Genetic and Epigenetic Factors in Mouse Radiation Induced Leukaemogenesis

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by

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ABSTRACT

Studies of radiation induced acute myeloid leukaemia (r-AML) susceptible CBA/H and r-AML resistant C57BL/6 inbred mice have provided an animal model that allows investigations into genetic contribution to r-AML. Genetic linkage analyses and loss of heterozygosity studies identified 2 r-AML predisposition and 2 tumour suppressor gene loci (Boulton et al., 2003; Cleary et al., 1999; 2001), suggesting radiation leukaemogenesis includes both direct effects (chromosomal deletions) and indirect genetic factors (target size, radiosensitivity and genetic instability). This study concentrates on (1) the r-AML susceptibility locus on distal chromosome 1 which overlaps with a genetically determined locus that modulates mouse bone marrow stem cell frequency and (2) a 3.4cM region on chromosome 4 that is deleted in many mouse leukaemias and lymphomas.

Fluorescence activated cell scanning of phenotypically defined bone marrow haemopoietic stem/progenitor cells showed that CBA/H mice have more stem/progenitor cells than C57BL/6 mice. Subsequent analyses of bone marrow cell death and recovery following whole body exposure to an in vivo single acute dose of 3Gy X-rays concluded that this difference is maintained post irradiation, so radiosensitivity is not a confounding factor. Analysis of candidate genes identified Ephx1 (chromosome 1) as a possible candidate although its role in radiation is unclear. Pax5 (chromosome 4) exhibited inactivation in radiation induced malignancies by chromosome deletion and/or Pax5 promoter methylation.

Radiation leukaemogenesis results from the accumulation of mutations over time. The target cell in r-AML is the haemopoietic stem cell (HSC) and this study proposes that not only is target cell number a cancer risk factor, but HSC proliferation in the immediate aftermath of exposure makes HSCs particularly susceptible to transformation. There is increasing evidence that aberrant differentiation gene expression contributes to leukaemogenesis, and this study also proposes Pax5 inactivation by allelic loss and promoter methylation confers a selective advantage in leukaemogenesis.
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ABBREVIATIONS

ALL    Acute Lymphoblastic Leukaemia
AML    Acute Myeloid Leukaemia
BCL    B Cell Leukaemia
BER    Base Excision Repair
BP     B Cell Progenitor
BSAP   B cell Specific Activator Protein
BTTL   Mixed B and T cell Thymic Lymphoma
BTL    Single Lineage B Cell Thymic Lymphoma
CAFC   Cobblestone Area Forming Cell
CFU-S  Spleen Colony Forming Unit.
CLL    Chronic Lymphocytic Leukaemia
CLP    Common Lymphoid Progenitor
CML    Chronic Myeloid Leukaemia
DNMT   DNA Methyltransferase
EAR    Excess Absolute Risk
Ephx1  Microsomal Epoxide Hydrolase 1
FA     Fanconi Anaemia
FACS   Fluorescence Activated Cell Scanning
Fancg  Fanconi Anaemia Complementation Group G
FITC   Fluorescein Isothiocyanate
GM-CSF Granulocyte-Macrophage Colony Stimulating Factor
Gy     Gray
HRR    Homologous Recombination Repair
HSC    Haemopoietic Stem Cell
ICF    Immunoeficiency, Centromeric Instability, Facial Anomalies.
IgH    Immunoglobulin Heavy chain
LET    Linear Energy Transfer
Lin    Lineage
L-ML   Early Pre B Lympho-Myeloid Leukaemia
LOH    Loss of Heterozygosity
LSC    Leukaemic Stem Cell
LTC-IC Long Term Culture Initiating Cell
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<td>LT-HSC</td>
<td>Long Term Haemopoietic Stem Cell</td>
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<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>MDR</td>
<td>Minimally Deleted Region</td>
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<tr>
<td>MM</td>
<td>Multiple Myeloma</td>
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<td>MP</td>
<td>Myeloid Progenitor</td>
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<td>Myeloperoxidase</td>
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<td>QTL</td>
<td>Quantitative Trait Locus</td>
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<td>r-AML</td>
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<td>RBMC</td>
<td>Red Bone Marrow Cell</td>
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<td>SCF</td>
<td>Stem Cell Factor</td>
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<td>SCFR</td>
<td>Stem Cell Frequency Regulator</td>
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<td>TL</td>
<td>Thymic Lymphoma</td>
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<tr>
<td>TSG</td>
<td>Tumour Suppressor Gene</td>
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<td>WBMC</td>
<td>White Bone Marrow Cell</td>
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1 Chapter 1: Introduction

1.1 Carcinogenesis.

Carcinogenesis is a multistage process primarily resulting from the accumulation of somatic mutations in a cell over time (Vogelstein & Kinzler, 1993). Transformation of a normal cell into a malignant cancer cell may require up to 3-7 independent mutations in one cell (Knudson 1971; Strachan & Read, 1999; reviewed Bertram, 2001). Mutations are rare (~10^{-6}/gene/cell cycle division; Strachan & Read, 1999) and therefore the accumulation of mutations is a time consuming process. As a result, there is a dramatic increase in the incidence of human cancer with age (Vogelstein & Kinzler, 1993).

Cancer risk is a combination of environmental, genetic and epigenetic factors. Exposure to environmental factors such as ionizing radiation, chemicals and ultraviolet rays, increases DNA damage which can become fixed and replicated into an inheritable change in DNA if incorrectly repaired (reviewed Bertram, 2001). Malignant transformation is promoted by mutations which confer a selective advantage to the cell such as those that increase proliferation or resist apoptosis (reviewed Bertram, 2001). Additional mechanisms contributing to malignancy are epigenetic changes including DNA methylation which have important roles in gene transcription (Plass, 2002).

1.1.1 Cancer Genes

Gene mutations that promote carcinogenesis can be sub-classified into mutations which cause ‘gain of function’ and mutations which cause ‘loss of function’ in the protein encoded by the gene. Proto-oncogenes promote cell proliferation in normal cells. Mutations that cause proto-oncogene activation promote the transformation of a normal cell into a malignant cell. These ‘gain of function’ oncogene mutations are dominant and therefore a mutation in one allele is sufficient to alter the functional activity of the gene product. Oncogenic ‘gain of function’ mutations often lead to increased proliferation, enhanced cell growth and reduced apoptosis – processes that would be predicted to promote cancer development. If there is a selective advantage for the mutation then the abnormal cell type will increase in number. The net result of
this is an increase in the number of target cells for subsequent mutations thus increasing the chance of malignant progression (Strachan & Read, 1999).

Tumour suppressor gene (TSG) products protect cells from malignant transformation by ensuring that DNA damage is repaired or that programmed cell death (apoptosis) is induced. TSG ‘loss of function’ mutations therefore reduce a cell’s ability to maintain genomic stability. Both alleles need to be inactivated to result in ‘loss of function’ (Knudson, 1971). In familial cancer susceptibility syndromes, loss of one TSG allele is generally silent and allows the germline inheritance of the mutated allele to be maintained in the population (reviewed Bertram, 2001). The mutation is recessive and therefore a second somatic ‘hit’ is required for gene inactivation. Malignant progression requires that loss of TSG function confers a selective advantage (Strachan & Read, 1999).

1.1.2 Genetic Predisposition to Cancer

Individual susceptibility to a particular cancer is influenced by the inheritance of high and/or low penetrance alleles that affect risk. Familial cancer predisposition syndromes are the result of the inheritance of high penetrance alleles transferred through the germline so that each cell in the body contains the mutation and thus increases the risk of any one cell developing malignancy (Strachan & Read, 1999). Chromosome breakage syndromes such as Fanconi Anaemia and Ataxia-Telangiectasia, are human autosomal recessive disorders characterised by inherited chromosomal instability and predisposition to cancer (Tischkowitz & Dokal, 2004; Boultonwood, 2001).

Fanconi Anaemia complementation group G (FANCG), one of the 11 separate complementation groups that make up the Fanconi Anaemia (FA) pathway, is a putative candidate gene in this study (Chapter 6). FA is characterised by bone marrow failure and a predisposition to Acute Myeloid Leukaemia (AML). The FA pathway functions as a DNA damage response system and FANCG is implicated in interstrand DNA cross-link repair. Double stranded DNA lesions in FA cells obstruct DNA replication and, because both strands are modified, there is no template for repair. This results in a block in both transcription and DNA replication. The inability of FA
cells to repair DNA damage or undergo apoptosis results in genetic instability and thereby results in accumulation in genetic lesions that promote cellular transformation (reviewed Wang & D’Andrea, 2004; Tischkowitz & Dokal, 2004).

Some alleles with low penetrance also confer a predisposition to cancer. Polymorphic variations have been identified in genes involved in carcinogen detoxification leading to susceptibility to chemotherapy/radiotherapy related AML (t-AML). Such genes encode detoxifying enzymes such as NAD(P)H:quinone oxidoreductase, cytochrome P450 3A4, epoxide hydrolase and glutathione S-transferase P1 (Larson et al., 1999; Felix et al., 1998; Lebailly et al., 2002; Allan et al., 2001). Microsomal epoxide hydrolase (Ephx1) is a putative candidate gene discussed in Chapter 5. The most common environmental compounds metabolized by Ephx1 are epoxide derivatives of polycyclic aromatic hydrocarbons such as benzene. Benzene induces chromosomal aberrations and AML (Catovsky & Hoffbrand, 1999) and distinct Ephx1 protein coding genetic polymorphisms have been genetically associated with several types of human cancer (Lebailly et al., 2002; Cajas-Salazar et al., 2003; Park et al., 2003).

1.1.3 Methylation.

DNA methylation is a heritable epigenetic modification of DNA. A methyl group from S-adenosyl methionine is transferred to the 5' position of the purine ring of cytosine in a reaction catalyzed by DNA methyltransferases (reviewed Worm & Guldberg, 2002). In mammalian cells, ~3-5% of genomic DNA cytosine residues are present as 5-methylcytosine and 70-80% of these are present in the dinucleotide sequence 5’- CpG – 3’ (reviewed Momparler & Bovenzi, 2000). Methylation of cytosine is mutagenic. Spontaneous deamination of 5-methylcytosine generates a cytosine (C) to thymine (T) transition mutation. Methylation also enhances ultra violet light absorption thus increasing the number of CC to TT mutations, as well as enhancing carcinogen binding causing DNA adducts and guanine to thymine transitions (reviewed Jones & Baylin, 2002). The net result is CpG dinucleotide depletion throughout evolution as demonstrate by the under representation of CpG dinucleotides in the genome (genome average ~40%) (reviewed Plass, 2002).
The distribution of CpG dinucleotides in the genome is unequal with 80% of all CpGs being found in repetitive DNA regions including Line 1 elements and Alu repeats (reviewed Chim et al., 2002; Robertson & Wolffe, 2000). Short (~0.2-1Kb) CpG dinucleotide rich genomic regions called CpG islands are also located in the genome (~37,000 in the mouse haploid genome) (Antequera & Bird, 1993). CpG islands constitute ~1% of the genome and contain more than 50% of the unmethylated CpG dinucleotides (reviewed Robertson & Wolffe, 2000). These CpG islands are often located in the 5' regulatory regions of genes and can extend into the first exon and intron. The methylation status of the CpG dinucleotides correlates with gene transcription. Unmethylated CpG dinucleotides result in the gene in a transcription-ready state while methylation of the CpG dinucleotides is associated with gene silencing (reviewed Robertson & Wolffe, 2000; Plass, 2002).

1.1.3.1 Importance of DNA Methylation

Differential DNA methylation is a critical signal for mammalian gene imprinting. Imprinting is the monoallelic expression of a biallelic gene whereby allelic exclusion has occurred according to the parent of origin. This means that the repressed allele always comes from the same parent. This monoallelic expression is faithfully transmitted to all daughter cells thus maintaining the same pattern of methylation throughout generations (Strachan & Read, 1999). Imprinting patterns are established during embryogenesis and sex-specific differences are observed. Supported by evidence that sperm DNA is methylated compared to oocyte DNA, methylation is considered a good candidate for differential imprinting (Sanford et al., 1987; Monk et al., 1987). An important role of DNA methylation is in X-inactivation. As females have two X chromosomes and males only one, then every normal female somatic cell must have one X chromosome silenced to compensate for dosage differences (sexual conflict). Unlike imprinting the inactivation of maternal or paternal X is independent of parental origin (reviewed Ehrlich, 2003).

DNA methylation also has a role in genome defence. Hypermethylation of genomic parasitic DNA elements (endogenous retrotransposons) has been implicated in the silencing of their expression and this restricts their spread throughout the genome. Strict control of retrotransposons is essential as retrotransposition can disrupt the
It is generally accepted that DNA methylation plays a major role in controlling gene expression. DNA promoter methylation effects histone modification and chromatin structure (reviewed Jones & Baylin, 2002). An open chromatin configuration permits transcription of a gene as the promoter region is accessible to transcription factors. Conversely methylation of a gene promoter condenses the chromatin and creates a closed chromatin conformation unaccessible to transcription factors and thus silencing transcription (reviewed El-Osta, 2003).

1.1.3.2 DNA Methyltransferases

The establishment and maintenance of DNA methylation is dependent on DNA methyltransferase enzymes (DNMTs). There are at least 4 DNA methyltransferases - DNMT1, DNMT2, DNMT3a and DNMT3b. DNA methyltransferases transfer the methyl group provided from S-adenosyl methionine to cytosine. They can be subclassified into (I) enzymes involved in 'de novo' methylation which occurs predominantly during early development and establishes embryonic methylation patterns, and (II) enzymes involved in 'maintenance' methylation and responsible for the methylation of hemi-methylated DNA following DNA replication (reviewed Robertson & Wolffe, 2000).

DNMT1 is a 'maintenance' methyltransferase and is the most abundant DNA methyltransferase in somatic cells. It has a preference for hemi-methylated DNA, localizes to replication foci and interacts with the proliferating cell nuclear antigen (PCNA). It also has an essential role in embryonic development, imprinting and X-inactivation (reviewed Chim et al., 2002; Robertson & Wolffe, 2000).

DNMT3a and DNMT3b are highly expressed in undifferentiated embryonic stem cells and at lower levels in somatic tissues (M.Okano et al., 1999). They exhibit equal preference for hemi- and unmethylated DNA and have a role in de novo methylation following embryo implantation and following the integration of retroviral sequences in mouse embryonic stem cells (reviewed Robertson & Wolffe, 2000). Although
DNMT3a/b exhibit redundancy in their de novo methylation function, they may demonstrate some functional specificity. DNMT3b is specifically required for methylation of centromeric minor satellite repeats and loss of this activity is characteristic of human ICF Syndrome (Immunodeficiency, centromeric instability, facial anomalies) where hypomethylation of centromeric satellites and centromeric heterochromatin instability is caused by a mutation in the DNMT3b gene (Okano et al., 1999).

The protein encoded by the DNMT2 gene contains many of the conserved DNA methyltransferase motifs but does not exhibit any enzyme activity required for de novo or maintenance DNA methylation (Okano et al., 1998). Purified human DNMT2 protein has a weak DNA methyltransferase activity but its function is currently unclear (Hermann et al., 2003).

1.1.3.3 Role of DNA Methylation in Cancer

Aberrant DNA methylation is found in many human tumours. It includes an overall global reduction in DNA methylation (hypomethylation) and a regional gene-specific increase in DNA methylation (hypermethylation). Analysis of the 5’methylycytosine content of genomic DNA of cancerous tissue compared to normal tissue has demonstrated that DNA hypomethylation is common in human cancer. Most of this hypomethylation occurs in repetitive DNA elements such as satellite repeat sequences in centromeric regions and endogeneous retrotransposable elements (i.e. Line-1 elements) (reviewed Erhlich, 2002).

Global hypomethylation influences genomic instability. Tumours showing global hypomethylation frequently display structural instability of the satellite DNA in the large juxta-centromeric heterochromatin regions of chromosome 1 and 16 as evidenced by karyotypic analysis of human ICF syndrome cells (reviewed Robertson & Wolffe, 2000). Genetic instability will accelerate the carcinogenic multi-stage process. Supportive evidence comes from transgenic mice engineered to express a hypomorphic Dnmt1 allele. This allele reduces Dnmt1 expression to 10% of that of wild-type levels and these transgenic mice show genome-wide hypomethylation of all tissues and the
development of aggressive T cell lymphomas with a high frequency of chromosome 15 trisomy (Gaudet et al., 2003).

Promoter hypermethylation of tumour suppressor genes (TSG) results in the inactivation of one allele. While promoter methylation may account for one 'hit', a mutation or deletion can account for the second 'hit'. Alternatively both 'hits' can be supplied by biallelic methylation (Veigl et al., 1998). Well documented examples of tumour suppressor gene inactivation by hypermethylation include *p15* (acute leukaemia and myelodysplasic syndrome), *p16* (non-Hodgkin's lymphomas), *E-Cadherin* (acute leukaemia), and *Calcitonin* (Acute Myeloid Leukaemia) (reviewed Chim et al., 2002; Ekmekci et al., 2004). Hypermethylation of specific gene promoters may confer a selective advantage during carcinogenesis and consequently enable the survival and subsequent progression from pre-malignant cell to full malignancy (reviewed Esteller, 2002).

Compared to normal tissues, DNA methyltransferases are over expressed in several solid and haematological malignancies (Robertson et al., 1999). The increased activity of DNA methyltransferases contrasts to the overall global hypomethylation seen in tumours (reviewed Esteller, 2002). However, increased DNA methyltransferase activity in cancer cells may simply reflect cell proliferation as tumours are by definition comprised of proliferating cells. DNA methyltransferases levels in cancer therefore require adjustment for cell proliferation rates. Many studies use PCNA (proliferation-associated marker) expression to compensate for cell proliferation and these studies indicated that DNA methyltransferases are over expressed in some tumours (Robertson et al., 1999) and down regulated in others (Kimura et al., 2003). In all studies tumour DNA methyltransferase expression was compared to the corresponding 'normal' cell type.

### 1.1.3.4 Aberrant DNA Methylation in Leukaemia

Aberrant gene promoter methylation has been studied in acute and chronic leukaemias, myelodysplasic syndrome and multiple myeloma, and a large number of different genes have been implicated (Chim et al., 2002; Ekmekci et al., 2004). Promoter methylation is not restricted to *de novo* malignancies with multiple genes in therapy related leukaemia also showing aberrant promoter methylation (Uehara et al., 2003).
DNMT1, 3a and 3b are all expressed in normal haematopoietic cells although expression level varies among cell types (Mizuno et al., 2001). Bone marrow cells extracted from patients with AML and CML (Chronic Myeloid Leukaemia) had increased expression of DNA methyltransferase transcripts ranging from 4-5 fold increases (DNMT1 and 3a) to an 11 fold increase (DNMT3b). The apparent up regulation of DNA methyltransferase genes may contribute to aberrant hypermethylation and consequently leukaemogenesis (Mizuno et al., 2001). Selective depletion of DNMT1 mRNA in human bladder cancer cells highlights the importance of DNA methyltransferases in malignancy. In the absence of DNMT1 mRNA, there is demethylation and reactivation of the silenced TSG CDKN2A (Robert et al., 2003).

