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PROSPECTIVE ISOLATION AND CHARACTERISATION OF MOUSE BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS

A thesis submitted to the National University of Ireland, Galway in fulfilment

of the requirements for the degree

Doctor of Philosophy

By

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ABSTRACT

Conventionally cultured mouse bone marrow (mBM) mesenchymal stromal cells (MSC) are a heterogeneous population that often initially contain contaminating haematopoietic cells. Variability in isolation methods, culture protocols and the lack of specific MSC surface markers might explain this heterogeneity. In this thesis it is shown that during early passaging of bone chip-derived MSC not only do haematopoietic cells disappear, but there is also a change in surface marker expression.

To further dissect bulk MSC populations, fluorescence activated cell sorting of mBM suspensions based on Sca-1 expression among non-hematopoietic cells was carried out and cells expanded in hypoxic conditions. During early passaging, there was a change in the surface phenotype of MSC affecting particularly CD44 and Sca-1 expression. It became evident that CFU-F frequencies and proliferation was greater among Sca-1^{+} compared with Sca-1^{-} cells. As evaluated by *in vitro* differentiation and qRT-PCR assays, both populations were capable of tri-lineage differentiation along osteocyte, chondrocyte, and adipocyte lineages.

By prospective isolation of Sca-1^{+}PDGFR$\alpha^{+}$$CD90^{+}$ non-hematopoietic mBM cells, clones of MSC could be isolated with a CFU-F frequency of 1/4. This is one of the highest CFU-F frequencies for mouse MSCS reported so far. Functional investigations demonstrated that these MSC clones had immuno-modulatory activity in that they inhibited T-lymphocyte proliferation.

"Inside every cynical person, there is a disappointed idealist." - George Carlin

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Finally, to Marta, for all the love, support and most importantly understanding all my silliness and flaws. I will try to limit the gaming and extend the Spanish learning. You are my anchor and I love you.

Dedicated to my parents, Hartmut and Gina Baustian.

ABBREVIATIONS

AMI	Acute myocardial infarction
Ang-1	Angiopoietin-1
APC	Antigen-presenting cell
B2M	Beta-2 microglobulin
BM	Bone marrow
BMP-2	Bone morphogenetic protein-2
BSA	Bovine Serum Albumin
CAR cell	CXCL12-abundant reticular cell
CaSR	Calcium-sensing receptor
cBM	Compact bone marrow
CFU-F	Colony forming unit – fibroblast
CVD	Cardiovascular disease
DC	Dendritic cell
ECM	Extracellular matrix
EMA	European Medicines Agency
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
fBM	Flushed bone marrow
FCM	Flow cytometry
FCS	Fetal calf serum
FDA	U.S. Food and Drug Administration
GM-CSF	Granulocyte macrophage-colony

	stimulating factor
GPI	Glycosylphosphatidylinositol
GVHD	Graft versus host disease
hESC	Human embryonic stem cells
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible transcription factor-1α
hMSC	Human mesenchymal stromal cells
HPC	Haematopoietic progenitor cells
HSC	Haematopoietic stem cells
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
iPSCs	Induced pluripotent stem cells
mAb	Monoclonal antibody
mBM-MSC	Mouse bone marrow mesenchymal stromal cells
MLR	Mixed Lymphocyte Reaction
mMSC	Mouse mesenchymal stromal cells
MSC	Mesenchymal stromal cells
NK cell	Natural killer cell
NO	Nitric oxide
OA	Osteoarthritis
OB	Osteoblast
OPN	Osteopontin

PGE2	Prostaglandin E2
PI	Propidium Iodide
pO_2	Partial pressure O_2
SCF	Stem cell factor
S-GAG	Sulphated glycosaminoglycans
Sca-1	Stem cell antigen-1
SSEA	Stage specific embryonic antigen
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor-β
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

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CHAPTER 1

Introduction

Mesenchymal stromal cells (MSC) are used in many research fields and have generated much interest for cell therapies due to their ability to differentiate into various cell types including osteocytes, chondrocytes and adipocytes [Pittenger et al. 1999]. While a lot is known about human MSC (hMSC) the understanding of mouse MSC (mMSC) biology is less advanced.

1.1 CLINICAL IMPACT AND USE

Stem cells have been used in clinical therapy since 1957 with the first bone marrow transplantation with haematopoietic stem cells (HSC) [Thomas et al. 1957].The bone marrow is still the main source for HSC today, but peripheral blood and the umbilical cord are used as well. Besides HSC, another type of stem cell the mesenchymal stromal cell (MSC) was identified in the bone marrow over 40 years ago and this cell type holds great promise for human therapy [Friedenstein, Gorskaja, and Kulagina 1976]. Over the last decade there has been an increased interest in this new type of stem cell as seen by an increased growth of publications regarding MSC (http://www.ncbi.nlm.nih.gov/pubmed). Also, the numbers of new clinical trials using MSCs have been increasing from 13 for 2004 to 135 for 2013 (www.clinicaltrials.gov). In addition, according to analysts, the market for stem cell therapies and products is expected to increase from $2.7 billion in 2011 to $8.8 billion in 2016 [Syed and Evans 2013]. Despite all these efforts, to date, there is no MSC therapy approved by the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA) (as of: 08/10/2014). Clinical trials for the exploration of the therapeutic potential of MSC focus on a wide range of applications (Figure 1.1). One of the main areas is the treatment of graft versus host disease (GvHD) a situation that arises following bone marrow transplantation and HSC engraftment.

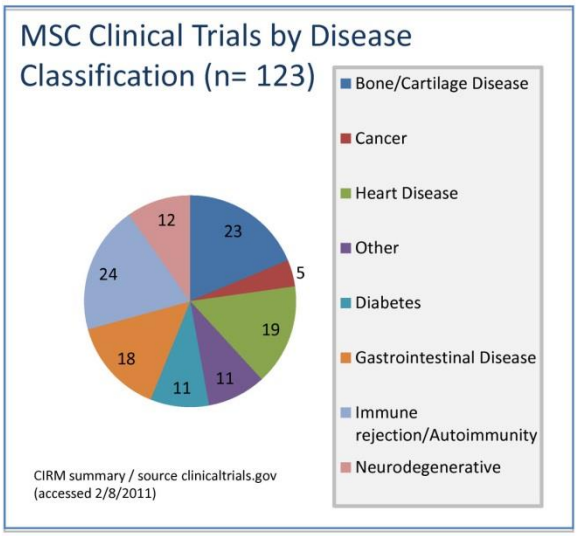

Figure 1.1 Diseases being addressed using MSCs for clinical trials (n = number of trials)[Trounson et al. 2011].

HSC transplantation is the gold standard for the treatment of haematological malignancies. One of the main complications of HSC transplantation is GVHD, which is caused by the reaction of donor's T lymphocytes against the recipient's antigens. Standard therapies against severe GVHD include the use of steroids, which are able to control the immunoreaction in about half of the cases. Unfortunately, steroid resistant-GVHD is associated with a very poor prognosis for the patient, with an expected survival of less than 10% [Mielcarek et al. 2003; Deeg 2007]. To address these problems, MSC have been used to treat these steroid-resistant GVHD [Ringdén et al. 2006; Fang et al. 2006]. Several clinical trials have investigated the potential benefits of MSC in the treatment of GVHD. One multicentre Phase II study investigated the effect of MSC in treatment of steroid-resistant, severe, acute GVHD [Le Blanc et al. 2008]. Patients were receiving mainly one or two infusions of *in vitro* expanded third-party HLA-mismatched BM-MSC. This treatment resulted in a higher overall survival two years after HSC transplantation and had no side-effects related to the infusions of MSC. Two Phase III clinical trials sponsored by Osiris Therapeutics, Inc., which is using their product Prochymal® to treat severe GVHD, failed to reach its primary clinical endpoint of

achieving a significant increase of complete response of steroid-resistant graft-versus-host disease lasting at least 28 days compared with placebo [Allison 2009; Galipeau 2013].

There is also much interest in the potential use of MSC in wound healing. MSC-based therapies offer a new treatment option for preventing morbidity and disability associated with chronic wounds. Healing of cutaneous wounds requires a well-orchestrated chain of events, both biological and molecular, which might be impaired in chronic diseases [Singer and Clark 1999]. The functional characteristic of MSC, such as their ability to migrate to the site of inflammation, stimulate resident progenitor cells to proliferate and differentiate, and their anti-inflammatory effects, may benefit wound repair. Studies have shown that treatment of cutaneous wounds with MSC can accelerate wound healing and increase epithelialization and angiogenesis [Wu et al. 2007; Falanga et al. 2007]. This suggests two main mechanisms by which MSC affect wound repair namely MSC differentiation and paracrine interactions with specific cell types within the wound [L. Chen et al. 2008]. Clinical studies have shown that the treatment of wounds with MSC is in general beneficial with no adverse effects [Lataillade et al. 2007; Yoshikawa et al. 2008]. One major field for wound healing in Western societies is diabetic foot ulcer, which affects 25% of diabetic patients and could result in amputation of the affected foot [Singh, Armstrong, and Lipsky 2014]. Current standard care only heals about 24% of wounds after 12 weeks of therapy [Markowitz et al. 2005]. A recent multi-centre study was investigating the efficacy of Grafix®, a human viable wound matrix from placental membrane containing living MSCs, compared with standard wound care [Lavery et al. 2014]. It showed that Grafix® treated patients had a significantly higher wound healing rate and less wound-related infections compared to standard treatment.

MSC are also considered for the biological repair of bone and articular cartilage. The osteogenic abilities were one of the first notions associated with MSCs [Ashton et al. 1985]. The differentiation of MSCs towards the osteogenic lineage has been shown *in vitro* and *in vivo* [Aslan et al. 2006]. Studies also confirmed that MSCs can be used to repair large bone defects *in vivo*, both in large animals [Kon

et al. 2000] and humans [Quarto et al. 2001]. Osteoarthritis (OA) is the most common form of degenerative joint disease with increasing numbers in the aging population worldwide [Lutz, Sanderson, and Scherbov 2008]. Unlike focal cartilage defects occurring in elite athletes, OA is a systematic, inflammatory disease which affects the whole joint, including articular cartilage, subchondral bone, synovium, muscle and tendon [Goldring and Goldring 2007]. It is characterized by the degeneration of cartilage, synovial inflammation and alterations in subchondral bone and surrounding soft tissues, leading to joint pain, stiffness and loss of function in the hands and the weight-bearing joints. So far, there are only symptom-modifying pharmacological treatments available, none of which are able to improve the overall disease outcome [Mobasheri et al. 2014]. This highlights the need for a non-surgical treatment option which will allow a more long-term improvement of disease. While the differentiation towards the osteogenic lineage is relatively straightforward, the formation of stable cartilage by MSCs is more challenging. Although *in vitro* pellet cultures are well established in human, the question remains whether this *in vitro* model represents the equivalent of stable or transient cartilage formation [Dell'Accio, De Bari, and Luyten 2001]. In fact, stable cartilage formation from synovial derived MSCs have proven unsuccessful [De Bari, Dell'Accio, and Luyten 2004].

Heart failure / Cardiovascular disease (CVD) is the leading causes of death in the world [Flynn and O'Brien 2011]. Acute myocardial infarction (AMI) is associated with ischaemia which leads to the death of cardiomyocytes by apoptosis and necrosis [Kajstura et al. 1996]. Even though it has been reported that cardiomyocytes exhibit some regenerative potential, this seems not be sufficient to replace the significant loss caused by AMI [Beltrami et al. 2001]. The only treatment option to counter the loss of cardiomyocytes is cardiac transplantation. Problems associated with this therapy include limited donor supply and need of life-long immunosuppressive therapy [Segers and Lee 2008]. The remodelling process is also associated with hypertrophy and fibrosis of cardiomyocytes which leads to reduced ventricular compliance, ventricular dilatation and eventually heart failure [Braunwald and Pfeffer 1991]. Current evidence indicates that MSCs

do not directly differentiate into cardiomyocytes, but deliver their effects through paracrine factors [Laflamme and Murry 2011]. It is worth noting that a recent, comprehensive study has shown that clinical trials with bone marrow derived adult stem cells, which do not show any factual discrepancies, do not benefit cardiac repair [Nowbar et al. 2014]. In this study 133 reports from 49 trials were investigated for discrepancies that could affect the degree of improvement of left ventricular function. It has been shown that only 5 trials were without discrepancies and these trials had no significant beneficial effect on the left ventricular function.

Overall it can be said that despite the enormous amount of clinical trials and research in recent years, no approved therapy has reached the commercial market. Although there is considerable encouraging *in vitro* data, in general, this has not translated into successful treatments.

Plastic-adherent culture condition is the traditional method for culturing MSCs *in vitro*. Single cell suspensions are obtained from tissues and cultured in media containing FCS. Cultures are expanded for a variable amount of passages to obtain sufficient numbers of cells and to remove contaminating haematopoietic cells. Still, the isolation and culture expansion of MSC is not standardized between research groups which have led to considerable variations in protocols. Some examples for different isolation techniques for BM-derived MSCs include density centrifugation with Ficoll^{TM} [Bara et al. 2014], retroviral selection [Kitano et al. 2000], culture in non-adherent conditions [Mendez-Ferrer et al. 2010], use of BM "plugs" [Suire et al. 2012] and FACS isolation [Jones et al. 2006; Jones et al. 2010]. But even by using the same isolation technique, differences in processing extracted cells, choice of density medium, wash and centrifugation steps, duration of cell attachment, media/serum type, additional growth factors and oxygen conditions further contribute to the diversity making it nearly impossible to compare studies from two different laboratories. This has been shown by a study from Seeger *et al.* [Seeger et al. 2007] which compared two similar randomized, placebo-based trials regarding the prevention of cardiac failure post-myocardial infarction [Lunde et al. 2006; Schächinger et al. 2006]. The MSCs used in these

trials differed in regard to the density media, centrifugation speeds, wash steps and storage conditions used for their preparation which affected the clinical outcome significantly. For example, it has also been shown that low density centrifugation, using Percoll^{TM} or Ficoll^{TM}, reduces the yield of mononuclear cells from bone marrow [Mareschi et al. 2012].

Besides the lack of knowledge, there are other obstacles for bringing stem cells into the clinic. These include the risk of immune rejection, possible pathogen contamination, change of phenotype during *in vitro* expansion, high costs, effectiveness of cell delivery and *in vivo* behaviour and safety of the long-term transplanted cells [Frenette et al. 2013]. Based on the 60 year history of HSC therapy, it has been argued that clinical trials with MSCs might be too far ahead of science and more basic/fundamental research needs to be done to fully understand the biology and therapeutic potential of MSC [Prockop, Prockop, and Bertoncello 2014].

1.2 THE BONE MARROW NICHE

The bone marrow (BM) has been studied intensively due to its important role in haematopoiesis. Due to the lifelong production of blood cells, haematopoietic stem cells (HSC) have the ability to self-renew and to asymmetrically differentiate towards haematopoietic progenitor cells (HPC). These progenitor cells then give rise to more specialized cells of the myeloid and lymphoid lineages. HSC are quiescent and differentiation as well as self-renewal has to be carefully regulated to not exhaust the stem cell pool. Therefore HSC reside in a special compartment within the bone marrow known as the HSC niche. The niche is regarded to be a physical construct which consists and transmits exogenous instruction to keep stem cells in a stem cell-like state [Potten and Loeffler 1990]. In this niche regulation is achieved by a number of means including cell-bound and soluble cytokines, cell - cell interactions and binding to extracellular matrix (ECM) components [Eliasson and Jönsson 2010]. In order to decipher the molecular code

of cell signals in the niche, researchers try to identify special instructor cells that interact with HSC. The hope is that the global niche effect can be brought down to a single cell with unique effector signals. To acknowledge the complex nature of stem cell biology this view has been extended from one single cell to a microenvironment of different, hierarchically organized "niche" progenitor cells [Bianco 2011]. In the mouse BM, a number of different anatomical locations (e.g. endosteal surfaces, sinusoidal walls, haematopoietic tissue proper) and cell types (e.g. osteoblasts, perivascular cells, adipocytes and stromal cells) have emerged over the last 10 years [Garrett and Emerson 2009; Kiel and Morrison 2008]. It is however possible that the niche is not a static location but instead dynamic and able to directly respond to injury or other cell signals [Milsom and Trumpp 2011]. One landmark study supporting this notion was done by Mendez-Ferrer *et al*. which showed that HSC supporting MSC, identified by nestin expression, are both near the endosteum and perivascular [Mendez-Ferrer et al. 2010]. Originally discovered in the developing central nervous system on neuroepithelial stem cells, the nestin gene transcribes intermediate filament protein type VI [Lendahl, Zimmerman, and McKay 1990]. It was later discovered that other progenitor cells from different anatomic locations also express nestin during development, e.g. muscle [Sejersen and Lendahl 1993; Kachinsky, Dominov, and Miller 1994], eye [Yang et al. 2000] and tooth development [Terling et al. 1995]. These nestin$^+$ MSC expressed high levels of HSC maintenance factor transcripts, such as CXCL12, stem cell factor (SCF), angiopoietin-1 (Ang-1), vascular cell adhesion molecule 1 (VCAM-1, also CD106), and osteopontin (OPN). Depletion of nestin$^+$ MSC led to the mobilisation of ~ 50% of HSC to the spleen. In addition homing to the bone marrow of HSC was impaired by ~90% in nestin depleted mice. In contrast, another study by Ding *et al*. has shown that depletion of SCF in nestin$^+$ cells and osteoblasts does not affect HSC numbers, but deletion in endothelial and perivascular cells significantly reduced HSC cells [Ding et al. 2012]. Another type of cell with many similarities to nestin$^+$ cells was described as CXCL12-abundant reticular cells (CAR) cells [Omatsu et al. 2010; Greenbaum et al. 2013]. These cells produce high amounts of CXCL12 and SCF which leads to an increase in HSC self-renewal and cycling and inhibits HSC differentiation. They are predominantly

found in the central BM but some are also located near the endosteum. Given that nestin^{+} cells are approximately four times less abundant than CAR cells, contain all colony-forming-unit fibroblast (CFU-F) activity within the marrow, have high self-renewal activity and are capable of tri-lineage differentiation, it has been speculated that nestin^{+} cells might be a more primitive subset of CAR cells [Ehninger and Trumpp 2011].

On the other hand, given that the process of haematopoiesis in adults is exclusively located in the BM, a relationship between HSC and bone has long been suggested [Lord, Testa, and Hendry 1975]. This is further supported by the close proximity of HSC and osteoblasts (OB) at the endosteum [Taichman and Emerson 1994; Calvi et al. 2003; Jung et al. 2007]. The endosteum is the inner surface of the bone next to the marrow space and is covered by a layer of bone-lining OB. It has been shown that Notch signalling from OB increases the number of HSC *in vivo* [Calvi et al. 2003]. HSC also express the calcium-sensing receptor (CaSR), enabling HSC to follow the Ca^{2+} gradient which results from bone re-modelling processes occurring at the endosteum and therefore support HSC engraftment at the endosteum [Adams et al. 2006]. Other factors produced by OB that are known to be involved in HSC maintenance and retention include CXCL12, OPN and N-Cadherin in addition to factors involved in keeping HSC in a quiescent state, such as Ang-1, membrane-bound SCF and thrombopoietin [Arai et al. 2004; Thoren et al. 2008; Yoshihara et al. 2007]. Interestingly, is has been suggested that osterix^{+} osteoprogenitor cells rather than mature osteocalcin^{+} OBs are needed for maintaining haematopoiesis [Raaijmakers et al. 2010]. Also HSC isolated from the endosteal region had a higher proliferated capacity and homing efficiency compared to their counterparts from the central BM [Grassinger et al. 2010]. Similar to nestin^{+} cells, depletion of OB leads to a mobilisation of HSC to the spleen and an increase of OB simultaneously leads to an augmentation of HSC [Visnjic et al. 2004; Calvi et al. 2003; Jung et al. 2006]. Furthermore, by transplanting osteoprogenitor cells under the kidney capsule, these cells were able to form a HSC niche *in vivo* through endochondral ossification [Chan et al. 2009]. It has been reported that stromal cells exist near the surface of the bone [Hisha et al. 1995].

This is no surprise as MSCs generate OBs that remain in close proximity. These OBs may not contribute directly to the niche activity, as osterix^{+} osteoprogenitors rather than mature osteocalcin^{+} OBs are required for the integrity of the niche [Raaijmakers et al. 2010]. This is further supported by the fact that MSCs seem to express higher levels of HSC maintenance factors (CXCL12, SCF, IL-7, VCAM1, and OPN) compared with OBs [Mendez-Ferrer et al. 2010]. A study by Siclari *et al*. has shown that MSCs isolated from the endosteal region contain more proliferate MSC and more immunosuppressive than their central BM counterpart [Siclari et al. 2013].

Taken together it can be said that HSCs can be found near the endosteum lined by OB or near sinusoidal endothelium. Depletion experiments have shown that even when various niche cells (MSC, CAR or OB) where ablated, not all HSC disappear or get mobilized [Visnjic et al. 2004; Mendez-Ferrer et al. 2010; Omatsu et al. 2010; J. Zhu et al. 2007]. This could be a result of inefficient experimental depletion systems or biological redundancy in the system. It could also indicate that there is more than one niche environment housing different subsets of HSC. These different subsets of HSC might include slow-cycling, dormant HSC near the endosteal region and fast-cycling, self-renewing HSC near the perivascular region [Trumpp, Essers, and Wilson 2010] (Figure 1.2).

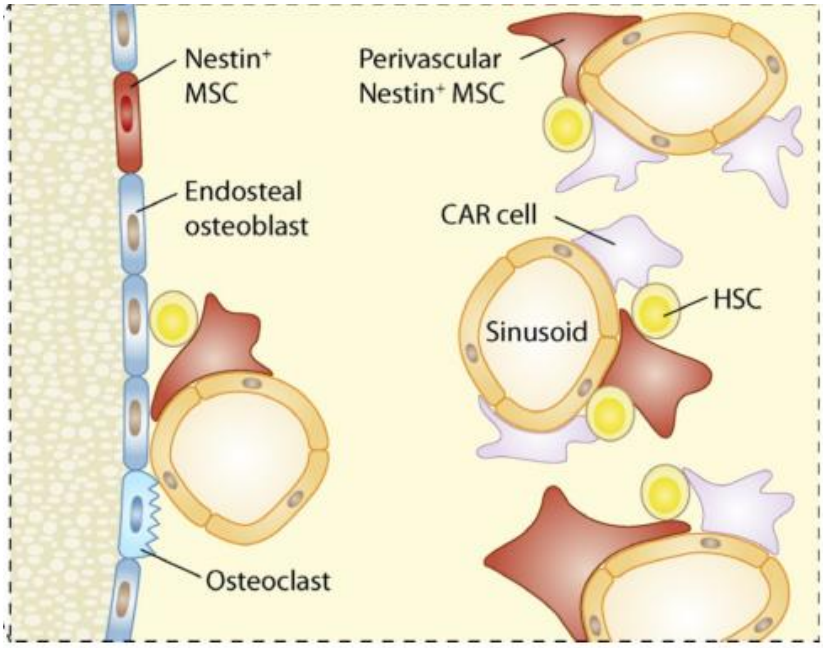

Figure 1.2 Location of HSC niches in long bones. HSCs are located at the endosteum, which is lined by OBs and osteoclasts. Vascular sinusoids are also found near the endosteum, but more frequently towards the centre of the BM. Adapted from [Ehninger and Trumpp 2011].

Another point to take into consideration is that HSC might not be as passive in the niche as someone might think. In this dynamic environment, HSC play an active part in the formation of the niche. As shown by Jung *et al.*, HSC do not rest passively in the niche but actively participate in its formation by directing bone formation of MSCs and participation in niche activities [Jung et al. 2008].

All these results could be result of overlapping of different kind of HSC supporting niche cells, confirming the earlier statement of a hierarchically organisation of the bone marrow niche.

1.3 EXTRACELLULAR MATRIX (ECM)

Based on the niche concept, it became clear that the plasticity of MSCs *in vivo* and *in vitro* is dependent on the microenvironment [Barry and Murphy 2004]. The bone marrow niche contains haematopoietic cells, stromal cells, adipocytes and vascular elements [Bianco et al. 2001]. All these cells are within a complex ECM composed of fine reticular fibres produced by the stromal cells [Campbell 1987]. Therefore the great variations in tissues or ECM microenvironments exhibit specific conditions that will guide cell maintenance and differentiation. Three variations of ECM can be distinguished based on their elasticity: 1) softer tissues such as the brain, 2) stiff tissues such as muscles, and 3) rigid tissues such as bones [Engler et al. 2006]. This elasticity seems to be vital for MSCs lineage differentiation. During cell-matrix interaction, adhesion complexes and the actin-myosin cytoskeleton transduce chemical signalling, which affects cytoskeletal organisation [Discher, Janmey, and Wang 2005]. In their niche, the stiffness of the matrix influences the mechanical forces that cells are exposed to. These forces trigger a variety of physiological responses such as cell motility, proliferation and differentiation [Griffith and Swartz 2006]. By influencing the focal- adhesion structure and the cytoskeleton, the stiffness of the matrix directly influences the lineage commitment of MSCs [Engler et al. 2006]. In addition to the matrix stiffness, soluble factors modulate MSC lineage commitment via RhoA signalling and Rho-kinase activity, which regulates the actin–myosin contractility [McBeath et al. 2004]. Analysis of BM, as well as the ECM made by cultured marrow stromal cells, has shown the presence of collagens I, III, IV, V, and VI, fibronectin, laminin, and other adhesive proteins, as well as large molecular weight proteoglycans and small leucine-rich proteoglycans [X. Chen et al. 2007]. Another study by Davis *et al.* looked at the influence of bone morphogenic protein-2 (BMP-2) and three-dimension (3D) osteoconductive substrates in osteogenesis. They observed enhanced effects in the osteogenic response of MSCs due to activation of multiple pathways mediated by the substrate and growth factors [Davis et al. 2011]. Still, most *in vitro* studies have been carried out using two-dimensional (2D) surfaces.

However, it was shown that cell types derived from *in vivo* settings quickly lose their differentiated phenotype when plated onto 2D surfaces [Raghavan et al. 2010]. In fact, studies revealed that chondrogenesis and osteogenesis can be induced by using 3D *in vitro* settings, using either collagen type II hydrogels or chorion-derived scaffolds [Jurgens et al. 2012; Mohr et al. 2010]. Not only can the differentiation of MSCs towards a specific lineage be influenced by ECM, but their ability to proliferate and preserve their stem cell phenotype is also dependent on ECM signals. By using murine bone marrow cell-derived ECM, it was shown that it inhibits "spontaneous" osteoblast differentiation and enhanced proliferate properties [X. Chen et al. 2007]. Additionally, mice lacking biglycan exhibit defects in the ability of marrow-derived progenitors to differentiate into osteoblasts, highlighting the role of ECM in the control of MSC behaviour [X.-D. Chen et al. 2002]. Another study showed that fibroblast derived ECM maintain human embryonic stem cells (hESC) in an undifferentiated state without inducing chromosomal aberrations [Escobedo-Lucea et al. 2012].

1.4 IMMUNOMODULATORY PROPERTIES

One of the reasons for the use of MSC in clinical therapy is their ability to mediate immunosuppressive and immunomodulatory effects on both adaptive and innate immunities [Marigo and Dazzi 2011]. Their effect on T lymphocytes is particularly well characterized. MSC suppress helper $CD4^+$ and cytotoxic $CD8^+$ T lymphocytes independently of whether the latter are naïve, antigen experienced, their functional state or the type of T lymphocyte receptor expressed [Krampera et al. 2003; Prigione et al. 2009]. This effect is also MHC independent, as it can be mediated by both autologous and allogeneic MSC. The Immunomodulatory effect is anti-proliferative, mediated by soluble factors acting in a paracrine fashion and is not a result of T-lymphocyte apoptosis [Di Nicola et al. 2002]. These paracrine signals include indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), nitric oxide (NO) and transforming growth factor-β (TGF-β) [Duffy, Ritter, et al. 2011].

This anti-proliferate effect is not restricted to T lymphocytes. Thus, the proliferation of enriched B lymphocytes from the spleen and BM is also inhibited [Glennie et al. 2005; Corcione et al. 2006]. In a mouse model of systemic lupus erythematosus, an autoimmune disease, MSC inhibited *in vivo* B lymphocyte proliferation, activation and IgG secretion [Deng et al. 2005]. Enigmatically, MSC are essential for B lymphopoiesis in the BM and have been shown to promote B lymphocyte function [Rasmusson et al. 2007; Traggiai et al. 2008].

MSC can modulate the activity of natural killer (NK) cells, a key player of the innate immunity, by suppressing interleukin (IL) -2 or IL-15 driven NK cell proliferation. This is however only true for resting NK cells, as cytotoxic activity of freshly isolated NK cells is not affected [Krampera et al. 2006; Sotiropoulou et al. 2006]. Similar to T lymphocytes, NK cell proliferation, cytotoxicity and cytokine production is inhibited by IDO, PGE2 and TGF-β secretion by MSC [Spaggiari et al. 2008; Sotiropoulou et al. 2006].

