UNIVERSITY OF BIRMINGHAM

Project One: Inhibitory and Immunomodulatory Properties of Human Choroid Plexus Epithelial Cells

and

Project Two: Characterisation of Human Mesenchymal Stromal Cells from Different Sources and the Effects of Therapeutic Ultrasound

Emma Rathbone

A thesis submitted to the University of Birmingham in partial fulfilment of the requirements for the award of the MRes

College of Medical and Dental Sciences

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Project One:

Inhibitory and Immunomodulatory Properties of Human Choroid Plexus Epithelial Cells

This project is submitted in partial fulfilment of the requirements for the award of the MRes

This project was carried out under the supervision of:

Dr John Curnow

Abstract

The blood-cerebrospinal fluid-barrier, including choroid plexus epithelial cells (CPEC), is the major site of homeostatic leukocyte trafficking into the CNS; however it is unknown whether CPEC can alter T cell function or recruit T cells as suggested by the dominance of central memory T cells in the CSF. This study therefore investigated; (1) whether CPEC inhibit $CD4^+$ T cell proliferation, and (2) if CPEC can influence $CD4^+$ T cell migration.

Using human amniotic epithelial cells (AEC) as a comparison, human CPEC or conditioned medium from the cells were incubated with $CD4^+$ T cells and proliferation assessed after 96 hours. The migration of $CD4^+$ T cell subsets through CPEC or AEC grown in inverted culture, to mimic physiologically relevant basolateral-apical migration, was also assessed.

Conditioned medium inhibited $CD4^+$ T cell proliferation by 67% (CPEC) and 65% (AEC), whereas direct contact inhibited proliferation by 90% (CPEC) and 71% (AEC). Inverted co-culture mediated a 20% (CPEC) and 26% (AEC) increase in central memory $CD4^+$ T cell migration.

Results demonstrate a strong immunosuppressive role for both CPEC and AEC. Both cell types preferentially recruit central memory $CD4^+$ T cells suggesting that cells from immune privileged sites may universally employ this mechanism of immune surveillance.

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Abbreviations

AEC	Amniotic Epithelial Cell
AICD	Activation-Induced Cell Death
α-MSH	α-Melanocyte Stimulating Hormone
BAB	Blood-Arachnoid-Barrier
BBB	Blood-Brain Barrier
BCSFB	Blood-Cerebrospinal Fluid-Barrier
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BTLA	B and T Lymphocyte Attenuator
CM	Conditioned Medium
CNS	Central Nervous System
CPEC	Choroid Plexus Epithelial Cell
CSF	Cerebrospinal Fluid
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
EAE	Experimental Autoimmune Encephalomyelitis
EGF	Epidermal Growth Factor
FasL	Fas Ligand
ICAM-1	Intercellular Adhesion Molecule-1

IDO	Indoleamine 2,3-Dixoygenase
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-15	Interleukin-15
IL-1Rα	Interleukin-1 Receptor α
IL-6	Interleukin-6
IL-7	Interleukin-7
iNOS	inducible Nitric Oxide Synthase
MSC	Mesenchymal Stem Cell
NGF	Nerve Growth Factor
PBMC	Peripheral Blood Mononuclear Cell
PD-L1	Programmed Death Ligand-1
PGE2	Prostaglandin E2
sHLA-G	soluble Human Leukocyte Antigen-G
Tcm	Central Memory $CD4^+$ T cells
TCR	T Cell Receptor
TEER	Trans-Epithelial Electrical Resistance
Tem	Effector Memory $CD4^+$ T Cell
TGF-β	Transforming Growth Factor-β

TNF-α	Tumour Necrosis Factor-α
TRAIL	TNF-Related Apoptosis-Inducing Ligand
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide
VSIG 4	V-Set and Ig Domain-Containing 4

Introduction

1.1 Properties of blood-CNS barriers

The central nervous system (CNS) has long been considered an immunologically privileged site owing to a number of unique anatomical features. Non-haematopoietic cells in the CNS lack MHC class I and class II expression, there is no distinctive lymphatic drainage system (thus no antigenic interactions), and physical and chemical protection is provided by endothelial barriers (Abbott *et al.*, 2009). Over the years the theory of CNS immune privilege has been extensively challenged and as increasing evidence of the requirement of immune cells in the CNS emerges, such as in bacterial or parasitic infections in the brain, it has been suggested that the CNS could more appropriately be termed an 'immunologically specialised site' (Ransohoff *et al.*, 2003).

The concept of biological barriers between the blood and CNS was established nearly 130 years ago in a seminal study by Paul Ehrlich; a finding later confirmed by Ehrlich's associate Edwin Goldmann who discovered that a water-soluble dye infused into the circulation was unable to stain brain tissue, but that the same dye infused into the cerebrospinal fluid (CSF) was able to stain brain tissue (Wolburg *et al.*, 2010). Though distinctly different, the blood-CNS barriers possess universal properties vital to the maintenance of CNS integrity including tight junctions, providing a physical barrier in which only permitted cells and molecules may pass, and channels regulating the transport of solutes in and out of the CNS (Abbott., 2003).

1.2 Leukocyte Trafficking in the CNS

There is limited entry of leukocytes into the CNS; however three main entry sites have been distinguished. The blood-brain-barrier (BBB), composed of tightly packed endothelial cells forming CNS microvessels, is the largest site for blood-CNS exchange and covers a total

surface area of between 12 and 18m^2 in an average human adult (Abbott *et al.,* 2009). Enclosing the CNS are arachnoid cells that constitute the avascular blood-arachnoid-barrier (BAB), and the final interface is the blood-cerebrospinal fluid barrier (BCSFB) formed by the choroid plexus, lining the cerebral ventricles. This barrier is composed of specialised epithelial cells known as choroid plexus epithelial cells (CPEC) that maintain brain homeostasis and secrete CSF into the ventricles. Due to the high rate of blood flow required for these activities and that the choroid plexus capillaries supplying the blood are fenestrated and lack tight junctions, opportunities for lymphocyte migration across the epithelium and into the perivascular space are more commonly presented, where contact with the choroid plexus can occur (Meeker *et al.,* 2012).

The sequential process of lymphocyte migration into the CNS under inflammatory conditions has been widely studied, particularly in the context of experimental autoimmune encephalomyelitis (EAE); the prototype animal model for multiple sclerosis (MS). Activation of $CD4^+$ T cells in the periphery against structural CNS auto-antigens leads to recruitment and infiltration into the CNS via activated endothelial cells of the BBB, where migration is facilitated by reduced tight junction integrity and inflammatory events are maintained (Wolburg *et al.,* 2010).

Conversely, lymphocytes also migrate into the CNS under 'normal' homeostatic circumstances to patrol the CNS for pathogens. This immune surveillance is distinct from the processes mediating lymphocyte migration in inflammation and is thought to occur across the BCSFB where selectins and adhesion molecules necessary for T cell migration are constitutively expressed, rather than across the BBB, where endothelial cells lack the necessary adhesion molecules required by T cells in the absence of inflammation (Goverman 2009). As the major site of immune cell trafficking between the periphery and the CNS, the

BCSFB has been described as a specialised gateway rather than a barrier (Shechter *et al.*, 2013).

1.3 Molecules Involved in Leukocyte Trafficking

A range of cytokines, chemokines and adhesion molecules are involved in leukocyte trafficking into the CNS. Adhesion molecules constitutively expressed by the choroid plexus include P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), suggesting their involvement in regulating leukocyte entry (Steffen *et al.*, 1996; Kivisakk *et al.*, 2003). Recent evidence has implicated the involvement of the pro-inflammatory cytokine interferon-γ (IFN-γ) in immune cell trafficking across the BCSFB, inducing the upregulation of adhesion molecules (Kunis *et al.*, 2013). In addition, the chemokine receptor CXCR3 also plays a role in leukocyte trafficking. CXCR3 is generally associated with lymphocyte migration into the CNS during inflammatory conditions but previous work has shown it is also involved in immune surveillance, with the majority of T cells in the CSF expressing CXCR3 regardless of disease status (Kivisakk *et al.*, 2002). The range of molecules involved in leukocyte trafficking may suggest that various combinations of these molecules are required to recruit certain cell types to the CNS under different conditions.

1.4 Immune Surveillance in the CNS

The high occurrence of $CD4^+$ central memory T cells in normal CSF (~90% of leukocytes compared to ~58% in peripheral blood) implies their involvement in CNS immune surveillance and that these cells may be granted entry into the CNS by selective recruitment (Wilson *et al.*, 2010; de Graaf *et al.*, 2011). This concept is generally accepted but it could

also be suggested that rather than simply acting as gatekeepers, CPEC actively function to modulate the phenotype of migrating lymphocytes as they traverse the BCSFB. In any case, activated $CD4^+$ T cells in the CNS must be amenable to rapid inhibition in order to prevent the damaging consequences of inflammation and maintain immune privilege. Excessive activation would override the mechanisms suppressing $CD4^+$ T cell proliferation resulting in inflammation and potentially fatal damage, as seen in infections such as meningitis. In other words, for CNS inflammation to occur a stimulus strong enough to exceed the threshold of $CD4^+$ T cell suppression is required.

1.5 Immunomodulation by Epithelial Cells

As CPEC constitute the barrier between blood and CSF and therefore the periphery and the CNS, it seems likely that they would provide (or at least contribute to) the 'switching off' signal required to keep activated $CD4^+$ T cells in check. Other types of epithelial cells, including colonic epithelial cells, retinal pigmented epithelial cells and amniotic epithelial cells have the ability to mediate localised immune privilege by suppressing the proliferation of activated $CD4^+$ T cells via an array of cell-bound and soluble mediators (Cruickshank *et al.,* 2004; Gregerson *et al.,* 2007; Qureshi *et al.,* 2010). For example, soluble factors known to promote ocular immune privilege by retinal pigmented epithelial cells include α-melanocyte stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP), interleukin-1 receptor α (IL-1Rα) and thrombospondin, which interact with the immune system through a range of mechanisms and could also be linked with immunomodulation mediated by other types of epithelial cells (Taylor *et al.,* 1994; Kennedy *et al.,* 1995; Sheibani *et al.,* 1997; Namba *et al.,* 2002).

Transforming growth factor-β (TGF-β) is an immunomodulatory molecule secreted by epithelial cells which inhibits $CD4^+$ T cell proliferation by inducing cell cycle arrest at the G1 phase (Howe *et al.,* 1991; Knuckey *et al.,* 1996; Li et al., 2005). Arachidonic acid-derived prostaglandin E2 (PGE2) is another immunoregulatory molecule and acts by inhibiting IL-2 production, thus preventing T cell activation and proliferation. PGE2 can be secreted by all cell types with epithelial cells constituting one of the major sources of PGE2 in the body (Liu *et al.,* 2012; Kalinski, 2012).

The induction of anergy, characterised by the inactivation of lymphocytes, is an important protective mechanism to prevent self-reactivity and autoimmunity. Two types have been described; (1) clonal anergy, arising from incomplete activation and growth arrest in previously activated cells, and (2) adaptive tolerance, induced in naïve T cells in an environment high in inhibitory molecules (such as Cytotoxic T Lymphocyte Antigen-4 (CTLA-4)) or low in co-stimulatory molecules required for complete activation (Schwartz, 2003). Such mechanisms of inhibiting T cell proliferation have been observed in renal tubular epithelial cells and thymic medullary epithelial cells and may therefore be a universal property of epithelial cells (Hoffmann *et al.,* 1992; Singer *et al.,* 1993).

As well as soluble molecules, epithelial cells also employ cell-bound factors to modulate the immune system. The death receptors Fas Ligand (FasL) and TNF-Related Apoptosis-Inducing Ligand (TRAIL) are known to cause Activation-Induced Cell Death (AICD) in T cells, possibly by upregulation following Tumour Necrosis Factor-α (TNFα) secretion by activated T cells (Green *et al.,* 2003). A hallmark of immune privilege, FasL and TRAIL induce caspase-dependent apoptosis and are expressed in numerous locations including the eye, testis and intestines (Griffith *et al.,* 1995; Xu *et al.,* 1999; Green *et al.,* 2001).

Another receptor known to modulate T cells is programmed death ligand-1 (PD-L1). Also known as B7-H1, PD-L1 is a counter-regulatory molecule of T cell stimulation and a member of the CD28 family which inhibits T cell proliferation by ligating PD-1 on activated T cells (Freeman *et al.,* 2000). PD-L1 is inducibly expressed by a range of cell types including colonic epithelial cells, gastric epithelial cells and airway epithelial cells, and is constitutively expressed in retinal pigmented epithelial cells (Cruickshank *et al.,* 2004; Beswick *et al.,* 2007; Heinecke *et al.,* 2008; Sugita *et al.,* 2009). Since PD-L1 is also expressed at the foetal-maternal interface, it is thought that this pathway may be important in regulating the immune responses at immune privileged sites (Guleria *et al.,* 2005).

Though strong evidence exists to support the immunoloduatory properties of epithelial cells, the difficulties in obtaining human CPEC means that this phenomenon has not been explored in these cells thus far.

1.6 Aims and Hypothesis of the Study

The hypothesis of this study was that CPEC inhibit the proliferation of $CD4^+$ T cells, and preferentially recruit central memory $CD4^+$ T cells.

In this study the interactions of $CD4^+$ T cells and CPEC were investigated, using AEC as a comparison, to determine whether CPEC can influence the proliferation and migration of $CD4^+$ T cells *in vitro*. Assays were based on the influence of CPEC in direct contact with $CD4^+$ T cells or using conditioned media to investigate the effects of soluble factors, and on the influence of $CD4^+$ T cell migration by employing an inverted culture system to mimic physiological basolateral to apical migration across the choroid plexus.

Methods

2.1 Human Amniotic Epithelial Cell (AEC) Isolation

Human AEC were isolated from placentas delivered by elective caesarean section following previously published protocols (Qureshi *et al.,* 2010). All procedures were carried out with fully informed ethical consent, following Local Research Ethics Committee guidelines.

2.2 Cell culture

Human CPEC (ScienCell Research Laboratories, Carlsbad, CA) were cultured in the recommended culture media EpiCM, supplemented with 10% foetal bovine serum (FBS), epithelial cell growth supplement and penicillin-streptomycin solution (ScienCell Research Laboratories). Culture surfaces were pre-coated with poly-l-lysine (2µg/cm^2, ScienCell Research Laboratories) to promote cell attachment, as instructed by the manufacturer. Medium was changed every 3-4 days and cells were passaged when 70-90% confluent. Cells were used at passage 3-6. AEC were cultured in complete RPMI supplemented with 10% FBS, 1% L-glutamine-penicillin-streptomycin solution (GPS) and 10ng/ml epidermal growth factor (EGF; Sigma Aldrich) with medium changes every 3-4 days. Cells were passaged when 70-90% confluent and used at passage 2-5.

2.3 Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMC were isolated from healthy consenting donors following ethics guidelines under study number UKCR4654. Blood was collected in tubes containing 50IU heparin sodium (LEO laboratories, Buckinghamshire, UK), diluted 1:1 with serum-free RPMI containing 1% GPS and 1% HEPES, layered onto ficoll-paque (GE Healthcare Bio-Sciences, Uppsala, Sweden) and centrifuged at 400xg for 30 min with no brake. Leukocyte layers were harvested and

washed for 8 min at 300xg, 200xg (to remove platelets) and 300xg in serum-free RPMI and re-suspended in 10ml RPMI after the final wash for counting.

2.4 Proliferation Dye Labelling

PBMC were re-suspended at $2x10^7$/ml and a 20µM solution of violet proliferation dye solution (eFluor 450, eBioscience, Hatfield, UK) was mixed 1:1 with cells to give a final dye concentration of 10µM. Cells were incubated for 10 min at 37°C in the dark prior to adding 5 times the volume of cold complete RPMI and incubating on ice for 5 min. Cells were washed 3 times in complete RPMI at 300xg for 8 min.

2.5 Positive Selection of $CD4^+$ T Cells

PBMC were kept cold during the following stages and 10µl of the PBMC suspension was retained for purity check. Cells were centrifuged at 300xg for 10 min and the supernatant was carefully aspirated completely, prior to re-suspending in 80µl MACS buffer (sterile phosphate buffered saline (PBS), 0.1% BSA and 2mM EDTA; Sigma Aldrich) per 10^7 PBMC. 20µl of CD4 Microbeads (Miltenyi Biotec, Surrey, UK) were added per 10^7 cells and the cell suspension was incubated for 15 min at 2-4°C. 1-2ml MACS buffer was added per 10^7 cells and the cell suspension was washed at 300xg for 10 min. PBMC were re-suspended in 500µl MACS buffer and were passed through a MS column (Miltenyi Biotec) on a pre-washed magnetic column separator. The unlabelled $CD4^+$ fraction was collected and the column was washed 3 times before removing from the magnetic column separator. 1ml MACS buffer was added to the column and the plunger was firmly pushed into the column over a universal tube to collect the $CD4^+$ cells. From each fraction, 10µl was retained and each (PBMC, CD4

negative cells and CD4 positive cells) stained with anti-CD4-APC antibody (clone 1/80 dilution in PBS/2%BSA; Miltenyi Biotec) to assess purity.

2.6 Anti-CD3/anti-CD28 Dynabead Titration

To determine the optimal number of anti-CD3/anti-CD28-coated stimulatory beads (Dynabeads; Life Technologies, Invitrogen, Paisley, UK) required to produce robust and reproducible $CD4^+$ T cell activation for the proliferation assays, a titration was carried out. Violet proliferation dye-labelled $CD4^+$ T cells were incubated with varying Dynabead ratios from 1:1 to 1:64 at $2x10^5$ cells/well in a 96 well plate. Cells were incubated for 96 hours and proliferation was assessed by flow cytometry.

2.7 Proliferation Assays

To investigate the influence of CPEC or AEC on proliferation, $CD4^+$ T cells were incubated with either conditioned media (CM) or CPEC or AEC directly.

For CM assays, CM was collected from confluent CPEC or AEC cultured in complete RPMI with no EGF for 72-96 hours and centrifuged at 1000xg for 10 min to pellet any debris. CM was decanted into new tubes and stored at -20°C until needed. Violet proliferation dye-labelled $CD4^+$ T cells were incubated in CM from CPEC or AEC, with or without anti-CD3/anti-CD28 bead stimulation (using a 1:8 cell-to-bead ratio). CM assays were carried out in flat-bottomed 96 well plates pre-coated with poly-l-lysine ($2\mu g/cm^2$) prior to adding CPEC and AEC at $2.5x10^4 cells/cm^2$ (cell density was determined by titration) and incubating for 72 hours. Plates were washed in sterile PBS and $1x10^5$ $CD4^+$ T cells were added per well. After a 96 hour incubation period $CD4^+$ T cells were retrieved, wells were washed 3x in PBS and $CD4^+$ T cell proliferation was assessed by flow cytometry. For co-culture assays, CPEC and

AEC were grown in a 24 well plate at $2.5x10^4$ cells/cm^2 for 72 hours. Violet proliferation dye-labelled $CD4^+$ T cells were added to the wells in complete RPMI at $6x10^5$ cells/well or $2x10^5$ cells/culture insert. Cells were then incubated for 96 hours at 37°C. For fixing, cells were washed in PBS/2% BSA and incubated in 50µl reagent A (FIX & PERM® cell fixation and cell permeabilisation kit; Life Technologies, Invitrogen, Paisley, UK) for 15 min at room temperature (RT) prior to a final wash. Cells were analysed immediately or stored at 2-8°C for later analysis.

