The effect of bone morphogenetic protein 4 on haematopoietic stem cells

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Declaration

I, Anil Abeyewickreme, confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

Abstract

Bone morphogenetic protein-4 (BMP4) is highly expressed at sites of haematopoietic stem cell (HSC) formation. During HSC formation in humans, BMP4 is strongly expressed by cells underlying the ventral floor of the dorsal aorta. BMP4 in combination with other factors has been shown to play a role in haematopoietic differentiation. The genes *Runx1*, *Scl*, *Gata2* and *Lmo2* are vital to the development of the haematopoietic system, and deletion of these genes produces an embryonic lethal phenotype due to the absence of blood formation. This study investigated whether BMP4 alone upregulates the expression of these genes. The role of BMP4 was explored during HSC development in an embryonic stem (ES) cell differentiation model and at later developmental stages using *ex vivo* foetal liver and bone marrow serum free cultures. Differentiating ES cells cultured in serum-free medium were found to express BMP4 and the BMP receptor endogenously. To establish a model for exogenous BMP4 addition in isolation, lentiviral vectors were used to deliver short hairpin RNA (shRNA) for sustained RNAi knockdown of endogenous *Bmp4* expression during ES cell differentiation. Differentiating shRNA treated ES cells were cultured and the expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* was measured over time by real time reverse transcription PCR. With the addition of exogenous BMP4 alone, expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* was unchanged at days 2 and 4 but increased at day 6 of differentiation. This demonstrates that BMP4 up regulates the expression of these genes which are critical to the development of the haematopoietic system. The use of lentiviral shRNA knockdown provides a model for the control of endogenous growth factors in future investigations of growth factors in ES cell differentiation.

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Abbreviations

AGM	Aorta gonad mesonephros
ALK	Activin like kinase
B-ME	Beta-mercaptoethanol
BFU-E	Erythroid blast forming units
BM	Bone Marrow
BMP4	Bone morphogenetic protein 4
BMPR	BMP receptor
BSA	Bovine serum albumin
CFC	Colony forming cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpc	days post coitum
EB	Embryoid Body
EC	Embryonic carcinoma cells
EDTA	Ethylenediaminetetraacetic acid
ES	Embryonic stem cells
FC	Flow cytometry
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
FL	Foetal liver
GFP	Green fluorescent protein
GM-CFU	Granulocyte macrophage colony forming units
GM-CSF	Granulocyte macrophage colony stimulating factor
HSCs	Haematopoietic Stem Cells
IGF1	Insulin like growth factor
IL	Interleukin
LIF	Leukemia inhibitory factor
LT-HSC	Long term haematopoietic stem cell
MHC	Major histocompatability complex
MMLV	Murine moloney leukaemia virus
MOI	Multiplicity of infection
MTG	Monothio-glycerol
mins	Minutes
mls	Millilitres
NK	Natural killer cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEI	Polyethylenimine
PFA	Paraformaldehyde
RNA	Ribonucleic acid
rpm	Revolutions per minute

RT	Reverse transcription
SCF	Stem cell factor
SF	Serum Free
SLAM	Signalling lymphocyte activation molecule
SR	Serum Replacement
ST-HSC	Short term haematopoietic stem cell
shRNA	Short hairpin RNA
TBST	Tris buffered saline tween 20
TPO	Thrombopoietin
UV	Ultra violet

Chapter 1 Introduction

Among the many different types of stem cells, haematopoietic stem cells (HSCs) are the most extensively studied. Probably the most successful therapy to have arisen out of stem cell research to date is the bone marrow transplant. As with all forms of transplant there is often inadequate supply of appropriately matched donors. For this reason research has focused on methods of expanding HSCs *ex vivo* and more recently the formation of HSCs from ES cells or alternative reprogrammed somatic cells.

1.1 Haematopoietic stem cells

1.1.1 Properties of stem cells

Stem cells are long lived cells, thought to be involved in repair processes within the body's tissues. They are unique in their ability to self renew, thereby surviving over long periods, and in their ability to give rise to specialised cell types. It is these properties that have drawn so much attention. It is hoped that the manipulation of these cells could one day give rise to an unlimited source of tissue that could be matched to a specific individual for transplantation.

Stem cells can be totipotent, pluripotent or multipotent (Figure 1.1). Totipotent cells are cells that are able to give rise to all the tissues necessary for foetal development including all the cells of the body and the placenta. Pluripotent cells can give rise to all the cells of

the body. Multipotent stem cells give rise to a more restricted range of cell types. HSCs give rise to the lymphoid, myeloid and erythroid constituents of the blood and are therefore considered to be multipotent stem cells. However there is data that suggests that the HSC containing fraction of bone marrow can give rise to a diverse range of tissues including: skeletal muscle fibres (Gussoni et al. 1999), cardiac muscle (Orlic et al. 2001), pneumocytes (Theise et al. 2002), renal cells (Kale et al. 2003) and neurones (Bonilla et al. 2002). However other experiments using more thoroughly purified HSC populations cast doubt on these results suggesting that the differentiation of non-haematopoietic cell types is a rarity if it happens at all (Wagers et al. 2002). Further experiments have suggested that the differentiation of non-haematopoietic cell types results from fusion after co-culture or *in vivo* injection (Terada et al. 2002;Vassilopoulos, Wang, & Russell 2003). Whether HSCs are in fact pluripotent or not is therefore a matter of debate.

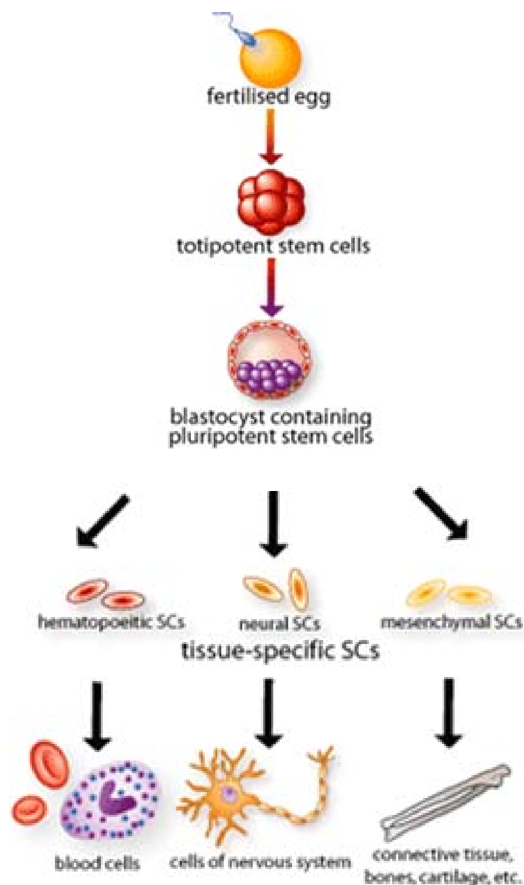


Figure 1.1 Types of stem cells
Totipotent, pluripotent and multipotent (tissue specific) stem cells differ in the range of tissues that they give rise to. HSCs are multipotent stem cells (Adapted from www.scq.ubc.ca).

1.1.2 Haematopoietic stem cells

Mature blood cells are replaced constantly by the process of haematopoiesis. This involves differentiation of multipotent HSCs to the erythroid, myeloid and lymphoid lineages as shown in Figure 1.2B.

The first evidence of the existence of HSCs came from experiments by Ray Owen and co-workers in 1945 which showed that bovine fraternal twins sharing a single placenta and

blood circulation retained production of blood cells genetically defined to be from both throughout their life.

1.1.3 Measuring haematopoietic stem cell activity

Over the many years since HSCs were first discovered a variety of ways have been found of identifying them and measuring their activity.

1.1.3.1 In vivo repopulation assays

HSCs are cells with the ability to self renew and to differentiate to produce cells of all the blood lineages. There can be no clearer demonstration of the presence of an HSC than by its ability to reconstitute the haematopoietic system in recipients whom have had the cells of the bone marrow destroyed by irradiation. Even before the advent of cell surface markers these assays were used to determine the presence of HSCs in a sample. Several different forms of repopulation assay have been developed for this purpose.

Competitive repopulation assays are used to acquire quantitative data about the stem cell content of a given population (Harrison 1980). In these assays irradiated mice are transplanted with limiting numbers of test cells and bone marrow that ensures short term engraftment and survival. The frequency of repopulating cells in these assays is back calculated using Poisson statistics and expressed as competitive repopulating units. For human HSCs non obese diabetic/severe combined immunodeficient (NOD/SCID) mice are used because they do not reject xenogenic cells (Bhatia et al. 1997).

Serial repopulation assays are used to obtain information about the ability of HSCs to self renew. In these assays bone marrow cells are transplanted to a recipient and are then used in a subsequent serial bone marrow transplant to a second recipient. The principle is that the transplant will only be successful if the HSCs were able to undergo substantial self renewal in the first recipient. The number of serial transplants that the original donor's bone marrow can undergo is a measure of the self renewal capacity of its HSCs (Harrison & Astle 1982).

The murine HSC population can be subdivided based on their self renewal capacity. Long-term stem cells (LT-HSC) have the ability to repopulate indefinitely. In contrast, short-term stem cells (ST-HSC) have a productive self renewal life span of approximately six to eight weeks and then disappear from the bone marrow. Multipotent progenitor (MPP) cells self-renew for less than two weeks (Morrison & Weissman 1994).

1.1.3.2 Cell surface markers

It was the advent of monoclonal antibody technology by Kohler and Milstein in 1975 and the development of the multiparameter fluorescence activated cell sorter by the Herzenberg group (Hulett et al. 1969) that facilitated the purification and analysis of the HSC. *In vivo* repopulation assays were used to quantify the number of HSCs in purified samples.

Initially bone marrow cells were depleted for differentiated blood cells using monoclonal antibodies shown in Figure 1.2B that identify erythroid, myeloid and lymphoid cells. These lineage negative (Lin^-) cells were enriched for marrow reconstituting activity. Work

done by Weissman and colleagues (Spangrude, Heimfeld, & Weissman 1988) identified the positive selection markers: stem cell antigen-1 (Sca-1) and low level expression of thymic antigen-1 (Thy-1 lo). Subsequently C-kit was also identified as a cell surface marker for the HSC (Ikuta & Weissman 1992). In human bone marrow, repopulating activity is enriched in the CD34 positive fraction of bone marrow (Bhatia et al. 1997). The cell surface marker phenotype of HSCs is summarised in Figure 1.2A.

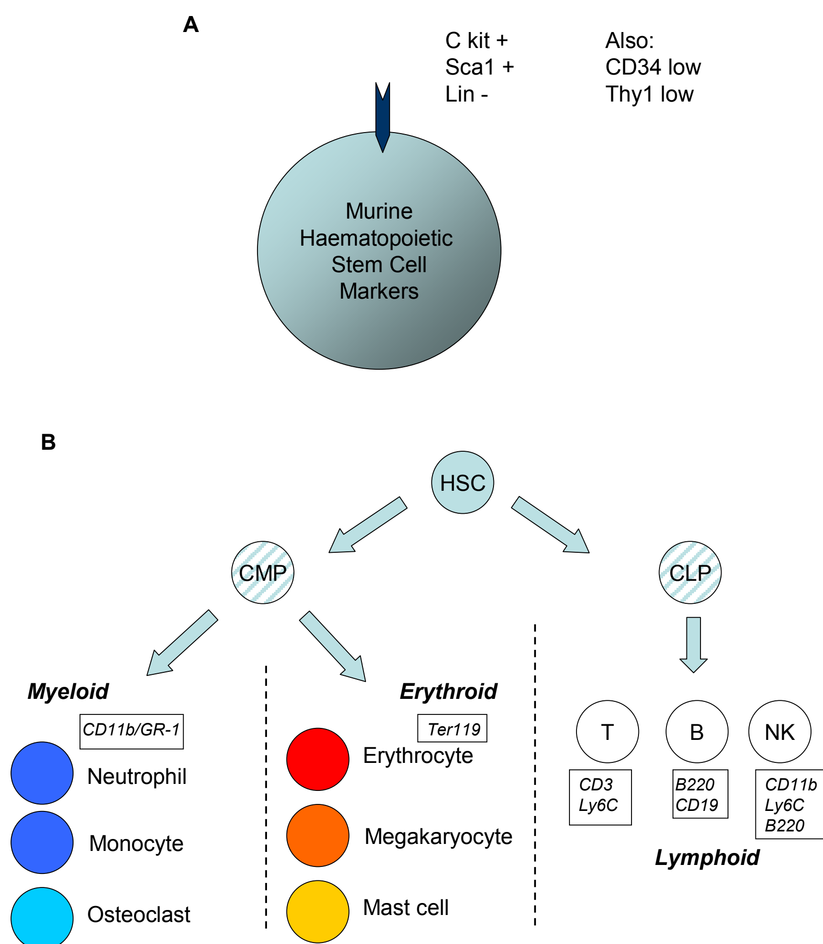


Figure 1.2 Cell surface markers of murine haematopoietic cells
A HSC cell surface markers (source information from *NIH stem cells registry*) **B** HSCs differentiate to produce the three main blood lineages: myeloid, erythroid and lymphoid cells. Lineage markers are shown in the boxes. It is the absence of these markers that identify a progenitor population. CMP: Common Myeloid Progenitor, CLP: Common Lymphoid Progenitor

HSCs giving rise to the early embryonic haematopoietic system appear to differ from adult HSCs in the cell surface markers they express. Embryonic HSCs express low levels of Sca-1 (de Bruijn et al. 2002) and are C-Kit, CD11b positive (Sanchez et al. 1996) and AA4.1 positive (Jordan et al. 1995).

1.1.3.3 Hoechst side population

When unpurified bone marrow cells are labeled with the membrane permeable DNA binding dye Hoechst 33342 a small fraction of cells are found to extrude this dye using a membrane pump. The cells can be separated using fluorescence activated cell sorting with an ultraviolet laser source. These cells have been found to be able to reconstitute the bone marrow of mice with fewer than 100 cells indicating a high level of enrichment for HSCs in this population (Goodell et al. 1996). The dye efflux has been found to be dependent on the ABC membrane protein ABCG2 (Zhou et al. 2001).

1.1.3.4 Colony assays

Colony assays are used to quantify the multipotent and lineage restricted progenitors. In this assay a single cell suspension is plated in methylcellulose culture in the presence of cytokines. This culture system supports the proliferation and differentiation of progenitor cells into recognisable progeny. Colony forming cells can then be counted and classified based on the types of mature cells visible within the colony. Colonies with two or more lineages present are thought to arise from a more primitive progenitor than those with only a single lineage present (Hara & Ogawa 1978).

1.1.4 The HSC cell cycle

HSCs are rare in the adult bone marrow (BM) typically numbering 1 HSC per 100,000 total murine BM cells (Szilvassy et al. 1990). Most HSCs *in vivo* are quiescent as demonstrated by their resistance to 5-fluorouracil compared to lineage-committed haematopoietic progenitor cells. 5-fluorouracil inhibits DNA synthesis targeting dividing cells in the S phase of the cell cycle causing cell death. Cells that are quiescent therefore are not affected by 5-fluorouracil.

When HSCs enter the cell cycle they can divide symmetrically or asymmetrically resulting in different HSC fates (Figure 1.3).

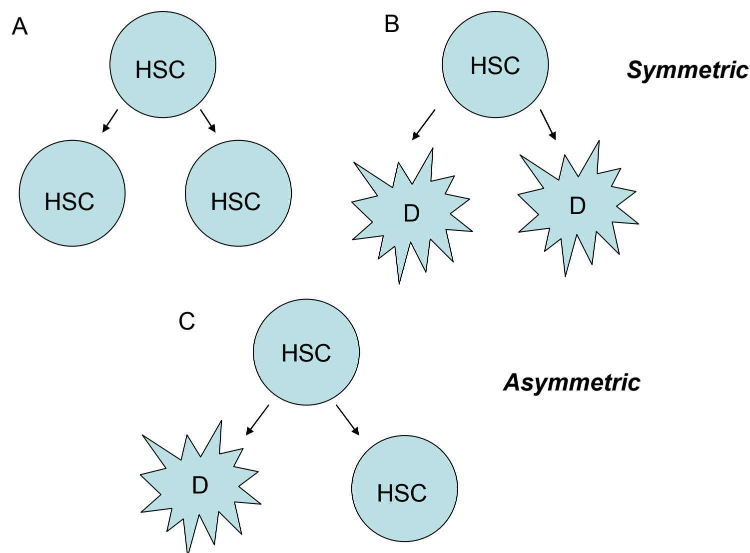


Figure 1.3 HSC Division
Symmetrical divisions give rise to two haematopoietic stem cells (A) or two differentiated cells (B). Asymmetric stem cell maintenance divisions give rise to one daughter HSC with identical properties and one committed daughter cell (C). The committed daughter cell then rapidly proliferates until exhaustion, withdraws from the cell cycle and progressively acquires the characteristics of a specialised blood cell (Lessard, Faubert, & Sauvageau 1994). HSC=Haematopoietic Stem Cell, D=Differentiated cell

HSCs have a slow rate of turnover and it is their descendent cells known as 'transit' or amplifying cells that are the drivers of large scale proliferation. It is these progenitor cells that provide the 10 billion blood cells per day necessary to maintain homeostasis (Attar & Scadden 2004).

1.1.5 Haematopoietic stem cell niche

The idea of the stem cell niche was first proposed by Schofield in 1978 (Schofield 1978). The stem cell niche was a defined anatomical site in which stem cells could self renew and differentiation was inhibited. At this site the space for stem cells would be limited thereby limiting the number of stem cells present. Most controversial of all he proposed that in this place, a more mature cell could revert back to a stem cell phenotype. The first evidence for this concept was provided by experiments in Drosophila (Xie & Spradling 1998). In this work, female germ cells were shown to reside in a location within the ovary necessary for the preservation of the stem cell phenotype. Signals including bone morphogenetic protein 4 were shown to be important to inhibiting differentiation. Evidence was also provided to show that cells introduced to this environment could gain stem cell characteristics.

Ex vivo studies showed that primitive human haematopoietic cells could be supported long term on primary osteoblasts (Taichman, Reilly, & Emerson 1996). This led to two separate studies in which animal models were generated. In one study the Bmp1a receptor was conditionally deleted (Zhang et al. 2003) and in the other Parathyroid hormone was constitutively expressed (Calvi et al. 2003). The aim of both of these animal models was to increase the number of osteoblasts. This resulted in an increase in the trabecular bone and

the number of HSCs. These experiments demonstrated that the osteoblast was a central participant in regulating the size of the stem cell compartment and therefore was a central component of the stem cell niche. N-cadherin and the Notch ligand Jagged 1 were identified as being critical to the interaction between the osteoblast and the stem cell.

From further experiments the HSC niche can be said to be comprised of four key components: the stem cell, the niche cell in this case the osteoblast, extra cellular matrix molecules and a link to the circulatory system (see Figure 1.4).

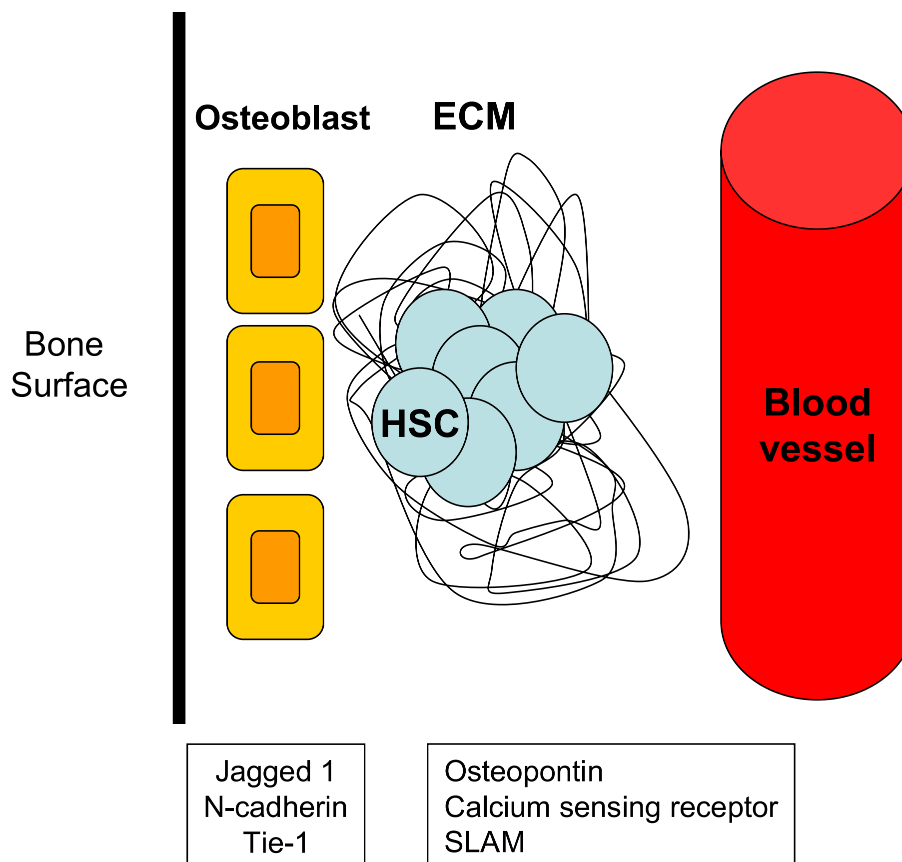


Figure 1.4 The main components of the bone marrow stem cell niche
HSC= Haematopoietic stem cell, ECM=Extracellular Matrix
Jagged 1 and N-cadherin have been shown to be critical to the interaction between the osteoblast and the stem cell. Other work has shown that Tie1 which is expressed on osteoblasts is important to regulating stem cell quiescence through interaction with Angiopoietin-1. Osteopontin an extracellular matrix protein in bone has been shown to negatively regulate stem cell number. Also a calcium sensing receptor expressed on stem cells has been shown to play a role in stem cell engraftment and localisation to the endosteal surface (Papayannopoulou & Scadden 2008). The signalling lymphocytic activation molecules (SLAM) family has been used to identify HSCs *in situ* in the bone marrow. Stem cells were found at greatest abundance around marrow blood vessels leading to suggestions that this is the site of the stem cell niche (Kiel et al. 2005). However stem cells are known to enter and exit the circulation and so there is some debate about whether this just reflects cells moving from their niche to the circulation.

1.2 Development of the mammalian haematopoietic system

After fertilisation the zygote undergoes a series of cell divisions with no significant growth known as cleavage. This results in the formation of a ball of cells known as the morula. As the morula grows, a fluid filled cavity forms known as the blastocoel. On one side of the blastocoel there is a layer of pluripotent cells known as the inner cell mass (see Figure 1.5). From these cells all of the tissues of the body are formed. A cell migration process known as gastrulation now occurs within the inner cell mass that results in the formation of the three germ layers: ectoderm, endoderm and mesoderm. It is the mesoderm that gives rise to blood cells. The extra-embryonic mesoderm gives rise to the yolk sac blood islands and the lateral plate mesoderm gives rise to the aorta gonad mesonephros (AGM) blood cells.

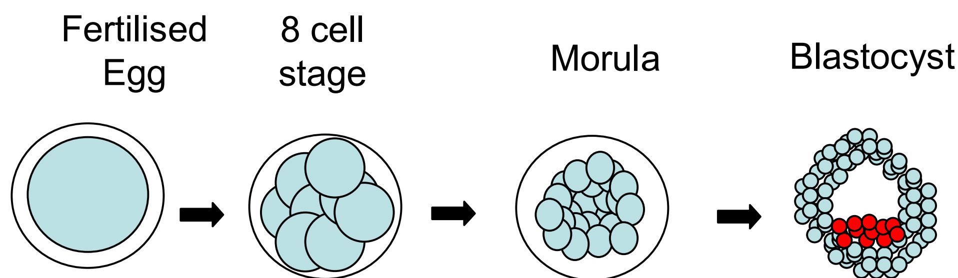


Figure 1.5 Early stages of embryo development
The fertilised egg undergoes a series of cleavage stages culminating in the formation of a ball of cells known as the morula. A fluid filled cavity forms within the ball of cells known as the blastocoel cavity. On one side of the blastocyst is the inner cell mass (shown in red) which after gastrulation gives rise to the endoderm, mesoderm and ectoderm. It is from the mesoderm that blood formation occurs.

1.2.1 Murine embryonic haematopoiesis

Yolk sac blood islands form by 7 days post coitum (dpc) and at 8.5 dpc they differentiate into endothelial and haematopoietic cells (Silver & Palis 1997). Mainly nucleated erythroid cells and macrophages form at this stage and vascular connections between the embryo and yolk sac are established. This initial wave of haematopoietic development that occurs in the yolk sac has become known as 'primitive' haematopoiesis. This is to provide spatial, functional and temporal distinction from 'definitive' haematopoiesis that gives rise to long-term multilineage reconstitution of irradiated recipients.

At 8.5dpc the first intra embryonic haematopoietic precursors appear in the para-aortic splanchnopleure (Godin, Dieterlen-Lievre, & Cumano 1995). This region gives rise to the aorta gonad mesonephros (AGM) region where HSC generation reaches its peak at 10.5 dpc with between 500 and 1000 cells being produced as clusters of cells associated with the vessel wall (Wood et al. 1997). It is these AGM cells that give rise to the long-term multilineage reconstituting HSCs (Muller et al. 1994;Cumano et al. 2001). Cumano and colleagues transplanted yolk sac or AGM cells into Ragγc deletion mutant mice which lack Natural Killer (NK) cells. Since embryonic cells have low levels of MHC class I expression they are susceptible to destruction by NK cells. The use of Ragγc deletion mutant mice prevents this from occurring. Six to eight months post transplantation AGM cells alone were able to provide long term multilineage reconstitution whereas yolk sac cells showed only short term erythro-myeloid reconstitution.

At 12 dpc there is cessation of HSC generation in the AGM and haematopoiesis is sustained in the foetal liver (Godin & Cumano 2002). AGM cells seed the foetal liver where there is expansion of the HSC pool (Kumaravelu et al. 2002). These HSCs subsequently establish the adult blood system. Evidence for this comes from experiments in which quail embryos were grafted onto the chick yolk sac before the emergence of blood cells and the onset of circulation. These experiments showed that the adult haematopoietic system was seeded by cells from within the embryo (Dieterlen-Lievre 1975). Similar previous experiments using amphibian embryos support this conclusion (Turpen, Knudson, & Hoefen 1981).

It likely however that the yolk sac and placenta contribute to the large numbers of HSCs found in the foetal liver (Gekas et al. 2005). More recent experiments in the avian system in which unvascularised quail allantoic rudiment was grafted in a chicken host showed that up to 8% of the host bone marrow was colonised by quail haematopoietic cells (Caprioli et al. 1998). This supports a role for the allantois in de novo generation of definitive haematopoietic stem cells. Thus sites traditionally thought to be involved in primitive haematopoiesis have been shown to contribute to definitive haematopoiesis as well. The anatomical sites of haematopoiesis in the embryo are summarised in Figure 1.6.

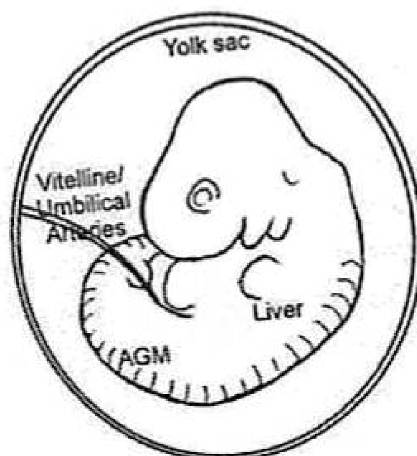


Figure 1.6 The sites of haematopoiesis in a mouse embryo
AGM cells colonise the foetal liver. However it is likely the yolk sac and allantois contributes to foetal liver colonisation as well (Dzierzak 2002).

In *Xenopus laevis,* experiments have provided evidence that the distinction between definitive and primitive haematopoiesis is based on environmental cues. Blood is specified in three compartments: the anterior ventral blood islands (the equivalent of the yolk sac), the posterior blood islands and the dorsal lateral plate (the intra-body region). They have been shown to encounter different amounts of bone morphogenetic protein 4 (Walmsley, Ciau-Uitz, & Patient 2002). By grafting the ventral blood islands or dorsal lateral plate cells to the reciprocal site the prospective haematopoietic cells can be reprogrammed to the alternative adult or primitive haematopoietic fate (Turpen et al. 1997).

1.2.2 Foetal liver haematopoiesis

It is thought that foetal liver activity is a consequence of colonisation and no further HSCs are generated *de novo* (Delassus & Cumano 1996;Dzierzak, Medvinsky, & de Bruijn 1998). Evidence for this is supported by the finding that HSC numbers increase up to 14 dpc but under normal conditions remains constant thereafter (Morrison et al. 1995;Sanchez et al. 1996). The foetal liver is therefore likely to be an environment conducive to the

expansion of HSCs by symmetric self-renewing divisions (Lessard, Faubert, & Sauvageau 1994;Takeuchi et al. 2002).

1.2.3 Adult haematopoiesis

In the mouse HSCs colonise the spleen as early as 12 dpc (Godin et al. 1999) and finally the bone marrow at 15-16 dpc (Isabelle Godin & Ana Cumano 2002). The adult system is generally static (Dzierzak 2002). The absolute number of HSCs does not seem to vary although during tissue damage there is some capacity for HSCs to expand (Iscove & Nawa 1997).

1.3 Genetic Control of Haematopoiesis

In the study of the genetic factors which underlie the processes of development already described, four genes have been identified as being critical. Murine gene deletion studies have shown that the expression of *Scl*, *Lmo2*, *Runx1* and *Gata2* are required for the development of the haematopoietic system and the absence of expression is lethal to the embryo (Figure 1.7). Recent studies have focused on developing a greater understanding of the effects caused by the absence of these factors and how these proteins interact.

Gene	***Deletion Mutant Survival***	***Phenotype of Deletion Mutant***
Scl	8.5-10.5 dpc	Bloodless – no yolk sac or liver haematopoiesis
Lmo2	10.5 dpc	No yolk sac haematopoiesis
Gata2	11.5 dpc	Almost bloodless
Runx1	12.5 dpc	Foetal liver anaemia

Figure 1.7 Gene deletion studies
The deletion of these genes is lethal at early time points due to the absence of blood formation. Analysis of the phenotype of the deletion mutants gives clues as to the role these genes play in haematopoiesis

1.3.1 Scl

The *Scl* (Stem Cell Leukaemia) gene is also known as *Tal-1* as it is the most frequent target of chromosomal rearrangements in children with T cell acute lymphoblastic leukaemia (Begley & Green 1999).

SCL is a transcription factor of the basic Helix-Loop-Helix family. These proteins heterodimerize with E-proteins in order to bind E-box DNA motifs. SCL can either activate or repress transcription depending on its integration within multifactorial complexes. *Scl* expression follows a differentiation-dependent gradient. It is expressed at high levels in HSCs and primitive progenitors and is down regulated as differentiation proceeds except in cells differentiating towards erythroid, megakaryocytic and mastocytic pathways (Lecuyer & Hoang 2004).

Scl deletion mutant mice do not survive beyond 8.5-10.5 dpc due to a complete absence of blood formation. *Scl* is therefore one of the earliest acting regulators of haematopoiesis, essential for primitive and definitive haematopoiesis (Shivdasani, Mayer, & Orkin 1995;Robb et al. 1995;Robb et al. 1996;Porcher et al. 1996).

Analysis of the zebrafish *Scl* deletion mutant reveals that *Scl* is critical to the development of arteries where HSCs emerge (Patterson, Gering, & Patient 2005). Ectopic expression of *Scl* in zebrafish embryos leads to the expansion of both haematopoietic and endothelial markers (Gering et al. 1998).

In *Xenopus* embryo animal cap explants, ectopic expression of *Scl* ventralised embryos and induced haematopoiesis throughout (Mead et al. 2001).

Using a conditional gene targeting approach, Orkin and co-workers have shown that *Scl* is critical to the formation of HSCs (Mikkola et al. 2003). However when *Scl* was deleted in adult mice, anaemia and thrombocytopenia developed as erythropoiesis and megakaryopoiesis was completely disrupted. *Scl* deleted HSCs exhibited stem cell activity on serial transplantation. This suggests that once HSCs are formed *Scl* is dispensable for adult function. However given the importance of the HSC pool to the life long production of blood cells, it seems likely that a network of genes would be involved in maintaining HSC function. The network probably adjusts as an adaptive response to the total absence of *Scl*. Proof that *Scl* does play a role in adult haematopoiesis is provided by experiments in which transgenic expression of *Scl* leads to expansion of the bone marrow Hoescht side

population which is known to be enriched for HSCs as previously described (Herblot, Aplan, & Hoang 2002).

1.3.2 *Lmo2*

Lmo2 (LIM domain only 2) was first discovered through its homology with *Lmo1*. Both of these genes are involved in chromosomal translocations that cause T cell leukaemia (Look 1997). It has recently gained attention due to retroviral insertion of the IL2 receptor γc chain into the *Lmo2* gene in patients being treated with gene therapy to correct X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al. 2003;Howe et al. 2008).

LMO proteins are comprised of zinc finger like domains that mediate protein interactions but lack any DNA binding capability. LMO proteins are therefore thought to function as adapters allowing the proper assembly of larger protein complexes.

Scl and *Lmo2* show overlapping expression patterns in early haematopoietic sites and in adult haematopoietic lineages. In *Lmo2* deletion mutant mice there is a complete absence of haematopoietic cells leading to death at 9.5 dpc. In addition there is abnormal blood vessel development where HSCs first emerge closely resembling the phenotype in *Scl* deletion mutant mice (Warren et al. 1994).

1.3.3 Gata2

Gata2, a member of the Gata family of transcription factors is a zinc finger transcription factor. These factors bind a common DNA sequence motif: T/A GATA A/G.

Gene expression analysis shows that *Gata2* is highly expressed in murine HSCs and adult haematopoietic progenitors. Immunohistochemistry shows that *Gata2* expression occurs in the murine AGM region as early as 8 dpc. At 11.5 dpc *Gata2* is expressed in the aortic endothelium and neighbouring mesenchymal cells where HSCs first emerge (Minegishi et al. 1999).

Gata2 deletion mutant murine embryos have impaired primitive erythropoiesis and a complete lack of any other committed progenitors causing death from anaemia at 10.5 dpc (Tsai et al. 1994). *Gata2* haploinsufficiency profoundly decreases the number of AGM HSCs but yolk sac HSCs are only slightly affected. Furthermore adult HSCs although normal in numbers were defective in competitive repopulation experiments. HSCs exhibited a delay in regeneration of the haematopoietic system implicating *Gata2* in HSC proliferation (Ling et al. 2004).

Gata2 is involved in the SCL – Lmo2 complex as already described in 1.3.2.

1.3.4 SCL-Lmo2-Gata complex

The similarities in expression patterns and deletion mutant phenotypes of *Scl* and *Lmo2* led to the hypothesis that SCL and Lmo2 worked together as a complex. This was demonstrated in extracts from leukemic cells (Valge-Archer et al. 1994) and more recently in zebrafish (Patterson et al. 2007). In fact, in addition to its association with SCL, Lmo2 has been shown to interact with the Gata family of proteins (Osada et al. 1995).

The SCL - Lmo2 - Gata2 complex is known to target the promoter of the tyrosine kinase receptor C-kit that is commonly used as a cell surface marker for HSCs. SCL is known to support survival of haematopoietic progenitors and this is thought to be due to its role in activating C-kit (Lecuyer et al. 2002). Unless all components of the transcription factor complex are present there is little if any effect on the C-kit promoter.

In the context of erythroid differentiation, SCL - Lmo2 complexes with GATA1. This complex has been shown to activate a key erythroid membrane glycoprotein glycophorin A.

SCL DNA binding defective mutant protein was able to rescue haematopoietic specification in SCL deletion mutant mice but was unable to restore differentiation of the erythroid and megakaryocytic lineages (Kassouf et al. 2008). SCL DNA binding activity was found to be dispensable for c-kit promoter activation but not for glycophorin A (Lecuyer et al. 2002). This data indicates that SCL in early haematopoietic cells acts as a protein that recruits other factors into a complex which provides DNA binding activity.

The role of the SCL-Lmo2-Gata complexes in HSCs and erythroid cells is summarised in Figure 1.8.

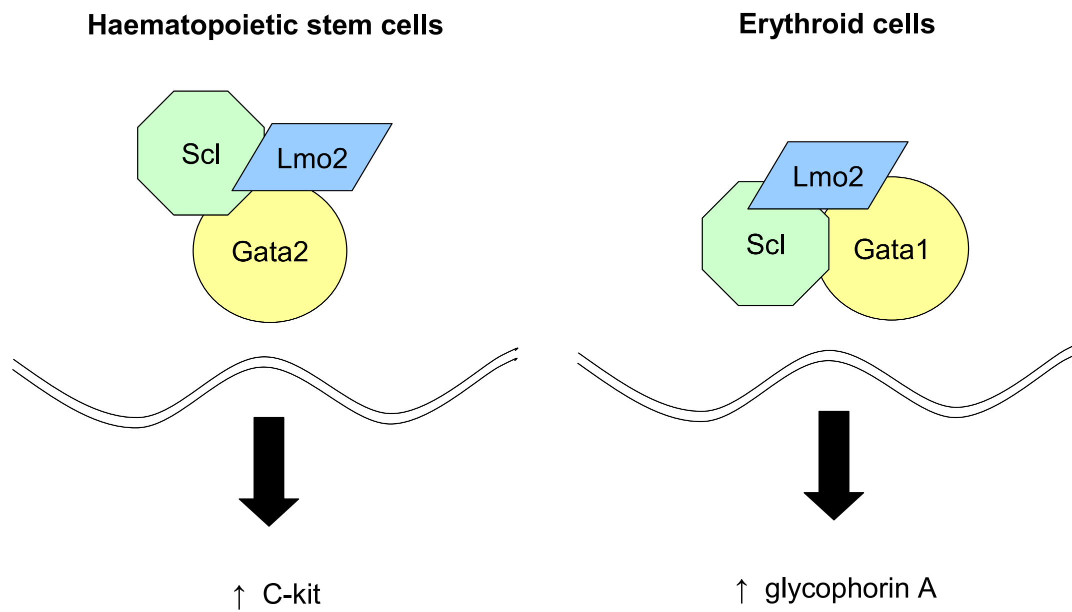


Figure 1.8 The role of the SCL-Lmo2-Gata complex in HSCs and erythroid cells
In HSCs the complex up regulates C-kit expression. Scl DNA binding activity is dispensable for this to occur. In erythroid cells this complex up regulates glycophorin A. Scl DNA binding activity is required for this to occur.

1.3.5 Runx1

Runx1 is the mouse homolog of *AML1*. This gene was initially identified in translocations in human leukaemia. Translocations at the *AML1* locus account for 30% of acute myeloid leukaemia and 25-30% of acute lymphoblastic leukaemia. AML1 is a member of a family of proteins known as core binding factors which are heterodimeric transcription factors consisting of a DNA binding subunit (CBFα) and a non DNA binding subunit (CBFβ). The CBFα subunit consists of a transactivation domain required for DNA binding and a runt domain required for association with the CBFβ subunit.

Complete ablation of *Runx1* leads to embryonic lethality at 12.5 dpc and foetal liver anaemia in mice (Okuda et al. 1996;Sasaki et al. 1996;Wang et al. 1996a;Wang et al. 1996b). Primitive yolk sac erythropoiesis is normal but definitive erythroid, myeloid and megakaryocytic cells are absent indicating a complete block in the definitive haematopoietic program. Thus no haematopoietic clusters are observed in the aorta or vitteline/umbilical arteries and no functional HSCs are found in the AGM at 10 or 11 dpc (Cai et al. 2000). *Runx1* is therefore thought to be important in the generation of adult HSCs and progenitors.

Since *Runx1* is embryonic-lethal it is not possible to determine the role of runx1 *in vivo* at later developmental stages using *Runx1* knockout mice. To overcome this, *Runx1 +/βgal* knock-in mice have been created by replacing one copy of *Runx1* with a gene encoding *β-galactosidase*. Tissue sections could be stained by adding β galactosidase substrate enabling the visualisation of *Runx1* expressing cells. *Runx1* expression was first seen at 7.5 dpc in the mesoderm precursors of primitive erythrocytes and then transiently in primitive erythrocytes of the developing blood islands at 8 dpc in the yolk sac. By day 8.5 only a small population of endothelial and haematopoietic cells retained *Runx1* expression in the yolk sac. *Runx1* expression in the dorsal aorta was seen at endothelial sites where haematopoietic cells emerge at 8.5 dpc prior to the emergence of haematopoietic clusters as shown in Figure 1.9 (North et al. 1999).

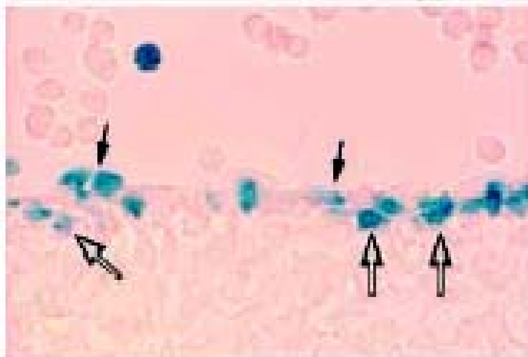


Figure 1.9 *Runx1* expression in the ventrolateral wall of dorsal aorta in a *Runx1+/βgal* mouse.
Runx1 is expressed in the cells lining the aorta (shown by arrows) at 8.5dpc prior to emergence of haematopoietic clusters in AGM region (North et al. 1999).

The expression of *Runx1* in emerging primitive erythrocytes is puzzling given that no defects are reported in primitive haematopoiesis in the *Runx1* deletion mutant mice. This may suggest *Runx1* is expressed but not essential to primitive haematopoiesis. Alternatively a defect in primitive haematopoiesis in the *Runx1* deletion mutant mice may not have been detected. Support for this theory is provided by work using transgenic mice expressing *CFBβ-MYH11*. In humans *CFBβ-MYH11* results from a translocation which causes leukemia. It is thought to function by sequestering Runx1 proteins in the cytoplasm (Adya et al. 1998). The expression of this protein impaired both primitive and definitive haematopoiesis (Miller et al. 2001).

In the amphibian system, *Xaml-1* is the Xenopus homolog of *Runx1*. Forced expression of the runt domain of Xaml-1 is thought to compete with Xaml-1 for DNA binding inhibiting its function. In these Xenopus embryos there is a block in both primitive and definitive haematopoiesis (Ciau-Uitz, Walmsley, & Patient 2000;Walmsley, Ciau-Uitz, & Patient

2002). This data again supports the idea that *Runx1* plays a role in both primitive and definitive haematopoiesis.

Most recently *Runx1* deletion mutant mice have been reanalysed and primitive erythrocytes did in fact display abnormal morphology and reduced expression of markers Ter119, Erythroid Kruppel-like factor and Gata1 (Yokomizo et al. 2008).

Inducible gene targeting experiments provide a further method of studying the effects of *Runx1 in vivo* at later developmental stages. Ichikawa and colleagues used the Cre-loxP recombination system to generate mutant mice in which exon 5 on the *Runx1* gene could be selectively deleted by the expression of Cre recombinase. Adult mutant mice were injected with polyinosinic-polycytidylic acid in order to induce expression of Cre recombinase and delete *Runx1* expression. These experiments suggest that *Runx1* was dispensable for adult HSC function (Ichikawa et al. 2004). In the absence of *Runx1* (AML-1), haematopoietic progenitors were maintained. However, the bone marrow showed inhibition of megakaryocytic maturation, increased haematopoietic progenitor cells and defective T- and B-lymphocyte development. They therefore concluded *Runx1* is required for maturation of megakaryocytes and differentiation of T and B cells, but not for maintenance of HSCs in adult haematopoiesis.

1.3.6 Integrated genetic control

The downstream genetics effects of *Scl* have been studied by introducing SCL protein into a *Scl* null murine yolk sac cell line and then performing global gene expression profiling. The results have shown that *Runx1* is a target of SCL. Furthermore there was evidence to suggest that binding of an SCL-Lmo2-Gata2 complex transactivated *Runx1* expression (Landry et al. 2008). This data suggests *Runx1* up regulation is downstream of these transcription factors in HSC development.

Studies in the sea urchin of transcription factor expression and the cis regulatory sequences that they bind, has led to the idea that there are gene regulatory networks. The hypothesis is that each gene network has a distinct regulatory function in development. The disruption of a transcription factor component leads to total loss of the particular body part that the network is involved in developing (Davidson & Erwin 2006). The genes *Gata2*, *Fli1* and *Scl* and their enhancers *Gata2*-3, *Fli1*+12 and *Scl*+19 are hypothesised to act as such a network in the specification of haematopoiesis in the mouse embryo. The three enhancers were found to be bound by each of these transcription factors forming a fully connected triad as shown in Figure 1.10 (Pimanda et al. 2007b).

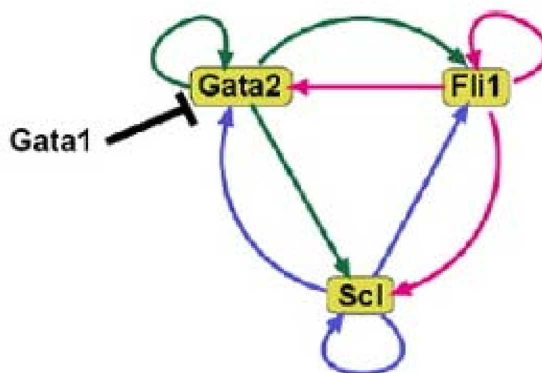


Figure 1.10 The *Gata2, Fli1, Scl* gene regulatory circuit
This triad of genes is proposed to form a gene regulatory circuit. The arrows indicate the ability of each gene to increase the expression of all the components of the circuit. This triad is hypothesised to be locked in an 'ON' state in a newly specified HSC so that it retains its multipotency in transition from its site of generation to the foetal liver. Gata1 was shown to disrupt Gata2 positive autoregulation (adapted from Pimanda et al. 2007).

Scl, *Lmo2*, *Runx1* and *Gata2* are therefore closely related both in the functions they perform and in the control of their expression.

1.4 Environmental control of haematopoiesis

Considerable attention has been given to the environment that supports the development of the haematopoietic stem cell. In particular there is great interest in the environmental stimuli that cause the expression of the genes *Scl*, *Lmo2*, *Gata2* and *Runx1*. Several different growth factors and intracellular pathways have been implicated in HSC specification. However a number of studies suggest that bone morphogenetic protein 4 (BMP4) plays an important role in early haematopoietic development. There is also some evidence of a role for BMP4 in adult haematopoiesis. Its precise function has yet to be fully understood.

1.4.1 Bone morphogenetic protein 4

BMP4 is a member of the transforming growth factor superfamily of growth factors. The potential role of BMP4 in haematopoietic development has been studied in a number of different species.

1.4.1.1 The BMP4 signalling pathway

BMP signals are transduced across the cell membrane via serine threonine protein kinase receptors (Figure 1.11). Type I receptors also known as activin receptor like kinases (ALKs) have an inactive kinase domain whilst type II receptors are constitutively active. When the ligand binds the type II receptor, a type I receptor is recruited and phosphorylation of the type I receptor by the type II kinase occurs resulting in activation of the type I receptor kinase domain (Wrana et al. 1994). There are several ALKs and different combinations of receptor allow the binding of different ligands to be distinguished (Massague 1996). In the case of BMP4, signals are transduced by ALK3 or ALK6 combining with Type II receptor (Shi & Massague 2003).

The type I kinase phosphorylates Smad intracellular signalling proteins. There are three different groups of Smads: receptor activated Smads, the common partner Smad (Smad4) and the inhibitory Smads. Receptor Smads are phosphorylated by activated type I receptors after which they oligomerise with Smad4 and translocate to the nucleus where they interact with other transcription factors to regulate gene expression. The receptor activated Smads for BMP signal transduction are Smads 1, 5 and 8 (Shi & Massague 2003).

The inhibitory Smads act by inhibiting the phosphorylation of receptor Smads, degrading activated type I receptors or by dephosphorylating activated type I receptor. Inhibitory Smads may also compete with other receptor Smads for binding the common partner Smad, Smad4. Inhibitory Smads include Smad7 which inhibits the activity of all receptor activated Smads and Smad6 which shows a more preferential inhibition of BMP Smads (Larsson & Karlsson 2005;Shi & Massague 2003).

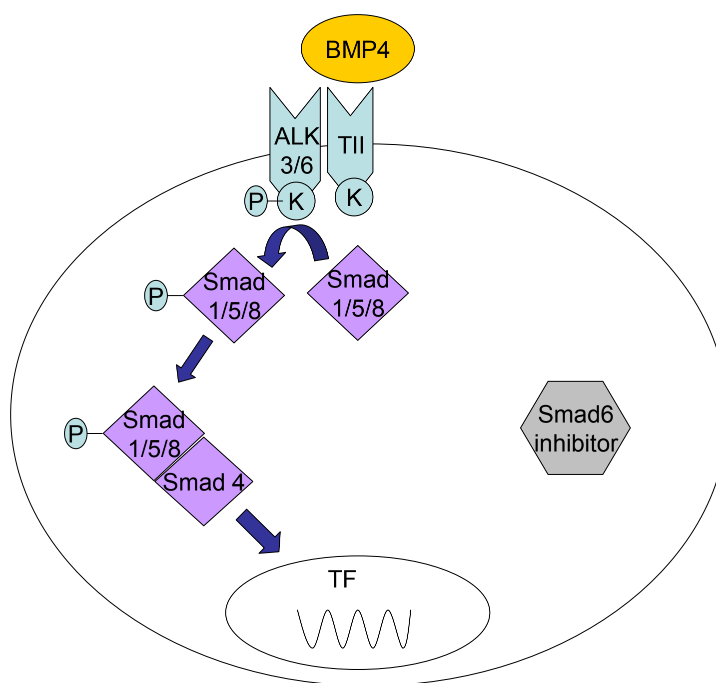


Figure 1.11 BMP4 signalling
BMPs activate heteromeric cell surface receptors consisting of type I (ALK) and type II serine threonine protein kinase subunits. Phosphorylation of the type I receptor by the constitutively active type II kinase results in activation of the type I receptor kinase domain. The type I kinase phosphorylates Smad intracellular signalling proteins. These phosphorylated receptor regulated Smads interact with co-Smad 4 resulting in translocation of the heteromeric complex to the nucleus where, by interaction with transcription factors, gene expression changes. Smad 6 is an inhibitory Smad. Inhibitory Smads are thought to act by inhibiting type I receptor mediated phosphorylation of Smads and also competing in binding co-Smad 4 (Nakao et al. 1997;Hata et al. 1998). ALK: Activin like kinase, TII: Type II, K:kinase, P:phosphate group, TF:Transcription factors

1.4.1.2 *Xenopus* experiments

Some of the earliest development experiments were carried out in the *Xenopus* frog. *Xenopus* produce large oocytes and therefore are well suited to manipulation and analysis during development.

Mesoderm specification occurs in the ventral region of the blastula. Mesoderm is induced by the secretion of BMP4. The 'Spemanns organiser' located in the dorsal region secretes molecules such as noggin and chordin which inhibits the action of BMP4 that result in the induction of dorsal fates (Figure 1.12A). Blood formation occurs in the ventral mesoderm and therefore requires high levels of BMP4 (Sadlon, Lewis, & D'Andrea 2004).

Thus *Xenopus* experiments have shown that BMP4 induces haematopoietic differentiation in *Xenopus* embryos (Kumano, Belluzzi, & Smith 1999). Furthermore, primitive and definitive haematopoiesis in *Xenopus* is inhibited by expression of a dominant negative BMP type I receptor in the ventral blood islands (Walmsley, Ciau-Uitz, & Patient 2002). BMP4 together with basic fibroblast growth factor and activin A can induce the formation of red blood cells from non-mesodermal structures (Huber et al. 1998).

In *Xenopus* BMP signalling induces a number of genes including the Vent (ventralizing) family members *Xvent1* and *2*. These genes induce ventral cell fate in addition to suppressing dorsal genes. BMP signalling also induces the haematopoietic specific genes such as *Gata1*, *Gata2*, *Scl*, *Lmo2* and *Xfli1* (Sadlon, Lewis, & D'Andrea 2004).

In *Xenopus*, BMP4 is proposed to lead to the formation of the complex of SCL-Lmo2-Gata1 complex which drives blood formation in developing blood islands (Mead et al. 2001). BMP4 may also drive the expression of the Xenopus Runx1 orthologue, Xaml (Tracey & Speck 2000). *Xaml* expression is lost when BMP4 signalling is inhibited in Xenopus embryos (Walmsley, Ciau-Uitz, & Patient 2002). The effects of BMP4 on haematopoietic genes are summarised in Figure 1.12B.

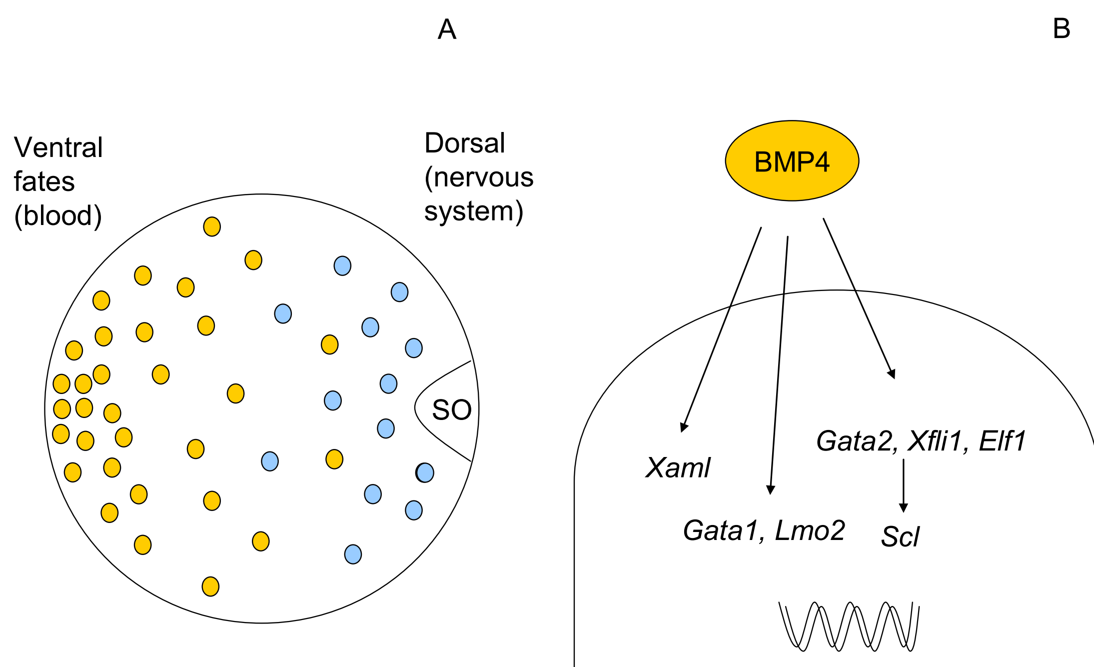


Figure 1.12 The role of BMP4 in *Xenopus* mesoderm patterning
A shows that secretion of BMP4 in the ventral region of the blastula induces ventral mesoderm fates whilst the Spemann's organiser (SO) in the dorsal region secretes inhibitors such as Noggin and Chordin which induces dorsal fates. **B** shows the haematopoietic genes shown to be induced by BMP4 in *Xenopus* experiments . The arrows indicate up regulation of gene expression.

1.4.1.3 Mouse experiments

Mice lacking BMP4 die in utero at the gastrulation stage. The few embryos that do develop show severe defects including disorganised and truncated structures (Winnier et

al. 1995). The rescue of some embryos is possibly due to the presence of related BMP ligands. Ablation of the BMP type I receptor (ALK3) or the type II receptor results in a more severe phenotype with no mesoderm formed (Beppu et al. 2000). Because death in these mutants occurs before blood formation, it is not possible to decipher the function of BMP4 in mammalian haematopoiesis in vivo. Research into the role of BMP4 in haematopoiesis has therefore mainly focused on lower vertebrates and *in vitro* studies.

In the AGM region of the mouse, BMP4 is highly expressed in the mesenchyme underlying the haematopoietic clusters that emerge from the ventral wall of the dorsal aorta during embryogenesis. BMP4 also increases the number of HSCs in AGM explants (Durand et al. 2007). This suggests that BMP4 is involved in HSC specification.

The co-expression of BMP4 and *Runx1* in the AGM has recently been demonstrated at 10.5 dpc. Furthermore Smad1 was shown to bind the *Runx1* promoter in a haematopoietic progenitor cell line transactivating *Runx1* expression. After 12 dpc *Runx1* expression is rapidly down regulated in the AGM whereas Smad6 continues to be expressed down regulating *Runx1* expression (Pimanda et al. 2007a).

1.4.1.4 Human experiments

BMPs are highly conserved and human homologues have been identified (Maeno et al. 1996). BMP4 is expressed by cells surrounding blood islands in the human yolk sac and is strikingly restricted to the two of three layers of stromal cells beneath the ventral floor of the dorsal aorta in 28 day old human embryos when intra-aortic haematopoietic clusters are

forming (Marshall, Kinnon, & Thrasher 2000). By day 34 a gradient of BMP4 expression has been established across the dorsal-ventral axis of the AGM region with expression polarised to the ventral wall of the dorsal aorta (Figure 1.13). This gradient of BMP4 expression disappears at the same time as the haematopoietic clusters at around 38 days gestation.

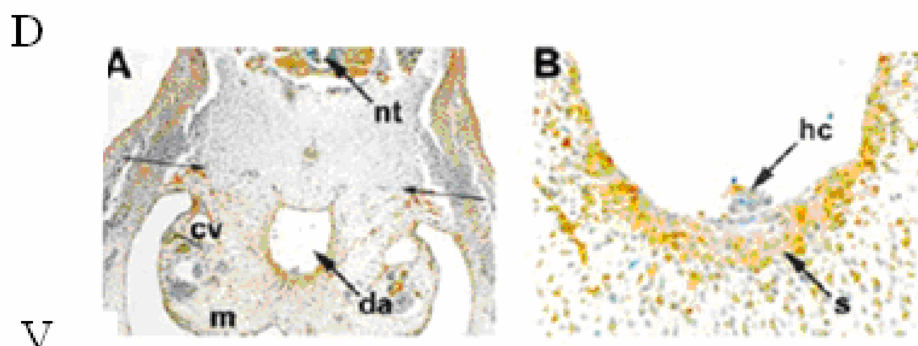


Figure 1.13 BMP4 staining in the AGM region of the human embryo
Transverse section through a 34 day human embryo (A) BMP4 is expressed in a gradient across the dorsal (D) – ventral (V) axis. (B) Higher magnification of (A). BMP4 is expressed at high levels underlying the ventral floor of the dorsal aorta in the AGM. Haematopoietic clusters can be seen within the dorsal aorta (Marshall, Kinnon, & Thrasher 2000) hc: haematopoietic clusters, s: stromal region underlying the clusters, da: dorsal aorta, m: mesonephros, cv: cardinal veins, nt: neural tube,

1.4.2 *In vitro* experiments – the role of BMP4 in adult haematopoiesis

Serum free expansion cultures suggest that high levels of BMP4 can enhance human bone marrow derived haematopoietic stem cell survival and increase engraftment in NOD SCID mice (Bhatia et al. 1999). Treatment of cord blood cells with high doses of BMP4 (25ng/ml) increased the length of time an HSC could be maintained in serum-free culture. BMP4 effects are highly dependent on concentration. A lower dose of BMP4 resulted in

complete differentiation of the CD34+ CD38- lineage- HSC population (Bhatia et al. 1999).

The observed stimulatory effects of other factors such as sonic hedgehog, which induce HSC proliferation, are mediated through modulation of BMP4 signalling pathways. Noggin, an inhibitor of BMP4, inhibited sonic hedgehog induced proliferation of primitive human progenitors (Bhardwaj et al. 2001).

Varying effects of BMP4 on the differentiation of CD34+ cells have been reported *in vitro*. In one study, BMP4 increased the frequency of erythroid burst forming units (BFU-E) in cord blood-derived CD34+ cells cultured in serum free conditions (Fuchs et al. 2002). However in a separate study using bone marrow-derived CD34+ cells, BMP4 had no effect on BFU-E formation (Maguer-Satta et al. 2003). The role of BMP4 in adult haematopoiesis has therefore yet to be fully defined.

1.5 Embryonic stem cells as a model for mammalian haematopoietic development

1.5.1 Embryonic stem cells

Teratomas are composed of a variety of tissues derived from all three germ layers. Skin, muscle, fat, gut epithelium, tooth structures, indeed any tissue of the body may be found. It was noted that the cells present represented those produced in embryonic development

except that they were present in a disorganised manner (DIXON & MOORE 1952). It was in the highly malignant form of teratoma that occurs in the testis known as teratocarcinoma that an undifferentiated cell type with a pluripotent phenotype was first identified. These cells became known as Embryonic Carcinoma (EC) cells (Kleinsmith & Pierce, Jr. 1964). During the 1970s it was found that murine EC cells had features similar to the inner cell mass of the early mouse embryo (Artzt et al. 1973;Jacob 1978). This culminated in 1981 with the isolation of the non-malignant version of these stem cells, the Embryonic Stem (ES) cell. These pluripotent stem cells were first isolated from early mouse embryos (Evans & Kaufman 1981;Martin 1981) and eventually from human embryos (Thomson et al. 1998).

Murine ES cells are isolated from the inner cell mass of blastocysts at day 3.5 of mouse development. When mouse blastocysts are cultured on a feeder layer of mitotically inactivated embryonic fibroblasts the outer layer of cells attach and the undifferentiated inner cell mass cells form clumps that can be passaged to yield ES cells (Evans & Kaufman 1981). These cells can be injected back into blastocysts and will contribute to the formation of all tissues (Bradley et al. 1984). Maintenance of ES cells *in vitro* is achieved by co-culture with irradiated fibroblasts (Martin 1981). Upon withdrawal of stromal contact the mouse ES cells differentiate into complex three dimensional cell aggregates called Embryoid bodies (EBs).

1.5.1.1 The molecular basis of pluripotency

Pluripotency of ES cells has been found to be dependent upon extrinsic growth factor signals and intrinsic transcription factor expression (Chambers 2004).

1.5.1.1.1 Growth factors

Although ES cells are typically maintained on mitotically inactive fibroblasts this co-culture is unnecessary if Leukemia Inhibitory factor (LIF) is added to the medium (Williams et al. 1988). LIF has been shown to activate transcription of STAT3. In the absence of LIF signalling ES cells differentiate to endoderm and mesoderm (Niwa et al. 1998).

The requirement for serum in the *de novo* isolation and expansion of ES cells has been shown to be replaceable by BMP4 (Ying et al. 2003). BMP4 appears to blocks neural differentiation. This occurs via the induction of *Id* genes. BMP4 is no longer required when cells express *Id1* transgene. Excision of a loxP flanked *Id1* transgene restores BMP4 dependence (Ying et al. 2003).

1.5.1.1.2 Transcription Factors

Several transcription factors have been implicated in pluripotency however the best characterised is Oct4. When *Oct4* was deleted *in vivo* although cells moved to the interior of the blastocyst they failed to adopt a pluripotent identity and instead differentiated to

trophectoderm (Nichols et al. 1998). Similarly when *Oct4* was deleted in ES cells, the cells differentiated to trophectoderm (Niwa, Miyazaki, & Smith 2000).

Oct4 acts on DNA in conjunction with Sox2 (Pevny & Lovell-Badge 1997). *Sox2* knockout ES cells differentiate to trophectoderm and do not have a pluripotent phenotype (Li et al. 2007).

Nanog a homeodomain protein maintains self renewal in the absence of STAT3 activation. ES cell self renewal however occurs with greatest efficiency with the expression of both STAT3 and Nanog. Nanog does not appear to be downstream of STAT3 or Oct4 or vice versa. Gene deletion of *Nanog* results in cell differentiation to primitive endoderm (Chambers et al. 2003).

The growth factors and transcription factors involved in maintaining the pluripotent state have been summarized in Figure 1.14.

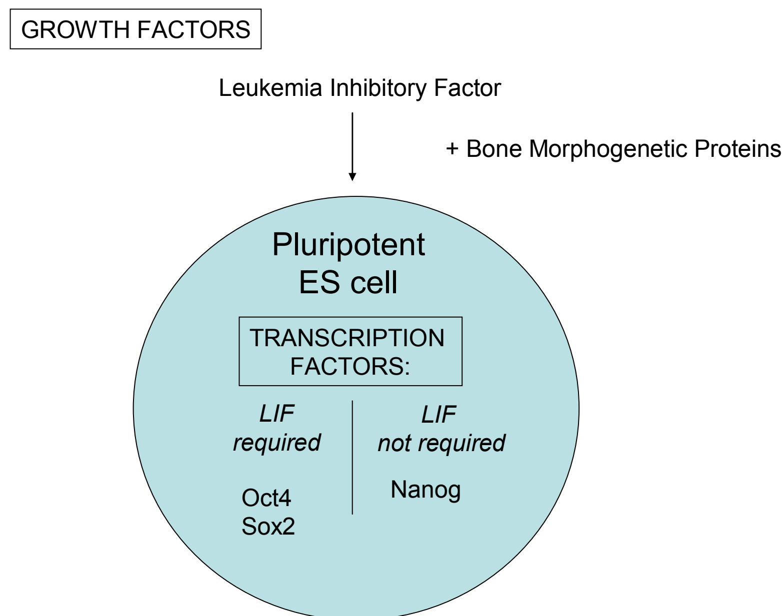


Figure 1.14 The molecular basis of pluripotency
Extrinsic growth factors and intrinsic transcription factors responsible for pluripotency in ES cells. While Oct4 and Sox2 require the presence of LIF to maintain self renewal and pluripotency Nanog does not.