By acting on antigen-presenting cells (APC), MSC can indirectly modulate the immune response. When dendritic cell (DC) precursors were exposed to differentiation factors, like granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4, in the presence of MSC, they failed to acquire a mature DC phenotype [Jiang et al. 2005; Spaggiari et al. 2009].

MSC-mediated immunosuppression is not only triggered by acting directly on immune effector cells, but is also a result of acting on immune regulating cells. It has been shown *in vitro* that MSC recruit regulatory T lymphocytes [Di Ianni et al. 2008; Prevosto et al. 2007]. MSC-induced Treg expansion has also been reported *in vivo*, e.g asthma [Nemeth et al. 2010] and diabetes [Madec et al. 2009], but it remains unclear if they are required for MSC activity as *in vitro* data suggests otherwise [Krampera et al. 2003].

Other lymphocytes affected by MSCs also include T-helper cells. Duffy *et al.* have shown that MSC inhibit Th17 lymphocyte differentiation from both naïve and memory T lymphocyte precursors [Duffy, Pindjakova, et al. 2011].

1.5 DIFFERENTIATION POTENTIAL OF CULTURE-EXPANDED MSC

As mentioned before one of the main criteria to define MSC is their ability to differentiate towards the three mesenchymal lineages bone, cartilage and fat *in vitro*.

The osteogenic differentiation is achieved by incubating cell monolayers in media with fetal calf serum (FCS), ascorbic acid, β-glycerophosphate and dexamethasone [Jaiswal et al. 1997]. This leads to an increase of alkaline phosphatase and calcium deposition. Chondrogenic differentiation requires a high cell density pellet, also called micromass culture, in the presence of TGF-β without FCS [Mackay et al. 1998]. Histological analysis and quantification is carried out to determine the levels of cartilage-specific production of sulphated proteoglycan and collagen type II. To induce adipogenic differentiation cell monolayers are treated with media containing FCS, dexamethasone, insulin, isobutyl methyl xanthine and indomethacin [Pittenger et al. 1999]. To determine successful adipogenic induction, cells are stained for lipid vacuoles. Notably not all cells in MSC culture system undergo differentiation. In fact, it has been shown that even in clonally derived populations, the differentiation potential varies with cells lacking differentiation of at least one lineage [Pittenger et al. 1999; Morikawa, Mabuchi, Kubota, et al. 2009].

In recent years it has become clear that MSC populations maintained *in vitro* contain coexisting subsets varying in their differentiation potential, regardless of their tissue of origin. A study from Karystinou *et al.* has shown that clonally-derived hMSC vary in their differentiation potential, with only 30% capable of differentiating towards all three lineages [Karystinou et al. 2009]. Similar results have been shown for hMSCs using high capacity *in vitro* assays, where only 50% of colony-forming cells were capable of tri-lineage differentiation [Russell et al. 2010].

In addition to the classical tri-lineage differentiation capabilities of MSC, other reports show the ability of MSC to differentiate into other mesenchymal lineages, such as myocytes and tenocytes (Figure 1.3) [Wakitani, Saito, and Caplan 1995; De Bari et al. 2001; Hoffmann et al. 2006].

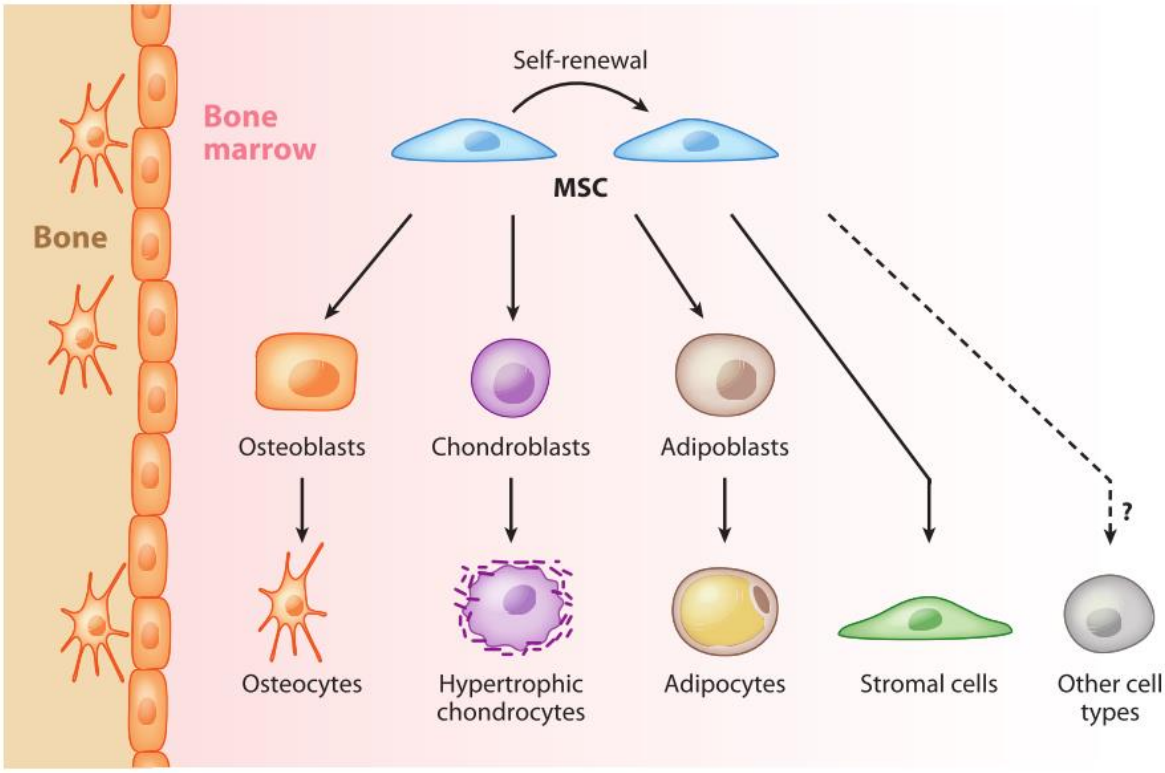

Figure 1.3 Differentiation of BM derived MSCs [Frenette et al. 2013].

However, most of the published claims for MSC multipotency are based entirely on *in vitro* differentiation assays. Therefore, these assays might not necessarily translate into the *in vivo* differentiation potential of MSCs. Furthermore, most of the published data only show a few cells able to differentiate into certain cell types, highlighting the notion of heterogeneity of *in vitro* MSC cultures.

1.6 HYPOXIA

Oxygen plays an important role in maintaining the stem cell niche. In fact it has been shown that low oxygen tensions greatly influence the biology of both embryonic and adult stem cells [Eliasson and Jönsson 2010; Silván et al. 2009; Krishnappa, Boregowda, and Phinney 2013]. Inhaled ambient air (21% oxygen) has partial pressure oxygen (pO_2) of 160mm Hg when it enters the lung. This pO_2 progressively decreases as it travels in the blood and has dropped to 2% - 9% (14 - 65mm Hg) when it reaches organs and tissues [Brahimi-Horn and Pouysségur 2007]. Therefore, ambient air (normoxic) *in vitro* conditions (21% O_2) do not reflect the physiological levels in the bone marrow, thereby exposing cells to a higher oxygen concentration than in their *in vivo* environment. Furthermore, due to its architecture of medullary sinuses and arteries, the oxygen tension in the bone marrow has been estimated to range from 1 – 7% [S.-C. Hung et al. 2007; Spencer et al. 2014]. The presence of these low oxygen tensions in stem cell niches might offer a selective advantage to their particular biological role [Cipolleschi, Dello Sbarba, and Olivotto 1993]. One of these advantages is the escape from oxidative stress which occurs during aerobic metabolism, where cells generate reactive oxygen species that can damage DNA [Fan et al. 2011]. Research has shown that mouse embryonic fibroblasts accumulate more mutations and senesce faster when cultured under 21% O_2 than cells cultured under 3% O_2 [Busuttil et al. 2003]. For several stem and progenitor populations, hypoxia is an important factor in stem cell biology, promoting an undifferentiated state [Mohyeldin, Garzón-Muvdi, and Quiñones-Hinojosa 2010; Prado-Lòpez et al. 2014]. Due to their location in the BM niche, MSC might also benefit from a hypoxic environment. Previous publications have shown that CFU-F frequency, growth and differentiation of mMSC were negatively affected by normoxic oxygen levels [Boregowda et al. 2012]. As mentioned earlier, differences in MSC isolation, culture and experimental design have made it difficult to make broad conclusion about MSC. The same is also true for the role of hypoxia on the biology of MSC. MSCs from human BM are one of the best characterised, and the influence of hypoxia on their

proliferation and differentiation have been intensively investigated. Unfortunately, even for MSCs from the same tissue, results from different groups differ in the reported effects of hypoxia (Table 1.1).

Table 1.1 Effects of hypoxia on mMSCs

Parameter	**Hypoxia decreases**	**Hypoxia increases**
Proliferation	[Holzwarth et al. 2010; S.-C. Hung et al. 2007]	[S.-P. Hung et al. 2012; Jin et al. 2010; Tsai et al. 2010; Fehrer et al. 2007]
Osteogenesis	[Holzwarth et al. 2010; Fehrer et al. 2007; S.-C. Hung et al. 2007]	[S.-P. Hung et al. 2012; Tsai et al. 2010]
Adipogenesis	[Holzwarth et al. 2010; Fehrer et al. 2007; S.-P. Hung et al. 2012; S.-C. Hung et al. 2007]	[Jin et al. 2010; Tsai et al. 2010]
Chondrogenesis	[S.-P. Hung et al. 2012]	[Jin et al. 2010; Khan et al. 2010; Markway et al. 2010; Tsai et al. 2010]

Molecular analyses show that hypoxia activates Akt signalling and the upregulation of c-Met, the receptor for hepatocyte growth factor (HGF) which leads to an increased migratory phenotype of MSCs [Rosová et al. 2008]. It also increases the expression of vascular endothelial growth factor (VEGF), CXCR4, CX3CR1 and promotes the phosphorylation of focal adhesion kinase (FAK) [S. H. Lee et al. 2010; S.-C. Hung et al. 2007]. In disease models, e.g. hind limb ischemia, hypoxic or ischemic tissues are known to produce cyto- and chemokines which are involved in the recruitment of MSCs to the site of injury [Ceradini and Gurtner 2005]. One important factor in mediating the cells' response to hypoxia is the hypoxia-

inducible transcription factor-1α (HIF-1α) [Semenza and Wang 1992]. When oxygen drops below 5%, HIF-1a stabilizes, translocates to the nucleus, heterodimerizes with the HIF-1β subunit and initiates a transcriptional program (Figure 1.4). HIF-1a is ubiquitous expressed and also plays an important role in fetal development [Compernolle 2003; Stroka et al. 2001]. Recently is has been shown that HIF-1α contributes to the increased radio-resistance of hypoxic cultured mMSCs by promoting the upregulation of DNA double strand break repair [Sugrue, Lowndes, and Ceredig 2014].

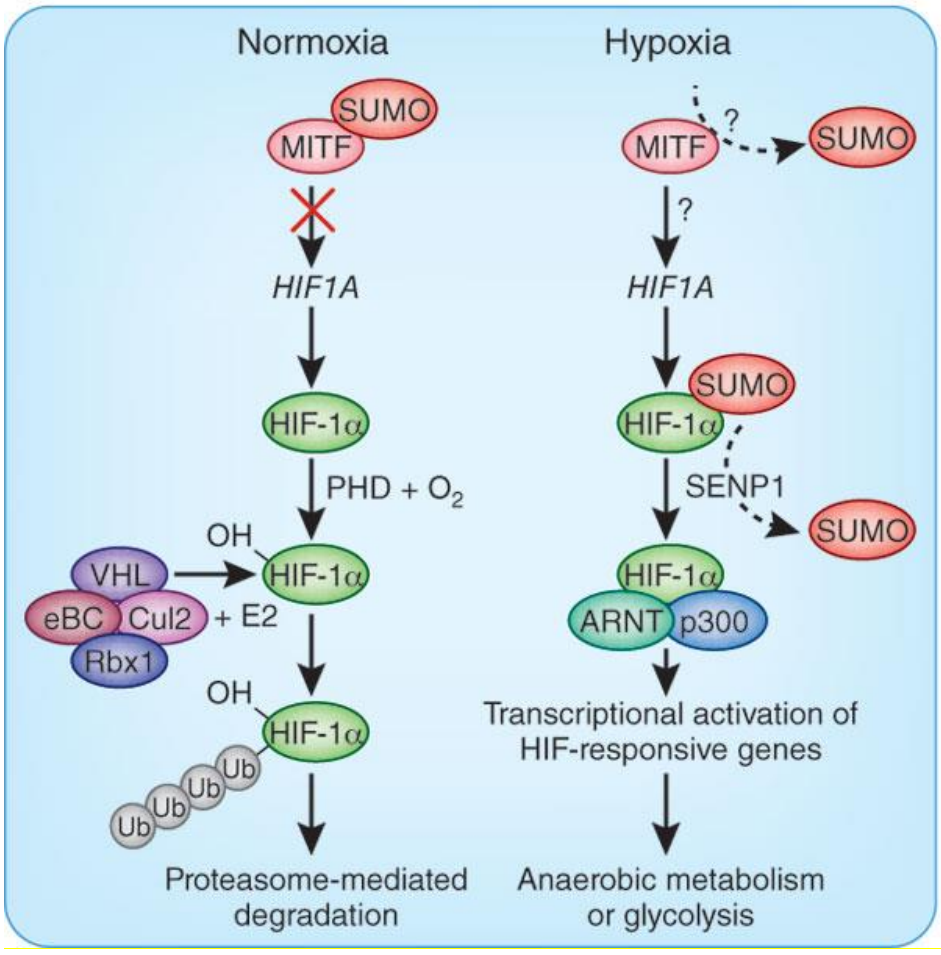

Figure 1.4 Regulation of HIF-1α [Ohh 2012].

In conclusion it can be said that oxygen tensions play an important role as a metabolic regulator of stem cell biology and represent an added dimension of

stem cell maintenance that influences the self-renewal and multi-lineage differentiation potential.

1.7 ISOLATION OF MSC

Bulk MSC have been isolated from a variety of different tissues including peripheral blood [Zvaifler et al. 2000], synovial membrane [De Bari et al. 2001], synovial fluid [Jones et al. 2004], periosteum [De Bari et al. 2006], articular cartilage, compact bone [Guo et al. 2006], cord blood [Erices, Conget, and Minguell 2000], skeletal muscle [Williams et al. 1999], adipose tissue [Zuk et al. 2002], dental pulp [S Gronthos et al. 2000] and placenta [Fukuchi et al. 2004]. Bone marrow has been in the focus of MSC isolation as a rich source HSCs as well as MSCs.

Cells derived from different tissues show a phenotypic heterogeneity and differ in their proliferation activity, but are similar in their tri-lineage differentiation capacity and expression of certain surface markers [Baksh, Yao, and Tuan 2007]. It is becoming clearer that the tissue of origin is a major factor in the variation of MSC biology [De Bari and Dell'accio 2008]. Within each tissue source, single-cell-derived clonal MSC population from limited dilution assays are highly heterogeneous in their proliferation and differentiation capacity [Donald G Phinney and Prockop 2007]. This variability hinders the clinical use of MSC due to unknown differentiation capacity of the used MSC population. It is therefore necessary to develop quantitative potency assays in order to measure the potency of the used MSC population [De Bari and Dell'accio 2007].

Criteria for the minimal identification of hMSC have been proposed by the International Society for Cell Therapy [Dominici et al. 2006]: i) adherence to plastic in standard culture conditions, ii) FACS analysis for the positive markers CD73, CD90 , CD105 and negative for CD34, CD45, HLA-DR , CD14 or CD11b, CD79a or CD19; iii) in vitro differentiation into osteoblasts, adipocytes and chondroblasts.

But even these criteria only allow a retrospective definition of hMSC and cannot be used to prospectively isolate hMSC. Furthermore due to differences between man and mouse, these criteria cannot be applied to mMSC. In fact it has been show that even in mice, marker expression and differentiation capacity differ between different mouse strains [Peister et al. 2004; Sung et al. 2008].

Difficulties associated with culturing mouse MSC (mMSC) as well as mouse strain variations in plating efficiency and the relative ease with which human cells can be cultured have resulted in comparatively more work being done with human than with mMSC [Peister et al. 2004; Anjos-Afonso, Siapati, and Bonnet 2004]. By culturing adherent cells from both species long-term, it became evident that their self-renewal and/or differentiation capacity became impaired [Kretlow et al. 2008; Wang et al. 2013]. Thus, the MSC-like properties of cells may not be retained after serial passaging *in vitro*. Another point to take into consideration is that findings in mice do not necessarily translate to man. Especially phenotypic marker expression not only varies between different mouse strains but also between man and mouse. For example, the well-known human MSC marker Stro-1 does not have an equivalent in mice [Baddoo et al. 2003; Simmons and Torok-Storb 1991] and reciprocally, the mouse marker Sca-1, does not have a human equivalent. Mouse MSC are also karyotypically unstable with shortened telomere length even in early cultures whereas human MSC are not [Miura et al. 2006; Bernardo et al. 2007; Wang et al. 2013].

Thus, the MSC-like properties of cells may not be retained after serial passaging *in vitro*. In order to try and improve the isolation of mMSCs, flow cytometry has been employed to positively select MSC. In these experiments several combinations of surface markers have been used, the most frequent being Sca-1 [Anjos-Afonso and Bonnet 2011].

1.8 THE SCA-1 MARKER

Discovered almost 30 years ago as antigens expressed by foetal thymocytes [Aihara et al. 1986], Stem cell antigen-1 (Sca-1, Ly-6A/E) and Sca-2 are members of the Ly-6 family of interferon-inducible lymphocyte activation proteins whose genes are located on mouse chromosome 15 [Malek, Danis, and Codias 1989][LeClair et al. 1986]. Sca-1 is an 18kDa mouse glycosylphosphatidylinositol (GPI) – linked cell surface protein and encoded by the mouse strain-specific Ly-6A/E allelic gene [van de Rijn et al. 1989]. Differing at the Ly-6 locus, Sca-1 is differentially expressed by lymphocytes from different mouse strains resulting in a 20-fold higher expression in C57Bl/6 mice (Ly-6^b) compared to BALB/c mice (Ly-6^a) [Spangrude and Brooks 1993]. In the cell membrane, Sca-1 is associated with protein tyrosine kinases and lipid rafts suggesting it may be involved in signal transduction [Stefanová et al. 1991][Suzuki 2012]. In C57Bl/6 mice, Sca-1 is a well-established marker of mouse HSCs and in conjunction with additional markers such as CD117 (c-kit) is routinely used for their isolation from BM [Ma et al. 2002]. Thus mouse HSC are frequently referred to as Lineage-negative, Sca-1-positive c-kit-high cells, often abreviated to "LSK". Outside the well-characterized haematopoietic system, Sca-1 is also expressed by a mixture of stem, progenitor and differentiated cell types in a variety of tissues. Sca-1 expression has been linked to stem/progenitor cell populations within the prostate [Burger et al. 2005], mammary gland [Welm et al. 2002], skeletal muscle [J. Y. Lee et al. 2000], heart [Matsuura et al. 2004], liver [Petersen et al. 2003] and skeletal system [B. Short and Wagey 2013]. This has led to the routinely use of Sca-1 in combination with negative selection for enrichment of stem and progenitor cells.

Likewise, for mMSC isolation, Sca-1 has been used in conjunction with other markers, but no systematic analysis of Sca-1 expression by cultured mMSCs has been reported so far. Sca-1 has already been used in combination with other marker to isolate mMSCs from bone marrow [Nakamura et al. 2010; Steenhuis, Pettway, and Ignelzi 2008] and a recent study was able to generate clonal subpopulations of mMSCs by combining Sca-1 and PDGFR-α staining which

showed tri-lineage differentiation capacity both *in vitro* and *in vivo* [Morikawa, Mabuchi, Kubota, et al. 2009].

Despite the extensive research on Sca-1 in mouse, it cannot be used for the isolation of human stem/progenitor cells, due to the fact that Sca-1 does not have a human homolog. This is a result of the evolutionary loss of a 500kb region of the Ly6 locus which included the Sca-1 gene in humans [Holmes and Stanford 2007]. Due to its important role in stem cell stress responses and its wide range of expression in mice, this might seem illogical. It is therefore likely that at least some of the functions exhibited by Sca-1 in mice are assumed by a number of human Ly6 proteins. Even though the exact human Ly6 proteins which could be a Sca-1 homolog are not known, the analysis of Sca-1 mutant mice have led to a variety of stem cell concepts which are equally relevant to human health and disease. This includes the involvement of MSC and progenitor cells in bone homeostasis, the relation between degenerative diseases and exhaustion of the stem cell pool and tissue maintenance by stem cells. Sca-1 null-mice showed decreased cardiac function [Rosenblatt-Velin et al. 2012] and age-dependent osteoporosis [Bonyadi et al. 2003], but only minor effects on adipose function [Staszkiewicz et al. 2012].

Thus, the study of Sca-1 in mice is still important to understand the function of Ly6 family proteins regarding stem cell biology.

1.9 HYPOTHESIS AND AIMS

Beside the broad use of hMSCs in clinical trials, fundamental knowledge about the basic biology and work mechanisms of these cells are still lacking. While hMSCs can be easily isolated based on surface marker expression, the isolation of mMSCs proves more difficult. Furthermore, standard isolation protocols for mMSC include plastic adherence and *in vitro* expansion which leads to a heterogeneous population of cells. It is therefore necessary to prospectively isolate single-cells to address fundamental questions about their basic biology and to take advantage of genetically altered mouse strains.

Therefore, the overall goals of my PhD studies were:

(i) To characterise the phenotype and to improve culture conditions of bone chip derived bulk mouse MSCs *in vitro* (Chapter 2).

(ii) To isolate MSCs from BM by FACS based on Sca-1 expression and investigate phenotypic changes during *in vitro* expansion (Chapter 3).

(iii) To clonally isolate a stromal cell population on a single cell level and investigate their immunoregulatory capacity (Chapter 4).

CHAPTER 2

Isolation of mMSCs from bone chips

2.1 INTRODUCTION

A key aspect in the investigation of mMSC is the isolation method employed. Normally, suspensions of bone marrow cells are cultured in plastic dishes and subsequently, non-adherent cells discarded. Two common problems associated with this isolation method are firstly that in early passages there is contamination with adherent haematopoietic cells and secondly, both mesenchymal and haematopoietic cells in such cultures are heterogeneous [D G Phinney et al. 1999]. Microscopic examination of the adherent mesenchymal cells shows them to be growing from individual foci, or colonies, and these colonies have been called colony forming unit – fibroblast (CFU-F) [Friedenstein, Gorskaja, and Kulagina 1976]. Difficulties associated with culturing mMSC as well as mouse strain variations in plating efficiency and the relative ease with which human cells can be cultured have resulted in comparatively more work being done with human than with mMSC [Peister et al. 2004; Anjos-Afonso, Siapati, and Bonnet 2004]. By culturing adherent cells from both species long-term, it became evident that their self-renewal and/or differentiation capacity became impaired [Kretlow et al. 2008].

It has been shown that human MSC from compact bone exhibit similar properties compared to those of their bone marrow counterparts [Sakaguchi et al. 2004] and that mouse compact bone is a richer source for MSC than mouse bone marrow [B. J. Short, Brouard, and Simmons 2009; Guo et al. 2006; H. Zhu et al. 2010]. It has also been shown that the yield as well as the lineage potential of MSC isolated from mouse bone marrow is dependent on donor age [Peister et al. 2004; Kretlow et al. 2008].

A common problem with all mouse MSC cultures is the contamination with haematopoietic cells [Meirelles and Nardi 2003; Suire et al. 2012; Krishnappa, Boregowda, and Phinney 2013]. Because most cells in the BM are haematopoietic in origin and adherent cells from the BM, including MSC support haematopoiesis, it is not surprising that contamination by haematopoietic cells is a feature of early MSC cultures [Calvi et al. 2003; Mansour et al. 2012]. It has been shown that this

contamination is reduced by sequential passaging [Nadri et al. 2007]. This is due to the fact that only cells which are best suited to the particular cell culture environment will dominate and proliferate. Among the important factors that contribute to this selection are: sensitivity to trypsin, nutrient or substrate limitation, growth rate, cell density and oxygen supply. The downsides of this approach include the i) change of phenotype, ii) bias of growth of a subpopulation within the heterogeneous bulk cultures and iii) senescence of MSC with extensive culture *in vitro* [Gregory, Ylostalo, and Prockop 2005; Kretlow et al. 2008; Anjos-Afonso, Siapati, and Bonnet 2004]. Several other protocols have been developed over the years to circumvent these problems including: i) antibody depletion of HSC before MSC culture [Baddoo et al. 2003], ii) stimulation of MSC growth by addition of basic fibroblast growth factor (bFGF) to the culture [D G Phinney et al. 1999; Sun et al. 2003], iii) adjusting the plating density *in vitro* [Sun et al. 2003; Meirelles and Nardi 2003; Peister et al. 2004], or iv) retroviral selection of cycling cells [Kitano et al. 2000; Tropel et al. 2004]. Still, all these protocols yielding either a heterogeneous population of cells or cannot be employed prospectively.

Similar to human MSC the definition of mouse MSC is based on i) plastic adherence in standard culture conditions, ii) positive and negative expression of certain surface markers, iii) tri-lineage differentiation capacity into osteoblasts, adipocytes and chondroblasts. In contrast to human MSC, there are no definitive surface markers for the direct isolation of mouse MSC [Baddoo et al. 2003]. There is, however, a general consensus that mouse MSC positively express CD106, CD105, CD73, CD29, CD44, and Sca-1 [Anjos-Afonso and Bonnet 2011]. These positive markers together with the absence of expression of the haematopoietic and endothelial markers Ter119, CD45, CD11b, and CD31 are routinely used for the definition of mouse MSC [R&D Systems].

There are commercially available surface marker cocktails for the *in vitro* definition of mouse MSC, which differ in their composition of antibodies [Abcam 2011; R&D Systems]. While the Abcam® cocktail contains the markers CD45, Sca-1, CD44, CD29 and CD90 to confirm the MSC phenotype, the R&D systems™ cocktail

additionally contains CD11b, CD73, CD105 and CD106 but lacks CD90. This shows the diversity of data regarding the mMSC phenotype *in vitro*.

Due to the difficulties associated with MSC isolation, characterisation and expansion protocols, bone chip derived MSC offered a way to easily obtain bulk MSC from C57Bl/6 mice. The aims of the current Chapter were therefore to show that currently used cell culture conditions can yield MSCs from mouse bone chips, purify them of hematopoietic cell contamination and characterise the MSC phenotype after short term *in vitro* expansion.

2.2 MATERIALS & METHODS

Animal strains and ethical approval

C57Bl/6 mice were bred in house and used at the age of 6 to 8 weeks. Experimental animals were housed in a specific pathogen-free facility and fed a standard chow diet. All animal procedures were carried out under license from the Irish Department of Health and Children by procedures approved by the NUI Galway Animal Care Research Ethics Committee (ID: 12/JULY/02).

Flushed and compact bone marrow

Femurs and tibias from 6 – 8 week old C57Bl/6 mice were dissected, bones were cleaned of muscle and adherent tissue, and bone marrow flushed out with an 18 gauge (G) needle and syringe containing α-MEM + GlutaMAX (Gibco). For breaking up cellular clumps, a 21G needle was used with gentle syringing. The resulting single cell suspension was called “flushed bone marrow” (fBM). Red cells lysis was performed on fBM using sterile H_2O for 5 seconds after which the reaction was quenched using FACS buffer (see appendix)[Houlihan et al. 2012].

To isolate endosteal lining cells, bones from fBM isolation were crushed with a pestle and mortar and gently washed with α-MEM + GlutaMAX. After extensive washing, bones were further chopped with a scalpel into 1 – 2mm pieces and

incubated for 45 minutes in 37°C in 4ml of α-MEM + GlutaMAX containing 2.5mg/ml Collagenase I (Sigma) and 100µg/ml DNAse I (Sigma-Aldrich). MSC Growth Medium (see appendix) was added to quench collagenase enzyme activity. The suspension was then filtered with a 70µm cell strainer to remove bone fragments and debris, and centrifuged at 400g for 5 minutes. This suspension was called "compact bone marrow" (cBM). An overview of isolated suspensions from long bones can be seen in Figure 2.1.