1.1.3.5 Evaluating DNA Methylation in single copy gene sequences

Various methods are used to evaluate the DNA methylation status of genes and genomes and each method has limitations (reviewed Momparler & Bovenzi 2000). The technologies commonly employed use restriction digestion enzymes or bisulfite modification.

1.1.3.5.1 Methyl-sensitive Restriction Enzyme digestion.

Methyl-sensitive restriction enzyme digestion exploits the inability of specific restriction enzymes to cut methylated genomic DNA and is used in conjunction with Southern blot analysis and/or PCR. Although methyl-sensitive restriction enzyme digestion is ideal for screening a large number of samples and gives a good indication of the average methylation status, there are limitations to this method. These include (1) limited tumour DNA stock - Southern blotting requires ~10μg DNA per sample therefore precious and irreplaceable tumour tissue can often become rapidly depleted, (2) complete restriction enzyme digestion of DNA is essential for both Southern blotting and PCR based techniques as incomplete DNA digestion complicates interpretation of the results and (3) this method only provide information about those CpG dinucleotide located within sequences recognised by the restriction enzymes (reviewed Momaler & Bovenzi, 2000).
1.1.3.5.2 Bisulphite Treatment for DNA

The methylation status of DNA cannot be detected using conventional techniques such as cloning and PCR without prior DNA modification. Bisulphite treatment discriminates between cytosine and 5-methylcytosine because their deamination products are different. Deamination converts cytosine to uracil but leaves the 5-methylcytosine intact (Frommer et al., 1992). Subsequent PCR analysis amplifies all uracils as thymines and all 5-methylcytosines as cytosines. Sequence differences between methylated and unmethylated DNA are therefore detectable. Successful bisulphite treatment results in complete conversion of all unmethylated cytosines into thymines and this includes all cytosines that are CpG dinucleotides. Verification of complete bisulphite conversion is important because it has been reported that the cytosine adjacent to the methylated CpG sites can be resistant to bisulphite treatment (Harrison et al., 1998). The PCR product can be either sequenced directly to give the average genome methylation, or cloned prior to sequencing to sample individual genomes.

Various other methods are available to identify sequence changes include methylation specific PCR (MSP), methylation-sensitive single nucleotide primer extension (Ms-SNuPE) and combined bisulphite restriction analysis (COBRA) (Herman et al., 1996; Gonzalgo & Jones, 1997; Xiong & Laird, 1997). All PCR based methods require careful primer design. PCR associated with cloning and sequencing may be labour intensive but it enables all CpG dinucleotides within a region to be analysed. In contrast, some primer based methods may supply results quicker but identify methylation only in the CpG dinucleotides located within the primer sequence. A distinct advantage of bisulphite treatment over methyl-sensitive restriction digestion is that less DNA is required (~1μg) (reviewed Momparler & Bovenzi, 2000).

1.1.3.5.3 Microarray based methylation assays

The methyl-sensitive restriction enzyme and bisulphite methods discussed above enable the methylation status of a specific gene to be analysed in a large number of samples but does not allow the analysis of a number of genes in parallel. Methylation specific oligonucleotide microarrays are based on bisulphite treatment (Gitan et al., 2001). Often for each gene under analysis, the microarray will contain two
oligonucleotides - one specific for the methylated form of the CpG dinucleotide and one specific for the unmethylated form of the CpG dinucleotide. Bisulphite treated DNA binds to the oligonucleotide corresponding to its methylation status. The hybridised glass slide is scanned and the intensity of the spot is indicative of the methylation rate (reviewed Novik et al., 2002). The main advantages of bisulphite based microarrays are (1) the use of unfractionate DNA enables numerous genes to be assessed at one time and (2) the methylation pattern of the CpG dinucleotides within the oligonucleotide probe can be accurately defined. A major limitation of bisulphite based microarrays is that lightly methylated DNA may not be able to hybridise to array oligonucleotides (reviewed Mockler & Ecker, 2005).

Methylation-sensitive restriction enzymes have also been exploited for methylation based arrays. Microarray oligonucleotides contain restriction sites for rare cutting methylation sensitive restriction enzymes. One example of a methylation-sensitive restriction enzyme microarray method is restriction landmark genomic scanning (RLGS) (Rush & Plass, 2002). RLGS uses the rare-cutting methylation-sensitive restriction enzymes NotI (GCGGCCGC) and AscI (GGCGCGCC). This sequence is mainly located in the 5' gene region thus enabling the global assessment of numerous promoter regions within a sample. A second digestion (often EcoRV, PstI or PvuII) produces smaller DNA fragments enables adequate separation in the first-dimension electrophoresis prior to a second digestion (HinfI, Mbol, or DpnII) and second-dimension electrophoresis. Comparison of hybridisation intensity between tumour and normal tissue enables DNA methylation in the NotI site to be identified – methylated DNA is not cut by the methylation-sensitive restriction enzyme and is not hybridised by radioactive endlabelling. RLGS has been exploited to identify methylation targets in de novo AML (Rush et al., 2001) and this technique has been used to identify ID4 promoter methylation in human leukaemia (Yu et al., 2005).

Additional methods for the global assessment of methylation using microarray include identification of methylated DNA by immunoprecipitation (using anti-5-methylcytosine antibodies) or affinity chromatography (methyl-binding domain agarose chromatography fractionates DNA according to the degree of CG methylation) prior to microarray hybridisation. The methods do not however precisely identify DNA methylation at specific cytosine residues (Mockler & Ecker, 2005).
of the capacity of methyl-CpG-binding domain protein-2 to bind specifically to methylated DNA sequences is seen in the methylated-CpG island recovery assay (Rauch & Pfeifer, 2005).

1.2 Radiation

Environmental and genetic interactions are factors in cancer risk. This study employs ionising radiation exposure as an initiating event in carcinogenesis. Ionising radiation can be sub-classified into electromagnetic (X-rays and γ-rays) radiations which have no mass/charge and particulate (electrons, protons, neutrons and α-particles) radiations which are charged. An alternative classification considers the amount of energy deposited along an ionisation track. X-rays and γ-rays are defined as low linear energy transfer (LET) as energy deposition is sparse, while high LET neutrons and α-particles produce dense ionisation tracks (reviewed Dainiak, 2002). Radiation dosimetry is measured using absorbed dose or effective dose. The absorbed dose is the amount of energy deposited in a unit mass of matter and this is measured in Gray (1Gy = 1J/kg). The effective dose is used to compensate for the ionisation densities of different types of radiation and tissue sensitivity. This is measured in Sieverts where 1Gy = 1Sv in sparsely ionising radiation (i.e. X-rays) and 1Gy = 20Sv in dense ionising radiation (i.e α-particles) (reviewed Wakeford, 2004).

1.2.1 Human exposure to Ionising Radiation

The average individual is exposed to an effective dose of 2-3 mSv/annum due to natural radiation. Some medical procedures also involve radiation exposure with doses varying from 3 mSv (single screening mammogram), 25 mSv (paediatric CT scan) to 2 Sv (single radiotherapy treatment). A high exposure to ionising radiation for humans is considered to be >100 mSv and human epidemiological data reports that the lowest dose associated with increased cancer risk is ~10-50 mSv for acute exposure and ~50-100 mSv for a chronic dose (Brennar et al., 2003). Approximately 50% of healthy adult humans exposed to an acute whole body dose of 4Gy of sparsely ionising radiation will die within 60 days from bone marrow failure (reviewed Wakeford, 2004).

Chronic or protracted exposures occur over a relatively long time and at low dose rates. A prime example is occupational exposure where exposure to low levels of
irradiation occurs over a period of years and is therefore difficult to relate to long term health problems. In contrast acute radiation exposure occurs over a short period of time and involves a relatively high dose. A common example is radiotherapy which is designed to deliver high doses to target cells while the rest of the body received either a low or no dose. Radiotherapy usually involves a fractionated dose whereby the individual is exposed to a series of treatments (~2 Sv/treatment) with the total exposure often being in excess of 20 Sv (reviewed Wakeford, 2004).

The most infamous and well documented acute human radiation exposure was the detonation of the atomic bombs over Japan in 1945. These detonations resulted in a large number of people receiving a wide range of doses which were estimated according to distance from the epicentre. The mean dose received by atomic bomb survivors was 200 mSv with approximately half of exposed individuals receiving bone marrow doses of 50 mSv or less (reviewed Brenner et al., 2003). Life Span Studies (LSS) of 86,000 atomic bomb survivors have monitored the health of the exposed individuals, and related illness to dose. An increased incidence of solid cancers and leukaemia has been detected. In haematological malignancies, the highest excess absolute risk (EAR) was for Acute Myeloid Leukaemia (1.1 cases per 10^4 person-years [PY] Sv) corresponding to an excess relative risk of 3.3 per Sv (Preston et al., 1994; Pierce et al., 1996). Therapy related AML (t-AML) is also a well documented consequence of radiation exposure (Leone et al., 1999) and this is further discussed in section 1.4.2.

1.2.2 Mouse models of Radiation Induced Leukaemia.

Mice are an important research tool for studying radiation leukaemogenesis because the induced leukaemias can be similar to those that arise in irradiated humans. The use of inbred mouse strains with a resistance or susceptibility to a particular disease reduces genetic complexity, and provides large numbers of genetically identical mice enabling statistical criteria to be met. The exact type of radiation-induced malignancy depends on the strain, type of exposure (acute or fractionated) and age of animal at exposure. The major type of haemopoietic malignancy that develops in mice following four weekly fractionated doses of 1.6-2.5 Gy at 4 weeks old is thymic lymphoma. This is a thymus-dependent T cell malignancy with no human counterpart,
which is characterised by an enlarged thymus and the presence of T-cell receptor β (TCRβ) gene rearrangements (Kaplan & Brown, 1952; Boulton et al., 2002).

Radiation induced acute myeloid leukaemia (r-AML) can be induced in various mouse strains including RF, SJL/J, C3H/HE, CBA/Ca and CBA/H (Festing, 1996). A good disease model however not only requires a relatively high incidence of induced disease but also a low spontaneous incidence. Acute exposure of 8-12 week old CBA/H mice to 3Gy X-rays results in a 20-25% incidence of r-AML with a mean latency of approximately 18 months (Major & Mole, 1978; Major, 1979; Wright & Lorimore, 1990; Plumb et al., 1998). To categorically attribute a malignancy to an environmental factor such as irradiation, there must be a low spontaneous incidence of disease in unexposed mice. The incidence of AML in un-irradiated CBA/H mice is <0.1%, much lower than the 2-6% background incidence in unirradiated RF mice (Major & Mole, 1978) and therefore the CBA/H mouse is the best model available. Exposure of 8-12 week old C57BL/6 mice to a single whole body dose of 3 Gy X-rays does not induce r-AML. C57BL/6 mice are therefore genetically resistant to r-AML.

Advances in diagnostic techniques have identified other haemopoietic malignancies in the irradiated mice. Studies of CBA/H x (CBA/H x C57BL/6)F1 and C57BL/6 x (CBA/H x C57BL/6)F1 backcross mice revealed mixed lineage Thymic lymphomas and also an early pre-B lympho-myeloid leukaemia (L-ML) (Cleary et al., 2001; Boulton et al., 2002). L-ML may not be attributed to radiation exposure as mice are naturally prone to B cell malignancies (Festing, 1996) and lymphoid malignancies were only rarely detected in irradiation studies (Major, 1979). Diagnosis of murine leukaemias is discussed in section 1.4.1.2.

1.2.3 Molecular and Cellular response to Irradiation

1.2.3.1 DNA Damage

Deposition of energy from ionising radiation results in the formation of excited and ionised molecules which cause DNA damage. Most DNA damage (~65%) is induced by the hydroxyl ·OH radical produced from the radiolysis of water while the remaining damage is direct. 1 Gy of low LET radiation results in 600-1000 single strand DNA breaks, 16-40 double strand DNA breaks and 250 damaged thymine residues. The
most biologically important DNA lesions in the irradiated cell are however attributed to DNA double strand breaks (reviewed Friedberg et al., 1995).

Cells respond to DNA damage by instigating DNA repair or apoptosis. There are five major DNA repair pathways which deal with a plethora of DNA lesions. Homologous recombination repair (HRR) and non homologous end joining (NHEJ) correct double strand breaks, base excision repair (BER) removes damaged bases, nucleotide excision repair (NER) removes thymine dimers and large chemical adducts while mismatch repair (MMR) corrects mismatched bases resulting from replication mistakes (reviewed Hoeijmaker et al., 2001). Cell cycle arrest allows the cell time to ensure all DNA damage is repaired and thereby prevents the conversion of genetic lesions into permanent mutations. However, if an individual cell is too damaged or DNA repair is unsuccessful, apoptosis is initiated and consequently defective apoptosis mechanisms can result in the survival of a cell with excess DNA damage. Subsequent replication of cells containing DNA damage can lead to carcinogenesis (reviewed Bernstein et al., 2002).

1.2.3.1.1 Poly (ADP-ribo) polymerase 1 (Adprt1)

Poly (ADP-ribo) polymerase 1 (Adprt1) is a putative candidate gene discussed in chapter 5. The poly (ADP-ribo) polymerase 1 (Parp1) protein has an important role in base excision repair (BER) where it acts as a ‘nick sensor’ and rapidly associates with single and double strand DNA breaks following exposure to genotoxic insults such as alkylating agents, ionising radiation or free radicals (reviewed Herceg & Wang, 2001). The enzymatic activity of Parp1 is enhanced by binding to DNA strand breaks and this enzymatic activity results in Poly (ADP-ribosyl)ation and the production of poly (ADP-ribose) polymers. The major recipient of poly (ADP-ribosyl)ation is Parp1 itself, resulting in a negatively charged protein which dissociates from the DNA enabling repair proteins to access the site of DNA damage (reviewed Chalmers, 2004). Parp1 also has the capacity to stimulate NHEJ which is the pathway by which most mammalian cells repair double strand breaks (reviewed Chalmers, 2004).

Adprt1 knock out mice are more susceptible to carcinogenesis than wild type mice and develop various types of malignancies including liver sarcomas, lung carcinomas and colon carcinomas – evidence that Parp1 has a key role in preventing cancer.
development (Tong et al., 2001). The rapid response of Parp1 following DNA damage gives transient protection of DNA strand breaks from nuclease attack and recombination events, enabling other repair proteins to reach the site before a mutation occurs. Extensive DNA damage and subsequent Parp1 activation can also induce apoptosis. Failure of Parp1 cleavage during apoptosis however results in necrosis and enhanced apoptosis (reviewed Herceg & Wang, 2001; Tong et al., 2001).

1.2.4 Genetic Consequences of DNA Damage

1.2.4.1 Chromosomal Deletions.
Radiation induced AML in both man and mouse exhibit chromosomal deletions thus implicating loss or reduced gene function in the radiation leukaemogenic multi-stage process. In man, chromosomal losses typically present in therapy related AML and therapy related Myelodysplastic syndrome include interstitial deletions on the long arm of chromosome 5 (5q-) and 7 (7q-) and the loss of the whole of chromosome 7 (monosomy 7) (Leone et al., 1999; Mhawech & Saleem, 2001).

More than 95% of mouse r-AMLs exhibit chromosome 2 interstitial and terminal deletions (Hayata et al., 1983; Alexander et al., 1995; Cleary et al., 1999). Loss of heterozygosity studies further defined a minimally deleted region on chromosome 2 (Alexander et al., 1995; Silver et al., 1999, Cook et al., 2004). Putative candidate genes located within the vicinity of the minimally deleted region included PU.1. A recent study of murine r-AMLs has identified dominant negative PU.1 mutations in 87% of leukaemic cell lines. 86% of these mutations resulted in the replacement of arginine 235 which is essential for DNA binding (Cook et al., 2004). The predominant C703T mutation has subsequently been identified in our stocks of r-AML leukaemic DNA (M.Plumb personal communication).

Loss of heterozygosity (LOH) studies reported that ~44% of thymic lymphomas that arose in X-irradiated (C57BL/6J x RF/J)F1 had chromosome 4 deletions (Santos et al., 1996). Chromosome 4 deletions were also detected in 75% of thymic lymphomas, all lymphatic leukaemias and 6% of r-AMLs in X-irradiated (CBA/H x C57BL/Lia)F1 mice (Meijne et al., 2001). LOH analyses have mapped a 3.4cM minimally deleted region (Cleary et al., 2001; Meijne et al., 2001). The 3.4cM MDR is located within the thymic lymphoma suppressor region 5 (TLSR5) which was mapped as a ~20cM
interval centred on 15.6 cM (D4mit21) on chromosome 4 (Meléndez et al., 1999) and near the Lyr2 (lymphoma resistance 2) locus (14 cM). Analysis of informative leukaemias/lymphomas demonstrated that 92% of thymic lymphomas, >95% of early pre-B lympho-myeloid leukaemias (L-ML) and 53% of r-AMLs that arose in X-irradiated CBA/H x (CBA/H x C57BL/6J) backcross mice exhibited LOH4 in the TLSR5 region (Cleary et al., 2001). Preferential loss of the maternally transmitted CBA/H allele was also observed in ~90% of TLs, r-AMLs and L-MLs (Cleary et al., 2001; Boulton et al., 2002).

Approximately 50% of r-AMLs that arose in X-irradiated CBA/H x (CBA/H x C57BL/6J) have both chromosome 2 and chromosome 4 deletions (Cleary et al., 1999). Although chromosome 2 deletions in r-AMLs have been readily documented by cytogenetics and LOH analysis (Hayata, 1983; Alexander et al., 1995; Silver et al., 1999; Cook et al., 2004), chromosome 4 deletions have not. It is possible that the large (>20 cM) chromosome 2 deletions are more readily detectable by cytogenetic studies than the smaller (<10 cM) chromosome 4 deletions (Cleary et al., 2001). Murine chromosomal deletions following irradiation exposure are not restricted to Chromosome 2 and 4. LOH studies of thymic lymphomas have also implicated loci on chromosome 5, 6, 11, 12, 14, 16 and 19 albeit in different genetic backgrounds (reviewed Boulton et al., 2002).