1.5.2 The potential use of ES cells in clinical therapy

In the clinical setting suitable donor bone marrow is often in short supply and cord blood contains a much lower absolute number of haematopoietic stem cells. Furthermore at present it is not possible to reliably expand *ex vivo* LT-HSCs. ES cells can be indefinitely expanded in vitro. This unlimited expansion allows the generation of large numbers of HSCs (Kyba & Daley 2003).

It has been demonstrated by Shinya Yamanaka and colleagues that mouse fibroblasts can be induced to a pluripotent phenotype via the retroviral mediated expression of *Oct4*, *Sox2*, *Myc* and *Klf1*. These cells are then known as induced pluripotent stem cells (Takahashi & Yamanaka 2006). The same has been demonstrated using human fibroblasts and other adult somatic tissues (Takahashi et al. 2007;Yu et al. 2007;Lowry et al. 2008). This is an

exciting discovery as this provides a means of generating pluripotent stem cells without the difficulties of nuclear transfer or the controversy of destroying embryos. Once there is an understanding of how to differentiate particular tissues, it seems a real possibility that patient specific tissue lines could be generated. This would circumvent the need for anti-rejection drugs following transplant.

1.5.3 ES cells as a model for haematopoietic development

In addition to these potential clinical applications ES cells provide a system in which haematopoietic development can be studied. Mammalian haematopoiesis commences at an early stage in development and the small size of embryos in addition to the limited number of haematopoietic cells at this stage make it difficult to study *in vivo*. The main advantage in using ES cells therefore is accessibility of the cellular material.

ES cells can be differentiated to form embryoid bodies (EBs), containing multiple cell types, by withdrawal of Leukaemia Inhibitory Factor (LIF) from the culture medium. Embryoid bodies develop blood islands *in vitro* (Doetschman et al. 1985).

Haematopoietic cells develop within the first six days of EB culture. By disrupting EBs at earlier time points, it is possible to investigate the development of haematopoietic precursors.

1.5.3.1 Primitive or definitive haematopoiesis?

The capacity of EBs to generate primitive erythrocyte colony forming cells, as well as the time course of 'blood island' formation, suggests that EB haematopoiesis recapitulates primitive haematopoiesis.

The many failed attempts to generate long-term adult haematopoietic repopulation using EB derived cells are thought to support this conclusion (Kyba & Daley 2003). However a few attempts have achieved repopulation successfully(Muller & Dzierzak 1993;Potocnik, Kohler, & Eichmann 1997). This may result from EBs that occasionally generate populations resembling AGM splanchnopleural mesoderm. The AGM arises as part of a highly organised process and its development in the relatively disorganised EB could only ever be haphazard and incomplete (Kyba & Daley 2003).

As an alternative to EB differentiation, ES cells can also be differentiated on stromal cell lines for efficient haematopoietic differentiation. Most stromal cell lines result in abundant macrophage growth. The OP9 stromal cell line is derived from a macrophage colony stimulating factor deficient (op/op) mouse. ES cells grown on an OP9 layer differentiate first to mesoderm colonies and then when replated on a fresh OP9 layer to haematopoietic blast colonies. These colonies contain erythroid, myeloid and lymphoid lineages (Nakano, Kodama, & Honjo 1994). This presence of lymphoid cells implies that definitive haematopoiesis is taking place. However ES cells cultured on OP9 cells are unable to engraft conditioned adult mice (Kyba, Perlingeiro, & Daley 2002).

The generation of lymphoid cells and the ability to produce long term adult repopulation are the two characteristics that separate definitive haematopoiesis from primitive haematopoiesis. A model therefore proposing the independent origin of primitive and definitive haematopoietic stem cells therefore seems inadequate. Instead it may well be the case that EB cells and yolk sac cells if given the appropriate environmental stimuli could take on a definitive stem cell phenotype.

Multilineage long-term repopulation by transplantation of day 9 and day 10 yolk sac tissue into the conditioned livers of new born mice has been achieved (Yoder, Hiatt, & Mukherjee 1997). Furthermore yolk sac cells presumably reprogrammed by several days exposure to an AGM-derived stromal line were able to produce long-term multilineage reconstitution (Matsuoka et al. 2001). This provides considerable support to the notion that in the right environment yolk sac cells can develop to give rise to definitive haematopoietic cells.

The inability of EB derived blood cells to achieve engraftment in adult recipients could either be the result of limited developmental potential or inability to survive in the adult environment. In order to study this ES cells were transduced with the oncogene responsible for Chronic Myeloid leukaemia, Bcr/Abl. This oncogene drives HSC proliferation blocking apoptosis whilst still allowing differentiation. These cells were able to engraft sub-lethally irradiated adult recipients causing a leukaemia with erythroid, myeloid and lymphoid components (Perlingeiro, Kyba, & Daley 2001). This suggests that

in fact ES derived HSCs do have definitive haematopoietic potential but it is cell survival in the adult environment that normally prevents this.

The screening of proteins involved in Bcr/Abl signalling pathways led to the identification of HoxB4 as a potential mediator of normal engraftment in the adult environment (Helgason et al. 1996). In order to investigate this further ES cells were created with tetracycline dependent *HoxB4* expression and adult repopulation of myeloablated recipients was attempted. *HoxB4* expressing cells engrafted generating both lymphoid and myeloid populations. The cells from primary animals could be transplanted into secondary animals suggesting the self renewal of a long term reconstituting haematopoietic stem cell. Furthermore continued expression of *HoxB4* expression was not required post-transplant for graft maintenance (Kyba, Perlingeiro, & Daley 2002).

This data suggests HoxB4 endows embryonic haematopoietic cells with adult haematopoietic characteristics. This provides further support for the idea that the primitive HSC can mature into a definitive HSC in response to the appropriate signal.

1.5.3.2 Haemangioblast

One of the main achievements of the ES system has been identification of the haemangioblast, a common precursor for both haematopoietic and endothelial lineages.

In murine embryos, histological analysis of developing blood islands in the yolk sac showed that haematopoietic and endothelial lineages developed in parallel from common

clusters of undifferentiated cells (Haar & Ackerman 1971). In avian embryos fate mapping experiments have shown that ventral mesoderm possesses both endothelial and haematopoietic potential (Pardanaud et al. 1996;Pardanaud & Dieterlen-Lievre 1999). Therefore the fact that endothelial and haematopoietic cells appear at similar time points and in close juxtaposition has resulted in the proposal that there may be a common mesodermal precursor for both the endothelial and haematopoietic lineages (Sabin 1920;Murray 1932).

The most direct evidence for the existence of the haemangioblast has come from studies using EB cultures. When grown in methylcellulose culture in the presence of vascular-endothelial derived growth factor (VEGF) these precursors emerge as blast colonies. The blast-colony forming cells (BL-CFCs) appear transiently, between day 2.5 and day 3.5 of differentiation. By replating BL-CFCs into other conditions it has been shown that these cells can differentiate into primitive and definitive erythroid and myeloid haematopoietic precursors as well as endothelial cells (Kennedy et al. 1997;Choi et al. 1998). BL-CFCs are *foetal liver kinase1 (Flk-1)* positive (Faloon et al. 2000).

Gene targeting experiments have provided further evidence for the existence of a common haemangiogenic precursor. Flk-1 is a tyrosine kinase receptor that is activated by vascular endothelial derived growth factor (VEGF). Ablation of *Flk-1* (Shalaby et al. 1995;Shalaby et al. 1997) and *Vegf* in mouse embryos result in an embryonic lethal phenotype and, most importantly, blocks both vascular and haematopoietic development (Carmeliet et al. 1996;Ferrara et al. 1996).

One day before the emergence of the Flk-1 positive haemangioblast, a more primitive cell type gives rise to transitional colonies. These transitional colonies express the mesodermal marker *Brachyury*. Transitional colonies give rise to BL-CFC (Robertson et al. 2000). This result has been reinforced by work in which a green fluorescent protein was targeted to the brachyury locus. GFP expression is seen to occur first and then later only in the GFP and Flk-1 positive population are BL-CFCs detected (Fehling et al. 2003).

Despite all of this work the haemangioblast has proved relatively difficult to find in vivo. However experiments in murine embryos in which Brachyury is coexpressed with GFP have demonstrated blast colony forming cells expressing Flk-1 can be found in the posterior region of the primitive streak prior to yolk sac blood island formation (Huber et al. 2004). These haemangioblasts are hypothesised to migrate to the yolk sac at which point they become committed haematopoietic and endothelial progenitors contributing to the formation of the blood island (Dzierzak & Speck 2008).

1.5.3.3 Subsequent Differentiation of Haemangioblasts

Scl null EBs are completely devoid of haematopoietic cells and give rise to BL-CFCs with endothelial but not haematopoietic potential (Robertson et al. 2000). Although this suggests *Scl* expression is necessary for haematopoietic cell formation, this contradicts evidence from studies in zebrafish which shows that *Scl* expression is upstream of the haemangioblast. In this study forced expression of *Scl* in *Xenopus* embryos resulted in expansion of haematopoietic and endothelial lineages (Gering et al. 1998).

In order to shed light on the relationship between Flk1, SCL and the formation of the haemangioblast a transgenic mouse in which one copy of the *Scl* gene has been replaced with a copy of the gene for the cell surface molecule CD4 has been generated (Chung et al. 2002). This has the advantage of allowing *Scl* expressing cells to be isolated via cell sorting for CD4 expression. BL-CFCs were highly enriched in this *Flk1/Scl* double-positive population. Later maintenance of *Flk1* expression was associated with endothelial differentiation whereas the maintenance of *Scl* expression was associated with haematopoietic differentiation.

Another group has generated an ES cell line with a CRE inducible allele of *Scl* on a null background and studied the time dependence of the requirement for *Scl*. While no haematopoietic cells were detected in *Scl* null ES cells, only *Scl* gene reactivation between days 2 to day 4 could rescue haematopoiesis. *Scl* reactivation at later stages was ineffective. Flk-1 positive cells were generated but in the absence of *Scl* expression they were unable to differentiate to haematopoietic cells (Endoh et al. 2002).

Taken together these results suggest that sustained high levels of *Scl* expression promote haematopoietic differentiation of Flk1 positive mesoderm at the expense of the endothelial program. The findings by Gering *et al.* (1998) however suggest that *Scl* can function in promoting haemangioblast differentiation although it is not a critical factor at this stage. This work has been summarised in Figure 1.15.

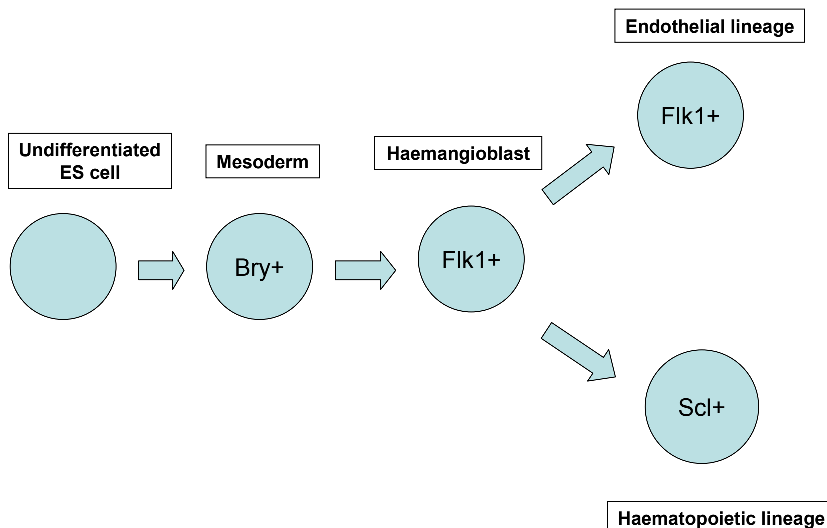


Figure 1.15 ES cell model of haematopoietic development
The undifferentiated ES cell differentiates to mesoderm. The *brachyury* positive mesodermal population are haemangioblast precursors. The haemangioblast is detected *in vitro* as a BL-CFC. These cells express Flk-1. Maintenance of Flk-1 expression is associated with endothelial differentiation. Scl expression is associated with haematopoietic differentiation.

1.5.4 The role of BMP4 in the haematopoietic development of ES cells

Johansson and Wiles were the first to demonstrate a role for BMP4 in haematopoietic differentiation of ES cells. In serum free chemically defined medium BMP4 induced haematopoietic differentiation in a concentration dependent manner (Johansson & Wiles 1995).

More recently BMP4 was shown to be required for Flk-1 and *Scl* expression. However VEGF in combination with BMP4 was necessary for optimal expansion and differentiation of *Scl* expressing haematopoietic progenitors. The effects of BMP4 were mediated by Smad1 and inhibited by Smad6 signalling molecules whilst the effects of VEGF were mediated by the mitogen activated protein kinase pathway (Park et al. 2004).

These results are in broad agreement with a more recent paper that has postulated the need for sequential exposure to growth factors: BMP4, activin A, basic Fibroblast growth factor and VEGF in order to achieve optimal haematopoietic differentiation. In this paper ES cells were differentiated as EBs initially with BMP4 which induced *Brachyury* expression. However BMP4 was needed in combination with Activin A and basic fibroblast growth factor at day 2.5 to achieve a maximal percentage of *Brachyury* and Flk-1 expressing cells, representing the haemangioblast. *Runx1*, *Hhex*, *Scl*, *Fli1* and *Lmo2* expression increased within the first six hours after addition of activin A and basic fibroblast growth factor. The addition of VEGF at day 2.5 was required to achieve maximal haematopoietic commitment measured by CD41 expression and numbers of haematopoietic colonies. VEGF maintained the expression of *Scl*, *Fli1*, and *Lmo2* but *Runx1* was not significantly affected. No effect was seen on *Gata2* with BMP4 and either VEGF or basic fibroblast growth factor and activin A added at day 2.5 (Pearson et al. 2008).

Lugus *et al.* (2007) however have provided evidence that BMP4 up regulates expression of *Gata2*. By generating an ES cell line with inducible *Gata2* expression they also showed that *Gata2* expression caused an increase in Flk-1 positive mesoderm even when the effect of BMP4 was blocked by the presence of the BMP4 antagonist noggin. This paper further proposes that *Gata2* induced Flk-1 Scl double positive cells independent of VEGF. This correlated to an increase in BL-CFCs which was maximal when *Gata2* expression was induced at day 2.75. Haematopoietic colony assays showed an increase in primitive

erythroid precursors (Lugus et al. 2007). This data contrasts with the results of Pearson *et al.* 2008.

BMP signals are transduced by the Smad family of transcriptional cofactors. *Smad1* knockout mice in a similar way to *Bmp4* knockout mice die too early to enable study of its role in the development of the haematopoietic system. An inducible *Smad1* ES cell line has therefore been generated. A pulse of *Smad1* expression between day 2 and 2.25 resulted in an increase in primitive erythrocyte and other haematopoietic colonies. Further analysis showed that *Smad1* caused an up regulation of *Gata2*, *Runx1* and *Scl* transcripts but not *Brachyury* or Flk-1. This correlated with an increase in BL-CFC formation. A pulse of *Smad1* expression therefore appears to result in an increase in haemangioblast formation. The authors of this paper stated that this alone accounted for the increase in the numbers of haematopoietic colonies seen (Zafonte et al. 2007).

Given that BMP4 is likely to signal via Smad1 it is unexpected that Brachyury and Flk-1 are not up regulated when *Smad1* expression is induced.

1.5.5 Human ES cell differentiation to haematopoietic cells

In recent years most work has focused on the differentiation of human ES cells to haematopoietic lineages. If cell replacement therapy is to become a reality it is important that the developmental pathways involved in human haematopoietic differentiation are understood.

Independent of other cytokines BMP4 appears to support haematopoietic differentiation consistently in several human ES cell lines (Chadwick et al. 2003).

1.5.5.1 The human haemangioblast

Recent work has characterised a transient population that appears in BMP4 stimulated EBs between 72 and 96 hours of differentiation that expresses KDR, the human homolog of Flk1. These cells form blast colonies with haematopoietic and vascular potential. These cells appear prior to the appearance of primitive erythroid cells (Kennedy et al. 2007). This population seems to correspond well to the haemangioblast population that appears in the mouse.

1.5.5.2 The repopulating potential of human ES cell derived haematopoietic cells

The *in vivo* repopulating potential of CD34+ve, CD45+ve cells derived from human ES cells has been assessed in NOD-SCID mice. Direct femoral injection allowed recipient survival and resulted in multilineage hematopoietic repopulation for up to 10 weeks. However analysis of the SCID repopulating cells revealed limited proliferative and migratory capacity. Ectopic expression of *HoxB4* had no effect on repopulating capacity (Wang et al. 2005).

This and other efforts made to replicate the strategy of ectopically expressing *HoxB4* to improve in vivo engraftment have failed. The genetic modification of human ES cells remains less advanced and less stable transgene expression could explain the lack of

benefit of *HoxB4* over expression (Papapetrou, Zoumbos, & Athanassiadou 2005). However this result could also reflect that the role of HoxB4 may vary in different species (Tian & Kaufman 2008).

The most successful repopulation to date using human ES cells has been achieved by differentiating cells in co-culture with the stromal cell line AM20.1B4 derived from the AGM region of 10dpc murine embryos. The whole cell mixture was transplanted after 12 days into NOD SCID mice achieving 2-16% engraftment efficiency (Ledran et al. 2008). This strategy has mimicked experiments that have shown mouse ES cells have definitive potential. Clearly by exposing ES cells during differentiation to an environment that promotes definitive haematopoiesis there is an increase in repopulating potential.

1.6 Summary

In order to expand HSCs ex-vivo, knowledge of the conditions in the bone marrow that allow their survival and expansion is necessary. The concept of the bone marrow niche is central to the future construction of a suitable environment where HSCs might be expanded for use in bone marrow transplant. Likewise if ES cells are to be differentiated to form HSCs it is necessary to understand the dynamic processes which lead to HSC formation.

There is strong evidence to support a central role for BMP4 in haematopoietic development. Perhaps the most persuasive evidence of this comes from immunohistochemistry staining that has shown that BMP4 is transiently expressed at high

levels in the human AGM region (Marshall, Kinnon, & Thrasher 2000). Experiments in the mouse also have shown that BMP4 becomes highly expressed just before Runx1 expressing haematopoietic clusters appear in the AGM (North et al. 1999;Durand et al. 2007). However the role BMP4 plays at this stage in haematopoietic differentiation remains unclear.

Experiments in the ES cell system have begun to investigate this. However thus far the consensus from experiments carried out using ES cells is that optimal haematopoietic differentiation requires a combination of factors including BMP4 (Park et al. 2004;Pearson et al. 2008). This implies BMP4 is necessary but not sufficient for optimal haematopoietic differentiation. These results suggest that BMP4 at least has a role to play in the up regulation of Flk-1 expressing cells and the haematopoietic genes *Scl*, *Lmo2*, *Gata2* and *Runx1*.

Scl, *Lmo2*, *Gata2* and *Runx1* clearly are essential to haematopoietic development. From research done so far it appears that they are closely related and the regulation of their expression is at least in part dependent on each other. Although the timing of lethality is clear in deletion mutant studies this only defines the processes for which these genes are critical. There remains a lack of clarity as to the kinetics of gene expression for each of these genes.

Few published experiments have been done to establish the effects of BMP4 on bone marrow and foetal liver cells. Of the few experiments that have been performed on mouse

bone marrow (Fuchs et al. 2002;Maguer-Satta et al. 2003) the results have been inconclusive.

1.7 Aims

Previous ES experiments have investigated optimal haematopoietic differentiation using a combination of growth factors. The main aim of this work was to determine the role BMP4 alone plays in early development. In particular these experiments were aimed at determining whether BMP4 up regulated the haematopoietic genes *Runx1*, *Scl*, *Gata2* or *Lmo2*.

Few experiments have investigated the role of BMP4 in developmentally more mature haematopoietic populations. Therefore in these experiments foetal liver and bone marrow serum free culture systems were developed in order to determine the effects of BMP4. In particular the aim was to see if there was up regulation of the same haematopoietic genes and thus if the effects of BMP4 were the same regardless of the developmental maturity of the haematopoietic population being investigated.

The genes *Runx1*, *Scl*, *Gata2* and *Lmo2* clearly are important to haematopoietic development. ES differentiation experiments sought to investigate the timing and sequence of expression in development. In the foetal liver and bone marrow cultures these experiments sought to characterise whether these genes play a role in HSC expansion or differentiation.

Chapter 2 Materials and Methods

2.1 Materials

Unless otherwise stated all tissue culture reagents were supplied by Gibco/Invitrogen, all general chemicals were supplied by Sigma and all plastics were from Nunc.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 ES cell culture

2.2.1.1.1 SNH stromal cell culture and irradiation

Murine SNH stromal cells (donated by Dr. Martinez Barbera, ICH) were maintained at low passage number in culture at 37ºC, 5% CO_2 and passaged every 3 -4 days at subconfluence. Cells were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% foetal calf serum (FCS). For use as feeder cells in embryonic stem (ES) cell culture, SNH cells were irradiated at 2500cGy using a ^{137}Caesium source.

Irradiated cells were either plated at $3x10^6$ cells/dish in 10cm tissue culture plates precoated with 0.1% gelatin (porcine 300 bloom) for use the following day or stored at -80ºC in FCS supplemented with 10% dimethyl sulfoxide (DMSO) for future use.

2.2.1.1.2 Undifferentiated ES cell culture

The murine CCE ES cell line (donated by Juan Pedro Martinez Barbera, Neural Development Unit, ICH) was maintained in ES medium. ES medium consisted of DMEM, 15% FCS, Penicillin (50µg/ml), 0.1mM MEM non essential amino acids, 1mM Sodium Pyruvate, 1000U Leukaemia Inhibitory Factor (LIF, Chemicon International) and 100 µM beta-mercaptoethanol (β-ME). Cells were incubated at 37ºC, 5% CO_2. Fresh medium was added each day and cells were passaged every 2-3 days. Prior to differentiation CCE ES cells were passaged onto gelatin coated tissue culture plates in the absence of irradiated SNH cells to remove feeder cells.

2.2.1.1.3 EB differentiation

CCE ES cells were suspended as 20µL 'hanging' drops on the lid of a non tissue culture treated plate (VWR International) in DMEM supplemented with either 10% FCS, 10% Serum Replacement (SR) or 10% SR and BMP4 (5ng/ml, R&D Systems). Cells were incubated at 37ºC, 5% CO_2. After 2 days developing EBs were washed from the lid, in the same medium as above, into a 10cm non tissue culture treated plate.

2.2.1.1.4 BMP4 titration experiments

Cells were differentiated as described above in DMEM supplemented with 15% SR and 0, 5, 10 and 25ng/ml of BMP4.

2.2.1.1.5 BMP receptor experiments

Cells were differentiated as described above in DMEM supplemented with 15% SR and 0, 25, 100 and 250ng/ml of BMP receptor 1α (R&D Systems).

2.2.1.2 Bone marrow Culture

2.2.1.2.1 Isolation of bone marrow (BM) cells

The hind legs of sacrificed adult CD1 mice were obtained and under sterile conditions the thigh, femur and tibia were dissected away. Bone marrow cells were flushed using CellGro stem cell growth medium (CellGenix, Germany) and passed through a 70µm cell strainer. 1ml of red cell lysis buffer (Qiagen) was added and incubated on ice for 10 minutes to remove erythrocytes.

2.2.1.2.2 Culture of bone marrow

The isolated bone marrow cells were seeded into a 6 well tissue culture plate at $1x10^6$ cells per ml in CellGro medium and 10^{-4}M beta-mercaptoethanol supplemented with 10% FCS or BMP4 (10ng/ml). Cells were incubated at 37ºC, 5% CO_2. In some experiments a further culture condition was added: BMP4 (10ng/ml) and BMP receptor (200ng/ml). In later experiments IGF-1 (20ng/ml) and TPO (20ng/ml) were added.

2.2.1.3 Foetal liver culture

2.2.1.3.1 Isolation of foetal Liver (FL) cells

Under sterile conditions foetal livers were dissected from day 14.5 CD1 mouse embryos. Liver cells were dissociated by placing them in 1ml of cell dissociation buffer for 10 minutes at 37ºC. They were then triturated using 21, 23 and 25 guage needles and passed through a 70 µm cell strainer. Red cell lysis buffer was added for 10 minutes and incubated on ice to remove erythrocytes.

2.2.1.3.2 Culture of foetal Liver Cells

$1x10^6$ cells/ml were seeded into a 6 well tissue culture plate in CellGro medium and 10^{-4}M beta-mercaptoethanol supplemented with 10% FCS or BMP4 (10ng/ml). In some experiments a further culture condition was added: BMP4 (10ng/ml) and BMP receptor (200ng/ml). The cells were incubated at 37ºC, 5% CO_2.

2.2.1.4 Maintenance of other cell lines

ST2 cells were cultured in RPMI medium supplemented with 10% FCS and penicillin (50µg/ml). SVEC and 293T cells were cultured in DMEM supplemented with 10% FCS and penicillin (50µg/ml). Cells were incubated at 37ºC, 5% CO_2. Cells were passaged at 80-90% confluency.

2.2.1.5 Long term storage of cell lines

Cell line cells were stored in vials containing $1x10^6$ cells in 1ml of freezing medium. Freezing medium contained 90% FCS and 10% DMSO.

In order to revive frozen cells, cells were thawed quickly in a 37ºC waterbath and then the appropriate culture medium was added slowly. Cells were then pelleted using a tabletop centrifuge (Heraeus) to remove the freezing medium and resuspended in culture medium.

2.2.2 Sample Analysis

2.2.2.1 Reverse transcription - PCR

2.2.2.1.1 Total RNA extraction

EBs were placed in cell dissociation buffer for 10minutes at 37ºC and triturated using a 23G needle. 1×10^6 cells per sample were collected in lysis buffer. RNA was then extracted using an RNAeasy mini kit (Qiagen). RNA was dissolved in 40µL RNase free water (Qiagen) and quantified using a Nanodrop ND-1000 spectrophotometer.

2.2.2.1.2 Reverse transcription

RNA was treated with DNase prior to the reverse transcription reaction in order to eliminate any contaminating genomic DNA. 16µL of the RNA sample was added to 2µL DNaseI and 2µL DNaseI 10x buffer and incubated at room temperature for 15 minutes. EDTA was used to inactivate the enzyme. The sample was incubated at 65ºC for 5 minutes to ensure DNase inactivation.

The reverse transcription (RT) reaction was performed on DNAse treated RNA by adding 8µL first strand buffer, 4µL DTT, 2µL random primers, 2µL dNTPs, 2µL RNase out and 2µL MMLV reverse transcriptase. The sample was incubated for 2 hours at 37ºC. Following incubation the reverse transcriptase enzyme was heat inactivated at 70ºC for 15 minutes.

2.2.2.1.3 PCR

The expression of *Hprt*, *Alk3*, *Bmpr2* and *Bmp4* was determined using PCR. A 25 µL reaction was performed using 100ng of cDNA template, 12.5µL of PCR mastermix, 2.5µL of upstream primer, 2.5µL of downstream primer and nuclease free water. 40 cycles of PCR were performed at 95ºC for 30 seconds, 56ºC for 30 seconds and 72ºC for 2 minutes in an Eppendorf Mastercycler PCR machine. *Alk3* and *BmprII* primer sequences were taken from Pesce *et al.* (2002) and *Bmp4* primer sequences were taken from Peretto *et al.* (2004). Primers were designed so that contaminant genomic DNA would not produce a band of equal size to those produced by amplification of cDNA. Primer sequences were as follows:

Hprt forward TCCCCAGACTTTTGATTTGC,
reverse GGAAAATACAGCCAACACTGC;

Alk3 forward ACTTTAGCACCAGAGGATACC,
reverse TTTTCACCACGCCATTTACCC;

BmprII forward TGCGGCTATAAGTGAGGTTGG,
reverse AGCAGTTGACATTGGGTTGAC;

Bmp4 forward CTCCCAAGAATCATGGACTG,
reverse AAAGCAGAGCTCTCACTGGT.

2.2.2.1.4 Real Time PCR

A 25µL reaction was performed using 1.4µL of cDNA sample, PCR mastermix (2x, Applied Biosystems), primers and probe (20x) and DNase free water. *Runx1*, *Scl*, *Gata2* and *Lmo2* expression was analysed. *Gapdh* was used as the internal standard. Primers and probes were obtained from Applied Biosystems. Each reaction was performed in triplicate. 45 cycles of PCR were performed in a Real Time ABI Prism 7000 PCR machine using default settings: cycling at 95º for 15 seconds and 60ºC for 1 minute. Data was analysed using ABI Prism software.

Relative quantification values were obtained using the comparative C_T method ($\Delta\Delta C_T$). For this calculation the C_T was calculated for the treated sample and the day 0 sample for the gene of interest. The C_T was also calculated for each sample for the endogenous

control. For each sample, reactions were performed in triplicate and the C_T value is calculated as an average of these values. The following calculations are then performed:

ΔC_T = C_T (gene of interest)- C_T (endogenous control)

$\Delta\Delta C_T$ = ΔC_T (gene of interest : treated sample) - ΔC_T (gene of interest : day 0)

Fold difference in gene expression = $2^{-\Delta\Delta C_T}$

2.2.2.2 Flow cytometry (FC) Analysis

2.2.2.2.1 4 colour flow cytometry

Where necessary, cells were dissociated in cell dissociation buffer for 10 minutes at 37ºC and triturated using a 23G needle. Samples collected (each containing up to $1x10^6$ cells) were suspended in PBS/10% bovine serum albumin (BSA) and divided between 3 flow cytometry (FC) tubes. Samples were incubated in the dark on ice for 30 minutes with fluorescence conjugated antibodies against murine stem cell markers: CD34 fluoroisothiocyanate (FITC), Flk-1 phycoerythrin (PE), Sca-1 PerCP and C-kit APC or murine haematopoietic differentiation markers: CD45 FITC, Ter119 PE, CD11b PerCP and CD19 APC. An isotype control tube contained IgG FITC, IgG PE, IgG PerCP and IgG APC. All antibodies used were directly conjugated. Antibody manufacturer details are as listed in table 2.1.

1μL of each antibody was added and the sample was diluted in PBS/10%BSA to 100μL. Samples were washed once and fixed in 1% paraformaldehyde (PFA, Polysciences Inc.). Samples were analysed on a CyAn ADP flow cytometer (Dako Cytomation) using Summit 4.2 software. A 488 nm argon laser was used to excite PE, FITC and PerCP fluorochromes and emission was collected. A 633 nm He Ne laser was used to excite APC which was collected at 675 nm. Cells were gated on the basis of their forward scatter and side scatter to exclude dead cells and debris. All antibodies were titrated prior to use and a minimum of 20000 events were collected. The isotype control was used for quadrant placement.

Table 2.1 Antibody Manufacturer Information

Specificity	***Catalogue Number***	***Source***	***Raised in***	***Type***
Stem Cell Markers				
CD34	553733	BD Pharmingen	Rat	FITC
CD117 (C-kit)	12-1171	eBioscience	Rat	PE
	553356	BD Pharmingen	Rat	APC
Flk-1	555308	BD Pharmingen	Rat	PE
Sca-1	12-5981	eBioscience	Rat	PE
	MSCA18	Caltag Labs	Rat	Cy5.5
Differentiation Markers				
CD45	553079	BD Pharmingen	Rat	FITC
CD11b	12-0112	eBioscience	Rat	PE
	550993	BD Pharmingen	Rat	Cy5.5
Ter119	553673	BD Pharmingen	Rat	PE
CD19	550992	BD Pharmingen	Rat	APC
Isotype Controls				
IgG2a	12-4321-81	BD Pharmingen	Rat	PE
IgG2a	555929	BD Pharmingen	Rat	FITC

2.2.2.2.2 Cell cycle analysis

Cells were collected and washed in phosphate buffered saline (PBS, Gibco/Invitrogen). Where necessary, cells were dissociated in cell dissociation buffer for 10 minutes at 37ºC and triturated using a 23G needle. Cells were then washed again and resuspended in Propidium Iodide buffer. Propidium Iodide was then added at 50µg/ml (BD Pharmingen). Cells were analysed on a CyAn ADP flow cytometer within 15 minutes for DNA levels. A minimum of 20,000 events were collected. Linear gates were set to quantify cells at each phase of the cycle.

2.2.2.2.3 Cell death analysis

Cells were collected and washed in PBS. Where necessary, cells were dissociated in cell dissociation buffer for 10 minutes at 37ºC and triturated using a 23G needle. Cells were then washed again and resuspended in Annexin V buffer (BD Pharmingen). Cells were then incubated with Annexin V antibody (BD Pharmingen) for 30 minutes in the dark on ice. After this Propidium Iodide was added and cells were analysed on a CyAn ADP flow cytometer within 15 minutes. A minimum of 20,000 events were collected.

2.2.2.3 Methylcellulose cultures:

2.2.2.3.1 Bone marrow and foetal Liver methylcellulose cultures

Bone marrow and foetal liver samples were replated at a density of 1500 cells in 1.5ml of complete methylcellulose medium containing haematopoietic cytokines (Methocult GF M3434, Stemcell Technologies Inc). Cultures were incubated for 14 days after which colonies were counted under the microscope and identified by morphology.

2.2.2.3.2 ES methylcellulose cultures

Embryoid body samples were replated at a density of 1500 cells/1.5 ml in 0.9% base methylcellulose medium (ES Cult M3120, Stem Cell Technologies Inc) containing FCS (15%), Insulin-transferrin (25µg/ml), MTG (4.5×10^{-4}M), IL1α (5ng/µl), IL3 (10ng/ml), IL6 (5ng/ml), IL11 (25ng/ml), SCF (25ng/ml), GM-CSF (5ng/ml) and Erythropoietin (2 units/ml). Cytokine and growth factor manufacturer details are as listed in table 2.2 Cultures were incubated for 14 days after which colonies were counted and identified by morphology.

Table 2.2 Cytokine and Growth Factor Manufacturer information

Cytokine/Growth Factor	***Catalogue Number***	***Source***
Erythropoietin	hBA-165	Santa Cruz Biotechnology
ES tested Foetal Calf Serum	06592	StemCell Technologies
GM-CSF	14-8331	eBioscience
IL1α		
IL3	14-8031	eBioscience
IL6	14-8061	eBioscience
IL11	418-ML	R and D systems
Insulin-Transferrin	51500-056	Invitrogen
MTG	M-3148	Sigma
SCF	14-8341	eBioscience

2.2.2.3.3 Cytospin and May Grumwald Giemsa Staining

Individual colonies were collected and transferred to microscope slides using a cytospin centrifuge (1000rpm for 5minutes). Cells were fixed using methanol (5 minutes, room temperature) and stained using May Grumwald Giemsa stain.

2.2.2.3.4 F4/80 immunocytochemical analysis of methylcellulose colonies

Individual colonies were collected and transferred to microscope slides using a cytospin centrifuge (1000rpm for 5 minutes). Cells were fixed in 4% PFA in PBS for 15 minutes at room temperature. Endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide in PBS for 30 minutes at room temperature. After washing in PBS non-specific antibody binding was blocked by incubating sections with 3% normal goat serum in PBS for 30 minutes at room temperature. Cells were then incubated with rat anti-mouse F4/80 primary antibody (eBioscience), diluted 1:50 with 0.1% BSA in PBS, overnight at 4°C. After washing cells in PBS, cells were incubated with biotinylated goat anti-rat antibody (Vector Laboratories) diluted 1:100 in PBS-BSA for 30 minutes at room temperature followed by avidin biotinylated peroxidase complex (ABC reagent, Vector Laboratories) diluted 1:200 in PBS-BSA for 1 hour at room temperature. Peroxidase activity was visualised using 0.025% 3,3'diaminobenzidine in PBS containing 0.03% hydrogen peroxide. After washing cells in PBS, slides were rinsed through a series of ethanol and Histoclear. They were then mounted with DPX and viewed using a Leica light microscope.

2.2.3 ES cell staining

2.2.3.1 Alkaline phosphatase staining

ES cells were cultured to confluency in 12 well plates. Culture media was aspirated and cells were fixed using 4% PFA in PBS for 2 minutes. Cells were then washed using Tris buffered saline tween 20 (TBST) composed of 20mM Tris-HCl, 0.15M NaCl, 0.05% Tween-20 at pH 7.4. Napthol phosphate and fast red violet solution (Chemicon International) was mixed in water in the ratio 2:1:1 and added to each well and incubated in the dark at room temperature for 15 minutes. After aspirating staining solution cells were washed with TBST and then PBS. The cells were visualised using a Nikon light microscope.

2.2.3.2 SSEA-1 staining

ES cells were cultured to confluency in 12 well plates. After aspirating culture media cells were fixed in 4% PFA in PBS for 15-20 minutes at room temperature. Cells were washed twice in TBST and permeabilised with 0.1% Triton in PBS for 10 minutes at room temperature. Cells were again washed twice using TBST. Non specific antibody binding was blocked by applying 4% normal goat serum in PBS for 30 minutes at room temperature. Primary anti-mouse SSEA-1 antibody (Chemicon International) was diluted 1in 20 in blocking solution and added to the cells for 1 hour at room temperature. Cells were then washed three times in TBST. Goat anti mouse FITC IgM antibody was diluted

1in 20 in PBS and incubated for 1 hour at room temperature. After washing cells three times in TBST cells were visualised using an Olympus fluorescent microscope.

2.2.4 Transfection

2.2.4.1 pEGFP-N1 transfection of ES cells

On the day of transfection ES cells were resuspended in EB differentiation medium containing DMEM and 15% SR in a 12 well plate. Cells were seeded at 200,000 cells per well in 1ml of EB differentiation medium. For each well 1.6µg of plasmid DNA was diluted in 100µL of Optimem medium and 4µl of lipofectamine 2000 was diluted in 100µL of Optimem. These dilutions were incubated for 5 minutes at room temperature and then mixed together to make a total volume of 200µL per well. The mixture was then incubated for 20 minutes at room temperature to allow formation of DNA-lipofectamine complexes. The complexes were then added to the medium and incubated for 4 hours at 37C, 5% CO2. After this the medium was replaced with EB differentiation medium. In each experiment the pEGFP-N1 plasmid was transfected. Also an untransfected control, plasmid DNA alone and lipofectamine 2000 alone control wells were included.

2.2.4.2 pKLO.1 transfection of ST2 cells

One day before transfection 100,000 cells were plated out in 500µL of culture medium per well without antibiotics in a 24 well plate. On the day of transfection lipofectamine – DNA complexes were prepared as follows. For each well 0.8µg of plasmid DNA was

diluted in 50μL of Optimem medium and 2μL of lipofectamine 2000 was diluted in 50μL of Optimem. These dilutions were incubated for 5 minutes at room temperature and then mixed together to make a total volume of 100μL per well and left for 20 minutes at room temperature. This mixture was then added to the medium and incubated for 4 hours at 37ºC, 5% CO_2. After this the medium was replaced with ST2 culture medium without antibiotics. In each experiment the 5 plasmids and a scramble sequence plasmid were transfected in separate wells. Also an untransfected control and lipofectamine 2000 alone control wells were included.

2.2.5 DNA manipulation

2.2.5.1 Restriction enzyme digestion

Plasmid DNA was single or double digested using restriction enzymes (Promega) added at no more than 10% of total reaction volume. An appropriate enzyme buffer was chosen depending on the restriction enzymes being used according to manufacturer's instructions. Total reaction volume was made up to 15-20μL. The reaction was incubated in a water bath at 37ºC for 2 hours.

2.2.5.2 Dephosphorylation of digested plasmid DNA ends

Digested plasmid DNA was treated with calf intestinal alkaline phosphatase (CIAP, New England Biolabs) to dephosphorylate the DNA ends prior to ligation. The reaction was performed in CIAP buffer incubated at 37ºC. 5 units of enzyme were added at the start of the reaction and after 30 minutes, a further 5 units of enzyme were added. The total

incubation period was for 1 hour. The enzyme was heat inactivated following the reaction by incubation for 15 minutes at 65ºC.

2.2.5.3 Agarose gel electrophoresis

DNA fragments were resolved by gel electrophoresis using 1% agarose gels in 1x TAE (40mM Tris acetate, 5mM ethylenediaminetetraacetic acid). Gels were prepared by dissolving agarose in 1x TAE by boiling the mixture in a microwave. After cooling ethidium bromide was added at 0.5μg/ml to enable visualization of DNA. Before loading DNA onto the gel, DNA samples were mixed with loading buffer (10mM Tris-HCl pH 7.5, 50mM EDTA, 10% Ficoll 400, 0.4% Orange G). A 1kb plus ladder was loaded onto each gel to enable size determination of DNA fragments. Gels were electrophoresed by applying a voltage of 100V across the gel. The separated fragments were visualized by exposure to UV light using an UVIdoc gel documentation system.

2.2.5.4 Gel purification of DNA

Following gel electrophoresis DNA fragments were visualised under UV light and excised using a scalpel blade. The DNA was then extracted from agarose using a QIAquick gel extraction kit (Qiagen) as per the manufacturer's instruction.

2.2.5.5 Ligation

Ligation was carried out using T4 DNA ligase (Promega) in T4 DNA ligase buffer. A vector insert ratio of 1:3 was used with 100ng of vector DNA added per reaction. Ligation reactions contained 1 unit of T4 DNA ligase and were made up to a total volume

of 10µL. The reaction was incubated overnight in a water bath at 16ºC. The ligation reaction was then immediately transformed into competent bacteria.

2.2.6 Bacterial manipulation

2.2.6.1 E. coli bacterial culture

E. coli were grown in flasks containing LB media incubated at 37ºC and agitated at 250 rpm or streaked out on LB plates containing 1.5% bacto agar (Merck) and incubated at 37ºC. E. coli carrying plasmids with ampicillin resistance were grown on media supplemented with ampicillin (50µg/ml, Stratagene).

For long term storage bacterial culture was made up with 15% glycerol and stored at -70ºC.

2.2.6.2 Preparation of electrocompetent bacteria

E. coli DH5α were cultured in LB media and incubated at 37ºC with agitation until the optical density was between 0.7 and 0.8. At this point the culture was pelleted by centrifugation at 5000 rpm for 15 minutes. The supernatant was discarded and the resulting pellet was resuspended in 1L of cold sterile 10% glycerol. Following this the bacteria were centrifuged again and resuspended in 1L of cold sterile glycerol. The bacteria were then centrifuged a final time and the supernatant was discarded and the bacteria resuspended in the small remaining volume of glycerol. The bacteria were transferred to a smaller centrifuge tube and centrifuged again at 7000 rpm for 10 minutes. The supernatant was removed and the cell pellet resuspended in 2ml cold sterile 10%

glycerol. The bacteria were snap frozen in dry ice into 100µL aliquots and stored at -70ºC.

2.2.6.3 Heat shock bacterial transformation

10µL of ligated DNA was incubated on ice with 100µL of competent bacteria for 30 minutes. The mixture was then incubated for 1 minute at 42ºC and then for a further 5 minutes on ice. 500µL of LB was then added to the transformed bacteria which were then incubated at 37ºC for 1 hour. After incubation the bacteria were spread onto an LB agar plate containing ampicillin (50µg/ml) and placed in an incubator at 37ºC overnight.

2.2.6.4 Plasmid DNA preparation

2.2.6.4.1 Small scale

Colonies were picked using 20µL pipette tips from LB agar plate cultures and grown overnight in 5ml liquid LB cultures containing ampicillin (50µg/ml). Plasmid DNA was then harvested from these cultures using a Qiagen mini-prep kit as per the manufacturer's instructions.

2.2.6.4.2 Large scale

Colonies were picked using 20µL pipette tips from LB agar plate cultures and grown overnight in 5ml liquid LB cultures containing ampicillin (50µg/ml). 500µL of this culture was then used to inoculate 500ml LB media containing ampicillin (50µg/ml). This culture was incubated overnight at 37ºC with agitation. A Qiagen mega-prep kit was used to harvest plasmid DNA as per manufacturer's instructions.

2.2.6.4.3 Measurement of DNA concentration

The plasmid DNA concentration was established using a NanoDrop ND-1000 spectrophotometer.

2.2.7 Lentivirus preparation and transduction

2.2.7.1 Lentivirus production

The day before transfection 1.2 x 10^7 293T cells were seeded into a 175 cm^2 tissue culture flask in culture media so that the next day cells were 80-90% confluent. On the day of transfection per flask 5mls of Optimem medium was prepared containing 50µg plasmid, 17.5µg pMDG2 (VSV-G) and 32.5µg p8.74 (gag-pol). Separately 5 ml of Optimem medium containing 1µL polyethylenimine (PEI, 10mM) was prepared. These two solutions were then filtered using a 0.22µm filter, mixed together and left for 20 minutes. The media on the 293T cells in culture was removed and the cells were washed using Optimem medium. The 10ml Optimem solution containing the PEI-DNA complexes were added to the flask of 293T cells. The cells were then incubated for 4 hours at 37ºC before replacing the media on the cells with culture media. After 48 hours the supernatant was harvested and fresh culture media was replaced on to the cells. The supernatant was centrifuged at 4000 rpm for 10 minutes and then filtered through a 0.22µm filter to remove any cell debris. The virus was concentrated by ultracentrifugation of the medium at 23,000rpm for 2 hours. After this the supernatant

was carefully decanted off so as not disturb the virus containing pellet. Virus was then resuspended in 150µL of Optimem which was stored as 20µL aliquots at -70ºC.

2.2.7.2 Titration of lentivirus supernatants

One day before transduction 100,000 293T cells were seeded per well in a 24 well plate in 500µl of culture medium. The next day a 5 fold series of dilutions of virus was prepared in 500µl aliquots of culture medium. The aliquots were used to replace the medium on the cells. Green fluorescent protein (GFP) expression was measured 48 hours after transduction by flow cytometry. Expression titre was then calculated by determining the well that produced 5-15% of GFP expression and then dividing the number of transduced cells in this well by the volume of virus used to transduce them.

2.2.7.3 Lentiviral ST2 transduction

100,000 ST2 cells in 500µL ST2 culture medium per well were seeded onto a 24 well plate the day before transduction. On the day of transduction virus was added at the required MOI to the culture media.

2.2.7.4 Lentiviral ES cell transduction

On the day of transfection ES cells were collected and resuspended in EB differentiation medium. Cells were transduced by adding virus at the required MOI to the medium. The ES cells were then differentiated by the hanging body differentiation method previously described in section 2.2.1.1.3. Briefly, 35,000 cells were suspended in 20µl droplets from the top of non-adherent tissue culture plates. After 2 days these droplets were washed off

the top of these plates into new non-adherent tissue culture plates. EBs were taken for PCR and flow cytometry analysis when necessary.

2.2.8 Statistical analysis

Where the data could be assumed to be normally distributed, a 2 tailed unpaired t-test was applied to determine statistical significance. The Wilcoxon Mann Whitney, a non parametric statistical test, was applied to rest of the data. The null hypothesis was that the treatment caused no difference.

The statistics software package SPSS Version 13.0 was used to perform the calculations. If $P<0.05$ then the result was judged to be significant.

For the real time PCR data the logarithmic values had to be converted to real values by raising 2 to the power of the $\Delta\Delta Ct$ value before statistical analysis was performed.

Chapter 3 The effect of BMP4 on haematopoietic stem cells

3.1 Introduction

In these experiments the role of BMP4 in three different culture systems was explored: ES cells as a model of yolk sac haematopoiesis, foetal liver cells and bone marrow cells. The main aim of these experiments was to discover whether BMP4 had an effect on the expression of *Runx1*, *Scl*, *Gata2* and *Lmo2*. Evidence from experiments in the ES cell system has suggested BMP4 up regulates the expression of these genes as already described in Section 1.5.6.

Serum is commonly used as a source of essential survival factors in cell culture. Foetal liver and bone marrow *ex vivo* culture systems had to be developed which would allow the survival of these cells in serum free conditions. Previous papers have already described serum free conditions for the differentiation of CCE ES cells (Adelman, Chattopadhyay, & Bieker 2002).

In taking samples for analysis the same time points were used across the different culture systems to determine if there were any similarities in the way the cells responded to BMP4. Samples were analysed by quantitative reverse transcription PCR for the

expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* and by flow cytometry for cell surface markers.

A common panel of antibodies was used for flow cytometry across all of the culture systems to establish any similarities in the response to BMP4. A stem cell panel of antibodies including CD34, Sca-1, Flk-1 and C-kit was used. These antigens are all known to be expressed on stem cells (see Section 1.1.3.2) with the exception of Flk-1 which is a marker for the haemangioblast (see Section 1.5.3.2). In the ES cell system BMP4 has been shown to increase the number of Flk-1 expressing cells (Park et al. 2004). In addition to attempting to verify this result, one of the aims of these experiments was to detect whether BMP4 up regulated Flk-1 expression in the developmentally more mature cells of the foetal liver and bone marrow.

In addition a differentiation panel was used to detect the presence of blood lineage markers including: CD45, Ter119, CD11b and CD19 (see Figure 1.2B). CD45 is a pan-haematopoietic marker (Hermiston, Xu, & Weiss 2003), Ter119 is an erythroid marker (Kina et al. 2000), CD11b is a myeloid marker (Springer et al. 1979) and CD19 is a lymphoid marker (Krop et al. 1996). This panel was used to detect differentiation of haematopoietic progenitors resulting from the presence of BMP4.

In the foetal liver and bone marrow systems methylcellulose colony assays were also performed to determine the number and type of progenitors present in culture.

Thus intracellular changes in gene expression could be correlated with changes in the type of cells present based on cell surface markers and colony analysis. The experimental approach is summarised in Figure 3.1.1.

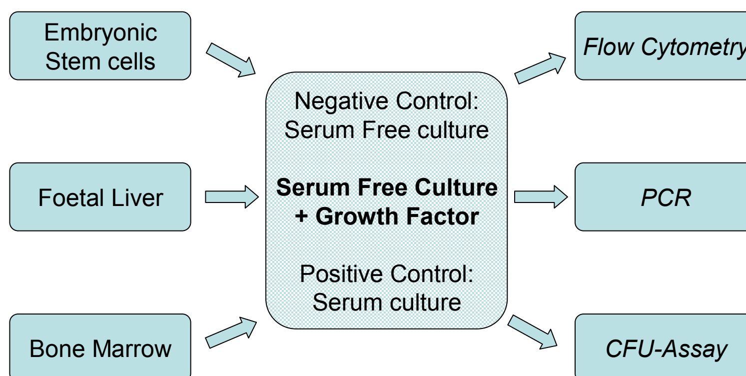


Figure 3.1.1 Experimental protocol in ES cell, foetal liver and bone marrow experiments
Cells were cultured in serum-free conditions (minimal growth conditions), with recombinant BMP4 or in the presence of foetal calf serum (maximal growth conditions). Analysis was carried out for changes in cell surface markers (flow cytometry), transcription factor expression (PCR) and haematopoietic output (CFU assay).

3.2 Embryonic Stem Cell Differentiation to Haematopoietic cells: The effect of BMP4

Many different types of murine ES cell line have been derived. For the purposes of these experiments a CCE embryonic stem cell line was used. This cell line is derived from the inbred 129/Sv mouse strain. These cells have previously been shown to differentiate into haematopoietic cells (Keller et al. 1993).

ES cells are known to differentiate to haematopoietic cells in the presence of 15% foetal calf serum (Keller et al. 1993). However serum contains variable amounts of factors and many other ill defined substances. These uncontrolled influences would have masked or at least complicated the analysis of a single growth factor (Johansson & Wiles 1995). For this reason ES cells were differentiated in serum replacement (SR) media which contains a minimal defined set of growth factors necessary to support the growth and survival of these cells. In order to determine the effects of BMP4, cells were differentiated in SR media with BMP4. Cells were differentiated in serum in addition as a positive control.

When ES cells are plated in EB differentiation medium they coalesce to form embryoid bodies. Within these embryoid bodies many different types of cells differentiate mimicking the processes that take place within the embryo. ES cells were differentiated in hanging drops suspended from the top of a petri dish (see Figure 3.2.1). Each 20 µL droplet contains 35000 ES cells and gives rise to a single embryoid body. Because each droplet contains the same number of cells there is greater consistency in the size of the embryoid bodies. Consistency in the size of the embryoid body aims to ensure greater consistency in the developmental stage between embryoid bodies and ultimately greater consistency in the results overall.

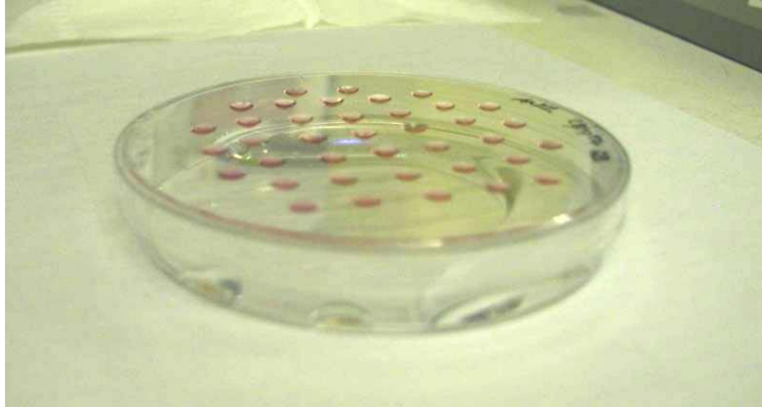


Figure 3.2.1 Embryoid body differentiation
Droplets on the top of the Petri dish contain 35,000 cells each. One embryoid body forms per droplet. This method aims to ensure greater consistency in the size of EBs that are formed and results overall.

Johansson and Wiles (1995) have tested BMP4 at concentrations between 0.1 and 10ng/ml for its ability to increase mesoderm and haematopoietic gene expression in CCE ES cells. Park *et al.* (2004) used BMP4 at 5ng/ml on R1 ES cells and found this was optimal for generation of Flk-1 and SCL expressing cells. Lugus *et al.* (2007) using R1 ES cells have also found this to be the optimal concentration of BMP4 for haematopoietic differentiation. Given this previous data I decided to use BMP4 at 5ng/ml.

Haematopoietic colony forming cells (CFCs) can only be detected from day 4 after differentiation in 15% serum. The frequency of CFCs increases up to day 6 when they comprise between 0.5 and 1% of the total EB population (Keller et al. 1993). For this reason samples were taken for analysis at day 0, day 3 and day 6. Sampling at these time points also allowed comparison of results obtained with the bone marrow and foetal liver cultures.

Cell samples were harvested as described in section 2.2.1.1 and then counted using trypan blue to check cell viability. Cell viability was 80-95% at all time points and under all conditions.

3.2.1 PCR Results

3.2.1.1 Reasons for using Real Time PCR

In reverse transcription PCR, mRNA is converted to cDNA by reverse transcriptase. Traditionally the quantity of mRNA can then be quantified by semi-quantitative PCR. In this process primers are chosen to amplify a specific sequence from the cDNA. PCR conditions, in particular, cycle number must then be determined such that a comparison can be made in the amount of DNA amplified from different samples at the end of the reaction.

In real time PCR in addition to the primers detecting a target sequence there is a fluorogenic probe. This probe has a 5' fluorescent dye and a quencher dye on the 3' end. While the probe is intact the quencher dye reduces the fluorescence emitted by the reporter dye. If the target sequence is present the probe anneals downstream of one of the primers and is cleaved by the 5' nuclease activity of Taq DNA polymerase as the primer is extended. The cleavage of the probe separates the reporter dye from the quencher dye increasing the fluorescence of the reporter dye. This cleavage also removes the probe

from the target strand such that primer extension can continue to the end of the template strand. With each cycle there is an increase in the amount of amplicon present and thus the intensity of fluorescence.

In real time PCR the fluorescence is measured and recorded at the end of each cycle. At the end of the PCR reaction a threshold is set at which point the PCR reactions were in the exponential phase and from this the cycle threshold or Ct is determined for each reaction. In these experiments relative quantification was used to determine the changes in gene expression. Thus in a given sample, gene expression was determined relative to the level of expression in the day 0 sample.

An endogenous control, in this case GAPDH, is used to normalize for differences in the amount of cDNA that is loaded into the reaction. Endogenous expression levels must be the same in all samples so that any differences between samples are only due to changes in the expression level of the gene under investigation.

Real time PCR has several advantages when compared with semi-quantitative PCR. Firstly because the level of amplicon is recorded at the end of each cycle this allows relative quantification to be carried out easily without the need for optimization experiments. Furthermore real time PCR is far more sensitive in that it can detect down to a 2 fold change in gene expression compared to agarose quantification which can only detect down to a 10 fold change. Gene expression values also control for the amount of

cDNA added to the reaction so that these quantities only reflect the levels of expression of the gene of interest.

3.2.1.2 Gene expression changes relative to day 0

In these experiments *Runx1*, *Scl*, *Gata2* and *Lmo2* expression was analysed at day 0, day 3 and day 6. *Gapdh* was used as an internal standard. *Gapdh* has been tested as an internal standard in differentiating ES cells and was found to remain relatively constant in comparison with HPRT and β-tubulin (Murphy & Polak 2002).

Data from 3 independent experiments is shown in Figure 3.2.2 and in the PCR tables in Appendix 1. The raw PCR data for experiment ES5 is also in Appendix 1. The data showed variability however some general trends were observed.

There were no statistically significant changes in *Runx1*, *Scl*, *Gata2* and *Lmo2* expression although the expression of each of these genes were generally at higher levels at day 3 and day 6 relative to day 0 under all culture conditions (see Appendix 1).

In experiment ES2 there were quite dramatic increases in *Lmo2* expression at day 6. Because these changes were present in all conditions it was not thought to be anomalous. ES cells are known to differentiate haphazardly and it is possible that in this experiment the cells were more predisposed to expanding an *Lmo2* expressing population of cells. *Lmo2* is expressed in cells of the erythroid and megakaryocytic lineage (Warren et al.

1994). This increase in expression may have reflected an expansion of this population however further analysis is required to prove this.

3.2.1.3 Gene expression changes dependent on culture condition

There were no differences between the serum replacement and BMP4 culture conditions. The lack of any clear difference between the BMP4 treated sample and the serum replacement samples was in contrast to previous evidence from experiments performed in ES cells suggesting that BMP4 is involved in up regulating the expression of these genes.

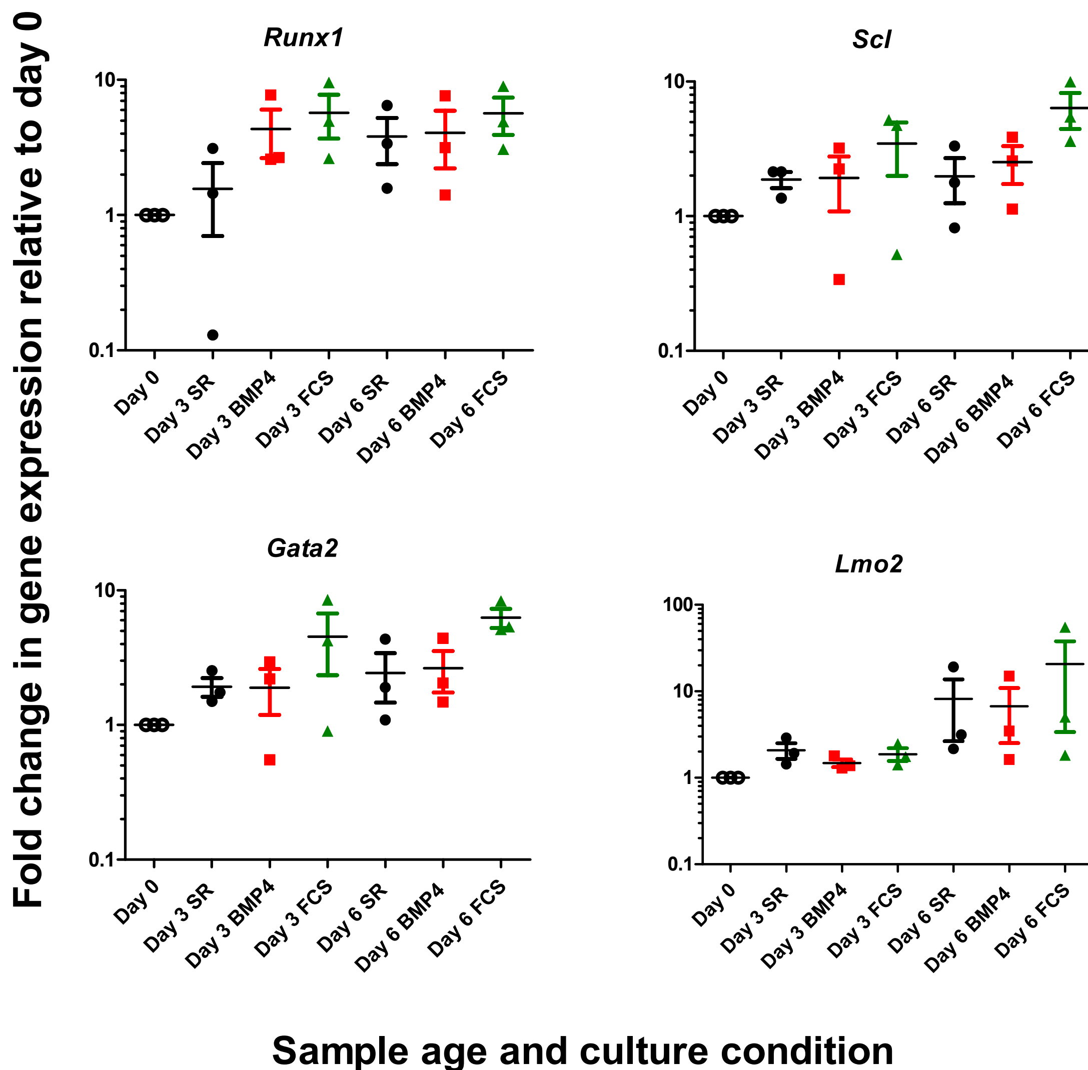


Figure 3.2.2 ES differentiation PCR results
Runx1, *Scl*, *Gata2* and *Lmo2* expression in EBs at day 0, day 3 and day 6. EBs were cultured in serum replacement (SR), with BMP4 and with serum (FCS). These logarithmic graphs show expression values standardised against a *Gapdh* control and are relative to Day 0. Results are from 3 independent experiments. The horizontal bars represent the mean and the standard error of the data set.

3.2.1.4 Flow Cytometry

Flow cytometry offers a rapid method for analysing the cell surface markers expressed on cells within a particular sample. Using different antibodies it is possible to perform analysis for the presence of multiple cell surface markers at one time. In addition flow cytometry can detect the size and granularity of cells using the forward scatter and side scatter characteristics of a cell. Different blood lineages can be distinguished using this method. Furthermore dead cells can be gated out of the analysis. For these reasons flow cytometry was the method of choice for analysing the expression of cell surface markers in these experiments.

EB cells were dissociated and labelled with a stem cell and differentiation panel of antibodies as described in section 2.2.2.1 at day 0, day 3 and day 6. The stem cell panel consisted of antibodies to CD34, Sca-1, Flk-1 and C-kit. The differentiation panel of antibodies consisted of antibodies to CD45, Ter119, CD11b and CD19. Data from 5 independent experiments is presented in the flow cytometry results tables in Appendix 1.

3.2.1.5 Stem Cell Markers

Figure 3.2.3 shows data from the stem cell marker analysis experiments and Figure 3.2.4 shows flow cytometry plots from a representative experiment (ES5).

Under serum replacement conditions at day 6 there was an increase in Flk-1 positive cells ($P<0.05$) when compared with day 0 levels. Overall however the trends were similar between serum replacement and BMP4 conditions. There was no statistically significant difference in stem cell marker expression between serum replacement and BMP4 conditions. This was surprising as BMP4 is well documented to cause an increase in the expression of Flk-1 expressing cells (Park et al. 2004).

Under serum conditions at day 6 there was an increase in Flk-1 expressing cells ($P<0.05$) relative to day 0.

The percentage of CD34 expressing cells was higher at day 6 ($P<0.05$) under serum conditions compared with serum replacement conditions. Flk-1 expressing cells were also higher at day 6 under serum conditions relative to serum replacement conditions ($P<0.05$). This most likely reflected the composition of factors in the serum promoting the differentiation of Flk-1 and CD34 positive populations.