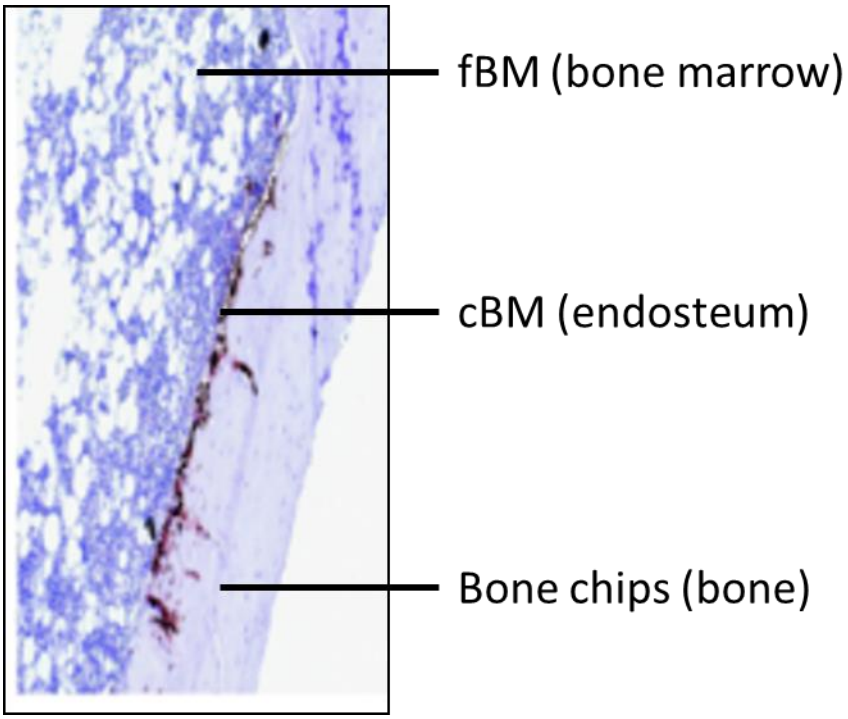

Figure 2.1 Anatomic locations of isolated bulk MSCs in long bones. Histological longitude section of mouse long bone. Cell suspensions were obtained from flushing out red BM ("fBM"), enzymatic release of endosteal lining cells ("cBM") and culturing "bone chips" of crushed long bones. Figure adapted from [Anjos-Afonso and Bonnet 2007].

Bone chip derived MSC

The bone chips left over from the isolation of cBM (see above) were washed with Dulbecco's phosphate buffered saline (DPBS), put in MSC Growth Medium and incubated in a humidified incubator with 5% CO_2 at 37°C. Cells were carefully

washed every 2 days with DPBS (Gibco) to remove the non-adherent fraction. Microscopic examination revealed that adherent cells grew out from these bone chips. Cells were harvested after 7 days by using 0.25% Trypsin/0.02% EDTA for 1min, filtered through a 70µm filter mesh (Sefar) and put in a new T175 flask containing pre-warmed MSC Growth Medium (Passage 1). After about 7 – 10 days, when the primary culture was nearly 80% confluent, cells were detached with Trypsin/EDTA for 1 min and seeded to new T175 flasks with pre-warmed MSC Growth Media. Culture medium was changed every 2 - 3 days and subsequent passages were performed when cells reached 60 – 80% confluence.

Flow cytometry

Adherent cells at the indicated passages were retrieved by treatment with Trypsin/EDTA and suspended in ice-cold PBS + 10% FCS at 10^6 cells/ml, and then stained for 30 min on ice with the following mAbs (see figure legends for details): biotinylated F4/80, MHC I (H2-D^b); APC-conjugated CD29, PDGFRα (APA5), Galectin-3; FITC-conjugated Sca-1 (Ly6A/E), CD45, CD49e, CD106; PE-conjugated CD31, CD44, CD73, CD90.2 and CD105; PE-Cy7-conjugated CD45; V450-conjugated CD45 and Ter119. Biotinylated antibodies were visualized with FITC-conjugated streptavidin (BD) following extensive washing. All mAbs, including isotype controls, were purchased from eBioscience except for CD90.2, CD45 and Ter119 (BD) and Galectin-3 (BioLegend) see appendix for details. Flow cytometric analysis was performed on a dual-laser BD Accuri C6 flow cytometer. Propidium Iodide (PI) fluorescence was measured in FL3, and a live cell gate was defined that excluded PI-positive cells. Additional gates were defined as positive or negative according to the isotype control fluorescence intensity.

MACS Purification

Adherent cells at passage 1 were retrieved by treatment with Trypsin/EDTA and suspended in ice-cold MACS buffer (see appendix), and then incubated with anti-mouse CD45 microbeads for 20 minutes on ice. Next, cells were washed in MACS buffer and separated using MS columns and a MiniMACS separator according to

manufacturer's instructions (Miltenyi Biotec. Inc.). The $CD45^-$ fraction was washed in MACS buffer and re-suspended in pre-warmed MSC growth medium.

Differentiation assays

To induce osteocyte differentiation, sub-confluent cells were cultured with Osteogenic Differentiation Medium (see appendix) for 14 days. The cells were then fixed with 95% methanol for 10 min and stained with Alizarin Red (Sigma-Aldrich).

To induce adipocyte differentiation, subconfluent cells were cultured for three cycles of Adipogenic Induction Medium / Adipogenic Maintenance Medium (see appendix). Each cycle consisted of feeding the subconfluent cells with the induction medium for 3 days, followed by 3 days of culture in the maintenance medium. After 14 days, the cells were fixed with 10% neutral buffered formalin for 30 min, and stained with Oil Red O (Sigma-Aldrich).

For chondrogenic differentiation, cultured cells were harvested using Trypsin/EDTA at passage 2 – 3 and 2.5×10^5 cells transferred to a 15-ml conical tube and washed with MSC medium. The tube was spun at 240g for 5 min at room temperature, and the supernatant was aspirated. The cells were resuspended in 1ml Incomplete Chondrogenic Medium (see appendix) into 1.5ml screw-cap microtubes, spun at 100g for 5 min, and the medium was aspirated. The cells were resuspended in 500µl of Complete Chondrogenic Medium (Incomplete Chondrogenic Medium supplemented with TGFβ-3 (10 ng/ml; Peprotech) and BMP-2 (100ng/ml; Peprotech)) and spun at 100g for 5 min at room temperature. The pellet was maintained with Complete or Incomplete Chondrogenic Medium changes every 3–4 days for 3 weeks. After 3 weeks, cell pellets were harvested, washed in DPBS and processed for sulphated glycosaminoglycans (S-GAG) measurement using dimethyl-methylene blue or stained with Safranin-O for sulphated proteoglycans.

Statistical analysis

All experiments were repeated at least 3 times with independently collected samples and all values are displayed as mean ± standard deviation ($\bar{X} \pm SD$) unless

stated otherwise. Statistical comparisons were analysed with the Student's t test using Excel 2010 and p-value less than 0.05 was considered statistically significant.

2.3 RESULTS

Early bone chip cultures contain cells of hematopoietic origin

For obtaining bone chip cultures, after flushing out the BM long bones, fragments were treated with Collagenase I. These pre-digested bone fragments were then put in culture and after 7 days out-growing cells were collected and passaged (Figure 2.2A). At this time they exhibited a fibroblast-like morphology typical for mMSC. It can also be seen that these cultures contain a high proportion of small, bright cells (Figure 2.2A) which are not seen in cultures at passage 3. It has been reported before that early passages of bulk MSC cultures contain hematopoietic cells [Peister et al. 2004] and that this contamination can be overcome by sequential passaging in hypoxia [Nadri et al. 2007]. Sequential passaging led to a significant reduction of the proportion of $CD45^+$ cells from 59% at Passage 1 to 4% at passage 3 (Figure 2.2B + 2.2C). In order to improve the removal of $CD45^+$ cell contamination, MACS purification with anti-mouse CD45 microbeads was performed according to the manufacturer's protocol. Passage 1 cells were taken which contained ~60% $CD45^+$ cells. Half were MACS purified and cultured until passage 2. At this time, the content of $CD45^+$ cells was 8% in MACS-purified cultures versus 18.2% in standard cultures (Figure 2.2D). Thus the removal of $CD45^+$ contaminating cells was better when using MACS beads compared to standard culture, but nevertheless still persisted. Unfortunately, the high amount of beads needed and the relatively low efficiency of removing $CD45^+$ cells meant that this method was not considered economical or efficient enough for further use.

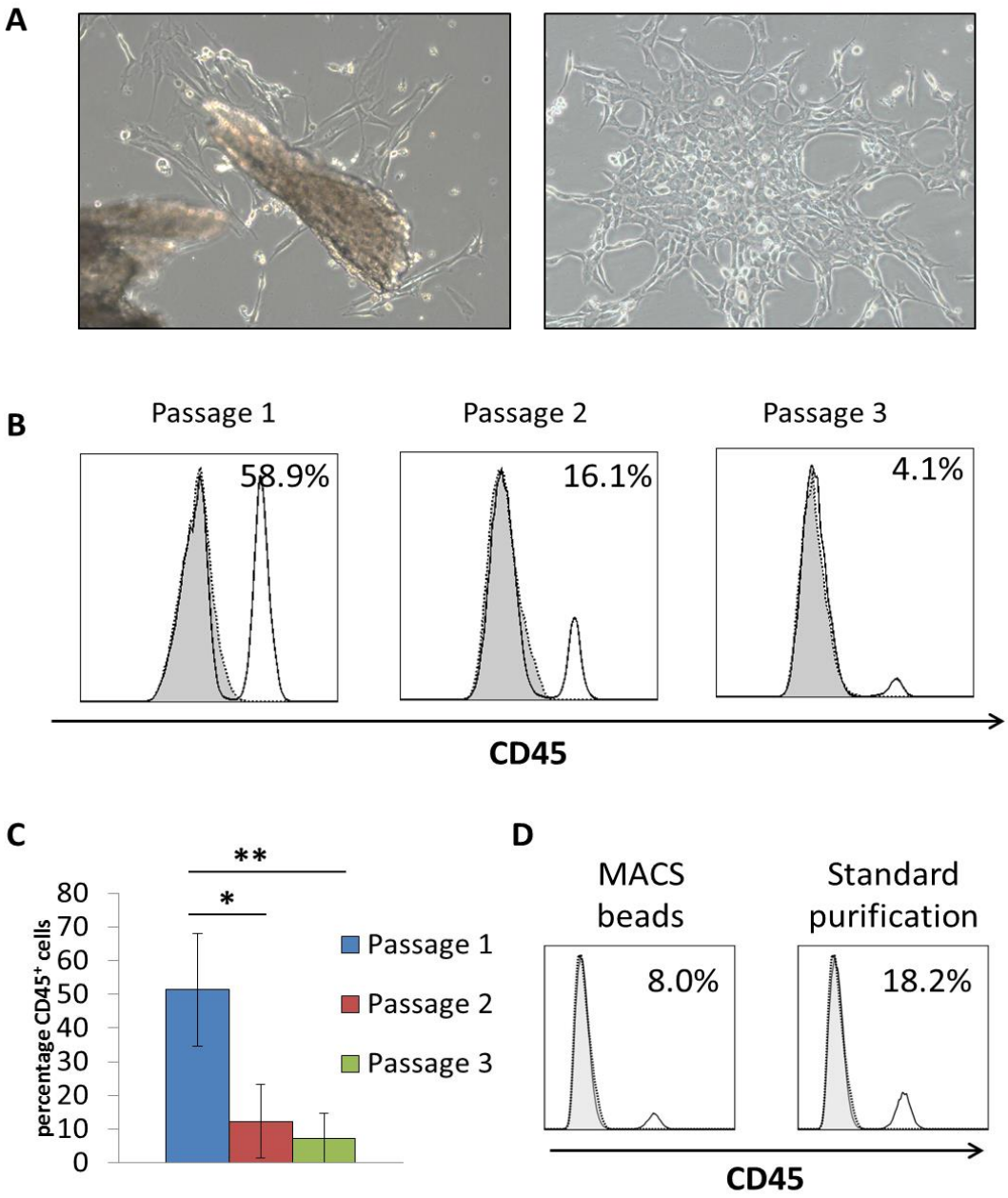

Figure 2.2 Purification of mMSCs by sequential passaging. (A) Brightfield images of cultured bone chips at passage 1. (B) Flow cytometric analysis of CD45 expression by bone chip cultures. Histograms show CD45 staining at the indicated passage numbers with the percent positive cells in each panel. (white, solid line = CD45; tinted, dashed line = isotype control). (C) Summary of CD45 distribution at different passages. Data are the means ± SD of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, Student's *t* test. (D) Flow cytometric analysis of MACS purified cells versus standard purification by sequential passaging at passage 2.

Additional haematopoietic markers were investigated for their expression in early bone chip cultures (Figure 2.3A). It can be seen that CD31, an endothelial marker, is not expressed throughout *in vitro* expansion. The reduction of $CD45^+$ by sequential passaging also resulted in a simultaneous/parallel reduction of $F4/80^+$ cells. Additional phenotypic analysis showed that $CD45^+$ cells in these early passage cultures were mostly $F4/80^+$ myeloid cells (Figure 2.3B). To determine other phenotypic characteristics, the size (FSC-A) and granularity (SSC-A) of these two cell populations were compared (Figure 2.3C). Although there was no difference in FSC-A, $CD45^-$ cells had a consistently higher SSC-A value than $CD45^+$ cells (Figure 2.3D).

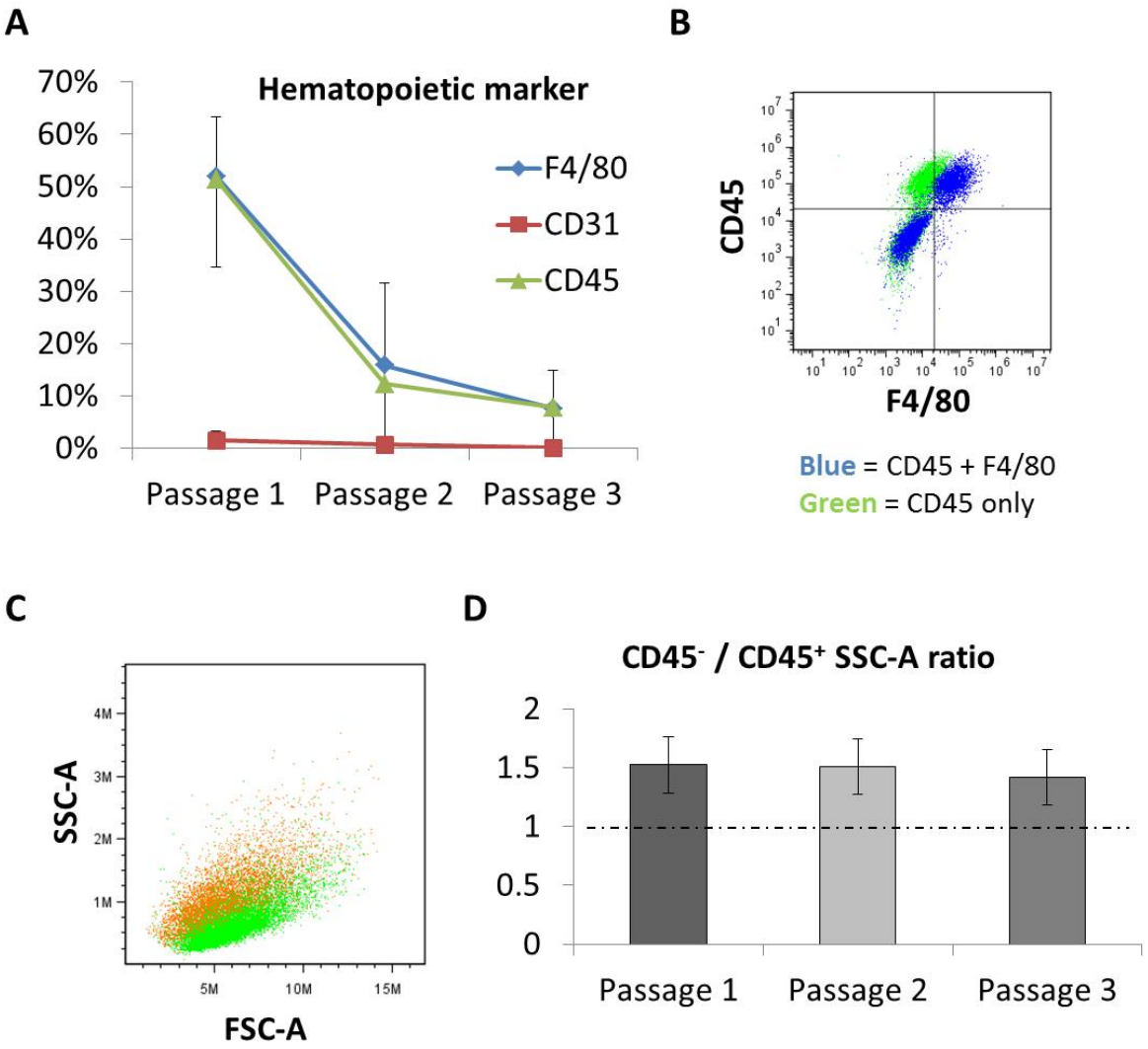

Figure 2.3 Characterisation of CD45 haematopoietic cells. (A) Flow cytometric analysis of hematopoietic marker expression during *in vitro* expansion. (B) Co-expression of CD45 and F4/80 (green, CD45 only; blue, CD45 $^{+}$ F4/80). (C) Cytogram display of FSC-A versus SSC-A for different CD45 subpopulations (orange, $CD45^{-}$; green, $CD45^{+}$). $CD45^{+}$ cells have lower SSC signals than $CD45^{-}$ cells. (D) Ratio of SSC-A between $CD45^{-}$ and $CD45^{+}$ cells. Data are the means ± SD of at least three independent experiments.

Bone chip MSCs exhibit heterogenous expression levels of human MSC markers

CD45$^-$ cells were investigated for expression of markers commonly, but not exclusively, used to characterize human MSC, namely CD73, CD90 and CD105 [Dominici et al. 2006]. The general gating strategy can be seen in Figure 2.4. In short, cells of interest were gated based on their FSC-A/SSC-A profile and doublets as well as dead cells were excluded. Gates were drawn based on fluorescence minus one (FMO) and isotype controls.

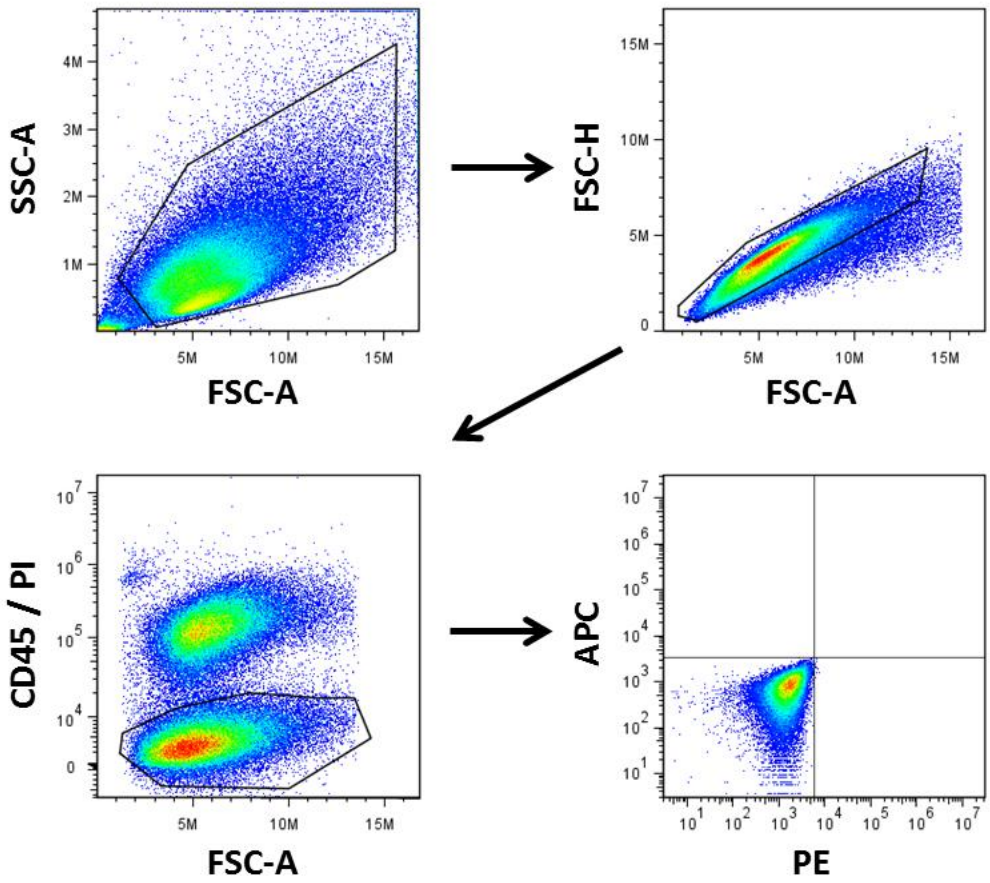

Figure 2.4 Representative gating strategy for flow cytometric analysis. Cells were gated based on their size and granularity (upper left dotplot) and doublets were excluded based on FSC-A vs FSC-H (upper right dotplot).CD45$^+$ and non-viable (PI$^+$) cells were gated out (lower left dotplot) and quadrants were drawn based on isotype controls (lower right dotplot).

Results obtained showed that there was a significant increase of $CD73^+$ cells from 6% at passage 1 to 47% at passage 3. At the same time $CD105^+$ cells increased significantly from 20% to 42%, whereas CD90 expression decreased slightly (from 92% to 85%) over 3 passages (Figure 2.5A). By simultaneous staining for CD73 and CD90, phenotypic heterogeneity was investigated further. As shown in Figure 2.5B, left panel, at passage 1, 88% cells were $CD90^+$ and $CD73^-$ with the $CD90^-$ cells being subdivided into 60% $CD73^+$ and 40% $CD73^-$ subsets. By passage 3 (Figure 2.5B, right panel) the 16% $CD90^-$ cells were $CD73^+$ and the $CD90^-$ $CD73^-$ cells had essentially disappeared. Thus there is clearly phenotypic evolution of surface marker expression in culture on $CD45^-$ cells and these are summarised in the pie graphs of Figure 2.5B. Similar phenotypic analysis was carried out on fresh compact (cBM) and flushed bone marrow (fBM). As shown in Figure 2.5C, most of gated $CD45^-$ /$Ter119^-$ cells were $CD90^-$ $CD73^-$ and of the $CD90^+$ cells, most were $CD73^-$. Taken together, this indicates that $CD90^-$ $CD73^-$ cells grow poorly under these *in vitro* culture conditions.

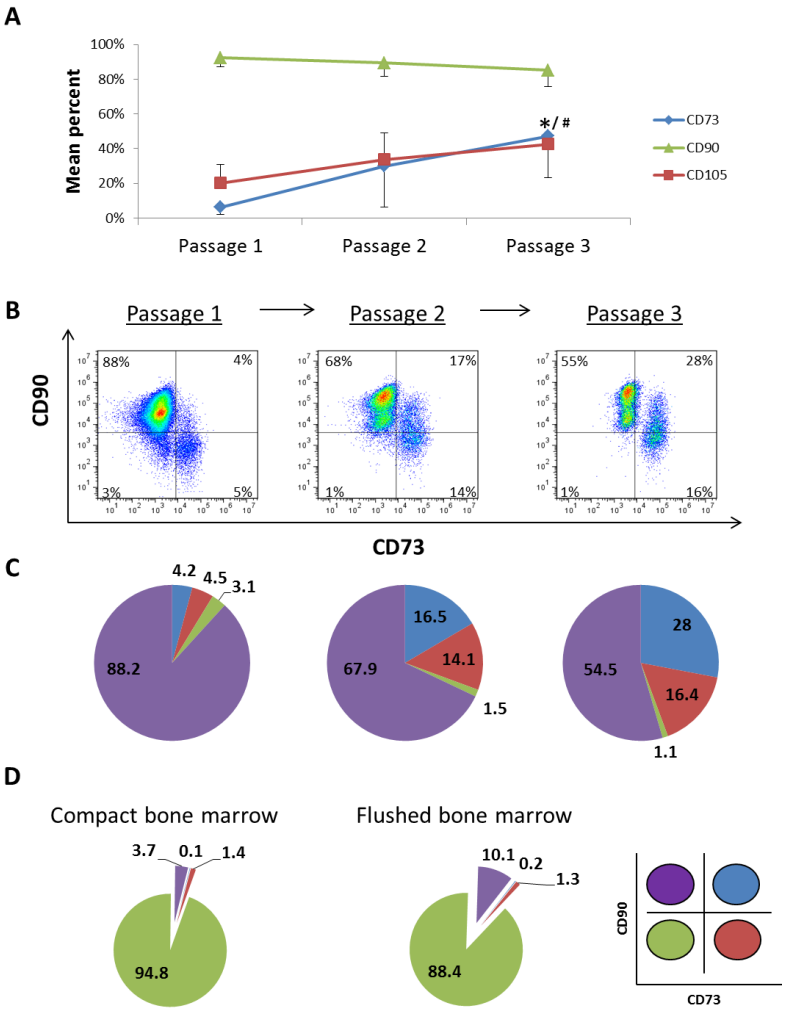

Figure 2.5 Expression of markers characteristic of human MSC. (A) Human MSC marker expression of gated CD45$^-$ cells from bone chips during *in vitro* passaging. Values represent means ± SD of at least three experiments. Significance testing was done between passage 1 and passage 3 for CD73 (#), CD90 and CD105 (*). (B) Cytogram displays of the distribution of CD73 vs CD90 over passaging is shown. Quadrants were placed based upon negative control samples in the lower left quadrant. The % positive cells in each quadrant are shown. (C) Pie chart representation of the distribution for CD73 vs CD90 *in vitro* at the passage numbers shown in B. (D) Pie chart representation of the distribution for CD73 vs CD90 by freshly-isolated compact and flushed BM. The lower right panel shows the colour codes used to define the four subpopulations expressing CD73 or CD90. Data pooled from at least three independent experiments. */# $p < 0.05$, Student's *t* test.

Expression of mouse markers by bone chip MSCs

Next, markers that have been tested on MSC (mouse and human), but are not exclusively found on them, were investigated. Galectin-3 is a marker ubiquitously expressed in adult tissues, but is mainly related to the epithelial and myeloid cells [Dumic, Dabelic, and Flögel 2006]. It has been reported that mMSC lines express Galectin-3 *in vitro* [Prado-Lòpez et al. 2014]. Results obtained indicate it is expressed in early passages but goes down simultaneously with CD45 expression and at passage 3 it is not expressed on $CD45^-$ cells (Figure 2.6A). CD49e is known to form non-covalent heterodimer with CD29, which then binds to fibronectin mediating cell migration and survival [Isaji et al. 2006]. It can be seen that CD49e is consistently expressed throughout passaging (Figure 2.6B). The same is true for CD106, also known as vascular cell adhesion molecule, which plays an important role in the retention of haematopoietic progenitor cells in BM [Ulyanova et al. 2005].

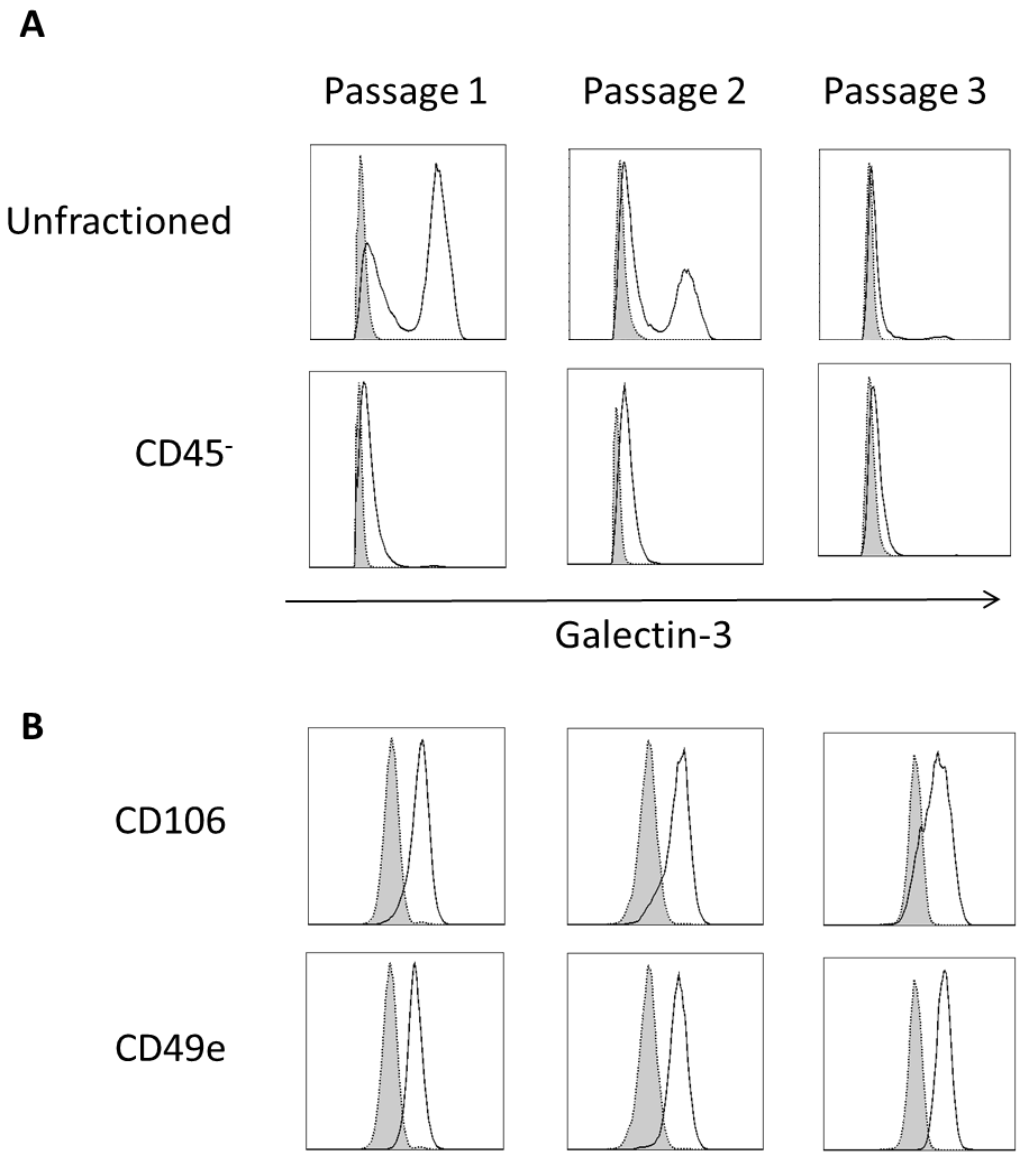

Figure 2.6 Phenotypic analyses of bulk MSC cultures. (A) Flow cytometric analysis of Galectin-3 expression by bone chip cultures in either unfractionated (upper panel) or in $CD45^-$ cells (lower panel). (B) CD106 (upper panel) and CD49e (lower panel) expression during *in vitro* expansion. Histograms show positive staining at the indicated passage numbers (white, solid line = mAB; tinted, dashed line = isotype control).

