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Pleiotrophin and its Role in Regulating Haemopoietic Stem Cell Regeneration

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Pleiotrophin and its role in regulating haemopoietic stem cell regeneration.

By

Rebecca Smith.

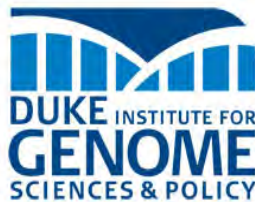


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M.Phil Biomedical Sciences

In conjunction with FAS Science challenge

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Abstract

Haemopoietic stem cells (HSCs) possess the unique capacity to self-renew and give rise to the entirety of the haematopoietic and immune systems throughout a lifetime (Dexter 1977). Patients with cancer are typically treated with high dose chemotherapy and/or radiation therapy which cause profound toxicity to the hematopoietic system resulting in high susceptibility to infection (Weiden 1973). Extensive research has been performed to identify genes/proteins which regulate hematopoietic self-renewal and repair in an attempt to develop therapies to mediate hematopoietic recovery from these therapies (Moore 1987, McNiece 2000, Holyoake 1996). To date there has been no soluble growth factor identified that acts on the HSC that may be used clinically. Therapeutic agents currently used clinically include erythropoietin (EPO) (Sieff, 1986) and Granulocyte-monocyte colony stimulating factor (GM-CSF) (Monroy 1987) which act on lineage progenitors to mediate a recovery response.

The Chute laboratory in Duke University, North Carolina, U.S.A, hypothesise that Pleiotrophin (PTN) is a novel growth factor for the HSC, and are currently working to understand the mechanism of this process.

In this research project we examined the effect of Pleiotrophin (PTN) on HSC regeneration both in vitro and in vivo post radiation injury. Mice treated with rPTN after a myelotoxic dose of radiation displayed increased short and long term haemopoetic progenitors indicating PTN can induce HSC regeneration in vivo. To determine if this induction of HSC regeneration was transferable clinically, two survival studies were performed, PTN treatment however did not prolong survival due to a failure to augment simultaneous mature blood cell reconstitution. PTN, through this research project is indicated as a regenerative factor for the HSC in vivo however its activity is localized to the HSC progenitor compartment.

Declaration

I certify that this thesis which I now submit for examination for the award of

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This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

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Abbreviations

- AGM (Aorta, gonads and mesonephrons)
- ALK (Anaplastic lymphoma kinase)
- Ang-1 (Angiopoietin-1)
- APC (adenomatosis)
- B.M (Bone Marrow)
- BFU-E (Burst forming unit-erythroid)
- CB (Cord Blood)
- CCIF (Cancer Centre Isolation Facility)
- CD (Cluster differentiation)
- cDNA (complementary DNA).
- cGy (Centigray units)
- CFC (Colony forming cell)
- CFDA-SE (Carboxy-fluorescein diacetate succinimidyl ester)
- CFU (Colony Forming Unit)
- CFU-GM (Colony Forming Unit Granulocyte Monocyte)
- CFU-GEMM (Colony Forming Unit-Granulocyte erythroid monocytic megakaryocyte)
- CLP (Common lymphoid progenitor)
- CMP (Common myeloid progenitor)
- CO₂ (Carbon Dioxide)
- DMSO (Dimethyl Sulfoxide)
- DNA (deoxyribonucleic acid)
- D-PBS (Dulbecco's Phosphate buffered Saline)
- EC (Endothelial cell)
- ECCM (Endothelial cell culture medium)
- EDTA (ethylenediaminetetraacetic acid)
- EPO (erythropoietin)
- FACS (Fluorescent activated cell sorting)
- FBS (Fetal Bovine Serum)
- FITC (Fluorescein isothiocyanate)

- GAPDH (Glyceraldehyde 3 phosphate dehydrogenase)
- G-CSF (Granulocyte Colony Stimulating Factor)
- GM-CSF (Granulocyte Monocyte Colony Stimulating Factor).
- GM-CSFR (Granulocyte Monocyte Colony Stimulating Factor Receptor)
- GR-1 (Granulocyte Receptor 1)
- GSK-3B (glycogen synthase kinase 3 beta)
- GVHD (Graft versus host disease)
- HB (haemoglobin)
- H&E (Haematoxylin and Eosin)
- hG-CSF (human Granulocyte Colony Stimulating Factor).
- HSC (Hematopoietic stem cell)
- HSCT (Hematopoietic stem cell Transplant)
- HUBEC (Human Brain Endothelial Cells).
- IMDM (Iscove's Modified Dulbecco's medium)
- IP (Intraperitoneal)
- IR (Irradiated)
- KSL (c-Kit positive, Sca-1 positive, Lineage negative)
- KTLS (c-Kit positive, Thy1.1, Sca-1 positive, Lineage negative)
- LDL (Low density lipoprotein)
- LEF (lymphoid enhancer binding factor)
- LFA-1 (lymphocyte function associated antigen-1).
- LIN (Lineage negative)
- LT-HSCs (Long-term Haemopoietic Stem Cells)
- Ly6A/E (Lymphocyte activation protein 6A)
- MACS (Magnetic cell sorting kit)
- MMP9 (Matrix metallopeptidase 9)
- mL (Millilitre)
- mm (millimeter)
- MNC (Mononuclear cells)
- NC (North Carolina)
- NK (Natural Killer)
- NMR (nuclear magnetic resonance)

- NOD/SCID (non-obese diabetic severe combined immunodeficient)
- OCT (Optimum cutting temperature)
- PAS (paraaortic splanchnopleura)
- PBS (Phosphate buffered saline)
- PCR (Polymerase chain reaction)
- PDGF (Platelet derived growth factor)
- PDGFR (Platelet derived growth factor receptor)
- PE (Phycoerythrin)
- PEN (Penicillan)
- PI3K (phosphoinositide 3 kinase)
- PTH (Parathyroid Hormone)
- PTHr (Parathyroid Hormone receptor)
- PTN (Pleiotrophin)
- *ptn* (pleiotrophin gene)
- RNA (Ribonucleic acid)
- RPM (revolutions per minute)
- rPTN (recombinant Pleiotrophin)
- RPTP $\beta\zeta$ (receptor proteinase tyrosine phosphatase beta zeta).
- RT (Reverse Transcription)
- R.T. (Room Temperature)
- RT-PCR (Real-Time Polymerase Chain Reaction).
- SDF (Stromal derived Factor)
- SRC (SCID repopulating cells)
- SNO (spindle shaped N-Cadherin⁺ CD45⁻ osteoblastic cells).
- STREP (Streptomycin)
- TCF (T-cell factor family)
- TCR (T cell receptor)
- TSF (Thrombopoietin, stem cell factor and Flt-3 ligand)
- VE-Cadherin (Vascular Endothelail Cadherin).
- VLA-4 (Very Late Antigen 4)
- 7-AAD (7-Amino-Actinomycin D)
- °C (degrees celcius)

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1.0 Introduction

1.1 Haemopoietic stem cell (HSC)

HSCs are cells that have the capacity to self renew, proliferate and differentiate, providing cells of the mature blood system throughout a lifetime. The concept of such a cell was first considered in 1961 subsequent to the injection of healthy marrow into a lethally irradiated recipient mouse which mediated the reconstitution of mature blood cells in the irradiated host (Till and Mc Culloch, 1961). This finding provided preliminary experimental evidence that all of the blood cells were derived from a single type of progenitor cell, also known as HSC. The majority of mature blood cells have a limited life span, red blood cells survive for 120 days and some white cells just hours, thus requiring their continuous replacement to sustain life. Replenishment of the mature blood cells does not occur as a direct single step but is in fact achieved through the production of progenitor cells that display diminished self-renewal capabilities with increased maturity and the assembly of HSC hierarchy. HSCs differentiate to multi-potent progenitors (Allen.D, 1984). These multi-potent progenitors have the ability to differentiate into either myeloid or lymphoid progenitor cells. Lymphoid progenitor cells give rise to T, B and Natural Killer (NK) cells while the myeloid progenitor cell gives rise to neutrophils, basophils, eosinophils, red cells, platelets and monocytes/macrophages (figure 1) all of which require constant replenishment to keep circulating blood levels constant. This method of mature blood cell production allows a large number of mature cells to be derived from a small population of HSCs. (Lajtha, 1979)

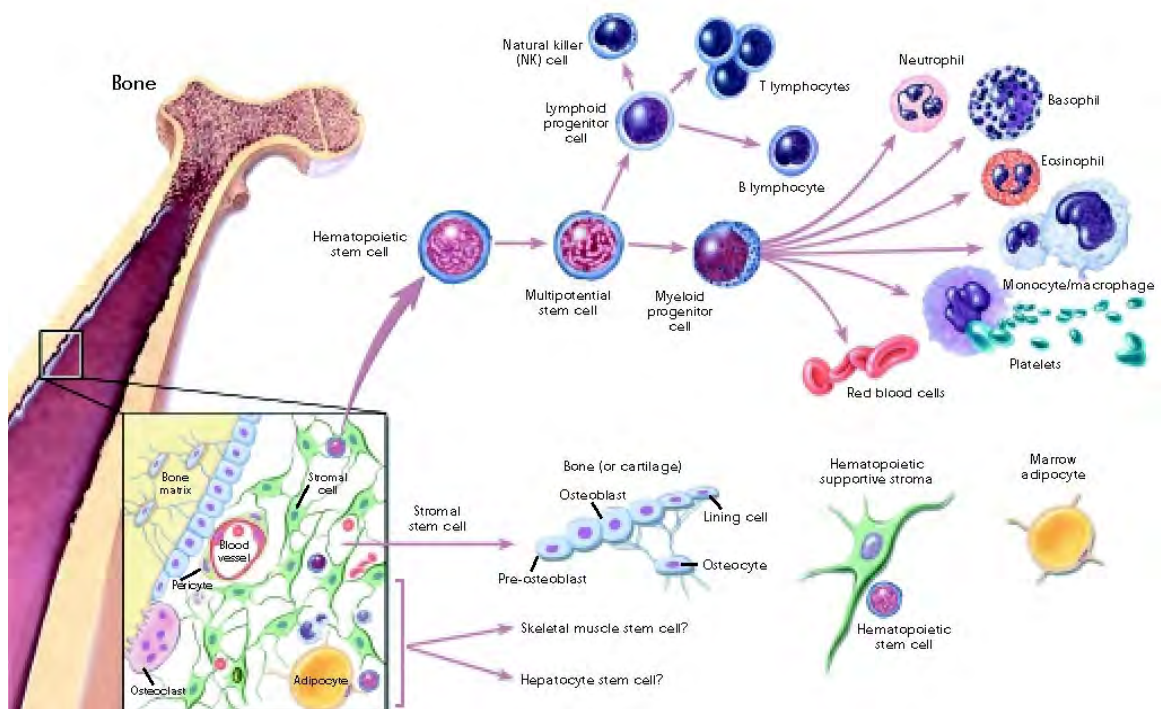


Figure 1: Haemopoietic stem cell (HSC) differentiation to mature blood cells. HSCs have the ability to self renew, proliferate and differentiate giving rise to all of the mature elements of the blood and immune systems throughout life. THE HSC differentiates to a less primitive multi-potent progenitor cell and which differentiates into either a lymphoid or myeloid progenitor and is said to be lineage committed as these that have differentiated to a lymphoid progenitor will give rise to B,T and natural killer (NK) cells whereas those multi-potent progenitor cells that have differentiated to a myeloid progenitor cell will give rise to neutrophils, basophils, platelets, red cells and monocytes/macrophages.

1.2 HSC origin

During foetal development haemopoietic activity begins in the yolk sac with the appearance of embryonic blood islands. The blood islands are primitive blood cells that are surrounded by endothelial cells, both of which are thought to originate from a common precursor or haemangioblast (Oberlin et al, 2002). These blood cells are generated through a process that is known as primitive haemopoiesis. HSCs originate in the aorta, gonads and mesonephrons (AGM) and paraaortic splanchnopleura (PAS) regions of the embryo proper which following the development of the circulatory system migrate to the liver of the embryo (Godin *et al*, 1995, Dzierzak.E, 1995). Subsequently the HSCs migrate to the spleen and lastly to the major site of haemopoiesis, the bone marrow. There are large numbers of stem cells within the foetal circulation but also in the umbilical cord (Wang, 1997). Haemopoietic activity at birth is widely distributed throughout the skeleton,

however this retreats with age so that haemopoiesis occurs in sternum, pelvis, skull and vertebrae in adulthood (Figure 2).

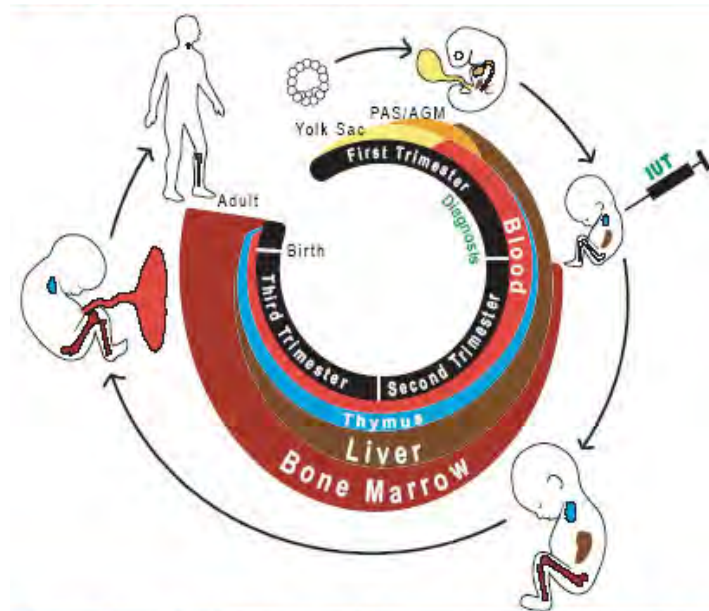


Figure 2: Sites of Haemopoiesis during development. Haemopoietic activity begins in the yolk sac with the formation of blood islands followed by the development of HSCs in the mesonephrons (AGM) and paraaortic splanchnopleura (PAS) regions of the embryo proper. With the development of the circulatory system stem cells transmigrate to the fetal liver. At the time of birth the primary site of haemopoiesis is the marrow and is widely distributed throughout the skeleton. In adults haemopoiesis is limited to marrow of the sternum, the skull , the vertebre and the pelvis.

Murine embryonic development is quite comparable to human embryonic development, however sites of haemopoiesis in the adult mouse differs from the adult human. Primary sites for murine haemopoiesis include the spleen, femur and tibia.

1.3 HSC niche

The stem cell niche or microenvironment, first hypothesised by Schofield, 1978 is the site at which HSCs reside and the process by which haemopoiesis occurs in vivo. The notion that a niche provided support to the HSC was further supported by in vitro co-culture experiments where increased HSC growth was noted (Dexter, 1977, Rios, 1990). The niche itself is composed of numerous cell types such as endothelial cells, fibroblasts, adipocytes and osteoblasts and displays both anatomical and functional properties. The anatomical structure provided by the cells and their products imparts physical support to the marrow which houses and protects the multi-potent stem cells. Functionally, the cells within the niche act to regulate stem cell proliferation, differentiation, survival and self-renewal which is achieved either directly or indirectly (Moore 1987). Figure 3

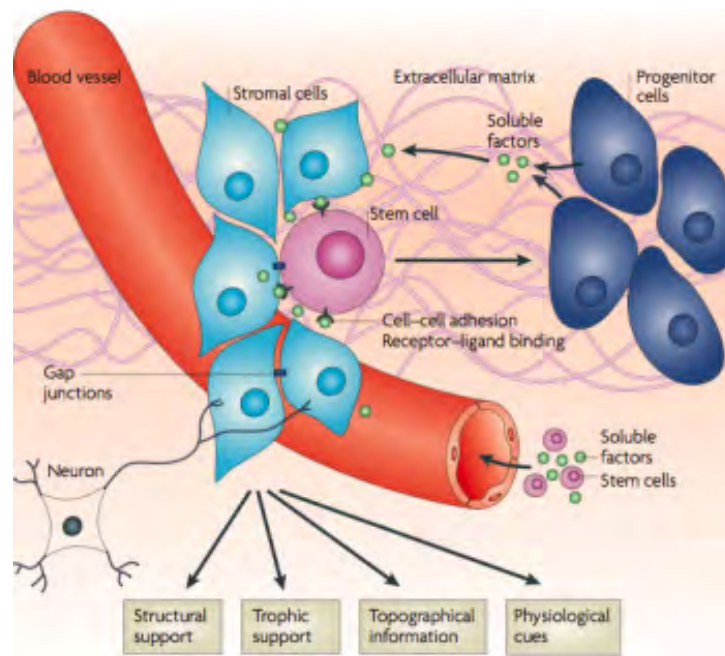


Figure 3: Haemopoietic stem cell niche. The niche is composed of numerous cell types such as endothelial cells, fibroblasts, adipocytes and osteoblasts which provide structural, trophic, topographical and physiological cues to the HSC.

The individual regulatory function performed by the cells of the niche are difficult to discern however research conducted over recent years has identified two diverse niches that support the bone marrow, the osteoblastic and vascular niche.

1.3.1 Osteoblastic niche

Early investigations into the role of osteoblasts indicated that they were an important component in skeletal development through bone generation and remodelling. The role of osteoblasts in HSC maintenance however is less well recognised. Mesenchymal stem cells which reside in the marrow give rise to a number of cells such as the chondrocytes, adipocytes, endothelial cells and osteoblasts in the marrow (Short, 2003 Murguruma, 2006) . Osteoblasts were thought to play a role in HSC maintenance initially through anatomical arrangement or location as HSCs reside along the endosteal surface of trabecular bone in close proximity to osteoblasts (Lord 1975). Also during embryogenesis primitive haemopoiesis occurs in the bone resorption centres suggesting perhaps osteoblast association was involved in HSC maintenance (Patt, 1972). In vitro co-cultures with osteoblasts supported the growth of HSCs (Tachiman 1994, Tachiman, 2000). Zhang et al outlined in vivo experiments whereby an increase in a particular sub-population of osteoblastic cells, spindle shaped N-cadherin⁺ CD45⁻ osteoblastic cells (SNO) that lined the bone increased the number of HSCs indicating this sub-population of osteoblasts as key components of the niche environment in vivo (Zhang, 2003). In addition to this, Calvi et al, performed a study whereby an increase in Parathyroid hormone (PTH) and activating Parathyroid hormone receptor (PTHr) in stroma cultures in vitro resulted in an increase in osteoblasts in turn increasing the number of long-term HSCs (Calvi,2003). Along with this study Calvi et al performed an in vivo study whereby the mice were genetically modified to produce activated parathyroid and parathyroid hormone related protein specific to osteoblasts which displayed an increase in long-term Haemopoietic stem cells (LT-HSCs) in vivo (Calvi, 2003). To further endorse this hypothesis Visnjic et al, 2004 investigated the depletion of osteoblasts and the effect this had on HSCs and the haemopoietic system. In order to perform this study a transgenic mouse was created whereby developing osteoblasts specifically expressed the herpes virus thymidine kinase gene that was under the control of a collagen promoter. The advantage of this mouse was that treatment with Ganciclovir, which activates thymidine kinase function, resulted in the eradication of the osteoblasts (Visnjic, 2004). The result observed was a decrease in marrow cellularity, a decrease in myeloid, lymphoid and erythroid progenitors and a decrease in the absolute numbers of HSCs. The discontinuance of Ganciclovir resulted in the re-emergence of osteoblasts and marrow cellularity

indicating quite strongly that osteoblasts play an important role in regulating haemopoiesis and HSCs in the marrow.

1.3.2 Vascular niche

HSCs not only localise along the endosteal surface of trabecular bone in close proximity to osteoblasts but also with the vascular endothelial cells from the earliest stages of embryogenesis through to HSC migration to the fetal liver (Oberlin, 2002). HSCs and progenitors also reside in close proximity to the BM sinusoidal vessels in the adult (Avecilla, 2004) and as the HSC and endothelial cells (EC) are derived from the same precursor or haemangioblast (Choi, 2002), investigations into a possible regulatory relationship between the HSC and ECs could be considered justifiable. HSCs when cultured in vivo with primary ECs maintained the HSC number and its repopulating ability in vivo (Yin, 2006). These findings are consistent with other studies including the experiment performed by Chute et al whereby human CD34+ CD38- haemopoietic stem cells when cultured in vitro with Human Brain Endothelial cells (HUBECs) (Chute et al, 2005). They found this increased the number of repopulating cells in non-obese diabetic Severe combined immune deficient (NOD/SCID) identifying the success of their engraftment. NOD/ SCID mice are a cross-breed of mice that have a genetic disorder whereby mice homozygous for the mutation display no mature T and B lymphocytes (SCID) (Bosma,1991) and mice that develop autoimmune T cell mediated insulin dependent diabetes mellitus (NOD) (Kataoka, 1983). The cross breeding results in a mouse which lacks T cells but that does not develop insulin dependent diabetes mellitus. These NOD/SCID mice are widely used to study human haemopoietic stem cell engraftment. (Greiner, 1998).The SCID repopulating cells are those that represent the long-term haemopoietic stem cells and their quantification is used as a technique to assess the reconstitution ability of in-vitro expanded haemopoietic stem cells (Larochelle, 1996, Bhatia, 1998). Co-culture of the CD34+ CD38- HSCs with HUBECs when transplanted into SCID mice displayed myeloid and B-lymphoid differentiation indicating a primitive haemopoietic stem cell was preserved during culture. Interestingly with this study Chute et al in 2002 outlines comparable SRC increases with both contact non-contact HUBEC cultures, indicating the regulation

of the HSC did not require direct contact (Chute, 2005). To further support the hypothesis Avezilla *et al* in 2004 demonstrated that administration of an antibody to vascular endothelium cadherin (VE-cadherin) caused endothelial cell removal resulting in haemopoetic failure. VE-cadherin is an adhesion molecule expressed on the surface of vascular endothelium (Lampugnani, 1995).

The vascular and osteoblastic niches although appear self governing do not exist independently. It is thought that the osteoblastic niche maintains the HSC in a quiescent state and that the vascular niche promotes differentiation and proliferation of HSCs through increased oxygen and growth factor availability accompanied by a loss of self-renewal capabilities (Kopp, 2005). This hypothesis is supported by a Thrombopoetin mutant mice study (Avezilla *et al*, 2004) whereby under stress thrombopoiesis was accomplished through the movement of HSCs from the osteoblastic niche to the sinusoidal endothelial cells where they differentiated into megakaryocyte progenitors and continued to mature to platelets.

1.4 HSC regulation

The majority of cells within the stem cell pool are quiescent, (G₀, phase of cell cycle) (Lajtha, 1979, Ogawa, 1993). A possible motive for this is to maintain a genetically pure pool of cells that can persist throughout life providing adequate mature blood cells. These quiescent cells however can respond to stress whereby they enter the cell cycle and differentiate to mature cells of the peripheral blood after which they return to a quiescent state. This observation implies HSCs must be responsive to positive and negative control factors. Stem cell regulation is a tightly controlled process that is achieved through complex intrinsic and extrinsic signals that are controlled by the surrounding environment. External signals in the form of adhesion molecules or secreted factors communicate to the HSC internal machinery to control HSC fate, be that self-renewal, differentiation or apoptosis.

HSC quiescence in the laboratory may be investigated through the use of Ki-67 antibody which recognises the Ki-67 antigen expressed in all active stages of the cell cycle therefore acting as an indicator for proliferation (Kubbutat, 1994).

1.4.1 Stem cell quiescence

Osteoblasts located at the endosteal surface of the trabecular bone regulate HSC using both adhesion molecules and secreted factors which act co-operatively to suppress HSC cell cycle (Arai, 2005). Adhesion molecules expressed by osteoblasts include N-cadherin and β 1-integrin, both of which act to anchor the HSC to the osteoblastic niche (Potocnik, 2000) maintaining them in an environment that promotes HSC quiescence (Arai, 2004). Angiopoietin-1 (Ang-1), secreted by the osteoblasts acts as a ligand for Tie 2 receptor expressed on the surface of HSCs. Ang-1 when secreted by the osteoblasts binds Tie 2 it activates β 1 integrin promoting adhesion of the HSCs to the osteoblastic niche and maintains the cell as a quiescent HSC (Arai, 2004). To support this hypothesis, mice deficient in Tie 2 failed to survive due to abnormal interaction of the HSC and osteoblastic niche (Suri, 1996).

1.4.2 Stem cell self renewal

The Wnt signalling pathway has been identified as an important pathway for HSC self-renewal (Reya, 2003). Wnt proteins are a family of signalling molecules that are defined by amino acid sequence as opposed to function (Nusse, 1992). The Wnt proteins bind one of two Wnt receptors, a seven transmembrane protein member of the frizzled family (Bhanot, 1996) and the low density lipoprotein (LDL) receptor related proteins (Duncan, 2005). The binding of the Wnt proteins to their receptors results in a cascade of signalling events that is transduced to β -catenin, a cytoplasmic phosphoprotein. β -catenin in the HSC is bound to a degradation complex composed of glycogen synthase kinase 3 beta (GSK-3B) and adenomatous polyposis coli (APC). In the absence of Wnt protein attachment the degradation complex acts to degrade β -catenin (Cadigan, 1997). Wnt attachment to its receptor dissociates the degradation complex ceasing β -catenin degradation resulting in the accumulation of undegraded β -catenin in the cytosol (Willert, 1998). The β -catenin relocates to the nucleus where it binds transcription factors of the lymphoid enhancer binding factor (LEF) and T-cell factor (TCF) family. This binding to the transcription factors activates them resulting in the active transcription of target genes myc and cyclin D which are involved in promoting self-renewal (Willert, 2003). The addition of Wnt3A in vitro promotes HSC self-renewal (Willert, 2003), indicating Wnt

signalling as a significant signalling pathway for HSCs. In contrast and conflicting to this finding is mice deficient in β -catenin displayed regular HSC self-renewal capabilities (Cobas, 2004) suggesting Wnt signalling is not a necessary pathway for HSC self-renewal regulation.

Notch signalling is also described as a regulatory pathway involved in HSC self-renewal (Domen, 1999). Notch a cell surface receptor expressed on HSCs, is cleaved and released into the cytosol when its ligand Jagged 1 is bound. The intracellular domain of the receptor relocates to the nucleus where it binds to the transcriptional repressor CSL transforming it to a transcriptional activator which acts to actively transcribe target genes. Supporting the hypothesis that Notch signalling is important in HSC self-renewal regulation is the observation that HSC differentiation and depletion accelerated with the addition of a notch signalling inhibitor (Duncan, 2005).

Although WNT and Notch signalling promote HSC self renewal in vitro (Reya, 2003, Willert, 2003), It appears these signalling pathways individually are not required for HSC self-renewal as in vivo deletion of the notch receptor and ligand (Mancini, 2005) and B-catenin (Koch, 2008) did not affect HSC maintenance. A possible explanation for this was provided by Morrison, 2008 where they express that maintenance of the HSC pool is so crucial for survival dependence on a single pathway would not be favourable for fear of a flaw. Signalling overlap would ensure no signalling pathway is solely responsible for HSC self-renewal imparting a more robust and adaptable method of HSC self-renewal regulation.

1.5 Stem Cell adhesion homing and migration

HSCs have the ability to move from one location to another in vivo demonstrated by the relocation of HSCs from the fetal liver to the marrow during development and also through the movement of transplanted HSCs to the bone marrow. This movement however is not a random process but is in fact a tightly regulated process that strikes a balance between HSC migration and retention, a process that is greatly influenced and mediated by the activities of adhesion and chemotactic molecules.

HSC movement from the peripheral blood through the vascular endothelium, into the marrow where it is retained and acts as a functional stem cell is termed homing. In contrast HSCs movement from the marrow, where retention has ceased, through the vascular endothelium to the peripheral blood is termed migration.

Adhesion molecules are expressed on the surface of the HSC, the vascular endothelium and stroma cells within the marrow. The adhesion molecules act to mediate entry and exit of HSC to and from the marrow and also to anchor HSCs to the niche to ensure quiescence and maintenance.

1.5.1 Homing

Homing of HSCs to the marrow involves a series of events which include adhesion, disengagement, trans-endothelial migration and engraftment into the niche (Mazo, 1999). The HSCs express a variety of adhesion molecules such as VLA-4, which is member of integrin superfamily, CD34 a member of the sialomucin family, CD31 and CD 50 members of the immunoglobulin superfamily and E and P selectins. Recognition and tethering of the HSC is achieved through the E and P selectins subsequent to which firm adhesion is achieved through VLA-4 and LFA-1. Once firm attachment of the HSC is achieved it squeezes through the endothelium and enters the marrow. A chemokine stromal derived factor 1 (SDF-1) expressed on human and murine marrow endothelial cells (Imai, 1999) and osteoblasts in the endosteal region of the marrow (Ponomaryov, 2000) acts to chemo-attract the HSCs which express CXCR4, the receptor for SDF-1 ligand, This attraction is not through kinetics but through the generation of a concentration gradient (Askenasy,2002). Providing support to this

hypothesis is an in vitro study where CD34+ and a FDCP stem cell line, both of which express CXCR4, moved in response to SCF-1 exposure (Aiuti, 1997). Further highlighting the importance of SDF-1 and CXCR4 in stem cell retention and maintenance was a knockout study where the mice died due to gross bone marrow failure (Tachibana, 1998, Zou, 1998). SDF-1 deficient mice highlight the requirement of SDF-1 ligand in homing, a requirement for effective myelopoiesis in the bone marrow. The SDF-1 deficient mice as embryos displayed typical myelopoiesis, however the SDF-1 deficient neonatal mice which require movement of HSCs from the fetal liver to the bone marrow with development, displayed incomplete bone marrow myelopoiesis. The movement of haemopoietic progenitors in the embryo and fetus from the aorta, gonads and mesonephrons (AGM) to the fetal liver was successful indicated by successful myelopoiesis. The impaired bone marrow myelopoiesis observed within the neonatal mice was due to impaired stem cell migration from the fetal liver to the marrow.

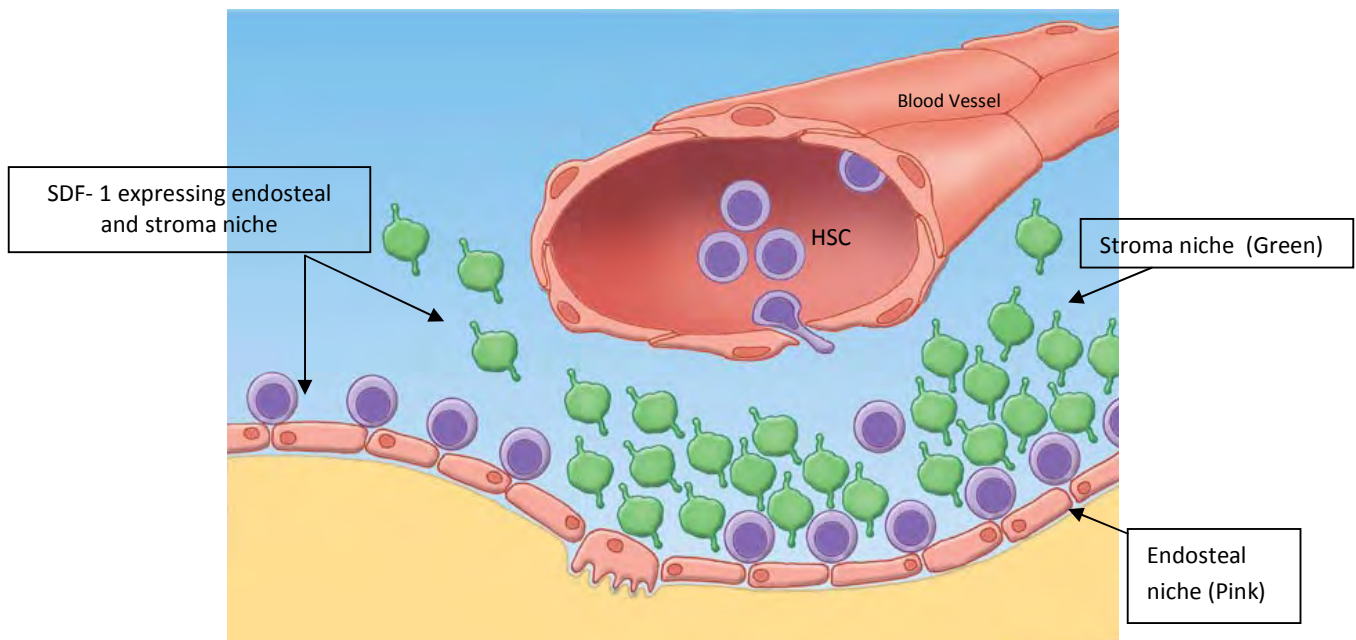


Figure 4: HSC homing. Movement of HSCs (purple) through the vessel is induced by a SDF-1 gradient whereby CXCR4 expressing HSCs are attracted to its receptor SDF-1 expressed on stromal cells (green) and endosteal niche cells (pink).

SDF-1 not only acts as a chemotactic factor for the homing of HSCs but also indirectly acts to maintain HSC quiescence in the marrow when they arrive, through immobility signals (Petit, 2005)

emitted from the osteoblastic niche. The study of HSC homing can be investigated in the laboratory using a dye that fluorescently stains the HSCs such as Carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE). This dye diffuses into the cells where it is retained and permits the ability of the stained cells to home to the marrow when transplanted and can be examined.

1.5.2 Migration

HSC movement from the marrow to the peripheral blood is termed mobilisation and it too is a tightly controlled process involving termination of stem cell anchorage and adhesion molecule activities. It is postulated that HSC mobilisation is involved in HSC homeostasis. It is thought the release of HSC to the peripheral blood is a regulatory mechanism *in vivo* whereby the amount of progenitors in the marrow is controlled so that excess amounts are removed due to the limited number of stem cell niches (Abkowitz, 2003). Bradford et al also demonstrated mobilisation of murine stem cells into the peripheral blood every 30 days is a requirement for cycling and stem cell turnover (Bradford, 1997).

Mobilisation *in vivo* occurs in response to stress signals such as the cytokine G-CSF or chemotherapy. These signals act to stimulate neutrophils and osteoblasts to release of membrane bound SCF-1 (Heissig, 2002) and trigger a transient increase of SDF-1 in the marrow (Petit, 2002). The increase in SDF-1 concentration results in the upregulation of proteolytic enzyme secretion such as cathepsin G, neutrophil elastase and MMP-9 in the marrow (Hessig, 2002, Levesque, 2003 and Petit, 2002). These proteolytic enzymes act to degrade SDF-1 through the cleavage of its NH₂ terminal sequence (McQuibban, 2001) resulting in increased CXCR4 expression (Petit, 2002) and extensive mobilisation of stem and progenitor cells to the peripheral blood.

G-CSF mobilises HSCs through the release of proteolytic enzymes from the neutrophil.

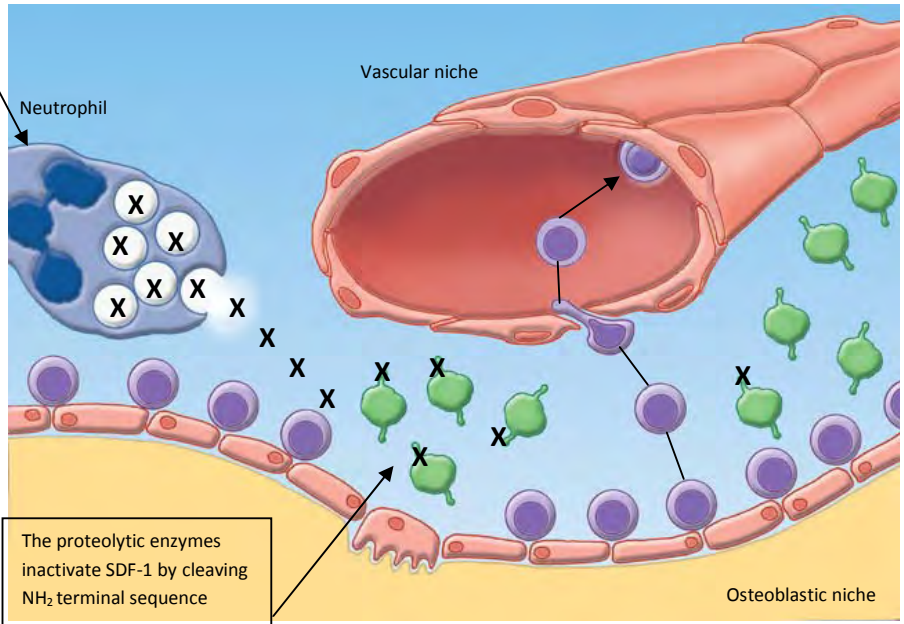


Figure 5: HSC migration. G-CSF activates the neutrophil to release proteolytic enzymes such as G, neutrophil elastase and MMP-9 in the marrow. These proteolytic enzymes then cleave the NH₂ terminal sequence of SDF-1 resulting its degradation and subsequent upregulation of CXCR4 and mobilisation of stem/progenitor cells to the peripheral blood. (Image: Physiology review Kopp, 2005)

Natural recruitment of HSC from the marrow to the peripheral blood through mobilisation is exploited clinically through the administration of hG-CSF (Welte et al 1987). This is widely used stem cell mobilising agent, used to mobilise stem cells to the peripheral blood where they are harvested and used as a major source of stem cells for autologous or allogeneic transplants. Similarly AMD3100 a CXCR4 antagonist is also used clinically as a stem cell mobilisation method in mice and humans (Broxmeyer, 2005).

1.6 Stem cell phenotype

Elucidation of a particular stem cell phenotype would provide a straightforward method of stem cell isolation, identification and quantification, useful both clinically and experimentally. A conclusive way to assess potential stem cell phenotypes is to examine the capability of the cell to engraft the marrow and repopulate the marrow and peripheral blood of an irradiated recipient after transplantation (Kamel-Reid 1998). Cell surface markers and the ability to exclude DNA binding dyes have led to the discovery of a panel of markers or surface antigens on the stem cells. The approach undertaken to identify the particular stem phenotypes in mice and humans have been quite similar, (competitive repopulation assays) (Spangrude, 1988), however the surface markers that identify human from murine haemopoietic stem cells differ.

1.6.1 Human stem cell phenotype

Adult human HSCs do not express many surface markers that are characteristic of differentiated or lineage committed haemopoietic cells (Muller- Sieburg, 1986). These surface antigens are termed lineage markers and are expressed on T and B cells, granulocytes, monocytes, macrophages, erythroid and natural killer cells, thus removal of these cells through negative selection results in the isolation of an immature population of haemopoietic cells that are said to be Lineage negative (Lin⁻). A sialomucin, CD34 was discovered to be expressed on the surface of haemopoietic progenitors with surface expression decreasing with differentiation to more mature cells (Civin, 1990) highlighting it as a potential marker for stem cells. In vitro studies demonstrated the ability of CD34⁺ cells to differentiate into various haemopoietic progenitor and lineage committed cells in long term culture provided support to this concept as well as in vivo repopulation studies. However not all stem cells express CD34 as highlighted by Zanjani et al, where Lin⁻ CD34⁻ cells transplanted into recipient sheep displayed long-term and multi-lineage differentiation. These cells however were incapable of providing short-term stem cells and repopulation capabilities. Interestingly however Zanjani observed

a high number of CD34⁺ cells in the marrow of these sheep suggesting the Lin⁻ CD34⁻ cells are more primitive than the CD34⁺ cells (Zanjani, 1998). One may use other markers with CD34 to determine a more primitive cell such as CD38. CD34⁺CD38⁻ cells displayed an increase in engraftment through the number of repopulating cells when compared to CD34⁺CD38^{low} cells (Bhatia, 1997) in NOD/SCID mice. Co-expression of CD10 or CD7 on CD34⁺ cells defines early lymphoid progenitors (Hao et al, 2001) while expression of IL-3R α ^{lo} on CD34⁺CD38⁺ cells defines early myeloid progenitors (Manz, 2002).

1.6.2 Murine stem cell phenotype.

Similar to the Human HSCs, the adult mouse HSCs do not express surface markers that are characteristic of differentiated or lineage committed haemopoietic cells. As outlined above these surface antigens are termed lineage markers and are expressed on T and B cells, granulocytes, monocytes, macrophages, erythroid and natural killer cells thus removal of these cells through negative selection results in the isolation of an immature population of haemopoietic cells that are said to be lineage negative (Lin⁻).

Murine haemopoietic progenitors were also found to express two cell surface glycoposphatidyl inositol linked immunoglobulin superfamilies, Thy-1.1 and stem cell antigen (SCA-1) (Spangrude et al, 1988). Aihara et al, 1986 produced monoclonal antibodies to pre-T hybridomas, one of which was directed against the cell surface antigen Sca-1 or stem cell antigen 1. The murine HSCs were deemed c-Kit⁺ and Thy1.1⁺ Lin⁻ Sca-1⁺ (KTLS). In contrast it has been shown that KTLS cells are not the only cells to radioprotect and reconstitute blood cell lineage in lethally irradiated mice as the Thy1.1^{lo}, Lin⁻ and Sca-1⁺ (T^{lo}LS) population of cells are capable of radioprotection and mature blood cell reconstitution in the C57BL/Ka-Thy-1.1 mouse (Uchida, Weissman, 1992). Co-expression of granulocyte monocyte colony stimulating factor receptor (GM-CSFR) and IL-7 receptor $\alpha\gamma$ defines the common myeloid progenitor (CMP) from the common lymphoid progenitor (CLP) (Kondo, 1997). The common myeloid progenitor expresses the GM-CSFR however it lacks IL-7R $\alpha\gamma$ and the common lymphoid progenitor expresses the IL-7R $\alpha\gamma$ and lacks the GM-CSFR (Kondo, 1997).

