The Role of BMP4 in the *Ex vivo* Expansion of Cord Blood Haemopoietic Stem Cells

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Statement of Originality

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Abstract

Establishment of conditions supporting haemopoietic stem cell (HSC) maintenance and expansion *ex vivo* is critical for wider clinical application of cord blood (CB) transplantation. AFT024 is a murine fetal liver cell line that expands primitive haemopoietic cells via a process that is not understood. Here we show that bone morphogenetic protein (BMP) 4, which is a member of the transforming growth factor-beta (TGFβ) super-family of pleiotropic regulators, is produced by AFT024 and contributes significantly to the maintenance of co-cultured CB-derived primitive cells. Significant amounts of BMP4 mRNA are produced by the supportive AFT024 stromal cell line and secreted BMP4 protein accumulates in AFT024 conditioned medium. Blockade of BMP4 activity in this co-culture model using neutralising BMP4 monoclonal antibody reduced expansion of primitive CB cells based on phenotypic ($CD34^+CD38^-$) and functional criteria (LTC-IC), and significantly reduced the capacity of the cultured CB stem cells to support repopulation in the NOD-SCID xenograft model. BMP4 is therefore an important growth factor for maintenance of HSC that contributes to the unique properties of the AFT024 stroma non-contact culture. Addition of supplemental BMP4 to clinical *ex vivo* cultures also increased maintenance of primitive haemopoietic cells in serum- and stroma-free defined culture conditions. Thus, BMP4 can contribute to HSC maintenance both in an established long-term co-culture model and in a clinical *ex vivo* expansion setting. On the basis of these studies BMP4 should be considered as a component in future CB *ex vivo* expansion protocols for use with other haemopoietic cytokines.

Abbreviations

AGM	aorta-gonad-mesonephrons
BMP	bone morphogenetic protein
BM	bone marrow
BMT	bone marrow transplantation
bp	base pair
BSA	bovine serum albumin
CB	cord blood
CD	cluster of differentiation
cDNA	complementary DNA
CFA	cobblestone forming area
CFC	colony forming cell
CFU	colony forming unit
CFU-GM	colony forming unit granulocyte-macrophage
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
Ct	cycle threshold
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DMSO	dimethylsulphoxide
dpc	days post coitum
DMEM	Dulbecco's modified eagle medium
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetra-acetic acid
EMT	epithelial-mesenchymal transition
FCS	fetal calf serum
FL	FMS like tyrosine kinase-3 ligand
GCSF	granulocyte colony stimulating factor
GMCSF	granulocyte-macrophage colony stimulating factor
GVHD	graft versus host disease
Gy	Grays
HLA	human leukocyte antigen

HSC	haemopoietic stem cell
HSCT	haemopoietic stem cell transplantation
IL	interleukin
LTC-IC	long term culture-initiating cell
MPP	multipotent progenitor
MQ	mill-Q
mRNA	messenger RNA
MSC	mesenchymal stem cell
MUD	matched unrelated donor
NOD-SCID	non-obese diabetic-severe combined immunodeficiency
OD	optical density
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SCF	stem cell factor
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SCT	stem cell transplantation
SRC	SCID repopulating cell
TE	tris-EDTA
TEA	triethanolamine
TGFβ	transforming growth factor-β
T_m	melting temperature
TPO	thrombopoietin
TRM	treatment related mortality
VBI	ventral blood island

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CHAPTER 1

INTRODUCTION

1.1 Haemopoietic stem cells

Mammalian blood formation originates in a small population of haemopoietic stem cells (HSC) through a process called haemopoiesis. The hallmark features of these cells are; (1) dramatic proliferative potential that is ultimately responsible for the production of large numbers of at least eight distinct lineages of mature blood cells [1], (2) self renewal capacity [2], and (3) the ability to engraft after being transplanted into an immuno-compromised host [3]. Considerable effort has been made in recent years to accurately determine the composition of the cell types that constitute the human HSC compartment. These studies have emphasised the heterogeneity of the human HSC in terms of proliferative and self-renewal capacities [4, 5]. Advances in flow cytometry have revealed important information on the phenotype of HSCs. Virtually all human haemopoietic progenitors and stem cells are found within the $CD34^+$ cell population [6], however the general consensus is that stem cells lack lineage markers (ie. antigens that identify mature granulocytes, macrophages, and lymphocytes). The $CD34^+CD38^-Lin^-$ cell population comprises less than 1% of the total $CD34^+$ cell population [7] and is highly enriched for *in vivo*-engrafting HSC by approximately 100-fold [7]. Other approaches for enriching HSC include efficient efflux pumping of rhodamine or hoescht dyes which identify side population cells (enriched 1000-fold for *in vivo* reconstitution activity) [8], or CD133 [9] (enriched for stem and progenitor cell subpopulations), have been described but are less characterised in humans than mice [10, 11].

1.2 Haemopoiesis

Haemopoietic stem cells go through a number of proliferative and commitment steps to give rise to all the myeloid and lymphoid lineages of the blood (**Figure 1.1**).

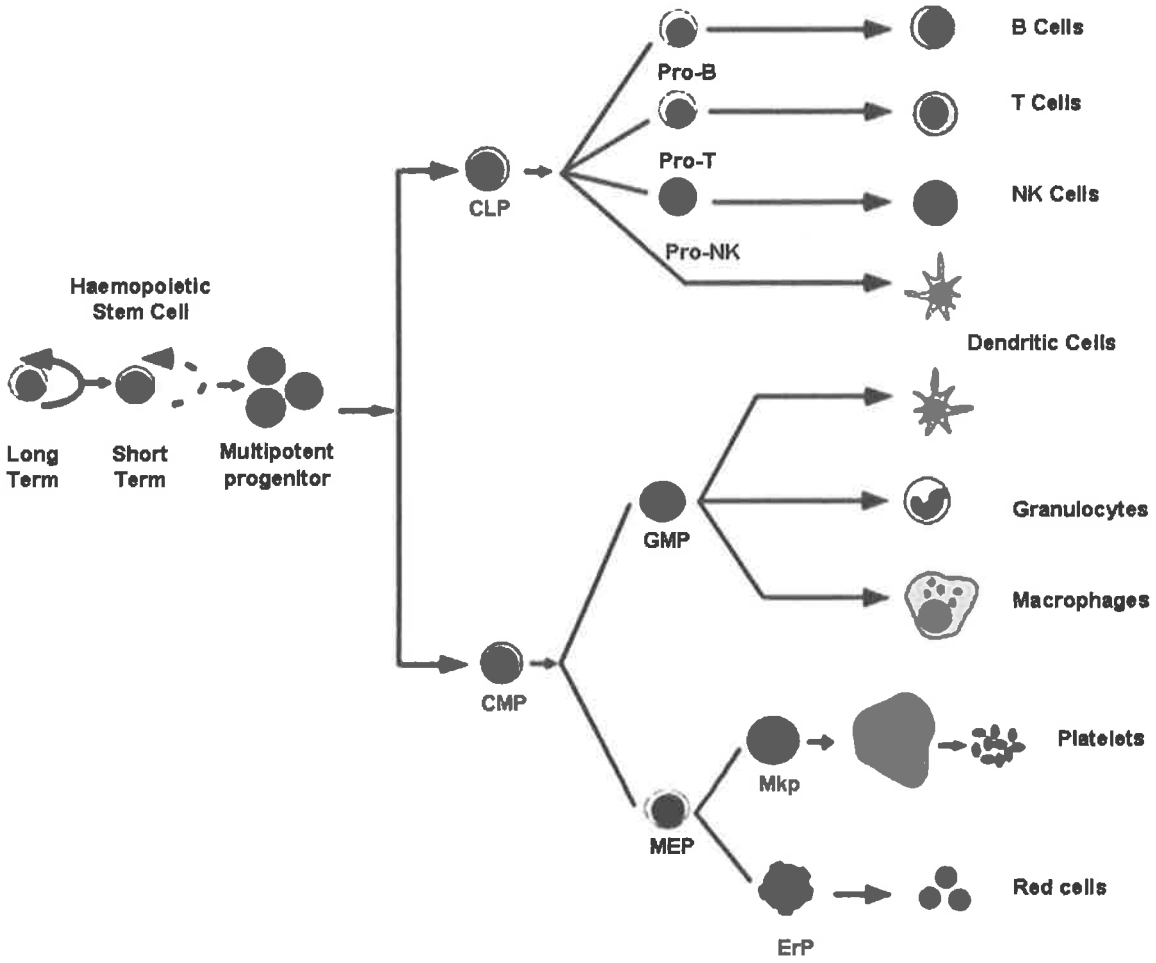


Figure 1.1 Haemopoiesis. Haemopoietic stem cells proliferative and differentiate to give rise to all adult blood cells (figure adapted from Reya *et al.*[114]).

Multipotent haemopoietic cells can be divided into three different populations: long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors (MPPs) without detectable self-renewal potential [12, 13]. These populations form a primitive cell lineage in which the long-term HSCs can give rise to short-term HSCs, which are able to differentiate into MPPs [12, 14]. As HSCs mature from the long-term self-renewing pool to MPPs, they progressively lose their potential to self-renew and become more mitotically active [15]. Whereas long-term HSCs can continuously and indefinitely give rise to mature haemopoietic cells, short-term HSCs and MPP reconstitute lethally irradiated mice for less than eight weeks [15]. The initial differentiation of MPPs will be along one of two major pathways, lymphoid or myeloid via the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP) cells respectively. These cells differentiate into lineage committed progenitor cells that in turn, form all mature blood cell types including erythrocytes, neutrophils, eosinophils, basophils, monocytes, mast cells, platelets and lymphocytes (**Figure 1.1**). Recently an alternative model of haemopoiesis has been proposed describing a population of cells with lympho-myelo differentiation potential that is unable to generate erythroid or megakaryocyte lineage committed cells [16]. This alternative model challenges the concept that the first lineage commitment step of adult MPPs is a strict separation into common myeloid and common lymphoid precursors.

The process of haemopoiesis is partly controlled by haemopoietic growth factors called cytokines and the ability of primitive cells to respond to these signals, as well as the particular microenvironment in which the progenitor cell resides [17, 18]. Haemopoiesis is a continuous process throughout adulthood whereby the production of mature blood cells must equal their loss. It is estimated that the average human produces approximately 3.7×10^{11} blood cells per day [18].

1.3 Haemopoietic stem cell transplantation

The repopulating ability of HSC makes them an important clinical resource. Bone marrow transplantation (BMT) is a procedure that restores stem cells that have been destroyed by high doses of chemotherapy and/or radiation therapy and is used extensively for the treatment of haematological malignancies, notably leukaemia, lymphoma and myeloma [19]. In addition non-malignant diseases such as aplastic anaemia, immunodeficiency, and enzymopathies often require BMT. Modern HSC transplantation originated from work showing that rodents could be protected against lethal haemopoietic injury by the intravenous infusion of bone marrow (BM) [20]. The subsequent identification of histocompatible antigens (the human leukocyte antigen (HLA) system in humans) and the development of cryobiologic techniques for freezing and thawing haemopoietic cells has brought BMT to its present state. There are two types of BMT which are now routinely used. In autologous transplants patients receive their own stem cells, whilst in allogeneic transplants patients receive donated stem cells from a matched related or matched unrelated donor (MUD). The success of allogeneic transplantation depends largely on how well the HLA antigens of the donor's marrow match those of the recipient's marrow. Whilst close relatives are more likely to have HLA matched BM, generally only 30-40% of patients will be able to locate suitably matched siblings or parents [21]. The remaining patients must try to identify potential donors using world wide BM donor registries, of which several exist. Advances in the supportive care of patients undergoing autologous or allogeneic stem cell transplantation have significantly reduced the mortality rates associated with these procedures [22]. However, both related and unrelated donor stem cell transplantation (SCT) remain associated with significant morbidity and mortality resulting from major

organ toxicity due to the conditioning regime, graft versus host disease (GVHD), graft failure or infectious complications [23].

1.4 Cord blood haemopoietic stem cell transplantation

In addition to the use of neonatal cord blood (CB) for allograft SCT, CB HSC have significant potential to help circumvent the problems associated with allogenic BMT and to increase the potential pool of available stem cells for transplantation. Gluckman *et al.* first reported the use of HLA-matched sibling umbilical CB stem cells to successfully reconstitute an infant with severe Fanconi anaemia in 1988 [24]. Since then over 7,000 CB transplantations have been performed worldwide, with the majority being in the unrelated setting [25, 26]. There are many advantages of using CB for SCT which is now accepted as an alternative source of HSC for transplantation in children [26-28]. As cryopreserved CB is readily available there is a reduction in time from initiating a search to performing the transplant procedure (given the ability to store fully tested and HLA-typed CB available for immediate use). This reduction in search time is critical in some patients. CB banks have been established in major transplant centers in Australia and worldwide with approximately 1 x 10^5 units currently available [29, 30]. Further to this, certain ethnic populations, for example, Australian Aboriginals, are vastly under-represented on BM donor registries. CB banks can target specific ethnic groups to address this imbalance.

It has been shown in early studies by Broxmeyer *et al.* [31] and others that CB contains a significantly higher number of HSC when compared with adult peripheral blood (PB) or BM allowing CB SCT to be performed with comparably lower volumes and reduced cell dose. CB is less immunogenic when compared to PB or BM due to the naivety of the immature T-cell population [31, 32]. Whilst B cell numbers are

comparable to adult peripheral blood [33], CB is up to 1,000 times less alloreactive in terms of proliferative T cells [34]. CB has a higher number of unprimed, naive T cells (CD45 RA^+) and lower populations of mature or primed T cells (CD45 RO^+) compared with that of adult peripheral blood [35]. As a result of this, the incidence and severity of GVHD following CB SCT is lower relative to MUD SCT. This has resulted in CB SCT being performed with a greater degree of HLA disparity than would normally be accepted for MUD SCT, with more patients able to identify potential donors. It is because of these advantages that CB has become an extremely attractive source of HSC with potential for broad application to treat many life-threatening haematological diseases.

1.5 Current limitations of cord blood haemopoietic stem cell transplantation

A significant limiting factor in the success of CB transplantation in adult patients is the limited cell dose that can be obtained from a single CB unit. This can result in delayed engraftment and graft failure [31]. The rate of engraftment is dependent on the number of infused stem cells, and the $CD34^+$ content of CB is at most 5% of the optimal dose for adults (2-4 x 10^6 $CD34^+$ cells/kg) [36]. As a result, the majority of CB SCT have been performed in children under 7 years of age or 20 kg in weight. Accordingly, a reproducible method for increasing the HSC population will increase CB application for adult HSC transplantation.

1.6 Cord blood stem cell transplantation in adult patients.

In recent years umbilical CB has been trialled as a source of HSC for adult SCT. A decreased risk of GVHD has made umbilical CB SCT an appealing alternative to BM

derived HSC, particularly where a MUD could not be found. Two recent studies have assessed the outcomes of adult patients receiving unrelated CB HSC for transplantation in large trials with encouraging results. In both of these reports, patients receiving CB HSC were on average younger, weighed less and had more advanced disease compared to recipients of MUD BM. CB recipients in the Rocha study [37] received a 10-fold lower cell dose and demonstrated a delayed neutrophil engraftment after 7 days compared to BM recipients. The rate of graft failure was 20% compared to 7% for the BM recipients. Importantly, incidence of GVHD, treatment related mortality, relapse, overall survival and leukaemia free survival were no different between the groups. Laughlin *et al.* [38] reviewed the outcome of 150 adults with leukaemia receiving a CB SCT and compared it to 450 patients receiving unrelated donor BM. Compared to the fully matched unrelated BM recipients, the CB recipients again had delayed engraftment but in contrast to the Rocha study had greater treatment related mortality (TRM) and lower survival. This is similar to earlier data showing survival following CB SCT is directly related to time to engraftment [36], illustrating the importance of achieving prompt engraftment. Together, these studies illustrate that CB SCT is feasible in adult patients, particularly if no suitable BM donor is identified. Delayed engraftment, rather than HLA disparity, has emerged as the limiting factor to more widespread use of CB as a source of HSC. This delay in engraftment may simply reflect the low cell number in CB grafts but may also reflect the immaturity of CB cells or the lack of facilitating cells in the graft. Current data indicates that engraftment is related to the total cell dose infused, (by either total nucleated cell dose [39] or $CD34^+$ cell dose [36]), and evidence shows positive outcome correlates with prompt engraftment. The data of Wagner *et al.* [36] showed favorable outcomes for neutrophil engraftment, TRM and survival in recipients of $CD34^+$ cell doses $> 1.7 \times 10^5$/kg compared to recipients of $\leq 1.7 \times 10^5$/kg.

Clearly, for wider applicability of CBT novel approaches to enhance both the rate and degree of engraftment are needed. It is likely that some improvements will come from clinical studies which utilise non-myeloablative conditioning regimens [40]. Technical improvements to increase the CB cell collection and reduce cell loss during processing and thawing will also result in some increase in cell dose. A variety of collection methods have now been proposed to optimise the collection volume and reduce the risk of microbial and maternal cell contamination [41].

Much research is currently aimed at increasing CB HSC numbers prior to transplantation. Promising results at least in experimental models, have been reported from the use of multiple CB units combined prior to SCT, co-transplantation with mesenchymal stem cells and *ex vivo* expansion of CB HSC.

1.7 Multi-cord blood unit haemopoietic stem cell transplantation

Early experience of double unit CB transplantation has demonstrated enhanced engraftment and neutrophil recovery. A recent study by Barker *et al.* [42] has reported encouraging results using CB units from two partially HLA-matched donors in high risk adult and adolescent patients. In a study of 23 patients it was observed that after transplanting two CB units, both units engrafted in only 5 of 21 patients at day 21 and even fewer at day 60 (2/19). By day 100, sustained engraftment was derived from only one unit in all patients. The predominating unit was not determined by nucleated cell dose, $CD34^+$ dose or degree of HLA-disparity but appeared to be related to T cell dose. Given the relatively small cell dose of the predominating unit, engraftment was superior to that predicted, suggesting the second unit facilitated engraftment by an, as yet, unexplained mechanism. However it was hypothesised by the authors that single-donor predominance may be caused by a graph versus graph reaction.

1.8 Mesenchymal stem cell co-transplantation

Mesenchymal stem cells (MSC) are derived from the non-haemopoietic elements of BM and are capable of *in vitro* differentiation into multiple mesodermal tissue types including osteoblasts, chondrocytes, myocytes and adipocytes [43]. MSC represent a major component of the BM microenvironment playing an important role in the regulation of haemopoiesis and providing a potential adjunct cellular therapy to enhance allogeneic haemopoietic engraftment [44-46]. MSC may promote HSC engraftment by supporting haemopoietic progenitor proliferation, enhancing haemopoietic growth factor production or facilitating homing of transplanted cells through adhesion molecules [47-51]. MSC have also been shown to be immunosuppressive and thus may promote engraftment by reducing the recipient alloimmune response and GVHD [52]. Preliminary studies evaluating MSC co-transplantation using the non-obese diabetic-severe combined immunodeficiency (NOD-SCID) model, demonstrate enhancement of engraftment. Using expanded MSC from different sources and at different doses, a number of groups have reported superior engraftment when analysed at 6 weeks post-transplantation [48, 53-55]. The differentiation potential of engrafted cells appears to be altered from predominant B lymphoid differentiation to myeloid and megakaryocyte lineages [56]. Similar results have been reported after *in utero* transplantation of BM derived stromal cells and HSC in fetal sheep [57, 58]. These observations led to the investigation of MSC administration as an adjunct to BMT. Limited clinical studies so far of MSC co-infusion have demonstrated it is both safe and feasible [59].

1.9 *Ex vivo expansion of haemopoietic stem cells*

While variations to the transplantation protocol, including the use of multiple CB units and non-myeloablative regimes have potential to improve outcome for adult patients, there is currently much interest in the *ex vivo* expansion of HSC from a single CB unit. A major challenge is the establishment of culture systems that facilitate *in vitro* HSC self renewal and expansion. One limiting factor has been finding an assay to quantitatively measure transplantable stem cells before and after culture. True stem cell markers that identify homogeneous populations of long-term repopulating HSC have not yet been discovered. As such phenotyping for stem cell markers (and excluding cells with lineage markers) is considered only a surrogate measure of stem cell properties. Early *ex vivo* expansion studies involving quantitative analysis of HSC were limited to *in vitro* colony forming assays. Committed and multipotent progenitors including colony-forming cells (CFC) and haemopoietic progenitors capable of initiating long term cultures (long-term culture initiating cells (LTC-IC) are used to quantitate the functional properties of cultured cells [60]. In these assays the endpoint is the presence of progenitor cells with colony-forming ability. This assay initially developed for studying human stem cell growth *in vitro,* is now commonly used to measure primitive murine and human haemopoietic progenitor cell frequencies, and can be altered to support growth of lympho-myeloid progenitors [61]. Until now, the CFCs and the LTC-IC assays are the only testing systems that have the ability to reliably measure mouse and human primitive haemopoietic stem/progenitor cell frequencies other than *in vivo* long-term engraftment studies. However, such assays give no information as to the self-renewal, multilineage differentiation, or engraftment capacity of expanded cells. Assays that are able to quantitate the most primitive and transplantable stem cells have been developed using animal transplantation models. Xenotransplantation assays for

primitive human haemopoietic cells capable of repopulating the BM of immune-deficient mice with myeloid and lymphoid lineages, provides a powerful approach to characterise the functional properties of expanded primitive cells [62]. NOD-SCID mice have proven to be a reliable recipient for detecting human haemopoietic-repopulating cells that differentiate into multilineage mature cell types, and are capable of self-renewal [3]. Human repopulating cells identified in this assay, defined as SCID-repopulating cells (SRC), have been shown to be enriched among an extremely rare $CD34^+CD38^-$ subfraction of lineage-depleted (Lin-) $CD34^+CD38^-$ CB cells [7].

1.10 Clinical ex vivo expansion of cord blood haemopoietic stem cells

The *ex vivo* expansion of HSC for clinical use is now recognised as a feasible and promising approach for SCT [63]. The unique challenges associated with clinical expansion of HSC involve the complete optimisation of multiple variables associated with culture conditions (such as combinations and concentrations of cytokines used, initial cell density and culture duration) that can have a profound influence on the functional properties of expanded cells [22]. Clearly clinical expansion requires serum free culture, defined media and clinical grade recombinant growth factors and cytokines. A number of clinical studies have now shown that it is feasible to culture BM or PB $CD34^+$ cells *ex vivo* and transplant them into patients without significant adverse events [64-67]. At least four groups using similar culture conditions have reported a reduction in the duration and severity of neutropenia following infusion of *ex vivo* expanded PB progenitor cells [68-71]. Experience assessing the utility of *ex-vivo* expanded CB is more limited. Shpall and colleagues have transplanted 37 patients with encouraging results [72]. CB aliquots of different sizes were thawed and selected $CD34^+$ cells cultured with defined media with supplemental stem cell factor (SCF), granulocyte-

colony stimulating factor (G-CSF) and thrombopoietin (TPO). The cells were expanded for 10 days and infused together with the unmanipulated fraction. All patients achieved neutrophil engraftment with a median time of 28 days (range 15 to 49 days). Considering the low numbers of cells infused per kg in these patients, the time to neutrophil engraftment was equivalent to or faster than previous reports in adult patients, suggesting a contribution of the expanded cells. Clearly these early trials were limited by availability of clinical grade cytokines and HSC selection devices which prevented optimal cytokine cocktails and culture conditions from being employed [40]. Current opinion suggests that future areas of investigation need to focus on refining preclinical and clinical studies in which cocktails of high grade cytokines, novel molecules/growth factors and sophisticated expansion systems will promote expansion of CB HSC/progenitor cells and reduce engraftment delay following CB SCT in adult patients [22, 33, 40, 73].

1.11 Experimental ex vivo expansion culture systems

Currently there is great interest in the development of novel culture systems capable of expanding repopulating cells that have self-renewal and multilineage differentiation capacity. Culture systems can be subdivided into two broad categories; (1) those relying on defined cytokine cocktail combinations as the only culture supplements, and (2) those relying on a pre-established stromal monolayer as an additional supportive component (with or without exogenously added cytokines) [74]. Those relying solely on cytokines have met with limited success. Most were restricted by the use of very short culture periods, with attempts at long-term culture resulting in a decrease in SRC frequency. The commonly studied cytokines for expansion cultures include G-CSF, SCF, TPO, granulocyte-macrophage colony stimulating factor (GM)-

CSF, interleukin (IL)-3, IL-6 and Flt-3 ligand (FL) [75], whilst more recently other molecules such as all-trans retinoic acid (ATRA) have been reported to maintain HSC in *ex vivo* cultures [76]. Combinations of cytokines can act in a synergistic manner to provide a strong mitogenic stimuli, allowing massive increases in cell number *ex vivo* [65]. However this usually results in a loss of primitive cells and engraftment capacity. The combination of SCF, FL, TPO and IL6 (and soluble IL6 receptor) has been shown to expand HSC over a period of many weeks in the presence of serum [77-79]. Conneally *et al.* [80] demonstrated that short-term culture (5-8 days) with a cocktail of cytokines (FL, SCF, IL3, IL6 & G-CSF) resulted in a 100-fold expansion of CFC with a 4-fold expansion of LTC-IC and a 2-fold expansion of competitive repopulation units (SRC equivalent). Studies by Bhatia *et al.* [81] involving engraftment assays of expanded CB $CD34^{+}CD38^{-}$ cells in NOD-SCID mice achieved moderate 2-fold increases in SRC after 4 days expansion. Cells were cultured in serum-free media containing FL, SCF, IL-3, IL- 6, and G-CSF. However, culture for longer periods (9 days) resulted in a decrease of SRC. The majority of studies in which progenitors are cultured in cytokine-supplemented conditions show that short-term culture results in a modest increase in SRC frequency, but loss of SRC if the cultures are extended for longer than 9 days [81]. This is most likely due to combinations of cytokines exerting potent stimulatory effects on HSC disrupting signalling pathways necessary for self renewal [82]. Thus, cytokine-mitogenic capacity induces massive expansion in cell number from primitive HSC cultures but fails to induce extensive HSC self renewal or expansion.

1.12 Stromal dependent culture systems

It has long been accepted that *in vivo* stem cells are found in close association with important cellular and extra-cellular components in discrete microenvironments [18, 83]. These observations suggest that *in vivo* stem cell regulatory mechanisms are likely to require cell-cell contact or short-range interactions [84]. Efforts to understand the features of the haemopoietic microenvironment began with the establishment of the Dexter long-term culture (LTC) system [85]. Although it was able to maintain long-term haemopoiesis, it exhibited a dramatic net decrease of stem cell activity over time [86]. Studies by Gan *et al.* [62], examined the maintenance of SRC of CB and BM $CD34^+$ cells when cultured with allogenic human BM stroma. It was shown that the number of SRC declined six-fold after 1 week of culture. A major drawback of these stroma-dependent culture systems is the heterogeneity present in the stromal cell monolayer [74]. Numerous studies have now been reported with cloned stromal cell lines [87-90], several of which have been shown to support long-term repopulating cells from murine, human, and primate sources. In 1999, Brandt *et al.* [91] reported successful expansion of human marrow HSC when co-cultured with a cloned porcine microvascular endothelial cell line (PMVEC). HSCs expanded in PMVEC co-culture were capable of competitive BM repopulation with multilineage progeny present eight weeks post-engraftment in SCID mice, and in a myeloablative, irradiated baboon model. Cells were transplanted after 10 days of *ex vivo* expansion culture, after which baboons became transfusion independent by day 23. BM derived stromal cell lines have also been used successfully to expand HSC. For example the FBMD-1 murine cell line was shown to expand $CD34^+$ cells capable of engrafting NOD-SCID mice 10-fold at day 14 [92]. Another murine BM cell line, AC6.21 demonstrated 150-fold total cell expansion and maintained long-term engrafting $CD34^+thy\text{-}1^+$ cells. Thalmeier *et al.* have

established two permanent human BM stromal cell lines (L87/4 and L88/5) that are both capable of supporting long-term proliferation of CB $CD34^+$ cells but not maintenance of HSC [93]. OP9 BM stromal cells (derived from an MCSF deficient mouse) have been shown to support haemopoiesis and induce the formation of $CD34^+$ cells from ES cells [94]. Recent studies have identified the membrane bound protein mkirre as an important component of OP9, with a soluble form of this protein reported to maintain cultured SRC in short-term stroma- and serum-free conditions [95]. Osteoblasts in the BM have recently been identified as key regulators of HSC self-renewal [96, 97]. In this environment, components of HSC adhesion to the endosteal bone surface, such as Tie2 and Osteopontin, regulate HSC proliferation [98-100]. The interaction of Tie2 with its ligand Angiopoietin-1 (Ang-1) has also been shown to block cell division and maintain long-term repopulating activity of HSCs cultured *in vitro* [98].

1.13 AFT024 stromal cell line

During embryonic development, the first definitive long-term repopulating HSC emerge and undergo rapid expansion in the embryonic aorta-gonad-mesonephrons (AGM) region [101] . Stromal cell lines from this area that support maintenance of HSC have recently been developed. Kusadasi *et al.* [102], have reported on several embryonic liver derived clones that expanded colony-forming unit granulocyte-macrophage (CFU-GM) 500-fold after 6 weeks culture. It is postulated that these stromal cell lines provide the unique microenvironment necessary for stem cell maintenance, producing unique growth factor combinations [84]. The AFT024 feeder is a murine fetal liver cell line derived by Lemishka *et al.* by imortalisation of fetal liver cells with a temperature-sensitive simian virus 40 T-antigen [103]. AFT024 cells have been shown to support repopulating murine stem cells for up to 2 weeks [104]. *Ex vivo*

culture of human CB $CD34^+Lin^-CD38^-$ cells in AFT024 cultures supplemented with FL, SCF & IL-7 resulted in a 3- to 5-fold expansion of LTC-IC after 2 and 5 weeks culture [105]. The same culture conditions increased SRC frequency by 1.3- to 2-fold after 7 and 14 days. Cultured SRC had multilineage differentiation ability, and were able to engraft secondary NOD-SCID mice, demonstrating self-renewal capacity. The AFT024 culture system can maintain primitive haemopoietic cells capable of long-term engraftment and self renewal in non-contact co-culture using transwells [104]. This *ex vivo* culture system has also been assessed using a fetal sheep xenograft model. Human $CD34^+CD38^-$ cells after 7 days of non-contact *ex vivo* culture with AFT024 cells (with supplemental IL-7, SCF and FL) were able to maintain long-term engraftment 6 months after transplantation. Furthermore, secondary and tertiary engraftment was also observed [104]. While these stroma-dependent co-cultures allow maintenance of HSC under laboratory conditions, future clinical development of *ex vivo* expansion protocols would not allow the use of murine stromal culture systems, even if it were non-contact. Thus it is important to understand the nature of signalling between the AFT024 and HSCs, and if possible to duplicate this in a defined culture system.

1.14 Molecular characterisation of AFT024

Recent studies have characterised AFT024 as a model of fetal liver haemopoiesis that supports HSC maintenance/expansion [106]. It was shown that AFT024 has mixed endodermal (epithelial) and mesodermal characteristics that fits within the description of cells in epithelial-to-mesenchymal transition (EMT). These cells correspond to a population often observed during development of the liver and associated with the development of haemopoiesis. This population disappears by the end of gestation, and is replaced by hepatocytes. Maturation of AFT024 and primary

fetal liver cells (with Oncostatin M treatment) reduced their LTC-IC supportive ability. Furthermore, this change in functional activity corresponded with a decreased EMT phenotype. It was concluded that AFT024 properties are consistent with those of EMT stromal cells that provide a suitable microenvironment to attract and nurture HSC during transient haemopoiesis in the fetal liver [106]. Other recent studies have focused on determining the molecular signals generated within the AFT024 stem cell niche. Hackney *et al.* [107] have undertaken a genomics approach that involved combining extensive sequence mining of an AFT024 subtracted cDNA library, high density array hybridisation and bioinformatics analysis. It was reasoned that molecules responsible for the stem cell supportive phenotype would be preferentially expressed in AFT024 compared with other non-stem cell supporting cell lines from the same tissue source. In support of this hypothesis, they have isolated the membrane bound protein delta-like/preadipocyte factor-1 (Dlk) [108] and the soluble factor proliferin-2 (plf-2) [109] which are preferentially expressed in AFT024 and contribute to maintenance of primitive haemopoietic cell populations *in vitro*. However, these molecules account for only a portion of the supportive capacity of AFT024 [107]. The authors concluded that there remains undiscovered HSC regulators provided by the stem cell niche [107].

1.15 Haemopoietic growth factors

To date the identification of molecules that regulate human HSC has focused mainly on cytokines, of which very few are known to act directly on stem cells. There is still a poor understanding of how initial HSC are expanded in the embryo to generate a pool of cells capable of supporting life-long haemopoiesis. It is likely that an understanding of the embryonic origin of HSC and the factors regulating their generation and expansion *in vivo* will facilitate further protocols for *ex vivo* HSC

expansion. More recently a number of soluble factors have been reported with varying abilities to support HSC self-renewal under *in vitro* conditions. A study by Bhardwaj *et al.* reported that the developmental growth factor sonic hedgehog (Shh) could expand pluripoptent HSC as measured in SRC assays after *ex vivo* serum free culture [110]. The highly specific BMP antagonist noggin was capable of inhibiting Shh-induced HSC proliferation. The authors concluded that Shh functions as a regulator of HSC, via downstream BMP signalling. Karanu *et al.* have demonstrated that notch signalling can regulate HSC [111]. It was reported that stem cell supportive stromal and endothelial cells express the notch receptor and its ligands delta-1 and delta-4, which when added to stroma free culture were able to expand pluripotent HSC *ex vivo*. The notch ligand, Jagged-1 (Jag1) has also been reported to expand primitive haemopoietic precursor cells up to 3-fold in stroma-free culture [112]. A study by Van Den Berg *et al.* demonstrated that Wnt5a is expressed in $CD34^{+}Lin^{-}$ primitive progenitor cells and that Wnt5a, Wnt2b and Wnt10b were able to expand early haemapoietic progenitor cells as determined by *in vitro* colony forming assays [113]. More recently it has been reported that soluble Wnt3a protein induces proliferation of highly purified human BM HSC in the absence of any other growth factor (through β-catenin stabilisation) [114] and that *ex vivo* culture with Wnt3a can expand murine HSC (from blc2 transgenic mice) up to 100-fold [115]. De Han *et al.* have shown that fibroblast growth factor-1 (FGF1) plays an important role in HSC homeostasis and that prolonged culture of BM cells in serum-free medium supplemented with only FGF1 resulted in significant expansion of multilineage, serially transplantable HSC [116]. Recent studies in lower organisms and the mouse have shown that BMPs play a critical role in the specification of haemopoietic tissue from the mesoderm germ layer (as discussed in detail below). BMP4 in particular emerges as an attractive candidate of HSC self-renewal. Indeed

Bhatia *et al.* have demonstrated that high levels of BMP4 can improve the limited maintenance of SRC observed in serum free, stroma free cultures [117] (as discussed below).