1.2.4.2 Genetic Instability

Exposure of mammalian cells to 1Gy X-rays increases the gene mutation rate 10 fold (Lorimore et al., 2003). Successful DNA repair should result in clonal descendants exhibiting normal cellular characteristics while inaccurate repair or replication may result in clonal progeny exhibiting radiation induced damage (Lorimore et al., 2003). It has been reported that radiation induced genomic instability is non-clonal (Huang et al., 2003; Lorimore et al., 2003). Post irradiation clonal expansion generates progeny comprising of heterogeneous subpopulations which are cytogenetically different. Some subpopulations may contain deleterious mutations present in genes encoding DNA repair or apoptotic proteins which would increase genomic instability (Huang et al., 2003). Selective proliferation of cells harbouring mutations with a growth advantage may progress to malignancy (Bertram, 2001).
1.3 Haemopoiesis

Haemopoiesis is the process which produces mature blood cells in vivo. In adult humans, haemopoietically active bone marrow is located primarily within the central skeleton and at the proximal ends of the long bones. When bone marrow blood cell production is impaired, the liver and spleen can resume their foetal haemopoietic role and become sites of extramedullary haemopoiesis (Hoffbrand & Pettit, 1993). The productive capacity of the haemopoietic system is huge, and maintaining cellular homeostasis in the blood requires a human adult to produce approximately $5 \times 10^{11}$ new blood cells each day to replace old and destroyed cells (Pallister, 1994).

Haemopoiesis is a hierarchal system. The critical cell in haemopoiesis is the pluripotential haemopoietic stem cell (HSC). HSCs constitute <0.01% of the total bone marrow cells in man and mouse (Müller-Sieburg & Riblet, 1996; Morrison et al., 1997). The HSC compartment is heterogeneous and contains stem cells with varying degrees of maturity. Most HSC are quiescent and only a small number of cells are proliferating at any one time. These proliferating HSCs have two major properties – the capacity for self renewal thus maintaining their cellularity in the bone marrow throughout life, and the potential to differentiate into different types of mature haemopoietic cells (reviewed Bell & Van Zant, 2004; Warner et al., 2004).

A schematic representation of haemopoiesis is shown in figure 1.1. Haemopoietic stem cells differentiate into haemopoietic progenitor cells. With their limited capacity for self-renewal and restricted ability to generate cell lineages, these cells expand cell populations prior to cell maturation (Pallister, 1994). Haemopoietic cellular maturation is a result of terminal differentiation and consequent reduced proliferative capacity. This reduced proliferative capacity during terminal differentiation reduces risk of malignant transformation (Bertram, 2001).
This representation of haemopoiesis is modified from a diagram in Blair & Pamphilon (2003). As cells progress through haemopoiesis, they become restricted in lineage options and committed to a cell lineage. The majority of total blood cells (>95%) are mature terminally differentiated haemopoietic cells. The following abbreviations are BFU-E (burst forming unit – erythroid), CFU (colony forming unit) and RBC (red blood cell).

Haemopoietic differentiation is strictly controlled by the expression of specific genes and extra-cellular signals (Pallister, 1994). During the course of this study, two genes were specifically analysed because of their potential role in differentiation and the leukaemogenic process. Pax5 is well characterised and is discussed in section 1.3.3 while less is known about the homeobox gene Hlx.

1.3.1.1 **Hlx**

**Hlx** (H2.0-like homeobox gene) is a murine homeobox gene expressed in haemopoietic cells (myeloid-macrophage lineage cells and early stages of B-cell development) and in embryogenesis with highest levels detected in the posterior (e.g. tail/posterior spinal cord) region (Allen *et al.*, 1991). The fact that within the haemopoietic system **Hlx** is
expression restricted to specific cell types raises the possibility that Hlx may be a commitment gene.

Homeobox genes encode transcriptional regulatory proteins that are vital for growth and differentiation. These genes are characterised by the presence of a common 183 nucleotide sequence which encodes a 61 amino acid homeodomain that is responsible for sequence specific interactions with DNA. Homeobox proteins influence haematopoietic growth and differentiation, and are intrinsic regulators of haemopoiesis. One well documented group of homeobox genes in leukaemogenesis are Hox genes which are characterised by a homeodomain and hexapeptide domain (reviewed Abate-Shen, 2002). Hox proteins have been implicated in HSC self-renewal (reviewed Bell & Van Zant, 2004). Aberrant expression of Hox proteins is associated with abnormal haemopoiesis with over expression of Hoxa10 in murine HSCs leading to myeloid leukaemia. Mice lacking Hoxa9 are leucopaenic while over expression of Hoxa9 leads to defective T cell development and AML (reviewed Owens & Hawley, 2002). Over expression of Human HLX (HB24) has been observed in acute leukaemia (Owens & Hawley, 2002), and compromises B and T cell development (Allen et al., 1995).

1.3.2 Transcriptional control of Lymphopoiesis

Characterisation of mouse leukaemic spleen/thymus from X-irradiated CBA/H x (CBA/H x C57BL/6) F1 backcross mice demonstrated that some myeloid leukaemias and thymic lymphomas had B cell heavy chain rearrangements (IgH^R) and expressed lineage inappropriate genes. Some leukaemias were therefore reclassified as early pre-B lympho-myeloid leukaemias (L-ML) based on the presence of IgH rearrangements and expression of lymphoid (VpreB1 and Rag1) and myeloid (MPO and LysM) genes (Cleary et al., 2001; Boulton et al., 2002). One aspect of my study was to identify potential candidate genes implicated within the chromosome 4 MDR detected in the L-MLs and r-AMLs (chapter 6) and therefore a gene with a role in the transcriptional control of lymphopoiesis would be a good candidate.

1.3.2.1 The Myeloid vs Lymphoid lineage decision.

The initial commitment to the lymphoid lineage is regulated by two main genes products – Ikaros and PU.1. Ikaros promotes lymphoid differentiation at the expense of myeloid development - Ikaros null transgenic mice fail to generate B, T or natural
killer cells (reviewed Busslinger, 2004). PU.1 regulates early B cell development in part by controlling the regulation of cytokine-dependent proliferation and differentiation. Low levels of PU.1 activate the Il-7Ra gene and induce B-cell development (DeKoter et al., 2002). PU.1 and C/EBPα have a major role in determining myeloid lineages. Myeloid development requires high levels of PU.1 expression with granulocyte differentiation requiring higher levels of PU.1 than monocyte differentiation. PU.1 is induced by C/EBPα (CCATT/enhancer binding protein alpha) which is upregulated during granulopoiesis and down regulated during monopoiesis. High levels of C/EBPα may therefore promote granulocyte differentiation over the monocytic pathway (Friedman, 2002).

1.3.2.2 Differentiation of lymphoid progenitors into B and T cells

Figure 1.2 summarises the transcriptional control of B and T lymphopoiesis. In the presence of Ikaros protein and low levels of PU.1 protein, the multipotent progenitor becomes a common lymphoid progenitor (CLP). The differentiation of the CLP to a committed pro-B cell is however critically dependent on three main transcription factors: E2A, EBF and Pax5. The E2A gene encodes two basic helix-loop-helix transcription factors, E12 and E47 and it is E12 that induces the expression of EBF (Kee & Murre, 1998), which binds and activates the Pax5 gene promoter (O’Riordan & Grosschedl, 1999). Activation of B cell specific genes and activation of V(D)J recombination by E2A and EBF is however not sufficient to commit B-cell progenitors to the B lineage. Pax5 is the critical B-lineage transcription factor that ensures that the CLP differentiates down the B cell lineage (reviewed Busslinger, 2004).

Notch1 has been implicated in the control of the T vs B cell lineage decision. Notch1 regulates T cell Vβ-DJβ rearrangements by controlling the chromatin accessibility of the Vβ genes in the TCRβ locus (Höflinger et al., 2004) and is also involved in the elimination of thymocytes that fail to pass the pre-T cell receptor checkpoint (reviewed Radtke et al., 2004). Activation of Notch1 by ligands of the Jagged or Delta families is essential for both thymus-dependent and thymus-independent T cell development and is sufficient for T-cell commitment. This implies that Notch1 signalling must be absent or negatively regulated to enable B-cell development. Pax5 is one known negative regulator of Notch1 and blocks T-cell development by repressing Notch1 while at the same time promoting B lymphopoiesis (Souabni et al., 2002). Notch1
itself can suppress B cell development by inhibiting E2A function (reviewed Schebesta et al., 2002A).

**Figure 1.2 – Transcriptional control of B and T lymphopoiesis**

B and T lymphopoiesis is modified from a diagram in Busslinger (2004). The following abbreviations are HSC (haemopoietic stem cell), CLP (Common lymphoid progenitor), MP (Myeloid progenitor). Transcription factor levels are critical for haemopoiesis. High levels of PU.1 promote the myeloid lineage and low levels the lymphoid lineage. A fine balance exists between positive regulation (solid arrows) and negative regulation (broken arrows).

### 1.3.3 Role of Pax5 in B-lymphopoiesis

*Pax5* is an essential B-lineage transcription factor and its role in mouse radiation leukaemogenesis is discussed in chapter 6. The *Pax5* gene encodes the B cell specific activator protein (BSAP) which regulates several B cell-specific genes (Adams et al., 1992). The absence of BSAP results in the down regulation of genes such as *CD19* and *mb-l(Iga)* because transcriptional initiation of these genes and recruitment of transcription factors is BSAP dependent (Nutt et al., 1997A; Nutt et al., 1998). Pax5 ensures progression down the B cell pathway by repressing transcription of lineage inappropriate genes such as *Notch1* and activating genes i.e. *BLNK* which enable B cells to respond to specific signals (Schebesta et al., 2002B).

The role of *Pax5* is not exclusive to early B cell development. Horcher et al (2001) used mice carrying a floxed *Pax5* allele that had been conditionally inactivated by CD19-cre or Mx-cre expression, to show that *Pax5* deletion resulted in preferential loss of mature B cells. This strongly implicated a role of *Pax5* throughout the whole
of B lymphopoiesis up to the point of plasma cell differentiation. Plasma cells are
defined as differentiated antibody-secreting cells derived from antigen-activated B
cells and it is only at this final B cell differentiation stage that repression of Pax5 is
actually required (Lin et al., 2003).

1.3.3.1 Pax5 and haemopoietic malignancy

Until recently Pax5 did not have a well documented role in malignant progression. A
rare translocation t(9;14)(p13;q32) in B-cell non-Hodgkin’s lymphoma translocates
Pax5 on human 9p13 within close proximity of the immunoglobulin heavy chain gene
(IgH), and results in increase Pax5 mRNA expression (Iida et al., 1996; Hamada et al.,
1998). Pax5 has also been reported to be fused to ETV6/TEL in a case of acute
lymphoid leukaemia with a t(9;12)(q11;p13) translocation (Cazzaniga et al., 2001) and
increased Pax5 mRNA levels have been reported in AMLs with t(8;21)/AML-1-ETO
rearrangements (Tiacci et al., 2004).

Aberrant expression of Pax5 mRNA levels has also been implicated in Multiple
Myeloma (MM). B cells from patients with MM exhibit premature expression of
Blimp-1 (B-lymphocyte-induced maturation protein 1) mRNA and reduced Pax5
mRNA levels. Blimp-1 represses mature B-cell gene expression programs prior to
plasma cell differentiation and therefore premature expression of Blimp-1 together
with low levels of Pax5, may cause the premature differentiation of proliferating B
cells producing plasma cells with the proliferative capacity of B cells (Borson et al.,
2002).

1.3.3.2 Pax5(-/-) B cells in normal and malignant haemopoiesis

To generate antibody diversity, B cells need to undergo successful heavy and light
chain rearrangements. Heavy chain rearrangements requires two sequential somatic
recombinations - D_h joining to the J_h segment to generate D_hJ_h, followed by V_h
joining with the D_hJ_h complex to generate a V_h D_hJ_h complex. Productive V_h D_hJ_h
recombination is required for the formation of a functional pre-B cell receptor and
subsequently initiation of light chain rearrangements (reviewed Busslinger, 2004).

In Pax5-deficient mice, B cell development is arrested at the early pro-B (also referred
to as Pre-B1 cell) stage. These Pax5 (-/-) early pro B cells have undergone D_hJ_h
rearrangements at normal frequency but V_hD_hJ_h rearrangements are reduced ~50 fold
(Nutt et al., 1997B). *Pax5* is therefore proposed to have a role in the progression of early pro B cells to the late pro B cell (also referred to as Pre-BII cell) stage (reviewed Busslinger, 2004).

Haemopoietic progenitors are multi-potent and exhibit lineage-promiscuous gene expression (Nutt et al., 1999A). To become committed down a specific cell lineage, alternate pathways needs to be blocked. This is partly achieved by controlling the activation and repression of particular genes and transcription factors. *Pax5* has been implicated in blocking survival and expansion of early myeloid cells by suppressing their response to myeloid growth factors (Chaing & Monroe, 1999; 2001). Various studies have shown that *Pax5* (-/-) early pro B cells can develop into other cell lineages following exposure to specific transcription factors or environmental conditions *in vitro* and *in vivo* (figure 1.3; Nutt et al., 1999A; Graf, 2002; Schaniel et al., 2002; Rolink et al., 2002). *Pax5* (-/-) early pro B cells have the pluripotential properties of a stem cell and can repopulate all bone marrow lineages except B cells (Graf, 2002; Rolink et al., 2002).

**Figure 1.3 - The pluripotent capacity of *Pax5*−/− pro B cells**

*Pax5*−/− early pro B cells can be manipulated into different lineages depending on exposure to specific transcription factors or environments. The shaded cells represent normal B cell haemopoiesis is the presence of *Pax5*. Abbreviations are GM-CSF (granulocyte-macrophage colony-stimulating factor), M-CSF (macrophage colony-stimulating factor), SCF (stem cell factor). ST2 are stromal cells and Trance is a cytokine. This diagram represents data in Nutt et al., (1999A).
1.4 Leukaemia

Leukaemia is characterised by the abnormal accumulation of white cells in the bone marrow and peripheral blood. Chronic lymphoid leukaemia (CLL) is the most common human leukaemia occurring mainly in the elderly, while the commonest form of childhood leukaemia is acute lymphoblastic leukaemia (ALL) (Hoffbrand & Pettit, 1993).

1.4.1 Classification of Leukaemia

1.4.1.1 Diagnosis of Human Leukaemia

Initial diagnosis of human leukaemias relies on the examination of peripheral blood and bone marrow using light microscopy. The leukaemic differentiation block is at an early stage in haemopoiesis where the cells have a high capacity for proliferation and this is represented by the immature nature of leukaemic cells seen microscopically (McCulloch et al., 1983; Greaves, 1997). Morphological analysis is supplemented by cytochemical staining, immunophenotyping, immunogenotyping and cytogenetics.

Cytochemical stains detect subcellular components which are often specific to a particular cell lineage. One example is Sudan Black which stains neutral lipids, phospholipids and lipoproteins and identifies granulocytic precursors (Pallister, 1994). Immunophenotyping is essential when cytochemistry is uninformative. Flow cytometry and immunocytochemistry exploit monoclonal antibodies against lineage specific cell surface antigens to identify different cell types and different stages of maturation. Panels of monoclonal antibodies against cell surface antigens are used to classify leukaemia (Hoffbrand & Pettit, 1993). Cytogenetic evaluations can also be informative on clonality of the leukaemia and abnormal karyotypes. Different leukaemias are characterised by different chromosomal abnormalities i.e. 90% of t(8;21) translocations are classified as AML-M2 (Smith et al., 2004).

Leukaemia diagnosis is critical to disease management and the techniques described above are both time consuming and subject to individual interpretation. Recent studies have suggested a role of gene expression profiling using DNA microarrays in leukaemia diagnosis (Haferlach et al., 2005; reviewed Kearney & Horsley, 2005).
DNA microarrays cluster patterns were used to distinguish between six different AML subgroups and 4 subgroups of ALL with high accuracy (Haferlach et al., 2005) and expression signatures could supply supportive data especially for leukaemias with normal karyotypes (reviewed Kearney & Horsley, 2005).

1.4.1.1.1 Single Lineage Leukaemia

There are two main criteria for sub-classifying leukaemias – Acute or Chronic and Myeloid or Lymphoid. Leukaemias are subdivided into acute or chronic leukaemia depending on the number of myeloblasts or lymphoblasts that are present in the bone marrow at clinical presentation (>30% blasts characterising acute leukaemia). Acute leukaemia is further classified using the FAB (French-American-British) scheme. This scheme distinguishes between types of acute leukaemia based on cell type and maturation. There are eight main subtypes of acute myeloid leukaemia – M0 to M7 and three main subtypes of acute lymphoid leukaemia – L1 to L3 (Hoffbrand & Pettit, 1993; Pallister, 1994).

1.4.1.1.2 Mixed Lineage Leukaemia

The increased use of cell surface antigen expression to classify haemopoietic malignancies in man and mouse has identified mixed lineage leukaemias expressing lymphoid and myeloid antigens (Catovsky & Hoffbrand, 1999; Cleary et al., 2001; Boulton et al., 2002; Smith et al., 2004). Often the monoclonal antibody panel used to confirm myeloid or lymphoid malignancy is biased by the initial morphological diagnosis. Consequently, many cases of mixed lineage leukaemia may be diagnosed as single lineage leukaemia due to the limited antibody repertoire. Two long standing models to explain mixed lineage leukaemias were ‘lineage infidelity’ and ‘lineage promiscuity’ (McCulloch et al., 1983; Greaves et al., 1986). These models have however been largely superseded by stem cell/progenitor plasticity which is discussed in section 1.4.4 (reviewed Hope et al., 2003; Warner et al., 2004).

1.4.1.1.3 Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) are a pre-leukaemic state as they often progress to acute myeloid leukaemia. Quantitative and qualitative abnormalities of all myeloid
cell lineages result in pancytopenia and often ineffective haemopoiesis, although bone
marrow may exhibit normal or increased cellularity (Hoffbrand & Pettit, 1994). In
humans, myelodysplastic syndromes are sub-classified based on the blast incidence in
the bone marrow, the frequency of ringed sideroblasts, and the proportion of
monocytes in the peripheral blood. This criteria is used to generate a FAB
classification that contains 5 subgroups ranging from refractory anaemia to chronic
myelomonocytic leukaemia (Mhawech & Saleem, 2001).