In human and mouse HSCs, markers expressed on the surface of the stem cell to date have been highlighted to be expressed on other cells types suggesting antigen expression may not correlate to stem cell function or potential. For example CD34 expressed on human HSCs (Bereson, 1991) is also expressed on vascular ECs (Fina, 1990) and some fibroblasts (Brown, 1991) suggesting its function may lie outside haemopoiesis. Isolation of stem cells, although rigorous nonetheless yields a heterogenous population of cells such that further efforts are required to determine a distinctive and unique phenotype from the most primitive stem cells throughout stem cell hierarchy to the least primitive stem cell within the stem cell pool.

1.7 Stem cell lineage selection

Stem cell division can be symmetric or asymmetric. Symmetric division is where a parent stem cell divides to produce two daughter cells or two parent cells. Asymmetric division describes the production of one parent and one daughter cell. Two theories exist as to the mechanism of asymmetric division, that being cell divisional and environmental asymmetry as outlined by Wilson and Thrupp, 2006. Divisional asymmetry describes the unequal distribution of stem cell fate determinants in the cytoplasm of a cell before it enters mitosis whereby subsequent to cell division the result is two non-identical daughter cells. One daughter cell receives the cell fate determinants retaining it as a stem cell while the other cell fails to receive any cell fate determinants and proceeds to cell differentiation. Environmental asymmetry describes the division of the parent cell to produce two identical daughter cells however, the exposure of these daughter cells differ. One daughter cell remains in the HSC niche conserving it as a HSC and the other daughter is exposed to a different niche promoting its differentiation. Divisional and environmental division although different result in the same net effect. Symmetric versus asymmetric division aims to promote HSC homeostasis and regulates HSC numbers.

1.8 Clinical applications of stem cells

Patients with haematological malignancies such as Multiple Myeloma or leukaemia have malignant cells in the marrow that have arisen from normal healthy haemopoietic tissue. These malignant cells, often with altered internal pathways proliferate rapidly independent of normal regulation. Treatment aimed at eliminating these malignant cells, include cytotoxic drugs and radiation which as an aside, destroy healthy cells of the marrow. The patient as a result is often left pancytopenic with low platelets and low red and white cells and very susceptible to infection. One therapy to reconstitute the marrow after radiation or cytotoxic injury is a haemopoietic stem cell transplant (HSCT). An autologous HSCT is one where the patients' own cells are harvested prior to myeloblastic therapy and are re-infused afterwards. Autologous transplants are used in the treatment of myeloma (Tricot, 1996 Barlogie, 2004 Child, 2003) and Hodgkins and non-Hodgkins Lymphoma (Pettengell, 2002 Reiser, 2002). Difficulties often encountered with the autologous HSCT are the failure to eradicate the disease in the harvested cells and the re-introduction of these cells to the marrow. The allogeneic HSCT is the use of donor cells which have the advantage of being free from disease and may aid elimination of malignant cells with a graft versus tumour response. However one limitation of the allogeneic HSCT is Graft Versus Host Disease (GVHD) whereby the transplanted cells begin to attack the host seeing it as foreign.

1.9 Radiation

Radiation is a process whereby energy is released from one source permeates through a medium and is absorbed by another. At a cellular level radiation exposure can cause damage to DNA and has been shown to be directly related to the level and dose of exposure (Muller, 1954). Damage occurs through both direct and indirect methods resulting in DNA breakage or damage (Michaels *et al*, 1978), (Schulte-Frohlinde, 1986). Direct injury is where radiation enters the cell and acts on the DNA itself resulting in mutations within the DNA, DNA strand breaks both single and double resulting sometimes in permanent and irreversible damage (Lea *et al*, 1942) finally resulting in cell death (Leenhouts *et al*, 1974) of both parent and progeny cells (Catcheside, 1946). Indirect damage caused by radiation is achieved through the hydrolysis of water contained within the cell resulting in the formation of free radical hydroxyl molecules (Dainton, 1948). These hydroxyl molecules can come together to form hydrogen peroxide and binds other molecules such as essential enzymes required for DNA damage repair within the cell with ease (Imlay *et al*, 1988). The result of this is the unavailability of these essential enzymes leading eventually to cell death. Interestingly, different cells within the body react differently to radiation with some cells being more sensitive while others are more resistant as first hypothesised by Bergonie and Tribondeau in 1906. Bergonie and Tribondeau were the first to suggest that cells that were actively dividing such as cells of the Gastrointestinal tract (Potten, 1998), reproductive organs (Stanford, 1955) and cells of the bone marrow (Till *et al* 1961) were more radiosensitive when compared to cells that were not dividing so rapidly such as cells of the nervous system and muscle cells (Rubin, 1984). Further supporting this concept is the observation that cells within the M phase of the cell cycle are more radio-sensitive when compared to when the cell is in the G₀, G₁ or S phase of the cell cycle and is thought to be due to the fact that the DNA is condensed to a single region available for injury (Chapman, 1999).

This knowledge of radiation and its effects were consequently exploited for therapeutic purposes.

Currently radiation is used to target and ablate rapidly dividing malignant, cancerous cells within tumours and treatment is associated with a wide range of cancers which include breast (Fisher, 1998) and prostate cancer (Reboul, 1965) for example.

Haematologically speaking radiation is used to treat malignancies of the bone marrow. It is used to ablate the patients' marrow which includes healthy cells, transformed malignant cells and immuno-competent cells. Subsequent to the removal of cells from the marrow the patient receives a Bone Marrow Transplant where donor cells enter the marrow and arrange themselves within the niche in a bid to repopulate the marrow with cells free from malignancy (Thomas, 1959), (Blume, 1980) (Powles 1980).

1.10 Pleiotrophin

Pleiotrophin (PTN) is a 17kDa protein which was first isolated from a bovine uterus and found to be a member of the heparin binding growth factor family due to its high affinity for heparin (Milner, 1989). It is encoded by the *ptn* gene and is highly expressed during embryogenesis (Bloch, 1992) however expression is limited in healthy adults to axons (Silos-Santiago, 1996) and endothelial cells (Yeh, 1998).

ptn gene expression is upregulated in cells stimulated by PDGF making it a member of the PDGF inducible gene family (Li, 1992). Yeh et al demonstrated that *ptn* expression in macrophages, endothelial cells and activated astrocytes in the brain of a rat was increased following ischemic injury. The PDGF A chain was also up regulated in these cells post injury in a time sequence that preceded PTN up regulation suggesting PTN signalling is a downstream consequence of PDGF signalling (Yeh et al, 1998). *ptn* gene expression has also

been shown to be hormone responsive (Vacherot, 1995) and is highly expressed in neural stem progenitor cells in mice (Jung, 2004).

1.10.1 Pleiotrophin structure

The structure of PTN was determined using the NMR technique and found to have two β -sheet domains, each of which have three anti-parallel β -strands connected by a flexible linker (Kilpeläinen, 2000). The N and C termini have lysine rich sequences but lack detectable structure and appeared to form random coils (Kilpeläinen, 2000). Binding of heparin to PTN is believed to induce a conformational change to the structure of PTN.

1.10.2 Mitogenesis.

PTN is a ligand for the receptor protein tyrosine phosphatase beta zeta (RPTP $\beta\zeta$) (Meng, 2000), Anaplastic lymphoma kinase (ALK) (Stoica, 2001) and Syndecan 3 (Landgraf, 2008). RPTP $\beta\zeta$ is an intrinsically activated tyrosine phosphatase whereby the binding of PTN to the RPTP $\beta\zeta$ results in its inactivation and the phosphorylation of a number of intracellular substrates such as AKT, β -Catenin (Meng, 2000) and β -adducin (Pariser, 2005) which remain un-phosphorylated through an active RPTP $\beta\zeta$ when PTN is not bound.

PTN has a diverse range of functions one of which is the promotion of mitogenesis in endothelial cells (Courty, 1991) and fibroblasts (Milner, 1989 , Fang, 1992), the mitogenesis is thought to be achieved through the activation of the PI3K pathway (Soultou, 1997). There are however conflicting studies that challenge PTN as a mitogenic factor. Raulo et al indicate rPTN in insects and bacterial cells lack mitogenic activity, however it is noted that PTN

supported the outgrowth of dendrites and axons in culture termed neurite outgrowth. Neurite outgrowth support from rPTN was also observed in cultures neuronal cells that were of embryonic and neuroblastoma origins and also in the neonatal rat brain (Rauvala, 1989).

1.10.3 Angiogenesis

Another important function for PTN is angiogenesis, or the formation of new vessels. PTN was first indicated as having angiogenic properties in a study where PTN expression was monitored in a rat brain for 14 days after ischaemic injury (Yeh, 1998). PTN levels increased moderately on day 1 and quite significantly on days 2 and 3, after which the levels began to decrease by day 14. What was noted in this study was the location of *ptn* upregulation as being at the sites of intense neovascularisation, suggesting PTN as having angiogenic properties. PTN may elicit an angiogenic response directly on the endothelial cells promoting proliferation or indirectly through the recruitment of pro-angiogenic cells to the site of angiogenesis. New vessel formation depends on the growth of existing vessels through the proliferation of resident cells but also on the recruitment of endothelial progenitor cells from the peripheral blood (Asahara, 1997). Heiss et al suggest PTN mediates angiogenesis through the induction of chemo-attraction of these endothelial progenitor cells and activates resident human vein endothelial cells (HUVECs) to proliferate in a manner which is dependent on nitric oxide (Heiss, 2008). PTN as an angiogenic factor has also been highlighted to play a role in the invasion of blood vessels into hypertrophic cartilage (Petersen, 2001) and is highly expressed in fetal and juvenile cartilage (Tapp, 1999).

1.10.4 Proto-oncogene

PTN is expressed at low levels in the healthy adult however, *ptn* when deregulated, can act as an oncogene promoting the transformation of healthy cells to those that display cancerous properties. Cells constructed to constitutively express *ptn*, when implanted into nude mice resulted in the formation of highly vascularised tumours (Chauchan, 1993). Further supporting this theory is the high level of *ptn* expression in a variety of malignant tumours such as prostate, neuroblastoma (Nakagawara, 1995), lung (Garver, 1993), melanoma (Chen, 2007) breast (Garver, 1994). This is also seen in the cell lines derived from these tumours (Fang, 1992).

1.10.5 Growth factor

Muramoto *et al* when examining cells of the stem cells niche found endothelial cells provided support to the stem cells post radiation injury (Muramoto 2006). In particular Human Brain Endothelial cells (HUBECs) were found to be supportive to the HSC resulting in 1-2 log expansion of human and murine stem cells in contact and non-contact cultures (Chute, 2005). To determine the HUBEC secreted factor that was responsible for the HUBEC mediated expansion of the HSCs in the non-contact cultures microarray analyses was performed (Himburg, accepted for publication *Nature Medicine* 2010). This analysis identified genes that were highly expressed in the supportive HUBECs that was lacking in endothelial cells that did not support HSC expansion when co-cultured. This micro-array analysis provided thirteen candidate genes, however PTN was found to be 25 fold higher in HUBECs than the non-supportive ECs (Himburg, accepted for publication *Nature Medicine* 2010). This finding and the previous literature indicating PTN as a growth factor for human embryonic stem cells (Soh, 2007) highlighted PTN as a candidate factor responsible for the expansion of

haemopoietic stem cells when in culture with HUBECs. Further to this PTN was found to induce HSC self-renewal in vitro in the absence of HUBECs, this was determined using a competitive repopulating assay which examines homing, engraftment and repopulating abilities of the transplanted cells (CD45.1) and found increased engraftment of cells treated in culture with recombinant PTN in the absence of HUBECs (Himburg, accepted for publication *Nature Medicine* 2010) in lethally irradiated recipients (CD45.2). Figure 6

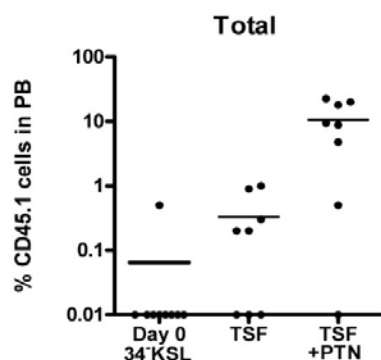


Figure 6: KSL cells were isolated from the femurs of a congenic donor mouse (CD45.1) were then transplanted into a lethally irradiated congenic recipient mouse (CD45.2) either un-manipulated on Day 0 or set up in culture for 7 days with TSF or TSF and 100ng/mL rPTN. After 12 weeks, peripheral blood from these mice was collected, stained with fluorescent antibodies and examined using flow cytometry. Increased engraftment of the PTN treated KSL cells compared to TSF treated alone was observed. The engrafted cells differentiated as normal as donor CD45.1 Macrophages, B and T cells were observed in the peripheral blood of the lethally irradiated recipient mouse CD45.2. This indicates that treatment with rPTN resulted in the replication of a multi-potent progenitor that has the capacity to differentiate into multiple lineages.

Further to this Anti-PTN when added to HUBECs and KSL cells was found to reduce HSC self-renewal in vitro in the absence of HUBECs, this was determined using a competitive repopulating assay which examines homing, engraftment and repopulating abilities of the transplanted cells (CD45.1) and found decreased engraftment of cells treated with in culture with HUBECs and Anti-PTN (Himburg, Accepted for publication *Nature Medicine* 2010) in lethally irradiated recipients (CD45.2). Figure 7

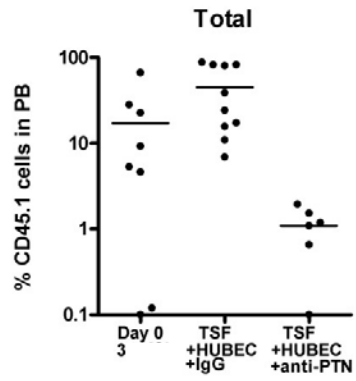


Figure 7: KSL cells isolated from the femurs of a congenic mouse (CD45.1), were transplanted into a recipient mouse either un-manipulated, with TSF and HUBEC for 7 days or with HUBECs and Anti-PTN for 7 days, after which the cells were transplanted. 12 weeks post transplantation peripheral blood from the recipient mice were collected and analysed using flow cytometry for donor engraftment (CD45.1). A decrease in engraftment was noted in those treated with HUBECs and Anti-PTN.

This research indicates rPTN as a growth factor for murine HSCs.

1.11 Mouse strains

The mouse strains used in this study were the C57BL/6 and Balb/c.

1.11.1 C57BL/6

C57BL/6 mice are currently the most widely used inbred strain of mice in research. This is due to the fact that they are easy breeders, are long-lived and congenic strains are widely available. They also display delayed Haemopoetic stem cell (HSC) senescence compared to other strains including Balb/c (Dykstra B,2008). The disadvantages associated with the C57BL/6 mice is that they have a reduced incidence of tumors (Kripke M.L, 1977) to examine and are easily irritable, making working with them difficult.



Figure 8: C57BL/6 mouse.

1.11.2 Balb/c

The Balb/c mice are an inbred strain of albino mice that are known to produce plasmacytomas when injected with mineral oil (Potter, M., and C. Boyce. 1962.) The plasmacytomas are often manipulated to be advantageous in a laboratory setting as they can produce a large number of monoclonal antibodies to a particular antigen. The Balb/c mice are both docile and small in size making working with them quite manageable.



Figure 9: Balb/c mouse

1.12 KSL cells

The identification and isolation of HSCs relies on surface protein expression. As discussed in section 1.6, no unique phenotype exists for human or murine HSCs although several combinations of surface proteins have been developed and new combinations of identification factors are continuously emerging. Challen *et al*, 2009 outline the numerous phenotypes and antigenic markers determined to highlight and isolate cells within the HSC pool of hierarchy. For this study the cells employed were the KSL population of cells. The KSL population of cells are those that are Lineage negative and that express both c-Kit and Sca-1 antigens. 10% of KSL cells are the most primitive long-term HSCs, the other 90% includes short-term HSCs and progenitor cells covering all stages of progenitor HSC hierarchy (Challen, 2009). KSL purification therefore allows the isolation of a heterogeneous population of cells that are highly enriched for HSCs.

1.12.1 Lineage negative cells

Lineage negative cells are cells that do not express antigens associated with cell maturity. Isolation of these cells requires the removal of mature haemopoetic cells such as B cells, T cells, monocytes, macrophages, granulocytes, erythrocytes in addition to their committed precursors all of which express antigens indicating them as lineage committed. Surface antigens associated with lineage commitment and mature cells include CD5 B220 CD11b Gr-1 and Ter 119. CD5 is a glycoprotein expressed on the surface of lineage committed T cells and a subset B cells (Huang, 1987) which acts as a negative regulator of T-Cell Receptor (TCR) signalling (Azzam, 1998). B220 is an antigen expressed on all cells committed to the B cell lineage (Coffman, 1981). CD11b is expressed on the surface of phagocytes (Springer, 1979) which includes macrophages and neutrophils. Granulocyte receptor 1 (Gr-1) is

expressed on granulocytes (Hestdal, 1991) while Ter119 is a monoclonal antibody directed against a glycoprotein on the surface of erythroid cells (Kina, 2000). The removal of mature cells expressing these surface antigens yields an immature population of marrow cells that are termed lineage negative (Lin⁻).

1.12.2 c-Kit

c-Kit or CD117 is a 145kDa transmembrane tyrosine kinase receptor (Ikuta, 1992) encoded by the proto-oncogene *Kit* (Chabot, 1988). It shares structural similarities to the receptor tyrosine kinases CSF-IR and PDGFR (Qui, 1988) and is expressed on mast cells (Mayrhofer, 1987), melanocytes (Nocha, 1989) and haemopoietic progenitor cells (Cambareri, 1988). The c-Kit ligand, stem cell factor (Zsebo, 1990) is a mitogenic factor for haemopoietic stem cells (Dexter, 1977), myeloid and erythroid progenitors (McNiece 1991) and mast cells (Tsai, 1991).

1.12.3 Stem cell antigen-1 (sca-1)

Sca-1 or Lymphocyte activation protein 6A (Ly6A/E), is an 18kDa phosphatidylinositol anchored protein, (Spangrude 1988) and is a member of the Ly-6 antigen family (Van de Run, 1989). It is encoded by two strain specific alleles of the Ly6 gene family (LeClair, 1986) where expression was determined initially on activated lymphocytes where activation resulted in upregulation (Yutoku, 1974). Expression of sca-1 has since extended to the HSC (Spangrude, 1988), stem and progenitor cells of other tissues and organs such as the heart (Matsuura, 2004), the prostate (Burger, 2005) and skin (Torna, 2001).

1.13 Murine KSL isolation

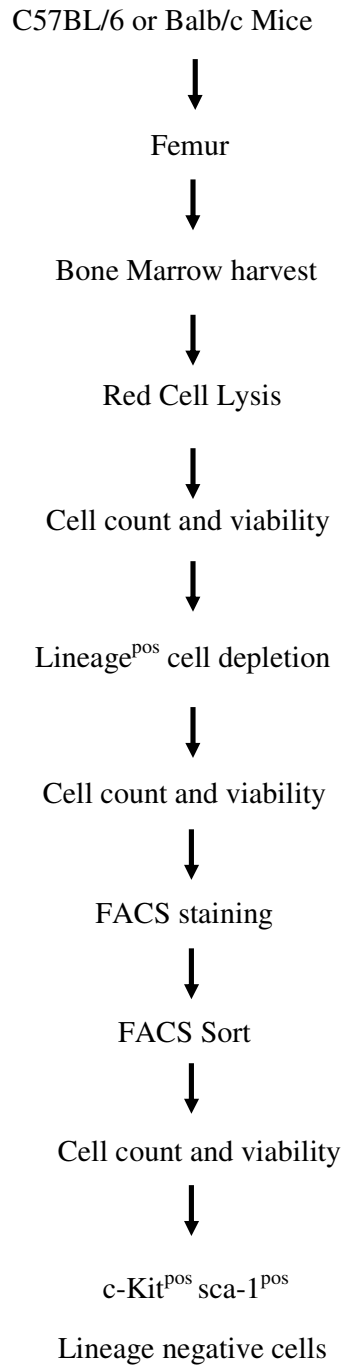


Figure 10: Flow chart of processing involved in murine stem cell isolation.

1.14 KSL isolation

The KSL cells are isolated from the femurs and tibias of the mice. The mice are sacrificed and the femurs and tibias removed and flushed for marrow contents. The red cells are removed using red cell lysis solution after which the cells are stained with antibodies labelled with magnetic beads directed against antigens associated with lineage specificity. The cells are passed through a magnetic field where cells labelled magnetic beads directed against lineage are retained. Cells that do not express lineage antigens are passed through the magnetic field resulting in the isolation of an immature population of cells. The lineage negative cells are stained by fluorescent labelled antibodies directed against c-Kit and Sca-1 allowing the immature cells to be separated into cells that express both c-Kit and Sca-1 and those that do not. The antibody directed against Sca-1 is a Fluorescein isothiocyanate (FITC) labelled anti-mouse Sca-1 antibody (BD Biosciences Pharmingen™). FITC is fluorescent dye that has an excitation wavelength of 488nm which is emitted of 530nm. The antibody directed against c-Kit is a Phycoerythrin (PE) labelled anti-mouse c-Kit antibody (BD Biosciences Pharmingen™). PE is excited at a wavelength of 488nm and is emitted at a wavelength of 628nm. Cells expressing both Sca-1 and c-Kit can be physically separated from those that do not using a FACS cell sorter. The lineage negative cells, when stained for Sca-1 and c-Kit are also stained with 7 Amino-Actinomycin D (7AAD). 7AAD is a DNA dye that can be used for cell viability. It is a specific G-C base intercalator (Cowden, 1981) allowing cells with intact cell membranes be distinguished from those with a degraded or damaged cell membranes. Intact cell membranes exclude the dye while damaged cell membranes are permeable to the dye. Upon entry into the cell, the dye forms a fluorescent complex with the DNA which can be excited and observed in the red portion of the spectrum at a wavelength of 488nm (Zelenin, 1984). The cells when isolated represent a live population of cells that are highly enriched for HSCs.

Aims

1. To investigate if Pleiotrophin causes expansion of Haemopoetic Stem Cells in vitro and at what stage of haemopoetic progenitor does it effect.
2. To investigate whether PTN signalling is necessary for HUBEC-mediated expansion and recovery of HSCs post radiation injury in vitro.
3. To examine if PTN arrests cells in the G0 phase of the cell cycle.
4. To examine the homing effect of PTN on Haemopoeitic Stem Cells.
5. To determine if PTN can induce Haemopoeitic Stem cell regeneration post radiation injury in vivo, by examining mature cell reconstitution and survival.
6. To examine histologically damage caused to the marrow spaces with PTN treatment.
7. To investigate if *ptn* gene in response to radiation injury is up-regulated in marrow mononuclear cells.

Experimental Design

In vitro experiments.

- Examination of HSC expansion with the addition of 100 ng/mL and 500ng/mL rPTN to radiated KSL cells in culture.
- Investigation into PTN signalling as a requirement for HUBEC mediated recovery of radiated KSL cells.
- Examination of rPTN as a possible arresting factor for KSL cells using Ki-67 antibody cell cycle staining.

In vivo experiments.

- Examination of the homing effect of rPTN on KSL cells.
- Examination of rPTN as a regenerative factor for HSCs in the marrow following radiation injury using MNC and KSL content with CFC assays.
- Survival studies.
- Mature blood cell reconstitution study.
- Examination of possible histological damage to marrow due to rPTN treatment.
- Investigation of *ptn* up-regulation in response to radiation injury.

2.0 Materials and Methods

2.1 Murine MNC Isolation

2.1.1 Mouse treatment

The strains used for this research project were the C57BL/6 and Balb/c, all of which were obtained from the Jackson Laboratory (Bar Harbor Maine, U.S.A) and weighed between 20-30g. All mice were housed in the Cancer Centre Isolation Facility (CCIF) of Duke University, Durham, North Carolina. The mice were housed in an environment with filtered air, were on a sixteen hour light, eight hour dark schedule and had an endless supply of food (Labchow) and antibiotic treated water. All experiments were approved by the Duke University Institutional Animal Care and Use Committee.

2.1.2 Murine Euthanasia

Carbon dioxide inhalation was achieved by placing the mice in a top opening CO₂ chamber. A slow flow of CO₂ was introduced into the chamber to achieve a high concentration of CO₂ at the base. After breathing ceased and the animal appeared unconscious, euthanasia was completed using cervical dislocation or dislocation of the neck. To perform cervical dislocation the animal was held by the tail. Fingers were placed at the back of the neck where a sharp pull at the base of the tail resulted in dislocation.

2.1.3 Femur and Tibia dissection

For sterile femur and tibia dissection the mice were set up in a sterile fume hood and sprayed with 70% ethanol. To fully ensure a sterile environment for BM collection, the dissection equipment (forceps and scissors) was also sprayed with 70% Ethanol.

To begin, a single incision was made at the lower abdomen of the mouse. An additional incision was made at the side of the leg leading to femur exposure. A further incision was made above the femur which was directed towards the abdomen thus removing excess muscle. Following this the scissors were placed underneath the femur at a right angle to the knee and an incision was made. The scissors was placed into the new incision and also on the outside of the knee (still at a right angle to the knee) where a cut is made through the knee. Following this the femur was detached from the hip joint using the scissors. Subsequent to femural removal, tibia removal commenced. The skin remaining on the lower leg was pulled to the ankle where an incision was made through the ankle. The tibia was then turned upside down so that the remaining knee joint was resting on the bench, allowing excess muscle to be removed. A final incision was made above the knee detaching the tibia from it.

2.1.4 Murine Bone Marrow harvest

The bone marrow from the femur and tibia was harvested using sterile techniques and was performed in the sterile environment of the fume hood. The ends of the femur and tibias were cut off just below the end of the marrow cavity using sterile scissors and forceps. A 5mL syringe filled with 10% Fetal Bovine Serum (FBS) (appendix) was then inserted into the spongy bone exposed by removal of the bone ends. The contents of the syringe were flushed through the bone marrow with reasonable force into a 50mL tube below containing 25mLs of 10% FBS diluted in Phosphate buffered serum (PBS). This process was repeated a number of times from each end of the femur and tibia allowing for maximum haemopoietic stem cell (HSC) collection. The approximate total cell yield per mouse is 20×10^6 cells.

2.1.5 Murine MNC isolation

Following bone marrow isolation, the contents were centrifuged at 1400rpm for 5 minutes resulting in the formation of a pellet of cells. The supernatant was aspirated and 10mL red cell lysis buffer (Sigma-Aldrich Chemical company Ltd St. Louis, MO) added to the cells, mixed using a vortex and left sitting for 5 minutes. The red cell lysis buffer removes contaminating red cells from the cell suspension by mediating the generation of a hypotonic environment. To return the solution to one that was isotonic, 40mL 10% FBS was added to bring the volume up to 50mL. Again the cells were spun and the supernatant aspirated. 20mLs of 10 % FBS was added to the cells to generate a dilute concentration of cells whereby an accurate cell count could be performed.

2.2 MNC viability and purification.

2.2.1 Cell counting

A cell count was performed using a haemocytometer and Nikon microscope to determine the concentration of cells in the cell suspension while the viability count determined the number of viable cells. The cell count and viability were determined simultaneously with the use of Trypan Blue (Sigma-Aldrich Chemical company Ltd.) and a haemocytometer. Cells were diluted to the desired concentration which was usually a 1 in 5. 10 μ l of this suspension was added to the haemocytometer and examined under the microscope (10x) where a cell count was determined. The Nuebauer haemocytometer was used and has a grid which is divided into 9 squares. Each of the nine squares is 1mm x 1mm x 0.1mm (depth under the coverslip). The cells in a single square would therefore equal the number of cells in 0.1 μ l, with 1 μ l equalling 1mm³ (taking the dilution factor into account) or if multiplied by 10,000 than the number of cells in 1ml.

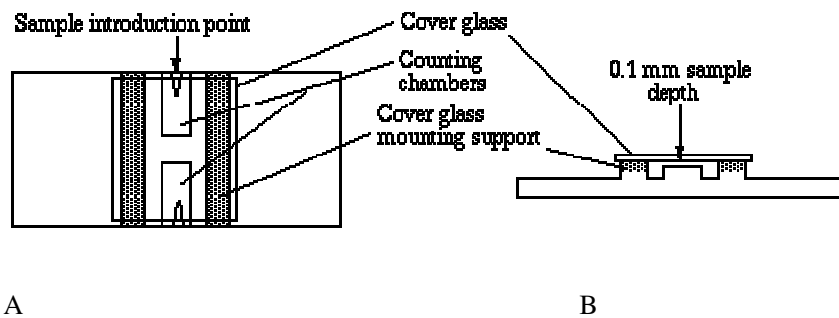


Figure 11: Haemocytometer

A: Looking down on haemocytometer.

B: Head on view of haemocytometer with coverslip attached.

www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

2.2.2 Cell Viability

Trypan Blue dye was added to the cells to determine cell viability. Degraded cells are permeable to the dye due to cell membrane damage, the viable cells however have intact cell membranes and as a result, exclude dye entry.

2.2.3 Column purification of Murine Lineage negative cells

To isolate a population of immature cells from the mononuclear cell (MNC) population the MACS magnetic cell sorting kit (Miltenyi Biotec Ltd. Bisley, United Kingdom) was used following the protocol suggested by the manufacturers. Murine HSCs do not express surface antigens associated with lineage or maturity, the MACS magnetic cell sorting kit allows selection of these cells, that do not express antigens associated with lineage or maturity, to be isolated. The cells once counted as outlined in section 2.6, were re-suspended in a volume of 40uL of 10% FBS per 10^7 cells. For every 10^7 cells 10uL of a Biotin-Antibody cocktail was added and incubated for 10 minutes at 4-8°C. The antibody cocktail includes antibodies directed against antigens associated with lineage and is the primary labeling reagent. The antibodies in the cocktail included Mac-1 Gr-1 Ter 119, B220 and CD5. Subsequent to this 30uL of 10% FBS was added per 10^7 cells. After which 20uL of anti-Biotin MicroBeads per 10^7 cells were added and incubated for 15 minutes at 4-8°C. The anti-Biotin Microbeads are the secondary labeling reagents which recognise the Biotin labeled primary antibody attached to lineage antigens resulting in the indirect magnetic labeling of lineage positive cells. To remove these lineage positive cells from the cell suspension, the cells were washed twice by adding 1-2mL of 10% FBS per 10^7 cells and spinning them for 10 minutes at 300g. The supernatant was removed and the cells bound and unbound (up to 10^8 cells) were re-suspended in 500uL of 10% FBS and added to the magnetic column for separation. However before the cells could be added to the column, it was primed firstly. Priming was achieved through the addition and passage of 3mls 10% FBS through the column. When passing through the column, magnetically labeled lineage positive cells were retained at the magnetic field, allowing lineage negative, unlabeled cells pass through the

column resulting in the formation and isolation of a pure suspension of lineage negative or immature cells, in which the HSCs are contained.

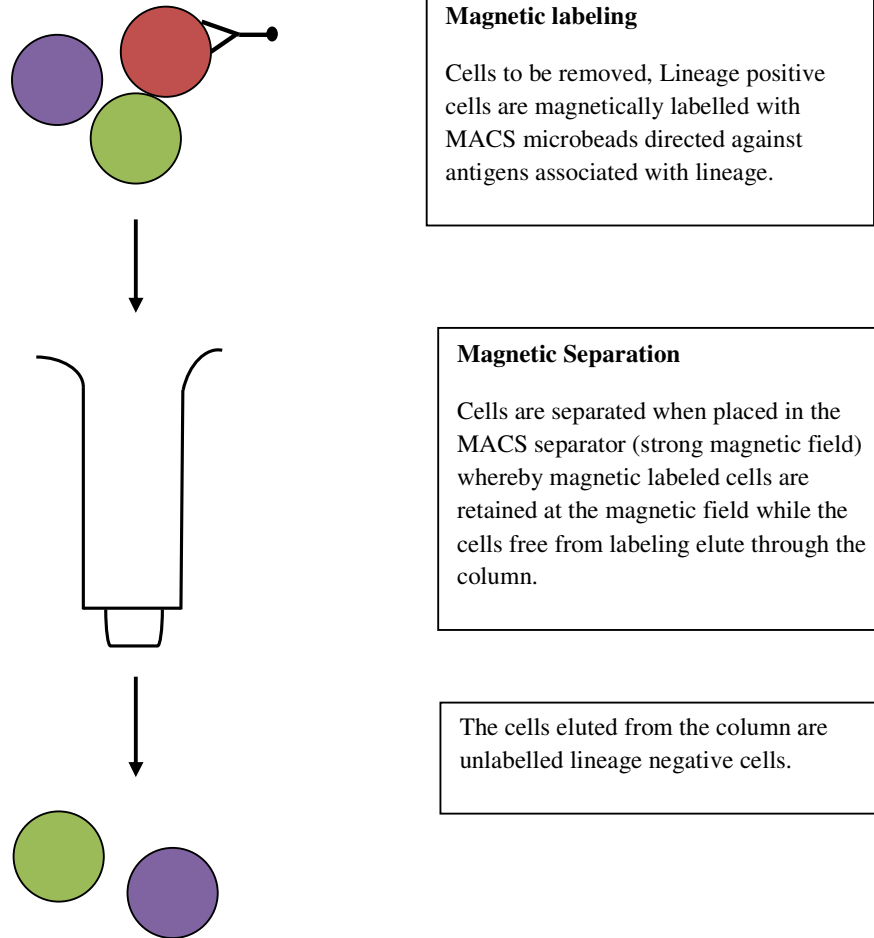


Figure 12: Magnetic cell sorting of Lineage negative cells.

2.3 MNC RNA isolation

2.3.1 MNC isolation Lymphoprep technique.

To isolate a population of MNCs from marrow cells 15mL of Lymphoprep™ was added to a 15mL tube. The contents of the marrow when flushed into 10% FBS as outlined in section 2.1.4 was spun at 1500rpm for 35 minutes. The the marrow contents were placed in on top of the Lymphoprep™, which has a density of 1.077g/mL, the red cell and granulocytes sink to the bottom while the MNC's remain at the sample medium interface. The MNC layer was then collected and diluted to 40mL with 10% FBS and spun at 1500rpm for 10 minutes. After centrifugation the MNC's formed a pellet at the base of the tube. In this pellet however some red cells remained so in order to isolate a pure polymorph suspension the contaminating red cells were removed as outlined in section 2.1.5.

2.3.2 MNC RNA isolation

MNC RNA isolation was achieved using the RNeasy Mini-Kit (Qiagen, Ambion Inc, Austin, Texas). The MNCs when isolated were lysed through the addition of 350µl of cell lysis solution RLT (<5x10⁶ cells) or 600uL RLT for cells that are 5x10⁶ - 1x 10⁶. To remove cell debris the cell solution was added to a tube which passed the cells through a filter unit when spun at 8000g for 2 minutes into a collecting tube. The filtered solution was then transferred to a RNeasy spin column, placed in a 2mL collection tube and centrifuged for 15 seconds at 8000g which mediates RNA binding. The RNA and DNA were then bound to the column while the protein passed through into the collecting tube. The flow through was discarded. The Rneasy spin column containing bound RNA and DNA was added to a 2mL collection tube and 700uL of RW1 buffer was added to the column and centrifuged at 8000g for 15 seconds in order to desalt the column membrane and result in more efficient DNase 1 digestion. 500uL RPE was added to the column and spun at 10,000rpm for 2 minutes to wash the column membrane. This washing step was performed twice. After this the column was added to a 1.5mL collection tube where 30-50uL of RNase free H₂O was added to the column and spun at 8000g for 1 minute, to elute highly pure RNA.

2.3.3 MNC RNA quantitation method.

Quantitation of RNA was achieved using the nanodrop® ND-1000 spectrophotometer. The nanodrop system is an electric spectrophotometer linked to a computer system, which provides RNA concentration and purity results from a sample in seconds. 1µl of sample was added to the nanodrop and closed after which the machine reports results via the computer screen.

2.3.4 Real-Time PCR

Real-time PCR performed was a two step reaction whereby cDNA was generated from RNA and amplified. The newly generated cDNA was used in the Real-time PCR analysis.

2.3.5 cDNA generation

cDNA was generated from the recently isolated RNA outlined in section 2.3.2 using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, California, USA). A 2x master mix was prepared on ice. The mix contains Kit reverse transcription products 2.0uL 10X RT buffer, 0.8uL 25X dNTP mix 100mM, 2.0uL 10x RT random primers, 1.0uL multiScribe™ Reverse Transcriptase and 4.2uL nuclease free H₂O. The mix was mixed gently and placed on ice. 10uL of this master mix was added to the required wells of a 96 well plate. 10uL of RNA sample was added to each well containing 10uL mix in the 96 well plate and pipetted up and down a few times to mix. The plate was sealed using cling film and centrifuged at 10,000 rpm for 10 minutes to spin the contents to the base of the well and to remove any bubbles. The plate was placed on ice until ready to load onto the thermocycler (2720 Thermocycler, Applied Biosystems). The Thermocycler was set up to run at 25°C for 10 minutes firstly 37°C for 120 minutes, 85°C for 5 seconds and to remain at 4°C when completed until ready to be removed from the cyclor. The plate was loaded onto the thermocycler and the reaction started.

2.3.6 Real-Time Polymerase Chain Reaction (PCR).

To perform a Real-time PCR reaction on the recently generated cDNA a master-mix containing reagents supplied in the Taqman Gene expression Assay Kit specific for the *ptn* gene was added to 1.5mL microcentrifuge tubes for each sample. For each sample 1.0uL of 20X Taqman Gene expression Assay, 9.0uL cDNA template diluted to the desired concentration (1-100ng) in Rnase free H₂O and 10uL 2X Taqman gene expression Mastermix, containing primers directed at the *ptn* gene were added. The tube was capped and centrifuged at 8,000g for 2 minutes. 20uL of the PCR reaction mix was added to the desired wells of a 96 well plate. The plate was sealed and centrifuged at 10000rpm to remove any bubbles within the wells. The plate was loaded onto the 7300 RT-PCR system, (Applied biosystems) and was set up to run at 50^oC for 2 minutes, 95^oC for 10 minutes and 40 cycles of 95^oC for 15 seconds and 60^oC for 1 minute. The 2^{-ΔΔCt} method was used to analyse gene expression in each sample relative to the housekeeping gene GAPDH.

2.4 Murine MNC separation by Flow Cytometry

2.4.1 Murine FACS sorting for c-Kit expressing, Sca-1 expressing, Lineage negative cells (KSL).

In order to purify the immature lineage negative cell population to a population of cells that are known to contain stem cells, flow cytometric sorting was employed. Flow cytometric sorting allows cells expressing particular surface antigens be physically separated from cells that do not using fluorescently labelled antibodies directed against the surface antigens of interest. The flow tubes were set up as outlined below in table 1. 20,000 cells were added to each of the compensation tubes (1-4). Compensation allows any spill over fluorescence to be detected and removed. 20,000 cells were also added to the isotype control (5) (Becton Dickinson and company Ltd.) ensuring no non-specific binding of the antibodies to the cells occurred providing false positive staining. The Lineage negative cells that were to be separated were added to tube (6) as were all of the fluorescently labelled antibodies.

Staining was as follows:

TUBE	FITC	PE	PE-CY5
1	---	---	---
2	Sca-1 (2) (<i>Becton, Dickinson and Company Ltd. Lot: 40174</i>)	---	---
3	---	c-Kit (2) (<i>Becton, Dickinson and Company Ltd. Lot:74612</i>)	---
4	---	---	7 AAD (5) (<i>Becton, Dickinson and Company Ltd. Lot: 93823</i>)
5	Rat IgG (1) (<i>Becton, Dickinson and Company Ltd. Lot: 52205</i>)	IgG (1) (<i>Becton, Dickinson and Company Ltd. Lot: 21237</i>)	---
6	Sca-1 (2) (<i>Becton, Dickinson and Company Ltd. Lot: 40174</i>)	c-Kit (2) (<i>Becton, Dickinson and Company Ltd. Lot:74612</i>)	7 AAD (5) (<i>Becton, Dickinson and Company Ltd. Lot: 93823</i>)

Table 1: Staining of Lineage negative cells for c-Kit and Sca-1 antigens for FACS sorting.

*Number in brackets () indicates the amount in uL added per 1 million cells.

The cells were stained at 4-8°C for 20-30 minutes in the dark. 7 AAD, which stains degraded cells, was not added at this point. During the 30 minute incubation two 15mL tubes were set up with 2ml 10% FBS to allow for the collection of cells that express c-Kit and Sca-1 into one tube and those that do not into the other tube. Following the 30 minute incubation the flow tubes were washed with the addition of 2ml PBS to each of the tubes, centrifuged at 1400rpm for 5minutes to pellet the cells and the supernatant aspirated. The compensation tubes were resuspended in 200µl PBS and the sort tube (tube 6) was resuspended in 500-1000 µl PBS. The PE-CY5 labelled 7AAD antibody (Becton, Dickinson and company Ltd) was added to the tubes outlined above in table 1. The flow tubes were then placed on ice and transported to the FACS cell sorter facility, Duke University, Durham, NC. The cell sorter employed was the Becton Dickinson FACSVantage SE cell sorter. When passed through the cell sorter a pure population of cells that were viable, Lineage negative (immature) and expressed the surface antigens c-Kit and Sca-1 was obtained.