1.16 Bone morphogenetic proteins

BMPs are members of the TGFβ super-family of signalling molecules. These pleiotropic cytokines are important for embryonic tissue development and in regulating cell proliferation, differentiation, morphogenesis and apoptosis in multiple systems [118, 119]. The BMP developmental signaling pathway is highly conserved in evolution, and is present in all animal species whose genomes have been sequenced (e.g. the worm C. elegans, the fruitfly Drosophila melanogaster, mice and humans) [120-124]. BMPs were first discovered as constituents of bone extracts that induced cartilage and bone formation when injected into rodents [125]. A family of at least 20 structurally related proteins have since been identified with multiple functions. [126, 127]. BMPs act via a family of heteromeric receptors referred to as the type I/type II serine, threonine kinases [128]. There are at least three types of TGFβ receptors; a 53 kDa type I receptor, a 70-85 kDa type II receptor and a 200-400 kD type III receptor [129]. Only type I and type II receptors appear to play significant roles in BMP binding and signalling and share similarities in the BMP system (as summarised in **Figure 1.2**) [128]. Both are considered to be serine/threonine kinase receptors, exist constitutively as homodimers in the absence of ligand and have relatively short extracellular regions containing a cysteine box [130]. The classical BMP-Smad pathway has been well described and can be summarised as follows. On BMP binding to specific cell surface type I and type II serine/threonine kinase receptors, receptor II phosphorylates receptor I

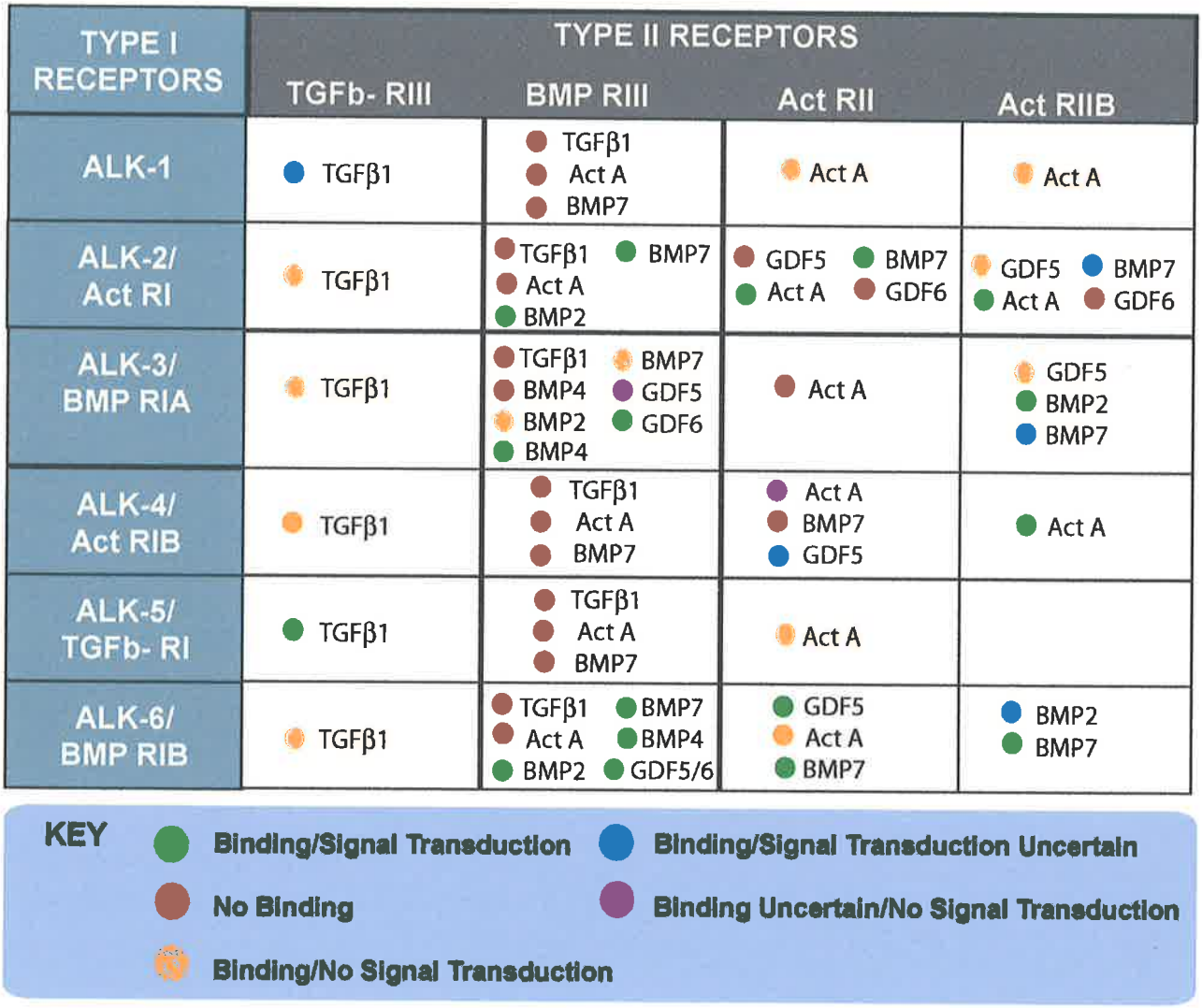


Figure 1.2 TGFβ family ligand/receptor summary. BMPs act via a family of heteromeric receptors referred to as the type I and type II serine, threonine kinases. Only type I and type II receptors appear to play significant roles in BMP binding and signalling and share similarities in the BMP system *(*figure adapted from Shi *et al.* [127]*)*.

serine and threonine residues, resulting in the activation of kinase activity and the phosphorylation of C-terminal serine residues on BMP-receptor regulated Smads, (Smad1, Smad5 and Smad8) [131, 132] (**Figure 1.3**). These phosphorylated Smads then interact with the co-Smad (Smad4) to form a complex which moves into the nucleus. Once in the nucleus, the Smad-co-Smad complex is involved in transcriptional regulation of target genes [131]. The activity of this pathway is further modulated by the inhibitory Smad's (6-8), which regulate TGFβ family signalling by interfering with the activation of pathway specific Smads [133].

1.17 BMP inhibitors

The biological activities of TGFβ ligands are negatively regulated by a number of specific binding proteins [134]. In particular, the BMPs are regulated by Chordin, noggin and follistatin. chordin and noggin have been shown to inhibit BMP4 activity by binding directly to BMP4 with high affinity, hence blocking interaction with cell-surface receptors [135, 136]. Follistatin forms a trimeric complex with BMP and its receptor [137] preventing Smad activation of the receptor-ligand complex.

1.18 BMP4 signalling during haemopoietic development

The first murine haemopoietic cells appear in the yolk sac blood islands with primitive erythroid cells identifiable at around embryonic day 7.5 [138]. By embryonic day 12 the liver is the predominant site of haemopoiesis [138]. This continues until postnatal week 6 when fetal liver haemopoiesis declines and BM becomes established as a life-long haemopoietic organ [139]. In the human, the fetal liver is colonised by blood cells at week 5 and continues as a major site of haemopoiesis until week 22. The

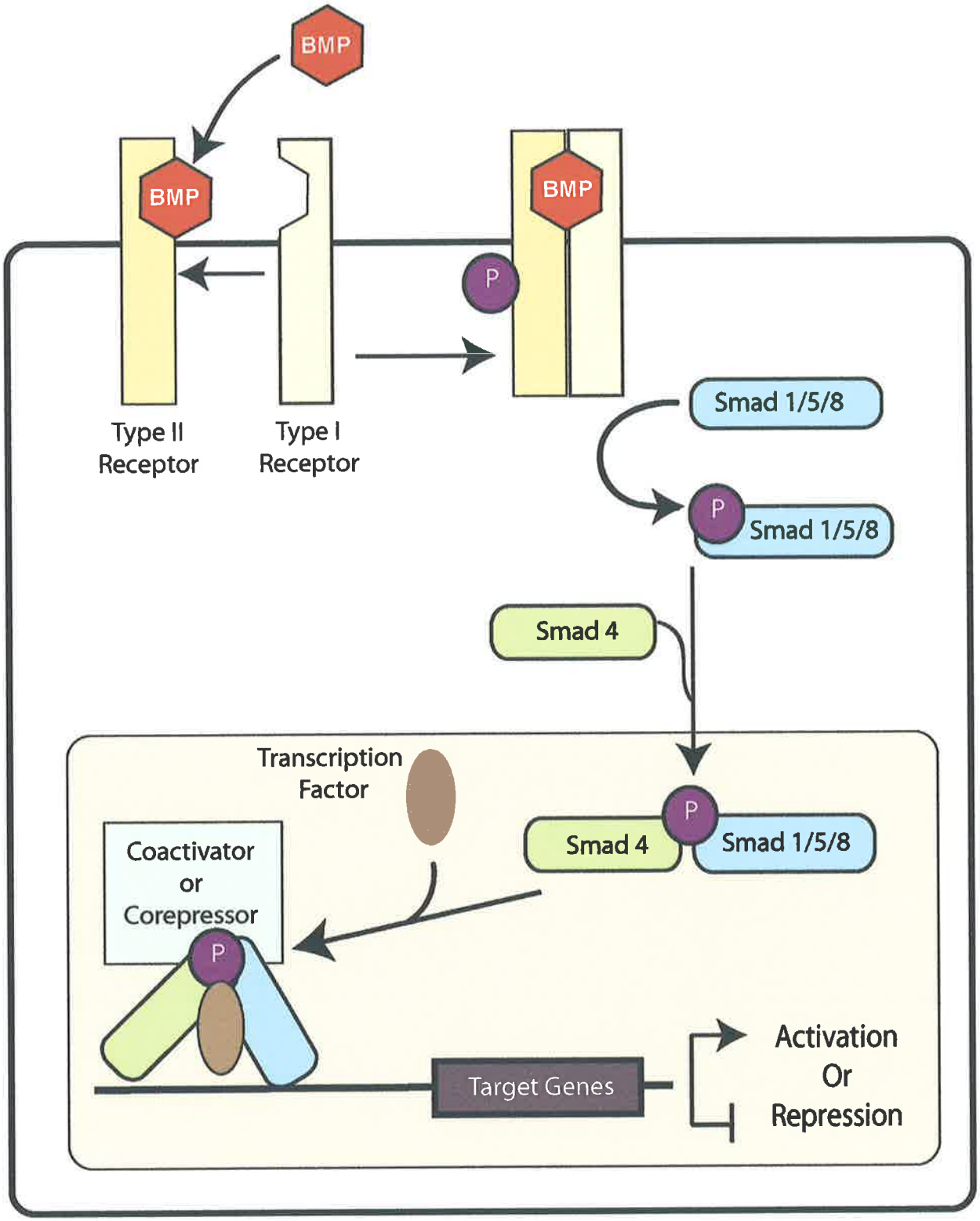


Figure 1.3 The classical BMP-Smad pathway. BMP signalling activates the Smad intracellular pathway to induce transcription of BMP target genes (figure adapted from; *Liu et al.* [131]).

fetal bones become colonised with haemopoietic cells between weeks 8-19 and by week 22 have become the major site of haemopoiesis [140]. It is now accepted that HSC arise in the embryo proper, prior to circulation, in the AGM region where the aorta, gonads and kidneys form [141]. Significant HSC activity is evident in the AGM region before any appearance of yolk sac or liver stem cell activity [141]. The formation of the HSC phenotype in embryonic development occurs as a result of complex intracellular signalling. Growth factor signals are likely to originate from the underlying stroma which forms a densely packed region of distinct morphology [142]. Importantly, Marshall *et al.* recently showed that BMP4 is expressed at high levels in this stromal region beneath the ventral wall of the dorsal aorta, suggesting it may provide a supportive signal [140]. BMP4 has been shown to act as a morphogen in the patterning of the dorsal-ventral axis of Xenopus embryo [140]. There exists a gradient of BMP4 expression across the Xenopus mesoderm with high concentrations specifying the most ventral tissues (such as blood) and lower concentrations specifying muscle [143]. This gradient of BMP4 activity forms due to the high expression of specific BMP inhibitors (noggin, chordin, follistatin) concentrated at the dorsal end [135]. In Xenopus, after gastrulation, HSC emerge from a region of embryonic tissue referred to as the ventral blood island (VBI), equivalent to the yolk sac in the mouse. The HSC of the VBI differentiate primarily into embryonic erythroid cells but also migrate to seed haemopoietic organs. The VBI region forms in response to the high level of BMP4, that is restricted to the ventral mesoderm [143]. Thus in Xenopus, high level BMP4 activity is critical for formation of ventral mesoderm derivatives such as blood. Together these findings suggest the possibility that a concentrated BMP4 signal may be critical for HSC formation and/or maintenance during development *in vivo*.

1.19 BMP target genes

Critical to our understanding of the role of BMPs in haemopoietic development, is the identification of direct target genes transcriptionally regulated in response to BMP receptor signalling in HSC precursors. BMP-induced homeobox (HOX) gene expression is likely to be an important downstream regulator of HSC formation. There are two major classes of HOX genes. The Class I HOX genes include HOXB4 which can promote large-scale *in vitro* expansion of HSC [144] and Hox9a which is required for HSC proliferation [145]. There is strong evidence to suggest that genes in class II also play an important role in haemopoiesis, consistent with their ability to regulate downstream genes in haemopoietic cells. Mixl1 is a HOX protein involved in the induction of ventral mesoderm [146]. A study by Mead *et al.* has shown that injection of Mixl1 mRNA into Xenopus results in extensive blood formation and transforms dorsal mesoderm to a ventral fate. Mixl1 expression is up-regulated by BMP4, consistent with the dominant negative mutant of Mixl1 blocking haemopoietic induction [146].

The Vent class of homeobox genes have also been shown to be directly downstream of BMP4. Xvent-2 is essential for the establishment of the dorsal-ventral body axis [147]. Henningfeld *et al.* have recently shown that the Xvent-2 gene promoter contains a BMP-responsive element (BRE) containing Smad1 and Smad4 binding sites and is also known to interact with the haemopoietic specific transcription factor GATA-2 which is also induced by BMP4 [148]. Injection of BMP4 mRNA into animal cap cells results in increased GATA-2 expression as well as stimulation of globin synthesis. Whilst a dominant negative BMP4 receptor injected into ventral mesoderm suppresses GATA-2 [148]. In Xenopus embryos, BMP signalling is known to result in the induction of a number of other haemopoietic specific genes including GATA-1, SCL

and LMO-2 [149-151]. Thus, downstream BMP signalling directs a cascade of transcription factors essential to haemopoietic induction.

1.20 BMP regulation of haemopoietic stem cells in vitro

Studies in lower organisms implicating a critical role of BMP activity in HSC development can now be extended to *ex vivo* culture systems to investigate the potential of BMP4 to influence HSC properties. An important study by Bhatia *et al.* has shown that BMPs are able to regulate the proliferation and differentiation of $CD34^+Lin^-CD38^-$ HSC during *in vitro* culture [117]. Here *ex vivo* cultures were performed stroma-free in media supplemented with 1% BSA (bovine serum albumin) with supplemental growth factors including Flt-3L (300 ng/ml), SCF (300 ng/ml), G-CSF (50 ng/ml) and IL3 (10 ng/ml). Expanded haemopoietic cells were assessed at day 2, day 4 and day 6 by immunophenotyping (for $CD34^+CD38^-$ primitive cells) as well as standard LTC-IC, CFU-GM and SRC analysis. They reported that high doses of exogenous BMP4 (25 ng/ml) extended the capacity of the serum free cytokine cocktail to maintain CB-derived HSC with the ability to repopulate NOD-SCID recipients in the SRC assay (83% maintenance of SRC after a 6 day culture period). These effects were shown to be highly concentration dependent, as at a lower dose (5 ng/ml), BMP4 promoted complete differentiation of the starting primitive $CD34^+Lin^-CD38^-$ population to $CD34^+CD38^+$, with resultant decreases in cell expansion and CFC output and a significant loss in the numbers of day 4 SRC. BMP2 and BMP7 were also tested in these assays, and shown to have a concentration dependent effect. Low doses (5 ng/ml) of BMP2 or BMP7 were tolerated without effect on day 4 SRC, however higher doses (50 ng/ml) resulted in populations acquiring a $CD38^+$ phenotype, a reduction in CFC and a significantly reduced level of SRC at day 4. Bhardwaj *et al.* reported that primitive CB populations

($CD34^+CD38^-Lin^-$) up-regulate expression of BMP4 as well as the BMP inhibitor noggin in response to cytokine [110]. Given that the balance of BMP concentration can dramatically effect HSC maintenance, detailed analysis of BMP production in stromal cell lines such as AFT024, and cytokine cultured CB cells is clearly warranted. Information derived from this type of investigation has potential to influence the development of culture systems for clinical *ex vivo* expansion of CB-derived HSC.

1.21 Project aims/rationale

A significant factor limiting the success of CB transplantation is the small cell harvest and associated HSC dose which reduces the current potential of this therapy to paediatric cases. Whilst HSC can not be easily expanded *in vitro* with stroma-free culture, some stromal cell lines can support long-term maintenance/expansion of HSC *ex vivo*. This project aims to identify important signals involved in maintenance of primitive haemopoietic cells during stromal non-contact cultures (focusing on the BMP family of growth factors) and apply these findings to the development of a clinically relevant *ex vivo* culture systems. BMP4 plays an important role in early embryonic patterning and the onset of haemopoiesis, is produced when CB stem cells are cultured, and has the capacity to alter the self-renewal and expansion properties of the HSC. We hypothesise that BMP proteins may have an important role in maintaining/expanding CB haemopoietic progenitor cells in stroma and stroma-free *ex vivo* expansion cultures. We therefore propose to further characterise/manipulate BMP4 activity in *ex vivo* cultures with the aim of enhancing HSC numbers. We will investigate this directly using the AFT024 co-culture model for CB HSC maintenance/expansion. In addition we aim to investigate the potential of BMP4 as a component in clinical *ex vivo* expansion cultures of CB $CD34^+$ cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines

Murine fetal liver-derived stromal cell lines used for *ex vivo* co-cultures with CB HSC were grown in DMEM supplemented with 20% fetal calf serum (FCS) and antibiotics. Cell lines used were AFT024, 2012, 2058, 2018 and 2012 (A kind gift of I. Lemishka, Princeton University) [74].

2.1.2 Primary cells

Fresh primary human CB was obtained under ethics approval from volunteer donors at the Women's & Children's Hospital Adelaide, South Australia. Whole CB was drained into Baxter CPDA-1 single blood packs (450 ml, ref FBR7110).

2.1.3 General chemicals

The following standard chemicals and reagents were used in this study. All chemicals were of analytical grade;

Acetone	Ajax, Australia
Acrylamide, bis-acrylamide:	Biorad Laboratories, USA
Agar bacterioloical	Oxoid, UK
Agarose	Progen, Australia
SeakKem GTG agarose	Cambrex, USA
Ampicillin	Boehringer, Germany
Calcium chloride	Ajax, Australia
Chloroform	Ajax, Australia
Diethylpyrocarbonate (DEPC)	Sigma, USA

Dimethyl formamide (DMF)	BDH, Australia
Dimethyl sulphoxide (DMSO)	Ajax, Australia
EDTA (ethylenediamine tetra-acetic acid)	Sigma, USA
Ethanol	BDH, Australia
Ethidium bromide (EtBr)	Sigma, USA
Glacial acetic acid	BDH, Australia
Glycerol	Ajax, Australia
Glycogen	Roche, Germany
Isoamyl alcohol	Sigma, USA
Lymphoprep	Axis-shield, Norway
Magnesium chloride ($MgCl_2.6H_20$)	Sigma, USA
Methanol	Ajax, Australia
Phenol (RNA), water saturated, pH 4.5	Amresco, USA
Phenol (DNA), water saturated pH 6.6	Armresco, USA
Potassium acetate	BDH, Australia
Potassium chloride	Ajax, Australia
Sodium acetate	Ajax, Australia
Sodium chloride	M&B, Australia
Sodium dihydrogen orthophosphate	Ajax, Australia
Sodium hydrogen orthophosphate	Ajax, Australia
Sodium hydroxide	Ajax, Australia
TEMED	Promega, USA
Tri-sodium citrate	BDH, Australia
Tris hydrochloride	Sigma, USA
Tryptone	Oxoid, UK

2.1.4 Tissue culture reagents

DMEM	JRH, USA
RPMI 1640	JRH, USA
HANKS buffer	JRH, USA
PBS buffer	JRH, USA
Fetal calf serum	CSL, NSW, Australia
HEPES buffered saline	Cambrex, USA
Penicillin/streptomycin	Gibco /Invitrogen, USA
Sodium pyruvate	Gibco /Invitrogen, USA
L-glutamine	Gibco /Invitrogen, USA
Trypsin	Gibco /Invitrogen, USA
SCF	Amgen, USA
TPO	Amgen, USA
FL	Amgen, USA
G-CSF	Amgen, USA
GM-CSF	Amgen, USA
IL-3	Amgen, USA
IL-6	Amgen, USA
EPO	Amgen, USA
rBMP4 (human)	R&D Systems, USA
rFollistatin (human)	R&D Systems, USA
rNoggin (human)	R&D Systems, USA
Monoclonal BMP4 neutralising antibody (clone 66119)	R&D Systems, USA
Methylcellulose medium (methocult)	SCT, Canada
X-Vivo10™	Cambrex, USA

2.1.5 *Molecular biology reagents*

AmpliTaq Gold™ Polymerase	Applied Biosystems, USA
DNA free DNAse I kit™	Ambion, USA
MMLV reverse transcriptase	Qiagen, USA
dNTPs (dATP, dCTP, dGTP, dTTP)	Roche, Germany
Oligo dT primer	Applied Biosystems, USA
Super-RaseIn™ (RNase inhibitor)	Ambion, USA
SYBR green	Molecular probes, USA
TRIZOL™	Life Technologies, USA
RNeasy kit (micro)™	Qiagen (USA)
Molecular weight standards	Gibco, USA

2.1.6 *Solutions and Buffers*

Solution/ buffer	**Preparation**
MACS buffer	PBS pH 7.2, supplemented with 0.5% bovine serum albumin and 2 mM EDTA. De-gas buffer with vacuum.
Chloroform (RNA extraction)	49:1 solution of chloroform with iso-amyl alcohol
Diethylpyrocarbonate (DEPC) water	0.1% (v/v) DECP with distilled water mixed for ≥ 5 hours at room temperature
DNA Loading buffer (6x)	40% (v/v) guanidinium isothiocyanate, 10% (v/v) sodium citrate pH 7.0, 10% N-lauroysarcosine, 0.1% (v/v) B-mercaptoethanol in DEPC water.

TAE electrophoresis buffer (50x)	0.2% (W/V) trizma base, of sodium acetate (anhydrous) 10mM, EDTA 5mM, adjusted to pH 7.2 with glacial acetic acid.
RNA denaturing buffer	10% deionized formamide, 8% formaldehyde, 1% MOPS buffer (Formamide is deionized by stirring with 20 g of Amberlite MB3 or MB1 ion exchange resin for 15 minutes).
10 x MOPS buffer	0.2 M MOPS (morpholinopropanesulphonic acid), 50 mM sodium acetate, 5 mM EDTA. Adjust to pH 7.0 with 1M NaOH.
Modified RIPA (lysis buffer)	1% NP40, 0.1% SDS, 0.1% NaDeoxycholate (DOC), 150 mM NaCl, Pefablock (complete tablet), 2.5 mM EDTA, 2.5 mM EGTA, 50 mM HEPES, pH 7.4
ELISA wash buffer	0.05% Tween 20 in PBS, pH 7.2 - 7.4
ELISA reagent diluent	1% BSA in PBS, pH 7.2 – 7.4
ELISA substrate solution	1:1 mixture of H_2O_2 and Tetramethylbenzidine
ELISA stop solution	2 N H_2SO_4
1 M tris pH 8.5	(12.1% w/v)Tris base in ~ 750 ml H_2O. Adjust pH to 8.5 with concentrated HCl.
0.5 M MOPS pH 7.0	MOPS free acid (10%) in d H_2O. Adjust pH to 7.0 with 10 N NaOH.
10X TBE	Tris base (0.5 Mm), boric acid (1mM), 0.5 M EDTA (0.25 mM) pH 8.0

Formamide loading dye	95% formamide, 20 mM EDTA pH 8.0, 0.05% bromophenol blue (w/v), 0.05% xylene cyanol (w/v).
10% ammonium persulphate (APS)	10% ammonium persulphate, aliquots stored at -20°C
Denaturing PAGE gel (20%)	7 M Urea, 20% acrylamide, 1% bisacrylamide (w/v) in TBE, polymerized with 0.1% TEMED & APS (v/v)
Buffered glycerol	10% glycerol in 1 mM HEPES pH 7.0, sterilized by autoclaving.
Non-denaturing PAGE gel (20%).	7M Urea, 20% acrylamide, 1% bis-acrylamide (w/v) in TBE buffer, polymerized by addition of 0.1% TEMED (v/v) and 1% APS solution (v/v).
Fetal Calf Serum (FCS)	Heat inactivated at 56°C for 1 hour.
TE	10 mM Tris-Cl, 1 mM EDTA, pH 7.5

2.1.7 Oligonucleotide PCR primers

Primers were designed to amplify PCR products with a melting temperature (T_m) of approx 65°C (Geneworks, Australia). All primers spanned introns.

Gene	Upstream primer (5' to 3')	Downstream primer (5' to 3')	Product (bp)
Cyclophilin A	GGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG	100
BMP4	GCCTGGGCACCTCATCACACGAC	AGGACCGCAGGGCTCACATCAAAA	92
FGF1	GCTTTTATACGGCTCGCAGA	CAGTTCTTCTCCGCGTGCTT	104
Shh	CCGACCGGGACCGCAGCAAGTA	TCGTCGCGGTCCAGGAAGGTGAGG	286
mKirre	CGGGGTGAATTCCAACAAGACTCG	CTGGCCGCTCAAGGTGCTGTAGAT	243
Wnt3a	GAACGCGACCTGGTCTACTACG	GTTAGGTTCGCAGAAGTTGGGT	80
Dlk	CCGCCTTCAGCAACCCCATCC	CTGTGCGGCCGCTACTGTGAAGG	204
Jag1	GACCAGAACGGCAACAAAACTTGATGGAA	TTGGTCTCACAGAGGCACTGCCAGGTTCA	224
Plf2	ACAGCTAAGCCTGGGTAGGACTCT	GATATTTCAGAAGCAGAGCACATG	699
BMP2	CCCCGGGGTATCACGCCTTTTA	GACACCCACAACCCTCCACAACCA	236
BMP7	AGCCCGGGTAGCGCGTAGAG	GCGCCGGTGGATGAAGCTCGA	202
Follistatin	TGCCACCTGAGAAAGGCTAC	ACAGACAGGCTCATCCGACT	201
Noggin	CCCTCTACGCCCTGGTGGTGGTC	AGGGTCTGGGTGTTCGATGAGGTC	127

2.2 Methods

2.2.1 RNA extraction (> 1 x 10^5 cells)

Total RNA was prepared from pelleted cells using TRIZOL™ (Life Technologies, USA) according to the manufactures instructions. Extracted RNA was resuspended in an appropriate volume of RNAse free water and stored at -80°C.

2.2.2 RNA extraction (< 1 x 10^5 cells)

Total RNA from low cell numbers was extracted using the *RNAeasy Micro kit*™ (Qiagen, USA) from rare sorted cell populations according to the manufacturers instructions. Extracted RNA was resuspended in an appropriate volume of RNAse free water and stored at -80°C.

2.2.3 *DNase digestion of RNA*

DNA contamination of RNA samples was removed by digestion with DNaseI using the DNA-free™ kit (Ambion, USA) according to the manufacturers instructions. Recovered RNA was stored at -80°C.

2.2.4 *Agarose-formaldehyde electrophoresis (RNA)*

Agarose was prepared by melting the required amount of agarose in milli-Q and adding 40% formaldehyde and 10 x MOPS respectively. Electrophoresis was performed in 1 x MOPS with 2.2 M formaldehyde. RNA samples were prepared with up to 25 mg of RNA in 5 µl RNAse free water, added to 15 µl RNA denaturation buffer. 1 µl of 10 mg/ml ethidium bromide was added to aid visualisation of RNA after electrophoresis. RNA samples were heated to 65°C for approximately 10 minutes to denature any secondary structure, cooled on ice for 2 minutes and 2 µl of sterile loading buffer added. Samples were loaded and electrophoresed at 5 V/cm. Short wave (254 nm) UV light was used to visualise RNA.

2.2.5 *Phenol/chloroform extraction of DNA*

Equal volumes of buffer-saturated phenol:chloroform (1:1) was added to the DNA solution and mixed well. DNA solutions were vortexed for 10 sec (except for high molecular weight DNA which was gently rocked). The sample was then microfuged on high for 3 mins. Following this, the aqueous layer was removed into a new tube (the above steps were sometimes repeated until an interface was no longer visible). To remove traces of phenol, an equal volume of chloroform was added to the aqueous layer

and microfuged on high for three minutes. Following this, the aqueous layer was removed to a new tube and the DNA was then ethanol precipitated.

2.2.6 Ethanol precipitation of DNA/RNA

To precipitate DNA, the volume of the solution was first measured and the salt concentration was adjusted by adding 1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M) or an equal volume of 5 M ammonium acetate (final concentration of 2.0-2.5 M) (if DNA is in a solution containing salt, the salt concentration was adjusted accordingly to achieve the correct final concentration). Following mixing, 2 to 2.5 volumes of cold 100% ethanol (calculated after salt addition) was added and mixed well. This solution was then place on ice or at -20°C for at least 20 minutes. The solution was then microfuged at maximum speed for 10 minutes and the supernatant carefully decanted. 1 ml 70% ethanol was then added, the solution mixed well and briefly centrifuged. The supernatant was then carefully decanted and the pellet briefly air or vacuumed dried. Finally, the pellet was resuspended in an appropriate volume of TE or water.

2.2.7 Nucleic acid quantitation

For solutions of DNA and RNA, the absorbance was measured at 260 nm where an OD of 1 in a 1 cm path length equalled 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA, and 20-33 μg/ml for oligonucleotides. Purity was estimated by measuring the absorbance ratio at 260 nm and 280 nm. Pure DNA and RNA solutions have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively.

2.2.8 *Quantitative RT-PCR*

2.2.8.1 *Reverse transcription*

RNA samples obtained from sorted CB $CD34^{+}38^{-}Lin^{-}$ and fetal-liver derived stromal cell lines cells were reverse transcribed using Omniscript™ or Sensiscript™ reverse transcriptase kits (Qiagen, USA) respectively. A 20 µl reaction mix was prepared on ice containing 1 µg of total RNA, oligo dT primer (10 µm), dNTP mix (10 mM), RNasin™ (5 Units), Omniscript™/Sensiscript™(5 Units) in RNase-free water up to 20 µl. The reaction was thoroughly mixed by vortexing for no more than 5 seconds. After this, the tube was centrifuged and incubated for 60 minutes at 37°C. The transcriptase was then inactivated by heating the reaction mixture to 93°C for 5 minutes followed by rapid cooling on ice. Samples were stored at -80°C.

2.2.8.2 *Real-time PCR*

PCR reactions were prepared on ice using the AmpliTaq Gold™ polymerase enzyme (Applied Biosystems, USA). A master mix was first prepared containing $MgCl_2$ (3 mM), dNTP mix (10 mM), SybR green (0.6 x), PCR buffer (1 x), primers (450 nM) and cDNA (50 ng) in MQ water. The reaction mixture was then split into two equal volumes (25 µl) and tested in duplicate. PCR tubes were placed in a rotorgene 2000 real-time PCR machine and heated to 95°C for 10 minutes and then cycled for 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds before a final extension step at 72°C for 8 minutes. Melt curve analysis was then performed (from 72°C to 99°C), before a final cycle of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Specific amplification of PCR products was determined by

ethidium agarose gel analysis and products confirmed by sequencing. Negative reverse transcription (RT) controls (without RT enzyme) and negative PCR controls (without template) were also included.

Cycle threshold (C_t) values were used to calculate relative expression using the Qgene™ software package [152]. Using this method, the normalised gene expression is calculated according to the following equation;

$$Normalised\ expression = \frac{(E_{target\ gene})^{Ct\ target\ gene}}{(E_{ref\ gene})^{Ct\ ref\ gene}}$$

Where E is efficiency of amplification

Before a gene could be quantitated an amplification efficiency plot was first performed on control cDNA template after serial dilution. These plots yield the amplification efficiencies of the target and the reference genes which are both crucial parameters for the calculation of normalised gene expression. An efficiency value was then calculated from the slope of the graph showing Ct value plotted against template input (log), according to the equation (as previously described [141]);

$$E = 10^{(-1/slope)}$$

2.2.9 ELISA test

Plates (96 well, Nunc, Norway) were coated with monoclonal BMP4 capture antibody (R&D Systems, USA) and incubated at room temperature overnight. Wells were then coated with supernatant from AFT024 cells (1 x 10^5 cells/ml). After saturation, BMP4 was detected using a monoclonal BMP4 antibody (R&D Systems)

and a horse radish peroxidase-conjugated secondary antibody to rat Ig (R&D Systems). Coloration was performed using O-phenylenediamine dihydrochloride (R&D Systems) and measured at 492 nm on a spectrophotometer.

2.2.10 Isolation of cord blood mononuclear cells

CB was first collected into 450 ml single blood packs (Baxter, USA) and then within 24 hr, diluted at 1:4 with PBS containing 2 mM EDTA. 35 ml of diluted cell suspension was then carefully underlayed with 15 ml of Ficoll-plaque (Amersham, UK) in 50 ml tubes. Following this, tubes were centrifuged for 35 minutes at 400 x g at 20°C in a swinging-bucket rotor. The upper layer was then aspirated leaving the mononuclear cell layer undisturbed at the interphase. Carefully the interphase was collected using a plastic Pasteur pipette. Cells in this layer were then washed twice in PBS containing 2 mM EDTA and centrifuged for 10 minutes at 200 x g at 20°C. Finally the cell pellet was resusupended in suitable media.

2.2.11 Freezing and thawing of mononuclear cell preparations

MNC preparations were pelleted by centrifugation, chilled on ice and resuspended at a concentration of 5 x 10^7 cells/ml in cold FCS containing 10% DMSO. Aliquots were frozen in nitrogen vapour phase for a minimum of 3 hours and stored in liquid nitrogen until required. Frozen MNC aliquots were thawed quickly by immersion in a 37°C water bath followed by gradual addition of 10 mls DMEM with 20% FCS. Cells were washed twice in the same medium by centrifugation at 450 x g for 5 minutes followed by removal of the supernatant.