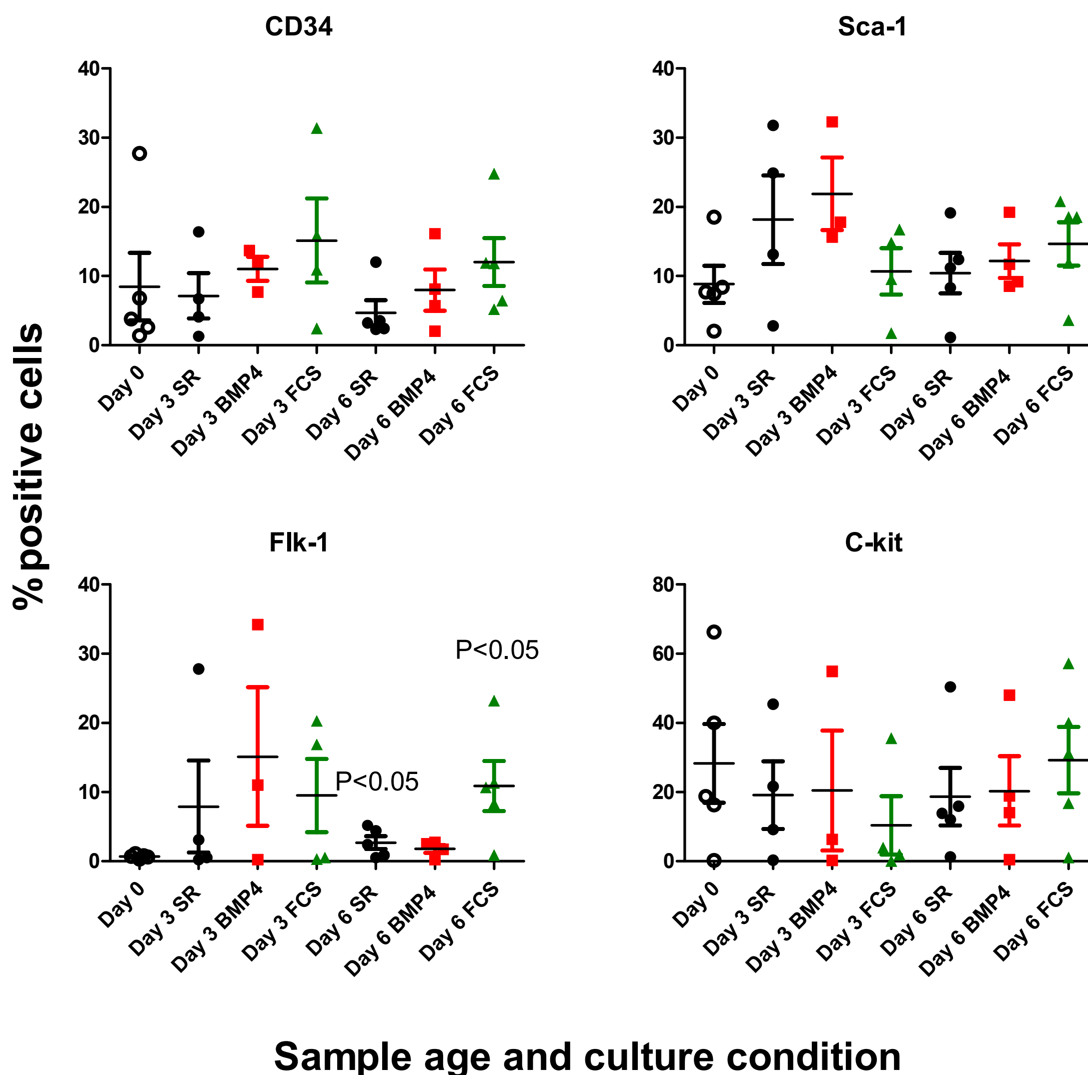


Figure 3.2.3 Flow cytometry analysis of stem cell surface markers
Stem cell markers: CD34, Sca-1 Flk-1 and C-kit expressed on EB cells at day 0 and after 3 and 6 days of culture with serum replacement media (SR), with BMP4 and with serum (FCS). Data is from 5 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

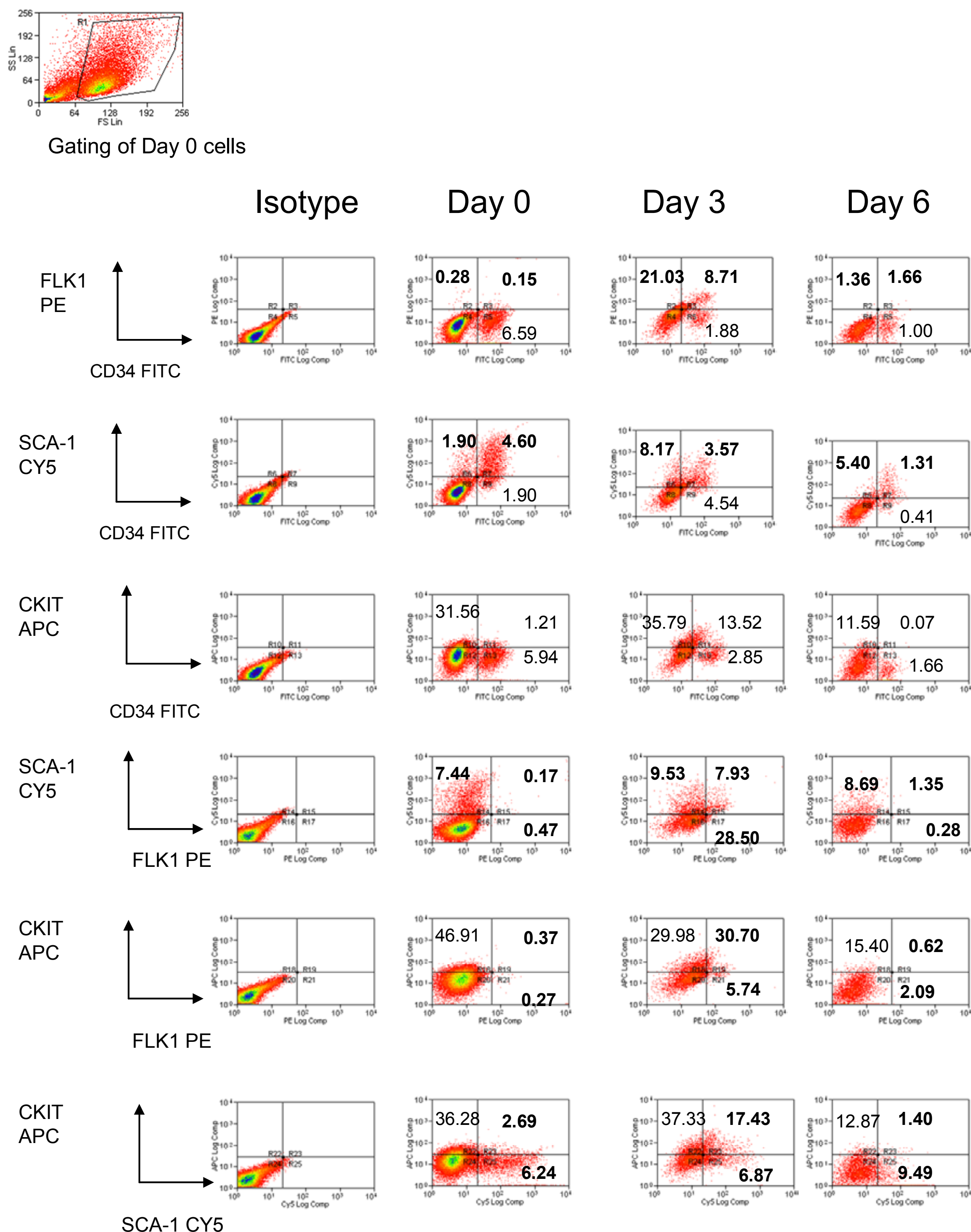


Figure 3.2.4 Representative stem cell panel flow cytometry plots
These plots from experiment ES5 show changes in stem cell markers for BMP4 culture conditions only at day 3. Day 0 data is included for comparison. Numbers indicate the percentage of cells in each quadrant. ES5 was chosen as a representative experiment as it best illustrated the general pattern of results.

3.2.1.6 Differentiation Markers

Figure 3.2.5 shows data from the differentiation marker analysis experiments and Figure 3.2.6 shows flow cytometry plots from a representative experiment (ES5).

Generally there were low levels of CD45, Ter119, CD11b and CD19 expressing cells under all conditions at Day 3 and Day 6. The low levels of differentiation were as expected given that haematopoietic differentiation only commences between day 3 and 6 (Park et al. 2004).

There were no statistically significant difference in differentiation marker expression between the serum replacement and BMP4 conditions. There was therefore no evidence for BMP4 promoting differentiation to any of the blood lineages.

Under serum conditions there was an increase in CD11b expressing cells at day 6 relative to day 0 ($P<0.05$). Serum conditions may promote myeloid differentiation.

Results from experiment ES5 at day 3 showed dramatically elevated CD19 positive cells under all conditions despite day 0 levels being similar to the other experiments. This once again is probably due to the haphazard differentiation of ES cells in this case promoting a CD19 positive cell population.

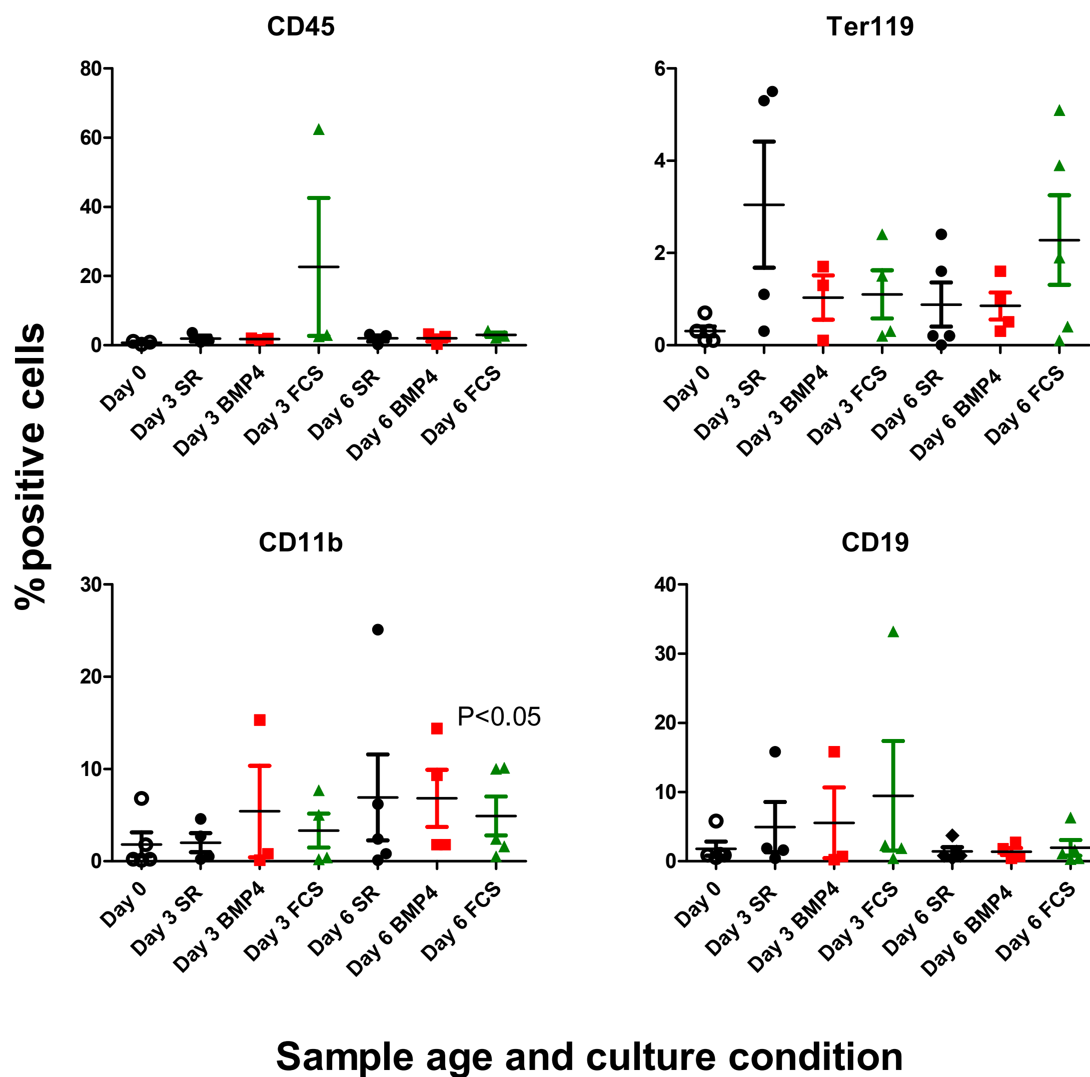


Figure 3.2.5 Flow cytometry analysis of differentiation markers
Differentiation markers: CD45, Ter119, CD11b and CD19 expressed on EB cells at day 0 and after 3 and 6 days of culture with serum replacement media (SR), with BMP4 and with serum (FCS). Data is from 5 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

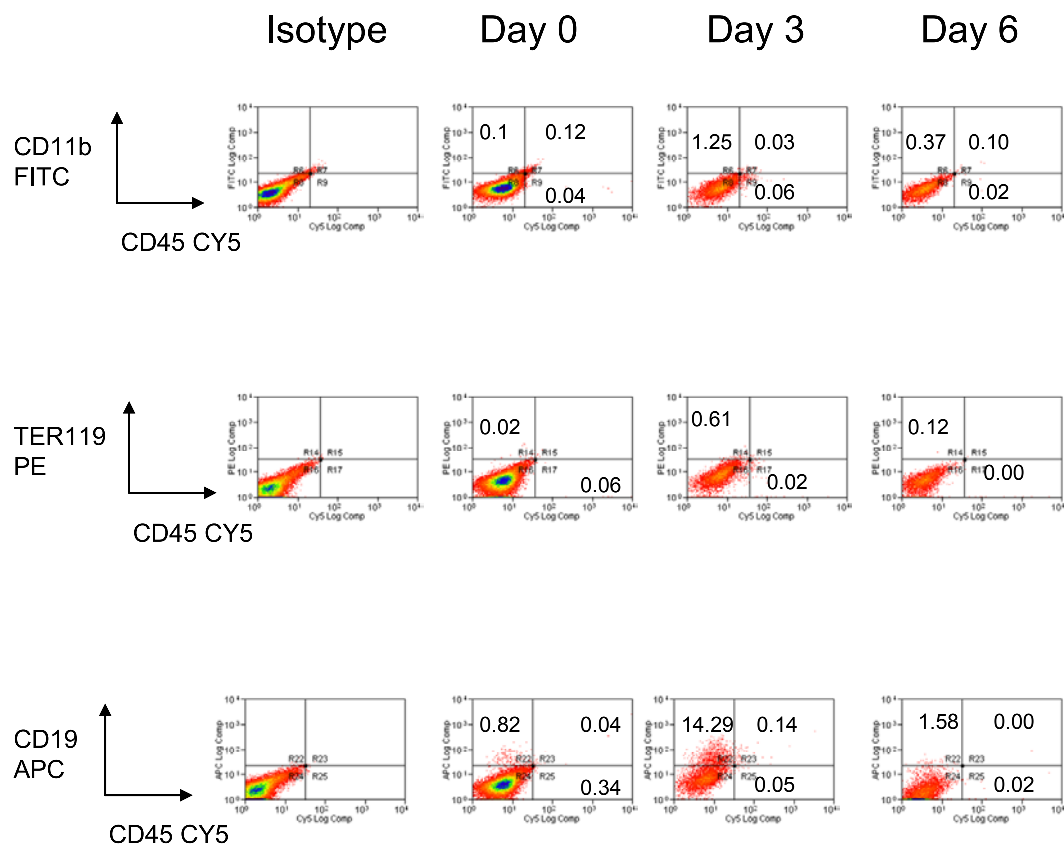


Figure 3.2.6 Representative differentiation panel flow cytometry plots
These plots from experiment ES5 show changes in differentiation markers for BMP4 culture conditions only at day 3. Day 0 data is included for comparison. Numbers indicate the percentage of cells in each quadrant. ES5 was chosen as a representative experiment as it best illustrated the general pattern of results.

3.2.2 Discussion of ES differentiation results

These initial experiments in the ES cell system were aimed at identifying trends that might be worth further investigation in both gene expression data and cell surface marker analysis resulting from the addition of BMP4.

Despite previous data, these experiments did not provide any evidence that BMP4 has a role in increasing the number of Flk-1 expressing cells or in the up regulation of the expression of *Runx1*, *Scl*, *Lmo2* and *Gata2*. This may have been due to the concentration of BMP4 used in the experiments. The concentration of 5ng/ml was chosen as this was the concentration that had been used in previous experiments for optimal haematopoietic differentiation (Park et al. 2004;Lugus et al. 2007). However these papers used R1 ES cells and it is possible that CCE ES cells respond to different concentrations of BMP4.

Another potential reason for the absence of any changes with the addition of BMP4 may have been the endogenous production of BMP4 by ES cells during differentiation.

In these experiments the data suggested that serum provided optimal conditions for haematopoietic differentiation. The increase in CD34 and Flk-1 positive cell populations was greatest under serum conditions. There was also an increase in CD11b expressing cells at day 6 under serum conditions.

Overall there were few markers for differentiated cells even at day 6. This is as expected as haematopoietic differentiation only begins between day 3 and day 6 (Park et al. 2004).

In both the gene expression and flow cytometry analysis inconsistent and dramatic results were produced in individual experiments. *Lmo2* expression in experiment ES2 and CD19 in experiment ES5 were dramatically increased over all culture conditions in contrast to the results achieved in the other experiments. This demonstrates the variability of ES cell differentiation and probably reflects the disorganisation of the developmental processes which are occurring in the embryoid bodies.

3.3 Foetal liver haematopoietic stem cells: the effect of BMP4

Little is known about the haematopoietic environment of the foetal liver except that there is a large expansion of HSCs in the murine foetal liver between 12-15dpc. Myelosupportive stromal cell lines have been derived from the foetal liver. However the molecular basis of HSC expansion in the foetal liver has yet to be identified.

These experiments sought to investigate whether BMP4 might play a role in the foetal liver environment. For this purpose foetal livers were obtained from 14.5dpc CD1 mouse embryos. Cells were then dissociated and grown in culture for a period of 3 days.

Culture conditions for growing foetal liver cells were optimised in serum and serum free conditions. In order to investigate the effect of BMP4 it was essential to develop serum free conditions in which a defined set of growth and survival factors could be added. In these primary cultures it proved difficult to develop serum free conditions which would allow sufficient survival after 3 days. In RPMI medium there was significant cell death and insufficient cells remained for analysis. However the use of CellGro medium with β-mercaptoethanol improved viability (data not shown). CellGro medium is a proprietary mixture based on DMEM with smaller amounts of RPMI and McCoys 5A medium containing minimal protein so as to facilitate serum free survival and experiments to determine the effects of added growth factors.

Cells were cultured serum free, with serum (10% FCS) and with BMP4 (10ng/ml) as described in Section 2.1.3. The concentration of 10ng/ml was chosen as this is within the range of concentrations used in studies of haematopoietic development in murine systems (Johansson & Wiles 1995). In experiments FL5 and FL6 BMP receptor culture conditions were added. BMP receptor was added at a concentration of 200ng/ml to antagonise the effects of BMP4. This concentration of BMP receptor has previously been shown to block the colony forming potential of CD34/c-kit^{high}cells derived from the AGM region (Marshall et al. 2007).

Samples were taken at 0 and 3 days for PCR, flow cytometry and methylcellulose assays. As before the aim was to analyse whether there were any affects on gene expression and how this correlated with cell types present measured by cell surface marker analysis in flow cytometry and colony forming potential in methylcellulose assays.

After 3 days there was some cell death under serum free and BMP4 culture conditions with cell viability at 60%. Cells grown under serum conditions however had 80-90% viability. $3x10^6$ cells were plated out at day 0 and under serum conditions there were similar cell numbers at day 3 but under BMP4 and serum free conditions there was on average $1.2x10^6$ live cells at day 3.

3.3.1 PCR results

The expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* was analysed. *Gapdh* was used as an internal standard. *Gapdh* has previously been used in foetal liver real time gene expression studies as an internal standard (Jochheim et al. 2004).

Data from 4 independent experiments is shown in Figure 3.3.1 and in the PCR tables in Appendix 1.

Under BMP4 conditions compared with day 0 values *Runx1*, *Scl*, *Gata2* and *Lmo2* expression remained at a similar level at day 3. Also under serum free conditions the expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* did not significantly change at day 3 compared with day 0. There were no significant differences between gene expression in day 3 serum free and BMP4 samples.

The pattern of gene expression under serum conditions after 3 days was similar to that under serum free conditions. There were no significant differences between day 3 serum and serum free samples.

The BMP receptor experiments showed at day 3 when compared with the BMP4 experiments a reduction in *Runx1*, *Scl*, *Gata2* and *Lmo2* expression (see Foetal liver PCR tables in Appendix 1). The reduction in gene expression was below the levels of even the

serum free conditions in experiment FL5 indicating that BMP4 may be produced by the cells. However further experiments are needed to clarify these results.

The pattern of changes was similar in serum free, BMP4 and serum conditions. This indicated that neither the addition of BMP4 nor the addition of serum changed the genetic response of these cells to being placed in culture.

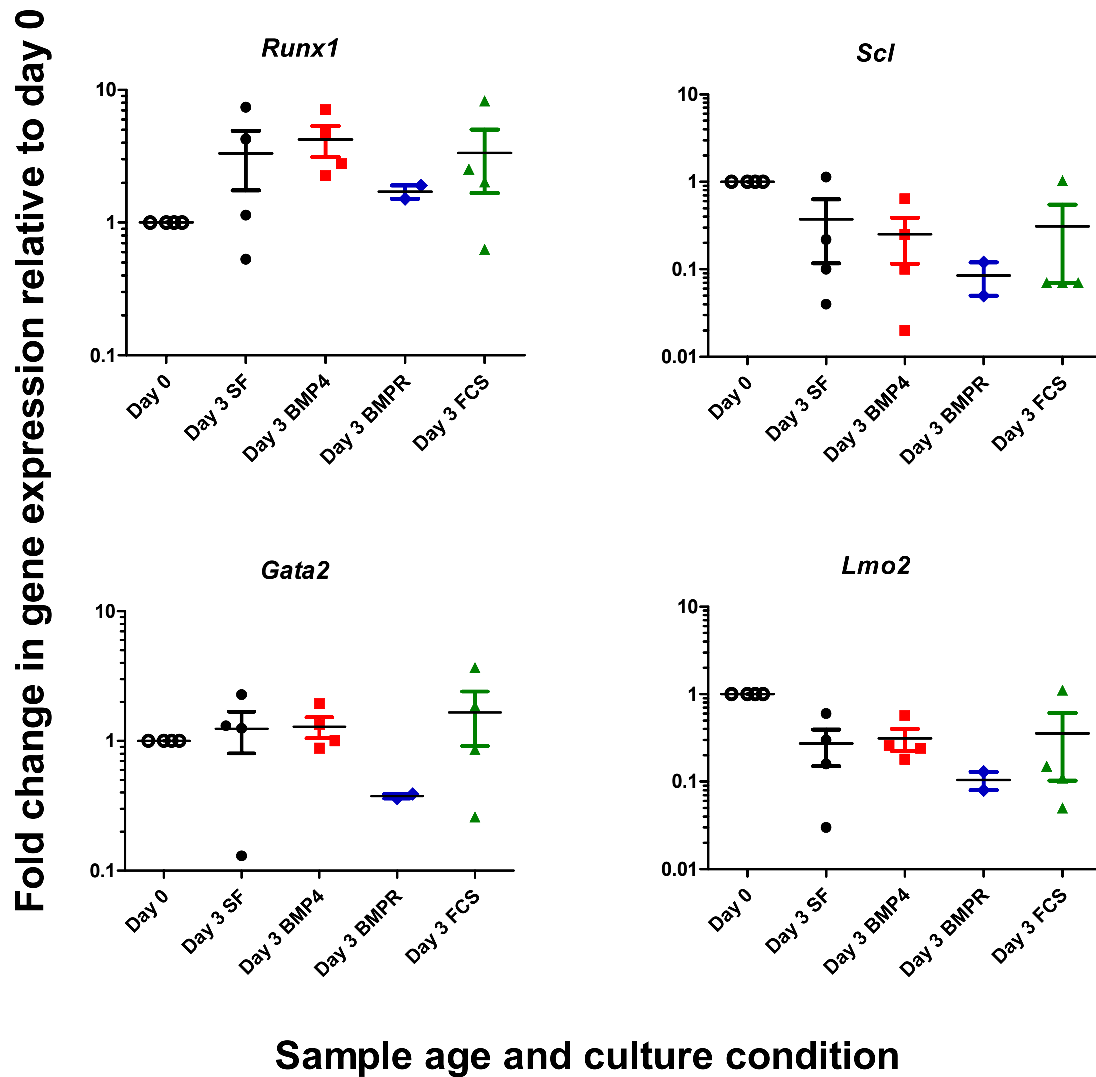


Figure 3.3.1 Foetal liver PCR results
Runx1, *Scl*, *Gata2* and *Lmo2* expression in unsorted foetal liver cells after 3 days in culture serum free (SF), with BMP4, with BMP receptor (BMPR) and in foetal calf serum (FCS). These logarithmic graphs show expression values relative to Day 0. Results are from 4 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

3.3.2 Flow Cytometry

Data from 6 experiments is presented in the flow cytometry results tables in Appendix 1.

3.3.2.1 Stem Cell Markers

Figure 3.3.2 shows data from the stem cell marker analysis experiments and Figure 3.3.3 shows flow cytometry plots from a representative experiment (FL5).

The pattern of change in stem cell marker expression was the same regardless of culture conditions.

At day 3 under BMP4 conditions there were similar levels of CD34, Sca-1, Flk-1 and C-kit expressing cells relative to day 0. Under serum free conditions there was an increase in Sca-1 expressing cells at day 3 relative to day 0 ($P<0.05$) although there were no statistically significant differences in stem cell marker expression between serum replacement and BMP4.

There were no significant increases in stem cell marker levels under serum conditions at day 3 relative to day 0. There were also no differences in comparison with the other culture conditions.

Also the addition of BMP receptor did not make any notable differences to stem cell marker expression at day 3 compared with the other culture conditions (see Foetal liver flow cytometry tables in Appendix 1).

The similarities observed in stem cell marker expression after 3 days in serum free, BMP4 and serum culture conditions suggests that the stem cells had the same response to being placed in culture regardless of culture condition.

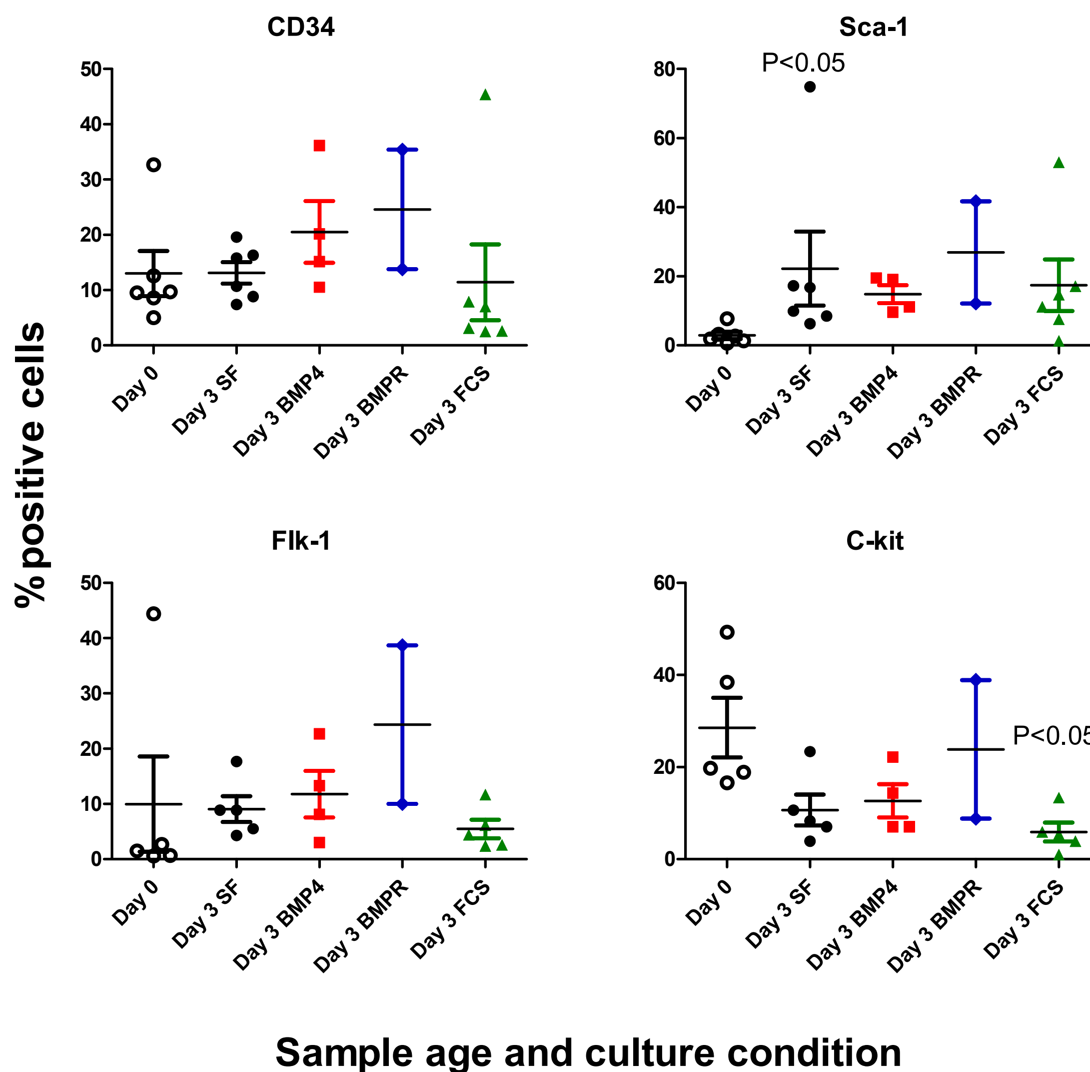


Figure 3.3.2 Flow cytometry analysis of stem cell markers
Stem cell markers CD34, Sca-1, C-kit and Flk expressed on unsorted foetal liver cells at day 0 and after 3 days of culture without serum (SF), with BMP4, with BMP receptor (BMPR) and with serum (FCS). Results are from 6 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

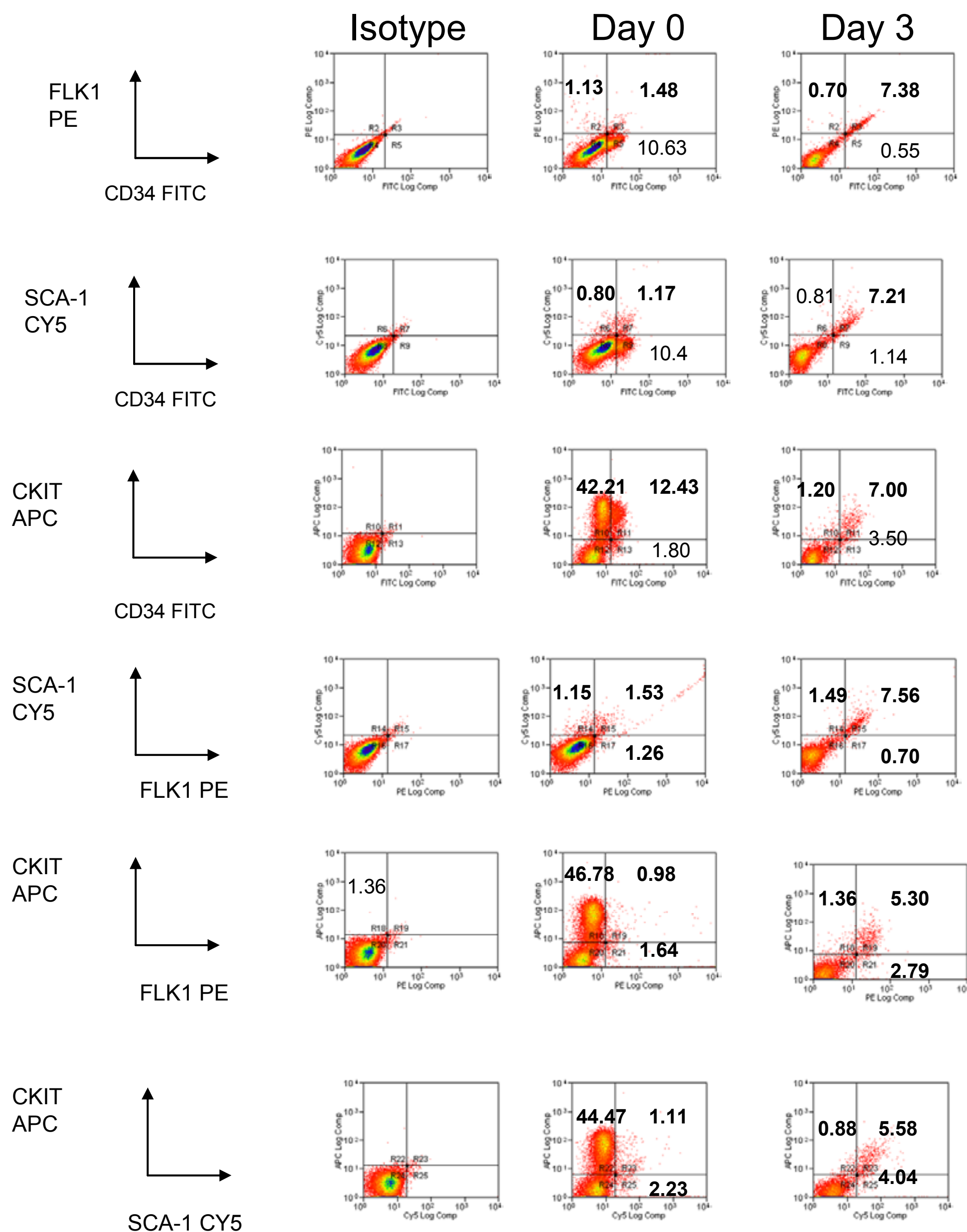


Figure 3.3.3 Representative stem cell panel flow cytometry plots
Flow cytometry plots from experiment FL5 showing changes in stem cell markers for BMP4 culture conditions only at day 3. Day 0 data is included for comparison. Numbers indicate the percentage of cells in each quadrant. FL5 was chosen as a representative experiment as it best illustrated the general pattern of results.

3.3.2.2 Differentiation Markers

Figure 3.3.4 shows data from the differentiation marker analysis experiments and Figure 3.3.5 shows flow cytometry plots from a representative experiment (FL5).

There were no significant changes in the levels of CD45, Ter119 and CD19 after 3 days relative to day 0 under any of the culture conditions. However there were significant increases in CD11b expressing cells relative to day 0 under serum free and serum conditions ($P<0.05$). Increases in CD11b expressing cells suggest that myeloid differentiation may be occurring.

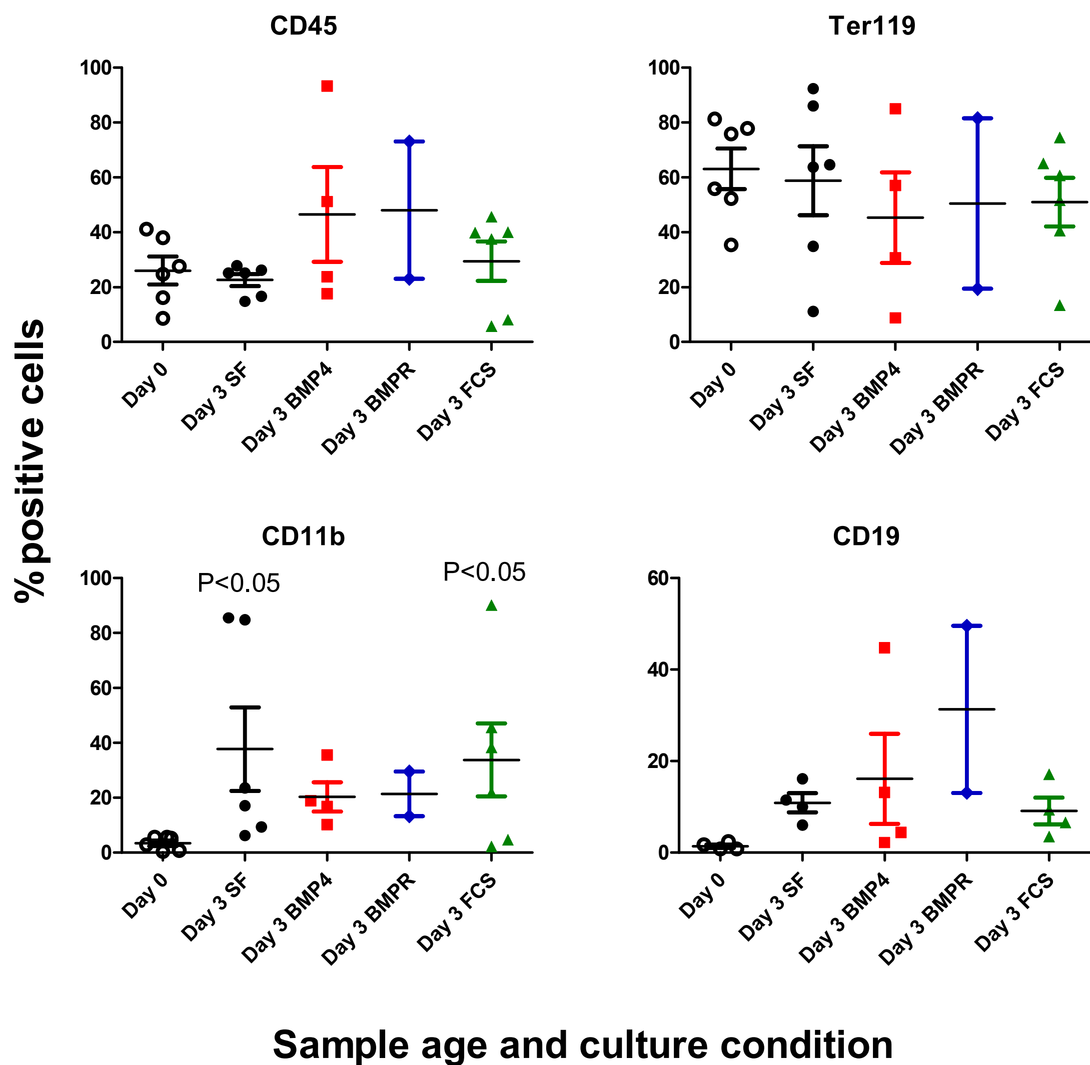


Figure 3.3.4 Flow cytometry analysis of differentiation markers
Differentiation markers: CD45, Ter119, CD11b and CD19 expressed on unsorted foetal liver cells at day 0 and after 3 days of culture without serum (SF), with BMP4, with BMP receptor (BMPR) and with serum (FCS). Results are from 6 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

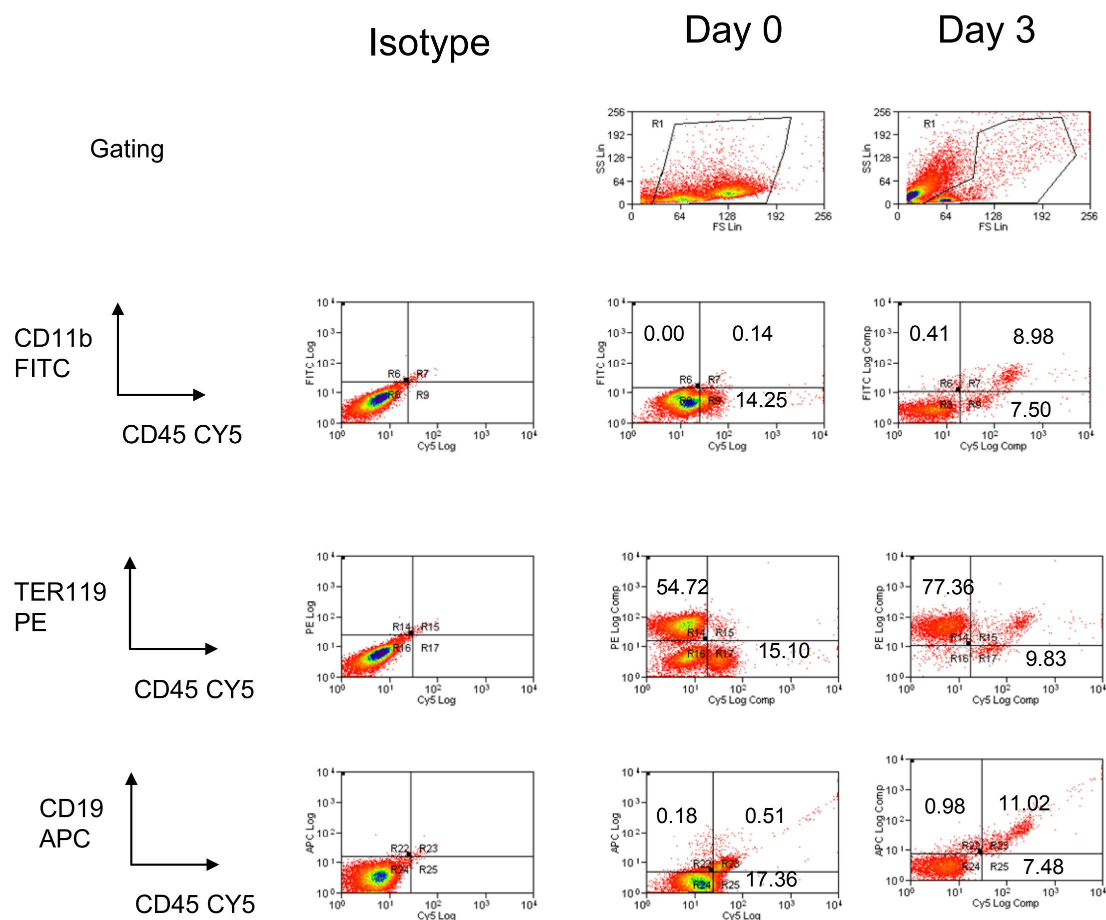


Figure 3.3.5 Representative differentiation panel flow cytometry plots
Flow cytometry plots from experiment FL5 showing changes in differentiation markers for BMP4 culture conditions only at day 3. Day 0 data is included for comparison. Numbers indicate the percentage of cells in each quadrant. FL5 was chosen as a representative experiment as it best illustrated the general pattern of results.

3.3.3 Colony Forming Unit Assays

Methylcellulose colony assays as described in Section 1.1.3.2.1 can be used to determine the number of multipotent and lineage restricted progenitors present in a sample. In this experiment samples were taken at day 0 and after culturing cells for 3 days in serum free, BMP4, BMP receptor and serum culture conditions. These cell samples were then placed in methylcellulose culture as described in Section 2.2.3.1. After 14 days visible colonies were counted and classified based on the mature cells present within the colony.

Colonies only formed consistently at day 3 from serum cultured samples (see Figure 3.3.6 and Table 3.3.1). At day 3 there were fewer colonies than at day 0. This suggests both serum free and BMP4 culture conditions either did not support the survival of progenitors or had caused differentiation of progenitors.

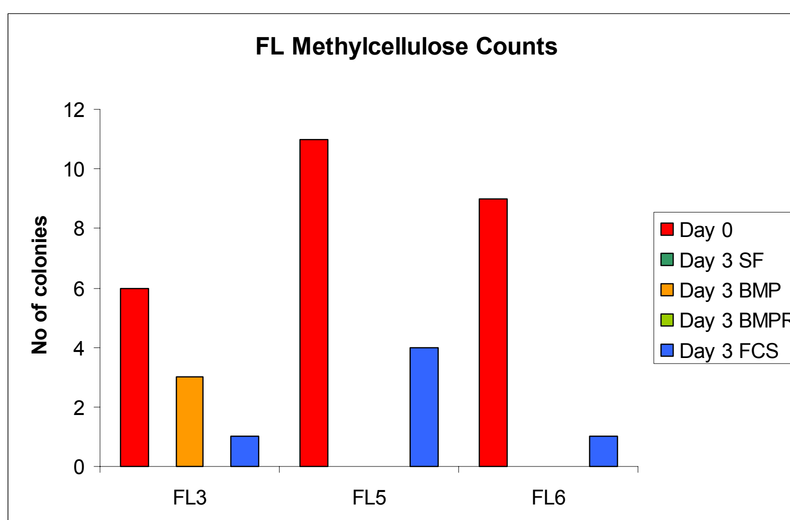


Figure 3.3.6 Methylcellulose colony assay results
Bar chart illustrating total number of haematopoietic colonies from samples of 1500 cells taken at Day 0 and after 3 days in culture serum free, with BMP4 and in foetal calf serum. Methylcellulose cultures were incubated for 2 weeks.

FL3	Total No. of colonies	GM colonies	M Colonies
Day 0	6	2	4
Day 3 SF	0	0	0
Day 3 BMP4	3	3	0
Day 3 FCS	1	1	0

FL5	Total No. of colonies	GM colonies	M Colonies
Day 0	11	11	0
Day 3 SF	0	0	0
Day 3 BMP4	0	0	0
Day 3 BMPR	0	0	0
Day 3 FCS	4	4	0

FL6	Total No. of colonies	GM colonies	M Colonies
Day 0	9	9	0
Day 3 SF	0	0	0
Day 3 BMP4	0	0	0
Day 3 BMPR	0	0	0
Day 3 FCS	1	1	0

Table 3.3.1 Methylcellulose assay tables
Tables show the total number of colonies and the constituent types of haematopoietic colonies obtained under each culture condition in 3 independent experiments. GM = Granulocyte macrophage and M = Macrophage

3.3.3.1 Cytospins

Cytospins allowed the type of cells e.g. granulocyte-macrophage present in the different colonies present to be determined.

Two different types of colony were identified in methylcellulose cultures. Colonies were identified by morphology as granulocyte macrophage colonies or macrophage colonies (Figure 3.3.7). Samples of the two types of colony were taken to confirm their identity. A cytospin was performed after which cells were stained using May Grumwald Giemsa stain as described in Section 2.2.3.3. Under a light microscope the cells were examined

(Figure 3.3.7). The granulocyte macrophage colonies were found to contain immature macrophages and granulocytes whilst the macrophage colony contained more mature macrophages.

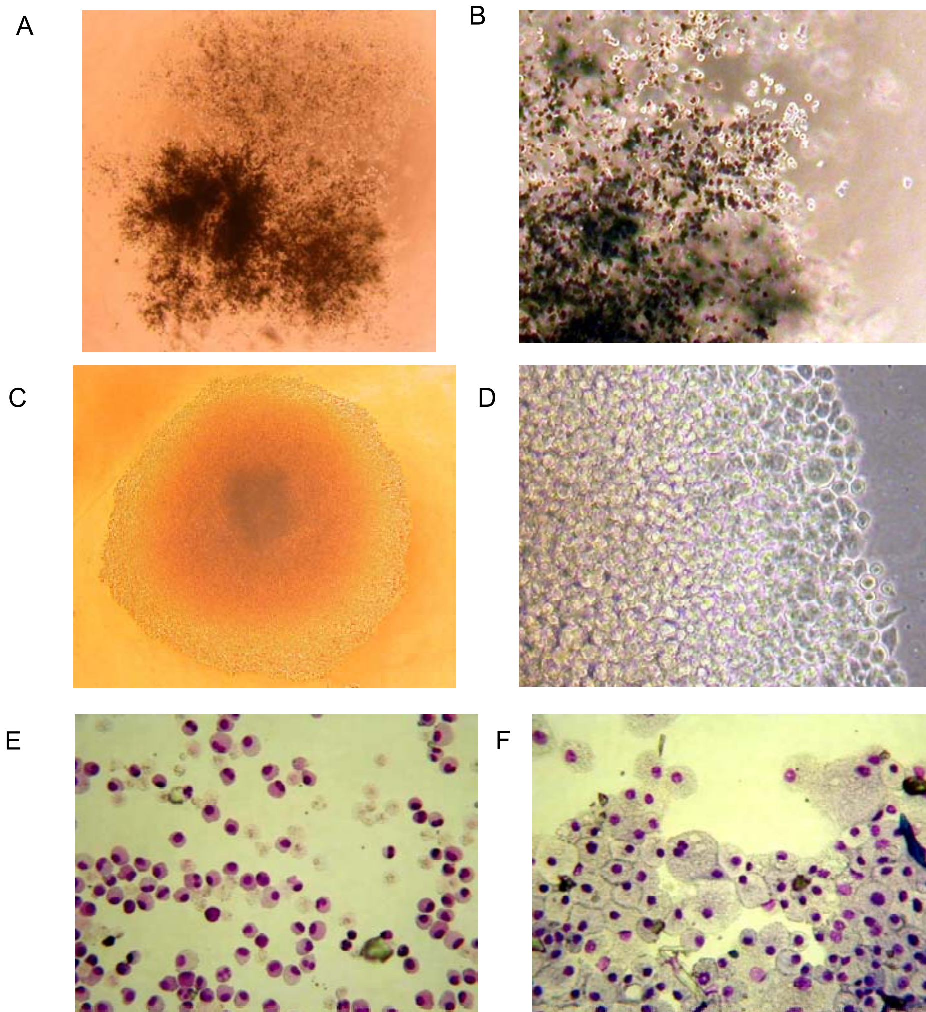


Figure 3.3.7 Colony micrographs and Cytospin Analysis
Micrographs showing granulocyte macrophage colonies (A and B) and the mature macrophage colony (C and D) obtained in methylcellulose cultures. Micrographs are at x4 (A and C) and x10 (B and D) magnification. Micrographs E and F (x20 magnification) show cells stained using May Grumwald Giemsa. stain from granulocyte-macrophage colonies (E) and mature macrophage colonies (F).

3.3.4 Discussion

The results in both gene expression and flow cytometry experiments showed the same pattern of change in serum free, BMP4 and serum conditions. The changes in gene expression and cell surface markers therefore reflect the response of the cells to culture *ex vivo*.

Cell death particularly under BMP4 and serum free conditions was high. Haematopoietic progenitors may either have died or have differentiated after 3 days and this is reflected in the results of the methylcellulose assays. If further experiments were to be performed in this system it would be necessary to further improve cell survival under serum free conditions.

There was variability in the results. This may reflect cell death and variations in the initial population.

There was some evidence suggesting that BMP receptor culture conditions reduced expression of *Runx1*, *Scl*, *Gata2* and *Lmo2*. These results suggest that the cells were producing BMP4 and BMP4 may have a role in inducing these genes. Future experiments should titrate the effects of BMP receptor to verify these results.

3.4 Bone Marrow Haematopoietic Stem Cells: The effect of BMP4

The aim of these experiments was to investigate the effect of BMP4 on mouse bone marrow derived HSCs. Previous research has investigated the role of BMP4 on mouse bone marrow derived haematopoietic progenitors. However the results of these experiments were conflicting. One experiment suggested BMP4 caused an increase in erythroid colony forming potential whilst the other experiment provided evidence contradicting this (Fuchs et al. 2002;Maguer-Satta et al. 2003). In human bone marrow derived HSC culture, BMP4 is said to increase HSC survival and engraftment of NOD-SCID mice (Bhatia et al. 1999). These experiments sought to characterise the effects of BMP4 and investigate whether these effects are mediated by an up regulation of the developmentally important genes: *Runx1*, *Scl*, *Gata2* and *Lmo2*.

Bone marrow was collected from the femurs of CD1 mice. It was necessary to find serum free conditions which maintained adequate survival. Initially RPMI medium was used however the viability of cells after 3 days was insufficient. The use of CellGro medium with β-mercaptoethanol improved viability.

The experimental set up was the same as with the foetal liver cells. Briefly, cells were cultured serum free, with BMP4 and with serum (10% FCS). BMP4 was added at 10ng/ml as this is within the range of concentrations used in studies of haematopoietic

development in murine systems (Johansson & Wiles 1995). In experiments BM6 and BM7 a BMP receptor culture condition was added. BMP receptor was added at 200ng/ml. This concentration was the same as used in the foetal liver studies.

Although there was 90% viability at day 0, there was between 40-50% viability at day 3 in BMP4 and serum free cultured samples. However in serum cultured samples the viability at day 3 was 70-80%. In order to increase cell survival TPO and IGF1 growth factors were added into serum free and BMP4 culture conditions. As a result cell viability after 3 days of culture increased to between 70-80% in BMP4 and serum free cultured samples. The cell numbers at day 3 were similar to those at day 0.

Samples were taken for analysis by PCR, flow cytometry and colony forming potential at day 0 and day 3.

3.4.1 PCR Results

Real time PCR was carried out to determine expression of *Runx1*, *Scl*, *Gata2* and *Lmo2*. *Gapdh* was used as an internal standard. *Gapdh* has previously been used as an internal standard in bone marrow real time gene expression studies (Gallagher et al. 2003). Data from 3 independent experiments is shown in Figure 3.4.1 and the PCR tables in Appendix 1.

Under serum free conditions after 3 days there were no significant changes in *Runx1*, *Scl* *Gata2* or *Lmo2* expression. Similarly under BMP4, BMP receptor and serum conditions

there were no significant changes in *Runx1*, *Scl, Gata2* or *Lmo2* expression. There were no statistically significant differences between culture conditions. The genetic response of cells was therefore the same regardless of culture condition.

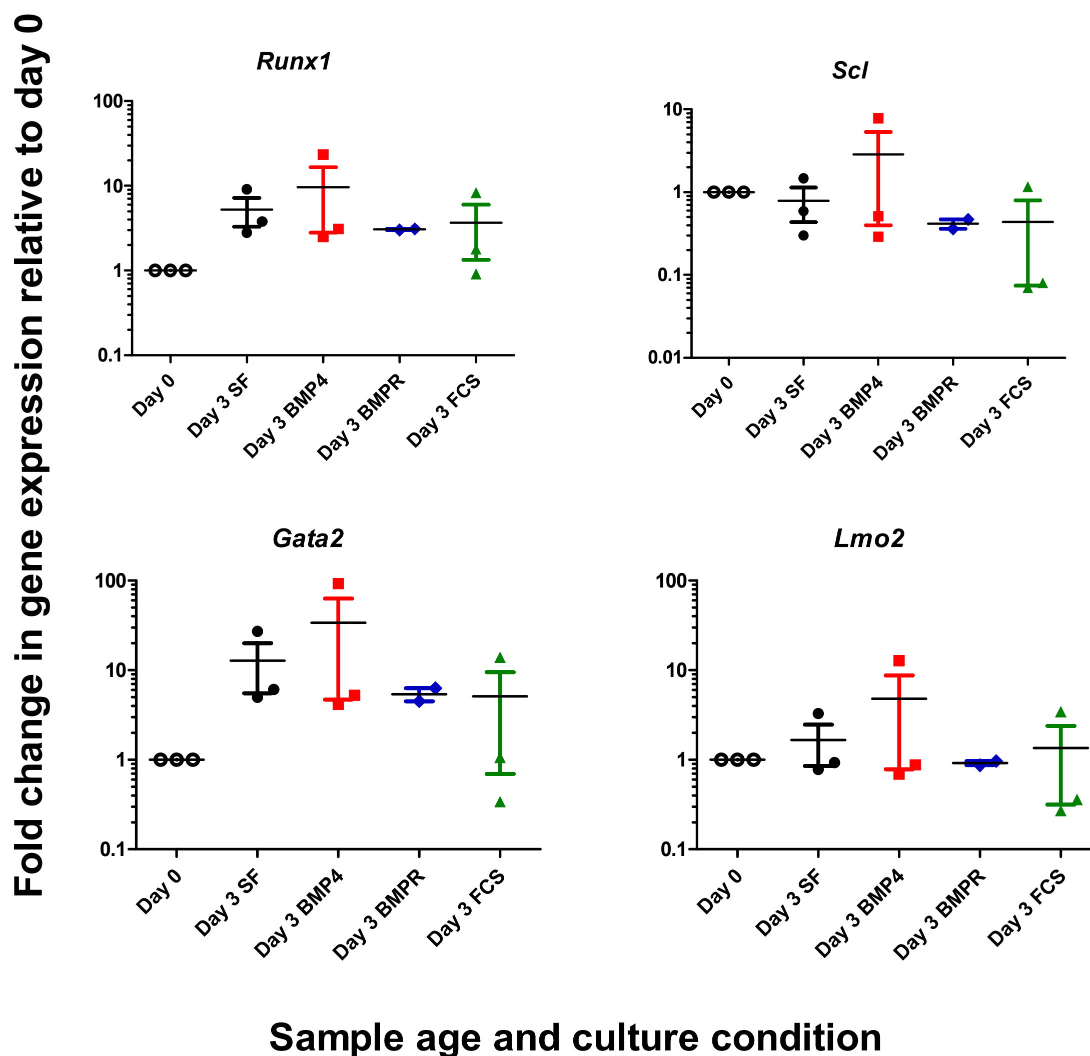


Figure 3.4.1 Bone Marrow PCR results
Runx1, *Scl*, *Gata2* and *Lmo2* expression in unsorted bone marrow cells after 3 days in culture serum free (SF), with BMP4, with BMP receptor (BMPR) and in foetal calf serum (FCS). These logarithmic graphs show expression values relative to day 0. Data shown is from 3 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

3.4.2 Flow Cytometry

Data from 3 experiments is presented in the flow cytometry results tables in Appendix 1.

3.4.2.1 Stem Cell Markers

Figure 3.4.2 shows results for the stem cell marker analysis experiments and Figure 3.4.3 shows flow cytometry plots from a representative experiment (BM6).

Under serum free conditions there were increases after 3 days in Sca-1 expressing cells ($P<0.05$) and C-kit expressing cells ($P<0.05$). The levels of CD34 and Flk-1 expressing cells however stayed about the same after 3 days.

Under BMP4 conditions the same pattern was seen with increases after 3 days in Sca-1 expressing cells ($P<0.05$) and C-kit expressing cells ($P<0.05$). However CD34 and Flk-1 expressing cells were at similar levels to those seen in serum free conditions after 3 days.

There were no significant differences at day 3 between serum replacement and BMP4 cultured cells. The increase in Sca-1 and C-kit expressing cells may suggest that there was an expansion of the HSC population in response to the serum free conditions.

With the addition of BMP receptor in experiment BM6, a reduction in CD34 and C-kit expressing cells occurred at day 3 (see Bone marrow flow cytometry results tables in

Appendix 1). Further experiments are required to see if this result can be repeated. However this result may provide an initial indication that BMP4 promotes the expression of stem cell markers CD34 and C-kit.

There were no changes seen in stem cell markers after 3 days under serum conditions. Levels of CD34, Sca-1, Flk-1 and C-kit all remained at similar levels at day 3 relative to day 0.

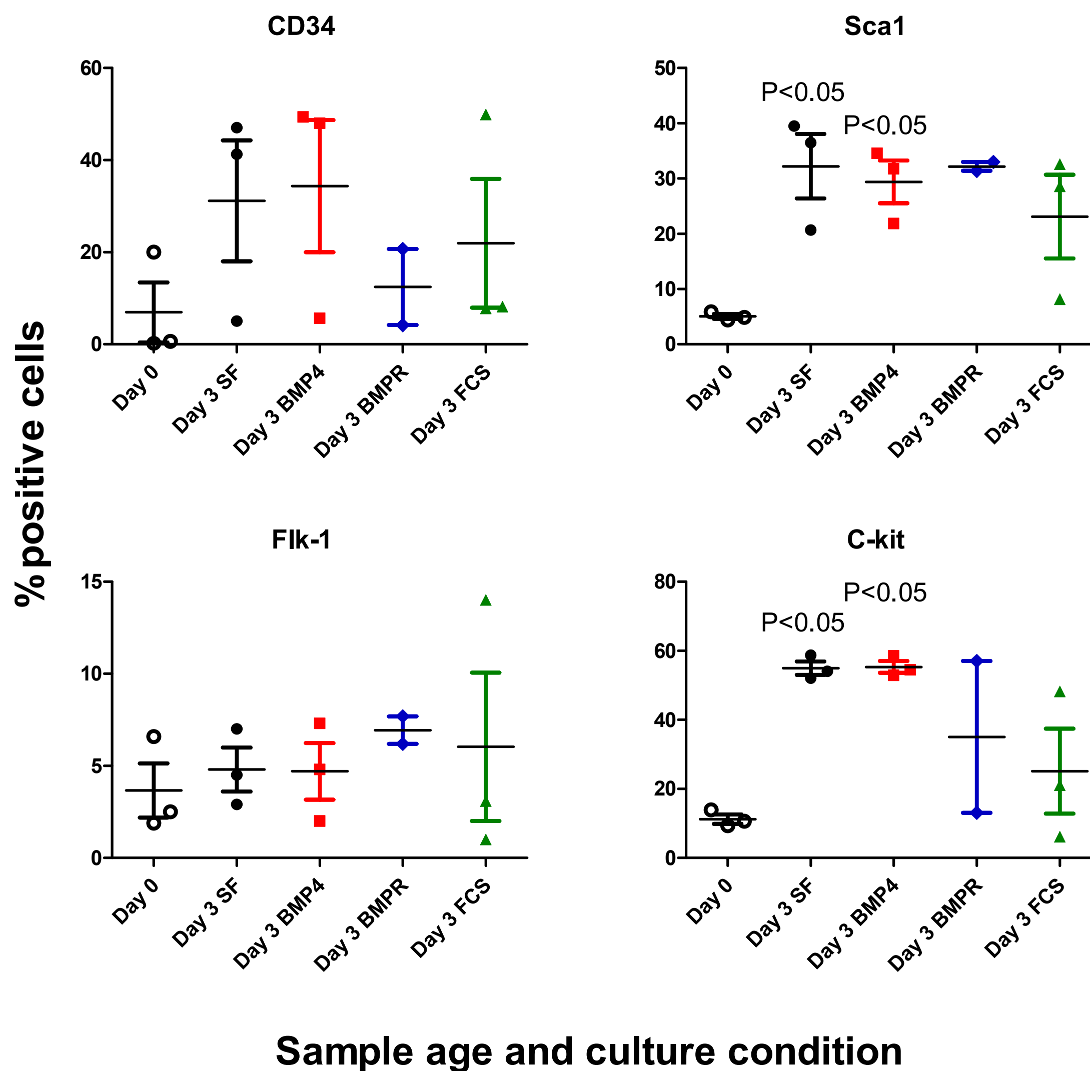


Figure 3.4.2 Flow cytometry analysis of stem cell markers
Stem cell markers: CD34, Sca1, C-kit and Flk-1 expressed on unsorted bone marrow cells at day 0 and after 3 days of culture serum free (SF), with BMP4, with BMP receptor (BMPR) and with serum (FCS). Results are from 3 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

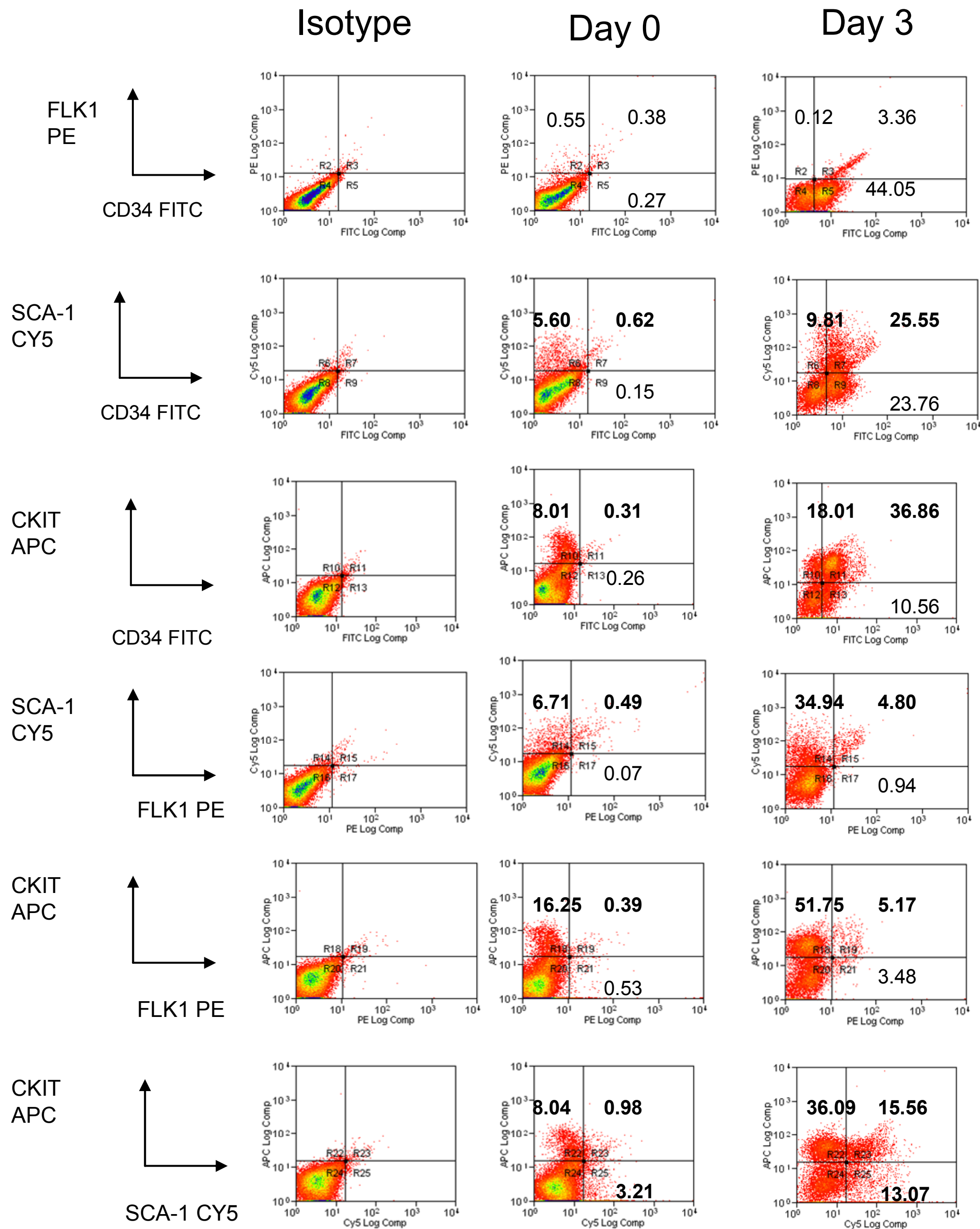


Figure 3.4.3 Representative stem cell panel flow cytometry plots
Flow cytometry plots from experiment BM6 showing changes in stem cell markers for BMP4 culture conditions only at day 3. Day 0 data is included for comparison. Numbers indicate the percentage of cells in each quadrant. BM6 was chosen as a representative experiment as it best illustrated the general pattern of results.

