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The Identification of Novel Genes Differentially Expressed in Haemopoietic Progenitor Cells

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B.Sc.

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

Deakin University, Australia

November 2001
I certify that the thesis entitled: **The Identification of Novel Genes Differentially Expressed in Haemopoietic Progenitor Cells**

submitted for the degree of: **Doctor of Philosophy**

is the result of my own research, except where otherwise acknowledged, and that this thesis in whole or in part has not been submitted for an award, including a higher degree, to any other university or institution.

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I have been fortunate during my PhD studies to be involved with and supported by exceptional scientists, friends and family.

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ABSTRACT

The biochemical and molecular processes that maintain the stem cell pool, and govern the proliferation and differentiation of haemopoietic stem/progenitor cells (HSPCs) have been widely investigated but are incompletely understood. The purpose of this study was to identify and characterise novel genes that may play a part in regulating the mechanisms that control the proliferation, differentiation and self-renewal of human HSPCs.

Reverse transcription differential display polymerase chain reaction (dd-PCR) was used to identify differences in gene expression between a HSPC population defined by expression of the CD34 phenotype, and the more mature CD34 depleted populations. A total of 6 differentially expressed complementary deoxyribonucleic acid (cDNA) sequences were identified. Four of these transcripts were homologous to well characterised genes, while two (band 1 and band 20) were homologous to unknown and uncharacterised partial gene sequences on the GenBank database and were thus chosen for further investigation.

The partial cDNA sequences for band 1 and band 20 were designated ORP-3 and MERP-1 (respectively) due to homologies with other well-characterised gene families. Differential expression of the ORP-3 and MERP-1 genes was confirmed using Taqman™ real-time polymerase chain reaction (PCR) with 3-4-fold and 4-10-fold higher levels in the CD34⁺ fractions of haemopoietic cells compared to CD34⁻ populations respectively. Additionally, expression of both these genes was down regulated with proliferation and differentiation of CD34⁺ cells further confirming higher expression in a less differentiated subset of haemopoietic cells.
The full coding sequences of ORP-3 and MERP-1 were elucidated using bioinformatics, rapid amplification of cDNA ends (RACE) and PCR amplification. The MERP-1 cDNA is 2600 nucleotides (nt) long, and localizes by bioinformatics to chromosome 7. It consists of three exons and 2 introns spanning an entire length of 31.4 kilobases (kb). The MERP-1 open reading frame (ORF) codes for a putative 344 amino acid (aa) type II transmembrane protein with an extracellular C-terminal ependymin like-domain and an intracellular N-terminal sequence with significant homology to the cytoplasmic domains of members of the protocadherin family of transmembrane glycoproteins. Ependymins and protocadherins are well-characterised calcium-dependant cell adhesion glycoproteins. Although the function of MERP-1 remains to be elucidated, it is possible that MERP-1 like its homologues plays a role in calcium dependent cell adhesion. Differential expression of the MERP-1 gene in haemopoietic cells suggests a role in haemopoietic stem cell proliferation and differentiation, however, its broad tissue distribution implies that it may also play a role in many cell types. Characterization of the MERP-1 protein is required to elucidate these possible roles.

The ORP-3 cDNA is 6631nt long, and localizes by bioinformatics to chromosome 7p15-p21. It consists of 23 exons and 22 introns spanning an entire length of 183.5kb. The ORP-3 ORF codes for a putative 887aa protein which displays the consensus sequence for a highly conserved oxysterol-binding domain. Other well-characterised proteins expressing these domains have been demonstrated to bind oxysterols (OS) in a dose dependant fashion. OS are hydroxylated derivatives of cholesterol. Their biological activities include inhibition of cholesterol biosynthesis and cell proliferation in a variety of cell types, including haemopoietic cells. Differential expression of the ORP-3 gene in haemopoietic cells suggests a possible role in the transduction of OS effects on haemopoietic cells, however, its broad tissue distribution implies that it may also play a role in many cell types.
Further investigation of ORP-3 gene expression demonstrates a significant correlation with CD34+ sample purity, and 2-fold higher expression in a population of haemopoietic cells defined by the CD34+38- phenotype compared to more mature CD34+38+ cells. This finding, taken together with the previous observation of down-regulation of ORP-3 expression with proliferation and differentiation of CD34+ cells, indicates that ORP-3 expression may be higher in a less differentiated subset of cells with a higher proliferative capacity. This hypothesis is supported by the observation that expression of the ORP-3 gene is approximately 2-fold lower in differentiated HL60 promyelocytic cells compared to control, undifferentiated cells.

ORP-3 expression in HL60 cells during normal culture conditions was also found to vary with expression positively correlated with cell number. This indicates a possible cell cycle effect on ORP-3 gene expression with levels highest when cell density, and therefore the percentage of cells in G0/G1 phase of the cell cycle is highest. This observation also correlates with the observation of higher ORP-3 expression in CD34+38+ cells, and in CD34+ and HL60 cells undergoing OS induced and camptothecin induced apoptosis that is preceded by cell cycle arrest at G0/G1. Expression of the ORP-3 gene in CD34+ HSPCs from UCB was significantly decreased to approximately half the levels observed in control cells after 24 hours incubation in transforming growth factor beta-1 (TGFβ1). As >90% of these cells are stimulated into cell cycle entry by TGFβ1, this observation further supports the hypothesis that ORP-3 expression is highest when cells reside in the G0/G1 phase of the cell cycle. Data obtained from investigation of ORP-3 gene expression in synchronised HL60 cells however does not support nor disprove this hypothesis.

Culture of CD34+ enriched HSPCs and HL60 cells with 25-OHC significantly increased ORP-3 gene expression to approximately 1.5 times control levels. However, as 25-OHC treatment also increased the percentage of apoptotic cells in these experiments, it is not valid to make any conclusions regarding the regulation of ORP-3 gene expression by OS. Indeed, the observation that camptothecin induced apoptosis also increased ORP-3 gene expression in HL60 cells raises the possibility
that up-regulation of ORP-3 gene expression is also associated with apoptosis.

Taken together, expression of the ORP-3 gene appears to be regulated by differentiation and apoptosis of haemopoietic progenitors, and may also be positively associated with proliferative and G0/G1 cell cycle status indicating a possible role in all of these processes.

Given the important regulatory role of apoptosis in haemopoiesis and differential expression of the ORP-3 gene in haemopoietic progenitors, final investigations were conducted to examine the effects OS on human HSPCs. Granulocyte/macrophage colony forming units (CFU-GM) generated from human bone marrow (ABM) and umbilical cord blood (UCB) were grown in the presence of varying concentrations of three different OS – 7keto-cholesterol (7k-C), 7beta-hydroxycholesterol (7β-OHC) and 25-hydroxycholesterol (25-OHC). Similarly, the effect of OS on HL60 and CD34+ cells was investigated using annexin-V staining and flow cytometry to measure apoptosis. Reduction of nitroblue tetrazolium (NBT) was used to assess differentiative status of HL60 cells. CFU-GM from ABM and HL60 growth was inhibited by all three OS tested, with 25-OHC being the most potent. 25-OHC inhibited >50% of bone marrow CFU-GM and >95% of HL60 cell growth at a level of 1 µg/ml. Compared to UCB, CFU-GM derived from ABM were more sensitive to the effects of all OS tested. Only 25-OHC and 7β-OHC significantly inhibited growth of UCB derived CFU-GM. OS treatment increased the number of annexin-V CD34+ cells and NBT positive HL60 cells indicating that OS inhibition of CFU-GM and HL60 cell growth can be attributed to induction of apoptosis and differentiation.

From these studies, it can be concluded that dd-PCR is an excellent tool for the discovery of novel genes expressed in human HSPCs. Characterisation of the proteins encoded by the novel genes ORP-3 and MERP-1 may reveal a regulatory role for these genes in haemopoiesis. Finally, investigations into the effects of OS on haemopoietic progenitor cells has revealed that OS are a new class of inhibitors of HSPC proliferation of potential relevance in vivo and in vitro.
PUBLICATIONS ARISING FROM RESEARCH CONDUCTED IN THIS STUDY

CHAPTERS 2, 3 AND 4.


CHAPTER 6


XIV
CHAPTER 1

LITERATURE REVIEW

1.1 THE DISCOVERY OF THE HAEMOPOIETIC STEM CELL

The path to the identification of the haemopoietic stem cell began in response to the clinical need for cells capable of protecting humans exposed to minimum lethal doses of irradiation or chemotherapy. Although death due to irradiation was associated with bleeding and infection, the precise cause was initially unknown (reviewed by Thomas 1991). The first insight came with the observation that lead shielding of haemopoietic tissues in the spleen prevented death from otherwise lethal doses of radiation (Jacobson et al 1949). Shortly after, Lorenz et al (1951) using mice and guinea pigs showed that intravenous infusion of syngeneic bone marrow (BM) after lethal irradiation also prevented death from irradiation. It was therefore proposed that chemotherapy and irradiation must ablate a population of cells in the BM that are essential for maintenance of normal short-term and long-term haemopoiesis in an organism. It soon became clear that it was only a small fraction of the donor BM cells that were able to reconstitute haemopoiesis in lethally irradiated hosts (Siminovitch et al 1963). These cells, termed haemopoietic stem/progenitor cells (HSPCs), when replaced by infusion of donor BM, were able to completely repopulate the hosts haemopoietic system for the entire lifetime of that organism (Maximow et al 1924, Till and McCulloch 1961, Siminovitch et al 1963).
The formal demonstration of HSPC function came when it was shown that a single chromosomally marked donor cell clone could repopulate the entire haemopoietic system of an irradiated recipient mouse (Ford et al 1956). Complete, long-term reconstitution of haemopoiesis in myelo-ablated hosts by infused donor HSPCs has since been confirmed by many workers using chromosomally marked donor cells and retroviral gene markers (reviewed by Graham and Wright 1997).

These observations provided the basis of the experimental evidence for pluripotential HSPCs, as in order for one cell to completely repopulate the haemopoietic system of an organism it is necessary for that cell to exhibit the properties of self-renewal and multilineage differentiation. Multilineage differentiation translates into the ability of one primitive cell to proliferate and differentiate into greatly expanded numbers of all the mature blood cell lineages. Self-renewal is "the ability of a pluripotent progenitor to produce daughter cells with identical pluripotent characteristics" (Till and McCulloch 1961, Siminovitch et al 1963, reviewed by Graham and Wright 1997 and Morrison et al 1997).
1.2 THE HAEMOPOIETIC SYSTEM

The ongoing production of mature blood cells during the lifetime of an animal is vital for survival as most blood cells have a limited lifespan. It is estimated that adult human needs to produce between $10^{11}$ and $10^{12}$ mature blood cells per day to compensate for the daily loss of differentiated cells. Haemopoiesis is the complex process whereby a small population of pluripotent HSPCs gives rise to all the more mature blood cell types with their specialised functions. The mature blood cells are produced as the HSPCs differentiate in a hierachial fashion and eventually become committed to a particular mature blood cell lineage (reviewed by Ogawa 1993, Metcalf 1993, Morrison et al 1995).

The haemopoietic system is therefore conceptualised as a hierarchy of progenitor cells at various levels of maturation with gradual overlap, and some movement between the compartments. In this model, the most mature cells are morphologically identifiable as belonging to a particular lineage (granulocytes, erythrocytes, monocytes, macrophages, lymphocytes) and have very limited proliferation potential. The cells in this compartment are derived from committed progenitors cells (CPCs) with a higher, but still finite proliferative potential. CPCs are in turn produced by a population of multipotential HSPCs with self-renewal potential (reviewed by Ogawa 1993, Metcalf 1989, 1993, Morrison et al 1995).

In order to prevent long-term depletion of stem cells and thus ensure an adequate supply of mature blood cells throughout life, the size of the stem cell pool must remain constant. Thus, in normal steady state haemopoiesis, the size of the stem cell population is maintained at a constant level by the balance between stem cell production by self-renewing cell divisions and stem cell loss by differentiation (commitment). This choice between self-renewal and commitment is just one of a number of decisions that punctuate blood cell production. Other options include survival versus apoptosis, proliferation versus quiescence, and periodic choice...
1.3 THE ONTOGENY OF HAEMOPOIESIS

It is currently reported that the earliest site for definitive haemopoiesis during human embryogenesis is the AGM region so designated to indicate the presence of the developing aorta, gonads and mesonephros (Tavian et al 1996, Cumano et al 1996, Medvinski and Dzierzak 1996). This region has been shown to be the richest site of pluripotent HSPCs in the early embryo (Muller et al 1994), and the pluripotent stem cells arising in this region carry many of the cell surface markers typical of adult HSPCs (Sanchez et al 1996). It is postulated that during development, these intra-embryonic stem cells subsequently migrate to the fetal liver (FL) which remains the major organ of blood cell production throughout fetal life. During the second and third trimester, circulating stem cells begin colonising bone cavities and gradually establish adult haemopoiesis in the BM which is maintained as the major adult organ of haemopoiesis (reviewed by Peault 1996). While these transitions occur, highly proliferative haemopoietic stem and progenitor cells migrate from one anatomical location to another in the fetal circulation (Gallacher et al 2000), and are subsequently found in abundance in umbilical cord blood (UCB) at birth (Clapp and Williams 1995).
1.4 SOURCES OF HSPC FOR TRANSPLANTATION

Treatment of certain cancers and leukemias requires the use of chemotherapy or radiotherapy which may destroy vital bone marrow HSPCs. In some cases, these cells must be replaced by equivalent cells from a donor. Additionally, stem cells transplants are the therapy of choice for certain hematological malignancies, bone marrow failure syndromes, immune deficiencies and genetic disorders (reviewed by Rodwell and Taylor 1997)

1.4-1 Adult Bone Marrow

Transplantable HSPCs can be obtained from the BM of the patient themselves pre-chemotherapy (autologous bone marrow transplant (BMT)), or from the BM of a consenting, HLA (histocompatibility loci antigens) matched, related adult donor (allogeneic BMT). Harvesting BM from a donor is a relatively painful and invasive procedure involving hospitalisation and samples taken under general anaesthetic.

Recent data from the International Bone Marrow Transplant Registry (IBMTR) has shown that since 1990, the number of autologous transplants has exceeded that of allogeneic transplants. However, patients with hematological malignancies, BM failure syndromes, immune deficiencies and genetic disorders specifically require allogeneic transplants. These patients are reliant on the donation of BM from a suitable HLA matched donor. The success rate for sibling donations is ~35% whilst that for searches via the volunteer bone marrow registries, is ~30% for caucasians, and is rarely successful for patients from ethnic minority groups who are under represented in the registry of volunteers. The process can take 3 - 6 months (for donor location, counselling and testing) from initiation of a search to transplant. This may be too long for patients with an aggressive or relapsed disease and hence
only 10% of patients for whom a search is performed via the unrelated registry actually go on to transplantation (reviewed by Rodwell and Taylor 1997, To et al 1997).

The use of less well-matched related or unrelated donors for allogeneic BMT increases the frequency of post-transplant complications. The most common and serious complication is acute graft versus host disease (GVHD). GVHD occurs when viable donor T cells recognise different host histocompatibility loci antigens and destroy the tissue of the immunocompromised recipient. GVHD occurs in 20 - 40% of HLA identical and 70 - 90% of HLA non-identical and unrelated transplants. Less common complications are graft rejection and delayed immune reconstitution (reviewed by Rodwell and Taylor 1997).

1.4-2 Mobilised Peripheral Blood (MPB)

In humans, HSPCs are present in low quantities in peripheral blood (PB) during steady state haemopoiesis post-natally. Additional HSPCs from the BM can be mobilised into the bloodstream of an adult by the use of a wide range of subcutaneously injected growth factors, either alone, or in combination. This process is far less painful than a bone marrow donation, and mobilises sufficient stem cells for successful transplant and haemopoietic rescue. These mobilised peripheral blood progenitors (MPB) HSPCs can be harvested by leukapheresis and re-infused into a suitable recipient (reviewed by To et al 1997).

Data from the IBMTR shows that since 1993, MPB has been used more often than BM as a source of HSPCs for transplantation as they offer several advantages. They can be harvested without general anaesthesia, the re-infused cells lead to faster haemopoietic and immune reconstitution compared to BM transplants, and there may be reduced likelihood of tumour contamination in the autologous setting.
Initial studies have suggested that the risk of graft rejection and the risk of developing severe GVHD are similar when using matched related allogeneic MPB as compared with matched related allogeneic BM stem cells. However, again, the major limitation in using HLA matched related sibling donors in transplantation has been that only 30 - 40% of potential recipients in need of such therapy have an HLA matched related family donor (reviewed by To et al 1997 and Cairo and Wagner 1997).

1.4-3 Fetal Liver

During fetal development, the liver is physiologically a part of the haemopoietic system. From the second to the seventh months of pregnancy, and ideally before the onset of lymphopoiesis, the fetal liver can be used for transplantation. It has been shown that FL stem cells can reconstitute successfully both haemopoietic and lymphopoietic systems as shown clinically in children with congenital immuno-deficiencies. Due to obvious ethical reasons, the future use of this source of HSPCs for human transplantation is uncertain (reviewed by Huss 2000).

1.4-4 Umbilical Cord Blood

Prior to birth, the circulation of the fetus is continuous within the fetal placental unit via the umbilical cord. The placenta, serving as the pre-natal site for gaseous, substrate and waste exchange with the supporting maternal circulation, represents a significant reservoir of fetal blood up until the time of birth (reviewed by Rodwell and Taylor 1997). Fetal blood, circulating throughout the cord and placenta, was first recognised as a significant source of haemopoietic stem cells by Knudtzon et al (1974). Subsequent studies confirmed this initial observation, and also demonstrated that the content of HSPCs in UCB greatly exceeds that found in adult
PB and is comparable to that in adult BM (ABM) (Nakahata and Ogawa 1982a and 1982b, Broxmeyer et al 1989 and 1990). This work resulted in an international and multi-institutional collaboration to perform the first successful UCB transplant that was undertaken in a child with Fanconis anaemia (Gluckman et al 1989).

Since then, there has been an expanding interest in the use of UCB as an alternate source of HSPC for transplantation and UCB is now recognised as a precious source of transplantable stem cells, easily obtained and in bountiful supply. To date, more than 1500 UCB transplants have been performed worldwide for children with different hematological and genetic diseases. Furthermore, an international UCB transplant registry has been planned and UCB banking is already in progress in many locations around the world. One of the many advantages of the development of these UCB banks has been the augmentation of the donor pool. However, despite increasing use of UCB as a source of transplantable stem cells, there is still a question as to whether there are sufficient cells in cord blood collections to routinely engraft an adult. Total collection volume and hence white cell content being the main variable affecting success (reviewed by Wagner 1994, Rodwell and Taylor 1997, Cairo and Wagner 1997, Gluckman 2000).
1.5 THE CHARACTERISATION OF THE HUMAN HAEMOPOIETIC STEM CELL

HSPCs are the subject of intense and increasing interest because of their biological properties and medical importance. Thus, over the years, there have been extensive efforts to purify and characterise this population for further research and clinical use. The rarity of HSPCs, and the lack of distinguishing physical characteristics and cell surface markers have hampered these efforts. Initially, physical means were used to enrich the progenitor fraction with limited success because of their low frequencies and lack of physical characteristics. Most HSPC measurements therefore rely on functional assays \textit{in vitro} and \textit{in vivo}. With the invention of fluorescence activated cell sorting (FACS), rare populations could be purified based on the expression of increasing sophisticated patterns of cell surface markers, and then characterised functionally (reviewed by Morrison et al 1995).

1.5-1 Characterisation of HSPCs by Morphology

Electron microscopic studies have revealed little differences in the ultrastructural appearance between stem cells and their immediate progeny of lesser potentiality and proliferative capacity (van Bekkum and Knaan 1978). HSPCs are small, lymphocyte-like, blastic mononuclear cells with few and small mitochondria, a single large nucleus with several nucleoli which occupies much of the cell, and little condensed chromatin (Radley et al 1999). These characteristics render them physically indistinguishable from other primitive cell types in the BM (Furukawa et al 1998). Hence, the purification methods reliant on morphology and other physical characteristics purify a very heterogeneous population of committed and uncommitted progenitors (Morrison et al 1995). Ficoll density centrifugation that separates heterogeneous cell suspensions on the basis of density is however, one
method widely used as a crude enrichment of haemopoietic mononuclear cells (MNCs) which contain HSPCs prior to subsequent manipulations

1.5-2 Characterisation of HSPCs with respect to cell-cycle status

The mammalian cell cycle is divided into M phase (mitosis) and interphase. M phase is composed of prophase, metaphase, anaphase and telophase during which cells condense their already duplicated chromosomes, align them onto microtubular spindle, segregate the sister chromatids, and finally split into two daughter cells. Interphase is divided into three phases;

- $G_1$ - the gap between mitosis and the onset of DNA replication
- $S$ - the period of DNA synthesis
- $G_2$ - the gap between S and subsequent M phases

A further phase ($G_0$) indicates a deeply arrested, metabolically inactive state in which cells are very refractory to re-entering the cell cycle (reviewed by Furukawa 1998). Cells in $G_0$ phase do not replicate DNA and synthesise little or no messenger ribonucleic acid (mRNA) and protein (Stein et al 1995). The $G_1$ and $G_2$ phases are the times not only for preparation of the forthcoming dynamic events (DNA replication in S phase and chromosome segregation in M phase), but during which regulatory decisions of cell fates are made. In $G_1$, cells decide whether to enter a state of quiescence ($G_0$) or to continue cell cycling (reviewed by Furukawa 1998).

Primitive HSPCs are resistant to the effects of cell cycle specific chemotherapeutic agents such as 5-flourouracil (5FU), 4-hydroxycyclophosphamide and radioactive thymidine ($^3$H-Thy) which selectively kill cells in the S phase of the cell cycle (Bruce and Meeker 1967, Fauser and Messner 1979). Additionally, HSPCs appear to be refractory to retroviral infection (Williams et al 1984). These observations indicate that the most primitive HSPC may be mitotically inactive (Varmus et al 1977), and hence residing in $G_0/G_1$ (Lajtha 1979a, Graham and Pragnell 1992). To
investigate this hypothesis, Fleming et al (1993) determined the long term reconstituting potential of BM HSPCs isolated on the basis of cell cycle status. Results demonstrated that cycling cells (S/G2/M) were less effective in long-term reconstitution of lethally irradiated hosts than the relatively mitotically inactive, and hence quiescent stem cells residing in G0/G1. This finding has been supported by many other studies, however only a distinction between cells in G0/G1 versus S/G2/M was possible leaving unanswered the question of whether the haemopoietic potential of cells in G0 is superior to that of cells residing in G1 (Reems and Torok-Storb, 1995, Leemhuis et al 1996, Gothot et al 1997). Wilpshaar et al (2000) demonstrated that UCB HSPCs residing in either G0 or G1 are equally effective in repopulating immune deficient mice suggesting that in this tissue, HSPC activity may not be tightly associated with mitotic quiescence.

The reason for this relative quiescence of the most primitive stem cells is unclear. It has been postulated however, that it may relate to requirements for maintenance of genetic purity of the immortal stem cell pool by acting to prevent myelotoxic insult. Spontaneous mutations in differentiating haemopoietic cells will often be of little consequence as the mutation bearing cell is programmed to die as a normal consequence of differentiation. In contrast, mutations in the stem cell pool would be regarded as permanent given the presumed immortality of this self-renewing population (Mauch et al 1989, Mauch et al 1995).

More recent data suggests that although the majority of haemopoietic stem cells appear to be in a quiescent state under normal circumstances, they may actually be very slowly proliferating rather than deeply arrested (Bradford et al 1997). These observations demonstrate that HSPCs regularly move in and out of the quiescent state which would allow for effective and rapid production of a committed progenitor population. Additionally, results by Habibian et al (1998) and Huttmann et al (2001) demonstrate that while entry into the cell cycle by stem cells may represent an early step in the commitment to differentiation and a decrease in self-renewal and long-term repopulating ability, HSPCs may also enter and exit cell
cycle without impairment to their "stem cell" properties. The ability of cycling haemopoietic progenitors in S/G2/M phases of the cell cycle to regain long-term repopulating and self-renewal ability when they return to G0/G1, further demonstrates the plasticity of the stem cell hierarchy (Habibian et al 1998).

1.5-3 Characterisation of HSPCs using Rhodamine 123 (Rho123) and Hoechst 33342 (Ho342)

Differences in metabolic properties and cell cycle status between HSPCs and more differentiated cells have been exploited in strategies for isolating early haemopoietic cells. Ho342 is a fluorescent dye that binds to the minor groove of DNA and allows the evaluation of DNA content in cells and helps to define the cell cycle phases (Gothot et al 1997, Leemhuis et al 1996). Metabolic activity of similar cells can be estimated by dyes such as Rho123 which stain the mitochondrial membrane (Leemhuis et al 1996).

Primitive HSPCs which stain weakly with both Ho342 and Rho123 are enriched for long term multipotent progenitors in vitro, have greater marrow repopulating ability and have the highest capacity to reconstitute haemopoiesis in lethally irradiated recipients than corresponding brightly stained populations (Baines and Visser 1983, Bertoncello et al 1985, Bertoncello et al 1991, Uchida et al 1996, Leemhius et al 1996, Ratajczak et al 1998a).

It has been postulated that uptake of Rho123 is minimal in dormant cells as a result of the presence of few or inactive mitochondria, whereas metabolically active cells, which require more energy, have a higher uptake of Rho123 (Leemhuis et al 1996). Interestingly however, it has also been found that the intensity of Rho123 staining of mitochondria is not only proportional to mitochondrial activation, but also to the expression of the multi-drug resistance (MDR-1) gene (Chaudhary and Roninson
1991). The MDR-1 gene encodes a membrane bound P-glycoprotein that efficiently pumps out Rho123, hence contributing to the observed phenotype.

A more recent flow cytometric assay for identifying HSPCs defines a side population (SP) of cells that display low Ho342 staining at two different wavelengths (Goodell et al 1996). This SP population is highly enriched for repopulating cells and is present in the BM of all species examined to date (Goodell et al 1997). Dull staining of HSPCs with Ho342 had been attributed to the predominantly G0/G1 status of these cells, however recent evidence suggests that it may be due to efflux of the Ho342 dye by the ABC transporter Bcrp1/ABCG2 (Zhou et al 2001).

1.5-4 Characterisation of HSPCs by Colony Forming Unit - Spleen (CFU-S)

The original assay for HSPCs was the CFU-S assay (Till and McCulloch 1961). This assay is based on the ability of HSPCs present in donor BM to rescue a potentially lethally irradiated recipient by colonising the ablated haemopoietic tissues. In brief, the method involves injection of HSPCs from donor haemopoietic tissues into lethally irradiated recipients. Within the first two weeks post-transplantation, primitive BM progenitors (operationally defined as CFU-S) from the donor tissue migrate to, and seed into the spleen of the irradiated recipient where they produce readily visible colonies containing differentiating haemopoietic cells of myeloid, erythroid, lymphoid and megakaryocytic origin (Till and McCulloch 1961). The linear relationship between the number of cells injected and number of spleen colonies produced provides a convenient quantitative assay for these multipotential cells. If the colonies are disaggregated and injected into secondary recipient mice, spleen colonies are produced (Siminovitch et al 1963) demonstrating that in generating the primary colonies, the CFU-S also produced
daughter cells with the properties of CFU-S and hence exhibit self-renewal (reviewed by Graham and Wright 1997).

The capacity for self-renewal and multi-lineage differentiation of spleen colony forming cells is however heterogeneous. Transplantation of day 14 (late CFU-S) rather than day 8 (early CFU-S) colonies produce a greater number of secondary colonies (Lewis and Trobaugh 1964), and colonies examined 7 - 9 days post transplantation generally contained cells of only one lineage (usually erythroid) while colonies examined at 12 - 14 days post-transplantation contained cells of more than one lineage (Curry and Trentin 1967). There are also demonstrable differences in the proliferative status of these early and late CFU-S. Many of the cells producing the early colonies were actively proliferating (Hodgson et al 1982) whereas the late appearing CFU-S were a minimally proliferating population (Becker et al 1965) with most cells in the G_0 state (Lajtha 1979a and b). These data are consistent with the “generation age hypothesis” (Rosendaal et al 1979) that proposes an age-structured hierachial CFU-S compartment. Developmentally early or young CFU-S with considerable self-renewal capacity, in subsequent divisions produce older CFU-S with decreasing self-renewal capacity and increasing probability of commitment to differentiation (reviewed by Graham and Wright, 1997).

For many years the cells giving rise to late CFU-S were considered equivalent to a HSPC population (Magli et al 1982). It is now thought that the most primitive, long term reconstituting HSPCs have “pre CFU-S” activity (Hodgson and Bradley 1979, Van Zant 1984, Lerner and Harrison 1990). They are too primitive to form a spleen colony within 12 days, but they give rise to progenitors that can do so. Accordingly, pre-CFU-S long-term marrow repopulating cells have been demonstrated to be resistant to 5-FU (Hodgson and Bradley 1979) and Rho123(dSSP) (Ploemacher and Brons 1988).
1.5-5 Characterisation of HSPCs by Direct Clonogenic Assays

Lifelong monitoring of multilineage haemopoietic repopulation in a transplanted animal or person constitutes the gold standard for HSPC assays. However, demonstration of complete and stable haemopoietic reconstitution requires a period of months to years, an obvious obstacle for researchers. To facilitate the study of early haemopoietic cells, many strategies have been developed for assaying these cells in vitro over shorter periods of time. However, none of these assays fully satisfies the criteria for HSPC analysis. Rather, it appears that the cells studied in these assays do not achieve the same degree of self-renewal and range of differentiation as they do in vivo. Consequently, results from alternative assays obviously must comply with in vivo repopulation studies (reviewed by Orlic and Bodine 1994, Morrison et al 1995).

HSPCs have been defined as cells that demonstrate the biological properties of self-renewal and multilineage differentiation into all the mature haemopoietic cell types. Multilineage potential can be identified functionally by the ability to produce all the mature blood cell lineages during cell culture. A range of cell culture assays have thus been defined and optimised for growth of all the mature haemopoietic lineages. Such assays involve culturing a suspension of BM, UCB or MPB cells in a semisolid nutrient medium that is supplemented with specific growth factors. If these cells are plated at a sufficiently dilute concentration, the progeny of each cell that is stimulated to grow will form a localised clone of recognisably differentiated cells that can be counted. The number of colonies counted can be extrapolated to divulge information regarding the number of progenitors termed colony-forming units (CFU) in the original sample. The different types of mature cells produced within each colony reflect the degree to which the original CFU has also been differentiatively restricted (how primitive it is in the stem cell hierarchy). Under optimal assay conditions, lineage restricted CFU can be further categorised according to;
a) size of the colonies they produce with the larger colonies indicating the presence of a more primitive progenitor with greater proliferative potential
b) time taken for the clonal progeny to complete their maturation with the more primitive progenitor cells requiring the longer time.

Additionally, by replating cells from these primary colonies and observing capacity for secondary growth under different assay conditions, information regarding self-renewal and the differentiation capacities of the original colony-forming cell (CFC) is also generated (reviewed by Bock 1997).

The proliferation potential of HSPC (defined as the capacity to divide and generate new daughter cells) as well as their expansion potential (defined as the capacity to produce more progenitor cells) appear to be biological features that depend on intrinsic factors. These are related to whether or not the cell is already committed to a particular lineage of differentiation, and if so, the specific haemopoietic lineage to which it belongs and its stage of maturation. However, the ability of a cell to exhibit such potential depends on extrinsic factors that include different cell types and cytokines that form part of the microenvironment in which that cell develops. In vitro proliferation and expansion of HSPCs also depends on variables in the microenvironment such as type of culture medium, concentration and type of growth factors added to the media, medium change schedules, temperature, presence or absence of serum, number of cells plated per culture etc. Hence, the conditions for each assay must be strictly defined and carefully adhered to in order for the results obtained to be meaningful (reviewed by Metcalf 1977, Metcalf 1993, Bock 1997).
Each clonogenic assay gives different information regarding the state of the stem cell compartment, and can be grouped into classes based on the characteristics of the cells produced in the colonies. Characteristics assessed include:

a) colour  
b) Colony morphology  
c) Cellular morphology  
d) Immunostaining (reviewed by Bock 1997)

1.5.5.1 Colony Forming Unit-Granulocyte/Monocyte (CFU-GM), Burst-Forming Unit-Erythroid (BFU-E) and Colony Forming Unit-Granulocyte/Erythrocyte/Monocyte/Megakaryocyte (CFU-GEMM)

Mature committed progenitors form colonies that reflect their differentiative pathways. For example, progenitors that are committed to the pathway of erythroid maturation will only form colonies containing this cell type under the appropriate growth conditions (CFU-E or BFU-E). Similarly, committed progenitors for the other mature blood cell lineages will only form colonies for their differentiated cell type (CFU-Megakaryocyte, CFU-Granulocyte). A slightly more immature committed progenitor cell is able to form colonies containing two mature cell types (CFU-GM) and a cell with higher multi-potential can form colonies in semisolid media comprising mixtures of granulocytes, erythroblasts, megakaryocytes, and macrophages (CFU-GEMM). Cells that give rise to CFU-GEMM survive short-term exposure to $^3$H-Thy indicating that they are not in active cell cycle (reviewed by Gordon 1993). In contrast, exposure to $^3$H-Thy kills 20 - 30% of the cells giving rise to CFU-GM and BFU-E suggesting that these committed progenitor cells are actively cycling (reviewed by Graham and Wright 1997, Bock 1997).
1.5-5.2 Colony Forming Unit-Blast (CFU-B)

Haemopoietic cells show no signs of cytoplasmic maturation during the entire stages of haemopoietic development, except for the last few cell divisions. These immature cells are termed “blast” cells and they form loosely arranged, occasionally clumped almost homogenous populations of small, round, refractile cells. Progenitors at a wide range of developmental stages may produce blast cell colonies if these colonies are identified before the constituent cells reach the terminal stages of maturation. Therefore, it is important to define the time frame at which these colonies are identified. A true CFU-B is identified late in the culture life when colonies present from more mature progenitor cells have already reached terminal maturation. Hence, under these conditions, a CFU-B represents a more primitive progenitor than, for example, a CFU-GM (reviewed by Gordon 1993, Bock 1997, Graham and Wright 1997).

Murine CFU-B were originally identified as small colonies consisting of only 50-500 blast cells on day 16 of culture (Nakahata and Ogawa 1982b). Further studies revealed that the growth of these colonies is delayed relative to the formation of CFU-GM and BFU-E because these colonies are derived from cell cycle dormant progenitors that were 5FU resistant and displayed high replating efficiencies (Nakahata and Ogawa 1982b, Koike et al 1986, Leary et al 1987). Colonies produced by secondary re-plating of CFU-B contained new CFU-B indicating that CFU-B cells reproduce themselves during colony growth and demonstrate some self-renewal. Alternative conditions for lineage commitment and differentiation can be used in the secondary culture in order to determine the functional spectrum of CFU-B (reviewed by Gordon 1993, Bock 1997, Graham and Wright 1997).
1.5-5.3 High Proliferative Potential Colony Forming Cells (HPP-CFC)

HPP-CFC are a heterogeneous population of cells resistant to 5FU toxicity, which generate very large colonies containing greater than 50,000 cells (>0.5mm in diameter) at 10 - 12 days of culture in agar (Bradley and Hodgson 1979, McNiece et al 1989, Bertoncello et al 1991). They have extensive proliferative capacity and possess some self-renewal potential as measured by their ability to be replated from secondary to tertiary and tertiary to quaternary plates (Lu 1993). As secondary replated murine and human HPP-CFC produce colonies that contain progenitors of granulocytes, erythrocytes, and megakaryocytes, HPP-CFC are considered be more primitive than the CFU-GEMM and closely correlated to the pre-CFU-S (McNiece et al 1990). Experiments by various researchers demonstrate that HPP-CFC can be discriminated further into functionally distinct populations on the basis of growth factor requirements and physical characteristics (McNiece et al 1986, Bertoncello et al 1991, Yoder et al 1993, Kriegler et al 1994, Lowry et al 1995). Those with the most demanding requirements for cytokines being associated with long-term repopulating cells (McNiece et al 1986).

1.5-5.4 Cobblestone-Area-Forming Cell (CAFC) Assay

When murine marrow is added to preformed stromal layers, colonies of adherent haemopoietic cells (cobblestone areas) can be observed (Cohen et al 1982). Following up this observation in the human system, Gordon et al (1985) described a human cell type that adhered to stroma and produced colonies of undifferentiated blast cells. Replating of these colonies showed limited self-renewal and the presence of CFU-GEMM, CFU-GM and BFU-E suggesting that CAFC are ancestral to CFU-GEMM (Gordon et al 1987). In the murine system, cells generating early appearing CAFC (days 7 - 10) appeared to co-enrich with CFU-S and committed progenitors. On the other hand, cells forming late appearing CAFC (Day 28) co-enriched with cells providing short-term haemopoietic reconstitution (Ploemacher et al 1989, 1991). Although CAFC determined at weeks 5 - 8 correlated closely with in vivo repopulating ability (Ploemacher et al 1991), there is
not sufficient evidence to support the hypothesis that CAFC represent a HSPC population (Dowding and Gordon 1992).

1.5-6 Characterisation of HSPCs by Secondary clonogenic Assays

This group of assays includes assays for progenitor cells that do not themselves form colonies in semisolid media, but instead can produce clonogenic progeny. Test populations are cultured for several weeks and then analysed for the number of clonogenic cells present (reviewed by Bock 1997).

1.5-6.1 Long-Term Culture (LTC) Assays

Stromal cell haemopoiesis has been extensively studied as a means for reproducing almost entirely the *in vivo* environment and thus the important inter-relationships between HSPCs and stromal cells of the microenvironment (Dexter et al 1977). Aspirated BM contains precursors of both the stromal components and haemopoietic cells and consequently, these cultures can be set up by inoculating tissue culture flasks with BM cells. Adherent stromal progenitors will adhere to the flasks and form a confluent stromal layer consisting of fibroblasts, fat cells, macrophages, and endothelial cells. Primitive HSPCs become trapped within the developing stromal layer and undergo haemopoiesis (Coulombel et al 1983). Alternatively, long-term BM cultures can be set up by growing the stromal layer to confluence, and then adding haemopoietic cell suspensions. In this case, the HSPCs adhere to the stromal layer (Verfaillie et al 1990). The two-stage system is useful in that it can be used to detect primitive HSPCs in sources such as blood that do not contain cells capable of producing a stromal layer. As in the CAFC assay, stromal cell lines have been employed to replace primary human marrow adherent feed layers (Tsai et al 1986, Sutherland et al 1991).
Several groups have established various LTC assays which detect an LTC initiating cell (LTC-IC). After 5-8 weeks, primary stroma supported cultures are scored for replatable CFUs that are released into the culture supernatant and assayed in semi-solid culture systems. The LTC-IC assay assesses a more primitive cell than any category of CFU by virtue of their ability to generate granulopoietic, erythroid and multilineage CFU for periods of 5 weeks or more when co-cultured with supportive marrow stromal cells in appropriate nutrient media LTC-IC and CAFC share a series of functional and phenotype qualities. A considerable proportion of both are resistant to cytotoxic drugs and virtually all the LTC-IC and CAFC activity is contained in the Rho123<sup>dim</sup> subset of human fetal and ABM (reviewed by Eaves 1998, Bock 1997).

Crooks and colleagues extended the period of standard long-term stromal co-cultures supplemented with interleukin-3 (IL-3) and stem cell factor (SCF) to over 60 days (Hao et al 1996). They observed two peaks of CFU production, the first during days 35-60 from LTC-IC and the second during days 60-100 from extended LTC-IC (ELTC-IC). Clones that appeared after 60 days from single late proliferating cells contained ten-fold more cells than from early proliferating cells. Whereas retroviral gene marking of LTC-IC showed efficient gene marking, ELTC-IC was not marked with vector. These studies suggest that ELTC-ICs represent a more primitive progenitor population than LTC-IC (reviewed by Bock 1997).

1.5-7 Characterisation of HSPCs by Transplantation Studies

*In vitro* assays have greatly expanded our knowledge of the phenotype of primitive human progenitor cells and their lineage relationships. Undoubtedly however, the gold standard for the definition of a HSPC is the ability of that cell to establish long term haemopoiesis in the whole animal (Morrison et al 1995). Transplantation
studies in mice (Williams et al 1984, Keller et al 1985) and cats (Abkowitz et al 1995) have demonstrated that with time, the progeny of a single cell may reconstitute the entire haemopoietic system (Turhan et al 1989). This would suggest that a single HSPC could establish a compartment of immature progeny sufficient for meeting the needs of the organism throughout its entire lifetime. Haemopoietic cells that possess long-term reconstituting (LTR) capacity when infused into lethally irradiated mice are termed LTR cells (LTRCs). LTRCs have been further characterised using serial transplantation whereby donor cells are infused into a lethally irradiated primary recipient. When reconstitution of the haemopoietic system has been established in the primary recipient, donor cells from the primary recipient are infused into a lethally irradiated secondary recipient, and so on into tertiary etc (reviewed by Bock 1997).

Assays for LTRC have been very useful for characterising putative HSPC subpopulations in mice. Characterisation of the human HSPC by measurement of LTR capacity poses obvious ethical and time constraints. Although some human haemopoietic cells characterised in vitro have the multipotential and proliferative properties of HSPCs, their capacity for LTR has not yet been established. Until this capacity has been confirmed, the human HSPC populations defined by in vitro assays cannot be considered the equivalent of a LTR HSPC. Consequently, various strategies have been pursued to develop an animal model for human haemopoiesis. Ideally, this model would provide the critical microenvironment for the engraftment of human haemopoietic cells without mediating rejection or permitting GVHD. Growth and differentiation of human cells would be supported and reflected by a physiological distribution of primitive and differentiated human cells in the host (reviewed by Bock 1997).

Over the past few years, novel chimeric animal models have been developed that may significantly enhance our ability to analyse the functions of human HSPC in vivo, but outside of the human body. These models include xenotransplantation of human haemopoietic cells in immunodeficient mice and pre-immune fetal sheep.
Recent results from these chimeric systems suggest that these animals provide a permissive environment for the study of human cells in vivo (reviewed by Bock 1997).

1.5-7.1 Characterisation of the human HSPC by transplantation into Immunodeficient Mice

Severe combined immuno-deficient (SCID) mice lack T cell and B cell function. Non-obese diabetic (NOD) mice lack T cell and B cell function, and show low natural killer (NK) cell activity and defective macrophage function. SCID mice have been backcrossed with NOD mice to produce SCID/NOD mutants. These mice are severely immunocompromised and provide a permissive microenvironment for the engraftment of human haemopoietic cells (Bosma et al 1983, Shultz et al 1995). Therefore they serve as excellent models for in vivo studies on the biology of human HSPC both from ABM and UCB. Engraftment of HLA mismatched human haemopoietic cells into SCID/NOD mice has been achieved from various sources including FL, UCB, ABM, or MPB (Nolta et al 1994, Vormoor et al 1994, Bock et al 1995, Larochelle et al 1996). Their developmental potential can be monitored based on the representation of donor-type cells over time and the ability to transfer progenitor activity to human fetal bone or thymus in secondary SCID/NOD recipients (Chen et al 1994).

Using transplantation of human haemopoietic cells into SCID/NOD mice, Dick and colleagues (Lapidot et al 1992, Larochelle et al 1996, Kamel-Reid and Dick 1988) identified human long-term SCID re-populating cells (SRC) based on their ability to initiate multilineage engraftment. Using cell purification, retroviral gene marking and limiting dilution approaches, several independent research groups have established that SRC capable of engrafting NOD/SCID mice are biologically distinct form LTC-IC and ancestral to these progenitors (Larochelle et al 1996, Bhatia et al 1997, Bhatia et al 1998).
1.5-7.2 Characterisation of the human HSPC by transplantation into pre-immune fetal sheep

In sheep, long term engraftment of human haemopoietic cells that have been injected into the peritoneal cavity of pre-immune fetuses has been achieved (Flake et al 1986, Srour et al 1992, Zanjani et al 1994). In utero transplanted cells from FL, ABM, and MPB reconstituted haemopoiesis. Human cells could be monitored in all haemopoietic tissues and the PB for at least several months, and human HSPC could be recovered from sheep BM more than 3 years after engraftment. The recovered human HSPC can then be used to engraft secondary recipients for up to several years (Srour et al 1992, Zanjani et al 1994). Engraftment was successful with as few as several hundred progenitor-enriched human BM cells (Kawashima et al 1996).