2.2.12 Cell counting

Cells were counted after staining with 0.2% trypan blue (Gibco, USA). Cell viability was determined as proportion of cells excluding dye. Total cell number was determined by standard haemocytometer counting procedure

2.2.13 $CD34^+$ isolation from cord blood mononuclear cell fraction

CB $CD34^+$ cells were isolated using MACS™ technology (Miltenyi Biotech, Germany). Firstly cell suspensions of CB MNC's were prepared at 10^8 cells per 300 ul in MACS buffer. Following this 100µl FcR blocking reagent (Miltenyi Biotech, Germany) was added (per 1 x 10^8 total cells). The cells were then labeled by adding 100 µl CD34 MicroBeads (Miltenyi Biotech, Germany) per 1 x 10^8 total cells, and incubated for 30 minutes at 4°C. Cells were then washed and resuspend in the appropriate amount of buffer for the MACS LS column™ (2 x 10^8 cells per ml). The MACS LS column™ (with column adapter) was then placed in the magnetic field of the MACS separator and rinsed with 3 ml of MACS buffer. Cells were passed through a pre-separation filter to remove clumps. The cell suspension was then applied to the column and allowed to pass through with MACS buffer (3 x 3 ml). The column was then removed from separator, placed on a suitable tube and with 5 ml MACS buffer the cells were flushed out. The magnetic separation step was then repeated using a second column with eluted cells washed in appropriate culture media.

2.2.14 Ex vivo expansion cultures

CB $CD34^+$ cells were co-cultured at 1 x 10^4 cells/ml on 4 µm polyester membrane permeable supports in 6 well transwells (Costar, USA) and maintained with pre-irradiated AFT024 (20 Gy) seeded at 1 x 10^5 cell/ml. Haemopoietic cells were co-

cultured for 14 or 28 days (having been split 1 in 4 at day 14). Co-cultures were maintained in 10% FCS/RPMI supplemented with human rFL, rSCF and rTPO all at 10 ng/ml (with fresh cytokine or recombinant BMP4 (100 ng/ml) added every 7 days). A BMP4 antibody (2 μg/ml), a matched isotype IgG2b control antibody (2 μg/ml) and human rNoggin (100 ng/ml) were also added to some cultures (all from R&D Systems, USA). Stroma-free *ex vivo* cultures were performed using CB $CD34^+$ cells cultured at 1 x 10^4 cells/ml in 24 well plates. Cultures were maintained in X-Vivo10™ media (Cambrex, USA) without serum, supplemented with SCF, TPO & FL (all at 10 ng/ml).

2.2.15 Cloning assays in semi-solid media

Human clonogenic progenitors were assayed by plating in 1 ml standard methylcellulose (Stem Cell Technologies, USA) supplemented with 2 U/ml EPO (Amgen, USA), 20 ng/ml IL-3 (Amgen, USA), 25 ng/ml SCF (Amgen, USA) and 15 ng/ml GCSF (Amgen, USA) followed by incubation for 7 days at 37°C/5% CO_2. Total CFU-GM expansion was calculated by multiplying total cell expansion by CFU-GM frequency after *ex vivo* culture, divided by CFU-GM frequency of the initial population prior to *ex vivo* culture. Absolute CFU-GM numbers were calculated by multiplying total cell number with CFU-GM frequency after *ex vivo* culture.

2.2.16 Long-term culture assays

LTC-IC assays using human CB haemopoietic cells (1 x 10^4 cells/ml) and 20 Gy pre-irradiated AFT024 cells as feeders (1 x 10^5 cells/ml) were performed under limiting-dilution conditions in long-term culture medium (RPMI supplemented with 12.5% FCS, 12.5% horse serum and 1 x 10^{-4} M β-mercaptoethanol). Statistical analysis and LTC-IC frequency was calculated using L-Calc™ software (Stem Cell Technologies, USA,

www.stemcell.com) as described in Section 2.2.19. Total LTC-IC expansion was calculated by multiplying total cell expansion by LTC-IC frequency after *ex vivo* culture, divided by LTC-IC frequency of the initial population prior to *ex vivo* culture. Absolute LTC-IC numbers were calculated by multiplying total cell number with LTC-IC frequency after *ex vivo* culture.

2.2.17 Engraftment assays

NOD-SCID mice of age 6-8 weeks were obtained from The Walter and Eliza Hall Institute of Medical Research Melbourne, Australia. Mice were kept in specific pathogen-free conditions and maintained on acidified water and autoclaved food. Pooled human CB $CD34^+$ cells (1×10^5) or cells harvested from *ex vivo* cultures (having been cultured at 1×10^5 CB $CD34^+$ cells in 5 ml co-cultures for 14 days, or having been split in fresh wells at day 14, for 28 days) were injected into the tail vein of irradiated NOD/SCID mice (mice received 250 cGy of total body irradiation at a dose rate of 325 cGy/min by parallel-opposed 4 MV x-rays2.5 Gy [153]). At 4-5 weeks after transplantation, nucleated BM cells from transplanted animals were analysed by flow cytometry for the presence of human CD45. Mice were considered to be positive for human HSC engraftment when at least 1.0% $CD45^+$ human cells were detected in a population of mouse BM cells. Cells from positive mice were further phenotyped using a range of antibodies, as described in Section 2.2.18. These experiments were performed with appropriate ethics approval.

2.2.18 Cytofluorometry

Fluorescein isothiocyanate (FITC)-conjugated antibody to CD38, phycoerythrin (PE)-conjugated antibody to CD34 (Becton, Dickinson and Company) were used for

immunophenotyping. Positive or negative populations were determined by their measured fluorescent intensity in relation to relevant matched isotype negative control antibodies according to standard protocols [117]. The presence of human cells in NOD-SCID mouse BM was determined using human CD45-FITC and mouse CD45 PE-Cy5 antibodies (Becton, Dickinson and Company), and immunophenotyping was performed with human CD3, CD33, CD19 and CD34 fluorescent-labelled antibodies (Becton, Dickinson and Company). Haemopoietic progenitor cell viability was determined by staining cultured cells with CD45-FITC and CD34-PE (Becton, Dickinson and Company) as well as 7-Amino-Actinomycin D (7 AAD) (Sigma, St Louis, MO) to detect dead cells.

2.2.19 Statistical analysis

All results are expressed as mean ± standard error of the mean (unless stated otherwise). Data were analysed using the students t-test as appropriate for the data set. $P<0.05$ was considered significant.

For LTC-IC (limiting dilution) calculations a linear relationship was obtained when the logarithm of the proportion of negative cultures was plotted against the total number of cells used to initiate each culture. The generalised linear model was fitted by finding the maximum likelihood using the Newton-Raphson method, and the "goodness-of-fit" to the model was assessed by the generalised Pearson Chi-square. The LTC-IC frequency in the HSC enriched CB cells corresponds to the derived cell dose at which 37% of the cultures yielded a negative response [60]. Statistical analysis and LTC-IC frequency was calculated using L-Calc™ software (Stem Cell Technologies, USA).

CHAPTER 3

CHARACTERISING THE AFT024 STEM CELL NICHE

3.1 Introduction

It is widely accepted that stem cells *in vivo* are intimately associated with discreet micro-environmental 'niches' [83, 154-156]. Adult BM, fetal liver and extra-embryonic placenta have all been shown to provide a specialised and supportive environment for HSC [17, 96, 97, 103, 155, 156]. Haemopoiesis can be mimicked *in vitro* by culturing progenitors on confluent layers of supportive stromal cells derived from adult, fetal or embryonic origin [62, 89, 91, 102, 157]. These stromal cells are useful tools for identifying growth factors that may mediate clinical *ex vivo* expansion of CB HSC. This project focuses on the murine fetal liver stromal cell line AFT024 which permits long-term maintenance and expansion of HSC as measured using *in vitro* assays (LTC-IC) and assays for multilineage engraftment using the myeloablated NOD-SCID mouse model [74, 158]. AFT024 therefore represents a manipulable *in vitro* model of fetal liver haemopoiesis [106] that can be used to dissect HSC regulation. It can be postulated that the AFT024 stromal cell line promotes haemopoiesis by producing unique soluble growth factors or growth factor combinations. However it has been recently shown that AFT024 do not express soluble haemopoietic cytokines previously associated with maintenance of LTC-IC (such as IL-3, IL-6, IL10, TNFα & TPO) [159]. Whilst Plf2 [109] and Dlk [108] have been shown to contribute to HSC support, these molecules account for only a portion of the supportive activity of AFT024 [107]. Here we determine expression by AFT024 of a number of soluble factors that have been recently reported to act on HSC *in vitro* and support self-renewal (these include Shh, BMP4, Wnt3a, notch ligands and FGF1) [110-112, 114, 116, 117] (see Introduction section 1.15). In particular we show an important role for BMP4 which is a member of the TGFβ super-family of pleiotropic regulators [160]. By

identifying and characterising factors that contribute to AFT024 support of HSC, we aim to further understanding of the stem cell niche and this in turn, will provide important insight relevant to the field of CB HSC *ex vivo* expansion.

3.2 *Growth factor mRNA expression in AFT024*

Here we determine mRNA expression in AFT024 for a number of growth factors that have been recently reported to act on HSC *in vitro* and support self-renewal (see above). Specific primers were designed to measure mRNAs encoding Shh, BMP4, Wnt3a, Jag1 and FGF1 (Section 2.1.7). Growth factor mRNA expression in *de novo* AFT024 was quantitated by real-time RT-PCR (see Methods section 2.18). Real-time quantitative PCR (QPCR) represents a highly sensitive and powerful technique for the quantitation of mRNA that allows for high-throughput analysis of gene expression [161]. For this project QPCR data was analysed and quantitated using Qgene™ analysis [152] (see Methods section 2.2.8). Of the growth factors measured in *de novo* AFT024, Shh, Fgf1 and Wnt3a mRNA were undetectable or present at relatively low levels (**Figure 3.1**). BMP4, Jag1 and mKirre mRNA were abundantly expressed suggesting a potential role in HSC self-renewal. Plf2 and Dlk, were detected, as reported previously [108, 109].

3.3 *Comparison of AFT024 to matched fetal liver stromal cell lines*

At a cellular level, the haemopoietic microenvironment consists of numerous distinct cell types. In BM, osteoblasts play a key role [96, 162], however, in fetal liver the key component cell types have not yet been identified. Previous studies have shown that stromal cell heterogeneity reflects a similarly broad range of haemopoietic

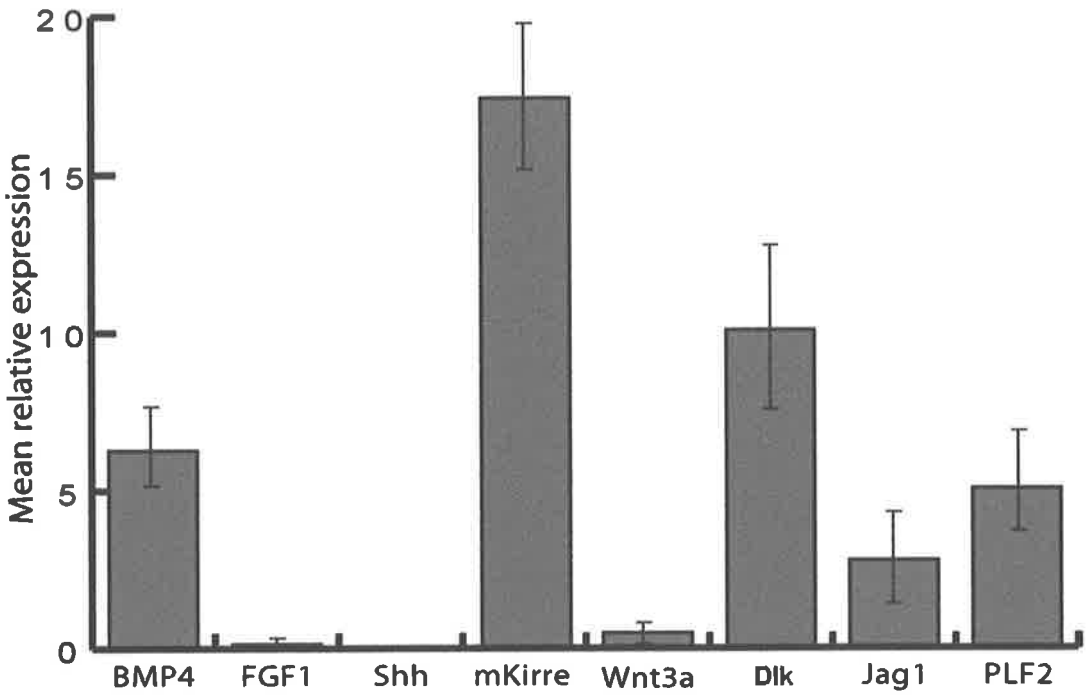


Figure 3.1 Expression profile of AFT024. Quantitaive PCR analysis of AFT024 total RNA to measure mRNA encoding specific growth factors known to act on HSC. Total RNA samples from *de novo* AFT024 were reverse transcribed and specific PCR products measured in duplicate real-time PCR reactions using *cyclophilin A* as control ($n=3$).

supportive abilities [103]. Some rare cloned stromal cell lines can support stem cell activity *in vitro*, while most are ineffective [103]. Experiments in this chapter compare growth factor production in a series of matched fetal liver derived stromal cell lines with varying levels of HSC support. Direct comparison of these cell lines to AFT024 may help dissect the mechanism of HSC regulation. AFT024 is one of a panel of matched stromal cell lines derived from murine fetal liver by immortalisation with a temperature-sensitive Simian virus 40 (SV40) T antigen [74]. All the stromal cell lines tested (including BFC012, 2012, 2018 and 2058) supported the proliferation and differentiation of myeloid cells in Dexter type BM cultures [103]. However, these cell lines differed markedly in their ability to maintain LTC-IC and stem cells with *in vivo* repopulating capacity [74, 103]. For this project the fetal liver cell lines BFC012, 2012, 2018 and 2058 (obtained from Dr I. Lemischka and Dr K. Moore of Princeton University) were initially compared against AFT024 to determine LTC-IC support (using freshly isolated CB $CD34^+$ cells) (see Methods section 2.2.16) (**Figure 3.2**). As described previously, AFT024 maintained the highest LTC-IC frequency (117.6 ± 17.6 LTC-IC per 10,000 input CB $CD34^+$ cells) (**Figure 3.2**). This was followed by 2058 (35.7 ± 5.4 per 10,000 cells), 2012 (25.0 ± 3.8 per 10,000 cells), 2018 (8.3 ± 1.3 per 10,000 cells), and finally BFC012 (0.2 ± 0.03 per 10,000 cells) which displayed negligible haemopoietic progenitor supportive activity (as previously described [74, 103, 158]).

The level of mRNA expression of BMP4, FGF1, Shh, mKirre, Wnt3a, Jag1 and Plf2 was then determined in these five fetal liver stromal cell lines (n=3) (**Figure 3.3**). Of the growth factors that had relatively low expression in AFT024, Shh was also not detected in any of the other cell lines, and FGF1 was shown to have similar expression in 2018 and BFC012. The relatively high expression of mKirre in AFT024 compared to

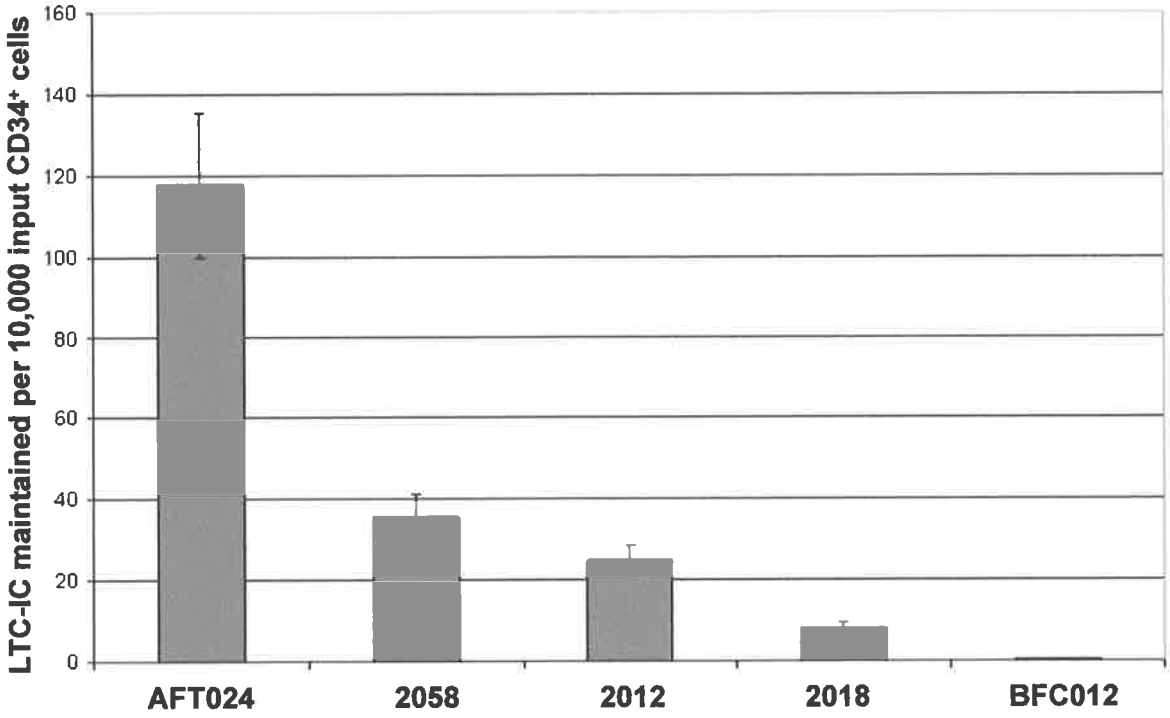


Figure 3.2 LTC-IC output from fetal liver stromal cell lines. LTC-IC assays were performed using fresh human CB CD34$^+$ cells and fetal liver stromal cells (AFT024, 2018, 2012, 2058 and BFC012) pre-irradiated at 20 Gy in long-term (5 week) culture (see Methods section 2.2.16). Graph shows average LTC-IC maintained from 10,000 fresh CB CD34$^+$ cells using different stromal feeders (n=2).

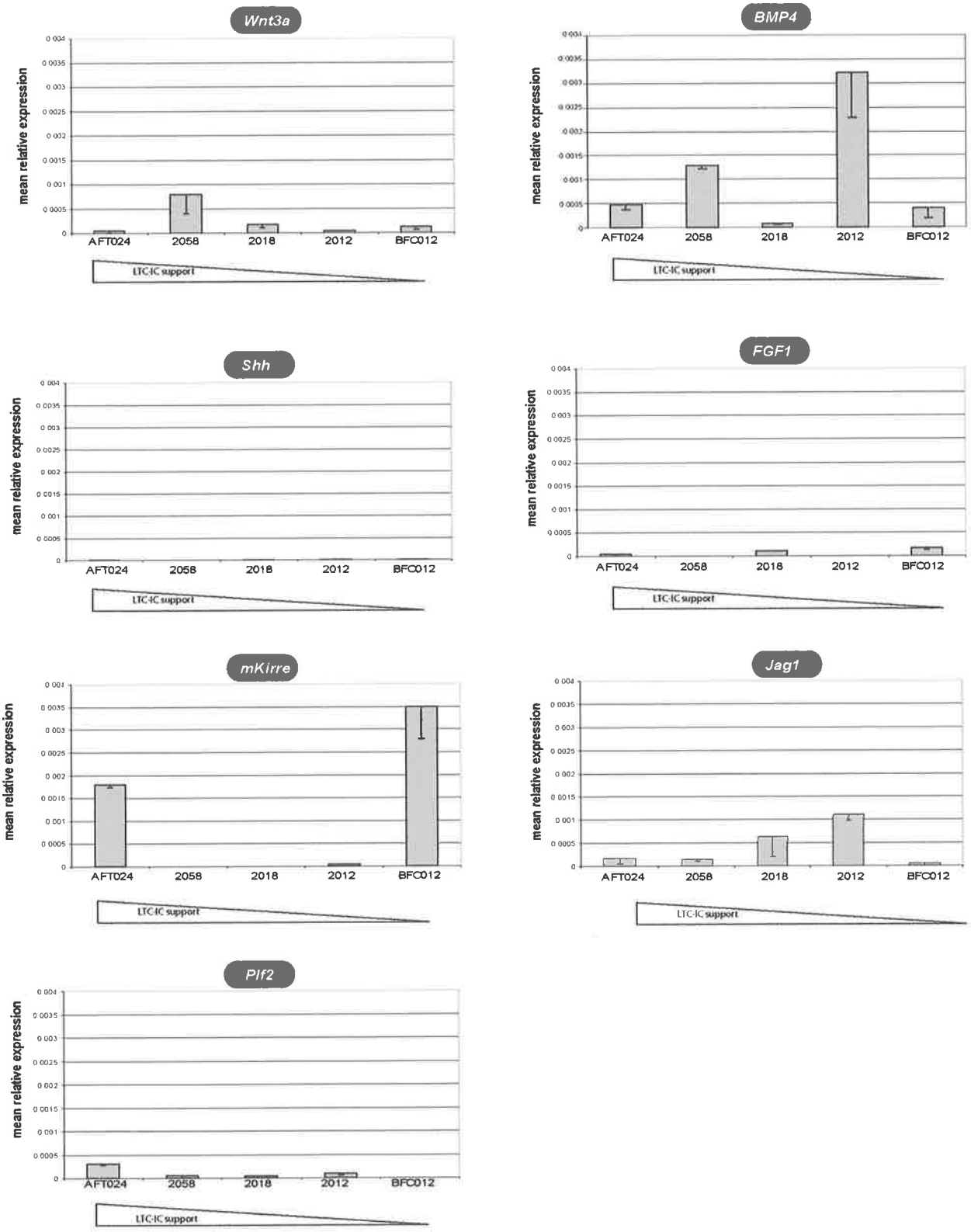


Figure 3.3 Growth factor mRNA expression profile of AFT024. Relative mean expression in stromal cell lines of growth factor mRNAs for Wnt3a, FGF1, Shh, BMP4, mKirre, Jag1 and Plf2 quantitated by QPCR. Total RNA samples from *de novo* fetal liver stromal cell lines (AFT024, 2018, 2012, 2058 & BFC012) were treated with DNaseI and reverse transcribed. Specific PCR products were measured in duplicate real-time PCR reactions and were quantitated against the control gene *Cyclophilin A,* using Q-gene™ software analysis (*n=3*).

most of the other cell lines suggests a potential role in AFT024 maintenance, although it was also expressed in BFC012. BMP4, Wnt3a, Jag1 and Plf2 were expressed in all supportive cell lines (albeit at diverse levels), and it is possible that these factors are key to HSC maintenance. The above expression analysis (as summarised in **Table 3.1**) demonstrated an overall poor correlation of LTC-IC maintenance with growth factor expression.

3.4 BMP expression in AFT024

From the above mRNA expression analysis, BMP4 was an attractive candidate to investigate further as BMP expression in AFT024 has not been characterised previously. BMP2 and BMP7 which have also been reported to exhibit primitive haemopoietic cell activity [117], as well as BMP8 which can regulate maintenance of germ line stem cell division [163], were also studied. We first determined the BMP mRNA expression profile in *de novo* AFT024. QPCR was performed using primers for BMP2, BMP7, BMP8 and the BMP antagonists follistatin and noggin (Methods section 2.1.7). BMP2 mRNA was not detectable by QPCR, whilst significant levels of BMP7 and BMP8 mRNA were observed (**Figure 3.4**). The BMP antagonists follistatin and low levels of noggin mRNA were also both expressed in AFT024. BMP4 mRNA production was also demonstrated in primary human osteoblast cells (known to secrete BMP4 at biologically active levels [164]) at similar levels to AFT024 (**Figure 3.4**).

		LTC-IC maintenance				
		AFT024	2058	2018	2012	BFC012
HSC growth factor mRNA expression	**Wnt3a**	+	++	+	+	+
	FGF1	+	-	+	-	+
	Shh	-	-	-	-	-
	BMP4	++	++	+	+++	++
	mKirre	+++	-	-	-	+++
	Jag1	+	+	+	++	+
	Plf2	++	+	+	+	-

Table 3.1 Summary of growth factor mRNA expression and LTC-IC maintenance. Comparison of stromal cell line support of LTC-IC (see Figure 3.2), with growth factor mRNA expression as determined by QPCR (see Figure 3.3).

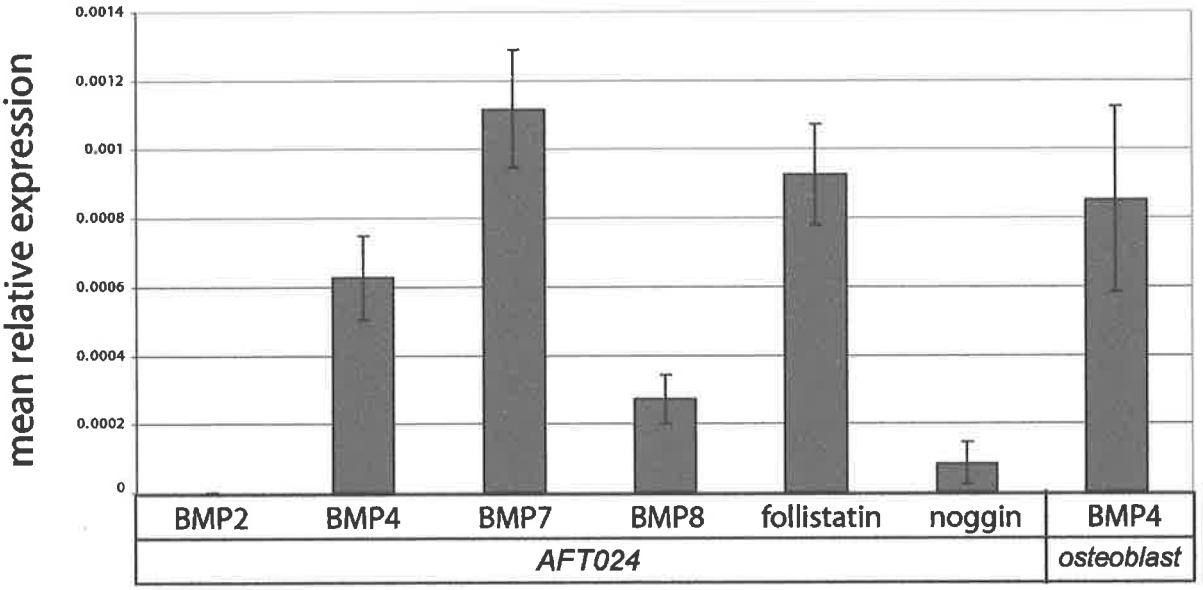


Figure 3.4 BMP mRNA expression profile of AFT024. Relative mRNA expression of BMP2, BMP4, BMP7 and BMP8 as well as the BMP antagonists noggin and follistatin in *de novo* AFT024 (and BMP4 expression in primary human osteoblast cells) by QPCR. Total RNA samples from *de novo* cells were treated with DNaseI and reverse transcribed. Specific PCR products were measured in duplicate real-time PCR reactions and were quantitated against the control gene *Cyclophilin A,* using Q-gene software analysis (*n=3*).

3.5 *BMP4 protein secretion by AFT024*

Having demonstrated BMP4 mRNA expression in AFT024, it was important to show that this resulted in BMP4 protein secretion. The level of AFT024-secreted BMP4 protein was therefore determined using a sandwich ELISA. AFT024 culture supernatant was collected over 72 hours and analysed (see Methods section 2.2.9). We found that detectable levels of BMP4 protein accumulated in AFT024 supernatant reaching 723.1 pg/ml (± 32.1 pg/ml) after 72 hr (**Figure 3.5**). Whilst this represents a concentration that is known to have biological activity in many systems [165-167], this level is thirty times lower than the amounts of BMP4 protein reported to have stem cell activity [117]. Bhatia *et al.* demonstrated that BMP4 at 25 ng/ml maintained HSC in *ex vivo* culture whilst a lower concentration of 5 ng/ml promoted primitive cell differentiation [117]. However, Bhatia *et al.* performed these assays in the absence of stroma and serum and therefore it is difficult to directly compare and make conclusions about potential haemopoietic activity. Experiments to address the role of BMP4 in HSC maintenance by AFT024 are discussed in the next chapter.

3.6 *BMP mRNA expression in fetal liver cell lines*

The level of BMP/BMP antagonist mRNA expression was also investigated in the non-supporting fetal liver cell lines (2018, 2058, 2012 & BFC012) (**Figure 3.6**). As described earlier, BMP4 mRNA expression was observed in AFT024, 2058, 2012 and BFC012. Whilst low levels of the BMP antagonist noggin were observed in all cell lines, high levels of follistatin mRNA were detected in 2018 and BFC012 (n=3). Compared to the other four cell lines, BMP7 was highly expressed in AFT024 and was not detected in 2012, or 2058. BMP2 (not expressed in AFT024), was detected in 2018,

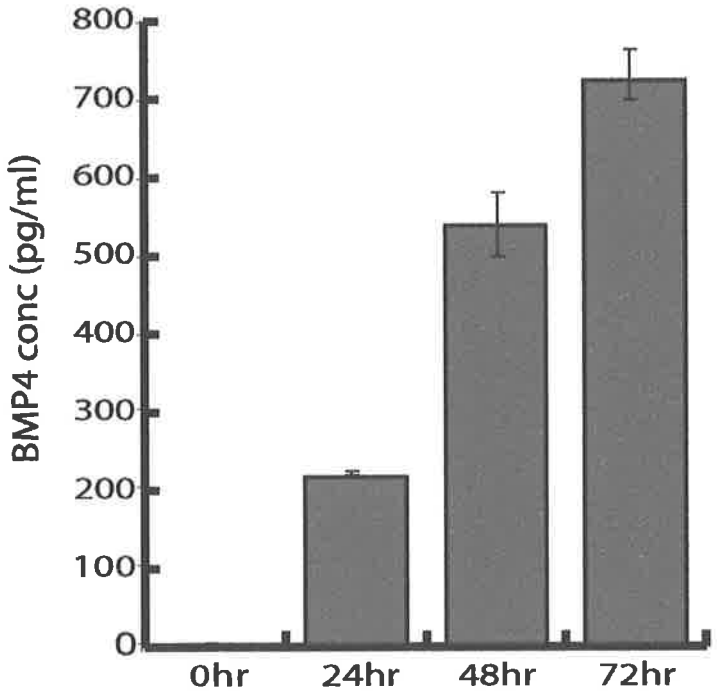


Figure 3.5 BMP4 detection in AFT024 media by ELISA. Accumulation of BMP4 protein in AFT024 supernatant was measured by ELISA after 24 hrs (210.3 ± 3.6 pg/ml), 48 hrs (538.9 ± 42.5 pg/ml) and 72 hrs (723.1 ± 32.1 pg/ml) culture. BMP4 was detected using a sandwich ELISA as described in Methods section 2.2.9 (*n=3*).

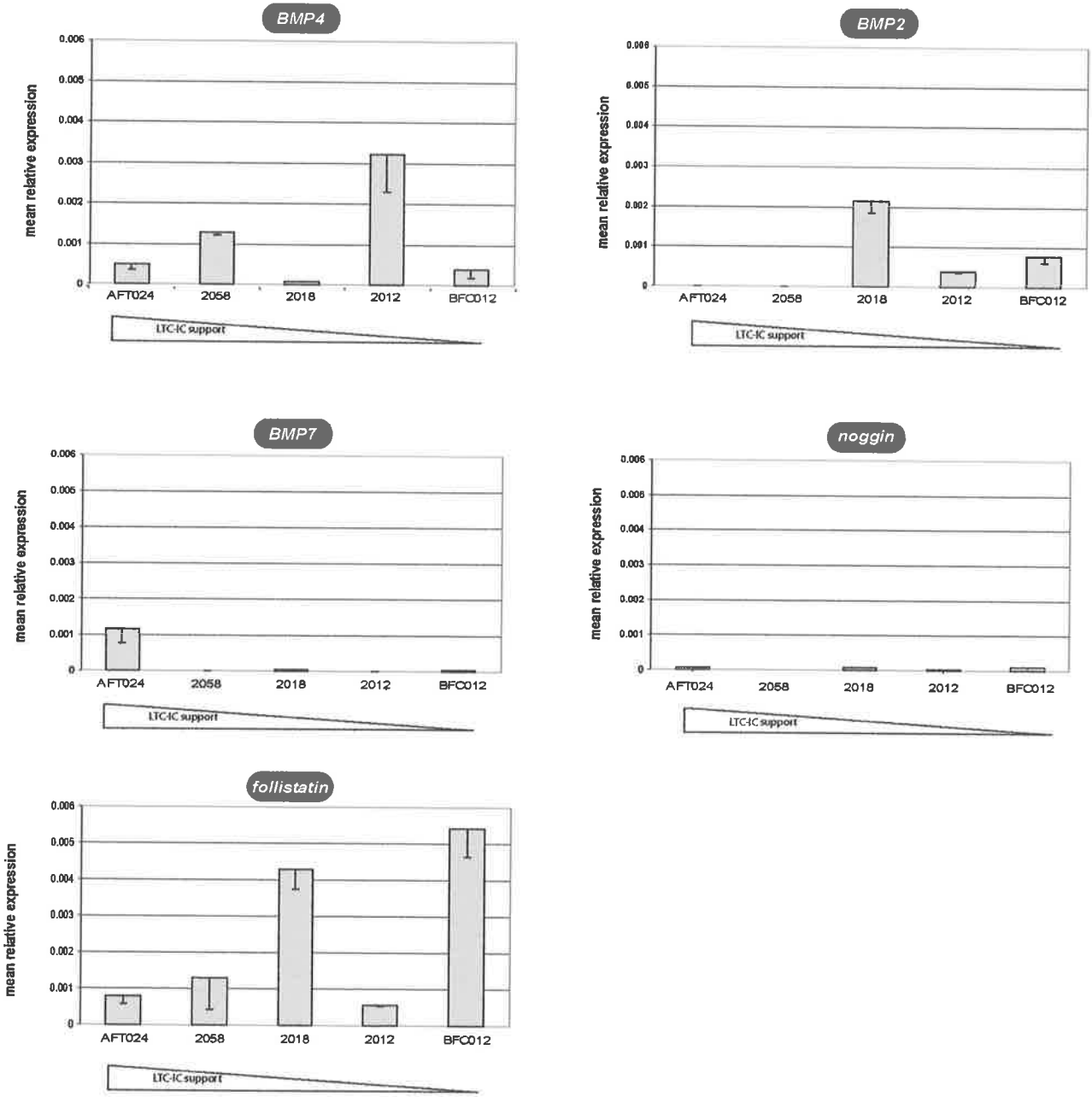


Figure 3.6 BMP mRNA expression profile of fetal liver stromal cell lines. Relative mean mRNA expression of BMP2, BMP4, BMP7, follistatin and noggin in fetal liver stromal cell lines determined by QPCR. Total RNA samples from *de novo* cells (AFT024, 2018, 2012, 2058 & BFC012) were treated with DNaseI and reverse transcribed. Specific PCR products were amplified in duplicate real-time PCR reactions and quantitated against the control gene *Cyclophilin A,* using Q-gene software analysis (*n=3*).

2012 and BFC012 but not 2058. These results describe a complex pattern of BMP and BMP antagonist expression in these stromal cell lines.

The level of BMP4 protein expressed in the non-supporting fetal liver cell lines (2012, 2018, 2058 & BFC012) was then analysed by ELISA (**Figure 3.7**). Stromal cell supernatant was collected and analysed at 24 hrs from each cell line. It was found that the relative level of protein expression generally correlated well with mRNA from *de novo* cells using QPCR. The cell line 2012 secreted the highest amount of BMP4 protein followed by AFT024, 2058, BFC012 and finally the non-supporter 2018 had the lowest BMP4 protein content in supernatant.

