HAEMOPOIETIC STEM CELL REPLICATIVE DYNAMICS IN NORMAL FETAL LIFE AND LEUKAEMOGENESIS

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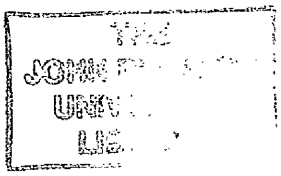

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ABBREVIATIONS

AF	alloimmunised fetuses
AGM	aorta-gonad-mesonephros
ALL	acute lymphoblastic leukaemia
AMKL	acute megakaryoblastic leukaemia
AML	acute myeloid leukaemia
APC	allophycocyanin
BFU-E	burst forming unit -erythroid
BSA	bovine serum albumin
CBS	cystathionine B synthase
CFC	colony forming cell
CFU-E	colony forming unit erythroid
CFU-GEMM	colony forming unit-granulocytic erythroid monocytic macrophage
CFU-GM	colony forming unit-granulocyte/macrophage
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
Ct	threshold cycle
dATP	deoxyadenine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DS	Down's syndrome
EDTA	ethylenediaminetetraacetic acid
EPP	eppendorf
ETP	early thymic progenitor
FACS	flow activated cell sorting
FITC	fluorescein isothiocyanate
FSC	forward scatter
GFP	green fluorescent protein
HSC	haemopoietic stem cell
IMDM	Iscove's Modified Dulbecco's Medium
LTC-IC	long term culture initiating cell
LT-HSC	long term haemopoietic stem cell

MNC	mononuclear cell
MPP	multipotent progenitors
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
NOD	non-obese diabetic
PBL	peripheral blood leucocyte
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PTI	preterm infants
qPCR	quantitative PCR
RCB	red cell lysis buffer
rpm	revolutions per minute
SCID	severe combined immunodeficiency
SOD	superoxide dismutase
SRC	SCID repopulating cell
SSC	side scatter
ST-HSC	short term haemopoietic stem cell
TE	tris-ethylene diamine tetra-acetate
TMD/TMD	transient myeloid dysplasia/transient abnormal myelopoiesis
TOP	termination of pregnancy
YS	yolk sac

ABSTRACT

Telomere shortening has been associated with loss of proliferative capacity of cells and, when critical, chromosomal instability. The loss of telomere in postnatal life has been extensively studied and shows that telomere loss is biphasic with rapid loss in early childhood, and a slower decline thereafter. Disorders such as aplastic anaemia, Fanconi's anaemia and Shwachman-Diamond syndrome all show accelerated telomere loss. This is associated with haemopoietic stem cell deficiency and increased risk of leukaemia. In Down Syndrome, a genetic disease due to the presence of an extra copy of part or all of chromosome 21, there is an increased risk of acute myeloid leukaemia which may manifest in the neonatal period as a transient abnormal myelopoiesis and there is accelerated telomere shortening of blood cells in DS children. This suggests that accelerated telomere shortening could be present already in fetal life in DS and lead to stem/progenitor cell deficiency. In this study I address the question of whether the accelerated telomere shortening is associated with progenitor/stem cell deficiency in DS individuals and whether this arises in fetal life.

As very little is known about telomere loss in fetal life, I firstly studied the telomere kinetics of HSCs in non–DS human fetuses. To achieve that I determined stem/progenitor changes in fetuses followed longitudinally for 8-12 weeks during the late second and third trimester of gestation and compared to a control group of premature infants of comparable gestational age and followed for a similar period of time. Fetuses and gestational age-matched preterm infants were comparable with respect to changes in the composition of peripheral blood cell populations and the frequency of circulating clonogenic precursors in the time period of the study. However, longitudinal assessment of mean telomere restriction fragment length in leucocytes revealed a striking difference in HSCs replication. No significant telomere shortening was documented in the fetuses, whereas marked telomere shortening was observed among the preterm infants. These results point to a profound difference in HSCs behaviour between fetal and early postnatal life which is independent from age of development but accompanies the event of birth. This poses new questions on

whether HSCs are intrinsically different in fetal life, or different populations of cells contribute to haemopoiesis during fetal and post-natal life.

Although fetal stem/progenitor cells seem to be protected from telomere shortening, this was not the case for fetal stem/progenitor cells in DS. An accelerated telomere shortening was found in fetal peripheral blood in keeping with my hypothesis. This was associated with stem/progenitor cell deficiency from a very early stage of development. GATA1 mutations in the activation domain in exon 2 have been found in most of DS leukaemia and in about 10% of randomly selected and otherwise normal DS neonates. This has led to the suggestion that GATA1 mutations could be a primary event in the emergence of leukaemia. However, as increased risk of leukaemia is seen in association with accelerated telomere shortening and stem cell deficiency, I examined the blood cells of the DS fetuses for the presence of GATA1 exon 2 mutations to establish whether those fetuses which presented a reduced number of stem/progenitor cells harboured this mutation. Absence of a concomitant GATA1 mutations in the DS fetuses examined here suggest that a reduced number of stem cells may be the initial predisposing factor to the development GATA1 mutations and of leukaemia.

DECLARATION

I declare that this thesis is my own composition and that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification to this or any other university or institute of learning.

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CHAPTER 1.

INTRODUCTION

1 INTRODUCTION

1.1 Haemopoiesis

Haemopoiesis is the process by which mature blood cells are generated. Mature and maturing blood cells represent greater than 95% of total haemopoietic cells of an individual (Lord 1988). Each has a specialised role and a finite lifespan and therefore need to be continually replaced during life (Geiger and Van Zant 2002). Replenishment of mature cells arises from proliferation and differentiation of more primitive precursors known as haemopoietic stem cells (HSCs). For haemopoiesis to occur two compartments are necessary, the haemopoietic compartment which comprises haemopoietic stem, progenitor and mature cells and the microenvironment where haemopoiesis takes place. The process of haemopoiesis involves a complex interaction between the intrinsic processes of the blood cells and their environment which changes with ontogeny – from yolk sac (YS) and aorta-gonad-mesonephros (AGM) in embryonic life, to liver in fetal life and bone marrow in postnatal life (Peault and Tavian 2003).

1.2. Haemopoietic stem and progenitor cells.

Haemopoietic stem cells are the base from which all blood cell lineages are derived. They are defined by their properties of extensive self renewal and proliferative capacity, coupled with the capacity to differentiate into erythroid, granulocytic, macrophagic, megakaryocytic and lymphoid cell lineages. HSCs are ultimately defined by their ability to reconstitute multilineage haemopoiesis in a lethally ablated host following transplantation (Orlic and Bodine 1994). The first evidence to indicate the existence of HSCs came from the experiments some forty four years ago by Becker, Till and McCulloch (Becker, *et al* 1963) when they discovered a population of clonogenic murine bone marrow cells capable of generating colonies of myeloid and erythroid cells in the spleens of lethally irradiated murine hosts. These spleen colonies occasionally contained clonogenic cells that could be further transplanted into secondary irradiated hosts and successfully reconstitute the haemopoietic system (Becker, *et al* 1963). These cells were proposed to be the HSCs and the assay was called the colony forming unit-spleen (CFU-S) assay. However, later experiments in the murine model revealed that there are actually two types of stem cell present –the

long term and short term haemopoietic stem cells (Morrison, *et al* 1995). CFU-S cells are now thought to represent the short term haemopoietic stem cells (ST-HSC) which reconstitute haemopoiesis for a period of weeks following transplantation. Long-term engraftment is attributed to a different cell type, which are more primitive than the CFU-S, and will only engraft and repopulate if the recipient also receives short term repopulating cells (ST-HSCs). A cell type known as the pre-CFU-S in *in vivo* assays is now considered to be responsible for long term marrow repopulating activity and hence correspond to the long term haemopoietic stem cell (LT-HSC) (Lord and Dexter 1995, Nardi and Alfonso 1999).

Using xenograft transplant experiments, the model of human haemopoiesis development has also been further refined and *in vivo* transplantation studies have confirmed the presence of two types of stem cells in humans. The most primitive is the long term haemopoietic stem cell (LT-HSC) which is capable of producing all haemopoietic cell types for the lifespan of the individual and can generate progeny that display similar properties in secondary transplant recipients (Morrison, *et al* 1995, Zanjani, *et al* 1992). The LT-HSC are thought to give rise to short term HSCs (ST-HSCs) which are capable of reconstituting myeloid and/or lymphoid compartments in individuals for a short period of time of approximately eight to twelve weeks (Morrison, *et al* 1995, Passegue, *et al* 2003).

Multipotent progenitors (MPPs) arise from the ST-HSC and have the capacity to differentiate into all myeloid and lymphoid lineages *in vitro* but little or no engraftment activity detectable by *in vivo* transplantation experiments (Morrison, *et al* 1995). They have the ability to differentiate into oligolineage restricted progenitors through a series of maturational steps known as commitment when they become increasingly restricted to a particular cell lineage. MPP differentiation gives rise to common lymphoid progenitors (CLPs) which become further restricted to generate either B, T and NK cells and common myeloid progenitors (CMPs) which generate myelo-erythroid lineages (Kondo, *et al* 1997, Manz, *et al* 2002). CMPs give rise to myelomonocytic progenitors (GMPs) and megakaryocytic/erythroid progenitors (MEPs) which subsequently yield granulocytes, macrophages,

erythrocytes and platelets. The generation of mature cells from HSCs is shown in figure 1.1.

It was initially thought that this process of commitment could only occur in this step wise and irreversible pattern but recent evidence of alternative lineage choices have been reported which question this concept. Rodriguez et al (Montecino-Rodriguez, *et al* 2001) described the presence in the bone marrow of a bipotent B-macrophage progenitor capable of producing both B cells and macrophages in *in vitro* cultures. Allman described an early T cell progenitor (ETP) in the thymus which retained a low myeloid potential (Allman, *et al* 2003) though one explanation of this is that the ETP may actually be upstream of the CLP rather than derived from the CLP (reviewed in (Bhandoola, *et al* 2003)) and thus the CLP may not be the common progenitor for B and T cells as was first thought. However, it is possible that there is a certain degree of flexibility at the level of the committed progenitors to face the demands of the organism in situations of emergency. CLPs, although thought to irreversibly committed to lymphoid differentiation in normal physiological conditions have been found to have a latent myeloid differentiation potential in the presence of exogenously expressed IL-2 in *in vitro* cultures (King, *et al* 2002, Kondo, *et al* 2000). Ectopic expression of GATA1 in progenitors committed to the neutrophil/monocyte pathway has also been shown to lead to the development of erythroid as well as basophilic and eosinophilic cells *in vitro* (Heyworth, *et al* 2002) which again suggests a degree of flexibility of committed progenitors.

1.3 Assays for human haemopoietic stem and progenitor cells.

There are two ways of quantifying human haemopoietic stem and progenitor cells. Firstly functional assays which can be used to retrospectively identify immature haemopoietic progenitors. Secondly, fluorescence activated cell sorting (FACS) for a range of cell surface markers is used to select for HSC as their expression of several surface markers changes with differentiation.

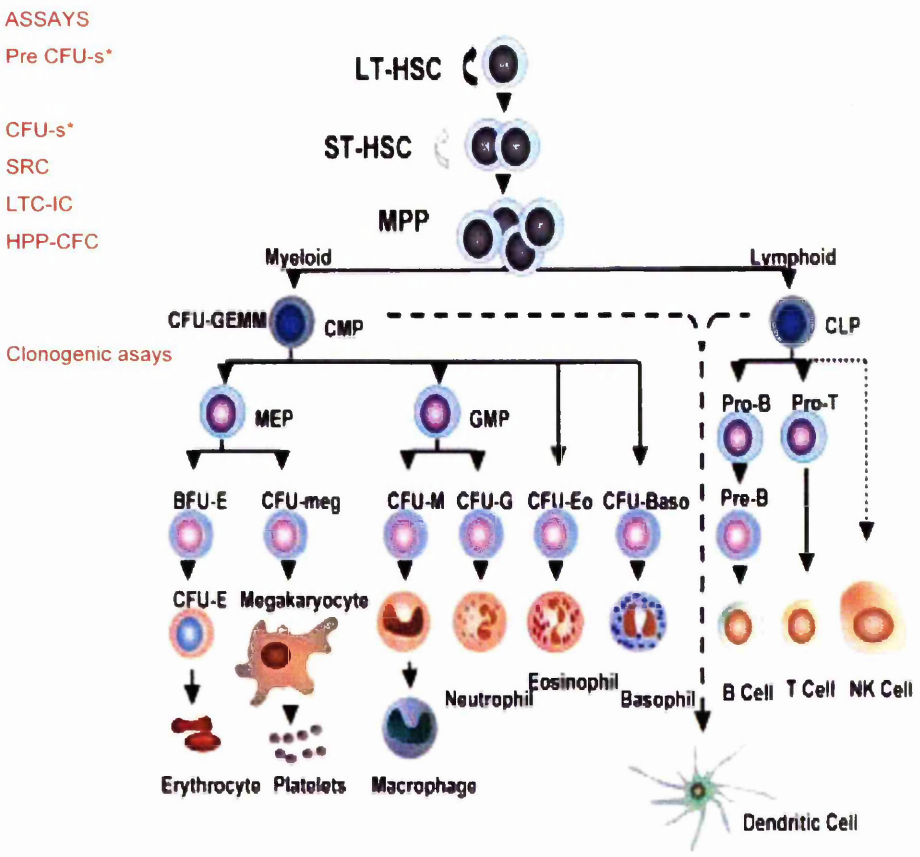


Figure 1.1: The haemopoietic tree
*Murine assay only

1.3.1 Functional assays of HSC and progenitors

HSCs are assayed by transplantation and assessment of multilineage repopulation in the recipient. Xenograft models have been developed involving transplantation of primitive human haemopoietic cells into immunodeficient animals. The most common animal used is the genetically immunodeficient mouse. Several mouse strains have been used and one of the most popular is the crossed non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse, which due to its severe immunodeficiency phenotype is capable of engraftment of human haemopoietic cells (Larochelle, *et al* 1996). NOD mice are susceptible to diabetes and in addition have a deficiency of natural killer cells and complement (Cashman, *et al* 1997). SCID mice lack functional B and T lymphocytes. Transplantation of human HSCs into the crossed NOD/SCID which has both sets of defects result in appearance of terminally differentiated human haemopoietic cells from multiple lineages including B-cells, mature erythroid cells and all types of myeloid cells. This model has been used to identify a pluripotent human stem cell termed SCID-repopulating cell (SRC) (Larochelle, *et al* 1996).

The main limitation of such assays is that human haemopoietic stem cell properties such as homing, engraftment, proliferation and differentiation might be very different in these models from endogenous *in vivo* haemopoiesis. If there is inefficient homing of HSCs then this may lead to underestimates of cell numbers by these assays. Moreover, the life span of immunodeficient mice is shortened to around five to eight months because they are susceptible to lymphoma and thus only the presence of ST-HSC can be detected and long term studies are not possible unless secondary transplants are performed (Cashman, *et al* 1997, de Wynter and Ploemacher 2001). Using a larger animal model allows more long term evaluation of human HSC. The fetal sheep HSC assay is based on the observation that the early gestational age sheep fetus allows long-term engraftment and multilineage expression of human HSCs in the absence of myeloablation (Srour, *et al* 1992, Zanjani 1997, Zanjani, *et al* 1992), possibly due to pre-immune status of the early gestation fetus. Notably, human HSCs engraft the host marrow and persist for long periods postnatally, allowing prolonged follow up and identification of LT-HSCs in this model (Srour, *et al* 1992). However, the *in vivo* xenograft models are cumbersome, time consuming and costly

and a number of *in-vitro* assays have been attempted in order to identify primitive haemopoietic progenitor cells. None of the *in vitro* assays described thus far isolate a pluripotent stem cell population, and there is increasing evidence that most *in-vitro* assays identify already committed progenitor cells (Bhatia, *et al* 1998, Larochelle, *et al* 1996).

The most primitive cell type identified by in vitro assay is the long-term culture initiating cell (LTC-IC). The long-term culture-initiating cell assay is based on the reconstitution of haemopoiesis within the microenvironment of a Dexter type long term bone marrow culture (de Wynter and Ploemacher 2001, Dexter, *et al* 1977). The rationale of the LTC-IC assay is based on the fact that haemopoiesis which occurs after five to eight weeks in culture must be established from very early cells, LTC-ICs, which have undergone proliferation and differentiation towards more committed progenitors. After five weeks the culture is characterised by foci of haemopoiesis represented by groups of cells which appear dark on phase contrast microscopy and are located underneath the stroma. These are named cobblestone areas and represent areas of primitive haemopoiesis. The number of cobblestone areas can be counted in a limited dilution assay and are a reflection of the number of LTC-IC present (Ploemacher, *et al* 1989, Weaver, *et al* 1997). However, this is a labour intensive process and so an indirect method of assessing the LTC-IC content by assessing the CFU-GM or BFU-E output as a result of LTC-IC differentiation after five to eight weeks of culture is also used. This is based on the concept that any CFU-GM or BFU-E obtained after five to eight weeks in culture are derived from the differentiation of LTC-IC in culture (Gunsilius, *et al* 2001). This indirect method, unlike the limiting dilution assay, is less quantitative of absolute number of LTC-IC present and can only be used to evaluate LTC-IC in comparative studies.

It is difficult at present to specify how much the *in vitro* LTC-IC population overlaps with *in vivo* long term repopulating cells. Certainly the frequency of mouse marrow cells that form LTC-IC *in vitro* correlates well with the frequency of bone marrow cells with *in vivo* long term repopulating ability (Ploemacher, *et al* 1991). However, gene marking studies of human haemopoietic cells reveal high numbers of LTC-IC containing the transgene but poor engraftment of those cells in a murine host

(Gunsilius, *et al* 2001). This suggests that part or all of the human LTC-IC compartment may be distinct from cells with marrow repopulating ability and thus do not have true HSC properties (Larochelle, *et al* 1996). These cells may represent the MPPs on figure 1.1

The colony forming cell (CFC) assays allows enumeration of more mature progenitors capable of forming colonies when cultured under appropriate conditions in semisolid media (methylcellulose, agar). These media reduce cell motility and thus allow single cells to proliferate (colony forming cells, CFC or colony forming units, CFU) and develop into cell clones which are identified as colonies (>50 cells) of differentiated cells after a culture period of seven to fourteen days. The composition of the colony determines which type of CFU has been seeded in the first place. CFU-GEMM corresponds to pluripotent myeloid progenitor cells which produce multilineage colonies (granulocytes, and erythrocytes, monocytes and megakaryocytes). CFU-GM is a more committed progenitor which gives rise to granulocyte/macrophage colonies. BFU-E corresponds to burst forming unit – erythroid, where burst describes the appearance of the colony and describes a primitive erythroid precursor (de Wynter and Ploemacher 2001).

1.3.2 Phenotypic identification of HSC

As all the functional assays of HSCs and their progenitors are retrospective in nature and not morphologically distinguishable (Gunsilius, *et al* 2001, Lord 1988), attempts have been made to identify primitive cells by immunophenotyping to allow prospective identification of cells. Despite exhaustive attempts, researchers have yet to find a single molecular marker expressed exclusively on HSCs. The current markers define a heterogenous population and at best allow us to enrich for a population of HSCs as well as progenitor cells committed to various lineages (Gunsilius, *et al* 2001). In humans, the CD34 antigen is most commonly used as an enrichment marker. CD34 is a sialomucin expressed on the surface of early progenitor cells but not on their more mature counterparts (Krause, *et al* 1996). Purified CD34 populations have been used in bone marrow transplants to effectively restore haemopoiesis (Brugger, *et al* 1994), confirming the status of CD34 as a 'stem cell marker'.

Attempts to improve the selection for HSCs have focused on using a combination of markers to enrich for more primitive cells. Other surface markers have been used in conjunction with CD34 to identify more primitive populations of cells such as CD34+38- cells which contain higher numbers of long-term repopulating cells when compared to CD34+38^{lo} (Bhatia, *et al* 1998). It was thought that the AC133 antigen (also known as CD133) may recognise a more primitive and less heterogeneous population than the CD34 antigen (Gunsilius, *et al* 2001). However,other studies show that while CD133+ cells have long term repopulating potential (Yin, *et al* 1997) there is as yet no real advantage over the more common CD34 marker.

Using a combination of four different cell surface markers, it has been demonstrated that the presence of CD34, CD133 and absence of CD38 and HLA-DR, defines a population with increased ability to restore haemopoiesis in recipients receiving myeloablative doses of chemoradiotherapy (Gunsilius, *et al* 2001). Recently the expression of KDR, a receptor for vascular endothelial growth factor, has been used in combination with CD34 to define a population of CD34+ KDR+ cells, of which only five to ten cells were necessary to restore full haemopoiesis in irradiated mice (Ziegler, *et al* 1999). Conversely CD34+KDR- populations are enriched for cells with no self renewal activity.

In 1996, Goodell et. al (Goodell, *et al* 1996) described a new method of obtaining an enriched HSC population from murine marrow by exploiting their ability to efflux the fluorescent dye, Hoechst 33342. The Hoechst-negative cells isolated by fluorescence-activated cell sorting are called "side population" (SP) cells and in the mouse were found to have *in vivo* repopulating ability. Further work demonstrated the presence of such SP cells in bone marrow of other mammalian species including humans (Goodell, *et al* 1996). The function of these SP cells in humans is less defined than their murine counterparts. *In vitro* studies of cord blood SP cells showed partitioning of lymphoid (NK cells) and myeloid progenitors in the CD34- and CD34+ subsets of SP cells (Storms, *et al* 2000). Later work by Uchida et al (Uchida, *et al* 2001) confirmed that SP cells were present in the liver of the second trimester human fetus and this population of cells contained a heterogeneous mixture of phenotypes. Cells with LTC-IC and CFC activity were found mostly in lin-

SP+CD34+38- fraction. Transplantation assays demonstrated the SP+CD34+CD38- fraction only showed repopulation in the NOD/SCID mouse and only 10% of CD34+38- cells were SP positive. This initially looked promising to further enrich transplantable populations of HSCs but the exposure of the cells to Hoechst 33342 may result in undesirable toxicity effects as seen in murine cells and limit the potential usefulness of this procedure (Siemann and Keng 1986).

In recent years there has been increasing evidence for a small sub-population of CD34 negative (CD34-) HSC (Bhatia, *et al* 1998, Zanjani, *et al* 1998). The use of xenograft assays has been central in the identification of such CD34- HSC, as little or no *in vitro* clonogenic (CFC) or LTC-IC activity has been demonstrated in the human CD34 negative lineage negative cell population (Bonnet 2002). Zanjani et. al. (Zanjani, *et al* 1998) transplanted populations of CD34+ or CD34- cells without detectable surface markers for multiple lineages (lin-), from the same normal human donors into preimmune fetal sheep and this resulted in long-term engraftment and multilineage hematopoietic cell/progenitor expression in both groups. Interestingly, the CD34-lin- cells as well as the CD34+lin- were able to repopulate secondary recipients –attesting to their self renewal ability. The observation that large numbers of CD34+ cells were found in repopulated sheep given CD34- cells suggests that the CD34- lin- population is more primitive than the CD34+lin- cells. At around the same time, Bhatia et. al. (Bhatia, *et al* 1998) also described a population of human CD34-lin- cells that they termed CD34-/SCID repopulating cells, or CD34-SRC that initiated multilineage haemopoiesis in NOD/SCID mice. Their CD34-SRCs were restricted to a Lin-CD34-CD38- population. The finding of lin-CD34-SRC establishes that the human HSC compartment is more complex than previously recognised and further work is required to establish the importance and the clinical significance of this CD34- SRC fraction.

1.4 Ontogeny of haemopoiesis.

Mammalian haemopoiesis occurs in successive waves in different haemopoietic organs during ontogeny and the requirements of the growing organism make differing demands on the haemopoietic system as gestation advances. In humans,

haemopoietic activity first appears in the extra-embryonic yolk sac (YS) in the middle of the third week of development at around day sixteen (Bloom 1940, Luckett 1978, Takashina 1987). This early haemopoiesis declines from week five in gestation and has virtually disappeared by week eight (Peault and Tavian 2003). This initial haemopoietic activity in the yolk sac is characterised by the production of primitive nucleated erythrocytes, which synthesise embryonic haemoglobins and macrophages (Peault and Tavian 2003, Tavian, *et al* 2001). The role of the macrophages formed is unclear, though it may be that they are important in providing a suitable microenvironment conducive to primitive haemopoiesis. In support of this, macrophage-like cells have been described in association with the generation of erythroblasts by La Pushin and Trentin in 1977 in erythroid spleen colonies (La Pushin and Trentin 1977). It has been postulated that this primitive haemopoiesis is designed to provide immune protection and maximal oxygen delivery to the early embryo (Orkin and Zon 2002).

The origin of HSCs within the YS and whether they are the source of HSCs for the establishment of definitive haemopoiesis is controversial. For many years it was accepted that HSCs were generated in the YS and subsequently migrated to fetal liver and bone marrow (Moore and Metcalf 1970) to provide definitive haemopoiesis for fetal and adult life. Indeed in the murine model when these YS HSCs were transplanted into busulphan conditioned newborn mice reconstitution of haemopoiesis was seen and secondary transplantation of these primary recipients into adult recipients showed donor contribution to long term haemopoiesis for at least six months suggesting that these cells do possess long term repopulating ability and self renewal capacity (Palis and Yoder 2001). However, the inability of YS HSCs to reconstitute all types of haemopoietic cells in lethally irradiated adult mice long term (Moore and Metcalf 1970, Palis and Yoder 2001) questions the concept of these YS HSCs being responsible for definitive haemopoiesis. In humans, the cells with haemopoietic potential which appear in the YS at day sixteen of development have been shown to give rise to only myeloid and NK cells (Peault and Tavian 2003, Tavian, *et al* 2001). Others have shown CFU-E and BFU-GM progenitors in the in YS (Migliaccio, *et al* 1986), though no cells with adult HSC properties are thought to

be produced at this stage and due to the limited nature of cells produced, this has been termed primitive haemopoiesis.

Later studies in mice which identified HSCs with long term repopulating activity in the aorta-gonad-mesonephros (AGM) region *before* the establishment of the circulation, also cast doubt on the yolk sac being the source of definitive HSCs and favoured the AGM region (Medvinsky and Dzierzak 1996, Palis and Yoder 2001). In contrast to YS HSCs, haemopoietic stem cells from the AGM region are capable of forming all types of mature blood cells and of restoring long term haemopoiesis in ablated adult mice (Medvinsky and Dzierzak 1996).

Haemopoietic activity is first seen in aorta-gonado-mesonephros (AGM) at around four weeks (day twenty-seven) of development in humans (Huyhn, *et al* 1995, Labastie, *et al* 1998, Peault and Tavian 2003). These haemopoietic cells from the AGM from days twenty-seven to forty give rise to long term haemopoietic cell cultures (Peault and Tavian 2003). One of the more striking findings of this work in early human development was that haemopoietic cells from day nineteen from the presumptive AGM, which is the splanchnopleura, were also capable giving rise to long term haemopoietic cell cultures (Peault and Tavian, 2003). Thus there is evidence of recognisable intra-embryonic HSCs two days before the embryo is connected to the yolk sac via blood circulation. As the YS HSCs have been found to only have predominantly myeloid potential, this led to the suggestion that definitive HSCs arise for the first time in the AGM and first migrate to the YS and liver and then around the time of birth from the liver to the bone marrow.

The AGM continues to provide haemopoietic cells until around day forty (week six) at which time its contribution diminishes and the fetal liver becomes the predominant site of haemopoiesis (Migliaccio, *et al* 1986) and remains the primary site of haemopoiesis for much of fetal life (Palis and Yoder 2001), although from about thirty weeks gestation, liver haemopoiesis declines. The first haemopoietic cells morphologically identifiable in the bone marrow are erythroblasts and macrophages and are found as early as week ten of gestation (Charbord, *et al* 1996). At this early stage there are few CD34+ haemopoietic cells in the bone marrow

though with increasing gestation the number of CD34+ cells increases and around twenty to twenty-two weeks there is evidence that bone marrow haemopoiesis is becoming established (Tavassoli 1991, Zon 1995). With the decline in fetal liver haemopoiesis there is a corresponding increase in bone marrow haemopoiesis which becomes the predominant site of haemopoiesis in late fetal life and remains so for the rest of prenatal and postnatal life under normal circumstances (Charbord, *et al* 1996).

Interestingly, the murine placenta was recently demonstrated to contain clonogenic hematopoietic progenitors, including CFU-GMs, CFU-GEMMs, BFU-Es, and HPP-CFCs (Alvarez-Silva, *et al* 2003). Moreover, between E10 and E12, these progenitors were present at numbers higher than those found in the YS and fetal liver. Furthermore these HSC were shown not to be maternally derived and to be capable of repopulating adult hosts (Ottersbach and Dzierzak 2005). This further adds to the complexity of HSC ontogeny, as the timing and origin of these placental HSC remains unknown and their contribution, if any, to definitive haemopoiesis in humans also remains unknown.

There is evidence that haemopoiesis differs in each anatomical site. As previously stated, YS HSC cannot repopulate adult ablated recipients in murine studies, whereas AGM, fetal liver and bone marrow HSCs are capable of long term bone marrow repopulation in adult animals (Palis and Yoder 2001). Fetal liver haemopoietic cells yield higher numbers of competitive repopulating units (CRU) and LTC-IC than either cord blood or adult bone marrow haemopoietic cells. In addition, cord blood yields more competitive repopulating units (CRU) and LTC-IC than adult BM (Holyoake, *et al* 1999). It is as yet unclear why these differences occur. The possibilities include changes in cell properties with age as they migrate from site to site, environmental influences or more controversially intrinsically different HSCs arising *de novo* at each site.

The sequential migration of HSCs from one site to another has never been conclusively demonstrated *in vivo* in mammalian species and is based on the measurement of haemopoietic activity in the YS and liver just after it is established in AGM and migration inferred because of the presence of significant numbers of

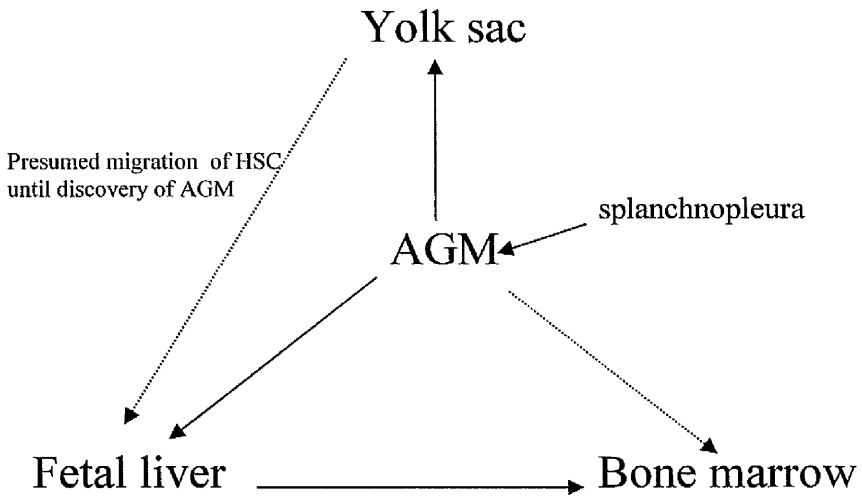
precursors in the blood stream (Delassus and Cumano 1996). Gene marking of fetal liver HSCs in the rat model by injecting retrovirus directly into the fetal liver also suggested that these cells did migrate to bone marrow *in vivo* (Clapp, *et al* 1995), however, the process of transfecting the cells with the gene may in itself alter their properties.

An alternative possibility challenges the idea of cells migrating and suggests they might be arising *de novo* in the endothelial compartment at each site. Some years ago it was observed that endothelial cells and haemopoietic cells often arose at the same time from the same area suggesting that a common precursor – the haemangioblast - might generate both endothelial and haemopoietic lineages and this proposition has received further attention in recent years (Peault and Tavian 2003).

In support of this hypothesis, both yolk sac vascular and blood systems are profoundly affected in defective knockout mice carrying targeted mutations in genes, such as transforming growth factor β and flk-1, a vascular endothelial growth factor receptor (Dickson, *et al* 1995, Shalaby, *et al* 1995). Secondly, insertion of β-galactosidase into the locus of Runx 1, a transcription factor necessary for AGM HSC development, showed localised expression of Runx 1 along the ventral wall of the dorsal aorta. Using transgenic mice which express green fluorescent protein (GFP) in the context of the Ly-6A/E locus, which contains all the regulatory elements of the HSC marker Sca-1, de Bruijn et. al.(de Bruijn, *et al* 2002) showed that GFP expression was localised to a few of the cells within the single layer of cells lining the dorsal aorta and these cells expressed CD31, CD34, c-kit and VE-cadherin endothelial/haemopoietic cluster markers.

More recently, Oberlin et al (Oberlin, *et al* 2002) described how endothelial cells from human yolk sac, AGM region, fetal liver and fetal bone marrow produced blood cells when cultured in the presence of stromal cells. Although this does not rule out the possibility of successive colonisation of haemopoietic areas by angio-haemopoietic precursors, these results could indicate the presence of fixed haemopoietic compartments during ontogeny. Figure 1.2 summarises the changes in haemopoietic sites with ontogeny.

ONTOGENY OF HUMAN HAEMOPOIESIS



Cells produced in each haemopoietic area

Day 16-?56	Yolk sac	nucleated red cell macrophages
Day 19	Splanchnopleura	HSCs
Day 21	Circulation between YS and embryo established	
Day 27-40	Aorta-gonad-mesonephros	HSCs
Day 40-?birth	Fetal liver	HSCs
Week 10	Bone marrow	erythroblast macrophages
Week 22	Bone marrow	HSCs

Fig. 1.2: Ontogeny of haemopoiesis
? Presumed cessation of haemopoiesis
A major haemopoietic stem cell (HSC) pool in mid-gestation mouse placenta has defined the placenta as yet another important anatomical site that participates in HSC development. The exact contribution of the placenta in mice and whether the placenta has a role in ontogeny of human haemopoiesis is yet to be defined.

1.5 HSC aging /self renewal.

The property of self renewal ascribed to HSCs implies that they maintain their numbers with age and the capacity of HSCs to maintain their numbers through self renewal replications implies that the stem cell population itself does not age. In support of this idea, it has been shown that primitive haemopoietic cells from one mouse can repopulate many mice and at least five serial transfers are possible without apparent loss of repopulating ability, suggesting their self renewal and proliferative capacity is enormous (Harrison 1979).

However, there is increasing evidence to suggest that the self renewal capacity of HSC is not infinite and other studies have demonstrated a large decline in repopulating ability with serial transplantation of HSCs in a murine model (Rosendaal, *et al* 1979, Ross, *et al* 1982). Although one could argue that the decline in regenerative potential may be a result of the transplantation procedure, further evidence to suggest that HSCs do show the effects of aging come from a number of studies. HSCs from older murine donors were at a significant disadvantage relative to HSCs from young murine donors in competitive repopulation studies (Korenberg, *et al* 1994, Rebel, *et al* 1996) suggesting a decreased replicative potential with age. Although this may be due to the decreased homing efficiency of old HSCs compared to HSCs from younger mice (Morrison, *et al* 1995), this in itself may be a possible change due to aging. Age related changes in the functional capacity of HSCs have also been reported in humans. There is a functional decline, as measured by colony output in *in-vitro* assays in human HSCs (CD34+ cells) from fetal liver, umbilical cord and adult bone marrow (Lansdorp, 1993). Ontogeny related functional differences in human HSCs have also been described in an *in vivo* xenogeneic model (Holyoake, *et al* 1999). Together, these results suggest that there is age-dependent diminished potential of HSCs and they therefore may have a limited replicative lifespan.

1.5.1 Telomere length and its regulation

Hayflick and Moorhead in 1961 suggested that there was some form of replicative lifespan operative in living cells, based on the observation that mammalian

fibroblasts during *in vitro* culture showed a decline and then a complete cessation of cell division. This replicative limit is known as the Hayflick limit and is defined as the point at which the cells can no longer proliferate even in the presence of mitogens (Hayflick and Moorhead 1961). This observation is open to the criticism of inadequate culture conditions but several observations point towards this *in vitro* phenomenon having biological significance. Firstly, there is a correlation between *in vitro* cell lifespan and the age of the cell donor (Bierman 1978, Bruce, *et al* 1986, Schneider and Mitsui 1976). Secondly there is reduced *in vitro* lifespan of cells from patients afflicted with premature aging syndromes (Metcalfe, *et al* 1996, Tahara, *et al* 1997).

One of the most widely studied factors involved in the cellular aging process is telomere regulation. The role of telomere shortening in particular has been extensively studied in the last fifteen years. Telomeres are DNA and protein containing structures present at the ends of eukaryotic chromosomes (McEachern, *et al* 2000). Telomeric DNA consists of series of six bases, TTAGGG, repeated thousands of times. They are essential for maintaining the integrity and stability of the chromosome. Telomeres shorten with each mitotic division of a cell. The initial work in this area described telomere loss with aging of cultured fibroblasts (Harley, *et al* 1990) and demonstrated that human fibroblasts *in vitro* lost telomeres with increasing proliferation and when they reached senescence, had significantly shorter telomeres than early passage cells (Harley, *et al* 1990, Vaziri, *et al* 1994).

It was then postulated that when telomeres reach a certain critical degree of shortening, corresponding to the Hayflick limit, the cell stops proliferating and becomes senescent (Harley 1991). Additional available evidence supports the hypothesis that telomeres fulfil the essential requirements as a replicometer for cells which 'count' cell divisions and trigger replicative senescence as several further studies have shown that loss of telomere length of cells is associated with a decrease in their replicative lifespan (Harley, *et al* 1992), Harley *et al,* 1990, Vaziri *et al*, 1994)

It was initially thought that the only contributor to telomere shortening was the inability of DNA polymerases to completely replicate a DNA template, the so called end replication problem (Kipling, *et al* 1999, Olovnikov 1973, Olovnikov 1996). In essence this problem arises as DNA polymerases are unidirectional (Levy, *et al* 1992) and can only synthesise DNA in 5'→3' direction as they move along the template strand. Somewhere along the 3' terminus of single stranded DNA, a specific RNA polymerase will synthesise a short stretch of nucleotides that is complementary to the DNA template. This RNA serves as a primer for DNA polymerase which will continue forming a new DNA strand from the template in the in the 5' to 3' direction of the newly formed strand only (fig 1.3).

For the complementary 5'→3' template strand, synthesis is discontinuous and sections of complementary strands called Okazaki fragments are made as the replication fork opens up. Subsequently the Okazaki fragments are stitched together by DNA ligase. Hydrolysis of the RNA primer and untranscribed nucleotides upstream of the 5' end of the RNA primer result in a region of untranscribed DNA at the 3' end of each DNA template strand. This part cannot be replicated as there is nowhere for the RNA primer to bind to allow DNA polymerase to work (Fig 1.3). Thus the 3' ends of all linear chromosomes are expected to shorten progressively with each round of DNA replication.

