Characterization of a stem cell population derived from human peripheral blood and its therapeutic potential in brain tumors

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Abstract

Adherent $CD34^+$ cells are a stem cell enriched population with a high frequency of primitive stem cells that are genetically primed to differentiate into tissue-specific lineages. These cells make up the adherent $CD34^+$ cells in peripheral blood which are the focus of this study. The first aim of this study was to transcriptionally characterize adherent CD34+ cells relative to non-adherent $CD34^+$ cells using a whole human genome Affymetrix U133 plus 2 array. The subsequent analysis demonstrated transcriptional differences in genes which are involved in quiescence, cell cycle, homing and adhesion, which are likely to be relevant to the suitability of adherent $CD34^+$ cells for clinical application. To verify the microarray analysis, a selection of representative transcripts was chosen and their relative expression was compared using real-time qPCR. To analyze further the stem cell characteristics of adherent $CD34^+$ cells, the DNA replication timing kinetics of pluripotency-associated genes in bone marrow-derived adherent CD34+ cells, non-adherent CD34+ cells, and foetal mesenchymal stem cells were conducted and compared to those of embryonic stem (ES) cells. This showed that pluripotency-associated genes Oct4 and Nanog replicate early in the cell cycle similar to ES cells. The second aim of this study was to examine the potential use of adherent CD34+ cells in neural injury. As adherent CD34+ cells are primitive stem cells, it was reasonable to hypothesize that these cells would differentiate into neural cells *in vitro*, but these experiments revealed that these cells are not neurogenic and, in our hands, do not differentiate into neural cells. The experiments revealed that adherent CD34+ cells differentiate efficiently and reproducibly into microglia-like cells (CD34MG cells). CD34MG cells upregulated microglia-associated antigens and demonstrated functional characteristics of microglia cells including TNFα and IL-6 release in response to lipopolysaccharide. CD34MG also stimulate responders in a mixed lymphocyte reaction, suggesting antigen presenting capabilities. To determine if these cells might be exploited for use in brain tumour therapy, it was shown that CD34MG migrate and invade in response to brain tumour glioma cells. Results also suggest that these cells are capable of presenting glioma specific antigen to immune cells in an autologous lymphocyte reaction *in vitro*. The combined results suggest that CD34MG cells might be exploited for therapeutic purposes in brain tumours. Stem cells are currently being explored for their therapeutic potential. This study demonstrates that adherent CD34+ cells and CD34MG cells may be useful for clinical use.

Declaration of originality

I declare that this is the result of my own work carried out between 2005 and 2008 in the Department of Haematology, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN. All the work, analysis, and illustrations were performed by me, unless otherwise specified in the text.

Catherine Flores

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Abbreviations

ACM	Astrocyte conditioned medium
BDNF	Brain-derived nerve factor
BHA	Butylated hydroxyanisole
BM	Bone marrow
BrdU	Bromodeoxyuridine
CCL2	Chemokine (CC motif) ligand 2
CD34MG	Adherent CD34+ cell derived microglia like cells
c-Myc	myelocytomatosis oncogene
CNS	Central nervous system
CTL	Cytotoxic T lymphocytes
CXCL10	Chemokine (CXC motif) ligand 10
CXCR4	Chemokine (CXC motif) receptor 4
DMEM	Dulbecco's minimum essential medium
DMEM:F12	DMEM supplemented with F12
DMSO	Dimethylsulfoxide
EBV	Epstein Barr virus
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ES cell	Embryonic stem cell
FBS	Foetal bovine serum
FGF2	Fibroblast growth factor 2
FGF8	Fibroblast growth factor 8
fhMSC	24XY foetal human mesenchymal stem cells
GBM	Glioblastoma multiforme
G-CSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hanks' buffered salt solution
hFib	HS27 primary human fibroblasts
HGF	Hepatocyte growth factor
HSC	Haematopoietic stem cell
IGF1	Insulin-like growth factor 1
IGFR	Insulin-like growth factor 1 receptor
IL-3	Interleukin 3
IL-4	interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
iPS	inducible plurirpotent stem cell
Klf4	Kruppel-like factor 4
LPS	Lipopolysaccharide
MAPC	Multipotent adult progenitor cells
MCP	monocyte chemoattractant protein
MIAMI	Marrow isolated adult multilineage inducible cells
MIF	Macrophage inhibitory factor
MIP1α	Macrophage inflammatory protein
MNC	Mononuclear cells
MSC	Mesenchymal stem cell
N2	Neural medium supplement
NGF	Nerve growth factor
Nkx2.2	NK2 homeobox2

NSC	Neural stem cell
OCT4	Octamer 4
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PNS	Peripheral nervous system
SCF	Stem cell factor
SCF	stem cell factor
SDF1	Stromal cell-derived factor-1
SHH	Sonic hedgehog
Sox2	SRY (sex determining region) box 2
SSEA	Stage specific embryonic antigen
SVZ	Subventricular zone of the hippocampal dentate gyrus
TGFβ	Transforming growth factor beta
TLR4	Toll like receptor 4
TNF	tumor necrosis factor
VEGF	Vascular endothelial growth factor
VSEL	Very small embryonic like cells
α-MEM	α-minimal essential medium

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Chapter 1 Introduction

1.1 Stem cells

Stem cells are undifferentiated cells defined by their capacities to self-renew indefinitely and to produce lineage defined progeny which can either be transamplifying progenitor cells or terminally differentiated effector cells. Stem cells are categorized by their developmental potential. Totipotent stem cells are able to give rise to all embryonic and extra-embryonic cells. Pluripotent stem cells are isolated from the inner cell mass of the blastocyst and are able to give rise to all cell types of the embryo proper. Multipotent cell types are able to give rise to a subset of cell lineages within the same germ layer. Oligopotent stem cells are able to give rise to a more restricted subset of cell lineages than multipotent cells. Unipotent cells are able to give rise to only a single mature cell type. In adults, stem cells were previously thought to be more lineage restricted, able to give rise to progeny cells of the corresponding tissue. Tissue-specific stem cells have been identified in the body and have been described in most tissues including the brain, intestine, heart, bone marrow, and blood[1-4]. The prospective identification of highly purified stem cell populations from adult tissues and the development of clonogenic assays for their function is essential for both understanding the capacity of these cells to regenerate tissues and for definitively measuring their plasticity.

Yamanaka and colleagues[166] (2006) were the first to demonstrate that somatic cells can be genetically reprogrammed into induced pluripotent stem (iPS) cells by transducing cells with four genes associated with pluripotent embryonic stem (ES) cells, Oct3/4, Sox2, Klf4, and c-Myc[166-168]. The same group has more recently reported that reprogramming does not require c-Myc, and that only Oct3/4, Sox2, and Klf4 are required to generate inducible pluripotent stem (iPS) cells in both mouse and human fibroblasts[169,170]. These iPS cells have identical properties to ES cells and give rise to chimeric mice[169,170]. Induced pluripotent stem cells are not tumor

forming and the absence of c-Myc increased the safety of these cells for potential clinical use. Inducing pluripotency of fibroblasts in humans for subsequent differentiation provides therapeutic potential of iPS for autologous cell therapy.

1.2 Stem cell division

In order for stem cells to simultaneously renew and give rise to lineage specific progeny, they must undergo asymmetric division. Asymmetric cell division is defined as any division that gives rise to daughter cells with different cell fates[5-8]. Asymmetric division ensures the balance between self-renewal and differentiation, and is required to maintain homeostasis, while symmetric division is required for population recovery after depletion[5-7].

Studies of *Drosophila* stem cell division have suggested two mechanisms of asymmetric stem cell division[5]. When division is governed by intrinsic mechanisms, regulators of self-renewal are localized asymmetrically during mitosis so they are inherited by only one daughter cell[5]. Cells orient themselves relative to surrounding tissue and use apical-basal or planar polarity of the surrounding tissue to set up an axis of polarity[5]. During mitosis, the axis is used to polarize the distribution of cell fate determinants and the mitotic spindle is oriented such that cell fate determinants are inherited by only one of the daughter cells[5]. Uneven distribution of these determinants leads to establishment of different cell fates.

If asymmetric division is governed by extrinsic niche mechanisms, the stem cell remains in close contact with the stem cell niche and depends on contact for maintaining self-renewal potential[5,9]. The cell orients its mitotic spindle parallel to the niche surface, thus only one daughter cell can maintain contact with the niche and retains self-renewal capability[5]. If the stem cell plane of division is perpendicular to the niche surface, two stem cells are generated[5].

An example of asymmetric stem cell division has been intensively studied in *Drosophila* neural tissue, and is becoming more defined in the adult human brain[5,7]. Studies have demonstrated that the orientation of the mitotic spindle influences post-natal human neural stem cell fate and the fate of daughter cells. Neural stem cells (NSC) remain in contact with the supporting ependymal layer on the lateral wall of the lateral ventricle[1,5] (Figure 1). These 'type B' cells are slowly proliferating stem cells which asymmetrically divide, both self-renewing and giving rise to rapidly dividing progeny 'type C' cells[1]. Type C cells in turn generate differentiated neuroblasts, 'type A' cells, which migrate and terminally differentiate into neurons[1] (Figure 1). Neuroblasts migrate as chains of cells along tunnels of glial cells formed by type B cells. Rapidly dividing precursor type C cells form focal clusters scattered along the network of glial tunnels[1].

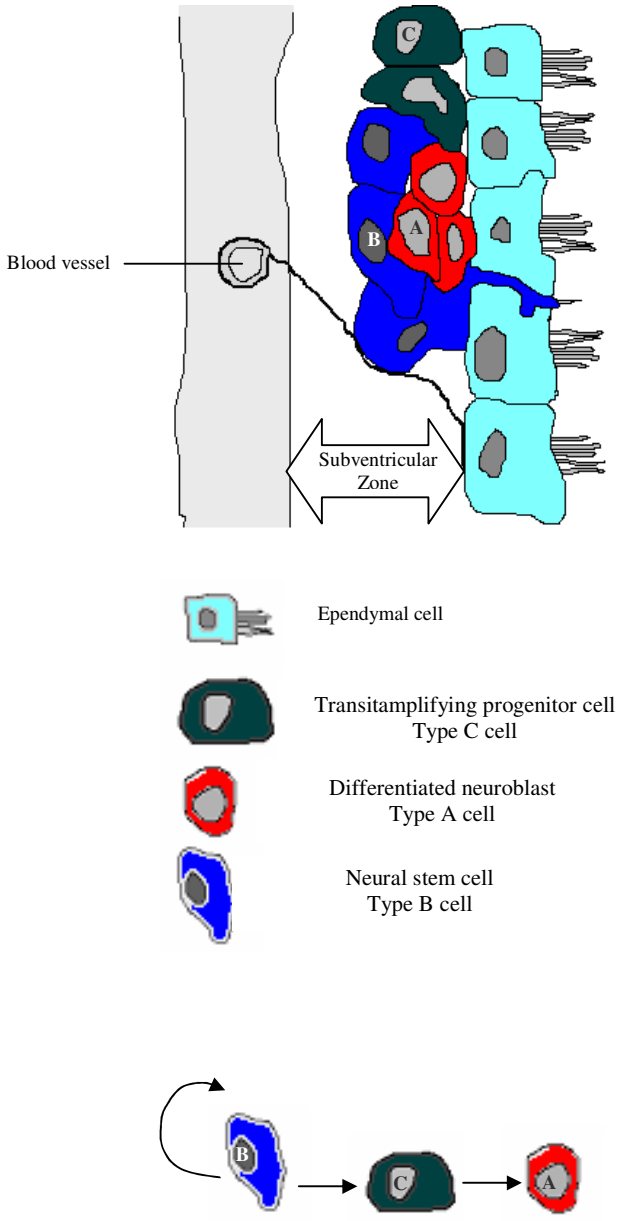


Figure 1. Asymmetric neural stem cell division. This model of neural stem cell division is an example of asymmetric division within the mammalian system. The neural stem cell niche is within the subventricular zone of the brain, which lies adjacent to the lateral wall of the lateral ventricle. Multiciliated ependymal cells line the wall of the ventricle. Neural stem cells, type B cells, slowly divide giving rise to rapidly dividing precursor type C cells which subsequently give rise to neuroblasts which are considered type A cells.

1.3 Stem cell multiplicity

Traditionally, there have been two broadly categorized stem cell types which reside in the bone marrow, hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC)[10,11]. Recently, different research groups have identified heterogeneous populations of stem cells in the bone marrow. These populations may or may not be overlapping, but have been assigned different names including multipotent adult progenitor cells[12] (MAPC), marrow-isolated adult multilineage inducible cells[13] (MIAMI), very small embryonic like cells[14-16] (VSEL), as well as precursor germ cells[17,18]. These cells have been demonstrated *in vitro* to be capable of upregulating phenotypic characteristics of various tissue specific lineages[10-14,16-18]. The differentiation of tissue-specific stem cells into effector cells of a different germ layer is highly controversial. Previous studies of stem cell differentiation remain under scrutiny as some results are found to be irreproducible and inconsistent. The irreproducibility of stem cell differentiation is inexplicable, but may be due to donor variability, detection strategies, cell purification techniques, or any number of reasons. Inconsistent results lead to alternative explanations for the observed differentiation of tissue-specific stem cells to unrelated cell types.

There are currently several theories to explain the multiplicity observed in stem cells from various adult tissues (Figure 2). First, direct stem cell transdifferentiation was proposed as the mechanism by which stem cells potentially contributed to cell types of different lineages[19]. Publications first suggested contributions of bone marrow and circulating stem cells to non-haematopoietic tissue as well as the differentiation of NSC into blood lineages[20,21]. These observations were thought to result from the transdifferentiation of developmentally plastic HSC and NSC to alternate lineage fates[20,21]. According to the second theory, lineage conversion was proposed to occur by the dedifferentiation of lineage defined stem cell to a more primitive multipotent cell, followed by redifferentiation along an alternative lineage.

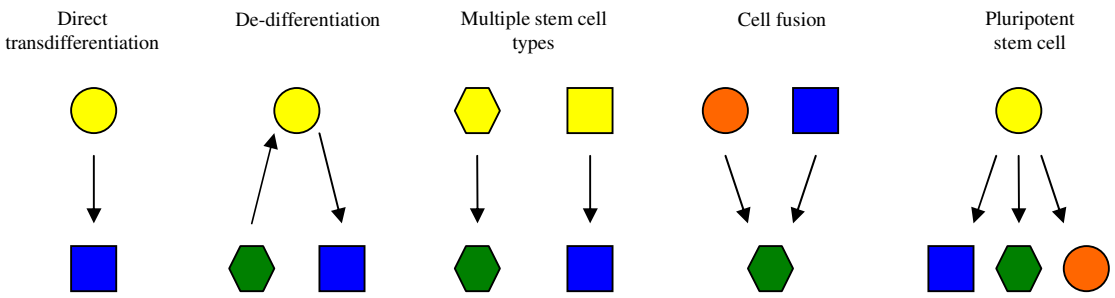


Figure 2. Stem cell multiplicity. Proposed mechanisms that may underlie observations of apparent adult stem cell plasticity including transdifferentiation of developmentally plastic stem cells, de-differentiation of a tissue-specific cell to a more primitive cell, presence of multiple lineage specific stem cell types within the isolated stem cell population, cell fusion or the presence of pluripotent stem cells.

The third explanation is that multiple types of tissue defined stem cells exist within a sample population. Many demonstrations of plasticity were inferred after in vitro differentiation and in vivo transplantation of unpurified heterogeneous cell populations such as unfractionated bone marrow (BM) derived cells. It is possible that unfractionated populations of BM cells contain multiple stem and progenitor cell populations, suggesting that distinct stem cells are instead accounting for observed transdifferentiation. Unfractionated BM contains multiple progenitor cell types including MSC, HSC, endothelial precursor cells and muscle progenitor cells[22]. Appropriate progenitor cells could contribute to differentiation of cells consistent with their intrinsic germ lineage, leading to observations suggestive of transdifferentiation. The fourth explanation of plasticity describes the highly controversial cell-cell fusion whereby the donor cell contributes to tissue regeneration by fusing with damaged cells from the host. Cell fusion has been implicated in contributions of transplanted BM cells to hepatocytes, cardiomyocytes, and Purkinje neurons. This was originally suggested after demonstrating the capability of embryonic stem (ES) cells to fuse to cultured neural cells or BM cells[23,24]. Although it has been proposed that heterokaryotic cell fusion in adults might exist as a physiological repair mechanism, the events are extremely rare in transplanted animal models.

Finally, contributions across multiple tissue types could arise from rare pluripotent stem cells found postnatally. Two groups have identified rare primitive stem cell populations in peripheral blood and bone marrow which express markers typically associated with pluripotent ES cells. Ratajczak and colleagues hypothesized that during development, a pool of VSEL cells are deposited in the bone marrow to maintain postnatal homeostasis and tissue regeneration of haematopoietic and nonhaematopoietic tissues[16,25]. Identified in the bone marrow and umbilical cord blood, VSEL cells express CXCR4+ and are enriched for mRNA for early muscle, heart, neural, liver, intestinal epithelium, epidermal, and pancreatic markers, similar to pluripotent ES cells[25]. Exposure of these cells to permissive environments leads to differentiation to neuroectoderm, cardiomyocytes, and pancreatic cells. A second stem cell enriched population was identified by Gordon and colleagues which are adherent CD34+ small lymphocyte like cells found in peripheral blood[11,26,27]. They are isolated from the bulk of CD34+ cells by their ability to adhere to tissue culture treated plastic, and are morphologically and transcriptionally distinct from non-adherent CD34+ cells[26]. Adherent CD34+ cells are quiescent, metabolically inert stem cells and express genetic messages which encode for liver, heart, epithelial, and pancreatic cells[26]. Adherent CD34+ cells are considered to be a population of primitive stem cells with high frequencies of stem cells which are genetically primed to differentiate into tissue-specific lineages[26]. These collective cells make up the adherent fraction of CD34+ cells in peripheral blood and are the main population of focus in this study.

1.4 Bone marrow stem cell niche

The stem cell niche can be defined as a spatial structure in which stem cells are housed and maintained in an undifferentiated state[10,28-30]. The haematopoietic stem cell niche is located in the sinusoidal endothelium in the trabecular bone of the bone marrow[10,11]. The balance between

self-renewal and lineage differentiation is governed by molecular cross-talk between the stem cells and the osteoblasts lining the sinusoidal endothelium[10,28].

1.4.1 Developmental migration

One interpretation of the multilineage capabilities of stem cells in the marrow is that bone marrow is a general stem cell repository for cells that can be mobilized for various purposes during adulthood. Signals within the microenvironment induce the mobilization of stem cells from the bone marrow into circulation. Ratajczak et al[31,32] hypothesized that during development, pluripotent stem cells are deposited in the bone marrow for future use, a phenomenon that has been termed 'developmental migration'. They hypothesized further that during ontogeny, stem cells are chemoattracted to the bone marrow which provides a microenvironment appropriate for a stem cell niche[14,25,33]. Chemokine, CXC motif, receptor 4 (CXCR4)-expressing stem cells migrate in response to stromal cell-derived factor-1 (SDF1), while c-Met+ stem cells migrate in response to hepatocyte growth factor (HGF)[33]. Studies have shown that both hematopoietic and non-hematopoietic stem cell migration into the bone marrow are CXCR4/SDF1 and c-Met/HGF dependent[34,35]. These cells remain in the bone marrow and are mobilized into the circulation during injury to aid in tissue repair and regeneration[14,35]. This theory is based on the detection of VSEL cells in adults which express ES cell-associated markers Oct4, Nanog, Rex1, and stage-specific embryonic antigen (SSEA)[14,16,25,33]. Adherent CD34+ cells and VSEL cells express a similar panel of markers.

1.4.2 Haematopoietic inductive microenvironment

Stem cell self-renewal and differentiation is determined by the inductive microenvironment within the stem cell niche. The haematopoietic inductive microenvironment was first described in a study by Wolf and colleagues[36,37] of the growth and differentiation of spleen colonies in

mice, arising from transplanted bone marrow. This study revealed that the differentiation of multipotent stem cells is influenced by their physical location in the spleen[36,37]. The inductive microenvironment of the anatomical location influences the probability of a stem cell differentiating into a specific lineage[11]. If the microenvironment induces lineage specification, it can be inferred that a stem cell niche must exist in which the molecular constituents control the balance between quiescence, self-renewal, and differentiation. The functional proof for the stem cell niche was provided by McCulloch and colleagues in 1965[38] and later by Barker[39,40]. Mutant *Sl/Sl*^{d} mice deficient in the gene which encodes for SCF show changes in the bone marrow stem cell niche and a consequent failure of HSC maintenance and reduction in normal bone marrow engraftment[10,38-40].

1.4.3 Multilineage priming

Enver and colleagues revealed that hematopoietic stem cells are 'multilineage primed', expressing low levels of mRNA of various hematopoietic lineages[11,41,42]. This implies that a single stem cell is capable of transcribing genes of multiple lineage specifications when placed in the appropriate inductive microenvironment[11,41,42]. Adherent CD34+ cells express genetic messages for multiple lineages including haematopoietic, hepatic, pancreatic, cardiac, and neural cells[11,26]. Applying the theory of multilineage priming, the corresponding genes should be available for transcription by adherent CD34+ cells, thus enabling them to contribute to the regeneration and repair of injured tissues. Since the cells are innately primed for multiple lineage specification, they lack the need for de-differentiation, transdifferentiation, or cell fusion[11].

1.5 Stem cell DNA replication timing

Genetic and transcriptional profiling of stem cells has revealed genes which are characteristic of stem cells. Gene profiling does not discriminate between genes subject to active repression and those that are not transcribed because activating proteins are limiting. Epigenetic and developmental studies have suggested that unique epigenetic markers may be valuable indicators of stem cell identity. Epigenetic correlations of gene expression are not absolute, but can be used collectively to gain insights into the underlying mechanisms that establish gene expression patterns through cell division, and suggest how stem cell gene expression is modulated. Traditionally, epigenetics is loosely categorized into DNA methylation [43], histone modification[44], and small interfering RNAs[45]. Other features of chromatin behaviour also correlate with gene transcription including DNA replication timing[46].

Replication timing is an indicator of chromatin state[47]. Recent studies have identified chromatin signatures and replication timing profiles of ES cells[46] and it has been demonstrated that changes in replication timing occur as ES cells differentiate[48]. The replication timing of genes depends on chromatin state and correlates with histone acetylation, and early replication of a gene does not necessarily reflect gene transcription[49]. Early replication of genes during S phase of the cell cycle is considered to be a feature of transcriptionally active regions of the genome, indicating chromatin accessibility, whereas late replication is characteristic of inactive gene regions and constitutive heterochromatin[46]. During development, pluripotent stem cells give rise to lineage-restricted progenitors from which more tissue-restricted cells are derived[50]. Thus in pluripotent ES cells, there is replication of lineage-associated genes early in the S phase, suggesting multilineage potential and pluripotency. Changes in replication timing have been shown to accompany differentiation, and activation of tissue specific genes correlates with a switch from early to late replication.

In ES cells, many genes that encode for transcription factors which are not expressed by ES cells but are important for later development, replicate early in ES cells[46]. In the same study, terminally differentiated T cells had significantly fewer early replicating genes[46]. This indicates that in tissue-defined cells, genes which are not actively expressed replicate late in the cell cycle, and it is likely that only actively transcribed genes replicate early. Azuara et al[46] found that genes encoding for neural-specific transcription factors replicated early in ES cells which retain neural potential, but replicated late in haematopoietic cells which do not have neural differentiation potential. This assumes that the chromatin structure governing gene expression which is not appropriate for a particular lineage undergo changes to switch replication from early to late replication as differentiation occurs. This indicates that chromatin changes occur in ES cells as they lose their pluripotency. Different laboratories have generated similar replication timing profiles of ES cell lines, indicating this technique may be potentially useful in defining degrees of 'stemness' [48,46,51].

1.6 The nervous system

The cells of the nervous system were first visualized in 1873 by Camillo Golgi using his silver impregnation staining method which stained only selected neurons, raising them above its surrounding tissue[52]. This was followed by the identification of glia, and microglia; by 1921, the various cells of the nervous system were identified[52]. In the central nervous system, neurons and glia have an interdependent relationship which is highlighted by the pathology of neurodegenerative diseases. Injuries of the nervous system involve damage to multiple cell types and often require the repair and regeneration of not only neurons, but also require repair of glial cells which provide support to neurons.

Macroglia is comprised of astrocytes and oligodendrocytes. Astrocytes are the predominant cells type in the central nervous system (CNS) and, based on morphology, are divided into two types: fibrous and protoplasmic[53]. The role of astrocytes is necessary for neuron function and survival. Astrocytes regulate the extracellular environment including the homeostasis of K+, H+, and Ca^{2+}. They synthesize and secrete essential growth factors in the CNS including nerve growth factor (NGF), brain-derived nerve factor (BDNF), vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGFβ). In addition to neuronal support, astrocytes are required for synaptogenesis and neurogenesis[53,54].

In the CNS, oligodendrocytes are responsible for myelinating axons by unidirectionally wrapping layers of myelin around a neuron's axon, initiated at the site of the axon-glial junction[55]. In the peripheral nervous system (PNS), Schwann cells produce myelin and are responsible for its maintenance. The myelin sheath consists of many wrappings of glial membrane around the axon with little or no cytoplasm remaining between adjacent wraps. This insulates the axon and allows for saltatory conduction and action potentials to propagate more efficiently along its length[55,56].

Microglia are unique in that they act as both supportive glia and as immunocompetent cells within the CNS[57]. The nervous system has historically been regarded as an immune privileged site, isolated from peripheral blood-born leukocytes and immune regulatory cells by the blood-brain barrier. The fragility of neurons requires protection from activated immune responses from peripheral leukocytes. Neurons also require physical and cytochemical support by glia. Microglia are responsible for the immunological and neuromodulatory responses which occur after CNS injury[58]. They are considered to be the resident tissue macrophages of the CNS, and when activated, can act as antigen-presenting cells[59]. There are two types of microglia, resident

and migrating microglia. Resident microglia make up the majority of the population within the CNS, while migrating phagocytes that enter the CNS, sometimes referred to as migrating microglia, are thought to be bone marrow derived and are able to cross the intact blood-brain barrier. CNS macrophages, or their precursors, which circulate in the peripheral blood enter the CNS and are recruited by chemokine (CC motif) ligand 2 (CCL2) and chemokine (CXC motif) ligand 10 (CXCL10)[58]. Under normal conditions, resident microglia are a fairly stable population which proliferates to maintain homeostasis.

1.6.1 Injury in the CNS

Stem cell biology in regenerative medicine is currently exploring two options in terms of neural cell replacement in neurodegenerative diseases. One method of therapy is transplantation of neural stem and progenitor cells derived from embryonic, foetal, and adult tissue. Another option is stimulating innate repair mechanisms in the CNS by exogenous means to accelerate endogenous neurogenesis. Although the potential of stem cell therapy in neurodegenerative diseases has shown therapeutic potential in the laboratory, many issues must be properly assessed prior to using stem cells in a clinical setting.

1.6.2 Bone marrow infiltrates in neural injury

Infiltration of bone marrow-derived microglia into the CNS occurs in two ways. The first is the progressive cellular infiltration that occurs in normal microglial turnover, but cell turnover is very low under normal conditions[58,60]. The second occurs during acute injury which triggers migration of bone marrow cells into the CNS and to the site of injury[58]. Studies have shown that bone marrow-derived cells contribute to the expansion of the microglial population in the CNS and are detectable during the acute phase of injury[61]. Infiltration and microglial transformation of bone marrow-derived cells occurs in models of major CNS injury such as focal cerebral ischaemia, as well as in models of discrete injury such as in anterograde axonal degeneration[60,61].

Studies have reported up to 13% of microglia are bone marrow-derived in lesioned areas after 7 days post injury[58], and up to 28% 12-14 days post injury[60].

Microglia share phenotypic characteristics with mononuclear phagocytes and are of haematopoietic origin[59,62]. In a study by Hess et al (2004), irradiated mice were transplanted with a single HSC expressing enhanced green fluorescent protein (EGFP) in order to observe haemopoietic reconstitution. Two months after transplantation, EGFP+ microglia were found in the brain parenchyma. Four months post-transplantation, many EGFP+ cells were found scattered throughout the brain parenchyma with morphological and antigenic characteristics of microglia. The EGFP+ cells were ramified in morphology and expressed RCA120 lectin. After 4 months, 12% of the total parenchymal microglia cells were EGFP+, and after 12 months, 40% of parenchymal microglia were EGFP+. Interestingly, this illustrates that microglia derived from bone marrow HSCs are able to cross the blood brain barrier when there is no CNS injury. After acute focal cerebral ischaemia, an influx of EGFP+ cells was observed in the area of infarction, and the cells were closely associated with blood vessel walls. In the ischaemic tissue, NeuN+ neuronal cells were seen to have been phagocytosed by EGFP+ cells[62]. This study makes three points. First, at least a proportion of parenchymal microglia originate and migrate from the HSC fraction of the bone marrow. Second, after focal ischaemia, HSCs which have the potential to differentiate into microglia migrate from the bone marrow to the ischaemic area. Third, microglia from the bone marrow have phagocytic macrophage-like capabilities. This evidence leads us to speculate that HSCs have potential to differentiate into microglial cells. It is noteworthy that the recipient mice were sublethally irradiated, and the effects of irradiation on the permeability of the blood-brain barrier are unclear.

1.6.3 Endogenous neurogenesis in injury

Although the components of the nervous system were identified by 1921, post-natal neurogenesis was not established until Eriksson et al (1998) reported the generation of neurons in the adult hippocampus[63]. The concept of post-natal neurogenesis was firmly established in the following years alongside research in stem cell biology. There are two neurogenic regions in the adult brain, the subventricular zone (SVZ) of the hippocampal dentate gyrus, and the olfactory bulb which may be associated with memory and smell respectively[52]. Adult CNS stem cells exhibit the cardinal characteristics of stem cells: self-renewal and pluripotency[64,65].

Investigations have shown an increase in cell proliferation and neurogenesis after insult[66,67], and a mounting number of studies seek to stimulate post-natal neurogenesis as an alternative to stem cell therapy. It has been demonstrated that endogenous neural stem cells proliferate in response to acute ischaemia in the forebrain, are recruited to the penumbra region, and give rise to hippocampal pyramidal neurons. Infusion of exogenous growth factors (fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF)) led to a 40% recovery of neurons lost due to ischaemia. Newly generated neurons showed functional synapse formation as well as recovery of brain function[66]. This points to the value of stimulating neurogenesis of endogenous neural stem cells using pharmaceutical agents as an alternative to stem cell transplantation.

1.6.4 Extra-cranial sources of stem cells for repair

The administration of non-neural cells in models of cerebral ischaemia has lead to the notion that non-neural cells provide neuroprotection and stimulate endogenous neurogenesis. It is likely that most if not all organs in the body have a reservoir of stem and progenitor cells which are mobilized after insult, thus expediting tissue repair and regeneration. It is known that haematopoietic stem and progenitor cells circulate in the peripheral blood. Kucia et al.[68] hypothesize that CXCR4+/CD45- tissue-specific stem cells also circulate in the non-

haematopoietic fraction of peripheral blood, maintaining a pool of cells which are easily distributed to areas of the body distant from the bone marrow. Fluorescence-activated cell sorting and mRNA analysis shows that within this population is a small population of neural-tissue committed stem cells which express neural markers GFAP, nestin, β-III tubulin, and NeuN. Of particular interest, after cerebral ischaemia, there is a marked increase in neural tissue-specific stem cells in the peripheral blood[15]. *In vitro* analysis of these cells shows that after a stroke, neural-tissue committed stem cells respond to chemoattractants SDF-1, HGF, and leukemia inhibitory factor (LIF)[15].

In vitro investigation has demonstrated that embryonic stem cells and foetal stem and progenitor cells, as well as cells from non-neural sources are capable of exhibiting phenotypic, genetic, and electrophysiological characteristics of neuronal cells (Figure 3). Animal models of neurodegenerative diseases demonstrate that stem cells are capable of restoring appropriate motor function. Molecular mechanisms of repair remain unknown making the long term effects of transplantations uncertain. The challenge which remains is to achieve the desired final outcome which is to restore lost organ function.

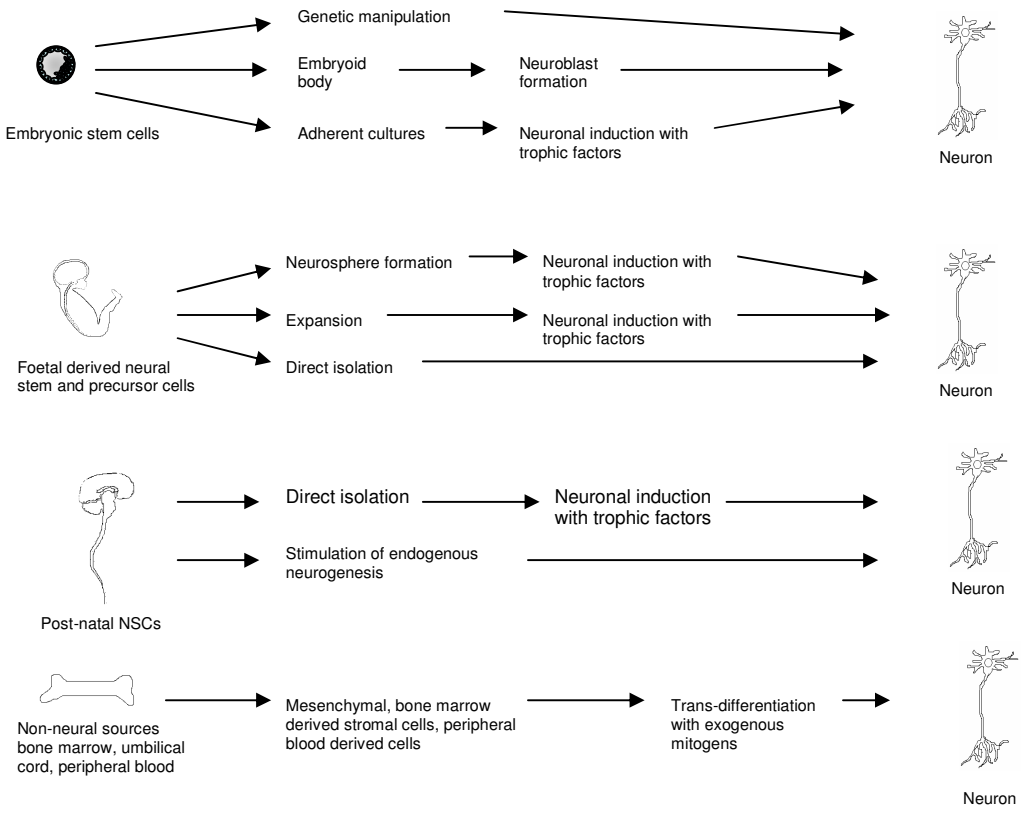


Figure 3. Stem cells for in vitro neuronal differentiation. There are several human stem cell sources that have demonstrated motor function in animal models. Neurons have been generated *in vitro* from ES cells, fetal tissue, and from cells derived from non-neural sources. Pluripotent ES cells are able to differentiate into cell types from all three germ layers and can be induced into all cells of the neural lineage by genetic manipulation or the addition of exogenous cytokines. Neural stem and precursor cells have been isolated from fetal and post-mortem adult brain tissue and has been demonstrated to differentiate into terminally differentiated neural cells. Adult bone marrow, umbilical cord and peripheral blood derived stem cells have been demonstrated to express genetic message for neural cells. Some of these stem cell types have been reported to promote motor function in animal models of neurological disease.

Literature suggests that bone marrow-derived cells have neural differentiation potential[12-15,19,75-77]. Previous data[26] demonstrated that adherent CD34+ cells express transcripts for dopamine, serotonin, and neurogenin-3, suggesting that adherent CD34+ cells may have the potential for

neuronal differentiation. It was thus reasonable to investigate the neuronal differentiation potential of adherent CD34+ cells *in vitro*, beginning with published neuronal differentiation protocols[75-77].

1.7 G-CSF mobilization

Autologous haematopoietic stem cell transplantation is currently used for treatment for haematological malignancies[19]. Granulocyte colony stimulating factor (G-CSF) mobilization with chemotherapy increases the number of CD34+ HSCs in the peripheral blood to levels higher than those in the bone marrow. During steady state conditions, the bone marrow contains a higher concentration of CD34+ HSCs than the peripheral blood[69,70]. During G-CSF mobilization, myeloid hyperplasia occurs in the bone marrow, expanding the haemopoietic system including mature and immature myeloid cells. After 4 to 5 days of G-CSF mobilization, the peak number of CD34+ haematopoietic stem cells (HSC) is released from the bone marrow into the circulation[70].

1.8 Stem cell therapy

One of the aims of stem cell biology in the context of regenerative medicine is to understand the mechanisms which govern self-renewal and differentiation so that stem cells can be used to instigate the regeneration of damaged tissues. In order for stem cells to be used for therapy in neurodegenerative diseases, a concerted regeneration of multiple neural cell types must occur[125,158,159]. Transplanted cells are also required to migrate to sites of injury, differentiate into multiple cell types, integrate with existing neural circuitry, and restore saltatory conductivity[125,158-160]. Animal models of disease have demonstrated the ability of stem cells to restore appropriate motor function[125,159,160]. Although stem cell transplants have demonstrated

restoration of physiological and motor function, it remains to be seen if regeneration due to stem cells can contribute to cognitive processes[160].

1.8.1 Requirements of stem cells for therapy

There are several factors which determine the suitability of stem cells for use in a clinical setting including accessibility of stem cells, quantity of cells available, isolation efficiency, cell safety, and ethical considerations[11,125,161,162].

1.8.1a Ethics

This includes the ethical acceptability of stem cells in the current regulatory environment. The derivation of pre-natal human tissue may be found offensive to certain communities and this is reflected by current legislation. Adult sources of stem cells are, however, less controversial[161,162].

1.8.1b Accessibility and numbers

Cells for therapeutic purposes must be derived from an accessible source[11,162,163]. The bone marrow and peripheral blood are self-renewing sources which can yield large numbers of cells with minimally invasive procedures. Although neural stem cells might be an ideal cell type for use in neurodegenerative disease, they are only accessible post-mortem, making donor transplants complicated and autologous transplants impossible[125]. In contrast, stem cells from the bone marrow and peripheral blood are highly accessible and acquired by minimally invasive procedures[11,162]. Bone marrow harvesting and mobilization are well established techniques which are routinely used clinically for therapy of haematological diseases. The number of stem cells derived from a tissue can prove rate limiting. For example, cell transplants from foetal tissues for Parkinson's disease require tissue from several donors[125,159]. This inaccessibility and low numbers of stem cells makes the use of this tissue unlikely to be established for routine

therapy. The harvesting of stem cells from blood and bone marrow is logistically advantageous and yields much larger numbers of stem cells for therapeutic use.

1.8.1c Tumorigenicity

Embryonic stem cells might be an ideal stem cell type for tissue regeneration since they can be induced to differentiate into tissue from all three germ layers[164]. Pluripotent ES cells can be kept undifferentiated in culture for prolonged periods of time and differentiated efficiently, but chromosomal aberrations have been demonstrated to occur in ES cells in prolonged culture[164,165]. Upon *in vivo* transplantation, teratomas can form which are benign masses of differentiated ES cells[164]. Masses with undifferentiated ES cells are called teratocarcinomas and malignant masses of undifferentiated cells are embryonic carcinomas[164]. In the development of tumors, genetic aberrations can affect apoptotic pathways, differentiation, or cell cycle control, consequentially leading to precancerous cells and uncontrolled proliferation[164]. Adherent CD34+ cells are isolated from the donor and are neither passaged nor kept in long term culture, decreasing chances of pro-oncogenic genetic alterations.