1.5-8 Characterisation of HSPCs by Cell Surface Antigens

A considerable variety of cell surface antigens have been identified that are expressed by populations of cells that express haemopoietic stem cell characteristics. These cell surface markers have provided an invaluable tool for enrichment of stem cell populations, and investigation of their biological properties both in vitro and in vivo. However, definitive identification of the most primitive stem cell, and the relationship of stem cells within the human haemopoietic hierarchy, has been difficult to clarify due to the heterogeneity of the stem cell compartment. This heterogeneity creates a major barrier in the isolation of discrete populations among putative stem cells for comparative analysis (Morrison et al 1995).
1.5-8.1 Characterisation of HSPCs by expression of the CD34 antigen

An important development was the purification of a CD34 Monoclonal Antibody (moAb) raised against the KG1 leukemic cell line (Civin et al 1984). The antibody recognises a 115kDa glycoprotein present on the cell surface of virtually all human stem and committed progenitor cells (Civin et al 1984, Civin and Loken 1987, Civin and Gore 1993). As cells mature and proliferate, the level of CD34 antigen expression declines so that more primitive stem and progenitor cells express the highest levels (Krause et al 1996).

CD34⁺ BM cells comprise only 1 - 3% of BM MNCs and 0.5% of MPB MNCs, but contain precursors for all haemopoietic lineages (Civin et al 1984, Civin and Loken 1987, Civin and Gore 1993). The CD34⁺ cell content of UCB has also been shown to be around 1% of nucleated cells (Kinniburg and Russell 1993, Fritsch et al 1994, Sutherland et al 1994). Interestingly, the frequency of CD34⁺ cells in UCB decreases with gestational age. At 17 weeks gestation CD34⁺ cell comprise 11% of all UCB MNCs, whereas by week 38 they comprise only 1% (Thilaganathan et al 1994).

The discovery of CD34 as a haemopoietic cell surface antigen has accelerated studies into developmental haemopoiesis. Immunoaffinity purified CD34⁺ BM, FL and UCB cells are 10 - 100 fold enriched for CFUs (CFU-M, CFU-G, CFU-GM, BFU-E, CFU-Meg CFU-mix, CFU-blast), LTC-IC, and HPP-CFC whereas CD34⁻ populations are depleted (reviewed by Krause et al 1996).

The expression of murine CD34 seems to parallel that of human CD34 with expression highest in the murine BM cells enriched for haemopoietic progenitors as assayed by CFU-GEMM, CFU-GM, BFU-E (Krause et al 1994). Just 200 CD34⁺ BM cells are capable of LTR of haemopoiesis in lethally irradiated recipients definitively indicating the presence of a HSPC (Krause et al 1994). Analogous BMT studies in baboons (Berenson et al 1988), rhesus monkeys (Wagemaker et al
1990), and humans (Berenson et al 1991) indicate that a CD34⁺ subpopulation of BM can provide durable donor derived long-term host haemopoietic reconstitution.

Taken together, these data demonstrate that CD34 expression is highest on the earliest haemopoietic progenitors and decreases to undetectable levels by the stage when maturing haemopoietic cell lose the ability to form colonies in vitro (Strauss et al 1986). The CD34⁺ population is heterogeneous and contains all cells that are needed for stable long-term multilineage haemopoiesis and those that are required for short-term production of functional blood cells of individual lineages (Strauss et al 1986, Berenson et al 1988, Andrews et al 1992).

Despite the importance of CD34 as a marker of early haemopoietic stem/progenitor cells, the function of CD34 is still not clear. Recent experiments indicate that CD34 expression may play a role in leucocyte adhesion and homing during the inflammatory response (Krause et al 1996), haemopoietic cell localisation/adhesion to stromal cells in the BM microenvironment (Healy et al 1995), and maintenance of the HSPC phenotype and function (Fackler et al 1995).

1.5-8.2 Characterisation of HSPCs by expression of the CD34 and CD38 antigen

The population of haemopoietic cells defined by expression of the CD34 antigen constitutes a very heterogeneous population of multipotential and lineage restricted cells. In an attempt to further characterise human HSPCs, haemopoietic cells have been further enriched by various cell surface marker combinations within CD34⁺ populations, and tested functionally by in vitro and in vivo methods (Morrison et al 1995).

CD38 is a Type II transmembrane glycoprotein distributed extensively on haemopoietic cells of mice and humans. It is an ectoenzyme and leucocyte activation antigen involved in numerous immune functions (Ferrero and Malavasi 1999). CD38 antigen expression is detectable on approximately 95% of CD34⁺ BM
cells (reviewed by Graham and Wright 1997), and increasing density of the CD38 antigen as well as decreasing levels of CD34 has been described as a feature of progenitor cell differentiation (Terstappen et al 1991, 1992).

More than 1000-fold enrichment of cells with stem cell characteristics can readily be achieved by positive selection for CD34 and negative selection against CD38 (Dick et al 1997). This CD34<sup>+</sup>CD38<sup>−</sup> immunophenotype has been demonstrated to be highly enriched for ELTC-IC, LTC-IC, LTRC and SRC (Terstappen et al 1991, Cardoso et al 1993, Waller et al 1995, Payne et al 1995, Reems and Torok-Storb 1995, Hao et al 1995, Randall et al 1996, Hao et al 1996, Bhatia et al 1997). Additionally, it has been established that human CD34<sup>+</sup>CD38<sup>−</sup> cells, but not CD34<sup>+</sup>38<sup>+</sup> cells, generate sustained re-transplantable multilineage human haemopoiesis in pre-immune fetal sheep transplant model (Civin et al 1996) and in NOD/SCID mice (Larochelle et al 1996).

Reems and Torok-Storb (1995) reported that the proportion of cells in G<sub>0</sub>/G<sub>1</sub> was 85.6% in CD34<sup>+</sup>CD38<sup>+</sup> cells, and 96.1% in CD34<sup>+</sup>CD38<sup>−</sup> cells. More recently, Dao et al (1998) reported that a higher percentage of these CD34<sup>+</sup>CD38<sup>−</sup> cells are in G<sub>0</sub>. As expected, unlike CD34<sup>+</sup>38<sup>+</sup> cells, CD34<sup>+</sup>38<sup>−</sup> cells do not readily respond to cytokines such as interleukins-3 (IL-3) IL-6, and stem cell factor (SCF) (Reems and Torok-Storb 1995). In fact, three days cultivation in liquid medium is necessary before a significant proportion of CD34<sup>+</sup>38<sup>−</sup> cells initiate growth and proliferation (Donahue et al 1996, Uchida et al 1997, Agrawal et al 1996). Accordingly, CD34<sup>+</sup>38<sup>−</sup> cells resist transduction by retroviral vectors (Dao et al 1998, Hao et al 1996). Taken together, this data supports the conclusion that unlike CD34<sup>+</sup>CD38<sup>+</sup> cells which are primed to enter active cell cycle and easily expand to committed progenitors, CD34<sup>+</sup>38<sup>−</sup> cells represent a more primitive, quiescent HSPC with higher potential for self-renewal.

While the exact function of CD38 is not known, ligation of CD38 suppresses human lymphopoiesis and myelopoiesis (Kumagai et al 1995, Todisco et al 2000).
Todisco et al (2000) caution that the lesser growth potential of sorted CD34+38+ cells may be at least in part, caused by signals mediated by anti-CD38 antibodies used in the sorting process. The significance of this finding remains to be elucidated.

1.5.8.3 Characterisation of HSPCs by expression of CD34 and c-kit

Another functionally and phenotypically important HSPC cell surface molecule is c-kit. The c-kit proto-oncogene encodes a transmembrane receptor (CD117) with tyrosine kinase activity that is expressed on haemopoietic cells (Yarden et al 1987, Geissler et al 1988). The ligand for c-kit, known variously as steel factor, mast cell growth factor or SCF, is a cytokine that plays a relevant role in haemopoietic progenitor cell viability and proliferation (Geissler et al 1988, Zsebo et al 1990).

Various studies have found that the majority of quiescent progenitors, multilineage colony forming cells, CFU-GEMM, LTC-IC and LTRC are found in the CD34+c-kitlo population of haemopoietic cells. Other studies rejected this population of cells because of their low proliferative response to growth factor stimulation (Gunji et al 1993, Laver et al 1995, Briddell et al 1992, Kawashima et al 1996, Simmons et al 1994).

However, it has been shown the expression of c-kit on purified CD34+c-kit+ HSPCs from UCB is clearly induced by differentiation (Sogo et al 1997). Studies in mice parallel this finding (Katayama et al 1993) and indicate that the c-kitlo cells are ancestral to the c-kitbright cells, and the expression of c-kit on these cells is a step in the maturation of these cells. The induction of c-kit expression would be accompanied by the acquisition of reactivity to SCF hence accounting for superior proliferative response. Taking the dormancy of HSPCs into consideration, it would be logical for HSPCs not to express c-kit to protect them from stimuli that would induce them into cell cycle. In this sense, c-kitlo cells would be more primitive than c-kit+ cells (Ikehara 2000).
1.5-8.4 Characterisation of HSPCs by the absence of expression lineage specific antigens

A mixture of lineage specific antibodies can also be used to purify primitive stem cell populations. These lineage specific antigens are a mixture of cell surface markers that are associated with more mature and committed progenitors (CD2, CD3, CD7, CD10, CD14, CD16, CD19, CD24, CD33, CD56, CD61, CD64, CD66b, CD71 and Glycophorin A). The lineage committed subsets are characterised by CD34⁺CD38⁻ and co-expression of one or more of the lineage specific antigens depending on the lineage commitment (Terstappen et al 1991, Huang and Terstappen 1994, Olweus et al 1995). Cell populations selected and enriched for repopulating cells (Goodell et al 1996, Goodell et al 1997) lack expression of these lineage markers and are termed Lin⁻.

1.5-8.5 Characterisation of HSPCs by expression of CD34 and Thy1/CD₉₀

The precise function of the cell surface antigen Thy-1 is currently unknown. It has been suggested that Thy-1 is involved in haemopoietic cell development possibly by mediating a negative signal that results in inhibition of cell proliferation (Mayani and Lansdorp 1994). A significant proportion of human UCB CD34⁺ cells express Thy-1 and these cells are reportedly stem cell enriched. In contrast, the majority of committed progenitors are CD34⁺Thy1⁻ (Craig et al 1993, Rappold et al 1997, Baum et al 1992).

A number of other studies indicate that candidate human HSPCs from fetal BM (Baum et al 1992), ABM (Murray et al 1994), MPB (Murray et al 1995, Sutherland et al 1996), UCB and FL (Craig et al 1993, Mayani and Lansdorp 1994) can reproducibly be defined phenotypically as CD34⁺Thy-1⁺Lin⁻. CD34⁺CD38⁻ cells can also be subdivided according to the presence of Thy-1, although this combination of cell surface markers appears to be a less reliable tool for separation of the HSPC population. For example, in fetal tissue and UCB, cells with high replating potential and/or stem cell activity co-exist in the CD34⁺CD38⁻Thy-1⁺ and CD34⁺CD38 Thy-
1- fractions. In contrast, stem cells from MPB were found exclusively in the CD34^+CD38^-Thy-1^+ cell fraction (Huang et al 1998, Korbling et al 1995).

1.5-8.6 Characterisation of HSPCs by expression of CD34 and HLADR, CD45RA, CD71, KDR or Flt-3

Additional studies investigating the expression of various cell-surface antigens allow further subdivision the CD34^+ HSPC population. As such, it is hypothesised that the more primitive progenitors express undetectable levels of CD71 (Lansdorp and Dragowska 1992, Mayani et al 1993), and CD45RA (Lansdorp et al 1990, Mayani et al 1993). In contrast, more primitive CD34^+ HSPCs from human ABM, UCB, MPB reportedly co-express KDR (vascular endothelial growth factor receptor 2) (Ziegler et al 1999) and the cytokine receptor Flt-3 (Rappold et al 1997, Gotze et al 1998, Xiao et al 1999).

It has been proposed that early haematopoietic progenitors express major histocompatibility complex class II molecules such as HLA-DR as a mechanism for the induction of self-tolerance during ontogeny (Agrawal et al 1991). However, it is still controversial whether the earliest identifiable HSPC is HLA-DR^+ or HLA-DR^+ (Sroor et al 1991). Huang and Terstappen (1992) reported that a CD34^+HLA-DR^+ pluripotent stem cell could give rise to CD34^+HLA-DR^+Lin^- HSPC. However, a CD34^+HLA-DR^+ cell is still able to develop cells of all lineages and is therefore considered to be representative of a stem cell population (Huang and Terstappen 1994). This was confirmed by Sroor et al (1993), who reported that CD34^+HLA-DR^+ marrow cells give rise to more differentiated precursors, while HPP-CFC are contained predominantly among the CD34^+HLA-DR^+ progenitors.

1.5-8.7 Characterisation of HSPCs by expression of CD34 and AC133

Miraglia et al (1997) described the production of AC133, a monoclonal antibody (moAb) that binds to a novel cell surface antigen present on a CD34^+ subset of HSPCs derived from human FL, ABM, and PB (Yin et al 1997). AC133 moAbs bind to
a glycosylated protein with 5 transmembrane domains. Although the precise function of AC133 is not known, it is postulated to be involved in cell-cell contact and cell signaling (Miraglia et al 1998).

*In vitro* clonogenic assays have demonstrated that the CD34\(^+\)/AC133\(^+\) population from ABM and UCB contains the majority of clonogenic cells, HPP-CFC and LTC-IC (Yin et al 1997, de Wynter et al 1998, Goussetis et al 2000). Additionally, *ex vivo* expansion of CD34\(^+\)/AC133\(^+\) cells causes a significant increase in CD34\(^+\) cells, CFU-C and HPP-CFC whereas culture of CD34\(^+\)/AC133\(^-\) cells shows a limited increase in committed progenitor cells only (Goussetis et al 2000, Matsumoto et al 2000). In contrast to their AC133\(^-\) counterparts, CD34\(^+\)/AC133\(^+\) cells have also been demonstrated to engraft successfully, and provide long-term repopulation in NOD/SCID mice and in fetal sheep (de Wynter et al 1998, Yin et al 1997).

1.5-9 The CD34\(^+\) stem cell

The use of CD34 antibodies for enumeration and isolation of HSPCs is well established both clinically and experimentally. Recent studies however indicate that the CD34\(^+\) cell fraction does not include all of the human stem cell activity.

Osawa et al (1996) demonstrated complete and long-term repopulation of the BM of a lethally irradiated mouse by transplantation of a single cell, which did not express CD34 or any other lineage markers. This CD34\(^-\)/Lin\(^-\) cell was however, devoid of short-term repopulating ability. CD34\(^-\)/Lin\(^-\) HSPCs have since been isolated from non-human primate and human haemopoietic tissue (Goodell et al 1997, Bhatia et al 1998). Unlike classically defined CD34\(^+\) HSPCs, the CD34\(^-\) Lin\(^-\) cells express neither Thy-1 nor HLA-DR antigens (Bhatia et al 1998). Additionally, in contrast to Lin\(^+\)CD34\(^+\)/CD38\(^-\) cells, the clonogenic activity of human CD34\(^-\)/CD38\(^-\) Lin\(^-\)cells has been reported to be negligible in short and long-term *in vitro*
assays (Goodell et al 1997, Bhatia et al 1998). Early reports indicated that the low clonogenic activity of these cells was not responsive to \textit{ex vivo} expansion with growth factors (Goodell et al 1997, Bhatia et al 1998). More recent results by Fujisaki et al (1999), Nakamura et al (1999) and Ando et al (2000) challenge these findings. The low \textit{in vitro} clonogenic activity of this primitive HSPC population does however, explain in part, why they were not discovered earlier.

Conflicting reports on the \textit{in vivo} repopulating ability of the CD34$^+$Lin$^-$CD38$^-$ cells have been obtained. Some researchers report comparable, if not superior engraftment and long-term repopulating ability of fresh CD34 Lin$^-$ cells compared to their CD34$^+$ counterparts (Bhatia et al 1998, Zanjani et al 1998, Gallacher et al 2000). Others report complete failure of engraftment (Fujisaki et al 1999, Kim et al 1999, Andrews et al 2000). Interestingly however, all studies indicate that the repopulating ability of the CD34 Lin$^-$ cells can be increased during \textit{ex vivo} culture. This is in contrast to the CD34$^+$Lin$^-$CD38$^-$ cells that rapidly lose their repopulating ability when cultured in the same conditions (Bhatia et al 1998, Ando et al 2000, Fujisaki et al 1999). Also, the CD34$^+$ Lin$^-$CD38$^-$ cells can be induced to differentiate into cells that express CD34 after \textit{in vivo} repopulation, or \textit{in vitro} cytokine stimulation (Bhatia et al 1997, Zanjani et al 1998, Nakamura et al 1999, Ando et al 2000, Gallacher et al 2000).

CD34$^+$Lin$^-$ stem cells have thus been hypothesised to be the most primitive blood cells identified to date (reviewed by Huss 2000). It is therefore surprising that this population comprises a 3 to 4-fold greater number of cells than all other CD34$^+$ sub-fractions combined. However, the frequency of repopulating cells within the CD34 Lin$^-$ fraction was shown to be far lower than that found in the CD34$^+$ subfraction (Bhatia et al 1998, Bhatia et al 1997). Therefore the biological function of CD34$^+$ stem cells more closely resembles the rare frequency expected of such a primitive cell and suggests heterogeneity within the CD34 Lin$^-$ stem cell populations used in these studies, and the need for more reliable markers of the stem cells within this compartment (reviewed by Huss 2000).
A more recent study by Gallacher et al (2000) further sub-divided the CD34-Lin\(^-\) population with the surface marker AC133. A unique subset expressing AC133 and lacking CD7 was found at low frequency within the CD34\(^+\)Lin\(^-\) fraction, which contained all of the progenitor capacity previously thought to be deficient in human CD34\(^+\) stem cells (Goodell et al 1997, Bhatia et al 1998). Furthermore, using defined in vitro culture conditions, these AC133\(^+\)CD34\(^-\)CD38\(^-\)Lin\(^-\) cells were capable of acquiring CD34 and possessed a clonogenic progenitor capacity equivalent to primitive CD34\(^+\) cells. Human cells were detected in the BM of the NOD/SCID mice after transplantation of ex vivo cultured AC133\(^+\)CD34\(^-\)CD38\(^-\)Lin\(^-\) cells, which suggests that this population contains primitive repopulating cells. A contrary report by Gao and colleagues (2001) indicating no engraftment of AC133\(^+\)CD34\(^-\) cells in NOD/SCID mice demonstrates the need for more data before the usefulness of AC133 as a marker of HSPCs can be fully assessed (Huss 2000).

1.5-10 The changing immunophenotype of HSPCs in the stem cell cycle

Clearly, both CD34\(^+\) and CD34\(^-\) primitive long-term repopulating cells have been identified, and the precise relationships between these cells needs to be elucidated. Huss et al (1998) hypothesised that the immunophenotype of the most primitive HSPCs change as part of a "stem cell cycle". Within this model, the most primitive HSPCs are CD34\(^-\) Lin\(^-\)HLA-DR\(^-\)Thy-1\(^-\)CD38\(^-\). These quiescent CD34\(^-\) HSPCs reside within the BM microenvironment until they can respond to external signals such as growth factors or cytokines. Eventually, the CD34\(^-\) stem cell expresses a receptor molecule and growth factor mediated signal transduction can occur. One mechanism of stem cell quiescence is the lack of activating the appropriate signal transduction pathways (Huss et al 2000). If a signal does occur, this stem cell can proliferate (self-renew and remain CD34\(^-\)) or differentiate into CD34\(^+\) haemopoietic progenitors. The CD34\(^+\) progenitors are more readily available to respond to
appropriate signals for expansion of the stem cell pool (self-renewal) or eventual terminal differentiation to mature haemopoietic cells (multi-lineage differentiation). Additionally, it has been demonstrated that circulating CD34⁺ stem cells can return to the BM stroma and down-regulate CD34 expression (Sato et al 1999) to return to a state of quiescence (reviewed by Huss et al 2000).

This model of the “changing stem cell immunophenotype” is also supported by the data of Ogawa et al (2001) and Storms et al (2000) who observed that the majority of stem cells in normal adult mice and human BM (respectively) are CD34⁻ whilst those activated by exposure to cytokines or mobilisation are CD34⁺. Additionally, this model should be extended to include the possibility that the most primitive HSPCs from human and murine fetal tissues are CD34⁻ (Uchida et al 2001, Ogawa et al 2001, Storms et al 2000).

The exact sequence of expression of other cell surface antigens on CD34⁺38⁻Lin⁺ progenitors is not known. Hence, neither is it known definitively which CD34⁺ subset represents the population with the highest self-renewal or multi-lineage potential. Possible candidates include CD34⁺38⁻ (Terstappen et al 1991), CD34⁺HLA-DR⁺ (Huang and Terstappen 1992), CD34⁺CD38⁻HLA-DR⁺ (Huang and Terstappen 1994, Huang et al 1998), CD34⁺Thy-1⁺Lin⁻ (Craig et al 1993) and CD34⁺CD45RA⁺CD71⁻ (Mayani and Lansdorp 1995). Collective interpretation of data is confounded by the quantitative differences in the proportion of candidate stem cells versus lineage committed progenitors among cells preparations derived from different sources. The significant functional differences among cells with the same phenotype from different ontogenic sources also contributes to the difficulty in definitively assigning a phenotype to the most pluripotent CD34⁺ population.

Nevertheless, the expression of HLA-DR seems to be one of the first steps of haemopoietic differentiation (Huang and Terstappen 1994, Huang et al 1998), followed by the acquisition of other cell surface antigens such as Thy-1, c-kit, KDR and Flt-3. Subsequent proliferation and maturation results in acquisition of such
cell surface antigens such as CD38, CD45RA, CD71 and the mature lineage specific antigens. Loss of CD34 expression on these Lin\(^+\) cells is considered a final maturational step in haemopoiesis. These CD34\(^-\)Lin\(^+\) cells express no HSPC characteristics and are the most abundant cell type in MPB, UCB and ABM.

Due to the quiescent nature, low clonogenic capacity and low frequency of true CD34\(^-\) stem cells in the CD34\(^-\)Lin\(^-\) population, concerns remain among haematologists regarding the nature and potency of CD34\(^-\) stem cells. As such, expression of CD34 is still considered a reliable marker for human haemopoietic progenitor cells and selection of the CD34\(^+\) cells from haemopoietic samples is still the most widely used method for enrichment of a HSPC population both for clinical and research use. *In vitro* and *in vivo* assays indicate that such a cell population, although heterogenous, is highly enriched for HSPC activity compared to the CD34\(^-\) population.
1.6 UCB VERSUS ABM HAEMOPOIETIC STEM AND PROGENITOR CELLS

The use of UCB as a source of transplantable stem cells has prompted many research groups to investigate the in vitro and in vivo characteristics of UCB cells with the ultimate goal of both optimising and expanding their clinical use. Results from these studies indicate that although a cord blood donation may not contain as many mature progenitors as a bone marrow donation, it may be of superior quality in many other ways (Cairo and Wagner 1997).

1.6-1 Immunity

Haemopoiesis and host defense in the neonate is developmentally immature compared with the adult and is a significant contributing factor to the increased susceptibility of the neonate to infections. Accordingly, one of the main advantages of UCB transplants over ABM is immunological immaturity of the accessory cells in the graft, including a lower expression of T-cell derived growth factors. In clinical practice, this property results in a reduced incidence and severity of GVHD, and may allow a higher degree of HLA disparity between donor and recipient. Completely HLA-mismatched FL progenitor cells have been used in the treatment of patients with severe combined immunodeficiency and aplastic anaemia (reviewed by Cairo and Wagner 1997, Rubinstein et al 1998).
1.6-2 Cell Cycle

Traycoff and colleagues (1994a) found a significantly higher percentage of UCB CD34\(^+\)HLA-DR\(^a\) in G\(_0\)/G\(_1\) (>97%) compared to only 89% of equivalent ABM. Nevertheless, these cells from UCB exited from dormancy more rapidly than BM cells such that after 36 hours incubation in the presence of SCF, only 55% of the UCB cells remained in G\(_0\)/G\(_1\) compared to 72% in ABM. Interestingly, even in the absence of exogenous cytokines, there was a significant decrease in the proportion of dormant cells in UCB, whereas the proportion of BM cells in G\(_0\)/G\(_1\) remained the same.

Similar studies have indicated that the majority of CD34\(^+\)CD38\(^-\) cells from BM are deeply quiescent (Reems and Torok-Storb 1995, Hao et al. 1995, Jordan et al. 1996). Most of these cells, including those identified functionally as LTC-IC, will enter meiosis within 7 days of exposure to high concentrations of cytokines, although a first division may not be seen before the third day (Reems and Torok-Storb 1995, Petzer et al. 1996a, Agrawal et al. 1996). In contrast, the same cytokines will stimulate human UCB CD34\(^+\)CD38\(^-\) cells to divide one day earlier and most, including the LTC-IC will complete at least one mitosis within the first 5 days (Glimm and Eaves 1999, Hennemann et al. 1999). Analogous experiments with FL CD34\(^+\)CD38\(^-\) cells suggest they can be recruited into cycle even faster (Glimm and Eaves 1999, Brummendorf et al. 1998).

Thus it seems that UCB contains a higher percentage of dormant (G\(_0\)/G\(_1\)) putative HSPCs, which also have the ability to exit from G\(_0\)/G\(_1\) more rapidly. This ability could be one of the mechanisms that give UCB HSPCs a higher proliferative/expansion potential as compared to BM HSPCs.
1.6-4 Frequency of Putative HSPCs according to immunophenotype

Many researchers have investigated the frequency of primitive progenitors, according to immunophenotype, from FL, FBM, pre-term UCB, UCB and ABM (in order of ontogenic age). Results consistently suggest that the more primitive ontogenic source of haemopoietic cells contain the highest percentage of primitive progenitors. This has been reported using the following phenotypes as markers for populations of primitive progenitors;

c) CD34⁺ Lin⁻ (Bhatia et al 1997)
d) CD34⁺HLA-DR⁺ (Cardoso et al 1993)
e) CD34⁺AC133⁺ (Majka et al 2000)

Therefore, although a cord blood donation may supply a smaller amount of MNCs compared to a BM donation for transplantation purposes, the supply of important primitive progenitors required for long-term reconstitution may be comparable, if not superior.

1.6-5 Long Term Culture and Clonogenic Assays

Results using clonogenic assays as a measure of HSPC activity also indicates that more primitive ontogenic source of haemopoietic cells contain the highest percentage of primitive progenitors. Compared to ABM, MNCs from UCB contain up to three times more BFU-E (Hows et al 1992, Steen et al 1994, Mayani et al 1998), a higher proportion of immature CFU-GM (Mayani et al 1998), more abundant immature CFU-MK (Hows et al 1992) and a significantly higher number

1.6-6 Spontaneous Colony formation

Schibler et al (1994) found that 6% of UCB CD34+ cells are capable of forming colonies in semi-solid cultures in the absence of exogenous cytokines. In contrast, BM CD34+ cells fail to produce any colonies under such conditions. Spontaneous growth of UCB CFU-Meg in the absence of added cytokines has also been documented by Deutsch et al (1995). In the former study, Schibler and colleagues (1994) individually analysed the growth factor independent colonies and found transcripts for both granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-3 suggesting that UCB HSPC are capable of producing some haemopoietic cytokines. Cytokine production by UCB CD34+ cells has also been documented by Watari et al (1994, 1996) who demonstrated both at the mRNA and protein levels the production of the cytokines IL-1β by CD34+ cells from UCB. When cells are separated on the basis of expression of CD34+38−, spontaneous colony formation in a classic semi-solid culture system was reproducible only in the ontogenically earliest cells from FL and not in cells from UCB and BM (Weekx et al 1998).
1.6-7 *Ex vivo* expansion

The proliferative capacity of HSPCs also appears to be inversely correlated with ontogenic age and many studies have indicated that *ex vivo* expanded FL and UCB HSPC samples contain more CD34+ cells, CFU and LTC-IC in short (< 1 week) and long-term (2 – 8 weeks) cultures than their adult counterparts. Traycoff et al (1994a,b,) investigated the *ex vivo* proliferative potential of UCB and ABM CD34+HLA-DR+ cells in 5 – 7 day liquid culture. They observed a 2 – 5 greater fold increase in the total number of *ex vivo* expanded CD34+ cells detected in UCB compared to ABM. Similarly, 1 – 4 week expansion of CD34+ cells isolated from UCB was significantly greater than that observed by ABM, as was the number of day 14 CFU-GM and CFU-Meg (Van de ven et al 1995). Similar results have been reported using UCB ABM or MPB cells separated on the basis of CD34+CD45RA+CD71+ (Lansdorp et al 1993), CD34+38+ (Cardoso et al 1993, Hao et al 1995) and CD34+Lin-38+ (Lewis and Verfaillie 2000). Accordingly, comparative measures of CFU after long-term culture also indicate that the proliferative capacity of HSPCs from FL exceeds that UCB and ABM (Nicolini et al 1999, Weekx et al 1998).

1.6-8 Response to cytokines

Weekx et al (1998) investigated ABM, UCB and FL CD34+CD38+ cells for differences in cytokine requirements and response to inhibitors. They found a direct response between ontogenic age and cytokine requirements; that is, FL and to a lesser extent UCB HSPCs have lower cytokine requirements for colony formation than equivalent cells from ABM. This is evidenced by spontaneous colony formation by FL and UCB HSPCs (as previously discussed), and also by the limited effect of exogenously added growth factors on clonogenic growth from FL and UCB. These results are consistent with those reported by other investigators
(Lansdorp et al 1993, Hao et al 1995, Rappold et al 1997, Muench et al 1995) and could be a result of autocrine production of growth factors (as previously discussed). Alternative hypotheses include higher sensitivity or higher expression of growth factor receptors, which might enable ontogenically younger HSPCs to respond to very low concentrations of cytokines (Weekx et al 1998). Presumably, clonogenic growth would be at/near maximal at lower growth factor concentrations therefore little/no response is seen at higher concentrations.

Results from in vivo transplantation studies parallel in vitro findings. Vormoor et al (1994) reported stable human long-term haemopoiesis in SCID mice transplanted with MNCs from human UCB. Interestingly, the authors observed no difference in the level of engraftment in UCB transplanted mice treated with or without the human cytokines granulocyte-colony-stimulating factor (G-CSF) and PIXY321. This was in contrast to studies with ABM cells in which there is a clear improvement on the level of engraftment in mice treated with human cytokines (Lapidot et al 1992). More recently, Hogan et al (1997) have transplanted NOD/SCID mice with purified CD34+ cells from human UCB observing engraftment and long-term reconstitution. Once again, treatment with human cytokines had no effect on the level of engraftment.

Interestingly, UCB CD34+ cells have been reported to be less responsive to suppressive cytokines in clonogenic assays (Lu et al 1993). In fact, macrophage inflammatory protein 1-α (MIP1α) - a cytokine known to inhibit BM clonogenic growth - has been reported to stimulate (de Wynter et al 1998a) or have no effect (Weekx et al 1998) on clonogenic growth of FL and UCB CD34+ cells. This differential response was postulated to be the result of differential expression of receptor subtypes for MIP1α by UCB and BM CD34+ cells (de Wynter et al 1998a). FL CD34+CD38- cells have also been reported to less sensitive to the inhibitory effects of transforming growth factor-β (TGFβ) (Weekx et al 1999) and Interferon-γ (IFN-γ) (Weekx et al 1998) than ABM.
1.6-9 *In vivo* transplantation

Ontogeny-associated differences in the re-populating ability of transplantable human HSPCs have also been reported and indicate that the functional differences between UCB and ABM HSPC are not only observed *in vitro*, but also *in vivo*.

Using the SCID/NOD xenotransplant model and limiting dilution analysis, Wang *et al* (1997) determined the frequency of the SRC in human UCB to be 3-fold higher than the frequency in ABM, and 6-fold higher than in MPB. This study is supported by results from Holyoake *et al* (1999) who developed a simple assay for comparing cells capable of re-populating NOD/SCID mice (competitive re-populating units [CRU]) from FL, UCB and ABM. Sub-lethally irradiated NOD/SCID mice were transplanted with varying numbers of CD34+ cell-enriched suspensions of human FL, UCB and ABM cells. The types and numbers of human cells present in the marrow of the mice were measured 6 to 8 weeks later using flow cytometry, *in vitro* progenitor assays, and secondary transplant endpoints. The average output per injected CRU of very primitive CD34+CD38- cells, LTC-IC, and secondary CRU was found to be highest for FL CRU and progressively decreased (up to >100-fold) for ontologically older CRU.

1.6-10 Homing

Despite the limitations imposed by the total number of stem cells available in a cord blood harvest, it is clear that fewer cells from this source are required per kilogram of the recipient than when ABM is used (Mayani and Lansdorp 1998). This could be because cord blood contains more progenitor cells, or because cord blood stem and progenitor cells have a greater proliferative capacity. An alternative hypothesis is that UCB HSPCs have an enhanced homing capacity which accounts for the faster regraftment potential of these cells, and which would also be in
accord with their physiological role in early life. The term homing refers to a series of events starting with the initial lodgement of stem cells within the bone marrow sinuses, followed by transmigration across the sinusoidal epithelium, and ending with their long-term establishment within the extravascular marrow spaces (reviewed by Yong et al 1999). In this homing process, chemokines such as stromal derived factor 1 (SDF-1) (Aiuti et al 1997) and adhesion molecules play an important role in governing the crucial step of transmigration of circulating stem cells across the vascular epithelium to arrive in the extravascular haemopoietic tissue.

Yong et al and Voermans et al (1999) reported that freshly isolated and growth factor stimulated UCB progenitors migrated more efficiently than MBP and ABM progenitors respectively. The UCB progenitors also responded rapidly to growth factor stimulation with an increase in migratory ability within 24 hours – whereas MPB cells responded only after 72 hours. Expression of CXCR4 - the chemokine receptor for SDF-1 - was lowest in MPB (Yong et al 1999) and equal in UCB and ABM. This difference may account for the relatively lower migratory ability of MPB CD34+ cells, but the higher migrational capacity of UCB CD34+ cells, in comparison to BM cells has a currently unknown intrinsic cause.

1.6-11 Cord Blood Plasma

There is considerable evidence to suggest that UCB plasma contains a factor, or factors, which may be contribute to the superiority of HSPCs from this source. UCB CFU-GEMM, unlike BM CFU-GEMM, gave rise to many secondary CFU-GEMM when replated. However, these secondary colonies were much smaller in size than the primary CFU-GEMM from which they derived (Carow et al 1993). When UCB plasma was added to the combination of growth factors in the culture media, the size of the secondary colonies were increased so that they were now
comparable to the size of the primary colonies. This effect was not seen when pooled adult plasma was added to the culture media. Similarly, the expansion and proliferation of UCB HSPCs has also been assessed in the presence of plasma from UCB and adult PB. It was found that UCB plasma induced significantly higher expansion/proliferation of CD34+ cells than adult plasma (Ruggieri 1994).

Traycoff et al (1994) examined the rate of exit of UCB CD34+ cells from the G0/G1 phase of the cell cycle in response to various cytokine combinations. While BM CD34+ cells failed to enter cell cycle unless stimulated by SCF, UCB CD34+ cells exited G0/G1 phases of the cell cycle in the presence of UCB plasma alone. The sensitivity of UCB cells to substances in UCB plasma was illustrated by their slow exit form G0/G1 when the UCB plasma was replaced by serum free media supplemented with appropriate growth factors. This finding was unique to UCB CD34+ cells. ABM CD34+ cells exited G0/G1 at the same rate whether incubated with serum free media or UCB plasma supplemented with growth factors. This suggests that CD34+ cells in UCB are sensitive to a factor/s in UCB plasma, and that this sensitivity is lost as the UCB HSPCs mature and traverse from fetal to adult stages of life. This sensitivity could be due to the presence of receptors on the UCB HSPC cell surface, or due to the presence of factors, novel or known, secreted into UCB plasma which are absent in PB plasma.

The factor or factors present in UCB plasma responsible for enhancing the proliferation and expansion of UCB progenitors is currently unknown. It may be attributable to a novel factor/s, or may represent the effects of other known cytokines alone or in a unique combination. Current data suggests that UCB plasma contains varying concentrations of IL-1β, IL-6 and tumour necrosis factor (TNF), but does not contain significant levels of IL-3, IL-4 and IL-5 (Carow et al 1993). The reported G-CSF and GM-CSF content of UCB is variable, with some reports indicating its presence in significant quantities (Biesecker and Emerson, 1993) and others not (Carow et al 1993).
1.7 THE GENETIC BASIS OF BIOLOGICAL DIFFERENCES BETWEEN HSPCS AND MORE MATURE, DIFFERENTIATED PROGENY

The characterisation of a stem cell and the mechanisms that control the decision for self-renwal versus differentiation are areas of intense investigation with major clinical relevance, especially for BMT. Clearly, a variety of control mechanisms are required to maintain steady state levels of mature blood cells, as well as to stimulate the rapid production of specific cell types as needed. Studies with cultured haemopoietic cells have shown that the growth, development, survival and death of haemopoietic cells is, in part, tightly regulated by a complex interaction between soluble and membrane bound stimulatory and inhibitory cytokines and their corresponding receptors within the micro-environment of that particular cell. The notion that the behaviour of haemopoietic cells is governed by extracellular signals has led to a frantic search for cytokines and their receptors with the possibility of useful manipulation of blood cell production in vitro and in vivo. Based on genomic studies, it has been estimated that there are 40 - 50 human chemokines (Rollins 1997). Many of these cytokines and many of their receptors, both inhibitory and stimulatory, have been cloned and identified over the last decade but many remain unknown (reviewed by Rollin 1997, Metcalf 1989, Metcalf 1993, Metcalf 1991, Ogawa 1993, Moore 1991).

There are a number of observations indicating that extracellular factors such as cytokines play only a part in control of haemopoiesis and do not appear to control lineage commitment and self-renewal. Instead, they seem to permit the exhibition of a predetermined cellular proliferative and differentiation potential (Ogawa et al 1989, Lansdorp et al 1993, Mayani et al 1993a). These findings suggest that the decisions that ultimately determine the fate of HSPCs are not dictated by the environment, but are instead controlled by currently ill defined intrinsic genetic mechanisms with a developmental component. Thus, it is likely that there are many
genes, both known and many still to be discovered, that affect stem cell function and fate (Shivdasani and Orkin 1996).

Using a variety of techniques, many investigators have begun to define some of the genes that play a part in specifying and controlling the activity of HSPCs. A number of relevant genes that encode transcription factors and receptors for ligands have already been identified. Whilst a thorough discussion of all these potential stem cell regulators is beyond the scope of this review, some of the data obtained from studies investigating a few of these genes is summarised.

1.7-1 The HOX family of transcription factors

HOX homeobox genes were first recognised as an evolutionary conserved family of transcription factors critical to the control of early embryonic development. Sauvageau et al (1994) used degenerate polymerase chain reaction (PCR) primers to detect the presence of HOX genes in purified human stem cell populations. Expression of multiple gene members of the HOX A, B and C clusters has since been demonstrated in haemopoietic cells (Sauvageau et al 1994, Lawrence et al 1996, Daga et al 2000). This expression appears to be restricted to the most primitive cells types such as human CD34+38 ABM cells, and is down regulated in more mature compartments (Sauvageau et al 1994, Lawrence et al 1996, Daga et al 2000). These patterns of expression are suggestive of HOX gene functional roles in early haemopoietic cells. Consistent with this, lack of functional HOXA9 gene led to impairment of myeloid and lymphoid differentiation (Izon et al 1998, Lawrence et al 1997). In contrast, numerous studies have revealed myeloproliferative effects of over expression of several different HOX genes (Thorsteinsdottir et al 1997, Sauvageau et al 1995, Sauvageau et al 1997). Interestingly, these studies have demonstrated HOX gene specific effects at multiple levels of haemopoiesis. For example, HOXB3 over expression impaired lymphoid but enhanced myeloid
development (Sauvageau et al 1997), HOXB8 over expression inhibited myeloid differentiation (Fujino et al 2001) while HOXA10 over expression altered megakaryocyte, macrophage, erythroid and B cell differentiation and was associated with a myeloproliferative syndrome (Thorsteinsdottir et al 1997, Buske et al 2001). Additionally, over expression of the C cluster homeobox gene HOXC4 induced in vitro expansion of committed as well as very early haemopoietic progenitors without perturbing the in vitro differentiation of these cells (Daga et al 2000).

HOX genes have also been implicated in the regulation of leukemic haemopoiesis (reviewed by Look 1997 and Van Oostveen et al 1999). Notably, aberrant expression of HOXA9 and HOXA10 has been identified as consistent diagnostic markers of AML in humans (Golub et al 1999, Kawagoe et al 1999). Indeed, over expression of these two A cluster homeobox genes in murine HSPCs leads to AML in transplant recipients (Thorsteinsdottir et al 1997, Kroon et al 1998).

Of particular interest in the context of HSPC regulation are findings by Sauvageau et al (1995) and Thorsteinsdottir et al (1999) which indicate that HOXB4 over expression leads to enhanced growth of primitive cells both in vitro and in vivo due to a marked increase in the level of HSPC regeneration. It is hypothesised that the potent enhancement of HOXB4 induced HSPC cell growth is due to an increase in self-renewal probability, without impairment of the production and maintenance of mature end cells (Antonchuk et al 2001). These studies highlight HOXB4 as a candidate HSPC regulator that can be exploited to enhance the growth of primitive HSPCs without deleterious effects on HSPC regulation and differentiation.
1.7-2 NOTCH receptors

NOTCH receptors are involved in a variety of cell-fate decisions that affect the development and function of many tissues including those of the muscular system, the peripheral nervous system, the eye, the vascular system (Xue et al 1999) and the haemopoietic system (Artavanis-Tsakonas et al 1999, Ellisen et al 1991). The human NOTCH receptor family consists of four members (NOTCH 1 – 4) (Larsson et al 1994, Sugaya et al 1997) which encode developmentally conserved transmembrane receptors expressed in both embryonic and adult cells. The ligands of the human NOTCH receptors (Jagged1, Jagged2 and Delta, Delta3) are also transmembrane proteins that are believed to activate NOTCH when expressed on adjacent cells. After binding, the intracellular domain of NOTCH (ICN) is cleaved and dissociates from the membrane. ICN is the active form of NOTCH and translocates to the nucleus where it directly binds to transcription factors activating the transcription of specific genes. These in turn regulate expression of tissue specific transcription factors that influence lineage commitment and other events (reviewed by Kojika and Griffin 2001).

The human NOTCH receptor family was first identified as a gene involved in the chromosomal translocation detected in some leukemias (Ellisen et al 1991, Capobianco et al 1997). This finding generated considerable interest as genetic studies in Drosophila melanogaster (D.melanogaster) suggest that the primary role of the NOTCH gene is to maintain cells in an undifferentiated state whether or not those cells are actively dividing (Artavanis-Tsakonas et al 1995). Subsequent studies have demonstrated that four NOTCH transcripts are expressed in haemopoietic cells (Larsson et al 1994), with CD34+Lin- cells expressing NOTCH1 and NOTCH2 (reviewed by Milner and Bigas 1999). Several studies indicate that human NOTCH receptors are involved in T-cell lineage development (reviewed by Kojika and Griffen 2001). However the role of NOTCH signaling in myelopoiesis is ambiguous. Milner et al (1996) and Carlesso et al (1999) showed that ectopic
expression of constitutively active forms of NOTCH1 inhibits differentiation of myeloid progenitor cell lines. In contrast, more recent data by Schroeder and Just (2000, 2000a) showed that activation of NOTCH1 promotes myeloid differentiation.

The role of NOTCH1 in the self-renewal and expansion of HSPCs is also uncertain. Data from some investigations indicate that activation of NOTCH1 reduces the probability of stem cell self-renewal (Schroeder and Just 2000, 2000a), whilst other data supports a role for the NOTCH1 ligands Jagged1, Delta1 and Delta3 in the expansion and survival of HSPCs (Karamu et al 2000, 2001). Notably, constitutive NOTCH1 signaling in haemopoietic progenitors established immortalized cytokine dependent cell lines that generated progeny with both lymphoid and myeloid characteristics in vitro and in vivo (Varnum-Finney et al 2000). More studies are therefore needed to clarify the role of NOTCH1 signaling in myelopoiesis and in the self-renewal and expansion of HSPCs.

1.7.3 The GATA family of Transcription factors

Among the GATA family of transcription factors, at least three are critically involved in haemopoiesis. Although all three are expressed early haemopoiesis, GATA2 is expressed more abundantly in HSPCs (Weiss and Orkin 1995), GATA1 in megakaryocyte/erythrocyte precursors and their progeny (Sposi et al 1992, Martin and Orkin 1990) and GATA3 in common lymphoid precursors and T-cells (Ho et al 1991, Ko et al 1991). Accordingly, the unique expression patterns of the GATA genes in HSPCs and different lineages corresponds to their functional identity. Gene ablation and embryonic stem (ES) cell in vitro differentiation reveals that GATA1 is required for maturation of erythroid and megakaryocyte lineages, and lack of GATA2 expression results in severe defects in early haemopoiesis (Pevny et al 1991, Pevny et al 1995, Shivdasani et al 1997). Although GATA3 null
mice die at embryonic day 11-12 (Pandolfi et al 1995), GATA3 null ES cells were able to develop all the haemopoietic lineages except T-cells (Ting et al 1996).

