3100 was recorded, while treating with VEGFR-2 blocking mAbs had no effect. Using VEGF isoforms specific for VEGFR-1 and VEGFR-2 (PlGF and VEGF-E respectively), it was determined that MSC mobilisation was dependant on activation of VEGFR-2 and not VEGFR-1.

PαS cells are a subset of MSCs found in the murine bone marrow that are PDGFRα+, Sca-1+, CD45-, Ter119-. Further characterisation of mobilised mMSCs by flow cytometric analysis of PαS cells, now provides a way to investigate the biology of MSCs, both in their steady state *in vivo* and in models of injury and inflammation. Molecular mechanisms lying downstream of VEGFR-2 have been explored and it has been shown that MMPs play a critical role in mobilisation. The use of drugs to mobilise MSCs into the blood may provide a cost effective, non-invasive treatment to promote tissue repair.

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List of Abbreviations.

ALS - Amyotrophic lateral sclerosis.

CFUs – Colony forming units.

DMEM - Dulbecco's modified eagle medium.

EPCs - Endothelial progenitor cells.

ES cells – Embryonic stem cells.

FACS – Fluorescence-activated cell sorting.

FCS – Fetal calf serum.

G-CSF - Granulocyte colony-stimulating factor.

hMSCs – Human mesenchymal stem cells.

HGF - Hepatocyte growth factor.

HPCs - Haematopoietic progenitor cells.

HSCs – Haematopoietic stem cells.

IDO - Indoleamine 2,3-dioxygenase.

IL – Interleukin.

i.p. – Intra-peritoneally.

iPS cells – Induced pluripotent stem cells.

ISCT – International society for cellular therapy.

i.t. – Intra-tracheally.

i.v. – Intra-venous.

MAPCs – Multipotent adult progenitor stem cells.

M-CSF – Macrophage colony-stimulating factor.

MFI – Mean fluorescence intensity.

MHC – Major histocompatibility complex.

MI – Myocardial infarction.

MIAMIs – Marrow-isolated adult multi-lineage inducible cells.

MPCs – Mesenchymal progenitor cells.

MSCs – Mesenchymal stem cells.

NK cells – Natural killer cells.

NO - Nitrous Oxide.

PDGF - Platelet derived growth factor.

PGE2 - Prostaglandin E2.

r.t. – Room temperature.

TGF- β - Transforming growth factor- β .

VEGF – Vascular endothelial growth factor.

VSELs – Very small embryonic-like cells.

1. Introduction.

1.1. Stem Cells.

The human body is constantly regenerating cells to replace those lost as a result of tissue damage or aging. Cells including erythrocytes, leukocytes, epithelial cells lining the gut and keratinocytes have a high rate of turnover as cells are constantly dying and tissue is being regenerated [1]. Organs rely on tissue specific adult stem and progenitor cells for tissue regeneration [2]. The most heavily researched of these stem cell populations are the haematopoietic stem cells (HSCs) which are multipotent cells capable of generating all blood cell types from the myeloid and lymphoid lineages, erythrocytes and platelets [3]. Autologous and allogeneic transplants of HSCs that are isolated from mobilised peripheral blood or from bone marrow have been used for >30 years clinically for bone marrow transplants [4,5,6,7]. Mesenchymal stem cells (MSCs) are a population of stem cells found predominantly in bone marrow and adipose tissue with the capacity to differentiate into adipose, bone and cartilage. MSCs are required for the maintenance of cartilage and bone and have been reported to promote regeneration of other tissues such as muscle [8,9,10], skin [11], lungs [12], heart [13], and more controversially, the nervous system [14,15] under homeostatic conditions when given i.v.

Embryonic Stem Cells (ES cells) are a pluripotent population of stem cells found in the inner cell mass of the early embryo [16,17,18]. ES cells possess the capacity to differentiate into all cell types of the adult body [19]. After long term culture *in vitro*, ES cells have been shown to maintain the developmental potential

to form derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) [16,20,21]. ES cells are capable of unlimited proliferation *in vitro* in their undifferentiated state [20]. Human ES cells express low levels of major histocompatibility complex (MHC)-I antigens, lack expression of MHC-II antigens and co-stimulatory molecules, are not recognized by natural killer cells and inhibit T-cell induced-stimulation by third-party antigen-presenting cells [22,23,24].

Generation of Induced pluripotent stem cells (iPS cells) was first reported by Takahashi and Yamanaka in 2006 [25]. iPS cells are created *in vitro* via the de-differentiation of adult cells into an undifferentiated state by upregulation of key genes, most commonly Oct4, Sox2, Klf4, and c-Myc [25]. This has been reported using several different methods of delivering these transgenes, including retroviral [26], lentiviral [27], transposon [28], Sendai virus [29], plasmid based [30], episomal plasmids [31], protein [32], minicircle vector [33], and mRNA [34,35]. iPS cells share many of the molecular, phenotypic and functional characteristics of embryonic stem cells (ES cells). Thus, they actively self-renew, proliferate, and divide at a rate equal to ES cells [36]. iPS cells express markers specific to ES cells, including SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, and Nanog [37]. Like ES cells, iPS cells express high telomerase activity to sustain self-renewal and proliferation [38]. In terms of pluripotency, iPS cells appear to be able to differentiate into all lineages including neural and cardiac differentiation [39].

MSCs, HSCs, ES cells and iPS cells are all potential candidates for use as stem cell therapies [40]. Whilst ES cells have the greatest potential in terms of

differentiation capacity and proliferation rate [41], there are ethical concerns about the cell source and evidence that ES cells in their undifferentiated state induce teratomas if injected i.v., as such they are used in their partially differentiated state. e.g. oligodendrocyte progenitors [10,42,43]. While in principle iPS cells could represent an alternative source of stem cells, at present technical issues are still being refined, for example, their efficiency of generation. Further, these cells are still undergoing rigorous characterisation at a molecular level. It has also been reported that abnormal gene expression in some cells differentiated from iPS cells can induce T-cell-dependent immune response in syngeneic recipients [44]. The immunogenicity of cells derived from patient-specific iPS cells should be further evaluated before they are used clinically. HSCs are currently used clinically to treat genetic blood disorders and leukaemia, and are currently being used in clinical trials with the aim of treating HIV [45,46].

1.2 Mesenchymal Stem Cells

While bone marrow and adipose contain large numbers of MSCs, they have also been isolated from nearly all human tissues [47]. These stem cells are multipotent and are therefore more restricted than ES cells with respect to the number of tissues they can differentiate into. However, the accessibility of MSCs combined with their rapid proliferation rate *in vitro* make them ideal candidates for cellular therapies on a clinical scale [48,49]. MSCs are a good alternative to ES cells for use clinically to treat specific diseases, as they lack the ethical problems

associated with ES cells. MSCs are currently being used for both autologous and allogeneic transplants due to their immuno-modulatory capacity [13,50].

The term mesenchymal stem cell was first termed by Caplan in 1991 to describe cells responsible for bone and cartilage formation in the embryo, and repair these tissues in the adult [51]. There had been previous studies investigating the transplantation of bone marrow to heterotopic anatomical sites, which resulted in *de novo* generation of ectopic bone and marrow [52]. Friedenstein *et al* went on to show that the osteogenic potential was associated with a minor sub-population of bone marrow cells [53]. These cells demonstrated rapid adherence to plastic tissue culture flasks and the formation of colonies generated from a single cell (colony-forming units, CFU-Fs). The original naming of MSCs was based on the hypothesis that they could differentiate into multiple cell types, beyond skeletal lineages [54]. However, this non-skeletal potential is yet to be consistently demonstrated either *in vitro* or *in vivo* [55,56]. The initial definition of MSCs referred to bone marrow derived stromal cells [53]. Further research has now led to the term being used to describe cell populations derived from other adult tissues [57]. The International Society for Cellular Therapy (ICST) established three minimal criteria for defining multipotent stromal stem populations as true MSCs. Human MSCs (hMSCs) must:

- (i) Be plastic-adherent when maintained in standard culture conditions,
- (ii) Express CD105, CD73, CD90 and lack CD45, CD34, CD14, or CD11b, CD79 α , or Cd19 and HLA-DR,
- (iii) Differentiate into osteoblasts, adipocytes and chondrocytes [58,59].

1.2.1. Sources of MSCs.

hMSCs were originally identified in postnatal bone marrow [53]. MSCs have since been isolated from many adult tissues. Commonly researched populations include adipose [60], dental pulp [61], umbilical cord blood [62], liver [63], lung [64], spleen [65], placenta [66], and amniotic fluid [67]. In order to isolate MSCs from solid tissues, enzymatic treatment with collagenase is required [68]. Bone marrow MSCs can also be harvested in this way [68]. Identification of MSCs in umbilical cord blood prompted research into the presence of MSCs in early fetal life [62]. MSCs are closely associated with haematopoietic stem cells (HSCs) in adult bone marrow. High levels of HPCs in first trimester fetal blood [69] suggested that due to their role in development, the frequency of stem cells in fetal tissue may be greater than that of adult human tissue [70]. As it is known that the number of MSCs in adult tissues decreases with advanced age, it is hypothesised that fetal tissue may be a better source for isolating large numbers of MSCs [71].

MSCs have not been consistently isolated from circulating adult blood [72], and yet they are found in highly vascularised organs. It has been proposed that there is a relationship between MSCs and pericytes, which support small vessels and hence are present in all vascularised organs [73]. It is also possible that pericytes are derived from MSCs, although this is yet to be proven. Importantly, not all MSCs can be defined as pericytes as it has been reported that within the bone marrow exists a population of MSCs which stain negative for pericyte markers such as NG2 [74].

Whilst cell populations derived from mouse brain, spleen, kidney glomeruli, liver, muscle, lung, bone marrow, pancreas and thymus all fit the required criteria to

be defined as MSCs, da Silva-Meirelles *et al* demonstrated that there was some variation in the tri-lineage differentiation capacity between each population. These differences involved the frequency of cells which actually differentiated in to the osteogenic or adipogenic phenotype, as well as on the degree of differentiation [72]. Furthermore, there have been reports of differences in marker expression of MSCs derived from different sources. For example, adipose tissue MSCs have been shown to express the required markers to define MSCs in addition to further markers which are not expressed on bone marrow derived hMSCs including CD49d [75], CD62e and CD31 [60]. It has been hypothesised that these differences are due to the influence of the local environment [76]. These findings are clinically relevant, in that MSC populations derived from different sources may be more suited to treating damage to the tissue from which they originate than cells isolated from other sources. Whilst the differentiation capacity of MSC populations from different sources has been investigated, to date very little research has been dedicated to comparing the functional differences of MSCs in terms of migratory ability, proliferative ability, and engraftment rate *in vivo*. One critical feature of an MSC population to be used in clinical trials is the ease by which MSCs can be harvested. Ideally, MSCs should be sourced from an ethically non-controversial and easily accessible tissue. The source must be rich in MSCs, which can be harvested with as little impact on patients as possible. For most fetal and adult tissues, including adult bone marrow, MSC collection represents an invasive procedure with a potential risk of infection. This has led to extensive research into umbilical cord blood [77,78,79]. Whilst banking of bone marrow and umbilical cord blood derived MSCs is becoming more common,

there are few facilities for storing MSCs from sources which would otherwise go unused, such as liposuction adipose and amniotic fluid from amniocentesis.

Stem cell therapies would ideally utilise stem cell populations with pluripotent differentiation and self renewal capacities, whilst not being tumourogenic. Fetal MSCs from 1st trimester blood and bone marrow fit these criteria, and have been reported to display several advantageous properties over their adult counterparts. Fetal blood and bone marrow derived MSCs express pluripotent markers such as Oct-4, SSEA-3, SSEA-4 and Nanog, which are not expressed on adult blood or bone marrow derived MSCs [80]. They also elicit a greater proliferative capacity and maintain longer telomeres during repeated passage than their adult counterparts [80]. Availability of MSCs from human fetal blood and bone marrow may be limited due to ethical objections. The human term placenta, a temporary organ with fetal contributions that is discarded post-partum has been shown to host a set of MSCs which exhibit many markers that are common to adult bone marrow derived MSCs [66,81]. These are an ethically uncontroversial and easily accessible source of MSCs for research and clinical purposes. Amniotic fluid has also been shown to be an abundant source of fetal MSCs. Amniotic fluid-derived MSCs are readily available from samples taken for amniocentesis and were first isolated from 2nd trimester amniotic fluid [82]. Further research into 1st trimester amniotic fluid-derived MSCs showed that they have a greater proven differentiation capacity than adult bone marrow derived MSCs, with the ability to differentiate into adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages. These cells were also shown to express ES cell markers, such as Oct-4 and SSEA-4 [83].

1.2.1.1. MSC Niche.

It was originally reported that MSCs reside around the microvasculature of their tissue of origin. This was determined via identification of MSCs using the human antibody STRO-1 [84]. However, STRO-1 is not exclusive to MSCs and its expression in hMSCs is gradually lost

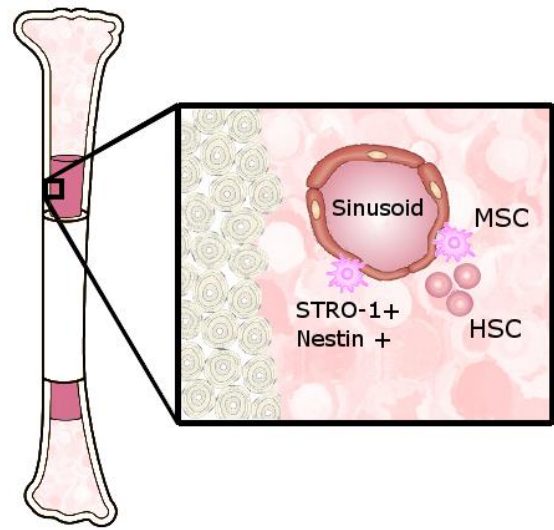


Fig. 1.1. Perivascular localisation of the MSC niche.

during culture expansion [85,86]. Within murine bone marrow, a sub-population of nestin+ MSCs has been identified which also occupy a perivascular localisation (Fig1.1). Nestin+ MSCs have also been shown to act as the functional components of the HSC niche [87,88], suggesting that the function and phenotype of MSCs may depend on their microenvironment/tissue of origin. MSCs support HSC homeostasis and have anti-proliferative features that are in common with physiological stromal niches [89].

1.2.2. Isolation of MSCs.

To date, there have been no minimal criteria for defining mMSCs [90]. For this reason, researchers investigating mMSCs tend to define mMSCs as murine cells which fit the criteria for hMSCs [91,92]. However, certain markers such as Sca-1,

have been identified as a positive marker for mMSCs but are not expressed on hMSCs as there is no human homologue. Conversely, Stro-1, a hMSC marker has no mouse counterpart. There is variation between mouse strains in the cell surface epitopes and *in vitro* differentiation capacities. mMSCs do not differentiate down the chondrogenic lineage as readily as hMSCs [93,94]. It has also been reported that mMSCs are karyotypically very unstable in culture, whereas human MSC are not [95,96,97]. By their nature, cell populations which can be classified as mMSCs in this way are heterogeneous when cultured *in vitro* [10]. Whilst human bone marrow stromal populations lose all CD45+ haematopoietic cell contamination after one passage *in vitro*, mMSCs require multiple passages (≥ 5) in order to generate a population of cells which can be defined as MSCs when isolated by plastic adherence alone [98]. Different laboratories have developed unique methods to isolate and expand the MSCs in culture. These varying isolation and culture techniques lead to differences in the phenotypic and functional characteristics of mMSCs [99].

MSCs make up a minor fraction of the cells in the bone marrow and other tissues [91]. Their rarity makes them difficult to isolate or identify *in vivo*. Studies in man have shown that the frequency of MSCs in bone marrow declines with age (1×10^{-4} of nucleated marrow cells in newborns compared to 1×10^{-6} in an 80 year old) [100]. Due to their rarity it is proposed that in order to use the cells as an efficient cellular therapy, MSCs must be isolated and expanded *in vitro*. hMSCs can be expanded *in vitro* to 1×10^7 cells from a 20ml bone marrow aspirate in a matter of weeks [101,102]. To date, there are few published results that assess how MSC numbers are related to their functional effects *in vivo*. However, if the beneficial effects of MSCs in damaged tissue are a result of factors produced by the MSCs, it

can be assumed that MSCs used as a cellular therapy will act in a dose dependent manner.

As MSCs are generally isolated from the bone marrow by plastic adherence, they are contaminated by haematopoietic cells, particularly macrophages. In order to purify populations of mMSCs which are uncontaminated with cells of the haematopoietic lineage at early passage, several research groups have outlined methodology for selectively sorting CD45-, CD34- and CD11b- populations using fluorescence-activated cell sorting (FACS) [103] or magnetic beads [104] to enrich the MSC levels in the populations. The density at which the cells are seeded on tissue culture flasks has also been identified as a variable which affects the heterogeneity of the cell population when isolating MSCs via plastic adherence alone. High density seeded bone marrow populations remain contaminated with CD45+ cells for longer periods of culture than low density seeded populations [105]. Different research groups also use different culture media in an attempt to enhance the proliferative rate of the MSCs and consequently get larger numbers in a shorter time, a critical consideration when wanting to perform autologous transplantations. Standard culture media for both human and murine MSCs such as DMEM, is supplemented with 10% fetal calf serum (FCS) which has some variation between batches. It has also been shown that increasing the percentage of FCS in culture media can increase the proliferative capacity of the cells [106]. Other specific media is available commercially where added factors are not defined. Efforts are currently being directed toward developing animal serum free media for cell culture purposes in order to remove any threat of disease. [107,108]. This variation in methodology for generating pure populations of MSCs and culture conditions between

laboratories creates a potential problem when comparing results between labs where MSCs are clearly distinct populations. The result of this can be seen in Table 1.1, which lists the discrepancies in surface marker profiles from different publications.

Surface Antigen	Human MSCs			Murine MSCs			References
	+	+/-	-	+	+/-	-	
Stro-1	8	1	0	0	0	0	[84,86,109,110,111,112,113,114]
CD13	5	0	0	1	0	1	[60,113,115,116,117,118,119]
CD29	5	0	1	12	0	0	[60,72,113,115,116,117,119,120]
CD44	11	0	0	10	1	0	[60,72,84,113,115,116,117,119,121]
CD73	5	0	0	0	0	0	[112,113,115,116,122]
CD105	7	0	2	2	0	0	[60,112,113,114,115,116,117,120,122]
CD106	5	0	3	4	1	0	[60,86,112,113,114,115,117,118,123]
CD11b	0	0	10	0	1	5	[60,84,115,117,119,123]
CD31	0	3	10	0	0	6	[60,84,113,115,116,117,118,119,121,123]
CD34	1	1	11	5	6	4	[60,72,84,113,115,116,117,118,120,121,123]
CD45	0	0	3	0	0	7	[60,84,113,115,116,117,118,120,121,123]
CD117	0	2	0	1	1	13	[60,72,84,115,117,118,119,123]
Sca-1	0	0	5	6	5	4	[72,117,119,123]
CD10	6	0	1	0	1	0	[60,113,116,117,118]
CD90	11	1	1	2	4	10	[72,84,115,116,117,118,119,121,123]
VEGFR-2	2	1	1	1	0	5	[84,118,123,124]

Table 1.1. Expression of MSC surface markers in different studies. (Adapted from [85])

Differences in human and murine bone marrow MSC populations may also be due to collection methods. Human bone marrow aspirate is most often harvested from the superior ileac crest and only a small volume is taken [125]. Murine bone marrow is most commonly harvested by flushing the complete bone marrow from femurs and tibias [53]. Due to difference in isolation methods between research groups, various publications have reported discrepancies in the expression of each of these non specific markers [126]. The heterogeneity of cultured MSCs is further

demonstrated by the set of markers which can be used to define them. Thus, very often positively expressed markers are not expressed constitutively on all cells within a population. Rather a sub-population of cells stain positively for said markers [127].

1.2.2.1. Phenotypic changes in MSCs upon culture.

It is apparent that culture-expanded MSCs differ from their *in vivo* progeny, as proliferation on plastic surfaces induces both phenotypic and functional changes [78,128,129]. This may be due to changes in cell function lending towards proliferation, at the same time reducing the capacity of MSCs to migrate. Due to a lack of specific MSC markers, MSCs are generally isolated via their ability to adhere to plastic, which involves repeated passage [130,131]. Upon long term culture, MSCs may be phenotypically and functionally altered, and not necessarily a true representation of MSCs in their natural state *in vivo* [41,132]. For example, MSCs have been reported to grow in size upon culture on plastic tissue culture flasks [133]. It has been reported that hMSCs at passage 2 express CXCR4, CXCR5, CXCR6, CCR1, CCR7 and CCR9 on the cell surface. However, when analysed at passage 12 – 16, hMSCs express very low levels or none of these chemokine receptors on the surface. These late passage hMSCs also lack the functional capacity to migrate towards the ligands of these receptors *in vitro* [134,135]. Moreover, down regulation of chemokine receptor expression is accompanied by a decrease in the expression of adhesion molecules such as ICAM-1, ICAM-2, VCAM-1 and CD157. These phenotypic changes have been shown to be associated with increasing cell cycle arrest and induction of the apoptotic pathway [76].

Currently, identification of MSCs is dependent on a combination of markers as there is no single marker that identifies this subset, as is the case for HSCs [136]. Identification of a specific marker for MSCs would enable pure populations of MSCs to be isolated without the need for expansion on plastic. Research in this area has proposed single markers such as SSEA-1 [137], SSEA-4 [138], Stro-1 [109] and the low affinity nerve factor receptor (CD271) [139] as specific markers for MSC populations within the human bone marrow. However, they have not been taken up by the scientific community at large. Research investigating the properties of these MSC populations has suggested that the bone marrow contains many sub-populations of stem or progenitor cells, each of which can be defined as MSCs [140,141].

1.2.3. Sub-populations.

Sub-populations of bone marrow derived MSCs are often identified by surface markers, clonality or homogeneous differentiation potential. Characterised populations include human multipotent adult progenitor cells (MAPCs), human marrow isolated adult multi-lineage inducible (MIAMI) cells, and very small embryonic like stem (VSEL) cells, which have been identified in mice and man. Murine nestin⁺ bone marrow cells have also been shown to be MSCs, which form a unique bone marrow niche with HSCs made of heterotypic stem-cell pairs [87]. MAPCs are identified via CD45 and Ter119 depletion along with selection via analysis in culture, including morphology and high Oct-4 mRNA levels. Whilst this set of cells has been highly researched and shown to have many favourable characteristics for

clinical therapy, they cannot be easily identified *in vivo*. This is also the case for MIAMIs, which are isolated due to their ability to form CFU-Fs *in vitro* [142]. VSELs are CD45⁻, Lin⁻, Sca-1⁺ cells in murine bone marrow and thus, can be sorted via fluorescence activated cell sorting (FACS). VSELs are very small in relation to other cells in the bone marrow population, significantly smaller than HSCs (3.63 + 0.69 versus 6.54 + 0.17µm in diameter) and express Oct-4, a marker of embryonic stem cells [143]. Differences in the phenotype of these sub-populations is characterised in table 1.2.

Cell Type	Species	Marker expression
MAPCs	Human	SSEA-1 ⁺ , CD13 ⁺ , VEGFR-2 ^{low} , Thy-1 ^{low} , CD34 ⁻ , CD44 ⁻ , CD45 ⁻ , CD117(c-kit) ⁻ , MHC I ⁻ , MHC II ⁻
MIAMIs	Human	CD29 ⁺ , CD63 ⁺ , CD81 ⁺ , CD122 ⁺ , CD164 ⁺ , c-met ⁺ , BMPR1B ⁺ , NTRK3 ⁺ , CD34 ⁻ , CD36 ⁻ , CD45 ⁻ , CD117 (c-kit) ⁻ , HLA-DR ⁻
VSELs	Mouse/ Human	CXCR4 ⁺ , AC133 ⁺ , CD34 ⁺ , SSEA-1 ⁺ (mouse), SSEA-4 ⁺ (human), AP ⁺ , c-Met ⁺ , LIF-R ⁺ , CD45 ⁻ , Lin ⁻ , HLA-DR ⁻ , MHC I ⁻ , CD90 ⁻ , CD29 ⁻ , CD105 ⁻

Table 1.2. Phenotype of MSC sub-populations. (adapted from [142])

Morikawa *et al* reported a further methodology for prospectively identifying and isolating MSCs from adult murine bone marrow based on marker expression alone, using flow cytometry [144]. This MSC population, termed PαS cells, are isolated as PDGFR-α⁺, Sca-1⁺, CD45⁻, Ter119⁻ bone marrow cells. As is true for hMSCs, mMSCs do not express many common surface antigens that are found on haematopoietic and endothelial cells. These include Ter119, an erythroid lineage marker [145], and CD45, a haematopoietic marker [123]. Both PDGFR-α and PDGFR-β are expressed by mesenchymal cells and the PDGF signalling pathway has been shown to be important in promoting survival and proliferation in mesenchymal cells

[146,147]. Several reports have shown that the tri-lineage potential of cells isolated based on Sca-1 expression alone [148,149,150]. Unfortunately, Sca-1 expression varies between mouse strains [98]. However, Morikawa *et al* demonstrated that P α S cells can be found in different mouse strains including DBA-1, C57BL/6 and BALB/c mice and that the level of Sca-1 expression was similar between all strains tested [124]. This sorted population gives rise to MSCs that are capable of *in vivo* engraftment into both the bone marrow and adipose tissue of mice, when transplanted systemically. P α S cells can also generate mesenchymal and neural crest lineage cells with or without *in vitro* expansion [144]. The identification of this marker combination enables *in vivo* examination of MSCs by flow cytometry. MSCs were found to be predominantly located in the arterial perivascular space near the bone adjacent to the vascular smooth muscle cells (vSMCs) [124]. A fundamental problem with *in vitro* culture of MSCs is their increase in size, causing them to become entrapped in the lungs when administered i.v. [130]. P α S cells expanded *in vitro* were also found to become entrapped in the lungs when injected i.v. [124]. This may be due to an increase in the size of the P α S cells upon 2D culture on plastic. P α S cells *in vivo* are very small relative to the rest of the cells in the bone marrow [124].

1.2.4. Differentiation Potential.

By definition MSCs must possess tri-lineage differentiation capacity, in that they must be able to differentiate into osteoblasts, adipocytes and chondrocytes

when cultured under set conditions *in vitro* [59]. There are reports that MSCs can differentiate into additional cell types of mesodermal origin such as skeletal muscle [139], smooth muscle [151] and endothelial cells [152]. More controversial is the differentiation into endodermal and neuroectodermal cell types such as cardiomyocytes [153], neurons and hepatocytes [154,155].

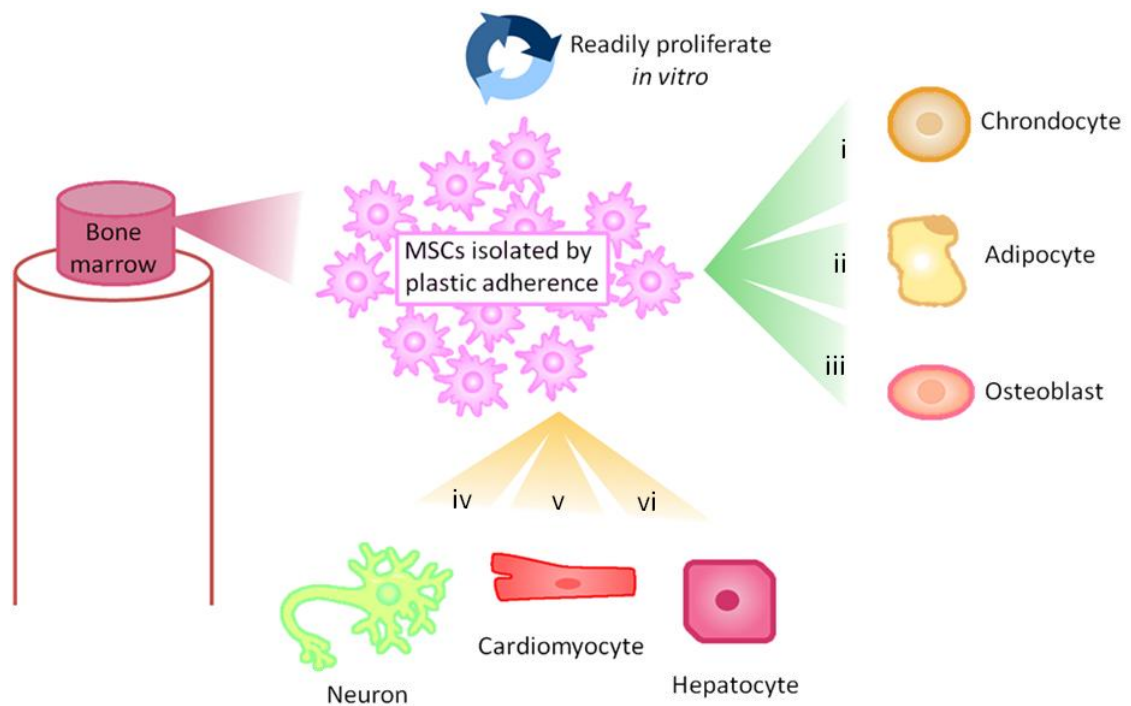


Fig. 1.2. Differentiation capacity of MSCs isolated by plastic adherence.

Fig 1.2 shows the published differentiation potential of bone marrow derived MSCs. Points i, ii, and iii show the classic defining differentiation potential of bone marrow derived MSCs down the chondrogenic, adipogenic and osteogenic lineages, respectively. Points iv, v and vi, show cell types which MSCs have been reported to differentiate into under set conditions *in vitro*, including neurons [156], cardiomyocytes [157] and hepatocytes [158].

Transdifferentiation describes the ability of MSCs to differentiate into other cell lineages as well as cells originating from other germ layers [125]. Under various

defined culture conditions, MSCs have been reported to exhibit transdifferentiation plasticity (Fig. 1.2). Transdifferentiation of MSCs into neurons has generated much interest due to the range of potential applications for currently untreatable neurological disorders and degenerative disorders [125]. *In vitro* differentiation of MSC into cells displaying neuronal characteristics has also been reported [155,159]. MSCs when injected directly into neonatal mouse brain, have been reported to differentiate into a neuronal phenotype [160]. This transdifferentiation is a topic of debate as it has been reported that the MSCs are transforming due to cell fusion [161] or contamination by other cell types and MSC sub-populations such as multipotent adult progenitor cells (MAPCs) [118], or due to cytotoxic changes induced by the media and staining procedures [162]. Much research is currently focussed on driving the MSCs down defined differentiation pathways *in vitro*, so that progenitor cells for the tissue of interest can be administered for specific diseases [163,164].

1.2.5. Immunosuppression.

In addition to their ability to differentiate, there are now a large number of studies reporting the immuno-modulatory effects of MSCs [165,166,167]. Following i.v. administration, MSCs can induce T-cell peripheral tolerance, home to inflamed tissues and exert a potent tissue-protective effect through the release of anti-inflammatory and anti-apoptotic molecules [49,89]. The wide range of factors and the roles that they play is summarised in Fig 1.3. This demonstrates why MSCs are currently being researched as candidates to treat a broad range of autoimmune and

inflammatory diseases. The known immuno-modulatory properties of MSCs increases the scope of diseases which can be potentially resolved using MSC therapy.

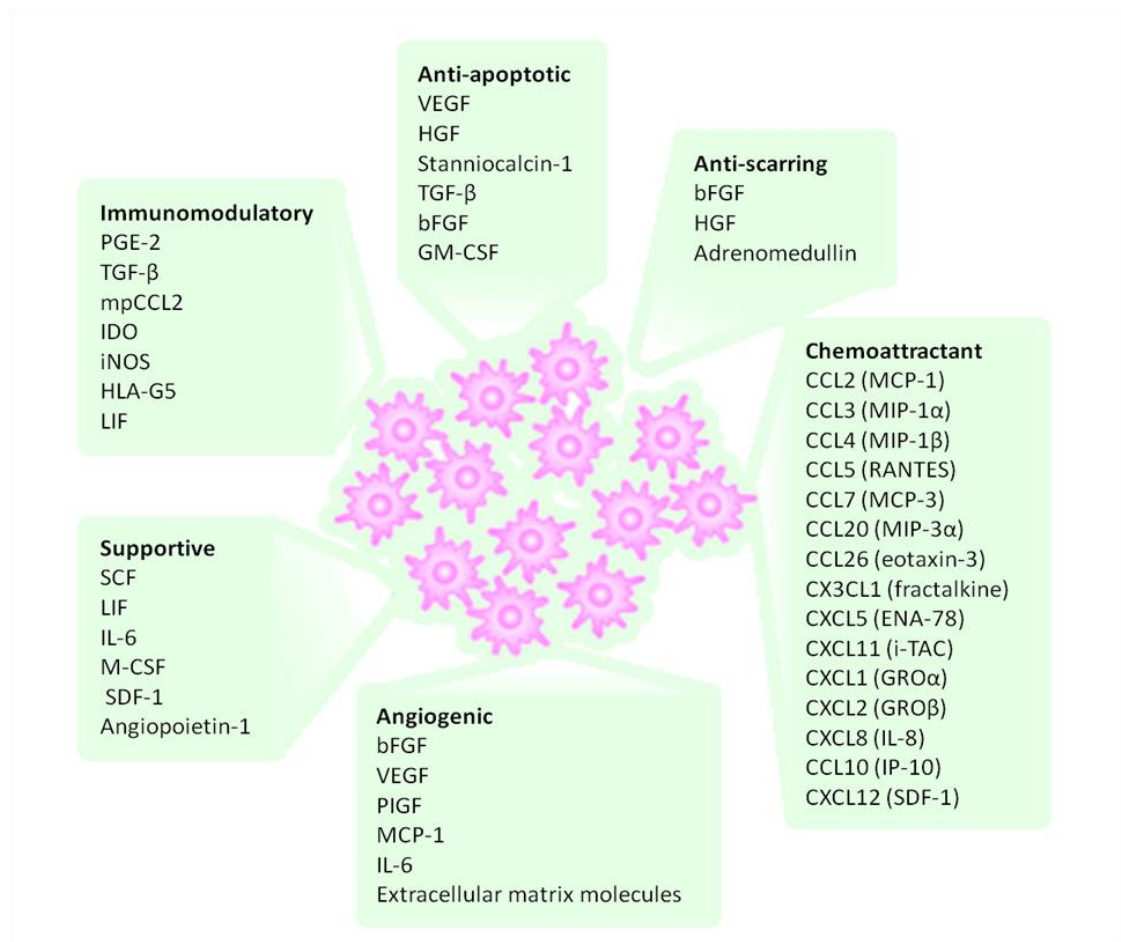


Fig 1.3. Factors secreted by MSCs in culture.

Cultured MSCs express low levels of MHC Class I and are MHC Class II–, CD40–, and CD86–, suggesting these cells would not elicit an immune response [168,169]. The immuno-modulatory capacities of MSCs have been demonstrated by several independent research groups, both *in vitro* and *in vivo*, in animal models and in humans [89,166,170]. The immuno-modulatory functions of MSCs were first demonstrated in their ability to suppress *in vitro* proliferation of T lymphocytes [171]. Since then, several studies have shown that MSCs can modulate many cell

types involved in the immune response *in vitro*. These include natural killer (NK) cells, B lymphocytes and dendritic cells [89,166,170]. The inhibition of T cell proliferation has been shown to be transient, whereby the removal of MSCs reinstates the T cell proliferative state [170]. T cell proliferation inhibition by MSCs is also known to act independently of cell-cell contact with the suppressive effect attributed to the secretion of either Transforming growth factor- β (TGF- β), Hepatocyte growth factor (HGF), Prostaglandin E₂ (PGE₂), Indoleamine 2,3-dioxygenase (IDO), Nitrous Oxide (NO) or Interleukin-10 (IL-10) [172,173]. This inhibition of proliferation is observed in both autologous and allogeneic responder cells [174]. By demonstrating that this action is not HLA restricted, MSCs are shown to be a good candidate therapy for use allogeneically [175]. MSCs interfere with functioning, differentiation and maturation of dendritic cells. Again, this is not dependent on cell-cell contact with the MSCs, but on soluble factors secreted by the MSCs including IL-6, IL-10, macrophage colony-stimulating factor (M-CSF) and PGE₂ [176]. The inhibition of B cells by MSCs is dependent on inflammatory conditions [177]. MSCs in the presence of IFN γ reduce B cell proliferation via the induction of IDO [178]. To inhibit NK cell proliferation, MSCs have been shown to require activation with either IL-2 or IL-15 [179,180].

As with much of the MSC functional analysis to date, the immunosuppressive mechanisms by which MSCs exert their effect have been established *in vitro*. However, it is not yet confirmed that these pathways of immunosuppression are conserved *in vivo* [181].

1.3. Stem Cell Therapies.

To date, iPS cells are yet to be used in clinical trials, ES cells have begun phase 1 clinical trials, and there are multiple reports of completed trials using MSCs [182].

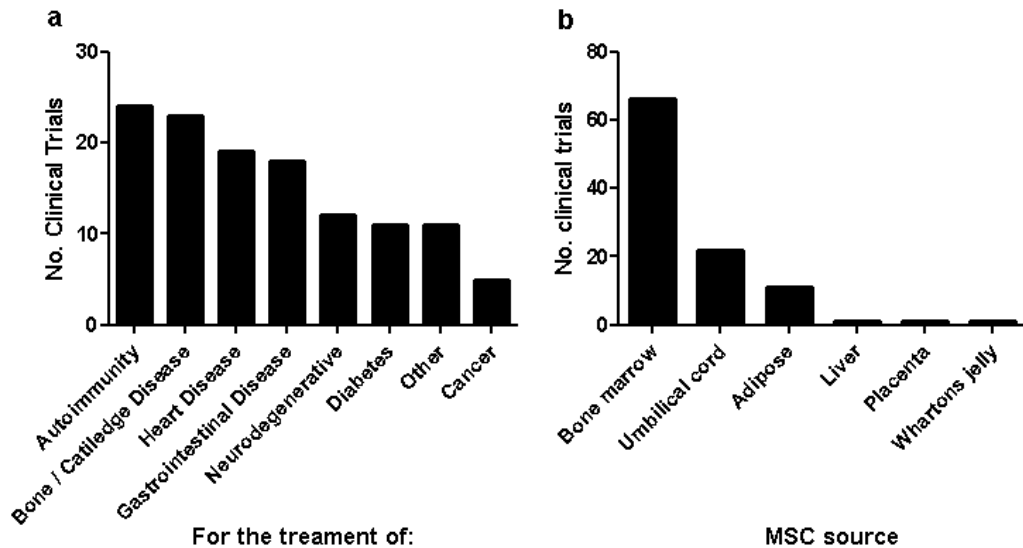


Fig. 1.4. Clinical trials using MSCs as a cell therapy.

The number of clinical trials currently listed on clinicaltrials.gov using MSCs is shown in Fig. 1.4, sorted depending on what disease or injury the MSCs are being used to treat (Fig. 1.4.a. adapted from [58]), or the source of the MSCs used in the clinical trials (Fig. 1.4.b). Generated by analysis of data on clinicaltrials.gov.

Currently 46% of MSC based cellular therapies utilise patients own bone marrow cells and 54% use allogeneic transplants (Fig. 1.5). Techniques currently employed to raise MSC numbers rely on *ex vivo* expansion, which is associated with practical, technical and regulatory hurdles. While none of these issues are insurmountable, the final cost of such a therapy will be reflective of this [72,183]. Such therapies have shown some success in phase II trials for the treatment of acute graft versus host disease (GvHD) and there are currently >170 clinical trials

registered on clinicaltrials.gov using MSCs to treat conditions including ischemic tissue (e.g. ischemic heart disease and critical limb ischemia in diabetic patients), autoimmune diseases (e.g. aGvHD, multiple sclerosis and ulcerative colitis) and to promote bone or cartilage regeneration (e.g. in the context of osteogenesis imperfecta and arthritis). Results for trials attempting to repair the damage caused by heart disease using MSCs have been inconsistent, though recent trials have shown a small but significant improvement in heart function [184].

In other areas of medicine, early clinical trials attempted to repair damage to bone caused by diseases such as osteogenesis imperfecta, or to speed up recovery from bone fractures. These trials relied on the ability of MSCs to differentiate into osteoblasts under set conditions [185]. Despite initially encouraging results, subsequent *in vivo* studies in both mouse

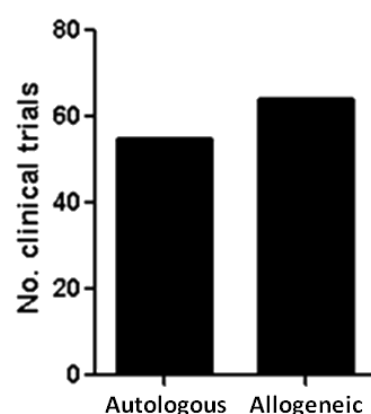


Fig. 1.5. Autologous vs. Allogeneic MSCs used in clinical trials to date.

and man have shown that MSCs typically exhibit low levels of engraftment and transdifferentiation within injured or diseased tissue and therefore do not contribute physically to tissue regeneration to a significant extent [186,187,188]. These findings initially cast doubt on the prospect of harnessing MSC plasticity to treat disease [54]. However, observations that the therapeutic application of MSCs resulted in reduced inflammation, apoptosis and fibrosis, led to trials designed to capitalise on the ability of the cells to suppress immune reactions and prevent tissue remodelling [189]. As MSCs appear to be immunologically inert [190], the numbers of allogeneic MSC transplants currently outweighs that of autologous transplants in

MSC clinical trials (Fig. 1.5). However there are some concerns about the administration of allogeneic MSCs, as reports have suggested that delivery to an inflamed site can result in gain of immune potency with accelerated damage due to a heightened immune mediated inflammatory response [191].

MSCs tend to be administered systemically. This is both less invasive and more convenient for multiple dosing regimens. One problem associated with systemic administration is reports of extremely low engraftment (<1%) at sites of injury or inflammation, and corresponding high levels of cell entrapment in the liver, spleen and lungs [186,192]. MSCs become entrapped in the lungs due to their large size and expression of cell surface adhesion receptors [193]. Despite the low engraftment rate and the complications caused by entrapment of cells in the lungs, numerous animal studies have reported a positive outcome following MSC administration i.v. [194,195]. As a result of this, in an attempt to improve endpoints further, MSCs administered during clinical trials are usually applied in doses of hundreds of millions for each infusion [189,192], or directly at sites of injury (e.g. Injected directly into myocardium following myocardial infarction (MI)). However, little research has been carried out investigating whether the beneficial effects of MSCs are dose dependent.

Clinical trials looking to exploit the immuno-modulatory qualities of MSCs include the treatment of amyotrophic lateral sclerosis (ALS), in which the MSCs are thought to improve the microenvironment for motor neurons, reducing cell degeneration rate [196]. In type 1 diabetes clinical trials, MSCs act by modulating the auto immune action and subsequent destruction or dysfunction of insulin producing beta cells in the pancreas [197].

Alternative approaches include the culture of MSCs *in vitro* under set conditions to generate replacement tissue *ex vivo*, or the genetic modification of MSCs to act as vehicles for treatments which will be recruited to sites of injury and inflammation. Recently, there have been reports of replacement tracheas built on decellularised or artificial scaffolds using chondrogenic progenitors derived from autologous bone marrow derived MSCs [198]. Researchers are now investigating the potential for culturing other tubular organs in this manner, as well as optimising current techniques to make the process more efficient [199]. Progress into artificial regeneration of other organs remains highly experimental as researchers still look to create decellularised tissue or suitable artificial scaffolds on which to seed the MSCs [200,201]. MSCs have been shown to home to tumour sites and could potentially act as a shield and vehicle for a tumouricidal gene therapy vector. To date, a number of anti-cancer genes have been inserted into MSCs which resulted in anti-cancer effects on various carcinoma models when transplanted *in vivo*, including TRAIL on breast cancer [202], glioma [203,204] and lung cancer [205], IFN- α on melanoma [206], IFN- β on pancreatic cancer [207], IFN- γ on leukaemia [208], IL-2 on glioma [209], IL-12 on melanoma and hepatoma [210], and NK4 on lung cancer [211].

Until recently, pharmacological methods to harness the regenerative capacity of MSCs have concentrated on mobilisation or directing cell fate *in vivo* [120,212]. Osteoporosis occurs due to a lack of bone mineral density. There are attempts to manipulate the fate of MSCs in the bone marrow (using statins) in order to drive the MSCs along an osteoblast differentiation pathway, thus increasing the numbers of bone forming cells in the affected area [213].

1.4. Chemokines and their receptors.

Chemokines (chemotactic cytokines) are small proteins which direct the movement of circulating leukocytes to sites of inflammation or injury. In addition to effects on cell locomotion, certain chemokines are capable of eliciting a variety of other responses affecting leukocyte adhesion, activation, degranulation, mitogenesis, and apoptosis [214]. There is evidence that MSCs respond to chemotactic signalling in order to home to sites of injury and inflammation [215,216]. Chemokine receptors and their ligands play an important role in tissue-specific homing of leukocytes and have also been implicated in trafficking of HPCs into tissues [217,218]. HPCs migrate to sites of injury and inflammation [219]. Under steady state, HPC populations are confined within the bone marrow, with basal release into the circulating blood. This retention is a result of constitutively produced CXCL12 binding to CXCR4 [220,221]. The CXCR4 antagonist AMD3100 inhibits CXCL12/CXCR4 binding, thus allowing the mobilisation of HPCs [218,220,221].

Systematically transplanted MSCs have been shown to preferentially home to sites of injury, where they support functional recovery [134,222,223]. This infers that MSCs also possess a migratory capacity. In terms of chemokine receptor expression, studies examining MSCs harvested from human bone marrow have reported conflicting data with respect to the profile of chemokine receptors expressed [134,224]. Due to variation in culture conditions and the known heterogeneity of MSC cultures, it is likely that differences in chemokine receptor

expression profiles are a reflection of these variables. There is also a suggestion that chemokine receptor profiles differ between human and murine MSC populations *in vitro*. To date hMSCs have been reported to express CXCR1, CXCR3, CXCR4, CXCR5, CXCR6, CX3CR1, CCR1, CCR3, CCR5, CCR7, CCR9, CCR10, when analysed at passage 2-9 [225]. mMSCs at passage 7-9 were shown to not express CCR3, CCR5, CCR7, CXCR4 or CXCR5 on the cell surface [134]. There is a growing body of evidence reporting on chemokine and chemokine receptor dependent modulation of MSC migration and proliferation [226,227,228,229,230]. In a murine model of MI, MSCs injected i.v, were shown to traffic to the heart in a CXCR4-dependent manner, with enhanced recruitment reported in mice where expression of CXCL12 was induced via the instillation of an adenoviral vector expressing CXCL12 into the myocardium [231]. Further, it has been shown that recruitment of MSCs to primary breast tumours was stimulated by CCL2 and it was subsequently shown that at a molecular level MSC migration via CCR2 was dependent on FROUNT [232,233]. Interestingly, MSCs also constitutively produce chemokines [229] (Fig. 1.3). There is also evidence that the proliferative capacity of MSCs is inversely linked to their ability to migrate [234]. It is important to investigate the mechanisms by which MSCs efficiently home to sites of injury and inflammation.

1.4.1. CXCL12.

The chemokine CXCL12 was first identified as Stromal Derived Factor (SDF)-1 α in the supernatant of bone marrow stromal cells [235,236]. It has since been shown to be expressed constitutively at high levels in the bone marrow. These high

levels are produced by osteoblasts, endothelial cells and reticular cells within the bone marrow stroma [237]. More recent studies have reported that CXCL12 levels in the bone marrow are regulated centrally by the sympathetic nervous system, noradrenalin acting via $\beta 2$ adrenoreceptors on osteoblasts and $\beta 3$ adrenoreceptors on nestin⁺ MSCs to reduce their production of CXCL12 [87,238]. There are two known chemokine receptors for CXCL12, CXCR4 and CXCR7 [239,240,241]. CXCR4 interacts with a range of signalling molecules and pathways and stimulates chemotaxis of leukocytes and stem cells. In contrast CXCR7 has a very high affinity for CXCL12, but does not couple to the signalling pathways that drive migration [242]. On binding to CXCR7, CXCL12 is internalised and subsequently degraded, thus CXCR7 appears to act as a sink for CXCL12 [243].

1.5. Stem Cell Mobilisation.

The bone marrow is a reservoir of progenitor cells including MSCs, HPCs, fibrocytes, and endothelial progenitor cells (EPCs). In response to disease or tissue injury, these cells are mobilised from the bone marrow and recruited into tissues where they contribute either to disease progression or tissue repair [244,245]. Infusion of HSCs and cultured EPCs has been shown to augment neovascularisation of tissue following ischemia and contribute to re-endothelialisation after injury [246]. HPC mobilising agents have had a profound impact in the field of haematological transplantation [217]. For over 30 years granulocyte colony-stimulating factor (G-CSF) and more recently CXCR4 antagonists, specifically

AMD3100, have been used to mobilise HPCs from the bone marrow into the blood so that they can be collected in a non-invasive manner in order to repopulate the bone marrow following radiotherapy [247]. A combination of G-CSF and CXCR4 antagonist treatment results in a synergistic mobilisation of HPCs [248].

It has been proposed that MSCs present in the bone marrow may act remotely to promote tissue repair. Hypoxia has been reported to be sufficient to increase circulating MSC levels in rats [249]. The effect of hypoxia is dependent on activation of HIF-1 α and thought to be due to increased levels of vascular endothelial growth factor (VEGF)-A¹⁶⁵ in the bone marrow and CXCL12 in the blood [249]. One study has reported an increase in circulating numbers of MSCs in the blood of patients with severe burns [250]. The detection of circulating MSCs in response to tissue injury suggest they can be mobilised from their niches upon appropriate signals and that they therefore target damaged tissue [250]. G-CSF has been classically applied to mobilise HSCs from the bone marrow which can then be easily isolated from peripheral blood. Although the protective effect of G-CSF in diseases such as stroke and ischemia has been proven [251,252], its capacity to mobilise MSCs into the peripheral blood is controversial [253,254]. Recent studies in mice have shown that G-CSF stimulated the proliferation of MSCs in the bone marrow as a result of an indirect effect of G-CSF, causing osteoclast resorption [120,255]. Direct mobilisation of MSCs from bone marrow after the unique combination of VEGF-A¹⁶⁵ and CXCR4 antagonist therapy has been reported, which suggests that particular molecular mechanisms do exist and can be exploited to boost MSC egress from the bone marrow [120].

The CXCR4 antagonist used in this set of experiments was AMD3100. The CXCL12/CXCR4 axis has been shown to be critical in the retention of HPCs, EPCs and MSCs following 4 day VEGF-A¹⁶⁵ treatment. Disrupting this axis using AMD3100 following VEGF-A¹⁶⁵ treatment, results in the mobilisation of EPCs and MSCs into the blood. VEGF-A¹⁶⁵ treatment alone results in no mobilisation [120]. Administration of VEGF-A¹⁶⁵ stimulates the entry of HPCs into the S/G2/M phase of the cell cycle *in vivo*, severely impairing their migratory capacity. Indeed, AMD3100 does not mobilise HPCs in mice pretreated with VEGF-A¹⁶⁵ [120]. VEGF-A¹⁶⁵ treatment does not stimulate the proliferation of EPCs. Therefore, EPCs are still mobilised in response to CXCR4 antagonism. In terms of G-CSF this pre-conditioning disrupts the CXCL12/CXCR4 retention pathway, while it is currently not known how VEGF-A¹⁶⁵ pre-treatment augments the mobilisation of EPCs and MSCs in response to AMD3100. A greater understanding of the molecular mechanisms that induce or control their mobilisation will help develop more efficacious therapies for MSC mobilisation.

The introduction of new mobilisation regimes such as this may also have detrimental effects under certain conditions such as cancer. Indeed, recruitment of vascular progenitors by tumours is observed in patients and drives the progression of cancer through increased tumour blood vessel formation in animal models [255].

1.6. VEGF.

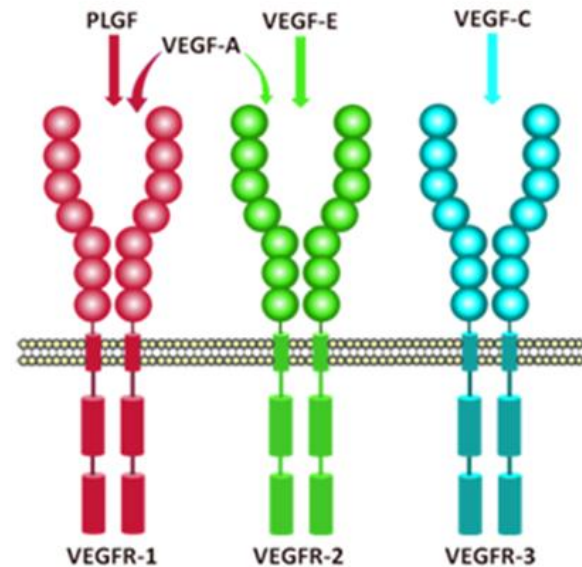
VEGF exists in several isoforms, the most commonly researched of which is VEGF-A¹⁶⁵. VEGF-A¹⁶⁵ plays a critical role in blood vessel formation and the migration of endothelial cells [256]. VEGF-A¹⁶⁵ was used in Pitchford *et al*'s experiments which first demonstrated MSC mobilisation, in order to assess its ability to mobilise EPCs [257]. Previously, it had been reported that it was necessary to treat mice with VEGF-A¹⁶⁵ for 4 days before EPCs were seen in the blood [257]. Treatment of immunosuppressed mice with a blocking mAb VEGFR-1 blocking mAb reduces cell cycling of HPCs and the survival of HSCs following engraftment [258,259]. This suggests that endogenous VEGF-A¹⁶⁵ acting via VEGFR-1 regulates the proliferation and survival of HPCs and HSCs in the bone marrow [258]. Pitchford *et al* showed that selective blockade of VEGFR-1 abolishes the ability of exogenous VEGF-A¹⁶⁵ to stimulate HPC entry into the cell cycle *in vivo* and restores the ability of CXCR4 antagonists to mobilise HPCs from the bone marrow. VEGF-A¹⁶⁵ promotes survival and stimulates the migration of endothelial cells through VEGFR-2 signalling [260]. The differential effects of VEGF-A¹⁶⁵ in regulating HPC and EPC mobilisation are mediated via VEGFR-1 for HPCs and VEGFR-2 for EPCs. The ability of VEGF-A¹⁶⁵ to differentially promote the cell cycling of HPCs as compared to EPCs explains why EPCs can be selectively mobilised by the CXCR4 antagonist in mice treated over 4 days with VEGF-A¹⁶⁵ [120].