3.4.2.2 Differentiation Markers

Figure 3.4.4 shows results for the differentiation marker analysis experiments and Figure 3.4.5 shows the flow cytometry plots from a representative experiment (BM6).

Under serum free, BMP4 and BMP receptor conditions the same pattern of changes in differentiation markers was seen. There was a decrease in Ter119 expressing cells over 3 days ($P<0.05$). Serum free conditions may have been inadequate to support the survival of Ter119 expressing cells. CD45, CD11b and CD19 expressing cells however remained at similar levels.

There were no statistical differences between serum free and BMP4 conditions. BMP4 therefore did not increase the differentiation of any particular lineage after 3 days in culture.

Cells grown under serum conditions had similar levels of CD45, CD11b, Ter119 and CD19 expressing cells after 3 days relative to day 0.

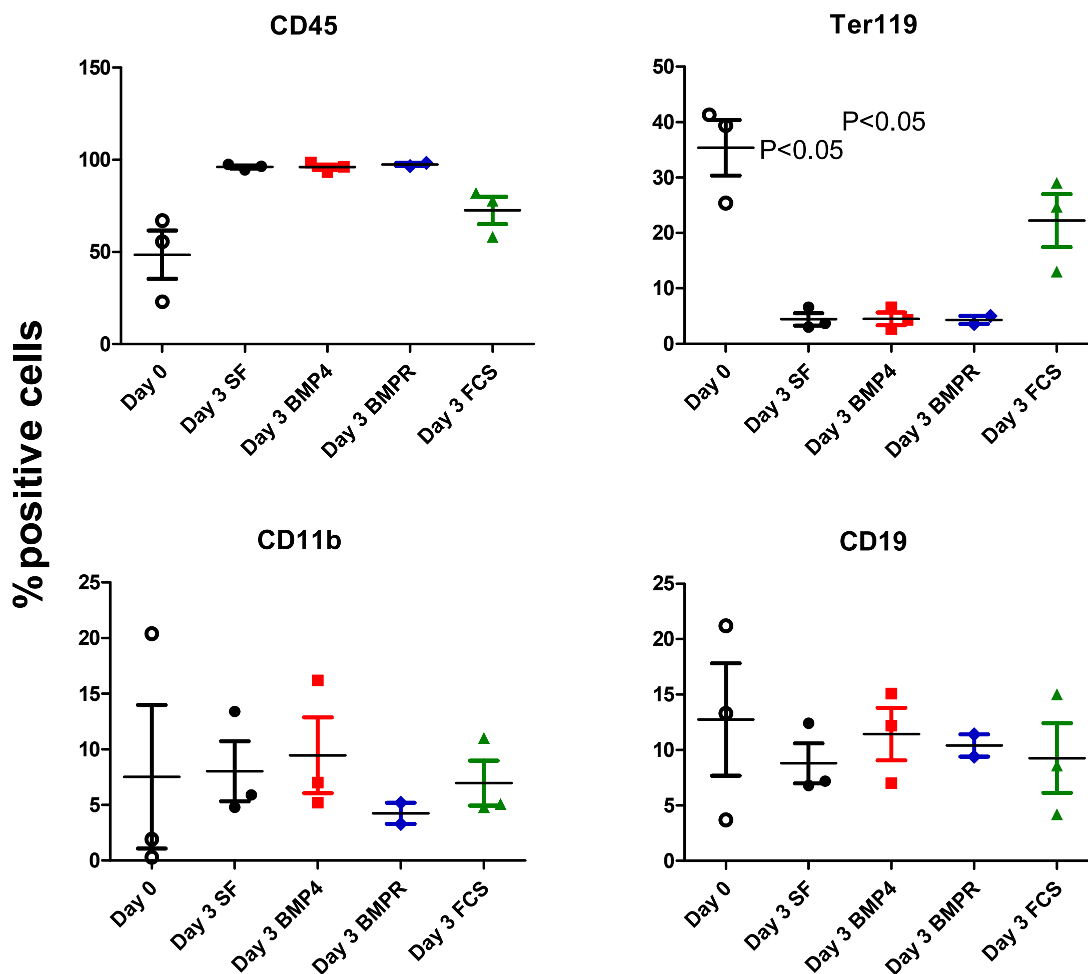


Figure 3.4.4 Flow cytometric analysis of differentiation markers
Differentiation markers: CD45, Ter119, CD11b and CD19 on unsorted bone marrow cells at day 0 and after 3 days of culture serum free (SF), with BMP4, with BMP receptor (BMPR) and with serum (FCS). Results are from 3 independent experiments. The horizontal bars represent the mean and the standard error of the data set. P values are shown where significant changes have occurred relative to day 0.

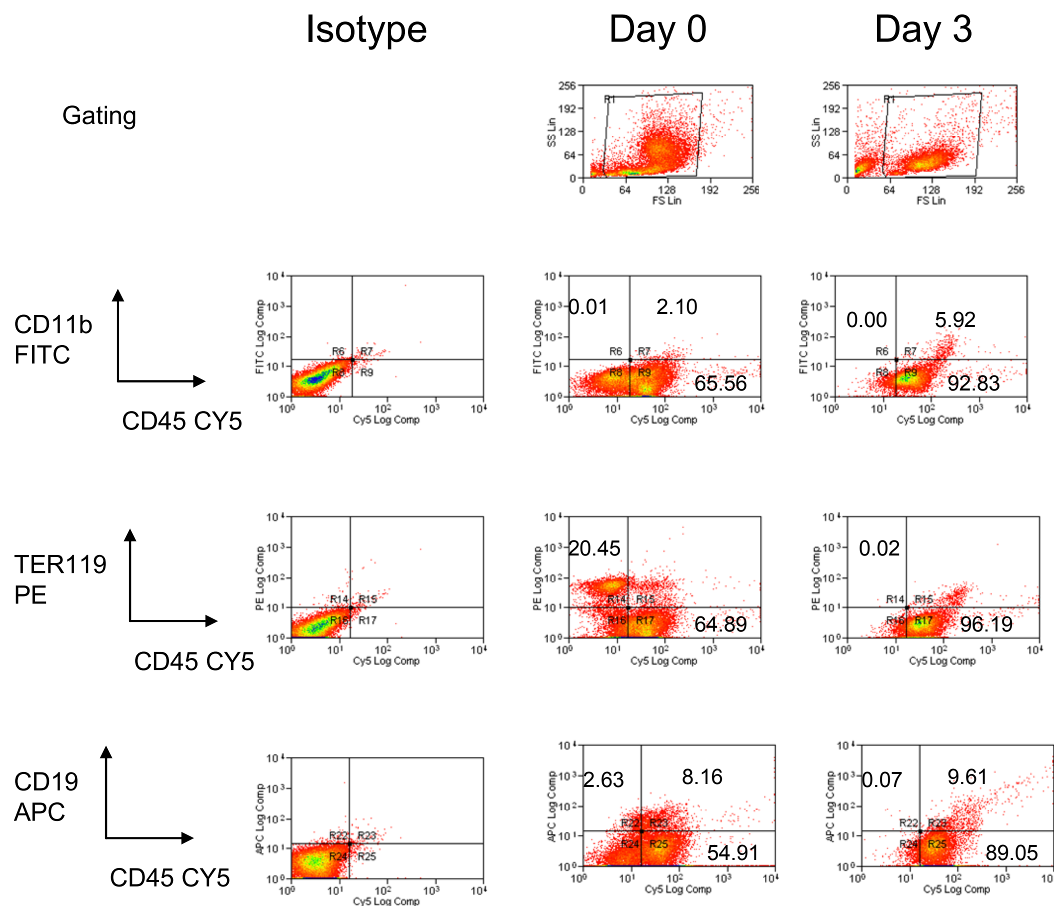


Figure 3.4.5 Representative differentiation panel flow cytometry plots
Flow cytometry plots from experiment BM6 showing changes in differentiation markers for BMP4 culture conditions only at day 3. Day 0 data is included for comparison. Numbers indicate the percentage of cells in each quadrant. BM6 was chosen as a representative experiment as it best illustrated the general pattern of results.

3.4.3 CFU assays and Cytospins

3.4.3.1 CFU assays

Samples were taken at day 0 and day 3 from each culture condition and were placed in methylcellulose culture as described in Section 2.2.3.1.

There were large increases in colonies under serum free and BMP4 conditions after 3 days (Figure 3.4.7). The addition of BMP4 to this mixture from the trends observed in experiments BM5 and BM6 did not cause a significant change in the numbers of haematopoietic progenitors.

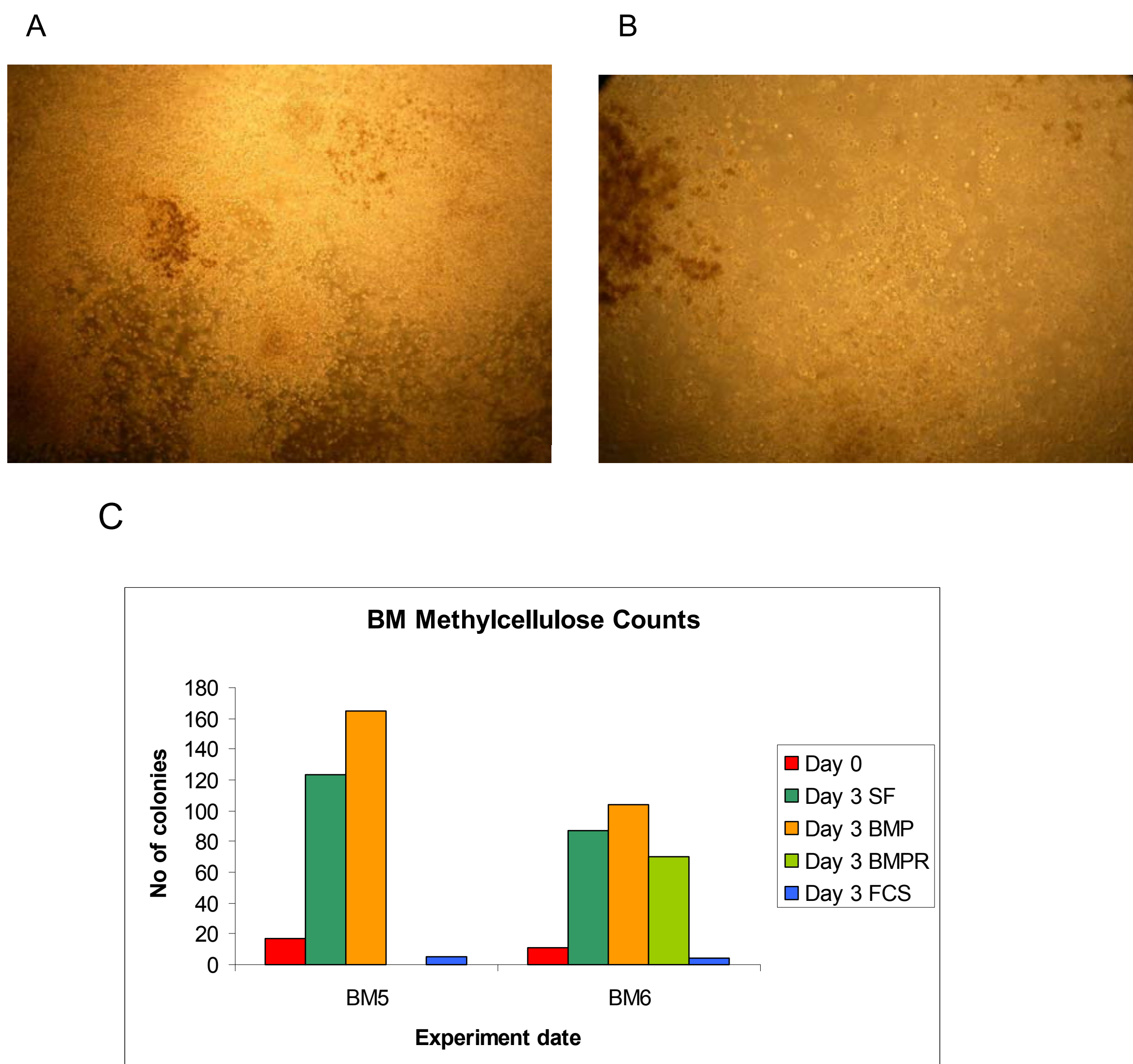


Figure 3.4.6 Methylcellulose colony results
Micrographs illustrating the dense colony formation obtained in methylcellulose cultures from Day 3 Serum Free and BMP4 samples after 2 weeks incubation. Micrographs are at x4 (A) and x10 (B) magnification. Bar chart (C) shows total number of haematopoietic colonies from samples of 1500 cells taken at Day 0 and after 3 days in culture serum free, with BMP4 and in foetal calf serum. BMP receptor culture conditions were added in BM6.

3.4.3.2 Cytospins

Two different types of colonies were present in methylcellulose cultures (Figure 3.4.8). The majority were identified visually as granulocyte macrophage colonies. Another type of colony was also seen. To confirm the nature of these two colony types cytospins were

performed and the cells were stained with May Grumwald Giemsa stain as described in Section 2.2.3.3. Under a light microscope the cells were examined (Figure 3.4.8). The colonies initially identified as granulocyte macrophage colonies actually contained macrophages and earlier cells including myeloblasts and erythroblasts. The other type of colony contained mature macrophages.

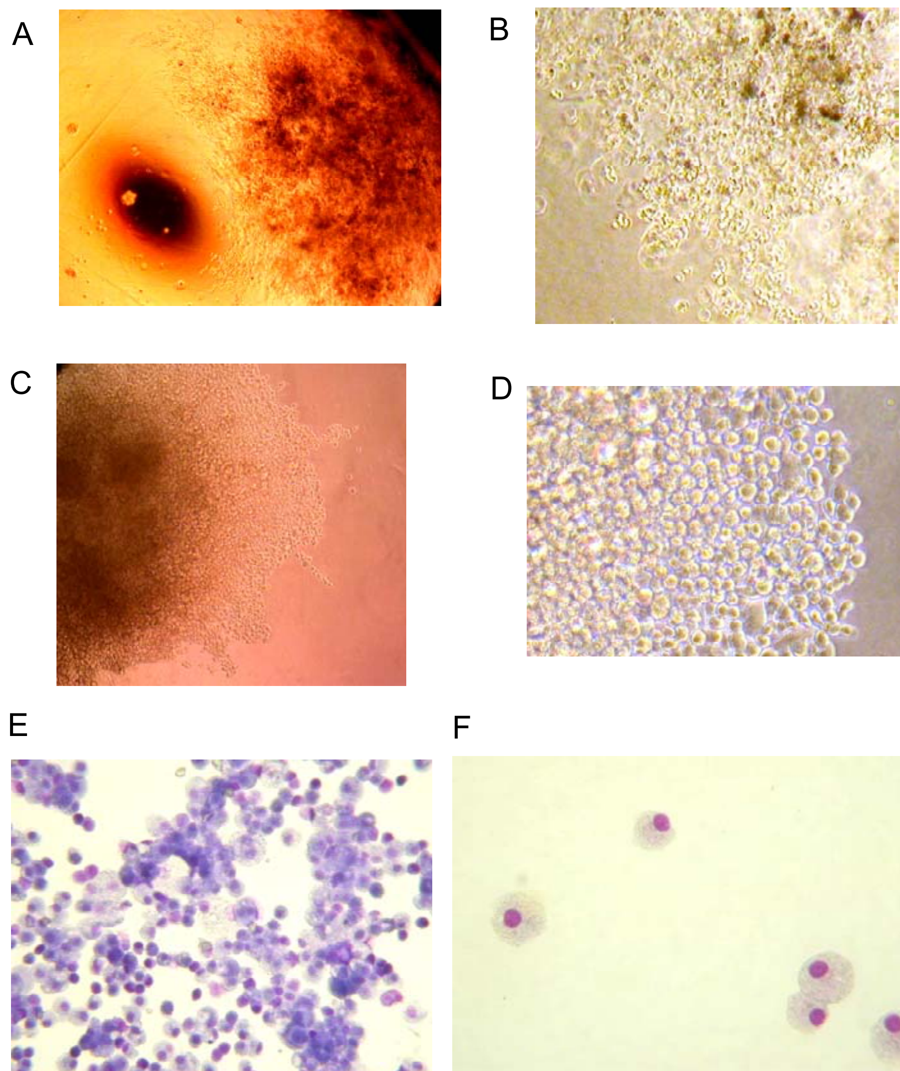


Figure 3.4.7 Colony micrographs and cytospin analysis
Micrographs showing granulocyte macrophage colonies (A and B) and the mature macrophage colony (C and D) obtained in methylcellulose cultures. Micrographs are at x4 (A and C) and x10 (B and D) magnification. Micrographs (x20 magnification) showing cells stained using May Grumwald Giemsa stain from granulocyte-macrophage colonies (E) and the macrophage colonies (F).

3.4.4 Discussion

Similar patterns were seen in PCR and flow cytometry in serum free and BMP4 conditions. There was a trend for an increase in Sca-1 and C-kit progenitors above day 0 levels under serum free and BMP4 conditions.

Further BMP receptor experiments are required to confirm if BMP receptor does reduce levels of CD34 and C-kit expressing cells as seen in BM6. BMP receptor did not show any effects in PCR analysis. This may have been due to the concentration of BMP receptor being too low. A titration of BMP receptor concentrations should be performed.

There was variability in the results probably due to variability in the bone marrow obtained which can be seen in the day 0 cell surface markers. In addition endogenous BMP4 produced by these variable populations of cells may have interfered with analysis. This may account for the similarities observed in the serum free and BMP4 culture conditions.

In experiment BM5 experiment there were large increases in *Runx1*, *Scl, Gata2* and *Lmo2* expression with the addition of BMP4. One distinguishing feature from flow cytometry was a high percentage of CD34 expressing cells in the initial population (20%). Clearly in this experiment a BMP4 responsive population was collected which was not present in the other repeat experiments. This illustrates the difficulty in drawing conclusions caused by the variability in the initial cell population.

3.5 Investigating the effect of BMP4 on a CD34 C-kit sorted bone marrow derived haematopoietic cell population

The viability of bone marrow cells remained high after 3 days with the addition of IGF1 and TPO. However there were concerns that the inconsistent initial population collected was causing variability in results. In order to resolve this, a specific cell population was sorted from collected bone marrow. The CD34 C-kit positive population was chosen as results from previous BMP receptor experiments had indicated that this population might be responsive to BMP4.

Bone marrow was collected from CD1 mice and then sorted for CD34 C-kit positive cells (Figure 3.5.1). The CD34 positive cells were also collected. In each experiment approximately 800,000 CD34 C-kit positive cells were collected and 500,000 CD34 positive cells were collected. These cells, along with S17 stromal cells which were included as a control, were then cultured in serum free conditions (with TPO and IGF1), with BMP4 (10ng/ml) and with BMP receptor (200ng/ml).

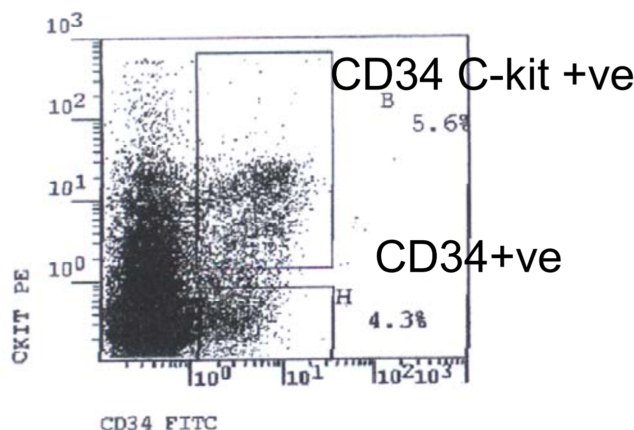


Figure 3.5.1 Cell sorting of adult murine bone marrow
FACS plot shows the populations of CD34 +ve and CD34 C-kit +ve cells that were sorted from adult murine bone marrow.

Cells were collected for analysis after 3 days by flow cytometry for CD34 C-kit positive cells and by PCR for *Gata2* expression only. This was because experiment BM5 had previously indicated that BMP4 may up regulate *Gata2* expression.

Cell survival was poor (Table 3.5.1). Unsorted bone marrow cells may contain cell populations that secrete growth factors and provide cell-cell interactions that support the survival of haematopoietic cells. The absence of these populations may have caused the increased levels of cell death.

At day 3 in $CD34^{+}C\text{-}kit^{+}$ cells	**Number of live cells**	**Viability at day 3**
Experiment 1	4.0×10^{5}	69%
Experiment 2	3.7×10^{5}	67%
Experiment 3	4.8×10^{5}	72%

Table 3.5.1 Cell survival table Cell survival at day 3 in CD34 C-kit positive cells in all 3 experiments.

In CD34 C-kit positive sorted cells there was a large reduction in CD34 C-kit expressing cells to between 20-30% of initial levels after 3 days (P<0.05, Figure 3.5.2 and Table 3.5.2). This occurred under all culture conditions. However the CD34 sorted cells, which were at day 0 CD34 C-kit negative, showed an increase in CD34 C-kit positive cells of between 20-30% to similar levels to that occurring in the CD34 C-kit positive cultured cells. This pattern of change occurred regardless of culture condition.

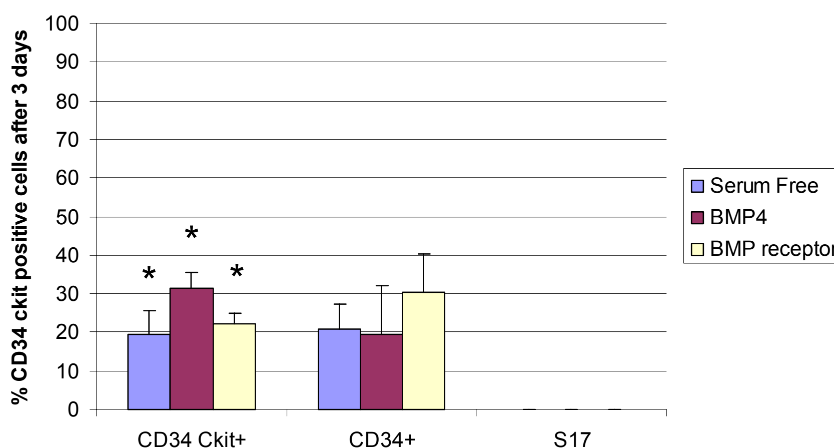


Figure 3.5.2 Effect of BMP4 on a CD34 C-kit expressing cells in sorted populations
CD34 +ve, CD34 C-kit +ve and S17 stromal cells were sorted and cultured. Graph shows the percentage of CD34 C-kit positive cells in each population after 3 days of culture under serum free, BMP4 and BMP receptor conditions. Data represents the average data from 3 experiments. Error bars represent the standard error. * indicates significant change in percentage of CD34 Ckit +ve cells relative to day 0 at P<0.05. At day 0 there was 100% CD34 C-kit +ve cells in the CD34+ C-kit population and 0% CD34 C-kit +ve cells in the CD34 population.

	CD34 Ckit+			CD34+			S17		
	SF	*BMP4*	*BMPR*	*SF*	*BMP4*	*BMPR*	*SF*	*BMP4*	*BMPR*
EXP 1	15.83	23.37	16.62	13.94	12.9	17.68	0	0	0
EXP 2	10.51	34.1	23.98	33.77	43.73	50.06	0	0	0
EXP 3	31.67	36.49	26.03	14.71	1.92	23.5	0	0	0
AV	**19.34**	**31.32**	**22.21**	**20.81**	**19.52**	**30.41**	**0**	**0**	**0**
P	**0.01**	**0.03**	**0.02**	**0.08**	**0.26**	**0.09**			

Table 3.5.2 Effect of BMP4 on a CD34 C-kit expressing cells in sorted populations
Table shows the data for each experiment and the overall average. P values indicate statistical change relative to day 0.

Gata2 expression was dramatically reduced after 3 days in the CD34 C-kit positive population across all culture conditions (P<0.05, Figure 3.5.3 and Table 3.5.3). A smaller reduction in *Gata2* expression occurred in CD34 population but this again occurred under all culture conditions.

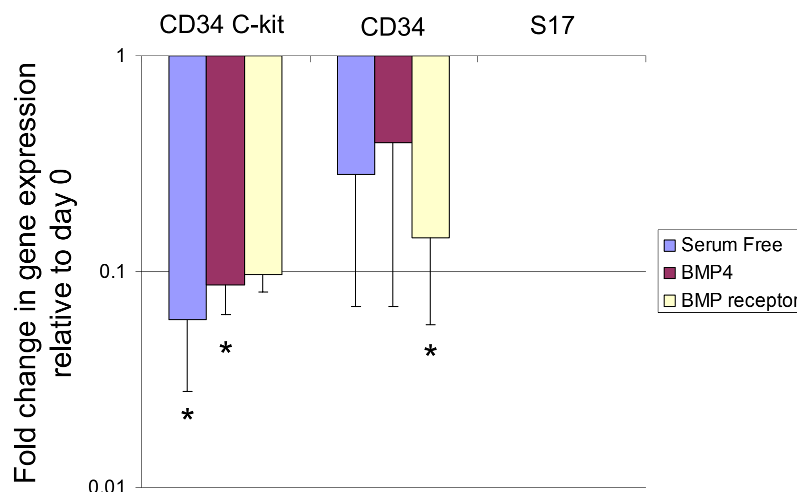


Figure 3.5.3 Effect of BMP4 on *Gata2* expression in sorted bone marrow populations
This is a logarithmic graph showing fold change in *Gata2* expression after 3 days in each sorted population. Data represents the average data from 3 experiments. Error bars represent the standard error. * indicates results significant at P<0.05.

	CD34 ckit			CD34			S17		
	SF	*BMP4*	*BMPR*	*SF*	*BMP4*	*BMPR*	*SF*	*BMP4*	*BMPR*
EXP 1	0.12	0.13	0.13	0.71	1.05	0.3	0	0	0
EXP 2	0.01	0.05	0.08	0.03	0.02	0	0	0	0
EXP 3	0.05	0.08	0.08	0.11	0.12	0.13	0	0	0
AV	**0.06**	**0.09**	**0.10**	**0.28**	**0.40**	**0.14**	**0**	**0**	**0**
P	**0.001**	**0.001**	**0.08**	**0.08**	**0.21**	**0.01**			

Table 3.5.3 Effect of BMP4 on *Gata2* expression in sorted bone marrow populations
Table shows the data for each experiment and the overall average. P values indicate statistical change relative to day 0.

The general pattern of change in CD34 C-kit positive cells and *Gata2* expression seems to reflect the response of cells to being placed in culture. Because cell survival was poor it is not possible to conclude whether BMP4 had any role to play in expanding a CD34 C-kit positive population or in up regulating *Gata2* expression.

3.6 Comparison of ES cell, Foetal Liver and Bone Marrow derived haematopoietic populations

A comparison of the haematopoietic populations derived from ES cells was made based on the PCR and flow cytometry data that was collected.

3.6.1 Gene Expression

Runx1, *Scl*, *Gata2* and *Lmo2* have all been shown to be essential to the development of the haematopoietic system. However there is little data on the relevance of these genes to the more mature haematopoietic populations of the foetal liver and bone marrow. Data from conditional gene targeting of *Scl* (Mikkola et al. 2003) and *Runx1* (Ichikawa et al. 2004) suggests that these genes are dispensable for adult haematopoietic function.

Previous experiments have shown that haematopoietic populations are formed in ES cells at day 6 under serum conditions. PCR data for day 0 bone marrow and foetal liver populations were therefore standardised against the haematopoietic population from ES

cells at day 6 under serum conditions. The level of gene expression was then compared (Figure 3.6.1).

The expression of *Scl* and *Lmo2* was at dramatically higher levels in both the foetal liver and bone marrow populations than in ES cells ($P<0.05$). Furthermore *Runx1* expression was also at higher levels in foetal liver and bone marrow populations than in ES cells ($P<0.05$). *Gata2* expression was however similar in all 3 populations.

The levels of expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* were similar in both the foetal liver and bone marrow populations.

Although a major role for these genes has been demonstrated in early development clearly they are expressed in the case of *Scl*, *Lmo2* and *Runx1* at higher levels in more mature populations. It seems likely therefore that these genes continue to have an important function at later stages of development and in the adult.

SCL and LMO2 are postulated to act in a complex with GATA factors in early development. The expression of both *Scl* and *Lmo2* at similarly high levels may suggest that these genes continue to act in a complex at later developmental stages and in the adult.

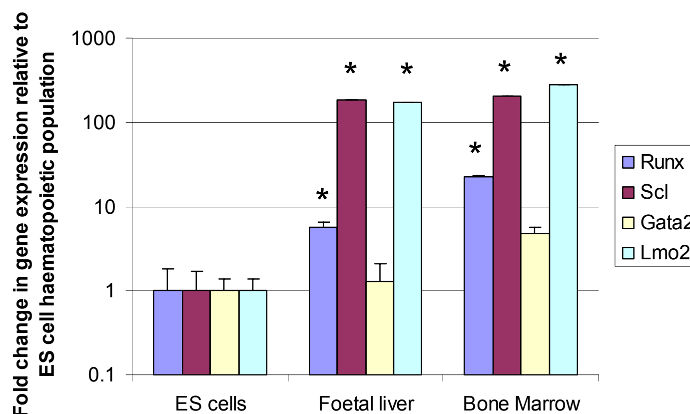


Figure 3.6.1 Gene expression comparison
A comparison of *Runx1*, *Scl*, *Gata2* and *Lmo2* gene expression in ES cells differentiated to day 6 in serum and day 0 cells from 14.5 dpc foetal livers and adult bone marrow. The results represent the average of the data collected. * indicates a statistically significant difference in gene expression relative to that in the ES cell population at P<0.05.

3.6.2 Stem Cell Markers

Bone marrow and foetal liver flow cytometry data show some differences in the initial populations (Figure 3.6.2). There were more Sca-1 positive cells in bone marrow than in foetal liver (P<0.05). However there were higher levels of C-kit expressing cells in the foetal liver (P<0.05).

ES cells after 6 days of differentiation in serum expresses C-kit at higher levels than in the bone marrow (P<0.05).

These stem cell markers have been shown to mark the haematopoietic stem cell (see section 1.1) and it is known that at different developmental stages these markers are

expressed at different levels. This may correlate with whether they are mostly quiescent as would be expected in the bone marrow or self renewing as would be expected in the foetal liver. It might therefore be hypothesised that the higher levels of C-kit expression found on foetal liver cells reflects HSC expansion and higher levels of Sca-1 found on bone marrow cells reflects HSC quiescence.

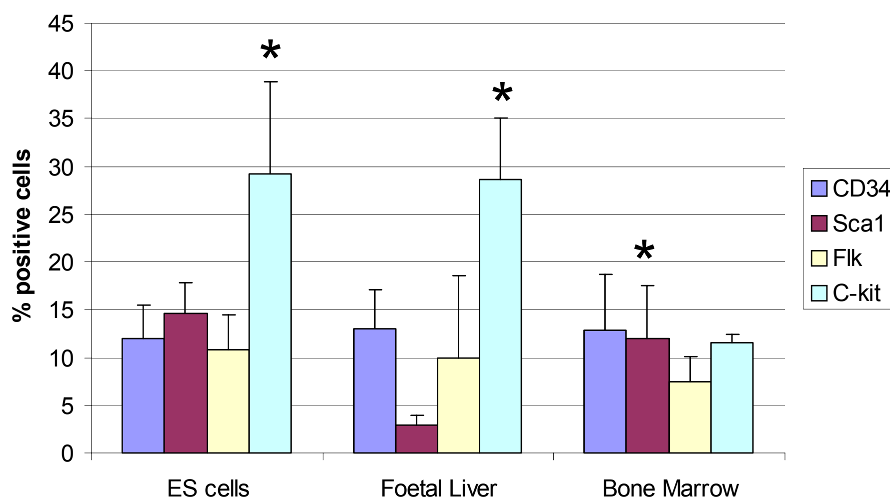


Figure 3.6.2 Stem cell surface marker comparison
A comparison of cells expressing stem cell markers in ES cells differentiated to day 6 in serum and day 0 cells from 14.5 dpc foetal livers and bone marrow. The results represent the average of the data collected. Statistically significant differences at $P<0.05$ are marked with *. C-kit is expressed at higher levels in ES cells after 6 days of differentiation and foetal liver cells than in the bone marrow. Sca-1 is expressed at higher levels in the bone marrow relative to the foetal liver.

3.6.3 Differentiation Markers

There were much higher levels of Ter119 expressing cells in the foetal liver than in the bone marrow ($P<0.05$, Figure 3.6.3). There were lower levels of CD11b expressing cells at day 0 in the foetal liver compared with the bone marrow ($P<0.05$). CD19 cells were present at higher levels at day 0 in bone marrow compared with foetal liver ($P<0.05$).

These differences in the presence of differentiated cells at day 0 may reflect that in the foetal liver environment haematopoietic stem cells are more prone to erythroid differentiation compared with the bone marrow environment where the environment favours myeloid and lymphoid differentiation. This may reflect differing priorities in the foetus where oxygenation is higher priority compared to the adult where defence becomes more important.

There were much lower levels of differentiated cells in the ES cells which was not surprising at this early developmental stage.

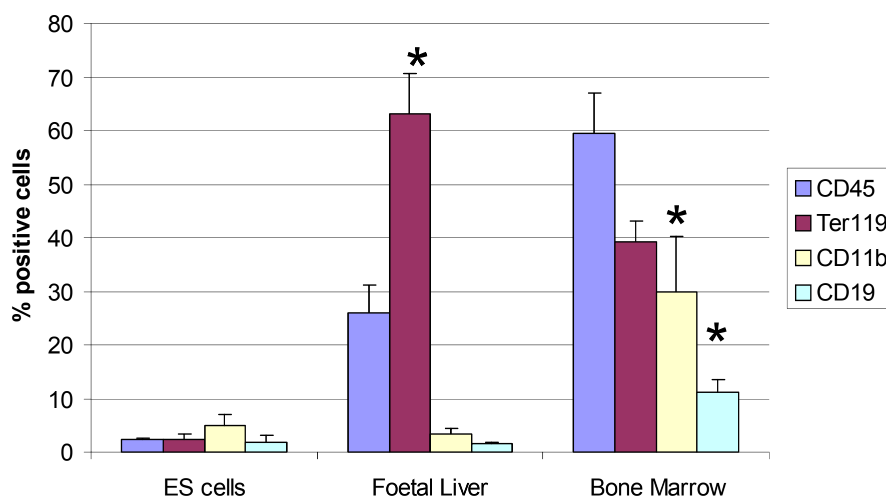


Figure 3.6.3 Differentiation surface marker comparison
A comparison of cells expressing differentiation markers in ES cells differentiated to day 6 in serum and day 0 cells from 14.5 dpc foetal livers and bone marrow. The results represent the average of the data collected. Statistically significant differences at $P<0.05$ are marked with *. There were more Ter119 cells in the foetal liver than in the bone marrow. There were more CD11b and CD19 cells in the bone marrow than in the foetal liver.

3.6.4 Discussion

Conditional gene targeting studies show that in adult *Scl* deletion mutants there is impaired erythroid and megakaryocytic differentiation (Mikkola et al. 2003) and in adult *Runx1* deletion mutant there is impaired megakaryocytic maturation and T and B lymphocyte differentiation (Ichikawa et al. 2004). The differences observed in the expression of these genes may therefore reflect the presence of more differentiated cells. Compared to the levels of differentiated cells at day 6 in ES cells, there were much higher levels of differentiated cells in the foetal liver and bone marrow. However between the foetal liver and bone marrow the patterns and levels of gene expression were very similar not reflecting differences in the types of differentiated cells present in the two populations.

These results may alternatively reflect higher levels of *Runx1*, *Scl* and *Lmo2* expression in the HSCs of these more mature populations. The reason few effects were seen on adult haematopoietic function in conditional gene mutants of *Runx1* and *Scl* maybe because other proteins with overlapping function can take over.

3.7 Cell cycle analysis of C-kit positive and Sca-1 positive bone marrow cells

Day 0 analysis showed that foetal liver cells expressed C-kit at higher levels than in the bone marrow. Sca-1 however was expressed at higher levels in the bone marrow than in the foetal liver. Given that the foetal liver environment is one of haematopoietic expansion (Lessard, Faubert, & Sauvageau 1994;Takeuchi et al. 2002) it was hypothesised that C-kit was expressed at higher levels on dividing cells. In the bone marrow most bone marrow HSCs are known to be quiescent (Szilvassy et al. 1990) and so it was hypothesised that Sca-1 was expressed on quiescent cells.

In order to investigate this fresh bone marrow isolated from CD1 mice was stained for Sca-1 and C-kit. These cells were then stained with PI and the cell cycle status of the Sca-1 and C-kit single positive cells was analysed.

PI fluoresces on binding double stranded DNA. The DNA content of cells can therefore be determined. Thus quiescent cells and cells entering the cycle (G0/G1 phase) can be distinguished from cells preparing for cell division (in S phase) which will have a greater DNA content. Furthermore cells about to undergo or undergoing mitosis (G2/M phase) can be distinguished as they contain double the normal quantity of DNA.

The C-kit population was found to have higher levels of both cells in S phase (P<0.01) and G2/M phase (P<0.01, see Figure 3.7.1). The Sca-1 population however had higher levels of cells G0/G1 phase (P<0.01).

Despite these clear differences there were still a large number of C-kit positive cells that were in G0/G1 phase. It would be interesting to analyse whether these cells are preparing for cell division.

Experiments by Till and McCulloch made it possible to identify HSCs based on the expression of C-kit, Sca-1 and lineage negative cell surface markers (Worton, McCulloch, & Till 1969;van Bekkum et al. 1979). This experiment supports the hypothesis that C-kit and Sca-1 expression is an indicator of cell cycle status. C-kit is present on dividing cells whereas Sca-1 is present on quiescent cells.

Previous data has found embryonic HSCs express low levels of Sca-1 (de Bruijn et al. 2002) and high levels of C-kit (Sanchez et al. 1996). In these experiments ES cells at day 6 of differentiation and foetal liver cells at 14.5 dpc were both found to contain a high level of C-kit cells relative to that seen in bone marrow. In the light of these findings this reflects the high level of cell division that is taking place at these early developmental stages. Sca-1 levels were higher in the bone marrow than in the foetal liver. This reflects the comparative quiescence of the bone marrow cells.

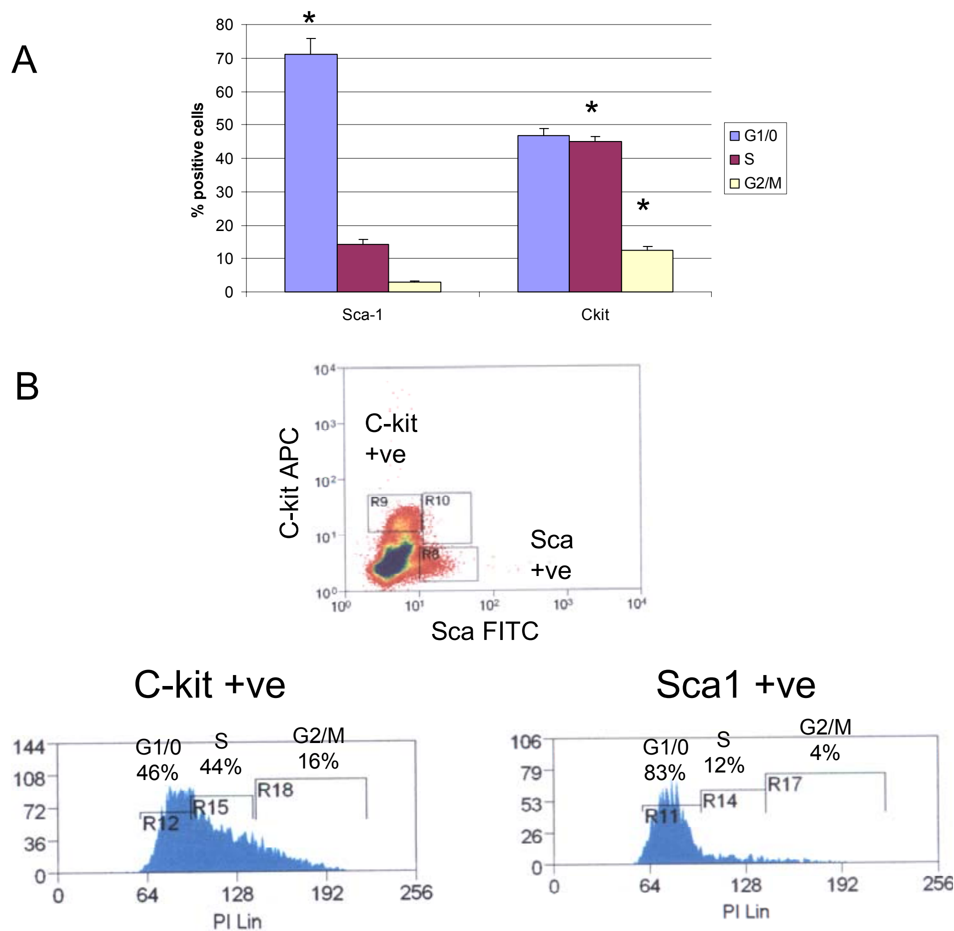


Figure 3.7.1 Cell cycle analysis in bone marrow C-kit and Sca-1 populations
A: The percentage of cells in G1/0, S and G2/M phase of the cell cycle in Sca1 and C-kit populations. Results are based on the average of 6 experiments. Error bars represent the standard error. Statistically significant results when comparing Sca1 and C-kit populations are marked with *. **B**: Results from a representative experiment showing the gating of Sca-1 and C-kit populations. Propidium Iodide staining for cell cycle analysis is shown for each population.

3.8 General Discussion

In these initial experiments much time was spent developing serum free cultures that contained minimal added growth factors. Following this, optimisation of the methods used for sample analysis was necessary. In the end several interesting observations were made from these experiments.

3.8.1 Comparison of Haematopoietic Populations

The simultaneous use of ES cells, foetal liver cells and bone marrow cells gave rise to an opportunity to compare haematopoietic populations at different developmental stages. *Runx1*, *Scl* and *Lmo2* which have critical roles in development were all found to have much higher levels of expression in the more mature populations of the foetal liver and bone marrow. This probably provides the best indication from the data obtained that these genes do play an important role in haematopoiesis at later developmental stages.

A comparison of stem cell markers found that there were higher levels of C-kit positive cells in the foetal liver and higher levels of Sca-1 positive cells in the bone marrow. Further investigation found that this correlated to cell cycle status with C-kit expressed on dividing cells and Sca-1 expressed on quiescent cells. This result sheds light on the role of these cell surface markers which since the first discovery of HSCs have been used to separate and identify them.

3.8.2 The effect of BMP4

The main aim of these experiments was to investigate the effect of BMP4 on haematopoietic populations at 3 stages of haematopoietic development.

In ES cell differentiation there was no evidence of BMP4 specific effects on the expression of *Runx1*, *Scl*, *Gata2* and *Lmo2* or any of the stem cell and differentiation markers studied. Given previous evidence indicating that BMP4 might play a role in the expression of these genes it may have been that the concentration of BMP4 was not optimal to produce these effects.

In foetal liver experiments there was no evidence of any BMP4 specific effects in PCR, flow cytometry and colony assays. There was a significant level of cell death in serum free conditions. The inadequate survival of cells may have been the reason that no effect was seen with the addition of BMP4. Further optimisation of serum free conditions therefore was needed. There was some evidence that BMP receptor did reduce the expression of all four genes studied. This may reflect that the foetal liver cells themselves were secreting BMP4.

In bone marrow experiments cell survival with the addition of TPO and IGF1 was much improved. Despite this there was still inconsistency in results which was thought to result from the variability of the initial population. One BMP receptor experiment showed a CD34 C-kit positive population might be BMP4 responsive. Based on this, in order to

reduce variability, experiments were conducted on a CD34 C-kit population that was sorted from the bone marrow. However cell survival was poor and no BMP4 specific effects were observed.

3.8.3 The next step

In the case of foetal liver cultures further optimisation of serum free conditions to improve cell survival was required in order that meaningful experiments could be conducted into the effects of BMP4. However even if this was achieved there were concerns about the variability in the initial populations and the problems this caused in conducting meaningful repeat experiments. One solution to this problem was to sort cell populations. Despite good cell survival in the unsorted bone marrow cultures, the culture of sorted populations proved significantly more difficult.

In order to continue experiments on bone marrow and foetal liver it would have been necessary to sort populations and then optimise culture conditions to allow cell survival. Given the results from experiments that had been carried out, there was no clear evidence of a population in either foetal liver or bone marrow that was BMP4 responsive.

However data from other studies had begun to provide evidence demonstrating a role for BMP4 in haematopoietic development. Furthermore it was known that the genes *Runx1*, *Scl*, *Gata2* and *Lmo2* had roles critical to haematopoietic development. It was therefore

decided to focus future experiments on whether BMP4 plays a role in up regulating these genes in early development using the ES cell differentiation model.

Chapter 4 Titration of BMP4 in ES cell haematopoietic differentiation

4.1 Introduction

Results from initial experiments in ES cells had shown no clear evidence of a BMP4 specific effect in either the PCR or flow cytometry studies that had been carried out. This was despite data from previous studies showing that BMP4 up regulated *Flk-1* and in combination with VEGF also up regulated SCL expression (Park et al. 2004). This may have been due to the concentration not being optimal to produce these effects. In order to find the optimal concentration a titration of the effects of BMP4 on *Runx1*, *Scl*, *Gata2* and *Lmo2* was performed.

In foetal liver experiments there was also no effect observed with the addition of BMP4. However with the addition of BMP receptor there was a reduction in the expression of all four genes studied. This implied that foetal liver cells were secreting BMP4. In order to allow comparison of serum free and BMP4 conditions it is necessary that serum free conditions are BMP4 free. There were concerns that ES cells, which are known to produce BMP4 as a mesoderm patterning factor, might also secrete BMP4. In order to address this, experiments were conducted to analyse the gene expression of BMP4 in ES cells.

In my initial ES cell experiments there had been some variability in results. This may have reflected normal variations in the developmental processes that take place in ES cells. However before carrying out further studies it was important to validate the experimental system in order to ensure that ES cells were in an undifferentiated state. In ES cell culture a small percentage of ES cells differentiate but over time this level can accumulate. This is why it is essential that ES cells used in haematopoietic differentiation are at a low passage number. The second purpose of validation was to ensure that these ES cells were capable of haematopoietic differentiation. Different ES cell lines are well known to have preferences in their differentiation potential. Some ES cell lines will be more prone to haematopoietic differentiation than others. This has been demonstrated in a recently published study using human ES cells (Chang et al. 2008).

4.2 Validation of ES cell culture

These experiments were aimed at ensuring that the ES cell line used was suitable for use in future experiments which would analyse the effects of BMP4 on haematopoietic differentiation. ES cells were first analysed in culture for the expression of markers associated with the undifferentiated phenotype. Then the capacity of these cells to differentiate into haematopoietic cells was analysed using PCR and CFU assays.

4.2.1 Alkaline phosphatase and SSEA-1 staining of undifferentiated ES cells

Mouse ES cells can be maintained in the undifferentiated state for extensive periods in the presence of leukaemia inhibitory factor (LIF). Upon removal of LIF mouse ES cells differentiate to produce cells of the three germ layers: endoderm, mesoderm and ectoderm (Evans & Kaufman 1981). However it is normal in culture even in the presence of LIF that a few ES cell colonies show signs of differentiation. It was therefore necessary to ensure that a sufficient level (at least 90%) of ES cell colonies were kept in an undifferentiated state.

Alkaline phosphatase is expressed at high levels by undifferentiated ES cells (Pease et al. 1990). A substrate, naphthol phosphate, was used to detect the presence of alkaline phosphatase. The precipitation of fast red chromogen at enzymatic sites resulted in

red/pink staining. Colonies with high levels of alkaline phosphatase activity were visible as seen in Figure 4.1A. 99% of colonies expressed high levels of alkaline phosphatase.

Stage-specific embryonic antigen 1 (SSEA-1) is a carbohydrate antigen that is expressed at the late cleavage stages of murine embryonic development (Solter & Knowles 1978). This antigen is strongly expressed by undifferentiated mouse ES cells. An antibody directed at mouse SSEA-1 antigen conjugated to FITC visible using a fluorescent microscope was used to stain ES cells. The expression of SSEA-1 on ES cell colonies can be seen in Figure 4.1B.

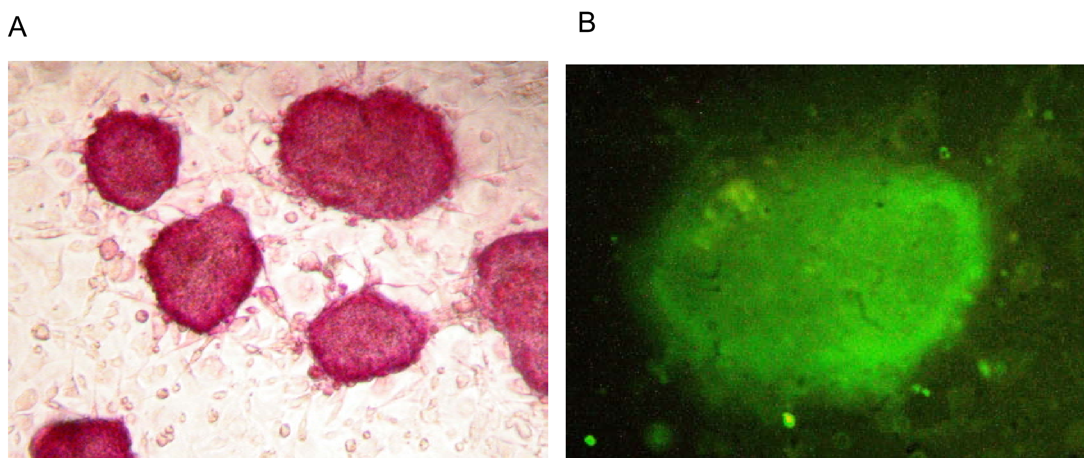


Figure 4.1 Alkaline phosphatase and SSEA-1 staining of ES cell colonies
Cells were cultured to high confluency and then fixed and stained for alkaline phosphatase (A) and SSEA1 (B) expression. In A the high level of alkaline phosphatase expression is in contrast to the background SNH stromal cell feeder layer (magnification 4x). B shows a single ES cell colony. SSEA-1 expression was visualised using a fluorescent microscope (magnification 10x).

4.2.2 QPCR analysis of *Nanog*, *Oct4* and *Sox2* expression

Several transcription factors have been implicated in pluripotency however the best characterised is *Oct4*. When *Oct4* is deleted *in vivo* cells failed to adopt a pluripotent

identity and instead differentiated to trophectoderm (Nichols et al. 1998). Similarly when *Oct4* was deleted in ES cells the cells differentiated to trophectoderm (Niwa, Miyazaki, & Smith 2000). Oct4 acts on DNA in conjunction with Sox2 (Pevny & Lovell-Badge 1997). *Sox2* knockout ES cells differentiate to trophectoderm and do not have a pluripotent phenotype (Li et al. 2007). Nanog a homeodomain protein maintains ES cell self renewal. Gene deletion of *Nanog* results in cell differentiation to primitive endoderm (Chambers et al. 2003).

Thus *Oct4*, *Sox2* and *Nanog* are crucial to ES cell identity. The expression of these genes were analysed in ES cells using quantitative PCR. These three genes were all found to be expressed (see Figure 4.2) confirming that these ES cells were pluripotent and in an undifferentiated state.

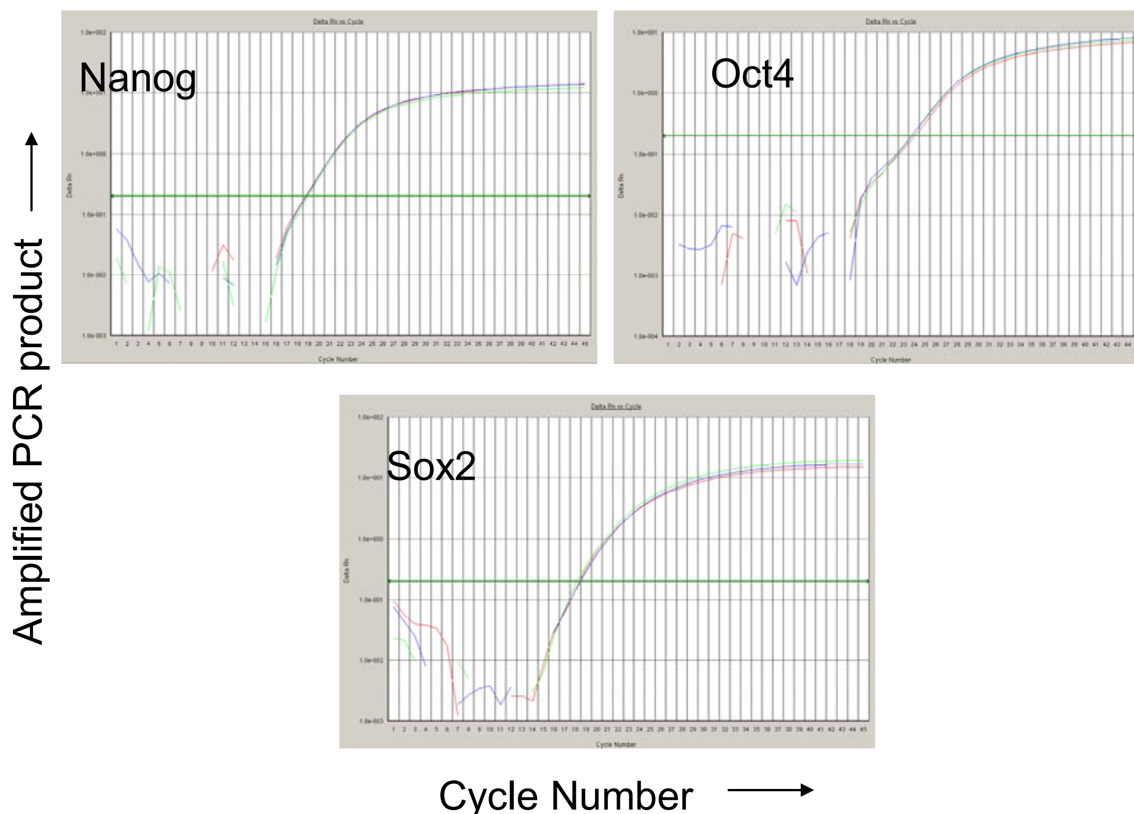


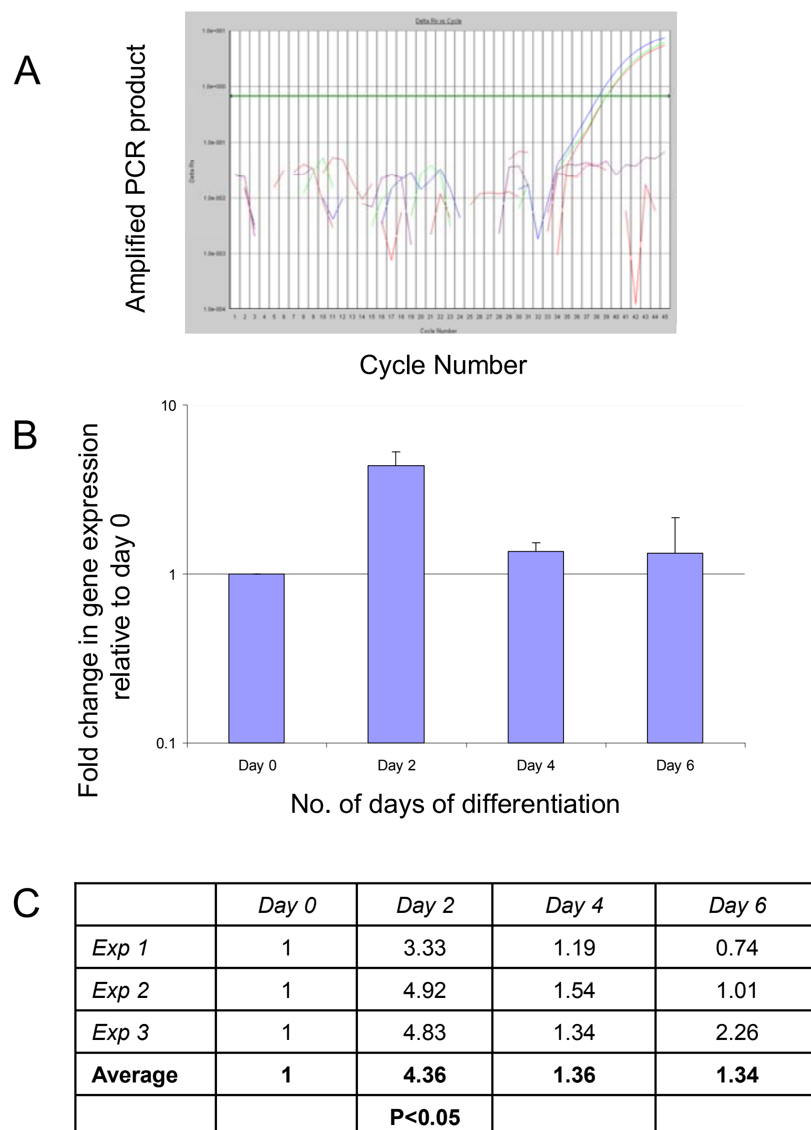
Figure 4.2 *Nanog*, *Oct4* and *Sox2* gene expression in undifferentiated ES cells
These logarithmic quantitative PCR plots show the amount of amplified PCR product plotted against the cycle number. In this RT-PCR the detection of PCR product represents the detection of mRNA and thus gene expression. *Nanog*, *Oct4* and *Sox2* gene expression was detected in these ES cells. The three lines in each plot represent the results of the triplicate repeats.

4.2.3 Flk-1 expression

Flk-1 is expressed on BL-CFCs which give rise to both endothelial and haematopoietic cells. These colonies arise between day 2.5 and day 3.5 of differentiation. Cells expressing Flk-1 represent the earliest progenitors of the haematopoietic system. Previous work by Park et al. using R1 ES cells has shown that under serum differentiation conditions *Flk-1* expression occurs at day 2 of differentiation peaking at day 4 and then reduces again after this (Park et al. 2004). This change in expression represents the transient appearance of the haemangioblast.

In order to investigate whether there was the transient appearance of a Flk-1 expressing population within differentiation cultures, real time PCR was carried out to determine *Flk-1* expression at time points day 0, 2, 4 and 6 as had been previously used by Park *et al*. *Flk-1* primers were successfully tested on the mouse endothelial cell line SVEC which had previously been shown to express *Flk-1* (Walter-Yohrling et al. 2004).

Results showed a four fold increase in *Flk-1* expression at day 2 ($P<0.05$) before a reduction in *Flk-1* expression levels at day 4 and day 6 (see Figures 4.3B and 4.3C). These results show the transient appearance of a *Flk-1* expressing population as has previously been described. The *Flk-1* expressing population peaked earlier in comparison with previous data at day 2 rather than day 4. However different ES cell lines may exhibit different patterns of differentiation. Since an R1 ES cell line was used previously and a CCE ES cell line was used in this experiment, this may explain the difference in results. These results overall confirm that a transient increase in *Flk-1* expression consistent with the appearance of a haemangioblast population occurs.



	Day 0	*Day 2*	*Day 4*	*Day 6*
Exp 1	1	3.33	1.19	0.74
Exp 2	1	4.92	1.54	1.01
Exp 3	1	4.83	1.34	2.26
Average	**1**	**4.36**	**1.36**	**1.34**
		P<0.05		

Figure 4.3 *Flk-1* expression in differentiating ES cells
A shows logarithmic quantitative PCR plot showing the detection of *Flk-1* expression on the control cell line SVEC. B shows the fold change in *Flk-1* expression relative to day 0 at each time point. This is a logarithmic graph and the results shown represent the average of 3 experiments. A transient increase in *Flk-1* expression can be seen at day 2. Error bars represent the standard error. C shows a table of the results obtained in each experiment.

4.2.4 Colony forming unit assays and F480 colony analysis

Methylcellulose assays are used to quantify the numbers of multipotent and lineage restricted progenitors in culture. In these experiments a sample of cells was taken from differentiation cultures after 6 days in the presence of serum. These cells were plated in methylcellulose medium with added cytokines (see Section 2.2.3.2) so as to allow the survival and maturation of progenitors. There are several variations of ES methylcellulose mixtures that have been used previously that allow colony formation, some of which use ingredients not readily available. Several rounds of optimisation were required in order to develop a media that would reliably allow colony formation.

After one week plates were analysed for the presence of haematopoietic colonies. Haematopoietic colonies were observed and pictures were taken (Figure 4.4). Granulocyte-macrophage, macrophage and erythroid-myeloid mixed colonies were present. This experiment provided visual evidence of the presence of haematopoietic cells in samples taken from differentiation cultures.

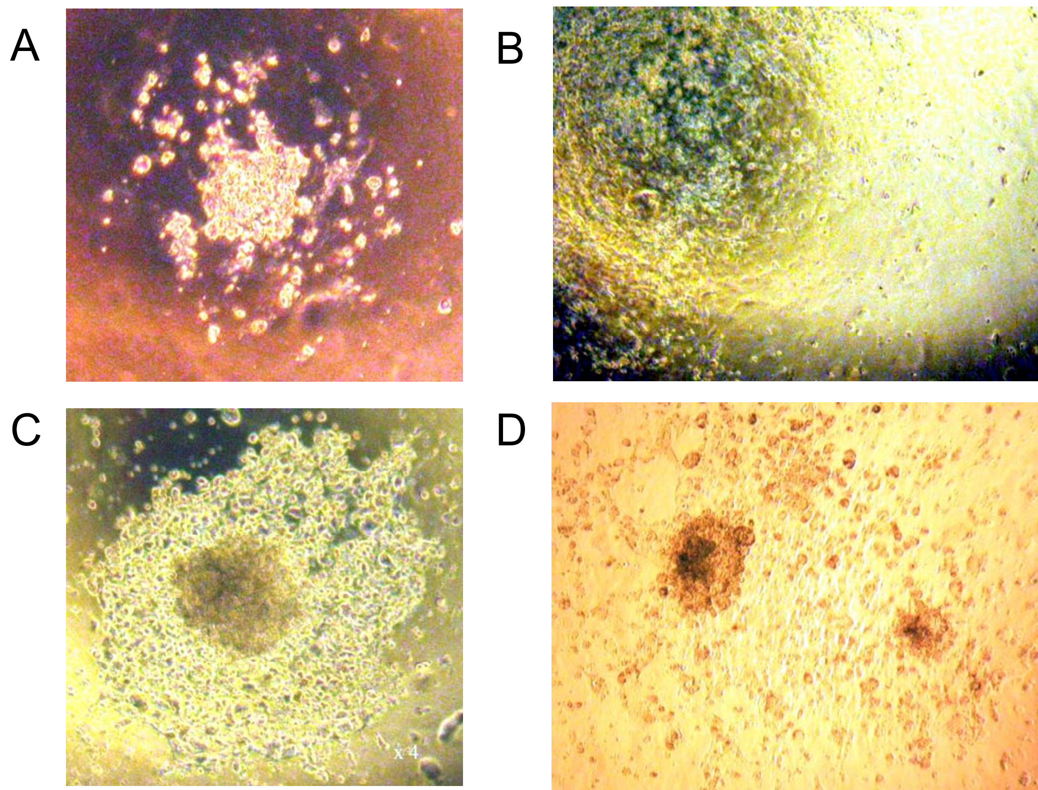


Figure 4.4 Colony forming potential of ES cells after 6 days of differentiation
Cells from day 6 EB differentiation cultures were plated in methylcellulose and after a further 7 days pictures were taken of colonies obtained. Granulocyte macrophage colonies (A and B), mature macrophage colonies (C) and mixed erythro-myeloid colonies (D) were observed. Micrographs were taken at x4 magnification.

Haematopoietic colonies which resembled macrophage colonies were harvested and further analysed for expression of the F4/80 cell surface marker. F4/80 is a 160kDa cell surface glycoprotein that is a member of the EGF TM7 family of proteins. F4/80 has been identified as a marker for mature mouse macrophages (Austyn & Gordon 1981). Harvested cells were fixed and stained with anti-mouse F4/80 antibody. A secondary antibody was then applied with peroxidase activity. Cells taken from macrophage colonies were found to express F4/80 (see Figure 4.5).

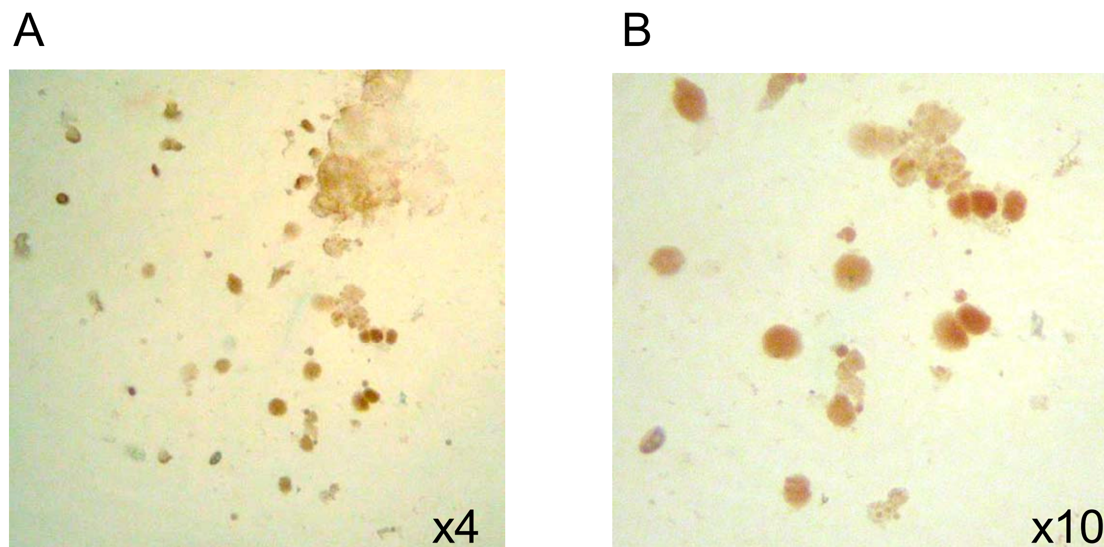


Figure 4.5 F4/80 staining of macrophage colony cells
Micrographs A and B show cells expressing the macrophage marker F4/80. Cells were stained with an anti-mouse F4/80 antibody and then a secondary antibody with peroxidase activity was applied. The addition of peroxidase substrate caused cells expressing F4/80 to become brown in colour.