More recent gene knockout studies demonstrate that both GATA2 and GATA3 were able to rescue the GATA1 null mutation to a certain degree suggesting a functional redundancy among them (Takahashi et al 2000). Functional redundancy is also suggested by data obtained by Chen and Zhang (2001) who demonstrate that although GATA3 expression is normally restricted to lymphoid precursors and committed T cells, over expression in HSPCs results in cessation of cell expansion and selective induction of megakaryocytic and erythroid differentiation, coupled with inhibition of myeloid and lymphoid development. However, numerous experiments with primary cells or haemopoietic cell lines seem to suggest otherwise. For example, enforced expression of GATA1 can induce erythrocyte, thrombocyte and eosinophil differentiation in early haemopoietic cell lines (Kulesza et al 1995, Visvader et al 1992, Yamaguchi et al 1998). In contrast, enforced expression of GATA2 in murine HSPCs was shown to block the differentiation and maintain cells in a more primitive stage in vivo (Persons et al 1999, Briegel et al 1993), or to induce myeloid differentiation in vivo (Heyworth et al 1999). The question therefore of whether there is functional redundancy within the GATA family of transcription factors remains to be addressed. Chen and Zhang (2001) however hypothesise that the specific lineage fate determination by individual GATA proteins is largely regulated at the level of expression in a lineage and developmental-stage restricted fashion, whereas the identity of the GATA factor may not be important.

1.7-4 c-Myb

The transcription factor c-Myb is the cellular counterpart of the v-Myb oncoprotein identified in the avian myeloblastosis virus (AMV) which causes mixed leukemia in chickens (reviewed by Blobel 2000). C-Myb expression is highest in progenitor
cells of the myeloid, lymphoid and erythroid lineages and is down regulated during maturation/differentiation of these cells (reviewed by Weston 1990). Forced expression of c-Myb blocks differentiation and maturation of erythroid and myeloid cell lines (Clarke et al 1988, McClinton et al 1990, Yanagisawa et al 1991), whereas treatment with antisense oligonucleotides reduces proliferation of immature cells of the erythroid, myeloid and T-lymphoid lineages (Gewirtz and Calabretta 1988, Anfossi et al 1989). Disruption of the c-Myb gene in mice leads to lethal anemia during fetal liver haemopoiesis (Mucenski et al 1991). Taken together, this data suggest that c-Myb functions in maintaining haemopoietic precursors in a proliferative state.

1.7-5 The mammalian Runt-domain family of transcription factors

Mammalian cells contain three genes that encode for proteins that share structural and functional similarity to the D.melanogaster protein Runt. These genes for these transcription factors have been designated AML1-3 due to the frequent rearrangement of AML1 in acute myelogenous leukemia (AML) (reviewed by Westendorf and Hiebert 1999). Consistent with its broad expression pattern and the presence of functionally important AML1 binding sites in the promoters and enhancers of myeloid and lymphoid expressed genes, knock-out studies reveal that AML1 is essential for the formation of all definitive blood lineages in the fetal liver (Okuda et al 1996, Niki et al 1997). Additionally, AML-ES cells fail to contribute to haemopoiesis in chimeric mice (reviewed by Westendorf and Hiebert 1999).

1.7-6 SCL/Tal-1

The SCL/Tal-1 gene encodes a basic-helix-loop-helix (bHLH) transcription factor first identified at the breakpoint of a chromosomal translocation with a T-cell acute

Mice homozygous for the deletion of the SCL gene die in utero as a result of the complete absence of blood formation (Robb et al 1995, Shivdasani et al 1995). Chimeric analysis also demonstrated that SCL^-/- ES cells were unable to contribute to any haemopoietic lineage (Robb et al 1996, Porcher et al 1996) suggesting that SCL acts as a critical regulator of haemopoiesis with a non-redundant function in the formation or behavior of HSPCs (Barton et al 1999).

Studies in cell lines and primary haemopoietic cells suggest distinct functions for SCL. Over-expression and antisense experiments in human and mouse erythroid cell lines support a role for SCL in the control of proliferation, self-renewal and terminal differentiation (Green et al 1991, Aplan et al 1992, Chiba et al 1993). Indeed cytokine induced erythroid differentiation of progenitor cells is associated with up-regulation of SCL mRNA whereas levels fall during granulocytic differentiation (Cross et al 1994). In human and mouse progenitor cell lines, enforced SCL expression is associated with both erythroid and megakaryocytic differentiation (Condorelli et al 1997, Valtieri et al 1998, Elwood et al 1998). In contrast, constitutive expression in progenitors prevents differentiation into non-expressing lineages (Tanigawa et al 1993, Hoang et al 1996). In M1 leukemic cell, over expression of SCL inhibits macrophage differentiation (Tanigawa et al 1995) and in normal human HSPCs, SCL over expression blocks granulocytic differentiation (Valtieri et al 1998). Evidence from other studies indicate that SCL may inhibit
apoptosis (Condorelli et al 1997, Leroy-Viard et al 1995, Bernard et al 1998), although the mechanism for this remains unclear.

1.7-7 Kit receptor and Steel/Stem cell Factor

The genes encoding the receptor tyrosine kinase c-kit, and its ligand SCF were identified by cloning of the loci involved in two naturally occurring single gene induced anemias in the mouse (reviewed by Ashman 1999). In normal haemopoietic tissues, c-kit expression appears to be restricted to the progenitor/stem cell compartment, however some differentiated blood cells express this receptor. Early experiments demonstrated expression of the c-kit mRNA or protein by mast cells (Mayrhofer et al 1987, Majumder et al 1988), megakaryocytes (Avraham et al 1992) and approximately 70% of CD34+ cells in the bone marrow including lineage restricted and primitive haemopoietic progenitors (Ashman et al 1991, Simmons et al 1994). A minor proportion of human and murine bone marrow MNCs with lymphoid markers co-express c-kit (Simmons et al 1994, Rico-Vargas et al 1994) consistent with the effect of SCF on lymphopoiesis in vitro and in vivo (Billips et al 1992, McNiece et al 1991, Godfrey et al 1992, Rodewald et al 1995). However, studies with a blocking moAb to c-kit indicate that although required for myelopoiesis, functional receptor is not required for lymphopoiesis (Ogawa et al 1991). C-Kit is down regulated with maturation of all haemopoietic lineages except mast cells that retain high levels of expression (To et al 1994). Peripheral blood cells are mostly negative for c-kit except for rare circulating CD34+ cells (To et al 1994) and a subset of natural killer cells (Matos et al 1993).

The cytokine SCF, the ligand for the c-kit receptor, is expressed throughout the body by stromal cells, fibroblasts and endothelial cells (Heinrich et al 1993). In vitro experiments have shown that it is a potent growth factor for primitive haemopoietic cells and multiple differentiating lineages acting in synergy with other cytokines (reviewed by Broudy 1997). SCF was shown to be a survival factor for primitive
HSPCs but not more differentiated cells (Borge et al 1997). Although it did not promote their self-renewal (Li and Johnson 1994), the effect on survival was seen even when cell proliferation was suppressed (Keller et al 1995). Several reports have also demonstrated suppression of apoptosis of haemopoietic cells by SCF (Carson et al 1994, Gommermann and Berger 1998). In synergy with other cytokines, it has also been shown to induce the proliferation of HSPCs for a limited time, without differentiation and loss of the capacity for haemopoietic reconstitution (Luens et al 1998).

SCF and erythropoietin co-operatively induce erythroid colonies (Wu et al 1997) whilst SCF acting alone can apparently induce mast cell development from immature HSPCs in human blood, ABM or fetal liver (Valent et al 1992, Irani et al 1992). Stromal membrane bound SCF has also been reported to be a chemotactic agent for HSPCs (Okumura et al 1996) promoting cell adhesion both directly by binding to HSPC bound e-kit (Kaneko et al 1991), and indirectly through upregulation of the avidity of β1-integrins for stromal cell components (Kinashi and Springer 1994, Dastych and Metcalf 1994). This adhesion response may be important in maintaining the HSPC in a permissive microenvironment (Bendall et al 1998).

1.7.8 Flt-3 and Flt-3 Ligand

The Flt-3 receptor tyrosine kinase was isolated independently by two different research groups using distinct cloning strategies. Rosnet et al (1991a, 1991b) used low stringency hybridisation and a DNA probe from the macrophage-colony stimulating factor (M-CSF) receptor to isolate a portion of a related DNA sequence. This partial clone was then used to isolate a full-length receptor (Rosnet et al 1991a, 1991b). Matthews et al (1991a, 1991b) used degenerate PCR oligonucleotides based on conserved regions within the kinase domain of tyrosine kinase receptors to isolate a full-length Flt-3 receptor clone.
The ligand for Flt-3 (FLig) has been cloned and experiments suggest that FLig is a cytokine that acts synergistically with a number of other growth factors to promote the proliferation of primitive cells (Lyman et al 1993, Hannum et al 1994). Gene expression studies have shown that FLig is produced by a wide variety of cells and tissues (Hannum et al 1994, Lyman et al 1994, Rasko et al 1995, Lyman and Williams 1995). In contrast, expression of Flt-3 is narrowly restricted. Thus it has been suggested that the cellular response to FLig may be controlled through regulated expression of Flt-3 (Lyman and Jacobsen 1998).

Unlike the expression pattern of c-kit, Flt-3 mRNA expression in human haemopoietic cells is relatively restricted to human lymphoid pre and pro-B cell lines human myeloid and monocytic cell lines and some megakaryocytic cell lines (DaSilva et al 1994, Brasel et al 1995, Meierhoff et al 1995). No Flt-3 expression is observed on erythroid cell lines, mast cells, natural killer (NK) cell lines and T-cell lines (DaSilva et al 1994, Lyman and Williams 1995, Brasel et al 1995, Meierhoff et al 1995). As for c-kit, the majority of adult AML samples are positive for Flt-3 receptor expression as are all B-lineage acute lymphoblastic leukemia (ALL) samples (Rosnet et al 1996, McKenna et al 1996, Carow et al 1996, Birg et al 1992).

Whilst conflicting reports exist Flt-3 expression data in normal haemopoietic progenitor cells appears to be largely restricted to the primitive progenitor cell compartment with expression in CD34+ HSPCs from UCB and ABM (Rappold et al 1997) and in the more primitive CD34+38- subfraction of progenitors (Xiao et al 1999). Expression of Flt-3 is down regulated with maturation of CD34+38- cells to CD34+38+ haemopoietic cells (Xiao et al 1999), with no expression of Flt-3 mRNA reported on mature B-cells and T-cells (Birg et al 1992). Furthermore, CD34+Flt-3+ cells do not up regulate Flt-3 expression (Gotze et al 1998). Conflicting results have also been obtained for Flt-3 expression on monocytes and granulocytes (Birg et al 1992, Rosnet et al 1993, Rappold et al 1997). The expression of Flt-3 in human haemopoietic cells is in agreement with observed effects of FLig on haemopoiesis. FLig enhances the proliferation and or maintenance of primitive CD34+38+ LTCIC

Flt-3 knockout mice have no defects in megakaryocyte and platelet production, but have reduced numbers of pro-B cells in the BM (Mackarehtschian et al 1995). These findings have been confirmed in FLig deficient mice (McKenna et al 2000) and are also validated by the lack of effect of FLig alone or in combination with other cytokines on murine megakaryocyte progenitor cells (Turner et al 1996). In contrast, FLig has been shown to promote growth of early murine B-cell progenitors with no effect on more differentiated B-cell progenitors (Hunte et al 1996). FLig also acts in synergy with Thrombopoietin (TPO) to enhance growth of candidate murine stem cells (Ramsfjell et al 1996) indicating an essential role for FLig and Flt-3 in B cell development from progenitor/stem cells and in the proliferation of primitive cells (Lyman and Jacobsen 1998).

1.7-9 Thrombopoietin (TPO) and its receptor C-mpl

The cytokine TPO has long been known to be the key factor in regulating megakaryocyte and erythroid proliferation and differentiation (de Sauvage et al 1996, Kobayashi et al 1995, Kaushansky et al 1995, Kaushansky et al 1995a). Although gene-targeting experiments of the TPO receptor c-mpl initially showed only loss of platelet forming capacity (Gurney et al 1994), subsequent studies demonstrated a proliferative defect in stem cells (Kimura et al 1998) with both TPO\(^-\) and c-mpl\(^-\) mice demonstrating reduced total haemopoietic progenitor cell numbers (Alexander et al 1996, Carver-Moore et al 1996). In addition, a recent report using flow cytometry to isolate c-mpl positive stem cell has shown that the receptor is present on very early haemopoietic progenitors from mice and humans (Solar et al 1998, Methia et al 1993). These data, combined with in vitro studies of cytokine activity suggests that TPO affects early developmental decisions.

1.7-10 MDR-1 and ABCG2

It is postulated that the Rho123<sup>lo</sup> Ho342<sup>lo</sup> phenotype of a putative stem cell population is due to higher expression of the MDR-1 and Berp1/ABCG2 genes (Chaudhary and Robinson 1991, Zhou et al 2001). The human MDR-1 gene encodes membrane bound P-glycoprotein that efficiently pumps out Rho123 (Chaudhary and Robinson 1991). Similarly, the Berp1/ABCG2 mRNA encodes an ABC transporter that is postulated to contribute to Ho342 efflux from cells (Zhou et al 2001). Expression of both MDR-1 and Berp1/ABCG2 mRNA is highest in primitive HSPCs and sharply down regulated with differentiation and proliferation leading to increased Rho123 and Ho342 retention respectively (Smeets et al 1999, Zhou et al 2001).

More recent experiments indicate that endogenously expressed transporters such as MDR-1 and ABCG2 may not only be conferring phenotypic characteristics to HSPCs, but also function. Results from experiments conducted by Bunting et al (1998 and 2000) indicate that enforced expression of MDR-1 in murine HSPCs causes not only expansion of the stem cell population <i>in vitro</i>, but also results in stem cell expansion and an associated myelo-proliferative syndrome <i>in vivo</i>. Similarly, enforced expression of the ABCG2 cDNA directly conferred the SP phenotype to BM cells, and caused a reduction in maturing progeny both <i>in vitro</i> and <i>in vivo</i> (Zhou et al 2001).
1.7-11 Telomerase and Telomere length

DNA replication in mammalian cells by the enzyme DNA polymerase is incomplete at the 3' end of linear chromosomes (Olovnikov 1996 and 1973). The effect of this “end replication problem” on DNA duplication is that the 3’ end of all linear chromosomes are expected to progressively shorten by approximately 50 – 100nt with each round of cell division (Harley et al 1990, Levy et al 1992). Such chromosome shortening can indeed be observed in all somatic cells to date (Harley et al 1990, Hastie et al 1990), including primitive haemopoietic cells (Vaziri et al 1994).

Telomeres, consisting of both DNA and proteins, are the physical ends of eukaryotic chromosomes. These sequences are approximately 2 – 15 kilobases (kb) long, and consist of a variable number of repeats of the nucleotide sequence 5’-TTAGGG-3’ (reviewed by Blackburn 1991). They play an important role in distinguishing intact from broken chromosomes (Sandell and Zakian 1993) and the stabilisation of chromosome ends thus protecting from nuclear degradation and end to end fusion. Additionally, they assist in the positioning of chromosomes during chromosome segregation and prevent loss of genetic information during cell division as a result of the “end replication” problem (Giraldo and Rhodes 1994).

The product of the telomerase gene is an enzyme that adds telomeric repeats to the ends of chromosomes, thereby maintaining their length. As such, expression of the telomerase gene in mammalian cells has attracted enormous attention in recent years as a gene that may affect genetic stability, and hence the proliferative potential of stem cells. Most human somatic cells do not express high levels of telomerase (reviewed by Blackburn 1991). As such, the telomeric length of the chromosomes within these cells decreases with cell divisions (Iwama et al 1997). In vitro, these cells enter replicative senescence after a finite proliferative life span; usually after 50 – 80 population doublings (reviewed by Harley 1991).
observation is the basis of the "telomere hypothesis of cellular aging" (Harley 1991, Harley et al 1992).

1.8 THE GENETIC BASIS OF BIOLOGICAL DIFFERENCES BETWEEN HSPCs FROM UCB AND ABM

While there has been progress in the identification of genes that appear to play important roles in conferring the stem cell phenotype to haemopoietic progenitors, considerably less is known regarding the actual mechanisms that account for the observed functional differences between UCB and ABM HSPCs. Ultimately, these differences will be due to the way in which genes are regulated in the stem cell compartment throughout the life cycle.

1.8-1 Telomerase gene

Recent studies suggest that the functional differences observed between UCB and BM HSPCs may be related to differences in telomere length. Vaziri et al (1994) purified CD34⁺CD38⁻ cells from UCB and ABM and measured their telomere length before and after 25 days in liquid culture with a selected cocktail of cytokines. They observed that, prior to the cultures, the mean length of telomere restriction fragments (TRF) of UCB cells was about 12kb which was significantly longer than that of BM cells (8kb). After the culture period, as a result of cell proliferation, the mean length of the TRF decreased 1.5 kb both in UCB and ABM cells. In this study, purified CD34⁺CD38⁻ cells from FL were also analysed and there mean TRF was even longer than that of the UCB cells. This study supports proliferation-dependant loss of telomeric DNA in cells of haemopoietic origin. Furthermore, the direct correlation between telomere length and developmental stage of human HSPC suggests that changes in proliferation/expansion potential could be related to changes in telomere length (Vaziri et al 1994, Lansdorp 1995, Lansdorp 1995a). It has not been demonstrated however, that the difference in telomere length between adult and UCB HSPCs is due to differential expression of
the telomerase gene. Interestingly, Engelhardt et al (1997) demonstrated higher telomerase activity in progenitors from ABM compared to those from MPB, UCB and PL progressively. In contrast, Iwama et al (1998) demonstrated a decrease in telomerase activity with age in PB. Any potential role of the telomerase gene in determination of the functional differences between UCB and ABM stem cells is therefore, uncertain.

1.8-2 Genes encoding growth factors and receptors

It is also possible that the superior clonogenic, re-populating and ex vivo expansion potential of UCB HSPCs compared to HSPCs from ABM, is due to differential expression of genes encoding known or novel growth factors and/or relevant receptors. Most studies however have demonstrated only modest or no differences in the expression of mRNA transcripts for several growth factors and receptors between these two populations both unstimulated and stimulated (Oh et al 2000). The level of TGFβ transcripts in unmanipulated ABM CD34+38 cells were consistently and significantly higher than phenotypically similar cells from fetal and neonatal sources (Oh et al 2000). In light of evidence that endogenous TGFβ production may serve as an autocrine inhibitor of primitive HSPC proliferation (Hatzfeld et al 1991, Cardoso et al 1993), it is possible that an up regulation of TGFβ expression in adult HSPCs could plays a role in promoting the superior properties of UCB HSPCs (Oh et al 2000). De Wynter et al (1998a) observed differential expression of the mRNA for two different receptors for MIP1α. The CCr1 and CCr5 receptors were expressed in CD34+ cells from ABM, but only CCr1 mRNA was seen in UCB CD34+ samples. The authors postulate that this differential expression may be responsible for the difference in MIP1α responsiveness between adult and fetal stem cells.
1.8-3 Transcription factors

Developmentally regulated expression of transcription factors may contribute to phenotypic differences in HSPCs from ontogenically different sources. Oh et al (2000) also demonstrated increased expression of c-fos and c-jun mRNA in ABM CD34⁺38⁻ cells compared to equivalent cells from human FL and UCB. This finding, taken together with evidence from Okada et al (1999) demonstrating inhibition of cell cycle progression with forced expression of c-fos, may contribute to the decreased expansion potential of these cells compared to ontogenically earlier cells.

1.8-4 Adhesion molecules

Finally, differentially expression of adhesion molecules may affect homing and migration, and hence re-populating ability of adult and fetal progenitors. Whilst expression of CXCR4 (the chemokine receptor for SDF-1) has been demonstrated to be lowest in MPB CD34⁺ cells (Yong et al 1999) thereby accounting for their relatively lower migratory ability, levels were equal in UCB and ABM. Thus, the higher migrational capacity of UCB CD34⁺ cells, in comparison to ABM cells has a currently unknown intrinsic cause.
1.9 THE IDENTIFICATION OF NOVEL GENES EXPRESSED BY HSPCS

Over recent years, a number of laboratories have initiated studies to identify novel genes whose products may play an important role in stem cell regulatory functions (reviewed by Lemischka 1999). The approaches used in these studies fall into three general categories; (i) identification of novel members of interesting gene families whose features suggest important stem cell regulatory functions (ii) high throughput sequence analysis of cDNA libraries constructed from at least partially purified stem cell populations, and surveying of human cDNA filter arrays (iii) identification of genes with gene expression profiles relatively restricted to at least partially purified stem cell populations (reviewed by Lemischka 2001).

1.9-1 The identification of novel members of interesting gene families

The identification of human homologues of D. melanogaster genes that have been demonstrated to play a role in germline stem cell (GSC) self renewal has provided a useful model to define candidate stem cell regulatory genes (Lewis 1978, Cox et al 1998). Sharma et al (2001) utilised this line of investigation to identify the human homologue of piwi, a D. melanogaster gene that plays an important role in GSC self-renewal (Cox et al 1998, Lin and Spradling 1997). This study demonstrated that the human homologue, termed hiwi is expressed in human CD34+ cells and is down regulated with differentiation of these cells. Transient expression of hiwi in a human leukemic cell line resulted in a dramatic reduction in cellular proliferation, and over expression led to programmed cell death. These studies suggest that hiwi may be an important negative developmental regulator of HSPCs.

Sassetti et al (2000) utilised homologies present in the cytoplasmic tails of the CD34 family sialomucins to identify the novel gene endoglycan. As for CD34, endoglycan
expression was detected in both endothelial cells and CD34⁺ bone marrow cells. Expression was however, also detected in mature monocytes. The role, if any of endoglycan in HSPC function is currently unknown.

1.9-2 High throughput sequence analysis of cDNA libraries constructed from at least partially purified stem cell populations, and surveying of human cDNA filter arrays

The construction and sequencing of HSPC cDNA libraries has generated an enormous number of partial and full length gene sequences, and is a relatively successful way of profiling gene expression in these cells and discovering novel genes. It is however, insufficient to reveal directly whether the genes identified are preferentially expressed, or play a regulatory role in haemopoietic progenitor cells. The challenge therefore is to isolate such meaningful sequences.

Yang et al (1996) made an EST database for CD34⁺ cells by single pass sequencing of 402 clones from a directional library. Thirty-five percent of these were from previously unknown genes, but none of these was differentially expressed in CD34⁺ cells. More recently, Zhang et al (2000a) undertook the characterization of 9866 and 4142 EST sequences obtained from UCB (Mao et al 1998) and ABM (Gu et al 2000) cDNA libraries. After eliminating sequences representing repetitive DNA elements, ribosomal RNA (rRNA) and mitochondrial DNA sequences, known and named genes they obtained 442 full-length open reading frames (ORF) representing undefined genes. They analysed the structural and functional characteristics of each ORF using bioinformatics, and determined differential expression using bioinformatics and macro-arrays. Of 285 genes expressed in UCB CD34⁺ cells, 35 that showed relatively restricted or preferential expression along a given orientation of differentiation were selected for future investigations.
Park et al (2001) utilised construction and sequencing of cDNA library constructed from mouse haemopoietic stem cells to identify a novel regulator of G-protein signaling (RGS18). Expression of human and mouse RGS18 was highest HSPCs, and was down regulated as these cells differentiated into more committed progenitors. Further studies are required however to verify the role of RGS18 in G-protein signaling in haemopoiesis.

By its ability to simultaneously detect and quantitate the expression of thousands of genes at on time, cDNA array technology is greatly improving our understanding of the complex patterns of gene expression in eukaryotic cells. Gomes et al (2001) utilised human cDNA filter arrays to survey the gene expression profile of CD34+ cDNA from human and baboon. A subset of 1554 cDNAs expressed at levels 100-fold and greater than background were described. This list included 595 named genes and 959 ESTs and uncharacterised cDNAs. A significant fraction of these genes are not unique to haemopoietic cells, but are also expressed in other tissues. This reinforces the concept that a tissue is not only defined by the genes that it expresses, but also by the overall pattern and relative abundance of these sequences. Therefore, the results from such studies provide a selection of some of most highly abundant genes expressed in HSPCs, and provide a starting point to develop a profile of the predominant cDNAs that define CD34+ cells (Gomes et al 2001).

1.9-3 Identification of genes with gene expression profiles relatively restricted to at least partially purified stem cell populations

Polymerase chain reaction (PCR) based methods have been used with variable success to investigate gene expression of a human HSPC population. Such methods utilize modifications that act as a screen to preferentially isolate differentially expressed genes. Graf and Torok-Storb (1995) utilized PCR based dd-PCR to identify differences between CD34+CD38hi and CD34+CD38lo cells and identified
one novel sequence that was expressed in the CD38<sup>hi</sup> population at approximately 2.5 times the concentration of the CD38<sup>hi</sup> population. The sequence contained no open reading frame and lacked a polyadenylation site. No further publications related to the characterisation of this gene have been found.

Two recent publications utilized PCR driven subtraction hybridization (SH) to investigate differential expression in HSPC. Zhang <i>et al</i> (2000b) identified four mRNAs expressed by CD34<sup>+</sup>CD38<sup>+</sup> cells that were either absent or present in reduced amounts in other CD34<sup>+</sup> cells. Two of these mRNAs encoded known genes whose expression in hematopoietic cells had not been reported previously, and the remaining two encoded unidentified cDNAs. Similarly, Liu <i>et al</i> (2000) identified 73 cDNA fragments, of which 37% were novel or homologous only to entries in EST databases. One such cDNA (C17) was expressed only in CD34<sup>+</sup> cells and encoded a potential cytokine.

Orlic <i>et al</i> (1999) utilised both dd-PCR and SH to identify stem cell specific cDNAs from highly enriched murine haemopoietic stem cell populations. A total of 25 differentially expressed transcripts were identified using dd-PCR compared to 151 from SH. Although SH was able to identify significantly more differentially expressed genes, only 37 (28.5%) of these sequences represented novel genes compared to 18 (90%) identified by dd-PCR. Further characterization of all the novel genes identified in these studies is required to determine their role in haemopoiesis.
1.10 AIMS OF THE STUDY

Thus whilst considerable progress has been made to identify genes which play a part in establishing and maintaining the stem cell properties of self-renewal and multilineage differentiation in UCB and ABM HSPCs, it is clear that there are still many more genes, both known and unknown, yet to be identified or characterised. Efforts to elucidate differences in gene expression between primitive haemopoietic progenitors from various sources should help to identify mechanisms by which stem cells are triggered into proliferation, differentiation and self-renewal. This knowledge may be used in the development of strategies to reliably exploit the proliferative capacity of UCB and ABM stem cells for use in clinical practice as it relates to stem cell transplantation and gene therapy.

The aims of the present study are;

1. To identify novel genes differentially expressed between a population of HSPCs defined by expression of the CD34 phenotype, and their more mature CD34- counterparts
2. To identify novel genes differentially expressed between UCB HSPCs and ABM HSPCs
3. To confirm differential expression of the novel gene/s identified in haemopoietic cells
4. To obtain the full coding sequence of the novel gene/s identified in haemopoietic cells
5. To investigate possible function/s of the proteins encoded by the novel gene/s identified
6. To investigate gene expression of the novel gene/s identified in physiologically relevant assays
7. To investigate possible functions of the novel gene/s identified in physiologically relevant assays
CHAPTER 2

THE USE OF DIFFERENTIAL DISPLAY POLYMERASE CHAIN REACTION TO IDENTIFY NOVEL GENES EXPRESSED IN HAEMOPOIETIC STEM/PROGENITOR CELLS

2.1 Abstract

The biochemical and molecular processes that maintain the stem cell pool, and govern the proliferation and differentiation of HSPCs have been widely investigated but are incompletely understood. The purpose of this study was to identify genes that may play a part in regulating the mechanisms that control the proliferation, differentiation, and self-renewal of human HSPC from UCB and ABM. dd-PCR was used as a screen to identify differences in mRNA expression between a HSPC population defined by expression of the CD34 phenotype, and more mature CD34 depleted populations. 6 differentially expressed cDNA transcripts were identified. 4 of these transcripts were homologous to well characterised genes, while two (band 1 and band 20) were homologous to unknown and uncharacterised partial gene sequences on the GenBank database. Bands 1 and 20 were designated ORP-3 and MERP-1 (respectively) on the basis of these homologous gene sequences. Differential expression of ORP-3 and MERP-1 was confirmed using Taqman™ real-time PCR with 3 - 4-fold and 4-10 -fold higher levels in the CD34+ fractions of haemopoietic cells compared to CD34+ populations respectively. Additionally, expression of both these genes was down regulated with proliferation and differentiation of CD34+ cells. Differential expression of both MERP-1 and ORP-3
in haemopoietic progenitors implicates a role in haemopoiesis. Further investigation is required to elucidate this possible role.
2.2 INTRODUCTION

The process of blood formation is sustained throughout an individual's life by the proliferation and differentiation of a small population of primitive HSPCs. HSPCs exhibit unique characteristics to ensure that under appropriate conditions, they are capable of both multilineage differentiation to produce all the mature blood types, and self-renewal which ensures that they are not depleted and able to continue to produce blood cells for an individual's entire lifespan. More mature, differentiated hematopoietic cells do not exhibit these characteristics and are restricted to one or more haemopoietic lineages with a limited capacity for self-renewal (reviewed by Morrison et al 1995, 1997). The biological differences between HSPCs and their more mature differentiated progeny are largely determined by the quantitative expression pattern of multiple genes (Shivdasani and Orkin 1996). Identification of these genes may lead to a better understanding of the mechanisms that regulate HSPC biology in normal and pathological haemopoiesis. This knowledge may also be used in the development of strategies to reliably exploit the proliferative capacity of HSPCs for use in clinical practice as it relates to stem cell transplantation and gene therapy.

Human HSPCs can be isolated from ABM and UCB, and are enriched in a rare population of cells that express the CD34 surface antigen. These CD34+ cells are enriched for the entire hierarchy of clonogenic hematopoietic cells and are able to regenerate and maintain lymphomyelopoiesis in vivo (reviewed by Krause et al 1996). Many studies have demonstrated that compared to ABM derived CD34+ HSPCs, equivalent cells from UCB have higher clonogenic and re-populating potential in vitro and in vivo. Furthermore, in vitro and in vivo studies have shown that UCB derived HSPCs possess higher proliferation and expansion potential than equivalent cells from ABM. They are more sensitive and respond more rapidly to growth factors, and are less sensitive to the effects of inhibitory cytokines (reviewed by Cairo and Wagner 1997).
Currently, it is not clear what the actual mechanisms are that account for the observed functional differences between UCB and ABM HSPCs. Ultimately, these differences will be due to the way in which genes are regulated in the stem cell compartment throughout the life cycle. Efforts to elucidate differences in gene expression between primitive haemopoietic progenitors from UCB and ABM should help to identify mechanisms by which stem cells are triggered into proliferation, differentiation and self-renewal. This knowledge may also be used to in the development of strategies to reliably expand HSPCs for use clinically and experimentally.

Whilst considerable progress has been made to identify genes which play a part in establishing and maintaining the stem cell properties of self-renewal and multilineage differentiation in UCB and ABM HSPC (reviewed by Shivdasani and Orkin 1996, Jordan and Van Zant 1998), there are still many more genes, both known and unknown, yet to be identified and characterised. The purpose of this study was two-fold. Firstly, to identify novel genes expressed in haemopoietic progenitor cells, and secondly, to identify novel genes differentially expressed in HSPCs from UCB and ABM. dd-PCR was utilised as a screen to identify differences in mRNA expression between CD34+ and CD34- cells from UCB and ABM. A number of differentially expressed transcripts, both novel and known, were identified. Differential expression of two of these novel transcripts, designated ORP-3 and MERP-1 was confirmed, and on the basis of preliminary nucleotide bioinformatics, these genes were chosen for further investigation.
2.3 MATERIALS AND METHODS

2.3-1 Ethics approval

All experimental procedures were conducted in accordance with the regulations and guidelines outlined by the National Health and Medical Research Council, and approved by the Deakin University (Ethics approval no. EC-82-97) and Geelong Hospital Ethics Committees (Ethics approval no. 97-14).

2.3-2 Cell sources

UCB samples were obtained after uncomplicated vaginal or cesarean delivery after clamping and cutting of the cord and drainage into 50ml sterile collection tubes containing 200 units of heparin (David Bull Laboratories, Mulgrave, Aust.) in 5mls of alpha medium (αMEM) (Trace Scientific, Noble Park, Aust.). ABM cells were obtained from cell scrapings of discarded rib and femur heads after cardiothoracic or hip-replacement surgery, or iliac crest aspirates. ABM cell scrapings were collected into 50ml sterile collection tubes containing 200 units of heparin in 20mls of αMEM. All samples were donated by volunteers at the Geelong Hospital, Barwon Health (Geelong, Victoria), according to approved institutional guidelines.

2.3-3 Depletion of bone fragments from ABM samples

All ABM samples were received as a slurry of bone fragments and marrow in heparin/media in one or more 50ml-collection tubes. Samples were kept gently mixing at room temperature on a Nutator and processed within 24 hours of
collection. Samples were mixed by vigorous inversion several times, then allowed to settle for a few minutes. When most/all of the large bone fragments had settled to the bottom of the collection tube, the supernatant containing mainly cellular material was removed into a clean/sterile collection tube. This process was repeated 3 – 5 times and cellular fragments pooled until virtually all haemopoietic cells were removed from the original ABM slurry (samples appear white in color). Pooled ABM samples depleted of bone fragments, were then spun at 480g for 5 minutes at 18 – 20°C. Supernatants were removed, cell pellets combined and made up to approximately 40mls (dependant on number of cells present) in phosphate buffered saline (PBS) containing 2mM ethylenediamine-tetra-acetic acid (EDTA) and 0.5% bovine serum albumin (BSA) (Sigma Aldrich, St.Louis, U.S.A.).

2.3-4 Red Cell Depletion of UCB samples

UCB samples were diluted to twice their original volume with PBS, mixed thoroughly by gentle inversion, and centrifuged for 25 minutes at 480g and 18 – 20°C. Centrifuge was set to low brake. After centrifugation, the lower density white blood cells were seen as a buff-colored carpet on top of the denser red cells. These were removed using a cannula and syringe and placed in a sterile 50-ml tube. Red cell depleted UCB samples were made up to a volume approximately equal to the original UCB sample with PBS.

2.3-5 Preparation of Mononuclear cells (MNCs) from UCB and ABM samples

UCB and ABM samples depleted of red cells and bone fragments respectively were carefully and gently layered onto an equal volume of Ficoll-Hypaque® (Amersham Pharmacia Biotech, Uppsala, Sweden) in 15 or 50ml sterile tubes. Great care was taken to ensure that there was minimal/no mixing of cell samples with Ficoll-
Hypaque®. Samples were centrifuged for 25 minutes at 440g and 18 – 20°C with centrifuge set to low brake. After centrifugation, the upper plasma/media layer was drawn off using a clean pasteur pipette. MNCs at the ficoll-plasma interface were carefully removed using a sterile 5 or 10-ml syringe and cannula. MNCs were washed twice with the addition of two volumes of PBS and centrifuged at 400g for 10 minutes at 18 – 20°C. After the second wash, the supernatant was removed, and the MNCs were resuspended in 300µl PBS/0.5%BSA/2mMEDTA per 1 x 10⁸ cells (minimum 300µl).

2.3-6 Immuno-magnetic separation of CD34⁺ cells from UCB and ABM MNCs

CD34⁺ cells were isolated from UCB and ABM MNCs using antibody labeling and a MiniMacs bead separation kit (Miltenyi Biotec, Becton Dickinson, Sunnyvale, U.S.A.) following the manufacturers instructions. Degassed PBS/0.5%BSA/2mMEDTA was used for washing VarioMacs and MiniMacs columns (Miltenyi Biotec) and MNCs. MNCs were resuspended in 500ul/10⁸ of 500units/ml DNase1 (Sigma Aldrich) before separation on a VarioMacs (for 2 – 20 x 10⁸ MNCs) or MiniMacs column (for ≤ 2 x 10⁸ MNCs). Immuno-magnetically-labeled CD34⁺ cells were eluted from the column with 2 – 5mls degassed PBS/0.5%BSA/2mMEDTA. Viability and cell number of the CD34 enriched and depleted cell populations was assessed using trypan blue (TB) (Sigma Aldrich) exclusion and manual cell counting.

2.3-7 Flow cytometric analysis of CD34 populations

Approximately 0.25 x 10⁶ cells from whole cord blood, ABM MNCs and CD34 depleted samples, and 0.01 x 10⁶ CD34⁺ cells were labeled with FITC-conjugated anti-CD45 and PE-conjugated anti-CD34 (Beckman Coulter, Fullerton, U.S.A.) according to the manufacturers instructions. The percentage of CD34⁺ cells in each
sample was determined by flow cytometric analysis (FacsCalibur, Becton Dickinson) following modified ISHAGE guidelines (Sutherland et al 1996a) using CELLQuest software (Becton Dickinson) (Figure 2.1). Equivalent numbers of cells labeled with FITC-conjugated anti-IgG1 and PE-conjugated anti-IgG1 were used as negative controls for the analysis.
Figure 2.1 Flow cytometric analysis of the percentage of CD34* cells in UCB and ABM samples following modified ISHAGE guidelines

Plot 1. Displays total events with a region (R1) around the lymphocyte area (G1)

Plot 2. Gate = Gate 1
Only events falling into R1 are displayed here. This dot plot is not part of the analysis but is used to check cytometer settings.

Plot 3. Displays total events.
A region (R2) is paced around the CD45* events.

Plot 4. Gate = Gate 2 (R2)
Displays events that are CD45* only. A region (R3) is placed around the CD34*, side scatter^b and forward scatter^b events.

Plot 5. Gate = Gate 3 (R2*R3)
Displays events that are CD45*/CD34*/side scatter^b/forward scatter^b. A region (R4) is placed around the CD45^dim events.

Plot 6. D=Gate = Gate 4 (R2*R3*R4)
Displays events that are CD45*/CD34*/side scatter^b/forward scatter^b/CD45^dim. A region (R5) is placed around the lymphocyte region.

Analysis
Gate 5 = (R2*R3*R4*R5)
CD45*/CD34*/side scatter^b/forwardscatter^b/CD45^dim/lymphocytes

Gate 2 = (R2)
CD45*

The percentage of cells that are CD34* is calculated as being:

(Gate 5 events/Gate 2 events) * 100
Plot 1. Total events
R1 = Gate 1 (lymphocyte area)

Plot 2. Gate 1
This dot plot is not part of the analysis. It is to check cytometer settings for gating strategy only.

Plot 3. Total events.
Gate 2 = R2 (CD45+)
Plot 4. Gate 2
Gate 3 = R2*R3 (CD45+CD34+)

Plot 5. Gate 3
Gate 4 = R2*R3*R4 (CD45+CD34+ lo-side scatter)

Plot 6. Gate 4
Gate 5 = R2*R3*R4*R5 (CD45+CD34+ lo-side & lo-forward scatter)
2.3-8 *Ex vivo* proliferation and differentiation of CD34⁺ cells

In selected experiments, isolated CD34⁺ cells were cultured for 1 week at 37°C in a humidified atmosphere flushed with 5% CO₂ in air, at a concentration of 0.5 x 10⁶ cells/ml in αMEM with 20% Foetal Bovine Serum (FBS) (CSL Biosciences, Parkville, Aust.), 2mM L-Glutamine (Sigma Aldrich), 200U/ml Penicillin/Streptomycin (Sigma Aldrich), 20ng/ml recombinant human SCF (rhSCF) (Amgen, Thousand Oaks, U.S.A.), 10ng/ml IL-1β (Endogen, Woburn, U.S.A.) 10ng/ml IL-3, 10ng/ml IL-6 and 10ng/ml G-CSF (Amrad, Boronia, Aust.). At the end of the culture period, viability and cell number were assessed using TB exclusion and manual cell counting.

2.3-9 Total RNA Isolation and Analysis

Total RNA was extracted by RNeasy™ Total RNA Isolation Kit (Qiagen, Clifton Hill, Aust.) and contaminating DNA removed by digestion with DNase1 MessageClean™ (GenHunter) according to the manufacturers instructions. DNA-free RNA was re-purified using RNeasy™ Total RNA Isolation Kit (Qiagen). RNA integrity was assessed by Formaldehyde Denaturing Gel Electrophoresis and quantity and purity determined by spectrophotometry. Total RNA of high integrity and purity was then used for subsequent dd-PCR or real-time PCR reactions. All PCR reactions were performed on a Perkin Elmer 9600 thermocycler (P.E. Applied Biosystems Foster City, U.S.A.) except where specified.
2.3-10 Reverse Transcription and Differential Display PCR

Reverse transcription (RT) and dd-PCR was carried out using cycling parameters and PCR conditions as specified in RNAimage Differential Display System (GenHunter Nashville, U.S.A.). Briefly, DNA-free RNA was reverse transcribed using standard RT-PCR conditions, Superscript Reverse Transcriptase II (Invitrogen, Carlsbaad, U.S.A), 50ngRNA/10μl reaction volume and one of three different one-base-anchored H-TM primers (where M is degenerate for A, G, C). 2μl cDNA was used as a template for a 20μl dd-PCR reaction using appropriate H-TM primer, one of 30 possible arbitrary primers, AmpliTaq DNA Polymerase (Qiagen) and dATP[32]. Amplified cDNAs were separated on a 6% denaturing polyacrylamide sequencing gel (Beckman Coulter, Sydney, Aust.) using a GENOMYX LR™ DNA Sequencing apparatus (Beckman Coulter) according to manufacturers instructions. Gels were dried without fixation, and exposed for 1 – 5 days to Kodak Biomax MR1 film (Eastman Kodak, Rochester, U.S.A.) for visualization.

2.3-11 Excision of Differentially Expressed cDNAs, Re-amplification and Sequencing

cDNAs that appeared differentially expressed in multiple comparisons (minimum of three in five comparisons) were excised from the dried acrylamide gel and re-amplified directly in a 40μl PCR reaction using AmpliTaq DNA Polymerase (Qiagen), and the appropriate anchored and arbitrary primer. Cycling parameters and PCR conditions were as specified in RNAimage Differential Display System (GenHunter). PCR products were visualised by standard agarose gel electrophoresis in a 1.5% low-melting point TAE gel and ethidium bromide staining. Bands to be sequenced were excised, purified using Qiaquick Gel Extraction Kit (Qiagen) and sequencing reactions carried out using ABI Prism BigDye™ Terminator Cycle
Sequencing Ready reaction Kit (P.E. Applied Biosystems) according to the manufacturers instructions and 3.2pmols of appropriate arbitrary primer (GenHunter). Sequencing PCR products were ethanol precipitated, air-dried, and sequences determined by staff at Monash University (Clayton, Aust.) using an ABI PRISM™ 373 DNA Sequencer (P.E. Applied Biosystems).

2.3-12 Reverse Transcription and Real-Time PCR Semi-Quantitation of novel differentially expressed genes

Reverse transcription was carried out using Reverse Transcription System (Promega, Madison, U.S.A.) following the manufacturers guidelines, random hexamers and 50ng DNA-free RNA/10μl reaction volume. cDNA was diluted 1:2 and used in subsequent Real-Time PCR reactions. All primers and probes for use in Real-Time PCR (Table 2.1) were designed using Taqman Primer Express™ Software (P.E. Applied Biosystems) and optimised for use as recommended by the manufacturer (Table 2.2). Real-Time PCR amplification was carried out using either Taqman™ Universal PCR Mastermix or SYBR™ Green PCR Master Mix (Table 2.2) on an ABI PRISM™ 7700 Sequence Detection System following the manufacturers guidelines (P.E. Applied Biosystem). Gene Expression was quantitated relative to expression of two housekeeping genes (β-actin and GAPDH) using Sequence Detector Software and the comparative Ct method (P.E. Applied Biosystem User Bulletin No. 2).
Table 2.1 Real-Time PCR Primer and Probe Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-gac aag atg cag aag gag att act-3'</td>
<td>5'-tga tcc aca tct gct gga agg t-3'</td>
<td>fam - atc att gct cct cct gag cgc aag tac tc - tamra</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ - cca cat cgc tca gac acc at - 3’</td>
<td>5’ - cca ggc ggc caa tag g - 3’</td>
<td>fam - aag gtg aag gtc gga gte aac gga ttt g - tamra</td>
</tr>
<tr>
<td>ORP-3</td>
<td>5’ - cca act gga cca tcc tgt ctt atg - 3’</td>
<td>5’ - aag cta agc aca agt gat cat cct aga - 3’</td>
<td>fam - aac att agt gta ttt ctc ctc tgc ttt c - tamra</td>
</tr>
<tr>
<td>MERP-1</td>
<td>5’ - ggg cct cag gag cag atc a - 3’</td>
<td>5’ - tgc cca tcc agg ttt cat agg - 3’</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>
**Table 2.2 Optimised Real-Time PCR Primer and Probe concentrations**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse primer</th>
<th>Probe</th>
<th>Real-Time Chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>600</td>
<td>600</td>
<td>100</td>
<td>Taqman™</td>
</tr>
<tr>
<td>GAPDH</td>
<td>300</td>
<td>300</td>
<td>100</td>
<td>Taqman™</td>
</tr>
<tr>
<td>ORP-3</td>
<td>900</td>
<td>900</td>
<td>100</td>
<td>Taqman™</td>
</tr>
<tr>
<td>MERP-1</td>
<td>100</td>
<td>100</td>
<td>Not Applicable</td>
<td>SYBR™ Green</td>
</tr>
</tbody>
</table>
2.3-13 Statistics

All gene expression results are expressed in arbitrary units (Mean ± SEM) and calculated from duplicate determinations. The Wilcoxon Signed Ranks Test and Paired Students T-Test were used to determine the statistical significance of the differences between related groups for non-parametric data and parametric data respectively. The Mann-Witney test was utilized to determine the statistical significance of the differences between non-related groups for non-parametric data. All statistical analyses were performed using SPSS (Statistics Package for the Social Sciences) version 10.0 software (Fullerton, CA, U.S.A.). In all instances, p ≤ 0.05 was considered statistically significant.