1.4.1.2 Diagnosis of Murine Leukaemia

Diagnosis of murine leukaemia is post-mortem as the emphasis is to study rather than
cure the disease. Clinical symptoms include weight loss, laboured breathing, pale feet,
piloerection and a palpable spleen. The predominant post-mortem feature is
splenomegaly often with the presence of lobulated white areas. Morphological
examination concentrates on bone marrow and peripheral blood smears and spleen
cross-sections. Morphologically, AML was originally diagnosed by the accumulation
of metamyelocytes in the spleen, peripheral blood and bone marrow with these
metamyelocytes having a distinct ringed nucleus (Major, 1979). Using a FAB-like
classification, the most common diagnosis for murine AML is M2 and M4 (Cleary et
al., 2001). M2 bone marrow contains myeloblastic cells demonstrating maturation and
often associated with promyelocytic forms, while M4 bone marrow has
myelomonocytic cells with evidence of both granulocytic and monocytic
differentiation.

Current diagnosis of murine leukaemias in M.Plumb's group also includes the
expression of lineage specific/restrictive markers and identification of gene
rearrangements. Northern blots of leukaemic spleen RNA are probed with
*Myeloperoxidase (MPO)*, *Lysozyme M (LysM)*, VpreB1 and *CD19* cDNAs to
distinguish between myeloid and B lymphoid malignancies. MPO is a myeloid
enzyme synthesized in azurophilic granules and exclusively found in promyelocytes
while Lysozyme M is a myeloid specific lineage enzyme abundantly expressed in
macrophages and monocytes, and detected in myelomonocytic and monocytic
leukaemias (Lübert et al., 1991). VpreB1 identifies early B cells as it constitutes a
component of the surrogate light chain and CD19 is present from the early pro-B stage
(Busslinger, 2004).
All leukaemic DNA samples (spleen and thymus) are routinely analysed for the presence of Immunoglobulin heavy chain (IgH) and T-cell receptor β (TCR β) rearrangements by southern blotting. IgH rearrangements are indicative of B cell malignancies and TCRβ of T cell malignancies. Characterisation of murine malignancies using both Southern and Northern blot analysis demonstrated that some leukaemias and lymphomas exhibited characteristics of mixed lineage disease (Cleary et al., 2001; Boulton et al., 2002). Previously diagnosed r-AMLs had to be reclassified into true r-AMLs (no IgH rearrangements detected; MPO/LysM mRNA present) and early pre-B lympho-myeloid leukaemias (detection of IgH rearrangements or VpreB1 mRNA; MPO/LysM mRNA present).

1.4.2 De novo vs Induced Leukaemia

The majority of de novo human leukaemias are characterised by the presence of chromosomal translocations that either create a fusion oncogene encoding a chimeric protein, or positions a proto-oncogene adjacent to an active gene thereby causing inappropriate transcription of the proto-oncogene. Numerous examples of leukaemic translocations have been identified in acute myeloid leukaemia (Smith et al., 2004) and in other haematological malignancies (Donner, 1997).

The risk of developing a second independent malignancy following successful radiotherapy/chemotherapy treatment for a primary malignancy is estimated to be 8-12% by 20 years after diagnosis of the first cancer with leukaemia presenting as the most frequent second malignancy (Leone et al., 1999). The predominant form of therapy related leukaemia is AML (t-AML) which is often preceded by myelodysplasia (MDS) and henceforth in this study, t-AML includes t-AML and t-MDS. The risk of t-AML depends on the primary malignancy and treatment, and t-AML accounts for 10-30% of all AMLs that present at clinic (Leone et al., 1999).

Numerous studies implicate therapeutic cytotoxic drugs and radiotherapy of primary malignancies as causal factors for the development of t-AML. t-AMLs post treatment with alkylating agents (i.e. Chlorambucil and Busulphan) tend to exhibit a preleukaemic MDS phase with pancytopenia and cytogenetic abnormalities of chromosome 5 and 7. In contrast, t-AMLs that arise following treatment with
topoisomerase II inhibitors (i.e. epipodophyllotoxins) often do not have a preleukaemic phase and frequently exhibit t(11;16) translocations (Leone et al., 1999; Mhawech & Saleem, 2001).

1.4.3 The Leukaemic stem cell

Although specific leukaemias are clonal, collectively as a group of malignancies leukaemias are extremely heterogenous. This heterogeneity is the basis of the FAB classification discussed in section 1.4.1.1.1. Two models are currently proposed to explain the heterogenous nature of AML and these have been extensively reviewed by Hope et al., (2003) and Warner et al., (2004). The first hypothesis is that all cells throughout the HSC/progenitor hierarchy are susceptible to transformation i.e some AMLs develop from HSCs and some from myeloid progenitors. The second and preferred hypothesis is that the initial mutations that result in transformation and progression are restricted to the HSC and that further mutations dictate disease type.

Analysis of bone marrow from AML patients demonstrates that differences exist between the normal HSC and the leukaemic stem cell (LSC). Both express common stem cell surface antigens i.e. CD34, CD38, HLA-DR and CD71 while the LSC lacks Thy-1 and c-Kit, and expresses IL-3Ra (CD123). IL-3Ra has a role in a number of signalling pathways including anti-apoptotic mechanisms suggesting that the up-regulation of IL-3Ra may be a survival mechanism in AML. The HSC and LSC do have common functional features as they both possess self-renewal characteristics (Hope et al., 2003; Warner et al., 2004).

1.4.4 Transcription factor levels in murine leukaemia

A locus demonstrates haploinsufficiency when the amount of gene product produced by a single copy is insufficient to produce a normal phenotype (Strachan & Read, 1999). Rosenbauer et al (2005) reviewed data obtained using hypomorphic alleles and knockout mice to suggest that graded down regulation of transcription factors is important in leukaemogenesis. Transcription factors assessed included PU.1, C/EBPa and GATA-1. C/EBPa has a major role in determining the myeloid lineage with high levels of C/EBPa reported to promote granulocyte differentiation over the monocytic
pathway (Friedman, 2002). C/EBPα mutations are frequently associated with human AML. Bi-allelic null mutations however are not detected and C/EBPα knockout mice do not develop AML. Unpublished data reviewed by Rosenbauer et al (2005) suggests that disruption of the 42 kD protein isoform and expression of the 30 kD protein isoform only, results in an AML-like disease in mice.

Further evidence of the significance of transcription factor levels in leukaemia is supplied from studies of GATA-1. GATA-1 regulates erythroid, megakaryocytic, mast cell and eosinophilic differentiation. Consistent with C/EBPα, leukaemia develops in GATA-1 knockdown but not in GATA-1 knockout mice. The development of leukaemia requires low levels of GATA-1 expression possibly ensuring sufficient protein level to support survival and proliferation of the cells, but not enough to promote differentiation (Shimizu et al., 2004).

A well documented dosage dependent haemopoietic transcription factor is PU.1 (section 1.3.2.1). Low levels of PU.1 in mice diagnosed with r-AML is characterised by deletion of one PU.1 allele and a missense mutation at codon 235 in the DNA binding Ets domain (Cook et al., 2004; section 1.2.4.1). No cases of murine r-AML where both PU.1 alleles had been deleted or where the remaining allele had a null-mutation were detected, implying that clones with minimal PU.1 activity were preferentially selected over complete loss of function (reviewed Rosenbauer et al., 2005).

PU.1 mutations are relatively rare in human AML (~7%) and in most reported cases a wild type allele is retained. Suraweera et al., 2005 failed to identify any PU.1 mutations in human therapy related AML and concluded that a potential role of PU.1 in therapy related AML would be restricted to interactions with other genes and not as consequence of PU.1 mutations. LOH2 and the missense mutation at codon 235 have been detected in various mouse studies (Cook et al., 2004; Suraweera et al., 2005; Mark Plumb, personal communication). However, it appears from analyses of human AML (both de novo and therapy related) that this mechanism is restricted to the murine model (Suraweera et al., 2005).
1.5 Characterisation of the Haemopoietic Stem Cell

Genome wide genetic linkage analysis of r-AMLs that arose in X-irradiated CBA/H x (CBA/H x C57BL/6) F1 backcross mice, identified a locus on distal chromosome 1 which overlaps with a genetically determined locus that modulates mouse bone marrow stem cell frequency (ScfrI) (Boulton et al., 2003). As ‘target cell’ number is a risk factor in malignant development, one aspect of my study was to establish a routine method to reproducibly measure the number of haemopoietic stem cells (HSC) in bone marrow. The methods available for analysing HSC number are described below.

1.5.1 In vivo Methods

1.5.1.1 Transplantation Studies

Transplantation studies are functional studies based on the ability of cell types to rescue mice from a dose of lethal irradiation. Animals are exposed to ~8 Gy of total body X-irradiation and then rescued with unexposed primitive cells (Szilvassy et al., 1990). The source of primitive cells can be spleen colony forming units (CFU-S) or cells obtained using fluorescence activating cell sorting (section 1.5.2.3). Long term repopulating HSC (LT-HSC) are defined as being those HSCs that have the capacity to repopulate the bone marrow of a lethally irradiated mouse for life. Short term repopulating HSC (ST-HSC) have a limited repopulating capacity and can only repopulated the bone marrow for 8-12 weeks post transplantation (reviewed Coulombel, 2004).

1.5.2 In vitro Methods

1.5.2.1 Culture Based Assays

Lineage-restricted progenitors can be identified and quantified using in vitro clonogenic assays. Colonies are cultured in a semi-solid medium and the number and type of colony established. Mixed colony forming cells (Mix-CFC) give rise to large colonies containing various haemopoietic cells representing their multi-potentiality while other colonies are lineage-specific i.e. BFU-E (burst colony-forming units, erythroid), granulocyte/macrophage-CFC, macrophage-CFC, eosinophilic-CFC, megakaryocytic-CFC, and basophilic-CFC (Testa et al., 1985).
Enumeration of more immature HSCs and immature progenitors requires the use of feeder cells which provide a substrate and a source of regulatory factors which can support the differentiation of immature cells into mature lymphoid and/or myeloid progeny. The HSC/progenitors that respond to the stroma by initiating long-term cultures are analysed by Cobblestone area-forming cell (CAFC) or long-term culture-initiating cell (LTC-IC) assays (Ploemacher et al., 1991; Sutherland et al., 1990). CAFCs are defined by the time of their appearance in culture with immaturity being directly associated with late appearing colonies - Day 35 CAFCs therefore originate from more immature cells than Day 7 CAFCs. The LTC-IC assay is a modification of the CAFC assay whereby CAFC progeny can be produced for more than 5 weeks when co-cultured with stromal fibroblasts. By engineering the fibroblast feeder layer to produce specific growth factors, various characteristics such as self-renewal capacity can be examined (Petzer et al., 1996).

1.5.2.2 HSC Surface Antigens

Clonogenic assays are labour intensive, technically demanding and genetic factors may influence the ability of the HSC to form colonies in vitro (Coulombel, 2004). A direct approach (which was used in this study) is the identification of HSC according to the expression of specific cell surface antigens. Exploitation of biotin-conjugated antibodies to mature cell markers in conjunction with streptavidin coated magnetic beads enables immunomagnetic separation of bone marrow into mature lineage positive (Lin+) and immature lineage negative (Lin-) cells. Lin- cells can then be stained using fluorescently labelled antibodies to HSC surface antigens and analysed by fluorescence activated cell scanning/sorting (FACS).

1.5.2.2.1 Stem cell Antigen (Sca-1)

Sca-1 (Ly-6A/E) is a widely accepted HSC marker and is routinely used to identify HSCs in murine studies (Okada et al, 1992; Spangrude & Brooks, 1992; Phillips et al., 1992; Uchida & Weissman, 1992; Osawa et al., 1996; Christensen & Weissman, 1999, Henchaerts et al., 2002). Sca-1 (Ly-6A/E) is a member of the Ly-6 antigen family of phosphatidylinositol-anchored membrane proteins. Although the role of Sca-1 in suppressing T-cell proliferation and differentiation is well established, it has recently
been reported that Sca-1 has a role in HSC/progenitor cell lineage fate, c-Kit expression, and possibly HSC homing (Bradfute et al., 2005). The role of Sca-1 is however not restricted to the haemopoietic system and Sca-1 has been shown to be functionally important in growth and differentiation of muscle cells (Mitchell et al., 2005) and Sca-1 expressing cells have been isolated in the heart (Matsuura et al., 2004).

The murine Sca-1 protein is encoded by strain-specific allelic genes Ly-6A and Ly-6E with the expression of Sca-1 being dependent on the haplotype of the Ly-6 locus. Haplotypes being defined as a series of alleles closely linked on a single chromosome (Strachan & Read, 1999). There are two allelic variants of Ly-6A/E, the Ly-6\textsuperscript{a} haplotype which expresses Ly-6E.1 and Ly-6\textsuperscript{b} which expresses Ly-6A.2. The existence of two different haplotypes complicates the use of anti Sca-1 in the characterisation of mouse HSCs. CBA/J, Balb/c, CeH/J, and A/J inbred mice express the Ly-6\textsuperscript{a} haplotype which is associated with low levels of Sca-1 staining while C57BL/6, AKR/J, DBA/J and SJL/L express the Ly-6\textsuperscript{b} haplotype and are therefore associated with high levels of Sca-1 staining (Spangrude & Brook, 1993).

1.5.2.2.2 c-Kit

c-Kit encodes a transmembrane tyrosine kinase receptor for stem cell factor (SCF). Primitive HSC with long-term repopulating activity have been shown to express c-Kit and indeed transplantation studies with Lin', c-Kit\textsuperscript+ cells resulted in recipient survival while transplantation of Lin', c-Kit\textsuperscript- cells resulted in death (Okada et al., 1991; Ikuta & Weissman, 1992). Transplantation of Lin', c-Kit\textsuperscript+, Sca-1\textsuperscript- cells did not protect mice from lethal irradiation while Lin', c-Kit\textsuperscript-, Sca-1\textsuperscript+ cells generated long-term repopulation of both myeloid and lymphoid lineages. Sca-1 expression therefore subdivided the Lin', c-Kit\textsuperscript+ cell population into Lin', c-Kit\textsuperscript-, Sca-1\textsuperscript- committed progenitors and Lin', c-Kit\textsuperscript+, Sca-1\textsuperscript+ HSCs and indicated that c-Kit expression is a valid HSC marker when used in conjunction with Sca-1 (Okada et al., 1992).

1.5.2.2.3 Thy-1.1

Thy-1.1 expression has also been used in the classification of mouse HSC as 98.7% of the long-term repopulating activity is found in Lin', Sca-1\textsuperscript+, Thy1.1\textsuperscript+ cells with loss of
Thy-1.1 expression occurring during maturation and differentiation. Like the Sca-1 locus, there are different haplotypes for the Thy-1 locus. Most inbred mice strains, including SJL/J and DBA/2, express the Thy-1.2 allele while a few divergent strains i.e. AKR/J express the Thy-1.1 allele. As few strains show Thy-1.1 expression then the use of antibodies against Thy-1.1 as a HSC marker is limited. The use of Thy-1.2 expression in HSC classification is also restricted as only 19.2% of the long-term repopulating activity is observed in Lin', Sca-1+, Thy1.2<sup>lo</sup> cells and HSC activity is found in the Thy1.2<sup>+</sup> cells (Spangrude & Brooks, 1992).

1.5.2.2.4 Foetal liver kinase 2 (Flk-2)

Flk-2 is a member of the Class III tyrosine kinase receptor family and is expressed in early haemopoietic progenitors (Gilliland & Griffin, 2002). Analysis of long term reconstituting activity of Lin', Sca-1<sup>+</sup>, c-Kit<sup>+</sup> Thy1.1<sup>lo</sup>, Flk-2<sup>-</sup> and Lin', Sca-1<sup>+</sup>, c-Kit<sup>+</sup> Thy1.1<sup>lo</sup>, Flk-2<sup>-</sup> bone marrow cells demonstrated that while the Flk-2<sup>-</sup> cells generated long term multi-lineage reconstitution, the Flk-2<sup>+</sup> cells exhibited only short term reconstitution properties (Christensen & Weissman, 2001). Flk-2<sup>+</sup> expression can therefore be used to define the presence of ST-HSC as Flk-2<sup>+</sup> expression is up-regulated as HSCs become more mature and correlates with the loss of long term activity and self renewal capacity (Adolfsson <i>et al.</i>, 2001). This enhanced expression of Flk-2<sup>+</sup> corresponds with the down regulation of Thy-1.1 enabling Flk-2<sup>+</sup> expression to replace Thy-1.1 expression in distinguishing between ST-HSCs and LT-HSCs (Christensen & Weissman, 2001).

1.5.2.2.5 CD34

Although CD34 is used to identify HSC for human bone marrow transplantation, recent studies have reported that it is Lin', Sca-1<sup>+</sup>, c-kit<sup>+</sup> CD34<sup>-</sup> bone marrow cells that are capable of long term haemopoietic reconstitution of lethally irradiated mice (Osawa <i>et al.</i>, 1996; Ogawa, 2002). As human recipients exhibit long term reconstitution of their bone marrow post transplantation of CD34<sup>+</sup> bone marrow cells, then human CD34<sup>+</sup> cells must either have long term repopulating capacity or pre-transplantation treatments are generating cell surface antigen modification. Peripheral blood stem cells mobilised by haemopoietic growth factor G-CSF (Granulocyte Colony Stimulating Factor) are CD34<sup>+</sup> and transplantation studies demonstrated that
once bone marrow homeostasis is achieved, CD34 expression is down-regulated and HSCs become CD34⁻ (Tajima et al., 2000). CD34 expression as an indicator of HSC is therefore dependent on the context in which the cells are analysed and while CD34⁺ expression may be a valid technique for obtaining human mobilised stem cells, in identification of murine HSCs, CD34⁺ expression should be used with caution.

1.5.2.3 **Fluorescence activated cell Scanning (FACS)**

The expression of cell surface antigens such as those described above can be exploited using fluorescence activated cell scanning/sorting (FACS). FACS enables the identification of specific cells located within a large cell population and is ideal for measuring HSC and progenitor cells which represent ~1% of bone marrow cells. Fluorescent activated cell scanning utilises fluorescently labelled antibodies to stain cell populations. Fluorescently stained cells are released individually through an ultrasonic vibrating nozzle in the form of a microdroplet. This droplet then travels through a laser light beam where it excites flurochrome and the intensity of the fluorescence emitted is monitored by a detector. Individual cells are represented on an oscilloscope display and can be analysed using compatible computer software. Numerous fluorescently labelled antibodies are commercially available including Fluorescein Isothiocyanate (FITC) and R-Phycocerythrin (PE) which are used in this study. Each fluorescent dye absorbs light at one wavelength and emits light at a longer wavelength enabling more than one cell surface antigen to be analysed at a time (Kuby, 1997).

