THE EFFECT OF PDGF, FGF AND TGF-β ON THE PROLIFERATION, DIFFERENTIATION AND IMMUNOMODULATORY FUNCTIONS OF PROSPECTIVELY-ISOLATED MURINE MESENCHYMAL STEM CELLS

by

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A thesis submitted to the

University of Birmingham

in partial fulfilment of the requirements for the degree of

MASTER OF RESEARCH

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August 2011

Abstract

Mesenchymal stem cells (MSCs) are a subset of non-haematopoietic, multipotent stem cells found in bone marrow (BM) that are able to differentiate into a variety of lineages. Their plasticity, combined with a potent immunosuppressive ability has led to MSCs being considered as a potential cell source for future therapies. However, progress in elucidating the mechanisms behind the clinical improvements seen in rodent models has been hampered by the heterogeneous populations of MSCs used in most studies, a direct consequence of the plastic-adherence method of MSC isolation.

In this study, we prospectively isolated MSCs (PDGFRα^{+}Sca-1^{+}CD45^{-}TER-119^{-}) from murine BM and examined the effect of PDGF, FGF and TGF-β signalling. Our results showed a marked increase in MSC proliferation in the presence of growth factors (GFs). Addition of PDGF or FGF skewed the differentiation of MSCs towards the adipogenic lineage but reduced their immunosuppressive capabilities. Conversely, TGF-β strongly inhibited both osteogenic and adipogenic differentiation while enhancing their immunomodulatory functions. The findings of this study show that it is possible to 'prime' MSCs towards distinct lineages while maintaining their proliferative capacity. This should facilitate research in identifying 'optimally therapeutic' populations of MSCs for use in future therapies.

Acknowledgements

I would like to take this opportunity to thank my supervisor, Dr Diarmaid Houlihan, for his help, guidance, training and friendship over the duration of this project. I would especially like to acknowledge his pro-active method of teaching, allowing me to achieve a deeper understanding of this field.

I would also like to express my gratitude to all members of the Centre for Liver Research for making me feel welcome and to Janine for her help with cryosectioning. In addition, I would also like to thank Kesley for performing the T-cell proliferation assays reported in this manuscript.

Finally, this project would not have been possible without the support provided by Dr Phil Newsome, who has given me the opportunity to work in his group.

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List of Abbreviations

β-gal	β-galactosidase
Ang-1	Angiopoietin-1
APC	Antigen presenting cell
BM	Bone marrow
CFU-F	Colony forming unit-fibroblastic
FACS	Fluorescence activated cell sorting
FGF2	Fibroblast growth factor 2
GF	Growth factor
HSC	Haematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem cell
NO	Nitric oxide
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PGE_2	Prostaglandin E2
PI	Propidium Iodide
Sca-1	Stem cell antigen-1
sHLA-G5	Soluble human leukocyte antigen-G5
Tconv	Conventional T cells
TGF-β	Transforming growth factor-β
Treg	T regulatory cell
vSMC	Vascular smooth muscle cell

Chapter 1

Introduction

1.1 Mesenchymal Stem Cells: A Historical Perspective

The term “Mesenchymal Stem Cell” (MSC) was first coined, somewhat prematurely, in 1991 by Arnold Caplan (Caplan, 1991). By altering the seeding density, he was able to push chick limb bud mesodermal cells down osteogenic or chondrogenic lineages, prompting him to hypothesise about the existence of multipotent mesenchymal precursors that were able to contribute to various mature mesenchymal lineages via distinct pathways (termed “The Mesengenic process”; Figure 1; Caplan, 1994). Caplan’s work can be traced back to a series of seminal papers by Friedenstein and colleagues who showed that cells with osteogenic potential resided in the non-haematopoietic section of bone marrow (BM; Friedenstein et al., 1974, Friedenstein et al., 1970). These cells were characterised by their ability to adhere to tissue culture plastic, spindle-shaped morphology, capacity to form colonies (colony forming unit-fibroblastic, CFU-F), and their ability to differentiate towards bone, cartilage and fat (Pittenger et al., 1999). Over 40 years later, the same four tenets (combined with the expression of certain surface markers) are still viewed as the benchmark criteria to define putative MSCs (Dominici et al., 2006), a system that has come under increasing scrutiny over the past decade (Bianco et al., 2008).

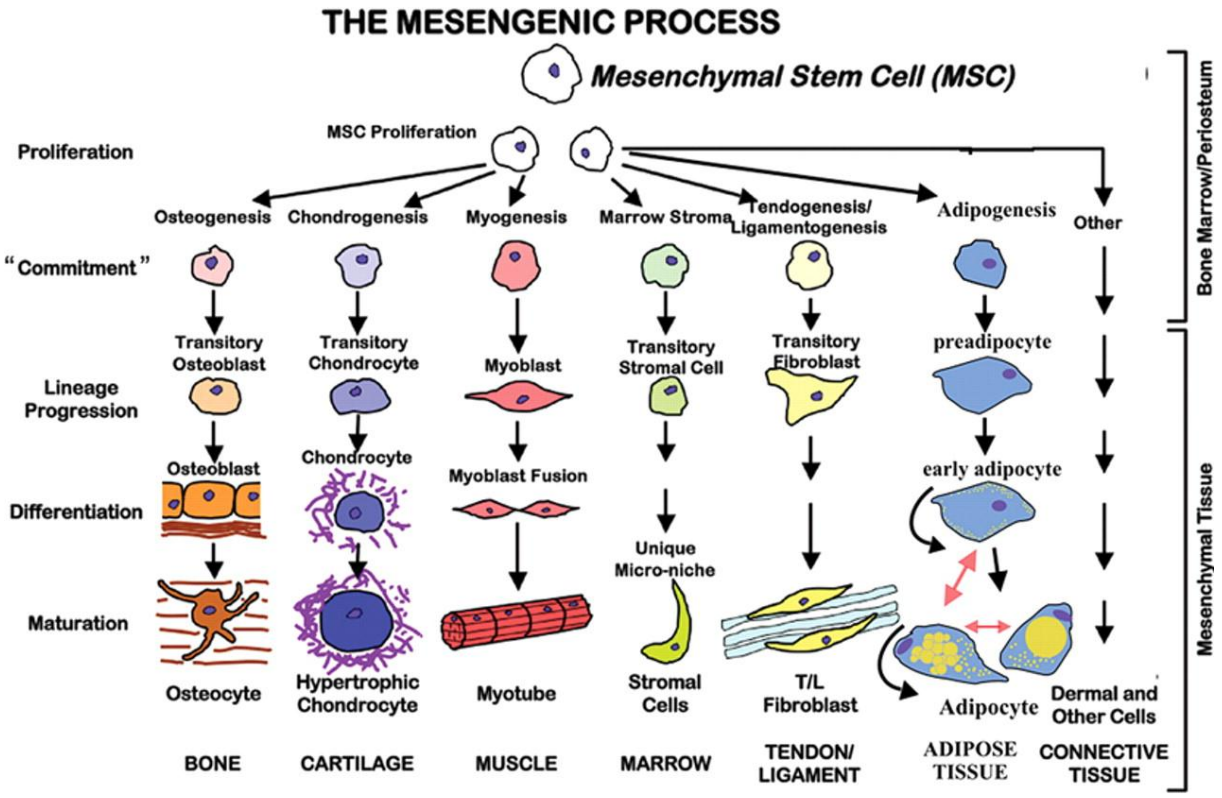

Figure 1 | **The Mesengenic Process**. This hypothetical pathway describes the ability of MSCs to differentiate via distinct pathways to form mature mesenchymal tissues. Figure taken from Caplan, 1991.

1.2 Therapeutic Potential of MSCs

In addition to the well characterised ability of MSCs to differentiate towards multiple mesenchymal lineages, they have also been shown to differentiate *in vitro* into a variety of clinically relevant non-mesenchymal lineages (Engler et al., 2006). Their plasticity, combined with a potent immunosuppressive ability has led to MSCs being considered as a potential cell source for stem cell therapies (Nombela-Arrieta et al., 2011).

Early rodent studies demonstrated the safety and efficacy of allogenic MSC infusions in multiple preclinical models, including myocardial infarction (Orlic et al., 2001) and renal disease (Kale et al., 2003). The clinical translation of this research has yielded positive results

in small-scale human trials looking at graft-versus-host disease (Le Blanc et al., 2008), osteogenesis imperfecta (Horwitz et al., 1999), and haematopoietic malignancies (Ning et al., 2008). However, preliminary reports from a large-scale, placebo-controlled trial utilising MSCs to treat chronic obstructive pulmonary disease showed no significant improvements in pulmonary function over controls, highlighting the urgent need to fully understand the fate of MSCs following infusion (Ankrum and Karp, 2010).

The current hypothesis states that MSCs migrate to injured sites and indirectly encourage tissue repair via the secretion of trophic factors (English et al., 2010). However, the mechanisms by which transplanted MSCs home and engraft are still unclear (Karp and Teol, 2009). Further studies on mechanisms are hampered by the heterogeneous nature of MSC populations used in different studies, a direct consequence of the isolation techniques employed. Additionally, the majority of studies have utilised human or rat MSCs due to difficulties in isolating and culturing their murine equivalents (Sun et al., 2003). These issues have, until recently, prevented the field from studying the basic biology of MSCs using the large number of transgenic mouse models available.

1.3 Isolation of Murine MSCs

The reason behind variability in MSC functions reported in previous literature is widely attributed to be the diverging isolation techniques used (Nombela-Arrieta et al., 2011). The isolation method, strain of mouse, seeding densities and medium formulations all have the potential to impact on MSC growth and function. In general, there are three main ways to isolate mouse MSCs: (1) plastic adherence; (2) immune depletion of haematopoietic cells; and (3) the prospective identification of MSCs.

1.3.1 Plastic Adherence

Initial attempts to isolate and culture murine MSCs took the same approach as their human equivalents, i.e. flushing BM onto plastic and culturing them for extended periods of time to remove contaminating cells. Immediately it was apparent that the plastic-adherent fraction displayed varying growth kinetics and differentiation capabilities, and up to 80% of the population were positive for CD11b and CD45, markers of leukocytes and haematopoietic cells (Peister et al., 2004, Phinney et al., 1999).

Nadri and colleagues attempted to remove contaminating haematopoietic cells on the basis of frequent medium changes and shorter trypsinisation times (Nadri et al., 2007). They demonstrate that changing the culture medium 3 hours after seeding and every 8 hours afterwards prevents the adherence of haematopoietic cells. Additionally, by shortening the trypsinisation time to 2 minutes, they were able to lift off MSCs while leaving behind firmly adherent macrophages.

A recent publication by Zhu *et al*. further refines the plastic adherence method (Zhu et al., 2010). It has long been thought that MSCs occupy an endosteal niche in cortical bone, and flushing the BM enriches the haematopoietic population while leaving MSCs behind. The authors combated this problem by digesting bones with collagenase before setting up an "explant culture system" where putative MSCs migrate out and grow (Zhu et al., 2010).

1.3.2 Immunodepletion of non-MSCs

In general, plastic adherence has failed to yield 100% pure MSC populations due to the fact that murine BM contains a relatively high percentage of haematopoietic cells (Sun et al., 2003). Additionally, the adherent stromal fraction of mouse BM has been shown to support B-cell lymphopoiesis and granulopoiesis without the addition of exogenous cytokines, resulting in the long term maintenance of contaminating cells (Phinney, 2008).

Baddoo and co-workers were the first to describe a protocol to alleviate this issue (Baddoo et al., 2003). The authors subjected 8-10 day old plastic-adherent cells to three rounds of immunodepletion with antibodies against CD11b (leukocytes), CD34 (haematopoietic progenitors), and CD45 (haematopoietic cells). The immunodepleted fraction (23±8% of plastic adherent cells) readily differentiated into bone, fat and cartilage and expressed typical markers of murine MSCs (Figure 2). However, immunodepleted cells displayed a prolonged doubling time of 5-7 days due to the downregulation of genes regulating cell cycle progression (Baddoo et al., 2003).