As part of the phenotypic analysis common markers expressed by mouse MSC, namely Sca-1, CD44 and CD29 were investigated[Baddoo et al. 2003]. It can be seen that Sca-1, CD44 and CD29 are consistently highly expressed throughout passaging (Figure 2.7A).

Bone chip MSCs are tri-lineage

The commonly used standard for identifying MSC is their capacity to differentiate into osteocytes, adipocytes and chondrocytes under the appropriate culture conditions [Pittenger et al. 1999]. To test whether bone chip MSC met these criteria, passaged cells were maintained in appropriate differentiation medium (see appendix) and cultured over 14 - 21 days. Adipogenic differentiation was characterized by the formation of lipid droplets stained by Oil Red O, osteogenic differentiation by Alizarin Red staining for calcium deposits and chondrogenic differentiation by Safranin-O staining for sulphated - glycosaminoglycans (S-GAG). As seen in Figure 2.7B, bone chip MSC are able to differentiate towards all three lineages proving their tri-lineage potential.

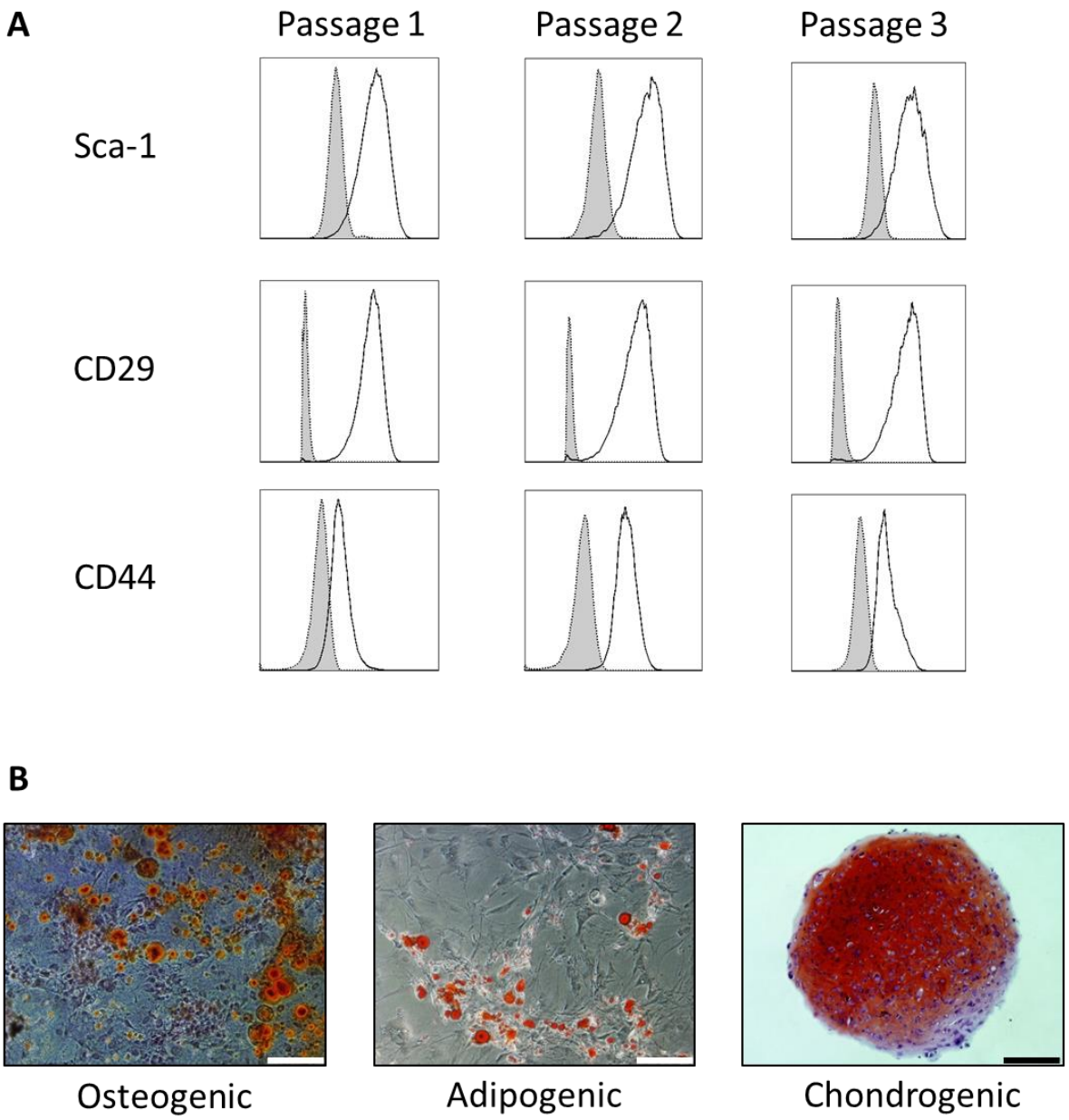

Figure 2.7 Bone chip cultures from C57Bl/6 mice contain MSCs. (A) Flow cytometric analysis of *in vitro* mouse MSC marker expression. Histograms show positive staining at the indicated passage numbers (white, solid line = mAB; tinted, dashed line = isotype control). (B) MSCs were cultured in osteogenic, adipogenic and chondrogenic differentiation media followed by Alizarin Red S, Oil Red O or Safranin O staining. Images bar = 200µm.

2.4 DISCUSSION

As major limiting factor in using early bulk MSC cultures is their contamination by a large number of haematopoietic $CD45^+$ cells (Figure 2.2B)[Jing et al. 2010; Meirelles and Nardi 2003]. This is no surprise, as MSC are thought to be part of the HSC niche *in vivo* [Charbord 2010; Frenette et al. 2013; Ehninger and Trumpp 2011]. This is also one of the reasons for their use as feeder cells in the *in vitro* expansion of HSC [Dexter, Moore, and Sheridan 1977]. A commonly used strategy to overcome haematopoietic cell contamination in MSC cultures is subsequent passaging which reduces the number of $CD45^+$ over a number of passages (Figure 2.3A)[Nadri et al. 2007]. These several passages are necessary, as $CD45^+$ cells can have different location/orientation/positions in the MSC monolayer which makes it difficult to remove them by passage 1. This is not surprising as cycling haematopoietic cells can migrate through the capillary walls into tissue via diapedesis. During this process, cells get attracted via chemokines to the tissue and are slowed down through rolling adhesion. Once they have tightly adhered, they start to transmigrate through the endothelial wall into the tissue. A similar process might happen *in vitro*, leading to different orientation of $CD45^+$ cells in regards to the $CD45^-$ monolayer. Additionally it has been shown that MSC form distinct microenvironments for HSC *in vitro*. These microenvironments include HSC that were non-adherent, cells adherent to the surface of the MSC layer, and cells that had migrated beneath the MSC feeder layer [Jing et al. 2010].

Human MSC have been intensively studied and are already used in human trials [Lv et al. 2014][Syed and Evans 2013]. CD73, CD90 and CD105, among others, are routinely used for the definition of human MSCs [Dominici et al. 2006]. Unfortunately these markers are not MSC specific and as recently reviewed [Bara et al. 2014] expression varies on passaging (Figure 2.4A). Mouse MSC also exhibit a heterogeneous staining for these markers making their use in prospective isolation problematic [Mabuchi et al. 2013]. $CD73^-/CD90^-$ cells represent a major population in fresh bone marrow, but only a minor (3%) in passage 1 expanded cells (Figure 2.4B). The low percentage of these cells in early passages suggests that they do

not survive well *in vitro*. As shown by others [Anjos-Afonso and Bonnet 2011], CD73 is barely detected on fresh cells, but is highly expressed on cultured cells. The slow increase over time of CD73 expression and reports of its up-regulation in hypoxia [Synnestvedt et al. 2002] support the assumption of marker up-regulation upon culturing. CD90 (Thy-1) was originally a prototypic T-cell marker [Reif and Allen 1964] but later it was found to be expressed on human MSC. However its expression on cells from C57Bl/6 is controversial with groups claiming its absence [Peister et al. 2004; Anjos-Afonso, Siapati, and Bonnet 2004] or its presence [Morikawa, Mabuchi, Kubota, et al. 2009; Nakamura et al. 2010] on mMSC.

Galectin-3 is part of the animal lectin family and has been reported to be expressed by hMSC [Mouldy Sioud et al. 2011]. Furthermore, the immunosuppressive capacity of hMSC is partially mediated by Galectin-3 [Hsu, Chen, and Liu 2009; M Sioud et al. 2010]. Analysis showed that Galectin-3 was not expressed by $CD45^-$ cells, but on $CD45^+$ cells (Figure 2.6A), which is consistent with reports claiming its expression on myeloid cells, in particular primary monocytes/macrophages [Liu et al. 1995]. On the other hand, it has been shown that Galectin-3 is expressed by MSC cell lines *in vitro* [Prado-Lòpez et al. 2014]. CD49e and CD106 are both expressed by bulk MSC *in vitro* (Figure 2.6B). CD49e expression has been reported for both human [Arpornmaeklong et al. 2011] and mouse MSC [Meirelles and Nardi 2003]. Its expression is somewhat expected, as CD49e associates with CD29 (integrin β_1 chain) to form the fibronectin receptor (VLA-5) [Kinashi and Springer 1994]. CD106 (VCAM-1) is a glycosylphosphatidylinositol (GPI)-linked transmembrane protein which is expressed by mouse bone marrow stromal cells and plays a key role in lymphocyte migration to the BM [Koni et al. 2001]. Deletion of CD106 leads to an increased level of circulating haematopoietic progenitor cells due to their release from bone marrow [Ulyanova et al. 2005]. The antigen markers CD29, CD44 and Sca-1, among others, are routinely used to define the presence of mouse MSCs *in vitro* [Abcam 2011; Qian, Le Blanc, and Sigvardsson 2012]. Phenotypic analysis showed that these markers were expressed as early as passage 1 and that there was stable expression until passage 3 (Figure 2.7A). Classical *in vitro* tri-lineage differentiation

assays confirm the notion that bone chip MSC cultures contain MSC (Figure 2.7B). It can be seen however, that despite homogenous expression of mouse MSC marker, not all cells differentiate towards the appropriate lineage.

Different mesenchymal progenitors share the same markers which might explain the heterogeneous differentiation capabilities *in vitro* [Muraglia, Cancedda, and Quarto 2000; Karystinou et al. 2009; Russell et al. 2010]. It has also been reported that MSC undergo phenotypic changes *in vitro* which results in a loss or gain of MSC marker expression [Jones et al. 2002; Bara et al. 2014]. The experiments presented in this Chapter were designed to see if culturing bone "chips" provide an easy way of isolating MSC relatively free of CD45 cell contamination without resorting to cell sorting. Although the culture system was simple and reliable, unfortunately, contamination by $CD45^{+}$ cells was still problematic. Heterogeneity withing the CD45-negative gated fraction was also noted. This highlights the need for a prospective isolation approach to target specific mesenchymal cell types.

Chapter 3

Characterisation of prospectively isolated Sca-1 populations

3.1 INTRODUCTION

As seen in chapter 2, there was a change in phenotype and surface marker expression in bulk cultures from bone chips during *in vitro* expansion. Due to the heterogeneous nature of bulk cultures, I wished to investigate if these changes were due to a bias of growth or a change in surface marker expression of certain cell subpopulations. It has been shown that *in vitro* conditions can alter the differentiation potential as well as cell surface marker expression [Kretlow et al. 2008; Qian, Le Blanc, and Sigvardsson 2012]. Nevertheless, the characterisation of *in vitro* expanded mMSCs using monoclonal antibodies is well accepted [Abcam 2011; R&D Systems]. However, it has been noted that cell populations tend to become more homogenous in terms of appearance with subsequent *in vitro* expansion [Bara et al. 2014].

Sca-1 was chosen as separation marker because of its wide use in stem cell isolation in mice and results from the previous chapter which showed a high Sca-1 expression during passaging (Figure 2.7A). It has been shown that Sca-1 expression is interferon-inducible and that Sca-1 is up-regulated *in vivo* after being immunologically challenged [Malek, Danis, and Codias 1989; Zhang et al. 2008].

As shown earlier, MACS purification was not able to completely remove $CD45^+$ cells (Figure 2.2D). Additionally reports have shown that flow cytometry (FCM) mediated cell sorting yields a higher purity than MACS [Li et al. 2013]. Another advantage of FCM is the simultaneous use of more than one fluorescent labelled antibody and therefore the pinpointing of distinct subpopulations. Similar to standard flow cytometry, FCM mediated cell sorting is using a single stream of liquid which is exposed to a laser beam. Single cells pass sequentially through the beam, and the fluorescent light from the labelled cells gives rise to electronic signals. By applying high frequency vibration, the stream is then broken into a series of uniform sized drops, each containing a single cell, at a fixed distance downstream of the laser. The signals from the fluorescence measurement are translated into electrostatic charges which are applied through the sheath fluid to

single drops. The individual charged drops then pass between two charged deflection plates and are deflected to appropriate containers [Bonner et al. 1972].

Additionally, the role of hypoxia during *in vitro* expansion was investigated. Traditionally, MSC are grown *in vitro* in "normoxic" conditions (21% oxygen). This level of oxygen does not reflect the physiological level in the bone marrow, thereby exposing cells *in vitro* to a higher oxygen concentration than in their native niche environment. Due to its architecture of medullary sinuses and arteries, the oxygen tension in the bone marrow has been estimated to range from 1 – 7% [S.-C. Hung et al. 2007; Spencer et al. 2014]. For several stem and progenitor populations, hypoxia is an important factor in stem cell biology, promoting an undifferentiated state [Mohyeldin, Garzón-Muvdi, and Quiñones-Hinojosa 2010; Prado-Lopez et al. 2010; Cipolleschi, Dello Sbarba, and Olivotto 1993]. Previous publications have shown that CFU-F frequency, growth and differentiation of mMSC were negatively affected by normoxic oxygen levels [Boregowda et al. 2012]. Moreover, oxygen leads to accumulation of mutation and senescence *in vitro* [Busuttil et al. 2003]. Expansion of mMSC in low-oxygen limits the accumulation of chromosomal aberrations, a common problem in mMSC cultures [Fan et al. 2011]. Due to contradicting/varying data in the literature (Table 1.1), the effect of hypoxic *in vitro* conditions on BM-derived MSCs remains uncertain.

3.2 MATERIALS & METHODS

Isolating primary chondrocytes

Femurs from 6 – 8 week old C57Bl/6 mice were dissected and articular cartilage was gently scraped from the femoral head and the condyles with a scalpel. Care was taken to not remove any subchondral bone. Cartilage chips were incubated in 2mg/ml Protease for 90 minutes at 37°C. Digestion was quenched using MSC Growth Medium (see appendix) and cartilage chips were centrifuged at 400g for 5 minutes. Medium was discarded and chips were washed with DPBS. After two

steps of washing, cartilage chips were put 2,5mg/ml Collagenase D and incubated at 37°C for 12 hours. Next cells were put in MSC Growth Medium and expanded in 37°C with 5% oxygen. Culture medium was changed every 2 - 3 days and subsequent passages were performed when cells reached 60 – 80% confluence.

Sorting

Flushed and compact BM was isolated as mentioned earlier (see chapter 2). Isolated cells were re-suspended in ice-cold FACS Buffer (see appendix) and stained for 30 min at 4°C with the following anti-mouse mAbs (see appendix for details): V450 conjugated CD45 and Ter119; FITC conjugated Sca-1. The labelled cells were washed twice, filtered through 40µm filter, re-suspended in SORT buffer (see appendix) and sorted using a BD Biosciences FACSAriaII® sorter fitted with a 70µm nozzle for fBM and an 85µm nozzle for cBM. Where appropriate, the purity of sorted cell subsets was determined by post-sorting analysis. The different subsets were gated based on their FMO and isotype controls. The fluorochrome channels for V450 and FITC were used, with Sytox blue for dead cell exclusion. Cells were sort purified with purities greater than 95% for each population and seeded on plastic or gelatine-coated plates at densities of 5 cells/mm^2.

Table 3.1 Excitation lasers and fluorochrome filters

Filter	**Fluorochrome**
Blue laser, 488nm	
530/30nm	FITC
585/30nm	PE
Red laser, 633nm	
660/20nm	APC
Violet laser, 405nm	
450/40nm	V450, Sytox Blue

Gelatine coating

10mm cell culture dishes were coated with 0.1% gelatine and incubated for 2 hours at room temperature. Gelatine was removed and the vessels were incubated for 1 hour at 37°C. Gelatine coated vessels were used right away or stored in DPBS for a maximum of 2 weeks at 4°C.

qRT-PCR

Total cellular RNA was extracted from Sca-1-defined subpopulations or articular chondrocytes using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription reactions were performed with total RNA at a concentration of at least 20ng/µl using the High Capacity cDNA Reverse Transcript Kit (Applied Biosystems) according to manufacturer's instructions. Real- time PCR (Lightcycler 480II; Roche) was performed with 2µl of the single-stranded cDNA sample with SYBR Green PCR master mix (Applied Biosystems). The PCR parameters and sequences of primers used are shown in Table 3.1. Each amplification reaction was checked to confirm the absence of nonspecific PCR product by melting curve analysis. The relative gene expression levels were calculated and presented using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen 2001]. Beta-2 microglobulin (β_2m) was used as a reference gene to normalize specific gene expression in each sample. Primers sequences from [Prado-Lòpez et al. 2014].

CFU-F assay

Sorted cells obtained from fresh fBM and cBM were plated into 10mm plates at different densities of 0.4 - 4 cells/mm^2 and incubated for 10 days in a humidified incubator at 37°C, supplied with 5% CO_2 and either 21%, 5% or 2% O_2. Subsequently, colonies were fixed with ice-cold 100% methanol, stained with 0.5% crystal violet (see appendix) and excessive crystal violet stain removed with H_2O. The numbers of colonies displaying fifty or more cells with spindled MSC morphology were scored using the Kodak Imager Station 4000MM. Colonies with

an area below 1mm^2 were scored as “small” and colonies with an area above 1mm^2 were scored as “large”.

Statistical Analysis

All experiments were repeated at least 3 times with independently collected samples and all values are displayed as mean ± standard deviation ($\bar{X}$ ± SD) unless stated otherwise. Statistical comparisons were analysed with the Student’s t-test and p-value less than 0.05 was considered statistically significant.

Table 3.2 PCR parameters and primer sequences. (A) PCR parameters for the Roche LC 480II. (B) Primer sequences

A

Program Name	Cycles	Analysis Mode
Pre-Incubation	1	None
Amplification	45 [1]	Quantification
Melting Curve	1	Melting Curves
Cooling	1	None

Temperature Targets				
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**
Pre-Incubation				
95	None	00:05:00 [3]	4.4	–
Amplification				
95	None	00:00:10	4.4	–
60	None	00:00:05 – 00:00:20 [4]	2.2 (Target °C ≥ 50°C) 1.5 (Target °C < 50°C) [7]	–
72	Single	00:00:05 – 00:00:30 [4] [5]	4.4	–
Melting Curve [8]				
95	None	00:00:05	4.4	–
65	None	00:01:00	2.2	–
97	Continuous	–	–	5 – 10 [6]
Cooling				
40	None	00:00:10	1.5	–

B

Target gene	Forward primer	Reverse primer
β_2m	5’- ATGGGAAGCCGAACATACTG -3’	5’- CAGTCTCAGTGGGGGTGAAT -3’
NANOG	5’- CACCCACCCATGCTAGTCTT -3’	5’- ACCCTCAAACTCCTGGTCCT -3’
TERT	5’- TCATGGGTGGTATGGGAGAT -3’	5’- AGGCTCCATGGTTAGGTGTG -3’
BMP2	5’- CCCCAAGACACAGTTCCCTA-3’	5’- GAGACCGCAGTCCGTCTAAG -3’
Myf5	5’-AGGAAAAGAAGCCCTGAAGC-3’	5’- GCAAAAAGAACAGGCAGAGG -3’
Col2α1	5’-GCCAAGACCTGAAACTCTGC-3’	5’- GCCATAGCTGAAGTGGAAGC -3’
Sox5	5’- CCAGGTAGATGCTGCTGACA -3’	5’- GAGCCAACATGTGCTGAAGA -3’
Sox6	5’- CTACCCTCAGCCAAGACAGC -3’	5’- TATGGATTCCCAAAGCAAGC -3’
Sox9	5’- AGCTCACCAGACCCTGAGAA -3’	5’- TCCCAGCAATCGTTACCTTC -3’
ACAN	5’- CGTGTGGAAATAGCTCTGTAGT -3’	5’- AACCTTCGCTCCAATGACTC -3’
Runx2	5’- CCCAGCCACCTTTACCTACA -3’	5’- TATGGAGTGCTGCTGGTCTG -3’
Col10α1	5’- GAGGAAGCCAGGAAAGCTG -3’	5’- GTACCGTTCAGCATAAAACATCC -3’

3.3 RESULTS

Expression of mouse MSC markers

Despite the heterogeneous expression of human MSC markers (CD73 and CD105), common mouse MSC markers, namely Sca-1, CD44 and CD29 [Baddoo et al. 2003], were highly and homogenously expressed by bulk cultures (Figure 2.6). I focussed particularly on the expression of so-called Stem cell antigen-1 (Sca-1). The Sca-1 distribution on gated $CD45^-/Ter119^-$ in freshly harvested bone marrow preparations was determined by flow cytometry. As shown in Figure 3.1A, 4.6% of cells processed from cBM and on gated $CD45^-/Ter119^-$ cells were Sca-1^+. Figure 3.1B summarises a series of 4 experiments where Sca-1 expression by cells isolated from fBM and cBM was compared. As shown, cells isolated from cBM are significantly enriched for Sca-1^+ cells. Next, the evolution of Sca-1, CD44 and CD29 expression on $CD45^-$ cells during culture was investigated (Figure 3.1C). Sca-1 expression increased from 4% in fresh cBM to 90% at Passage 3. CD44 expression increased from 45% in fresh cBM to 97% at passage 3. CD29 expression was consistently elevated at around 95% (Figure 3.1C). Thus, after 3 passages, cells are fairly homogeneous for Sca-1, CD44 and CD29 (Figure 3.1C) yet are distinctly heterogeneous for CD73 and CD105 (Figure 2.4A). It also became evident that Sca-1^- sorted cells are a mix of phenotypic heterogeneous cells as seen in brightfield images of passage 1 cultures which showed fibroblastic-like cells as well as small, bright cells (Figure 3.1D). Sca-1^+ sorted cells on the other hand seem to be more homogenous even in early passages.

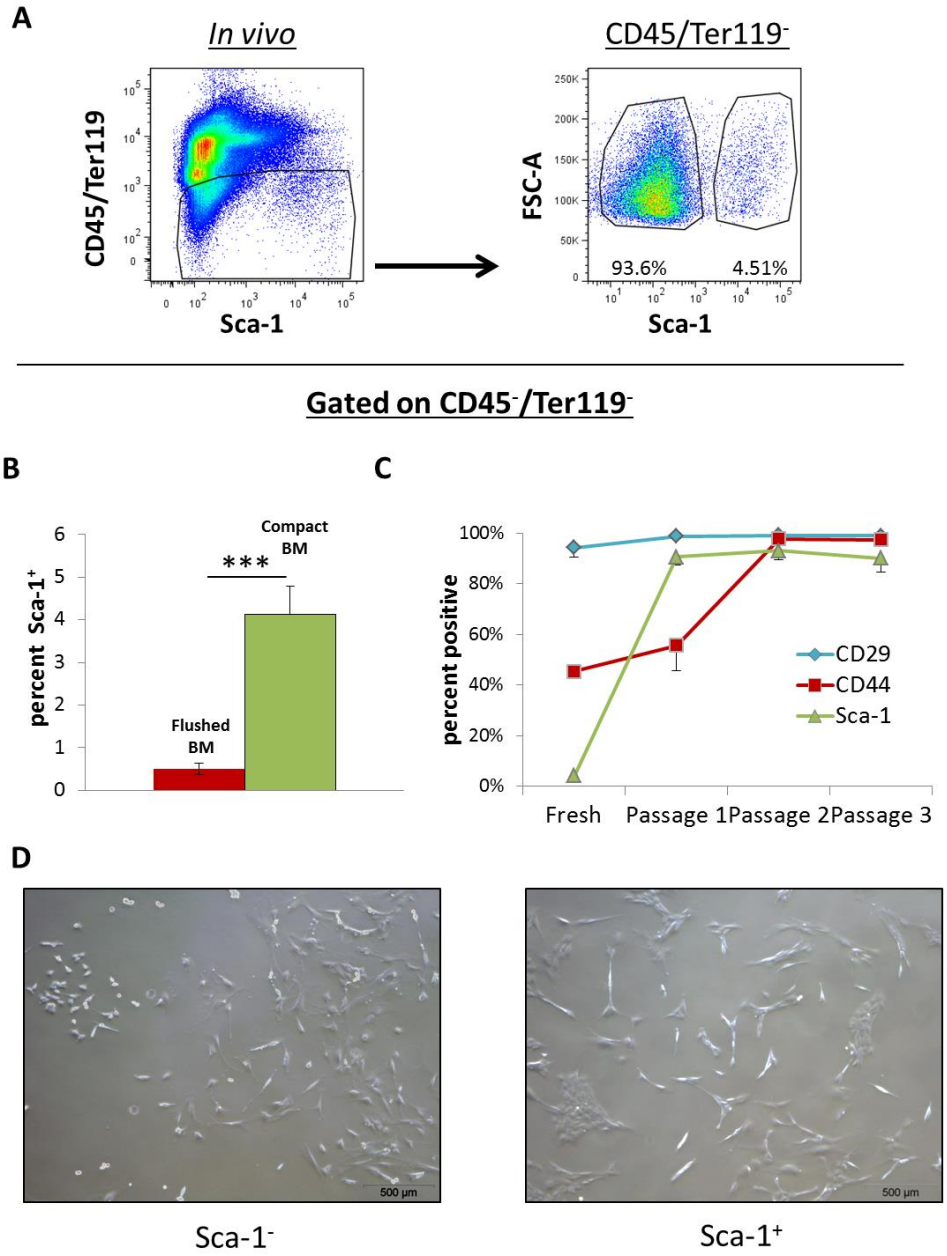

Figure 3.1 Evolution of mouse MSC markers expression. (A) The left cytogram display is of Sca-1 versus CD45$^-$/Ter119$^-$ expression by cells in freshly harvested cBM preparations. The right panel shows the FSC versus Sca-1 cytogram of cells gated in the left panel. (B) Histograms show a comparison of Sca-1 distribution in fBM versus cBM. (C) Evolution of Sca-1, CD44 and CD29 expression on cBM derived CD45$^-$ cells *in vitro*. (D) Brightfield images of passage 1 cultures form sorted cells. Data are the means ± SD of at least three independent experiments. ***p < 0.001, Student's *t* test.