3.7 AFT024 respond to human cytokine

Studies using xenotransplantation as a measure of stem cell function have indicated that SCF, FL and TPO are the most important cytokines for promoting HSC expansion in *ex vivo* culture [22, 77, 80]. These early acting cytokines were therefore supplemented into AFT024 *ex vivo* expansion co-culture studies described in Chapter 4. Previous reports have shown that human cytokines can stimulate AFT024 factor secretion and enhance LTC-IC support (compared to culturing in AFT024 conditioned media, with these additional cytokines) [159]. It was next investigated whether FL, SCF and TPO, when supplemented in AFT024 co-culture, induced BMP mRNA production in AFT024 during culture. AFT024 were cultured for 12 hrs with and without addition of cytokine and compared to *de novo* cells. mRNA expression profiles of BMPs and BMP antagonists were then determined by QPCR. In three separate experiments, there was no significant increase in BMP4 mRNA (average 1.38-fold of control) after 12 hrs co-culture compared to *de novo* cells (and cytokine-free cultures) (n=3) (**Figure 3.8**). BMP2 was not detected in cytokine free culture, however in three experiments there

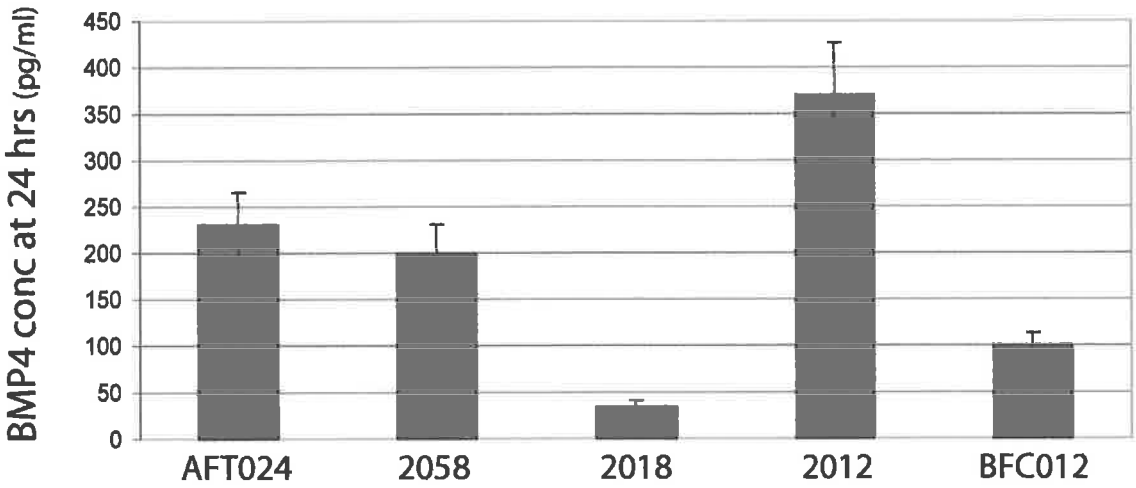


Figure 3.7 BMP4 detection in stromal cell media by ELISA. Accumulation of BMP4 protein in AFT024, 2058, 2018, 2012 and BFC012 supernatant measured by ELISA after 24 hrs culture. BMP4 was measured in duplicate cultures using a sandwich ELISA as described in Methods section 2.2.9 (*n=3*).

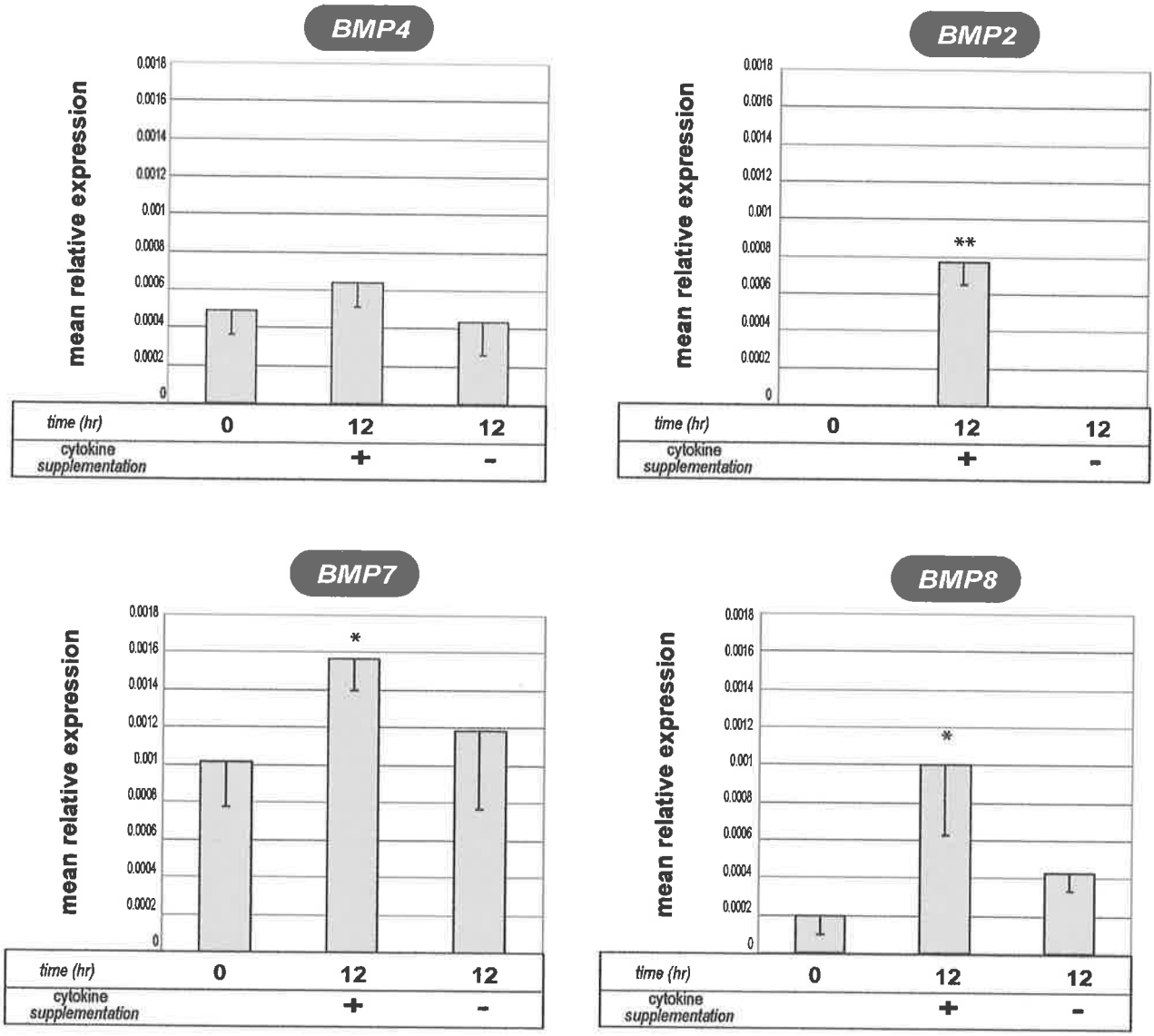


Figure 3.8 BMP mRNA quantitation in AFT024 after cytokine treatment. Relative mean expression of BMP mRNA detected using QPCR in AFT024, treated with or without cytokine stimulation (TPO, FL & SCF at 10 ng/ml) for 12 hrs. Total RNA samples from *de novo* and cultured cells were treated with DNaseI and reverse transcribed. Specific PCR products were amplified in duplicate real-time PCR reactions and quantitated against the control gene *Cyclophilin A,* using Q-gene™ software analysis. BMP2 and BMP8 mRNA were significantly up-regulated following cytokine stimulation (*=P<0.05, **=P<0.005 compared to 0 hr time points, n=3).

was significant upregulation of BMP2 after culture with cytokine ($P<0.005$). BMP7 and BMP8 were also up-regulated following 12 hrs culture in cytokine supplemented media (1.7-fold and 4.1-fold respectively, $P<0.05$, $n=3$). Given that BMP2 and BMP7 have been implicated with a loss of SRC during *in vitro* culture [117], the induction of these factors is not likely to enhance primitive cell maintenance in this system.

3.8 Haemopoietic stem cell cross talk with AFT024

Recent studies have shown that soluble factors released by $CD34^+$ cells can influence the secretion of cytokines, by stromal cells, which may be important for stem/ progenitor cell growth [162, 168, 169]. In addition, it was demonstrated that culturing HSC in AFT024 conditioned media did not reproduce the same level of LTC-IC maintenance observed in AFT024/HSC co-culture [159]. We therefore investigated the cellular cross-talk between CB stem cells and AFT024 cells during *in vitro* co-culture. To test whether co-culture influenced expression of BMPs we measured BMP mRNA expression in AFT024 with and without exposure to HSC (with phenotype $CD34^+CD38^-Lin^-$). Haemopoietic cell populations with phenotype $CD34^+CD38^-Lin^-$ have been shown to be highly enriched for cells with *in vivo*-repopulating ability [170, 171]. Cells with this phenotype were sorted from a CD34 positive fraction of CB using flow cytometry to select for cells lacking CD38 and lineage marker expression (**Figure 3.9a**). To confirm high HSC purity within the sorted population, LTC-IC frequency was determined. It was found that using $CD34^+CD38^-Lin^-$ cells in a serial dilution assay, an LTC-IC frequency of 385 (± 109) per 1 x 10^3 cells was observed (**Figure 3.9b**). This frequency is consistent within the literature for LTC-IC frequency of cells with this

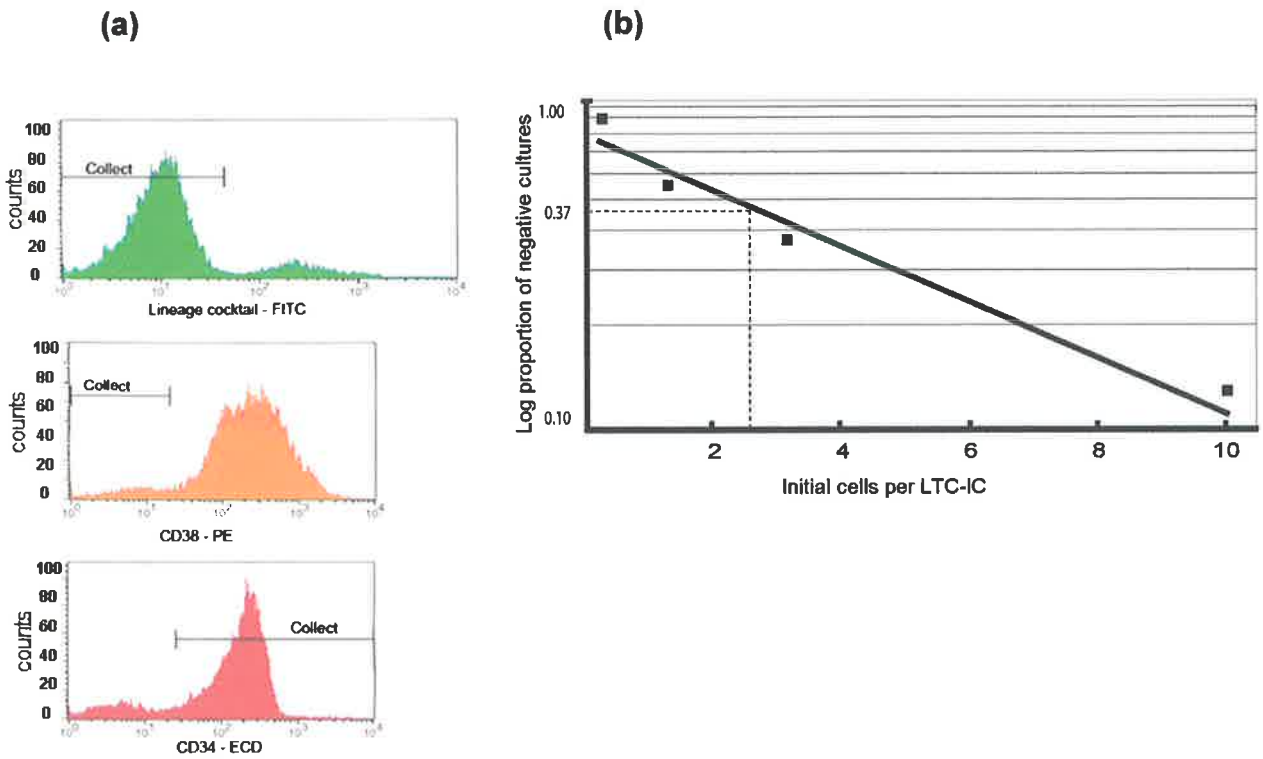


Figure 3.9 Isolation of purified primitive haemopoietic cell populations. (a) Flow cytometry was used to obtain HSC with phenotype $CD34^+CD38^-Lin^-$ from a $CD34^+$ enriched fraction of CB MNC using monoclonal antibodies for CD34, CD38 and a mixed lineage cocktail (CD3, CD4, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, CD2 and CD235a). (b) High primitive cell purity was confirmed by LTC-IC analysis. 1000 $CD34^+CD38^-Lin^-$ cells were cultured on pre-irradiated AFT024 feeder cells under limiting-dilution conditions in long-term (5 week) culture. The LTC-IC frequency of input cells corresponded to the derived cell dose at which 37% of the cultures yielded a negative response (see Methods section 2.2.16). Graph shows an LTC-IC frequency of 1 in 2.6 cells or 385 (± 109) per 10^3 input cells, confirming a highly enriched primitive haemopoietic cell population.

phenotype [80]. Sorted populations of these primitive cells were then co-cultured on AFT024. It was found that although the levels of BMP2 and BMP7 (and to a lesser extent BMP4) mRNA expression increased in AFT024 over 12 hrs co-culture with cytokine (as shown in Section 3.7), the fold increase was not significantly different in cultures with or without HSC (**Figure 3.10**). This result suggests that BMP mRNA production in AFT024 is not regulated by signalling from the HSC. This observation is consistent with previous studies that did not detect differences in cytokine levels in AFT024 supernatants cultured with or without human $CD34^+$ cells [159]. However, Gupta *et al.* have shown that human BM $CD34^+$ cells can regulate stromal production of IL-6 and G-CSF as well as increasing the colony stimulating activity of cloned BM stromal cell lines [168]. Whilst a similar mechanism of crosstalk may occur in AFT024 co-cultures, recent efforts to identify factor(s) induction by haemopoietic progenitors upon interaction with AFT024 stroma, have identified genes mainly associated with cell division and DNA stabilisation and repair [172].

3.9 Induction of BMP4 mRNA in HSC during AFT024 co-culture

We next tested whether HSC respond to external cytokines or AFT024 signalling, and then alter BMP production. A previous study has shown that exposing HSC ($CD34^+CD38^-Lin^-$) to a cytokine cocktail (SCF, G-CSF, Flt-3L, IL3 and IL6) up-regulated BMP4 expression [110]. To test for BMP4 induction by HSC in the AFT024 co-culture system, BMP4 mRNA expression levels were measured in co-cultured primitive populations of CB HSC with $CD34^+$ Lin^-CD38^- phenotype. The $CD34^+Lin^-$ $CD38^-$ population represents less than 1% of the $CD34^+$ population and we obtained only small numbers of cells from each CB donation. mRNA was extracted (as described in Methods section 2.2.2) from small populations of $CD34^+Lin^-CD38^-$ cells (average of

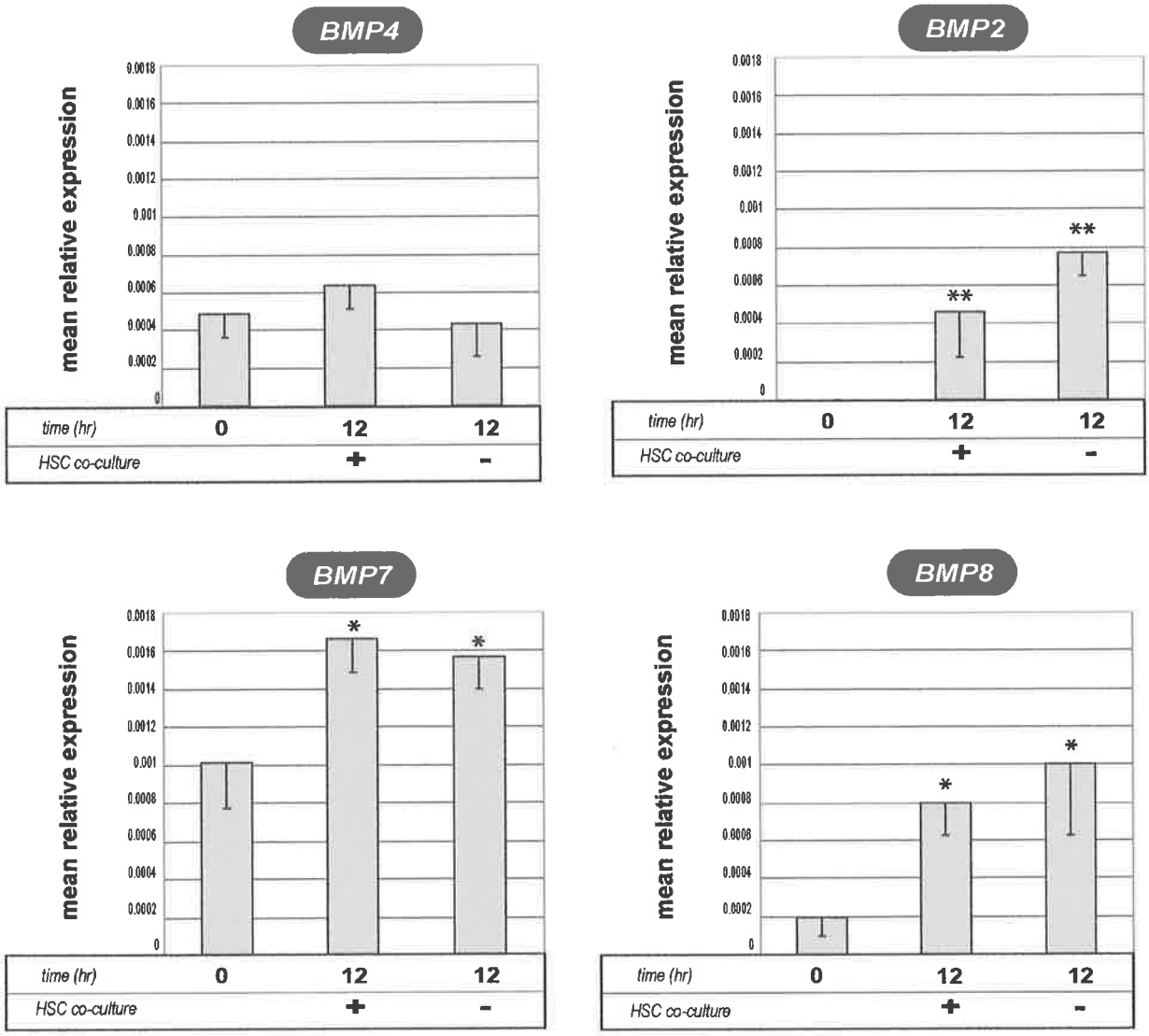


Figure 3.10 BMP mRNA quantitation in AFT024 after co-culture with HSC. Relative mean expression of BMP mRNA by QPCR in *de novo* AFT024 after 12 hr culture with or without CB HSC, and with or without cytokine supplementation (FL, SCF & TPO at 10 ng/ml). Total RNA samples from *de novo* and cultured cells were treated with DNaseI and reverse transcribed. Specific PCR products were amplified in duplicate real-time PCR reactions and quantitated against the control gene *Cyclophilin A,* using Q-gene™ software analysis (*= $P<0.05$, **=$P<0.005$ in comparison to 0 hr time-points, n=3).

8,000 sorted cells) and BMP4 was successfully detected in as few as 5 cells using this method (**Figure 3.11a**). We then established co-cultures using 5 x 10^3 sorted $CD34^+Lin^-CD38^-$ cells in transwells with and without the addition of cytokines TPO, FL and SCF for 12 hrs, and with or without AFT024 co-culture. Significant levels of BMP4 mRNA expression were detected in freshly sorted $CD34^+Lin^-CD38^-$ cells (**Figure 3.11b**). Furthermore, it was found over three experiments that the level of BMP4 mRNA expression was greatly increased (11.1-fold ± 2.9-fold, $P<0.05$) after 12 hours culture with cytokine supplementation. No significant change was observed without addition of cytokine and there was no apparent AFT024-dependent effect on BMP4 mRNA production. mRNA expression for other BMP genes was not measured due to limited RNA availability from these rare primitive cell populations.

3.10 BMP4 expression in mouse primary haemopoietic tissue

The above results suggest the possibility that BMP4 is produced by both HSC and fetal liver stroma and therefore may be important in fetal liver haemopoiesis. To further investigate a potential role in the fetal liver we next measured the level of BMP4 mRNA expression in primary mouse fetal liver cells (at a developmental time-point that is consistent with significant fetal liver HSC expansion [173]) and compared this to AFT024 expression. Using RT-PCR, BMP4 mRNA was found to be expressed in whole murine 14.5 dpc fetal liver (FL) at similar levels to AFT024 (**Figure 3.12**). BMP4 mRNA was also detected in other primary haemopoietic tissues, such as whole mouse spleen, and at lower levels in whole mouse BM and thymus (which include many non-stromal components) and not detected in PB MNC (**Figure 3.12**). High BMP4 expression in primary fetal liver cells suggests it may contribute to fetal haemopoiesis and be associated with HSC expansion in the fetal liver during embryonic development,

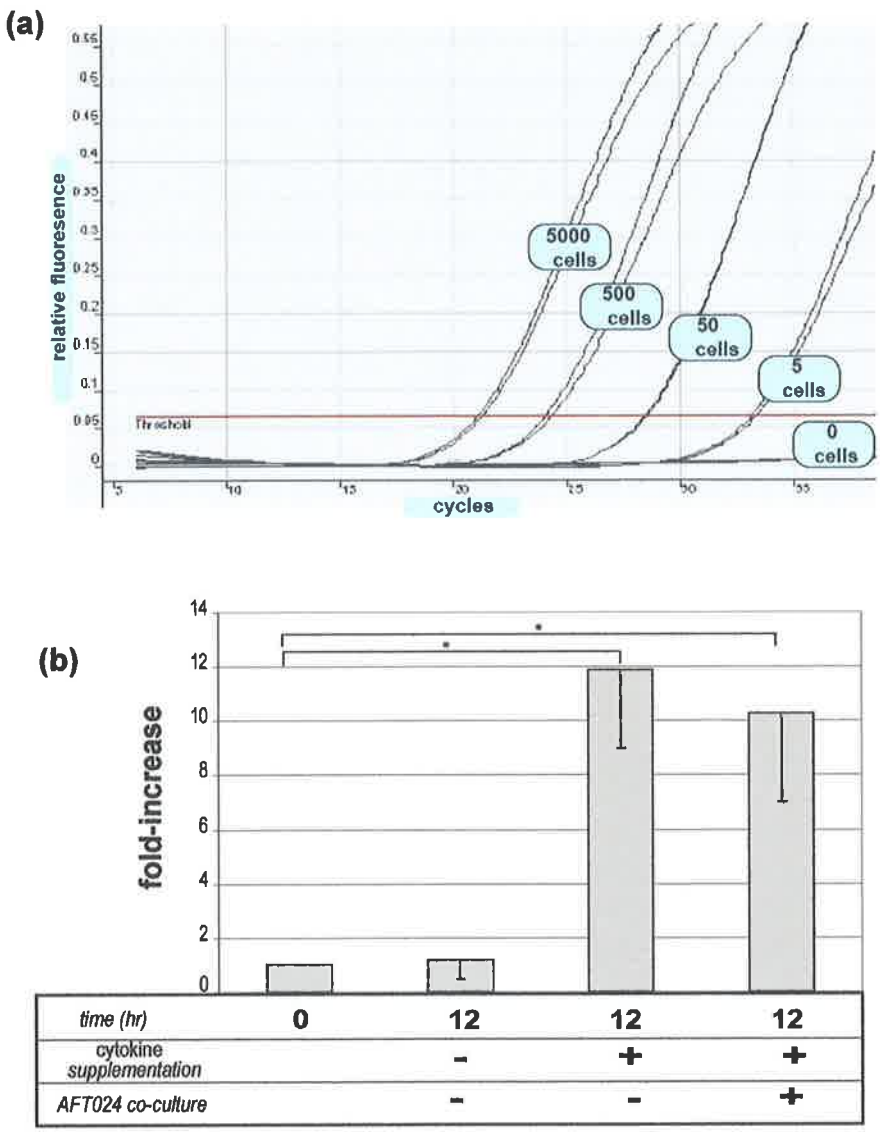


Figure 3.11 BMP4 mRNA detection in HSC. (a) QPCR amplification curves showing high sensitivity of BMP4 mRNA detection from serially diluted suspensions of $CD34^{+}Lin^{-}CD38^{-}$ cells. (b) Mean relative expression of BMP4 mRNA measured in $CD34^{+}Lin^{-}CD38^{-}$ cells with or without co-culture with AFT024 or cytokine stimulation (TPO, FL & SCF all at 10 ng/ml) for 12 hrs. Total RNA samples from *de novo* and cultured cells were treated with DNaseI and reverse transcribed. Specific PCR products were amplified in duplicate real-time PCR reactions and quantitated against the control gene *Cyclophilin A,* using Q-gene™ software analysis. BMP4 mRNA was significantly up-regulated with cytokine stimulation after 12 hrs (*=$P<0.05$ compare to 0 hr time-points, n=3).

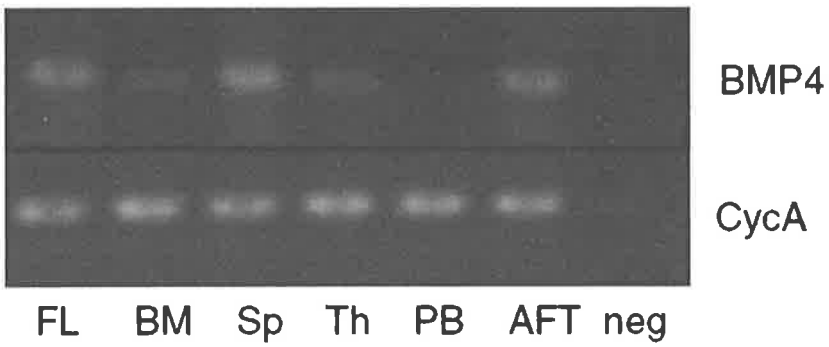


Figure 3.12 BMP4 mRNA detection in murine primary haemopoietic tissue. RT-PCR showing BMP4 expression (at 36 cycles) in primary murine hematopoietic tissue including fetal liver (FL), bone marrow (BM), spleen (Sp), thymus (Th) peripheral blood (PB) as well as AFT024 (AFT) compared to the control gene *cycliphilin A*.

in a mechanism that may be mimicked by AFT024.

3.11 AFT024 as a model of fetal liver haemopoiesis

Recent studies have validated AFT024 as a model of fetal liver haemopoiesis where there is a specialised environment that supports HSC expansion [106]. It has been shown that AFT024 has mixed endodermal (epithelial) and mesodermal characteristics consistent with cells in epithelial-to-mesenchymal transition (EMT). Such characteristics are observed in developing liver and associated with haemopoiesis. This population disappears by the end of gestation, and is replaced by hepatocytes. It has been reported for both primary fetal liver, AFT024, and other stromal cell lines (2018 and BFC012), that maturation of these cells (through Oncostain M treatment) reduced their LTC-IC supportive ability [106]. This change in functional activity corresponded to a decreased EMT phenotype (cells became more epithelial and less mesenchymal). EMT stromal cells may provide a suitable microenvironment which attracts and nurtures HSC during transient haemopoiesis in the fetal liver. We next performed experiments to examine if BMP mRNA induction by cytokines (FL, SCF & TPO as observed in Section 3.7) is associated with a change in EMT phenotype by AFT024 cells, that could potentially alter their supportive capacity. For this we used markers of both epithelial cells and mesenchymal cells that could be detected in AFT024 by flow cytometry. We were unable to detect the mesenchymal marker Stro-1 or the epithelial marker E-cadherin in AFT024 (data not shown) (as reported by Chargouri *et al.*) using this approach, however we did detect cytokeratin-8 (epithelial) and collagen-1 (mesenchymal), consistent with AFT024 displaying characteristics of an EMT phenotype (**Figure 3.13**). After two or four days culture with or without supplemental cytokine (FL, SCF & TPO), or culture with CB $CD34^{+}Lin^{-}CD38^{-}$ cells, the staining for

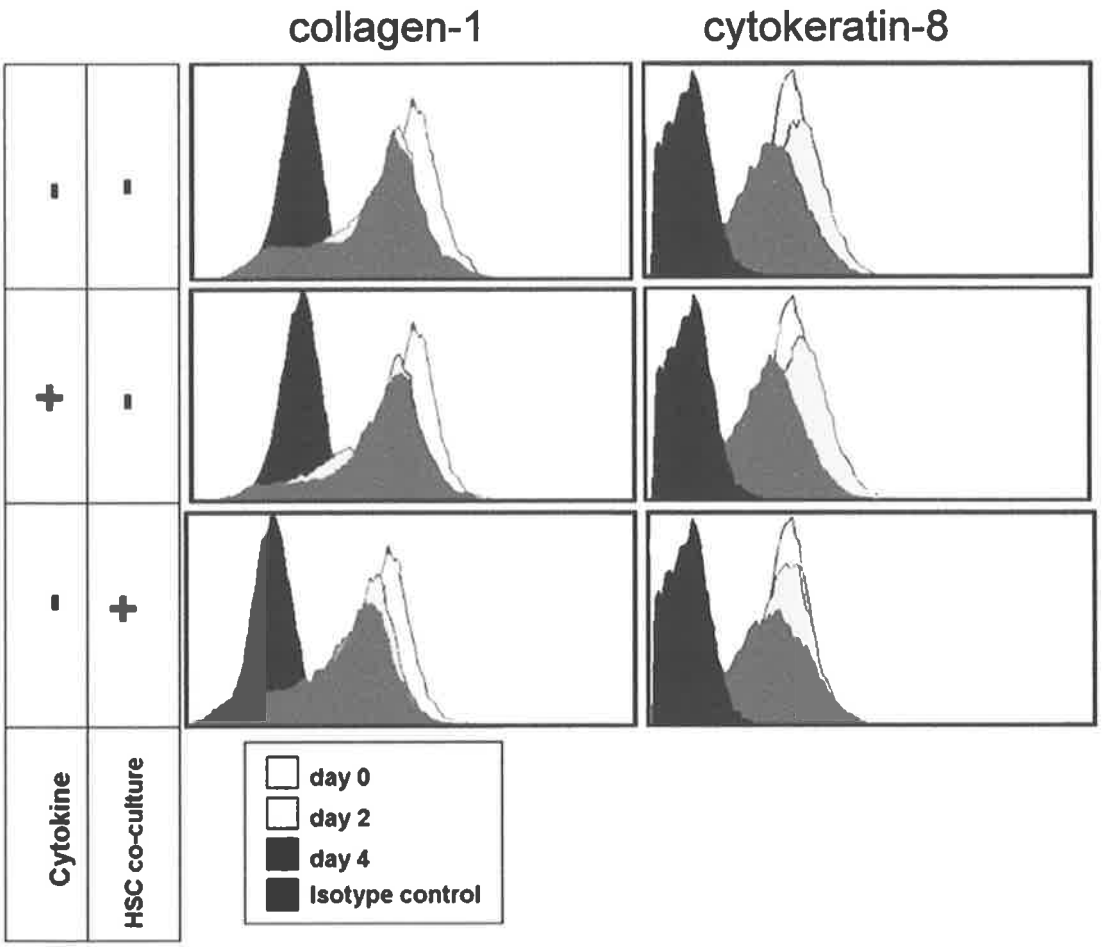


Figure 3.13 Analysis of EMT phenotype in AFT024. Analysis of epithelial and mesenchymal markers in AFT024 cells after culture for 2 and 4 days with and without supplemental cytokines (FL, SCF & TPO at 10 ng/ml) or CB $CD34^{+}Lin^{-}CD38^{-}$ cells in trans-wells. Cultured and *de novo* AFT024 cells were permeabilised and stained for the EMT markers, collagen-1 (epithelial) or cytokeratin-8 (mesenchymal) as well as isotype control antibodies. Changes in EMT phenotype of cultured AFT024 was then assessed using flow cytometry (representative of 3 experiments).

these markers did not significantly change compared to *de novo* AFT024. We conclude that AFT024 do respond to human cytokine (SCF, FL & TPO as shown in Section 3.7) however this does not appear to be related to changes in the EMT phenotype as demonstrated by surface marker staining.