2.8 Migration Assays

The underside of cell culture inserts (pore diameter 3.0µm, surface area 0.33cm^2; Sigma Aldrich) were coated with 35µl laminin (50µg/ml; Sigma Aldrich) for 2 hours at 37°C and washed 3x in distilled water. CPEC/AEC were applied to the underside of the inserts in 50µl complete RPMI at $2.5x10^4$ cells/cm^2 for 1-2 hours to enable loose adherence. Culture inserts were gently placed in media the correct way round in a 24 well plate and incubated for 72 hours at 37°C. Some culture inserts were also used without any cells grown on the underside. Culture inserts were placed in fresh wells and $2x10^5$ $CD4^+$ T cells added per insert. Some culture inserts were also placed in wells containing CPEC CM or AEC CM, or with CPEC or AEC grown in the bottom. Cells were incubated for 4 hours at 37°C and migrated fractions were collected. For antibody staining, cells were washed in PBS/2% BSA and for phenotypic analysis were incubated with anti-human CD4-PE-Vio770 (clone VIT4, 1/80; Miltenyi Biotec), anti-human CD45RA-PE-CF594 (clone HI100, 1/400; BD Biosciences, Oxford, UK) and anti-human CCR7-FITC-AlexaFluor 488 (clone G043H7, 1/20; Biolegend, London, UK) in PBS/2%BSA + 2.5µM vitamin C for 20 min at RT. Cells were washed in PBS/2%BSA and

fixed in 50µl reagent A (Life Technologies, Invitrogen) for 15 min at RT. Cells were washed again and analysed immediately or stored at 2-8°C for later analysis.

2.9 Flow Cytometry

Cells were analysed on a Beckman Coulter CyAn ADP flow cytometer and data was processed using Kaluza Flow Cytometry Analysis software v1.2 (Beckman Coulter, High Wycombe, UK). $CD4^+$ T cell populations were analysed as percentages rather than actual numbers to account for the difficulty in retrieving all of the original cells.

Statistics

Data were analysed using Graphpad Prism software v5.0 and are presented as mean ± SEM.

P values of less than 5% ($P < 0.05$) were considered statistically significant.

Results

3.1 CPEC and AEC differ in Morphology

In vivo CPEC constitute a cuboidal epithelium, however in culture they appeared elongated and fibroblast-like; a characteristic generally not associated with epithelial cells (Fig. 1A & Fig. 1B). In contrast AEC were typically epithelial, exhibiting the polygonal, cobblestone appearance commonly associated with epithelial cells (Fig. 1C).

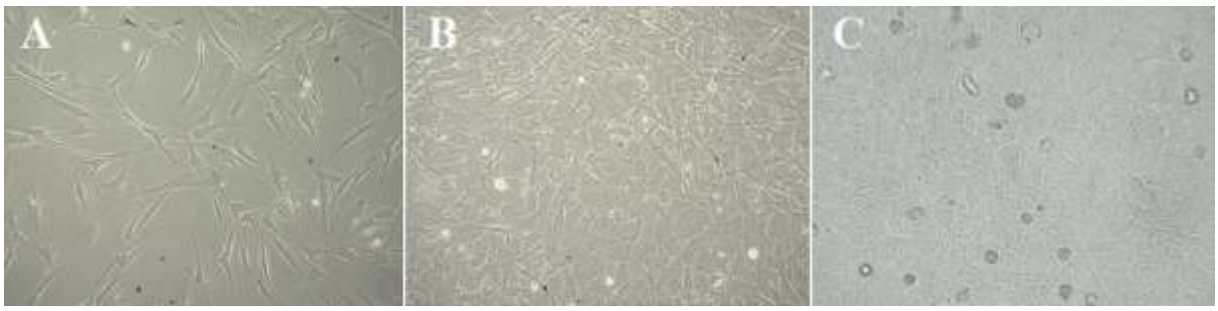

Figure 1. Morphology of CPEC and AEC in culture. Human CPEC at low density (A) and high density (B) cultured in EGF-supplemented media exhibit a fibroblast-like morphology. Human AEC cultured in EGF-supplemented media display the typical cobblestone morphology associated with epithelial cells (C). Photographs taken at x10 magnification.

3.2 Dynabead Titration to Determine the Most Appropriate Cell-to-Bead Ratio

Anti-CD3/anti-CD28-coated stimulatory beads were used to stimulate $CD4^+$ T cells by ligating CD3 and CD28 receptors, mimicking stimulation by antigen presenting cells. To determine the most appropriate number of beads to produce robust and reproducible $CD4^+$ T cell proliferation, a preliminary titration study was carried out using 1:1 to 1:64 cell-to-bead ratios (Fig. 2). This also enabled smaller changes in proliferation to be noticeable by preventing over-stimulation of $CD4^+$ T cells, which would presumably also cause an increase in cell death.

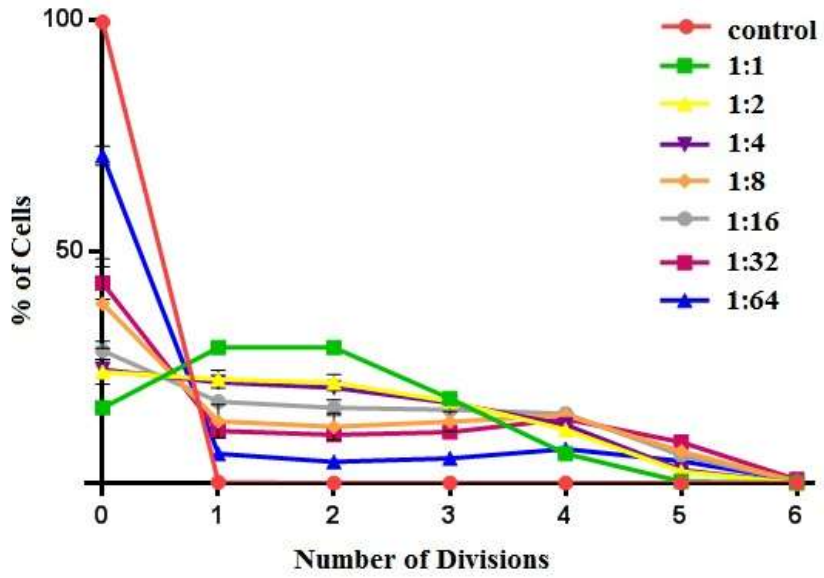

Figure 2. Titration of anti-CD3/anti-CD28 stimulatory beads. The proliferation of $CD4^+$ T cells in response to varying dilutions of stimulatory beads over a 96 hour period was determined. A 1:8 cell-to-bead ratio was selected as the most appropriate ratio to induce sufficient CD4+ T cell proliferation, enabling stimulated cells to undergo more divisions than higher cell-to-bead ratios such as 1:1 (n=1, triplicate wells, mean ± SEM).

As expected, the number of undivided $CD4^+$ T cells increased incrementally with each bead dilution. The higher cell to bead ratios such as 1:1 and 1:2 resulted in more of the original cell population proliferating, however they also culminated in less cell divisions. Conversely, the lower cell-to-bead ratios meant that less $CD4^+$ T cells proliferated, but those that did underwent more divisions.

With the 1:8 ratio, 61% of $CD4^+$ T cells proliferated at least once and 7% of cells underwent a total of 5 divisions. Therefore this ratio provided an intermediate level of proliferation sensitive to change and was therefore selected for use in future assays.

3.3 CPEC Inhibit CD4$^+$ T Cell Proliferation via Secreted Factors

The purity of isolated CD4$^+$ T cell populations for each isolation was between 95-98% (Fig. 3). CD4$^+$ T cells constituted approximately 47-50% of the PBMC population from peripheral blood, with approximately 15% of CD4$^+$ T cells constituting the non-retained fraction; probably due to incomplete labelling by the CD4 Microbeads.

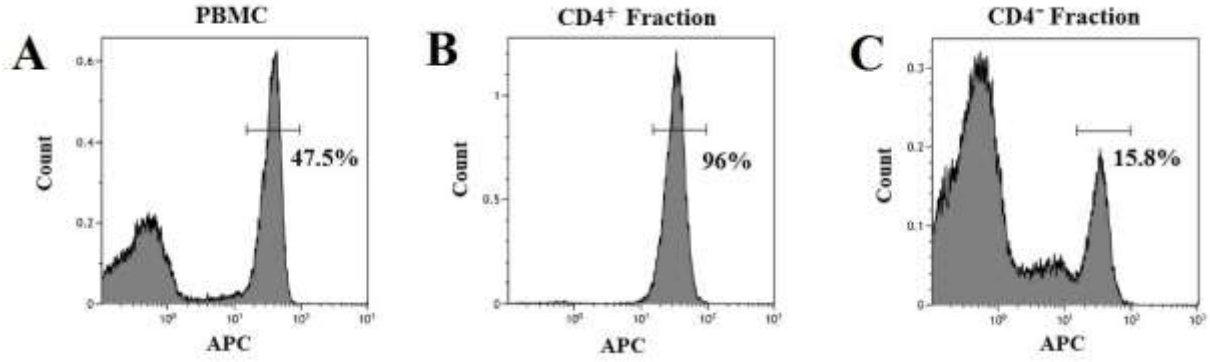

Figure 3. Purity of CD4$^+$ T cell fraction post isolation. Representative histograms showing proportion of CD4$^+$ T cells in whole PBMC (A) and CD4$^+$ T cell purity post-positive selection (B) from the same donor. CD4$^+$ expression in the negative fraction post-positive selection was also assessed (C). Gated on total viable lymphocytes from forward scatter vs side scatter density plots (n=6).

To assess the influence of factors secreted by CPEC and AEC on CD4$^+$ T cell proliferation, proliferation dye-labelled CD4$^+$ T cells were incubated in complete RPMI (control) or CM from CPEC or AEC with or without CD4$^+$ T cell activation (Fig. 4A). Cultures were incubated for 96 hours prior to analysis of cell proliferation by flow cytometry. In combining the data from individual experiments, the positive control (i.e. CD4$^+$ T cell proliferation alone) was converted to 100% and all other values converted to a percentage of the control, such that any inhibition in proliferation is proportional to the control (Fig. 4B). When incubated in CM from CPEC, CD4$^+$ T cell proliferation decreased by 66.7% compared to the positive control. Similarly, when incubated in CM from AEC, CD4 cell proliferation shows a decrease of 65.4%. With the proliferation dye, each peak to the left of the resting cells peak represents a

cell population division. The percentage of cells in the viable population in each cell division was gated on and converted to average number of divisions.

Flow cytometry histograms from one representative experiment (Fig. 4C-F) show that the percentage of $CD4^+$ T cell proliferation in the positive control (i.e. stimulated $CD4^+$ T cells alone) was 93.5% with an average of 2.7 divisions. For stimulated $CD4^+$ T cells in CPEC CM proliferation was reduced to 80.7% with 1.9 divisions and for stimulated $CD4^+$ T cells in AEC CM proliferation was reduced to 86.9% with 2 divisions. In the resting (i.e. unstimulated) $CD4^+$ T cell populations (alone or with CPEC CM or AEC CM) proliferation was <1% and the average number of divisions was 0.02 or less.

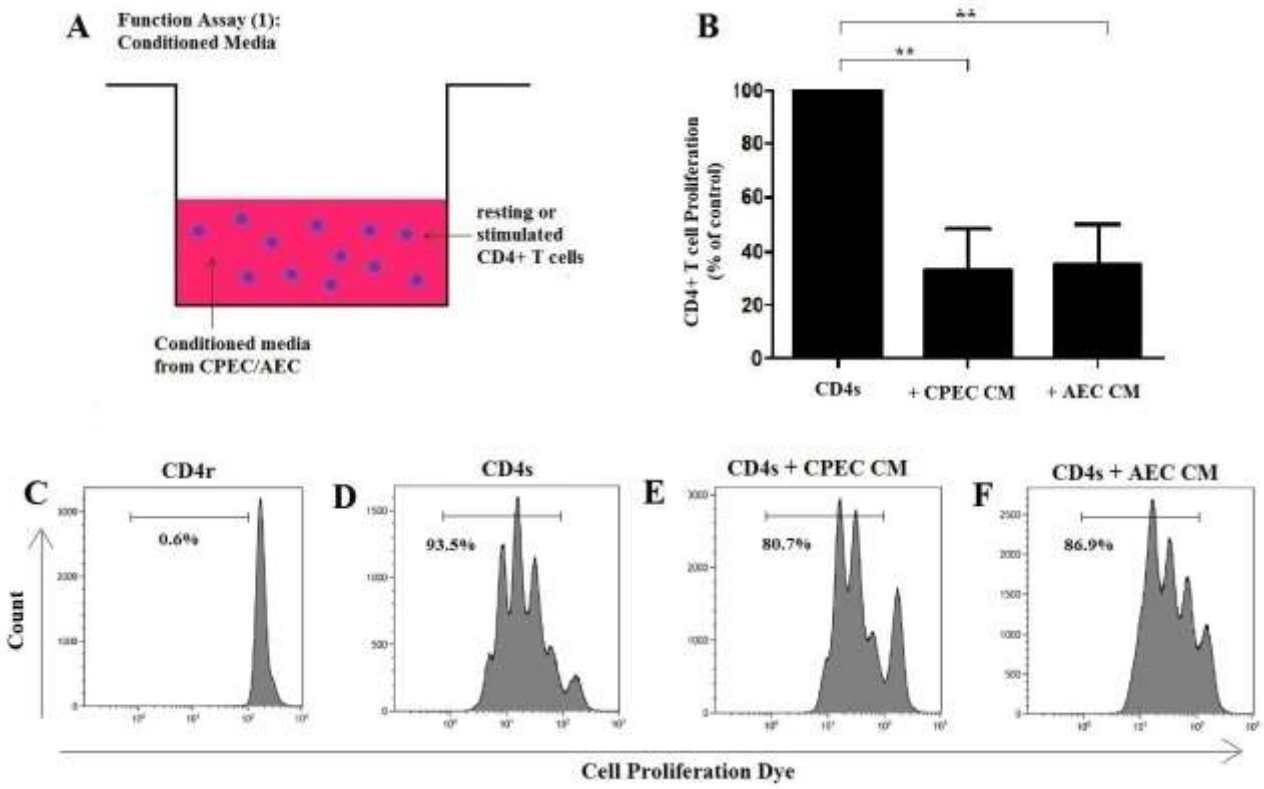

Figure 4. Soluble factors secreted by CPEC and AEC suppress $CD4^+$ T cell proliferation. Resting or stimulated $CD4^+$ T cells were incubated in EGF-free complete RPMI collected from CPEC or AEC for 96 hours (A). Culture of stimulated CD4 cells in the presence of conditioned media (CM) resulted in a 66.7% decrease (CPEC) and a 65.4% decrease (AEC) in $CD4^+$ T cell proliferation (B). Data are mean ±SEM and are significant to ** (P= <0.01) using a one-way ANOVA and Dunnet's post-hoc test (independent assays repeated in triplicate, n=3-4). Representative frequency histograms showing the proliferation of resting $CD4^+$ T cells (C), stimulated $CD4^+$ T cells (D), stimulated $CD4^+$ T cells in CPEC CM (E) and stimulated $CD4^+$ T cells in AEC CM (F) after a 96 hour incubation period. Cell divisions are represented by peaks increasing from right to left.

3.4 CPEC Abrogate CD4$^+$ T Cell Proliferation via Cell Contact-Mediated Mechanisms

The influence of direct contact-mediated mechanisms on CD4$^+$ T cell proliferation was determined by culturing proliferation dye-labelled CD4$^+$ T cells directly with CPEC or AEC with or without stimulatory beads, and determining proliferation after a 96 hour incubation period (Fig. 5A).

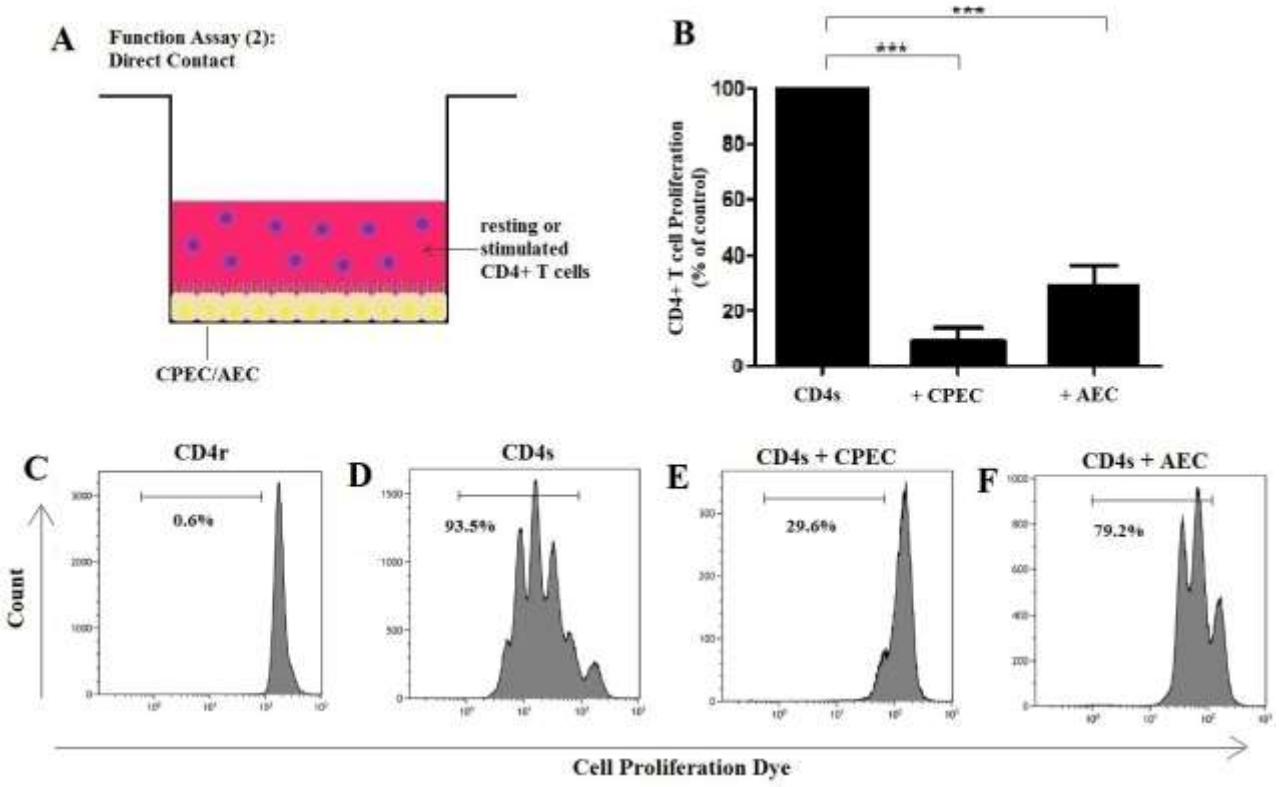

Figure 5. Direct co-culture with CPEC or AEC reduces activated CD4$^+$ T cell proliferation. Resting or stimulated CD4$^+$ T cells were co-cultured with CPEC or AEC for 96 hours (A). Direct contact with CPEC or AEC significantly inhibited activated CD4$^+$ T cell proliferation (B). Data are mean ±SEM and are significant to *** (P= <0.001) using a one-way ANOVA and Dunnet's post-hoc test (independent assays repeated in triplicate, n=3-4). Representative frequency histograms showing the proliferation of resting CD4$^+$ T cells (C), stimulated CD4$^+$ T cells (D), stimulated CD4$^+$ T cells cultured in direct contact with CPEC (E) and stimulated CD4$^+$ T cells cultured in direct contact with AEC (F). Cell divisions are represented by peaks increasing from right to left.