4.2.5 Discussion

ES cells expressed high levels of alkaline phosphatase and SSEA-1 antigen consistent with an undifferentiated phenotype. Furthermore gene expression data showed that *Oct4*, *Sox2* and *Nanog* which are markers of a pluripotent phenotype were also expressed. This data therefore confirms that these ES cells were both undifferentiated and pluripotent.

The ability of these ES cells to differentiate to haematopoietic cells was also analysed. Flk-1 expression marks the haemangioblast which is the earliest progenitor of the haematopoietic system. A transient increase in *Flk-1* expression occurred at day 2 which is consistent with the appearance of a haemangioblast population.

In previous experiments flow cytometry analysis for the expression of differentiation markers was performed. Cells expressing Ter119 and CD11b corresponding to erythroid and myeloid differentiation were found after 6 days of differentiation. In these experiments methylcellulose assays found visual evidence of erythroid and myeloid colonies. The presence of mature macrophages in macrophage colonies was confirmed by F4/80 staining. Thus these ES cells were shown to be capable of haematopoietic differentiation.

4.3 BMP4 and BMP receptor expression in differentiating ES cells

There were concerns about BMP4 being secreted by differentiating cells in the 'negative control' serum free differentiation conditions. This endogenous BMP4 would make it difficult to detect the effect, if any, of BMP4 added into the culture medium. In order to address these concerns RT-PCR analysis was carried out for the expression of *Bmp4*.

BMP4 can only produce a cellular response if cell surface receptor is expressed. BMP receptor has two components: ALK3 and BMPR2. BMP4 binds BMPR2 and then ALK3 is recruited resulting in the activation of intracellular kinase activity that activates the Smad signaling pathway (Larsson & Karlsson 2005). Thus both component receptors must be expressed for BMP4 signaling to take place. RT-PCR was therefore also carried out to detect the expression of *Alk3* and *Bmpr2*.

Primer sequences to *Bmp4*, *Alk3* and *Bmpr2* were taken from previous papers (Pesce, Gioia, & De Felici 2002;Peretto et al. 2004). A BLAST search was done to ensure that the primers were specific to *Bmp4*, *Alk3* and *Bmpr2*. Primers were also checked to ensure that they were designed to cross intron-exon boundaries and therefore would not detect genomic DNA contamination.

The murine stromal cell line ST2 had previously been shown to express *Bmp4*, *Alk3* and *Bmpr2* (Otsuka et al. 1999). Primers were therefore tested on the ST2 cell line to confirm their ability to detect BMP4 and BMP receptor expression (see Figure 4.6).

To analyse *Bmp4*, *Alk3* and *Bmpr2* expression ES cells were differentiated under serum replacement conditions and samples taken at day 0, 2, 4 and 6 for RT-PCR analysis. *Hprt* which is a housekeeping gene was used as a control for the reverse transcription reaction.

At all time points cells expressed both components of BMP receptor: *Alk3* and *Bmpr2* (see Figure 4.6). The cells therefore had the capacity to detect the presence of BMP4 added into the culture medium. In addition *Bmp4* was expressed by the cells at all time points (see Figure 4.6). The endogenous expression of BMP4 meant that further experiments needed to take into account that the serum replacement 'negative control' could not be assumed to be BMP4 free. This endogenous expression of BMP4 may also have explained why previous experiments had yielded few differences between the BMP4 treated samples and 'negative control' serum free samples.

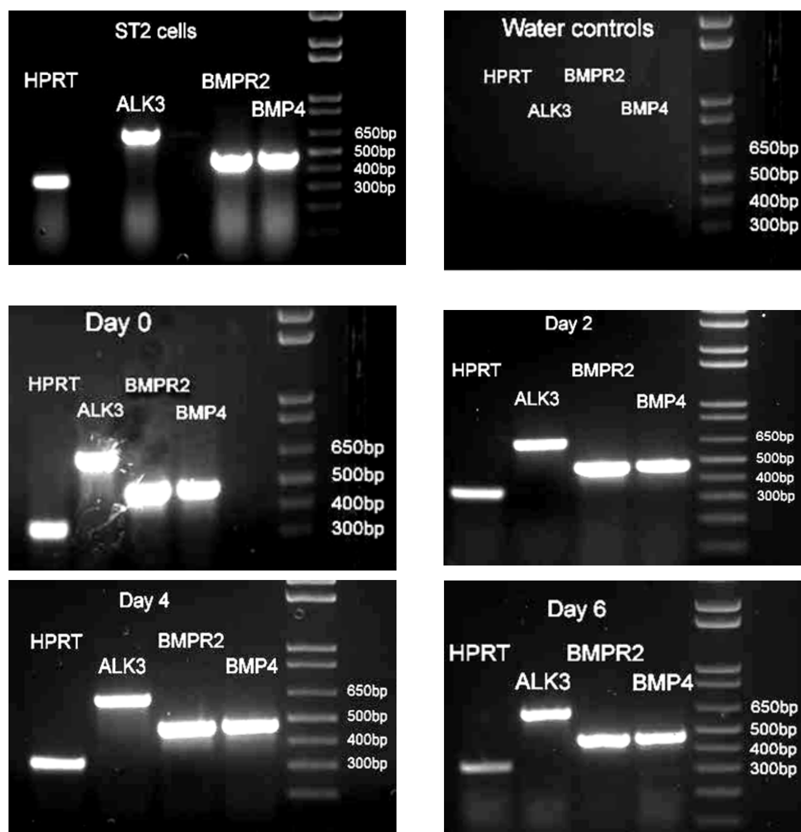


Figure 4.6 RT-PCR analysis of the expression of *Alk3*, *Bmpr2* and *Bmp4* in the ST2 control cell line and in EB differentiation cultures at days 0, 2, 4 and 6.
Differentiation was under serum free conditions. *Hprt* is a housekeeping gene and was included as a control for the reverse transcription reaction. Water controls were included as a control for contamination. The size of PCR products were as follows: *Hprt*- 324 base pairs (bp), *Alk3* - 626bp, *Bmpr2* – 460bp and *Bmp4* – 468bp. Bmp4 and both components of the BMP receptor were detected at all time points.

4.4 BMP4 titration

Other published work had shown a difference in untreated and BMP4 treated samples despite any endogenous BMP4 expression that may have been present. Work done by Park *et al*. showed that BMP4 had a role in the up-regulation of SCL in combination with VEGF in R1 ES cells (Park et al. 2004). However in my previous experiments there was no clear evidence of a BMP4 specific role in the regulation of *Runx1*, *Scl*, *Gata2* or *Lmo2*. It was possible that an effect wasn't observed because the concentration of BMP4

was not optimal to change the expression of these genes. In order to determine whether a different concentration of bmp4 might more optimally induce haematopoietic gene expression a BMP4 titration was carried out.

Concentrations of BMP4 between 2 and 25ng/ml had been used with different types of ES cells in other work (Bhatia et al. 1999;Park et al. 2004). Therefore in these experiments ES cells were differentiated in serum replacement medium containing 0, 5, 10 and 25ng/ml BMP4 and samples were taken at day 0, 2, 4 and 6. Samples were analysed by quantitative RT-PCR for *Runx1*, *Scl*, *Gata2* and *Lmo2* expression. The experimental method has been summarised in figure 4.7.

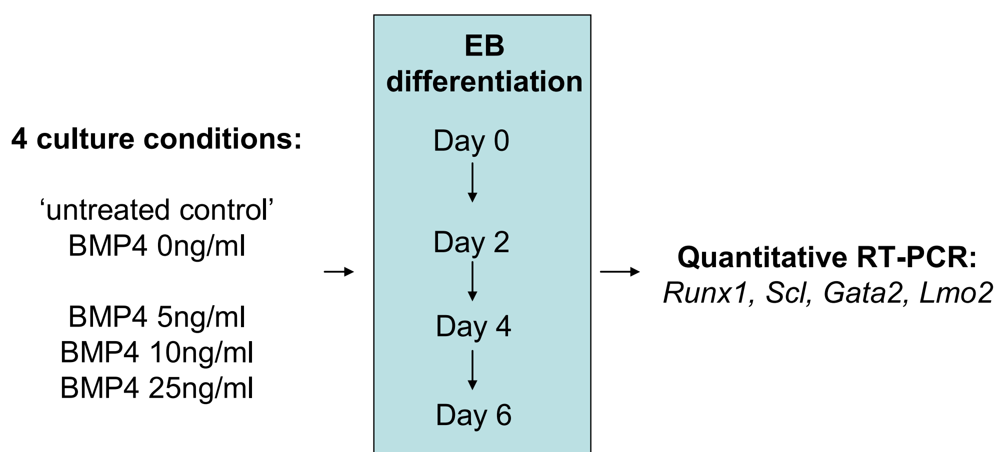


Figure 4.7 Experimental protocol used in BMP4 titration experiments.

4.4.1 Analysis of Data

In previous experiments fold change in gene expression was calculated relative to day 0. This data is useful to determine the direction of change in gene expression relative to day 0. Since the expression levels of the haematopoietic genes at day 0 would be expected to

be low only an increase in expression above day 0 levels indicates that haematopoietic development is taking place.

Previous variability in gene expression data came about because in each experiment gene expression increased to a different level relative to day 0. With this variability it is difficult to make conclusions about the effect of BMP4. In order to control for this, gene expression values were calculated relative to the age matched untreated control.

Data was therefore analysed relative to day 0 to determine whether any increase in haematopoietic gene expression had taken place indicating haematopoietic development. Also gene expression relative to the age-matched untreated control was calculated to determine the effect of BMP4.

4.4.2 Effects of BMP4 concentration on gene expression

Tables showing the data for each experiment are in Appendix 2.

Runx1 expression increased at day 2 in serum free conditions above day 0 levels ($P<0.05$, Figure 4.8). Although the general trend across all culture conditions was for an increase in *Runx1* expression at day 2.

At day 4 BMP4 reduced *Runx1* expression at 5ng/ml relative to the untreated control (P<0.05). At the other concentrations BMP4 did not produce any notable changes in *Runx1* expression compared with the untreated control.

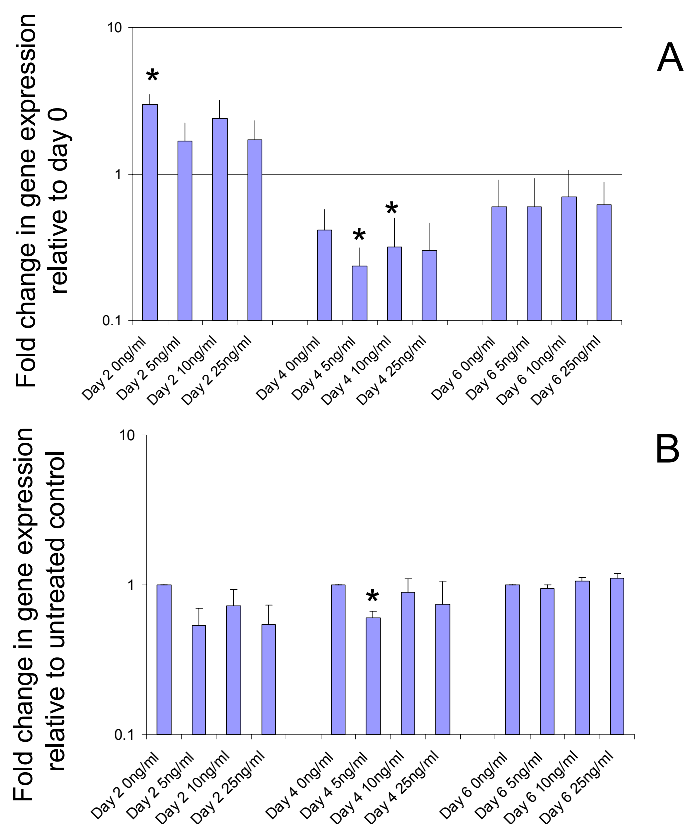


Figure 4.8 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Runx1*.
Samples were taken at day 2, 4 and 6 and were differentiated with BMP4 at concentrations of 0, 5, 10 and 25ng/ml. Gene expression values represent the average achieved over 5 experiments. The error bars show standard error. * indicates statistically significant results at P<0.05.

There were no statistically significant changes in *Scl* expression at any of the time points relative to day 0 (Figure 4.9). There were however higher levels overall of *Scl* expression at day 4 relative to day 0.

At day 6 BMP4 at 10ng/ml increased *Scl* expression relative to the untreated control (P<0.05).

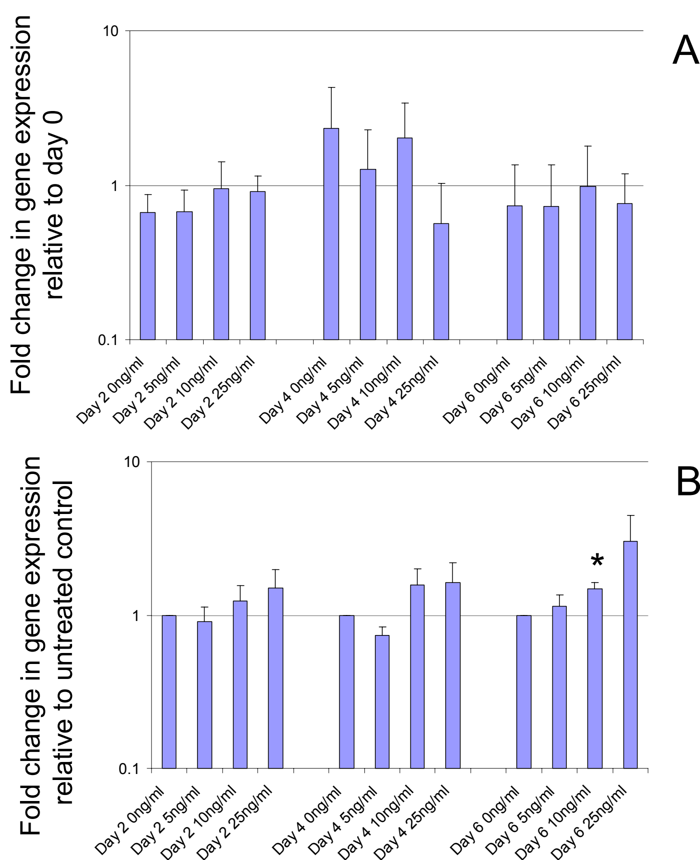


Figure 4.9 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Scl.*
Samples were taken at day 2, 4 and 6 and were differentiated with BMP4 at concentrations of 0, 5, 10 and 25ng/ml. Gene expression values represent the average achieved over 5 experiments. The error bars show standard error. * indicates statistically significant results at P<0.05.

There were no statistically significant changes in *Gata2* expression at any of the time points but there was a definite trend for higher levels of *Gata2* expression at day 4 relative to day 0 (Figure 4.10).

At day 4 with 10ng/ml and 25ng/ml BMP4 there was an increase in *Gata2* expression relative to the untreated control (at 10ng/ml $P<0.05$ and 25ng/ml $P<0.05$). This increase in expression was striking particularly in experiments 2 and 3.

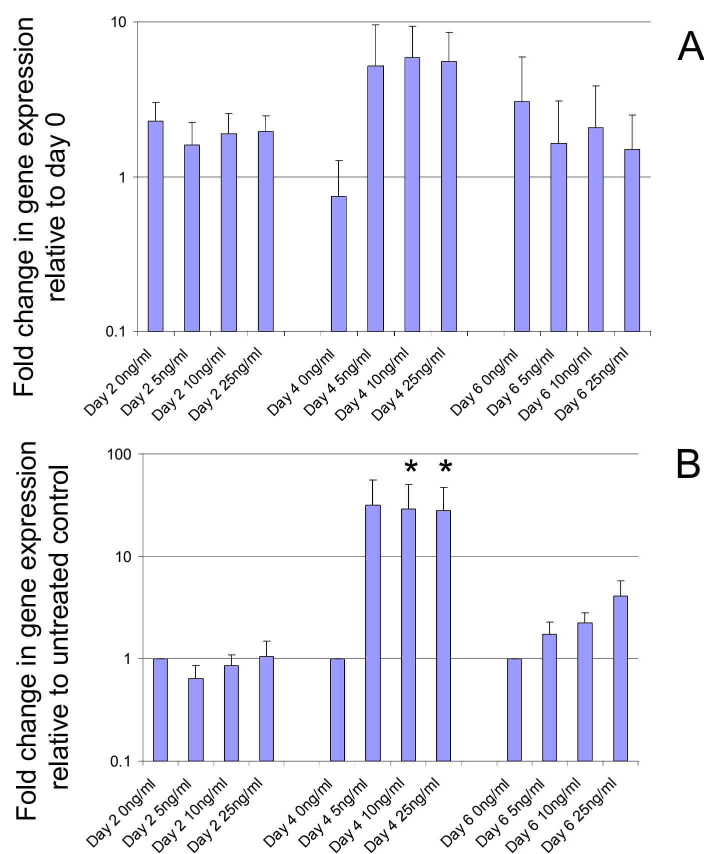


Figure 4.10 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Gata2*.
Samples were taken at day 2, 4 and 6 and were differentiated with BMP4 at concentrations of 0, 5, 10 and 25ng/ml. Gene expression values represent the average achieved over 5 experiments. The error bars show standard error. * indicates statistically significant results at $P<0.05$.

There were no statistically significant changes in *Lmo2* expression at any time points (Figure 4.11). There was however an average increase in *Lmo2* expression at all time points.

At day 6 there was evidence of a concentration dependent increase in *Scl*, *Lmo2* and *Gata2* gene expression relative to the untreated control. Although these increases were not statistically significant it is possible that if higher concentrations of BMP4 are used larger statistically significant increases in gene expression might occur.

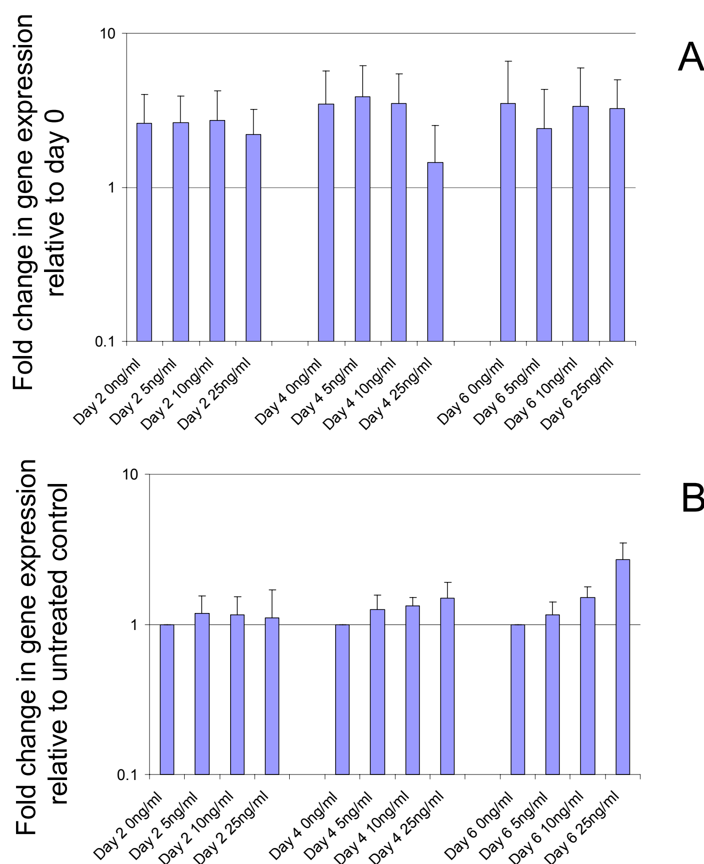


Figure 4.11 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Lmo2*.
Samples were taken at day 2, 4 and 6 and were differentiated with BMP4 at concentrations of 0, 5, 10 and 25ng/ml. Gene expression values represent the average achieved over 5 experiments. The error bars show standard error. * indicates statistically significant results at $P<0.05$.

4.4.3 Discussion of BMP4 titration data:

The data was still variable. Although in some experiments there were large increases in the expression of one gene there were large increases in other genes in other experiments. This variability probably reflects the chaotic nature of the developmental processes that are occurring as the ES cells differentiate due to the artificial environment in which they are differentiating. Therefore the data was analysed with reference to the average changes in gene expression and the statistical significance of the results.

The most prominent result was an increase in *Gata2* expression at day 4 with the addition of BMP4 at 10 and 25ng/ml. This increase in *Gata2* expression was above day 0 levels. This data therefore supports work previously done by Lugus *et al.* which showed that *Gata2* was a direct target of BMP4 in R1 ES cells (Lugus et al. 2007).

It is possible given the apparent concentration dependence of average gene expression changes at day 6 in *Scl*, *Gata2* and *Lmo2* that higher concentrations would have produced larger changes in gene expression.

This data further suggests BMP4 maintains rather than up regulates *Scl* expression at 10ng/ml at day 6. Furthermore BMP4 actually reduced *Runx1* expression at 5ng/ml at day 4. These results differ from data obtained in previous work. However Park *et al.* and Pearson *et al.* used combinations of growth factors and this may account for the difference in results. Specifically in the experiments performed by Park *et al.* VEGF in combination with BMP4 produced an increase in SCL expressing cells. In Pearson *et al.*

BMP4 was used in combination with Activin A, basic fibroblast growth factor and VEGF to produce increases in *Runx1*, *Scl* and *Lmo2* expression. These are therefore the first experiments to describe the effects of BMP4 alone on the expression of *Runx1*, *Scl*, *Gata2* and *Lmo2*.

4.5 BMP receptor titration

The expression of BMP4 in the 'negative control' serum free samples posed several problems to investigating the role of BMP4 in haematopoietic development in ES cells. Firstly calculations of gene expression relative to untreated control assumed that the untreated control was BMP4 free. Therefore accurate calculations as to the change in gene expression resulting from the addition of BMP4 could not be carried out. The second problem was that there was an unknown quantity of BMP4 secretion in each experiment which could have been adding to the variability of results.

Previous experiments in the foetal liver and bone marrow had used soluble BMP receptor to antagonize the action of BMP4. An experiment was therefore carried out to determine if soluble BMP receptor would reduce haematopoietic gene expression. Previous experiments in human ES cells had used BMP receptor at concentrations of 250ng/ml (Kennedy et al. 2007). BMP receptor was added at concentrations of 0, 25, 100 and 250ng/ml.

Samples were taken at day 0, 2, 4 and 6 and analysed by quantitative RT-PCR. Gene expression values were calculated relative to day 0 and relative to the age matched untreated control. The experimental method is summarized in Figure 4.12.

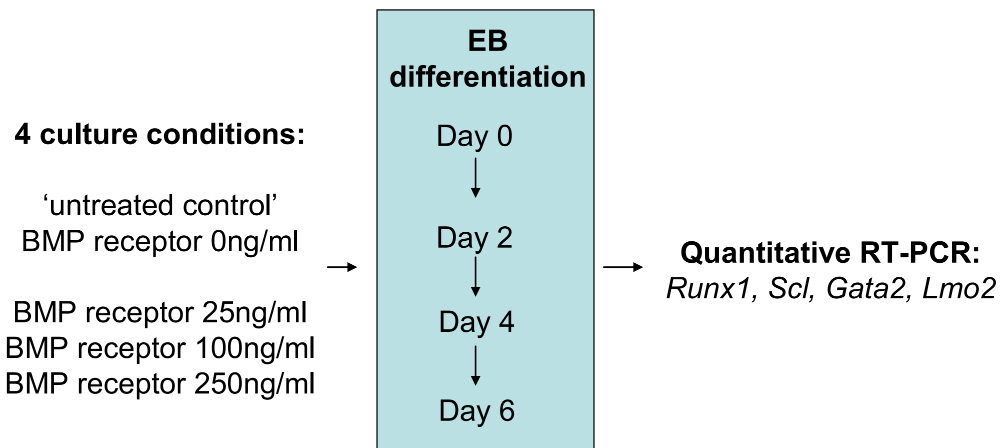


Figure 4.12 The experimental protocol used in BMP receptor titration experiments.

Tables showing the data for each experiment are in Appendix 1.

Although not statistically significant *Runx1* expression was highest at day 2 (Figure 4.13). At all BMP receptor concentrations there was no effect on Runx1 expression.

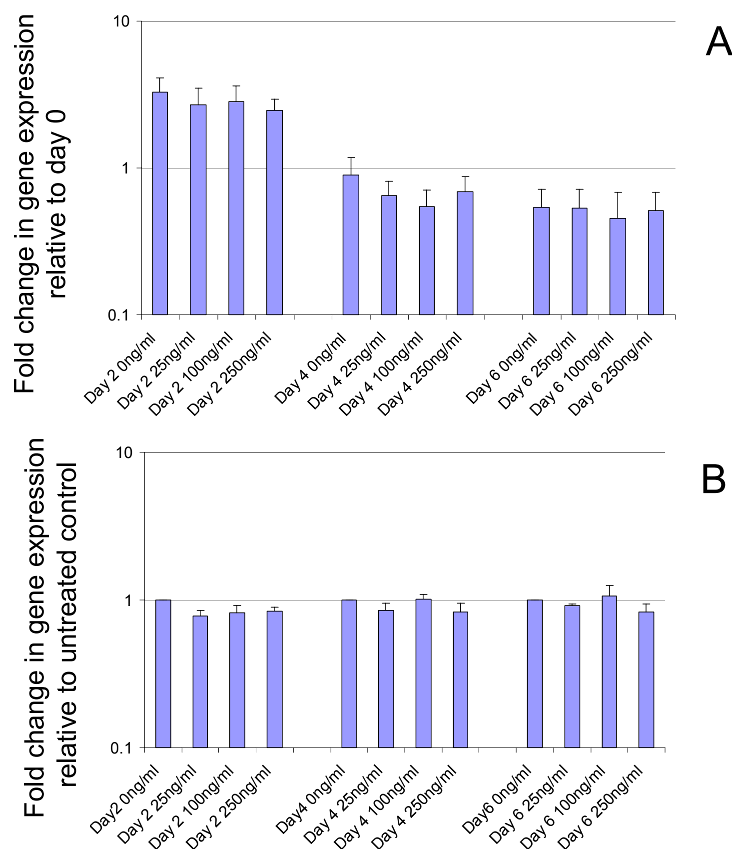


Figure 4.13 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Runx1*.
Samples were taken at day 2, 4 and 6 and were differentiated in the presence of soluble BMP receptor at concentrations of 0, 25, 100 and 250ng/ml. Gene expression values represent the average achieved over 4 experiments. The error bars show standard error.

Scl expression was at its highest at day 2 but did not increase significantly above day 0 levels (Figure 4.14). There was no change in *Scl* expression at any concentration of BMP receptor at any of the time points.

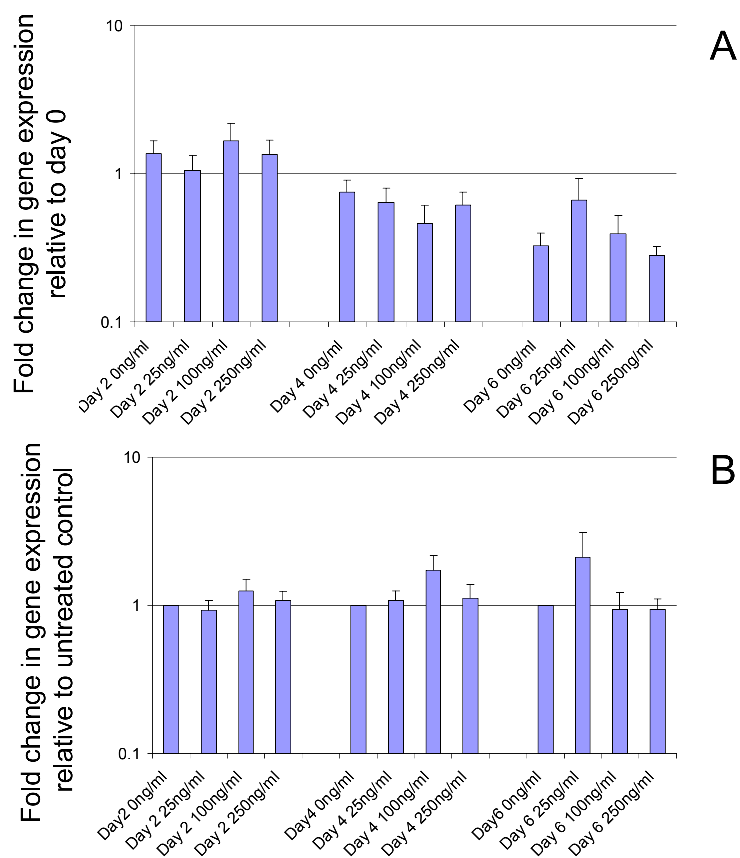


Figure 4.14 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Scl*.
Samples were taken at day 2, 4 and 6 and were differentiated in the presence of soluble BMP receptor at concentrations of 0, 25, 100 and 250ng/ml. Gene expression values represent the average achieved over 4 experiments. The error bars show standard error.

At day 2 *Gata2* expression was at its highest relative to day 0 (Figure 4.15). There was no change in *Gata2* expression at any concentration of BMP receptor at any of the time points.

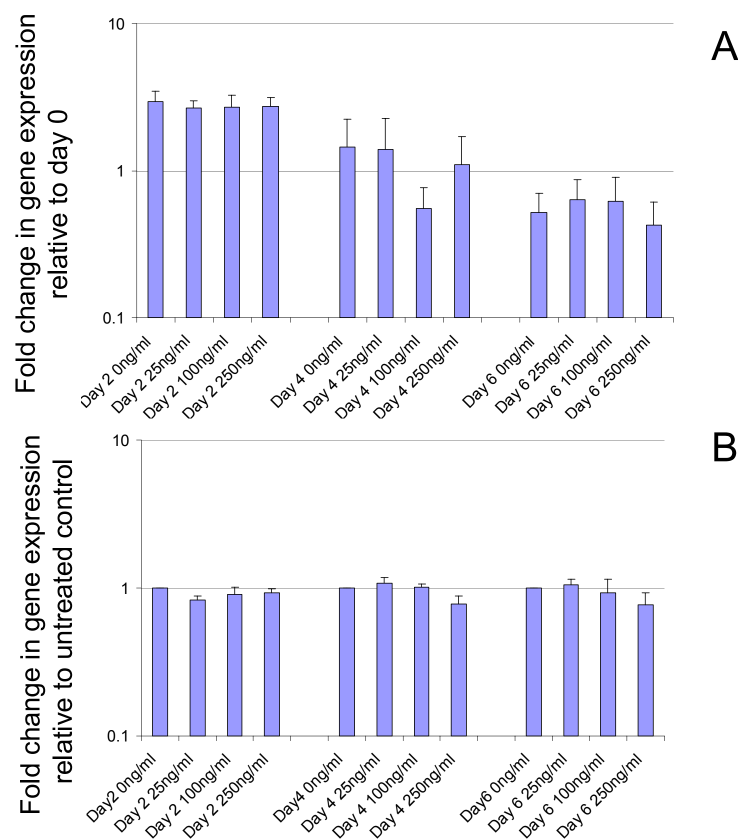


Figure 4.15 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Gata2*.
Samples were taken at day 2, 4 and 6 and were differentiated in the presence of soluble BMP receptor at concentrations of 0, 25, 100 and 250ng/ml. Gene expression values represent the average achieved over 4 experiments. The error bars show standard error.

Lmo2 expression was at its highest at day 2 relative to day 0 levels (Figure 4.16). *Lmo2* expression then reduced at days 4 and 6 to day 0 levels. BMP receptor did not change *Lmo2* expression at any concentration at any of the time points.

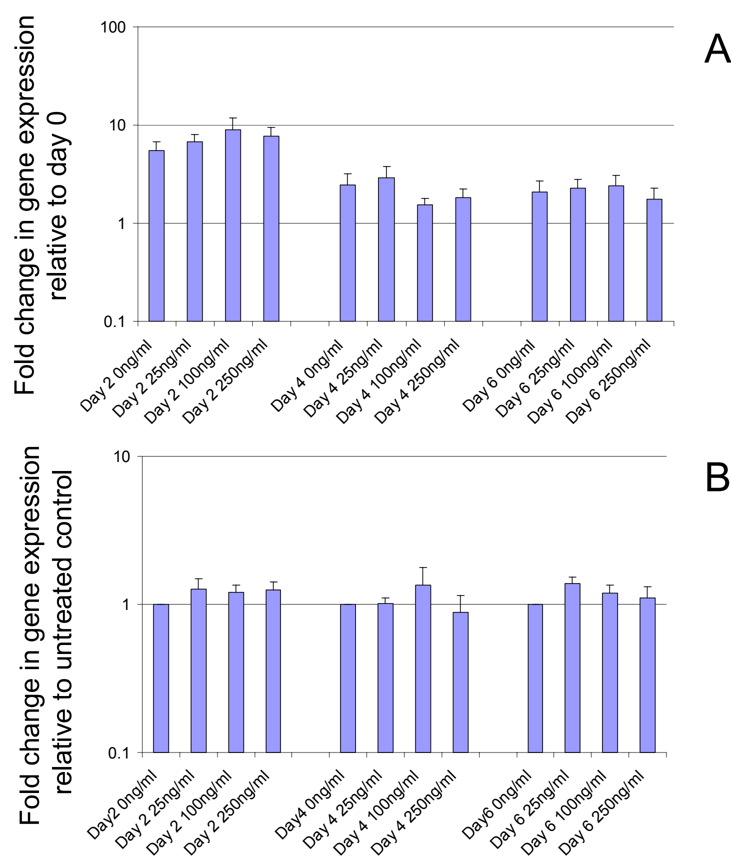


Figure 4.16 Graphs showing the fold difference in gene expression relative to: (A) day 0 and (B) the untreated age matched control for *Lmo2*.
Samples were taken at day 2, 4 and 6 and were differentiated in the presence of soluble BMP receptor at concentrations of 0, 25, 100 and 250ng/ml. Gene expression values represent the average achieved over 4 experiments. The error bars show standard error.

4.5.1 Discussion

The addition of BMP receptor had been expected to result in a reduction in *Runx1*, *Scl*, *Gata2* and *Lmo2* gene expression. However no reduction in gene expression occurred. This may be because the concentration of BMP receptor used was inadequate to

sufficiently antagonize the action of BMP4 so as to achieve a reduction in gene expression.

4.6 General discussion

ES cell differentiation experiments in chapter 3 produced variable results and did not show any effect for BMP4 in haematopoietic differentiation despite results showing otherwise in other published work (Park et al. 2004;Pearson et al. 2008). In order to address these issues the ES cell system was tested to ensure that: 1) the cells had an undifferentiated and pluripotent phenotype and 2) the cells could differentiate into haematopoietic cells. Cells were found to express SSEA-1 and high levels of alkaline phosphatase consistent with an undifferentiated phenotype. Cells expressed *Oct4*, *Sox2* and *Nanog* consistent with a pluripotent phenotype. The transient expression of the haemangioblast marker *Flk-1*, the demonstration of methylcellulose haematopoietic colony formation combined with differentiation cell surface marker data from previous experiments proved that these cells were capable of haematopoietic differentiation.

In previous foetal liver experiments there was evidence of secretion of BMP4 by cells in culture. BMP4 is a mesoderm patterning factor and therefore is secreted during development. It was therefore a concern that BMP4 was being secreted in 'negative control' serum free conditions. The expression of BMP4 and the two components of the BMP receptor: *Alk3* and *Bmpr2* were analysed. At all time points: Day 0, 2, 4 and 6 there was expression of both *Bmp4* and the two components of the BMP receptor.

Nevertheless other published work had indicated that BMP4 had a role in haematopoietic differentiation despite the endogenous expression of BMP4 by differentiating cells. With this in mind a BMP4 titration was carried out to find out whether other concentrations of BMP4 might produce changes in the expression of *Runx1*, *Scl*, *Lmo2* and *Gata2*. A significant increase in *Gata2* expression occurred with the addition of BMP4. This result is in agreement with work done by Lugus *et al*. which has produced similar findings (Lugus et al. 2007).

However there continued to be variability in the results from these experiments. This was probably in part due to the chaotic nature of the differentiation processes occurring in EBs. This may also have resulted from the variable expression of BMP4 by differentiating cells. A BMP receptor titration was carried out. However the concentration of BMP receptor may have been inadequate and no effects were seen on gene expression.

The general pattern of gene expression was similar in both the BMP4 and BMP receptor titration results. There were increases in *Runx1*, *Gata2* and *Lmo2* expression at day 2. The expression of *Scl* increased in the BMP4 experiments at day 4 whereas in the BMP receptor experiments there were smaller increases only at day 2. The early expression of *Runx1* in combination with *Gata2* and *Lmo2* probably indicates that *Runx1* has some role in the formation of the haemangioblast cells and primitive haematopoiesis. This would concur with data from Lacaud *et al.* that found a reduction in BL-CFCs and fewer

primitive erythroid cells produced from *Runx1* deletion mutant ES cells (Lacaud et al. 2002).

The secretion of BMP4 by serum free samples may have been variable between repeat experiments. In addition calculations comparing BMP4 treated samples to serum free samples assumed that serum free samples were BMP4 free. Thus the secretion of BMP4 by differentiating cells posed a major hurdle to further investigation of the role of BMP4 in haematopoietic development.

Chapter 5 Knockdown of *Bmp4* expression to investigate haematopoietic differentiation of ES cells

5.1 Introduction

In order to resolve the issues caused by the secretion of BMP4 by differentiating ES cells attempts were made to use short hairpin RNA (shRNA) to knock down BMP4 expression. The aim of these experiments was to provide a BMP4 free experimental system in which the effect of added BMP4 could be determined.

Homologous recombination could be used to knock down gene expression completely by generating a null mutation of the gene. This method has previously been used by Winnier *et al.* to produce BMP4 deletion mutant mice. Most embryos developed only to the egg cylinder stage showing little or no mesoderm differentiation. There was an absence of the expression of the mesodermal marker *Brachyury*. Embryos died between 6.5 and 9.5 dpc, too early for study of the effects of BMP4 on haematopoietic development (Winnier et al. 1995). These experiments were aimed at taking these results further by effectively rescuing the null phenotype in differentiating ES cells.

5.1.1 RNAi – triggering the pathway using siRNA versus shRNA

In recent years RNA interference (RNAi) has been developed as a technique for gene silencing. Experiments originally performed in C. elegans have shown that double stranded RNA (dsRNA) can be used to specifically and efficiently inhibit gene expression by causing the degradation of mRNAs with sequences homologous to that of the dsRNA (Fire et al. 1998). This occurs via a two step mechanism (Figure 5.1). First dsRNA is cleaved to 21-25 nucleotide small interfering RNAs (siRNAs) by Dicer enzymes, part of the RNase III ribonuclease family. The siRNAs are then unwound and incorporated into a multicomponent nuclease known as RNA induced silencing complex (RISC) which recognises and destroys mRNAs with homologous sequences (Hannon 2002).

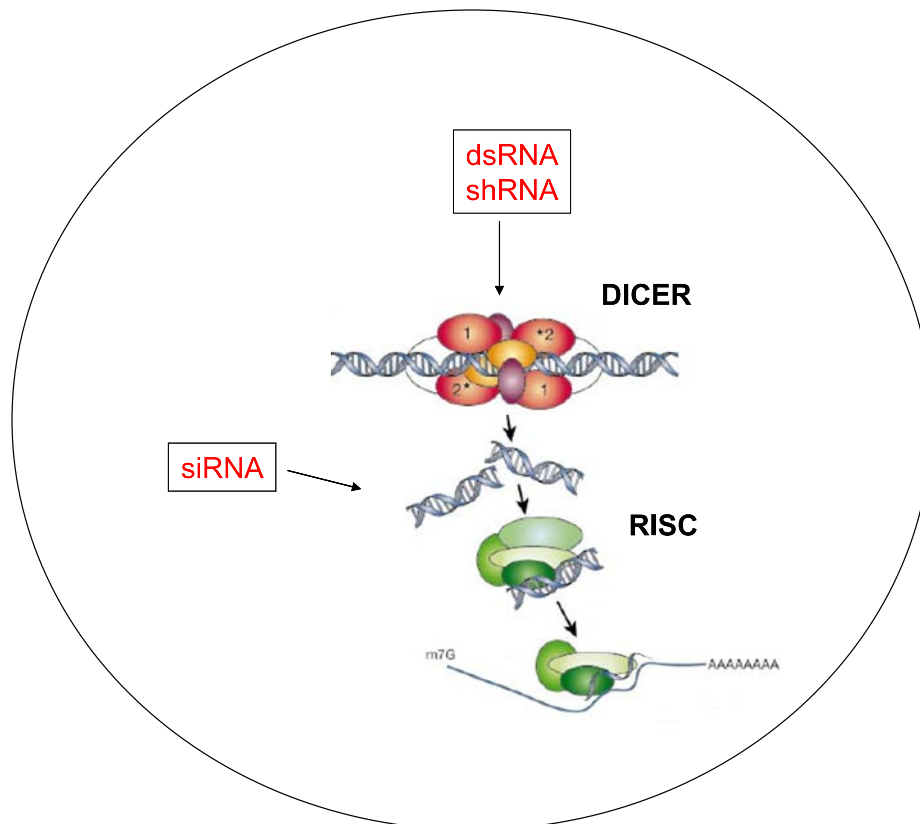


Figure 5.1 The RNAi pathway
dsRNA and shRNA is processed into 21 nucleotide fragments by the enzyme dicer. These fragments are bound by the RISC and unwound. mRNAs with homologous sequences are then recruited to the RISC and destroyed (adapted from Hannon et al. 2002).

Most mammalian cells have a potent antiviral response that is triggered by the presence of dsRNA mediated by dsRNA activated protein kinase (PKR). The ultimate outcome of this response is cell death via apoptosis. It was therefore a welcome surprise that dsRNA could be introduced to a range of cells inducing sequence specific silencing without producing a generalised cellular response (Paddison, Caudy, & Hannon 2002). However restriction of RNAi to these particular cell culture systems would have severely limited the usefulness of this technology.

Tuschl and colleagues were the first to demonstrate that short RNAs designed to mimic the products of Dicer could trigger RNA interference (Tuschl et al. 1999). These siRNAs enter the silencing pathway without triggering PKR dependent cellular changes. This has led to the use of chemically synthesised siRNAs to induce gene silencing in a wide range of human and mouse cell lines (Caplen et al. 2001). However the major disadvantage of siRNA is that the effects are transient.

Although the original purpose of the RNAi pathway was thought to be as a cellular defence mechanism against viruses, endogenous triggers of RNAi pathways have been described more recently. This was initially discovered due to the similarities in phenotype of *Dicer* deletion mutants and *Let-7* deletion mutants. The *Let-7* gene encodes a small highly conserved RNA species that regulates the expression of endogenous genes in worm development. The active RNA species is initially transcribed as a 70 nucleotide precursor and then is processed into a 21 nucleotide form by Dicer (Reinhart et al. 2000;Grishok et al. 2001). The *Let-7* gene has been discovered as founding member of a large group of small RNAs known as miRNAs thought to be involved in the post transcriptional regulation of gene expression (Lagos-Quintana et al. 2001). This mechanism has been exploited to induce sequence specific gene silencing. These short hairpin RNAs (shRNAs) can be cloned into a plasmid and synthesised from a RNA polymerase III promoter to induce long term stable suppression of a target gene (Paddison et al. 2002).

The aim of these experiments was to use plasmids expressing shRNA to knockdown *Bmp4* expression in differentiating ES cells so as to create a BMP4 free experimental system.

5.1.2 pLKO.1 plasmid

A set of five plasmids containing different shRNA sequences to knockdown BMP4 expression were obtained from Sigma Aldrich. The shRNA sequences were designed using Sigma's proprietary algorithm that scores potential sequences for efficient knockdown and sequence specificity to minimise off target effects. The shRNA sequences were designed to produce a hairpin structure consisting of a 20-21 base pair stem and a 6 base loop that would be recognised and cleaved by Dicer upon expression via a U6 RNA polymerase III promoter in the host cell. The resulting siRNA duplex could then incorporate into the RISC to suppress BMP4 expression. The plasmid contained an ampicillin resistance marker to allow for plasmid propagation in E. coli and a puromycin resistance marker for stable selection in mammalian cells. The expression of puromycin resistance was driven by a *PGK* promoter.

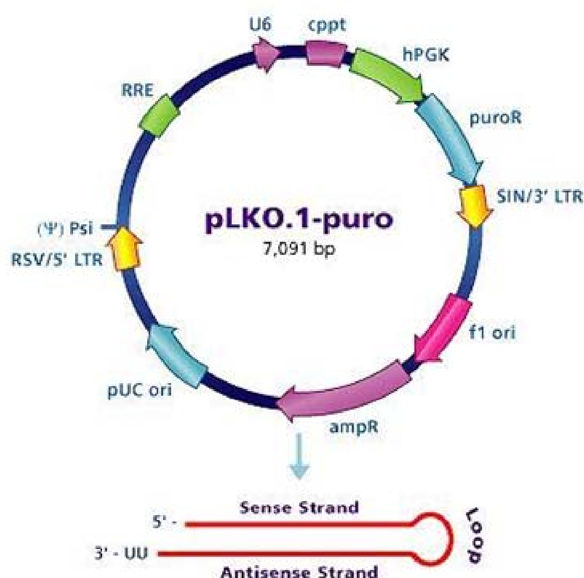


Figure 5.2 Features of the pLKO.1 puro plasmid
This plasmid contains a U6 promoter to drive shRNA expression, a puromycin resistance gene (*puroR*) driven by a human PGK promoter (hPGK) and an ampicillin resistance (*ampR*). The pUC origin (pUC ori) is an origin of replication allowing propagation of the plasmid in E. coli. The F1 origin (f1 ori) allows single stranded DNA synthesis. The 5'LTR, 3'LTR, RRE and Ψ signal are all features taken from lentiviruses explained in section 5.3. The diagram illustrates the structure of a short hairpin RNA. This diagram is adapted from the Sigma Aldrich website.

5.2 Transfection of ES cells

DNA can be delivered to cells using viral and non-viral methods. Viral methods are more time consuming and costly than non viral methods. shRNA expressing plasmids were therefore delivered to ES cells by transfection using a non viral method. Transfection can be achieved by electroporation and lipofection. A comparison of electroporation and lipofection in ES cells showed that lipofection achieved a 5.8 fold higher transfection efficiency than electroporation (Ma et al. 2004). Lipofection was therefore used in preference to electroporation.

5.2.1 Lipofection

Lipid molecules can be used to mediate gene delivery in a process known as lipofection. When lipids are mixed with DNA the lipids form spheres called liposomes with DNA trapped in the aqueous centre. When added to cells cultured in vitro these liposomes fuse with cell membranes and are taken up into cells via endocytosis. The use of lipid molecules with a positively charged head linked to a hydrophobic tail allows the formation of lipoplexes when mixed with DNA. The positive charge of lipoplexes allows more efficient binding to negatively charged cell membranes. Once taken up into cells by endocytosis there would normally be fusion of the vesicle contents with lysosomes and the degradation of DNA contained within. However the inclusion of the molecule dioleoylethanolamine (DOPE) allows entrapped DNA to escape the endosomes. The DNA can then go to the nucleus of the cell where it can use the transcriptional machinery of the cell (Kobayashi et al. 2005).

Ma *et al*. transfected murine ES cells with an enhanced green fluorescence protein (eGFP) expression plasmid by lipofection and quantified transfection efficiency. They found that 5.75% of cells expressed GFP two days after transfection. They went on to generate knock in mice using stably transfected cells. Thus lipofection can be used to stably transfect ES cells.

Lipofection of shRNA expressing plasmids has been tested using a GFP reporter system in murine ES cells (Tang et al. 2004). The reagent lipofectin was used to transfect ES cells. Transfection efficiency was around 15%. Cells were harvested two hours after

transfection and GFP expressing cells quantified. Knockdown of GFP expression was achieved. The transfected ES cells showed no difference in morphology or growth rate compared with untransfected ES cells.

However there are few papers where successful lipofection of ES cells has been carried out. The generation of stably transfected ES cells and knock in mice by Ma *et al.* shows that ES cells can maintain the phenotype brought about by the expression plasmid through differentiation. This however has not previously been shown with shRNA plasmids in the context of lipofection. Furthermore Tang *et al.* only quantified knockdown after 2 hours. In order to study haematopoietic differentiation it was necessary to induce knockdown over 6 days.

5.2.2 Lipofection of ES cells with pEFGP-N1

The first step was to analyse whether transfection in ES cells would be efficient enough to allow its use for the delivery of shRNA expression plasmids in future experiments. In order to achieve this ES cells were transfected with the pEGFP-N1 plasmid which expresses GFP (Figure 5.3A).

The pEGFP-N1 plasmid was amplified in *E. coli* bacterial culture and in order to test plasmid integrity a restriction digest was performed using *NdeI* and *NotI* (Figure 5.3B).

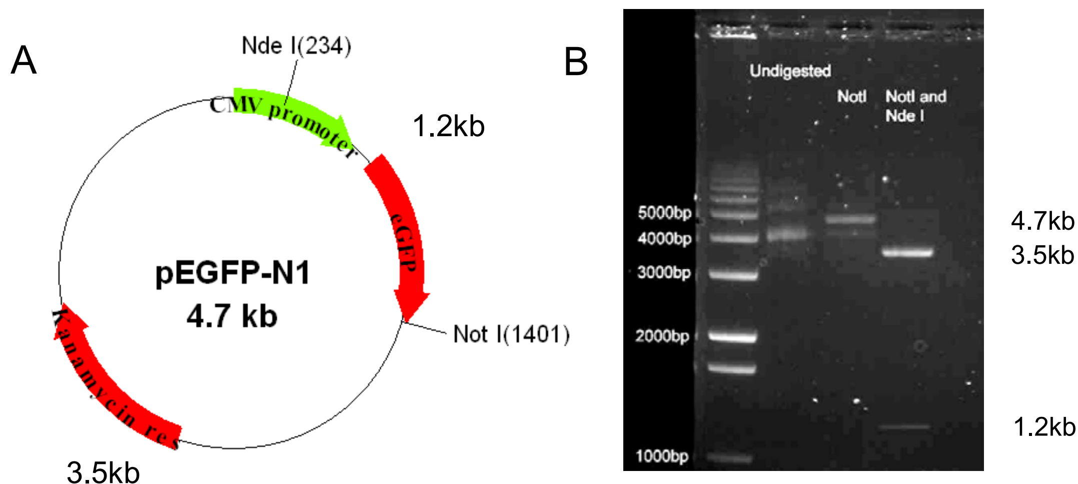


Figure 5.3 pEGFP-N1 plasmid
A shows the features of the pEGFP-N1 plasmid (4.7kb). eGFP expression is driven by a CMV promoter. The plasmid also carries a kanamycin resistance gene. Plasmid integrity was checked after bacterial propagation by restriction digest with *NotI* and *NdeI* as shown in **B**. This double restriction resulted in the formation of 3 fragments: 3.5kb and 1.2kb in size.

Lipofectamine has previously been used to transfect GFP expression plasmids into murine ES cells (Ma et al. 2004). Cells were transfected by adding 1.6µg of plasmid DNA to 4µl of lipofectamine 2000 on 200,000 cells. For comparison cells were treated with lipofectamine alone, DNA alone and untreated. The transfection efficiency was determined by flow cytometry at days 2, 4 and 6 of differentiation. The percentage of GFP expressing cells and the expression of GFP per cell was quantified at each time point. The experimental protocol is summarised in Figure 5.4.

Future experiments using shRNA expression plasmids would seek to suppress expression of BMP4 over 6 days of differentiation, it was therefore important to quantify transfection efficiency over this period.

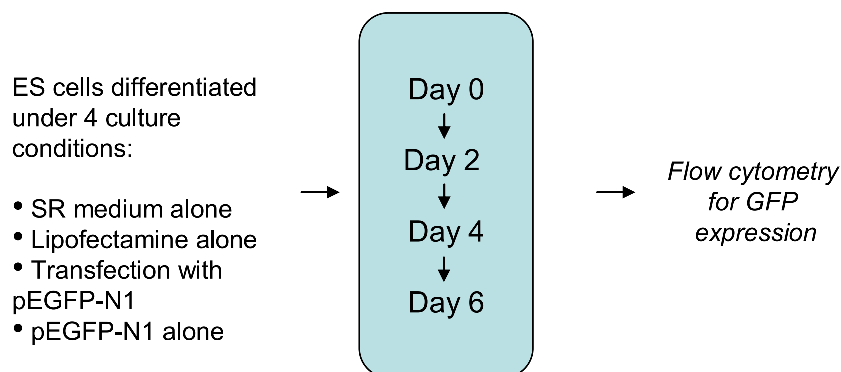


Figure 5.4 Experimental protocol used in testing the transfection efficiency of ES cells.

Three repeat experiments were carried out. At days 2, 4 and 6 GFP expression was visualised in differentiating EBs using fluorescent microscopy. As can be seen in Figure 5.5 transfected EBs at day 2 and 4 could be seen to brightly express GFP however at day 6 no GFP expression could be seen.

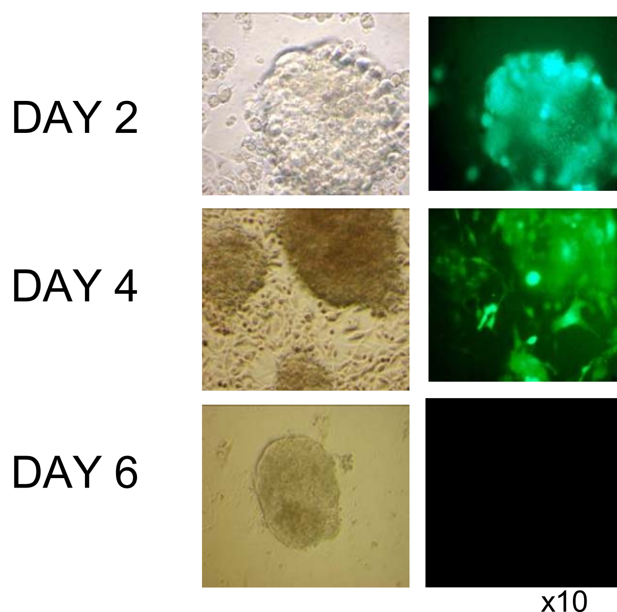


Figure 5.5 GFP expression in transfected ES cells
Micrographs of EBs taken at days 2, 4 and 6 after transfection with a light microscope (left) and with a fluorescent microscope to detect GFP expression (right) at 10x magnification.

Flow cytometry revealed that in transfected day 2 EBs 44.7% of cells expressed GFP (Figure 5.6). This was a significantly higher percentage than in the untreated cells ($P<0.05$). However the percentage of GFP expressing cells dropped back to 9.6% at day 4 and 6.2% at day 6. These levels were not significantly different compared with untreated cells at the same time points.

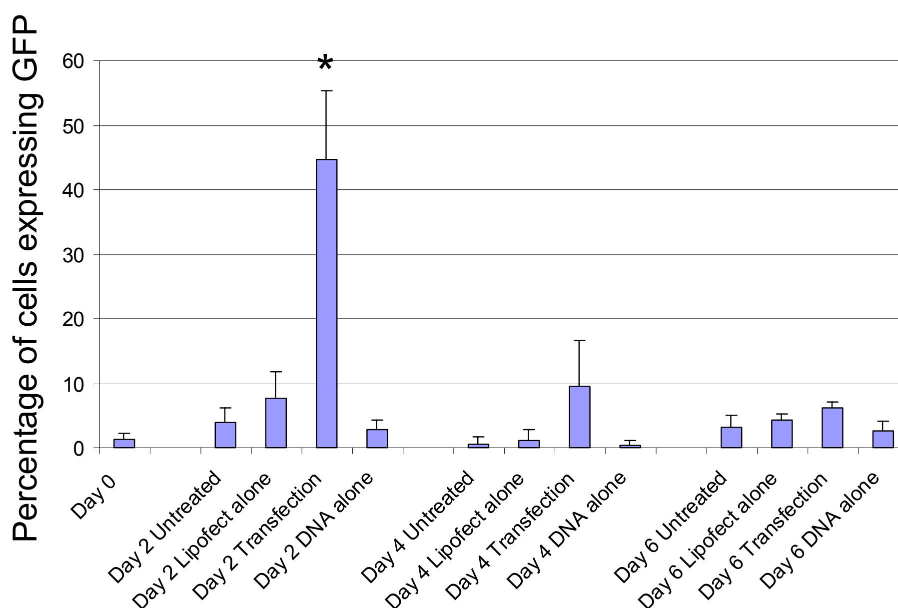


Figure 5.6 Lipofection of ES cells with pEGFP-N1 – GFP expression
The percentage of GFP positive cells as detected by flow cytometry at day 2, 4 and 6 when untreated, with treated with lipofectamine alone, with plasmid DNA alone and when transfected. Results are the average of 3 experiments. Error bars indicate the standard error. * indicates results significant at $P<0.05$.

The mean fluorescence intensity measures the amount of GFP expression per cell providing another measure of plasmid uptake. Although the mean fluorescence intensity was on average higher at day 2 this increase was not statistically significant when compared with untreated cells (Figure 5.7).

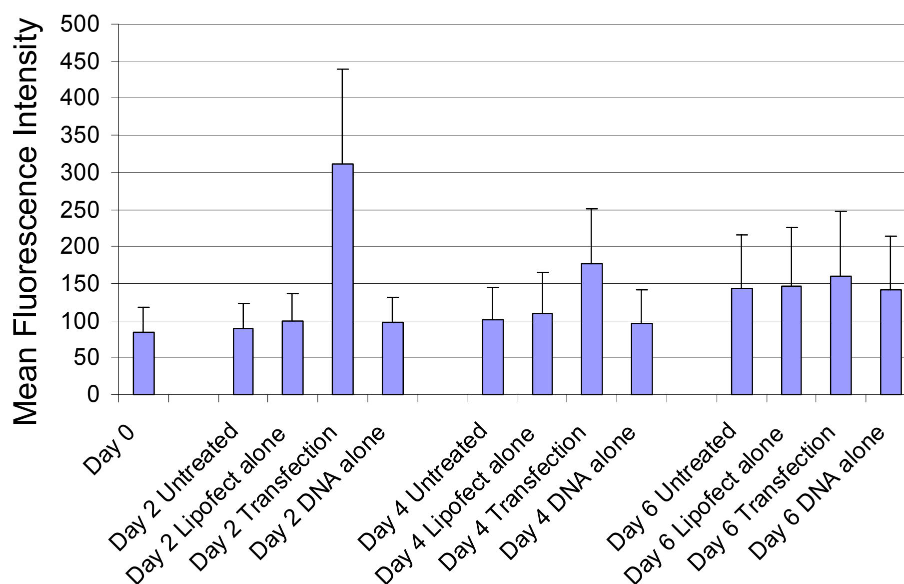


Figure 5.7 Lipofection of ES cells with pEGFP-N1 – Mean Fluorescence Intensity
The mean fluorescence intensity at day 2, 4 and 6 when untreated, with treated with lipofectamine alone, with plasmid DNA alone and when transfected. Results are the average of 3 experiments. Error bars indicate the standard error. * indicates results significant at $P<0.05$.

These results showed that it was possible to achieve transfection of ES cells using lipofection. The transfection efficiency of 44.7% at day 2 was higher than recorded in previous experiments. This may be due to the cells being transfected at the onset of differentiation compared to previous studies which have transfected undifferentiated cells. The effect however was transient with the percentage of GFP expressing cells being comparable with that in the untreated control at day 4 and day 6.

5.2.3 Lipofection of ES cells with pLKO.1 shRNA plasmids

Given the success of transfection using the pEGFP-N1 plasmid further experiments were carried out to determine the kinetics of knockdown using the set of five shRNA expressing plasmids obtained from Sigma. Although the shRNA sequences in these plasmids were predicted to knockdown *Bmp4* this can only be determined by testing. Therefore the aim of these experiments was to determine which shRNA sequence produced the highest knockdown of *Bmp4* expression. This plasmid would then be used in subsequent ES cell differentiation experiments.

The murine stromal cell line ST2 was previously used to test *Bmp4* primers as it is known to express *Bmp4* (see Section 4.3). In these experiments the SNH cell line was used to assay *Bmp4* knockdown with each of the five plasmids. Therefore SNH cells underwent lipofection with each of the five plasmids and then were cultured for 6 days. An untreated control was cultured for comparison. A further control group of cells was treated with lipofectamine alone to check that the transfection reagent was not affecting cell behaviour. Also a control pLKO.1 plasmid with no shRNA sequence was transfected. This was to ensure that the plasmid alone was not altering cell behaviour.

Samples were taken for analysis at day 0, 2, 4 and 6. This was to determine knockdown at each of the time points that would be analysed in future experiments. Samples were analysed by RT-PCR for *Bmp4* expression. The experimental protocol is summarised in Figure 5.8.

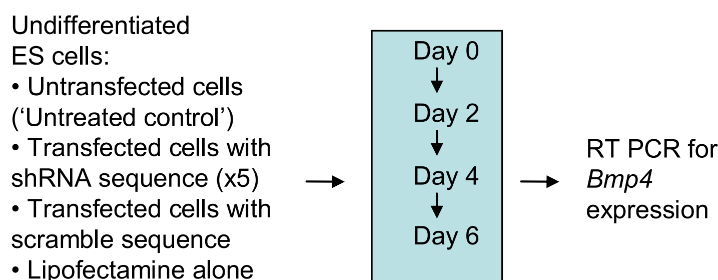


Figure 5.8 The experimental protocol used in testing shRNA expressed from pLKO.1 plasmids on ST2 cells

The knockdown of *Bmp4* was initially determined using semi-quantitative PCR. *Bmp4* primers had already been tested and used in previous experiments (see Section 4.3). Furthermore at this point the main aim was to choose the plasmid which would produce the best knockdown. Thus precise quantification was unnecessary.

The quantity of expression was estimated by computer analysis based on the brightness of the band visualised on the gel. As can be seen in Figure 5.9 there were no differences in band intensity using any of the sequences at any of the time points. This may have been due to saturation of the PCR. That is to say the amount of amplified product appeared the same because the PCR reaction had not been stopped during the exponential phase of the reaction. In order to optimise this it would have been necessary to conduct further experiments to determine the number of cycles necessary for the PCR to be in the exponential phase. This would have been time consuming. It was therefore decided that real time PCR was a faster and superior technique for determining *Bmp4* knockdown.

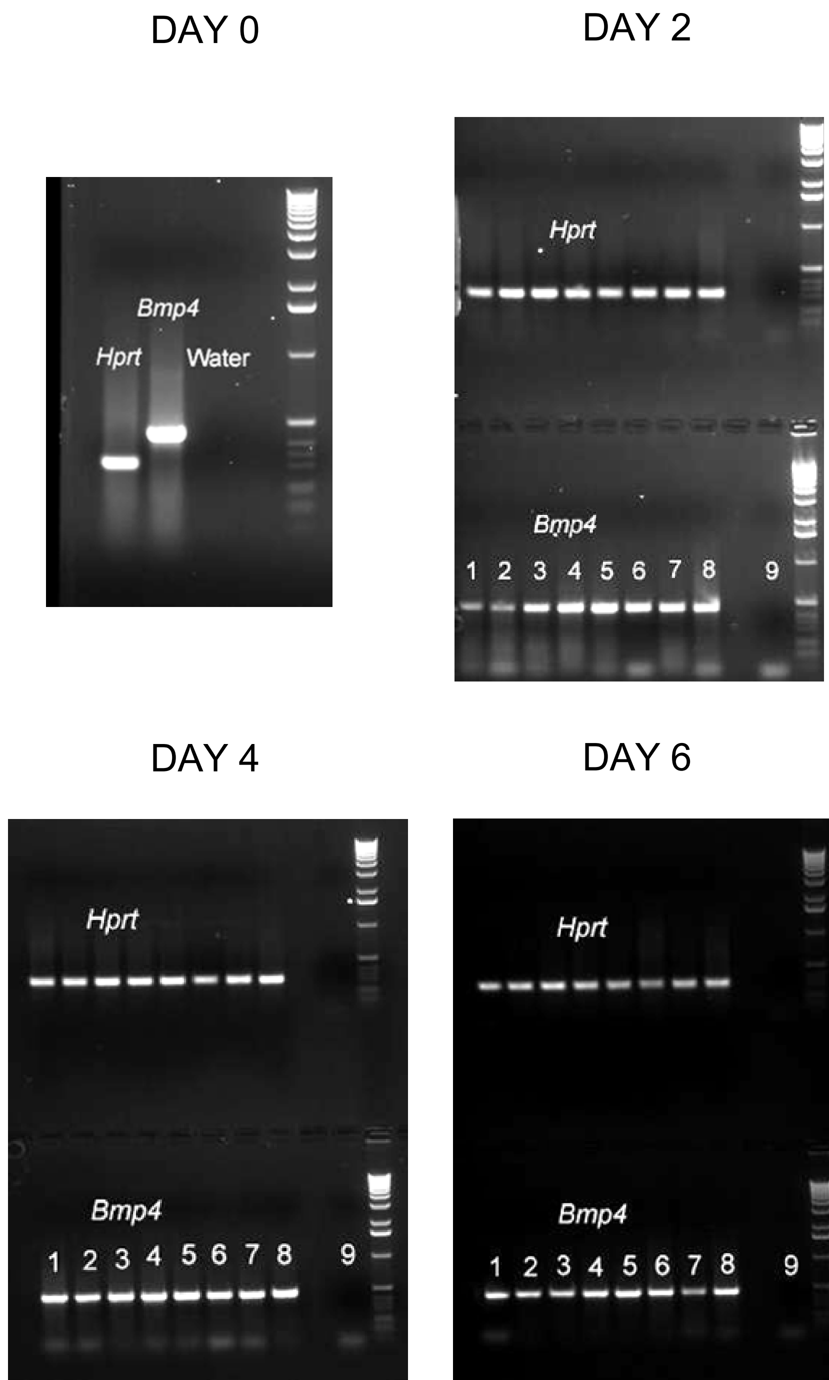


Figure 5.9 Testing shRNA expressed from pLKO.1 plasmids on ST2 cells using RT-PCR
The expression of *Bmp4* and the housekeeping gene *Hprt* at Day 0, 2, 4 and 6 in ST2 cells was determined by semi-quantitative PCR. Lane 1 shows expression in untreated cells. Lanes 2-6 show expression in cells treated with each of the 5 shRNA plasmids. Lane 7 shows expression in cells treated with pLKO.1 plasmid with no shRNA sequence. Lane 8 shows expression in cells treated with lipofectamine alone and Lane 9 shows expression in the water control.