The mobilisation of stem and progenitor cells out of the bone marrow is thought to be a multi-step process. The cells are released from their respective niches within the bone marrow, followed by migration through the bone marrow stroma to the sinusoidal endothelium [261]. In addition to its potent angiogenic functions VEGF-A¹⁶⁵ can stimulate proliferation, delay senescence, suppress

apoptosis and promote survival of various cells [262]. VEGF-A¹⁶⁵ has also been shown to decrease MSC expression of cell cycle inhibitors such as p16INK and p21, and increase MSC proliferation rates, and hence VEGF-A¹⁶⁵ can mitigate culture-induced stress of MSCs [263].

There are 3 known subtypes of VEGFRs, VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1) and VEGFR-3 (Flt-4) [264]. VEGFR-1 promotes the survival and proliferation of haematopoietic cells whilst VEGFR-2 promotes the proliferation of endothelial cells [265] and VEGFR-3 is involved in lymphangiogenesis

Fig.1.6. VEGFRs and their ligands.



[266]. With regards to mMSC mobilisation via VEGF-A¹⁶⁵ and AMD3100 treatment, Kolonin and Simmons state that it is likely VEGF-A¹⁶⁵ is exerting an indirect effect as MSCs do not express VEGFRs [255], however contradicting studies have shown MSCs to express both VEGFR-1 [267,268] and VEGFR-2 [124,268]. Various VEGF isoforms exist including VEGF-A¹⁶⁵, placental growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D [269]. VEGF related proteins have also been discovered including VEGF-E from the parapoxvirus Orf Virus [270] and VEGF-F in snake venom [262,271]. In particular, PLGF has been shown to act specifically on VEGFR-1 [271] and VEGF-E is known to activate VEGFR-2 (Fig. 1.6.) [262].

In cancer stem cells, which share many of the defining characteristics of MSCs, an autocrine positive loop exists where VEGF-A¹⁶⁵ binds to VEGFR-1 and

VEGFR-2 resulting in the release of matrix metalloprotease (MMP)-2 and MMP-9 [272]. Migration of leukocytes through extra-cellular matrix is facilitated by ECM-degrading enzymes such as MMPs, and thus they may be playing a critical role in the mobilisation of MSCs [214]. The role of MMPs in MSC mobilisation have yet to be investigated.

1.7. Hypothesis and Aims.

Trafficking of endogenous MSCs involves their mobilisation from the bone marrow and chemokine-dependent recruitment into tissues.

The main aims are:

1. To define a reproducible methodology for isolating a pure population of mMSCs at low passage number in order to characterise them in terms of chemokine receptor expression and determine the effects of continued *in vitro* culture on chemokine receptor expression levels.
2. To compare chemokine receptor expression on hMSCs derived from a range of fetal tissues.
3. To further characterise CD45⁻, Ter119⁻, PDGFR- α ⁺, Sca-1⁺ (P α S) cells as a sub-population of homogeneous murine bone marrow derived MSCs.
4. To optimise previously reported selective mobilisation regimes for MSCs in order to enhance mobilisation.

5. To explore alternative methods of mobilising endogenous MSCs and further interrogate existing regimes in order to elucidate the molecular mechanisms by which MSCs are released into the blood.

2. Materials and Methods.

2. Materials and Methods.

2.1. Materials.

General laboratory chemicals and cell-culture reagents were purchased from either Invitrogen (Paisley, United Kingdom) or Sigma-Aldrich (Poole, United Kingdom). Recombinant murine chemokines/cytokines IFN- γ , TNF- α , and CXCL12 were purchased from PeproTech EC (London, United Kingdom). The CXCR4 antagonist AMD3100 was obtained from Sigma-Aldrich (Poole, United Kingdom). FACS antibodies were purchased from the following companies; anti-mCXCR3 PE, anti-mCCR10 PE, anti-mCXCR4 PE, anti-mCXCR2 PE, anti-mCCR10 PE, anti-mSca-1 PE, anti-mCCR9 FITC, anti-mCXCR2 APC, anti-mCXCR6 PE, anti-mCCR3 FITC, anti-mCCR6 FITC, anti-mCD105 PE – R & D Systems (Abingdon, UK); anti-mCCR1 PE – Santa Cruz Biotechnologies (Santa Cruz, USA); anti-mCXCR4 PE, Fc-block, anti-mCD45 PE, anti-mCD11b PE – BD Biosciences (Oxford, UK); anti-mCD45 FITC, anti-mCXCR4 FITC, anti-mCD140a APC – BD Pharmingen (Becton, USA); anti-mCCR7 APC, anti-mVEGFR-1 APC - ebioscience (San Diego, USA).

2.2. Animals.

Female BALB/c mice were purchased from Harlan. Mice were used at the age of 3-30 weeks. UK Home Office guidelines for animal welfare, based on the Animals (Scientific Procedures) Act of 1986 were strictly observed.

2.3. Statistical Analysis.

Data are expressed as mean $\pm$ SEM. Data were analysed using paired t-tests or one-way analysis of variance (ANOVA), followed by Bonferroni multiple-comparisons test. All analyses were conducted using the GraphPad Prism statistical package (version 4.0; Graph Pad, San Diego, CA). P values less than 0.05 were considered significant. P < 0.05 denotes *, P < 0.01 denotes **, P < 0.001 denotes ***.

2.4. Cell isolation and culture.

2.4.1. Harvest of murine bone marrow MSCs.

Femurs and tibias were isolated from mouse hind legs (m=10). The bone marrow was then flushed from the femurs and tibias with 2ml of DMEM +10% FCS per mouse, using a syringe. The cell suspension was then centrifuged at 1200rpm for 5 minutes at room temperature (r.t.). The pellet was re-suspended in Mesencult (Stem Cell Technologies) and cells were incubated at 37°C, 5% CO₂. Half of the media was replaced after 3 days. After 1 week of culture, the cells became confluent and were passaged and reseeded into new T75 flasks. These were designated passage 1 (P1) cells.

2.4.2. Human fetal blood derived MSC isolation.

Fetal blood collection was approved by the Research Ethics Committee of Hammersmith & Queen Charlotte's Hospital in accordance with the Declaration of

Helsinki. Blood (gestation 10 weeks and 4 days) was collected by cardiocentesis under ultrasound guidance. Cells were selected by adherence, cultured in DMEM–high glucose (Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (BioSera, Ringmer, United Kingdom), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Invitrogen), and expanded at 10 000 cells/cm² at 37°C with 5% CO₂.

2.4.3. Human fetal bone marrow derived MSC isolation.

Bone marrow samples were obtained from 4 fetuses (median gestational age, 13 weeks). Single-cell suspensions of fetal bone marrow were prepared by flushing the bone marrow cells out of the humeri and femurs using a syringe and 22-gauge needle into DMEM (Sigma-Aldrich, United Kingdom) supplemented with 10% fetal bovine serum (FBS) (Stem Cells Technology, Vancouver BC, Canada), 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco BRL, Life Technologies, Paisley, United Kingdom). Fetal liver samples were taken from 4 fetuses (median gestational age, 11 weeks). Single-cell suspensions were prepared by mincing the organ through a 70-mm nylon filter (Becton Dickinson, United Kingdom), and cells were re-suspended as for the bone marrow samples.

2.4.4. Human placenta derived MSC isolation.

Human second-trimester placenta tissue was obtained after informed consent from 10 women undergoing socially indicated termination of pregnancy at a mean gestational age (GA) of 19 weeks.

2.4.5. Human 1st and 2nd trimester amniotic fluid-derived MSC isolation.

Amniotic fluid was derived from human first-trimester and second-trimester pregnancies after informed consent. Amniotic fluid was collected by ultrasound guided transabdominal puncture using a 22-gauge spinal needle (Vygon, Ecouen, France).

2.4.6. Tracheal fibroblast culture.

Murine tracheas were minced to pieces of around 1 to 2 mm³. The minced pieces were transferred to eppendorfs and washed in PBS by vigorous mixing with a vortex mixer. The excess supernatant was removed and the pieces were re-suspended in DMEM + 10% FCS and plated in T75 tissue culture flasks. The flasks were incubated at 37°C, 5% CO₂. Half of the media was replaced after 3 days. Upon reaching confluence, the fibroblasts were passaged and reseeded into new T75 flasks. These were designated passage 1 (P1) cells.

2.5. FACS analysis.

2.5.1. Flow cytometry analysis of MSC marker and chemokine receptor expression on mMSCs and hMSCs.

To perform flow cytometric (FACS) analysis the cells were detached by trypsinisation, and were allowed to recover for 2hrs at 37°C in culture media before staining. Cells were then re-suspended in FACS buffer (PBS, 1% Bovine Serum Albumin (BSA), 0.1% sodium azide; 5×10^5 cells/100µl FACS-buffer), pre-incubated with murine Fc block (rat anti-mouse CD16/32 mAb) for 10min at 4°C and then stained with specific antibodies for 20 min at 4°C in the dark. For intracellular FACS staining cells were fixed in 2% formaldehyde for 20 min at rt, washed once in FACS buffer and then re-suspended in Saponin buffer at 4°C (PBS, 1% Bovine Serum Albumin (BSA), 0.1% sodium azide, 0.5% saponin). Fc block and specific antibody stains were performed in Saponin buffer. Afterwards cells were washed and re-suspended in FACS-buffer. Relative chemokine receptor expression intensity levels were measured using MFI levels recorded on CellQuest software.

2.5.2. PαS cell identification and mobilisation.

For bone marrow PαS staining [124], femurs and tibias were isolated from mouse hind legs. The bone marrow was then flushed from the femurs and tibias of mice with 2ml of DMEM +10% FCS per mouse, using a syringe. The cell suspension was then centrifuged at 1200rpm for 5 minutes at room temperature (r.t.). The excess supernatant was removed and the pellet was re-suspended in FACS buffer (PBS, 1% Bovine Serum Albumin (BSA), 0.1% sodium azide; 5×10^5 cells/100µl FACS-buffer)[145]. Cardiac bleeds were taken from treated and control mice, grouped in 2

for each experiment. RBCs were lysed using ACK buffer (0.15 M ammonium chloride, 1 M potassium hydrogen carbonate and 0.01 mM EDTA, pH 7.2) for 4 minutes at room temperature. The cell suspension was then centrifuged at 1200rpm for 5 minutes at room temperature (r.t.). The excess supernatant was removed and the pellet was re-suspended in FACS buffer. Cells were pre-incubated with murine Fc block (rat anti-mouse CD16/32 mAb) for 10 min and then stained with the following antibodies for 20 min in the dark: allophycocyanin (APC) anti-mouse CD140a (PDGFR α), fluorescein isothiocyanate (FITC) anti-mouse CD45, fluorescein isothiocyanate (FITC), anti-mouse TER-119 (ebioscience), phycoerythrin (PE)-conjugated monoclonal anti-mouse Sca-1 (R&D Systems). Afterwards cells were washed and re-suspended in FACS-buffer. All samples were run on a BD FACSaria cell sorter (BD Biosciences). Gates were defined as positive for PDGFR α and Sca-1 and negative for CD45 and TER119, according to the isotype control fluorescence intensity.

2.5.3. P α S cells from collagenase treated bone marrow.

Femurs and tibias were dissected and crushed with a pestle. For collagenase treated experiments, bone and bone marrow fragments were incubated for 1h at 37°C in 0.2% collagenase (Wako)/DMEM (Gibco) containing 1% Penicillin-Streptomycin (P/S). Cell suspensions were collected and bone and bone marrow fragments were crushed. The suspension was filtered with a cell strainer, and collected by centrifugation at 1200rpm for 7 min at 4°C. Both collagenase-treated or -untreated bone marrow cells were collected in the pellet and soaked in 1 ml water (Sigma) for

5–10 seconds to burst red blood cells, after which 10ml of DMEM + 10% FCS was added, and the suspension was filtered through a cell strainer [144]. The excess supernatant was removed and the pellet was re-suspended in FACS buffer. Cells were pre-incubated with murine Fc block (rat anti-mouse CD16/32 mAb) for 10 min and then stained with the following antibodies for 20 min in the dark: allophycocyanin (APC) anti-mouse CD140a (PDGFR α), fluorescein isothiocyanate (FITC) anti-mouse CD45, fluorescein isothiocyanate (FITC), anti-mouse TER-119 (ebioscience), phycoerythrin (PE)-conjugated monoclonal anti-mouse Sca-1 (R&D Systems). Afterwards cells were washed and re-suspended in FACS-buffer. All samples were run on a BD FACSaria cell sorter (BD Biosciences). Gates were defined as positive for PDGFR α and Sca-1 and negative for CD45 and TER119, according to the isotype control fluorescence intensity.

2.5.4. Expression of markers and chemokine receptors on PaS cells.

Specific markers and chemokine receptors were examined on PaS cells by staining the cell populations with the following antibodies for 20 min in the dark: allophycocyanin (APC) anti-mouse Sca-1, fluorescein isothiocyanate (FITC) anti-mouse CD45, fluorescein isothiocyanate (FITC), anti-mouse TER-119 (ebioscience), PE-Cy7 anti-mouse CD140a (PDGFR α), and all other markers of interest stained with phycoerythrin (PE)-conjugated mAbs.

2.5.5. Size comparison of uncultured and cultured PaS cells.

P α S cells in uncultured bone marrow and P25 bone marrow derived mMSCs were compared by staining with the following antibodies for 20 min in the dark: allophycocyanin (APC) anti-mouse CD140a (PDGFR α), fluorescein isothiocyanate (FITC) anti-mouse CD45, fluorescein isothiocyanate (FITC), anti-mouse TER-119 (ebioscience), phycoerythrin (PE)-conjugated monoclonal anti-mouse Sca-1 (R&D Systems). Cells gated as CD45-, Ter-119-, PDGFR α +, Sca-1+ were then compared using forward and side scatter.

2.5.6. Cell cycle analysis of P α S cells using TO-PRO.

Cells were stained for P α S cell markers as described above. The cells were then fixed using ice cold ethanol added drop-wise. Cells were incubated in 50 μ l of TO-PRO (100 μ g/ml), and 50 μ l RNase (100 μ g/ml) before running the cells on a BD FACSaria cell sorter (BD Biosciences). Histograms of the gated cells showed the first peak to represent cells in G0/G1 and subsequent peaks represent actively dividing cells.

2.6. Differentiation of MSCs.

2.6.1. *In vitro* differentiation of MSCs.

To induce differentiation of MSCs into adipogenic and osteogenic lineages, differentiation media was added to confluent MSC cultures. A standard differentiation media containing 10% FBS, penicillin/streptomycin and fungizone was used, to which specific compounds were then added. For osteogenic differentiation, 50 μ g/ml ascorbic acid-2-phosphate, 10nM dexamethasone and 10mM β -glycerol

were added to the standard media. Adipogenic differentiation was induced by adding 50µg/ml indomethacin, 100nM dexamethasone and 10ng/ml insulin to the standard. The micromass culture technique was used to induce chondrogenic differentiation. Confluent MSC cultures were trypsinised and 2×10^5 cells were pelleted in a 15ml falcon tube. To the basic media, 10ng/ml TGF-β, 50nM ascorbic acid-2-phosphate and 6.25µg/ml insulin were added. The chondrogenic differentiation media was changed every 3 days for 3 weeks. The media were replaced every 3 days for 3 weeks.

2.6.2. Staining of differentiated MSCs.

To confirm differentiation of MSCs into osteocytes, adipocytes and chondrocytes, the cell populations were stained with cell type specific dyes as reported in previous publications [115]. MSCs cultured in osteogenic media were stained with Alizarin red (Sigma-Aldrich). This dye is used to demonstrate the presence of calcium. Interaction of Alizarin dye with calcium ions results in a bright red stain. MSCs cultured in adipogenic media were stained with Oil red O (Sigma-Aldrich) which is a fat soluble dye that stains triglycerides and lipids of fixed cells with a deep red colour. Cells cultured in chondroinductive media were stained using Toluidine Blue, which stains the background blue (orthochromatic staining) and the areas of cartilage matrix red-purple (metachromatic staining). Slides were mounted using Histomount mounting medium (VWR International Ltd.). Images were obtained using a Nikon DMX1200 digital camera and processed using LUCIA software (Jasc® Software Inc.).

2.7. Mobilisation of endogenous MSCs into the blood.

2.7.1. CFU-MSC assay.

5×10^5 cells were added to Mesencult media including supplements (Stemcell Technologies) in 30mm petri dishes. Dishes were incubated for 7 days before media was changed, and then incubated for a further 14 days before the enumeration of MSC colonies. Colonies were defined as containing ≥ 30 cells and counted at X40 magnification.

2.7.2. Bone marrow colony assays.

1×10^5 cells are added to Mesencult media including supplements (Stemcell Technologies). Dishes are incubated for 7 days before media is changed, and then incubated for a further 14 days before the enumeration of MSC colonies.

2.7.3. Mobilisation of mMSCs via VEGF-A¹⁶⁵ + AMD3100 treatment.

Eight- to ten-week old female BALB/c mice (Harlan, Oxford, UK) were administered VEGF-A¹⁶⁵ (2.5 μ g/mouse, 1.25 μ g/mouse, or 0.625 μ g/mouse i.p.)(PeproTech) or vehicle on 4 consecutive days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100, 5 mg/kg i.p) or vehicle and blood was collected via cardiac puncture 60 min later for enumeration of circulating MSC levels.

2.7.4. Repeated VEGF-A¹⁶⁵ + AMD3100 administration.

Mice in group A were pre-treated with vehicle (PBS) once daily for 9 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Mice in group B were pre-treated with VEGF-A¹⁶⁵ once daily for 4 days (100µg/kg i.p.). On day 5 they received a CXCR4 antagonist (AMD3100 5mg/kg i.p.). On days 6-9 they were treated with vehicle (PBS) once daily. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Mice in group C were pre-treated with VEGF-A¹⁶⁵ once daily for 4 days (100µg/kg i.p.). On day 5 they received a CXCR4 antagonist (AMD3100 5mg/kg i.p.). On days 6-9 they received further treatment with VEGF-A¹⁶⁵ once daily (100µg/kg i.p.) Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Blood was taken for analysis of circulating MSCs 60 minutes post final AMD3100 administration. MSC numbers are shown as number of colonies per ml blood.

2.7.5. MSC mobilisation following treatment with VEGF-A¹⁶⁵ + CCL27, CXCL11 or PDGF.

Eight to ten-week old female BALB/c mice (Harlan, Oxford, UK) were administered VEGF (2.5 µg/mouse i.p.) or vehicle on 4 consecutive days. Twenty-four hours after the last injection, mice were administered a vehicle (PBS), CCL27, CXCL11 or PDGF (250µl 50nM i.v.) or a CXCR4 antagonist (AMD3100 5mg/kg i.p.) and blood was collected via cardiac puncture either 1 hour, 6 hours, or 24 hours later for enumeration of circulating MSC levels.

2.7.6. VEGF-A¹⁶⁵ + CXCL12 challenge.

Mice were pre-treated with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.), CXCL12 (50nM, 200µl, i.v.), or vehicle (PBS, 200µl, i.v.). Sixty minutes later, blood was taken for analysis of circulating MSCs.

2.7.7. Administration of Anti-VEGFR1 + anti-VEGFR2 Antibodies.

Mice were pre-treated with VEGF for 4 days, as previously described. On days 1 and 3, mice were administered anti-VEGFR1, anti-VEGFR2, a combination of anti-VEGFR1 + anti-VEGFR2, or control IgG (2.5 mg/kg i.p.) [273] 30 min before VEGF administration. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100, 5 mg/kg i.p) or vehicle and blood was collected via cardiac puncture 60 min later for enumeration of circulating MSC levels.

2.7.8. Treatment with different VEGF isoforms and PlGF.

Mice were treated with 100µg/kg of VEGF-A¹⁶⁵, PlGF or 1000µg/kg VEGF-E, or vehicle (i.p.) on 4 consecutive days. On day 5 mice were administered AMD3100 (5mg/kg, i.p.) or vehicle. Cardiac bleeds were taken 60 minutes later for enumeration of mobilised MSCs.

2.7.9. Blocking protease activity during VEGF and AMD3100 treatment.

Mice were administered marimastat (30mg/kg)(Tocris) in PBS.0.01% Tween (vehicle), vehicle or PBS 60 min before VEGF administration daily during the 4 day VEGF treatment protocol. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100, 5 mg/kg i.p) or vehicle and blood was collected via cardiac puncture 60 min later for enumeration of circulating MSC levels.

2.8. Enzyme-linked immunosorbent assays (ELISA).

2.8.1. Analysis of CXCL12 levels in cell supernatants.

To determine the amount of CXCL12 in the cell-free culture supernatant, commercial murine ELISA kits (R & D systems) were used according to the manufacturer's instructions and read at 450nm.

2.8.2. Analysis of CXCL12 levels in blood plasma.

Following different treatment regimens, blood was taken via cardiac bleed. The blood samples were then transferred to eppendorfs and centrifuged at 1000rpm for 3 minutes. The top layer of blood plasma was subsequently removed and the amount of CXCL12 in each sample was compared using commercial murine ELISA kits (R & D systems).

2.9. P5 bone marrow derived mMSC population doubling times.

Cells were seeded at densities of 5×10^4 , 1×10^5 and 2.5×10^5 cells per well in a 6 well plate. At 24, 48, 72 and 96 hours cell numbers were counted using a haemocytometer. Population doubling times (Td) were calculated using $Td = \log(2)/(\log(1 + (r/100)))$.

2.10. Propidium Iodide (PI) staining.

Cell cycle analysis was performed on 2×10^5 cells suspended in 50µl RNase (100µg/ml), 50µl Propidium Iodide (PI:100µg/ml and azide), and 150µl 0.1% Triton X, and then analysed immediately in FACSCalibur (BD) flow cytometer. A dot-plot of PI width against PI area was recorded. Aggregates of cells were detected as events having a larger PI-W profile and were not included in the gate. A PI-A histogram of the gated cells showed the first peak to represent cells in G0/G1, the second peak in G2/M, and the in-between area S phase cells.

2.11. Collagen 1 immunofluorescent staining.

1×10^4 cells of each analysed cell type were plated in each well of an 8 well chamber slide. Cells were cultured as previously described until confluent. Cells were then stained with Collagen-1 FITC conjugated antibodies (Millipore) and visualised under a fluorescence microscope.

2.12. Boyden chamber chemotaxis assay.

Harvested cells were incubated for 2-3hrs at 37°C in DMEM + 10% FCS. Simultaneously, the filter/membrane (8µm pore size, NeuroProbe) was placed in 10µg/ml fibronectin for 1hr at 37°C, after which it was washed twice in PBS. The whole assembled Boyden chamber (except filter) was blocked by adding 90µl of buffer (HBSS – 30mM HEPES, 0.25% BSA) and incubating for 1 hr at 37°C, before rinsing once with buffer. 28µl of buffer ± chemoattractant was added to each of the lower wells. The polycarbonate filter/membrane was then positioned on top. 50µl of cells were added to each of the top wells ($1-2 \times 10^4$ cells/well). The chamber was incubated for 15 hrs at 37°C in a humidified box. After incubation, the contents of the upper wells were carefully removed with a pipette. Filters were removed, wiped off on the upper side and air dried. The filters were fixed for 15 minutes in 4% PFA, stained with Diffquick and the underside of the filters scanned for migrated cells under a light microscope. MSCs were counted in 5 fields of view (100 x magnification).

2.13. Indium¹¹¹ labelling of P5 bone marrow derived mMSCs.

0.1mCi (approx 4MBq) of Indium¹¹¹ radionucleotide was chelated in 100µl 2-mercaptopyridine-N-oxide. 1ml of a cell suspension containing 1×10^6 MSCs were added to the ¹¹¹InCl₃-chelate for 15 mins at r.t. The cells were washed with 10ml PBS/Tyrodé's and centrifuged (1200rpm, 5 mins, r.t.) 3 times. After the final wash,

the cells were re-suspended in 1ml PBS/Tyrode's. 100µl of ¹¹¹In-labelled MSCs were injected either via the i.v. or i.p. route. After incubating for 4 hours, the mice were culled under terminal anaesthesia and dissected into the following sections; blood, thymus, heart, lungs, liver, spleen, kidneys, femurs, intestinal tract, mesenteric lymph nodes and tail. Blood and tissue levels of Indium¹¹¹ were determined using a gamma counter with a 100-500 KeV energy range.

2.14. Murine model of LPS induced lung inflammation.

Mice were challenged intratracheally with 40µl of 50µg/ml LPS, 30 mins later 2×10^5 Indium¹¹¹ MSCs were injected i.p. After 5.5 hrs mice were anaesthetised, blood collected by cardiac puncture, peritoneum lavaged with 1ml PBS, tissues collected, and radioactivity in each measured as previously described.

2.15. Quantitative PCR of PαS cells, mES cells and mMSCs.

RNA isolation and DNase treatment was performed using a liquid handling system (BiomekFxp, Beckman Coulter) to automate and integrate protocols from Agencourt RNAdvance Cell v2 (Beckman Coulter) and TURBO DNA-free (Applied Biosystems). Reverse transcription was performed using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems) and Veriti thermal cycler (Applied Biosystems). Quantitative PCR was performed on a 7900HT (Applied Biosystems) using TaqMan gene expressions assays including FAM™ dye-labelled TaqMan® MGB probe and primers set spanning 2 exons each specific for the target genes and the loading

controls (Hmbs and Gapdh). Data analysis was performed using DataAssist Software v1.0 (Applied Biosystems) using the geometric mean of two loading controls (Hmbs and Gapdh) for normalization. The acquisition of this data was carried out by Dr. Michela Nosedà.

2.16. Matrigel protocol.

Matrigel is a gelatinous material that is a liquid at 0°C and a solid at body temperature. After anesthetization the skin was shaved on the back of the neck and Matrigel plugs (250µl) were injected subcutaneously. MSCs were mobilised from the bone marrow via chronic VEGF treatment and AMD3100 challenge, as previously described. Matrigel plugs were excised at days 5 and 10. Upon excision, Matrigel plugs were weighed and then digested by incubation with 2.5 ml of dispase for 90 min at 37°C. Cell suspensions were then centrifuged at 1200rpm for 5 minutes at r.t. The excess supernatant was removed and the pellet was re-suspended in mesencult. MSC numbers were quantified by colony assays.

3.

**Isolating and characterising a population of
murine bone marrow MSCs.**

3. Isolating and characterising a population of murine bone marrow MSCs.

3.1 Introduction.

Human MSCs are defined by the ICST [59] as:

- Plastic adherent under standard culture conditions.
- Positive and negative for a range of defined cell surface markers.
- Capable of differentiation into osteoblasts, adipocytes and chondrocytes under specific conditions [59].

MSCs have been isolated from nearly all human and murine tissues. While all grow on plastic and possess tri-lineage differentiation capacity, considerable variation has been reported with respect to their phenotype (Table 1.1). It does not appear that this variation is due to tissue source of MSCs, more likely due to methods for isolation and expansion. Further, it is unclear whether these different phenotypes impact on the functionality of the MSCs. However, there have been some disappointing results in some high profile clinical trials using allogeneic MSC, where the nature of MSCs administered to patients have not been disclosed. This makes interpretation of these clinical trials difficult. Further understanding on how MSC isolation and culture conditions impact on phenotype and functions are absolutely necessary. With respect to murine MSCs, a similar situation exists where many labs have developed individual isolation and expansion protocols and MSCs that are studied are experimental populations and clearly different phenotypically.

This chapter focuses on exploring some of the basic mechanisms by which mMSCs act both *in vitro* and *in vivo*. The phenotype of mMSCs has been shown to be highly conserved when compared to that of hMSCs [134]. However, in comparison to hMSCs, purification and characterisation of mMSCs are still relatively poorly documented [274].

Whereas all mMSCs must possess tri-lineage differentiation capacity and the ability to adhere and proliferate on plastic surfaces, there is debate over the range of cell surface markers which mMSCs express. Table 1.1 lists published reports of mMSCs staining both positively and negatively for CD34, Sca-1, CD90, CD13 and CD117. This is most likely due to the difference in isolation techniques between laboratories. A clear difference between murine and human cultures of bone marrow stromal cells is the levels of contamination of CD45+ haematopoietic cells that represent a high percentage in mouse and persist over several passages of culture [93]. This may be in part due to the way in which the cells are isolated. The human bone marrow from which hMSCs are derived is specifically extracted from the iliac crest and only a small amount is taken, relative to the total femoral bone marrow. In contrast, in the mouse, the whole femoral bone marrow is flushed. This means that there are potential differences in the isolated stem cell populations due to differences in their anatomical localisation, from which MSCs are cultured. CD45+ murine bone marrow cells of the haematopoietic lineage appear to be more persistent than their human counterparts in their ability to adhere to plastic and the stromal cells cultured upon it. For this reason, various laboratories use different methods to deplete CD45+ cells as well as increasing the number of passage in order to isolate a pure population of mMSCs, which stains negative for CD45

[268,275,276]. These different methodologies have led to phenotypic differences in the MSC populations isolated by different laboratories. It is also clear from Table 1.1. that mMSCs cannot be identified by any one specific marker and they do not express a defined set of markers. Due to a lack of a specific marker to identify, mMSCs are a heterogeneous population containing within its' number, cells at different stages of the cell cycle and with differing levels of plasticity [277]. The first aim of this project was to develop methodology to reproducibly isolate a population of MSCs from murine bone marrow.

There are various factors that must be considered in order to create a reproducible methodology. An early report suggested that extensive passaging of mMSCs was required to obtain a pure population of stem cells. Thus in this chapter, the phenotypic and functional characteristics of low and high passage MSCs were assessed. Phinney *et al* compared bone marrow derived mMSCs from various strains of mice to assess differences between strains and to identify the most suitable strain for mMSC isolation and culture *in vitro* [278]. They found that of the strains investigated, BalbC were optimum for mMSC isolation and culture, in that they had the highest proliferative activity of the strains investigated and cells within the MSC population could readily differentiate down osteogenic, adipogenic and chondrogenic lineages. However, it has been reported that proliferating cells will not migrate as readily as those in a quiescent state [120].

Chemokine receptors and their ligands play an important role in tissue specific homing of leukocytes [134]. An important feature of stem cells to be used for cellular therapies is that they must either: a) migrate to sites of injury and

inflammation, or b) if applied locally, must be retained at sites of injury and inflammation [279]. A common feature found when administering MSCs *in vivo* is the low engraftment rate at the target site [167]. We hypothesised that this may be due to a loss of chemokine receptors or adhesion molecules on the surface of the cells as a result of *in vitro* culture conditions. This chapter characterises the chemokine receptor profiles of mMSCs having undergone different levels of passage. Having assessed chemokine receptor levels, it is then critical to quantify the functionality of these receptors in terms of migratory potential. Due to the heterogeneous nature of MSC populations, it is likely that receptors will not be expressed on all of the cells in a given population [85]. Therefore, subtle changes in receptor levels may have functional relevance in terms of chemotactic ability. Recent studies have reported that chemokine receptor expression profiles are similar between mouse and human bone marrow MSCs [134]. Knowing this, it can be hypothesised that mMSCs can act as a good model for further study of chemokine receptors and the role they play in directing migration of MSCs in man. It is important to assess whether the reported conflicting characterisation of mMSC markers between laboratories extends to external chemokine receptor levels.

It is currently thought that upon reaching a site of injury or inflammation, MSCs may differentiate into tissue specific cells to replace damaged or dead cells, or alternatively, resolve the damage in a paracrine manner [280,281]. A considerable amount of studies investigating the role of these cells in disease, suggest that a very low proportion of the MSCs actually differentiate at the site and repair the tissue via cell replacement [282]. Instead, the role of MSCs in modulating the immune response appears to be the main driving force in guiding the repair of tissues [283].

MSCs have been shown to avoid allorecognition [284]. MSCs have also been shown to modulate the function of T cells and lack expression of MHC II [285]. This means that MSCs are ideal for transplantation into allogeneic hosts.

Having established methodology to culture mMSCs and characterised their chemokine receptor expression, experiments were performed to assess tissue distribution of MSCs *in vivo* in naïve mice and in a model of acute lung inflammation. The impact of the route of administration and the cell numbers injected was investigated.

3.2 Aims.

- To develop a reproducible method for isolating early passage bone marrow derived mMSCs.
- Characterisation of early passage bone marrow derived mMSCs in terms of antigen phenotype, internal and external chemokine receptor profile and chemotactic ability.
- Examination of migratory patterns of cultured early passage bone marrow mMSCs when administered in different ways, both in naïve mice and in a model of acute inflammation.

3.3 Results.

In order to develop methodology and confirm reproducibility of an early passage bone marrow mMSC population, the easiest way to analyse the cell populations was to investigate their antigen phenotype. The antigen phenotype of MSCs is widely reported to be negative for the haematopoietic lineage markers CD45 and CD11b, but positive for CD29 and CD105 [59,286,287]. Fig. 3.1. shows the expression of these markers on cell populations cultured in different ways in order to ascertain whether these cell populations fit the antigen profile to be classed as MSCs. This also illustrates how different culture conditions can affect the phenotype of the cell populations. The cell populations analysed in Fig. 3.1.a. and 3.1.b. both show CD45 and CD11b expression. These figures show P2 bone marrow populations before and after CD45 depletion using magnetic beads, respectively. Before CD45 depletion, CD29 expression is low at $12.67 \pm 4.16\%$ and the haematopoietic lineage markers are very highly expressed ($75.53 \pm 4.88\%$ expression). As expected, the number of cells expressing CD45 or CD11b following CD45 depletion drops to $29.00 \pm 25.24\%$. P5 and P25 bone marrow populations, can be seen in Fig. 3.1.c. and 3.1.d. respectively. These populations have not been CD45 depleted. The antigen phenotype for these cells is similar with $<5\%$ CD45/CD11b expression (P5 $3.31 \pm 2.04\%$, $2.95 \pm 2.21\%$ P25 $0.33 \pm 0.33\%$, $0.33 \pm 0.33\%$), $\sim 80\%$ CD29 expression (P5 $85.20 \pm 8.34\%$, P25 $79.17 \pm 3.02\%$) and $\sim 40\%$ CD105 expression (P5 $49.50 \pm 7.55\%$, P25 $36.03 \pm 10.06\%$). From this, it is clear that, in terms of marker expression, P2 bone marrow populations with or without CD45 depletion cannot be classified as a

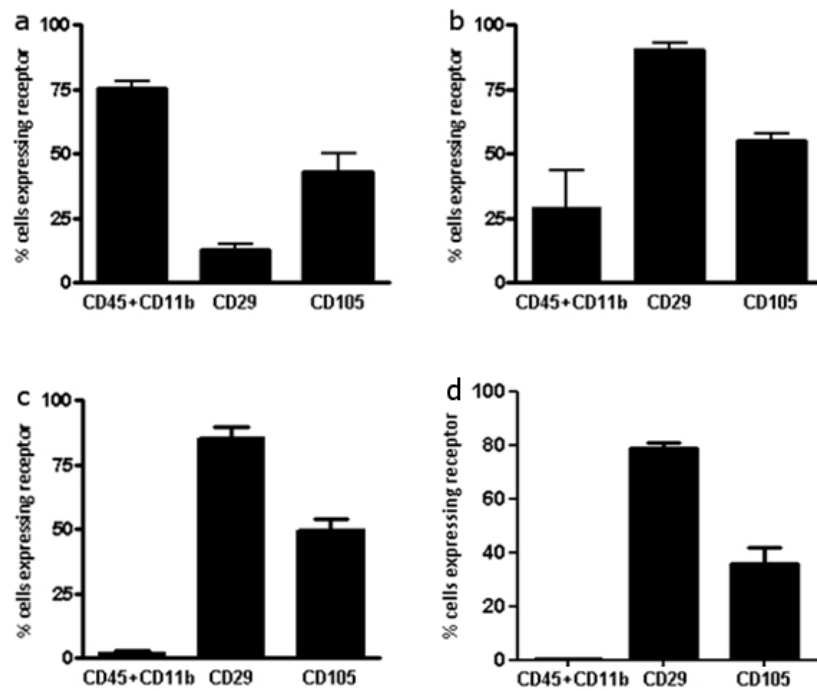


Fig. 3.1. Creating a population of CD45- MSCs.

Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ and passaged 2 or 5 times. FACS analysis of P2 bone marrow populations for the haematopoietic markers CD45 and CD11b, and MSC positive markers CD29 and CD105 (Fig 3.1.a). FACS analysis of a P2 bone marrow population after depletion using Miltenyi CD45-binding magnetic beads (MACs:Miltenyi Biotech) (Fig 3.1.b). FACS analysis of a P5 bone marrow population (Fig 3.1.c). FACS analysis of a P25 bone marrow population (Fig 3.1.d). Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3-4.

population of MSCs. However, P5 and P25 bone marrow populations express marker profiles consistent with those of MSCs. In order to further assess their MSC-like phenotype, the presence or absence of further antigen markers on P5 bone marrow populations was analysed (Fig. 3.2). P5 bone marrow cells isolated via plastic adherence alone are CD45-, CD11b-, CD34-, CD29+, CD105+, Sca-1+. The histogram plots in Fig. 3.2.b. are representative of each bar in Fig. 3.1.a. This shows that, of the positively expressed markers, only CD29 appears to be constitutively expressed. It is apparent that CD105 and Sca-1 are only positively expressed on a subset of the total bone marrow derived population.

In order to be classified as MSCs, this cell population must also show trilineage differentiation potential and proliferate on plastic surfaces *in vitro*. P5 bone marrow cells cultured *in vitro* can differentiate into osteoblasts, chondrocytes and adipocytes. (Fig. 3.3.a). Population doubling times of P5 and P25 bone marrow populations in plastic culture flasks *in vitro* are compared in Fig. 3.3.b. Doubling times have been reported to decrease with increased passage number. Consistent with this doubling times of P25 bone marrow mMSCs were reduced from 49.71 ± 10.87 hours to 35.50 ± 8.82 hours in the first 96 hours of culture, when compared to P5 bone marrow mMSCs.

Late passage bone marrow mMSCs also fulfil MSC criteria in terms of the antigen profile. Fig. 3.4 shows the expression and the representative histogram plots of several markers on P25 bone marrow cells. These cells are CD45-, CD11b-, CD34-, CD29+, CD105+, Sca-1+. In comparison to the P5 bone marrow cells, the only

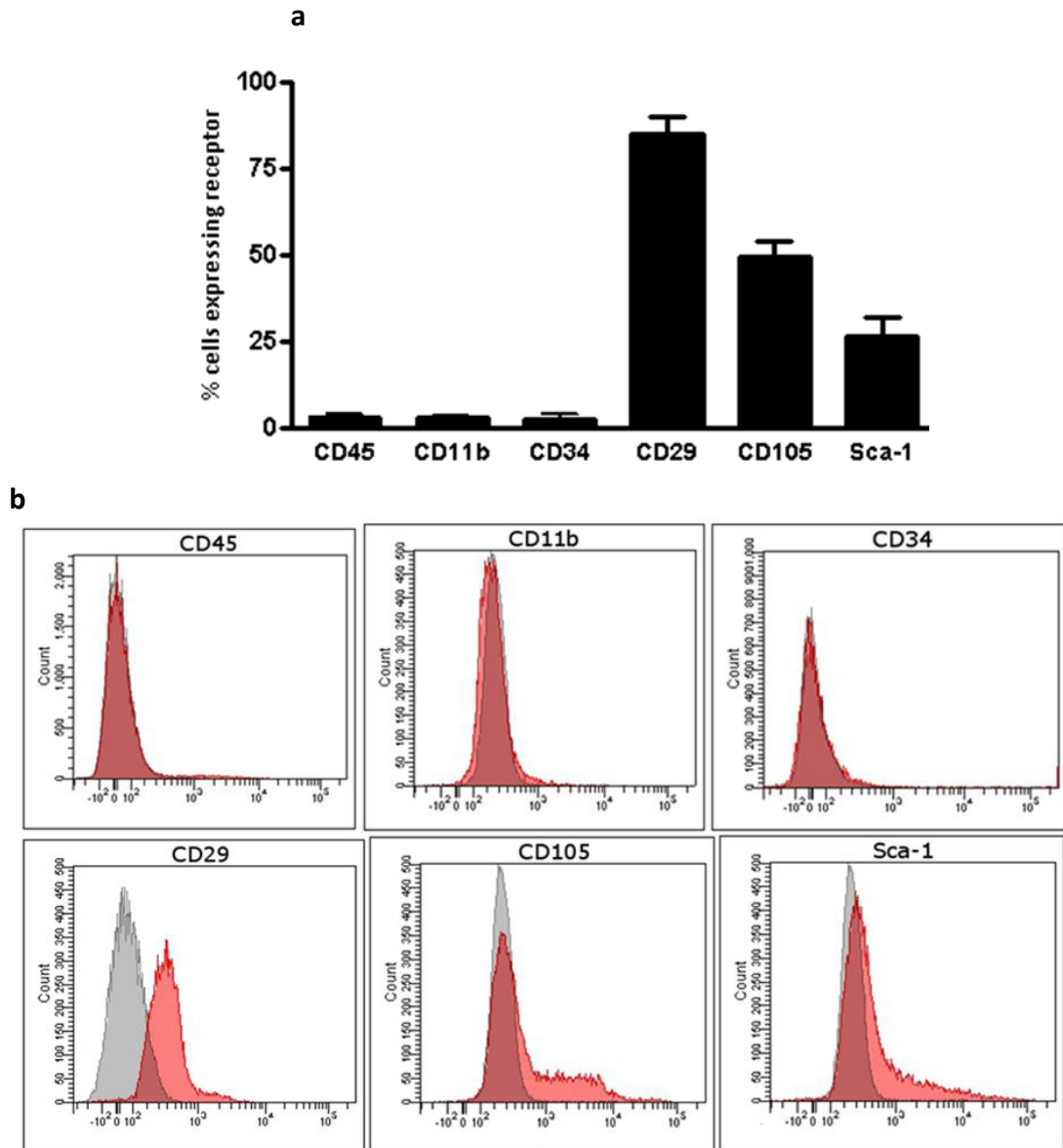


Fig. 3.2. Cell marker expression on P5 mMSCs.

Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ and passaged 5 times. (Fig 3.2.a) FACS analysis of P5 bone marrow populations for the haematopoietic markers CD45 and CD11b, EPC and haematopoietic marker CD34, MSC markers CD29, CD105 and Sca-1. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3-6. (Fig 3.2.b). FACS histogram plots of selection of markers on bone marrow mMSCs at passage 5 isolated by plastic adherence (grey plots = isotype controls, red plots = marker of interest on cell population). Data shown is representative.

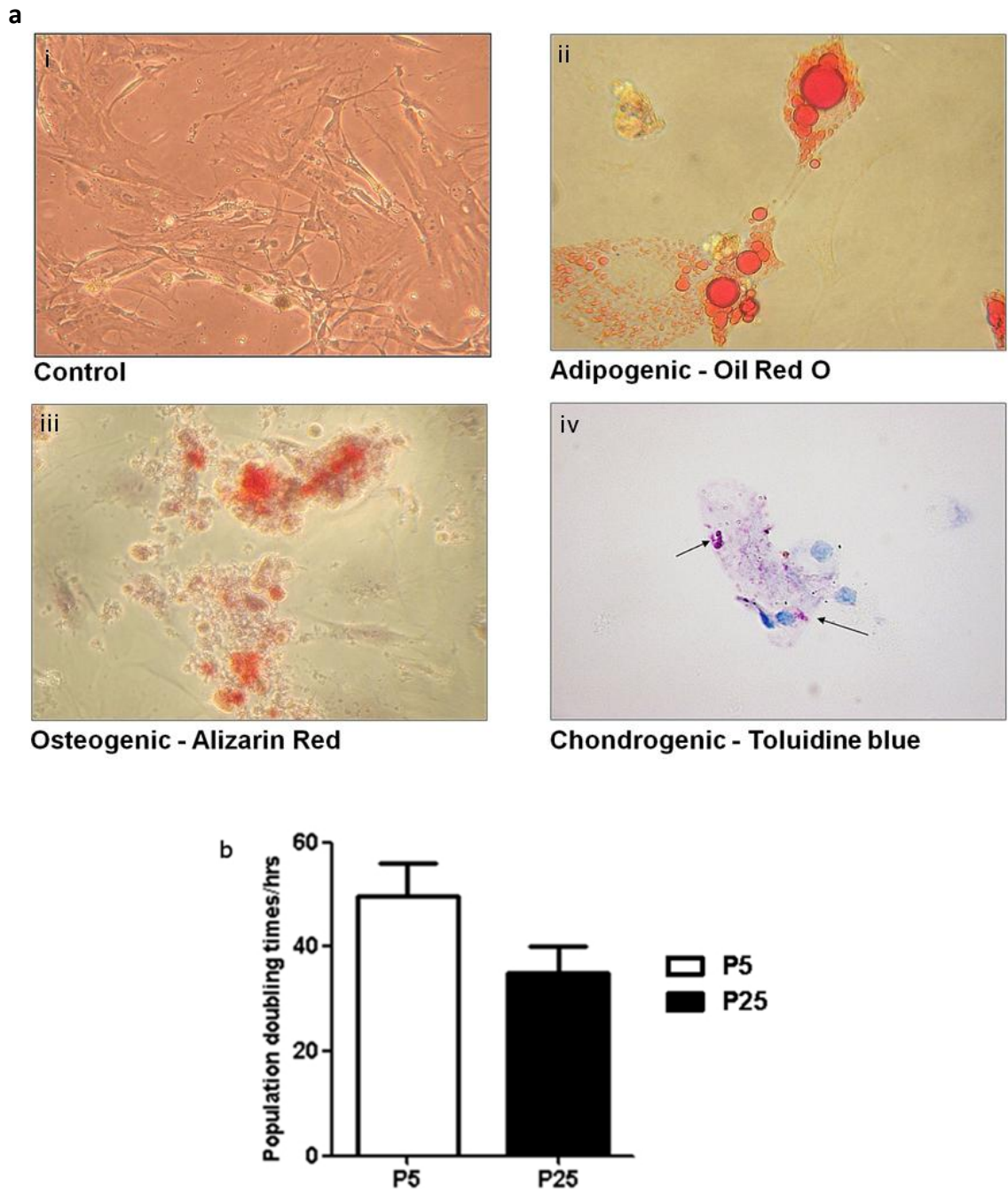


Fig 3.3. Differentiation and population doubling times of P5 and P25 mMSCs.

Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ and cultured to passage 5. 3×10^5 P5 mMSCs were plated in each well of a 6-well plate. Different culture media was added to each depending upon published differentiation protocols as described in the methods [115]. Untreated control cultures (Fig 3.3.a.i). The presence of lipid-rich vacuoles stained with Oil Red O confirmed adipogenic induction (Fig

3.3.a.ii). Alizarin Red S staining shows mineralization of the extracellular matrix confirming osteogenic lineage (Fig 3.3.a.iii). Toluidine Blue (arrows) staining revealed the deposition of proteoglycans and lacunae confirming differentiation into the chondrogenic lineage (Fig 3.3.a.iii). Fig 3.3.b. Cells were seeded at densities of 5×10^4 , 1×10^5 and 2.5×10^5 cells per well in a 6 well plate. At 24, 48, 72 and 96 hours cell numbers were counted using haemocytometers. Population doubling times (Td) were calculated using $Td = \log(2)/(\log(1 + (r/100)))$. n = 3. (Fig 3.3.c)

significant difference is the expression levels of Sca-1. $67.20 \pm 21.72\%$ of P25 bone marrow cells express Sca-1, as opposed to $26.37 \pm 11.47\%$ of P5 bone marrow cells.

If there are functional differences between the early and late mMSC populations, it is likely to be related to migratory and chemotactic ability. Chemokine receptor profiles often correlate with the trafficking ability of the cell populations [288]. The external receptor levels of several chemokine receptors on the P5 bone marrow mMSC population can be seen in Fig 3.5.a. Fig. 3.5.b. shows representative histogram plots for each receptor. CXCR3 ($41.48 \pm 16.81\%$), CXCR4 ($46.33 \pm 15.57\%$), CCR9 ($40.91 \pm 14.16\%$) and CCR10 ($34.31 \pm 11.85\%$) are the highest expressed surface chemokine receptors of those analysed, all of which are expressed on >30% of the P5 bone marrow mMSC population. CCR3 ($3.25 \pm 0.50\%$) and CCR6 ($3.06 \pm 1.92\%$) are both expressed by <5% of the cells. The histogram plots suggest that there are sub-populations of cells within the P5 bone marrow mMSC population, expressing either CXCR1, CXCR2, CXCR3 or CXCR4 on the cell surface.

Knowing that repeated passage can affect the properties of the MSC population, late passage (P25) bone marrow mMSCs were also characterised in terms of their chemokine receptor profile. The external receptor levels of several chemokine receptors on the P25 bone marrow mMSC population are shown in Fig. 3.6. Fig. 3.6.b. shows representative histogram plots for each receptor. CXCR3, CCR7, and CCR10 are the highest expressed of all the receptors on the cell surface, each expressed by ~20% of the P25 bone marrow mMSCs ($21.48 \pm 13.54\%$, $22.32 \pm 1.27\%$, $19.60 \pm 17.06\%$). CXCR2, CXCR4, CXCR6, CXCR7, CCR3 and CCR6 are all expressed by <10% of P25 bone marrow mMSCs ($5.87 \pm 3.50\%$, $6.54 \pm 2.86\%$, $8.67 \pm 5.28\%$, $1.83 \pm$

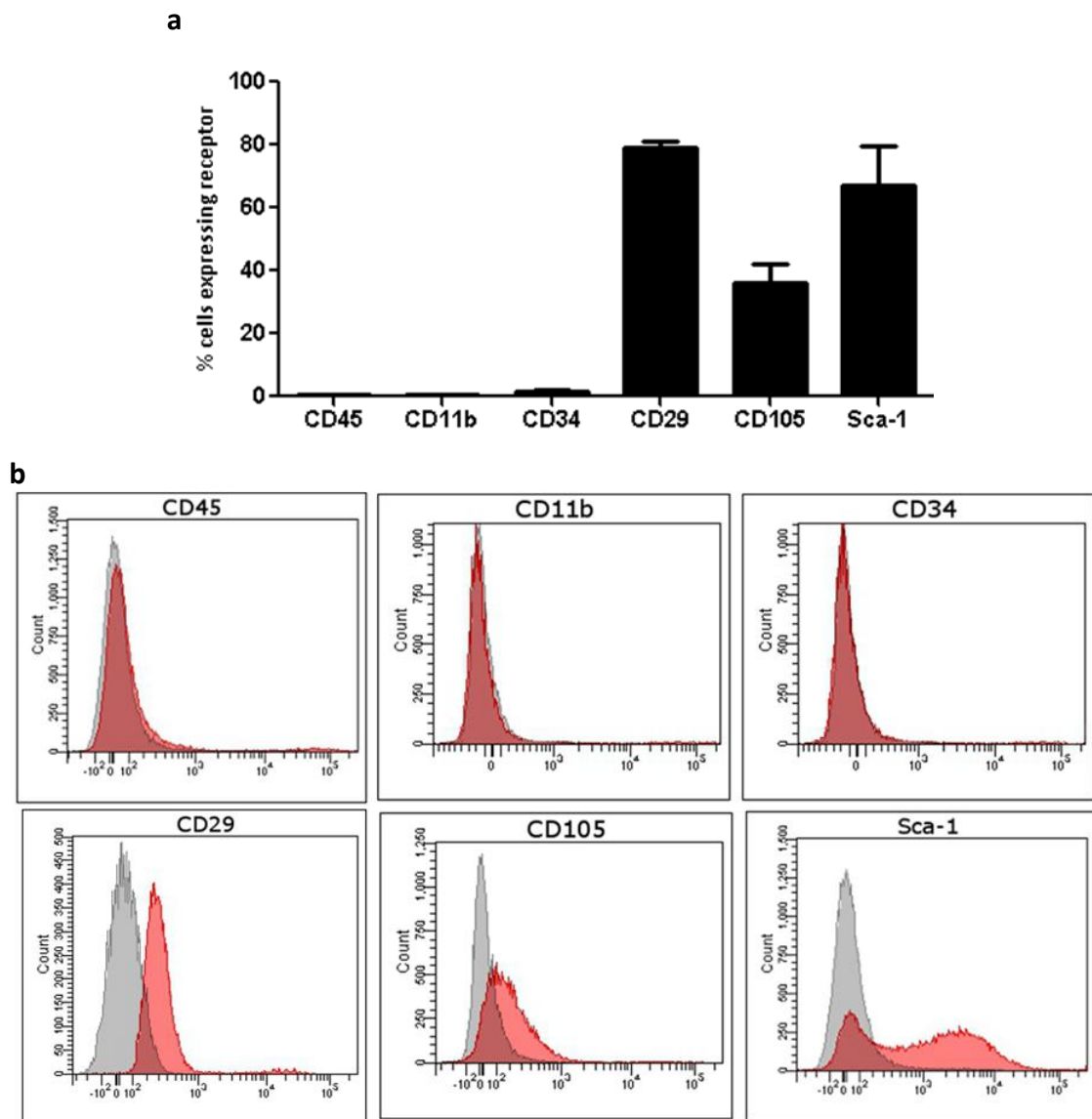
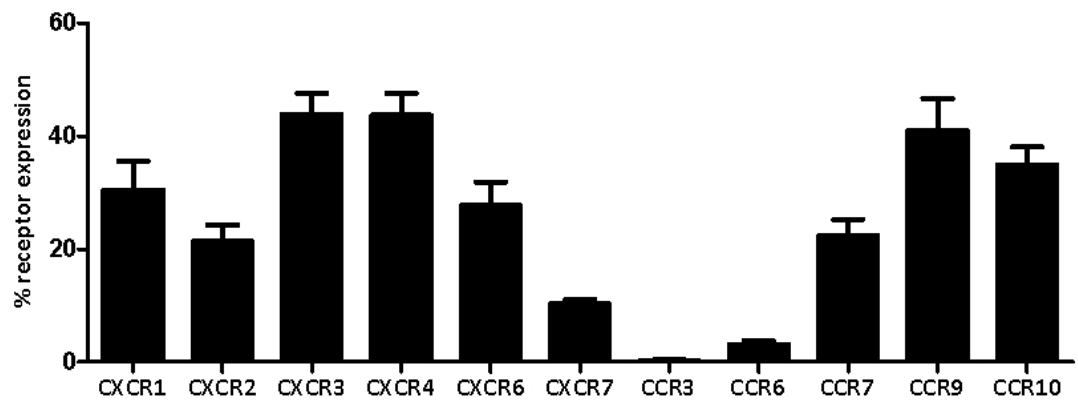


Fig. 3.4. Cell marker expression on P25 mMSCs.

Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ and passaged 25 times. Fig 3.4.a FACS analysis of P25 bone marrow populations for the haematopoietic markers CD45 and CD11b, EPC and haematopoietic marker CD34, MSC markers CD29, CD105 and Sca-1. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3. Fig 3.4.b. FACS histogram plots of selection of markers on bone marrow mMSCs at passage 25 isolated by plastic adherence. Data shown is representative.

a



b

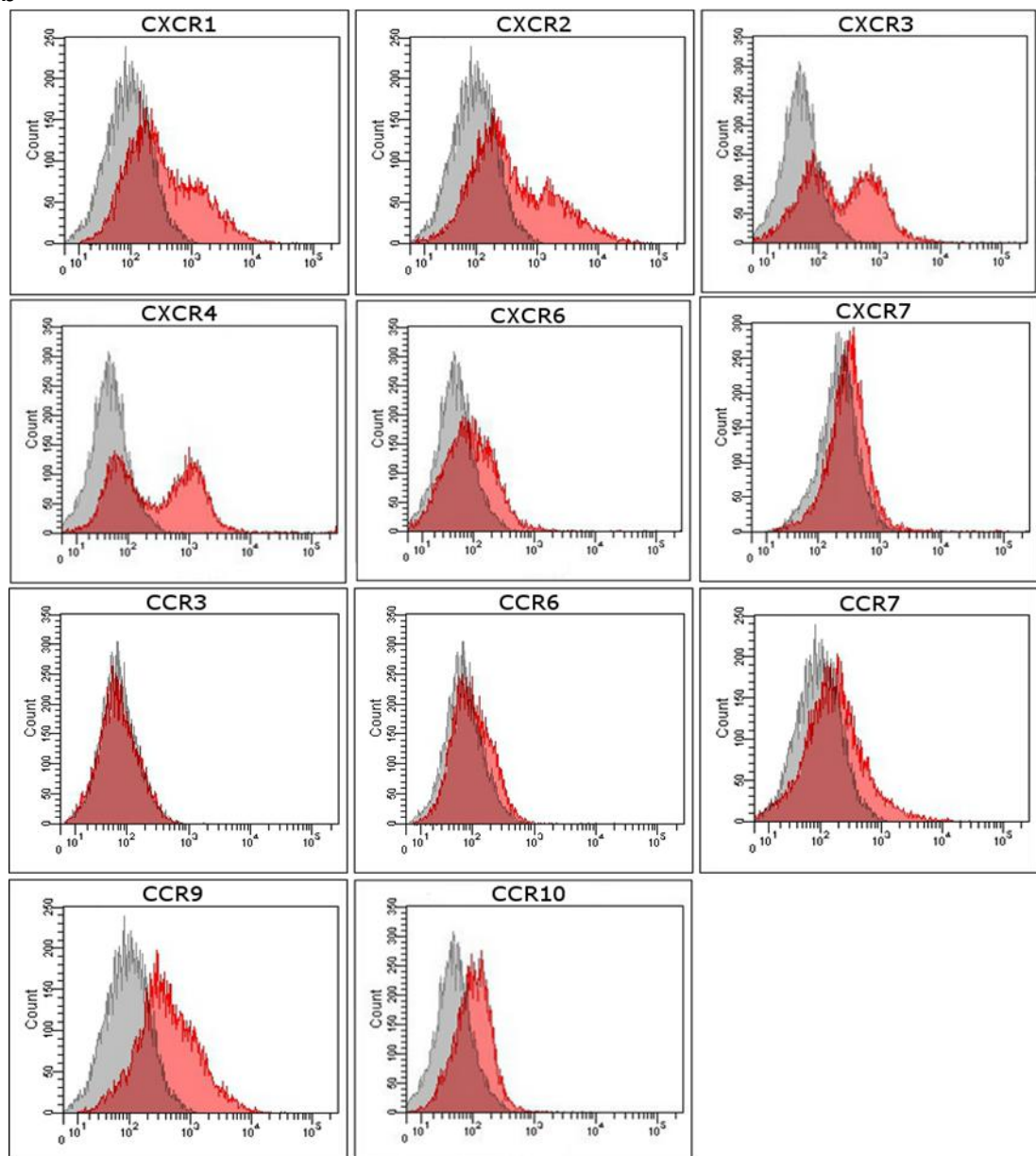
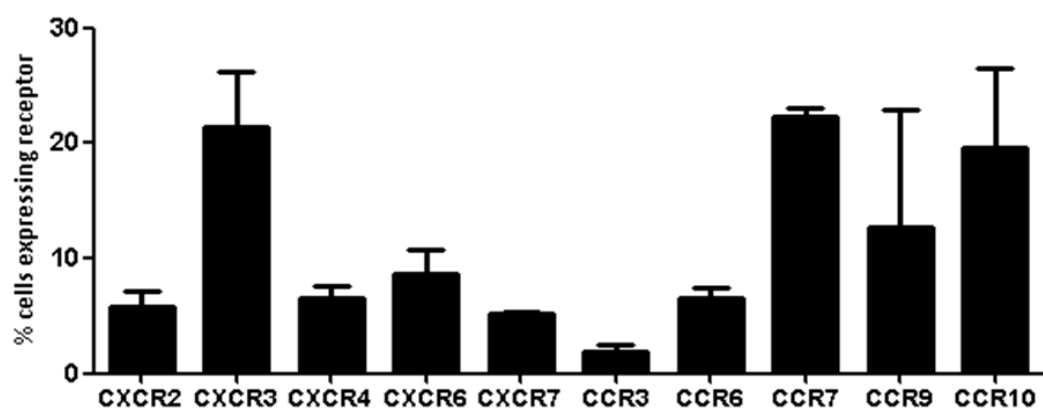


Fig. 3.5. External chemokine receptor expression on expression on P5 mMSCs.

Fig 3.5.a. Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ to passage 5. Cells were trypsinised, recovered and analysed for external chemokine receptor expression. 1×10^5 cells were used to stain for each receptor. Representative external chemokine receptor expression levels of P5 mMSCs were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3-13. Fig 3.5.b. FACS histogram plots of external chemokine receptor expression on bone marrow mMSCs at passage 5 isolated by plastic adherence. Data shown is representative.

a



b

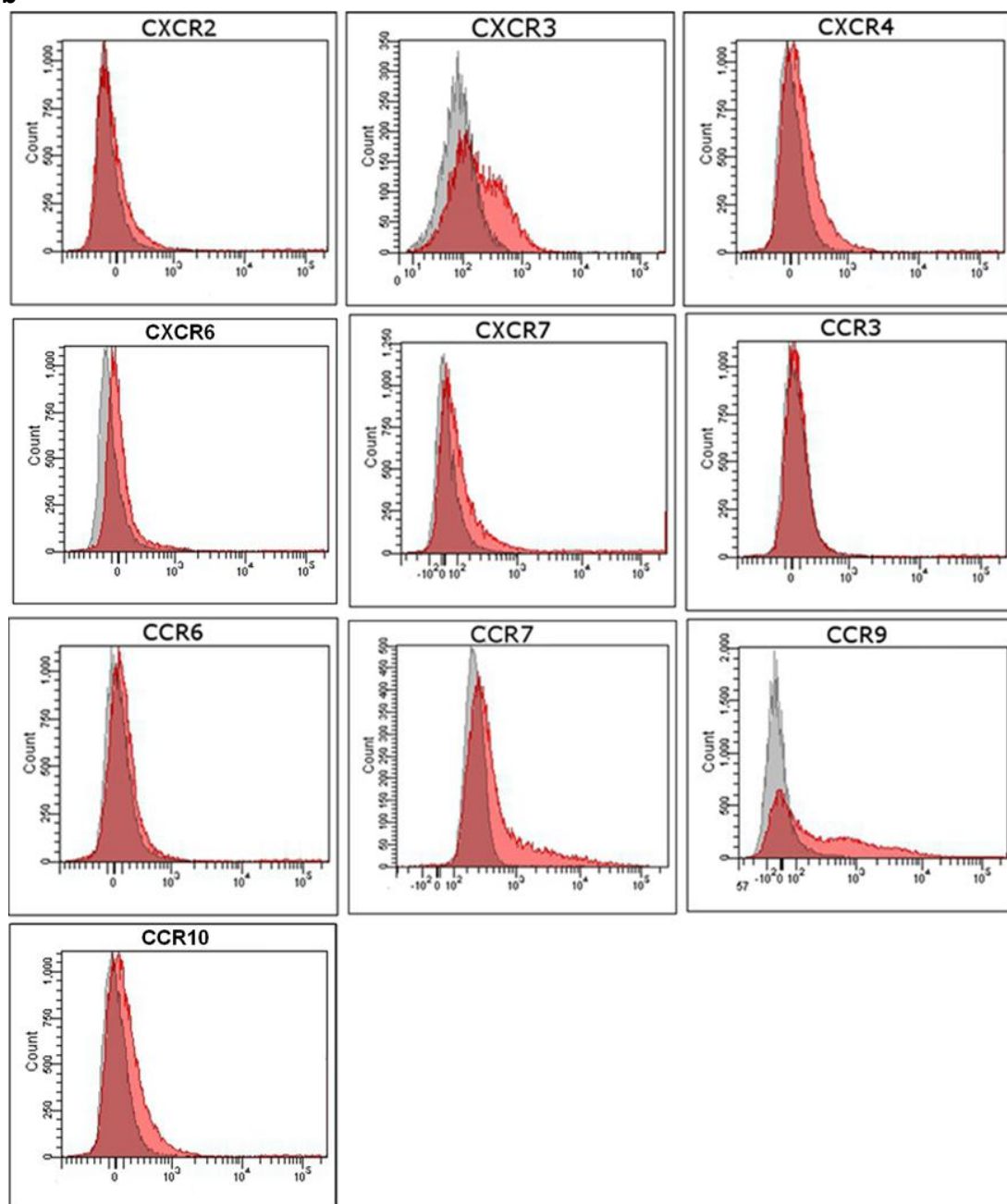


Fig. 3.6. External chemokine receptor expression on expression on P25 mMSCs.

Fig 3.6.a. Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ to passage 25. Cells were trypsinised, recovered and analysed for external chemokine receptor expression. 1×10^5 cells were used to stain for each receptor. Representative external chemokine receptor expression levels of P25 mMSCs were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. Levels were recorded by subtracting the isotype control expression levels from the marker stained population expression levels. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3-8. Fig 3.6.b. FACS histogram plots of external chemokine receptor expression on bone marrow mMSCs at passage 25 isolated by plastic adherence (grey plots = isotype controls, red plots = marker of interest on cell population). Data shown is representative.

1.27%, $6.50 \pm 2.31\%$). Interestingly, bone marrow mMSCs at late passage (P25) have significantly reduced surface receptor expression levels of CXCR2, CXCR3, CXCR4, CXCR6 and CCR9 when compared to early passage (P5) bone marrow mMSCs (Fig. 3.7.). The most significant of these reductions in surface receptor expression is CXCR4, reduced from $46.33 \pm 15.57\%$ at P5 to $6.54 \pm 2.86\%$ at P25.

If chemokine receptors are present intracellularly in the MSC population, there is the potential to upregulate cell surface expression levels which may in turn increase the migratory capacity of the MSCs. It has previously been reported that mature neutrophils expressing low levels of CXCR4 on their surface rapidly upregulate expression in culture over a matter of 8 hours [220]. These cells were shown to have large intracellular stores of CXCR4. By comparing the intracellular levels of chemokine receptors, it is possible to see whether any changes externally are due to increases in the intracellular expression levels. Fig. 3.8. shows the intracellular chemokine receptor levels of P5 and P25 bone marrow mMSCs. In general, intracellular levels of chemokine receptors are much higher than external levels for both P5 and P25 bone marrow mMSCs. Over 40% of P5 bone marrow mMSCs express CXCR3 ($97.32 \pm 1.17\%$), CXCR4 ($78.12 \pm 17.37\%$), CXCR6 ($46.64 \pm 11.39\%$), CCR6 ($53.47 \pm 10.70\%$), CCR7 ($74.33 \pm 7.23\%$), CCR9 ($91.49 \pm 6.44\%$) or CCR10 ($87.78 \pm 9.09\%$) intracellularly (Fig. 3.8.a). Nearly 100% of cells in the population expressed CXCR3, CCR9, and CCR10 intracellularly. There was little or no expression of CXCR2 ($3.60 \pm 2.07\%$) or CCR1 ($0.33 \pm 0.58\%$) intracellularly in this population. Over 60% of P25 bone marrow mMSCs expressed CXCR2 ($60.01 \pm 19.62\%$), CXCR3 ($97.14 \pm 3.62\%$), CXCR4 ($79.68 \pm 18.92\%$), CCR6 ($82.14 \pm 10.11\%$), CCR7 ($89.63 \pm 1.01\%$), CCR9

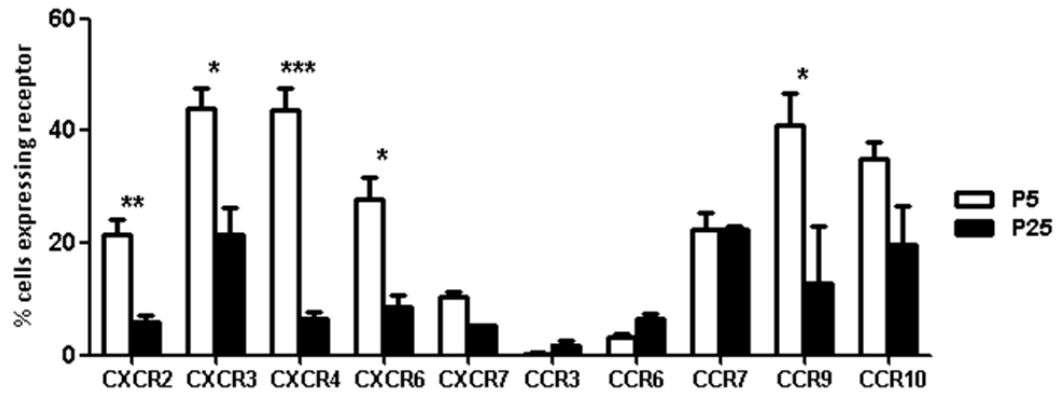


Fig. 3.7. Comparison of external expression of chemokine receptors on passage 5 and passage 25 murine bone marrow derived MSCs.

Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ to passage 5 or passage 25. Cells were trypsinised, recovered and analysed for external chemokine receptor expression. 1 x 10⁵ cells were used to stain for each receptor. Representative external chemokine receptor expression levels of P5 and P25 mMSCs were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. Expression levels are illustrated as the mean FACS measurements ± SEM. n = 3-13. *p < 0.05, **p < 0.01, and ***p < 0.001 between selected groups.

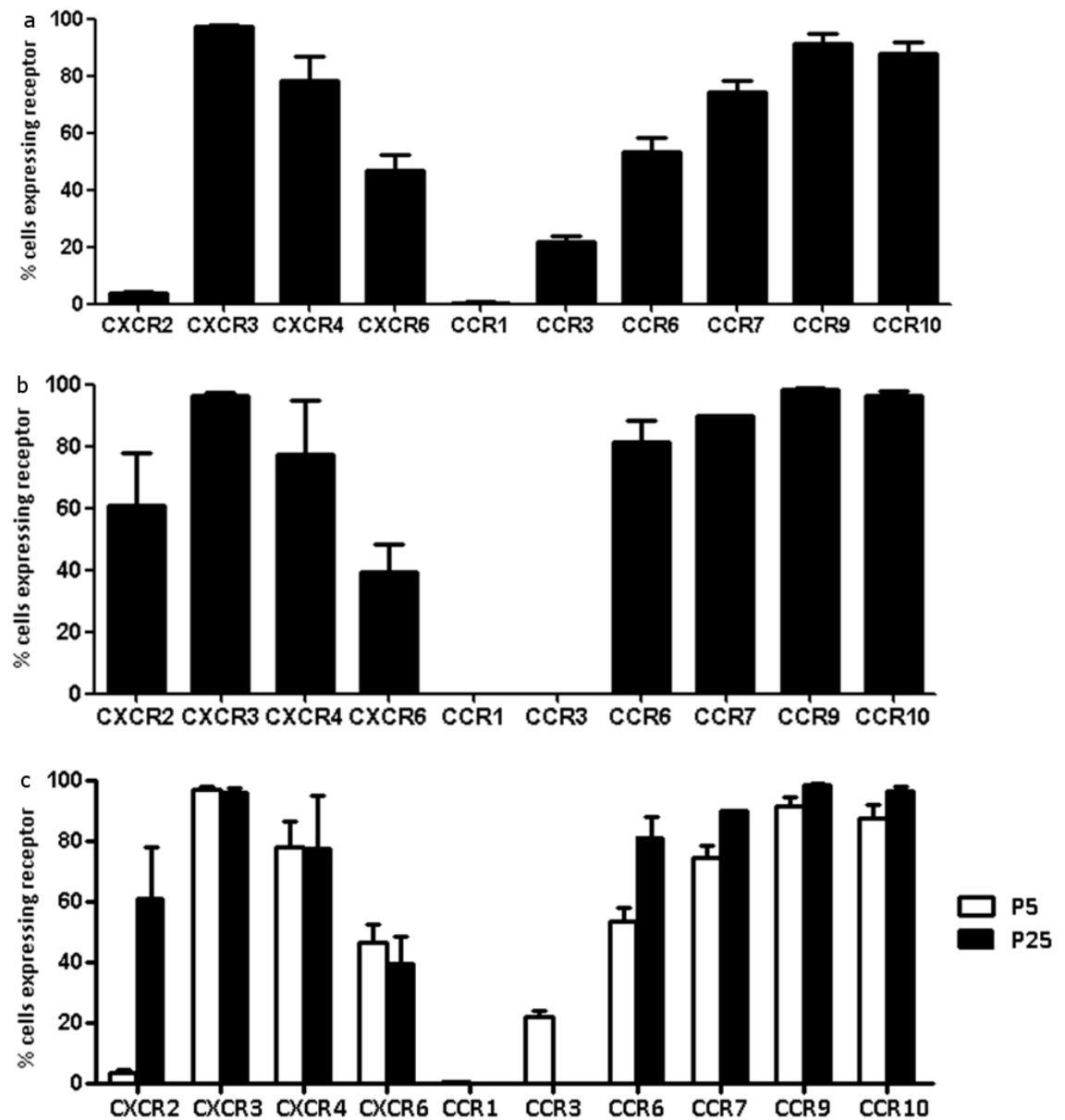


Fig. 3.8. Comparison of intracellular chemokine receptor expression on murine bone marrow MSCs at differing passage numbers.

Fig 3.8.a. Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ to passage 5. Cells were trypsinised, recovered and analysed for intracellular chemokine receptor expression. 1 x 10⁵ cells were used to stain for each receptor. Representative intracellular chemokine receptor expression levels of P5 mMSCs were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. Expression levels are illustrated as the mean FACS measurements ±

SEM. n = 3-5. Fig 3.8.b. Bone marrow cell populations were cultured in Mesencult (StemCell technologies) at 37°C, 5% CO₂ to passage 25. Cells were trypsinised, recovered and analysed for intracellular chemokine receptor expression. 1×10^5 cells were used to stain for each receptor. Representative intracellular chemokine receptor expression levels of P25 mMSCs were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 1-3. Fig 3.8.c. Comparison of representative intracellular chemokine receptor expression levels of P5 and P25 mMSCs. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 1-5.

($98.62 \pm 1.15\%$) or CCR10 ($96.85 \pm 2.07\%$) intracellularly. CCR1 and CCR3 were not expressed (Fig. 3.8.b). When these two populations were compared (Fig. 3.8.c), there were similar intracellular expression levels of CXCR3, CXCR4, CXCR6, CCR1, CCR7, CCR7, CCR9 and CCR10. However, early passage (P5) bone marrow mMSCs expressed significantly more intracellular CCR3 ($22 \pm 3.46\%$ vs. 0%), whereas late passage (P25) bone marrow mMSCs expressed significantly more intracellular CXCR2 ($60.01 \pm 19.62\%$ vs. $3.60 \pm 2.07\%$).

P5 bone marrow mMSCs have a different external chemokine receptor profile to that of P5 tracheal fibroblasts. The varying external chemokine receptor profiles of these 2 cell populations can be seen in Fig. 3.10.a. While both P5 bone marrow mMSCs and P5 tracheal fibroblasts had similar expression levels of CXCR2 ($21.20 \pm 9.33\%$, $13.45 \pm 2.79\%$), CXCR3 ($41.48 \pm 16.81\%$, $54.94 \pm 13.46\%$) and CCR10 ($34.31 \pm 11.85\%$, $45.86 \pm 20.85\%$), there were significant differences in the levels of CXCR4 ($46.33 \pm 15.75\%$, $11.42 \pm 9.56\%$), CXCR6 ($40.91 \pm 14.16\%$, $9.16 \pm 5.44\%$) and CCR6 ($3.06 \pm 1.92\%$, $41.17 \pm 11.73\%$). Other populations of murine fibroblasts were also found to have different chemokine receptor profiles (data not shown). Importantly, P5 murine tracheal fibroblasts expressed high levels of collagen-1 intracellularly whereas P5 bone marrow mMSCs did not express collagen-1 (Fig. 3.10.b).

The functionality of these receptors, specifically the chemokine receptor levels on the surface of the cells was then tested. MSCs were cultured for 48h in the absence of serum to arrest the cells in G₀ of the cell cycle prior to performing chemotaxis assays. As shown in Fig. 3.9.a. The number of actively dividing cells

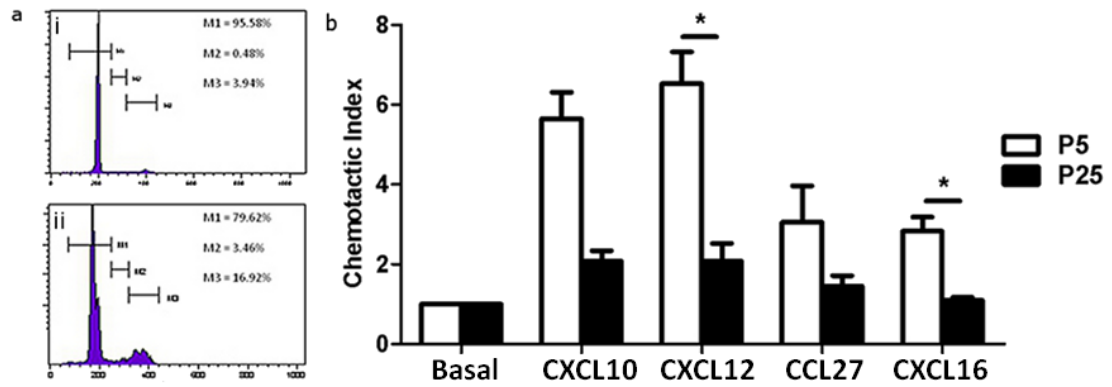


Fig 3.9. Varying chemotaxis of P5 and P25 mMSCs towards CXCL10, CXCL12, CCL27 and CXCL16.

P5 mMSCs were seeded at a density of 3×10^5 cells/well. After 24 hours recovery in Mesencult culture media was replaced with either DMEM + 0.1% FCS or DMEM + 10% FCS. Fig 3.9.a. PI staining to illustrate the percentage of cells in each stage of the cell cycle having been cultured for 48 hours in either; DMEM + 0.1% FCS (Fig. 3.9.a.i: M1=95.58%, M2=0.48%, M3=3.94%) or DMEM + 10% FCS (Fig. 3.9.a.ii: M1=79.62%, M2=3.46%, M3=16.92%). PI staining analysis was carried out using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software. Fig 3.9.b. Cells were cultured to confluence at P5 or P25 in Mesencult. Cells then had the Mesencult replaced with DMEM + 0.1% FCS for 48 hours to enter the cells into a quiescent state. Cells were trypsinised, recovered and placed in the top wells of a Boyden chamber. CXCL10, CXCL12, CCL27 or CXCL16 (30nM) were added to the bottom chamber as indicated. Migration of mMSCs is expressed as the mean number of cells transmigrated to the lower side of the membrane $\pm$ SEM. $n=3$. Statistical comparisons were made between indicated columns using a paired student T –test ($P < 0.05$ denotes *) (P5 vs. P25 mMSCs towards CXCL10, $P=0.0568$)

(3.94%, Fig. 3.9.a.i) is significantly lower than that of bone marrow MSCs cultured in media containing 10% serum for 48 hours (16.92%, Fig. 3.9.a.ii). The relative chemotactic ability of P5 and P25 bone marrow mMSCs is shown in Fig. 3.9.b. P5 mMSCs show a higher chemotactic response to the ligands of all 4 receptors investigated. P5 bone marrow mMSCs showed a significant increase above controls in the level of migration to the CXCR4 ligand CXCL12 (6.34 ± 0.511 vs. 2.26 ± 0.31) and the CXCR6 ligand CXCL16 (3.10 ± 0.38 vs. 1.08 ± 0.18). Overall, while P5 mMSCs exhibited robust chemotactic responses to all of these chemokines, P25 MSCs exhibited very low levels of migration, consistent with low levels of CXCR3, CXCR4, CXCR6 and CCR10 expressed by the cells.

To enable accurate determination of the distribution of MSCs *in vivo*, MSCs were labelled with ^{111}In and then injected either i.v. or i.p. These administration routes were chosen as they have been used by others to examine the therapeutic effects of MSCs in animal models [289,290]. When P5 bone marrow mMSCs were injected into mice i.p. and i.v., they showed different localisation patterns after 4 hours. When 2.5×10^5 cells were injected i.v., the majority ($76.75 \pm 6.84\%$) of cells were located in the lungs after 4 hours, with a small population in the liver ($12.34 \pm 1.41\%$ Fig. 3.11.a). However, when 2.5×10^5 P5 bone marrow mMSCs are injected i.p., the majority ($66.48 \pm 13.11\%$) were associated with the intestine after 4 hours. There are also relatively small but significant numbers found in the liver, spleen and bone marrow ($9.167 \pm 4.53\%$, $8.08 \pm 5.33\%$, $7.09 \pm 3.01\%$, respectively (Fig. 3.11.b). A second experiment was performed injecting fewer cells to see whether that would reduce the acute entrapment and more tissues were assessed to determine tissue distribution more accurately. When lower numbers of P5 bone marrow mMSCs were

injected i.p. or i.v. (1×10^5 cells) there was a greater tissue distribution after 4 hours (Fig. 3.12.a & 3.12.b). When injected i.v. The majority of P5 bone marrow mMSCs were still found in the lungs ($40.83 \pm 14.13\%$), but markedly less when compared to the previous experiment. Significant percentages of the cells were found in the liver, bone marrow, intestine, skin and ribcage ($14.51 \pm 3.45\%$, $3.92 \pm 2.35\%$, $6.76 \pm 3.15\%$, $5.56 \pm 4.14\%$, $10.39 \pm 8.76\%$). When injected i.p., the most notable difference was that $24.25 \pm 9.25\%$ of cells had homed to the skin. Significant numbers of the cells were also found in the peritoneal lavage, the liver, the bone marrow and the spine ($8.66 \pm 1.72\%$, $7.44 \pm 1.56\%$, $4.34 \pm 1.47\%$, $7.54 \pm 4.07\%$). Next, the tissue distribution of injected P5 bone marrow mMSCs was examined under inflammatory conditions, in which chemokines would be released at relevant tissue sites. Mice therefore received a single dose of LPS i.t. ($40 \mu\text{l}$, $50 \mu\text{g/ml}$) 1 hour prior to i.p. injection of 1×10^5 bone marrow mMSCs. There were no MSCs found in the lungs after 4 hours. The distribution was not significantly different at this time point to the distribution when injected i.p. into naïve mice.

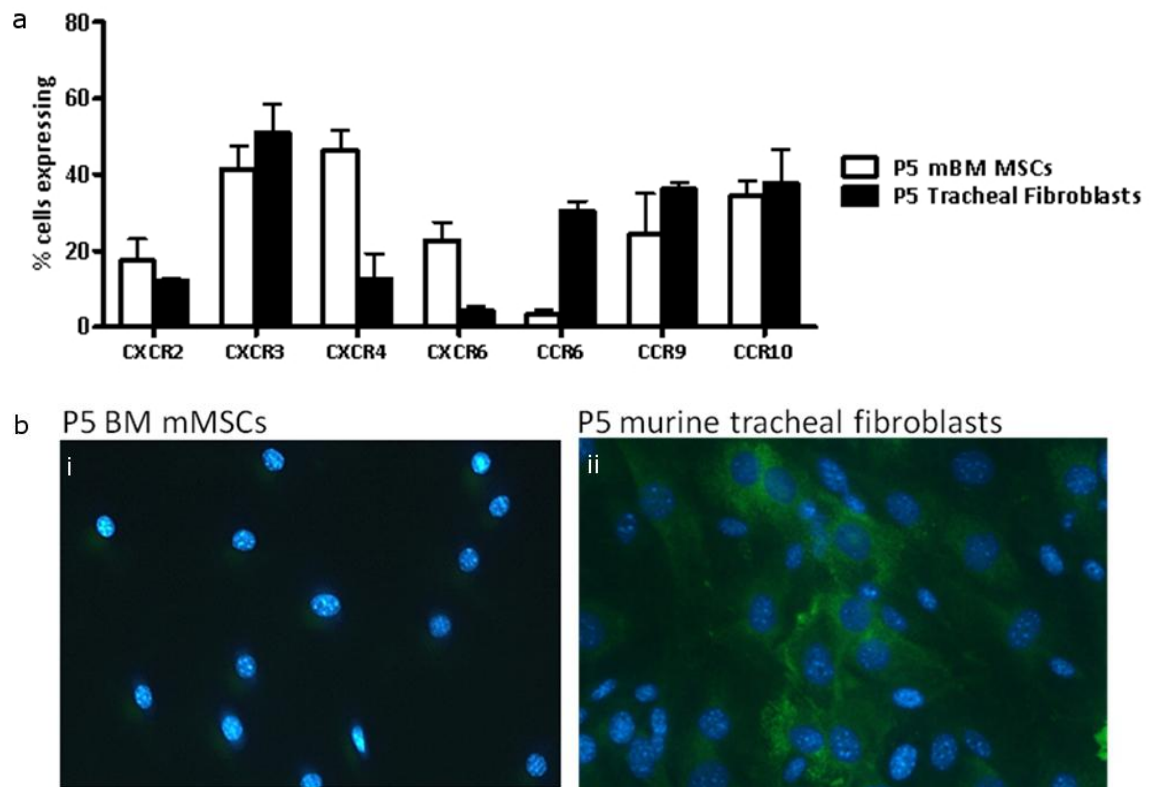


Fig 3.10. Different characteristics of passage 5 bone marrow derived mMSCs and passage 5 murine tracheal fibroblasts.

Fig 3.10.a. The two different cell populations were cultured in DMEM + 10% FCS at 37°C, 5% CO₂ to passage 5. Cells were trypsinised, recovered and analysed for external chemokine receptor expression. 1 x 10⁵ cells were used to stain for each receptor. Representative external chemokine receptor expression levels of P5 mMSCs and P5 murine tracheal fibroblasts were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. Expression levels are illustrated as the mean FACS measurements ± SEM. n = 2-8. Fig 3.10.b. Varying expression of collagen-1 in passage 5 bone marrow derived mMSCs (Fig 3.10.b.i) and P5 murine tracheal fibroblasts (Fig 3.10.b.ii). The two different cell populations were cultured in DMEM + 10% FCS at 37°C 5% CO₂ to passage 5. Cells were then stained as described in the methods for Collagen 1 and assessed using an immunofluorescence microscope.

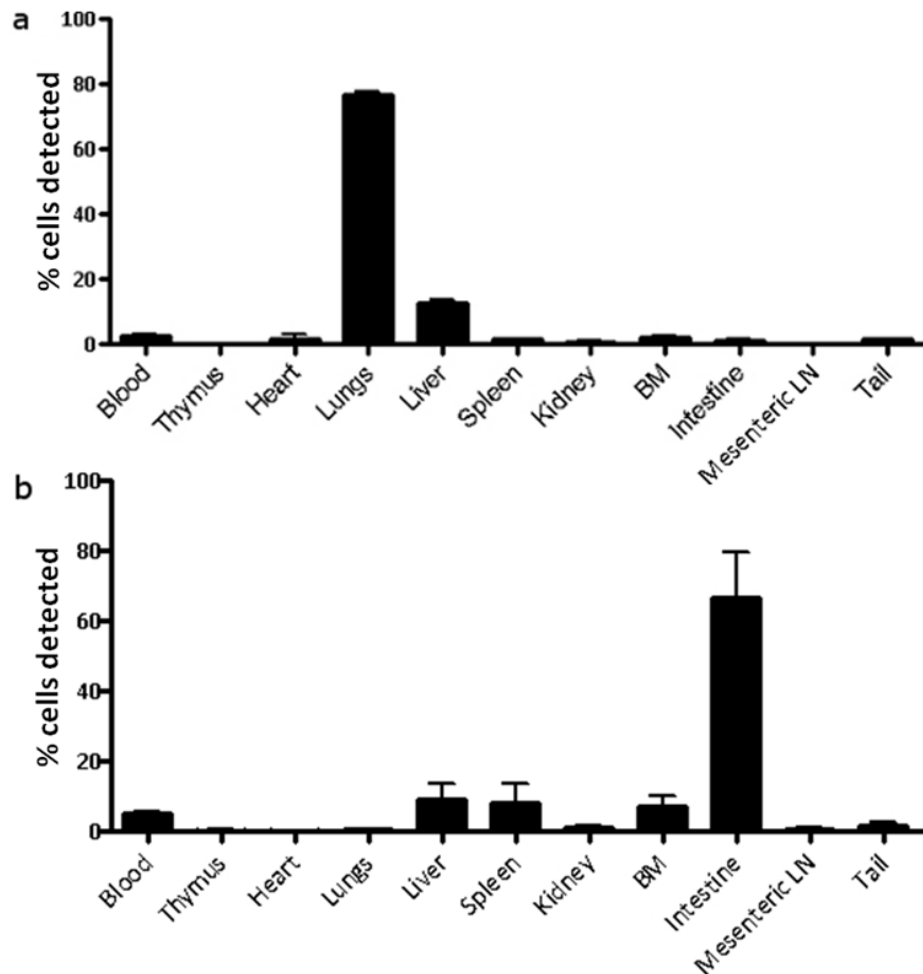


Fig 3.11. 2.5×10^5 Indium¹¹¹ labelled MSCs injected i.v. or i.p. into naïve mice.

Fig 3.11.a Indium¹¹¹ labelled MSCs were injected i.v. 4 hours after administration, the relative levels of radioactive cells were measured throughout the mice. Integrated absorbance of segmented specimens was determined using a gamma counter over 100-500 KeV energy range. Mice were divided into 12 sections. Distributed percentages of MSCs throughout the body are shown in each case. Results are expressed as the mean levels $\pm$ SEM. n=3. Fig 3.11.b. Indium¹¹¹ labelled MSCs were injected i.p. 4 hours after administration, the relative levels of radioactive cells were measured throughout the mice. Integrated absorbance of segmented specimens was determined using a gamma counter over 100-500 KeV energy range. Distributed percentages of MSCs throughout the body are shown in each case. Results are expressed as the mean levels $\pm$ SEM. n=3.

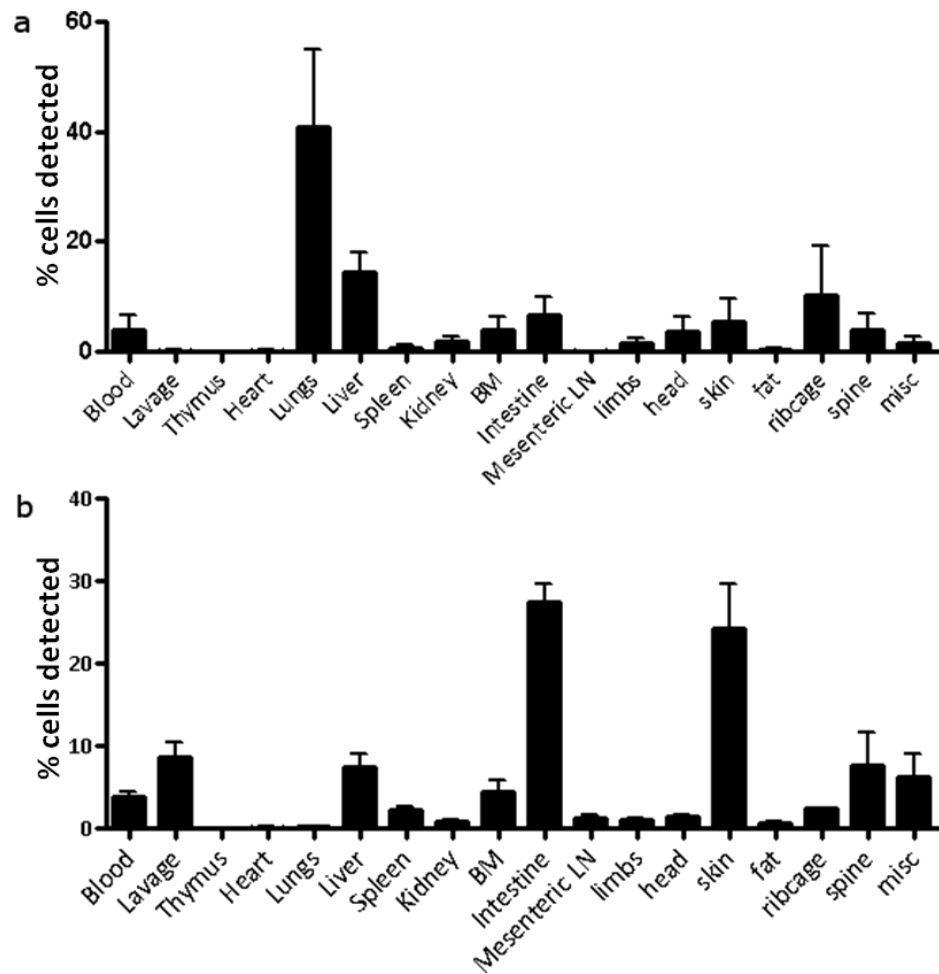


Fig 3.12. 1×10^5 Indium¹¹¹ labelled MSCs injected i.v. or i.p. into naïve mice.

Fig 3.12.a. Indium¹¹¹ labelled MSCs were injected i.v. 4 hours after administration, the relative levels of radioactive cells were measured throughout the mice. Integrated absorbance of segmented specimens was determined using a gamma counter over 100-500 KeV energy range. Mice were divided into 19 sections. Distributed percentages of MSCs throughout the body are shown in each case. Results are expressed as the mean levels $\pm$ SEM. n=4. Fig 3.12.b. Indium¹¹¹ labelled MSCs were injected i.p. 4 hours after administration, the relative levels of radioactive cells were measured throughout the mice. Integrated absorbance of segmented specimens was determined using a gamma counter over 100-500 KeV energy range. Distributed percentages of MSCs throughout the body are shown in each case. Results are expressed as the mean levels $\pm$ SEM. n=3.

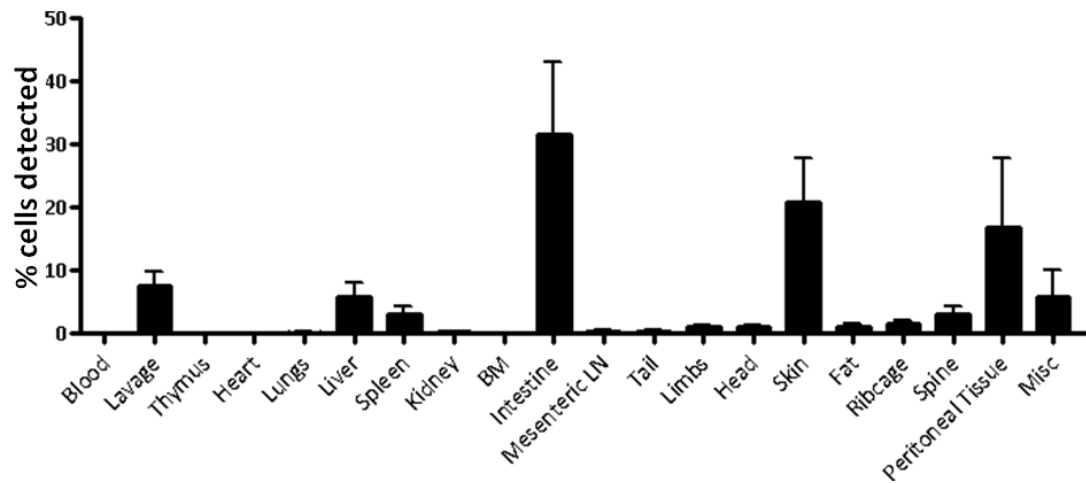


Fig 3.13. Injection of Indium¹¹¹ labelled MSCs into an LPS mouse model.

LPS was administered i.t. 1 hour before i.p injection of 2×10^5 MSCs. 4 hours after administration, the relative levels of radioactive cells were measured throughout the mice. Integrated absorbance of segmented specimens was determined using a gamma counter over 100-500 KeV energy range. Distributed percentages of MSCs throughout the body are shown in each case. Results are expressed as the mean levels $\pm$ SEM. n=4.

3.4 Discussion.

MSCs are generally isolated from tissues by adherence to plastic and expanded in a standard cell culture medium (e.g. DMEM) containing FCS. It is clear from the work presented here and what has been published previously that this generates a heterogeneous population of cells. The work presented here shows that for MSCs derived from bone marrow, by passage 5 the MSCs were devoid of contaminating haematopoietic cells. However, while these MSCs expressed markers of MSCs they were clearly not homogeneous.

Generally mMSCs are known to be CD45-, CD11b-, CD34-, CD29+, CD105+, Sca-1+ [287,291]. These markers were therefore used in order to ensure that mMSCs isolated in this chapter could reproducibly fit these criteria. Figs. 3.1 and 3.2 show representative marker expression on populations isolated using different methodology. These figures highlight the need for repeated passage in order to purify and deplete the population of CD45+ cells of the haematopoietic lineage. When culturing human bone marrow derived MSCs, the non-adherent CD45+ cells are lost following a single passage [115]. Fig 3.1.a illustrates how this is not the case with murine bone marrow, which at passage 2 has $75.33 \pm 4.88\%$ of its population staining positive for CD45. The CD45+ cells in murine bone marrow appear to be more persistent and it takes further passaging to deplete the population of these cells. This phenomenon may also be due to differences in methodology between human and murine bone marrow extraction. It was found that, at passage 5, the level of cells that stain positive for CD45 is at an acceptable level of $<5\%$ ($3.314 \pm$

2.039%, $2.95 \pm 2.21\%$, respectively). Attempts to deplete the population of CD45+ and CD11b+ cells using magnetic beads proved inefficient, with a high level of variability between depleted populations and passage 2 CD45 depleted bone marrow populations still containing within their number $29 \pm 25.24\%$ of cells that were either CD45+ or CD11b+. The P5 bone marrow mMSCs do not express CD45, CD11b or CD34, but do express CD29, CD105 and Sca-1. However, CD105 and Sca-1 only appear to be expressed on a sub-population of these cells, demonstrating that the population remains heterogeneous. This is consistent with previous reports stating that mMSCs are a heterogeneous population [127,292].

Continued culture of MSCs *in vitro* to increase population numbers may have detrimental consequences with regards to maintaining the beneficial properties of the MSCs for use in clinical therapies. These properties include the ability of MSCs to differentiate into specific lineages, migrate to sites of injury, and their immunomodulatory capacity [293]. In order to be classified as MSCs, as well as the correct marker profile, the cells must also proliferate on plastic surfaces and differentiate into chondrocytes, adipocytes and osteoblasts. Thus, the status of P5 bone marrow mMSCs was confirmed by their proliferative capacity and ability to differentiate down the 3 required lineages upon correct stimulus. However, of note it was clear that not all of the MSCs differentiated, again showing that not all of the cells in each population are true stem cells. Thus, it is most likely that MSCs isolated and expanded in this way are a mixture of stem and progenitor cells.

The finding that all chemokine receptors expressed on these populations are only found on a percentage of the cells further suggests that this is a heterogeneous

population. Late passage bone marrow mMSCs express much lower levels of chemokine receptors on their surface when compared to their early passage counterparts. This can be seen in Fig. 3.7 in which a number of chemokine receptors show a significantly reduced level of expression on the surface of P25 bone marrow mMSCs in comparison to P5 bone marrow mMSCs (CXCR2, CXCR3, CXCR4, CXCR6, CCR9). The most notable of these reductions was CXCR4. As previously noted, the CXCL12/CXCR4 axis plays a critical role in the trafficking of several sets of stem cells around the body [294]. This reduced chemokine receptor expression may be a result of changes to their phenotype due to excessive passaging, resulting in an increased number of cells actively dividing which in turn inhibits their ability to migrate [168]. Preliminary data looking at P5 bone marrow mMSC populations stained with mAbs for both CXCR3 and CXCR4 suggest that there is no double staining. This infers that the mMSCs that are CXCR4 positive are a distinct population to mMSCs which are CXCR3 positive. Further studies to investigate and compare the phenotypes of these sub-populations may help identify homogeneous populations of MSCs for use in treating disorders with different chemokine expression profiles.

Interestingly, while there were dramatic differences with respect to external expression of chemokine receptors, internal expression levels were consistent. One hypothesis for future research is that these intracellular levels can be up-regulated to be expressed on the surface of the cells, thus, creating a population of MSCs that can proliferate rapidly to obtain sufficient numbers for clinical applications efficiently, whilst maintaining their ability to home to sites of injury or inflammation. Preliminary studies carried out, did not reveal rapid up-regulation of this pool of chemokine receptors when MSCs were stimulated with a range of inflammatory

cytokines (data not shown). Chamberlain *et al* reported high levels of CXCR3 and CXCR6 on the surface of bone marrow derived mMSCs, consistent with levels reported here [134]. However, they found no expression of CXCR4 on the cell surface. This discrepancy is likely to be due to different culture protocols, in particular the use of DMEM as a culture medium and not Mesencult.

The functionality of the external receptors on P5 and P25 bone marrow mMSCs was compared in Fig. 3.9. Here, it was confirmed that the loss of chemokine receptor expression on the surface of P25 bone marrow mMSCs does result in a loss of chemokine dependent motility. From this it is apparent that cells which have undergone excessive passaging are not suitable for systemic administration in clinical therapies because they may lose their ability to migrate into specific areas of injury within tissue. They may function if administered locally, but it has not yet been assessed whether this *in vitro* culture methodology results in the loss of further surface molecules, including adhesion molecules, such as VCAM-1 [295]. These factors play a critical role for the engraftment of MSCs at target sites and are thus important to consider when choosing the population of stem cells to use for therapies [296]. In Fig. 3.9. it is clear that P5 bone marrow mMSCs have a greater capacity to migrate to the ligands for CXCR3 and CXCR4 (CXCL10 and CXCL12, respectively) than the ligands for CXCR6 and CCR10 (CXCL16 and CCL27, respectively). This finding may have implications in directing MSCs to sites of different injuries which propagate the release of different chemokines at the site.

There has been much debate in the literature as to the exact nature of MSCs. In particular some reports suggest that both pericytes [74] and fibroblasts [297]

have MSC-like functionality when cultured under certain conditions. Whilst the nomenclature of the cells is not particularly important in comparison to their beneficial effects as cellular therapies, fibroblasts and in particular fibrosis can be detrimental in tissue repair [298]. Fig 3.10. confirms that the P5 bone marrow mMSCs that have been investigated in this chapter are phenotypically distinct from fibroblasts. There are obvious differences in expression levels of CXCR4, CXCR6 and CCR6, while levels of CXCR3, CCR9 and CCR10 are comparable. Most importantly, P5 bone marrow mMSCs do not express collagen-1, whereas P5 tracheal fibroblasts do (Fig. 3.10.b), suggesting that the P5 bone marrow mMSC are not fibroblast like. Of note, while collagen-1 is synthesised by chondrocytes and osteoblasts, it is not expressed by their progenitors [299]. Whilst P5 bone marrow mMSCs can differentiate into chondrocytes and osteoblasts, a lack of collagen-1 suggests that a percentage of the cells within the P5 bone marrow mMSCs are undifferentiated.

The *in vitro* data on P5 bone marrow mMSCs suggests that the cells should readily migrate to specific sites and organs *in vivo*. To investigate whether this *in vitro* data is applicable in *in vivo* models, P5 bone marrow mMSCs were radioactively labelled with Indium¹¹¹ and injected i.v. or i.p. into naïve mice (Fig. 3.11 and 3.12) and a murine model of acute lung inflammation (Fig 3.13). Due to the large size of mMSCs cultured on plastic (>10µm), there have been reports of mMSCs getting caught in the lungs following i.v. administration [134]. Consistent with this, 4 hours post administration of 2.5×10^5 P5 bone marrow mMSCs i.v., the majority of cells were found in the lungs ($76.75 \pm 6.84\%$), with $12.34 \pm 1.41\%$ also found in the liver. 4 hours post administration of 2.5×10^5 P5 bone marrow mMSCs i.p., the majority of cells ($66.48 \pm 13.11\%$) were associated with the intestines, with significant levels also

detected in the liver, spleen and bone marrow. It is known that the lungs, liver, lymph nodes, spleen and bone marrow all express high levels of CXCL12 [300]. Therefore, consistent with the original hypothesis, these data suggest that CXCL12 generated at sites of inflammation and injury may aid MSC recruitment and suggest that one strategy to enhance recruitment could be to stimulate CXCL12 expression at these sites, or deliver exogenous chemokines to these sites. The high levels in the lungs may be due to cellular entrapment in the pulmonary capillary network, which may be problematic if repeated in man. The large numbers associated with the intestines of mice which received the cells i.p. may be a result of the cells not migrating but adhering locally. The variation in mMSC distribution patterns demonstrates that the way in which mMSCs are administered is critical were these cells to be used as clinical therapies and reach the desired target organ in man. In an attempt to overcome the potential problem of large cell numbers in the lungs after 4 hours when administered i.v., lower mMSC numbers were given both i.v. and i.p. and the distribution patterns compared. In addition, more tissues were assessed to try and account for all the MSCs injected. The results of this can be seen in Fig. 3.12. Importantly, in Fig 3.12.a. it is notable that the number of mMSCs in the lungs 4 hours after administration was $40.83 \pm 14.13\%$, a significant reduction when compared to levels following i.v. administration of 2.5×10^5 mMSCs ($76.75 \pm 6.84\%$). The distribution among other tissues in both figures (Fig. 3.12.a. and 3.12.b.) is markedly increased. Therefore, administration of lower numbers of MSCs may be beneficial for clinical applications, as higher numbers of MSCs may reach the preferred target. Again, significant levels of mMSCs are found in the liver, bones and bone marrow in both experiments, suggesting that CXCL12 may be guiding mMSC

migration due to surface expression of CXCR4. As illustrated in Fig. 3.5., only $46.33 \pm 15.57\%$ of the P5 bone marrow mMSCs express CXCR4 on the cell surface. This may explain why not all cells are migrating to these organs. Interestingly, similar levels of mMSCs were found in the blood of mice when administered i.p. or i.v. This shows that the cells are actively migrating even 4 hours post administration. This also infers that any effect of the mMSCs may not be apparent in the 4 hour timeframe which these experiments were limited to due to the terms of the licence. Also of note is the high levels of mMSCs found in the skin of mice that were given the cells i.p. It is known that the skin expresses high levels of CXCL10 [301], a ligand of CXCR3. Fig 3.9 demonstrated that P5 bone marrow mMSCs could migrate in response to CXCL10 in an efficient manner. One explanation could be that mMSCs which express CXCR3 on the surface ($41.48 \pm 16.81\%$) preferentially migrate to tissue expressing ligands of CXCR3, such as the skin. However, this was only seen when cells were given i.p. Therefore, point of administration clearly impacts on distribution pattern. These findings have implications with regards to mMSC sub-population selection, with a view to treating different site-specific injuries and diseases. An example of this is psoriasis, a skin disease and potential target for MSC therapies. By sorting the P5 bone marrow mMSCs which positively express CXCR3 on their surface, the number of mMSCs recruited to the skin may be increased. Likewise, for diseases of the liver, lungs, spleen and bones, P5 bone marrow MSCs which express CXCR4 on their surface could offer greater levels of engraftment at the site of injury.