2.3-14. Bioinformatics

Nucleic acid and protein sequences were analysed using software available from the Australian National Genomic Information Service (TRANSLATE, BESTFIT, ECLUSTALW) and the National Center for Biotcchnology Information (BLASTN, BLASTP, TBLASTX).
2.4 RESULTS

2.4-1 Enumeration and Purification of CD34+ cells from UCB and ABM

FACS analysis using a modified ISHAGE protocol (Figure 2.1) of all the UCB whole blood sample (WB) and ABM MNC samples used in this study showed that 0.47 ± 0.13 % (n = 45) and 3.70 ± 0.89% (n = 25) were CD34+ respectively. Following CD34 enrichment with the MACs system, 60.93 ± 2.41% of the UCB, and 55.02 ± 5.01% of ABM populations were CD34+ (Table 2.3) The percentage of CD34+ cells in the CD34- fractions was < 0.01% in UCB and ABM. Average viability of the CD34+ and CD34- populations from ABM and UCB did not vary significantly.
Table 2.3  FACS Analysis of UCB and ABM CD34⁺ populations - Pre- and Post MACS enrichment

All data is expressed as (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>UCB (n = 45)</th>
<th>ABM (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCB % 34⁺</td>
<td>0.47 ± 0.13 %</td>
<td>3.70 ± 0.89%</td>
</tr>
<tr>
<td></td>
<td>60.93 ± 2.41%</td>
<td>55.02 ± 5.01%</td>
</tr>
<tr>
<td></td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>MNC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MACS enriched 34⁺ population</td>
<td>93.8 ± 0.9%</td>
<td>94.3 ± 0.9%</td>
</tr>
<tr>
<td>MACS depleted 34⁺ population</td>
<td>95.7 ± 0.9%</td>
<td>95.2 ± 1.0%</td>
</tr>
</tbody>
</table>
2.4-2 Isolation of cDNAs differentially expressed between CD34⁺ and CD34⁻ haemopoietic cells from ABM and UCB using dd-PCR

Gene expression of 10 UCB and 5 ABM samples was assessed by dd-PCR using 10 primer combinations. A total 20 differentially expressed cDNAs (present in CD34⁺ cells from UCB and ABM) were excised from the acrylamide gel. Of these, 12 were successfully re-amplified and 6 successfully sequenced. At the time of sequencing and BLAST analysis, 2 these 6 cDNA sequences (designated band 1 and band 20) were homologous to partial sequences for unknown and uncharacterised genes, whilst 4 represented partial fragments of known and well-characterised genes (Table 2.4). Of the two unknown genes, band 1 appeared to be more highly expressed in CD34⁺ cells from ABM compared to CD34⁺ cells from UCB, while band 20 expression appeared to be equal across the two populations. Both band 1 and band 20 were chosen for further investigation.
Table 2.4 cDNA sequences differentially expressed between CD34+ and CD34- haemopoietic cells

cDNA size is in nucleotides (nts)

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Size</th>
<th>Sequence information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>603nts</td>
<td>Accession No. ABO14604. <em>Homo sapiens</em> mRNA for KIAA0704 protein, partial cds</td>
</tr>
<tr>
<td>4</td>
<td>414nts</td>
<td>Accession No. AF004340. <em>Homo sapiens</em> ATPase6/8 gene, mitochondrial gene encoding mitochondrial protein</td>
</tr>
<tr>
<td>10</td>
<td>345nts</td>
<td>Accession No. V00662. <em>Homo sapiens</em> mitochondrial genome</td>
</tr>
<tr>
<td>14</td>
<td>450nts</td>
<td>Accession No. AF017456. <em>Homo sapiens</em> lysosomal pepstatin insensitive protease (CLN2) mRNA, complete cds</td>
</tr>
<tr>
<td>16</td>
<td>340nts</td>
<td>Accession No. M26700. <em>Homo sapiens</em> ubiquinone-binding protein, mRNA, complete cds</td>
</tr>
<tr>
<td>20</td>
<td>208nts</td>
<td>Accession No. BC000686 - <em>Homo sapiens</em> mRNA for UCC1 protein</td>
</tr>
</tbody>
</table>
2.4-3 Identification of ORP-3, a novel gene expressed in HSPCs using dd-PCR

Blast analysis of the 603nt band 1 sequence (Figure 2.2) revealed 98.6% homology to a 5066nt partial gene sequence in the GenBank database (Accession No. AB014604. *Homo sapiens* mRNA for KIAA0704 protein, partial cds) (Figure 2.3 & 2.4). Translation of the open reading frame (ORF) of this partial nucleotide sequence using TRANSLATE programs on the ANGIS database results in an amino acid sequence with homology to human oxysterol binding protein (OSBP-Hm). The partial gene sequence for the putative KIAA0704 protein was first identified by Ishikawa et al (1998) in their search for novel human genes expressed in the brain. More recently, Laitinen et al (1999) identified it as part of an expressed sequence tag (EST) search for cDNAs displaying homology to the oxysterol (OS) binding domain of OSBP. This group designated the sequence oxysterol binding protein-related protein-3 (ORP-3).

The KIAA0704/ORP-3 cDNA sequence contained a putative stop codon at position 2004 but a poly-adenylation signal and poly-A tail were not evident (Figure 2.4). Additionally, this sequence lacked a putative start codon and the sequence 5' of the stop codon was ORF. The KIAA0704/ORP-3 gene sequence was thus incomplete. Elucidation of the additional 5' and 3' sequence of ORP-3 is the subject of investigation in Chapter 4.
Sequence matching ABO14604 (*Homo sapiens* mRNA for KIAA0704 protein, partial cds) depicted in red.

```
1  nccagcctnn nocctttctc tttgatacqm taencttttgc gaancggtta nctntgggaaq
61  ggaactnaga aagaagtgaa atacaaaagc agaggattga acaacagtgg anttttaagge
121  gycgggtott agaagaaaat cagttggagc accagocctcg gtttttcagg aatcctcgacg
181  atgactccttg ggtggagcaac ggcaactattt ggaacttataa aagatactttg gtttttccaa
241  actggaacca cctgctttat gttgaaaaag taaaagaaaa agataacttt aaggttttcttc
301  tccctgtgctt ggctctctgaa gttggcacaata cctgcttttta tatactttaa aagatactctta
361  gggagataac cggagcagc ttaagcatgtt taacttctttt aagtttatt tttccagtgtg
421  cgtntctttta caatttccaa tgttnaaccttg aatgttttata tntcaatgtg aacaccttgg
481  cattctctct tattaaactttatattaaaaatgagatgttt cagtggtggg
541  ttaaatt accccctacac ttttnttcct cttntnttctc cttntnttct cctntnttct
601  tct
```
Figure 2.3 Best-fit of original ORP-3 cDNA sequence obtained by DD-PCR (603nt) with AB014604 (5066nt)

Gap Weight: 5.000  Average Match: 1.000  Length Weight: 0.300
Average Mismatch: -0.900  Quality: 470.1  Length: 496
Ratio: 0.950  Gaps: 2  Percent Similarity: 98.583
Percent Identity: 96.964

| AB014604 1794 | gaggaggggaacttaagaagaagctgaaatcacaasagcagcggattgcaca     |
| ORP-3 54     | ntgagaggggaacttaagaagaagctgaaatcacaasagcagcggattgcaca    |
| AB014604 1844 | actggaagagaagggcgcggtcttagaagaagatctcagttggaggagcaccc    |
| ORP-3 104    | acttgtctttaagggcgcggtcttagaagaagatctcagttggaggagcaccc    |
| AB014604 1894 | agcctcggttcttcggaaatcgcagctcctccttggtggcgcacgcgcg     |
| ORP-3 154    | agcctcggttcttcggaaatcgcagctcctccttggtggcgcacgcgcg     |
| AB014604 1944 | acctatttggaaaccttgaagatctttgcttttcccacaaactggacactcc    |
| ORP-3 204    | aacctatttggaaaccttgaagatctttgcttttcccacaaactggacactcc    |
| AB014604 1994 | tgtctttctggtaaaagtatgaagagttgataatgtgattttctctc       |
| ORP-3 253    | tgtctttctggtaaaagtatgaagagttgataatgtgattttctctc       |
| AB014604 2044 | ctgtgcttccttcgactggagctgggcaccaaccctctgtgattttatatttaaag |
| ORP-3 303    | ctgtgcttccttcgactggagctgggcaccaaccctctgtgattttatatttaaag |
| AB014604 2094 | atacctcgtagtatacttgaccttagrcgactcgtgaacctttcaag       |
| ORP-3 353    | atacctcgtagtatacttgaccttagrcgactcgtgaacctttcaag       |
| AB014604 2144 | ttctatatttatctagcgctatcctttttatcataatttcacaattttcagttatcctgat |
| ORP-3 403    | ttctatatttatctagcgctatcctttttatcataatttcacaattttcagttatcctgat |
| AB014604 2194 | tgtttatatagaaaagatgcactgagccttagctatcttttcatatatataact |
| ORP-3 453    | tgtttatatagaaaagatgcactgagccttagctatcttttcatatatataact |
| AB014604 2243 | atttataaaatatgaaagattctactttggtttatttttttttttattttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Figure 2.4 ABO14604. Homo sapiens mRNA for KIAA0704 protein, partial cds (5066nt)

Sequence matching Original ORP-3 DD-PCR transcript depicted in red. Putative stop codon (boxed and shaded in yellow) at position 2004.

1   aaaaaatgcat ccaagaaccgt ggcgcaagtgc aatgctaatc ggtagaaatg agccagctcc
61  tgcacaagcat ggaagctcctg cactggacat atcctgcacc agctatcacc gcctatccagq
121  tgcagctcttt tgaagcctcgg aaaaaaggaaa aaaaaaaaaa cagagagtttg ggctccagag
181  cttatggtgga agatgctaaa ggaacactgg gcgctccgga aacctttctt ggcagctagaa
241  gacccacact ccaccacctt aattttgtcag actatagatttt tggagaagag aaaaaatttt
301  ctagatgcttc tgaacaccct gcaagagtttt ttaaattttg cagacagctg tggcatatgtg
361  gccataagtt ttactctcct ttaaggttcag ctttaaatat cttgctgagc qggcagaaqga
421  aacttgaagg ccgtgactggag cagatgctgc cctgcgtcctc gctctctcag gctatggtgc
481  tggagaagagc cccctctcctc gcctagcccc aaaaaagaggc ttttttttttt tgcctcaagga gtttctttct
541  gaaaacctct gcgctcctcg cctcccagct gctacagctg gatgcacacc
601  tggcagagga aacaccaccag gatttcagcc cagaccttgg attattatttt ctttagatgatc cttccaaa
661  tggcagtcttc cactacgct gcctctctttt gttttttttt gtttctctgg tatttttttttt
721  ctctagacac ctaactgacgc ctctctctctt gtttttttttt ttcctgtctg tatttttttttt
781  atatactcttt cctagatata atttggattttt ctttctatttt ctttctatttt
841  ctagctcttgag tttgctctgtt ctcggagtgg cactctctgg cactctctgg cactctctgg
ggactggactgc gatgtgctgc ggttaacttc gtttttttttt ttttttttttt
901  acgctcgtctgcc gtttccgccc cgttccgccc cgttccgccc cgttccgccc cgttccgccc
cactctctgg gctctctctg ggttaacttc ctttttttttt ttttttttttt
961  tttgctctctg gctctctctg gtttccgccc cgttccgccc cgttccgccc cgttccgccc
cactctctgg gctctctctg ggttaacttc ttttttttttt ttttttttttt
1021 gcgctcgtctgcc gtttccgccc cgttccgccc cgttccgccc cgttccgccc cgttccgccc
cactctctgg gctctctctg ggttaacttc ttttttttttt ttttttttttt
1081 aagagattttt attttttttttt ttttttttttt ttttttttttt ttttttttttt
1141 ctttaaatttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
1201 aggttttttttt gtcagctgctg ctttttttttt ttttttttttt ttttttttttt
1261 ctgctctctg aaaaaaatttt ttctggagaag atgagagaga gaaaaaataaa atttttgcca
1321 aatctcgactg aatttcagcag tggagcaata cctctctcct ttttttttttt
1381 atatttttttt ggttaacttc atttttttttt ttttttttttt ttttttttttt
1441 tggagaagagc cggcagctgctg gtttccagagc gtttccagagc gtttccagagc gtttccagagc
ttttttttttt ttttttttttt ttttttttttt
1501 ggaagctctttlc gtaatttttt ttttttttttt ttttttttttt ttttttttttt
1561 ttgacagcagc cggcagctgctg gtttccagagc gtttccagagc gtttccagagc gtttccagagc
ttttttttttt ttttttttttt ttttttttttt
1621 ggcgcagctgctg gtttccagagc gtttccagagc gtttccagagc gtttccagagc gtttccagagc
ttttttttttt ttttttttttt ttttttttttt
1681 cgttctctctg cgcctcctctg ggcagctgctg gtttccagagc gtttccagagc gtttccagagc
ttttttttttt ttttttttttt ttttttttttt
1741 aggttttttttt gtcagctgctg ctttttttttt ttttttttttt ttttttttttt
1801 ggaagctctttlc gtaatttttt ttttttttttt ttttttttttt ttttttttttt
1861 ggcccagcagc cggcagctgctg gtttccagagc gtttccagagc gtttccagagc gtttccagagc
ttttttttttt ttttttttttt ttttttttttt
1921 atatttttttt ggttaacttc gggactctgtt cggcagctgctg gtttccagagc gtttccagagc
ttttttttttt ttttttttttt ttttttttttt
1981 aacacactttgc ttttttttttt ttttttttttt ttttttttttt ttttttttttt

93
Figure 2.4 (continued)
Figure 2.4 (continued)

ttttaattct caagtttaaa taatgctaat ccctgttcca ttgsgtttaa atgttttaac
ttttttttt ttctctaatta ttccatccaa aacctctgtat gaattacaca gaatattttg
gaatggggcata tttgtgccag taacaccctacctcagga ttaaatctc ctaaccagctg
tcaacccag cactgtcagc gaattaacctt ctcaactgctt ccagttgttac
gagcagcct ctaactccct ccocagggcc ccaagcaacc caaaggcttg tattctttga
taataggttaattaataaa aagcctcaat gcaagttctc eacttgttc otctctcaac
tacggggggt attctgttcc taactaagaag gatgggggtt caoccagggg caaagaatc
cagggtcag ccaacagcgg caaggtgtcct tttgttccttctcctgttaaaggtgacat tctgggggg
ataatacaac taatatatt tttggttttt acgtcatta caagtgtgct ttctctatat
ttggttttt tattttcatta gtaactgttt cttttggtgct tcaacactgg tttctctctc
gagttctcct gatrtatctt gattgtatcgtattactgtct aagaatgtg taaagcttataa
agaattaaag tttttatatgg gctgtt
Identification of MERP-1, a novel gene expressed in HSPCs using dd-PCR

The 208nt band 20 cDNA contained a poly-adenylation signal and polyA tail (Figure 2.5). This sequence did not contain an ORF and BLAST analysis of the nucleotide sequence revealed 96.0% homology to a 2367nt partial gene sequence in the GenBank database (Accession No. BC000686 – Homo sapiens mRNA for UCC1 protein) (Figure 2.6 & 2.7). The UCC1 sequence was first identified by Nimmrich et al (2001) in their search for novel human genes differentially expressed in colon cancer. Translation of this nucleotide sequence using TRANSLATE programs on the ANGIS database resulted in an amino acid sequence with significant homology to an ependymin family of proteins described in teleost fish (Hoffman and Schwarz 1996). It is likely therefore that the band 20/UCC1-gene sequence is the first mammalian gene encoding an ependymin-related protein in humans. This sequence was thus designated mammalian ependymin related protein –1 (MERP-1).

The UCC1/MERP-1 cDNA was complete at the 3’end as a polyadenylation signal and a poly-A tail were clearly evident (positions 2319 and 2349 respectively) (Figure2.7). As per the KIAA0704/ORP-3 cDNA, there was a putative stop codon at position 805 and the sequence 5’ of this stop codon was ORF. The UCC1/MERP-1 sequence was thus incomplete. Elucidation of the additional 5’ sequence of MERP-1 is the subject of investigation in Chapter 3.
Figure 2.5 Original MERP-1 sequence identified by DD-PCR (208 nt)

Sequence matching BC000686 (*Homo sapiens* mRNA for UCC1 protein) depicted in red.

1   ccgggtgtaa tgtgtagcct ccatattata atgaaatcag tctattact
51  ttagggcttat ccatattata caaatcta tgccttttaa gnttttttg
101 atatggttga tttggaata atatggataa tntggnttat aaggggttat
151 aaaaaatgnt gntaaaata aagnnttcna tgygatcnaa aaaaaaaaang
201 tttttaaaa
Figure 2.6 Best-fit of original MERP-1 cDNA sequence obtained by DD-PCR (208nt) with BC000686 (2367nt)

Gap Weight: 5.000  Average Match: 1.000  Length Weight: 0.300
Average Mismatch: -0.900  Quality: 175.2  Length: 203
Ratio: 0.872  Gaps: 2  Percent Similarity: 96.020
Percent Identity: 90.547

MERP-1
8  aatgtgtgacccctatatatatagttatatcactttattatagtt 57
BC000686 2162  aatgtgtgacccctatatatatagttatatcactttattatagtt 2211

MERP-1
58  tatcttatattaaacaaatatttacctgtatgatatggtgatggtggtgatggtggtgatggtggtg 106
BC000686 2212  tatcttatattaaacaaatatttacctgtatgatatggtgatggtggtgatggtggtg 2261

MERP-1
107  ntggttttgnaataatatggatatnttgntataaggtgtatataaaaca 155
BC000686 2262  tgggttttgnaatatatatggatatnttgntataaggtgtatataaaaca 2311

MERP-1
156  atgntgtaataaaactaatnnttntgnntctcactttctcactttaaacaagnttaaaa 205
BC000686 2312  atgntgtaataaaactaatnnttntgnntctcactttctcactttaaacaagnttaaaa 2361

MERP-1
206  aa 208

BC000686 2362  aa 2364
Figure 2.7 BC000686 - *Homo sapiens* mRNA for UCC1 protein (2367nt)

Sequence matching original MERP-1 cDNA obtained by DD-PCR depicted in red. Putative stop codon, polyadenylation signal and poly-A tail boxed in yellow, blue and pink (respectively).

```
1  ggccagaggg tttggtccag accacactcc cggccacagtgc cggaaagac
51  oggogggagc cactctgatc cggacgcct ccagcggcccc ttgggctttg
101  gctttccctc gggcagggga agaatgaccg cagatgcaggg acgggccccc
151  cttccagacgc tccccggggtc cctgggtggc tgtgctgtgg gggggctcgg
201  ggctctggacc ctgtagcggcc tgtgcagcct gggggcgggt gggggccgcc
251  gcccgtgcca ggccgccgag cagatggaggg cggggtgacc tttgtacccag
301  ccggggctcg ccggtgcgctt ggggtgcgtg ggggcgggaa gggggatgtg tccctgacca
351  gccgacgggt cggctgatgcttgatttggat agtgctatgg ttttcacgat
401  gccacggcgag cccctgacgtt tccacacagat cccctacgt aagtaaaggtg
451  ggtgttttga caatccacgtg ggtatattaa agcctcttggatttacggg
501  cccacgacgt gcacagtgcc caatcgggag aagatgcgg aagacgtgtg
551  cttgggttcc tgtcagctgg gaaaggggcag ccaggagagt ataatacctg
601  cgggcccagc ccctcggccag attgccccttc ctgcagctag ccggtttgat
651  gtattcttta gtgcacagctt attagcagtc cccaaggtat caaatggcct
701  gtttttttga gtttttttta gttttttttt gttttttttt gttttttttt
751  cttgggttcc tgtcagctgg gaaaggggcag ccaggagagt ataatacctg
801  ccctcggtg cccccgccag cccgtggtcc gctccgtggt cccgtggtcc
851  gggggtgtgg ctgacagctc tgtgctgtgg ggggtggtgg cccccgtgg
901  tggagacagaa tagctttttttataatggcagattgtaattgtagtctacgat
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1451  gggggtgtgg ctgacagctc tgtgctgtgg ggggtggtgg cccccgtgg
```
Figure 2.7 (continued)
2.4.5 Quantitation of ORP-3 Gene Expression Using Taqman™ Real-Time PCR in CD34⁺ and CD34⁻ hematopoietic cells from UCB and ABM

To confirm differential expression of ORP-3, Taqman™ real-time PCR technology was employed to quantitate gene expression relative to β-actin and GAPDH (Figure 2.8). Results confirm higher expression of ORP-3 in CD34⁺ hematopoietic cells compared to equivalent CD34⁻ cells from ABM (11.2 ± 2.2 versus 2.6 ± 1.1, n = 14, p ≤ 0.001) and UCB (6.3 ± 0.9 versus 2.0 ± 0.4, n = 21, p ≤ 0.0001). Additionally, this data indicates a 1.8-fold increase in ORP-3 expression in ABM CD34⁺ cells compared to UCB CD34⁺ cells (p ≤ 0.05).
Figure 2.8 ORP-3 Gene Expression (relative to β-actin & GAPDH and expressed in arbitrary units) as measured by Taqman™ Real-Time PCR

ORP-3 gene expression in CD34⁺ and CD34⁻ hematopoietic cells from UCB (n=21) and ABM (n=14) * p ≤ 0.001, # p ≤ 0.0001
2.4-6 Quantitation of MERP-1 Gene Expression Using SYBR Green™ Real-Time PCR in CD34+ and CD34- hematopoietic cells from UCB and ABM

To confirm differential expression of MERP-1, SYBR Green™ real-time PCR technology was utilized to quantitate gene expression relative to two housekeeping genes β-actin and GAPDH (Figure 2.9). Results confirm higher expression of MERP in CD34+ hematopoietic cells compared to equivalent CD34- cells from ABM (23.4 ± 4.9 versus 5.4 ± 1.5, n = 10, p ≤ 0.005) and UCB (12.3 ± 1.1 versus 1.2 ± 0.2, n = 10, p ≤ 0.005). No significant difference was seen for MERP-1 gene expression in CD34+ cells from UCB and ABM.
Figure 2.9 MERP-1 Gene Expression (relative to β-actin & GAPDH and expressed in arbitrary units) as measured by SYBR Green™ Real-Time PCR

MERP-1 gene expression in CD34+ and CD34- hematopoietic cells from UCB (n=10) and ABM (n=10). * p ≤ 0.005
2.4-7 Quantitation of ORP-3 Gene Expression Using Taqman™ Real-Time PCR in CD34⁺, CD34⁺ and CD34⁺ culture haemopoietic cells – post one week culture (CD34⁺culture)

To further explore expression of ORP-3, CD34⁺ cells were isolated from UCB and ABM, and the populations divided into two samples. One subpopulation of CD34⁺ cells underwent RNA extraction and reverse transcription immediately (CD34⁺) along with the CD34⁺ population. The second subpopulation of CD34⁺ was cultured for one week in the presence of growth factors (2.3-8) that are known to potentiate the proliferation (indicated by an increase in cell number) and differentiation (indicated by a decrease in the percentage of cells that are CD34⁺) of progenitor cells (reviewed by Graham and Wright 1997, Ogawa 1993, Metcalf 1993). These cells were designated (CD34⁺culture). At the end of the culture period, these were also RNA extracted and reverse transcribed.

FACS analysis of the CD34⁺ and CD34⁺culture samples showed that after one-week culture under the conditions described, the percentage of cells that were CD34⁺ decreased significantly by approximately 40% and 33% in UCB and ABM respectively (p ≤ 0.001). Total cell number as measured by Trypan blue staining increased by approximately 9% and 8% in UCB and ABM respectively, although this increase was only significant for UCB samples (p ≤ 0.03). Viability of the CD34⁺ and CD34⁺culture samples from UCB and ABM (>90%) did not vary significantly (Table 2.5).

Gene expression data again confirms higher expression of the ORP-3 gene in CD34⁺ cells compared to CD34⁺ cells. Results also indicate that ORP-3 expression in CD34⁺ cells is down regulated after one week culture in UCB (6.2 ± 0.9 versus 2.5 ± 0.6, n = 14, p ≤ 0.01) and ABM samples (10.8 ± 3.5 versus 3.6 ± 1.1, n = 9, p ≤ 0.02) (Figure 2.10).
Table 2.5 FACS Analysis of UCB and ABM CD34⁺ populations - Pre- and Post 7-day culture

All data is expressed as (mean ± SEM). *p < 0.001, #p < 0.032

<table>
<thead>
<tr>
<th></th>
<th>Pre-culture</th>
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<th></th>
<th>Post-culture</th>
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<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%34⁺</td>
<td>%Viability</td>
<td>Cell No. (× 10⁶)</td>
<td>%34⁺</td>
<td>%Viability</td>
<td>Cell No. (× 10⁶)</td>
</tr>
<tr>
<td>UCB (n = 14)</td>
<td>55.98 ± 1.39</td>
<td>94.4 ± 1.0</td>
<td>2.08 ± 0.47</td>
<td>33.39 ± 3.29</td>
<td>92.1 ± 1.8</td>
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<tr>
<td>% change</td>
<td></td>
<td></td>
<td></td>
<td>*40.4</td>
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<tr>
<td>ABM (n = 11)</td>
<td>59.53 ± 5.96</td>
<td>93.6 ± 1.62</td>
<td>0.62 ± 0.23</td>
<td>39.89 ± 7.12</td>
<td>89.7 ± 1.4</td>
<td>0.87 ± 0.20</td>
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<tr>
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<td></td>
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</tbody>
</table>
Figure 2.10 ORP-3 Gene Expression (relative to β-actin & GAPDH and expressed in arbitrary units) as measured by Taqman™ Real-Time PCR

ORP-3 gene expression in CD34−, CD34+ and CD34+cultured hematopoietic cells after one week culture (CD34+cultured) from UCB (n=14) and ABM (n=9). * p ≤ 0.01, # p ≤ 0.02
2.4-8 Quantitation of MERP-1 Gene Expression Using SYBR Green™ Real-Time PCR in CD34^+ , CD34^+ and CD34^- haemopoietic cells – post one week culture (CD34^-culture)

Expression of MERP-1 was also investigated in UCB samples under the culture conditions previously described (2.4-7). Data again confirms higher expression the MERP-1 gene in CD34^+ cells compared to CD34^- cells, and also indicates that MERP-1 expression in CD34^- cells is down regulated after one week culture (7.8 ± 1.2 vs. 4.2 ± 1.0, n = 10, p ≤ 0.007). (Figure 2.11).
Figure 2.11 MERP-1 Gene Expression (relative to β-actin & GAPDH and expressed in arbitrary units) as measured by SYBR Green™ Real-Time PCR

MERP gene expression in CD34⁻, CD34⁺ and CD34⁺ hematopoietic cells after one week culture (CD34⁺culture) from UCB (n=10). * p ≤ 0.007
2.5 DISCUSSION

The ideal comparison for isolating gene sequences specifically involved in determining HSPC function would be the comparison of cDNA isolated from a pure HSPC population versus cDNA isolated from the same population depleted of HSPCs. The source of the cDNA used in this study was from CD34 enriched and depleted cell population. While expression of the CD34 antigen defines a population of cells which is enriched for haemopoietic progenitor activity, it is a heterogeneous population of lineage restricted and multi-potential cells (Morrison et al 1995). However, at the inception of this project, isolation of cDNA from a more primitive population of progenitors was not viable for several reasons. Firstly, the instrument required to isolate such a population (Fluorescence Activated Cell Sorter) was unavailable. Additionally, preliminary experiments indicated that the limited cell number obtained from such an isolation strategy would prove restrictive for an extensive gene discovery program. As such, it was decided that cDNA from CD34 enriched and depleted cell populations would be used in gene discovery protocols, and more primitive progenitor populations would be used for subsequent exploration of gene expression after confirmation of differential expression.

Immuno-magnetic isolation of HSPC populations defined by expression of the CD34 antigen resulted in a mean purity of approximately 61 and 55% from UCB and ABM samples (respectively). cDNA from these samples was then compared to corresponding cDNA from samples that were however, almost entirely depleted of were CD34+. Subsequent dd-PCR results indicate that the lower than ideal purity of the CD34+ enriched populations provided no barrier to success in identifying differentially expressed genes. This is probably at least partially due to the ability of dd-PCR to identify up and down-regulation of gene expression and not just the presence or absence of expression. Additionally, false positives were minimized by the excision of genes that appeared differentially expressed in multiple comparisons.
Using this method of dd-PCR, six differentially expressed transcripts were identified in CD34* enriched HSPC populations, with low expression in CD34 depleted populations. The cDNA sequences of four of these differentially expressed transcripts were homologous to well characterised genes. Interestingly, two of these transcripts (bands 4 and 10) were homologous to different regions of the mitochondrially encoded gene for ATPase 6 (ATPase6).

ATPase6 gene codes for the large, integral membrane subunit of mitochondrial ATP synthase (F1F0-ATPase) which is the key enzyme for ATP production in mammalian cells. It catalyses ADP phosphorylation for respiratory ATP formation (reviewed by Saraste 1999). Mutations in the ATPase6 have been implicated in several serious human diseases including Leighs syndrome, a fatal encephalopathy of infancy and a multi-systemic syndrome characterised by neuropathy, ataxia and retinosa pigmentosa (NARP) (reviewed by Graeber and Muller 1998). Additionally, many studies demonstrate increased expression of the ATPase6 gene or ATP synthase activity as a feature of cell immortalisation (Kim et al 2001) and neoplastic transformation (Torroni et al 1990, Chen et al 1998, Perona and Serrano 1988). In contrast, decreased expression of, and mutations in the ATPase6 gene have been described as a feature of aging (Edris et al 1994, Marin-Garcia et al 1997, Marin-Garcia et al 1994), and the anti-proliferative and anti-tumorigenic actions of interferon and angiotatin have been postulated to be at least partially due to inhibition of ATPase6 gene expression and subsequent inhibition of ATP synthase activity (Moscr et al 1999, Lou et al 1994). Taken together, this data demonstrates increased expression of ATPase6 in cells with a high proliferative capacity that is consistent with differential expression of ATPase in human HSPCs. Additional data by Mills et al (1999) demonstrating that inhibition of ATP synthase activity by the specific inhibitor oligomycin increases differentiation and apoptosis of a human promyelocytic cell line supports this hypothesis and raises interesting possibilities as to the role of ATPase6 in haemopoietic progenitors. While it is clear that this is worthy of future investigation, as the aim of this study was to identify novel differentially expressed genes, this particular line of investigation was not pursued. Similarly,
further investigations of bands 16 and 14 that also encoded well-characterised gene sequences were not initiated.

Bands 1 and 20 however were found to be homologous to two uncharacterised, partial gene sequences. Additional sequence of both these transcripts was obtained using BLAST searching of the GenBank EST and NR database. This strategy however did not complete these sequences and additional coding nucleotides remained to be identified. These transcripts were designated ORP-3 and MERP-1 on the basis of homology between their conceptual protein products and other well characterised proteins (discussed further in Chapters 3 and 4 of this dissertation).

Taqman™ and SYBR Green real-time PCR was employed to confirm differential expression of the ORP-3 and MERP-1 genes. Results confirm higher expression of both these genes in a population of HSPCs defined by expression of the CD34 phenotype. Expression of ORP-3 was found to be 2.5 to 4-fold higher in CD34+ cells from UCB and ABM (respectively) than corresponding CD34- populations. Expression of MERP-1 was 10 and 4-fold higher in equivalent populations respectively. Interestingly, we also observed significantly higher expression of ORP-3 in CD34+ cell populations from ABM compared to equivalent cell populations from UCB. The CD34+ cells from these two sources are known to exhibit various differences in their in vitro and in vivo characteristics. Compared to ABM, UCB CD34+ cells contain a higher number of clonogenic cells in short and long-term cultures with a higher re-plating potential and ex vivo proliferative potential (reviewed by Cairo and Wagner 1997). Differential expression of ORP-3 in HSPCs from UCB and ABM implies a potential role in the regulation of their different biological properties.

The CD34+ population of cells is heterogeneous and contains multi-potential cells that are needed for stable long term multilineage haemopoiesis, and more mature lineage restricted cells that are required for short term production of functional blood cells of individual lineages (Morrison et al 1995). Therefore, we investigated ORP-3
gene expression in this population before and after 7 days culture in media containing growth factors that promote the differentiation and proliferation of hematopoietic progenitors (reviewed by Graham and Wright 1997, Ogawa 1993, Metcalf 1993). Our results indicate that under these culture conditions, mean ORP-3 and MERP-1 gene expression in freshly isolated, uncultured CD34+ cells is 2 to 5-fold higher than in these same cells after 7 days culture.

Taken together, ORP-3 and MERP-1 expression data indicates higher expression in a less differentiated subset of hematopoietic cells. The physiological significance of this differential expression and the function of ORP-3 and MERP-1 in these cell populations require further investigation. Elucidation of the full sequence of both these genes, and computer assisted proteomics as discussed in Chapters 3 & 4 of this dissertation will be useful in designing functional assays that may reveal these functions.
CHAPTER 3
ELUCIDATION OF THE FULL MERP-1 SEQUENCE AND BIOINFORMATICS

3.1 ABSTRACT

A partial sequence for MERP-1 was identified by dd-PCR in CD34⁺ haemopoietic progenitor cells, with low expression in CD34⁻ haemopoietic cells. This transcript had 100% homology with a previously identified partial gene sequence for the putative UCC1 protein (Nimmrich et al 2001). Using RACE and PCR, additional 5' sequence (257 nucleotides) compared to the UCC1 mRNA has been identified. When combined with the UCC1 cDNA, this novel sequence codes for additional 173 amino acids. This additional sequence completes the full coding region of the MERP-1 gene. The MERP-1 cDNA is 2600nt long, and localizes by bioinformatics to Chromosome 7. It consists of three exons and 2 introns spanning an entire length of 31.4kb. The MERP-1 open reading frame codes for a putative 344 amino acid type II transmembrane protein with an extracellular C-terminal ependymin like-domain (Hoffmann and Schwarz 1996) and an intracellular N-terminal sequence with significant homology to the cytoplasmic domains of members of the protocadherin family of transmembrane glycoproteins (Angst et al 2001). Ependymins and protocadherins are well-characterised calcium-dependant cell adhesion glycoproteins. Although the function of MERP-1 remains to be elucidated, it is possible that MERP-1 like its homologues plays a role in calcium dependent cell adhesion. Differential expression of the MERP-1 gene in haemopoietic cells suggests role in haemopoietic stem cell proliferation and differentiation, however, its broad
tissue distribution implies that it may also play a role in many cell types. Characterization of the MERP-1 protein is required to elucidate these possible roles.
INTRODUCTION

Using the technique of dd-PCR to compare the gene expression profiles of CD34\(^+\) and CD34\(^-\) hematopoietic cells, a differentially expressed transcript with 100% homology to the recently identified partial gene sequence for the putative UCC1 protein (Accession No. BC000686 - *Homo sapiens* mRNA for UCC1 protein) (Nimmrich et al., 2001) was isolated and sequenced. Conceptual translation of the ORF of the UCC1 cDNA identified an amino acid sequence with an ependymin-like domain. Until recently, no other ependymin-like proteins have been identified in any other species (Hoffmann and Schwarz 1996). This sequence is a mammalian ependymin homologue and we have termed it mammalian ependymin related protein 1 (MERP-1). Sequences for additional mammalian homologues from monkey and mouse have recently been added to the GenBank database and it is now clear that a new family of MERP-1-like genes exists.

The purpose of this study was to elucidate the complete coding sequence of MERP-1, to characterise the putative MERP-1 protein by bioinformatics and to compare this sequence to its ependymin and protocadherin homologues. Additionally, expression of MERP-1 in multiple tissues was further investigated by northern hybridization.
3.3 METHODS

3.3-1 Ethics approval

All experimental procedures were conducted in accordance with the regulations and guidelines outlined by the National Health and Medical Research Council, and approved by the Deakin University (Ethics approval number EC-82-97) and Geelong Hospital Ethics Committees (Ethics approval number 97-14).

3.3-2 Cell sources

UCB samples were obtained after uncomplicated vaginal or cesarean delivery after clamping and cutting of the cord and drainage into 50ml sterile collection tubes containing 200 units of heparin (David Bull Laboratories, Mulgrave, Aust.) in 5mls of alpha medium (αMEM) (Trace Scientific, Noble Park, Aust.). All samples were donated by volunteers at the Geelong Hospital, Barwon Health (Geelong, Victoria), according to approved institutional guidelines.

3.3-3 Red Cell Depletion of UCB samples

UCB sample was diluted 1: 2 with PBS, mixed thoroughly by gentle inversion centrifuged for 30 minutes at 480g and 18 - 20°C. Centrifuge was set at low brake. After centrifugation, the low-density white blood cells were seen as a buff-colored carpet on top of the denser red cells. These were removed using a cannula and syringe and placed in a sterile 50-ml tube. Red cell depleted UCB samples were made up to a volume approximately equal to the original UCB sample with PBS.
3.3-4 Preparation of Mononuclear cells from UCB and ABM samples

UCB and ABM samples depleted of red cells and bone fragments respectively were carefully and gently layered onto an equal volume of Ficoll-Hypaque® (Amersham Pharmacia Biotech, Uppsala, Sweden) in 15 or 50ml sterile tubes. Great care was taken to ensure that there was minimal/no mixing of cell samples with Ficoll-Hypaque®. Samples were centrifuged for 25 minutes at 440g and 18 – 20°C. Centrifuge was set to low brake. After centrifugation, the upper plasma/media layer was drawn off using a clean pasteur pipette. Mononuclear cells (MNCs) at the ficoll-mononuclear cells (MNCs) were carefully removed using a sterile 5 or 10-ml syringe and cannula. MNCs were washed twice with the addition of two volumes of PBS and centrifugation at 480g for 10 minutes at 18 – 20°C. After the second wash, the supernatant was removed, and MNCs resuspended. Viability was assessed using Trypan Blue staining.

3.3-5 mRNA Extraction and Rapid Amplification of cDNA Ends (RACE) to obtain additional MERP-1 sequence

Nested MERP-1 5’ RACE primer sequences (Table 3.1) optimised for use with the Marathon™ cDNA Amplification Kit (Clontech, La Jolla, U.S.A.) were designed using PRIME software on the ANGIS database. Primers 1 and 2 were designed according to the UCC1 cDNA sequence. Primers 3 and 4 were designed consensus to novel MERP-1 sequence as it was revealed (Figures 3.1 and 3.2).

mRNA was extracted from UCB MNC using Oligotex™ Direct mRNA Kit (Qiagen, Clifton Hill, Aust.) and 5’ RACE PCR was carried out using a Marathon™ cDNA Amplification Kit following manufacturers guidelines. All PCR reactions were performed on a Perkin Elmer 9600 thermocycler (P.E. Applied Biosystems Foster City, U.S.A.) using touchdown cycling. 5’ RACE PCR products were visualised by
standard agarose gel electrophoresis in a 1.5% low-melting point TAE gel and ethidium bromide staining. Bands to be sequenced were excised, purified using Qiaquick Gel Extraction Kit (Qiagen) and sequencing reactions carried out using ABI Prism BigDye™ Terminator Cycle Sequencing Ready reaction Kit (P.E. Applied Biosystems) according to the manufacturers instructions and 3.2pmols of appropriate RACE primer. Sequencing PCR products were ethanol precipitated, air-dried, and sequences determined by staff at Monash University (Clayton, Aust.) using an ABI PRISM™ 373 DNA Sequencer (P.E. Applied Biosystems).
Table 3.1 MERF-1 5’ Nested RACE PCR Primer Sequences

<table>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>1</td>
<td>5’- ccg agg gca agc cca agc cca ag-3’</td>
</tr>
<tr>
<td>2</td>
<td>5’- cac tgt gcc ggg agt gtg gtc tg -3’</td>
</tr>
<tr>
<td>3</td>
<td>5’- ccg ggg aag gct cca gtg aat cg -3’</td>
</tr>
<tr>
<td>4</td>
<td>5’- cca cta gca tcc tgg gag gca tcc tg -3’</td>
</tr>
</tbody>
</table>
Figure 3.1 Schematic diagram of MERP-1 and UCC1 cDNA and protein sequences.

3' and 5' UTRs indicated by open boxes, ORFs by shaded boxes, and protein sequence by black boxes. Regions amplified by RACE and liver library PCR indicated in brackets.
Figure 3.2 The complete MERP-1 cDNA (2066nt)

Original DD-PCR transcript depicted in black, additional sequence obtained by consensus with UCC1 (blue), novel sequence obtained by 5’ RACE and PCR amplification from a liver library depicted in red. Nested reverse RACE primers 1 - 4 (nrrp 1-4) are indicated (open boxes). Positions of forward and reverse primers used for amplification of MERP-1 amplicon 1 (fp1 and rp1) and 2 (fp2 and rp2) from a human liver library are also indicated (yellow boxes). Positions of putative start and stop codons highlighted by *.
3.3-6. cDNA library PCR to confirm additional MERP-1 sequence

Using PRIME software on the ANGIS database, primers were designed to amplify two PCR products. Forward and reverse primers for amplicon 1 were designed consensus to the existing UCC1/MERP-1 cDNA (Figures 3.1 and 3.2). The reverse primer for amplicon 2 was designed consensus to the existing UCC1/MERP-1 cDNA. The forward primer for amplicon 2 was designed consensus to nucleotides on a genomic clone 5' to an area that the UCC1/MERP-1 cDNA mapped to (Figures 3.1, 3.2 and 3.3). Visual analysis of this region on the genomic clone revealed a putative transcription initiator (Figure 3.3).

Primers for amplicons 1 and 2 (Table 3.2) were used to obtain a 1003 nucleotide (nt) amplicon (amplicon 1) and a 553nt amplicon (amplicon 2) from a human liver cDNA library. These two PCR products spanned the entire MERP-1 coding region (with 298-nucleotide overlap) (Figure 3.1 and 3.2). PCR was performed with PCR Enhancer, Platinum Taq Polymerase (Invitrogen, Carlsbad, U.S.A.) and touchdown cycling on a Perkin Elmer 9600 thermocycler (P.E. Applied Biosystems Foster City, U.S.A.). PCR products were visualised and sequence fidelity verified as previously described in this chapter (section 3.3-5).
Figure 3.3 5’ Sequence of MERP-1 demonstrating two possible start codons and putative transcription initiator.

Alignment of the 5’ cDNA sequence of MERP-1 with the chromosome 7 genomic sequence (Accession no. AB018634) (bold) demonstrates the putative transcription initiator (highlighted in gray nts –4 to +5), 5’UTR, two possible start codons (boxed and yellow at positions 20 and 32) and kozak sequences (open box).
Table 3.2 Primer Sequences used to amplify the complete MERP-1 coding region.

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<td><strong>Forward primer</strong></td>
<td>5'- act ttg gtc cag acc aca ctc ccg -3'</td>
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<tr>
<td><strong>Reverse primer</strong></td>
<td>5'- cca cac cct cgc cat tca att ttc -3'</td>
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<table>
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<td><strong>Forward primer</strong></td>
<td>5'- tcc ccc ctc tta aaa c - 3'</td>
</tr>
<tr>
<td><strong>Reverse primer</strong></td>
<td>5'- act ttg cta cta cat aac ctg g - 3'</td>
</tr>
</tbody>
</table>
3.3-7. Northern Hybridization

A 1003nt MERP-1 amplicon (amplicon 1, section 3.3-6) was labeled using $[\alpha^{-32P}]dATP$ and a Strip-Eze$^\text{TM}$ DNA probe synthesis kit (Ambion Inc, Austin, U.S.A.). Unincorporated label was removed using Probe Quant G50 microspin columns (Amersham Pharmacia Biotech.). The labeled MERP-1 probe was hybridized to a Human Multiple Tissue Northern Blot$^\text{TM}$ (Clontech) containing equal amounts of poly(A)$^+\text{RNA}$ from 12 different tissues, with ULTRAhyb$^\text{TM}$ hybridization buffer (Ambion Inc.) according to the manufacturers instructions, and was visualized by autoradiography.