3.12 Conclusion

The stromal haemopoietic microenvironment provides a complex molecular milieu that regulates the self-renewal and differentiation activities of stem cells [17]. This chapter demonstrated expression for a number of growth factors known to regulate HSC activity, at mRNA level in the supportive fetal liver stromal cell line AFT024. Some of these factors including mKirre, BMP4, and the notch ligand Jag-1 could potentially be contributing to the long-term HSC maintenance reported with AFT024 (lack of commercially available recombinant protein or antibody at the time of this study limited further characterisation of mKirre). BMP4 mRNA was found to be expressed at equivalent levels to Dlk and Plf-2 mRNA raising the possibility that BMP4 protein could be produced and contribute to AFT024 maintenance of HSC [108, 109]. Analysis of mRNA for other BMP and BMP antagonists was also performed on AFT024, showing significant expression for BMP7, BMP8 and follistatin. Furthermore, AFT024 responded to cytokine treatment (TPO, FL & SCF) by up-regulation of BMP 2, 7 and 8. Thus there is a complex pattern of BMP expression in the AFT024 culture system and this is affected by the presence of haemopoietic growth factors (as summarised in **Figure 3.14**). Previous attempts to identify molecular signals provided by AFT024 have used microarray analysis and *de novo* AFT024 [107]. It is possible that key factors required for HSC support are dependent on growth factor stimulation on AFT024 and these may not be revealed in such studies. In an effort to correlate supportive capacity

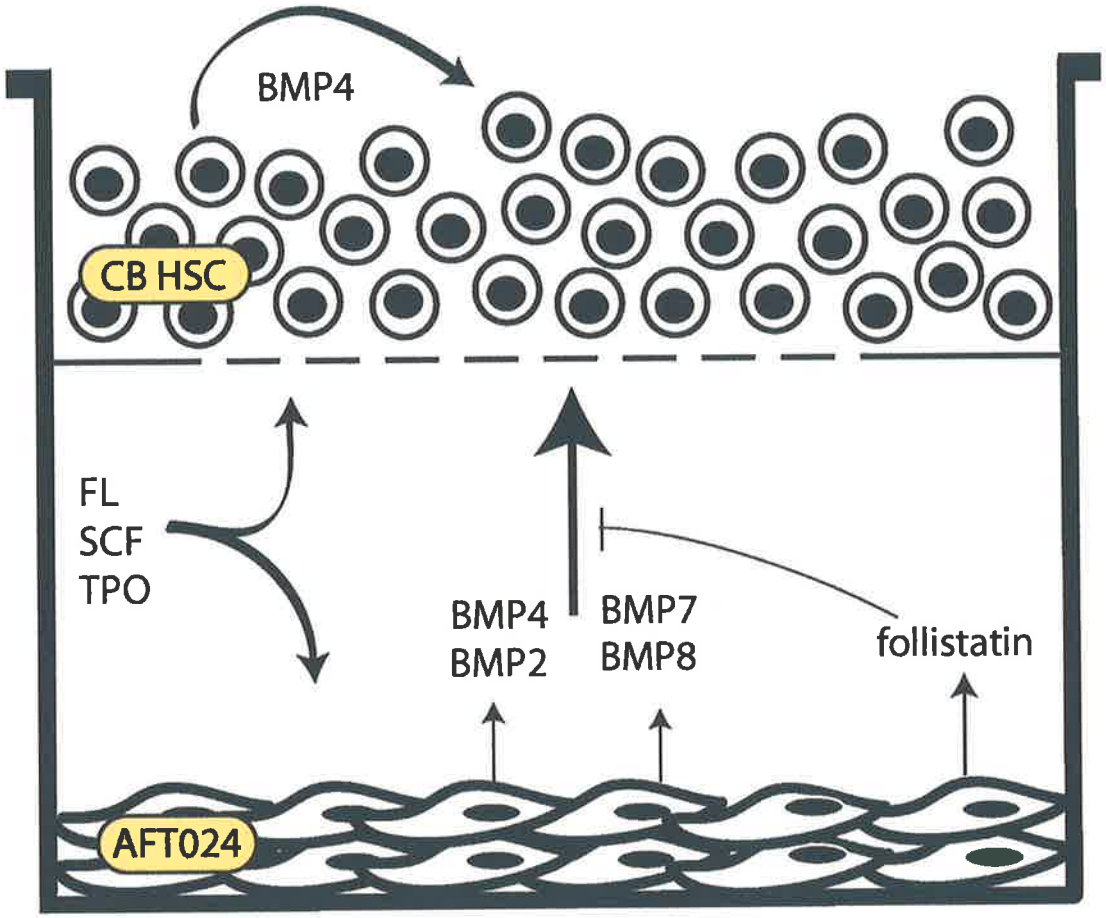


Figure 3.14 Proposed model of BMP activity in the AFT024/HSC co-culture system. Supplemental human cytokine (FL, SCF & TPO) induces BMP4 mRNA in CB HSC and BMP2, 7 & 8 in AFT024. AFT024 also express BMP4 and the BMP antagonist follistatin mRNA.

with production of growth factors, expression profiles were then determined for a range of matched stromal cell lines with different levels of HSC supportive activity. It was observed that all supportive cell lines expressed BMP4, Wnt3a, and Jag-1 at varying levels, however there was generally poor correlation between growth factor expression at a transcript level and HSC supporting ability. Thus it is likely that no one growth factor is sufficient for HSC support and a number of different growth factor combinations may provide the required signals that allow HSC self renewal and survival.

In the literature there is now increasing evidence to suggest that BMP4 not only induces the development of early blood formation, but also may regulate self renewal of the HSC *in vivo* in the adult BM stem cell niche [96, 117, 143, 148, 174, 175]. The studies in this chapter suggest the possibility that BMP4 may contribute to CB HSC maintenance in AFT024 *ex vivo* co-culture. However, while BMP4 activity may be important for AFT024 maintenance it may not be necessary or sufficient in all cases where stromal support is observed. BMP4 is not differentially expressed at a transcript or protein level in AFT024 compared to other non-supporting fetal liver cell lines (Section 3.3, Section 3.7 & Hackney *et al.* [107]). Whilst higher levels of secreted BMP4 protein were detected in AFT024 supernatant at 24 hr compared to the non-supporting fetal liver cell lines BFC012 and 2018, the non-supporting lines 2058 and 2012 produced levels of BMP4 equivalent to or greater than AFT024. AFT024 may therefore represent a supportive stem cell niche that is characterised by the concerted action of a number of soluble factors, rather than any individual component. Whilst BMP4 was also induced by the growth factors in the HSC population during co-culture, there are 1000-fold fewer of these cells compared to AFT024 in this co-culture system.

It is likely that the HSC derived BMP4 acts predominantly in an autocrine manner, however any BMP4 produced is not sufficient to expand HSC cultured *ex vivo* without stroma, compared to AFT024 co-culture (as demonstrated in Chapter 5). We however found BMP4 protein was secreted by AFT024 reaching 723 pg/ml after 72 hrs. As previous studies have shown BMP4 concentration-dependent effects on stem cell activity [117], it is difficult to predict the role of BMP4 activity in AFT024 co-culture based on expression analysis. To address this question directly we performed experiments to manipulate the BMP4 concentration axis in AFT024 co-culture. The results of this are reported in the next chapter.

CHAPTER 4

BMP4 AS A HAEMOPOIETIC GROWTH FACTOR IN AFT024 CO-CULTURE

4.1 Introduction

The extrinsic signals that promote HSC self-renewal are likely to be produced by the cellular components of the fetal liver microenvironment [107]. A better understanding of the role of growth factors in the AFT024 *in vitro* model of fetal liver haemopoiesis may lead to the development of defined culture systems for clinical HSC expansion [106, 107]. We hypothesise here that BMP4 may contribute to AFT024 primitive haemopoietic cell activity. This would be consistent with the endogenous production of BMP4 protein by AFT024 (Section 3.5). In addition we test the ability of supplemental BMP4 to further enhance maintenance/expansion of HSC from CB in co-culture with AFT024. To address these questions we used phenotypic and functional assays. Phenotypic assessment of primitive haemopoietic cells is commonly used for routine assessment of blood products used for transplantation and in clinical trials [19, 31, 40]. Haemopoietic progenitors are commonly defined by their expression of CD34 [176-179], while the more primitive population of cells with a $CD34^{+}CD38^{-}$ phenotype is enriched for HSC that can exhibit long-term engraftment and self renewal following transplantation into an immuno-compromised host [81]. This phenotype is therefore commonly used as a surrogate stem cell marker [180-182]. Primitive haemopoietic cell maintenance after *ex vivo* culture is better assessed using the CFU-GM and LTC-IC functional assays for committed and primitive haemopoietic progenitor cells [60, 183]. Stem cell engraftment assays using the myeloablative NOD-SCID mouse model provide an important complementary *in vivo* assay which measures the multilineage engraftment capacity of a test population [170]. Although it has recently been shown that distinct hematopoietic stem/progenitor cell populations may be responsible for repopulating NOD/SCID mice compared with nonhuman primates [184] this assay is used

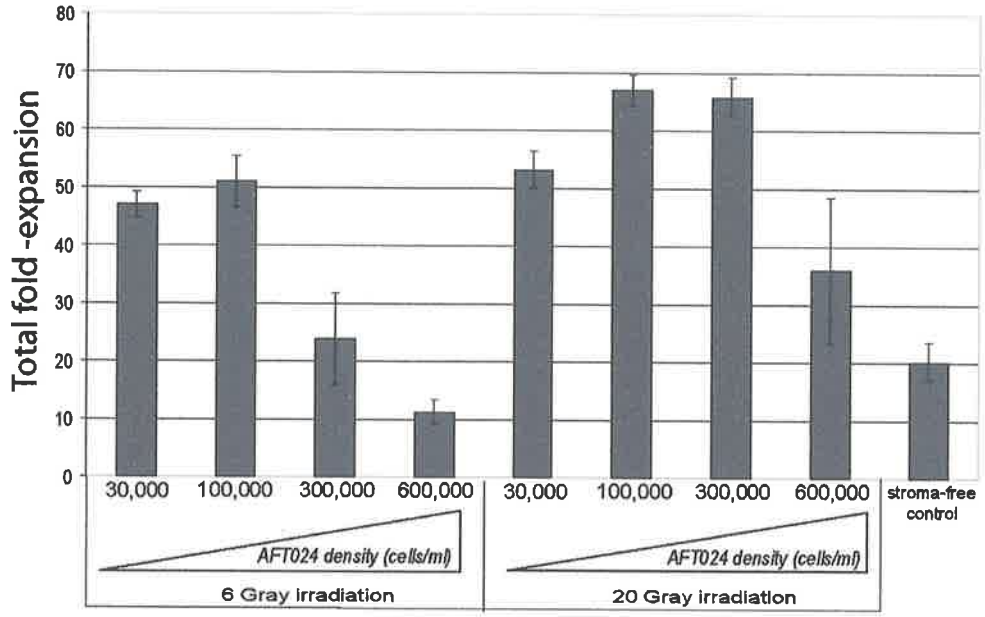
extensively to quantitate human HSC engraftment potential. Here these standard phenotypic and haemopoetic functional assays for primitive cells are employed to define the role of BMP4 in AFT024 dependent expansion of CB.

4.2 AFT024 ex vivo maintenance/expansion of cord blood HSC

For CB stem cell transplantation, $CD34^+$ cells can be enriched from the mononuclear cell fraction and administered intra-veneously [19]. This procedure has been used extensively in the clinic and is approved in many countries [19]. For this project $CD34^+$ CB cells were sorted using Ficoll and MACS reagents (see Methods section 2.2.10 & 2.2.13) to isolate a $CD45^+CD34^+$ population of cells which was used as the starting population for *ex vivo* expansion cultures. The AFT024 culture system provides a unique microenvironment that promotes haemopoietic cell growth yet maintains primitive progenitors [74]. It was determined by several optimisation experiments testing different AFT024 seeding densities and pre-irradiation conditions (summarised in **Figure 4.1a** and **4.1b**), that optimal *ex vivo* expansion in the AFT024 co-culture was achieved with a seeding density of 1×10^5 cells/ml after 20 Gy irradiation and co-cultured with CB $CD34^+$ cells seeded at 1×10^3 cells/ml (**Figure 4.1c** and **4.1d**). Under these optimised conditions for co-culture, total haemopoietic cell expansion and maintenance of primitive cell phenotype was consistent with previous reports of AFT024 activity [74, 104]. Supplemental human cytokines SCF, FL and TPO at 10 ng/ml (which have been shown to dramatically expand both CFC and SRC over several weeks [77, 92]) were included in the co-culture to further enhance primitive cell maintenance.

We initially confirmed that AFT024 support of primitive haemopoietic cells does not require cell contact (as reported previously [104]). For these experiments, CB

(a)



(b)

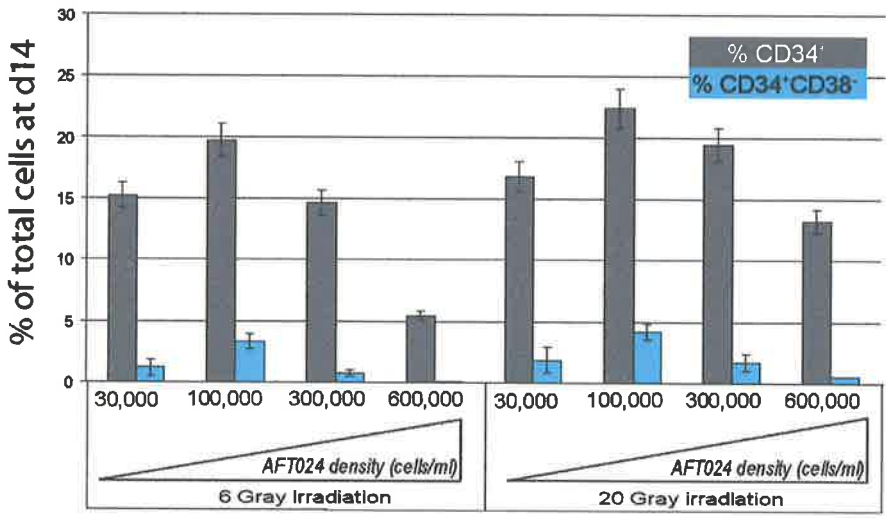
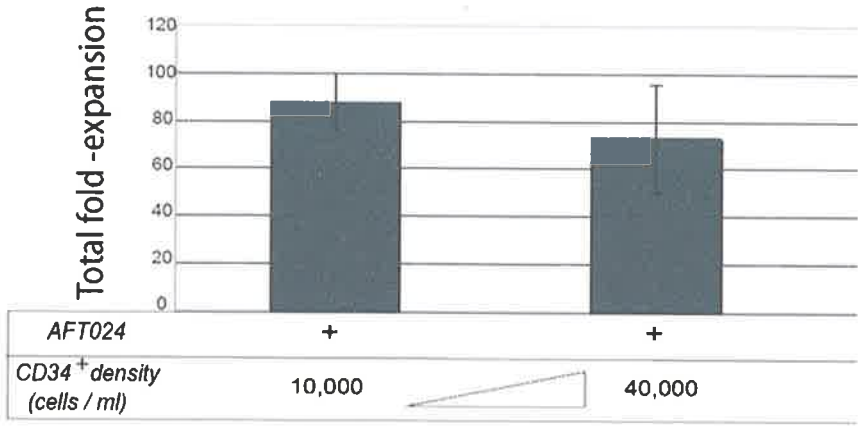


Figure 4.1 Optimisation of AFT024 co-culture. (a) Total haemopoietic cell fold-expansion and **(b)** percentage of cells maintaining primitive haemopoietic cell phenotype ($CD34^+$ grey bars & $CD34^+CD38^-$ blue bars) with different AFT024 seeding densities of 3 x 10^4, 1 x 10^5, 3 x 10^5 or 6 x 10^5 cells/ml and pre-irradiation at 6 or 20 Gy (see Appendix A for flow cytometry histograms). **Figures 4.1c** and **4.1d** are shown on following page.

(c)



(d)

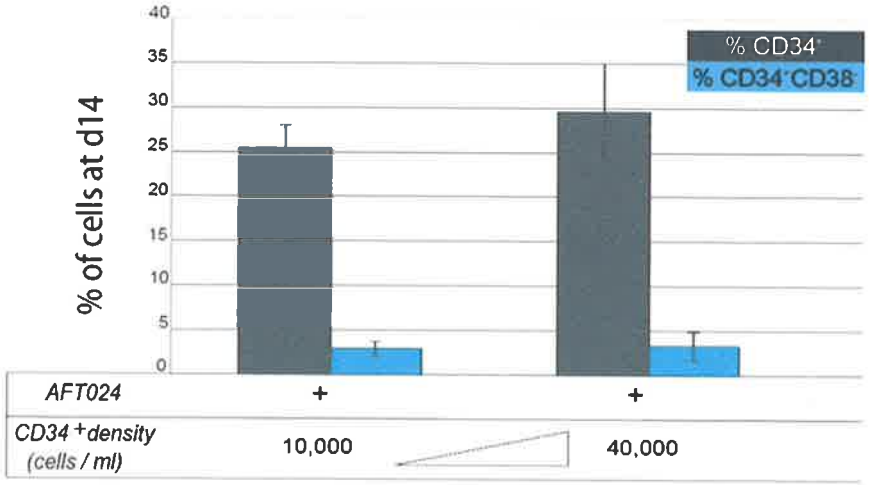
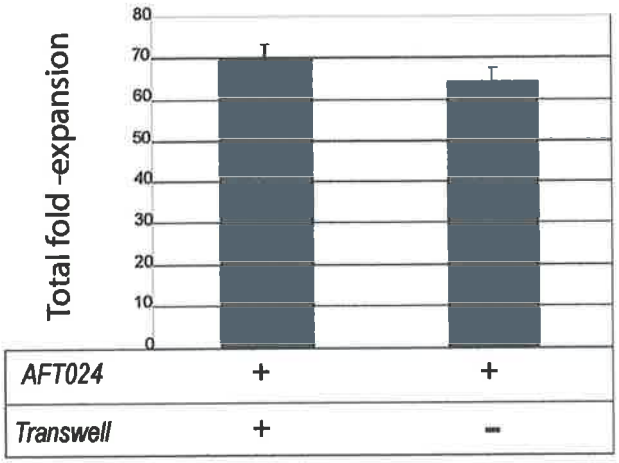


Figure 4.1 Optimisation of AFT024 co-culture. **(c)** Total haemopoietic cell fold-expansion and **(d)** percentage of cells maintaining primitive haemopoietic cell phenotype ($CD34^+$ grey bars & $CD34^+CD38^-$ blue bars) was equivalent with CB $CD34^+$ cells seeded at either $1x10^4$ or $4x10^4$ cells/ml after 14 days culture on AFT024 with supplemental FL, SCF & TPO (see Appendix B for flow cytometry histograms).

(a)



(b)

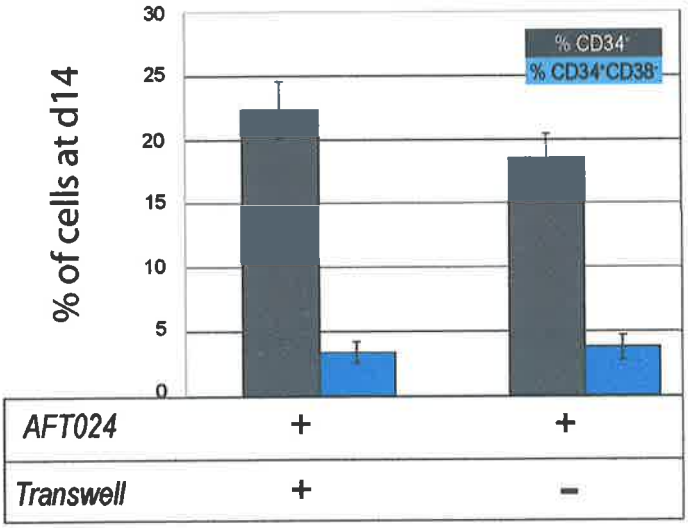


Figure 4.2 Analysis of AFT024/HSC non-contact culture (a) Total haemopoietic cell fold-expansion and **(b)** percentage of cells maintaining primitive haemopoietic cell phenotype ($CD34^+$ grey bars & $CD34^+CD38^-$ blue bars) following culture of CB $CD34^+$ cells in non-contact (trans-wells) or in direct contact with AFT024 (see Appendix B for flow cytometry histograms).

$CD34^+$ cells were cultured with AFT024 in transwells (non-contact cultures) or in direct contact with AFT024. After 14 day *ex vivo* culture there was no significant difference in total haemopoietic cell expansion or cell viability (Methods section 2.2.12) **(Figure 4.2)**. There was also no significant difference in the levels of $CD34^+$ cells, or cells with the more primitive $CD34^+CD38^-$ phenotype.

4.3 Expansion of cord blood $CD34^+$ cells with AFT024 and BMP4 supplementation

Experiments in Chapter 3 demonstrated that BMP4 was being secreted by AFT024 at levels thirty times lower than that described by Bhatia *et al.* as being supportive for HSC in culture [117]. It was next determined whether enhanced primitive haemopoietic cell maintenance or cell expansion could be achieved by supplementing BMP4 in AFT024 co-culture. Three independent experiments were performed using fresh CB samples cultured in duplicate wells. CB $CD34^+$ cells were co-cultured with AFT024 (using culture conditions described in Section 4.2) with or without rBMP4 supplementation (and with appropriate stroma-free controls). BMP4 was supplemented over a range of concentrations (1, 2, 5, 10, 25 & 50 ng/ml) including concentrations previously shown to exhibit HSC activity (5 and 25 ng/ml [117]) and added every third day of culture over 14 days. The haemopoietic cell fraction was then removed and assayed for total cell expansion and viability (Methods section 2.2.12). Over three independent experiments the average level of total expansion varied from 81.1-fold (± 22.0-fold) to 104.0-fold (± 10.8-fold) in wells containing AFT024 stroma (stroma-free expansion was significantly lower at 33.2-fold ± 2.3-fold). There with no significant difference in total expansion or in total cell viability with or without supplementation of BMP4 (**Figure 4.3**). Expanded cells were then assessed for maintenance of primitive cell phenotype. Over three experiments, the percentage of $CD34^+$ cells remaining after

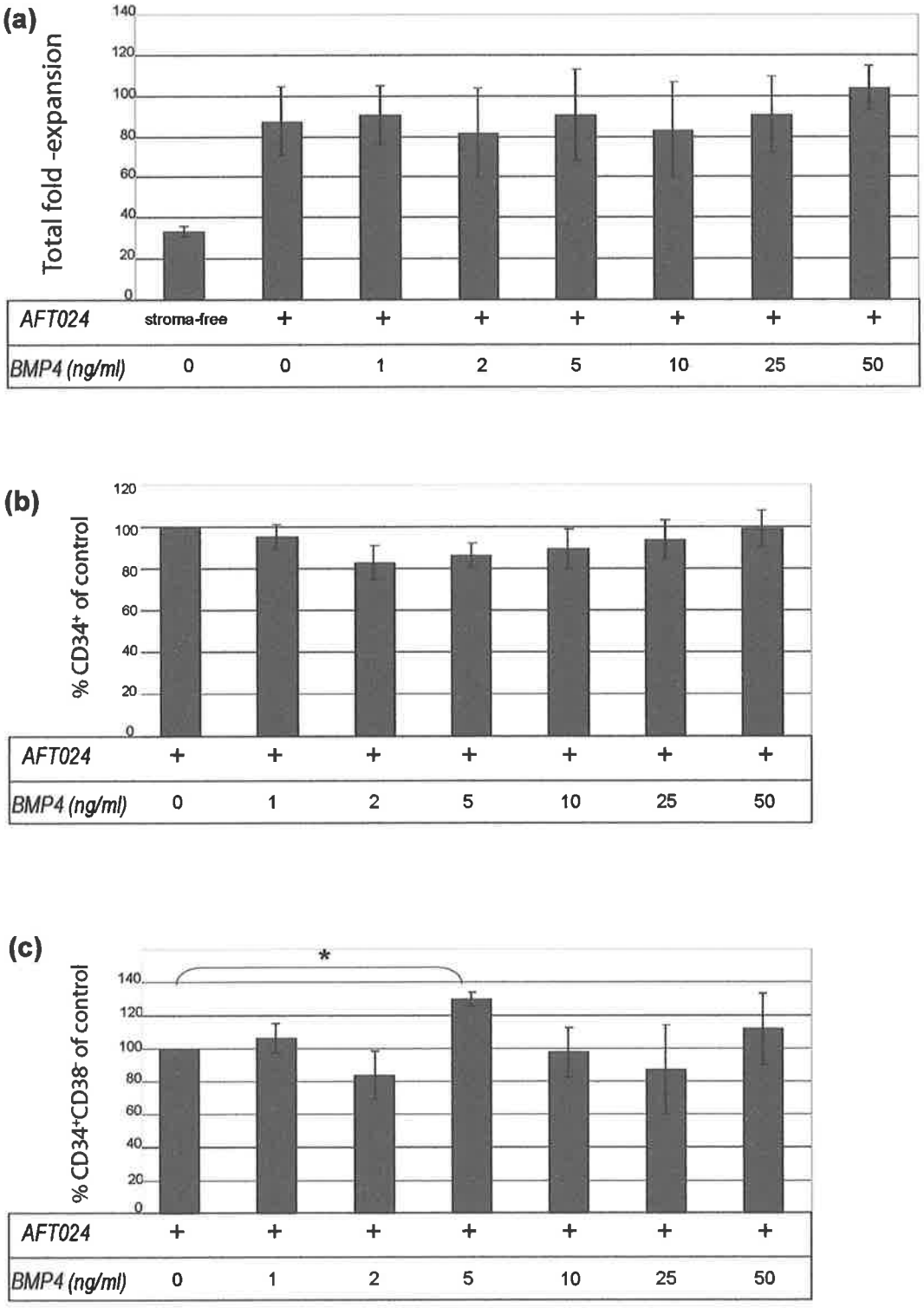


Figure 4.3 BMP4 supplementation in AFT024/HSC co-culture. (a) Total haemopoietic cell fold-expansion, **(b)** percentage of cells maintaining $CD34^+$ phenotype & **(c)** percentage of cells maintaining $CD34^+CD38^-$ phenotype with or without addition of rBMP4 at 1, 2, 5, 10, 25 and 50 ng/ml in 14 day co-culture with AFT024. Cultures were supplemented with FL, SCF & TPO. A stroma-free control culture was included for comparison (*=$P<0.05$ compared to untreated stromal-control, n=3 experiments performed in duplicate) (see Appendix C for flow cytometry histograms).

14 days varied considerably in control AFT024 supported cultures, ranging from 17.3% to 32.3% (which is consistent with previous reports [104, 185]). High variation of $CD34^+$ cell maintenance after *ex vivo* culture from multiple CB donors has been revealed previously [67]. To reveal any BMP4-dependent effect on primitive cell phenotype over multiple experiments, we assessed primitive phenotype as a percentage of the untreated stromal control, and averaged results over three experiments. Whilst the level of $CD34^+$ cell maintenance was not significantly different with addition of BMP4 to co-cultures, a significantly increased proportion of cells (29.8% ± 4.1%) maintained the $CD34^+CD38^-$ phenotype in co-culture supplemented with BMP4 at 5 ng/ml ($P < 0.05$) (all other concentrations of BMP4 supplementation showed no significant difference to control). *In vitro* assays for primitive cell function were then used to investigate this result further.

4.4 ***Haemopoietic progenitor cell analysis of expanded cord blood $CD34^+$ cells with AFT024 and BMP4 supplementation***

Although phenotypic analysis provides a means to quantify progenitor and stem cell content, this assay does not provide information on the functional capacity of these cells [186]. To more effectively characterise and dissect AFT024 maintenance/ expansion of primitive CB cells, functional stem/progenitor cell assays were used. The most common approach to assaying multilineage or single-lineage committed haemopoietic progenitors, is a clonal assay in semi-solid media with human growth factors which supports growth of the clonal progeny of a single progenitor cell [187-189]. Culture media have been optimised for outgrowth of erythroid, monocyte/ macrophage, granulocytic, megakaryocytic and multipotent progenitor cells [187, 190, 191]. In this project we used methyl cellulose based colony forming unit (CFU) assays

to quantitate myeloid progenitor (CFU-GM) numbers after *ex vivo* expansion (see Methods section 2.2.15) [190]. The relative levels of myeloid progenitor (CFU-GM) expansion in AFT024 co-culture with BMP4 supplementation were then determined. Over three independent experiments the average CFU-GM frequency of freshly isolated CB $CD34^+$ cells was found to be 0.0112 ± 0.025 (or $11.2\% \pm 2.5\%$) (which is consistent with past reports [104, 192, 193]). As previously described we observed expansion of CFU-GM in 14 day co-cultures of AFT024 with CB $CD34^+$ cells [74, 104, 185] (22.7-fold CFU-GM expansion ± 2.9-fold) (**Figure 4.4a**). With BMP4 supplementation at 1 ng/ml, CFU-GM expansion decreased significantly to 14.6-fold expansion (± 2.0-fold, $P< 0.05$). In addition to calculating progenitor cell expansion (see Methods section 2.2.15) the absolute numbers of CFU-GM after 14 day *ex vivo* culture were also determined. At day 14 the absolute number of CFU-GM with BMP4 treatment at 1 ng/ml was not significantly different to the AFT024 stromal control (see Appendix D). Other concentrations of BMP4 supplementation did not significantly affect CFU-GM expansion or absolute CFU-GM number compared to the stromal controls over 14 day co-culture with AFT024.

The functional assay for long-term culture initiating cells (LTC-IC) is a standard assay used to quantify the most primitive haemopoietic cells that can be detected *in vitro* [194]. In this assay, cells that can initiate and sustain haemopoiesis and generate secondary colony forming cells for a minimum of 5 weeks in stroma-based cultures, represent primitive progenitors termed LTC-IC [195, 196]. When cells are plated in limiting dilutions, calculation of the absolute frequency of LTC-IC can be determined [195, 197] (as well as LTC-IC expansion by comparison to the starting population). Here we used limiting dilution analysis to determine the frequency of LTC-IC in both

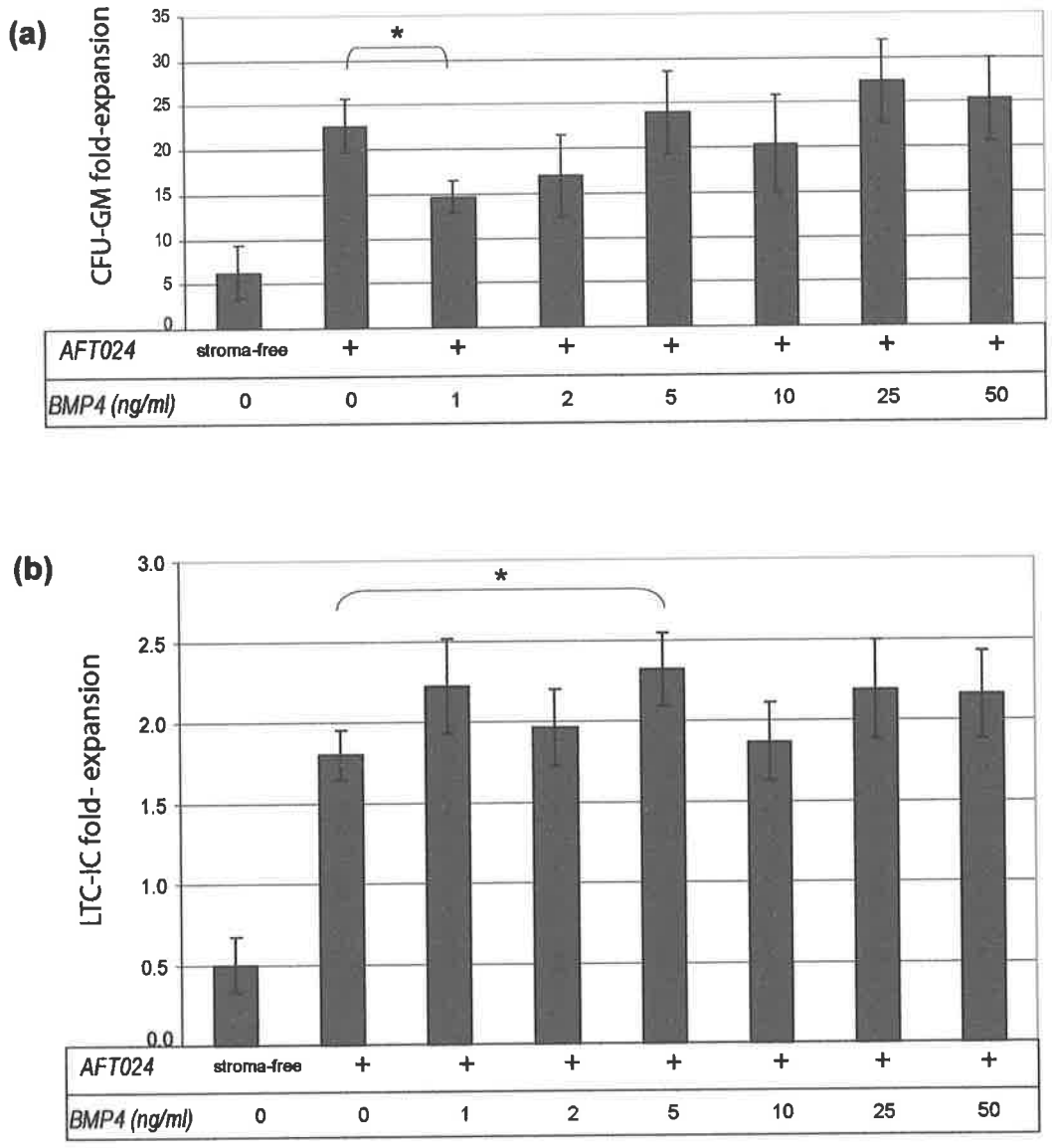


Figure 4.4 Haemopoietic progenitor cell expansion in AFT024 co-culture with BMP4 supplementation. Fold-expansion of **(a)** CFU-GM and **(b)** LTC-IC from 14 day *ex vivo* co-culture of CB $CD34^+$ cells on AFT024 with supplemental FL, SCF & TPO with or without rBMP4 supplementation at 1, 2, 5, 10, 25 and 50 ng/ml (or cultured stroma-free) (*=$P<0.05$ compared to untreated-stromal control, n=3 experiments performed in duplicate).

fresh and AFT024 expanded CB $CD34^+$ cells with and without BMP4 supplementation (see Methods section 2.2.16). The reported LTC-IC frequency in human CB populations varies considerably (ranging from 0.2 to 56% [196], [162, 169]). Differences in the stromal feeder used in the LTC-IC assay and cell culture method, such as frequency of media change, initial plating cell density, starting cell phenotype or cytokines added, can influence frequency [198-202]. For this project, using three independent CB samples the LTC-IC frequency of freshly isolated CB $CD34^+$ cells averaged 0.0251 ± 0.0021 (or 2.5% ± 0.21%) which is consistent with past reports using similar LTC-IC assay conditions [104, 158]. After two weeks co-culture with AFT024, LTC-IC were expanded 1.80-fold (± 0.15-fold) consistent with the reported ability of AFT024 to expand primitive haemopoietic cells during *in vitro* culture [74, 158] (**Figure 4.4b**). In comparison, stroma-free cultures resulted in a 45% net loss of LTC-IC over 2 weeks. With BMP4 supplementation at 5 ng/ml we observed a modest but statistically significant enhancement of LTC-IC expansion (2.45-fold expansion ± 0.23-fold, $P<0.05$) (**Figure 4.4b**) and absolute LTC-IC number (see table in Appendix D). Other concentrations of supplemental BMP4 did not significantly change LTC-IC expansion or absolute LTC-IC number compared to control cultures. These experiments demonstrate that the supportive AFT024 microenvironment can significantly expand LTC-IC during *ex vivo* co-culture as reported previously.

Whilst addition of BMP4 to AFT024 co-culture at 5 ng/ml appeared to enhance both maintenance of primitive cell phenotype ($CD34^+CD38^-$) and expansion of LTC-IC, the effects observed were modest and an overall concentration-dependent effect of BMP4 was not demonstrated. We concluded further investigation to assess the maintenance of engrafting cells during AFT024 co-culture with supplemental BMP4

was not warranted. Further experiments focused on elucidating the role of endogenous BMP activity in the AFT024 culture system.

4.5 *Blocking endogenous BMP4 activity in AFT024 co-culture*

BMP4 protein is secreted from AFT024 cells (Section 3.5) and BMP4 mRNA expression is induced in HSC in response to cytokine (Section 3.9 & Bhardwaj *et al.* [110]). Experiments were next performed to assess the contribution of this endogenous BMP4 activity. We used two independent biological agents to block BMP4 signalling in the AFT024 co-culture system. Noggin has been shown to be a high-affinity BMP binding protein that antagonises BMP bioactivities [203] (see Introduction section 1.17). rNoggin (R&D systems) was exogenously added to AFT024/$CD34^+$ co-culture at 100 ng/ml (this concentration has been shown to block BMP4 activity in culture [204, 205]). In addition to this, a monoclonal BMP4 neutralising antibody (R&D systems) was also used to block BMP4 activity. This antibody is a potent inhibitor of BMP4 activity both *in vitro* and *in vivo* [206]. To monitor the effect of BMP4 blockade in the AFT024 culture system we measured total cell expansion, maintenance of primitive phenotype, CFU-GM output and LTC-IC expansion. Expanded cells were also assessed *in vivo* for stem cell engraftment and multilineage repopulation in the NOD-SCID mouse model.