To investigate whether culture expanded MSCs could traffic to a site of inflammation, P5 bone marrow mMSCs were injected i.p. into an murine model of acute inflammation in the lung. LPS administered i.t. induces chemokine production

in the lungs [302]. In Fig. 3.11.b. and 3.12.b., it was demonstrated that when P5 bone marrow mMSCs are administered i.p., no cells are found in the lungs 4 hours post administration. This may however be due to the short time period given to allow cells to migrate from the peritoneal cavity. Future research could focus on the distribution of P5 bone marrow mMSCs at later time points and in response to different models of injury or inflammation.

4.

**Characterisation and comparison of human MSCs
from different tissues.**

4. Characterisation and comparison of human MSCs from different tissues.

4.1 Introduction.

Having established the profile of chemokine receptors expressed by mMSC, comparisons were made with hMSCs isolated from different sources. MSCs have been isolated from various tissues, but questions remain as to whether these different subsets can actually be classified as the same cell type and whether their suitability for clinical therapies is consistent across populations from different sources. Identification of mesenchymal progenitors in adult human blood [303] and subsequent identification in term cord blood [62] prompted research into the presence of MSCs in early fetal life. MSCs are closely associated with haematopoietic stem cells (HSCs) in adult bone marrow. High levels of HPCs in first trimester fetal blood [69] suggested that due to their role in development, the frequency of stem cells in fetal tissue may be greater than that of adult human tissue [70]. Campagnoli *et al* originally isolated MSCs from human 1st trimester blood, liver and bone marrow [63]. Since then, 2nd trimester fetal liver, lungs, spleen, pancreas and kidney have also been found to be rich sources of MSCs [304]. In this chapter, MSC populations are assessed that were derived from human fetal bone marrow, fetal blood, placenta, 1st and 2nd trimester amniotic fluid.

Stem cell therapies would ideally utilise stem cell populations with pluripotent differentiation and self renewal capacities, whilst not being

tumorigenic [305]. Fetal MSCs from 1st trimester blood and bone marrow have been reported to display several advantageous properties over their adult counterparts. Fetal blood and bone marrow MSCs have been shown to express pluripotency markers such as Oct-4, SSEA-3, SSEA-4 and Nanog, which are not expressed by adult blood or bone marrow derived MSCs [306]. They also elicit a greater proliferative capacity and maintain longer telomeres during repeated passage than their adult counterparts [80]. However, availability of MSCs from human fetal blood and bone marrow may be limited due to ethical objections. The human term placenta, a temporary organ with fetal contributions that is discarded post-partum has also been shown to host a set of MSCs which exhibit many markers that are common to adult bone marrow derived MSCs [66,81]. These are an ethically uncontroversial and easily accessible source of MSCs for research and clinical purposes.

Amniotic fluid has also been shown to be an abundant source of fetal MSCs. Amniotic fluid MSCs were first isolated from 2nd trimester amniotic fluid [82]. Amniotic fluid-derived MSCs are readily available from samples taken for amniocentesis. Further research into 1st trimester amniotic fluid-derived MSCs showed that they have a greater proven differentiation capacity than adult bone marrow derived MSCs, with the ability to differentiate into adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages [83]. These cells were also shown to express ES cell markers, such as Oct-4 and SSEA-4 [307], but importantly do not form teratomas *in vivo* [308]. These cells may be cultured under specific conditions to provide specific progenitor cells which may have higher success rates for resolving organ specific injuries and diseases.

There has been a large amount of research assessing the fate of MSCs administered *in vivo* and how they affect disease progression in both animal models and human clinical trials [309]. Typically, these MSCs exhibit low levels of engraftment within diseased or injured tissue [310]. However, the paracrine effect of these cells has been shown to alter the tissue micro-environment, which may be affecting tissue repair [311]. Therefore, the ability to enhance MSC numbers at the site of injury or inflammation is a highly advantageous property. In Chapter 3, it was established that chemokine receptor levels and the subsequent ability of MSCs to migrate towards chemokines vary according to how MSCs are passaged *in vitro*. This may impact on whether MSCs reach their desired target when used as cell therapies. Currently, the majority of clinical trials carried out using MSCs have used adult bone marrow derived MSCs (Fig. 1.4). However, there is increasing awareness of the stem cell populations in other tissues. This can be seen with an increased use of adipose derived MSCs in recent trials. Several government and commercial institutions are now banking human umbilical cord and tissue derived MSCs. There are currently no banking facilities for any of the MSC sources described in this chapter.

Fetal MSCs have been shown to proliferate at a greater rate than their adult counterparts [312]. For this reason, they could represent a better source for any therapeutic application in which large cell numbers are needed. However, the ability of these cells to home to, and be retained at the appropriate target site has yet to be fully investigated. Different injuries and diseases result in the production of different inflammatory mediators and chemokines. The likelihood that MSCs will reach the site of injury in different disease states may depend on the chemokine receptor profile of the population. To date, the chemokine receptor profile of MSCs derived

from these sources has not been investigated. This data should inform future researchers on the suitability of these different hMSC populations for their use in clinical trials for the treatment of diseases with different inflammatory profiles.

Studies were performed in collaboration with Dr. P. Guillot's lab, who has ethical approval for collection and has established methodology for expansion of these hMSC populations. All MSCs have been characterised and were shown to be CD105+, CD73+, CD29+, CD44+ with the potential to engraft and differentiate *in vivo* [313]. MSCs from different sources were all isolated via plastic adherence analysed at passage 5 so as not to be affected by the changes to proliferation rate and external chemokine receptor expression associated with long term passaging as described in Chapter 3.

4.2 Aims.

- To characterise populations of hMSCs derived from different tissues in terms of chemokine expression.
- To compare chemokine receptor profiles of P5 hMSCs to P5 bone marrow mMSCs.
- To investigate functionality of any differences in chemokine receptor expression between MSC populations.

4.3 Results.

In order to investigate the migratory potential of the hMSCs of differing fetal origin, the chemokine receptor profiles for each set of MSCs was characterised using flow cytometry. In Fig. 4.1.a. it can be seen that over 45% of 1st trimester amniotic fluid-derived hMSCs express either CXCR1 ($45.07 \pm 22.18\%$), CXCR3 ($64.02 \pm 5.99\%$), CXCR4 ($48.02 \pm 13.43\%$), CXCR6 ($49.39 \pm 18.77\%$), CCR9 ($60.16 \pm 15.30\%$) or CCR10 ($54.60 \pm 9.77\%$) on the cell surface. There was expression of CXCR2 ($16.54 \pm 8.87\%$) and CCR3 ($10.07 \pm 1.42\%$) on these cells, although at much lower levels. There was little/no expression of CCR2, CCR4, CCR6 and CCR7 on the surface of the cells. The receptors expressed on the highest proportion of cells was CXCR3 and CCR9, which were both expressed by >60% of the 1st trimester amniotic fluid-derived MSC population. Representative histogram plots for each chemokine receptor investigated can be seen in Fig. 4.1.b. For each of the highly expressed receptors (>45%) it is a sub-population of MSCs which are expressing the receptor on the cell surface.

The chemokine receptor profile on P5 2nd trimester amniotic fluid-derived hMSCs can be seen in Fig. 4.2.a. This shows that CXCR3 ($52.12 \pm 13.37\%$), CXCR4 ($43.79 \pm 1.33\%$), CXCR6 ($41.80 \pm 13.13\%$), CCR9 ($61.95 \pm 10.34\%$), and CCR10 ($42.02 \pm 10.32\%$) were all expressed by >40% of the hMSC population. There was expression at a lower level for CXCR1 ($29.93 \pm 7.93\%$), CXCR2 ($18.15 \pm 6.81\%$) and CCR7 ($17.92 \pm 9.60\%$). There was low expression of CCR3 ($11.58 \pm 3.81\%$) and CCR4 ($6.07 \pm 3.44\%$) on this MSC population and there was no expression of CCR2 and

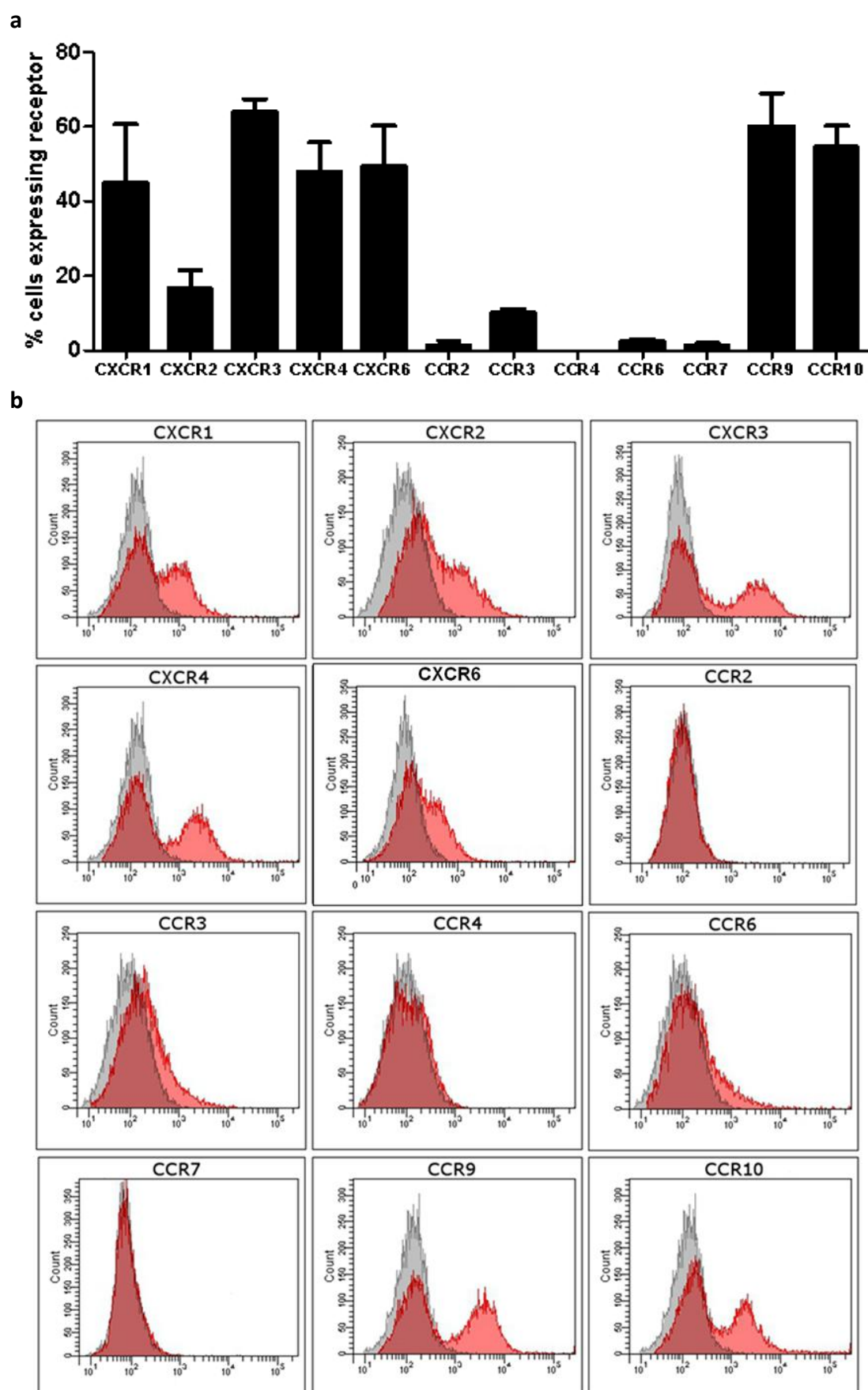


Fig. 4.1. Comparative external chemokine receptor expression and binding intensity of 1st trimester amniotic fluid-derived hMSCs.

Fig. 4.1.a shows the external chemokine receptor profile for 1st trimester amniotic fluid-derived hMSCs, determined by FACS analysis. Fig. 4.1.b shows representative FACS histograms for each of the external chemokine receptors. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3.

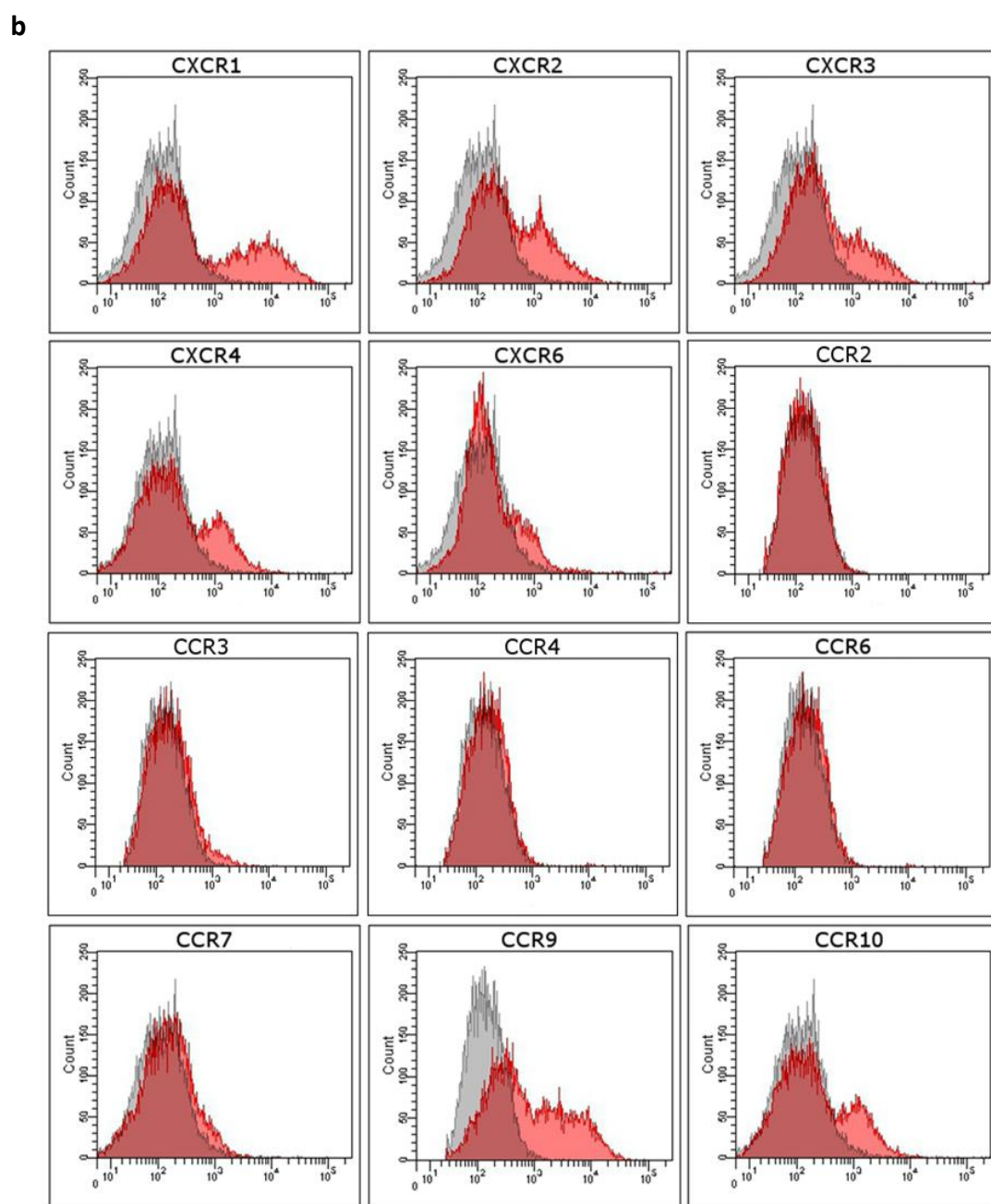
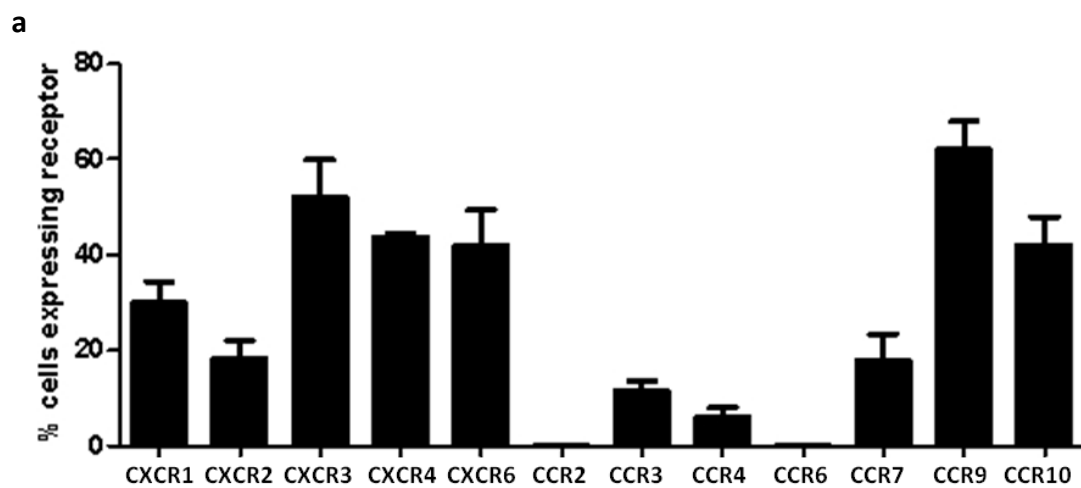


Fig. 4.2. Comparative external chemokine receptor expression and binding intensity of 2nd trimester amniotic fluid-derived hMSCs.

Fig. 4.2.a shows the external chemokine receptor profile for 2nd trimester amniotic fluid-derived hMSCs, determined by FACS analysis. Fig. 4.2.b shows representative FACS histograms for each of the external chemokine receptors. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3.

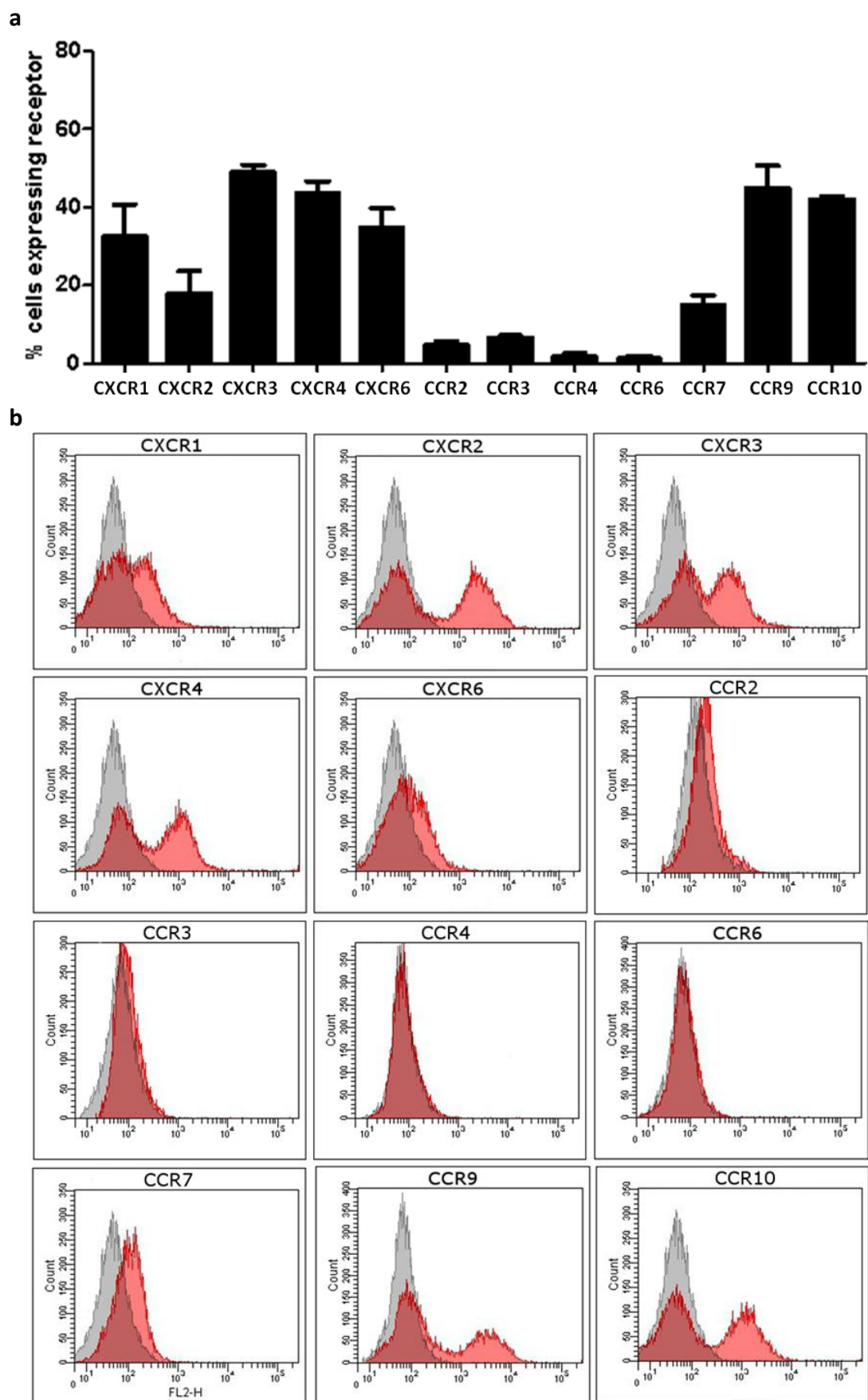


Fig. 4.3 Comparative external chemokine receptor expression and binding intensity of fetal bone marrow derived hMSCs.

Fig. 4.3.a shows the external chemokine receptor profile for fetal bone marrow derived hMSCs, determined by FACS analysis. Fig. 4.3.b shows representative FACS histograms for each of the external chemokine receptors. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3.

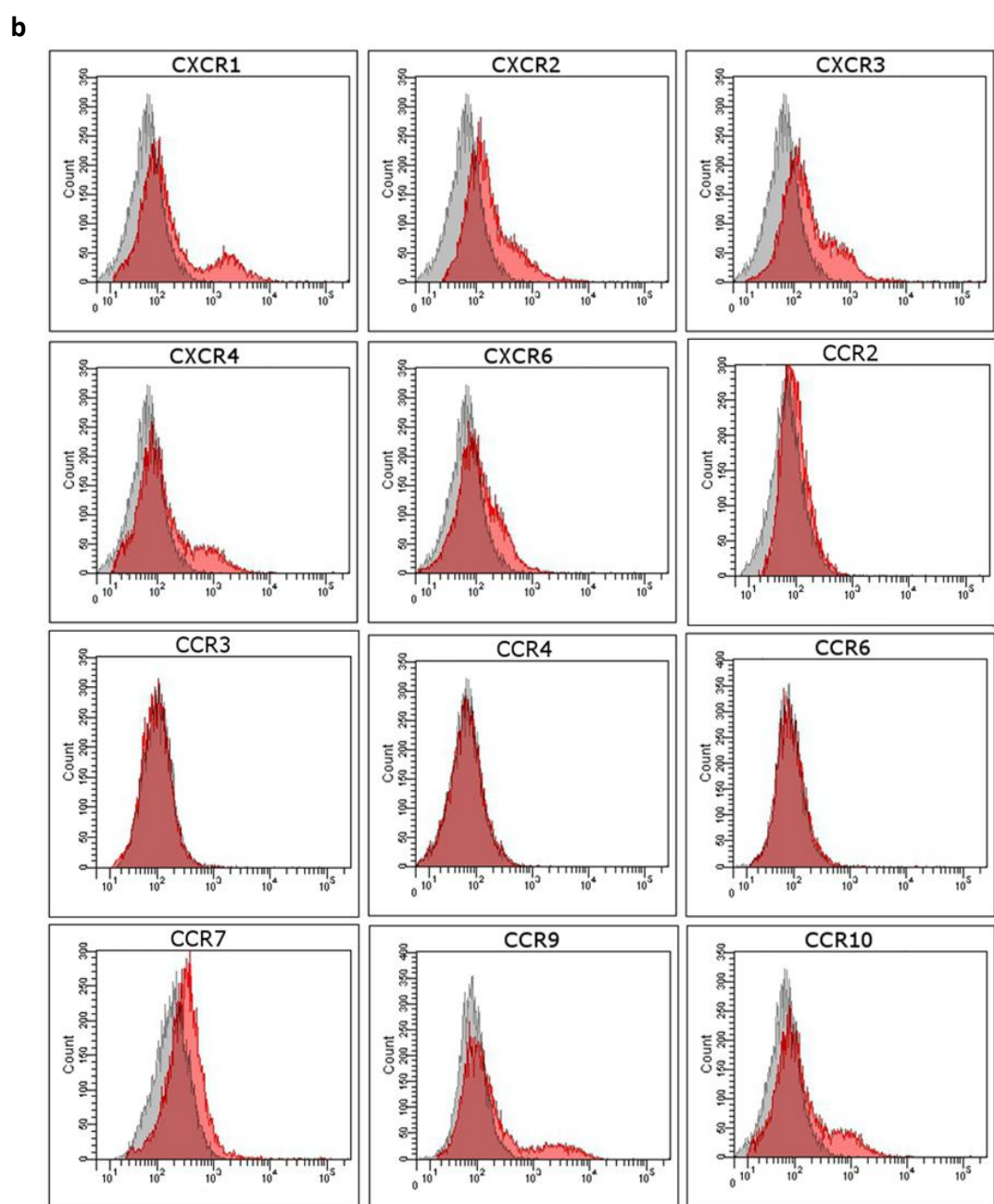
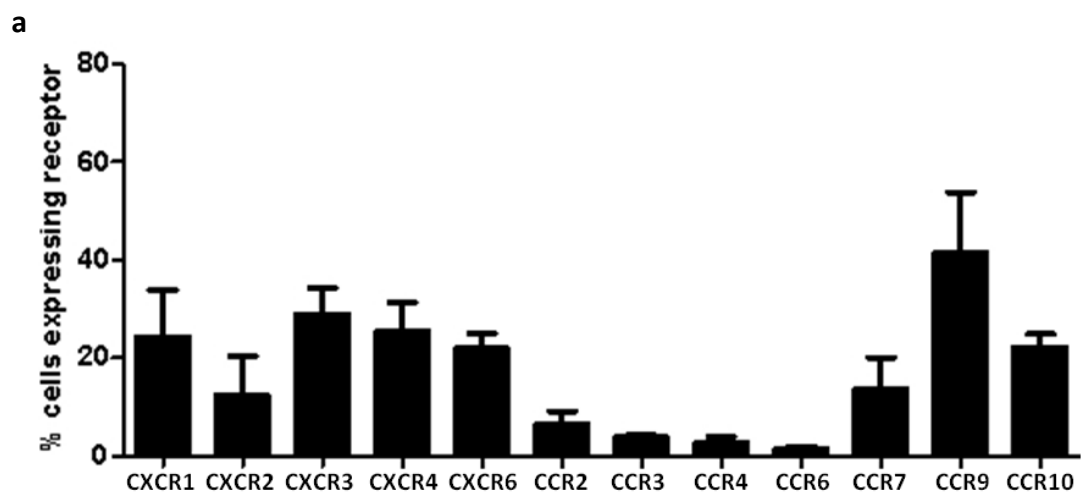


Fig. 4.4. Comparative external chemokine receptor expression and binding intensity of fetal blood derived hMSCs.

Fig. 4.4.a shows the external chemokine receptor profile for fetal blood derived hMSCs, determined by FACS analysis. Fig. 4.4.b shows representative FACS histograms for each of the external chemokine receptors. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3.

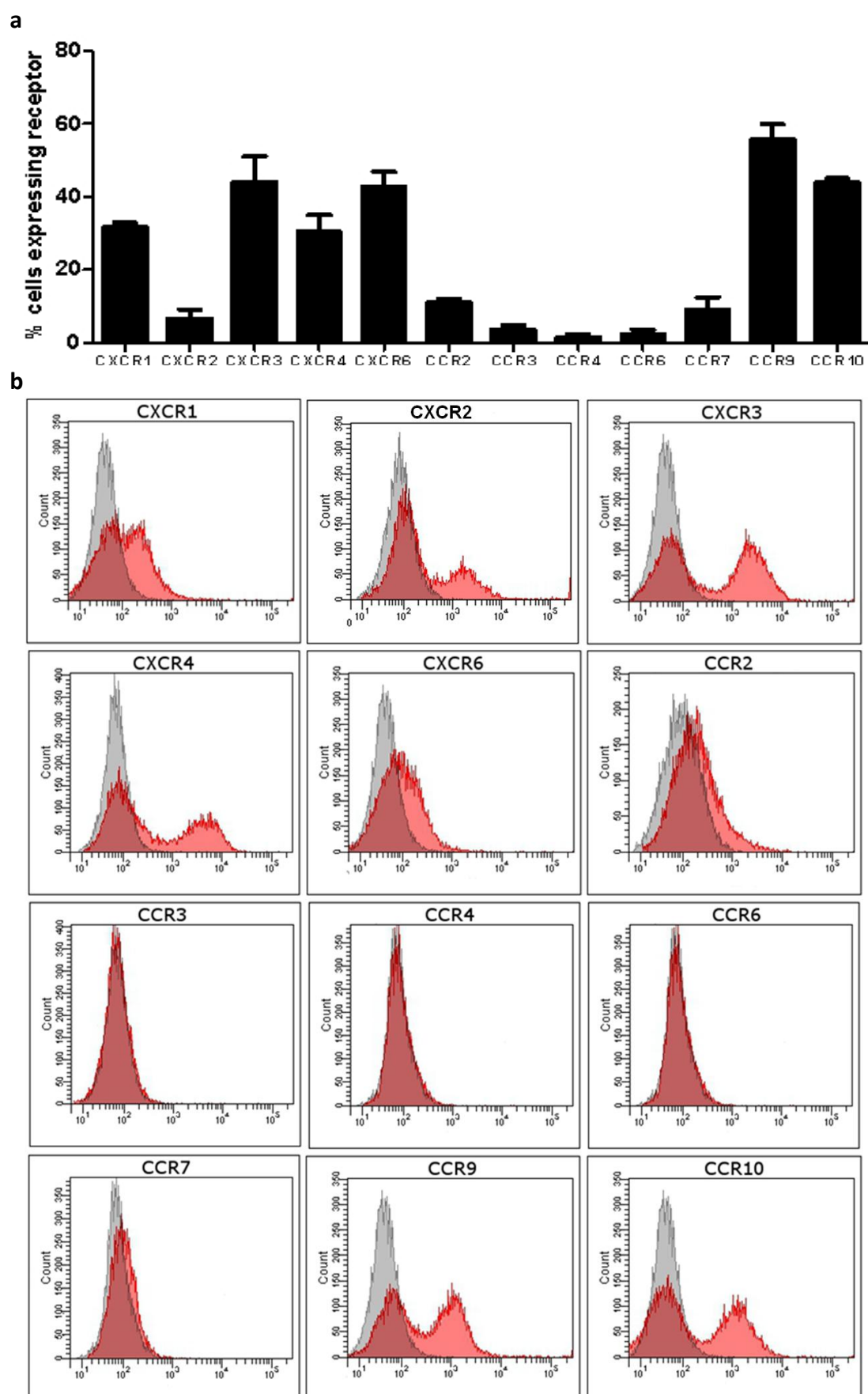


Fig. 4.5. Comparative external chemokine receptor expression and binding intensity of placental derived hMSCs.

Fig. 4.5.a shows the external chemokine receptor profile for placental derived hMSCs, determined by FACS analysis. Fig. 4.5.b shows representative FACS histograms for each of the external chemokine receptors. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 3.

CCR6. The highest expressed receptors on this population were CXCR3 and CCR9, both being expressed by over 50% of the MSC population. Fig. 4.2.b. shows representative histogram plots for each of the receptors analysed. Each of the highly expressed receptors (CXCR3, CXCR4, CXCR6, CCR9, CCR10) appear to be expressed by a defined sub-population of the 2nd trimester amniotic fluid-derived hMSCs. The chemokine receptors expressed at lower levels (CXCR2, CCR3, CCR4, CCR7) did not appear to be expressed on a defined sub-population.

The comparative external chemokine receptor expression on fetal bone marrow derived MSCs isolated by plastic adherence alone at P5 can be seen in Fig. 4.3.a. This showed that CXCR3 ($48.96 \pm 3.27\%$), CXCR4 ($43.62 \pm 5.36\%$), CCR9 ($46.97 \pm 9.88\%$) and CCR10 ($42.10 \pm 1.14\%$) were expressed on the surface of these hMSCs. CXCR1 ($32.44 \pm 11.66\%$) and CXCR6 ($34.91 \pm 8.40\%$) were also expressed on a significant proportion of the cells. There was low levels of expression of CXCR2 ($17.97 \pm 9.99\%$) and CCR7 ($15.07 \pm 3.41\%$), and very low or no expression of CCR2, CCR3, CCR4 and CCR6 on the surface of these cells. The most highly expressed receptors on this population are CXCR3 or CCR9 which were both expressed by nearly 50% of the population. Fig. 4.3.b. shows representative histogram FACS plots for each of the chemokine receptors analysed. Of the 12 plots, 6 show that the expression of the respective chemokine receptors is on a defined sub-population of the fetal bone marrow derived hMSCs, as illustrated by a double peak in their respective histograms. These 6 histogram plots represent the 6 most highly expressed receptors on this population, namely CXCR1, CXCR3, CXCR4, CXCR6, CCR9 and CCR10. The comparative external chemokine receptor expression on P5 fetal blood derived hMSCs isolated by plastic adherence alone can be seen in Fig. 4.4. Fig.

4.4.a. shows that there is significant expression of CXCR1 ($24.36 \pm 13.48\%$), CXCR3 ($28.82 \pm 9.60\%$), CXCR4 ($25.41 \pm 10.40\%$), CXCR6 ($21.96 \pm 5.52\%$), CCR9 ($41.59 \pm 21.25\%$), CCR10 ($22.25 \pm 4.70\%$), each of which are expressed on the surface of more than 20% of the cell population. There is expression, at a lower level, of CXCR2 ($12.40 \pm 13.75\%$) and CCR7 ($13.57 \pm 9.33\%$), each of which are present on the surface of more than 10% of the population. There is little or no expression of CCR2, CCR3, CCR4 and CCR6. CCR9 is expressed on a higher proportion of the population than any of the other chemokine receptors, with over 40% of the cells expressing the receptor on the cell surface. Fig. 4.4.b. shows representative histogram FACS plots for each of the chemokine receptors analysed. Of the 12 plots, 4 show clearly that the expression of the respective chemokine receptor is on a defined sub-population of the fetal blood derived hMSCs, as illustrated by a double peak in the FACS histogram plots. These 4 histogram plots are that of CXCR1, CXCR4, CCR9 and CCR10. The respective histogram plots of CXCR3 and CXCR6 however show a definite shift but no clear double peak and thus, it cannot be suggested that these receptors are solely expressed on a sub-population of this set of hMSCs.

The external chemokine receptor expression on P5 placental derived hMSCs isolated by plastic adherence alone can be seen in Fig. 4.5. Fig. 4.5.a shows that CXCR3 ($44.13 \pm 12.08\%$), CXCR6 ($42.82 \pm 6.95\%$), CCR9 ($55.77 \pm 7.10\%$) and CCR10 ($44.03 \pm 1.99\%$) are expressed by over 40% of the hMSC population. CXCR1 ($31.64 \pm 2.26\%$) and CXCR4 ($30.4 \pm 7.92\%$) are also significantly expressed with over 30% of the cells expressing each of the receptors. The remaining 6 chemokine receptors which were analysed; CXCR2, CCR2, CCR3, CCR4, CCR6 and CCR7, are not significantly expressed on the surface of these cells. All 6 are expressed by <10% of

the population. Of all the receptors analysed, CCR9 is expressed on the highest proportion of the placental derived hMSCs. Fig. 4.5.b. shows representative histogram plots for the external chemokine receptors analysed. Of the 6 receptors that stained positively, CXCR1, CXCR3, CXCR4, CCR9 and CCR10, all have double peaks showing that the positive cells represent a sub-population within the hMSC population. CXCR6 is the only positive receptor which doesn't appear to be solely expressed on a sub-population.

Having obtained external chemokine receptor profile datasets for hMSCs isolated from 5 separate sources using the same culture conditions, the profiles can now be compared. In doing this, the classification of these cells as MSC populations with matching phenotypes could be assessed, and any differences would provide insight into the suitability of each source for clinical application. This can be seen in Fig. 4.6. Fig. 4.6.a. shows the external chemokine receptor profile of the 5 sets of hMSCs. The general pattern of receptor expression is maintained across all subsets. There are however, variations in the levels of expression between the hMSCs from different sources. Of note is the lack of CCR7 expression on hMSCs derived from 1st trimester amniotic fluid. Of the 5 most abundantly expressed receptors across the hMSC sets, hMSCs derived from amniotic fluid show the highest percentage of positive cells, with 1st trimester amniotic fluid-derived hMSCs expressing the highest receptor levels for 4 out of 5 of them (CXCR3, CXCR4, CXCR6, CCR10). For all 5 of the highly expressed receptors, fetal blood derived hMSCs had the lowest percentage of cells positive for each receptor. The binding intensity of the chemokine receptor marker antibodies, as measured via the mean fluorescence intensity (MFI) has also been compared (Fig. 4.6.b). This gives an indication of the comparative levels of each

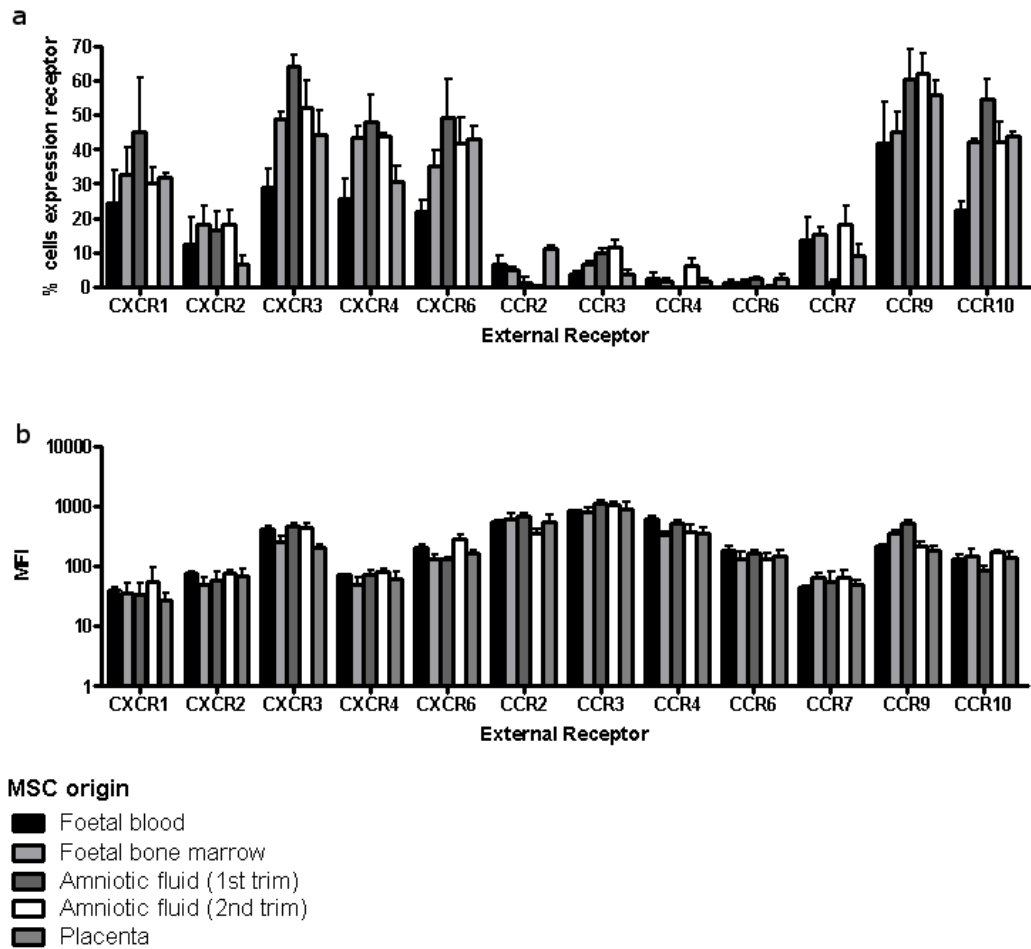


Fig. 4.6. Comparative external chemokine receptor expression and binding intensity of fetal hMSCs.

Fig. 4.6.a. shows the external chemokine receptor profile for various sources of fetal derived hMSCs, determined by FACS analysis. Fig. 4.6.b shows the pattern of external chemokine receptor binding intensity for the various receptors on the different cell sources. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. $n = 2-3$.

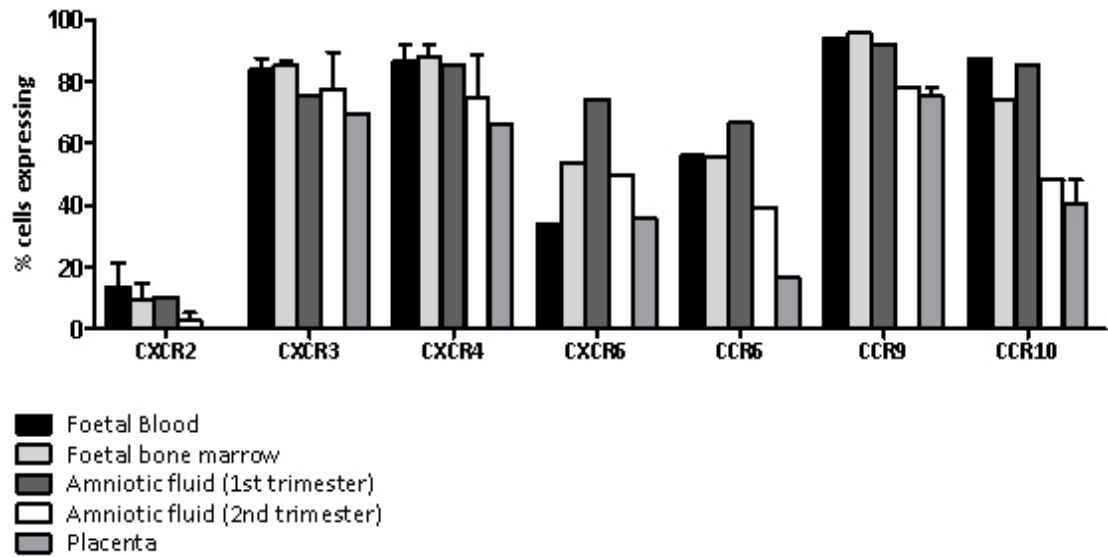


Fig. 4.7. Comparative intracellular chemokine receptor expression of fetal hMSCs.

Intracellular chemokine receptor profile for various sources of fetal derived hMSCs, determined by FACS analysis. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. n = 1-3.

chemokine receptor on individual cells. As with the percentages of cells expressing each receptor, the MFI levels are preserved across the hMSC sets for each receptor. There is no notable variation between the hMSCs derived from different cell sources. However, these MFI levels are a representation of the whole cell population and not necessarily representative of the chemokine expressing sub-population.

In Chapter 3, intracellular levels were investigated and demonstrated that mMSCs express high levels of chemokine receptors intracellularly. If these sets of hMSCs also display high levels of chemokine receptors intracellularly, there is the potential for upregulation of chemokine receptors on the cell surface. It is hypothesised that increased levels of chemokine receptors on the cell surface could lead to increased migratory activity toward the ligand for each relevant receptor. This has implications for clinical therapies where greater levels of MSCs may be directed to target sites via chemokine administration at the site. Fig. 4.7. shows a comparison of intracellular chemokine receptor expression on the 5 sets of hMSCs isolated from different sources. Of the 7 chemokine receptors analysed, 3 of the receptors were expressed at high levels (>70%) of all 5 hMSC subsets (CXCR3, CXCR4, CCR9). Fetal blood, fetal bone marrow and 1st trimester derived hMSCs expressed high levels of CCR10 (>70%), whereas a lower, but still significant percentage (40-50%) of 2nd trimester amniotic fluid and placental derived hMSCs expressed CCR10 intracellularly. CXCR2 was expressed at low levels on cells (>20%) in all hMSC groups. CXCR6 and CCR6 showed varying levels of intracellular expression across the 5 hMSC sets. CXCR6 was expressed on 80% of 1st trimester amniotic fluid-derived hMSCs, ~60% of fetal bone marrow and 2nd trimester amniotic fluid-derived hMSCs, and ~40% of fetal blood and placental derived hMSCs. CCR6 was expressed on 60-70% of fetal blood, fetal

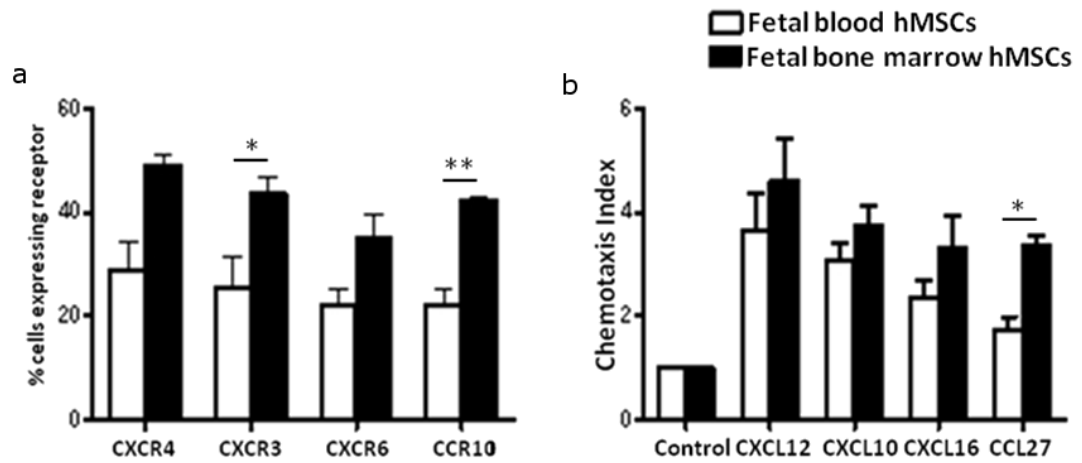


Fig. 4.8. Boyden chamber chemotaxis of human fetal blood and bone marrow derived MSCs towards CXCL12, CXCL10, CXCL16 & CCL27.

Fig. 4.8.a. Comparison of CXCR3, CXCR4, CXCR6 and CCR10 expression on the surface of fetal blood and fetal bone marrow derived hMSCs. Fig. 4.8.b. Boyden chamber chemotaxis of human fetal blood and human fetal bone marrow derived MSCs towards ligands of 4 highest expressed chemokine receptors (10ng/ml), as determined by external chemokine receptor FACS analysis. Expression levels are illustrated as the mean FACS measurements $\pm$ SEM. Statistical analysis using students t-test. * $p < 0.05$, and ** $p < 0.01$ between selected groups. $n = 3$.

bone marrow and 1st trimester amniotic fluid-derived hMSCs, 40% of 2nd trimester derived hMSCs and <20% of placental derived hMSCs.

The relevance of any subtle differences in external chemokine receptor levels in Fig. 4.6. must be assessed in order to demonstrate the clinical relevance of this novel data. To compare functionality of external chemokine receptor expression levels on hMSCs derived from different sources, chemotaxis assays were carried out. The chemotactic ability of hMSCs derived from fetal bone marrow and fetal blood at P5 can be seen in Fig. 4.8. Fig. 4.8.a. shows a comparison of 4 of the highest expressed receptors on the hMSCs between the fetal blood and fetal bone marrow derived hMSCs. Fetal bone marrow derived hMSCs express significantly higher levels of CXCR3 and CCR10 on their surface than fetal blood derived hMSCs. Fig. 4.8.b. illustrates the functionality of CXCR4, CXCR4, CXCR6 and CCR10 in chemotaxis assays to their respective ligands. Greater numbers of the fetal bone marrow cells migrate to each of the ligands (CXCL12, CXCL10, CXCL16 and CCL27). The difference is only significant for CCR10 migration to CCL27. However, for both sets of cells the number of cells that have chemotaxed to the 4 ligands is higher than basal levels.

For both sets of cells, the highest level of chemotaxis is to CXCL12. For both sets of these cells the corresponding receptor for CXCL12, CXCR4 is the highest expressed receptor. The functional relevance of this remains unclear. However, in order to further confirm the validity of mMSCs as a research model, the ability of the 5 sets of fetal hMSCs to produce CXCL12 was investigated. CXCL12 levels in the culture media of P5 cells cultured for 72 hours were measured (Fig. 4.9). All 5 sets of cells create large amounts of CXCL12. Of note, there are no significant differences in

the levels of CXCL12 produced by the 5 sets of hMSCs isolated from different sources, as compared by one-way ANOVA followed by Bonferroni multiple-comparisons test.

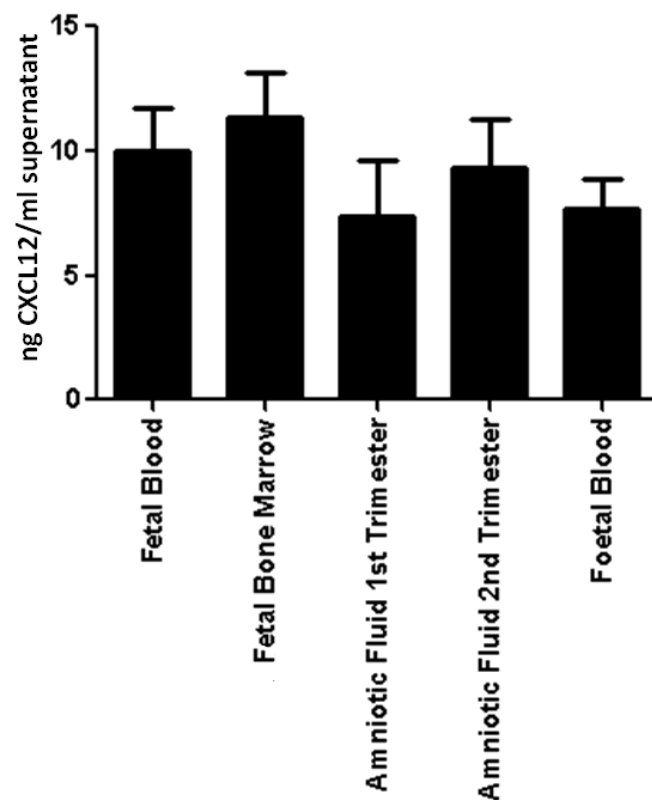


Fig. 4.9. CXCL12 production by P5 hMSCs obtained from different sources.

CXCL12 levels in hMSC supernatants were measured using ELISAs after 3 days culture *in vitro* in T75 flasks. Levels are quantified per ml of supernatant. Expression levels are illustrated as the mean ELISA measurements $\pm$ SEM. $n = 4$.

4.4 Discussion.

Having determined that mMSCs express a distinct profile of chemokine receptors, comparisons were made with human adult bone marrow MSCs and then MSCs from a range of fetal tissue sources [134]. The external chemokine receptor profile has been characterised for all 5 sets of hMSCs investigated in this chapter. Strikingly, each set of P5 hMSCs displays a similar external chemokine receptor profile. This general trend can be seen in Table. 4.1.

Table. 4.1. Expression of external chemokine receptors on hMSCs.