3.3-8 Bioinformatics

Nucleic acid and protein sequences were analysed using software available from the Australian National Genomic Information Service (TRANSLATE, BESTFIT, ECLUSTALW), National Center for Biotechnology Information (BLASTN, BLASTP, TBLASTX), EXPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (PredictProtein, ProfileScan, PSORT II, ProtParam, Tmpred, ProtScale, Signal IP) and the TRANSFAC database of the Research Group Bioinformatics/AG Bioinformatik (MatInspector).
3.4 RESULTS

3.4.1 Elucidation of additional 5' MERP-1 sequence by RACE and PCR, and localization to Chromosome 7 by Bioinformatics

Using a combination of RACE and PCR, a further 257nt of the 5' sequence of the UCC1/MERP-1 cDNA was identified to complete the full coding region (Figure 3.1). The MERP-1 cDNA is 2600nt long (Figure 3.2). This sequence has been confirmed by PCR amplification and sequencing of two overlapping MERP-1 amplicons from a liver cDNA library (Figure 3.1) and by alignment to a genomic clone (GenBank Accession No. AC018634).

Alignment of the current 5' region of MERP-1 with its genomic sequence on chromosome 7 (Accession No. AC018634) reveals a possible transcription initiator at position -4 to +5 (Figure 3.3). No TATA box is observed within 30nts of the transcription initiator.

A 5' untranslated region (UTR) of 19nt precedes a possible translation start site at position 20 that is defined by a consensus Kozak sequence (Kozak, 1995). Because the 5' UTR of MERP-1 is quite short, it is possible that the 40S ribosomal subunit will bypass the first ATG and initiate translation at a second possible translation start site at position 32 which also has a consensus Kozak sequence (Figure 3.3). A third possible start site identified previously in the partial UCC1 gene sequence (Nimmrich et al 2001) is located at position 380 and translation from this position significantly truncates the encoded protein (Figure 3.4). However, as most eukaryotic genes only require approximately 20nts of 5' UTR and adhere to the first ATG rule, it is more likely that translation will begin at position 20 or 32 (Kozak 1995). Characterization of the MERP-1 protein is required to confirm this.
The 5' sequence of MERP-1 has been difficult to obtain due to its unusually high GC-content and a region of low complexity. In particular, analysis of this region using Taqman Primer Express™ software reveals an area of low-complexity (nt 95-136) capable of forming stable secondary structures that have made RACE and sequencing difficult (Figure 3.5). Reflecting this, the GenBank expressed sequence tag (EST) database contains several partial MERP-1 ESTs that differ in this region (Accession Nos. AW409509, BG765975 and BG762982 contain two repeats in this region. Accession Nos. BG707397, BG490552, and BG721912 contain one) (Figure 3.6)

Sequences for additional mammalian homologues from monkey and mouse have recently been added to the GenBank database and it is clear that a new family of MERP-like genes exists (Apostoulopolous et al 2001). Currently, the nucleotide sequences of a monkey ependymin related protein (mo-MERP-1) (Accession No. AB063094) and two-mouse ependymin related proteins (mu-MERP-1 and mu-MERP-2) (Accession Nos. AY027861 and AF353717) have been cloned. The 5' sequences of these genes, although shorter, contain significant homology (~82%) to the novel coding sequence we have identified for MERP-1 (Figure 3.7). The mu-MERP-1 sequence is currently ORF however the mo-MERP-1 & mu-MERP-2 sequences deviate from the MERP-1 sequence over the region of low complexity which terminates the ORF. It is possible that the same factors contributing to the difficulty obtaining the 5’ sequence of MERP-1 are applicable to the murine and monkey nucleotide sequences.

The MERP-1 coding region terminates at position 1054 and is followed by a polyadenylation signal and a poly-A tail at positions 2518 and 2590 respectively (Figure 3.4). The 3' UTR of 1546nt (Figure 3.4) is approximately twice the mean length for all known human cDNAs (Pesole et al 1999).

The genomic MERP-1 sequence maps to chromosome 7 (Accession No. AC018634) and consists of 3 exons and 2 introns spanning an entire length of 31.4kb (Figure 3.8)
and Table 3.3). Intron/exon boundaries were found to conform to the GT-AG rule. The additional novel coding sequence reported here is contiguous with exon 1 of the previously reported gene sequence for the putative UCC1 protein (Nimmrich et al 2001).
Figure 3.4 The complete MERP-1 cDNA (2600nts)

Depicting positions of exon boundaries (exons alternating between blue and green). Putative start codons displayed (boxed yellow) at positions 20 and 32. The start codon previously identified by Nimmrich et al. (2001) in the partial UCC1/MERP-1 sequence is indicated at position 380. Consensus Kozak sequences highlighted in gray, stop codon (boxed blue), and poly adenylation signal and poly-A tail boxed in pink and green respectively.
Figure 3.4. (cont)

1301 ttctctatgc cttgttcaggg ctgtttttct gcattgcaagg gtataacacat
1351 aaaggcgagtgc ctagcacta gggagggcgt agtaagagaa gtgtgcttctg
1401 gcagcgttacta ttatttgtgg acataatattc gatttggtaga taataaaaaag
1451 cagagtagatt ttgtgcaatt ttattataaa ttcttaattt cctcgcagag
1501 aatgcococct ttatgctgc accaggggtg ggacttgctc ccctgagccc
1551 taatccaccc tgcctctgca cctcccttgtgt gcgcaaaaaa atgataacttt
1601 aatactcttc cagacaccaag atattttatgg ctaggceccas ttagatataaa
1651 catttaggaag gasatgaaaaa gtaaaaaag gtaaataatc ttctctataaa
1701 gccacacttt gagcaaggca gtttagagaa ttcttaagtc tacaatggcac
1751 tagacacgag cattgtagct tctttttttt ctatgcaaga gattgtgtgt
1801 atgtgtctgaa ttcttcacaga cttgcaata cccaggcagt attcataaat
1851 aggccgtaac aggagtctag gacactattt tctctttaaaa cccaggactag
1901 atgttcctgag tattgtcact cctttggtca ttaaatgcac ttggcttgctc
1951 ccgctcttgg cctctctagca acgtcttttc ataaacctctc tattgtgtgt
2001 cttgcacctcc ttctcaggtca gctcttctac aagagttgct cccagctgtcy
2051 gattgctttg accttgcatc ttgagatctg atcttctcaca ataatatatata
2101 agtctgttga taattttcctgc ttctgagaca ctctacctct attcgtgtgtc
2151 tgagtctttt ggctacgggt accttttagg tttataattt tatactctagc
2201 aacaaagcag ccctgtggcaac atagtcagtg ccctaagtat ttgtagagty
2251 aagaaagcca gctctctctg tccttctgct ctattgtgtg tgaatgtgtgt
2301 tggatgctgg ccctgctagg gagagacctt gataaataaa atttttatttt
2351 ctgtgtgctt ccctgtgtat gtttattcaca ccattttatc ttctttagtt
2401 accagagaga atgttgagac cctttatattt aagaaaca atctttatc
2451 ttatatattta ttatattata aacaaattttaa ttgccatattaa agatctttttc
2501 gatattgttt ctttgcaggt aatattggtat attctttggtt aatagggagtgt
2551 taaaaaactag cttgtaatata taatagtttt ctatgtctaa caaaaaaaaaa

132
Figure 3.5 Nucleotides 95 - 136 of the MERP-1 gene

Analysis of nucleotides 95 – 136 of the MERP-1 region of low complexity using Taqman™ Primer Express software demonstrates the ability of this region to form stable secondary structures thus eluding reverse transcription and making sequencing difficult.
Alignment of MERP-1 with several ESTs on the GenBank database using ECLUSTALW demonstrates the sequence ambiguity created by the region of low complexity from nucleotides 95 – 136.

AW409709
BG490562
BG707397
BG721912
BG765975
BG762982
MERP-1

------------- gacg -------------
------------- ggtg --------------
------------- ccagcactg --------------
------------- gcagcactg --------------
------------- gcagcactg --------------
------------- gcagcactg -------------

------------- tggcgcacccagtggcgcagcagcagt -------------
------------- gtggagcctgagcagtgcacccagtggcgcagcagcagt -------------
------------- acgcagcactg --------------
------------- gcagcactg --------------
------------- gcagcactg -------------
------------- gcagcactg -------------

------------- gcagcagcagcagtggcgcagcagcagtggcgcagcagcagtggcgcagcagcagtggcgcagcagcagt -------------
------------- gcagcagcagcagtggcgcagcagcagtggcgcagcagcagtggcgcagcagcagtggcgcagcagcagt -------------
------------- gcagcagcagcagtggcgcagcagcagtggcgcagcagcagtggcgcagcagcagt -------------
------------- gcagcagcagcagtggcgcagcagcagtggcgcagcagcagt -------------
------------- gcagcagcagcagtggcgcagcagcagtggcgcagcagcagt -------------

***************
Figure 3.7 Alignment of the nucleotide sequences of Mammalian MERP-1 homologues using ECLUSTALW.

Mo-MERP-1 (ABO63094), mu-MERP-1 (AYO27861) and mu-MERP-2 (AF353717).

mo - MERP-1  ...
mu - MERP-1  ...
hu - MERP-1  tcccccctctctttttaaacaagatgcctccccaggtgtctgtctgtgcaaaatgtgccttccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  cctgtggccagcaggtgacgtgacgtgaaatcagctcaggtgatcttccccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  ggcctctggtctcgtgctcaggtgagctggtctcgagctcacaagcacaggggtgccttccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  ggcctctggtctcgtgctcaggtgagctggtctcgagctcacaagcacaggggtgccttccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  ggcctctggtctcgtgctcaggtgagctggtctcgagctcacaagcacaggggtgccttccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  ggcctctggtctcgtgctcaggtgagctggtctcgagctcacaagcacaggggtgccttccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  ggcctctggtctcgtgctcaggtgagctggtctcgagctcacaagcacaggggtgccttccc

mo - MERP-1  ...
mu - MERP-2  ...
hu - MERP-1  ggcctctggtctcgtgctcaggtgagctggtctcgagctcacaagcacaggggtgccttccc
Figure 3.8 Schematic diagram of the genomic structure of the MERP-1 gene and encoded protein.

Exons 1 – 3 of the genomic MERP-1 sequence are depicted in black boxes with a schematic of the protein shown below. The putative MERP-1 protein sequence demonstrates the position of the signal sequence (black), transmembrane domain (gray) and ependymin like region (lined). N-terminal sequence homologous to the cytoplasmic tail of three members of the protocadherin family indicated by an open box.
Table 3.3 Localisation of MERP-1 to Chromosome 7 (Accession No. AC018634) by bioinformatics.

<table>
<thead>
<tr>
<th>Exon/Intron</th>
<th>From</th>
<th>To</th>
<th>Reverse &amp; Complement</th>
<th>Length (nts)</th>
</tr>
</thead>
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<tr>
<td>Exon 1</td>
<td>72023</td>
<td>72670</td>
<td>yes</td>
<td>648</td>
</tr>
<tr>
<td>Intron 1</td>
<td>44391</td>
<td>72022</td>
<td>yes</td>
<td>27632</td>
</tr>
<tr>
<td>Exon 2</td>
<td>44183</td>
<td>44390</td>
<td>yes</td>
<td>209</td>
</tr>
<tr>
<td>Intron 2</td>
<td>43032</td>
<td>44182</td>
<td>yes</td>
<td>1151</td>
</tr>
<tr>
<td>Exon 3</td>
<td>41299</td>
<td>43031</td>
<td>yes</td>
<td>1733</td>
</tr>
</tbody>
</table>
3.4-2 MERP-1 Northern Hybridization

Investigation of MERP-1 expression by northern hybridization revealed the presence of one transcript in various normal human tissues measuring approximately 2.6kb (Figure 3.9). Highest levels of expression were observed in brain, heart and skeletal muscle with lower expression in kidney, spleen, placenta and lung. Expression of MERP-1 in colon and peripheral blood leukocytes was below detectable levels.
Figure 3.9 Expression of MERP-1 in multiple tissues as detected by Northern Hybridization.

Lanes 1 - 12 = brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, peripheral blood leukocytes (respectively).
3.4.3 MERP-1 Protein Bioinformatics

Conceptual translation of the full MERP-1 ORF yields a putative 344 amino acid protein with a MW of 38.1kD and pI of 9.66 (unglycosylated). Blast analysis of this protein reveals significant homology to two families of calcium dependant cell-adhesion molecules – ependymins and protocadherins (Shashoua 1991, Angst et al. 2001).

Both protocadherins and piscine ependymins are synthesized via precursors containing a hydrophobic signal sequence typical of secretory proteins. Similarly, MERP-1 contains an N-terminal hydrophobic signal peptide that is predicted to be cleaved between S21 and E22 to result in a 323 amino acid mature protein with a MW of 35.9kD and a pI of 9.59 (Figure 3.10). The hydrophobic N-terminal signal peptide sequence is evident (high hydrophobicity index) on a hydropathy plot (Figure 3.11) for the first 21 amino acids of MERP-1.

The hydropathy of the theoretical MERP-1 protein plot displays an additional stretch of 22 amino acids with a high hydrophobicity index from A133 to G154 indicating the presence of a possible transmembrane domain (Rost 1996) (Figure 3.11). Further analysis of this transmembrane-like domain using software available on the EXPASY database (TMpred) designates MERP-1 as a Type II membrane protein (N-terminal inside) (Figure 3.12). This is analogous to protocadherins which are integral transmembrane glycoproteins (Suzuki, S.T. 2000). In contrast, fish ependymins do not contain transmembrane domains and form the major soluble glycoprotein in cerebrospinal fluid. The transmembrane-like domain of MERP-1 corresponds in position to the N-terminal hydrophobic signal sequence of teleost ependymins (Figure 3.13) and suggests that the additional 5' sequence of MERP-1 is evolutionarily divergent.

Comparison of the predicted amino acid sequence of MERP-1 with seven other well-characterized teleost ependymins (for alignment with two teleost ependymins, see
Figure 3.13) reveals several conserved features. The identity between MERP-1 and the teleost ependymins is approximately 50% and because MERP-1 is on average 128 amino acids longer than the teleost sequences, all of this identity occurs in the C-terminal extracellular part of the sequence from M\(^{121}\) (Figure 3.13).

In most teleosts, ependymins exist as dimeric proteins encoded by two highly homologous genes (Muller-Schmid et al 1992). The dimeric proteins are held together by disulfide links possibly utilizing four highly conserved cysteine residues (Muller-Schmid et al 1992). Alignment of fish ependymins and their mammalian homologues demonstrates conservation of these cysteine residues, and also potentially reveals three additional sites found only in mammalian homologues (Figure 3.13). Whether these are utilized for the formation of inter-molecular disulphide bonds incorporating another related protein remains to be elucidated.

We have also compared the putative MERP-1 protein sequence with the amino acid sequences obtained from theoretical translation of the entire mo-MERP-1, mu-MERP-1 and mu-MERP-2 ORF. Identity with the three-mammalian homologues is considerably higher at approximately 96, 99 and 83% respectively (Figure 3.13).

The amino acid sequence of MERP-1 contains four potential N-linked glycosylation sites. Two of these are positioned within the putative intracellular protocadherin-like domain (N\(^{23}\)G\(^{26}\) and N\(^{63}\)R\(^{40}\)) and two are positioned within the extracellular ependymin-like domain, (N\(^{250}\)F\(^{253}\) and N\(^{302}\)V\(^{305}\)) (Figure 3.13). N-linked glycosylation sites are also found in fish ependymins where have been demonstrated to bind oligosaccharides (Ganss and Hoffmann, 1993). Thus, like teleost ependymins and protocadherins, the mature MERP-1 is likely to be glycosylated. Interestingly, the position of these glycosylation sites is highly conserved amongst the mammalian ependymin-like proteins only (Figure 3.13).

BLAST analysis of the N-terminal intracellular region of MERP-1 reveals approximately 43% homology with the intracellular cytoplasmic tails of three
members of the protocadherin family – *Mus musculus* Cadherin-related neural receptor 5 (BAA29050), *Homo sapiens* Protocadherin alpha 13 precursor (Q9Y510) and *Mus musculus* Protocadherin alpha 12 (AAK26048) (Figure 3.14). Protocadherins are also postulated to function in calcium-dependant cell adhesion. Unlike fish ependymins however, this feature is dependent upon a variable number of extracellular repeat sequences characteristic of all cadherins termed ectodomains which lie outside of the region of homology with MERP-1 (Gumbiner 1996) (Figure 3.12).

The N-terminal sequence of MERP-1 also displays the consensus sequence for two glycosaminio-attachment sites at positions S^{38} and S^{113} (Figure 3.13). Such sites are a defining feature of proteoglycans and act as binding sites for glycosaminoglycan chains such as heparin sulfate and chondroitin sulfate (Kolset and Gallagher 1990, Bourdon et al 1987). Glycosaminio-attachment sites were not found in any of the teleost ependymin and protocadherin sequences examined.
Figure 3.10 MERP cDNA and encoded protein displaying intron/exon boundaries.

Exon 1 (nucleotides 1 – 648), exon 2 (nucleotides 649 – 858) and exon 3 (nucleotides 859 – 2600) displayed in red, blue and black respectively. Hydrophobic residues within the putative signal sequence (amino acids 1 – 21) and transmembrane domain (amino acids 133 – 154) highlighted in gray. N-linked glycosylation sites highlighted in pink and glycosaminoglycan attachment sites in blue. Putative C-terminal extracellular ependymin –like domain (boxed in green) and N-terminal intracellular protocadherin –like domain (boxed in red) are also displayed.
Figure 3.11 Hydropathy plot of the putative MERP-1 protein

Hydrophobic signal sequence (amino acids 1 – 21) and putative transmembrane domain (amino acids 133-154) are demonstrated.
Figure 3.12 Schematic diagram of the putative Type II MERP-1 protein depicting extracellular region of homology to Teleost ependymins and intracellular region of homology to protocadherins.

Putative Type II MERP-1 protein sequence demonstrating possible transmembrane domain (gray), ependymin like-region (green) and N-terminal sequence homologous to cytoplasmic tail of three members of the Type I protocadherin family (red). Cadherin-like repeats of protocadherin ectodomain indicated (blue). Figure is not to scale.
Figure 3.13. Alignment of the putative MERP-1 protein with two teleost and three mammalian homologues.

Goldfish ependymin (G.fish, Accession No.AAA49165), Zebrfish ependymin (Z.fish, Accession No. CAA36742), Human MERP1 (hu-MERP1, Accession No. AAK15788), Monkey MERP1 (mo-MERP1, Accession No.BAB01585), Murine MERP1 (mu-MERP1, Accession No.AAK15787) and Murine MERP2 (mu-MERP2, Acession No. AAK29146). Amino acid identity for identical (*), conservative substitutions (.) and moderately conserved substitutions (.) are as indicated. N-glycosylation sites are highlighted by pink boxes and glycosaminoglycan attachments sites by blue boxes. Hydrophobic residues within the signal sequence and transmembrane-like domain are shaded grey. Cysteine residues conserved in mammalian MERPs only are indicated by a grey arrow, whilst a black arrow indicates those conserved amongst all depicted sequences.
Figure 3.14 Alignment of the N-terminal sequence of the putative MERP-1 protein with three protocadherins

Alignment of amino acids 1-109 of human MERP-1 (hu-MERP1, AAK15788), amino acids 727-843 of Homo sapiens Protocadherin alpha 13 precursor (hu-procad α13, Q9Y510), amino acids 691-807 of Mus musculus Cadherin-related neural receptor 5 (mu-CNR 5, BAA29050) and amino acids 726-843 of Mus musculus Protocadherin alpha 12 (mu-procad α12, AAK26048). Amino acid identity for identical (*), conservative substitutions (:) and moderately conserved substitutions (.) are as indicated. N-glycosylation sites are highlighted by pink boxes, conserved cysteine residues by arrows, and glycosaminoglycan attachments sites by blue boxes. Hydrophobic residues within the signal sequence and transmembrane-like domain are shaded gray.
3.5 DISCUSSION

A partial sequence for MERP-1 was identified by dd-PCR in CD34⁺ haemopoietic progenitor cells, with low expression in CD34⁺ hematopoietic cells. This transcript had 100% homology with a previously identified partial gene sequence for the putative UCC1 protein. Using RACE and PCR, additional 5' mRNA sequence (257 nucleotides) has been identified. The full MERP-1 cDNA sequence of 2600nt has been confirmed by sequencing of two overlapping amplicons from a human liver library. Additionally, the size of the MERP transcript we have identified compares well to the size of the MERP-1 transcript (2.6kb) observed by northern hybridization using a commercially prepared multiple tissue blot. Nimmrich et al (2001) reports the size of UCC1/MERP-1 transcript as approximately 2.4kb. As only one transcript was identified in both studies, it is possible that the difference in reported transcript size is due to the use of different size markers. In this study, the MERP-1 transcript was situated between the 2.4kb and 4.4kb markers.

Analysis of the MERP-1 gene reveals several interesting features. The 3' UTR of 1546nt (Figure 3.4) is approximately twice the mean length for all known human cDNAs (Pesole et al 1999). This finding is consistent with previous reports (Zhang et al. 2000b, Gregorio-King et al 2001) and supports the hypothesis that long UTR's are in some way characteristic of genes expressed by developing hematopoietic cells (Zhang et al 2000b). The physiological significance of this increasingly common finding is unknown. The first exon of the MERP-1 gene is also unusually large (648nt) compared to the average size of human first exons (284nt) (Zhang 1998). Interestingly, this exon codes for a putative transmembrane domain and signal sequence (Figure 3.8) which is similar to the organisation of the large first exons reported for protocadherins (Wu and Maniatis 1999).

Northern hybridization revealed MERP-1 expression in various normal human tissues. Highest levels of expression were observed in brain, heart and skeletal
muscle with lower expression in kidney, spleen, placenta and lung. In agreement with our observation of low expression in CD34+ HSPCs, MERP expression was not detected in peripheral blood leukocytes. Consistent with a previous report (Nimmrich et al 2001) we observed no MERP-1 transcript by northern hybridization in normal colon. In contrast to our observation of MERP-1 expression in a variety of normal human tissues, the expression of teleost ependymins has been limited to the brain (Hoffmann and Schwarz 1996, Sterrer et al 1990). This suggests that MERP-1 may have a role other than as described in fish.

The MERP-1 ORF encodes a putative Type II transmembrane protein. The C-terminal extracellular portion of MERP-1 has significant homology to an ependymin family of proteins described in teleost fish (Hoffmann and Schwarz 1996) and indeed contains a characteristic ependymin-like domain.

Teleost ependymins were originally discovered due to their increased synthesis in studies investigating memory consolidation and regeneration of nerves in the goldfish (Shashoua 1976, Shashoua 1976a, Thormodsson et al 1992). Although the precise function of piscine ependymins is unknown, it is postulated that they have a role in calcium dependant cell-cell contact between neural cells (Shashoua 1991, Shashoua 1988, Shashoua and Moore 1978, Piront and Schmidt 1988). Structurally, they share several characteristics with glycoproteins mediating cell contact phenomena, and they bear the L2/HNK-1 epitope (Shashoua 1986) that has been established as a typical feature of many neural cell adhesion molecules (Kruse et al 1984, Kruse et al 1985). Additionally, a peptide fragment of goldfish ependymin has been demonstrated to activate the AP-1 transcription factor (Shashoua et al 2001) which is postulated to have a functional role in the proliferation, differentiation and survival of many cell types, including haemopoietic cells (reviewed by Liebermann et al 1998).

The N-terminal intracellular portion of MERP-1 has homology to the C-terminal intracellular portions of three members of a Type I transmembrane protein family.
termed protocadherins. Protocadherins are members the cadherin superfamily of cell adhesion molecules known to be involved in many biological processes, such as cell recognition, cell signaling, cell communication, morphogenesis, angiogenesis and possibly even neurotransmission (Angst et al 2001). Although the precise function of the cytoplasmic region of protocadherins is unknown, it is postulated to function in intracellular signaling and diversity in this moiety imparts specificity to their interactions with cytoplasmic components (Angst et al 2001).

Comparison of MERP-1 with its protein homologues reveals several conserved features. Both teleost ependymins and protocadherins are synthesized via precursors containing a hydrophobic signal sequence typical of secretory proteins (Sterrer et al 1990, Muller-Schmid et al 1992). Theoretical translation of the MERP-1 ORF yields a protein that also contains a predicted N-terminal hydrophobic signal sequence. Analogous to protocadherins, MERP-1 is predicted to be a transmembrane protein. In contrast, ependymin protein is the major glycoprotein component of the cerebrospinal fluid on various orders of teleost fish (Hoffmann and Schwarz 1996). Interestingly however, despite the observation that all ependymins share features with secretory molecules, a bound form associated with the extracellular matrix has been reported (Hoffmann and Schwarz 1996) and it is postulated that this bound form is the functional form (Schwarz et al 1993).

The amino acid sequence of MERP-1 contains 4 N-glycosylation sites. Such sites in fish ependymins have been demonstrated to bind crucial oligosaccharides that in turn confer calcium-binding capabilities to the ependymins (Ganss and Hoffmann 1993). It is hypothesized that this is essential for interaction with the extracellular matrix (ECM) (Hoffmann and Schwarz 1996). Interestingly, cadherins are also postulated to function in calcium-dependant cell adhesion. Unlike fish ependymins however, this feature is dependent upon a variable number of extracellular repeat sequences characteristic of all cadherins termed ectodomains (Gumbiner 1996) which are not present in MERP-1.
Alignment of fish ependymins and MERP-1 demonstrates conservation of five cysteine residues. In teleosts, these cysteine residues are postulated to be utilised for the formation of disulphide links with other, highly homologous proteins (Muller-Schmid et al 1992, Konigstorfer et al 1989). The conservation of these cysteine residues, and an additional three found only in the mammalian homologues, raises the possibility that MERP-1 also exists as a dimeric protein. The related discovery of two highly homologous murine MERP genes (Apostolopolous et al 2001) supports this hypothesis and also indicates that an additional human MERP gene exists. Current experiments however have been unable to isolate such a sequence (unpublished data).

The N-terminal intracellular sequence of MERP-1 also displays the consensus sequence for two glycosaminoglycan-attachment sites which in proteoglycans, act as binding sites for glycosaminoglycan chains such as heparin sulfate and chondroitin sulfate (Kolset and Gallagher 1990, Bourdon et al 1987). Although the significance of this finding is at present unclear, given the important role such molecules play in the control of hematopoiesis in the BM microenvironment (Kolset and Gallagher 1990), it is interesting to speculate on the possible role of MERP-1 in haemopoiesis.

Thus, on the basis of protein bioinformatics, it appears that the putative MERP-1 protein is a Type II transmembrane glycoprotein. The C-terminal extracellular region has significant homology to ependymins and contains glycosylation sites that may render calcium-binding capabilities, and hence possible calcium dependent adhesion properties. The putative intracellular N-terminal region of MERP-1 has significant homology to the cytoplasmic domains of three members of the protocadherin family of Type I transmembrane glycoproteins. This region is postulated to function in intracellular signaling.

It is well accepted that homologous proteins often share similarities at the functional level. Although the function of MERP-1 remains to be elucidated, it is possible that MERP-1, like teleost ependymins and mammalian protocadherins, plays a role in
calcium dependent cell adhesion that may ultimately affect the differentiation, survival and proliferation of cells. Differential expression of the MERP-1 gene in haemopoietic cells suggests a possible role in HSPC proliferation and differentiation, however, its broad tissue distribution, implies that it may play a role in many different cell types. Characterization of the MERP-1 protein is required to elucidate these possible roles.
CHAPTER 4
ELUCIDATION OF THE FULL ORP-3 SEQUENCE AND BIOINFORMATICS

4.1 ABSTRACT

A partial sequence for ORP-3 was identified by dd-PCR in CD34⁺ haemopoietic progenitor cells, with low expression in CD34⁻ hematopoietic cells. This transcript had 100% homology with a previously identified partial gene sequence for the putative protein KIAA0704. Using RACE and PCR, additional 5' (964 nucleotides) and 3' (601 nucleotides) sequence compared to the KIAA0704 mRNA has been identified. When combined with KIAA0704, this novel sequence codes for additional 219 amino acids and completes the full coding region of the ORP-3 gene. The ORP-3 cDNA is 6631 nt long, and localizes by bioinformatics to Chromosome 7p15-p21. It consists of 23 exons and 22 introns spanning an entire length of 183.5kb. The ORP-3 open reading frame codes for a putative 887 amino acid protein which displays the consensus sequence for a highly conserved oxysterol-binding domain. Other well-characterised proteins expressing these domains have been demonstrated to bind oxysterols (OS) in a dose dependant fashion. OS are hydroxylated derivatives of cholesterol. Their biological activities include inhibition of cholesterol biosynthesis and cell proliferation in a variety of cell types, including haemopoietic cells. Differential expression of the ORP-3 gene in haemopoietic cells suggests a possible role in the transduction of OS effects on haemopoietic cells, however, its broad tissue distribution implies that it may also play a role in many cell
types. Characterization of the ORP-3 protein is required to elucidate these possible roles.
4.2 INTRODUCTION

Using the technique of dd-PCR to compare the gene expression profiles of CD34⁺ and CD34⁻ hematopoietic cells, a differentially expressed transcript with 100% homology to the recently identified partial gene sequence KIAA0704 was isolated and sequenced. Conceptual translation of the KIAA0704 ORF identified an amino acid sequence with a highly conserved oxysterol-binding domain. The partial gene sequence for KIAA0704 was first identified by Ishikawa et al (1998) in their search for novel genes expressed in the brain. More recently, Laitinen et al (1999) identified it as part of an EST search for cDNAs displaying homology to the OS binding domain of OSBP. This group designated the partial gene sequence oxysterol related protein 3 (ORP-3). On the basis of partial and complete gene sequences on the GenBank database, it is clear that ORP-3 belongs to a family of at least 12 oxysterol binding protein related proteins (Moreira et al 2001).

The purpose of this study was to elucidate the complete coding sequence of ORP-3, to characterise the putative ORP-3 protein by bioinformatics and to compare this sequence to other ORP homologues. Additionally, expression of ORP-3 in multiple tissues was further investigated by northern hybridization.
4.3 METHODS

4.3-1 Ethics approval

All experimental procedures were conducted in accordance with the regulations and guidelines outlined by the National Health and Medical Research Council, and approved by the Deakin University (Ethics approval number EC-82-97) and Geelong Hospital Ethics Committees (Ethics approval number 97-14).

4.3-2 Cell sources

UCB samples were obtained after uncomplicated vaginal or cesarean delivery after clamping and cutting of the cord and drainage into 50ml sterile collection tubes containing 200 units of heparin (David Bull Laboratories, Mulgrave, Aust.) in 5mls of alpha medium (αMEM) (Trace Scientific, Noble Park, Aust.). All samples were donated by volunteers at the Geelong Hospital, Barwon Health (Geelong, Victoria), according to approved institutional guidelines.

4.3-3 Red Cell Depletion of UCB samples

UCB sample was diluted 1:2 with PBS, mixed thoroughly by gentle inversion centrifuged for 30 minutes at 480g and 18 – 20°C. Centrifuge was set at low brake. After centrifugation, the low-density white blood cells were seen as a buff-colored carpet on top of the denser red cells. These were removed using a cannula and syringe and placed in a sterile 50-ml tube. Red cell depleted UCB samples were made up to a volume approximately equal to the original UCB sample with PBS.
4.3-4 Preparation of mononuclear cells from UCB samples

UCB samples depleted of red cells were carefully and gently layered onto an equal volume of Ficoll-Hypaque\textsuperscript{R} (Amersham Pharmacia Biotech, Uppsala, Sweden) in 15 or 50ml sterile tubes. Great care was taken to ensure that there was minimal/no mixing of cell samples with Ficoll-Hypaque\textsuperscript{R}. Samples were centrifuged for 25 minutes at 440g and 18 – 20°C. Centrifuge was set to low brake. After centrifugation, the upper plasma/media layer was drawn off using a clean Pasteur pipette. Mononuclear cells (MNCs) at the ficoll-mononuclear cells (MNCs) were carefully removed using a sterile 5 or 10-ml syringe and cannula. MNCs were washed twice with the addition of two volumes of PBS and centrifugation at 480g for 10 minutes at 18 – 20°C. After the second wash, the supernatant was removed, and MNCs resuspended. Viability was assessed using Trypan Blue staining.

4.3-5 mRNA Extraction and Rapid Amplification of cDNA Ends (RACE) to obtain additional ORP-3 sequence

Nested ORP-3 5’ and 3’ RACE primer sequences (Table 4.1) optimised for use with the Marathon\textsuperscript{TM} cDNA Amplification Kit (Clontech, La Jolla, U.S.A.) were designed using PRIME software on the ANGIS database. 5’ nested RACE primers 1 and 2 were designed according to the KIAA0704 cDNA sequence. 5’ nested RACE primers 3, 4 and 5 were designed consensus to novel ORP-3 sequence as it was revealed (Figures 4.1 and 4.2). 3’ nested RACE primers 1 and 2 were designed according to the KIAA0704 cDNA sequence. 3’ nested RACE primer 3 was designed consensus to novel ORP-3 sequence as it was revealed (Figures 4.1 and 4.2).

mRNA was extracted from UCB MNC using an Oligotex\textsuperscript{TM} Direct mRNA Kit (Qiagen, Clifton Hill, Aust.) and 5’ RACE PCR was carried out using a Marathon\textsuperscript{TM}
cDNA Amplification Kit following manufacturers guidelines. All PCR reactions were performed on a Perkin Elmer 9600 thermocycler (P.E. Applied Biosystems Foster City, U.S.A.) using touchdown cycling. 5' RACE PCR products were visualised by standard agarose gel electrophoresis in a 1.5% low-melting point TAE gel and ethidium bromide staining. Bands to be sequenced were excised, purified using a Qiaquick Gel Extraction Kit (Qiagen) and sequencing reactions carried out using an ABI Prism BigDye™ Terminator Cycle Sequencing Ready reaction Kit (P.E. Applied Biosystems) according to the manufacturers instructions and 3.2pmols of appropriate RACE primer. Sequencing PCR products were ethanol precipitated, air-dried, and sequences determined by staff at Monash University (Clayton, Aust.) using an ABI PRISM™ 373 DNA Sequencer (P.E. Applied Biosystems).
Table 4.1 ORP-3 5’ and 3’ Nested RACE Primer sequences.

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<th>3’ nested RACE primers</th>
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Figure 4.1 The complete ORP-3 cDNA (6631)

Original dd-PCR transcript is depicted in black, additional sequence obtained by consensus with KIAA0704 (blue), novel sequence obtained by 5' and 3' RACE depicted in red. Nested 5' RACE primers 1 - 5 (nrrp 1-5) and nested 3' RACE primers 1 - 3 (nfrp1 - 3) are indicated (open boxes). Positions of forward and reverse primers used for amplification of ORP-3 amplicon (fpl1 and rpl1) used as a probe for northern hybridization indicated (yellow boxes). Positions of putative start and stop codons highlighted by *.
Figure 4.2 Schematic diagram of elucidation of the full ORP-3 sequence by RACE and Bioinformatics.

Depicting Start and Stop codons (black boxes), original 581bp dd-PCR transcript (black), sequence obtained by consensus to KIAA0704 (blue), sequence obtained by 5’ and 3’ RACE (red).
4.3-6. Northern Hybridization

A PCR product spanning 1426bp of the ORP-3 coding region was amplified from UCB MNC cDNA in a 20μl standard PCR reaction using Herculase Enhanced DNA Polymerase (Stratagene, La Jolla, U.S.A.), and forward (5'-GCT CAG GAA GTT CTG TTA TCT CC -3') and reverse primers (5'-GCT AAG CTA AGC ACA AGT GAT CA-3') (Figure 4.1). The generated amplicon was subcloned by blunt ended ligation into pCR-Script Amp SK(+) (Stratagene). Sequence fidelity was verified by restriction enzyme digest and sequencing. The ORP-3 insert was isolated by BssH II digest, gel cleaned using Qiaquick Gel Extraction Kit (Qiagen) and labeled using [α-32P]dATP and a Strip-Ez™ DNA probe synthesis kit (Ambion Inc, Austin, U.S.A.). Unincorporated label was removed using Probe Quant G50 microspin columns (Amersham Pharmacia Biotech.). The labeled ORP-3 probe was hybridized to a Human Multiple Tissue Northern Blot™ containing equal amounts of poly(A)+RNA from 12 different tissues (Clontech), with ULTRAhyb™ hybridization buffer (Ambion Inc.) according to the manufacturers instructions, and was visualized by autoradiography.

4.3-7 Bioinformatics

Nucleic acid and protein sequences were analysed using software available from the Australian National Genomic Information Service (TRANSLATE, BESTFIT, ECLUSTALW), National Center for Biotechnology Information (BLASTN, BLASTP, TBLASTX), EXPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (PredictProtein, ProfileScan, PSORT II, ProtParam, Tmpred, ProtScale, Signal IP) and the TRANSFAC database of the Research Group Bioinformatics/AG Bioinformatik (MatInspector).
4.4 RESULTS

4.4-1 Elucidation of the full ORP-3 cDNA Sequence, and localization to Chromosome 7p15-21 by RACE and Bioinformatics

Using RACE, a further 1565nts of the ORP-3 cDNA was amplified and sequenced. This novel sequence included 964nts of the remaining 5' coding and untranslated region, and 601nts of the remaining 3'UTR including the poly-A tail. We have confirmed this sequence using BLAST software on the NCBI database (Figure 4.1 and 4.2).

The entire ORP-3 cDNA is 6631 bp long and maps to chromosome 7p15-21 (Accession No. AC004008-Human PAC clone RP5-899B21, Accession No. AC004239 - Homo Sapiens BAC clone CTB-34F8, Accession No. AC004016 - Homo Sapiens BAC clone RG424N05, and Accession No. AC003093 - Homo Sapiens BAC clone RG38F02). The genomic ORP-3 sequence contains 23 exons and 22 introns spanning an entire length of 183.5kb (Figure 4.3 and 4.4). The ORP-3 intron/exon boundaries were found to conform to the GT-AG rule and were confirmed by alignment of the genomic sequences from the BAC clones and the ORP-3 cDNA (Table 4.2).

A 5' UTR of 306 nucleotides precedes the coding region. No obvious TATA box sequence is observed within 100bp of a possible transcription initiator sequence contained between positions -3 to +6 (Figure 4.5). No sequences resembling a sterol regulatory element were observed in the 5' flanking region. The translation start site at position 307 is defined by a possible Kozak sequence (Figure 4.3).

The ORP-3 coding region terminates at position 2968 and is followed by a polyadenylation signal and a poly-A tail at positions 6593 and 6607 respectively (Figure 4.3). The 3' UTR of 3.661kb is approximately four-times the mean length for all known human cDNAs (Pesole et al 1999).
Figure 4.3 ORP-3 Full mRNA Sequence (length 6631nts).

Depicting positions of exon boundaries (exons alternating between blue and green), start codon (boxed yellow) with kozak sequence (shaded gray), stop codon (boxed blue), poly-adenylation signal (boxed pink) and poly A tail (boxed green).