4.6 *Expansion of cord blood $CD34^+$ with AFT024 and BMP4 neutralisation*

AFT024 non-contact co-cultures with supplemental FL, SCF & TPO were initially established using fresh CB $CD34^+$ cells in trans-wells as described in the previous section (Section 4.2). Comparisons were made between this control culture and

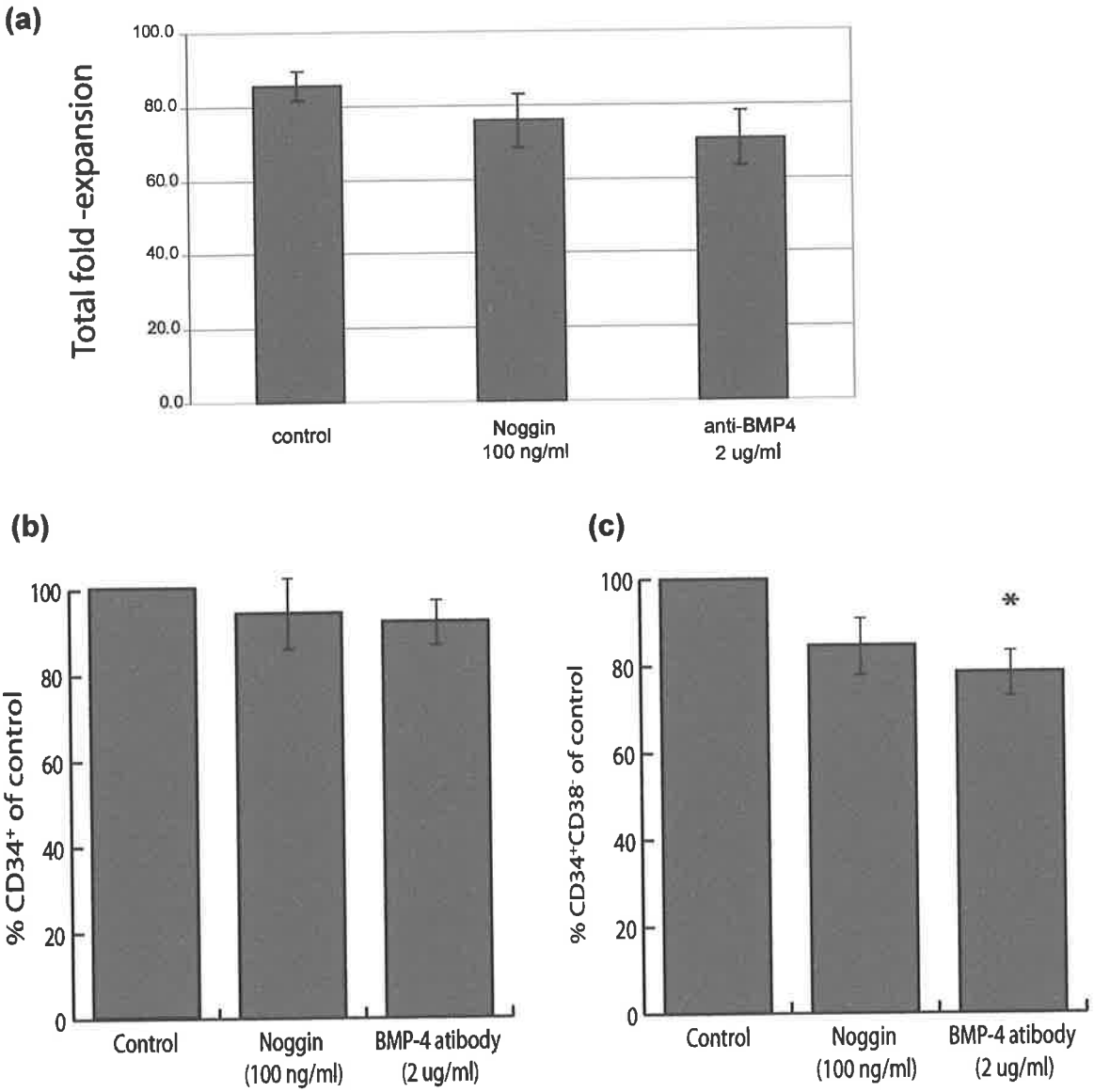


Figure 4.5 Blocking BMP4 in AFT024/HSC co-culture. (a) Total haemopoietic cell fold-expansion, **(b)** percentage of cells maintaining $CD34^+$ phenotype & **(c)** $CD34^+CD38^-$ phenotype with or without BMP4 blockade using rNoggin (100 ng/ml) or a neutralising BMP4 antibody (2 μg/ml) with AFT024 and supplemental FL, SCF & TPO at d 14 (*= $P<0.05$, n=3 experiments performed in duplicate) (see Appendix E for flow cytometry histograms).

identical AFT024 cultures with either noggin at 100 ng/ml or BMP4 antibody at 2 μg/ml. Cultures were set up in duplicate for 14 days with fresh antibody or noggin added every third day of culture. At day 14 there were no significant differences in total cell expansion (**Figure 4. 5a**) or cell viability (see Methods section 2.21) amongst these different culture conditions over three independent experiments performed in duplicate. Expanded cells were then analysed by immunophenotyping to determine levels of primitive cell maintenance. For this we monitored immunophenotype with the CD34 and CD38 antibodies as described previously. In three independent experiments no significant difference was detected in $CD34^+$ cell maintenance with or without BMP4 blockade. However, the proportion of cells at day 14 with phenotype $CD34^+CD38^-$, was reduced by 15.3% (± 6.5%, P=0.07) with noggin and by 21.54% (± 4.9%, P<0.05) with the BMP4 antibody (**Figure 4. 5c**).

4.7 *Haemopoietic progenitor cell analysis of AFT024 expanded cord blood $CD34^+$ cells after BMP4 blockade*

In addition to monitoring cell number and phenotype we performed functional assays with the expanded cells following 14 day co-culture in the presence or absence of noggin or neutralising BMP4 antibody. Cells were plated in methylcellulose and CFU-GM colonies scored (see Methods section 2.2.15). In three experiments the net level of CFU-GM progenitor expansion in control co-cultures of AFT024 and CB $CD34^+$ cells was 19.2-fold (± 2.6-fold) (**Figure 4.6a**). With BMP4 blockade, the level of net CFU-GM expansion was reduced to 14.8-fold (± 3.6-fold) and 13.2-fold (± 2.2-fold, P< 0.05) using noggin and anti-BMP4 respectively. The absolute number of CFU-GM present at 14 days culture was also reduced with BMP4 blockade (see table in Appendix F). The

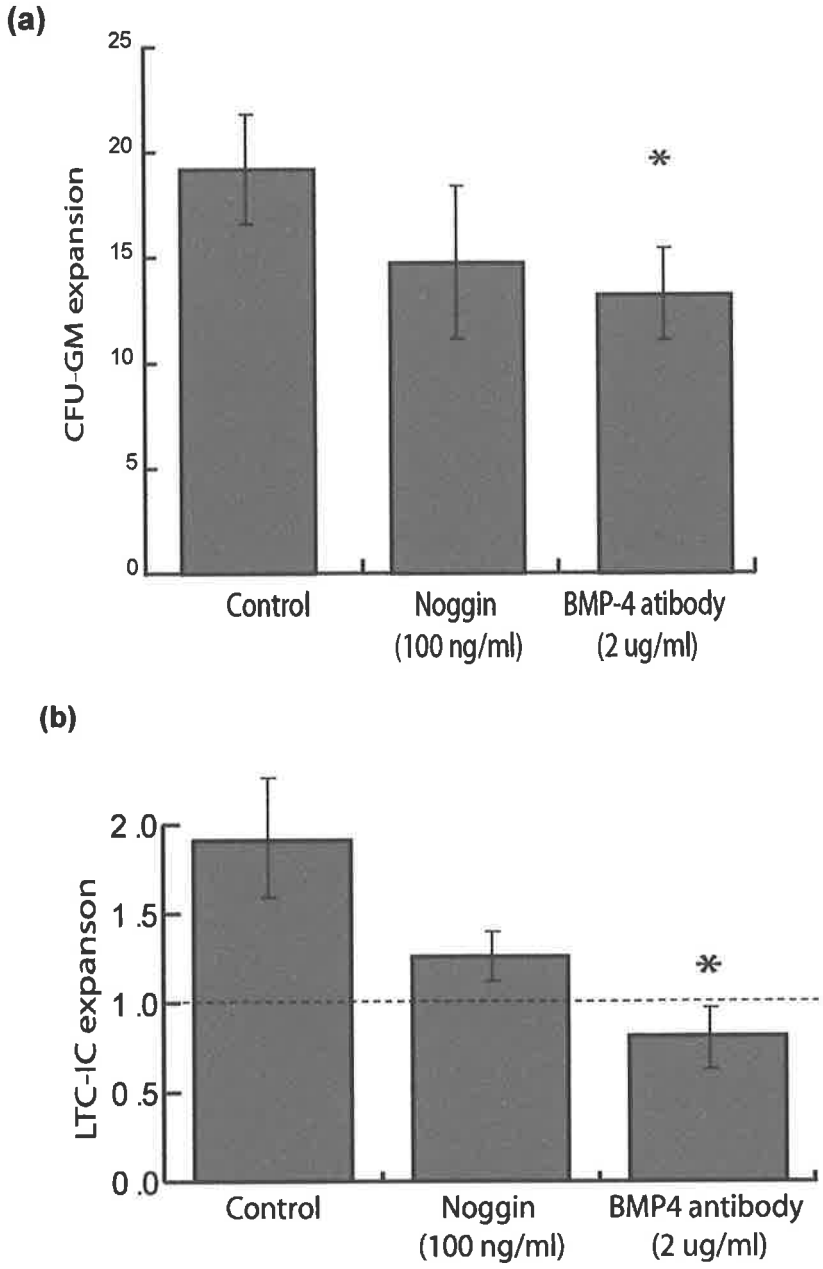


Figure 4.6 Haemopoietic progenitor cell expansion in AFT024 co-culture with blockade of BMP4 Fold-expansion of **(a)** CFU-GM and **(b)** LTC-IC from 14 day *ex vivo* co-culture of CB $CD34^+$ cells on AFT024 with supplemental FL, SCF & TPO with or without BMP4 blockade using rNoggin (100 ng/ml) or a neutralising BMP4 antibody (2 ug/ml) (* = $P< 0.05$, n=3 experiments performed in duplicate).

contribution of BMP4 to primitive cell maintenance and expansion of CB HSC in AFT024 co-culture was also measured using the LTC-IC assay (Methods section 2.2.16). Over the three separate AFT024 co-culture experiments the net expansion of LTC-IC in control cultures (1.91-fold ± 0.32-fold) was consistent with that reportedpreviously [104] (**Figure 4.6b**). Importantly, both methods of BMP4 blockade consistently reduced expansion of LTC-IC. In fact we observed a significant net loss of LTC-IC (0.71-fold expansion ± 0.2-fold, $P<0.05$) in cultures supplemented with BMP4 neutralising antibody (n=3). This compared to noggin supplemented cultures where expansion was reduced to 1.26-fold (± 0.14-fold). To rule out any non-specific antibody effects, an isotype control antibody (IGg2b) (R&D systems) was added to AFT024 co-cultures over two weeks and shown not to affect LTC-IC output in *ex vivo* culture (see Appendix F).

4.8 Analysis of progenitor cell survival of AFT024 expanded cord blood with BMP4 blockade

It has been demonstrated in some culture systems that enhanced primitive haemopoietic cell output is due to increased survival [207]. We performed experiments to determine if BMP4 was promoting haemopoietic progenitor cell survival rather than maintenance/expansion. CB $CD34^+$ progenitor cells were co-cultured with AFT024 for 14 days with and without BMP4 blockade using noggin and a BMP4 antibody. After 14 days the expanded cells were analysed by flow cytometry to determine the level of CD45, CD34 and cell viability (using 7AAD staining, see Methods section 2.2.18). Progenitor cell survival was determined as the proportion of $CD45^+CD34^+7AAD^-$ cells (compared to $CD45^+CD34^+7AAD^+$ cells) as described previously [208]. An equivalent

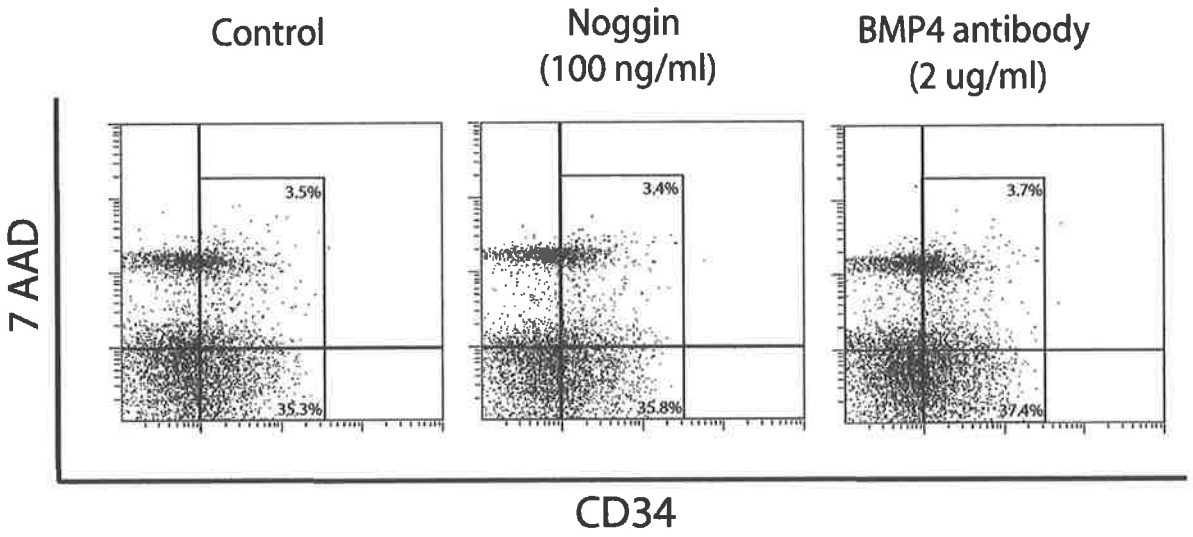


Figure 4.7 BMP4 does not effect survival of haemopoietic progenitor cells in AFT024 co-culture. Viability of $CD34^+$ haemopoietic progenitor cells after 14 d co-culture on AFT024 with and without BMP4 blockade using noggin and BMP4 antibody. Hematopoietic progenitor cell viability was determined by staining cultured cells with CD45-FITC and CD34-PE as well as 7 AAD (data shown representative of 3 experiments).

level of progenitor cell survival was detected in control cultures (90.13% ± 1.39%) compared to cultures supplemented with noggin (90.34% ± 1.38%) or BMP4 antibody (90.17% ± 1.25%)(n=3) (**Figure 4.7**). Thus blockade of BMP4 activity with neutralising antibody induces loss of LTC-IC but has negligible effect on survival of haemopoietic progenitors in *ex vivo* culture. Whilst we cannot rule out a selective effect of BMP4 on survival of very primitive cells (eg HSC), methods to measure HSC survival were not available.

4.9 Engraftment and multilineage repopulation of expanded cord blood CD34$^+$ cells after BMP4 neutralisation.

An important property of HSC is their ability to repopulate conditioned recipients [209]. The development of systems permitting transplantation of human haemopoietic cells into immune-deficient mice provides such an experimental repopulation assay for human HSC [7]. These repopulating cells, termed SCID-repopulating cells (SRC), are distinct from most of the progenitors that are detected using short and long-term *in vitro* culture assays [32, 62, 81, 170]. These cells are considered to be the most primitive haemopoietic cells experimentally identifiable in humans. SRC frequency can be affected by conditions that alter HSC growth and viability and also by changes to the HSC phenotype that affect the capacity of these cells to home to their niche in BM [7, 79, 192, 210]. We used a stem cell engraftment assay to investigate whether blockade of BMP4 activity, during co-culture of AFT024 and CB HSC, compromised the engrafting ability or multilineage capacity of HSC in the cultured CD34$^+$ population (see Methods section 2.2.17). Engraftment was assayed at day 0, and after 2-week and 4-week co-cultures with and without supplemental

noggin or neutralising BMP4 antibody. A matched isotype control antibody for the neutralising BMP4 antibody (IGg_{2a}) was also used at the same concentration (2 μg/ml). The large size of this experiment required many CB samples to be collected, stored and then pooled prior to commencement. To isolate the required 2 x 10^7 $CD34^+$ cells, 24 individual CB units were collected and processed. CB $CD34^+$ cells from individual cords were stored in liquid nitrogen (at 5 x 10^6 cells/ml). Whilst the average yield of mononuclear cells collected from CB samples correlated well with volume, the $CD34^+$ yield varied considerably (as previously described [211]) (see Appendix G). Once sufficient $CD34^+$ cells had been collected, they were thawed, pooled and analysed by flow cytometry. The pooled cells were 95.4% positive for CD34 and CD45 with 98% viability after trypan blue staining analysis (Methods section 2.2.12).

4.10 Engraftment after 14 day ex vivo expansion co-culture

Parallel AFT024 co-cultures were set up using permeable trans-well supports in 6 well plates. Pre-irradiated AFT024 (20 Gy) at 1 x 10^5 cells/ml were co-cultured with 1 x 10^6 $CD34^+$ cells for 14 and 28 days with or without BMP4 blockade. To determine engraftment capacity of the starting population, an initial population of 1 x 10^5 $CD34^+$ uncultured CB cells was administered to each of 6 mice. To determine engraftment capacity after *ex vivo* culture under each condition the expanded progeny from a single transwell containing 1 x 10^5 $CD34^+$ cells was then injected into a single NOD-SCID, with a total of 6 mice injected for each of the four co-culture conditions (as summarised in **Figure 4.8**).

In the mouse, the number of progenitors involved in short-term reconstitution is large, while that of primitive cells operating months after the transplantation is reduced [209]. Whilst engraftment monitored at 4-5 weeks will primarily measure short-term

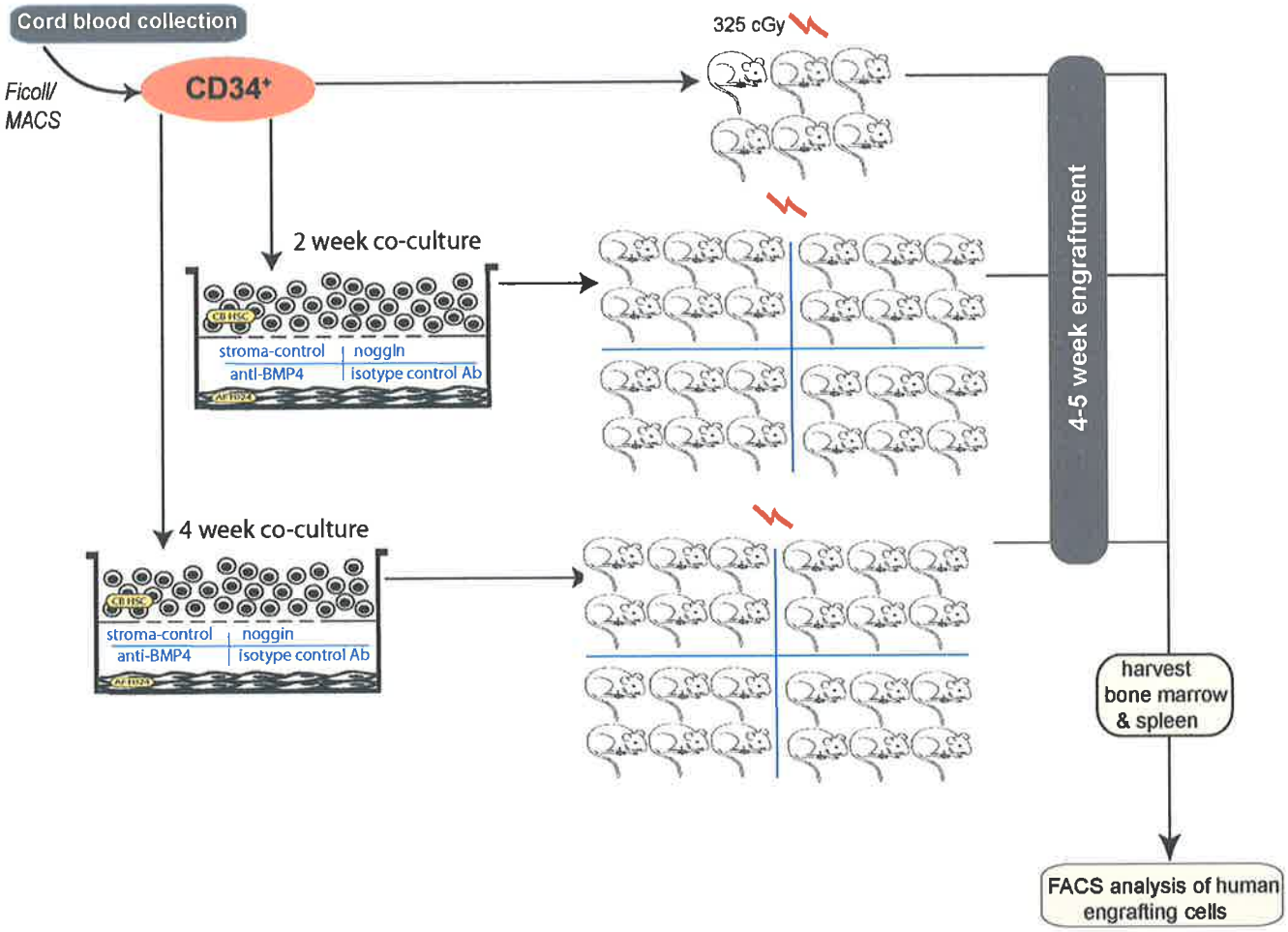


Figure 4.8 HSC engraftment assay. CB CD34$^+$ cells were transfused via tail vein injection into pre-irradiated (3.25 cGy) NOD/SCID mice before and after *ex vivo* co-culture on AFT024, with or without BMP4 blockade. Mice were sacrificed after 4-5 weeks engraftment and whole bone marrow and spleen analysed for human engrafted (CD45$^+$) cells.

repopulation, this time-point is sufficient to measure HSC engrafting cells [62, 171] and was used here. BM and spleen were harvested from sacrificed mice to determine levels of human cell engraftment and the proportion of lineage committed progeny derived from engrafting cells. After 14 and 28 days *ex vivo* culture, high levels of total haemopoietic cell expansion were achieved in each condition with high cell viability maintained (Methods section 2.2.12) (**Figure 4.9**). As described in previous studies [104] engraftment capacity of the $CD34^+$ population was maintained after 14 days co-culture with AFT024 (74.75% at d0 and 76.45% at d14, were positive for human CD45) (**Figure 4.10**). A potential contribution of BMP4 to self renewal of cultured human repopulating cells is suggested by the reduced chimerism in mice transplanted with cells from 14 day cultures supplemented with reagents which neutralise BMP4 activity (**Figure 4.10**). After co-culture supplemented with BMP4 neutralising antibody, engraftment at 4.5 weeks post-transplantation was significantly reduced to 50.15% ($P<0.05$). In presence of noggin, engraftment was also reduced but did not reach significance. Engraftment in untreated control co-cultures (76.45%) and cultures that were supplemented with an isotype-matched control antibody (84.03%) were not significantly different from that observed with the starting population. Thus, when BMP4 activity is specifically targeted we observed a decrease in engraftment. Supplemental noggin resulted in consistent reductions in LTC-IC and in engraftment, however changes in both cases were not significant.

4.11 Engraftment after 28 day ex vivo expansion co-culture

Prior to this study, the level of SRC maintenance in AFT024 co-culture beyond 14 days was unknown. We investigated whether blockade of BMP4 activity during co-culture of AFT024 and CB HSC compromised total expansion, the engrafting ability or

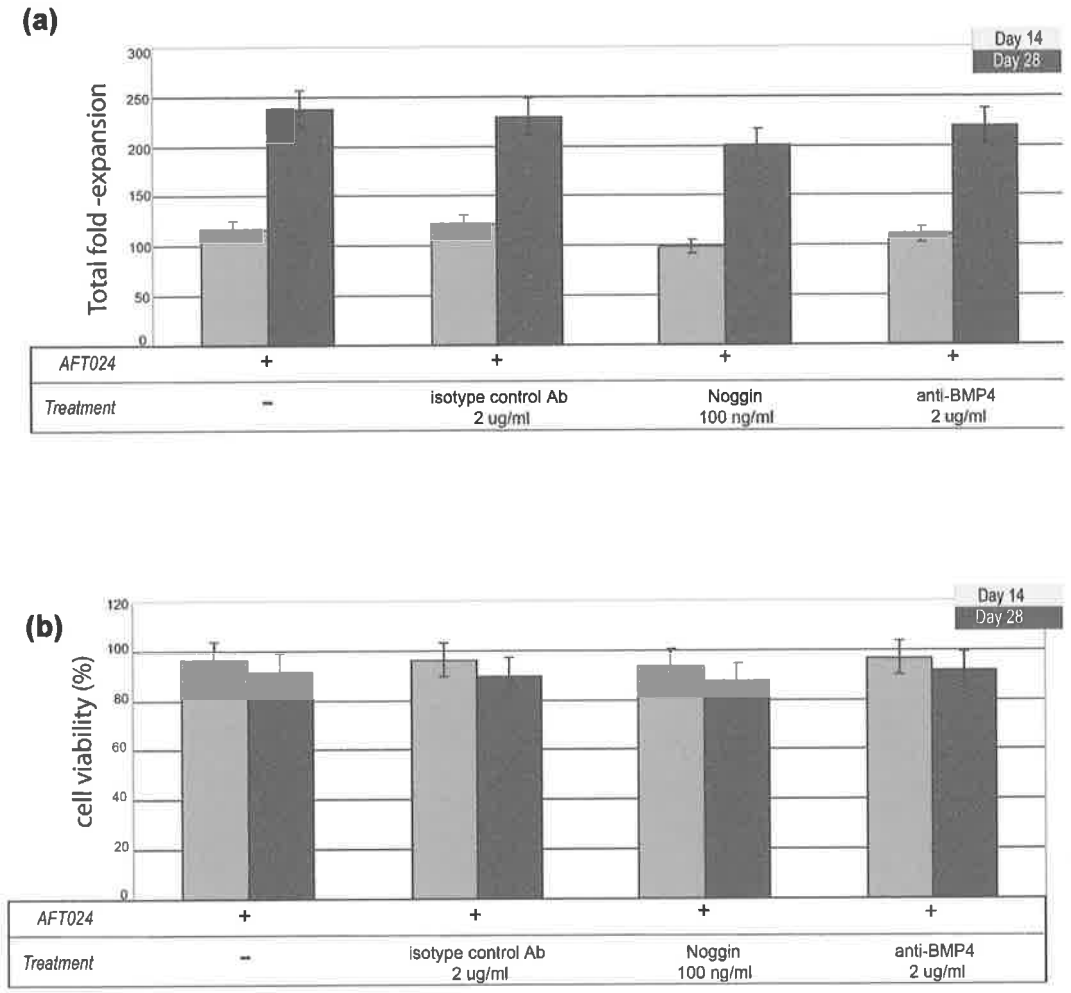


Figure 4.9 Blocking BMP4 in AFT024/HSC co-culture prior to engraftment assay. (a) Total haemopoietic cell fold-expansion and **(b)** viability of expanded haemopoietic cells after 14 day (light grey bars) and 28 day (dark grey bars) co-culture on AFT024 with or without BMP4 blockade using rNoggin (100 ng/ml) or a neutralising BMP4 antibody (2 μg/ml).

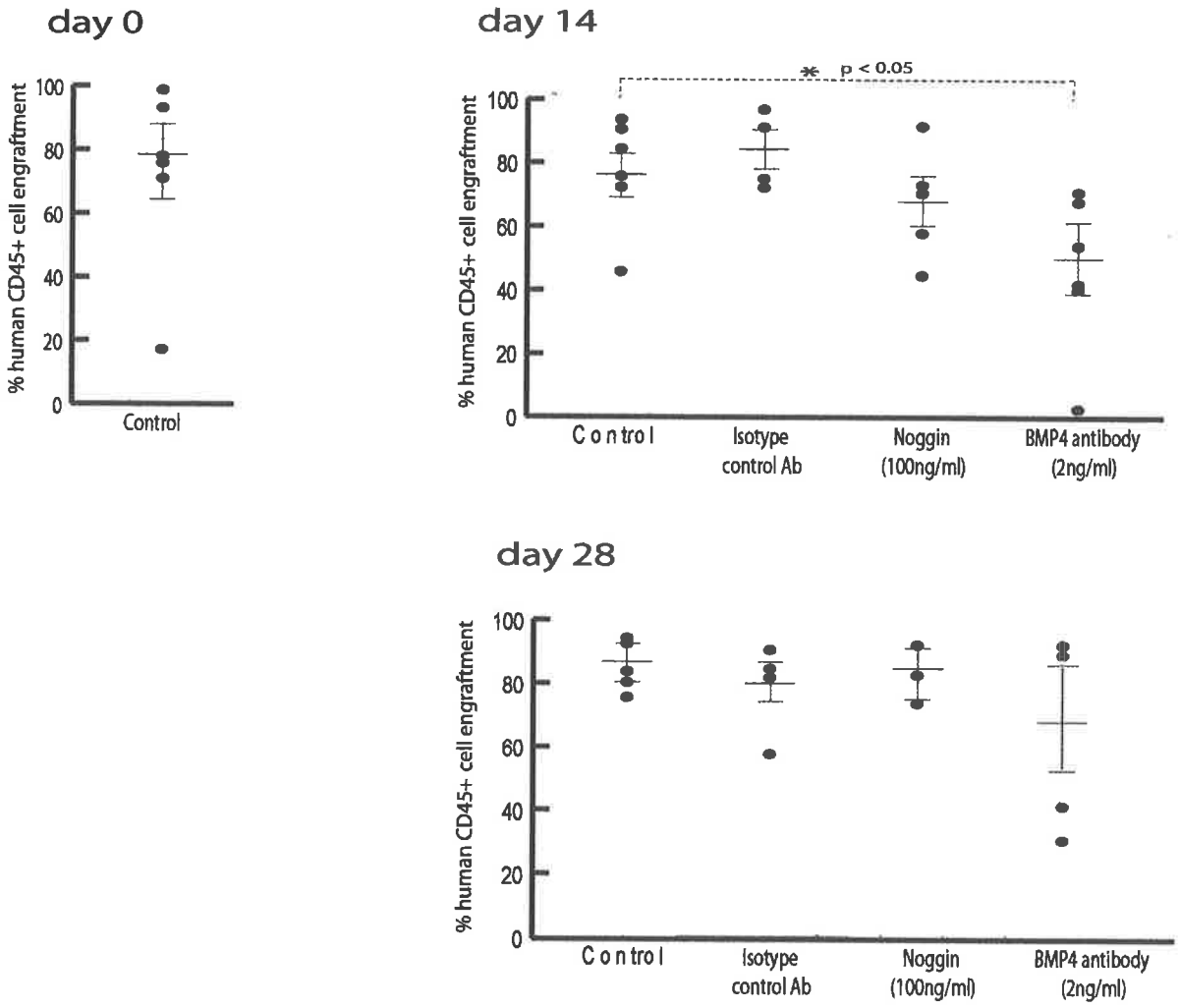


Figure 4.10 Engraftment potential following AFT024 co-culture with BMP4 blockade. Detection of human repopulating cells in NOD-SCID mouse bone marrow transplanted with expanded cord blood $CD34^+$ cells with and without BMP4 blockade after 14 and 28 days co-culture on AFT024. Graph shows amount of human chimerism in the bone marrow of NOD-SCID mice detected 4-5 weeks post-transplantation with the total progeny from 1 x 10^5 initial human cord blood $CD34^+$ cells, co-cultured on AFT024 for 14 days with and without noggin or a BMP4 neutralising antibody (* = $P<0.05$).

multilineage capacity of repopulating cells over a four week culture period. After 28 days co-culture high levels of total haemopoietic cell expansion were achieved in each condition with high cell viability (2.2-fold higher expansion in control cultures at d28 compared to d14 cultures) (**Figure 4.9**) The expanded cells were administered to NOD-SCID mice and engraftment allowed to proceed for a period of 4-5 weeks. Several mice died (due to bacterial infection) prior to the experimental end, however we could still draw important conclusions from the experiment. In the untreated control group (n=5), it was observed that AFT024 successfully maintained engrafting cells for 28 days *ex vivo* co-culture (89.5%, ± 2.7% human $CD45^+$ cell detection) and there had been no significant loss of repopulating capacity compared to fresh $CD34^+$ cells. Addition of BMP4 antibody to the co-culture resulted in a decreased level of engraftment (66.9% ± 17.7%), however this did not reach significance, possibly due to the small sample size (n=4). Engraftment in the noggin (n=3) group and the isotype control group (n=4) also were not significantly different to the untreated control group.

4.12 Phenotypic analysis of engrafted cells

We performed phenotypic analysis of engrafted cells using lineage-specific antibodies. This revealed that blockade of the BMP4 pathway following 14 and 28 day *ex vivo* co-culture did not alter the multilineage differentiation of haemopoietic progenitors *in vivo* (see Methods section 2.2.18). **Figure 4.11** shows the levels of day 14 engrafted myeloid ($CD33^+$), B-lymphoid ($CD19^+$), T-lymphoid ($CD3^+$) and $CD34^+$ cells from mice receiving expanded cells from noggin or antibody containing cultures, and the control cultures (untreated and isotype-matched antibody), were not significantly different.