More recently it has become clear that cell division is not the only contributor to telomere shortening. The extent of telomere shortening can vary according to different conditions present in the environment. In extreme conditions such as chronic hyperoxia where cells are subjected to increased oxidative stress Von Zglinicki et. al. (von Zglinicki, *et al* 1995) showed telomere loss of fibroblasts cultured *in vitro* to be five times faster than normal.

In addition, another contributor to telomere length is the enzyme telomerase, a ribonucleoprotein reverse transciptase that synthesises telomeric repeats onto the 3' end of chromosomes, thus counteracting losses of telomere length occurring with cell replication (Blasco, *et al* 1997). In humans telomerase activity is detectable in stem

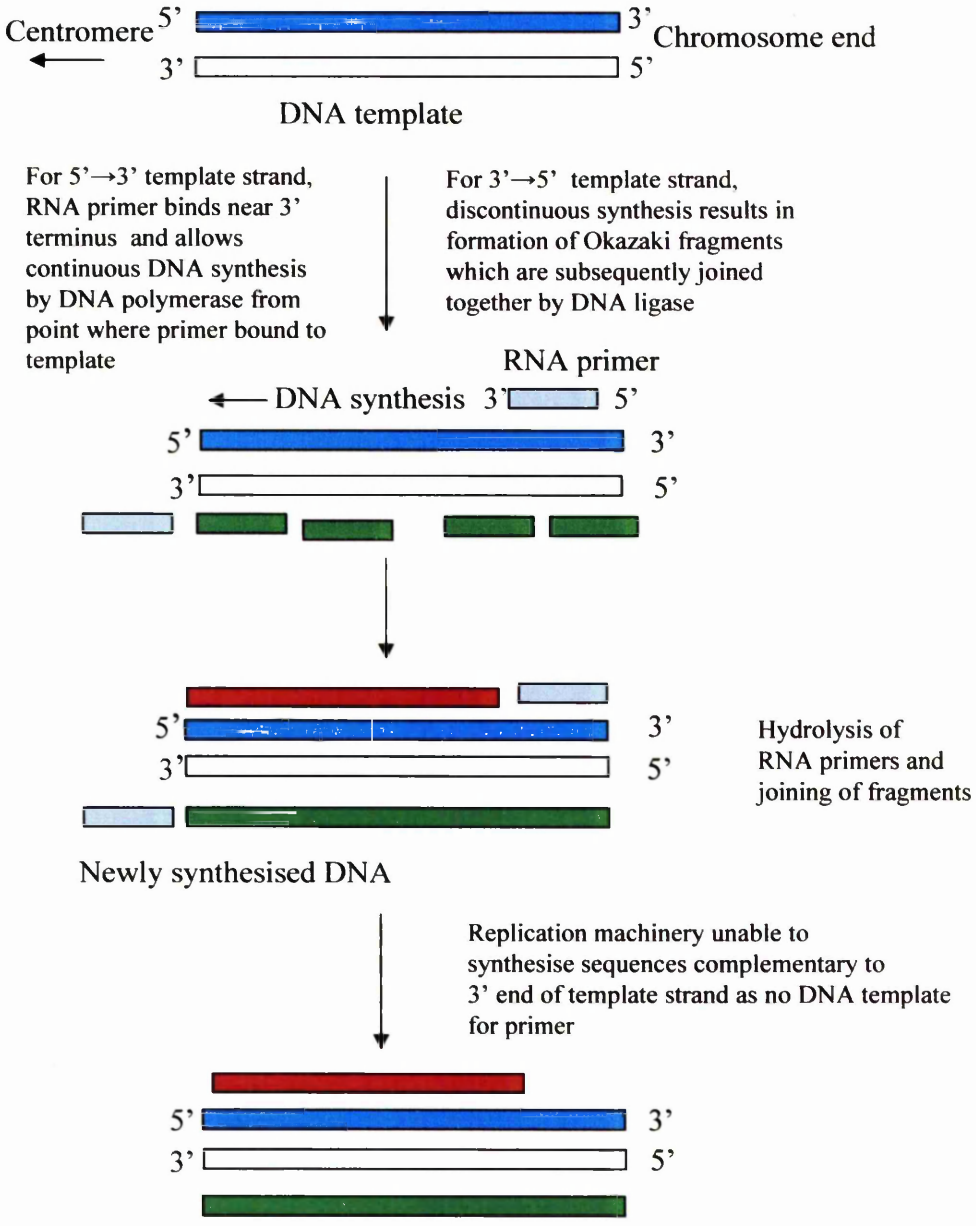


Figure 1.3 The end replication problem.
Figure show replication of telomere at one end of a chromosome pair

cells of germline in the testis and ovary and in embryonic stem cells during early development and in HSCs, but not in most somatic tissues (Morin 1989). Some adult somatic cells express telomerase for example, telomerase is transiently upregulated by activated human T cells (Buchkovich and Greider 1996). However, both HSCs and human T cells lose telomere with replication suggesting that telomerase expression in these cells is insufficient to prevent telomere erosion (Lansdorp 1995b).

1.5.2 Telomere length in normal HSC.

At present telomere length is measured by Southern Blotting or Flow FISH techniques which require high numbers of cells (10^6), thus making in it difficult to measure telomeres in small subpopulations of cells HSCs. Most studies therefore have assessed telomere length in peripheral blood leucocytes (PBLs) and therefore they measure the mean telomere length of the population of study. These studies are based on the assumption that the number of divisions between stem cell and mature cell are constant and therefore any changes in PBLs are a reflection of changes occurring in HSC population. Indeed there is evidence of good correlation of telomere length of CD34+ cells and neutrophils and CD34+ cells and T cells from same blood samples (Robertson, *et al* 2000).

It would be expected if HSCs self renew and generate daughter cells with equal properties, telomere length in HSCs would remain unchanged with time. However, studies of telomere length dynamics in PBLs carried out in postnatal life show loss of telomere with aging in keeping with the hypothesis that HSC lifespan is finite. HSC telomere loss in humans occurs in two distinct phases. There an initial rapid loss of telomere length in the first four years of life of up to 1000bp/year (Frenck, *et al* 1998, Rufer, *et al* 1999). This has been interpreted as reflecting the proliferation of stem cells in early life to establish a stem cell pool and maintain haemopoiesis in the rapidly growing infant (Frenck, *et al* 1998, Robertson, *et al* 2000). Subsequently, there is a more gradual loss of telomere length in PBLs of around 40bp/year for the remainder of life (Frenck, *et al* 1998, Robertson, *et al* 2000, Rufer, *et al* 1999). This has been interpreted as reflecting the turnover required and decreased proliferative work from the HSC by having only to maintain normal haemopoiesis.

1.5.3 Accelerated telomere shortening, aging & HSC deficiency

It has now been shown that accelerated shortening of telomere length is associated with increased incidence of leukaemia. In Fanconi's anaemia, there is an accelerated rate of telomere length shortening seen and an increased incidence of leukaemia (Leteutre 1999). Similarly in Shwachman–Diamond syndrome (SDS), an inherited disorder with impaired haemopoiesis and an increased risk of myelodysplasia and/or acute myeloid leukaemia has been seen. A study by Thornley (Thornley, *et al* 2002a) demonstrated that patients with SDS had shorter telomeres in lymphoid and myeloid cells than age matched controls, implying accelerated telomere loss. In aplastic anaemia, there is a well recognised risk of developing clonal disorders such as paroxysmal nocturnal haemoglobinuria and myelodysplastic syndrome (Narayanan, *et al* 1994, Tichelli, *et al* 1994) and also accelerated telomere shortening (Ball, *et al* 1998). In disorders such as CML, a form of myeloproliferative disorder, shortening of telomere length is associated with the onset of blast crisis (Boultwood, *et al* 1999, Boultwood, *et al* 2000) again suggesting an association between shortening of telomere and onset of leukaemia.

Conditions which exhibit an accelerated/premature aging phenotype are associated with accelerated telomere loss, HSC deficiency and increased incidence of leukaemia. Ataxia telangiectasia (AT), a rare autosomal recessive disorder is clinically characterised by progressive neuronal degeneration, ocular telangiectasia, immunodeficiency, premature aging and predisposition to cancer in particular leukaemia and lymphomas. Metcalfe et al.(Metcalfe, *et al* 1996) showed increased telomere shortening in peripheral blood leucocytes from AT patients and occurrence of stem cell deficiency has also been documented (Wong, *et al* 2003).

In aplastic anaemia accelerated telomere shortening has been associated with a stem cell deficiency as evidenced by a decrease in both CD34+ cells and *in vitro* progenitor cell frequency (Ball, *et al* 1998, Maciejewski, *et al* 1994). Also in Fanconi's anaemia, where accelerated telomere shortening has been described, there is progressive bone marrow failure characterised by progressive pancytopenia and *in vitro* clonogenic assays and long term cultures of Fanconi cells show a decrease in progenitor cell frequency (Stark, *et al* 1993). In SDS there is evidence of decreased

numbers of HSCs in bone marrow (Thornley, *et al* 2002a). Thus many of the disorders associated with increased leukaemia have both accelerated telomere loss and stem cell deficiency.

Dyskeratosis congenita (DC) is a rare inherited bone marrow failure syndrome characterised by abnormal skin pigmentation, nail dystrophy and oral leukoplakia. Patients have been reported as having a premature ageing phenotype and have a predisposition to malignancy. The disease has both X-linked and autosomal inheritance patterns. The gene responsible for X-linked DC (DKC1) encodes a highly conserved protein called dyskerin that is believed to be essential in ribosome biogenesis and may also be involved in telomerase assembly and correct function of the telomerase complex (Mason 2003). In the autosomal form of DC, mutations in the RNA component of telomerase have been described (Mason 2003). Cells from DC patients can show reduced telomerase activity and there is also evidence of accelerated telomere shortening in peripheral blood cells (Vulliamy, et al 2001) confirming that DC is a disease of telomere maintenance. A deficiency in haemopoietic progenitors has also been documented (Ball, *et al* 1998) confirming a stem cell deficiency in addition to the accelerated telomere shortening. Interestingly, the majority of the malignancies seen in DC are epithelial in origin, although malignancies of the haemopoietic system have also been reported (Dokal 2000). The increase in epithelial malignancies may be a result of the accelerated aging of tissues that show rapid turnover, though why this is not more apparent in the haemopoietic system is not clear. It may be that these individuals are succumbing to other complications of the disease before malignancies in the haemopoietic have time to manifest.

From the evidence above, one can postulate that situations of stem cell deficiency are associated with accelerated telomere loss and thus accelerated aging of the haemopoietic system and increased risk of leukaemia.

1.6 Down syndrome as a model of accelerated aging.

Down syndrome (DS, trisomy 21) is a genetic disease caused by the presence of an extra copy of part or the whole of chromosome 21. Clinically, individuals with this disorder have a characteristic phenotype and clinical manifestations including cardiac

defects, learning difficulties and haematological abnormalities and they also develop conditions associated with aging such as ocular cataracts and Alzheimers disease at a much earlier age than the normal population (Korenberg, *et al* 1994). The differences seen in the haemopoietic system can be apparent even in the neonatal period on a routine blood count and include a decreased platelet count in DS neonatal individuals compared to normal term neonates, a higher erythrocyte count and a peripheral blood macrocytosis (Roizen and Amarose 1993). Together these suggest disturbances in the haemopoietic system arising in early life.

DS has been quoted as the most common factor predisposing to childhood leukaemia as children with DS have a ten to twenty fold excess risk of developing leukaemia (Lange 2000). New-born DS infants frequently present with a transient disorder characterized by the presence of primitive haemopoietic cells in the peripheral blood, liver and bone marrow with the ultrastructural and cytochemical characteristics of megakaryoblasts (Lazarus, *et al* 1981). The incidence of this disorder, most commonly known as transient abnormal myelopoiesis (TAM) or transient myeloproliferative disorder (TMD), may be 10% of newborn DS individuals (Zipursky, *et al* 1997). Interestingly, there are often more blasts in the peripheral blood than the bone marrow and the disease may present with hepatic failure due to massive infiltration of the liver with blast cells. TMD is a clonal disorder (Miyashita, *et al* 1991) which in the majority of infants can disappear with little or no therapy (Lange, 2000) or which can result in death in around 17% of infants, mainly those with evidence of hepatic infiltaration. Up to 30% of individuals with resolved TMD will go on to develop acute myeloid leukaemia in the first four years of life (Zipursky, *et al* 1992) with the total incidence of AML between ten and twenty-fold higher than in normal children, the risk for the individual is as high as 1%. The majority of cases of acute myeloid leukaemia (AML) in DS are of M7 FAB subtype – acute megakaryoblastic leukaemia (AMKL). Most of these AML cases are preceded by a myelodysplastic (MDS) pre-phase (Lange, 2000). In contrast to AML seen in adult subjects such a myelodysplastic illness is exceptionally uncommon in children. The incidence of Acute Lymphoblastic Leukaemia (ALL) is also increased in DS individuals but to a lesser degree than that of AML. This increased risk of

malignant change is restricted to haematological malignancy, and is not a general property of DS (Hasle, *et al* 2000).

Data from an experimental murine model suggest that the haemopoietic disturbances seen in DS may be due to HSC abnormalities arising in fetal life. Indeed in a murine model of DS (trisomy 16) a reduction in cells possessing the phenotype of HSC was found in the fetal liver together with a reduction in the number of mature blood cells in liver, spleen and thymus. More importantly fetal liver HSC from trisomic mice were unable to restore normal haemopoiesis to irradiated normal hosts, (Epstein, *et al* 1985) suggesting that the peripheral blood abnormalities encountered are likely to be due to HSC abnormalities arising early during fetal development and the development of clonal disorders may be due to premature aging of the stem cell pool. It must be borne in mind that one of the shortcomings of this murine T16 model is that it may not accurately reflect the human situation as not all the genes seen in the human Down syndrome critical region on chromosome 21 have murine counterparts on murine chromosome 16 (Toyoda, *et al* 2002). Very recently a new mouse model has been developed containing almost all of human chromosome 21 and this may prove useful in future studies of the haemopoietic system in DS in fetal life (O'Doherty, *et al* 2005).

To date there is very little data available on haemopoiesis and HSC numbers or their functional capacity in human DS fetuses. In postnatal life, telomere length studies have found that PBLs from individuals with DS showed greater rates of loss with aging than normal individuals (Vaziri 1993) suggesting that there may be accelerated HSC aging in these individuals and one could hypothesise that this may possibly result from increased cell turnover if there is an HSC deficiency.

Thus the available evidence supports the idea that DS may be a suitable model of accelerated aging of haemopoietic system and because these individuals can be identified before birth due to their trisomy 21, haemopoietic tissue can be obtained and used to study HSC numbers and replicative dynamics before the clinical manifestation of TMD or leukaemia. With the increasing evidence that childhood leukaemias are fetal in origin (Wiemels, *et al* 1999), the study of the development of

leukaemia in DS fetuses may serve as a useful model for attempting to identify factors contributing to the onset of childhood leukaemia in general.

1.7 Aims

The hypothesis underpinning this work is that accelerated HSC aging in DS begins in fetal life before the onset of clonal disorders and is associated with HSC deficiency which may contribute to the increased risk of leukaemia in this condition. There is very little data on haemopoiesis in human fetal life *in vivo* and corresponding telomere length changes during this time of maximal growth and no data on HSC replication in DS in humans in fetal life

The overall aim is to determine whether accelerated HSC aging is a contributing factor by determining HSC numbers and replicative dynamics in DS in fetal life.

To achieve my aim I will

1. Firstly determine HSC replicative dynamics and properties of haemopoietic cells in fetal life and perinatal life in non DS individuals. To accurately determine telomere dynamics of HSCs, the rate of decline of telomere in longitudinal and cross-sectional samples of human fetal blood and longitudinal samples from preterm infants of similar gestational age range to fetal group will be measured.

2. Determine and compare HSC, progenitor cell numbers and HSC replicative dynamics in DS and non DS fetuses.

CHAPTER 2.

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Subjects.

Human fetal blood was obtained after ethical approval from Salford and Trafford and Liverpool Research Ethics Committee from two groups of fetuses at St Mary's Hospital, Manchester and Liverpool Women's Hospital, Liverpool. The first group consisted of fetuses undergoing feticide by administration of intra-cardiac potassium chloride prior to termination of pregnancy (TOP) for a variety of reasons including cardiac defects, neural tube defects, cytogenetic abnormalities and social reasons. Due to the limited availability of fetal samples, all samples were utilised even though it was not possible to completely sure that they were not affected by haematological disorders. Peripheral blood was taken from these fetues immediately prior to intra-cardiac potassium administration. This cross-sectional group are referred to as TOP group in subsequent text. The second group consisted of fetuses affected with Rhesus alloimmunisation or neonatal/fetal alloimmune thrombocytopenia and sequential peripheral blood samples were obtained prior to intrauterine transfusion at time of clinically indicated ultrasound guided cordocentesis for monitoring and treatment of alloimmune disorders. Umbilical cord blood was also obtained from the same fetuses immediately after delivery. This group are subsequently referred to as AF group.

Fetal samples received ranged from 0.5-1.0ml in volume. An initial count of nucleated cells was made using a haemocytometer after eliminating enucleated red blood cells by dilution with a 3% acetic acid solution (VWR, Dorset, UK). Where total cell number was greater than $3x10^6$, the sample was divided as follows 0.3-0.5ml for DNA extraction ($0.9\text{-}1.5x10^6$ cells), 0.4 ml for immunophenotyping ($2\text{-}5x10^4$ cells per tube for all markers) and 0.3ml for mononuclear cell separation and colony assay. Where cell quantity was less than $3x10^6$, sample was used for either DNA extraction or immunophenotyping and if possible progenitor cell assay

Sequential peripheral blood samples from preterm infants (PTI) were obtained following ethical approval from Liverpool Research Ethics Committee at time of clinically indicated blood tests from a group of eight normal clinically well infants in the Special Care Baby Unit, Liverpool Women's Hospital. Samples ranged from

0.4-1.0ml in volume. Samples for each infant were only utilised if the infant was clinically well with no clinical or laboratory evidence of infection and with stable respiratory and cardiovascular parameters. Initial count of nucleated cells was performed as above. Similar to fetal samples, the initial nucleated cell count of samples was determined and experiments performed according to cell count.

2.2 Immunophenotyping of fetal and infant peripheral blood cells.

Fetal or infant blood cells were stained with a panel of antibodies to cell surface antigens chosen to represent both mature cells and earlier progenitor cells. The antibodies used, their dilution and appropriate isotype controls are as listed in table 2.1. The cell types determined and their corresponding immunophenotype are listed in table 2.2.

All antibodies and their corresponding isotype controls were from Caltag, Silverstone, UK, except for CD34 and CD34 isotype match control which were from Pharmingen, (Oxford, UK). For Caltag antibodies optimum dilution of antibody was determined as 1:20 per staining (which was 5 µl of antibody or control for total staining volume of 100 µl). For Pharmingen antibodies, optimum dilution was 1:10 (10 µl per staining).

Around 50000-80000 cells were incubated with appropriate dilutions of primary antibodies as indicated in table 2.2 and total volume of samples made up to 100µl with sodium azide buffer (0.1% sodium azide from Sigma, Poole, UK, 2% FCS , in phosphate buffered saline both from Gibco, Paisley UK) and mixed well. Samples were then left on ice for 45 minutes and at the end of the incubation, 3ml of red cell lysis buffer (RCB, 10mM potassium carbonate, 150mM ammonium chloride, 0.1mM EDTA, pH 8.0) was added to each tube and mixed with the cell solution which was then incubated at room temperature for 5 minutes to allow red cell lysis. Cells were then centrifuged at 800g for 5 minutes, followed by one wash with 2ml azide buffer. Following another 5 minute centrifugation at 800g, cells were resuspended in 300µl of 2% formaldehyde solution (BDH Laboratory Supplies, UK) containing 0.02% BSA (Gibco, UK), in PBS and stored at 4°C until acquisition was carried out using a

Marker	Dilution	Fluorochrome	Isotype control
CD4	1:20	Phycoerythrin (PE)	IgG2a
CD8	1:20	PE	IgG2a
CD14	1:20	PE	IgG2a
CD15	1:20	Fluorescein (FITC)	IgM
CD19	1:20	FITC	IgG1
CD20	1:20	PE	IgG3
CD33	1:20	PE	IgG2b
CD34	1:10	PE	IgG1
CD45	1:20	FITC or Allophycocyanin (APC)	IgG1
Glycophorin A	1:20	PE	IgG1

Table 2.1: Mouse monoclonal antibodies directed against human haemopoietic cell antigens used for staining whole blood form fetal and preterm subjects.

Immuno-phenotype	Cell types
CD4	T cells
CD8	T cells
CD19/20	B cells
CD33+/15-	Myeloblasts
CD33+/15+	Promyelocytes, myelocytes
CD33-/15+	Neutrophils
CD14	Monocytes
CD45+/glycophorin+	Erythroblasts
CD45-/glycophorin+	Pronormoblast, normoblast, reticulocyte
CD34+	Stem/progenitor cells

Table 2.2: Cell types determined in human fetal and preterm infant peripheral blood by immunophenotyping.

Becton Dickinson FACScalibur instrument. A minimum of 20000 total events (30000-75000 for CD34) were acquired and analysed using CellQuest software. For analysis, events acquired by the flow cytometer were plotted on dotplots and forward light scatter of cells (FSC) and right angle light scattering properties of cells (SSC) were used to define regions containing lymphoid cells (R1), monocytes (R2) and granulocytes (R3) and erythroid progenitors (R4) as shown on figure 2.1. Events from R1, R2, R3 and R4 were displayed in a dot plot and quadrants positioned in such a manner that 98% cells stained with isotype control were in the first log of fluorescence intensity. Any event with fluorescence superior to the first log was considered positive. For the lymphoid markers, events acquired in R1 were analysed (lymphocyte gate). For myeloid markers, events acquired in R1, R2 and R3 were analysed. For erythroid markers, events acquired in gate R1 and R4 were analysed. For CD34 cells, events acquired in R1 were analysed. The frequency of positive cells labelled for each cell type acquired in the appropriate gate was expressed as a percentage of the total number of nucleated cells present in the sample.

2.2.1 Measurement of CD34+ cells by ISHAGE method

In addition in some samples, where cell numbers permitted, a second method of analysing CD34+ cell frequency was employed, as described in ISHAGE guidelines for CD34 frequency by dual staining with CD34PE and CD45 (Barnett, *et al* 1999, Sutherland, *et al* 1996). This was done to check the accuracy of the previous method as CD34+ cells are rare cells and it was important to determine their numbers accurately. In this method, at least 70000 events were collected per sample and an initial gate (R1) is set on a CD45+ versus side scatter plot (figure 2.2a); this gate contains all CD45+ events and will exclude CD45- events (erythrocytes, platelets and other debris). The events in R1 are then displayed on a second dot plot of CD34 vs SSC and a second gate, R2, is produced to include the cluster of CD34+ events (figure 2.2b). On a third plot of CD45 vs side scatter, the events fulfilling R1 and R2 are plotted. Cells forming a cluster with characteristic low SSC and low to intermediate CD45 fluorescence are then gated on this third plot to produce region R3 which contains CD34+ cells (figure 2.2c). Finally, the events fulfilling criteria of R1 and R2 and R3 are plotted on a dot plot of FSC vs SSC (figure 2.2d). The lymphocyte/blast region of this plot, R4, identifies the cluster of events meeting all

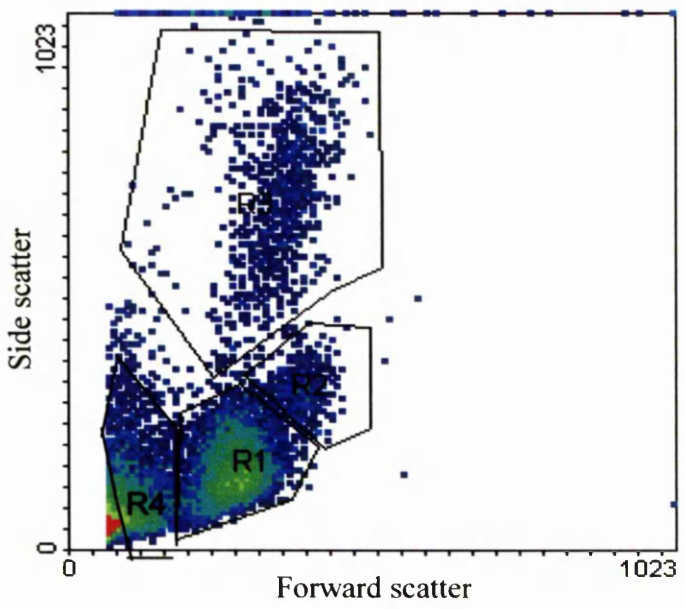


Figure 2.1: A representative profile of light scatter characteristics of fetal peripheral blood cells on flow cytometry.

R1 =lymphocyte gate,
R2 =monocyte gate
R3 = granulocytes
R4 = erythroid progenitors.
All samples were gated in similar manner.

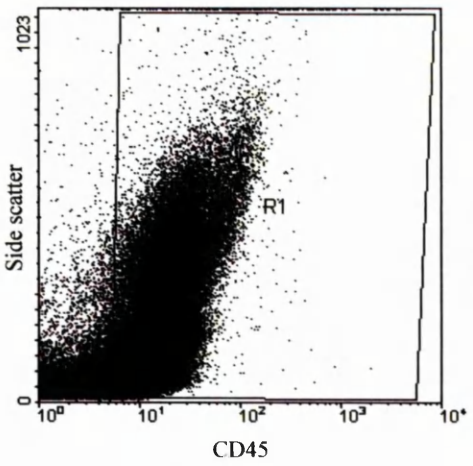


Fig 2.2a

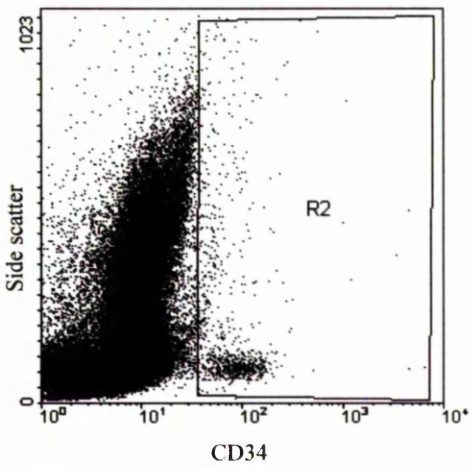


Fig 2.2b

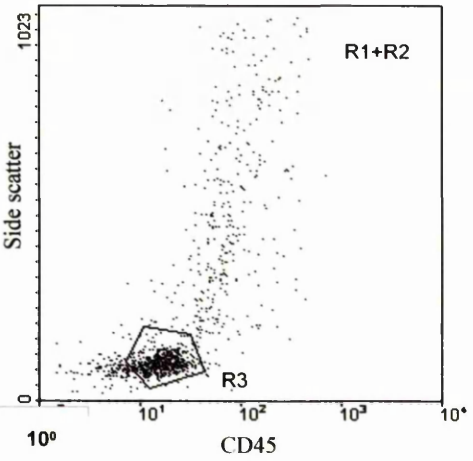


Fig 2.2c

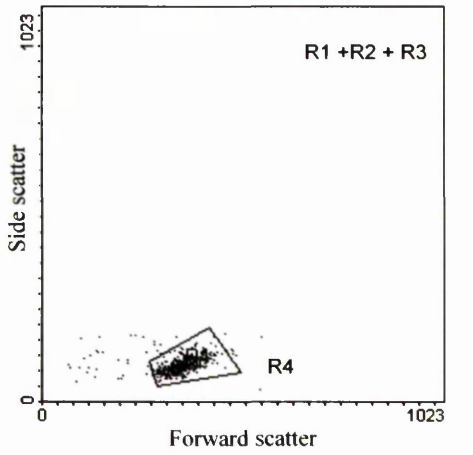


Fig 2.2d

Figure 2.2: Peripheral blood sample analysed for CD34+ cells by ISHAGE gating strategy. Figure 2.2a, cells gated on CD45+ population. Figure 2.2b, CD45+ cells gate for CD34+ cells. Figure 2.2c, CD45+ and CD34+ cells with low side scatter demonstrating CD34+ stem cells have low to medium CD45+ staining. Figure 2.2d, cells fulfilling fluorescent and light scatter criteria form a distinct population (R4) in lymphoid area of Forward vs side scatter plot.

the fluorescence and light scatter criteria of CD34+ haemopoietic stem/progenitor cells. Similar analysis can be performed on a second staining sample from the same individual with CD45 APC and isotype control to CD34 PE to determine numbers non-specifically stained cells (Sutherland, *et al* 1996). However, because the gating strategy excludes cells which non-specifically bind CD34 (Barnett, *et al* 1999), this step is not necessary. The results of CD34+ frequency measured by the ISHAGE method were compared to results obtained on same samples using the method described in the previous section. Table 2.3 shows CD34+ cell frequency measured by both methods which gave similar values for CD34+ cell frequency, confirming that method described in section 2.2, paragraph 4 was reliable.

2.3 Progenitor cell assays

Mononuclear cells (MNCs) were separated from whole blood by layering fetal or infant blood made up to 800µl with PBS onto 400µl of lymphozyten (d=1.077g/L, Lymphozyten, PAA Laboratories, Austria) in 1.5ml Eppendorf containers and centrifuging at 5000rpm for 5 minutes. Cells from interface and above were removed and washed with sterile PBS. Cells were counted with haemocytometer and resuspended in RPMI medium (Gibco, Paisley, UK) at a concentration of $2.5x10^4$ in 100µl. One hundred microlitres of this solution ($2.5x10^4$ cells) was added to 0.9ml of methocult H4434 (Stem Cell Technologies, Vancouver, Canada) consisting of 0.9% methylcellulose with Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% fetal bovine serum, 1% BSA (Gibco, UK), 10^{-4}M 2-mercaptoethanol, 2mM L-gluTMDine, 50ng/ml recombinant human (rh) stem cell factor, 10ng/ml rh granulocyte-macrophage colony-stimulating factor, 10 ng/ml rh interleukin-3 and 3 unit/ml rh erythropoietin). Then 0.25ml of methylcellulose/cell suspension was placed in 3 central wells of a 24 well plate (Fred Baker, Cheshire, UK). The surrounding wells were half-filled with double distilled water and the plates incubated at 37°C in 5% $C0_2$ humidified atmosphere for 14 days. After 14 days all colonies consisting of more than 50 cells were scored according to morphology as burst-forming unit erythroid (BFU-E), colony forming unit granulocyte-macrophage (CFU-GM) or colony forming unit granulocyte-erythroid-monocyte-macrophage (CFU-GEMM).

Sample	CD34+ Frequency (%) ISHAGE method	CD34+ Frequency (%) Dotplot method
FB11	0.26	0.2
FB110	0.33	0.29
NB1	0.45	0.59
NB5	0.32	0.34
NB7	0.55	0.63
NB15	0.4	0.34
NB19	0.06	0.09
NB24	0.08	0.1
NB29	0.1	0.07

Table 2.3: Comparison of CD34+ cell frequency measured by ISHAGE and dotplot methods

2.4 DNA extraction from fetal and infant haemopoietic cells.

Enucleated red cells were removed by lysis with RCB. Following lysis, remaining cells were washed in PBS and incubated with DNA lysis buffer (1% sodium dodecyl sulphate, 10mM Tris-HCl, 100mM NaCl) and proteinase K (40µg per $3x10^6$ cells) overnight at 37°C. Blunt pipette tips were used thereafter to prevent shearing of DNA. Genomic DNA was purified from cell lysate by firstly adding an equal volume of phenol (VWR Ltd, Dorset) and the sample mixed until an emulsion formed. Following centrifugation at 5000rpm for 15 minutes, the aqueous phase was removed without disturbing the interface and equal volume of a 1:1 phenol and chloroform (VWR Ltd, Dorset) mix added. The sample was mixed to form an emulsion and centrifugation was again performed at 5000rpm for 10 minutes and the aqueous phase removed. Thirdly, an equal volume of chloroform was added, mixed until an emulsion formed and centrifuged at 5000rpm for 5 minutes. Again the aqueous phase was collected and added to 1/10 volume of 5M NaCl and 2.5 volumes of 100% ethanol to precipitate genomic DNA. The precipitate was washed with 70% ethanol, collected with a tissue culture hook and redissolved in 200µl of TE (10 mM Tris-Cl, pH 7.5 1 mM EDTA) buffer. The quality and quantitiy of the DNA was determined by measuring the OD_{260} of the DNA solution using a spectrophotometer.

2.5 Telomere length measurement by Southern Blotting.

2.5.1 Genomic DNA digestion

In order to leave telomeres intact, genomic DNA was digested with restriction enymes which do not cleave telomeric DNA. The intact DNA left following digestion is the terminal restriction fragment (TRF) which contains the intact telomeres and can be measured to assess the mean telomere length of the sample. Ten micrograms of each DNA sample was digested to completion overnight at 37°C with 30 units of **Rsa-I** and 30 units **Hinf-I**. (Roche Diagnostics, East Sussex, UK). Quantity of digested DNA was checked by spectrophotometry. To assess integrity of genomic DNA and completeness of digestion, 1µg undigested and digested DNA was run on a 0.6% agarose gel (Sigma, UK). All samples found to have intact genomic DNA and complete digestion with restriction enzymes were used for telomere length measurement.

2.5.2 Manufacture of radiolabelled size marker

For each telomere gel, Lambda Hind III digest (Sigma, UK) was used as a size marker and radiolabelled with $\alpha^{32}P$ using the Random Primed DNA Labelling Kit (Roche, East Sussex, UK). Briefly, the following were incubated for 10 minutes at room temperature: 0.5μg Lambda DNA Hind-III digest, 1μl water, 5μl (50μCi) $^{32}P\alpha$-dATP (Amersham, UK), 0.5μl 10x concentrate 'H' buffer 1.5μl of a mix of DNA nucleotides cytosine, thymidine and guanosine and 1μl of Klenow enzyme from random primed DNA labelling kit according to manufacturers instructions. After 10 minutes, 0.5μl unlabelled dATP was added and the reaction incubated for a further 10 minutes at room temperature. The reaction was then terminated by adding 2μl of 0.5M EDTA and 20μl TE. Radiolabelled size marker was stored at 4-8°C for up to 1 week.

2.5.3 Agarose gel electrophoresis of digested genomic DNA samples

Digested DNA samples were run in a 0.5% agarose gel (Seachem, Biowhittaker,Wokingham, UK) in tris-acetate-EDTA buffer (1xTAE, Sigma UK), with 0.5μg/ml of ethidium bromide (Sigma) to allow visualisation of DNA under U.V. light. Gels were set and run at 4°C for 48 hours. Samples were loaded in sets of no more than four to six adjacent lanes in gel. Each set of lanes was separated by radiolabelled size marker.

2.5.4 Drying of the 0.5% agarose gel after electrophoresis.

The gel was vacuum dried at 65°C for 1 hour 40 minutes or until gel was perfectly flat and dry. The dried gel was then denatured in 1.5M NaCl and 0.5M NaOH for 20 minutes. Following denaturation, gel was neutralised for 20 minutes in 1M Tris-Hcl pH 8.0 and 1.5M NaCl and rinsed briefly in water before placing flat and heat sealing in plastic bag.

2.5.5 Telomere probe radiolabelling

In-gel hybridisation of telomereic DNA was done using a phosporus 32 labelled 5'-$(CCCTAA)_3$. The probe was bought commercially from Invitrogen (Paisley, UK) and upon receipt the probe was diluted to make a 20μM solution. The enzyme polynucleotide kinase (Roche, East Sussex, UK) was used in the radiolabelling

reaction of the probe with γ-^{32}P of γ-ATP. This enzyme catalyses the transfer of the terminal phosphate group on (γ position of supplied ATP) to the 5'hydroxylated terminus of the telomere probe. The probe was labelled fresh prior to every use according to manufacturer's instructions. Briefly, the following were incubated for 30 minutes at 37°C: 1µl oligonucleotide telomere probe (20 nmol), 1µl 10x concentrated PNK buffer (Roche, East Sussex, UK), 2µl deionised water, 5µl (50µCi) γ^{32}P ATP (Amersham, UK) and 1µl polynucleotide kinase (10 units) (Roche, East Sussex UK)

Enzyme activity was then terminated by transferring the reaction mixture to a 65°C water bath for 10 minutes. The probe was then precipitated to remove unincorporated γ-^{32}ATP by adding 75µl TE, 10µl 3M NaOAc, 1µl $MgCl_2$, 5µg of 1mg/ml tRNA (all from Sigma, Dorset) solution in TE and 200µl 95% ethanol.

Precipitation was carried out by placing the mixture in dry ice for 15 minutes and then centrifuging for 30 minutes at 13000rpm at 4°C. The supernatant containing was carefully removed and the probe pellet washed in 70% ethanol. The pellet was then dissolved in 200µl T.E. and checked for even distribution in solution of radioactivity with scintillation counter. The desired activity of the probe was 125000-250000 counts per minute per millilitre of hybridisation buffer.

The gel was heat sealed in a bag with 10ml hybridisation buffer (7% sodium dodecyl sulphate (SDS), 1% bovine serum albumin (BSA), 500mM Na_2HPO_4, 10mM EDTA, 10µl herring sperm at 10mg/ml) containing the radiolabelled probe. The SDS was always added last and the buffer warmed in a 65°C waterbath before adding it to the gel. The bag was agitated and monitored with scintillation to ensure even distribution of the probe. It was incubated and constantly agitated overnight at 37°C in a hybridisation oven.

At completion of the hybridisation period the gel was opened in 1 litre of 0.24% x SSC and then washed three times in 500ml of 0.1% SSC for 15 minutes. Following this, the gel was again heat sealed in plastic bag and placed in a Phosphor Imager cassette (Molecular Dynamics, Amersham, UK). The gel was exposed on the

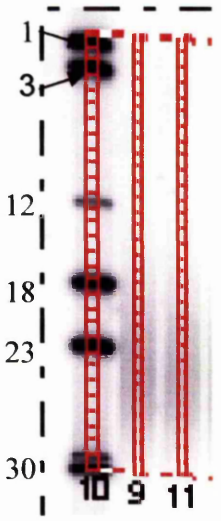
cassette for 24 hours and cassette then analysed on a phosphoimager (Molecular Dynamics). The analysis information was stored using Image Quant© software.

2.5.6 Analysis of gel

ImageQuant© software was used to determine the mTRF length of each digested DNA sample run on each gel. Initially, the DNA size markers across the gel were identified and a line drawn joining the top of all the 2027kb size marker bands and a second line drawn joining the bottom of all 23.23kb size marker bands. Between these two lines, individual grids consisting of 30 rows and 1 column were drawn to cover the length of both the DNA size marker ladder and each of the DNA samples using the ImageQuant program. (fig 2.3). The region number corresponding to each band of the DNA size marker was then determined and the region number, molecular weight (MW) and logarithm of MW of corresponding band were entered in three separate columns of a table (fig 2.3a). Then a best fit line of regression of y on x was calculated where y was the logarithm of the MW of each band and x was the corresponding region number. Graph 2.3b shows the linear relationship between log MW and distance travelled in the gel and from it the intercept (c) and gradient (m) were calculated using the equation of a straight line:

$y=mx + c$ where y= logarithm of molecular weight, x = region number.