Bmp4 knockdown was calculated relative to the untreated control. Results are shown in Figure 5.10. Overall *Bmp4* knockdown begun at day 2 and was at its maximum at day 4. The knockdown in expression was transient and by day 6 there was no reduction in *Bmp4* expression. Plasmids 2 and 4 produced the greatest knockdown at day 4 ($P<0.05$). However there was also a reduction in *Bmp4* expression in both the plasmid without shRNA control and transfection reagent alone control ($P<0.05$). In order to determine whether this was an anomaly or a genuine result the experiment was repeated.

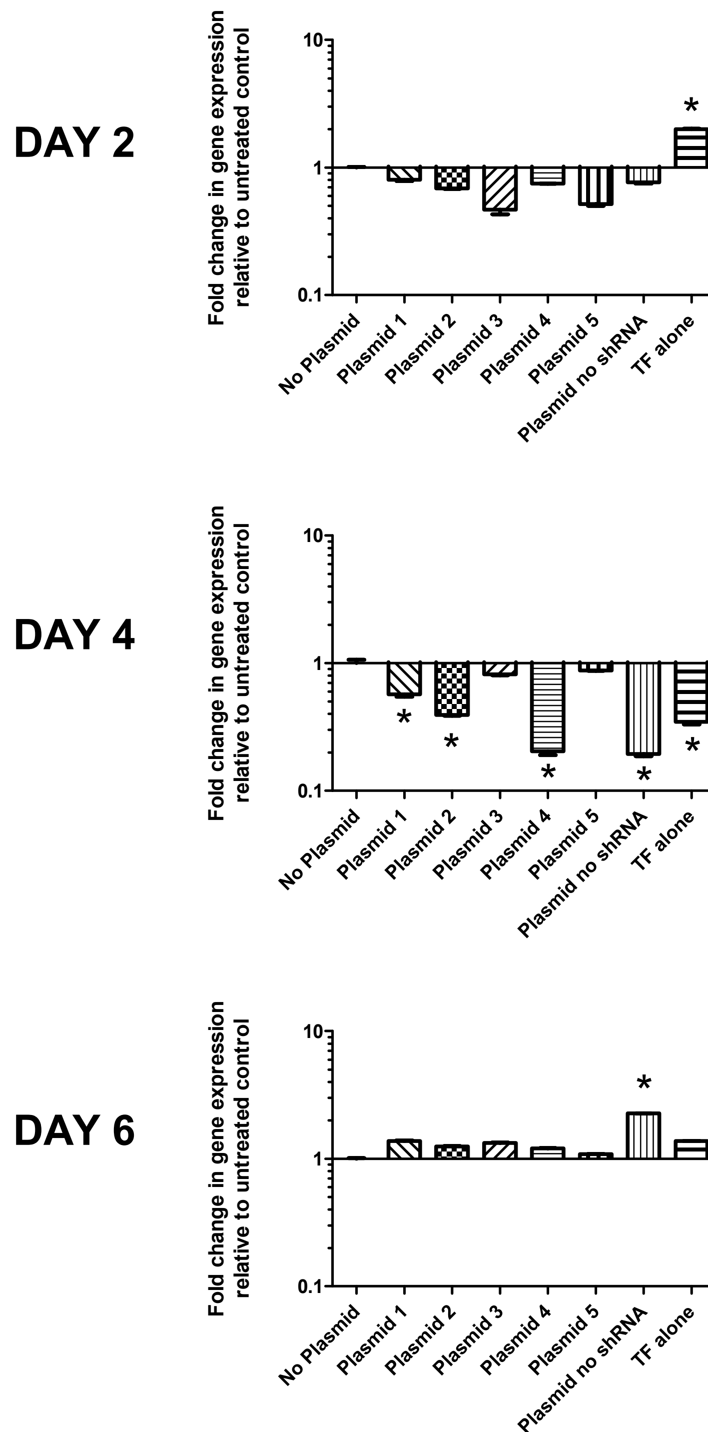


Figure 5.10 Testing shRNA expressed from pLKO.1 plasmids on ST2 cells using real time PCR – Experiment 1
Logarithmic graphs showing fold change in *Bmp4* expression relative to the untreated control in ST2 cells in cells treated with each of the 5 shRNA plasmids, in cells treated with pLKO.1 plasmid with no shRNA sequence, in cells treated with lipofectamine (TF) alone and in the water control. Results are the average of 3 experiments. Error bars indicate the standard error. * indicates change in BMP4 expression relative to the untreated control at $P<0.05$.

In the second experiment (Figure 5.11) overall the highest level of knockdown was achieved at day 2. The level of knockdown reduced from these levels at day 4 and day 6. Plasmid 4 produced the highest level of knockdown at day 2 and plasmid 2 produced the highest level of knockdown at day 4 ($P<0.05$). There was again knockdown of *Bmp4* expression in the transfection reagent alone control at day 2 ($P<0.05$). However there wasn't a reduction in *Bmp4* expression in the plasmid without shRNA control as well.

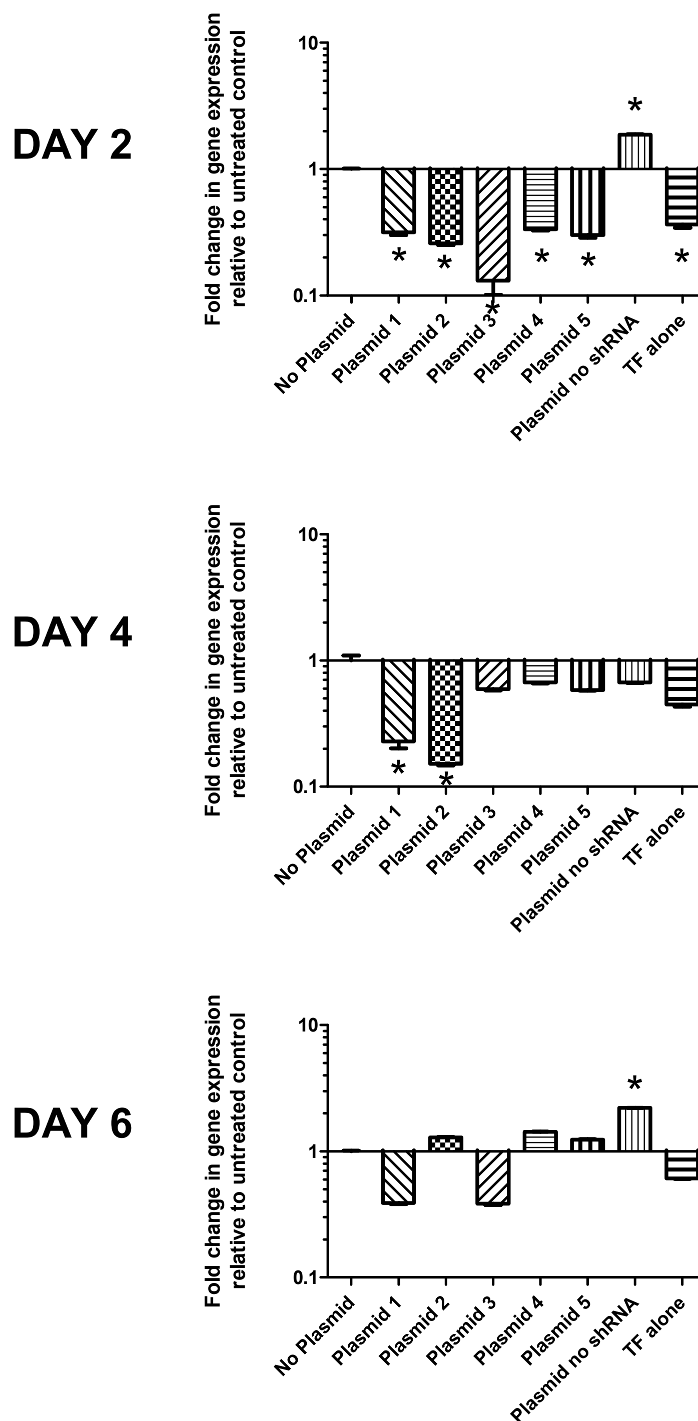


Figure 5.11Testing shRNA expressed from pLKO.1 plasmids on ST2 cells using real time PCR – Experiment 2

The experiment was repeated to confirm whether initial results showing knockdown with transfection reagent alone was accurate. These logarithmic graphs show fold change in *Bmp4* expression relative to the untreated control in ST2 cells in cells treated with each of the 5 shRNA plasmids, in cells treated with pLKO.1 plasmid with no shRNA sequence, in cells treated with lipofectamine (TF) alone and in the water control. Results are the average of 3 experiments. Error bars indicate the standard error. * indicates change in BMP4 expression relative to the untreated control at $P<0.05$.

In both experiments plasmid 2 induced the highest level of knockdown at day 4. However these results also indicated that the transfection reagent was changing the behaviour of the cells causing a reduction in *Bmp4* expression. It was a concern that ES cells might be even more sensitive to the transfection reagent. It was therefore necessary to either use a different transfection reagent or method of DNA delivery in further knockdown experiments.

This result in addition to previous work that suggested that high toxicity was caused by lipid based transfection reagents in ES cells led to a decision not to pursue the use of other transfection reagents for plasmid delivery (Ma et al. 2004;Kobayashi et al. 2005).

5.3 Development of Lentiviruses expressing shRNA for knockdown of BMP4

Lentiviral vectors are derived from the human immunodeficiency virus, HIV-1. HIV-1 is a retrovirus and has an RNA genome that is replicated via a DNA intermediate. In the host cell, reverse transcriptase is used to create the DNA intermediate that can then be integrated into the host genome using integrase. HIV-1 alternates between two forms: the provirus and the virion. The provirus describes the double stranded DNA that has integrated into the host genome. Viral RNA and proteins are produced from the provirus using the host's transcription and translation apparatus. Assembled viruses then bud off from the host's cell membrane as virions capable of infecting other cells.

Lentiviral vectors have several characteristics that make them excellent mediators of DNA delivery (Kobayashi et al. 2005). Firstly they have been shown to be capable of infecting many different types of cells including dividing and non-dividing cells. They generally exhibit low cytotoxicity. Also they integrate their genetic material into host chromosomes with high efficiency resulting in stable expression of transgenes (Naldini et al. 1996).

Lentiviral vectors in comparison to other types of retroviral vectors are less susceptible to silencing in ES cells (Ellis 2005). ES cells genetically modified with retroviruses shut down expression of integrated transgenes. The mechanisms are thought to include DNA methylation, histone deacetylation and binding of negative trans-acting factors to integrated transgenes (Chilton & Le Doux 2008).

Several papers have now been published in which lentiviruses have been used to achieve stable expression of lentiviral transgenes in murine ES cells. Pfeifer *et al.* have achieved stable transgene expression in undifferentiated murine ES cells. The transgene expression was maintained during EB differentiation and also *in vivo*. EB differentiation was studied for a period of 8 days and occurred normally. ES cells carrying the transgene were used to generate transgenic mice successfully (Pfeifer et al. 2002). Kosaka *et al.* have also shown that lentiviruses are capable of efficient gene delivery in undifferentiated murine ES cells. They add that there was no transduction associated cytotoxicity (Kosaka et al. 2004). Hamaguchi *et al.* have transduced murine ES cells with GFP using a lentiviral vector and shown that GFP was expressed through to day 6 in EB

differentiation. Haematopoietic colonies derived from these EBs also expressed GFP. Thus lentiviruses are capable of mediating gene delivery in ES cells. Furthermore stable transgene expression can be maintained in EB differentiation and EB derived haematopoietic cells.

Liu *et al.* have used lentiviral vectors expressing shRNA targeting *Sox17* expression to investigate cardiac muscle formation in differentiating murine ES cells. The knockdown of *Sox17* expression lasted for 12 days of differentiation (Liu et al. 2007). This experiment provides proof of principle for the use of shRNA expressing lentiviral vectors to knockdown gene expression in differentiating murine ES cells.

The pLKO.1 plasmids obtained from Sigma had features allowing the generation of lentiviral particles (see Figure 5.2). Within the plasmid there was a 5' long terminal repeat (LTR), a 3'LTR, Ψ encapsidation signal, central polypurine tract and Rev response element all of which are lentiviral features. The 5'LTR acts as a promoter of viral transcription by RNA polymerase II. Although the 3'LTR has the same sequence arrangement as the 5'LTR it does not normally act as a promoter instead it is involved in transcription termination and polyadenylation. Each LTR has a U3, R and U5 region. In this vector the U3 region of the 3'LTR had been engineered with a 400 base pair deletion. Because in reverse transcription the U3 region of the 3'LTR is used as a template to generate the U3 regions of the 3' and 5'LTR, modifications in 3'LTR are copied to the 5'LTR. These vectors are therefore self-inactivating in that once they integrate they are incapable of synthesising vector transcripts and rely on internal promoter activity. These

self inactivating (SIN) vectors are replication incompetent. The SIN mutation also alleviates concerns regarding cellular non-specific gene activation caused by promoter insertional mutagenesis.

Other features included the Ψ encapsidation signal which normally ensures only full length transcripts are packaged into virions. The central polypurine tract is thought to be involved in the nuclear import of lentiviral DNA. The Rev response element normally binds Rev to allow nuclear export of viral RNA.

Thus in order to prepare competent virus it was necessary to transfect these plasmids into a packaging cell line.

5.3.1 Cloning GFP-WPRE into pKLO.1

In order to facilitate easier virus titring and analysis of transduction efficiency, green fluorescent protein and the woodchuck hepatitis virus post-transcriptional regulatory element (GFP-WPRE) was cloned into plasmid 2 (Figure 5.12). The shRNA sequence from plasmid 2 had induced the highest level of knockdown compared with other plasmids in the previous transfection experiments and so was chosen for use in further experiments. By cloning GFP-WPRE into the lentiviral construct virus titring and transduction efficiency could be determined quickly and easily by analysing the percentage of GFP expressing cells using flow cytometry.

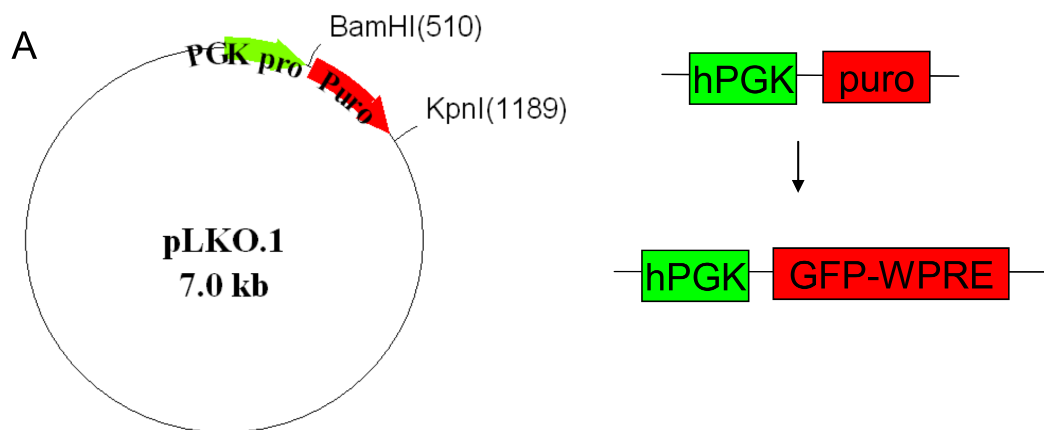


Figure 5.12 Cloning strategy
Puromycin resistance gene was excised and a GFP-WPRE fragment from the SEW plasmid was cloned in.

WPRE acts to increase transgene expression in this case GFP. It is thought to act at a post-transcriptional level however the exact mechanism is not known (Zufferey et al. 1999).

A restriction digest was performed to excise GFP-WPRE, a 1.4 kilobase (kb) fragment from the SEW plasmid using *BamHI* and *EcoRI*. The SEW plasmid was chosen only because it contained the GFP-WPRE fragment and any plasmid containing this fragment could have been used. Also the puromycin resistance gene a 679 base pair (bp) fragment was excised from the pLKO.1 plasmid using the same enzymes, *BamHI* and *EcoRI*. The 6.3kb pLKO.1 backbone and 1.4kb GFP-WPRE fragments were resolved by gel electrophoresis. These bands were cut out of the gel and the DNA gel extracted (Figure 5.13).

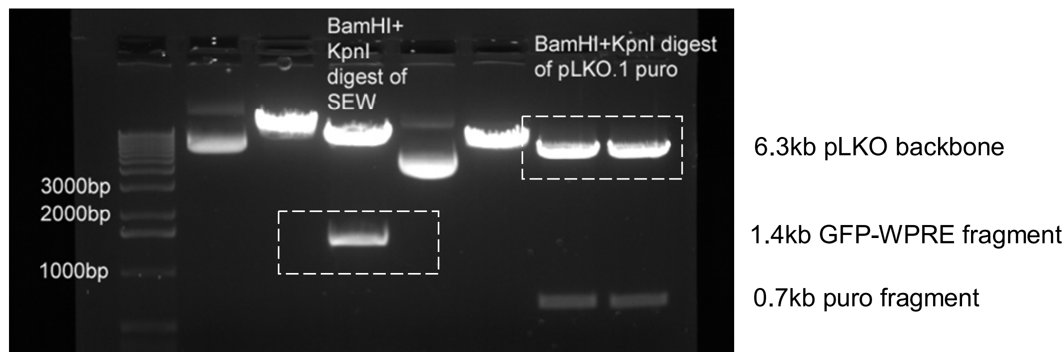
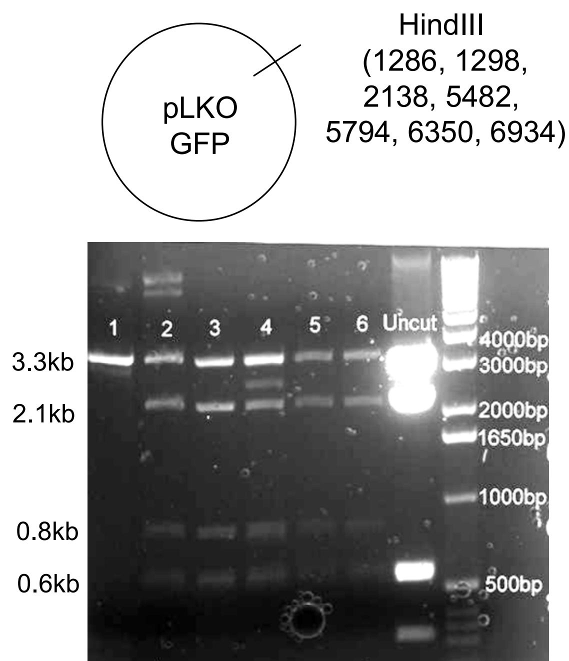


Figure 5.13 SEW and pLKO.1 restriction digest
Restriction digest of SEW and pLKO.1 puro plasmids with BamHI and KpnI in order to obtain pLKO.1 backbone and GFP-WPRE insert (highlighted fragments). The puro fragment was removed from the pLKO.1 plasmid.

The pLKO.1 backbone was then phosphatase treated in order to remove phosphates group that can allow vectors that have been single digested to religate without taking up the GFP-WPRE insert. The aim was to reduce background colonies following transformation.

The phosphatase treated pLKO.1 backbone and GFP-WPRE fragment was then ligated. After overnight incubation competent bacteria were transformed by heat shock and then were cultured on plates selective for ampicillin resistant bacteria.

In order to confirm that resulting colonies contained pLKO.1-GFP-WPRE, plasmid DNA from six colonies was expanded by miniprep and then a series of restriction digests were performed. This included a single restriction digest with *HindIII* and also a double digest with *BamHI* and *KpnI* (Figure 5.14). This confirmed that GFP-WPRE had been successfully ligated into pLKO.1.



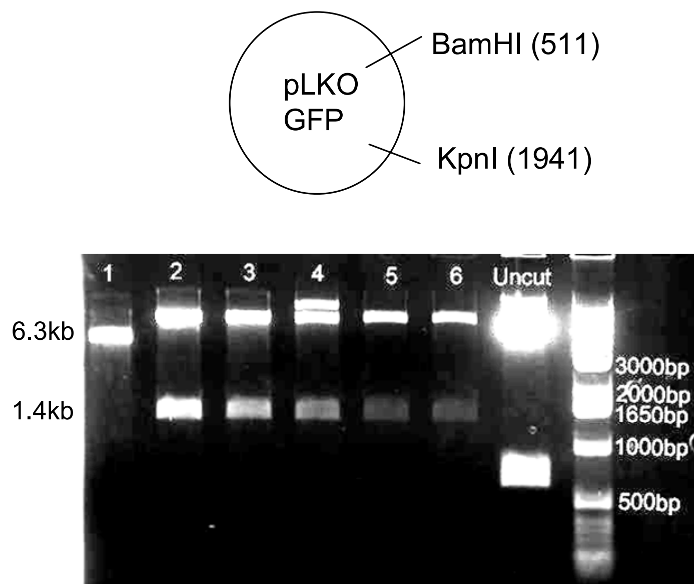


Figure 5.14 Restriction digests to test cloning
Following ligation and transformation, bacteria were cultured on ampicillin plates. 6 colonies were picked for restriction digest analysis to confirm successful insertion of GFP-WPRE into pLKO.1. Restriction digests used *HindIII* and *BamHI* with *KpnI*. Plasmid diagrams indicate sites at which enzymes cut the plasmid. Expected fragment sizes are indicated at the side of the gel.

5.3.2 Generating lentivirus

Lentiviral particles carry a coat protein that affects the ability of the virus to infect certain cell types. This viral coat is known as the pseudotype of the lentivirus. Previous experiments have used lentivirus vector pseudotyped with vesicular stomatitis virus G protein (VSV-G) to efficiently transduce murine ES cells (Hamaguchi et al. 2000). Thus VSV-G pseudotyped lentivirus was generated in these experiments.

In order to produce lentiviral particles the pLKO.1-GFP-WPRE plasmid was transfected into the human 293T packaging cell line along with the pMDG2 plasmid which expresses the VSV-G viral coat protein and the p8.74 plasmid that expresses the Gag and Pol proteins necessary for functional lentivirus production. Two days after transfection, the supernatant was harvested. The virus was then concentrated by ultracentrifugation and frozen down for future use.

A pLKO.1-GFP-WPRE plasmid carrying a 'scrambled' sequence (kindly donated by J. Metelo, Institute of Child Health) was also used to generate lentivirus. The 'scrambled' sequence consisted of 4 base pairs mismatched to any known mouse gene. The purpose of this plasmid was for use as a control in future experiments. This enabled testing of whether lentivirus alone could change the expression of BMP4 and cell behaviour in general.

The 293T cell line was observed to express GFP using a fluorescent microscope two days after transfection for both the pLKO.1-GFP-WPRE shRNA (Figure 5.15A). This provided confirmation of successful ligation of the GFP-WPRE fragments.

To determine the virus titre, 293T cells were transduced with a 5 fold dilution series of virus. The transduction efficiency was then measured by analysing the percentage of GFP expressing cells using flow cytometry. The dilution at which 5-15% of cells were transduced was determined (Figure 5.15B). The virus titre was then calculated by dividing the number of transduced cells in this well by the volume of virus used to transduce them. The titre for shRNA lentivirus was calculated as 2.8×10^8 and for scramble sequence lentivirus 4.325×10^8 virus particles per millilitre.

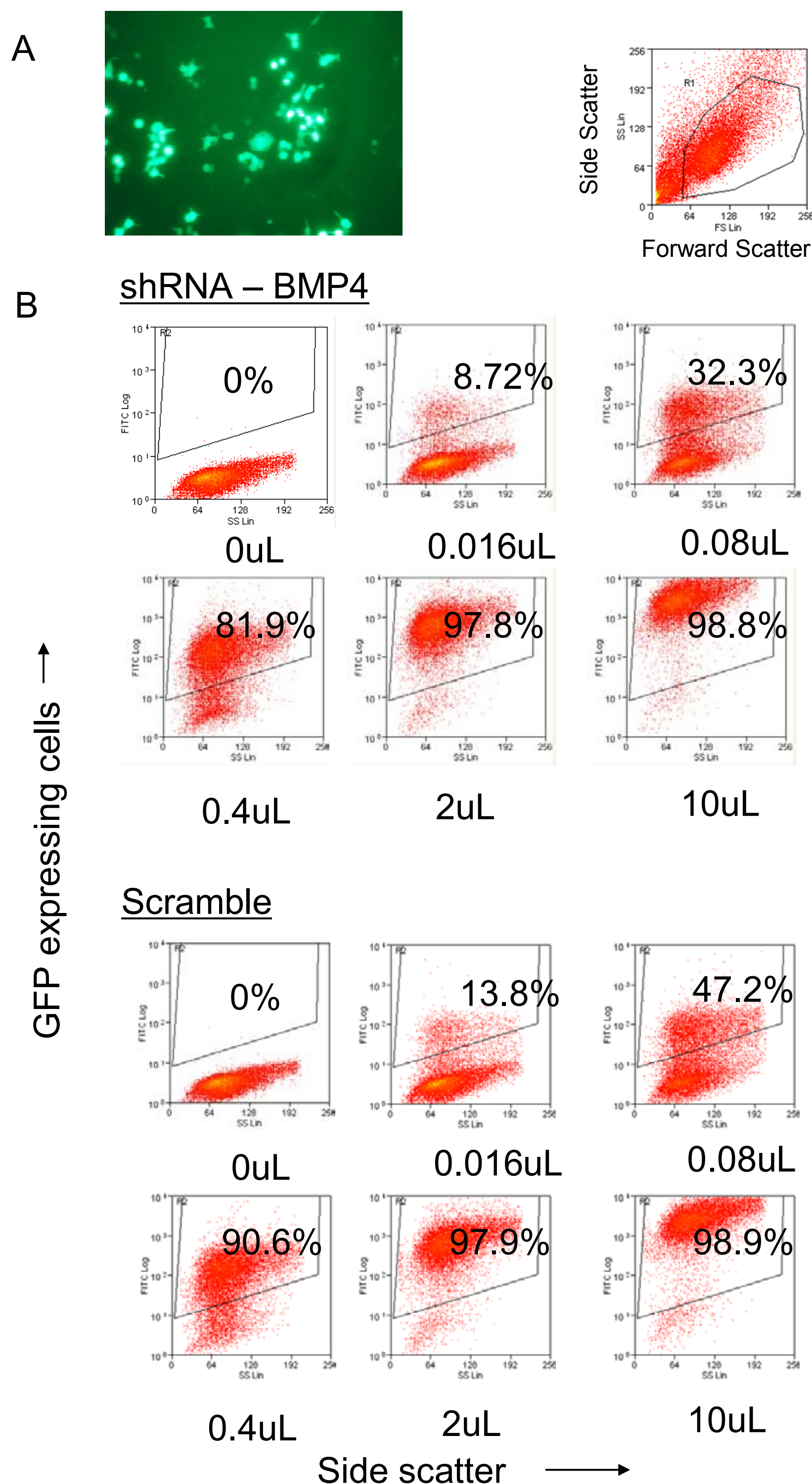


Figure 5.15 Generating shRNA and scramble sequence lentivirus
A shows GFP expressing 293T cells following transfection with shRNA GFP-WPRE pLKO.1 plasmid. **B** shows virus titre data for both the shRNA and scramble sequence lentiviruses. Virus titre was determined by transducing lentivirus with a 5 fold dilution series. The virus titre was calculated using the dilution at which 5-15% of cells were transduced.

5.3.3 Testing lentiviral shRNA in ST2 cells

Initial experiments were aimed at determining the ability of shRNA expressing lentiviruses to transduce cells and to knockdown *Bmp4* expression. The control scramble sequence lentivirus was included in experiments to determine whether the lentivirus alone would cause a reduction of *Bmp4* expression that might be indicative of non-specific cellular effects caused by the introduction of lentivirus.

ST2 cells, cells previously shown to express *Bmp4*, were seeded into a 24 well plate. The following day these cells were transduced with virus at a range of multiplicity of infection's (MOIs): 0, 0.16, 0.8, 4, 20 and 100. The MOI is defined as the number of lentiviral particles per cell introduced to the culture.

After 2 and 4 days samples were taken for analysis by flow cytometry to determine the percentage of GFP positive cells and therefore the transduction efficiency. Also samples were taken for quantitative RT-PCR so that the knockdown of BMP4 could be quantified. The experimental protocol is summarised in Figure 5.16. 3 repeat experiments were carried out.

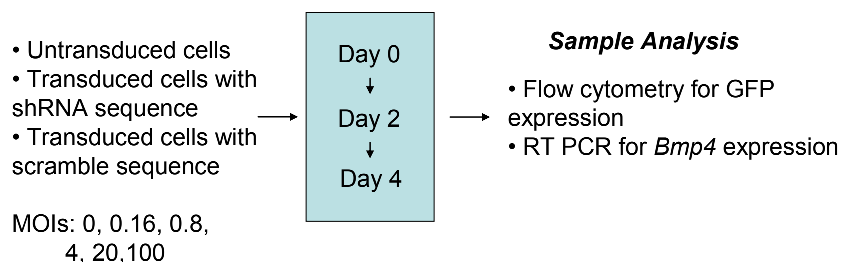
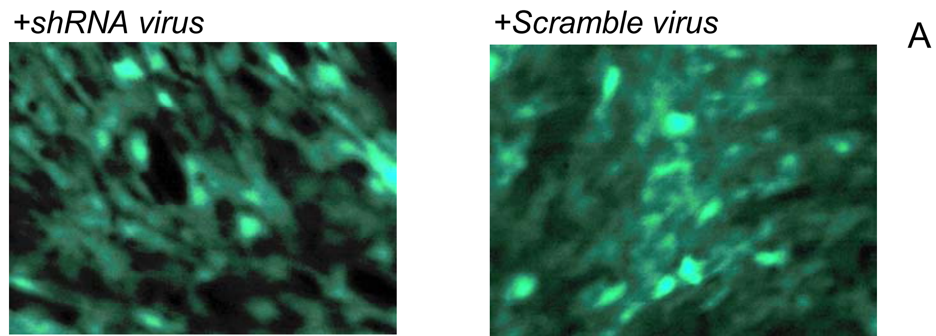


Figure 5.16 Experimental protocol for testing shRNA lentivirus

At day 2 GFP expression was visualised using a fluorescent microscope and was observed in both the shRNA and scramble treated ST2 cells (Figure 5.17A). Flow cytometry demonstrated that at day 2 an MOI of 4 and above was sufficient to produce a significant increase in GFP expressing cells above the level of untransduced cells with both the scramble and shRNA lentiviruses ($P<0.05$, Figure 5.17B). At day 4 the levels of transduced cells remained similar compared to day 2 levels. However an MOI of 0.8 and above was sufficient to produce an increase in GFP expressing cells above the level of untransduced cells with both scramble and shRNA sequences ($P<0.05$).



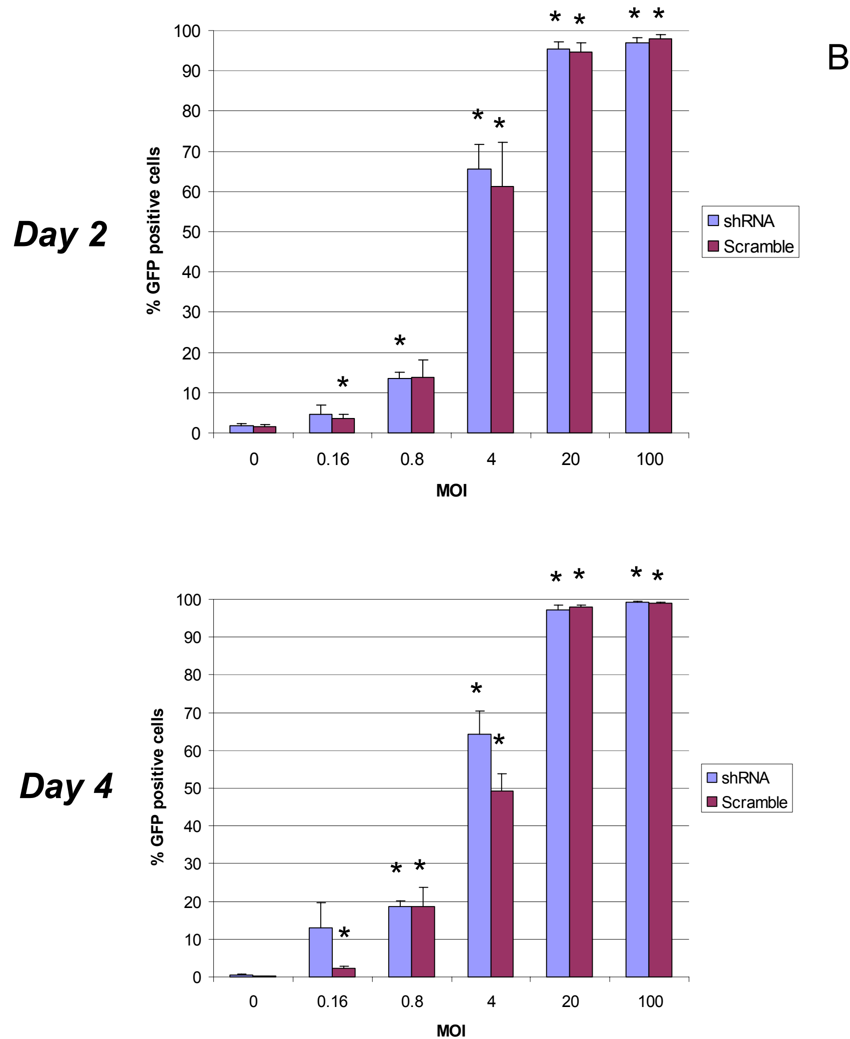


Figure 5.17 Testing shRNA lentivirus on ST2 cells – Transduction efficiency
A shows micrographs (10x magnification) obtained using a fluorescent microscope showing ST2 cells transduced with both shRNA and scramble sequence lentivirus (MOI 20) at day 2. **B** shows transduction efficiency measured as the percentage of cells expressing GFP. Cells were transduced with shRNA and scramble sequence lentiviruses at MOIs of 0, 0.16, 0.8, 4, 20 and 100. Results shown are the average of 3 experiments. Error bars represent the standard error. * indicates significant change at $P<0.05$ relative to untransduced cells.

Bmp4 expression analysis revealed that *Bmp4* knockdown was increased at higher MOIs. However at higher MOIs there was also *Bmp4* knockdown with the scramble sequence lentivirus (Figures 5.18 and Table 5.1). At day 2 an MOI of 20 produced significant knockdown only with the shRNA lentivirus ($P<0.05$) but at an MOI of 100 there was significant knockdown with both the shRNA and scramble lentiviruses ($P<0.05$). At day 4 an MOI of 0.8 was sufficient to produce a significant knockdown with the addition of shRNA lentivirus ($P<0.05$). However significant knockdown occurred in both the shRNA and scramble treated cells at MOIs of 4 and above ($P<0.05$).

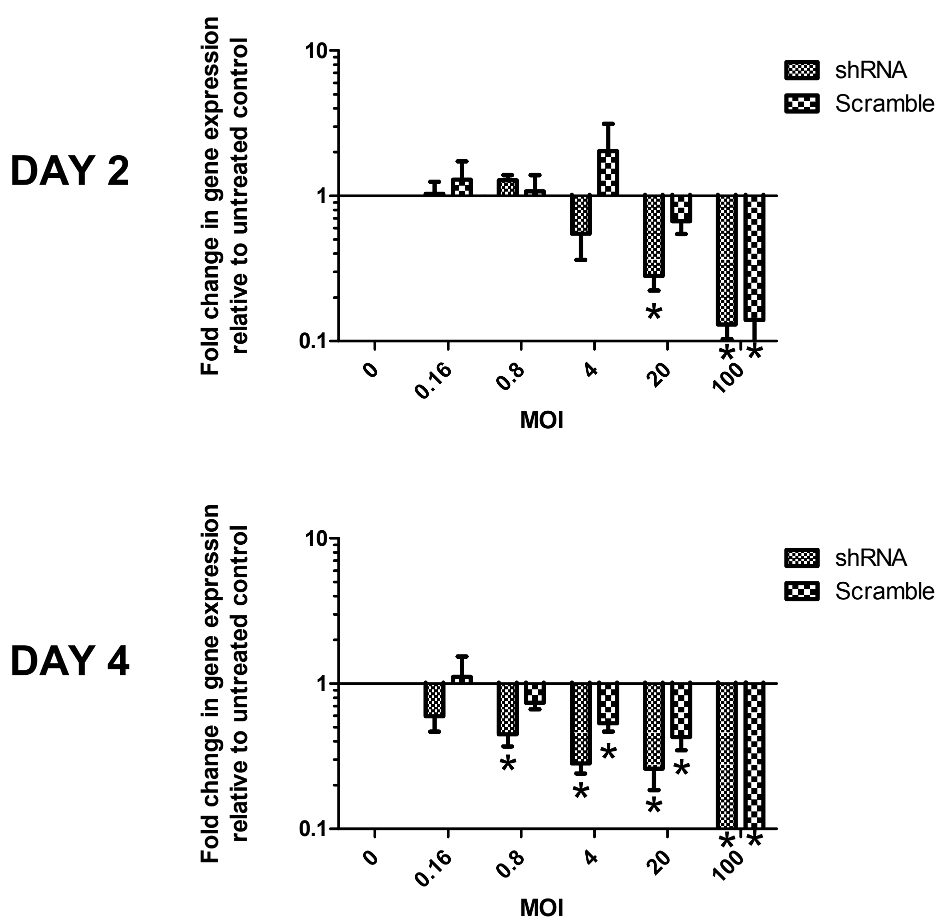


Figure 5.18 Testing shRNA lentivirus on ST2 cells – *Bmp4* expression
Logarithmic graphs showing fold change in *Bmp4* expression relative to the untreated control at day 2 and day 4. Cells were transduced with shRNA and scramble sequence lentiviruses at MOIs of 0, 0.16, 0.8, 4, 20 and 100. Results shown are the average of 3 experiments. Error bars represent the standard error. * indicates results significant at $P<0.05$ relative to untreated cells.

shRNA results

DAY 2	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	0.92	1.09	0.50	0.39	0.09
Exp 2	1	1.45	1.30	0.25	0.21	0.18
Exp 3	1	0.73	1.46	0.90	0.24	0.12
Average	**1**	**1.03**	**1.28**	**0.55**	**0.28**	**0.13**

DAY 4	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	0.59	0.38	0.33	0.41	0.18
Exp 2	1	0.82	0.60	0.32	0.18	0.04
Exp 3	1	0.37	0.36	0.20	0.19	0.05
Average	**1**	**0.59**	**0.45**	**0.28**	**0.26**	**0.09**

Scramble results

DAY 2	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	2.02	1.70	4.24	0.56	0.06
Exp 2	1	1.33	0.83	0.82	0.91	0.23
Exp 3	1	0.53	0.70	1.04	0.54	0.13
Average	**1**	**1.29**	**1.08**	**2.03**	**0.67**	**0.14**

DAY 4	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	1.94	0.89	0.66	0.27	0.11
Exp 2	1	0.85	0.68	0.53	0.52	0.08
Exp 3	1	0.55	0.65	0.42	0.50	0.08
Average	**1**	**1.11**	**0.74**	**0.53**	**0.43**	**0.09**

Table 5.1 Bmp4 expression tables
Fold change in *Bmp4* expression relative to the untreated control at day 2 and day 4. Cells were transduced with shRNA and scramble sequence lentiviruses at MOIs of 0, 0.16, 0.8, 4, 20 and 100. Results shown are from 3 experiments. P values indicate the statistical significance of results relative to untreated cells.

These experiments showed that both shRNA and scramble sequence lentiviruses were capable of transducing cells and achieving knockdown of BMP4 expression. With higher MOIs there was a greater level of transduction and knockdown. However knockdown in scramble treated cells occurred at higher MOIs. This may indicate toxicity caused by the introduction of lentivirus. Thus in ES experiments it would be important to use an MOI which would achieve optimal knockdown with the lowest toxicity.

5.3.4 Testing lentiviral shRNA in ES cells

The next step was to determine the optimal MOI at which to transduce ES cells with shRNA lentivirus. ES cells were also transduced with lentivirus expressing scramble sequence to assess non specific effects caused by the introduction of lentivirus.

In ES cell differentiation a large range of tissue types is generated. This is likely to involve large scale changes in gene expression which could have affected the expression of the shRNA. It was therefore important to assess over time whether there were any changes in transduction efficiency or *Bmp4* knockdown.

ES cells were transduced and differentiated on the same day. Samples were taken at day 2 and day 4 of differentiation. Samples were analysed by flow cytometry for the percentage of GFP expressing cells and by quantitative RT-PCR for *Bmp4* knockdown. The experimental protocol was as shown in Figure 5.16.

At day 2 GFP expression was visualised using a fluorescent microscope for EBs transduced using both the scramble and shRNA lentiviruses (Figure 5.19).

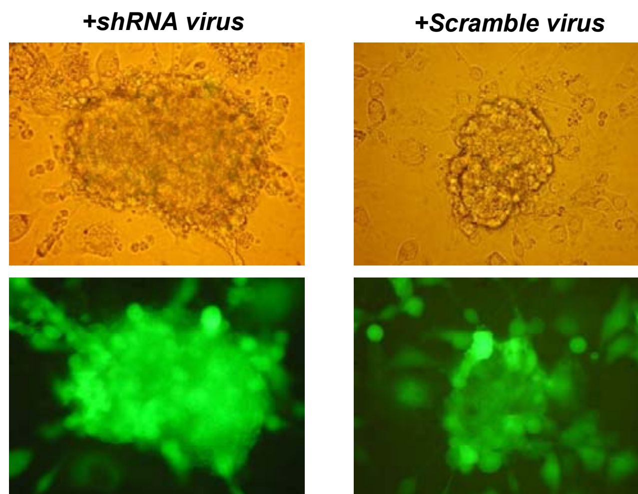


Figure 5.19 Micrographs of EBs at day 2 of differentiation obtained using light microscope (top) and fluorescent microscope (bottom) at 10x magnification (MOI 20).

At day 2 a higher MOI correlated with an increase in the percentage of GFP expressing cells (Figure 5.20). The shRNA lentivirus at an MOI of 0.8 produced a significant increase in GFP expressing cells relative to untransduced cells ($P<0.05$). At an MOI of 4 both the shRNA and scramble sequence lentivirus produced an increase in GFP expressing cells relative to untransduced cells ($P<0.05$).

At day 4 the pattern was similar with an MOI 0.8 producing a significant increase in GFP expressing cells only with shRNA transduced cells relative to untransduced cells ($P<0.05$). However at an MOI of 4 both the shRNA and scramble sequence lentivirus produced an increase in GFP expressing cells relative to untransduced cells ($P<0.05$). A higher MOI correlated with an increase in GFP expressing cells. However at an MOI of 100 there was a suspicious decrease in GFP expressing cells which may have reflected cell death caused by uptake of the virus. At such a high MOI it is possible that multiple copies of the virus are taken up by each cell overloading the cell causing cell death.

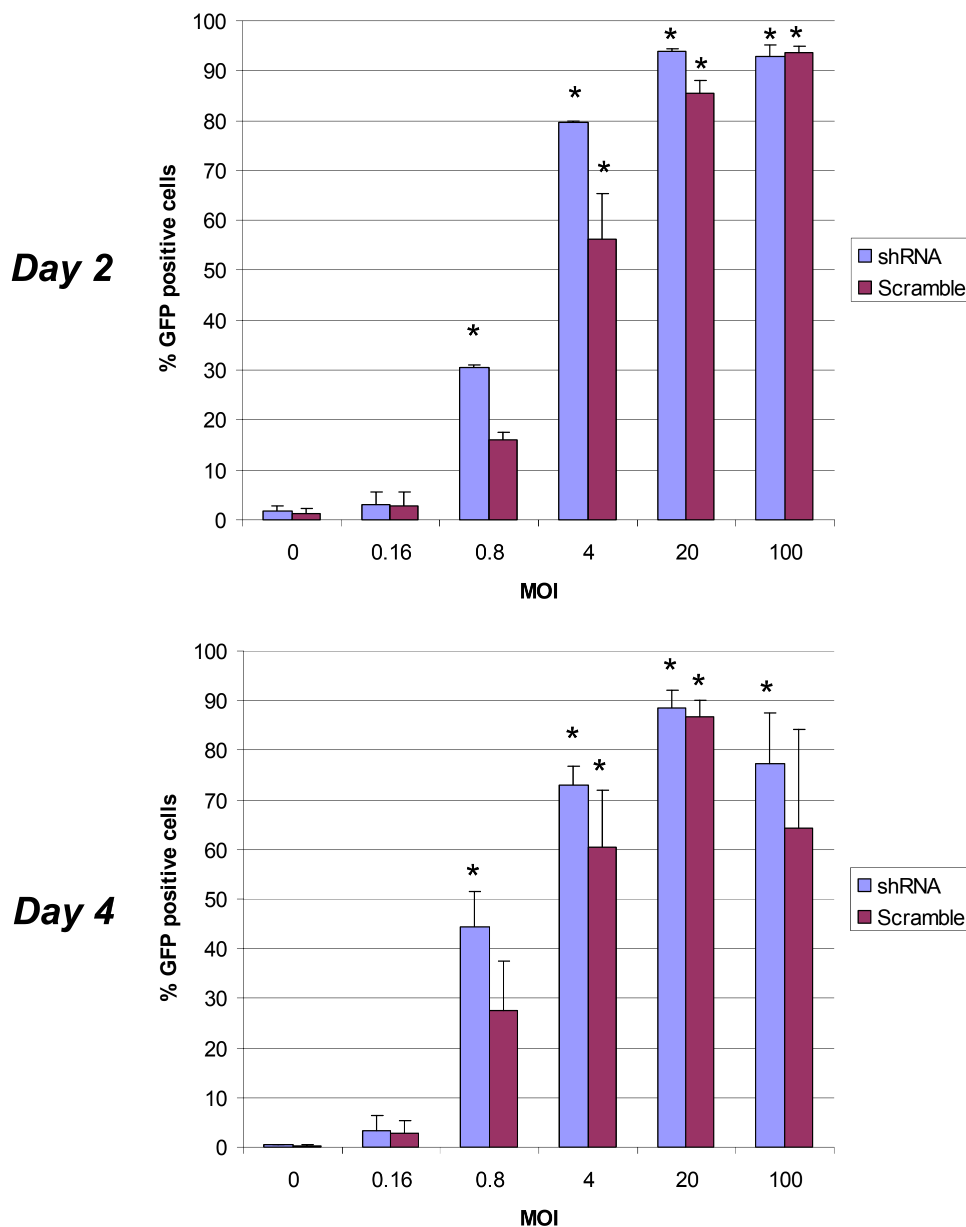


Figure 5.20 Testing shRNA lentivirus on ES cells – Transduction efficiency
Transduction efficiencies measured as percentage of GFP positive cells at day 2 and day 4. Cells were transduced at MOIs of 0, 0.16, 0.8, 4, 20 and 100 using shRNA and scramble sequence lentivirus before differentiation. Graph show average values obtained in 3 experiments. Error bars are the standard error. * indicates significant results at $P<0.05$ relative to the untransduced cells.

At day 2 at MOIs of 20 and 100 there was a significant reduction in *Bmp4* expression with shRNA lentivirus ($P<0.05$, Figure 5.21 and Table 5.2). However there was no significant change in *Bmp4* expression with scramble sequence lentivirus at any of the

MOIs used. At day 4 there was significant *Bmp4* knockdown with shRNA lentivirus at MOIs of 4 and 100 (P<0.05). It was surprising that an MOI of 20 did not produce significant knockdown. However this was probably as a result of the larger range of expression values in the three experiments conducted at this MOI. Scramble sequence shRNA only produced significant knockdown of *Bmp4* expression at an MOI of 100.

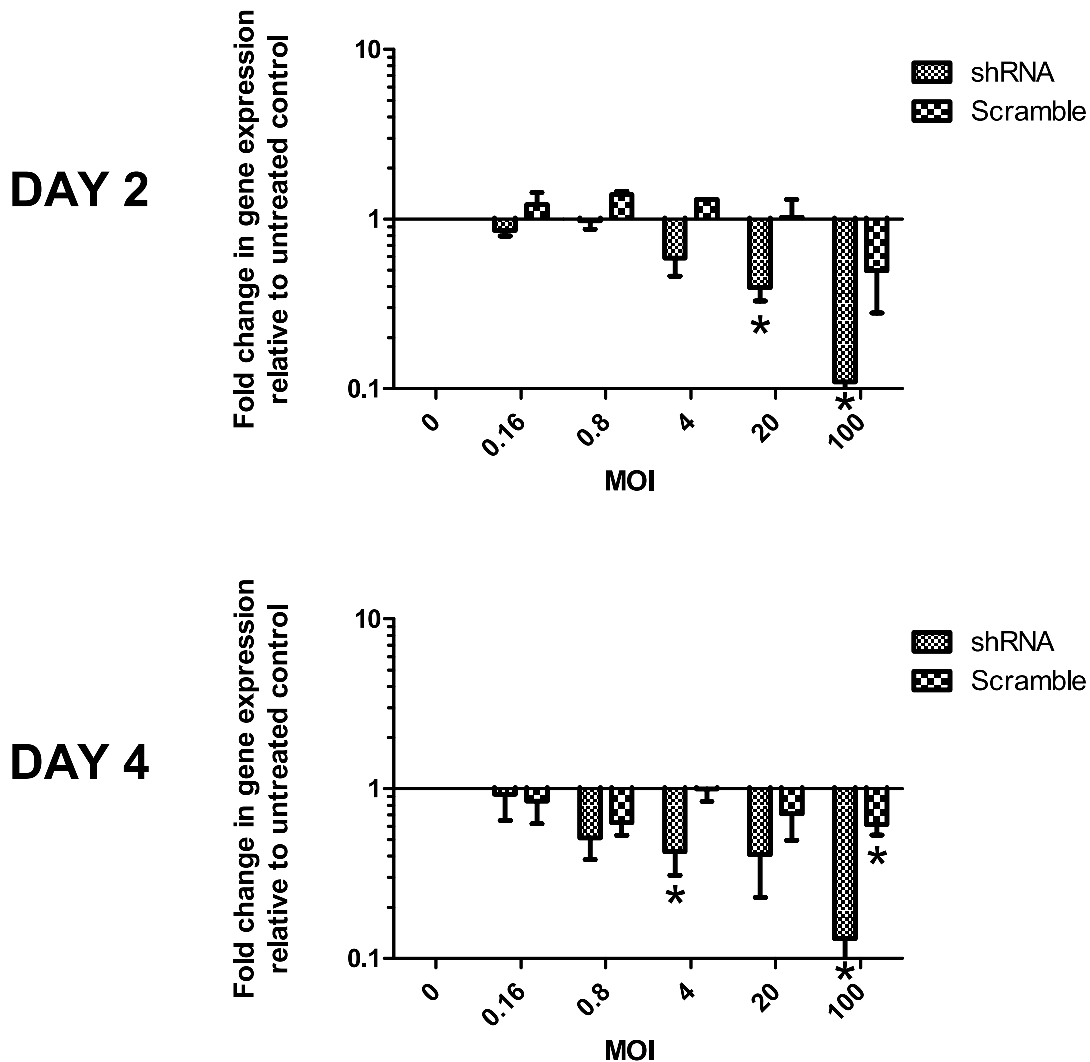


Figure 5.21 Testing shRNA lentivirus on ES cells – *Bmp4* expression
Fold change in Bmp4 expression relative to the untreated control at day 2 and day 4. Logarithmic graph shows average values obtained in 3 experiments. Error bars are the standard error. * indicates significant results at P<0.05 relative to the untreated control.

shRNA results

DAY 2	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	0.98	0.99	0.84	0.51	0.17
Exp 2	1	0.82	0.79	0.52	0.40	0.07
Exp 3	1	0.77	1.15	0.41	0.28	0.08
Average	**1**	**0.86**	**0.97**	**0.59**	**0.39**	**0.11**

DAY 4	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	0.37	0.38	0.64	0.18	0.24
Exp 2	1	0.39	1.26	0.23	0.76	0.07
Exp 3	1	0.77	1.15	0.41	0.28	0.08
Average	**1**	**0.51**	**0.93**	**0.43**	**0.41**	**0.13**

Scramble results

DAY 2	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	1.27	1.51	1.30	0.54	0.14
Exp 2	1	1.58	1.39	1.31	1.50	0.89
Exp 3	1	0.80	1.28	1.31	1.04	0.46
Average	**1**	**1.22**	**1.39**	**1.31**	**1.03**	**0.50**

DAY 4	*0*	*0.16*	*0.8*	*4*	*20*	*100*
Exp 1	1	0.45	0.65	0.79	0.30	0.65
Exp 2	1	0.64	0.60	0.90	0.80	0.74
Exp 3	1	0.80	1.28	1.31	1.04	0.46
Average	**1**	**0.63**	**0.84**	**1.00**	**0.71**	**0.62**

Table 5.2 Bmp4 expression tables
Fold change in *Bmp4* expression relative to the untreated control at day 2 and day 4. Cells were transduced with shRNA and scramble sequence lentiviruses at MOIs of 0, 0.16, 0.8, 4, 20 and 100. Results shown are from 3 experiments. P values indicate the statistical significance of results relative to the untreated control.

In order to determine if the shRNA lentivirus caused cytotoxicity, ES cells were transduced at the same range of MOIs and differentiated to day 4 at which point cells were stained with Annexin V and Propidium Iodide to assess cell death (Figure 5.22). Annexin V staining identifies apoptotic cells and staining with both propidium iodide and annexin V identifies necrotic cells. There were significant increases in cell death in particular apoptotic cells at MOIs of 20 and 100 ($P<0.05$). This was probably caused by the uptake of multiple lentivirus particles overloading the cells causing cell death.

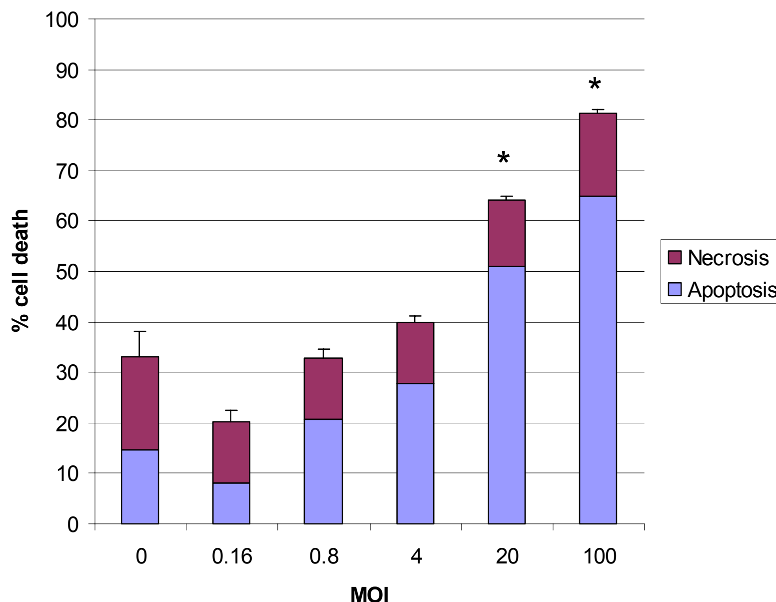
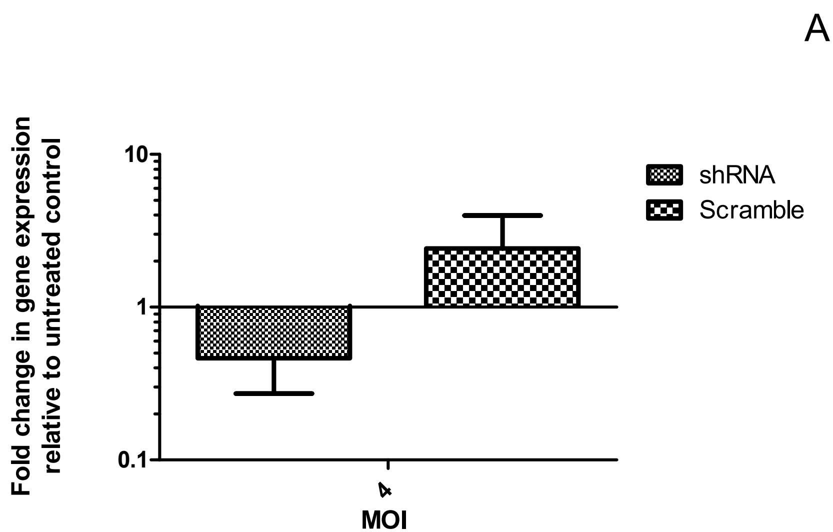


Figure 5.22 Cell death in transduced ES cells
Necrosis and apoptosis at each MOI with the addition of shRNA lentivirus at day 4. Graph shows average values obtained in 3 experiments. Error bars are the standard error. * indicates significant results at $P<0.05$.

The conclusion from these experiments was that an MOI of 4 would produce the highest knockdown of *Bmp4* with the lowest cytotoxicity. This MOI was therefore used in following experiments. The average knockdown over 3 experiments at day 2 was 41% and at day 4 was 57%. There would therefore be some limited expression of *Bmp4* in further experiments however this would be at much lower level.

Given that further haematopoietic differentiation experiments would continue through to day 6, knockdown of *Bmp4* expression at an MOI of 4 was assayed at day 6 as well (figure 5.9F). There was an average reduction of *Bmp4* expression by 54% with shRNA treated cells in line with the reduction of *Bmp4* expression at day 4.

A



B

DAY 6 MOI 4	*shRNA*	*Scramble*
Exp 1	0.67	1.43
Exp 2	0.64	0.39
Exp 3	0.08	5.45
Average	**0.46**	**2.42**

Figure 5.23 Testing shRNA lentivirus on ES cells – Day 6 *Bmp4* expression
A shows logarithmic graph showing fold change in Bmp4 expression relative to the untreated control at day 6. Graph shows average values obtained in 3 experiments. Error bars are the standard error. * indicates significant results at $P<0.05$ relative to the untreated control. **B** shows values obtained in each experiment.

5.4 The effects of lentiviral shRNA mediated BMP4 knockdown on the haematopoietic differentiation of ES cells

In these experiments shRNA was used to knockdown the expression of *Bmp4*. This would provide a *Bmp4* free culture condition so that the effects of added BMP4 could be determined. The primary aim of these experiments was to determine whether BMP4 caused a change in the expression on the haematopoietic stem cell genes *Runx1*, *Scl*, *Gata2* and *Lmo2*.

The expression of *Bmp4* is important to the undifferentiated phenotype as it blocks neural differentiation through the induction of *Id* genes (Ying et al. 2003). The knockdown of *Bmp4* expression may therefore have fundamentally affected the undifferentiated ES cell phenotype. For this reason *Bmp4* knockdown was only induced at the start of differentiation in ES cells. Data from shRNA testing experiments showed that induction of *Bmp4* knockdown occurred from day 2 which is when haematopoietic differentiation begins. This was sufficient for the purpose of assessing the effect of BMP4 in haematopoietic differentiation.

Thus ES cells were differentiated for 6 days in serum free culture, with shRNA lentivirus, with shRNA lentivirus plus BMP4 (25ng/ml) and with scramble sequence lentivirus. BMP4 was added at 25ng/ml because in previous experiments there was evidence that this concentration caused an increase in *Scl*, *Gata2* and *Lmo2* expression (see Section

4.4.2). ES cells were transduced with scramble sequence lentivirus to detect any non-specific cellular effects caused by uptake of the lentivirus. The experimental protocol is summarised in Figure 5.24.

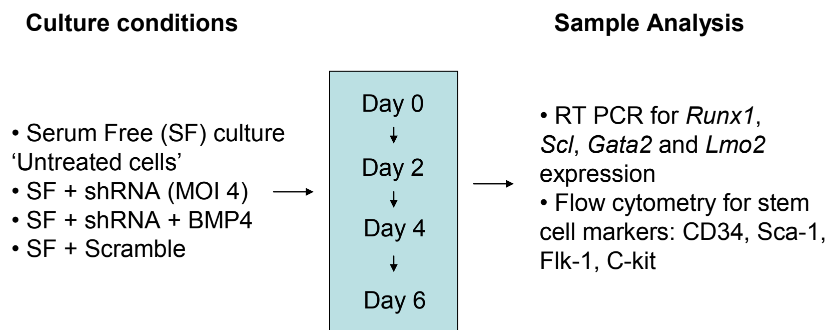


Figure 5.24 Experimental protocol for determining the effects of BMP4 in ES cell haematopoietic differentiation.

5.4.1 Quantitative PCR analysis

Samples were taken for quantitative PCR analysis of *Runx1*, *Scl*, *Gata2* and *Lmo2* expression at days 0, 2, 4 and 6. The experiment was repeated 5 times. Tables showing the results obtained in each experiment are in Appendix 3. Results were interpreted relative to day 0 to determine whether positive changes reflecting haematopoietic differentiation were occurring and relative to the untreated control to determine the effect of added BMP4 as described in Section 4.4.1.

At day 2 and day 4 under all culture conditions *Runx1* expression stayed at day 0 levels (Figure 5.25A). However at day 6 there was a reduction in *Runx1* expression in serum free culture conditions, with shRNA and with scramble lentiviruses ($P<0.05$). However with the addition of BMP4 to shRNA treated cells *Runx1* expression was maintained at day 0 levels ($P=0.138$).

The most striking change in *Runx1* expression relative to the untreated control was at day 6 (Figure 5.25B). There was an increase in *Runx1* expression relative to the untreated control with added BMP4 ($P<0.05$). Taking the expression relative to day 0 and the expression relative to the untreated control into account, at day 6 BMP4 maintained *Runx1* expression at day 0 levels.

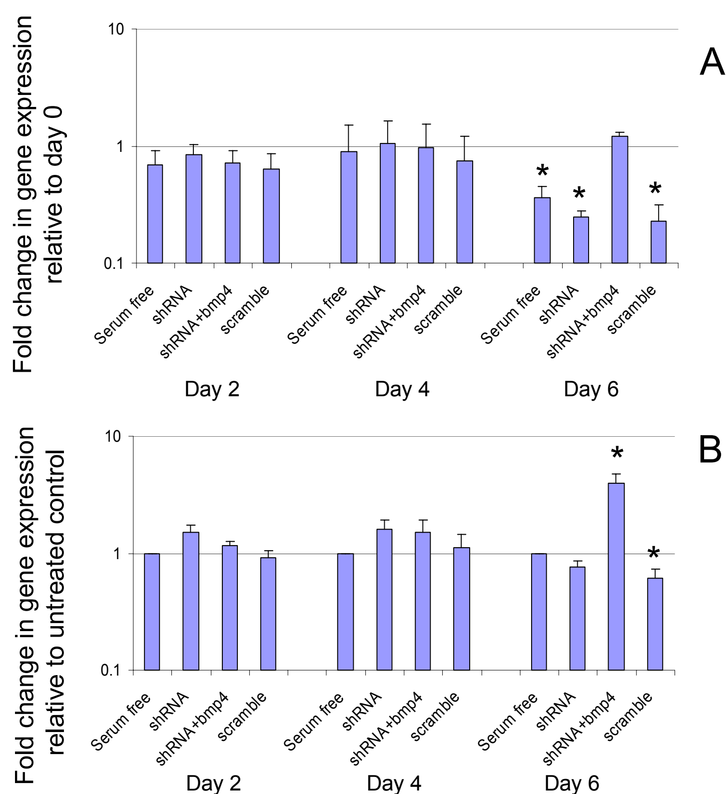


Figure 5.25 QPCR analysis – *Runx1*
Graphs show fold change in *Runx1* expression. Gene expression has been calculated relative to: (**A**) day 0 and (**B**) the untreated control. Cells were differentiated under serum free conditions, with shRNA, with shRNA plus BMP4 and with scramble lentivirus. Samples were taken at day 0, 2, 4 and 6. The results shown are the average of 5 experiments. The error bars indicate standard error. * indicates results significant at $P<0.05$.