The combined data from repeated experiments shows that direct contact with CPEC reduced CD4$^+$ T cell proliferation by 90.2% whereas direct contact with AEC promoted a 70.8% reduction in CD4$^+$ T cell proliferation (Fig. 5B). The average number of CD4$^+$ T cell divisions

in resting (i.e. unstimulated) populations alone or in contact with CPEC or AEC was below 0.05, therefore resting $CD4^+$ T cell populations were not included in the final analysis. Representative frequency histograms show an average number of $CD4^+$ T cell divisions of 2.7 with 93.5% proliferation for activated $CD4^+$ T cells alone, 0.3 (29.6% proliferation) for activated $CD4^+$ T cells in contact with CPEC and 1.1 (79.2% proliferation) for activated $CD4^+$ T cells in contact with AEC (Fig. 5C-F).

3.5 Selective Central Memory T Cell Migration in Inverted Culture

To mimic the migration of $CD4^+$ T cell subsets in the basolateral-apical direction as observed *in vivo* across the BCSFB, epithelial cells were grown on laminin-coated inserts for 1-2 hours prior to returning to the correct orientation for a further 72 hours (Fig. 6A). $CD4^+$ T cells were added to the inserts and the phenotypes of cells that had migrated through the epithelial layers after a 4 hour incubation period was determined by flow cytometry. Since the majority of $CD4^+$ T cells in the CSF are of the central memory phenotype, the ability of CPEC to recruit central memory $CD4^+$ T cells was investigated, using AEC as a comparison.

Migrated cells from whole $CD4^+$ T cell populations were characterised by staining for CD45RA and CCR7 antigens and assessing by flow cytometry (Fig. 6C-E). Effector memory $CD4^+$ T cells (Tem) were classified as $CD45RA^-$ $CCR7^-$ and central memory $CD4^+$ T cells (Tcm) were classified as CD45RA- $CCR7^+$. Naïve $CD4^+$ T cells were classified as being both $CD45RA^+$ and $CCR7^+$.

On average, the relative migration of Tcm was significantly increased from 35% (± 5.1) to 55% (±7.3) with CPEC on the culture insert and 61% (±2.1) with AEC on the culture insert, suggesting preferential recruitment of Tcm. The relative migration of Tem was insignificantly affected in the presence of CPEC (11.8% ±3.0) and AEC (12.3% ±2.3) on the culture insert

compared to the control, i.e. spontaneous migration (11.4% ±0.8). The relative migration of naïve $CD4^+$ T cells was reduced from 55% (±5.3) to 33% (±9.6) when CPEC were grown on the underside of the culture insert, and 27% (±4.4) when AEC were grown on the underside of the culture insert (Fig. 6B).

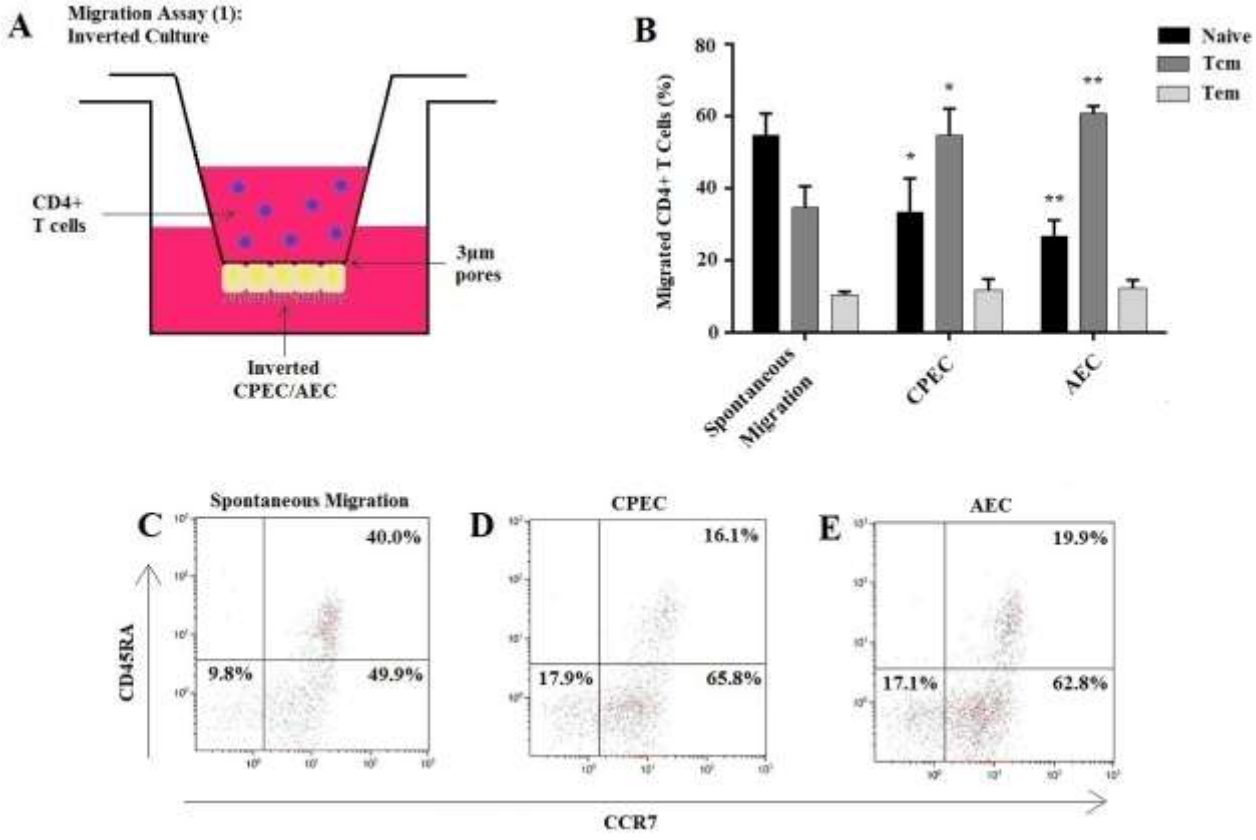

Figure 6. Inverted culture of CPEC and AEC increased central memory $CD4^+$ T cell migration. The phenotype of migrated $CD4^+$ T cells through CPEC or AEC grown on the underside of laminin-coated transwell inserts was determined after 4 hours (A). The presence of CPEC or AEC increased Tcm migration compared to the control (spontaneous migration; B). Data are means ± SEM and are significant to * (P= <0.05) and ** (P= <0.01) using a two-way ANOVA and Tukey's multiple comparisons test (independent assays repeated in singular or duplicate wells, n=3). Representative flow cytometry quadrants of an individual experiment showing the proportions of naïve, Tem and Tcm $CD4^+$ cells migrated spontaneously through a culture insert (C) or through CPEC (D) or AEC (E) grown on the underside of culture inserts.

3.6 Conditioned Media from CPEC and AEC Induces Tcm Recruitment

The influence of CPEC, AEC or soluble factors secreted by both cell types on the migration of $CD4^+$ T cell subsets was investigated using transwell culture inserts set up in 24 well plates. CPEC or AEC-derived CM was added to the wells (Fig. 7A) or epithelial cells were grown in the wells (Fig. 7B) and migrated $CD4^+$ T cells were analysed after 4 hours.

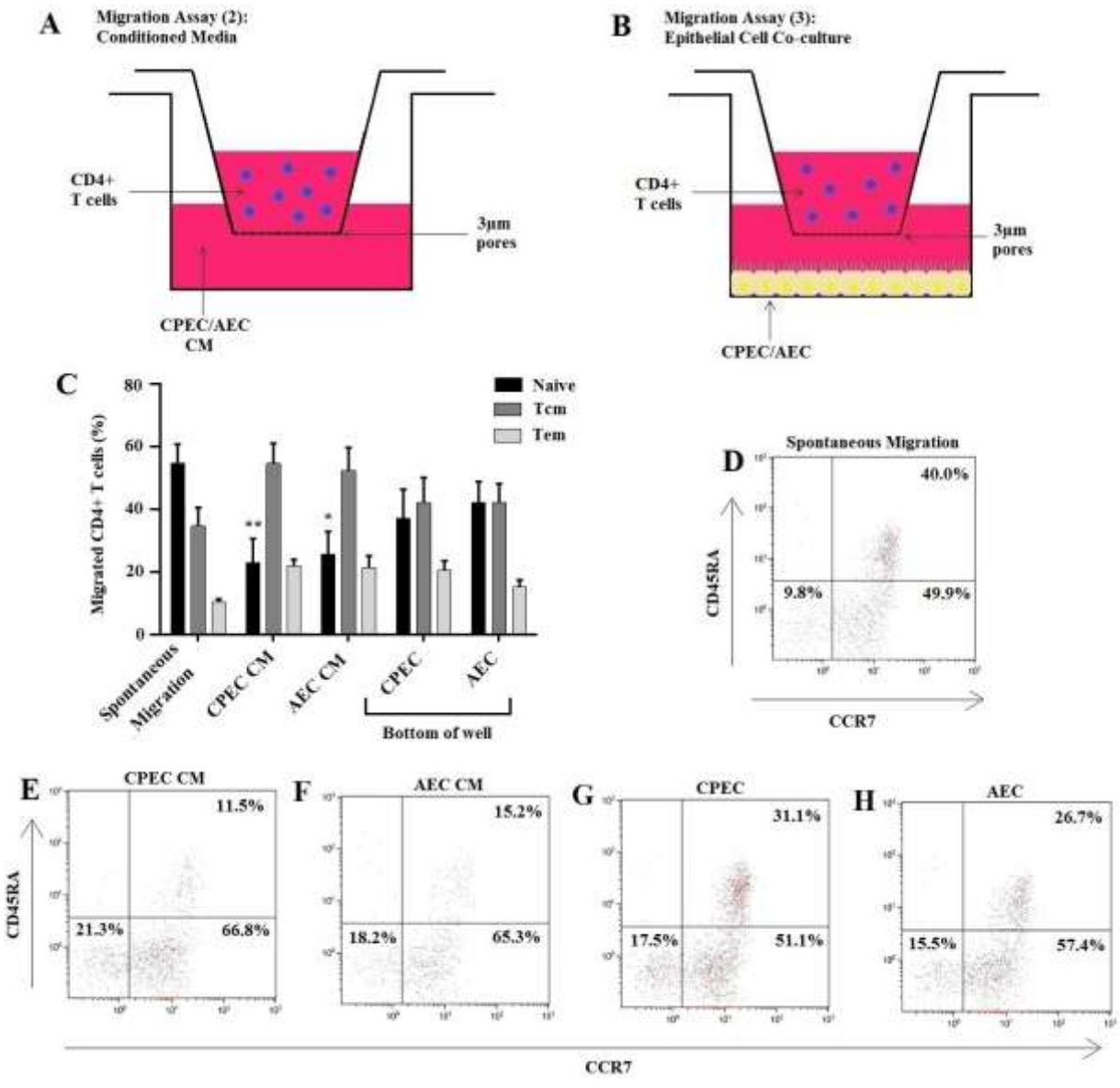

Figure 7. Secreted factors play a role in epithelial cell-mediated Tcm recruitment. The migration of $CD4^+$ T cells towards CPEC or AEC-derived CM (A) or towards CPEC or AEC growing in the wells (B) was investigated. Graph show the average migration of $CD4^+$ T cells through transwell inserts towards CPEC or AEC-derived CM, or towards CPEC or AEC in the wells (C). Data are mean ± SEM and are significant to * (P= <0.05) and ** (P= <0.01) using a two-way ANOVA and Tukey's multiple comparisons test (independent assays repeated in single or duplicate wells, n=3-4). Representative flow cytometry quadrants of an individual experiment showing the proportions of naïve, Tem and Tcm $CD4^+$ cells migrated spontaneously through a culture insert (D), towards CPEC CM (E) or AEC CM (F) or towards CPEC (G) or AEC (H) in the wells.

Spontaneous migration, i.e. the migration of $CD4^+$ T cells through the culture insert with no cells or CM in the well, showed that the migrated cell population constituted 55% (±5.3) naïve CD4 cells, 35% (±5.1) Tcm and 11% (±0.8) in a 4 hour period.

Results show that CM from both CPEC and AEC recruit Tcm and Tem to a lesser extent but show a reduction in naïve $CD4^+$ T cell migration (Fig. 7C). The presence of CPEC-derived CM in the wells increased Tcm migration to 55% (±7.0; a 20% increase) and Tem migration to 22% (±3.9; an 11% increase) of the total migrated $CD4^+$ T cell population. Relative naïve $CD4^+$ T cell migration was 23% (±7.4; a 32% decrease).

Addition of AEC-derived CM to the wells showed similar results, with the migrated population constituting 26% (±12.6) naïve CD4 cells (a 29% decrease), 52% (±13.3) Tcm (a 17% increase) and 21% (±0.7) Tem (a 10% increase). This appears to suggest that epithelial cells employ mechanisms to actively recruit Tcm and Tem.

Discussion

This study sought to investigate the potential of CPEC, which *in vivo* constitute the BCSFB, to influence $CD4^+$ T cell proliferation and migration in direct contact or in the presence of secreted soluble factors. AEC were used alongside CPEC as a positive control due to their well-documented immunomodulatory properties. Results indicate that both CPEC and AEC (1) can modulate the immune response, and (2) influence the migration of $CD4^+$ T cell subsets.

4.1 CPEC and AEC Morphology

In culture, CPEC appeared fibroblast-like and elongated; a characteristic not generally associated with epithelial cells. These cells were procured commercially and although they had only been previously passaged once, their unusual phenotype could have been a potential artefact of culture caused by a high number of cell doublings, which may have promoted reversion to a less differentiated phenotype of neuronal origin. These cells had also been previously subjected to freezing in liquid nitrogen for transport purposes, which may have affected their phenotype. In contrast, AEC exhibited the typical cobblestone morphology associated with epithelial cells. AEC were sourced from primary human tissue shortly prior to use and were not frozen, which could have contributed to them retaining their phenotypic characteristics.

Interestingly a change in morphology was observed in AEC in contact with activated $CD4^+$ T cells; however in the same conditions there was no morphological change in CPEC. The cobblestone-like morphology of AEC under resting conditions became star shaped and fibroblast-like with numerous protrusions- a previously unreported phenomenon. This could reflect the inhibitory capacity of the cells, as this study has shown that AEC-mediated

inhibition of activated $CD4^+$ T cells is less potent than CPEC, which appear to readily inhibit activated $CD4^+$ T cells. It could be that on stimulation, AEC require the up-regulation of inhibitory receptor(s) and signalling proteins required to dampen the immune response which could (1) be responsible for the apparently lower inhibitory capacity of AEC, and (2) cause the observed change in morphology. This would also imply that CPEC constitutively express the molecules required to inhibit $CD4^+$ T cell proliferation, also reflected by the results of this study.

4.2 Mechanisms of Inhibiting $CD4^+$ T Cell Proliferation

This study has demonstrated that immunosuppressive factors secreted by both CPEC and AEC inhibit $CD4^+$ T cell proliferation to a similar degree (approximately 66%), however direct contact resulted in an even greater degree of inhibition, with CPEC mediating a 90% and AEC a 70% reduction in proliferation. This suggests that the secreted factors responsible for this phenomenon could be common to epithelial cells, in part agreeing with previous publications documenting inhibition mediated by different types of epithelial cells (Cruickshank *et al.*, 2004; Sugita *et al.*, 2009; Qureshi *et al.*, 2010). Direct contact with AEC resulted in only a slight increase in $CD4^+$ T cell inhibition, suggesting that AEC-mediated effects could be entirely soluble and that the observed improvement in direct contact could be a technical effect of using CM, rather than a biological effect. However with such significant attenuation of $CD4^+$ T cell proliferation, CPEC-mediated inhibition is difficult to dispute and suggests a potent immune-regulatory role for CPEC in terms of both secreted soluble factors and direct interaction with $CD4^+$ T cells.

4.2.1 Secreted Factors

The mechanisms mediating $CD4^+$ T cell inhibition remain elusive and are likely complex and multi-factorial. Established molecules playing a role in immunomodulation include TGF-β and PGE2, however it is possible that other molecules play a role.

Arginine is required by T cells for a number of important biological functions, including proliferation and expression of the T cell receptor (TCR) complex (Ochoa *et al.,* 2001; Popovic *et al.,* 2007). One candidate molecule that may contribute to epithelial cell-mediated $CD4^+$ T cell inhibition is inducible nitric oxide synthase (iNOS), which catalyses the production of nitric oxide from arginine. iNOS is a major mediator in the suppression of T cell proliferation by some cell types such as mesenchymal stem cells (MSC) and its production has been reported in retinal pigmented epithelial cells in response to activated T cells, thus it is plausible that CPEC and/or AEC may employ iNOS to the same effect (Liversidge *et al.,* 1994; Sato *et al.,* 2007). Arginine depletion is also mediated by arginase and though the induction of iNOS or arginase blocks T cell proliferation, simultaneous induction of both enzymes results in T cell apoptosis, which appeared to be increased in CPEC and AEC CM (Bronte *et al.,* 2003). Analysing the CM for these metabolites or enzymes would indicate whether any of these mechanisms are at play.