1.8.1d Isolation efficiency

Several stem cell populations have been identified in various organs, but their rarity makes them difficult to isolate from the bulk of the tissue. Stem cell populations are likely heterogeneous, requiring multi-parameter separation techniques. Mesenchymal stem cells are usually isolated with plastic adherence and long term culture with defined cytokines. Stem cells in haematopoietic tissue are more easily isolated than those in solid tissues and organs because blood and bone marrow are liquid suspensions of cells. Methods that have been applied include FACS sorting, including selection by size, Rhodamine 123 exclusion, or Hoescht expression. For example, VSEL cells and side population stem cells are isolated by these criteria. However,

flow cytometry is not acceptable for current good manufacturing practice (cGMP) requirements. Stem cells can also be isolated using antibody conjugated magnetic bead isolation. CD34+ cells are routinely isolated by magnetic separation for transplants in haematological diseases. Adherent CD34+ cells are isolated magnetically and separated from the bulk of the CD34+ cells by adherence to tissue culture treated plastic. These cells can be isolated in a closed system for clinical use with minimal manipulation to donor tissue, adding to their desirability for clinical use.

1.8.1e Safety

Although there is increasing knowledge about stem cells and their molecular control mechanisms, there are key factors that exclude the majority of stem cell populations from clinical use, particularly tumorigenicity and immune tolerance. ES cells are pluripotent and their differentiation is well controlled *in vitro*. In the body, MSCs and ES cells have epigenetic mechanisms in place which prevent uncontrolled proliferation. Their artificial expansion in culture is thought to alter these intrinsic epigenetic checkpoints of self-renewal, thus MSC and ES cells implanted into animal models have been shown to cause teratoma formation. Immune rejection is also a consideration in donor cell transplants. In the case of blood or bone marrow cell therapy, autologous cell transplants are ideal, but patients may not be well enough to undergo G-CSF mobilization for autologous transplants, or may be haematologically depleted and yield insufficient cells. Use of tissue mismatched donors might risk immune rejection and the onset of graft versus host disease. Mesenchymal stem cells are immunosuppressive, but may lose this characteristic in mismatch transplants[171]. For example, a recent study using MSC to prevent graft-versus-host disease (GvHD) in patients with haematological malignancies subjected to allogeneic HSC transplant found that without mesenchymal stem cells prevent graft-versus-host disease, they increase graft-versus-leukemia reactions[172,173].

1.9 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most common and most lethal primary malignant type of brain tumor in adults. Prognosis is very poor with a median survival time of less than 15 months. A fundamental challenge in the management and treatment of gliomas is the propensity for single tumor cells to invade peritumoral and distant brain tissue. These invasive tumor cells evade surgical removal, chemotherapy, and radiation exposure. Current treatment is surgical removal followed by radiotherapy and/or chemotherapy with Temozolomide or Gliadel. Both drugs are generally well tolerated because of their limited systemic toxicity.

Gliomas of astrocytic origin are classified into four grades as defined by the World Health Organization. Pilocytic astrocytomas (grade I tumor) are usually indolent, have a limited invasive capacity, and rarely undergo anaplastic progression[71]. Pleomorphic xanthoastrocytomas (grade II tumor) are low-grade tumors with cellular pleomorphism and have a limited capacity for brain tissue invasion. Diffuse-type astrocytomas have the capacity to disperse into surrounding tissue and a high frequency of anaplastic progression[71]. Grade II astrocytomas[71] are well differentiated and have mild to moderate nuclear pleomorphism. Grade III astrocytomas are highly proliferative and commonly have a moderate degree of cellular pleomorphism and are more heterogeneous in cell type. Grade IV glioblastoma multiforme (GBM) is the most aggressive glial tumor and is pathologically distinctive with geographic necrosis and microvascular hyperplasia, and has marked cellular pleomorphism[71].

There has been no significant increase in survival of GBM patients in the 30 years of brain tumor research[71-74]. Therefore, the development of novel therapeutic approaches is essential. Genetic mutations have been identified in various types of gliomas, most of which are involved in

autocrine and paracrine signaling. These include genes amplifications which lead to overexpression of mitogens and their specific receptors including EGF and its receptor (EGFR), platelet-derived growth factor (PDGF) and its receptor (PDGFR), insulin-like growth factor 1 (IGF-1) and its receptor (IGFR)[74]. Several angiogenic factors are also overexpressed in gliomas including FGF, interleukin 8 (IL-8), PDGF, TGF, and VEGF[71]. The combined genetic variations of these factors and mitogens lead to subsequent aggressive cellular proliferation, invasion, and angiogenesis, rendering malignant gliomas, especially GBM, resistant to current therapies.

Several factors including the immunosuppressive environment of the tumor and the low avidity of the T cells for the tumor antigen and may contribute to the poor antitumor efficacy of the endogenous immune response[135]. Although tumor specific endogenous immunity can be elicited in cancer patients by overexpressing tumor antigens, it cannot prevent tumor growth[200]. Cancer immunotherapy has become a recent focus for the development of glioma treatment. Tumor-associated antigens recognized by tumor-reactive cytotoxic T lymphocyes (CTLs) have been identified[198]. Tumor antigens expressed by human malignant neoplasms can be categorized loosely into three different groups; the differentiation antigens; the products of viral, mutated, differentially spliced or overexpressed genes; and housekeeping or metabolic pathway antigens[135, 198-200].

1.9.1 Current clinical cell therapy in GBM

Active immunotherapy is a strategy where anti-tumor immunity is initiated following immunization with tumor antigen, and studies have demonstrated that peripherally administered tumor antigen-based immunizations are capable of inducing intracranial anti-tumor responses[73,139,198]. Dendritic cell therapy is a form of active immunotherapy where large numbers of autologous dendritic cells are expanded *in vitro* from monocytes and loaded with tumor antigens. These are then administered as a vaccine to induce anti-tumor

immunity[139,199,200]. Clinical trials using dendritic cell vaccination demonstrated increases in tumor specific cytotoxicity[73,139]. Another cell therapy taken to clinical trial is the use of interleukin 4 (IL-4) transfected fibroblasts for autologous transplant, but patients had recurrent tumor formation[201].

1.10 Aims

Previous studies have demonstrated the ability of adherent CD34+ cells to increase engraftment in bone marrow transplant as well as give rise to cells of haematopoietic lineage[174, 175]. This project contributed to known data about adherent CD34+ cells. The aims of this project were to create a transcription profile of adherent CD34+ cells and compare transcript expression relative to non-adherent CD34+ cells. Previously published transcription data[26] showed that adherent CD34+ cells express transcription factors which encode for neurons, thus the next aim of this project was to determine if adherent CD34+ cells are capable of neuronal differentiation *in vitro*. As these cells are haematopoietic cells, the following aim was to determine if adherent CD34+ cells differentiate into microglia, which are of haematopoietic developmental origin. The final aim of this project was to conduct preliminary experiments to determine if either adherent CD34+ cells or adherent CD34+ cell derived microglia have potential uses for cell therapy in glioblastoma multiforme.

Chapter 2 Materials and methods

2.1 Source of material for study

2.1.1 Leukaphereses

Granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood cells were obtained from leukaphereses processed by the Stem Cell Laboratory, Hammersmith Hospital, in excess of clinical requirements. Informed consent and local research ethics committee approval were granted in all cases. Ethics reference number 06/Q0406/78.

The leukapherised blood sample was diluted in Hanks' Buffered Salt Solution (HBSS) (Invitrogen, Paisley, UK), layered over Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 1800 rpm for 30 minutes. Mononuclear cells (MNC) were aspirated from the interface and washed in HBSS. Cells were then counted using a haemacytometer after diluting in 3% glacial acetic acid (Sigma-Aldrich Company Ltd, Gillingham, UK) to lyse red blood cells.

2.1.2 Fetal human mesenchymal stem cells and human fibroblast cells

24XY fetal human mesenchymal stem cells (fhMSC) and HS27 human fibroblast (hFib) cells were originally obtained from Professor Nick Fisk, and consented use was allowed in collaboration with Dr. Deanna Taylor. Cells were primary cells used between passage 8-13 and cultured in D10 medium which consisted of Dulbecco's minimum essential medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% FBS (Invitrogen, Paisley, UK), 1% L-glutamine (Invitrogen, Paisley, UK) and 1% penicillin-streptomycin (Invitrogen, Paisley, UK).

2.1.3 Neural stem cells

Human neural stem cells R197V (NSC) were provided by Re-Neuron (Surrey, UK) and used in collaboration with Dr. Deanna Taylor and Dr. Reaz Vawza. NSC were used to observe neuronal differentiation and as positive staining controls for neuronal differentiation experiments. Cells were cultured in suspension neurospheres and plated onto poly-D-lysine/fibronectin coated wells for differentiation purposes. Cells were maintained and expanded in DMEM supplemented with F12 (DMEM:F12) (Invitrogen, Paisley, UK) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 1% B27 without vitamin A (Invitrogen, Paisley, UK), 10ng/ml epidermal growth

factor (EGF; Peprotec, London, UK), and 10ng/ml fibroblast growth factor 2 (FGF2)(Peprotec, London, UK). Neural stem cells were used as neural differentiation positive controls to ensure that differentiation protocols did induce neurogenesis in cells known to differentiate into neural cells.

2.1.4 Glioblastoma cell lines

Glioblastoma cell lines U253 and U118 were a kind gift from Dr. Tracy Warr (Institute of Neurology, The National Hospital of Neurology and Neurosurgery, Queen Square, London). The lines U87, U253, and U118 were cultured in DMED:F12 medium, supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were passaged and detached from culture dishes with trypsin/EDTA (Sigma-Aldrich Company Ltd, Gillingham, UK) for 8 mins at 37°C, and subcultured as needed.

2.1.5 N9 microglia cell line

N9 rat microglia cells were provided by Dr. Deanna Taylor. The cells were cultured in DMEM supplemented with 5% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. To subculture, cells were gently scraped from the flask with a plastic cell scraper.

2.1.6 Embryonic stem cells

Embryonic stem (ES) cells E9 were a kind gift from Dr. Wei Cui as a positive control for Oct4 and Sox2 protein expression. The cells provided were plated and fixed for immunofluorescent microscopy.

2.2 Isolation of $CD34^+$ cells

MNCs were labelled for CD34+ isolation using the MiniMacs $CD34^+$ positive isolation kit (Miltenyi Biotec Camberley, UK) as detailed in the manufacturer protocol. For every 10^8 cells, PBMCs were suspended in 300µl Macs buffer (PBS (Invitrogen, Paisley, UK), 0.5% bovine serum albumin (PAA laboratories, Pasching, Austria) and 2mM EDTA (Sigma-Aldrich Company Ltd, Gillingham, UK), 100µl FcR blocking reagent, and 50µl of monoclonal hapten-conjugated CD34 antibody. This was incubated at 4-8ºC for 15 minutes, and washed in Macs buffer by centrifugation at 1800 rpm for 3 minutes. The cells were then resuspended in 500µl Macs buffer per 10^8 cells and passed through a pre-wetted LS MiniMacs column (Miltenyi Biotec Camberley, UK) mounted on a Miltenyi magnet. The column was then washed with 3ml Macs buffer three times. The column was then removed from the magnet and the $CD34^+$ cells eluted with 5ml Macs buffer.

2.3 Isolation of plastic adherent CD34+ cells

Isolated $CD34^+$ cells were plated on 35-mm^2 Petri dishes in α-minimal essential medium (α-MEM) (Invitrogen, Paisley, UK) and incubated for 30 minutes at 37°C and 5% CO_2. The non-adherent cell fraction was removed by washing the plates three times with HBSS. Standard culture conditions for plastic adherent $CD34^+$ cells include culture in α-MEM supplemented with 30% FBS and cytokines (20 ng/ml stem cell factor [SCF], 1 ng/ml GM-SCF, 5 ng/ml IL-3, and 100 ng/ml G-CSF) at 37°C in 5% CO_2 in air.

2.4 Neural Differentiation

The details of differentiation protocols are outlined below. Initial attempts to induce neuronal differentiation were conducted using published protocols as described and referenced below. Protocols were then derived from published work, using various combinations of cytokines and growth factors as detailed below.

Prior to neural differentiation, plastic adherent CD34+ cells were kept in culture in α-MEM supplemented with 30% FBS and cytokines (20 ng/ml stem cell factor [SCF], 1 ng/ml GM-SCF, 5 ng/ml IL-3, and 100 ng/ml G-CSF) at 37°C in 5% CO_2 in air for 5 days. The medium was then removed, and replaced with the appropriate experimental neural induction medium as detailed below. For fMSC neural differentiation, fMSCs were plated onto poly-D-lysine coated glass coverslips overnight in DMEM medium supplemented with 10% FBS. The medium was then replaced with the desired neural induction medium.

2.4.1 Neuronal induction protocol 1

Based on the neuronal induction protocol by Woodbury et al[75], adherent CD34+ cells were cultured in DMEM, 20% FBS, and 1mM β-mercaptoethanol for 24 hours. The medium was then replaced with medium consisting of DMEM, 2% dimethylsulfoxide (DMSO), and 200 µM butylated hydroxyanisole (BHA) and cultured for 2 days.

2.4.2 Neuronal induction protocol 2

Based on the neuronal induction by Tao et al[76], adherent CD34+ cells were plated onto fibronectin coated plastic tissue culture treated plates and cultured in DMEM/nutrient mixture F12 (DMEM/F12; Invitrogen, Paisley, UK), 25µg/ml insulin, 100 µg/ml transferring, 100 µM

putrescine, 0.02µM progesterone, and 0.03 µM sodium selenite (Sigma-Aldrich Company Ltd, Gillingham, UK). The medium was supplemented with 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF), and 1 ng/ml platelet derived growth factor (PDGF) (R&D Systems, Minneapolis, MN, USA). The cultures were incubated at 37°C in 5% CO_2 in air with daily additions of the above stated growth factors and the medium changed every 2 days.

2.4.3 Neuronal indiction protocol 3

Based on the neuronal induction protocol by Muñoz-Elias et al[77], adherent CD34+ cells were plated on poly-D-lysine (Sigma-Aldrich Company Ltd, Gillingham, UK) coated plates and cultured in DMEM, 20% FBS supplemented with 5 ng/ml bFGF for 24 hours. The medium was then replaced and supplemented with 100 µM BHA, 10 µM forskolin, N2 medium supplement (Invitrogen, Paisley, UK) and incubated at 37°C in 5% CO_2.

2.4.4 Neuronal induction protocol 4

Derived from a neuroectodermal differentiation protocol by Jiang et al[12], adherent CD34+ cells were cultured in DMEM/F12 medium with N2 medium supplement, 1% glucose (Sigma-Aldrich Company Ltd, Gillingham, UK), 1% L-glutamine (Sigma-Aldrich Company Ltd, Gillingham, UK). This was then supplemented with 10 nM bFGF, 10 nM sonic hedgehog (Shh; Peprotec, London, UK), 10 nM fibroblast growth factor 8 (FGF8; Peprotec, London, UK), and 1 nM retinoic acid (Sigma-Aldrich Company Ltd, Gillingham, UK). Cells were incubated at 37°C in 5% CO_2.

2.4.5 Neuronal induction protocol 5

Adherent CD34+ cells were cultured in DMEM/F12 medium with B27 medium supplement (Invitrogen, Paisley, UK) and 1% L-glutamine, supplemented with an additional 10 nM bFGF. Cells were incubated at 37°C in 5% CO_2.

2.4.6 Neuronal induction protocol 6

Based on the neural stem cell expansion and differentiation methods of Svendsen et al[78], Adherent CD34+ cells were cultured in DMEM/F12 medium with B27 medium supplement and

1% L-glutamine, supplemented with an additional 10 nM bFGF, and 10 nM EGF. Cells were incubated at 37°C in 5% CO_2.

2.4.7 Neuronal induction protocol 7

Derived from a differneitation protocol by Shihabuddin et al[79], adherent CD34+ cells were cultured in DMEM/F12 medium with N2 medium supplement, 1% L-glutamine and 1% glucose, supplemented with an additional 10 nM bFGF. Cells were incubated at 37°C in 5% CO_2.

2.4.8 Neuronal induction protocol 8

Adherent CD34+ cells were cultured in DMEM/F12 medium with N2 medium supplement, 1% L-glutamine and 1% glucose, supplemented with an additional 10 nM bFGF. Cells were incubated at 37°C in 5% CO_2 for 7 days, then the medium replaced with DMEM/F12 medium with N2 medium supplement, 1% L-glutamine and 1% glucose. Cells were incubated at 37°C in 5% CO_2.

2.4.9 Neuronal induction protocol 9

Adherent CD34+ cells were cultured in DMEM/F12 medium with N2 medium supplement and 1% L-glutamine, supplemented with an additional 10 nM bFGF, and 10 nM EGF. Cells were incubated at 37°C in 5% CO_2.

2.5 Microglial differentiation

Differentiation of bone marrow derived cells was performed as described by Davaust et al (2006). To induce microglial differentiation, cells were cultured in 50% astrocyte conditioned medium (ACM) and 50% DMEM/F12 and incubated at 37°C in 5% CO_2. Half the medium in culture was replaced every 3-5 days.

2.5.1 Astrocyte conditioned medium

Four day old rat pups were decapitated and their heads placed in dishes with HBSS. The skin was cut to reveal the skull which was cut along the midline and sides and removed. Next, the cerebellum was removed and discarded. The cortex and midbrain were removed and placed in a dish with HBSS (without Phenol red) on ice. Under a dissecting microscope, the hemispheres

were separated and the meninges and midbrain were removed and discarded. The hippocampus was dissected out and the cortices placed in a petri dish in a solution containing 100ml HBSS, 300mg bovine serum albumin (BSA) (Sigma-Aldrich Company Ltd, Gillingham, UK), and 2mg DNAse (Qiagen, Crawley, UK). The hippocampus was physically dissociated with sterile scissors and pipetted up and down with a 1 ml Gilson pipette. The suspension was transferred to a 50ml Falcon tube and centrifuged at 2000rpm for 5 minutes at 4ºC. The supernatant was removed and pellet resuspended in a solution of 20ml HBSS, 600µl BSA, 3.400mg DNAse, and 5mg trypsin. This was passed through a Gilson pipette to remove clumps, then incubated at 37ºC for 5 minutes, then 1ml FBS was added to neutralize the trypsin. The tissue was collected and centrifuged at 2000rpm for 5 minutes at 4ºC. The pellet was resuspended in 10ml HBSS, 30mg BSA, and 0.2mg DNAse and left for 2 minutes to allow heavy clumps to settle and the supernatant replaced into a new Falcon tube. The debris was resuspended in 10ml HBSS, 30mg BSA, and 0.2mg DNAse. After a further 2 minutes to allow heavy clumps to settle, the supernatant was collected. This was repeated once more. The pooled supernatants were centrifuged at 2000rpm for 5 minutes at 4ºC. The resulting pellet was resuspended in MEM with L-valine, passed through a nylon mesh filter, and plated out in tissue culture treated T75 flasks. On day 7, flasks were shaken for 18 hours at 200 on a rocking platform and each flask was split. The medium on the remaining astrocytes was changed and left uninterrupted for 4 weeks. Medium was harvested from the culture after 4 weeks (astrocyte conditioned medium, ACM), filtered, and used in differentiation experiments.

2.6 RNA Isolation

RNA was isolated from newly isolated adherent CD34+ cells and non-adherent CD34+ cells using the RNeasy minikit according to the manufacturer's instructions (Qiagen, Crawley, UK). For each sample to be analyzed, cells were spun down into a pellet and the supernatant removed. For each 100µl of Buffer RLT used, 1µl β-mercaptoethanol (BME) was added. A total 350µl of RLT/BME lysis buffer was added to the cell pellet, and a needle and syringe was used to shred the lysate, and it was resuspended in 70% ethanol. The mixture was applied to an RNeasy spin column and centrifuged for 15sec at 10,000 rpm, and the flow through discarded. 700ul of Buffer RW1 was added to the column, centrifuged, and the flow through discarded. To the column, 80µl of DNAse (Promega, Southampton, UK) was added and incubated for 15min at room temperature. To wash, 350µl Buffer RW1 was added and centrifuged. The column was then washed with 500µl Buffer RPE twice. The RNA was eluted by adding 50µl RNAse free

water (Qiagen, Crawley, UK) to the column, and centrifuged for 1min at 10,000rpm. Eluted RNA quality was assessed using the RNA 6000 Pico Assay (Agilent Technologies) on the Bioanalyser 2100.

2.6.1 RNA salt precipitation

To concentrate RNA in dilute samples, the salt precipitation technique was used. First, 3M sodium acetate (pH5.2) in amounts equal to 1/10th of the volume of the diluted RNA was added to the RNA. Three volumes of 100% ethanol was added, mixed, and stored overnight at -20ºC. The mixture was spun at 13,000rpm for 10min at 4ºC. The supernatant was aspirated and the pellet washed in 70% ethanol and spun down. The ethanol was aspirated and the pellet dried, and resuspended in an appropriate amount of RNAse free water to give the desired concentration.

2.6.2 Agilent 2100 Bioanalyser, RNA 6000 Pico/Nano Assay

Total RNA quality was measured using the RNA 6000 Pico Assay or the RNA 6000 Nano Assay on the Agilent 2100 bioanalyzer (Aglient technologies, Santa Clara, CA, USA). To prepare the gel-dye for the RNA 6000 Pico Chip, RNA 6000 Pico dye concentrate was vortexed for 10sec and spun down. 1µl of RNA 6000 Pico dye concentrate was added to 65µl of filtered gel. The mixture was spun for 10min at 13000g. The RNA Pico chip was loaded using the Chip Priming Station, where 9µl of the gel-dye mix was dispensed into the designated well, and plungered for 30sec. Additional gel-dye mix and the RNA 6000 Pico Conditioning Solution were placed into the appropriate well. 5µl of the RNA 6000 Pico marker was added into all remaining wells. 1µl diluted RNA 6000 ladder was added to the ladder well, and 1µl of each sample was added to appropriate wells. The RNA 6000 Pico chip was analyzed with the Bioanalyzer 2100.

2.7 Microarray small sample preparation protocol

Total RNA extracted from cell samples was prepared for cDNA microarray analysis using the Gene expression small sample protocol 1 as described by the MRC Microarray Centre (http://microarray.csc.mrc.ac.uk).

2.7.1 First round amplification: First strand cDNA synthesis

For each sample, preparation of RNA for microarray analysis began with 100ng of total RNA in a 0.2ml PCR tube, 5pmol T7 Oligod(T) primer (provided by the microarray center), and 0.5µl small sample prep (SSP) diluted Poly A+ Cocktail (provided by the microarray center). This was mixed by pipetting and incubated in a thermal cycler at 70ºC for 6 mins, cooled to 4º for 2 mins, and spun. The master mix for each reaction consisted of 0.375µl RNase free dH_2O, 1µl 5X First Strand Buffer, 0.5µl (0.1M) DTT, 0.375µl (10mM) dNTP Mix (Invitrogen, Paisley, UK), 0.25µl (40U/µl) RNase Inhibitor (Ambion, Warrington, UK), and 0.5µl (200U/µl) Superscript II (Invitrogen, Paisley, UK). This master mix was mixed and spun before adding 3µl to each reaction tube, and incubated at 42ºC in a thermal cycler with a heated lid for 1 hour. The sample was brought up to 70ºC in the thermal cycler for 10 mins to inactivate the Superscript II, spun and cooled to 4ºC before proceeding.

2.7.2 First strand cDNA synthesis

For each reaction, the master mix for this reaction consisted of 22.75µl RNase free dH_2O, 7.5µl 5X Second Strand Buffer, 0.75µl (10mM) dNTPs Mix, 0.25µl (10U/µl) DNA ligase, *E.coli,*, 1µl (10U/µl) DNA polymerase, *E.coli,* and 0.25µl (2U/µl) RNase H (Invitrogen, Paisley, UK) to make up 32.5µl master mix per reaction. This was mixed and 32.5µl was added to each reaction tube from the First Strand Synthesis, mixed by pipetting and spun down. This was incubated at 16ºC in a thermal cycler without a heated lid for 2 hours and placed in ice before proceeding to the clean-up of double stranded cDNA.

2.7.3 Clean-up of double stranded cDNA

The product from the second strand synthesis (37.5µl) was transferred into a clean RNase/DNase free 1.5ml microfuge tube. Each product reaction tube was diluted by adding 82.5µl RNase free dH_2O and mixed by pipetting. Each reaction then received 2µl Glycogen (5mg/ml), 0.6X volumes 5M NH_4OAc (72µl) (Ambion, Warrington, UK), 2.5X volumes cold 100% ethanol (480µl), then mixed by pipetting. This was incubated for 1 hour at -20ºC to precipitate the double stranded cDNA. To collect the cDNA, the reaction tubes for each sample were centrifuged at 14,000 rpm for 20 mins at 4ºC, and the supernatant was gently aspirated with a pipette. The remaining pellet was washed with 800µl of 70% cold ethanol and centrifuged at 14,000 rpm for 20 mins at 4ºC, the supernatant aspirated, and all traces of ethanol removed by air

drying the pellet for 5-10 mins at room temperature. The tubes were then placed on ice to proceed to the in vitro transcription reaction.

2.7.4 In vitro transcription

The reagents used in this step was from the MEGAscript T7 Kit (Ambion, Warrington, UK). For each reaction, the master mix consisted of 4µl RNase free dH_2O, 1µl T7 ATP Solution (75mM), 1µl T7 CTP Solution (75mM), 1µl T7 GTP Solution (75mM), 1µl T7 UTP Solution (75mM), and 1µl T7 10X Reaction Buffer. Then 9µl of the master mix was added to each purified cDNA pellet from the previous step, and mixed thoroughly by pipetting to completely dissolve the pellet. Next, 1µl T7 10X Enzyme Mix was added to each sample, mixed, and spun briefly. This was then incubated at 37ºC for 5 hours in a thermal cycler with a heated lid. For every hour of this incubation, the reactions were gently mixed by tapping the tube and spinning briefly.

2.7.5 Clean-up of amplified cRNA

The reagents used for this step were from the RNeasy Mini Kit (Qiagen, Crawley,UK). Before beginning the reaction the RLT Buffer was prepared by adding 1µl β-mercaptoethanol for each 100µl RLT Buffer to be used. For the *in vitro* transcription, into each reaction tube, 90µl RNase free dH_2O and 350µl RLT Buffer was added and mixed by pipetting. Next 250µl of 100% ethanol was added, the entire mixture was transferred onto a RNeasy Spin Column, and spun at maximum speed for 15 seconds at room temperature. The collection tubes were emptied and 500µl RPE Buffer was added to the column. This was spun at maximum speed for 2 minutes at room temperature, the collection tube replaced with a new one, and spun again for 1 minute at room temperature. The spin column was again transferred to a fresh collection tube. To elute the cRNA, 30µl RNase free dH_2O was pipetted onto the middle of the spin column membrane, incubated for 4 minutes at room temperature, and spun at maximum speed for 1 minute. The cRNA was eluted again by adding a further 20µl RNase free dH_2O onto the middle of the membrane of the spin column, incubated for 4 minutes at room temperature, and spun for 1 minute at maximum speed. The total eluate (~50µl) was taken and pipetted onto the middle of the membrane of the same spin column, incubated for 4 minutes at room temperature, and spun for 1 minute at maximum speed.

An aliquot of the eluate was diluted with RNase free dH_2O and the absorbance at 260nm and 280nm was measured with the NanoDrop device as well as the final yield. Acceptable yields

after this first round amplification was between 1-2µg. Lower yields indicated problems with the reactions. Quality control was measured using the RNA 6000 Pico Assay on the Agilent 2100 Bioanalyser to identify any genomic degradation, product contamination, and measure cRNA quantity.

2.7.6 Second round amplification: First strand cDNA synthesis

The starting amount of cRNA for each sample in this reaction was 1µg cRNA in a total volume of 4µl. The volume was adjusted with RNase free dH_2O as necessary. To each cRNA sample (1µg in 4µl), 1µl Random Primers (0.2µg/µl) (provided by the microarray center) was added, mixed thoroughly, incubated at 70ºC for 10 minutes, then cooled on ice for 2 minutes. For each sample, the master mix for this reaction consisted of 2µl 5X First Strand Buffer, 1µl (0.1M) DTT, 0.5µl (10nM) dNTP Mix, 1µl (200U/µl) SuperScript II (Invitrogen, Paisley, UK), and 0.5µl (40U/µl) RNase Inhibitor (Ambion, Warrington, UK), to make up a total volume of 5µl master mix per sample. To each sample, 5µl of master mix was added to the amplified cRNA/primer mixture at room temperature, mixed, spun briefly, and incubated at 42ºC for 1 hour in a thermal cycler with a heated lid. Next 1µl RNase H (2U/µl) (Invitrogen, Paisley, UK) was added, pipetted, spun briefly, and incubated at 37º for 20 minutes. The sample was then heated to 95ºC for 5 minutes to inactivate the RNase H, then cooled on ice for 2 minutes and spun down. This was kept on ice until the next step.

2.7.7 Second strand cDNA synthesis

To the first strand reaction tubes from the previous step, 2µl (5pmol/µl) T7 Oligo (dT) Primer (provided by the microarray center) was added, mixed by pipetting, and spun briefly. This was incubated at 70ºC for 6 minutes, cooled on ice for 2 minutes, and spun down. For each reaction, the master mix consisted of 43.5µl Nuclease-free water, 15µl 5X Second Strand Buffer, 1.5µl (10mM) dNTP Mix, 2µl (10U/µl) DNA Polymerase I, *E. coli* (Invitrogen, Paisley, UK) for a final volume of 62µl for each sample. Next, 62µl master mix was added to the sample, mixed, and incubated at 16ºC for 2 hours in a thermal cycler without a heated lid.

2.7.8 Clean-up of double stranded cDNA

To each sample reaction tube, 2µl (5mg/ml) glycogen, 45µl (0.6X volumes) NH_4OAc (Ambion, Warrington, UK), 300µl (2.5X volumes) of cold 100% ethanol was added and mixed by

pipetting. The ds cDNA was precipitated at -20ºC for 30 minutes. The samples were then centrifuged at 14,000 rpm for 20 minutes at 4ºC. The supernatant was removed, the pellet washed with 800µl of 70% cold ethanol, and centrifuged at 14,000 rpm for 5 minutes at 4ºC. All traces of ethanol was aspirated and the pellet left to air dry at room temperature for 5-10 minutes.

2.7.9 In vitro transcription

The reagents used for this *in vitro* transcription was from the Enzo Bioarray High Yield RNA Transcript Labelling Kit (Affymetrix, Santa Clara, CA, USA). For each reaction, the master mix consisted of 22µl nuclease free dH_2O, 4µl (10X) HY Reaction Buffer, 4µl (10X) Biotin-labelled ribonucleotides, 4µl (10X) DTT, and 4µl (10X) RNase Inhibitor Mix. Into each reaction tube, 38µl master mix was added to the purified ds cDNA from the previous step and mixed by pipetting. Next 2µl (20X) T7 RNA Polymerase was added and the samples mixed and spun briefly. This was then incubated at 37ºC for 5 hours in a thermal cycler with a heated lid. At each hour of the incubation, the reaction was gently mixed by tapping the tube and spinning briefly.

2.7.10 Clean-up of amplified cRNA

The regents used in this step were from the RNeasy Mini Kit (Qiagen, Crawley, UK). Prior to proceeding, 1µl β-mercaptoethanol was added to each 100µl RLT Buffer to be used. For the *in vitro* transcription, each sample tube from the previous step received 60µl RNase free dH_2O and 350µl Buffer RLT (with β-mercaptoethanol). Next 250µl of 100% ethanol was mixed into the reaction, and the entire sample was then pipetted onto the RNeasy Spin Column and spun at maximum speed for 15 seconds at room temperature. The collection tube was emptied, then 500µl RPE Buffer was added to the column, spun at maximum speed for 15 seconds, the collection tube emptied, and an additional 500µl Buffer RPE added to the column. This was spun at maximum speed for 2 minutes and the spin column transferred to a fresh collection tube and spun for an additional 1 minute. The spin column was transferred to a new collection tube. To elute the cRNA, 30µl RNase free dH_2O was pipetted onto the middle of the membrane of the spin column, incubated at room temperature for 4 minutes, and spun at maximum speed for 1 minute. An additional 20µl of RNase free dH_2O was added to the membrane of the spin column, incubated for 4 minutes at room temperature, and spun at maximum speed for 1 minute.

An aliquot of cRNA eluate was diluted with RNase free dH_2O to assess the quality on the NanoDrop. The absorbance at 260nm and 280nm was measured and the acceptable ratio of the absorbances ranged from 1.9 to 2.1. The final cRNA yield from 1µg cRNA from the first round amplification was between 40-100µg. An aliquot of each sample was run for quality on the RNA 6000 Nano Assay on the Agilent 2100 Bioanalyser as previously described. The minimum concentration was 1.25µg/µl.

2.7.11 Bioinformatics

Analysis of the gene arrays were analyzed using Rosetta Resolver program. This was validated by the Bioinformatics Support Centre using Genespring and R Statistical programme. Further analysis and classifications of biological functions were complete using the Database for Annotation, Visualization, and Integration Discovery (DAVID) (http://david.abcc.ncifcrf.gov) to classify functional annotation clustering using the Gene Ontology terms.

2.8 Immunofluorescence

2.8.1 Immunofluorescence staining of live cells

For immunofluorescence using live cells, cells were rinsed once with modifies eagle's medium (MEM), and placed on ice for the remainder of the staining process. Both primary and secondary antibodies were diluted in 3% bovine serum albumin (BSA) in MEM and incubated for 20 minutes on ice. After 20 mins, excess liquid on the cells was gently removed and the cells then rinsed 3 times for 5 minutes with 3% BSA in MEM. Cells were then incubated in secondary antibody for 30 minutes, washed 3 times, and then fixed in 4% PFA for 10 minutes. Fixed cells were washed twice in PBS for 5 minutes. For nuclear staining, cells were incubated in DAPI 1:1000 for 2 minutes. Cells were again washed 3 times and mounted on glass slides.

2.8.2 Immunofluorescence staining of fixed cells

For immunofluorescence using fixed cells, the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed three times in PBS for five minutes. Cells were then permeabilized and blocked in one step using a solution of 5% normal goat serum and 0.1% Triton-X 100 for 30 minutes at room temperature. The primary antibodies were added immediately afterwards and incubated for one hour at room temperature. The cells were washed 3 times in 5% normal goat serum in PBS for 5 minutes. They were then incubated in secondary

antibody for 45 minutes, washed 3 times in 5% normal goat serum in PBS for 5 minutes. For nuclear staining, cells were incubated in DAPI 1:1000 for 2 minutes. Cells were again washed 3 times in 5% normal goat serum in PBS, then washed once in PBS for 5 minutes. Cells on slides were then mounted with Vectashield (Vector Labs, Peterborough, UK). Cells in 96 well plates were covered with 50µl PBS and immediately analysed with microscopy.

The details of the antibodies used for immunofluorescent staining are detailed below. Immunofluorescent stained cells were visualized on a Zeiss fluorescent microscope and analyzed using the Metamorph and Photocapture programs. Quantification of results was statistically analyzed using Student's t-test.

Table 1. Summary of antibodies used and their working dilutions.

Antibody	Species	Manufacturer	Dilution	Cell type by which antigen is expressed
Anti-nestin	Mouse	Abcam	1:100	Stem cell
Anit-nestin	Mouse	Chemicon	1:100	Stem cell
Anti-Oct4	Mouse	Abcam	1:50	Stem cell
Anti-SSEA3	Mouse	Santa Cruz	1:50	Stem cell
Anti-SSEA4	Mouse	Santa Cruz	1:50	Stem cell
Anti-Cripto1	Mouse	Abcam	1:100	Stem cell
Anti-Sox2	Mouse	Abcam	1:100	Stem cell
Anti-olig2	Mouse	Chemicon	1:100	Early oligodendrocyte
Anti-galC	Rabbit	Chemicon	1:25	Late oligodendrocyte
Anti-O4	Mouse	*	Not diluted	Oligodendrocyte
Anti-A2B5	Mouse	*	Not diluted	Oligodendrocyte
Anti-O1	Mouse	*	Not diluted	Oligodendrocyte
Anti-PSA-NCAM	Mouse	*	Not diluted	Neuronal
Anti-GFAP	Rabbit	Dako	1:500	Astrocyte
Anti-B-III tubulin	Mouse	Chemicon	1:200	Early neuron
Anti-S100β	Mouse	Abcam	1:100	Astrocyte
Anti-Ox42	Mouse	Abcam	1:50	Microglia
Anti-Ox42	Mouse	*	Not diluted	Microglia
Anti-Iba-1	Mouse	Abcam	1:50	Microglia
Anti-CCR1	Rabbit	Abcam	1:300	
Anti-BMPR2	Goat	Santa Cruz	1:100	Early B cell receptor
Anti-CD34	Mouse			Stem cell
Anti-CD68	Mouse	Abcam	1:100	Microglia
Anti-Iba1	Rat	Abcam	1:50	Microglia
Anti-CD80	Mouse	Abcam	1:100	B7.1/co-stimulatory
Anti-HLA-DR	Mouse	BD	1:100	MHCII
Anti-CD133	Rabbit	Abcam	1:100	Stem cell
Anti-TLR4	Goat	Abcam	1:100	Activation marker
Anti-Ki-67	Mouse	Abnova	1:100	Cell cycle
Alexa 488	Rabbit	Invitrogen	1:500	Secondary
Alexa 594	Rabbit	Invitrogen	1:500	Secondary
Anti-mouse IgG whole molecule,	Goat	Sigma	1:100	Secondary against mouse

FITC conjugated				
Anti-rat whole molecule, TRITC conjugated	Goat	Abcam	1:100	Secondary against rat
Anti-goat whole molecule FITC conjugated	Mouse	BD	1:100	Secondary against goat
Anti-rabbit IgG whole molecule, TRITC conjugated	Goat	Sigma	1:100	Secondary against rabbit

*Supernatant antibodies were supplied by laboratory of Prof Richard Reynolds

2.8.3 Fluorescent activated cell sorting

Cells prepared for FACS analysis were labelled using the Fix and Perm® cell labelling kit (Caltag Laboratories, Buckingham, UK). For labelling of extracellular antigens, cells were incubated with the appropriate antibody for 20 minutes at room temperature. They were then washed in 3ml PBS and centrifuged at 1800rpm for 3 minutes. Cells were then incubated in Reagent A for 15 minutes at room temperature, washed in 3ml PBS, centrifuged at 1800rpm for 3 minutes, and re-suspended in 300µl PBS for analysis. For intracellular antigens, cells were first fixed with Reagent A for 15 minutes and washed as above. Then cells were incubated with the permeabilization Reagent B and the appropriate amount of primary antibody for 15 minutes at room temperature. The cells were then washed with 3ml PBS and centrifuged at 1800rpm for 3 minutes. Cells were then incubated with Reagent B and the appropriate amount of secondary antibody for 15 minutes at room temperature, washed, centrifuged, and re-suspended in 300µl PBS. Labelled cells were analyzed with the BD FACSCalibur (BD Bioscience) with Cell Quest Pro software.

2.9 Cell function

2.9.1 Lipopolysaccharide stimulation of cytokine production

To measure cell activation, cells were stimulated with lipopolysaccharide (LPS) (Axxora, Nottingham, UK). LPS was added to culture medium at a working concentration of 1µl/ml and left in culture medium for 8, 16, or 32 hours, then the cells and supernatants were collected for analysis.