1 aatcccccct ccaacctctg eggccgggtc ggaggctgct aagctgcaagt
tgcgggtcta cccgagaacg cgggttgcct ttgctccgct tctgttgct
101 aaccccgag cacgcccggag ggaactagg gacagctcct ggtgcaacag
tgctgaggg ttcgggaaac cggcttact gggacctact atcctctggg
201 ggtacgggtg ggtacggaag tttgagttg tgtgacgg ccgtgagctt
301 tgtgctgtg ctcggagcga cggactctt ctgatgctg ctctggagcga
351 atccttcg cggccagca gtcgctgc ccgccagca ggggagacag
401 aggcacgtg ggaaagttgg gcaagagctg ggggggagat gaattcaccg
451 cagggcgcac cgcctcaggc aggttttgg cttgaaagag aggaagggc
501 cttttaaggc tggcataaag gatgcttcttc ttggtcagc ggaattttga
551 aatctgctc ggaactattg gatagaggc gatagggcgc gcattgtgggc
601 attggatggc gtcttctcagtt cagatggtcg aagaaatgct cacaatgcgat
651 agaccttgac acggagagcg acatctacca tctgaaagtc aagctcaagag
701 aagatctggc tgcgagttct gcagcctgcct gcgtcctggc ggtgctgcgtc
751 cctgtgagtc tgtggcctgg ttcaggtgtgc gcagcaatgc ttcctcggg
801 ggtcaggtttg gtcacgtcta aagagcaatg tttttaacc ccaggaatac
851 gttatcatttt cttgttggttg tgtgacagc tttcagcgatt ctgacagctg
901 ttcatggggt ggtccctaaag gttgacttttt cttgtctcagct cgaggtgctc
951 ctcaggggct cttgaaaaat gtctcaagag ccggcggcac tgtgccgtct
tatggataggc gtctggcgtc ctgacgatatt gctgagtcgct
1001 aatctgctcgt ggcaggtttc ccggagaccag tgtttgagag ccctgggtctt
ttctggcagttt ccggagaccag tgtttgagtc
1051 ttgagaagat cggctcgtcag tgtggcgttc ggagcttcagtt tggagctctg
1101 atccttcg cggccagca gtcgctgc ccgccagca ggggagacag
1151 gcaaaaaatg tgggctgctc agagagcag cggctcgtcag tgtttgagag
1201 gggacctggt gatagagggc ggtgctgcgtc
1251 tgtggcctgg ttcaggtgtgc gcagcaatgc ttcctcggg
1301 ggtcaggtttg gtcacgtcta aagagcaatg tttttaacc ccaggaatac
1351 gttatcatttt cttgttggttg tgtgacagc tttcagcgatt ctgacagctg
1401 ttcatggggt ggtccctaaag gttgacttttt cttgtctcagct cgaggtgctc
1451 atccttcg cggccagca gtcgctgc ccgccagca ggggagacag

Exon 4
Exon 5
Exon 6
Exon 7
Exon 8
Exon 9
Exon 10
Exon 11
Exon 12
Figure 4.3 (cont)

1601 ctggaactct atctgtgact atcgctactc gaactctgcc ctggtgccaa
1651 ctggtgccac atcgctggag cggaaacttc cagagatgaa aacgagctc
1701 tagttccatac gctttctaat gaaagtagac tcctccatcact tgaactccctt
1751 tctgagtttt tttgagctga tttcagttcg ttatctccaa gcttttccaga
1791 aaacgagactt ctgcaaggt actctattgt tgcggaactta atgtgaaactc
1831 ttccttctgc ttaaactctg tctaagttctg gcaaggagact caagggcttg
1881 cctggtggcc cccctgcccc agcagctgaa acatgagcctg tggaaactcc
1921 tgggaaacaa cacaagcagg gaccaatcct aagttgccct gcccgtggag
1971 cccgagccgg ccctgacagg ctgcagcagg ttcctggcagg agcttggagta
2011 cccgagcttc ctggcagaaag ccggcagat cccggccccc ctcgggaagga
2061 tgggtaagtt ggcctgcttt gctgataggt gctatggcac tagatcactcd
2101 cgacggtggg gccagtacgtg gataggtc ttcgggacaa caaaggggcag
2151 cttcggcggg gaaagctgaa tggcggagct gttcagcacc
2201 atccgctcct ctctgcgtgt cagctgagct cttgaaactt ttgcttgtgg
2251 cagcaggtgg gtcgcaatgg gaaatggga aacggtgtgg ggaacttoaa tgggaattgt
2301 tggatgggga agaagactac ttcatacgata acatgatccag cggccagagg
2351 ggctgctagtg aagctggtat gttataagcc gaaatcagtg agcactataq
2401 cctgactcgga aagagcataa tttataagcc aaggtcataag ggggtgtatc
2451 cccgctgata cggctgcttg gataggtc ttcgggacaa caaaggggcag
2501 cgggtcctgg gaaagctgaa tggcggagct gttcagcacc
2551 cttcggcggg gaaagctgaa tggcggagct gttcagcacc
2601 cccgctgata cggctgcttg gataggtc ttcgggacaa caaaggggcag
2651 cttgtaaggtc gttataagcc gaaatcagtg agcactataq
2701 tgcggttctt ctcggttcac ccagctgtta ccagctgtta ccagctgtta
2751 gatgctgtga gacacgggaa tggcggagct gttcagcacc
2801 tgcggttctt ctcggttcac ccagctgtta ccagctgtta ccagctgtta
2851 gatgctgtga gacacgggaa tggcggagct gttcagcacc
2901 cccgctgata cggctgcttg gataggtc ttcgggacaa caaaggggcag
2951 cccgctgata cggctgcttg gataggtc ttcgggacaa caaaggggcag
3001 tgcggttctt ctcggttcac ccagctgtta ccagctgtta ccagctgtta
3051 gatgctgtga gacacgggaa tggcggagct gttcagcacc
3101 cttgtaaggtc gttataagcc gaaatcagtg agcactataq
3151 gatgctgtga gacacgggaa tggcggagct gttcagcacc
3201 cttgtaaggtc gttataagcc gaaatcagtg agcactataq
3251 cccgctgata cggctgcttg gataggtc ttcgggacaa caaaggggcag

Stop codon
Figure 4.3 (cont)

3301 atttatgcct tataaaatta tgggttagaa aaaaaatcaaa cctctcccaag
3351 gtgtgatttga gtataagaaat tccccagggtt acctaaacacat ggttaagcataa
3401 ccccaagttta atttgggtgct gaaaaatctct ttttcccctca gacagctttt
ttgcgctggcttttggtgt ctggagagct gagaatatttt
3451 gattagttcc tataagaacaa ttctgtcctggct gtaatcctttgct gacaagattt
3501 ttctatctgcta ttctatggtt tagggagaaag atataagatt atatggaatgt
3551 atatattttat atataactgta taagggacct tctttgaaaag gaagactattg
3601 aaccgattccc tctcctatagta ctcactcgctt ctagagtctcttatagaaataccttct
3651 gtgctctcaca gataaatattt atagaagagaa agaatccaa ttaattaattat
3701 atacaattag ccaccccagag agacagtaaggg aatatttccc ttagtggaag
3751 ctgagtctcctt gagaagtttac gagaagttttct cctgctcccc aaccceacccc
3801 gtttttttttcct cctccctcagttt atcacccttccc ttggttcatgta gaaattcccc
3851 eggttaatat aaaaactctaca gttttatctcc caacagcagtt tgacatatgta
3901 aaggtttgctt aaaaaactcg taatttgggt agatacttaac atgttttctct
3951 taattaactgctttttcctctt aataagccttctt atatatattata
4001 ttcgtatgtaa aaggttagccttg ctcgctttttc aagacaaatgt agagaatgtga
4051 ttctctgcttttttcttg tttggttaaagc gcttttactg gttcttttac
4101 cctctctcctt ttctttttctg ttataaatcatttcttttg ttagtggttgct
4151 acagaggctttgg aataatacattttaatttgaatgtgaggg acctcttcctc
4201 ttttttaatag tggctatccctt cattatcataa atatgttagct tagttgatatt
4251 gtggatttggcag aagagtttcgca aacttttgcctt agatgacatt aaaaaccaag
4301 cctataatttgaagctttcctt tcttttaagttc gttgtggataaag cttttggctt
4351 tcaatgtaaa aaggtgtagttt aagaggggtc tcctgagaaac aaccttggtgat
4401 acctttaagaa atccagacag tttgcaattgt ttagaatgaa gagaataatata
4451 attacaggaattcagctgttccctcatggaatggctta aataaccaagttgacaagt
4501 tggacaatt tggacatggata actagaaatgg aacacaaatgtaatatcattt
4551 gctctcttggtattctggtatg gatggctttttcttttttttgcactggtgtctt
4601 ttgtattcatttttcttggagaaagctctctgactgaatagatgttggccttttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Figure 4.3 (cont)

5101 tcttagtcagt acctgcatct ataagggct tgttgtgaat ttaaagagct
5151 tgtgtagcga tcctgatatt ataagtttaa aacatatcttg agggggaat
5201 gacctcttttt ctctctctgt cccctttetc aaagagccct tcccccaca
5251 tgtatagctct taggacctttt tatagtttta aaggaagaa aacagaaacg
5301 tggcatacatc tgtggaagac ccaggttagaa ttaatatttc tgggacttta
5351 atctctgcttgt taataaatgc taatacatact aacatattgtg ttaactgttt
5401 taacccctctt tttttttctta attattccat cccaaaatttc tggggaatta
5451 cacagaaatc tttggaatag ggccttttgag cagttaaaaa cccctgcactt
5501 caggtattgt tcctgctcaca gctgtgcaact aaccccagcct gcaggaatct
5551 catccagaaaa tgaagctacaaga gtctccagt gtacaggaga gcacagctact
5601 tccccccccc gcgccccccc ccccccaggg ctggtattcc tcgggtgatna
5651 ggtattataa ataaagccct caatgcaagct ttacctactga gtctctcttee
5701 tccacagcag cggggttctgt gtcttaacta aagagatggg agttccactg
5751 aggccagagaa gtccaggaga gcccccacacgc tcgggaaggt tgtctgtgac
5801 ttcctgtgag aacctctgggg gcggatatct aactctaatat attttatat
5851 gtttaagcctc tcctctctgc gttttttttct aattctcttg tttttttact
5901 tgtgatataat cttttgttgg ggtttaagac acgtttctcc atctgcaatct
5951 tccagatatc tataagtggag cggatctctca tttttattaa gtcagagctt
6001 aaaaaatcta aagttttttttttttttttttt gactctggat gtagcccota
6051 ctacataacc tacaagtttat gctttctggc tcaactcata tccctcgaga
6101 ccctttagtt atagagttaga gcattaagaa ggttgtctta tgggttttagg
6151 aataaatttt gctagtttagt ccataaatttt gttgagatgt cttttatatgg
6201 atattaacact gcggataaat ctaattttggc tccaggagta tagctatat
6251 cttctgcct tttctctctct cggctgtcct aactggtttttc taactgttagg
6301 tctttcatct tctgtgtggg aaacctgggt catgttaata tctggaattc
6351 atgagctcag catgaaaccct ccattttcag agacgtggtt ggtaagctgat
6401 atcagaatgt gtaattttat ttattttttg attattttat ccagggtgta
6451 ctcttttttc cttatatttt gattttttttt aatcttccat tcctacacct gaaaatattt
6501 tttttttttt ctctcttcacactctcc tctgtgcttc gcggggggtc Poly-adenylation signal
6551 ctaatttttt cttttctttcagct ctagtgact gtagtagaag tataaatc
6601 acatggaaaa aaaaaa aaaaaaaa a

Poly-A Tail
Figure 4.4 Schematic diagram of the genomic structure of the ORP-3 gene and localisation to chromosome 7p15-21 by bioinformatics

Exons numbered 1 – 23 are depicted in black boxes.

Start codon

Stop codon & poly A signal

5kb
Alignment of the 5' cDNA sequence of ORP-3 (red) with the chromosome 7p-15-21 genomic sequence (black) (Accession no. AC004008) demonstrates the putative transcription initiator (highlighted in gray nts -2 to +6) and TATA-less promoter.

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-23 cccagggg ccagggggtg gggggggtg cccagggg ccagggggtg gggggggtg
 38 ggtccaggg ccagggggtg gggggggtg cccagggg ccagggggtg gggggggtg
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Table 4.2 Localisation of ORP-3 to Chromosome 7p15-p21 (Accession Nos. AC004008, AC004239, AC004016, and AC003093).

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4.4-2 ORP-3 Northern Hybridization

ORP-3 expression was investigated using a Human Multiple Tissue Northern Blot™ (Clontech) containing poly(A)+RNA from various normal human tissues normalized for β-actin expression. Three ORP-3 mRNA transcripts were detected measuring approximately 7.0, 4.4 and 3.6 kb. The 4.4 and 3.6 kb ORP-3 transcripts were highly expressed in heart and skeletal muscle, with lower expression in kidney. Minimal levels of these transcripts could also be detected in spleen, thymus and brain. The 7.0 kb transcript was detected predominantly in brain, skeletal muscle and kidney. Peripheral blood leukocytes expressed only the 7.0 and 4.4 kb transcript (Figure 4.6).
Figure 4.6 Expression of ORP-3 in multiple tissues as detected by Northern Hybridization.

Lanes 1 – 12 = brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, peripheral blood leukocytes (respectively).
4.4-3 ORP-3 Protein Bioinformatics

Translation of the ORP-3 ORF yields an 887 amino acid protein with a calculated pI of 6.42 and a theoretical molecular mass of 101.2236 kD (Figure 4.7). ORP-3 is predicted to be a soluble protein devoid of any significant hydrophobic signal sequences or potential transmembrane sequences. It does not contain a bipartite nuclear localisation signal but does contain two other possible nuclear localisation signals at positions 60 and 266 and has 65% prediction of nuclear localisation.

Amino acid sequence analysis of ORP-3 reveals the presence of a pleckstrin homology (PH) domain (95 residues), putative leucine zipper region (LZR) (1 methionine and 3 leucine residues spaced exactly 7 amino acids apart), putative dimerisation region (17 amino acids) at the NH$_2$-terminus, and a well conserved oxysterol binding protein (OSBP) signature pentapeptide (E(KQ)xSH(HR)PPx(STACF)A) contained within a more moderately conserved OSBP domain (388 residues) at the COOH terminus (Figure 4.7). A number of evolutionary related eukaryotic proteins that seem to be involved with mediating the actions of OS on cells have been found to contain this signature and domain (Jiang et al 1994).

Comparison of the amino acid sequences of ORP-3 with 3 other well-characterised OSBP homologues - human OSBP (OSBP-Hm), drosophila OSBP (OSBP-Dm) and rabbit OSBP (OSBP-Oc) reveals approximately 56%, 55% and 56% overall identity respectively (Figure 4.8). The four proteins appear well conserved and harbor the same modular domains previously mentioned (Figure 4.9).
Figure 4.7 Amino acid sequence encoded by ORP-3.

Conceptual translation of the ORP-3 ORF results in an 887 amino acid protein which displays a PH domain (boxed red), consensus nuclear localisation signals (shaded yellow), putative leucine zipper region (boxed blue), putative dimerisation region (boxed pink) and an OSBP signature (shaded green) contained within a more moderately conserved OSBP domain (boxed green).
Figure 4.8 Alignment of the amino acid sequence of ORP-3 with OSBP-Hm, OSBP-Oc and OSBP-Dm using ECLUSTALW.

Amino acid residues residing within the PH domain are indicated in red, putative leucine zipper and dimerisation regions in blue and pink respectively, and OSBP domain in green.
Figure 4.9 Schematic diagram of the amino acid sequences of ORP-3 with OSBP-Hm, OSBP-Oc and OSBP-Dm.

Amino acid residues residing within the PH domain are indicated in red, putative leucine zipper and dimerisation regions in blue and pick respectively, and OSBP domain in green.
4.5 DISCUSSION

A partial sequence for ORP-3 was identified by dd-PCR in CD34+ hematopoietic progenitor cells, with low expression in CD34- hematopoietic cells. This transcript had 100% homology with a previously identified partial gene sequence KIAA0704. Conceptual translation of the full KIAA0704 ORF revealed significant homology to human oxysterol binding protein (OSBP-Hm). On the basis of ESTs in the GenBank database, a family of up to 12 human OSBP related genes are known to exist and these transcripts have been designated oxysterol related proteins (ORPs) (Laitinen et al 1999, Moreira et al 2001). Our OSBP related gene has been designated ORP-3 (Laitinen et al 1999).

The full ORP-3 mRNA of 6.631kb was amplified using RACE technology. This sequence includes a 5' UTR of 306bp, and a very long 3' UTR of 3.661kb. The 3' UTR of ORP-3 is among the largest known human 3' UTR's (Pesole et al 1999). Zhang et al (2000b) identified four human cDNAs differentially expressed by early hematopoietic progenitors with very long 3' UTRs and suggests that long UTR's are in some way characteristic of genes expressed by developing hematopoietic cells, and may play a role during development. Interestingly, the MERP-1 gene discussed in Chapter 3 of this thesis also has a long 3' UTR. The physiological significance of this increasingly common finding is unknown.

Using bioinformatics, ORP-3 is localised to chromosome 7p15-21. Although a variety of lymphoid and myeloid malignancies are associated with deletions on chromosome 7, currently, there are no reported deletions of the 7p15-21 area specifically. The genomic structure is 183.5kb long with 23 exons and 22 introns. The translation start codon is located in exon 2 and stop codon in exon 23. The upstream promoter region of ORP-3 appears to be TATA-less which is consistent with findings by Levanon et al (1990) regarding the promoter region of OSBP-Hm. Additionally, as with OSBP-Hm, no sequences resembling a sterol regulatory
element (Smith et al 1988) were observed in the 5' flanking region of ORP-3. Translation of the ORP-3 open reading frame yields an 887 amino acid protein that has a high degree of homology (56%) to three other well-characterised OSBP proteins- OSBP-Hm, OSBP-Dm and OSBP-Oc. The four homologues appear well conserved and harbor the same modular domains: a PH domain, a putative LZR and dimerisation region at the -NH₂ terminus, and a highly conserved OSBP signature contained within a larger, more moderately conserved OSBP domain at the -COOH terminus.

OSBP is a well-characterised protein that has been demonstrated to bind oxysterols (OS) in a dose dependant fashion (Kandutsch and Shown 1981, Kandutsch and Thompson 1980). This cytosolic protein has been extensively purified and characterised from many tissues and cells of the mouse (Kandutsch and Shown 1981), chinese hamster (Kandutsch and Thompson 1980), rat (Kandutsch and Thompson 1980), rabbit and human (Levanon et al 1990, Dawson et al 1989) and is remarkably well conserved across all species. Deletion mutagenesis studies reveal that the C-terminal half of the OSBP, containing the OS binding domain, is required for OS binding (Ridgway et al 1992). All OSBP homologues contain this signature and domain and it can be assumed, given the high degree of conservation amongst all species, that all proteins bearing this signature mediate, to some degree, the actions of OS on cells.

The N-terminal part of OSBP-Hm and ORP-3 contains a PH domain. PH domains are sequences of approximately 100 amino acids sharing some primary sequence and a lot of secondary and tertiary structure (Saraste and Hyvonen 1995, Musacchio et al 1993). Many studies have demonstrated that the PH domain of OSBP-Hm is required for translocation of the liganded protein to the Golgi apparatus (GA) and its subsequent effects on cholesterol homeostasis (Ridgway and Lagace 1995, Lagace et al 1997, Levine and Munro 1998). The ability to recognise the GA was also demonstrated by the PH domain of Osh1p, a yeast homologue of OSBP-Hm (Levine and Munro 1998). It is highly probable therefore, that the PH domain of ORP-3 is also involved in GA specific targeting upon OS binding.
Dawson et al (1989) analysed the predicted amino acid sequence of OSBP-Hm and identified an apparent LZR. This motif is conserved in ORP-3, OSBP-Dm, and OSBP-Oc. While LZRs may be associated with DNA binding (Landschulz et al 1988, Kouzarides and Ziff 1988, Turner and Tijan 1989, Gentz et al 1989), this is generally seen when the LZR is flanked by a cluster of basic amino acids adjacent to the NH$_2$ terminal (Hurst 1995, Turner and Tijan 1989). The putative LZR$s$ of OSBP-Hm, OSBP-Oc and OSBP-Dm are flanked by acidic residues and this finding may correlate with the inability of many groups to demonstrate specific DNA binding with OSBP-Hm and OSBP-Oc (Dawson et al 1989, Taylor et al 1984, Taylor et al 1989). Though the region preceding the LZR of ORP-3 is basic rather than acidic, it does not contain the classic basic domain signature characteristic of DNA binding LZR$s$. The ability of ORP-3 to bind DNA via the LZR therefore remains uncertain.

LZR motifs have also been reported to play a role in hetero and homo-dimerisation. Thus, although the LZR of OSBP-Hm, OSBP-Oc, OSBP-Dm and ORP-3 may not be sufficient to allow DNA binding, it may allow these proteins to form homodimers or heterodimers with other, as yet unidentified proteins (Buckland and Wild 1989). This is consistent with the hypothesis that OSBP-Hm exists in various forms based on frequent observations that its molecular mass varies from 57 – 280kDa (reviewed by Smith 1996). In this manner, proteins that do not bind DNA can still be involved in regulation of transcription by modifying the activity of transcription factors through protein-protein interactions (Mendel and Orti 1988, Yoshinaga et al 1992). Ridgway et al (1992) confirmed homo-dimerisation of OSBP-Oc, however they demonstrated that the putative LZR was neither necessary nor sufficient for this dimerisation. Instead, dimerisation of OSBP-Oc required a sequence element of 18 residues in the amino acid interval of 261-296. This region is completely conserved in OSBP-Hm and OSBP-Dm, but only moderately conserved in ORP-3.
The size of the complete ORP-3 cDNA sequence has been confirmed by northern hybridization with the presence of a transcript at approximately 7.0kb. Our results also revealed the presence of two other transcripts at approximately 4.4 and 3.6Kb. The presence of these alternative ORP-3 mRNA transcripts has also been reported by Laitinen et al (1999) who found a low level of expression of all three in all tissues investigated with 7.0kb most prominent in spleen and peripheral blood leukocytes. In contrast, our results suggest differential expression of the transcripts across the various tissues. This discrepancy may be due to the use of probes complementary to different areas of the ORP-3 transcript. These splice variants may be produced as a result of post-transcriptional modification or alternative poly-adenylation signals and warrant further investigation to determine their possible roles in cell/tissue specific functions. Interestingly, recent publications of the characterisation of ORP-4 (Moreira et al 2001) also report the presence of multiple splice variants.

It is well accepted that homologous proteins often share similarities at the functional level. It is therefore highly probable that ORP-3, like OSBP-Hm, plays a role in the transduction of OS effects on cells, in particular, hematopoietic cells. OS are a family of widely occurring hydroxylated derivatives of cholesterol. They have been demonstrated to be atherogenic, immunosuppressive and cytotoxic to a variety of normal and tumour cell types. Various studies have demonstrated that their cytotoxicity results from induction of apoptosis, that is believed to be, at least in part, via a bcl-2 independent mechanism that requires the de novo synthesis of RNA (reviewed by Guardiola et al 1996, Smith and Johnson 1989, Thompson and Ayala-Torres 1999). Additionally, investigations in the present study have demonstrated that OS are potent inhibitors of CD34+ cell growth (discussed further in Chapter 6 of this dissertation).

Many studies have implicated a role for OSBP-Hm in OS regulation of cholesterol balance (Taylor et al 1984, Lagace et al 1997). However, the role of OSBP in OS induced apoptosis is unclear (Guardiola et al 1996, Smith and Johnson 1989, Thompson and Ayala-Torres 1999) and suggests a role for other OS binding
proteins, such as ORP-3, in the mediation of OS effects on cells. To date, the
complete coding sequence of seven human oxysterol-binding proteins (OSBP-Hm,
ORP-1, 2, 3, 4, 6, 7) have been added to the GenBank database, and at least another
six are postulated to exist (Laitinen et al 1999, Moreira et al 2001). Laitinen et al
(1999) studied the gene expression patterns of ORP 1 – 6 in various human tissues.
Each of the cDNAs was found to exhibit a unique tissue distribution demonstrating
that they code for separate proteins that may play functionally different roles in
cells.

Publications related to the investigation of other human OSBP homologues indicate
the possibility of additional roles for OS and their binding proteins. The partial
sequence for the human OSBP homologue ORP-4 identified by Laitinen et al
(1999) was also identified by Fournier et al (1999). They designated the sequence
HeLa Metastatic gene (HLM) due to its specific expression in HeLa cells. HLM
gene expression has been correlated to metastatic potential in lung, colon and breast
cancer (Fournier et al 1999), and has also been demonstrated to be up regulated in
the PB of patients with chronic myeloid leukemia (CML) (Silva et al 2001).

Recently, Moreira et al (2001) reported the elucidation of the complete ORP-4 gene
sequence and OS binding of the ORP-4. Interestingly, initial investigations of the
ORP-1 and 2 proteins (Xu et al 2001) indicate that these two proteins were unable
to bind the OS 25-hydroxycholesterol, but instead bound the phospholipids
phosphatidic acid and phosphatidylinositol-3-phosphate. Additionally, over
expression of ORP-2 resulted in a dramatic decrease in cell growth and a block in
Golgi derived vesicle transport (Xu et al 2001).

The identification of OSBP homologues in lower species suggests additional
functions for these proteins. OSBP-Dm was identified in a screen for D.
melanogaster cDNAs capable of overcoming the blockade of cell cycle progression
induced by over expression of the Wee1p gene in Schizosaccharomyces pombe
(Alphey et al 1998). This suggests that OSBP-Dm may act as a positive regulator
of cell cycle progression (Gonzalez et al 1989) rather than as a regulator of cholesterol biosynthesis since insects do not synthesise sterols (Svoboda et al 1994). Similarly, two oxysterol binding proteins designated OBPa and OBPx have been recently identified within a highly conserved chromosomal locus of Candida albicans thought to be instrumental in controlling fungal meiosis, cell growth and viability (Hull et al 1999). More recently, Sugawara et al (2001) identified an OSBP-homologue (BIP) from Caenorhabditis elegans and Xenopus laevis which is postulated to function in TGFβ signalling and regulation of body length.

The Saccharomyces cerevisiae genome encodes seven OSBP homologues (OSH1 – 7) each of which have been postulated to play a role in various aspect of sterol metabolism (reviewed by Beh et al 2001). Whilst individual deletion of each of the OSH genes produced specific sterol related defects, none have been found to be essential for yeast viability. Each OSH gene therefore performs distinct non-essential functions that contribute to a common essential function (Beh et al 2001). It remains to be determined whether members of the mammalian family of OSBP homologues perform similar, yet distinct and overlapping functions.

Given the homology between ORP-3 and OSBP-Hm, a protein which has been well characterised and is known to bind oxysterols and play a part in mediating their effects on cells, it is likely that ORP-3 also plays a role in mediating OS effects on cells. OS are hydroxylated derivatives of cholesterol, which have been demonstrated to inhibit the transcription of many genes involved in cholesterol biosynthesis and cell replication, and to be apoptotic to a variety of cell types. Given the importance of these processes in haemopoiesis, characterization and investigation of the function of ORP-3 may provide useful insights into a possible regulatory role for OS and their binding proteins in HSPC proliferation, differentiation and self-renewal.
CHAPTER 5

ORP-3 GENE REGULATION

5.1 ABSTRACT

Expression of the ORP-3 gene was previously found to be 3 - 4-fold higher in the CD34+ fractions of haemopoietic cells compared to CD34- populations from UCB and ABM respectively. Further investigation of ORP-3 gene expression demonstrates a significant correlation with CD34+ sample purity, and 2-fold higher expression in a population of haemopoietic cells defined by the CD34+38- phenotype compared to more mature CD34-38+ cells. This finding, taken together with the previous observation of down-regulation of ORP-3 expression with proliferation and differentiation of CD34+ cells (Chapter 2), indicates that ORP-3 expression may be higher in a less differentiated subset of cells. This hypothesis is supported by the observation that expression of the ORP-3 gene is approximately 2-fold lower in differentiated HL60-v cells compared to control, undifferentiated cells.

ORP-3 expression in HL60 cells during normal culture conditions was also found to vary with expression positively correlated with cell number. This indicates a possible cell cycle effect on ORP-3 gene expression with levels highest when cell density, and therefore the percentage of cells in G0/G1 phase of the cell cycle is highest. This observation also correlates with the observation of higher ORP-3 expression in CD34+38- cells, and in CD34+ and HL60-v cells undergoing OS induced and camptothecin induced apoptosis. Data obtained from investigation of ORP-3 gene expression in synchronized HL60-v cells does not support nor disprove this
hypothesis with no significant difference in ORP-3 gene expression observed when 63, 75 and 71% of the cells were in G₀/G₁, S and G₂/M respectively.

Expression of the ORP-3 gene in CD34⁺ HSPCs from UCB was significantly decreased to approximately half the levels observed in control cells after 24 hours incubation in 10ng/ml TGFβ1. As >90% of these cells are stimulated into cell cycle entry by TGFβ1, this observation further supports the hypothesis that ORP-3 expression is highest when cells reside in the G₀/G₁ phase of the cell cycle.

Culture of CD34⁺ enriched HSPCs and HL60-v cells with 25-OHC significantly increased ORP-3 gene expression to approximately 1.5 times control levels. However, as 25-OHC treatment also increased the percentage of apoptotic cells in these experiments, it is impossible to make any conclusions regarding the regulation of ORP-3 gene expression by OS. Indeed, the observation that camptothecin induced apoptosis also increased ORP-3 gene expression in HL60-v cells raises the possibility that up-regulation of ORP-3 gene expression is also associated with apoptosis. However, as both camptothecin and OS induced apoptosis is preceded by G₀/G₁ cell cycle arrest, this data also supports higher expression of ORP-3 in this cell cycle phase.

Taken together, this data indicates higher expression of the ORP-3 gene in a less differentiated subset of cells, and/or during the G₀/G₁ phase of the cell cycle.
5.2 INTRODUCTION

A partial sequence for the ORP-3 gene was identified using the technique of dd-PCR to compare the gene expression profiles of CD34 enriched and depleted populations. Semi-quantitation by real-time PCR has confirmed higher expression of this gene in a population of HSPCs defined by expression of the CD34 phenotype. Additionally expression of ORP-3 is down regulated with culture of CD34⁺ HSPCs using a combination of growth factors known to promote proliferation and expansion.

The full coding sequence of the ORP-3 gene has been obtained using a combination of RACE and computer assisted bioinformatics. The ORP-3 ORF encodes a 887 amino acid protein with significant homology to human OSBP, a well characterised protein that has been demonstrated to bind OS in a dose dependant fashion. This binding is hypothesised to mediate at least some of the actions of OS on cells (Kandutsch and Shown 1981, Kandutsch and Thompson 1980). OS are hydroxylated derivatives of cholesterol, which have been demonstrated to inhibit the transcription of many genes involved in cholesterol biosynthesis and cell replication, and to be apoptotic to a variety of cell types (reviewed by Guardiola et al 1996, Smith and Johnson 1989, Thompson and Ayala-Torres 1999). Given the importance of these processes in haemopoiesis, and differential expression of the ORP-3 gene in haemopoietic progenitors, characterization and investigation of the function of ORP-3 may provide useful insights into a possible regulatory role for OS and their binding proteins in HSPC proliferation, differentiation and self-renewal.

Insights into the possible role of ORP-3 in haemopoiesis may be obtained by investigation of ORP-3 gene expression in a variety of experimental situations. The purpose of this study was therefore to investigate some aspects of regulation of the expression of the ORP-3 gene.
5.3 METHODS

PART 1: REGULATION OF THE ORP-3 GENE IN CD34+ HSPCS FROM UCB

5.3-1 Ethics approval

All experimental procedures were conducted in accordance with the regulations and guidelines outlined by the National Health and Medical Research Council, and approved by the Deakin University (Ethics approval no. EC-82-97) and Geelong Hospital Ethics Committees (Ethics approval no. 97-14).

5.3-2 Cell sources

UCB samples were obtained after uncomplicated vaginal or cesarean delivery after clamping and cutting of the cord and drainage into 50ml sterile collection tubes containing 200 units of heparin (David Bull Laboratories, Mulgrave, Aust.) in 5mls of alpha medium (αMEM) (Trace Scientific, Noble Park, Aust). All samples were donated by volunteers at the Geelong Hospital, Barwon Health (Geelong, Victoria) according to approved institutional guidelines.

5.3-3 Red Cell Depletion of UCB samples

UCB sample was diluted 1: 2 with PBS, mixed thoroughly by gentle inversion centrifuged for 25 minutes at 480g and 18 – 20°C. Centrifuge was set at low brake.
After centrifugation, the lower density white blood cells were seen as a buff-colored carpet on top of the denser red cells. These were removed using a cannula and syringe and placed in a sterile 50-ml tube. Red cell depleted UCB samples were made up to a volume approximately equal to the original UCB sample with PBS.

5.3-4 Preparation of Mononuclear cells from UCB and ABM samples

UCB samples depleted of red cells were carefully and gently layered onto an equal volume of Ficoll-Hypaque® (Amersham Pharmacia Biotech, Uppsala, Sweden) in 15 or 50ml sterile tubes. Great care was taken to ensure that there was minimal/no mixing of cell samples with Ficoll-Hypaque®. Samples were centrifuged for 25 minutes at 440g at 18 – 20°C. Centrifuge was set to low brake. After centrifugation, the upper plasma/media layer was drawn off using a clean pasteur pipette. Mononuclear cells (MNCs) at the ficoll-plasma interface were carefully removed using a sterile 5 or 10-ml syringe and cannula. MNCs were washed twice with the addition of two volumes of PBS and centrifugation at 400g for 10 minutes at 18 – 20°C. After the second wash, the supematant was removed, and MNCs were resuspended in 300µl PBS/0.5%BSA/2mMEDTA per 1 x 10⁸ cells (minimum 300µl).

5.3-5 Immuno-magnetic separation of CD34⁺ cells from UCB MNCs

CD34⁺ cells were isolated from UCB MNCs using antibody labeling and a MiniMacs bead separation kit (Miltenyi Biotec, Becton Dickinson, Sunnyvale, U.S.A.) following the manufacturers instructions. Degassed PBS/0.5%BSA/2mMEDTA was used for washing VarioMacs and MiniMacs columns (Miltenyi Biotec) and MNCs. MNCs were resuspended in 500ul/10⁸ of 500units/ml DNase1 (brand, manufacturer) before separation on a VarioMacs (for 2
- 20 x 10^8 MNCs) or MiniMacs column (for ≤ 2 x 10^8 MNCs). Immunomagnetically-labeled CD34^+ cells were eluted from the column with 2 - 5mls degassed PBS/0.5%BSA/2mMEDTA. Viability and cell number of the CD34 enriched and depleted cell populations was assessed using trypan blue (Sigma Aldrich) exclusion and manual cell counting. Purity was determined using flow cytometry (section 5.3-6).

5.3-6 Flow cytometric analysis of CD34 populations

Approximately 0.25 x 10^6 cells from whole cord blood, ABM MNCs and CD34 depleted samples, and 0.01 x 10^6 CD34^+ cells were labeled with FITC-conjugated anti-CD45 and PE-conjugated anti-CD34 (Beckman Coulter, Fullerton, U.S.A.) according to manufacturers instructions. The percentage of CD34^+ cells in each sample was determined by flow cytometric analysis (FacsCalibur, Becton Dickinson) following modified ISHAGE guidelines (Sutherland et al 1996a) using CELLQuest software (Becton Dickinson) (See Chapter 2. Figure 2.1). Equivalent numbers of cells labeled with FITC-conjugated anti-IgG1 and PE-conjugated anti-IgG1 were used as negative controls for the analysis.

5.3-7 Fluorescence Activated Cell Sorting of CD34^+ 38^- and CD34^+ 38^+ subsets

In selected experiments, immunomagnetically selected UCB CD34^+ cells were labeled with FITC-conjugated anti-CD38, PE-conjugated anti-CD34 and PE-Cy5-conjugated anti-CD45 (Beckman Coulter, Fullerton, U.S.A.) according to manufacturers instructions. Purity of CD34^+ populations was determined by flow cytometric analysis (FacsCalibur, Becton Dickinson) following modified ISHAGE guidelines (Sutherland et al 1996a) using CELLQuest software (Becton Dickinson). Equivalent numbers of cells labeled with FITC-conjugated anti-IgG1, PE-conjugated
anti-IgG₁ and PECY5-conjugated anti-IgG₁ were used as negative controls for the analysis. CD34⁺38⁻det cells were sorted using a FacsCalibur Cell Concentrator Sorting Module using sort gates constructed to include approximately 10% of the total CD34⁺ population (Figure 5.1). Viability and cell number of the CD34⁺38⁻ and CD34⁺38⁺ cell populations was assessed using trypan blue (Sigma Aldrich) exclusion and manual cell counting. Cells were then immediately homogenized in TRIZOL® reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturers instructions, and stored at -80°C until RNA extraction and reverse transcription.
Figure 5.1 Gating strategy for fluorescence activated cell sorting and analysis of CD34⁺38⁻CD3⁺ cells from CD34⁺ immunomagnetically purified UCB samples

Analysis dot plots only depicted here. Gating strategy is similar although all gates are rectilinear.

Plot 1. Displays total events with a region (R1) around the lymphocyte area (G1)

Plot 2 Gate = Gate 1
Only events falling into R1 are displayed here. This dotplot is not part of the analysis but is used to check cytometer settings.

Plot 3 Displays total events.
A region (R2) is paced around the CD45⁺ events.

Plot 4 Gate = Gate 2 (R2)
Displays events that are CD45⁺ only. A region (R3) is placed around the CD34⁺, side scatterlo and forward scatterlo events.

Plot 5 Gate = Gate 3 (R2*R3)
Displays events that are CD45⁺/CD34⁺/side scatterlo/forward scatterlo. A region (R4) is placed around the CD45dim events

Plot 6 Gate = Gate 4 (R2*R3*R4)
Displays events that are CD45⁺/CD34⁺/side scatterlo/forward scatterlo/CD45dim. A region (R5) is placed around the lymphocyte region.

Plot 7 Gate = Gate 5 (R2*R3*R4*R5)
Displays events that are;
CD45⁺/CD34⁺/side scatterlo/forward scatterlo/ CD45dim/lymphocytes. A region (R6) is placed around the CD38⁻ cells and another (R7) around the CD38⁺ cells.
Figure 5.1 (cont)

Analysis

Gate 6 = (R2*R3*R4*R5*R6)  
(CD45+/CD34+/side scatter<sub>lo</sub>/forwardscatter<sub>lo</sub>/CD45<sub>dmi</sub>/lymphocytes/CD38<sup>-</sup>)

Gate 7 = (R2*R3*R4*R5*R7)  
(CD45+/CD34+/side scatter<sub>lo</sub>/forwardscatter<sub>lo</sub>/CD45<sub>dmi</sub>/lymphocytes/CD38<sup>+</sup>)

Gate 2 = (R2)  
CD45<sup>+</sup>

The percentage of cells that are CD34<sup>-</sup>38<sup>-</sup> is calculated as being;  
(Gate 6 events/Gate 2 events)*100

The percentage of cells that are CD34<sup>+</sup>38<sup>+</sup> is calculated as being;  
(Gate 7 events/Gate 2 events)*100

Sorting protocol

Events that fall into Gate 6 = (R2*R3*R4*R5*R6)  
(CD45+/CD34+/side scatter<sub>lo</sub>/forwardscatter<sub>lo</sub>/CD45<sub>dmi</sub>/lymphocytes/CD38<sup>-</sup>)  
are sorted.

Events that fall into Gate 7 = (R2*R3*R4*R5*R7)  
(CD45+/CD34+/side scatter<sub>lo</sub>/forwardscatter<sub>lo</sub>/CD45<sub>dmi</sub>/lymphocytes/CD38<sup>+</sup>)  
are sorted.
Plot 1. Total events
R1 = Gate 1 (lymphocyte area)

Plot 2. Gate = R1 (lymphocyte area)
This dot plot is not part of the analysis. It is to check cytometer settings for the gating strategy only

Plot 3. Total events
Selection of CD45pos and CD45dull events
Gate 2 = R2 (CD45+)
Plot 4. Gate 2
Selection of the lo-side scatter, CD34pos population
Gate 3 = R2\textsuperscript{*}R3 (CD45+CD34+)

Plot 5. Gate 3
Selection of the lo-side scatter, CD45dull, CD34pos population
Gate 4 = R2\textsuperscript{*}R3\textsuperscript{*}R4 (CD45+CD34+lo-side scatter)

Plot 6. Gate 4
Re-selection of the lo-side scatter, lo-forward scatter, CD45-dull, CD34pos population
Gate 5 = R2\textsuperscript{*}R3\textsuperscript{*}R4\textsuperscript{*}R5 (CD45+CD34+lo-side & lo-forward scatter)
Plot 7. Gate 5
Selection of the CD38neg and CD38pos population from the CD34 pos, CD45-dull, lo-side scatter and lo-forward scatter population
Gate 6 = R2*R3*R4*R5*R6 (CD45+CD34+lo-side & lo-forward scatterCD38-)
Gate 7 = R2*R3*R4*R5*R7 (CD45+CD34+lo-side & lo-forward scatterCD38+)
5.3-8 Culture of CD34⁺ cells with selected growth factors

In selected experiments, isolated CD34⁺ cells were resuspended to a concentration of 0.5 x 10⁶ cells/ml in αMEM (Trace Scientific, Noble Park, Aust.) with 20% Foetal Bovine Serum (FBS) (CSL Biosciences, Parkville, Aust.), 2mM L-Glutamine (Sigma Aldrich), 200U/ml Penicillin/Streptomycin (Sigma Aldrich). Each CD34⁺ sample was then divided into 9 equal volumes. One sample was immediately homogenized in TRIZOL® reagent according to the manufacturers instructions, and stored at 80°C until RNA extraction and reverse transcription. The remaining eight samples were cultured for 24 hours at 37°C in a humidified atmosphere flushed with 5% CO₂ in air with one of the following growth factors; 50ng/ml rbSCF (Amgen, Thousand Oaks, U.S.A.), 20ng/ml IL-3 (Amrad, Boronia, Aust.), 20ng/ml G-CSF (Amrad), 100ng/ml FLT-3 (Stem Cell Technologies Inc. Vancouver, Canada), 20ng/ml GM-CSF (Amrad), 10ng/ml TGFβ1 (Invitrogen) and 100ng/ml MIP1α (Endogen, Woburn, MA. U.S.A.). Control cells were incubated under the same conditions with no growth factor/inhibitors. At the end of the culture period, viability and cell number were assessed using trypan blue exclusion and manual cell counting. Cells were then immediately homogenized in TRIZOL® reagent according to the manufacturer instructions, and stored at 80°C until RNA extraction and reverse transcription.

5.3-9 OS treatment of CD34⁺ cells

Isolated CD34⁺ cells were cultured for 96 hours at 37°C in a CO₂ incubator, at a concentration of 0.5 x 10⁶ cells/ml in RPMI 1640 (Trace Scientific, Noble Park, Aust.) with 10% FBS (CSL Biosciences, Parkville, Aust.), 2mM L-Glutamine and 200U/ml Penicillin/Streptomycin (Sigma Aldrich). 25-OHC was added to treatment tubes at a final concentration of 2μg/ml in media. All treatments and controls contained an equal amount of carrier (ethanol at 0.5%). At various time points (see
results), 0.1 x 10⁶ CD34⁺ cells were removed from culture, immediately homogenized in TRIZOL® reagent according to the manufacturers instructions, and stored at 80°C until RNA extraction and reverse transcription.

PART 2: ORP-3 GENE REGULATION IN HL60 CELLS

5.3-10 Cell lines

Two stable transfected subclones of the human promyelocytic leukemia cell line HL60 were used: HL60 cells transfected and over-expressing human bcl-2 (HL60-bcl2) and HL60 cells transfected with vector alone (HL60-v) (generously supplied by Dr. G. Vairo). The cells were maintained in HL60 base media consisting of RPMI 1640 (Trace Scientific) supplemented with 10% FBS (CSL Biosciences), 200U/ml Penicillin/Streptomycin and 2mM L-Glutamine (Sigma Aldrich). Additionally, all cultures were supplemented with 2μg/ml Puromycin (Sigma Aldrich) to ensure stable vector transfection. HL60-v and HL60-bcl2 cells were maintained in asynchronous exponential growth for all experiments.

5.3-11 Expression of ORP-3 in HL60-v and HL60-bcl2 cells

Exponentially growing cells were split to a concentration of 0.25 x 10⁶ cells/ml in media and cultured in a CO₂ incubator at 37°C. After 48 hours, cell number and viability were assessed using trypan blue exclusion, and 1.0 x 10⁶ cells were immediately homogenized in TRIZOL® reagent according to the manufacturers instructions, and stored at 80°C until RNA extraction and reverse transcription.
5.3-12 Expression of ORP-3 in HL60-v cells over 6 days in normal culture conditions

Exponentially growing cells were split to a concentration of $0.25 \times 10^6$ cells/ml in media and cultured for 6 days in a CO$_2$ incubator at 37°C. Cell number and viability were assessed every 24 hours using Trypan Blue exclusion. $1.0 \times 10^6$ cells were removed daily and immediately homogenized in TRIZOL$^\text{®}$ reagent according to the manufacturers instructions, and stored at `80°C until RNA extraction and reverse transcription.

5.3-13 OS treatment of HL60-v cells

Exponentially growing cells were split to a concentration of $0.5 \times 10^6$ cells/ml in media and treated with 2μg/ml of 25-OHC for 48 hours. Cells were cultured in a CO$_2$ incubator at 37°C. All treatments and controls contained an equal amount of carrier (ethanol at 0.5%) and were performed in duplicate. Cell number and viability were assessed daily using trypan blue exclusion (Sigma Aldrich). At various time points (see results), $1.0 \times 10^6$ HL60-v cells were removed from culture, immediately homogenized in TRIZOL$^\text{®}$ reagent according to the manufacturers instructions, and stored at `80°C until RNA extraction and reverse transcription. At various time points (see results), $1.0 \times 10^6$ HL60-v cells were removed from culture and the percentage of apoptotic cells analysed using flow cytometry (5.3-14).
5.3-14 Flow cytometric evaluation of the percentage of HL60-v cells undergoing apoptosis

Cells were resuspended to a final concentration of 1 x 10^6/ml in Annexin Binding Buffer and labeled with 5.0μg/ml Propidium Iodide (PI) (Molecular Probes) and FITC-conjugated annexin-V according to manufacturers instruction (Pharmingen). The percentage of viable (PI<sup>−</sup>) apoptotic cells (annexin-V-FITC<sup>+</sup>) was determined by flow cytometric analysis (FacsCalibur, Becton Dickinson) using CELLQuest software (Becton Dickinson) (Figure 5.2).
Figure 5.2 Flow cytometric evaluation of the percentage of HL60-v cells undergoing apoptosis
Plot 1. Total events
R1 = Gate 1 (all cellular events)

Plot 2. Gate = R1
FL1 = Annexin-V-FITC
FL2 = PI
LL = Viable, non-apoptotic cells
LR = Viable, apoptotic cells
UR = Non-viable cells
5.3-15 Initiation of apoptosis in HL60-v cells by OS and Camptothecin

Exponentially growing cells were split to a concentration of $0.3 \times 10^6$ cells/ml in HL60 base media and treated with 1.0μg/ml of 25-OHC or 15nM camptothecin (Sigma Aldrich) for 72 hours. Cells were cultured in a CO$_2$ incubator at 37°C. All treatments and controls contained an equal amount of carrier (ethanol at 0.5%) and were performed in duplicate. Cell number and viability were assessed daily using Trypan Blue exclusion (Sigma Aldrich). At various time points (see results), $1.0 \times 10^6$ cells were immediately homogenized in TRIZOL® reagent according to the manufacturers instructions, and stored at $80^\circ$C until RNA extraction and reverse transcription. At various time points (see results), $1.0 \times 10^6$ cells were removed from culture and the percentage of apoptotic cells determined using flow cytometry (section 5.3-14).

5.3-16 Differentiation of HL60 cells

Exponentially growing cells were split to a concentration of $0.3 \times 10^6$ cells/ml in media and treated with 1μM retinoic acid (Sigma Aldrich) for 96 hours. Cells were cultured in a CO$_2$ incubator at 37°C. All treatments and controls contained an equal amount of carrier (DMSO at 0.1%) and were performed in duplicate. At various time points (see results) cell number, viability and differentiative status were assessed using trypan blue exclusion and nitroblue tetrazolium (NBT) (Sigma Aldrich) reduction (5.3-17). At various time points (see results), $1.0 \times 10^6$ cells were immediately homogenized in TRIZOL® reagent according to the manufacturers instructions, and stored at $80^\circ$C until RNA extraction and reverse transcription.
5.3-17 Assessment of differentiative status of HL60-v cells by nitroblue tetrazolium (NBT) reduction

Differentiative status of HL60-v cells was assessed by the ability to reduce NBT (Sigma Aldrich) as previously described (Blair et al 1985). Briefly, 250μl of cell suspension (at approximately 0.5 x 10^6 cells/ml) was mixed with an equal volume of 0.1% NBT in PBS. Five microlitres of 100μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) in dimethyl-sulphoxide (DMSO) (Sigma Aldrich) was added, and the cells incubated at 37°C 20 minutes. The percentage of NBT positive (cells containing dark/blue granules) was assessed using manual cell counting.

5.3-18 Cell synchronization of HL60 cells by double thymidine block

Exponentially growing HL60-v cells were split to a concentration of 0.35 x 10^6/ml 24 hours before cell synchronisation by double thymidine block as previously described (Ooka and Daillie 1974, Bruno and Olson 1992). Cells were cultured in a CO₂ incubator at 37°C. All treatments and controls were performed simultaneously in duplicate. Briefly, exponentially growing cells were blocked for 16 hours in HL60 base media with 2mM thymidine (Sigma Aldrich). Cells were then washed with warmed PBS. The washed cells were resuspended to a final concentration of 0.5 x 10^6 cells/ml in pre-warmed HL60 base media with 24μM deoxythymidine (Sigma Aldrich), and released for 10 hours. Cells were then washed in warmed PBS, resuspended to a final concentration of 0.5 x 10^6 cells/ml, and blocked for 16 hours in pre-warmed HL60 base media with 2mM thymidine. After the second block, cells were washed in warmed PBS and released by resuspension to a final concentration of 0.5 x 10^6 cells/ml in pre-warmed HL60 base media with 24μM deoxythymidine. At various time points after this second release (see results), 1.0 x 10^6 cells were removed and immediately homogenized in TRIZOL® reagent according to the manufacturers instructions, and stored at -80°C until RNA extraction and reverse
transcription. At various time points during the release (see results), 0.25 x 10^6 cells were also removed for cell cycle analysis by flow cytometry (see section 5.3-19).