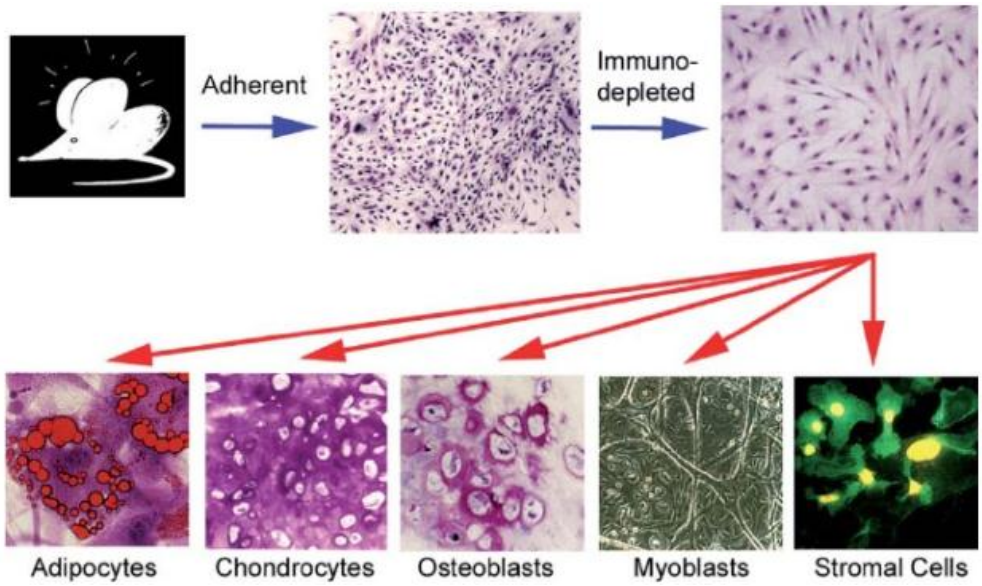

Figure 2 | **Immunodepletion of non-MSCs.** This schematic shows how primary cultures of BM, after immunodepletion, yield a relatively homogenous population of spindle-shaped MSCs. These MSCs have the potential to differentiate *in vitro* into the lineages depicted. Figure taken from Phinney, 2008.

1.3.3 Prospective Isolation of Murine MSCs

Over the past decade, countless parallels between MSCs and the more extensively characterised haematopoietic stem cells (HSCs) have been drawn. HSCs are viewed as the "prototypical stem cell", as a single, prospectively isolated murine HSC has the ability to serially reconstitute the haematopoietic system in irradiated mice (Krause et al., 2001). In doing so, HSCs meet the two benchmarks used to define stem cells: multipotency and self-renewal. Their multipotency has been proven at a single-cell level by *in vivo* transplantation, and their self-renewal by their ability to serially reconstitute a tissue compartment identical in function to the one explanted (Bianco et al., 2008). In contrast, MSCs have, until recently, failed to meet these strict criteria since the biology of MSCs has been inferred from the study of *in vitro* cultured cells (Meirelles et al., 2008). This has prevented us from studying the functions of MSCs *in vivo*, a limitation which had remained in place until the first papers detailing prospective methods to isolate MSCs were published.

In a landmark study, Morikawa and colleagues were the first to publish a method to prospectively identify and isolate murine MSCs (Morikawa et al., 2009). The authors used fluorescence activated cell sorting (FACS) to select for non-haematopoietic ($CD45^-TER119^-$) BM cells that co-expressed platelet derived growth factor receptor alpha (PDGFRα) and stem cell antigen-1 (Sca-1). This double-positive population (PαS cells) showed robust proliferation and 120,000-fold higher CFU-F frequency than whole BM, and was also able to undergo tri-lineage differentiation at the clonal level. PαS cells resided in the perivascular space of cortical bone *in vivo* and expressed both angiopoietin-1 (Ang-1) and CXCL12, two necessary factors for haematopoiesis. This suggests that BM MSCs might function as haematopoietic niche cells *in vivo*, as previously hypothesised (Meirelles et al., 2008). Most importantly, the authors demonstrated that PαS cells could reconstitute their *in vivo* niche. Systemic co-transplantation of 10,000 PαS cells alongside 100 CD34-KSL HSCs restored haematopoiesis in irradiated mice. Significant numbers of transplanted PαS cells were located in the perivascular region of cortical bone alongside vascular smooth muscle cells (vSMC; Figure 3). Some cells expressed CXCL12 and Ang-1, and others were found to give rise to functioning osteoblasts or adipocytes. Furthermore, the authors were able to isolate secondary CFU-Fs from recipient mice and show they retained their tri-lineage differentiation potential. Thus, this paper was the first to convincingly show the *in vivo* self-renewal and multipotency of a specific cell type that displays many of the hypothesised functions of MSCs.

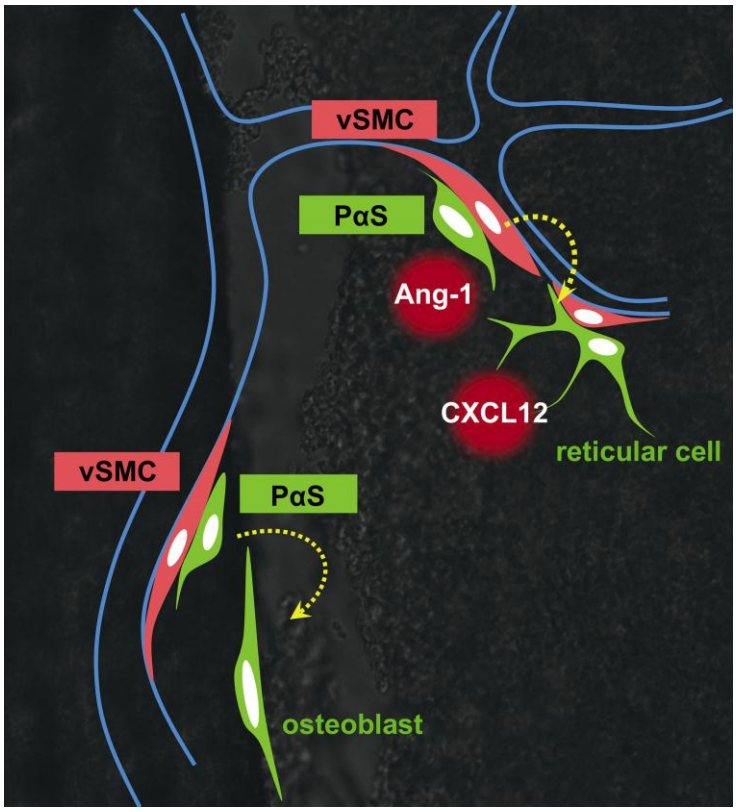

Figure 3 **| *In vivo* location of PαS cells in BM**. PαS cells can be found in the perivascular space alongside vSMCs. They can give rise to both osteoblasts and reticular cells that function as haematopoietic niche cells. Figure taken from Morikawa et al., 2009.

A more recent paper identified that Nestin^{+} MSCs in BM formed an essential haematopoietic niche (Mendez-Ferrer et al., 2010). Nestin^{+} cells were found expressing high levels of CXCL12 and Ang-1 in the perivascular region of cortical bone. These cells underwent tri-lineage differentiation at the clonal level, and were able to form multipotent, self-renewing "mesenspheres" *in vitro*. The authors convincingly show *in vivo* self-renewal activity at the single cell level using a heterotrophic bone ossicle assay (Figure 4). They also showed, for the first time, that Nestin^{+} MSCs contribute to the physiological skeletal remodelling via differentiation into osteoblasts, osteocytes and chondrocytes *in vivo*. Even more

impressively, the authors proved that Nestin^{+} MSCs are essential for the maintenance of HSCs in BM. The selective deletion of Nestin^{+} cells resulted in a 50% reduction in CD34-KSL HSCs two weeks after treatment. Depletion of Nestin^{+} cells also significantly reduced (by 90%) the homing of HSCs to the BM in lethally irradiated mice.

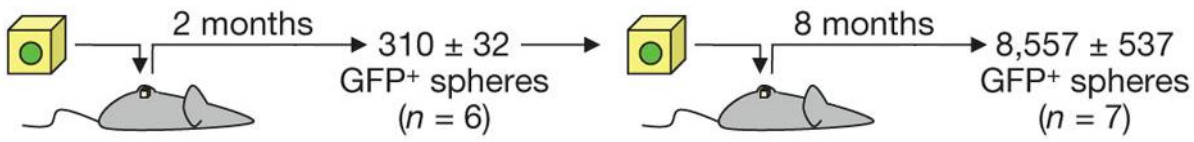

Figure 4 | **Experimental schematic of the *in vivo* self-renewal of BM Nestin^{+} MSCs.** Single clonal mesenspheres attached onto calcium phosphate hydroxyapatite cubes were transplanted subcutaneously into recipient mice and left for two months. From these, 310 Nestin^{+} secondary mesenspheres were isolated, of which 38% showed multilineage differentiation. Single secondary spheres were transplanted into recipient mice and left for 8 months. A total of 8,557 Nestin^{+} tertiary mesenspheres could be isolated, demonstrating the remarkable self-renewal properties of these cells in serial transplantations. Figure taken from Mendez-Ferrer et al., 2010.

1.4 MSC-mediated Immunosuppression

One of the most exciting and clinically-relevant findings of MSC biology is their potent immunomodulatory functions (Uccelli et al., 2008). This finding prompted rapidly growing interest in the use of MSCs as a cellular therapy for autoimmune diseases alongside the more "traditional" uses of MSCs in regenerative medicine.

1.4.1 Mechanisms of Immunosuppression

The mechanisms behind the effects of MSCs on the innate and adaptive immune systems are not well understood (Ben-Ami et al., 2011). So far, we know that MSCs need to be in an inflammatory environment to "switch on" certain immunomodulatory functions (Ryan et al., 2007, Krampera et al., 2006). Once activated, MSCs can secrete a plethora of soluble factors that mediate immunosuppression (summarised in Figure 5). Neutralisation of one or more of these factors does not result in complete reversal of immunosuppression, suggesting that there are other (possibly non-soluble) factors in play (Nauta and Fibbe, 2007). To this end, a degree of cell-cell contact might be required as the immunosuppressive effects were weaker when transwell culture systems were used (Ben-Ami et al., 2011).

The innate immune system is the first line of defence against pathogens in vertebrates (Parkin and Cohen, 2001). The effect of MSCs on the innate system has not been explored in detail, but we know that secretion of interleukin (IL)-6 and prostaglandin E2 (PGE_2) by activated human MSCs inhibits the maturation and function of monocyte-derived dendritic cells, impairing their ability to function as antigen presenting cells (APC; Nauta et al., 2006). MSCs can also inhibit natural killer (NK) cell proliferation via the secretion of PGE_2 and

indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the breakdown of tryptophan (an essential amino acid required for leukocyte proliferation; Spaggiari et al., 2008). Finally, MSCs have also been shown to inhibit the production of free radicals by neutrophils via IL-6 secretion, thereby dampening the respiratory burst response (Raffaghello et al., 2008).

The adaptive immune system is the second, more specific line of defence against pathogenic attack (Parkin and Cohen, 2001). Studies focusing on the effects of MSCs on the adaptive immune system are more numerous and detailed than their innate counterparts (Uccelli et al., 2008). A variety of soluble factors have been implicated in MSC-mediated $CD4^+$ T cell suppression, including PGE_2, IDO, and iNOS (Ben-Ami et al., 2011). This suppression is mediated by the arrest of T cells in the G_0/G_1 phase of the cell cycle (Glennie et al., 2005). Additionally, MSC co-culture reduces IFN-γ production by T_H1 cells and increases IL-4 production by T_H2 cells, skewing the immune response towards an anti-inflammatory state (Aggarwal and Pittenger, 2005). MSCs that were pulsed with peptides from viral antigens were resistant to $CD8^+$ T cell-mediated lysis *in vitro* (Rasmusson et al., 2007). This was due to the constitutive release of soluble HLA-G5 (sHLA-G5; Morandi et al., 2008). Finally, MSCs have been shown to have a positive effect on T regulatory cell proliferation (Treg; Selmani et al., 2008) while inhibiting B cell antibody release (Corcione et al., 2006).