2.9.2 Measurement of cytokines

2.9.2.1 Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) were performed according to manufacturer's protocols, using commercial kits for specified cytokines (R7D Systems, Abingdon, UK). Briefly, supernatants to be assayed were collected, spun down, and filtered to remove contaminating cells. After reconstitution and preparation of reagents, a 96well plate was prepared first by adding 50µl of Assay Diluent to each well. Next, 200µl of standard, control, or sample supernatant was added to appropriate wells and incubated for 2hrs. The plate was washed four times with Wash Buffer, and blotted before 200µl of Antibody Conjugate was added to each well, incubated for 1hr, and washed four times. 200µl of Substrate Solution was added and incubated for 20mins at room temperature in the dark. To stop the reaction, 50µl of Stop Solution was added to each well. Optical density was determined using an OPTImax Tunable microplate reader with absorbance set at 450nm.

2.9.2.2 Human cytokine array

All reagents used in the human cytokine array constituted a component of the Proteome Profiler Human Cytokine Array Panel A Array Kit (R&D Systems, Minneapolis, MN, USA). Into each well of the 4-well multi-dish provided, 2ml Array Buffer 4 and array membranes were placed and the dish incubated for 1 hour at room temperature. Samples were prepared by adding 1ml of each sample to 0.5ml of Array Buffer 4. Next, 15µl of reconstituted Cytokine Array Panel A Detection Antibody Cocktail was added to each prepared sample and incubated for 1 hour at room temperature. After one hour, Array Buffer 4 was aspirated off the membranes in the multi-dish and the sample mixture was added to the membrane. This was incubated overnight at 2-8ºC on a rocking platform. Each membrane was removed and washed in 20ml of 1X Wash Buffer for 10 minutes on a rocking platform shaker. Membranes were washed three times. The streptavidin-HRP provided was diluted in Array Buffer 5, and 1.5ml of diluted streptavidin-HRP was added into each well of the multi-dish, and the membranes placed into each well of the dish. This was incubated for 30 minutes at room temperature and washed three times as previously described. Each membrane was removed from the wash container and incubated with a chemiluminescent detection reagent and placed protein side up in an X-ray film cassette and exposed to film for 1-5 minutes.

2.9.3 Migration assays

Migration assays were conducted using polycarbonate membrane inserts (Nunc, Roskilde, Denmark) which were used in standard 24 well cell culture plates. Appropriate cells were prepared and seeded into the bottom of the 24 well plates at 5 x 10^3 cells per well. Polycarbonate membrane inserts were placed above the plated cells and 500 µl of stem cell suspension (1x10^5 cells/ml) was placed within the insert. After 5-8 hours, the inserts were discarded and the cells in the 24 well plates were fixed and analysed.

2.9.4 Invasion assays

Invasion assays were conducted using the BD BioCoat™ Matrigel™ Invasion Chamber (BD Bioscience, Oxford, UK). The chambers were allowed to rehydrate using PBS for 2 hours at 37ºC. After rehydration, either cancer cell lines were plated into the bottom well, or conditioned medium or culture medium was put in the bottom well. Cell suspensions were then prepared at seeding densities of 1x10^5 cells/ml and placed within the transwell. The entire chamber system was incubated at 37ºC for 24 hours. After incubation, the non-invading cells were removed from the upper surface of the Matrigel membrane by using a cotton tipped swab to scrape the cells off the membrane with firm but gentle pressure. This was repeated with a swab moistened with culture medium. Cells on the lower surface of the membrane were fixed with 100% methanol for 30 seconds and stained with 1% Toluidine blue for 2 minutes. The membranes were then washed in water and air dried. To count the cells, the membranes were removed from the insert housing using a scalpel and mounted on a microscope slide. To calculate the percent invasion of cells, the following formula was used

% invasion = (mean # stem cells invading through Matrigel insert membrane/ mean # cells migrating through control Matrigel insert membrane) x 100

The invasion index = (% invasion stem cell/ % invasion control cell)

2.9.5 Mixed Lymphocyte Reaction

Two samples of peripheral blood mononuclear cells (PBMC) were derived from whole blood of excess clinical samples and suspended in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% FCS and 1% penicillin/streptomycin. The PBMCs were then diluted to a final concentration of 5 x 10^5 cells/ml (100µl = 5 x 10^4 cells). The stimulator cells were then irradiated to 60cGy. In a 96 well round bottom plate, 100µl of medium was dispensed into three wells as background control, 100µl of the responder cell suspension was dispensed into three

wells, 100µl of the stimulator cell suspension was dispensed into three wells, 100µl of each responder and stimulator cell suspension was dispensed into three wells. An additional 100µl of medium was added to ensure there was 200µl in each well. The assay was incubated at 37ºC, 5% CO_2 for five days. On day five, each well in the assay was pulsed with 25µl of tritiated ^{3}H-Thymidine (1µCi). The assay was incubated for an additional 18 hours and the cells harvested in a cell harvester in filter-mat and the scintillaion results read in the beta reader.

2.10 DNA replication timing

2.10.1 BrdU labeling

Replicating cells were grown in culture until they reached 80% confluence. After a change of culture medium, 1.5 mg/ml BrdU (Beckton Dickson, Oxford, UK) per 10 ml culture medium was added (final concentration of 50µM) and incubated at 37ºC for 90 minutes. Cell cultures were trypsinized until a single-cell suspension was achieved

2.10.2 Flow cytometric sorting

The ethanol fixative was washed out with cold PBS Ca^2-, Mg^2- by centrifuging at 1700 rpm for 10 minutes at 4ºC. The cells were washed twice. After the last wash, 1ml of staining buffer per 10^6 cells was added. Each 10ml of staining buffer was made up of 9.4ml buffer 40/8/21, 0.5ml (1mg/ml) propidium iodide (PI), 0.1ml 5% NP-40, 1ml (10mg/ml) RNasaA. The cells were declumped by squeezing them through a G25 needle with a 1ml syringe and left for 10 minutes on ice in the dark. After staining, the cells were filtered through a mesh to remove clumps, and placed in a new tube for cell sorting. Cell sorting was conducted as a service by the MRC FACS suite. Cells were sorted and fractionated according to cell cycle status, resulting in six fractions corresponding to cell cycle stage G0/G1, S1, S2, S3, S4, G2/M. Each fraction contained between 20,000 -50,000 cells.

After sorting, each fraction was resuspended in 4ml of lysis buffer which consisted of 800µl 5M MaCl, 80µl 0.5M EDTA pH8, 200µl 1M Tris-Cl pH8, 100µl 20% SDS, 2.46ml dH_2O, 160µl (10mg/ml) proteinase K, 200µl sonicated salmon sperm. The cells were incubated in lysis buffer for 2 hours at 50ºC.

2.10.3 DNA extraction and precipitation

After incubation in lysis buffer, each tube of fractionated cells received 7.5ng/10^4 cells of control Drosoph BrdU labelled DNA, 400µl Phenol, shaken for 1 minute, then centrifuged at maximum speed for 5 minutes. This resulted in a separation of the organic and aqueous phases of the solution, and the organic phenol layer was removed. Next, 400µl of Chloroform was added, shaken for 1 minute, and spun at maximum speed for 5 minutes. The aqueous phase of the solution was removed, transferred to a new collection tube, and 800µl ice cold 100% ethanol was added. This was incubated at -20ºC for 2 hours to allow the DNA to precipitate. The samples were then centrifuged at maximum speed for 30 minutes at 4ºC, and the ethanol was aspirated. An additional 1ml of 70% cold ethanol was added and the samples were centrifuged at maximum speed for 30 minutes at 4ºC. The ethanol was aspirated and the pellets were resuspended in 480µl TE buffer pH8 and incubated at 37ºC for 30 minutes. Next, 20µl sonicated salmon sperm DNA (10mg/ml) was added and the samples were incubated for an additional 30 minutes at 37ºC.

2.10.4 BrdU Immunoprecipitation

Samples were each sonicated for 45 seconds at 30 Hz.

Sonicated samples were heated to 95ºC for 5 minutes, transferred to ice for 2 minutes, and spun down. Next, 50µl adjuster buffer and 80µl anti-BrdU (Becton Dickinson, Oxford, UK) were added, and the samples incubated for 20 minutes on a rotating platform. Then 35µg anti-mouse IgG (Sigma-Aldrich, Dorset, UK) was added, samples incubated for 20 minutes on a rotating platform, and spun at maximum speed for 20 minutes at 4ºC. The supernatant was aspirated, the pellets washed with 1ml wash buffer, and centrifuged for an additional 20 minutes at 4ºC. The wash buffer was aspirated, the BrdU labelled DNA pellet resuspended in 300µl lysis buffer, and incubated for 2 hours at 50ºC.

2.10.5 DNA purification

This step was completed using components of the Qiagen Gel Extraction Kit (Qiagen, Crawley, UK). First, 900µl Buffer was added to the samples and mixed. The solutions were transferred to mix columns and spun at maximum speed for 1 minute. The columns were then washed with 750µl Buffer PE, spun for 1 minute, the collection tubes emptied, and the columns spun again for 1 minute 30 seconds. To elute the DNA, TE buffer pH8 was used at 1µl per 150-200 cells in the original sorting fraction. Half the volume of TE was placed on the spin column, incubated for 1

minute, spun at maximum speed for 1 minute, and repeated. The DNA was then stored at 4ºC until SybrGreen analysis.

2.11 Real-time quantitative PCR

Quantitative SyberGreen PCR was conducted as described by the manufacturer's protocol (Sigma-Aldrich, Dorset, UK). Briefly, a master mix was made consisting of 15µl SyberGreen (Sigma-Aldrich, Dorset, UK) and 11.2µl H_2O for each replicate. For each gene, 0.9µl forward primer and 0.9µl reverse primer was added. Finally, 2µl cDNA of the appropriate sample was added. Analysis was conducted using an Opticon II plate reader and its accompanying software.

Chapter 3 Characterization of adherent CD34+ cells

Chapter summary

In this chapter it is demonstrated that adherent CD34+ cells are transcriptionally distinct from their non-adherent CD34+ cell counterparts. Analysis of genes associated with stem cell function support previous data that adherent CD34+ cells are more primitive than non-adherent CD34+ cells. DNA replication timing analysis various stem cells revealed that adherent CD34+ cells replicate genes associated with pluripotency in a similar pattern to ES cell controls, further validating their primitive state. The following results are revealed and discussed in this chapter:

- cDNA microarray analysis of transcriptional expression
- Transcriptional analysis of stem cell associated genes
- Transcriptional analysis of cell surface receptors
- DNA replication timing of stem cells

3.1 Transcriptional profiling

The detection of various stem cell populations in adult humans has lead to studies which have attempted to define and subsequently to isolate putative stem cell populations *in vitro*. In the haematopoietic system, there has been a search for markers which are selectively expressed by primitive haematopoietic stem/progenitor cells. They include markers of efficient efflux pumping of Rhodamine 123 or Hoescht dyes, isolation by immune antigens such as CD34 and CD133, and cell sorting of the side-population of haematopoietic stem cells. There have been efforts to understand molecular mechanisms and genetic regulation of the stem cell state by analyzing transcriptional profiles of stem cell populations using cDNA microarrays.

Expression profiling provides information about the genes that are expressed by a particular cell type and their relative abundance. Transcriptional and genetic profiling of stem cell populations has not yet unequivocally identified a common signature of 'stemness' outside the context of embryonic stem cells, and a definition of the molecular nature of pluripotency in adult derived

stem cells remains ambiguous. Transcriptional information was scanned for those documented to be involved in stem cell function, including genes involved in quiescence and cell cycle, transendothelial migration, and adhesion to bone marrow which are likely to be relevant to clinical stem cell application.

Microarrays are typically used to screen for large numbers of genes expressed in a tissue of interest. Here the Affymetrix U133 plus 2 whole human genome array was used to compare the entire genome of adherent CD34+ cells and non-adherent CD34+ cells. To use the Affymetrix U133 plus 2 array platform, RNA was first isolated from the target tissue, processed and amplified, then added to the chip surface for hybridization. The chip consists of thousands of probes, each of which is a distinct DNA sequence. The basic principle is that of selective binding of nucleic acid molecules to complementary sequences. When the tissue-derived pool of RNA is added to the surface of the chip, a highly sensitive search and sorting of molecular partners, probes with their complementary targets, is performed. Each single location on the array is made up of large numbers of probes (about 10 million), thus enabling quantification of expression. The larger the number of copies of a particular nucleic acid sequence in a sample, the more the binding to the particular matching probes on the chip. Fluorescence-based detection schemes use lasers to excite fluorescently labeled RNA molecules in order to quantify bound RNA transcripts.

3.1.1 DNA replication timing

DNA replication timing can be used to indicate whether particular cell types are more or less likely to differentiate into one or another lineage. The replication timing of genes depends on chromatin state and correlates with histone acetylation, and early replication of a gene does not necessarily reflect gene transcription[49]. Early replication of genes during S phase of the cell

cycle is considered to be a feature of transcriptionally active regions of the genome, indicating chromatin accessibility, whereas late replication is characteristic of inactive gene regions and constitutive heterochromatin[46]. During development, pluripotent stem cells give rise to lineage-restricted progenitors from which more tissue restricted cells are derived[50]. Thus, in pluripotent ES cells, there is replication of lineage-associated genes early in the S phase, suggesting multilineage potential and pluripotency[46,50].

Based on previously observed differences in function[26], including adhesion and proliferation, it is reasonable to hypothesize that adherent CD34+ cells are genetically distinct from their non-adherent CD34+ cell counterparts. The aims of this study were to identify genes which are more or less highly expressed by adherent CD34+ cells relative to non-adherent CD34+ cells, and to identify genes which may play a role in stem cell function within this gene subset. DNA replication timing was also conducted to determine if the chromatin state of adherent CD34+ cells and non-adherent CD34+ cells allows the active transcription of pluripotent stem cell-associated genes.

3.2 Results

3.2.1 cDNA Microarray analysis of gene expression

3.2.1.1 Quality control

Total RNA was extracted from adherent CD34+ cells and corresponding non-adherent CD34+ cells from three blood samples. Total RNA was subjected to cDNA synthesis, amplification, double strand cDNA clean up, and cRNA synthesis and clean-up. A number of quality control measures were performed throughout the procedure in order to assess the quality of the total RNA, cDNA, and cRNA samples prior to hybridization onto chip arrays. The strict quality control measures were required by the MRC Microarray Centre. These requirements comprised (*i*) the ratio of the absorbance at 260nm and 280nm (A_{260}/A_{280}) to be within a range of 1.9 and 2.1; (*ii*) a cRNA yield after first round amplification from 100ng total RNA between 1-2µg; (*iii*) bioanalyser traces of total RNA, first round cRNA and second round cRNA with no ribosomal bands.

Quality control entailed analysing total RNA using the Agilent 2100 Bioanalyser (Agilent Technologies). The Bioanalyser is a microfluidics-based platform which uses electrophoretic separation to create an electropheretogram of nucleic acid samples. 250ng total RNA was loaded onto a Nano Assay LabChip (Agilent Technologies) to create electropheretograms of total RNA from the samples. The process was repeated after both the first round of amplification and the second round of amplification to create a gel electropheretogram of cRNA products. Poly A+ controls were used to monitor genomic stability during the processing of samples. All quality control measures were assessed by staff at the MRC Microarray Centre before proceeding to the next stage. Figure 4 shows Bioanalyser traces which verify the good quality total RNA extraction and successful cRNA synthesis and yield.

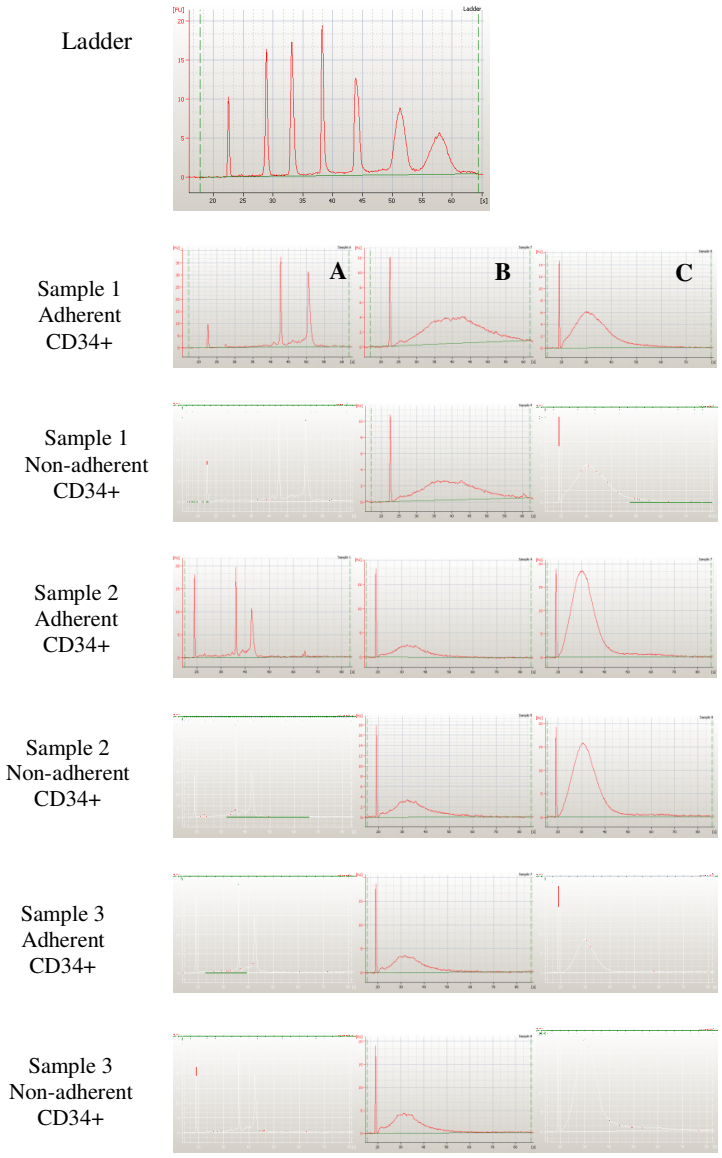


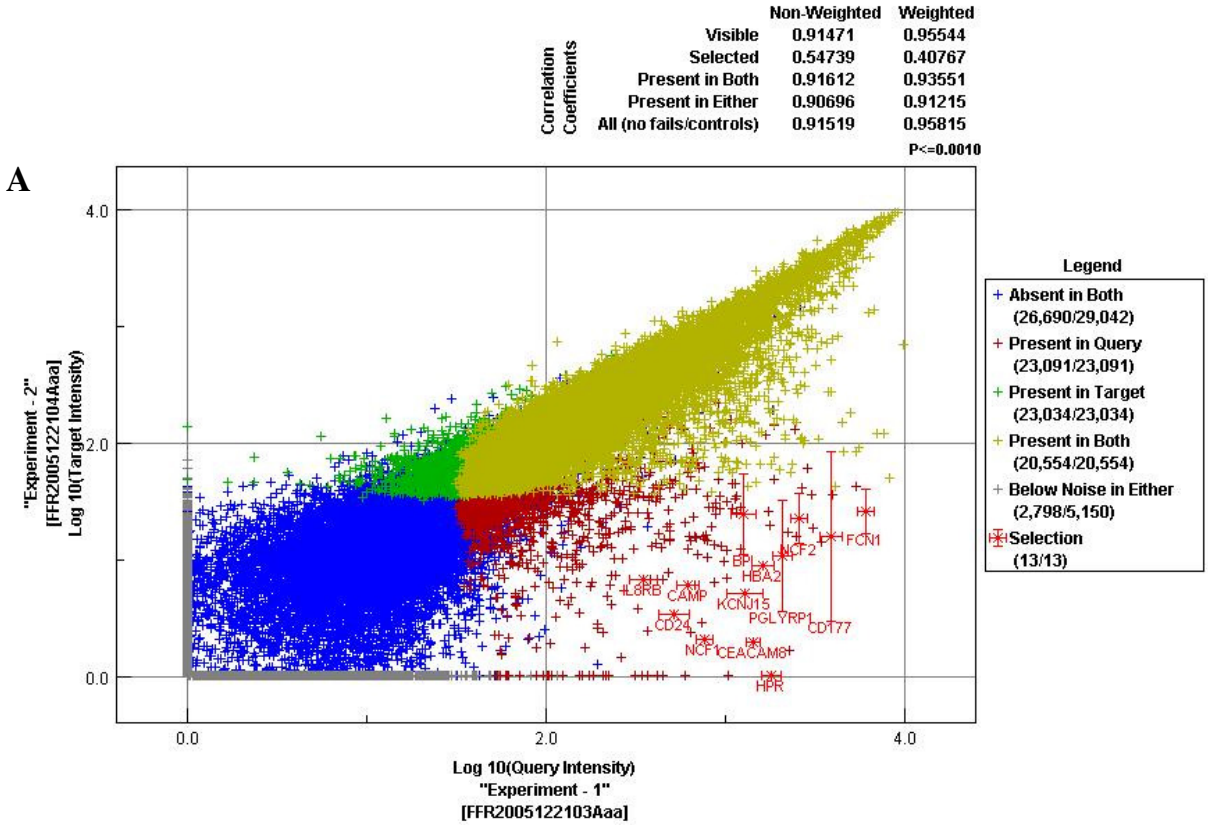
Figure 4. RNA quality control. To ensure the highest quality of the total RNA isolated from donor CD34+ cells, total RNA analyzed with the Bioanalyser 2100 (Agilent) for contamination, degradation, and proper 260/280 ratio. Samples were analyzed to ensure no contamination from PCR product. The cDNA products after the first and second rounds of amplification were examined for quality and quantity. These traces show no degradation of total RNA (A), proper amplification and quality after the first round amplification (B), and adequate amplification and quality after the second round amplification (C).

3.2.1.2 Adherent CD34+ cells are genetically distinct from non-adherent CD34+ cells

To determine differences in gene expression between adherent CD34+ cells and non-adherent CD34+ cells, CD34+ cells were isolated from the mobilized peripheral blood of samples. Gene trend analysis was done using Rosetta Resolver. Transcripts that were differentially expressed by ≥2 fold by adherent CD34+ population relative to non-adherent CD34+ cells, and were within a 95% confidence level by Student's t-test were defined as significantly differentially expressed. The upregulation of some genes and downregulation of others is important because of positive and negative influences on cell function. Figure 5a shows a graphic representation of resulting gene expression profiles for each sample. Each spot represents the relative expression of each gene of both adherent CD34+ and non-adherent CD34+ expression. Adherent CD34+ cell expression is on the x-axis, and non-adherent CD34+ cell expression is on the y-axis. Each spot represents one of 47,000 gene probes on the array. Blue spots represent those genes not expressed by either CD34+ population. Red spots are genes more highly expressed by adherent CD34+ cells by ≥2 fold relative to non-adherent CD34+ cells. Green spots are genes expressed by non-adherent CD34+ cells by ≥2 fold relative to adherent CD34+ cells. Yellow spots are those not statistically significantly more highly expressed by one population or the other. Filtering by Rosetta Resolver revealed that 562 genes were consistently more highly upregulated in adherent CD34+s, and 338 genes were more highly upregulated in non-adherent $CD34^+$ cells (Figure 5b and c). Consequently, genes which were upregulated by non-adherent CD34+ cells were downregulated by the adherent CD34+ cells. The adherent CD34+ cells and non-adherent CD34+ cells expressed equivalent levels of 10,669 genes. The complete gene expression data are available at the MRC Microarray Centre, Imperial College London (http://mimironline.csc.mrc.ac.uk/ experiment EXP_CFMG_1006_01). The final results were validated and re-analysed using the R software by the MRC Bioinformatics support center. The re-analysis of results by professionals with different software strengthens the validity of the data. Only consistent results amongst the three samples were used.

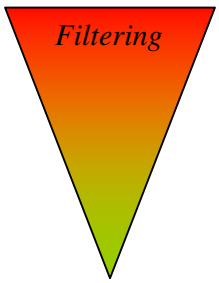
3.2.1.3 Intensity subsets of genes

Following analysis of gene expression using Rosetta Resolver software, the resulting molecular profiles (562 genes preferentially expressed by adherent CD34+ cells, and 338 genes preferentially expressed by non-adherent CD34+ cells) were divided into functional categories as defined by the Gene Ontology database (www.geneontology.org). It was presumed that adherent CD34+ cells are a primitive stem cell population, and subsequent analysis focused primarily on genes which are involved in quiescence, cell cycle, homing and adhesion which are likely to be relevant to adherent CD34+ cells' suitability for clinical application.



47,000 probes on the Affymetrix U133 plus 2 array

B



562 genes more highly expressed by adherent CD34+ cells

C

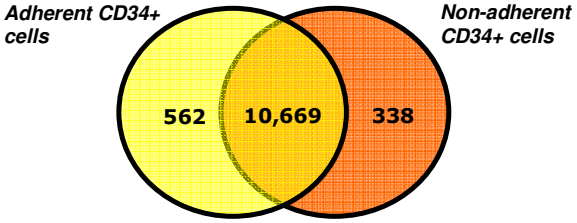


Figure 5. Differential gene expression by adherent CD34+ cells and non-adherent CD34+ cells. A representation of the relative gene expression by adherent CD34+ and non-adherent CD34+ cells as analysed with Rosetta Resolver (A). Transcripts were filtered from 47,000 possible expressed genes by identifying those which were expressed by ≥2 fold by one population relative to the other and were within a 95% confidence level by Student's t-test. Adherent CD34+ cells more highly expressed 562 genes by ≥2 fold relative to non-adherent CD34+ genes (B). The Venn diagram (C) depicts the number of genes differentially expressed in adherent and non-adherent CD34+ cells. There were 562 genes significantly upregulated in plastic adherent CD34+ cells which were not highly expressed by non-adherent CD34+ cells. There were 338 genes significantly upregulated in the non-adherent fraction of CD34+ cells that were not highly expressed in the plastic adherent fraction. The number of genes expressed by both populations was 10,669.

3.2.1.4 Stem cell genes

Genetic and transcriptional profiling of stem cells has revealed genes which are characteristic of stem cells. Although there are no discrete stem cell genes, some genes are associated with pluripotent stem cells, specifically *POU5F1* (Oct4), *NANOG, ZFP42* (Rex1), *SOX2*, *SSEA1, SSEA3,* and *SSEA4*[80-82]. The microarray data was examined for expression of these pluripotent stem cell-associated genes, and the expression of *POU5F1*, *NANOG, ZFP42*, *SOX2*, *SSEA1, SSEA3,* and *SSEA4*[80-82] were not high enough in either adherent CD34+ cells or non-adherent CD34+ cells to be detected by the microarray.

To determine if adherent CD34+ cells and non-adherent CD34+ cells express low levels of *SOX2, NANOG, ZFP42* (Rex1) and *POU5F1* (Oct4), Syber green real-time PCR was conducted and relative expression in between the populations was compared (Figure 6). Adherent CD34+ cells expressed relatively higher levels of *SOX2, NANOG,* and *POU5F1* than non-adherent CD34+ cells, but non-adherent CD34+ cells expressed slightly higher levels of *ZFP42* than adherent CD34+ cells. This was an average of only two experiments and is not very convincing. Although the trend seems to indicate that adherent CD34+ cells may express slightly higher levels of the genes in question, there are not enough replicates to indicate significance. These real-time PCR results indicate that adherent CD34+ cells express some of the genes closely associated with pluripotent stem cells.

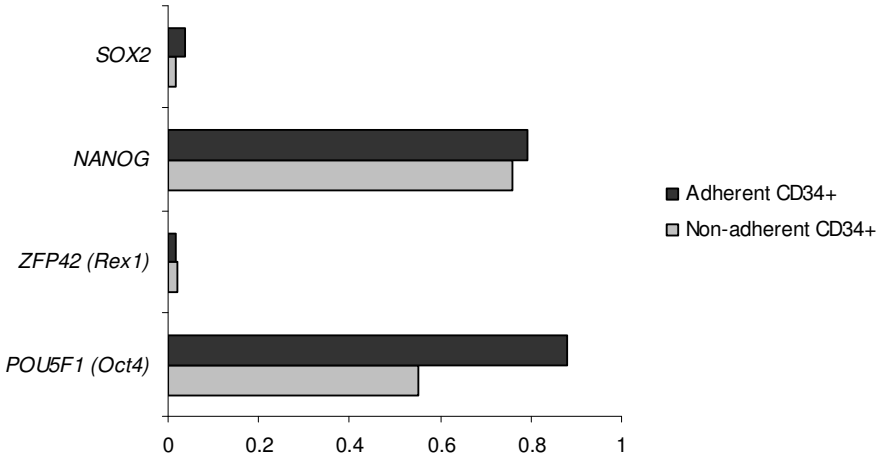
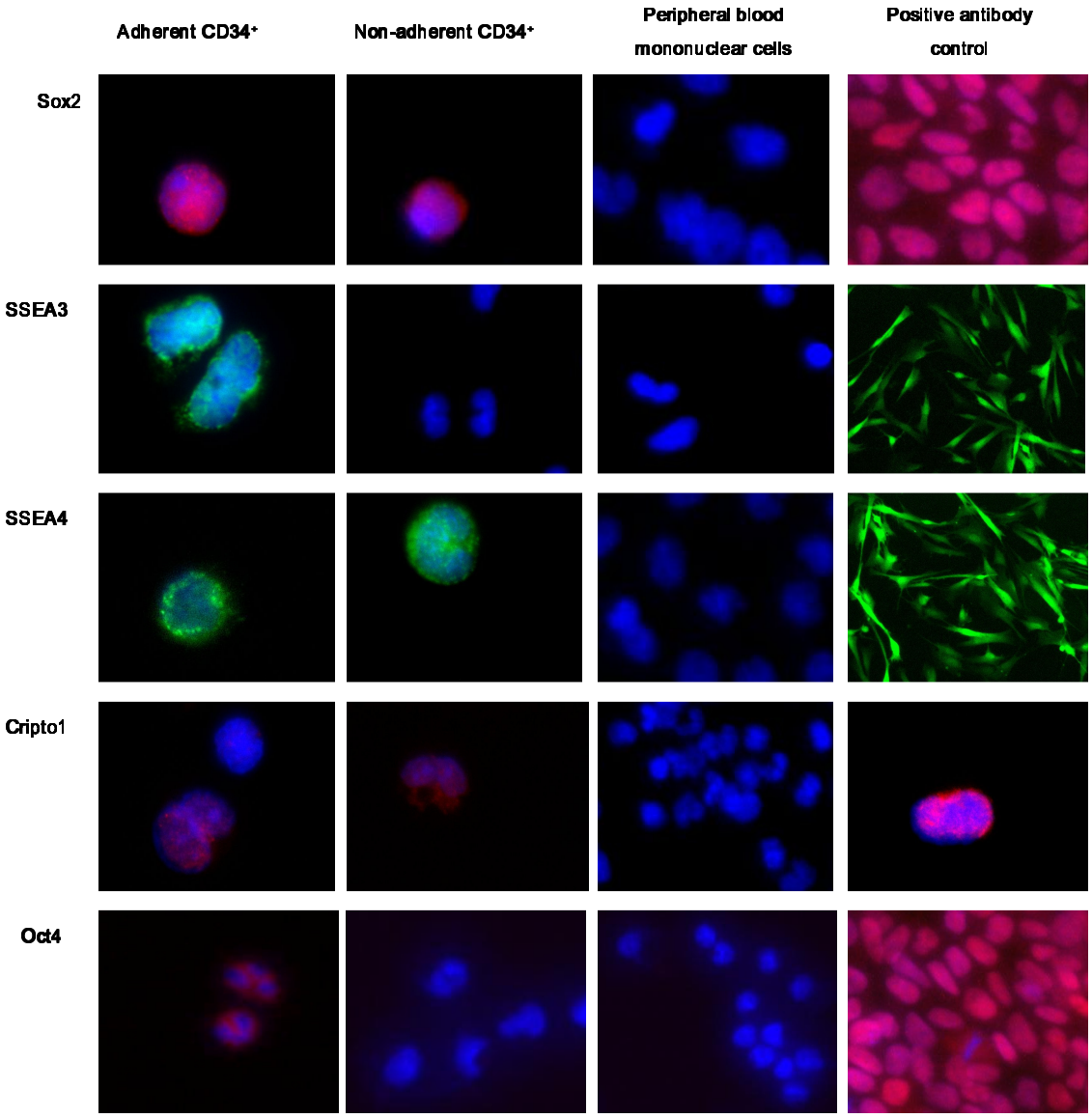


Figure 6. Real-time qPCR for pluripotency associated genes in adherent CD34+ cells and non-adherent CD34+ cells. Relative expression of *SOX2, NANOG, ZFP42* (commonly known as Rex1), and *POU5F1* (commonly known as Oct4) was compared between adherent CD34+ and non-adherent CD34+ cells were measured with Syber green real-time PCR. Levels of expression were normalized to levels of housekeeping genes *GAPDH* and *HPRT*.

To confirm the expression of stem cell associated genes on the protein level, immunofluorescence microscopy for stem cell associated antigens Sox2, SSEA3, SSEA4, Cripto1, and Oct4 was conducted (Figure 7). The results revealed that 63% ± 8% of adherent CD34+ cells expressed Oct4 protein, relative to 0% ± 3% of non-adherent $CD34^+$ cells. Developmental stem cell marker Sox2 was expressed in 67% ± 12% of adherent CD34+ cells versus 31% ± 7% of non-adherent CD34+ cells. SSEA3 was expressed on 62% ± 11% of adherent CD34+ cells, but only on 8% ± 4% of non-adherent CD34+ cells. SSEA4 was not discriminatory between the two populations as it was expressed by 88% ± 13% of adherent CD34+ cells and by 83% ± 9% of non-adherent CD34+ cells. Cripto1, a transcription factor

associated with embryonic stem cells also did not discriminate between adherent and non-adherent CD34+ cells, and was expressed by 34% ± 6% of adherent CD34+ cells (Cripto1^{dim}) and 31% ± 9% of non-adherent CD34+ cells (Cripto1^{dim}). The final panel of candidate markers specific for adherent CD34+ cells are comprised of Oct4, Sox2, SSEA3, and BMPR2 (Figure 7b). The analysis of each marker was conducted on at least three biological samples, but markers were not necessarily immunostained in parallel in all instances. These immunofluorescence results did not agree with the previously conducted real-time qPCR results which indicated that both adherent CD34+ cells nor non-adherent CD34+ cells express relatively low amounts of *SOX2* and *ZFP42*. The real-time qPCR results also indicate that non-adherent CD34+ cells express *POU5F1*, but relatively much less than adherent CD34+ cells. This suggests that non-adherent CD34+ cells might transcribe *POU5F1*, but that it is not translated to the Oct4 protein.

A


Adherent CD34+
Non-adherent CD34+
Peripheral blood mononuclear cells
Positive antibody control
Sox2
SSEA3
SSEA4
Cripto1
Oct4

B

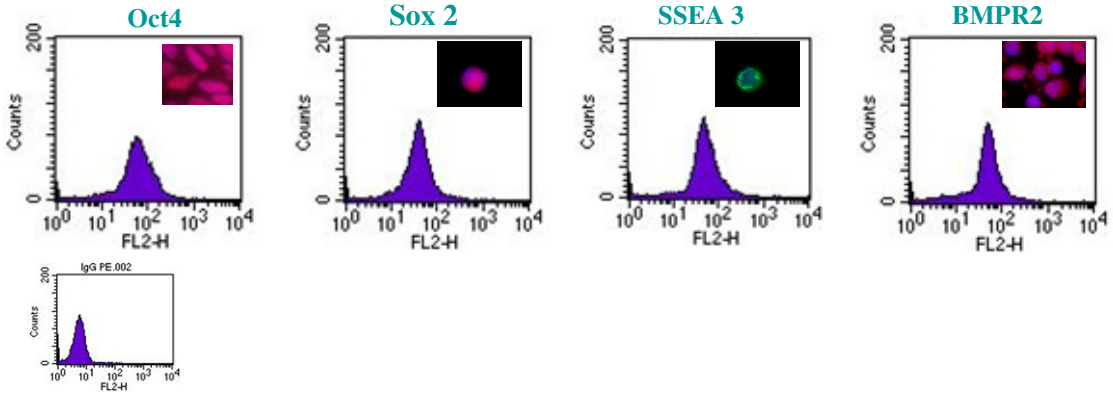

Oct4
Sox 2
SSEA 3
BMPR2
Counts
FL2-H
IgG PE.002

Figure 7. Protein expression of stem cell associated markers by adherent CD34+ cells. To further validate the stem cell qualifications of adherent CD34+ cells, immunofluorescent microscopy and FACS analysis were used to detect if a panel of markers known to be associated with stem cells is expressed by adherent $CD34^+$ cells. We immunostained for Oct4, Sox2, SSEA3, SSEA4, Cripto1 and BMPR2 and found that adherent CD34+ cells more highly express Oct4, Sox2, SSEA3, and BMPR2 relative to non-adherent CD34+ cells. The frequency of protein expression was analysed with microscopy (**A**) and validated with FACS (**B**). Antibodies were IgG monoclonal antibodies, and the FACS histogram represents the IgG control for all antibodies used for FACS in panel **B**. Positive antibody controls for Oct4, Sox2, and Cripto1 were human embryonic stem cells provided by Dr. Wei Cui, and positive antibody controls for SSEA3 and SSEA4 were foetal mesenchymal stem cells

3.2.1.5 Cell Cycle Regulation

Compared to non-adherent CD34+ cells, plastic adherent CD34+ cells expressed significantly higher levels of 6 genes involved in the negative regulation of progression through the cell cycle*, MTSS1, GAS7, CDKN1C* (p57), *GAS2L1, PHB,* and *FOXN3* (also known as CHES1). The absence or downregulation of metastasis suppressor 1 (*MTSS1* or *MIM*) is associated with bladder and hepatocellular carcinomas[83,84]. *GAS7* is predominantly expressed in the brain and its expression is associated with differentiating, non-proliferating cells[85]. *CDKN1C,* commonly known as p57, is correlated with cell cycle arrest in side-population haematopoietic stem cells[86]. *FOXN3* encodes for checkpoint suppressor 1 which induces cell cycle arrest at G_1 and G_2[87]. *PHB* (prohibitin) regulates E2F1 function and binds to Rb family members[88].

The GO mapping analysis of cell cycle regulatory mechanisms revealed 23 genes that are more highly expressed by non-adherent CD34+ cells and less by adherent CD34+ cells which are involved in the regulation of the cell cycle. These genes are *TOP3A, ANAPC5, PAPD5, EML4, NUSAP1, RAD54L, CIT, TPX2, CDC25A, CDC25C, CCNA1* (cyclin A1)*, NEK4, APBB2, GTSE1, CHAF1B, MCM2, UHRF1, NERCC1, CCNE1* (CyclinE1)*, SQSTM1* (p60)*, E2f1, KIF15,* and *MYC*. Cyclin A1, which is encoded by *CCNA1*, binds to cell cycle regulators Rb family proteins and p21 family proteins[89]. MYC is a transcription factor linked to cell cycle regulation, proliferation, and cell fate decision[90].

Conversely, these proliferation-promoting genes are downregulated in the adherent CD34+ cells, indicating a potential difference in cell proliferation kinetics between adherent CD34+ and non-adherent CD34+ cells.

In addition to cell cycle promoting genes, non-adherent $CD34^+$ cells overexpressed 6 genes involved in DNA replication. These were *PRIM1, RRM2, CHAF1B, MCM2, POLG*, and *POLE*. In addition, non-adherent CD34+ cells differentially expressed CD135 (fms-related tyrosine kinase 3) which, when co-expressed with CD34, may represent early cycling, but not quiescent stem cells. CD135 was not significantly upregulated by plastic adherent CD34+ cells, further suggesting that plastic adherent CD34+ cells may be quiescent stem cells. This observation supports the hypothesis that plastic adherent CD34+ cells may be suspended in G0, while non-adherent CD34+ cells are mitotically active.