Expression Level	External Receptors
High (>40%)	CXCR3, CXCR4, CXCR6, CCR9, CCR10
Mid (10-40%)	CXCR1, CXCR2, CCR7
Low/Negative (<10%)	CCR2, CCR3, CCR4, CCR6

Not only does this novel data suggest that hMSCs from different sources are highly conserved in their phenotype with respect to chemokine receptor expression, the subtle differences between populations may be important to consider when deciding which hMSC populations are to be used for different cell therapies. Of the 5 highest expressed receptors across the hMSC populations, amniotic fluid-derived hMSCs show the highest expression levels, with 1st trimester amniotic fluid-derived hMSCs expressing the highest levels of CXCR3, CXCR4, CXCR6 and CCR10 of all 5 populations. These 4 receptors were investigated in terms of chemotactic ability on P5 bone marrow mMSCs due to the high levels of expression on the surface of the

mMSCs. Of the 5 hMSC populations, fetal blood derived hMSCs have the lowest expression levels for each of the receptors.

The variations in chemokine receptor levels across the hMSC populations investigated in this chapter were not shown to be functional when comparing the ability of human fetal blood and human fetal bone marrow derived hMSCs to chemotax to CXCL12, CXCL10, CXCL16 (ligands for CXCR4, CXCR3, and CXCR6 respectively). Representative histogram plots of these populations show that the cells which stain positive for CXCR1, CXCR2, CXCR3, CXCR4, CCR9 and CCR10 represent a sub-population of the total hMSC populations. It is unknown whether these sub-populations express multiple chemokine receptors or a single receptor. Future experiments could investigate whether greater numbers of hMSCs will migrate to media containing multiple chemokines of interest. This has clinical relevance when hoping to direct high numbers of hMSCs to a defined region [314]. Further investigation of these chemokine receptor positive subsets may also identify homogeneous sub-populations of hMSCs or mesenchymal progenitor cells (MPCs). In terms of hMSCs from different sources, the subtle differences in chemotactic ability between fetal blood and fetal bone marrow derived hMSCs suggests that stem cell populations which express higher levels of chemokine receptors will migrate more efficiently to sites of injury or inflammation. Considering this, it can be hypothesised that human amniotic fluid hMSCs, particularly 1st trimester amniotic fluid-derived hMSCs are the most suitable of the 5 populations analysed for use in clinical therapy. Although there were no notable differences in the MFI of whole populations, further analysis of the chemokine receptor positive sub-populations

and the changes in MFI between sources may show differences in levels of expression at a cellular level.

Whilst the external chemokine receptor profile of hMSCs is consistent with that of mMSCs reported in Chapter 3, there are some differences to previously reported levels on adult bone marrow derived hMSCs. Chamberlain *et al* reported high levels of CCR3 and CCR2 on the cells surface, which were not expressed on the fetal hMSCs described here. They did however see high levels of CXCR3, CXCR6 and CCR9, consistent with levels reported here [134]. Sordi *et al* also report expression of CXCR3, CXCR6 and CCR9 on the cell surface, although at much lower levels [315]. These differences can be attributed to differences in the duration of culture and the seeding density.

Currently umbilical cord blood and adult bone marrow derived MSCs are the most commonly banked MSC populations [77]. Of the 5 sources of hMSCs described in this chapter, there are ethical concerns regarding the clinical use of fetal blood and fetal bone marrow derived hMSCs. However, amniotic fluid and placental derived hMSCs are generally discarded materials. Currently thousands of pregnant women undergo amniocentesis procedures annually [316]. Unused amniotic fluid could be used to culture cells for banking to provide an alternative source of potentially viable hMSCs for future cell therapies.

The paracrine effects of MSCs are thought to be playing a role in resolving injury and inflammation. For this reason, CXCL12 levels produced by hMSCs from the 5 different sources are compared. All 5 populations produce large amounts of CXCL12 when cultured *in vitro*. However, there are no significant differences in

levels between the populations. Future experiments will examine the secretome of MSCs derived from different sources. The expression of chemokine receptors on the surface of all MSC populations investigated, subsequent chemotactic ability and paracrine effects suggest that any one of these populations is a good candidate for clinical use. Further interrogation of the heterogeneous hMSC populations may provide homogeneous populations of MSCs which are better suited for the treatment of specific diseases, depending on the specific range of chemokines produced as a result of said diseases or injuries. All of the data from the hMSC populations in Chapter 4 is highly consistent with the data on P5 bone marrow mMSCs in Chapter 3, adding further confidence in the use of these cells as a model for hMSCs.

Of note these studies indicate that the profile of chemokine receptors expressed by hMSCs is consistent across different tissue sources of these MSCs. Moreover this pattern is consistent across species. These studies indicate that MSCs isolated by this methodology are not a homogeneous population as there are clear sub-populations expressing specific chemokine receptors. Assuming that chemokine receptors orchestrate the recruitment of MSCs to sites of injury or inflammation, it can be deduced that specific sub-populations of MSCs may be recruited to sites expressing defined chemokines. It will be important therefore to further characterise these sub-populations with respect to their ability to differentiate into specific lineages and their secretome.

5.

Characterisation of P α S cells.

5. Characterisation of PαS cells.

5.1 Introduction.

There are no specific markers for murine MSCs. Thus different research groups often have conflicting data as they compare heterogeneous and therefore different populations. This is often as a result of variations in isolation techniques [275,276,317]. To date the most consistent way to isolate and characterise mMSCs has been isolation via depletion assays in addition to plastic adherence, for analysis at early passage. This is problematic, as it is also known that persistent passaging of MSCs may cause phenotypic and functional changes in MSCs [129]. Plastic is an unnatural material for cells to grow on, and culture conditions are generally optimised for proliferation rather than the maintenance of MSCs. As demonstrated in Chapters 3 and 4, these culture methods result in a heterogeneous population of adherent cells that contain a percentage of unidentified, putative stem cells. This makes it very difficult to predict how MSCs are acting *in vivo* [115]. This is not the case for other stem cells. HSCs can be identified via surface markers, sorted and transplanted without ever undergoing culture. This has resulted in a rapid growth of knowledge of HSC biology and how they function *in vivo* [318,319]. It can be hypothesised that identification of specific mMSC markers will result in a similar rapid expansion in the knowledge of MSC biology. mMSCs are far more difficult to isolate and more susceptible to haematopoietic cell contamination than hMSCs [320]. For this reason, there has been more research on hMSCs than mMSCs. This

lack of research into how MSCs act in mouse models has greatly hindered the exploitation of the therapeutic potential of MSCs.

Recent research identifying MSC specific markers has reported specific marker combinations to identify homogeneous populations of murine bone marrow derived MSCs [321,322,323,324]. Of note, Morikawa *et al* reported methodology for prospectively identifying and isolating MSCs from murine bone marrow using flow cytometry [124]. This sorted population gives rise to MSCs that are capable of *in vivo* engraftment when transplanted systemically and can generate mesenchymal and neural crest lineage cells with or without *in vivo* expansion [144]. This MSC population, termed P α S cells, are isolated as PDGFR- α +, Sca-1+, CD45-, Ter119- murine bone marrow cells. mMSC cultures are often contaminated with haematopoietic cells [325]. CD45 is expressed at high levels on the cell surface of all nucleated haematopoietic cells and their precursors, and Ter119 is expressed on cells of erythroid lineage [326,327]. By selectively sorting for cells which are negative for these two markers, the mMSC population is haematopoietic cell free. Via a screening process, platelet derived growth factor receptor- α (PDGFR- α an early mesodermal marker [328]) and stem cell antigen-1 (Sca-1 [329]), a murine stem cell marker, were found to be good selective markers for a population of homogeneous MSCs. The identification of this marker combination enables *in vivo* examination of MSCs which were found to be predominantly located in the arterial perivascular space near the bone adjacent to the vascular smooth muscle cells (vSMCs) [124]. Interestingly, i.v. administration of uncultured P α S cells, were found to integrate into both the bone marrow and adipose tissue of mice [124]. Two fundamental problems with *in vitro* culture of MSCs is their loss of migratory capacity with

continued culture [129] and their increased size, causing them to become entrapped in the lungs when administered i.v. [330]. P α S cells expanded *in vitro* were also found to become entrapped in the lungs [124]. This may be due to changes in cell morphology and increase in the size of the P α S cells upon 2D culture on plastic. P α S cells isolated directly from murine bone marrow are very small relative to the rest of the cells in the bone marrow. These results add further weight to the argument that in order to recruit to target sites *in vivo*, MSCs should not be expanded *ex vivo*.

Other homogeneous stem cell sub-populations have been identified in murine bone marrow. These include marrow isolated adult multilineage inducible cells (MIAMIs) [324], multipotent adult progenitor cells (MAPCs) [322], and very small embryonic like cells (VSELs) [321]. MAPCs are isolated via CD45 and Ter119 depletion, along with selection via analysis in culture, including morphology and high Oct-4 mRNA levels. Whilst this set of cells has been highly researched and shown to have many favourable characteristics for clinical therapy, they cannot be identified *in vivo* [322]. This is also the case for MIAMIs, which are isolated due to their ability to form CFU-Fs *in vitro* [142]. VSELs are CD45-, Lin-, Sca-1+ cells and thus, can be sorted in a very similar manner to P α S cells. VSELs are also found to be very small indeed, significantly smaller than HSCs (3.63 ± 0.69 versus $6.54 \pm 0.17\mu\text{m}$ in diameter) and express Oct-4, a marker of embryonic stem cells [143]. Interestingly, P α S cells have been shown to express surface markers common to P5 bone marrow mMSCs including CD105 and VEGFR-2, and differentiate robustly into adipocytes, chondrocytes and osteoblasts. As yet, P α S cells have only been identified in the

bone marrow, but clearly it is possible to investigate whether these cells are present in other tissues.

5.2 Aims.

- To identify and further characterise the PαS cell population found in murine bone marrow.
- To investigate the effects of *in vitro* culture on the PαS cell population.
- To compare the external chemokine receptor expression profile of PαS cells with that of P5 bone marrow mMSCs.

5.3 Results.

A lack of common markers for mMSCs has led to discrepancies in the standardisation of mMSC characterisation [85]. By sorting P α S cells, a clearly defined population of mMSCs defined by antigen expression alone can be used for *in vivo* detection. In order to further characterise P α S in terms of chemokine receptor profiling and the effects of *in vitro* culture on this population, the previously published isolation techniques were reproduced [124]. The results of P α S cell sorting can be seen in Fig. 5.1. Figs. 5.1.a.i, ii and iii are FACS dot plots of whole femoral bone marrow stained with fluorescent antibodies for CD45 (FITC), Ter119 (FITC), PDGFR- α (APC) and Sca-1 (PE). By gating the CD45- and Ter119- (FITC-) cells and subsequently gating the PDGFR- α +, Sca-1+ cells, a population of P α S cells was isolated. These P α S cells are a rare population, making up only $0.08 \pm 0.04\%$ of the total bone marrow (Fig. 5.1.b.ii). In Figs. 5.1.b.i and 5.1.b.ii, it can be seen that there were large numbers of cells in the bone marrow which were either CD45-, Ter119-, PDGFR- α +, Sca-1- or CD45-, Ter119-, PDGFR- α -, Sca-1+, consistent with previous findings [124]. It has been reported that a greater number of P α S cells can be sorted in a shorter time period by treating crushed bone with collagenase [124]. Having isolated P α S cells from murine bone marrow, murine femurs were crushed and treated with different isoforms of collagenase. The resulting relative percentages of P α S cells in the bone marrow or bone populations is illustrated in Fig. 5.2.a and representative dot plots can be seen in Fig. 5.2.b. Collagenase treatment of crushed bones resulted in $0.27 \pm 0.06\%$ P α S cells compared to $0.08 \pm 0.04\%$ of the murine

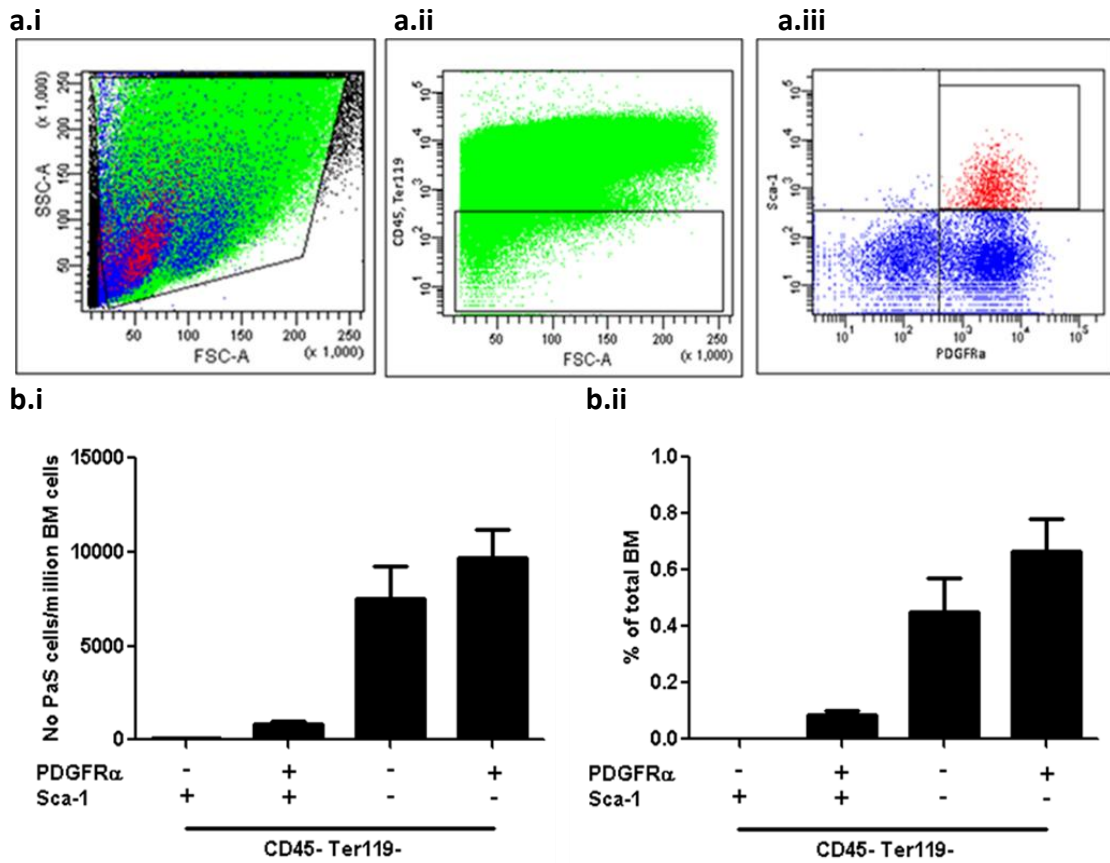


Fig. 5.1. Identification and isolation of PaS cells from murine bone marrow.

Fig. 5.1.a. PaS cells are isolated from the bone marrow (Fig. 5.1.a.i) and defined as CD45- Ter119- (Fig. 5.1.a.ii) PDGFRα⁺ Sca-1⁺ (Fig. 5.1.a.iii). Quantification of levels of PaS cell numbers found in murine bone marrow per million cells (Fig. 5.1.b.i) and as a percentage of the total bone marrow (Fig. 5.1.b.ii).

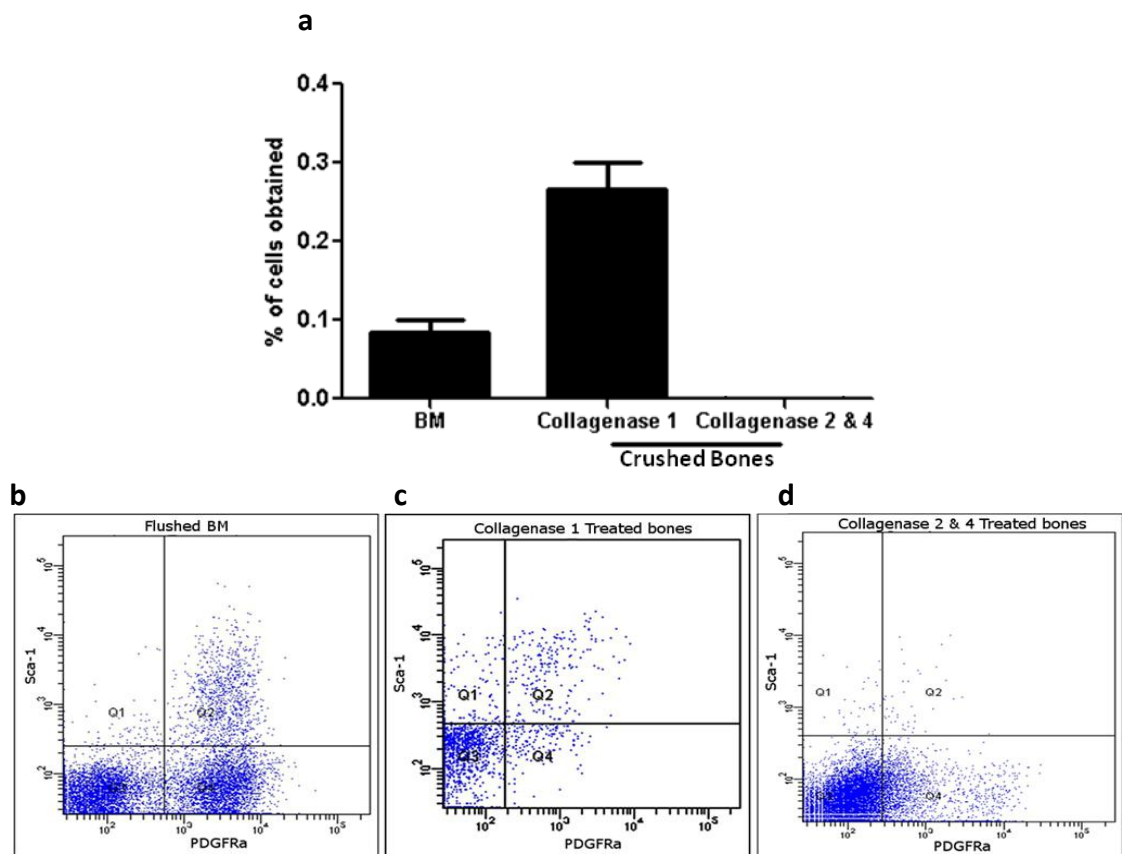


Fig. 5.2. Isolation of PαS cells from murine bone marrow or bone via collagenase treatment.

Fig. 5.2.a. Quantification of PαS as a percentage of the total number of cells sorted, when isolated using different protocols. Fig. 5.2.b. PαS cells isolated from the bone marrow via flushing with DMEM + 10% FCS. Fig. 5.2.c. PαS cells isolated from flushed murine femurs and tibias after crushing with a pestle and mortar and collagenase 1 treatment for 1 hour in a 37°C shaking water bath. Fig. 5.2.d. PαS cells isolated from flushed murine femurs and tibias after crushing with a pestle and mortar and collagenase 2 & 4 treatment for 1 hour in a 37°C shaking water bath.

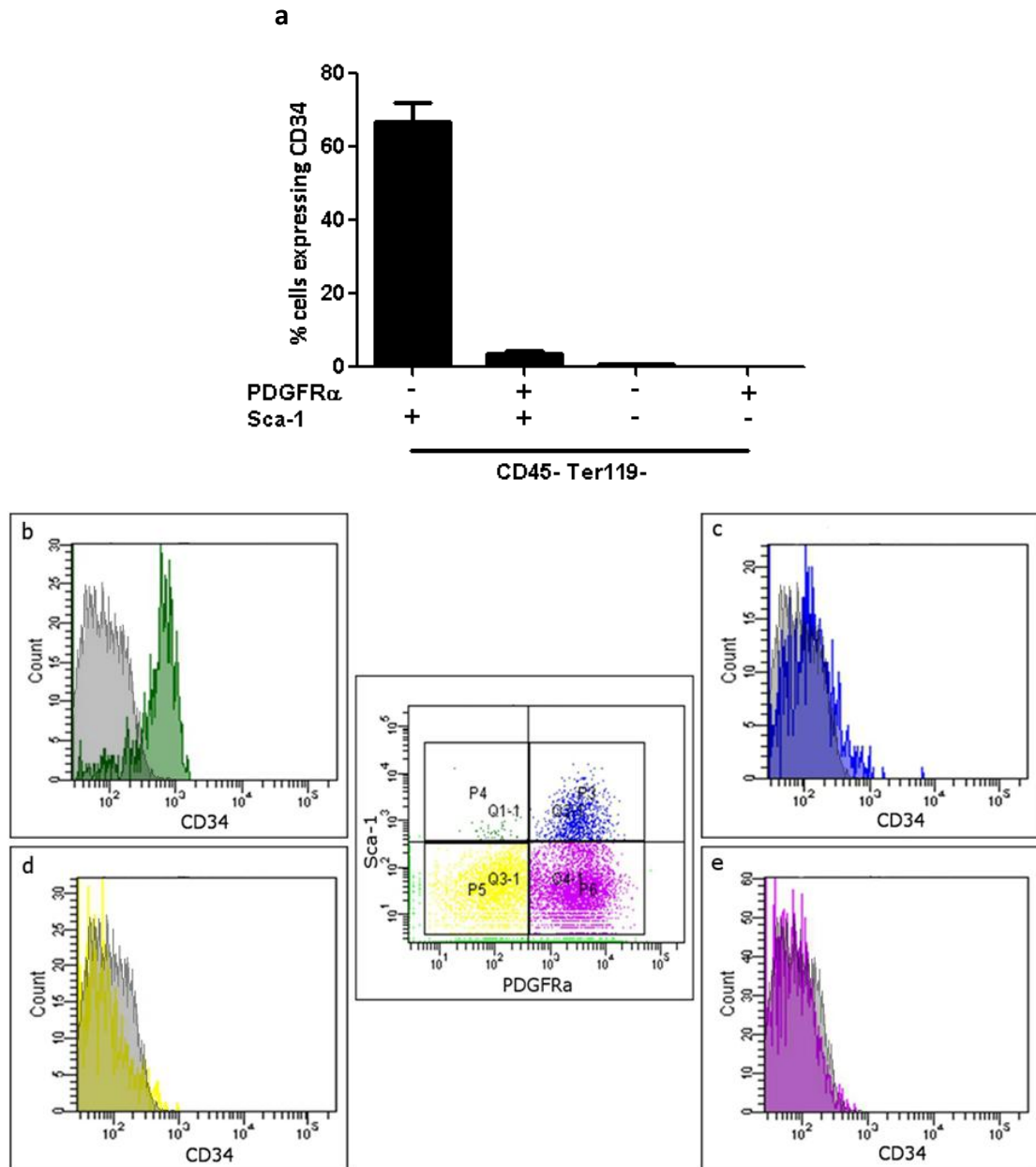


Fig. 5.3. Differential expression of CD34 on CD45- Ter119- bone marrow subgroups as defined by PDGFR α and Sca-1 expression.

Fig. 5.3.a. Quantification of CD34 expression on CD45- Ter119- bone marrow subgroups as defined by PDGFR α and Sca-1 expression. n = 3. Fig. 5.3.b. Expression of CD34 on CD45-, Ter119-, PDGFR α -, Sca-1+. Fig. 5.3.c. Expression of CD34 on CD45-, Ter119-, PDGFR α +, Sca-1-. Fig. 5.3.d. Expression of CD34 on CD45-, Ter119-, PDGFR α -, Sca-1-. Fig. 5.3.e. Expression of CD34 on CD45-, Ter119-, PDGFR α +, Sca-1-.

bone marrow and $4.6 \times 10^{-4} \pm 7.07 \times 10^{-7}\%$ of flushed bones treated with collagenases 2 and 4. But importantly, absolute numbers of P α S cells that could be harvested by these procedures were e.g. 12720 per femur by flushing and 10530 per femur by crushing the bones, suggesting that while P α S cells represent a significantly higher percentage of cells in bone, similar numbers can be isolated from both sources.

Having consistently identified and sorted P α S cells from murine bone marrow and crushed bone, further characterisation of uncultured P α S cells was carried out by investigating the expression of other cellular markers in a 4th FACS channel. This was the case for CD34 expression on P α S cells as illustrated in Fig. 5.3. which shows uncultured P α S cells stained with CD34 in the PE-Cy-7 channel. Of the 4 sub-populations in Fig. 5.3, it can be seen that P α S cells (blue, upper right quadrant), CD45⁻, Ter119⁻, PDGFR- α ⁻, Sca-1⁻ cells (yellow, bottom left quadrant) and CD45⁻, Ter119⁻, PDGFR- α ⁺, Sca-1⁻ (purple, bottom right quadrant) cells expressed no CD34 on the cell surface. However CD45⁻, Ter119⁺, PDGFR- α ⁻, Sca-1⁺ cells (green, top left quadrant) showed high levels of CD34 on the cell surface. In order to compare P α S cells with the P5 bone marrow mMSC population isolated and characterised in Chapter 3, various markers on the cell surface of P α S cells were analysed including murine MSC markers, VEGFRs (Fig. 5.4) and chemokine receptors (Fig. 5.5). P α S cells were negative for CD45, CD11b, CD34, AC133 and VEGFR-3 on the cell surface. P α S cells expressed high levels of CD105, Sca-1, VEGFR-1, and an intermediate percentage of the population expressed VEGFR-2. It appears that there was a sub-population expressing VEGFR-1 and VEGFR-2. Uncultured P α S cells expressed high levels of CXCR3 ($88.20 \pm 1.98\%$), CXCR4 ($87.72 \pm 3.58\%$), and CCR10 ($54.55 \pm 7.14\%$).

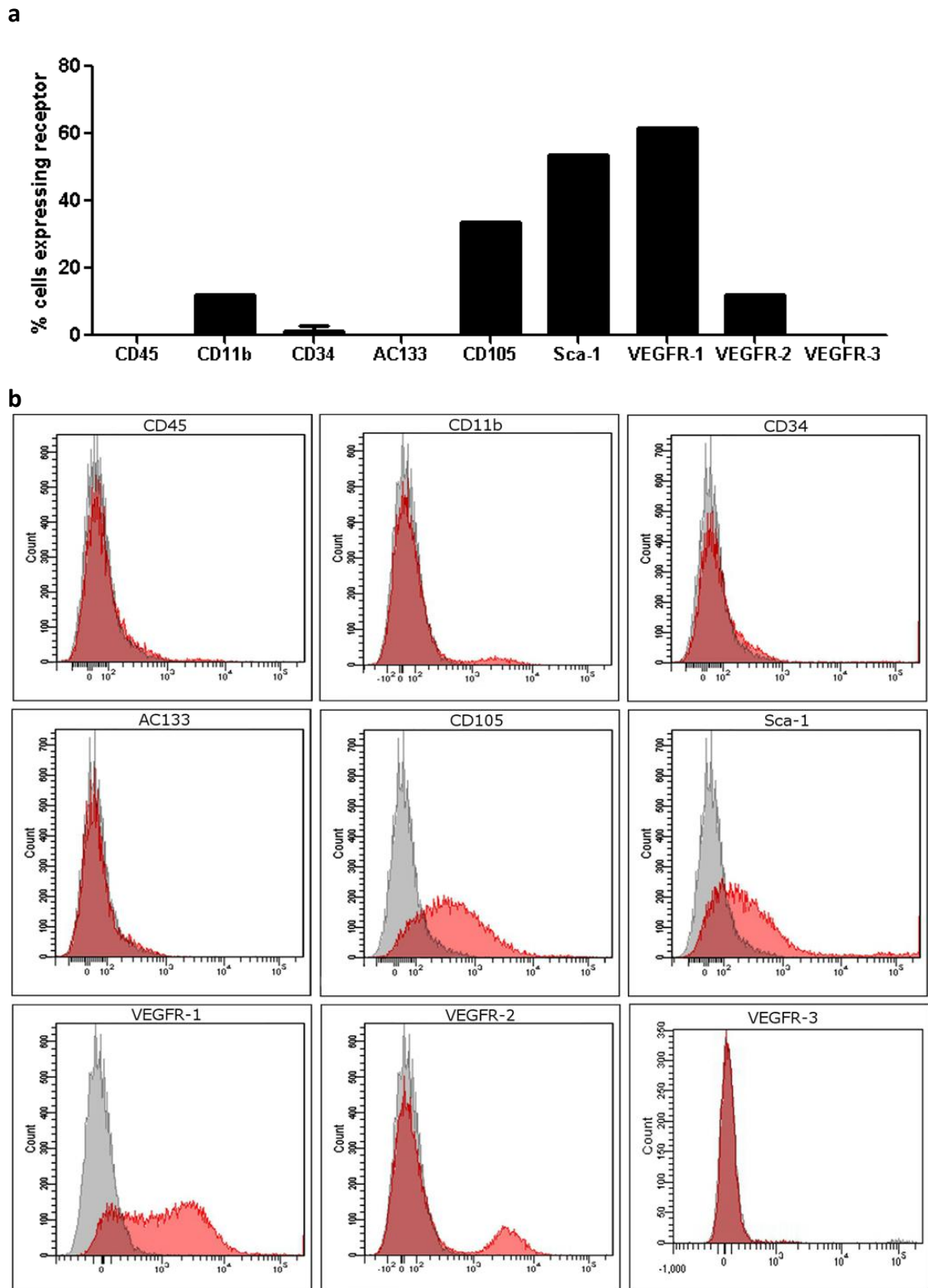
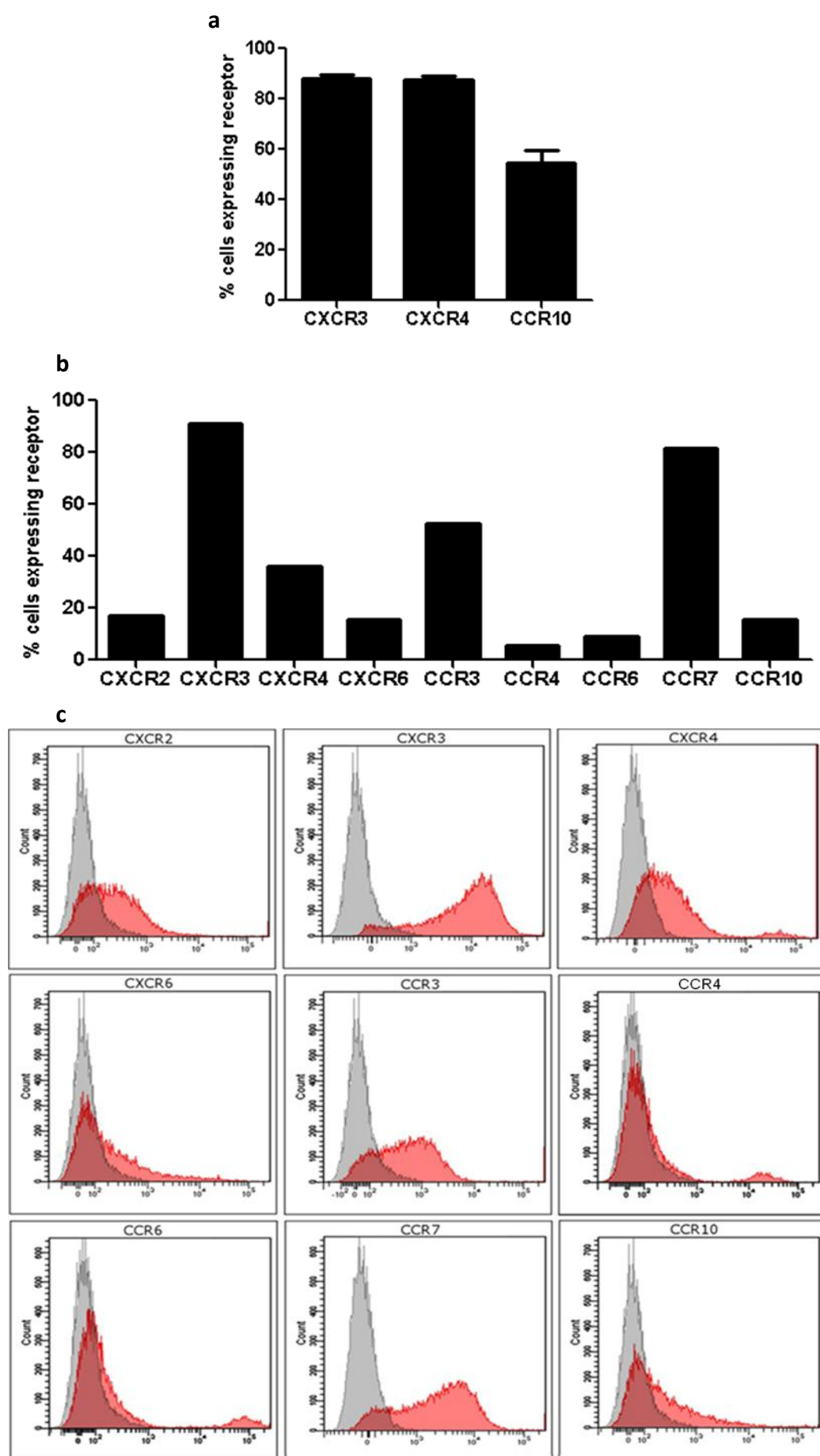


Fig. 5.4. Expression of murine MSC markers on P5 PaS cells.

Fig. 5.4.a. Quantification of MSC markers on P5 PaS cells. n=1-2. Fig. 5.4.b. Representative histogram plots of MSC markers on P5 PaS cells.



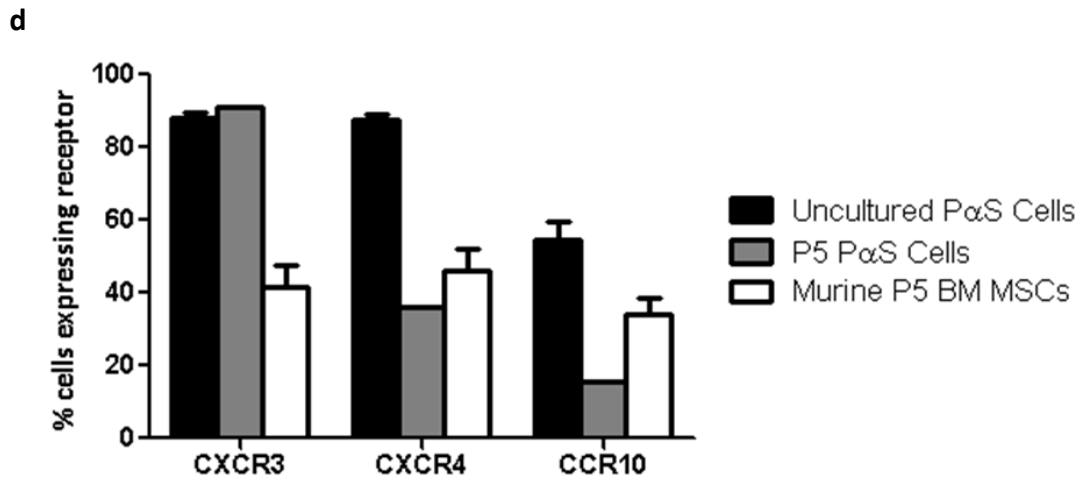


Fig. 5.5. External chemokine receptor expression on P5 PαS cells.

Fig. 5.5.a. Flushed bone marrow cells were stained for CD45, Ter119, PDGFR α , Sca-1 and either CXCR3, CXCR4 or CCR10. Expression levels of CXCR3, CXCR4 and CCR10 were recorded on cells that were CD45⁻ Ter119⁻ PDGFR α ⁺ Sca-1⁺. n = 3. Fig. 5.5.b. Sorted PαS cell populations were cultured in Mesencult (StemCell technologies) at 37°C. 5% CO₂ to passage 5. Cells were trypsinised, recovered and analysed for external chemokine receptor expression. 1 x 10⁵ cells were used to stain for each receptor. Representative external chemokine receptor expression levels of P5 PαS cells were measured using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software. n = 1. Fig. 5.5.c. FACS histogram plots of external chemokine receptor expression on PαS cells at passage 5. Data shown is representative. Fig. 5.6.d. A comparison of CXCR3, CXCR4 and CCR10 expression on uncultured PαS cells, PαS cells cultured to P5 and murine bone marrow MSCs isolated by plastic adherence alone. n=1-3.

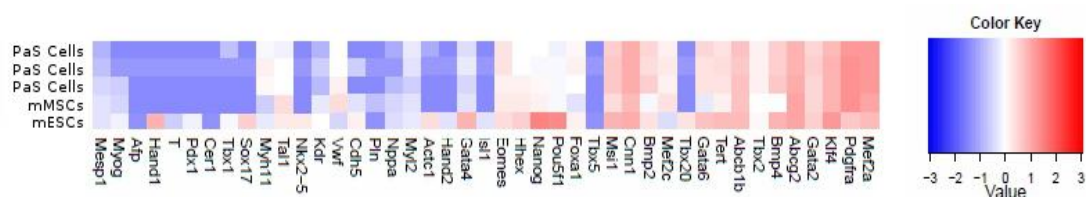


Fig. 5.6. Quantitative PCR of PaS cells, mES cells and mMSCs.

Quantitative PCR of P5 PaS cells, P5 mES cells and P5 mMSCs. RNA isolation and DNase treatment was performed using a liquid handling system (BiomekFxp, Beckman Coulter) to automate and integrate protocols from Agencourt RNAdvance Cell v2 (Beckman Coulter) and TURBO DNA-free (Applied Biosystems). Reverse transcription was performed using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems) using Veriti thermal cycler (Applied Biosystems). Quantitative PCR was performed on a 7900HT (Applied Biosystems) using Taqman gene expressions assays including FAM™ dye-labelled TaqMan® MGB probe and primers set spanning 2 exons each specific for the target genes (Fgfr1, Fgfr2, Fgfr3 and Fgfr4) and the loading controls (Hmbs and Gapdh).

Upon culture to passage 5 in the same culture conditions in which P5 bone marrow mMSCs were cultured in Chapter 3, P α S cells expressed high levels of CXCR3, CXCR4, CCR3 and CCR7. They expressed lower levels of CXCR2, CXCR6, CCR4, CCR6 and CCR10. When the external CXCR3, CXCR4 and CCR10 levels are compared in Fig. 5.5.d. between P5 bone marrow mMSCs, uncultured P α S cells and P5 P α S cells, there were notable differences. P5 bone marrow mMSCs and P5 P α S cells showed comparable expression of CXCR4 and CCR10. Uncultured P α S cells expressed higher levels of CXCR4 on the cell surface than P5 P α S cells and P5 bone marrow mMSCs. There was no difference in CXCR3 expression between uncultured and P5 P α S cells. However CXCR3 was expressed on a much lower percent of P5 bone marrow mMSCs. Due to the rarity of P α S cells in the bone marrow, less than 1×10^5 cells can be sorted at any one time, at great cost. For this reason, only preliminary experiments investigating the culture of P α S cells have been carried out to date.

P α S cells can differentiate into cells of the neural lineage and express markers which are used to identify ES cells such as AbcG2 and Tbx2. It has been hypothesised that P α S cells may be phenotypically closer to mES cells than mMSCs [124]. To compare P α S, mMSCs and mES cells in more detail, QRT-PCR was carried out on the three cell populations to compare the expression of 45 relevant genes. In Fig. 5.6. it can be seen that P α S cells were phenotypically closer to mMSCs and that there are notable differences in the gene expression profile between P α S cells and mES cells, including Nanog, a transcription factor critically involved with self renewal of undifferentiated ES cells [24].

The isolation of a homogeneous population of mMSCs is very appealing with respect to their characterisation *in vivo*. However, investigation into their therapeutic potential may require larger numbers than those which can be efficiently sorted. To achieve this, the cells must be cultured *ex vivo*. Bone marrow derived MSCs isolated by plastic adherence alone have been shown to be a heterogeneous population that undergo phenotypic and functional changes with continued culture *in vitro* [129]. To ascertain whether this is also true for PαS cells, PαS cells were cultured to passage 5 and compared with P5 and P25 murine bone marrow mMSCs for expression of CD45, Ter119, PDGFR-α and Sca-1 (Fig. 5.7). At passage 5, only 41.8% of cultured PαS cells stain as CD45-, Ter119-, PDGFR-α+, Sca-1+, meaning that only 41.8% of this population is a homogeneous population of MSCs and the remaining 58.2% have undergone phenotypic changes due to *in vitro* culture conditions. In Fig. 5.7.e. the levels of PαS cells within uncultured bone marrow, P5 bone marrow mMSCs, P5 PαS cells and P25 bone marrow mMSCs are compared. There is no significant difference in the percentage of the cells that are PαS cells in the P5 PαS, P5 bone marrow mMSC, P25 bone marrow mMSC cultures. There is no difference in the levels of CD45-, Ter119-, PDGFR-α+, Sca-1- cells and CD45-, Ter119-, PDGFR-α-, Sca-1+ cells in the P5 and P25 bone marrow mMSC populations. P5 PαS cells have within their number higher levels of CD45-, Ter119-, PDGFR-α+, Sca-1- and lower levels of CD45-, Ter119-, PDGFR-α-, Sca-1+ cells when compared to P5 and P25 bone marrow mMSCs. Interestingly, the dot plots in Figs. 5.7.a-d highlight differences in the profiles of the PαS cells within the cultured populations (e.g. PαS cells in the P5 bone marrow mMSC population are Sca-1^{lo} whereas PαS cells in the P25 bone marrow mMSC

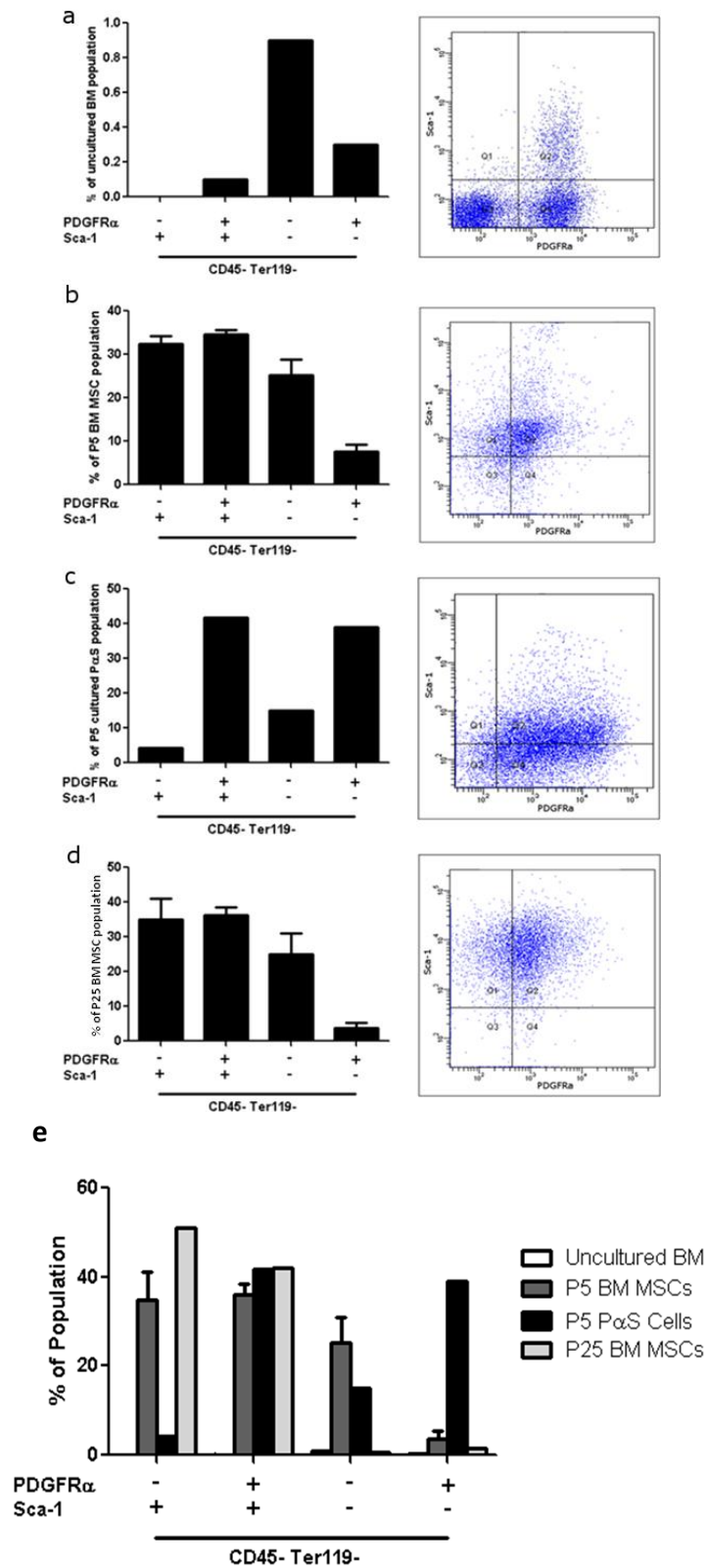


Fig. 5.7. Percentage of PαS cells in different cell populations.

Cells from different sources were stained for CD45, Ter119, PDGFRα and Sca-1. Fig. 5.7.a.

Quantification of PαS levels in bone marrow cell populations isolated by flushing of murine

femurs and tibias measured by FACS (Fig. 5.7.a.i). Representative dot plot of P α S levels in bone marrow cell populations isolated by flushing of murine femurs and tibias measured by FACS (Fig. 5.7.a.ii). Fig. 5.7.b. Quantification of P α S levels in P5 mMSCs isolated by plastic adherence alone, measured by FACS (Fig. 5.7.b.i). Representative dot plot of P α S levels in P5 mMSCs isolated by plastic adherence alone, measured by FACS (Fig. 5.7.b.ii). Fig. 5.7.c. Quantification of P α S levels in FACS sorted P α S cultured to P5 in Mesencult, measured by FACS (Fig. 5.7.c.i). Representative dot plot of P α S levels in FACS sorted P α S cultured to P5 in Mesencult, measured by FACS (Fig. 5.7.c.ii). Fig 5.7.d. Quantification of P α S levels in P25 mMSCs isolated by plastic adherence alone, measured by FACS (Fig. 5.7.d.i). Representative dot plot of P α S levels in P5 mMSCs isolated by plastic adherence alone, measured by FACS (Fig. 5.7.d.ii). Fig. 5.7.e. Comparison of sub-populations between sources of P α S cells as a percentage of the source population. n = 1-3.

population are Sca-1^{hi}), demonstrating the impact of continued culture on the PαS cells.

It has been reported that cultured MSCs when administered to mouse models i.v. can result in death, as well as causing pulmonary and hemodynamic alterations, thus preventing the intended access to other organs. [193]. We propose that the increase in the size of the MSCs upon culture leads to entrapment of the cells within the lungs. Upon their characterisation, the small size of VSELs was seen as an advantageous quality as these cells can circumvent any entrapment problems when administered [331]. In Fig. 5.8.a. the size of cultured PαS cells is compared to VSELs. Both sets of cells are of similar size and are very small in relation to other cells residing in the bone marrow. To confirm whether or not cells grow in size upon continued culture on plastic surfaces, the size of uncultured PαS cells has been compared to PαS cells within the P25 bone marrow mMSC population. (Fig. 5.8.b) Whilst there is some overlap between the two sets of cells, there is a lot more variation in the size of the PαS cells cultured to P25. The vast majority of PαS cells in the P25 bone marrow mMSC population are much larger than the uncultured PαS cells.

As PαS cells are a newly characterised population of cells, there are still several variables which are yet to be investigated. In humans, adults have lower levels of MSCs in the bone marrow than infants [71]. This has implications when deciding what age mice to use for future research on PαS cells. To see if this difference is maintained for PαS cells, levels were compared between young (2.5

weeks) and old (30 weeks) mice. There are more PαS cells in the bone marrow of young mice (1086 ± 80.16) than old mice (568.3 ± 179.6) (Fig. 5.9).

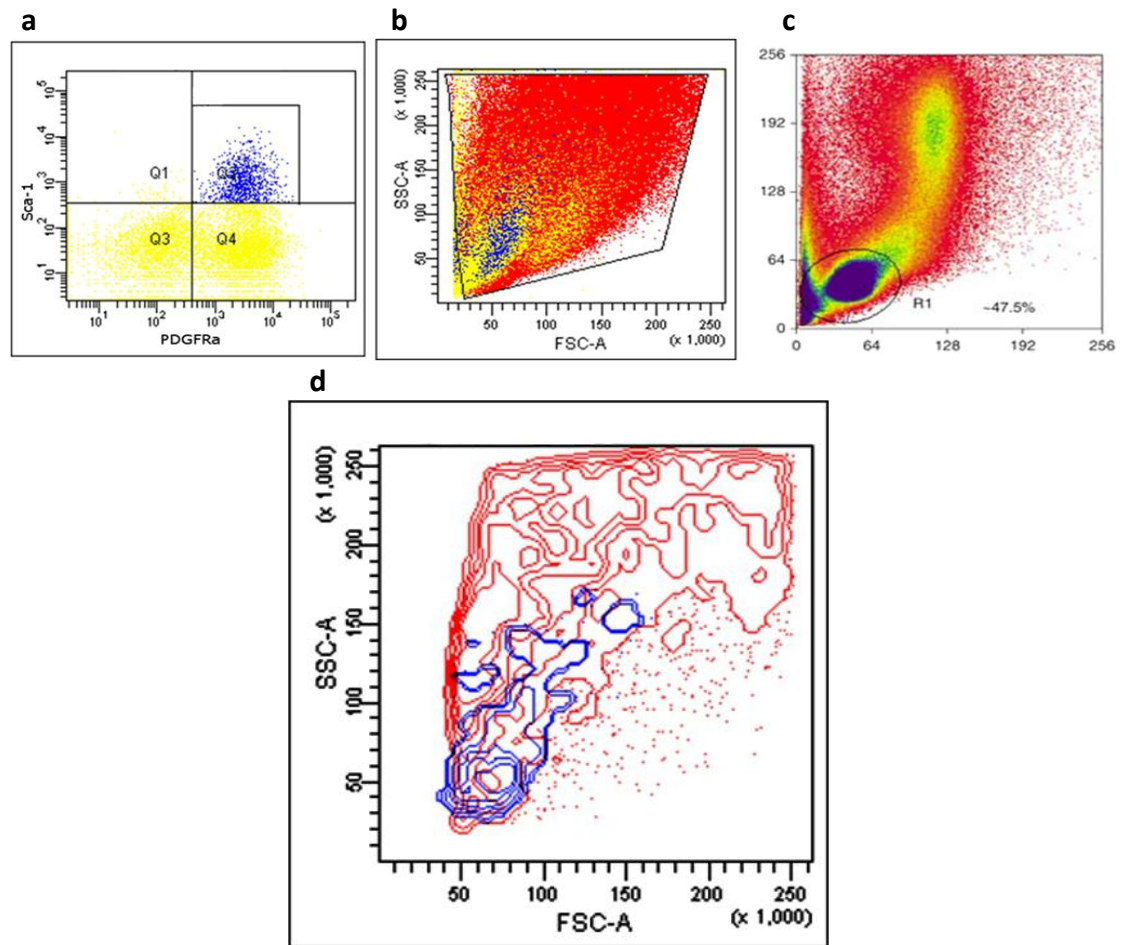


Fig. 5.8. Comparison of PaS cells with VSELs.

Fig. 5.8.a. PaS cells were identified using FACS analysis (blue). Fig. 5.8.b. The PaS cells were back-gated in order to visualise their size in comparison with the rest of the cells of the bone marrow. Fig. 5.8.c. Data from Kucia *et al*, 2006 [321] illustrating the size of very small embryonic like (VSEL) cells in comparison to the rest of the bone marrow. Fig. 5.8.d. Density plot to compare the size of uncultured PaS cells (blue) to PaS cells in the population of late passage (P25) cultured bone marrow derived mMSCs (red).

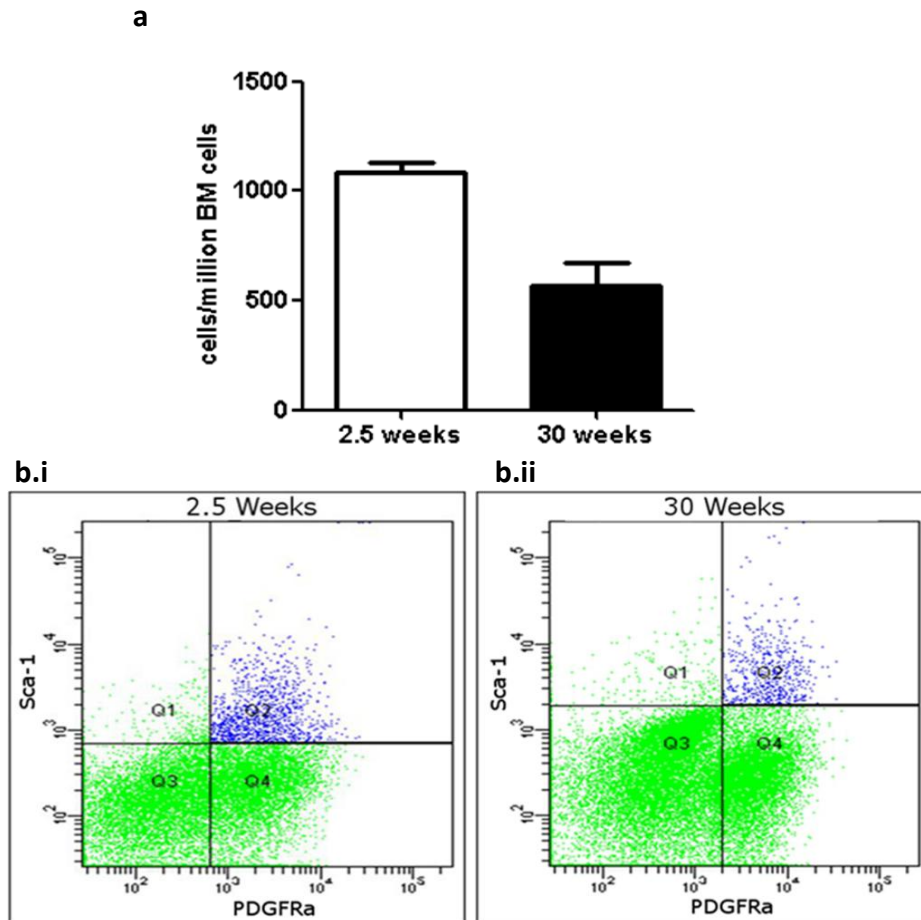


Fig. 5.9. Comparison of PaS cells in the bone marrow of young (2.5 weeks old) and old (30 weeks old) mice.