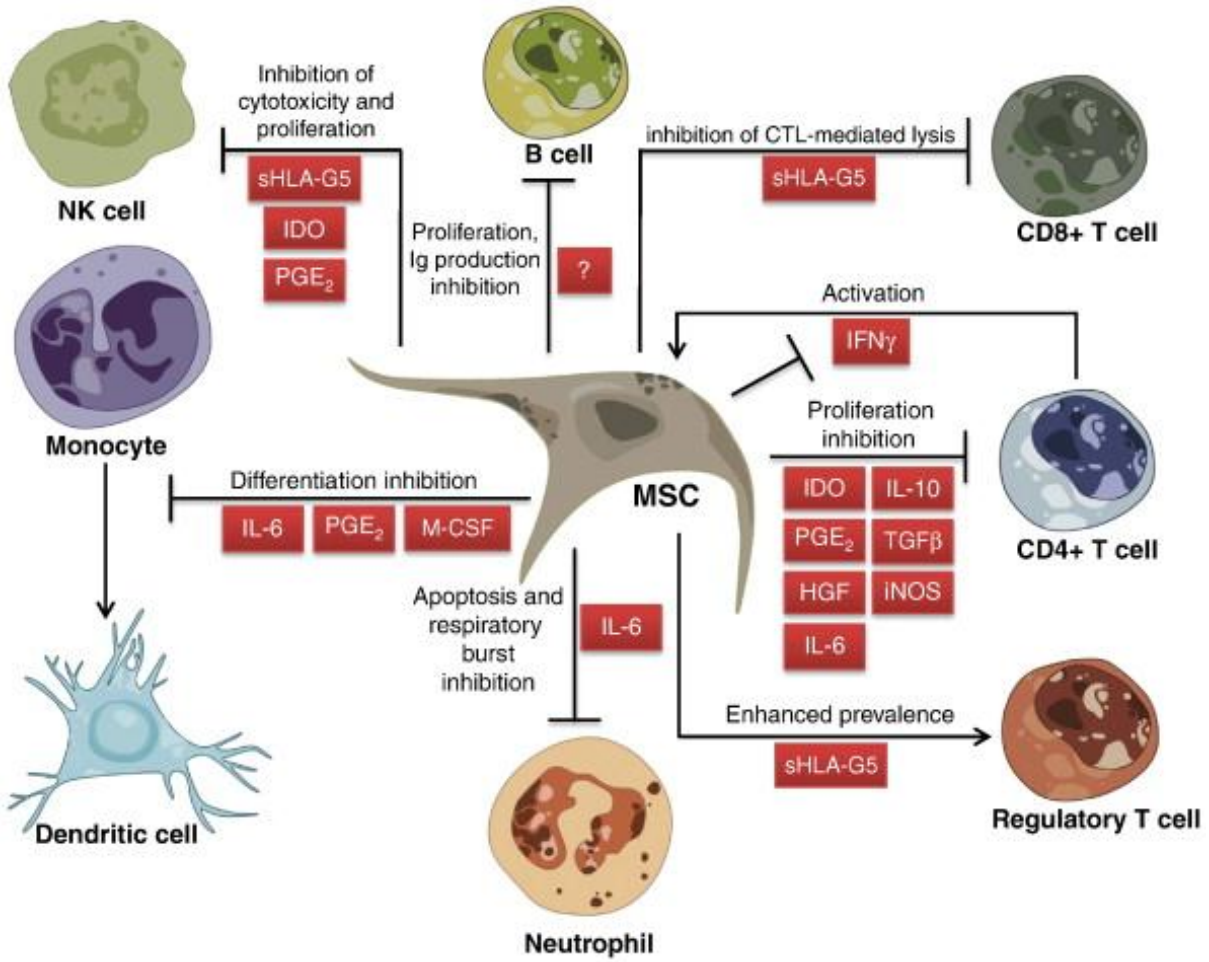

Figure 5 | **Mechanisms of MSC-mediated immunosuppression**. This diagram summarises most reported mechanisms by which MSCs regulate both the innate and adaptive immune systems. Figure taken from Ben-Ami et al., 2011. Abbreviations (in addition to those found in text): M-CSF, macrophage colony stimulating factor; HGF, hepatocyte growth factor; iNOS, inducible nitric oxide synthase.

1.4.2 Caveats to MSC-mediated Immunosuppression

While the results described above seem promising, it is important to note the ratios of MSC:leukocytes used in most studies (1:1 to 1:100) are much higher than physiological levels (Le Blanc and Ringden, 2007). Some groups have reported a stimulatory effect on T cell proliferation with low (1:100) numbers of MSCs (Le Blanc et al., 2003), while others report

an inhibitory response at the same concentration (Ding et al., 2009). Reasons for conflicting data include the heterogeneity of MSC populations, species-specific differences, culture conditions and the method of leukocyte stimulation. Despite these discrepancies, it is widely accepted that MSCs need to be at high concentrations (>1:10) to fully inhibit lymphocyte proliferation, and such high numbers are unfeasible for therapeutic uses.

One potential risk of MSC-mediated immunosuppression is their ability to promote tumour growth by dampening immune responses against the tumour (English et al., 2010). MSCs are known to constitutively secrete various pro-angiogenic and matrix degrading proteins (Meirelles et al., 2009). Studies on the effect of MSCs on tumour growth have yielded contradictory results (Aboody et al., 2008). An interesting study by Ramasamy *et al*. identified that while human MSCs inhibited tumour cell proliferation *in vitro*, they promoted growth when co-transplanted *in vivo* (Ramasamy et al., 2006). Zhu and co-workers also showed that co-transplantation of human MSCs with tumour cells favoured tumour growth (Zhu et al., 2006). Pathological examination of the tumour revealed rich angiogenesis and extensive invasion into surrounding tissue, suggesting a role for MSC-derived VEGF or metalloproteinases (MMPs). Conversely, MSC infusion inhibited tumour growth in an established Kaposi's sarcoma model (Khakoo et al., 2006). These conflicting results again highlight the urgent need to move to purer populations of prospectively-identified MSCs in future studies.

1.5 Lineage Priming of MSCs

Lineage priming can be defined as a model of stem cell differentiation in which a given stem cell expresses a subset of genes related to the lineage they are already committed to differentiate into (Delorme et al., 2009). This model has been extensively studied and proven in the HSC field (Månsson et al., 2007), but there is only one paper that has applied this concept to MSCs (Delorme et al., 2009). In this study, Delorme and colleagues show that clonal populations of human and mouse MSCs are primed towards the osteogenic, adipogenic and chondrogenic lineages independent of any differentiation-inducing stimuli. The importance of this finding is that MSC differentiation into non-primed lineages (e.g. hepatocytes) would require extensive epigenetic reprogramming to turn off the "core program" and switch on novel pathways (Delorme et al., 2009).

The concept of lineage-primed MSCs is also important when looking at therapeutic uses of these cells. For example, a surgeon would ideally want MSCs primed towards the osteogenic lineage to treat patients with skeletal defects, while chondrocyte-primed MSCs would be ideal in patients with cartilage disorders. When transplanted, these lineage-primed MSCs would readily differentiate into bone and cartilage respectively, reducing the risk of differentiating towards an unwanted cell type.

To this end, Ng *et al*. used a broad transcriptomics approach to identify platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), and transforming growth factor-β1 (TGF-β1) as key molecules in human MSC proliferation and differentiation (Ng et al., 2008).

PDGF signalling was active during adipogenesis and chondrogenesis; TGF-β signalling was active during chondrogenesis; and FGF signalling was active during osteogenesis, adipogenesis and chondrogenesis. Inhibition of any of the 3 pathways resulted in an altered differentiation potential. In addition to their roles in MSC differentiation, PDGF, FGF and TGF-β were also important for MSC proliferation. Inhibition of these pathways resulted in cell death or significantly increased population doubling times, while the addition of all three growth factors (GFs) was sufficient to maintain MSCs in serum-free media (Ng et al., 2008). The finding of Ng and co-workers enables researchers to prime MSCs towards distinct lineages while maintaining MSCs in a proliferative state. The use of a lineage-primed population would allow us to infuse lower numbers of MSCs into patients to reach the same beneficial outcome, thereby reducing the risk of pulmonary emboli seen in high-dose MSC infusions (Furlani et al., 2009). The potential benefits of lineage-primed MSCs forms the basis of this present study, as described in the following section.

1.6 Project Aims and Objectives

MSCs hold great promise in future uses as a regenerative or immunosuppressive therapy. However, there is plenty of conflicting data in published literature that has fuelled speculation about the potency of these cells (Bianco et al., 2008). The major confounding factor is widely attributed to be the isolation technique used. As described previously, most experiments to date have utilised heterogeneous populations of plastic-adherent cells that fail to meet the requirements of a true stem cell. Two recent publications detailing the prospective isolation of murine MSCs (Mendez-Ferrer et al., 2010, Morikawa et al., 2009) have the potential to revolutionise this field, but the uptake of these techniques by the wider scientific community has been slow. If the claims made by both groups are reproducible, we would venture that all further studies on murine MSCs utilise these cells exclusively.

In this study, we set out to prospectively identify and isolate murine MSCs based on the Morikawa method (Morikawa et al., 2009). We then attempted to lineage-prime MSCs, following on from previous work identifying PDGF, FGF and TGF-β as key factors in MSC proliferation and differentiation (Ng et al., 2008). One key strength of this study is that we are able to observe the effects of the aforementioned GFs on a pure population of MSCs directly after isolation, thereby avoiding any bias associated with a culture-manipulated starting population. Finally, we investigated the immunosuppressive properties of lineage-primed PαS cells in T cell proliferation assays.

The successful completion of this project should contribute to the current “state of the art” regarding MSC biology. The use of prospectively identified MSCs would allow us to tease out the functional characteristics of MSCs and the mechanisms behind MSC-mediated immunosuppression using a homogenous starting population. By increasing our understanding of these elusive cells, we can hopefully facilitate the creation of more effective therapies for patients suffering from skeletal defects or autoimmune diseases.

Chapter 2

Methods

2.1 Prospective Isolation of Murine MSCs

The prospective isolation of PαS cells was performed as described previously (Morikawa et al., 2009). Briefly, 8-10 week old C57BL/6 (Harlan, Oxon, UK) or BALB/c (Jackson Laboratory, Bar Harbor, USA) mice were sacrificed and their femurs and tibias collected. Cleaned bones were gently crushed using a pestle and mortar and incubated in DMEM (Invitrogen, Paisley, UK) supplemented with 0.2% Collagenase A (Wako Chemicals, Neuss, DE) and 1x Penicillin/Streptomycin/Glutamine (P/S/G) solution (Invitrogen) for 60 minutes at 37°C. Following incubation, the cell suspension was filtered through a cell strainer (BD Biosciences, Oxford, UK) and kept on ice. Larger bone fragments were collected in a pestle and washed repeatedly with ice-cold Hank's balanced salt solution (HBSS; Invitrogen) supplemented with 2% foetal bovine serum (FBS; Invitrogen), 10mM HEPES (Sigma-Aldrich, Dorset, UK) and 1X P/S/G solution to remove any firmly adherent cells lining the endosteum. The resulting cell suspension was pelleted by centrifugation at 280 *g* for 7 minutes at 4°C. The pellet was immersed in 1ml H_2O (Sigma-Aldrich) for 5 seconds followed by 2xPBS supplemented with 4% FBS to lyse red blood cells (RBCs). This suspension was pelleted (280 *g* for 5 minutes at 4°C) and resuspended in 1ml supplemented-HBSS prior to antibody staining.

Single-cell suspensions of BM were labelled with conjugated antibodies to CD45 (PE; 1µl/mouse), Ter-119 (PE; 1µl/mouse), Sca-1 (FITC; 1µl/mouse) and PDGFRα (APC; 2µl/mouse) for 30 minutes on ice. All antibodies were purchased from eBiosciences (Hatfield, UK). Following one wash in an excess of supplemented-HBSS, the sample was resuspended in 2µg/ml Propidium Iodide (PI) solution (eBiosciences) prior to sorting.

Fluorescence activated cell sorting was performed by Dr. Diarmaid Houlihan on a MoFlo XDP Cell Sorter (Figure 6; Beckman Coulter, High Wycombe, UK). Relevant gates were applied to select the live cell population, exclude PE-CD45^{+}/PE-Ter-119^{+} haematopoietic cells and purify APC-PDGFRα^{+}/FITC-Sca-1^{+} cells of interest.

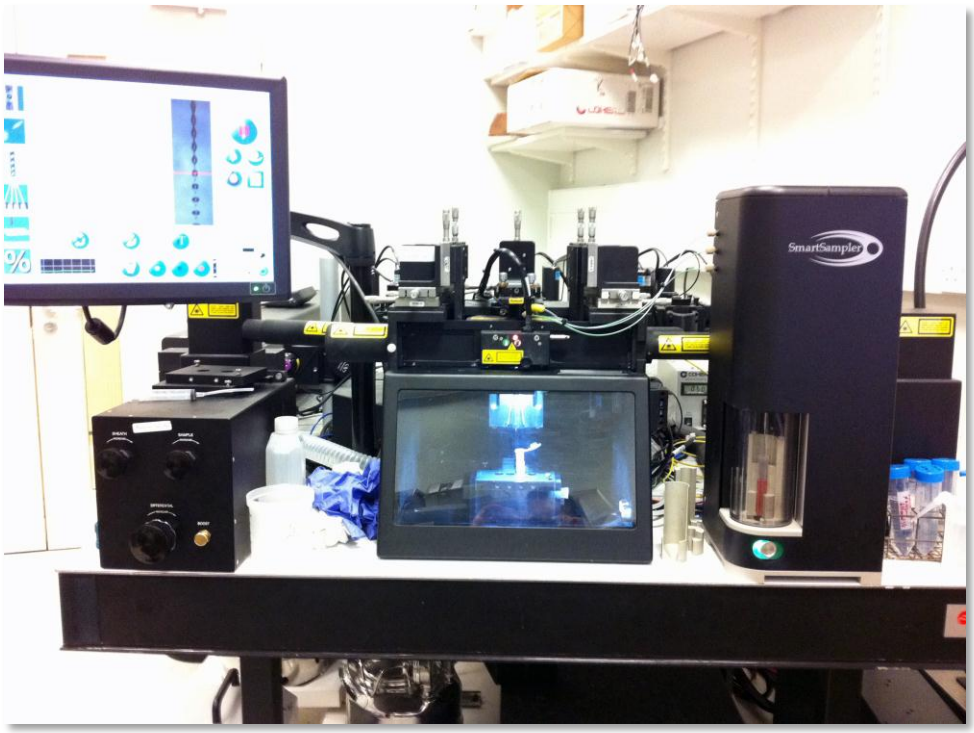

Figure 6 | **MoFlo XDP Cell Sorter**. A front-on view of the sorter showing the control panel, fluid pressure control valves, sorting chamber and the sample input chamber.