At day 2 and day 4 there was no change in *Scl* expression relative to day 0 (Figure 5.26A). At day 6 there was a reduction in *Scl* expression relative to day 0 in serum free culture, with the addition of shRNA and scramble sequence lentiviruses ($P<0.05$). However there was an increase in *Scl* expression above day 0 levels with the addition of BMP4 ($P<0.05$).

Relative to the untreated control *Scl* expression did not change at day 2 or day 4 (Figure 5.26B). At day 6, *Scl* expression increased dramatically relative to the untreated control with the addition of BMP4 ($P<0.05$). Under all other culture conditions at day 6, *Scl* expression was at similar levels to the untreated control. Putting all the data together, this indicates that at day 6 BMP4 increased *Scl* expression.

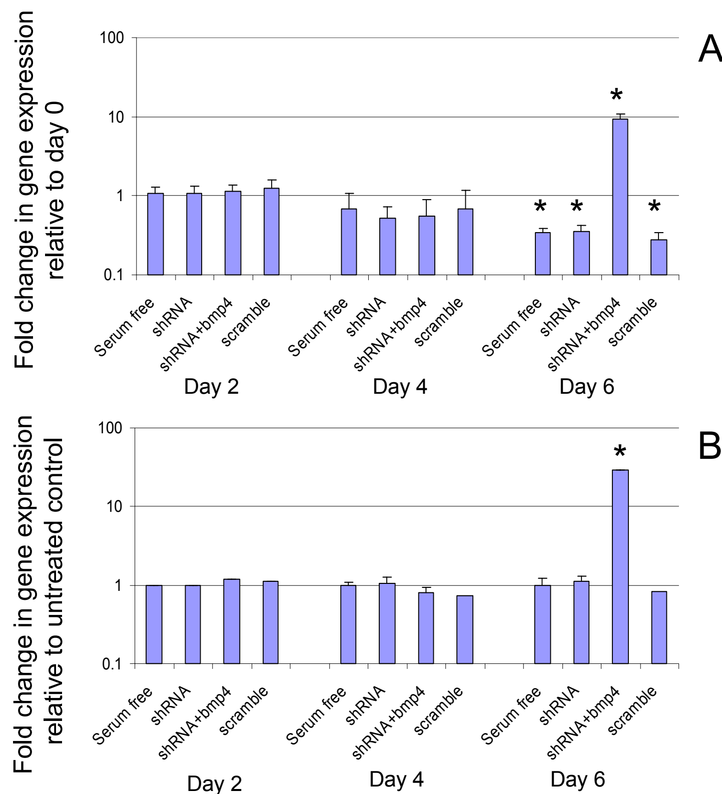


Figure 5.26 QPCR analysis – *Scl*
Graphs show fold change in *Scl* expression. Gene expression has been calculated relative to: (**A**) day 0 and (**B**) the untreated control. Cells were differentiated under serum free conditions, with shRNA, with shRNA plus BMP4 and with scramble lentivirus. Samples were taken at day 0, 2, 4 and 6. The results shown are the average of 5 experiments. The error bars indicate standard error. * indicates results significant at P<0.05.

Gata2 expression reduced below day 0 levels at days 2, 4 and 6 (P<0.05, Figure 5.27A). The only exception to this was at day 6 where the addition of BMP4 caused the maintenance of *Gata2* expression at day 0 levels (P=0.893).

Relative to the untreated control *Gata2* expression levels were similar at day 2 and at day 4 under all culture conditions (Figure 5.27B). At day 6 *Gata2* expression stayed the same with the addition of shRNA lentivirus but reduced with the addition of scramble lentivirus (P<0.05) relative to the untreated control. With the addition of BMP4 however

there was an increase in Gata2 expression levels relative to the untreated control at day 6. This data indicates that BMP4 maintains *Gata2* expression at day 0 levels.

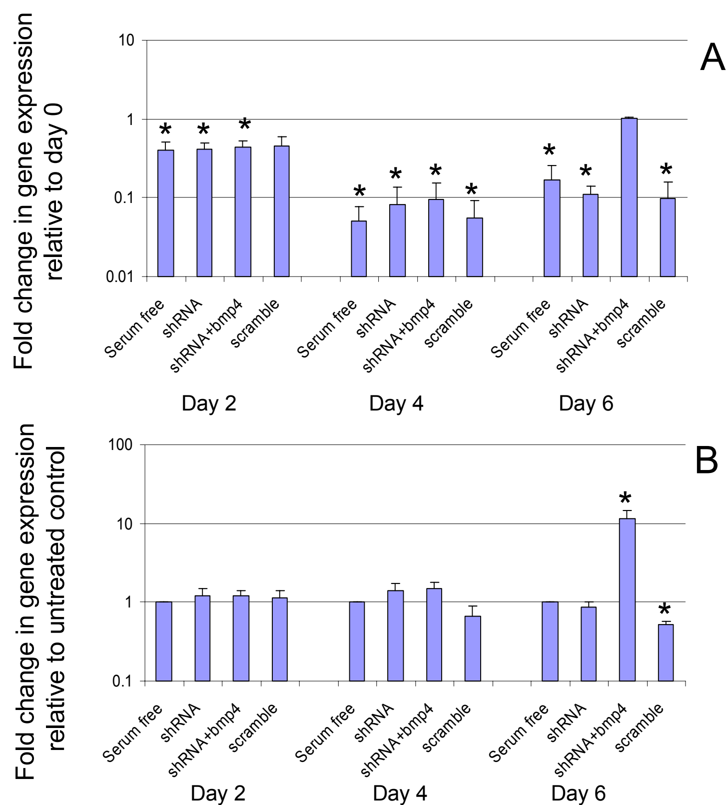


Figure 5.27 QPCR analysis – *Gata2*
Graphs show fold change in *Gata2* expression. Gene expression has been calculated relative to: (**A**) day 0 and (**B**) the untreated control. Cells were differentiated under serum free conditions, with shRNA, with shRNA plus BMP4 and with scramble lentivirus. Samples were taken at day 0, 2, 4 and 6. The results shown are the average of 5 experiments. The error bars indicate standard error. * indicates results significant at $P<0.05$.

Overall *Lmo2* expression levels stayed at similar levels at day 2 relative to day 0 (Figure 5.28A). There was a general increase in *Lmo2* expression levels at day 4. Then at day 6 there was a reduction back to day 0 levels. There was an increase in *Lmo2* expression at day 6 under serum free conditions and with the addition of BMP4 relative to day 0 ($P<0.05$).

Relative to the untreated control *Lmo2* expression was at reduced levels with the addition of BMP4 at day 4 (P<0.05, Figure 5.28B). However at day 6 there was a striking increase in *Lmo2* expression relative to the untreated control with the addition of BMP4 (P<0.05). Under all other conditions *Lmo2* expression levels were similar to the untreated control at day 6. This data together indicates that at day 6 BMP4 increases *Lmo2* expression.

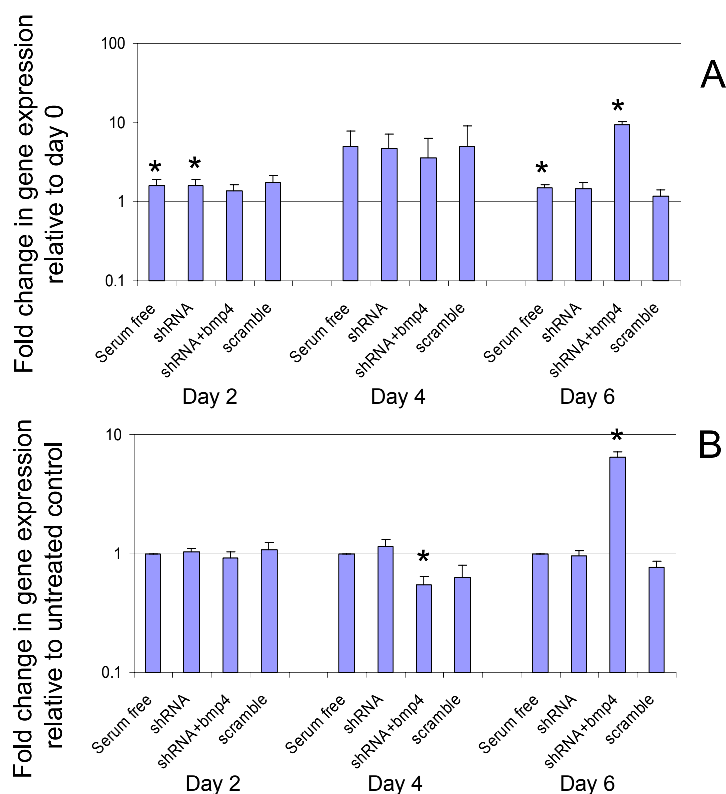


Figure 5.28 QPCR analysis – *Lmo2*
Graphs show fold change in *Lmo2* expression. Gene expression has been calculated relative to: (**A**) day 0 and (**B**) the untreated control. Cells were differentiated under serum free conditions, with shRNA, with shRNA plus BMP4 and with scramble lentivirus. Samples were taken at day 0, 2, 4 and 6. The results shown are the average of 5 experiments. The error bars indicate standard error. * indicates results significant at P<0.05.

The most striking effects of BMP4 were seen at day 6. These results showed that at day 6 BMP4 maintained *Runx1* and *Gata2* expression at day 0 levels. However BMP4 increased the expression of *Scl* and *Lmo2* expression above day 0 levels.

5.4.2 Flow cytometry

Samples were taken at day 6 to determine if the changes seen in gene expression at this time point correlated with changes in stem cell surface markers. Cells cultured in serum free conditions, with shRNA, with shRNA plus BMP4 and with scramble sequence lentivirus were stained with the same stem cell panel of antibodies as used in initial experiments (see Section 3.2.2.1). This included antibodies to detect CD34, Sca-1, Flk-1 and C-kit. Results shown in Figure 5.29A show the average results from 4 experiments and in Figure 5.29B flow plots from a representative experiment are shown (experiment 4). Tables showing the data obtained in each experiment are in Appendix 3.

There was an increase in CD34 expressing cells with the addition of shRNA relative to the untreated control ($P<0.05$). This was surprising as a reduction in BMP4 would have been expected to reduce the number of CD34 expressing cells.

However the addition of BMP4 to shRNA transduced cells caused an increase in C-kit expressing cells ($P<0.05$). There was also a trend for an increase in Flk-1 expression with the addition of BMP4 which did not reach statistical significance.

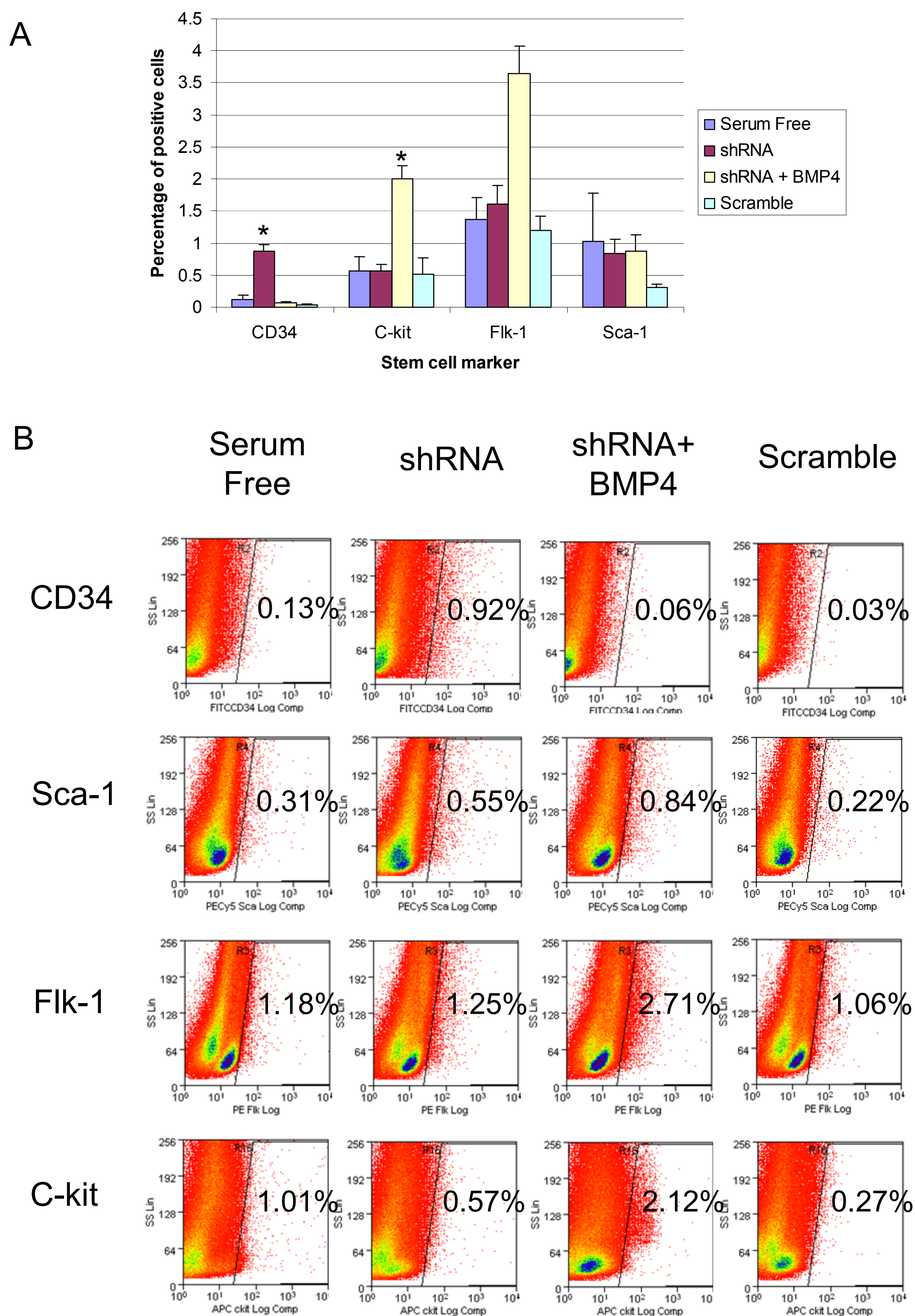


Figure 5.29 Stem cell marker analysis
A ES cells were differentiated to day 6 under serum free conditions, with shRNA, with shRNA plus BMP4 and with scramble sequence lentivirus. The cells were stained with a stem cell panel of antibodies: CD34, Sca-1, Flk-1 and C-kit. Results shown are the average of 4 experiments. The error bars represent the standard error. * indicates results significant at $P<0.05$. **B** shows flow cytometry plots from a representative experiment (Experiment 4). The results from all experiments are tabulated in Appendix 2.

5.4.3 Discussion

This experiment investigated the role of BMP4 in haematopoietic differentiation by measuring changes in haematopoietic stem cell gene expression and stem cell surface marker expression. Lentivirus expressing shRNA was used to knockdown *Bmp4* expression so as to reduce the production of BMP4 by cells in serum free culture. It is known that without the expression of BMP4 mesoderm formation is defective (Winnier et al. 1995) and thus this experiment aimed to investigate the role of exogenous BMP4 in rescuing a knockdown phenotype.

These results have shown that BMP4 maintains *Runx1* and *Gata2* expression and up regulates expression of *Scl* and *Lmo2* at day 6 of differentiation. Previous experiments have suggested that BMP4 combined with VEGF was necessary to increase *Scl* expressing cells (Park et al. 2004). This experiment shows that BMP4 alone is sufficient to up regulate *Scl* expression. Pearson *et al*. have suggested that BMP4 alone is required for efficient mesoderm formation but is insufficient for haematopoietic specification (Pearson et al. 2008). In this paper a combination of growth factors including BMP4 were used to produce increases in *Runx1*, *Scl* and *Lmo2* expression. In the current experiment BMP4 alone has been shown to be capable of producing increases in *Scl* and *Lmo2* expression.

Previous titration experiments (Section 4.4) had shown that BMP4 caused an increase in *Gata2* expression levels. The general trend in the data suggested that there was an increase above day 0 levels. However in this experiment exogenous BMP4 only resulted

in the maintenance of *Gata2* expression levels at day 0 levels. It is possible that in the titration experiments although up to 25ng/ml of BMP4 was added, the actual concentration in the EBs was higher due to the production of BMP4 by the differentiating cells. The use of a higher concentration of BMP4 might therefore increase the expression of *Gata2* above day 0 levels as seen in previous experiments.

Runx1 and *Gata2* expression relative to the untreated control was reduced at day 6 when transduced with scramble sequence lentivirus at $P<0.05$. However this pattern was not seen with the shRNA expressing lentivirus and therefore it seems unlikely that this change in gene expression resulted from non-specific cellular effects caused by the uptake of the lentivirus.

Flow cytometry analysis showed increases in C-kit expressing cells with the addition of BMP4. However given that C-kit is a marker for haematopoietic stem cells in early development (Sanchez et al. 1996), this may represent the expansion of a HSC population. Further analysis of this population is required to prove this. There was a trend for an increase in Flk-1 expressing cells in line with previous data that has shown the same result in differentiating ES cells (Park et al. 2004).

The timescale of BMP4 dependent increases in haematopoietic gene expression which only occurred at day 6 seems slower compared to earlier titration experiments where changes were seen at day 2 and day 4. This may result from slower less efficient mesoderm formation resulting from the knockdown of BMP4 expression.

There was no observed reduction in gene expression in the shRNA treated cells relative to the untreated cells. The shRNA treated cells are likely to have had low mesoderm formation and little if any haematopoietic specification. This result reveals that the serum free 'untreated' cells probably also differentiated little without additional stimulus.

5.5 General Discussion

Knockdown of *Bmp4* expression can be achieved using siRNA. However the effects are only transient. In order to achieve more sustained knockdown short hairpin RNA which also causes knockdown via the RNAi pathway was expressed from a plasmid.

The main challenge of shRNA delivery in ES cells is to achieve knockdown without producing non-specific cellular effects or cytotoxicity. Transfection is generally less expensive and less time consuming. Published literature suggests that it is possible to transfect ES cells (Ma et al. 2004). In order to determine whether this was a feasible technique to deliver shRNA plasmids, ES cells were transfected with the pEGFP-N1 plasmid to determine transfection efficiency. In comparison with previous studies using lipofection, higher transfection efficiency was observed. At day 2 44.7% of transfected ES cells expressed GFP. This compares with 5.75% in Ma et al. and 15% in Tang et al. In both papers different ES cell lines were used (Tang et al. 2004;Ma et al. 2004). It maybe that different ES cell lines have different transfection potentials.

Transfection reagents however are known to cause toxicity (Kim et al. 2008). shRNA plasmids were transfected into the control stromal cell line ST2. The transfection reagent alone caused a reduction in *Bmp4* expression. Concerns that this may have resulted from cytotoxicity and that this might be an issue in ES cells led to a search for alternatives to transfection for shRNA delivery.

The plasmids came with lentivirus components. The plasmid with the most effective shRNA sequence was chosen and lentivirus was generated. In order to facilitate easier virus titering and analysis of transduction efficiency GFP-WPRE was cloned into the plasmid.

shRNA expressed from lentivirus has previously been used as a means to investigate the function of the transcription factor Sox17 in cardiac muscle differentiation of ES cells (Liu et al. 2007). However it was important to test the ability of the shRNA expressed from lentivirus to knockdown *Bmp4*. Testing was carried out at a variety of MOIs in ST2 cells. Transduction efficiency was high at MOIs of 4 and above. The shRNA transduced cells were shown to produce up to 89% knockdown. However at higher MOIs the scramble sequence lentivirus was shown to cause *Bmp4* knockdown. Since the scramble sequence is designed to have no effect on *Bmp4* expression this suggested that at higher MOIs uptake of the lentivirus was causing cytotoxicity.

Testing of the shRNA expressing lentivirus in ES cells was therefore aimed at finding the optimal MOI which would produce the highest level of *Bmp4* knockdown with the

minimum cytotoxicity. An MOI of 4 produced 41% knockdown at day 2, 57% knockdown at day 4 and 54% at day 6. Transduction efficiency was high with over 70% transduction at day 2 and day 4. Annexin V, Propidium Iodide staining revealed that there was not significant cytotoxicity at day 4 at an MOI of 4. Higher MOIs however did produce significant cytotoxicity.

shRNA at MOI of 4 was therefore used to investigate the role of BMP4 in haematopoietic gene expression. The addition of BMP4 at 25ng/ml led to the up regulation of *Scl* and *Lmo2* genes at day 6. *Runx1* and *Gata2* expression was maintained at day 0 levels. Further flow cytometric analysis of day 6 EBs showed that this correlated with an increase in C-kit expressing cells.

These results were far more robust and consistent than in previous experiments. This probably resulted from the absence of uncontrolled endogenous expression of *Bmp4*. Previous work in the area has not taken into account endogenous expression of *Bmp4*. Furthermore previous work has suggested that additional factors are needed to up regulate these haematopoietic genes (Park et al. 2004;Pearson et al. 2008). This experiment has therefore clarified the role of BMP4 showing that it produces an up regulation of *Scl* and *Lmo2* expression and maintains expression of *Runx1* and *Gata2*. This series of experiments illustrates the importance of taking into account endogenous expression in determining the effects of growth factors in differentiation in the ES cell system.

Scl and Lmo2 along with Gata2 have been shown to act in a complex together. This complex has been shown previously to up regulate expression of C-kit in murine adult bone marrow (Lecuyer et al. 2002). Given the results in this experiment it may be that in early development BMP4 up regulates the formation of this complex which can then up regulate C-kit expression.

Furthermore the Scl-Lmo2-Gata2 complex has been shown to activate *Runx1* expression in a murine yolk sac cell line (Landry et al. 2008). BMP4 may also maintain *Runx1* expression through the up regulation of this complex.

Future experiments should investigate whether BMP4 up regulates the formation of this complex and whether this is Smad dependent given that BMP4 signaling is thought to occur via the Smad pathway (Larsson & Karlsson 2005).

Scl and *Lmo2* have similar deletion mutant phenotypes and expression patterns in early and adult haematopoietic lineages. Given the results in this experiment showing that both of these genes were up regulated, this data together suggests that these genes may share the same transcriptional control mechanisms.

Chapter 6 Final Discussion

This work sought to determine the effect of BMP4 on the developing haematopoietic populations in ES cell differentiation, the foetal liver and the adult haematopoietic populations of the bone marrow. In doing this several noteworthy observations have been made.

BMP4 in haematopoietic development

Since the initial finding that BMP4 is expressed by stromal cells beneath the ventral floor of the dorsal aorta in 28 day old human embryos where intra-aortic clusters form (Marshall, Kinnon, & Thrasher 2000), several studies have implicated BMP4 in the initial stages of haematopoietic development. Much of this work has been done *in vitro* using ES cell differentiation which is accepted as a model of haematopoietic development (Keller et al. 1993). The accessibility of cellular material makes this model easy to manipulate in comparison with any *in vivo* developmental system.

This work sought to further investigate the role of BMP4 in ES cell haematopoietic differentiation by specifically defining the haematopoietic genes that are up regulated in response. Knockout studies have shown that *Runx1*, *Scl*, *Gata2* and *Lmo2* are all critical to haematopoietic development. The deletion mutant phenotype for all of these genes is lethal due to the absence of blood development. At the time of completing this thesis two

papers were published further elucidating the roles of *Scl* and *Runx1* in the development of definitive haematopoietic cells. These studies show that haemangioblasts give rise to the haemogenic endothelium which then gives rise to definitive haematopoietic cells. Furthermore these studies demonstrate that *Scl* is necessary for the establishment of the haemogenic endothelium and *Runx1* is critical in the generation of definitive haematopoietic cells (Lancrin et al. 2009;Chen et al. 2009). The aim of this study was to establish whether BMP4 would up regulate the expression of any of these critical haematopoietic genes: *Runx1*, *Scl*, *Gata2* and *Lmo2*.

Previous studies have shown that BMP4 in combination with other factors can up regulate these genes. Park *et al.* have shown that whilst BMP4 alone is sufficient for the differentiation of Flk-1 expressing mesoderm, a combination of VEGF and BMP4 dramatically enhances *Scl* up regulation (Park et al. 2004). Pearson *et al.* have developed this work adding that BMP4 in combination with activin A, basic fibroblast growth factor and VEGF is required for optimal haematopoietic differentiation (Pearson et al. 2008). In this study they showed that this combination of growth factors could increase *Runx1*, *Scl* and *Lmo2* expression within a 24 hour period. Lugus *et al.* have shown that after 2 days of differentiation cells treated for 1 hour with BMP4 showed an increase in *Gata2* expression (Lugus et al. 2007). This was in contrast to results in Pearson *et al.* in which no increase in *Gata2* expression was observed.

Unlike previous studies which have attempted to produce optimal gene expression and haematopoietic differentiation, this work was aimed exclusively at discovering the role that BMP4 plays in this process.

There was variability in the results achieved from ES cell haematopoietic differentiation experiments. Despite efforts to make the process as consistent as possible, for example using the technique of hanging body differentiation, there was still variability in the results obtained. This made it difficult to draw conclusions about the effects of BMP4. This was further complicated by endogenous *Bmp4* expression in differentiating ES cells. The use of lentiviral shRNA to knockdown *Bmp4* expression very much improved the consistency of data. This suggests that in part the variability seen was due to inconsistent BMP4 production between experiments.

Attempts are currently being made to differentiate specific tissues from ES cells. In order to do this a clear understanding of the role of specific growth factors in the differentiation process is necessary. It is essential that studies take account of endogenous growth factor production. Otherwise endogenous growth factor production in untreated samples will mask the effects seen in treated samples.

In this case lentiviral shRNA was used to knockdown *Bmp4* expression. This provided a serum free system in which *Bmp4* was not being expressed allowing the effect of added BMP4 to be determined. This experiment revealed that BMP4 increased *Scl* and *Lmo2*

expression whilst maintaining levels of *Runx1* and *Gata2* expression. Furthermore there was an increase in C-kit expressing cells.

In the BMP4 titration experiments there were increases in *Gata2* expression above day 0 levels with the addition of BMP4. The endogenous BMP4 in addition to the exogenous BMP4 may have exceeded the levels present in the shRNA experiments. If a higher concentration of BMP4 was used in the shRNA experiment this may have caused a larger increase in *Gata2* expression.

BMP4 is thought to produce intracellular changes via the Smad signalling pathway in particular Smad1. Zafonte *et al.* have generated Smad1 inducible ES cells (Zafonte et al. 2007). The activation of Smad1 expression caused an increase in *Flk-1*, *Runx1*, *Scl* and *Gata2* expression. This data is in line with the data presented in this study. However Zafonte *et al.* have presented their quantitative PCR data as relative to an untreated age matched control and therefore it is difficult to tell whether these changes represent changes above day 0 levels or the maintenance of gene expression at day 0 levels. This illustrates the utility of determining gene expression relative to both day 0 and an age matched untreated control.

DNA delivery in ES cells

The need to deliver shRNA to ES cells provided an opportunity to investigate the efficacy of different gene delivery techniques in ES cells. The two methods used were lipofection

and lentiviral transduction. In comparison with previous studies which have used lipofection, higher transfection efficiencies were observed in these experiments. At day 2 there was 44.7% transfection efficiency as compared to 5.75% in studies by Ma *et al.* (Ma et al. 2004). This could be due to differences in both the transfection reagent used and the ES cell line used. The major concern with the use of lipofection for delivering shRNA plasmids was the potential for cytotoxicity. Several papers suggest that this is significant problem in the transfection of ES cells (Ma et al. 2004;Kobayashi et al. 2005). In addition knockdown only lasted to day 4 which was insufficient for these experiments.

Lentiviruses integrate into the genome and therefore can produce long lasting effects. Given the gene expression changes presumably are occurring as differentiation proceeds, it was a concern that there might be inactivation of the knockdown. However experiments showed that shRNA knockdown in transduced cells increased from day 2 to day 4 and then remained steady to day 6. However at high MOIs of 20-100, cytotoxicity was significant. The MOI was therefore optimised to produce a high level of knockdown with a low level of cytotoxicity.

Thus lentiviruses are effective vectors with which DNA delivery can be achieved in differentiating ES cells especially if long term effects are required.

C-kit, Sca-1 and the cell cycle

In initial experiments attempts were made to discover what effect if any BMP4 had on haematopoietic populations of the foetal liver and bone marrow. There was variability in results caused by variation in the initial populations detected. One method of resolving this issue was to perform experiments on a sorted population of cells. A CD34 C-kit positive population was sorted from bone marrow. There were difficulties however with maintaining adequate cell survival over the culture period.

These initial experiments brought about an opportunity to compare the gene expression and cell surface marker characteristics of haematopoietic cell populations from ES cells, foetal liver and bone marrow populations. These experiments showed that *Runx1*, *Scl* and *Lmo2* which have been shown to be critical in the genesis of HSCs are expressed at much higher levels in the foetal liver and bone marrow. This suggests that these genes continue to have an important function in more mature haematopoietic populations. Conditional knockout has shown that deletion of *Runx1* in the adult impairs megakaryocytic differentiation and T and B lymphocyte development (Ichikawa et al. 2004) and deletion of *Scl* in the adult impairs erythroid and megakaryocytic differentiation (Mikkola et al. 2003). The increased level of expression of these genes may reflect the increased presence of these cells in these more mature haematopoietic populations.

The cell surface markers C-kit and Sca-1 have been used to identify the HSC since its first discovery (Worton, McCulloch, & Till 1969). In this work analysis of cell surface markers revealed a higher level of C-kit expressing cells in ES cell derived and foetal liver haematopoietic populations compared with bone marrow. There was however higher levels of Sca-1 expressing cells in the bone marrow compared with the ES cell derived and foetal liver populations. It was therefore hypothesised that C-kit was expressed on dividing cells whereas Sca-1 was expressed on quiescent cells. In order to test this, bone marrow cells were stained for C-kit and Sca-1 and the cell cycle status of these populations was determined. These experiments showed that there were much higher levels of G2/M and S phase cells in the C-kit population and there were much higher levels of G1/0 cells in the Sca-1 positive population. The expression of these markers does therefore appear to be related to cell cycle status.

Scl and Lmo2

SCL and LMO2 show overlapping expression patterns in early haematopoietic sites and in adult haematopoietic lineages. The deletions mutants of *Scl* and *Lmo2* both cause similar phenotypes with a complete absence of blood formation and death around 9.5 dpc (Warren et al. 1994;Shivdasani, Mayer, & Orkin 1995). In the shRNA experiments both *Scl* and *Lmo2* were up regulated to similar levels above day 0 expression. Furthermore in the comparison of day 0 populations there were increases in both *Scl* and *Lmo2* to similar levels in both the foetal liver and bone marrow populations. This data together suggests that *Scl* and *Lmo2* may share the same transcriptional control mechanisms.

Timing of gene expression in ES differentiation

The patterns of gene expression between experiments were too inconsistent to draw any overall conclusions as to what the kinetics of gene expression was for each gene. However in the shRNA experiment, in which the results were most consistent, at day 6 when the cells responded to BMP4 increases in gene expression for *Runx1*, *Scl*, *Gata2* and *Lmo2* occurred together. The regulation of gene expression between these genes has been shown previously to be interconnected. Pimanda *et al.* have described a gene regulatory network comprising of *Gata2*, *Fli1* and *Scl* and Landry *et al.* have provided evidence that the SCL-Lmo2-Gata2 complex can transactivate Runx1 expression (Pimanda et al. 2007b;Landry et al. 2008). The increase in expression of all these genes at the same time therefore is in line with previous data.

Future work

In order to determine the effect of BMP4 in the foetal liver and bone marrow populations it is necessary to sort consistent populations and find appropriate culture conditions so as to maintain an adequate level of cell survival. Furthermore future experiments should take into account endogenous BMP4 production.

Future experiments could also investigate the signalling pathways that are activated by BMP4. It is known that there is a close relationship in the control of the expression of *Runx1*, *Scl*, *Gata2* and *Lmo2*. Given that the transcriptional control mechanisms of the 4 genes investigated in this study are so closely related, it is possible that BMP4 only

triggers one component which causes the up regulation of the other genes. In particular given the results in the shRNA ES experiments it is possible that BMP4 could directly up regulate *Scl* and *Lmo2* triggering indirect up regulation of *Runx1* and *Gata2*. Future experiments could explore this hypothesis.

A recent paper has compared Brachyury$^+$Flk1$^+$ populations and Brachyury$^+$Flk1$^-$ populations based on mRNA changes and protein changes (Williamson et al. 2008). mRNA changes were measured using microarray and quantitative PCR. Protein changes were analysed by quantitative proteomics. In this study it was found that 95% of protein changes were not associated with mRNA changes and that there was more protein changes than mRNA changes. This illustrates the potential importance of post transcriptional regulation in protein expression. Thus future studies should verify the gene expression findings in this study with protein analysis.

BMP4 as a mesoderm patterning factor is involved in the development of many different tissue types. It remains a puzzle as to how this one molecule could have so many different effects. It may be that the effects of BMP4 seem diverse but in fact they are just part of a multitude of inputs including cell-cell interactions and other locally produced factors that cause a cell to differentiate down a particular pathway. Recent work in *Xenopus* has shown that exposure to BMP4 even for 20 minutes causes Smad1 phosphorylation and within half an hour effects are seen on the transcription of downstream genes (Simeoni & Gurdon 2007). Cells are therefore able to quickly respond to changes in extracellular BMP4 concentration which is reflected by the steady state

Smad1 concentration in the nucleus. Cells in culture are traditionally exposed to one concentration of BMP4 however it maybe that in development as the embryo changes shape, cell populations are exposed to different concentrations of BMP4 at different time points. Future work should investigate whether the timing of the addition of BMP4 as well as the concentration of BMP4 is important.

There are significant differences between the phenotypes of murine ES cells and human ES cells. Human ES cells are SSEA3 +ve, SSEA4 +ve and SSEA1 –ve. Murine ES cells however are SSEA1 +ve and are both SSEA3 and SSEA4 –ve. In addition human ES cells express two keratin sulphate related antigens, TRA-1-60 and TRA-1-81 (Andrews 2002). It is likely that the differentiation processes will be different as well. For this reason it is important that future experiments discover the extent to which the lessons learnt in the murine ES cell system in regard to haematopoietic differentiation can be extrapolated to the human ES cell system. In particular it is important to determine whether the human homologs of *Runx1*, *Scl*, *Gata2* and *Lmo2* play the same roles in the haematopoietic differentiation of human ES cells. Also future experiments should establish whether BMP4 induces the expression of these genes.

Conclusion

In order to use ES cells in the clinic it is essential to understand the environmental and genetic triggers that cause differentiation down specific pathways. This study illustrates some of the difficulties that must be overcome in order to understand these pathways and

also some potential solutions. The differentiation of ES cells in EBs mimics the processes that occur in developing embryos in many ways except that it is considerably more disorganised. When the aim is to develop one specific type of tissue, the processes occurring in EBs which are aimed at developing all the tissues of the body will be in direct contradiction. Thus in order to differentiate a particular type of tissue it may be necessary in addition to promoting the differentiation of that particular tissue to also inhibit differentiation down other pathways.

In this study where it was only necessary to investigate the role of a particular growth factor the first step was to suppress the expression of BMP4 using shRNA. The effect of added BMP4 could then be determined. Previous ES cell differentiation studies do not take into account the endogenous production of growth factors and thus untreated controls in experiments may not provide fair comparison by which effects can be determined. This study provides a novel method of establishing the effects of growth factors in ES cell differentiation.

Appendix 1

ES differentiation - PCR tables

Results are expressed as fold difference in gene expression relative to Day 0. All results are correct to 2 decimal places. SR = Serum Replacement, FCS = Foetal calf serum

Runx1

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES2	1	1.45	2.60	2.63	6.48	7.62	8.98
ES4	1	3.12	2.67	4.94	3.38	3.16	4.91
ES5	1	0.13	7.75	9.58	1.58	1.41	3.07
P		0.446	0.400	0.398	0.363	0.401	0.384

Scl

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES2	1	1.36	0.34	0.52	1.78	2.58	9.96
ES4	1	2.13	2.24	4.73	0.82	1.13	5.45
ES5	1	2.13	3.20	5.18	3.32	3.87	3.61
P		0.099	0.314	0.202	0.347	0.268	0.393

Gata2

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES2	1	1.50	0.55	0.90	1.09	2.05	5.36
ES4	1	1.75	2.20	8.48	1.90	1.48	8.32
ES5	1	2.53	2.94	4.22	4.34	4.40	5.11
P		0.159	0.287	0.396	0.367	0.339	0.301

Lmo2

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES2	1	2.89	1.29	2.47	19.16	15.03	54.82
ES4	1	1.43	1.38	1.75	2.16	1.63	4.99
ES5	1	1.92	1.79	1.41	3.15	3.48	1.82
P		0.206	0.117	0.166	0.423	0.422	0.423

ES differentiation - Flow cytometry results

Data is from 5 independent experiments. Tables show stem cell and differentiation marker data. Results are expressed in the form of percentage of positive cells detected. ND indicates where an experiment has not been done. All results are correct to 1 decimal place. P values indicate statistical significance relative to day 0 result.

Stem Cell Markers

CD34

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	1.4	1.3	ND	2.4	2.4	ND	6.4
ES3	2.6	6.7	7.7	10.9	3.5	16.1	5.2
ES4	3.8	ND	ND	ND	2.3	5.7	11.8
ES5	6.8	4.1	11.7	15.8	3.2	2.0	11.9
ES6	27.7	16.4	13.7	31.4	12.0	8.1	24.8
P		0.465	1.00	0.068	0.225	0.715	0.138

Sca1

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	2.0	2.8	ND	1.7	1.1	ND	3.6
ES3	7.4	31.8	32.3	14.8	8.3	8.5	18.5
ES4	8.4	ND	ND	ND	12.4	19.2	20.8
ES5	7.7	13.1	17.8	9.5	11.2	9.2	11.9
ES6	18.5	24.9	15.6	16.7	19.1	11.7	18.5
P		0.068	0.285	0.581	0.176	0.465	0.068

Flk1

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	0.7	0.5	ND	0.5	5.2	ND	8.3
ES3	1.1	27.8	11.0	20.3	2.4	1.7	11.3
ES4	0.8	ND	ND	ND	0.9	2.7	23.2
ES5	0.6	3.1	34.2	16.9	4.4	2.5	10.7
ES6	0.3	0.2	0.2	0.3	0.5	0.2	0.9
P		0.465	0.285	0.285	0.043	0.141	0.043

C-kit

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	16.3	21.6	ND	3.9	13.8	ND	16.8
ES3	18.8	9.2	6.3	1.9	15.9	18.9	31.0
ES4	66.3	ND	ND	ND	50.4	48.0	57.2
ES5	40.0	45.4	54.9	35.6	12.0	14.0	40.0
ES6	0.2	0.3	0.2	0.1	1.2	0.4	1.1
P		0.715	0.655	0.068	0.080	0.465	0.465

Differentiation Markers

CD45

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	x	x	x	x	x	x	X
ES3	0.2	0.9	1.5	2.5	2.7	2.4	4.1
ES4	x	x	x	x	X	x	X
ES5	0.8	1.3	1.9	62.5	0.2	0.3	2.1
ES6	1.1	3.6	2.0	2.9	3.0	3.2	2.7

Ter119

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	0.1	0.3	ND	0.3	0	ND	0.4
ES3	0.7	5.3	1.3	1.5	2.4	1.6	5.1
ES4	0.1	ND	ND	ND	1.6	0.5	1.9
ES5	0.3	5.5	1.7	2.4	0.2	0.3	3.9
ES6	0.3	1.1	0.1	0.2	0.2	1.0	0.1
P		0.068	0.285	0.144	0.680	0.109	0.080

CD11b

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	0.1	0.5	ND	0.2	0.1	ND	0.5
ES3	1.8	4.6	15.3	7.7	6.2	9.3	10.0
ES4	6.8	ND	ND	ND	25.1	14.4	10.1
ES5	0.2	0.2	0.1	5.0	0.8	1.8	1.6
ES6	0.2	2.7	0.8	0.4	2.4	1.8	2.4
P		0.109	0.285	0.068	0.068	0.066	0.043

CD19

Experiment Date	Day 0	Day 3			Day 6		
		SR	BMP4	FCS	SR	BMP4	FCS
ES1	0.9	1.9	ND	1.9	0.8	ND	1.1
ES3	0.5	1.6	0.7	2.3	1.4	0.7	0.4
ES4	5.8	ND	ND	ND	3.7	2.7	6.3
ES5	1.0	15.8	15.8	33.2	0.8	1.8	1.6
ES6	0.9	0.4	0.2	0.4	0.4	0.4	0.3
P		0.144	0.593	0.144	0.345	0.715	0.588

Foetal liver PCR tables

Results are expressed as fold difference in gene expression relative to Day 0. All results are correct to 2 decimal places. P values indicate statistical significance relative to day 0 result. SR = Serum Replacement, FCS = Foetal calf serum.

Runx1

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL3	1	1.14	2.26	ND	2.02
FL4	1	4.26	2.78	ND	2.53
FL5	1	7.41	7.13	1.91	8.26
FL6	1	0.53	4.73	1.51	0.63
P		0.273	0.068		0.144

Scl

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL3	1	0.10	0.02	ND	0.07
FL4	1	0.22	0.10	ND	0.07
FL5	1	1.13	0.64	0.12	1.03
FL6	1	0.04	0.25	0.05	0.07
P		0.144	0.068		0.131

Gata 2

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL3	1	1.25	1.00	ND	0.86
FL4	1	1.31	0.88	ND	1.85
FL5	1	2.28	1.94	0.39	3.68
FL6	1	0.13	1.34	0.36	0.26
P		0.715	0.285		1.00

Lmo2

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL3	1	0.16	0.24	ND	0.11
FL4	1	0.30	0.18	ND	0.15
FL5	1	0.60	0.57	0.13	1.11
FL6	1	0.03	0.26	0.08	0.05
P		0.068	0.068		0.144

Foetal Liver - Flow cytometry results

Data is from 6 independent experiments. Tables show stem cell and differentiation marker data. Results are expressed in the form of percentage of positive cells detected. ND indicates where an experiment has not been done. All results are correct to 1 decimal place. P values indicate statistical significance relative to day 0 result.

Stem Cell Markers

CD34

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	32.7	8.8	ND	ND	7.9
FL2	9.5	16.3	ND	ND	2.6
FL3	5.0	19.6	15.2	ND	2.5
FL4	8.6	15.8	20.2	ND	7.0
FL5	12.6	10.7	10.5	13.8	45.4
FL6	9.7	7.4	36.2	35.4	3.1
P		0.753	0.144		0.345

Sca1

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	0.6	74.9	ND	ND	17.1
FL2	7.7	6.3	ND	ND	1.2
FL3	3.2	17.2	19.5	ND	7.6
FL4	1.9	16.8	19.1	ND	14.6
FL5	2.7	9.9	9.6	12.2	11.2
FL6	1.3	8.5	11.2	41.7	53.0
P		0.046	0.068		0.075

Flk

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	44.4	5.5	ND	ND	2.6
FL2	ND	ND	ND	ND	ND
FL3	1.5	17.7	13.3	ND	4.4
FL4	0.6	8.9	3.0	ND	11.7
FL5	2.7	8.9	8.1	10.0	6.2
FL6	0.7	4.3	22.7	38.7	2.4
P		0.5	0.068		0.5

C kit

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	ND	ND	ND	ND	ND
FL2	16.6	23.4	ND	ND	1
FL3	19.7	8.3	14.4	ND	5.9
FL4	18.9	3.9	7.1	ND	3.9
FL5	49.3	7.1	7.1	8.8	13.4
FL6	38.4	10.7	22.2	38.9	5.4
P		0.080	0.068		0.043

Differentiation Markers

CD45

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	41.1	25.1	ND	ND	5.8
FL2	38.0	25.1	ND	ND	8.1
FL3	27.6	16.6	23.8	ND	39.8
FL4	8.6	27.8	93.3	ND	40.0
FL5	16.2	14.8	17.6	23.0	37.5
FL6	24.7	26.2	51.2	73.0	45.6
P		0.6	0.273		0.917

Ter119

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	77.9	92.4	ND	ND	51.6
FL2	81.3	34.9	ND	ND	74.5
FL3	75.88	64.64	57.0	ND	40.6
FL4	52.3	11.1	8.8	ND	13.4
FL5	55.9	86.0	85.0	81.5	65.1
FL6	35.4	63.7	30.6	19.4	60.7
P		0.753	0.465		0.249

CD11b

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	5.3	84.8	ND	ND	21.9
FL2	2.9	85.5	ND	ND	90.1
FL3	5.6	23.5	18.9	ND	38.4
FL4	5.6	17.1	16.8	ND	4.7
FL5	0.4	9.4	10.2	13.3	45.6
FL6	0.8	6.2	35.5	29.5	2.2
P		0.028	0.068		0.046

CD19

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
FL1	ND	ND	ND	ND	ND
FL2	ND	ND	ND	ND	ND
FL3	0.84	11.5	4.4	ND	3.5
FL4	1.7	6.0	2.2	ND	6.6
FL5	0.8	10.0	13.2	13.1	17.1
FL6	2.4	16.1	44.8	49.6	9.3
P		0.068	0.068		0.068

Bone marrow PCR tables

Results are expressed as fold difference in gene expression relative to Day 0. All results are correct to 2 decimal places. P values indicate statistical significance relative to day 0 result. SR = Serum Replacement, FCS = Foetal calf serum.

Runx1

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	1	9.11	23.37	ND	8.26
BM6	1	3.79	3.10	3.01	1.79
BM7	1	2.80	2.50	3.10	0.91
P		0.163	0.334		0.371

Scl

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	1	1.47	7.78	ND	1.16
BM6	1	0.59	0.51	0.47	0.08
BM7	1	0.30	0.29	0.36	0.07
P		0.606	0.529		0.260

Gata2

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	1	27.22	92.63	ND	13.96
BM6	1	4.97	4.16	4.50	1.06
BM7	1	6.13	5.22	6.29	0.34
P		0.245	0.377		0.450

Lmo2

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	1	3.29	12.79	ND	3.44
BM6	1	0.93	0.88	0.87	0.36
BM7	1	0.78	0.69	0.97	0.27
P		0.498	0.444		0.765

Bone marrow - Flow cytometry results

Data is from 7 independent experiments. Results are expressed in the form of percentage of positive cells detected. ND indicates where an experiment has not been done. + denotes addition of TPO and IGF1. All results are correct to 1 decimal place. P values indicate statistical significance relative to day 0 result.

Stem Cell Markers

CD34

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	20.0	47.1	49.4	ND	7.8
BM6	0.7	41.3	48.0	20.7	49.9
BM7	0.2	5.1	5.7	4.2	8.2
P		0.146	0.152		0.494

Sca1

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	4.4	20.7	21.9	ND	8.2
BM6	5.9	39.5	34.6	33.0	28.6
BM7	4.9	36.5	31.8	31.4	32.6
P		0.038	0.02		0.131

Flk

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	2.5	2.9	2.0	ND	1.0
BM6	1.9	4.5	4.8	7.7	3.1
BM7	6.6	7.0	7.3	6.2	14.0
P		0.262	0.408		0.464

C kit

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	10.6	52.1	52.9	ND	21.1
BM6	13.9	54.1	54.5	13.1	48.2
BM7	9.3	58.7	58.6	57.0	6.1
P		0.004	0.004		0.333

Differentiation Markers

CD45

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	55.6	96.4	93.3	ND	81.9
BM6	66.9	97.4	98.4	98.3	77.7
BM7	22.9	94.6	96.2	96.5	58.0
P		0.061	0.068		0.077

Ter119

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	41.3	3.7	4.3	ND	13.0
BM6	25.4	3.0	2.6	3.6	24.7
BM7	39.4	6.6	6.6	5.0	29.0
P		0.02	0.018		0.246

CD11b

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	20.4	13.4	16.2	ND	11.0
BM6	1.9	4.8	5.2	3.3	4.8
BM7	0.3	5.9	7.0	5.2	5.1
P		0.908	0.609		0.910

CD19

Experiment Date	Day 0	Day 3			
		SF	BMP4	BMPR	FCS
BM5	3.7	7.2	7.0	ND	4.2
BM6	13.3	6.8	15.1	9.4	8.6
BM7	21.2	12.4	12.2	11.4	15.0
P		0.407	0.769		0.23

Raw PCR data

Data is from representative ES cell Real Time PCR experiment (ES5) in Chapter 3.

NTC = Non template control

Sample Name	Detector	CT	Average	Delta CT	DD CT	FOLD CHANGE
EB D0	mouse gapdh vic	14.06				
EB D0	mouse gapdh vic	14.1				
EB D0	mouse gapdh vic	14.09	14.08333			
EB D3 FCS	mouse gapdh vic	14.47				
EB D3 FCS	mouse gapdh vic	14.33				
EB D3 FCS	mouse gapdh vic	14.62	14.47333			
EB D3 SR	mouse gapdh vic	18.41				
EB D3 SR	mouse gapdh vic	18.64				
EB D3 SR	mouse gapdh vic	18.78	18.61			
EB D3 BMP4	mouse gapdh vic	17.01				
EB D3 BMP4	mouse gapdh vic	17.11				
EB D3 BMP4	mouse gapdh vic	17.24	17.12			
EB D6 FCS	mouse gapdh vic	15.24				
EB D6 FCS	mouse gapdh vic	15.2				
EB D6 FCS	mouse gapdh vic	15.17	15.20333			
EB D6 SR	mouse gapdh vic	15.88				
EB D6 SR	mouse gapdh vic	15.9				
EB D6 SR	mouse gapdh vic	15.98	15.92			
EB D6 BMP4	mouse gapdh vic	15.81				
EB D6 BMP4	mouse gapdh vic	15.89				
EB D6 BMP4	mouse gapdh vic	16.14	15.94667			

EB D0	Runx	26.59				
EB D0	Runx	26.47				
EB D0	Runx	26.39	26.48333	12.4	0	1
EB D3 SR	Runx	29.68				
EB D3 SR	Runx	29.95				
EB D3 SR	Runx	29.83	29.82	15.34667	2.946667	0.13
EB D3 BMP4	Runx	28				
EB D3 BMP4	Runx	28.05				
EB D3 BMP4	Runx	28.12	28.05667	9.446667	-2.95333	7.75
EB D3 FCS	Runx	26.16				
EB D3 FCS	Runx	26.25				
EB D3 FCS	Runx	26.37	26.26	9.14	-3.26	9.58
EB D6 SR	Runx	26.88				
EB D6 SR	Runx	27.1				
EB D6 SR	Runx	26.84	26.94	11.73667	-0.66333	1.58
EB D6 BMP4	Runx	28.12				
EB D6 BMP4	Runx	27.69				

EB D6 BMP4	Runx	27.67	27.82667	11.90667	-0.49333	1.41
EB D6 FCS	Runx	26.76				
EB D6 FCS	Runx	26.62				
EB D6 FCS	Runx	26.8	26.72667	10.78	-1.62	3.07

EB D0	Scl	27.93				
EB D0	Scl	28.06				
EB D0	Scl	27.86	27.95	13.86667	0	1
EB D3 FCS	Scl	26.32				
EB D3 FCS	Scl	25.92				
EB D3 FCS	Scl	25.66	25.96667	11.49333	-2.3733	5.18
EB D3 SR	Scl	31.13				
EB D3 SR	Scl	31.52				
EB D3 SR	Scl	31.5	31.38333	12.77333	-1.0933	2.13
EB D3 BMP	Scl	29.15				
EB D3 BMP	Scl	29.37				
EB D3 BMP	Scl	29.41	29.31	12.19	-1.6767	3.20
EB D6 FCS	Scl	27.2				
EB D6 FCS	Scl	27.14				
EB D6 FCS	Scl	27.31	27.21667	12.01333	-1.8533	3.61
EB D6 SR	Scl	28.11				
EB D6 SR	Scl	28.02				
EB D6 SR	Scl	28.04	28.05667	12.13667	-1.73	3.32
EB D6 BMP	Scl	27.79				
EB D6 BMP	Scl	27.76				
EB D6 BMP	Scl	28.03	27.86	11.91333	-1.9533	3.87

EB D0	M gata2	24.26				
EB D0	M gata2	24.46				
EB D0	M gata2	24.39	24.37	10.28667	0	1
EB D3 FCS	M gata2	22.68				
EB D3 FCS	M gata2	22.62				
EB D3 FCS	M gata2	22.75	22.68333	8.21	-2.0767	4.22
EB D3 SR	M gata2	27.45				
EB D3 SR	M gata2	27.63				
EB D3 SR	M gata2	27.6	27.56	8.95	-1.3367	2.53
EB D3 BMP4	M gata2	25.81				
EB D3 BMP4	M gata2	25.93				
EB D3 BMP4	M gata2	25.81	25.85	8.73	-1.5567	2.94
EB D6 FCS	M gata2	23.14				
EB D6 FCS	M gata2	23.11				
EB D6 FCS	M gata2	23.16	23.13667	7.933333	-2.3533	5.11
EB D6 SR	M gata2	24.11				
EB D6 SR	M gata2	24.23				
EB D6 SR	M gata2	23.93	24.09	8.17	-2.1167	4.34
EB D6 BMP	M gata2	24.14				
EB D6 BMP	M gata2	24.02				

EB D6 BMP4	M gata2	24.13	24.09667	8.15	-2.1367	4.40

EB D0	Lmo2 mouse	28.04				
EB D0	Lmo2 mouse	28.06				
EB D0	Lmo2 mouse	28.15	28.08333	14	0	1
EB D3 FCS	Lmo2 mouse	27.89				
EB D3 FCS	Lmo2 mouse	27.99				
EB D3 FCS	Lmo2 mouse	28.06	27.98	13.50667	-0.4933	1.41
EB D3 SR	Lmo2 mouse	31.58				
EB D3 SR	Lmo2 mouse	31.55				
EB D3 SR	Lmo2 mouse	31.88	31.67	13.06	-0.94	1.92
EB D3 BMP4	Lmo2 mouse	30.28				
EB D3 BMP4	Lmo2 mouse	30.41				
EB D3 BMP4	Lmo2 mouse	30.15	30.28	13.16	-0.84	1.79
EB D6 FCS	Lmo2 mouse	28.32				
EB D6 FCS	Lmo2 mouse	28.33				
D6 FCS	Lmo2 mouse	28.37	28.34	13.13667	-0.8633	1.82
EB D6 SR	Lmo2 mouse	28.49				
EB D6 SR	Lmo2 mouse	28.12				
D6 SR	Lmo2 mouse	28.18	28.26333	12.34333	-1.6567	3.15
EB D6 BMP	Lmo2 mouse	28.14				
EB D6 BMP	Lmo2 mouse	28.16				
D6 BMP	Lmo2 mouse	28.14	28.14667	12.2	-1.8	3.48

Runx	NTC	Undetermined
Runx	NTC	Undetermined
Runx	NTC	Undetermined
Scl	NTC	Undetermined
Scl	NTC	Undetermined
Scl	NTC	Undetermined
M gata2	NTC	Undetermined
M gata2	NTC	Undetermined
M gata2	NTC	Undetermined
mouse gapdh vic	NTC	Undetermined
mouse gapdh vic	NTC	Undetermined
mouse gapdh vic	NTC	Undetermined
Lmo2 mouse	NTC	Undetermined
Lmo2 mouse	NTC	Undetermined
Lmo2 mouse	NTC	Undetermined

Values are fold difference in gene expression correct to 2 decimal places. The statistical significance of results is given by the P values.

Runx1 (BMP4 titration)

Relative to Day 0

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	4.68	2.10	3.60	1.68
Exp 2	2.17	1.20	1.13	1.21
Exp 3	1.80	0.32	0.20	0.03
Exp 4	3.23	3.59	4.52	3.72
Exp 5	3.01	1.18	2.49	1.91
Average	**2.98**	**1.68**	**2.39**	**1.71**
P	**0.04**	**0.23**	**0.14**	**0.14**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	1.03	0.50	0.99	0.75
Exp 2	0.30	0.15	0.15	0.13
Exp 3	0.14	0.08	0.06	0.03
Exp 4	0.40	0.32	0.39	X
Exp 5	0.19	0.13	0.00	0.30
Average	**0.41**	**0.24**	**0.32**	**0.30**
P	**0.08**	**0.04**	**0.04**	**0.5**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	1.78	1.88	2.13	1.56
Exp 2	0.10	0.13	0.12	0.12
Exp 3	0.08	0.09	0.09	0.12
Exp 4	0.52	0.51	0.65	0.69
Exp 5	0.49	0.37	0.48	0.58
Average	**0.59**	**0.60**	**0.69**	**0.61**
P	**0.07**	**0.07**	**0.07**	**0.07**

Relative to untreated control

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	1	0.45	0.77	0.36
Exp 2	1	0.55	0.52	0.56
Exp 3	1	0.18	0.11	0.01
Exp 4	1	1.11	1.40	1.15
Exp 5	1	0.39	0.83	0.63
Average	**1**	**0.54**	**0.72**	**0.54**
P		**0.08**	**0.35**	**0.08**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	1	0.49	0.97	0.73
Exp 2	1	0.51	0.49	0.45
Exp 3	1	0.52	0.44	0.18
Exp 4	1	0.79	0.96	X
Exp 5	1	0.69	1.60	1.60
Average	**1**	**0.60**	**0.89**	**0.74**
P		**0.04**	**0.5**	**0.72**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	1	1.06	1.20	0.87
Exp 2	1	1.07	0.99	0.99
Exp 3	1	0.88	0.88	1.18
Exp 4	1	0.96	1.25	1.32
Exp 5	1	0.75	0.98	1.19
Average	**1**	**0.94**	**1.06**	**1.11**
P		**0.5**	**0.69**	**0.23**

Scl (BMP4 titration) Relative to Day 0

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	1.42	1.49	2.80	1.39
Exp 2	0.59	0.48	0.57	0.93
Exp 3	0.39	0.11	0.12	0.03
Exp 4	0.65	1.05	0.65	1.29
Exp 5	0.31	0.24	0.61	0.91
Average	**0.67**	**0.67**	**0.95**	**0.91**
P	**0.23**	**0.35**	**0.5**	**0.89**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	1.30	0.94	2.68	1.97
Exp 2	0.08	0.06	0.08	0.05
Exp 3	0.09	0.10	0.09	0.10
Exp 4	10.17	5.25	7.13	X
Exp 5	0.04	0.03	0.12	0.13
Average	**2.34**	**1.27**	**2.02**	**0.56**
P	**0.69**	**0.5**	**0.69**	**0.69**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	3.21	3.26	4.17	2.41
Exp 2	0.03	0.05	0.05	0.05
Exp 3	0.06	0.09	0.09	0.53
Exp 4	0.34	0.19	0.54	0.71
Exp 5	0.06	0.06	0.07	0.11
Average	**0.74**	**0.73**	**0.98**	**0.76**
P	**0.5**	**0.5**	**0.5**	**0.5**

Relative to untreated control

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	1	1.05	1.97	0.98
Exp 2	1	0.82	0.97	1.58
Exp 3	1	0.29	0.31	0.07
Exp 4	1	1.62	1.01	2.00
Exp 5	1	0.76	1.94	2.90
Average	**1**	**0.91**	**1.24**	**1.51**
P		**0.69**	**0.5**	**0.23**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	1	0.72	2.05	1.51
Exp 2	1	0.72	0.97	0.63
Exp 3	1	1.08	1.03	1.10
Exp 4	1	0.52	0.70	X
Exp 5	1	0.67	3.07	3.26
Average	**1**	**0.74**	**1.57**	**1.62**
P		**0.08**	**0.35**	**0.14**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	1	1.02	1.30	0.75
Exp 2	1	1.74	1.99	1.83
Exp 3	1	1.49	1.48	8.65
Exp 4	1	0.56	1.59	2.08
Exp 5	1	0.94	1.07	1.85
Average	**1**	**1.15**	**1.48**	**3.03**
P		**0.5**	**0.04**	**0.08**

Gata2 (BMP4 titration) Relative to Day 0

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	4.94	2.78	3.92	2.40
Exp 2	2.48	1.30	1.19	1.94
Exp 3	0.92	0.21	0.19	0.05
Exp 4	2.24	3.35	2.84	3.11
Exp 5	0.89	0.36	1.35	2.30
Average	**2.29**	**1.60**	**1.90**	**1.96**
P	**0.23**	**0.5**	**0.23**	**0.14**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	2.85	0.27	5.80	4.58
Exp 2	0.11	2.82	3.03	2.80
Exp 3	0.17	22.37	19.38	14.32
Exp 4	0.45	0.49	0.71	X
Exp 5	0.15	0.10	0.32	0.40
Average	**0.74**	**5.21**	**5.85**	**5.53**
P	**0.5**	**0.69**	**0.23**	**0.08**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	14.66	7.45	9.13	5.46
Exp 2	0.03	0.10	0.010	0.09
Exp 3	0.02	0.06	0.08	0.22
Exp 4	0.33	0.34	0.69	1.14
Exp 5	0.19	0.21	0.33	0.62
Average	**3.05**	**1.63**	**2.06**	**1.50**
P	**0.5**	**0.69**	**0.69**	**0.69**

Relative to untreated control

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	1	0.56	0.79	0.49
Exp 2	1	0.53	0.48	0.78
Exp 3	1	0.23	0.20	0.05
Exp 4	1	1.50	1.27	1.39
Exp 5	1	0.40	1.51	2.58
Average	**1**	**0.64**	**0.85**	**1.06**
P		**0.35**	**0.89**	**0.35**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	1	0.87	2.04	1.61
Exp 2	1	27.00	28.84	26.66
Exp 3	1	127.85	110.79	81.86
Exp 4	1	1.10	1.60	X
Exp 5	1	0.66	2.14	2.74
Average	**1**	**31.45**	**29.08**	**28.22**
P		**0.5**	**0.04**	**0.04**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	1	0.51	0.62	0.37
Exp 2	1	3.01	2.98	2.62
Exp 3	1	3.05	3.81	10.65
Exp 4	1	1.02	2.08	3.42
Exp 5	1	1.15	1.77	3.31
Average	**1**	**1.75**	**2.25**	**4.08**
P		**0.69**	**0.89**	**0.69**

Lmo2 (BMP4 titration)

Relative to Day 0

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	8.11	7.00	8.54	4.70
Exp 2	0.63	1.47	1.61	1.95
Exp 3	1.08	0.27	0.31	0.05
Exp 4	2.35	3.81	2.41	4.28
Exp 5	0.77	0.66	0.70	0.01
Average	**2.59**	**2.64**	**2.71**	**2.20**
P	**0.5**	**0.35**	**0.35**	**0.42**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	5.06	11.03	7.57	4.66
Exp 2	0.23	0.40	0.48	0.39
Exp 3	0.19	0.19	0.36	0.50
Exp 4	11.61	7.52	8.86	X
Exp 5	0.15	0.10	0.18	0.22
Average	**3.45**	**3.85**	**3.49**	**1.44**
P	**0.72**	**0.72**	**0.72**	**0.72**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	15.82	10.08	13.61	9.76
Exp 2	0.27	0.55	0.63	1.29
Exp 3	0.27	0.35	0.37	0.67
Exp 4	1.03	0.83	1.87	4.20
Exp 5	0.20	0.21	0.23	0.31
Average	**3.52**	**2.40**	**3.34**	**3.25**
P	**0.69**	**0.5**	**0.69**	**0.35**

Relative to untreated control

	Day 2 0ng/ml	*Day 2 5ng/ml*	*Day 2 10ng/ml*	*Day 2 25ng/ml*
Exp 1	1	0.86	1.05	0.58
Exp 2	1	2.31	2.54	3.07
Exp 3	1	0.25	0.29	0.05
Exp 4	1	1.63	1.03	1.82
Exp 5	1	0.85	0.90	0.01
Average	**1**	**1.18**	**1.16**	**1.11**
P		**0.69**	**0.89**	**0.69**

	Day 4 0ng/ml	*Day 4 5ng/ml*	*Day 4 10ng/ml*	*Day 4 25ng/ml*
Exp 1	1	2.18	1.50	0.92
Exp 2	1	1.75	1.21	0.97
Exp 3	1	1.00	1.88	2.62
Exp 4	1	0.648	0.763	X
Exp 5	1	0.69	1.27	1.50
Average	**1**	**1.25**	**1.32**	**1.50**
P		**0.5**	**0.08**	**0.47**

	Day 6 0ng/ml	*Day 6 5ng/ml*	*Day 6 10ng/ml*	*Day 6 25ng/ml*
Exp 1	1	0.64	0.86	0.62
Exp 2	1	2.07	2.38	4.83
Exp 3	1	1.28	1.35	2.46
Exp 4	1	0.80	1.81	4.07
Exp 5	1	1.02	1.12	1.53
Average	**1**	**1.16**	**1.51**	**2.70**
P		**0.69**	**0.14**	**0.08**

Runx1 (BMP receptor titration)

Relative to Day 0

	Day 2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	2.61	2.54	2.99	1.83
Exp 2	2.32	1.31	1.27	2.21
Exp 3	2.45	1.96	2.15	1.98
Exp 4	5.71	5.01	4.91	3.88
Average	**3.27**	**2.70**	**2.83**	**2.47**
P	**0.07**	**0.07**	**0.07**	**0.07**

	Day 4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	0.23	0.20	0.23	0.21
Exp 2	0.89	0.69	0.78	1.02
Exp 3	1.61	0.97	X	0.94
Exp 4	0.84	0.75	0.62	0.57
Average	**0.89**	**0.65**	**0.54**	**0.69**
P	**0.72**	**0.07**	**0.11**	**0.14**

	Day 6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	0.74	0.73	X	0.78
Exp 2	0.44	0.40	0.36	0.42
Exp 3	0.88	0.92	0.89	0.79
Exp 4	0.10	0.09	0.11	0.06
Average	**0.54**	**0.53**	**0.45**	**0.51**
P	**0.07**	**0.11**	**0.07**	**0.07**

Relative to untreated control

	Day2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	1	0.88	0.86	0.68
Exp 2	1	0.57	0.55	0.95
Exp 3	1	0.80	0.88	0.81
Exp 4	1	0.86	0.99	0.90
Average	**1**	**0.78**	**0.82**	**0.84**
P		**0.07**	**0.07**	**0.07**

	Day4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	1	1.05	1.01	0.89
Exp 2	1	0.97	1.15	0.70
Exp 3	1	0.77	0.88	1.15
Exp 4	1	0.60	X	0.59
Average	**1**	**0.85**	**1.01**	**0.83**
P		**0.27**	**0.59**	**0.27**

	Day6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	1	0.90	0.74	0.68
Exp 2	1	0.99	1.58	1.06
Exp 3	1	0.89	0.81	0.96
Exp 4	1	0.87	1.10	0.64
Average	**1**	**0.91**	**1.06**	**0.83**
P		**0.07**	**1**	**0.27**

Scl (BMP receptor titration)

Relative to day 0

	Day 2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	0.64	0.91	1.67	1.21
Exp 2	1.18	0.94	1.27	1.70
Exp 3	1.96	0.55	0.61	0.44
Exp 4	1.71	1.84	3.10	2.02
Average	**1.37**	**1.06**	**1.66**	**1.34**
P	**0.27**	**0.72**	**0.27**	**0.27**

	Day 4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	0.44	0.25	0.31	0.31
Exp 2	0.70	0.51	0.76	0.73
Exp 3	1.17	0.99	X	0.93
Exp 4	0.69	0.81	0.31	0.50
Average	**0.75**	**0.64**	**0.46**	**0.62**
P	**0.14**	**0.07**	**0.11**	**0.07**

	Day 6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	0.28	1.42	0.47	0.32
Exp 2	0.16	0.18	0.17	0.21
Exp 3	0.50	0.66	0.73	0.38
Exp 4	0.37	0.39	0.21	0.22
Average	**0.33**	**0.66**	**0.40**	**0.28**
P	**0.07**	**0.27**	**0.07**	**0.07**

Relative to untreated control

	Day2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	1	1.07	1.81	1.18
Exp 2	1	0.79	1.07	1.44
Exp 3	1	1.27	1.40	1.01
Exp 4	1	0.60	0.71	0.71
Average	**1**	**0.93**	**1.25**	**1.08**
P		**0.72**	**0.27**	**0.47**

	Day4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	1	1.32	1.47	0.76
Exp 2	1	1.41	2.60	1.89
Exp 3	1	0.72	1.09	1.04
Exp 4	1	0.85	X	0.79
Average	**1**	**1.07**	**1.72**	**1.12**
P		**0.47**	**0.11**	**1.00**

	Day6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	1	1.17	0.44	0.72
Exp 2	1	5.08	1.67	1.15
Exp 3	1	1.14	1.06	1.28
Exp 4	1	1.03	0.56	0.59
Average	**1**	**2.46**	**1.06**	**1.05**
P		**0.07**	**1**	**0.72**

Gata2 (BMP receptor titration)

Relative to day 0

	Day 2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	1.96	2.41	1.97	1.80
Exp 2	3.50	2.52	2.17	3.40
Exp 3	2.16	2.05	2.34	2.36
Exp 4	4.10	3.61	4.33	3.40
Average	**2.93**	**2.65**	**2.70**	**2.74**
P	**0.07**	**0.07**	**0.07**	**0.07**

	Day 4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	0.33	0.25	0.29	0.28
Exp 2	1.02	0.80	0.97	0.96
Exp 3	3.74	3.94	X	2.83
Exp 4	0.71	0.61	0.41	0.31
Average	**1.45**	**1.40**	**0.56**	**1.09**
P	**1.00**	**0.72**	**0.11**	**0.72**

	Day 6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	0.80	1.03	1.25	0.92
Exp 2	0.37	0.40	0.26	0.33
Exp 3	0.82	1.01	0.90	0.41
Exp 4	0.08	0.08	0.07	0.05
Average	**0.52**	**0.63**	**0.62**	**0.43**
P	**0.07**	**0.47**	**0.27**	**0.07**

Relative to untreated control

	Day2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	1	0.88	1.06	0.83
Exp 2	1	0.72	0.62	0.97
Exp 3	1	0.95	1.08	1.09
Exp 4	1	0.77	0.89	0.85
Average	**1**	**0.83**	**0.91**	**0.93**
P		**0.07**	**0.47**	**0.27**

	Day4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	1	1.23	1.10	0.50
Exp 2	1	1.23	1.01	0.92
Exp 3	1	0.78	0.95	0.94
Exp 4	1	1.05	X	0.76
Average	**1**	**1.07**	**1.02**	**0.78**
P		**0.27**	**0.59**	**0.07**

	Day6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	1	0.86	0.57	0.44
Exp 2	1	1.29	1.57	1.14
Exp 3	1	1.08	0.70	0.91
Exp 4	1	0.99	0.86	0.61
Average	**1**	**1.06**	**0.92**	**0.77**
P		**0.72**	**0.72**	**0.27**

Lmo2 (BMP receptor titration)

Relative to day 0

	Day 2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	7.19	9.02	15.85	11.06
Exp 2	3.62	3.70	4.57	6.09
Exp 3	3.13	5.30	4.21	3.74
Exp 4	8.09	8.80	11.37	10.17
Average	**5.51**	**6.70**	**9.00**	**7.77**
P	**0.07**	**0.07**	**0.07**	**0.07**

	Day 4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	1.64		1.37	1.42
Exp 2	2.16	1.81	2.04	2.26
Exp 3	4.62	4.69	X	2.77
Exp 4	1.45	2.21	1.17	0.82
Average	**2.47**	**2.90**	**1.53**	**1.82**
P	**0.07**	**0.11**	**0.14**	**0.11**

	Day 6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	1.77	2.27	2.45	2.84
Exp 2	0.56	0.95	0.59	0.59
Exp 3	3.62	3.50	3.26	1.26
Exp 4	2.27	2.40	3.39	2.37
Average	**2.06**	**2.28**	**2.42**	**1.77**
P	**0.14**	**0.14**	**0.14**	**0.27**

Relative to untreated control

	Day2 0ng/ml	*Day 2 25ng/ml*	*Day 2 100ng/ml*	*Day 2 250ng/ml*
Exp 1	1	1.09	1.40	1.26
Exp 2	1	1.02	1.26	1.68
Exp 3	1	1.69	1.34	1.20
Exp 4	1	X	0.84	0.86
Average	**1**	**1.27**	**1.21**	**1.25**
P		**0.11**	**0.14**	**0.14**

	Day4 0ng/ml	*Day 4 25ng/ml*	*Day 4 100ng/ml*	*Day 4 250ng/ml*
Exp 1	1	0.97	0.90	0.35
Exp 2	1	1.25	2.20	1.54
Exp 3	1	0.84	0.95	1.05
Exp 4	1	1.02	X	0.60
Average	**1**	**1.02**	**1.35**	**0.88**
P		**1.00**	**1.00**	**1.00**

	Day6 0ng/ml	*Day 6 25ng/ml*	*Day 6 100ng/ml*	*Day 6 250ng/ml*
Exp 1	1	1.53	0.81	0.57
Exp 2	1	1.28	1.38	1.60
Exp 3	1	1.69	1.06	1.05
Exp 4	1	1.06	1.50	1.21
Average	**1**	**1.39**	**1.18**	**1.11**
P		**0.07**	**0.27**	**0.47**

Runx1 (shRNA experiment) Relative to Day 0

Values are fold difference in gene expression correct to 2 decimal places. The statistical significance of results is given by the P values.