Next ImageQuant© was used to calculate the average MW of each DNA smear. For each sample, the telomere is seen as a smear on the gel and the same 30 region grid was placed over each smear to exactly match that placed over size marker bands. The equation generated above from the relationship between logarithm of molecular weight and distance migrated by size marker (in the example log MW = 0.03469x (Region number) + 0.243 was used to calculate the logarithm of the molecular weight for each of 30 regions in each sample (see table 2.4). The molecular size (in base pairs) of DNA in each region was determined by calculating the inverse logarithm from the value of logMW obtained.



grid no	mw	log mw
1	2.027	0.306854
3	2.322	0.365862
12	4.361	0.639586
18	6.557	0.816705
23	9.416	0.973866
30	23.13	1.364176

Fig. 2.3a

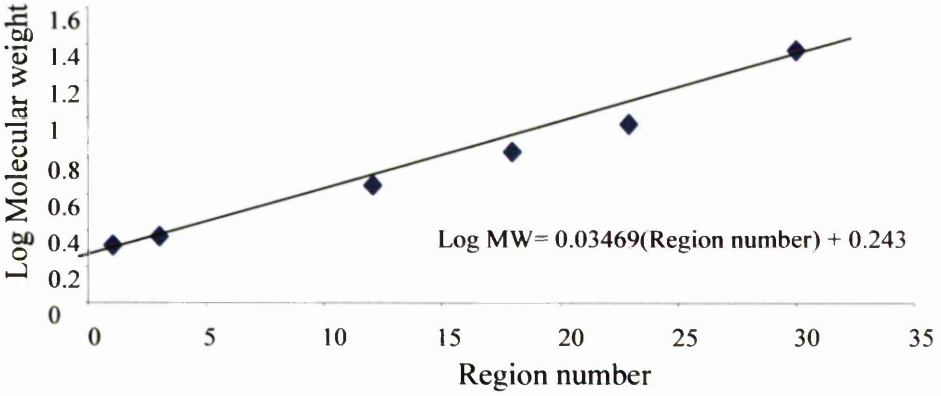


Fig. 2.3b

Fig 2.3: Measurement of mTRF from telomere gel. (a) Divison of size marker and sample lanes into 30 equal regions using ImageQuant. The logarithm of known molecular weight (mw) of size marker fragment and their corresponding positions in grid were used to calculate the linear relationship between them.

b Linear relationship between log of molecular weight of size marker and distance migrated through gel.

Grid number	Log MW	Molecular Weight (MW)	Optical density (OD)	MW * OD
1	0.277453	1.894318	327.434	620.2643
2	0.31205	2.051398	268.193	550.1707
3	0.346647	2.221503	252.495	560.9185
4	0.381244	2.405714	250.598	602.8671
5	0.415841	2.6052	219.801	572.6255
6	0.450438	2.821227	256.127	722.5924
7	0.485035	3.055167	274.638	839.065
8	0.519632	3.308507	247.945	820.3277
9	0.554229	3.582853	263.917	945.5758
10	0.588826	3.879949	284.382	1103.388
11	0.623423	4.20168	300.554	1262.832
12	0.65802	4.55009	337.019	1533.467
13	0.692617	4.927391	377.254	1858.878
14	0.727214	5.335978	429.316	2290.821
15	0.761811	5.778445	514.849	2975.027
16	0.796408	6.257603	630.43	3944.981
17	0.831005	6.776493	791.679	5364.807
18	0.865602	7.33841	957.942	7029.772
19	0.900199	7.946923	1153.668	9168.111
20	0.934796	8.605894	1430.171	12307.9
21	0.969393	9.319508	1759.795	16400.42
22	1.00399	10.0923	2056.859	20758.43
23	1.038587	10.92917	2353.714	25724.13
24	1.073184	11.83543	2505.023	29648.02
25	1.107781	12.81684	2636.928	33797.09
26	1.142378	13.87963	2670.101	37060.02
27	1.176975	15.03055	2448.998	36809.8
28	1.211572	16.27691	1983.457	32284.55
29	1.246169	17.62662	1535.662	27068.53
30	1.280766	19.08824	1180.334	22530.5
			Σ OD=30699.28	ΣOD * MW =337155.9

mTRF (kb) = $\frac{\Sigma(\text{MW of region} * \text{optical density of region})}{\Sigma(\text{optical density of region})}$

=337155.9/30699.28

=10.98253

Table 2.4. Calculation of mean telomere length from one DNA sample with ImageQuant© software.

The telomere signal for each region was quantified by the program as the optical density (OD) and recorded from each region in the grid. Then for each sample the telomere length was calculated by determining the sum of the optical density from each region multiplied by the molecular weight of the same region and dividing by the sum of optical densities of whole sample (table 2.4).

$$mTRF\ (kb) = \Sigma\ \frac{(MW\ of\ region\ (kb)^*\ optical\ density\ of\ region)}{\Sigma\ (optical\ density\ of\ region)}$$

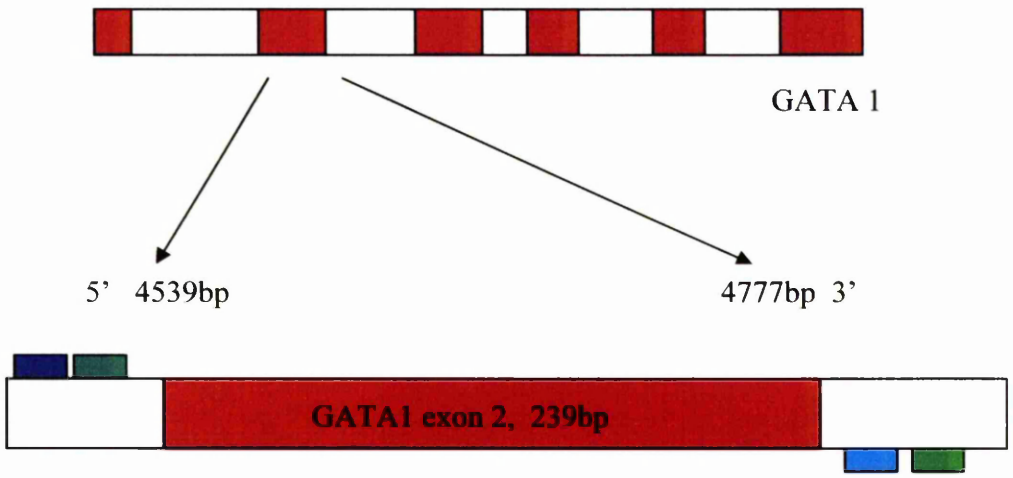
2.7 Sequencing of GATA 1 exon 2.

2.7.1 PCR amplification of exon 2 of GATA1

Genomic DNA corresponding to exon 2 of GATA1 (genomic DNA accession no. AF196971, complementary DNA accession no. NM_002049) was amplified by polymerase chain reaction using two sets of flanking oligonucleotide primers purchased from Invitrogen, UK (figure 2.4) as previously described by Rainis,*et al* 2003, Hitzler *et al* 2003. For each DS DNA sample, fetal DNA or cell line controls (K562, Jurkat), 500ng of genomic DNA in 5µl of DDH_2O was added to 45µl PCR mastermix containing 0.2µM of each GATA 2F/2R primers, 0.2µM dNTPs, 50mM magnesium chloride and 0.5µl of TAQ polymerase. Each tube was then vortexed briefly and pulse microfuged to collect all sample at base. Polymerase chain reaction conditions for GATA1 2F/2R were 95°C for 5 minutes, and then 35 cycles of 94°C for 10 seconds, 60°C for 10 seconds and 68°C for 60 seconds. Following PCR, 3µl of each sample was added to separate EPPs containing 15µl of 6x loading buffer and run on a 2% agarose gel to confirm that reaction had worked and expected PCR product seen.

2.7.2 Purification of GATA1 exon 2 PCR products

GATA1 exon 2 PCR products from each DS, fetal or cell line sample were purified to remove unused primers using QIAquick kit (Qiagen, Sussex, UK, Cat no. 28104). Firstly, 5 volumes (225µl) of buffer PB were added to 1 volume of the sample (45µl) and mixed by vortexing. For each sample a labelled QIAquick spin column was placed in a 2ml collection tube. The sample and buffer mixture were placed in a



GATA1-1F	5'-GGAAGGATTTCTGTGTCTGAG-3'
GATA1-1R	5'-TCTTGGCATTGGCTGAGTGC-3'
GATA1-2F	5'-AAAGGAGGGAAGAGGAGCAG-3'
GATA1-2R	5'GACCTAGCCAAGGATCTCCA-3'

Primers GATA1 1F/1R give a PCR product of 319bp
Primers GATA1 2F/2R give a PCR product of 392bp

GATA1 1F/1R Rainis et al,2003, Blood,102(3):981.
GATA1 2F/2R Hitzler et al, 2003, Blood,101(11);4301

Figure 2.4: Primers used to amplify and sequence GATA1 exon 2 of genomic DNA in DS DNA samples

QIAquick spin column and centrifuged for 60 seconds at 13000rpm to bind the DNA to the column. The flow-through was discarded and QIAquick column placed back into same collection tube. To wash the DNA, 750µl of PE buffer was added to the column and then centrifuged for 60 seconds. The flow-through was discarded and column centrifuged for a further 60 seconds. To elute, the column was then placed in a clean 1.5ml EPP and 50µl of EB buffer (10mM Tris-Cl, pH 8.5) was added to the centre of the QIAquick membrane and the column centrifuged for 60 seconds. The purified DNA in the elution buffer was stored at -20°C until used in the sequencing reaction.

2.7.3 Sequencing reaction of GATA1 exon 2 PCR products

The sequencing was carried out at the Molecular Biology Core Facility, Paterson Institute of Cancer Research, Manchester by Dr Stuart Pepper. Bi-directional sequencing of purified PCR products from GATA1 2F/2R reactions was performed using GATA1 1F or 1R primers. For each sequencing reaction, the following reagents were added to a separate EPP tube: Terminator Ready Reaction Mix 4µL, PCR product at 100ng, primer (GATA1 1F or GATA1 1R) at a concentration of 15pmol and finally deionised water to bring the final volume of each reaction to 20µL. The contents of each tube were mixed well and briefly microcentrifuged to ensure all the reaction mix was at the bottom of the tube. To sequence the GATA1 exon 2 DNA, the samples were placed in a thermal cycler and 25 cycles of the following were performed: 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes. Following the final cycle, the samples were held at 4°C until ready to purify.

2.7.4 Purification of sequencing extension products

Firstly, the tubes were briefly microcentrifuged to spin down the contents. Next, the following were added to each sample:16 µL of deionized water and 64 µL of non-denatured 95% ethanol so that the final ethanol concentration was 60 ± 3%. The tubes were closed and vortexed briefly and then left at room temperature for 15 minutes to precipitate the extension products. Next the tubes were placed in a microcentrifuge with their orientations marked and spun for 20 minutes at maximum speed. The supernatants were carefully aspirated with a separate pipette tip for each

sample and discarded to leave only extension product pellets. Then 250 µL of 70% ethanol was added to each tube and the tubes were vortexed briefly. Then the tubes were microcentrifuged for 10 minutes at maximum speed. Again the supernatants were aspirated carefully and discarded. Finally the samples were dried in a vacuum centrifuge for 10–15 minutes and resuspended in formamide. They were then transferred to 96 well plates and briefly microcentrifuged to ensure all reaction mix was contained at bottom of each well.

2.7.5 Electrophoresis and analysis of extension products

The plates were placed in the autosampler of an Applied Biosystems 3100 capillary sequencer. The 50 cm capillaries are filled with 3100 POP-6 polymer, a medium that separates the DNA fragments using standard conditions of run times, run voltage and run temperature as recommended by the manufacturer. The autosampler positions capillaries into the sample wells and the fluorescently labeled DNA is loaded into the capillary by a short period of electrophoresis called electrokinetic injection. The capillaries are then rinsed with water to remove sample adhering to the capillary sides. The autosampler then moves and positions the capillaries into the buffer chamber for electrophoresis. When the DNA fragments reach the detection window, the laser beam excites the dye molecules and causes them to fluoresce. The fluorescence emissions from the samples are collected simultaneously and spectrally separated by a reflective spectrograph. The 3100 sequencing Data Collection software reads and interprets the fluorescence data, then displays the data as an electropherogram.

2.8 Measurement of telomere length by real time PCR

2.8.1 Preparation of mastermix stock for real time PCR

Eurogentec qPCR Core Kit for Sybr Green I (Cat No. RT-SN10-05) was used to prepare mastermix stocks for use with telomere (T) or 36b4 (S) PCRs. The mastermix was prepared in a dedicated laboratory area with designated pipettes and tips. Firstly the 1 in 2000 dilution of Sybr Green was prepared by briefly microcentrifuging the 5µl of 1/10 of Sybr Green provided, then adding 995µl of DMSO and vortexing briefly to give 1 in 2000 solution. Then the complete master

mix (except primers) was prepared by adding reagents according to table 2.5 to a 15ml Falcon tube in the order specified. The tube was vortexed for 15 seconds to mix, pulse centrifuged to collect droplets and then aliquoted into 1ml EPPs

2.8.2 Preparation of primers for real time PCR

To quantify the amount of telomere in an unknown sample, two separate real time PCR reactions were performed on each sample. The first reaction uses a reference gene to standardise/normalise for differences in the amount of total template added to each reaction. Any autosomal single copy gene can serve as the basis for the reference. For this project, 36b4 was used as this had already been described and optimised for use in real time PCR work (Boulay, *et al* 1999). The sequences of the 36b4 primers are shown in figure 2.5. The second PCR reaction amplifies telomere sequence in the sample. The primers for the telomere PCR reaction are shown in fig 2.5. Both telomere and 36B4 primers were provided by Invitrogen (Paisley, UK). For each primer a concentrated stock solution of 1mM was prepared by diluting with DDH_20. A working solution of 100μM was prepared for each primer by diluting the concentrated stock solution with TE^{-4} (Tris-EDTA (TE) buffer, 10mM Tris-HCl, 0.1mM EDTA, pH 8.0). At time of performing PCR appropriate amounts of each set of primers were added to mastermix stocks and mixed by vortexing to give final concentrations of reagents as listed in table 2.6

2.8.3 Preparation of DNA samples for real time PCR

All DNA samples were prepared in a different area of the laboratory to mastermix area using dedicated pipettes and tips. Firstly, the concentration of genomic DNA (gDNA) was measured by spectrophotometry. This concentrated genomic DNA was sonicated and diluted to a final concentration of 1.75ng/μl in 10^{-4} TE. (TE^{-4} prepared by adding 2μl of 1X TE to 20ml of DDH_2O). Aliquots of each diluted DNA sample were stored at -20°C until used in PCR.

2.8.4 Preparation of DNA dilutions for standard curve.

Genomic DNA from K562 cells was diluted to initial concentration of 10ng/μl with TE^{-4} (S1). From this starting concentration, serial dilutions were performed to generate decreasing concentrations of the reference DNA sample (S2-S5). Each

Reagent	Volume added (µl)	Final concentration
DDH_20	8991.5	
10X reaction buffer	2450	1X
$MgCl_2$ (50mM)	1715	3.5mM
dNTPs (5mM)	980	0.2mM
Sybr Green (1:2000)	490	1:66000
Taq polymerase (5U/µl)	122.5	0.025U/µl
Total volume	14749µl	

Table 2.5: Table showing volumes of reagents required and final concentrations of reagents in mastermix stock for real time PCR

Tel 1: 5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3'

Tel 2: 5'TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'

36B4u: 5'-CAGCAAGTGGGAAGGTGTAATCC-3'

36B4d: 5'-CCCATTCTATCATCAACGGGTACAA-3'

Figure 2.5: Primers used for telomere and 36B4 PCRs.
Primers described by Cawthon, 2002, Nucleic Acids Res, 30(10)e47

Reagent	Volume per reaction	Final Concentration
10X reaction buffer	2.5µl	1x
$MgCl_2$ (50mM)	1.75µl	3.5mM
dNTPs (5mM)	1µl	200µM
Sybr Green (1:2000)	0.5µl	1:66000
Taq polymerase (5U/µl)	0.125µl	0.025µl/reaction
36b4D	0.075µl	300nM
36B4U	0.125µl	500nM
DDH_20	8.92µl (36B4)	
Tel 1	0.0675µl	270nM
Tel 2	0.225µl	900nM
DDH_20	8.83µl (tel)	

Table 2.6: Table showing final concentrations of reagents required in each well for each real time PCR reaction for both telomere and 36B4 PCRs

dilution was stored in aliquots at -20°C until time PCR performed. At time when PCR performed, 10µl of each dilution was placed in three adjacent wells in 96 well plates.

2.8.5 Setting up 96 well plate for real time PCR

For each type of PCR (T or S), 10µl (17.5ng) aliquots of sample DNA are placed in triplicate in corresponding positions in 2 PCR plates (Eurogentec, Cat No RT-PL96-01N). Each plate also contained standard curve samples in triplicate and appropriate no template controls.

2.8.6 PCR conditions for telomere and 36B4 PCR

All real time PCR reactions were carried out on Applied Biosystems Sequence Detection System. When setting up the machine for each reaction, the conditions of the reaction and the file name are entered before placing plate in the machine. The conditions set for telomere PCR were 50°C for 2 minutes then 95° C for 10minutes and then 30 cycles of 95°C for 15 seconds and 54°C for 2 minutes. For 36b4 plates reaction conditions were 50°C for 2 minutes, 95°C for 10 min and then 35 cycles of 95°C for 15 seconds and 58°C for 1min. Following this the plate is added and lid secured before commencing the reaction. Upon completion of the reaction, the data from each 96 well plate was saved onto Zip disc. Wells containing standard curve samples were labelled appropriately with concentration and correct dye layer. Wells containing samples were labelled as unknown and unused wells were labelled not in use from drop down menu.

2.8.7 Analysis of PCR data using SDS software.

Analysis of the PCR data was performed using SDS software. Firstly, for each 96 well plate, an amplification plot was generated which shows change in fluorescence in each well on y-axis versus cycle number of PCR reaction on the x-axis. (figure 2.6). On this plot two variables need to be determined in each reaction. The first is the baseline. The baseline is set as the number of cycles of the reaction where there is no change in fluorescence above a background level. The default setting on the system is 3-15 cycles however, Applied Biosystems recommend subtracting two cycles from cycles when amplification first occurs and enter this value as the stop

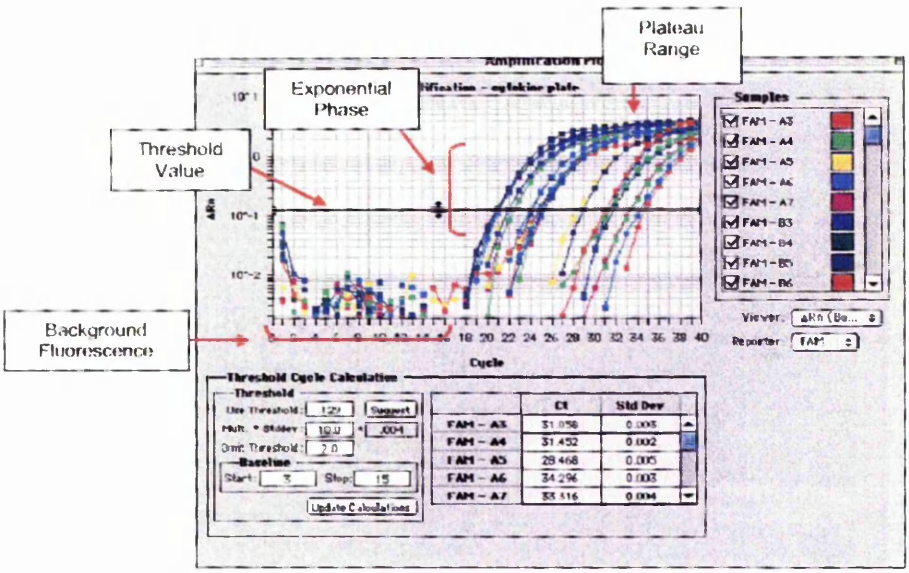


Fig 2.6. Graph showing change in fluorescence of each sample with cycle number for a typical real time PCR experiment.

point. For telomere PCR plates the baseline was adjusted to 3-12 cycles as amplification first seen around cycle 14. For 36b4 PCR, baseline remained on the default setting of 3-15. These baseline settings were used in all subsequent PCR reactions.

An increase in fluorescence above the baseline indicates detection of accumulated PCR product. A fixed fluorescence value known as the threshold can be set above the baseline in the exponential phase of the reaction. The parameter C_T (threshold cycle) is defined as the cycle number at which the fluorescence from a sample passes the fixed threshold. The higher the amount of target, the sooner the fluorescence passes the threshold and hence the lower the Ct value. Thresholds for telomere and 36B4 reaction plates were set in the middle third of the visible part of the exponential phase of amplification. The same thresholds were used in all telomere PCRs and in all 36b4 PCRs.

Once the baseline and threshold have been set, a standard curve plot was generated using the software. This is a plot of the straight line relationship between the logarithm of initial target copy number (amount of target) for a set of dilutions against the C_T value for each dilution. Quantitation of the amount of target in unknown samples is achieved by measuring C_T of unknown and using the standard curve to determine the equivalent starting copy number/amount of standard. The SDS software automatically computes the quantity of unknowns using the equation generated by the standard curve. For each sample, as the reactions was performed in triplicate, the average amount was calculated and this was stored as an Excel document.

2.8.8 Calculating the T/S ratio for each sample

The values obtained above from both telomere and reference gene PCRs were pasted into a new Excel document and the average telomere amount was divided by the 36B4 amount to give a T/S ratio for each sample. Then the T/S ratio for each sample was divided by the T/S ratio of the chosen calibrator sample (DNA from Jurkat cells) to give a relative T/S ratio which is the measure of telomere content of the sample.

2.8.9 Calculating efficiency of real time reactions

The efficiency of the reaction is calculated from the linear relationship between the logarithm of the starting amount of standard curve dilutions and the cycle number at which the fluorescence of the reaction passes a given threshold. The efficiency of the reaction (E) is given by the equation:

$$E = 10^{(-1/\text{gradient})}$$

For accurate quantitation of unknowns, the efficiency of the reaction in the exponential phase should be as near to 2 as possible. In other words there should be an exact doubling of the product with each cycle. Thus the gradient of the line should be -3.32.

2.8.10 Real Time PCR performed on cells directly

K562 cells, cultured in RPMI with 10% fetal calf serum, were sorted into a 96 well PCR plate using a FACSvantage cell sorter. The machine was set up to deposit 500 cells in each well of row A, 100 cells in each well of row B, 50 cells in each well of row C, 10 cells in each well of row D, 5 cells in each well of row E, 2 cells in each well of row F and 1 cell in each well of row G. For row A, the volume of 500 cells was 2.5μL and each well had 7.5μL of TE^{-4} added to give final volume of 10μL. Each well of all other rows had TE^{-4} added with sterile, separate pipette tip for each well to make up volume to 10μL. Following the cell sorting procedure, plates were sealed with adhesive film and stored at 4°C or -20°C until used for PCR reaction. At the time of performing PCR, the plates were defrosted if necessary, briefly centrifuged to collect all droplets at bottom of each well and 15μL of telomere or 36B4 PCR mastermix was added to each well in designated area of laboratory with designated PCR tips. After addition of master mix, plates were sealed with PCR adhesive film and kept on ice while transported to ABI 7700 SDS equipment. On arrival, each plate was briefly centrifuged and immediately commenced on the appropriate PCR cycle

CHAPTER 3.

HSC REPLICATIVE DYNAMICS IN FETAL LIFE

3 HSC REPLICATIVE DYNAMICS IN FETAL LIFE

3.1. Introduction

Whilst HSC aging has been extensively studied in postnatal life by functional studies showing decreased proliferative capacity with age and by studies of telomere dynamics, very little is known of normal HSC aging in fetal life. There is evidence that HSC have different functional properties in fetal life, they have increased cell cycle activity (Christensen 1988, Lansdorp 1995a, Peschle, *et al* 1981) and they respond differently to cytokine stimulation (Hao, *et al* 1995, Weekx, *et al* 1998). The proliferative capacity of CD34+ cells has been suggested to be inversely correlated with ontogenic age (Weekx, *et al* 1998) with fetal liver CD34+ HSC having a greater proliferation than either cord blood or adult bone marrow CD34+ HSC. Ontogeny related differences in haemopoiesis have also been demonstrated in an *in vivo* xenogeneic model and show greater self renewal activity in fetal liver HSC compared to cord blood or adult bone marrow HSC (Holyoake, *et al* 1999).

To date, the telomere changes in fetal life are relatively unknown. One study which does address this looked at telomere length in cord blood samples from newborn preterm infants and found that there was a decrease in telomere length with gestational age but a wide variation between individuals (Friedrich *et al*, 2001). However, this was a cross sectional study and the large variation between individuals even at the same age limits the analysis of telomere length. As yet nothing is known of the *in vivo* telomere changes in fetal life in humans and thus HSC replicative dynamics at this time of life.

The aim of this chapter is to further increase our knowledge by determining the dynamics of telomere length changes in normal fetal life both by cross sectional analysis and by longitudinal analysis of sequential samples from a group of fetuses undergoing regular intra-uterine blood sampling.

3.2 Cross sectional analysis of replicative dynamics in fetal blood cells.

3.2.1 Phenotypic characterisation of fetal blood cells

To ascertain that no significant changes in cell type were present with advancing gestation, which may bias the telomere length analysis, peripheral blood samples from eight fetuses undergoing termination of pregnancy (TOP) with gestational ages ranging from 23-32 weeks taken at time of feticide were used for immunophenotyping. As sample volumes were small, between 0.5 and 1ml, it was not always possible to carry out a complete panel for each sample (see table 3.1). To determine if there were any significant changes in the proportion of each cell type due to gestation, data was analysed using Spearman's rank correlation coefficient. Data are expressed as median value and range of values observed for each cell type.

CD4 and CD8 were used as representative T cell markers. The frequency of CD4+ cells in whole blood samples, calculated as described in chapter 2, section 2.2 from TOP fetuses (n=6) was 13.3% (median) and ranged from 2.7% to 21.2%. The frequency of CD8+ cells in TOP group (n=8) was 7.9% (range 4.6% to13.9%).

The frequency of B cells, assessed in a similar way to CD4 and CD8 was measured by co-expression of CD19/CD20 and was 4.9% (range 3.5-9.2%, n=7) in TOP group.

Figure 3.1 and table 3.2 summarises the proportions of lymphoid cells with increasing gestation. CD4 and CD8 showed no statistically significant trend with increasing gestation. CD19 showed a statistically significant decrease in frequency in whole blood with increasing gestation.

CD33 and CD15 dual staining was performed to identify CD33+/15- (myeloblasts) CD33+/15+ (promyelocytes, myelocytes) and CD33-/15+ (neutrophils). CD14 was used as a representative monocyte cell marker. The proportions of each cell type are shown in figure 3.2 and table 3.3. None of the myeloid markers exhibited a statistically significant change in proportion with increasing gestation from 23-32 weeks (table 3.3).

Sample	TOP 29	TOP 3	TOP 18	TOP 11	TOP 1	TOP 23	TOP 32	TOP 63
Gestation (weeks)	23	24	26	26	27	28	29	32
CD4	Y	Y	Y	N	Y	Y	Y	N
CD8	Y	Y	Y	Y	Y	Y	Y	Y
CD19/20	Y	Y	N	Y	Y	Y	Y	Y
CD14	Y	Y	Y	Y	Y	Y	Y	Y
CD33+/15+	Y	Y	Y	Y	Y	Y	Y	Y
CD33+/15-	Y	Y	Y	Y	Y	Y	Y	Y
CD33-/15+	Y	Y	Y	Y	Y	Y	Y	Y
Glyco+/45+	Y	Y	N	Y	N	Y	Y	Y
Glyco+/45-	Y	Y	N	Y	N	Y	Y	Y

Table 3.1 Immunophenotyping experiments performed on whole blood cells from eight TOP samples.
Glyco=glycophorin

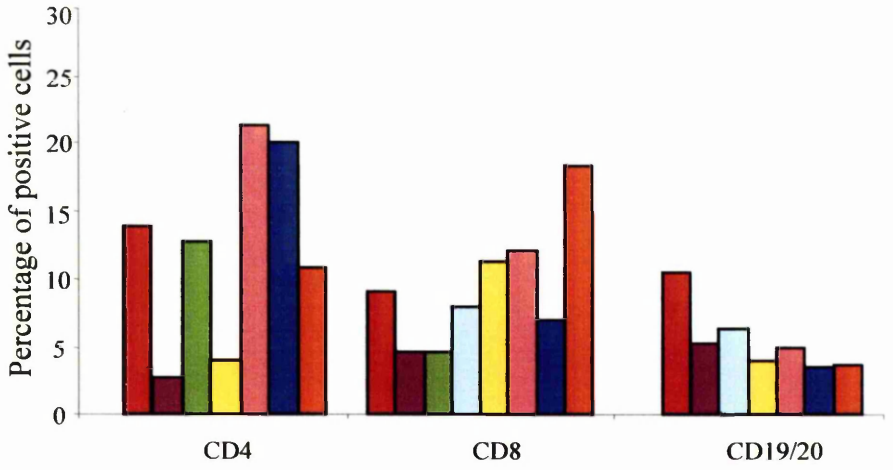


Figure 3.1: Percentage of lymphoid cells in fetal blood in TOP fetuses. Samples were obtained from fetuses of 23,24,26,27,28,29 and 32 weeks gestation. Bars for each cell type show from left to right, youngest to eldest gestational age.

Cell type	Median (range)	Number of samples	Correlation coefficient	P value
CD4	13.3% (2.7-21.2%)	6	0.54	0.27
CD8	7.9% (4.6-13.9%)	8	0.3	0.52
CD19/20	4.9% (3.5-9.2%)	7	-0.78	0.04*

Table 3.2. Statistical analysis of changes in lymphoid cells with age in TOP using Spearman's rank correlation coefficient.

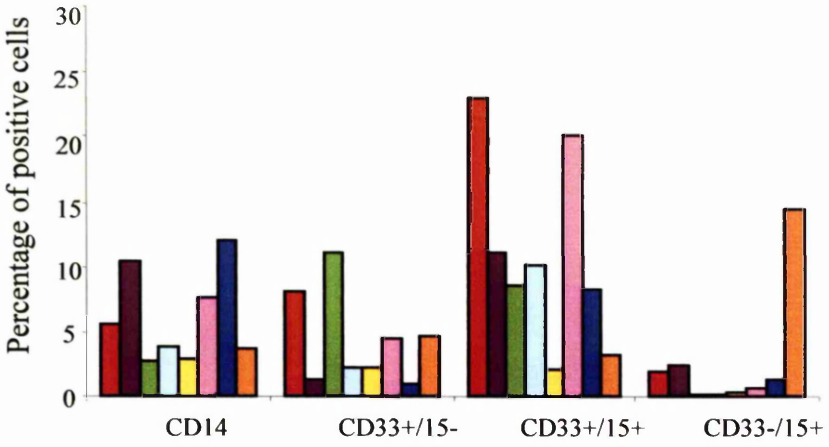


Figure 3.2: Percentage of myeloid cells in fetal blood in TOP fetuses. Samples were obtained from fetuses of 23,24,26,27,28,29 and 32 weeks gestation. Bars for each cell type show from left to right, youngest to eldest gestational age.

- TOP 1, 23 weeks
- TOP 2, 24 weeks
- TOP 3, 26 weeks
- TOP 4, 26 weeks
- TOP 5, 27 weeks
- TOP 6, 28 weeks
- TOP 7, 29 weeks,
- TOP 8, 32 weeks

Cell type	Median (range)	Number of samples	Correlation coefficient	P value
CD14	6.2% (2.7-12%)	8	0.12	0.78
CD33+/15-	2.2% (0.9-11.1%)	8	0.12	0.77
CD33+/15+	8.5% (2.1-16.6%)	8	0.23	0.56
CD33-/15+	1.5% (0.1-8.8%)	8	0.35	0.4

Table 3.3. Statistical analysis of changes in myeloid cells with age in TOP using Spearman's rank correlation coefficient.

A combination of glycophorin A and CD45 was used to measure the proportions of cells in the erythroid lineage. CD45+/glycophorin+ contains mainly erythroid progenitors at the erythroblast stages, just after CFU-E stage where they can no longer form erythroid colonies and are committed to becoming erythrocytes. Cells at later stages of erythroid development - pronormoblast, normoblast and reticulocytes are contained within the CD45-/glycophorin+ population. The distribution of erythroid cells in cross sectional samples is shown in figure 3.3. No statistically significant trend was observed in the number of CD45+/glycophorin+ cells or in the numbers of CD45-/glycophorin+ cells with increasing gestation (table 3.4).

3.2.2 Telomere length measurement in cross sectional samples

DNA from the peripheral blood of sixteen TOP fetuses (six of whom were used in immunophenotyping studies above) between 21 and 32 weeks gestation was extracted as described in section 2.4 and 2.5.1 and non-telomeric DNA was removed by specific restriction enzymes leaving intact telomeres. Completion of digestion was assessed by gel electrophoresis of matched undigested and digested DNA samples. Figure 3.4 shows a representative example of an electrophoresis gel of matched undigested and digested DNA samples. Undigested genomic DNA samples show a high molecular weight band near the proximal (loading) end of the gel. Digested samples do not show this high molecular weight band but have a smear of lower molecular weight telomere restriction fragments towards the top of the gel. Only samples that were completely digested – i.e. no evidence of high molecular weight band on digested DNA sample on gel electrophoresis were selected for telomere length analysis.

The mean telomere restriction fragment length (mTRF) for each sample compared to gestational age is shown in figure 3.5. Telomere lengths showed an increasing trend with gestation but this was not significant by correlation analysis ($r = 0.24$, $p = 0.65$, figure 3.5). Variation of telomere length between fetuses was high and differences up to 1.2 kb between individuals of the same gestation were seen.

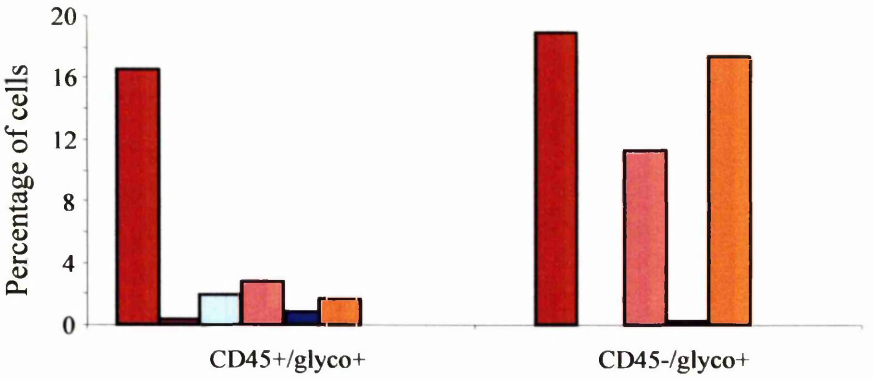


Figure 3.3: Percentage of erythroid cells in fetal blood in TOP fetuses. Samples were obtained from fetuses of 23,24,26,28,29 and 32 weeks gestation. Bars for each cell type show from left to right, youngest to eldest gestational age

- TOP 1, 23 weeks,
- TOP 2, 24 weeks
- TOP 4, 26 weeks,
- TOP 6, 28 weeks
- TOP 7, 29 weeks
- TOP 8, 32 weeks

Marker	Median (range)	Number of samples	Correlation coefficient	P value
CD45+/ glyco+	1.4% (0.3-16.5%)	6	-0.6	0.21
CD45- /glyco+	0.55% (0-18.9%)	5	-0.06	0.91

Table 3.4. Statistical analysis of changes in erythroid cells with age in TOP using Spearman's rank correlation coefficient.
(glyco=glycophorin)

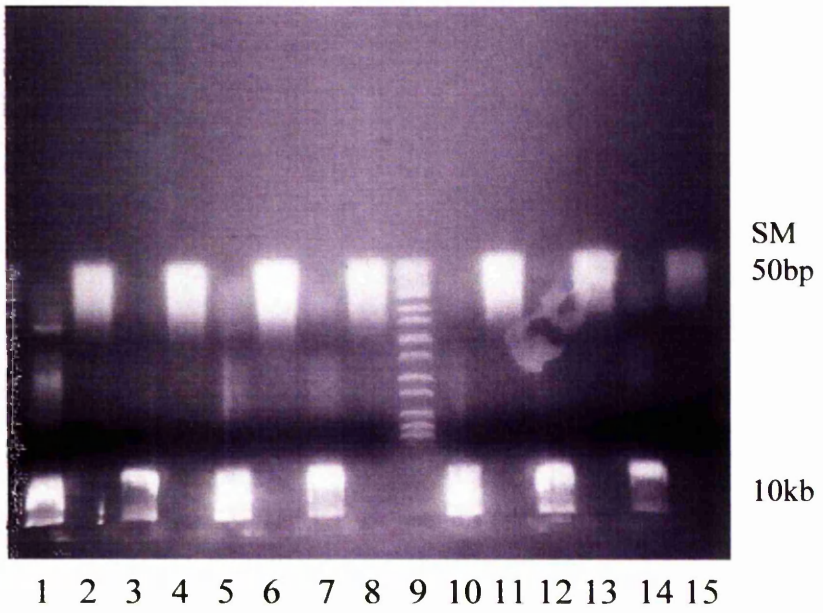


Figure 3.4: Gel showing undigested and digested genomic DNA samples. Lanes 1,3,5,7,10,12 and 14 contain intact genomic DNA while lanes 2,4,6,8,11,13 and 15 contain matched digested samples. Lane 9 contains size marker

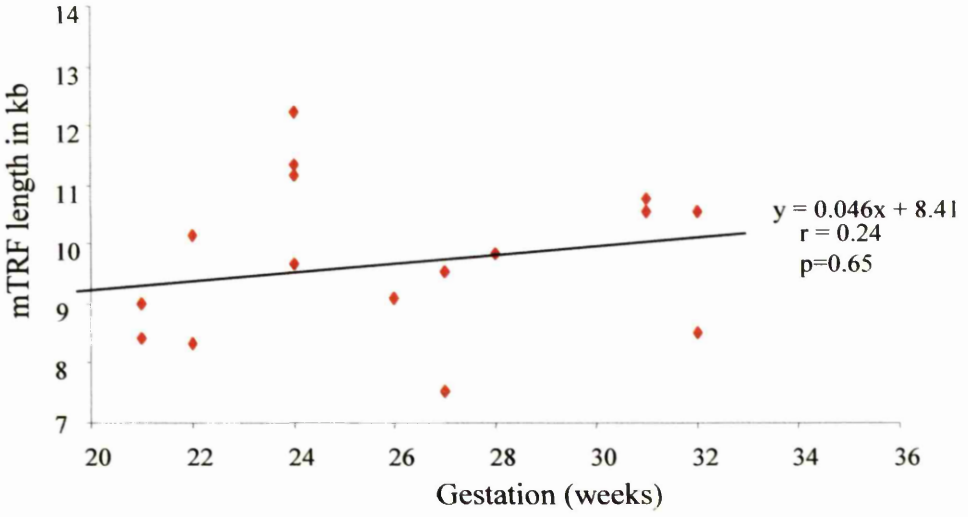


Figure 3.5: Mean TRF length measurement in DNA samples from sixteen TOP fetuses of gestational ages ranging from 21 to 32 weeks.