2.2 Cell Culture

Sorted PαS cells were cultured in αMEM+GlutaMAX (Invitrogen) supplemented with 10% FBS and 1x P/S/G solution. Flasks were incubated at 37°C, 5% CO_2 with medium changed every 3-4 days. To investigate the influence of cytokine stimulation, some MSC cultures were cultured in αMEM maintenance medium supplemented with 10ng/ml of one of the following: murine PDGF-AA, PDGF-BB, FGF2 (all Peprotech EC, London, UK) or TGF-β1 (New England Biolabs, Hitchin, UK).

2.3 Growth Curve Analysis and Senescence Staining

At day 0, PαS were seeded at a density of 4,000 cells per well of a 48-well plate (Corning, Amsterdam, NL) in the presence of αMEM maintenance (*n*=2) or growth factor (GF) supplemented medium (*n*=2). Once confluent, cells were passaged into larger wells at a 1:2 ratio. After 30 days an aliquot taken to estimate the final cell number and samples were stained for the expression of β-galactosidase (β-gal) using a senescence detection kit (BioVision, California, USA) according to manufacturer's instructions. β-gal staining was quantified by counting the number of positive cells from 12 randomly selected fields of view per sample.

2.4 Differentiation Cultures

2.4.1 Osteogenic Differentiation

To induce osteogenic differentiation, subconfluent MSCs were cultured in osteogenic basal medium (Lonza, Cologne, DE) for 17 days. Cultures were then fixed in 10% formal saline for 15 minutes and stained for the presence of calcium deposits using an Alizarin Red Osteogenic Assay Kit (Millipore, Watford, UK) following manufacturer's instructions.

2.4.2 Adipogenic Differentiation

To induce adipogenic differentiation, confluent MSCs were cultured for four days in adipogenic induction medium (Lonza), followed by four days in adipogenic maintenance medium (Lonza). Differentiated cultures were fixed in 10% formal saline for 5 minutes and stained for adipogenesis using Oil Red O (Sigma-Aldrich).

2.4.3 Chondrogenic Differentiation

To induce chondrogenic differentiation, cell pellets of 250,000 MSCs at passage 3 were cultured in chondrogenic basal medium (Lonza) and 10ng/ml human TGF-β3 (Peprotech). Cultures were maintained for 3 weeks with medium changes every 3 days. Differentiated pellets were gently aspirated, embedded in optimal cutting temperature compound and snap-frozen in liquid nitrogen. 10µm serial sections were cut by Janine Youster and fixed for 10 minutes in acetone. The proteoglycan content of cartilage pellets was visualised by staining with 0.1% toluidine blue (Sigma) made up in a pH 4.0 acetic acid/sodium acetate buffer solution.

2.5 Lymphocyte Proliferation Assay

The isolation of lymphocytes and T cell proliferation assays were performed by Kesley Attridge to investigate the immunosuppressive properties of PαS cells *in vitro*.

2.5.1 Isolation of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T regulatory cells

Mononuclear cells (MNCs) were isolated from BALB/c mice by mashing the spleen and lymph nodes through a fine wire mesh. $CD4^+$ T cells were negatively identified using a magnetic labelling system (Miltenyi Biotec, Bisley, UK). Splenocytes were incubated in a biotin-conjugated antibody cocktail (10µl per 10^7 cells) at 4°C for 10 minutes followed by incubation with anti-biotin microbeads (Miltenyi; 20µl per 10^7 cells) and PE-CD25 (Miltenyi; 10µl per 10^7 cells) for 15 minutes. Labelled samples were then run through an LD column, where the $CD4^+$ cells were enriched in the run-off. Purified $CD4^+$ cells were then further labelled with anti-PE microbeads (Miltenyi; 10µl per 10^7 cells) and run through MS columns. $CD4^+CD25^-$ T cells did not adhere to the column and were collected, while $CD4^+CD25^+$ Tregs were retained in the column and had to be eluted.

2.5.2 Isolation of $CD19^+$ B cells

MNCs were isolated as described above. Single cell suspensions were labelled with CD19 microbeads (Miltenyi; 10µl per 10^7 cells) and run through an LS column. $CD19^+$ B cells were retained in the column and had to be eluted.

2.5.3 *In vitro* Suppression Assays

$CD4^+CD25^-$ T cells were labelled with 1µM CFSE (Invitrogen) for 10 minutes prior to use. T cells were cultured in RPMI 1640 (Invitrogen) supplemented with 1x P/S/G solution and 2-mercaptoethanol at a density of 25,000 cells/well in round-bottomed 96-well plates with or without graded numbers of MSCs (at passages 2-3). $CD19^+$ B cells (2:1 ratio of B cells:T cells) were added for co-stimulation (via CD86) and cultures were supplemented with 0.8µg/ml of anti-mouse CD3e (BD Biosciences) to ligate the T cell receptor and stimulate T cell proliferation. In a subset of experiments, purified $CD4^+CD25^+$ Tregs were used as a comparison to MSC-mediated immunosuppression. Cultures were maintained for 72 hours before the absolute number of $CFSE^+$ cells was quantified by flow cytometry.

Chapter 3

Results

3.1 Isolation of PαS cells

Single cell suspensions of murine BM were labelled with conjugated antibodies against CD45, TER119, PDGFRα and Sca-1 and analysed using FACS (Morikawa et al., 2009). PI was used to exclude non-viable cells from downstream analysis. PI^{low} cells typically represented 85-90% of the total population (Figure 7A). Next, the haematopoietic cells ($CD45^{+}TER119^{+}$) were excluded by gating on PE-negative cells (<0.5% viable cells; Figure 7B). This PE-negative fraction was then analysed for PDGFRα and Sca-1 expression, and a gate was applied around the double-positive subpopulation (Figure 7C). PαS cells typically represented 10-15% of PE-negative cells, or 0.05-0.10% of all BM cells. Cell yields from C57BL/6 and BALB/c mice were similar (data not shown). We routinely achieved a typical purity >95% and a yield approaching 5,000-8,000 PαS cells/mouse.

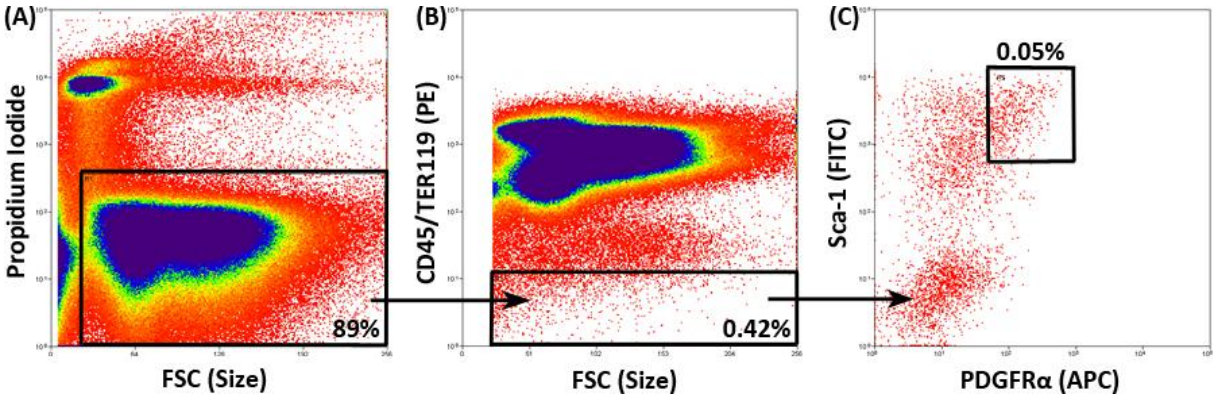

Figure 7 | **Representative FACS plots of PαS cell isolation from C57BL/6 mice**. (A) A live gate is applied around PI^{low} cells. (B) The non-haematopoietic fraction is then purified and (C) analysed for PDGFRa/Sca-1 expression.

3.2 Effect of PDGF, FGF and TGF-β on PαS cell Morphology and Growth

Freshly isolated PαS cells displayed characteristic spindle-shaped morphology and adhered to tissue culture plastic (Figure 8A). We then investigated the effect of PDGF, FGF2 and TGF-β1 signalling on PαS cell growth, as these pathways have been implicated in MSC proliferation (Ng et al., 2008). The addition of GFs caused a change in morphology (Figure 8B,C,D). The addition of PDGF or FGF caused PαS cells to display an even more spindly, almost neuron-like morphology. TGF-β caused PαS cells to grow in discrete colonies that were tightly packed with cells of varying sizes. No obvious cell death or spontaneous differentiation was seen in the presence of GFs.

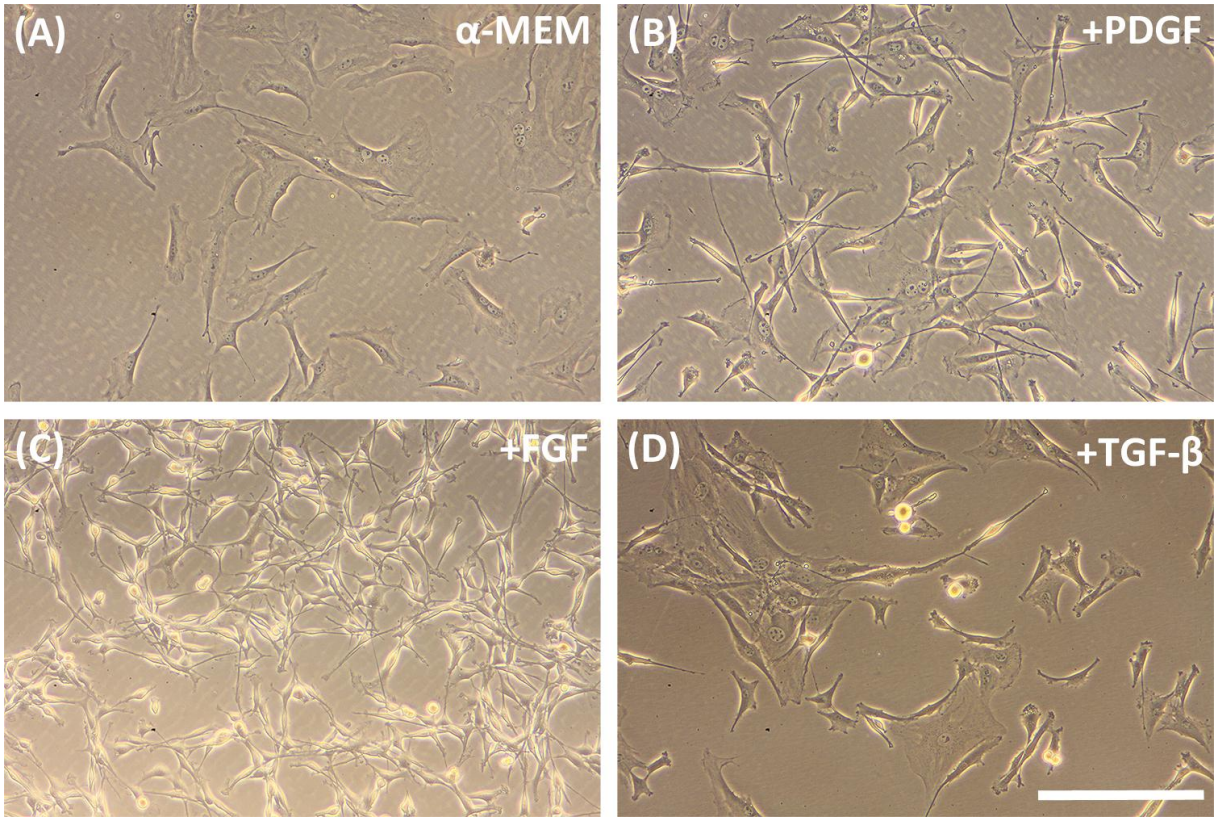

Figure 8 | **Morphologies of freshly isolated PαS cells**. Representative images of passage 0 PαS cells cultured in (A) α-MEM maintenance medium, (B) α-MEM+10ng/ml PDGF-BB, (C) α-MEM+10ng/ml FGF2, and (D) α-MEM+10ng/ml TGF-β1. All images taken at 100x magnification. Bar, 25µm.

The ability of MSCs to undergo serial passaging *in vitro* without senescence is one of the criteria used to define MSCs (Dominici et al., 2006). To this end, growth curves were constructed for PαS cells cultured in α-MEM (*n*=2) and GF-supplemented (*n*=2) medium (Figure 9). FGF-supplemented cells reached 10 population doublings (PDs) over a 30 day period, closely followed by PDGF, TGF-β and α-MEM. To account for differences in cell size between groups, a final cell count was taken on day 30 (Table 1). This once again demonstrated the remarkable *in vitro* proliferation of FGF-supplemented PαS cells, which yielded nearly 10^7 cells from the original 4000 cells seeded.