5.3-19 Flow cytometric evaluation of the cell cycle status of HL60-v cells

0.25 x 10^6 cells were centrifuged at 480g for 5mins and the supernatant was removed. Cells were resuspended and fixed in 65μl ice cold PBS and 195μl of ice-cold 95% ethanol, and incubated room temperature for 5 – 10 minutes. Cells were re-centrifuged, supernatant removed and re-suspended to a final concentration of 0.25 x 10^6 cells/ml in 15μg/ml PI and 2μg/ml RNase Cocktail™ (Bresatec, Adelaide, Aust.). The percentage of cells in G0/G1, S and G2/M phases of the cell cycle was analysed by flow cytometric analysis (FacsCalibur, Becton Dickinson) using MODFIT software (Becton Dickinson) (Figure 5.3).
Figure 5.3 Flow cytometric evaluation of the cell cycle status of HL60-v cells

a) The percentage of cells in G0/G1, S and G2/M phases of the cell cycle in control cells

b) The percentage of cells in G0/G1, S and G2/M phases of the cell cycle in synchronized cells at T=2

c) The percentage of cells in G0/G1, S and G2/M phases of the cell cycle in synchronized cells at T=6

d) The percentage of cells in G0/G1, S and G2/M phases of the cell cycle in synchronized cells at T=13
FL2 = PI

Control unsynchronised cells

M1 = G0/G1
M2 = S
M3 = G2/M
FL2 = PI

Synchronised cells

T=2

M1 = G0/G1
M2 = S
M3 = G2/M
HL60 Cell Synch 2.046

FL2 = PI
Synchronised cells
T=6

M₁ = G0/G1
M₂ = S
M₃ = G2/M
Synchronised cells

$T=13$
PART 3: QUANTITATION OF ORP-3 GENE EXPRESSION BY TAQMAN REAL-TIME PCR

5.3-20 Total RNA Isolation and Reverse Transcription

Total RNA was extracted by TRIZOL® reagent and contaminating DNA removed by digestion with DNaseI Amplification Grade (Invitrogen) according to the manufacturers instructions. Quantity and purity of the RNA was determined by UV Spectrophotometry. Reverse transcription was carried out on a Perkin Elmer 9600 thermocycler (P.E. Applied Biosystems Foster City, U.S.A.) using Reverse Transcription System (Promega, Madison, U.S.A.) following the manufacturers guidelines, random hexamers and 50ng DNA-free RNA/10μl reaction volume. cDNA was diluted 1:2 and used in subsequent real-time PCR reactions.

5.3-21 Real-Time PCR Semi-Quantitation of ORP-3 and bcl2

All primers and probes for use in Real-Time PCR (Table 5.1) were designed using Taqman™ Primer Express Software (P.E. Applied Biosystems) and optimised for use as recommended by the manufacturer (Table 5.2). Real-Time PCR amplification was carried out using either Taqman™ Universal PCR Mastermix or SYBR™ Green PCR Master Mix (Table 5.2) on an ABI PRISM™ 7700 Sequence Detection System following the manufacturers guidelines (P.E. Applied Biosystem). Gene Expression was quantitated relative to expression of two housekeeping genes (β-actin and GAPDH) using Sequence Detector Software and the comparative Ct method (P.E. Applied Biosystem User Bulletin No. 2).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td><code>5'- gac agg atg cag aag gag att act-3'</code></td>
<td><code>5'- tga tcc aca tct gct gga aag t -3'</code></td>
<td><code>fam- aca att gct cct cct gag gcc aag tac tc- tamra</code></td>
</tr>
<tr>
<td>GAPDH</td>
<td><code>5'- cca cat cgc tca gac acc at-3'</code></td>
<td><code>5'- cca ggc gcc caa tag g -3'</code></td>
<td><code>fam- aag gtg aag gtc gga gtc aac gga ttg g - tamra</code></td>
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<tr>
<td>ORP-3</td>
<td><code>5'-aca gcc agc tcc tgg aca a -3'</code></td>
<td><code>5'-ctg cca cat ata cca tcc ttt cc -3'</code></td>
<td><code>fam- cca ggc aga ttc cca gcc c - tamra</code></td>
</tr>
<tr>
<td>Bcl2</td>
<td><code>5'- cat gtt tgt gga gag cgt cca -3'</code></td>
<td><code>5'-gcc cgt tca ggt act cag tca -3'</code></td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>
### Table 5.2 Optimised Real-Time PCR Primer and Probe concentrations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse primer</th>
<th>Probe</th>
<th>Real-Time Chemistry</th>
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<tr>
<td>β-actin</td>
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<td>100</td>
<td>Taqman™</td>
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<td>GAPDH</td>
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<td>Taqman™</td>
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<tr>
<td>Bcl2</td>
<td>50</td>
<td>50</td>
<td>N/A</td>
<td>SYBR Green™</td>
</tr>
</tbody>
</table>
5.3-22 Statistics

All gene expression results are expressed in arbitrary units (Mean ± SEM) and calculated from duplicate determinations. The Wilcoxon Signed Ranks Test and Paired Students T-Test were used to determine the statistical significance of the differences between two related groups for non-parametric data and parametric data respectively. The Mann-Witney and Students T-test was utilized to determine the statistical significance of the differences between two non-related groups for non-parametric and parametric data respectively. One Way Analysis of Variance (Tukeys and Tamhanes Post-Hoc tests) was utilised for statistical analysis of multiple samples from the same source, Two Way Analysis of Variance (Tukeys and Tamhanes Post-Hoc tests) for statistical analysis of multiple samples from different sources. For correlations, a Pearson correlation coefficient was used for parametric data. All statistical analyses were performed using SPSS (Statistics Package for the Social Sciences) version 10.0 software (Fullerton, CA, U.S.A.). In all instances, p ≤ 0.05 was considered statistically significant.
5.4 RESULTS

PART 1: REGULATION OF THE ORP-3 GENE IN CD34+ HSPCs FROM UCB

5.4-1 Correlation of ORP-3 gene expression with viability and purity of UCB CD34+ HSPCs

To investigate whether ORP-3 expression in UCB CD34+ HSPCs correlates with the purity or viability of the samples, ORP-3 expression in each sample was plotted against purity and viability (Figures 5.4a and b). A total of 34 UCB CD34+ enriched cell populations, with varying degrees of purity and viability were used in this analysis. Mean viability, purity and ORP-3 gene expression of the samples was 95% (± 1.0%), 55.9% (± 3.6%) and 8.7 (± 1.2) respectively. Results indicate that ORP-3 expression was not significantly correlated with viability of the samples, but was significantly correlated with sample purity (p ≤ 0.05).
Figure 5.4 ORP-3 gene expression in CD34⁺ haemopoietic cells from UCB

a) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and GAPDH and expressed in arbitrary units) versus sample viability (n = 34)

b) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and GAPDH and expressed in arbitrary units) versus sample purity (n = 34, p ≤ 0.047, Pearson correlation coefficient)
5.4-2 Quantitation of ORP-3 Using Taqman™ Real-Time PCR in CD34⁺38⁺ and CD34⁺38⁻ haemopoietic cells from UCB

The CD34⁺ population constitutes a very heterogeneous cell population of multipotential and lineage restricted cells (Morrison et al 1995, Graham and Wright 1997). To investigate ORP-3 gene expression in a more primitive subfraction of cells, CD34⁺ enriched UCB samples were sorted according to expression of the CD38 antigen (Xiao and Dooley 2000, Reems and Torok-Storb 1995). ORP-3 gene expression in the more primitive sub-population of cells defined by the CD34⁺38⁻ phenotype, was 2 – fold higher than in the more mature CD34⁺38⁺ cells (17.6 ± 3.8, 8.0 ± 2.9, n= 5, p ≤ 0.04) (Figure 5.5). Mean viability (>80%) of the two populations did not vary significantly (results not shown).
Figure 5.5 ORP-3 gene expression in CD34-, CD34+CD38+ and CD34+CD38- haemopoietic cells from UCB

ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and GAPDH, and expressed in arbitrary units) in CD34+38- cells (17.6 ± 6.5) is 2-fold higher than in CD34+38+ cells (8.0 ± 5.9, n = 5, p ≤ 0.04, Wilcoxon signed ranks test)
5.4.3 The effect of growth factors and inhibitors on ORP-3 gene expression in CD34+ HSPCs

Of the cytokines investigated in this study, only transforming growth factor-beta1 (TGFβ1) significantly altered expression of the ORP-3 gene from control levels. Expression of the ORP-3 gene in CD34+ HSPCs from UCB was significantly decreased after 24 hours incubation in 10ng/ml TGFβ1 (6.9 ± 0.4) compared to expression in paired controls (15.4 ± 1.5, n = 6, p ≤ 0.02) (Figure 5.6). Viability of the control and TGFβ1 incubated cells did not vary significantly (87.7 ± 3.0% versus 85.6 ± 5.3% respectively). Cell number of the control and TGFβ1 incubated cells also did not vary significantly (0.41 ± 0.13 x 10^6 versus 0.26 ± 0.06 x 10^6 respectively).
Figure 5.6 ORP-3 gene expression in CD34\(^+\) haemopoietic cells from UCB exposed to different cytokines.

ORP-3 gene expression (measured by Taqman\textsuperscript{TM} real-time PCR, calculated relative to β-actin and expressed in arbitrary units) in CD34\(^+\) cells is was significantly decreased by incubation with 10ng/ml TGFβ1 (6.9 ± 0.4) compared to paired controls (15.4 ± 1.5, n = 6, p ≤ 0.024, One-way ANOVA).
5.4-4 The effect of OS on ORP-3 gene expression in CD34⁺ HSPCs from UCB

There are many instances where the transcription of genes encoding receptors or binding proteins are regulated by the presence of their ligands (Murthy et al 1989, Brizzi et al 1990). Homology between ORP-3 and OSBP-Hm suggests that ORP-3 may have a role in OS binding and mediation of OS effects. Culture of CD34⁺ enriched HSPCs from UCB with 2μg/ml of 25-OHC significantly increased ORP-3 gene expression to approximately 1.5 times control levels at 48 hours (14.3 ± 3.3 versus 9.0 ± 1.8, n = 5, p ≤ 0.05) (Figure 5.7). At 48 hours, the percentage of viable apoptotic CD34⁺ cells was also increased by OS treatment to approximately 1.3 times the levels seen in control CD34⁺ cells (see Chapter 6, 6.4-2). The apoptotic effect of OS on CD34⁺ cells, although interesting and worthy of further investigation, introduces another variable to the experiment. Thus, although expression of the ORP-3 gene was up regulated after the addition of OS to the culture media, it is not possible to make any conclusions regarding the regulation of ORP-3 gene expression by OS.
Figure 5.7 The effect of 2μg/ml 25-OHC on ORP-3 gene expression in CD34^+ haemopoietic cells from UCB.

2μg/ml 25-OHC significantly increased ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) in CD34^+ cells (14.3 ± 3.3 versus 9.0 ± 1.8 at 48 hours, n = 5, p ≤ 0.05, Two-way ANOVA).
PART 2: ORP-3 GENE REGULATION IN HL60 CELLS

5.4.5 The effect of bcl2 over expression on ORP-3 gene expression in HL60 cells

To further investigate regulation of ORP-3 gene expression, the human pro-myelocytic cell line HL60 was used. This bipotent cell line can be induced to differentiate into granulocytes and monocytes in response to specific external stimuli, and is therefore routinely used as a model of haemopoiesis (Collins 1987, Ahmed et al 1991). Two stable transfected subclones of HL60 were utilised; HL60 cells transfected and over-expressing human bcl-2 (HL60-bcl2) and HL60 cells transfected with vector alone (HL60-v). Bcl-2 is an integral mitochondrial membrane protein that functions to repress induction of apoptosis (Vaux et al 1988, reviewed by Chao and Korsmeyer 1998). Over-expression of bcl-2 in transfected HL60-bcl2 compared to HL60 cells transfected with vector alone was confirmed using SYBR Green™ real-time PCR. (285.0 ± 49.9 versus 21.4 ± 6.3, n = 5, p ≤ 0.03). Expression of ORP-3 in these cells was not significantly different (9.9 ± 2.3 versus 10.0 ± 1.2, n = 5) (Figure 5.8).
Figure 5.8 The effect of bcl2 over expression on ORP-3 gene expression in HL60 cells

Over-expression of bcl-2 in transfected HL60-bcl2 compared to HL60 cells transfected with vector alone was confirmed using SYBR Green™ real-time PCR (285.0 ± 49.9 versus 21.4 ± 6.3, n = 5, p ≤ 0.025). Expression of ORP-3 in these cells was not significantly different (9.9 ± 2.3 versus 10.0 ± 1.2, n = 5).
5.4-6 Expression of ORP-3 in HL60-v cells during six days culture

The expression of many genes is known to vary with cell cycle (Chen and Feldman 1981, Jakesz et al 1984, Crabtree et al 1980). As such, the expression of these genes will also vary over time during normal culture conditions. HL60-v cells were split whilst still in exponential growth (1.07 ± 0.11 x 10^6 cells/ml) and cultured for 140 hours (Figure 5.9a, b). A significant difference in the viability of these cells was only observed between time = 0, when cell were just passaged, and time = 144 (Figure 5.9a) (99.6 ± 0.4% versus 97.2 ± 0.1%, p ≤ 0.03, n = 5). There was no correlation between cell viability and ORP-3 gene expression (n = 35) (Figure 5.10a).

Expression of the ORP-3 gene was found to not vary significantly in HL60-v cells from 0 – 120 hours in culture (Figure 5.9c). During this time, cells were in exponential growth phase with cell number approximately doubling every 24 hours (Figure 5.9b). Expression of ORP-3 at 144 hours was approximately 2 – 3 times higher than levels observed from 0 – 120 hours (p ≤ 0.001, n = 5) (Figure 5.9c). At this time point, HL60-v cells were no longer in exponential growth and cell number at 144 hours was only 13% higher than the preceding time point (1.19 ± 0.06 x 10^6 cell/ml versus 2.17 ± 0.09 x 10^6 cell/ml, n = 5) (Figure 5.9b). Interestingly, an increase in ORP-3 gene expression was significantly correlated with an increase in cell number (n = 35, p < 0.004) (Figure 5.10b).
Figure 5.9 ORP-3 gene expression and growth of HL60-v cells over six days normal culture

a) Viability of HL60-v cells as measured by trypan blue exclusion (n = 5, p ≤ 0.029, Time = 144 compared to Time = 0 only, One-way ANOVA)

b) Cell No. of HL60-v cells as measured by trypan blue exclusion

c) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) is significantly increased in HL60-v cells after 144 hours in culture (n = 5, p ≤ 0.001, Time = 144 compared to all other time points, One-way ANOVA)
Figure 5.10 ORP-3 gene expression in HL60-v cells

a) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) versus sample viability (n = 35)

b) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) versus cell number (n = 35, p < 0.004, Pearson correlation co-efficient)
5.4-7 The effect of OS on ORP-3 gene expression in HL60-v cells

Culture of HL60-v cells with 2μg/ml of 25-OHC significantly increased ORP-3 gene expression to approximately 1.5 times control levels at 24 hours (7.3 ± 0.7 versus 5.1 ± 0.5, n = 6, p ≤ 0.04) (Figure 5.11a). At 24 hours, the percentage of viable apoptotic HL60-v cells was also increased by OS treatment to approximately 1.6 times the levels seen in control HL60-v cells (2.52 ± 0.41% versus 1.56 ± 0.30%, n = 5, p ≤ 0.001) (Figure 5-11b). The apoptotic effect of OS on HL60-v cells parallels the observation of the apoptotic effect of OS on CD34⁺ cells from UCB (Chapter 6). This observation validates (at least in part) the use of HL60-v cells as a model for investigating regulation of ORP-3 expression in haemopoietic progenitors. However, as discussed in section 5.4-4, the increase in apoptosis of HL60-v cells incubated with OS although interesting and worthy of further investigation, introduces another variable into the experiment. Thus, although expression of the ORP-3 gene in HL60 cells was up regulated after the addition of OS to the culture media, it is unclear whether this up-regulation is a direct, or indirect effect resulting from increased apoptosis.
Figure 5.11 The effect of 2μg/ml 25-OHC on ORP-3 gene expression in HL60-v cells

a) 25-OHC (2μg/ml) induced a 1.4-fold increase in ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) compared to controls (7.3 ± 0.7 versus 5.1 ± 0.5 at 24 hours, n = 6, p ≤ 0.04, Two-way ANOVA).

b) 25-OHC (2μg/ml) induced a 2.8-fold increase in the percentage of viable HL60-v apoptotic cells (annexin-V<sup>pos</sup>, PI<sup>neg</sup>) compared to controls (4.33 ± 0.52 versus 1.56 ± 0.36 at 36 hours, p ≤ 0.001, n = 6, Two-way ANOVA)
5.4.8 The effect of Camptothecin and OS induced apoptosis on ORP-3 gene expression in HL60-v cells

Camptothecin induces apoptosis in cells by inhibition of DNA topoisomerase 1 (Hsiang and Liu 1988). The mechanism by which OS such as 25-OHC induce apoptosis is unclear. The purpose of this experiment was to investigate regulation of ORP-3 gene expression in HL60-v cells exposed to both these apoptotic agents.

Treatment of HL60-v cells with 15nM camptothecin (9.2 ± 0.7% at 48 hours, n = 4, p ≤ 0.001) and 1.0μg/ml 25-OHC (8.6 ± 1.2% at 48 hours, n = 4, p ≤ 0.001) induced an 8-fold increase in the percentage of cells undergoing apoptosis (measured by annexin-v staining and flow cytometry) compared to controls (1.9 ± 0.7 at 48 hours, n = 4) (Figure 5-12a). This increase in apoptosis was accompanied by an increase in ORP-3 gene expression in both the camptothecin treated cells (15.2 ± 2.2 at 24 hours, n = 4, p ≤ 0.02) and 25-OHC treated cells (15.1 ± 0.8 at 24 hours, n = 4, p ≤ 0.001) compared to controls (8.8 ± 0.6 at 24 hours, n = 4) (Figure 5-12b).

Although ORP-3 gene expression in OS treated cells at 36 hours was approximately 2-fold higher than in camptothecin treated cells (21.5 ± 2.6 versus 10.5 ± 1.5), this difference was not significant at the p ≤ 0.05 level (Figure 5-12b).
Figure 5.12 The effect of 1μg/ml 25-OHC and 15nM camptotheclin on ORP-3 gene expression in HL60-v cells

a) Both 15nM camptotheclin (15.2 ± 2.2 at 24 hours, n = 4, p ≤ 0.024, Two-way ANOVA) and 1.0μg/ml 25-OHC (15.1 ± 0.8 at 24 hours, n = 4, p ≤ 0.001, Two-way ANOVA) increased ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) in HL60-v cells approximately 1.7- fold compared to controls (8.8 ± 0.6 at 24 hours, n = 4)

b) Both 15nM camptotheclin (9.2 ± 0.7% at 48 hours, n = 4, p ≤ 0.001, Two-way ANOVA) and 1.0μg/ml 25-OHC (8.6 ± 1.2% at 48 hours, n = 4, p ≤ 0.001, Two-way ANOVA) increased the percentage of annexin-v⁺ HL60-v cells approximately 8- fold compared to controls (1.9 ± 0.7 at 48 hours, n = 4)
5.4-9 The effect of HL60-v differentiation on ORP-3 gene expression

In response to specific external stimuli, HL60 cells can be induced to differentiate into granulocyte, monocyte or macrophage-like cells (Collins 1987, Ahmed et al 1991). NBT staining is commonly used to detect reactive oxygen species formed by granulocytes in the presence of endotoxin (Harris and Ralph 1985). To investigate the effect of differentiation on ORP-3 gene expression, HL60-v cells were induced to differentiate along the granulocyte pathway by treatment with 1μM retinoic acid (RA). Differentiation was confirmed by an increase in the percentage of NBT positive cells compared to controls (45.0 ± 1% versus 13.6 ± 1.1% at 48 hours, n = 5, p < 0.001) (Figure 5-13a). Cell number and viability of the control and RA treated cells did not vary significantly over the course of the experiment (1.4 ± 0.06 x 10^6/ml versus 1.2 ± 0.07 x 10^6/ml and 98.7 ± 0.3% versus 98.4 ± 0.5% at 96 hours respectively, n = 5).

ORP-3 gene expression in control HL60-v cells at 96 hours increased approximately 2.7-fold compared to control cells at time = 0 (11.3 ± 1.0 versus 4.2 ± 0, n = 5, p ≤ 0.001, n = 5) (Figure 5-13b). This increase in ORP-3 gene expression in HL60-v cells over time in normal culture confirms observations reported in section 5.4-6. In contrast, ORP-3 gene expression at 96 hours in HL60-v cells treated with 1μM RA remained the same as observed at time = 0 (5.9 ± 0.9 versus 4.2 ± 0, n = 5). As such, ORP-3 gene expression in differentiated HL60-v cells was significantly lower than in control cells at 96 hours (5.9 ± 0.9 versus 11.3 ± 1.0 at 96 hours, n = 5, p ≤ 0.001, n = 5) (Figure 5-13b).
Figure 5.13 The effect of HL60-v differentiation on ORP-3 gene expression

a) HL60-v cells treated with 1μM retinoic acid displayed a higher percentage of NBT positive cells compared to controls (45.0 ± 1% versus 13.6 ± 1.1% at 48 hours, n = 5, p < 0.001, Two-way ANOVA).

b) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) in retinoic acid treated HL60-v cells was significantly lower than in control cells (5.9 ± 0.9 versus 11.3 ± 1.0 at 96 hours, n = 5, p ≤ 0.001, n = 5, Two-way ANOVA).
5.4.10 The effect of cell cycle status on ORP-3 gene expression in HL60-v cells

To investigate the effect of cell cycle on ORP-3 gene expression, HL60-v cells were synchronized by double thymidine block and ORP-3 gene expression was measured at three phases of the cell cycle. Two hours after release from the second thymidine block, approximately 63.1% (± 2%) of the synchronized cells were in S phase indicating that the synchronization was successful (Figure 5-14a). The synchronized cells then progressively moved through subsequent stages of the cell cycle. At 6 and 13 hours post release, 74.6% (± 2.9) and 71.3% (± 1.9%) were in G2/M and G0/G1 respectively (Figure 5-14b and c). In contrast, the percentage of control unsynchronized cells in each phase of the cell cycle did not vary significantly over the times investigated (Figure 5-14a,b and c). Viability of the synchronized and unsynchronized cells did not vary significantly (96.5 ± 1.0 versus 97.2 ± 1.1, n = 4). Analysis of ORP-3 gene expression in unsynchronized and synchronized cells at T = 2, 6 and 13 demonstrated a trend to lower ORP-3 gene expression in cells synchronized at G2/M (4.8 ± 1.2 versus 6.7 ± 0.9, n = 4) (Figure 5-14d). This difference however did reach statistical significance (Figure 5-14d).
Figure 5.14 The effect of cell synchronization on ORP-3 gene expression in HL60-v cells

a) The percentage of control unsynchronized cells in G0/G1 did not vary significantly 2, 6 and 13 hours post release (47.9 ± 1.1%, 47.8 ± 0.6%, 48.2 ± 0.6%, n = 4, One-way ANOVA). The percentage of synchronized cells in G0/G1 at 13 hours post-release was approximately 6-fold higher than at 2 and 6 hours post release (63.1 ± 2.0% versus 11.6 ± 1.2% and 7.0 ± 0.7%, n = 4, p ≤ 0.001, One-way ANOVA).

b) The percentage of control unsynchronized cells in S phase did not vary significantly 2, 6 and 13 hours post release (25.4 ± 1.7%, 24.9 ± 0.8%, 25.6 ± 1.1%, n = 4, One-way ANOVA). The percentage of synchronized cells in S phase at 2 hours post-release was approximately 7-fold higher than at 6 and 13 hours post release (74.6 ± 2.9% versus 15.6 ± 2.7% and 6.3 ± 0.6%, n = 4, p ≤ 0.001, One-way ANOVA).

c) The percentage of control unsynchronized cells in G2/M phase did not vary significantly 2, 6 and 13 hours post release (20.6 ± 1.9%, 22.5 ± 0.8%, 22.1 ± 1.6%, n = 4, One-way ANOVA). The percentage of synchronized cells in G2/M phase at 6 hours post-release was approximately 5-fold higher than at 2 and 13 hours post release (71.3 ± 1.9% versus 10.2 ± 1.3% and 24.1 ± 3.2%, n = 4, p ≤ 0.001, One-way ANOVA).

d) ORP-3 gene expression (measured by Taqman™ real-time PCR, calculated relative to β-actin and expressed in arbitrary units) in control unsynchronized and synchronized cells did not vary significantly at 2 (7.4 ± 0.8 versus 10.1 ± 0.6), 6 (6.7 ± 0.9 versus 4.8 ± 1.2) and 13 hours post-release (8.3 ± 1.2 versus 7.3 ± 0.7) (n = 4, One and Two-way ANOVA).
Figure 5.14 (cont)
5.5 DISCUSSION

In previous studies, expression of the ORP-3 gene was found to be 3 - 4-fold higher in the CD34+ fractions of haemopoietic cells compared to CD34- populations from UCB and ABM respectively (Chapter 2). It is therefore not surprising that expression of the ORP-3 gene is significantly correlated with purity of the CD34 samples.

The CD34+ population however constitutes a very heterogeneous cell population of multipotential and lineage restricted cells (reviewed by Morrison et al 1995, Graham and Wright 1997). Additional monoclonal antibodies have been used to further sub-divide CD34+ cells into populations based on the expression of other cell surface antigens. The CD34+38- immunophenotype reportedly defines a more primitive subpopulation of haemopoietic progenitors than the CD34+ immunophenotype alone (reviewed by Graham and Wright 1997, Xiao and Dooley 2000). ORP-3 gene expression in the more primitive sub-population of cells defined by the CD34+38- phenotype, was 2 - fold higher than in the more mature CD34+38+ cells. This finding, taken together with the previous observation of down-regulation of ORP-3 expression with proliferation and differentiation of CD34+ cells (Chapter 2), indicates that ORP-3 expression may be higher in a less differentiated subset of cells. This hypothesis is supported by the observation that expression of the ORP-3 gene is approximately 2-fold lower in differentiated HL60-v cells compared to control, undifferentiated cells.

Interestingly, ORP-3 expression in HL60 cells during normal culture conditions was also found to vary with expression positively correlated with cell number. As ORP-3 gene expression did not correlate with sample viability, this may indicate a cell cycle effect on ORP-3 gene expression with levels highest when cell density, and therefore the percentage of cells in G0/G1 is highest (Rubin and Steiner et al 1975). This observation also correlates with the observation of higher ORP-3 expression in CD34+38- cells which have been reported to have a higher proportion of cells in
G0/G1 than corresponding CD34^+38^- haemopoietic cells (Reems and Torok-Storb 1995, Donahue et al 1996, Uchida et al 1997). Cell cycle dependent changes in the level of the OSBP-Hm protein have also been reported (Beseme et al 1985). In this study, Beseme et al (1985) hypothesized that levels of OSBP-Hm protein were lowest during G0/G1, and increased through S to peak at G2/M. This finding has not been confirmed nor has its significance been reported.

Data obtained from investigation of ORP-3 gene expression in synchronized HL60-v cells demonstrates a trend towards lower expression when HL60 cells are actively dividing and synchronized in G2/M. While this is consistent with previous observations of higher expression in CD34^+38^- HSPCs and HL60 that are not actively dividing, this difference did not reach statistical significance and interpretation of this data is thus limited. It is clear therefore, that a possible correlation between ORP-3 expression and cell cycle cannot be positively excluded and thus warrants further investigation.

Haemopoiesis is regulated, at least in part, by a series of growth factors that positively and negatively control proliferation, differentiation and survival of haemopoietic progenitor stem cells and their progenies committed to a more restricted differentiation pathway (reviewed by Metcalf 1989, Moore 1991, Ogawa 1993). These haemopoietic growth factors and inhibitors act by inhibiting or up-regulating the transcription of genes whose products ultimately play a part in determining HSPC fate (Becker et al 1999, Pirelli et al 2000, Ducos et al 2000).

Of the cytokines investigated in this study, only TGFβ1 significantly altered expression of the ORP-3 gene from control levels. Expression of the ORP-3 gene in CD34^+ HSPCs from UCB was significantly decreased to approximately half the levels observed in control after 24 hours incubation in 10ng/ml TGFβ1. Interpretation of this data is however complicated by the heterogeneous nature of the CD34^+ haemopoietic cells used in this study, and the bi-directional effects of TGFβ1 on such a population (Keller et al 1994). Indeed, TGFβ1 is capable of inhibiting the
growth of the most primitive haemopoietic progenitors, and stimulating the less primitive cells (Van Ranst et al 1996). The TGFβ1 inhibition and stimulation of primitive and more committed progenitors, is reflected by the increased and decreased percentage of cells in G0/G1 respectively (Ando and Griffin 1995, Batard et al 2000). As the majority of HSPCs (95 – 80%) in the CD34+ population of UCB samples are of the more committed CD34+38+ phenotype (reviewed by Xiao and Dooley 2000), it could be concluded that ORP-3 expression is down-regulated as these cells are stimulated into cell cycle (Ando and Griffin 1995, Batard et al 2000, Van Ranst et al 1996). Although this hypothesis is consistent with previous observations, this data cannot be considered conclusive. More definitive results regarding the effect of TGFβ1 and other growth factors on ORP-3 gene expression would be obtained using a less heterogeneous population of cells.

There are many instances where the transcription of genes encoding receptors or binding proteins are regulated by the presence of their ligands (Murthy et al 1989, Brizzi et al 1990). Homology between ORP-3 and OSBP-Hm suggests that ORP-3 may have a role in OS binding and mediation of OS effects. Culture of CD34+ enriched HSPCs and HL60-v cells with 25-OHC significantly increased ORP-3 gene expression to approximately 1.5 times control levels. However, as 25-OHC treatment also increased the percentage of apoptotic cells in these experiments, it is impossible to make any conclusions regarding the regulation of ORP-3 gene expression by OS. Indeed, the observation that camptothecin induced apoptosis also increased ORP-3 gene expression in HL60-v cells raises the possibility that up-regulation of ORP-3 gene expression is associated with apoptosis.

Conclusions from the data obtained in this study regarding regulation of expression of the ORP-3 gene are inhibited by the complex and intricately linked nature of the cell cycle dynamics of quiescence, apoptosis, proliferation and differentiation of haemopoietic cells. The most primitive haemopoietic progenitors are reported to be quiescent and have a higher percentage of cells in G0/G1 (Reems and Torok-Storb 1995, Leemhuis et al 1996, Donahue et al 1996, Ghotot et al 1997, Uchida et al
1997). These cells are able to respond to cytokines and given the right stimulus, begin actively proliferating and progressively move through the cell cycle phases of G0/G1, S and G2/M (reviewed by Furakawa et al 1998, Ladd et al 1997). Terminal differentiation of these cell results in blockade of the cells in the G0/G1 phase and eventual cell death by apoptosis (Studzinski et al 1997, Boyd and Metcalf 1984, reviewed by Furakawa et al 1998). At any time during this continuum, cells may receive apoptotic stimuli, cell cycle arrest, and die. The phase in which cell cycle arrest occurs prior to apoptosis is dependent on the nature of the apoptotic signal. Camptothecin and OS induced apoptosis have been reported to be preceded by arrest at the G0/G1 phases of the cell cycle (Christ et al 1993, Hyun et al 1997, Lizard et al 1996, Del Bino et al 1994, Traganos et al 1996, Nair et al 2000).

The CD34+38- population of primitive haemopoietic progenitor cells is also known to have a higher percentage of cells in G0/G1 than more mature CD34+38+ cells (Reems and Torok-Storb 1995, Leemhuis et al 1996, Donahue et al 1996, Gothot et al 1997, Uchida et al 1997) raising the possibility that expression of ORP-3 is highest in the G0/G1 phase of the cell cycle. This hypothesis is supported by down regulation of ORP-3 gene expression in CD34+ HSPCs exposed to stimulatory cytokines, positive correlation of ORP-3 gene expression with increasing cell density and up regulation of expression in HL60 cells undergoing OS and camptothecin induced apoptosis. Additionally, expression of ORP-3 appears to be highest in a less differentiated subset of cells with gene levels highest in more primitive undifferentiated haemopoietic cells (CD34+38- and undifferentiated HL-60), and down regulated with differentiation. This is consistent with observations that gene expression of the OSBP homologue HLM/ORP-4 is up regulated in solid tumors and in the PB of patients with CML (Fournier et al 1999, Silva et al 2001). Taken together, this data raises interesting questions regarding a possible role for ORP-3 in cell cycle progression and differentiation of haemopoietic progenitor cells.
CHAPTER 6

THE EFFECTS OF OXysterols ON HAEMOPOIETIC PROGENITOR CELLS

6.1 ABSTRACT

Differential expression of the ORP-3 gene implicates a role in haemopoiesis. The ORP-3 protein is predicted to contain an extremely well conserved oxysterol (OS) binding domain. OS are widely occurring hydroxylated derivatives of cholesterol detected in blood, cells and tissues. They exhibit a number of biological activities including inhibition of cellular proliferation and cytotoxicity that has been associated with induction of apoptosis. Given the important regulatory role of apoptosis in haemopoiesis, the aim of this study was to investigate the effects OS on human haemopoietic stem/progenitor cells (HSPCs). CFU-GM generated from human bone marrow (ABM) and umbilical cord blood (UCB) were grown in the presence of varying concentrations of three different OS - 7keto-cholesterol (7k-C), 7beta-hydroxycholesterol (7β-OHC) and 25-hydroxycholesterol (25-OHC). Similarly, the effect of OS on HL60 and CD34+ cells was investigated using annexin-V staining and flow cytometry to measure apoptosis. Reduction of nitroblue tetrazolium (NBT) was used to assess differentiative status of HL60 cells. CFU-GM from ABM and HL60 growth was inhibited by all three OS tested, with 25-OHC being the most potent. 25-OHC inhibited >50% of bone marrow CFU-GM and >95% of HL60 cell growth at a level of 1 µg/ml. Compared to UCB, CFU-GM derived from ABM were more sensitive to the effects of all OS tested. Only 25-OHC and 7β-OHC
significantly inhibited growth of UCB derived CFU-GM. OS treatment increased the number of annexin-V positive cells and NBT positive cells indicating that OS inhibition of CFU-GM and HL60 cell growth can be attributed to both induction of apoptosis and differentiation. These investigations have revealed that OS are a new class of inhibitors of HSPC proliferation of potential relevance in vivo and in vitro.
6.2 INTRODUCTION

Expression of the novel oxysterol binding protein homologue ORP-3 was found to be 4 – 5 fold higher in CD34+ hematopoietic cells compared to equivalent CD34- cells from adult bone marrow (ABM) and umbilical cord blood (UCB). Additionally, expression of this gene was 2-fold higher in the more primitive subfraction of cells defined by the CD34+38- phenotype, and was down regulated with proliferation and differentiation. The ORP-3 protein belongs to a family of proteins that contain a highly conserved oxysterol-binding domain (Laitinen et al 1999). Well-characterised proteins expressing this domain bind oxysterols (OS) in a dose dependent fashion (Ridgway et al 1992) and it is postulated that this binding plays a part in mediating the actions of OS on cells.

OS form a large family of hydroxylated derivatives of cholesterol that have been detected in the blood, cells and tissues of animals and humans (reviewed by Brown and Jessup 1999). They have been demonstrated to be immunosuppresive (Kucuk et al 1992, Kucuk et al 1994), and accumulation in a variety of tissues during pathological processes such as atherosclerosis (Brown and Jessup 1999), inflammation (Yui and Yamazaki 1990), trauma (Demopoulos et al 1982) oxidative stress (Kostyuk et al 1985), coronary heart disease (Zieden et al 1999), Alzheimer’s disease (Lutjohann et al 2000), and cataract formation (Girao et al 1998) has been observed. Particular attention has been paid to OS in the field of atherosclerosis research, mainly due to their abundance in lipid laden foam cells, and because they display many potent pro-atherogenic properties in vitro and in vivo (Brown and Jessup 1999). One of their pro-atherogenic properties includes cytotoxicity to vascular smooth muscle. Indeed, OS have been found to be cytotoxic toward many normal and tumour cell types (reviewed by Guardiola et al 1996).

Cytotoxicity of OS was initially thought to be due to inhibition of endogenous cholesterol biosynthesis by the transcriptional repression of several critical genes
involved in the cholesterol pathway (reviewed by Edwards and Ericsson 1998). In particular, OS inhibition of the rate limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) in actively dividing cells could result in deficient cholesterol synthesis, impaired membrane formation and hence, inhibit proliferation (Chen et al 1974). However, studies by various researchers demonstrate that the cytotoxic effects of OS do not correlate with their ability to inhibit HMG-CoAR, and are not abrogated by addition of cholesterol or mevalonate (a cholesterol intermediate) to culture media (Taylor et al 1984, Bakos et al 1993, Ayala-Torres et al 1997, O'Callaghan et al 1999). Therefore OS cytotoxicity cannot exclusively be a result of inhibition of cholesterol synthesis.

Taken together, these results demonstrate that OS are potent inducers of apoptosis capable of regulating genes and gene products important for cell viability. More recent reports also indicate that OS induce differentiation of keratinocytes (Hanley et al 2000). Given the important regulatory role of both these processes in hematopoiesis and differential expression of ORP-3 in haemopoietic progenitors, the aim of this study was to investigate the effects of several OS on the growth of HSPCs.
6.3 METHODS

PART 1: THE EFFECT OF OS ON HSPCS FROM UCB AND ABM

6.3-1 Ethics approval

All experimental procedures were conducted in accordance with the regulations and guidelines outlined by the National Health and Medical Research Council, and approved by the Deakin University (Ethics approval no. EC-82-97) and Geelong Hospital Ethics Committees (Ethics approval no. 97-14).

6.3-2 Cell sources

UCB samples were obtained after uncomplicated vaginal or cesarean delivery after clamping and cutting of the cord and drainage into 50ml sterile collection tubes containing 200 units of heparin (David Bull Laboratories) in 5mls of alpha medium (αMEM) (Trace Scientific). ABM cells were obtained from cell scrapings of discarded rib and femur heads after cardiothoracic or hip-replacement surgery, or iliac crest aspirates. ABM cell scrapings were collected into 50ml sterile collection tubes containing 200 units of heparin in 20mls of αMEM. All samples were donated by volunteers at the Geelong Hospital, Barwon Health (Geelong, Victoria), according to approved institutional guidelines.
6.3-3 Depletion of bone fragments from ABM samples

All ABM samples were received as a slurry of bone fragments and marrow in heparin/media in one or more 50ml-collection tubes. Samples were kept gently mixing at room temperature on a Nutator and processed within 24 hours of collection. Samples were mixed by vigorous inversion several times, then allowed to settle for a few minutes. When most/all of the large bone fragments had settled to the bottom of the collection tube, the supernatant containing mainly cellular material was removed into a clean/sterile collection tube. This process was repeated 3 – 5 times and cellular fragments pooled until virtually all haemopoietic cells were removed from the original ABM slurry (samples appear white in color). Pooled ABM samples depleted of bone fragments, were then spun at 480g for 5 minutes at 18 – 20°C. Supernatants were removed, cell pellets combined and made up to approximately 40mls (dependant on number of cells present) in PBS containing 2mM EDTA and 0.5% BSA (Sigma Aldrich).

6.3-4 Red Cell Depletion of UCB samples

UCB sample was diluted 1: 2 with PBS, mixed thoroughly by gentle inversion centrifuged for 25 minutes at 480g and 18 – 20°C. Centrifuge was set at low brake. After centrifugation, the lower density white blood cells were seen as a buff-colored carpet on top of the denser red cells. These were removed using a cannula and syringe and placed in a sterile 50-ml tube. Red cell depleted UCB samples were made up to a volume approximately equal to the original UCB sample with PBS.
6.3-5 Preparation of Mononuclear cells from UCB and ABM samples

UCB and ABM samples depleted of red cells and bone fragments respectively were carefully and gently layered onto an equal volume of Ficoll-Hypaque\textsuperscript{R} (Amersham Pharmacia Biotech) in 15 or 50ml sterile tubes. Great care was taken to ensure that there was minimal/no mixing of cell samples with Ficoll-Hypaque\textsuperscript{R}. Samples were centrifuged for 25 minutes at 440g at 18 – 20°C. Centrifuge was set to low brake. After centrifugation, the upper plasma/media layer was drawn off using a clean pasteur pipette. MNCs at the ficoll-plasma interface were carefully removed using a sterile 5 or 10-ml syringe and cannula. MNCs were washed twice with the addition of two volumes of PBS and centrifugation at 400g for 10 minutes at 18 – 20°C. After the second wash, the supernatant was removed the MNCs resuspended and viability and cell number was assessed using trypan blue staining. MNCs to be used for CFU-GM studies (5.3-7) were resuspended in αMEM (Trace Scientific) to a final concentration of 2.3 x 10\textsuperscript{5} cells/ml. MNCs to be used for immuno-magnetic enrichment of CD34\textsuperscript{+} cells (5.3-6) were resuspended in to a final in 300µl PBS/0.5%BSA/2mMEDTA per 1 x 10\textsuperscript{8} cells (minimum 300µl).

6.3-6 Immuno-magnetic separation of CD34\textsuperscript{+} cells from UCB and ABM MNCs

CD34\textsuperscript{+} cells were isolated from UCB and ABM MNCs using antibody labeling and a MiniMacs bead separation kit (Miltenyi Biotec) following the manufacturers instructions. Degassed PBS/0.5%BSA/2mMEDTA was used for washing VarioMacs and MiniMacs columns (Miltenyi Biotec) and MNCs. MNCs were resuspended in 500µl/10\textsuperscript{8} of 500units/ml DNase1 (brand, manufacturer) before separation on a VarioMacs (for 2 – 20 x 10\textsuperscript{8} MNCs) or MiniMacs column (for \( \leq 2 \times 10^8 \) MNCs). Immuno-magnetically-labeled CD34\textsuperscript{+} cells were eluted from the column with 2 – 5mls degassed PBS/0.5%BSA/2mMEDTA. Viability and cell number of the CD34
enriched and depleted cell populations was assessed using trypan blue (Sigma Aldrich) exclusion and manual cell counting.

6.3-7 Flow cytometric analysis of CD34 populations

Approximately 0.25 x 10⁶ cells from whole cord blood, ABM MNCs and CD34 depleted samples, and 0.01 x 10⁶ CD34⁺ cells were labeled with FITC-conjugated anti-CD45 and PE-conjugated anti-CD34 (Beckman Coulter) according to manufacturers instructions. The percentage of CD34⁺ cells in each sample was determined by flow cytometric analysis (FacsCalibur, Becton Dickinson) following modified ISHAGE guidelines (Sutherland et al 1996a) using CELLQuest software (Becton Dickinson) (see Chapter 2, Figure 2.1). Equivalent numbers of cells labeled with FITC-conjugated anti-IgG₁ and PE-conjugated anti-IgG₁ were used as negative controls for the analysis.

6.3-8 OS treatment of Colony-forming unit granulocyte-macrophage (CFU-GM) growth

For all experiments, 25-OHC, 7β-OHC and 7α-C (Sigma Aldrich) stock solutions were freshly prepared in ethanol as previously described (Lizard et al 1996). MNCs from ABM and UCB were plated at a concentration of 2 x 10⁴/ml in methylcellulose medium containing 0.9% methylcellulose in Iscoves MDM, 30% Fetal Bovine Serum (FBS), 1% BSA, 10⁻⁴M 2-Mercaptoethanol, 2mM L-Glutamine, 50ng/ml rh Stem Cell factor, 10ng/ml rh GM-CSF, 10ng/ml rh IL-3 (Stem Cell Technologies, Vancouver, Canada) and various concentrations of OS (see section 6.4-1). All treatments and controls contained an equal amount of carrier (ethanol at 0.5%) and were performed in duplicate. Each sample was plated into 8 wells of a 24 well standard tissue culture plate. Plates were incubated at 37°C in a CO₂ incubator.
Colonies (> 40 cells/aggregate) were assessed using an inverted microscope after 14 days.

6.3-9 OS treatment of CD34+ cells

Isolated CD34+ cells were cultured for 96 hours at 37°C in a CO2 incubator, at a concentration of 0.5 x 10^6 cells/ml in RPMI 1640 (Trace Scientific) with 10% FBS (CSL Biosciences), 2mM L-Glutamine and 200U/ml Penicillin/Streptomycin (Sigma Aldrich). 25-OHC was added to treatment tubes at a final concentration of 2μg/ml in media. All treatments and controls contained an equal amount of carrier (ethanol at 0.5%). At various time points (see results), 0.1 x 10^5 cells were removed from culture and the percentage of cells undergoing apoptosis determined by flow cytometry (6.3-10).