3.3 Longitudinal analysis of replicative dynamics in fetal blood cells.

As the inter-individual variation between fetuses was very wide, even at the same age and thus may mask any changes in mTRF occuring with gestation in this group of fetuses, I decided to determine mTRF changes in fetuses followed longitudinally. To achieve that I used peripheral blood samples from a total of ten alloimmunised fetuses (AF) undergoing serial blood sampling in utero. Eight fetuses were affected by rhesus isoimmunisation (RhD), AF1, 3, 4, 5, 7, 11, 12, 13. Two fetuses had isoimmunisation to platelet antigens (neonatal/fetal alloimmune thrombocytopenia, NAIT, AF2 and AF6). The gestational period sampled in this group of fetuses was 23-36 weeks. The experiments performed on each sample from each fetus are summarised in table 3.5.

RhD and NAIT are caused by a maternal immune reaction to an incompatible antigen on the developing fetus's blood cells. Such fetuses are at risk of developing haemolytic anaemia or thrombocytopenia in utero and therefore may regularly receive red cell or platelet transfusions. Both red cell transfusions and platelet transfusion products were leucodepleted and irradiated before administration to recipient fetuses. Although the presence of nucleated donor cells (leucocytes) in transfusion products was unlikely, two separate red cell (erythroid) transfusion products were tested for presence of contaminating donor leucocytes, which could interfere with the assessment of telomere length analysis of these fetuses by immunostaining with anti-CD45. The number of CD45+ cells was determined by acquiring 50000 events in both of the samples and each was found to contain less than 0.01% CD45+ cells (see figure 3.6).

3.3.1 Progenitor cell frequency of longitudinal samples

To ascertain that haemopoiesis was not affected by the underlying immune disorder and these fetuses could be considered representative of the normal fetal population. I enumerated the number of progenitor cells by CD34+ immunostaining and progenitor cell assays and compared this to the progenitor cell proportions found in the cross sectional, haematologically normal, TOP group over a similar time period.

Allo-immunised fetuses	Sampling points (gestational age in weeks)	Time point of experiments		
		mTRF	Immunostaining	Progenitor assays
AF 1	23,25,26,28,31,34	23,26,28,31	25,26,28,31	28,31
AF 2	26,27,28,29,30	26,27,30	26,27,28,29,30	26,28,29,30
AF 3	28,31,34,36	28,34,36	28.31.34.36	28,31,34,36
AF 4	23,24,26,28,31	23,24,26,31	23,24,26,28,31	23,24,26,31
AF 5	29,32,34,35	32,35	ND	29,34,35
AF6	26,30,34	26,30,34	ND	26
AF7	28,30,32,33,35,	32,35	30,33	28,30,32,32,35
AF11	25,28,30,33,35	25,28,30,33,35	25,33	25,28,30,33,35
AF12	32,34,36	ND	32,34	32,34,36
AF13	30,33,35	33,35	35	30,33,35

Table 3.5: Sampling points and experiments performed on each blood sample from fetal longitudinal group. ND indicates experiment not done.

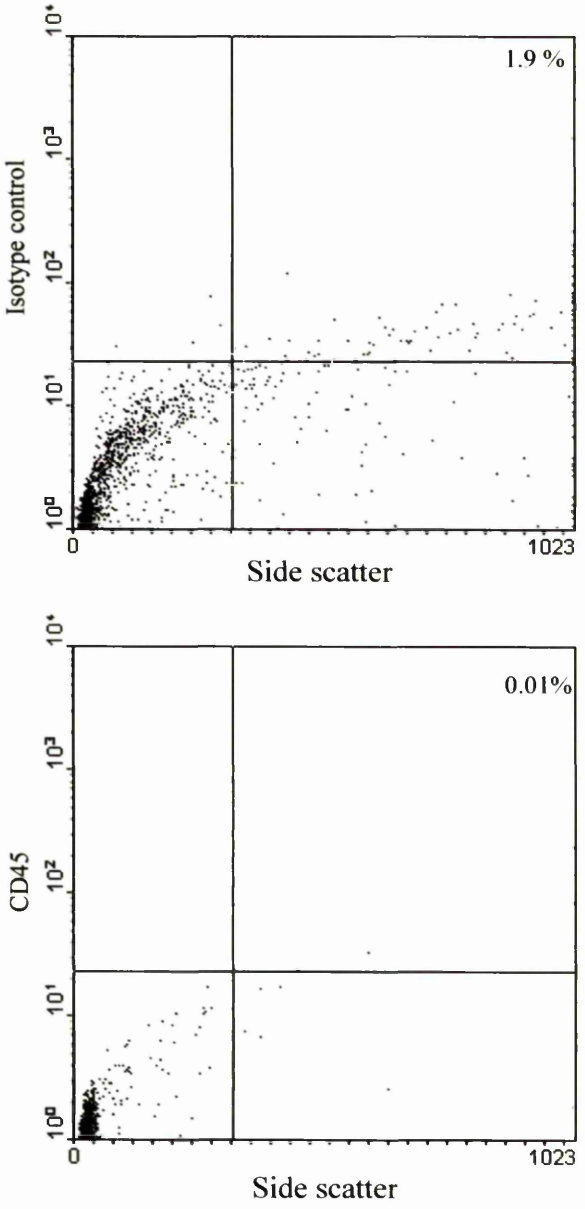


Figure 3.6: Analysis of transfusion products for CD45+ cells.
The top panel shows events obtained for isotype control and bottom panel shows events obtained with anti-CD45 antibody staining of packed red cell product.

A minimum of 30000 total events were acquired to measure CD34 frequency and calculated as described in section 2.2. The median proportion of CD34+ cells in the longitudinal group was 0.33% (0.04-1.23%, n=25). Spearman's rank correlation coefficient was used to detect any changes in CD34+ cells due to increasing age. Proportions of CD34+ cells showed no statistically significant correlation with increasing gestational age (r =0.04, p=0.84) in the longitudinal group (figure 3.7).

The proportion of CD34+ cells in peripheral blood of TOP group between 23 and 35 weeks was 0.75% (range 0.05-1.7%). As the data from the two groups was not normally distributed, the Mann Whitney U test was used to detect any significant difference in proportion of CD34+ cells between the two groups in the time period of 23-36 weeks and no statistically significant difference was found (p=0.84, see table 3.6).

To further compare the number of progenitors in alloimmunised and TOP fetuses, samples were assayed for number of clonogenic progenitors. To establish the optimum concentration of cells to use in the progenitor cell assays, five samples were used to perform a titration analysis. The samples were plated in triplicate at concentrations of $1x10^4$, $2x10^4$, $2.5x10^4$, $3x10^4$, $4x10^4$ and $5x10^4$ mononuclear cells per assay and after 14 days the numbers of BFU-E, CFU-GM and CFU-GEMM colonies were counted. The average colony number at each concentration was plotted and is shown in figure 3.8. As can be seen in figure 3.8, there is increase in colony number with increasing cell numbers up to a plating concentration of $3x10^4$/millilitre. I then chose to plate all samples at concentration of $2.5x10^4$ cells per millilitre. At this concentration colony growth did not reach a plateau and colonies are relatively easy to score as they remain distinct in the plate.

Sequential samples were collected and assayed from ten of the AF group between 23 and 36 weeks and compared to assay results from seventeen TOP fetuses from a similar gestational period (23-35 weeks) using the one way analysis of variance (ANOVA) test, as the data was normally distributed. As expected, great variation was found between samples.

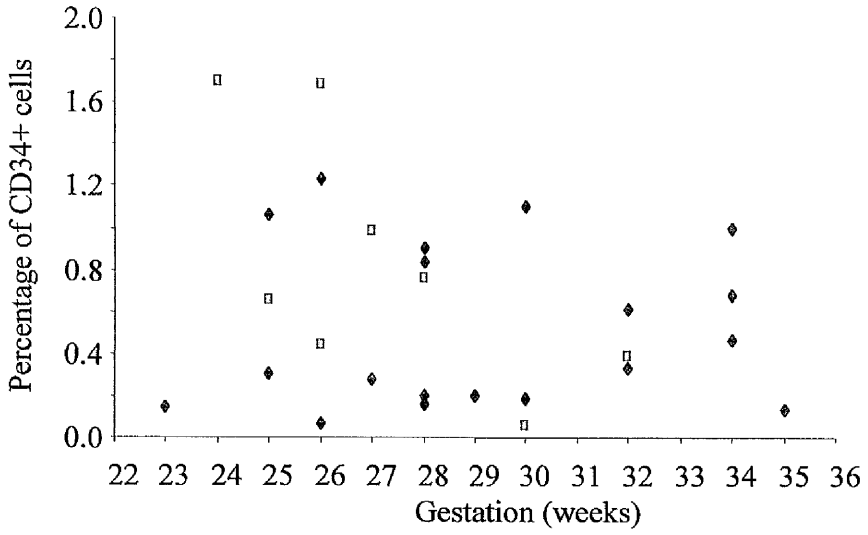
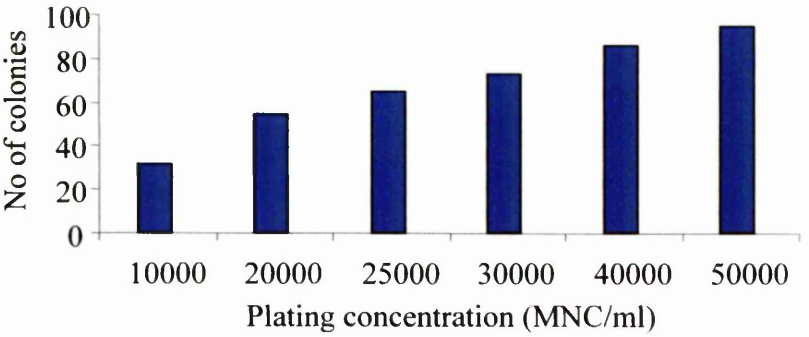
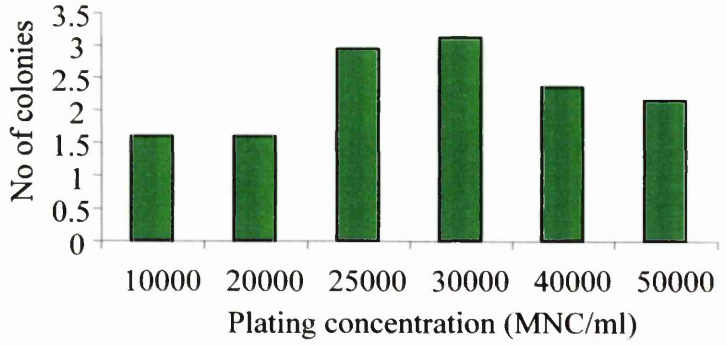


Figure 3.7: Percentage of CD34+ cells in peripheral blood from TOP(▫)and alloimmunised (♦) fetuses between 22 and 35 weeks gestation.)

Cell type	AF median (range)	Effect of gestation in AF group	TOP median (range)	AF vs TOP
CD34+	0.33% (0.04-1.23) n=25	0.84	0.75% (0.05-1.7) n=8	0.84

Table 3.6 Assessment of CD34 cell frequency in AF group and comparison of AF and TOP groups.





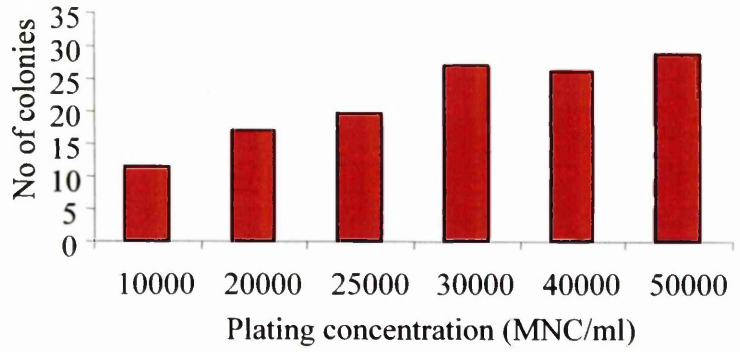


Figure 3.8: Titration of progenitor cell assay. The number and type of clonogenic cells at each plating concentration of mononuclear cells is shown

■ BFU-E ■ CFU-GM ■ CFU-GEMM

The number of BFU-E, CFU-GM and CFU-GEMM in alloimmunised fetuses showed similar ranges as the TOP group as shown in figures 3.9, 3.10, 3.11. The median (and range) of each progenitor type is shown in table 3.7. As data were normally distributed, statistical analysis by ANOVA was performed and showed there was no significant difference in the number of BFU-E, CFU-GM and CFU-GEMM between AF and TOP groups (Table 3.7, column 5). In addition there were no significant changes in BFU-E, CFU-GM and CFU-GEMM with increasing gestational age (Table 3.7, column 3).

3.3.2 Phenotypic characterisation of longitudinal samples

Samples from each fetus were monitored throughout gestation to detect any changes in cell types due to increasing age of the fetus, which may affect changes in telomere length. The data was analysed for significant changes with gestation using Spearman's rank correlation coefficient. In addition the proportion of each cell type determined by immunophenotyping have been compared to those previously found in the haematologically normal TOP group using Mann Whitney U test to detect any statistically significant differences between groups.

At least 17000-20000 events were collected in total for each cell type and FACS analysis performed in a similar way to samples from TOP fetuses in section 3.2.1. The majority of samples from alloimmunised fetuses showed proportions of lymphoid markers in a similar range to TOP group as shown in Figure 3.12 and 3.13. The dashed lines represent the range seen in the TOP group for each cell type. None of the lymphoid markers showed statistically significant age related trend in any of the fetuses (table 3.8). There was no statistically significant difference in the number of CD8 or CD19/20 cells between the TOP and alloimmunised groups. There was a statistically significant decrease in the number of CD4 cells in the TOP group.

As already described for TOP group, CD33 and CD15 dual staining was performed to identify CD33+/15- (myeloblasts), CD33+/15+ (promyelocytes, myelocytes) and CD33-/15+ (neutrophils) and CD14 was used as a representative monocyte cell markers

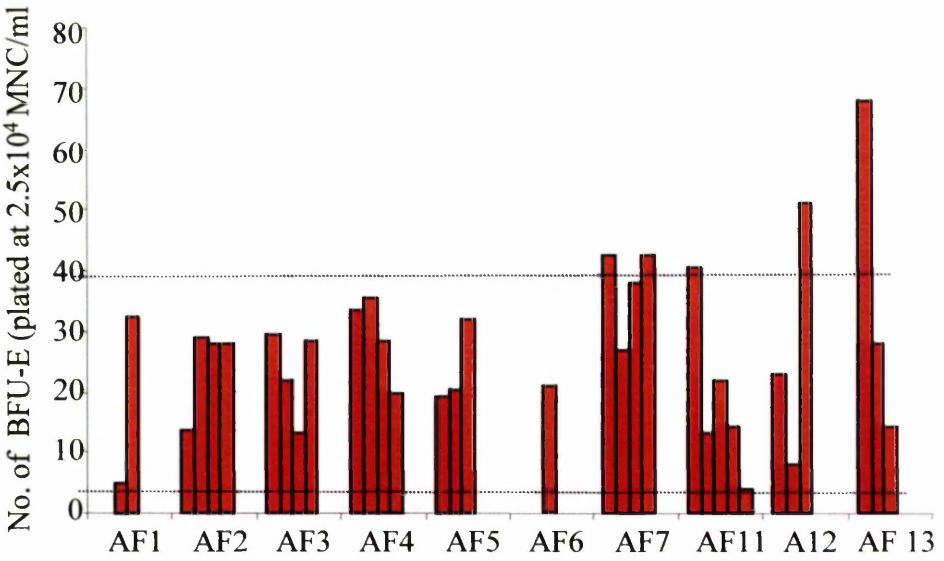


Figure 3.9: Frequency of BFU-E with increasing gestation in AF group. The range of BFU-E frequency in TOP group is indicated by dotted lines. In all cases, assays were plated at cell concentration of $2.5x10^4$ mononuclear cells per millilitre of methocult medium and scored on day fourteen.
AF1 was assayed at 28 and 31 weeks,
AF2 at 26,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24,26 and 31 weeks,
AF5 at 29, 34 and 35 weeks,
AF6 at 26 weeks,
AF7 at 28, 30, 32 and 35 weeks,
AF11 at 25, 28, 30, 33 and 35 weeks,
AF12 at 32, 34 and 36 weeks and
AF13 at 30, 33 and 35 weeks.

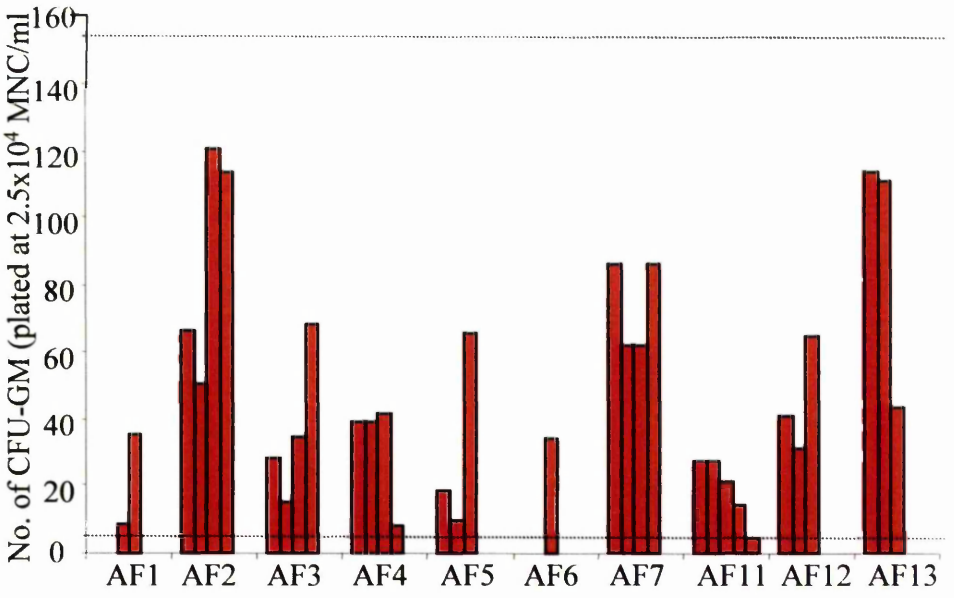


Figure 3.10: Frequency of CFU-GM with increasing gestation AF group. The range of CFU-GM frequency in TOP group is indicated by dotted lines. In all cases, assays were plated at cell concentration of $2.5x10^4$ mononuclear cells per millilitre of methocult medium and scored on day fourteen.
AF1 was assayed at 28 and 31 weeks,
AF2 at 26,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24,26 and 31 weeks,
AF5 at 29, 34 and 35 weeks,
AF6 at 26 weeks,
AF7 at 28, 30, 32 and 35 weeks,
AF11 at 25, 28, 30, 33 and 35 weeks,
AF12 at 32, 34 and 36 weeks and
AF13 at 30, 33 and 35 weeks.

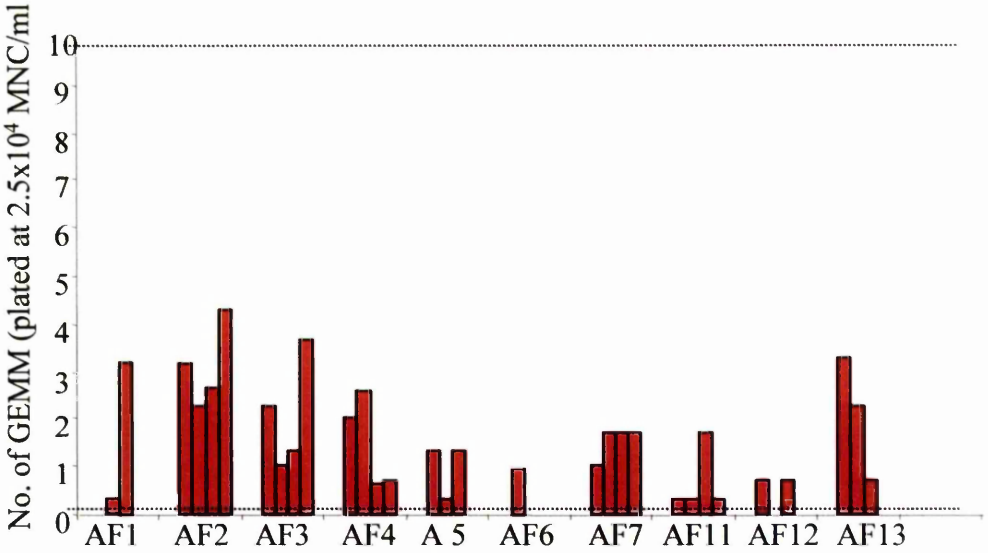


Figure 3.11: Frequency of CFU-GEMM with increasing gestation in AF group. The range of CFU-GEMM frequency in TOP group is indicated by dotted lines. In all cases, assays were plated at cell concentration of $2.5x10^4$ mononuclear cells per millilitre of methocult medium and scored on day fourteen.
AF1 was assayed at 28 and 31 weeks,
AF2 at 26,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24,26 and 31 weeks,
AF5 at 29, 34 and 35 weeks,
AF6 at 26 weeks,
AF7 at 28, 30, 32 and 35 weeks,
AF11 at 25, 28, 30, 33 and 35 weeks,
AF12 at 32, 34 and 36 weeks and
AF13 at 30, 33 and 35 weeks.

Progenitor	AF median (range)	Effect of gestation in AF group	TOP median (range)	AF vs TOP
BFU-E	27.7 (2-67) n=35	0.12	20.8 (3-38) n=14	0.19
CFU-GM	38.7 (3-120) n=35	0.31	39.8 (5-154) n=14	0.89
CFU-GEMM	1.3 (0-4) n=35	0.66	2.3 (0-10) n=14	0.23

Table 3.7: Assessment of differences in numbers of clonogenic progenitors in AF and TOP fetuses. In all cases, assays were plated at cell concentration of 2.5x104 mononuclear cells per millilitre of methocult medium. Data analysed using ANOVA.

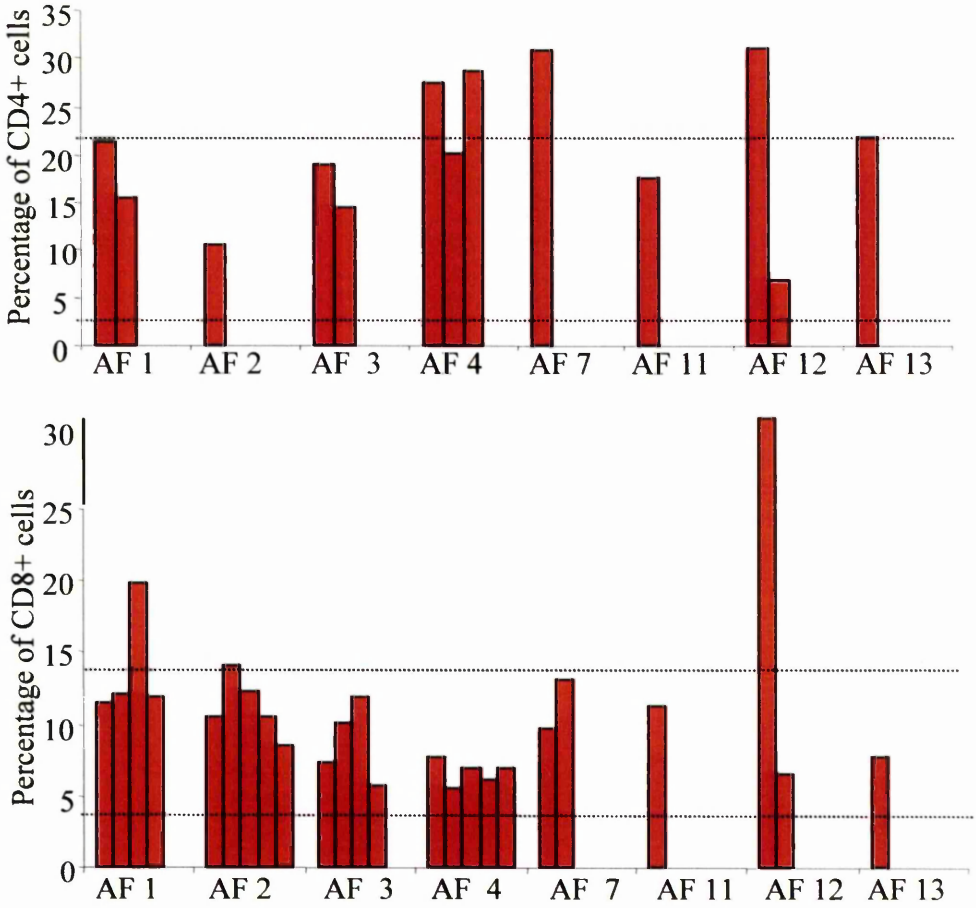


Figure 3.12: Percentage of CD4 and CD8 lymphoid cells in whole blood in AF group. Dotted lines indicate range of values observed in TOP group.

For CD4,
AF1 was assayed at 28 and 31 weeks,
AF2 at 30 weeks,
AF3 at 34 and 36 weeks,
AF4 at 23,26 and 31 weeks,
AF7 at 30 and 35 weeks,
AF11 at 25 weeks,
AF12 at 32 and 34 weeks and
AF13 at 33 weeks.

CD8 was assayed in
AF1 at 25,26 28 and 31 weeks,
AF2 at 26,27,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24,26, 28 and 31 weeks,
AF7 at 30 and 33 weeks,
AF11 at 25 weeks,
AF12 at 32 and 34 weeks and
AF13 at 33 weeks.

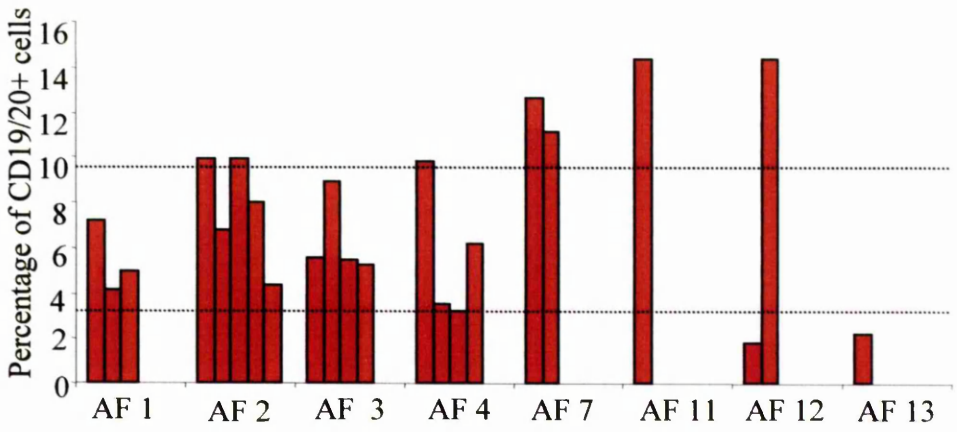


Figure 3.13: Percentage of CD19 + B cells in whole blood in AF group. Dotted lines indicate range of values observed in TOP group.

CD19/20 was assayed in
AF1 at 25, 28 and 31 weeks,
AF2 at 26,27,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24, 28 and 31 weeks,
AF7 at 30 and 33 weeks,
AF11 at 25 weeks,
AF12 at 32 and 34 weeks and
AF13 at 33 weeks.

Cell type	AF median (range)	Effect of gestation	TOP median (range)	AF vs TOP
CD4	20.2% (7.9-34.8%) n=15	0.46	13.3% (2.7-21.2%) n=6	0.023
CD8	10.6% (5.6-31.4%) n=29	0.96	7.9% (4.6-13.9%) n=7	0.16
CD19/20	4.8 (2.4-12.7%) n=29	0.12	4.9% (3.5-9.2%) n=7	0.78

Table 3.8: Assessment of lymphoid cell frequency in fetal blood cells and comparison of lymphoid cell proportions in whole blood in AF and TOP groups. Effect of gestation in AF group was assessed by Spearman's rank correlation coefficient and groups were compared by Mann Whitney U test.

At least 16000-20000 total events were collected for each cell type and analysed as described in section 3.2.2. Figure 3.14 and 3.15 show the frequency of myeloid markers in the alloimmunised group with increasing gestation. The dashed lines represent the range seen in the TOP group for each cell type. The median and range of each cell type are shown in table 3.9.

There were no statistically significant differences in the proportions of myeloid markers between the TOP and alloimmunised groups (table 3.9). The alloimmunised fetuses had proportions of myeloid markers in same ranges as age matched TOP samples. In addition, no statistically significant trends in CD33+/15+ and CD33+/15- cell types cell frequencies were observed with increasing age (table 3.9). There was a statistically significant increase in the number of CD15+ neutrophils with increasing age ($p=0.03$), however, the correlation was very weak, $r=0.4$.

CD45 and glycophorinA dual staining were used to distinguish CD45+/glycophorin+ and CD45-/glycophorin+ erythroid cells. As with other markers, a minimum of 16000-20000 events were collected for measurement. The proportions of erythroid cells found in each fetus with increasing gestation is shown in figure 3.16 and table 3.10. No difference in proportions of erythroid cells was found between TOP and alloimmunised groups. There was no significant change in CD45-/glycophorin+ cells with increasing age ($p=0.32$) and there was a statistically significant weak correlation ($p=0.05$, $r=0.4$) between CD45+/glycophorin+ cells with increasing gestational age (table 3.10).

Table 3.11 summarises al the immunophenotyping data and statistical comparison of AF and TOP groups.

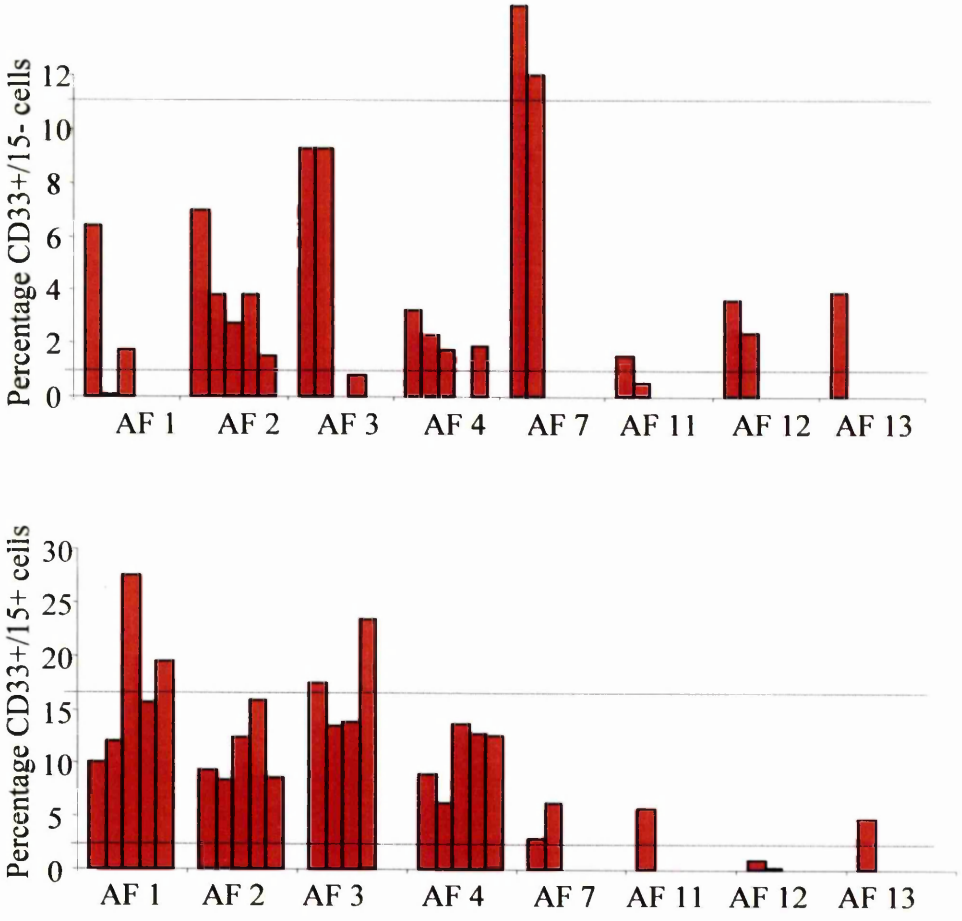


Figure 3.14. Percentage of CD33+15+ and CD33+15- myeloid cells in AF group. Dashed horizontal lines indicate lowest and highest values seen in TOP group. For both CD33+/15- and CD33+/15+,
AF1 was assayed at 25, 26,28,31 and 34 weeks,
AF2 at 26,27,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24, 26,28 and 31 weeks
AF7 at 33 and 35 weeks
AF11 at 25 and 33 weeks
AF12 at 34 and 36 weeks
AF13 at 35 weeks

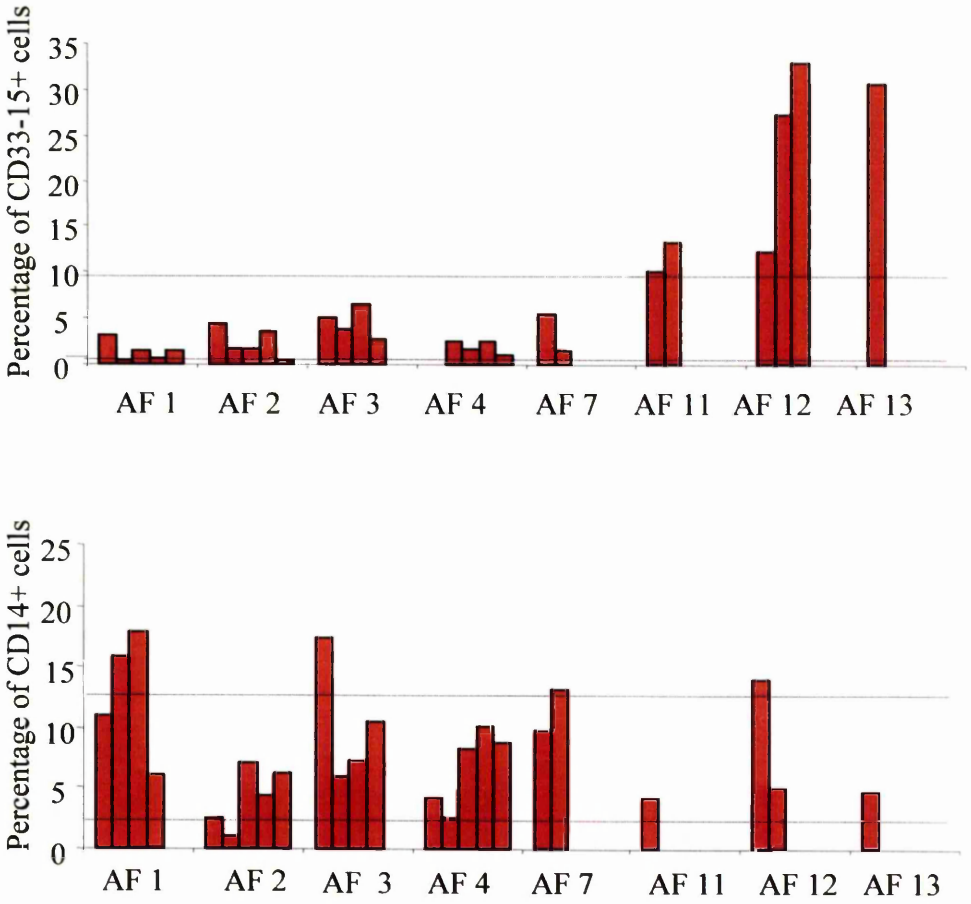


Figure 3.15. Percentage of CD33-15+ and CD14+ myeloid cells in AF group. Dashed lines indicate range of markers in TOP group.

For CD33-/15+,
AF1 was assayed at 25, 26,28,31 and 34 weeks,
AF2 at 26,27,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24, 26,28 and 31 weeks
AF7 at 33 and 35 weeks
AF11 at 25 & 33 weeks
AF12 at 34 and 36 weeks
AF13 at 35 weeks

CD14 was assayed in
AF1 at 23, 25, 28 and 31 weeks,
AF2 at 26,27,28,29 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks,
AF4 at 23,24,26, 28 and 31 weeks,
AF7 at 30 and 33 weeks,
AF11 at 25 weeks,
AF12 at 32 and 34 weeks and
AF13 at 33 weeks.

Cell type	AF median (range)	Effect of gestation p value	TOP median (range)	AF vs TOP p value
CD33+/ CD15-	2.6% (0-14.6%) n=25	0.91	13.3% (0.9-11.1%) n=6	0.48
CD33+/ CD15+	12% (0.1-27.4%) n=25	0.55	7.9% (2.1-16.6%) n=7	0.16
CD33-/ CD15+	1.5 (0.1-32.9%) n=26	0.03 corr =0.4	4.9% (0.1-8.8%) n=7	0.11
CD14	6.7% (1-17.9%) n=28	0.19	5.4% (2.7-12%) n=8	0.44

Table 3.9: Assessment of myeloid cell frequency in fetal blood cells and comparison of myeloid cell proportions in AF and TOP groups. Effect of gestation in AF group was assessed by Spearman's rank correlation coefficient and groups were compared by Mann Whitney U test.