To verify the microarray analysis, a selection of cell cycle genes was chosen and their relative expression was compared using SybrGreen real-time PCR. Real-time PCR was conducted with cDNA synthesised from the same total RNA isolated for microarray experiments above. The data below show the average results from three samples (Figure 8). The cell cycle data produced by the microarray imply that adherent CD34+ cells may be slowly cycling or arrested in G_0, whilst the non-adherent CD34+ fraction may be actively cycling. Real-time qPCR of 9 cell cycle-regulating genes, identified by microarray, was conducted. *GAS2L1, PHB, CHES1,* and *p57* are involved in the negative regulation of the cell cycle, while *MYC, CDC25a, CDC25c, MCM2,* and *CCNA1* (CyclinA1*)* promote cell cycle progression (Figure 3). The genes involved in the negative regulation of the cell cycle, *GAS2L1, PHB, CHES1,* and *p57,* were more highly expressed by ≥2 fold by the adherent CD34+ cells relative to the non-adherent CD34+ cells. The genes involved in cell cycle progression *MYC, CDC25a, CDC25c, MCM2,* and *CCNA1* (CyclinA1) were more highly expressed by the non-adherent CD34+ cells by ≥2 fold.

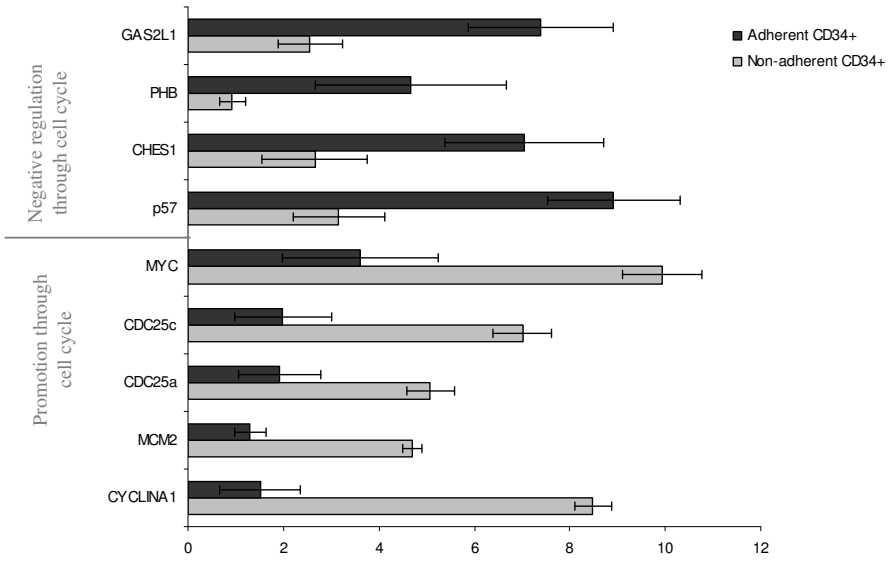


Figure 8. Real-time qPCR relative expression of cell cycle regulation genes. SybrGreen real-time PCR was conducted on a subset of genes identified by the Affymetrix U133 plus 2 gene array to validate gene expression in both adherent CD34+ cells and non-adherent CD34+ cells.

3.2.1.6 Adherent CD34+ cells lack Ki-67

In addition to molecular screening and characterization, Ki-67 expression was investigated at the protein level. Subpopulations of CD34+ HSCs have been demonstrated to lack Ki-67 expression, indicating that they are suspended in Go[91]. The Ki-67 protein is present during the G_1, S, G_2, and M phases of the cell cycle and is not expressed during G_0 phase[92]. Thus, cells negative for Ki-67 are assumed to be arrested in G_0. Only 24% of plastic adherent CD34+ cells were Ki-67+, while 61% of non-adherent CD34+ cells were Ki-67+ (Figure 9). Positive expression of Ki-67 confirms on the protein level that most that non-adherent CD34+ cells were mitotically active. The absence of Ki-67 protein in plastic adherent CD34+ cells indicates that

most plastic adherent CD34+ cells were arrested in G_0. This experiment validated gene expression data which indicated that the non-adherent CD34+ cells over express genes involved with the initiation of the cell cycle.

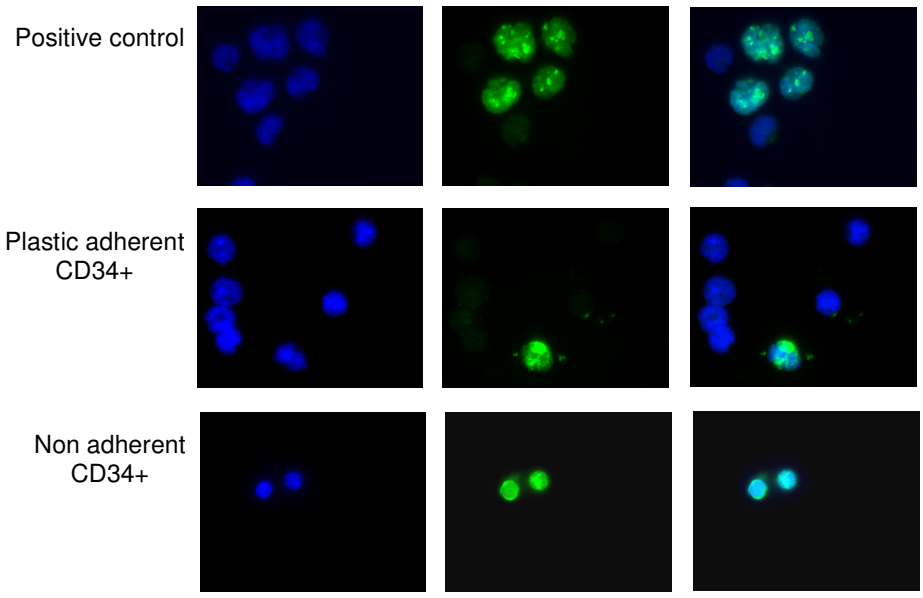


Figure 9. Quiescent adherent CD34+ cells do not express Ki67 protein. Immunofluorescent staining shows that only 24% of plastic adherent CD34+ cells are Ki-67+, while 61% of non-adherent CD34+ cells express Ki-67. This may be an indication that plastic adherent CD34+ cells are in a quiescent state. K562 cell line was used as a positive staining control for Ki-67, as cell lines are expected to express Ki-67. Ki-67 expression was quantified with immunofluorescent microscopy by counting 300 cells across 5 fields of view for each condition. Results are representative of three replicate experiments.

3.2.1.7 Cell adhesion

Adherent CD34+ cells highly expressed 22 genes implicated in cell adhesion to the bone marrow microenvironment. These are *CTNND1, CCR1, CCL2, FN1, ADAMDEC1, CSPG2, DSC3, SLAMF7, CD61, BMPR2, PCDH9, CD62, ITGA7, TAOK2, ITFG2, THBS1, CD41, GP5, PGM5, CTNNA1* and *Tie2*. Tie2 is an important adhesion molecule since it is involved in retaining $CD34^{+}$ cells in the stem cell niche in the bone marrow and is implicated in the

maintenance of stem cell quiescence[93,94]. Non-adherent CD34$^+$ cells do not significantly express these adhesion molecules, according to the microarray results.

3.2.1.8 Transendothelial migration

Tropism to sites of injury is a desirable property of stem cells. The homing and migration potential of these cells at the genetic level was examined. 22 genes related to leukocyte transendothelial migration were examined, of which 16 were more highly expressed by adherent CD34+ cells. These genes were *CTNND1, CXCL4, PIK3CB, PIK3R5, CXCL7, PRKCB1, MSN, NCF2, CYBB, CCR1, ACTN4, CXCL16, MMP9, MAPK13, CTNNA1*, and *CCRL2*. The chemokines CXCL4 and CXCL7 promote immune cell adhesion and transendothelial migration [95]. Only 6 genes related to leukocyte transendothelial migration were more highly expressed by non-adherent CD34$^+$ cells. These were *RASSF5, ITGAL, CXCL13, PTK2B, PIK3R2*, and *PXN*.

3.2.1.9 Chemokine activity

GO term analysis revealed that adherent CD34+ cells highly expressed genes which encode for chemokines *CXCL4, CCL2, CXCL16, CXCL5, CCL3, CCL8, CXCL7, CCL3L1, IL8,* and *CXCL3*. Non-adherent CD34+ cells did not preferentially express genes which encode for chemokines. Chemokines are essential for recruitment of immune cells to aid tissue repair and regeneration. These data suggest that adherent CD34+ cells may be attracted towards sites of injury by the synthesis and release of chemokines.

3.2.1.10 Apoptosis

Plastic adherent CD34+ cells more highly expressed 23 genes associated with the regulation of apoptosis. These genes were *IFG1R, CASP10, CCL2, SERPINB2, DIDO1, DNAJA3, IER3, SERPINB9, BIRC4, STK17A, ALK7, DAPK1, CTSB, SLAMF7, HIPK2, CARD9, MAPK1, PHB, HSPA1A, MTCH1, DPF2, PRKCE,* and *CASP2*. The non-adherent CD34+ cells highly

expressed 8 genes which regulate apoptosis, but did not share the same genes as the adherent CD34+ cells. These genes were *FOXO3A, NF346, FAS, MPO, RNASE3, DOCK1, CD38,*and *NALP1*. In either population, it remains unclear if expression of these genes induce self-apoptosis, or induce the cell to cause apoptosis of neighboring cells.

3.2.2 Cell surface receptors

An interest of the group is to identify surface markers expressed on plastic adherent CD34+ cells. Identifying such ligands or receptors is desirable as they may provide alternative methods for identifying and isolating plastic adherent CD34+ cells. Accordingly, the microarray results were examined for cell surface markers which were more highly expressed in plastic adherent CD34+ cells relative to non-adherent CD34+ cells. Arrays revealed that CCR1, BMPR2, IL1R, and CCRL2 were differentially upregulated in plastic adherent CD34+ cells. To validate this, immunofluorescence microscopy was used to determine the presence of these antigens at the protein level. Only BMPRII was expressed at statistically different levels between the two cell populations. BMPs belong to the TGFβ superfamily of growth factors. BMPs bind to two kinds of serine/threonine kinase receptors, type 1 and type 2. Activation of BMP receptors stimulates MAP kinase and Smad dependent pathways[96]. BMPR-II forms a complex with c-Kit which regulates differentiation of haemopoietic cells. Downstream activation of the Smad-dependent signalling results from BMPR-II activation. Smad activation requires stimulation of BMPR-II, but Smad signalling is increased when both c-Kit and BMPR-II are activated[96].

In comparing BMPR2 expression between plastic adherent and non-adherent CD34+ cells, a significantly greater percentage of plastic adherent CD34+ cells was found to express BMPR2 relative to non-adherent CD34+ cells ($p=0.0006$). Quantifying immunofluorescence images for BMPR2 expression, 79.7±2.6% of plastic adherent CD34+ expressed BMPR2+, 12.6±2.2% of

non-adherent CD34+ cells expressed BMPR2+, 10.4±3.43% of CD34- cells expressed BMPR2+, and 2.8±0.63% of total CD34+ cells expressed BMPR2+ (Figure 10). The relative expression of BMPR2 in different fractions of the mononuclear fraction of mobilized peripheral blood help illustrate that BMPR2 could potentially be used as a specific marker for plastic adherent CD34+ cells.

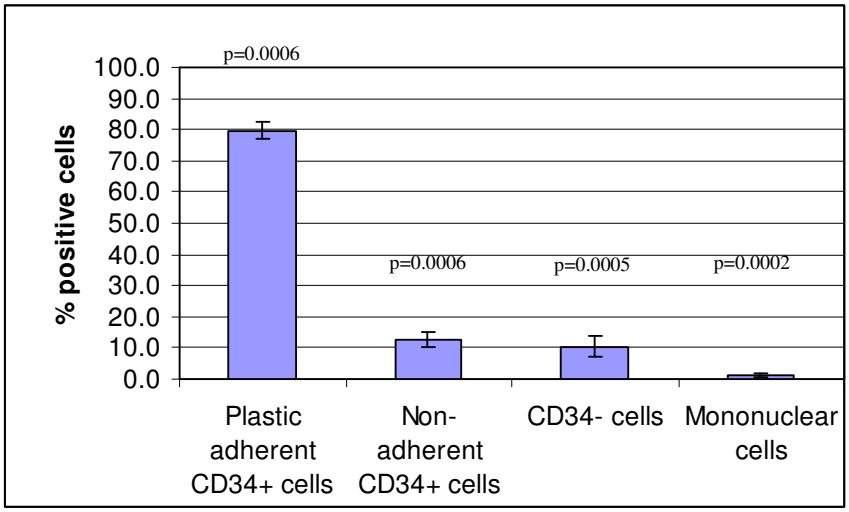


Figure 10. BMPRII protein expression by various fractions within PBMCs. To find cell surface markers which would help identify plastic adherent CD34+ cells, BMPRII was immunocytochemically analyzed to validate its protein expression. In viewing these results, it is important to acknowledge that the total proportion of plastic adherent CD34+ cells is only 0.2% of the mononuclear cells in peripheral blood. Analysis of different fractions of mobilized peripheral blood for BMPR2 indicated that only a small percentage (1.3%) of total mononuclear cells expressed BMPR2, indicating that this receptor may be an appropriate marker for plastic adherent CD34+ cells. Results are representative of four replicates. P-values refer to significant differences from adherent CD34+ cells and were determined using the Student's t-test.

To validate the immunofluorescence quantification data, FACS analysis of adherent CD34+ cells versus non-adherent CD34+ cells for relative BMPRII expression was conducted (Figure 11). It was observed that 87% of plastic adherent CD34+ cells are BMPR2+, while 41% of non-adherent CD34+ cells are BMPR2+, supporting the previous observation although the difference was less clear cut. By flow cytometry, the mean fluorescence intensity was ~50 fold greater than that in the non-adherent CD34+ fraction. The disparity in BMPR2 expression between the immunofluorescence data and the FACS analysis may be due to the different sensitivities of the methodologies, or may represent variations between donor samples. Both results demonstrate that plastic adherent CD34+ cells more highly express BMPR2 than the non-adherent CD34+ cells, making BMPR2 a possible candidate marker for plastic adherent CD34+ cells.

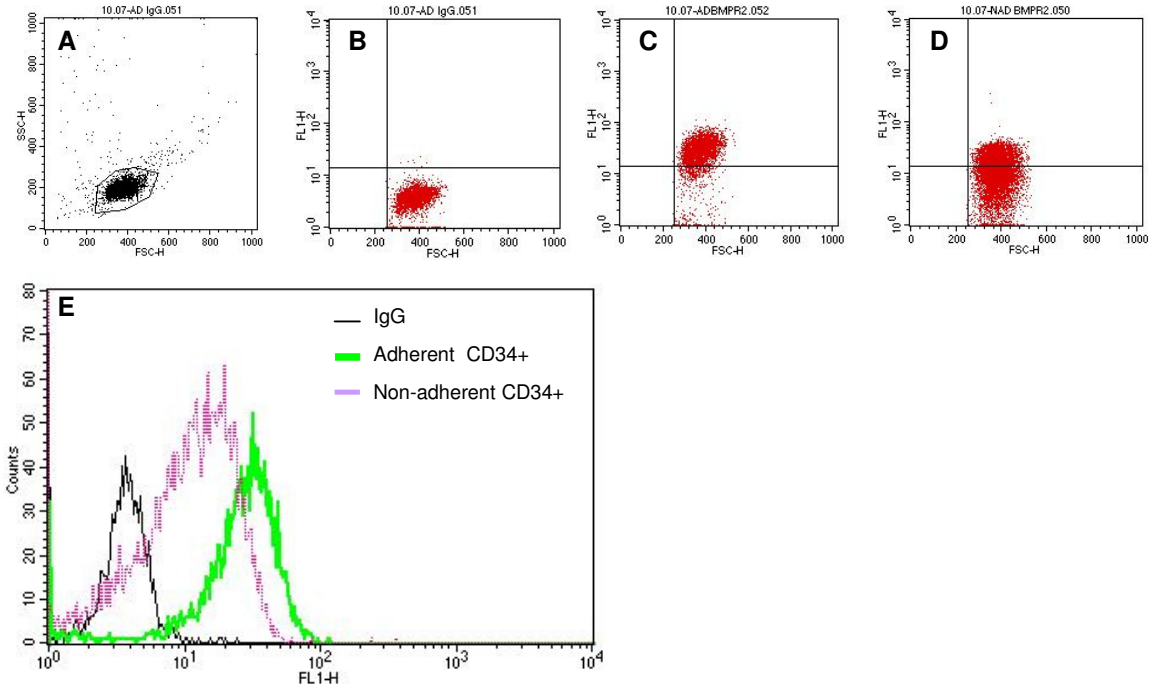


Figure 11. FACS analysis of BMPRII expression in adherent CD34+ cells versus non-adherent CD34+ cells. The CD34+ cells found in mobilized peripheral blood are small and lymphocyte-like in morphology (**A**). FACS analysis of relative BMPR2 expression in plastic adherent CD34+ cells versus non-adherent CD34+ cells show that 87.88% of plastic adherent CD34+ cells express BMPR2 (**C**), while 41.29% of non-adherent CD34+ cells express BMPR2 (**D**). Panel **B** represents the IgG control used to set the gate. This data is also represented in the histogram (**E**) which shows a clear shift in the peak fluorescence between the adherent and non-adherent CD34+ cells. *FACS was conducted by Dr. Stephen Marley*

3.2.3 Chromatin state of bone marrow derived cells

Early replication of genes during S phase of the cell cycle is considered to be a feature of transcriptionally active regions of the genome, indicating chromatin accessibility, whereas late replication is characteristic of inactive gene regions and constitutive heterochromatin[46].

Total CD34+ cells, adherent CD34+ cells, and non-adherent CD34+ cells were examined to determine if these cells have epigenetic features which associate them with pluripotent embryonic stem (ES) cells, or with more restricted neural stem cells (NSC). The timing of DNA replication was assessed by pulse-labeling cell populations with BrdU, and fractionating them according to cell cycle stage (Figure 12a). The relative abundance of chosen sequences within newly synthesized DNA are then determined using real-time qPCR (Figure 12b). Early replication during G1/S1 phase is considered to be associated with transcriptionally active regions of the genome, and indicative of accessible chromatin. Late replication is associated with constitutively expressed genes and genes that are actively supressed[46]. Replication timing is represented as a color code with green representing early replication and red as late replication (Figure 12c).

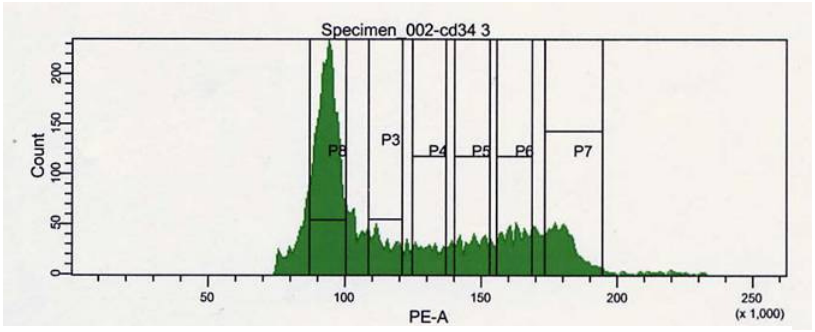
3.2.3.1 Bone marrow derived cells and NSC replicate Oct4 and Nanog early

Figure 13 represents the replication profiles for total CD34+ cells, adherent CD34+ cells, non-adherent CD34+ cells, and ES cells. The CD34+ cells have similar replication timing profiles of genes associated with pluripotency. Appropriate controls of early replication (*α-globin*) and late replication (*β-amylase*) were used during analysis of all cell populations. The *α-globin* gene is replicated early in the cell cycle.

Genes associated with ES cells *POU5F1* (Oct4) and *Nanog* replicated early in G1/S1 in total CD34+ cells, adherent CD34+ cells, non-adherent CD34+ cells, and ES cells. *ZFP4* (Rex1) did not replicate until middle-late/late part of the cell cycle. This indicates that these cells have a propensity for expressing the *POU5F1* and *Nanog* genes, but not *ZFP4* (Figure 13 and Figure 14).

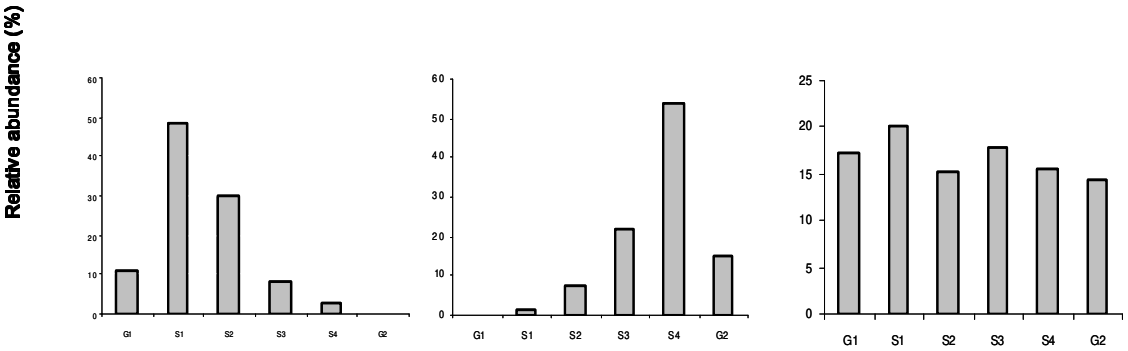
Replication timing data is supported by previously conducted real-time qPCR for relative expression of *SOX2, NANOG, ZFP42*, and *POU5F1* by adherent CD34+ cells and non- adherent CD34+ cells (Figure 6). The gene expression data validates the positive expression of these genes in adherent CD34+ cells, but none of the genes tested were expressed early by adherent CD34+ cells compared to non-adherent CD34+ cells.

A



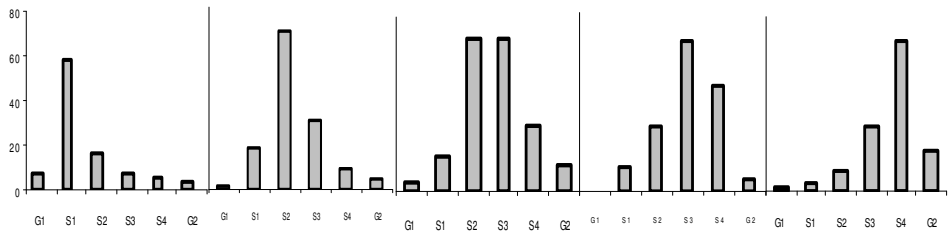
PI Intensity

B



Cell cycle fraction

C



Cell cycle fraction

Early Mid Early Middle Mid Late Late

Figure 12. Explaination of DNA replication timing. Stem cells in question were pulse-labelled with BrdU, stained with PI, and separated by cell sorting into six fractions corresponding to cell cycle stages (G1, S1, S2, S3, G2/M) (A). The abundance of locus-specific DNA was compared between cell cycle fractions using real-time PCR analysis of anti-BrdU purified templates. Constitutively early replicating (α-globin) and late replicating (β-amylase) loci were used as controls (B). The replication timing of each gene was defined according to its peak abundance in G1/S1 (early), S2 (mid-early), S2 and S3 (middle), S3 (mid-late), and S4/G2 (late) phases and represented as a colour code (C).

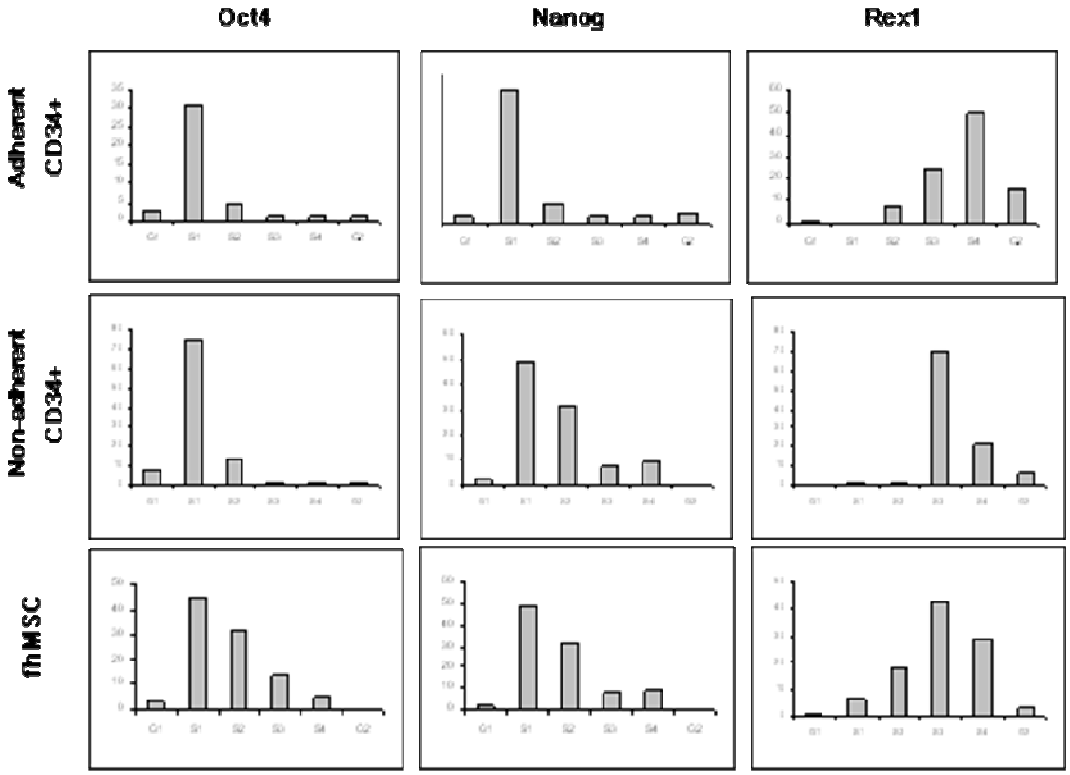
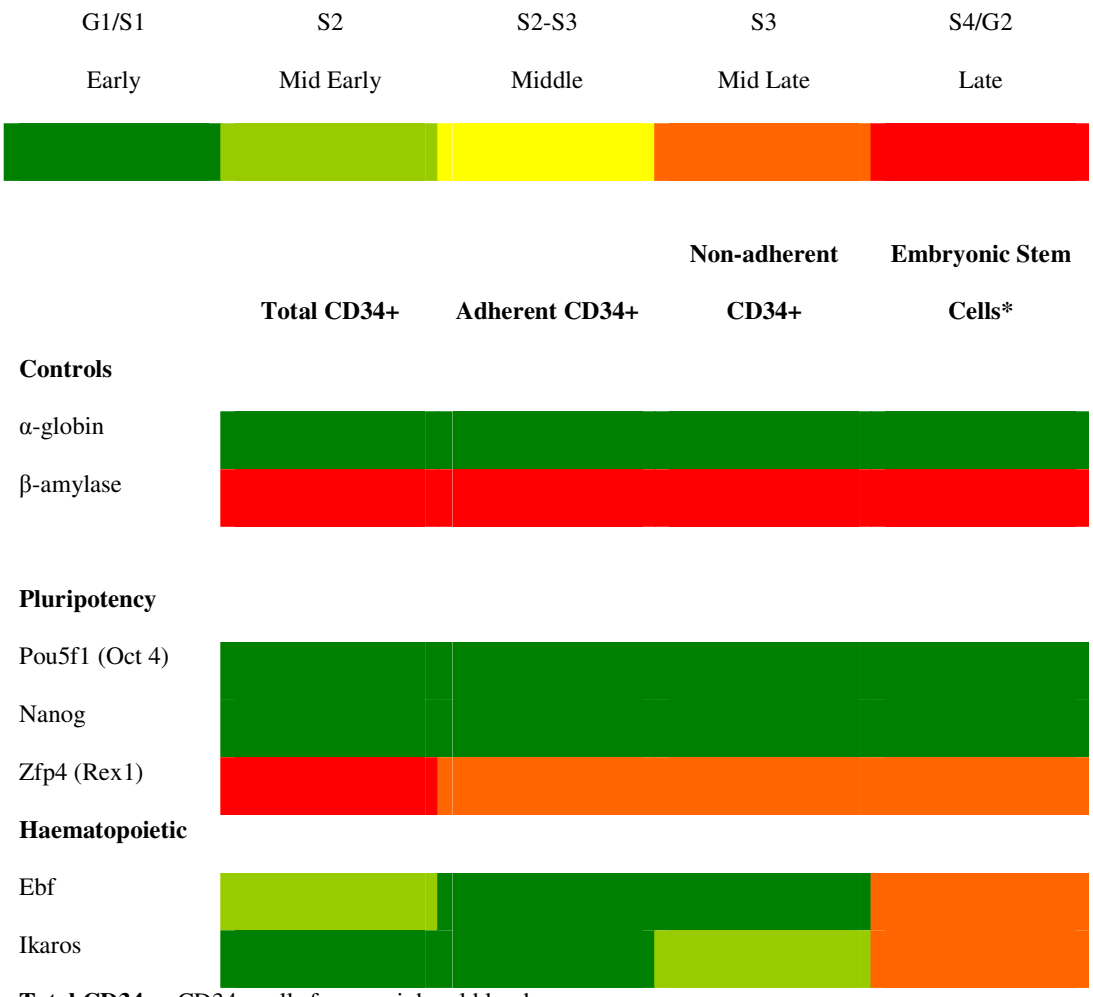


Figure 13. Replication timing ofOct4, Nanog, and Rex1 by bone marrow derived stem cells. Adherent CD34+ cells, non-adherent CD34+ cells, and fhMSCs were labelled with BrdU, stained with PI and separated into six fractions corresponding to cell cycle stages. The abundance of Oct4, Nanog, and Rex1 DNA was compared between cell cycle fractions. The replication timing of each gene was defined according to its peak abundance. Oct4 and Nanog were replicated early in G1 by adherent CD34+ cells, non-adherent CD34+ cells and fhMSCs indicating that the chromatin states of these genes allow their transcriptional accessibility in these cells. The relative abundance of Rex1 was highest late in the cell cycle in all three cells, at S4 in adherent CD34+ cells, and at S3 in non-adherent CD34+ cells and fhMSCs indicating this gene in not likely to be transcribed.



Total CD34+ : CD34+ cells from peripheral blood

Ad CD34+: Adherent CD34+ cells, primitive quiescent stem cells

NA CD34+ : Non-adherent CD34+ cells (to compare with the adherent CD34+ cells)

ES: Human embryonic stem cells **Replication timing of ES cells prepared by Sandra Pinho*

Figure 14. Summary of DNA replication timing results. DNA replication timing is summarized as a colour code. The figure summarizes the findings which indicate that ES cells and CD34+ cells replicate genes associated with pluripotent ES cells, *POU5F1* and *NANOG,* early in the cell cycle, but not *ZFP4* (Rex1). The subpopulations of CD34+ cells replicate haematopoietic genes *EBF* and *IKAROS* early in G0/G1 or G2, but ES cells replicate these genes late at S3. Analysis of the DNA replication timing of haematopoietic genes by CD34+ cells provides a control for the assay.

3.3 Discussion

Adherent CD34+ cells are a transcriptionally distinct subpopulation of CD34+ G-CSF mobilized peripheral blood. This was supported in this study by the identification of subsets of 562 transcripts which are more highly expressed, and 338 transcripts which are less highly expressed by adherent CD34+ cells relative to non-adherent CD34+ cells. Within these subsets of genes are genes which are involved in stem cell functions including quiescence, migration, and adhesion to bone marrow.

3.3.1 Adherent CD34+ cells express pluripotency associated markers

Molecular analysis of stem cell and pluripotent cell associated markers has lead to protein analysis and identification of an adherent CD34+ cell phenotype. Although it was assumed that adherent CD34+ cells were more primitive than non-adherent CD34+ cells[97,98], PCR and immunofluorescence microscopy revealed that adherent CD34+ cells express developmental markers Oct4 (encoded by *POU5F1*), Sox2, and Nanog. Oct4, Sox2 and Nanog are currently considered to be core regulators in pluripotent stem cells[99,100]. In studies of embryonic stem cells, the quantitative expression of Oct4 maintains pluripotency, and its down-regulation in pluripotent embryonic stem cells leads to trophectoderm differentiation [82,101]. Nanog promotes pluripotent cell self-renewal[100]. Although Sox2 is not exclusive to embryonic stem cells, it is necessary in the maintenance of pluripotency[100].

DNA replication timing introduces another method for determining potential cell plasticity by examining an epigenetic control mechanism of transcription. The replication timings of the genes which encode for Oct4, Nanog, and Rex1 were examined in adherent CD34+ cells, non-adherent CD34+ cells, and fhMSCs. Oct4 was replicated early by all three stem cell types and this result supports the results of real-time qPCR (Figure 6). The immunofluorescence data

indicated that Oct4 is expressed by the majority of adherent CD34+ cells, but not by non-adherent CD34+ cells. This discrepancy in results suggest that perhaps although Oct4 was replicated early in all populations tested, the message was only translated into protein by the adherent CD34+ cells.

Primitive cells thought to be precursors of germ cells, including oocytes and spermatogonial cells, have been identified in the bone marrow[17,18]. One interpretation of the multiplicity of stem cell types in the marrow is that bone marrow is a general repository for stem cells that can be mobilized for various purposes during adulthood[25]. The high frequency of $Oct4^+$, $Sox2^+$, and $Nanog^+$ cells in the adherent CD34+ cell fraction suggests that these cells may be the result of "developmental migration[25]" and residual cells from development. Stem cell genes which include *NOTCH1, WNT1, CXCR4, GATA2,* and members of the HoxA and HoxB families were equally highly expressed in adherent and non-adherent CD34+ cells. These genes are associated with proliferation, differentiation regulation, and migration of embryonic, neural, mesenchymal, and haematopoietic stem cells[102-109]. Our data indicate that there is no difference in transcription expression between adherent CD34+ cells and non-adherent CD34+ cells in the expression of transcripts which encode for stem/progenitor proliferation.

3.3.2 Cell cycle control

Our genetic analysis of the genes involved in cell cycle control revealed that adherent CD34+ cells more highly expressed genes involved in the negative regulation of the cell cycle, while non-adherent CD34+ cells more highly expressed genes which promote progression through the cell cycle (Figure 15). Non-adherent CD34+ cells highly express genes which encode for cyclin a1 and cyclin e1. Conversely, adherent CD34+ cells expressed significantly lower levels of these genes. At the same time, adherent CD34+ cells express high levels of p57, whose

expression results in the inhibition of cyclin e1 and cyclin a1, leading to a decrease in DNA synthesis and cell proliferation[110]. This finding was validated by the Ki-67 immunofluorescence data which indicated that adherent CD34+ cells are arrested in G_0, while non-adherent CD34+ cells are actively cycling.

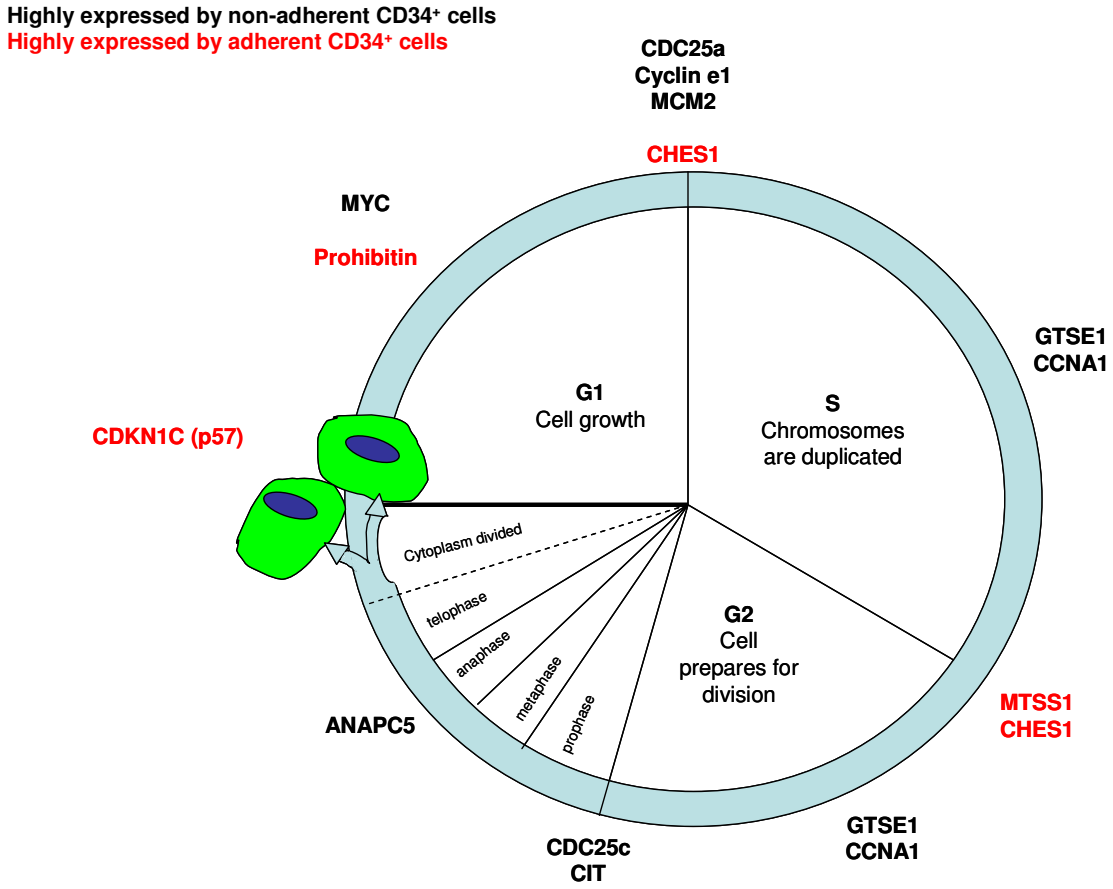


Figure 15. Cell cycle-associated genes are differentially expressed by adherent CD34+ cells and non-adherent CD34+ cells. Genes whose expression is higher in adherent CD34+ cells and genes involved in the negative regulation of the cell cycle are in red. Those more highly expressed by non-adherent CD34+ cells and cell cycle promoting genes are in black.

3.3.3 Mitotic quiescence is a property of plastic adherent CD34+ cells

Cell cycle distribution studies on peripheral blood CD34+ cells and bone marrow CD34+ cells confirm that there are low numbers of CD34+ cells in the S, G_2, and M phases of the cell cycle[111,112]. Previous studies using PI FACS analysis demonstrated that the majority of CD34+ stem cells from the peripheral blood are in G_0/ G_1 and CD34+ cells were presumed to be quiescent[113]. The caveat to this conclusion is that PI analysis does not discriminate between G_0 and G_1. Later DNA analysis revealed that the majority, but not all, of the peripheral blood CD34+ cells are in G_1[112]. When combined with previous studies mentioned above, it can be assumed that it is the adherent CD34+ cell fraction that is arrested in G_0, while the majority of the non-adherent CD34+ cells are in G_1.

These data were confirmed by immunofluorescent staining for Ki-67 which demonstrated that the majority of adherent CD34+ cells were Ki-67$^-$ and the majority of non-adherent CD34+ cells were Ki-67$^+$. The Ki-67 protein is present in the nuclei of cells in the G_1, S, G_2 and mitotic phases of the cell cycle. Quiescent cells or cells arrested in G_0 lack Ki-67 antigen, thus indicating that adherent CD34+ cells are arrested in G_0. The majority of non-adherent CD34+ cells are Ki-67 positive and are thus in the cell cycle, probably in G_1. Overall the data suggest that mitotic quiescence is a property of plastic adherent CD34+ cells.