Bone marrow was flushed from murine femurs and tibias. Fig. 5.9.a. Quantification of the levels of PaS cells in the bone marrow of young (2.5 weeks old) and old (30 weeks old) mice per million bone marrow cells. Fig. 5.9.b. Representative dot plot of PaS cells (blue) in young (2.5 weeks old) mice (Fig 5.9.b.i). Representative dot plot of PaS cells (blue) in old (30 weeks old) mice (Fig. 5.9.b.i).

5.4 Discussion.

In identifying P α S cells as bone marrow derived mMSCs, the properties of MSCs in their natural state *in vivo* can be investigated. As P α S cells are a newly characterised population, isolation of the cells using reported methodology needed to be shown to be reproducible [124]. P α S cells cannot be identified in humans due to the lack of a Sca-1 homolog. The homogeneity of the population combined with the availability of mouse models of disease and inflammation makes P α S cells a good candidate for the interrogation of MSC biology *in vivo*. P α S cells are very small in relation to the rest of the cells in the bone marrow. This may be a functional requirement of MSCs in order to traffic efficiently around the body. It is known that cultured P α S cells when injected into mice i.v., become entrapped in the lungs whereas uncultured cells migrate to the bone marrow and adipose tissue therefore being able to circulate unhindered [124]. The rarity of the P α S cells is not surprising, at 0.01% of the bone marrow. This is consistent with previous reports of MSC cell numbers [278,332]. Previous reports interrogating the functionality of the 4 subsets of CD45⁻ Ter119⁻ bone marrow cells, as defined by their PDGFR α and Sca-1 expression, showed that both CD45⁻, Ter119⁻, PDGFR α ⁺, Sca-1⁻ cells and CD45⁻, Ter119⁻, PDGFR α ⁻, Sca-1⁺ cells can be classified as heterogeneous populations of MSCs. This is because they fulfil all of the criteria to be classified as MSCs [278]. They adhere and proliferate on plastic surfaces and a percentage of the population can be differentiated along osteogenic, adipogenic and chondrogenic lineages. It was hypothesised that these subsets may be mesenchymal progenitor cells as opposed

to the homogeneous population of P α S cells which have greater levels of plasticity and proliferation [144]. Two methods of isolating P α S from the femurs of mice have been reported, with differing levels of efficiency. By treating flushed and crushed femurs with collagenase, a higher percentage of P α S cells were isolated [124].

Flushed and crushed femurs were treated with different forms of collagenase. These results show that P α S cells harvested from crushed femurs is dependent on collagenase-1 and that treatment with collagenase 2 and 4 does not result in the isolation of these cells. Fig. 5.2.a. shows that when using collagenase-1 treatment on crushed femurs, 0.3% of the cells isolated were P α S cells. Although this is significantly higher than the percentage obtained from untreated flushed bone marrow, the levels were not consistent with previous reports, which showed higher levels of P α S cells isolated [124]. Significantly, absolute numbers of P α S cells were higher in the bone marrow of naïve mice when compared to collagenase-1 treated crushed bones. This is due to differences in the total number of cells from which P α S cells can be sorted e.g. 15.9×10^6 cells from the bone marrow of one femur versus 3.9×10^6 cells from a collagenase-1 treated crushed femur. In attempting to further characterise the subsets of CD45⁻ Ter 119⁻, as defined by PDGFR α and Sca-1 expression, CD34 expression levels on the sub-populations were investigated. CD34 is generally used as a marker for haematopoietic and endothelial progenitor cells, however subsets of MSCs which are CD34⁺ have been reported [85]. Analysis of the 4 subsets shows that the CD45⁻, Ter119⁻, PDGFR α ⁻, Sca-1⁺ cells express high levels of CD34, whereas the other 3 subsets including P α S cells do not. This CD34⁺ subset is one of the two that Morikawa *et al* referred to as a progenitor subset. Morikawa

et al hypothesised that these cells have slightly less plasticity than the P α S cells, and are more likely to be progenitors for osteoblasts [124].

Identification of these cells within the bone marrow allowed the opportunity to check whether the P5 bone marrow mMSCs characterised in Chapter 3, were a true population of MSCs in comparison to their *in vivo* state. Having illustrated the detrimental effect continued passage has on chemokine receptor expression on these cells, the effects of culture on the phenotype of MSCs was investigated. When looking at stem cell markers, the P α S cells displayed a similar phenotype to the P5 bone marrow mMSCs isolated and characterised in Chapter 3. Interestingly, P α S cells also express high levels of VEGFR-1, an intermediate population express VEGFR-2 and there is no surface expression of VEGFR-3. This data is consistent with previous reports which state that VEGFR-2 is expressed at an intermediate level on the cell surface and found no expression of VEGFR-3 [124]. The VEGFR-2 expression on a sub-population of P α S cells demonstrates that there are differences in the phenotype at the individual cell level. In order to look further at any differences there may be, Quantitative PCR of P5 P α S cells, P5 mES cells and P5 mMSCs was carried out, looking at 45 genes of interest. The pattern of the gene profiles of P5 bone marrow mMSCs and P α S cells is highly conserved. Comparison of P α S cells and P5 bone marrow mMSCs to mES cells shows there are many genes expressed on mES cells that are not expressed on P α S or P5 bone marrow mMSCs. Taking all of this information together we can conclude that although P α S cells may be a useful set of stem cells to be used therapeutically due to their homogeneity, they are much closer phenotypically to classically defined MSCs than ES cells. One of the features that make MSCs attractive as a candidate for cell therapy is their ability to home to

sites of injury and inflammation. As chemokine receptors on the cell surface of MSCs play a critical role in this pathway and chemokine receptor levels decreased with repeated passage, chemokine receptor levels on the P α S cells were analysed before the cells were cultured. By sorting the P α S cells, chemokine receptor profile on the P α S cells following culture *in vitro* could also be assessed. On uncultured P α S cells, CXCR3, CXCR4 and CCR10 are all highly expressed. Upon culture *in vitro*, due to the highly proliferative nature of the cells and a resultant larger population at passage 5, a wider range of chemokine receptors could be investigated. When comparing the 2 sets of data, it is clear that culture of the cells has led to a dramatic decrease in CXCR4 and CCR10 on the cell surface. When both sets were compared to the P5 bone marrow mMSC external receptor profile analysed in Chapter 3, the levels of CXCR4 and CCR10 were similarly expressed by both sets of cultured cells at a much lower level than the uncultured cells. This provides data to support the hypothesis that MSCs, when extracted from the bone marrow and cultured, lose chemokine receptors on the cell surface which may impact on the efficiency with which they home to sites. The recent characterisation work carried out on P α S cells by Morikawa *et al* [144] suggests that these are a true MSC population. The chemokine receptor expression levels reported here, may explain the low levels of engraftment at sites of damage in human clinical trials. Interestingly, CXCR3 levels remain high when compared to P5 bone marrow mMSCs. This difference is another example of how differences in culture techniques can lead to variation in MSC populations between labs. This may be due to the paracrine effects of other bone marrow cells on the MSCs upon initial culture.

Having observed that cultured P α S cells were phenotypically different to their uncultured counterparts, maintenance of the CD45⁻, Ter119⁻, PDGFR α ⁺, Sca-1⁺ phenotype in cultured P α S cells was investigated. The result of this can be seen in Fig. 5.7.a. and 5.7.b. When cultured to passage 5, although 100% of the cells are CD45⁻ and Ter119⁻, only 40% of cells retain both PDGFR α and Sca-1 expression. 10% of cells are negative for both of these markers, meaning nearly 50% of the cells are now falling into the gates which represent the MPCs. This means that either, the adherence of the cells to plastic, the proliferation of cell population, or factors in the media are causing the cells to differentiate down specific lineages. The majority of the cells in the cultured P α S cell population are CD45⁻, Ter119⁻, PDGFR α ⁺, Sca-1⁻ MPCs as analysed by FACS. If time permitted, analysis of whether the cells express any specific differentiation markers over time may provide information about how these culture conditions influence differentiation potential on cultured P α S cells.

Interestingly, this set of experiments illustrated differences between P α S cells and MSCs isolated by plastic adherence alone. Both P5 and P25 bone marrow mMSCs have a subset of P α S cells which represent 30-35% of the total population. The main difference in the distribution of the PDGFR α [±], Sca-1[±] subsets between cultured P α S cells and the MSCs isolated by plastic adherence is that cultured P α S have nearly 40% PDGFR α ⁺, Sca-1⁻ and <10% PDGFR α ⁻, Sca-1⁻ cells, whereas P5 and P25 bone marrow mMSCs have <10% PDGFR α ⁺, Sca-1⁻ and nearly 40% PDGFR α ⁻, Sca-1⁻ cells. This difference in the number of the two progenitor subsets, between cultured P α S and bone marrow mMSCs illustrates that the other cells within the bone marrow that are initially cultured with the bone marrow mMSCs drive differentiation of a population of these stem cells down a specific lineage to become

PDGFR α -, Sca-1+ mesenchymal progenitor cells. MSCs isolated by plastic adherence alone do indeed represent a heterogeneous population of progenitor cells with a sub-population of MSCs, while P α S cells may start off as stem cells but will inevitably divide and differentiate upon culture.

It has been suggested that culture of MSCs changes their size [129]. To investigate this further, the size of the P α S cell population in uncultured bone marrow and P25 bone marrow mMSCs isolated by plastic adherence alone was compared. Fig. 5.8.b. is a density plot which compares the size of these two subsets directly. Uncultured P α S cells are very small whilst the vast majority of the P25 P α S cells are significantly larger in size. Whether this would affect their functional ability to migrate around the body, is yet to be verified. However, considering the mortality reports due to airway blockages caused by the i.v. administration of cultured MSCs, it can be hypothesised that uncultured P α S cells may traffic more efficiently. Development of culture conditions to maintain 'stemness' whilst driving proliferation is thus desirable for use of MSCs as therapeutic tools.

The relevance of the size of bone marrow stem cells has been previously referred to by Zuba Surma *et al* in their paper which classifies another subset of bone marrow derived mMSCs, VSELs [142]. These cells are CD45-, Lin-, Sca-1+, as are P α S cells. VSELs were shown to be mobilised following myocardial infarction [143]. They demonstrate a marked increase in the trafficking of VSELs in the peripheral blood early after myocardial infarction, which raises the possibility that these pluripotent cells may contribute to myocardial repair in this setting. Knowing that the P α S cells share the markers which define the VSELs and are originally located in

the murine bone marrow, P α S cells were compared with VSELs in terms of size relative to the rest of the bone marrow. Fig. 5.8.a. illustrates the FACS plots of this analysis. This reveals that the two cells types are indeed similar in terms of size, in relation to the rest of the bone marrow. This also suggests that the two populations may have a large amount of overlap in their characterisation. Future experiments could investigate the similarities of these cells further to clarify whether they are indeed the same cells or if P α S cells are a subset of VSELs as the data to date suggests.

Stem cells do not appear to function as well in the elderly as they do in children in terms of tissue repair [71]. This may be due to alterations to stem cells caused by an aged micro-environment [333], or a reported decrease in adult stem cell numbers. Following the identification of P α S cells, we can now see if this is true for this subset of MSCs. Upon comparing numbers in young mice aged 2.5 weeks to older mice aged 30 weeks and over it was found that younger mice did have a greater percentage of P α S cells in the bone marrow. However, due to their smaller size, young mice have lower absolute numbers of P α S cells in the bone marrow. This has implications for the isolation of P α S cells and the importance of using age matched mice in comparative studies.

The work described in this chapter is ongoing. Although P α S cells offer an easy way to characterise MSCs in their natural state, advances in technology are constantly refining the best practice for MSC analysis. By further characterising P α S cells, it has been shown that only a sub-population of P α S cells express VEGFR-2.

Future characterisation will focus on comparing subsets of PαS cells and their potential for use in cell therapies.

6.

Mobilisation of endogenous mMSCs from the bone marrow into the peripheral blood.

6. Mobilisation of endogenous mMSCs from the bone marrow into the peripheral blood.

6.1 Introduction.

In previous chapters, MSC populations isolated from different sources using different isolation techniques have been characterised *in vitro*. However, there are both practical and technical complications associated with the harvesting, isolation, *ex vivo* expansion, and delivery of these cells [334,335]. Upon culture, all MSC populations examined here were shown to be heterogeneous. Continued culture and passage of bone marrow mMSCs *in vitro* has resulted in a reduction in external chemokine receptor levels. Upon i.v. administration of *in vitro* cultured PaS cells, large numbers becoming entrapped in the lungs. Conversely, uncultured PaS cells traffic efficiently and integrate into the bone marrow and adipose tissue [124]. For this reason, it has been postulated that a more efficient form of regenerative medicine may involve the mobilisation of endogenous MSCs [129]. However, MSCs have been reported to home to tumours and contribute to tumourigenesis [336], a factor that needs to be taken into consideration when increasing circulating numbers of MSCs. The bone marrow contains many progenitor cell populations, including MSCs, HPCs, fibrocytes, and EPCs. In response to disease or tissue injury, these cells are mobilised from the bone marrow and recruited into tissues where they can contribute to disease progression or tissue repair [244,245]. Infusion of HSCs and cultured EPCs has been shown to augment neovascularisation of tissue

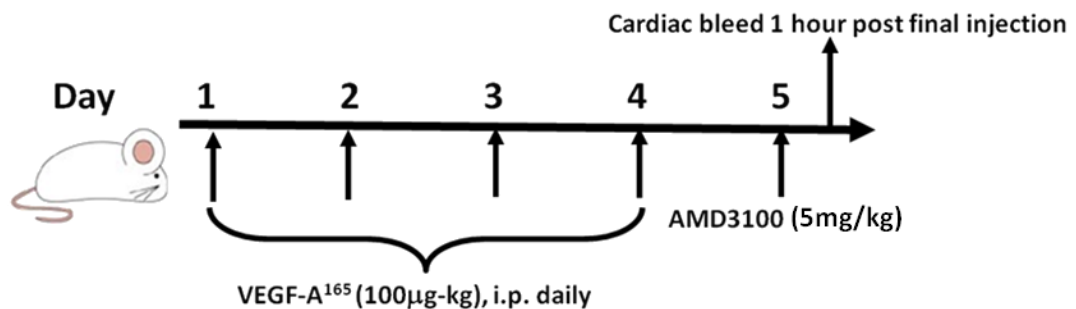
following ischemia and contribute to re-endothelialisation after injury [337]. Due to the known immuno-modulatory effects and multipotent differentiation potential of MSCs, mobilisation of these cells into the peripheral blood could aid repair of tissue in response to injury and inflammation. HPC mobilising agents have had a profound impact in the field of haematological transplantation [338]. If MSCs can be efficiently mobilised into the blood in a similar manner, there is great potential for directing these cells for therapeutic benefit. This may also be useful as a method for harvesting MSCs in a non-invasive manner.

The critical aspect when mobilising cells from the bone marrow is to ensure specific mobilisation so that only cells which will enhance regenerative capacity are mobilised. Knowledge of inducible, selective mobilisation of sets of stem and progenitor cells from the bone marrow was expanded upon by Pitchford *et al* in 2009 [120]. This research documented the specific mobilisation of HPCs, EPCs and MSCs. The study described a mechanism for the mobilisation of EPCs and HPCs, but no explanation for the mechanism by which MSCs are mobilised. Further, there was no experimental evidence that MSCs that are mobilised are functional and effective in enhancing the body's ability to repair. The protocol for this mobilisation can be seen in Fig. 6.i.

VEGF exists in several isoforms, the most commonly researched of which being VEGF-A¹⁶⁵, which is often referred to as VEGF. VEGF-A¹⁶⁵ plays a critical role in blood vessel formation and the migration of endothelial cells. VEGF-A¹⁶⁵ was used as a test agent in Pitchford *et al*'s experiments due to its role in stimulating EPC mobilisation [257]. The mobilisation of stem and progenitor cells out of the bone

marrow is thought to be a multi-step process [339]. The cells are released from their respective niches within the bone marrow, followed by migration across the bone marrow to the sinusoidal endothelium [261]. With regards to MSC mobilisation, Kolonin and Simmons state that it is likely VEGF plays a role in mobilising MSCs from the bone marrow in an indirect manner as MSCs do not express VEGFRs [255]. However, in Chapter 5 cultured PaS cells were shown to express VEGFR-1 and a sub-population expressed VEGFR-2.

Fig 6.i. Protocol for mobilising MSCs from the blood into the bone marrow.



The CXCL12/CXCR4 axis is critical for the retention of HPCs in the bone marrow [340]. Inhibition of this axis by CXCR4 antagonism using AMD3100 rapidly releases HPCs into the circulation in naïve mice. In contrast, while MSCs express CXCR4, administration of the antagonist alone does not stimulate mobilisation of MSCs. However, disrupting this axis using AMD3100 following VEGF-A¹⁶⁵ treatment, resulted in the mobilisation of MSCs into the blood. VEGF-A¹⁶⁵ treatment alone results in no mobilisation [120]. Administration of VEGF-A¹⁶⁵ was shown to stimulate the entry of HPCs into the S/G2/M phase of the cell cycle *in vivo*, severely impairing the migratory capacity. As such AMD3100 did not mobilise HPCs in mice pre-treated

with VEGF-A¹⁶⁵. The mechanisms by which MSCs are mobilised using this treatment regimen remain unclear. There are thought to be several stem cell niches other than the bone marrow from which stem cells can be mobilised. Notable examples include: myocardial tissue, adipose tissue, spleen, liver and lungs [341]. However, by perfusing the murine femoral bone marrow in isolation, MSCs were shown to be mobilised via VEGF-A¹⁶⁵ and AMD3100 in a manner consistent with mobilisation and enumeration by cardiac bleed. Whilst the MSCs could be being mobilised from the tissue surrounding the femoral bone marrow, the higher numbers of MSCs in the bone marrow compared to muscle suggests that the bone marrow is the major source of mobilised MSCs [341].

By mobilising MSCs into the blood using pharmacological agents, it is hypothesised that the healing process could be ramped up, resulting in a quicker recovery time and/or a greater level of repair. This hypothesis infers that the speed and level of recovery is dependent upon the number of MSCs mobilised into the circulating blood. VEGF-A¹⁶⁵ and AMD3100 have been identified as potential pharmacological agents for the mobilisation of MSCs. It is therefore hypothesised that optimisation of the dosage and duration of treatment with each of these agents could result in a greater level of MSC mobilisation into the blood. Previous work demonstrated that pre-treatment with VEGF-A¹⁶⁵ was critical for MSC mobilisation, in that acute administration of VEGF-A¹⁶⁵ alone or in combination with AMD3100 did not elicit MSC mobilisation.

As well as optimisation of the mobilisation regimen, it is also important to investigate if this mobilisation can be achieved using other factors. Chemokines have

been shown to act as a chemoattractant guiding migration of cells throughout the body [342,343]. CXCR4 is one of the highest expressed external chemokine receptors on all sets of MSCs investigated and its ligand CXCL12 is a potent chemoattractant for MSCs [230]. Other highly expressed receptors include CXCR3 and CCR10. CXCR3 and its ligand CXCL11, modulate leukocyte trafficking and CCR10 with its ligand CCL27 direct homing of T cells to the skin in immuno-modulatory pathways [344,345]. Both VEGF-A¹⁶⁵ and PDGF have been shown to stimulate cells to migrate [346,347]. PDGF has been shown to be involved in ligand-dependent chemotactic signalling [346]. It was hypothesised that each of these factors may play a role in the mobilisation of mMSCs by inducing mobilisation following VEGF-A¹⁶⁵ pre-treatment. Interruption of the CXCR4/CXCL12 axis following VEGF-A¹⁶⁵ treatment results in mMSC mobilisation. This may be due in part to the subsequent CXCL12 gradient causing cells to migrate from low CXCL12 concentrations in the bone marrow to elevated levels in the peripheral blood [294]. Further interrogation of this pathway via the direct administration of CXCL12 to the bloodstream may also result in MSC mobilisation.

Once the cells have been mobilised, it is important to investigate the functionality of the MSCs. That is, whether the mobilised bone marrow mMSCs can efficiently home to sites of injury and inflammation. Experiments have been performed to set up a model to investigate trafficking of endogenous MSCs to establish whether MSCs mobilised pharmacologically from the bone marrow can traffic to sites of tissue injury and inflammation.

6.2 Aims.

- Optimisation of mMSC mobilisation from the bone marrow into the peripheral blood using VEGF-A¹⁶⁵ and AMD3100.
- To investigate the pharmacokinetics of the MSC mobilisation with respect to:
 - Duration of enhanced mobilisation.
 - Ability to induce multiple mobilisation time points.
- To investigate other factors that may be able to mobilise endogenous MSCs *in vivo*.
- To determine the functionality of mobilised MSCs in terms of homing ability.

6.3 Results.

First experiments were performed to optimise the mobilisation of MSCs using the VEGF-A¹⁶⁵/AMD3100 combined therapy. Specifically, to optimise the dose of VEGF-A¹⁶⁵ or number of pre-treatments. Mice were therefore pre-treated with VEGF-A¹⁶⁵ for 2 or 4 days with 10, 50 or 100µg/kg VEGF-A¹⁶⁵ followed by a single dose of AMD3100 on day 5. After 4 days treatment using differing VEGF-A¹⁶⁵ daily doses (10, 50 or 100µg/kg i.p.) followed by a single dose of AMD3100 (5mg/kg), MSCs were mobilised from the bone marrow into the blood in a dose dependent manner (Fig. 6.1. 0µg/kg 0, 10µg/kg 5.30 ± 5.30 , 50µg/kg 18.36 ± 11.66 , 100µg/kg 32.25 ± 8.76 MSCs/ml). The greatest numbers are mobilised after 4 daily doses of 100µg/kg VEGF-A¹⁶⁵ followed by a single dose of AMD3100 (5µg/kg). After 2 days VEGF-A¹⁶⁵ pre-treatment at various doses (10, 50 or 100µg/kg i.p.), followed by a single dose of AMD3100 on day 3, MSCs were not mobilised in significant numbers (Fig. 6.1).

Since AMD3100 is fast acting with a half life of 3-5 hours, it is not known how long the MSC mobilisation persists for [348]. To investigate the kinetics of mobilisation, MSC numbers in the blood were determined at 0, 1, 6 and 24 hours post AMD3100 administration in VEGF-A¹⁶⁵ treated mice. As shown in Fig. 6.2. MSC numbers in the blood are highest 1 hour post AMD3100 injection (32.25 ± 12.37 MSCs/ml) with MSC numbers returning to basal levels 6 hours post AMD3100 administration (3.07 ± 3.07 MSCs/ml).

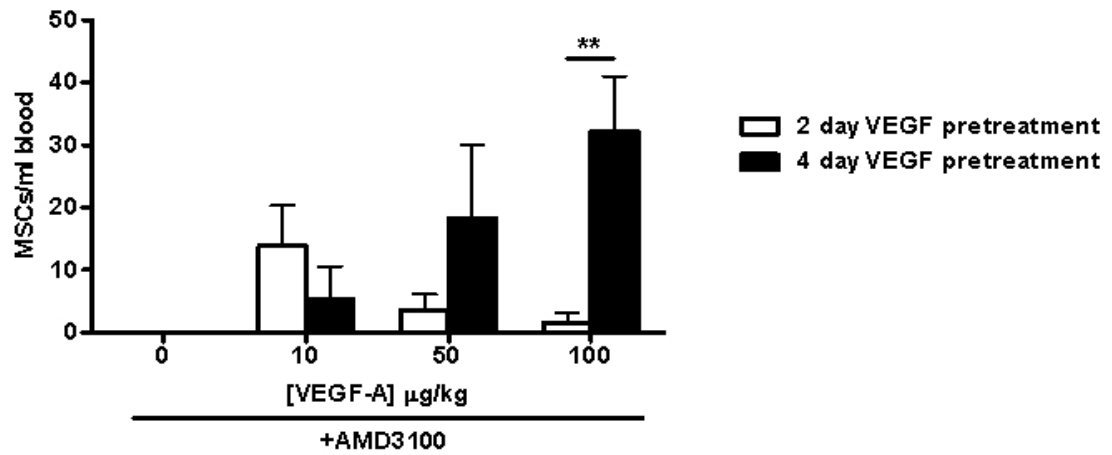


Fig. 6.1. Optimisation of dose and duration of VEGF-A¹⁶⁵ pre-treatment for MSC mobilisation by AMD3100.

Mice were pre-treated with VEGF-A¹⁶⁵ (10, 50 or 100µg/kg i.p.), or vehicle (PBS) once daily for 2 or 4 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or vehicle (PBS). Sixty minutes later, blood was taken for analysis of circulating MSCs. MSC numbers are shown as number of colonies per ml blood. n = 5–14 mice per group. Data are means ± SEM. **p < 0.01, between selected groups.

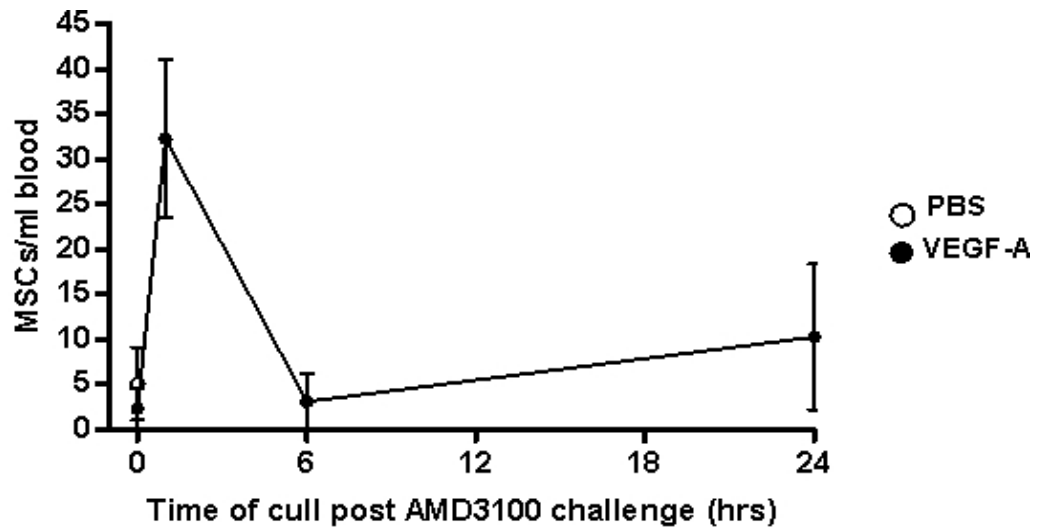


Fig. 6. 2. Kinetics of MSC mobilisation by AMD3100 in VEGF-A¹⁶⁵ pre-treated mice.

Mice were pre-treated with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or vehicle (PBS). Blood was taken for analysis of circulating MSCs at 0, 1, 6, or 24hours post AMD3100 administration. MSC numbers are shown as number of colonies per ml blood. n = 6–14 mice per group. Data are means ± SEM.

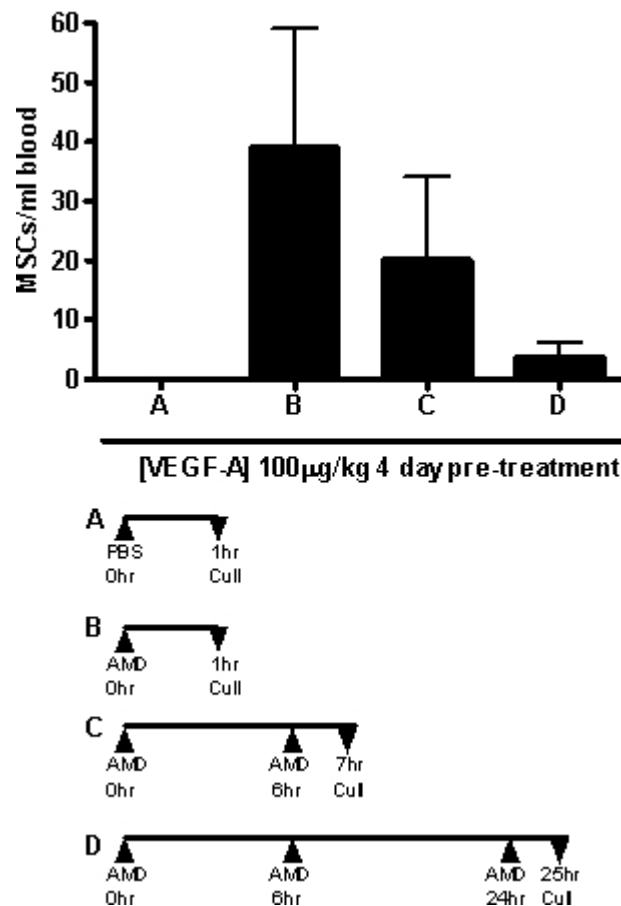


Fig. 6.3. Effect of repeated AMD3100 administration on MSC mobilisation in VEGF-A¹⁶⁵ pre-treated mice.

Mice were pre-treated with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or vehicle (PBS). 6 hours post initial AMD3100 administration, 2 of the groups were administered AMD3100 (5mg/kg i.p.) 24 hours post initial AMD3100 administration, 1 of the groups was administered AMD3100 (5mg/kg i.p.). Blood was taken for analysis of circulating MSCs 60 minutes post final AMD3100 administration. MSC numbers are shown as number of colonies per ml blood. n = 6 mice per group. Data are means ± SEM.

It is currently not known whether a single peak of MSCs would be sufficient to induce a therapeutic effect (e.g. following tissue ischemia) or whether a more sustained increase in MSC numbers would be desirable. Experiments were designed to ascertain whether MSCs could be mobilised repeatedly by multiple dosing with AMD3100 following single pre-treatment with VEGF-A¹⁶⁵. MSCs are most effectively mobilised after a single AMD3100 dose, following 4 daily doses of VEGF-A¹⁶⁵ (39.02 ± 20.01 MSCs/ml). MSCs were also present in the blood following a 2nd AMD3100 injection, 6 hours after the 1st AMD3100 dose, although to a lesser extent (20.08 ± 14.07 MSCs/ml). There was no significant mobilisation of MSCs when mice were given 3 AMD3100 doses at 1, 6 and 24 hours following VEGF-A¹⁶⁵ administration and culled 1 hour post the final AMD3100 injection (3.72 ± 2.43 MSCs/ml), as determined by one way-ANOVA and bonferroni post test. Attempts were also made to alter the timing and chronicity of VEGF-A¹⁶⁵ administration. In Fig. 6.4. it can be seen that neither administration of a single dose of AMD3100 5 days post initial dosing regimen, or a subsequent administration of 4 days VEGF-A¹⁶⁵ and a single dose of AMD3100 at day 10, could mobilise MSCs to a comparable level to that of 4 days VEGF-A¹⁶⁵ treatment and a single dose of AMD3100 on day 5. There does appear to be some mobilisation having re-administered VEGF-A¹⁶⁵ + AMD3100, but this mobilisation was not significant.

Chemokines are involved in the directed migration of cells [349]. CXCR3 and its ligand CXCL11, modulate leukocyte trafficking and CCR10 with its ligand CCL27 direct homing of T cells to the skin in immuno-modulatory pathways. PDGF has been shown to be involved in ligand-dependent chemotactic signalling [346]. Receptors for these chemokines were demonstrated to be expressed on MSCs, therefore Fig.

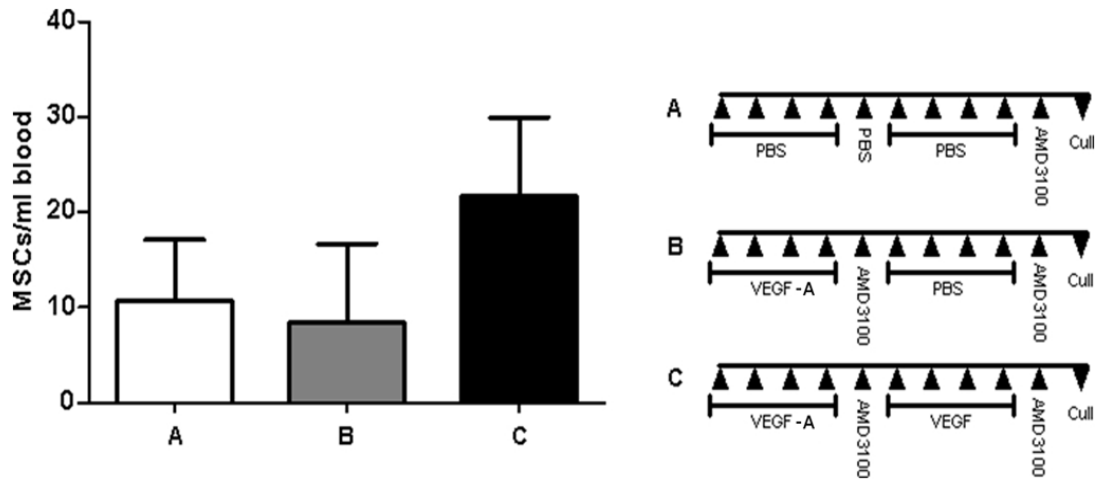


Fig. 6.4. Effect of repeated VEGF-A¹⁶⁵ + AMD3100 administration on MSC mobilisation.

Mice in group A were pre-treated with vehicle (PBS) once daily for 9 days (100µg/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Mice in group B were pre-treated with VEGF-A¹⁶⁵ once daily for 4 days (100µg/kg i.p.). On day 5 they received a CXCR4 antagonist (AMD3100 5mg/kg i.p.). On days 6-9 they were treated with vehicle (PBS) once daily. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Mice in group C were pre-treated with VEGF-A¹⁶⁵ once daily for 4 days (100µg/kg i.p.). On day 5 they received a CXCR4 antagonist (AMD3100 5mg/kg i.p.). On days 6-9 they received further treatment with VEGF-A¹⁶⁵ once daily (100µg/kg i.p.) Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Blood was taken for analysis of circulating MSCs 60 minutes post final AMD3100 administration. MSC numbers are shown as number of colonies per ml blood. n = 6 mice per group. Data are means ± SEM.

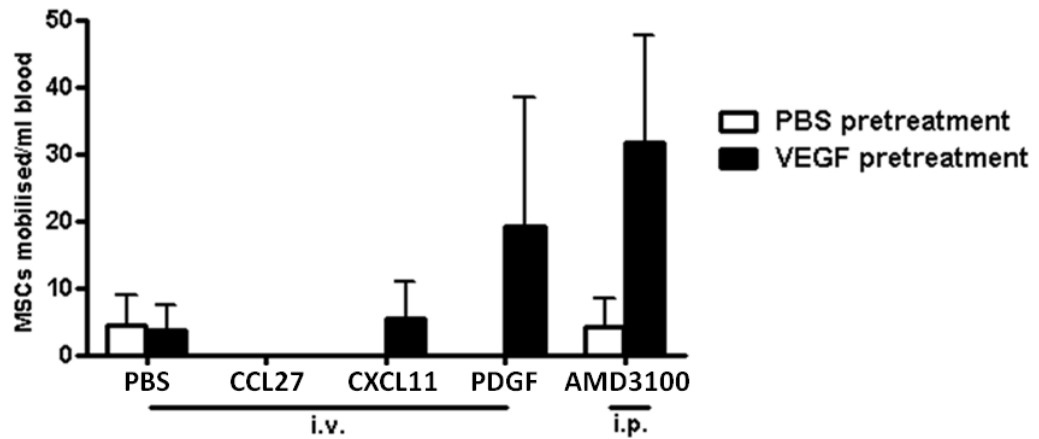


Fig. 6.5. MSC mobilisation following VEGF-A¹⁶⁵ pre-treatment and chemokine or growth factor challenge i.p. or i.v.

Mice were pre-treated with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). Twenty-four hours after the last injection, mice were administered a vehicle (PBS), CCL27, CXCL11 or PDGF (250µl 50nM i.v.) or a CXCR4 antagonist (AMD3100 5mg/kg i.p.). Sixty minutes later, blood was taken for analysis of circulating MSCs. Data are means ± SEM. n=3.

6.5 shows the effects and efficiency of CCL27, CXCL11 and PDGF in mobilising MSCs, either alone or in combination with 4 days VEGF-A¹⁶⁵ pre-treatment. CCL27 and CXCL11, ligands for CXCR3 and CCR10 respectively did not cause mobilisation of MSCs into the blood either when administered alone or injected iv 24 hours after 4 daily doses of VEGF-A¹⁶⁵ (i.p. 100 mg/kg). PDGF, when administered i.p (100µg/kg) 24 hours after 4 days of daily intra-peritoneal injections of PBS did not result in any MSC mobilisation. Combining a single dose of PDGF (i.p. day 5) with 4 day VEGF-A¹⁶⁵ pre-treatment resulted in MSC mobilisation (19.20 ± 19.20 MSCs/ml), although there is a high level of variability between mice and the numbers mobilised are not significant over controls.

The CXCR4/CXCL12 axis is important in the retention of stem cells in the bone marrow [294]. (Fig. 6.6). It has previously been shown that delivery of CXCL12 via an adenovirus stimulated HPC mobilisation into the blood [348]. To investigate whether CXCL12 could similarly mobilise MSCs, mice pre-treated with VEGF-A¹⁶⁵ over 4 days were administered a single i.v. dose of CXCL12 or AMD3100. While disrupting the CXCR4/CXCL12 axis using a CXCR4 antagonist (AMD3100, i.p. 5mg/kg) resulted in mobilisation into the blood, CXCL12 did not mobilise MSCs (Fig. 6.6). To determine whether AMD3100 displaced CXCL12 into the plasma, CXCL12 levels were measured following treatment with VEGF-A¹⁶⁵ (100µg/kg i.p.) or PBS once daily for 4 days and a single administration of AMD3100 on day 5. There was no significance between the levels of CXCL12 in the blood plasma when comparing each of these groups.

Injection of matrigel sub-cutaneously causes an inflammatory response in

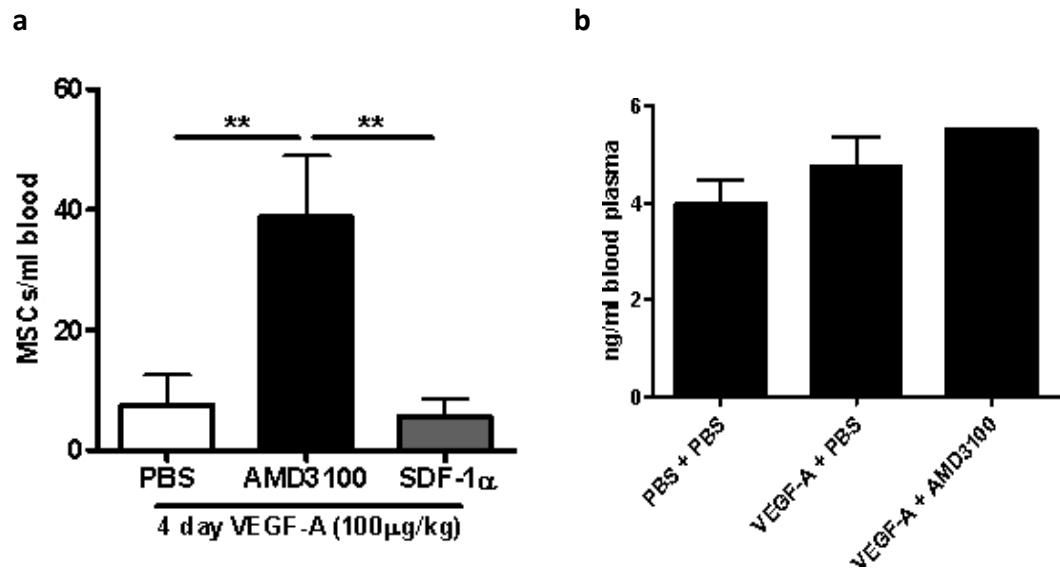


Fig. 6.6. The role of CXCL12 in the mobilisation from the bone marrow following VEGF-A¹⁶⁵ and AMD3100 treatment.

(Fig. 6.6.a) Mice were pre-treated with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100 μ g/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.), CXCL12 (50nM, 200 μ l, i.v.), or vehicle (PBS, 200 μ l, i.v.). Sixty minutes later, blood was taken for analysis of circulating MSCs by colony assays. Data are means $\pm$ SEM. **p < 0.01, between selected groups. n = 6-18. (Fig. 6.6.b) CXCL12 levels in blood plasma following treatment with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100 μ g/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.), CXCL12, or vehicle (PBS). Sixty minutes later, blood was taken from which plasma was obtained for analysis of CXCL12 levels via ELISA.

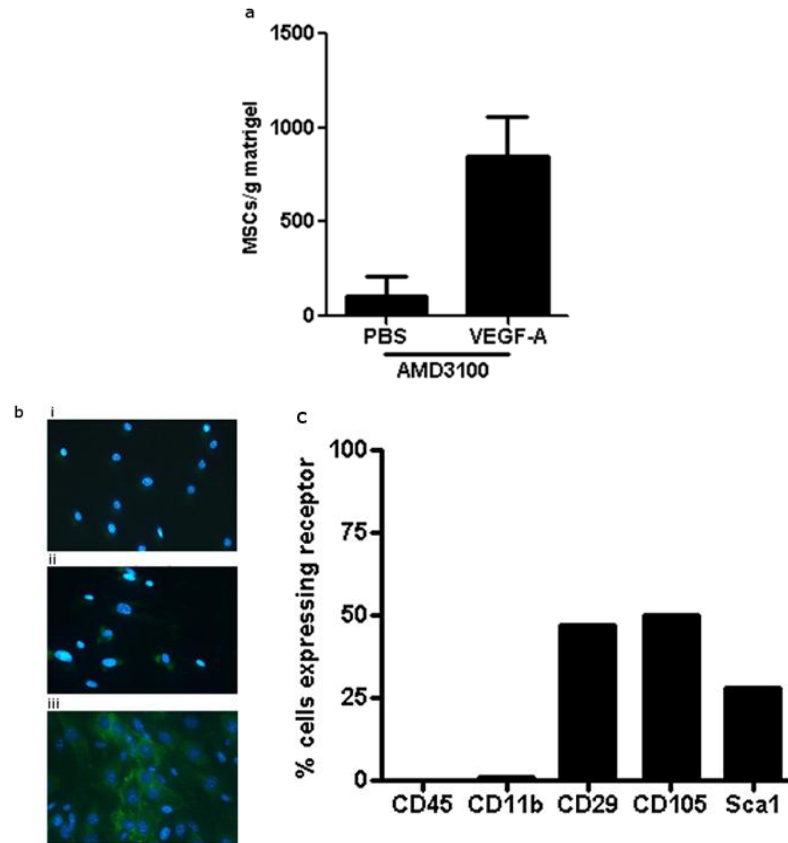


Fig. 6.7. MSC recruitment *in vivo* to matrigel injected subcutaneously, following pre-treatment with VEGF-A¹⁶⁵ and AMD3100.

Fig. 6.7.a. Matrigel was injected subcutaneously under the dorsal skin. Mice were pre-treated with VEGF-A¹⁶⁵ or vehicle (PBS) once daily for 4 days (100 mg/kg i.p.). Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5 mg/kg i.p.). Mice were culled and the matrigel harvested 5 days post the final injection. Data are means $\pm$ SEM. n = 2-4. Fig. 6.7.b. Upon culture the MSCs obtained from the matrigel were tested for their collagen 1 expression Fig. 6.7.b.ii. using immunohistochemistry and compared with P5 bone marrow mMSCs (Fig. 6.7.b.i) and murine tracheal fibroblasts (Fig. 6.7.b.iii). (Fig. 6.7.c) FACS analysis of P5matrigel derived MSCs for a selection of mMSC markers (CD45, CD11b, CD29, CD105, Sca-1) n=1.

mice [350]. MSCs injected into animals or patients have been shown to home to sites of injury *in vivo* [134,351]. A model was set up to investigate whether endogenous MSCs mobilised into the blood could traffic to sites of tissue injury. Thus Matrigel was injected sub-cutaneously and then mice were treated with VEGF-A¹⁶⁵ (100µg/kg i.p.) or PBS for 4 days followed by a single injection of AMD3100 on day 5. Matrigel plugs were harvested on day 10. The recruitment of MSCs into matrigel plugs at the site of inflammation *in vivo* is shown in Fig. 6.7.a. Matrigel plugs were dissolved in dispase and subsequent MSC numbers in the matrigel were quantified using CFU-F assays *in vitro*. MSCs were quantified per g/matrigel plug excised from the mouse. Mice who were treated with PBS for 4 days (250µl i.p.) followed by a single dose of AMD3100 on day 5 (5 mg/kg i.p.) had low levels of MSCs present in the matrigel plugs on day 5 (105.3 ± 105.3 MSCs/g matrigel). Matrigel plugs in mice treated with the MSC mobilising regimen (4 daily VEGF-A¹⁶⁵ injections i.p., 100 mg/kg, single AMD3100 injection on day 5, i.p., 5 mg/kg) had higher levels of MSCs present (847.5 ± 213.0 MSCs/g matrigel). The significance of this finding cannot be calculated due to low n numbers. To further characterise CFU-Fs obtained from matrigel plugs, immunohistochemistry for collagen-1 was performed. As shown in Fig. 6.7.b, cells cultured from the Matrigel plugs did not express collagen-1 while murine tracheal fibroblasts did express collagen-1. Further characterisation of the matrigel derived MSCs can be seen in Fig. 6.7.c, which shows that the cells did not express the haematopoietic markers CD45 and CD11b, but did express the MSC surface markers CD29, CD105 and Sca-1.

6.4 Discussion.

Identifying molecular mechanisms by which MSCs egress from the bone marrow and the physiological role of these distinct stem and progenitor cell subsets remain to be major hurdles for regenerative medicine. Clinically, G-CSF has been used to mobilise HPCs for more than 30 years for bone marrow transplants [352,353]. G-CSF induces HPC mobilisation by decreasing bone marrow CXCL12 levels and increasing CXCR4 levels in the bone marrow [354]. AMD3100 is a bicyclam molecule that selectively and reversibly antagonises the binding of CXCL12 to its receptor CXCR4 causing subsequent egress of HPCs from the bone marrow to the peripheral blood [355]. However, this has been shown to be ineffective as a mobilising agent in 20% of patients. When patients are treated with a combination of G-CSF and AMD3100, the number of responsive patients is increased further, compared to G-CSF alone [356].

The detection of circulating MSCs in response to tissue injury suggests that MSCs can be mobilised from their niches upon appropriate signals in order to recruit to damaged tissue [250]. Treatment with VEGF-A¹⁶⁵ for 4 days followed by a single dose of AMD3100 is a reproducible strategy for mobilising MSCs from the bone marrow into the blood [120]. In Pitchford *et al's* study, distinct pathways were identified regulating the differential mobilisation of progenitor cells. Furthermore, significant numbers of MSCs were detected in peripheral blood when the CXCR4 antagonist, AMD3100, was administered to mice pre-treated with VEGF-A¹⁶⁵ but not G-CSF [120]. In this chapter I first sought to establish the kinetics of this response

and develop strategies to enhance MSC numbers in the blood over a more sustained period, as might be required for effective treatment of a chronic disease or serious acute injury (e.g. myocardial infarction). Attempts to mobilise MSCs from the bone marrow with G-CSF treatment have been largely disappointing [357].

There are several VEGF isoforms, of which the most heavily researched is VEGF-A [358]. VEGF-A¹⁶⁵ has been shown to play a role in angiogenesis, vasodilatation (indirectly by NO release), is chemotactic for macrophages and granulocytes, and notably, stimulates EPC migration [257,359]. Upon pre-treatment with VEGF-A¹⁶⁵ and the acute administration of AMD3100, MSCs and EPCs are mobilised, whereas HPC and neutrophil mobilisation is suppressed.

To date, the pharmacokinetics of the dosing regimen and its clinical relevance have not previously been investigated. It was first important to establish the lowest dose of VEGF-A¹⁶⁵ required to mobilise MSCs, to reduce cost and the potential for side-effects when research is translated into man. Investigation into lower dosing regimens of VEGF-A¹⁶⁵ looking at whether MSC mobilisation levels equal to or greater than those previously described (4 days VEGF-A¹⁶⁵ 100µg/kg i.p., day 5: AMD3100 5mg/kg i.p.) could be achieved can be seen in Fig. 6.1. Differing daily doses and time courses of 2 or 4 days were investigated. When VEGF-A¹⁶⁵ was administered for 2 days at 10, 50 or 100µg/kg followed by a single dose of AMD3100 (5mg/kg) on day 3, there was no significant mobilisation of MSCs into the blood. This suggests that the bone marrow needs to be exposed to a prolonged administration of VEGF-A¹⁶⁵ in order for the mMSCs to be released following CXCR4 antagonism. Lowering the dose of VEGF-A¹⁶⁵ from 100 to 50 or 10µg/kg substantially reduced the

mobilisation of MSCs. Indeed at the lower concentrations of VEGF-A¹⁶⁵, MSC levels were not significantly elevated. From this, it can be seen that the described VEGF-A¹⁶⁵ treatment protocol is the optimum dose of the 4 investigated. Interestingly, the apparent dose dependent response in the 4 day VEGF-A¹⁶⁵ treated mice suggests that a greater level of mobilisation could be achieved if the dose was increase above 100µg/kg. Similarly, the fact that there is no mobilisation in the 2 day experiments but there is significant mobilisation in the 4 day treatments, suggests that even longer dosing regimens e.g. 6, 8 days may result in an enhanced mobilisation over that caused by 4 day treatment with VEGF-A¹⁶⁵. Experiments using longer treatments or higher concentrations of VEGF-A¹⁶⁵ were not performed due to the cost implications.

Analysing MSC numbers in this way gives representative circulating levels at a single time point. AMD3100 has been shown to have rapid effects and a short half life in experiments investigating HPC mobilisation from the bone marrow [356]. The optimum time point for HPC mobilisation has been shown to be 60 minutes post intra-peritoneal AMD3100. In order to understand the kinetics of this mobilisation and to see if the analysis was taking place at the appropriate time point, circulating MSC numbers were quantified at various time points post AMD3100 administration. Within 6 hours of administration, MSC numbers in the peripheral blood have returned to basal levels. This is likely to be due to MSCs homing back to the bone marrow or redistributed to other tissues with local stem cell niches such as the liver, spleen and lungs [330]. Having returned to basal levels, MSC numbers in the circulating blood remain at basal levels 24 hours after AMD3100 administration. Fig. 6.2. provides significant evidence that the time point of 1 hour proves optimum as a

model to be used to characterise the mechanism of mobilisation further. Having previously noted that using a combination of VEGF-A¹⁶⁵ and AMD3100 mobilises MSCs but not HPCs, it is known that the AMD3100 is causing mobilisation of MSCs via a different pathway to that of HPC mobilisation [120]. Having optimised the conditions by which MSCs are mobilised, future studies could investigate the potential of different CXCR4 antagonists in this mobilisation regimen.

With regards to the clinical applications of mobilising MSCs from the bone marrow into the blood, many candidate diseases for treatment would require multiple applications as opposed to a single curative treatment. In particular, degenerative diseases such as multiple sclerosis [360] in which the MSCs will act only in a temporary manner as opposed to having a curative effect. For this reason, repeated mobilisation of MSCs into the circulating blood via CXCR4 antagonism was investigated. The effect of repeated AMD3100 administration in VEGF-A¹⁶⁵ pre-treated mice can be seen in Fig. 6.3. Dosing regimen A is a control and B shows the optimum dosing regimen identified to date. Additional doses of AMD3100 (5mg/kg) in the hours that follow, as can be seen in C, do not provide a second peak of mobilisation. From this, it can be assumed that the number of MSCs primed for mobilisation by AMD3100 has been exhausted. 24 hours may not be sufficient for the niche from which the MSCs are mobilised to re-populate. Alternatively, the bone marrow may be generating new MSCs which have not received the prior VEGF-A¹⁶⁵ treatment. This latter point was investigated via re-treating with VEGF-A¹⁶⁵. Alternatively, the bone marrow could benefit from a longer recovery period before repeated AMD3100 dosing. MSC levels in the blood of mice treated with the optimum mobilisation regimen followed by a second AMD3100 application on day

10 is shown in Fig. 6.4.b. A lack of a second peak indicates that allowing time for the bone marrow to re-populate is not sufficient for repeated MSC mobilisation by AMD3100 after 5 days. In Fig. 6.4.c. mice were given the optimum dosing regimen twice in succession. The numbers mobilised suggest that a second mobilisation may be possible, however any increase over control is not significant. Due to the nature of MSCs, it is also possible that there is only a small subset of MSCs that can be mobilised from the bone marrow which, following initial release have homed back to the bone marrow where they have been primed for a second release. In order to investigate this further using existing technology, mice would need to receive blood transfusions following initial MSC mobilisation to remove the mobilised MSCs and prevent them homing back to the bone marrow. For the purposes of this investigation this is not feasible in terms of time or cost.