6.3-10 Flow cytometric evaluation of the percentage of CD34+ cells undergoing apoptosis

Cells were resuspended to a final concentration of 0.25 x 10^5 cells/ml in annexin-V binding buffer (Pharmingen) and labeled with PE-conjugated CD34, 2.5μg/ml 7-Amino-actinomycin D (7AAD) (Molecular Probes, Eugene OR.) and FITC-conjugated annexin-V according to manufacturers instructions (Pharmingen). The percentage of viable (7-AADneg), CD34+ (side scatterlo, forward scatterlo) apoptotic cells (annexin-V-FITCpos) was determined by flow cytometric analysis (FacsCalibur, Becton Dickinson) using CELLQuest software (Becton Dickinson) (Figure 6.1).
Figure 6.1 Flow cytometric evaluation of the percentage of CD34⁺ haemopoietic cells undergoing apoptosis
Plot 1. Total events
R1 = Gate 1 (all cellular events)

Plots 2, 3 and 4. Gate = R1
These dot plots are not part of the analysis. They are to check cytometer settings for the gating strategy only.
Plot 4. Ungated
Selection of CD34+/lo side scatter events

Plot 5. Gate = R2 (CD34+/lo side scatter)
LL = Viable non-apoptotic CD34+/lo side scatter cells
LR = Viable apoptotic CD34+/lo side scatter cells
UR = Non-viable CD34+/lo side scatter events
PART 2: THE EFFECT OF OS ON HL60 CELLS

6.3-11 Cell lines

Two stable transfected subclones of the human promyelocytic leukemia cell line HL60 were used: HL60 cells transfected and over-expressing human bcl-2 (HL60-bcl2) and HL60 cells transfected with vector alone (HL60-v) (generously supplied by Dr. G. Vairo). The cells were maintained in RPMI 1640 (Trace Scientific) supplemented with 10% FBS (CSL Biosciences), 200U/ml Penicillin/Streptomycin and 2mM L-Glutamine (Sigma Aldrich). Additionally, all cultures were supplemented with 2μg/ml Puromycin (Sigma Aldrich) to ensure stable vector transfection. HL60-v and HL60-bcl2 cells were maintained in asynchronous exponential growth for all experiments.

6.3-12 OS treatment of HL60-v & HL60-bcl2 cells

Exponentially growing cells were split to a concentration of 0.5 x 10^6 cells/ml in media and treated with various concentrations of 25-OHC, 7β-OHC and 7α-C for 96 hours. Cells were cultured in a CO2 incubator at 37°C. All treatments and controls contained an equal amount of carrier (ethanol at 0.5%) and were performed in duplicate. At various time points (see Results), cell number, viability, differentiative status and the percentage of cells undergoing apoptosis were assessed using trypan blue exclusion, NBT reduction and flow cytometry.
6.3-13 Assessment of differentiative status of HL60-v cells by Nitroblue Tetrazolium (NBT) reduction

Differentiative status HL60-v cells was assessed by the ability to reduce NBT (Sigma Aldrich) as previously described (Blair et al 1985). Briefly, 250μl of cell suspension (at approximately 0.5 x 10^6 cells/ml) was mixed with an equal volume of 0.1% NBT in PBS. 5μl of 100μg/ml Phorbol 12-Myristate 13-Acetate (PMA) (Sigma Aldrich) in Dimethyl-sulphoxide (DMSO) (Sigma Aldrich) was added, and the cells incubated at 37°C 20 minutes. The percentage of NBT positive (cells containing dark/blue granules) was assessed using manual cell counting.

6.3-14 Flow cytometric evaluation of the percentage of HL60-v cells undergoing apoptosis

Cells were resuspended to a final concentration of 1 x 10^6/ml in Annexin Binding Buffer and labeled with 5.0μg/ml Propidium Iodide (PI) (Molecular Probes) and FITC-conjugated annexin-V according to manufacturers instruction (Pharmingen). The percentage of viable (PI^-) apoptotic cells (annexin-V-FITC^+) was determined by flow cytometric analysis (Faescalibur, Becton Dickinson) using CELLQuest software (Becton Dickinson) (see Chapter 5, Figure 5.2).

6.3-15 Statistics

All results are expressed as (mean ± SEM). We utilized One Way Analysis of Variance (Tukeys and Tamhanes Post-Hoc tests) for statistical analysis of multiple samples from the same source, Two Way Analysis of Variance (Tukeys and Tamhanes Post-Hoc tests) for statistical analysis of multiple samples from different
sources, and Paired and Non-Paired T-Test to determine differences between two related and non-related groups (respectively). All statistical analyses were performed using SPSS (Statistics Package for the Social Sciences) version 10.0 software (Fullerton, CA, U.S.A.). In all instances, $p \leq 0.05$ was considered statistically significant.
6.4 RESULTS

PART 1: THE EFFECT OF OS ON HSPCS FROM UCB AND ABM

6.4-1 The effect of OS on CFU-GM growth

To investigate the effects of OS on HSPC growth and proliferation, we examined
growth of CFU-GM in the presence of 25-OHC, 7β-OHC and 7κ-C. All results are
expressed as a percentage of control to normalise for the variation in colony growth
from UCB and ABM. OS dose dependently inhibited growth of ABM CFU-GM with
a significant approximately 50% reduction at 0.8μg/ml for 25-OHC (p ≤ 0.001, n = 8), 2.5μg/ml for 7β-OHC (p ≤ 0.01, n = 8) and 4.2μg/ml for 7κ-C (p ≤ 0.004, n = 8).
CFU-GM growth from UCB was also inhibited by all OS tested, however,
statistically significant inhibition was observed in the presence of 25-OHC and 7β-
OHC only. 25-OHC was able to inhibit approximately 50% of UCB CFU-GM
growth at a concentration of 1.7μg/ml (p ≤ 0.001, n = 8). Interestingly, for all OS
tested, the inhibition of ABM colony formation was greater than for UCB, indicating
the cells from the UCB were more resistant (n = 8, p ≤ 0.001, 0.001, 0.02 for 7β-
OHC, 7κ-C and 25-OHC respectively) (Figure 6.2a, b, c and d).

As the reported plasma levels of 25-OHC are significantly lower than those reported
for 7β-OHC and 7κ-C (Brown and Jessup 1999, Babiker and Diczfalusy 1998,
Dzeletovic et al 1995), we also investigated the effect of 0 – 1μg/ml 25-OHC on
CFU-GM growth. Our results confirm inhibition of CFU-GM growth by 25-OHC
over doses that more closely approximate physiological values (Figure 6.2d) with
statistically significant inhibition at 0.5 and 1.0μg/ml for ABM and UCB
respectively (p ≤ 0.04, n = 12). Again UCB cells appeared more resistant than the
equivalent cells from ABM.
Figure 6.2 The effect of OS on UCB and ABM CFU-GM growth.

UCB and ABM CFU-GM were grown in the presence of various concentrations of OS (as indicated). Colonies were counted on Day 14 and data is expressed as % of control to normalise for variation in CFU-GM growth from UCB and ABM.

a) $\beta$-OHC induced a dose dependent decrease in CFU-GM growth from UCB ($56.7 \pm 10.2\%\ n=8,\ p \leq 0.02$ at $6.7\mu g/ml$, One-way ANOVA) and ABM ($62.8 \pm 7.7\%\ n=8,\ p \leq 0.01$ from $1.7\mu g/ml$, One-way ANOVA). ABM CFU-GM growth was significantly more sensitive to the inhibitory effect of $\beta$-OHC than UCB CFU-GM ($n=8,\ p \leq 0.001$, Two-way ANOVA).

b) 7-κC induced a dose dependent decrease in CFU-GM growth from UCB ($n=8$), and ABM ($70.9 \pm 4.9,\ n=8,\ p \leq 0.004$ at $1.7\mu g/ml$, One-way ANOVA). ABM CFU-GM growth was significantly more sensitive to the inhibitory effect of 7-κC than UCB CFU-GM ($n=8,\ p \leq 0.001$, Two-way ANOVA).

c) 25-OHC induced a dose dependent decrease in CFU-GM growth from UCB ($47.0 \pm 10.6,\ n=8,\ p \leq 0.001$ at $1.7\mu g/ml$, One-way ANOVA), and ABM ($51.4 \pm 4.6\%,\ n=8,\ p \leq 0.001$ at $0.84\mu g/ml$, One-way ANOVA). ABM CFU-GM growth was significantly more sensitive to the inhibitory effect of 25-OHC than UCB CFU-GM ($n=8,\ p \leq 0.02$, Two-way ANOVA).

d) 25-OHC induced a dose dependent decrease in CFU-GM growth from UCB ($66.1 \pm 6.0\%,\ n=12,\ p \leq 0.001$ at $1.0\mu g/ml$, One-way ANOVA), and ABM ($65.1 \pm 6.8\%,\ n=12,\ p \leq 0.04$ at $0.5\mu g/ml$, One-way ANOVA).
Figure 6.2 (cont)
6.4-2 OS induce apoptosis of CD34\(^+\) cells

The majority of hematopoietic progenitors responsible for CFU growth are contained within the CD34 expressing cell fraction of UCB and ABM (Krause et al 1996). To confirm the hypothesis that impaired survival of haemopoietic progenitors contributes to the observed decreased in CFU-GM growth, purified CD34\(^+\) HSPC were cultured with 25-OHC and the percentage of cells undergoing apoptosis was analysed by annexin-V staining performed simultaneously with 7-AAD. This method is commonly used to detect early apoptotic cells (Koopman et al 1994) and allows discrimination between apoptosis and necrosis (Majno and Joris 1995). Results indicate that after 48 hours there was a significant increase in the percentage of OS treated cells undergoing apoptosis compared to paired controls (4.9 ± 0.6\% versus 3.7 ± 0.4\%, \(n = 5\), \(p \leq 0.01\)). By 72 hours, the percentage of OS treated cells undergoing apoptosis had increased to approximately twice that in controls (3.4 ± 0.2\% Vs 6.5 ± 0.6\%, \(p \leq 0.001\)) (Figure 6.3).
Figure 6.3 The effect of OS on CD34$^+$ HSPCs from UCB as measured by annexin-V staining and flow cytometry

25-OHC (2μg/ml) induced an increase in the percentage of viable CD34$^+$ apoptotic cells (annexin-V$^{\text{pos}}$, 7AAD$^{\text{neg}}$, side scatter$^{\text{lo}}$, forward scatter$^{\text{lo}}$) compared to controls (4.87 ± 0.55 versus 3.68 ± 0.43 at 48 hours, p ≤ 0.01, n = 5, Two-way ANOVA).
6.4-3 OS inhibit HL60-v cell number and viability in a dose dependent fashion

To further investigate the effect of close to physiological doses of OS on hematopoietic cells, the human pro-myelocytic cell line HL60-v was utilized (Collins 1987). 7κ-C, 7β-OHC and 25-OHC significantly inhibited growth of HL60-v cells in a dose dependent fashion when compared to controls (Figure 6.4 a, b and c). At 96 hours, approximately 50% (or greater) inhibition of cell growth by 5.0 μg/ml 7β-OHC (0.40 ± 0.02 x 10⁶ cells/ml versus 1.46 ± 0.12 x 10⁶ cells/ml, p ≤ 0.001, n = 5), 5.0 μg/ml 7κ-C (1.13 ± 0.11 x 10⁶ cells/ml versus 1.85 ± 0.01 x 10⁶ cells/ml, p ≤ 0.001, n = 5) and 0.25μg/ml 25-OHC (0.81 ± 0.01 x 10⁶ cells/ml versus 2.05 ± 0.21 x 10⁶ cells/ml, p ≤ 0.001, n = 5) was observed. This decrease in cell growth was accompanied by a comparable decrease in cell viability (results not shown). As in the clonogenic experiments, 25-OHC was found to have the most potent inhibitory effect on cell growth and viability, with 1.0ug/ml resulting in total inhibition at 96 hours compared with 45 and 15% inhibition for equivalent doses of 7κ-C and 7β-OHC respectively (Figure 6.4a, b and c).
Figure 6.4 The effect of OS on HL60-v cell growth

HL60-v cells were cultured in the presence of various concentrations of OS (as indicated). Cell number was assessed at the times indicated.

a) 7β-OHC induced a dose dependent decrease in HL60-v growth (n= 5, p ≤ 0.009 at 1.0μg/ml, One-way ANOVA).

b) 7-αC induced a dose dependent decrease in HL60-v growth (n = 5, p ≤ 0.03 at 2.5μg/ml, One-way ANOVA).

c) 25-OHC induced a dose dependent decrease in HL60-v growth (n = 5, p ≤ 0.01 at 0.5μg/ml, One-way ANOVA).
6.4-4 OS induce apoptosis in HL60-v cells in a dose dependent fashion

To test whether the observed inhibition of HL60-v growth could be attributed, at least in part, to an increase in apoptosis, flow cytometry and annexin-V staining was utilized to measure the percentage of viable cells (PI\textsuperscript{neg}) undergoing apoptosis. At 48 hours, treatment with 0.5\(\mu\)g/ml 25-OHC (8.9 ± 0.5% versus 1.8 ± 0.3%, p ≤ 0.001, n = 5), 5.0\(\mu\)g/ml 7\(\alpha\)-C (2.8 ± 0.3 versus 0.9 ± 0.2, p ≤ 0.03, n = 5) and 5.0\(\mu\)g/ml 7\(\beta\)-OHC (5.6 ± 1.3% versus 1.1 ± 0.1%, p ≤ 0.03 n = 5) yielded a 5, 3 and 5.5-fold significant increase (respectively) in the percentage of apoptotic cells compared to controls (Figure 6.5).
Figure 6.5 The Effect of OS on HL60-v cells as measured by annexin-V staining and flow cytometry.

At 48 hours, 0.5µg/ml 25-OHC (8.9 ± 0.5% versus 1.8 ± 0.3%, p ≤ 0.001, n = 5, Paired Students T-Test), 5.0µg/ml 7κ-C (2.8 ± 0.3 versus 0.9 ± 0.2, p ≤ 0.03, n = 5, Paired Students T-Test) and 5.0µg/ml 7β-OHC (5.6 + 1.3% versus 1.1 + 0.1%, p ≤ 0.027 n = 5, Paired Students T-Test) induced an increase in the percentage of viable apoptotic cells (annexin-V<sup>pos</sup>, PI<sup>neg</sup>) compared to controls.
6.4-5 OS induce differentiation of HL60-v cells

In response to specific external stimuli, HL60 cells can be induced to differentiate into granulocyte, monocyte or macrophage-like cells (Collins 1987, Ahmed et al 1991). NBT staining is commonly used to detect reactive oxygen species formed by granulocytes in the presence of endotoxin (Harris and Ralph 1985). At 48 hours, 0.5ug/ml 25-OHC (0.44 ± 0.16 versus 0.09 ± 0.02 x 10^5 cells/ml, n = 4, p ≤ 0.005), 5.0ug/ml 7β-OHC (0.45 ± 0.15 versus 0.04 ± 0.04 x 10^5 cells/ml, n = 4, p ≤ 0.007), and 5.0ug/ml 7α-C (0.38 ± 0.12 versus 0.06 ± 0.01 x 10^5 cells/ml, n = 4, p ≤ 0.05), showed a 5, 6 and 11-fold significant increase (respectively) in NBT positive cells compared to control. (Figure 6.6). This data demonstrates that these OS are capable of inducing differentiation of the pro-myelocytic cell line along a granulocytic path, and indicates that OS induced decrease in cell growth of HL60-v cells can be partially attributed to an increase in differentiation.
Figure 6.6 The Effect of OS on HL60-v cells as measured by NBT reduction.

HL60-v cells were cultured in the presence of various concentrations of OS (as indicated). At 48h the capacity of cells to reduce NBT was assessed and total cell number counted.

a) 7-kC induced an increase in NBT positive HL60-v cells at 48 hours (0.38 ± 0.12 versus 0.06 ± 0.01 x 10^5 cells/ml at 5.0μg/ml, n = 4, p ≤ 0.05, One-way ANOVA).

b) 7β-OHC induced an increase in NBT positive HL60-v cells at 48 hours (0.45 ± 0.15 versus 0.04 ± 0.04 x 10^5 cells/ml at 5.0μg/ml, n = 4, p ≤ 0.007, One-way ANOVA).

c) 25-OHC induced an increase in NBT positive HL60-v cells at 48 hours (0.44 ± 0.16 versus 0.09 ± 0.02 x 10^5 cells/ml at 0.5μg/ml, n = 4, p ≤ 0.005, One-way ANOVA).
6.4-6 Over expression of bcl-2 significantly reduces OS effects on HL60-v cells

A HL60 cell line has been utilised to investigate the effect of over expression of the apoptosis inhibitory protein bcl-2 (Vaux et al 1988, Vaux et al 1992) on OS induced apoptosis. The constitutive over expression of bcl-2 in cells affects progression of the cell cycle (Mazel et al 1996, Linette et al 1996). As a result, the HL60-bcl2 cells used in this experiment have a significantly increased growth rate and viability when compared to the null vector cell line HL60-v (results not shown). All results are therefore presented as percent of control to enable direct comparison between OS induced cytotoxicity of HL60-v and HL60-bcl2 cells. Results indicate that over-expression of bcl-2 partially abrogated the OS induced decrease in cell growth (Figure 6.7a) and viability (Figure 6.7b) for all three OS tested. This abrogation was statistically significant for all three OS tested.
Figure 6.7 The effect of OS on HL60-v and HL60-bcl2 cell growth and viability

a) Over expression of bcl-2 in partially abrogated the OS induced decrease in viability after 48 hours culture in the presence of 1μg/ml 25-OHC (68.7 ± 5.7% versus 82.2 ± 4.4%, n = 5, p ≤ 0.05, Independent Samples T-test), 4μg/ml 7-κC (91.7 ± 1.3% versus 99.5 ± 0.8%, n = 5, p ≤ 0.03, Independent Samples T-test) and 4μg/ml 7β-OHC (90.7 ± 1.6% versus 100.4 ± 0.5%, n=5, p ≤ 0.008, Independent Samples T-test).

b) Over expression of bcl-2 partially abrogated OS induced inhibition of cell growth after 48 hours culture in the presence of 1μg/ml 25-OHC (24.8 ± 5.0 versus 38.4 ± 6.8 x 10⁶ cell/ml, n = 5, p ≤ 0.05, Independent Samples T-test), 4μg/ml 7-κC (72.8 ± 6.1 versus 90.1 ± 5.9 x 10⁶ cell/ml, n = 5, p ≤ 0.05, Independent Samples T-test) and 4μg/ml 7β-OHC (54.0 ± 2.8 versus 93.9 ± 9.1 x 10⁶ cell/ml n=5, p ≤ 0.003, Independent Samples T-test).
A

B

25-OHC  7-kC  7b-OHC

25-OHC  7-kC  7b-OHC

Mean Viability (% of control)

Mean Cell No. (% of control)
6.4-7 Over expression of bcl-2 significantly reduces OS induced apoptosis of HL60-v cells

We utilized annexin-V staining to investigate whether the decreased sensitivity of HL60-bcl2 cells to OS cytotoxicity is due to a decrease in the percentage of cells undergoing apoptosis. As the basal rate of apoptosis in these two cell systems is significantly different (results not shown), results were normalized by subtracting the percentage of annexin-V$^{\text{pos}}$ cells in the control, from the value obtained after OS treatment. In this manner, apoptotic rate between HL60-v and HL60-bcl2 can be directly compared. Our results indicate that after 24 hours treatment with $1\mu\text{g/ml 25-OHC}$, there was a 4-fold difference in the percentage of viable (P$^{\text{neg}}$) apoptotic HL60-v and HL60-bcl2 cells ($0.13 \pm 0.07\%$ versus $0.60\% \pm 0.17\%$ n = 5, p<0.001). These results indicate that bcl-2 induced resistance to 25-OHC can be attributed, at least in part, to a significant decrease in the percentage of apoptotic cells (Figure 6.8). Although it cannot be stated definitively, it is possible that bcl-2 induced resistance to the apoptotic effects of the OS 7\kappa-C and 7\beta-OHC occurs by the same mechanism.
Figure 6.8 The effect of OS on the percentage of apoptotic HL60-v and HL60-bcl2 cells as measured by annexin-V staining and flow cytometry.

Over expression of bcl-2 partially abrogated the percentage of HL60 cells undergoing apoptosis in the presence of 1μg/ml 25-OHC (0.13 ± 0.07% versus 0.60 ± 0.17% at 24 hours, n = 5, p ≤ 0.001, Independent Samples T-test).
6.5 DISCUSSION

Normal haemopoiesis is characterised by a dynamic balance between the proliferation and differentiation of HSPCs (Morrison et al 1995). To maintain homeostasis, apoptosis plays the important role of removing redundant cells (McKenna and Cotter 1997, Park 1996). Dysregulation of differentiation and apoptosis has been implicated in many haematological disorders (McKenna and Cotter 1997, Yoshida and Mufti 1999, Maru 2001, Philpott et al 1995, Dive and Hickman 1991), and has frustrated efforts to ex vivo expand stem cells for transplantation (Traycoff et al 1998). The identification of substances capable of triggering or modulating both these processes is important to our understanding of haemopoiesis.

The results of this study demonstrate that OS significantly inhibit clonogenic growth of HSPCs from UCB and ABM. Although it cannot be said definitively which CD34^+ cells are responsible for clonogenic growth, results indicate that OS inhibition of clonogenic growth is at least partially due to an increase in apoptosis of human CD34^+ progenitor cells (Krause et al 1996). Given our observation of OS induced differentiation of HL60 cells, it may also be due to an increase in differentiation of HSPCs and hence loss of proliferative potential (Boyd and Metcalf 1984). The observation that the OS 7β-OH-C and 7k-C are potent stimulators of IL-1β secretion (Lizard et al 1998) and TGFβ transcription (Leonarduzzi et al 2001) indicates that OS may also affect growth of haemopoietic progenitors by modulating the release of inhibitory cytokines from accessory cells. Finally, a recent study by Tamasawa et al (2001) demonstrating 7k-C induced release of vascular cell adhesion molecule-1 from human umbilical vein endothelial cells raises the possibility that OS may modulate HSPC function by affecting the expression of surface adhesion molecules.
The types and levels of OS reported in human plasma vary widely and have been reported to be between $1 - 4700\text{nmol/L (0.4ng/ml - 1.9μg/l)}$ for 7κ-C, $2 - 2500\text{nmol/L (0.8ng/ml - 1.0μg/ml)}$ for 7β-OHC and $0 - 27.5\text{nmol/L (0 - 1ng/ml)}$ for 25-OHC (Babiker and Diczfalusy 1998, Dzeletovic et al 1995, reviewed by Brown and Jessup). 7κ-C inhibited ABM CFU-GM growth at levels within the reported physiological range for plasma, and 7β-OHC at approximately twice the plasma levels quoted in literature. However, circulating plasma concentrations may not be an accurate indicator of OS concentrations in the HSPC microenvironment. Additionally, standard conditions for CFU-GM growth include addition of growth factors to the media that are above physiological levels. It is therefore possible that under more limited growth factor conditions, OS inhibition would be more potent. Ares et al (2000) observed enhanced cytotoxicity of 25-OHC in smooth muscle cells with addition of tumour necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) suggesting that the presence of inhibitory cytokines may also potentiate OS induced apoptosis.

Interestingly, compared to UCB, the growth of ABM CFU-GM was more sensitive to the effects of all the OS tested. The CD34$^+$ cells from these two sources are known to exhibit various differences in their in vitro and in vivo characteristics. Compared to ABM, UCB CD34$^+$ cells contain a higher number of clonogenic cells in short and long term cultures with a higher re-plating potential and ex vivo proliferative potential (reviewed by Cairo and Wagner 1997). Additionally, it has been demonstrated that single UCB HSPCs are much less sensitive to the inhibitory effects of the suppressive cytokines TGFβ and macrophage inhibitory protein-alpha (MIP1α) (Lu et al 1993, de Wynter et al 1998a). It is therefore possible that the decreased sensitivity of UCB HSPCs to apoptotic and differentiating factors such as OS may contribute to their increased clonogenicity. Additionally, it has been demonstrated that OS are most potent added in vitro when cells are exponentially growing (Beseme et al 1986) and at G2/M (Astruc et al 1983). Thus it is equally possible that the increased sensitivity of ABM CFU-GM is a reflection of their faster entry into cell cycle in culture (Lu et al 1993). Interestingly, the CD34$^+$ cells
from UCB express higher levels of the OS binding protein homologue ORP-3 than equivalent cells from ABM (Gregorio-King et al 2001). A link between the relative resistance of UCB cells to OS, and differential expression of ORP-3 is at this time, purely speculation.

The study of the effects of OS on haemopoietic progenitors has been extended by utilizing the human pro-myelocytic cell line HL60. Results indicate that 7κ-C, 7β-OHC and 25-OHC significantly inhibit growth, induce apoptosis and increase granulocytic differentiation of HL60 cells. Although significant inhibition of cell growth was observed at concentrations lower than those required to induce differentiation, it is difficult at this stage to de-lineate between an increase in apoptosis as a result of differentiation (Collins 1987), or as a result of a separate apoptotic program in these cells (Park et al 1994). The observed OS induced apoptosis of CD34⁺ progenitor cells indicates however that OS are capable of both apoptosis and differentiation. Additional experiments to address the mechanism by which different OS induce differentiation and their ability to induce monocytic differentiation are in progress.

Two previous studies by Aupeix et al (1995, 1997) investigated the effects of 4 – 12µg/ml 7β-OHC and 25-OHC only on HL60 growth (Table 6.1). This study demonstrated inhibition of cell growth at doses approximately 8 – 40 fold less than those used in previous studies, and which are closer to levels reported in plasma. Additionally, we have demonstrated that OS induced decrease in cell growth is partially abrogated by over expression of the anti-apoptotic protein bcl-2. This is in line with observations by Lizard et al (1997) and Harada et al (1997) utilizing a human promonocytic leukemic cell line and murine macrophage-like cells, and supports the hypothesis that OS induced cell death involves two pathways, “bcl-2 inhibitable and bcl-2 uninhibitable”. Further studies are required to investigate whether other OS such 7κ-C and 7β-OHC invoke cell death by a similar pathway.
It is speculated that OS are generated endogenously \textit{in vivo} by simple autoxidation of cholesterol (non-enzymic) or by enzymatic reactions involving specific hydroxylases. Tissues and cells known to express hydroxylases include the liver, brain and macrophages (Brown and Jessup 1999). Additionally, preliminary results within our laboratory indicate that 25-hydroxylase is expressed in primary cultured BM stroma, and that expression of this enzyme is modulated by cytokines (unpublished data). This raises the possibility that OS production by the stroma may occur enzymatically, and that haemopoietic growth factors and inhibitors may physiologically regulate enzymic production.

It is postulated that the actions of OS are mediated by binding to oxysterol binding proteins (Ridgway et al 1992). Previous work carried out as part of this dissertation has revealed that gene expression of one of these proteins, ORP-3 is up regulated in CD34\(^+\) HSPCs (Chapter 4). In this study it has been demonstrated that OS inhibit CFU-GM growth, and induce apoptosis of CD34\(^+\) and HL60 cells. Additionally, data indicates that they induce granulocytic differentiation of HL60 cells. Together, these findings suggest that OS may be important regulators of HSPC cell growth both \textit{in vitro} and \textit{in vivo}. Further studies are required to elucidate the mechanisms of OS induced differentiation and apoptosis.
Table 6.1. Previous studies demonstrating the apoptotic effects of OS on haemopoietic cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>OS</th>
<th>Concentration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7k-C</td>
<td>300nm (0.12ug/ml)</td>
<td>Johnson et al 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7b-OHC</td>
<td>30uM (12ug/ml)</td>
<td>Aupeix et al 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7k-C</td>
<td>5-80ug/ml (12.5 - 200uM)</td>
<td>Lizard et al 1997 &amp; 1998</td>
</tr>
<tr>
<td>HL60</td>
<td>Pro-myelocytic</td>
<td>7b-OHC</td>
<td>10 - 30uM (4 - 12ug/ml)</td>
<td>Aupeix et al 1995 &amp; 1997</td>
</tr>
<tr>
<td>THP-1</td>
<td>Macrophage</td>
<td>25-OHC</td>
<td>5ug/ml (12.5uM)</td>
<td>Rusinol et al 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25-OHC</td>
<td>30uM (12ug/ml)</td>
<td>Aupeix et al 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7b-OHC</td>
<td>30uM (12ug/ml)</td>
<td>Aupeix et al 1997</td>
</tr>
<tr>
<td>K562</td>
<td>Myeloid</td>
<td>7b-OHC</td>
<td>5 - 30uM (2 - 12ug/ml)</td>
<td>Hyun et al 1997</td>
</tr>
<tr>
<td>Murine lymphocytes</td>
<td>7b-OHC</td>
<td>5 - 25uM (2 - 10ug/ml)</td>
<td>Hyun et al 1997</td>
<td></td>
</tr>
<tr>
<td>RDM4</td>
<td>Murine lymphoma</td>
<td>7b-OHC</td>
<td>5 - 30uM (2 - 12ug/ml)</td>
<td>Hyun et al 1997</td>
</tr>
</tbody>
</table>
7.1 THE IDENTIFICATION OF NOVEL GENES EXPRESSED IN HAEMOPOIETIC PROGENITOR CELLS USING DD-PCR.

The first aim of this dissertation was the identification of novel genes expressed in haemopoietic progenitor cells. The identification of ORP-3 and MERP-1 using dd-PCR demonstrates that this technique can be used successfully to isolate novel gene sequences which are differentially expressed in CD34 enriched and depleted cell populations. It is therefore also possible that this technique could be used to isolate novel genes expressed in a more primitive population of progenitors such as CD34^-38^- HSPCs. Additional preliminary experiments performed also indicate that differentially expressed gene sequences can be isolated from CD34^+ cells before and after incubation with cord serum. Further investigation of these partial sequences could reveal information regarding the mechanisms by which cord serum enhances the proliferation and expansion of UCB progenitors.
7.2 IDENTIFICATION OF MERP-1, A NOVEL MAMMALIAN EPENDYMIN RELATED PROTEIN GENE EXPRESSED IN CD34+ PROGENITOR CELLS

A partial sequence for MERP-1 was identified in CD34+ hematopoietic progenitor cells, with low expression in CD34- hematopoietic cells. The complete MERP-1 cDNA sequence was obtained by bioinformatics, RACE and PCR. Theoretical translation of the MERP-1 ORF reveals a protein with significant homology to an ependymin family of proteins that until recently, have only been described in teleost fish (Hoffmann and Schwarz 1996). Indeed, MERP-1 is the first ependymin like gene sequence identified in mammals (Nimmrich et al 2001). Additional mammalian homologues have subsequently been identified and it appears that MERP-1 belongs to a family of homologous gene sequences (Apostolopolous et al 2001). There are however significant differences between the MERP-1 gene and these additional mammalian homologues, which raises the possibility that they may not be complete and correct as they currently exist in the GenBank database. Additional work is planned to clarify this and enable confident characterisation of the complete gene family of mammalian ependymin related proteins in mammals.

Investigation of MERP-1 gene expression indicates higher expression in a population of HSPCs defined by expression of the CD34 phenotype. Additionally, expression in CD34+ cells is down regulated with differentiation and proliferation of these cells. Interestingly, Nimmrich et al (2001) identified a partial sequence for MERP-1 due to higher expression in colon cancer compared to normal colon. Taken together, MERP-1 expression data indicates higher expression in a less differentiated subset of cells. The physiological significance of this differential expression and the function of MERP-1 in these cell populations requires further investigation. Specifically, investigation of MERP-1 gene expression in normal and malignant tissues, and in the more primitive populations of haemopoietic progenitor and embryonic stem cell populations as they are stimulated to differentiate along specific pathways may provide further information regarding the role of the MERP-1 gene. Studies
investigating regulation of MERP-1 gene expression in haemopoietic cells by growth factors and inhibitory cytokines are also in progress.

On the basis of protein bioinformatics, it appears that the putative MERP-1 protein is a Type II transmembrane glycoprotein. The C-terminal extracellular region has significant homology to ependymins and contains glycosylation sites that may render calcium-binding capabilities, and hence possible calcium dependent adhesion properties (Ganss et al 1993). The putative intracellular N-terminal region of MERP-1 has significant homology to the cytoplasmic domains of three members of the protocadherin family of Type I transmembrane glycoproteins. This region is postulated to function in intracellular signaling (Angst et al 2001). It is therefore possible that MERP-1, like its teleost homologues and protocadherins, plays a role in calcium dependent cell adhesion. As teleost ependymins have been demonstrated to activate the transcription factor AP-1 (Shashoua et al 2001), MERP-1 mediated cell adhesion may ultimately affect the differentiation, proliferation and survival of cells (Liebermann et al 1998). Given the important role these processes play in the regulation of haemopoiesis, MERP-1 functional studies should be conducted to examine the role of the MERP-1 in HSPC adhesion and homing to the stroma, colony formation, differentiation and proliferation.

Production of the MERP-1 protein and characterisation of its physical properties is the crucial next step. This will not only confirm the translated product of the MERP-1 gene, but will also allow the investigation of other proteins that MERP-1 may associate with and activate. Subsequent production of a MERP-1 antibody will allow western blotting for characterisation of the in vivo MERP-1 protein, confirmation of gene expression data at the protein level, immunohistochemistry for cellular localisation, and flow cytometry for investigation of MERP-1 expression in purified populations of haemopoietic cells. The antibody may also be used for functional studies aimed at investigating the effects of inhibition of MERP-1 function in cell culture. Conversely, transient transfection of the MERP-1 gene in an immortalised or multipotent cell line will allow investigation of the effects of over-expression of the MERP-1 gene.
7.3 IDENTIFICATION OF ORP-3, A NOVEL OXYSTEROL BINDING PROTEIN RELATED PROTEIN GENE EXPRESSED IN CD34⁺ PROGENITOR CELLS

A partial sequence for ORP-3 was identified in CD34⁺ haemopoietic progenitor cells, with low expression in CD34⁺ haemopoietic cells. The full ORP-3 mRNA was obtained using RACE technology and bioinformatics and conceptual translation of the full ORF reveals a protein with significant homology to human oxysterol binding protein (OSBP-Hm). On the basis of partial and complete gene sequences in the GenBank database, it is clear that ORP-3 belongs to a family OSBP-Hm homologous gene sequences. Given differential expression of the ORP-3 gene in haemopoietic progenitor cells, it is possible that other ORP family members also play a role in haemopoiesis. Analysis of the expression of the other ORP family members by northern hybridisation will determine if any of these other ORPs is expressed predominantly in haemopoietic cells, and hence potentially plays an important role in haemopoiesis. Such investigations have already revealed that ORP-5 expression is relatively restricted to haemopoietic cells (Laitinen et al 1999), and preliminary investigations also indicate that ORP-5 is also differentially expressed in haemopoietic progenitors (unpublished data).

It is also clear that at least 8 ORP-3 splice variants exist (unpublished data). The existence of splice variants has also been reported for other ORP family members (Laitinen et al 1999, Moreira et al 2001) and it is possible that these splice variants perform cell/tissue specific functions. Current investigations are directed at determining which of the ORP-3 splice variants is predominantly expressed in CD34⁺ HSPCs. This information is required not only to allow investigation of the specific expression of this splice variant, but also to elucidate which ORP-3 mRNA sequence should be used for transfection and protein production.

Translation of the ORP-3 open reading frame yields an 887 amino acid protein that
has a high degree of homology other well-characterised OSBP proteins. Studies have demonstrated that the C-terminal half of these OSBP-homologues containing the OS binding domain is required for OS binding (Ridgway et al 1992). The N-terminal PH domain of ORP-3, like OSBP-Hm, is most probably required for translocation of the liganded protein to the GA. As for OSBP-Hm (Dawson et al 1989), the predicted amino acid sequence of ORP-3 also contains a possible LZR.

Given homology between ORP-3 and other OSBP-homologues, it is probable that ORP-3, like OSBP-Hm, plays a role in the transduction of OS effects on cells, in particular, haemopoietic cells. While this is yet to be determined, it does potentially raise the prospect that ORP-3 may play a regulatory role of in OS induced regulation of cholesterol metabolism, cell growth, apoptosis, and differentiation. Functional studies should therefore be directed at elucidating this possible role.

Production of the ORP-3 protein will allow determination of its ability to bind both specific OS and DNA, and will also allow the investigation of other proteins that ORP-3 may associate with and activate. Subsequent antibody production will enable western blotting to examine whether ORP-3, as does OSBP-Hm, forms homodimers or heterodimers with other, as yet unidentified proteins (Smith 1996) utilising the putative dimerisation region described in OSBP-Oc (Ridgway et al 1992). Depending on its specificity, the ORP-3 antibody can also be utilised for western blotting for characterisation of the in vivo ORP-3 protein, for confirmation of ORP-3 gene expression data at the protein level, and immuno-histochemistry for cellular localisation.

The ORP-3 antibody may also be used in functional studies investigating the effect of inhibition of ORP-3 in cell culture. Specifically, the role of ORP-3 in the transduction of OS effects on haemopoietic progenitor cells and cell lines should be investigated. Conversely, transfection of the ORP-3 gene into a haemopoietic cell line such as HL60 will allow investigation of the effects of over expression of ORP-3 in the transduction of OS effects. Additionally, as expression of the ORP-3 gene
appears to also be regulated by proliferation, differentiation, apoptosis and cell cycle, over expression and inhibition of ORP-3 and subsequent effects on all these processes can be investigated.
7.4 ORP-3 GENE REGULATION

Expression of the ORP-3 gene was found to be 3 - 4-fold higher in the CD34+ haemopoietic cells compared to CD34- populations from UCB and ABM, and was down regulated with differentiation and proliferation of CD34+ cells. Furthermore, expression in the more primitive sub-population of cells defined by the CD34+38- phenotype, was 2 - fold higher than in the more mature CD34+38- cells. These findings indicate that ORP-3 expression may be higher in a less differentiated subset of cells. This hypothesis is supported by the observation that expression of the ORP-3 gene is approximately 2-fold lower in differentiated HL60-v cells compared to control, undifferentiated cells. Whilst the reason for this differential expression currently remains elusive, further investigations of ORP-3 expression in haemopoietic cells induced to differentiate along specific paths will be of interest. Additionally, the use of multipotent embryonic stem cells induced to differentiate along other pathways will determine if ORP-3 differential expression is specific to the haemopoietic system.

Studies by Fournier et al (1999) and Silva et al (2001) indicate that expression of the ORP-4 gene is significantly associated with metastatic potential and CML. Preliminary studies also indicate that expression of the ORP-3 and ORP-5 genes is higher in MNCs from the BM of patients with AML when compared to expression in equivalent cells from non-AML BM (unpublished data). As patients with AML demonstrate altered apoptotic, differentiative and OS mediated cholesterol regulatory mechanisms (Vitolis et al 1994, Ahmed et al 1999, Ratajczak et al 1998b, Tatidis et al 1997), this finding, raises the possibility that ORPs may be involved in the pathogenesis of hematological malignancies. Current and future investigations will determine whether up-regulation of the ORP-3 and ORP-5 genes is specific to only AML, or extends to other hematological and non-hematological malignancies. If so, it is possible that expression of ORP-3 is related to cellular proliferative capacity. This possibility is in agreement with the observed higher expression in CD34+38-
HSPCs, and in undifferentiated HL60 cells.

Higher expression of the ORP-3 gene in CD34^-38^- cells may also be a cell cycle phenomenon. Whilst preliminary studies investigating ORP-3 gene expression in unsynchronised HL60 cells support this hypothesis, data obtained from investigation of ORP-3 gene expression in synchronized HL60 cells was inconclusive. Given previous reports indicating that the levels of OSBP-Hm protein also vary during cell cycle, this line of investigation warrants further examination. Incubation of HL60 cells with camptothecin and OS was also found to increase ORP-3 gene expression. Whilst this may be due to cell cycle arrest at G_0/G_1, the possibility that ORP-3 is regulated by, or plays a part in, the apoptotic program cannot be excluded. The effect of cytokines that act by inhibiting or stimulating the cell cycle in purified populations of CD34^-38^- and CD34^-38^ haemopoietic cells are planned, as are additional HL60 cell synchronization experiments.

UCB derived CD34^+ cells are reported to have a higher proliferative potential and a higher percentage of cells in G_0/G_1 than equivalent cells from ABM (Traycuff et al 1994, reviewed by Cairo and Wagner 1997). The finding therefore, of significantly higher expression of ORP-3 in CD34^+ cell populations from ABM does not support the hypothesis that ORP-3 expression is positively associated with proliferative capacity and G_0/G_1 phase of the cell cycle. It is possible that the heterogeneous nature of the CD34^+ cells from these two cell populations has made it difficult to interpret this data. The preliminary observation of differential ORP-3 expression between UCB and ABM HSPCs therefore requires confirmation by determination of the relative ORP-3 gene expression in purified populations of more primitive progenitors.

Combined with results obtained from characterisation of the ORP-3 protein and functional studies using ORP-3 antibodies and ORP-3 transfectants, additional gene expression data information obtained from future studies will assist in the elucidation of the function of the ORP-3 protein in the haemopoietic system.
7.5 THE EFFECTS OF OS ON HAEMOPOIETIC CELLS

Whilst the role of ORP-3 in the mediation of OS effects remains purely speculative, investigation of the effects of OS on haemopoietic cells has provided some interesting and significant results. In particular, the observations that OS significantly inhibit growth of human progenitor cells raises the possibility that OS may act as physiologically relevant inhibitors of haemopoiesis in vitro and in vivo. Results from this study indicate that the observed inhibition of haemopoietic cell growth can be attributed to both OS induced of apoptosis and differentiation. Further studies are required to de-lineate the pathways through which OS mediate both these processes.

Whilst the OS 7k-C inhibited ABM CFU-GM growth at levels within the reported physiological range for plasma, additional work is required to ascertain whether other OS produce these effects in vitro at concentrations more closely approximate physiological. This requires investigations conducted with growth factors concentrations more closely approximating physiological, and also in the presence of inhibitory cytokines that may potentiate OS effects.

Current studies are addressing the question of whether in vivo production of OS may be physiologically regulated and therefore an important regulator of haemopoiesis. To this end, the expression of genes that encode specific hydroxylases which produce OS in vivo are currently being investigated in primary and immortalised haemopoietic and stromal cells. Additional investigation of possible growth factor modulation of the expression of these genes will also clarify the physiological role, if any of OS on haemopoiesis.

Experiments utilising specific caspase and PKC inhibitors can provide insight into the mechanism by which OS induce both apoptosis and differentiation in haemopoietic cells. As previous studies indicate that OS can affect the transcription,
secretion and release of inhibitory cytokines and surface adhesion molecules (Lizard et al 1998, Leonarduzzi et al 2001, Tamasawa et al 2001), further investigations can also investigate the effects of OS on transcriptional regulation of the genes that encode these molecules in haemopoietic progenitor cells.

Finally, as differential expression of the ORP-3 and ORP-5 genes has been observed (unpublished data) functional studies to investigate whether patients with AML demonstrate altered responses to OS in culture will be conducted.
7.6 FINAL CONCLUSION

In summary, results obtained from this study have revealed that dd-PCR is a useful tool to investigate differential expression and identify novel genes expressed by haemopoietic stem/progenitor cells. Two novel genes, ORP-3 and MERP-1 were identified in CD34+ cells from UCB and ABM. Differential expression of both these genes was confirmed using Taqman™ real-time PCR, and was found to be down regulated with differentiation and proliferation of haemopoietic progenitor cells. Additionally, ORP-3 was found to be more highly expressed in HSPCs from ABM than equivalent cells from UCB.

The full coding sequences of MERP-1 and ORP-3 were obtained using RACE and PCR, and bioinformatics of the predicted proteins revealed potential function roles for these genes. The MERP-1 gene encodes a putative transmembrane cell adhesion molecule with a potential role in the differentiation, proliferation and survival of haemopoietic progenitor cells.

The ORP-3 gene encodes a putative OS binding protein and most probably plays a role in the transduction of OS effects on cells. Expression of the ORP-3 gene was further investigated and found to be regulated by differentiation and apoptosis of haemopoietic progenitors, and may also be positively associated with proliferative status and G0/G1 cell cycle status indicating a possible role in all of these processes.

Finally, OS have been demonstrated to be potent inhibitors of progenitor cell growth by induction of apoptosis and differentiation. While the role of ORP-3 in the transduction of OS effects on HSPCs is purely speculative, these studies have revealed OS are a new class of inhibitors of HSPC proliferation of potential relevance in vivo and in vitro. Further studies may also elucidate important regulatory roles for the ORP-3 and MERP-1 gene in the regulation of HSPC self-renewal, proliferation and differentiation.
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