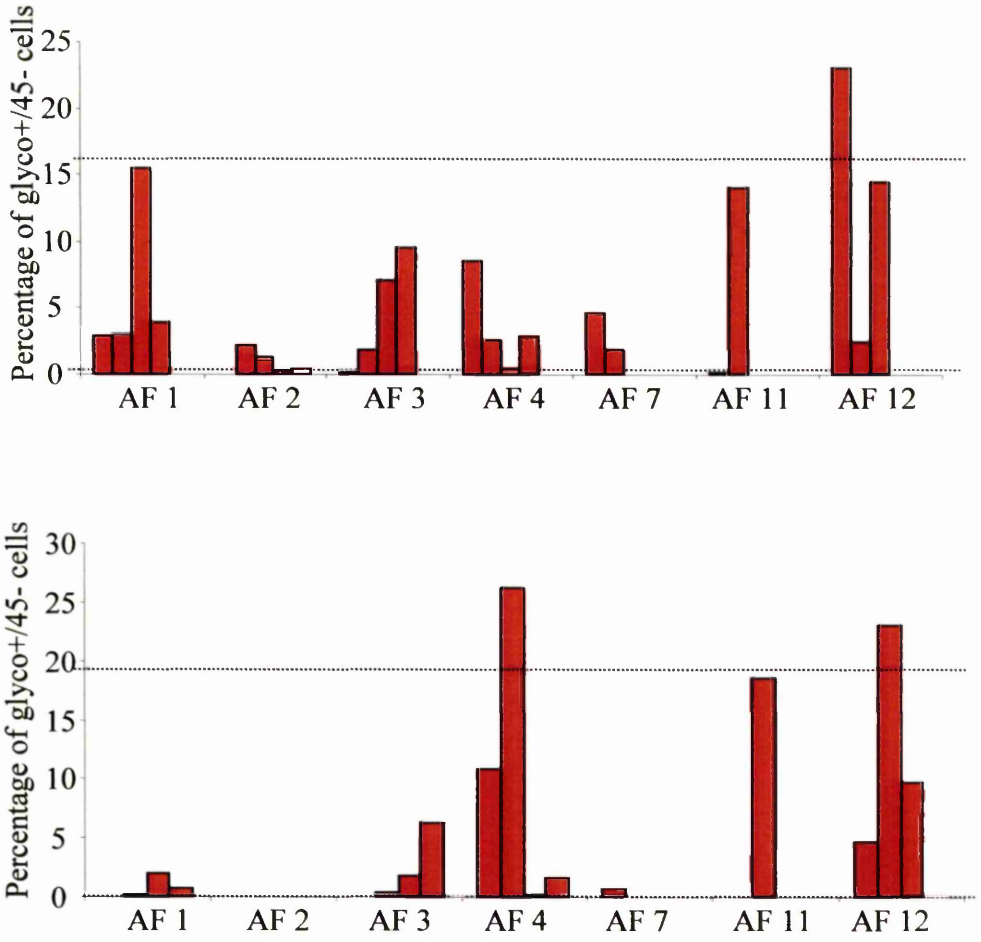


Figure 3.16: Percentage of erythroid cells in peripheral blood from in AF group. Dashed horizontal lines indicate lowest and highest values seen in TOP group. For CD45+/glycophorin+ and CD45-glycophorin+,
AF1 was assayed at 25, 26, 31and 34 weeks,
AF2 at 27,28,28 and 30 weeks,
AF3 at 28, 31, 34 and 36 weeks
AF4 at 23, 24, 26 and 28 weeks
AF7 at 33 and 35 weeks
AF11 at 28 and 33 weeks
AF12 at 32,34,36 weeks

Cell type	AF median (range)	Effect of gestation p value	TOP median (range)	AF vs TOP p value
CD45+/ Glyco+	2.7% (0-23%) n=24	0.05 r=0.4	1.5% (0-16.5%) n=6	0.46
CD45-/ Glyco+	0.8% (0-26.3%) n=21	0.32	0.55% (0-18.9%) n=7	0.84

Table 3.10: Assessment of erythroid cell frequency in fetal blood cells and comparison of erythroid cell proportions in AF and TOP groups. Effect of gestation in AF group was assessed by Spearman's rank correlation coefficient and groups were compared by Mann Whitney U test. (Glyco =glycophorin)

Immuno-phenotype	Cell types	TOP Median	AF Median	AF vs TOP (Mann Whitney U test) (p value)	Effect of gestation (p value)
CD4	T cells	13.3%	20.2%	0.023*	0.46
CD8	T cells	7.9%	10.6%	0.16	0.96
CD19/20	B cells	4.9%	4.8%	0.78	0.12
CD33+/ CD15-	Myeloblasts	2.2%	2.6%	0.48	0.91
CD33+/ CD15+	Promyelocytes myelocytes	8.4%	12.0%	0.16	0.55
CD33/ CD15+	Neutrophils	1.5%	2.8%	0.113	0.03* r = 0.4
CD14	Monocytes	5.4%	6.7%	0.44	0.19
CD45+/ Glyco phorin+	Erythrocyte progenitors	1.35%	2.75%	0.46	0.05* r = 0.4
CD45-/ Glyco phorin+	Pronormoblast Normoblast reticulocyte	0.55%	0.8%	0.84	0.32
CD34+	Stem/ progenitor cells	0.75%	0.33%	0.84	0.84

Table 3.11: Comparison of cell frequencies in TOP and AF groups. Data was analysed by Mann Whitney U Test to look for differences between TOP and alloimmunised groups and by Spearman's rank correlation coefficient to look for differences due to gestational age

3.3.3 Telomere length measurement

To define the variabilty of the southern blot technique in determining mTRF, I assessed the mTRF length of the same sample run in multiple adjacent lanes on two gels. One microgram aliquots of digested DNA from this sample were run in each well across the gel in two separate gels and mTRF length measured to ascertain the difference in telomere length due to position of sample in gel. To mimic the loading of samples from fetuses, in each gel every fifth lane contained labelled size marker. All subsequent gels containing fetal samples were run in a similar way. Figure 3.17 shows mTRF measurement from sets of adjacent lanes in one of these gels. The mean difference in mTRF length between identical samples was 168bp and the SD was 125bp. The maximum variation between identical samples found was 780bp and changes greater than this value were considered to be significant.

To define the variability between gels, aliquots of one microgram of DNA extracted from cell lines Jurkat and K562 were run in identical adjacent positions in each gel. Table 3.12 shows the value of mTRF for Jurkat and K562 samples in twelve gels performed. Gels were only considered for further analysis of mTRF in unknown samples if the values obtained from Jurkat and K562 were consistent within each gel.

DNA from peripheral blood of each of eight fetuses in AF group was extracted as described in section 2.4 & 2.5.1 and run on an agarose gel following digestion of non-telomeric DNA. To optimise accuracy of results all samples from the same fetus were run on adjacent lanes on the same gel. Each sample was run on two separate gels to confirm results. To compare the changes occurring over the entire period of study for each fetus, the first and last samples were compared and results are as shown in table 3.13. No overall significant change was seen in six of the eight fetuses during the gestational period analysed. Only 2 fetuses (AF3 and AF 11) had overall change above the resolution of the gel. AF3 showed an overall increase and AF11 an overall decrease in telomere length. However, significant fluctuations were observed in four fetuses within the period of observation (figure 3.18). In fetuses AF1, AF4, AF3 and AF11 there were changes in telomere length exceeding the limits of gel resolution. For example, in AF4 the telomere length changes from 9.48kb at 25 weeks to 11.47kb at 28 weeks, a difference of 1990bp in 3 weeks. In the next two

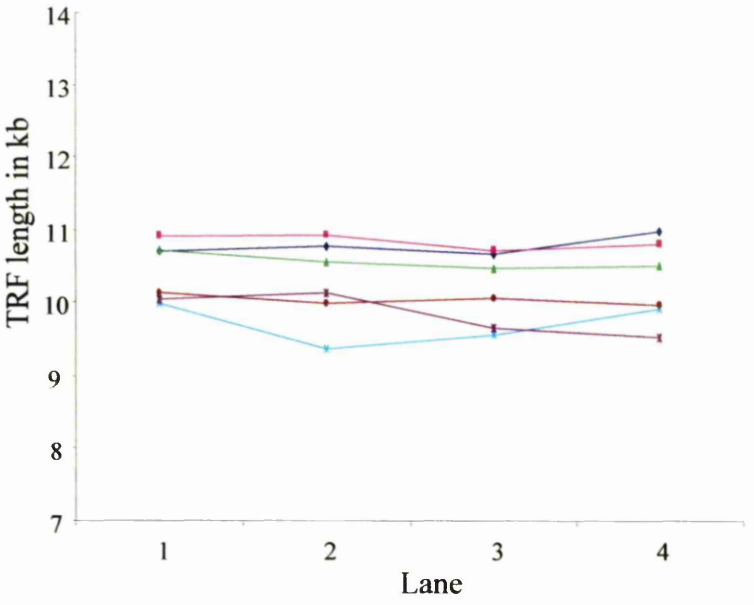


Figure 3.17: mTRF length of same sample run in adjacent lanes in gel. The graph shows the mTRF lengths measured in sets of four adjacent lanes across the gel. Size marker occupied lanes adjacent to each set.

Gel	mTRF Jurkat (Kb)	mTRF K562 (Kb)	Gel suitable for analysis
280704	8.62	6.94	Yes
210704	8.2	6.91	Yes
240604	9.11	7.88	No
220604	8.6	7.50	Yes
230504	8.47	6.82	Yes
300404	6.38	-	No
120903	8.41	8.1	No
Telden 9.	8.17	7.1	Yes
Telden 8	8.18	7.35	Yes
Telden 6	8.18	7.42	Yes
Telden 3c	8.48	7.21	Yes
Telden 1	8.28	7.39	Yes
Mean±SD	8.25±0.64	7.33±0.4	

Table 3.12 Values of mTRF from Jurkat and K562 cells lines used in each of 12 gels to show inter-gel variability of Southern Blotting technique when measuring telomere length.

The mean and standard deviation of values is shown on the bottom row. The fourth column indicates whether the gel was considered suitable for analysis of mTRF in unknown samples.

Fetus	Gestational age range	Weeks between 1st and last sample	Differences between 1st and last samples (bp)
AF 1	23-31	8	+540
AF 2	26-30	4	+280
AF 3	28-36	8	+1390*
AF 4	23-31	8	-110
AF 5	32-35	3	+610
AF 6	26-34	8	+580
AF 7	32-35	3	-100
AF 11	25-35	10	-1200*

Table 3.13: Overall difference in mTRF length between first and last time points sampled in eight fetuses followed longitudinally.

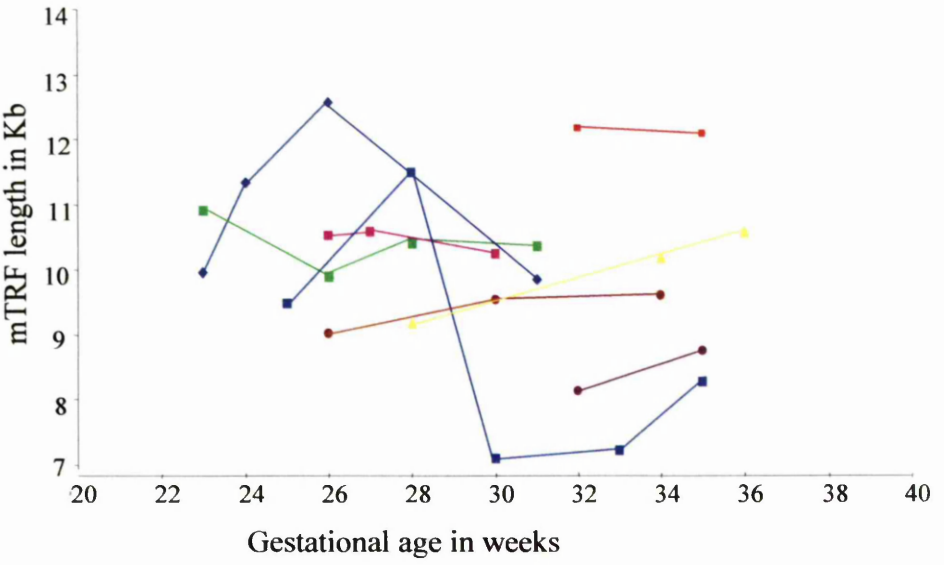


Figure 3.18 : Mean TRF length of DNA samples from 8 fetuses in AF group.

- AF 1
- AF 2
- AF 3
- AF 4
- AF 5
- AF 6
- AF 7
- AF 11

weeks, this fetus shows a decline in telomere length of 4380bp then a further increase of 1060bp between 33-35 weeks. AF11 initially shows an increase of 1570bp between 23 and 24 weeks and a further increase in telomere length of 1170bp between 24 and 26 weeks. However, in the period between 26-31 weeks this fetus exhibits a decline in telomere length of 2650bp.

3.4 Discussion

The aim of this chapter was to establish HSC telomere length changes in fetal life. To achieve this I have used mTRF measurement in peripheral blood from fetuses from 22-32 weeks gestation undergoing feticide prior to termination of pregnancy and fetuses of 23-36 weeks undergoing sequential peripheral blood sampling in utero. Fetal life is a time of enormous growth and development in all body systems, exceeding even that in early childhood and thus one would anticipate vast changes occurring in the haemopoietic system.

Firstly I have analysed the telomere length changes in the peripheral blood cells of a cross sectional groups of fetuses undergoing TOP. There is a good correlation between mTRF in mature blood cells and their more primitive counterparts, progenitors and stem cells as shown by various investigators (Robertson, *et al* 2000, Sakoff, *et al* 2002). Robertson et al also found that T cells had consistently longer telomeres than neutrophils, the explanation of which is thought to be that lymphoid precursors have fewer divisions to become mature cells whereas myeloid precursors require more divisions between progenitor and mature cell level and thus lose more telomere. However, the rate of loss of telomere was similar in both cell types and in CD34 cells, a finding also reported by Rufer et al. (Rufer, *et al* 1999) suggesting that the rate of change reflects turnover of stem cells. Thus there is convincing evidence that the telomere length in mature blood cells can be considered representative of changes occurring in HSC compartment as long as the blood cell type composition remains constant.

To take this factor into account in this study, I evaluated mature cell markers on cells and their distribution throughout gestation from TOP fetuses of 22-32 weeks gestation. No consistent changes were seen in the myeloid and erythroid compartments and in the T cell fraction of the lymphoid compartment with advancing gestation in the TOP group. Statistical analysis detected a significant decrease in B cells with increasing gestation, though this may be biased by the high value seen in the 23 week fetus, as all other values were between 3.5 and 6.4% of total cell count.

This cross sectional study looked at only a small number of fetal samples and in order to maximise the number of samples analysed, fetuses were included who may have had haematological abnormalities and thus the results here should be interpreted with caution as some of the fetuses may not be normal. In addition, the lack of statistical significance in cell markers with age in this small and variable group may reflect an inadequate sample size. It is also important to mention the limitations of the immunophenotyping panel used to detect different cells types. The panel used is by no means exhaustive and due to the changing levels of expression of different antigens on different cells with cell development, it may not give a completely true picture of cell types in fetal blood. One way to overcome this would have been to perform automated full blood counts in conjunction with morphological analysis of fetal blood cells and immunophenotyping.

Analysis of telomere lengths in this cross-sectional group showed no significant trend and certainly no decrease in telomere length as gestation advances as one might have expected on the basis of previous studies in neonates, children and adults (Frenck, *et al* 1998, Friedrich, *et al* 2001, Robertson, *et al* 2000, Rufer, *et al* 1999, Zeichner, *et al* 1999). The interpretation of the results is again limited as there are some fluctuations in cell types between the different fetuses, however, my findings are in agreement with findings from a study from Schonland et al (Schonland, *et al* 2003) which documented no change in telomere length in CD4 T cells and neutrophils from cord blood samples from healthy infants born between 32 weeks and term and suggests that despite the limitations of the immunophenotyping in determining changes in cells types, there is no demonstrable difference in telomere length in haemopoietic cells in the last trimester in cross sectional studies.

However, it is possible that the inter-individual variation between the fetuses, as seen in B cell numbers and telomere length may biase the analysis of this cross sectional group. A large variability of up to 1.2 Kb between fetuses at the same gestation has been observed in this study. Indeed, it has been shown by others that there is considerable variation in telomere length between individuals at any given age (Friedrich, *et al* 2001, Vaziri, *et al* 1993).

To overcome the problems of variation between individuals I then went on to examine telomere length changes in longitudinal samples obtained from fetuses undergoing blood sampling in utero at regular intervals. Rhesus isoimmunisation and alloimmune thrombocytopenia are both disorders where there is a maternal immune reaction to an incompatible antigen on the developing fetus's blood cells (Kaplan 2001, Kaplan 2002, Urbaniak and Greiss 2000). Such fetuses are at risk of developing haemolytic anaemia or thrombocytopenia in utero. The clinical outcome of these pregnancies has been much improved by the development of ultrasound guided fetal blood sampling and intrauterine transfusion into umbilical blood vessels at point of insertion of umbilical vessels into placenta (Berkowitz, *et al* 1986, Grannum, *et al* 1988, Kaplan 2002, Rodeck, *et al* 1984, Schumacher and Moise 1996). This gave me the unique opportunity to access sequential fetal blood samples in the same fetus.

As the fetuses received red cell and platelet transfusion products from adults it was important to establish that there was no contamination with donor leucocytes as this could interfere with the assessment of mTRF length. This was unlikely to happen as all donor products are leucodepleted before use. The pan-leucocyte marker, CD45+, was used and CD45+ cells were found to account for less than 0.01% of total cell count in each donor sample tested suggesting that contaminating donor cell telomere length measurements were unlikely to affect the outcome of the telomere analysis.

Furthermore, as the immune response of the mother adversely affects the cells of the blood system in the fetus, it was also important to establish whether the process of haemopoiesis was significantly affected by the immunological insult to the haemopoietic system of the fetus. This is an important consideration as it highlights a possible limitation of using these samples to represent the normal fetal population. Both the immune disease process and its treatment may affect haemopoiesis in the fetus. Work done by a group in the Netherlands looking at proportions of different leucocyte subsets in transfused and non-transfused fetuses with NAIT concluded that transfused fetuses were no different to controls suggesting that platelet transfusions do not alter leucocyte subsets (Radder, *et al* 2004). A study comparing haemoglobin levels, reticulocyte counts and serum erythropoietin levels in transfused and non-

transfused fetuses with Rhesus alloimunisation found no significant differences between the two groups which the authors conclude suggests that there is no or only partial suppression of endogenous erythropoiesis in transfused fetuses (Dallacasa, *et al* 1996). In this work, comparison of the number of progenitor cells and mature blood cells in alloimmunised fetuses and fetuses undergoing TOP for reasons other than rhesus disease or NAIT showed no significant differences in progenitor cell numbers in colony assays. This is in accordance with studies quoted above and also with Vaughan et al (Vaughan, *et al* 1998), who demonstrated that Rhesus anti D antibody did not inhibit or increase *in vitro* colony production from fetal blood cells. The progenitor cell frequency as measured by CD34+ immunostaining showed no overall difference between the TOP and alloimmunised groups. No difference in the CD34 cell numbers with age was seen in the AF group. This is in contrast to the findings of others who have reported a decrease in CD34+ cells with age in cross sectional fetal blood samples from 13 and 17 weeks to term (Gasparoni, *et al* 2000, Shields and Andrews 1998). However, it is important to note that other studies have looked at CD34 numbers over a longer time period than this study and secondly, the previous studies have all been cross sectional, which introduces inter-individual variability. It is also important again to highlight that the the groups in this work were small and showed much variability which may affect the statistical test and thus a lack of statistical significance may not represent equivalence of the groups but rather an inadequate sample.

When considering changes with increasing gestational age, only CD45-Glycophorin+ erythroid cells and CD15+ neutrophils showed any change with age and both showed and increase with advancing gestational age. None of the other myeloid or erythroid, nor any of the lymphoid markers showed any significant change with gestational age

Although CD45-Glycophorin+ showed an increase in frequency with increasing gestation, the correlation was weak ($r=0.4$) an increase in red cell precursors with age was not seen in the BFU-E assays. As mentioned previously, immunophenotyping alone may not have been the optimum way to determine red cell types and thus this result should be considered with caution. Previously published data suggests that red cell progenitor numbers peak in second trimester and then may decrease in third

trimester though a statistical analysis comparing the two time periods was not performed (Campagnoli, *et al* 2000). In addition, work by Forestier et al (Forestier, *et al* 1991) showed that although BFU-E numbers tended to decrease from 18-30 weeks, there was great variability in numbers and no corresponding decrease in the number of normoblasts or mature red cells. As immunophenotyping was performed to determine if changes in proportions of cell types affected telomere length, it was important to look at whether changes seen with immunophenotyping correlated with mTRF changes. In this project, changes in CD45-/glycophorin+ cells did not correlate with changes in telomere length. For example AF4 showed a large increase in CD45-/glycophorin+ cells between 23 and 24 weeks followed by a decrease at 26 weeks. In the same time period the telomere length did not show parallel changes and steadily increased from 23 to 26 weeks. Likewise, AF1 showed an increase in red cell progenitors between 28 and 34 weeks while telomere length in time period of 28 -31 weeks remained stable.

The increase in CD15+ neutrophils with age is in accordance with previously published data (Forestier, *et al* 1991) where neutrophils were shown to increase from 6% to 30% of total nucleated cells from 18-30 weeks and my results also show similar percentages of neutrophils by immunophenotyping (CD15+) in most AF fetuses compared to the differential counts quoted by Forestier on normal fetuses. A couple of the fetuses (AF12 & AF13) show percentages of neutrophils above the normal range established by Forestier – this again may reflect problems with using these individuals as representative of the normal fetal population or it may reflect limitation of the immunophenotyping in discriminating between cell types as CD15 will also be expressd on eosinophils and monocytes as well as neutrophils. The correlation observed of CD15+ cells with age was weak ($r = 0.4$) and thus the increase in CD15+ cells is unlikely to bias the telomere length analysis.

In the lymphoid lineage there was no significant increase in the numbers of lymphocytes seen in the alloimmunised group with increasing age and likewise Forestier observed that lymphocyte numbers remained constant between 18 weeks and term (Forestier, *et al* 1991).

In considering differences in cell type proportions between the TOP and AF groups, the only statistically significant difference was seen in the number of CD4 T cells which was increased in the AF group. As CD4 is a mature cell marker and no other differences were found in the mature or the progenitor compartments, it is unlikely to represent a difference in the process of haemopoietic development between the AF and TOP groups. I therefore concluded that the longitudinal model could be considered a representative view of normal fetal haemopoiesis and that the telomere length studies performed on fetal peripheral blood will reflect changes in replicative dynamics of HSCs as gestation advances.

The Southern Blotting technique was chosen to measure mTRF length in these samples for a number of reasons. Firstly, it is a robust and reliable technique which remains the gold standard for telomere length assays. Secondly, the southern blotting technique uses DNA from all nucleated cells in sample with no cell wastage due to sorting procedures which is advantageous when sample size is small, as it permits the best use of the available sample. Using this technique, the results obtained within and between gels showed good uniformity and the variability seen (mean +SD) was similar to that described by others (Thornley, *et al* 2002b). I went on to further assess the accuracy of the technique by looking at the effect of sample position across the gel on the mTRF length measurement. This allowed me to determine the maximum difference (780bp) between identical samples across the gel which I then considered to be the maximum variability of the technique. Although not commonly used by others describing this technique to measure mTRF, determining the maximum variability attributable to the technique allowed me to be confident that the changes in mTRF that I saw in my samples were genuine when they were greater than 780bp. As all samples from each fetus were run on the same gel and repeated on second gel, this further improved the reliability of the technique.

No overall change in mTRF above the resolution of the technique was observed in six of the eight fetuses. Of the two fetuses which did show overall change, one fetus showed an overall increase and the other a decrease. One would expect that if telomere length changes are regulated in utero as they are postnatally, then because of the enormous growth of the fetus and more rapid turnover of haemopoietic cells,

there would be large losses in telomere length. One possibility is that the technique used may not be sensitive enough to pick up the changes or the time period (13 weeks) observed may have been too short to draw any conclusion. This seems unlikely as studies carried out in children have demonstrated significant changes in as little as the first two months of life (Zeichner, *et al* 1999). The loss of telomere in fetal life would be expected to exceed that in childhood as the growth rate in fetal life is greater. The average newborn will double their birth weight and blood volume in the first five to six months of life (Behrman RE 2000) whereas in the time period observed the fetus increase more than 3 fold in weight (Moore and Persaud, The Devloping Human, Ch.6) and the fetoplacental blood volume increase 5 times (Leduc, *et al* 1990). Moreover cell turnover is faster. Studies measuring red cell lifespan showed that the lifespan of red cell in term infants to be around 60-70 days and in preterm infants around 35-50 days (Pearson 1967, Wranne 1967).

It is possible that mechanisms such as activation of telomerase, a ribonucleoprotein polymerase that synthesizes telomeric repeats onto the 3' ends of eukaryotic chromosomes and thus may prevent telomeric shortening, can only partially restore telomere length in adult HSC following intense proliferation but are much more effective in fetal HSC. Indeed higher levels of telomerase have been detected in fetal liver HSC compared to adult bone marrow HSC (Yui, *et al* 1998).

However, the surprising findings are the significant fluctuations in telomere length seen in four of the fetuses during gestation. The fluctuations observed are greater than the maximum variability of the southern blotting technique and therefore are significant. These large point to point variations may be due to clonal fluctuation. The contribution of individual HSCs is determined stochastically and depending on the relative contributions of HSCs with longer or shorter replicative histories, telomere length fluctuates in a seemingly random manner. The presence of clonal fluctuation has previously been described in relation to mTRF length changes seen in first 2 years following BMT (Thornley, *et al* 2002b). Immediately following engraftment of a bone marrow transplant there is maximal proliferation of infused HSCs in the recipient. Different HSC clones contributing to haemopoiesis at different times could result in telomere length fluctuation similar to what it was

observed in the fetal samples examined in this study. However, study of gene marking and mapping of retroviral insertions in baboon following BMT showed how haemopoiesis is maintained by several HSC clones contributing to specific lineages continuously for more than two years, questioning the concept of clonal fluctuation (Mazurier, *et al* 2004). Moreover in the study by Thornley et al an overall decrease in mTRF is observed with time but no overall loss is observed in fetal samples. This is suggestive of fetal HSCs having different properties than adult HSCs.

Interestingly most fluctuations occurred before 28 weeks gestation and after this time there was usually either no change or an increase in telomere length. The explanation of these findings at present is not clear but one possibility, although highly speculative at this stage, is that the variation may be the result of the contribution of different HSC pools from different sites. The telomere analysis in this project has been carried out on fetal blood samples in the last trimester of pregnancy. It is possible that in this gestational time period the mature blood cells derive from both bone marrow and fetal liver HSC. The liver is the main organ responsible for haemopoiesis in fetal life but there is also increasing contribution from the bone marrow as gestation advances (Zon 1995). The bone marrow HSCs contribution starts around week 20 of gestation. If bone marrow HSCs are generated *de novo*, then they will have undergone fewer divisions than their liver counterparts and therefore will have longer telomeres. It is therefore possible that most of the mTRF fluctuation seen in the period between 20 and 30 weeks are the result of the liver and bone marrow contributing alternatively to mature cell production in the peripheral blood. After that time, bone marrow contribution is progressively prevailing resulting in an apparent lengthening/stabilisation of telomere length.

This questions the accepted explanation of migration of HSCs from one site to another to account for the change in haemopoietic sites. Recent studies have isolated populations of cells expressing only endothelial markers in known haemopoietic sites (embryonic YS and AGM and fetal liver and bone marrow) and evaluated HSC activity (Oberlin, *et al* 2002). Strikingly, endothelial cells from each site are capable of producing multilineage haemopoiesis when grown on a stromal cell line. This raises the possibility of participation of fixed compartments to haemopoiesis during

development. In many ways it would be advantageous to have separate stem cell pools fulfilling different tasks according to the developmental requirements of the organism. Transferring cells which have already undergone substantial proliferation from one environment to another for reprogramming would seem less advantageous than having a newly formed pool of cells with different capabilities according to needs of organism and greater replicative capacity.

CHAPTER 4

HSC REPLICATIVE DYNAMICS IN PRETERM INFANTS

4. HSC REPLICATIVE DYNAMICS IN PRETERM INFANTS

4.1 Introduction

One possible explanation for the absence of telomere shortening observed in fetal life is that HSC replicative dynamics are differently regulated in fetal life, protecting the individual from the effects of HSC replicative stress at a time when HSC undergo great expansion. The available evidence in postnatal life from time of normal term delivery shows accelerated telomere shortening in early childhood of up to 1000bp/year in the first four years of life in cross-sectional studies (Frenck, *et al* 1998, Rufer, *et al* 1999) and up to 170bp/year in a longitudinal study following a group of infants from the age of one month to three years (Zeichner, *et al* 1999). There is very little data available on telomere length changes in HSC during the second and third trimester gestation, or at the equivalent time in preterm infants. Indeed the only information available is the cross sectional analysis of mTRF in cord blood of preterm babies by Friedrich et al. (Friedrich *et al*, 2001). In this study the outcomes are dubious as a significant decrease in mTRF was seen only between 27 and 32 weeks gestation. This was obtained using a very low number of samples and with a study which was cross sectional in design and thus showed great inter-individual variation. When a higher number of samples were used and the analysis was extended to a period between 26 and 42 weeks gestation, a decreasing trend was observed although this was not significant and presented a very poor correlation.

To ascertain that the lack of telomere shortening seen in fetal HSC and described in chapter 3 is due to an intrinsic property of fetal HSCs and not due to inadequacy of the study design, I decided to compare longitudinal mTRF changes in fetal blood cells to mTRF changes in preterm babies of equivalent gestational age followed longitudinally over a similar equivalent time period of twelve weeks. To compare the data obtained from the analysis of fetal samples, I used the same methodology to exactly characterise telomere length changes in mature peripheral blood cells from preterm infants.

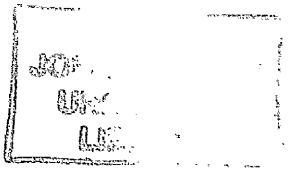
4.2 Phenotypic characteristics of blood cells from preterm infants

A total of thirty blood samples were available from eight preterm infants (PTI) with equivalent gestational ages ranging from 23-35 weeks. The first sample from each infant was taken at the time of delivery or within 24 hours of delivery, and subsequent samples were taken on the neonatal unit at the time of routine blood tests. Samples were only utilised if the infant was clinically well with no clinical or laboratory evidence of infection and no change in clinical condition. For ease of comparison with the AF group, the age of each infant is given as gestational age. The experiments performed on each sample from each infant are summarised in table 4.1. Twenty one blood samples were assayed for a panel of surface markers for myeloid, lymphoid and erythroid cells.

CD4 and CD8 were used as representative T cell markers and CD19/20 dual staining was used to determine B cell frequency. The median proportion of CD4 and CD8 cells was 17.2% and 5.4% and the median B cell proportion was 9.7%. The distribution of each lymphoid cell type in each infant with age is shown in figure 4.1a-c. The median proportion of CD4+ and CD8+ cells are shown in table 4.2.

Percentages of myeloid cells types were determined using dual staining with CD33 and CD15 for CD33+/15- (myeloblasts) CD33+/15+ (promyelocytes, myelocytes) and CD33-/15+ (neutrophils). CD14 was used as a monocyte marker. The distribution of each cell type in each infant at each time point sampled is shown in figure 4.2 and 4.3. The median proportion of each cell type expressed as percentage of total number of cells and range of values found is summarised in table 4.3.

A combination of glycophorin A and CD45 was used to measure proportions of CD45+/glycophorin+ erythroid progenitors at the erythroblast stages, just after CFU-E stage, and CD45-/glycophorin + pronormoblasts, normoblasts and reticulocytes. The median percentage of CD45+/glycophorin+ was 8.2% and median percentage of CD45-/glycophorin+ cells was 29.5%. Figure 4.4 shows the proportion of erythroid cells in each sample at each time point sampled in this group of infants from 23-35 weeks. Table 4.4 summarises the median and range of erythroid cells



Infant	Sampling Points (equivalent gestational age)	Time points of experiments		
		Immuno phenotyping	Progenitor assays	mTRF
PT1	28,29,30,31,32	28,29,31,32	28,29,30, 31,32	28,29,30, 31,32
PT2	23	23	23	ND
PT3	28,29,30,32,33, 34	28,29,30 32	28,30,32, 33,34	28,29,30, 33
PT4	32,33	32,33	32,33	32,33
PT5	29,32,33,35	32,33,35	29,33,35	32,33,35
PT6	32,33,35	35, 33(CD34)	32,35	32,33,35
PT7	33,34	33,	33	33,34
PT8	23,25,26,27,28, 32,35	23,26,27,33, 35	23,25,26,27 28,33,35	23,25,33, 35

Table 4.1: Sampling time points and experiments performed on each blood sample from each infant in PTI group.

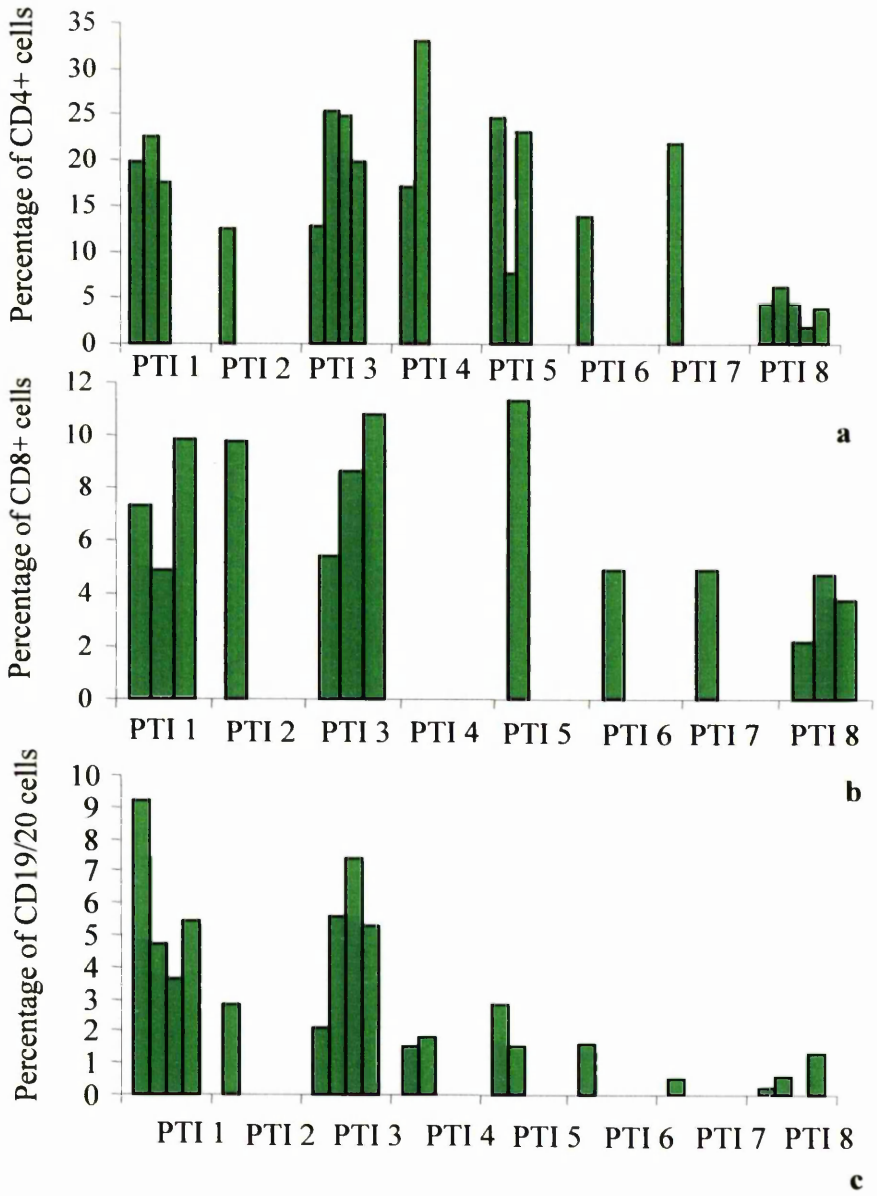
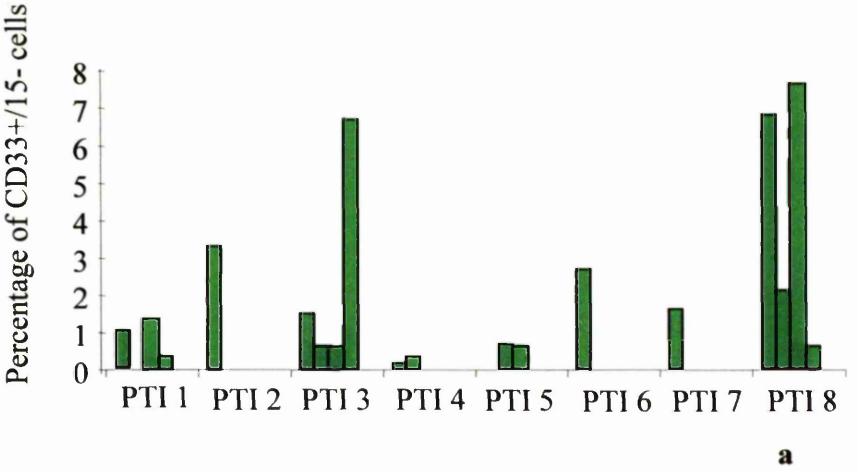


Figure 4.1: Percentage of CD4 T Cells (a) and CD8 T cells (b) and CD19/20 B cells (c) in sequential blood samples from PTI

PTI 1 assayed at 28, 29, 31 & 32 weeks
PTI 2 at 23 weeks
PTI 3 at 28,29,30 and 32 weeks
PTI 4 at 32 and 33 weeks
PTI 5 at 32,33,&35 weeks
PTI 6 at 35 weeks
PTI 7 at 33 weeks
PTI 8 at 23,26,27,33,&35 weeks

Cell type	Median Range
CD4	17.2% (1.7-25.4%)
CD8	5.4% (2.2-12.4%)
CD19/20	2.5% (0-9.2%)

Table 4.2: Median and range for each lymphoid cell type in PTI group.