2.5 HUBEC isolation and culture.

2.5.1 Endothelial cell culture

To examine the effect Human brain endothelial cells (HUBECs) had on irradiated KSL cells in vitro, the 1 million HUBECs were placed in a flask and incubated at 37°C with 5% CO₂ for two or three days thus ensuring sufficient numbers were generated so as to conduct the experiment. The 30mL cell culture flask was gelatinized by adding enough 10% Gelatin (Sigma-Aldrich Chemical company Ltd.), solution (appendix) to sufficiently coat the base and incubated at RT for one hour. This gelatin allows attachment of the HUBECs to the flask surface promoting endothelial cell growth. While the gelatin was coating the flask the Endothelial Cell Culture Medium (ECCM), (appendix) was heated in the water bath (37°C) after which the endothelial cells were thawed or trypsinized.

To thaw the Human Brain Endothelial cells (HUBECs) that had been stored in liquid nitrogen without causing damage to them they were thawed quickly to remove all ice crystals. In order to achieve this, the cells were kept on dry ice pellets until they were ready to be placed into the water bath, maintained at 37°C, where they remained until the ice crystals disappeared. Following this the cells were added to 5mL 10% FBS, pelleted, counted and resuspended in 5mL ECCM.

Alternatively to trypsinize cells that are ready for passage, the media was aspirated and the flask washed twice with sterile PBS. Sufficient trypsin was added to coat the bottom of the flask which detached the cells from the surface of the flask. The flask was incubated at 37°C for three minutes in the humidified CO₂ incubator. Following three minutes incubation the trypsin was inactivated through the addition of 5mL 10% FBS. The trypsin must be inactivated soon after this time as it can be toxic to the cells. Following this treatment the cells were collected and washed twice with 5mL ECCM. The cells were pelleted, counted and resuspended in 5mL ECCM.

When the HUBECs were thawed or trypsinized they were counted and viability was determined via Trypan Blue exclusion as outlined in previous section 2.2. Concurrently to this the gelatin coating the flask (above) was aspirated and the flask washed once with phosphate buffered Saline (PBS) and was subsequently aspirated. Endothelial cell culture medium (ECCM) was added to

the flask, 12mL for a flask that supports 75cm² of growth (T75) and 25mL for a flask that supports 175cm² of growth (T175). The endothelial cells were added to the flask whereby 1-5x10⁵ cells were added to the T75 flask and 0.5-1x10⁶ cells were added to the T175 flask. The cells were incubated in the humidified CO₂ incubator at a temperature of 37^oC for up to seven days where they grow attached to the flask. Following seven days in culture the endothelial cells were re-passed.

2.6 KSL cell and progeny flow cytometry staining and analysis.

2.6.1 Flow cytometric staining.

Isolated KSL cells were added to the wells of a 12 well plate under different conditions and incubated for 7 days at 37°C with 5% CO₂. The wells after seven days contained a heterogenous population of cells such as KSL cells, their progeny and fully mature cells. The cells were collected into tubes containing 5mLs 10% FBS, spun down and resuspended in 200µl 10% FBS for cell count and viability staining using Trypan blue (Sigma-Aldrich Chemical company Ltd). In order to quantify the KSL cells within the heterogenous population of cells flow cytometry was performed. This does not separate the cells based on phenotypes but analyses the quantity of particular cells within a mixed population based on surface antigen expression. Compensation performed was identical to that outlined in section 2.4.1. Flow cytometry sorting as and 7ADD staining both of which follow the same principle. In order to identify the immature population of cells within the mixed population APC fluorescently labeled antibodies directed against lineage were employed instead of MACS cell sorting, as cells do not need to be separated for analysis. 20,000 cells from each treatment well was stained with an isotype antibody (5) which ensures no non-specific binding of the antibody to the cells resulting in false positive staining. The remainder of the cells from each well were added to separate tubes (6) for analysis and each of the antibodies (Anti-Sca-1, Anti-c-Kit and Anti-Lin) and DNA dye 7 AAD added to each tube. Table 2 below summarises the staining protocol.

TUBE	FITC	PE	PE-CY5	APC
1	---	---	---	---
2	Sca-1 (2) <i>Becton, Dickinson and Company Ltd</i> Lot : 40174	---	---	---
3	---	c-Kit (2) <i>Becton, Dickinson and Company Ltd</i> Lot: 74612	---	---
4	---	---	7 AAD (5) <i>Becton, Dickinson and Company Ltd</i> Lot: 93823	---
5	Rat IgG (1) <i>Becton, Dickinson and Company Ltd</i> Lot : 21237	IgG (1) <i>Becton, Dickinson and Company Ltd</i> Lot : 52205	---	LIN (20) <i>Becton, Dickinson and Company Ltd</i> Lot : 19744
6	Sca-1 (2) <i>Becton, Dickinson and Company Ltd</i> Lot : 40174	c-Kit (2) <i>Becton, Dickinson and Company Ltd</i> Lot: 74612	7AAD (5) <i>Becton, Dickinson and Company Ltd</i> Lot: 93823	LIN (20) <i>Becton, Dickinson and Company Ltd</i> Lot : 19744

Table 2: Staining for c-Kit and Sca-1 antigens on Lineage negative cells for FACS analysis following incubation for 7 days. Numbers in brackets indicate volume in μl per 1 million cells.

Staining duration, incubation and washing of the cells was identical to the staining procedure outlined in section 2.2.5 for flow sorting, one difference is that all of the tubes are resuspended in 200 μL PBS for analysis. The cells were analysed on the FACSCanto II as outlined in section 2.6.1 and the % KSL content between the treatment groups determined

2.6.2 CFDA-SE staining

The staining of KSL and MNCs was achieved using a Vybrant® CFDA SE Cell Tracer Kit (Molecular Probes™, Invitrogen detection technologies, Eugene, Oregon, USA). Staining was performed as per manufacturers' instructions. The kit provided vials of 10mM CFDA-SE stock solution (Component A) and DMSO (Component B) which are stored at -20°C. Prior to the staining of cells the reagent must be prepared whereby 1 vial of Component A and Component B are defrosted. Into the 10mM CFDA-SE stock solution, 90uL of DMSO or Component B was added. The solution was then diluted to 0.1uM using Phosphate Buffered Saline (PBS). In order to label cells with the CFDA-SE dye in suspension, the cells were centrifuged at 1400rpm for 5minutes and a pellet formed. The supernatant was aspirated from the cells which were re-suspended in 1mL pre-warmed 10% FBS containing the probe prepared previously. The cells and labeling reagent were incubated in a water bath at 37°C for 15 minutes after which they were centrifuged at 1400rpm for 5minutes and re-suspended in 1mL fresh pre warmed 10% FBS. The cells were incubated for 30 minutes in a water bath at 37°C to ensure complete staining of the cells. The cells were centrifuged at 1400rpm for 5minutes and resuspended in 800uL 10% FBS.

2.6.3 Flow cytometry analysis

2.6.3.1 FACS set up

When start up of the FACS CantoII (Becton, Dickinson and company Ltd.) was completed, cytometer set up and tracking beads (Becton, Dickinson and company Ltd.) were run on the machine to ensure it was working correctly and met quality control requirements. One drop of beads (stored at 4°C) was added to 250uL PBS in a flow tube, vortexed and loaded manually onto the cytometer. When the beads had passed quality control requirements the cytometer was then prepared for sample analysis. Preparations include the generation of a new workfile and compensation of the fluorescent labeled antibodies. Samples for analysis are then vortexed and loaded manually or in a rack.

2.7 Murine Colony-Forming Cell Assays

2.7.1 Colony forming cell assay (Short-term).

In order to generate a medium that promotes colony formation 400uL of a mix containing 356uL IMDM (Iscove's Modified Dulbecco's medium) 40uL FBS and 4uL Pen/Strep is added to a 15mL tube. To this mix 4mL Methocult (Stem cell technologies Inc. Vancouver Canada) is added. To this tube containing the mix and 4mL Methocult, 2,000 cells were added and mixed vigorously. The number of cells added allow for four dishes to be prepared with approximately 500 cells per dish, although the required amount was three. The extra cells allow for those lost due to the viscosity of the methocult mixture. 1.2mL of mix containing IMDM, FBS, Pen/Strep Methocult and cells was added to each of the 35mm gridded colony forming cell (CFC) dishes (Thermo Fisher Scientific MA, U.S.A) where complete coating of the entire dish was ensured. All of the 35mm CFC dishes were added to a single large cell culture dish, to which a single CFC dish filled with sterile water was added. This was to prevent drying out of the cultures during the 14 day incubation at 37^oC in 5% CO₂. After two weeks, the hematopoietic colonies in each plate were counted and scored

2.7.2 Long-term culture initiating cell Assay.

Murine M2-10B4 (ATCC CRL-1972) bone marrow stromal cells were plated in a 24 well dish and irradiated with 1500 cGy (Cs-137 source) as outlined in section 2.9.1. 45,000, 90,000, and 180,000 of bone marrow mononuclear cells isolated from the femurs of mice as outlined in section 2.1 and were added to the stromal cell layers. 20,000 stromal cells were added to the wells and incubated at 37^oC with 5% CO₂ in 1mL long term culture medium for two days prior to MNC addition. The MNCs, when added to the stromal layer were incubated at 37^oC with 5%CO₂ in 1mL long term culture medium (StemCell Technologies) with weekly half-medium changes for 4 weeks.

Following four weeks in incubation the cells from each well were collected counted using the procedure outlined in section 2.2 and the MNCs, progeny and stromal cells in each well divided between three 35mm methocellulose dishes (MethoCult, StemCell Technologies) as outlined in section 2.7.1.

2.8 Murine Bone Marrow Histology

2.8.1 Bone Marrow slide preparation

Murine femurs were dissected from recently euthanized mice outlined in section 2.1 and collected into a 15mL tube containing 5mLs 10% FBS solution. Excess tissue was removed from the femurs by means of gauze. Subsequent to this the femurs were placed in 5ml Cal-Ex Decalcifying solution (Thermo Fisher Scientific Inc. MA, U.S.A) on ice for 30 minutes. This step aims to decalcify the bones to allow for successful sectioning. The femurs were placed in increasing sucrose gradients of 1mL of 10% 15% and 20% solutions (appendix) each for three minutes. Following this each of the femurs was placed in 1mL 1:1 Optimum cutting temperature (OCT) medium (Sakura Finetek Ltd.): 20% sucrose solution (appendix) and incubated overnight at 4°C. The following day the femurs were removed and placed in a labeled Tissue-Tek cryomould (Sakura Finetek Ltd Chicago IL U.S.A) and OCT (Sakura Finetek Ltd.) is added to cover the femur. Freeze embedding of the femur was achieved by placing the cryomould on dry ice until frozen. Samples were stored at -80°C until required for sectioning. The sections were cut using the cryostat (Leica Ltd. Solms Germany) following which they were fixed in cold (-20°C) acetone for 5-10 minutes. The slides were stored at -80°C until required for staining.

2.8.2 Bone Marrow staining

The sections were fixed in 10% Formalin solution (appendix) and washed under running tap water. To stain with Haematoxylin the slides were immersed into a container filled with Harris Haematoxylin (Sigma-Aldrich chemical company Ltd.) for 3 seconds. The slides were rinsed in running H₂O. To differentiate the slides were added to a 0.5% acid water solution for five seconds and rinsed in running H₂O. The slides were added to Scotts tap water substitute concentrate (Sigma-Aldrich chemical company Ltd), an alkaline solution, to blue and again rinsed with running H₂O. To counterstain the sections, the slides were added to an acidified Eosin stain (Sigma-Aldrich chemical company Ltd.) for two minutes and rinsed in running H₂O. To dehydrate the sections they were added to three grades of alcohols 80% first, 95% and 100% alcohol with one minute per solution. Following this the slides were added to three xylene solutions in sequence with 90 seconds per solution. To mount the slides cryoseal was added to the slides and a coverslip placed on top.

2.9 Murine in vivo and Murine KSL cell in vitro radiation

2.9.1 Irradiation

Radiation exposure of the KSL cells ex vivo or the mice was achieved through the use of the Cesium 137 irradiator (MDS Nordion, Ottawa, Ontario, Canada), which is a small gamma irradiator. The mice were placed into circular containers (4 or 5 mice per container). Filter paper was added to the slots to allow air flow into the containers and an elastic band was placed around the container to keep it closed and the filter paper in position. The circular containers were added to a rectangular carrying case (4 containers per carrying case). The carrying case was placed into three plastic bags and transported to the irradiator room. The three bag wrap-up aims to ensure no air exchange between the external environment and the mice and vice versa occurs. On entry into the irradiator room the containers were removed from the carrying case and placed inside the unlocked irradiator. The timer was set to achieve the desired radiation dose and 'reset' was pressed. The irradiator door was closed

and 'source raise' pressed. When radiation was complete the mice were wrapped up in plastic bags and transported back to the mouse housing facility.

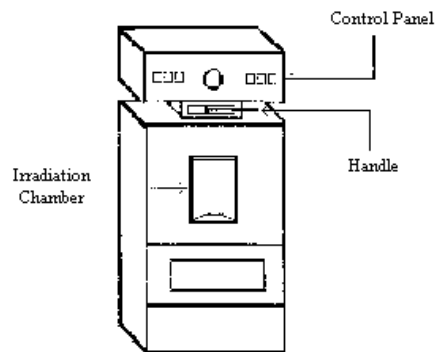


Figure 13: Illustration of the Cesium 137 gamma irradiator displaying the control panel, the handle and the irradiation chamber.

<http://ehs.columbia.edu/Images/Cs137Irradiator.gif>

2.10 Murine intraperitoneal and intravenous injections.

2.10.1 Murine intraperitoneal injections (IP)

To begin IP injections the working area on the mouse was firstly cleaned down with ethanol and an extra cage obtained to house mice following injection. The syringe was unwrapped and loaded with the appropriate volume for injection. The volume for injection was 200uL with a concentration of 100ng/mL rPTN or saline. The mouse was picked up by the tail with the right hand and scruffed behind the neck with the left hand. Once a secure grip was achieved the mouse was turned so the abdomen directed upwards. The mouse was tilted back so that the head was directed downwards. The abdomen was sprayed with ethanol and the contents of the syringe injected into the peritoneal cavity.

2.10.2 Murine intravenous tail vein injections

To begin tail vein injections the mice were placed under a heat lamp to increase blood flow to the tail vein and dilate the vessels, aiding vein injections. The mice were placed into a plastic cylindrical restriction device whereby the body was restrained but their tail is free at one end. The tail was cleaned with an alcohol wipe and the lateral veins located. Injections occurred at the top of the tail (nearest the body) and pressure applied to the site for a few seconds to stop the bleeding. The mice were returned to their cages.

2.11 Murine peripheral blood collection and analysis.

2.11.1 Submandibular bleed

The method used for submandibular bleeding was that which is outlined by Golde *et al*, 2005. 5uL EDTA (BD) was added to Eppendorf tubes which were labeled appropriately. The lancet was removed from sterile packaging ready for use. Subsequent to this the mouse was picked up by the tail with the right hand and scruffed with the left hand with the abdomen facing downwards. According to Golde *et al*, 2005 the lancet is directed towards the jugular vein where the retro-orbital and submandibular veins, which drain the face, meet (Figure 14).

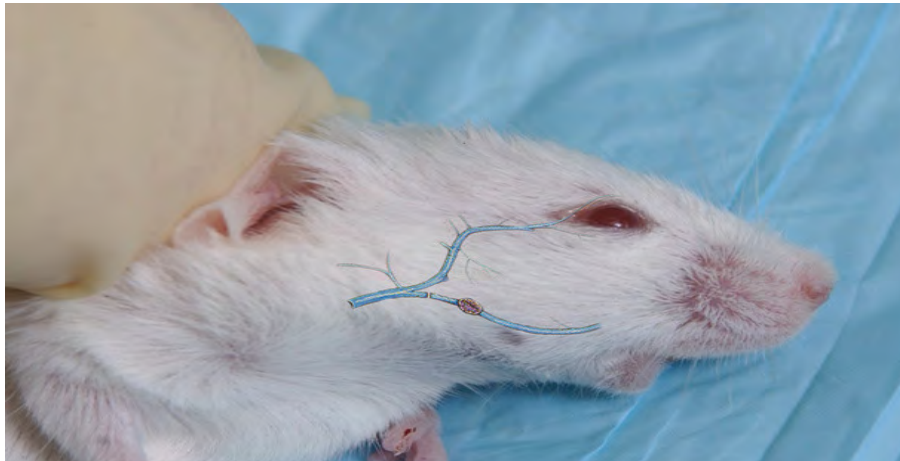


Figure 14: Bleeding from the mouse targets the Jugular vein where the retro-orbital and submandibular veins originate. (Golde *et al.*, Technique 2005)

The lancet was directed into the cheek of the mouse with a small amount of force to create a hole from which the blood was discharged in droplets (Figure 15). The droplets were collected into an eppendorf tube which contained 5uL EDTA and mixed vigorously to prevent clot formation. To stop bleeding from the cheek, sterile gauze was applied with some pressure to the source. The mice self groomed shortly afterwards and no evidence of the puncture existed subsequently.

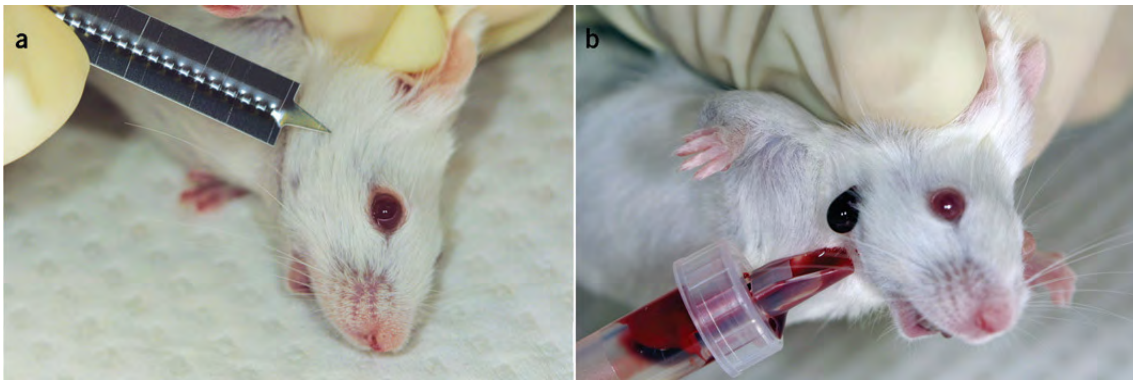


Figure 15: Blood collection using Submandibular bleed method.

A: Incision of the cheek with Lancet

B: Collection of blood from the cheek

Golde *et al.*, Technique 2005

2.11.2 Full blood count analysis.

Full blood counts were performed on the HEMAVET 950FS haematology analyzer (Drew Scientific Inc., Oxford CT, U.S.A). The machine was standardised using mouse control (Drew Scientific Inc.) prior to sample analysis. Once sample analysis was complete the Hemavet produced a report containing a variety of parameters including white cell count, red cell count and platelet count. On completion of required tests the HEMAVET was cleaned and powered off.

2.12 Ki-67 Proliferation assay.

2.12.1 Ki-67 7AAD Assay

The Ki-67 7AAD assay is one that is performed over two days.

Day 1

KSL cells when isolated as outlined in previous section (2.1) were added to wells under different conditions and incubated for 7 days at 37^oC with 5% CO₂. On day seven the cultures were collected and added to 2.5mL eppendorf tubes. The cells were spun down at 1000rpm for 10 minutes to form a pellet. The supernatant was removed and the pellet resuspended in 2mL 10% FBS. The cells were centrifuged and resuspended in 100uL 10% FBS where a cell count was performed and cell viability examined for each well using Trypan Blue exclusion. The cells were stained using fluorescently labeled antibodies, a PE labeled antibody directed against c-Kit, a PE Cy7 labelled antibody directed against Sca-1 and Lineage APC directed against antigens associated with lineage as outlined in section. The cells when stained were resuspended in 2mL Phosphate Buffered Saline, centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and the cells were resuspended in 400uL 4.0% Formaldehyde (Appendix). The cells were put on ice for 1 hour after which 400uL of 0.2% triton X-100 was added to the cells on top of the 400uL 4.0% Formaldehyde the Triton-X permeabilises the cells. The cells were incubated overnight at 4^oC covered in tinfoil, to prevent photobleaching.

Day 2

1.2mL 1% FBS (appendix) was added to the cells and centrifuged at 1000rpm for 10 minutes. The supernatant was removed and the cells resuspended in 100uL 1% FBS. 10uL of FITC labeled IgG antibody was added to the isotype tubes. The isotype tubes are used to control any non-specific binding. 20uL of FITC labelled Ki-67 antibody is added to the tubes containing the cells of interest. The cells were incubated for 20-30 minutes at room temperature (covered in foil). Following this incubation 2mL 1% FBS is added to the tubes and spun at 1000 rpm for 10 minutes. The supernatant

was removed and the cells were resuspended in 500uL 1% FBS and incubated at 4°C for one hour allowing Ki-67 to diffuse into the cells. The cells after one hour were centrifuged at 1000rpm for 10 minutes after which the supernatant was removed and the cells resuspended in 150uL 1% FBS. To this 150uL 1.5uL 7AAD was added. The tubes were then loaded onto the Flow cytometer and analysed as outlined in previous section (2.6.3).

2.13 Statistical analysis.

The statistical analysis method employed for this thesis was the T-Test method. This method looks to examine the mean of two groups of data to determine if they are significantly different. Significance applies where $p < 0.05$.

3.0 Results

3.1 PTN in vitro irradiation study

3.1.1 This experiment was to assess whether the addition of rPTN was sufficient for the ex vivo expansion of HSCs in liquid suspension cultures.

Mice

20 C5BL/6 mice (8-9 weeks old) from the Jackson Laboratory (Bar Harbor Maine, U.S.A) were used.

Bone Marrow collection

The mice were sacrificed by CO₂ euthanasia and cervical dislocation. The femurs and tibia from each of the mice were dissected and flushed as outlined in section 2.1.

KSL isolation

The contents of the bone marrow underwent red cell lysis to achieve a mononuclear cell (MNC) population. Lineage positive cells were removed from the MNC population by means of magnetic cell sorting as outlined in section 2.2.3. This resulted in a pure Lineage negative population. This population of cells underwent further processing by means of Flow cytometry whereby cells that positively expressed c-Kit and sca-1 were collected to achieve a population of cells that were negative for antigens associated with maturity/lineage (Lineage negative), c-kit and sca-1 positive (KSL) and enriched for stem cells outlined in section 2.4.

Radiation

The c-kit^{pos} Sca-1^{pos} Lin⁻ cells, once isolated were exposed to 300cGy of radiation at a rate of 0.56 cGy per minute as outlined in section 2.9.

Cell culture

5000 c-kit^{pos} Sca-1^{pos} Lin⁻ cells were plated in a 12-well plate with 1mL of TSF (appendix), 100ng/mL rPTN in TSF (Appendix) or 500ng/mL rPTN in TSF (Appendix). The KSL number per well in a 12-well plate varies from 1000 cells (Varnum-Finney, 2003) to 10,000 cells (DeHart, 2005) ensuring adequate cell contact that is supportive to growth. The Chute laboratory however has determined an in-house optimum range of 5000-20,000 KSL cells per well in a 12 well plate. The experiment layout was as follows each of which were performed in triplicate:

IR KSL + TSF	IR KSL + TSF 100ng/mL rPTN	IR KSL + TSF 500ng/ml rPTN
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Table 3: Experiment set up to test whether the addition of rPTN is sufficient for the ex vivo expansion of HSCs in liquid suspension cultures

IR: Irradiated cells.

NI: Non-irradiated cells

TSF: Thrombopoietin, stem cell factor and Flt 3 ligand (cytokine reagent).

rPTN: Recombinant Pleiotrophin.

The cells were cultured for seven days in 5% CO₂ at 37°C.

Seven day culture

Following seven days in culture the cells were collected from the wells into 15mL tubes. The wells were washed to ensure maximum cell collection and a cell count and viability analysis was performed as outlined in section 2.2. The cultures were analysed individually for cell count, KSL content using flow cytometry and quantification of lineage committed progenitors using a colony forming cell assay.

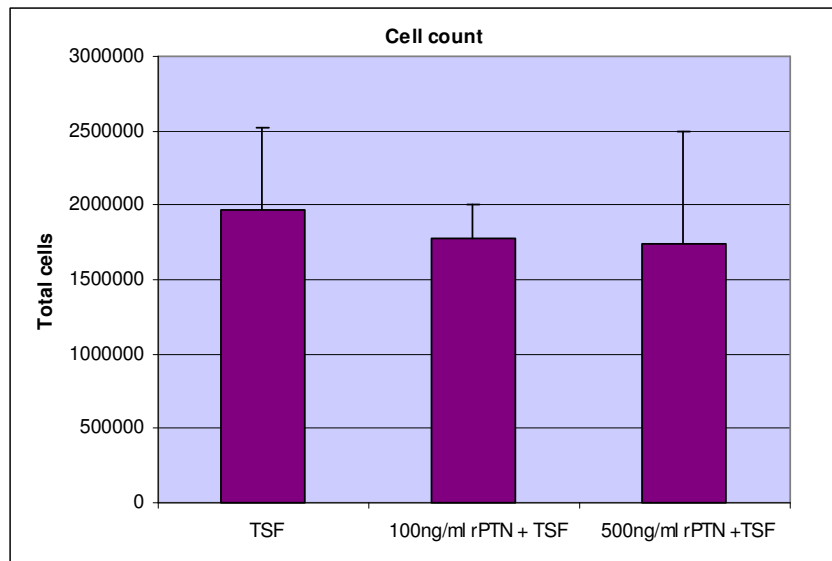


Figure 16: The average cell counts of KSL cells and progeny both mature and immature after 7 days in culture with TSF, TSF and 100ng/mL rPTN or TSF plus 500ng/mL rPTN, each of which were performed in triplicate. The cell count reflects the entire cell population in the well including the stem cells, stem cell progeny, lineage committed progenitors and cells at all stages of differentiation including those that have fully matured. There is no significant difference in cell counts between the treated cultures with a p value of 0.3 for TSF against 100ng/mL rPTN and a p value of 0.34 for TSF against 500ng/mL rPTN.

To identify the specific stem cell enriched population within the cultures the KSL cells of the cultures were stained with antibodies labelled with fluorescent probes and analysed using flow cytometry.

100,000 cells from each well was analysed initially through forward and side scatter allowing the population be centred around a live population of cells where the KSL population exists, denoted P1 in figures 17,18 and 19. Subsequently this population was narrowed further when the cells expressing antigens associated with lineage or maturity were excluded, allowing the Lineage negative (Lin-) population to be analysed, denoted in Figures 17,18 and 19 as P2. This Lineage negative population was further categorised into a population of cells that were positive for both sca-1 and c-Kit denoted as P3 in Figures 17, 18 and 19. The content of cells that displayed a phenotype that was negative for lineage markers and positive for the sca-1 and c-Kit antigens could be compared between the cultures treated with TSF (Figure 17), 100ng/mL (Figure 18) or 500ng/mL rPTN (Figure 19).

TSF treated KSL cells.

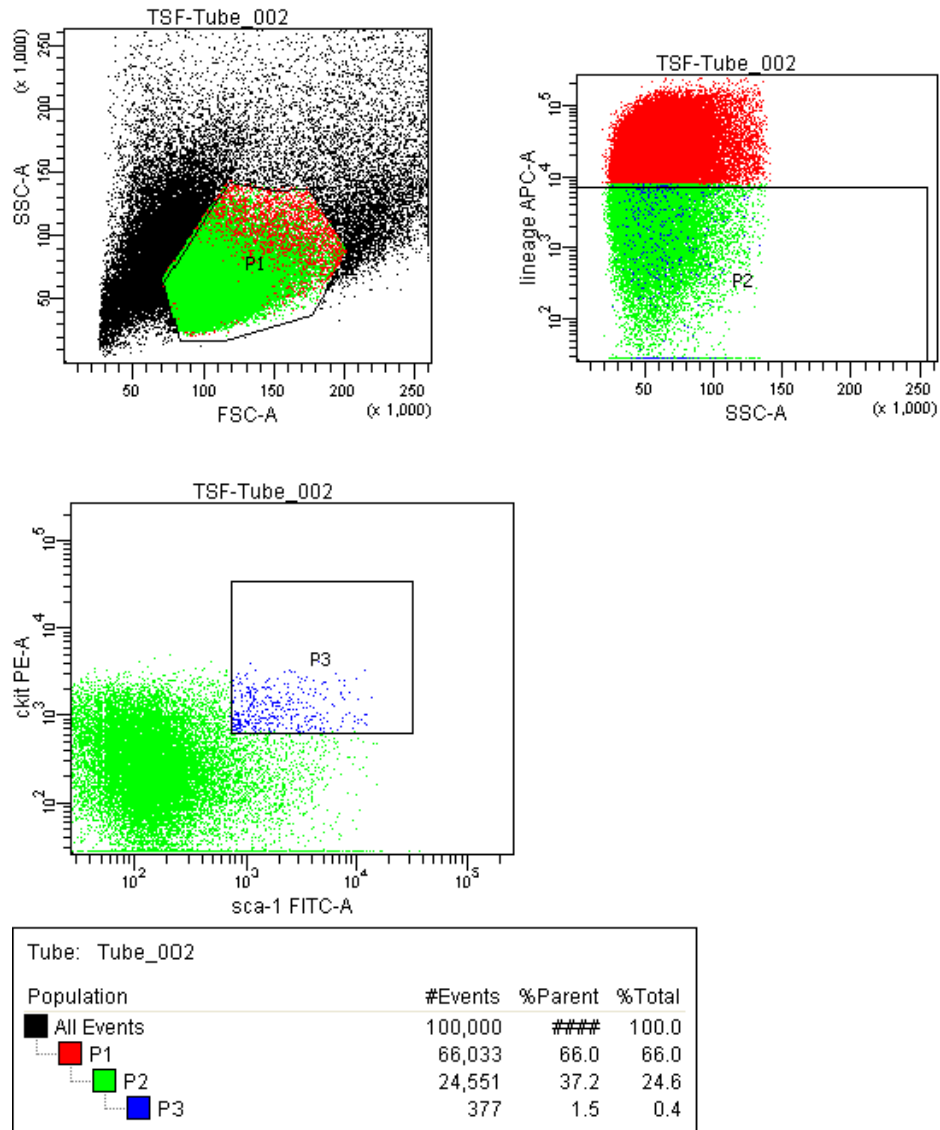


Figure 17: Flow cytometry report to determine the KSL population in a TSF treated culture. 18,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate and suspended in TSF. The culture was incubated for seven days with 5% CO₂ at 37°C. On day seven 100,000 cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a live population of cells where the KSL cells reside. P2 denotes immature cells within the culture as these represent the cells that are negative for antigens associated with maturity or lineage. P3 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The KSL cell percentage within the Lineage negative population was 1.5%.

rPTN (100ng/mL) + TSF treated KSL cells.

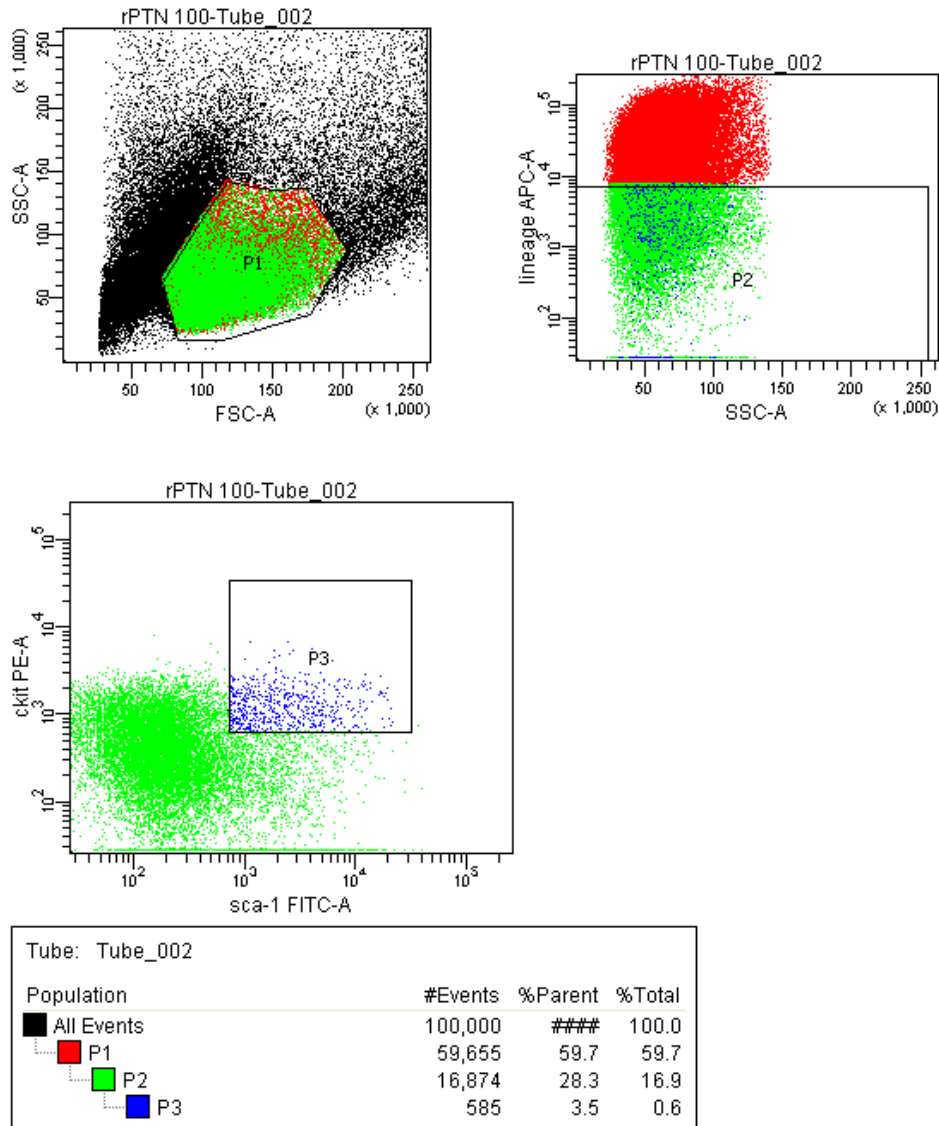


Figure 18: Flow cytometry report to determine the KSL population in a rPTN treated culture. 18,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate and suspended in 100ng/mL rPTN and TSF. The culture was incubated for seven days with 5% CO₂ at 37OC. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a live population of cells where the KSL cells reside. P2 denotes immature cells within the culture as these represent the cells that are negative for antigens associated with maturity or lineage. P3 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The KSL cell percentage within the Lineage negative population was 3.5%.

rPTN (500ng/mL) + TSF treated KSL cells.

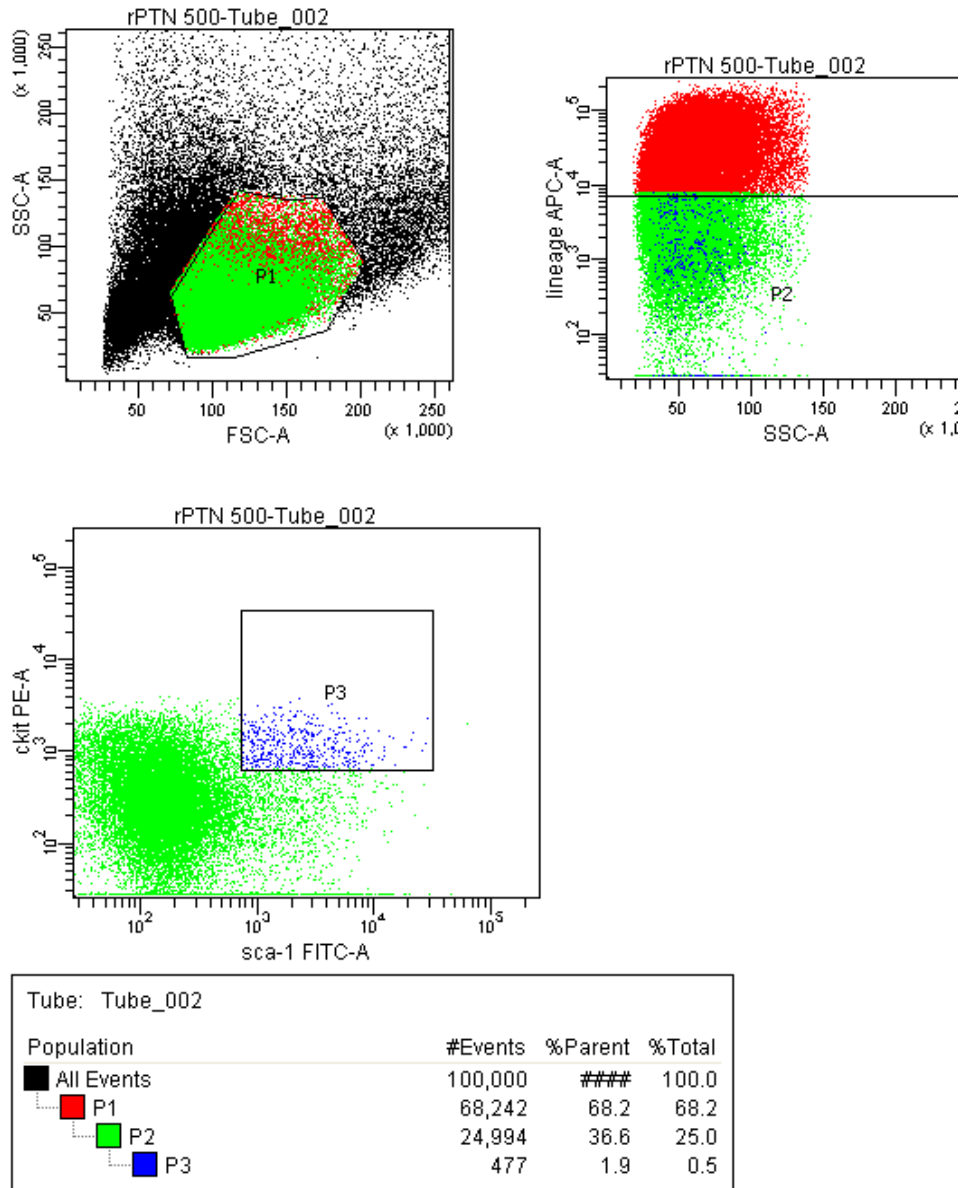


Figure 19: Flow cytometry report to determine the KSL population in a rPTN treated culture. 18,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate and suspended in 500ng/mL rPTN and TSF. The culture was incubated for seven days with 5% CO₂ at 37OC. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a live population of cells where the KSL cells reside. P2 denotes immature cells within the culture as these represent the cells that are negative for antigens associated with maturity or lineage. P3 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The KSL cell percentage within the Lineage negative population was 1.9%.

KSL population percentages were determined using Flow cytometry within the TSF, TSF and 100ng/mL rPTN and TSF and 500ng/mL rPTN cultures, each of which were performed in triplicate. In Figure 20 the % Sca-1 c-Kit Lin- is the percentage of cells that express both c-Kit and Sca-1 within the population of cells that are negative for antigens associated with Lineage or maturity. The Lineage negative percentage is the percentage of cells within the 100,000 analysed cells that are negative for lineage antigens. The % KSL is the number of cells within the 100,000 cells analyzed that are negative for antigens associated with Lineage and are c-Kit positive and Sca-1 positive.

Condition	% sca c-kit+ Lin-	Lin- %	% KSL
TSF	1.5	37.2	0.558
TSF	1.4	38.8	0.5432
TSF	1.6	38.7	0.6192
100ng/ml rPTN + TSF	3.5	28.3	0.9905
100ng/ml rPTN + TSF	1.7	38.5	0.6545
100ng/ml rPTN + TSF	1.2	40.1	0.4812
500ng/ml rPTN + TSF	1.9	36.6	0.6954
500ng/ml rPTN + TSF	1.5	37.6	0.564
500ng/ml rPTN +TSF	1.6	38.8	0.6208

Table 4: Table of results obtained from Flow cytometric report acquired from the analysis of 18,000 irradiated KSL cells added to a well containing either TSF, TSF and 100ng/mL rPTN or TSF and 500ng/mL rPTN in 1mL. The cultures were incubated at 37°C with 5% CO₂ after which 100,000 cells from each well was stained with Fluorescent labeled antibodies directed against the antigens Sca-1 and c-Kit and those associated with lineage. The % Sca-1 c-Kit Lin- is the percentage of cells that express both c-Kit and Sca-1 within the population of cells that are negative for antigens associated with Lineage or maturity. The Lineage negative percentage is the percentage of cells within the 100,000 analyzed cells that are negative for lineage antigens. The % KSL is the number of cells within the 100,000 cells analyzed that are negative for antigens associated with Lineage, are c-Kit positive and Sca-1 positive.

The KSL data outlined in Table 4 when compiled into a bar graph, Figure 20 displayed no significant difference between all of the cultures. There was however a modest increase in the % KSL in cultures treated with PTN, in particular 100ng/mL rPTN, suggesting an increase in the stem cell enriched population within this culture.