Another molecule implicated in immune regulation is indoleamine 2,3-dixoygenase (IDO). IDO expression has been detected in the brain and at the maternal-foetal interface, thus it is possible that CPEC and AEC limit T cell proliferation by producing this enzyme, which restricts the availability of the essential amino acid tryptophan in the local environment (Ligam *et al.,* 2005; Kwidzinski *et al.,* 2007). Analysing the extent of apoptosis by quantification of dead cells wouldn't address which molecules are involved but may provide some information on the mechanisms involved.

AEC also secrete interleukin-10 (IL-10), vascular endothelial growth factor (VEGF) and soluble human leukocyte antigen-G (sHLA-G), a non-classical class I antigen exclusively expressed by placental and thymic cell types with a role in immune tolerance (Bogic *et al.,* 2000; Lefebvre *et al.,* 2000; Li *et al.,* 2005). Reflecting their function as facilitators of neuronal growth, CPEC have been documented to secrete nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and other neurotrophins as well as interleukin-6 (IL-6) and basic fibroblast growth factor (bFGF) (Yamamoto *et al.,* 1996; Vallieres *et al.,* 1997; Chodobski *et al.,* 2001; Borlongan *et al.,* 2004). Thus although both CPEC and AEC likely secrete a range of common immunomodulatory factors, it is difficult to know how conserved the immunosuppressive response is amongst epithelial cell types. The developmental origin, location and function of the cells *in vivo* could therefore influence the molecules that are secreted. Furthermore, a distinction must be made between molecules the cells *can* secrete and molecules the cells *do* secrete on contact with T cells.

4.2.2 Cell-bound Mechanisms

In addition to soluble factors, negative signals delivered to T cells by cell-bound counter-regulatory molecules contribute to the tight regulation of the immune system. Such rapid and deliberate responses are vital to maintaining the delicate balance between resting and inflammatory conditions.

4.2.2.1 Induction of Anergy

Dysregulated lymphocyte migration across the BCSFB could be of potential danger so must be tightly regulated. Though it is possible that both types of anergy (clonal anergy and adaptive tolerance) may exist in the same environment, the findings in this report suggest that

the $CD4^+$ T cells are inhibited on activation, since resting $CD4^+$ T cells in contact with CPEC or AEC do not appear to be adversely affected compared to the control. Also, the anti-CD3/anti-CD28-coated stimulatory beads provide both signals required for complete $CD4^+$ T cell activation; thus clonal anergy can be disregarded as a mechanism of inhibition.

Naïve $CD4^+$ T cells constitute approximately 30% of the total $CD4^+$ T cell population so their inhibition would theoretically still enable 70% of $CD4^+$ T cells to proliferate. In this study direct contact with CPEC inhibited the proliferation of over 90% of the total CD4 cell population, thus ruling out adaptive tolerance as the major mechanism of $CD4^+$ T cell inhibition. This supports findings from a previous study, which demonstrated that colonic epithelial cells inhibit $CD4^+$ T cell proliferation but do not induce anergy (Cruicksank *et al.*, 2004).

4.2.2.2 Death Receptors

Previous work has shown that AEC mediate apoptosis by FasL and TRAIL signalling, thus maintaining a state of immune privilege (Li *et al.*, 2005). Though FasL is expressed in the brain and spinal cord, it is not expressed in the choroid plexus (French *et al.*, 1996; Bechmann *et al.*, 1999), leading to the suggestion that different areas of the CNS may employ different mechanisms of regulating the immune system. This could offer a fail-safe mechanism such that if one of these regulatory pathways was defective, an alternative would be available; a logical possibility considering the damaging consequences of CNS inflammation.

4.2.2.3. PD-1-PD-L1 Signalling

Since it appears that FasL probably does not play a role in CPEC-mediated inhibition, other receptor-dependent mechanisms must be explored.

As the counter-regulatory molecule PD-L1 has been previously implicated as a mechanism of immunomodulation by various epithelial cell types, it is possible that this molecule is also responsible for regulating immune responses by CPEC (Cruickshank *et al.,* 2004; Beswick *et al.,* 2007; Heinecke *et al.,* 2008; Sugita *et al.,* 2009). Blocking the PD-L1 target PD-1 in mice promotes the development of autoimmune diseases by inducing a chronic breakdown of peripheral self-tolerance, suggesting that the PD-1-PD-L1 pathway is important in preventing autoimmune conditions (Nishimura *et al.,* 1999; Nishimura *et al.,* 2001). Further evidence for the involvement of this pathway in the CNS comes from Salama *et al.,* who found that blocking PD-1 resulted in increased autoreactive T cells in the CNS which contributed to EAE (Salama *et al.,* 2003). Though their results show that PD-L1 is expressed in the brains of mice, the specific location of PD-L1 expression is not established so it remains unknown whether this receptor is expressed by CPEC.

4.2.2.4 Alternative Mechanisms of Inhibition

It could be that CPEC employ unidentified mechanisms to inhibit T cell proliferation. Recently identified members of the CD28/B7 superfamily, including V-set and Ig domain-containing 4 (VSIG4), B and T lymphocyte attenuator (BTLA), B7-H3 and B7-H4, are co-inhibitors of T cell proliferation and give an insight into the complexity of cell signalling pathways but also raise the possibility of other previously unidentified inhibitory receptors playing a role (Watanabe *et al.,* 2003; Vogt *et al.,* 2006; Kryczek *et al.,* 2006; Leitner *et al.,* 2009).

4.3 Preferential Immune Cell Recruitment Mediated by Epithelial Cells

This study sought to better understand the relationship between naïve, central memory and effector memory $CD4^+$ T cell subsets with CPEC to help elucidate the complex nature of lymphocyte trafficking across the choroid plexus. As a well-established immunomodulatory cell type providing immune privilege *in vivo*, AEC were used as a comparison. Results from this study suggest that CPEC and AEC actively recruit Tcm and to a lesser extent Tem.

The findings that less Tcm migrated towards CPEC and AEC growing in the wells than towards CM is difficult to explain. These effects were presumably due to soluble factors, and it may be that the presence of $CD4^+$ T cells induced a downregulation in the chemotactic molecules secreted by the epithelial cells. Inverted culture appeared to promote Tcm migration more than standard culture, suggesting that the mechanisms involved may be more strongly mediated via the basolateral surface of epithelial cells rather than the apical surface.

4.3.1 Promoting Tcm Recruitment

As previously mentioned, CM contains a host of soluble factors and cytokines which may have promoted specific chemoattraction of Tcm. This would be advantageous in the context of immune privilege for a number of reasons: (1) memory T cells in general are experienced and have previously encountered antigen, (2) Tcm have less effector function at rest (as compared to Tem) and (3) Tcm can rapidly proliferate and differentiate into effector cells on encountering antigen (Berard & Tough, 2002). The idea that AEC could differentially recruit Tcm has not previously been explored but considering their requirement to promote immune privilege at the foetal-maternal interface, preferential Tcm recruitment would appear beneficial.

4.3.1.1 Chemokines involved in Selective Tcm Recruitment

Tcm express the homing chemokine CCR7 on their surface, which can be used to gain access to the CNS via CCL21 expressed constitutively on the choroid plexus (Kivisakk *et al.,* 2003). However, CCR7 is also expressed by naïve T cells and transiently by Tem on activation, so although this pathway is certainly a route for lymphocyte trafficking, it seems unlikely to be responsible for selective Tcm recruitment (Sallusto *et al.,* 1999; Rot *et al.,* 2004).

IFN-γ signalling, which has recently been implicated in leukocyte trafficking across the BCSFB, may play a role in Tcm migration into the CNS (Kunis *et al.,* 2013).

It is reported that the majority of T cells in the CSF express the chemokine receptor CXCR3, which is involved in immune surveillance, regardless of disease status (Kivisakk *et al.,* 2002). Since the majority of T cells in the CSF are $CD4^+$ Tcm, it is possible that these cells are granted entry into the CNS through CXCR3-dependent mechanisms mediated by cytokines secreted by epithelial cells. CXCL9 and CXCL10, two of the chemokines that bind CXCR3, are constitutively expressed by human intestinal epithelial cells and CXCL9 is also expressed constitutively by epithelial cells lining the male urogenital tract (Linge *et al.,* 2008), raising the possibility of CXCL9 or CXCL10 expression by CPEC or AEC.

4.3.1.2 Adhesion Molecules involved in Selective Tcm Recruitment

The presence of a P-selectin-expressing subset of memory $CD4^+$ T cells in the peripheral blood suggests that this subpopulation may be granted unlimited access to the CNS via P-selectin-mediated trafficking across the choroid plexus (Kivisakk *et al.,* 2003). As tissue-specific lymphocytes have previously been described this raises the possibility that CNS-specific lymphocytes also exist, and it may help explain the distinction between immune

surveillance, where tissues are routinely patrolled, and inflammation, mediated by T cells non-specific to the CNS and thus have never encountered CNS antigens (Cose 2007).

In the BCSFB, CPEC are polarised so that lymphocyte trafficking occurs in the basolateral to apical direction. Selective recruitment would imply that CPEC exert this function from their basolateral side; however CPEC are adhered to a basal lamina thus presumably imposing restrictions on the secretion or expression of regulatory molecules. As such, it could be that initial migration is non-specific and that phenotypic modulation occurs during migration, ensuring that any cell entering the CNS is modified to the requirements dictated by CPEC.

Because of the range of chemokines, cytokines and adhesion molecules known to contribute to homeostatic leukocyte trafficking into the CNS, it is likely that a combination of molecules is required to produce the correct 'entry code' for specific immune cell subsets.

4.4 Assay Refinements and Future Work

4.4.1 Effects of Culture Conditions on Epithelial Cells

Though not relevant to the *in vivo* characteristics of the cells, understanding the influence of extended passage number or prolonged culture on the phenotype and function of CPEC and AEC would help identify artefacts induced by these conditions, which can be addressed and rectified in future studies to make them more physiologically relevant. Ideally, prolonged culture should be avoided and cells should be used at low passage but practically this may be difficult so understanding more about the effects of prolonged culture is important for optimising *in vitro* studies.

4.4.2 Improving the Proliferation Assays

Some unrecoverable adherent $CD4^+$ T cells were observed in co-culture were observed, possibly causing selective harvesting and leading to experimental bias. However prior to harvesting the $CD4^+$ T cells there was no visible $CD4^+$ T cell proliferation in contact with CPEC, suggesting that the biological mechanisms are true and that further protocol refinements are required. Harvesting the epithelial cells from each well and then purifying the $CD4^+$ T cells may ensure a better recovery rate.

4.4.3 Real-Time Analysis of the Influence of Secreted Factors on Proliferation

Demonstrating a direct biological effect rather than a metabolic effect, i.e. caused by the depletion of nutrients, may be difficult. To analyse the effects of secreted factors in real-time, a culture insert permitting the diffusion of soluble factors but preventing cell migration could be used, with the epithelial cells grown in the well and the $CD4^+$ T cells added to the culture insert. These inserts are available commercially and typically come with a pore size of 0.4μm. Though this current study has shown that factors secreted by CPEC and AEC significantly reduce $CD4^+$ T cell proliferation, the described transwell system would likely provide a more accurate account of the degree of inhibition and could be employed in future studies.

4.4.4 Investigating the Expression Characteristics of Inhibitory Molecules

Future work could investigate the time course of $CD4^+$ T cell inhibition which may help to elucidate the expression characteristics of the inhibitory molecules involved, i.e. whether their expression is constitutive or induced. To analyse the potency of secreted factors, $CD4^+$ T cells

could be incubated in varying concentrations of CM to determine the minimum threshold required to inhibit $CD4^+$ T cell proliferation. Multiplex ELISA or mass spectrometry could be employed to elucidate the components of CPEC and AEC-derived CM, and gene expression analysis of CPEC and AEC in contact with resting and stimulated $CD4^+$ T cells may reveal the expression characteristics of any inhibitory molecules or receptors.

4.4.5 Investigating the Mechanisms of Epithelial Cell-Mediated Inhibition

Though inhibition of $CD4^+$ T cell proliferation by epithelial cells has been widely reported, the precise mechanisms remain elusive. Further studies could investigate whether inhibition is reversible by culturing stimulated $CD4^+$ T cells with CPEC for a period of time, harvesting the $CD4^+$ T cells and restimulating in the absence of CPEC. This would demonstrate whether the $CD4^+$ T cells have been rendered anergic or if they are still responsive and are able to proliferate. The latter case would suggest that CPEC enable $CD4^+$ T cells to retain their functions but are temporarily 'dampened' in their presence.

It is possible that certain $CD4^+$ T cell subsets, such as naïve $CD4^+$ T cells, are more amenable to inhibition than others. Repeating the assays with purified $CD4^+$ T cell subsets and restimulating the $CD4^+$ T cells after an initial stimulation period would show whether epithelial cells are selective in their inhibition.

4.4.6 Improving the Inverted Co-Culture Model

Inverted co-culture is intended to be physiologically representative of the BCSFB so that $CD4^+$ T cell migration occurs in the basolateral to apical direction, as it occurs *in vivo*. An important feature of the BCSFB is the presence of tight junctions (TJ), providing a physical

barrier between the CNS and the periphery with proteins such as occludin, claudin-1 and ZO-1 (Johanson *et al.,* 2011). Ideally, a monolayer of epithelial cells would have formed on the underside of the insert. The integrity of the epithelial layer can be assessed by measuring the trans-epithelial electrical resistance (TEER) or by assessing the permeability of the barrier to macromolecules such as dextran (Tenebaum *et al.,* 2013), and monolayer formation may be improved by reducing serum and calcium concentrations in the media (Chang *et al.,* 1997). Also, cutting out the culture insert and staining the epithelial cells for TJ proteins would have revealed more information on the barrier characteristics and the arrangement of epithelial cells.

Though this study modelled lymphocyte migration across a model of the BCSFB, trafficking into the CNS is comprised of multiple barriers and a multi-tiered transwell system mimicking endothelium, a matrigel-like component to replace the stroma and finally the CPEC of the BCSFB may be more accurate in replicating the complex *in vivo* system and may also demonstrate a role for endothelium.

Conclusions

A number of candidate molecules exist that could be responsible for CPEC-mediated $CD4^+$ T cell inhibition, though it is unlikely that any one molecule is entirely accountable for this effect. Rather, it appears that CPEC and AEC exhibit a multi-factorial approach to regulating the immune system with evidence for the involvement of both soluble factors and direct contact-dependent mechanisms. Though evidence exists for the inhibition of T cell proliferation by other types of epithelial cells, it is unclear whether epithelial cells from different sources harbour similar abilities to modulate the immune response or whether those derived from immune privileged sites are more highly specialised.

Due to the high numbers of $CD4^+$ Tcm present in the CSF, preferential recruitment of Tcm has been implicate for CPEC, but this phenomenon is previously undocumented for AEC (de Graaf *et al.,* 2011). Thus it seems that immune privileged sites may universally employ this mechanism of immune surveillance, giving a new dimension to the term 'immune privilege'.

Understanding a biological system under normal conditions is equally as important as understanding the same system in disease. A greater appreciation of lymphocyte trafficking and the events that occur at the BCSFB may enhance our understanding of inflammatory conditions of the CNS, such as multiple sclerosis. Furthermore, readily available immunomodulatory cells have generated interest for their therapeutic potential, providing a great deal of scope for further studies.

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UNIVERSITY OF BIRMINGHAM

Project Two:

Characterisation of Human Mesenchymal Stromal Cells from Different Sources and the Effects of Therapeutic Ultrasound

This project is submitted in partial fulfilment of the requirements for the award of the MRes

This project was carried out under the supervision of:

Dr Ben Scheven

Abstract

Mesenchymal stromal cells (MSC) from different sources may possess enhanced properties over others. Furthermore, ultrasound is a potential tool to influence cell behaviour. MSC from adult (bone marrow, adipose and dental pulp) and foetal (amnion) sources were compared and the effects of ultrasound were investigated.

The proliferation, osteogenic and adipogenic differentiation potential, expression of immunomodulatory (TGF-β1, IL-10, COX-2) and stemness (Oct-4, nanog) genes and secretion of VEGF, BDNF and TGF-β1 was assessed. Proliferation, gene expression and cytokine secretion 48 hours after a single 5 min ultrasound treatment (1MHz, 250mWcm^{-2}) was also determined.

Dental pulp-MSC had the highest proliferative capacity but the lowest differentiation potential. Bone-marrow-MSC had the lowest proliferative capacity but highest differentiation potential along with adipose-MSC, and secreted high levels of VEGF. Amnion-MSC possessed the highest osteogenic potential, expressed both immunomodulatory and stemness genes moderately and secreted highest levels of TGF-β1 and BDNF. Ultrasound has no effect on proliferation and had mostly opposing effects on gene expression in dental pulp-MSC and adipose-MSC, but increased VEGF secretion.

MSC from different sources possess specialised characteristics, and may be preferentially selected based on the desired application. Ultrasound may enhance immunomodulatory and stemness properties of some MSC types for experimental or therapeutic use.

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List of Abbreviations

ADSC	Adipose Tissue-Derived MSC
AMSC	Amnion-Derived MSC
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BMSC	Bone Marrow-Derived MSC
CFU-F	Colony Forming Unit-Fibroblasts
CM	Conditioned Media
COX-2	Cyclooxygenase-2
DPSC	Dental Pulp-Derived MSC
EGF	Epidermal Growth Factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-10	Interleukin-10
IL-8	Interleukin-8
LIPUS	Low Intensity Pulsed Ultrasound
MSC	Mesenchymal Stromal Cell
Oct-4	Octamer-Binding Transcription Factor-4
PBMC	Peripheral Blood Mononuclear Cell

PDGF	Platelet-Derived Growth Factor
PGE2	Prostaglandin E2
SATA	Spatial Average Temporal Average
TGF-β	Transforming Growth Factor-β
VEGF	Vascular Endothelial Growth Factor

Introduction

1.1 What are Mesenchymal Stromal Cells?

Mesenchymal stromal cells (MSC) are a heterogeneous population of multipotent cells well known for their self-renewing abilities, cytoprotective properties and potent immunomodulatory capacity, interacting with both innate and adaptive arms of the immune system through numerous soluble and cell-bound factors (Uccelli *et al.,* 2008; Le Blanc *et al.,* 2012).Originally identified by Friedenstein *et al* in 1968, MSC isolated from the bone marrow were described as colony forming unit-fibroblasts (CFU-F) and have since been extensively characterised, though their precise nature remains elusive (Friedenstein *et al.,* 1968).