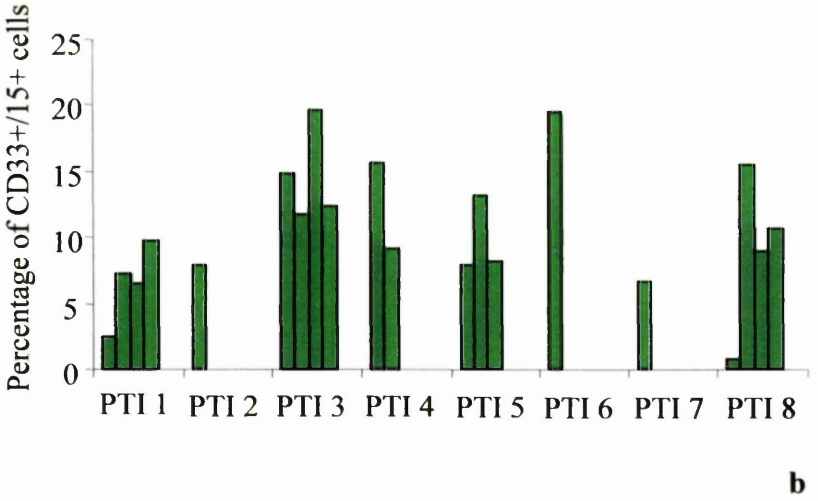
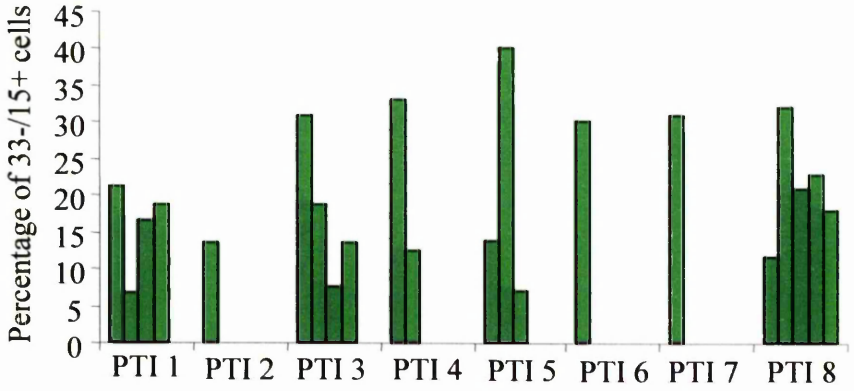
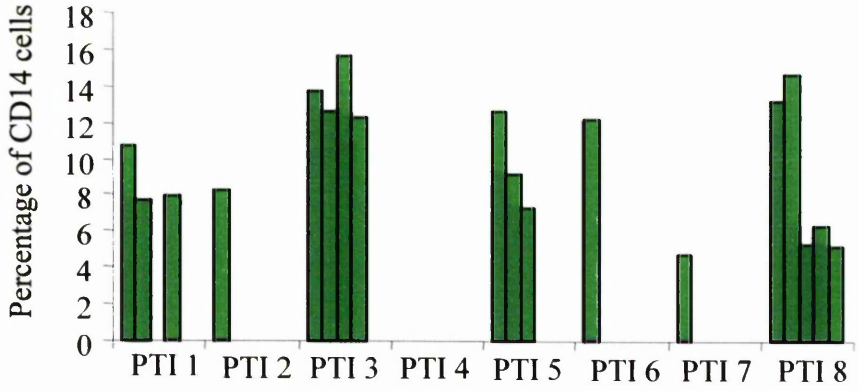


Figure 4.2: Percentage of CD33+/15- cells (a) and CD33+/15+ cells (b) in sequential blood samples from PTI

PTI 1 assayed at 28, 29, 31 & 32 weeks
PTI 2 at 23 weeks
PTI 3 at 28,29,30 and 32 weeks
PTI 4 at 32 and 33 weeks
PTI 5 at 32,33,&35 weeks
PTI 6 at 35 weeks
PTI 7 at 33 weeks
PTI 8 at 23,26,27,33,&35 weeks



a



b

Figure 4.3: Percentage of CD33-/15+ cells (a) and CD14 cells (b) in sequential blood samples from PTI group

PTI1 assayed at 28, 29, 31 & 32 weeks
PTI2 at 23 weeks
PTI3 at 28,29,30 and 32 weeks
PTI4 at 32 and 33 weeks
PTI5 at 32,33,&35 weeks
PTI6 at 35 weeks
PTI 7 at 33 weeks
PTI 8 at 23,26,27,33,&35 weeks

Cell type	Median Range
CD33+/15-	9.1% 0-19.6%) n=21
CD33+/15+	0.7% (0-7.3%) n=21
CD33-/15+	18.6% (6.7-40%) n=21
CD14	10.3% (4.7-15.6%) n=18

Table 4.3: Median and range for each myeloid cell type in PTI group.

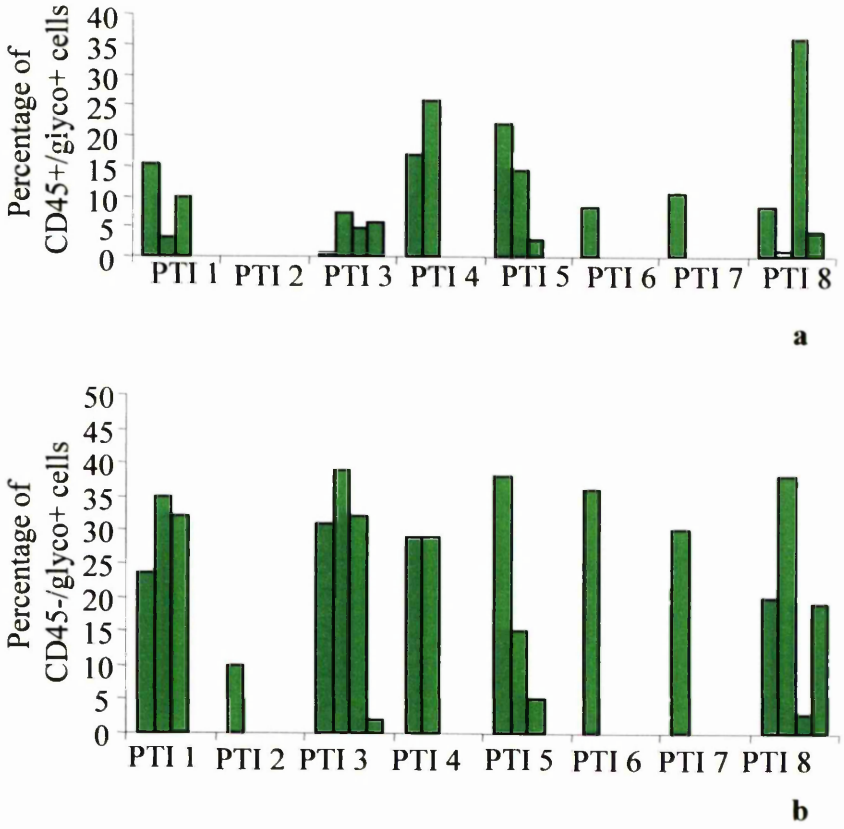


Figure 4.4: Percentage of CD45+/glyco+ cells (a), CD45-/glyco+ cells (b) in sequential blood samples from PTI.

PTI 1 assayed at 28, 29, 31 & 32 weeks
PTI 2 at 23 weeks
PTI 3 at 28,29,30 and 32 weeks
PTI 4 at 32 and 33 weeks
PTI 5 at 32,33,&35 weeks
PTI 6 at 35 weeks
PTI 7 at 33 weeks
PTI 8 at 23,26,27,33,&35 weeks

Cell types	Median Range
CD45+/glyco+	8.2% (0.8-30%)
CD45-/glyco+	29.5% (2-39%)

Table 4.4: Median and range of erythroid cells in PTI
(glyco =glycophorin A)

4.3. Progenitor cell frequency

CD34 was used as a marker of progenitor/stem cells and was measured as described in section 2.2. CD34+ cell frequency in PTI between 23 and 35 weeks equivalent gestational age ranged from 0.05-1.16% and the median value was 0.12%. Figure 4.5 shows the change in CD34+ cells with increasing age in each of the infants in the study. To further quantify progenitor numbers, progenitor cell assays were performed on sequential samples collected from eight PTI between 23 and 35 equivalent gestational age, a time period of 12 weeks. The number of BFU-E, CFU-GM and CFU-GEMM progenitors in PTI with increasing age is shown in figure 4.6. The median and range for each progenitor type is summarised in table 4.5.

4.4 Statistical analysis of cell type proportions

To determine whether there was any difference in cell types between the AF and PTI groups, univariate analysis of variance was used to determine any statistically significant difference in blood cell type composition and in the changes occurring with increasing time in gestation. The latter is important because any change in time between the two groups may bias the telomere analysis.

4.4.1 Comparison of means: PTI and AF

In the lymphoid lineage, there was no difference in CD4 cell numbers between the AF and PTI groups ($p=0.12$), neither was there a significant difference in CD19/20+ cell numbers between groups ($p=0.1$). There were significantly more CD8 cells in the AF group compared to PTI ($p=0.001$). When the overall proportions of myeloid cell types in each group over the time period were compared, the AF group had significantly more CD33+/15+ cells ($p=0.02$) than the PTI group and the PTI group had significantly more CD33+/15- cells ($p=0.01$), CD15+ neutrophils ($p=0.004$) and CD14+ monocytes ($p=0.02$). This is summarised in table 4.6, column 5. No significant difference in numbers of erythroid cells between fetal and infant groups was seen.

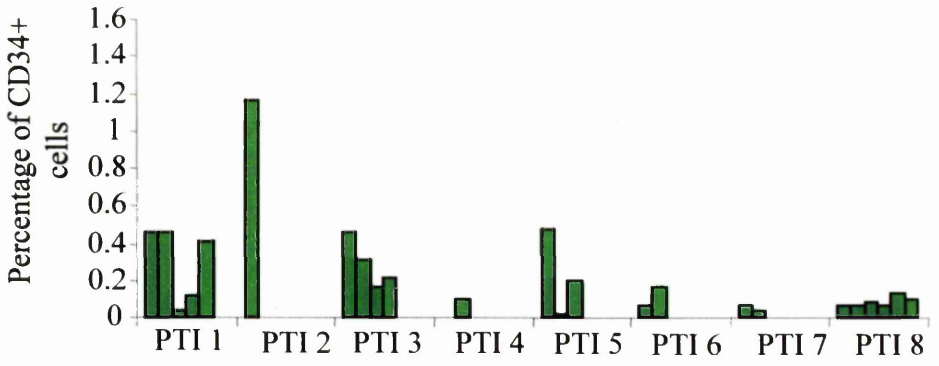


Figure 4.5: Proportion of CD34+ cells in whole blood in PTI group with increasing age.

PTI 1 assayed at 28, 29, 31 & 32 weeks
PTI 2 at 23 weeks
PTI 3 at 28,29,30 and 32 weeks
PTI 4 at 32 weeks
PTI 5 at 32,33,&35 weeks
PTI 6 at 33, 35 weeks
PTI 7 at 33 weeks
PTI 8 at 23,26,27,33,&35 weeks

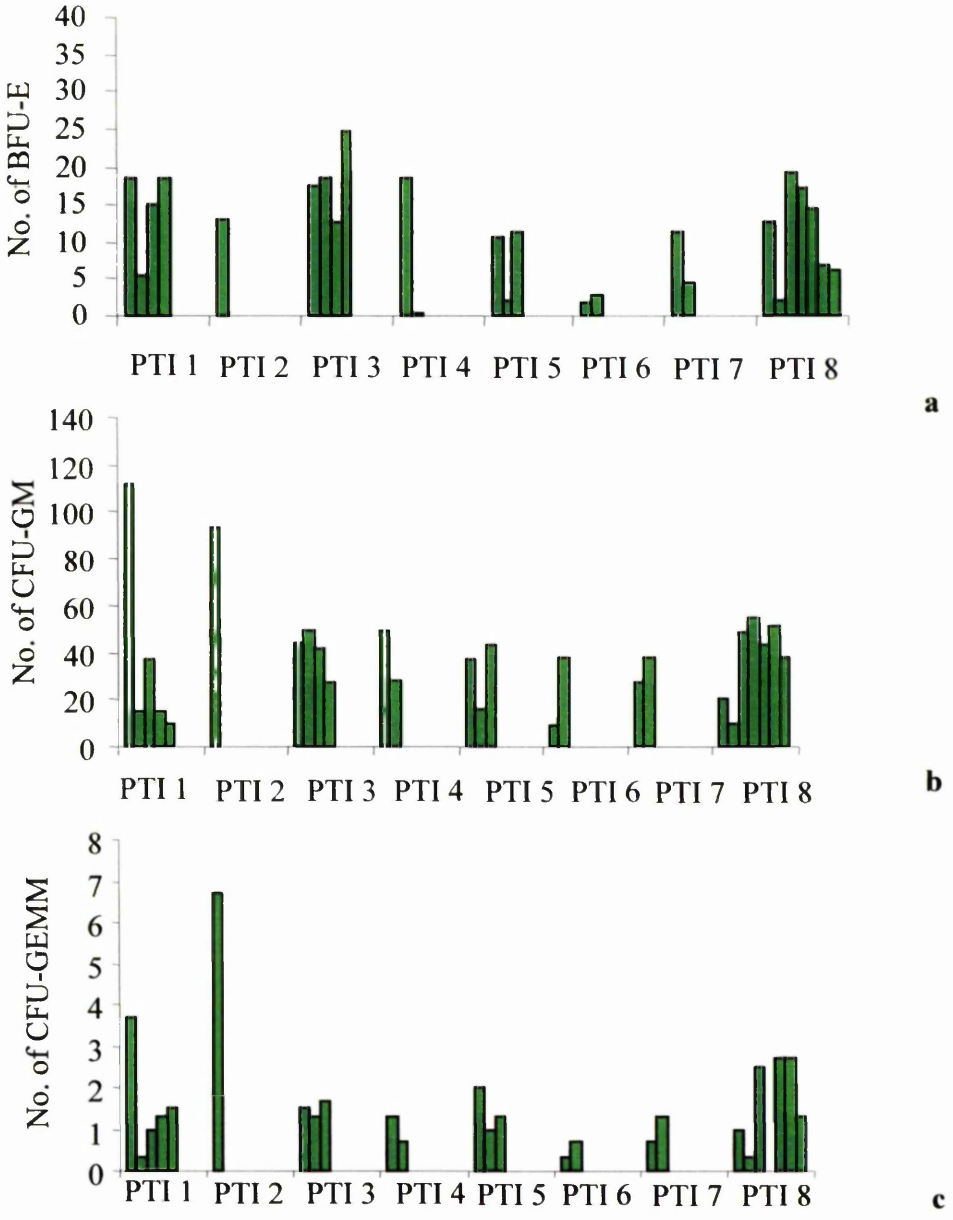


Figure 4.6: Number of BFU-E (a) and CFU-GM (b) and CFU-GEMM progenitors in peripheral blood samples from PTI plated at $2.5x10^4$ MNC/ml

PTI 1 assayed at 28, 29, 31 & 32 weeks
PTI 2 at 23 weeks
PTI 3 at 28,29,30 and 32 weeks
PTI 4 at 32 weeks
PTI 5 at 32,33,&35 weeks
PTI 6 at 35 weeks
PTI 7 at 33 weeks
PTI 8 at 23,26,27,33,&35 weeks

Progenitor type	Median Range
BFU-E	12 (0-24.5)
CFU-GM	38 (9-111)
CFU-GEMM	1.3 (0-2.7)
CD34	0.12% (0.05-1.16%)

Table 4.5: median and range for each progenitor type in PTI (plating density $2.5x10^4$ MNC/millilitre of methocult)

Cell type	AF Median mean Range (%)	PTI Median mean Range (%)	Effect of age in PTI p value	AF v PTI Effect of group p value	Effect of age PTI v AF p value
CD4	20.2 20.9 7.9-34.8	17.2 15.6 1.7-25.4	0.48	0.12	0.24
CD8	10.6 12.2 5.6-31.4	5.4 6.9 2.2-12.4	0.02	0.001	0.26
CD19/ CD20	4.8 5.5 2.1-12.7	2.5 3.7 0-9.2	0.23	0.1	0.07
CD33+/ CD15-	2.6 4.2 0-14.6	9.7 9.9 0-19.6	0.49	0.01	0.41
CD33+/ CD15+	12 11.3 0.1-27.4	0.7 3.3 0-19.6	0.78	0.02	0.35
CD33-/ CD15+	2.8 6.5 0.1-32.9	18.2 19.4 6.7-40	0.59	0.004	0.7
CD14	6.7 7.8 1-17.9	10.3 10.2 4.7-15.6	0.7	0.02	0.63
CD45+/ glyco+	2.8 5.1 0-23	8.2 10.4 0.8-36.4	0.12	0.29	0.36
CD45-/ Glyco+	0.8 5.1 0-26.3	29.5 24.9 0-38.7	0.65	0.1	0.48

Table 4.6: Summary of cell type proportions by immunophenotyping and comparison with AF group by ANOVA. Glyco =glycophorin

Comparison of progenitor cell content by measuring the content of CD34+ cells between AF and PTI showed that there were significantly more CD34+ cells in AF subjects (p=0.046). Functional analysis of progenitor cells by clonogenic assay showed that the PTI group had significantly fewer BFU-E (p=0.001, table 4.7) than the AF. No significant differences in the mean numbers of CFU-GM (p=0.52, table 4.7) or CFU-GEMM (p=0.82, table 4.7) were seen between PTI and AF groups.

4.4.2 Analysis of effect of age on cell types

There were no age related changes in most of the cell types measured by immunophenotyping within the PTI group or when the age related trends were compared between AF and PTI. Only CD8 cells showed an increase with age (p=0.02) in the PTI group. However, when the age related trends in CD8 in the PTI group were compared with AF group there was no significant difference between the two groups. There was no significant trends in colony numbers with increasing age within the PTI group or when AF and PTI were compared for BFU-E (p=0.86), CFU-GM (p=0.83) nor CFU-GEMM (p=0.75). Table 4.6 and 4.7 and figures 4.7 and 4.8 summarise the statistical analysis.

4.5 Telomere length measurement

Sequential samples from seven PTI were used to determine mTRF length. As for fetal blood samples, to optimise accuracy of mTRF measurements all samples from the same infant were run on adjacent lanes on the same gel. Sample lanes and size marker lanes were distributed in a similar manner to that described in section 3.1.2. Figure 4.9 shows the mTRF changes with time in each infant in the study. The mTRF length in this group of infants ranged from 7.2Kb to 11.2Kb, similar to range of values seen in fetal group which ranged from 7.22 -12.6Kb. As can be seen on this graph, there is an overall decrease in mTRF with increasing age. Unlike the fetal group, large fluctuations do not occur and infants followed for longer than two weeks all show a decline in telomere length. For example, PTI 3 had mTRF of 11.2Kb at 28 weeks, 9.94Kb at 30 weeks and 8.77Kb at 33 weeks equivalent gestational age. PTI 8 had mTRF length of 9.924Kb at 23weeks, 9.19Kb at 33 weeks and 8.19Kb at 35 weeks. The mTRF changes are summarised in table 4.8 which shows the overall changes in mTRF length for each infant as well as the rate of change of telomere length.

Cell type	AF Median mean Range (%)	PTI Median mean Range (%)	Effect of age in PTI p value	AF v PTI Effect of group p value	Effect of age PTI v AF p value
CD34	0.33 0.4 0.04-1.23	0.12 0.24 0.05-1.16	0.75	0.05	0.34
BFU-E	26.5 27.7 (2-67)	11 12 (0-20.5)	0.86	0.001	0.89
CFU-GM	47.4 38.7 (3-120)	41.7 38 (9-111)	0.83	0.52	0.49
CFU-GEMM	1.8 1.3 (0-4)	1.5 1.3 (0-2.7)	0.75	0.82	0.53

Table 4.7: Summary of progenitor cell numbers in PTI and comparison with AF group by ANOVA.
Glyco =glycophorin

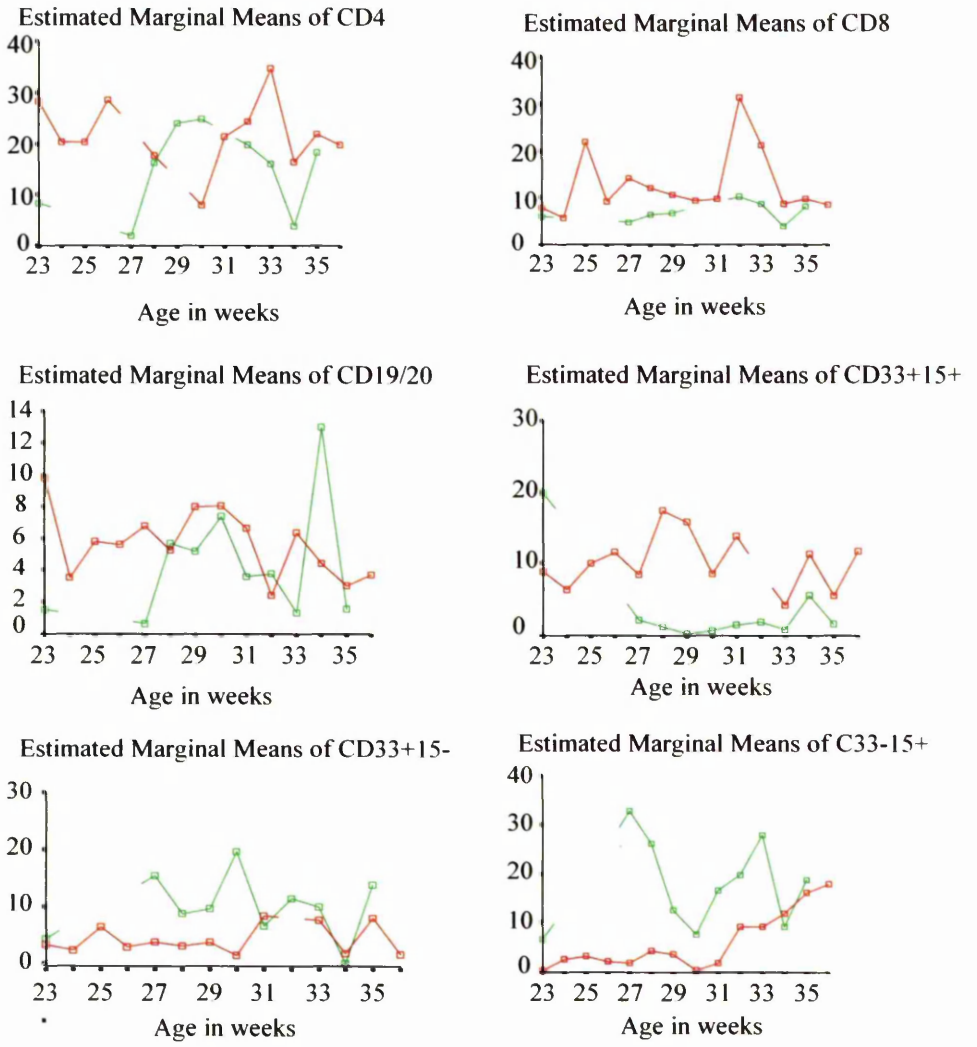


Figure 4.7: Statistical comparison of lymphoid and myeloid cell types in PTI and AF. Y-axis show estimated marginal mean proportion for each cell type

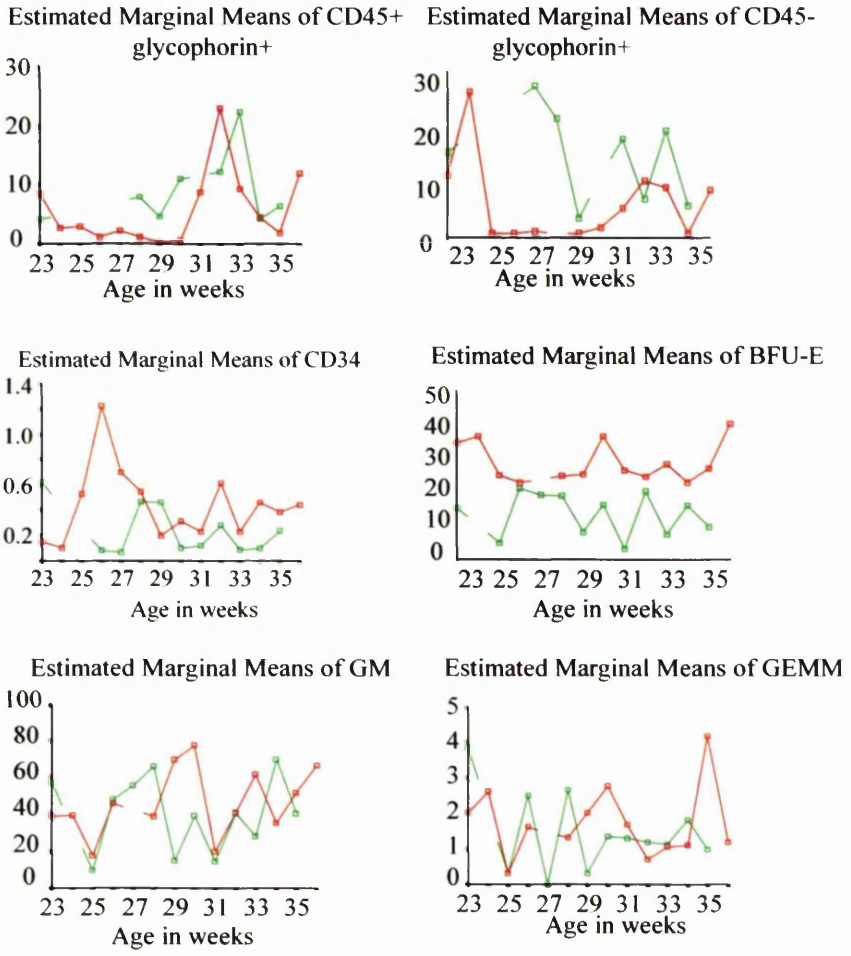


Figure 4.8: Statistical comparison of erythroid and progenitor cell types in PTI and AF. Y-axis show estimated marginal mean proportion for each cell type

AF PTI

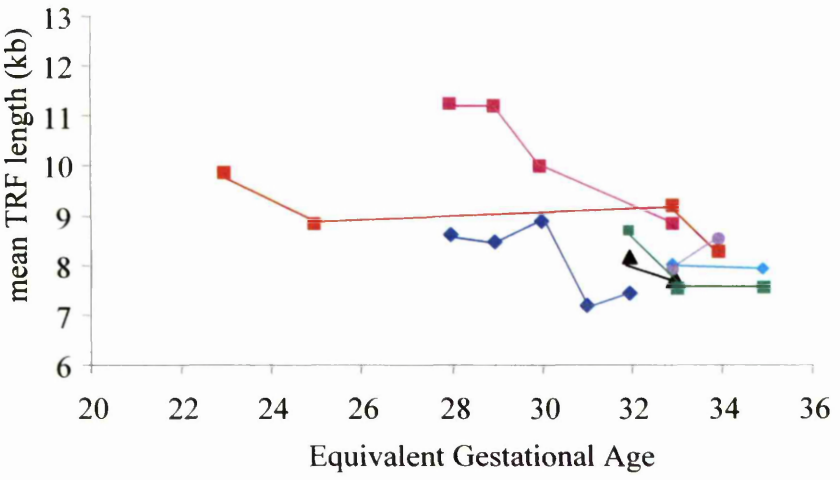
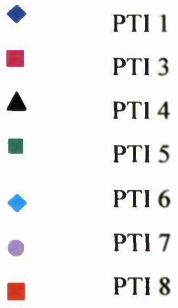


Figure 4.9: Mean TRF length of serial DNA samples from seven preterm infants as measured by Southern Blotting



Infant	Equivalent Gestational age range	Weeks between first and last sample	mTRF difference between first and last sample	Rate of change of mTRF in bp/week
PTI 1	28-32	4	-1214bp*	-303.5
PTI 3	28-33	5	-2430bp*	-486
PTI 4	32-33	1	-443bp	-443
PTI 5	32-35	3	-1128bp*	-376
PTI 6	32-35	3	+430bp	-143
PTI 7	32-33	1	+550bp*	+550
PTI 8	23-35	12	-1600bp*	-133

Table 4.8: Overall change in mTRF length in each PTI studied

4.6 Discussion

The aim of this chapter was to determine if HSCs in preterm infants differ in their telomere length changes from HSCs in age matched fetuses. To achieve this aim, I have measured mTRF length in sequential peripheral blood samples from preterm infants of equivalent gestational age and followed for a similar period of time and I have compared to the data obtained from fetal blood as described in chapter 3.

When comparing the mean content of myeloid, lymphoid and erythroid maturing and mature cells, several differences have been found between the two groups. In the lymphoid compartment there was a statistically significant decrease in the mean proportion of CD8 T cells in the preterm infants compared to fetal group. There were differences in the overall content of myeloid cell types. The AF group had significantly higher numbers of maturing myeloid cells (CD33+15+) compared to PTI and PTI had higher numbers of myeloblasts (CD33+15- cells), neutrophils (CD15+ cells), and monocytes (CD14+ cells) compared to the fetal group. This is not unexpected as changes occurring with birth have been previously described, both in the myeloid and lymphoid compartment. The absolute number of neutrophils have been seen to rise in both preterm and term infants in the first 24 hours of life. Following this surge, neutrophils counts in peripheral blood then decrease to normal childhood levels in the first month of life (Engle, *et al* 1997, Manroe, *et al* 1977, Mouzinho, *et al* 1994). Indeed in three of the five infants in this study with more than one CD15 immunophenotyping result, the first sample –which was usually cord blood or peripheral blood collected within 24 hours of birth, had the highest proportion of neutrophils. This pattern of increase in neutrophils numbers suggests birth rather than gestational age is associated with a surge in neutrophils numbers in the peripheral circulation in the first day of postnatal life. It has been shown that newborns have increased plasma concentration of G-CSF (Gessler and Dahinden 2003) which one could postulate may be responsible for this surge in neutrophils and account for why the PTI group had a higher concentration of mature neutrophils leading to a dilution of more immature myeloid cells

Differences have been seen also in the stem/progenitor cell compartment. When the PTI group was compared to the AF group with respect to mean numbers of

progenitors, there were significantly less CD34+ cells in PTI compared to AF subjects and progenitor assays showed that peripheral blood from PTI group contained fewer BFU-E progenitors than AF group (p=0.001). This is in agreement with previously published work. Decrease in number of CD34+ cells following birth has been reported by a number of groups (Gasparoni, *et al* 2000, Li, *et al* 2001). Erythropoiesis has been seen to diminish with birth and the production of red cells in the newborn has been quoted to decrease by a factor of two to three in the first few days after birth, possibly triggered by the change in tissue oxygen content at birth (Nathan, 1993).

Although these changes are important they are unlikely to bias the telomere length analysis if the changes remain constant in the PTI samples used for telomere analysis. For the telomere length analysis on the mature blood cells to be a reflection of mTRF in the HSC compartment with increasing age, it is important that the cell type composition of the blood remained constant during the sampling time. Therefore I used a statistical test to allow me to determine if there were any significant changes in cell proportions with increasing age which could influence mTRF length analysis in this group of preterm infants and to compare age related trends between the two groups.

None of the myeloid or erythroid cell types demonstrated any statistically significant change in proportion with increasing age in PTI by statistical analysis, nor did they show any age related differences to the AF group. In the lymphoid compartment, there was no significant change in CD4 T cells or CD19 B cells with time. When the age related trends for each group were compared, there was no difference in CD4 or CD19 numbers with age. This is similar to the findings of Juretic et al. (Juretic, *et al* 2000). The only significant change was seen in CD8 T cells with the PTI group showing an increase in CD8 cells with age. It is important to consider the effect of T cells numbers as this could potentially be a source of bias in telomere studies. The available data on T cells numbers in peripheral blood in the time period corresponding to the second and third trimester of gestation are limited and often in disagreement probably because of great inter-individual variation. An increase in CD8 T cells in term compared to premature newborns has previously been described

(Juretic, *et al* 2000) although a decrease in CD8 T cells with increasing gestational age in cord blood samples from late second trimester, third trimester and term has also been reported (Schultz, *et al* 2000). When I compared the changes in CD8 proportion with age in the PTI group to the AF group, I found the increase in CD8 seen in preterm infants was not significantly different to changes in proportion seen in the fetal group. Previous analysis of changes in CD8 frequency with gestation in fetal group in Chapter 3 indicated that though CD8 tended to increase with age this was not statistically significant. This suggests that the increase seen with age in preterm infants, although statistically significant, is small and unlikely to bias the telomere length studies. Moreover naïve T cells have been shown to have longer telomeres than memory and effector T cells (Weng, *et al* 1997). The major element of bias is thought to be due to the massive proliferation that occurs when a naïve T cell is activated in response to antigenic stimulation and telomerase can be activated. However, this is unlikely to affect the telomere analysis of blood samples in this study as it has been previously described that there are few (<1%) activated T cells in peripheral blood of preterm and term neonates (Juretic, *et al* 2000) and the vast majority of T cells are naïve (Szabolcs, *et al* 2003). In this work, the blood samples from infants were only utilised if the infant was clinically well with no evidence of infection and thus the effect of antigenic stimulation of T cells should have been minimised.

I therefore concluded that there were no age related differences between the groups which could sufficiently influence analysis and render it invalid. Using the same telomere length assay as was used for the fetal samples, in all infants studied for longer than two weeks there was decline in mTRF length with increasing age. In contrast, as was described in Chapter 3, there were no consistent trends in mTRF in fetal group with advancing age. The telomere loss was of 231bp/week and in the same range that the one reported by Friedrich et al.(Friedrich, *et al* 2001) in a cross sectional study where telomere length decreased with age at a rate of around 240bp/week between 27 and 32 weeks gestation.

One possible reason for such differences in HSC behaviour is that parturition itself has a role in changing behaviour of haemopoietic cells. Certainly, birth has a

profound effect on other systems, the lungs expel and absorb excess fluid and immediately commence process of gas exchange; the increase in oxygen tension causes functional closure of ductus arteriosus in the heart which has an important role in allowing correct flow of blood to and from the systemic and pulmonary circulations (Behrman, *et al* 2000). This increase in oxygen tension in the blood may have a role in haemopoietic cell function. It is known that HSCs undergo expansion in conditions of hypoxia (Danet, *et al* 2003). There is accumulating evidence that oxidative stress is an important factor in telomere biology; several studies have shown that cultured fibroblasts show greater rates of decline of telomere length when subjected to conditions of increased oxidative stress (von Zglinicki 2002, von Zglinicki, *et al* 1995). The increase in oxygen levels results in greater production of reactive oxygen species and thus leads to increased likelihood of DNA damage and telomere loss. Thus in fetal life, in the relative hypoxia of the intra-uterine environment, the fetus is protected against oxidative stress and its effects on telomere length. However, in postnatal life, this protection is lost, and it could be postulated that the haemopoietic cells therefore begin to lose telomeres. Protection from telomere loss in fetal HSC could be enhanced by increased telomerase levels. Telomerase expression has been found higher in fetal liver HSC than cord blood and adult HSC (Yui, *et al* 1998). Moreover it has very recently been reported that under hypoxic conditions, telomerase may be upregulated (Anderson, *et al* 2006), providing an hypothesis for a potential mechanism of telomere maintenance in fetal life.

Lastly, a further possibility, although highly speculative at this stage is that different populations of haemopoietic stem cells are responsible for prenatal and postnatal haemopoiesis. Normally in the last trimester of gestation, there is contribution from both hepatic and bone marrow haemopoietic cells to haemopoiesis. The net result of turnover of hepatic HSCs and bone marrow HSCs is fluctuating telomere length but no overall decrease or increase in mTRF trend. However, hepatic haemopoiesis ceases very quickly after birth (Brauner, *et al* 2001) and the bone marrow therefore becomes the only anatomic location for haemopoiesis. While the hepatic environment may be more suitable for the rapid proliferation in haemopoietic cells without telomere loss as seen in fetal life, it may only be able to perform this function in fetal life thus necessitating a switch in haemopoietic sites at time of birth. The increase in

oxygen tension at birth may render the hepatic environment unsuitable for continuing haemopoiesis as it may expose the HSCs to increasing levels of oxygen and thus predisposes them to oxygen free radical damage (Chow, *et al* 2001) thus necessitating an immediate cessation of the fetal liver as a haemopoietic site. Although the bone marrow has significant numbers of HSCs and progenitors at birth, the amount of marrow tissue continues to expand requiring turnover of existing cells to produce the expansion. The changes seen in the infant group in this project may reflect the sudden cessation of hepatic haemopoiesis and the turnover of BM HSCs to expand the stem cell compartment and provide mature haemopoietic cells.

Regardless of the mechanisms responsible for the different telomere regulation this work highlights fetal HSC as good model to study expansion in absence of aging, important in any cell and gene therapy transplantation protocols.

CHAPTER 5

HAEMOPOIESIS IN DOWN SYNDROME IN FETAL LIFE

5 HAEMOPOIESIS IN DOWN SYNDROME IN FETAL LIFE

5.1 Introduction

Individuals affected with Down syndrome (DS) show features which suggest that DS could represent a model of accelerated HSC aging. Firstly there is increased risk of acute myeloid leukaemia in childhood (Lange 2000) which is normally preceded by a myelodysplastic phase – a feature uncommon in childhood leukaemia and usually seen in the elderly. Secondly there is evidence of immune dysfunction (Cuadrado and Barrena 1996, Park, *et al* 2000). Immune dysfunction has also been linked with accelerated aging of the haemopoietic system (Rossi, *et al* 2005) as aging has recently been found to be accompanied by down-regulation of lymphoid mediating genes and upregulation of myeloid specific genes. Thirdly, accelerated telomere shortening which is associated with aging has also been seen in haemopoietic cells in DS in postnatal life (Vaziri, *et al* 1993). Accelerated telomere loss is associated with increased HSC replicative stress and HSC deficiency as seen in aplastic anaemia and ataxia telangiectasia (Ball, *et al* 1998, Metcalfe, *et al* 1996). Indeed data from a murine model suggests that in DS there is also a deficiency of HSC (Epstein, *et al* 1985).

Intriguingly, the increased incidence of leukaemia in DS is largely confined to early childhood whereas one might expect that in the face of HSC deficiency, the risk of leukaemia would continue to increase into adulthood, particularly as other conditions associated with aging such as ocular cataracts and Alzheimers disease occur at a younger age in DS compared to normal individuals. There are a number of possible reasons which may help to explain this. Firstly, the occurrence of leukaemia in childhood may reflect abnormalities in haemopoietic cells arising in fetal life, when cellular proliferation is maximal and HSCs therefore at their most active. The occurrence of transient myeloproliferative disorder in newborns with DS (Lange 2000) and the presence of an HSC deficiency in fetal liver HSC in the trisomy 16 murine model of DS (Epstein, *et al* 1985) are strong indicators that there may be potential abnormalities in the haemopoietic system in these individuals which do arise in fetal life. That the incidence of leukaemia then decreases may reflect decreasing levels of replicative stress on the HSCs as cell turnover slows down after the first few years of life.

Following on from chapter 4, if there are separate pools of HSCs for fetal (liver HSCs) and postnatal (bone marrow HSCs) life, then the increased risk of leukaemia may relate to only acclerated aging of fetal HSCs and as individuals get older, other complications of DS such as recurrent infections and cardiac disease may be more likely causes of morbidity and mortality before the effects of aging are apparent in bone marrow HSCs

In this chapter, I sought to investigate HSC biology in DS in fetal life before the onset of haematological disorders, to ascertain whether accelerated telomere shortening and decreased HSC numbers are apparent in fetal life. Using fetal DS as a model of accelerated aging offers the significant advantage that the properties of stem cells could be investigated before the effects of the aging process are apparent (i.e. before onset of leukaemia). To achieve this aim, I collected peripheral blood from DS fetuses and compared composition by immunophenotyping, progenitor cell content by progenitor cell assays and immunophenotyping and HSC replication by mTRF measurement to age matched control fetuses without DS.

In addition, while this work was in progress, a number of sources documented mutations in the GATA1 gene in TMD and AMKL blasts, suggesting that this mutation was a major contributing event in leukaemogenesis in DS (Mundschau, *et al* 2003, Wechsler, *et al* 2002). I therefore sought to investigate whether this mutation was present in blood cells in fetal life in same fetal samples before the manifestation of clonal disorders.

5.2 Comparison of immature and mature cell types in DS and non-DS individuals in fetal life.

5.2.1 Sample collection and cellularity of samples

Peripheral blood samples from five fetuses with Trisomy 21 (Down syndrome, DS) confirmed by karyotype analysis with gestational ages ranging from 23-35 weeks were collected at time of termination of pregnancy by intra-cardiac administration of potassium chloride. Blood from a group of age matched fetuses of similar gestational age from 23-35 weeks undergoing termination of pregnancy for reasons other than Trisomy 21 were collected as a control group (TOP group). Sample volumes were between 0.5 and 1ml.