1.5.2.4 **Exclusion of fluorescent dyes**

Rhodamine 123 (Rh-123) and Hoechst 33342 are examples of exclusion dyes that are used to identify HSCs. Fluorescent dye staining of HSCs is often preceded by FACS or wheat germ agglutination (Phillips et al., 1992) thus removing mature cells prior to staining. Fluorescence dyes such as rhodamine 123 (Rh-123) depend on the different properties of cycling and non-cycling cells. Rh-123 has a relatively high affinity for mitochondrial membranes and is retained more efficiently in cycling cells than in quiescent cells. Primitive HSCs are typically non-cycling and therefore exhibit low accumulation of Rh-123 and low Hoechst 33342 staining. This enables primitive HSCs to be distinguished from more mature HSCs/progenitors which exhibit a high accumulation of Rh-123 by comparison (reviewed Okada et al., 1991).
1.5.2.5 **Defining the HSC using different assays.**

Equating data obtained using such a wide range of techniques is difficult in a dynamic system such as haemopoiesis where cell differentiation and maturation is progressive. Summarising the data from *in vitro* and *in vivo* studies has established the following broad classification (de Hann *et al.*, 1997A; Passegué *et al.*, 2003).

- Stem cells which have the ability to generate long term multi-lineage reconstitution in a lethally irradiated mouse are: Lin\(^-\), Sca-1\(^++\), c-Kit\(^++\) Thy1.1\(^lo\), Flk-2\(^-\), CD34\(^lo\), Rh-123\(^lo\), Hoechst 33342\(^lo\); CAFC-day 35, LT-CICs.

- Stem cells which have the ability to generate short term multi-lineage reconstitution in a lethally irradiated mouse are: Lin\(^-\), Sca-1\(^++\), c-Kit\(^++\) Thy1.1\(^hi\), Flk-2\(^++\), CD34\(^+\), Rh-123\(^hi\), Hoechst 33342\(^hi\); CAFC-day 28.

1.5.3 **HSC Global Gene Expression - DNA Array Systems**

DNA array systems have been exploited to assess global gene expression profiles in HSCs. These systems enable the simultaneous detection of numerous genes in one experimental approach and enable analysis of HSCs which are rare and difficult to maintain in culture – factors that have hindered biochemical and molecular characterisation of these cells. DNA array profiling has been used to analyse the HSC genetic regulatory pathways of different types of stem cell (Ramalho-Santos *et al.*, 2002) and also different cell stages throughout haemopoiesis (Ivanova *et al.*, 2002). Ivanova *et al.*, (2002) reported upregulation of specific genes corresponded to specific cell types. *Hoxa5* and *Hoxa10* (role in stem cell differentiation) are specific to LT-HSCs while genes associated with cell cycle initiation (*Weel, Cdk4, Mcmd* and *Myb*) are restricted to the ST-HSCs and early progenitors.

Gene expression patterns have been compared between HSCs and leukaemic cells. Kronenwett *et al.*, (2005) reported that compared to normal CD34+ cells, GATA-2 was upregulated in CML CD34+ cells while GATA-3 and C/EBP\(\alpha\) (lineage commitment genes) were not expressed. This suggests CML CD34+ cells have a greater self-renewal ability compared to normal CD34+ cells. DNA array systems are
powerful tools in establishing differential expression between enriched haemopoietic and leukaemic cell populations (Park et al., 2002; Kronenwett et al., 2005).

1.6 Aims of PhD

This project is part of an ongoing study to identify factors that confer a susceptibility to radiation induced AML in the CBA/H inbred mouse. It represents the second phase and therefore continuation of the work undertaken by Helen Cleary (Cleary et al., 2002) and Emma Boulton (Boulton et al., 2002; Boulton et al., 2003). Prior to commencing this PhD, two genetic loci had been implicated in the mouse radiation leukaemogenic process. A genome wide genetic linkage analysis had identified among others a locus on distal chromosome 1 (Boulton et al., 2003), while LOH studies had indicated a locus on chromosome 4 (Boulton et al., 2002; Cleary et al., 2002). This thesis focuses on the further characterisation of these two loci.

1.6.1 Distal Chromosome 1

Genome wide genetic linkage analysis of r-AMLs that arose in X-irradiated CBA/H x (CBA/H x C57BL/6)F1 backcross mice identified a locus on distal chromosome 1 (92.3-100cM) which overlaps with a genetically determined locus (90-113cM) that modulates mouse bone marrow stem cell frequency (Scfrl) (Boulton et al., 2003). Although various studies have reported that significant differences in HSC numbers exist between different inbred strains (Henckaerts et al., 2002; 2004; de Hann & Van Zant, 1997B; Morrison et al., 2002; Müller-Sieburg & Riblet, 1996), the CBA/H inbred mouse strain has not been analysed. As the target cell in r-AML is the HSC, an initial aim of this project was to establish an 'in house' method to measure HSCs, compare the number of HSCs in the r-AML susceptible CBA/H and the r-AML resistant C57BL/6 mouse strains, and test the hypothesis that HSC number is a genetically determined risk factor in r-AML (Chapter 3).

r-AML is however an induced malignancy and therefore susceptibility may be determined by the number of HSCs that survive a leukaemogenic dose of X-rays and not a function of the number of HSCs present before exposure. The second aim of this project was to test the hypothesis that it is the number of HSCs that survive irradiation that determines the risk factor in r-AML by monitoring HSC cell death and recovery in
the two inbred mice strains following exposure to a single acute dose of 3Gy X-rays in vivo. As haemopoiesis is hierarchical, then differences in HSC numbers or response to ionising radiation could be reflected throughout the haemopoietic system and consequently several aspects of total bone marrow cellularity were included in these analyses (Chapter 4).

Although this thesis concentrates the HSC number and survival post exposure to an in vivo leukaemogenic dose of X-rays, the above ‘stem cell frequency’ hypotheses may be incorrect. Other putative candidate genes located within the chromosome 1 r-AML susceptibility locus were also analysed with priority being given to those genes that had a potential role in haemopoiesis/leukaemogenesis and/or response to ionising radiation (Chapter 5).

1.6.2 Chromosome 4

A 3.4cM minimally deleted region (14.5-17.9cM) on chromosome 4 was identified by LOH studies of mouse leukaemias and lymphomas that arose in informative 3Gy X-irradiated CBA/H x (CBA/H x C57BL/6)F1 backcross mice (Cleary et al., 1999; 2001; Boulton et al., 2002). A candidate gene approach was also used on genes located in the MDR implicated by LOH4. As with the chromosome 1 genes, candidate genes were prioritised based on either their response to ionising radiation or role in haemopoiesis. The aim of this study being to determine firstly whether candidate genes were inactivated during the mouse leukaemogenic process, and secondly to determine whether this inactivation was genetic or epigenetic (Chapter 6).
## 2 Chapter 2: Materials and Methods

### 2.1 Materials

#### 2.1.1 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Kb DNA ladder size marker</td>
<td>Invitrogen UK (Paisley, UK)</td>
</tr>
<tr>
<td>ΦX 174 DNA/Hae III marker</td>
<td>ABGene (Epsom, UK)</td>
</tr>
<tr>
<td>Agar</td>
<td>Biogene Ltd (Kimbolton, UK)</td>
</tr>
<tr>
<td>Agarose</td>
<td>Cambrex Bioscience Rocklands Inc (Rockland ME, USA)</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma-Aldrich Company Ltd (Poole, UK)</td>
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<tr>
<td>Boric Acid</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA) Fraction V</td>
<td>Sigma-Aldrich Company Ltd (Poole, UK)</td>
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<tr>
<td>Bromophenol Blue</td>
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<tr>
<td>Chloroform</td>
<td>Fisher Scientific (Loughborough, UK)</td>
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<tr>
<td>Deoxynucleotide Triphosphates (dNTPs)</td>
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</tr>
<tr>
<td>DePex Mountant</td>
<td>Fluka Biochemika (Bucks, UK)</td>
</tr>
<tr>
<td>Diethyl Pyrocarbonate</td>
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</tr>
<tr>
<td>Ethanol</td>
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</tr>
<tr>
<td>Ethidium Bromide Tablets</td>
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</tr>
<tr>
<td>Ethylenediaminetetra-acetic Acid (EDTA)</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Ficoll</td>
<td>Amersham Biosciences (Little Chalfont, UK)</td>
</tr>
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<td>Formaldehyde 37%</td>
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</tr>
<tr>
<td>Formamide</td>
<td>Sigma-Aldrich Company Ltd (Poole, UK)</td>
</tr>
<tr>
<td>Giemsa Stain</td>
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</tr>
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<td>Glacial Acetic Acid</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
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<td>Hydrochloric Acid</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Isoton II Azide free balanced electrolyte solution</td>
<td>Beckman Coulter UK Ltd (High Wycombe, Bucks)</td>
</tr>
<tr>
<td>Jenners Stain</td>
<td>BDH Laboratory Supplies (Poole, UK)</td>
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<tr>
<td>DNA Lysis Buffer</td>
<td>Applied Biosystems (Warrington, UK)</td>
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<tr>
<td>Magnesium Chloride</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
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<tr>
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<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Pharmingen Stain Buffer</td>
<td>Pharmingen BD (San Diego, USA)</td>
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<td>Chemicals</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
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<td>Phenol</td>
<td>Fisher Scientific (Loughborough, UK)</td>
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<tr>
<td>Phosphate Buffered Saline Tablets (+Mg and Ca)</td>
<td>ICN Pharmaceutical (Ohio, USA)</td>
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<tr>
<td>Proteinase K</td>
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</tr>
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<td>RNAbee Lysis Buffer</td>
<td>Biogenesis (Poole, UK)</td>
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<tr>
<td>RNase A</td>
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<td>Slide Buffer pH 6.4</td>
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<tr>
<td>Sodium Chloride</td>
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<tr>
<td>Sodium Dodecylsulphate (SDS)</td>
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<td>Sodium Hydrophosphate</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>Fisher Scientific (Loughborough, UK)</td>
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<tr>
<td>Tris Acetate</td>
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<td>Tri Sodium Citrate</td>
<td>Fisher Scientific (Loughborough, UK)</td>
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<td>Trizma Base</td>
<td>Sigma-Aldrich Company Ltd (Poole, UK)</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Oxoid Ltd (Basingstoke, UK)</td>
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<tr>
<td>X-Galactoside</td>
<td>Melford Laboratories Ltd (Ipswich, UK)</td>
</tr>
<tr>
<td>Xyelene Cyanole FF</td>
<td>Sigma-Aldrich Company Ltd (Poole, UK)</td>
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<tr>
<td>Yeast Extract</td>
<td>Oxoid Ltd (Basingstoke, UK)</td>
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### 2.1.2 Kits

<table>
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<tr>
<th>Kit</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit</td>
<td>ABI Prism (Warrington, UK)</td>
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<tr>
<td>CpGenome DNA Modification Kit</td>
<td>Intergen Company (Oxford, UK)</td>
</tr>
<tr>
<td>One step RT-PCR Kit</td>
<td>Qiagen Ltd (Crawley, UK)</td>
</tr>
<tr>
<td>Qiaprep Maxiprep Kit</td>
<td>Qiagen Ltd (Crawley, UK)</td>
</tr>
<tr>
<td>Qiaprep Miniprep Kit</td>
<td>Qiagen Ltd (Crawley, UK)</td>
</tr>
<tr>
<td>Qiaquick Gel extraction Kit</td>
<td>Qiagen Ltd (Crawley, UK)</td>
</tr>
<tr>
<td>Random Primer DNA labelling system</td>
<td>Invitrogen Ltd (Paisley, UK)</td>
</tr>
<tr>
<td>Topo TA cloning Kit with one shot Top10 chemically competent <em>E.Coli.</em></td>
<td>Invitrogen Ltd (Paisley, UK)</td>
</tr>
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</table>
2.1.3 Solutions

All solutions were stored at room temperature unless indicated.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Preparation</th>
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</thead>
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<tr>
<td>Column Wash</td>
<td>1 X TE, 0.1% SDS</td>
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<tr>
<td>Church and Gilbert Prehybridisation Buffer</td>
<td>5g BSA, 250ml 14% SDS, 250 ml Na₂HPO₄, 1ml 0.5M EDTA pH 8.0, Preheat overnight 65°C, filtered 0.45μm syringe filter. Stored at -20°C.</td>
</tr>
<tr>
<td>DNA Loading Dye</td>
<td>10ml 50X TAE, 12.48g Ficoll, 0.1g Bromophenol Blue. Final volume 100ml with sterile water.</td>
</tr>
<tr>
<td>DEPC water</td>
<td>10μl Diethyl Pyrocarbonate, 200ml sterile water. Autoclaved.</td>
</tr>
<tr>
<td>10X MOPS</td>
<td>0.2M MOPS, 0.05M NaAc (Anhydrous) 0.01M EDTA. pH 7.0 with 10M NaOH.</td>
</tr>
<tr>
<td>Luria-Bertini (LB) Agar</td>
<td>15g/l Agar added to LB medium prior to sterilization.</td>
</tr>
<tr>
<td>Luria-Bertini (LB) Medium</td>
<td>10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl, made up to 1 litre with sterile water. pH 7 with 5M NaOH.</td>
</tr>
<tr>
<td>PBS/BSA</td>
<td>1 tablet dissolved in 100ml sterile water, 0.2% Fraction V BSA, 65°C overnight, Filter 0.2μm. Stored at 4°C.</td>
</tr>
<tr>
<td>RNA Denaturing Dye</td>
<td>3.75ml Formamide, 1.2ml Formaldehyde (37%), 0.75ml 10X MOPS, 0.5ml Glycerol, 0.25ml Ethidium bromide (1mg/ml), colour with bromophenol blue and xylene cyanol. Stored at -20°C.</td>
</tr>
<tr>
<td>Southern Depurinating Solution</td>
<td>0.25M HCL.</td>
</tr>
<tr>
<td>Southern Denaturing Solution</td>
<td>0.5M NaOH, 1M NaCl.</td>
</tr>
<tr>
<td>Southern Neutralising Solution</td>
<td>0.5M Tris-HCL pH 7.5, 3M NaCl</td>
</tr>
<tr>
<td>20X SSC</td>
<td>3M Sodium chloride, 0.3M tri-sodium citrate.</td>
</tr>
<tr>
<td>10X TAE</td>
<td>0.4M Tris-Ac, 0.05M NaAc, 0.001M EDTA, pH 7.8 with acetic acid.</td>
</tr>
<tr>
<td>10X TBE</td>
<td>0.9M Tris borate, 1mM EDTA, pH8.3.</td>
</tr>
<tr>
<td>10X TE</td>
<td>100mM Tris HCL, pH 8.0 10mM EDTA</td>
</tr>
<tr>
<td>Wash solution 1</td>
<td>2X SSC, 0.1% SDS</td>
</tr>
<tr>
<td>Wash solution 2</td>
<td>0.2X SSC, 0.1% SDS</td>
</tr>
<tr>
<td>Wash solution 3</td>
<td>0.1X SSC, 0.1% SDS</td>
</tr>
</tbody>
</table>
2.1.4 Primers

All gene sequences were obtained from http://www.nlm.nih.gov/entrez/query.fcgi. Tables 2.4 shows the primers used to generate probes and sequence DNA. These primers were chosen using http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi. Table 2.5 shows the primers used to analyse the methylation status of the Pax5 gene promoter and these were chosen using http://www.urogene.org/methprimer. All primers were purchased freeze dried from Thermohybaid Interactiva (Ashford, UK).

2.1.5 Available Probes

Various probes were available ‘in house’ including diagnostic probes for VpreB1, CD19, LysozymeM (LysM) and Myeloperoxidase (MPO), and house keeping gene probes Glutathione Peroxidase (GPX) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). These had previously been generated as described in Cleary et al (2001).

2.1.6 Restriction Enzymes

All restriction enzymes used in this study were purchased from New England Biolabs (Hitchin, UK) and digested as per the manufacturers’ recommendations.

2.1.7 Statistical Analyses

Results were compiled as a database using Microsoft excel 2000 and statistically analysed using STATISTICA data analysis software system, version 6 (2001) Statsoft Inc by Professor Yuri Dubrova.
Table 2.4 – Synthetic primers – Gene specific probes and sequencing primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Product Size</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adprtl</td>
<td>NM_007415</td>
<td>1209</td>
<td>ADRPT1F1 CACCCTCCAAGAAGAGCAAG&lt;br&gt;ADRPT1R1 CACTTTTGGAACACCATTGTGCG</td>
</tr>
<tr>
<td>DDB1</td>
<td>NM_015735</td>
<td>1116</td>
<td>XPEF2 GTGTCTCAAGAGCCAAAGC&lt;br&gt;XPER2 GACCACAATTACAGCCATTGCT</td>
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<tr>
<td>Dnmt1</td>
<td>NM_010066</td>
<td>1463</td>
<td>DNMT1F1 CGGACAGTGACACCCCTTT&lt;br&gt;DNMT1R1 ATAGACCAGCTTGTTGTTG</td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>NM_007872</td>
<td>1478</td>
<td>DNMT3AF1 ACTTGGAGAAGCGGAGTGAA&lt;br&gt;DNMT3AR1 CATGTAGCAGTTCAGGGGT</td>
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<tr>
<td>Dnmt3b</td>
<td>NM_010068</td>
<td>1152</td>
<td>DNMT3BF1 TACAGGAGCTGTCCACACAT&lt;br&gt;DNMT3BR1 CTCCAGTGATGCCCTAAA</td>
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<tr>
<td>Ephl</td>
<td>NM_010145</td>
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<td>EPFXF1 AAGCCATCAGCCAAGAAGA&lt;br&gt;EPFRX1 GCTAAAGCAGTGGTTGTTG</td>
</tr>
<tr>
<td>Fancg</td>
<td>NM_053081</td>
<td>466</td>
<td>FANCGL0 AAAGTGGGAAAAACCTGTGC&lt;br&gt;FANCGR1 GAAGCAGAGAGAAGGTACGG</td>
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<tr>
<td></td>
<td></td>
<td>670</td>
<td>FANCGL7 TACAGGACCTGCCTGCTGGCT&lt;br&gt;FANCGR9 GCACAGGTGGTCCTCCACTTT</td>
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<tr>
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<td></td>
<td></td>
<td>739</td>
<td>HLXF2 GCAGAAAGGACACAGGAGAAG&lt;br&gt;HLXR2 AGTCTCTATGCACAGACCCC</td>
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<td></td>
<td>943</td>
<td>HLXF3 GCTGTCTCCGCTCTGCTG&lt;br&gt;HLXR3 ACTGAAGCTGTTACACTCCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>474</td>
<td>HLXF4 ATCACCACCGCAGCAACAG&lt;br&gt;HLXR4 AGTGGAGAAGAAGACGACAG</td>
</tr>
<tr>
<td>mtDNA</td>
<td>J01420</td>
<td>720</td>
<td>mDNAFL1 ACACACCGCCGTCACCTCC&lt;br&gt;mDNAR1 GGCTGCTTTAGGCTGAATTT</td>
</tr>
<tr>
<td>Pax5</td>
<td>NM_008782</td>
<td>957</td>
<td>PAX5L5 GAACTTGCCCACATCAAGG&lt;br&gt;PAX5R1 GGGAACCTCAGAAATCATT</td>
</tr>
<tr>
<td>Pax5</td>
<td>AL772319</td>
<td>3419</td>
<td>PAX5F9 GAGAAAGATACAGCACGAC&lt;br&gt;PAX5R4 TGATGGAGTATGAGGAGCC</td>
</tr>
<tr>
<td>Pax5</td>
<td></td>
<td>2726</td>
<td>PAX5FA GTCCGATGGTTGTGCTT&lt;br&gt;PAX5RA GGACTGCTGGGATCTTAG</td>
</tr>
<tr>
<td>Pax5</td>
<td>AF157014</td>
<td>505</td>
<td>P5ProF1 ATGACCCCGACAGCAGCCTATG&lt;br&gt;P5ProR1 AAGAACCCAGGGAAAGGTTG</td>
</tr>
<tr>
<td>Sca-1</td>
<td>NM_010738</td>
<td>682</td>
<td>SCA1F1 CCCCTTCTCGAGGAGTCC&lt;br&gt;SCA1R1 CTTCACTTGCTGTCGTGGT</td>
</tr>
</tbody>
</table>
Table 2.5 – Synthetic primers - Methylation primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Product size</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax5</td>
<td>AF157014</td>
<td>222</td>
<td>C39F1: TATCCAAAAATTTTACCTATTCCTC</td>
</tr>
<tr>
<td>Promoter</td>
<td></td>
<td></td>
<td>C39R1: TTGGGGTTTATTTTTTAGGTTAGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>222</td>
<td>C39F2: ACCCTAACTCAAATTCTCCTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C39R2: AGAATTAGGGAAAAGGTGTTAGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>185</td>
<td>C39F3: CTCTCTAACCTCTTACACACTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C39R3: GTTATGTGAGATTATGTGTGT</td>
</tr>
</tbody>
</table>

2.2 Treatment of Animals

2.2.1 Animal Welfare

CBA/H mice were from the Harwell colony and were bred at the Biomedical Services Department at Leicester Royal Infirmary. C57BL/6 mice were purchased from Harlan UK Limited (Bicester, UK). All mice were maintained under standard conditions in the Biomedical Services Department, Leicester University.