1.2 Properties of MSC from Different Sources

Due to their presence in the stromal compartment of almost all tissues (da Silva Meirelles *et al.,* 2006), MSC can be isolated from a wide variety of sources, the most common of which are bone marrow (Quiroz *et al.,* 2008), adipose tissue (Fraser *et al.,* 2006) and peripheral blood (Chong *et al.,* 2012) with alternative sources including muscle (Young *et al.,* 2001), heart (Beltrami *et al.,* 2003), synovium (de Bari *et al.,* 2001) and lung (Griffiths *et al.,* 2005). MSC have more recently been derived from birth-associated 'discard' tissues including the placenta, umbilical cord and foetal membranes and with simple non-invasive retrieval, plentiful supply and no ethical constraints, holds a number of benefits over conventionally sourced MSC (Soncini *et al.,* 2007). Furthermore, there is evidence that MSC of a foetal origin possess enhanced proliferative capacity, lower immunogenicity and superior immunosuppressive properties compared to adult MSC, making them more attractive for cell-based therapies (Kern *et al.,* 2006; Deuse *et al.,* 2011). Donor age also affects MSC plasticity,

with the differentiation potential of MSC decreases as donor age increases (D'Ippolito *et al.*, 1999).

MSC secrete a wide range of growth factors and cytokines which are largely responsible for their immunomodulatory and cytoprotective qualities. These include angiogenic growth factors such as vascular endothelial growth factor (VEGF), neurotrophins such as brain-derived neurotrophic factor (BDNF), and immunomodulatory cytokines such as transforming growth factor-β1 (TGF-β1) amongst many others (Hass et al., 2011; Lopatina et al., 2011). However it is unknown whether the location of MSC *in vivo* influences the MSC 'secretome', or whether all MSC secrete the same molecules to the same degree.

The proliferative capacity of MSC may depend on their location and function *in vivo*. A previous study found that adipose-derived MSC (ADSC) from the subcutaneous adipose region proliferated faster compared to those derived from the omental region, suggesting that there are variations even within MSC derived from the same source (van Harmelen *et al.*, 2004). Cell density *in vivo* could also determine proliferative capacity – if one tissue has relatively high numbers of MSC then proliferation may be lower, but another tissue with relatively low numbers of MSC may require higher turnover. Furthermore, proliferation of some cell types such as bone marrow-derived MSC (BMSC) is limited by contact inhibition in culture, whereas other cell types such as dental pulp-derived MSC (DPSC) appear to grow by multi-layering, thus enabling cell number to rapidly increase (Baksh *et al.*, 2007). The lower proliferative capacity of BMSC has been reported on multiple occasions, and it has been suggested that other MSC such as ADSC may be preferable over BMSC where large numbers are required (D'Ippolito *et al.*, 1999; Kern *et al.*, 2006).

Because amnion is a temporary tissue existing for only nine months *in vivo*, amnion-derived MSC (AMSC) may have a different proliferative capacity compared to adult MSC. During development AMSC must proliferate rapidly as the amnion grows and by the third trimester are likely very slow at proliferating or even cease to proliferate completely, again providing evidence that MSC derived from the same tissue may exhibit variable characteristics (Guillot et al., 2007). However there is conflicting evidence in the literature regarding AMSC proliferation. A number of groups have reported that AMSC from term amnion undergo cell death after only 4-5 passages in culture (Portmann-Lanz et al., 2006; Soncini et al., 2007) however others have reportedly maintained AMSC for over 15 passages and describe a high proliferative capacity (Kim et al., 2007, Ilancheran et al., 2009), thus it is likely that donor variability and tissue age is important to this phenomenon.

1.3 Expanding MSC in Culture

Often, the isolation of MSC alone is not sufficient to generate enough cells for experimental or therapeutic use; therefore MSC must be readily expandable in culture. This may be achieved by supplementing culture media with growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF); a practice known as '*ex vivo* expansion', which has been applied to increase MSC proliferation (Lindenmair *et al.*, 2012).

Basic FGF (bFGF) is the most common media supplement for MSC and in addition to increasing proliferation, is said to increase the immunomodulatory potential of MSC by upregulating HLA-class I and HLA-DR expression; however it is also reported that bFGF leads to a limited differentiation capacity and shortening of MSC in culture (Sotiropoulou *et al.*, 2006). EGF is also a common media supplement for MSC expansion and studies have

shown that EGF not only enhances MSC proliferation, but also the differentiation potential, motility and paracrine activities of MSC, potentially making MSC more valuable for therapeutic use (Tamama *et al.,* 2010). It is unclear whether MSC from different sources respond to mitogenic stimulation to the same degree.

1.4 Clinical Applications of MSC

MSC are an exciting prospect for cell-based therapies and an extensive list of on-going clinical trials using MSC-based therapy to ameliorate diseases exists. Most commonly, MSC are studied as potential treatments for bone and cartilage disease, heart disease and graft-versus-host disease, but also liver disease, brain disease, diabetes and multiple sclerosis (Wang & Zhao, 2012; Kim & Cho, 2013). Clinical interest in MSC is generally attributed to their immunomodulatory and cytoprotective properties but also to their ability to home to tumours when injected intravenously, making them an exciting prospect for anti-cancer treatment (Reagan & Kaplan, 2011). Furthermore, even though alternative cell types such as embryonic stem cells possess a superior proliferative and regenerative capacity to MSC, their embryonic nature means they are less differentiated and more susceptible to teratoma formation, whereas MSC are generally considered safe for use in humans (Sohni & Verfaillie, 2013). However, differences in the tissue source and culture conditions of MSC has sometimes led to differing or even conflicting evidence in the literature, posing a challenge for the therapeutic use of MSC (Sohni & Verfaillie, 2013).

1.5 Therapeutic Ultrasound

Ultrasound is a mechanical force which can stimulate a range of biological effects in cells and tissues. It consists of mechanical vibrations of an inaudible frequency generated by the conversion of electrical energy to acoustic energy through a piezoelectric transducer, resulting in the transmission of acoustic waves (Speed 2001). The therapeutic potential of ultrasound and its ability to induce lasting changes in biological systems was first identified in 1927 by Wood and Loomis (Wood & Loomis, 1927) and since then, extensive research has culminated in the use of ultrasound in over a million NHS treatments annually (Ter Haar *et al.*, 1985).

Ultrasound is generally divided into two powers; high intensity (~1000Wcm^{-2}), which obliterates focussed regions of cells or tissues by generating high temperatures, and low intensity (~100mWcm^{-2}), which generates less focussed pulses that penetrate tissues more deeply (Ter Haar 2007). Low intensity pulsed ultrasound (LIPUS) is considered therapeutic when administered in the range of 0.75-3MHz (Speed 2001) and in contrast to high intensity ultrasound is associated with non-thermal effects such as acoustic streaming and cavitation (Dyson 1982).

1.6 Cellular Effects of Therapeutic Ultrasound

Aside from its clinical use, mainly in treating bone fractures but also in tissue healing, cancer therapy and soft tissue stimulation, ultrasound has also been used on cell cultures to stimulate a variety of responses (Claes & Willie 2007; Paliwal & Mitragotri 2008). Ultrasound has been applied extensively to induce chondrogenesis in MSC for tissue engineering purposes or to increase proliferation as a means of *ex vivo* expansion, usually using BMSC but more recently using ADSC as an alternative because of their abundance and accessibility (Gimble 2003). As

well as structural alterations on a cellular level, ultrasound is associated with a range of stimulatory effects including increased secretion of enzymes, growth factors, chemicals and structural proteins in a variety of cell types (Nolte *et al.*, 2001; Chang *et al.*, 2002; Altland *et al.*, 2004; Chiu *et al*., 2008). For example, it is widely reported that ultrasound increases VEGF and TGF-β1 secretion, with a dose-dependent effect of TGF-β1 secretion observed (Reher *et al.*,1999; Li *et al.*, 2003; Harle *et al.*, 2005; Leung *et al.*, 2006; Lu *et al.*, 2008; Khanna *et al.*, 2009)

Previous studies have reported that ultrasound increases cell proliferation to varying degrees, however because of the variability in frequency, intensity, exposure times and cell types, this data is difficult to compare (Man *et al.*, 2012; Ghorayeb *et al.*, 2013). One study found that exposing human umbilical cord-derived MSC to a single application of ultrasound increased cell proliferation by 3 fold during a 5 day period, but repetitive treatments reduced proliferation and viability (Yoon *et al*., 2009). A more recent study reported similar findings; that shorter, single applications of ultrasound increased proliferation whereas long or repetitive treatments reduced proliferation in human DPSC (Al-Daghreer *et al*., 2012).

Many reports have indicated that ultrasound has no adverse effect on cell viability, however some reports have indicated an enhancement of viability after ultrasound treatment, including in human BMSC, by inhibiting the expression of apoptosis-related genes (Zhang *et al*., 2003; Lee *et al*., 2007; Choi *et al*., 2011; Kang *et al.*, 2011; Man *et al*., 2012).

It is clear that much of the literature surrounding the effects of ultrasound on cell cultures is inconsistent and conflicting due to the wide range of frequencies, intensities, exposure times, treatment pattern (single or repetitive) and cell types used and is thus difficult to compare.

1.7 Aims and Hypothesis of the Study

The hypothesis of this study was that foetal-derived MSC possess better proliferative and differentiation capacities than adult MSC, and that administration of therapeutic ultrasound to MSC would alter their stemness and immunomodulatory properties.

This study aimed to characterise MSC derived from adult sources (bone marrow, adipose tissue and dental pulp) and from a foetal source (amnion) by investigating characteristics in culture (i.e. morphology and growth characteristics), *ex vivo* expansion potential using media supplements and multi-lineage differentiation potential. The influence of therapeutic ultrasound on the expression of immunosuppressive and stem cell genes (including TGF-β1 and Oct-4) and the secretion of immunomodulatory growth factors was also investigated.

Methods

2.1 Isolation of Human Amniotic Mesenchymal Stromal Cells (AMSC)

Human AMSC were isolated from placentae collected from healthy donors following elective caesarean section with informed written consent and Local Research Ethics Committee approval. Isolation was conducted following a well-established protocol (Soncini *et al*, 2007).

2.2 Cell Culture

Pooled BMSC, ADSC (both Lonza, Basel, Switzerland), DPSC (AllCells L.L.C., Alameda, CA, USA) and AMSC were cultured in high glucose Dulbecco's Modified Eagle Medium (HG-DMEM; Biosera, Boussens, France) supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, 1% amphotericin B and 200mmol/L L-glutamine (all Sigma Aldrich, St Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2. Medium was changed every 3-4 days and cells were sub-cultured when 70-90% confluent.

2.3 Proliferation Assay to assess *Ex Vivo* Expansion Potential

Cells were grown in 6 well plates at $9.25x10^4$ cells/well in standard growth media described above either alone or with the addition of 10ng/ml human Epidermal Growth Factor (EGF; Peprotech, London, UK) or 10ng/ml human basic Fibroblast Growth Factor (bFGF; Peprotech). Cultures were maintained at 37°C for 7 days and cell number was determined microscopically.

2.4 Multi-lineage Differentiation Assays

For osteogenic differentiation, cells were grown in 12 well plates in osteogenic media containing 10^{-9} dexamethasone, 10mM β-glycerophosphate and 50µg/ml ascorbic acid (all Sigma Aldrich) for 21 days at 37°C, with medium changes every 3-4 days. After one week, media was changed from growth media containing 10% FBS to maintenance media containing 5% FBS. At day 21 cultures were washed 3x in phosphate buffered saline (PBS), fixed in 10% v/v neutral buffered formaldehyde (Leica Biosystems Ltd, Peterborough, UK) and incubated in 2% Alizarin Red S (BDH Laboratory Supplies, Poole, UK) at room temperature for 20 min with gentle agitation. Alizarin Red was made up in distilled water to pH 4.2 using acetic acid. Wells were washed 3x in distilled water and visualised using a Carl Zeiss Primo Vert microscope equipped with an AxioCam ERc5s camera (Carl Zeiss Microscopy Ltd, Cambridge, UK). Mineralisation was quantified by counting the number of nodules in four fields of view per well.

For adipogenic differentiation, cells were grown in 12 well plates in adipogenic media containing 0.5mM 3-isobutyl-1-methylxanthine, 0.5µM hydrocortisone and 1µg/ml insulin (all Sigma Aldrich) at 37°C, with medium changes every 3-4 days. After one week, media was changed from growth media containing 10% FBS to maintenance media containing 5% FBS. On day 21, cultures were fixed in 10% formaldehyde, washed 3x in PBS and stained with Oil Red O (BDH Laboratory Supplies). To make the stock solution, 0.25g Oil Red O was dissolved in 100ml isopropanol (VWR International S.A.S, Fontenaye-sous-Bois, France) for 1 hour at 56°C and filtered when cool to remove any undissolved dye. A working solution consisted of 2 parts distilled water mixed with 3 parts Oil Red O stock solution. Finally, wells were washed 3x in PBS and visualised under the microscope.

2.5 Ultrasound Treatment

Cells were grown in 6 wells plates at $1.25x10^5$ cells/well for 48 hours. Medium was replaced with 9ml fresh medium per well to enable immersion of the transducer head. Plates were placed in a custom-made silicone support filled with sterile, room temperature distilled water. Ultrasound was generated by a DuoSon; a commercially available therapeutic device programmed to transmit ultrasound at different frequencies and intensities (SRA Developments Ltd & Orthosonics Ltd, Devon, UK). For this study the DuoSon was used in 'mode 1', emitting a frequency of 1MHz and an intensity of $250mWcm^{-2}$ spatial average temporal average (SATA). Prior to ultrasound treatment, the transducer head was submerged in 70% ethanol for a minimum of 1 hour, washed with sterile medium and allowed to air dry under sterile conditions. The transducer was then placed in a metal ring clamped to a stand to enable insertion into the culture wells. Sham control plates were subjected to the same treatments and were placed in the water bath for the same time are their matched control plate, but with the ultrasound transducer switched off.

To investigate the effects of different ultrasound exposure times on cell proliferation and viability, cells were grown in 6 well plates at $9.25x10^4$ cells/well and exposed to ultrasound for 1, 3 or 5 min. Cells were harvested after 96 hours and proliferation and viability assessed by microscopy and trypan blue staining.

To investigate the effects of ultrasound treatment on gene expression and cytokine secretion, cells were grown in 6 well plates at $1.25x10^5$ cells/well and allowed to adhere for 48 hours prior to ultrasound exposure. Cells were exposed to 5 min ultrasound per well and 5ml medium was removed to leave 4ml per well. Cells were incubated for 48 hours at 37°C,

conditioned media (CM) was collected for analysis of cytokine secretion and cells were harvested and pooled for PCR (see below).

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

CM was collected from cells growing in 6 well plates 48 hours after ultrasound treatment, with corresponding sham controls. CM was also collected from confluent cell cultures growing in standard culture flasks. To obtain CM, supernatants were centrifuged at 3000rpm for 10 min, decanted into a fresh tube and stored at -20°C until needed. VEGF, BDNF and TGF-β1 secretion was analysed by ELISA (R&D Systems, Abingdon, UK) following the manufacturer's instructions.

2.7 Semi-Quantitative Polymerase Chain Reaction (PCR)

RNA isolation was achieved using the RNeasy Mini Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. cDNA was synthesised from the RNA templates using a Tetro cDNA synthesis kit (Bioline, Taunton, MA, USA) and cDNA was concentrated by washing in RNase-free water in microcon filters (Merck Millipore, Darmstadt, Germany). PCR was achieved by mixing an optimal volume of cDNA with RedTaq ReadyMix (Sigma Aldrich) and forward and reverse primers (Life Technologies, Invitrogen, Paisley, UK) to obtain a total reaction volume of 25µl and samples were processed on a GeneAmp PCR System 2700 (Life Technologies, Invitrogen). cDNA was normalised using the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure uniform band intensities where possible before performing PCR with the primers of interest.

Samples were run on a 1.5% agarose gel containing ethidium bromide (Sigma Aldrich) for 20-30 min at 120 volts. Semi-quantification of bands was achieved using GeneSnap and GeneTools image analysis software (Syngene, Cambridge, UK).

2.8 Statistics

Data were analysed using Graphpad Prism software v5.0 and are presented as mean ± SEM where n= >1, or mean ± standard deviation where n=1 and repeats were within a single experiment. P values of less than 5% ($P < 0.05$) were considered statistically significant.

Results

3.1 MSC Morphology and Growth Characteristics in Culture

At low density DPSC appear long, thin and small in comparison to the other MSC (Fig. 1A). However, they proliferated very quickly and as a confluent monolayer appeared homogeneous, forming hair-like, wavy patterns. In contrast, BMSC appeared spindly and curvy when sparse but when confluent formed a mixture of irregular-shaped cells, suggesting a heterogeneous population (Fig. 1B). ADSC (Fig. 1C) and AMSC (Fig. 1D) also appeared heterogeneous in nature, composed of cells differing in size and shape – particularly ADSC where some cells were long and wavy whereas others were round or polygonal in shape.

The growth of DPSC, BMSC, ADSC and AMSC in culture was determined by counting the number of cells obtained after passaging on a weekly basis (Fig. 1E). Although DPSC exhibited the biggest cell loss after reviving from liquid nitrogen, they appeared to have the highest proliferative capacity of all cell types. BMSC had the lowest proliferative capacity meaning that less were available for experiments compared to the other cells. ADSC appeared to have a generally constant proliferative capacity but took longer to become established after reviving from liquid nitrogen, and AMSC, the only cell type not exposed to liquid nitrogen, appeared highly proliferative for only a short period of time. All MSC began to plateau with increasing passage number.