Mature and immature cell types were determined by immunophenotyping. Progenitor cell assays were used to enumerate BFU-E, CFU-GM and CFU-GEMM progenitors and Southern Blotting was used to determine telomere length. Each of the experiments performed on each DS sample are summarised in table 5.1. For any variable where there were few observations in either group ($n<4$) no statistical analysis was performed. In this case, the value of each variable at each gestational age is described in the text. Where there was sufficient data in each group, data were compared using Student's t-.test as data for each variable followed an approximately normal distribution, and this is summarised by mean and p value.

An initial count of nucleated cells was made using a haemocytometer after eliminating enucleated red blood cells by dilution with a 3% acetic acid solution. The average (and SD) of total cell count of peripheral blood samples from four fetuses with DS was $3.57\pm1.75 \times 10^6$cells per millilitre (ml) of blood (range $1.2\text{-}5.17\times10^6$/ml) and in TOP group average count was $4.17\pm3.43\times10^6$/ml (range $1.14\text{-}9.49\times10^6$ per ml, n=6, see figure 5.1). There was no statistical difference in total peripheral blood cell numbers between the two groups by Student's t test (p=0.73).

Sample	Gestation	Immuno-phenotyping	Progenitor assay	Telomere length	Sequencing
DS1	Term	No	No	No	Yes
DS2	35 weeks	Yes	Yes	No	Yes
DS47	30 weeks	Yes	Yes	Yes	No
DS52	32 weeks	Yes	No	Yes	No
DS4	23 weeks	Yes	Yes	Yes	Yes
DS5	23 weeks	No	Yes	Yes	Yes

Table 5.1: Experiments performed on each DS peripheral blood sample.

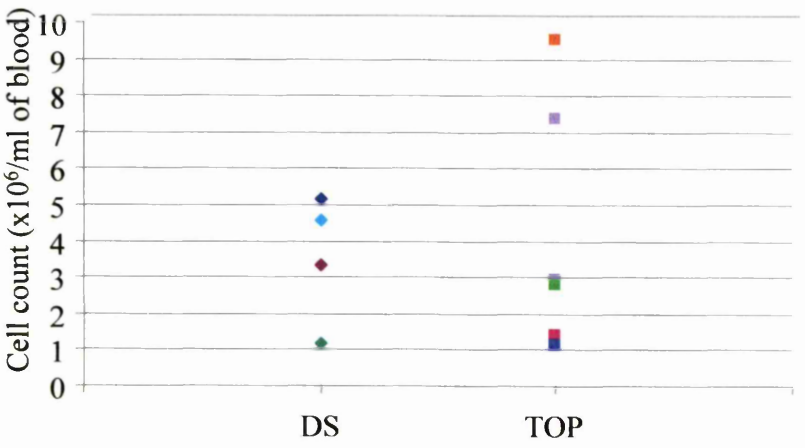


Figure 5.1: Total nucleated cell count of DS and TOP blood samples.

- DS 4, 23 weeks
- DS 47, 30 weeks
- DS 52, 32 weeks
- DS 2, 35 weeks
- TOP, 23 weeks
- TOP, 24 weeks
- TOP, 28 weeks
- TOP, 30 weeks
- TOP, 32 weeks

5.2.2 Immunophenotype analysis of lymphoid markers

Peripheral blood from four of the DS group and six non-DS (control) fetuses was assayed for surface markers expressed on mature and progenitor blood cell types to determine if there were any differences in peripheral blood composition between the DS fetuses and their age matched controls. Due to small size of samples it was not always possible to carry out analysis of complete set of markers for each sample. At least 16000-20000 total events were collected for each sample and the percentage of each cell type determined as described in section 2.2.

CD4 and CD8 were used as representative T cell markers. The results are summarised in figure 5.2. Cells numbers permitted the analysis of the frequency of CD4+ cells in only two of the DS samples and the frequencies found were 6.9% at 32 weeks and 0.7% at 35 weeks. In contrast, CD4 was measured in four of the control fetuses at 23, 24 28 and 29 weeks and frequencies obtained were 13.8%, 12.7%, 20% and 21.2% respectively. CD8 was measured in two of the DS fetuses at 32 and 35 weeks and frequencies of 5.7% and 3.1% were found respectively. CD8 was measured in five of the control group (23, 23, 24, 28 and 29 weeks) and values of 9%, 4.6%, 9.6%, 7% and 12% were observed.

The proportion of B cells was measured by CD19/20 expression in all four DS fetuses and all six control fetuses. The proportions of CD19/20 in each fetus are shown in figure 5.3. Comparison of groups by students t-test showed there was a significant difference in frequency of CD19/20 cells between the two groups ($p=0.03$).

5.2.3 Immunophenotype analysis of myeloid markers

CD33 and CD15 dual staining was performed to determine CD33+/15- myeloerythroid progenitor and myeloblasts, CD33+/15+ myeloid progenitors, promyelocytes and myelocytes and CD33-/15+ neutrophils. CD14 was used as monocyte marker. The proportion of each cell type at each time point for both DS and non-DS are shown in figures 5.4 and 5.5.

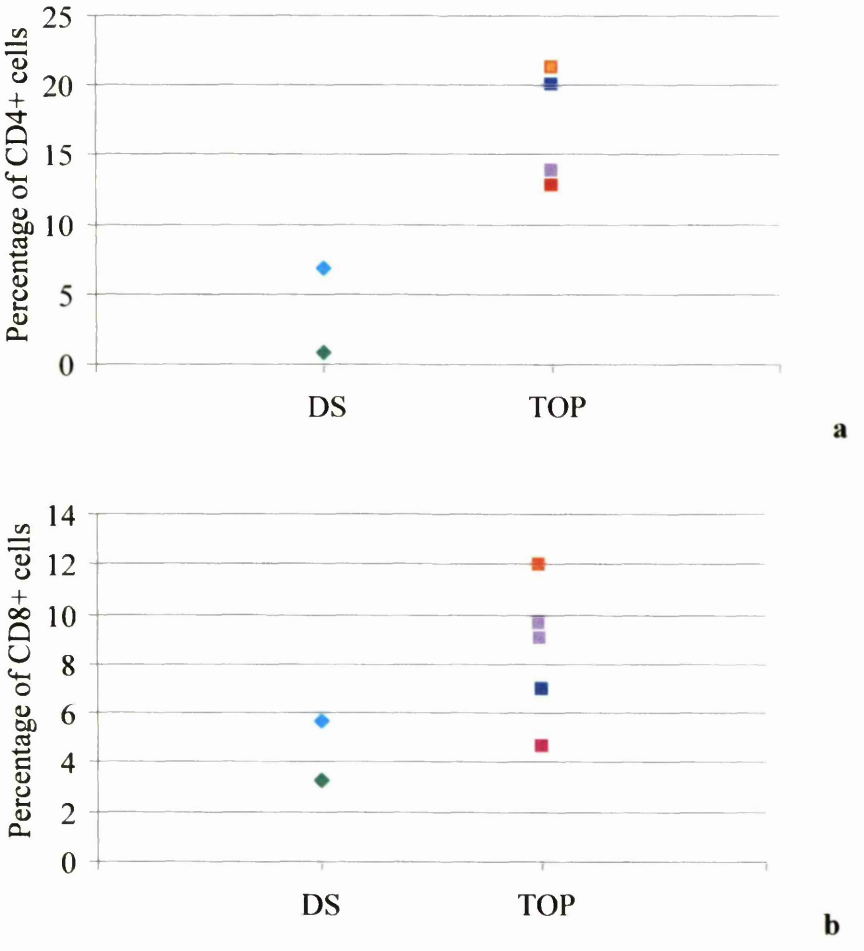


Figure 5.2: Percentage of CD4+ cells (a) and CD8+ cells (b) in peripheral blood in DS and control fetuses by immunophenotyping

- DS 52, 32 weeks
- DS 2, 35 weeks
- TOP, 23 weeks
- TOP, 24 weeks
- TOP, 28 weeks
- TOP, 30 weeks
- TOP, 32 weeks

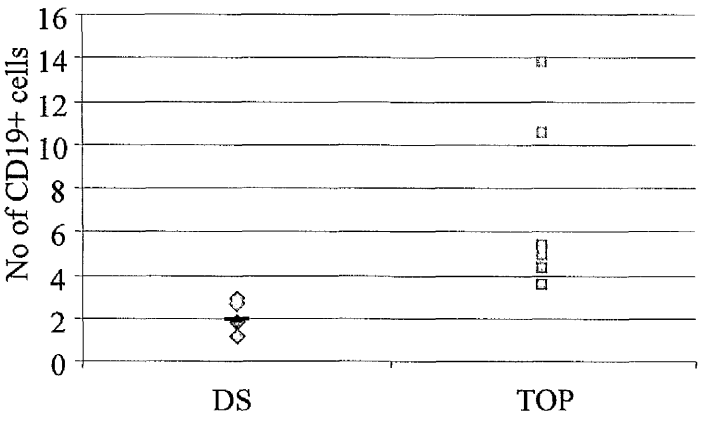


Figure 5.3: Percentage of CD19/20+ B cells in peripheral blood from DS and age matched TOP controls.

- DS 4, 23 weeks
- DS 47, 30 weeks
- DS 52, 32 weeks
- DS 2, 35 weeks
- TOP, 23 weeks
- TOP, 24 weeks
- TOP, 28 weeks
- TOP, 30 weeks
- TOP, 32 weeks

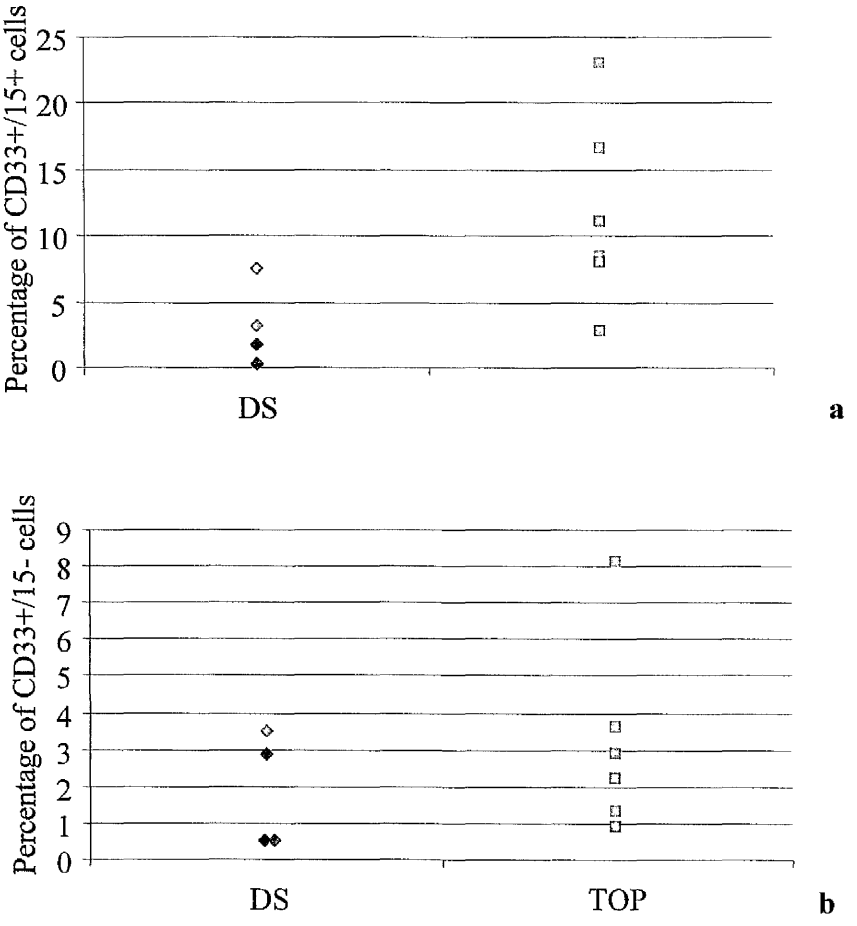


Figure 5.4: Percentage of CD33+15+ cells (a) and CD33+15- cells (b) in peripheral blood in DS and control TOP fetuses

- ◇ DS 4, 23 weeks
- ◇ DS 47, 30 weeks
- ◇ DS 52, 32 weeks
- ◇ DS 2, 35 weeks
- ▫ TOP, 23 weeks
- ▫ TOP, 24 weeks
- ▫ TOP, 28 weeks
- ▫ TOP, 30 weeks
- ▫ TOP, 32 weeks

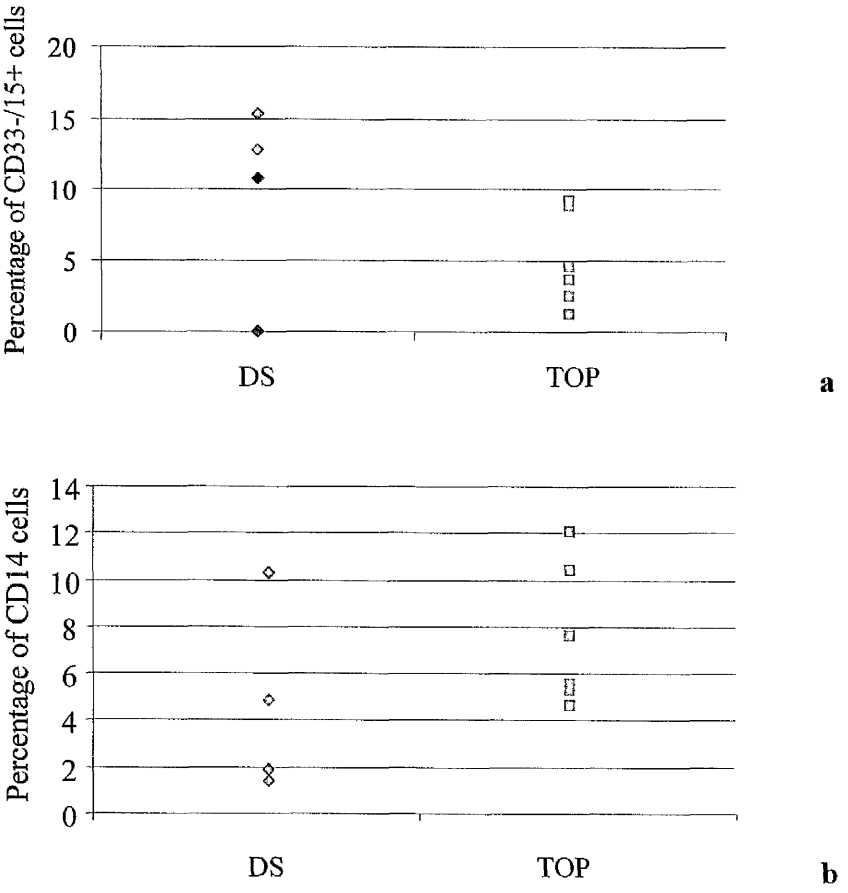


Figure 5.5: Percentage of CD33-15+ cells (a) and CD14+ cells (b) in peripheral blood in DS and control TOP fetuses

- ◇ DS 4, 23 weeks
- ◇ DS 47, 30 weeks
- ◇ DS 52, 32 weeks
- ◇ DS 2, 35 weeks
- ▫ TOP, 23 weeks
- ▫ TOP, 24 weeks
- ▫ TOP, 28 weeks
- ▫ TOP, 30 weeks
- ▫ TOP, 32 weeks

Comparison of the frequency of each of the cell types in the two groups by Student T test showed that there was a significant difference in proportion of CD33+/15+ cells (p=0.03). No differences were found in proportions of CD33+/15-, CD33-/15+ or CD14+ cells (p=0.45, p=0.12, p=0.41).

5.2.4 Immunophenotype analysis of erythroid markers

A combination of glycophorin A and CD45 was used to measure proportions of cells in erythroid lineage in each sample. Figure 5.6 shows the proportion of CD45+/glycophorin+ and CD45-/glycophorin+ erythroid cells in each sample. No statistically significant difference was found between groups by students t-test (Glycophorin+/CD45+, p=0.33 glycophorin+/CD45-, p= 0.46).

5.3 Comparison of progenitor cell frequency in DS to normal fetal life

5.3.1 Immunophenotype analysis of CD34+ cells

CD34 was used as a marker of progenitor/stem cells. A minimum of 30000 total events was acquired to measure CD34 frequency as described in section 2.2. The proportion of CD34+ cells in each sample is shown in figure 5.7. Although not statistically significant, there were fewer CD34+ cells in DS group compared to TOP (0.32% and 0.97% respectively, p=0.08).

5.3.2 Progenitor cell assay

Peripheral blood samples from four DS fetuses (gestational ages 23, 23, 30 and 35weeks) and seven control samples (gestational ages 23, 23, 24, 29, 30, 32, 35 weeks) were collected and used for enumeration of BFU-E, CFU-GM and CFU-GEMM progenitors. The average number of colonies and range values for each progenitor type are as shown in table 5.2. Figure 5.8 summarises the comparison of progenitor cell proportions in the DS and non-DS control blood samples. A statistically significant difference in the number of CFU-GM was found (p=0.03, Students t test) but no statistically significant differences were found in BFU-E numbers (p=0.9) or CFU-GEMM numbers (p=0.2)

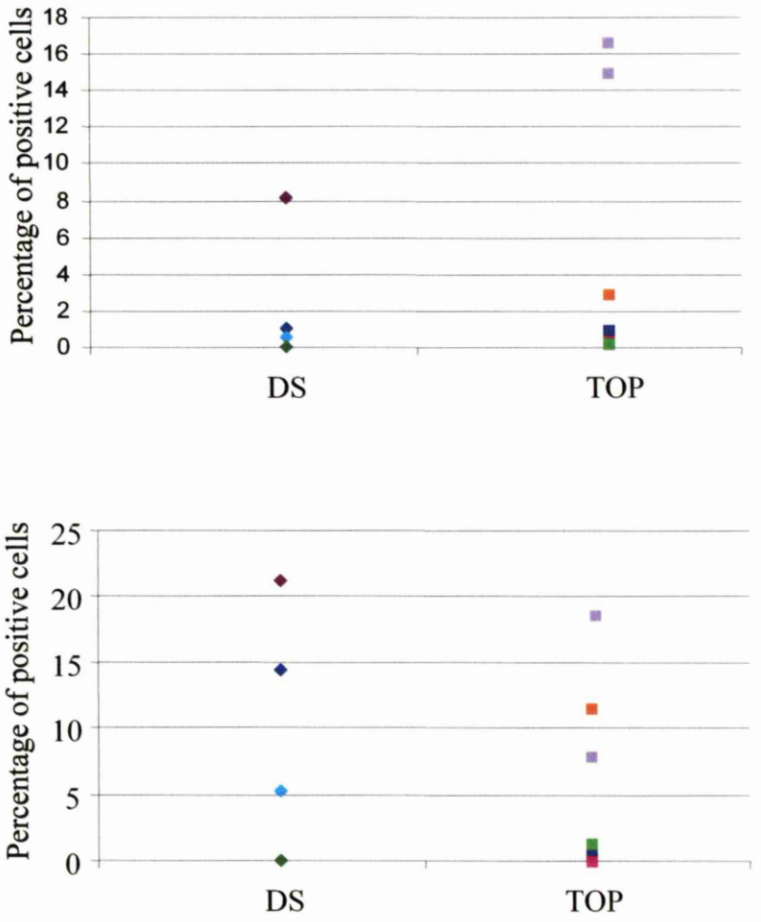


Figure 5.6:Percentage of glycophorin+/CD45+ (a) and glycophorin+/CD45- (b) erythroid cells in peripheral blood from DS and age matched TOP controls.

- DS 4, 23 weeks
- DS 47, 30 weeks
- DS 52, 32 weeks
- DS 2, 35 weeks
- TOP, 23 weeks
- TOP, 24 weeks
- TOP, 28 weeks
- TOP, 30 weeks
- TOP, 32 weeks

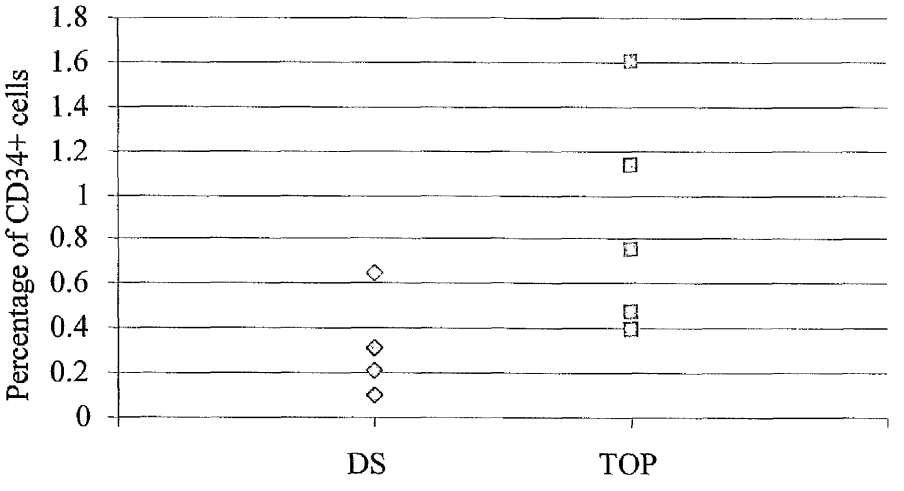


Figure 5.7: Percentage of CD34+ cell frequency in peripheral blood of fetuses with DS and age matched TOP controls

- ◇ DS 4, 23 weeks
- ◇ DS 47, 30 weeks
- ◇ DS 52, 32 weeks
- ◇ DS 2, 35 weeks

- ▫ TOP, 23 weeks
- ▫ TOP, 24 weeks
- ▫ TOP, 28 weeks
- ▫ TOP, 30 weeks
- ▫ TOP, 32 weeks

Progenitor type	DS Mean (range)	Termination of pregnancy (TOP) Mean (range)	DS v TOP Student t test (p value)
BFU-E	20.2 (5.7-40)	19.6 (3-38.3)	0.9
CFU-GM	21.2 (11.3-36)	69.8 (13-154)	0.03
CFU-GEMM	2.3 (0-7.4)	4.6 (1.3-10.3)	0.2

Table 5.2: Comparison of proportions of progenitor cells in peripheral blood from DS and TOP samples . (Plating density $2.5x10^4$ MNC/millilitre of methocult

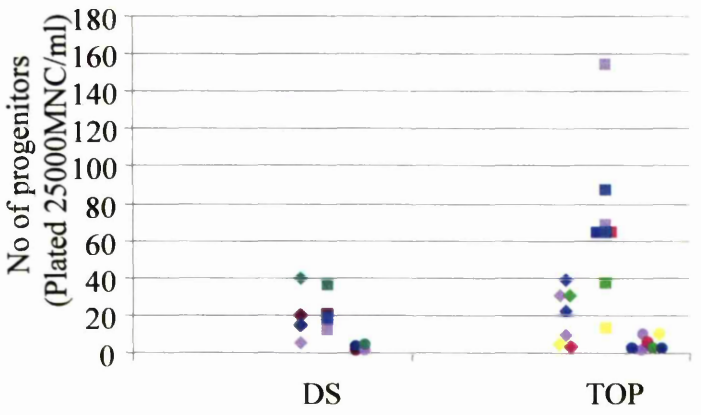


Figure 5.8: Comparison of frequency of clonogenic progenitor cells in DS and age matched control fetuses.

◆ = BFU-E ■ = CFU-GM ● = CFU-GEMM

◆◆ DS 4, 23 weeks
◆ DS 47, 30 weeks
◆ DS 52, 32 weeks
◆ DS 2, 35 weeks

■ TOP, 23 weeks
■ TOP, 24 weeks
■ TOP, 28 weeks
■ TOP, 30 weeks
■ TOP, 32 weeks
■ TOP, 35 weeks

5.4 Telomere length analysis of peripheral blood cells from fetuses with DS in fetal life.

DNA from peripheral blood of four fetuses with DS and control fetuses was extracted as described in section 2.5.1 and mTRF length of DNA was measured by Southern blotting as described in sections 2.5.2 -2.5.6. Figure 5.9 shows mean TRF length in DS and age matched control samples. The mean TRF length in DS subjects was significantly reduced compared to TOP group (8.3±0.3Kb and 10.5 ±0. 51Kb respectively, p=0.03).

5.5 Sequencing of GATA 1, exon 2 in DS subjects

5.5.1 GATA 1 exon 2 primers

As subjects affected by TMD/AMKL have been demonstrated to have a mutation in exon two of GATA1 (Rainis, *et al*, 2003, Hitzler, *et al*, 2003), polymerase chain reaction of GATA1 exon 2 was carried out using two distinct sets of primers already described (Hitzler *et al*, 2003, Rainis *et al*, 2003). Genomic DNA from three fetuses affected with DS with a low number of CD34+ cells and CFU-GM as described in experiments above, one newborn with DS and TMD, two normal fetuses and Jurkat cell line were used. Using the first set of primers, labelled GATA1 2F/1R, a PCR product was 392bp was seen in all samples on electrophoresis as expected (figure 5.10)

5.5.2 Direct sequencing of GATA1 exon 2 from DS samples

Bi-directional sequencing of purified PCR products from GATA1 2F/2R reactions from all 3 fetal DS subjects was performed using GATA1 1F /1R primers. A further sample from a newborn affected with DS and TMD/acute leukaemia was also subjected to sequencing following PCR amplification of GATA 1 exon 2 with 2F/2R primers and purification of PCR product. The sequencing results show no mutations in the 3 DS fetuses with no evidence of a clonal disorder and a low number of stem/progenitor cells. However, a mutation was found in the newborn with DS and overt AML resulting in a premature stop codon as expected (Figure 5.11).

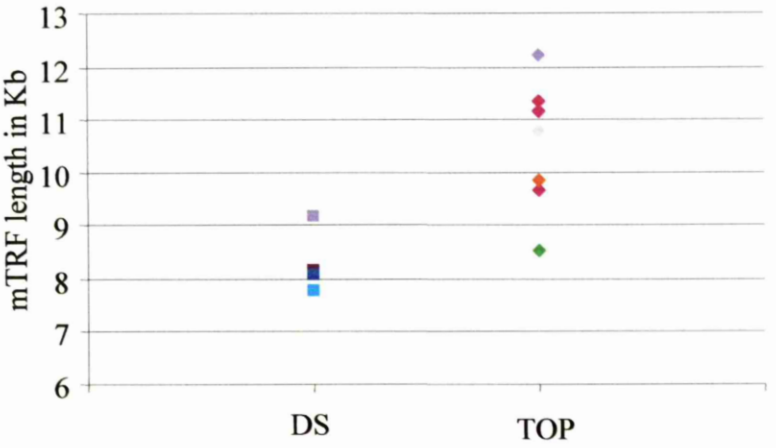
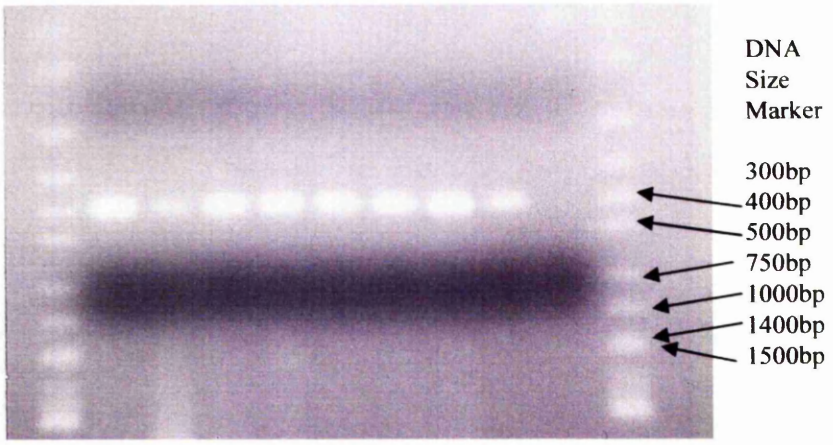


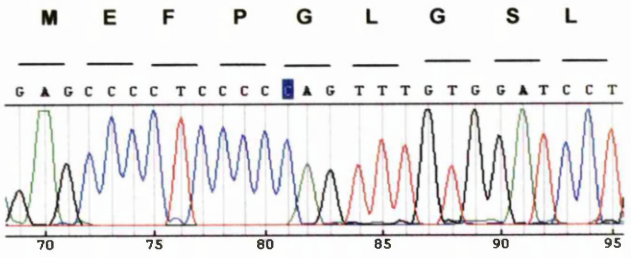
Figure 5.9: Comparison of mTRF length in DS and age matched TOP controls.

- DS 4, 23 weeks
- DS 47, 23 weeks
- DS 52, 30 weeks
- DS 2, 32 weeks
- TOP, 23 weeks
- TOP, 24 weeks
- TOP, 28 weeks
- TOP, 30 weeks
- TOP, 31 weeks
- TOP, 32 weeks

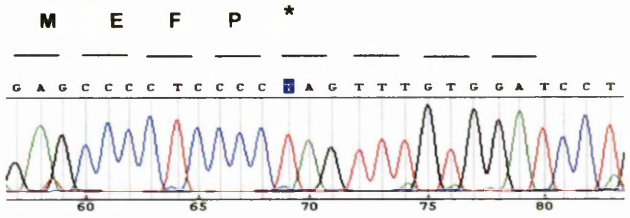


1	DS 1	7	Jurkat
2	DS 2	8	FB 78
3	DS 47	9	NTC
4	DS 52	SM	Size
5	DS 4		marker
6	DS 5		

Figure 5.10: Gel showing PCR products with GATA 1 2F/2R primers. DS=samples from DS individuals, FB =fetal blood from non DS individual NTC=no template control.



a



b

Figure 5.11: DNA sequencing GATA1 exon 2 in DS fetuses. A representative example of part of GATA-1 exon 2 sequencing in leucocytes of peripheral blood of a DS fetus, which results in a normal GATA-1 protein sequence (a) and sequence of the blasts of a DS newborn affected by AML showing a mutation in exon 2 of the GATA-1 gene, which results in a stop codon and in the translation of a truncated GATA-1 protein (b)

5.6 Discussion

The aim of this chapter was to determine whether there was accelerated telomere loss and HSC deficiency in DS starting as early as fetal life. Fetal life was chosen as there is evidence of leukaemia at birth in some individuals with DS (Lange 2000) indicating that the changes leading to leukaemia must have arisen in fetal life. Indeed in the murine model of DS there is evidence of an HSC deficiency in fetal liver HSC (Epstein, *et al* 1985). Access to samples of fetal haemopoietic tissue can be difficult. For my PhD, the availability of haemopoietic cells from fetal tissue was peripheral blood obtained at the time of termination of pregnancy by injection of intracardiac potassium chloride. I was able to collect peripheral blood from 5 fetuses with DS between 23 and 35 weeks gestation and one term newborn with DS and TMD. Sample size although limited to 1 millilitre of blood were sufficient to perform experiments as noted above.

Immunophenotyping revealed several differences between the DS and control group. In the lymphoid compartment, there were fewer CD4, CD8 and a statistically significant decrease in B cells in peripheral blood in DS fetuses. In interpreting this, it should be noted that one of the control fetuses (TOP, 28 weeks) had a high nucleated cell count and also a high percentage of CD4 and CD8 T cells suggesting that this fetus may have been ill and therefore not a normal control. Secondly one of the DS fetuses had an abnormally low total neucleated count which may reflect unknown problems in this fetus and this may also biase the comparison of the two groups. However, despite this and although it was not possible to perform statistical analysis due to small numbers of results, my findings are in keeping with those of Gjertson et. al. (Gjertson, *et al* 1999) who found decreased numbers of CD8+ T cells in fetal thymus in Trisomy 16 mice, the murine model of human trisomy 21. Epstein et al.(Epstein, *et al* 1985) also documented an overall decrease in both total haemopoietic cell numbers and T lymphocytes in fetal thymus in the Ts16 mouse model compared to normal controls. Although data describing the proportions of T cells in DS in fetal life are scant, the available evidence from 17-24 weeks gestation does show reduced T cell numbers in fetal blood (Thilaganathan, *et al* 1993). In postnatal life in DS diminished T lymphocyte numbers in thymus and peripheral circulation have been described in childhood (Musiani, *et al* 1990, Noble and Warren

1987). The decrease in B cell numbers in human fetal DS is similar to the findings in the murine model of DS where fewer pre-B and B cells in fetal liver in Ts 16 mice have been reported (Gjertson, *et al,* 1999 and Epstein, *et al* 1985). In humans, the available data shows a decrease in B cell numbers in childhood *(*Cossarizza, *et al 1990,* Cossarizza, *et al 1991,* Thilaganathan, *et al 1993).*

In the myeloid compartment, I found a statistically significant decrease in CD33+15+ precursors by immunophenotype analysis. There was also a decrease in CD33+/15- myeloblasts, but this did not reach statistical significance. It is interesting to note that these fetuses show reduced number of CD33+ cells as the infants and children with TMD or AMKL show an increase in CD33+ cells by immunophenotyping as this marker is expressed on blast cells (Lange, 2000). As there was no increase in number of CD33+ cells seen in this group, compared to the control, this reassures that these DS fetuses are unlikely to be affected by TMD/AMKL. Moreover the CD33+/15- population contains both CFU-GM and BFU-E clonogenic progenitors, whereas the CD33+/15+ contains only the CFU-GM populations of cells. Indeed these results mirror the data found with the clonogenic assay. The most striking finding in the progenitor compartment was the significant decrease in the number of CFU-GM progenitor in the DS group but not in the number of BFU-E or CFU-GEMM. Again the limitation of this result is that it may be affected by the abnormally high cell numbers in one of the controls and the abnormally low numbers in one of the DS fetuses. However, the data are in part consistent with data from the Ts16 mouse model which showed decreased granulocyte-macrophage stem cells defined by CFU-S assay from fetal liver HSCs at day 14-18 gestation (Epstein, *et al,* 1985).

In TAM and AMKL, abnormalities are seen in megakaryocyte lineage with arrested development at the megakaryoblast stage. An assay measuring megakaryocyte erythroid progenitors may have helped to further elucidate the haemopoeitic abnormalities in the DS fetuses by determining whether there were demonstrable abnormalities in megakaryocytes in this group. However, the small size of the samples received did not permit the use of this assay.

Surprisingly at first was that no statistical difference in the number of erythroid progenitors was observed between DS and TOP fetuses in my study. This is in contrast to the decreased proportions of erythroid cells found in fetal liver of the Ts16 mouse and highlights one of the differences between this mouse model and human DS. While there are similarities between Ts16 mice and DS in particular decreased numbers of lymphocytes and myeloid progenitors there are also differences between the two in that, the murine model also has profound abnormalities of the erythroid cells with severe anaemia and the trisomic mice die in utero (Dierssen, *et al* 2001, Epstein, *et al* 1985). This may be because Ts16 mice are not trisomic for all the genes found on the DS critical region on human chromosome 21. The new mouse model of DS which has recently been described may prove to be more representative of the human situation (O'Doherty, *et al* 2005), though as yet there has been no description of the haematological parameters in this model. However, our findings are supported by the fact that infants with DS do not show similar defects in erythroid cells and do not have such a severe phenotype as the trisomy 16 murine model. It is not unreasonable to suggest that in conditions of increased replicative stress and stem cell deficiency, the erythroid progenitor may be privileged in its maintenance at the expense of other progenitor types. In support of this is recent evidence suggesting that the Megakaryocyte-Erythroid progenitor might develop directly from the short term repopulating stem cell population without a common myeloid progenitor intermediate and well before the lymphoid progenitor (Adolfsson, *et al* 2005, Singh 1996, Takano, *et al* 2004). Also in humans Notch activation through Delta 4 in conditions of hypoxia induced fetal liver CD34+38- cells to generate erythroid progenitors, while maintaining their LTC-IC activity and thus preventing progenitor cell exhaustion (Dando, *et al* 2005), suggesting that there are mechanisms which would privilege the erythroid lineage.

To assess the number of stem/progenitor cells I used CD34 as a marker. Although not statistically significant at 95% level the fetuses with DS did have fewer CD34 positive cells than the non DS controls. These data are in agreement with data from the murine model, where there are fewer HSCs capable of bone marrow reconstitution upon transplantation (Epstein, *et al* 1985) and are supported by other findings in our laboratory where a significant decrease in CD34 numbers and in

LTC-IC in bone marrow in DS children compared to normal controls were also found (Ilaria Bellantuono, personal communication).

The results considered thus far demonstrate deficiencies in mature, precursor and progenitor cell types already apparent in fetal life in DS. Telomere length measurements by the same mTRF length assay used in chapter 3 and 4 showed that compared to age matched non DS controls, DS subjects had shorter telomeres in fetal life. This analysis may have been biased by the reduced number of lymphoid cells present in the peripheral blood of DS fetuses compared to non DS. As T cells have longer telomeres than neutrophils it is of concern that the accelerated telomere shortening may be the result of a different blood cell composition and the analysis should be repeated on CD34+ population of cells. However, the differences in proportions of cells are well below 10% and the accelerated telomere shortening is vastly reduced, making it unlikely that the reduction is exclusively the result of a change in blood composition. This finding is also supported by the available data from postnatal life, which also showed increased telomere shortening in haemopoietic cells from individuals with DS (Vaziri, *et al* 1993).

There are two possible explanations whereby this HSC deficiency resulting in accelerated telomere length shortening could arise. Firstly a smaller HSC pool could be generated during HSC development resulting in increased proliferation of the existing cells in an effort to maintain normal haemopoiesis. An alternative explanation is that there is a relatively normal HSC pool at the start but there is increased cell turnover, leading to exhaustion of the HSC pool. In support of this, there is evidence in postnatal life in DS of certain mature cell types such as neutrophils having shorter lifespans (Yasui, *et al* 1999) thus requiring an increased cell turnover to maintain production. Combined with this, in DS, the extra copy of chromosome 21 results in increased expression of the gene encoding for the enzyme CuZn superoxide dismutase (SOD) which results in an increase of up to 50% in activity of this enzyme. Increased levels in various haemopoietic cells have been found in DS including erythrocytes, lymphocytes, platelets and granulocytes (Bjorksten, *et al* 1984, Muchova, *et al* 2001). Aberrant SOD expression in cells has been linked with decreased granulocyte and macrophage colony production,

increased oxidative stress due to higher levels of reactive oxygen species and increased susceptibility of cells to apoptosis (Peled-Kamar, *et al* 1995). Increased oxidative stress itself has recently been shown to contribute to accelerated telomere loss *in vitro* (von Zglinicki 2002) and one could speculate that this may contribute to increased telomere loss in DS.