All animal procedures were carried out under guidance issued by the Medical Research Council in its publication ‘Responsibility in the use of animals for medical research’ (July 1993) and Home Office Project license number PPL 80/1565 and 80/1564.

2.2.2 X-Ray

Eight to 12 weeks old male mice were exposed to a single acute dose of 3.0 Gy X-rays at 0.5Gy/min (constant potential, 250 kV, 1.2 mm Cu) using a Pantak industrial X-ray machine (Connecticut, USA). Animals were killed at specific time points by a Home Office Schedule One approved method (Appropriate methods of humane killing (Appendix 1 - Schedule 1) 1990).

2.2.3 Dissection

Mice were killed by cervical dislocation. Blood was extracted by inserting a 25 gauge needle directly into the heart. Where appropriate, blood was smeared onto a glass slide and the remaining sample transferred either directly into an eppendorf or expelled into RNAbee lysis buffer for RNA preparations and stored on ice. Solid tissues (spleen, kidney, liver, brain and tail) were then removed and stored directly on dry ice before
being transferred to −80°C until required. The leg was cut on the knee joint and into the hip bone. The ends of the long bones were trimmed to expose the interior of the marrow shaft. Femoral bone marrow cells were flushed into PBS containing BSA using a 25 gauge needle and a 1ml syringe, and stored on ice. Bone marrow slides were made by smearing femoral bone marrow over a glass slide.

2.2.4 Staining Smears

Slides were air dried and fixed in 100% Ethanol for 15 minutes. Slides were stained initially with 56% (v/v) Jenners stain and then with 15% (v/v) Giemsa stain - both these stains being diluted with pH 6.4 buffer. Once dried, the slide was mounted using DePex and a coverslip. The slides were then viewed using an Olympus BH2 microscope with attached Olympus camera (Olympus America Inc, NY) at X570 magnification.

2.3 Extraction of Tissues

2.3.1 Genomic DNA from spleen/kidney/bone marrow.

2.3.1.1 Proteinase K digestion of tissue sample

Dissected solid tissues were removed from storage at −80°C and placed in liquid nitrogen. The tissue was pulverized using a mortar and pestle before being spooned into room temperature Lysis Buffer (volume dependent on tissue size). Total bone marrow and lineage negative bone marrow DNA extraction was performed on fresh samples, which had been resuspended in 1 ml and 50μl of PBS respectively. Equal volumes of Lysis Buffer were added to each resuspended sample.

200μl (10μl in the case of lineage negative bone marrow) of Proteinase K (10mg/ml) was added to each tube and the tube incubated at 55°C overnight (~16 hours). In the case of solid tissues, following this incubation 50μl RNase A (10mg/ml) was added to the resultant mixture, the solution was gently mixed and incubated for a further 30 minutes at 37°C. All samples were transferred to 4°C until required.
2.3.1.2 Phenol/Chloroform extraction

1 volume of phenol was added to each sample and centrifuged (3000 rpm, 5 minutes, 21°C). The phenol layer was removed and the process repeated initially with another phenol extraction. This was followed by a 1:1 (v/v) phenol/chloroform extraction and finally a 1 volume chloroform extraction to remove all traces of phenol. The DNA was then ethanol precipitated.

2.3.1.3 Ethanol Precipitation

0.1 Volume 3M Sodium Acetate and 1 Volume Isopropanol were added consecutively to the aqueous layer from the extraction. Gentle inversion of the sample precipitates the DNA which is removed using a plastic stick, transferred into 70% (v/v) Ethanol and left at room temperature for 3-4 hours. After centrifugation (3000rpm, 15 minutes, 21°C), the Ethanol was decanted and the pellet left to dry for ~30 minutes. The DNA was then dissolved in 1XTE (volume dependent on precipitated DNA) and stored at 4°C.

2.3.1.4 Quantification and confirmation of DNA.

DNA integrity was confirmed by running aliquots of extracted DNA on a 1% (w/v) agarose/1XTBE gel (1g LE agarose, 100ml 1XTBE) containing ethidium bromide (10μg/μl) at 80V. DNA was viewed using a Gene Genius Bioimaging system and GeneSnap computer package (Syngene Ltd, Cambridge, UK). 1:200 dilutions of DNA solutions were made using sterile water. DNA quantification was carried out using an Ultrospec 2100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, UK) with measurements in μg/μl.

2.3.2 RNA extraction from spleen/bone marrow/blood.

2.3.2.1 Preparation of tissues for extraction.

Dissected solid tissues were removed from storage at −80°C and placed in liquid nitrogen. Pulverized tissue was spooned into RNAbee lysis buffer (volume dependent on tissue size). The RNAbee/tissue tube was mixed by shaking and the RNA sheared by repeat pipetting before being placed on ice. Bone marrow RNA extraction was performed on fresh bone marrow, which had been suspended in 250μl PBS buffer.
This bone marrow suspension was added to 2ml RNAbee lysis buffer. Whole blood was expelled directly into 1 ml RNAbee lysis buffer at dissection.

2.3.2.2 Extraction and precipitation of RNA.

1/10th volume of Chloroform was added to the RNAbee/tissue falcon tube and incubated on ice for 15-20 minutes. The tube was then centrifuged (20 minutes, 6000g, 4°C) and the clear supernatant transferred to a new tube. 1 Volume of Isopropanol was added, the solution mixed and incubated on ice for 20 minutes. Following centrifugation (15 minutes, 6000g, 4°C), the supernatant was discarded, 5ml 70% Ethanol added and the tube vortexed with care. The tube was then recentrifuged (10 minutes, 6000g, 4°C), the supernatant discarded and the tube left to dry inverted on tissue. The resultant pellet was resuspended in 50% (v/v) Ethanol/DEPC water to inhibit Ribonucleases.

2.3.2.3 Quantification and confirmation of RNA.

RNA integrity was confirmed by running denatured aliquots of the RNA solutions on 1% (w/v) agarose/1X MOPS gels containing ethidium bromide (10μg/μl). Samples were denatured by adding 3μl RNA to 20μl RNA denaturing loading dye and heating for 4-5 minutes at 65°C before being snap chilled on ice. 1% (w/v) agarose/1X MOPS gels were made by dissolving 1g LE agarose in 10ml 10X MOPS and 72ml sterile water, ethidium Bromide (10μg/μl) was added and once cooled 18ml 37% formaldehyde was added. The gel was run in a tank containing 1X MOPS at 80 volts. The gel was viewed using a Gene Genius Bioimaging system and GeneSnap computer package. Quantification of ethidium bromide staining was established using the Syngene GencTool computer package. 1:200 dilutions of RNA solutions were made using sterile water. RNA quantification was carried out using an Ultrospec 2100 pro UV/Visible spectrophotometer with measurements in μg/μl.
2.4 Gene Cloning

2.4.1 Reverse Transcriptase PCR

The Qiagen one step RT-PCR protocol uses 2 types of enzymes. The first type of enzyme is 'reverse transcriptase' which is activated immediately during the reaction thus ensuring efficient and specific reverse transcription. The second is 'HotStarTaq' DNA polymerase which is activated at 95°C and which inactivates the reverse transcriptase. The polymerase ensures specificity and reproducibility by eliminating extension from non-specifically annealed primers and primer-dimers. Efficiency is also aided by the 5X Qiagen 1 Step Buffer (KCL, (NH₄)₂SO₄) which minimises optimisation.

<table>
<thead>
<tr>
<th>Volumes</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase Free Water 29µl</td>
<td>-</td>
</tr>
<tr>
<td>5X Qiagen 1 Step Buffer 10µl</td>
<td>1X</td>
</tr>
<tr>
<td>DNTP Mix 2µl</td>
<td>400 µM each dNTP</td>
</tr>
<tr>
<td>Primer A 3µl</td>
<td>0.6µM</td>
</tr>
<tr>
<td>Primer B 3µl</td>
<td>0.6µM</td>
</tr>
<tr>
<td>Qiagen 1 step Enzyme 2µl</td>
<td>-</td>
</tr>
<tr>
<td>Template RNA 1µl (10µl if Poly A+)</td>
<td>1pg-2µg/reaction</td>
</tr>
<tr>
<td>TOTAL 50µl</td>
<td></td>
</tr>
</tbody>
</table>

The Qiagen one step RT-PCR protocol was used to set up each reaction and in all experiments parallel negative controls (RNase free water replacing template RNA) were performed. All reactions were performed using 0.2mmI tubes in a MJ Research PTC-2000 Peltier Thermal Cycler (Waltham, UK). The samples were initially heated for 30 minutes at 50°C, followed by 15 minutes at 95°C. Each PCR cycle then consists of denaturing at 94°C (1 minute), primer annealing at 58°C (1 minute) and extension at 72°C (90 seconds) for a total of 40 cycles. This was followed by a final extension step of 72°C (10 minutes). PCR products were resolved on a 1-2% (w/v)agarose/1XTBE gel against 1KB DNA ladder or a ΦX174 DNA/HaeIII Marker to confirm product size.

2.4.2 Polymerase Chain Reaction.

2.4.2.1 PCR Buffers

10X Reaction Buffer (750 mM Tris-HCL (pH8.8), 200 mM (NH₄)₂SO₄, 0.1% (v/v) Tween20) was used with an appropriate quantity of MgCl₂ (25mM), both supplied with Taq polymerase (ABgene, Epsom, UK).
11. IX PCR Buffer was produced in the Genetics Department, Leicester University by R. Neumann using the components outlined below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration of stock solutions</th>
<th>Final Concentration in the PCR reaction.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCL pH 8.8</td>
<td>1 M</td>
<td>45 mM</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>1 M</td>
<td>11 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 M</td>
<td>4.5 mM</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>100%</td>
<td>0.045%</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>10 mM</td>
<td>4.4 μM</td>
</tr>
<tr>
<td>dATP</td>
<td>100 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>dCTP</td>
<td>100 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>dGTP</td>
<td>100 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>dTTP</td>
<td>100 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>BSA</td>
<td>50 mg/ml</td>
<td>13 μg/ml</td>
</tr>
</tbody>
</table>

2.4.2.2 PCR Protocol

All reactions were performed using 0.2mmol tubes in a MJ Research PTC-2000 Peltier Thermal Cycler.

Amplification of small PCR products (<2 Kb)

50 ng of genomic DNA was amplified in a 10μl reaction using 0.125mM of dNTPs (Amersham Biosciences, Little Chalfont, UK), 0.375μM of primers, 0.5 units of Taq polymerase, 1μl 10X reaction buffer and 1.5 μM MgCl₂. Following denaturation at 94°C for 1.5 minutes, the PCR reactions were cycled at 94°C for 45 seconds, 58°C for 45 seconds and 72°C for 45 seconds for at total of 35 cycles, ending with a 10 minutes incubation at 72°C.

Amplification of larger PCR products (>2 Kb)

50 ng of genomic DNA was amplified in a 10μl reaction using 0.4μM of primers, 0.9μl 11.1X reaction buffer, 12mM Tris Base, 0.5 Units of Taq polymerase and 0.05 units of PFU Taq (Stratagene, Amsterdam, Netherlands). Standard PCR conditions were denaturation at 96°C for 1 minute, 32 cycles of 96°C for 20 seconds, 60°C for 30 seconds and 65°C for 3 minutes, followed by a final incubation of 72°C for 10 minutes. Conditions were altered to suit specific primers.
2.4.2.3 **PCR Confirmation**

PCR products were resolved on a 1-2% (w/v) LE agarose/1X TBE gel against the appropriate ladder to confirm the product size.

2.4.3 **Ligation and Transformation.**

Ligation and transformation was carried out using the TOPO-TA cloning kit and following the manufacturers’ instructions.

2.4.3.1 **Ligation**

Ligation involved incubating 2μl of PCR product (section 2.4.2.2) or gel extracted product (section 2.4.7) with 1μl salt solution (final concentration 200mM NaCl, 10mM MgCl$_2$) and 1μl of TOPO vector in a total volume of 6μl. Ligation of vector with the PCR product occurs spontaneously when incubated at room temperature for 5 minutes. The number of transformants produced can be increased 2-3X by using the salt solution or extending the incubation time of 5 minutes.

2.4.3.2 **Transformation**

The vector containing the PCR insert was then added to TOP10 competent cells and incubated at 4°C for 30 minutes before being transformed by heat shock at 42°C for 30 seconds. SOC Media (2% Tryptone, 0.5% Yeast Extract, NaCl, KCl, MgCl$_2$, MgSO$_4$, 20mM Glucose) was added to the cells and incubated for 1 hour shaking horizontally. The transformed reaction was then spread on to pre-warmed Luria-Bertini (LB) plates containing ampicillin (10μg/μl) and covered with 40μl X-gal (40μg/μl) before being incubated overnight at 37°C.

The pCR2.1-TOPO vector contains an ampicillin resistance gene and the LacZ’ gene which encodes the α-peptide portion of the enzyme β-galactosidase. This ensures that only transformed cells with the ampicillin resistance gene can grow on the plates. X-gal is a lactose analogue which is broken down by β-galactosidase to form a blue colony. Insertion of PCR product into the plasmid disrupts the LacZ open reading frame and results in inactivation of LacZ. As LacZ encodes β-galactosidase, active β-galactosidase is not expressed and therefore X-gal is not broken down. This results in white colonies. White colonies can therefore be selected from the plates using sterile
procedures and resuspended in LB media containing 10μg/μl Ampicillin. Incubation overnight at 37°C enables scaling up of the culture.

2.4.4 Small scale preparation of plasmid using Qiagen Miniprep.

The Qiagen protocol uses modified alkaline lysis whereby DNA is exposed to a narrow alkaline range (pH 12.0 – 12.5) by adding NaOH-SDS based lysis buffer. This results in denaturation of linear bacterial chromosomal DNA but not covalently closed circular (CCC) plasmid DNA. Subsequent neutralisation results in chromosomal DNA aggregating to form an insoluble network whilst the plasmid DNA remains in solution.

3ml of bacterial culture was pelleted by centrifuging for 2 minutes, 13000g and the manufacturers’ protocol followed. Briefly this involves resuspending the bacterial cells in a buffer containing RNAse A. A NaOH-SDS based lysis buffer was then added followed by a neutralising buffer, and the bacterial debris pelleted by centrifuging for 10 minutes. Passing the supernatant through a QIAprep spin column results in purification as the plasmid DNA binds to the silica-gel column in high salt concentrations. RNA and soluble proteins are washed out using a medium salt buffer. The purified plasmid DNA was then eluted with 50μl of 10mM Tris.HCL pH 8.5 to yield 10-25μg of plasmid DNA.

2.4.4.1 Restriction Digestion of Minipreps

The pCR®2.1-TOPO vector has an EcoR1 site on either side of the insertion site of the PCR product. This enables restriction digestion of the plasmid using EcoR1 to be used to establish the presence of a correctly sized insert. Approximately 0.2-0.5μg of eluted DNA was incubated with 1μl (20 U/μl) EcoR1 and 2μl EcoR1 react buffer in a total volume of 20μl for 2 hours at 37°C. The resultant solution was then run on a 1-2% (w/v) agarose/1X TBE ethidium bromide (10μg/μl) gel against either a 1Kb DNA ladder or a φX174 DNA/HaeIII Marker depending on the expected size of the insert. Clones that gave the correct size insert were then sequenced for confirmation.

2.4.5 Large scale preparation of plasmid using Qiagen Maxiprep.

Flasks containing 400ml of LB media (0.1 mg/ml ampicillin) were inoculated with 4.5ml of bacterial culture containing the appropriate plasmid and were grown overnight (37°C, continuous shaking). The Qiaprep Maxiprep (Qiagen) kit was used
and the manufacturers’ protocol followed. This kit works on the alkaline lysis principle described previously (section 2.4.4). Purified plasmid DNA was eluted in 100-200μl 1X TE dependent on pellet. The plasmid was converted to 1mg/ml prior to restriction digestion with EcoRI.

2.4.5.1 Restriction Digestion of Plasmid

As described in section 2.4.4.1.