3.3.4 Genetic attributes of adherent CD34+ cells which make them desirable for use in regenerative medicine

The gene arrays conducted in this study revealed that adherent CD34+ cells highly upregulate genes which are involved in cell adhesion and transendothelial migration. These two properties are important for stem cell mobilization to sites of tissue injury if the bone marrow serves as a reservoir for stem cells which maybe released to aid tissue repair and regeneration[94]. Quiescent stem cells adhere to osteoblasts in the bone marrow niche[28]. Quiescent stem cells in the bone

marrow express the receptor tyrosine kinase Tie2, which interacts with its ligand Angiopoietin-1 (Ang1) expressed by osteoblasts[28,94]. Tie2 is an important adhesion molecule since it is involved in retaining $CD34^+$ cells in the stem cell niche in the bone marrow and is implicated in the maintenance of HSC quiescence[93,94]. According to the transcriptional data, adherent CD34+ cells highly express Tie2, but non-adherent $CD34^+$ cells do not significantly express this quiescence-related adhesion molecule.

Gene analysis revealed that adherent CD34+ cells more highly express genes related to cell migration and transendothelial migration. Regulated migration and proper localization is critical for stem cell function in aiding tissue repair and maintaining homeostasis[114]. Migration through endothelial tight junctions is necessary for proper stem cell homing.

Adherent CD34+ cells also express genes involved in the migration and recruitment of other effector cells. These genes include MMP9 which is important for T cell migration through basement membrane[115]. MMP9 is also important for recruiting other CD34+ progenitors[116], and blocking MMP9 reduces T cell trafficking to injury[115]. Adherent CD34+ cells also highly express genes related to the synthesis of 10 chemokines including anti-microbial CXCL8/IL-8. This implies that adherent CD34+ cells are involved in chemotactic recruitment of immune and tissue specific cells. Given all the above evidence, it is reasonable to speculate that adherent CD34+ cells are those stem cells which home in immediate response to injury.

Table 2. Subset of genes highly up-regulated by adherent CD34+ cells.

Functional Catergory			
Cell cycle	MTSS1	MIM, MIMA, MIMB	metastasis suppressor 1
	GAS7	MLL/GAS7	growth arrest-specific 7
	p57	BWCR, BWS, KIP2, WBS, p57	cyclin-dependent kinase inhibitor 1c (p57, kip2)
	WWOX	FOR, FRA16D, HHCMA56, PRO0128, WOX1, WWOX v8	putative oxidoreductase
	EXT1	EXT, LGCR, LGS, ttv	exostoses (multiple) 1
	GAS2L1	GAR22	growth arrest-specific 2 like 1
	HEXIM1	CLP1, EDG1, FLJ13562, HIS1, MAQ1	hexamethylene bis-acetamide inducible 1
	NIPBL	CDLS, CDLS1, DELANGIN, IDN3, IDN3-B	nipped-b homolog (drosophila)
	IGF1R	CD221, IGFIR, JTK13	insulin-like growth factor 1 receptor
	PDGFa	PDGF1	platelet-derived growth factor alpha polypeptide
	RAD21	HR21, MCD1, NXP1, SCC1, hHR21	rad21 homolog (s. pombe)
	CDC2L5	CDC2L, CHED	cell division cycle 2-like 5 (cholinesterase-related cell division controller)
	PHB	PHB1	prohibitin
	PRMT5	IBP72, JBP1, SKB1	protein arginine methyltransferase 5
	PDGFb	PDGF2, SIS, SSV, c-sis	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
	CHES1	FOXN3	checkpoint suppressor 1
	MAPK13	PRKM13, SAPK4, p38delta	mitogen-activated protein kinase 13
	HECA	HDC, HDCL, HHDC	headcase homolog (drosophila)
	PIK3CB	PI3K, PI3KCB, PI3Kbeta, PIK3C1, p110-BETA	phosphoinositide-3-kinase, catalytic, beta polypeptide
	CDK9	C-2k, CDC2L4, CTK1, PITALRE, TAK	cyclin-dependent kinase 9 (cdc2-related kinase)
	CXCL7	PPBP, B-TG1, Beta-TG, CTAP-III, CTAP3, CTAPIII, CXCL7, LA-PF4, LDGF, MDGF, NAP-2, PBP, SCYB7, TC1, TC2, TGB, TGB1,	pro-platelet basic protein (chemokine (c-x-c motif) ligand 7)

		THBGB, THBGB1	
	HIPK2		homeodomain interacting protein kinase 2
		ERK, ERK2, ERT1, MAPK2, P42MAPK, PRKM1, PRKM2, p38,	
	MAPK1	p40, p41, p41mapk	mitogen-activated protein kinase 1
	DUSP6	MKP3, PYST1	dual specificity phosphatase 6
		NHPX, OTK27, SNU13	nhp2 non-histone chromosome protein 2-like 1
	NHP2L1		(s. cerevisiae)
		EFO1, ESO1	establishment of cohesion 1 homolog 1 (s.
	ESCO1		cerevisiae)
Cell adhesion	CTNND1	p120, CAS, CTNND	catenin (cadherin-associated protein), delta 1
		CD191, CKR-1, CMKBR1, HM145,	
	CCR1	MIP1aR, SCYAR1	chemokine (c-c motif) receptor 1
		GDCF-2, HC11, HSMCR30, MCAF, MCP-1, MCP1, MGC9434, SCYA2,	
	CCL2	SMC-CF	chemokine (c-c motif) ligand 2
		CIG, ED-B, FINC, FN, FNZ, GFND2, LETS,	
	FN1	MSF	fibronectin 1
	ADAMDEC1		adam-like, decysin 1
		CSPG2, DKFZp686K06110, ERVR, PG-M, WGN,	
	CSPG2	WGN1, VCAN	chondroitin sulfate proteoglycan 2 (versican)
		CDHF3, DSC, DSC1,	
	DSC3	DSC2, DSC4, HT-CP	desmocollin 3
		19A, CD319, CRACC,	
	SLAMF7	CS1	slam family member 7
		ITGB3, GP3A, GPIIIa	integrin, beta 3 (platelet glycoprotein iiia, antigen
	CD61		cd61)
	TEK	Tie2	TEK tyrosine kinase, endothelial
		BMPR-II, BMPR3, BMR2, BRK-3, PPH1,	
	BMPR2	T-ALK	bone morphogenic protein receptor 2
	PCDH9		protocadherin 9
		SELP, CD62P, FLJ45155, GMP140, GRMP, LECAM3,	selectin p (granule membrane protein 140kda,
	CD62	PADGEM, PSEL	antigen cd62)

	ITGA7		integrin, alpha 7
	TAOK2	MAP3K17, PSK, PSK1, TAO1, TAO2	tao kinase 2
	ITFG2		uncharacterized hematopoietic stem/progenitor cells protein mds028
	THBS1	THBS, TSP, TSP1, thrombospondin-1p180	thrombospondin 1
	CD41	ITGA2B, CD41B, GP2B, GPIIb, GTA, HPA3	integrin, alpha 2b (platelet glycoprotein iib of iib/iiia complex, antigen cd41)
	GP5	CD42d	glycoprotein v (platelet)
	PGM5		phosphoglucomutase 5
	CTNNA1	alpha-E-catenin; alpha-catenin	catenin (cadherin-associated protein), alpha 1, 102kda
Chemokine activity	CXCL4	SCYB4, PF4	platelet factor 4 (chemokine (c-x-c motif) ligand 4)
	CCL2	GDCF-2, HC11, HSMCR30, MCAF, MCP-1, MCP1, MGC9434, SCYA2, SMC-CF	chemokine (c-c motif) ligand 2
	CXCL16	CXCLG16, SR-PSOX, SRPSOX	chemokine (c-x-c motif) ligand 16
	CXCL5	ENA-78, SCYB5	chemokine (c-x-c motif) ligand 5
	CCL3	MIP-1-alpha, MIP1A	chemokine (c-c motif) ligand 3
	CCL8	HC14, MCP-2, MCP2, SCYA10, SCYA8	chemokine (c-c motif) ligand 8
	CXCL7	B-TG1, Beta-TG, CTAP-III, CTAP3, CTAPIII, CXCL7, LA-PF4, LDGF, MDGF, NAP-2, PBP, SCYB7, TC1, TC2, TGB, TGB1, THBGB, THBGB1	pro-platelet basic protein (chemokine (c-x-c motif) ligand 7)
	CCL3L1		chemokine (c-c motif) ligand 3-like 1
	IL8	3-10C, AMCF-I, CXCL8, GCP-1, GCP1, K60, LECT, LUCT, LYNAP, MDNCF, MONAP, NAF, NAP-1, NAP1, SCYB8, TSG-1, b-ENAP	interleukin 8 (CXCL8)
	CXCL3	CINC-2b, GRO3, GROg, MIP-2b, MIP2B,	chemokine (c-x-c motif) ligand 3

		SCYB3	
Leukocyte transendothelial migration		p120, CAS, CTNND	
	CTNND1		catenin (cadherin-associated protein), delta 1
	CXCL4	PF4, SCYB4	platelet factor 4 (chemokine (c-x-c motif) ligand 4)
	PIK3CB	PI3K, PI3KCB, PI3Kbeta, PIK3C1, p110-BETA	phosphoinositide-3-kinase, catalytic, beta polypeptide
	PIK3R5	FOAP-2, P101-PI3K, p101	phosphoinositide-3-kinase, regulatory subunit 5, p101
	CXCL7	B-TG1, Beta-TG, CTAP-III, CTAP3, CTAPIII, CXCL7, LA-PF4, LDGF, MDGF, NAP-2, PBP, SCYB7, TC1, TC2, TGB, TGB1, THBGB, THBGB1	pro-platelet basic protein (chemokine (c-x-c motif) ligand 7)
	PRKCB1	PKC-beta, PKCB, PRKCB, PRKCB2	protein kinase c, beta 1
	MSN		moesin
	NCF2	NCF-2, NOXA2, P67-PHOX, P67PHOX	neutrophil cytosolic factor 2 (65kda, chronic granulomatous disease, autosomal 2)
	CYBB	CGD, gp91-1, gp91-Phox, NOX2, p91-Phox	cytochrome b-245, beta polypeptide (chronic granulomatous disease)
	CCR1	CD191, CKR-1, CMKBR1, HM145, MIP1αR, ScyaR1	RANTES receptor; Chemokine receptor 1
	ACTN4	ACTININ-4, FSGS, FSGS1	actinin, alpha 4
	CXCL16	CXCLG16, SR-PSOX, CLG4B, GELB	chemokine (c-x-c motif) ligand 16
	MMP9		matrix metallopeptidase 9 (gelatinase b, 92kda gelatinase, 92kda type iv collagenase)
	MAPK13	SAPK4, p38 delta, PRKM13	mitogen-activated protein kinase 13
	CTNNA1	CAP102	catenin (cadherin-associated protein), alpha 1, 102kda
	CCRL2	CKRX, CRAM-A, CRAM-B, HCR	Chemokine receptor like 2
Apoptosis	IFG1R	CD64, CD64A, FCRI	insulin-like growth factor 1 receptor
	CASP10	ALPS2, FLICE2, MCH4	caspase 10
	CCL2	GDCF-2, HC11, HSMCR30,	chemokine (c-c motif) ligand 2

	MCAF, MCP-1, SCYA2, SMC-CF	
	PAI, PAI-2, PLANH2	serpin peptidase inhibitor, clade b (ovalbumin),
SERPINB2		member 2
	BYE1, DATF1,	
DIDO1	DIDO2, DIDO3, DIO-1	death inducer obliterator 1
DNAJA3	TID1	dnaj (hsp40) homolog, subfamily a, member 3
	DIF2, GLY96, IEX-1,	
IER3	IEX-1L, PRG1	immediate early response 3
	CAP3, p19	serpin peptidase inhibitor, clade b (ovalbumin),
SERPINB9		member 9
	AP13, ILP1,	
BIRC4	MIHA, XLP2	baculoviral iap repeat-containing 4
STK17A	DRAK1	serine/threonine kinase 17a (apoptosis-inducing)
ALK7		activin A receptor type 1c
DAPK1		death-associated protein kinase 1
CTSB	APPS, CPSB	cathepsin b
	19A, CD319, CRACC,	
SLAMF7	CS1	slam family member 7
HIPK2		homeodomain interacting protein kinase 2
CARD9		caspase recruitment domain family, member 9
	ERK, ERK2, ERT1, MAPK2, P42MAPK, PRKM1, PRKM2, p38,	
MAPK1	p40, p41, p41mapk	mitogen-activated protein kinase 1
PHB		prohibitin
	HSP70-1, HSP70-1A, HSP72, HSPA1,	
HSPA1A	HSPA1B	heat shock 70kda protein 1a
MTCH1	PIG60, PSAP	mitochondrial carrier homolog 1 (c. elegans)
DPF2	REQ, UBID4	d4, zinc and double phd fingers family 2
PRKCE		protein kinase c, epsilon
	ICH-1L, ICH-1L/1S,	
CASP2	ICH1, NEDD2	Caspase 2, apoptosis-related cysteine peptidase

Table 3. Subset of genes highly up-regulated by non-adherent CD34+ cells.

Functional Catergory			
M Phase and cell cycle	TOP3A		topoisomerase (dna) iii alpha
	ANAPC5	APC5, PDL-108	anaphase promoting complex subunit 5
	PAPD5	TRF4-2	pap associated domain containing 5
	EML4	C2orf2, , ELP120, ROPP120	echinoderm microtubule associated protein like 4
	NUSAP1	ANKT, BM037, LNP, SAPL	nucleolar and spindle associated protein 1
	RAD54L	HR54, RAD54A, hHR54, hRAD54	rad54-like (s. cerevisiae)
	CIT	CRIK, STK21	citron (rho-interacting, serine/threonine kinase 21)
	TPX2	DIL2, HCA519, HCTP4, REPP86, p100	tpx2, microtubule-associated, homolog (xenopus laevis)
	CDC25a	CDC25A2	cell division cycle 25a
	CDC25c	CDC25	cell division cycle 25c
	CCNA1		cyclin a1
	NEK4	NRK2, STK2	nima (never in mitosis gene a)-related kinase 4
	APBB2	FE65L, FE65L1	amyloid beta (a4) precursor protein-binding, family b, member 2 (fe65-like)
	GTSE1	B99	g-2 and s-phase expressed 1
	CHAF1B	CAF-1, CAF-IP60, CAF1, CAF1A, MPP7	chromatin assembly factor 1, subunit b (p60)
	SEPT9	MSF, MSF1, NAPB, PNUTL4, SINT1, SeptD1	septin 9
	MCM2	BM28, CCNL1, CDCL1, MITOTIN, cdc19	mcm2 minichromosome maintenance deficient 2, mitotin (s. cerevisiae)
	UHRF1	Np95, RNF106, hNP95	ubiquitin-like, containing phd and ring finger domains, 1
	MYC	c-Myc	v-myc ptoto-oncogene80
DNA replication	PRIM1	P49	primase, polypeptide 1, 49kda
	RRM2	R2, RR2M	ribonucleotide reductase m2 polypeptide
	CHAF1B	CAF-1, CAF-IP60, CAF1, CAF1A, CAF1P60, MPP7	chromatin assembly factor 1, subunit b (p60)
	POLG		polymerase (dna directed), gamma

	POLE		polymerase (dna directed), epsilon
Leukocyte transendothelial migration	RASSF5		ras association (ralgds/af-6) domain family 5
	ITGAL	LFA-1, LFA1A	integrin, alpha l (antigen cd11a (p180)
	CXCL13	ANGIE, ANGIE2, BCA-1, BCA1, BLC, BLR1L, SCYB13	chemokine (c-x-c motif) ligand 13 (b-cell chemoattractant)
	PTK2B	CADTK, CAKB, FADK2, FAK2, FRNK, PKB, PTK, PYK2, RAFTK	ptk2b protein tyrosine kinase 2 beta
	PIK3R2	P85B, p85, p85-BETA	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)
	PXN		paxillin
Apoptosis	FOXO3A	FOXO3	forkhead box o3a
	ZNF346	JAZ	zinc finger protein 346
	FAS	ALPS1A, APO-1, APT1, CD95, FAS1, FASTM, TNFRSF6	fas (tnf receptor superfamily, member 6)
	MPO		myeloperoxidase
	RNASE3		ribonuclease, rnase a family 3
	DOCK1	DOCK180, ced5	dedicator of cytokinesis 1
	CD38	T10	cd38 antigen (p45)
	NALP1	CARD7, CLR17.1, DEFCAP, DEFCAP-L/S, DKFZp586O1822, KIAA0926, NAC, NALP1, PP1044, SLEV1, VAMAS1	nacht, leucine rich repeat and pyd (pyrin domain) containing 1

Chapter 4 Neural differentiation of bone marrow derived stem cells

Chapter summary

Various laboratories have published data which suggest that bone marrow derived cells are capable of differentiating into neural lineage cells[12, 13, 15, 75-77]. Based on these data along with data that suggests adherent CD34+ cells are primitive stem cells, it was reasonable to hypothesize that adherent CD34+ cells might be capable of neurogenic differentiation *in vitro*. Published neural differentiation protocols of bone marrow derived cells were first used to determine if they were capable of inducing adherent CD34+ cells to differentiate into neural cells. This chapter outlines *in vitro* neural differentiation experiments of bone marrow derives stem cells and uses neural stem cells as positive differentiation controls. No neural differentiation was observed, thus retrospective DNA replication timing analysis was conducted to determine if bone marrow derived cells are epigenetically primed for neural differentiation. The collective results revealed that bone marrow derived stem cells including adherent CD34+ cells do not differentiate into neural cells *in vitro*. This chapter describes the following:

- Neural differentiation patterns of human neural stem cells
- Bone marrow derived stem cells do not differentiate into neural cells
- DNA replication timing of neurogenicity associated genes by bone marrow derived stem cells

4.1 Bone marrow-derived cells in the brain

It is known that bone marrow and peripheral blood derived cells migrate through the blood brain barrier during CNS injury. Results of experiments using radiation bone marrow chimeras have indicated that 0.5% to 2% of astrocytes are derived from the bone marrow[117]. The caveat of this study is that radiation may affect the permeability of the blood brain barrier. Several groups have independently identified stem/progenitor cells which show neural characteristics; however, these instances are highly controversial as the sources for such cells are non-neural

tissues[68,118,119]. In addition, bone marrow derived cells have been induced in vitro to express mature neuron phenotypes[75,77,120,121]. There remains a need for scrutiny as to the reproducibility and functionality of the cells used for these types of experiments. This appreciation has forced more rigorous and thorough investigation of stem/progenitor cells in the bone marrow, umbilical cord, and peripheral blood. It is possible that discrepancies in the results are due to a lack of uniformity in bone marrow cell isolation. For example, Priller et al[60] used bone-marrow from 5-fluoruracil treated mice stimulated with IL-3 and IL-6. The cells isolated by D'Ippolito and colleagues (MIAMI)[13] cells are isolated by adherence to fibronectin and colony isolation.

The administration of non-neural cells in animal models of cerebral ischaemia has lead to explorations of non-neural cells providing neuroprotection and stimulating endogenous neurogenesis. There is a school of thought which believes that most if not all organs in the body have a reservoir of stem and progenitor cells which are mobilized after insult, thus expediting tissue repair and regeneration. Moreover, it is known that haematopoietic stem and progenitor cells circulate in the peripheral blood. Ratajczak and colleagues[16,25,68] hypothesized that CXCR4+/CD45- tissue-specific stem cells also circulate in the peripheral blood, maintaining a pool of cells which are easily distributed to areas of the body distant from the bone marrow. Fluorescence activated cell sorting and mRNA analysis have shown that a small population of neural-tissue committed stem cells which express neural markers (GFAP, nestin, β-III tubulin, and NeuN) is found in the mononuclear fraction of peripheral blood. Furthermore, blood from G-CSF-mobilized patients contains a higher number of tissue-specific stem cells than steady-state blood. Of particular interest, after cerebral ischaemia, there is a marked increase in neural tissue-specific stem cells in the circulation[15]. *In vitro* analyses of these cells show that after a stroke, neural-tissue committed stem cells respond to chemoattractants SDF-1, HGF, and LIF[15]. This leads to the hypothesis that the bone marrow contains a population of non-haematopoietic

stem/precursor cells that express early neural specific markers, and are mobilized into the peripheral blood after CNS injury[15]. These findings suggest a quiescent reservoir of neural lineage cells in the bone marrow, which may be triggered after CNS damage. Alternatively, there exists a population of cells which has a high potential for differentiating into neural cells and responding to chemoattractants released after cerebral ischaemia.

4.1.1 DNA replication timing

During development neural specification occurs early in embryogenesis. Once specified, progenitors generate distinct neural cell types. This process requires establishing patterns of neural-specific gene expression and a concerted restriction of alternative cell fates[122]. Although regulators of neural commitment have been identified, only recently have chromatin based studies of neural commitment been studied and little is known about the epigenetics of neural lineage restriction[46,48]. One contributing factor is the availability of neural cells and difficulties in acquiring a homogenous population of neural tissue. The terminally differentiated nature of neural cells which are out of cycle creates a problem in DNA replication timing studies, as cells are required to be in the cell cycle in order for BrdU incorporation.

The previous chapter validated that adherent CD34+ cells are primitive stem cells and previously published data demonstrated that adherent CD34+ cells express transcriptional message for neural associated markers NeuroD, Pax6, and Nkx2.2[11], thus it was reasonable to hypothesize that adherent CD34+ cells might have neural differentiation potential. Here the primary aim was to evaluate the ability of bone marrow-derived cell populations to differentiate into neural lineages, using neural stem cells (NSC) as a model for differentiation. The DNA replication timing kinetics of human neural stem cells was defined and compared with the kinetics of

adherent CD34+ cells and fhMSCs to determine whether they have potential neurogenic capabilities at the chromatin level.

4.2 Results

4.2.1 Neuronal differentiation of neural stem cells

Neural stem cells or primary neural cells were used to provide a reference for comparison in neuronal differentiation experiments using various types of stem cells. To investigate antigenic expression of neural markers during NSC differentiation, R197 NSCs were cultured in an established neuronal induction medium. Briefly, NSCs were grown in neural basal media supplemented with N2, EGF, and bFGF. At days 0, 10, and 21, differentiating cells were stained for neuronal marker βIII-tubulin, astrocytic marker GFAP, oligodendrocyte marker olig2, and neural stem cell marker nestin. Antigenic expression was compared between NSCs in standard culture conditions and NSCs treated in neuronal differentiation conditions (Figure 16). Untreated NSCs had an increase in nestin expression and a sustained expression of GFAP indicating astrocytic differentiation. In neuronal differentiation conditions, Nestin expression was strongest after 10 days, but decreased after 21 days in differentiation conditions. This downregulation of nestin, a stem cell marker, validates differentiation of NSCs.

NSCs in neuronal differentiation conditions upregulated βIII tubulin at 10 days, and began growing in an observable pattern as seen in Figure 10b. β-III tubulin positive cells grow in large clusters and extend processes towards adjacent, evenly spaced clusters of similar size and shape. A typical cell will keep its cell body close to adjacent cell bodies, and extend long axons towards dendrites of another cell. The growth patterns mimic those seen in human neural stem cell differentiation into neurons (Figure 16).

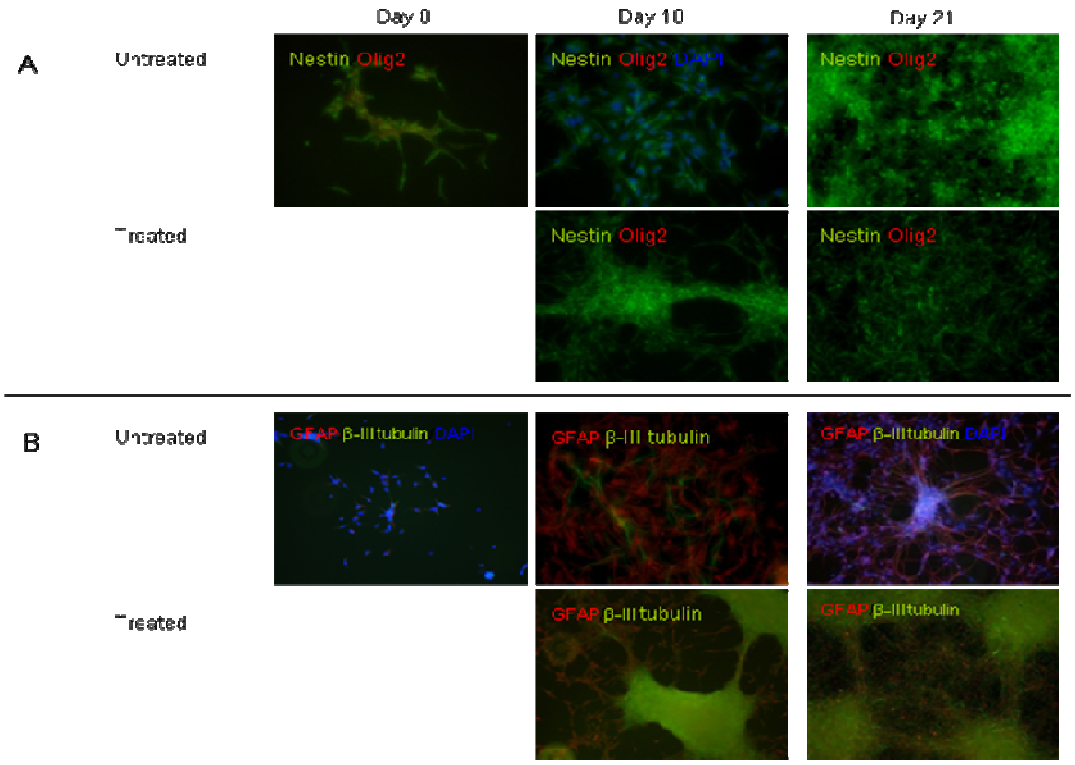


Figure 16. Neural lineage differentiation of NSC. Antigenic expression of neural stem cell marker nestin and early oligodendrocyte marker olig2 were followed at three time points during neuronal differentiation of human neural stem cells (A). In standard culture conditions, nestin expression increased over time at day 10 and day 21. In neuronal differentiation conditions, nestin expression increased at day 10, but was dim at day 21. Olig2 expression was absent in both treated and untreated conditions by day 10. Antigenic expression of astrocyte marker GFAP and immature neuronal marker β-III tubulin were also followed during neuronal differentiation (B). In standard culture conditions, NSCs upregulate GFAP and express little or no β-III tubulin at both day 10 and 21. Under neuronal differentiation conditions, a proportion of cells retain GFAP expression. β-III tubulin is expressed by day 10 and retained at day 21. At day 21, cells that express β-III tubulin begin to exhibit growth patterns whereby large clumps of β-III tubulin positive cells cluster and extend processes towards adjacent clusters of similar cells. These immunofluorescent investigations help give an idea of an appropriate pattern of antigenic expression to be expected during differentiation experiments with various stem cell types. Results are representative of three replicates.

4.2.2 Non-neural stem cell differentiation into neural lineage cells

4.2.2.1 Adherent CD34+ cells

To determine if plastic adherent CD34+ cells differentiate into neural lineage cells *in vitro*, previously described neuronal differentiation methods were first used[12,75-77]. These published protocols were used for bone marrow-derived cells, the details of which are described in chapter 2 materials and methods, neural induction protocols 1-4[12,75-77]. Woodbury and colleagues previously observed neuronal differentiation capabilities of bone marrow-derived cells by treating them with DMSO, followed by bFGF for 24 hours (neural induction protocol 1)[75]. Tao et al[76] induced neural differentiation of bone marrow derived cells with cytokines known to be present in the brain microenvironment, bFGF, EGF, and PDGF (neural induction protocol 2). Munoz-Elias and colleagues[77] employed a serum free method to differentiate plastic adherent mesenchymal stem cells (MSC), using a pre-induction step with BHA, followed with cytokines bFGF, EGF, and PDGF (neural induction protocol 3). Jiang and colleagues[12] induced bone marrow derived cells into neuro-ectodermal cells by using DMEM/F12 with N2 supplement, and additional growth factors basicFGF, sonic hedgehog, FGF8, and retinoic acid (neural induction protocol 4). When cultured using these protocols, adherent CD34+ cells died within 24-72 hours. Variations of these protocols were then used in attempts to induce neural differentiation of adherent CD34+ cells. These protocols are described in chapter 2, neural induction protocols 5-9. Neural differentiation was not observed under any of these conditions (Figure 17). Positive controls were used to ensure that the antibodies used for immunofluorescent microscopy were functioning properly.

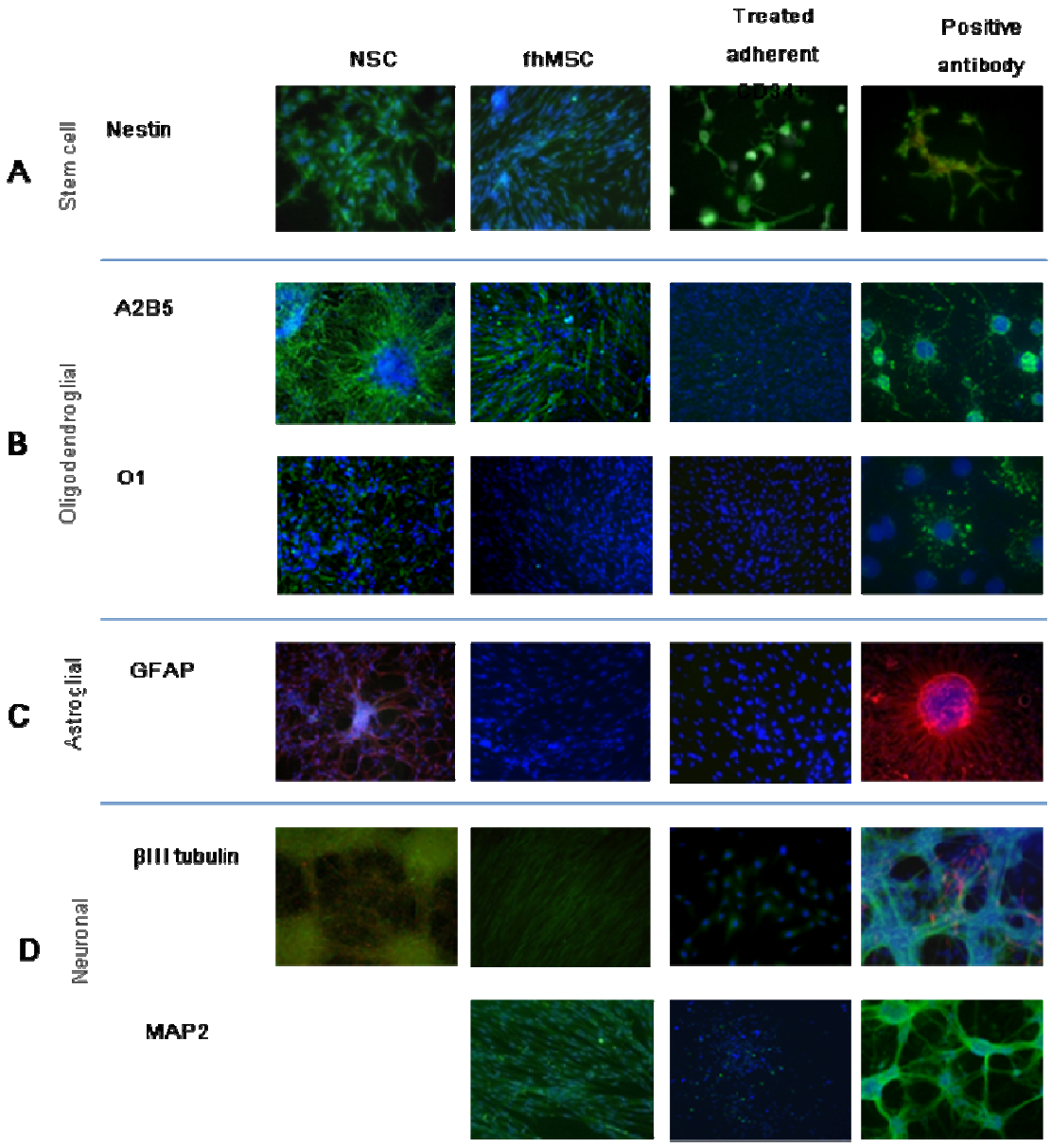


Figure 17. Neural differentiation of bone marrow-derived cells. The stem cell marker nestin is expressed by NSC, fhMSC, and adherent CD34+ cells (**A**). Neural induction of adherent CD34+ cells and fhMSC revealed that these bone marrow derived stem cells do not differentiate into oligodendrocytes (**B**), astrocytes (**C**), nor neurons (**D**), *in vitro*. Human or rat neural cells were used as positive staining controls for neural antibodies. Results are representative of three replicate experiments.

4.2.2.2 Foetal mesenchymal stem cells

A similar experimental protocol was used to induce neural differentiation of foetal human mesenchymal stem cells (fhMSC). The cells were plated onto poly-D-lysine/fibronectin coated plastic 96 well plates. As a positive control for differentiation, NSCs were plated and treated in parallel. Human fibroblasts (hFibs) were used as a negative control, treated in parallel to fhMSCs. The hFibs were not expected to differentiate into neural cells nor to express neural antigens. After 10 days in culture, fhMSCs expressed low levels of nestin. After 21 days, both treated and untreated fMSCs express nestin. Treated cells also exhibited morphological changes, assuming a bipolar morphology under neural differentiation conditions rather than the fibroblast-like morphology of the untreated fhMSCs. At 21 days, untreated and treated fhMSCs expressed β-III-tubulin at low levels, with no significant difference in expression (Figure 18). At 10 and 21 days of treatment, fhMSCs did not express oligodendrocyte specific antigens (A2B5, O4, Olig2, GalC) nor astrocyte specific antigens (GFAP) (Figure 18).

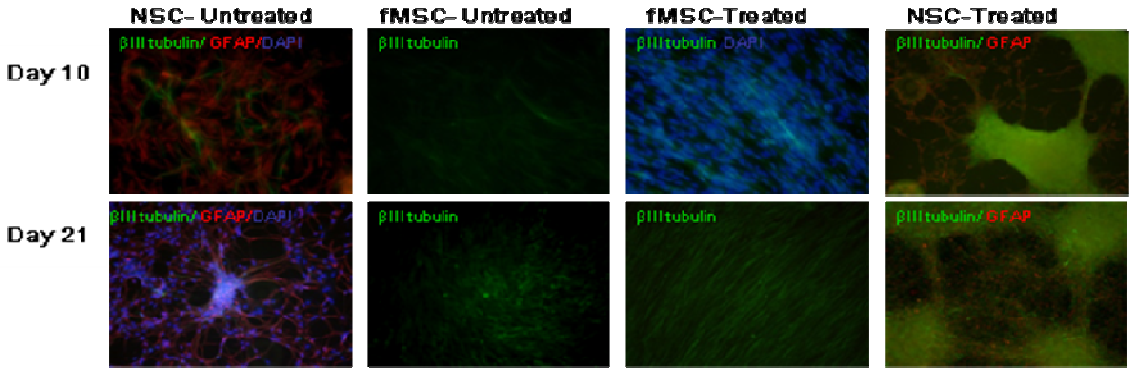


Figure 18. Foetal human mesenchymal stem cells express low levels of β-III-tubulin in culture. Under neural differentiation treatment conditions, fhMSCs have higher expression of β-III-tubulin after 10 and 21 days. Neural stem cell controls cultured and treated in parallel to fhMSCs express little or no β-III-tubulin under standard conditions, but are positive for the neuronal marker after 10 and 21 days in treatment. Untreated, NSCs express astrocytic GFAP, but GFAP expression seems to be downregulated under neuronal differentiation treatment. The fhMSCs do not express GFAP with or without treatment conditions in neither 10 nor 21 days. Results are representative of three replicate experiments.

4.2.3 Chromatin state of bone marrow derived cells

The results described above suggest that adherent CD34+ cells and fMSCs do not differentiate into neural lineage cells *in vitro*. To substantiate this view, DNA replication timing experiments were conducted to determine if genes associated with neural cell differentiation are replicated early by CD34+ cells, adherent CD34+ cells, non-adherent CD34+ cells and fhMSC. As controls for the replication timing of neurogenic genes, NSCs and ES cells were also analysed. Early replication of genes during S phase of the cell cycle is considered to be a feature of transcriptionally active regions of the genome, indicating chromatin accessibility, whereas late replication is characteristic of inactive gene regions and constitutive heterochromatin[46].

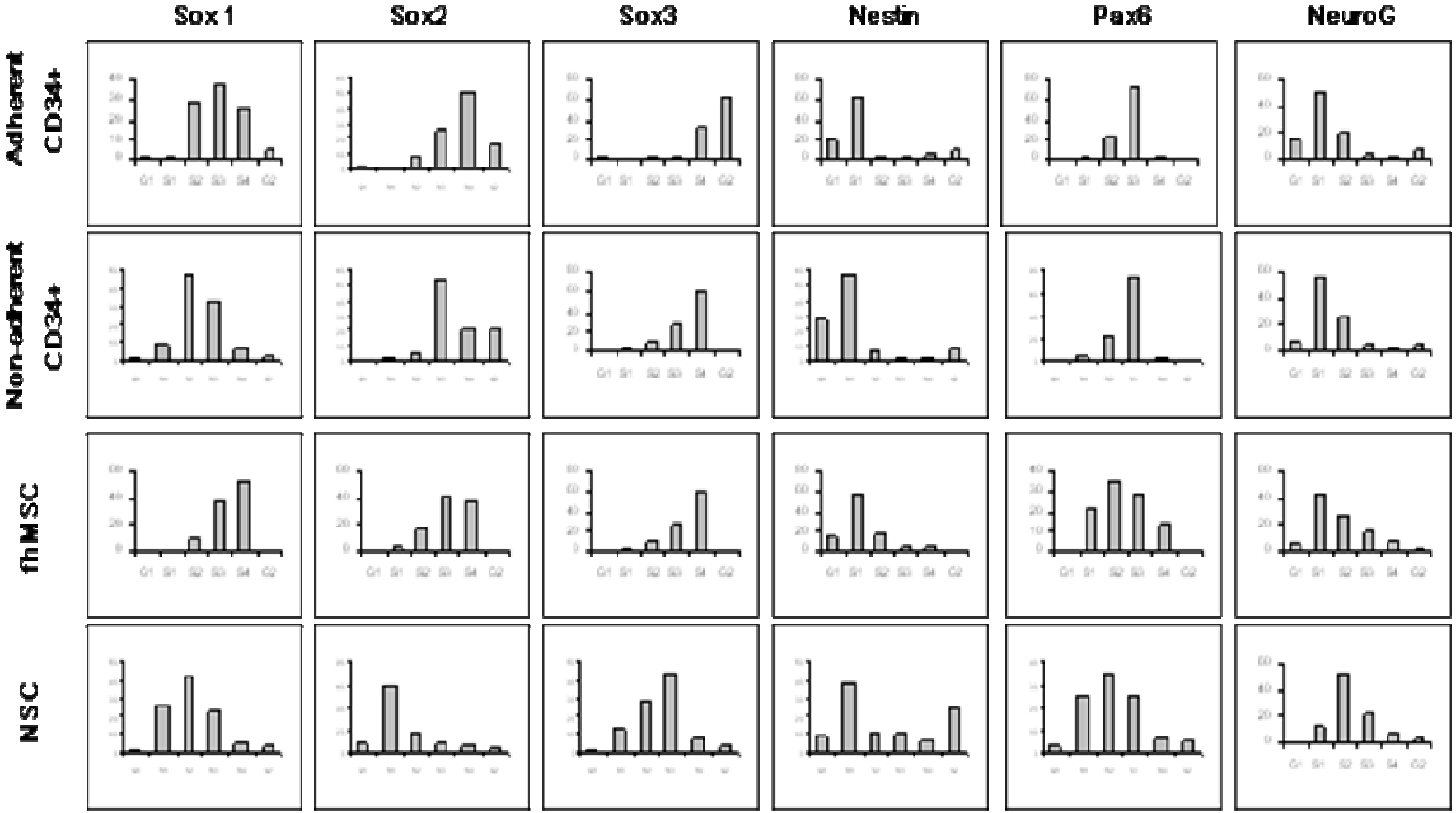
These experiments examined whether total CD34+ cells, adherent CD34+ cells, non-adherent CD34+ cells and fMSCs have epigenetic features which associate them with neural lineage restricted NSCs. The timing of DNA replication was assessed as before (section 3.2.3). The relative abundance of chosen sequences within newly synthesized DNA then determined using real-time qPCR (Figure 12) and replication timing colour coded as described above (Figure 12).