Although the association between HSC deficiency and leukaemia is established, the mechanisms which lead to leukaemogenesis are unclear. It is accepted that HSC deficiency will lead to accelerated telomere shortening which has been implicated in in genomic instability and increased risk of mutagenesis. If there is also an impaired DNA repair mechanism, then this could further increase the risk of mutagenesis. Individuals with DS have a functional folate deficiency due to increase in cystathionine B synthase (CBS) (figure 5.12) which is also encoded on chromosome 21 (Pogribna, *et al* 2001). The effect of increased levels of this enzyme are alterations in nucleotide biosynthesis which can result in increased DNA fragility and DNA strand breaks and decreased ability to repair damaged DNA, one could postulate that this be a further possible cause of increased predisposition to mutations in DS.

It should be noted that the over expression of SOD and CBS have thus far only been evaluated in postnatal life. Apart from one report of increased SOD in erythrocytes in fetal life (Aliakbar, *et al* 1993) the contribution of SOD and CBS levels to disturbances in cells in fetal life in DS has not been evaluated.

While this project was in progress, several papers were published documenting the presence of GATA1 mutations in the blasts seen in AMKL and TMD (Gurbuxani, *et al* 2004, Hitzler, *et al* 2003, Mundschau, *et al* 2003, Rainis, *et al* 2003). GATA 1 is a DNA binding transcription factor encoded on the X chromosome expressed primarily in erythroid cells, megakaryocytes, mast cells, and eosinophils where it is responsible for maturation of such cells (Hitzler, *et al* 2003). Identical mutations of GATA1 have been described in blasts in cases of TMD with subsequent development of AMKL (Miller, *et al* 1986). When the TMD regresses, the blasts disappear and the mutation is no longer detected but when AMKL develops the mutation reappears in blast cells.

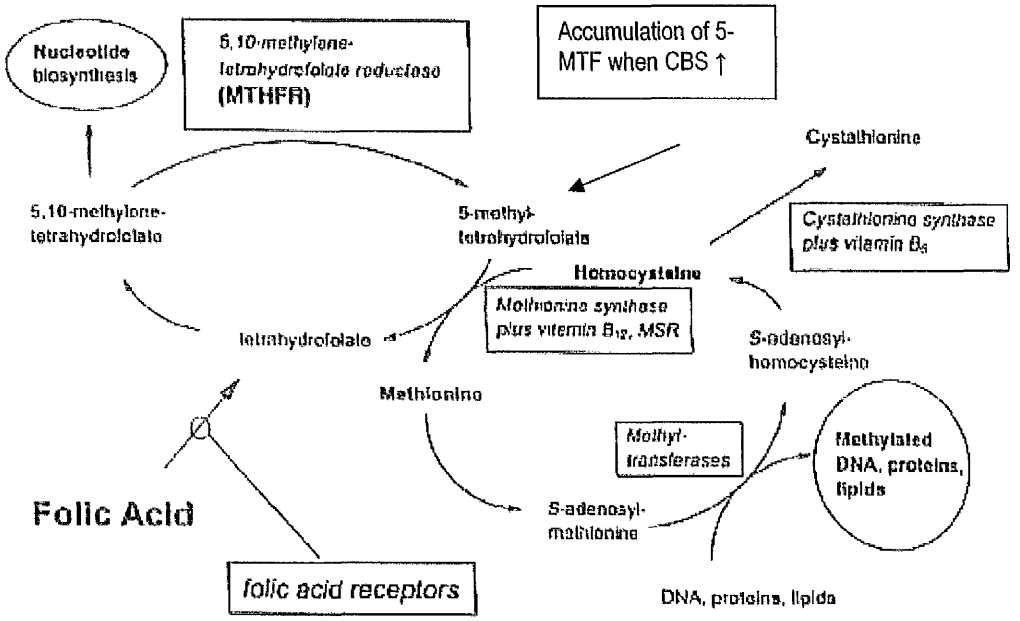


Image from Homocysteine metabolism in children with down syndrome: in vitro modulation, Pogribna M, Melnyk S, Pogribny I, Chango A, Yi P, James SJ Am J Hum Genet 2001 Jul;69(1):88-9. http://www.ds-health.com/abst/a0108.htm

Figure 5.12: Folate metabolism in Downs syndrome

In DS there is increased expression of the enzyme cystathionine synthase (CBS) which results in increased formation of cystathionine and depletion of homocysteine and methionine. Thus there is an accumulation of 5-methyl-tetrahydrofolate – the so called methyl trap – and less production of tetrahydrofolate leading to a functional folate deficiency even in the face of normal folate levels. Folate deprivation has been shown to lead to imbalance in the deoxynucleotide pool resulting in an increase in folate sensitive fragile sites and DNA strand breaks.

Moreover the same GATA-1 mutation was found in 2 out of 11 normal fetuses other than DS. This led to the hypothesis that GATA1 mutations were involved in the initiation of leukaemogenesis in DS.

To ascertain whether decreased stem cell numbers were associated with GATA1 mutations, I determined whether any of the fetuses in my study had mutations of GATA1 clustered within the first coding exon (exon 2). None of the DS fetal subjects in my study had demonstrable GATA1 exon 2 mutations but the term newborn with TMD/AMKL did have a mutation detectable. Although more fetuses should be examined to strengthen the correlation between low number of stem/progenitor cells and absence of mutations and different subset of cells should be analysed for mutations in GATA-1 to increase sensitivity of the assay. This result raises the interesting possibility that HSC deficiency in DS may predispose to an increased risk of mutations and that the appearance of the GATA1 mutation may be a secondary event arising in the face of HSC deficiency which confers a selective advantage to the clone in which it occurs and initiates leukaemia. A flow diagram of this proposed model is shown in figure 5.13.

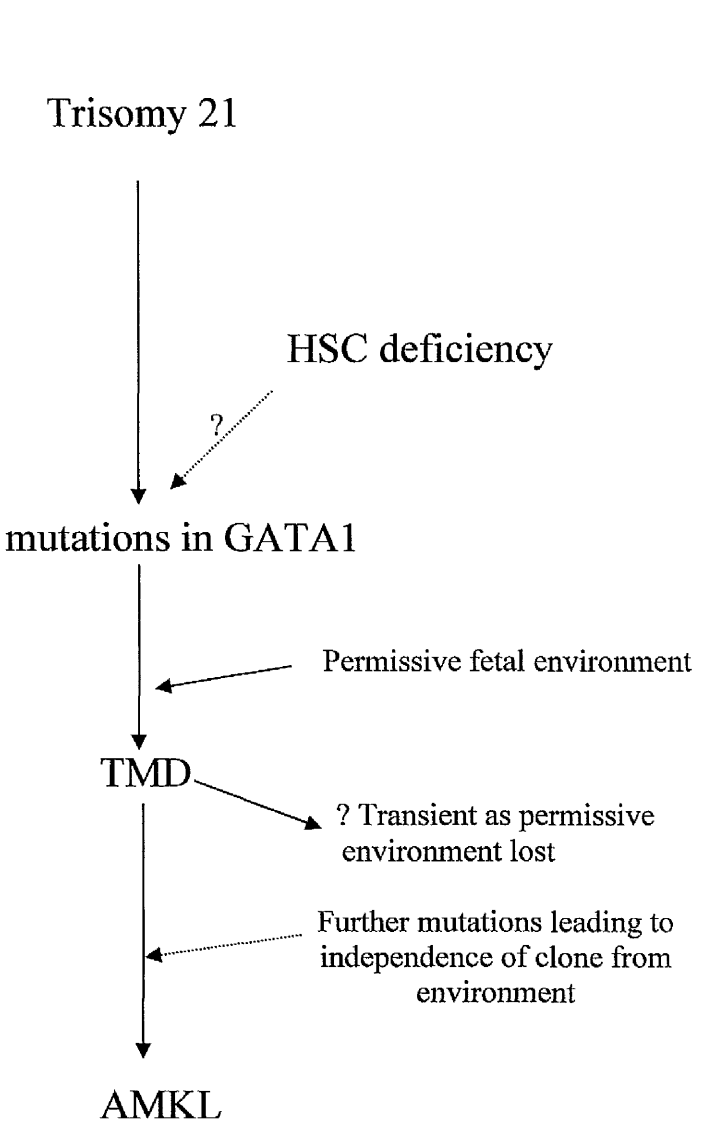


Figure 5.13. Possible model of leukaemogenesis in DS

Trisomy 21 occurs early in embryogenesis and is the proposed likely initiating event in leukaemogenesis (Guxburani et al, 2004). The second event to occur in fetal life is the GATA1 mutation leading to a block in differentiation of megakaryocytes. In addition, individuals with Trisomy 21 have reduced progenitor cell numbers. The combination of Trisomy 21 and the GATA1 mutation in HSCs in fetal life is sufficient to promote transient expansion of immature megakaryoblasts seen in TMD. The transient nature of the clone may be due to the loss of the supportive fetal liver environment after birth. However, the clone may still be present at a low level and acquisition of further mutations results in clone being less dependent on the fetal environment eventually results in AMKL

CHAPTER 6

EVALUATION OF MEASUREMENT OF TELOMERE LENGTH BY REAL TIME PCR

6 EVALUATION OF MEASUREMENT OF TELOMERE LENGTH BY REAL TIME PCR

6.1 Introduction

In previous chapters I have used a Southern Blotting technique to measure telomere length in all nucleated blood cells in a sample. While this technique remains useful, it cannot be used to determine telomere length in subsets of cells within the peripheral blood unless large samples are available. Thus there is a need for a robust technique which can be used to determine telomere length in small subsets of cells (e.g. CD34+ cells) within small samples. This would be helpful to further refine telomere dynamics in HSCs as it eliminates the bias due to changes in cell composition of blood in telomere analysis.

In 2002, a new way of measuring telomere using the quantitative real time PCR technique was described (Cawthon 2002). This PCR technique potentially offers a fast, relatively inexpensive method of measuring telomere length and only requires a very small amount of genomic DNA, a feature which makes this technique especially attractive in projects such as this where the sample size is small. It may also have the potential to be used to measure telomere length directly on very small cell numbers. This could help improve our knowledge of telomere biology as it may facilitate further studies in fetal and early life of HSC behaviour in different organs in fetal life.

The question addressed in this chapter is whether real time PCR offers significant advantages over the well established Southern Blotting technique which is the current gold standard, particularly in the setting of fetal and neonatal telomere length measurements where the sample size and thus cell numbers are small. In addition, this chapter will address whether this technique can permit us to look at telomere length in cells directly without the need for prior extraction of the DNA from the cells.

6.2 Real time PCR for telomere measurement

6.2.1 Confirmation of PCR product formation by primers

To test the specificity of the primers amplifying the telomere sequence of repeats, conventional PCR was used on DNA extracted from cells lines Jurkat and K562. As a control, reactions were set up containing E. Coli DNA which does not contain telomere sequence repeats. As a reference, to control for the amount of target DNA used, amplification of a housekeeping gene, the 36B4 gene, was also carried out. For the telomere primers, smears were seen as expected beginning at the smallest predicted size of 76bp, which is the combined length of the two primers and progressively fading up to 500bp, which is largest possible amplicon with real time PCR (figure 6.1). With the 36B4 primers a 74bp product was found as expected (figure 6.2). There was no amplification detected when E. Coli DNA was added to the reaction.

6.2.2 Optimisation of Standard Curve for real time PCR

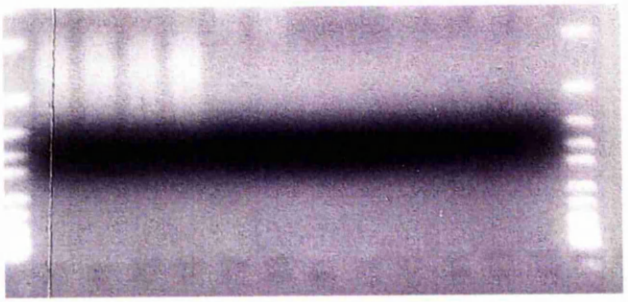
One of the most crucial steps in using the real time PCR technique for quantitation is to have an accurate standard curve, as the quantity of the target and references in each experiment are determined from this. Standard curves are generated by amplifying serial dilutions of a chosen sample and assessing the relationship between the logarithm of the amount of sample used and the Ct value obtained. A good standard curve is measured by two parameters. One is the efficiency of the amplification (which should be as near to two as possible). This depends mainly on the efficiency of annealing of the primers to the target DNA which in turn varies depending on the quality of the target DNA and the primer sequence. The second parameter is the correlation coefficient (which should be as near to one as possible) which establishes the correlation between input DNA and Ct. The efficiency of the reaction was calculated from the gradient of the line using the equation in section 2.8.9; the correlation coefficient of the relationship between the logarithm of the starting amount of DNA and Ct was also calculated. For reliable results there two parameters have to be highly reproducible among experiments.

I used two different dilution series of serial dilutions of DNA from K562 cells to establish the optimum dilution of standards to generate standard curves, a two fold

1 2 3 4 5 6 7

Lane
1 Size marker
2 Jurkat + 36B4 primers
3 K562 + 36B4 primers
4 E.Coli DNA + 36B4 primers
5 Jurkat –no primers
6 Jurkat –no Taq polymerase
7 Size marker

Figure 6.1: Gel electrophoresis of 36B4 PCR products



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lane	
1	Size marker
2+ 3	Jurkat + telomere primers
4+ 5	K562 + telomere primers
6+7	E. Coli + telomere primers
8+9	Jurkat – no primers
10+11	Jurkat –no taq polymerase
12+13	Water + telomere primers
14	Size marker

Figure 6.2: Gel electrophoresis of telomere PCR Products

dilution and a five fold dilution. With the two fold dilution, the final amounts placed in each well of the PCR plate ranged from 50ng to 3.125ng. Figure 6.3 shows the results of experiments using these dilutions of K562 DNA to generate standard curves for both telomere and 36b4 PCR reactions. In the example above, for 36b4 and telomere PCRs the gradients of the lines are -3.79 and -3.793 respectively. Using the equation, $E= 10^{(-1/gradient)}$, it can be seen that with this two fold dilution, the reactions were not of optimum efficiency (E= 1.85 for 36B4 and E=1.84 for telomere PCR and correlation coefficients were 0.93 and 0.96 respectively).

With the five fold dilution, the range of starting quantities was extended from 100ng to 0.16ng which represents a 600 fold total difference. This resulted an efficiency of 2.03 for 36b4 PCR and efficiency of 2.02 for the telomere PCR. There is also an improvement in the correlation coefficients of the standard curves with the 36b4 now having a correlation coefficient of 0.997 and the telomere 0.983 (figure 6.4). This sixteen fold dilution was then used in all subsequent PCR reactions.

6.3 Comparison of Southern Blotting and optimised quantitative PCR

Figure 6.5 shows correlation of relative T/S ratios determined by quantitative PCR and mean TRF lengths determined by Southern blot analysis in DNA samples from 16 individuals. The correlation coefficient is 0.71 indicating a strong positive linear relationship and the p value is 0.001.

6.4 Variability of real time PCR method for measuring telomere length

To test the reproducibility of telomere length measurement from well to well across the 96 well plate used for the PCR reactions, 17.5ng of DNA from K562 cells was added to each well of two 96 well plates and 36b4 and telomere PCRs carried out. Data was analysed according to formula outlined in Applied Biosystems methods and Cawthon 2003. The standard deviation of this relative T/S ratio, reflecting the well to well variation across the plate was 0.165. This gives a coefficient of variation of 16.5% which is satisfactory. This experiment was repeated at three different times and the results are summarised in table 6.1.

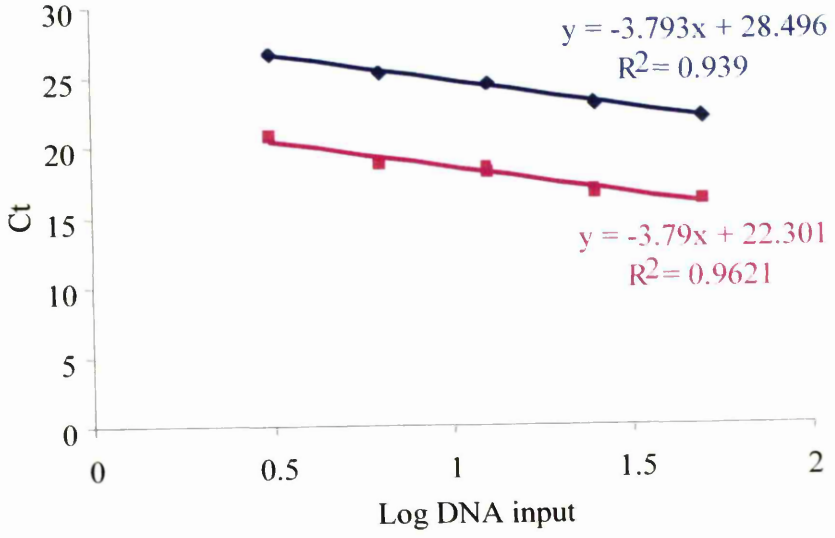


Figure 6.3: Standard curves using two fold dilutions of K562 standard in quantitative PCR experiment.

◆ telomere ■ 36B4

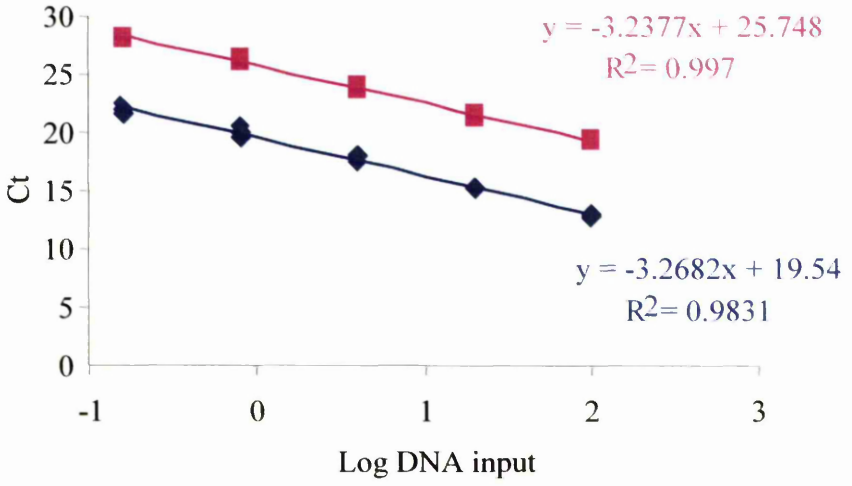


Figure 6.4: Standard curves using five fold dilution of K562 standards in quantitative PCR reaction.

◆ telomere ■ 36B4

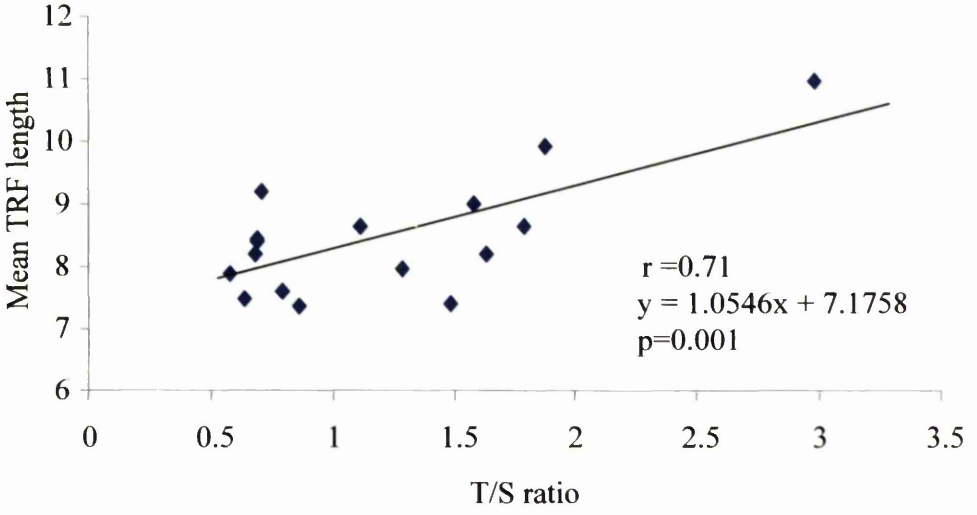


Figure 6.5: Comparison of real time PCR method and Southern blotting for measurement of telomere length in 16 DNA samples

Expt No	Mean ± SD Ct telomere Baseline (B) Threshold (T)	Mean ± SD Ct 36b4	Ave $2^{-\Delta\Delta Ct}$	SD of $2^{-\Delta\Delta Ct}$	Coefficient of variation
Expt 1 K562	16.31±0.18 B=3-12 T=0.1	24.5±0.2 B=3-15 T=0.09	1.03	0.165	16.5%
Expt 2 Jurkat	16.23±0.29 B=3-12 T=0.1	25.05±0.18 B=3-15 T=0.09	1.004	0.26	26%
Expt 3 K562	15.78±0.24 B=3-12 T=0.035	22.62±0.18 B=3-15 T=0.15	1.02	0.197	19.3%

Table 6.1: Reproducibility of real time PCR technique.

6.5 Real time PCR performed on cells directly

The potential advantage of performing real time PCR on cells directly without prior extraction of DNA is that it avoids any loss of DNA associated with the extraction procedure. Conventional Pars were performed on serial dilutions of cells (500 per well down to 1 per well) using 36B4 or telomere primers at the PCR conditions described in section 2.8.10. This confirmed that the primers did amplify DNA in cells, as decreasing amount of products of the expected size were seen with decreasing number of cells down to 10 cells/well visualised by gel electrophoresis in both cases (data not shown).

In view of the success of the conventional PCR using cells directly added to the PCR tube, I went on to perform real time PCR on cells by sorting cells directly into the PCR plates as described in section 2.8.10. Numerous experiments were performed and a typical standard curve for the reference gene is shown in figure 6.6. Despite multiple attempts, the efficiency of the reaction was always too low and I was unable to attain suitable correlation coefficients, suggesting that the technique at present cannot be accurately used on cells directly.

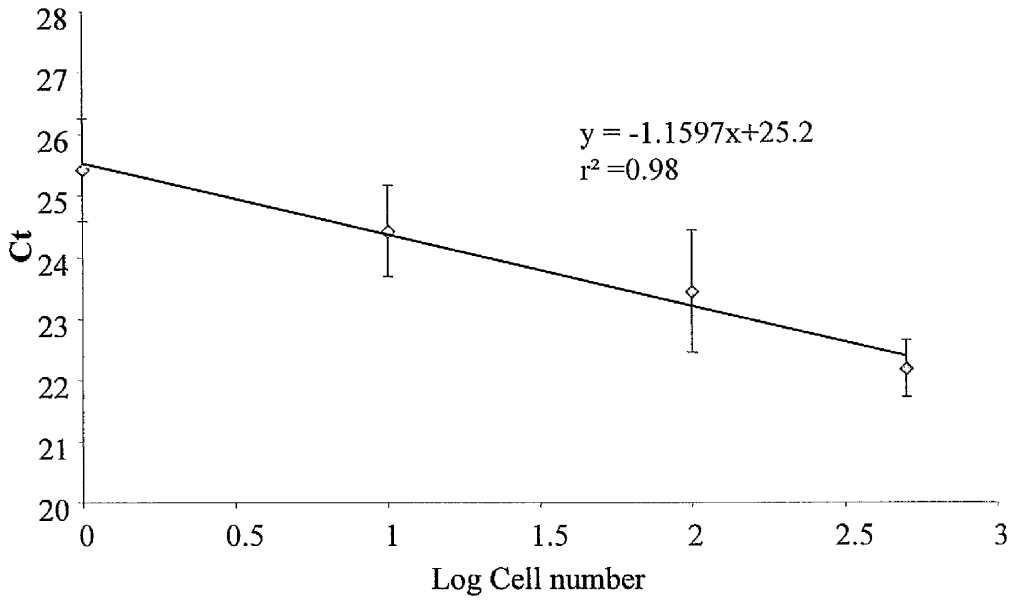


Figure 6.6: Standard curve of 36B4 PCR performed on cells directly.

6.6 Discussion

Although a powerful tool, real time PCR as shown in this chapter needs to be approached with extreme care. The PCR product increases exponentially and thus any errors in the set up of the reaction will increase with cycle number. When the quantification calculations are performed, the transformation of linear values further increases the variability. Thus critical and strict evaluation of the assay is needed to obtain reliable data. Having established that the primers for both the housekeeping gene (36b4) and telomere formed the expected PCR products, the first hurdle encountered on using this technique for measuring telomere length was generating a reliable and reproducible standard curve for quantitation. The importance of the standard curve is two-fold; firstly it allows relative quantitation of unknown samples because quantities of unknowns are expressed relative to a basis sample – the calibrator; secondly it provides an integral method of measuring the efficiency of the PCR reaction in each particular PCR plate which when considered with appropriate controls allow one to decide whether the plate is suitable for analysis. Five fold dilution of the standard from 100ng/well down to 0.16ng/well resulted in the greatest accuracy (in terms of efficiency and correlation coefficient) and reproducibility between different PCR reactions. Other steps to improve accuracy included storing standard curve DNA dilutions in small volume aliquots containing sufficient DNA for performing reaction in triplicate and allowing only one freeze/thaw cycle of this aliquot. Similarly, DNA from unknown samples was stored in high concentration and when diluted to 1.75ng/µl for use in the assay, small volume aliquots were stored and only one freeze/thaw cycle permitted. This is in accordance with trouble shooting guideline issued by Eurogentec (suppliers of Sybr Green core kit) and Applied Biosystems (suppliers of ABI7700 Taqman equipment).

With the standard curve optimised, a set of criteria were established for each PCR type to determine whether a plate was accurate enough for quantitation analysis. For 36b4 PCR plates were accepted if the slope of standard curve was -3.32±0.18, if thr correlation coefficient equal to, or greater than 0.99 and there was no amplification in the no template control wells with settings of baseline of 3-15 and threshold of 0.15. For telomere PCR, plates were accepted if the slope of standard curve was -3.32±0.2, and the correlation coefficient equal to or greater than 0.98 and there was no

amplification in the no template control wells with a baseline setting of 3-12 and a threshold of 0.035. The slightly lower value of correlation coefficient in the telomere PCR may reflect the slight inaccuracy involved with a bigger amplicon. The recommended amplicon size is 80-150bp (Applied Biosystems, Product literature). The telomere amplicon may actually be longer than this as indicated by the gel electrophoresis in figure 6.2. When the first telomere primer binds to complementary DNA, the second primer may bind at either the first available adjacent site or slightly further downstream due to the repeating TTTAGG sequences of telomeres. At the beginning of the PCR reaction when all components of the reaction including the primers are in excess, then one would expect that both primers would bind to all available sites – thus the most abundant product will be 76bp product but there will also be products of varying increasing lengths up to 500bp which is maximum length of amplification product expected with real time PCR.

With fully optimised conditions, the real time PCR method of measuring telomere length showed acceptable reproducibility (CV =16.5%) and good correlation with Southern Blot analysis for the same DNA samples. A correlation coefficient of 0.7 with a p value of 0.001 indicates a significant positive correlation between T/S ratios (PCR telomere quantity measurement) and mTRF length measured in the 16 samples compared. This value is similar to that achieved by Cawthon ($r = 0.677$, $p<0.0001$) on a larger sample of 95 individuals. This compares favourably with correlation of other methods to Southern Blotting. The Q-FISH method of telomere length measurement has been shown to have correlation of 0.79 with mTRF by southern blotting in the study by Ferlicot et al (Ferlicot, *et al* 2003) and a variability of around 12%. Techniques such as automated Flow-FISH have been shown to have a similar correlation of 0.87 when compared to southern blot TRF length (Baerlocher and Lansdorp 2003) and a coefficient of variation of around 4%. However, the initial cell input required with these techniques is in the order of a million cells. Thus one can conclude that the real time PCR method would be advantageous for measuring telomere length of DNA from haemopoietic cells.

Having established a good correlation between the techniques, this quantitative PCR method was further investigated to determine whether it could be used to perform

telomere length measurements on cells directly without having to extract DNA from the cells. Although the pilot experiments performed by conventional PCR to determine whether cells could be added directly to the PCR reaction showed promising results, the experimental findings with the real time PCR were not reproducible. My first attempt to improve the efficiency and accuracy of the results was to perform the PCR immediately following the cell sorting procedure. As a control, I included K562 DNA standards in each 96 well plate as well as decreasing cell numbers. However, despite good efficiency being noted in wells containing DNA standard curve samples, the reactions occurring in wells containing just cells did not show efficient amplification and hence did not demonstrate accurate standard curves.

There are several reasons why the reaction may have reduced efficiency in this situation. If the DNA in cells in each well of the plate is not fully accessible to primers, then reduced binding of primers will result in suboptimal amplification, and by the time the reaction has got to a stage where there is doubling of product with each cycle, other reagents are limiting and thus efficiency is poor. Thus, in an effort to make DNA more accessible, I tried freezing plates at -20°C overnight or for five days before performing PCR. The rationale behind this step was that freezing may cause shearing of DNA into fragments which may improve reaction by allowing primers to find binding sites more readily but again I was unable to demonstrate any improvement in reaction efficiency. The next consideration was whether the medium in which the cells were suspended for sorting could contain elements which interfere with PCR reaction. Cells were suspended in RPMI with 10% fetal calf serum. Although the amount of medium added to each well in a cell sorting procedure is very small, one possibility is that the small amount of protein present interfered and inhibited the PCR reaction. As already stated real time PCR is an incredibly sensitive technique, thus even tiny amounts of interfering substances may have significant effects. To try and prevent this, cells were washed and suspended in PBS rather than medium prior to cell sorting. However, there was no improvement in reaction efficiency.

Finally I tried combining all the above modifications, thus suspending cells in PBS, performing cell sort, sealing plates with adhesive seal and foil to prevent loss of contents and freezing for 5 days at -20°C as each of these measures individually seemed to improve the reaction slightly. However, this combination of procedures did not result in an improvement in the reaction.

Thus in the time available for this project it was not possible to fully optimise the real time PCR technique for use on cells directly without prior extraction of DNA. Inability to appropriately sonic ate the DNA and most importantly the cell debris released from the burst of the cells in the PCR tube during the reaction may be two major factors contributing to the lack of success. However, extraction of DNA is now possible with as little as 15000 cells using specialised kits which yield 100ng of DNA. This represents a great improvement to previously described techniques.

CHAPTER 7

CONCLUDING REMARKS

7 CONCLUDING REMARKS.

The main hypothesis of this work is that in DS accelerated HSC aging begins in fetal life before the onset of clonal disorders and leads to HSC deficiency which may contribute to the increased risk of leukaemia. This was based on the observation that TMD can be present in newborns with DS (Lange 2000), suggesting that any disturbance in the haemopoietic system arises in fetal life. Moreover increased telomere shortening was reported in leucocytes of children affected by DS and a deficiency of fetal liver HSCs was previously described in the murine model of DS (Epstein, *et al* 1985). This suggests that in DS the HSCs undergo accelerated aging. In order to study HSC aging I used telomere length studies as a surrogate marker of HSC replication as measurement of telomere length has been shown to correlate with aging and replicative lifespan (Harley, et al 1990, Vaziri et al 1994). Although there are various factors contributing to telomere length in addition to losses encountered by cell division, it is still a good marker to determine the replicative lifespan of a cell and thus study cellular aging.

As there is very little data available on HSC replicative dynamics in fetal life I have studied the telomere kinetics in fetal peripheral blood leucocytes during the second and third trimester of gestation. Previous studies have shown that as long as the blood cell composition remains constant, changes in telomere length in blood cells with time reflect changes in HSCs. I demonstrated that in fetal life, there was no change in telomere length with increasing gestational age, a somewhat surprising finding considering the enormous growth rates of the fetus at this time of life, which exceeds even the growth rates in infancy and childhood. The lack of telomere decline seen in this time period is all the more intriguing when one considers that cell turnover is faster in fetal life and other investigators have shown decline in telomere length in adult bone marrow haemopoietic cells compared to cord blood or fetal liver haemopoietic cells using a similar Southern Blotting technique to measure telomere length (Vaziri, *et al* 1994). This was a phenomenon typical of fetal life and not due to an inadequate technique. Indeed I demonstrated there was telomere shortening above the limit of detection of the technique in a similar time period in postnatal life, similar to findings of previous investigators (Friedrich, *et al* 2001)

It has previously been described that fetal and postnatal HSCs have different functional properties by study of their cell cycle activity, response to cytokines and their proliferative capacity *in vitro* and *in vivo,* (Christensen 1988, Hao, *et al* 1995, Holyoake, *et al* 1999, Lansdorp 1995a, Peschle, *et al* 1981, Weekx, *et al* 1998) however, this was thought to be the result of ontogenic age. The novelty of this work is that changes in HSC replicative dynamics are not linked to ontogenic age but to birth. It is unclear whether this change in behaviour is caused by a change in the properties of HSCs triggered by the event of birth or if it is due to switch to a different population of HSCs at the time of birth.

Telomere length studies may be useful in determining whether two populations of HSCs generated separately at different sites contribute to fetal and adult haemopoiesis or the same population sequentially migrate from the liver where most of fetal haemopoiesis occurs to the bone marrow where adult haemopoiesis resides. The argument for use of this technique is as follows, if the same population are responsible for haemopoiesis at each site then the HSC migrate sequentially. This population of HSCs would therefore have a similar replicative and environmental history and thus similar mTRF values. If, however, HSC populations are generated *de novo* at each site, then it is possible that in fetal life while one site such as the liver is the main organ of haemopoiesis, the mTRF of liver HSCs would be shorter than mTRF of fetal bone marrow HSC which are being laid down for future use but are not actively proliferating. So far there has been little done in this area. Very little direct comparison has been made between HSCs in fetal liver and fetal bone marrow. Of interest is a study by Lim et al (Lim, *et al* 2005) where similar numbers of CD34+ cells or CD34+38-cells were found in the fetal liver and bone marrow in the second trimester of gestation. However, cells in the fetal liver showed far greater clonogenic potential than cells from fetal bone marrow suggesting that fetal bone marrow HSCs are in a quiescent state. Telomere kinetics has never been directly compared between the two sites. The only data available are from Vaziri et al (Vaziri, *et al* 1994). This group performed a cross sectional study comparing telomere length in haemopoietic cells from fetal liver, cord blood and adult bone marrow. Cells were obtained from pooled samples due to the small size of the fetal samples. To have more pertinent information mTRF measurement in HSCs from fetal liver and bone marrow in same

fetus at same gestational age points should be examined to ascertain whether differences are present between the two sites. However, to achieve this in addition to the challenges of obtaining the appropriate material to work with, the present technology is unable to obtain significant results with the small numbers of cells obtained from fetal liver and bone marrow samples. Hence I optimised a method of measuring telomere length by using a real time PCR technique. As the quantity of DNA needed for the procedure is less than other methods, smaller sample sizes should be adequate. I was able to demonstrate that I could obtain reliable results with as little as 105ng of DNA using this method in contrast to the 2 micrograms needed for Southern Blotting. Although I was not successful in optimising this real time PCR method to measure telomere length in individual cells, with the current optimised method, telomere length could be measured in as little as 15000 cells which should be in the range of the number of CD34+ cells that could be obtained from a fetal liver or fetal bone marrow sample.

The telomere studies performed here were a background to investigate the HSC replicative dynamics in DS individuals in fetal life. Although telomere length seems to be protected from shortening in fetal life, a marked decrease was seen in DS peripheral blood leucocytes compared to haematologically normal controls suggesting that accelerated telomere shortening was present in DS in fetal life. Moreover, similar to DS individuals in postnatal life, fetal subjects with DS had fewer stem/progenitor cells than age matched non-DS controls, suggesting that stem/progenitor cell deficiency with increased turnover of remaining cells is present in this condition. Unfortunately, due to small sample sizes and time limitations it was not possible to perform more primitive stem cells assays such as the long term culture initiating cells (LTC-IC) assay or repopulation assays. However, similar studies performed in our laboratory on bone marrow of children with DS show decreased number of LTC-IC (Ilaria Bellantuono, personal communication). One caveat of this study is that the peripheral blood cell composition in DS differs from the TOP controls and this may potentially bias the study. This is unlikely to change the result of the study as the difference is mainly confined to the lymphoid cells and is small whereas the difference in telomere length is quite pronounced. However, it would also be important to determine the telomere length in the CD34 cell subset in

these individuals to confirm accelerated telomere loss in the stem/progenitor cell population. With the optimisation of the real time PCR technique this may now be feasible.

The accelerated telomere shortening and decreased numbers of haemopoietic stem/progenitor cells in DS in fetal life suggests that the mechanisms underlying haemopoietic disturbance in DS are similar to disorders such as aplastic anaemia, Fanconi's anaemia and ataxia telangiectasia which are associated with an increased occurrence of leukaemia. In DS in the last few years mutations in the inactivation domain in exon 2 of GATA-1 have been found in most of DS leukaemia and in about 10% of randomly selected and otherwise normal DS neonates. This has led to the suggestion that GATA-1 mutations could be an early event in the emergence of leukaemia (Ahmed, *et al* 2004). However, the absence of a concomitant GATA-1 mutations in the DS fetuses examined here suggest that, although GATA-1 mutation is an important step in DS leukaemia development, it may not be the primary event and a reduced number of stem cells may be the initial predisposing factor to the development of leukaemia.

As yet, it is unclear why GATA1 mutations lead to TMD and why the TMD undergoes spontaneous regression with only supportive treatment. One possibility, which is highly speculative, is that the truncated GATA1 protein produced as a result of mutations in GATA1 offers a proliferative advantage only to fetal liver HSC and not postnatal HSCs. In support of this recent work in a transgenic murine model by Orkin's group demonstrated that mutated GATA1, which results in a short form of GATA1 (GATA1s), led to hyperproliferation of a yolk sac and fetal liver but not bone marrow haemopoietic progenitors. They hypothesis that the presence of GATA1s in fetal progenitor cells confer a proliferative advantage to the haemopoietic progenitors which are responsible for the occurrence of TMD and its transient nature (Ginzinger 2002) as the clone switches off when fetal haemopoiesis ceases.

However, this study does not explain why the leukaemia re-emerges in some children with DS who presented with DS in infancy. There are two possibilities; either the

same clone of cells is responsible for both TMD and subsequent AMKL or that there are separate clones responsible for the TMD and subsequent AMKL. Of the evidence available, the clonal evolution model is favoured (Hitzler, *et al* 2003, Rainis, *et al* 2003). One possibility is that following the resolution of TMD there are small numbers of the clone remaining which do not have a proliferative advantage in postnatal life. However, there is then accumulation of further mutations which makes the clone progressively less dependent on its environment and eventually results in AMKL. This is in line with the way tumorigenesis is thought to occur as a multi-step process, with mutations accumulating in tumour cells that favour environmental independence (Smalley, *et al* 2005).

In conclusion, this work has demonstrated that telomere length changes in fetal peripheral blood leucocytes undergo very little or no overall loss in a period of great HSC expansion and this differs from postnatal life suggesting that fetal HSCs may be a good model to study stem cell expansion in the absence of aging. This would represent a great advance in cell transplantation and gene therapy. More importantly this has highlighted DS as a good model to study HSC aging and its role in leukaemogenesis since accelerated telomere shortening associated with a stem/progenitor cell deficiency has been found before the onset of clonal evolution or GATA1 genetic mutation which has been proposed to be associated with the initiation of leukaemia in DS.

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