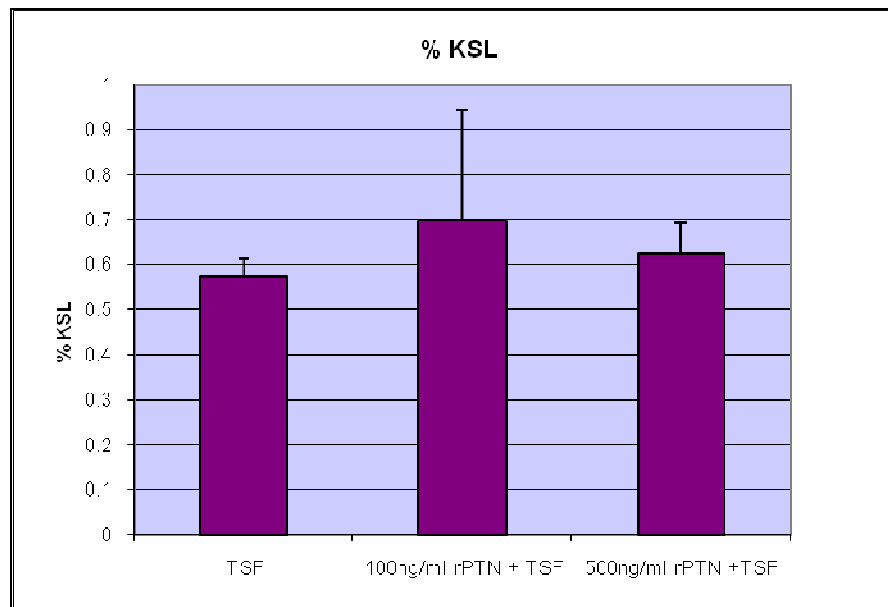


Figure 20: Percentage KSL cells of the total cell count after 7 days in culture with TSF, TSF and 100ng/mL rPTN or TSF plus 500ng/mL rPTN. There is no significant difference in KSL percentage between the treated cultures with a P value of 0.2 for TSF against 100ng/mL rPTN and 0.15 for TSF against 500ng/mL rPTN. There does however appear to be a modest trend in the KSL percentage with the cultures treated with 100ng/mL rPTN.

To examine the self-renewal activity of the KSL cells within the cultures one may observe the fold expansion of KSL cells between the TSF, TSF and 100ng/mL rPTN and the TSF and 500ng/mL rPTN cultures. The input of KSL cells to each culture was 18,000 cells. The total number of KSL cells was determined from the % KSL figures from Table 4 divided by 100 multiplied by the total cell count for each culture. This KSL number is then divided by the input cells (18,000) to determine fold expansion.

Condition	Total cell count	% KSL	KSL no.	Fold Expansion
TSF	1387500	0.558	7742.25	0.430125
TSF	2050000	0.5432	11135.6	0.618644444
TSF	2475000	0.6192	15325.2	0.8514
100ng/ml rPTN + TSF	2025000	0.9905	19484.55	1.082475
100ng/ml rPTN + TSF	1575000	0.6545	10308.38	0.572687778
100ng/ml rPTN + TSF	1712500	0.4812	8240.55	0.457808333
500ng/ml rPTN + TSF	1862500	0.6954	12951.83	0.719546111
500ng/ml rPTN + TSF	2425000	0.564	13677	0.759833333
500ng/ml rPTN +TSF	937500	0.6208	5820	0.323333333

Table 5: Table of data outlining deduction of KSL fold expansion from cell count and Flow cytometric analysis. Cell count was performed using Trypan blue exclusion method. The %KSL is provided by Flow cytometric analysis and is outlined in Figure 5. The total number of KSL cells was determined from the % KSL figures from Figure 5 divided by 100 multiplied by the total cell count for each culture. This KSL number is then divided by the input cells (18,000) to determine fold expansion.

The KSL fold expansion data outlined in Table 5 when compiled into a bar graph, Figure 21 displayed no significance between all of the cultures with a p value of 0.3 for TSF versus 100ng/mL rPTN and 0.4 for TSF versus 500ng/mL rPTN when examined statistically.

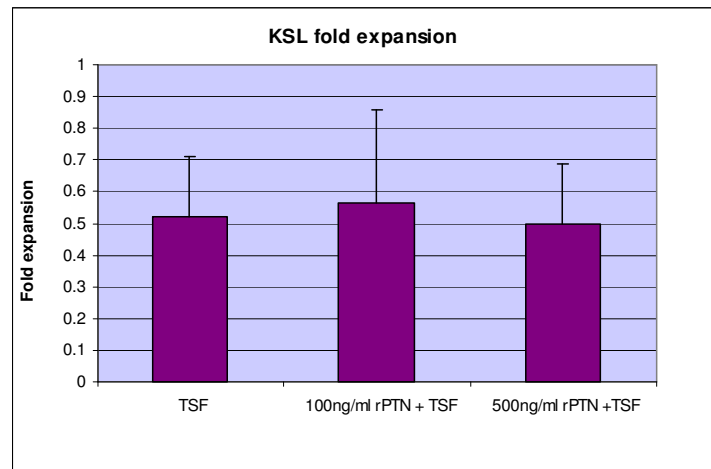


Figure 21: KSL fold expansion after 7 days in culture with TSF, TSF and 100ng/mL rPTN or TSF plus 500ng/mL rPTN relative to the input of KSL cells on Day 0. There is no significant difference in KSL fold expansion between the treated cultures, a modest increase is noted however with 100ng/ml rPTN.

To identify the lineage committed progenitors in the cultures a colony forming cell assay was set up. Stem cell progeny that enter a particular lineage, myeloid or erythroid can be quantitated when placed on a methocellulose medium that promotes differentiation and proliferation of stem cells to form progenitor cells that have short-term repopulating capabilities but have lost long-term repopulating abilities.

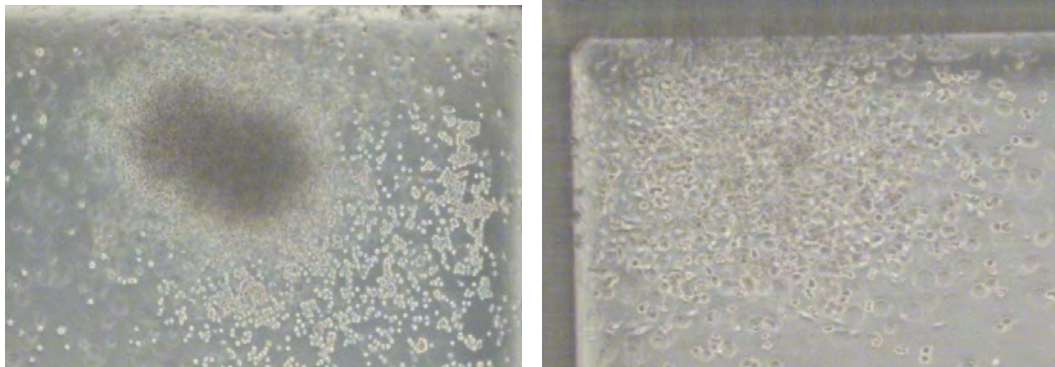


Figure 22: CFU-GM (Colony forming unit-granulocyte monocyte). Monocytic colonies are oval/round in shape often have grey/granulocytic centre while granulocytic colonies are bright round, smaller and more uniform in size.

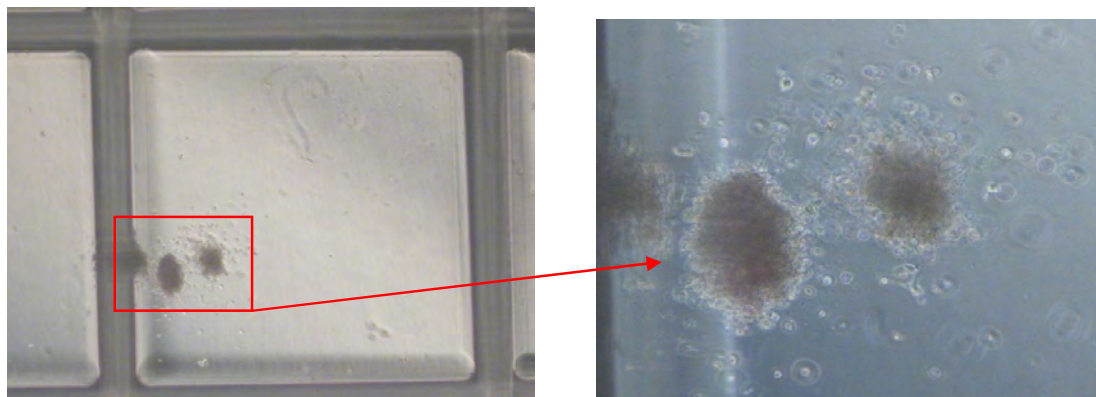


Figure 23: BFU-E (Burst forming unit-erythrocyte). These colonies contain erythroid clusters and individually appear tiny, irregular in shape and size, appear fused together and have a reddish/brown tint.

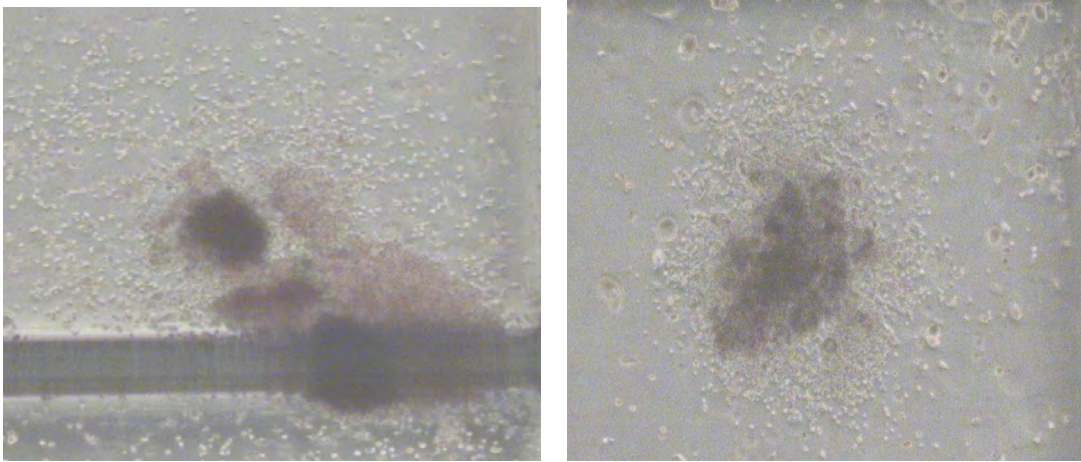


Figure 24: CFU-GEMM: Colony forming unit-granulocyte erythroid monocytic and megakaryocytic lineage. These colonies are often quite large, have indistinct borders and a reddish/brown tint.

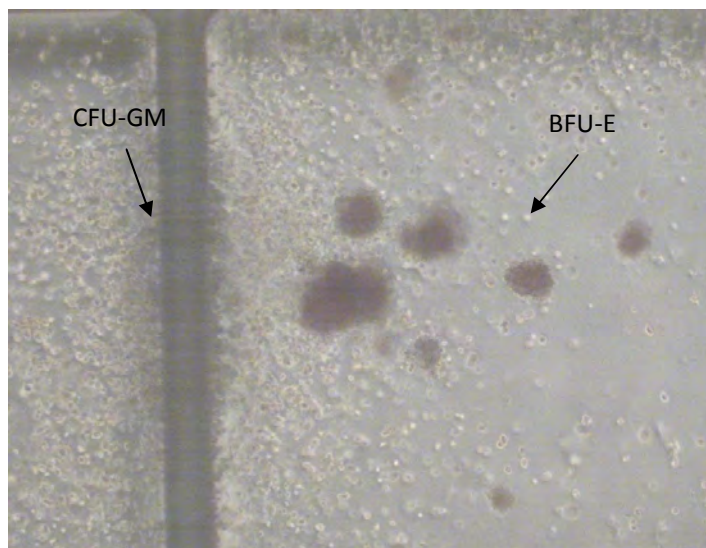


Figure 25: CFU-GM and BFU-E morphology contrast. CFU-GM with oval to round colonies that are bright and uniform in size compared to a BFU-E which is small, irregular, appear fused together and with a reddish/brown tint.

Each CFC assay was performed in triplicate for each treatment group where 500 cells were added to each CFC dish containing methocellulose medium which was incubated at 37°C with 5% CO₂ for 14 days. Colony formation was examined microscopically and quantitated for each treatment group, TSF, TSF plus 100ng/mL rPTN and TSF plus 500ng/mL rPTN whereby a colony is deemed to have 40 or more cells. The colony quantitation was divided into those that form Granulocyte Monocyte (GM) colonies or those that have entered myeloid lineage, those that form Burst Forming Units-Erythroid

(BFU-E) or are of erythroid lineage, and those that are mixed or of mixed lineage. Table 6 outlines the average number of total colonies from the 500 cells added to each dish for each treatment group.

Condition	GM	BFU-E	Mixed (CFU-GEMM)	Total colonies/500 cells
TSF	33	0	0	33
TSF	22	1	0	23
TSF	13	0	0	13
100ng/ml rPTN + TSF	12	0	0	12
100ng/ml rPTN + TSF	7	0	0	7
100ng/ml rPTN + TSF	8	0	0	8
500ng/ml rPTN + TSF	12	0	0	12
500ng/ml rPTN + TSF	11	1	1	13
500ng/ml rPTN +TSF	10	1	0	11

Table 6: Raw data from Colony Forming Cell Assay. Granulocyte/Monocyte (GM) indicates the cells of myeloid Lineage. Burst Forming Unit-Erythrocyte (BFU-E) indicates those of erythroid lineage and Mixed indicates those that are of mixed myeloid lineage (CFU-GEMM). 18,000 KSL cells were added to a well with TSF, TSF and 100ng/mL rPTN and TSF with 500ng/mL rPTN and incubated at 37°C with 5% CO₂. On day 7 the cultures were collected and counted by Trypan Blue exclusion. 500 cells from each culture (each in triplicate) were added to a methocellulose medium and incubated for 14 days at 37°C with 5% CO₂. On day 14 the colonies were quantified microscopically. The total number of colonies per 500 cells was the number of colonies present on the dish on day 14.

The raw data in table 6 when compiled into a bar graph (Figure 26) displays borderline significance in colony number between the TSF and 100ng/ml rPTN treatment groups with a p value of 0.05.

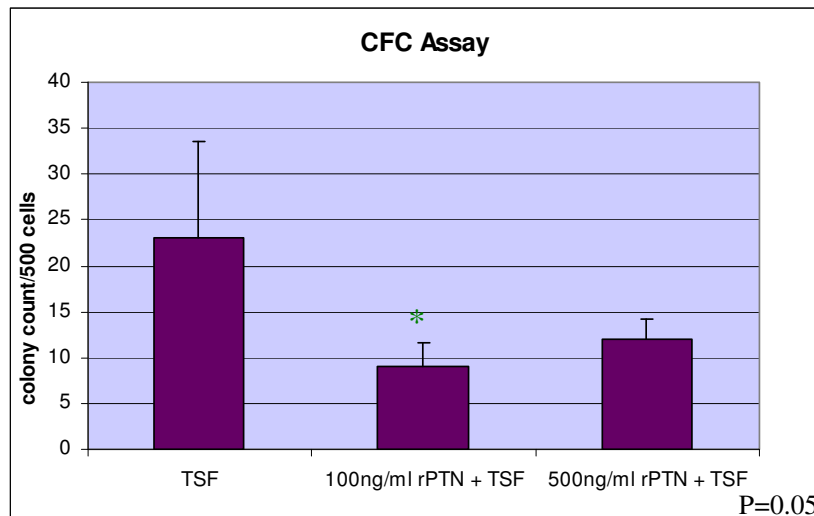


Figure 26: Colony Forming cell assay where 500 cells from the three samples per treatment group were added to a methocellulose medium for two weeks and incubated at 37⁰C with 5% CO₂, after which the colonies formed are quantified and averaged for each treatment group. The number of colony forming cells in the TSF and the 100ng/mL rPTN group is boarderline significant with a p value of 0.05. This result indicates a of a number of possibilities, one being there are less lineage committed progenitors in the PTN treated groups, which may be explained by either a stop in differentiation of the KSL cells or that they have entered self renewal or both. Another explanation for this result is those treated with rPTN may have differentiated so far that more cells have matured and are passed the colony forming stage.

Colony forming cell count may also be analysed when the total cell count is taken into consideration whereby the number of colonies formed from 500 cells is translated to the number of potential colonies from the total cell count, Table 7.

Condition	GM	BFU-E	Mixed	Total colonies/500 cells	Total cell count	Total no. colonies
TSF	33	0	0	33	1387500	63825
TSF	22	1	0	23	2050000	53300
TSF	13	0	0	13	2475000	59400
100ng/ml rPTN + TSF	12	0	0	12	2025000	28350
100ng/ml rPTN + TSF	7	0	0	7	1575000	25200
100ng/ml rPTN + TSF	8	0	0	8	1712500	41100
500ng/ml rPTN + TSF	12	0	0	12	1862500	48425
500ng/ml rPTN + TSF	11	1	1	13	2425000	53350
500ng/ml rPTN +TSF	10	1	0	11	937500	20625

Table 7: Raw data from Colony Forming Cell Assay. Granulocyte/Monocyte (GM) indicates the cells of myeloid Lineage. Burst Forming Unit-Erythrocyte (BFU-E) indicates those of erythroid Lineage and Mixed indicates those that are of mixed myeloid lineage (CFU-GEMM). 18,000 KSL cells were added to a well with TSF, TSF and 100ng/mL rPTN and TSF with 500ng/mL rPTN and incubated at 37°C with 5% CO₂. On day 7 the cultures were collected and counted by Trypan Blue exclusion. 500 cells from each culture (each in triplicate) were added to a methocellulose medium and incubated for 14 days at 37°C with 5% CO₂. On day 14 the colonies were quantified microscopically. The total number of colonies per 500 cells was the number of colonies present on the dish on day 14. The total number of colonies takes into account the total cell count of the cultures on day 7.

The results as outlined in table 7 are achieved through the multiplication of the number of colonies per 500 cells and taking into to account the actual number of cells isolated for each treatment group outlined in figure 1. When the total cell count is taken into consideration and the total colony number normalised, the number of colonies in the PTN treated groups versus the TSF treated group is significant in that the pvalue is < 0.05 for both the rPTN 100ng/mL (where p =0.0081) and 500ng/mL (where p = 0.0315) versus TSF as outlined and highlighted in Figure 26.

The total cell count in the cultures treated with 100ng/mL rPTN was although not significant displayed a trend of being moderately increased over those treated with 500ng/mL rPTN. A trend of increase was also noted in the KSL content of the cells in the 100ng/mL rPTN over the 500ng/mL rPTN and TSF alone treated cultures. Less lineage committed progenitors displayed in the cultures treated with 100ng/mL rPTN when compared to 500ng/MI rPTN or TSF through the colony forming cell assay. This experiment as a whole broadly suggests 100ng/ml rPTN treatment of irradiated KSL cells results in the increase of KSL cells.

3.1.2 To test whether PTN signaling was necessary for HUBEC-mediated expansion of HSCs in vitro. The technique was similar to that outlined in section 3.1.1.

HUBEC

Human brain endothelial cells were passed as outlined in section 2.5. 10,000 HUBECs were added to the required wells in a 12-well plate and allowed to settle for three days before the KSL cells were added.

The experiment layout was as follows:

IR KSL TSF (1mL)	IR KSL HUBEC + ECCM + IgG (1mL)	IR HUBEC (ECCM 1mL) + Anti-PTN in TSF (100ug/mL) (1mL) Anti-PTN final conc. 50ug/mL
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Table 8: Experiment lay-out to test whether PTN signaling is necessary for HUBEC-mediated expansion of HSCs in vitro. IgG antibody directed against goat was added to the HUBECs to control for any effects seen with the addition of the Anti-PTN antibody, which was goat anti-human to the cultures.

Input of HUBECs 10,000/well

Input of KSL cells 8000/well

ECCM: Endothelial cell culture medium.

TSF: Thrombopoietin, stem cell factor and Flt-3 ligand (Cytokine reagent)

HUBEC: Human brain endothelial cells

KSL: c-Kit +, sca-1+ lineage negative cells.

Note: The HUBECs and KSL cells are not in direct contact in culture. They are set up in transwell plates whereby the EC act indirectly on the radiated KSL cells.

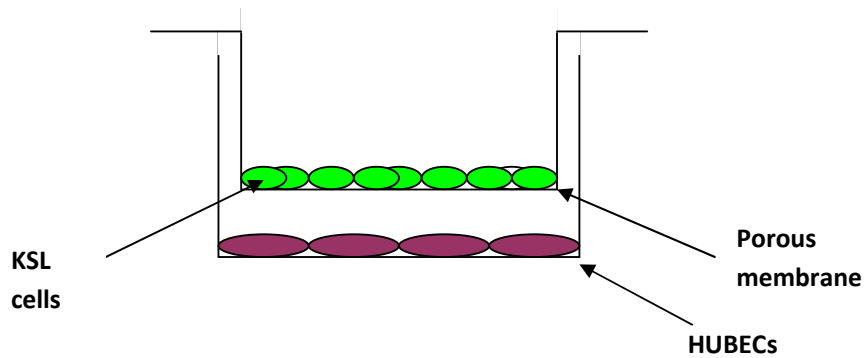


Figure 27: Transwell plate set-up for HUBEC (at the base), KSL (in the inserted well) and Anti-PTN antibody added to the ECCM mixture that flows between the HUBECs and KSL cells.

The cells were cultured for seven days (5% CO₂ at 37°C).

Seven day culture

Following seven days in culture the cells were collected from the wells into 15mL tubes. The wells were washed to ensure maximum cell collection and a cell count and viability analysis was performed as outlined in section 2.2. Flow cytometry (section 2.4.1) and Colony Forming cell assays (Section 2.7) were also performed.

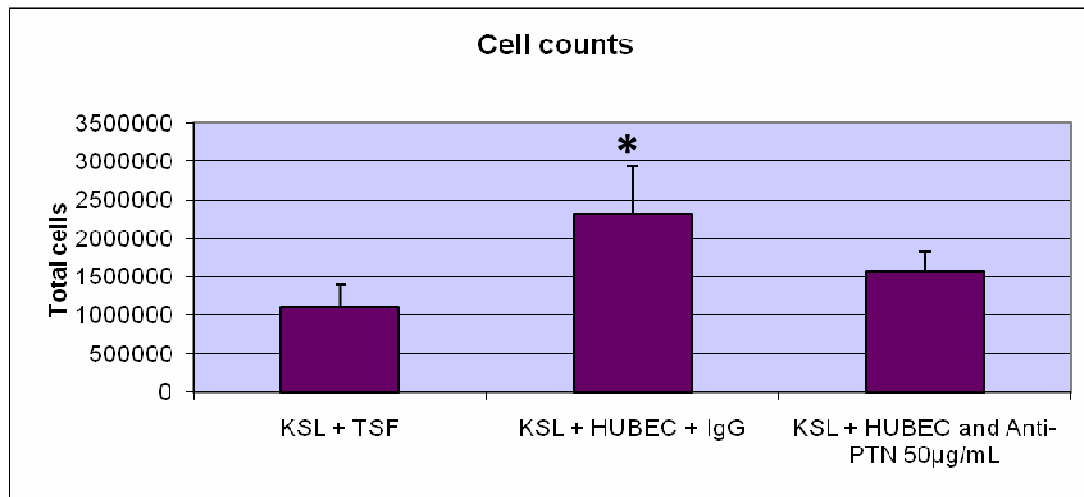


Figure 28: The average cell counts of KSL cells and progeny after 7 days in culture with TSF, HUBECs and HUBECs with 50ng/mL Anti-PTN, each of which were performed in triplicate. There is a significant increase in the total cell number of the cultures with HUBECs alone where $p < 0.05$ when compared to TSF alone. Anti-PTN although not statistically significant is border-line with a P value of 0.050 when compared to TSF alone.

To examine the specific stem cell enriched population within the cultures the KSL cells of the cultures were stained with antibodies labeled with fluorescent probes and analysed using flow cytometry. 100,000 cells from each well was analysed initially through forward and side scatter allowing the population be centered around a live population of cells where the KSL population exists, denoted P1 in figures 29,30 and 31. Subsequent to this the population was further characterised into live cells denoted as P2 in figures 29, 30 and 31. Following this the live population of cells was further classified by identifying the cells expressing antigens associated with lineage or maturity and excluding them. This allowed the immature cells that are Lineage negative be analysed, denoted in Figures 29, 30 and 31 as P3. This Lineage negative population was then further categorised into a population of cells that were positive for both sca-1 and c-Kit denoted as P4 in Figures 29, 30 and 31. The content of cells that displayed a phenotype which was negative for lineage markers and positive for the sca-1 and c-Kit antigens could be compared between the cultures treated with TSF (Figure 29), HUBECs (Figure 30) or HUBECs and 50ng/mL Anti-PTN (Figure 31).

TSF treated KSL cells.

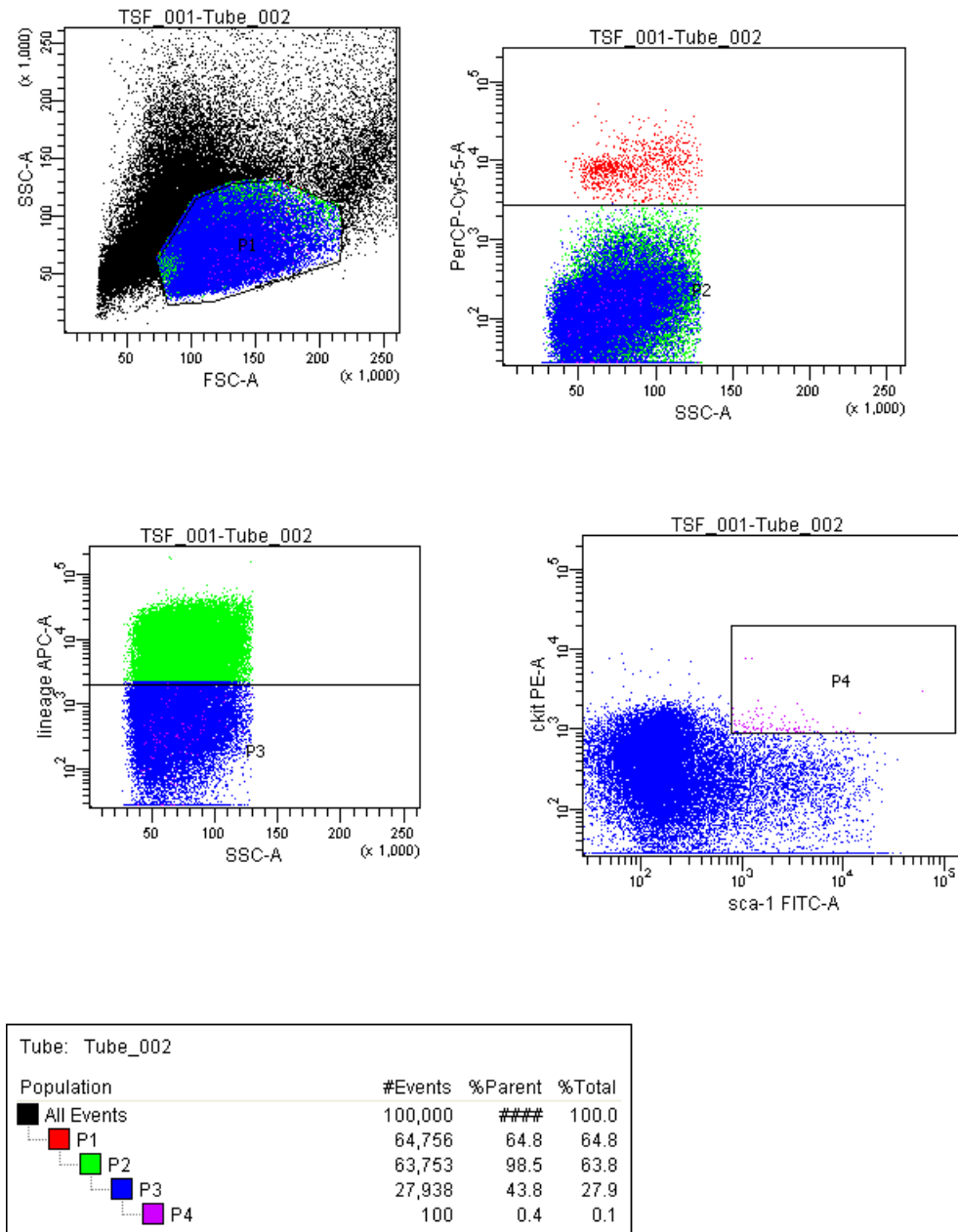


Figure 29: Flow cytometry report to determine the KSL population in a TSF treated culture. 8,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate and suspended in 1mL TSF. The culture was incubated for seven days with 5% CO₂ at 37OC. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a population of cells where the KSL cells reside. P2 denotes live cells within the P1 population. P3 denotes immature cells within the culture as these represent the cells that are negative for antigens associated with maturity or lineage. P4 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The percentage KSL cell percentage within the Lineage negative population was 0.4%.

HUBEC and IgG antibody treated KSL cells.

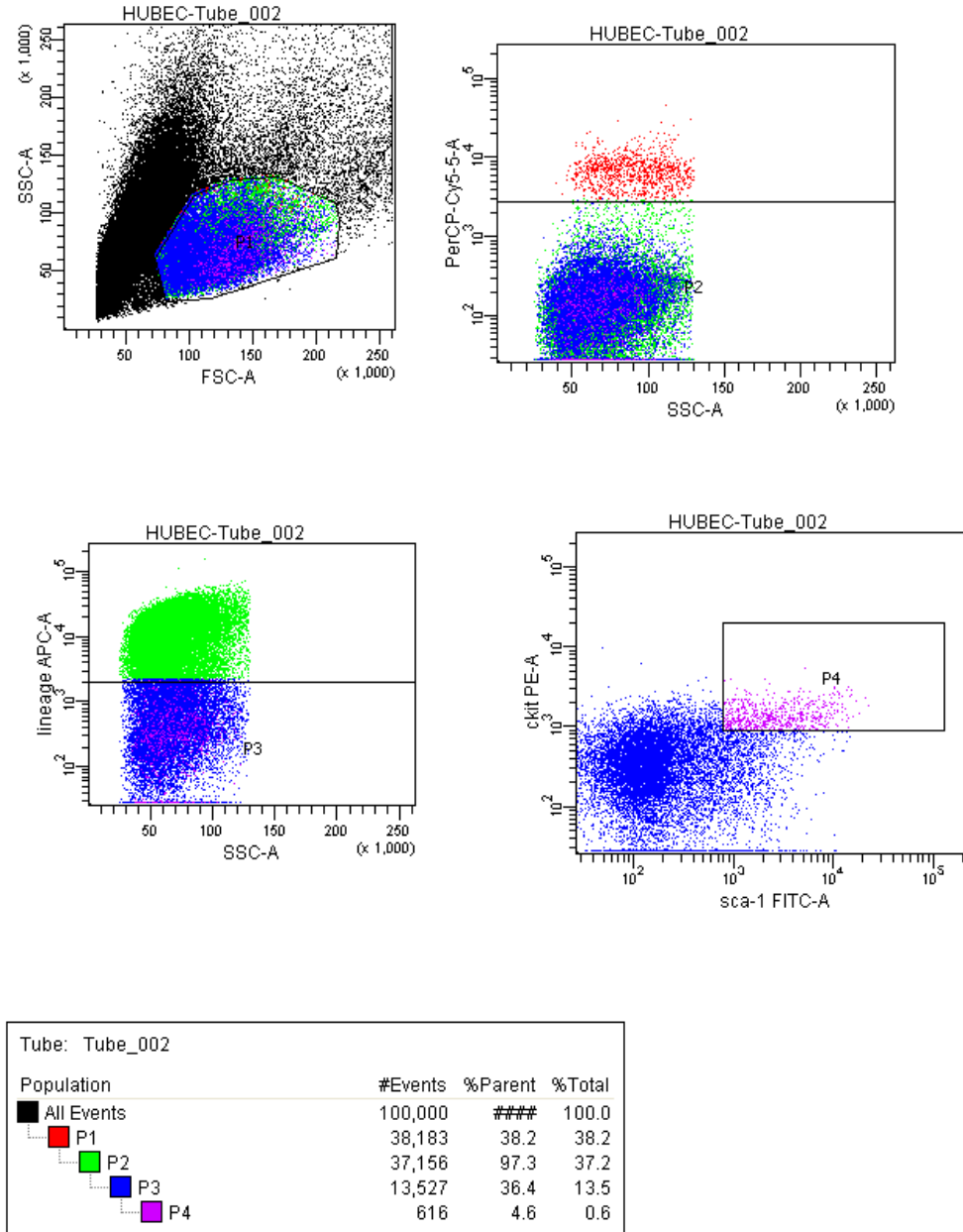


Figure 30: Flow cytometry report to determine the KSL population in a HUBEC treated culture. 8,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate containing 10,000 HUBECs and suspended in 1mL ECCM. The culture was incubated for seven days with 5% CO₂ at 37OC. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a population of cells where the KSL cells reside. P2 denotes live cells within the P1 population. P3 denotes immature cells within the P2 population as these represent the cells that are negative for antigens associated with maturity or lineage. P4 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The percentage KSL cell percentage within the Lineage negative population was 4.6%.

HUBEC and Anti-PTN (50ng/mL) treated KSL cells.

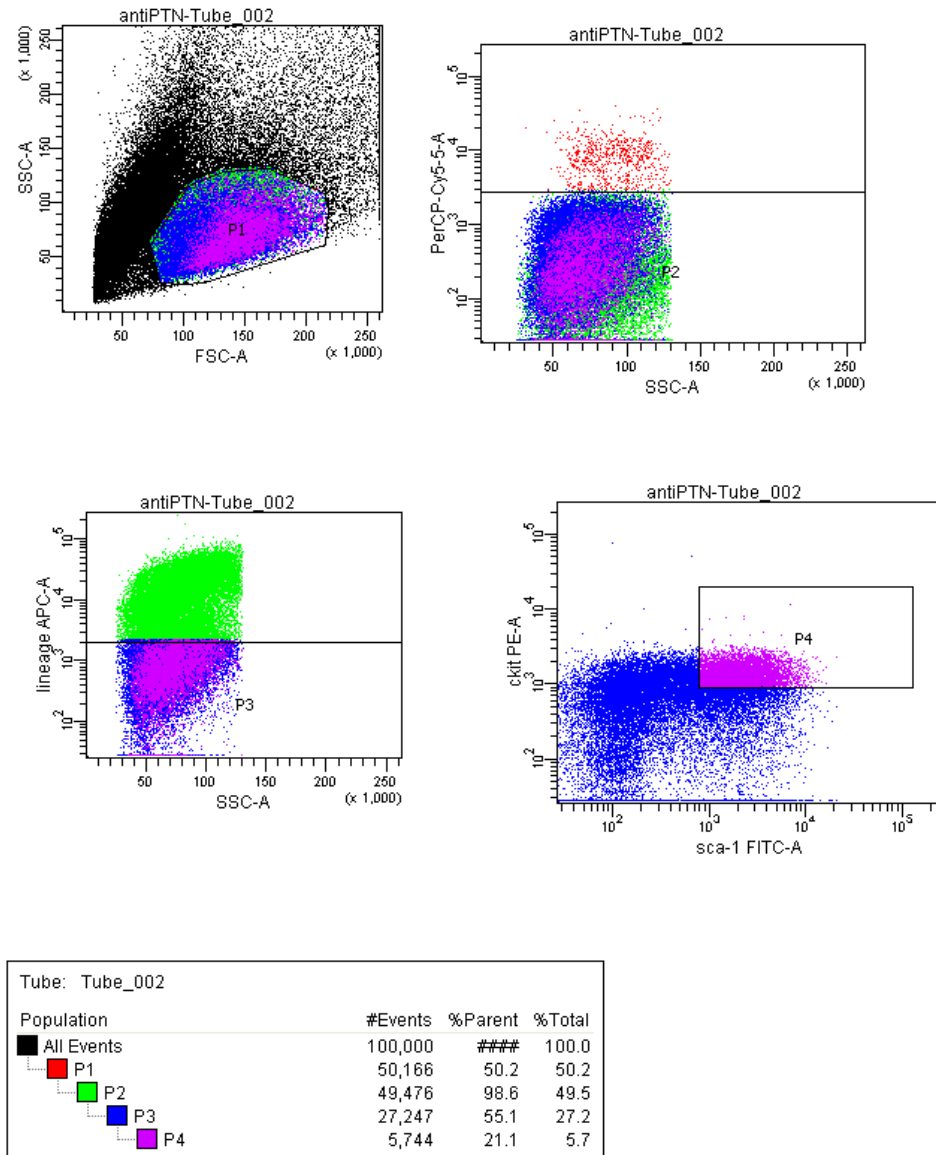


Figure 31: Flow cytometry report to determine the KSL population in a HUBEC and Anti-PTN treated culture. 8,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate containing 10,000 HUBECs and 50ng/mL Anti-PTN and suspended in 1mL ECCM. The culture was incubated for seven days with 5% CO₂ at 37OC. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a population of cells where the KSL cells reside. P2 denotes live cells within the P1 population. P3 denotes immature cells within the P2 population as these represent the cells that are negative for antigens associated with maturity or lineage. P4 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The percentage KSL cell percentage within the Lineage negative population was 21.1%.

KSL population percentages were determined using Flow cytometry within the TSF, HUBEC and HUBEC plus 50ng/mL Anti-PTN100ng/mL rPTN cultures, each of which were performed in triplicate are outlined in Table 9.

Condition	%sca c-kit+ /lin-	Lin - %	% KSL
TSF	0.4	44.5	0.178
TSF	0.4	43.8	0.1752
TSF	0.3	48	0.144
HUBEC	4.6	36.4	1.6744
HUBEC	5.3	31.2	1.6536
HUBEC	4.3	31.1	1.3373
Anti-PTN (50ng/mL) + HUBEC	21.1	55.1	11.6261
Anti-PTN (50ng/mL)+ HUBEC	22.5	66.2	14.895
Anti-PTN (50ng/mL)+ HUBEC	17.7	56.2	9.9474

Table 9: Table of raw data obtained from Flow cytometric report acquired from the analysis of 18,000 irradiated KSL cells added to a well containing either TSF, HUBECs and HUBECs plus 50ng/mL Anti-PTN 50ng/mL in 1mL ECCM. The cultures were incubated at 37°C with 5% CO₂ after which 100,000 cells from each well was stained with Fluorescent labeled antibodies directed against the antigens Sca-1 and c-Kit and those associated with lineage. The % Sca-1 c-Kit Lin- is the percentage of cells that express both c-Kit and Sca-1 within the population of cells that are negative for antigens associated with Lineage or maturity. The Lineage negative percentage is the percentage of cells within the 100,000 analyzed cells that are negative for lineage antigens. The % KSL is the number of cells within the 100,000 cells analyzed that are negative for antigens associated with Lineage, are c-Kit positive and Sca-1 positive.

The KSL data outlined in Table 9 when compiled into a bar graph, Figure 32 demonstrated there was a significant difference in KSL percentage between the treated cultures after 7 days incubation.

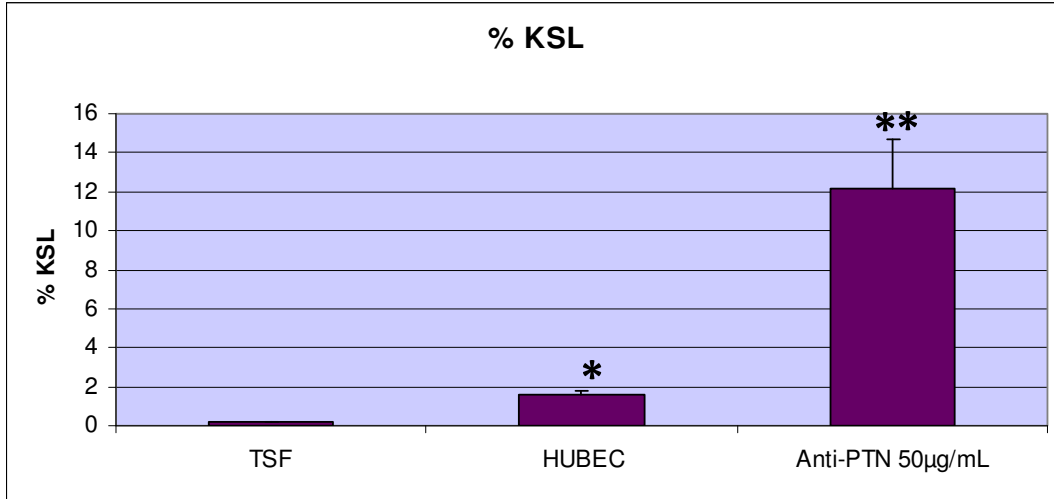


Figure 32: Percentage KSL cells of the total cell count after 7 days in culture with TSF, HUBECs alone and HUBECs with Anti-PTN (50ng/mL). There is a significant difference in KSL percentage between the treated cultures. Firstly there are more KSL cells within the HUBEC alone treated group when compared to TSF alone ($p=0.0028$). Secondly there are many more KSL cells in the HUBEC and Anti-PTN treated group when compared to TSF ($p=0.0072$). Lastly, when one compares the KSL content within the HUBEC treated group versus the HUBEC and Anti-PTN group there is also significance ($p=0.009$). This data suggest that when KSL cells are set up in culture with HUBECs and PTN is blocked using Anti-PTN that there is an increase in the KSL content within the cultures, therefore suggesting that PTN is not required for HUBEC mediated expansion of stem cells in culture.