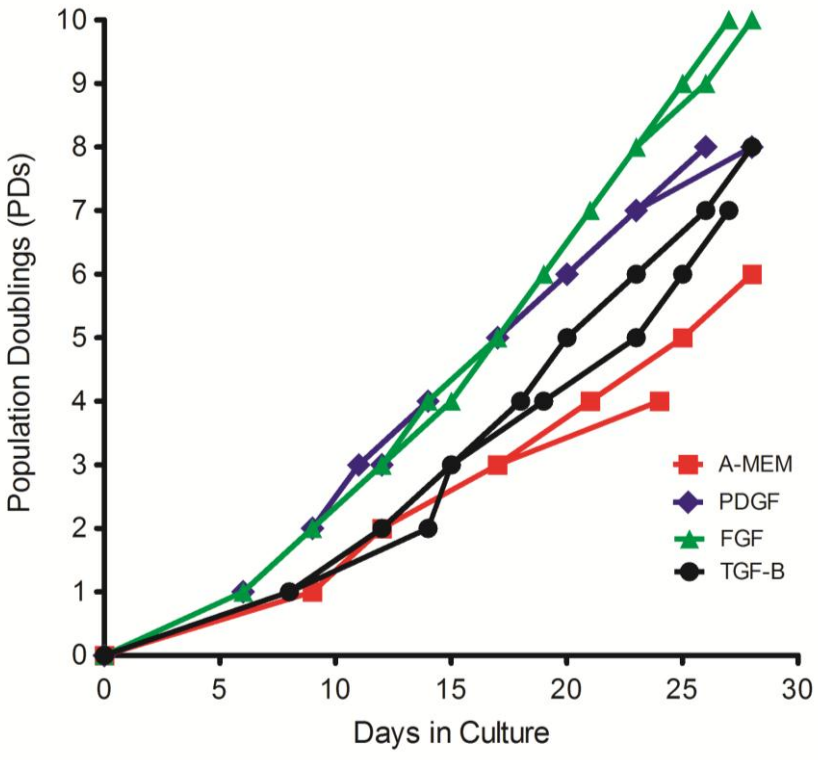

Figure 9 | **Growth curve analysis of PαS cells**. Population doublings over a 30 day period were noted. Each line represents an independent experiment.

Table 1 | **Final cell counts after growth curve analysis**.

Condition (*n*=2)	Number of cells seeded (Day 0)	Average number of cells harvested (Day 30)
α-MEM	4000	215,000
PDGF-BB	4000	1,020,000
FGF2	4000	9,760,000
TGF-β1	4000	480,000

3.3 Effect of GFs on PαS cell Senescence

Recent publications have identified that both human and murine MSCs undergo replicative senescence upon prolonged *in vitro* culture (Wagner et al., 2008). In this study, we analysed 30 day old cultures for the expression of β-gal using a senescence staining kit (Figure 10). Changes in morphology towards larger, flatter cells could be observed. β-gal staining was quantified by counting the number of positive cells from 12 randomly selected fields of view per sample (Figure 10E). Thirty day old α-MEM cells were 17.54% (±9.7%, total cells counted = 1775) positive for β-gal, while PDGF-supplemented cultures were 6.73% (±0.08%, total cells counted = 934) positive, FGF-supplemented cultures were 0.5% (±0.5%, total cells counted = 1112) positive, and TGF-β cultures were 15.9% (±1.5%, total cells counted = 1676) β-gal positive.

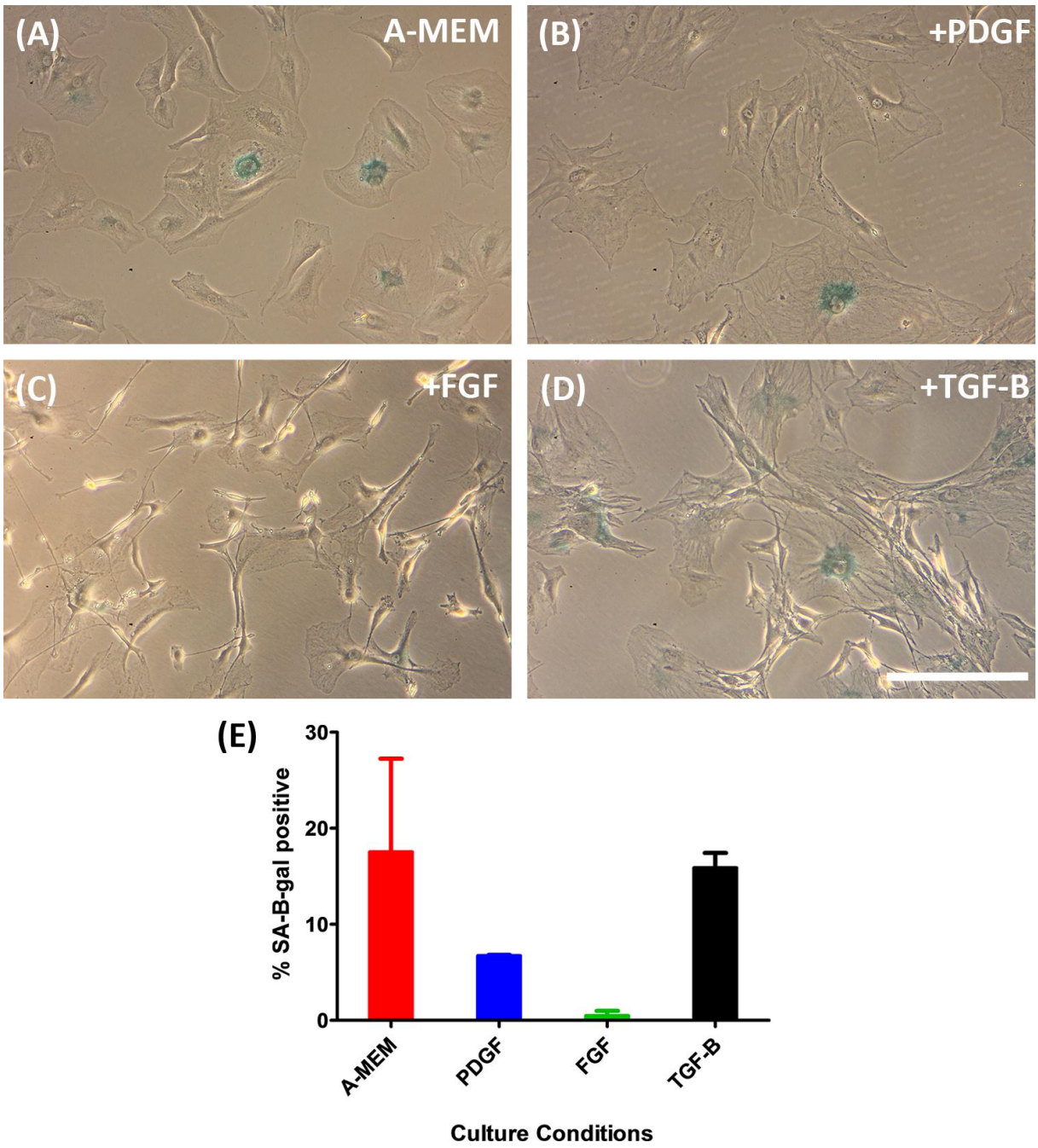

Figure 10 | **β-galactosidase expression of 30 day old PαS cells**. (A-D) Representative images of senescence associated β-gal staining. Images were taken at 100x magnification. Bar, 25µm. (E) Quantification of β-gal positive cells. Data represented as mean±SEM.

3.4 Effect of GFs on PαS cell Differentiation

Previous work by Ng and colleagues had identified PDGF, FGF and TGF-β as key signalling pathways controlling the differentiation of human MSCs towards osteoblasts, adipocytes or chondrocytes (Ng et al., 2008). Here, we repeated their experiment using prospectively isolated PαS cells which allows us to study the effects of GFs from the moment of isolation.

3.4.1 Osteogenic Differentiation

Osteogenic differentiation was induced by culturing PαS cells in commercially available differentiation medium. After 17 days of culture, samples were fixed and stained using alizarin red (Figure 11A). After imaging, the dye was extracted from the stained monolayer and quantified against known calcium concentration standards (Figure 11B).

PαS cells grown in standard α-MEM showed robust osteogenic differentiation across all passages. There seems to be an increase in calcium deposition at later passages, a finding that has been reported previously (Wagner et al., 2008). It would seem that tissue culture plastic is an independent promoter of osteogenic differentiation, as the propensity for osteogenic differentiation increased at later passages in GF-supplemented medium as well.

The addition of GFs to maintenance medium resulted in reduced bone differentiation across all passages. PDGF and FGF attenuated osteogenic differentiation by up to 50% compared to controls. TGF-β strongly inhibited osteogenic differentiation by up to 80% compared to controls. The presence of adipocyte-like cells could be observed in FGF differentiation cultures, suggesting that FGF may prime PαS cells towards the adipogenic lineage.

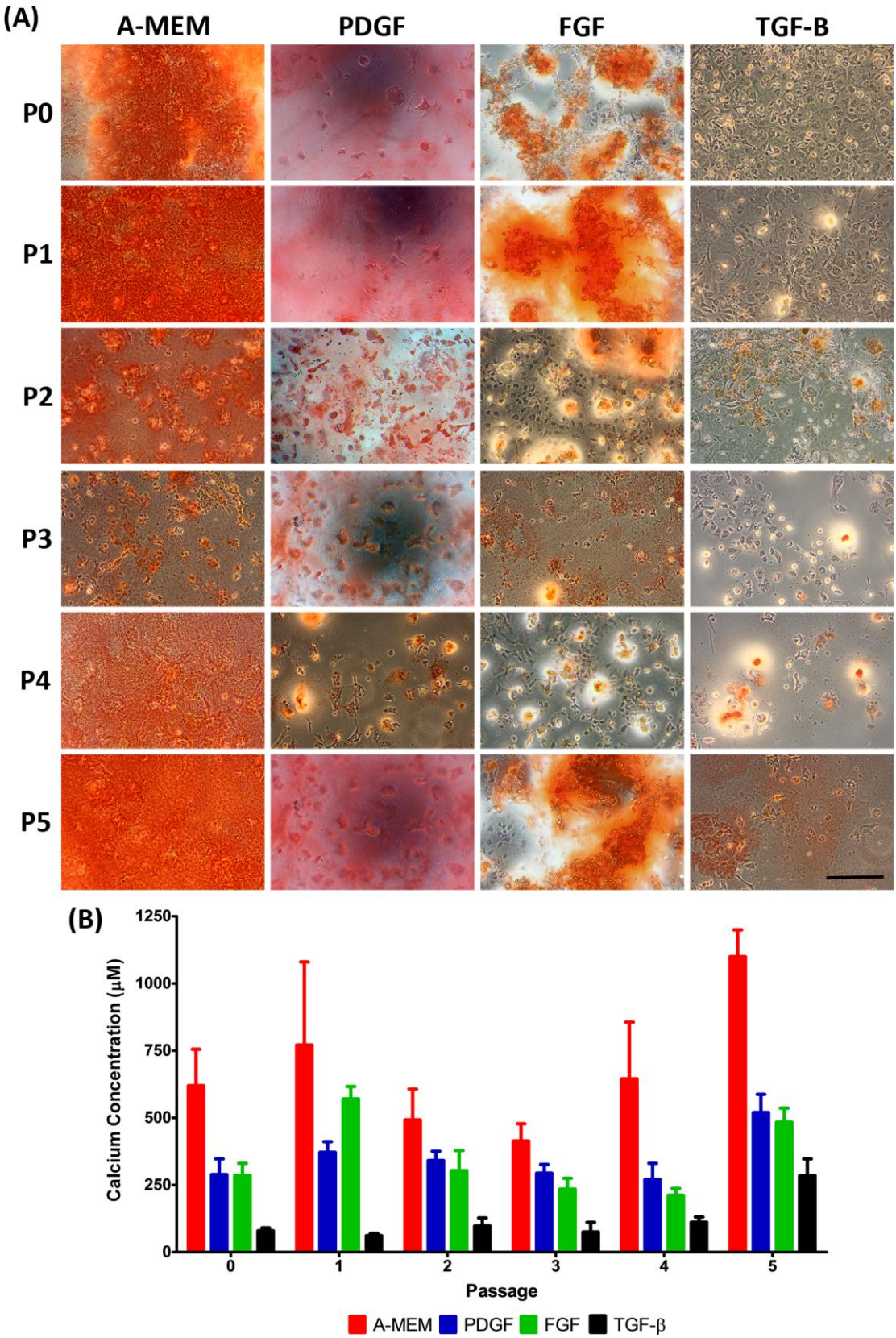

Figure 11 | **Osteogenic differentiation of PαS cells**. (A) Representative images of alizarin red staining, 100x magnification. Bar, 25µm. (B) Quantification of alizarin red staining in α-MEM (*n*=6), PDGF-BB (*n*=3), FGF2 (*n*=6) and TGF-β1 (*n*=3) supplemented PαS cells. Data represented as mean±SEM.

3.4.2 Adipogenic Differentiation

Adipogenic differentiation was induced in one cycle of adipogenic induction and maintenance medium. This was shorter than the manufacturer's recommendation of three cycles, as the addition of GFs had a profound impact on adipogenic differentiation and cultures were undergoing necrosis due to lipid droplet formation. After differentiation, cultures were fixed and stained using oil red O (Figure 12).