Phenotypic evolution in Sca-1 sorted subpopulations

Given the increase of both CD44 and Sca-1 expression by cultured cells, further investigation of this phenotypic evolution was done by flow cytometry. For this purpose $CD45^-/Ter119^-$ cells were further separated into $Sca\text{-}1^-$ and $Sca\text{-}1^+$ cells. $Sca\text{-}1^+$ cells initially showed a quite broad staining distribution for Sca-1 in both fBM (Figure 3.2, upper panels) and cBM (Figure 3.2, middle panels). After culturing for 2 passages, the expression of Sca-1 became more uniform in both sorted subpopulations (Figure 3.2, lower panels). $Sca\text{-}1^-$ cells on the other hand were initially clearly negative for Sca-1, but after 2 passages the expression was heterogeneous (Figure 3.2, lower left panel), including about 10% of $Sca\text{-}1^-$ cells. The simplest interpretation of these results is that many $Sca\text{-}1^-$ sorted cells became $Sca\text{-}1^+$ upon culture. For both flushed and compact BM, CD44 expression was initially negative on $Sca\text{-}1^+$ sorted cells but weakly expressed by $Sca\text{-}1^-$ sorted cells (Figure 3.2, middle column). Upon culture there was an increase in CD44 expression by both sorted subpopulations. CD29 was expressed on both $Sca\text{-}1^+$ and $Sca\text{-}1^-$ sorted cells, with a broader staining on the freshly-isolated $Sca\text{-}1^+$ cells (Figure 3.2, upper right panels). After culturing, CD29 was uniformly highly expressed by both sorted subpopulations (Figure 3.2, lower panels).

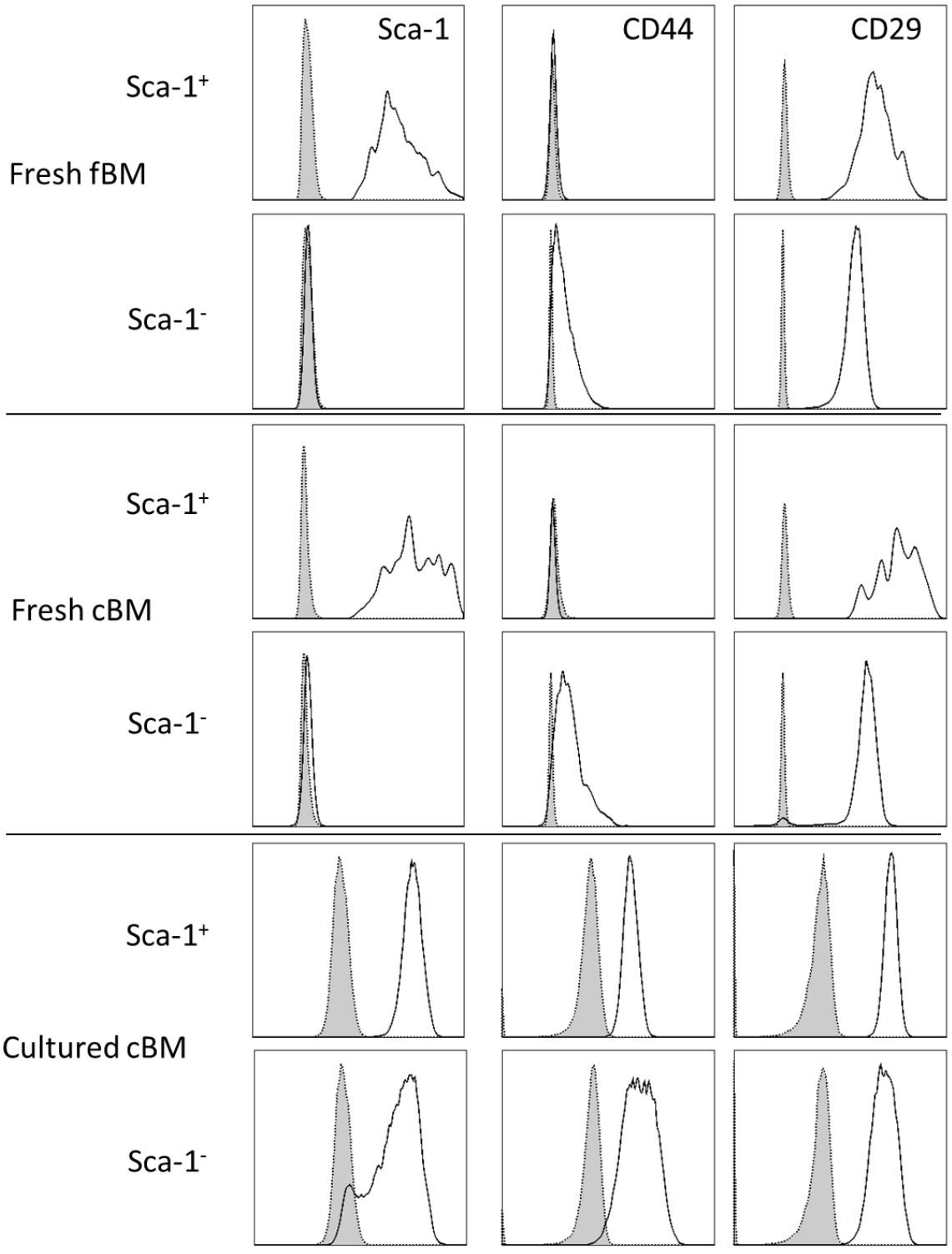

Figure 3.2 *In vitro* cultures up-regulate mouse MSC marker. Phenotypic evolution of mouse MSC markers Sca-1 (left column), CD44 (middle column) and CD29 (right column) in Sca-1 sorted subpopulations in the BM preparations indicated on the left. Cultured cBM (lower panels) has been expanded *in vitro* for 2 passages. White, solid line = mAB; tinted, dashed line = isotype control.

CFU-F frequencies among Sca-1 sorted subpopulations

When sorting and expanding Sca-1 subpopulations, it became evident that these cells differ in their ability to form initial colonies. To measure these differences, CFU-F frequencies among Sca-1 sorted subpopulations of flushed versus compact BM was analysed. As shown in Figure 3.3A, compared with unsorted ("bulk") $CD45^-$ /$Ter119^-$ cells, the CFU-F frequency was always higher among $Sca\text{-}1^+$ versus $Sca\text{-}1^-$ sorted cells in both BM preparations. Subpopulations isolated from compact BM always had a higher CFU-F frequency compared with their flushed BM counterparts (Figure 3.3A). Recently, hypoxia has been used to improve *in vitro* growth of MSC [Prado-Lòpez et al. 2014]. Therefore, cells were cultured in normoxic (21% oxygen) and hypoxic (5% oxygen) conditions and CFU-F frequencies were compared. For fBM, hypoxia improved CFU-F frequencies in bulk and $Sca\text{-}1^-$ sorted subpopulations (Figure 3.3B). Given that the overall CFU-F frequencies were higher among cBM, two hypoxic concentrations (2% and 5% oxygen) were compared on cells from cBM. As shown in Figure 3.3C there was a progressive increase in CFU-F correlated with the degree of hypoxia for the bulk and $Sca\text{-}1^-$ cells, but a difference between 2% and 5% oxygen was not seen for $Sca\text{-}1^+$ cells. To further improve the CFU-F frequencies, plastic dishes were coated with 0.1% gelatine (see appendix) and used for expansion of sorted subpopulations in 2% oxygen. As seen in Figure 3.3C gelatine had no effect on the CFU-F frequencies of $Sca\text{-}1^+$ cells but benefits the $Sca\text{-}1^-$ CFU-F frequencies.

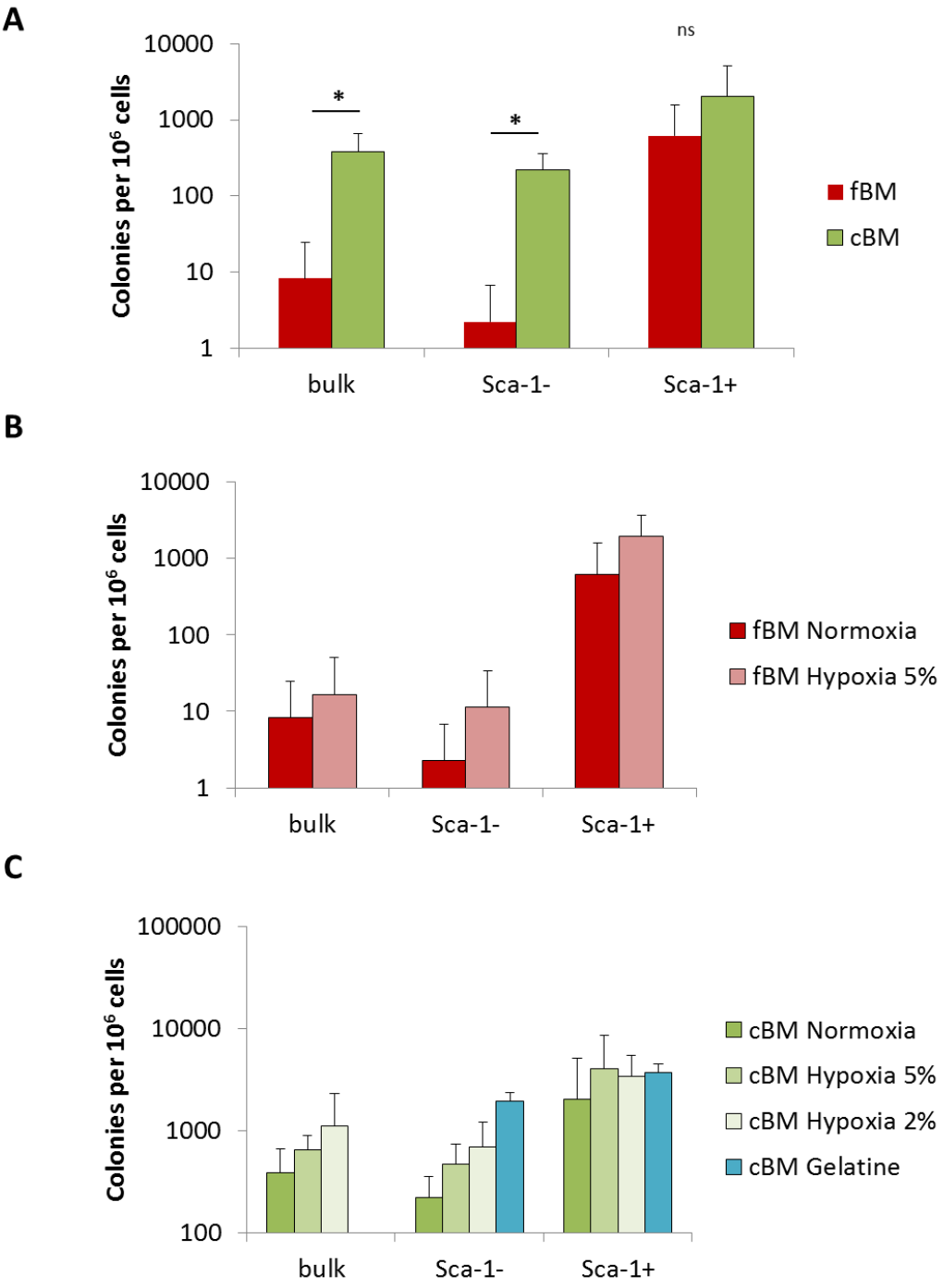

Figure 3.3 CFU-F frequencies among Sca-1 sorted subpopulations. (A) CFU-F frequencies among Sca-1 sorted subpopulations of fBM versus cBM. (B) CFU-F frequencies in subpopulations from fBM cultured in 21% O_2 (Normoxia) or 5% O_2 (Hypoxia). (C) CFU-F frequencies in subpopulations from cBM cultured in 21%, 5% or 2% O_2 with or without 0.1% gelatine. Data are the means ± SD of at least three independent experiments. *$p < 0.05$, Student's *t* test.

Increased colony size in Sca-1$^+$ cells

When counting CFU-F, it became evident that there were size differences between colonies of Sca-1$^-$ and Sca-1$^+$ sorted cells. Whereas "bulk" CD45$^-$/Ter119$^-$ cells showed heterogeneity in CFU-F colony size, there was a dramatic difference between Sca-1$^-$ (small colonies) and Sca-1$^+$ (large colonies)(Figure 3.4A). To quantify this difference in colony size, individual colonies were measured using the Kodak Imager 4000 software (Carestream). Colonies were marked using the manually ROI setting and the area was measured in mm^2 (Figure 3.4B). Raw data of the size distribution of >100 colonies from 4 independent experiments are visualized in histograms (Figure 3.4C). It can be seen that the size of Sca-1$^-$ colonies are concentrated on the left of the histogram, while Sca-1$^+$ colonies cover a wide area of different sizes. Therefore, to simplify the analysis colonies were grouped in "small" (< 1mm^2) and "large" (> 1mm^2) colonies.

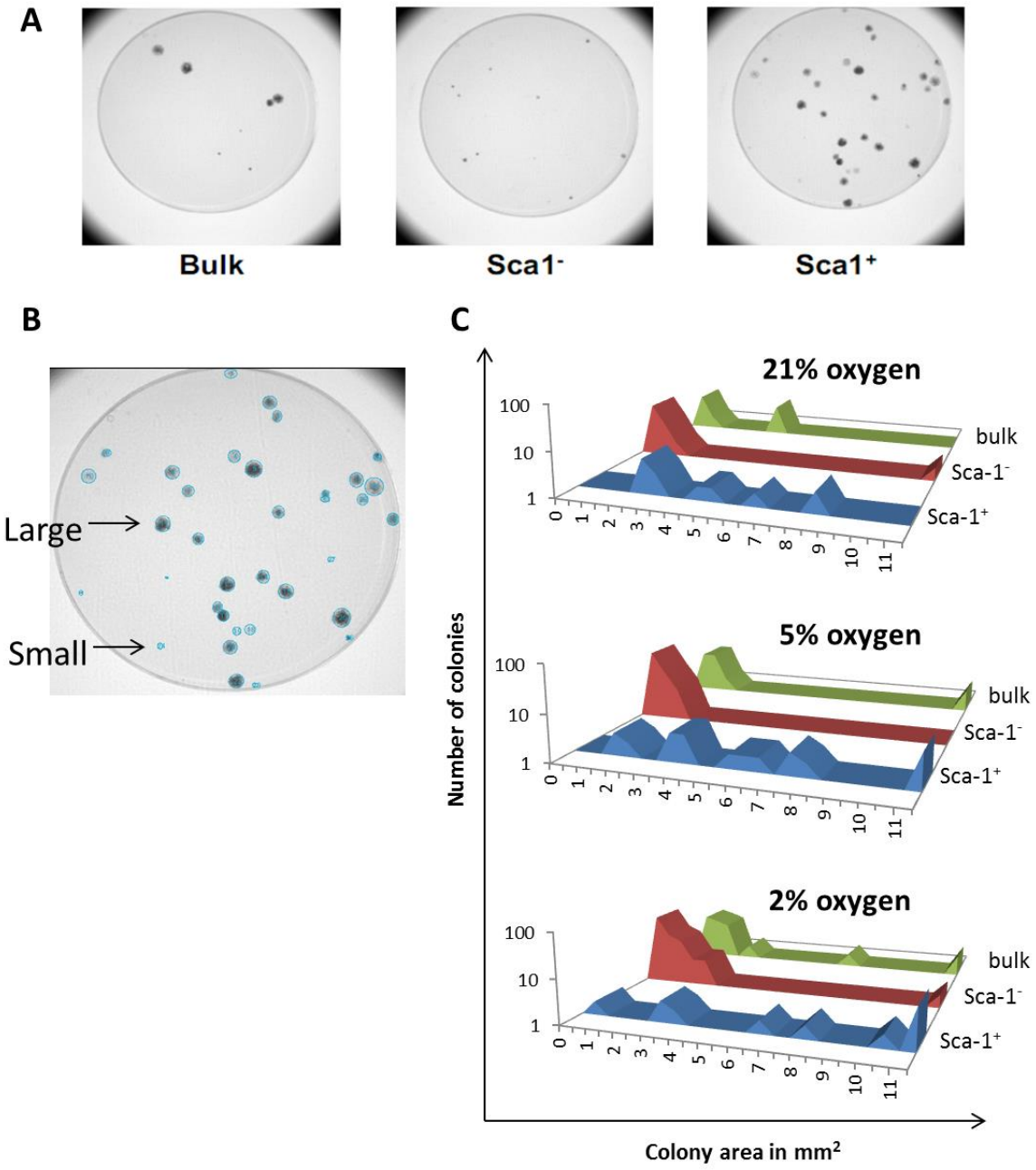

Figure 3.4 Increased colony sizes in Sca-1$^+$ sorted cells. (A) Shown are images of colonies from the indicated sorted populations of sorted cBM. "Bulk" refers to unsorted cells. (B) Manually selected ROI gates for area measurement. (C) Raw colony size for the different sorted subpopulation in 21%, 5% or 2% oxygen. Data are pooled of >100 colonies from at least three independent experiments.

The "bulk" CD45$^-$/Ter119$^-$ population consists of ~ 50% colonies which are smaller than 1mm^2, as well as ~ 50% large colonies which an area above 1mm^2 (Figure 3.5A). This pattern of size distribution is true for normoxic (21% O_2) and hypoxic (2% and 5% O_2) oxygen conditions. The majority of colonies in the Sca-1$^-$ population are small in size (~76% of total colonies) with only a few large colonies. This is almost completely reversed in Sca-1$^+$ cells, with a minority of small colonies (less than 10%) and over 90% large colonies. In both populations it can be seen that oxygen conditions have no significant effects on numbers of small or large colonies. While scoring the size of colonies it became clear that oxygen conditions did not affect the number of colonies but rather their size. As can be seen in Figure 3.5B, the mean colony size of Sca-1$^+$ cells is significantly increased with the degree of hypoxia. Sca-1$^-$ cells on the other hand seem to be little or not affected by oxygen levels. Bulk cells are a mixture of Sca-1$^-$ and Sca-1$^+$ cells and don't seem to be affected by oxygen levels.

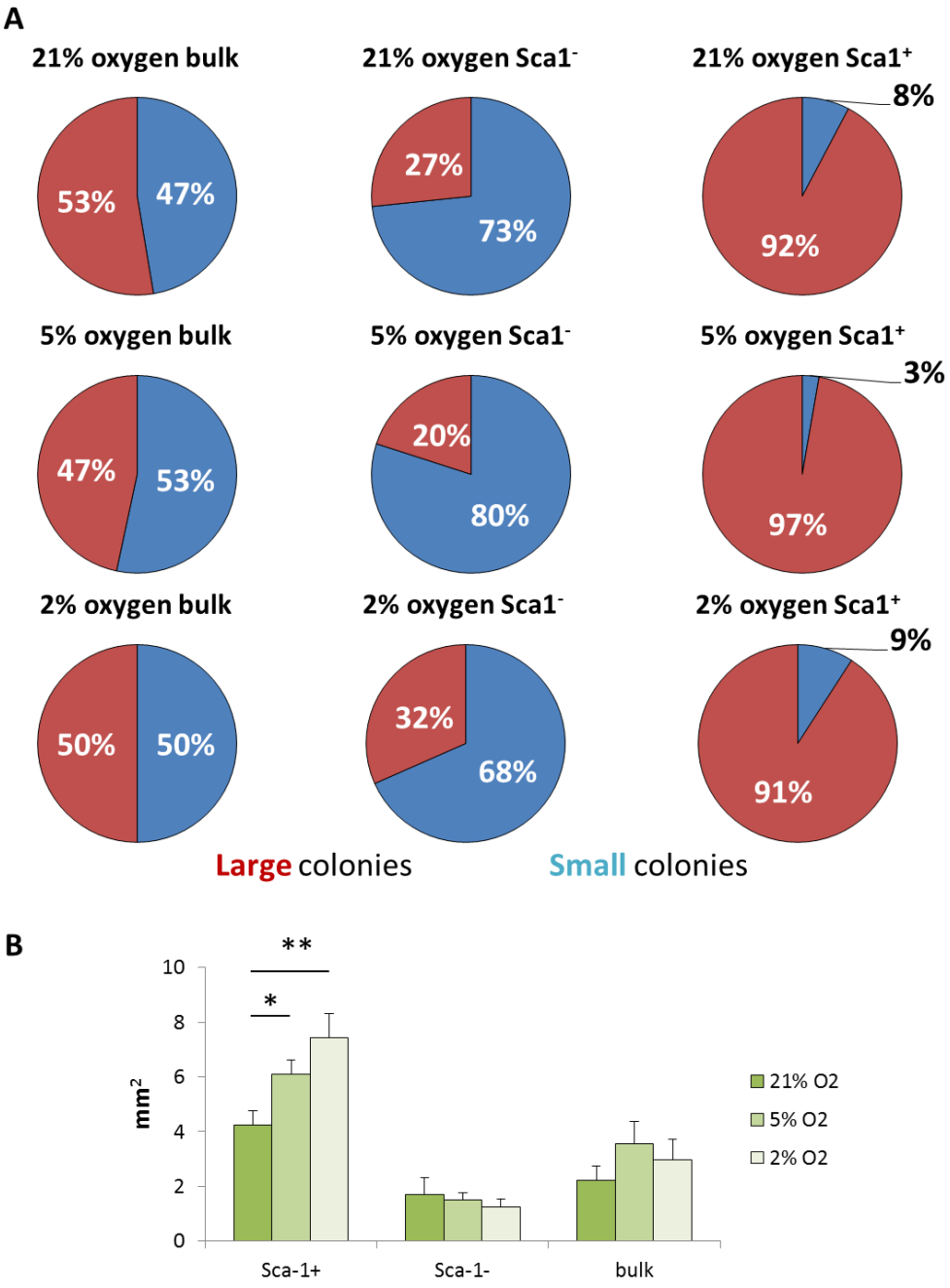

Figure 3.5 Hypoxia increases colony sizes of Sca-1$^+$ sorted cells. (A) Ratio of large versus small colonies in 21%, 5% or 2% O_2. (B) Summarises the colony size for the different sorted subpopulation in 21%, 5% or 2% O_2. The numbers of colonies displaying fifty or more cells with spindled MSC morphology were scored using the Kodak Imager Station 4000MM. Colonies with an area below 1mm^2 were scored as "small" and colonies with an area above 1mm^2 were scored as "large". Data are the means ± SEM of >100 colonies from at least three independent experiments. *$p < 0.05$, **$p < 0.01$, Student's *t* test.

As seen in Figure 3.6A, freshly isolated Sca-1^{+} cells are significantly larger than their Sca-1^{-} counterparts and also have a wider size distribution as shown by FSC-A histograms. During *in vitro* expansion, these differences lose significance and the size of Sca-1^{+} sorted cells is more evenly distributed (Figure 3.6B). Morphologically, Sca-1^{+} and Sca-1^{-} sorted cells showed differences in phenotype, with Sca-1^{-} cells exhibiting a small, cobblestone-like phenotype versus a large, fibroblast-like phenotype for Sca-1^{+} cells (Figure 3.6C).

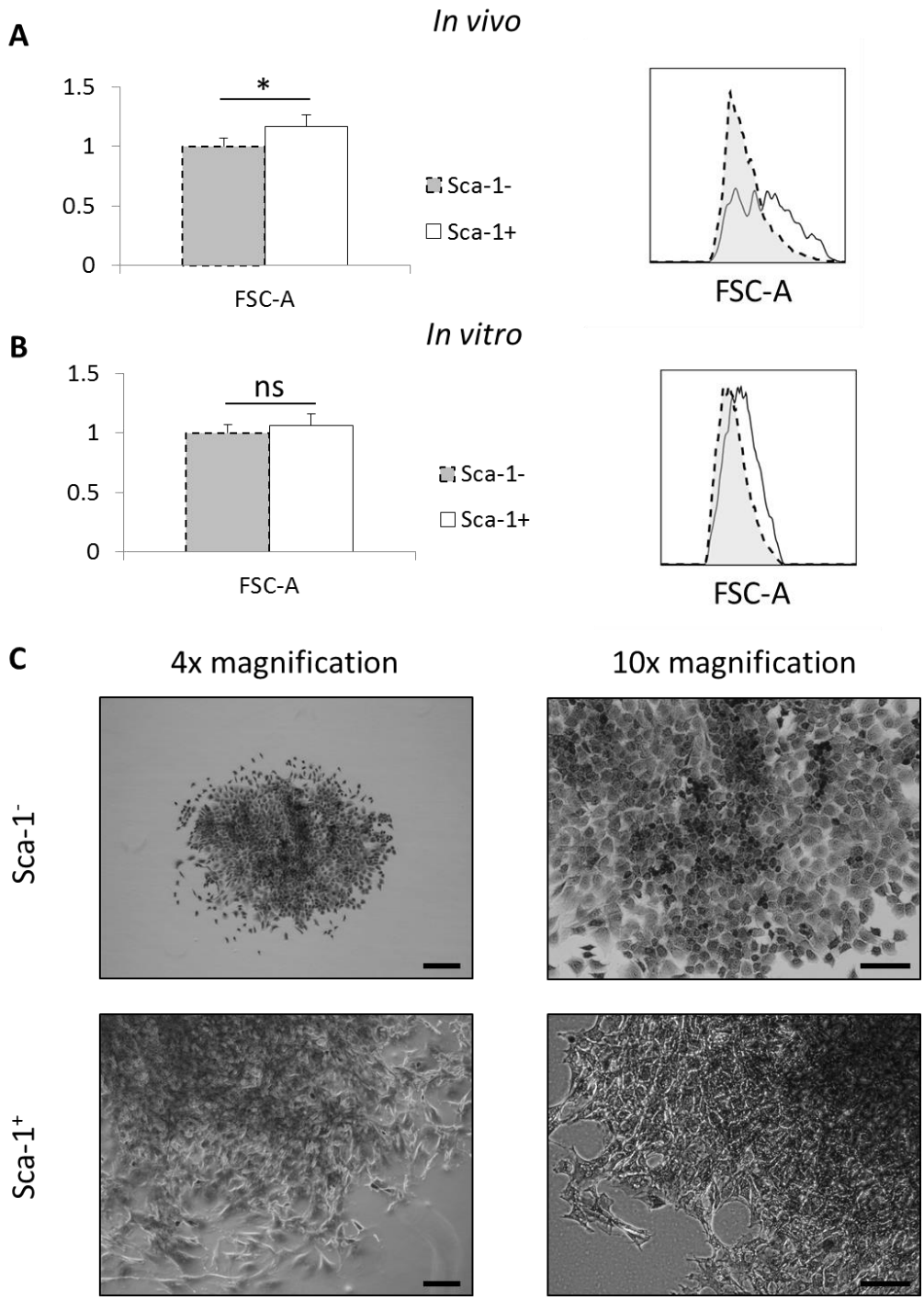

Figure 3.6 Size and colony appearance of Sca-1 sorted subpopulations. (A) Size differences in freshly isolated Sca-1 subpopulations (white, solid line = Sca-1$^+$; tinted, dashed line = Sca-1$^-$). (B) Size differences in cultured Sca-1 subpopulations. (C) Brightfield images of Sca-1$^-$ and Sca-1$^+$ expanded colonies after 10d of culture. First column = 4x magnification (bar = 200µm) and second column 10x magnification (bar = 100µm). Data are the means ± SD of at least three independent experiments. *p < 0.05, Student's *t* test.

Sorted subpopulations differ in their differentiation potency

To see if these differences in CFU-F frequencies and colony sizes affect the potential of tri-lineage differentiation, the differentiation capacity of Sca-1^+ and Sca-1^- sorted cells to the osteo-, adipo- and chondrocyte lineages was investigated. After two rounds of culture expansion, cells were transferred to the corresponding differentiation conditions. After 14 days, cells were analysed for osteo- and adipocyte differentiation; chondrocyte differentiation was measured after 21 days. As shown in Figure 3.7A, both Sca-1^+ and Sca-1^- sorted cells differentiated along the osteo- and adipocyte linages, however Sca-1^- cells showed increased differentiation to chondrocytes as demonstrated by Safranin-O staining for proteoglycans (Figure 3.7A, lower panel). Quantification for sulphated glycosaminoglycans (S-GAG) confirmed the difference in chondrocyte differentiation was significant with a ~3-fold greater S-GAG content in Sca-1^- cells (Figure 3.7B). This result suggested that cultured Sca-1^- cells had a superior chondrocyte differentiation capacity.