4.2.3.1 Adherent CD34+ cells and fhMSCs do not replicate neurogenicity genes early

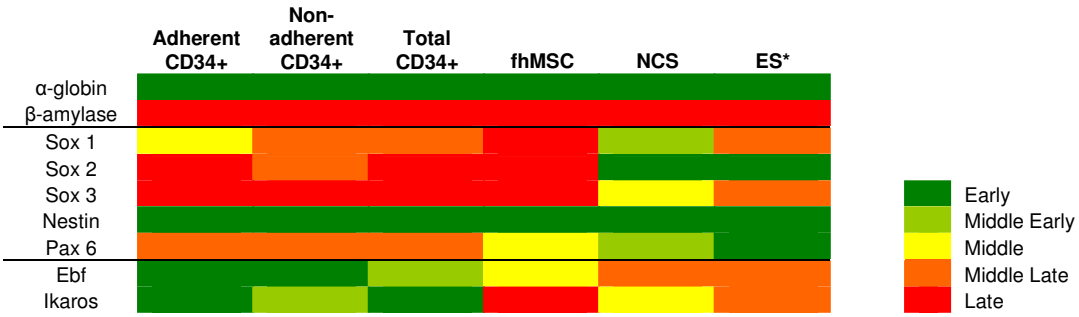
To determine when genes involved in neural cell fate determination are replicated in NSC, DNA replication timing for loci of *SOX1, SOX2, SOX3, NESTIN,* and *PAX6* in NSC was conducted (Figure 19). *SOX1, SOX2, SOX3* and*, PAX6* were replicated late in the cell cycle. *NESTIN* was replicated at G1/S1 early stages of the cell cycle. *SOX3* was not replicated until S2-S3. To ensure that early replication was specific to neural related genes, we also probed for haemopoietic genes Ebf and Ikaros expression in NSCs. Both haemopoietic genes were replicated middle (S2-S3) to middle-late (S3) in NSCs, as predicted.

Ensuring that neurogenic genes replicate early in NSCs, the study continued by determining if bone marrow derived cells have the propensity to express neurogenic genes. The replication timing of *SOX1, SOX2, SOX3, NESTIN,* and *PAX6* in total CD34+ cells, adherent CD34+ cells, non-adherent CD34+ cells, and fhMSCs relative to known neurogenic NSC. Genes involved in neural specification during development replicated late in the cell cycle of the bone marrow derived populations (Figure 19). *SOX1, SOX2,* and *SOX3* replicated during S2-S4/G2, middle to late during the cell cycle. The stem cell marker *NESTIN* replicated early in G1 in all cell populations, indicating the propensity of *NESTIN* expression. Nestin is a broadly expressed gene and protein, thus its early replication and expression is not unexpected. All bone marrow derived cells have a similar pattern of DNA replication timing of neurogenic genes. This suggests that the chromatin structure of the loci for neurogenic genes in total CD34+, adherent CD34+, non-adherent CD34+, and fhMSC makes neural expression unlikely.

A


Sox1
Sox2
Sox3
Nestin
Pax6
NeuroG
Adherent CD34+
Non-adherent CD34+
fhNSC
NSC

B



Adherent CD34+: Adherent CD34+ cells which are thought to be primitive quiescent stem cells
Non-Adherent CD34+ : Non-adherent CD34+ cells (to compare to the asherent CD34+ cells)
Total CD34+ : CD34+ cells from peripheral blood
fhMSC: Foetal human mesenchymal stem cells
NSC: Human neural stem cells
ES: Human embryonic stem cells **Replication timing prepared by Sandra Pinho*

Figure 19. DNA replication timing of nerogenicity-associated genes by different stem cell populations. Cells were fractionated by cell cycle phase and probed for the loci in question. Relative abundance of genes in each fraction was analysed using real-time qPCR (**A**). The fraction with the highest gene expression is said to be the time in the cell cycle when the gene is replicated. Genes replicated early in the cell cycle are more accessible and its transcription likely. BrdU purified templates from cell cycle fractions of adherent CD34+ cells, non-adherent CD34+ cells, total CD34+ cells, fhMSCs, and NSC were probed for neurogenicity genes Sox1, Sox2, Sox3, Nestin, and Pax6. The peak relative abundance of Sox1, Sox2, Sox3, and Pax6 genes were late in the cell cycle suggesting that these cells are not likely to be neurogenic. The histogram for the replication timing of each gene is represented as a coulor code (**B**).

To validate replication timing data, real-time qPCR was conducted to determine relative expression of *SOX1, SOX2, SOX3, PAX6, EBF, REX1, NANOG,* and *OCT4* in adherent CD34+, non-adherent CD34+, fetal mesenchymal stem cells (24XY fMSC), and neural stem cells (R197) (Figure 20). This epigenetic evidence validates the previous findings that adherent CD34+ cells and fhMSCs do not differentiate into neural cells.

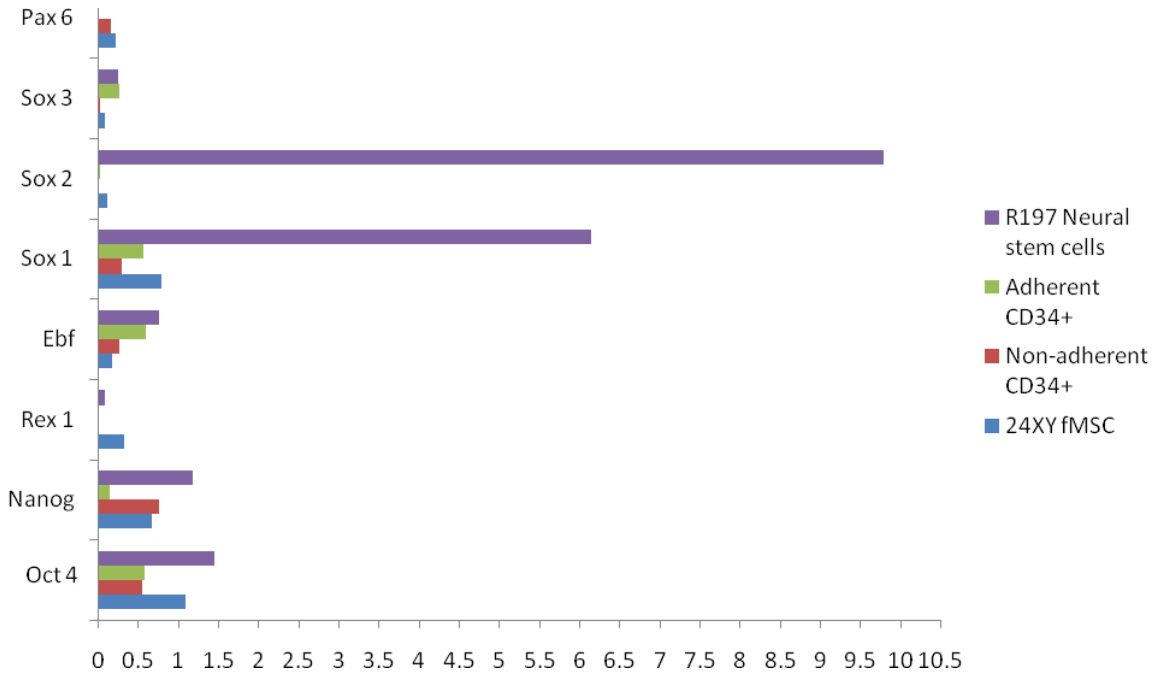


Figure 20. Real-time qPCR of pluripotency and neurogenicity-associated genes expressed by bone marrow-derived stem cells. This histogram shows relative expression of pluripotent and neurogenic genes expressed in adherent CD34+ cells, non-adherent CD34+ cells, fhMSC and NSC. The low expression of neural genes supports previous replication timing data. Relative expression was normalized to the expression of housekeeping genes *GAPDH* and *HPRT*. Results are an average of two replicate experiments, thus any statistical significance in expression among populations cannot be determined.

4.3 Discussion

Investigations have shown an increase in neural cell proliferation and neurogenesis after insult[66,67], and a mounting number of studies attempt to stimulate post-natal neurogenesis as an alternative therapy to transplantation. It has been demonstrated that in response to acute ischaemia in the forebrain, endogenous neural stem cells proliferate, are recruited to the penumbra region, and give rise to hippocampal pyramidal neurons. Infusion of exogenous growth factors FGF2 and EGF led to 40% recovery of the neurons lost due to ischaemia. Newly generated neurons show functional synapse formation as well as recovery of brain function[66]. This points to the possibility of stimulating neurogenesis of endogenous neural stem cells using pharmaceuticals as an alternative to transplantation.

There are two main lines of investigation which address neural lineage differentiation of cells from non-neural tissue. First, several groups have independently identified stem and progenitor cells in peripheral blood and bone marrow which express neural lineage markers[15,68,118,119,123]. Second, cells derived from non-neural tissue such as umbilical cord, peripheral blood and bone marrow have been induced to express a neural phenotype[12,75,77,121]. This leaves the question: are there pre-determined neural committed precursor cells outside the CNS/PNS, or are there adult derived stem cells which are truly plastic?

Here previously defined protocols[12,75-77] were employed for neural differentiation of bone marrow derived cells in order to differentiate adherent CD34+ cells and fMSCs into neural cells. To ensure protocols used were appropriate for neuronal or glial differentiation, NSCs were cultured in the differentiation media to ensure that the culture conditions induced lineage specified differentiation. This study found that adherent CD34+ cells do not differentiate into neurons or oligodendrocytes under the conditions used. Adherent CD34+

cells did not reproducibly differentiate into astrocyte-like cells. In three of five experiments, adherent CD34+ cells upregulated markers S100β and GFAP, and demonstrated morphological changes (data not shown). The reproducibility of these observations was questionable, and it is not possible to conclude that neural differentiation occurred. The literature reveals that bone marrow-derived cells have been observed to spontaneously differentiate into astrocyte like cells[12,75,77,121]. The observed differentiation of adherent CD34+ cells may be spontaneous and an artefact of culture. It is possible that the neural differentiation medium provided an appropriate inductive microenvironment, inducing the differentiation of adherent CD34+ cells into astrocyte-like cells. Inconsistencies may also be due to variations in donors.

Similar results were observed when fhMSCs were cultured in neural induction medium. There was no morphological or antigenic evidence of glial differentiation. In a single instance, in neuronal differentiation conditions, fhMSC upregulated the neuronal markers PSA-NCAM and MAP2. This result was irreproducible and observed only once in five replicate experiements. It is noteworthy that both adherent CD34+ cells and fhMSC upregulated neural markers in single instances indicating that it might be a rare event. The cellular mechanisms which govern the spontaneous differentiation of bone marrow-derived cells may be the key to *in vitro* differentiation across germ lineages. With this in mind, previous irreproducible results from other studies may have been artefacts or random spontaneous events.

As cells replicate their DNA, some sequences replicate early in S phase (G1-S1), while others replicate late in S phase (S3-S4). Early replication is associated with chromatin accessibility and the active transcription of the gene. In this study the replication kinetics of genes which

encode transcription factors important during neural development, *SOX1, SOX2, SOX3,PAX6,* and *NESTIN* were examined. Non-neural adherent CD34+ cells and fhMSCs were compared to neurogenic NSCs. The genes which control developmental neurogenesis are replicated early in NSCs, but late in adherent CD34+ cells and fhMSCs. The replication timing findings validate the above *in vitro* neural differentiation experiments. Replication timing revealed that the chromatin structure of bone marrow-derived cells may decrease the propensity of these cells to actively transcribe neural genes. As the chromatin structure does not allow genes involved in neurogenesis to be easily transcribed, neural differentiation of adherent CD34+ cells and fhMSCs is unlikely.

It was proposed that adherent CD34+ cells are multilineage primed stem cells which express lineage specific genes associated with liver, pancreas, and neural tissues. It was hypothesized that the genetic expression of neural markers enables adherent CD34+ cells to differentiate into neural lineage cells when placed in a neural inductive microenvironment. However, the results of the differentiation and replication timing experiments suggest that adherent CD34+ cells and fhMSCs do not have neurogenic potential *in vitro*. Although real-time qPCR shows that adherent CD34+ cells and fhMSCs express low levels of neurogenic genes, and spontaneously upregulate neural associated proteins, the DNA replication timing data suggests that these cells may not have a definitive potential for neural differentiation. Studies have revealed that bone marrow-derived cells affected during CNS injury, such as recruitment during ischaemia, immunosuppressive properties, promote/inhibit angiogenesis[124,125]. Perhaps direct differentiation of bone marrow and peripheral blood derived adherent CD34+ cells are not an appropriate means for cell therapy; instead it may be possible to exploit the indirect effects of adherent CD34+ cells.

Chapter 5 Microglial differentiation of adherent CD34+ cells

Chapter summary

It is known that the immune cells of the CNS, called microglia, are of haematopoietic developmental origin[57, 61, 62, 117, 130]. Davoust and colleagues[130] demonstrated that a proportion of bone marrow derived cells differentiated into microglia when exposed to astrocyte conditioned medium. As peripheral blood and bone marrow derived CD34+ cells are of haematopoietic origin, it was hypothesized that adherent CD34+ cells are capable of differentiating into microglia. Astrocyte conditioned medium was used to induce microglial differentiation of adherent CD34+ cells. Adherent CD34+ cells exposed to astrocyte conditioned medium upregulated antigens associated with microglia. Adherent CD34+ cells derived microglia also released TNFα and IL-6 in response to LPS, which is a functional property of microglia. This chapter describes the following:

- Adherent CD34+ cells upregulate morphological features and antigenic expression of microglia
- Adherent CD34+ cell derived microglia respond to LPS
- Adherent CD34+ cell derived microglia secrete TNFα and IL-6 in response to LPS

5.1 Role of microglia

Glial cells provide physiological support for neurons and maintain homeostasis by aiding in neuronal repair during injury or disease. Glial cells are classified as macroglia, which are the astrocytes and oligodendrocytes, and microglia, which are the immune effector cells of the central nervous system (CNS). Macroglia are derived from neuroectoderm, but the developmental origin of microglia remains debatable. Although traditional concepts state that microglia are derived from neuroepithelial cells, current studies suggest that microglia residing in the CNS are of mesodermal origin[62,117,126]. In the adult brain, microglia are recruited from peripheral blood monocytes, originating from the bone marrow[62,126,127].

Several studies have demonstrated that, in irradiated chimeric mice, neural injury induced an increase in microglial recruitment and engraftment of GFP+ bone marrow-derived cells in the brain[60,62,117,128,129]. Furthermore, models of neural injury include those characterized by an influx of leukocytes from the blood as a result of an altered blood-brain barrier; those characterized by region-specific neurodegeneration such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis; and models of focal or acute neural degeneration such as facial nerve axotomy[129,130]. Under specific conditions, bone marrow-derived cells are able to cross the blood brain barrier and differentiate into microglial cells[129,131]. This observation implies that the adult bone marrow contains a subpopulation of cells with microglial differentiation potential.

As microglia are suggested to be of haemopoietic developmental origin, the aim of this part of the study was to determine if adherent and/or non-adherent CD34+ cells can differentiate into microglia. Several factors suggest that adherent CD34+ cells might be microglial precursors including (*i*) adherent CD34+ cells are more primitive than non-adherent CD34+ cells, in terms of their potential for differentiation, (*ii*) the previously described microarray study revealed that adherent CD34+ cells highly express genes which encode for transendothelial migration, indicating that they are more likely to respond to injury than their non-adherent CD34+ counterparts, and (*iii*) adherent CD34+ cells more highly express CCL2, the ligand thought to be responsible for adhesion to, and migration through the blood brain barrier[130]. Here, different subpopulations of bone marrow-derived cells were placed under microglial differentiation conditions previously described by Davoust et al[130]. This study revealed that mesenchymal stem cells die when placed in microglia differentiation conditions, and that non-adherent CD34+ cells do not demonstrate any characteristic of microglia-like cells. Adherent CD34+ cells demonstrate morphological and antigenic

evidence of microglial differentiation. Functionality of these adherent CD34+ cell derived microglia-like cells (CD34MG) was assessed by examining baseline cytokine secretion, cytokine secterion in response to LPS activation, and antigen presentation capabilities.

5.2 Results

5.2.1 Adherent CD34+ cells differentiate into cells with microglia-like morphology

Adherent CD34+ cells or non-adherent CD34+ cells were cultured in astrocyte conditioned medium (ACM) for 7, 10, or 21 days. No significant morphological changes were observed in the adherent CD34+ cells cultured for 7 days, but after 10 days, approximately 40% of them assumed the morphology of microglia, including a ramified appearance and small neurite spines (Figure 21). After 21 days, approximately 80% of the adherent CD34+ cell-derived microglia-like cells (henceforth referred to as CD34MG) had multiple cellular processes (Figure 1). In contrast, no morphological changes were observed in non-adherent CD34+ cells cultured in ACM after 7, 10 and 21 days.

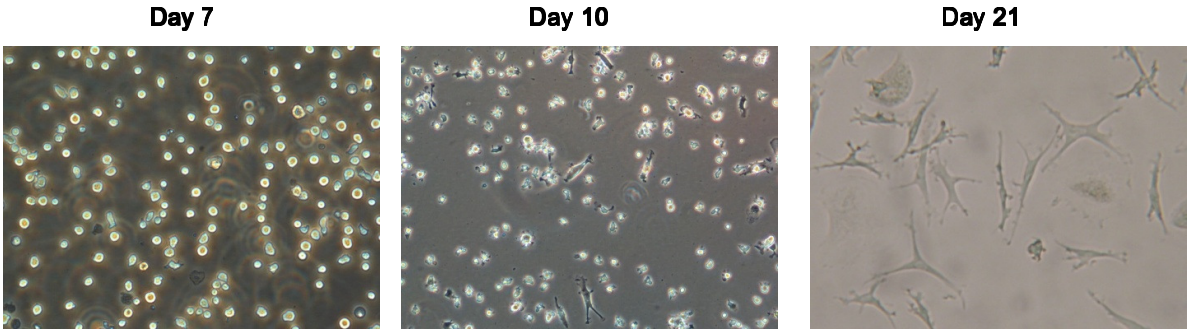
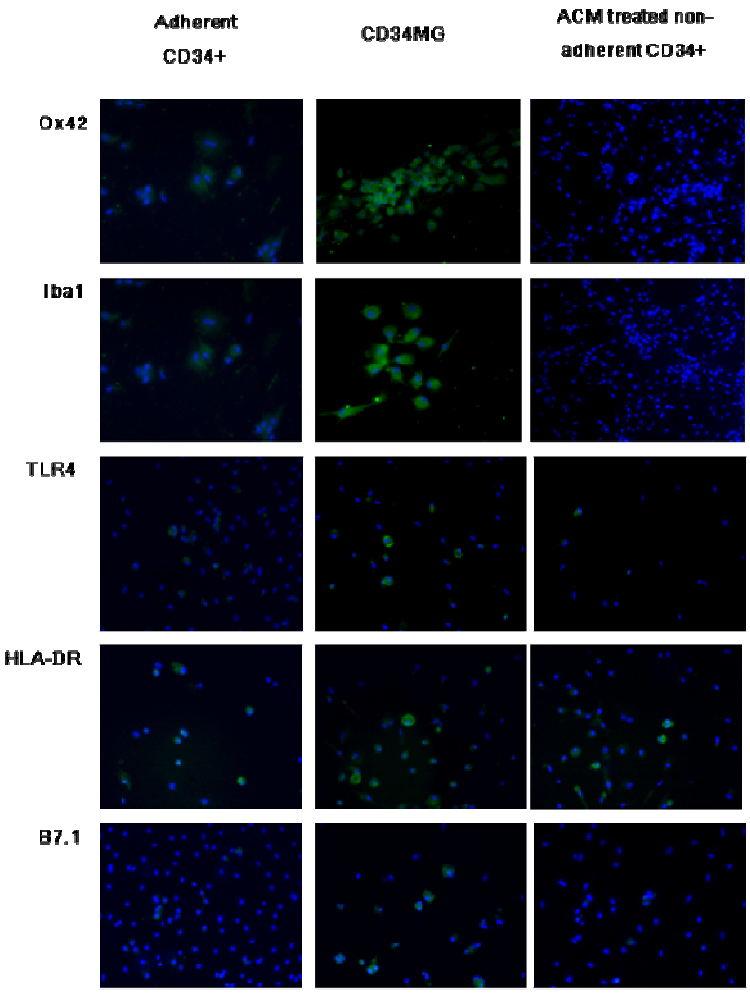


Figure 21. Adherent CD34+ cells upregulate morphological changes in response to astrocyte conditioned medium. To induce microglial differentiation, adherent CD34+ cells were treated cultured in ACM 24hrs after isolation. After 7 days in ACM, no morphological changes were observed, but after 10 days, cells had similar morphology to the bipolar microglia in a resting state. Twenty one days in ACM treatment induced a ramified resting microglial morphology with multipolar processes.

5.2.2 CD34MG upregulate microglial markers

Expression of antigens associated with microglia, Ox42, Iba1, and HLA-DR was investigated by immunofluorescence microscopy (Figure 22). Newly isolated adherent CD34+ cells expressed low levels of microglial markers. About 9 ± 4.2% expressed Ox42, 5 ± 2.7% expressed Iba1, 1 ± 0.7% expressed TLR4, about 9 ± 2.8% expressed HLA-DR, and none expressed B7.1. Since morphological changes were not observed until after 10 days of culture in ACM, immunofluorescence microscopy was conducted at this time. A significantly higher frequency of CD34MG cells than adherent CD34+ cells expressed these microglial markers; 81 ± 10.4% of adherent CD34+ cells which expressed Ox42, 71 ± 9.6% expressed Iba1, 16 ± 7.3% expressed TLR4, 35 ± 8.1% expressed HLA-DR, and 16 ± 6.4% upregulated the co-stimulatory molecule B7.1. Non-adherent CD34+ cells expressed very low levels of Ox42, Iba1, and TLR4. Interestingly, 38 ± 6.2% of non-adherent CD34+ cells expressed HLA-DR and 21 ± 12.6% expressed co-stimulatory molecule B7.1. Exposure to ACM for 10 days caused a decrease in HLA-DR and B7.1 expression by non-adherent CD34+ cells (Figure 22)



Antigen	Adherent CD34+ cells	CD34MG	Non-adherent CD34+ cells	ACM treated non-adherent CD34+ cells	n=
Ox42	9 ± 1.3	81 ± 2.9	3 ± 2.9	7 ± 4.3	5
p= (compared to adherent CD34+ cells)*		0.0001	0.25	0.29	
Iba1	5 ± 2.8	72 ± 7.9	7 ± 1.7	13 ± 5.6	5
p= (compared to adherent CD34+ cells)*		0.0012	0.55	0.003	
TLR4	1 ± 1.8	16 ± 3.5	7 ± 0.33	3 ± 2.4	4
p= (compared to adherent CD34+ cells)§		0.02	0.004	0.014	
HLA-DR	9 ± 4.2	35 ± 3.6	38 ± 3.2	27 ± 5.2	5
p= (compared to adherent CD34+ cells)*		0.002	0.0014	0.02	
B7.1	2 ± .9	16 ± 0.6	21 ± 4.4	8 ± 2.4	3
p= (compared to adherent CD34+ cells) §		0.001	0.037	0.31	

*p-value determined using the Wilcoxon Signed Rank nonparametric test on SPSS software

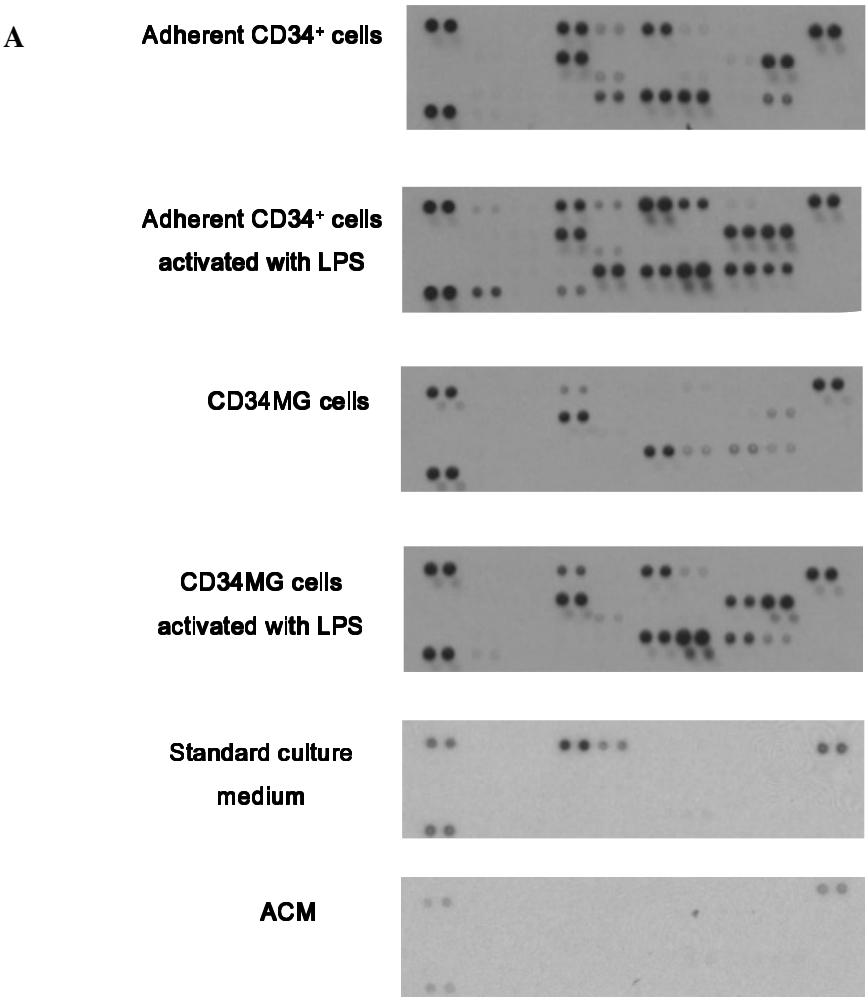
§ p-value determined using paired student's t-test on SPSS software

Figure 22. CD34MG cells upregulate microglia-associated markers. Adherent CD34+ cells and non-adherent CD34+ cells were treated with ACM for 10 days and microglial antigen expression of Ox42, Iba1, TLR4, HLA-DR and B7.1 was assessed with immunofluorescence microscopy. Upregulation of microglia associated markers by CD34MG was statistically more significant than untreated adherent CD34+ cells. ACM treated non-adherent CD34+ cells did not significantly upregulate the panel of markers used to identify microglia.

5.2.3 Cytokine secretion by adherent CD34+ cells and CD34MG

To determine if adherent CD34+ cells and CD34MG secrete cytokines, a semi-quantitative human cytokine array was used to measure relative amounts of release of 36 cytokines which were pre-determined by the manufacturer. Supernatant from adherent CD34+ cells (5 days after medium change) and supernatant from CD34MG (5 days after medium change), as well as fresh standard culture medium and ACM was collected and analyzed. Adherent CD34+ cells secrete CXCL1, IL1ra, IL8, IL16, MCP1, MIP1α, MIP1β, and SerpinE1. After treatment in ACM for 10 days, there was a downregulation of all cytokines, except for an increase in SerpinE1 release. CD34MG secrete relatively lower levels of IL1ra, IL8, MIF, MIP1α, MIP1β, and SerpinE1 (Figure 23). This downregulation of cytokine secretion may suggest that as adherent CD34+ cells differentiate into CD34MG, they downregulate Th1 cytokine secretion. This is consistent with the literature which reports that microglia are less immunocompetent than peripheral blood macrophages and other immune cells found in the circulation.

A


Adherent CD34+ cells
Adherent CD34+ cells activated with LPS
CD34MG cells
CD34MG cells activated with LPS
Standard culture medium
ACM

B

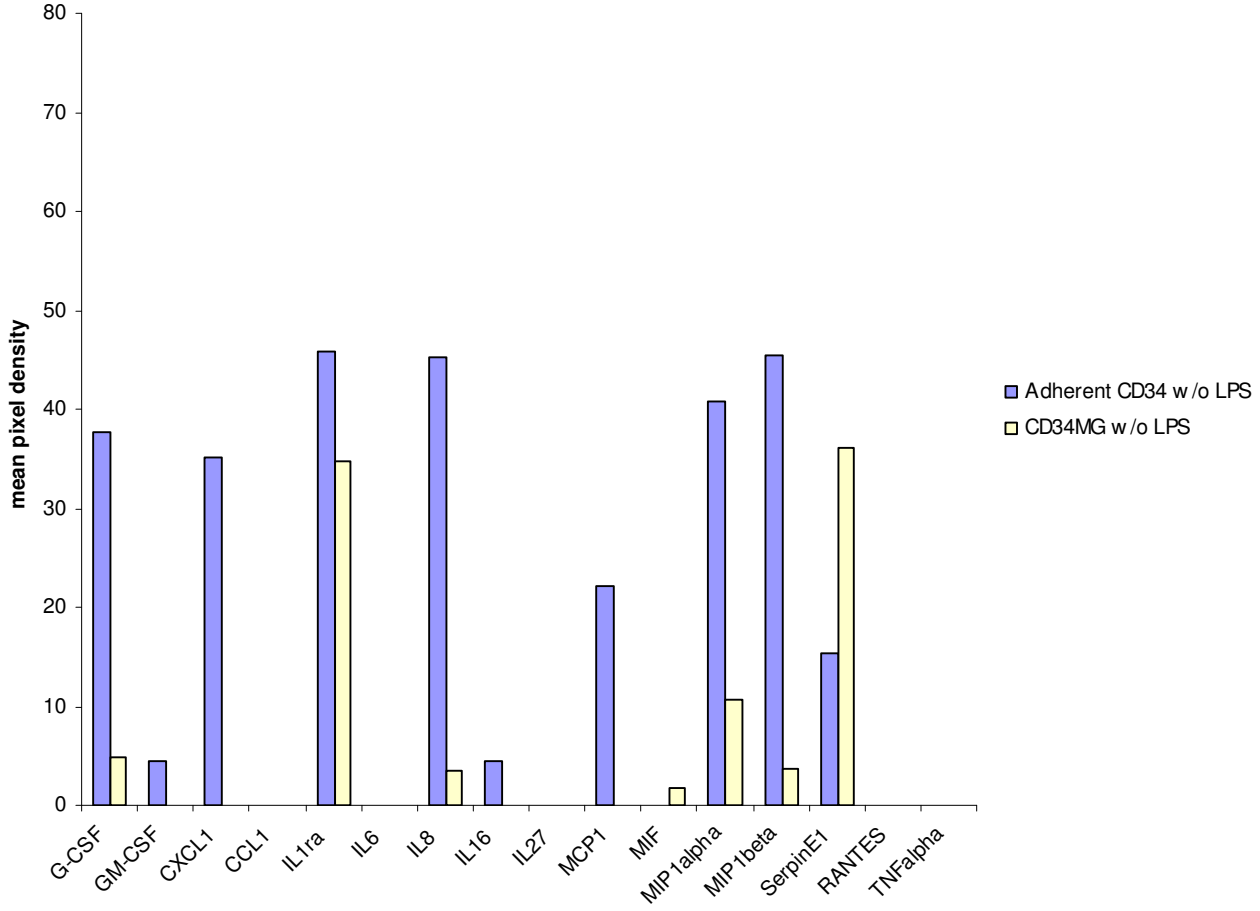


Figure 23. Cytokine released by adherent CD34+ cells and CD34MG. Supernatants were collected from cultures of adherent CD34+ cells and CD34MG cells, and analyzed for cytokine content using a semi-quantitative cytokine array (**A**). The array measured presence of cytokine and its relative abundance between samples which was captured on X-ray film. Locations of spots on the array corresponded with specific cytokines pre-defined by the manufacturer. Densitometry measurements are represented in histogram form (**B**). Densitometry of relative cytokine abundance between samples was measured and recorded as mean pixel density of each spot on the array. Adherent CD34+ cells secrete more pro-inflammatory cytokines CXCL1, IL-1ra, IL-8, IL-16, MCP-1, MIF, MIP1α, and SerpinE1. CD34MG secrete relatively lower levels of IL-1ra, IL-8, MIF, MIP1α, MIP1β, and SerpinE1. These results represent an average of two experiments.

5.2.4 LPS activation

Previous immunofluorescence analysis revealed that a low frequency of adherent CD34+ cells and CD34MG express TLR4, the receptor through which gram negative bacteria activate an immune response in certain cells including microglia. To determine if adherent CD34+ cells and CD34MG become activated in response to LPS, 1µg of LPS was added to cell cultures for two hours and the supernatants were collected and analyzed as before. The results revealed that adherent CD34+ cells released greater amounts of CXCL1, CCL1, IL6, IL8, MCP1, MIF, MIP1α, SerpinE1, RANTES, and TNFα in response to LPS (Figure 24a). There was downregulation of IL1ra, IL16, and MIP1β. This upregulation of pro-inflammatory cytokines indicates activation via TLR4. When exposed to LPS for 2 hours, CD34MG released more amounts of G-CSF, CXCL1, IL1ra, IL6, IL8, IL27, MIF, MIP1α, and MIP1β when exposed to LPS for 2 hours relative to CD34MG without LPS activation (Figure 24b). This indicates pro-inflammatory activation of CD34MG via TLR4. LPS activation induced an increase in SerpinE1 release, but the opposite pattern was observed in the CD34MG cells where LPS induced a decrease in SerpinE1 secretion (Figure24a and b).

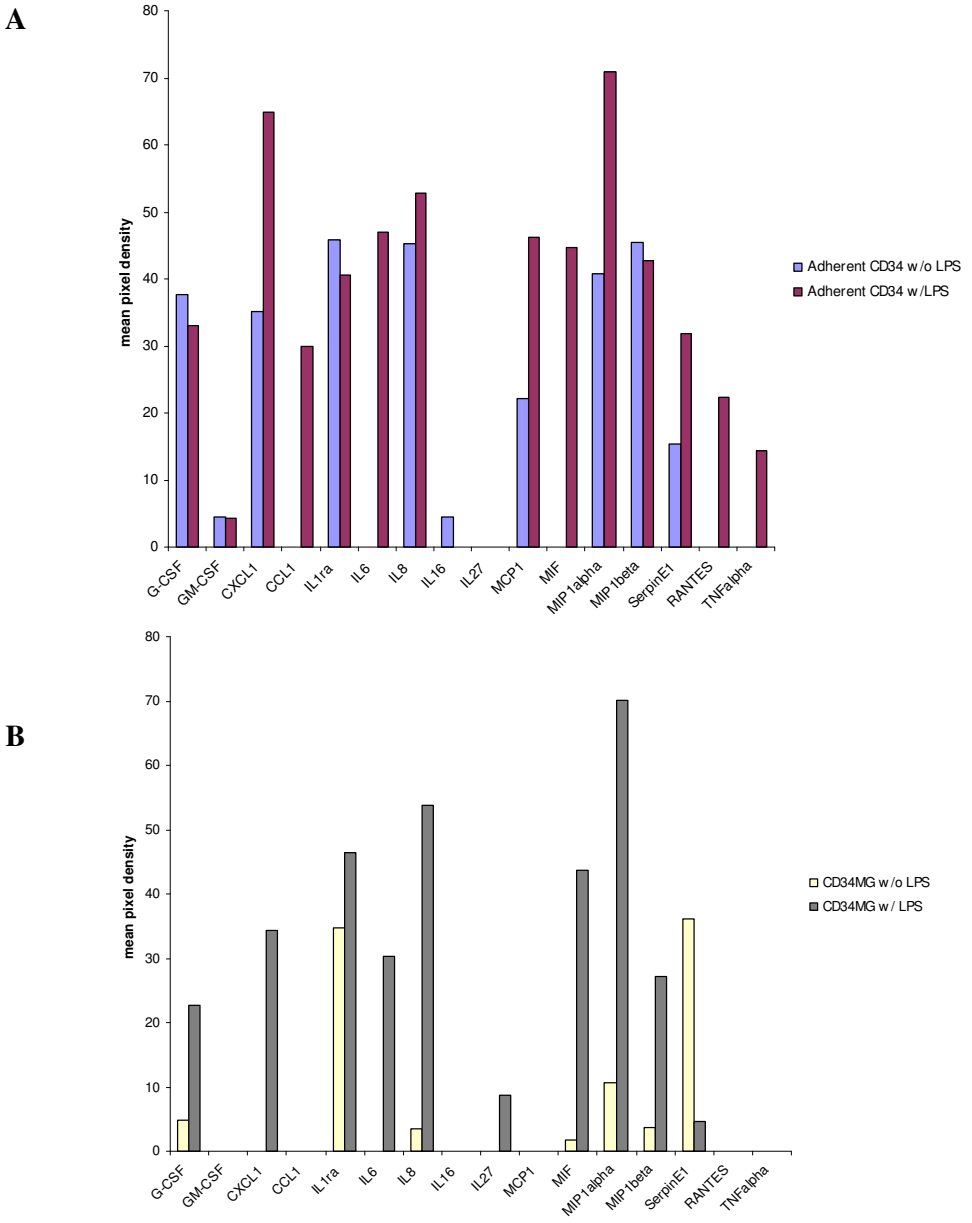


Figure 24. Exposure to LPS alters cytokine secretion by adherent CD34+ cells and CD34MG. Exposure of adherent CD34+ cells and CD34MG to LPS caused a pro-inflammatory activation in both populations. Adherent CD34+ cells secreted relatively more amounts of cytokine after activation with LPS, as well as a greater number of pro-inflammatory cytokines (**A**). CD34MG cells secreted significantly greater amounts of pro-inflammatory cytokines as well as a greater number of cytokines

relative to their non-activated counterparts (B). These results represent an average of two experiments.

Release of TNFα and IL-6 is a hallmark of microglial LPS activation. Neither adherent CD34+ cells nor CD34MG cells released TNFα nor IL-6 in their resting states. The semi-quantitative cytokine array revealed that LPS activation induced TNFα and IL-6 release by adherent CD34+ cells, and IL-6 release by CD34MG.

A quantitative analysis of TNFα and IL6 secretion by adherent CD34+ cells and CD34MG cells with and without LPS activation was then performed. Both adherent CD34+ cells and CD34MG were exposed to LPS for 2, 4, 8, 16, 32, and 48 hours, and supernatant and cell lysates were collected. This procedure was repeated 7, 10, and 21 days after the beginning of ACM treatment.