PαS cells cultured in α-MEM underwent sporadic adipogenic differentiation at earlier passages, but this ability was lost from P3 onwards. This disappointing result might be due to the one week differentiation protocol used or the finding that MSCs lose their adipogenic differentiation potential at later passages (Wagner et al., 2008)

The addition of GFs had a profound effect on the ability of PαS cells to differentiate into adipocytes. Stimulation with TGF-β seemed to completely inhibit adipogenic differentiation at all passages. Conversely, PDGF-supplemented PαS cells readily differentiated into adipocytes and this effect was maintained up to P5. The addition of FGF caused almost 100% differentiation into adipocytes at earlier passages, and the effect was maintained until P5. These findings agree with Ng *et al*., who showed that PDGF and FGF signalling pathways were active during adipogenesis (Ng et al., 2008). The fact that PDGF and FGF inhibit osteogenesis and strongly induce adipogenesis, even with a shortened protocol, suggests that they might prime PαS cells towards the adipogenic lineage.

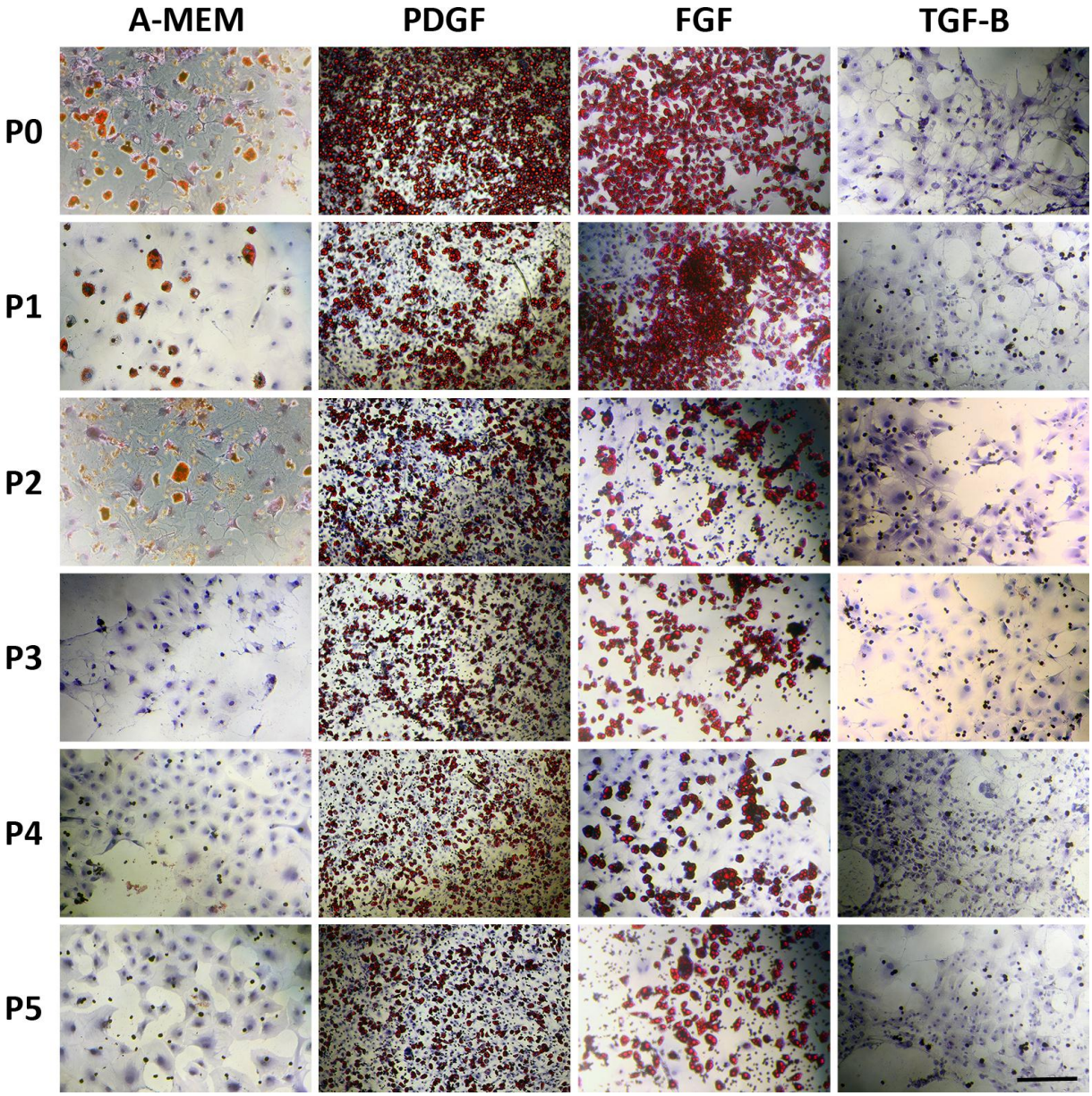

Figure 12 | **Adipogenic differentiation of PαS cells**. Representative images of adipogenic differentiation as visualised by oil red O staining. Wells were counterstained with haematoxylin prior to imaging. *n*=3 for all medium conditions at each passage. All images were taken at 100x magnification. Bar, 25µm.

3.4.3 Chondrogenic Differentiation

Chondrogenic differentiation was induced by culturing pellets of 250,000 PαS cells (passage 3) in chondrogenic medium supplemented with TGF-β3. After 3 weeks in culture, pellets were embedded, sectioned, and stained with toluidine blue to visualise proteoglycan content. Due to time constraints, we were only able to complete chondrogenic differentiation for PαS cells cultured in α-MEM medium. Widespread proteoglycan secretion can be observed (red-purple colour) within the pellet micromass, suggesting efficient differentiation down the chondrogenic lineage (Figure 13A). As a negative control, cytospun peripheral blood macrophages were also stained with toluidine blue. No proteoglycan deposition could be observed, confirming the specificity of the stain.

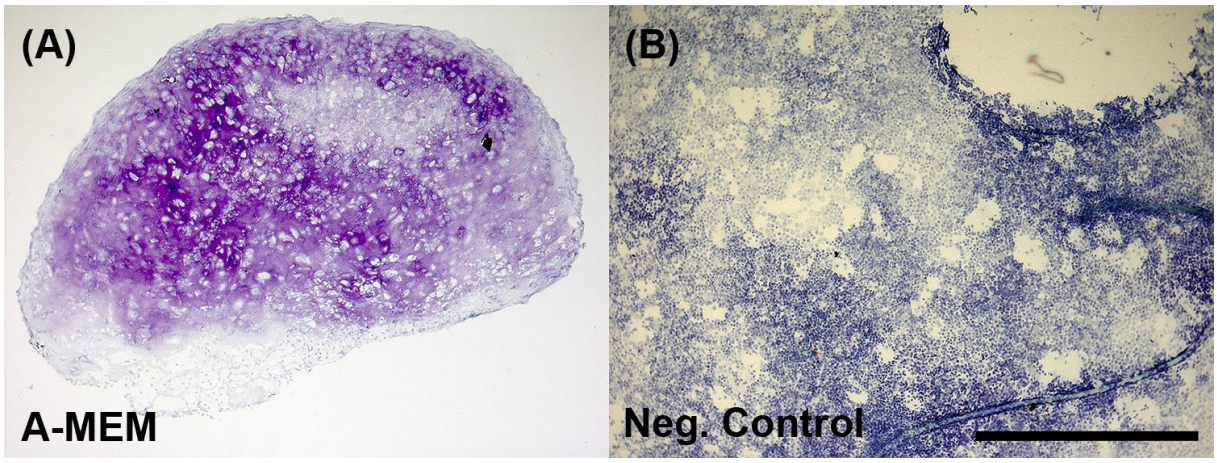

Figure 13 | **Chondrogenic differentiation of α-MEM PαS cells**. (A) Representative image of a chondrogenic pellet stained with 0.1% toluidine blue. (B) Peripheral blood macrophages stained with toluidine blue as a negative control. All images taken at 100x magnification. Bar, 50µm.

From reading previous literature, we expect TGF-β treatment to increase chondrogenic differentiation and proteoglycan secretion (Ng et al., 2008, Xu et al., 2008). Due to the strong adipogenic differentiation seen with PDGF and FGF treatment, we do not expect these GFs to prime PαS cells towards the chondrogenic lineage.

3.5 Mechanism of PDGF-mediated Differentiation

The PDGF ligand family consists of four members that form homo- or heterodimers to become active (Betsholtz, 2004). So far, five isoforms have been recorded: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD. Binding of these ligands to PDGF tyrosine kinase receptors causes receptor dimerisation (PDGFRα, PDGFRβ, PDGFRαβ) and activation of downstream pathways (Fredriksson et al., 2004). PDGF-BB (the ligand used in this study) binds to all receptor isoforms, and is therefore used as a promiscuous activator of PDGF signalling. PDGF-AA is selective for PDGFRα only (Figure 14; Fredriksson et al., 2004).

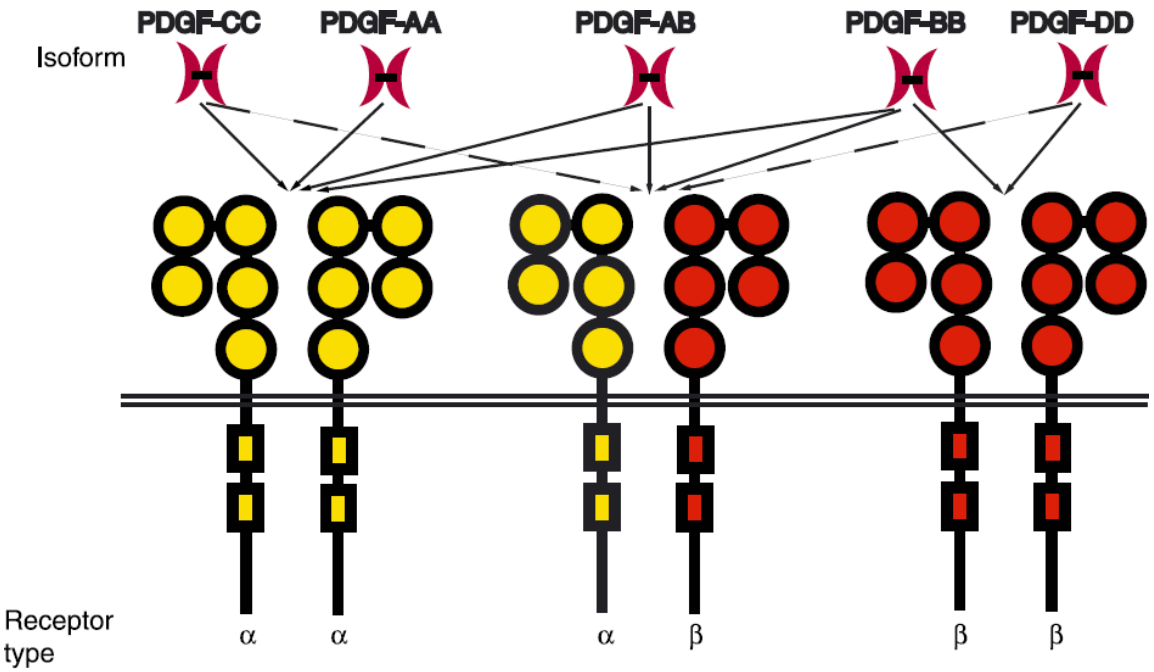

Figure 14| **PDGF receptor signalling**. The receptor specificities of the five PDGF ligands are shown. Of these, PDGF-BB can signal through all three receptor combinations, making it a promiscuous activator of PDGF signalling. Conversely, PDGF-AA signals exclusively through the PDGFRα homodimer. Figure taken from Fredriksson et al., 2004.

To identify whether PDGFRα was responsible for the priming of PαS cells towards the adipogenic lineage, we repeated the osteogenic (Figure 15) and adipogenic (Figure 16) differentiation experiments in the presence of 10ng/ml PDGF-AA (*n*=3). PαS cells cultured in PDGF-AA readily differentiated into osteoblasts at earlier passages. The calcium concentrations at P0/P1 were comparable to α-MEM cells at the same stages. In contrast, PDGF-BB cells did not readily differentiate into osteoblasts across all passages. Interestingly, the osteogenic differentiation propensity of PDGF-AA was lost by P2. It would be interesting to see whether this picks up again at later passages.

PαS cells grown in PDGF-AA lost the propensity to differentiate into adipocytes compared to cells supplemented with PDGF-BB. Adipocyte differentiation had diminished by P2 in PDGF-AA cells, which is similar to α-MEM cells. Meanwhile, PDGF-BB supplemented cells readily underwent adipogenic differentiation across all passages tested. These preliminary results suggest that the adipogenic priming seen using PDGF is not exclusively mediated by the PDGFRα, as PDGF-AA was not able to recreate the same effects seen with PDGF-BB.