A CXCR4 antagonist is used as a short acting pharmacological agent in this series of experiments due to the known action of the CXCR4/CXCL12 in the retention of stem and progenitor cells in the bone marrow. However, a lot of research has been published on the role that other chemokines and their receptors play in the trafficking of leukocytes throughout the body [361,362]. Having elucidated in Chapter 3 that CXCR4 is one of several highly expressed chemokine receptors on the surface of P5 bone marrow mMSCs, an obvious route to investigate other pathways which can cause MSC mobilisation is to target these other receptors and analyse their ability to cause MSC egress. Administration of AMD3100 has been shown to increase levels of CXCL12 in the blood [363] such that a chemokine gradient is created along which cells can migrate into the blood. CXCR3 and CCR10 are both highly expressed on the surface of MSCs. However, when their ligands CXCL11

(CXCR3) and CCL27 (CCR10), were administered to mice systemically, either alone or following 4 day VEGF-A¹⁶⁵ treatment of cells, there was no significant mobilisation of MSCs above basal levels. This suggests that VEGF-A¹⁶⁵ treatment and a chemokine gradient down which MSCs can chemotax *in vitro*, are not sufficient for mobilisation. This finding is confirmed in Fig. 6.6. which shows that administration of CXCL12 to the blood directly following VEGF-A¹⁶⁵ pre-treatment, does not result in MSC mobilisation. VEGF-A¹⁶⁵ was initially investigated due to its role in angiogenesis. PDGF supports vascular development and angiogenesis in a number of organs [364] and has been shown to be a potent chemoattractant for bone marrow derived hMSCs *in vitro* [365,366]. As shown in Fig. 6.5. there was some indication that PDGF could mobilise MSCs, but the data was not significant. This will be worth further investigation in the future.

In Chapters 3 & 4, it was confirmed that both human and murine MSCs can migrate towards chemokines using *in vitro* chemotaxis assays. It is well documented that chemokines are produced at sites of injury and inflammation [228,367]. However, following systemic administration of bone marrow mMSCs in Fig 3.13. to an LPS model of inflammation in the lungs in mice, there was no recruitment of MSCs to the lungs in the time period analysed. Having successfully mobilised the MSCs into the circulating blood, it was critical to test whether these cells could be recruited to sites of inflammation. To do this, a model of injury was used, whereby matrigel plugs were injected subcutaneously in mice which were subsequently treated with the VEGF-A¹⁶⁵ and AMD3100 mobilisation regimen, or PBS and AMD3100. Matrigel is a gelatinous protein mixture that resembles the complex extracellular environment found in many tissues. 5 days post AMD3100

administration, matrigel plugs were removed and the MSC numbers (per g of Matrigel), were quantified using colony assays. Mice treated with the MSC mobilising regimen had greater numbers of MSCs at the site of injury in the matrigel, than mice treated with PBS and AMD3100. This provides evidence to support that these MSCs can migrate to sites of tissue injury, having been mobilised from the bone marrow. This has far reaching implications in terms of clinical applications. We know now that MSCs mobilised using this pharmacological approach will both migrate to, and be retained at sites of injury without the need for further direction via physical or pharmacological interventions. The MSCs harvested from the matrigel plugs formed CFU's *in vitro* and stained negatively for collagenase-1, confirming that they are not fibroblasts. This model system may be used to further investigate MSC trafficking from bone marrow to sites of inflammation *in vivo*.

7.

**Interrogating the molecular mechanisms of MSC
mobilisation from the bone marrow.**

7. Interrogating the molecular mechanisms of MSC mobilisation from the bone marrow.

7.1 Introduction.

Having ascertained that VEGF-A¹⁶⁵ plays a critical role in priming bone marrow for the mobilisation of MSCs, it was important to investigate the molecular mechanisms underlying this response. There are 3 known subtypes of VEGFRs, VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1) and VEGFR-3 (Flt-4) [368]. VEGFR-1 promotes the migration and proliferation of haematopoietic cells whilst VEGFR-2 promotes the proliferation of endothelial cells [265] and VEGFR-3 is involved in lymphangiogenesis [266]. It has been reported that hMSCs express mRNA for VEGFR-1, VEGFR-2 and VEGFR-3 [347] and preliminary data in Chapter 5 suggested that PαS cells express VEGFR-1 and a sub-population express VEGFR-2. It was therefore important to characterise P5 bone marrow MSCs in terms of their external VEGFR expression levels. VEGF-A¹⁶⁵ only binds to VEGFR-1 and VEGFR-2, with a higher affinity for VEGFR-1 [271]. The high affinity of VEGFR-1 for VEGF-A¹⁶⁵ is critical in angiogenesis, where VEGFR-1 acts as a decoy receptor for VEGF-A¹⁶⁵, thereby preventing it from binding to and activating VEGFR-2 [369]. Pitchford *et al* [120] investigated selective binding to VEGFRs when looking at the mechanism of HPC release from the bone marrow following 4 day VEGF-A¹⁶⁵ treatment. It was found that selective blockade of VEGFR-1 abolishes the ability of exogenous VEGF-A¹⁶⁵ to stimulate HPC entry into the cell cycle *in vivo*, thus, restoring the ability of CXCR4 antagonists to mobilise

HPCs from the bone marrow [120]. Previous mouse studies which have blocked VEGFR-1 have also reported a strong inhibition of inflammation and atherosclerotic plaque formation [370]. Given that VEGF-A¹⁶⁵ is acting on these cells via VEGFR-1 and/or VEGFR-2 binding, VEGFR-1 and VEGFR-2 blocking experiments and their subsequent effect on MSC mobilisation will be investigated in this chapter. Various VEGF isoforms exist including VEGF-A¹⁶⁵, PlGF, VEGF-B, VEGF-C and VEGF-D [269]. VEGF related proteins have also been discovered named VEGF-E and VEGF-F [262,271]. PlGF has been shown to act specifically on VEGFR-1 [271] and VEGF-E is known to activate VEGFR-2 [262]. Experiments in this chapter will focus on the isoforms of VEGF that bind specifically to VEGFR-1 and VEGFR-2 in order to determine their ability to mobilise MSCs from the bone marrow into the peripheral blood. These experiments will provide novel mechanistic data as to the roles of VEGFRs in MSC mobilisation. Future studies could involve the optimisation of specific VEGFR binding in the mobilising pathway in order to enhance MSC numbers in the blood, as has been investigated in Chapter 6 with VEGF-A¹⁶⁵.

As detailed in Chapter 5, the characterisation of PαS cells and specifically their surface marker profile (CD45- Ter119- PDGFRα+ Sca-1+) means that the identification of MSCs *in vivo* is now possible [124]. This chapter aims to identify PαS cells in the blood and confirm whether this highly homogeneous population of MSCs can be mobilised via VEGF-A¹⁶⁵ and AMD3100 treatment. Confirmation of this result means that analysing the effects of VEGF-A¹⁶⁵, VEGFR blocking and VEGF isoform treatment on the phenotype of the MSCs is possible. In terms of identifying a mechanism by which the cells are primed for mobilisation, there are several pathways which could be playing a critical role. If VEGFRs are present on the cell

surface as is the case for P α S cells, the action of VEGF-A¹⁶⁵ may result in changes to VEGFR surface expression levels. This is the case with hMSCs, which when treated with VEGF-C demonstrate an upregulation of VEGFR-2 on the cell surface [371]. HPCs are mobilised from the bone marrow in G₀ [120]. Mobilisation of MSCs may be dependent on their cell cycle status. Chapter 6 illustrated that no MSCs are mobilised via VEGF-A¹⁶⁵ treatment alone, confirming that CXCR4 antagonism drives the mobilisation of primed cells. It could be hypothesised that VEGF is affecting CXCR4 expression levels on the surface of the MSCs, making them more susceptible to mobilisation. Each of these parameters will be investigated by analysing the bone marrow P α S cells of mice treated with different VEGF regimens over 4 days, prior to AMD3100 treatment. As with the effects of MSCs at sites of injury and inflammation, VEGF-A¹⁶⁵ may be acting on MSCs by affecting their paracrine function. Migration of leukocytes through extra-cellular matrix is facilitated by ECM-degrading enzymes such as matrix metalloproteases (MMPs) [214]. In cancer stem cells, which share many of the defining characteristics of MSCs, an autocrine positive loop exists where VEGF-A¹⁶⁵ binds to VEGFR-1 and VEGFR-2 resulting in the release of MMP-2 and MMP-9 [372]. If VEGF-A¹⁶⁵ is acting in a similar way on MSCs, this may explain how the cells are being primed in such a manner that MSCs can move along a CXCL12 gradient into the blood upon AMD3100 treatment.

If a therapeutic benefit is to be achieved using endogenous MSCs mobilised into the blood using pharmacological agents, a key factor may be the numbers of cells that are mobilised. It is not yet known what level of MSC numbers is sufficient to provide an enhanced repair. It is not yet known if any of the therapeutic effects of MSCs are acting in a dose dependent manner *in vivo*. Cellular treatments thus far

have shown low engraftment levels, yet still had a therapeutic benefit [373]. This suggests that this enhancement may be achieved by relatively low numbers. However, in order to ensure this mobilisation of endogenous MSCs is sufficient to pass this threshold, further optimisation of this mobilisation regimen must be investigated.

7.2 Aims.

- To characterise P5 bone marrow mMSCs in terms of VEGFR surface expression.
- Interrogate VEGF biology with respect to mobilisation.
- To investigate whether PαS cells can be mobilised from the bone marrow to blood in a quantifiable manner.
- To elucidate a mechanism by which MSCs are mobilised from the bone marrow following VEGF and AMD3100 treatment.

7.3 Results.

It has been reported that mMSCs do not express VEGFRs on the cell surface [255]. However, this proved not to be the case for P5 bone marrow mMSCs. Fig. 7.1. shows the varying levels of external VEGFR expression on P5 bone marrow derived mMSCs. In particular, high numbers of mMSCs express VEGFR-1 ($69.26 \pm 7.86\%$), an intermediate number of cells ($23.95 \pm 8.17\%$) express VEGFR-2 and none of the cells express VEGFR-3. Figs. 7.1.b,c,d. show representative histogram FACS plots for each of the receptors analysed. From this it can be seen that VEGFR-1 is expressed on a defined sub-population of P5 bone marrow mMSCs. Fig. 7.1.d. is a FACS dot plot showing both VEGFR-1 and VEGFR-2 expression on the same set of P5 bone marrow mMSCs. This illustrates that of the cells which are VEGFR-2 positive, nearly all (98%) are VEGFR-1 positive.

VEGF-A¹⁶⁵ is acting on these cells via VEGFR-1 and/or VEGFR-2 binding. VEGFR-1 and VEGFR-2 blocking experiments were conducted to investigate the subsequent effect on MSC mobilisation. Enhanced mobilisation of MSCs into the peripheral blood using VEGFR-1 blocking antibodies (mAbs) was observed (Fig. 7.2). The numbers of MSC CFU-Fs/ml of blood, formed after 21 days culture for blood taken from mice treated with different pharmacological agents can be seen in Fig. 7.2.a. Mice treated with PBS for 5 days had little or no circulating MSCs in the circulating blood 60 minutes after the final PBS injection (7.64 ± 4.28 MSCs/ml blood). Mice treated for 4 days with IgG and VEGF-A¹⁶⁵, in combination with a single

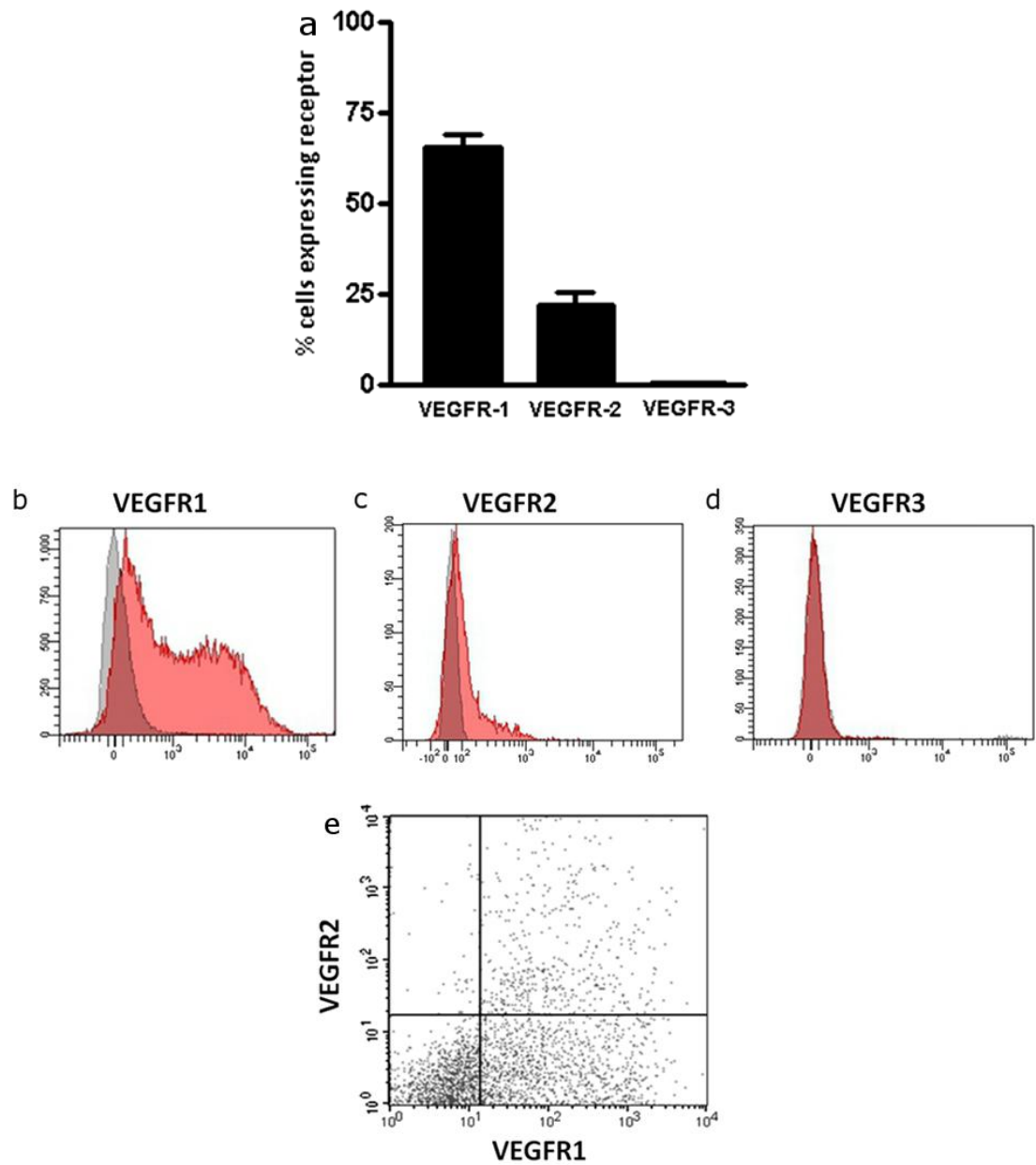


Fig. 7.1. External VEGFR1, VEGFR2 and VEGFR3 expression on passage 5 bone marrow derived murine MSCs.

Fig 7.1.a. Quantification of VEGFR expression on P5 bone marrow derived mMSCs. n = 3. FACS histogram plots of VEGFR1 (Fig 7.1.b) and VEGFR2 (Fig 7.1.c) and VEGFR3 (Fig 7.1.d) on bone marrow MSCs at passage 5 isolated by plastic adherence. (Fig 7.1.e). FACS dot plot analysis of passage 5 bone marrow MSCs co-stained for VEGFR1 in APC (FL4-H) and VEGFR2 in PE (FL2-H).

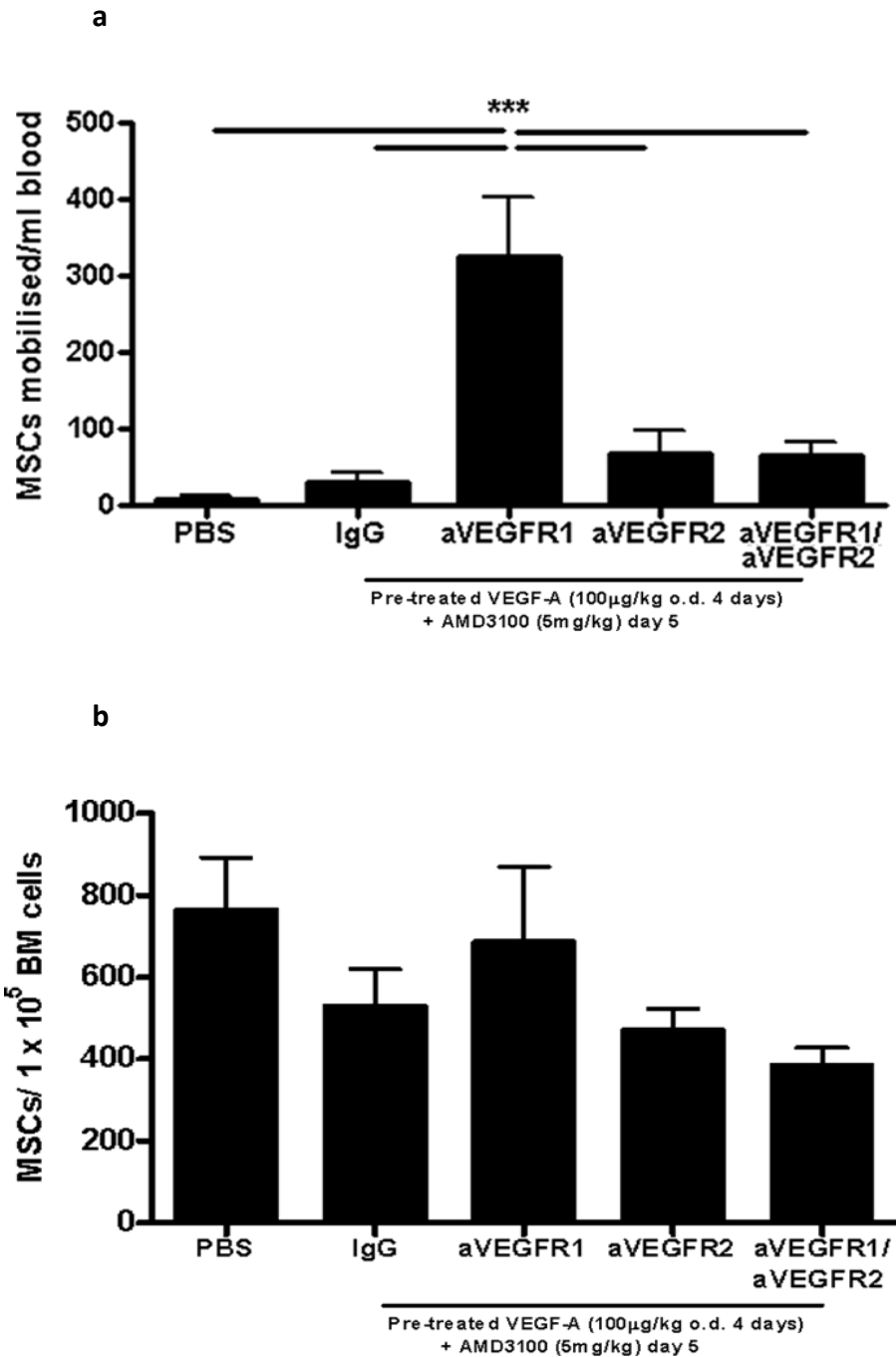


Fig. 7.2. Enhanced mobilisation of MSCs using VEGFR blocking antibodies.

Mice were pretreated with VEGF-A¹⁶⁵ (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. At day 0 and 2, groups were treated with IgG, anti VEGFR1 +/- or anti VEGFR 2 blocking antibodies. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or vehicle (PBS). Sixty minutes later, blood was taken for analysis of circulating MSCs and hind leg femurs were flushed with 1ml DMEM + 10% FCS for

analysis of bone marrow MSCs. MSC numbers are shown as number of colonies per ml blood (Fig 7.2.a) and MSCs in the bone marrow / 1×10^5 bone marrow cells (Fig 7.2.b). $n = 5$ mice per group. Data are means $\pm$ SEM. *** $p < 0.001$ between selected groups.

AMD3100 dose on day 5, had an increased number of MSCs in the circulating blood than the PBS controls (30.78 ± 12.75 MSCs/ml blood). Mice treated with VEGF-A¹⁶⁵ for 4 days, anti-VEGFR-1 mAbs on days 1 and 3 and a single dose of AMD3100 on day 5 showed very high levels of MSC mobilisation into the blood 60 minutes post AMD3100 injection (326.5 ± 76.17 MSCs/ml blood). Mice treated with either anti-VEGFR-2 mAbs, or both anti-VEGFR-1 and anti-VEGFR-2 mAbs in combination with VEGF-A¹⁶⁵ over 4 days and a single dose of AMD3100 dose on day 5, resulted in significantly increased mobilisation of MSCs, above that of control levels, but an order of magnitude below that of mice treated with VEGF-A¹⁶⁵, anti-VEGFR-1 mAbs and AMD3100 (anti-VEGFR-1 68.25 ± 29.07 MSCs/ml blood, anti-VEGFR-1 + anti-VEGFR-2 65.57 ± 17.18 MSCs/ml blood). Mice in the group treated with VEGF-A¹⁶⁵, a VEGFR-1 mAbs and AMD3100 had significantly more MSCs in the blood than mice in all 4 of the other treatment groups. Fig. 7.2.b. shows the MSC number in the bone marrow of mice for each of the treatment groups explained above (PBS 763.7 ± 288.2 , IgG 529 ± 200.8 , anti-VEGFR-1 686.1 ± 409.7 , anti-VEGFR-2 470.4 ± 118 , anti-VEGFR-1 + anti-VEGFR-2 384 ± 98.06 MSCs/ 1×10^5 bone marrow cells). There were no significant differences in MSC numbers in the bone marrow, as quantified by colony assay, between each of the 5 groups.

PlGF has been shown to act specifically on VEGFR-1 [271] and VEGF-E is known to specifically activate VEGFR-2 [262]. In order to compare activation of either VEGFR-1 or VEGFR-2 individually, PlGF and VEGF-E were administered over 4 days and the resulting MSC mobilisation was compared to VEGF-A¹⁶⁵. MSC numbers in peripheral blood for groups of mice treated with PBS or growth factors for 4 days

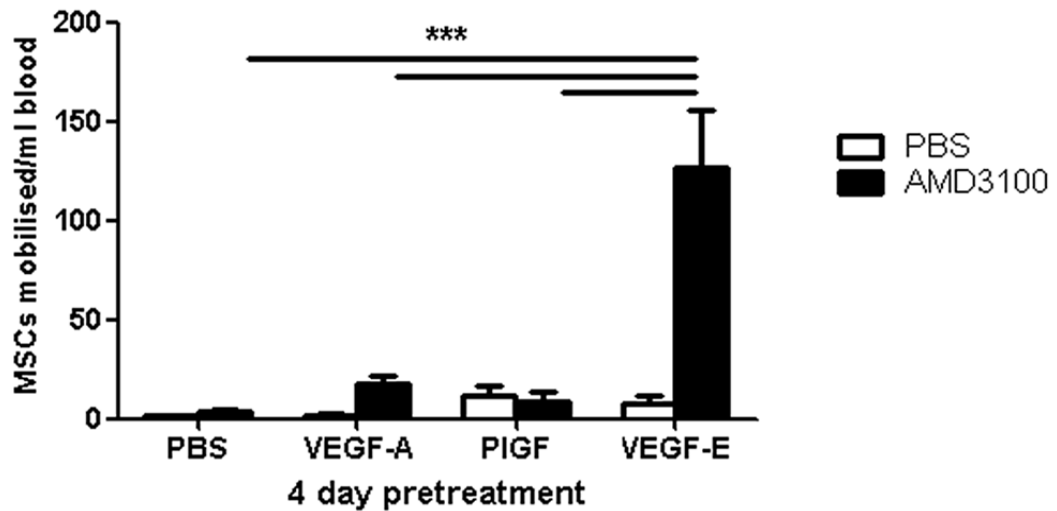


Fig. 7.3. MSC mobilisation into blood following growth factor pre-treatment and challenge with AMD3100.

Mice were pretreated with VEGF-A¹⁶⁵ (100µg/kg i.p.), PlGF (100µg/kg i.p.), VEGF-E (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or PBS. Sixty minutes later, blood was taken for analysis of circulating MSCs. MSC numbers are shown as number of colonies per ml blood. n = 5-12 mice per group. Data are means ± SEM. ***p < 0.001 between selected groups.

followed by a single dose of either PBS or AMD3100 on day 5 can be seen in Fig. 7.3. For mice treated with PBS, VEGF-A¹⁶⁵, PlGF or VEGF-E for 4 days followed by a single dose of PBS on day 5, there were no significant numbers of MSCs mobilised into the blood (2.50 ± 1.71 , 2.33 ± 1.42 , 11.96 ± 5.16 , 7.50 ± 4.67 MSCs/ml blood, respectively). Mice treated for 4 days with PBS followed by a single dose of AMD3100 on day 5 did not display an elevated number of MSCs in the peripheral blood above that of control (5.39 ± 4.10 MSCs/ml blood). Mice treated with VEGF-A¹⁶⁵ for 4 days followed by a single dose of AMD3100 showed significant mobilisation of MSCs into the peripheral blood when compared to control (32.56 ± 11.24 MSCs/ml blood). Mice treated with PlGF for 4 days followed by a single dose of AMD3100 showed no significant mobilisation of MSCs into the peripheral blood when compared to control (9.94 ± 8.13 MSCs/ml blood). Mice treated with VEGF-E for 4 days followed by a single dose of AMD3100 showed significant mobilisation of MSCs into the peripheral blood when compared to control (141.9 ± 33.35 MSCs/ml blood). Mice treated with VEGF-E and AMD3100 showed significant enhanced mobilisation of MSCs into the peripheral blood than mice treated with VEGF-A¹⁶⁵ and AMD3100 (141.9 ± 33.35 vs. 32.56 ± 11.24 MSCs/ml blood).

Fig. 7.4. compares MSC numbers in the blood of mice treated with single growth factors and AMD3100 in comparison to mice treated with both VEGF-E and PlGF plus AMD3100. Mice treated for 4 days with PBS followed by a single dose of AMD3100 on day 5 did not result in an elevated number of MSCs in the blood above that of control (1.25 ± 0.91 MSCs/ml blood). Mice treated with VEGF-A¹⁶⁵ for 4 days followed by a single dose of AMD3100 showed significant mobilisation of MSCs into

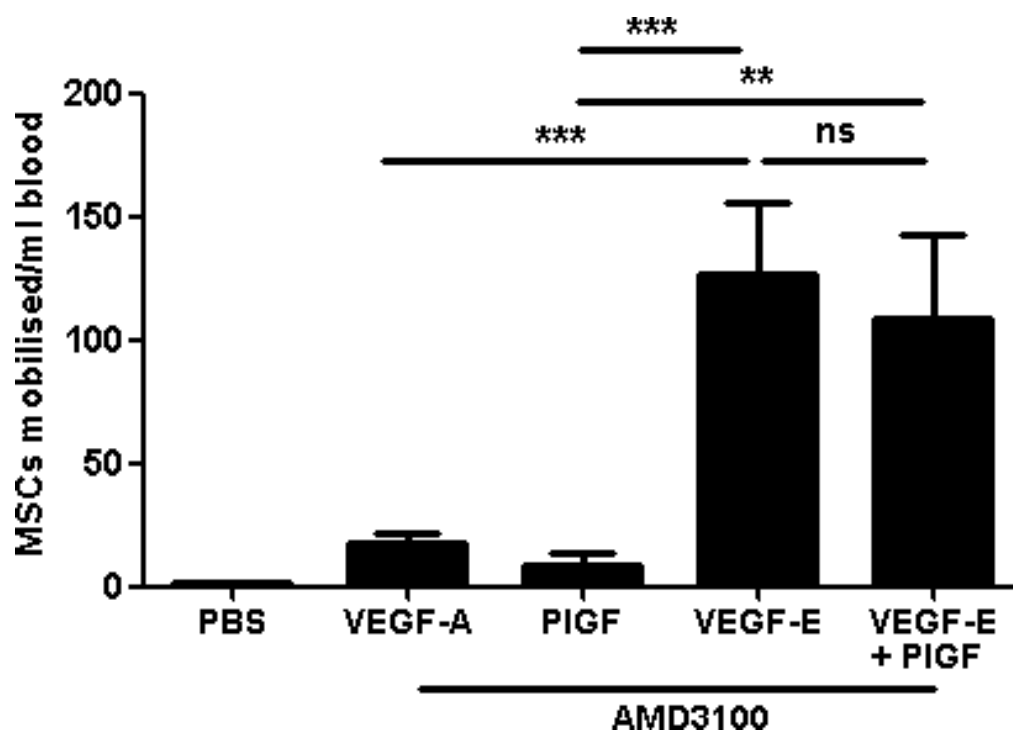


Fig. 7.4. MSC mobilisation into blood following growth factor pre-treatment and challenge with AMD3100.

Mice were pretreated with VEGF-A¹⁶⁵ (100µg/kg i.p.), PIGF (100µg/kg i.p.), VEGF-E (100µg/kg i.p.), PIGF (100µg/kg i.p.) + VEGF-E (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or PBS. Sixty minutes later, blood was taken for analysis of circulating MSCs. n = 5-12. Data are means ± SEM. **p < 0.01, and ***p < 0.001 between selected groups.

the peripheral blood when compared to control (17.37 ± 4.43 MSCs/ml blood). Mice treated with PlGF for 4 days followed by a single dose of AMD3100 showed no significant mobilisation of MSCs into the peripheral blood when compared to control (8.964 ± 4.26 MSCs/ml blood). Mice treated with VEGF-E for 4 days followed by a single dose of AMD3100 showed significant mobilisation of MSCs into the peripheral blood when compared to control (127.1 ± 28.61 MSCs/ml blood). Mice treated with VEGF-E and PlGF for 4 days followed by a single dose of AMD3100 on day 5 again showed significant enhanced mobilisation of MSCs into the peripheral blood, consistent with mice treated with VEGF-E + AMD3100 (108.9 ± 33.78 vs. 127.1 ± 28.61 MSCs/ml blood). (Fig. 7.3)

The characterisation of P α S cells and specifically their surface marker profile (CD45- Ter119- PDGFR α + Sca-1+) means that murine MSCs can be identified *in vivo*. When comparing Figs. 7.5.a. and 7.5.b., it is evident that the P α S cells and MPCs identified in murine bone marrow (shown in red) are found in very low numbers in the blood of PBS treated mice. In contrast, the blood of mice treated with VEGF-A¹⁶⁵, anti-VEGFR-1 and AMD3100, as illustrated in Fig 7.5.c. contains significant numbers of P α S cells and both sets of MPCs (CD45- Ter119- PDGFR α + Sca-1-, CD45- Ter 119- PDGFR α - Sca-1+). The differences in the numbers of CD45- Ter119- PDGFR α /Sca-1 $\pm$ cells in the blood of PBS treated mice when compared to VEGF-A¹⁶⁵, anti-VEGFR-1 mAb, AMD3100 treated mice, are quantified in Figs. 7.5.d,e,f and g. The enhanced numbers of P α S cells in the blood of VEGF-A¹⁶⁵, anti-VEGFR-1 mAbs, AMD3100 treated mice when compared to PBS treated mice (51.57 ± 12.77 vs. 8.02 ± 1.18 P α S cells/ml blood) can be seen in Fig. 7.5.d. Fig 7.5.e. The enhanced numbers of CD45-

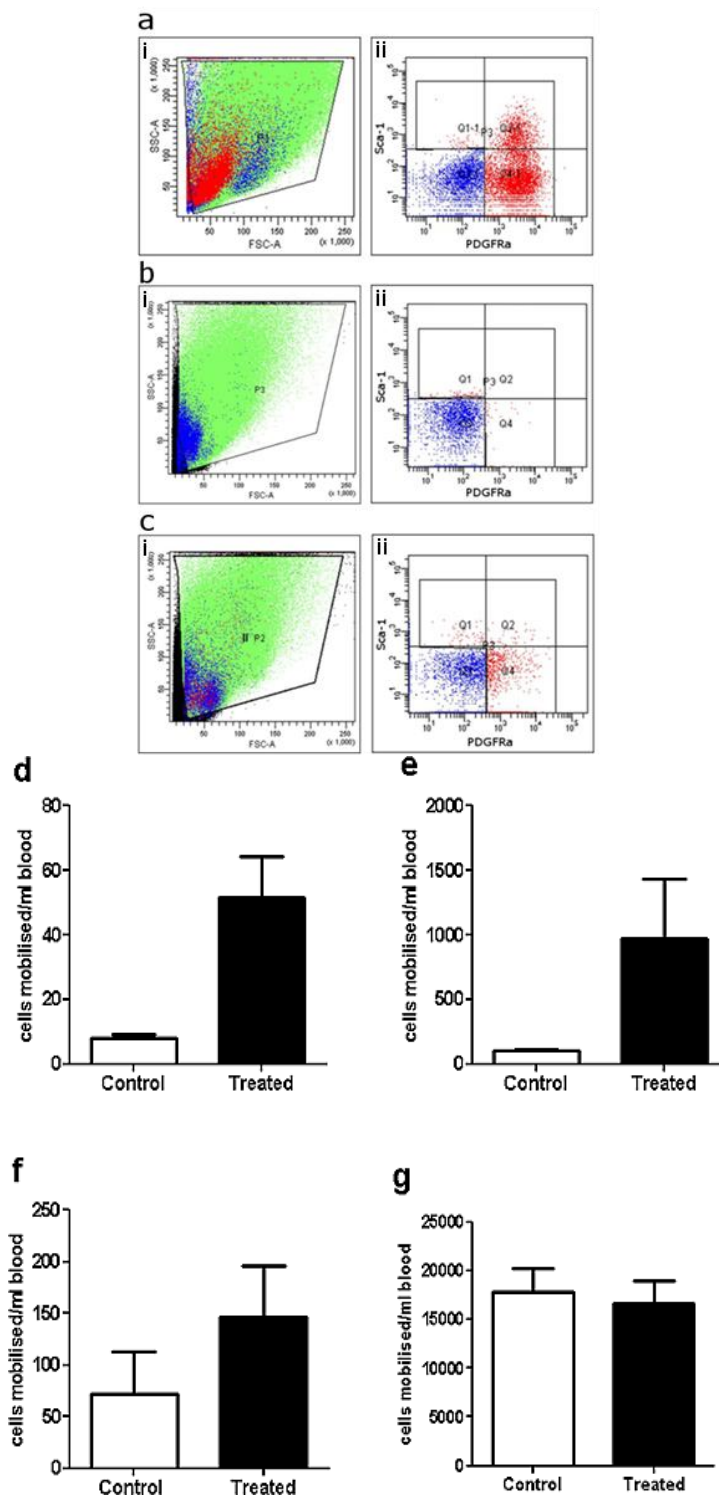


Fig. 7.5. PaS cell mobilisation into blood following growth factor and blocking antibodies pre-treatment and challenge with AMD3100.

Mice were pretreated with VEGF-A¹⁶⁵ (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. At day 0 and 2, mice treated with VEGF-A¹⁶⁵ were treated with anti VEGFR1 blocking

antibodies. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or vehicle (PBS). Sixty minutes later, blood was taken for analysis of circulating PαS cells. FACS analysis shows levels of PαS cells mobilised into the blood per ml blood, as determined by staining for CD45, Ter119, PDGFRα and Sca-1. Fig. 7.5.a. Dot plot showing PαS cells, CD45 - Ter119 - PDGFRα + Sca-1 - cells, and CD45 - Ter119 - PDGFRα - Sca-1 + cells (red) in murine bone marrow from naïve mice. Fig. 7.5.b. Dot plot showing PαS cells, CD45 - Ter119 - PDGFRα + Sca-1 - cells, and CD45 - Ter119 - PDGFRα - Sca-1 + cells (red) in blood of mice treated for 4 days with PBS and a single dose of AMD3100. Fig. 7.5.c. Dot plot showing PαS cells, CD45 - Ter119 - PDGFRα + Sca-1 - cells, and CD45 - Ter119 - PDGFRα - Sca-1 + cells (red) in blood of mice treated for 4 days with VEGF-A¹⁶⁵ combined with anti VEGFR1 blocking antibodies on day 2 and 4 and AMD3100 on day 5. Fig. 7.5.d. Quantification of PαS cells in blood in mice treated for 4 days with VEGF-A¹⁶⁵ combined with anti VEGFR1 blocking antibodies on day 2 and 4 and AMD3100 on day 5. Fig. 7.5.e. Quantification of CD45 - Ter119 - PDGFRα + Sca-1 - cells in blood in mice treated for 4 days with VEGF-A¹⁶⁵ combined with anti VEGFR1 blocking antibodies on day 2 and 4 and AMD3100 on day 5. Fig. 7.5.f. Quantification of CD45 - Ter119 - PDGFRα - Sca-1 + cells in blood in mice treated for 4 days with VEGF-A¹⁶⁵ combined with anti VEGFR1 blocking antibodies on day 2 and 4 and AMD3100 on day 5. Fig. 7.5.g. Quantification of CD45 - Ter119 - PDGFRα - Sca-1 - cells in blood in mice treated for 4 days with VEGF-A¹⁶⁵ combined with anti VEGFR1 blocking antibodies on day 2 and 4 and AMD3100 on day 5. n = 3. Data are means ± SEM.

Ter119- PDGFR α + Sca-1- cells in the blood of VEGF-A¹⁶⁵, anti-VEGFR-1 mAbs, AMD3100 treated mice when compared to PBS treated mice (969 ± 462.9 vs. 104.0 ± 13.61 cells/ml blood) can be seen in Fig. 7.5.e. Fig. 7.5.f. illustrates the enhanced numbers of CD45- Ter119- PDGFR α - Sca-1+ cells in the blood of VEGF-A¹⁶⁵, anti-VEGFR-1 mAbs, AMD3100 treated mice when compared to PBS treated mice (146.6 ± 49.02 vs. 71.82 ± 40.73 cells/ml blood). There are no differences between the two groups in terms of CD45- Ter119- PDGFR α - Sca-1- cell numbers in the blood (17848 ± 2392 vs. 16719 ± 2301 cells/ml blood) (Fig. 7.5.g). Quantification of P α S cell numbers in the blood of mice treated with various combinations of VEGF isoforms, anti-VEGFR-1 mAbs, AMD3100 and PBS can be seen in Fig. 7.6.a. Mice treated with VEGF-A¹⁶⁵ or VEGF-E for 4 days and a single dose of PBS on day 5 do not show a significant increase in the numbers of the P α S cells in the peripheral blood. Mice treated with VEGF-A¹⁶⁵ for 4 days $\pm$ anti-VEGFR-1 mAbs on days 1 and 3 and AMD3100 on day 5, show a trend towards enhanced levels of P α S cells, however this is not significant. Mice treated with VEGF-E for 4 days and a single dose of AMD3100 on day 5 showed significantly enhanced numbers of P α S cells in the blood over PBS treated mice, mice treated with VEGF-A¹⁶⁵ and AMD3100 and mice treated with VEGF-E and AMD3100. Numbers of P α S cells in the bone marrow of mice treated with PBS, VEGF-A¹⁶⁵ or VEGF-E for 4 days and a single dose of PBS or AMD3100 are quantified in Fig 7.6.b. There are no significant differences between P α S cell numbers in the bone marrow of any of the groups.

If a therapeutic benefit is to be achieved using endogenous MSCs mobilised into the blood using pharmacological agents, a key factor may be the numbers of cells that are mobilised. In order to enhance this mobilisation, it is critical to

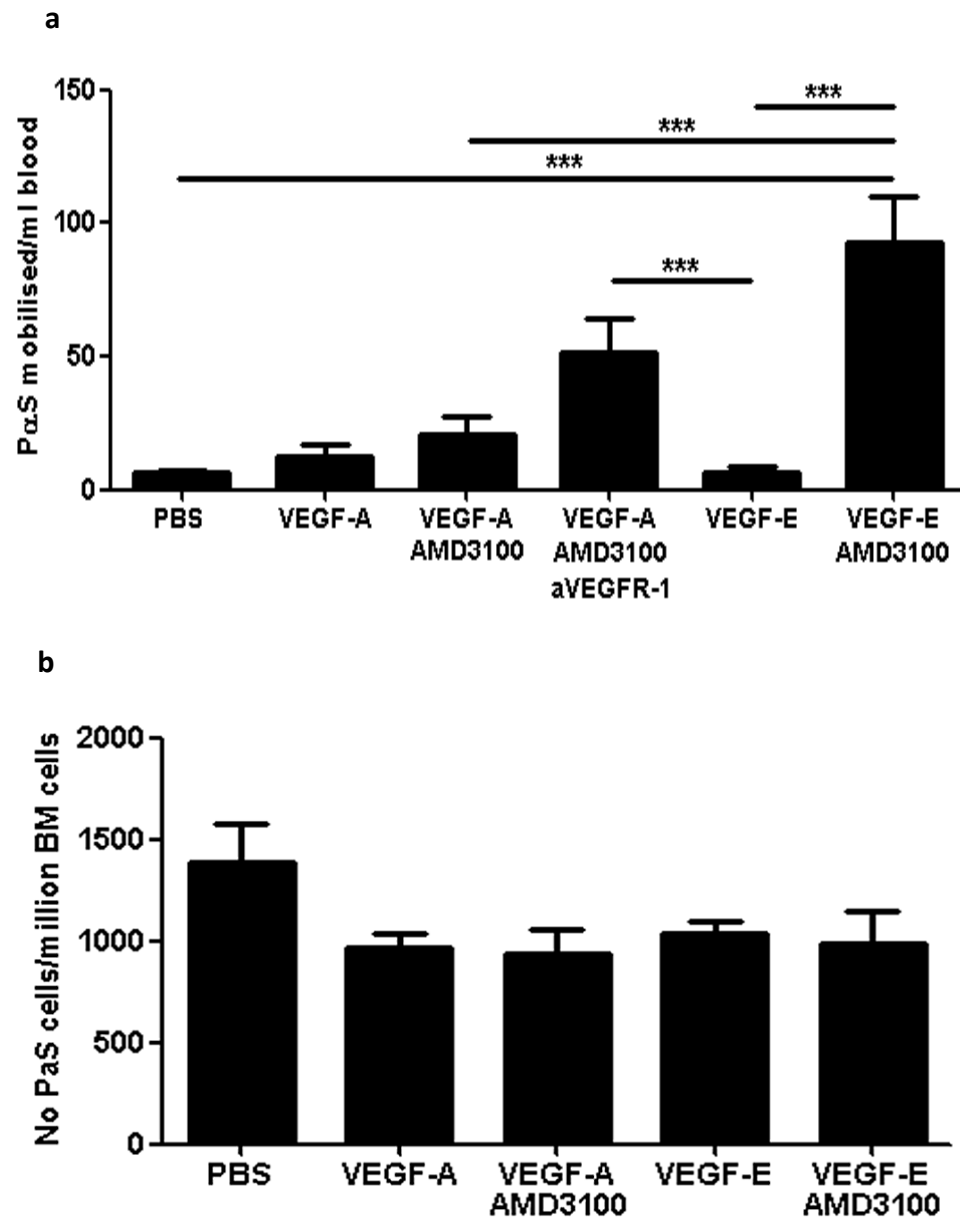


Fig. 7.6. PaS Cells mobilised into the blood following VEGF isoform pre-treatment and challenge with AMD3100.

Mice were pretreated with VEGF-A¹⁶⁵, VEGF-E (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. At day 0 and 2, groups were treated with IgG, anti VEGFR1 blocking antibody. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.) or vehicle (PBS). Sixty minutes later, blood and bone marrow were taken for analysis of circulating PaS cells. FACS analysis shows levels of PaS cells mobilised

into the blood per ml blood (Fig. 7.6.a) and PαS numbers in the bone marrow/million bone marrow cells (Fig. 7.6.b), as determined by staining for CD45, Ter119, PDGFRα and Sca-1. n = 3-12. Data are means ± SEM. ***p < 0.001 between selected groups.

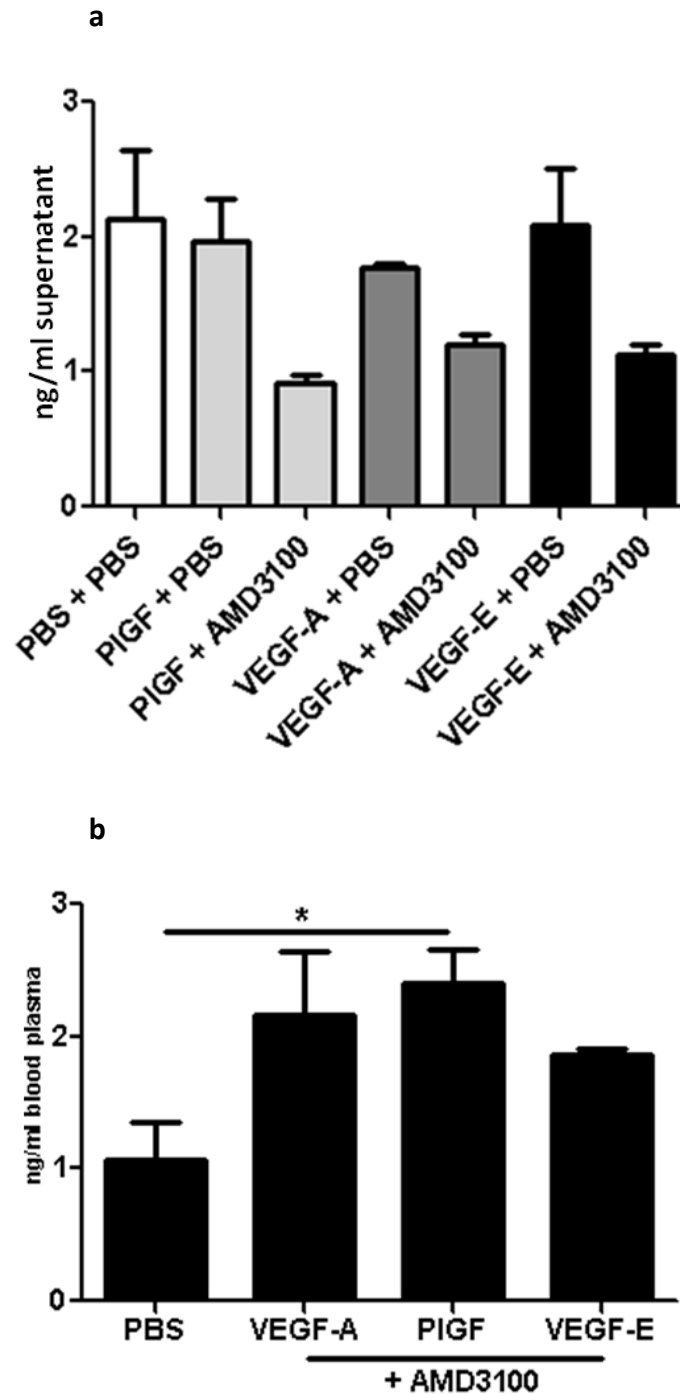


Fig 7.7. CXCL12 levels in blood and bone marrow following growth factor pre-treatment and challenge with AMD3100.

Mice were pretreated with VEGF-A¹⁶⁵ (100µg/kg i.p.), PIGF (100µg/kg i.p.), VEGF-E (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.), or vehicle (PBS). Fig. 7.7.a.

Sixty minutes after the final injection, femurs were flushed with 0.5ml PBS. These cell suspensions were spun down and the supernatant analysed for CXCL12 levels via ELISAs. n = 3-10 mice per group. Data are means $\pm$ SEM. Fig. 7.7.b. Sixty minutes after the final injection, blood was taken via cardiac bleed. The blood was spun down and the blood plasma analysed for CXCL12 levels via ELISAs. n = 5-6 mice per group. Data are means $\pm$ SEM. *p < 0.05 between selected groups.

understand the mechanism by which the MSCs egress the bone marrow. Therefore, comparison of the levels of the CXCR4 ligand CXCL12 in the bone marrow and blood of mice treated for 4 days with growth factors $\pm$ AMD3100 on day 5 were compared. CXCL12 in the bone marrow of mice in these groups are compared in Fig. 7.7.a. Mice treated for 4 days with VEGF-A¹⁶⁵, PlGF or VEGF-E for 4 days followed by a single dose of PBS on day 5 showed no difference in CXCL12 levels when compared to mice treated with PBS alone. However, groups that received AMD3100 on day 5 (PlGF + AMD3100 91.49 $\pm$ 14.75pg, VEGF-A¹⁶⁵ + AMD3100 120.8 $\pm$ 22.72pg, VEGF-E + AMD3100 112.1 $\pm$ 18.65pg) all displayed ~50% reduction in CXCL12 levels in the bone marrow in comparison to PBS treated mice (PBS 213 $\pm$ 143.4pg, IgG 196.8 $\pm$ 55.18pg, VEGF-A¹⁶⁵ 176 $\pm$ 6.5pg, VEGF-E 208.1 $\pm$ 60.08pg). In Fig. 7.7.b., CXCL12 levels in the blood plasma of mice treated with growth factors + AMD3100 were compared to PBS treated mice. In each of the different treatment groups, (VEGF-A¹⁶⁵ + AMD3100, PlGF + AMD3100, VEGF-E + AMD3100), there appeared to be an increase in the amount of CXCL12 present in the blood plasma, which amounted to about a doubling (PBS 106.7 $\pm$ 68.8pg, VEGF-A¹⁶⁵ 216.6 $\pm$ 115.4pg, PlGF 240.2 $\pm$ 62.58pg, VEGF-E 186.5 $\pm$ 9.21pg). However, this increase was only significant in the group of mice treated for 4 days with PlGF followed by a single dose of AMD3100. VEGFR expression on bone marrow P α S cells in mice treated for 4 days with PBS or VEGF-E was compared in Fig 7.8. There was no significance in the difference in the percentage of P α S cells expressing VEGFR-1 (PBS 68.97 $\pm$ 7.36%, VEGF-E 82.5 $\pm$ 4.57%) or VEGFR-2 (PBS 40.9 $\pm$ 16.66%, VEGF-E 55.4 $\pm$ 19.12%) on the surface between the two groups (Fig. 7.8.a). Representative histogram FACS plots of both VEGFR-1 and VEGFR-2 in mice treated with PBS or VEGF-E can be seen in Fig. 7.8.b.

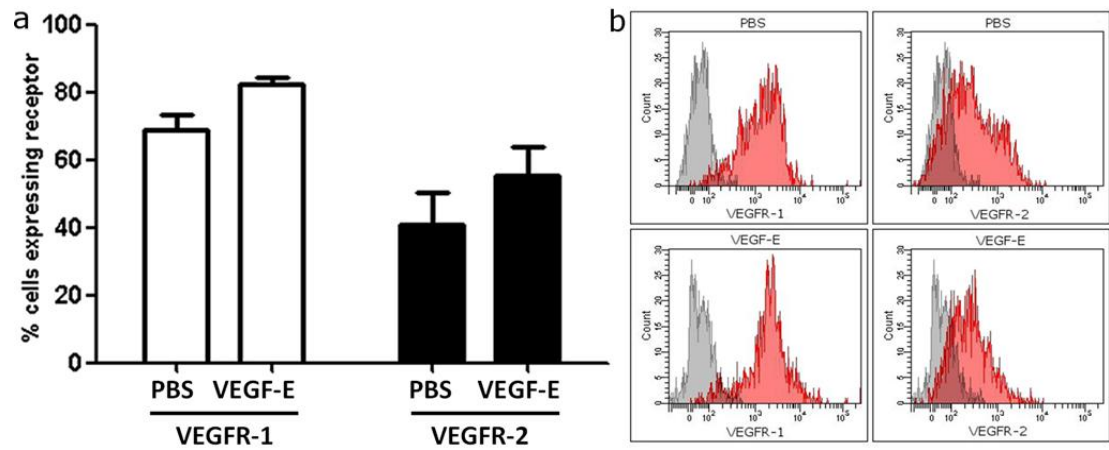


Fig. 7.8. VEGFR expression on PαS cells in the bone marrow following chronic VEGF-E treatment.

Fig 7.8.a. Mice were pretreated with VEGF-E (100μg/kg i.p.), or vehicle (PBS) once daily for 4 days. 24 hours after the 4th injection, bone marrow was analysed via FACS to quantify the expression of VEGFR-1 and VEGFR-2 on PαS cells. n = 3. Data are means ± SEM. Fig. 7.8.b FACS histogram of VEGFR-1 and VEGFR-2 expression on bone marrow PαS cells in mice treated with PBS or VEGF-E (100μg/kg i.p.), for 4 days.