To examine the self-renewal activity of the KSL cells within the cultures one may observe the fold expansion of KSL cells between the TSF, HUBEC and HUBECs and Anti-PTN (50ng/mL) cultures. The cell fold expansion was examined and normalised for each of the culture conditions as outline in Figure 33.

Conditions	Cell count	% KSL	KSL no.	Fold Expansion
TSF	1150000	0.178	2047	0.225875
TSF	787500	0.1752	1379.7	0.1724625
TSF	1362500	0.144	1962	0.24525
HUBEC	1887500	1.6744	31604.3	3.9505375
HUBEC	3025000	1.6536	50021.4	6.252675
HUBEC	2000000	1.3373	26746	3.34325
Anti-PTN (50ng/mL) + HUBEC	1862500	11.6261	26536.1	27.06701
Anti-PTN (50ng/mL)+ HUBEC	1475000	14.895	219701.3	27.4626625
Anti-PTN (50ng/mL)+ HUBEC	1387500	9.9474	138020.2	17.252519

Table 10: Table of data outlining deduction of KSL fold expansion from cell count and Flow cytometric analysis. Cell count was performed using Trypan blue exclusion method. The %KSL is provided by flow cytometric analysis and is outlined in Figure 21. The total number of KSL cells was determined from the % KSL figures from Figure 18 divided by 100 multiplied by the total cell count for each culture. This KSL number is divided by the input cells (8,000) to determine fold expansion.

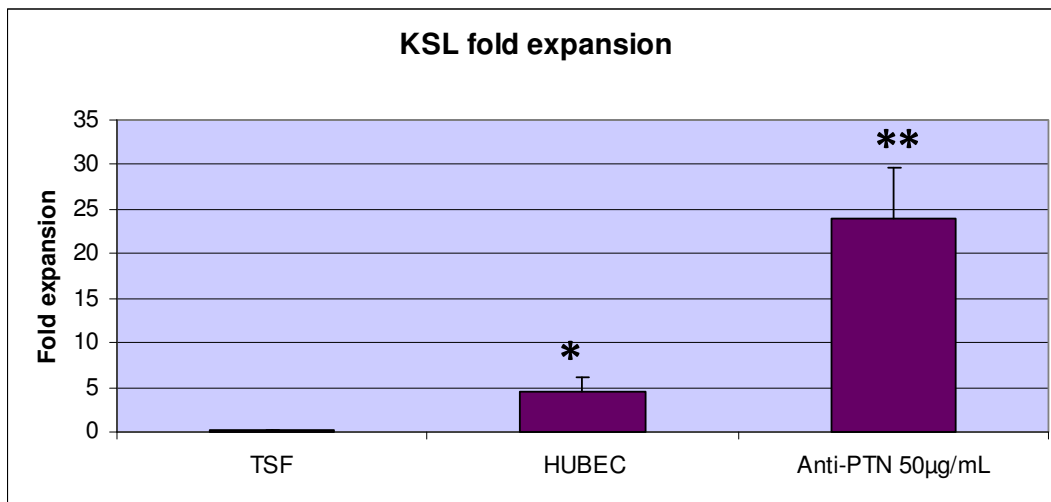


Figure 33: The KSL fold expansion between different treatment groups is statistically significant ($p < 0.05$) and indicated with the asterix. The HUBEC alone treated group when compared to TSF alone has a p value of 0.02 indicating a difference in KSL fold expansion between these treatment groups. The Anti-PTN and HUBEC treated group is significantly higher when compared to TSF alone ($p = 0.009$) and to the HUBEC alone treated group ($p = 0.011$) indicating a difference in KSL fold expansion between these treatment groups.

To identify the lineage committed progenitors in the cultures a colony forming cell assay was set up. 500 cells from the treated cultures TSF, HUBEC and IgG and HUBEC and Anti-PTN (50ng/mL), when collected on Day 7 were added to the methocellulose medium to allow for analysis of lineage committed progenitors after treatment. Each CFC assay was performed in triplicate for each treatment group. The 500 cells were added to each CFC dish containing methocellulose medium which was incubated at 37°C with 5% CO₂ for 14 days. Colony formation was examined microscopically and quantitated for each treatment group, TSF, HUBECs and HUBECs plus 50ng/mL Anti-PTN whereby a colony is deemed to have 40 or more cells. The colony quantitation was divided into those that form Granulocyte Monocyte (GM) colonies indicating those that have entered myeloid lineage, those that form Burst Forming Units-Erythroid (BFU-E) or are of erythroid lineage, and those that are CFU-GEMM are of granulocyte, erythroid, monocyte and megakaryocyte lineage. Table 11 outlines the average number of total colonies from the 500 cells added to each dish for each treatment group.

Conditions	GM	BFU	GEMM	Total colonies/500 cells
TSF	5	0	0	5
TSF	7	0	0	7
TSF	5	0	0	5
HUBEC	1	0	0	1
HUBEC	4	0	0	4
HUBEC	2	0	0	2
Anti-PTN (50ng/mL) + HUBEC	6	0	0	6
Anti-PTN (50ng/mL)+ HUBEC	10	0	0	10
Anti-PTN (50ng/mL)+ HUBEC	13	0	0	13

Table 11: Raw data from Colony Forming Cell Assay. Granulocyte/Monocyte (GM) indicates the cells of myeloid Lineage. Burst Forming Unit-Erythrocyte (BFU-E) indicates those of erythroid lineage and Mixed indicates those that are of mixed myeloid lineage (CFU-GEMM). 18,000 KSL cells were added to a well with TSF, HUBECs and and HUBECs plus 50ng/mL Anti-PTN and incubated at 37°C with 5% CO₂. On day 7 the cultures were collected and counted by Trypan Blue exclusion. 500 cells from each culture (each in triplicate) were added to a methocellulose medium and incubated for 14 days at 37°C with 5% CO₂. On day 14 the colonies were quantified microscopically. The total number of colonies per 500 cells was the number of colonies present on the dish on day 14.

The raw data in Table 11 when compiled into a bar graph (Figure 34) displays a significant difference in the number of colony forming cells between the different treatment groups.

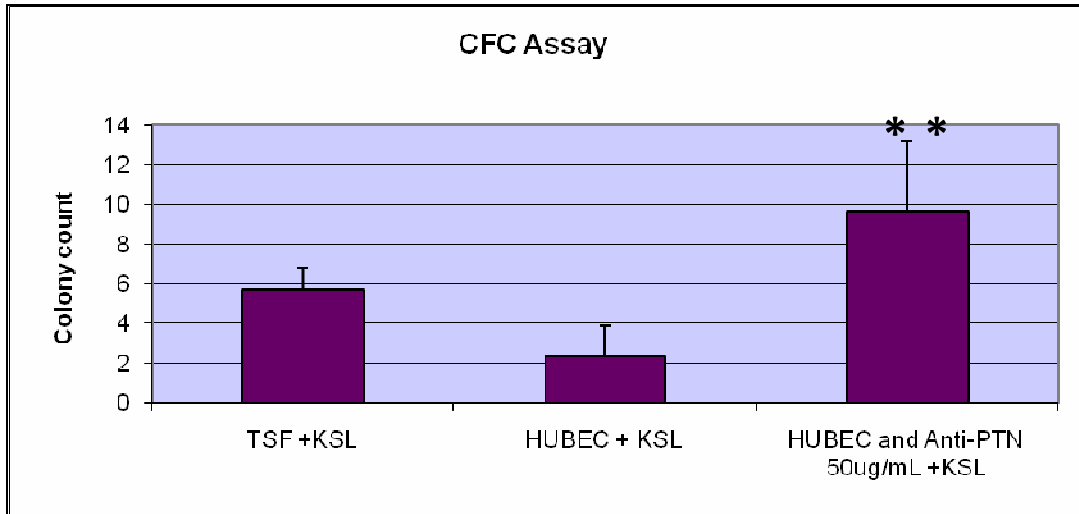


Figure 34: Colony Forming cell assay where 500 cells from the three samples per treatment group were added to methocellulose medium for two weeks and incubated at 37°C with 5% CO₂, after which the colonies formed are quantified and averaged for each treatment group. The number of colony forming cells in the different treatment groups is significantly different with significance indicated with a p value <0.05. TSF versus HUBEC and Anti-PTN (p=0.022) and HUBEC versus HUBEC and Anti-PTN (P=0.02).

Colony forming cell count may also be analysed when the total cell count is taken into consideration whereby the number of colonies formed from 500 cells is translated to the number of potential colonies from the total cell count, Table 12.

Conditions	GM	BFU	GEMM	Total colonies/500 cells	Total cell count	Total no. colonies
TSF	5	0	0	5	1150000	11500
TSF	7	0	0	7	787500	11025
TSF	5	0	0	5	1362500	13625
HUBEC	1	0	0	1	1887500	3775
HUBEC	4	0	0	4	3025000	24200
HUBEC	2	0	0	2	2000000	8000
Anti-PTN (50ng/mL) + HUBEC	6	0	0	6	1862500	22350
Anti-PTN (50ng/mL)+ HUBEC	10	0	0	10	1475000	29500
Anti-PTN (50ng/mL)+ HUBEC	13	0	0	13	1387500	36075

Table 12: Raw data from Colony Forming Cell Assay. Granulocyte/Monocyte (GM) indicates the cells of myeloid Lineage. Burst Forming Unit-Erythrocyte (BFU-E) indicates those of erythroid Lineage and mixed indicates those that are of granulocyte erythroid monocytic and megakaryocytic lineage (CFU-GEMM). 8,000 KSL cells were added to a well with TSF, HUBECs alone and HUBECs plus 50ng/mL Anti-PTN and incubated at 37°C with 5% CO₂. On day 7 the cultures were collected and counted by Trypan Blue exclusion. 500 cells from each culture (each in triplicate) were added to a methocellulose medium and incubated for 14 days at 37°C with 5% CO₂. On day 14 the colonies were quantified microscopically. The total number of colonies per 500 cells was the number of colonies present on the dish on day 14. In order to normalise for the difference in cell count in each of the wells the colony number is also presented as the total number of colonies.

When the total cell count is taken into consideration statistical difference exists in the number of colonies between the treated groups (Table 12). When PTN released from the HUBECs was blocked with Anti-PTN a significant increase in the number of colonies relative to both TSF (p= 0.022) and the HUBEC treated groups (p= 0.045) was noted. Highlighting further the findings previously outlined with regards to Figure 34.

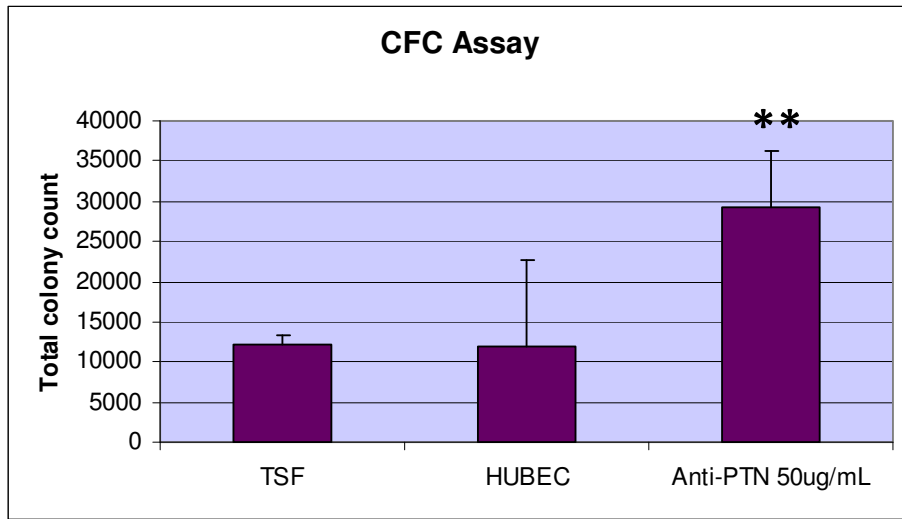


Figure 35: When the total cell count is taken into consideration statistical difference exists in the number of colonies between the treated groups. When PTN released from the HUBECs is blocked there is a significant increase in the number of colonies relative to both TSF ($p= 0.022$) and the HUBEC treated groups ($p= 0.045$).

This experiment indicates PTN signalling is not required for HUBEC-mediated expansion and recovery of HSCs post radiation injury

3.1.3 This experiment was to investigate if PTN arrested cells in the G0 phase of the cell cycle in culture.

KSL cells were isolated and plated in a similar manner to that outlined in section 3.1.1. The cells for this experiment however were not exposed to any radiation prior to incubation with 5% CO₂ at 37°C for seven days. On day seven the cell population within the wells including stem cells, stem cell progeny, lineage committed progenitors and cells at all stages of differentiation including those that had fully matured were collected and counted. The cells were then stained for those that were negative for antigens associated with lineage or maturity, and that expressed c-Kit and Sca-1 antigens on their surface (KSL). The cells were subsequently subjected to a two-day Ki-67 antibody staining schedule outlined in section 2.12 and examined using flow cytometry using the Ki-67 antibody to allow for cell cycle analysis within the Lineage negative and KSL populations in the TSF treated group (Figure 36) and the rPTN treated group (Figure 37).

100,000 cells from each well was analysed initially through forward and side scatter allowing the population be centered around a live population of cells where the KSL population exists, denoted P1 in figures 36 and 37. Subsequently this population was narrowed further for the cells expressing antigens associated with lineage or maturity were excluded, allowing the Lineage negative population be analysed, denoted in Figures 36 and 37 as P2. The staining with Ki-67 allowed the analysis of cell cycle status of these cells. P6 represents cells within the lineage negative population (P2) that are in the G0 phase of the cell cycle. P7 indicates cells within the lineage negative population that are in the G1 phase of the cell cycle. P8 indicates cell within this population that have entered the G2/S/M phases of the cell cycle.

This Lineage negative population was then further categorised into a population of cells that were positive for both Sca-1 and c-Kit denoted as Q1, Q2, Q3 and Q4. The amount of live cells in G0 stage within this population is represented by P3. P4 represents cells within the KSL population that have entered the G1 phase. P5 denotes live cells within the KSL population that are in the G2/S/M stages of the cell cycle in figure 36 and 37.

Cell cycle analysis of cells incubated with TSF.

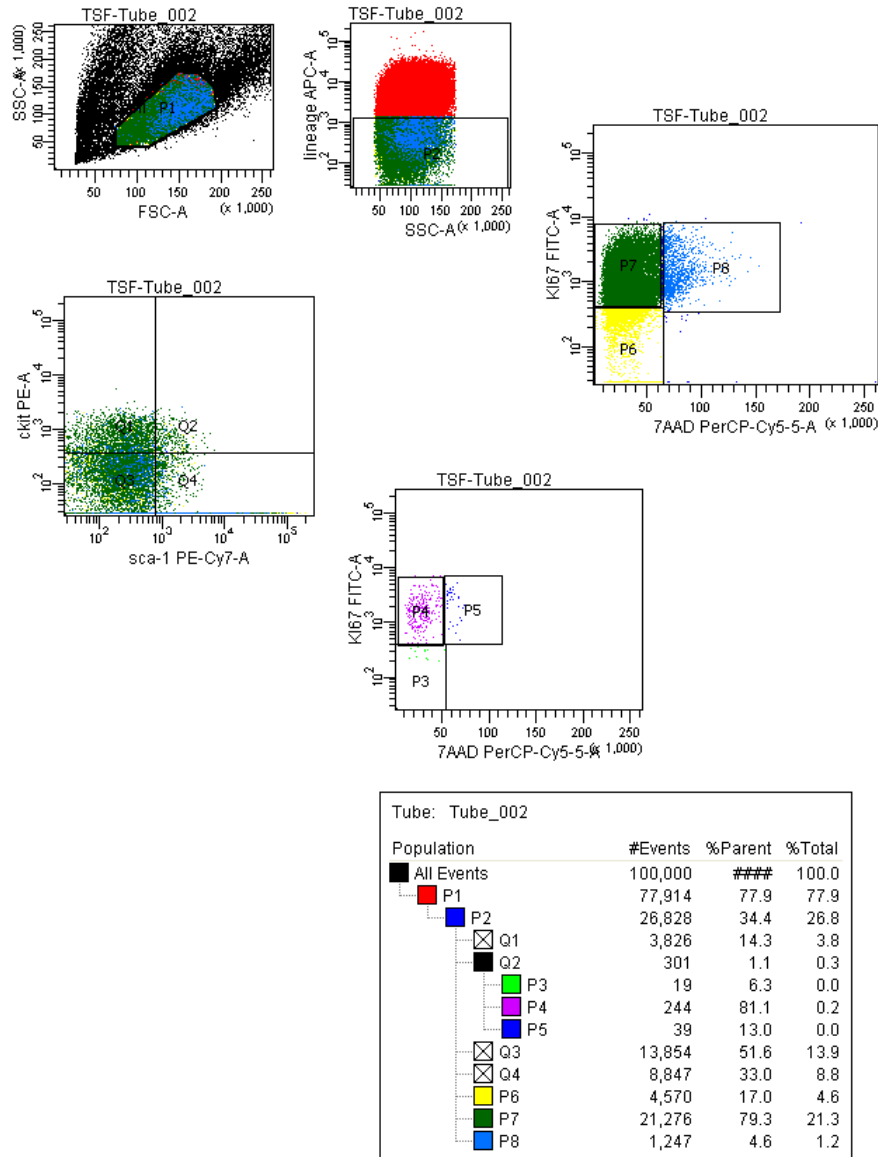


Image 36: Flow cytometry report to determine the cell cycle status of cells within the Lineage negative and KSL cell populations. 20,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate containing 1mL TSF. The culture was incubated for seven days with 5% CO₂ at 37°C. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a population of cells where the KSL cells reside. P2 denotes immature cells within the P1 population as these represent the cells that are negative for antigens associated with maturity or lineage. P6 denotes cells within the lineage negative population that are in the quiescent or G₀ phase of cell cycle. P7 denotes cells within the lineage negative population that are in the G₁ phase of the cell cycle while P8 denotes cells within the lineage negative population that are in the G₂/S/M phase of cell cycle. Q1 Q2 Q3 Q4 indicates the cells within the Lineage negative population that are positive for c-Kit and Sca-1 expression (KSL). P3 denotes live cells within the KSL population that are in the G₀ phase of cell cycle (6.3%). P4 indicates live cells within the KSL population that are in the G₁ phase of cell cycle (81.1%). P5 indicates live cells within the KSL population that are in the G₂/S/M phase of cell cycle (13%).

Cell cycle analysis of cells incubated with 100ng/mL rPTN

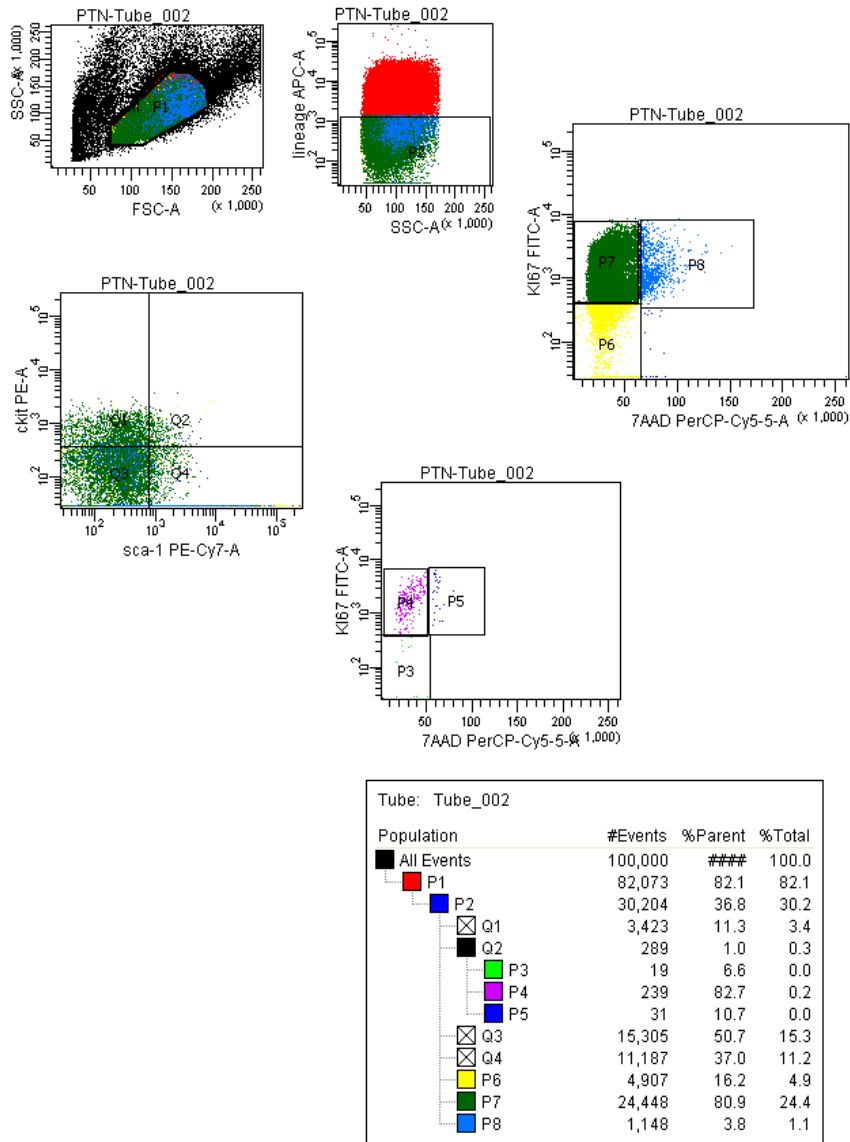


Image 37: Flow cytometry report to determine the cell cycle status of cells within the Lineage negative and KSL cell populations. 20,000 purified KSL cells were irradiated with 200cGy radiation and added to a 12 well plate containing 100ng/mL rPTN. The culture was incubated for seven days with 5% CO₂ at 37°C. On day seven the cells were stained with fluorescent labeled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P1 denotes a population of cells where the KSL cells reside. P2 denotes immature cells within the P1 population as these represent the cells that are negative for antigens associated with maturity or lineage. P6 denotes cells within the lineage negative population that are in the quiescent or G0 phase of cell cycle. P7 denotes cells within the lineage negative population that are in the G1 phase of the cell cycle while P8 denotes cells within the lineage negative population that are in the G/S/M phase of cell cycle. Q1 Q2 Q3 Q4 indicates the cells within the Lineage negative population that are positive for c-Kit and Sca-1 expression (KSL). P3 denotes live cells within the KSL population that are in the G0 phase of cell cycle (6.3%). P4 indicates live cells within the KSL population that are in the G1 phase of cell cycle (81.1%). P5 indicates live cells within the KSL population that are in the G2/S/M phase of cell cycle (13%).

The flow cytometry data obtained for the TSF and 100ng/mL rPTN and TSF treated cultures are outlined in Table 13 below.

	Lineage negative			KSL		
Condition	%G0	%G1	%G2/S/M	%G0	%G1	%G2/S/M
TSF + KSL	16.9	78.6	5.4	3.4	78.8	16.5
TSF +KSL	9.6	87.8	2.8	2.3	84.6	11
TSF +KSL	17	79.3	4.6	6.3	81.1	13
KSL + 100ng/mL rPTN +TSF	25.1	71.4	5.2	5.5	83.2	11
KSL + 100ng/mL rPTN +TSF	21.4	77.9	2.1	5.1	90.1	5.1
KSL + 100ng/mL rPTN +TSF	16.2	80.9	3.8	6.6	82.7	10.7

Table 13: Raw data obtained from Flow cytometric reports acquired from the analysis of 20,000 irradiated KSL cells added to a well containing 1mL TSF or 1mL 100ng/mL rPTN and TSF. There is no significant difference in the cell cycle analysis between the treated cultures, however it does appear that there is a modest increase in the percentage lineage negative cells in the G0 or quiescent stage of the cell cycle in those that were treated with rPTN when compared to TSF alone. There is also a very modest increase in the percentage KSL cells in the G0 stage of the cell cycle in the rPTN treated groups.

The raw data outlined in Table 13 when compiled into a bar chart appears as follows in figure 38.

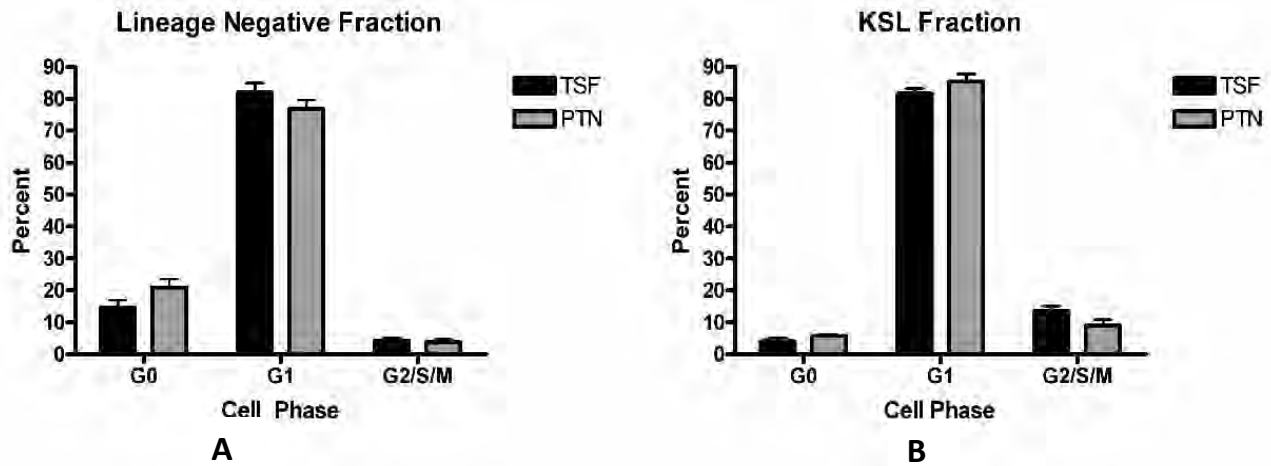


Figure 38: Cell cycle analysis. 20,000 KSL cells were added to a well plate containing TSF or 100ng/mL rPTN plus TSF and incubated for 7 days at 37°C with 5% CO₂.

A: Cell cycle analysis of the lineage negative population of cells. The lineage negative cells are those that lack antigens associated with maturity and represent an immature population of cells. These findings primarily indicate that most cells within the Lineage negative population are in the G1 phase of the cell cycle. There is no significant difference between the rPTN and the TSF treated groups in the G0, G1, G2/S and M stages of the cell cycle where $p=0.07$, $p=0.13$ and $p=0.136$ respectively.

B: Cell cycle analysis of the KSL population of cells. The KSL cell population of cells are a population of cells that are highly enriched for stem cells. These findings primarily indicate that most cells within the KSL population are also in the G1 phase of the cell cycle. No significant difference between the rPTN and the TSF treated groups exists in the G0, G1, G2/S and M phases of the cell cycle with a p values of $p=1.4$, $p=1.3$ and $p=0.07$ respectively.

These results indicate no difference in the number of cells that are quiescent or in G0 between the TSF and rPTN treated groups within the KSL population indicating no stop in differentiation within the Lineage negative of KSL populations between the TSF and rPTN treated groups. These results indicate that rPTN treatment does not induce cell quiescence within the Lineage negative or KSL populations.

3.2 PTN in vivo irradiation study.

3.2.1 This experiment was to examine the homing effect of PTN on HSCs to the marrow.

To begin the homing experiment a trial experiment to was performed whereby CFDA-SE staining of MNCs occurred, this tested the ability of the dye to enter the cells and be detected using flow cytometry. Recently isolated cells from the marrow of a murine femur which underwent red cells lysis was stained with CFDA-SE dye as per manufacturer's protocol. The cells were run on the flow cytometer at a wavelength of 450nm to test for positive cells. Figure 38 represents the negative control or a population of cells not stained with CFDA-SE dye where fluorescent emittance is negative. Figure 39 represents the experimental cells or those stained with CFDA-SE dye where fluorescent emittance is positive.

Negative control used for the optimisation of CFDA-SE staining in marrow MNCs.

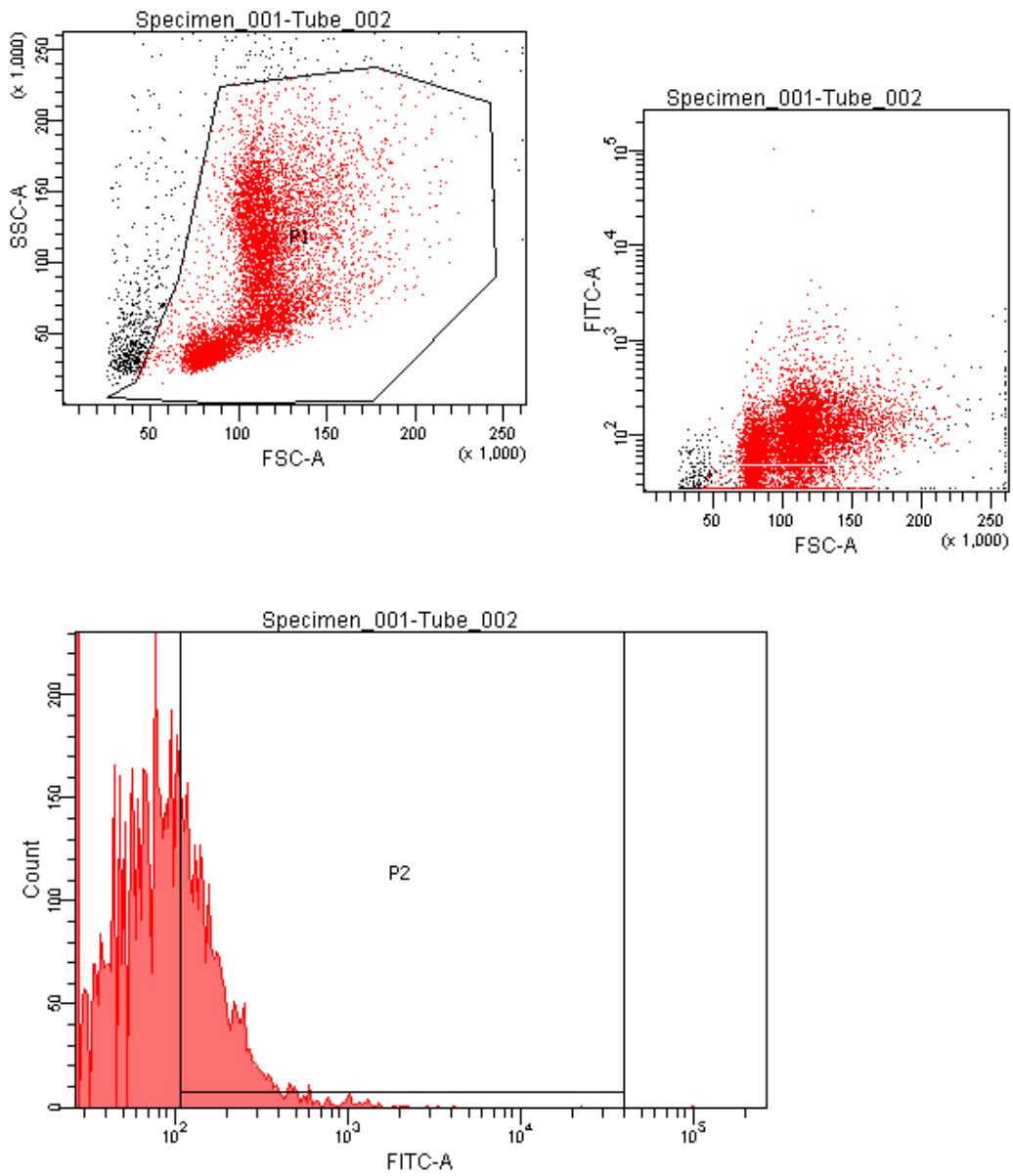


Figure 39: Negative control for carboxy-fluorescein succinimidyl ester (CFDA-SE) staining test. The peak to the left and entering P2 represents the population of test cells. FITC-A represents a wavelength of 450nm, where CFDA-SE positive cells are visible. As the cell population (red peak) is to the left these cells are negative.

Positive control used for the optimisation of CFDA-SE staining in marrow MNCs.

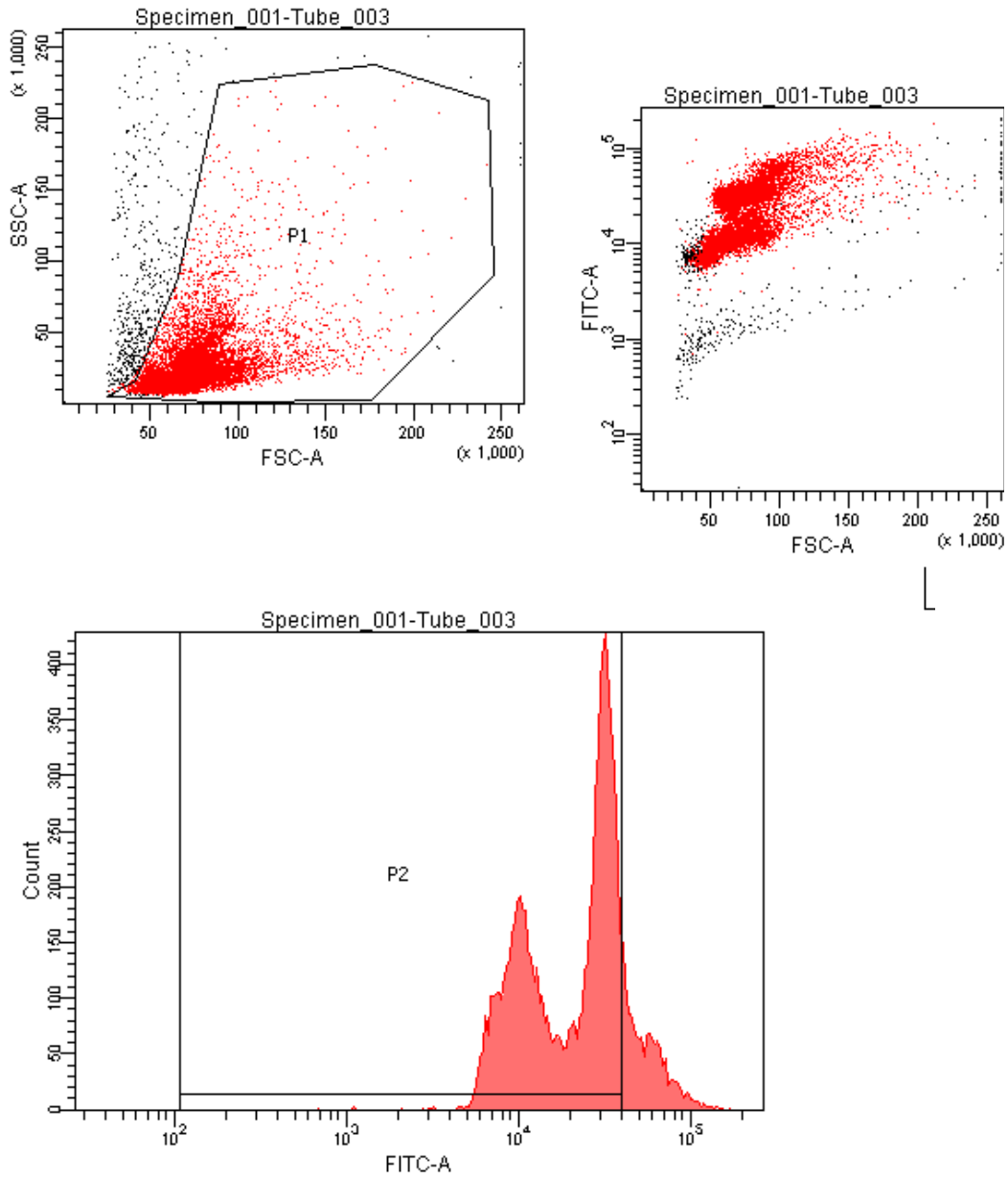


Figure 40: Positive control for CFDA-SE staining test. Peaks in P2 represent population of test cells. Two peaks represent different cell generations with the far right peak representing the parent cells with the left peak representing the daughter cells. FITC-A represents a wavelength of 450nm, where CFDA-SE positive cells are visible. As the cell population (red peak) is to the right these stained cells are positive for CFDA-SE staining indicating staining was successful.

As the optimisation of the CFDA staining proved successful it could be applied to the following study.

Mice

30 male C5BL/6 mice (8-9 weeks old) from the Jackson Laboratory (Bar Harbor Maine, U.S.A).

KSL isolation

KSL cells were isolated from donor mice using a similar method outlined in previous section 2.1. The KSL were set up in a 12 well transwell plate with 20,000 KSL/well and 20,000 HUBECs/well in a similar method to that outlined in section 3.1.2.

The experiment was set up in triplicate as follows;

KSL + TSF (1mL)	KSL+ HUBEC + ECCM (1mL)	KSL + HUBEC (ECCM 1mL) + 100ng/mL rPTN in TSF (1mL)	KSL + HUBEC (ECCM 1mL) + Anti-PTN in TSF (100ug/mL) (1mL) Anti-PTN final conc. 50ug/mL
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Table 14: Experiment lay-out to examine the homing effect of PTN on HSCs.

TSF: Thrombopoietin, stem cell factor and Flt 3 ligand (cytokine reagent).

rPTN: Recombinant Pleiotrophin.

HUBECs: Human Brain Endothelial cells.

KSL and progeny staining

Following seven days in culture the cells were collected from the wells into 15mL tubes. The wells were washed to ensure maximum cell collection and a cell count and viability analysis was performed as outlined in section 2.2. The cells were stained with CFDA-SE staining dye outlined in section 2.6.2.

Radiation

16 recipient C5BL/6 mice were exposed to a single fraction lethal dose of 850cGy using Cesium 137 irradiator as outlined in section 2.9.1.

Injections

Each lethally irradiated recipient mouse (n=4 per group) received 20,000 CFDA-SE stained treated KSL and progeny cells via tail vein injections (2.10.2).

Marrow collection

The recipient mice, injected with stained KSL and progeny cells were sacrificed 16 hours after transplantation and their marrows harvested. The mice were sacrificed using a similar method to that outlined in previous section 3.1.1. The femurs from these mice were flushed using 10% FBS and the bone marrow contents isolated. Following red cell lysis the cells were filtered and examined for CFDA positive cells within the marrow using Flow cytometry.

The CFDA positive cell percentage was determined for each mouse taking variabilities in cell counts from each mouse into account. The % CFDA positive cells were averaged for each group and there relative values outlined in Figure 41.

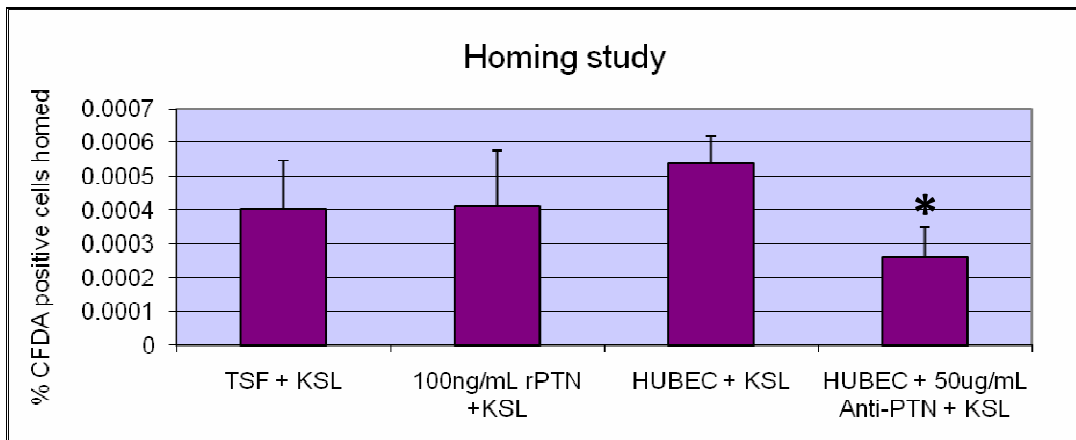


Figure 41: 20,000 KSL cells were cultured with TSF, 100ng/mL rPTN, HUBEC and HUBEC plus 50ng/mL Anti-PTN. Following 7 days in culture the cells were collected and stained with CFDA dye that fluoresces at 450nm. The cells and progeny were transplanted into lethally irradiated recipient mice. 16 hours consequent to this the mice were sacrificed and the bone marrow harvested. Following a red cell lysis, the marrow cells were filtered and run directly on the flow cytometer. The % CFDA positive cells homed to the marrow is outlined above. There is no significant difference in homing with those treated with TSF or rPTN. There is however a significant difference in homing between those that were treated with HUBECs and HUBECs plus Anti-PTN ($p= 0.001$).

3.2.2 To determine if rPTN can induce HSC regeneration post radiation injury in vivo by examining mature blood cell reconstitution and mouse survival.