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	0.93	1.28	1.24	1.30
Exp 2	1.40	1.21	1.10	1.06
Exp 3	0.58	0.72	0.67	0.35
Exp 4	0.37	0.71	0.41	0.31
Exp 5	0.15	0.31	0.20	0.15
Average	**0.68**	**0.85**	**0.72**	**0.64**
P	**0.225**	**0.5**	**0.225**	**0.225**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	0.32	0.71	0.54	0.37
Exp 2	3.37	3.46	3.30	2.62
Exp 3	0.35	0.40	0.30	0.12
Exp 4	0.28	0.29	0.29	0.26
Exp 5	0.14	0.36	0.42	0.33
Average	**0.89**	**1.04**	**0.97**	**0.74**
P	**0.5**	**0.5**	**0.5**	**0.5**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.69	0.34	1.32	0.56
Exp 2	0.42	0.30	1.05	0.19
Exp 3	0.23	0.20	0.95	0.12
Exp 4	0.20	0.21	1.10	0.20
Exp 5	0.28	0.20	1.60	0.09
Average	**0.36**	**0.25**	**1.20**	**0.23**
P	**0.043**	**0.043**	**0.138**	**0.043**

Runx1 (shRNA experiment)   Relative to untreated control

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	1	1.37	1.33	1.40
Exp 2	1	0.87	0.79	0.76
Exp 3	1	1.25	1.15	0.61
Exp 4	1	1.93	1.11	0.85
Exp 5	1	2.10	1.37	0.98
Average	**1**	**1.50**	**1.15**	**0.92**
P		**0.08**	**0.225**	**0.5**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	1	2.26	1.71	1.18
Exp 2	1	1.03	0.98	0.78
Exp 3	1	1.15	0.86	0.35
Exp 4	1	1.11	1.04	0.91
Exp 5	1	2.51	2.98	2.35
Average	**1**	**1.61**	**1.51**	**1.11**
P		**0.043**	**0.345**	**0.893**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	1	0.49	1.91	0.80
Exp 2	1	0.72	2.51	0.44
Exp 3	1	0.84	4.06	0.51
Exp 4	1	1.07	5.58	0.99
Exp 5	1	0.71	5.66	0.33
Average	**1**	**0.77**	**3.94**	**0.61**
P		**0.08**	**0.043**	**0.043**

Scl (shRNA experiment) Relative to Day 0

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	1.60	1.82	0.88	1.65
Exp 2	0.85	0.60	0.79	0.94
Exp 3	0.40	0.46	0.75	0.31
Exp 4	1.25	1.30	1.47	1.25
Exp 5	1.33	1.15	1.82	2.12
Average	**1.09**	**1.06**	**1.14**	**1.26**
P	**0.5**	**0.893**	**0.686**	**0.345**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	0.41	0.54	0.30	0.36
Exp 2	2.24	1.26	1.89	2.68
Exp 3	0.18	0.17	0.14	0.07
Exp 4	0.25	0.44	0.35	0.24
Exp 5	0.33	0.23	0.06	0.07
Average	**0.68**	**0.53**	**0.55**	**0.68**
P	**0.5**	**0.08**	**0.5**	**0.5**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.44	0.56	7.24	0.54
Exp 2	0.20	0.46	5.83	0.21
Exp 3	0.34	0.19	9.51	0.19
Exp 4	0.45	0.33	11.79	0.22
Exp 5	0.30	0.23	12.79	0.24
Average	**0.35**	**0.35**	**9.43**	**0.28**
P	**0.043**	**0.043**	**0.043**	**0.043**

Scl (shRNA experiment) Relative to untreated control

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	1	1.14	0.55	1.03
Exp 2	1	0.71	0.94	1.12
Exp 3	1	1.15	1.88	0.77
Exp 4	1	1.04	1.18	1.01
Exp 5	1	0.86	1.37	1.59
Average	**1**	**0.98**	**1.18**	**1.10**
P		**0.893**	**0.345**	**0.345**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	1	1.31	0.73	0.87
Exp 2	1	0.56	0.85	1.20
Exp 3	1	0.95	0.79	0.42
Exp 4	1	1.74	1.39	0.95
Exp 5	1	0.71	0.19	0.21
Average	**1**	**1.05**	**0.79**	**0.73**
P		**0.893**	**0.345**	**0.225**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	1	1.25	16.30	1.21
Exp 2	1	2.26	28.84	1.05
Exp 3	1	0.57	28.25	0.56
Exp 4	1	0.73	26.36	0.48
Exp 5	1	0.78	43.41	0.83
Average	**1**	**1.12**	**28.63**	**0.83**
P		**0.893**	**0.043**	**0.345**

Gata2 (shRNA experiment)

Relative to Day 0

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	0.58	0.68	0.73	0.82
Exp 2	0.74	0.41	0.44	0.57
Exp 3	0.27	0.25	0.34	0.20
Exp 4	0.28	0.40	0.52	0.20
Exp 5	0.18	0.36	0.17	X
Average	**0.41**	**0.42**	**0.44**	**0.45**
P	**0.043**	**0.043**	**0.043**	**0.068**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	0.13	0.30	0.32	0.20
Exp 2	0.10	0.08	0.13	0.07
Exp 3	0.01	0.01	0.01	0.002
Exp 4	0.01	0.01	0.01	0.004
Exp 5	0.01	0.01	0.01	0.002
Average	**0.05**	**0.08**	**0.09**	**0.06**
P	**0.043**	**0.043**	**0.043**	**0.042**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.53	0.22	0.97	0.34
Exp 2	0.12	0.14	0.87	0.05
Exp 3	0.08	0.06	0.98	0.04
Exp 4	0.07	0.08	1.12	0.04
Exp 5	0.06	0.06	1.10	0.03
Average	**0.17**	**0.11**	**1.01**	**0.10**
P	**0.043**	**0.043**	**0.893**	**0.043**

Gata2 (shRNA experiment)   Relative to untreated control

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	1	1.18	1.26	1.42
Exp 2	1	0.55	0.60	0.78
Exp 3	1	0.92	1.27	0.73
Exp 4	1	1.47	1.91	0.74
Exp 5	1	2.01	0.95	2.07
Average	**1**	**1.23**	**1.20**	**1.15**
P		**0.345**	**0.5**	**0.686**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	1	2.23	2.39	1.46
Exp 2	1	0.84	1.30	0.75
Exp 3	1	0.92	1.10	0.20
Exp 4	1	2.21	1.93	0.66
Exp 5	1	0.86	0.68	0.27
Average	**1**	**1.41**	**1.48**	**0.67**
P		**0.686**	**0.138**	**0.225**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	1	0.43	1.85	0.64
Exp 2	1	1.14	7.09	0.37
Exp 3	1	0.70	12.24	0.47
Exp 4	1	1.06	15.60	0.56
Exp 5	1	1.04	20.07	0.61
Average	**1**	**0.87**	**11.37**	**0.53**
P		**0.686**	**0.043**	**0.043**

Lmo2 (shRNA experiment) Relative to Day 0

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	1.73	1.38	1.15	1.71
Exp 2	1.31	1.22	0.98	1.36
Exp 3	0.70	0.86	0.79	0.43
Exp 4	2.71	2.86	2.23	2.66
Exp 5	1.41	1.54	1.73	2.41
Average	**1.57**	**1.57**	**1.38**	**1.72**
P	**0.08**	**0.08**	**0.345**	**0.138**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	3.09	2.75	1.25	1.36
Exp 2	16.60	14.62	14.25	21.16
Exp 3	1.27	1.50	0.77	0.47
Exp 4	1.55	2.78	0.90	1.08
Exp 5	2.16	1.94	0.58	0.80
Average	**4.93**	**4.72**	**3.55**	**4.97**
P	**0.043**	**0.043**	**0.893**	**0.686**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	1.97	2.34	8.69	2.06
Exp 2	1.37	1.55	6.93	1.10
Exp 3	1.44	0.99	11.26	1.13
Exp 4	1.50	1.53	11.31	1.05
Exp 5	1.14	0.88	8.28	0.55
Average	**1.48**	**1.46**	**9.30**	**1.18**
P	**0.043**	**0.225**	**0.043**	**0.345**

Lmo2 (shRNA experiment) Relative to untreated control

	Day 2 Serum free	*Day 2 shRNA*	*Day 2 shRNA +BMP4*	*Day 2 Scramble*
Exp 1	1	0.80	0.67	0.99
Exp 2	1	0.94	0.75	1.04
Exp 3	1	1.22	1.13	0.62
Exp 4	1	1.06	0.82	0.98
Exp 5	1	1.09	1.22	1.71
Average	**1**	**1.02**	**0.92**	**1.07**
P		**0.686**	**0.5**	**0.893**

	Day 4 Serum free	*Day 4 shRNA*	*Day 4 shRNA +BMP4*	*Day 4 Scramble*
Exp 1	1	0.89	0.41	0.44
Exp 2	1	0.88	0.86	1.28
Exp 3	1	1.19	0.61	0.38
Exp 4	1	1.79	0.58	0.69
Exp 5	1	0.90	0.27	0.37
Average	**1**	**1.13**	**0.54**	**0.63**
P		**0.686**	**0.043**	**0.138**

	Day 6 Serum free	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	1	1.19	4.41	1.04
Exp 2	1	1.13	5.08	0.81
Exp 3	1	0.69	7.84	0.78
Exp 4	1	1.02	7.55	0.70
Exp 5	1	0.77	7.24	0.49
Average	**1**	**0.96**	**6.42**	**0.76**
P		**0.686**	**0.043**	**0.08**

Flow cytometry tables (shRNA experiment)

Values are percentage of positive cell correct to 2 decimal places.

CD34	*Day 6 Serum free*	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.03	0.65	0.04	0.04
Exp 2	0.03	1.11	0.11	0.03
Exp 3	0.32	0.80	0.05	0.06
Exp 4	0.13	0.92	0.06	0.03
Average	**0.13**	**0.87**	**0.07**	**0.04**
P		**0.01**	**0.47**	**0.26**

Sca-1	*Day 6 Serum free*	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.22	0.42	0.40	0.24
Exp 2	2.54	1.15	0.69	0.49
Exp 3	X	1.26	1.57	0.26
Exp 4	0.31	0.55	0.84	0.22
Average	**1.02**	**0.85**	**0.88**	**0.30**
P		**0.62**	**0.66**	**0.40**

Flk-1	*Day 6 Serum free*	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.89	1.60	3.90	0.68
Exp 2	2.03	1.18	3.32	1.28
Exp 3	X	2.41	4.68	1.75
Exp 4	1.18	1.25	2.71	1.06
Average	**1.37**	**1.61**	**3.65**	**1.19**
P		**0.96**	**0.07**	**0.21**

C-kit	*Day 6 Serum free*	*Day 6 shRNA*	*Day 6 shRNA +BMP4*	*Day 6 Scramble*
Exp 1	0.32	0.31	1.76	0.24
Exp 2	0.35	0.50	1.63	1.30
Exp 3	X	0.85	2.53	0.25
Exp 4	1.01	0.57	2.12	0.27
Average	**0.56**	**0.56**	**2.01**	**0.52**
P		**0.63**	**0.01**	**0.94**

References

Adelman, C. A., Chattopadhyay, S., & Bieker, J. J. 2002, "The BMP/BMPR/Smad pathway directs expression of the erythroid-specific EKLF and GATA1 transcription factors during embryoid body differentiation in serum-free media", *Development*, vol. 129, no. 2, pp. 539-549.

Adya, N., Stacy, T., Speck, N. A., & Liu, P. P. 1998, "The Leukemic Protein Core Binding Factor beta á(CBFbeta)-Smooth-Muscle Myosin Heavy Chain Sequesters CBFalpha 2 into Cytoskeletal Filaments and Aggregates", *Molecular and Cellular Biology*, vol. 18, no. 12, pp. 7432-7443.

Andrews, P. W. 2002, "From teratocarcinomas to embryonic stem cells", *Philos.Trans.R Soc Lond B Biol Sci.*, vol. 357, no. 1420, pp. 405-417.

Artzt, K., Dubois, P., Bennett, D., Condamine, H., Babinet, C., & Jacob, F. 1973, "Surface antigens common to mouse cleavage embryos and primitive teratocarcinoma cells in culture", *Proc Natl Acad Sci U.S.A.*, vol. 70, no. 10, pp. 2988-2992.

Attar, E. C. & Scadden, D. T. 2004, "Regulation of hematopoietic stem cell growth", *Leukemia*, vol. 18, no. 11, pp. 1760-1768.

Austyn, J. M. & Gordon, S. 1981, "F4/80, a monoclonal antibody directed specifically against the mouse macrophage", *Eur J Immunol.*, vol. 11, no. 10, pp. 805-815.

Begley, C. G. & Green, A. R. 1999, "The SCL Gene: From Case Report to Critical Hematopoietic Regulator", *Blood*, vol. 93, no. 9, pp. 2760-2770.

Beppu, H., Kawabata, M., Hamamoto, T., Chytil, A., Minowa, O., Noda, T., & Miyazono, K. 2000, "BMP type II receptor is required for gastrulation and early development of mouse embryos", *Dev Biol.*, vol. 221, no. 1, pp. 249-258.

Bhardwaj, G., Murdoch, B., Wu, D., Baker, D. P., Williams, K. P., Chadwick, K., Ling, L. E., Karanu, F. N., & Bhatia, M. 2001, "Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation", *Nat Immunol.*, vol. 2, no. 2, pp. 172-180.

Bhatia, M., Bonnet, D., Wu, D., Murdoch, B., Wrana, J., Gallacher, L., & Dick, J. E. 1999, "Bone Morphogenetic Proteins Regulate the Developmental Program of Human Hematopoietic Stem Cells", *The Journal of Experimental Medicine*, vol. 189, no. 7, pp. 1139-1148.

Bhatia, M., Wang, J. C. Y., Kapp, U., Bonnet, D., & Dick, J. E. 1997, "Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 10, pp. 5320-5325.

Bonilla, S., Alarcon, P., Villaverde, R., Aparicio, P., Silva, A., & Martinez, S. 2002, "Haematopoietic progenitor cells from adult bone marrow differentiate into cells that express oligodendroglial antigens in the neonatal mouse brain", *Eur J Neurosci.*, vol. 15, no. 3, pp. 575-582.

Bradley, A., Evans, M., Kaufman, M. H., & Robertson, E. 1984, "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines", *Nature.*, vol. 309, no. 5965, pp. 255-256.

Cai, Z., de Bruijn, M., Ma, X., Dortland, B., Luteijn, T., Downing, R. J., & Dzierzak, E. 2000, "Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo", *Immunity.*, vol. 13, no. 4, pp. 423-431.

Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., Martin, R. P., Schipani, E., Divieti, P., Bringhurst, F. R., Milner, L. A., Kronenberg, H. M., & Scadden, D. T. 2003, "Osteoblastic cells regulate the haematopoietic stem cell niche", *Nature*, vol. 425, no. 6960, pp. 841-846.

Caplen, N. J., Parrish, S., Imani, F., Fire, A., & Morgan, R. A. 2001, "Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 17, pp. 9742-9747.

Caprioli, A., Jaffredo, T., Gautier, R., Dubourg, C. +., & Dieterlen-Lievre, F. 1998, "Blood-borne seeding by hematopoietic and endothelial precursors from the allantois", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 4, pp. 1641-1646.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., & Nagy, A. 1996, "Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele", *Nature*, vol. 380, no. 6573, pp. 435-439.

Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A., & Bhatia, M. 2003, "Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells", *Blood*, vol. 102, no. 3, pp. 906-915.

Chambers, I. 2004, "The molecular basis of pluripotency in mouse embryonic stem cells", *Cloning Stem Cells.*, vol. 6, no. 4, pp. 386-391.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., & Smith, A. 2003, "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells", *Cell.*, vol. 113, no. 5, pp. 643-655.

Chang, K. H., Nelson, A. M., Fields, P. A., Hesson, J. L., Ulyanova, T., Cao, H., Nakamoto, B., Ware, C. B., & Papayannopoulou, T. 2008, "Diverse hematopoietic

potentials of five human embryonic stem cell lines", *Exp Cell Res.*, vol. 314, no. 16, pp. 2930-2940.

Chen, M. J., Yokomizo, T., Zeigler, B. M., Dzierzak, E., & Speck, N. A. 2009, "Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter", *Nature*, vol. 457, no. 7231, pp. 887-891.

Chilton, J. M. & Le Doux, J. M. 2008, "Quantitative analysis of retroviral and lentiviral gene transfer to murine embryonic stem cells", *J Biotechnol.*, vol. 138, no. 1-2, pp. 42-51.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., & Keller, G. 1998, "A common precursor for hematopoietic and endothelial cells", *Development*, vol. 125, no. 4, pp. 725-732.

Chung, Y. S., Zhang, W. J., Arentson, E., Kingsley, P. D., Palis, J., & Choi, K. 2002, "Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression", *Development*, vol. 129, no. 23, pp. 5511-5520.

Ciau-Uitz, A., Walmsley, M., & Patient, R. 2000, "Distinct origins of adult and embryonic blood in Xenopus", *Cell.*, vol. 102, no. 6, pp. 787-796.

Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P., & Godin, I. 2001, "Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution", *Immunity.*, vol. 15, no. 3, pp. 477-485.

Davidson, E. H. & Erwin, D. H. 2006, "Gene regulatory networks and the evolution of animal body plans", *Science.*, vol. 311, no. 5762, pp. 796-800.

de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J., & Dzierzak, E. 2002, "Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta", *Immunity.*, vol. 16, no. 5, pp. 673-683.

Delassus, S. & Cumano, A. 1996, "Circulation of hematopoietic progenitors in the mouse embryo", *Immunity.*, vol. 4, no. 1, pp. 97-106.

Dieterlen-Lievre, F. 1975, "On the origin of haemopoietic stem cells in the avian embryo: an experimental approach", *J Embryol Exp Morphol.*, vol. 33, no. 3, pp. 607-619.

DIXON, F. J. & MOORE, R. A. 1952, "Tumors of the testicle", *Acta Unio.Int.Contra.Cancrum.*, vol. 8, no. 2, pp. 310-315.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., & Kemler, R. 1985, "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium", *J Embryol Exp Morphol.*, vol. 87:27-45., pp. 27-45.

Durand, C., Robin, C., Bollerot, K., Baron, M. H., Ottersbach, K., & Dzierzak, E. 2007, "Embryonic stromal clones reveal developmental regulators of definitive hematopoietic

stem cells", *Proceedings of the National Academy of Sciences*, vol. 104, no. 52, pp. 20838-20843.

Dzierzak, E. 2002, "Hematopoietic stem cells and their precursors: developmental diversity and lineage relationships", *Immunol Rev.*, vol. 187:126-38., pp. 126-138.

Dzierzak, E., Medvinsky, A., & de Bruijn, M. 1998, "Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo", *Immunol Today.*, vol. 19, no. 5, pp. 228-236.

Dzierzak, E. & Speck, N. A. 2008, "Of lineage and legacy: the development of mammalian hematopoietic stem cells", *Nat Immunol*, vol. 9, no. 2, pp. 129-136.

Ellis, J. 2005, "Silencing and variegation of gammaretrovirus and lentivirus vectors", *Hum.Gene Ther.*, vol. 16, no. 11, pp. 1241-1246.

Endoh, M., Ogawa, M., Orkin, S., & Nishikawa, S. 2002, "SCL/tal-1-dependent process determines a competence to select the definitive hematopoietic lineage prior to endothelial differentiation", *EMBO J.*, vol. 21, no. 24, pp. 6700-6708.

Evans, M. J. & Kaufman, M. H. 1981, "Establishment in culture of pluripotential cells from mouse embryos", *Nature.*, vol. 292, no. 5819, pp. 154-156.

Faloon, P., Arentson, E., Kazarov, A., Deng, C. X., Porcher, C., Orkin, S., & Choi, K. 2000, "Basic fibroblast growth factor positively regulates hematopoietic development", *Development*, vol. 127, no. 9, pp. 1931-1941.

Fehling, H. J., Lacaud, G., Kubo, A., Kennedy, M., Robertson, S., Keller, G., & Kouskoff, V. 2003, "Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation", *Development*, vol. 130, no. 17, pp. 4217-4227.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., & Moore, M. W. 1996, "Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene", *Nature*, vol. 380, no. 6573, pp. 439-442.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. 1998, "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans", *Nature*, vol. 391, no. 6669, pp. 806-811.

Fuchs, O., Simakova, O., Klener, P., Cmejlova, J., Zivny, J., Zavadil, J., & Stopka, T. 2002, "Inhibition of Smad5 in human hematopoietic progenitors blocks erythroid differentiation induced by BMP4", *Blood Cells Mol Dis.*, vol. 28, no. 2, pp. 221-233.

Gallagher, R. E., Yeap, B. Y., Bi, W., Livak, K. J., Beaubier, N., Rao, S., Bloomfield, C. D., Appelbaum, F. R., Tallman, M. S., Slack, J. L., & Willman, C. L. 2003, "Quantitative real-time RT-PCR analysis of PML-RARalpha mRNA in acute promyelocytic leukemia:

assessment of prognostic significance in adult patients from intergroup protocol 0129", *Blood*, vol. 101, no. 7, pp. 2521-2528.

Gekas, C., Dieterlen-Lievre, F., Orkin, S. H., & Mikkola, H. K. 2005, "The placenta is a niche for hematopoietic stem cells", *Dev Cell.*, vol. 8, no. 3, pp. 365-375.

Gering, M., Rodaway, A. R., Gottgens, B., Patient, R. K., & Green, A. R. 1998, "The SCL gene specifies haemangioblast development from early mesoderm", *EMBO J.*, vol. 17, no. 14, pp. 4029-4045.

Godin, I., Dieterlen-Lievre, F., & Cumano, A. 1995, "Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 3, pp. 773-777.

Godin, I. & Cumano, A. 2002, "The hare and the tortoise: an embryonic haematopoietic race", *Nat Rev Immunol*, vol. 2, no. 8, pp. 593-604.

Godin, I., Garcia-Porrero, J. A., Dieterlen-Lievre, F., & Cumano, A. 1999, "Stem Cell Emergence and Hemopoietic Activity Are Incompatible in Mouse Intraembryonic Sites", *The Journal of Experimental Medicine*, vol. 190, no. 1, pp. 43-52.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., & Mulligan, R. C. 1996, "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo", *J Exp Med.*, vol. 183, no. 4, pp. 1797-1806.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G., & Mello, C. C. 2001, "Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing", *Cell.*, vol. 106, no. 1, pp. 23-34.

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M., & Mulligan, R. C. 1999, "Dystrophin expression in the mdx mouse restored by stem cell transplantation", *Nature*, vol. 401, no. 6751, pp. 390-394.

Haar, J. L. & Ackerman, G. A. 1971, "A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse", *Anat.Rec.*, vol. 170, no. 2, pp. 199-223.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J. I., de Saint, B. G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L. E., Wissler, M., Prinz, C., Rabbitts, T. H., Le Deist, F., Fischer, A., & Cavazzana-Calvo, M. 2003, "LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1", *Science.*, vol. 302, no. 5644, pp. 415-419.

Hamaguchi, I., Woods, N. B., Panagopoulos, I., Andersson, E., Mikkola, H., Fahlman, C., Zufferey, R., Carlsson, L., Trono, D., & Karlsson, S. 2000, "Lentivirus Vector Gene Expression during ES Cell-Derived Hematopoietic Development In Vitro", *The Journal of Virology*, vol. 74, no. 22, pp. 10778-10784.

Hannon, G. J. 2002, "RNA interference", *Nature.*, vol. 418, no. 6894, pp. 244-251.

Hara, H. & Ogawa, M. 1978, "Murine hemopoietic colonies in culture containing normoblasts, macrophages, and megakaryocytes", *Am J Hematol.*, vol. 4, no. 1, pp. 23-34.

Harrison, D. E. 1980, "Competitive repopulation: a new assay for long-term stem cell functional capacity", *Blood*, vol. 55, no. 1, pp. 77-81.

Harrison, D. E. & Astle, C. M. 1982, "Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure", *The Journal of Experimental Medicine*, vol. 156, no. 6, pp. 1767-1779.

Hata, A., Lagna, G., Massague, J., & Hemmati-Brivanlou, A. 1998, "Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor", *Genes Dev*, vol. 12, no. 2, pp. 186-197.

Helgason, C. D., Sauvageau, G., Lawrence, H. J., Largman, C., & Humphries, R. K. 1996, "Overexpression of HOXB4 enhances the hematopoietic potential of embryonic stem cells differentiated in vitro", *Blood*, vol. 87, no. 7, pp. 2740-2749.

Herblot, S., Aplan, P. D., & Hoang, T. 2002, "Gradient of E2A Activity in B-Cell Development", *Molecular and Cellular Biology*, vol. 22, no. 3, pp. 886-900.

Hermiston, M. L., Xu, Z., & Weiss, A. 2003, "CD45: a critical regulator of signaling thresholds in immune cells", *Annu.Rev Immunol*, vol. 21, pp. 107-137.

Howe, S. J., Mansour, M. R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M. H., Pike-Overzet, K., Chatters, S. J., de Ridder, D., Gilmour, K. C., Adams, S., Thornhill, S. I., Parsley, K. L., Staal, F. J., Gale, R. E., Linch, D. C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D. K., Schmidt, M., Von Kalle, C., Gaspar, H. B., & Thrasher, A. J. 2008, "Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients", *J Clin Invest.*, vol. 118, no. 9, pp. 3143-3150.

Huber, T. L., Kouskoff, V., Joerg Fehling, H., Palis, J., & Keller, G. 2004, "Haemangioblast commitment is initiated in the primitive streak of the mouse embryo", *Nature*, vol. 432, no. 7017, pp. 625-630.

Huber, T. L., Zhou, Y., Mead, P. E., & Zon, L. I. 1998, "Cooperative Effects of Growth Factors Involved in the Induction of Hematopoietic Mesoderm", *Blood*, vol. 92, no. 11, pp. 4128-4137.

Hulett, H. R., Bonner, W. A., Barrett, J., & Herzenberg, L. A. 1969, "Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence", *Science.*, vol. 166, no. 906, pp. 747-749.

Ichikawa, M., Asai, T., Saito, T., Seo, S., Yamazaki, I., Yamagata, T., Mitani, K., Chiba, S., Ogawa, S., Kurokawa, M., & Hirai, H. 2004, "AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis", *Nat Med.*, vol. 10, no. 3, pp. 299-304.

Ikuta, K. & Weissman, I. L. 1992, "Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 4, pp. 1502-1506.

Isabelle Godin & Ana Cumano 2002, "The Hare and the Tortoise Haematopoietic Race", *Nature Reviews Immunology*, vol. 2, pp. 593-604.

Iscove, N. N. & Nawa, K. 1997, "Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion", *Curr Biol.*, vol. 7, no. 10, pp. 805-808.

Jacob, F. 1978, "The Leeuwenhoek Lecture, 1977. Mouse teratocarcinoma and mouse embryo", *Proc R Soc Lond B Biol Sci.*, vol. 201, no. 1144, pp. 249-270.

Jochheim, A., Hillemann, T., Kania, G., Scharf, J., Attaran, M., Manns, M. P., Wobus, A. M., & Ott, M. 2004, "Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes", *Int.J Dev Biol.*, vol. 48, no. 1, pp. 23-29.

Johansson, B. M. & Wiles, M. V. 1995, "Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development", *Molecular and Cellular Biology*, vol. 15, no. 1, pp. 141-151.

Jordan, C. T., Astle, C. M., Zawadzki, J., Mackarehtschian, K., Lemischka, I. R., & Harrison, D. E. 1995, "Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation", *Exp Hematol.*, vol. 23, no. 9, pp. 1011-1015.

Kale, S., Karihaloo, A., Clark, P. R., Kashgarian, M., Krause, D. S., & Cantley, L. G. 2003, "Bone marrow stem cells contribute to repair of the ischemically injured renal tubule", *J Clin Invest.*, vol. 112, no. 1, pp. 42-49.

Kassouf, M. T., Chagraoui, H., Vyas, P., & Porcher, C. 2008, "Differential use of SCL/TAL-1 DNA-binding domain in developmental hematopoiesis", *Blood*, vol. 112, no. 4, pp. 1056-1067.

Keller, G., Kennedy, M., Papayannopoulou, T., & Wiles, M. V. 1993, "Hematopoietic commitment during embryonic stem cell differentiation in culture", *Molecular and Cellular Biology*, vol. 13, no. 1, pp. 473-486.

Kennedy, M., D'Souza, S. L., Lynch-Kattman, M., Schwantz, S., & Keller, G. 2007, "Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures", *Blood*, vol. 109, no. 7, pp. 2679-2687.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., & Keller, G. 1997, "A common precursor for primitive erythropoiesis and definitive haematopoiesis", *Nature*, vol. 386, no. 6624, pp. 488-493.

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., & Morrison, S. J. 2005, "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells", *Cell*, vol. 121, no. 7, pp. 1109-1121.

Kim, J. M., Lee, M., Kim, K. H., Ha, Y., Choi, J. K., Park, S. R., Park, H., Park, H. C., Ahn, C. H., Kim, S. W., & Choi, B. H. 2008, "Gene therapy of neural cell injuries in vitro using the hypoxia-inducible GM-CSF expression plasmids and water-soluble lipopolymer (WSLP)", *J Control Release.*, vol. 133, no. 1, pp. 60-67.

Kina, T., Ikuta, K., Takayama, E., Wada, K., Majumdar, A. S., Weissman, I. L., & Katsura, Y. 2000, "The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage", *Br J Haematol*, vol. 109, no. 2, pp. 280-287.

Kleinsmith, L. J. & Pierce, G. B., Jr. 1964, "Multipotentiality of Single Embryonal Carcinoma Cells", *Cancer Research*, vol. 24, no. 9, pp. 1544-1551.

Kobayashi, N., Rivas-Carrillo, J. D., Soto-Gutierrez, A., Fukazawa, T., Chen, Y., Navarro-Alvarez, N., & Tanaka, N. 2005, "Gene delivery to embryonic stem cells", *Birth Defects Res C.Embryo.Today.*, vol. 75, no. 1, pp. 10-18.

Kosaka, Y., Kobayashi, N., Fukazawa, T., Totsugawa, T., Maruyama, M., Yong, C., Arata, T., Ikeda, H., Kobayashi, K., Ueda, T., Kurabayashi, Y., & Tanaka, N. 2004, "Lentivirus-based gene delivery in mouse embryonic stem cells", *Artif.Organs.*, vol. 28, no. 3, pp. 271-277.

Krop, I., de Fougerolles, A. R., Hardy, R. R., Allison, M., Schlissel, M. S., & Fearon, D. T. 1996, "Self-renewal of B-1 lymphocytes is dependent on CD19", *Eur J Immunol*, vol. 26, no. 1, pp. 238-242.

Kumano, G., Belluzzi, L., & Smith, W. C. 1999, "Spatial and temporal properties of ventral blood island induction in Xenopus laevis", *Development*, vol. 126, no. 23, pp. 5327-5337.

Kumaravelu, P., Hook, L., Morrison, A. M., Ure, J., Zhao, S., Zuyev, S., Ansell, J., & Medvinsky, A. 2002, "Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver", *Development*, vol. 129, no. 21, pp. 4891-4899.

Kyba, M. & Daley, G. Q. 2003, "Hematopoiesis from embryonic stem cells: lessons from and for ontogeny", *Exp Hematol.*, vol. 31, no. 11, pp. 994-1006.

Kyba, M., Perlingeiro, R. C., & Daley, G. Q. 2002, "HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors", *Cell.*, vol. 109, no. 1, pp. 29-37.

Lacaud, G., Gore, L., Kennedy, M., Kouskoff, V., Kingsley, P., Hogan, C., Carlsson, L., Speck, N., Palis, J., & Keller, G. 2002, "Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro", *Blood*, vol. 100, no. 2, pp. 458-466.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., & Tuschl, T. 2001, "Identification of novel genes coding for small expressed RNAs", *Science.*, vol. 294, no. 5543, pp. 853-858.

Lancrin, C., Sroczynska, P., Stephenson, C., Allen, T., Kouskoff, V., & Lacaud, G. 2009, "The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage", *Nature*, vol. 457, no. 7231, pp. 892-895.

Landry, J. R., Kinston, S., Knezevic, K., de Bruijn, M. F. T. R., Wilson, N., Nottingham, W. T., Peitz, M., Edenhofer, F., Pimanda, J. E., Ottersbach, K., & Gottgens, B. 2008, "Runx genes are direct targets of Scl/Tal1 in the yolk sac and fetal liver", *Blood*, vol. 111, no. 6, pp. 3005-3014.

Larsson, J. & Karlsson, S. 2005, "The role of Smad signaling in hematopoiesis", *Oncogene*, vol. 24, no. 37, pp. 5676-5692.

Lecuyer, E. & Hoang, T. 2004, "SCL: from the origin of hematopoiesis to stem cells and leukemia", *Exp Hematol.*, vol. 32, no. 1, pp. 11-24.

Lecuyer, E., Herblot, S., Saint-Denis, M., Martin, R., Begley, C. G., Porcher, C., Orkin, S. H., & Hoang, T. 2002, "The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1", *Blood*, vol. 100, no. 7, pp. 2430-2440.

Ledran, M. H., Krassowska, A., Armstrong, L., Dimmick, I., Renstrom, J., Lang, R., Yung, S., Santibanez-Coref, M., Dzierzak, E., Stojkovic, M., Oostendorp, R. A., Forrester, L., & Lako, M. 2008, "Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches", *Cell Stem Cell.*, vol. 3, no. 1, pp. 85-98.

Lessard, J., Faubert, A., & Sauvageau, G. 1994, "Genetic programs regulating HSC specification, maintenance and expansion", *Oncogene*, vol. 23, no. 43, pp. 7199-7209.

Li, J., Pan, G., Cui, K., Liu, Y., Xu, S., & Pei, D. 2007, "A Dominant-negative Form of Mouse SOX2 Induces Trophectoderm Differentiation and Progressive Polyploidy in Mouse Embryonic Stem Cells", *Journal of Biological Chemistry*, vol. 282, no. 27, pp. 19481-19492.

Ling, K. W., Ottersbach, K., van Hamburg, J. P., Oziemlak, A., Tsai, F. Y., Orkin, S. H., Ploemacher, R., Hendriks, R. W., & Dzierzak, E. 2004, "GATA-2 Plays Two Functionally Distinct Roles during the Ontogeny of Hematopoietic Stem Cells", *The Journal of Experimental Medicine*, vol. 200, no. 7, pp. 871-882.

Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R. J., & Schneider, M. D. 2007, "Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells", *Proceedings of the National Academy of Sciences*, vol. 104, no. 10, pp. 3859-3864.

Look, A. T. 1997, "Oncogenic transcription factors in the human acute leukemias", *Science.*, vol. 278, no. 5340, pp. 1059-1064.

Lowry, W. E., Richter, L., Yachechko, R., Pyle, A. D., Tchieu, J., Sridharan, R., Clark, A. T., & Plath, K. 2008, "Generation of human induced pluripotent stem cells from dermal fibroblasts", *Proceedings of the National Academy of Sciences*, vol. 105, no. 8, pp. 2883-2888.

Lugus, J. J., Chung, Y. S., Mills, J. C., Kim, S. I., Grass, J. A., Kyba, M., Doherty, J. M., Bresnick, E. H., & Choi, K. 2007, "GATA2 functions at multiple steps in hemangioblast development and differentiation", *Development*, vol. 134, no. 2, pp. 393-405.

Ma, H., Liu, Q., Diamond, S. L., & Pierce, E. A. 2004, "Mouse embryonic stem cells efficiently lipofected with nuclear localization peptide result in a high yield of chimeric mice and retain germline transmission potency", *Methods.*, vol. 33, no. 2, pp. 113-120.

Maeno, M., Mead, P. E., Kelley, C., Xu, R. H., Kung, H. F., Suzuki, A., Ueno, N., & Zon, L. I. 1996, "The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in Xenopus laevis", *Blood*, vol. 88, no. 6, pp. 1965-1972.

Maguer-Satta, V., Bartholin, L., Jeanpierre, S., Ffrench, M., Martel, S., Magaud, J. P., & Rimokh, R. 2003, "Regulation of human erythropoiesis by activin A, BMP2, and BMP4, members of the TGFbeta family", *Exp Cell Res.*, vol. 282, no. 2, pp. 110-120.

Marshall, C. J., Sinclair, J. C., Thrasher, A. J., & Kinnon, C. 2007, "Bone morphogenetic protein 4 modulates c-Kit expression and differentiation potential in murine embryonic aorta-gonad-mesonephros haematopoiesis in vitro", *Br J Haematol.*, vol. 139, no. 2, pp. 321-330.

Marshall, C. J., Kinnon, C., & Thrasher, A. J. 2000, "Polarized expression of bone morphogenetic protein-4 in the human aorta-gonad-mesonephros region", *Blood*, vol. 96, no. 4, pp. 1591-1593.

Martin, G. R. 1981, "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells", *Proc Natl Acad Sci U.S.A.*, vol. 78, no. 12, pp. 7634-7638.

Massague, J. 1996, "TGFbeta signaling: receptors, transducers, and Mad proteins", *Cell*, vol. 85, no. 7, pp. 947-950.

Matsuoka, S., Tsuji, K., Hisakawa, H., Xu, M. j., Ebihara, Y., Ishii, T., Sugiyama, D., Manabe, A., Tanaka, R., Ikeda, Y., Asano, S., & Nakahata, T. 2001, "Generation of definitive hematopoietic stem cells from murine early yolk sac and paraaortic splanchnopleures by aorta-gonad-mesonephros region-derived stromal cells", *Blood*, vol. 98, no. 1, pp. 6-12.

Mead, P. E., Deconinck, A. E., Huber, T. L., Orkin, S. H., & Zon, L. I. 2001, "Primitive erythropoiesis in the Xenopus embryo: the synergistic role of LMO-2, SCL and GATA-binding proteins", *Development*, vol. 128, no. 12, pp. 2301-2308.

Mikkola, H. K. A., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y., & Orkin, S. H. 2003, "Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene", *Nature*, vol. 421, no. 6922, pp. 547-551.

Miller, J. D., Stacy, T., Liu, P. P., & Speck, N. A. 2001, "Core-binding factor {beta} (CBF{beta}), but not CBF{beta}-smooth muscle myosin heavy chain, rescues definitive hematopoiesis in CBF{beta}-deficient embryonic stem cells", *Blood*, vol. 97, no. 8, pp. 2248-2256.

Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J. D., & Yamamoto, M. 1999, "The Mouse GATA-2 Gene is Expressed in the Para-Aortic Splanchnopleura and Aorta-Gonads and Mesonephros Region", *Blood*, vol. 93, no. 12, pp. 4196-4207.

Morrison, S. J., Hemmati, H. D., Wandycz, A. M., & Weissman, I. L. 1995, "The purification and characterization of fetal liver hematopoietic stem cells", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 22, pp. 10302-10306.

Morrison, S. J. & Weissman, I. L. 1994, "The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype", *Immunity.*, vol. 1, no. 8, pp. 661-673.

Muller, A. M. & Dzierzak, E. A. 1993, "ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients", *Development*, vol. 118, no. 4, pp. 1343-1351.

Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F., & Dzierzak, E. 1994, "Development of hematopoietic stem cell activity in the mouse embryo", *Immunity.*, vol. 1, no. 4, pp. 291-301.

Murphy, C. L. & Polak, J. M. 2002, "Differentiating embryonic stem cells: GAPDH, but neither HPRT nor beta-tubulin is suitable as an internal standard for measuring RNA levels", *Tissue Eng.*, vol. 8, no. 4, pp. 551-559.

Murray 1932, "The development in vitro of the blood of the early chick embryo", *Proc R Soc London*, vol. 11, pp. 497-521.

Nakano, T., Kodama, H., & Honjo, T. 1994, "Generation of lymphohematopoietic cells from embryonic stem cells in culture", *Science*, vol. 265, no. 5175, pp. 1098-1101.

Nakao, A., Afrakhte, M., Morn, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., & Dijke, P. t. 1997, "Identification of Smad7, a TGF[beta]-inducible antagonist of TGF-[beta] signalling", *Nature*, vol. 389, no. 6651, pp. 631-635.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., & Trono, D. 1996, "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector", *Science.*, vol. 272, no. 5259, pp. 263-267.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., & Smith, A. 1998, "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4", *Cell.*, vol. 95, no. 3, pp. 379-391.

Niwa, H., Miyazaki, J., & Smith, A. G. 2000, "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells", *Nat.Genet.*, vol. 24, no. 4, pp. 372-376.

Niwa, H., Burdon, T., Chambers, I., & Smith, A. 1998, "Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3", *Genes and Development*, vol. 12, no. 13, pp. 2048-2060.

North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M., & Speck, N. A. 1999, "Cbfa2 is required for the formation of intra-aortic hematopoietic clusters", *Development*, vol. 126, no. 11, pp. 2563-2575.

Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., & Downing, J. R. 1996, "AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis", *Cell.*, vol. 84, no. 2, pp. 321-330.

Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D. M., Leri, A., & Anversa, P. 2001, "Mobilized bone marrow cells repair the infarcted heart, improving function and survival", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 18, pp. 10344-10349.

Osada, H., Grutz, G., Axelson, H., Forster, A., & Rabbitts, T. H. 1995, "Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 21, pp. 9585-9589.

Otsuka, E., Yamaguchi, A., Hirose, S., & Hagiwara, H. 1999, "Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid", *AJP - Cell Physiology*, vol. 277, no. 1, p. C132-C138.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., & Conklin, D. S. 2002, "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells", *Genes and Development*, vol. 16, no. 8, pp. 948-958.

Paddison, P. J., Caudy, A. A., & Hannon, G. J. 2002, "Stable suppression of gene expression by RNAi in mammalian cells", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 3, pp. 1443-1448.

Papapetrou, E. P., Zoumbos, N. C., & Athanassiadou, A. 2005, "Genetic modification of hematopoietic stem cells with nonviral systems: past progress and future prospects", *Gene Ther*, vol. 12, no. S1, p. S118-S130.

Papayannopoulou, T. & Scadden, D. T. 2008, "Stem-cell ecology and stem cells in motion", *Blood*, vol. 111, no. 8, pp. 3923-3930.

Pardanaud, L. & Dieterlen-Lievre, F. 1999, "Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo", *Development*, vol. 126, no. 4, pp. 617-627.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M., & Dieterlen-Lievre, F. 1996, "Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis", *Development*, vol. 122, no. 5, pp. 1363-1371.

Park, C., Afrikanova, I., Chung, Y. S., Zhang, W. J., Arentson, E., Fong, G. h., Rosendahl, A., & Choi, K. 2004, "A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells", *Development*, vol. 131, no. 11, pp. 2749-2762.

Patterson, L. J., Gering, M., Eckfeldt, C. E., Green, A. R., Verfaillie, C. M., Ekker, S. C., & Patient, R. 2007, "The transcription factors Scl and Lmo2 act together during development of the hemangioblast in zebrafish", *Blood*, vol. 109, no. 6, pp. 2389-2398.

Patterson, L. J., Gering, M., & Patient, R. 2005, "Scl is required for dorsal aorta as well as blood formation in zebrafish embryos", *Blood*, vol. 105, no. 9, pp. 3502-3511.

Pearson, S., Sroczynska, P., Lacaud, G., & Kouskoff, V. 2008, "The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF", *Development*, vol. 135, no. 8, pp. 1525-1535.

Pease, S., Braghetta, P., Gearing, D., Grail, D., & Williams, R. L. 1990, "Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF)", *Dev Biol.*, vol. 141, no. 2, pp. 344-352.

Peretto, P., Dati, C., De Marchis, S., Kim, H. H., Ukhanova, M., Fasolo, A., & Margolis, F. L. 2004, "Expression of the secreted factors noggin and bone morphogenetic proteins in the subependymal layer and olfactory bulb of the adult mouse brain", *Neuroscience.*, vol. 128, no. 4, pp. 685-696.

Perlingeiro, R. C. R., Kyba, M., & Daley, G. Q. 2001, "Clonal analysis of differentiating embryonic stem cells reveals a hematopoietic progenitor with primitive erythroid and adult lymphoid-myeloid potential", *Development*, vol. 128, no. 22, pp. 4597-4604.

Pesce, M., Gioia, K. F., & De Felici, M. 2002, "Derivation in culture of primordial germ cells from cells of the mouse epiblast: phenotypic induction and growth control by Bmp4 signalling", *Mech Dev.*, vol. 112, no. 1-2, pp. 15-24.

Pevny, L. H. & Lovell-Badge, R. 1997, "Sox genes find their feet", *Curr Opin.Genet.Dev.*, vol. 7, no. 3, pp. 338-344.

Pfeifer, A., Ikawa, M., Dayn, Y., & Verma, I. M. 2002, "Transgenesis by lentiviral vectors: Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 4, pp. 2140-2145.

Pimanda, J. E., Donaldson, I. J., de Bruijn, M. F. T. R., Kinston, S., Knezevic, K., Huckle, L., Piltz, S., Landry, J. R., Green, A. R., Tannahill, D., & Gottgens, B. 2007a, "The SCL transcriptional network and BMP signaling pathway interact to regulate RUNX1 activity", *Proceedings of the National Academy of Sciences*, vol. 104, no. 3, pp. 840-845.

Pimanda, J. E., Ottersbach, K., Knezevic, K., Kinston, S., Chan, W. Y. I., Wilson, N. K., Landry, J. R., Wood, A. D., Kolb-Kokocinski, A., Green, A. R., Tannahill, D., Lacaud, G., Kouskoff, V., & Gottgens, B. 2007b, "Gata2, Fli1, and Scl form a recursively wired gene-regulatory circuit during early hematopoietic development", *Proceedings of the National Academy of Sciences*, vol. 104, no. 45, pp. 17692-17697.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W., & Orkin, S. H. 1996, "The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages", *Cell.*, vol. 86, no. 1, pp. 47-57.

Potocnik, A. J., Kohler, H., & Eichmann, K. 1997, "Hemato-lymphoid in vivo reconstitution potential of subpopulations derived from in vitro differentiated embryonic stem cells", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 19, pp. 10295-10300.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R., & Ruvkun, G. 2000, "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans", *Nature*, vol. 403, no. 6772, pp. 901-906.

Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D., & Begley, C. G. 1996, "The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse", *EMBO J.*, vol. 15, no. 16, pp. 4123-4129.

Robb, L., Lyons, I., Li, R., Hartley, L., K+¦ntgen, F., Harvey, R. P., Metcalf, D., & Begley, C. G. 1995, "Absence of yolk sac hematopoiesis from mice with a targeted

disruption of the scl gene", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 15, pp. 7075-7079.

Robertson, S. M., Kennedy, M., Shannon, J. M., & Keller, G. 2000, "A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1", *Development*, vol. 127, no. 11, pp. 2447-2459.

Sabin 1920, "Studies on the origin of blood vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation", *Contib.Embryol.Carnegie Institute of Washington*, vol. 9, p. 214.

Sadlon, T. J., Lewis, I. D., & D'Andrea, R. J. 2004, "BMP4: Its Role in Development of the Hematopoietic System and Potential as a Hematopoietic Growth Factor", *Stem Cells*, vol. 22, no. 4, pp. 457-474.

Sanchez, M. J., Holmes, A., Miles, C., & Dzierzak, E. 1996, "Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo", *Immunity.*, vol. 5, no. 6, pp. 513-525.

Sasaki, K., Yagi, H., Bronson, R. T., Tominaga, K., Matsunashi, T., Deguchi, K., Tani, Y., Kishimoto, T., & Komori, T. 1996, "Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 22, pp. 12359-12363.

Schofield, R. 1978, "The relationship between the spleen colony-forming cell and the haemopoietic stem cell", *Blood Cells.*, vol. 4, no. 1-2, pp. 7-25.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., & Rossant, J. 1997, "A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis", *Cell.*, vol. 89, no. 6, pp. 981-990.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., & Schuh, A. C. 1995, "Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice", *Nature*, vol. 376, no. 6535, pp. 62-66.

Shi, Y. & Massague, J. 2003, "Mechanisms of TGF-beta signaling from cell membrane to the nucleus", *Cell*, vol. 113, no. 6, pp. 685-700.

Shivdasani, R. A., Mayer, E. L., & Orkin, S. H. 1995, "Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL", *Nature*, vol. 373, no. 6513, pp. 432-434.

Silver, L. & Palis, J. 1997, "Initiation of Murine Embryonic Erythropoiesis: A Spatial Analysis", *Blood*, vol. 89, no. 4, pp. 1154-1164.

Simeoni, I. & Gurdon, J. B. 2007, "Interpretation of BMP signaling in early Xenopus development", *Dev Biol.*, vol. 308, no. 1, pp. 82-92.

Solter, D. & Knowles, B. B. 1978, "Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1)", *Proc Natl Acad Sci U.S.A.*, vol. 75, no. 11, pp. 5565-5569.

Spangrude, G. J., Heimfeld, S., & Weissman, I. L. 1988, "Purification and characterization of mouse hematopoietic stem cells", *Science*, vol. 241, no. 4861, pp. 58-62.

Springer, T., Galfre, G., Secher, D. S., & Milstein, C. 1979, "Mac-1: a macrophage differentiation antigen identified by monoclonal antibody", *Eur J Immunol*, vol. 9, no. 4, pp. 301-306.

Szilvassy, S. J., Humphries, R. K., Lansdorp, P. M., Eaves, A. C., & Eaves, C. J. 1990, "Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 22, pp. 8736-8740.

Taichman, R. S., Reilly, M. J., & Emerson, S. G. 1996, "Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures", *Blood*, vol. 87, no. 2, pp. 518-524.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. 2007, "Induction of pluripotent stem cells from adult human fibroblasts by defined factors", *Cell.*, vol. 131, no. 5, pp. 861-872.

Takahashi, K. & Yamanaka, S. 2006, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors", *Cell.*, vol. 126, no. 4, pp. 663-676.

Takeuchi, M., Sekiguchi, T., Hara, T., Kinoshita, T., & Miyajima, A. 2002, "Cultivation of aorta-gonad-mesonephros-derived hematopoietic stem cells in the fetal liver microenvironment amplifies long-term repopulating activity and enhances engraftment to the bone marrow", *Blood*, vol. 99, no. 4, pp. 1190-1196.

Tang, F. C., Meng, G. L., Yang, H. B., Li, C. J., Shi, Y., Ding, M. X., Shang, K. G., Zhang, B., & Xue, Y. F. 2004, "Stable Suppression of Gene Expression in Murine Embryonic Stem Cells by RNAi Directed from DNA Vector-Based Short Hairpin RNA", *Stem Cells*, vol. 22, no. 1, pp. 93-99.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., & Scott, E. W. 2002, "Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion", *Nature*, vol. 416, no. 6880, pp. 542-545.

Theise, N. D., Henegariu, O., Grove, J., Jagirdar, J., Kao, P. N., Crawford, J. M., Badve, S., Saxena, R., & Krause, D. S. 2002, "Radiation pneumonitis in mice: a severe injury model for pneumocyte engraftment from bone marrow", *Exp Hematol.*, vol. 30, no. 11, pp. 1333-1338.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. 1998, "Embryonic Stem Cell Lines Derived from Human Blastocysts", *Science*, vol. 282, no. 5391, pp. 1145-1147.

Tian, X. & Kaufman, D. S. 2008, "Differentiation of embryonic stem cells towards hematopoietic cells: progress and pitfalls", *Curr Opin.Hematol.*, vol. 15, no. 4, pp. 312-318.

Tracey, W. D. & Speck, N. A. 2000, "Potential roles for RUNX1 and its orthologs in determining hematopoietic cell fate", *Semin Cell Dev Biol.*, vol. 11, no. 5, pp. 337-342.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., & Orkin, S. H. 1994, "An early haematopoietic defect in mice lacking the transcription factor GATA-2", *Nature*, vol. 371, no. 6494, pp. 221-226.

Turpen, J. B., Kelley, C. M., Mead, P. E., & Zon, L. I. 1997, "Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo", *Immunity.*, vol. 7, no. 3, pp. 325-334.

Turpen, J. B., Knudson, C. M., & Hoefen, P. S. 1981, "The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment", *Dev Biol.*, vol. 85, no. 1, pp. 99-112.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., & Sharp, P. A. 1999, "Targeted mRNA degradation by double-stranded RNA in vitro", *Genes and Development*, vol. 13, no. 24, pp. 3191-3197.

Valge-Archer, V. E., Osada, H., Warren, A. J., Forster, A., Li, J., Baer, R., & Rabbitts, T. H. 1994, "The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 18, pp. 8617-8621.

van Bekkum, D. W., van den Engh, G. J., Wagemaker, G., Bol, S. J., & Visser, J. W. 1979, "Structural identity of the pluripotential hemopoietic stem cell", *Blood Cells.*, vol. 5, no. 2, pp. 143-159.

Vassilopoulos, G., Wang, P. R., & Russell, D. W. 2003, "Transplanted bone marrow regenerates liver by cell fusion", *Nature*, vol. 422, no. 6934, pp. 901-904.

Wagers, A. J., Sherwood, R. I., Christensen, J. L., & Weissman, I. L. 2002, "Little evidence for developmental plasticity of adult hematopoietic stem cells", *Science.*, vol. 297, no. 5590, pp. 2256-2259.

Walmsley, M., Ciau-Uitz, A., & Patient, R. 2002, "Adult and embryonic blood and endothelium derive from distinct precursor populations which are differentially programmed by BMP in Xenopus", *Development*, vol. 129, no. 24, pp. 5683-5695.

Walter-Yohrling, J., Morgenbesser, S., Rouleau, C., Bagley, R., Callahan, M., Weber, W., & Teicher, B. A. 2004, "Murine Endothelial Cell Lines as Models of Tumor Endothelial Cells", *Clinical Cancer Research*, vol. 10, no. 6, pp. 2179-2189.

Wang, L., Menendez, P., Shojaei, F., Li, L., Mazurier, F., Dick, J. E., Cerdan, C., Levac, K., & Bhatia, M. 2005, "Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression", *The Journal of Experimental Medicine*, vol. 201, no. 10, pp. 1603-1614.

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H., & Speck, N. A. 1996a, "Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 8, pp. 3444-3449.

Wang, Q., Stacy, T., Miller, J. D., Lewis, A. F., Gu, T. L., Huang, X., Bushweller, J. H., Bories, J. C., Alt, F. W., Ryan, G., Liu, P. P., Wynshaw-Boris, A., Binder, M., Marin-Padilla, M., Sharpe, A. H., & Speck, N. A. 1996b, "The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo", *Cell.*, vol. 87, no. 4, pp. 697-708.

Warren, A. J., Colledge, W. H., Carlton, M. B., Evans, M. J., Smith, A. J., & Rabbitts, T. H. 1994, "The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development", *Cell.*, vol. 78, no. 1, pp. 45-57.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., & Gough, N. M. 1988, "Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells", *Nature.*, vol. 336, no. 6200, pp. 684-687.

Williamson, A. J. K., Smith, D. L., Blinco, D., Unwin, R. D., Pearson, S., Wilson, C., Miller, C., Lancashire, L., Lacaud, G., Kouskoff, V., & Whetton, A. D. 2008, "Quantitative Proteomics Analysis Demonstrates Post-transcriptional Regulation of Embryonic Stem Cell Differentiation to Hematopoiesis", *Molecular Cellular Proteomics*, vol. 7, no. 3, pp. 459-472.

Winnier, G., Blessing, M., Labosky, P. A., & Hogan, B. L. 1995, "Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse", *Genes and Development*, vol. 9, no. 17, pp. 2105-2116.

Wood, H. B., May, G., Healy, L., Enver, T., & Morriss-Kay, G. M. 1997, "CD34 Expression Patterns During Early Mouse Development Are Related to Modes of Blood Vessel Formation and Reveal Additional Sites of Hematopoiesis", *Blood*, vol. 90, no. 6, pp. 2300-2311.

Worton, R. G., McCulloch, E. A., & Till, J. E. 1969, "Physical separation of hemopoietic stem cells differing in their capacity for self-renewal", *J Exp Med.*, vol. 130, no. 1, pp. 91-103.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., & Massague, J. 1994, "Mechanism of activation of the TGF-beta receptor", *Nature*, vol. 370, no. 6488, pp. 341-347.

Xie, T. & Spradling, A. C. 1998, "decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary", *Cell.*, vol. 94, no. 2, pp. 251-260.

Ying, Q. L., Nichols, J., Chambers, I., & Smith, A. 2003, "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3", *Cell.*, vol. 115, no. 3, pp. 281-292.

Yoder, M. C., Hiatt, K., & Mukherjee, P. 1997, "In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 post coitus", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 13, pp. 6776-6780.

Yokomizo, T., Hasegawa, K., Ishitobi, H., Osato, M., Ema, M., Ito, Y., Yamamoto, M., & Takahashi, S. 2008, "Runx1 is involved in primitive erythropoiesis in the mouse", *Blood*, vol. 111, no. 8, pp. 4075-4080.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, I. I., & Thomson, J. A. 2007, "Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells", *Science*, vol. 318, no. 5858, pp. 1917-1920.

Zafonte, B. T., Liu, S., Lynch-Kattman, M., Torregroza, I., Benvenuto, L., Kennedy, M., Keller, G., & Evans, T. 2007, "Smad1 expands the hemangioblast population within a limited developmental window", *Blood*, vol. 109, no. 2, pp. 516-523.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W. G., Ross, J., Haug, J., Johnson, T., Feng, J. Q., Harris, S., Wiedemann, L. M., Mishina, Y., & Li, L. 2003, "Identification of the haematopoietic stem cell niche and control of the niche size", *Nature*, vol. 425, no. 6960, pp. 836-841.

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., & Sorrentino, B. P. 2001, "The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype", *Nat Med.*, vol. 7, no. 9, pp. 1028-1034.

Zufferey, R., Donello, J. E., Trono, D., & Hope, T. J. 1999, "Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors", *The Journal of Virology*, vol. 73, no. 4, pp. 2886-2892.