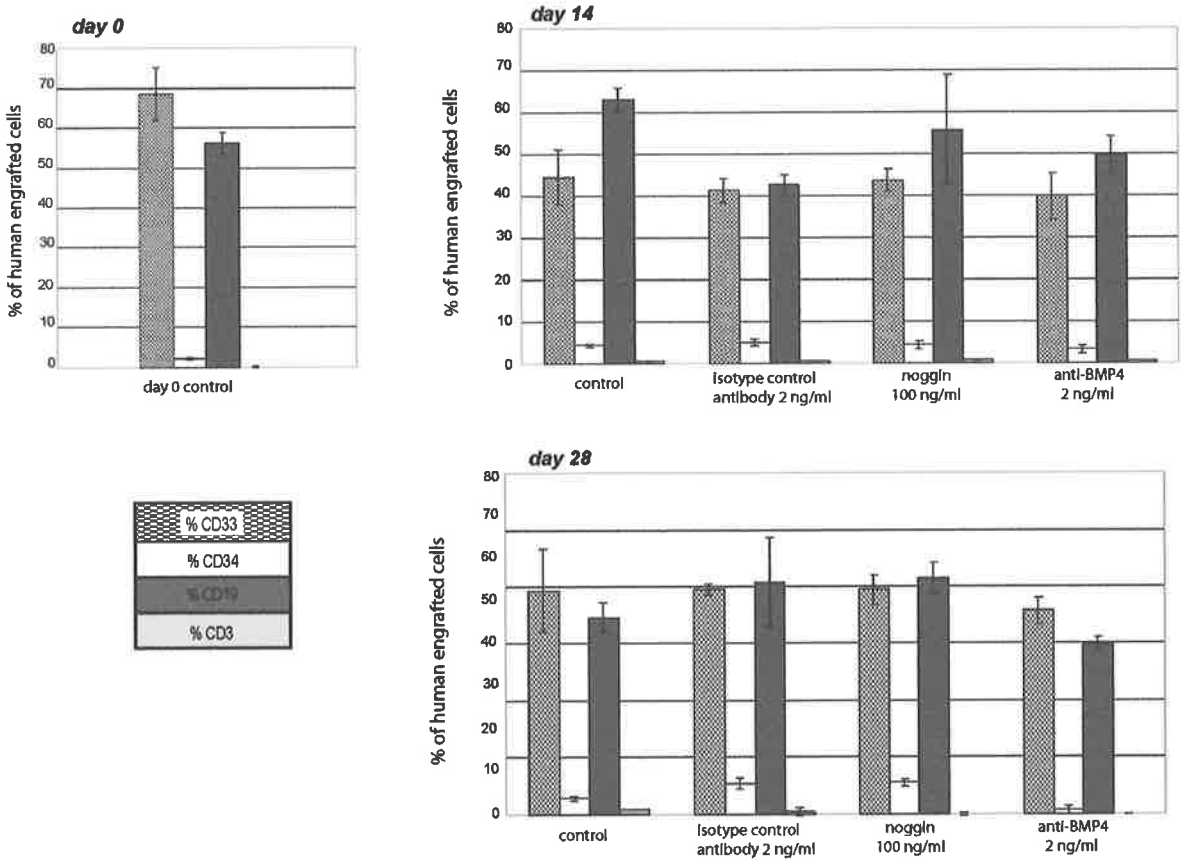


Figure 4.11 Multilineage repopulation of human engrafting cells in NOD-SCID mice. Flow cytometric analysis of multilineage repopulation of mouse bone marrow by human engrafted cells, before (day 0) and after (days 14 and 28) *ex vivo* culture on AFT024 with or without BMP4 blockade. Graph shows the average proportion of human CD33, CD19, CD3 and CD34 positive cells as a percentage of total human detected cells (n=6).

4.13 Spleen engrafting cells after ex vivo expansion co-culture of cord blood CD34$^+$ cells

The level of engrafting cells after AFT024 co-culture with and without BMP4 blockade was also analysed in recipient spleen (**Figure 4.12**). A significantly smaller proportion of human CD45$^+$ cells were detected in spleen compared to BM, consistent with preferential homing to the BM niche. In contrast to BM repopulating cells, HSC migrating to the spleen are more likely to differentiate to repopulate the injured haemopoietic system in this model, than to self-renew [79, 210]. This is consistent with reduced spleen engraftment typically observed when performing repopulation assays [79, 210]. We observed no significant differences in the level of engraftment in NOD-SCID spleen following *ex vivo* AFT024 co-culture with or without BMP4 blockade **(Figure 4.12).**

4.14 Conclusion

The AFT024/HSC co-culture system has been optimised to maintain the most primitive stem cell compartment including engrafting/repopulating cells [104] and supports expansion of at least some other primitive members of the stem/progenitor cell compartment (eg LTC-IC) [158]. The microenvironment provided by AFT024 is thus sufficient for maintaining stem cells in an undifferentiated state while allowing commitment, progenitor expansion and differentiation to take place. This chapter focused on defining the contribution of BMP4 within this complex system, in particular defining the role of BMP4 with regard to long-term maintenance of HSC and primitive progenitors. Supplementing BMP4 in this co-culture system to levels reported previously to enhance HSC maintenance (25 ng/ml [117]), did not significantly improve

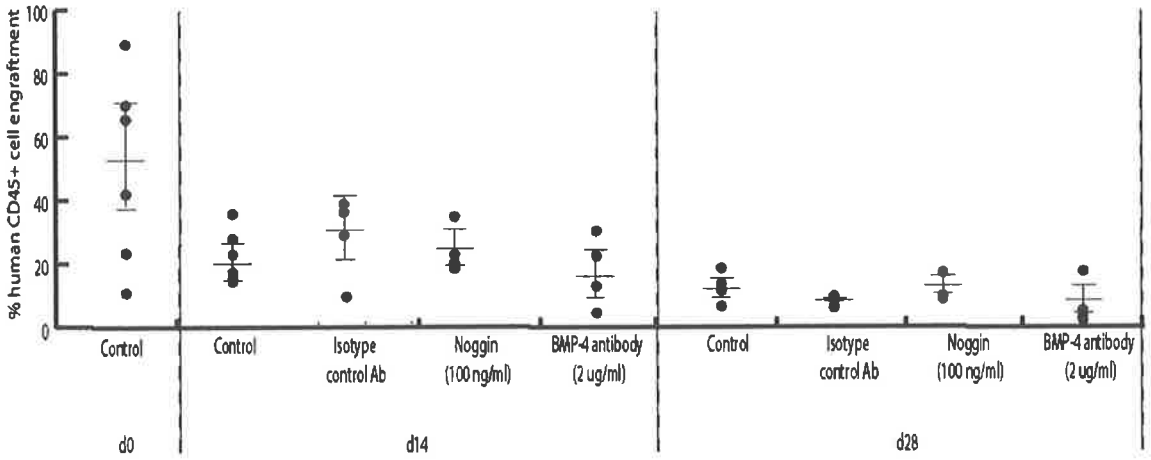


Figure 4.12 Spleen engrafting cells. Detection of human repopulating cells in NOD-SCID mouse spleen transplanted with expanded cord blood $CD34^+$ cells with and without BMP4 blockade after 14 and 28 days co-culture on AFT024. Graph shows amount of human chimerism in the spleen of NOD-SCID mice detected 4-5 weeks post-transplantation with the total progeny from 1 x 10^5 cultured human cord blood $CD34^+$ cells (or d0 unexpanded cord blood $CD34^+$ cells).

primitive cell maintenance, however our data is consistent with BMP4 being a component of the AFT024 supportive niche. As reported previously [104] we found that non-contact co-culture with AFT024 resulted in expansion of LTC-IC over 14 days (mean fold increase in LTC-IC of 1.9-fold). Consistent with this, we observed high levels of NOD-SCID engraftment using $CD34^+$ cells expanded after 14 and 28 days culture with AFT024. Specific inhibition of BMP4 activity with a neutralising BMP4 antibody reduced output of primitive haemopoietic cells significantly based on phenotypic ($CD34^+CD38^-$) and functional (LTC-IC) criteria. Blockade with this BMP4 antibody resulted in a net loss of LTC-IC over the 14 day co-culture. Inclusion of noggin in the co-culture resulted in consistent but less dramatic decreases in maintenance of primitive phenotype ($CD34^+CD38^-$), CFU-GM and LTC-IC expansion which did not reach significance. Noggin may however be acting to inhibit other BMPs present during AFT024 co-culture. For example activity of BMP2 and BMP7, which have been shown to promote primitive cell differentiation [117], can also be blocked by noggin but is not inhibited by the anti-BMP4 antibody.

In vivo experiments measuring the multilineage engraftment capacity of cultured $CD34^+$ cells were consistent with a significant effect of the BMP4 antibody on primitive cell maintenance. In a xeno-transplantation assay for HSC based on BM repopulation ability in the NOD-SCID mouse, BMP4 blockade with the neutralising antibody resulted in a significant reduction in engraftment. The d14 antibody-treated group displayed reduced engraftment compared to both the untreated control and the group receiving cells from cultures supplemented with an isotype matched antibody. There was no significant difference in myeloid, erythroid or lymphoid differentiated

progeny of engrafted cells between the groups indicating that BMP4 is acting at the stem cell level.

Stem cell transplantation in the SRC assay uses the intravenous (IV) route of cell administration, which requires homing of the injected cells to reach the BM or spleen haemopoietic microenvironment. We observed less human cell engraftment in mouse spleen compared to BM and differences in BM and spleen homing stem cells have recently been reported [210, 212]. Stem cells which home to BM are significantly enriched for cells capable of secondary multilineage engraftment following serial transplantation and express significantly more BM adhesion associated surface proteins [212]. Whilst we can not rule out a BMP4 effect on HSC homing in this study, the consistency between the *in vitro* LTC-IC data and the transplantation assay are most consistent with BMP4 activity contributing to maintenance and self-renewal of HSC in this co-culture system. In Chapter 5, these results are considered in the context of other reports of BMP4 activity on stem cell maintenance and self renewal.

This chapter has provided evidence supporting a role for BMP4 as an active regulator of HSC maintenance/expansion in the AFT024 co-culture model. It has been suggested previously that BMP4 may have the capacity to support maintenance of HSC from CB [117] and together with the data presented here it is clear that BMP4 should be considered as an important component when establishing conditions for clinical *ex vivo* expansion culture of CB-derived HSC (as discussed in Chapter 5).

CHAPTER 5

CLINICAL EX VIVO EXPANSION OF CORD BLOOD HSC WITH BMP4

5.1 Introduction

The microenvironment of the stem cell niche is crucial for maintenance of the self-renewal capacity of early stem cells [83, 97, 107, 156]. Studies focused on defining stromal cell signalling may lead to the isolation of molecules that can be used in a clinically suitable culture system for HSC *ex vivo* expansion [104]. Due to the possible transmission of infectious diseases, the safety of graft tissue expanded in the presence of xenogeneic stromal cells (such as AFT024) can not be guaranteed [22, 31]. For an *ex vivo* expansion system to have wide applicability in the clinic it must provide reliable and reproducible results and comply with regulatory restrictions. It must also be serum-free and stroma-free and use clinically approved cytokines and media [22, 31]. A number of serum-free media preparations have been developed and these have been shown to promote haemopoietic cell proliferation [22, 63]. CB, BM and PB $CD34^+$ cells expand when cultured in the serum-replacement cell medium X-Vivo10™ (Cambrex). The majority of studies in which progenitors are cultured in stroma-free, serum-free, conditions with cytokine supplementation show that culture for less than 6 days results in a modest increase in SRC frequency, but loss of SRC is observed if the cultures are extended for longer periods [81]. Individual cytokines are known to trigger many intra-cellular signal transduction pathways which activate multiple gene transcription factors. Thus, large combinations of many cytokines can potentially have complex effects on HSC and defining a combination that permits CB stem cell expansion is a significant challenge [40]. Increasing evidence suggests that it may be possible to develop defined stroma-free systems containing primitive acting growth factors or soluble fusion proteins that focus on specific pathways involved in expansion of HSC [112, 114, 116, 175]. It will be important to optimise use of these in clinical *ex vivo* expansion cultures. Here we assess the ability of BMP4 to enhance haemopoietic progenitor cell

maintenance using the X-Vivo10™ stroma- and serum-free culture system with supplemental FL, SCF & TPO (see Methods section 2.2.14).

5.2 *Defined ex vivo culture of cord blood haemopoietic stem cells*

Cultures were established using 1 x 10^4 CB $CD34^+$ cells cultured in X-vivo10™ with SCF, TPO and FL at 10 ng/ml (without serum). Haemopoietic cells were cultured for 7 and 14 days and assessed for maintenance of haemopoietic progenitors using *in vitro* functional assays. Over three separate experiments (using duplicate cultures of freshly isolated CB $CD34^+$ cells) total nucleated cells were expanded 8.3-fold ± 2.1-fold at d7 and 25.5-fold ± 3.3-fold at d14 (**Figure 5.1a**). Analysis of expanded cells revealed a significant increase in absolute CFU-GM numbers at both time-points compared to d0 (**Figure 5.1b**) (representing 6.1-fold ± 1.2-fold expansion at d7 and 16.8-fold ± 3.5-fold expansion at d14). Importantly it was demonstrated that absolute numbers of primitive LTC-IC were maintained at high levels over 14 days *ex vivo* culture (71.2% ± 11.5% at d7 and 70.5% ± 11.2% at d14) (**Figure 5.1c**). This level of haemopoietic progenitor output is significantly lower than that generated in AFT024 co-culture (as reported in Chapter 4; e.g. in AFT024 co-culture, LTC-IC were expanded 1.9-fold and CFU-GM were expanded 22.5-fold) and demonstrates the difficulty of expanding primitive haemopoietic cells without stroma and serum. However, these results are comparable to previous reports using defined culture systems which are clinically relevant [200, 213-215].

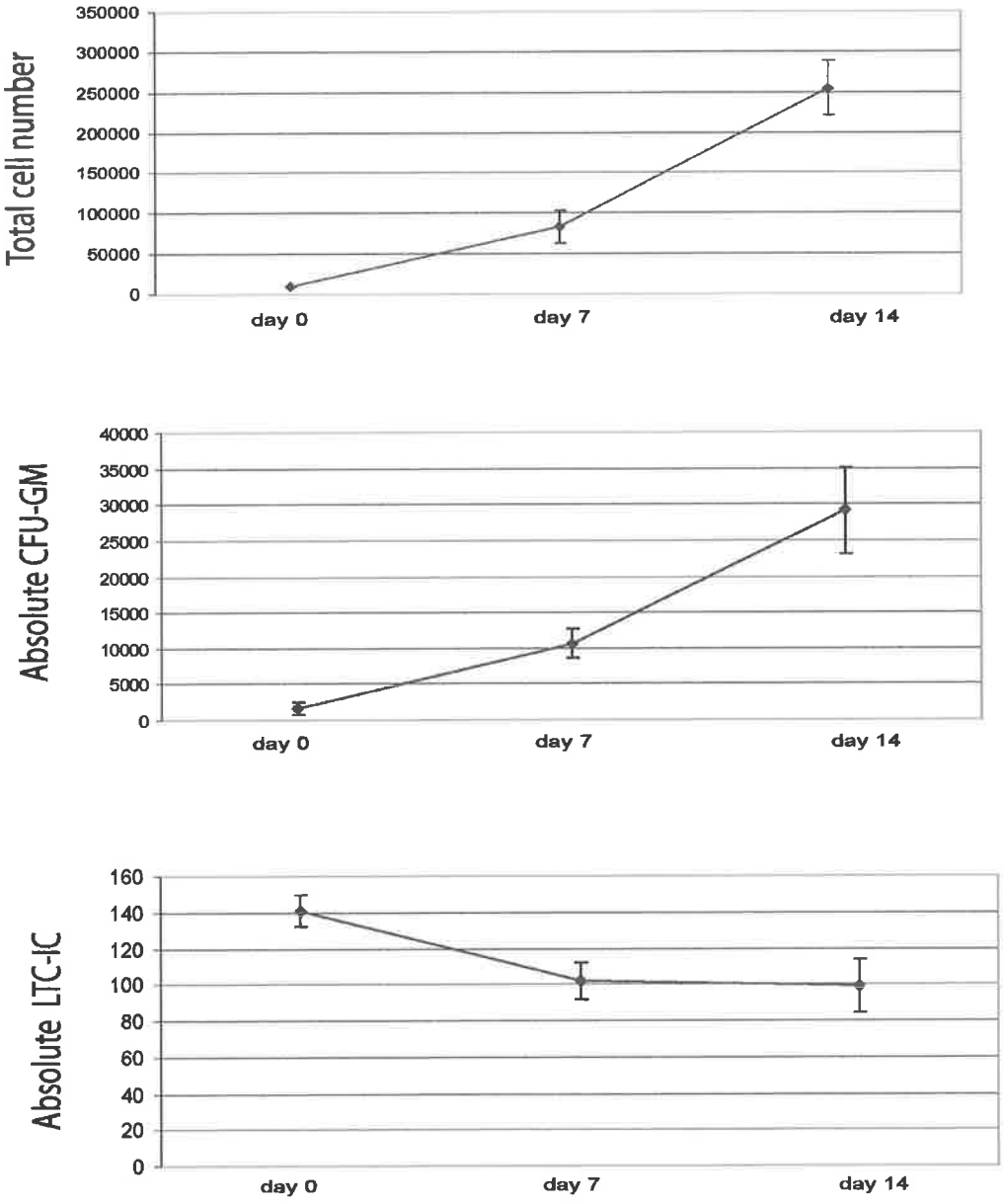


Figure 5.1 Stroma- and serum-free *ex vivo* expansion culture. (a) Total haemopoietic cell number **(b)** absolute CFU-GM and **(c)** absolute LTC-IC at d0, d7 and d14 of stroma- and serum-free culture of cord blood $CD34^+$ cells with supplemental FL, SCF & TPO, cultured in X-Vivo10™ media. Absolute progenitor (CFU-GM or LTC-IC) content was determined by multiplying total haemopoietic cell number with progenitor cell frequency (n=3 experiments).

5.3 *Clinical expansion of cord blood CD34$^+$ cells with supplemental BMP4*

It was next investigated whether output of the above stroma-free culture system could be improved with addition of rBMP4. We focused on haemopoietic output (i.e. CFU-GM) and maintenance of primitive cells (i.e. LTC-IC). BMP4 was added over a range of concentrations from 1 ng/ml (which is comparable to endogenous BMP4 levels in AFT024 culture, see Section 3.5) to 50 ng/ml. Previous reports of BMP4 supplementation supporting SRC maintenance in a serum-based culture system used 25 ng/ml [117]. Fresh CB CD34$^+$ cells were cultured for 7 and 14 days at 1 x 10^4 cells/ml, and evaluated using immunophenotyping, CFU-GM and LTC-IC assays as previously described (see Methods section 2.2.15 and 2.2.16). Total cell viability in control cultures (see Methods section 2.2.12) decreased significantly at day 14 of culture (85% ± 2.3%) compared to d7 (95% ± 1.8%, $P< 0.05$) and d0 (96% ± 1.1, $P<0.05$). There was no significant difference in cell viability with BMP4 treatment. After culture for 7 and 14 days, the average level of total cell expansion with supplemental BMP4 was not significantly different to controls (**Figure 5.2**). Maintenance of primitive haemopoietic cell phenotype after 7 and 14 days *ex vivo* culture with or without BMP4 was then determined. In all conditions the proportion of cells with a CD34$^+$CD38$^-$ phenotype was significantly higher than previous experiments using AFT024 co-culture (as described in Chapter 4); e.g. in AFT024-supported cultures the percentage of CD34$^+$CD38$^-$ cells at d14 was 2.7% (see Appendix C), compared to control stroma-free cultures of 8.2% (see Appendix H). It has been recently reported that CD38 surface protein can be down-regulated during stroma-free culture of haemopoietic cells (due to a depletion of retinoids that occurs over time during *in vitro* culture) resulting in a poor correlation

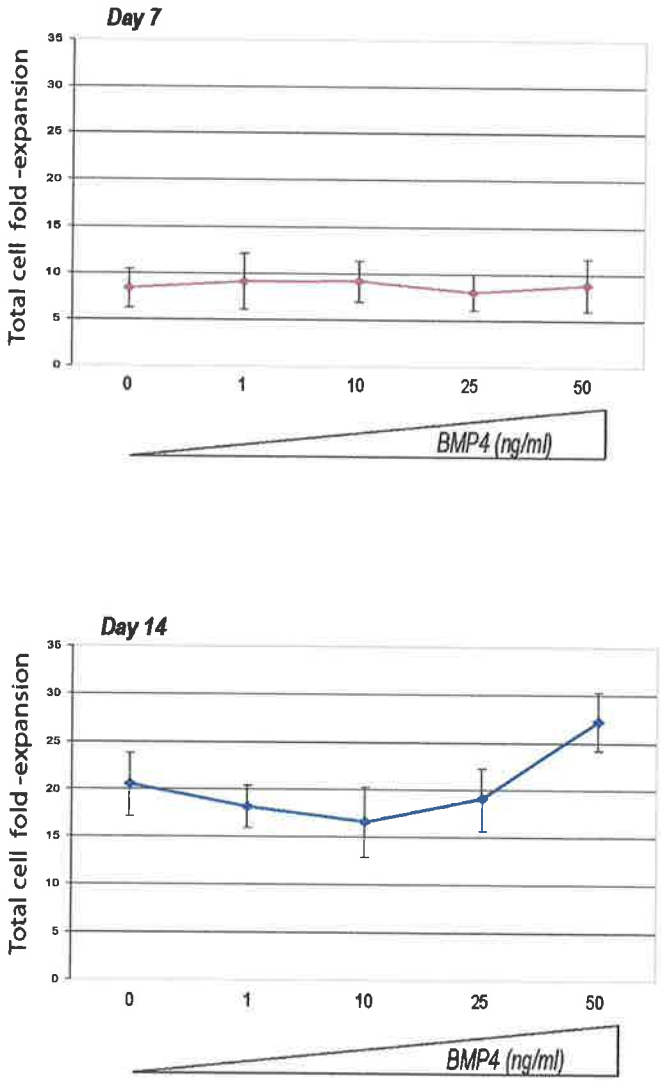


Figure 5.2 Stroma- and serum-free *ex vivo* expansion culture with rBMP4. Total haemopoietic cell fold-expansion of **(a)** d7 and **(b)** d14, stroma- and serum-free cultures of cord blood $CD34^+$ cells with supplemental FL, SCF & TPO in X-Vivo10™ media, with or without rBMP4 at 1, 10, 25 or 50 ng/ml (n=3 experiments).

between cultured SRC and $CD34^+CD38^-$ phenotype [186, 216-218]. This may not occur in stromal co-culture, for example a study by Chute *et al.* [219], demonstrated that CD38 down-regulation in $CD34^+CD38^-$ cells co-cultured with stromal cells (human brain endothelial cell line (HUBECS) was prevented by soluble factors secreted by the stroma. In this system there is therefore a good correlation between cultured SRC and $CD34^+CD38^-$ phenotype [219]. Our observations of higher $CD34^+CD38^-$ detection in stroma-free cultures compared to co-culture with AFT024 are consistent with this report and suggest a similar mechanism that prevents down-regulation of CD38 surface expression in AFT024 co-culture. The analysis in **Figure 5.3** compares the proportion of cells with $CD34^+CD38^-$ phenotype with or without BMP4 treatment in stroma-free cultures. A BMP4 dose-dependent effect was observed at both day 7 and 14 of culture (**Figure 5.3**). Supplemental BMP4 at 50 ng/ml (at d14) and 25 ng/ml (at d7) significantly enhanced the proportion of $CD34^+CD38^-$ cells remaining in culture by 207.8% (± 29.1%, $P<0.05$) and 151.0% (± 12.8%, $P<0.05$) respectively compared to untreated controls. However, we cannot conclude that phenotype is associated with increased SRC as $CD34^+CD38^-$ does not always correlate with SRC in stroma free cultures and we can not exclude the possibility that BMP4 affects shedding of the CD38 extra-cellular protein.

5.4 Maintenance of haemopoietic progenitors in defined ex vivo culture with BMP4 supplementation

In vitro assays (CFU-GM, and LTC-IC) were then performed to assess haemopoietic progenitor cell output with BMP4 supplementation in stroma-free cultures. BMP4 had no significant effect on CFU-GM expansion or absolute CFU-GM

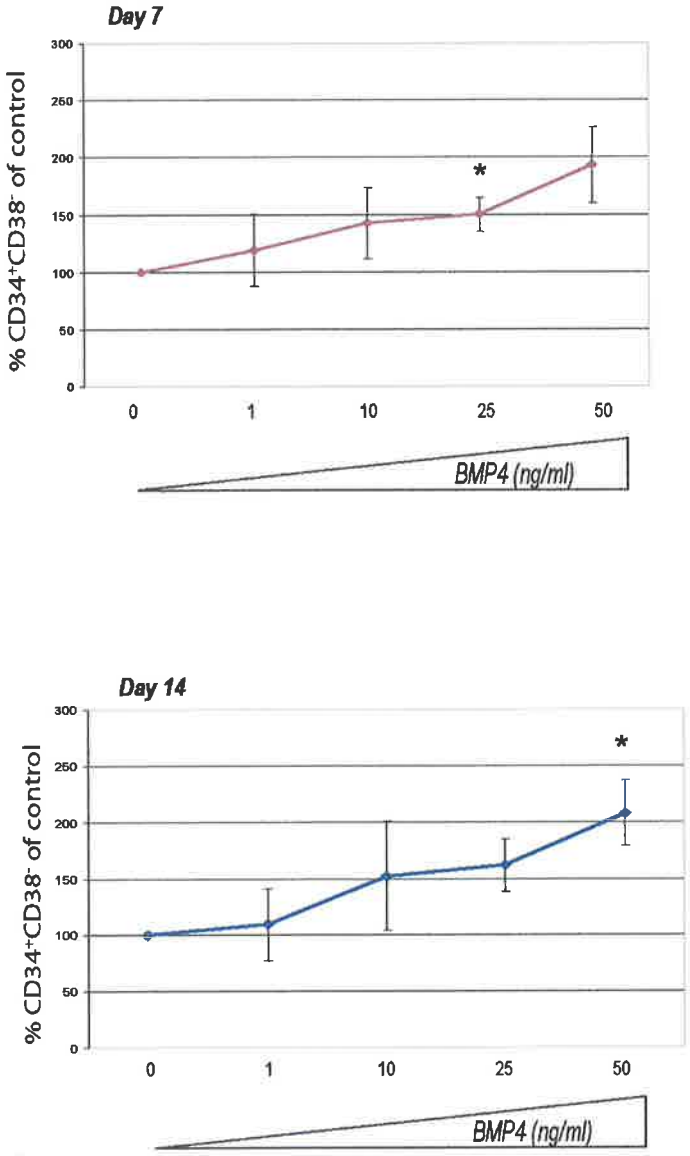


Figure 5.3 Maintenance of primitive cell phenotype in stroma- and serum-free *ex vivo* expansion culture with rBMP4. Percentage of cells maintaining primitive cell phenotype ($CD34^+CD38^-$) at **(a)** d7 and **(b)** d14, in stroma- and serum-free cultures of cord blood $CD34^+$ cells with supplemental FL, SCF & TPO in X-Vivo10™ media, with or without rBMP4 at 1, 10, 25 and 50 ng/ml (n=3 experiments, * =$P< 0.05$) (see Appendix H for flow cytometry histograms).

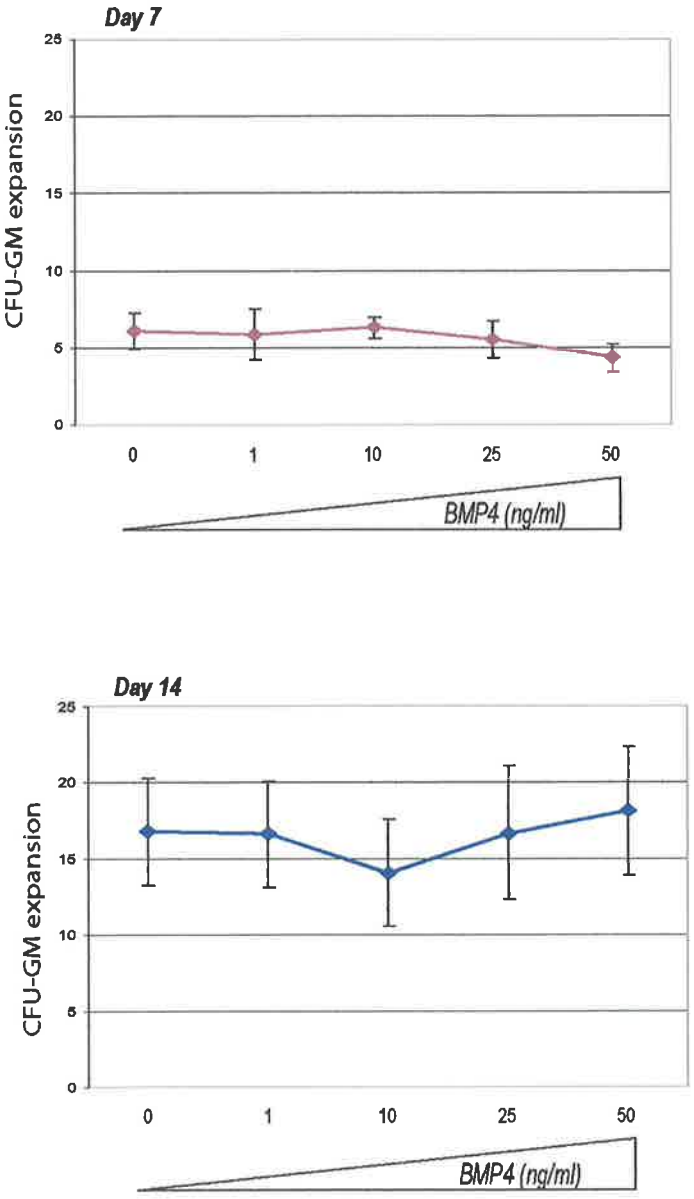


Figure 5.4 CFU-GM output in stroma- and serum-free *ex vivo* culture with BMP4. Total CFU-GM fold-expansion of **(a)** d7 and **(b)** d14, stroma- and serum-free cultures of cord blood $CD34^+$ cells with supplemental FL, SCF & TPO in X-Vivo10™ media, with or without rBMP4 at 1, 10, 25 or 50 ng/ml (n=3 experiments).

numbers over three experiments (a large variation in CFU-GM frequency was observed in all conditions) (**Figure 5.4** & see Appendix I). Over three separate experiments we observed an average 71.2% (± 10.0%) maintenance of LTC-IC at day 7 in control cultures. In contrast to CFU-GM, BMP4 supplementation at 1 ng/ml and 25 ng/ml significantly enhanced LTC-IC maintenance at d7 compared to untreated control cultures (**Figure 5.5** & see Appendix I). Both 1 and 25 ng/ml doses were significantly different from controls (1.10-fold expansion ± 0.13-fold, $P<0.05$ and 1.17-fold expansion ± 0.16-fold, $P<0.05$ respectively) and 10 ng/ml showed the same trend. Following 14 days culture, LTC-IC were maintained at 0.71-fold expansion ± 0.22-fold (or 70.5%) in control cultures and there was no significant difference for cultures supplemented with BMP4.

5.5 Conclusion

Cytokine cocktails have been used to clinically expand haemopoietic cell fractions with limited success [81]. The commonly studied cytokines include G-CSF, GM-CSF, IL-3, IL-6, SCF, FL and TPO. Combinations of cytokines can act in a synergistic manner allowing massive increases in cell number *ex vivo*, but these lead to loss of primitive cells and engraftment capacity over time [65]. The work in this chapter demonstrated prolonged maintenance of LTC-IC during *ex vivo* culture with supplemental cytokines SCF, TPO and FL in defined XVivo-10™ media. Haemopoietic output was significantly reduced compared to cultures containing stroma and serum (discussed in Chapter 4). Whilst removing serum from *ex vivo* culture is required for regulatory compliance, expansion of primitive cells without serum is difficult to achieve. For example Piacebello *et al.*, using long-term serum containing cultures,

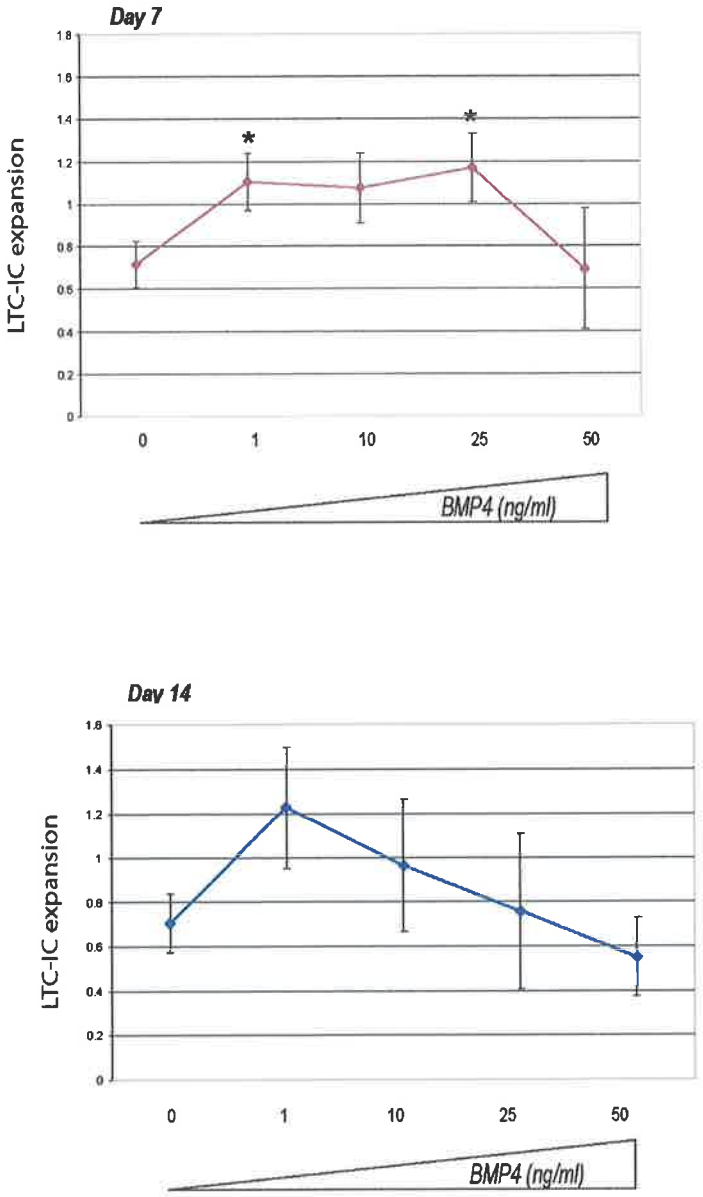


Figure 5.5 LTC-IC output in stroma- and serum-free *ex vivo* culture with BMP4. Total LTC-IC fold-expansion of **(a)** d7 and **(b)** d14, stroma- and serum-free cultures of cord blood $CD34^+$ cells with supplemental FL, SCF & TPO in X-Vivo10™ media, with or without rBMP4 at 1, 10, 25 or 50 ng/ml (* =$P< 0.05$, n=3 experiments).

demonstrated high levels of CFU-GM and LTC-IC expansion [220], however, the result could not be repeated using serum replacement media preparations with a similar cytokine cocktail [213]. Here we observed a net loss of LTC-IC activity over 14 days without support from serum or stromal cells. This *ex vivo* protocol was chosen as it mirrors closely culture systems that have been used for clinical trials. For example, Williams *et al.* expanded CB $CD34^+$ cells for 12 days in serum-free X-Vivo10™ medium supplemented with 1% HSA and a GM-CSF/IL3 fusion protein [221]. An overall median 26-fold total cellular expansion was observed prior to transplant (compared to 20-fold at d14 in our culture system, **Figure 5.5**). No toxicity was attributed to the expanded cells, and clinical benefit of reduced neutropenia following high-dose chemotherapy was observed.