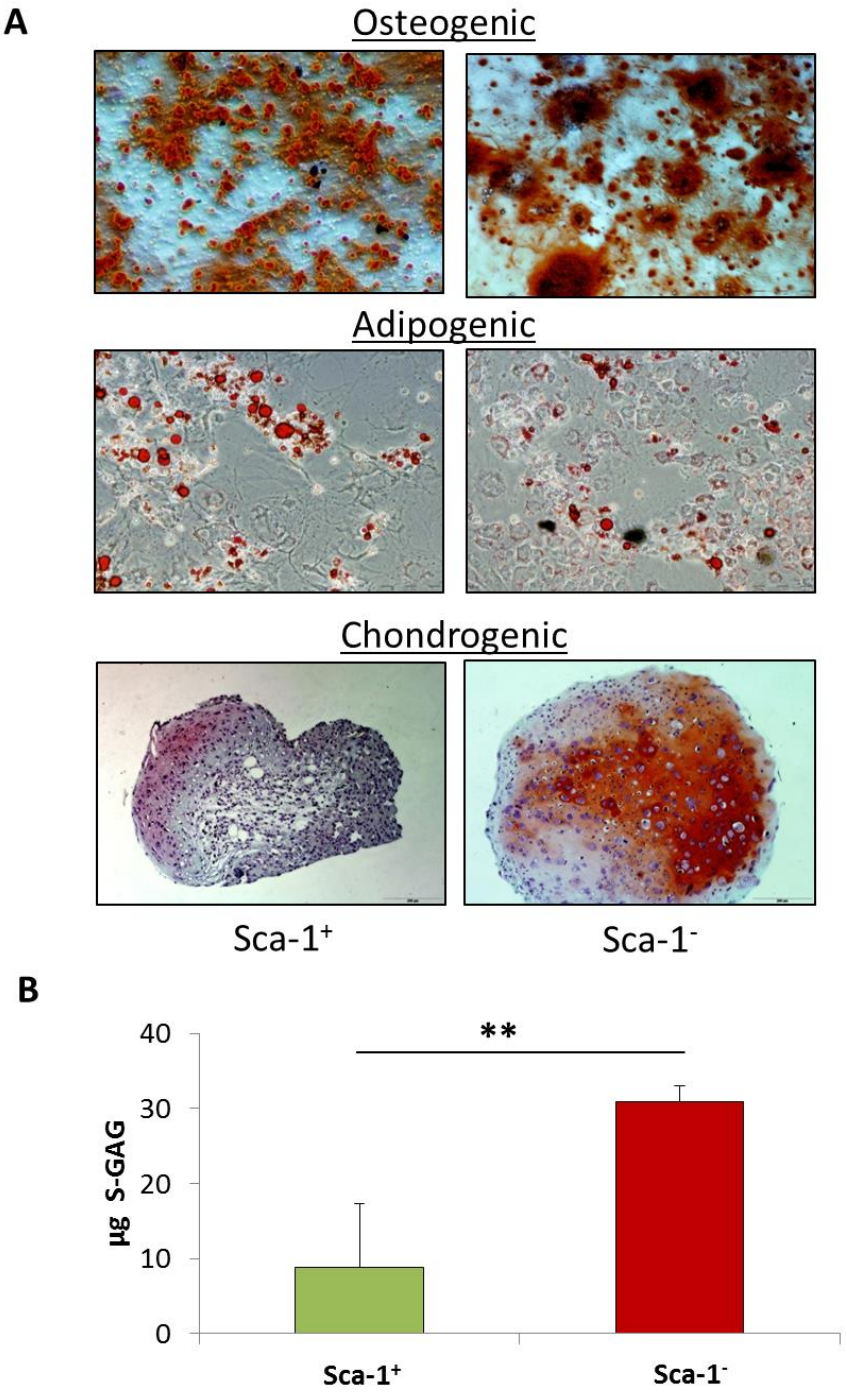

Figure 3.7 Subpopulations differ in their tri-lineage differentiation potential. (A) MSCs were cultured in osteogenic (upper A), adipogenic (middle A) and chondrogenic (lower A) differentiation media followed by Alizarin Red S (upper A), Oil Red O (middle A) or Safranin O staining (lower A). Osteogenic and adipogenic images bar = 100µm; and chondrogenic images bar = 200µm. (B) Chondrogenic pellet cultures from the indicated subpopulations assayed for S-GAG content.

To address whether freshly-sorted cells were likewise chondrocyte "primed", the expression of transcripts characteristic of stem cell (NANOG, TERT), osteo- (BMP2), myogenic/adipo- (Myf5) and chondrocytes (Col2a1) in both subpopulations were analysed by qRT-PCR with β_2m as internal reference gene. In freshly-isolated BM cells, expression of transcripts for NANOG, TERT, BMP2 and Myf5 were considerably higher (300-fold) among Sca-1$^+$ sorted cells (Figure 3.8A). In contrast, expression of Col2a1, a chondrocyte specific gene, was considerably higher (50-fold) among Sca-1$^-$ sorted cells. Upon culture in hypoxia, the differences in transcript levels became less distinct for NANOG, TERT, BMP2 and Myf5 and the difference in Col2a1 expression became higher (100-fold) (Figure 3.8B). To investigate further the chondrogenic capacity of Sca-1$^-$ sorted cells, analysis for transcripts of genes specific for chondrocyte differentiation was carried out. In general, freshly isolated Sca-1$^-$ cells showed a higher expression of transcripts for chondrocyte differentiation (Figure 3.8C). The chondrogenic genes for Col2a1, ACAN, Sox9, Col10a1 and Sox6 were at least 5-fold higher in Sca-1$^-$ compared to Sca-1$^+$ cells. The differences in Runx2 and Sox5 expression levels were less distinctive. To compare levels of chondrocyte-specific genes in sorted cells with mature chondrocytes, primary chondrocytes were isolated from the knees of C57Bl/6 mice and were cultured in 5% hypoxia. Then their level of transcripts were analysed and compared with Sca-1$^-$ sorted cells. Primary articular chondrocytes (AC) showed a higher expression for all chondrogenic transcripts compared to Sca-1$^-$ sorted cells, except Col10a. (Figure 3.8D). Expression of Sox5, Sox6 and ACAN were ~4-fold higher in ACs compared to Sca-1$^-$ sorted cells. ACs also had ~8-fold higher levels of transcripts for Sox9, Col2a and Runx2, and 11-fold less expression of transcripts for Col10a.

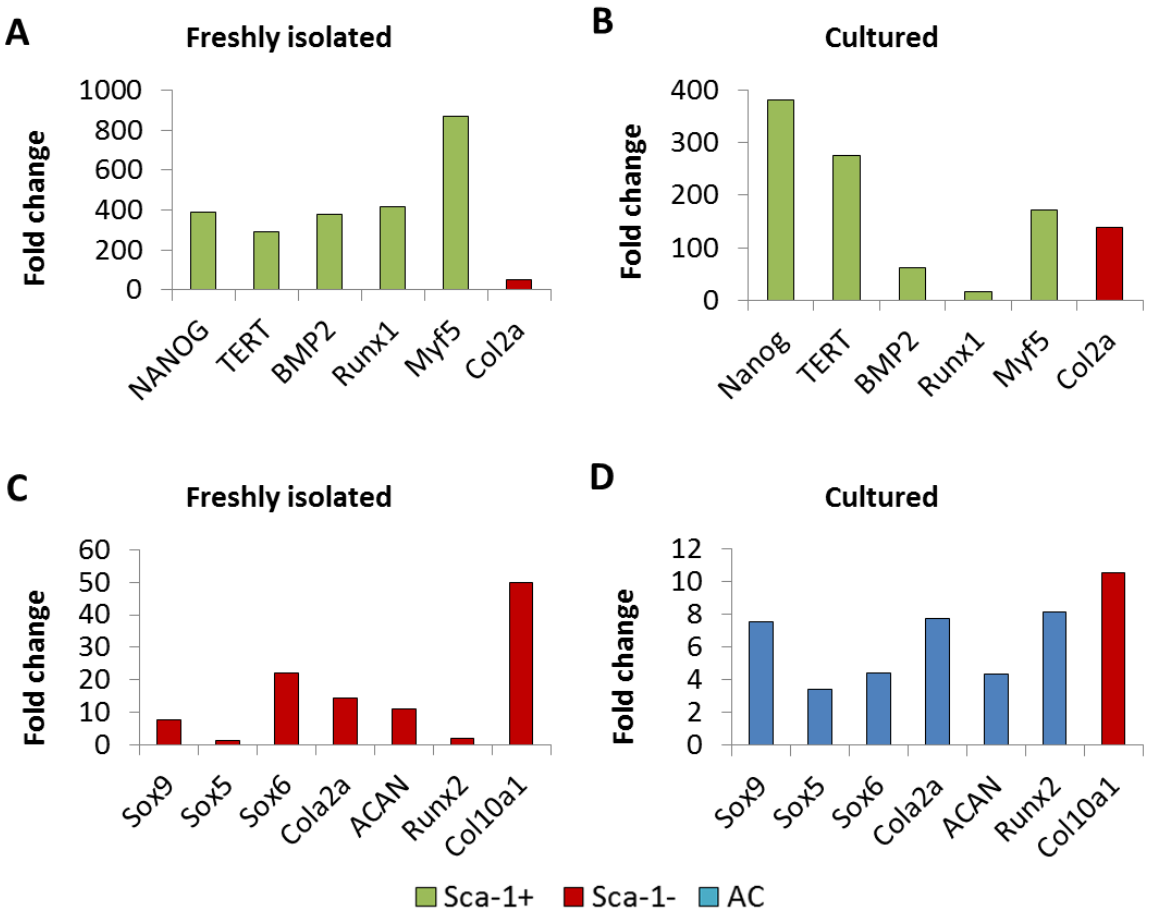

Figure 3.8 Sca-1$^-$ cells display a more chondrogenic transcriptomic profile. (A) Fold change of relative expression of stem and lineage specific gene transcripts in freshly isolated and (B) cultured Sca-1 sorted subpopulations. Fold change is shown of displayed population against Sca-1$^+$ or Sca-1$^-$ sorted cells. (C) Fold change of relative expression of transcripts associated with chondrogenesis in freshly isolated Sca-1$^-$ sorted cells compared against Sca-1$^+$ sorted cells and (D) cultured articular chondrocytes compared with Sca-1$^-$ cells.

3.4 DISCUSION

Analysis showed that expression of both CD44 and Sca-1 antigens increased upon culturing. This is somewhat expected given that expression of both antigens can be regulated upon cell signalling [Haegel and Ceredig 1991][Malek, Danis, and Codias 1989] and CD44 has been known for a long time to be an activation marker on mouse and human T-cells [Haegel and Ceredig 1991]. CD44 is used frequently as a marker to define mouse MSC [Abcam 2011][Qian, Le Blanc, and Sigvardsson 2012]. Recent research has however shown the CD44 might not be a useful marker for the isolation of MSC as i) MSC could be isolated from sorted $CD44^-$ cells and ii) CD44 was acquired *in vitro* [Qian, Le Blanc, and Sigvardsson 2012]. Herein it was shown that gated $CD45^-/Sca\text{-}1^+$ fresh flushed and compact BM cells were also $CD44^-$ and again CD44 was acquired *in vitro*. Additional experiments focused on Sca-1 expression showing that Sca-1 was acquired by sorted $Sca\text{-}1^-$ cells (Figure 3.2) [Steenhuis, Pettway, and Ignelzi 2008][Anjos-Afonso, Siapati, and Bonnet 2004]. Given that the possible contamination of sorted $Sca\text{-}1^-$ cells by $Sca\text{-}1^+$ was at most 2%, and given that both subpopulations grew at a similar rate, the most likely interpretation is that Sca-1 was acquired by cultured $Sca\text{-}1^-$ cells rather than outgrowth of $Sca\text{-}1^+$ contaminants.

Two major isolation methods are used today to isolate mMSC from bone marrow. The traditional method consists of flushing out the red bone marrow (fBM) and the use of mechanical sheer force to obtain single cell suspensions. This may leave behind potential stem/progenitor cells residing at the endosteum. By collagenase digestion of bone fragments, endosteum residing cells (cBM) can be harvested and purified using FCM. A general conclusion from these experiments was that the CFU-F frequency was considerably higher among compact BM cells (Figure 3.3). There is general agreement that in the BM, MSC and the HSC their support can be found at different anatomical locations that differ also in oxygen availability (reviewed in [Mohyeldin, Garzón-Muvdi, and Quiñones-Hinojosa 2010]). Many such MSC/HSC “niches” are found close to cortical bone in a relatively hypoxic environment and therefore, for maximum recovery of CFU-F, crushing bones

followed by collagenase digestion is clearly advantageous. Surprisingly, when gated $CD45^-/Ter119^-$ BM cells from either flushed or compact BM were sorted based upon Sca-1 expression, CFU-F colony size varied significantly between subpopulations (Figure 3.4B). Although differences in CFU-F colony size have been previously reported [Siclari et al. 2013], this is the first study indicating differences in colony size among freshly isolated, sorted mMSC subpopulations. Additionally, hypoxia had an effect both on CFU-F frequency (Figure 3.3) and average colony size (Figure 3.4) which proved significant for the colony size of $Sca\text{-}1^+$ sorted cells (Figure 3.4). This might mean that the majority of $Sca\text{-}1^+$ cells are located close to the endosteum where oxygen levels are physiologically hypoxic [Mohyeldin, Garzón-Muvdi, and Quiñones-Hinojosa 2010]. In summary, hypoxia improved CFU-F frequency among $Sca\text{-}1^-$ sorted cells but had little effect on colony size. In contrast, hypoxia did not improve CFU-F frequency among $Sca\text{-}1^+$ sorted cells but did improve colony size. "Bulk" MSCs which comprise a mixture of both $Sca\text{-}1^-$ and $Sca\text{-}1^+$ cells showed an improvement in both CFU-F frequencies and colony size in hypoxia compared to normoxia. The poor chondrogenic differentiation capacity of bulk mMSC might also be a result of the low CFU-F frequency of the more chondrogenic $Sca\text{-}1^-$ population.

Taken together, the smaller size of colonies, the lower CFU-F frequency and lack of a significant response of $Sca\text{-}1^-$ sorted cells to hypoxia might suggest they reside in a different anatomical location and represent less differentiated cells. In analogy with haematopoiesis, differences in mean colony size could indicate different stem/progenitor subpopulations [Magli, Iscove, and Odartchenko 1982]. Thus, slowly-proliferating stem cells would form smaller colonies appearing later in culture than rapidly-proliferating progenitors forming early larger colonies. However, our transcriptomic analysis of freshly isolated $Sca\text{-}1^+$ and $Sca\text{-}1^-$ cells did not confirm this hypothesis, with higher levels of NANOG and TERT expression among $Sca\text{-}1^+$ sorted cells (Figure 3.8A + B). However, it has to be mentioned that transcript levels do not always correlate with protein levels. Both $Sca\text{-}1^+$ and $Sca\text{-}1^-$ sorted cells have the capability to give rise to osteoblasts, chondrocytes and adipocytes. In addition, and confirming results from differentiation assays, the

Sca-1$^-$ sorted subpopulation appeared to have more chondrogenesis related transcripts (Figure 3.8C). Prior to exposure to chondrogenic stimuli they expressed relatively elevated levels of a collection of transcripts characteristically expressed by chondrocytes at different stages of differentiation.

The *in vitro* differentiation of the Sca-1$^-$ sorted subpopulation to chondrocytes was superior to that of Sca-1$^+$ sorted cells as measured by GAG accumulation. It has been shown that Sca-1$^{-/-}$ mice developed age-related osteoporosis due to reduced numbers of osteoprogenitors and osteoblasts. Furthermore they displayed a weakened bone structure and bone material caused by the reduced number of MSC, which also resulted in impaired adipogenesis *in vitro* [Bonyadi et al. 2003]. Although Sox5 and Runx2 were quite well expressed by Sca-1$^+$ cells, the partner Sox genes Sox6 and Sox9 (Sox trio)[Ikeda et al. 2004] were poorly expressed and could indicate that their poor chondrogenic potential was due to limited Sox6 and Sox9 expression [Han and Lefebvre 2008; Lunj et al. 2005]. Interestingly, Sca-1$^-$ cells have a higher expression of Col10a compared to primary articular chondrocytes (Figure 3.8D). Col10a is a late chondrogenesis marker for hypertrophic chondrocytes [Shen 2005] which could indicate that Sca-1$^-$ cells represent a less-differentiated population of committed chondrocytes. In addition, the high expression of Col10a could also be an interim stage towards a more osteogenic development [Pelttari et al. 2006].

CHAPTER 4

Prospective isolation of clonal populations of mMSC

4.1 INTRODUCTION

As mentioned before the isolation of mMSCs from the C57Bl/6 mouse strain is more difficult compared to other mouse strains. However, to take advantage of the broad genetically modified C57Bl/6 strains available, it is necessary to have a reliable isolation method and culture conditions for BM-derived mMSCs from this particular mouse strain.

One major obstacle that hinders the true identity of mouse MSC is the lack of specific markers for distinguishing them *in vivo* [Bianco 2011; Jones and McGonagle 2008]. Even though there are surface markers for the identification of mMSC *in vitro*, due to the removal from their *in vivo* environment and exposure to artificial culture conditions, their expression could be altered, thus excluding them for prospective isolation [Jones et al. 2002]. As seen in chapter 3, there was a clear change in surface marker expression of *in vitro* expanded cells. Additionally, certain cell culture conditions may maintain the typical MSC tri-lineage differentiation potency but they may not preserve the naive MSC phenotype [Jo et al. 2007; Morikawa, Mabuchi, Niibe, et al. 2009].

As shown in chapter 3, hypoxia had a positive effect on the CFU-F frequency of Sca-1^{+} cells. To further enhance culture conditions for single cell isolation, the effect of gelatine coating and cell free extracellular matrix (ECM) coating was investigated. It has been shown that ECM coating improved MSC proliferation and maintained their differentiation capacity [X. Chen et al. 2007]. Furthermore ECM is an important factor in the maintenance of stem cells *in vivo* and plays a role in their fate regulation [Trappmann et al. 2012; Vidane et al. 2013].

Intense investigation for the isolation of MSC focus on the use of monoclonal antibodies to prospectively enrich MSC based on their surface phenotype. Jones *et al.* isolated $CD45^{low}CD271^{+}$ cells from human trabecular bone which were capable of tri-lineage differentiation [Jones et al. 2010]. Another study has shown that the use of CD146 could distinguish between perivascular versus endosteal localization of BM-derived hMSC [Tormin et al. 2011]. Other markers for the isolation of a

homogenous BM stromal cell population include STRO-1 and VCAM-1 [Stan Gronthos et al. 2003]. Unfortunately, due to the differences between man and mouse, these markers cannot be used for the prospective isolation of mMSCs.

Several marker combinations have been used to isolate mouse MSCs, e.g. Stage specific embryonic antigens (SSEA)-1 and 4 have been used to single-cell sort mMSCs [Anjos-Afonso and Bonnet 2007; Gang et al. 2007]. However, single-cell sorting was done with *in vitro* expanded cultures and not directly from mouse BM. Méndez-Ferrer *et al.* used a transgenic mouse reporter line expressing GFP under the control of promoter of the *nestin* gene to prospectively isolate mMSC [Mendez-Ferrer et al. 2010]. PDGFRα in combination with CD51 expression has been shown to overlap with nestin^{+} cells and are capable of supporting HSC growth [Pinho et al. 2013]. Sca-1 has already been used in combination with other marker to isolate mMSC from bone marrow [Nakamura et al. 2010; Steenhuis, Pettway, and Ignelzi 2008] and a recent study was able to generate clonal subpopulations of mMSC by combining Sca-1 and PDGFRα staining which showed tri-lineage differentiation capacity both *in vitro* and *in vivo* [Morikawa, Mabuchi, Kubota, et al. 2009]. However, not all isolated clones were able of tri-lineage differentiation.

Therefore the isolation and expansion of single cells and their immunomodulatory capacities were under investigation.

4.2 MATERIALS & METHODS

MS-5 cell lines

MS-5 MSC line was provided by Prof. Antonius Rolink (Department of Biomedicine, University of Basel). This adherent cell line was isolated from γ-irradiated mouse bone marrow [Itoh et al. 1989]. Cell lines were cultured in Cell Line Growth Medium (Appendix II) in humidified incubators at 37°C containing either 21%, 5% or 2% oxygen. Culture medium was changed every 3-4 days and subsequent

passages were performed when cells reached 60 – 80% confluence. Attached cells were washed with sterile Dulbecco's phosphate buffered saline (DPBS) and cells were split using trypsin/EDTA for 2 minutes at 37°C. Trypsin/EDTA was neutralized with Cell Line Growth Medium and cells were centrifuged at 400g for 5 minutes. Cells were then plated in new T75 flasks.

Extracellular matrix (ECM)

96-well flat bottom plates were coated with 0.1% gelatine as described before (Chapter 3). 7500 MS-5 cells were seeded per well and cultured in Cell Line Growth Medium. Once they were confluent media was removed and replaced with Cell Line Growth Medium containing 10µg/ml Mitomycin C. Cells were incubated for 3 hours at 37°C and then washed 3 times with DPBS. Cell Line Growth Medium was added and cells were placed in a humidified incubator at 37°C for 5 days. Media was removed and cells were washed 2 times with DPBS. ECM Lysis Buffer (Appendix III) was added and cells were incubated at 4°C for 12 hours on a plate rocker. ECM Lysis Buffer was then removed and plates were washed very gently 5 times with DPBS. ECM plates were stored in DPBS at 4°C for a maximum of 4 weeks.

Immunofluorescence Staining

ECM plates were fixed in 10% formalin for 15 minutes at room temperature and washed with DPBS. Plates were then incubated with DPBS containing 4% FBS for 30 minutes following staining with biotinylated antibody to collagen I for 1 hour. After washing with DPBS, Streptavidin-FITC and DAPI were added for 30 minutes at 4°C. After washing with DPBS, images were captured using an IX71 Olympus fluorescent microscope with Olympus Cell P software.

Single-cell sorting

Single-cell sorting was carried out using a BD FACS Aria II machine. The general protocol for sample preparation is identical to sorting Sca-1 subpopulations (Chapter 3). Cells were sorted in 0.1% gelatine coated 96-well flat bottom plates containing 200µl MSC Growth Medium.

Colony counting

96-well plates containing single sorted cells were cultured for 7 days in a humidified incubator at 37°C with 2% oxygen. Counting was done using a regular light microscope and colonies displaying fifty or more cells with spindled MSC morphology were scored.

MLR assay

Cultured cells from single-cell sorts were added in graded numbers of 1:50, 1:100 and 1:200 MSC/splenocytes ration into 96-well round bottom plates and allowed to adhere for 24h prior to addition of splenocytes. Splenocytes were extracted from C57Bl/6 mice by processing the spleen between two pieces of nylon gauge with 150µm pore diameter (Sefar). Red cell lysis was performed with ACK lysis buffer (see appendix) and cells were re-suspended in T-cell medium (see appendix). Cells were labelled with CellTrace CFSE cell proliferation kit (Invitrogen) according to manufacturer's instructions and stimulated with 0.5µg/ml purified anti-mouse CD3 and CD28 antibodies (eBioscience). 10^5 splenocytes of labelled splenocytes were added to each well and cultured for 3 days in T-cell medium (see appendix). After staining with anti-mouse CD4-PE and CD8-APC (BD), flow cytometry analysis was carried out as mentioned before. Difference in percentage of CFSE staining intensity between stimulated and MSC co-cultured splenocytes was used to calculate inhibition of T-cell proliferation.

4.3 RESULTS

Improving *in vitro* cell culture conditions

Based on the results from the previous chapter, the effects of oxygen supply and gelatine coating on CFU-F frequencies for single cell sorted cells was investigated. MS-5 cells were used for a proof of concept, due to their rapid growth and very robust response to *in vitro* conditions [Sugrue, Lowndes, and Ceredig 2014]. MS-5 cells were sorted at frequencies from 30cells/well to 1cell/well and expanded for 7

days in corresponding oxygen conditions. It can be seen that hypoxia always improved the numbers of colonies compared to normoxia and that gelatine coating had a beneficial effect especially at higher sorted cell numbers (Figure 4.1).

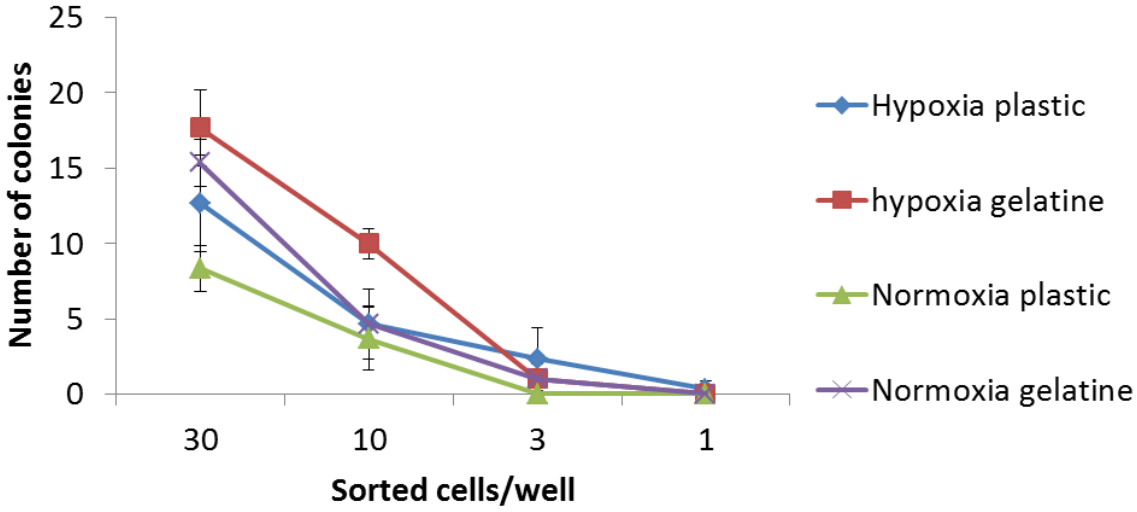

Figure 4.1 Number of colonies from MS-5 sorted cells. MS-5 cells were sorted and outgrown colonies were counted after 7 days *in vitro* culture.

To further improve CFU-F frequencies, 96-well plates were coated with extracellular matrix (ECM) produced by MS-5 cells. To enhance production of ECM and to control the unrestricted growth of MS-5 cells, cultures were treated with Mitomycin C, a DNA crosslinker, to inhibit cell division but not viability and production of ECM proteins. Due to reports that indicate that Mitomycin C toxicity is dependent on the cell line, the effect of Mitomycin C on proliferation was tested [Rauth, Mohindra, and Tannock 1983]. As seen in Figure 4.2A, Mitomycin C successfully inhibits the proliferation of MS-5 cells at concentrations ranging from 100µg/ml to 10µg/ml. As shown in Figure 4.2B, Mitomycin C inhibits proliferation of MS-5 cells, but not the production of ECM. To confirm matrix production ECM plates were stained with DAPI and collagen I. Figure 4.2C shows stained ECM plates. Despite osmotic shock induce lysis of MS-5 cells and intensive washing, DAPI staining shows irregular shape of cell nuclei, so-called nuclear ghosts. However, collagen I staining show stable expression after ECM lysis buffer

treatment. Storage in DPBS at 4°C for up to 4 weeks had no effect on ECM phenotype (Figure 4.2D). Even though there was no proliferation of potentially leftover of lysed MS-5 cells after 10 days in culture, the phenotypic appearance of the ECM and the presence of nuclear ghosts made it impossible to reliable count CFU-F frequencies from sorted cells (Figure 4.2C). Therefore, it was not possible to use ECM-coated plates for cloning experiments.