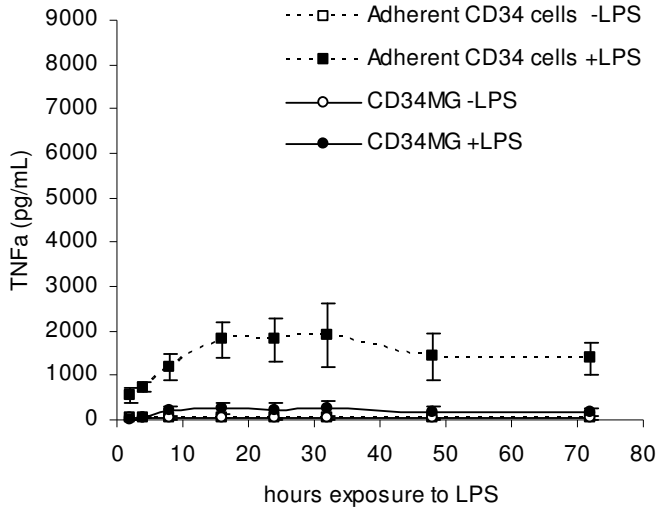
5.2.5 TNFα

The quantitative assay revealed that without LPS activation, adherent CD34+ cells do not constitutively secrete TNFα at 7, 10, or 21 days of culture (Figure 25). After LPS activation, day 7 adherent CD34+ cells secrete peak levels of 1887.4pg/ml TNFα after 32 hours. LPS activation of day 10 adherent CD34+ cells induced peak TNFα release only 4 hours after activation, releasing 1262.56pg/ml. Day 21 cells peak secretion of TNFα was also after 4 hours of LPS activation, secreting 1082pg/ml (Figure 25).

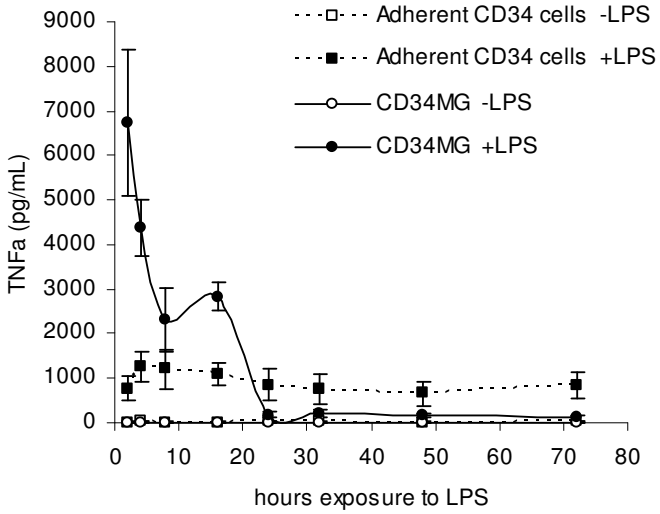
CD34MG cells in an unactivated resting state constitutively release low amounts of TNFα. Analysis of ACM medium alone confirmed that it did not contain any TNFα. Day 7 CD34MG supernatants were found to contain 31.2pg/ml TNFα; day 10 and 21 CD34MG secreted lower amounts of 3.09- 8.3pg/ml TNFα (Figure 25).

LPS activated day 7 CD34MG cells secreted 2pg/ml after 2 hours to 244pg/ml after 16 hours of LPS exposure. Day 10 and 21 CD34MG demonstrated a release of TNFα after 2 hours of LPS exposure with peak cytokine levels of 4385pg/ml and 4319pg/ml respectively. After peak cytokine release at 2 hours, TNFα levels decline. This may reflect an initial response of TNFα release and its subsequent degradation or metabolism by the cells (Figure 25). The limitation of this assay is that cytokine secretion is not measured until 2 hours of exposure to LPS. A measurement of immediate cytokine secretion over shorter periods of time may be necessary in the future to confirm cytokine secretion.

A


Adherent CD34 cells -LPS
Adherent CD34 cells +LPS
CD34MG -LPS
CD34MG +LPS
TNFa (pg/mL)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
hours exposure to LPS

B


Adherent CD34 cells -LPS
Adherent CD34 cells +LPS
CD34MG -LPS
CD34MG +LPS
TNFa (pg/mL)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
hours exposure to LPS

C

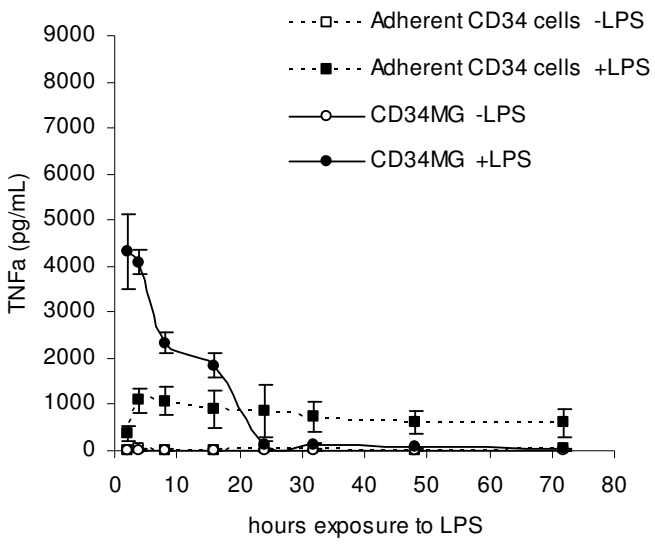


Figure 25. TNFα secretion by adherent CD34+ cells and CD34MG. (**A**) LPS exposure commenced on the 7th day of culture and continued for 72 hours. Untreated adherent CD34+ cells were used as controls. (**B**) LPS exposure commenced on the 10th day and (**C**) 21st day of culture. Unstimulated resting adherent CD34+ cells did not constitutively secrete TNFα in culture, but secreted cytokine after LPS activation. CD34MG cells did not constitutively secrete TNFα in culture. LPS activation of CD34MG cells in the 10th (**B**) and 21st (**C**) day of culture induced peak TNFα secretion after 2 hours of exposure. Results represent five replicate experiments.

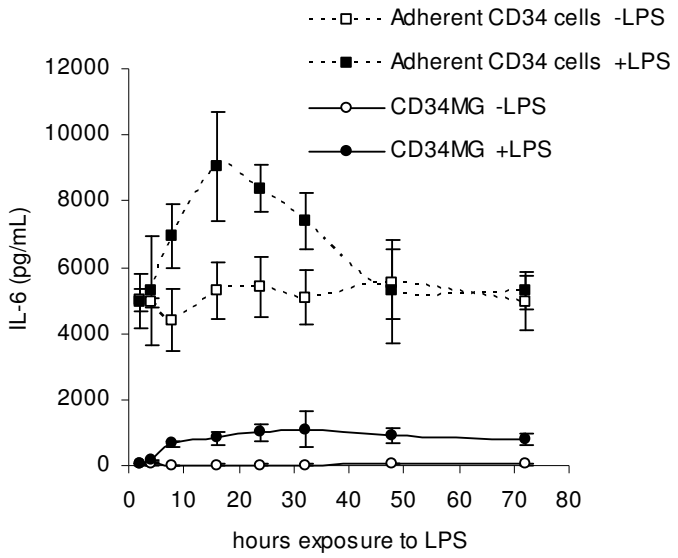
5.2.6 IL-6

Adherent CD34+ cells cultured for 7 days in standard culture conditions secrete constitutive amounts of IL6, between 4389 to 5503pg/ml (Figure 26a). After 10 days in culture, IL6 levels decreased to levels from 72 to 300pg/ml (Figure 26b). At day 21, IL6 release was as low as 16 to 194pg/ml (Figure 26c).

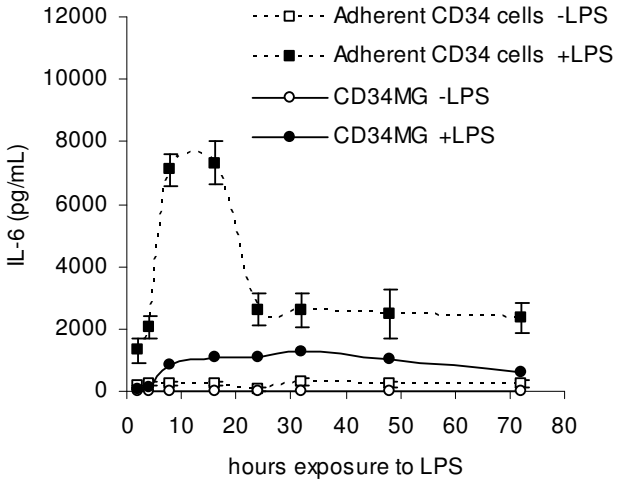
When activated with LPS, day 7 adherent CD34+ cells secreted up to 9055pg/ml IL-6 after 16 hours to exposure to LPS, but levels returned to constitutive levels after 48 hours in culture (Figure 26a). LPS-activated day 10 adherent CD34+ cells had maximum IL-6 secretion at 16 hours of LPS exposure, secreting 7310pg/ml IL-6 (Figure 26b). By day 21 of culture, adherent CD34+ cells only secreted a maximum of 6259pg/ml IL-6 after 8 hours of LPS exposure (Figure 26c).

CD34MG cells secreted very low amounts of IL-6 in the resting state. CD34MG cells after 7, 10 and 21 days of culture secreted constitutively low levels of IL-6 between 16 and 52pg/ml at all three time points (Figure 26a-c). LPS-activated day 7 CD34MG cells secreted up to 1087pg/ml IL-6 32 hours after commencement of LPS exposure. Day 10 CD34MG secreted 1265pg/ml IL-6 after 32 hours of LPS exposure (Figure 26b), and day 21 CD34MG cells secreted 1349pg/ml after 32 hours of exposure (Figure 26c). The time course shows that IL-6 secretion of LPS activated CD34MG cells increases slightly over time, which is the inverse of the pattern seen in TNFα secretion which showed a decrease in TNFα with time.

A


Adherent CD34 cells -LPS
Adherent CD34 cells +LPS
CD34MG -LPS
CD34MG +LPS
IL-6 (pg/mL)
12000
10000
8000
6000
4000
2000
0
0 10 20 30 40 50 60 70 80
hours exposure to LPS

B


Adherent CD34 cells -LPS
Adherent CD34 cells +LPS
CD34MG -LPS
CD34MG +LPS
IL-6 (pg/mL)
12000
10000
8000
6000
4000
2000
0
0 10 20 30 40 50 60 70 80
hours exposure to LPS

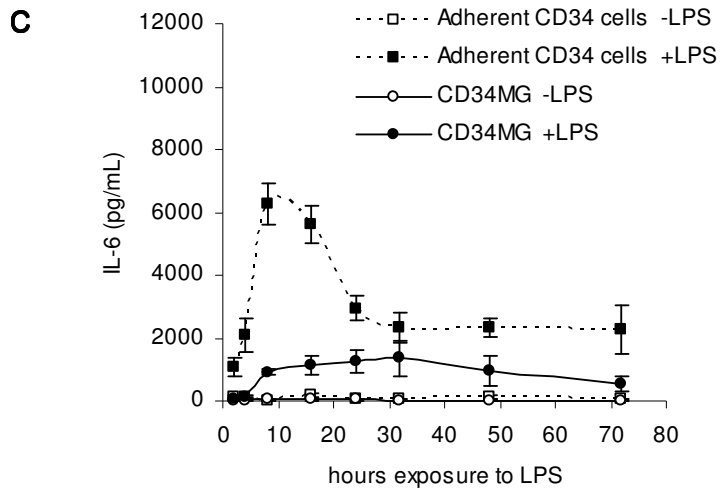


Figure 26. IL-6 secretion by adherent CD34+ cells and CD34MG. Adherent CD34+ cells constitutively secreted IL-6 at 7 days in culture, but levels of IL-6 decreased by 10 and 21 days in standard culture. LPS activation of adherent CD34+ cells caused massive spikes in IL-6 secretion at 8 hours after LPS exposure. Although levels decreased by 24 hours, a constitutive amount of IL-6 remained in culture up to 48 hours. ACM treated CD34MG cells did not secrete constitutive amounts of IL-6, but LPS activation caused relatively mild IL-6 secretion after 2 hours exposure to LPS. This level of activation decreased as the age of cell cultures increased. This observed IL-6 release is similar to documented patterns seen in microglia. Results represent five replicate experiments.

5.2.7 Antigen presentation by CD34MG

Antigen presentation experiments were conducted with the help of Stefania Lymperi (Prof Francesco Dazzi's lab). To determine if CD34MG are antigen presenting cells, mixed lymphocyte reactions were conducted using autologous leukocytes. CD34MG (from donor A) was loaded with EBV peptide by pulsing overnight. Loaded CD34MG was then cultured in a round bottom 96-well plate with PBMC (also from donor A). PBMC proliferation was then measured by thymidine uptake. CD34MG loaded with EBV peptide caused a moderate proliferation of PBMCs, indicating antigen presenting cell function. Peptide alone did not cause PBMC proliferation. CD34MG alone caused a minimal PBMC proliferation. Adherent CD34 loaded with EBV peptide did not cause a significant proliferation of PBMCs, indicating that adherent CD34 are not antigen presenting cells. Although proliferation caused by peptide loaded CD34MG was not as much as that caused by an HLA mismatched reaction, it was considerably greater than that caused by peptide pulsed adherent CD34+ cells or CD34MG cells that were not pulsed with peptide. This corroborates the immunofluorescence data (Figure 22) which showed that although some (9%) adherent CD34+ cells express HLA-DR, they do not express the costimulatory molecule B7.1 (Figure 27).

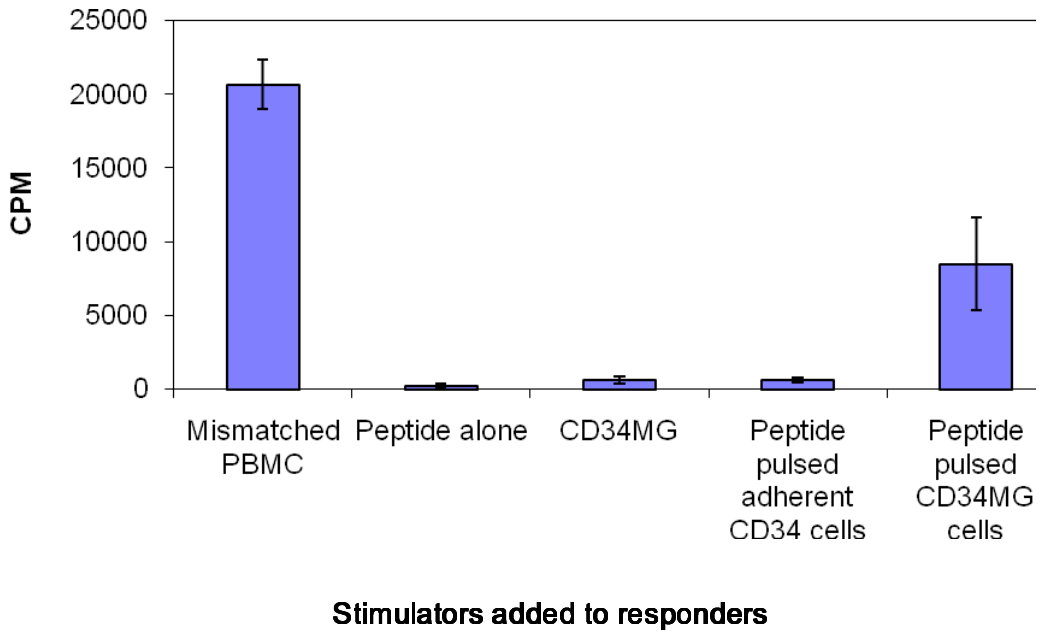


Figure 27. Mixed lymphocyte reactions determine if CD34MG cells are capable of antigen presentation. To determine if CD34MG cells are capable of presenting antigen, CD34MG cells were loaded with EBV peptide overnight, washed and the cells irradiated. PBMC from the same donor from which CD34MG cells were produced were introduced to the EBV loaded CD34MG cells. An immune response was determined by measuring proliferation of PMBC with ^{3}H tritiated thymidine uptake. The graph shows that CD34MG induced more proliferation than adherent CD34+ cells in an autologous reaction, indicating that CD34MG was able to present EBV peptide to peripheral immune cells. Results represent three replicate experiments.

5.3 Discussion

Although it has been well documented that microglia are of haematopoietic origin, and are recruited from the bone marrow during neural injury, the precise identity of the microglial precursors has not yet been determined[62,131,132]. Here it was proposed that adherent CD34+ cells differentiate into microglial cells *in vitro*. Four subpopulations of bone marrow cells were exposed to ACM in order to induce microglial differentiation. It was observed that fhMSC and adult MSC died relatively quickly under these conditions.

Non-adherent CD34+ cells did not show morphological evidence of differentiation or upregulate microglial markers in ACM. It was previously described that known that adherent CD34+ cells and non-adherent CD34+ cells are genetically and morphologically different. As migrating immune cells of the nervous system, bone marrow-derived microglia must tether to and adhere to the endothelium of the blood brain barrier. Adherent CD34+ cells also upregulate the gene which encodes for CCL2, the ligand that allows migration through the tight junctions of the blood brain barrier.

Adherent CD34+ cells secrete pro-inflammatory cytokines, and increase cytokine levels in response to LPS, as an *in vitro* model of immune response. There was an inverse relationship between the age of the cells and the amount of cytokine secretion. As time in culture increased, the amount of cytokine release decreased. Differences in cytokine levels through the culture period may indicate that the adherent CD34+ cells may either be regulating cytokine secretion with time in culture, metabolising the cytokines, or that the secretion is temporarily released by the cells as an acute response.

The lower levels of cytokines secreted by CD34MG may reflect the immunosuppressive effects of the astrocyte conditioned medium on the adherent CD34+ cells or reflect differentiation. It is widely known that astrocytes secrete factors that cause immunosuppression[10-12] and that infiltrating peripheral monocytes are immunosuppressed in the brain[13,14]. Based on morphological, antigenic, and functional evidence, it is assumed that CD34MG cells are microglia like cells. The modes of microglia reaction to various pathological stimuli include pro-inflammatory cytokine release including TNFα and IL-6. LPS activation of CD34MG revealed large amounts of TNFα and IL-6 secretion, similar to patterns of cytokine release by microglia[133]. The release of these pro-inflammatory cytokines further validates microglial characteristics of CD34MG cells.

Interestingly, LPS activation of adherent CD34+ cells and CD34MG cells induced different patterns of cytokine secretion. Figure 8 illustrates that adherent CD34+ cells (Figure 29a) rapidly release increasing amounts of IL-6 up to 8 and 16 hours of LPS exposure, and relatively higher levels of IL-6 secretion are sustained in the presence of LPS. In contrast, CD34MG secreted 7 times less IL-6 than adherent CD34+ cells in response to LPS activation and relatively low amounts of IL-6 in culture in the presence of LPS.

Although LPS activation of adherent CD34+ cells caused a spike in IL-6 secretion, the amount of TNFα secretion was relatively low and remained at constitutive levels in the presence of LPS (Figure 28a). LPS activation had the opposite effect on CD34MG cells which showed an immediate and relatively high release of TNFα within 2 hours of LPS exposure. After an initial spike of cytokine release, levels decreased and were down to negligible levels within 24 hours.

In both cell populations, inverse patterns of cytokine secretion were observed in response to LPS. Adherent CD34+ cells secreted relatively large amounts of IL-6, but low amounts of TNFα (Figure 28a). In contrast, CD34MG released high amounts of TNFα immediately in response to LPS and relatively low levels of IL-6 (Figure 28b).

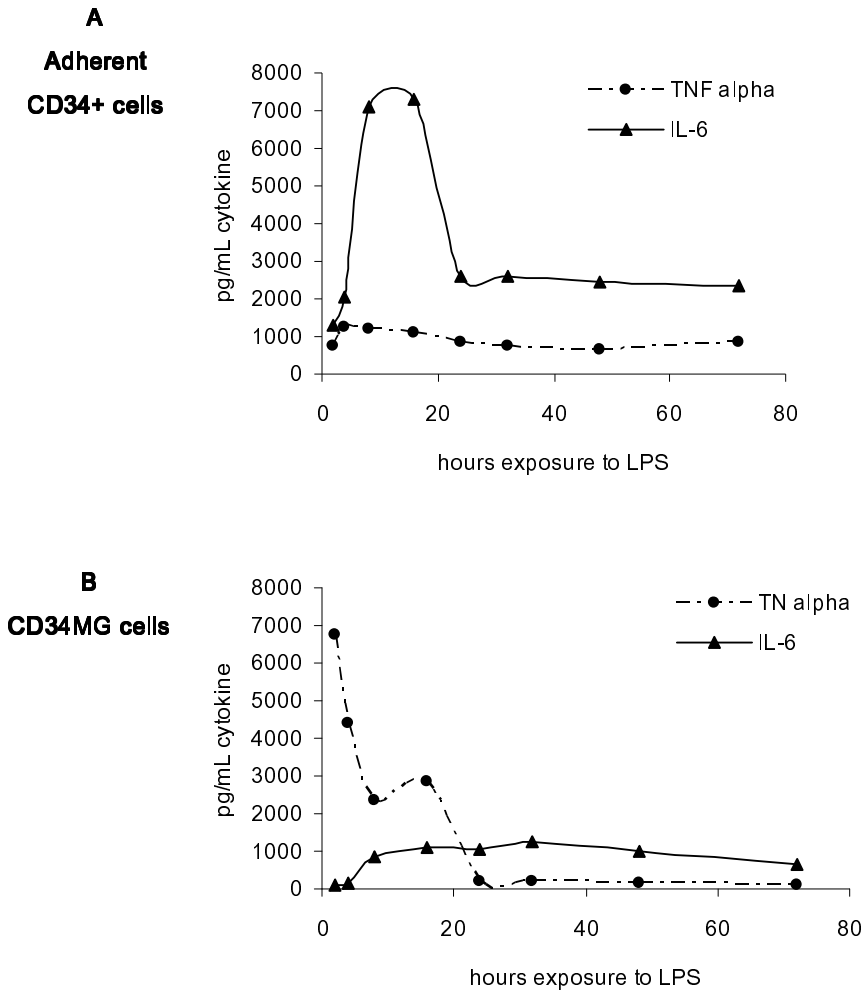


Figure 28. Cytokine release in response to LPS activation. LPS activation was used to determine if either adherent CD34+ cells (A) or CD34MG cells (B) released cytokine in response to pathogens, in this case LPS. Adherent CD34+ cells and CD34MG in 10 days of culture were exposed to LPS for 2 to 72 hours and TNFα and IL-6 levels were measured. Patterns of cytokine secretion by adherent CD34+ cells are the opposite of those for CD34MG cells. In response to LPS, adherent CD34+ cells (A) secreted almost 7 times more IL-6 within 8 to 16 hours than CD34MG, but adherent CD34+ cells secreted 6 times less TNFα than CD34MG in response to LPS. In both cell populations, when one cytokine was released in relatively high amounts, the other cytokine was released in relatively low amounts.

The results of experiments investigating antigen presentation revealed that CD34MG have moderate antigen presenting activity relative to adherent CD34+ cells *in vitro*, suggesting that they may be functional immune effecter cells. The results described above suggest that CD34MG cells upregulate microglial characteristics and functionality. It is widely known that microglia are of haematopoietic developmental origin, thus it is reasonable to expect that primitive bone marrow-derived adherent CD34+ cells are capable of differentiating into microglia-like cells. The next part of this study examines the potential use of adherent CD34+ cells and CD34MG in therapy.

Chapter 6 Stem cell therapy of glioblastomas

Chapter summary

The previous chapter described the ability of adherent CD34+ cells to differentiation into microglia like cells called CD34MG cells. In this chapter, the therapeutic potential of CD34MG cells were explored for use in brain tumors, particularly glioblastoma multiforme (GBM). Experiments revealed that adherent CD34+ cells and CD34MG cells have tropism towards glioblastoma cell lines. CD34MG cells also demonstrated possible effector function, and cause apoptosis of glioblastoma cell lines *in vitro*. This chapter describes the following in detail:

- Adherent CD34+ cells and CD34MG cells have tropism towards glioblastoma cell lines
- CD34MG cells are antigen presenting cells
- CD34MG cells are capable of presenting glioblastoma cell antigen
- CD34MG cells cause apoptosis of glioblastoma cell lines in a dose dependent manner

6.1 Brain tumors

During the later stages of glioblastoma multiforme (GBM), single cell infiltrates begin to diffuse into the peritumoral region[134-138]. Although the bulk of the tumor can be surgically removed, the diffuse infiltrates remain embedded within healthy brain tissue. These cells are inaccessible to current therapeutic strategies, thus leading to tumor recurrence[138]. Viral vectors have not been proven to deliver apoptotic agents to tumor-specific regions, and have lead to damage of healthy brain tissue[74,136]. The lack of known glioma-specific antigens has lead to poor outcomes of antibody mediated drug and gene delivery[74]. Other experimental strategies include immunotherapy with autologous dendritic cells whereby the patient's dendritic cells are isolated and pulsed with peptide derived from their own tumor[74,135,139,140]. The peptide-loaded dendritic cells are then re-infused into the patient. Although the results from animal models have lead to the prevention of tumor growth, patients in clinical trials

have developed autoimmunity[135,139,140]. In the previous chapter, it was described that CD34MG cells are capable of acting as antigen presenting cells, presenting viral peptide and causing PBMC proliferation in an autologous reaction. Experiments were then conducted to determine if CD34MG was able to present glioblastoma specific antigen in an autologous reaction. Induction of PBMC proliferation would suggest that CD34MG cells might have potential use in immunotherapy by a similar way as dendritic cells.

6.1.1 Uses of stem/progenitor cells in GBM

Cell-based gene delivery is emerging as a strategy for brain tumor therapy. Current chemotherapeutic treatments for brain tumors are not tumor specific and cause large scale toxicity including damage to non-tumorigenic brain tissue, leading to further brain damage [136,137]. The major advantage of using stem cells as vehicles for gene or drug delivery is their potential for therapeutic specificity[136,137]. Embryonic stem cells, NSCs and MSCs have demonstrated tumor trophic properties in animal models and they infiltrate solid tumors[134,136-138,141]. Stem and progenitor cell-mediated drug and gene delivery is being studied to improve the efficacy of the delivery of therapeutic agents.

6.1.2 Immune evasion

Endogenous microglia and peripheral leukocytes have been observed within human gliomas[74,142]. Regardless of leukocyte migration and tumor infiltration, gliomas are able to evade innate immune surveillance[74,142,143]. Gliomas express HLA class I and II molecules, but do not express the costimulatory molecule B7-1 (CD80) and express low amounts of B7-2 (CD86)[74,142-144]. The lack of costimulation leads to failure of antigen presentation and subsequent T cell activation[74,143]. Gliomas also secrete immunosuppressive cytokines TGF-β, VEGF, and interleukin 10[145,146], and recruit T regulatory cells which play a role in tumor

tolerance[74]. Vaccines created to augment tumor-specific cellular immunity have been found to partially overcome glioma immunosuppression by boosting low level immunity and stimulating the proliferation of higher avidity T cells[74,139,140,147]. Recent investigations showed that dendritic cell (DC) and peptide antigens are capable of inducing an antitumor response within the CNS, emphasizing that the brain is accessible to systemic immunotherapy[74,135,139,148-150]. This opens the door for immunotherapeutic approaches for glioma.

The aim of the experiments described herein was to explore the potential of adherent CD34+ cells and CD34MG for therapeutic purposes in glioblastoma. This study sought to determine if adherent CD34+ cells and CD34MG had tumor trophic properties, invasive properties or immune properties which might be exploited for therapy. The studies described in previous chapters revealed that CD34MG cells have antigen-presentating capabilities, inducing proliferation of immune cells *in vitro* in an autologous lymphocyte reaction. The following experiments were conducted to determine if CD34MG cells might be potentially useful for future immunotherapy in gliomas. As antigen-presenting cells, the ability of CD34MG cells to present glioma-specific antigen was observed which might be exploited in the future to mimic dendritic cell therapy and boost low level immune responses.

6.2 Results

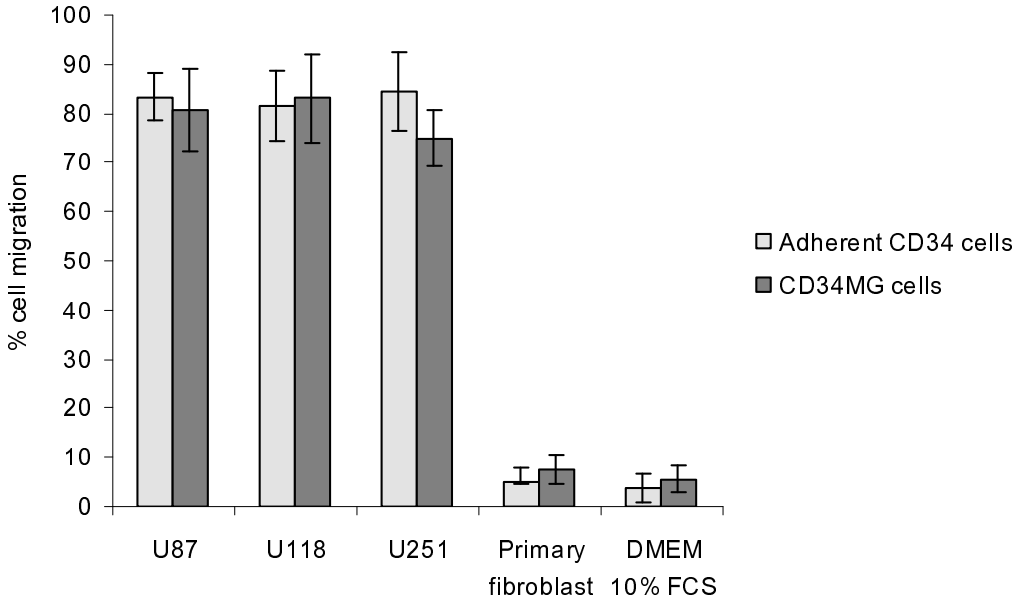
6.2.1 Migration

Stem cells and microglia are tumor-tropic. A transwell migration assay was used to determine if adherent CD34+ cells and CD34MG have tropism towards glioblastoma cells *in vitro*. Approximately 5 x 10^3 GFP transfected U251, U118, or U87 glioblastoma cells (GFP transfection was performed by Dr. Faisal Altaf) were plated in the bottom wells of a 24 well plate transwell system (as described in materials and methods) and 2 x 10^4 either adherent

CD34+ cells or CD34MG cells were placed into the 6µm nylon mesh transwell insert for 5 hours. After 5 hours, the transwell was removed and the remaining cells within the transwell insert were trypsinized and scraped, collected, centrifuged, and counted. Five replicate experiments were performed. The adherent CD34+ cells and CD34MG cells were obtained from the same donor samples for each replicate.

The results indicated that 83 ± 4.8% of adherent CD34+ cells migrated through the transwell membrane in response to trophic factors released by U87glioma cells, 81 ± 7.13% in response to U118, and 84.4 ± 8.02% in response to U251. Similar patterns of migration were observed with CD34MG cells where 80.8 ± 8.35% of cells migrated in response to U87 glioma cells, 83 ± 9.03% migrated in response to U118 cells, and 75 ± 5.48% migrated in response to U251 cells (Figure 29). There was no observed difference in migration in response to the different glioma cell lines. As shown in Figure 30, 5 ± 3.16% of adherent CD34+ cells and 7.4 ± 2.97% of CD34MG cells migrated in response to primary fibroblasts. This was significantly less migration than in response to the glioma cell lines ($p=0.009$). This result indicated that the adherent CD34+ cells and CD34MG cells migrate in response to soluble trophic factors secreted by glioma cells *in vitro*.

A



B

Migration of cells in response to glioma cell lines relative to fibroblasts:	Adherent CD34 cells	CD34MG cells
U87	p = 0.009	p = 0.009
U118	p = 0.009	p = 0.009
U251	p = 0.009	p = 0.009

Figure 29. Migration assays demonstrate tropism of adherent CD34+ cells and CD34MG towards glioma cells. Transwell migration assays were conducted to determine if adherent CD34+ cells and CD34MG cells have tropism towards glioma cell lines U87, U118, or U251 (A). The majority of both adherent CD34+ cells and CD34MG cells migrated through the transwell membrane in response to trophic factors released by glioma cells. There was no significant difference in the percent of adherent CD34+ cells and CD34MG cells which migrated in response to glioma cells. Comparing the migration potential between the two cell populations, the p values were calculated using the Wilcoxon Signed Rank nonparametric test (n=5 biological replicates) on SPSS software (migration to U87 p=0.223, U118 p= 0.686, U251 p=0.08) (B). The migration levels of adherent CD34+ cells and CD34MG cells in response to the glioma cells were compared to the percent of cells which migrated in response to primary fibroblasts using the Mann-Whitney nonparametric test. There were

significantly more adherent CD34+ cells and CD34MG cells which migrated in response to glioma cells than in response to normal primary fibroblasts.

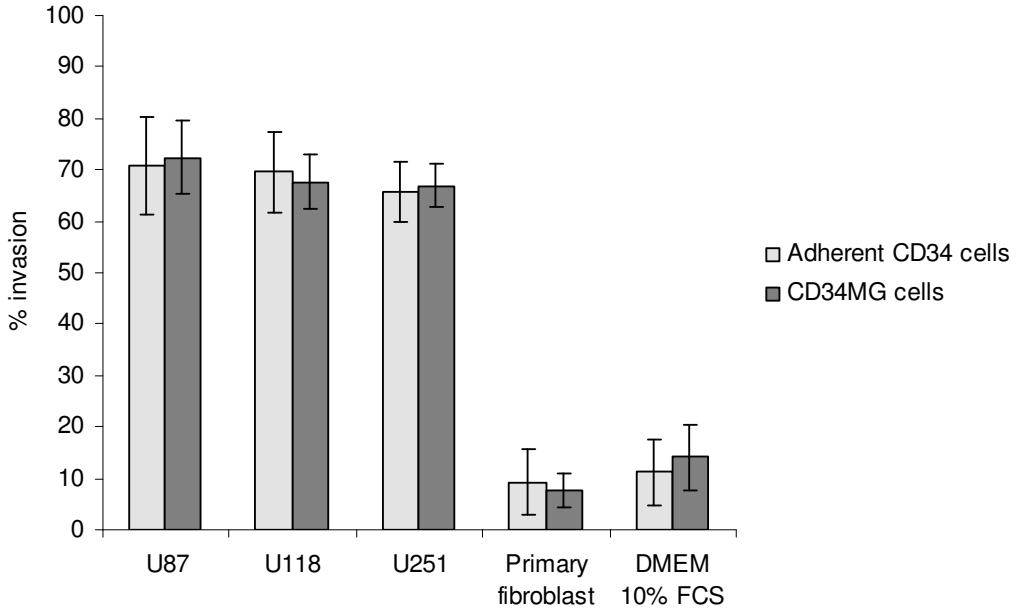
6.2.2 Invasion

Experiments similar to the migration assay were conducted to determine if either adherent CD34+ cells or CD34MG cells can digest and migrate through a Matrigel extracellular matrix. Active digestion of extra cellular matrix would suggest that these cells could be invasive *in vivo*. A 24 well plate transwell system for invasion assays was used where the transwell inserts were coated with Matrigel extra cellular matrix. 5,000 glioma cells U87, U118, or U251 cells were plated in the bottom well of the system, and an equal number of either adherent CD34+ cells or CD34MG cells were placed in the transwell insert. After 24 hours (as per the manufacturer's instructions), the number of adherent CD34+ cells or CD34MG cells which passed through the Matrigel were counted. The cells were counted under a fluorescent microscope. The GFP-transfected glioma cells showed as green; the non-fluorescing cells were assumed to be the adherent CD34+ cells or CD34MG cells which had migrated through the transwell. To ensure that the cell migration was in response to trophic factors released by the glioma cells, primary fibroblasts were plated instead of glioma cells as a control.

Like the pattern observed in the migration assay, the invasion assay revealed that 70.8 ± 9.47% of adherent CD34+ cells invaded in response to U87 glioma cells, 69.6 ± 7.83% invaded in response to U118 cells, and 65.8 ± 5.81 in response to U251 cells. CD34MG cells were also invasive, with 72.4 ± 7.16% invading through the matrix in response to U87 cells, 67.6 ± 5.37 in response to U118 cells, and 66.8 ± 4.21% in response to U251 cells. There was no significant difference in invasion due to different glioma cell lines U87, U118, or U251 (Figure 30). Only 9.2 ± 6.38% of adherent CD34+ cells and 7.6 ± 3.21% of CD34MG cells invaded in response to primary fibroblasts.

Approximately 7 times fewer adherent CD34+ cells and 10 times fewer CD34MG cells invaded in response to primary fibroblasts than in response to glioma cells. The data suggest that both adherent CD34+ cells and CD34MG cells are invasive, and that this response can be mediated by tropic factors released by glioma cells.

A



B

Invasion of cells in response to fibroblasts relative to:	Adherent CD34 cells	CD34MG cells
U87	p = 0.009	p = 0.009
U118	p = 0.009	p = 0.009
U251	p = 0.009	p = 0.009

Figure 30. Invasion assays demonstrate tropism of adherent CD34+ cells and CD34MG towards glioma cells. Invasion assays using Matrigel matrix coated transwell system were conducted to determine if adherent CD34+ cells and CD34MG cells are invasive. The active digestion of extra cellular matrix suggests that cells would be invasive through tissue *in vivo*. Patterns of invasion are similar to that seen in migration assays above. The majority of both adherent CD34+ cells and CD34MG cells actively digested through the extra cellular matrix in response to either U87, U118, or U251 glioma cells. Comparing the migration potential between the two cell populations, the p values were calculated using the Wilcoxon Signed Rank nonparametric test (n=5) using SPSS software (migration to U87 p=0.893, U118 p= 0.588, U251 p=0.786). The invasion of adherent CD34+ cells

and CD34MG cells in response to the glioma cells was compared to the percent of cells which invaded in response to primary fibroblasts using the Mann-Whitney nonparametric test (B). Significantly more adherent CD34+ cells and CD34MG cells invaded through extra-cellular matrix in response to glioma cells than in response to normal primary fibroblasts.

6.2.3 Potential of CD34MG cells to present glioma antigen

Experiments reported in the previous chapter revealed that the microglia-like CD34MG cells are antigen presenting cells. To determine if CD34MG cells are able to present tumor specific antigen in an autologous leukocyte reaction, CD34MG cells were cultured overnight with U251 lysate to "load" them with tumor specific antigen. Antigen loaded CD34MG cells were then irradiated and incubated for 5 days with fresh PBMCs from the same donor. Leukocyte proliferative responses were determined with ^{3}H (tritiated) thymidine incorporation during the last 18 hours of culture. Cells were harvested for liquid scintillation counting and the results expressed as counts per minute. The results are shown in Figure 31. To demonstrate professional antigen presentation, macrophages were isolated as stimulators and loaded with tumor antigen (macrophage isolation was performed by Allison Russell, Dr. N Dibb's laboratory). Macrophages were irradiated and cultured with PBMCs from the same donor. Stimulators in the experiment consisted of lysate pulsed macrophages, lysate pulsed adherent CD34+ cells, CD34MG cells alone, and CD34MG cells pulsed with tumor lysate. Peripheral blood macrophages loaded with tumor lysate induced proliferation of PBMCs and served as a positive control for the purposes of these experiments. This provided a control that the overnight pulsing of antigen presenting cells with U251 tumor lysate was sufficient to "load" the cells with tumor antigen and that proper antigen presenting cells are able to present the tumor antigen. Lysate pulsed adherent CD34+ cells did not induce responder proliferation. Lysate pulsed CD34MG cells induced proliferation of autologous PBMCs, but

responder proliferation was not as much as that induced by macrophages. These reactions were conducted using samples from three normal donors. As glioma cells theoretically do not upregulate MHC molecules, and tumor cell lysate did not induce proliferation, the observed proliferation is likely to result from antigen presentation by macrophages and CD34MG, and not from the glioma cells themselves (Figure 31).