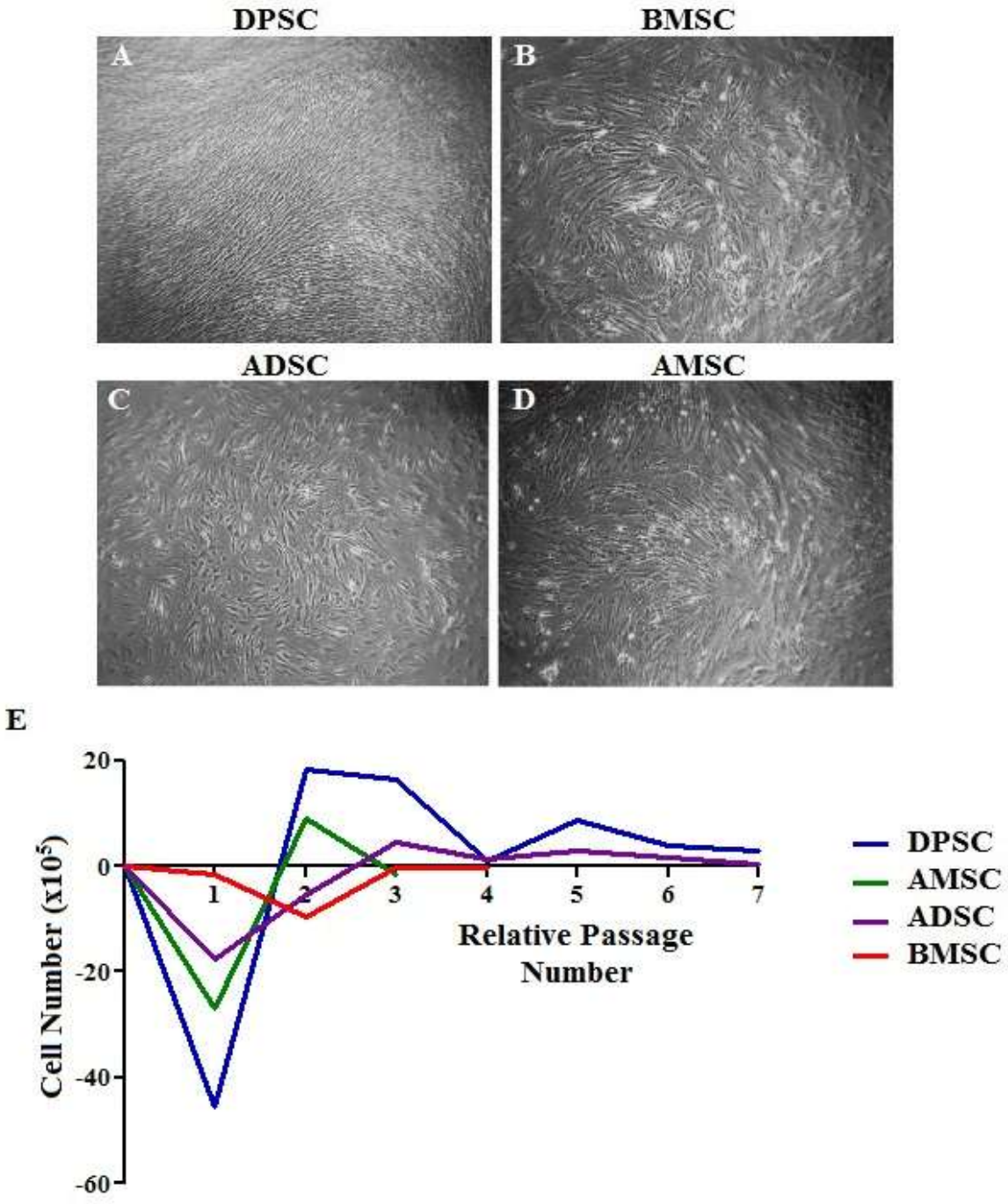


Figure 1. Morphology and Growth Characteristics of MSC in culture. The morphology of DPSC (A), BMSC (B), ADSC (C) and AMSC (D) at confluence is shown under standard culture conditions (x4 magnification). Cell number in culture was determined on a weekly basis when cells were passaged (E). Because the cells were of various passage numbers, P0 represents the initial number the cells were frozen at (DPSC, BMSC and ADSC) rather than the actual cell passage number, except in the case of AMSC which were obtained by primary isolation. Cells used for experiments were accounted for.

3.2 *Ex Vivo* Expansion Potential of MSC

For cells to be used therapeutically or for large scale experiments, they must be readily expandable in order to obtain large numbers. The *ex vivo* expansion potential of MSC derived from dental pulp, adipose tissue and amnion was assessed by comparing those grown in standard media to those grown in EGF and bFGF after 7 days in culture (Fig. 2). Addition of both EGF and bFGF increased proliferation in all cell types, particularly DPSC and ADSC. In all cell types bFGF appeared to have a greater mitogenic effect than EGF.

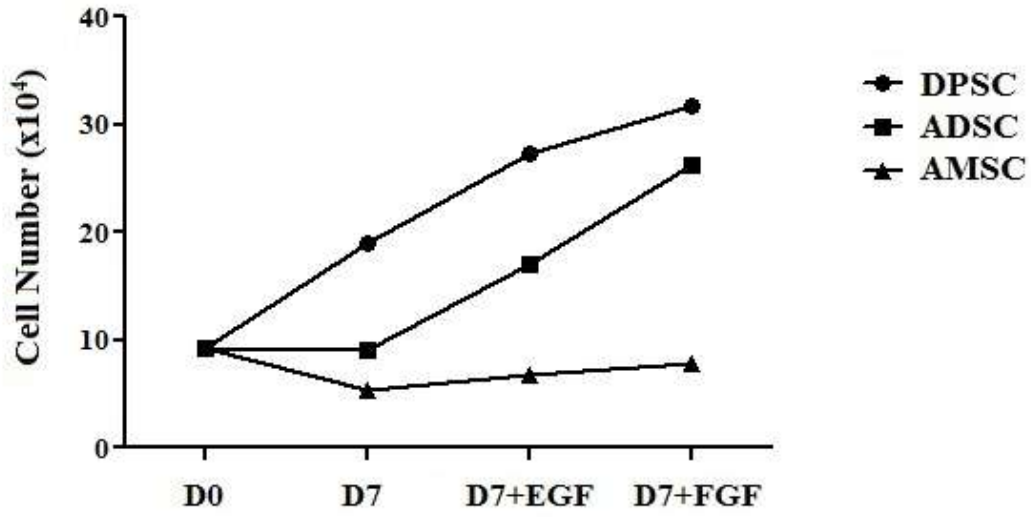


Figure 2. EGF and FGF increase MSC proliferation. DPSC, ADSC and AMSC were cultured in standard media, EGF-supplemented media or FGF-supplemented media for 7 days and proliferation was determined (n=1, individual wells). D0= day 0 (seeding number), D7= day 7.

3.3 Multi-lineage Differentiation Potential

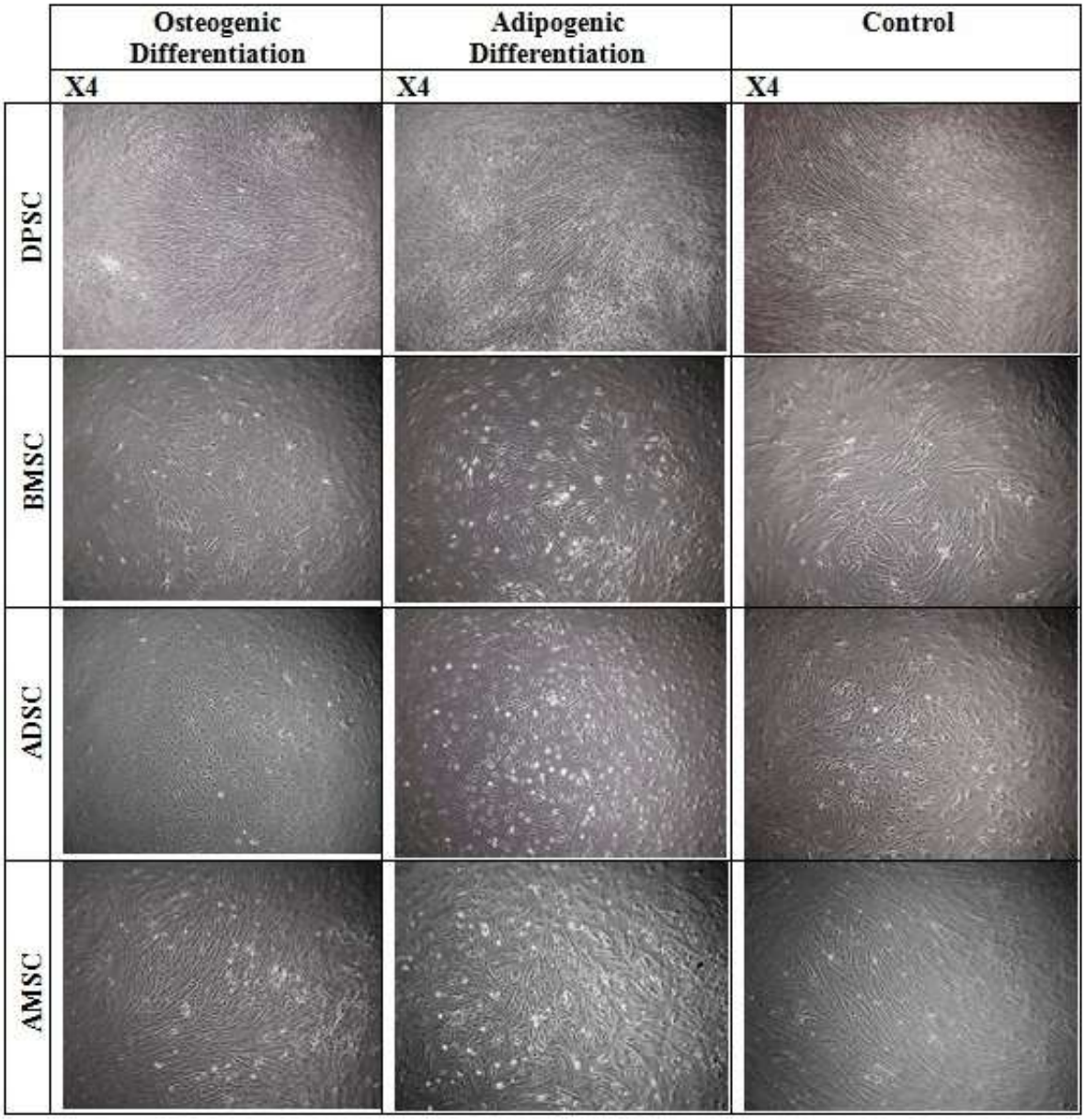
MSC are renowned for their ability to differentiate into cells of a mesodermal linage, i.e. those of an osteogenic, adipogenic and chondrogenic phenotype. To compare the multi-lineage differentiation potential of MSC, each cell type was grown in osteogenic and

adipogenic media for 21 days. After only 7 days in culture BMSC, ADSC and AMSC showed morphological differences in both osteogenic and adipogenic treated groups compared to untreated groups, whereas DPSC in both osteogenic and adipogenic treated groups appeared the same as the untreated group (Fig. 3A). Vacuole formation, characteristic of adipogenic conversion, was visible in the cytoplasms of BMSC and particularly ADSC after 7 days and stellate, protruding cells characteristic of osteocytes were present in BMSC and ADSC. At this early stage, nodule formation was not observed in any of the groups.

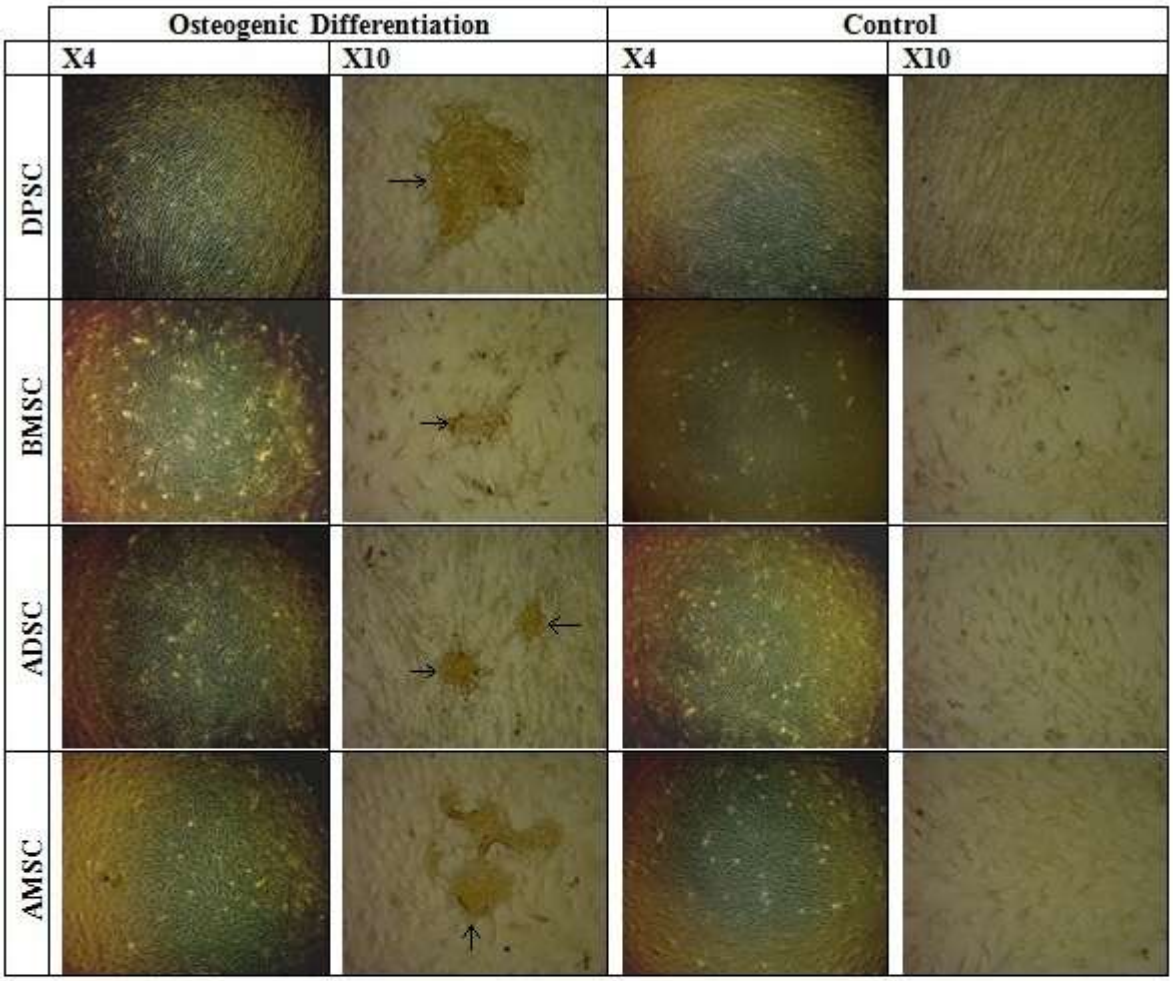
After 21 days cells were stained with Alizarin Red and visualised (Fig. 3B). Osteogenic conversion was observed in all cell types, indicated by the presence of calcification or 'nodules' which absorb the dye. Nodules observed in DPSC and AMSC cultures appeared much larger than those in BMSC and ADSC. Mineralisation was semi-quantified by counting the number of Alizarin Red-positive nodules in four fields of view (Fig. 3C). Results show that BMSC and AMSC stained positive for a significantly higher number of nodules than DPSC. BMSC and AMSC also had more nodules than ADSC but this was not statistically significant.

To investigate adipogenic conversion, MSC were cultured in adipogenic media for 21 days and were stained with Oil Red O (Fig. 3D). Unfortunately, technical issues prevented the stain from working and many of the cells lifted up, meaning that semi-quantification could not be achieved. However, evidence of adipogenic conversion, assessed by observing vacuole formation, was observed in BMSC and ADSC but not DPSC or AMSC.

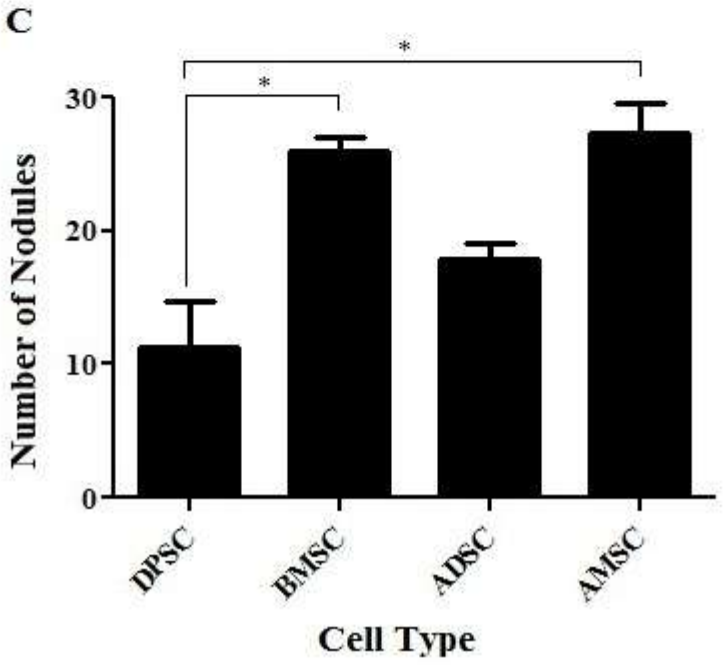
A


Osteogenic Differentiation
Adipogenic Differentiation
Control
X4
X4
X4
DPSC
BMSC
ADSC
AMSC

B


Osteogenic Differentiation
Control
X4
X10
X4
X10
DPSC
BMSC
ADSC
AMSC

C


*
*
30
20
10
0
Number of Nodules
DPSC
BMSC
ADSC
AMSC
Cell Type

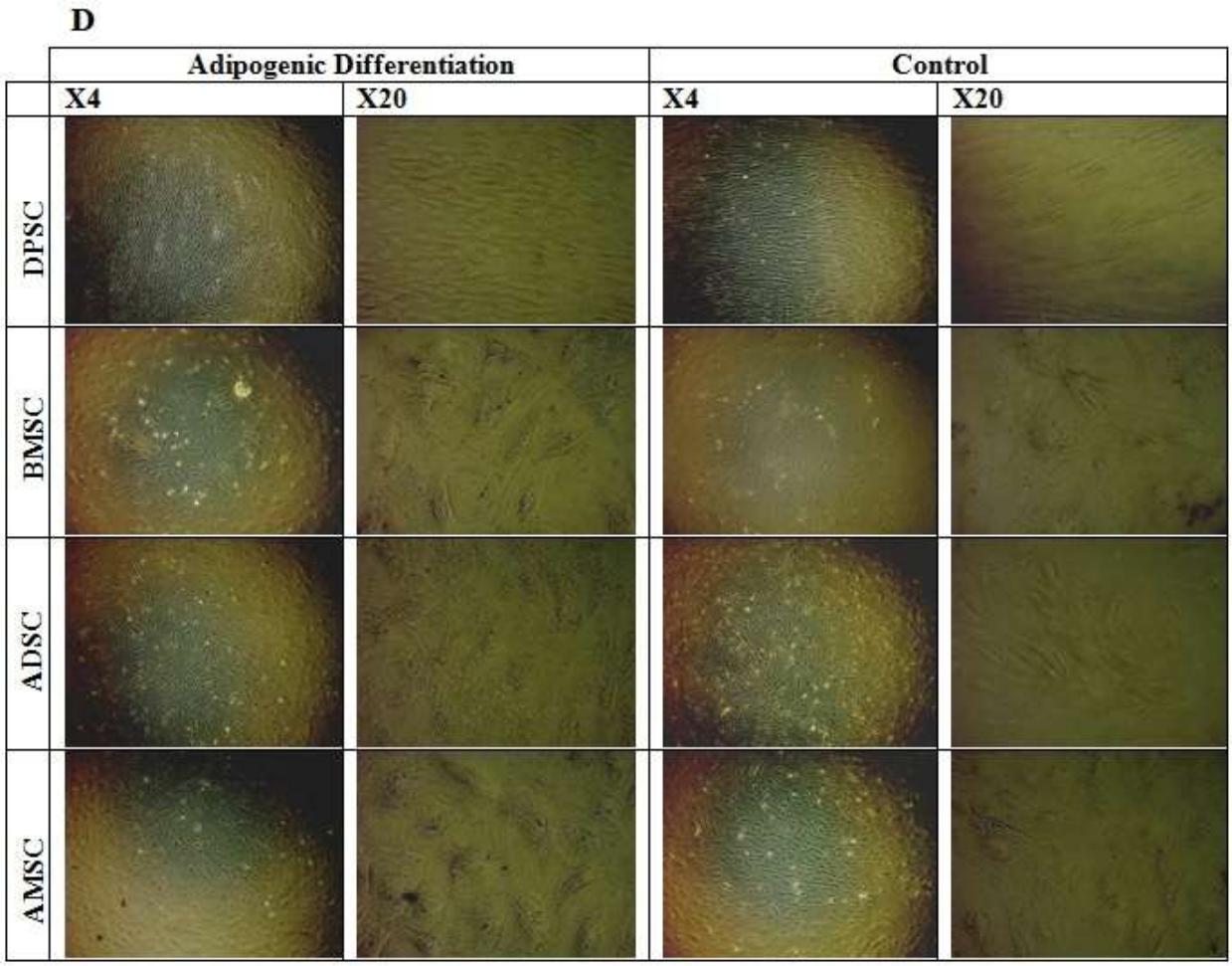


Figure 3. Osteogenic and adipogenic potential of MSC. MSC were cultured in adipogenic and osteogenic media and analysed after 7 days in culture (A; x4 magnification). The osteogenic differentiation potential of MSC after 21 days was assessed by alizarin red staining (B). Osteogenic conversion was indicated by the presence of calcification or 'nodules' which take up the dye (x4 and x10 magnification; shown by black arrows). Mineralisation was semi-quantified by counting the number of alizarin red positive nodules in four fields of view after 21 days culture in osteogenic media (C). Data are means ± SEM and were significant to * (P= <0.05) using a one-way ANOVA with Tukey's post-hoc test (n=2, triplicate wells except BMSC which were individual wells for each experiment). The ability of all four cell types to differentiate into adipocytes was determined by culture in adipogenic medium for 21 days (D). Cells were stained in Oil Red O but technical issues meant that the stain didn't work properly and much of the cells lifted up, meaning that semi-quantification could not be achieved (x4 and x20 magnification).