3.2.2.1 This experiment was to examine if PTN could induce HSC regeneration post myelotoxic radiation injury

Mice

10 male C5BL/6 mice (8-9 weeks old) were from the Jackson Laboratory (Bar Harbor Maine, U.S.A).

Radiation

Radiation was achieved using Cesium 137 irradiator. 20 mice were exposed to a single fraction myelotoxic dose of 700cGy as outlined in section 2.9.

Injections

5 C5BL/6 mice were injected intraperitoneal (IP) with 100ng/g body weight or 2ug rPTN (appendix) for 7 consecutive days as 8-9 week old C5BL/6 mice weigh approximately 20g. 5 control mice were injected intraperitoneal (IP) with saline for 7 consecutive days as outlined in section 2.10.1

MNC

On day seven the mice were sacrificed firstly by CO₂ euthanasia followed by cervical dislocation. The femurs from these mice were flushed using 10% FBS and the bone marrow contents isolated. Following red cell lysis the cells were MNCs were counted using Trypan Blue exclusion as outlined in section 2.2. The MNC content would give an indication as to whole BM recovery.

KSL

A portion of the MNC cells were stained for KSL content and analysed using Flow cytometry outlined in section 2.6.1. The KSL content in the femurs represents haemopoetic progenitor recovery post radiation injury.

CFC

A colony forming cell assay was set up with MNCs isolated from the femurs of the rPTN and Saline treated mice. The CFC was set up using the procedure outlined in previous section 2.7.1. The CFC would indicate quantity of lineage committed progenitors between the rPTN and Saline treated groups.

LT-CIC

A long term colony initiating cell assay was set up with MNCs isolated from the femurs of the rPTN and Saline treated mice. The LT-CIC was set up using the procedure outlined in previous section 2.7.2. The LT-CIC would indicate the quantity of more primitive long-term haemopoetic lineage committed progenitors in the rPTN and Saline treated groups.

On day 7 of injections the femurs from these mice were collected and analysis for stem cell regeneration performed. To identify bone marrow recovery as a whole MNC counts were performed Table 15 and Figure 42. The MNC counts were performed by flushing the marrow from the femurs of the treated mice and separated them using the Lymphoprep technique. Trypan blue exclusion was the method by which the MNC count and viability was determined (Table 15).

Condition	MNC count/mouse
Saline	75,000
Saline	145,000
Saline	375,000
Saline	205,000
Saline	122,500
Average	184,500
100ng/g PTN	520,000
100ng/g PTN	415,000
100ng/g PTN	700,000
100ng/g PTN	300,000
100ng/g PTN	182,500
Average	423500

Table 15: MNC count. Irradiated mice were injected (IP) for seven consecutive days with Saline or 100ng/g body weight rPTN. The marrow from the femurs was flushed and the MNCs isolated using the Lymphoprep technique. The MNC count was achieved using Trypan Blue exclusion. Healthy mice have MNC counts of approximately 1×10^6 . The diminished MNC counts in these mice are a result of the myelotoxic dose of radiation. The results show that mice treated with 100ng/g PTN had a 2/3 fold higher MNC recovery when compared to Saline.

These results Table 15 when compiled into a bar graph appear as follows (Figure 42).

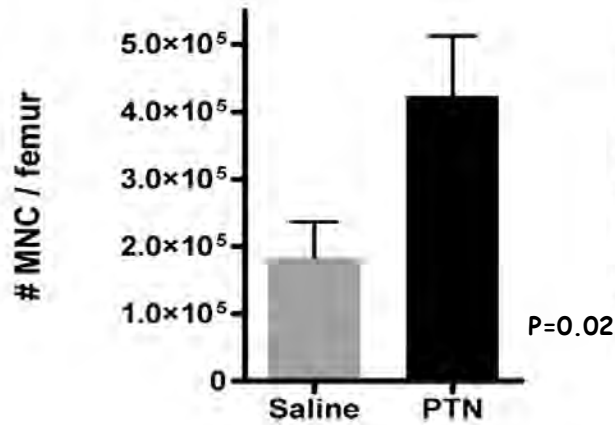


Figure 42: Mononuclear cell count in the femurs of mice that were exposed to a myelotoxic dose of radiation (700cGy) subsequent to which they received 100ng/g body weight rPTN or saline for seven consecutive days after which the MNC count was performed. (N=5/group)

To examine a possible difference in stem cell enriched population in the femurs of the mice in the saline treated and rPTN treated groups the KSL counts for each mouse was determined using Flow Cytometry.

Cells from each femur was analysed initially through forward and side scatter allowing the population be centered around a live population of cells where it was thought the KSL population exists, denoted P1 (not displayed in figures 43 or 44). Subsequently this population was narrowed further when the cells expressing antigens associated with lineage or maturity were excluded, allowing the Lineage negative population be analyzed, denoted in Figures 43 and 44 as P2. This Lineage negative population was then further categorized into a population of cells that were positive for both sca-1 and c-Kit denoted as P3 in Figures 43 and 44. The content of cells that displayed a phenotype that was negative for lineage markers and positive for the sca-1 and c-Kit antigens could be compared between the cultures treated with TSF (Figure 43) or rPTN (Figure 44)

KSL content in a lethally irradiated mouse treated with Saline for 7 consecutive days.

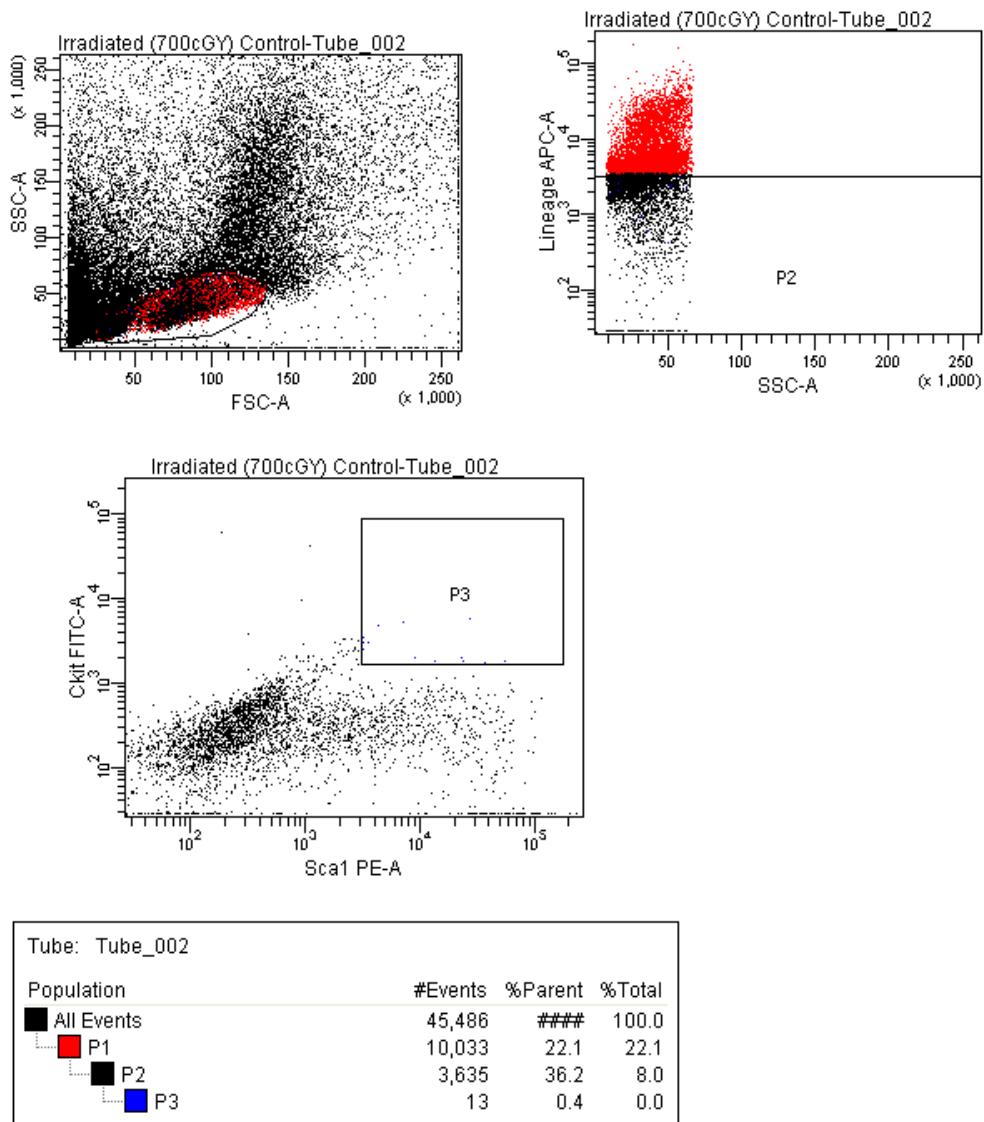


Figure 43: Flow cytometry report to determine the KSL population in an irradiated mouse treated for 7 consecutive days with saline. On day seven cells isolated from the femurs of these mice were stained with fluorescent labelled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P2 denotes a population of immature cells as these represent the cells that are negative for antigens associated with maturity or lineage. P3 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoetic stem cells are known to express these antigens. The KSL cell percentage within the Lineage negative population was 0.4 %.

KSL content in a lethally irradiated mouse treated with 100ng/g body weight rPTN for 7 consecutive days.

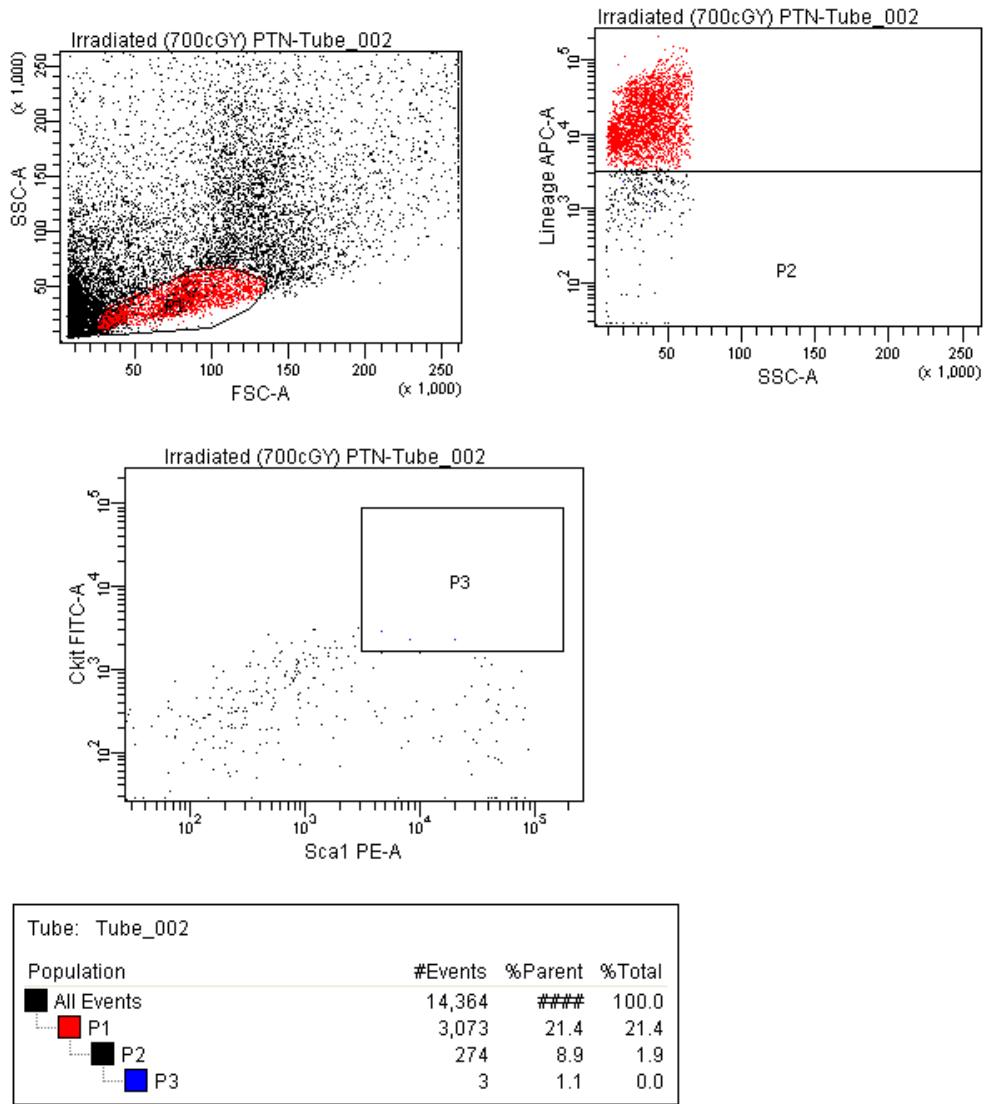


Figure 44: Flow cytometry report to determine the KSL population in an irradiated (700cGy) mouse treated for 7 consecutive days with 100ng/g body weight rPTN. On day seven cells were isolated from the femurs of these mice and stained with fluorescent labelled probes directed against antigens associated with maturity (Lineage) and Sca-1 and c-Kit. P2 denotes a population of immature cells as these represent the cells that are negative for antigens associated with maturity or lineage. P3 denotes a population of cells within the lineage negative or immature population that display the antigens sca-1 and c-Kit as the haemopoietic stem cells are known to express these antigens. The KSL cell percentage within the Lineage negative population was 1.1%.

KSL population percentages were determined using Flow cytometry within the TSF and rPTN treated groups (n=5 per group).

Condition	Cell count	% Lineage -	% Sca-1 + c-Kit	% KSL	# KSL
Saline	75,000	36.2	0.4	0.144	108.6
Saline	145,000	21.6	0.3	0.0648	93.96
Saline	375,000	5.3	0	0	0
Saline	205,000	6.2	0.5	0.031	63.55
Saline	122,500	9	0.7	0.063	77.18
PTN	520,000	8.9	1.1	0.0979	509.08
PTN	415,000	10.4	0	0	0
PTN	700,000	15.6	0.7	0.1092	764.4
PTN	300,000	7.2	1.8	0.1296	388.8
PTN	182,500	9.6	1.5	0.1344	245.28

Table 16: Table of raw data obtained from Flow cytometric report acquired from the analysis of mice injected for 7 days with Saline of 100ng/mL rPTN. The marrow cells were stained with Fluorescent labelled antibodies directed against the antigens Sca-1 and c-Kit and those associated with lineage. The % lineage negative represents the percentage lineage negative cells within the population of cells analysed that are negative for antigens associated with maturity. The % Sca-1 c-Kit is the percentage of cells that express both c-Kit and Sca-1 within the population of cells that are negative for antigens associated with Lineage or maturity. The % KSL is the number of cells within the cells analyzed that are negative for antigens associated with Lineage, are c-Kit positive and Sca-1 positive.

The KSL content within the marrow indicates haemopoietic progenitor recovery post radiation injury. The mice treated with rPTN displayed a 7/8 fold higher KSL count than the control treated group, indicating increased progenitor recovery with rPTN treatment. This data when displayed as a bar graph, figure 45, highlights the difference in haemopoietic progenitor recovery in the marrow as a whole between the saline and rPTN treated groups.

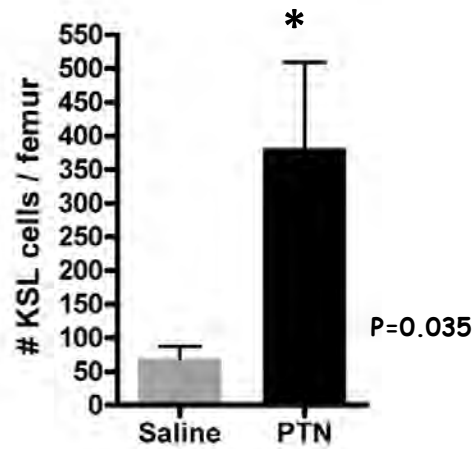


Figure 45: KSL count in the femurs of mice that were exposed to a myelotoxic dose of radiation (700cGy) subsequent to which they received 100ng/g body weight rPTN or saline for seven consecutive days after which the KSL count was performed.(N=5/group)

To determine the difference in lineage committed progenitors between the Saline and 100ng/g body weight rPTN treatment groups a Colony Forming Cell assay and a long-term colony initiating cell assay were set up on day 7. In the Colony forming cell assay, the MNC's from the marrow were added to a methocellulose medium which promotes proliferation and differentiation of lineage committed progenitors into colonies and can be quantified (Figure 46).

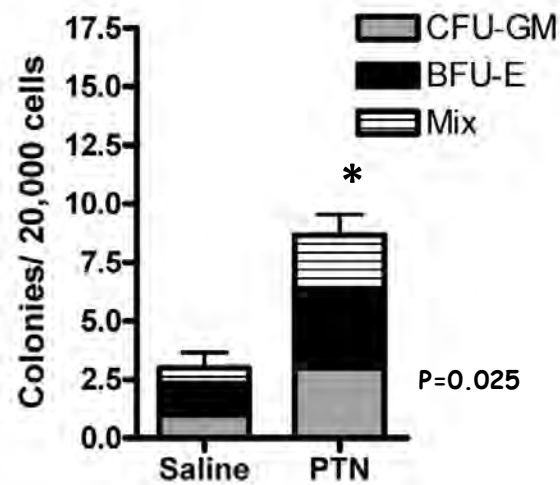


Figure 46: Colony forming cell assay from 20,000 MNCs from the femurs of mice that received a myelotoxic dose of radiation (700cGy) subsequent to which they received 100ng/g body weight rPTN or saline for seven consecutive days. The granulocyte/monocyte (GM) indicates cells that are of myeloid lineage. The burst forming unit-erythroid (BFU-E) indicates cells that are of erythroid lineage and Mix indicates a colony of cells that are of mixed myeloid lineage, granulocytic, myeloid and erythroid (CFU-GEMM). CFC assay outlines a higher number of lineage committed progenitors in erythroid and myeloid lineages in those that were treated with PTN when compared to the saline control indicating preservation of the progenitor cell content. (N=5/group)

To analyse the effect PTN had on more primitive progenitors, the MNC were added to a layer of murine BM stromal cells and incubated at 37°C with 5% CO₂ for four weeks. Following this the cells were added to the methocellulose medium for two weeks, again promoting proliferation and differentiation of lineage committed progenitors that could be quantified microscopically (Figure 47)

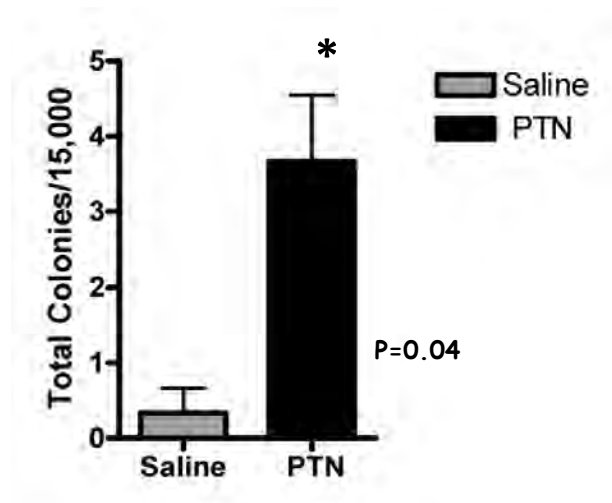


Figure 47: Long-term colony initiating cell assay of 15,000 MNCs from the femurs of mice that received a myelotoxic dose of radiation (700cGy) consequent to which they received 100ng/g body weight rPTN or saline for seven consecutive days. The LT-CIC assay outlines a higher number of long term lineage committed progenitors in those that were treated with PTN when compared to the saline control indicating an increase in the number of long-term colony initiating cells with rPTN. (N=5/group)

The six week assay highlighted an 11 fold increase in the long-term primitive progenitors capable of initiating colonies four weeks after isolation from the marrow in mice that received rPTN post radiation injury. This indicates that indicating PTN is not only a growth factor for stem cells in vitro but is a regenerative factor for stem cells in vivo.

3.2.2.2 This experiment is to test if rPTN provides radioprotection to HSCs in vivo prior to ionizing radiation exposure.

Mice

30 female Balb/c mice (8-9 weeks old) were from the Jackson Laboratory (Bar Harbor Maine, U.S.A).

Radiation

Radiation was achieved using Cesium 137 irradiator. The mice were exposed to a single fraction lethal dose of 850cGy (Borsotti, 2009) (Whartenby, 2002), indicated by Day 0. A lethal dose is one that results in 100% death by day 30 (Section 2.9).

Injections

15 Balb/c mice were injected intraperitoneal (IP) with 100ng/g body weight or 2ug rPTN (appendix) for 7 consecutive days as 8-9 week old Balb/c mice weigh approximately 20g. The control mice were injected intraperitoneal (IP) with saline for 7 consecutive days (Section 2.10.1). For this experiment injections were performed for 7 days prior to radiation exposure.

Survival

The mice were monitored for survival post radiation exposure (Day 0) over a 30 day period.

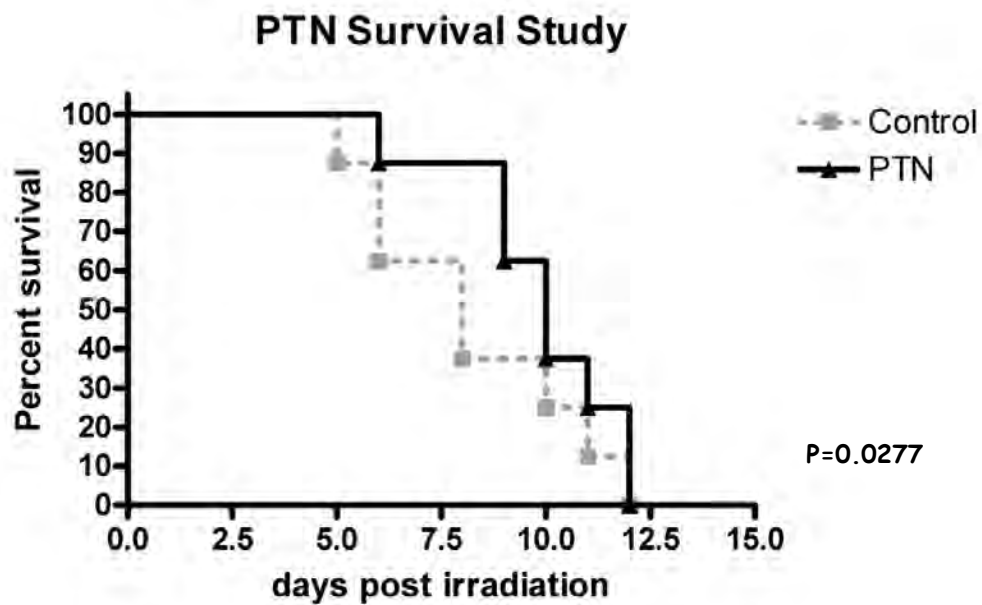


Figure 48 Survival study whereby lethally irradiated mice (850cGy) were injected (IP) with either 100ng/g body weight rPTN or saline for 7 days prior to radiation exposure. Survival was monitored from radiation exposure on Day 0. (N=15/group)

Treatment with rPTN post radiation injury did not enhance survival in lethally irradiated mice.

3.2.2.3 This experiment was to examine if rPTN is a reparative factor for HSCs in vivo following ionizing radiation exposure.

Mice

24 female Balb/c mice (8-9 weeks old) from the Jackson Laboratory (Bar Harbor Maine, U.S.A) were used.

Radiation

Radiation was achieved using Cesium 137 irradiator. The mice were exposed to a single fraction lethal dose of 850cGy (Borsotti, 2009) (Whartenby, 2002) indicated by Day 0 (Figure 49). A lethal dose is one that results in 100% death by day 30 and is outlined in Procedure 2.9.

Injections

12 Balb/c mice were injected intraperitoneal (IP) with 100ng/g body weight or 2ug rPTN (appendix) for 14 consecutive days as 8-9 week old Balb/c mice weigh approximately 20g. The control mice were injected intraperitoneal (IP) with saline for 5 consecutive days as outlined in section 2.10.1. For this experiment injections were performed for 5 days after radiation exposure (Day 0).

Survival

The mice were monitored for survival post radiation exposure (Day 0) over a 30 day period.

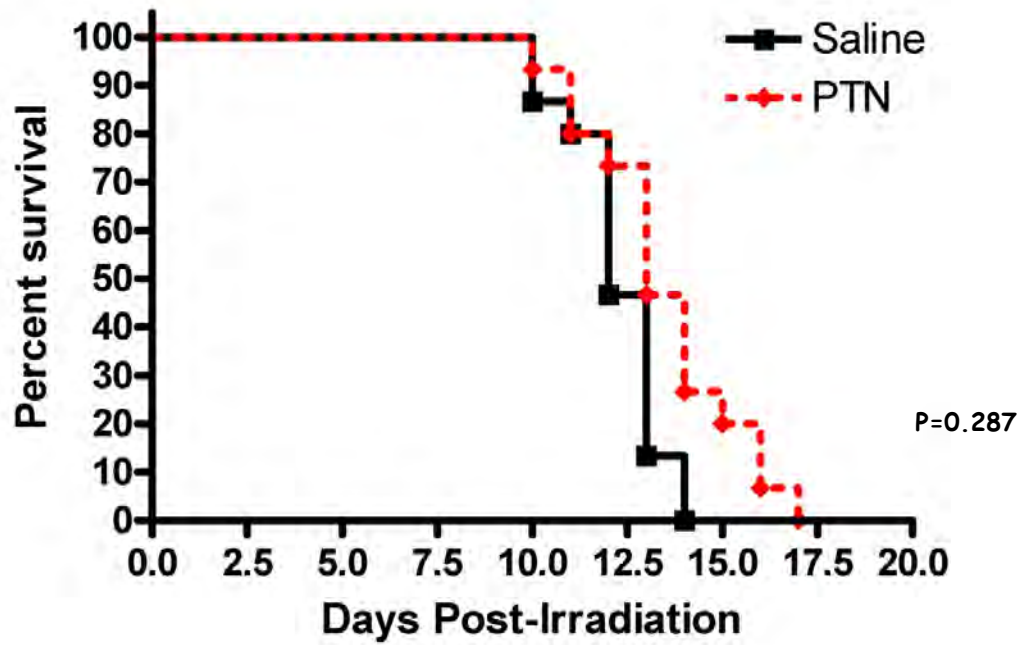


Figure 49: Mice (N=12/group) were injected (IP) for 5 consecutive days with either 100ng/g body weight rPTN or saline, exposed to lethal radiation (850cGy) and their survival monitored

Administration of PTN before radiation injury did not enhance survival of lethally irradiated mice also.

3.2.2.4 This experiment was to examine whether rPTN is a reparative factor for HSCs in vivo following ionizing radiation exposure via mature blood cell repopulation

Mice

24 female Balb/c mice (8-9 weeks old) were from the Jackson Laboratory (Bar Harbor Maine, U.S.A).

Radiation

Radiation was achieved using Cesium 137 irradiator. The mice were exposed to a single fraction sublethal dose of 550cGy. (Given, 1994), (Gong, 2005). A sub-lethal dose is where 100% of mice exposed are alive at day 30. (Section 2.9)

Injections

12 Balb/c mice were injected intraperitoneal (IP) with 100ng/g body weight or 2ug rPTN (appendix) for 14 consecutive days as 8-9 week old Balb/c mice weigh approximately 20g. The control mice were injected intraperitoneal (IP) with saline for 14 consecutive days as outlined in procedure 2.10.1.

Blood collection

On days 10, 14, 18, 22, 25 and 29 the 24 mice were bled using the submandibular bleeding technique (Golde W. T, 2005) and the blood collected into tubes containing EDTA outlined in section 2.11.1.

Blood analysis

Full blood counts were performed for each mouse on the Hemavet 950FS as outlined in section 2.11.2. Parameters measured included White cells (Figure 50), red cells (Figure 51), haemoglobin (Figure 52) and platelets (Figure 53).

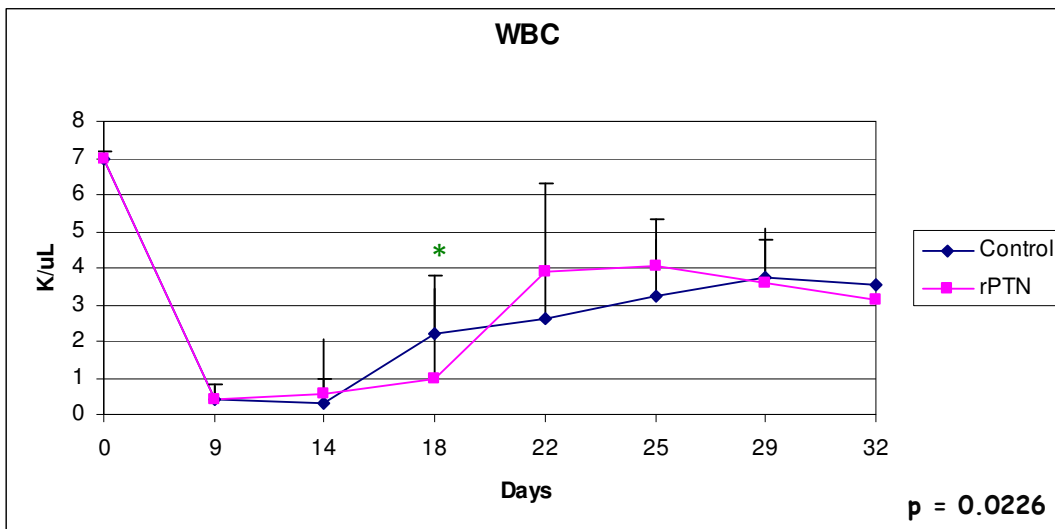


Figure 50: White cell count in sub lethally irradiated (550cGy) balb/c mice (n=12/group), treated fourteen days Day 0 – Day 14 with either saline (blue) or 100ng/g body weight rPTN (pink). No significant difference in white cell count between treated groups.

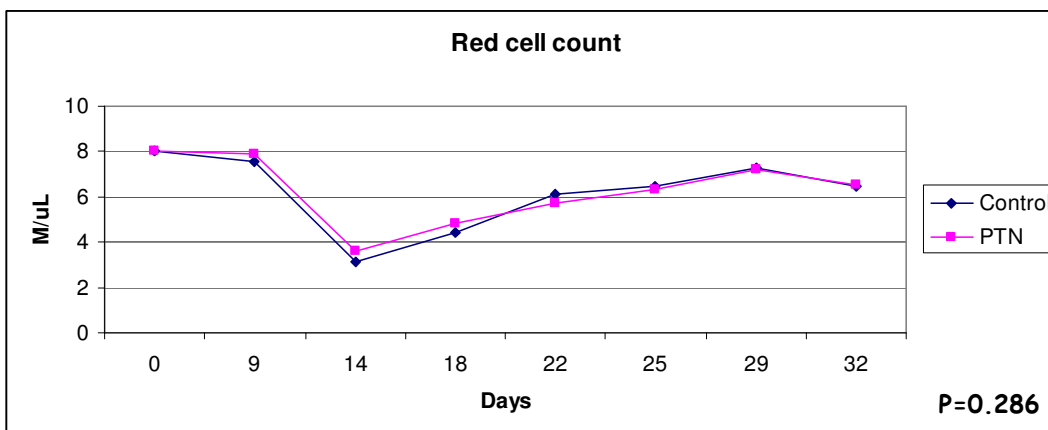


Figure 51: Red cell count in sub lethally irradiated (550cGy) balb/c mice (n=12/group), treated for fourteen days Day 0 – Day 14 with either saline (blue) or 100ng/g body weight rPTN (pink). No significant difference in white cell count between treated groups.

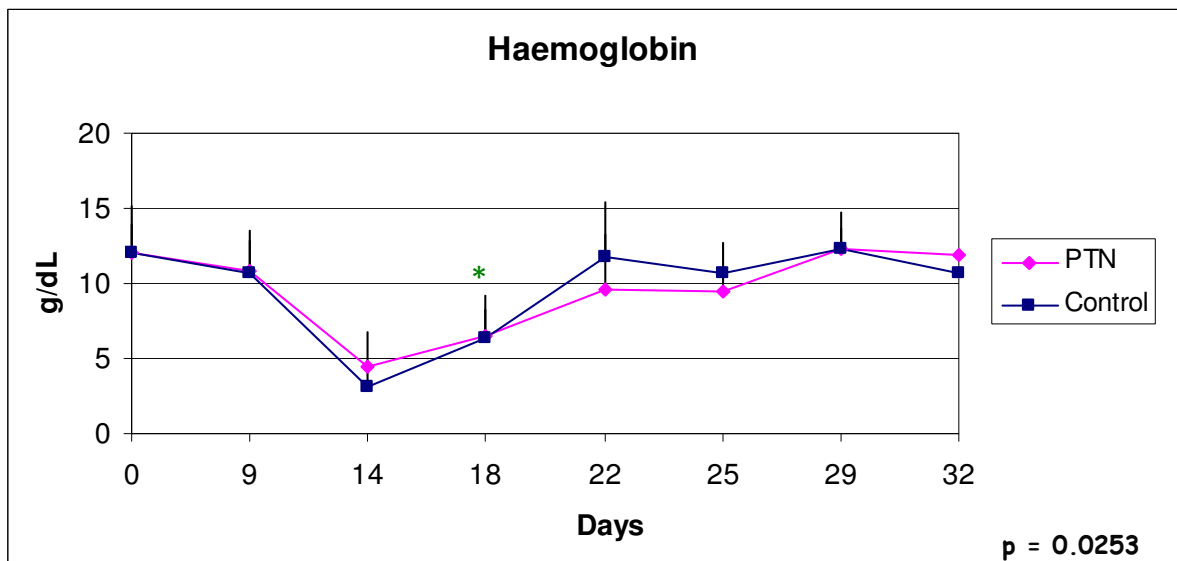


Figure 52: Haemoglobin values in sub lethally irradiated (550cGy) balb/c mice (n=12/group), treated fourteen days Day 0 – Day 14 with either saline (blue) or 100ng/g body weight rPTN (pink). No significant difference in haemoglobin values between experimental groups.

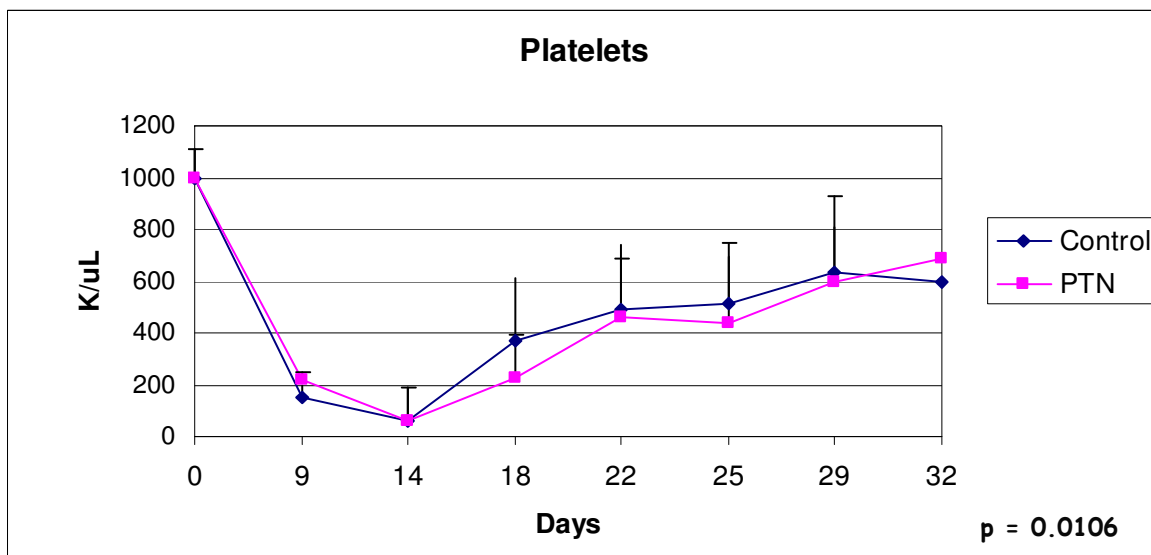


Figure 53: Platelet count in sub lethally irradiated (550cGy) balb/c mice (n=12/group), treated fourteen days Day 0 – Day 14 with either saline (blue) or 100ng/g body weight rPTN (pink). No significant difference in platelet count between treated groups

The full blood counts indicate that although there is an increase in stem/progenitor cells in the marrow, it is not reflected in the mature blood cells of the peripheral blood.

3.2.3 This experiment was to examine could PTN induce marrow toxicity with prolonged treatment (Experimental design).

To investigate if treatment with rPTN resulted in histological damage in the marrow spaces by PTN induced toxicity 8-9 week old healthy Balb/c mice were treated with the same dose and injection schedule used for the mature blood cell reconstitution study 3.2.2.4. On day 7 and 14 femurs from these mice were isolated, paraffin embedded, stained with H&E and examined for marrow structure and overall architecture (Figure 54).

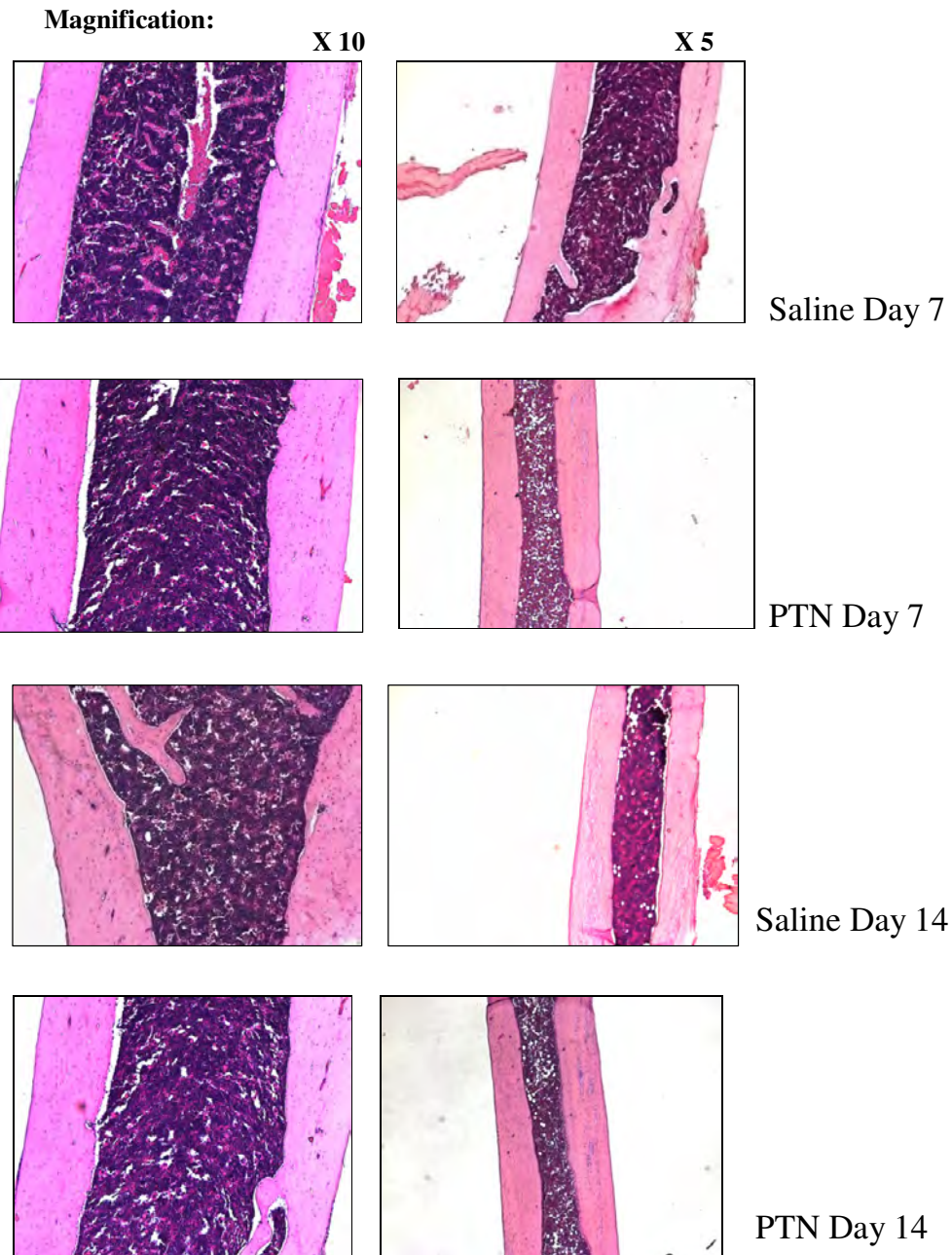


Figure 54: H&E stained femurs (x5 and x10 magnification) from healthy Balb/c mice (n=2/group) treated for either 7/14 days with either rPTN or Saline.

The haematoxylin and eosin stained femurs in figure 48 display no significant differences in architecture between the saline and the rPTN treated groups indicating that the failure of mature blood cell reconstitution with rPTN was not due to PTN induced toxicity.

3.2.4 This experiment is to examine if *ptn* is up-regulated in the marrow in response to radiation injury.

Mice

6 female Balb/c mice (8-9 weeks old) from the Jackson Laboratory (Bar Harbor Maine, U.S.A) were used.