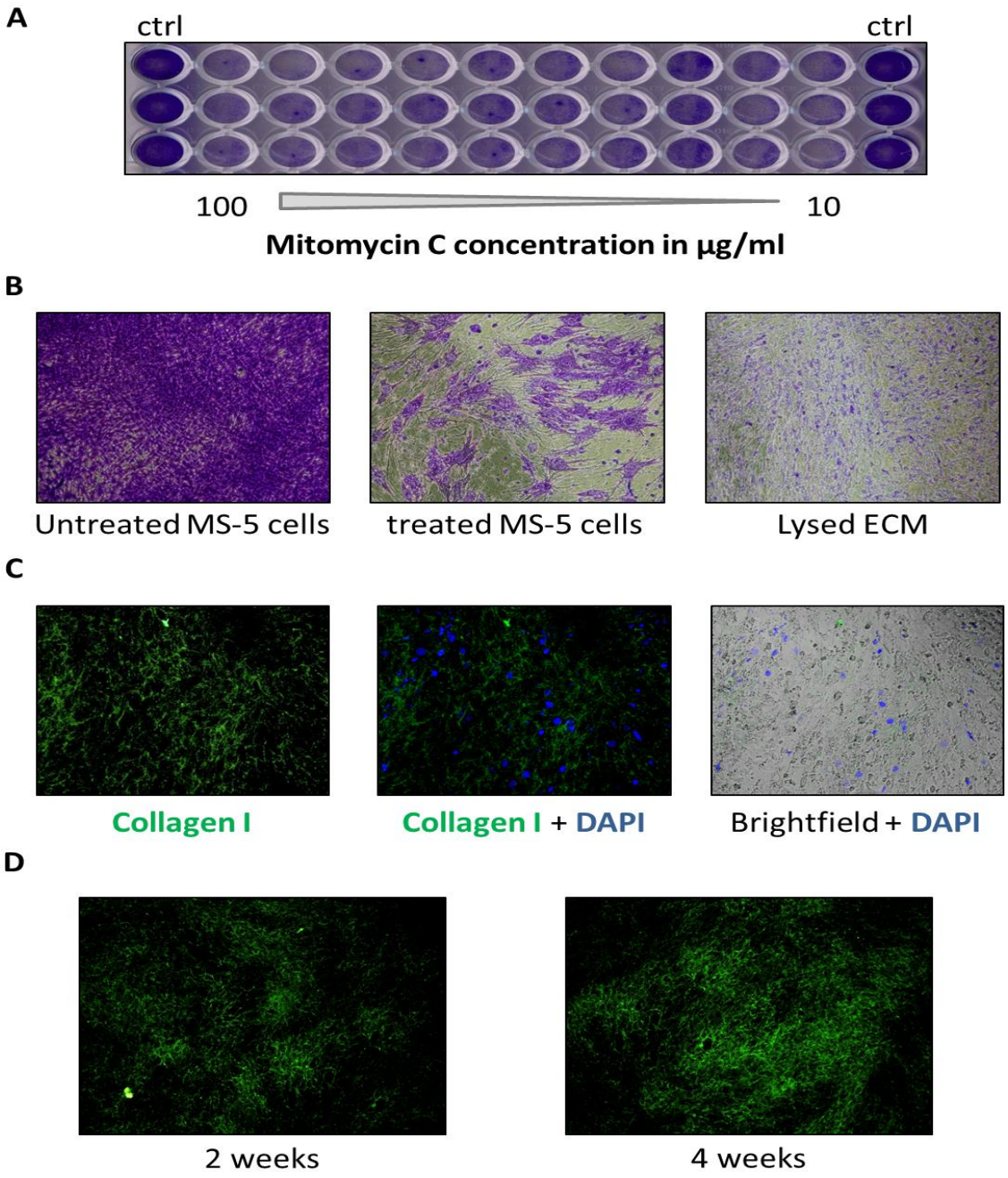

Figure 4.2 ECM production by MS-5 cells. (A) MS-5 cells were treated with Mitomycin C at various concentrations and stained with Crystal violet. (B) Crystal violet staining of Mitomycin C treated (10µg/ml) and non-treated cells and ECM appearance after lysis. (C) Immunohistochemistry of ECM with collagen I (green) and DAPI (blue). (D) Collagen I (green) staining of ECM after 2 - 4 weeks storage at 4°C. Magnification 200x.

PDGFRα$^+$/CD90$^+$ subpopulation shows an increased CFU-F frequency

Based on the results from chapter 3, Sca-1$^+$ cells were clearly enriched for transcripts of stem cell genes NANOG and TERT, and showed increased CFU-F frequencies and proliferation. PDGFRα in conjunction with Sca-1 has been used to purify mouse MSC from cBM on a single cell level with a 1/22.5 CFU-F frequency [Morikawa, Mabuchi, Kubota, et al. 2009]. To further increase the CFU-F frequency of freshly single-cell sorted mouse BM, cells were additionally stained with CD90, a marker used for the characterisation of human MSCs and consistently expressed by freshly-isolated mMSC (Figure 2.5). The CD45$^-$/Ter119$^-$/Sca-1$^+$ population was subdivided into four distinct subpopulations of PDGFRα/CD90 expressing cells (Lower right panel, Figure 4.3).

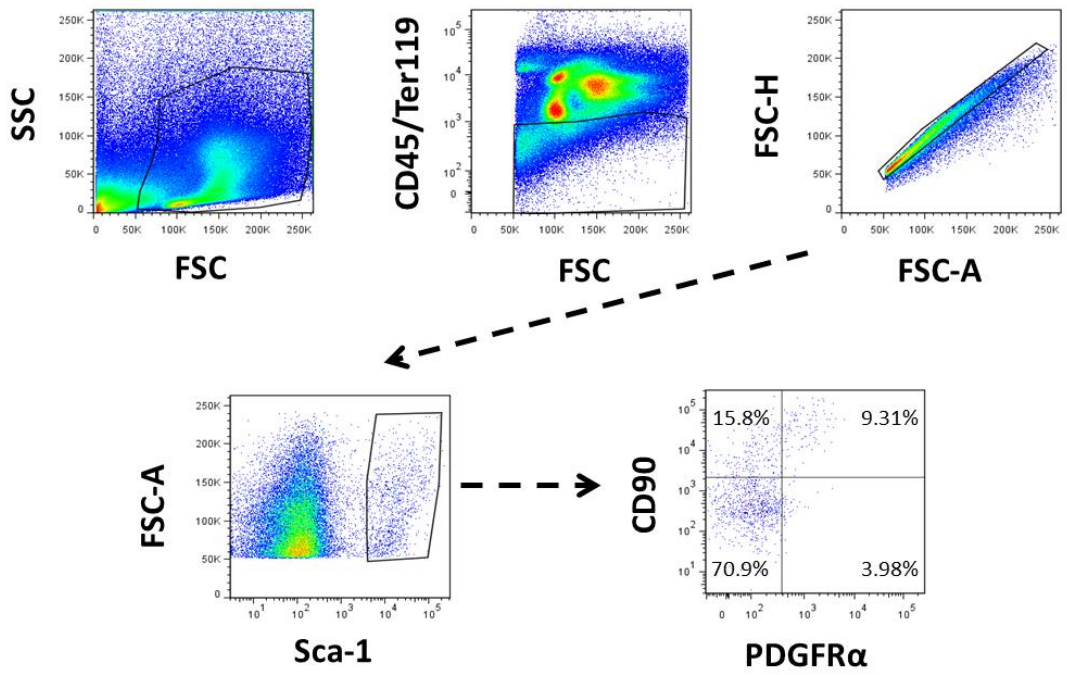

Figure 4.3 Representative gating strategy for single-cell sorting (A) Gating strategy for the single cell isolation of PDGFRα$^+$/CD90$^+$ cells. Cells were initially gated on FSC and SSC signals followed by CD45$^-$/Ter119$^-$ cells and doublet exclusion (upper cytograms). Gated Sca-1$^+$ cells among CD45$^-$/Ter119$^-$ cells (lower left panel) are then subdivided into the four CD90/PDGFRα subpopulations (lower right panel) where percentages in each quadrant are indicated.

As shown in Figure 4.4A, the overall PDGFRα staining of $CD45^-/Ter119^-/Sca\text{-}1^+$ cells is lower in fBM compared to cBM. It also became evident that fBM had a significant lower expression of $Sca\text{-}1^+/PDGFR\alpha^+/CD90^+$ cells (Figure 4.5B) than cBM in total bone marrow (Figure 4.4B). The other subpopulations showed no significant differences in total bone marrow distribution.

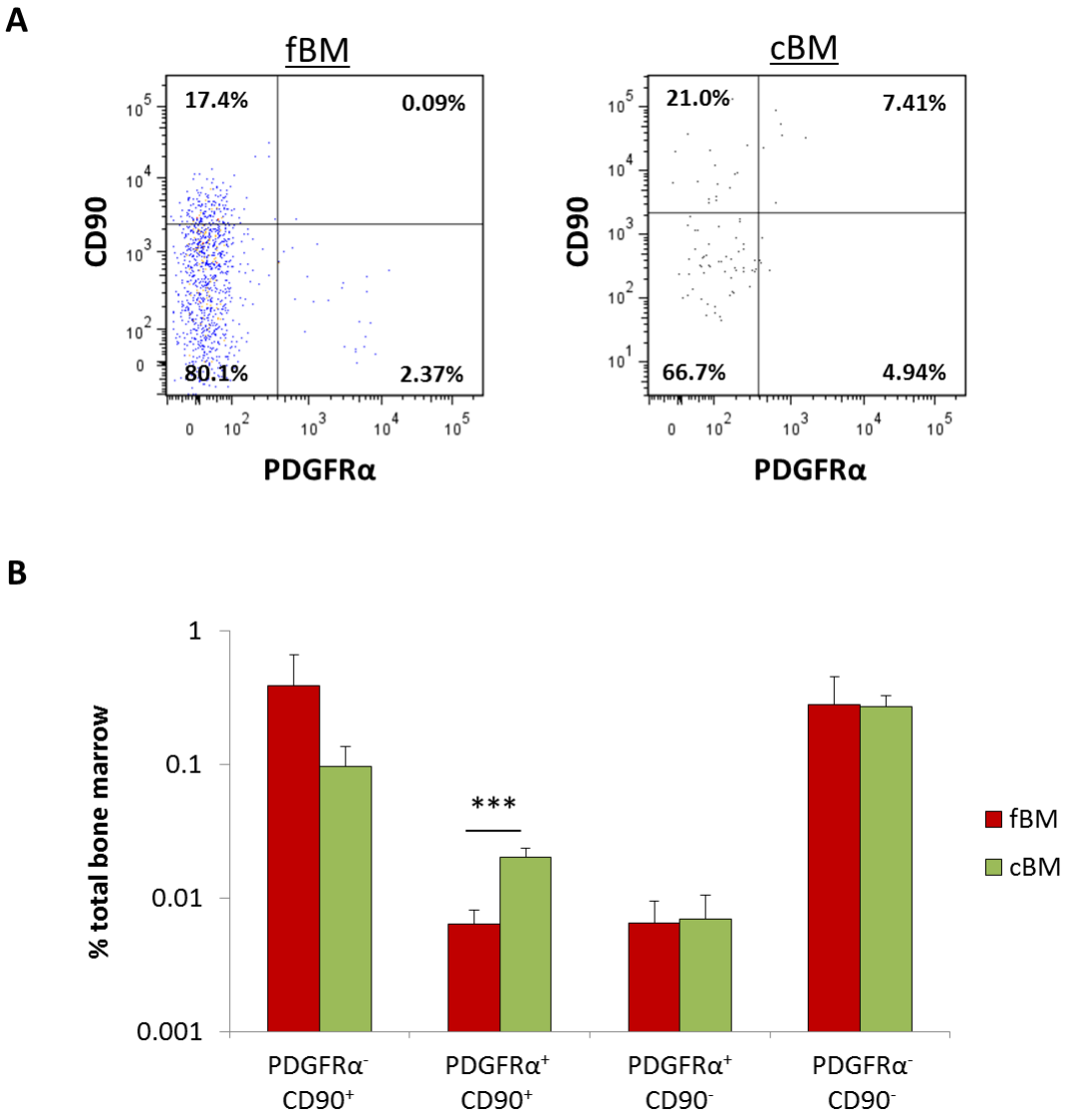

Figure 4.4 Distribution of PDGFRα and CD90 in fresh BM. (A) Cytogram display of the distribution of PDGFRα vs CD90 in fBM (left cytogram) and cBM (right cytogram). The % positive cells in each quadrant are shown. (B) Percentage positive subpopulations in total bone marrow of fresh fBM and cBM. Data are the means ± SD of at least three independent experiments. ***$p < 0.001$, Student's *t* test.

Taking a closer look within the two Sca-1 subpopulations of cBM, it can be seen that there is a significant higher proportion of PDGFRα^{+}/CD90^{+} cells in Sca-1^{+} cells (Figure 4.5A). This difference is about 50 times higher in Sca-1^{+} than Sca-1^{-} sorted subpopulations and there is an overall higher expression of PDGFRα and CD90 in Sca-1^{+} sorted cells (Figure 4.5B).

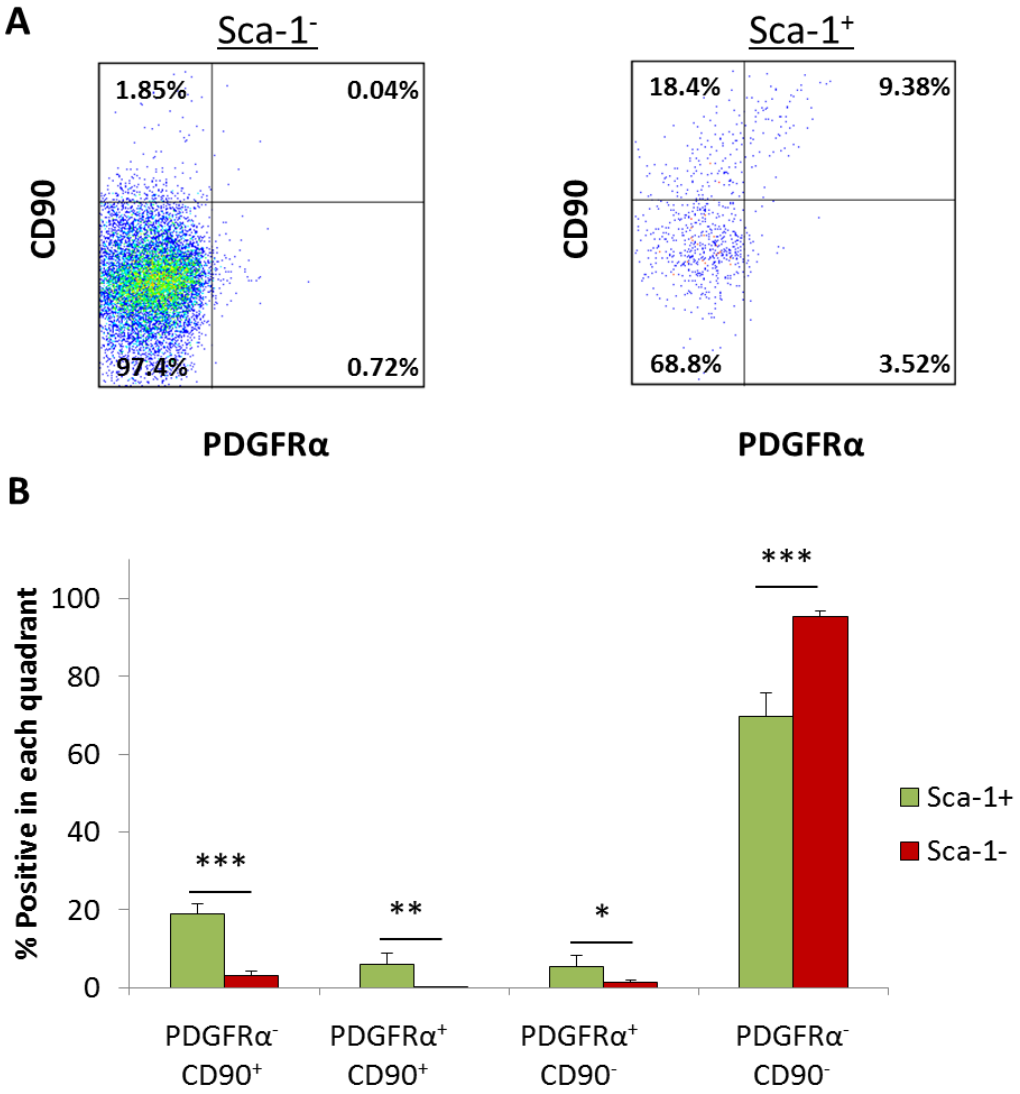

Figure 4.5 Distribution of PDGFRα and CD90 in Sca-1^{-} versus Sca-1^{+} cells. (A) Cytogram display of the distribution of PDGFRα vs CD90 in Sca-1^{-} (left cytogram) and Sca-1^{+} cells (right cytogram). The % positive cells in each quadrant are shown. (B) Percentage positive in each quadrant for Sca-1^{-} and Sca-1^{+} cells. Data are the means ± SD of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student's *t* test.

When expanded *in vitro,* PDGFRα^{+}/CD90^{+} from cBM showed a spindle-shaped, fibroblastic morphology typical for MSCs (Figure 4.6A), whereas the two PDGFRα^{-} cell populations generated cobble-stone like morphologies. Interestingly, under these growth conditions PDGFRα^{+}/CD90^{-} cells failed to grow as adherent cells. Compared with unfractionated Sca-1^{+} cells, where the CFU-F frequency in fBM and cBM was 1/513 and 1/251 respectively (Figure 4.6B), in four experiments the mean frequencies among single-cell sorted PDGFRα^{+}/CD90^{+} /Sca-1^{+} from cBM was 1/4. This is amongst the highest frequencies of CFU-F so far reported for C57Bl/6 mice. Following their initial isolation and identification, individual clones were expanded for several days and passaged at 10, 14 and 18 days. During this time, not all clones continued growing and by 18 days, only ~40% of initially-plated clones were growing (Figure 4.6C).

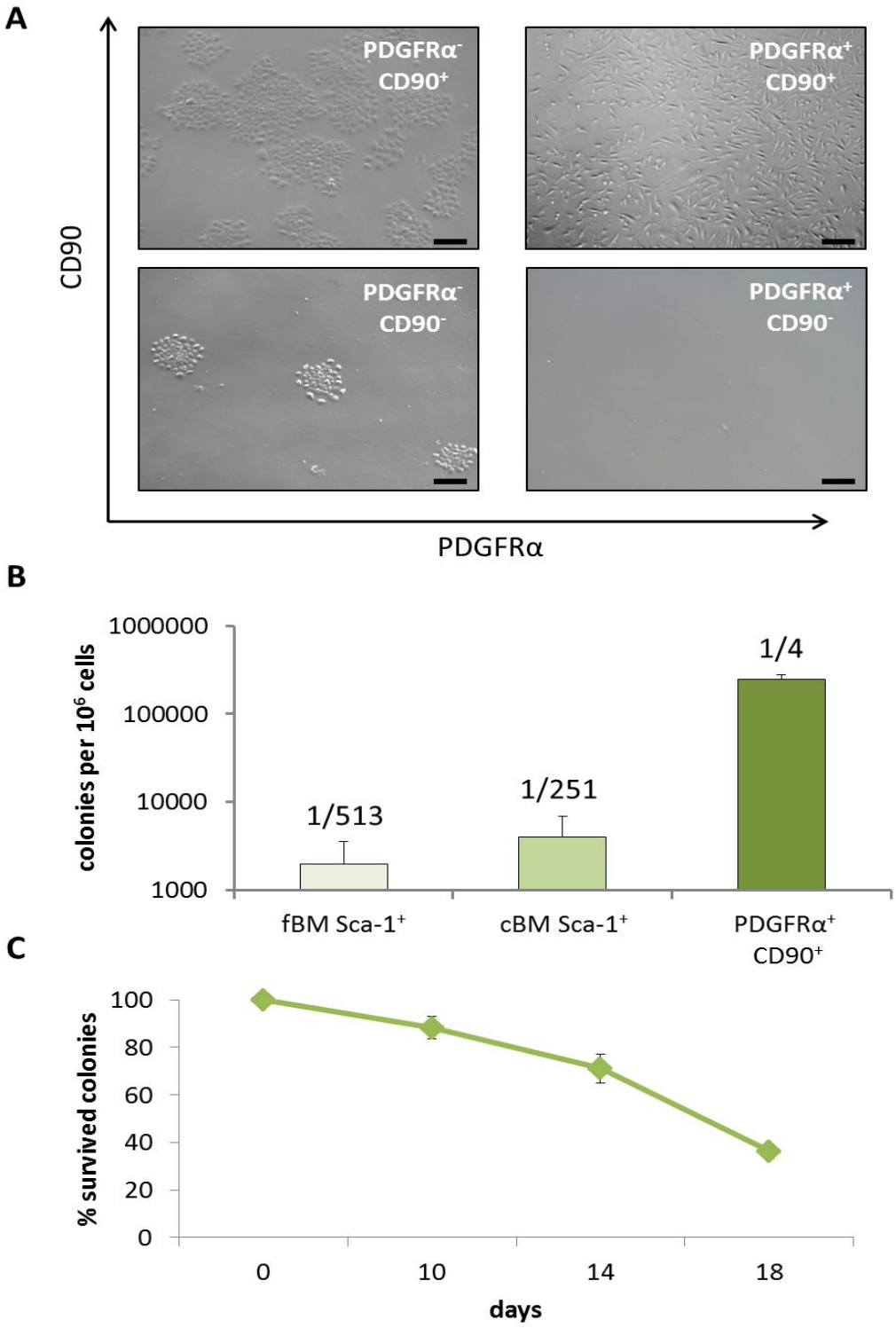

Figure 4.6 CFU-F frequencies and colony survival of PDGFRα$^+$/CD90$^+$ cells. (A) Brightfield images of expanded cells of the indicated subpopulation after 10d of culturing. (B) CFU-F frequencies of Sca-1$^+$ cells in fBM (left), cBM (middle) and PDGFRα$^+$ CD90$^+$ Sca-1$^+$ cBM (right) cells. (C) Survival curve for PDGFRα$^+$/CD90$^+$ colonies during *in vitro* expansion.

Clone expansion

Clones were expanded in MSC Growth Media, but many of them stopped proliferating (Figure 4.7A). Surviving colonies from fBM as well as cBM showed typical fibroblastic-like phenotype (Figure 4.7B and C). Surviving colonies were exposed to media for osteo- and adipogenic differentiation, but unfortunately, none of the cells survived after 14 or 21 days of *in vitro* differentiation. Interestingly though, after 7 days *in vitro* differentiation, lipid vacuoles could be seen in osteo- and adipogenic differentiating cultures (Figure 4.8A and B).

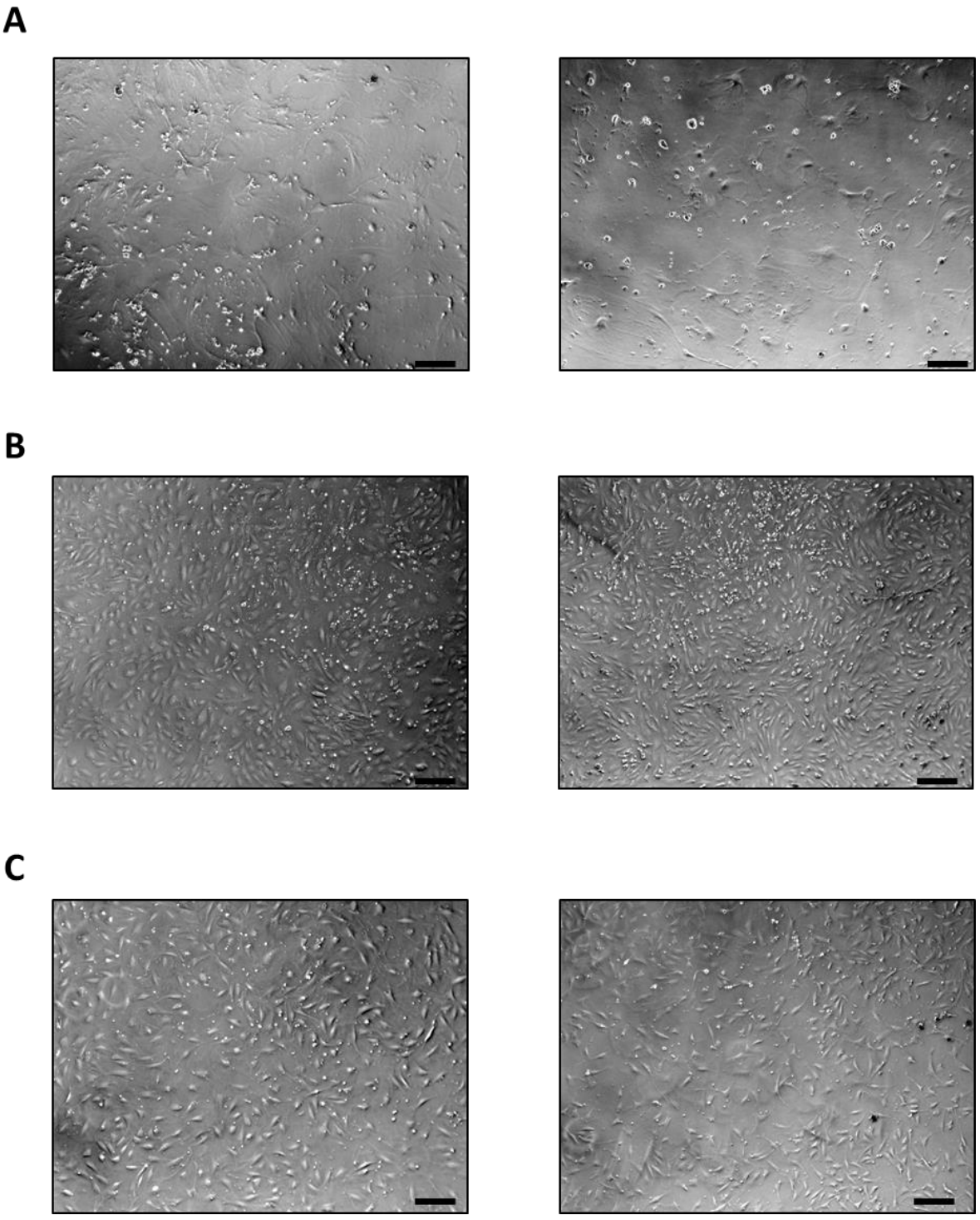

Figure 4.7 PDGFRα^{+}/CD90^{+} derived colonies exhibit a fibroblast-like phenotype.
(A) Non-surviving colonies from cBM in MSC Growth Media. (B) Surviving colonies with fibroblastic-like phenotype from fBM and (C) cBM. Image bar = 200µm.

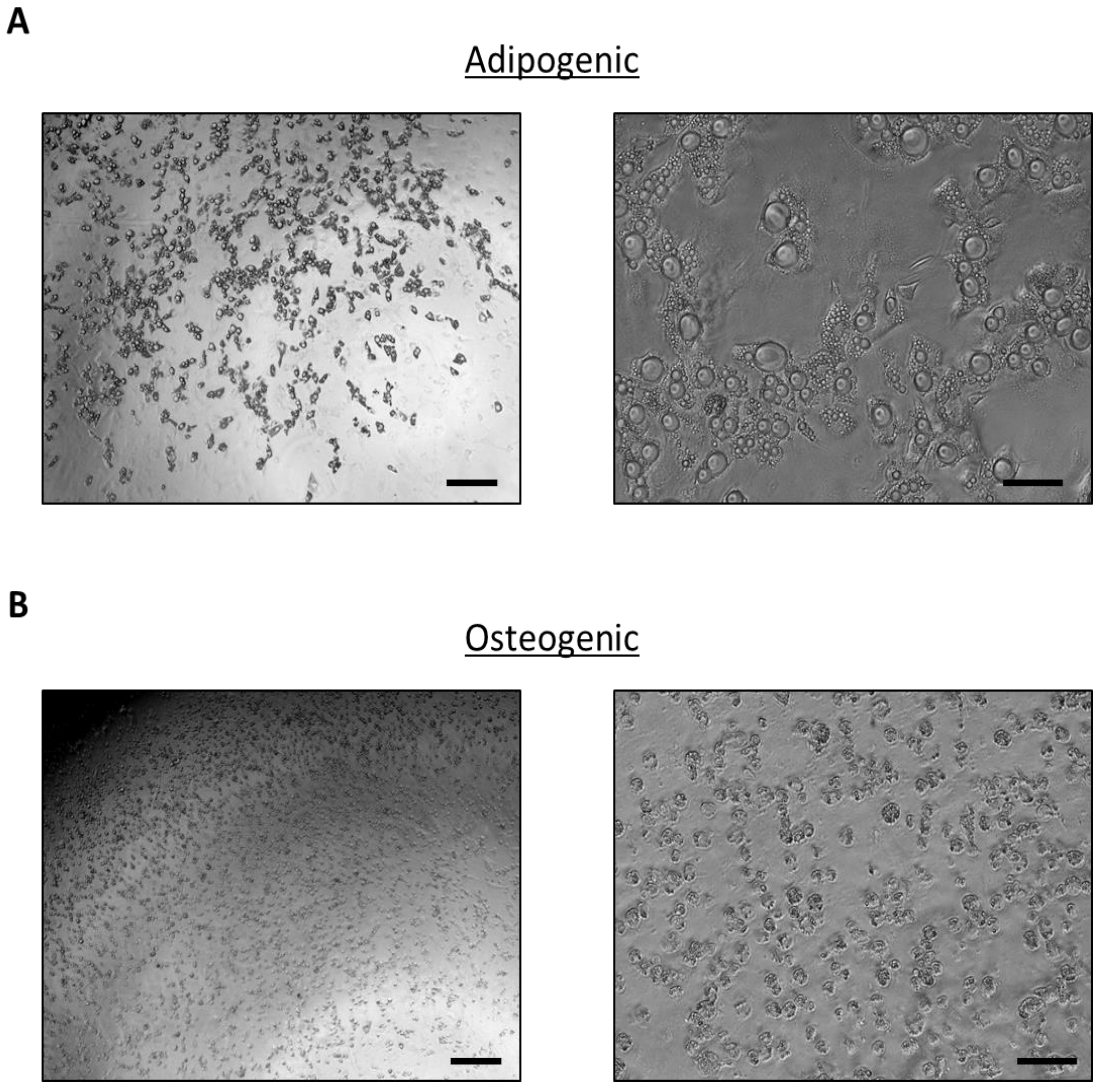

Figure 4.8 Differentiation capacities of PDGFRα^{+}/CD90^{+} cells. (A) Expanded PDGFRα^{+}/CD90^{+} cells after 7 days in adipogenic and (B) osteogenic differentiation media. First column = 4x magnification (image bar = 200µm) and second column 10x magnification (image bar = 100µm).