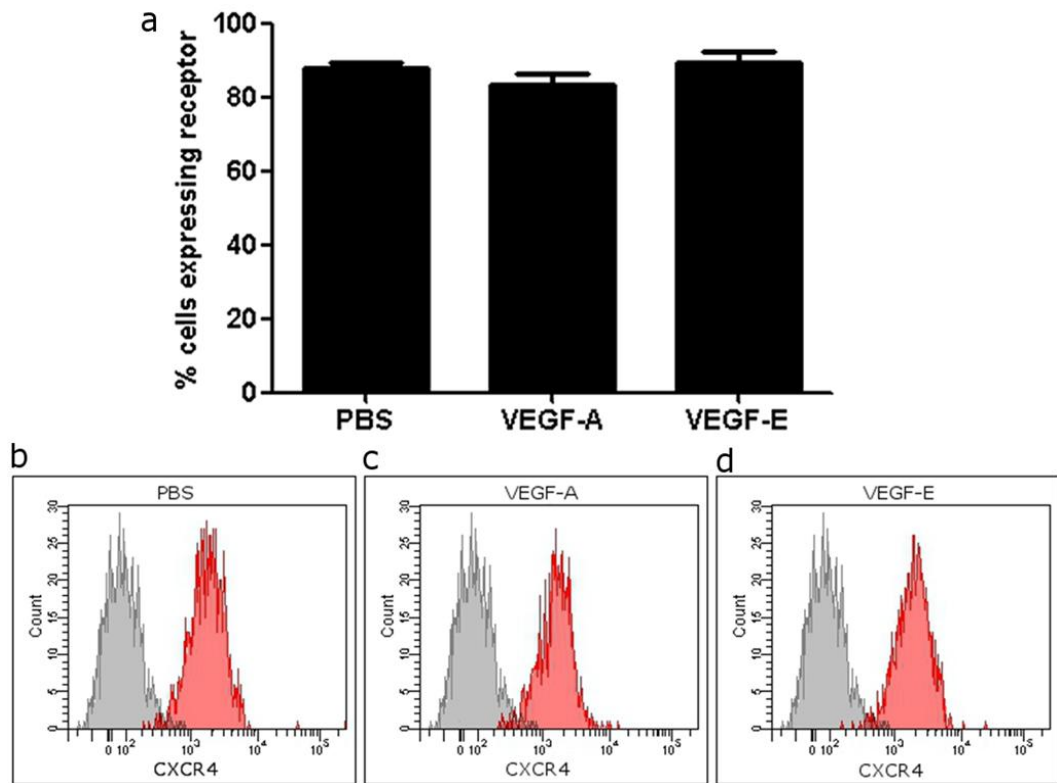


Fig. 7.9. CXCR4 expression on PaS cells after chronic VEGF isoform treatment.

Mice were pretreated with VEGF-A¹⁶⁵, VEGF-E (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. 24 hours after the 4th injection, bone marrow was analysed via FACS to quantify the expression CXCR4 on PaS cells. (Fig. 7.9.a) $n = 3$. Data are means $\pm$ SEM. (Fig. 7.9.b) FACS histogram of CXCR4 expression on bone marrow PaS cells in mice treated with PBS for 4 days. (Fig. 7.9.c) FACS histogram of CXCR4 expression on bone marrow PaS cells in mice treated with VEGF-A¹⁶⁵ (100µg/kg i.p.), for 4 days. (Fig. 7.9.d) FACS histogram of CXCR4 expression on bone marrow PaS cells in mice treated with VEGF-E (100µg/kg i.p.), for 4 days.

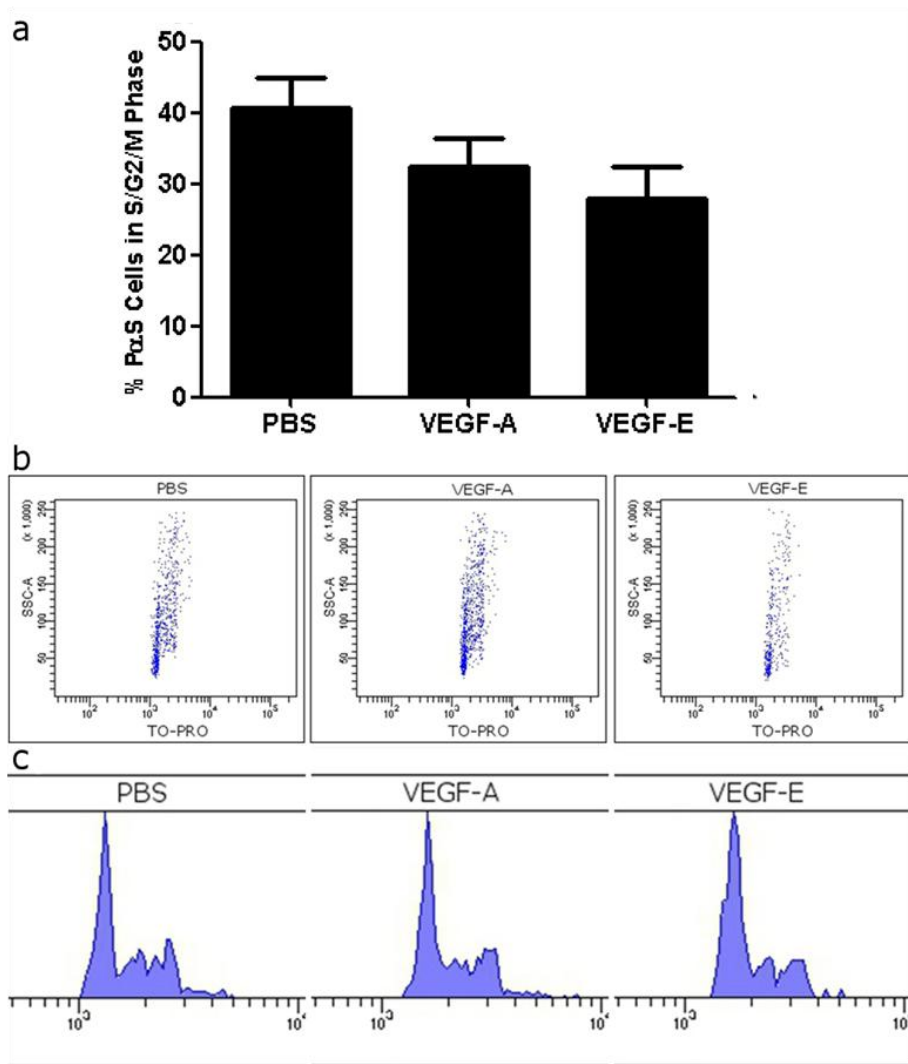


Fig. 7.10. Cell cycling of PaS cells in bone marrow following chronic VEGF treatment.

Fig. 7.10.a Mice were pre-treated with VEGF-A¹⁶⁵, VEGF-E (100µg/kg i.p.), or vehicle (PBS) once daily for 4 days. Twenty-four hours after the last injection, bone marrow was analysed to see what population of PaS cells were in the S/G2/M phase of the cell cycle. n = 6. Data are means ± SEM. Fig. 7.10.b Dot plots of TO-PRO staining of bone marrow PaS cells in mice treated with VEGF-A¹⁶⁵, VEGF-E (100µg/kg i.p.), or PBS for 4 days. Fig. 7.10.c FACS histograms of TO-PRO expression on bone marrow PaS cells in mice treated with VEGF-A¹⁶⁵, VEGF-E (100µg/kg i.p.), or PBS for 4 days.

There were no differences in MFI levels (as illustrated by the shift in peak) in VEGFR-1 expression or VEGFR-2 expression between mice treated with PBS or VEGF-E.

CXCR4 expression on bone marrow PαS cells in mice treated with PBS, VEGF-A¹⁶⁵, or VEGF-E is compared in Fig. 7.9. High percentages (PBS 87.72 ± 3.54%, VEGF-A¹⁶⁵ 83.48 ± 6.6%, VEGF-E 59.50 ± 5.88%) of the PαS cells from each group express CXCR4. There is no difference in terms of percentage of cells expressing CXCR4 (Fig. 7.9.a.) and the amount of receptors each cell is expressing (MFI, Fig. 7.9.b.) between mice treated with PBS, VEGF-A¹⁶⁵, or VEGF-E.

The cell cycle status of the PαS cells, and more specifically the number of PαS cells actively dividing in the bone marrow of mice treated with either PBS, VEGF-A¹⁶⁵ or VEGF-E for 4 days is compared in Fig. 7.10. There is no significant difference in the number of PαS cells in the S/G2/M phase of the cell cycle between mice treated with PBS, VEGF-A¹⁶⁵ or VEGF-E (PBS 40.73 ± 10.35%, VEGF-A¹⁶⁵ 32.37 ± 10.09%, VEGF-E 28.03 ± 10.69%). This is further confirmed in Fig. 7.10.c. which shows representative histogram plots of the cell cycle status for each bone marrow PαS cell population. The fact that the histogram plots are very similar in terms of the shift and height of each peak suggests that the cell cycle status of PαS cells is not affected by treatment with VEGF-A¹⁶⁵ or VEGF-E over 4 days.

MMPs have been cited as a factor involved in the mobilisation of other undifferentiated cells through the ECM [214]. MSC markers in circulating blood after VEGF isoform treatment combined with a single dose of AMD3100, with or without the application of the broad-spectrum MMP inhibitor, marimastat were compared

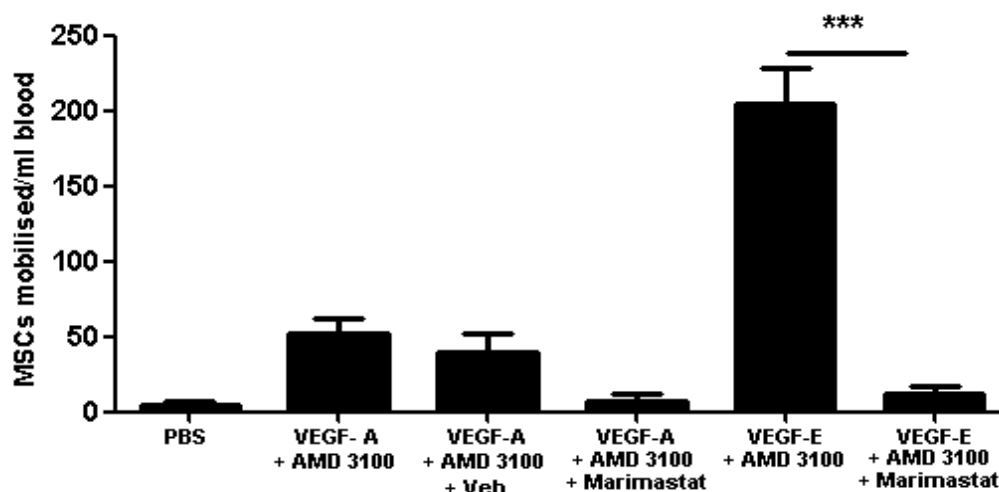


Fig 7.11. Inhibition of MSC mobilisation using a broad-spectrum matrix metalloproteinase inhibitor.

Mice were treated with PBS, vehicle (0.01% tween) or a broad-spectrum matrix metalloproteinase inhibitor (Marimastat 30mg/kg) once daily for 4 days. Mice were treated with VEGF-A¹⁶⁵ (100µg/kg i.p.), VEGF-E (100µg/kg i.p.), or vehicle (PBS) 1 hour after first injection, once daily for 4 days. Twenty-four hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p.), or PBS. Sixty minutes later, blood was taken for analysis of circulating MSCs. n = 7-9. Data are means ± SEM. ***p < 0.001 between selected groups.

(Fig. 5.11). Mice treated with VEGF-A¹⁶⁵ (10.69 ± 14.88 cells/ml blood) or VEGF-E (10.64 ± 16.55 cells/ml blood) and marimastat for 4 days followed by a single injection of AMD3100 on day 5 did not show any enhanced mobilisation of MSCs into the blood over levels in PBS treated mice (5.85 ± 4.53 cells/ml blood), as quantified by colony assay. Mice treated with VEGF-A¹⁶⁵ and the marimastat vehicle (0.01% tween) mobilised a number of MSCs into the blood (45.55 ± 15.43 cells/ml blood) that was not significantly lower than numbers found in the blood of mice treated with VEGF-A¹⁶⁵ plus AMD3100 alone (55.64 ± 14.48 cells/ml blood). Mice treated with VEGF-E and AMD3100 showed mobilisation levels that were significantly higher than P α S cell levels in the blood of mice treated with VEGF-E + AMD3100 in combination with marimastat (220 ± 29.99 vs. 10.64 ± 7.40 cells/ml blood).

7.4 Discussion.

Previous work by Pitchford *et al* and the results presented in Chapter 6 indicate that VEGF pre-treatment of mice is necessary for AMD3100 induced mobilisation of MSCs. In this chapter the molecular mechanisms whereby VEGF promotes mobilisation were further investigated. Firstly, it was important to establish which VEGFRs are expressed by MSCs. While some studies have reported that MSCs express mRNA for VEGFR-1, 2 and 3, others suggest that MSCs do not express VEGFRs [255,374]. FACS analysis of P5 bone marrow mMSCs showed a high level of VEGFR-1 expressed by 69.29% of the mMSC population, while only 23.95% expressed VEGFR-2 at low levels. Of relevance, 98% of mMSCs expressing VEGFR-2 expressed VEGFR-1. There was no expression of VEGFR-3 on the surface of the mMSCs. This evidence, combined with previous reports that VEGF-A¹⁶⁵ can only bind VEGFR-1 or VEGFR-2 [262] meant that the focus of the investigation could be narrowed to these 2 receptors. To further analyse the role of these 2 receptors in MSC mobilisation via VEGF-A¹⁶⁵ and AMD3100 treatment, mice were treated with this MSC mobilisation regimen whilst also blocking either VEGFR-1, VEGFR-2 or both VEGFR-1 and VEGFR-2 using anti-VEGFR mAbs [120]. As Fig. 7.2.a shows, using the existing mobilisation regimen whilst blocking VEGFR-1 results in a 10 fold (30.78 ± 12.75 vs. 325.54 ± 76.17 CFU-Fs/ml blood) amplification of MSC numbers in the circulating blood over the previously established optimum mobilising regimen. This enhancement is negated when VEGFR-2 is blocked, or both VEGFR-1 and VEGFR-2 are blocked in combination with the mobilising regimen. This suggests a model that

when VEGF-A¹⁶⁵ is administered in the absence of blocking antibodies, the majority binds to VEGFR-1 which is expressed at high levels and has a high affinity for VEGFR-1, but does not elicit the signal for mobilisation. The remaining VEGF-A¹⁶⁵ binds to VEGFR-2 which is required for mobilisation. Blocking VEGFR-1 permits VEGF-A¹⁶⁵ to bind to VEGFR-2, thereby increasing the signal for mobilisation. This is highly indicative that the priming of MSCs in the bone marrow for release via AMD3100 administration is due to VEGF-A¹⁶⁵ acting solely through VEGFR-2. It is also evident that VEGF-A¹⁶⁵ acting through VEGFR-1 is not responsible for this mobilisation. The fact that the mobilised MSC numbers are not reduced to basal levels in the treatment groups which were given anti-VEGFR-2 mAbs or both anti-VEGFR-1 and anti-VEGFR-2 mAbs, may be due to sub-optimal blocking conditions. As the blocking mAbs were only administered on days 1 and 3, there may not have been a complete saturation of the receptors with the blocking mAbs. This means that VEGF-A¹⁶⁵ may still be acting through VEGFR-2, although at much lower levels. This may also explain why there is mobilisation when mice are treated with VEGF-A¹⁶⁵ and AMD3100 alone, as VEGFR-1 has a much higher affinity for VEGF-A¹⁶⁵ than VEGFR-2.

The remaining numbers of MSCs present in the bone marrow following the MSC mobilisation treatment do not differ between any of the aforementioned groups. The bone marrow origin of these cells has been previously confirmed using perfusion assays by Pitchford *et al* [341]. This indicates that the treatment is not exhausting MSC numbers in the bone marrow. This also supports the theory that multiple mobilisations are possible without any long term effects on the health of the bone marrow. This could suggest that the MSCs are being mobilised from a specific bone marrow niche and as yet, the mobilisation regimen is not accessing the

remaining niches. Alternatively, further optimisation of the regimen and more specific binding may enable further boosting of MSC mobilisation into the blood.

PlGF is a ligand which binds specifically to VEGFR-1 [271]. Importantly, while PlGF is a natural ligand which has been shown to stimulate chemotactic migration of human MPCs [347], VEGF-E does not occur in man [375] but acts as a specific ligand for VEGFR-2 [262]. As previously noted, the mobilisation regimen may be further optimised by using specific VEGFR ligands. The aim of using specific ligands was to enable mobilisation of MSCs in a more efficient manner in terms of pharmacological dosing, level of mobilisation, or both. Following 4 day treatment with VEGF-E and AMD3100 challenge on day 5, there is enhanced mobilisation of MSCs as compared to VEGF-A¹⁶⁵ (112.3 ± 50.62 vs. 15.07 ± 4.58 CFU-Fs/ml blood). This mobilisation is 7 fold higher than that seen with VEGF-A¹⁶⁵ and AMD3100. 4 days treatment with PlGF followed by AMD3100 challenge on day 5 does not result in any mobilisation of MSCs above that of basal levels. This is further confirmation that priming of MSCs for mobilisation by AMD3100 is a VEGFR-2 dependent event. The doses of PlGF and VEGF-E used in these experiments are the optimal doses for VEGF-A¹⁶⁵ dependent mobilisation. In future experiments, optimisation of VEGF-E dosing could result in further enhancement of MSC levels in the blood and/or a reduction in the amount of ligand needed to elicit the same effect. Following the analysis of this experiment, it was still not clear whether VEGFR-1 serves simply as a scavenger for VEGF-A¹⁶⁵ on MSCs or actually delivers a negative signal inhibiting the MSC mobilisation. To test this, the experiment was repeated with an extra treatment group in which mice were pre-treated with PlGF and VEGF-E in combination (Fig. 7.4). Importantly, when mice were treated with both PlGF and VEGF-E in combination, mobilised MSC levels

were not significantly different to those in mice treated with VEGF-E alone, in combination with AMD3100. This demonstrates that VEGFR-1 interaction is not playing an inhibitory role in the mobilisation of endogenous MSCs and therefore, mobilisation is solely dependent on activation of MSCs through VEGFR-2. We can speculate that the high level of VEGFR-1 may operate as an effective homeostatic mechanism to prevent MSC mobilisation when presented with small fluctuations of VEGF-A¹⁶⁵ levels in the bone marrow. As noted above, there are no selective VEGFR-2 agonists in man. Having optimised the mobilisation protocol, it was critical to investigate the mechanisms by which these cells are released. In knowing the mechanism by which VEGF isoforms acting on VEGFR-2 primes the cells, more targets may become apparent which could help improve this technique in terms of efficient dosing and increased mobilisation of MSCs into the blood.

The publication of a set of markers (CD45- Ter119- PDGFR α + Sca-1+, P α S cells) which identify a homogeneous population of mMSCs, makes investigating whether true stem cells are mobilised during this treatment regimen possible [144]. Having ascertained in previous chapters that P5 bone marrow mMSCs are a heterogeneous population made up of $34.96 \pm 10.66\%$ P α S cells, confirmation was needed that these cells are amongst the MSCs which are being mobilised via VEGF and AMD3100 administration. To do this, P α S cell numbers were quantified in the blood of mice treated with the different variations of treatment regimens in comparison to PBS treated control mice. In Figs. 7.5 and 7.6, it can be seen that P α S cells are mobilised using these treatment regimens, and that the P α S cells are mobilised in a quantitative manner similar to that observed using colony assay analysis. Furthermore, cell populations defined by Morikawa *et al* to be

mesenchymal progenitor cells (CD45⁻ Ter119⁻ PDGFR α ⁺ Sca-1⁻, CD45⁻ Ter119⁻ PDGFR α ⁻ Sca-1⁺) were also mobilised into the blood following VEGF-A¹⁶⁵, anti-VEGFR-1 mAbs and AMD3100 treatment. It has been reported that the successful treatment of degenerative diseases such as retinal degeneration [376] are dependent upon the differentiation state of the cells administered to the site of injury or inflammation. By increasing circulating numbers of stem and progenitor cells, the chances of a suitable cell type homing to the site of injury or inflammation is increased. Of note, the small size of the cells in relation to the rest of the bone marrow (and cultured MSCs Fig. 5.8) is maintained after mobilisation, as can be seen in Fig. 7.5.c. in which the P α S and MPCs are highlighted in red. This increases the likelihood that these cells will not become trapped in the pulmonary circulation, and they therefore may have an enhanced rate of successful recruitment to sites of injury or inflammation when compared to cultured MSCs or indeed culture expanded P α S cells. There is no significant depletion of P α S cells in the bone marrow suggesting that there is therefore the potential for increased levels of mobilisation from the bone marrow. There may also be other MSC and P α S cell niches that are not accessed using this mobilisation regimen e.g. the perivascular niche of MSCs thought to be present in almost all adult tissues [84,377].

By analysing the P α S cells in the bone marrow and blood, it is possible to analyse the effects of these treatment regimens on the cells *in vivo*. Having identified that MSC mobilisation is VEGFR-2 dependent, and that VEGFR-2 is only expressed on a small population of MSCs, it was hypothesised that treatment with VEGF isoforms may be upregulating VEGFR-2 expression, or down-regulating VEGFR-1 expression on the surface of these cells. However, as Fig. 7.8. illustrates, there was

no change in the expression levels of VEGFR-1 or VEGFR-2 on PαS cells in the bone marrow following treatment with the VEGFR-2 specific ligand VEGF-E when compared to PBS treated mice. Strikingly, VEGFR-2 was only expressed by a sub-population of the PαS cells in the bone marrow in both treatment groups (PBS 40.9 ± 16.66 , VEGF-E 55.4 ± 19.12). Knowing that the CXCL12/CXCR4 axis is critical in the retention of several types of stem cell in the bone marrow [120,221,294,378], it was hypothesised that changes to CXCR4 expression levels on the PαS cells, or CXCL12 levels in the blood or bone marrow may play a role in the release of PαS cells and MSCs. However, there was no change in the CXCR4 expression on PαS cells in the bone marrow between mice treated with PBS, VEGF-A¹⁶⁵ or VEGF-E (Fig 7.9.a). There is also no shift in MFI of CXCR4 expression of PαS cells between these groups (Fig. 7.9.b). The CXCL12 levels in the bone marrow following treatment with PlGF, VEGF-A¹⁶⁵ or VEGF-E alone were not significantly different from that of PBS. Interestingly, treatment with AMD3100 reduced levels of CXCL12 in the bone marrow and increased levels in the blood irrespective of the VEGF isoforms pre-treatment, although changes in bone marrow did not reach significance. These data suggest AMD3100 displaces CXCL12 from bone marrow into blood thereby reversing the chemokine gradient.

Given that the levels were altered in both blood and bone marrow for all groups following AMD3100 treatment, it can be assumed that the priming effect of VEGFR-2 binding isoforms does not impact on, and is therefore not regulated by CXCR4 expression or CXCL12 levels in the bone marrow and the blood. This is further confirmed when comparing the results of PlGF pretreatment and subsequent AMD3100 challenge in Fig. 7.7. to VEGF-A¹⁶⁵ or VEGF-E plus AMD3100 treatment.

The CXCL12 gradient going from a reduction in the bone marrow to the significant increase in the blood is a feature of this treatment regimen, and yet we observe no mobilisation of MSCs into the blood. Cell cycle has been reported to regulate HPC mobilisation [120], in that HPCs mobilised into the blood are in G₀ and HPC proliferation reduces the extent of their mobilisation. It was therefore hypothesised that the mobilisation of MSCs and PαS cells from the bone marrow could be a result of VEGFR-2 induced quiescence. Fig. 7.10. suggests this is not the case. When the cell cycle status is compared between PαS cells in the bone marrow of mice treated with PBS, VEGF-A¹⁶⁵ or VEGF-E, there are no significant differences. Between 60% and 70% of PαS cells in the bone marrow are in G₀. Potentially, a separate pharmacological agent could be added to the treatment regimen in order to promote MSC quiescence.

This enhanced mobilisation may also be due to VEGF-A¹⁶⁵ or VEGF-E binding the heparin sulphate binding glycosaminoglycans (GAG) chains of the extracellular matrix and the cellular basement membranes upon VEGFR-1 blocking. VEGF-A¹⁶⁵ also binds to neuropilin-1 (NRP-1), however this is a non-signalling receptor. MMPs have been implicated in the mobilisation of HPCs from the bone marrow [379,380,381]. It was therefore of interest to investigate whether MMPs regulated MSC mobilisation. Marimastat is a broad-spectrum MMP inhibitor that has previously been used *in vivo* in attempts to inhibit tumour metastasis [382]. Mice were treated with VEGF-A¹⁶⁵ or VEGF-E with or without accompanying marimastat application and AMD3100 challenge. Strikingly, marimastat inhibited MSC mobilisation driven by both VEGF-A¹⁶⁵ and VEGF-E. These data suggest that MMPs may act downstream of VEGFR-2 eliciting MSC mobilisation. It can be hypothesised

that treatment over 4 days with VEGF isoforms, increases MMP activity via VEGFR-2 binding. These MMPs then prime the MSCs and P α S cells for release by breaking down the surrounding extracellular matrix. AMD3100 treatment creates a CXCL12 gradient, down which MSCs and P α S cells can migrate out of the bone marrow into the blood. CXCR4 expression levels on P α S cells were shown to be unchanged following VEGF isoform treatment. This suggests that P α S cells mobilised via these treatment regimens retain the capacity to efficiently migrate to sites of injury and inflammation.

Current therapies rely upon the application of MSCs which have been cultured *ex vivo*. These culture techniques have had significant effects on the functional capacity of these cells, in particular their chemokine receptor expression levels, size and capacity to differentiate [383,384]. Using VEGFR-2 specific ligand treatment over 4 days in combination with a single AMD3100 challenge, significant numbers of MSCs and P α S cells were mobilised into the circulating blood. Having demonstrated that this mobilisation does not exhaust MSC numbers within the bone marrow and identified the key mechanisms by which the cells are mobilised, further research and optimisation of this treatment regimen should boost mobilised MSC levels further still. In terms of clinical applications, this is a non-invasive treatment which has huge significance in terms of tissue repair following disease or injury. Following optimisation of the protocol, the next step would be to investigate the effectiveness of this treatment protocol in models of disease and injury to identify potential targets for clinical trials.

8. Discussion.

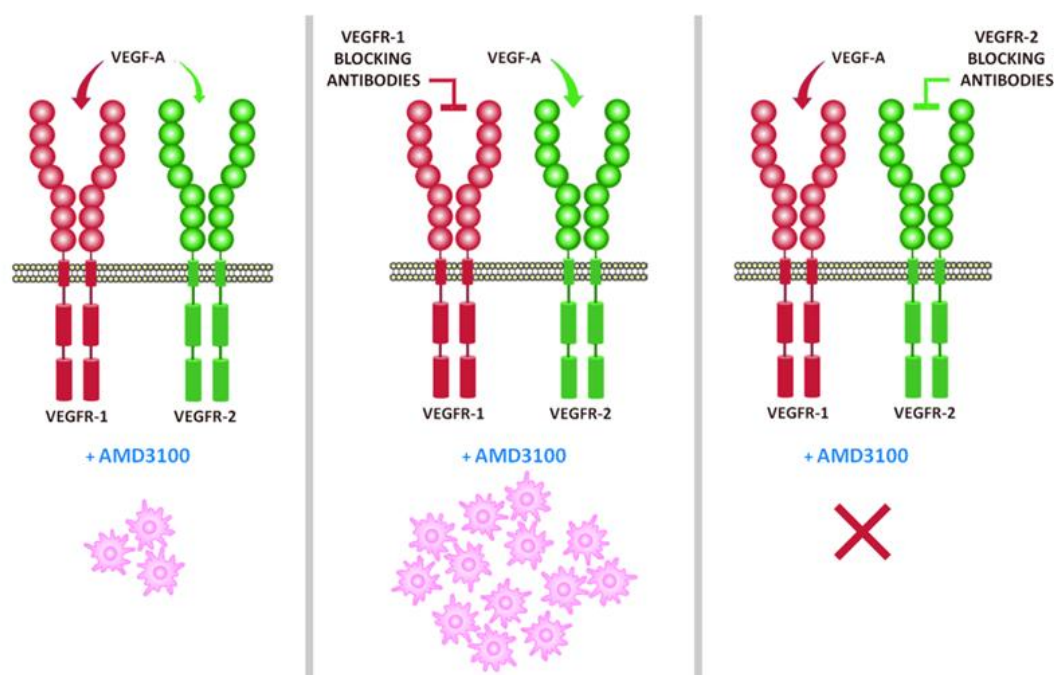
8. General Discussion.

8.1. Ten-fold enhancement of endogenous MSC mobilisation.

An alternative way of harnessing MSCs for cellular therapy rather than extracting, culturing and re-injecting them either locally or systemically, is to selectively mobilise MSCs from the bone marrow into the blood directly using a pharmacological approach. To date, selective mobilisation of endogenous MSCs from the bone marrow to the blood using pharmacological agents has only been reported using VEGF-A¹⁶⁵ in combination with the CXCR4 antagonist, AMD3100, or G-CSF alone [120,254]. However, there have been several papers reporting that MSC mobilisation is not detected in the peripheral blood when mice are treated with G-CSF [120,253]. Studies in mice treated with VEGF-A¹⁶⁵ and AMD3100 in combination showed low but significant numbers of MSCs in the peripheral blood one hour post AMD3100 administration, when compared to PBS treated mice [120]. Further interrogation of this selective mechanism of mobilisation investigated here has enhanced this mobilisation ten-fold to levels significantly higher than previously reported using any MSC mobilisation regimen. It is hypothesised that a greater number of MSCs in the circulating blood as a result of this treatment regimen will result in greater levels of MSC engraftment at sites of injury or inflammation, and a subsequent enhanced resolution of the damaged tissue. This enhanced selective mobilisation of mMSCs requires VEGFR-2 stimulation and CXCR4 antagonism. The dependence on VEGFR-2 is illustrated in Fig. 8.1., which shows the resultant

mobilisation of MSCs following specific VEGFR blocking and treatment with VEGF-A¹⁶⁵ and the CXCR4 antagonist, AMD3100.

Fig. 8.1. Enhanced mobilisation of MSCs via blockade of specific VEGFRs.

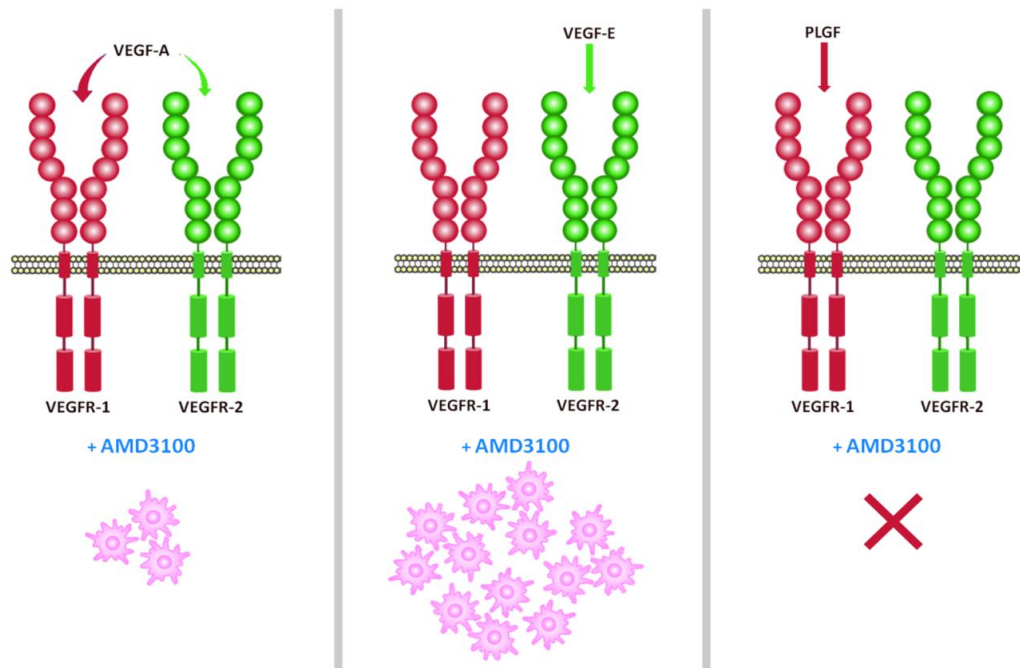


8.2. Enhanced mobilisation of MSCs using specific VEGF isoforms.

By targeting VEGFR-1 and VEGFR-2 using the specific ligands PlGF and VEGF-E respectively, the enhanced mobilisation of MSCs occurred through VEGFR-2 interaction directly and the redundancy of VEGFR-1 in the mobilisation was confirmed (Fig. 8.2). Importantly, when mice were treated with both PlGF and VEGF-E in combination, mobilised MSC levels were not significantly different to those in mice treated with VEGF-E alone, in combination with AMD3100. This demonstrates that VEGFR-1 interaction is not playing an inhibitory role in the mobilisation of endogenous MSCs and therefore, mobilisation is solely dependent on activation of MSCs through VEGFR-2. Optimisation of the protocols using VEGFR-2 specific ligands

in combination with a CXCR4 antagonist means that 2 receptors, VEGFR-2 and CXCR4 must play a role at some level.

Fig. 8.2. Enhanced mobilisation of MSCs using VEGFR-2 specific ligands.



8.3. Mechanism of MSC mobilisation from the bone marrow to the peripheral blood.

Interestingly, VEGFR-1 and VEGFR-2 are both expressed on a percentage of P5 bone marrow mMSCs and PaS cells. Treatment with any of the VEGF isoforms did not affect the levels of VEGFR-1 or VEGFR-2 expression by PaS cells in the bone marrow, indicating that this mechanism was not involved in mobilisation. The CXCR4/CXCL12 chemokine axis is critical for the retention of HPCs in the bone marrow and it has been shown that G-CSF stimulates HPC mobilisation through a down-regulation of CXCL12 levels in the bone marrow [218]. In contrast, treatment of mice with the different isoforms of VEGF did not change levels of CXCL12 in the

bone marrow. Further, expression of CXCR4 was not altered by VEGF pre-treatment, suggesting that changes in this chemokine axis are not involved in MSC mobilisation by VEGF. It is not yet known whether VEGF isoforms acting through VEGFR-2 are acting directly on the MSCs themselves in the bone marrow or through VEGFR-2 on other resident cells in the bone marrow that might affect cell egress. If VEGF is activating other cell populations in the bone marrow, these cells may then be producing factors which result in MSC mobilisation. VEGF-E interacts specifically with VEGFR-2, whereas VEGF-A¹⁶⁵ binds to both VEGFR-1 and VEGFR-2, with a higher affinity for VEGFR-1. The fact that MSCs do express VEGFR-2 means that VEGF-E and VEGF-A¹⁶⁵ may be having a direct effect on MSCs, making them more accessible in the bone marrow for mobilisation down a CXCL12 gradient. There was no difference in CXCL12 levels in the bone marrow following 4 day treatment with PBS, VEGF-A¹⁶⁵ or VEGF-E. Levels are only altered for all groups following AMD3100 treatment. This suggests that the priming effect of VEGFR-2 binding isoforms is not through an effect on the CXCR4/CXCL12 chemokine axis.

It has been reported that upon 4 day treatment with VEGF, HPCs within the bone marrow enter the cell cycle. It is thought that these actively dividing cells cannot migrate and hence HPC mobilisation by subsequent CXCR4 antagonism is negated [120]. This is not the case for MSCs. The percentage of PαS cells actively dividing in the bone marrow is comparable for mice treated with PBS, VEGF-A¹⁶⁵ or VEGF-E for 4 days suggesting that changes in MSC proliferation are not key to MSC mobilisation by VEGF. While the bone marrow origin of these cells has been previously confirmed using *in situ* perfusion of the bone marrow [341], there is no significant depletion of PαS cells in the bone marrow following VEGF/AMD3100

treatment. This suggests that a very small proportion of the total MSC population has been mobilised and indicates that there is potential for increased levels of mobilisation. This also supports the theory that multiple mobilisations are possible without any long term effects on the health of the bone marrow. Recently, prolonged treatment of mice with G-CSF has been shown to increase MSC numbers in the bone marrow [385]. It is possible therefore that by increasing MSC numbers in the bone marrow, with such a G-CSF treatment prior to administration of VEGF-E and AMD3100, the numbers of MSCs which are mobilised to the peripheral blood may be increased yet further.

Analysis of CXCR4 and cell cycle status of MSCs and PαS cells following VEGF isoform treatment suggests that these cells retain their migratory capacity and should in theory migrate to sites of injury and inflammation. Current therapies rely upon the application of MSCs which have been cultured *ex vivo* [386]. These culture techniques have had significant effects on the functional capacity of these cells, in particular their chemokine receptor expression levels, size and capacity to differentiate.

8.3.1. The role of MMPs in the mobilisation of MSCs using VEGF isoforms in combination with AMD3100.

MMPs have been implicated in playing a role in angiogenesis, tissue repair and even metastasis [379,387]. MMP-2, -3, -10, -11, -13, and -14, as well as TIMP-2, have been detected in MSCs at the mRNA and protein level [388]. Indeed hMSCs have been shown to secrete MMP-2 in response to co-culture with damaged corneal

epithelial cells [389]. hMSCs have been shown to degrade and penetrate type I collagen networks in tandem with the expression MMPs. Specifically, MT1-MMP is required for hMSC-mediated collagenolysis, involved in invasion [381]. Taken together, this suggests that MMPs may play a role in making MSCs in the bone marrow mobilisable. Marimastat is a broad spectrum MMP inhibitor. Administering marimastat during treatment with the mobilising regimen results in no MSCs found in the circulating blood 1 hour post AMD3100 administration, suggesting that MMPs are playing a critical role in this mobilisation. Without formerly treating with VEGF, MSCs are not mobilised following AMD3100 administration. VEGF must be acting in a way which alters the state of the MSCs in the bone marrow. It is likely that MMPs play a role in this mechanism. Taking this data together, it is hypothesised that treatment over 4 days with VEGF isoforms increases MMP activity via VEGFR-2 interaction. These MMPs then prime the MSCs and P α S cells for their release by breaking down the surrounding extracellular matrix prior to AMD3100 treatment, and the subsequent formation of a CXCL12 gradient out of the bone marrow into the blood. However it is not yet known whether the VEGF acts directly through VEGFR-2 on the MSCs themselves, which then secrete MMPs as a result, or whether there are other cell populations in the bone marrow involved which may be secreting MMPs or activated in some other way via their VEGFR-2 expression.

8.4. P α S cells as a source for cell therapy.

P α S cells have been characterised as a highly homogeneous population of bone marrow mMSCs [124]. By demonstrating their induced mobilisation using a

pharmacological method it becomes easier to investigate mobilised MSC populations, how they traffic to, and act at sites of injury and inflammation in a regenerative manner. P α S cells have been further characterised in Chapter 5. P α S cells are very small and have high levels of external chemokine receptors. This makes them an ideal candidate for use as a cell therapy. Their small size relative to cultured MSCs reduces their chance of being entrapped in the respiratory vasculature or other highly branching capillary networks. High chemokine receptor expression on the cell surface of MSCs directly correlates with their ability to migrate to ligands of said receptors. Chemokines such as IL-8 and CXCL12 are produced at high levels in sites of injury or inflammation [390,391]. In a normal repair model, the number of cells local to the injury or inflammation is increased over time via recruitment of cells through the blood stream. By mobilising the P α S cells into the blood, it is hoped the amount of stem cells local to injured tissue over time will be increased further. It can be hypothesised that the high levels of chemokine receptors on these P α S cells will result in efficient homing and greater numbers of P α S cells recruited to the site of injury or inflammation. The benefits of uncultured P α S cells over cultured P α S cells have been investigated previously. Uncultured P α S cells homed efficiently to the bone marrow and adipose tissue, both of which express high levels of CXCL12, whereas cultured P α S cells become entrapped in the respiratory system [144].

8.5. Pharmacokinetics of MSC mobilisation.

Dose and timing of VEGF-A¹⁶⁵ were originally determined to compare directly with G-CSF, as this dosing regimen had been successfully used to show EPC mobilisation [257]. Experiments performed to determine whether the dose or pre-treatment could be reduced showed that bone marrow needs to be exposed to a prolonged administration of VEGF-A¹⁶⁵ in order for the mMSCs to be released following CXCR4 antagonism.

The adult body contains many stem cell niches other than the bone marrow from which stem cells can be mobilised. Notable examples include: myocardial tissue, adipose tissue, spleen, liver, and lungs [392,393,394,395]. Previously *in situ* perfusion of the femoral vasculature in mice treated with VEGF-A¹⁶⁵ and AMD3100 has shown that MSCs are mobilised directly from the bone marrow using the regimen [341]. Once mobilised, stem cells can be rapidly recruited to the site of injury, home back to the bone marrow (within minutes) or redistribute to other tissues with local stem cell niches such as the liver, spleen, and lungs [391,392,396,397]. By investigating the pharmacokinetics of mobilisation using VEGF and AMD3100 in Chapter 6, it is now evident that MSC mobilisation is rapid with optimum mobilisation occurring 1 hour post AMD3100 administration. However, due to the rapid homing of MSCs to stem cell niches around the body, further studies are required to determine strategies to provide a more sustained mobilisation of MSCs.

8.6. Pharmacologically mobilised MSCs vs. *ex vivo* cultured MSCs.

The favourable characteristics of MSCs for cellular therapy documented *in vitro* have not yet been translated into man [98]. Although this area has been heavily researched of late, a lack of understanding of the basic biology through which these cells exert their beneficial properties may explain the underwhelming results in clinical trials [386]. This project has attempted to increase the understanding by investigating isolation methodology, the effects of *in vitro* culture on MSCs, and alternative strategies for administering MSCs as a cellular therapy.

8.6.1. Phenotypic switching of cultured MSCs.

There are reports suggesting that MSCs should only be used clinically at early passage numbers due to phenotypic and functional changes that occur as the result of repeated passaging [59,168]. There have been previous reports of both human and murine MSCs losing migratory capacity and ability to migrate to sites of injury or inflammation *in vivo*, following culture *in vitro* [168,335]. However, the phenotypic changes which result in this reduction have not previously been characterised. It has been hypothesised that an increase in proliferation rate leads to a decrease in migratory capacity, with the suggestion that the actively dividing cells will not migrate efficiently due to their increased size and a phenotypic switching from migratory action to a proliferative action [398]. In Chapter 3, mMSCs were shown to display reduced external chemokine receptor profiles following 25 passages, when compared to mMSCs which had been passaged 5 times. However, both sets of cells appeared morphologically similar and displayed the same antigen phenotype. Loss of chemokine receptor expression on the surface of P25 bone marrow mMSCs

resulted in a loss of chemokine dependent motility. From this it is apparent that cells which have undergone excessive passaging are not suitable for systemic administration in clinical therapies because they may lose their ability to migrate into specific areas of injury within tissue. This corresponds with previous studies where MSCs could home to bone marrow with high efficiency but lost homing ability following culture [129]. MSCs may function if administered locally, but it has not yet been assessed whether this *in vitro* culture methodology results in the loss of further surface molecules, including adhesion molecules such as VCAM-1, which may be critical for engraftment at sites of administration [295]. This loss of chemokine receptor expression upon culture may explain why the *in vitro* promise of MSCs has not been realised *in vivo*. For example, the beneficial properties for MSCs in treating graft versus host disease *in vitro* [399,400] have not been mirrored in the recent Osiris phase III clinical trials evaluating Prochymal (*in vitro* cultured hMSCs), for the treatment of GvHD, where Prochymal recipients showed no statistical benefit at primary endpoints [386]. In contrast, recent studies showed a positive effect using early passage MSCs to treat GvHD [401]. The difference may be a mass produced, possibly extensively expanded MSC product versus individual MSC preparations. Alternatively, endogenously mobilised MSCs provide a more efficacious approach to cell therapy.

Once the cells are recruited to the site of injury or inflammation, it is not yet known how the MSCs are exerting their beneficial effects. Various reports have stated that this is due to the immuno-modulatory capacity of MSCs [165,166,191,194]. In culture, hMSCs were shown to produce high levels of CXCL12. It is not known whether CXCL12 is being produced in order to maintain the MSCs in

a proliferative state or whether MSCs constitutively produce CXCL12 and this aids the repair and regeneration of tissues. CXCL12 is a well recognised chemoattractant for stem cells [7,351,402]. By producing large amounts of CXCL12 at the site of injury, MSCs may be attracting other leukocytes and stem cells that express CXCR4, including CD34+ haematopoietic progenitors [391], T lymphocytes, monocytes, pro and pre-B cells [79] or greater numbers of MSCs to aid regeneration via alternative pathways. Chemokines acting upon MSCs may determine functionality through various pathways. Angiogenesis during wound healing is regulated by numerous biological mediators, including chemokines [403]. It is also known that the immunosuppressive function of MSCs relies on chemokines [404]. MSCs in the presence of IFN- γ plus TNF- α , IL-1 α , or IL-1 β promotes the expression of high levels of chemokines and NO. Chemokines drive T cell migration into proximity with MSCs, where T cell responsiveness is suppressed by NO [405].

8.6.2. Heterogeneity of cultured MSCs.

P25 mMSCs were found to have a higher proliferation rate than P5 mMSCS. The increased proliferative capacity may be a result of culture conditions. MSCs are cultured *in vitro* to increase cell numbers. The optimum culture conditions are therefore set for proliferative action as opposed to maintenance of cellular phenotype. This apparent phenotypic switching upon culture is also apparent when culturing a homogeneous population of MSCs, PaS cells. Assuming PaS cells are stem cells, with culture a mix of stem cells and progenitors are produced.

The percentage of P α S cells in all cultured mMSC populations investigated (P5 P α S, P5 bone marrow mMSCs, P25 bone marrow mMSCs) showed no significant difference between populations. Even within their niche in the bone marrow, mMSCs are surrounded by many other cell types, all of which are maintained in a steady state by their interaction with other cells and the factors that these cells produce. The heterogeneous population may come about as a result of mMSCs producing factors to revert to the steady state conditions of the bone marrow. Herein lies a major obstacle for the use of MSCs as a cellular therapy. By treating patients with heterogeneous cell populations, it is difficult to assess which cell types are responsible for the resulting outcome. Designing experimental conditions to allow MSCs to proliferate in an undifferentiated state is one of the current challenges for the scientific community.

In order to increase the likelihood of MSCs reaching and acting at the site of interest, the MSCs need to be administered as a homogeneous population in which the migratory capacity is maintained. It is not yet known whether the loss of chemokine receptor on MSCs cultured *in vitro* is due to differentiation of MSCs into cell types which subsequently do not express chemokine receptors on the cell surface, or a loss of chemokine receptors of the cell surface of MSCs directly. The percentage of P α S cells in the P5 bone marrow mMSCs and P25 bone marrow mMSCs is not significantly different, suggesting that mMSCs are losing chemokine receptors on the cell surface. The migratory potential may also be limited by an increase in cell size upon culture, reported here. Previous reports of cultured MSCs becoming entrapped in the lungs [130,193,406] are consistent with the results reported here, in which P5 bone marrow mMSCs when administered intravenously

were also found in large numbers in the lungs 4 hours post administration. These findings suggest that MSCs should not be cultured at all, or a method of culture should be found in which the size of MSCs is maintained if the cells are to be used safely in clinical applications. Comparison of

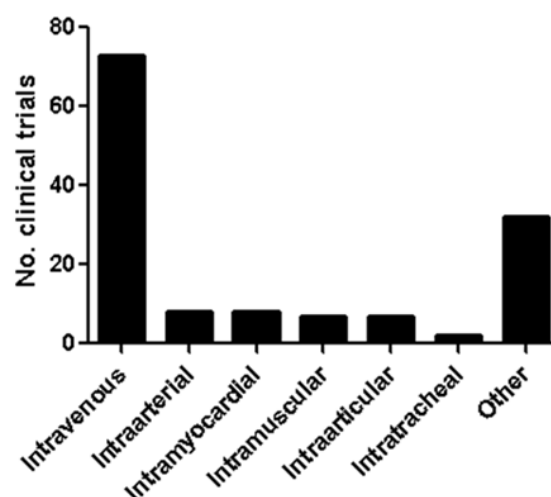


Fig. 8.3. Route of administration in first 175 clinical trials using MSCs.

mMSC distribution when administered i.v. or i.p. in mice demonstrated how the route of application of MSCs plays a critical role in MSCs reaching clinical targets. Analysis of the clinical trials reported on clinicaltrials.gov reveals that in the majority of studies MSCs are administered i.v. (Fig. 8.3). For these cells to successfully treat the damaged tissue, they must first migrate to sites of injury or inflammation.

As there are differences in the manner in which mMSCs and hMSC are obtained, there may be some differences in the initial heterogeneity of the cell populations which may be amplified upon prolonged culture *in vitro*. In his thesis, I have shown that chemokine receptor expression is consistent across species and between tissue sources, suggesting that with respect to trafficking properties mMSCs may represent a good model of their human counterparts. But importantly, chemokine receptor profiles reveals distinct sub-populations of MSCs and future work will explore the functional attributes of these sub-populations, with respect to trafficking but also their differentiation and immuno-modulatory properties.

8.7. Murine MSCs as a model for human MSCs.

It has previously been reported that mMSCs act as a good representative population for hMSCs [134]. The chemokine receptor profile has been characterised for all 5 sets of hMSCs investigated here. Strikingly, when comparing the chemokine receptor profiles of P5 MSCs derived from human fetal blood, fetal bone marrow, 1st trimester amniotic fluid, 2nd trimester amniotic fluid and placenta, it was found that the overall internal and external chemokine receptor profile was conserved across all sources. There were however differences in expression levels of certain chemokine receptors between sources e.g. Fetal bone marrow derived MSCs expressed higher levels of CXCR3, CXCR4, CXCR6 and CCR10 on the cell surface than fetal blood derived MSCs. Initial results investigating the functional relevance of these differences showed that fetal bone marrow did not show a significantly greater chemotactic response to CXCL12, CXCL16, CCL27 and CXCL11 than fetal blood. This may have clinical relevance when selecting MSCs for future clinical trials. Following culture, MSCs which retain the greatest chemotactic ability should be used if the MSCs are to be administered systemically. This should allow a greater number of MSCs to migrate to sites of injury where their therapeutic benefit is increased. The hMSC populations used in this study were all investigated at passage 5 so that their phenotype could be compared directly to the P5 bone marrow mMSCs isolated in Chapter 3. However, the findings in Chapter 5 regarding repeated passage and culture of a homogeneous population of MSCs (PαS cells) shows that any *in vitro* culture results in a reduction in the surface chemokine receptor levels on MSCs. Although this has only been shown thus far for mMSCs, it can be assumed that the same is true for hMSCs due to the conserved chemokine receptor profiles across

species. In order to be confirmed as MSCs, the cells must possess the ability to proliferate on plastic surfaces. Whilst this may help to confirm the cells populations as MSCs, it may in turn be having immediate detrimental effects on the MSCs should they ever be used for systemic administration clinically.

8.8. Summary.

This project determines chemokine receptor profile on murine bone marrow MSCs at early and late passage numbers and human MSCs derived from a range of fetal tissues including fetal blood, bone marrow, amniotic fluid and placenta. The overwhelming result from this analysis is the consistency across species and tissue source with respect to chemokine receptor profiles. In addition it is clear that expression of specific chemokine receptors defines sub-populations of MSCs. It has previously been shown that pre-treatment of mice with VEGF-A¹⁶⁵ supports the subsequent mobilisation of MSCs by the CXCR4 antagonist AMD3100. In this thesis the VEGF biology with respect to this response has been investigated and it was shown that mMSCs express high levels of VEGFR-1 and a sub-population express VEGFR-2, but signalling via VEGFR-2 is required for mobilisation. Indeed VEGF-A¹⁶⁵ in combination with aVEGFR-1 mAbs enhanced mobilisation 10 fold, suggesting that under normal homeostatic conditions VEGFR-1 acts as a sink for VEGF-A¹⁶⁵. To exploit this phenomenon we used VEGF-E, a selective VEGFR-2 agonist, and were able to show 7 fold enhanced mobilisation of mMSCs over VEGF-A¹⁶⁵. Further characterisation of mobilised mMSCs by flow cytometry on PαS cells, as elaborated upon in Chapter 5 now provides a way to investigate the biology of MSCs, both in their steady state *in vivo* and in models of injury and inflammation. Molecular mechanisms lying downstream of VEGFR-2 have been explored and it has been shown that MMPs play a critical role in mobilisation. Pharmacological mobilisation of MSCs may provide an alternative strategy to stem cell therapy to promote tissue regeneration or immune suppression. Such a strategy may be more commercially viable for financial and technical reasons. Culture of MSCs generates a

heterogeneous population of stem and progenitor cells with poor trafficking abilities, it will therefore be of interest in the future to compare pharmacological mobilisation, versus stem cell therapy with respect to tissue regeneration.

8.9. Future Work.

- Develop methodology for sustained MSC mobilisation via the optimisation of the current protocol along with other approaches, such as administration of adenoviruses expressing VEGF-isoforms.
- Investigate which MMPs are activated in response to treatment with different VEGF isoforms.
- Further investigate the role of the CXCR4/CXCL12 axis in mobilisation, specifically whether blocking CXCL12 inhibits mobilisation.
- Assess resolution of tissue damage in mouse models of injury and inflammation when endogenous MSCs are mobilised pharmacologically.
- Characterise and compare sub-populations of MSCs as defined by the expression of extracellular chemokine receptors, in terms of:
 - Differentiation potential.
 - Chemotactic ability *in vitro* and *in vivo*.
 - Immuno-modulatory capacity.
- Compare the chemotactic ability *in vitro* and pattern of distribution of MSCs isolated from different tissues, when administered *in vivo*.

9. Bibliography.

9. Bibliography.

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