Radiation

Radiation was achieved using Cesium 137 irradiator. The mice were exposed to a single fraction lethal dose of 850cGy. A lethal dose is where 100% of mice exposed are deceased at day 30. (Section 2.9)

Bone Marrow collection

The mice were sacrificed by CO₂ euthanasia and cervical dislocation. The femurs and tibia from each of the mice were dissected and flushed as outlined in section 2.1. Two mice were sacrificed at 6 and 24 hours post radiation injury, while two mice received no radiation.

MNC isolation

MNCs were isolated from the marrow using the lymphoprep technique outlined in section 2.3.1.

Real-Time PCR

RNA was isolated from the marrow MNCs using the technique outlined in section 2.3.2. The RNA was quantified and reverse transcribed to cDNA using the methods outlined in sections 2.3.3 and 2.3.5. The cDNA was amplified and compared to the house-keeping gene (Section 2.3.6).

To determine active transcription of the *ptn* gene post radiation exposure a two step real-time polymerase chain reaction was performed whereby isolated RNA is reverse transcribed to cDNA and the cDNA is then amplified using the Real-time PCR method and quantified at 6 and 24 hours post injury. The RNA isolated from the marrow MNCs for each mouse at the various time points were quantitated using the Nanodrop and are outlined in Table 17.

Condition	ng/uL RNA isolated
Non-irradiated	207.8
Non-irradiated	101.2
6 hrs post radiation	81.0
6 hrs post radiation	138.9
24 hrs post radiation	58.9
24 hrs post radiation	62.5

Table 17: RNA quantitation using the Nanodrop. Marrow MNCs isolated from the femurs of mice exposed to no radiation, 6hrs post radiation exposure and 24hrs post radiation exposure.

The amount of RNA isolated from the marrow of the treated mice decreases with time post radiation injury reflecting marrow destruction and cell damage. After RNA isolation the RNA was reverse transcribed to cDNA and amplified to quantifiable levels using real-time PCR. The levels of cDNA obtained were quantified using the comparative threshold method against the house-keeping gene GAPDH. The results when compiled into a bar graph are outlined in Figure 55.

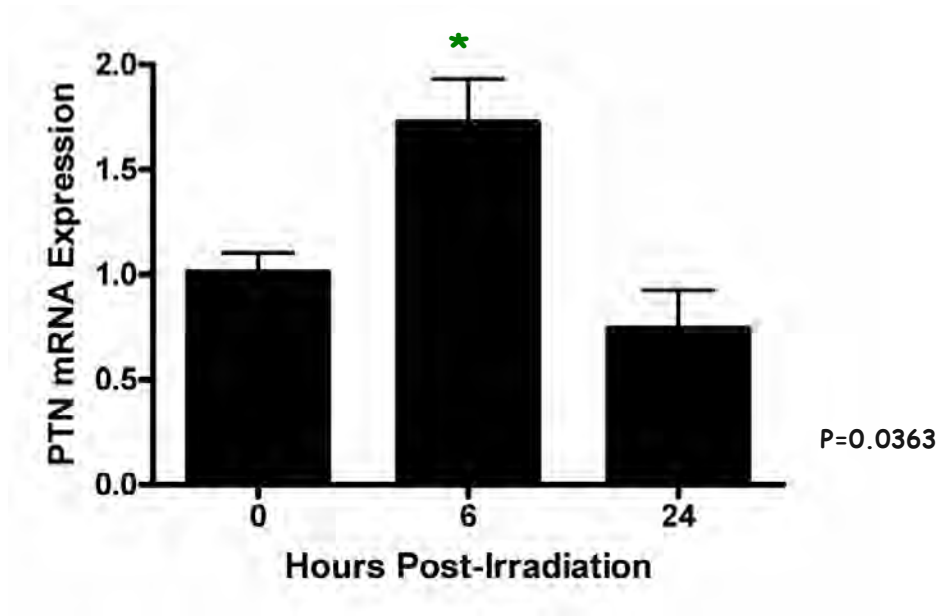


Figure 55: PTN mitigation study. One group of mice were exposed to no radiation (indicated 0) (n=2 per group) and another 2 groups were exposed to a lethal dose of radiation (850cGy) 6 and 24 hours post radiation injury. To determine active transcription of the *ptn* gene post radiation exposure a two step real-time polymerase chain reaction was performed whereby isolated RNA is reverse transcribed to cDNA and the cDNA is then amplified using the Real-time PCR method. Quantification of *ptn* expression between the treated groups was determined using the comparative threshold method relative to the house keeping gene GAPDH.

The Real-time PCR bar graph (Figure 55) highlights PTN as a possible autocrine factor, released by MNCs within the marrow to act on the marrow in order to mitigate marrow recovery post radiation injury. The *ptn* gene appears to be down regulated 24 hours post radiation injury suggesting PTN in its role as a mitigator post radiation injury is immediate and short-lived.

4.0 Discussion

Pleiotrophin is a 17kDa protein which is a member of the Heparin Binding Growth Factor Family (HBGF) (Milner 1989). It is expressed highly expressed during embryogenesis (Bloch 1992), but is however limited in the healthy adult (Yeh, 1998). It has a myriad of functions which include mitogenesis (Courty 1991), angiogenesis (Yeh, 1998) and promotion of neurite outgrowth (Rauvala, 1989). PTN has also been described as having oncogenic properties as increased PTN expression is noted in various cancers such as lung (Garver 1993), breast (Garver 1994) and melanoma (Chen 2007).

Endothelial cells, as part of the vascular niche have been shown to provide support to the HSC both in vitro (Chute, 2002) and in vivo (Muramoto, 2006 and Salter, 2009). Chute et al describe Human Brain Endothelial Cells as providing a supportive role for HSCs compared to endothelial cells of alternative origins in both direct and interestingly in-direct contact (Chute 2002) suggesting a factor released from the HUBECs is regulating the HSCs in some way. Further to these studies Chute outlines a molecular profile for novel endothelial cell derived growth factors released from the HUBECs which is lacking in endothelial cells of other origins which regulate haemopoiesis using microarray analysis (Chute, 2006). Additional microarray, quantitative real-time PCR and Elisa studies performed in the Chute laboratory highlighted Pleiotrophin as candidate growth factor for HSCs (Himburg, accepted for publication *Nature Medicine* 2010). From this Pleiotrophin was investigated as a regulatory factor for HSCs in the absence of HUBECs whereby HSCs were cultured with rPTN in vitro and transplanted into irradiated recipients and showed to have increased numbers of HSC engraftment to the marrow of these cells using competitive repopulating assays (Himburg, accepted for publication *Nature Medicine* 2010). To further support this hypothesis Himburg displayed decreased HSC engraftment of HSCs treated with HUBECs and Anti-PTN, thus blocking any PTN signalling from the HUBECs further highlighting PTN as a regulatory factor for HSCs.

This research project aimed to investigate Pleiotrophin as regenerative factor for HSCs post radiation injury using both in vitro and in vivo methods. In order to study this we firstly examined the ability of Pleiotrophin to cause the expansion of HSCs in vitro post radiation injury. This was followed by a study which examined PTN signalling as a requirement for HUBEC-mediated expansion and recovery of HSCs post radiation injury in vitro. We also examined the ability of Pleiotrophin to cause the expansion of HSCs in vivo post radiation injury by examining MNC content, KSL cell content and Lineage committed cell content. We examined the effect of PTN on mature blood cell reconstitution and overall survival of the mice pre and post radiation exposure. The homing effect of PTN on HSCs was also investigated as too was cell cycle analysis of marrow cells post PTN exposure. The *ptn* gene was also examined to determine its regulation in the marrow immediately post radiation exposure.

In order to investigate the ability of Pleiotrophin to cause the expansion of HSCs in vitro post radiation injury rPTN was added to irradiated HSCs at two concentrations, 100 and 500ng/mL and incubated at 37°C for seven days. On day seven the wells containing cells at all stages of differentiation including stem cells, stem cell progeny, lineage committed progenitors and those that had fully matured were counted. The cell count in the culture treated with 100ng/mL rPTN was modestly increased over those treated with 500ng/ml despite there being no statistical significance. To identify the stem cell enriched population within the cultures Flow cytometry was used and again a modest increase in the KSL content within the 100ng/ml rPTN treated group was noted over the 500ng/ml rPTN and TSF treated groups, suggesting KSL self-renewal. To identify cells within these cultures that may have committed to a particular lineage with differentiation, colony forming cell assays were performed. The colony forming cell assay outlined less lineage committed progenitors in cultures treated with 100ng/ml rPTN when compared to cultures treated with 500ng/ml rPTN or TSF, the decrease in lineage committed progenitors may be due to KSL self-renewal or a stop in differentiation. The decrease found in CFC colonies may also be due to the HSC differentiation promotion through the colony forming stage to fully mature blood cell. The increase in KSL content within the 100ng/ml rPTN treated groups however suggests one of the former theories. The experiment suggests 100ng/ml rPTN treatment of radiated KSL cells results in the increase of KSL cells.

To determine if PTN signalling was necessary for HUBEC mediated expansion and recovery of HSCs in vitro radiated KSL cells were exposed to HUBECs and Anti-PTN. The Anti-PTN aimed to block PTN signalling from the HUBECs to the KSL cells to answer this question.

The cell count reflected the entire cell population in the well including differentiated and undifferentiated cell types. These include the stem cells, stem cell progeny, lineage committed progenitors and cells at all stages of differentiation including those that have fully matured. There was a significant difference in the total cell counts between the different treatment conditions with more cells in the HUBEC alone treated cells ($p=0.0307$) when compared to TSF alone. Treatment with HUBECs and Anti-PTN, although not statistically significant, is border-line with a P value of 0.050 when compared to TSF alone. To identify the specific stem cell enriched population within the cultures, the KSL content was determined using flow cytometry. The results however are conflicting as one may anticipate that with increased KSL that there is increased self-renewal and therefore less differentiation.

There were many more KSL cells in the HUBEC and Anti-PTN treated group when compared to TSF ($p=0.0072$) and the HUBECs alone ($p=0.009$). This data suggests that when KSL cells are set up in culture with HUBECs and PTN is blocked using Anti-PTN that there is an increase in the KSL content within the cultures, therefore indicating that PTN is not required for HUBEC mediated expansion of stem cells in culture. To identify the lineage committed progenitors in the colonies, a colony forming cell assay was performed. The HUBEC alone treated group has significantly less colonies/500 cells when compared to TSF ($p=0.021$). Cells treated with HUBEC and 50ng/mL Anti-PTN has significantly higher colony forming cells when compared to HUBEC alone ($p=0.025$). TSF versus the HUBECs is however not significant ($p=0.08$). This result suggests the KSL cells that were treated with the HUBECs have less lineage committed progenitors as there are less colonies formed. The less colonies is suggestive of a number of possibilities, one being there are less lineage committed progenitors in the HUBEC treated groups, which may be explained by either a stop in differentiation of the KSL cells or that they have entered self renewal or both. Another explanation for this result is

those treated with HUBECs alone may have differentiated so far that more mature cells exist and are passed the colony forming stage. The results also suggested that when PTN (thought to be released from HUBECs as a soluble factor) is blocked through Anti-PTN that there are more lineage committed progenitors suggesting the cells are progressing in development to the lineage committed cells and are therefore no longer multi-potent cells. This CFC result agrees with previous studies with PTN acting as a self-renewal factor, the cells when treated with HUBECs and PTN signalling blocked multi-potent progenitors are fewer and lineage committed progenitors greater. The increased KSL content within the HUBEC and Anti-PTN treated cultures remains a cause for investigation. Perhaps when PTN is blocked another gene within the HUBECs is upregulated in response to the blocking, that the HUBECs are themselves responding, perhaps through the up-regulation of another gene, the protein of which is a potential HSC growth factor resulting in an increase in stem/progenitor cells. Also KSL cells, although highly enriched for HSCs are not a pure population of stem cells, that perhaps the increase of cells within the cultures is due to a cell that has the KSL phenotype, but is not a stem cell. Previous experiments in the Chute lab noted a similar response when non-irradiated KSL cells were set up in culture with HUBECs, TSF and HUBECs with Anti-PTN (50ng/mL), that being a significant increase in the KSL% in the cultures where PTN activity of HUBECs was blocked and also an increase in lineage committed progenitors, determined through the Colony forming Cell Assay

To investigate if PTN arrests HSC differentiation cell cycle analysis using flow cytometry was performed. The cells were treated with TSF and 100ng rPTN in vitro and any differences in cell cycle investigated in the KSL population and Lineage negative populations within the cultures. No significant difference existed between the rPTN and the TSF treated groups in the percentage of cells in the G₂/S/M phases of the cell cycle in the lineage negative population. The lineage negative population examines a broad population of immature cells, however to examine a population enriched for stem cells, cells that are within the lineage negative population were classified into those that express c-Kit and Sca-1 antigens was performed. There was no significant difference in the KSL percentage of cells in the different phases of the cell cycle in the treated cultures however there is a very modest increase in the percentage of cells within the G₀ phase of the cell cycle in the cultures

treated with 100ng/mL rPTN. There is a modest increase in the percentage KSL cells within the G1 phase of the cell cycle in cultures treated with rPTN when compared to TSF treated cultures. There is also a moderate decrease in the percentage of KSL cells in the G2/S/M phases of the cell cycle between the rPTN and the TSF treated groups. These results indicate no difference in the number of cells that are quiescent or in G0 between the TSF and rPTN treated groups within the KSL population indicating no stop in differentiation within this population between the TSF and rPTN treated groups. Interestingly however there is a difference in the amount of cells that are in the G0 phase of the cells cycle between the TSF and rPTN treated cells within the lineage negative population indicating rPTN induces modest amounts of cell quiescence within the lineage negative or immature population.

The first in vivo experiment performed investigated the homing effect of rPTN on KSL cells. This experiment hoped to provide support to previous studies in the Chute laboratory indicating PTN as a growth factor capable of inducing stem cell self renewal. It also aimed to provide some answers as to the capabilities of the KSL cells within the HUBEC and Anti-PTN treated cultures. The homing study results indicated two things, firstly, when KSL cells were treated with HUBECs plus Anti-PTN (50ng/mL), there was a decrease in homing to the marrow. This may lend itself as an explanation therefore as to why Dr. Himburg noted a decrease in engraftment with these cells when transplanted into lethally irradiated mice. That perhaps there may be an increase in multi-potent progenitors when PTN released from the HUBECs is blocked but that they do not home to the marrow efficiently thus resulting in a failure of these cells to engraft and re-populate the irradiated mice in the initial Competitive re-populating study, which examines homing, engraftment and cell repopulation.

Secondly, when KSL cells are treated with TSF and TSF plus 100ng/mL rPTN there was no increase in homing to the marrow with one over the other. This indicates the increased engraftment observed with the addition of rPTN to the KSL cultures is due in fact to an increase in multi-potent progenitors, and not due to an increased ability to home. Some may argue that the increased engraftment observed by Dr. Himburg may have been due to an increase in homing perhaps through the addition of an adhesion molecule such as CXCR4 and not necessarily due to an increase in multi-

potent cells with the ability to home, engraft and repopulate. This study provides further evidence to the hypothesis of PTN as a novel growth factor for HSCs.

The next study aimed to determine if PTN could induce HSC regeneration post myelotoxic injury in vivo, in light of the encouraging in vitro results. Mice received a myelotoxic dose of radiation, after which they were treated with either 100ng/g body weight rPTN or Saline. The marrow from these mice were then collected and examined for mononuclear and KSL cell content. The MNC count indicates broadly whole bone marrow recovery. The mice treated with rPTN, post a myelotoxic dose of radiation displayed a 2/3 fold higher MNC count than the control treated group, indicating increased marrow recovery with rPTN treatment highlighting the difference in BM cellularity as a whole between the saline and rPTN treated groups. To examine haemopoietic progenitor recovery post radiation injury in the mice the KSL content was examined using Flow cytometry. The mice treated with rPTN displayed a 7/8 fold higher KSL count than the control group indicating PTN was responsible for haemopoietic progenitor recovery post radiation injury. To determine the lineage committed progenitors between the rPTN and saline treated groups a CFC assay was performed. The CFC assay outlines a higher number of lineage committed progenitors in erythroid, myeloid and mixed lineages in those that were treated with PTN when compared to the saline control group indicating preservation of the lineage progenitors in the rPTN treated group post radiation injury. To analyse the effect of rPTN on more primitive long term progenitors a long-term colony initiating cell assay was performed. This assay highlighted an 11 fold increase in the long-term primitive progenitors post radiation injury in mice that received rPTN compared to the control group. This study indicates that PTN is not only a growth factor for stem cells in vitro but is a regenerative factor for stem cells in vivo.

To investigate if this increase in primitive long and short term progenitors with increased lineage committed progenitors is transferable clinically two survival studies were performed. For these studies 100ng/g body weight of rPTN was administered before and after radiation exposure, it was found the rPTN did not enhance survival of these lethally irradiated mice. As survival is directly correlated to

the reconstitution of mature blood cells a study to examine mature blood cell reconstitution with rPTN was performed. The parameters examined included white cells, red cells platelets and Haemoglobin. The full blood counts when analysed displayed no increase in circulating mature blood cells in mice treated with rPTN compared to the control group indicating although there is an increase in stem/progenitor cells within mice treated with rPTN that it is not reflected in the mature blood cells, that a disconnect exists between repopulation and regeneration of the marrow and the lack of mature cells in the peripheral blood.

To investigate if the difference in between the marrow repopulation and mature blood cell reconstitution studies was due to the prolonged treatment regimes in the reconstitution study a further study to examine if prolonged treatment with rPTN caused damage to the marrow in vivo. Mice were treated with the same dose of as that in the stem cell/progenitor repopulation study and also the mature blood cell reconstitution study. The femurs were collected and the marrow stained with haematoxylin and eosin to determine histologically if any had damage occurred to the bone marrow. No damage between the saline and treatment group was noted indicating the results observed were valid, not due to rPTN induced toxicity.

Finally to investigate PTN as a mechanism used by the body to mitigate or relieve radiation damage incurred post radiation injury naturally. We investigated MNCs of the marrow to determine if the *ptn* gene is up-regulated in the marrow in response to radiation damage and if so was it immediately or was it a more delayed response. A sharp increase in the active transcription of the *ptn* gene was noted in the MNCs of the marrow six hours after radiation injury occurred highlighting PTN as a possible autocrine factor released by MNCs of the marrow to act on the marrow to mitigate marrow recovery.

Conclusions

PTN is a self renewal factor for stem/progenitor cells in vitro and is a regenerative factor for stem cells in vivo. PTN activity however is localised to the HSC compartment highlighted by the failure to reconstitute circulating mature blood cells and in turn the failure to prolong survival of lethally irradiated mice when administered before or after radiation injury.

Future directions for this study would include the administration of PTN and GM-CSF post radiation injury to aid increase survival and mature blood cell reconstitution. The PTN would regenerate the stem/progenitor cells and preserve the lineage committed cells while the GM-CSF would aim to elevate the disconnect between the marrow and mature cells in the peripheral blood by promoting the differentiation of the granulocyte and monocyte lineage committed cells.

Another future direction for PTN would be to study if its use in expanding HSCs in vivo with the goal of generating more cells for harvest for use in bone marrow transplants. PTN would increase the stem cell content in the marrow, so treatment with a mobilising agent such as G-CSF or AMD3100 would lead to a more fruitful stem cell harvest with a corresponding increase in transplanted and engrafted cells.

5.0 References

- Abkowitz J.L., Robinson A.E, Kale S, Long M.W, Chen J. Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. *Blood*, 15 August 2003, Vol. 102, No. 4, pp. 1249-1253
- Aihara Y, Buhning H.J, Aihara M, Klein J. An attempt to produce 'pre T' cell hybridomas and to identify their antigens. *European Journal of Immunology* Vol 16 Issue 11 1391-1399, 1986.
- Aiuti A, Webb I.J, Bleul C, Springer T, Gutierrez-Ramos J.C. The Chemokine SDF-1 Is a Chemoattractant for Human CD34⁺Hematopoietic Progenitor Cells and Provides a New Mechanism to Explain the Mobilization of CD34⁺ Progenitors to Peripheral Blood. *J. Exp. Med* Volume 185 January 1997 111-120
- Allen.D, Dexter T.M, The essential cells of the hemopoietic microenvironment. *Experimental Hematology* 1984, Vol 12 no.7 517-521.
- Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh G.Y, Suda T. Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche. *Cell* Vol 118 Issue 2 149-162 2004.
- Arai F. Regulation of hematopoietic stem cells by the niche. *Trends in Cardiovascular medicine*. Vol 15 Issue 2 2005 75-79.
- Arai F. Regulation of haemopoietic stem cells and interactions with stem cell niche. *J.Oral Biosci.* 48(1): 22-29, 2006
- Asahara T, Murohara T, Sullivan A, Silver M, Van Der Zee R, Li T, Witzenbichler B, Schatteman G, Isner J.M. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis *Science* 14 February 1997: Vol. 275. no. 5302, pp. 964 - 966
- Ashman L.K. Review. The biology of stem cell factor and its receptor C-Kit. *The International Journal of Biochemistry & Cell Biology* 31 (1999) 1037±1051
- Askenasy N, Zorina T, Farkas D.L, Shalit I. Transplanted hemopoietic cells seed in clusters in recipient bone marrow in vivo. *Stem Cells* 2002; 20(4):301-10.
- Avecilla S.T, Hattori K, Heissig B, Tejada R, Liao F, Shido K, Jin DK, Dias S, Zhang F, Hartman TE, Hackett NR, Crystal RG, Witte L, Hicklin D.J, Bohlen P, Eaton D, Lyden D. De Sauvage F, Rafii S. Chemokine mediated interaction of haemopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature Medicine* 10: 64-71, 2004.
- Azzam H. S, Grinberg A, Lui K, Shen H, Shores E.W, Love P.E. CD5 Expression Is Developmentally Regulated By T Cell Receptor (TCR) Signals and TCR Avidity. *J. Exp. Med.*, Volume 188, Number 12, December 21, 1998 2301-2311
- Barlogie B, Shaughnessy J, Tricot G, Jacobson J, Zangari M, Anaissie E, Walker R, Crowley J. Treatment of Multiple Myeloma. *Blood*, 1 January 2004, Vol. 103, No. 1, pp. 20-32.
- Bellantuono I., Haemopoietic stem cells. *The International Journal of Biochemistry & Cell Biology* 36 (2004) 607–620
- Bergonie J, Tribondeau L, Interpretation de quelques resultats de la radiotherapie et ssai de fixation d'une technique rationelle. *Compt Rend Acad Sci*, 1906 143 pp. 983-985 1906

Berenson R.J, Bensinger W.I, Hill, R.S, Andrews R.G, Garcia-Lopez J, Kalamaz D.F, Still B.J, Spitzer G, Buckner C.D, Bernstein I.D. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. *Blood* Volume 77, Issue 8, pp. 1717-1722, 1991.

Bhanot P, Brink M, Samos C.H, Hsieh J.C, Wang Y, Macke J.P, Andrew D, Nathans J, Nusse R. A new member of the Frizzled Family from *Drosophila* functions as a wingless receptor. *Nature* 382(6588):225-30. 1996.

Bhatia M, Bonnet D, Kapp U, Wang J.C.Y, Murdoch B, Dick J.E. Quantitative Analysis Reveals Expansion of Human Hematopoietic Repopulating Cells After Short-term Ex Vivo Culture. *J. Exp. Med.* Volume 186, Number 4, August 18, 1997 619-624

Bhatia M, Bonnet D, Murdoch B, Gan O.I, Dick J.E. A newly discovered class of human hematopoietic cells with SCID repopulating activity. *Nature Medicine* 4 1038-1045 1998.

Blank U, Karlsson G.O, Karlsson S. Signaling pathways governing stem-cell fate. *Blood*, 15 January 2008. Volume 111, Number 2.

Bloch B, Normand E, Kovesdi I, Bohlen P. Expression of the Heparin Binding neurite promoting Growth Factor gene in the brain of fetal, neonatal and adult rat: an in-situ hybridization study. *Brain research. Developmental brain research.* 1992 Dec 18;70 (2):267-78

Blume KG, Beutler E, Bross K.J, Chillar R.K, Ellington O.B, Fahey J.L, Farbstein M.J, Forman S.J, Schmidt G.M, Scott E.P, Spruce W.E, Turner M.A, and Wolf J.L. Bone-marrow ablation and allogeneic marrow transplantation in acute leukemia. *The New England Journal Of Medicine.* Vol 302: 1041-1046 19 1980

Bonnet D. Biology of human bone marrow stem cells. *Clin Exp Med* (2003) 3:140–149

Borsotti C, Franklin A.R.K, Lu S.X, Kim T.D, Smith O.M, Suh D, King C.G, Chow A, Liu C, Alpdogan O, van den Brink M.R.M. Absence of donor T-cell-derived soluble TNF decreases graft-versus-host disease without impairing graft-versus-tumor activity *Blood*, 15 July 2007 Volume 110, Number 2.

Bosma M.J. Carroll A.M. The SCID mouse mutant; Definition characterisation and potential uses. *Annual review of Immunology.* Vol 9 323-350. 1991

Bradford G.B, Williams B, Rossi R, Bertoncello I. Quiescence, cycling and turnover in the primitive hematopoietic stem cell compartment. *Experimental Haematology* 1997 May ;25 (5):445-53.

Broudy V.C. Stem Cell Factor and Hematopoiesis. *Blood*, Vol 90, No 4 (August 15), 1997: pp 1345-1364

Brown J, Greaves M.F, Molgaard H.V. The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts. *International Immunology*, Vol. 3, No. 2, pp. 175-184, February 1991.

Broxmeyer H.E, Orschell C.M, Clapp D.W, Hangoc G, Cooper S, Artur Plett P, Liles W.C, Li X, Graham-Evans B, Campbell T.B, Calandra G, Bridger G, Dale D.C, Srour E.F. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *JEM*, Volume 201, Number 8, 1307-1318 2005

Burger P.E, Xiong X, Coetzee S, Salm S.N, Moscatelli D, Goto K, Wilson E.L. Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue 7180–7185 *PNAS* May 17, 2005 vol. 102 no. 20.

Cadigan K.M, Nusse R, Wnt signaling: a common theme in animal development. *Genes & Development*. 1997. 11:3286-3305

Calvi L.M, Adams G.B, Weibrecht K.W, Weber J.M, Olson D.P, Knight M. C, Martin R.P, Schipani E., Divieti P, Bringhurst F.R, Milner L.A, Kronenberg H.M Scadden D.T Osteoblastic cells regulate the haematopoietic stem cell niche *Nature* Vol 425 No 23 October 2003

Cambareri. A.C, Ashman L.K, Cole S.R, Lyons A.B. A monoclonal antibody to a human mast cell/myeloid leukaemia specific antigen binds to normal haemopoietic progenitor cells and inhibits colony formation *in vitro*. *Leukemia Research* Vol. 12, Nos. 11/12, pp. 929-939, 1988.

Catcheside D.G. Genetic effects of radiation. *British Medical Bulletin*, Vol 4 18-24 (1946).

Chabot B, Stephenson D.A, Chapman V.M, Besmer P, Bernstein A. The proto-oncogene c-Kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature* Vol 335 1 September 1988.

Challen G.A, Boles N, Lin K.K.Y, Goodell M.A. Mouse haemopoetic stem cell identification and analysis. *Cytometry Part A* 75A: 14-24, 2009 15

Chapman J.D, Stobbe C.C, Gales T, Das I.J, Zellmer J.L, Biade S, Matsomoto Y. Condensed chromatin and cell inactivation by single hit Kinetics. *Radiation Research*. 151 433-451 (1999).

Chauchan A.K, Li Y.S, Deuel T.F. Pleiotrophin transforms NIH 3T3 cells and induces tumors in nude mice. *Proceedings of the National Academy of Sciences*, Vol. 90, pp. 679-682, January 1993

Chen H, Campbell R.A, Chang Y, Li M., Wang C.S, Li J, Sanchez E, Share M, Steinberg J, Berenson A, Shalitin D, Zeng Z, Gui D, Perez-Pinera P, Berenson R.J, Said J, Bonavida B. Deuel T.F, Berenson J.R. Pleiotrophin produced by multiple myeloma induces transdifferentiation of monocytes into vascular endothelial cells: a novel mechanism of tumor-induced vasculogenesis. *Blood*, 26 FEBRUARY 2009 Volume 113, Number 9

Chen H, Gordon M.S, Campbell R.A, Li M, Wang C.S, Lee H.J, Sanchez E, Manyak S.J, Gui D, Shalitin D, Said J, Chang Y, Deuel T.F, Baritaki S, Bonavida B, Berenson J.R. Pleiotrophin is highly expressed by myeloma cells and promotes myeloma tumor growth. *Blood*, 1 July 2007, Vol. 110, No. 1, pp. 287-295.

Child J.A, Morgan G.J, Davies F.E, Owen R.G, Bell S.E, Phil D, Hawkins K, Brown J, Drayson M.T, Selby P.J. High-Dose Chemotherapy with Hematopoietic Stem-Cell Rescue for Multiple Myeloma. *The new England Journal of Medicine*. Volume 348:1875-1883 May 8, 2003 Number 19.

- Choi K, The hemangioblast: A common progenitor of hematopoietic and endothelial cells. *Journal of Hematotherapy and Stem Cell research* 11:91-101 2002.
- Choudhuri R, Zhang H.T, Donnini S, Ziche M Bicknell R. An Angiogenic Role for the Neurokines Midkine and Pleiotrophin in Tumorigenesis. *Cancer research* 57. 1814-1819. May I. 1997
- Chute J.P, Muramoto G.G, Fung J, Oxford C. Soluble factors elaborated by human brain endothelial cells induce the concomitant expansion of purified human BM CD34_CD38_ cells and SCID-repopulating cells. *Blood*, 15 January 2005. Vol 105, Number 2.
- Chute .J.P , Muramoto G.G., Dressman H.K, Wolfe G, Chao N.J,Lin S.Molecular profile and partial functional analysis of novel endothelial cell-derived growth factors that regulate hematopoieses. *STEMCELLS* 2006;24:1315–1327
- Civin C.I, Strauss L.C, Fackler M.J,Trichmann T.M, Wiley J.M, Loken M.R. Positive stem cell selection-basic science. *Progress in clinical and Biological Research*. 1990:333: 387-401.
- Cobas M, Wilson A, Ernst B, Mancini S.J.C, MacDonald H.R, Kemler R, Radtke F. β -Catenin Is Dispensable for Hematopoiesis and Lymphopoiesis. *Exp Med*. 2004 January 19; 199(2): 221–229.
- Coffman R. L, WEISSMAN I.L. B220: a B cell-specific member of the T200 glycoprotein family. *Nature* 289, 681 - 683 19 February 1981
- Cottler-Fox M.H, Lapidot T, Petit I, Kollet O, John F. DiPersio, Dan Link, and Steven Devine. Stem cell mobilisation. *Hematology* 2003
- Courty J, Dauchel M.C, Caruelle D, Perderiset M, Barritault D. Mitogenic properties of a new endothelial growth factor related to Pleiotrophin. *Biochemical and Biophysical Research Communications*. 1991 Oct 15;180(1);145-51.
- Cowden R.R, Curtis S.K. Microfluorometric Investigations of chromatin structure. Evaluation of Nine DNA-Specific Fluorochromes as Probes of chromatin organization. *Histochemistry* (1981) 72:11-23
- Dainton F.S. On the Existence of Free Atoms and Radicals in Water and Aqueous Solutions Subjected to Ionizing Radiation *J. Phys. Chem.*, 1948, 52 (3), pp 490–517
- Dakstra B, Haan G.D Hematopoietic stem cell aging and self-renewal. *Cell Tissue Res* (2008) 331:91–101
- Dar A. Kollet O, Lapidot T. Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice *Experimental Hematology* 34 (2006) 967–975
- DeHart S.L, Heikens M.J, Tsai S. Jagged2 promotes the development of natural killer cells and the establishment of functional natural killer cell lines. *Blood*, 1 May 2005 Volume 105, number 9.
- Deuel T.F, Zhang N, Yeh H.J, Santiago I.S, Wang Z.Y. Pleiotrophin: A Cytokine with Diverse Functions and a Novel Signaling Pathway. *Archives of Biochemistry and Biophysics* Vol. 397, No. 2, January 15, pp. 162–171, 2002.
- Dexter T.M ,Allen T.D,Lajtha L.G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *Journal of physiology* Vol 91 issue 3 335-344 1977.

Dexter T.M, Moore M.A.S. In vitro duplication and 'cure' of haemopoietic defects in genetically anaemic mice. *Nature* Vol 269 29 September 1977.

Dexter T.M. Garland J, Scott D, Scolnick E, Metcalf D. Growth of factor-dependent hemopoietic precursor cell lines. *Journal of Experimental Medicine*, Vol 152, 1036-1047 1980.

Domen J, Weissman I.L. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Molecular Medicine Today*. Vol 5 Issue 5 201-208 1999.

Duncan A.W, Rattis F.M, DiMascio L.N, Congdon K, Pazianos G, Zhao C, Yoon K, Cook J.M, Willert K, Gaiano N. Reya T. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nature Immunology* 6, 314 - 322 (2005)

Dykstra B, de Hann G. Hematopoietic stem cell aging and self-renewal. *Cell and Tissue Research*. Vol 331 Jan 2008.

Dzierzak E, Medinsky A. Mouse embryonic hematopoiesis. *Trends in genetics* Volume 11 Issue 9 September 1995.

Fadok V.A, Laszlo D.J, Noble P.W, Weinstein L, Riches D.W.H, Henson P.M. Particle Digestibility Is Required for Induction of the Phosphatidylserine Recognition Mechanism Used by Murine Macrophages to Phagocytose Apoptotic Cells. *The journal of Immunology* Vol 151, 4274-4285, No. 8, October 15, 1993

Fang W, Hartman N, Chow D.T, Raiteelg A.T Wellstein A Pleiotrophin Stimulates Fibroblasts and Endothelial and Epithelial Cells and Is Expressed in Human Cancer. *The Journal of Biological Chemistry*. Vol. 261, No. 36, Issue of December 25, pp. 26889-25897 1992.

Fina L, Molgaard H.V, Robertson D, Bradley N.J, Monaghan P, Delia D, Sutherland D.R, Baker M.A ,Greaves M.F. Expression of the CD34 gene in vascular endothelial cells. *Blood* Volume 75, Issue 12, pp. 2417-2426, 1990

Fisher B, Dignam J, Wolmark N, Mamounas E, Costantino J, Poller W, Fisher ER, Wickerham D.L, Deutsch M, Margolese R, Dimitrov N and Kavanah M. Lumpectomy and radiation therapy for the treatment of intraductal breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-17. *Journal of Clinical Oncology*, Vol 16, 441-452, 1998

Gallagher L, Murdoch B, Wu D.M, Karanu F.N, Keeney M, Bhatia M. Isolation and characterization of human CD34²Lin² and CD34¹Lin² hematopoietic stem cells using cell surface markers AC133 and CD7. *Blood*, 1 May 2000. Vol 95, Number 9

Garcia-Porrero J.A, Godin I.E Dieterlen-Lièvre F. Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. *Anat Embryol* (1995) 192:425-435.

Garver R.I, Chan C.S, Milner P.G. Reciprocal expression of Pleiotrophin and Midkine in normal versus malignant lung tissues. *American Journal of respiratory cell and molecular biology*. 1993 Nov. 9(5):463-6

Garver R.I, Radford D.M, Donis-Keller H, Wick M.R, Milner P.G. Midkine and Pleiotrophin expression in normal and malignant breast tissue. *Cancer* 1994 Sep 1;74 (5):1584-90.

Givon T, Revel M, Slavin S. Potential Use of Interleukin-6 in Bone Marrow Transplantation: Effects of Recombinant Human Interleukin-6 after Syngeneic and Semiallogeneic Bone Marrow Transplantation in Mice. *Blood*, Vol83, No 6 (March 15), 1994: pp 1690-1697

Godin I, Dieterlen-Lievre F, Cumano A. Emergence of multipotent hemopoietic cells in the yolk sac and paraortic splanchnopleura in mouse embryos, beginning at 8.5 days post-coitus. *Proc. Natl. Acad. Sci. USA* Vol. 92, pp. 773-777, January 1995

Golde W.T, Gollobin P, Rodrigue L.L. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Technique* Volume 34, No. 9 October 2005.

Gong J.K., Endosteal marrow: a rich source of haemopoietic stem cells. *Science* Vol 199 Issue 4336 1443-1445 1978.

Gong H, Shen P, Jin Li, Xing C, Tang F. Therapeutic Effects of *Lycium barbarum* Polysaccharide (LBP) on Irradiation or Chemotherapy-Induced Myelosuppressive Mice. *Cancer Biotherapy & Radiopharmaceuticals*. Volume 20, Number 2, 2005

Graham G., Wright E. Haemopoietic stem cells: Their heterogeneity and regulation. *The International Journal of experimental pathology* (1997), 78 197-218.

Greiner D.L, Hesselton R.A, Shultz L.D. SCID Mouse Models of Human Stem Cell Engraftment. *Stem Cells* 1998;16:166-177.

Hall P.A. Watt F.M. Stem cells the generation and maintenance of cellular diversity. *Development* 106, 619-633 (1989).

Hao Q.L, Zhu J, Price M.A, Payne K.J, Barsky L.W, Crooks G.M. Identification of a novel, human multilymphoid progenitor in cord blood. *Blood*, 15 June 2001, Vol. 97, No. 12, pp. 3683-3690.

Hawley R.G, Ramezani A, Hawley T..S Hematopoietic Stem Cells *Methods In Enzymology*, Vol. 419 2006.

Heiss C, Wong M.L, Block V.I, Lao D, Real W.M, Yeghiazarians Y, Lee R.J, Springer M.L. Pleiotrophin induces nitric oxide dependent migration of endothelial progenitor cells. *Journal of Cellular Physiology*. 2008;215 (2): 336-73.

Heissig B, Hattori K, Dias S, Friedrich M, Ferris B., Hackett N.R., Crystal R.G, Besmer P, Lyden D, Moore M.A.S, Werb Z, Rafii,S. Recruitment of Stem and Progenitor Cells from the Bone Marrow Niche Requires MMP-9 Mediated Release of Kit-Ligand. *Cell* Vol 109 Issue 5. 2002 625-637.

Hestall K, Ruscetti F.W, Ihle J.N, Jacobsen S.E, Dubois C.M, Kopp W.C, Longo D.L, Keller J.R. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *The Journal of Immunology*, Vol 147, Issue 1 22-28, 1991.

Holmes C, Stanford W.A. Concise Review: Stem Cell Antigen-1: Expression, Function, and Enigma. *STEM CELLS*. 2007;25:1339-1347

- Holyoake T.L, Freshney M.G, McNair L, Parker A.N, McKay P.J, Steward, Fitzsimons E, Grahame G.J, Pragnell I.B. Ex vivo expansion with stem cell factor and interleukin-11 augments both short-term recovery posttransplant and the ability to serially transplant marrow. *Blood*. Volume 87, Issue 11, pp. 4589-4595, 1996
- Huang H.J, Jones N.H, Strominger J.L, Herezenberg L.A. Molecular cloning of Ly-1, a membrane glycoprotein of mouse T lymphocytes and a subset of B cells: molecular homology to its human counterpart Leu-1/T1 (CD5). *Proceedings of the National Academy of Sciences*, Vol. 84, pp. 204-208, January 1987
- Huang X, Cho S, Spangrude G.J Hematopoietic stem cells: generation and self-renewal. *Cell Death and Differentiation* (2007) 14, 1851–1859.
- Ikuta K, Weissman I.L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation *Proc. Natl. Acad. Sci. USA* Vol. 89, pp. 1502-1506, February 1992
- Imai K, Masanobu K, Jingxin W, Noriaki S, Hideaki Y, Juncichi H, Masanobu S, Fumihiro H, Junji T, Masahiro A, Masuo H. Selective secretions of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitors to the bone marrow. *British Journal of Haematology* September 1999 106:4-11.
- Imlay J.A Linn S. DNA damage and oxygen radical toxicity. *Science*, Vol 240, Issue 4857, 1302-1309
- Ivanova N.B, Dimos J.T, Schaniel C, Hackney J.A., Moore K.A, Lemischka I.R. A Stem Cell Molecular Signature. *Science* Vol 298 18 October 2002
- Jackson K.A, Majka . S.M, Wulf G.G., Goodell M.A. Stem Cells: A minireview. *Journal of Cellular Biochemistry Supplement* 38:1–6 (2002)
- Jones D.L, Wagers A.J. No place like home: anatomy and function of stem cell niche. *Nature reviews molecular cell biology* volume 9 January 2008.
- Jung C.G, Hida H, Nakahira K, Ikenaka K, Kim H.J, Nishino H. Pleiotrophin mRNA is highly expressed in neural stem (progenitor) cells of mouse ventral mesencephalon and the product promotes production of dopaminergic neurons from embryonic stem cell-derived nestin-positive cells. *The FASB Journal*. 2004;18:1237-1239
- Kamel-Reid S, Dick J.E. Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* 23 December 1988 Vol. 242. no. 4886, pp. 1706 - 1709
- Kataoka S, Satoh J, Fujiya H, Toyota T, Suzuki R, Itoh K, Kumagai K. Immunologic aspects of the non-obese diabetic (NOD) mouse. Abnormalities of cellular immunity. *Journal of American Diabetes association* Vol 32 no.3 247-253. 1983.
- Kennedy M., Firpo M., Choi K, Wall. C, Robertson S, Kaburn N, Keller G. A common precursor for primitive erythropoiesis and definitive haemopoiesis. *Nature* Vol 386 No. 3 April 1997.
- Kiel M.J, Morrison S.J. Uncertainty in the niches that maintain haematopoietic stem cells. *Nature reviews immunology* volume 8 April 2008.