3.4 Gene Expression and Growth Factor Secretion of MSC

Gene expression of selected immunomodulatory (transforming growth factor-β1 (TGF-β1), interleukin-10 (IL-10) and cyclooxygenase-2 (COX-2)) and stem cell-related genes (octamer-binding transcription factor-4 (oct-4) and nanog) was investigated under standard culture conditions by PCR (Fig. 4A & Fig. 4B). Gene expression was semi-quantified by normalising to the housekeeping gene, GAPDH, which was converted to 100% (not shown). Primer sequences are shown in table 1. TGF-β1 expression in DPSC was 19%, 2.3% in AMSC, 0.5% in BMSC and 0% in ADSC. COX-2 was highly expressed by DPSC (44.0%) and AMSC (34.0%), and also expressed by BMSC (8.7%) but not expressed by ADSC. Oct-4 was not expressed by DPSC but was expressed by BMSC (18.3%), ADSC (9.6%) and AMSC (9.2%). Nanog was highly expressed by BMSC (54.8%) and ADSC (51.3%) and was expressed to a lesser extent by AMSC (21.1%). Very low nanog expression was observed in DPSC (5.3%) in comparison to the other cell types. IL-10 was not expressed by any of the cell types.

Cytokine secretion was analysed by ELISA after collecting supernatants from confluent MSC cultures. VEGF production (Fig. 4C) was highest in BMSC (3421 ± 112) and DPSC (2655 ± 136) and lowest in ADSC (337 ± 152) and AMSC (228 ± 205). BDNF production (Fig. 4D) was highest in AMSC (184 ± 112) and much lower in BMSC (78 ± 37), DPSC (73 ± 8) and ADSC (53 ± 24). Secretion of both active and inactive forms of TGF-β1 (Fig. 4E) was highest in AMSC (17730 ± 5834), and again much lower in BMSC (8163 ± 1853), DPSC (8707 ± 2023) and ADSC (5497 ± 2061).

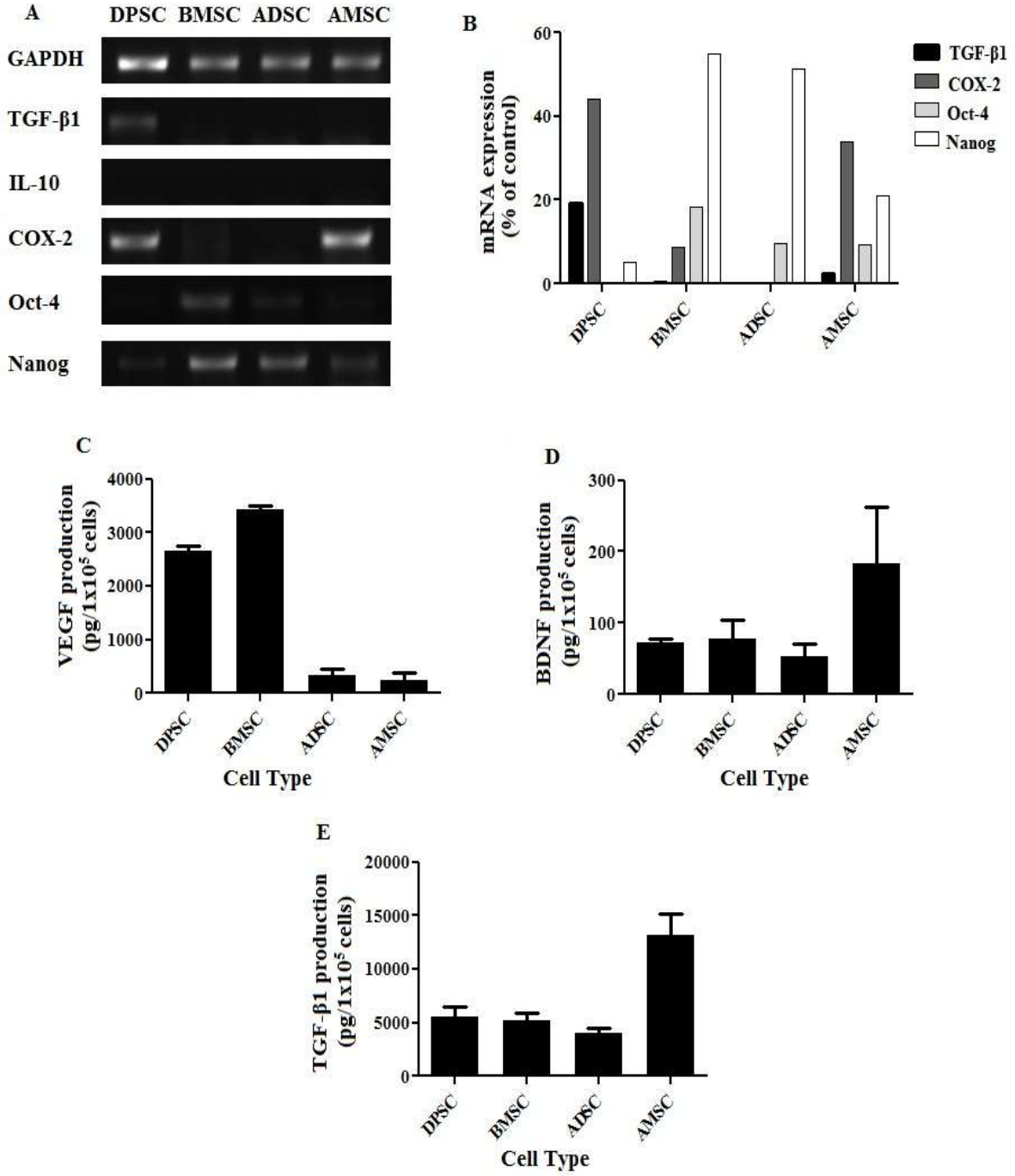


Figure 4. Gene expression and cytokine secretion of DPSC, BMSC, ADSC and AMSC in standard culture conditions. An agarose gel showing amplified cDNA fragments produced by PCR (A). Gene expression of TGF-β1, COX-2, Oct-4 and nanog by MSC normalised against the housekeeping gene, GAPDH (B). Results are displayed as a percentage of GAPDH expression (n=1). Confluent cell cultures were grown in complete media for 5 days and supernatants were collected and analysed for VEGF (C), BDNF (D) and TGF-β1 (E) secretion by ELISA. Experiments were repeated in duplicate from individual supernatants (mean ± standard deviation, n=1). Statistical analyses were not performed due to lack of repeated experiments.

3.5 The Effects of Ultrasound on MSC Proliferation

Ultrasound has widely been reported to influence the function of a variety of cell types, but there has been no direct comparison of MSC from different sources thus far. The ultrasound machine was set up under sterile conditions applied to cells grown in six well plates (Fig. 5A) and following the application of ultrasound, some cells (AMSC, and ADSC on one occasion) had lifted up (Fig 5B; x4 magnification).

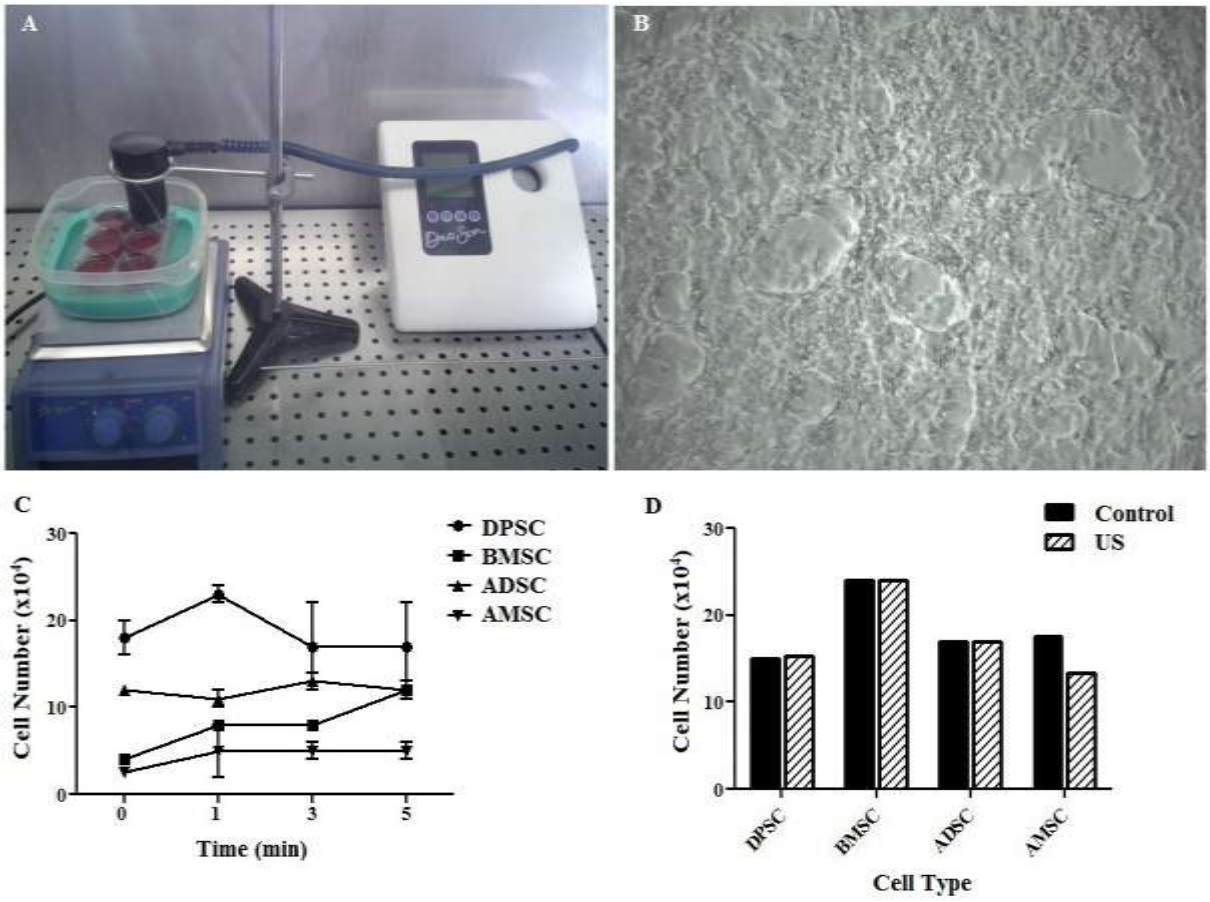


Figure 5. The effects of ultrasound on MSC proliferation. The ultrasound machine was set up under sterile conditions with the transducer submerged in culture media supported by a metal clamp, and six well plates were supported by a custom-made silicone mould (A). Some cell types lifted up following ultrasound treatment (B). Cells were exposed to ultrasound for 1, 3 or 5 minutes and proliferation determined after 96 hours (C). Data are means ± standard deviation (n=1) from duplicate wells (DPSC, ADSC and AMSC) or individual wells (BMSC). MSC were exposed to ultrasound for 5 minutes and proliferation determined after a 48 hour incubation period (D). Graph shows average number of cells per well from 3 pooled wells (n=1).

The effect of various exposure times on the proliferation of MSC was investigated by determining proliferation after a 96 hour period (Fig. 5C). Cells were plated at a low enough density to enable proliferation, but high enough for the cells to remain viable. For the three time periods assessed (1, 3 and 5 min) results show that ultrasound had no significant effect on MSC proliferation or viability (assessed by trypan blue staining - data not shown).

Using the 5 minute exposure time, the proliferation of MSC 48 hours after ultrasound exposure was also determined (Fig. 5D). Again, ultrasound appeared to have no significant effect on proliferation compared to the controls, although there was a slight reduction in cell number after ultrasound was applied to AMSC – possibly due to some of them lifting up.

3.6 The Effects of Ultrasound on MSC Gene Expression and Cytokine Secretion

Due to low cell numbers, only the gene expression of DPSC and ADSC were analysed. Gene expression was investigated 48 hours after a single 5 min ultrasound treatment (Fig. 6A & Fig 6B). Primer sequences are shown in table 1.

TGF-β1 expression in ADSC decreased by 20.6% (45.3-24.7%) but increased by 26.7% (7.4-34.1%) in DPSC. COX-2 expression in ADSC was very low and decreased slightly after ultrasound treatment (3.2%), also decreasing in DPSC (21.2%). Oct-4 expression increased in ADSC by 15.2% (14.8-30.0%) and decreased in DPSC by 9% (22.4-13.4%) and nanog expression increased in ADSC by 7.4% (9.8-17.2%) and decreased in DPSC by 4.9% (14.9-10.0%). IL-10 expression was not expressed by any of the cell types.

Gene	Sequence (5' to 3')	Annealing Temp	Product Size (bp)
GAPDH	F- TCTAGACGGCAGGTCAGGTCC R- CCACCCATGGCAAATTCCATG	60	597
TGF-β1	F- CGCCTTAGCGCCCACTGCTCCTGT R-0 GGGGCGGGACCTCAGCTGCAC	60	533
IL-10	F- TGCCTTCAGCAGAGTGAAGA R- GGGAAGAAATCGATGACAGC	60	255
COX-2	F- CAAATTGCTGGCAGGGTTGC R- GGGACAGCCCTTCACGTTAT	60	444
Oct-4	F- GACTGAGCTGGTTGCCTCAT R- GGTCTTCACCTGTTTGTAGCTG	60	599
Nanog	F- GAGTAGTCCCTTCGCAAGCC R- TTGATGTCCTGGGACTCCTCC	60	481

Table 1. PCR primer sequences, annealing temperature and product size (bp). GAPDH, gyceraldehyde 3-phosphate dehydrogenase; TGF-β1, transforming growth factor-β1; IL10, interleukin-10; COX-2, cyclooxygenase-2; Oct-4, octamer-binding transcription factor-4. Primers were designed using the Information Hyperlinked over Proteins (IHoP) website (http://www.ihop-net.org/UniPub/iHOP) and the nucleotide-BLAST and primer-BLAST features of the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/pubmed).

Cytokine secretion was analysed by ELISA after collecting cell supernatants 48 hours after ultrasound treatment. Ultrasound appeared to increase VEGF production in DPSC and BMSC but decreased VEGF production in ADSC and AMSC (Fig. 6C). BDNF production (Fig. 6D) was generally low and showed no apparent trend, and TGF-β1 production (Fig. 6E) also appeared to show no trend.

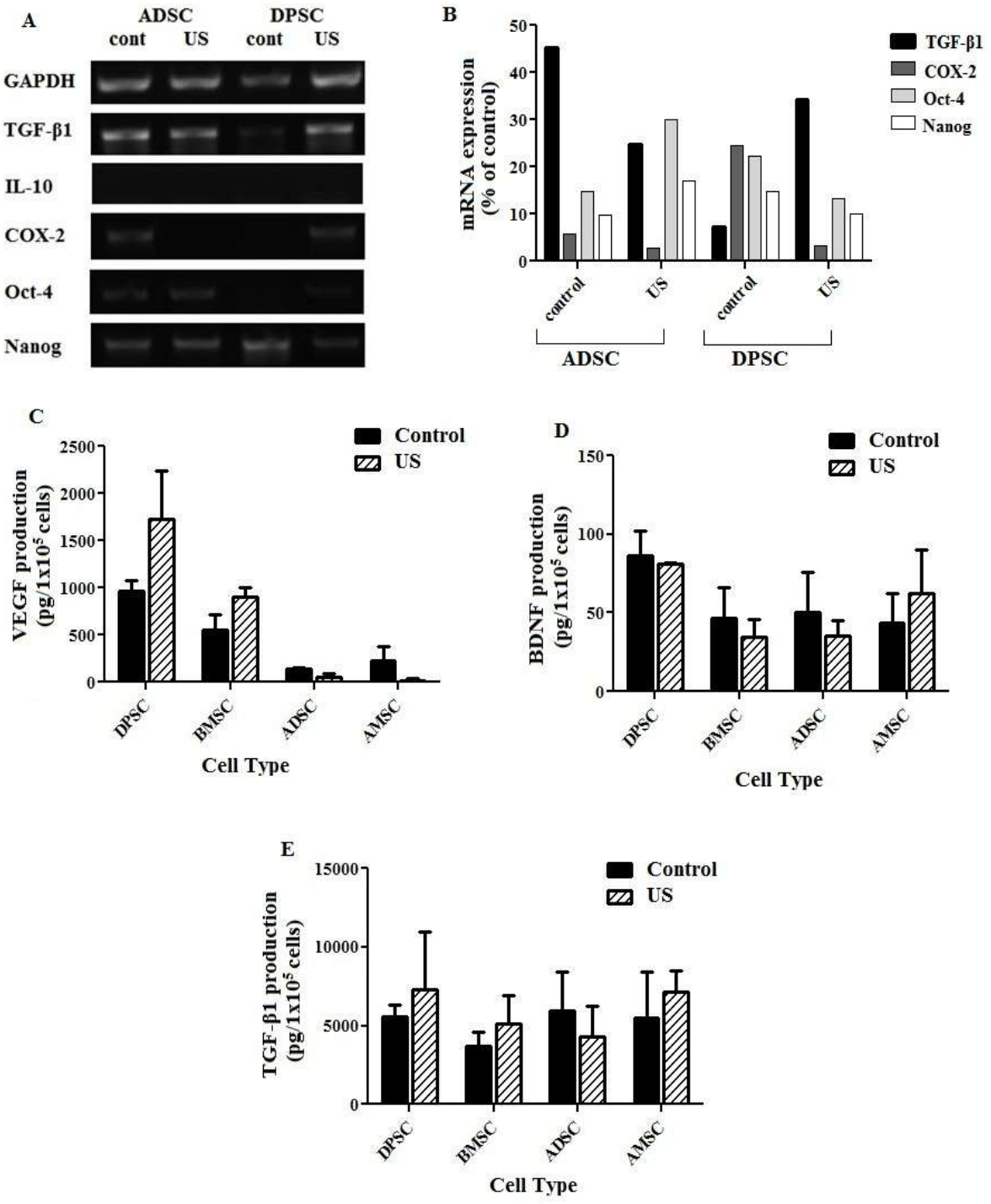


Figure 6. Gene expression of DPSC and ADSC and cytokine secretion of all MSC types after ultrasound treatment. An agarose gel showing amplified cDNA fragments produced by PCR (A). Gene expression of TGF-β1, COX-2, Oct-4 and nanog by DPSC and ADSC normalised against the housekeeping gene, GAPDH (B). Results are displayed as a percentage of GAPDH expression (n=1). Supernatants were collected 48 hours after ultrasound treatment and analysed for VEGF (C), BDNF (D) and TGF-β1 (E) secretion by ELISA. Experiments were repeated in duplicate from individual supernatants (mean ± standard deviation, n=1). Statistical analyses were not performed due to lack of repeated experiments.