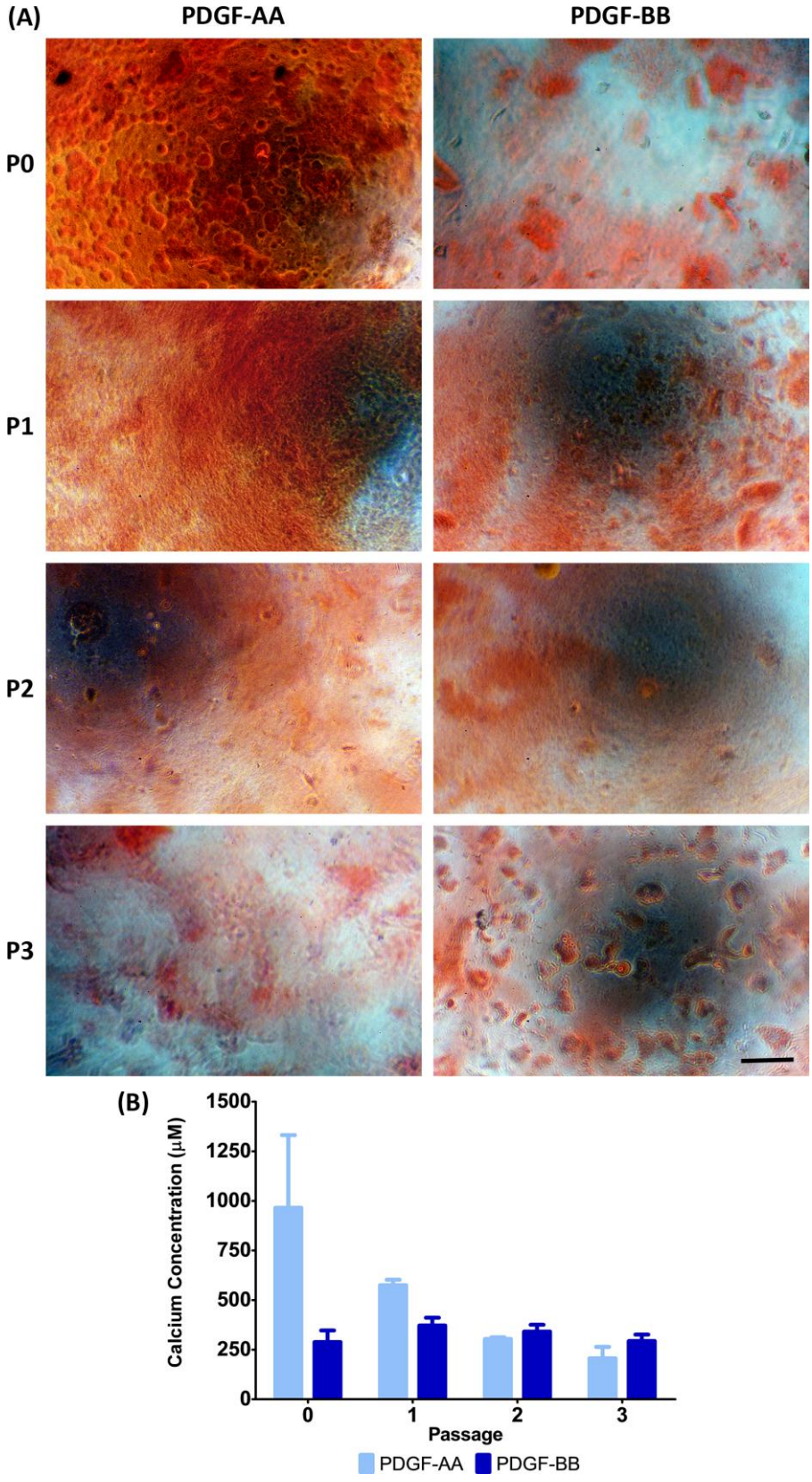

Figure 15 | **Osteogenic differentiation of PαS cells supplemented with PDGF-AA**. (A) Representative images of alizarin red staining, 100x magnification. Bar, 25µm. (B) Quantification of alizarin red staining in PDGF-AA (*n*=3) and PDGF-BB (*n*=3) supplemented PαS cells. Data represented as mean±SEM.

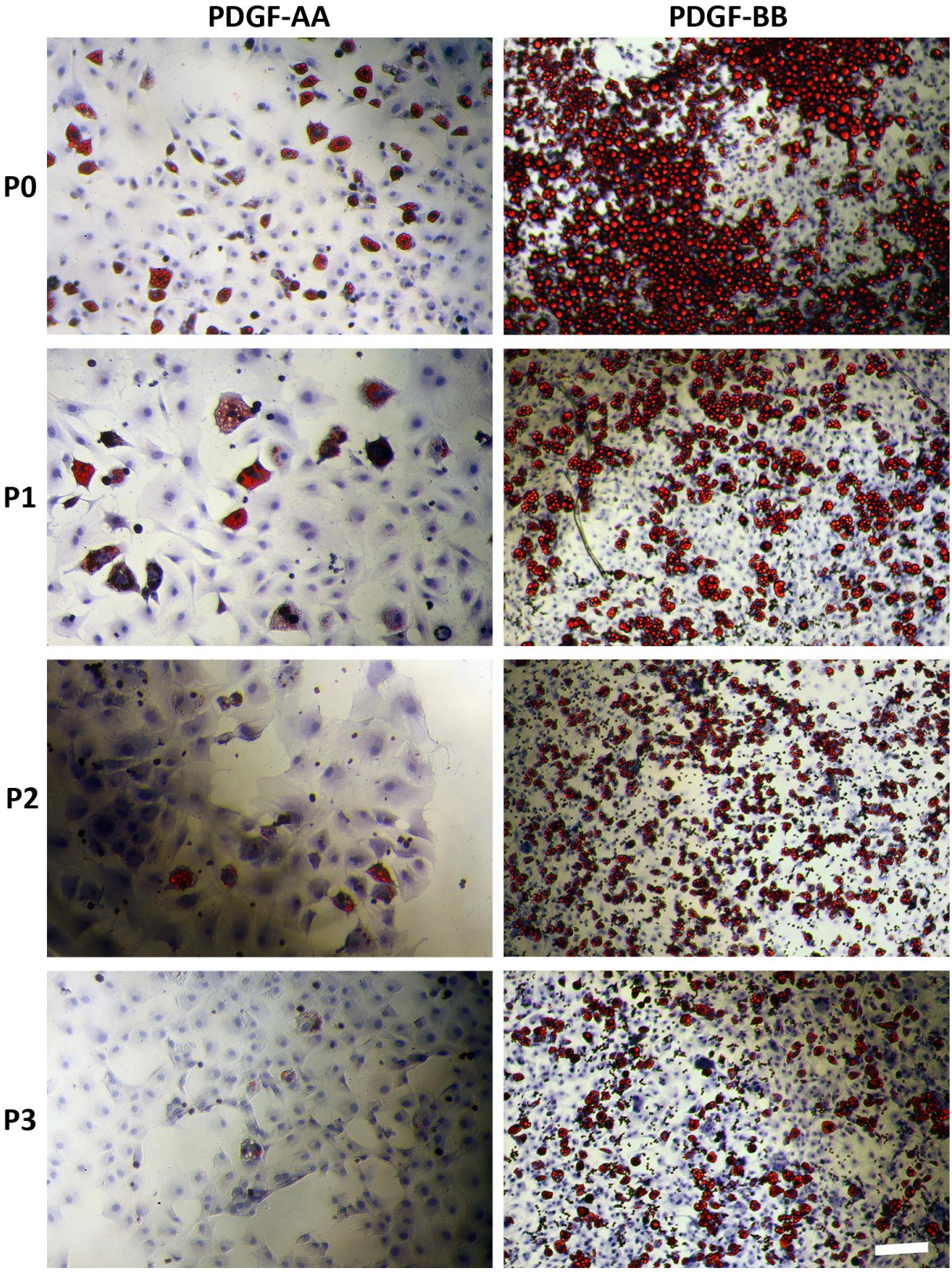

Figure 16 | **Adipogenic differentiation of PαS cells supplemented with PDGF-AA**. Representative images of adipogenic differentiation as visualised by oil red O staining. Wells were counterstained with haematoxylin prior to imaging. All images were taken at 100x magnification. Bar, 25µm

3.6 Immunomodulatory Functions of PαS cells

Preliminary *in vitro* T cell proliferation assays were performed to try and identify whether lineage-primed PαS cells had varying capacities of immunomodulation. CFSE-labelled $CD4^+$ conventional T cells (Tconv) were stimulated with anti-CD3 and $CD19^+$ B cells in the presence of graded numbers of MSCs. After 72 hours' co-culture, the total number of CFSE-labelled $CD4^+$ cells was quantified.

We first assessed the ability of high concentrations (>1 PαS cell:4 Tconv) of PαS cells grown in α-MEM and PDGF to suppress T cell proliferation (Figure 17). In this assay, $CD4^+CD25^+$ Tregs were added as a comparison to MSC-mediated immunosuppression. α-MEM PαS cells caused an 80% drop in total $CD4^+$ cell counts at the 1:4, 1:2 and 1:1 concentrations. PDGF-supplemented PαS cells seemed to attenuate the immunomodulatory response at 1:4 compared to α-MEM cells, but the full inhibitory response was re-established at higher concentrations. Our preliminary results also suggest that MSCs are more potent in terms of immunomodulation than Tregs at all concentrations tested.

Due to time constraints, we opted out of performing a T cell proliferation assay using FGF-supplemented PαS cells as our findings show that FGF primes MSCs towards the adipogenic lineage, as seen with PDGF-supplemented cells. Instead, TGF-β supplemented MSCs were used as we think TGF-β primes MSCs towards the chondrogenic lineage. For this experiment, the concentration of MSC:Tconv was diluted down to 1:32, allowing us to compare the effects of MSCs at more 'physiological' levels. Once again, the addition of MSCs inhibited T cell proliferation in a dose-dependent manner (Figure 18). At 1:32 dilutions, TGF-β MSCs

appeared to be more suppressive compared to α-MEM controls (40% and 15% suppression, respectively). This trend was repeated at 1:16, 1:8 and 1:4 dilutions. By 1:2 dilutions, the inhibitory effects of TGF-β and α-MEM MSCs were similar.

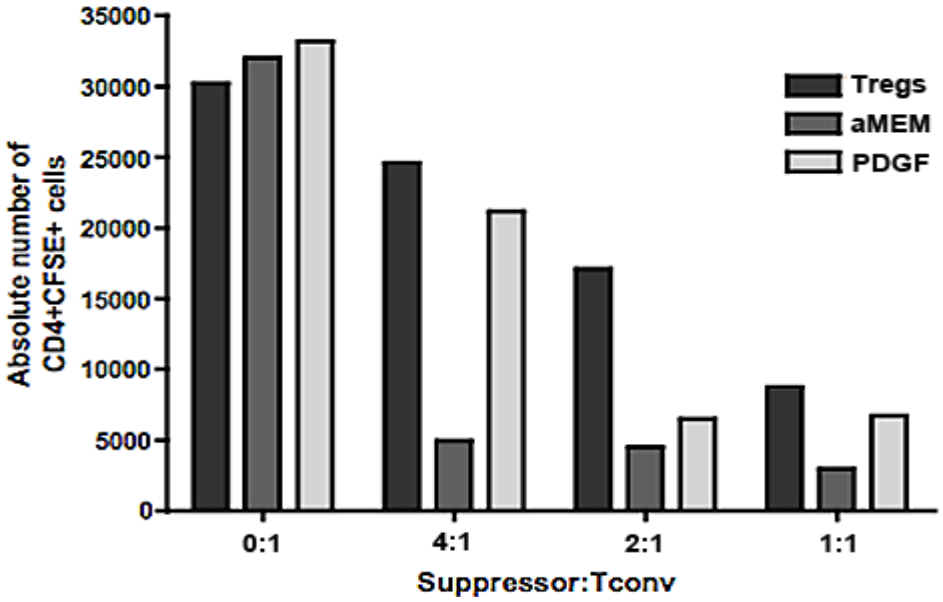

Figure 17 | **Immunomodulation by α-MEM and PDGF-supplemented PαS cells**. The immunomodulatory functions of PαS cells were tested by culturing 2.5x10^4 stimulated CFSE-labelled Tconv cells in the presence of graded numbers of MSCs/Tregs (*n*=1 at each concentration) as indicated above.