- Kilpeläinen I, Kaksonen M, Kinnunen T, Avikainen H, Fath M, Linhardt R.J, Raulo E and Rauvala H. Heparin-binding Growth-associated Molecule Contains Two Heparin-binding β -Sheet Domains That Are Homologous to the Thrombospondin Type I Repeat. *J Biol Chem*, Vol. 275, Issue 18, 13564-13570, May 5, 2000
- Kina T, Koichi I, Eiji T, Katsuya W, Majumdar A.S, Weissman I.L, Katsura Y. The monoclonal antibody TER-119 recognises a molecule associated with Glycophorin A and specifically marks the late stage of murine erythroid lineage. *British Journal of Haematology*. 109:2 2000
- Koch U, Wilson A, Cobas M, Kelmer R, MacDonald H.R, Radtke F. Simultaneous loss of β - and γ -catenin does not perturb hematopoiesis or lymphopoiesis. *Blood*, 1 January 2008, Vol. 111, No. 1, pp. 160-164
- Kondo M, Weissman I.L Akashi K. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell*, Vol. 91, 661–672, November 28, 1997
- Kopp H.G, AVECILLA S.T, Hooper A.T, Rafii S. The Bone Marrow Vascular Niche: Home of HSC Differentiation and Mobilization. *Physiology* 20: 349-356 2005.
- Kripke M.L Immunological Unresponsiveness Induced by Ultraviolet Radiation. *Immunological Reviews* Vol 80 Issue 1 87-102.
- Kripke M.L. Latency, Histology, and Antigenicity of Tumors Induced by Ultraviolet Light in Three Inbred Mouse Strains. *Cancer Research* 37, 1395-1400, May 1977
- Kubbutat M.H, Key G, Duchrow M, Schluter C, Flad H.D, Gerdes J. Epitope analysis of antibodies recognising the cell proliferation associated nuclear antigen previously defined by the antibody Ki-67 (Ki-67 protein). *Journal of Clinical Pathology* 1994; 47:524-528.
- Lai S, Czubayko F, Riegel A.T., Wellstein A. Structure of the human heparin-binding growth factor gene pleiotrophin . *Biochemical and Biophysical Research Communications*. Vol. 187, No. 2, 1992
- Lajtha L.G. Stem cell concepts. *Differentiation* Volume 14 Issue 1-3 23-33. 1979
- Lampugnani M.G, Corada M, Caveda L, Breviario F, Ayalon O, Greiger B, Dejana E. The molecular organization The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). *The Journal of Cell Biology*, Vol 129, 203-217 1995
- Landgraf P, Wahle P, Pape H.C, Gundelfinger E.D, Kreutz M.R. The Survival-promoting Peptide Y-P30 Enhances Binding of Pleiotrophin to Syndecan-2 and -3 and Supports Its Neuritogenic Activity. *J. Biol. Chem.*, Vol. 283, Issue 36, 25036-25045, September 5, 2008
- Lapidot T, Dar A, Kollet O. How do stem cells find their way home? *Blood*, 15 September 2005 Vol 106, Number 6
- Larochelle A, Vormoor J, Hanenburg H, Wang J.C.Y, Bhatia M, Lapidot T, Moritz T, Murdoch B, Xiao X.L, Kato I, Williams D.A, Dick J.E. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy *Nature Medicine* Vol 2 1329-1337 (1996).

- Lea D.E, Catcheside D.G. The mechanism of the induction by radiation of chromosome aberrations in *Tradescantia* *Journal of genetics*. Vol 44 (2-3): 216-245 (1942)
- LeClair K.P., Palfree R.G.E., Flood P.M, Hammerling U, Bothwell A. Isolation of a murine Ly-6 cDNA reveals a new multigene family. *The EMBO Journal* vol.5 no. 12 pp.3227 -3234, 1986
- Leenhouts H.P. Chadwick K.H. Radiation induced DNA double strand breaks and chromosome aberrations. *Theoretical and applied genetics*. Vol 44(4): 167-172 (1974)
- Lévesque J.P, Hendy J, Takamatsu Y, Simmons P.J Bendall L.J. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *Journal of clinical investigation*. 111(2): 187-196 (2003).
- Li Y.S, Gurrieri M, Deuel T.F. Pleiotrophin Gene Expression is highly restricted and is regulated by platelet-derived growth factor. *Biochemical and Biophysical research communications*. 1992 Apr 15:184 (1):427-32.
- Li Y.S, Milner P.G, Chauachan A.K, Watson M.A, Hoffman R.M, Kodner C.M, Milbrandt J, Deuel T.F. Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science* Vol. 250 1690-1694 1990
- Li Y.S, Hoffman R.M, Le Beauin M.M, Espinosa R Jenkins N.A, Gilbert D.J Copeland N.G , Deuel T.F. Characterization of the Human Pleiotrophin Gene. *The Journal of biological chemistry* Vol. 267, No. 36, Issue of December 25, pp. 26011-26016, 1992
- Lord B.I, Testa N.G, Hendry J.H. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* Vol. 46 65-72 1975
- Maeda N, Noda M. Involvement of Receptor-like Protein Tyrosine Phosphatase RPTP and Its Ligand Pleiotrophin/Heparin-binding Growth-associated Molecule (HB-GAM) in Neuronal Migration. *The Journal of Cell Biology*, Volume 142, Number 1, July 13, 1998 203–216
- Majumder S, Brown K, Qiu F.H, Besmer P. c-kit Protein, a Transmembrane Kinase: Identification in Tissues and Characterization. *Molecular and Cellular biology*, Nov. 1988, p. 48964903
- Mancini S.J.C, Mantei N, Dumortier A, Suter U, MacDonald H.R, Radtke. *aged1*-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood*, 15 March 2005, Vol. 105, No. 6, pp. 2340-2342.
- Manz M.G, Miyamoto T, Akashi K, Weissman I.L. Prospective isolation of human clonogenic common myeloid progenitors. *Proceedings of the National Academy of Sciences*, 2002 vol. 99 no. 18 11872-11877
- Matsuura K, Nagai T, Nishigaki N, Oyama T, Nish J, Wada H, Sano M, Toko H, Akazawa H, Sato T, Nakaya H, Kasanuki H, and Komuro I. Adult Cardiac Sca-1-positive Cells Differentiate into Beating Cardiomyocytes. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* Vol. 279, No. 12, Issue of March 19, pp. 11384–11391, 2004.
- Maximow A.A, Relation of blood cells to connective tissue and endothelium *Physiological Reviews*, Vol. IV, No. 4

- Mayrhofer G, Gadd S.J, Spargo L.D.J and Ashman L.K Specificity of a mouse monoclonal antibody raised against acute myeloid leukaemia cells for mast cells in human mucosal and connective tissues. *Immunol. Cell Biology.*, 65 (Pi. 3) 241-250 (1987)
- Mazo I.B, Andrian U.H.V. Adhesion and homing of blood-borne cells in bone marrow microvessels. *Journal of Leukocyte Biology* Volume 66, July 1999
- McNiece I.K, Langley K.E, Zsebo K.M. The role of recombinant stem cell factor in early B cell development, synergistic interaction with IL-7. *The Journal of Immunology* Vol. 146.3785-3790. No.11. Jun 1. 1991
- McNiece I, Jones R, Bearman S.I, Cagnoni P, Nieto Y, Franklin W, Ryder J, Steele A, Soltz J, Russell P, McDermitt J, Hogan C, Murphy J, Shpall E.J. Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer. *Blood*, 1 November 2000, Vol. 96, No. 9, pp. 3001-3007
- McQuibban A.G., Butler G.S, Gong J.H, Bendall L, Power C, Clark-Lewis I, Overall C.M Matrix Metalloproteinase Activity Inactivates the CXC Chemokine Stromal Cell-derived Factor-1. . *Biol. Chem.*, Vol. 276, Issue 47, 43503-43508, November 23, 2001
- Metcalf D. *The Haemopoietic Colony Stimulating Factors*, published by Elsevier, Amsterdam. (1984)
- Meng K, Rodríguez-Peña A, Dimitrov T, Chen W, Yamin M, Noda M, Deuel T.F. Pleiotrophin signals increased tyrosine phosphorylation of β -catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase β/ζ . *Proc Natl Acad Sci U S A*. 2000 March 14; 97(6): 2603–2608.
- Michaels HB, Hunt JW. A model for radiation damage in cells by direct effect and by indirect effect: a radiation chemistry approach. *Radiation research*. 1978 Apr;74(1):23-34.
- Milner P.G, Li Y.S, Hoffman R.M Kodner C.M, Siegel N.R, Deuel T.F A novel 17kDa Heparin-binding growth factor (HBGF-8) in bovine uterus: purification and n-terminal amino acid in bovine uterus: purification and n-terminal amino acid sequence E310 *chemical and biophysical research communications* Vol. 165, No. 3, 1989 December 29, 1989 Pages 1096-1103.
- Mitsiadis T.A, Salmivirta M, Muramatsu T, Muramatsu H, Rauvala H, Lehtonen E, Jalkanen M and Thesleff I. Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development* 121, 37-51 (1995)
- Monroy R.L, Skelly R.R, MacVittie T.J, Davis T.A, Sauber J.J, Clark S.C, Donahue R.E. The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. *Blood* Volume 70, Issue 5, pp. 1696-1699 1987
- Moore K.A, Ema H, Lemischka, I.R. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. *Blood* Vol 89 Issue 12 4337-4347. 1987
- Morrison S.J, Uchida N, Weissman I.L. The biology of hematopoietic stem cells. *Annu. Rev. Cell Dev. Biol.* 1995. 11:35-71.

Morrison S.J. Spradling A.C. Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance throughout Life. *Cell* 132, 598–611, February 22, 2008.

Muller H.J. The manner of dependence of the 'permissible dose' of radiation on the amount of genetic damage. *Acta Radiologica* 41(1) 5-20 January 1954

Muramoto G.G, Chen B, Cui X., Chao N.J., Chute J.P. Vascular Endothelial Cells Produce Soluble Factors That Mediate the Recovery of Human Hematopoietic Stem Cells after Radiation Injury. *Biology of Blood and Marrow Transplantation* 12:530-540 (2006)

Muller-Sieburg C.E., Whitlock C.A., Weissman I.L. Isolation of two early B lymphocyte progenitors from mouse marrow: A committed Pre-Pre-B cell and a clonogenic Thy-1^{lo} hematopoietic stem cell. *Cell*, 28 February 1986, Volume 44, Issue 4, 653 – 662.

Murguruma Y, Yahata T, Miyatake H, Sato T, Uno T, Itoh J, Shunichi K, Ito M, Tomomitsu H, Ando K. Reconstitution of the functional human haemopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood* Vol 107 No. 5 1878-1887 2006.

Muramatsu T., Midkine and Pleiotrophin: Two Related Proteins Involved in Development, Survival, Inflammation and Tumorigenesis. *J. Biochem.* 132, 359-371 (2002)

Nakagawara A, Milbrandt J, Muramatsu T, Deuel T.F, Zhao H, Cnaanand A, Brodeur G.M. Differential Expression of Pleiotrophin and Midkine in Advanced Neuroblastomas. *Cancer Research* 55, 1792-1797, April 15, 1995

Nocha K, Majumder S, Chabot , Ray P, Cervone M, Bernstein A, Besmer P. Expression of *c-kit* gene products in known cellular targets of W mutations in normal and W mutant mice evidence for an impaired *c-kit* kinase in mutant mice. *Genes & development* 3:816-826 _9 1989

Nusse R. Varmus H.E. WNT genes. *Cell* Vol 69 Issue 7 1073-87. 1992

Nusse R. Wnt signaling and stem cell control. *Cell Research* (2008) 18:523-527.

Oberlin E, Tavian M, Blazsek I, Peault B. Blood forming potential of vascular endothelium in the human embryo. *Development* 129, 4147-4157 (2002)

Orkin S.H. Hematopoiesis: how does it happen? *Current Opinion in Cell Biology* 1995, 7:870-877

Orkin S.H. Transcription factors and hematopoietic development. *The Journal of Biological Chemistry*. Vol 270 No. 10, March 4955-4958, 1995.

Ogawa M, Matsuzaki, Y, Nishikawa S, Hayashi S.I, Kunisada T, Sudo T, Kina K, Nakauchij H and Nishikawa S.I. Expression and Function of *c-kit* in Hemopoietic Progenitor Cells. *J. Exp. Med.* © The Rockefeller University Press Volume 174 July 1991 63-71

Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood*. Volume 81, Issue 11, pp. 2844-2853. 1993.

Pariser H, Perez-Pinera P, Ezquerra L, Herradon G, Deuel T.F. Pleiotrophin stimulates tyrosine phosphorylation of β -adducin through inactivation of the transmembrane receptor protein tyrosine phosphatase β/ζ . *Biochemical and Biophysical research communications*. Vol 335 Issue 1 232-239.2005.

Patt H.M, Maloney M.A. Relationship of bone marrow cellularity and proliferative activity: a local regulatory mechanism. *Cell Tissue Kinet* 5 (4): 303-9 1972.

Peterson W, Wildemann B, Pufe T, Raschke M, Schmidmaier G. Immunolocalization of the angiogenetic factor pleiotrophin (PTN) in the growth plate of mice. *Archives of orthopaedic and trauma surgery*. Vol 121 November 2001.

Petit I, Szyper-Kravitz M, Nagler A, Lahav M, Peled A, Habler L, Ponomaryov T, Taichman R.S, Arenzana-Seisdedos F, Fujii N, Sandbank J, Zipori D, Lapidot T. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nature Immunology* 3, 687 - 694 (2002)

Petit I, Goichberg P, Spiegel A, Peled A, Brodie C, Seger R, Nagler A, Alon R, Lapidot T. Atypical PKC- ζ regulates SDF-1-mediated migration and development of human CD34⁺ progenitor cells. *J Clin Invest*. 2005 January 3; 115(1): 168–176.

Pettengell R. Autologous stem cell transplantation in follicular non-hodgkins lymphoma. *Bone Marrow transplant* 2002 29 Sup1:S1-4.

Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, Arenzana-Seisdedos F, Magerus A, Caruz A, Fujii N, Nagler A, Lahav M, Szyper-Kravitz M, Zipori D, Lapidot T. Induction of the chemokine stroma derived factor-1 following DNA damage improves human stem cell function. *Journal of Clinical Investigation*. Volume 112, Issue 2 (July 15,2000)

Potocnik A.J, Brakebusch C, Fassler R. Fetal and Adult Hematopoietic Stem Cells Require β 1 Integrin Function for Colonizing Fetal Liver, Spleen, and Bone Marrow. *Immunity* Vol 12 Issue 6 653-663 2000.

Potten C.S Grant H.K. The relationship between ionizing radiation-induced apoptosis and stem cells in the small and large intestine. *British Journal of Cancer*. 1998 October; 78(8): 993–1003.

Potter, M., and C. Boyce. 1962. Induction of plasma cell neoplasms in strain BALB/c mice with mineral oil and mineral oil adjuvants. *Nature (Lond.)*. 193:1086

Powles R. L. , Clink H.M, Bandini G, Watson J.G, Spence D, Jameson B, Kay H.E.M, Morgenstern G, Hedley D, Lumley H, Lawson D, Barrett A, Lawler S and Mcelwain T.J. The Place of Bone Marrow Transplantation in Acute Myelogenous Leukaemia. *The Lancet* Volume 315, Issue 8177, 17 May 1980, Pages 1047-1050

Pytela R., Pierschbacher M.D., Ginsberg M.H, Plow E.F, Ruoslahti E. Platelet Membrane Glycoprotein IIb/IIIa: Member of a Family of Arg-Gly-Asp-Specific Adhesion Receptors. *Science*, Vol. 231 1986.

Qiu F, Ray P, Brown K, E.Barker P, Jhanwarl S, Ruddle F.H Besmer P. Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family-oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *The EMBO Journal* vol. 7 no.4 pp. 1 003 - 1011, 1 988

- Rafii S, Avezilla S, Shmelkov S, Shido K, Tejada R, Moore M.A, Heissig B, Hattori K. Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. *Annals of the New York Academy of Sciences* 996:49-60 May 2003.
- Raulo E, Julkunen I, Merenmies J, Pihlaskari R, Rauvala. H. Secretion and biological activities of heparin-binding growth- associated molecule. Neurite outgrowth-promoting and mitogenic actions of the recombinant and tissue-derived protein. *Journal of Biological Chemistry* Vol. 267, Issue 16, 11408-11416, 06, 1992
- Rauvala H. An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. *The EMBO Journal* vol.8 no.10 pp.2933-2941, 1989
- Raynal P, Pollard H.B. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium and phospholipid-binding proteins. *Biochimica et Biophysica Acta* 1197 (1994) 63-93
- Reboul J, Ballanger F, Delorme G, Teisser JP. Radiotherapy in cancer of the prostate. *Annales de radiologie*. 1965;8(5):353-8.
- Reiser M, Diehl V. Current treatment of follicular non-Hodgkins Lymphoma. *European Journal of Cancer* Vol 38 issue 9, 2002 1167-1172.
- Reya T. Regulation of Hematopoietic Stem Cell Self-Renewal the *Endocrine Society* 2003. 283-295.
- Reya T, Morrison S.J, Clarke M.F, Weissman I.L. Stem cells, cancer and cancer stem cells. *Nature* Vol 414 November 2001
- Rios M, Williams D.A, Systemic analysis of the ability of stromal cell lines derived from different murine adult tissues to support maintenance of haemopoietic stem cells in vitro. *Journal of cellular physiology*. Vol 145 Issue: 3 434-443. 1990
- Roberts R.A, Spooncer E, Parkinson E.K, Lord B.I, Allen T.D Dexter. T.M. Metabolically Inactive 3T3 Cells Can Substitute for Marrow Stromal Cells to Promote the Proliferation and Development of Multipotent Haemopoietic Stem Cells. *Journal of cellular physiology* 132:203-214 1987
- Rubin D.B, Drab E.A, Ward W, Smith L.J, Fowell S. Enzymatic responses to radiation in cultured vascular endothelial and smooth muscle cells. *Radiation Research*. Vol 99 420-432 (1984).
- Salter A.B ,Meadows S.K ,Muramoto G.G Himburg H, Doan P, Daher P, Russell L, Chen B, Chao N.J and John P. Chute. Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood*, 26 February 2009, Vol. 113, No. 9, pp. 2104-2107
- Savill J, Fadok V, Henson P, Haslett C. Phagocyte recognition of cells undergoing apoptosis. *Immunology Today* 131 Vol. 14 No. 3 1993
- Scadden D.T. The stem cell niche as an entity of action. *Nature* Vol 441 June 2006.
- Schulte-Frohlinde D. Mechanism of radiation-induced strand break formation in DNA and polynucleotides. *Advances in space research*. 1986;6(11):89-96.

- Schmid I, Krall W.J, Uittenbogaart C.H, Braun J, Giorgi J.V. Dead Cell Discrimination with 7-Amino-Actinomycin D in Combination with Dual Color Immunofluorescence in Single Laser Flow Cytometry. *Cytometry* 13:204-208 (1992)
- Schofield R. The relationship between the spleen colony forming cell and the haemopoietic stem cell. *Blood cells* 1978;4 (1-2):7-25.
- Short B, Brouard N, Occhiodoro-Scott A. Mesenchymal Stem Cells. *Archives of Medical Research* Vol 34 Issue 6 565-571 2003
- Sieff C.A, Emerson S.G, Mufson A, Gesner T.G, Nathan D.G. Dependence of Highly Enriched Human Bone Marrow Progenitors on Hemopoietic Growth Factors and Their Response to Recombinant Erythropoietin. *J. Clin. Invest.* Volume 77, January 1986, 74-81
- Silos-santiago I, Yeh H.J, Gurrieri M.A, Guillerman R.P, Li Y.S, Wolf J, Snider W., Deuel T.F. Localization of pleiotrophin and its mRNA in subpopulations of neurons and their corresponding axonal tracts suggests important roles in neural-glial interactions during development and in maturity. *Journal of Neurobiology.* Volume 31 Issue 3 283-296. 1996
- Soh .B.S, Song C.M, Vallier L, Li. P, Choong C, Huat Yeo B. Pleiotrophin Enhances Clonal Growth and Long-Term Expansion of Human Embryonic Stem Cells. *STEM CELLS* 2007;25:3029–3037
- Souttou B, Ahmad S, Riegel A.T, Wellstein A. Signal Transduction Pathways Involved in the Mitogenic Activity of Pleiotrophin. *Journal of Biological Chemistry* Volume 272, Number 31, Issue of August 1, 1997 pp. 19588-19593
- Spangrude G.J, Heimfeld S, Weissman I.L. Purification and characterization of mouse Hematopoietic stem cells. *Science*, New Series, Vol. 241, No. 4861 (Jul. 1, 1988), pp. 58-62
- Springer T, Galfré G, Secher D.S, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *European Journal Of Immunology.* Vol 9 Issue 4 301-306. 1979
- Stall F.J.T, Luis T.C, Tiemessen M.M. WNT signalling in the immune system: WNT is spreading its wings. *Nature reviews Immunology.* Vol 8 August 2008.
- Stanford R.W Vance J. The Quantity of Radiation Received by the Reproductive Organs of Patients during Routine Diagnostic X-ray Examinations. *British Journal of Radiology* (1955) 28, 266-273 (1955).
- Stoica G.E, Kuo A, Aigner A, Sunitha I, Souttou B, Malerczyk C, Caughey D.J, Wen D, Karavanov A. Riegel A.T, Wellstein A. Identification of Anaplastic Lymphoma Kinase as a Receptor for the Growth Factor Pleiotrophin. *J. Biol. Chem.*, Vol. 276, Issue 20, 16772-16779, May 18, 2001
- Suri C, Jones P.F, Patan S, Bartunkova S, Maisonpierre P.C, Davis S, Sato T.N, Yancopoulos G.D. Requisite Role of Angiopoietin-1, a Ligand for the TIE2 Receptor, during Embryonic Angiogenesis. *Cell*, 27 December 1996, Volume 87, Issue 7, 1171 - 1180
- Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 393, 591-594 (11 June 1998)

- Taichman R.S, Emerson S.G. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *Journal of Experimental Medicine*, Vol 179, 1677-1682 1994.
- Taichman R.S, Reilly M.J, Emerson S.G. Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. *Blood* Volume 87, Issue 2, pp. 518-524 2000
- Taichman R.S Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood*, 1 April 2005. Vol 105, No.7
- Tait J.F, Gibson D, Fujikawa K. Phospholipid Binding Properties of Human Placental Anticoagulant Protein-I, a Member of the Lipocortin Family. *The Journal of Biological Chemistry* Vol. 264, No. 14, Issue of May 15, pp. 7944-7949, 1989
- Tapp H, Hernandez D.J, Neame P.J, Koob T.J. Pleiotrophin inhibits chondrocyte proliferation and stimulates proteoglycan synthesis in mature bovine cartilage. *Matrix Biology Journal of the international society for matrix biology*. 1999 18(6):543-56.
- Thomas E.D, Lochte H.L, Cannon J.H, Sahler O.D, Ferrebee J.W. Supralethal whole body irradiation and isologous marrow transplantation in man. *The Journal of Clinical Investigation*. 1959 Oct; 38:1709-16.
- Throwbridge I.S, Omary M.B. Molecular complexity of leukocyte surface glycoproteins related to the macrophage differentiation antigen Mac-1. *Journal of experimental medicine*. Vol 154 November 1981.1517-1524.
- Till J.E, McCulloch E.A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14**:213-22, 1961.
- Torna J.G, Akhavan M, Fernandes K.J.L, Barnabé-Heider F, Sadikot A, Kaplan D.R and Miller F.D Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nature cell biology* VOL 3 September 2001 778-784.
- Tricot G, Vesole D.H, Jagannath S, Hilton J, Munshi N, Barlogie B. Graft-versus-myeloma effect: proof of principle. *Blood* 1996 87: 1196-1198
- Tsai M, Takeishi T, Thompson H, Langley K.E, Zsebo K, Metcalfe D.D, Geissler E.N, Gallis S.J. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proc. Natl. Acad. Sci. USA* Vol. 88, pp. 6382-6386, July 1991.
- Uchida N, Weissman I.L. Searching for Hematopoietic Stem Cells: Evidence That Thy-1.1⁺ Lin⁻ Sca-1⁺ Cells Are the Only Stem Cells in C57BL/Ka-Thy-1.1 Bone Marrow. *J. Exp. Med.* Volume 175 January 1992 175-184.
- Vacherot F, Laaroubi K, Caruelle D, Delbe J, Barritault D, Caruelle J.P, Courty J. Upregulation of the Heparin-Affin regulatory peptide by Androgen. *In Vitro Cell Development Biology-Animal* 31:647-648 1995.
- Varnum-Finney B, Brashem-Stein C, Bernstein I.D. Combined effects of *Notch* signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood*, 1 March 2003 Volume 101, number 5.

- Van De Run.M, Heimfeld.S, Spangrude G.J, Weissman I.L Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. *Immunology* Vol. 86, pp. 4634-4638, June 1989.
- Veiby O.P, Lyman S.D, Jacobsen S.E.W. Combined signaling through interleukin-7 receptors and flt3 but not c-kit potently and selectively promotes B-Cell commitment and differentiation from uncommitted murine bone marrow progenitor cells. *Blood*, Vol 88, No 4 (August 15). 1996: PP 1256-1265
- Visnijić D, Kalajzić Z, Rowe D.W, Katavić V, Lorenzo J., Aguila H.L Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood*, 1 May 2004 Vol 103, No 9
- Wang J.C.Y. Doedens M., Dick J.E. Primitive Human Hematopoietic Cells Are Enriched in Cord Blood Compared With Adult Bone Marrow or Mobilized Peripheral Blood as Measured by the Quantitative In Vivo SCID-Repopulating Cell Assay *Blood*, Vol. 89 No. 11 (June 1), 1997: pp. 3919-3924
- Weiden P.L, Lerner K.G, Gerdes A, Heywood J.D, Fefer A, Thomas E.D. Pancytopenia and Leukemia in Hodgkin's Disease: Report of Three Cases. *Blood*, 1973, Vol. 42, No. 4, pp. 571-577
- Welte K, Bonilla M.A, Gillio A.P, Boone T.C, Potter G.K, Gabilove J.L, Moore M.A, O'Reilly R.J , Souza L.M. Recombinant human granulocyte colony-stimulating factor. Effects on hematopoiesis in normal and cyclophosphamide-treated primates. *Journal of Experimental Medicine*, Vol 165, 941-948, 1987
- Whartenby K.A , Straley E.E, Kim H, Racke F, Tanavde V, Gorsk K.S, Cheng L, Pardoll D.M, Civin C.I. Transduction of donor hematopoietic stem-progenitor cells with Fas ligand enhanced short-term engraftment in a murine model of allogeneic bone marrow transplantation. *Blood*, 1 November 2002. Volume 100, Number 9.
- Whetton A.D, Spooncer E. Role of cytokines and extracellular matrix in the regulation of haemopoietic stem cells. *Current opinion in Cell Biology*, 1998, 10:721-726.
- Whetton A.D, Graham G.J. Homing and mobilization in the stem cell niche. *Cell Biology* (Vol. 9) June 1999.
- Willert K, Nusse R. b-catenin: a key mediator of Wnt signalling. *Current opinion in genetics & development*, 1998
- Willert K, Brown J.D, Danenburg E, Duncan A.W, Weissman I.L, Reya T, Yates J.R, Nusse R. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448-452 (22 May 2003)
- Wilson A., Trumpp A. Bone-marrow haematopoietic stem-cell niches *Nature reviews Immunology* Vol 6 February 2006.
- Wissman I.L, Anderson D.J, Gage F. Stem and Progenitor Cells: Origins, Phenotypes, Lineage commitments and Transdifferentiations. *Annual. Rev. Cell Dev. Biol.* 2001. 17:387-403.
- Yeh H, He Y.Y, Xu J, Hsu C.Y, Deuel T.F Upregulation of Pleiotrophin Gene Expression in Developing Microvasculature, Macrophages, and Astrocytes after Acute Ischemic Brain Injury. *The Journal of Neuroscience*, May 15, 1998, 18(10):3699-3707

Yin T and Li L., The stem cell niches in bone *The Journal of Clinical Investigation* Volume 116 Number 5 May 2006.

Yuhas J.M, Storer J.B. On mouse strain differences in radiation resistance: Hematopoietic death and endogenous colony forming unit. *Radiation research* 39,608-622 (1969)

Yutoku M, Grossberg A.L, Pressman D. Preparation and properties of rabbit anti-mouse plasmacyte serum purified by in vivo absorption: Further evidence for a new antigenic determinant on plasmacytes. *The Journal of Immunology*. Vol, 112, No. 3, March 1974

Zanjani E.D, Almeida-Porada G, Livingston A.G, Flake A.W, Ogawa M. Human Bone Marrow CD34-cells engraft in vivo and undergo multi-lineage expression that includes giving rise to CD34+ cells. *Experimental Hematology* 1998 (26)4:353-60.

Zelenin A.V, Poletaev A.I, Stepanova N.G, Barsky V.E, Kolesnikov V.A, Nikitin S.M, Zhuze A.L, Gnutchev N.V 7-Amino-Actinomycin D as a Specific Fluorophore for DNA Content Analysis by Laser Flow Cytometry. *Cytometry* 5:348-354 (1984)

Zhang J, Niu C, Ye Ling, Huang H, He ,Tong W, Ross J, Haug J, Johnson T., Feng J.Q, Harris S, Wiedemann L.M1., Mishina Y ,Li 1,4 Identification of the haematopoietic stem cell niche and control of the niche size *Nature* Vol 425 No. 23 October 2003.

Zon L. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature* Vol 453 15 May 2008

Zou Y.R, Kottmann A.H, Kuroda M, Taniuchi I, Littman D.R. Function of the chemokine receptor CXCR4 in haematopoiesis and in Cerebellar development. *Nature* Vol 393 11 JUNE 1998.

Zsebo K.M, Williams D.A, Geissler E.N. Broudy V.C, Martin F.H, Atkins H.L, Hsu R.Y, Birkett N.C, Oklno K.H, Murdock D.C, Jacobsen F.W, Langley K.E, Smith K.A, Takeishi T, Cattanach B.Y, Galli S.J, Suggs S.V. Stem Cell Factor Is Encoded at the Sl Locus of the Mouse and Is the Ligand for the c-kit Tyrosine Kinase Receptor. *Cell*, Vol. 63, 213-224, October 5, 1990, Copyright 0 1990

Appendices

Laboratory equipment

- Cell culture hood (SterilGard III. The Baker Company Sanford U.S.A)
- CO₂ incubator (Sanyo Scientific Indianapolis IN U.S.A)
- Centrifuge (Sorvall RT refrigerated centrifuge)
- Waterbath (Precision)
- Microscope
 - Nikon Eclipse TS100 (Nikon Instrument Inc. N.Y, U.S.A)
 - Zeiss Axiovert 200 (Carl Zeiss Microimaging Inc. Boston MA, U.S.A)
- Vortex (Vortex Genie® Scientific Industries NY, U.S.A)
- FACS CantoII (Becton, Dickinson and Company Ltd)
- Cryostat (*Leica* CM 3050 Solms, Germany)
- -80°C freezer (Sanyo scientific Indianapolis IN,U.S.A)
- Cesium 137 irradiator (MDS, Nordia, Ottawa, Ontario, Canada)
- -20°C freezer (Fisher Scientific Pittsburgh PA, U.S.A)
- 4-8°C fridge (Fisher Scientific Pittsburgh PA, U.S.A)
- Automatic pipette (pipetman® Gilson Middleton WI, U.S.A)
- Pipet aid (Drummond)
- Cell sorter (Beckton Dickenson FACSVantage SE cell sorter).
- Nuebauer Haemocytometer.
- Cell Counter
- Aerated circular perspex containers
- Haemavet 950FS Hematology Analyser (Drew Scientific Oxford CT, U.S.A)
- Nanodrop ND-1000 spectrophotometer (ThermoScientific, Nanodrop products Wilmington D.E U.S.A)
- 2720 Thermocycler (Applied Biosystems)
- 7300 RT-PCR system (Applied Biosystems)
- Sterile scissors and forceps

Laboratory consumables

- 15cc tube (Falcon® Becton Dickinson and company Ltd.)
- 50cc tube (Falcon® Becton Dickinson and company Ltd.)
- Flow tubes (Falcon® Becton Dickinson and company Ltd.)
- 1.5mL Eppendorf tubes (Eppendorf Ltd. Hamburg, Germany)
- 16.5 gauge needle (Becton Dickinson and Company Inc.)
- 5mL syringe (Becton Dickinson and Company Inc.)
- 35mm gridded CFC dish (Thermo Fisher Scientific MA, U.S.A)
- Large cell culture dish (Thermo Fisher Scientific)
- Kendall Curity Sterile sponge gauze (Kendall Healthcare Ltd. Mansfield M.A U.S.A)
- Slide rack
- Coverslips (VWR international Inc. West Chester, PA, U.S.A)
- Filter paper (VWR international Inc.)
- Elastic band
- Plastic bags
- Animal lancet GoldenRod® (MEDIpoinc Inc., Mineola, NY, U.S.A)
- Pipette tips (Sharp® Denville Scientific Inc. Metuchen, NJ, U.S.A)
- 5ml, 10ml, 25ml Sterile pipette (Falcon® Becton Dickinson and company Ltd)
- 0.2um Nalgene bottle (Thermo Fisher Scientific)
- Cling Film
- 96 well plate

Laboratory Reagents

- Red Cell lysis Buffer (Sigma-Aldrich Chemical company Ltd St. Louis, MO)
- Trypan Blue (Sigma-Aldrich Chemical company Ltd.)
- MACS magnetic cell sorting kit (Miltenyi Biotec Ltd. Bisley, United Kingdom)
 - Biotin Antibody cocktail
 - Anti-Biotin Microbeads
- 2% Gelatin (Sigma-Aldrich Chemical company Ltd.)
 - Catalogue no. G1393
- Tracking beads (Becton, Dickinson and company Ltd.)
- Vybrant ® CFDA –SE Cell Tracer Kit (Molecular Probes, Invitrogen detection technologies, Eugene, Oregon, U.S.A)
- Phosphate buffered Saline (Gibco® Invitrogen Corporation San Diego CA, U.S.A)
- Iscove's Modified Dulbecco's medium/IMDM (Invitrogen Corporation)
 - Catalog no. 12440
- Methocult (Stem cell technologies Inc. Vancouver, Canada)
 - Catalog no. GF M3434
 - Lot: 08G26993
 - Expiry: 07/2010
- Cal-Ex Decalcifying solution (Thermo Fisher Scientific Inc. MA, U.S.A)
 - Catalog no. CS510-1D
- Sakura Tissue-Tek Optimum Cutting Temperature (OCT) Compound (Sakura Finetek Ltd.)
- Murine M2-10B4 (American Tissue Culture Corporation ATCC number: CRL-1972, Stem Cell Technologies Inc. Vancouver Canada)
- Harris Haematoxylin (Sigma-Aldrich chemical company Ltd.)
 - Lot: HTS-2-32
- Scotts tap water substitute concentrate (Sigma-Aldrich chemical company Ltd)
 - Lot: 078K4366
 - Expires: 07/10
- Eosin stain (Sigma-Aldrich chemical company Ltd.)
 - Lot: HT110-2-32
- Cryoseal XYL mounting medium (Thermo Fisher Scientific Inc)

- Lot: 105167
- Expires 06/10
- Ethylenediaminetetra-acetic acid/ EDTA (Vacutainer® Becton Dickinson and company Ltd)
- HEMAVET 950FS mouse control (Drew Scientific Oxford CT, U.S.A)
- 100% Heat-inactivated Fetal Bovine Serum (Hyclone® Thermo Fisher Scientific Inc. MA, U.S.A)
 - Catalogue no. SH30071.03
- Penicillan/streptomycin (Gibco® Invitrogen Corporation)
 - Catalogue no. 15140-122
- Stem Cell Factor (Research & Diagnostics Systems, Inc. Minneapolis, MN USA).
 - Catalog no. 455-MC
- Flt 3 ligand (Research & Diagnostics Systems, Inc)
 - Catalog no. 427-FL
- Thrombopoietin (Research & Diagnostics Systems, Inc)
 - Catalog no. 488-TO
- Recombinant Pleiotrophin (Research & Diagnostics Systems, Inc).
 - Catalog no. 252-PL
- Anti-Pleiotrophin antibody (Research & Diagnostics Systems, Inc)
 - Catalog no. AF-252-PB
- Heparin (Sigma-Aldrich chemical company Ltd)
 - Catalog no. H3149
- Endothelial Cell Growth Supplement/ECGS (Sigma-Aldrich chemical company Ltd)
 - Catalog no. E0760
- L-Glutamine (Invitrogen Corporation)
 - Catalogue no. 25030-081
- Medium 199 (Invitrogen Corporation)
- Lymphoprep™ (Axis-Shield Oslo Norway)
- Sucrose (Sigma-Aldrich chemical company Ltd)
 - Catalog no. S1888
- FITC labeled anti-mouse Ly-6A/E (sca-1) (Becton, Dickinson and Company Ltd.)
 - Lot: 40174
 - Expiry: 09/2011
- FITC labeled rat IgG1, κ isotype control (Becton, Dickinson and Company Ltd.)

- Lot: 52205
- Expiry: 06/2009
- PE anti-mouse CD117 (c-kit 2B8) (Becton, Dickinson and Company Ltd.)
 - Lot: 74612
 - Expiry: 11/2012
- PE Rat IgG2a, κ isotype control (Becton, Dickinson and Company Ltd.)
 - Lot: 21237
 - Expiry: 05/2014
- APC mouse lineage antibody cocktail (CD3e, CD11b, CD45R/CD220, erythroid cells, Ly-6G and Ly-6c) (Becton, Dickinson and Company Ltd.)
 - Lot: 19745
 - Expiry: 09/2010
- APC mouse lineage isotype control cocktail (Becton, Dickinson and Company Ltd.)
 - Lot: 19744
 - Expiry: 11/2010
- PE-CY5 labeled 7AAD (Becton, Dickinson and Company Ltd.)
 - Lot: 93823
 - Expiry: 11/2009
- FITC mouse Anti-human Ki-67 set ((Becton, Dickinson and Company Ltd.)
 - 51-36524x clone; B56
 - 51-35404x-clone MOPC-2
 - Lot ; 556026
 - Expiry 30/04/2010
- RNeasy Mini Kit (Qiagen, Ambion Inc. Austin Texas U.S.A)
 - RLT lysis solution
 - RNeasy spin column
 - RW1 buffer
 - RPE
 - RNase free H₂O
- cDNA Reverse Transcription Kit (Applied Biosystems, California U.S.A)
 - 10xRT buffer
 - 25x dNTP mix
 - 10x RT primers
 - MultiScribe Reverse Transcriptase

- Nuclease free H₂O
- Taqman *ptn* gene expression assay kit.
 - Taqman gene expression mastermix (Applied Biosystems, California U.S.A)

Laboratory solutions

Cytokine Resuspension Medium

Iscove's Modified Dulbecco's medium

1% FBS

1% Pen/Strep

(Filter sterilize through 0.2um Acrodisc syringe filter or Nalgene bottle)

Endothelial Cell culture Medium

50mL FBS

5mL Pen/Strep

5mL L-Glutamine (Invitrogen Corporation)

0.5mL 1000x Heparin

2 vials ECGS (60mg/L; Dissolve each vial in 5mL M199 and mix before adding)

Bring up to 500mL with Medium 199

10% FBS

500mL DPBS (Dulbecco's Phosphate Buffered Saline)

55mL 100% Heat-inactivated Fetal Bovine Serum

0.5mL Penstrep

10% Gelatin Solution

Melt 2% Gelatin in 37°C water-bath

Add 55mL 2% Gelatin to 500mL PBS.

IMDM base medium

Iscove's Modified Dulbecco's medium 10% FBS

1% Pen/Strep

(Filter sterilize through 0.2um Nalgene bottle)

100ng/mL rPTN

Stock rPTN: 50ug lyophilized powder

50ug rPTN (stock) in 500ul TSF → 100ng/uL rPTN

10uL (100ng/uL) in 10mL TSF → 100ng/mL.

500ng/mL rPTN

Stock rPTN: 50ug lyophilized powder

50ug rPTN (stock) in 500ul TSF → 100ng/uL rPTN

50uL (100ng/uL) in 10mL TSF → 500ng/mL

100ng/g rPTN

250ug lyophilized stock solution in 25mL PBS: 10ug/mL or 2ug/200uL.

Average Balb/c 20g. 20g x 100ng: 2000ng or 2ug.

So ach mouse injected with 200uL of 10ug/mL solution or 2ug rPTN.

10% Sucrose

333uL 30% sucrose stock solution

666uL PBS

15% Sucrose

500uL 30% stock solution

500uL PBS

20% Sucrose

666uL 30% Sucrose Solution

333uL PBS

30% Sucrose solution (Stock)

150uL 100% Sucrose

350uL PBS

TSF

97mL Iscove's Modified Dulbecco's medium

1mL 100X stem cell factor

1mL 100X Flt 3

1mL 100X Thrombopoietin