Discussion

4.1 Comparison of MSC from Different Sources

4.1.1 Morphology and Proliferative Capacity

MSC reportedly display a fibroblast appearance in culture, which in this study was exhibited by all four MSC types analysed (Horwitz *et al.,* 2005). DPSC and BMSC appeared long and spindle-like, whereas ADSC and AMSC appeared more stellate. This might reflect the origins of these cells and could suggest that DPSC and BMSC share a more similar developmental origin than ADSC and AMSC. The similarities between DPSC and BMSC have been confirmed by a previous analysis of over 4,000 human genes, which found that gene expression was remarkably similar between the two cell types (Shi *et al.,* 2001). Therefore the large difference in proliferative capacity of DPSC and BMSC may be artefacts caused by external factors such as isolation protocol, length of time frozen, culture conditions and donor variability.

Consistent with previous publications, BMSC displayed a lower proliferative capacity than MSC from other sources, indicating that *ex vivo* expansion is required or alternatively sourced MSC should be used (D'Ippolito *et al.,* 1999; Kern *et al.,* 2006). AMSC also displayed a comparatively low proliferation capacity; therefore it appears that donor variability such as age and developmental stage of the tissue plays a significant role in the proliferation of AMSC, and indeed other types of MSC.

MSC are also characteristically heterogeneous in culture, which was displayed by BMSC, ADSC and AMSC but not DPSC – this phenomenon is unclear but could suggest that DPSC have been highly purified to the extent where only one cell type in this population exists, or that culture conditions have caused conversion to the predominating cell type. It is unlikely

that this morphological variation is a result of culture conditions as DPSC did not appear to change in morphology during their time in culture, and all four cell types were cultured in the same media. Conversely, DPSC may appear homogeneous morphologically but could be heterogeneous on a genetic level – a characteristic not revealed by appearance alone. Further work, including comparison of procured versus freshly isolated cells, would enable this phenomenon to be elucidated.

4.1.2 *Ex Vivo* Expansion Potential of MSC

The major limitation in this study is the generally low proliferative capacity of MSC. Many research groups supplement MSC culture media in order to enhance cell yield and viability. The findings in this study are in line with previous work that showed bFGF as a superior cell culture supplement to EGF, but with a side-by-side comparison of MSC from three different sources (Gharibi & Hughes 2012).

An interesting observation was that during removal of adherent cells, those cultured in bFGF detached much quicker than cells in complete media or in EGF, to the extent that these cells were almost fully detached by the time the cells in the other groups had started to become loose. This suggests that bFGF may down-regulate the expression of adhesion molecules, supported by a recent report which demonstrated that bFGF significantly affects the expression levels of a range of adhesion molecules including the down-regulation of fibronectin and some integrins (Kashpur *et al.,* 2013).

Although addition of mitogens such as EGF and bFGF indeed increase MSC proliferation, effects on other aspects of MSC function should be carefully considered. There are certainly documented effects on gene expression and cell function during culture with mitogens

(Kashpur *et al.*, 2013) but there may also be lasting effects continuing after their removal which could influence cell behaviour, ultimately impacting on the outcome of experiments, *in vivo* work or clinical trials. Therefore mitogens should only be used when necessary and selecting MSC types with a high proliferative capacity, such as DPSC, may abolish the need for mitogens.

4.1.3 Differentiation Potential

This study has shown that some MSC types are highly amenable to differentiation, exhibiting morphological alterations after only a few days in differentiation-inducing media. Specifically, BMSC, ADSC and AMSC displayed an altered morphology after only 7 days in both osteogenic and adipogenic media, and vacuole formation was observed in BMSC and ADSC after 7 and 21 days. Mineralisation was observed in all four cell types suggesting that all MSC are particularly amenable to osteogenic conversion. On semi-quantification mineralisation was greatest in BMSC and AMSC, however counting the number of nodules may not be entirely representative of osteogenic conversion as the size of nodules was not considered. On closer inspection nodules in AMSC and DPSC cultures were much larger than in BMSC and ADSC cultures, so a more sensitive method of semi-quantification such as eluting and measuring the dye absorbed by each cell type or assessing alkaline phosphatase activity would better enable osteogenic conversion to be compared between MSC. A previous report has suggested that instead of the standard three week incubation period in differentiation media, nodule development is better seen after five weeks in culture; therefore it may not necessarily be that one MSC type is more plastic than another- it may just be that some MSC types are quicker at differentiating into other linages than others (Baksh *et al*.,

2007). This could also apply to adipogenic conversion where it appeared that DPSC and AMSC did not exhibit vacuole formation.

Interestingly, AMSC displayed very strong osteogenic conversion but no adipogenic conversion. This reluctance to convert to the adipogenic lineage has been widely reported, and adipogenic potential is thought to be displayed, albeit weakly, by very few donors (Wolbank *et al.*, 2010, Lindenmair *et al.*, 2012). This restricted differentiation potential has also been observed in umbilical cord-derived MSC which also fail to convert to the adipogenic lineage, whereas ADSC and BMSC could (Kern *et al.*, 2006).

Unsurprisingly, ADSC displayed stronger adipogenic conversion than other cell types and BMSC displayed stronger osteogenic conversion than all other cell types, suggesting that as with proliferative capacity, the differentiation potential of MSC strongly depends on their location *in vivo.* This is presumably a reflection of their developmental origin and suggests that MSC are site-specific and tailored to their location, with roles in repopulating specific cells (such as adipocytes for ADSC) within the stem cell niche.

As donor age affects MSC plasticity, this variable, along with MSC source, should be considered where MSC are to be used in differentiation studies (D'Ippolito *et al.*, 1999).

4.1.4 Gene Expression and Growth Factor Secretion

Results from this study show that immunomodulatory genes (TGF-β1 and COX-2, an enzyme which generates prostaglandin E2; PGE2), were expressed the highest by DPSC and AMSC, with very little to no expression in BMSC and ADSC. However, TGF-β1 was highly secreted by AMSC in comparison to the other cell types, which exhibited similar, comparably low

secretion levels. This suggests that although DPSC express TGF-β1 there is downstream regulation modulating its secretion, also implying that large-scale secretion may be rapidly induced. These results correlate with a previous report suggesting that foetal-derived MSC secrete more TGF-β1 than adult-derived MSC (Deuse *et al.,* 2011).

COX-2 expression was highest in DPSC and then in AMSC, suggesting that these two cell types may be the most potent immunomodulators; however as previously discussed, increased gene expression does not necessarily mean that more protein is secreted– downsteam regulation could be occurring so these results must be carefully interpreted. IL-10 expression was not observed in any cell type, suggesting its expression is induced, for example by inflammatory stimuli.

The pattern of VEGF secretion could be largely down to the role of different MSC *in vivo.* DPSC secrete VEGF to regulate dentine tissue repair and VEGF is important in BMSC-mediated vascularisation, repair and cell signalling within the bone marrow, which could be responsible for their higher secretion (Mayer *et al.,* 2005; Scheven *et al.,* 2009). Alternatively, AMSC and ADSC may primarily secrete other growth factors to promote angiogenesis such as platelet-derived growth factor (PDGF) or interleukin-8 (IL-8) and indeed, TGF-β1 is also angiogenic, which is highly secreted by AMSC.

A previous study showed that AMSC secrete more neurotrophic factors (such as BDNF), are more amenable to neuronal differentiation and express neural stemness markers compared to adult MSC which supports the finding that BDNF was highly secreted by AMSC in comparison to the other cell types (Yan *et al.,* 2013).

The genes for oct-4 and nanog, transcription factors associated with pluripotency and self-renewal, were expressed highest by BMSC and ADSC, AMSC to a lesser extent and barely at

all by DPSC. Interestingly, this correlates with findings from the differentiation study, where BMSC and ADSC converted to both osteogenic and adipogenic lineages whereas AMSC, and to a lesser extent DPSC, only displayed osteogenic conversion. This suggests that BMSC and ADSC are more pluripotent than the other cell types and may be preferentially selected for differentiation studies; however this conflicts with a previous study which found that only foetal MSC express oct-4 and nanog (Guillot 2006). A later study also found that oct-4 was not expressed in adult MSC but that nanog was, and that nanog expression was not expressed in freshly isolated cells but took time in culture to become detectable, which may explain the apparently lower expression in AMSC (Pierantozzi *et al.*, 2011). This group also found that nanog was only expressed in proliferating MSC; therefore nanog expression may have been influenced by other factors.

4.2 The Effects of Ultrasound on MSC

4.2.1 Viability and Proliferation

In this study trypan blue staining indicated no apparent difference between control and ultrasound-treated groups, which is in agreement with previous reports (Zhang *et al*., 2003; Kang *et al.,* 2011; Man *et al*., 2012). Conflicting data in the literature make it difficult to draw solid conclusions on the influence of ultrasound on cell proliferation, though it appears that short, single applications of ultrasound at the very least do not reduce proliferation. This is in agreement with the findings from this study that a single, 5 minute treatment does not influence MSC proliferation. Therefore, ultrasound appears not to be useful in affecting these parameters but may be applied to MSC cultures for other purposes with no adverse effects on proliferation and viability.

4.2.2 Cytokine Secretion and Gene Expression

With the great variety in methods of ultrasound application and exposure times, it is difficult to directly compare the outcomes of previous ultrasound studies and data is sometimes conflicting. That gene expression in MSC is different in standard culture conditions compared to the control for the ultrasound study suggests that recent passaging and plating out influences gene expression compared to confluent, established cultures. Results from this study indicate that TGF-β1 expression was increased in DPSC but decreased in ADSC after ultrasound treatment, with secretion of active and inactive forms of TGF-β1 appearing to show the same phenomenon. In agreement with the literature, there was increased TGF-β1 secretion by BMSC and AMSC. Though reduced TGF-β1 expression has not previously been described after ultrasound treatment, there has been a report of ultrasound having no significant influence on TGF-β1 expression, demonstrating variable findings in the literature (Al-Daghreer *et al.*, 2012). Therefore, it would seem likely that its expression is not reduced after ultrasound treatment.

Expression of stemness genes after ultrasound treatment also showed opposite trends in both ADSC and DPSC, increasing in ADSC but decreasing in DPSC after ultrasound treatment. This therefore suggests that ultrasound promotes self-renewal and pluripotency in ADSC, promoting stem cell-like properties, but causes DPSC to become less pluripotent. The effects of ultrasound on stemness gene expression has not been previously described, but it seems unlikely that ultrasound would have completely opposing effects in two similar cell types. This polar variation in gene expression could be due to contributions from external factors such as donor age or cell density (which may impact on the availability of nutrients), or a switch in gene expression to alternative but similar genes. Also, the values derived from this study are only relative as they are calculated based on GAPDH expression and compared to

'resting' control groups, so they may not be entirely representative of the true effects of ultrasound.

Therapeutic ultrasound reportedly stimulates PGE2 production (Reher *et al.,* 2002) but in this study, expression of the enzyme responsible for PGE2 synthesis, COX-2, decreased in both DPSC and ADSC after ultrasound treatment. It may be that COX-2 expression is initially upregulated to increase PGE2 synthesis before decreasing to compensate for the increased PGE2. Analysing gene expression at a single time point (48 hours) means that fluctuations in COX-2 expression are undetectable; therefore measuring total PGE2 secretion may be more indicative of the overall effect of ultrasound on COX-2 expression.

Results show no apparent trend in BDNF secretion following treatment, though VEGF is increased in DPSC and BMSC, and decreased in ADSC and AMSC; however VEGF secretion was so low by ADSC and AMSC that this apparent decrease is likely insignificant. Also, because DPSC have a high proliferative capacity they quickly become confluent after plating out, whereas other cell types such as AMSC and BMSC struggled to become fully confluent, which may skew the results. Differences in VEGF secretion could be due to the location and function of the cells *in vivo.* For example, MSC in bone marrow niches are constantly subjected to mechanical forces associated with physiological activity (i.e. external forces) and the mechanical environment of the bone marrow (i.e. internal forces) acting upon the bone marrow microenvironment (Gurkan *et al.,* 2008). As such, external stressors such as ultrasound that influence biochemical pathways may be processed differently by MSC depending upon their location and function.

4.3 Assay Refinements and Future Work

4.3.1 Cell Source and Procurement

In this study, MSC were either purchased from commercial companies which involved pooling and freezing (DPSC, BMSC and ADSC) or were isolated from single donors with no freezing (AMSC), meaning that the cells were not directly comparable. Using freshly isolated cells from a known source is more reliable so if possible, future studies should use freshly isolated MSC.

4.3.2 Improving the Reliability of the Findings

Much of the data presented in this report is not technically reliable due to the lack of replicates within individual experiments and lack of repeated experiments, meaning that statistical analyses could not always be applied. The major limitations were cell number, hence why the *ex vivo* expansion potential of the cells was investigated, and lack of time. Repeating the experiments would provide a better indication of the true biology occurring.

4.3.3 The Influence of Culture Conditions on MSC Function

The addition of mitogens or other supplements such as vitamins to standard growth culture medium is controversial, but their effects on MSC proliferation are well documented. If MSC are expanded using mitogens with the intention of being used for a cellular therapy then the long term effects must be properly validated, hence rigorous testing must take place. As both EGF and bFGF were used at 10ng/ml in this study, the effects of higher or lower

concentrations of both growth factors is unclear. Growth factor titration may provide more information, and assessing proliferation by an alternative method rather than cell counting may be more appropriate for this purpose. For example, a colourimetric assay such as MTT, XTT or WST-1 which takes a 96 well plate format would enable high throughput analysis of viable cells and is often available as a ready-to-use kit.

4.3.4 Expanding the Ultrasound Study

Though therapeutic ultrasound has been used since the 1940's, the application of ultrasound to influence cell function *in vitro* is relatively recent and though many publications report that ultrasound can affect cell behaviour and function, its precise biochemical mechanisms are generally still elusive (Paliwal & Mitragotri 2008). The wide variations in intensity and frequency make it difficult to collate and translate experimental data, and many studies use limited combinations (if at all) of intensity and frequency to deliver ultrasound to cells, often leading to repetition of results. Also, the ultrasound exposure time and culture period of cells post-treatment is variable and is likely to show variations in gene expression depending on when the cells are harvested, providing massive scope for future work. Ideally, a publicly accessible database or review document containing results from previous ultrasound studies would be available to enable researchers to efficiently progress in this field. Future studies should look at the effects of a wider range of ultrasound treatments on cell behaviour, factoring in different exposure times and analysis times of cells post-treatment.

4.3.5 Direct Analysis of Immunosuppressive Properties

Analysing gene expression or cytokine secretion as markers of immunosuppressive capacity is a biased approach as immunosuppression is a complex process mediated by a host of soluble and cell-bound factors and as such, upregulation of one particular immunosuppression-related gene does not necessarily relate to more potent immunosuppression. Assessing immunosuppressive capacity by proliferation assay, which measures the ability of MSC to inhibit peripheral blood mononuclear cell (PBMC) proliferation, may provide a more representative indicator of immunosuppression.

Conclusions

Sourcing of MSC has important implications for the function and availability of cells for research and therapeutic purposes. Because BMSC exhibit a low proliferative capacity they may not be the cell of choice if other MSC are available, however the addition of media supplements such as bFGF or EGF may improve cell yield for large-scale experiments or cell therapies.

The data in this report provides a snapshot of gene expression and cytokine secretion of MSC which *in vitro* may be influenced by cell culture conditions, and *in vivo* may be influenced by external factors such as mechanical stressors. On the whole, AMSC expressed reasonable levels of both immunomodulatory and stemness genes analysed, suggesting they are versatile and ideal for all-round purposes. Though it is unclear from this study whether AMSC possess enhanced properties over adult MSC, birth-associated tissues are an excellent source of MSC due to their simple retrieval and plentiful supply. DPSC have a very high proliferative capacity and may also be better applied for immunomodulatory purposes, however immunomodulation is a complex and multi-factorial process influenced by many factors and is difficult to summarise by analysing such few cytokines. Expression of 'stemness' genes appeared to reflect the differentiation potential of MSC and suggested that BMSC and ADSC may be better used for differentiation studies for example across linage boundaries, such as in conversion to insulin-secreting cells for a cell-based diabetes therapy. Therefore, researchers could select the type of MSC based on their requirements.

Ultrasound is a promising avenue of research with great potential not only for enhancing cells *in vitro* and for cell based therapeutics, but also as a therapy in itself by direct application to damaged tissues. Ultrasound may or may not influence gene expression and cytokine

secretion in different cell types as it is difficult to confidently evaluate this based on the current literature. Therefore much work is required to evaluate the effects of different intensities, frequencies, exposure times and long-term effects on cell cultures for ultrasound to be reproducibly and effectively applied in the future.

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