2.4.6 Sequencing

2.4.6.1 Sequencing Minipreps

Sequencing was carried out using an Applied Biosystems Model 377 DNA Sequencing System, with the ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit in accordance with the manufacturers’ instructions. 10-20ng/KB of plasmid DNA was incubated with 3.2μM primer and Big Dye Reaction mix in a total volume of 10μl. The reaction was amplified using the following PCR conditions. Samples were initially denatured at 95°C for 5 minutes. PCR reactions were then cycled at 95°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes for a total of 25 cycles.

The PCR products were vortexed with 10μl sterile water, 2μl 3M NaAc ph5.5 and 50μl 100% ethanol before being incubated on ice for exactly 7 minutes. After a 20 minute spin, the tubes were washed twice with 70% ethanol before being left to dry. Gel running was performed by the Protein and Nucleic Acid Laboratory (PNACL), University of Leicester. Sequence data was analysed using Factura™ Release 1.2.0 (Applied Biosystems, Warrington, UK) and Autoassembler Release 1.4.0 on Apple Macintosh computers. Resultant sequences were then blasted against the appropriate gene using the NCBI blast programme to confirm their authenticity.

2.4.6.2 Sequencing Maxipreps.

500ng/ml of plasmid DNA was incubated with 15μM primer and Big Dye Reaction mix in a total volume of 10μl. The reaction was amplified using the following PCR conditions. Samples were initially denatured at 95°C for 5 minutes. PCR reactions were then cycled at 95°C for 30 seconds, 53°C for 30 seconds and 60°C for 4 minutes.
for a total of 80 cycles. The resultant PCR product was precipitated and sequenced as per section 2.4.6.1.

2.4.7 DNA purification

PCR purification was carried out using the QIAquick gel extraction kit. Essentially, the correctly sized bands were cut out of a 1-2% (w/v) agarose/1XTAE gel under fluorescence and incubated at 50°C for 10 minutes with a solubilization Buffer. Passing this solution through a silica membrane spin column binds DNA enabling the removal of impurities. Any remaining salts were washed from the column before eluting the DNA under low salt and basic conditions (50μl 10mM Tris HCL).

2.5 Southern and Northern Blotting.

2.5.1 Treatment of Samples.

2.5.1.1 Preparation of DNA for a Southern Blot.

10-15μg DNA was digested with 7μl Restriction Enzyme (10-20U/μl) overnight at the optimum temperature for the appropriate enzyme. A further 7μl of Enzyme was added the following day and left to digest for more than 5 hours. The solution was then ethanol precipitated. This involved incubating the digested DNA with 0.1 Volume of 3M NaAc and 3 Volumes of 100% Ethanol for 10 minutes on dry ice before removing the ethanol and resuspending the resultant DNA in 20μl of 1XTE. DNA that required a double enzyme digestion was resuspended in 150μl water and digested with a second enzyme. The double-digested DNA was precipitated and resuspended in 20μl of 1XTE as before.

DNA digestion was checked by electrophoresing ~1μg of the digested DNA on a 1-2% (w/v) agarose/1XTBE gel for ~1 hour at 100V. Once digestion had been confirmed, 10μg of digested DNA was run on a 1-2% (w/v) agarose/1XTBE gel containing ethidium bromide overnight at 40V. Each southern blot had one lane containing a 1Kb Ladder to enable subsequent sizing. DNA visualisation required a Gene Genius Bioimaging system and GeneSnap computer package. The gel was then subjected to a sequence of washes (2 x 10 minutes Depurinating buffer, 2 x 20 minutes Denaturing
buffer, 2 x 20 minutes Neutralising buffer) before being transferred to the blotting apparatus.

2.5.1.2 Preparation of RNA for a Northern Blot.

RNA preparation required 20μg of RNA and 20μl RNA loading buffer to be denatured for 4-5 minutes at 65°C before being snap chilled on ice. The RNA was then run on a 1% (w/v) agarose/1XMOPS gel in a 1X MOPs solution overnight at 40 volts. The RNA was soaked in a 10X SSC high salt solution for 40 minutes.

2.5.2 Transfer of RNA or DNA onto a nylon membrane.

The blotting apparatus consisted of a tray of 10X SSC which had a perspex plate placed on top. This plate was covered with buffer soaked Whatmann's 3MM chromatography paper (Whatmann's International Ltd, Maidstone, UK). The gel was placed on top of the chromatography paper and covered initially with a MAGNA nylon membrane (MSI, Osmonics Laboratory Products) before more chromatography paper and paper towels were added. At each stage that paper was added, care was taken to squeeze out air bubbles which would interfere with the transfer of the RNA. Weights were then placed on top of a second perspex plate and the blot left overnight with intermittent changing of the paper towels to aid transfer. The apparatus was then dismantled and the membrane then baked for 1 hour at 80°C before being crosslinked by exposing the membrane to 7x10⁴ J/cm² of UV light in a RPN 2500 ultraviolet crosslinker (Amersham Biosciences, Little Chalfont, UK).

2.5.3 Pre-Hybridising the Membrane.

The nylon membrane was soaked in 2X SSC and transferred into a pre-warmed hybridisation bottle. 12ml of Church & Gilbert prehybridisation buffer/BSA was added and the bottle prehybridised in 65°C Mini 10 hybridisation oven (ThermoHybaid, Ashford, UK) for >5 hours.

2.5.4 Radiolabelling a probe.

Random Priming DNA labelling kit was used to radiolabel various probes. 20-50ng of probe was added to sterile water (total volume 23μl), denatured by boiling for 6 minutes and immediately immersed in ice. 15μl of Random primer buffer, 6μl of dNTPs (equal volumes of all dNTPs excluding dCTP which is incorporated into α-³²P), 5μl α-³²P-dCTP (1000 Ci/mmol, NEN, Belgium) and 1μl of Klenow Fragment (DNA
Polymerase I) were added to the probe and incubated at room temperature for 1 hour. DNA synthesis was stopped by adding 30μl of Column Wash (1X TE/0.1% (w/v) SDS). G50Nic columns (Amersham Biosciences) were equilibrated twice with Column Wash after which the probe was passed through the column. Un-incorporated dNTPs were removed by passing 400μl of column wash through the column. Eluted probe was collected by adding a further 400μl of column wash through the column. After being denatured at 100°C for 6 minutes and cooled on ice for 5 minutes, the resultant probe was added to a prehybridised membrane and incubated overnight at 65°C.

2.5.5 Washing excess probe from a membrane.

The membrane was initially washed twice at 65°C (2 x 30 minutes) in low stringency washing solution (2 x SSC/0.1% SDS) and then twice at 65°C (2 x 30 minutes) in high stringency washing solution (0.2 x SSC/0.1% SDS). The membrane was then removed from the bottle and checked for radioactivity using a Gieger counter. Counts suggesting the presence of unbound probe resulted in a further increased stringency wash (0.1 x SSC/0.1% SDS) of 30 minutes 65°C. The membrane was then wrapped in Saran Wrap and placed in Hypercassette autoradiographic cassettes (Amersham Biosciences) which incorporate an intensifying screen. Cassettes were then exposed using either Kodak imaging film Biomax MS (Autorads, Lutterworth, UK) or Fugi RX100 X-ray film (Genetic Research Instrumentation, Braintree, UK) for varying amounts of time at -80°C depending on the strength of signal. The pattern of hybridisation was visualised by autoradiography using Xograph Imaging System Compact X4. The need for either further washes or further exposures was then established.

2.5.6 Cyclone procedure.

Radioactive expression of Northern blots was quantified using a Cyclone Storage and Phosphor Screen Phosphoimager (Packard Biosciences Ltd, Pangbourne, Berks, U.K.). Supersensitive type ST cyclone storage phosphor screens were placed against radioactive blots for varying amounts of time at room temperature depending on the strength of signal. Signals were quantified using OptiQuant™ software and blanked against the background signal.
2.5.7 Reusing a membrane.

Used membrane were stripped in a solution of 1000ml boiling water containing 10ml 10% SDS and 5ml 20X SSC for 15-20 minutes. Following confirmation that the blot was no longer radioactive, the membrane was either re-probed or stored at 4°C.

2.6 Pax5 gene promoter Methylation Status.

2.6.1 Restriction digestion of the Pax5 gene promoter region.

2.6.1.1 Southern Blot Analysis

As per section 2.5.1.1, 10µg genomic DNA was double digested with 7µl EcoR1 (10-20U/µl) and 5µl HpaII (10U/µl) and resuspended in 20µl 1X TE. Restriction digested DNA was resolved on a 1.8% (w/v) agarose/1XTBE gel and treated as per section 2.5.

2.6.2 Bisulphite Treatment.

2.6.2.1 Preparation of samples.

10µg of genomic DNA was digested with 4µl EcoR1 (10-20U/µl) as per section 2.5.1.1. In the case of lineage negative DNA extraction, the whole aliquot of Lin' DNA was digested with EcoR1. DNA was purified using 1:1 (v/v) phenol/chloroform extraction followed by 1 chloroform extraction. This was followed by incubation with 0.1 Volume of 4M NaCl and 3 volumes of 100% Ethanol on dry ice for 15 minutes or -80°C overnight. Once the ethanol was removed, the DNA was washed with 70% ethanol before being resuspended in 1X TE.

2.6.2.2 Bisulphite Treatment of DNA.

The CpGenome modification kit was used to bisulphite treat DNA. The manufacturers’ instructions for the preparation of reagents were followed and the samples treated as per the following protocol. Bisulphite treatment can be subdivided into two procedures DNA Modification and DNA clean up.

2.6.2.2.1 DNA modification

A solution of 1µg DNA in 100µl sterile water (10ng/µl) was denatured by adding 7µl 3M NaOH. This was incubated for 10 minutes at 37°C before being neutralised by
adding 550μl freshly prepared DNA modification Reagent I. The resultant solution was then vortexed and incubated at 50°C for 16-20 hours.

5μl of well-suspended DNA modification Reagent III was added to the DNA suspension along with 750μl DNA Modification Reagent II. The sample was then mixed briefly and incubated at room temperature for 5-10 minutes. The tubes were centrifuged (10 seconds, 5000g) to pellet the DNA modification Reagent III and the supernatant discarded.

2.6.2.2 DNA Clean up.

The pellet was washed 3 times in 1ml of 70% ethanol (10 seconds, 5000g). Following the removal of the final wash, 50μl 20mM NaOH/90% Ethanol was added to each sample. This wash was then followed by two 1ml 90% ethanol washes before finally 30μl TE (10mM Tris/0.1 mM EDTA, ph 7.5) was added to each tube. DNA was incubated for 15 minutes at 50-60°C and spun for 2-3 minutes before the eluted DNA was transferred to a new eppendorf.

2.6.2.3 Pax5 Promoter Methylation status

~1μg bisulphite treated DNA was amplified in triplicate as per section 2.4.2.2. (annealing temperature 58°C). The PCR product was run on a 1.8% (w/v) low melting point agarose/1X TAE Ethidium Bromide Gel against a FX174 DNA/Hae III marker thus enabling the assessment of the size of the PCR products. PCR purification was carried out using the QIAquick gel extraction kit (section 2.4.7.). Purified DNA was ligated and transformed and the resultant white colonies were grown and DNA eluted using the miniprep procedure (section 2.4.4). Miniprep sequencing was used to confirm methylation (section 2.4.6.1).

2.7 Fluorescence Activated Cell Scanning (FACS)

2.7.1 Establishing bone marrow counts.

Extracted bone marrow was initially washed in Pharmingen stain buffer (centrifuge 4000g, 5 minutes, 20°C) before being resuspended in 1ml of Pharmingen stain buffer. Bone marrow was then diluted 1:2000 in Isoton II and the cells counted using a
Schärfe System Casy® 1 counter (Reutlingen, Germany). The Schärfe System Casy® 1 counter principle is based on electrolyte resistance and pulse area analysis. Cells suspended in a weak electrolyte (Isoton II) solution pass individually through an electrically supplied capillary. This capillary has a defined electrical resistance level and cells displace the electrolyte solution equivalent to the cell’s volume. Changes in resistance levels are seen as a ‘pulse’ enabling the diameter-linear size distribution to be computed by the analyser.

2.7.2 Analysis of immature haemopoietic cell compartment.

2.7.2.1 Antibodies

All antibodies were purchased from Pharmigen BD (San Diego, USA). Cocktail antibodies to remove mature cells were biotin-conjugated rat anti-mouse monoclonal antibodies at the concentration 0.5mg/ml. Antibodies used for fluorescence staining are either Fluroscin Isothiocyanate (FITC) or R-phycoerythrin (PE) conjugated rat anti-mouse monoclonal antibodies and their concentrations are stated below. FITC conjugated Rat IgG\(_2\), \(\kappa\) and PE conjugated IgG\(_2\), \(\kappa\) monoclonal immunoglobulin isotype controls were used as isotype-matched negative controls for immunofluorescent staining as per manufacturers’ instructions.

Biotin-labelled antibodies are expensive and therefore antibodies were titrated to establish the minimum volume of antibody required to ensure complete binding of all relevant cells. Optimisation of the cocktail utilised the binding efficiency of each conjugated antibody to establish the volume of antibody required to bind a million cells. The cocktail below is appropriate for a bone marrow suspension containing ~10 million white cells and ~8 million red cells per ml.

<table>
<thead>
<tr>
<th>Biotinylated antibody</th>
<th>Cell type removed</th>
<th>Standard Cocktail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ter119</td>
<td>Red Cells</td>
<td>16(\mu)l</td>
</tr>
<tr>
<td>Ly6G+Ly6C</td>
<td>Myeloid</td>
<td>6(\mu)l</td>
</tr>
<tr>
<td>CD11b (Mac-1)</td>
<td>Granulocytes/Monocytes</td>
<td>6(\mu)l</td>
</tr>
<tr>
<td>CD45R/B220</td>
<td>B Lymphocytes</td>
<td>6(\mu)l</td>
</tr>
<tr>
<td>CD3(\varepsilon)</td>
<td>T Lymphocytes</td>
<td>6(\mu)l</td>
</tr>
<tr>
<td>Pharmingen stain buffer</td>
<td></td>
<td>460(\mu)l</td>
</tr>
</tbody>
</table>
2.7.2.2 **Preparation of Magnetic Beads**

Each mouse required 200μl of Streptavidin Ultra-load Magnetic Beads (Metachem Diagnostics, Northampton UK) to be washed 3 times in Pharmingen stain buffer and made up to a final volume of 500μl.

2.7.2.3 **Antibody mediated removal of cells using magnetic beads.**

Using the white count obtained from the Schärfe System Casy® 1 counter, the volume of resuspended bone marrow needed to be analysed to give a total count of 10 million white cells per ml was established. This volume was added to the appropriate amount of Pharmingen stain buffer needed to give a total volume of 4500μl. 500μl of the appropriate antibody cocktail was then added, the resultant solution mixed and incubated on ice for 40 minutes.

Following the incubation, excess antibody was removed by centrifuging the solutions (6 minutes, 4°C, 400 rcf) and removing the supernatant. The pellet was then resuspended in 500μl Pharmingen stain buffer and an additional 4 ml of buffer added. 500μl of pre-washed magnetic streptavidin coated beads were then added to each tube and the tubes gently mixed before incubating on ice for 30 minutes.

The tubes were then stood against a magnet for 10 minutes to allow the beads to attach. The supernatant was then transferred to another tube and the process repeated with an extra 1ml of buffer to ensure complete removal of unbound cells. The cells were then pelleted (centrifuged 10 minutes, 4°C, 500g) and the supernatant discarded. The pellet was resuspended and transferred to an eppendorf tube where again the cells were subjected to a magnetic force to remove any remaining magnetic beads. Further washes with Pharmingen stain buffer and magnetic removal continued until the pellet had a straw-like coloured appearance. The pellet was then finally resuspended in a volume of 400μl Pharmingen stain buffer.

2.7.2.4 **Fluorescence staining of cells.**

The 400μl aliquot from the previous stage was divided into two 200μl aliquots. 1μl of FITC-labelled anti Sca-1 (0.5mg/ml) and 1μl of PE-labelled anti c-Kit (0.2mg/ml) were added to one aliquot while 1μl of FITC-labelled anti Sca-1 and 1μl of PE-labelled anti Flk-2 (0.2mg/ml) were added to the other aliquot. These aliquots were
then incubated on ice for 30 minutes to allow staining. The aliquots were then spun (6 minutes, 3000g, 4°C) and supernatant discarded. The pellets were then washed twice in 200µl Pharmingen stain buffer before being resuspended in 500µl Pharmingen stain buffer and transferred into a FACS tube ready for analysis.

### 2.7.3 Analysis of Total Bone Marrow.

FACS analysis routinely required an unstained aliquot of bone marrow cells to act as a control. This control was made by adding 10µl of bone marrow cells to 490µl Pharmingen stain buffer.

#### 2.7.3.1 Fluorescence staining of total bone marrow.

Two aliquots of 10µl total bone marrow diluted in 190µl Pharmingen stain buffer were made. 2µl of FITC-labelled anti Mac-1 (0.5mg/ml) and 2µl of PE-labelled anti Flk-2 were added to one aliquot while 2µl of FITC-labelled anti Sca-1 and 2µl of PE-labelled anti c-Kit were added to the other. These aliquots were then incubated on ice for 30 minutes to allow staining. The aliquots were then centrifuged (6 minutes, 3000g, 4°C) and supernatant discarded. The pellets were then washed twice in 200µl PBS/BSA before being resuspended in 500µl Pharmingen stain buffer and transferred into a FACS tube ready for analysis.

### 2.7.4 Analysis of Fluorescence stained cells.

5000 cells per suspension were analysed using a Becton Dickinson Facscan in conjunction with the CellQuest pro computer package. Unstained bone marrow and isotypes (FITC and PE) were used to establish and calibrate the experimental parameters (Chapter 3). If necessary, experimental parameters were altered to compensate for changes in antibody batches. An example of an analysis set up is shown below.

- **Detectors/Amps**

<table>
<thead>
<tr>
<th>Detector</th>
<th>Voltage</th>
<th>Amp Gain</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 FSC</td>
<td>E00</td>
<td>1.32</td>
<td>Lin</td>
</tr>
<tr>
<td>P2 SSC</td>
<td>444</td>
<td>1</td>
<td>Lin</td>
</tr>
<tr>
<td>P3 FL1</td>
<td>566</td>
<td>1</td>
<td>Log</td>
</tr>
<tr>
<td>P4 FL2</td>
<td>551</td>
<td>1</td>
<td>Log</td>
</tr>
<tr>
<td>P5 FL3</td>
<td>150</td>
<td>1</td>
<td>Log</td>
</tr>
</tbody>
</table>

- **Threshold - FSC-H** 52
- **Compensation - F12** 23.6 %
Chapter 3: Comparison of CBA/H and C57BL/6 Bone Marrow cellularity.