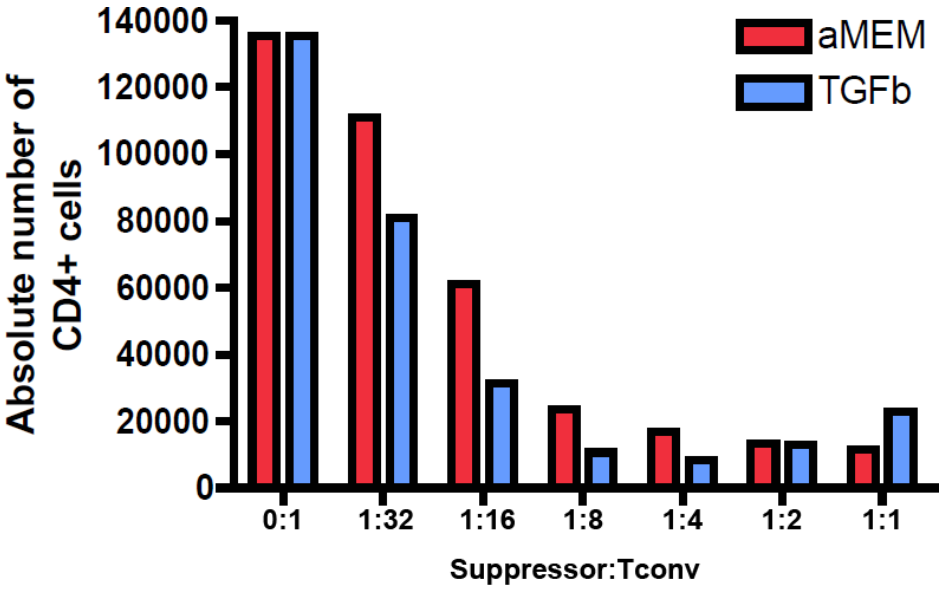

Figure 18 | **Immunomodulation by α-MEM and TGF-β supplemented PαS cells**. The immunomodulatory functions of PαS cells were tested by culturing 2.5x10^4 stimulated CFSE-labelled Tconv cells in the presence of graded numbers of MSCs (*n*=1 at each concentration) as indicated above.

Chapter 4

Discussion

4.1 Isolation, Proliferation and Senescence of PαS cells

In this study, we were able to prospectively isolate MSCs according to the Morikawa method (Morikawa et al., 2009). This method was chosen as it was successful in isolating MSCs from 6 commonly used strains of mice, while the Nestin^{+} MSC isolation method requires the use of a Nestin-GFP mouse (Mendez-Ferrer et al., 2010, Morikawa et al., 2009). The PαS yields we achieved from C57BL/6 and BALB/c mice were similar in number and morphology to the ones reported in Morikawa *et al.* (2009). The benefits of prospectively isolating MSCs are substantial, as we can now study MSC function in the absence of contaminating cells or the prerequisite of *ex vivo* expansion. Thus, it seems puzzling that no primary research paper published since 2009 has utilised prospectively-isolated PαS cells in their studies.

The effect of cytokine stimulation on PαS cell proliferation was analysed by measuring the population doublings over a 30 day period. PαS cells grown in standard α-MEM yielded over $2x10^5$ cells from the 4000 seeded, which is similar to the Morikawa study (Morikawa et al., 2009). FGF-supplemented PαS cells have a remarkable capacity to expand *in vitro*, yielding nearly 10^7 cells. The mitogenic effect of FGF2 has been shown in previous MSC literature (Lai et al., 2011, Tsutsumi et al., 2001), and it is no surprise that FGF2 has a mitogenic effect on

PαS cells as well. A shorter time period for *ex vivo* expansion is favourable for future therapies, as *in vitro* cultured murine MSCs have previously been shown to lose their homing ability when systemically infused (Javazon et al., 2004, Rombouts and Ploemacher, 2003).

PDGF-stimulated PαS cells also proliferated faster than α-MEM controls. PDGFs have historically been shown to act as mitogens for various mesenchymal cells (Betsholtz, 2004). PDGF-BB is crucial for the survival and proliferation of pericytes during embryonic development (Lindahl et al., 1997). The isolation of MSCs from a perivascular niche have led many to hypothesise that pericytes are the *in vivo* 'MSC' (Meirelles et al., 2008). Thus, it would be logical if cultured MSCs are stimulated by PDGF in the same way as their *in vivo* counterparts. Further evidence comes from a study that showed that MSCs cultured in serum-free medium containing fresh frozen plasma (rich in PDGFs) exhibited enhanced growth characteristics (Müller et al., 2006).

TGF-β stimulated PαS cells also showed increased proliferation compared to α-MEM controls. Jian *et al*. previously showed that TGF-β1 stimulated human MSC proliferation via a novel form of cross-talk with the Wnt signalling pathway (Jian et al., 2006). The pro-proliferative effect of all three GFs on PαS cells backs up the findings of Ng *et al*., who identified these pathways as key regulators of human MSC proliferation. It would be interesting to see, in future studies, whether GF supplementation increases CFU-F capability of PαS cells as well.

After growth curve analysis, 30 day old cultures were stained for the expression of SA-β-gal as a marker of senescence. The accumulation of senescent cells may limit the therapeutic applications of MSCs, especially cases where large numbers of MSCs are required to see a clinical benefit. Our results show that "aged" MSCs increase in size and spread further, a common phenotype of senescent cells (Sethe et al., 2006). The systemic infusion of larger, older MSCs carries a risk of them being trapped in small capillaries (Furlani et al., 2009, Toma et al., 2009). Promisingly, <1% of cells in FGF-stimulated cultures were β-gal^{+}, even after they underwent extensive proliferation. PDGF (6%), TGF-β (16%) and α-MEM (17%) cells were slightly more β-gal^{+}. Although these values may seem high after 5-10 PDs, an estimated 7-9 PDs would have occurred during initial colony formation (Wagner et al., 2008). Future studies looking at longer time periods and examining the karyotype of late-passage MSCs would yield more information about PαS cell senescence (Ueyama et al., 2011).

4.2 Lineage Priming of PαS cells

As described previously, lineage priming can be defined as a model of stem cell differentiation in which a given stem cell expresses a subset of genes related to the lineage they are already committed to differentiate into (Delorme et al., 2009). Thus, when a stem cell is exposed to differentiation-inducing conditions, they readily differentiate down that lineage. We investigated the effects of PDGF, FGF and TGF-β on PαS cell differentiation to try identify whether they lineage-prime our MSCs. Unfortunately, chondrogenic differentiation was not completed due to time constraints, so we are currently unsure whether GF treatment primes MSCs towards the chondrogenic lineage as well.

PDGF and FGF treatment seems to prime PαS cells towards the adipogenic lineage. A marked reduction in osteogenic (compared to α-MEM controls) differentiation was seen in both samples across all passages. The PDGF pathway has been shown to be active during adipogenesis and chondrogenesis (Ng et al., 2008, Kratchmarova et al., 2005). However, the decreased osteogenic differentiation seen in FGF PαS cells is surprising, as FGF has been shown to support osteogenic differentiation in previous studies (Ng et al., 2008, Minamide et al., 2007, Tsutsumi et al., 2001). Additionally, preliminary studies on the mechanism of PDGF-supplemented PαS cell differentiation revealed that the adipogenic priming seen was not mediated exclusively by PDGFRα. Human MSCs express both forms of the PDGF receptor (Ball et al., 2007). Culturing PαS cells in α-MEM causes a decrease in PDGFRα expression, while PDGF-BB supplemented medium maintains the expression of PDGFRα (Diarmaid Houlihan, unpublished observations). The functional consequences of PDGFRα maintenance are not clear. The use of small molecule inhibitors of specific PDGFR isoforms would help elucidate how PDGF exerts its effect on PαS cells.

In our hands, TGF-β supplemented PαS cells failed to differentiate into osteoblasts or adipocytes. The effect of TGF-β on MSC differentiation is unclear in previous literature, with some publications suggesting that TGF-β1 is a pro-osteogenic factor (Roelen and Dijke, 2003), and others implicating TGF-β1 in promoting chondrogenesis (Ng et al., 2008, Xu et al., 2008). Furthermore, TGF-β has been shown to inhibit adipogenesis in MSCs (Kim et al., 2009, Ng et al., 2008) and pre-adipocyte cell lines (Zamani and Brown, 2011). Due to their failure to form bone or fat, we suggest that TGF-β may induce chondrogenic differentiation instead.

4.3 Immunomodulatory Phenotype of PαS cells

The immunomodulatory functions of PαS cells have not been previously reported in literature. Here, we performed T cell proliferation assays with our lineage-primed cells. One limitation of these studies was that they were only performed once, and it remains to be seen if our findings are reproducible.

We showed that α-MEM PαS cells have a potent immunosuppressive activity *in vitro* at concentrations >1:10 (MSC:Tconv). At lower dilutions, the inhibitory effect is lessened. Similar findings have been reported previously, with most studies using ratios of 1:10 or higher (Le Blanc and Ringden, 2007). Furthermore, α-MEM PαS cells showed greater immunosuppression than Tregs at the concentrations tested. This could be due to the $CD4^+CD25^+$ Treg population used in this study. $CD4^+CD25^+$ could also mark activated, effector, or memory T cells (Parkin and Cohen, 2001). The transcription factor Foxp3 is specifically expressed in $CD4^+CD25^+$ Tregs and can be used to isolate a purer population of Tregs (Hori et al., 2003). It would be interesting to see the immunomodulatory capacities of $CD4^+CD25^+Foxp3^+$ Tregs compared to PαS cells in future studies.

Our findings show that PαS cells primed towards the adipogenic lineage had weaker immunosuppressive properties compared to α-MEM cells. Conversely, TGF-β supplemented MSCs (potentially primed towards the chondrocyte lineage) showed increased immunomodulatory functions compared to α-MEM cells. TGF-β1 is a well-known anti-inflammatory protein secreted by MSCs that has been previously shown to inhibit T cell proliferation (Di Nicola et al., 2002). PαS cells were cultured in TGF-β medium for weeks

prior to performing T cell proliferation assays. Thus, any residual TGF-β1 that remains membrane-bound on MSCs prior to co-culture with T cells could have an inhibitory effect in the assay. Future work to address this issue could utilise multiple washes in PBS to ensure residual TGF-β does not get carried over.

4.4 Future Prospects

This project has partially met the initial aims and objectives. We have been able to reproducibly isolate PαS cells in our lab, and we have comprehensively characterised the effects of PDGF, FGF and TGF-β signalling on PαS cell differentiation towards the osteogenic and adipogenic lineages. Future work should address the effect of the aforementioned GFs on chondrogenic differentiation. We have also begun to characterise the immunomodulatory phenotype of PαS cells, but more repeats are necessary to determine whether our results are truly representative.

4.4.1 Mechanisms of GF Action

Our results show that the addition of GFs skews the differentiation of PαS cells down distinct lineages. Firstly, we would want to confirm that PαS cells express the necessary receptors to mediate signal transduction. The next stage would be to unravel a mechanism for these changes by perturbing the signalling pathways involved. Since we have a well-defined starting population and a quantifiable end-point readout, any inhibitors we add should give us a clear indication of whether a specific pathway is involved or not. Small molecule inhibitors and antibodies against various receptors and intracellular signalling cascades are commercially available and have been used in previous MSC literature.

4.4.2 Mechanisms of Immunosuppression

Our basic T cell proliferation assays showed that PαS cells do exert an immunomodulatory phenotype. We are currently working on identifying the soluble factors that mediate this process by using small molecule inhibitors of previously reported factors. For example, IDO secretion by human MSCs results in the local depletion of tryptophan and the build-up of immunomodulatory tryptophan metabolites (Ren et al., 2009). Use of a competitive IDO-inhibitor, 1-methyl tryptophan, alongside human MSCs in T cell proliferation assays reversed the suppressive response (Ren et al., 2009). A previous paper from the same group identified that murine MSCs express very little IDO but exert their immunomodulatory functions by the local release of nitric oxide (NO) in response to inflammatory cytokines (Ren et al., 2008). NO production is mediated by inducible nitric oxide synthases (iNOS). The addition of a non-selective inhibitor of all NOS isoforms, L-NMMA, resulted in a complete reversal of the immunosuppressive response (Ren et al., 2008). As NO plays a key role in murine MSC immunosuppression, it would be a good molecule to start our analysis with. Other soluble factors that can be inhibited include: PGE_2 (inhibited by Indomethacin; Nemeth et al., 2009), MMP2/9 (inhibited by SB-3CT; Ding et al., 2009), TGF-β1 (inhibited by neutralising antibodies; Di Nicola et al., 2002) and IL-10 (inhibited by neutralising antibodies; Yang et al., 2009). Finally, another interesting experiment would be to determine whether cell-cell contact is necessary for PαS cells to suppress proliferation. This can be done by using MSC supernatants or transwell culture systems.

4.4.3 CTGF mediated fibroblast differentiation

A landmark study by Lee and co-workers identified that clonal MSC populations could be differentiated into fibroblasts with high doses of connective tissue growth factor (CTGF; Lee et al., 2010). Their finding hints at a possible MSC origin of fibroblasts that is responsible for the repair of damaged connective tissues and effective scar tissue formation. Another study identified that cardiac $CD44^{+}$ mesenchymal progenitors differentiated into fibroblasts that mediated scar formation following myocardial infarction in mice (Carlson et al., 2011). It will be interesting to study the fibroblastic differentiation of PαS cells in murine models displaying reduced fibrosis. Our lab has an established vascular adhesion protein-1 (VAP-1) knockout mouse model that displays reduced liver fibrosis upon injury (Weston and Adams, 2011). We could isolate PαS cells from VAP-1 knockouts to see whether the reduced fibrosis observed is due to a defect in MSC differentiation or other factors.

Chapter 5

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