Imunnomodulatory properties

Surviving clones were then tested for their ability to inhibit the proliferation of CD3/CD28-stimulated T-cells. All of twelve clones tested showed potent inhibitory activity on T-cell proliferation at a 1:100 MSC : T-cell ratio. A result from a representative clone is shown in Figure 4.9A. Additional staining with CD4 and CD8

mAb revealed a simultaneous degree of inhibition of proliferation in both T lymphocyte subsets mediated by PDGFRα^{+}/CD90^{+} cells (Figure 4.9B).

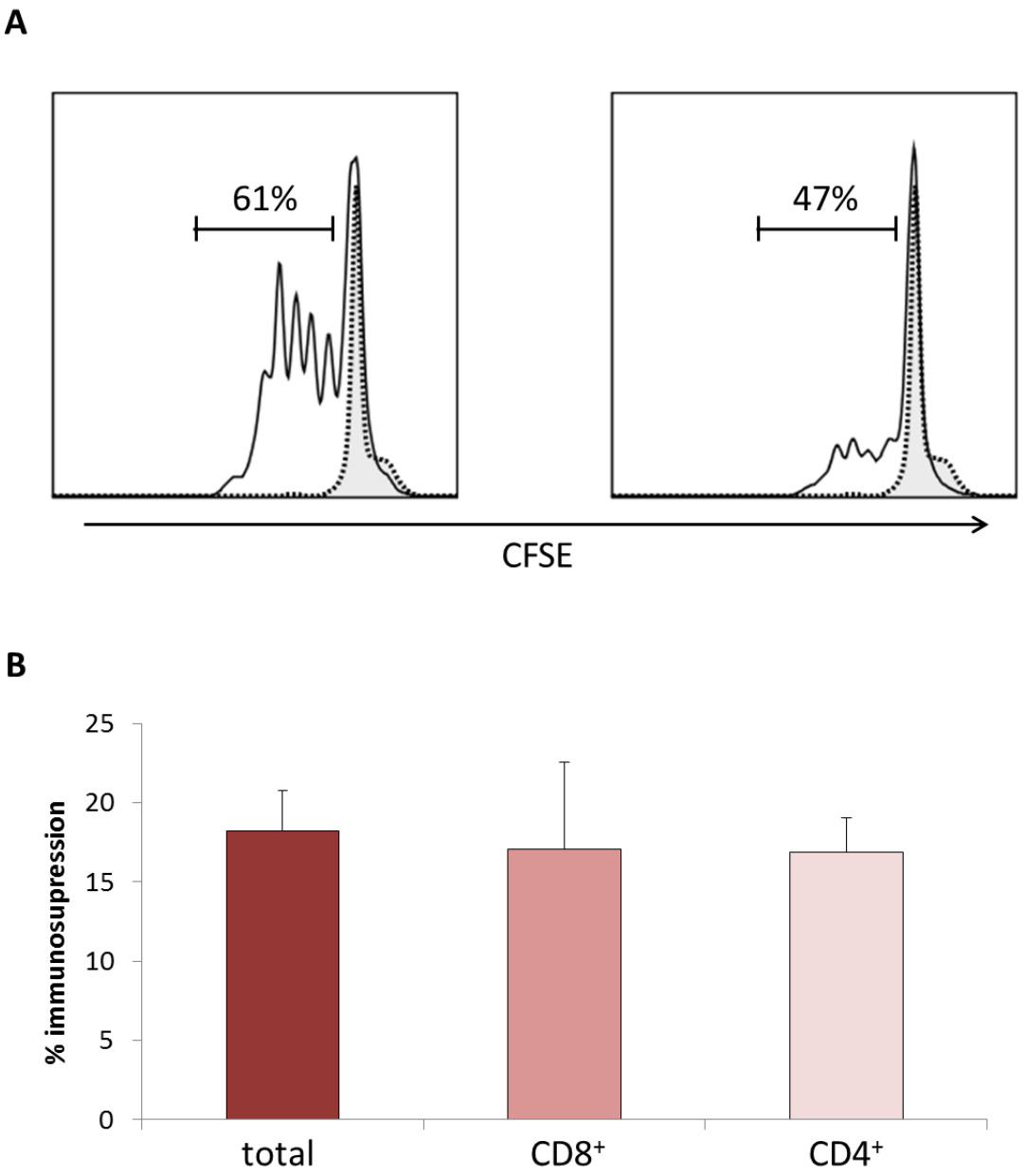

Figure 4.9 PDGFRα^{+}/CD90^{+} cells inhibit splenocyte proliferation. (A) Representative histogram of CFSE staining among gated T cells of CD3/CD28 stimulated splenocytes with and without addition of cloned PDGFRα^{+}/CD90^{+} cells (white, solid line = stimulated splenocytes; tinted, dashed line = unstimulated splenocytes). (B) Percentage immunosuppression among different subsets of T lymphocytes.

4.4 DISCUSSION

Most isolation protocols and *in vitro* culture systems for mouse MSCs utilize uncoated plastic vessels. While these offer an easy method for the expansion of most cell types, it might not be the best way for the maintenance of *ex vivo* stem cells. Due to the fine regulation of MSCs in their native niche, *in vitro* systems might therefore benefit from a more *in vivo*-like coating to expand these cells. As shown in Figure 4.1, expansion of MSCs on gelatine coated plates coupled with hypoxic oxygen condition yielded a number of colonies. Reports have shown that ECM and the stiffness of the matrix interact with collagen fibres and thereby affecting the stem cell-fate of hMSC [Trappmann et al. 2012]. Even though the generation of matrix was successful (Figure 4.2B) the presence of so-called nuclear ghosts made it unfeasible for the expansion of single-cell derived colonies. It is therefore necessary to improve the ECM protocol before it can be used in future experiments. It has been shown by Sugrue *et al.* that MS-5 is a very robust cell line, especially when cultured in hypoxia [Sugrue, Lowndes, and Ceredig 2014]. The solution could be the use of another MSC cell line which is easier to lyse but still produce ECM. Also the lysis itself could be enhanced by adding DNAse to the lysis buffer and therefore reducing the appearance of nuclear ghosts. Additionally UV-light could be used to lyse the ECM producing cells, unfortunately that could also affect the functional properties of ECM proteins. Finally, using an immortalized cell line engineered to undergo apoptosis triggered by a chemical inducer, could result in a more intact and active ECM [Bourgine et al. 2013].

In the seminal paper of Morikawa *et al.,* compact BM-derived cells were sorted using a combination of $CD45^-$, $Ter119^-$, $Sca\text{-}1^+$ and $PDGFR\alpha^+$ (PαS markers) and a CFU-F frequency of 1/22.5 obtained [Morikawa, Mabuchi, Kubota, et al. 2009]. From such cultures, individual colonies were isolated and some had *in vitro* tri-lineage differentiation potential even following re-isolation from *in vivo*. Similar to results from Morikawa *et al.,* PDGFRα expression is increased in cBM (Figure 4.4). In this thesis, CD90 expression as a selection marker in addition to PDGFRα was used to directly sort single cells from BM. As shown in Figure 4.6, this combination

of markers allows the isolation of CFU-F with a frequency of 1/4 among PDGFRα^{+}/CD90^{+} cells. This represents a higher frequency than previously reported by Morikawa *et al*, most likely due to the omission of the CD90^{-} subpopulation of Sca-1^{+}/PDGFRα^{+} cells, which failed to grow *in vitro* (Figure 4.6). In addition to the increase in CFU-F frequencies, morphological differences between subpopulations of sorted cells were also evident. Even though the CFU-F frequencies were one of the highest reported so far, the failure of these cells to thrive *in vitro* hinders future studies. The full implication of these differences in cell morphology requires additional investigation beyond the scope of this thesis.

The standard methods for *in vitro* differentiation assays require a certain cell number and density to drive the cells to a particular lineage, especially chondrogenic density pellet cultures [Pittenger et al. 1999; Peister et al. 2004; Russell et al. 2010]. It is therefore necessary to expand these cells beforehand in order to have sufficient numbers for the *in vitro* assays. Most *in vitro* culture and expansion protocols, including this one, are based on a basic medium supplemented with 10–20% of Foetal Bovine Serum, which might not be the most suitable way to maintain the undifferentiated state of MSCs. This has been supported by studies which show that the differentiation capacity of cultured MSCs is gradually reduced upon cell passaging [Kretlow et al. 2008]. It also became clear that cells undergo phenotypic changes with passaging resulting in a change of surface marker expression (Chapter 3). In fact, it has been shown that MSCs expanded under conditions that maintain MSC differentiation capacity might not preserve the naïve MSC phenotype [J. Zhu et al. 2007].

Another explanation might be the lying in the niche concept mentioned earlier (Chapter 1). In this concept, supporting cells keep the naïve stem cell phenotype through a soluble and cell-contact signalling network like the bone marrow environment. This highlights the notion that the *in vivo* and *in vitro* plasticity of MSCs greatly depends on the microenvironment [Barry and Murphy 2004]. By clonally isolating these cells, the niche is disrupted and cells might undergo phenotypic changes in response to missing signals. This is further supported by the fact, that bulk cultures from cBM are fully capable of tri-lineage differentiation

(Chapter 2). In order to minimise these effects the use of ECM to mimic niche signals might be beneficial in not only improving CFU-F frequencies but also in stem cell maintenance.

The maintenance of MSCs *in vitro* and *in vivo* is regulated by a combination of intrinsic and extrinsic mechanisms. Intrinsic mechanisms include specific transcription factors expressed by the cells. A change in these transcription factors due to prolonged expansion *ex vivo* can lead to a cell crisis resulting in impaired proliferation and differentiation. Extrinsic mechanisms are signals provided by the local microenvironment (niche). These signals include growth factors, the extracellular matrix (ECM) and contact with other cells. Interactions with the niche are dynamic, as stem cells are able to remodel the niche in response to the signals they receive from it. In connective tissue, like fibroblasts in the dermis and chondrocytes in cartilage, cells tend to be completely surrounded by the ECM and adhere via cell surface receptors like integrin [Hynes 2002]. This close connection allows the cell to sense mechanical forces from the ECM and respond via a process known as mechanotransduction [Puklin-Faucher and Sheetz 2009]. This process can result in changes in cell shape and size and responses such as differentiation and proliferation.

CHAPTER 5

Final Discussion

5.1 WHAT HAVE WE LEARNED

One aim of this project was to establish viable cell culture conditions and to show MSC-like properties for bulk MSC cultures derived from C57Bl/6 bone chips. The rationale for this was to address the possibility that by culturing bone chips, contamination by haematopoietic cells would be minimised. Even though the extraction of mMSCs from bone chips has been described before [H. Zhu et al. 2010], phenotypic characterisation was only carried out after the third passage *in vitro* and could therefore not detect early changes in surface marker expression. As shown in Figure 2.2, haematopoietic cell contamination was present in early passages, but was reduced by sequential passaging and absent in passage 3 *in vitro* cultures. This highlights the fact that even bulk cultures from bone chips, which were several times washed and pre-treated with collagenase, still contain significant numbers of haematopoietic cells and need to be expanded *in vitro* for at least 3 passages in order for this contamination to be diluted out. The surface phenotype of these cells characterised by monoclonal antibody staining showed homogenous expression of markers commonly used for *in vitro* expanded mMSCs (Figure 2.7), but a heterogeneous expression for other markers (Figure 2.6). *In vitro* assays for tri-lineage differentiation potency showed stable differentiation towards all three lineages, confirming the fact that bone chip-derived cells exhibit MSC-like features.

Following observations in chapter 2, namely that Sca-1 was a marker consistently expressed by bone chip cells, bone marrow cells were sorted based on Sca-1 expression and the surface marker expression changes during *in vitro* expansion were further investigated. Surface marker expression changed significantly for commonly used mouse MSC markers, which questions their usefulness in the validation of the mMSC phenotype *in vitro* (Figure 3.2). Additional analysis focused on the effect of hypoxia on CFU-F frequencies and colony size. Hypoxia improved CFU-F frequency among Sca-1^- cells but had little effect on colony size. In contrast, hypoxia did not improve CFU-F frequency among Sca-1^+ cell but did improve colony size. “Bulk” MSC which comprise a mixture of both Sca-1^- and Sca-1^+ cells

showed an improvement in both CFU-F frequencies and colony size in hypoxia compared to normoxia (Figures 3.3 and 3.5). In general it became clear that hypoxia had beneficial effects on both parameters compared to normoxia and differed in the extent depending on the Sca-1 subpopulation.

Differentiation assays confirm tri-lineage differentiation capacity for both subpopulations, but also revealed a higher degree of chondrogenic differentiation in Sca-1$^-$ cells (Figure 3.7). Furthermore, transcriptomic analysis of the different subpopulations revealed more chondrogenic gene transcripts in Sca-1$^-$ cells (Figure 3.8).

To overcome the limitations based on non-clonal isolated cell populations, cells were clonally isolated using a novel and unique marker combination (Figure 4.3). Isolated clones showed one of the highest CFU-F frequencies reported for mMSCs so far (Figure 4.6). Unfortunately *in vitro* conditions for expansion and differentiation proved insufficient and need to be improved for further analysis of this BM subpopulation (Figure 4.7 and 4.8). Nevertheless, surviving individual clones suppressed T-cell proliferation, thereby revealing immunosuppressive properties.

5.2 OBSTACLES

An important factor that has emerged over the years in stem cell research is the role of oxygen levels during *in vitro* culturing. Not only is it necessary to culture MSC *in vitro* to obtain sufficient numbers for *in vivo* assays, but also for confirming MSC phenotype using *in vitro* differentiation assays. Despite several studies on the role of hypoxia on differentiation, it remains controversial if it is beneficial, as different groups obtain very different results for the influence of hypoxia on differentiation of BM-derived mMSCs (Table 1.1). One reason might be the lack of standardization in isolation and culture protocols which may lead to a selective expansion of subsets of cells [Park et al. 2012].

In this thesis stable differentiation of bulk MSC cultures and Sca-1 sorted cells could be achieved by using hypoxic conditions (Figure 2.6 and 3.7). It has to be noted though, that even Sca-1 sorted subsets are still a heterogeneous populations of cells. Another aspect to take into consideration is that these assays do not necessarily correlate with *in vivo* differentiation potential of MSCs. In fact it has been shown that induced pluripotent stem cells (iPSCs) could successfully differentiate to form bone and cartilage *in vivo*, but that they failed to do so using *in vitro* assays [Phillips et al. 2014].

Even though early questions were raised about the homogeneity of cultured cells and how many of these cultured stromal cells are in fact true stem cells, bulk isolated MSCs are still the most used today. The International Society for Cellular Therapy (ISCT) tried to define minimal criteria for the identification of cultured hMSCs in an effort to make results from different research groups more comparable [Dominici et al. 2006]. These criteria include plastic adherence, flow cytometric analysis and differentiation assays (see Chapter 1 for details). Although these criteria marked an important step in standardizing cell preparations among different research groups, it did little to help dissect the complex nature of bulk cell preparations. Also, due to money restrictions and personal experience many researcher use their own panel of cell surface markers. Furthermore, these markers are only valid for hMSCs and no standardized marker arrays are available for mMSCs.

Therefore multipotency must be evaluated using clonal assays rather than bulk population. Non-clonal assays cannot prove that the different lineages arose from the same progenitor. Unfortunately, most of the publications so far involving MSC are based on *in vitro* assays using non-clonal bulk MSC cultures.

Another point to take into consideration is the fact that even single cell-derived colonies from *in vitro* cultures can differ significantly in their differentiation potential. DiGirolamo *et al.* observed that clonally derived MSCs from a single mother colony could be expanded in separate cultures and subjected to identical osteogenic conditions [DiGirolamo et al. 1999]. However, some of these daughter

cells efficiently differentiated into osteoblasts whereas others did not, demonstrating that even when derived from a single cell, the differentiation potential might be affected by *in vitro* conditions. In fact it has been shown that based on the location of the cells within a single colony, single-cell derived clones from different locations within that colony result in distinctive *in vitro* differentiation potential [Gregory, Ylostalo, and Prockop 2005]. This shows that even single cells-derived colonies form their own microenvironment and preconditioning daughter cells within.

The C57Bl/6 strain is the most widely used inbred strain in the world, and the first which genome has been fully sequenced [Consortium 2002]. This has led to their wide use in all research areas and they are commonly used for generating transgenic mice. Unfortunately, as reported by others, the C57Bl/6 mouse strain is one of the hardest for obtaining MSCs from BM [Pittenger et al. 1999]. Therefore, due to its wide use and the large amount of transgenic models available, it will be necessary to improve isolation protocols to fully understand the basic biology before attempting the transition into the clinical setting.

The standard method for verifying MSC phenotype is flow cytometric analysis of cell surface markers and differentiation assays to confirm tri-lineage differentiation capacity of *in vitro* cultures. Both these methods might be significantly biased based on the culturing process the cells have to undergo in order to obtain sufficient numbers for *in vitro* assays [Nadri et al. 2007]. When validating the phenotype of bulk MSC cultures it became clear that the reported surface markers differ significantly between freshly isolated and *in vitro* expanded cells. As shown in Figure 3.2, expression of markers characteristic of mMSC changed during *in vitro* expansion. This is even more worrying/alarming as these markers are well accepted in the literature and are advertised by companies as *in vitro* mMSC marker [Abcam 2011; R&D Systems]. A possible explanation lies in the fact that these mMSC cell surface markers are targeting adhesion receptors which might be naturally expressed and up-regulated by adherent cells. For example, Qian *et al.* have shown that freshly isolated BM-derived mMSCs do not express

CD44, but gain expression during adherent *in vitro* expansion [Qian, Le Blanc, and Sigvardsson 2012]. This was confirmed herein. Another study has shown that human MSCs and skin fibroblasts express the same markers *in vitro*, e.g CD44 and CD29 [Whitney et al. 2009]. It is therefore necessary to establish cell surface markers which are not affected by adherent *in vitro* conditions. Also, even with homogenous expressed markers, bulk cultures are still heterogeneous. This is further supported by *in vitro* differentiation assays, where only subsets of cells undergo differentiation. In Figure 2.7 it can be seen that in osteo- and adipogenic *in vitro* differentiation assays, not all cells undergo differentiation.

For the definition of MSCs, it is generally accepted that there have to undergo tri-lineage differentiation. Unfortunately, the differentiation of mMSCs is more difficult than it is for hMSCs. This is particularly the case for chondrogenic differentiation capacity which seems to be less in C57Bl/6 mice compared to other mouse strains [Peister et al. 2004]. Due to this, not all researchers include all three lineages in their differentiation assays and frequently only focus on osteo- and adipogenic differentiation assays [Ooi et al. 2013; Siclari et al. 2013]. One reason for this is that the ideal conditions for the efficient differentiation of chondrocytes from mMSC have yet to be found.

Another problem is the naturally low abundance of stem cell-like cells in murine bone marrow. Even though a high CFU-F frequency was achieved using the Sca-1^+/PDGFRα^+/$CD90^+$ marker combination, cells needed to be passaged and expanded *in vitro*, to generate sufficient numbers for further analysis. Unfortunately, due to insufficient *in vitro* conditions these strategies can affect the differentiation and clonogenic potential as well as senescence of MSCs [Kretlow et al. 2008; Gruber et al. 2012].

Based on the results from Chapter 3, *in vitro* culture condition may alter the phenotype of cells isolated by FCM. Clonally isolated Sca-1^+/PDGFRα^+/$CD90^+$ cells became senescent with further passaging which restricted the number of clones analysed. Especially the differentiation media seemed to negatively affect the cells and their proliferation. Based on the assumption that these cells are homogenous,

the lack of supporting cells (i.e. the niche) might have a negative impact on maintaining the true state of these cells. It is therefore necessary for future research to optimise culture conditions for clonally isolated cells. This could be done by the use of ECM, which could mimic certain niche features.

Due to the difficulties in the translation of MSCs into the clinical setting, some researchers suggest overhauling the classical approach of market approval and regulation of stem cell products by health professionals and scientists, to a more consumer choice based model [Salter, Zhou, and Datta 2014]. On the other hand, scientists believe that based on the 60 years of research it took to establish HSC therapy, more fundamental research needs to be done to fully understand the risks and benefits of MSCs, before the application of new MSC products [Prockop, Prockop, and Bertoncello 2014]. I too believe that more systematic and thoroughly based research needs to be done, not only to fully understand the working mechanisms of MSCs, but also to avoid false hopes in patients.

MSCs are a very versatile subset of cells able of tri-lineage differentiation, immunomodulation and homing to different anatomic regions. This inherent "plasticity" of MSCs might be the key to most of the beneficial effects of MSCs in animal disease models and human clinical trials. Therefore, it might be more effective to identify subsets of epitopes or a combination of known surface markers that can distinguish functional differences between population rather than identifying "the one" MSC.

5.3 FUTURE DIRECTIONS

In this thesis it was shown that hypoxia influences the CFU-F frequencies and proliferation of isolated MSCs (Chapter 3). However, the differences between normoxic and hypoxic conditions ranged over a wide spectrum (21% vs 5% vs 2%) and were not consistent during expansion. Reasons for this include the fact that the incubators were shared with other researchers, and therefore a lot of opening and closing of incubator doors which changed the oxygen conditions for a short

time. Furthermore, passaging and feeding the cells was done in a standard cell culture hood and therefore in normoxic conditions. These small changes in oxygen conditions might be important, as a recent study has shown that oxygen concentrations in the mouse BM might only differ by a small percentage [Spencer et al. 2014]. Using two-photon phosphorescence lifetime microscopy, Spencer *et al.* have shown that despite its high vascularity, the oxygen concentrations in the BM is less than 4.2% O_2. Furthermore, dependent on the location within the BM niche, oxygen concentrations showed subtle differences which could not be mimicked by our *in vitro* conditions. It might therefore be necessary to establish more accurate control of oxygen concentrations in cell cultures in order to mimic the native niche of mouse MSCs.

Additionally, the BM niche is a dynamic environment not only influenced by hypoxia, but also by other environmental factors including matrix proteins and cytokines (Chapter 1). It has been shown that MSCs express and produce HSC maintenance factor transcripts like CXCL12 and SCF [Mendez-Ferrer et al. 2010; Greenbaum et al. 2013]. It might therefore be necessary to supplement MSC cell culture medium with certain factors to mimic niche feedback mechanisms. This is especially important for clonally isolated cells, as they do not have any supporting microenvironment like bulk isolated MSCs. The same is true for the use of ECM, which could mimic certain niche features and therefore help to maintain the native phenotype of isolated MSCs. By culturing MS-5 cells and following gentle lysis by osmotic shock, I was able to generate ECM which is rich in collagen (Figure 4.2). However, functional analysis of matrix proteins and refinement of the ECM lysis protocol is necessary to obtain a homogenous cell product for the use in MSC *in vitro* protocols.

APPENDIX I: Media

GROWTH MEDIA

MSC Growth Medium

α-MEM with GlutaMAX

FBS (10%)

Equine serum (10%)

Penicillin/Streptomycin (100 units/µg/ml)

T-cell Medium

High Glucose DMEM

FBS (10%)

L-Glutamine (1%)

HEPES (1%)

Non-essential amino acids (1%)

2-mercaptoethanol (0.1%)

Penicillin/Streptomycin (100 units/µg/ml)

Cell Line Growth Medium

High Glucose DMEM

FBS (10 %)

Penicillin/Streptomycin (100 units/µg/ml)

DIFFERENTIATION MEDIA

Osteogenic Differentiation Medium

α-MEM with GlutaMAX

Dexamethasone (100nM)

Ascorbic acid-2-phosphate (50µM)

β-glycerophosphate (20mM)

L-thyroxine (50ng/ml)

FBS (10%)

Equine serum (10%)

Adipogenic Induction Medium

High glucose DMEM

Dexamethasone (1µM)

Insulin (10µg/ml)

Indomethacin (200µM)

3-Isobutyl-1-Methyl-Xanthine (500µM)

FBS (10%)

Penicillin/Streptomycin (100

Penicillin/Streptomycin (100 units/µg/ml)

Adipogenic Maintenance Medium

High glucose DMEM

Insulin (10µg/ml)

FBS (10%)

Penicillin/Streptomycin (100 units/µg/ml)

Chondrogenic Differentiation Medium

High Glucose DMEM

Dexamethasone (100nM)

Ascorbic acid-2-phosphate (50µg/ml)

L-Proline (40µg/ml)

Bovine insulin (6.25µg/ml)

Transferrin(6.25µg/ml)

Selenous acid(6.25µg/ml)

Linoleic acid (5.33µg/ml)

Bovine Serum Albumin (1.25mg/ml)

Sodium pyruvate (1mM)

Penicillin/Streptomycin (100 units/µg/ml)

APPENDIX II: Buffers

FACS Buffer

PBS

FBS (2%)

Sodium azide (0.5mg/ml)

MACS Buffer

PBS

Bovine Serum Albumin (0.5%)

EDTA (0.2mM)

SORT Buffer

Ca^{2+}/Mg^{2+}-free PBS

FBS (1%)

HEPES (25mM)

EDTA (2mM)

ECM Lysis Buffer

Tris (10mM)

EDTA (1mM)

pH 7.4

APPENDIX III: Reagents

Reagent	Supplier	Catalogue number
0.25% trypsin/EDTA	Sigma-Aldrich	T4049
10% neutral buffered formalin	Sigma-Aldrich	HT501128
2-mercaptoethanol	Sigma-Aldrich	M3148
α-MEM	Gibco-Invitrogen	32561
Anti-mouse CD45 microbeads	Miltenyi Biotec Inc.	130-052-301
Bovine serum albumin	Sigma-Aldrich	A2153
High Capacity cDNA reverse transcription kit with RNase inhibitor®	Applied Biosystems	4374966
CellTrace CFSE cell proliferation kit®	Molecular Probes-Invitrogen	C34554
Chloroform	Sigma-Aldrich	496189
Collagenase I	Sigma-Aldrich	C9891
Collagenase D	Roche	11088858001
Dimethyl sulfoxide(DMSO)	Sigma-Aldrich	D2650
Disodium EDTA	Sigma-Aldrich	E5134
DMEM	Sigma-Aldrich	D6429
DNase I	Sigma-Aldrich	DN25
Dulbecco's PBS	Gibco-Invitrogen	14190
Ethanol	Sigma-Aldrich	E7023
Fast green	Sigma-Aldrich	F7252
Glycerol	Sigma-Aldrich	G5516
HEPES	Sigma-Aldrich	H0887
Hyclone equine serum	Sigma-Aldrich	H1270
Hyclone fetal bovine serum (FBS)	Fisher Scientific Ireland	SV30143.03

Indomethacin	Sigma-Aldrich	I7378
Isopropanol	Sigma-Aldrich	I9516
Methanol	Sigma-Aldrich	34860
Non-essential amino-acids	Sigma-Aldrich	M7145
Penicillin/streptomycin	Gibco-Invitrogen	15140-122
Phosphate buffered saline tablets	Sigma-Aldrich	P4417
Protease	Sigma-Aldrich	P5147
RNA Later® solution	Applied Biosystems	AM7020
RNA zap	Applied Biosystems	AM9780
Sodium azide	Sigma-Aldrich	A2152
SYBR green I master	Roche	04707516001
Sytox® Blue	Invitrogen	S10349
Trizol®	Invitrogen	15596018

APPENDIX IV: Antibodies

Antibody preparation	Species/Isotype	Clone	Supplier
Biotin-conjugated			
Collagen I	Rabbit	polyclonal	Antibodies-online
F4/80	Rat $IgG_{2a,\kappa}$	BM8	eBioscience
APC-conjugated			
CD29	Armenian Hamster IgG	HMb1-1	eBioscience
PDGFRα	Rat $IgG_{2a,\kappa}$	APA5	eBioscience
Galectin-3	Rat $IgG_{2a,\kappa}$	M3/38	BioLegend
FITC-conjugated			
Sca-1 (Ly6A/E)	Rat $IgG_{2a,\kappa}$	D7	eBioscience
CD45	Rat $IgG_{2b,\kappa}$	30-F11	eBioscience
CD49e	Armenian Hamster IgG	HMa5-1	eBioscience
CD106 (VCAM-1)	Rat $IgG_{2a,\kappa}$	429	eBioscience
PE-conjugated			
CD31	Rat $IgG_{2a,\kappa}$	390	eBioscience
CD44	Rat $IgG_{2b,\kappa}$	IM7	eBioscience
CD73	Rat IgG_1	TY/11.8	eBioscience
CD90.2	Rat $IgG_{2a,\kappa}$	53-2.1	BD Biosciences
CD105	Rat $IgG_{2a,\kappa}$	MJ7/18	eBioscience
PE-Cy7-conjugated			
CD45	Rat $IgG_{2b,\kappa}$	30-F11	BD Biosciences
V450-conjugated			
CD45	Rat $IgG_{2b,\kappa}$	30-F11	BD Biosciences
Ter119	Rat $IgG_{2b,\kappa}$	TER-119	BD Biosciences
Purified			
CD3	Armenian Hamster IgG	145-2C11	eBioscience
CD28	Syrian Hamster IgG	37.51	eBioscience

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