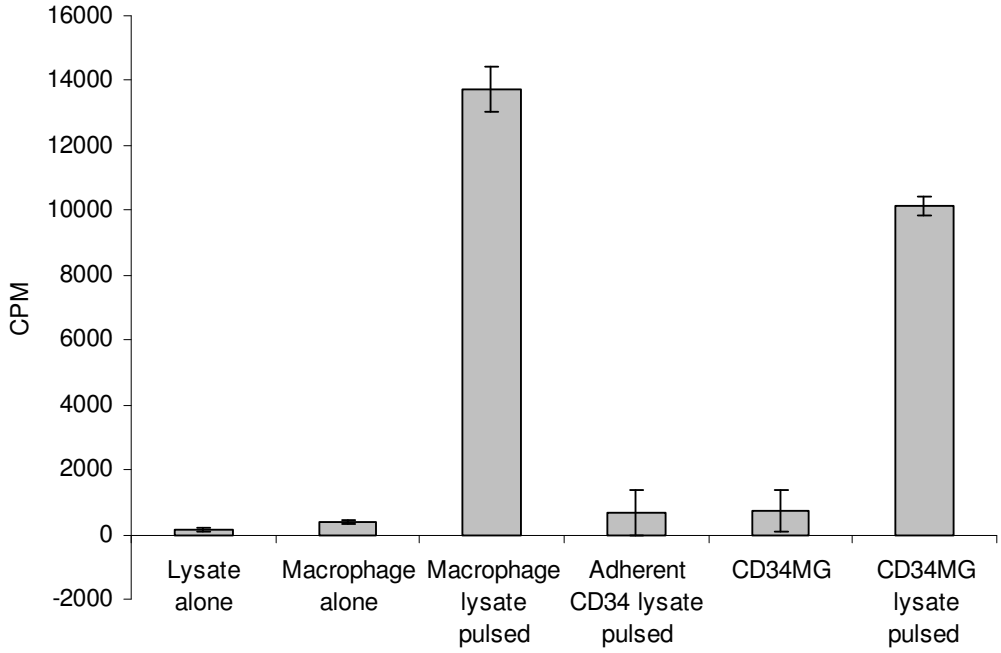
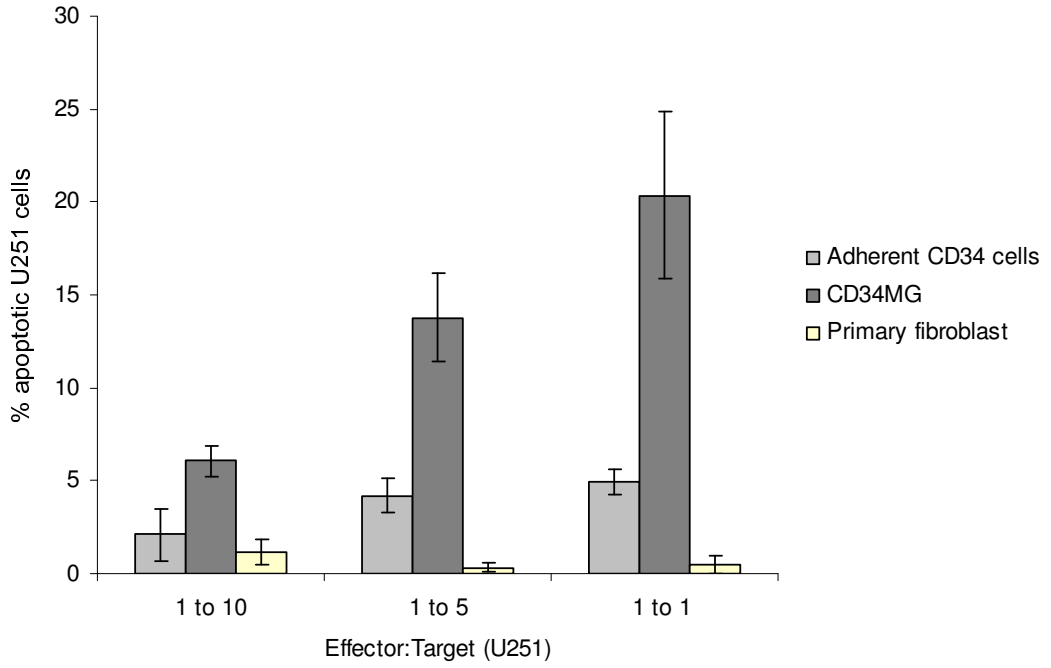


Figure 31. Determination of glioma-specific antigen presentation by CD34MG. CD34MG cells were pulsed overnight with U251 lysate to load them with tumor specific antigen. The cells were then cultured with PBMCs from the same donor, the proliferative response measured, and the results expressed as counts per minute (CPM). Macrophages, which are professional antigen presenting cells and were used as a positive control, induced proliferation of immune cells, presumably T cells. Adherent CD34+ cells loaded with antigen did not induce proliferation, but CD34MG cells loaded with tumor specific antigen induced proliferation of leukocytes. In this autologous reaction, there was no significant difference in responder proliferation due to exposure to tumor lysate alone versus naive macrophages ($p=0.0841$), but lysate pulsed macrophages induced a significant proliferation when compared to naive macrophages ($p=0.0027$), lysate pulsed adherent CD34+ cells ($p=0.0029$), and CD34MG cells alone ($p=0.0025$). There was no significant difference in proliferation in responders exposed to lysate pulsed macrophages versus lysate pulsed CD34MG ($p=0.0067$). The graph represents results from three replicate experiments. Paired student's t-test was conducted to determine significance.

6.2.4 Possible effector function of CD34MG

To determine if either adherent CD34+ cells or CD34MG cells have effector functions, either cell population was co-cultured with U251-GFP glioma cells in effector:target ratios of 1:10, 1:5, or 1:1. After 10 hours, apoptosis of U251-GFP cells was detected by trypan blue exclusion. All the cells in the co-culture were trypsinized and diluted with trypan blue, loaded onto a haemacytometer, and visualized under a fluorescence microscope. Cells which fluoresced green and took up the trypan blue were assumed to be the apoptotic U251-GFP cells. As a control, primary fibroblasts were also co-cultured with U251-GFP cells to ensure that cell death was due to effector function, not due to co-culture conditions. The results demonstrated that adherent CD34+ cells did not cause significantly more apoptosis than the fibroblast control at any of the three cell ratios (Figure 32). Comparisons of the means were conducted with t-tests on SPSS, and the details of the statistical results are seen in Figure 33b. CD34MG cells caused significantly more U251 cell death than the control fibroblasts at all three cell ratios. At a 1:10 (CD34MG:U251) dilution, 6.08 ± 0.82% of U251 cells were apoptotic after 10 hours. At a 1:5 dilution, 13.77 ± 2.4% of U251 cells were apoptotic, and in a 1:1 culture, up to 20.33 ± 4.5% of U251 cells were apoptotic. When comparing levels of apoptosis caused by adherent CD34+ cells and CD34MG cells, there was no difference in U251 apoptosis at a 1:10 effector-target ratio. At 1:5 and 1:1 co-cultures of CD34MG and U251 cells, there was significantly more U251 apoptosis than in the adherent CD34+ cell and U251 co-cultures with the same ratios. These results are an average of four replicate experiments. They indicate that adherent CD34+ cells do not cause apoptosis of U251 glioma cells in co-culture. CD34MG cells induced increasing amounts of U251 cell death as the effector-target ratio decreased. This evidence indicates that CD34MG cells are cytotoxic to U251 cells *in vitro*, but their effects on gliomas *in vivo* remain unknown.



	Adherent CD34+	CD34MG	Fibroblast
1 to 10			
mean	2.08	6.08	1.17
standard error	1.38	0.82	0.65
P value (compared to adherent CD34 cells)		0.14*	0.58**
P value (comparison of CD34MG with fibroblast)			0.004**
1 to 5			
mean	4.21	13.77	0.33
standard error	0.91	2.4	0.24
P value (compared to adherent CD34 cells)		0.05*	0.02**
P value (comparison of CD34MG with fibroblast)			0.004**
1 to 1			
mean	4.92	20.33	0.5
standard error	0.67	4.5	0.5
P value (compared to adherent CD34 cells)		0.02*	0.002**
P value (comparison of CD34MG with fibroblast)			0.004**

**Paired t-test on SPSS* ***Independent-samples t-test on SPSS*

Figure 32. Effector function of adherent CD34+ cells and CD34MG cells. Adherent CD34+ cells, CD34MG or primary fibroblasts were co-cultured with U251-GFP glioma cells for 10 hours. The cells were then trypsinized, collected, and apoptosis of U251-GFP cells was assessed by trypan blue dye exclusion under a fluorescence microscope. Adherent CD34+ cells did not cause significantly more

apoptosis than the fibroblast control at any cell ratio. CD34MG cells caused significantly more U251 cell death than the control fibroblasts at all three cell ratios. There was no difference in apoptosis caused by adherent CD34+ cells and CD34MG cells in co-cultures in a 1:10 ratio. In 1:5 and 1:1 co-cultures of CD34MG and U251 cells, there was significantly more U251 apoptosis than in the adherent CD34+ cell and U251 co-cultures with the same ratios.

6.2.5 Possible mechanism of killing

Previous experiments revealed that adherent CD34+ cells and CD34MG cells secrete pro-inflammatory cytokines and increase the amounts of cytokine release in response to foreign pathogens. One of these cytokines is TNFα which is a well documented inducer of apoptosis of glioblastoma cells[151-153]. To determine if CD34MG-mediated apoptosis might be due to TNFα release, preliminary experiments were conducted where either adherent CD34+ cells or CD34MG cells were co-cultured with GFP transfected U251 glioma cells. Cells were co-cultured in a 1:1 ratio for 8 hours (similar to the above mentioned killing experiment) and the supernatant collected. TNFα was measured quantitatively with a Luminex multiplex assay as in previous experiments (Figure 33). From previous experiments, it was revealed that adherent CD34+ cells and CD34MG cells secrete low amounts of TNFα in standard cultures. In these experiments, adherent CD34+ cells secreted 32 ± 12pg/ml TNFα and CD34MG cells secreted 176 ±28pg/ml TNFα. U251 cells in standard culture produced negligible amounts of TNFα, (8 ± 5pg/ml TNFα). When either cell type was co-cultured with U251 cells, relatively higher levels of TNFα were measured in the supernatant. In adherent CD34+ cell and U251 co-culture supernatant, 359 ± 82pg/ml TNFα was present. In CD34MG and U251 co-culture supernatant, 1487 ± 294pg/ml TNFα was measured. As TNFα is cytotoxic to glioma cells, it is assumed that it is unlikely that U251 cells would upregulate the mechanisms to synthesize and secrete TNFα in this co-culture condition. Thus, the TNFα found in the co-culture supernatants is assumed to be released by the adherent CD34+ cells or the CD34MG cells.

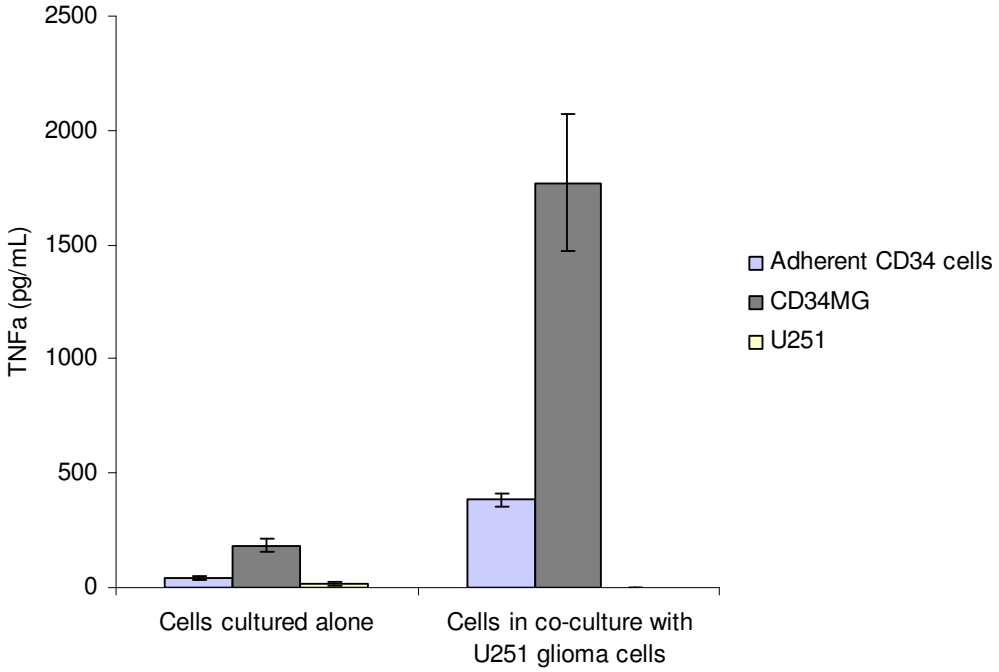


Figure 33. **TNFα secretion by adherent CD34+ cells and CD34MG when co-cultured with glioma cells.** Adherent CD34+ cells in standard culture conditions secrete 38.3 ± 8.5pg/ml TNFα , and CD34MG cells secret 184 ± 29.91 pg/ml TNFα. U251 cells in standard culture have negligible amounts of TNFα in the supernatant, 14.6 ± 6.5 pg/ml TNFα. When either adherent CD34+ cells or CD34MG cells are co-cultured with U251 glioma cells, relatively larger amounts of TNFα is found in the supernatant. Supernatant collected from co-cultures of adherent CD34+ cells and U251 cells contained 396.3 ± 21.03 pg/ml TNFα. Co-cultures of CD34MG cells and U251 cells contained 1771.33 ± 298.94 pg/ml TNFα. This suggests that the levels of TNFα may contribute to observed U251 cell death.

6.3 Discussion

One of the aims of the overall project was to explore the potential use of adherent CD34+ cells in neurodegenerative disease neurological malignancy. Here it was observed that adherent CD34+ cells and CD34MG cells have tropism for glioma cells *in vitro*, and that CD34MG cells are able to present glioma antigen in a lymphocyte reaction. These properties might be further studied and exploited for possible therapeutic use in glioblastoma.

Evidence from the literature indicates that stem cells isolated from various tissues possess inherent tumor tropism, and that systemically administered stem and progenitor cells migrate to and infiltrate primary and metastatic solid tumors[136]. This trophic property has been exploited by other workers to deliver multiple therapeutic genes including IL-4, IL-12, IL-23, TRAIL, and IFNβ[136,137]. Tropism of stem cells to tumors is likely to be mediated by multiple cell-surface and secreted proteins. Cytokine/receptor pairs include SDF-1/CXCR4[154], SCF/c-kit[155] and MCP1/CCR2[156]. Adherent CD34+ cells have been previously demonstrated to express CXCR4[26], while glioma cells express CXCL12/SDF1, the ligand for CXCR4, one possible factor contributing to the tumor tropism of adherent CD34+ cells. The present study demonstrated that adherent CD34+ cells are potentially tumor-tropic as they migrated in response to U251 glioma cells *in vitro*. The *in vitro* migration and invasion assays demonstrate that motility of adherent CD34+ cells and CD34MG cells can be mediated by gliomas, suggesting the potential use of these for cell-based therapy in gliomas.

Preliminary experiments were conducted to determine if either adherent CD34+ cells or CD34MG cells were functional immune effector cells. CD34MG cells co-cultured with U251 glioma cells and resulted in apoptosis of the glioma cells. Previous experiments revealed that adherent CD34+ cells and CD34MG cells secrete TNFα in response to a model

of exposure to pathogen, namely LPS activation. TNFα is known to cause apoptosis to glioma cells including U251 cells. Analysis of TNFα in standard conditions and co-culture conditions revealed that TNFα release is induced by the direct co-culture of adherent CD34+ cells or CD34MG cells with U251 glioma cells. As all cell types released relatively low amounts of TNFα when cultured alone, it cannot be definitively deduced which cell type is responsible for the cytokine synthesis and release. One likely explanation is that CD34MG cells are activated by the presence of non-autologous cells, causing them to synthesize and release TNFα. CD34MG cells activate TNFα receptors on U251 cells, causing apoptosis. The mechanisms of cell death are of considerable interest and should be pursued further.

The observed cytokine release may explain the glioma cell apoptosis in co-culture conditions. Apoptosis was only observed in co-cultures with at 1:1 effector to target ratio. This result suggests that apoptosis of U251 cells in co-culture with CD34MG cells may require either direct cell to cell contact or co-culture with the cells for a constant supply of cytokine. Thus TNFα release could be induced when CD34MG reach a tumor *in vivo*, but it is noteworthy that TNFα is toxic to normal brain tissue[157]. Further analysis of the apoptotic mechanisms would require apoptosis assays when cells are in direct contact cultures compared with apoptosis when cells are in transwell cultures. This would determine if apoptosis was contact-mediated or due to secreted factors.

Glioma cells downregulate the co-stimulatory molecules required to initiate activation of innate immunity[139]. The goal of cancer immunotherapy is to boost immunity and stimulate T cell response of those which home to the tumor[74,139]. Animal and human studies have demonstrated that immunotherapy with dendritic cells and peptide antigen is capable of inducing an anti-glioma response[74,140,149]. This suggests that the systemic immune response can be altered to mediate intracranial anti-tumor responses. The experiments described in

this chapter explored the possibility of exploiting the immune properties of adherent CD34+ cells and CD34MG cells for immunotherapy. The work reported in previous chapters revealed immunological properties of CD34MG cells including the secretion of TNFα in response to pathogens, tumor-tropism, and antigen presenting capabilities. Overall, these experiments suggest that CD34MG cells might have immunotherapeutic potential in gliomas.

Chapter 7 Discussion

7.1 Adherent CD34+ cells

Adherent mononuclear cells from bone marrow were first identified in a study by Gordon and colleagues[98]. which demonstrated that plastic adherent mononuclear cells were a heterogeneous population that includes stromal and hematopoietic progenitor cells which can be sustained in long term culture[98]. The primitive nature of these cells was demonstrated by the ability of adherent cells to produce several granulocyte-macrophage colony-forming cells (CFU-GM) and erythroid burst-forming units (BFU-E)[97,174]. The majority of adherent cells were identified as CD34+ and comprised of 5-10% of the total CD34+ cells[174]. Further characterization of adherent CD34+ cells was conducted which revealed that these cells are quiescent and out of cell cycle[175], and simultaneously express tissue specific genes[26]. The combined data qualify adherent CD34+ cells as primitive stem cells derived from bone marrow and peripheral blood.

In order to deliver effective stem cell based clinical therapy, it is important to understand the basic biological and molecular mechanisms which govern how and when lineage specification occurs. The present study contributed to defining the stem cell biology of adherent CD34+ cells which are a bone marrow-derived population by (*i*) identifying genetic differences between adherent CD34+ cells and non-adherent CD34+ cells, (*ii*) validating the stem cell qualifications of adherent CD34+ cells by examining gene expression profiles, antigen expression, and DNA replication timing, (*iii*) determining if the adherent CD34+ cells have *in vitro* neural differentiation potential, (*iv*) phenotypically and functionally characterizing adherent CD34+ cell-derived microglia-like cells CD34MG, and (*v*) exploring

the potential of adherent CD34+ cells and CD34MG cells for cellular therapy in glioblastoma.

The comparison of gene expression between adherent CD34+ cells and non-adherent CD34+ cells revealed that adherent CD34+ cells more highly express genes which are involved in cell cycle control, adhesion to the bone marrow, and transendothelial migration. These characteristics contribute to their quiescent stem cell qualifications. Real-time qPCR and immunoflurescence microscopy validated the gene data and further revealed that adherent CD34+ cells express antigens associated with pluripotent ES cells. Previously published data revealed that adherent CD34+ cells express tissue specific transcription factors[26], indicating that this primitive stem cell population maybe multilineage primed.

When cultured in an *in vitro* neural microenvironment of astrocyte conditioned medium, adherent CD34+ cells differentiated into microglia/brain macrophages, CD34MG cells. It is documented that there is an increase of CD34+ cells in circulation after neural injury[176,177]. If it is assumed that adherent CD34+ cells lie quiescent in the bone marrow and migrate during injury, it is a reasonable to speculate that during neural injury, a higher frequency of adherent CD34+ cells are mobilized from the bone marrow. These cells might then migrate to the site of neural damage and differentiate into microglia, the immune cells of the CNS.

Functional experiments revealed that CD34MG cells secrete pro-inflammatory cytokines TNFα and IL-6 in response to LPS, an *in vitro* model of pathogens. These cells also demonstrated antigen presentation capabilities. The potential of these cells for autologous cell based therapy in glioblastoma was then explored. These cells are tumor-tropic and secrete TNFα which is known to cause apoptosis in glioblastoma cells. Although *in vitro*

studies demonstrate the anti-glioblastoma potential of CD34MG, the molecular mechanisms of action remain unknown. As cells which demonstrate immune capable functions, it is important to examine the immune responses triggered by the presence of these cells in an animal model before they are considered for stem cell therapy.

Gordon and colleagues[26] demonstrated through nested-PCR that adherent CD34+ cells express neural associated message including Nkx2.2, NeuroD, and Pax6 suggesting that adherent CD34+ cells might have neural differentiation potential. The results revealed by this study demonstrated that adherent CD34+ cells are not capable of neural cell differentiation *in vitro*. This lack of neurogenic potential was further validated by DNA replication timing which indicated that neurogenic genes are not likely to be expressed by bone marrow derive stem cells including adherent CD34+ cells. Although nested-PCR results show expression of neural associated genes, their translation may not necessarily occur. Although neural genes are expressed, other regulatory molecules might be present, preventing neural differentiation.

7.2 Stem cell hierarchy

The 'stemness' continuum is anchored at one end by plastic totipotent stem cells, and at the other end by terminally differentiated cells. Within the stem cell developmental hierarchy, adherent CD34+ cells are believed to be more similar to pluripotent stem cells than to germ lineage committed multipotent stem cells. The original data on adherent CD34+ cells revealed that these cells can give rise to cells of the haematopoietic lineage, but further analysis demonstrated that adherent CD34+ cells express mRNA for multiple tissue specific transcription factors. Adherent CD34+ cells also express genes and antigens closely

associated with ES cells, strengthening their relatively primitive status. Within the bone marrow stem cell compartment, a hierarchy can be extracted from current literature available. Mesenchymal stem cells are multipotent stem cells which give rise to cells of the mesodermal germ layer. Haematopoietic stem cells are lineage restricted unipotent stem cells which give rise to the various cells of the circulatory and immune systems. Within the bone marrow there are other stem cells whose lineage differentiation restrictions are uncertain. Stem cells that resemble mesenchymal stem cells which have been identified recently are the MAPC and MIAMI cells[12,13,178]. The MAPCs do not appear until several passages in an artificial culture, raising uncertainty about their place in the natural bone marrow[12,179]. They differentiate into cells of mesoderm, but have not been proven to reproducibly differentiate into cells of a different germ layer. MIAMI cells express antigens associated with pluripotent stem cells, Oct4, Rex1, SSEA4 and have been demonstrated to differentiate into cells of mesodermal, ectodermal, and endodermal lineage[13,178]. These cells are also only characterized and identified after several artificial passages and expansion for over 50 cell doublings[13,178]. Although MAPC and MIAMI cells express pluripotency markers, the fact that they are only identifiable after artificial culture suggests that these populations may result from prolonged culture rather than being natural populations which are identifiable in the bone marrow. VSEL cells are small embryonic like cells that are proposed to have been deposited in the bone marrow during ontogeny[14,25]. They are identifiable in peripheral blood, bone marrow, and umbilical cord blood, and express pluripotency markers as well as tissue-specific markers[14,16,25,33,180]. These cells share similar properties with adherent CD34+ cells and could be the same population of stem cells.

Within the bone marrow stem cell continuum, VSEL cells and adherent CD34+ cells are likely to be the most primitive cells thought to have been deposited during development.

Ratajczak et al[31,32] hypothesized that during development, pluripotent stem cells are deposited in the bone marrow for future use. This phenomenon that has been termed 'developmental migration' whereby stem cells are chemoattracted during ontogeny, to the bone marrow which provides a microenvironment appropriate for a stem cell niche[14,25,33]. The developmental origins of adherent CD34+ cells are evidenced by fetal cell microchimerism dwhich is the presence of fetal cells in maternal tissue[27]. Fetal cells persist for many years after pregnancy and have been identified in multiple organs. In a study of maternal blood, cells from male offspring were detected among maternal adherent CD34+ cells[27]. In addition, studies using murine models have demonstrated that fetal microchimeric cells are recruited in response to injury. This provides evidence that adherent CD34+ cells are residual of developmental cells and supports their primitive status.

Enver and colleagues revealed that hematopoietic stem cells are 'multilineage primed', expressing low levels of mRNA of various hematopoietic lineages[11,41,42]. This implies that a single stem cell is capable of transcribing genes of multiple lineage specifications when placed in the appropriate inductive microenvironment[11,41,42]. Adherent CD34+ cells express genetic messages for multiple tissue lineages including haematopoietic, hepatic, pancreatic, cardiac, and neural cells[11,26]. Applying the theory of multilineage priming, the corresponding genes should be available for transcription by adherent CD34+ cells, thus enabling these cells to contribute to the regeneration and repair of injured tissue. Since the cells are innately primed for multiple lineage specification, they lack the need for de-differentiation, transdifferentiation, or cell fusion[11].

7.3 Potential use of adherent CD34+ cells in neurological deficits

Although the timely limitations of this study restrict the exploration of the potential therapeutic use of adherent CD34+ cells in glioblastoma multiforme, further studies may reveal their use for other neurological disease. Two neurological diseases often explored by other laboratories for stem cell therapy are Parkinson's disease and cerebral ischaemia. It is speculated that stem cells migrate in response to injury in order to aid in tissue repair and regeneration[11, 15, 16,19], including during cerebral ischaemia[15, 177]. Studies have reported increases in stem cell frequency in the peripheral blood after cerebral ischemia[15, 177], based on these observations, it is a reasonable assumption that microenvironmental cues within the site of injury either induces stem cells to mobilize into the peripheral blood, or quiescent stem cells into the cell cycle, or induces the rapid proliferation of transitamplifying cells to give rise to as many cells as possible to aide repair[8]. Diseases in which this rapid proliferation of cells is desirable are those associated with tissue death. In the CNS, diseases involving focal cell death are cerebral ischemia and Parkinson's Disease. Parkinson's Disease requires a specific neuronal regeneration and re-circuitry, thus far stem cell therapy has not proven to either replace degenerated dopaminergic neurons nor restore innervations to the site of injury. It is a reasonable suggestion that stem cells which are capable of differentiating into endothelial cells are most suitable for therapy in cerebral ischemia. Previous data from this group demonstrated that adherent CD34+ cells are capable of differentiating into endothelial cells[201]. The endothelial potential of adherent CD34+ cells suggests that they might be able of contributing to angiogenesis, further suggesting they might be useful in cerebral ischaemia. This section discusses the potential additional uses of adherent CD34+ cells in the two neurological diseases where these cells would likely be most beneficial. Although this study focused only on glioblastoma multiforme, the potential use of adherent CD34+ cells in cerebral ischemia is also discussed for future studies.

7.3.1 Cellular requirements for cell therapy in cerebral ischemia

In three types of animal models of cerebral ischemia, global forebrain ischemia, focal ischemia, and middle cerebral artery occlusion, endogenous neurogenesis is induced in the two neurogenic regions of the brain[67,160]. BrdU labeling experiments revealed that middle cerebral artery occlusion of an animal model is followed by a marked increase in neural cell proliferation in the subventricular zone and neuroblast migration to the site of injury[67,158]. This observation suggests that the brain has the capacity to regenerate neural cells after damage. Despite this regeneration, there is limited permanent replacement of lost neurons. About 80% of new neurons die within 6 weeks of the ischemic event, and only about 0.2% of lost neural cells are replaced[67,158]. Although new neural cells are regenerated, the lack of blood supply to the area leads to death of the new cells. In order for stem cell replacement therapy to be effective, there must be (*i*) revascularization of the damaged area and reestablishment of the blood supply, (*ii*) migration of transplanted stem cells through site of injury, (*iii*) concerted regeneration and differentiation of stem cells into multiple types of neural cells, and (*iv*) establishment of saltatory conductivity with existing circuitry[67,125,158-160].

The angiogenic properties of bone marrow derived stem cells including adherent CD34+ cells, MSC, MAPC and HSC would contribute to neovascularization of the ischemic penumbra. After ischemia, the blood brain barrier is compromised, allowing extra-cranial delivery of stem cells injected into the carotid artery. Stem cell replacement therapy using bone marrow derived stem cells would be ideal for cerebral ischemia to aid revascularization and prevention of further neural cell damage. Using adherent CD34+ cells would allow for an autologous transplant of stem cells. Although this study has demonstrated that adherent CD34+ cells might not differentiate into functional neurons, if they aide angiogenesis, this

would allow the regeneration and establishment of endogenous neurogenesis. A Phase I clinical trial using CD34+ cells in patients with acute cerebral ischemia has begun. To date, one patient with focal ischaemia was infused with autologous adherent CD34+ cells. This patient has demonstrated recovery of motor and cognitive function, not typically associated with acute ischaemia. The results are promising in this one patient, but further patients must be recruited. Retrospective studies of the possible mechanisms of recovery must be derived in an appropriate animal model. One speculated mechanism is by adherent CD34+ cell contributions to intracranial angiogenesis, possibly restoring partial blood supply to the ischaemic area.

7.3.2 Potential use of stem cells in brain tumors

Bone marrow derived stem cells are tumor trophic and can contribute to existing vasculature and home to sites of neovascularization[181]. Although this property is extremely useful in ischaemic injuries such as myocardial infarct, cerebral ischaemia, or ischaemic limbs[179,182-186], increasing vasculature in malignant settings contributes to tumor growth and development[187]. Both MSC and MAPCs contribute to tumor angiogenesis and neovascularization of solid tumors[181,187,188]. In the case of brain tumors, MSCs are recruited to the peritumoral region by glioma cells which release angiogenic factors. These MSCs then contribute to angiogenesis and tumor vasculature[138,188]. HSCs are also known to contribute to the vasculature in brain tumors[189,190].

The specificity of stem cell migration to solid tumors is either taken as a negative indication as they contribute to angiogenesis and vascularization of the tumor, or as an indication that they can be exploited for therapeutic purposes such as the delivery of anti-tumor genes and apoptotic molecules. Adherent CD34+ cells may be seen in the same light. Work by

previous members of the group demonstrated that adherent CD34+ cells are capable of differentiating into vascular endothelial cells, upregulating von Willebrand factor, EGFR and VEGF. In an animal model of myocardial infarction, adherent CD34+ cells administered to the site of injury induced functional recovery. Based on the *in vitro* expression of pro-angiogenic factors, the functional recovery in the animal might be due to revascularization of the ischemic tissue by mechanisms induced by adherent CD34+ cells. The pro-angiogenic effect of adherent CD34+ cells may be valuable in treatment of ischemic injuries, but likely detrimental in tumor therapy. In contact co-cultures of adherent CD34+ cells with glioblastoma cell line U251 in this study, the U251 cells persisted. Thus adherent CD34+ cells do not prevent the survival of glioma cells *in vitro*, and this has also been observed with other cancer cell lines by other members of the group. However when adherent CD34+ cells were differentiated into CD34MG cells, cell dose dependent apoptosis of U251 cells was observed in contact co-cultures with CD34MG. This result demonstrated that naïve adherent CD34+ cells do not cause apoptosis of glioblastoma cells, but their differentiated derivatives do. The pro-apoptotic effect might be due to TNFα release by CD34MG cells which was observed in the presence of pathogens. Whether similar results will be seen in brain tumor bearing animal models will give a more accurate view of the potential therapeutic value of CD34MG cells in gliomas.

7.3.3 Potential use of adherent CD34+ cells in glioblastoma therapy

Conventional therapies for glioblastomas are non-specific and often lead to incapacitating damage to surrounding normal tissue[73,190]. There is a need for the development of tumor specific therapeutic strategies. The tumor specificity of stem cells including NSC, MSC, and umbilical cord derived MSC, has been exploited to deliver pro-apoptotic genes, such as IFNβ and TNFα, and anti-tumor drugs, to glioblastomas in animal models[136,141,191]. These studies

have found tumor regression in murine models of glioblastoma. Having demonstrated tropism towards glioma cells *in vitro*, adherent CD34+ or CD34MG cells might be a useful tool for drug or gene delivery for glioblastoma and should be further explored in future studies. Evidence from the literature concludes that naïve stem cells, especially those which are capable of long term culture and expansion, should not be used as therapy for cancer as they are more likely to contribute to malignancy[187,188,190]. Conversely, genetically manipulated stem cells with tumor tropic features can be exploited to deliver anti-tumor genes and drugs[136]. Adherent CD34+ cells are likely to contribute to tumor vascularization and growth, but perhaps either their manipulation into CD34MG cells, or transfecting them with anti-tumor molecules might prove therapeutic.

One caveat of stem cell therapy is the risk of teratoma formation by the stem cells themselves[164,181,187,188,190]. Adherent CD34+ cells are not likely to form tumors themselves. Adherent CD34+ cells are a slowly cycling or quiescent cell population. The genetic characterization of adherent CD34+ cells and non-adherent CD34+ cells revealed that adherent CD34+ cells more highly express genes involved in cell cycle checkpoints and suppression, while non-adherent CD34+ cells highly express proliferation genes including c-Myc. Although it is speculated they are not tumor forming, adherent CD34+ cells might contribute to tumor angiogenesis.

7.3.4 The potential usefulness of CD34MG

This work demonstrated that adherent CD34+ cells differentiate into microglia-like cells, CD34MG. After insult or injury, endogenous microglia, the immune cells in the nervous system, are the first cells to migrate and respond to the site of injury. Microglia become activated, proliferate, and secrete pro-inflammatory cytokines such as TNFα, IL6, and IL8.

It was previously thought that the blood brain barrier was impermeable, but recent evidence shows that the blood brain barrier is compromised during neural injury[192,193]. Murine models of cerebral ischemia demonstrated that after ischemia, CD34+ cells from the bone marrow are recruited and differentiate into microglia and astrocytes[176,177]. Brain tumors recruit peripheral blood derived monocytes which infiltrate the solid tumor[73,194]. These infiltrates include immune cells such as T cells, dendritic cells, and macrophages, but these cells are immunosuppressed within the glioma environment. This immunosuppression is part of the immune evasion strategy of gliomas. Although glioblastoma cells express HLA Class I and II, they lack co-stimulatory molecules B7.1 and B7.2[143], thus CD4+ T cells which infiltrate solid tumors are not activated and no anti-tumor immune response is induced. Glioblastoma associated T cells can be activated by the addition of exogenous B7 stimulatory molecules, anti-CD28 antibody[143].

Tumor surveillance is a function of the immune system mediated by natural killer T cells (NKT) which mount an antitumor immune response by activating natural killer cells and cytotoxic T lymphocytes (CTL)[143]. Either the level of activation is insufficient in the prevention of malignancy, or the immune escape mechanisms of the tumor are effective enough to overcome innate anti-tumor immunity. Tumor-specific antibodies and CTLs have been isolated from the peripheral blood of glioblastoma patients[73]. Antigens drain from the CNS to the cervical lymph nodes where they can be presented by antigen presenting cells to naïve T cells. Although naïve T cells cannot cross the blood brain barrier, activated T cells migrate to sites of inflammation in the CNS[73]. Professional antigen-presenting cells have not been documented in the CNS and it is thought that the microglia are the antigen presenting

cells in the brain[195,196]. Microglia are bone marrow derived and can present antigen to T cells in a mixed lymphocyte reaction[197].

There was demonstrable evidence in this study that CD34MG cells are functional microglia that are capable of presenting antigen and tumor specific antigen. There may be value in exploring the potential of CD34MG for use in active immunotherapy. The caveat with dendritic cell therapy is that it induces such a strong anti-tumor response that autoimmunity is a great risk. *In vitro* lymphocyte reactions in this study demonstrated that although capable of glioma tumor antigen presentation, CD34MG cells do not induce as robust a responder proliferation as professional antigen presenting cell, peripheral macrophages. This result suggests that CD34MG *in vivo* activation of anti-tumor immunity may not be as strong as that induced by professional antigen presenting cells. It will be important to determine whether the immune response will be sufficient to induce CD8+ cytotoxic T cell activation and tumor clearance.

7.4 Future studies

Gordon and colleagues[11] have previously successfully demonstrated the clinical safety of adherent CD34+ cells and have begun their use in a neurodegenerative disease. This project was explore the potential use of adherent CD34+ cells for cell therapy in an *in vitro* setting. Future studies should next focus on creating pre-clinical models of neurological diseases in murine models. Possible studies are discussed below.

Neural differentiation experiments determined that these cells o not have neurogenic potential *in vitro*. Given time and resources, this finding should have been confirmed *in vivo*. A

possible experiment would be to induce neural cell death in an immunodeficient mouse model, such as acute stroke. The adherent CD34+ cells should then the transplanted, and cell migration and localization tracked. After assessment of possible functional recovery in the animal model, histology should be conducted to determine the final fate of the transplanted adherent CD34+ cells. These results might then reveal a possible mechanism of functional regenerative capacity in the animal, regardless of neuronal differentiation of the cells.

Adherent CD34+ cells were capable of differentiating into microglia like cells CD34MG, and their potential for use in therapy in glioblastoma muliforme explored. Here it was demonstrated that the cells migrate and invade in specific response to glioma cells, and that CD34MG cells are antigen presenting cells, capable of presenting glioma specific antigen in an autologous reaction. It was further demonstrated that CD34MG secrete TNFα, a cytokine known to induce apoptosis in glioma cells[152-153]. This may be the mechanism by which CD34MG cells caused apoptosis to glioma cell line U251 cells in a dose dependent manner. Given time and resources, this study should continue in an intracranial tumor bearing immunodeficient murine model. Two main studies can be conducted, the first would be to examine if transfusion of CD34MG cells causes tumor regression or prevents tumor growth. The second possible study would be based on immunotherapy. CD34MG have demonstrated their antigen presenting capabilities. *In vitro*, CD34MG cells can be pulsed with glioma lysate to load them with glioma specific antigen. The loaded cells would then be infused into tumor bearing murine models, and possible tumor regression measured. Monitoring for tumor specific immune cells (such as cytotoxic CD8+ cells) would also be analysed.

7.5 Overview

Adherent CD34+ cells are a primitive stem cell-enriched population which is distinct from the non-adherent CD34+ population. This study contributed to their genetic, antigenic, and functional characterization, and further supported their status as a primitive quiescent stem cell population. Adherent CD34+ cells are capable of differentiating into microglia like cells, upregulating antigenic markers and functional characteristics of microglia. As the ultimate aim of this project was to exploit adherent CD34+ cells for use in neurological deficits, *in vitro* investigations were conducted to determine if the adherent CD34+ cell derived microglia-like cells had properties which might be useful for therapy against glioblastomas. Tumor trophic characteristics, TNFα secretion and antigen presentation are properties that were observed by this study and may prove useful for translational use. Adherent CD34+ cells have been proven safe for clinical use, and although their potential for use in stem cell based therapy in neurodegenerative disease has only been explored *in vitro*, they demonstrate potential for autologous cell transplant in glioblastoma cell therapy.

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