While we observed a net loss of LTC-IC in this culture system over 14 days, we have shown an ability of BMP4 at 1 and 25 ng/ml to restore maintenance of LTC-IC *ex vivo* in stroma- and serum-free culture. This is consistent with previous reports of BMP4 activity. For example BMP4 has been shown to mediate the self-renewal of primitive haemopoietic progenitors in embryoid bodies [222] and Bhatia *et al.* [117] demonstrated an ability of BMP4 at 25 ng/ml to promote short-term maintenance of SRC in culture (83% maintenance over 6 days in IMDM with 1% BSA and growth factors). Taken together, all of these studies suggest BMP4 has the ability to contribute to maintenance of HSC in *ex vivo* cultures. Maintaining cultured HSC *ex vivo* represents a critical step towards developing culture systems that may be able to expand HSC from CB, as well as for developing gene therapy strategies [223]. It appears likely that *ex vivo* expansion of immature haemopoietic cell compartments by as little as 10-fold will be sufficient for widespread clinical application [22]. However, despite many attempts in recent years, a

defined *in vitro* system capable of more than a two-fold expansion of true engrafting HSC has not been described. Even in situations where growth factors such as Wnt and notch ligands have been used in tandem, their observed *in vitro* effects to date have been modest [223, 224]. All reported clinically trialled systems have been unable to expand HSC at comparable levels to stromal-dependant co-cultures. To recreate the supportive stem cell niche in defined culture, the precise combination and dose of all contributing components must first be identified. We show here that BMP4 represents an important factor in stromal cell maintenance of primitive cells (Chapter 4) and also exhibits primitive haemopoietic cell maintenance activity in stroma-free culture (this Chapter). Future experiments testing BMP4 in combination with other growth factors and measurement of engrafting cell frequency will be important for the development of successful protocols for *ex vivo* expansion of CB HSC.

CHAPTER 6

FINAL DISCUSSION

6.1 Stroma-dependent ex vivo expansion of cord blood HSC

Umbilical CB has gained acceptability as an alternative transplant modality for patients who lack suitable BM donors [23]. However, delayed haemopoietic recovery and higher rates of graft failure compared to BM or PB SCT remain the major limitations of the procedure [23]. Both complications relate to the limited cell dose in a CB collection. There is currently much interest in the *ex vivo* expansion of CB HSC and clinically approved *ex vivo* expansion cultures have been established [22]. Whilst HSC maintenance can be supported during short-term, defined, *in vitro* culture with a combination of haemopoietic cytokines [81], HSC cannot be expanded significantly in long-term culture in the absence of stroma [103, 157]. Adult BM, fetal liver and extra-embryonic placenta have however all been shown to provide a specialised and supportive niche for HSC [17, 96, 97, 103, 155, 156]. In the developing embryo the fetal liver provides a unique environment allowing significant expansion in HSC number prior to establishment of haemopoiesis in the BM. The ability of murine fetal liver-derived cell lines to maintain and expand HSC in long-term cultures has been well documented [225, 226], however the mechanism involved remains largely unknown.

Here we focus on the supportive capacity of the murine fetal liver cell line AFT024 which permits long-term maintenance of HSC [74, 158]. We first optimised co-culture conditions that would stimulate total cell expansion while supporting the most primitive haemopoietic cells (Section 4.2). We found that a non-contact AFT024 co-culture was able to significantly expand LTC-IC over 14 days (mean fold increase in LTC-IC 1.9-fold) as reported previously [104]. Consistent with this, we observed high levels of NOD-SCID engraftment using $CD34^+$ cells expanded after 14 days culture with AFT024. We determined the AFT024 mRNA expression profile for proteins known to act on HSC and stimulate self-renewal. This analysis demonstrated that Jag-1,

mKirre and BMP4 mRNA all are abundantly expressed by AFT024. These results are consistent with combinations of soluble factors acting synergistically to maintain the self-renewal capacity and multipotency of HSC.

6.2 *BMP4 as a haemopoietic growth factor*

Here a key aim was to investigate the specific role of BMP4 in the maintenance of primitive CB cells during co-culture in the AFT024 *in vitro* model, and in a defined clinically relevant culture system. We focused on BMP4 for a number of reasons. The BMP proteins are members of the TGFβ super-family of signalling molecules. These pleiotropic cytokines are important for embryonic tissue development and for regulation of cell proliferation, differentiation, morphogenesis and apoptosis in multiple systems [160]. BMP4 in particular, has been shown to regulate the differentiation of embryonic mesoderm into haemopoietic tissues in Xenopus [143, 174], and can regulate the haemopoietic transcription factors GATA-1, GATA-2, SCL, LMO-2 and MixL1 [148-151, 165]. In this study we observed expression of BMP4 mRNA and secretion of soluble BMP4 by AFT024 that is capable of maintaining HSC in long-term co-culture [74]. We have also detected BMP4 mRNA expression in mouse 14.5 dpc fetal liver. Thus, we focused on defining the contribution of BMP4 to long-term, primitive haemopoietic cell maintenance in the AFT024 *ex vivo* culture system. We first supplemented AFT024 cultures with BMP4 at concentrations equivalent to that reported to have HSC supportive capacity [117]. Whilst supplementation with ATRA in AFT024 co-cultures has been shown to increase expansion of primitive haemopoietic progenitors in a stromal cell-dependent fashion [185], addition of BMP4 to AFT024 co-cultures did not increase LTC-IC expansion. To test whether BMP4 produced in the AFT024 co-culture is sufficient for HSC maintenance we used two specific neutralising reagents

(rNoggin and neutralising BMP4 antibody) to block BMP4 activity during co-culture. Inhibition of BMP4 activity with neutralising antibody reduced output of primitive haemopoietic cells based on phenotypic criteria ($CD34^+CD38^-$) and led to a net loss of LTC-IC. These results were mirrored in experiments measuring the multilineage engraftment capacity of cultured $CD34^+$ cells using the NOD-SCID transplantation assay. In this assay for HSC with BM repopulation ability, the BMP4 antibody-treated group displayed a significant reduction in engraftment compared to both the untreated control and the group receiving cells from cultures supplemented with an isotype matched antibody. Whilst additional information could be drawn from using limiting dilution analysis of transplanted HSC (to determine SRC frequency [81]) and measuring later time-points of engraftment (e.g. > 7 weeks engraftment to assess long-term repopulating cells [14, 81]), the consistency between the *in vitro* LTC-IC data and the transplantation assay are most consistent with BMP4 activity contributing to maintenance of HSC in this co-culture system. Supplemental noggin resulted in consistent reductions in LTC-IC, and in engraftment, compared to untreated cultures, however changes in both cases were not significant.

BMP4 mRNA was shown to be up-regulated in the cultured HSC (Section 3.9) and this may provide an autocrine signal. However compared to AFT024 cells in this system, HSC are rare and do not expand without stromal support. It may be possible to determine the precise contribution of AFT024 secreted BMP4 (as opposed to HSC derived BMP4) using RNAi technology targeting BMP4 mRNA production in AFT024.

To test further the capacity of BMP4 to directly act on CB HSC, we tested the ability of BMP4 to contribute to the maintenance of LTC-IC in a stroma-free culture, based on a current clinical expansion protocol [221]. In this system BMP4 supported cells with $CD34^+CD38^-$ phenotype and maintained LTC-IC levels after 7 days culture

equivalent to fresh CB. A previous study by Bhatia *et al.* [117] has demonstrated an ability of BMP4 at 25ng/ml to promote short-term maintenance of SRC in culture. There is therefore an accumulation of evidence that BMP4 has the ability to support maintenance of HSC/haemopoietic progenitors in various *ex vivo* cultures.

6.3 Mechanism of BMP4 activity

BMP4 may be acting to support HSC maintenance by multiple mechanisms. BMP4 can promote both proliferation and survival signals [117, 227, 228] however, we did not detect BMP4-induced survival in this system suggesting BMP4 may affect the cycling or self-renewal capacity of primitive haemopoietic cells. Whilst our cell survival assay was limited by the heterogeneity of the $CD34^+$ cell population analysed, methods to assess cell death in more primitive haemopoietic populations were not available. We cannot rule out an indirect BMP4 action mediated by the induction of other soluble factors in AFT024 or HSC. For example, BMPs have been shown to positively regulate Wnt3a expression in primitive neuronal progenitor cells [229] and the BMP4 gene has been reported to be a downstream target of both Shh and Wnt3a signalling [110, 230]. However we show here that both Shh and Wnt3a mRNA are undetectable or poorly expressed in AFT024, suggesting that BMP4 is acting independently of these signalling pathways in this model. Furthermore, a direct role for BMP4 in HSC maintenance in the AFT024 co-culture model is consistent with previous data. For example BMP4 has been shown to mediate the self-renewal of primitive haemopoietic progenitors in embryoid bodies [222] and promote short-term maintenance of SRC in culture [117].

Whilst the role in haemopoiesis of haemopoietic cytokines and their associated signal transduction pathways has been well characterised *in vivo*, much less is known about haemopoietic functions of other growth factors such as the BMPs. Mice lacking

BMP4 die *in utero* before blood formation [231], making it difficult to directly test the function of BMP4 in mammalian haemopoiesis *in vivo*. However, there is now accumulating evidence from both *in vivo* and *in vitro* experiments indicating that BMP4 can act at multiple points in mammalian haemopoiesis [165, 175, 205]. The BMP activated Smad complex, which translocates into the nucleus is thought to function by associating with DNA binding transcription factors *in vivo* to control the expression of haemopoietic genes such as AML1, Runx1, Cbfa2 and Gata-2 [232]. However, much evidence suggests that the BMP/Smad cascade can also interact with other signalling systems that may have a role in regulating haemopoiesis. Several studies have provided evidence for cross-talk between the BMP-Smad and the JAK-STAT pathways. Leukemia inhibitory factor (LIF) can synergise with BMP2 by means of a complex containing Smad1, STAT3 and p300 [233]. In the frog, BMP and gp130 receptor signalling pathways exhibit synergistic activity in ventral mesoderm patterning [234, 235]. Whilst STAT5 activation can promote HSC self-renewal [236, 237], it is unknown whether the BMP and JAK-STAT signalling pathways can cooperate to regulate HSC. Recent evidence has also linked BMP signalling with the notch pathway. Notch signalling has been implicated in lineage decision making processes in a variety of cell types. Notch-1 knockout mice (but not notch-2) experience severely impaired definitive haemopoiesis and have significantly lower BMP4 expression *in vitro* and *in vivo* [238]. Addition of BMP4 protein to the notch1 deficient cultures failed to rescue the haemopoietic defect, indicating that both notch and BMP signalling are required in this system. Such synergy between supportive factors has also been demonstrated in murine embryonic stem (ES) cells where LIF and BMP4 act in concert to maintain mouse ES cell self-renewal [239]. BMP4 was shown to act as a "stem-cell pluripotency factor", and induced members of the Id family via Smad dependent mechanisms [239]. This

activity is consistent with an ability of BMP4 to expand epithelial progenitors by upregulation of Id proteins [240] and a key role of BMP4 signalling may be to block differentiation. This activity has been reported in mouse ES cells where BMP4 inhibits the Erk and p38 MAP kinase pro-differentiation pathways [241], while it induces differentiation in human ES cells [242, 243]. This suggests a complex role for BMP4 in regulating stem cell properties [244]. A greater understanding of the regulation and expansion of murine and human ES cells, which undergo extensive self-renewal in culture (and defining the precise role of BMP4 in this process), may provide further insight into the regulation of HSC self-renewal [245].

Recent studies have also suggested an important role for BMP4 in regulating BM derived adult HSC. Zhang *et al.* demonstrated that BMP4 signalling indirectly controls the number of HSC *in vivo* by regulating the size of the stem cell niche [96]. Bhardwaj *et al.* has shown that the proliferation and differentiation of HSC i*n vivo* can be regulated by both BMP4 and Shh [110]. These factors were shown to collaborate with cytokines in the BM to create an environment that maintains stem cell homeostasis. There is therefore an accumulation of evidence that BMP4 has the ability to support maintenance of HSC both *in vitro* and *in vivo* via multiple mechanisms. Further characterisation of downstream signalling cascades in primitive haemopoietic and ES cells will help elucidate the mechanism of BMP4 activity in these systems.

6.4 Future research in cord blood ex vivo expansion

Increasing our understanding of stem cell biology and signalling is critical for a wider therapeutic use of CB [22]. Recent data from clinical trials has strengthened the feasibility of CB for transplantation in adult patients, particularly if no suitable BM donor is identified [38]. While variations to the transplantation protocol, including the

use of multiple CB units and non-myeloablative regimes have potential to improve outcome for adult patients [40], there is still currently much interest in the clinical *ex vivo* expansion of CB HSC. Clinical trials of *ex vivo* expansion have thus far focused on expanding both the number of short-term repopulating cells and mature cells with the goal of limiting the period of pancytopenia observed following high-dose chemotherapy or chemoradiotherapy [22]. Studies to date have not addressed whether true long-term repopulating cells have been expanded and transfused and contribute to the improved overall rate of engraftment [22]. This can be attributed to a lack of appropriate assays to accurately quantitate long-term HSC, and to regulatory restrictions and difficulty in obtaining high grade reagents/growth factors suitable for clinical use [22, 40]. None of the clinical trials reported to date have utilised combinations of the cytokines required for maximal LTC-IC or SRC expansion *in vitro* (such as a cocktail of FL, SCF & TPO), or have trialled *ex vivo* culture with developmentally important growth factors (such as BMPs, Wnts, hedgehogs or notch ligands [22, 246]) or intracellular molecules such as HoxB4, Bmi-1, C/EBP-alpha or gfi-1 that can expand HSC *in vitro* [247-251]. Thus, true clinically useful stem cell expansion strategies that would allow the use of very small numbers of stem cells for therapy are yet to be determined and important pre-clinical and clinical trials are still needed. However, *ex vivo* expansion is a promising technology that is likely to have a significant impact on the future of SCT, where insufficient cell dose is a major limitation to their utility for adult transplantation. This study demonstrated a BMP4 contribution to primitive CB cell maintenance in stroma-dependent and stroma-free, serum-free cultures. The identification of further regulators of HSC maintenance (including primitive haemopoietic growth factors and other molecules such as ATRA) represents a major step toward the goal of expanding HSC populations *ex vivo*, thereby expanding their clinical potential in cell replacement and

gene therapy. We have shown that BMP4 can contribute to HSC maintenance, both in an established long-term co-culture model and in a clinical *ex vivo* expansion setting (and have recently published these findings, see Appendix J). On the basis of these studies BMP4 should be considered as a supplement in future CB *ex vivo* expansion protocols and for development of future cell replacement and gene therapy approaches.

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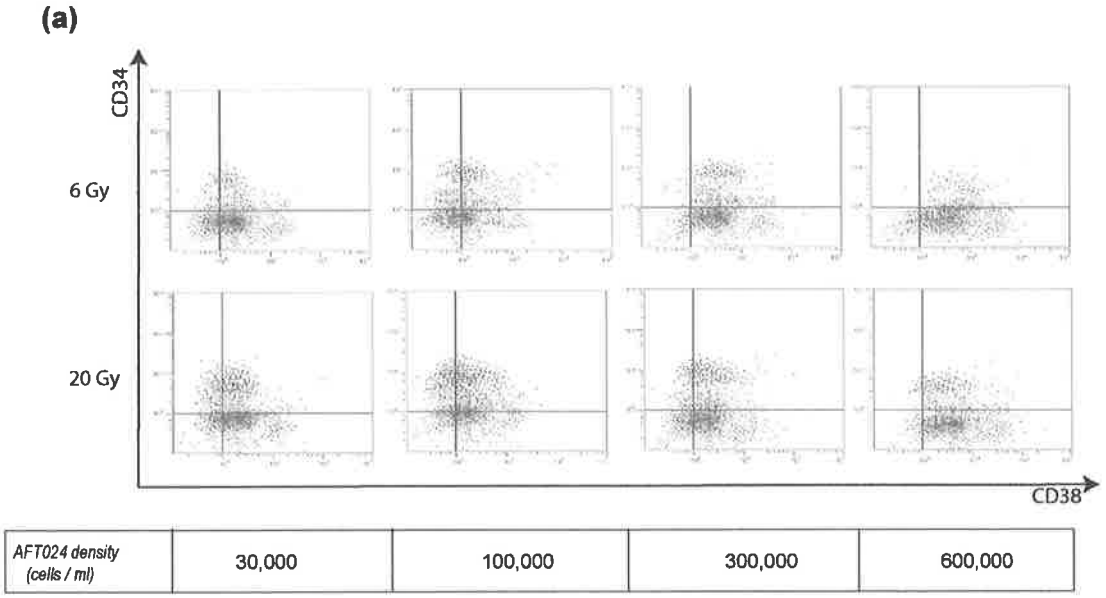
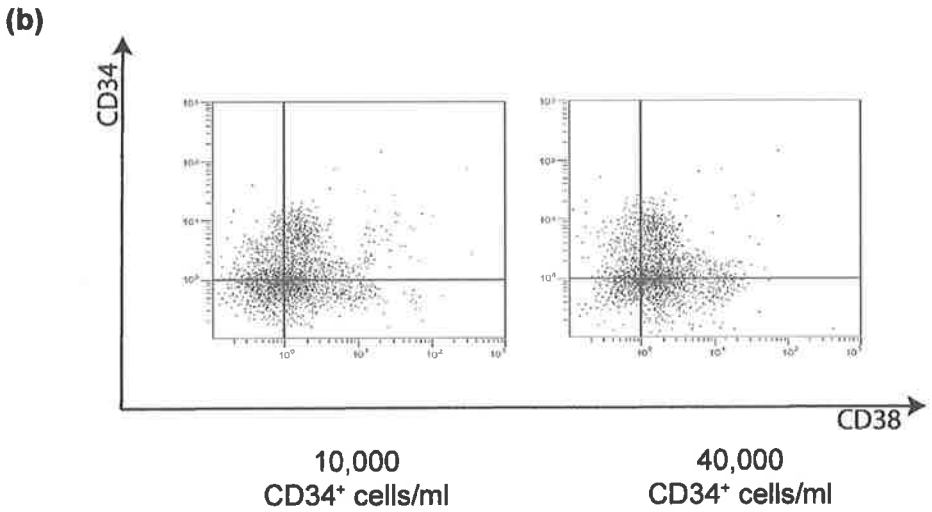
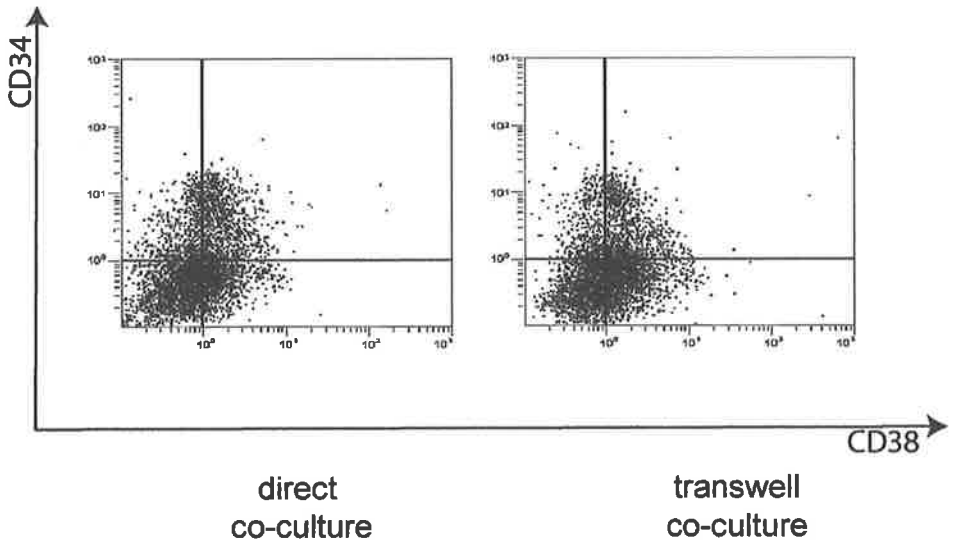
Appendix A





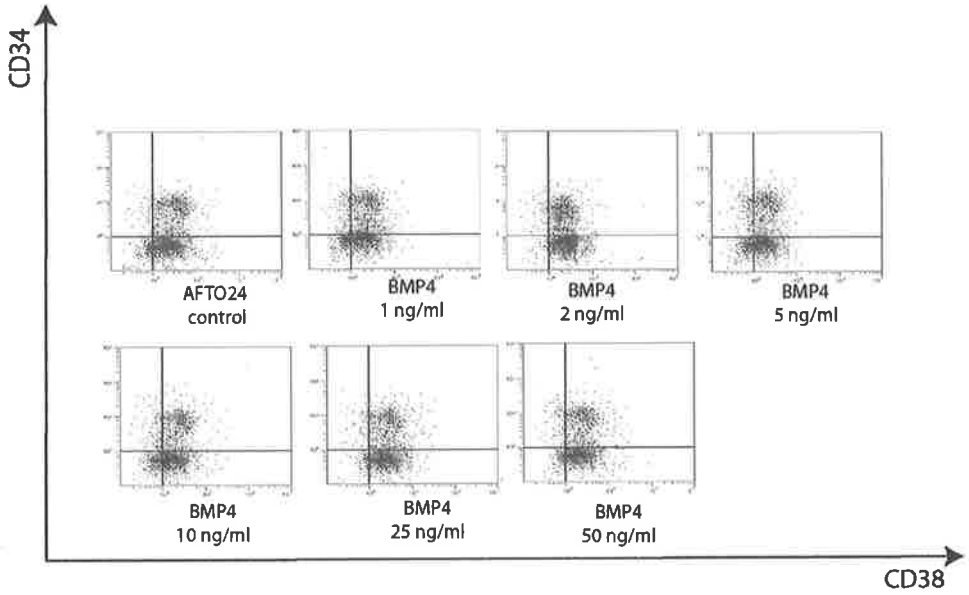
Flow cytometry histograms showing primitive haemopoietic cells maintained on AFT024 under optimised conditions. CD34 and CD38 expression analysis of expanded cord blood $CD34^+$ cells using flow cytometry after **(a)** co-culture with AFT024 seeded at 3 x 10^4, 1 x 10^5, 3 x 10^5 or 6 x 10^5 cells/ml and pre-irradiated at 6 or 20 Gy, and **(b)** CB $CD34^+$ cells seeded at $1x10^4$ or $4x10^4$ cells/ml after 14 days culture.

Appendix B



Flow cytometry histograms showing maintenance of primitive haemopoietic cells in non-contact co-culture with AFT024. CD34 and CD38 expression analysis of expanded cord blood $CD34^+$ cells using flow cytometry after being cultured non-contact (using trans-wells) or in direct contact with AFT024 for 14 days.

Appendix C



Flow cytometry histograms showing maintenance of primitive haemopoietic cells cultured on AFT024 with BMP4. CD34 and CD38 expression on *ex vivo* expanded CB $CD34^+$ cells cultured on AFT024 with or without rBMP4 at 1, 2, 5, 10, 25 and 50 ng/ml at day 14. Cultures were supplemented with FL, SCF & TPO.

Appendix D

(a)

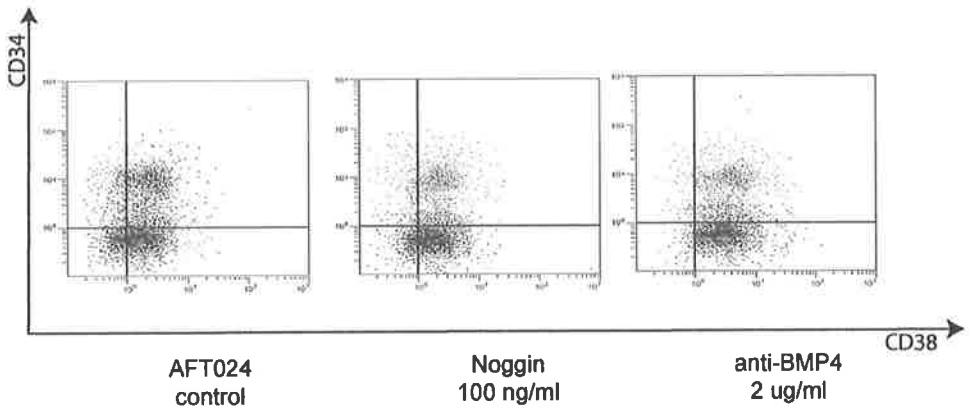
	Day 0	Day 14							
	CB CD34+	*Stroma-free*	*AFT024 control*	*BMP4 1 ng/ml*	*BMP4 2 ng/ml*	*BMP4 5 ng/ml*	*BMP4 10 ng/ml*	*BMP4 25 ng/ml*	*BMP4 50 ng/ml*
Absolute CFU-GM	1,736	8,5456	24,213	16,591	19,942	29,045	23,768	31,184	28,949
s.e.m ±	250	5,287	3,611	4,104	6,766	11,763	7,969	8,501	8,077
significance # P>0.05 * P<0.05 ** P<0.005	-	*	-	#	#	#	#	#	#

(b)

	Day 0	Day 14							
	CB CD34+	*Stroma-free*	*AFT024 control*	*BMP4 1 ng/ml*	*BMP4 2 ng/ml*	*BMP4 5 ng/ml*	*BMP4 10 ng/ml*	*BMP4 25 ng/ml*	*BMP4 50 ng/ml*
Absolute LTC-IC	246	117	443	534	485	587	456	549	526
s.e.m ±	21	38	59	23	88	26	65	122	69
significance # P>0.05 * P<0.05 ** P<0.005	-	*	-	#	#	*	#	#	#

CFU-GM and LTC-IC output in AFT024 co-culture with BMP4 supplementation. Absolute number of **(a)** CFU-GM and **(b)** LTC-IC from 14 day *ex vivo* co-culture of CB $CD34^+$ cells on AFT024 with supplemental FL, SCF & TPO with or without rBMP4 supplementation at 1, 2, 5, 10, 25 and 50 ng/ml (or cultured stroma-free) (n=3 experiments performed in duplicate).

Appendix E



Flow cytometry histograms showing primitive haemopoietic cells co-cultured on AFT024 with BMP4 blockade. CD34 and CD38 expression analysis of expanded cord blood $CD34^+$ cells using flow cytometry after co-culture on AFT024, with or without BMP4 blockade using rNoggin (100 ng/ml) or a neutralising BMP4 antibody (2 ug/ml) with supplemental FL, SCF & TPO at day 14.

Appendix F

(a)

	Day 0	Day 14		
	CB CD34+	*AFT024 control*	*Noggin 100 ng/ml*	*Anti-BMP4 2 ug/ml*
Absolute CFU-GM	933	17,913	14,223	9,253
s.e.m ±	88	4,254	4,139	2,897
significance # P>0.05 * P<0.05 ** P<0.005	-	-	#	*

(b)

	Day 0	Day 14			
	CB CD34+	*AFT024 control*	*Noggin 100 ng/ml*	*Anti-BMP4 2 ug/ml*	*Isotype Matched antibody*
Absolute LTC-IC	229	437	295	193	372
s.e.m ±	88	4,254	4,139	2,897	23
significance # P>0.05 * P<0.05 ** P<0.005	-	-	#	*	#

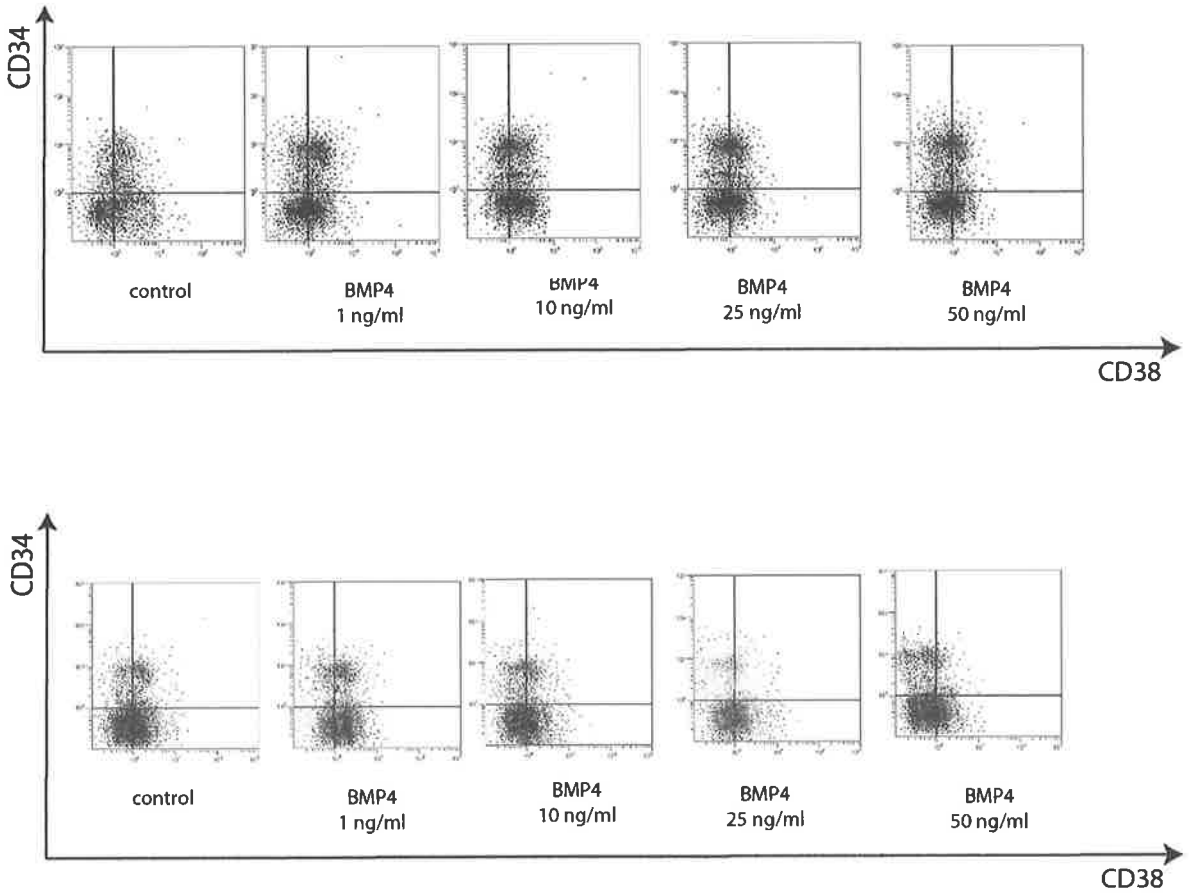
CFU-GM and LTC-IC output in AFT024 co-culture with BMP4 blockade. Absolute number of **(a)** CFU-GM and **(b)** LTC-IC from 14 day *ex vivo* co-cultures of CB $CD34^+$ cells on AFT024 with supplemental FL, SCF & TPO with or without BMP4 blockade using rNoggin (100 ng/ml) or a neutralising BMP4 antibody (2 ug/ml) at d14 (n=3 experiments performed in duplicate).

Appendix G

CB donor	CB volume (mls)	Total MNC	Total $CD34^+$	$\%CD34^+$ (of MNC)
1	51	$2.5x10^8$	800,000	0.32
2	31	$2.1x10^8$	1,550,000	0.74
3	49	$2.0x10^8$	580,000	0.29
4	18	$1.8x10^8$	950,000	0.53
5	46	$2.3x10^8$	950,000	0.41
6	50	$1.0x10^8$	500,000	0.50
7	81	$4.5x10^8$	330,000	0.07
8	40	$2.1x10^8$	233,000	0.11
9	56	$2.0x10^8$	1,000,000	0.50
10	51	$1.65x10^8$	500,000	0.30
11	25	$2.0x10^8$	366,000	0.18
12	50	$1.0x10^8$	210,000	0.21
13	56	$2.0x10^8$	320,000	0.16
14	51	$2.0x10^8$	2,600,000	1.30
15	58	$2.85x10^8$	1,100,000	0.39
16	94	$1.65x10^8$	1,550,000	0.94
17	86	$4.0x10^8$	2,790,000	0.70
18	38	$1.2x10^8$	600,000	0.50
19	26	$0.8x1^8$	920,000	1.15
20	33	$1.1x10^8$	450,000	0.41
21	29	$1.2x10^8$	290,000	0.24
22	35	$1.3x10^8$	850,000	0.65
23	80	$1.86x10^8$	920,000	0.49
AVERAGE	**49.3 ml ± 20.2 ml**	**$1.87x10^8$ ± $0.8x10^8$**	**Total= $2.4x10^7$**	**Average= 0.43 % ± 0.37 %**

Umbilical cord blood collection and processing. Fresh primary human cord blood was obtained following ethics approval from volunteer donors from the Women's & Children's Hospital Adelaide, South Australia. Mononuclear cells (MNC) were isolated by density gradient centrifugation over lymphoprep (Axis-Sield, Oslo Norway) and purified for $CD34^+$ cells using magnetically activated cell sorting (MACS) using Direct CD34 Progenitor Cell Isolation Kit and LS Separation Columns (Miltenyi Biotech, Auburn, CA) (see Methods section 2.2.10-2.2.13) to isolate a total of 2 x 10^7 $CD34^+$ cells for the engraftment assay (see Section 4.9).

Appendix H



Flow cytometry histograms showing primitive haemopoietic cells maintained in clinical expansion cultures with BMP4. CD34 and CD38 expression on CB CD34$^+$ cells cultured stroma- and serum-free with supplemental FL, SCF & TPO in X-Vivo10™ media, with or with supplementation of rBMP4 at 1, 10, 25 and 50 ng/ml after **(a)** 7 day and **(b)** 14 day *ex vivo* culture (n=3 experiments).

Appendix I

(a)

	Day 0	Day 7					Day 14				
	CB CD34+	*control*	*BMP4 1 ng/ml*	*BMP4 10 ng/ml*	*BMP4 25 ng/ml*	*BMP4 50 ng/ml*	*control*	*BMP4 1 ng/ml*	*BMP4 10 ng/ml*	*BMP4 25 ng/ml*	*BMP4 50 ng/ml*
Absolute CFU-GM	1,736	10,606	7,686	8,918	9,293	7,186	29,112	32,780	30,452	29,095	36,966
s.e.m ±	807	2,079	1,635	786	4,447	1,011	6,039	8,607	10,199	5,490	5,366
significance # P>0.05 * P<0.05 ** P<0.005	-	-	#	#	#	#	-	#	#	#	#

(b)

	Day 0	Day 7					Day 14				
	CB CD34+	*control*	*BMP4 1 ng/ml*	*BMP4 10 ng/ml*	*BMP4 25 ng/ml*	*BMP4 50 ng/ml*	*control*	*BMP4 1 ng/ml*	*BMP4 10 ng/ml*	*BMP4 25 ng/ml*	*BMP4 50 ng/ml*
Absolute LTC-IC	141	102	155	157	168	100	100	175	137	112	82
s.e.m ±	9	10	5	33	30	44	47	62	30	18	21
significance # P>0.05 * P<0.05 ** P<0.005	-	-	*	#	*	#	-	#	#	#	#

CFU-GM and LTC-IC output in stroma- and serum-free *ex vivo* culture with rBMP4. Total CFU-GM & LTC-IC fold-expansion of d7 **(a)** and d14 **(b)** stroma- and serum-free cultures of cord blood $CD34^+$ cells with supplemental FL, SCF & TPO in X-Vivo10™ media, with or with supplementation of rBMP4 at 1, 10, 25 and 50 ng/ml (n=3 experiments).

Appendix J

Hutton, J.F., Rozenkov, V., Khor, F.S.L., D'Andrea, R.J. and Lewis, I.D. (2006) Bone morphogenetic protein 4 contributes to the maintenance of primitive cord blood hematopoietic progenitors in an ex vivo stroma-noncontact co-culture system. *Stem Cells and Development, v. 15 (6), pp. 805-813, December 2006*