3.1 Introduction

Different inbred mouse strains have been bred for a number of reasons including a susceptibility to spontaneous or induced disease and are therefore invaluable tools in identifying genetic susceptibility genes. r-AML susceptible CBA/H and r-AML resistant C57BL/6 inbred mice (section 1.2.2) were used to try and identify genetic factors that confer a susceptibility or resistance to r-AML. A genome wide screen of r-AML affected CBA/H x (CBA/H x C57BL/6)F1 backcross mice had previously identified two suggestive predisposition loci - a CBA/H susceptibility locus on chromosome 1 (92.3-100 cM; Relative Risk 2.17) (figure 3.1) and a CBA/H resistance locus on chromosome 6 (20.5-38.5 cM; Relative Risk of 2.75) (Boulton et al., 2003). This thesis concentrates on the CBA/H susceptibility locus on chromosome 1.

Figure 3.1 – r-AML susceptibility locus located on murine chromosome 1

Figure 3.1 illustrates genetic linkage data for r-AML affected CBA/H x (CBA/H x C57BL/6) backcross mice published by Boulton et al., 2003. LOD >3.0 is suggestive of linkage. The r-AML susceptibility locus is located at ~100cM, a region that overlaps with the Stem Cell Frequency Regulator 1 locus (Scfr1).
The Chromosome 1 r-AML susceptibility locus maps within the genetically
determined Stem cell frequency regulator 1 (Scfrl) QTL (http://www.informatics.
jax.org; 90-113cM; figure 3.1). The Scfrl locus determines the frequency of bone
marrow long term culture-initiating cells (LTC-IC) in mice (Müller-Sieburg & Riblet,
1996). LTC-IC are functionally and phenotypically indistinguishable from long term
haemopoietic stem cells (LT-HSC) which have the ability to repopulate and maintain
the bone marrow for life following transplantation into a lethally irradiated mouse
(reviewed Coulombel, 2004).

One factor in the risk of cancer is target cell number, and the target cell in myeloid
leukaemogenesis is the haemopoietic stem cell (HSC) (reviewed Hope et al., 2003;
Warner et al., 2004). This raises the possibility that genetically determined HSC
number is a risk factor in r-AML. Various studies using different assays have
indicated that there are significant differences in the frequency of bone marrow HSCs
in inbred mouse strains (Henckaerts et al., 2002, 2004; de Hann & Van Zant., 1997B;
Morrison et al., 2002; Müller-Sieburg & Riblet., 1996) with many studies reporting
that the r-AML resistant C57BL/6 mice have a low number of HSC compared to other
inbred mouse strains. Most studies have compared C57BL/6 and DBA/2 mice.
Although DBA/2 mice are related to CBA mice (Festing, 1996), no detailed studies
have examined CBA/H mice. This study aimed to compare the number of bone
marrow HSCs in the CBA/H and C57BL/6 mouse strains and to test the hypothesis
that CBA/H mice have a higher frequency of HSCs and therefore ‘target cells’ than
C57BL/6 mice.

3.2 Experimental Design

3.2.1 Choice of antibodies

HSCs constitute ~0.01% of white bone marrow cells. To make this population
amenable to analysis, mature bone marrow cells are removed using an immuno-
magnetic system of biotinylated antibodies in conjunction with streptavidin coated
magnetic beads. This study used a lineage (Lin+) cocktail of 5 antibodies – CD3ε,
CD45R/B220, CD11b, Ly6G+Ly6C and Ter119 to remove mature T lymphocyte, B
lymphocytes, granulocytes/monocytes, myeloid cells, and red cells respectively. The
Ter119 antibody reacts with a 52-kDa molecule associated with glycoporphin A on
erthroid lineage cells and is expressed from pro-erythroblasts to mature RBC stages.
The use of the lineage cocktail enables the selection of Lin' bone marrow cells which are enriched for more immature bone marrow cells.

Flow cytometry using fluorescently labelled antibodies was used to identify phenotypically defined bone marrow cells both within total bone marrow and Lin' bone marrow. Rat anti-mouse monoclonal antibodies were conjugated to either Fluroescein Isothiocyanate (FITC) or R-phycoerythrin (PE), and the excited fluorochromes detected using a Becton Dickinson Facscan (section 1.5.2.3). Lin' bone marrow cells were stained with antibodies to cell surface antigens Sca-1, c-Kit and Flk-2, and total bone marrow with antibodies to Mac-1, Sca-1, c-Kit and Flk-2. These antigens were chosen because they are expressed on HSCs or have an association with AML.

Sca-1, c-Kit and Flk-2 are HSC cell surface markers (section 1.5.2.2). Lin', Sca-1++, c-Kit++ Flk-2' bone marrow cells have long term reconstituting ability and can rescue a lethally irradiated mouse for life, while Lin', Sca-1++, c-Kit++, Flk-2++ bone marrow cells are only capable of short term bone marrow repopulation (Christensen & Weissman, 2001). Both Flk-2 and c-Kit have been implicated in AML, as ~70% of human AMLs show high expression of Flk-2 and Flk-2 mutations are detected in 30% of these malignancies (Gilliand & Griffin, 2002). c-Kit is expressed in up to 80% of AML cases (Warner et al., 2004). Mac-1 was chosen to stain total bone marrow because it is expressed in myeloid progenitors (Morrison et al., 1997) and AML is a myeloid disease.

3.2.2 Experimental parameters

Cells analysed by flow cytometry are visualised on a cytogram which reflects their staining intensity (figure 3.2). Unstained bone marrow cells were scanned to establish background fluorescence. Altering the forward scatter (FSC) and side scatter (SSC) detector settings (section 2.7.4) enabled bone marrow cells to be displayed and gated. Adjustments of the FL1 and FL2 detector settings ensured that the unstained bone marrow cells congregated in the bottom left hand corner of the cytogram (figure 3.2). The majority of unstained cells were located in the 30 x 40 log scale quadrant with background staining outside this region calculated to be 1.02% (n=8; ± 0.16 s.e.m).
Isotype matched negative controls (antibodies conjugated to the fluorochrome only) were used to confirm that the staining patterns were not the result of non-specific binding of FITC and PE conjugated antibodies to bone marrow cells. Both FITC and PE utilise the same excitation wavelength of 488nm. FITC emissions are measured by the FL1 detector (green signal - fluorescence peak 530nm) and PE by the FL2 detector (orange signal – fluorescence peak 575nm). These signals are however not exclusive with a significant amount of orange fluorescence being present in the FITC emission and green in the PE emission. Isotype controls are used to compensate for the overlapping of the different fluorochromes thus reducing false positives (BD Biosciences). Background staining of bone marrow for single PE isotype was 0.8% (n=5; ± 0.22 s.e.m) and single FITC isotype was 0.38% (n=5; ± 0.11 s.e.m) (M. Plumb, personal communication).

Bone marrow is composed of heterogeneous cell populations and therefore to maximise information gained from FACS analysis, cytogram regions were arbitrarily defined as they emphasised differences between CBA/H and C57BL/6 bone marrow cells. This resulted in 9 cytogram regions (figure 3.3). The analysis of bone marrow white cells expressing individual cell surface antigens is further complicated by the presence of red cells in the total bone marrow. Red cells can be removed from bone marrow by lysis using buffered ammonium chloride but this was not recommended as it reduced the effectiveness of the antibody staining (BD Biosciences). Therefore to specifically analyse the white cell population in total bone marrow it is necessary to compensate for the red cell count because all cells are counted during FACS analysis. The following formula was used in an excel work sheet to calculate the percentage of stained white bone marrow or Lin⁻ white cells within a particular cytogram quadrant/region as a percentage of the total white bone marrow/ Lin⁻ cell count:

\[
\text{White count} + \text{Red count} \times \frac{\% \text{ cells stained with specific white count antibody in cytogram region}}{\text{White count}}
\]

The Mann Whitney U-test was used to test differences in the two independent samples – CBA/H and C57BL/6 bone marrow. The Mann Whitney U-test is a non parametric test that is not dependent on a normal population distribution and analyses the degree of separation between two samples (Sokal & Rohlf, 1994).
Quadrants were defined by the presence of the unstained total bone marrow cells. As shown most bone marrow cells are located in the 30 x 40 log quadrant and this is subsequently represented as -/- (figure 3.3). Counting the percentage of cells within the remaining three quadrants established background staining of 1.02% (n=8; ± 0.16 s.e.m). Background staining has been confirmed using unstained Lin- and isotype controls (Data not shown).

Flow cytometry cytogram sections were based on staining intensity. The unstained region (-/-) was established using unstained bone marrow and isotype controls (figure 3.2). The fluorescence antibodies used were FITC (Fluorescein Isothiocyanate) and PE (R-Phycoerythrin) conjugated as represented on the y and x axis respectively. Red symbols represent the FITC and blue the PE staining intensity. The distinction between + and ++ was arbitrary.
3.3 Results

3.3.1 Total Bone Marrow

3.3.1.1 Bone Marrow White and Red cell counts.

White (WBMC) and red (RBMC) cells numbers in bone marrow were counted using a Schärfe System Casy® 1 cell counter which can be set to count cells according to size: red cells were defined as 3-6μm whilst white cells were defined as >6μm (figure 3.4A). The red cell size was confirmed by analysing blood (figure 3.4B; analysed by M Jawad). In addition to supplying information concerning bone marrow cellularity, counting was essential to estimate the antibody concentration required to prepare Lin− cells by immuno-magnetic separation.

Figure 3.4 - Schärfe System Casy® 1 cell counter profiles

As illustrated in table 3.1, no statistically significant difference ($p=0.47$) was observed in the recovery of WBMC from 2 femur between the two inbred mouse strains. There was however a very reproducible and statistically significant difference in the recovery of red cells in the bone marrow ($p=0.0002$) with CBA/H mice having more bone marrow red cells than C57BL/6 mice (Table 3.1).
Table 3.1 – Cellularity of bone marrow extracted from 2 mouse femurs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CBA/H</th>
<th>C57BL/6</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBMC</td>
<td>30.85 ± 0.36 (n=17)</td>
<td>32.29 ± 0.33 (n=19)</td>
<td>p = 0.47</td>
</tr>
<tr>
<td>RBMC</td>
<td>24.5 ± 0.86 (n=24)</td>
<td>19.4 ± 0.65 (n=18)</td>
<td>p = 0.0002</td>
</tr>
<tr>
<td>WBMC/RBMC ratio</td>
<td>1.34 ± 0.01 (n=30)</td>
<td>1.68 ± 0.01 (n=19)</td>
<td>p = 0.0001</td>
</tr>
</tbody>
</table>

8-12 week old male CBA/H and C57BL/6 mice were sacrificed and the number of bone marrow cells extracted from both femur were counted using a Schärfe System Casy® 1 counter (figure 3.4A). White bone marrow cell (WBMC) and red bone marrow cell (RBMC) numbers obtained were statistically analysed using the Mann-Whitney U-test. Results are expressed as x10^6 cells/2 femur (± s.e.m). To reduce technical error, counts were also expressed as a ratio.

To compensate for the experimental error involved in harvesting bone marrow from individual mice, WBMC and RBMC counts were expressed as a ratio (WBMC divided by RBMC). The WBMC/RBMC ratio (table 3.1) was also very reproducible and gave a statistically significant difference between the two mouse strains (p=0.0001). There is no significant difference in peripheral red cell count between C57BL/6, CBA/CaJ and CBA/J mice (Kile et al., 2003) suggesting that strain-specific difference in red cell numbers is specific to the bone marrow and possibly represents differences in bone marrow architecture.

3.3.1.2 Phenotypically defined Bone Marrow cells

Additional mouse strain specific differences in bone marrow cells were also analysed by flow cytometry. Aliquots of CBA/H and C57BL/6 total bone marrow were stained with: (1) FITC-labelled anti Mac-1 and PE-labelled anti Flk-2; and (2) FITC-labelled anti Sca-1 and PE-labelled anti c-Kit. Examples of the cytograms obtained are shown in figure 3.5. The percentage of WBMCs that stained for each antibody and the relative staining intensity (++ and +++; figure 3.3) after correction for RBMC count is summarised in table 3.2 where statistical difference between the two stains is analysed using the Mann Whitney U-test.
Figure 3.5 – FACS contour cytograms obtained from staining total bone marrow cells with fluorescently labelled antibodies.

Total bone marrow was stained with fluorescently labelled anti Mac-1, anti Flk-2, anti Sca-1 and anti c-Kit. Contour plots were used to emphasize the differences between the CBA/H and C57BL/6 mouse strains. In all cytograms 5000 bone marrow cells were analysed. Percentages for the lower left hand regions are A (77.81%), B (58.71%), C (93%), D (75.76%).

A & B represent Mac-1 and Flk-2 staining for CBA/H and C57BL/6 respectively. The solid arrow in cytogram B indicates the higher number of Mac-1++ cells seen in the C57BL/6 inbred strain when compared to the arrow in cytogram A.

C & D represents Sca-1 & c-Kit staining for CBA/H and C57BL/6 respectively. The solid arrow in cytogram D indicates the presence of Sca-1++ cells in the C57BL/6, a population that is not identifiable in CBA/H cytogram C. The broken arrow represents a discrete population of c-Kit++ cells that appear to be located in an identical position in both mice strains.
Table 3.2 – WBMC staining for Mac-1, c-Kit, Sca-1 and Flk-2

<table>
<thead>
<tr>
<th>Cytogram Region</th>
<th>Cell surface antigen</th>
<th>% white bone marrow cells expressing cell surface antigen</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CBA/H</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>A</td>
<td>Mac-1++ (% WBMC)</td>
<td>34.62 ± 1.07 (n=10) &amp; 45.99 ± 2.07 (n=14)</td>
<td>( p=0.0004 )</td>
</tr>
<tr>
<td>B</td>
<td>Mac-1++/+ (% WBMC)</td>
<td>51.42 ± 1.82 (n=10) &amp; 69.67 ± 2.5 (n=14)</td>
<td>( p=0.0001 )</td>
</tr>
<tr>
<td>C</td>
<td>c-Kit++ (% WBMC)</td>
<td>11.84 ± 0.42 (n=10) &amp; 14.09 ± 0.42 (n=12)</td>
<td>( p=0.003 )</td>
</tr>
<tr>
<td>D</td>
<td>c-Kit++/+ (% WBMC)</td>
<td>28.06 ± 0.88 (n=10) &amp; 32.15 ± 1.07 (n=12)</td>
<td>( p=0.0176 )</td>
</tr>
<tr>
<td>E</td>
<td>Sca-1++ (% WBMC)</td>
<td>1.229 ± 0.15 (n=10) &amp; 4.58 ± 0.25 (n=12)</td>
<td>( p=0.0001 )</td>
</tr>
<tr>
<td>F</td>
<td>Sca-1++/+ (% WBMC)</td>
<td>8.61 ± 0.38 (n=10) &amp; 16.97 ± 0.75 (n=12)</td>
<td>( p=0.0001 )</td>
</tr>
<tr>
<td>G</td>
<td>Flk-2++ (% WBMC)</td>
<td>2.43 ± 0.39 (n=10) &amp; 3.73 ± 0.37 (n=12)</td>
<td>( p=0.0192 )</td>
</tr>
<tr>
<td>H</td>
<td>Flk-2++/+ (% WBMC)</td>
<td>10.17 ± 0.76 (n=10) &amp; 13.86 ± 0.82 (n=14)</td>
<td>( p=0.0049 )</td>
</tr>
</tbody>
</table>

Comparison of both high intensity stained cells (++) and all stained cells (++/+) in the cytogram regions described in figure 3.3. All results are expressed as a percentage of bone marrow white cells (± s.e.m) expressing specific cell surface antigens using FACS and corrected for the RBMC content. Statistical analysis uses the Mann-Whitney \( U \)-test and ‘n’ indicates the number of mice analysed.
Although the WBMC number is comparable in the two mouse strains, significant differences exist in the cellular composition of bone marrow. CBA/H bone marrow has on average more red cells than C57BL/6 bone marrow and this is supported by the WBMC/RBMC ratio which excludes experimental error. Analysing staining intensity exclusively in the total bone marrow white cells, demonstrates that C57BL/6 mice have more Sca-1, Mac-1, c-Kit and Flk-2 staining WBMCs than CBA/H mice. Differences in the Sca-1 WBMCs are consistent with the Ly6A/E haplotypes of the inbred strains – C57BL/6 mice express the Ly6b haplotype which is associated with a high Sca-1 expression, and CBA/H mice express the Ly6a haplotype which is associated with a low Sca-1 expression (Spangrude & Brook, 1993). Statistically significant differences in Mac-1, c-Kit and Flk-2 staining WBMCs between the two inbred mice strains indicates haplotype differences may exist in these haemopoietic cell markers and laboratory experiments are currently underway to map QTLs that determine these differences. Analysis of the c-Kit++ staining intensity identified a discrete population of strongly staining Sca-1', c-Kit++ cells (figure 3.5C/D; broken arrow) which is present in both mice strains. Further characterisation of these cells through immunophenotyping should enable identification of this cell population.

3.3.2 Lineage Negative Cells

Lin' bone marrow cells are isolated by immuno-magnetic separation using biotinylated antibodies and streptavidin coated magnetic beads. Magnetic removal of mature bone marrow cells results in a >95% pure lineage negative (Lin') bone marrow fraction (BD Biosciences Pharmingen) which facilitates the analysis of the less abundant immature HSC compartment by FACS. The efficiency of the antibody cocktail to remove mature bone marrow cells was monitored by calculating the percentage of total bone marrow cells remaining post immuno-magnetic separation. Lin' cells were counted using a Schärfe System Casy® 1 cell counter, and no statistically significant difference (p=0.216) was detected between the number of Lin' white cells harvested from CBA/H and C57BL/6 bone marrow (table 3.3).

In this study the Lin' cells constituted 2.01% (± 0.15 s.e.m; n=23) of CBA/H and 2.63% (± 0.27 s.e.m; n=24) of C57BL/6 WBMCs. This demonstrates that >97% of bone marrow cells are removed using our antibody cocktail and this is well within the
>95% removal estimated by BD Biosciences Pharmingen who supply the antibodies.
Mature bone marrow cell removal associated with other studies range from ~95% (Okada et al., 2001), ~91.5% (Okada et al., 1992) and 88-94% (de Hann et al., 2000) and this difference probably represents the use of different antibodies within the antibody cocktail.

The recovery of Lin' cells was an important measure that enabled the identification of problems with the protocol. Technical problems that arose during this study included improved bone marrow harvesting ability (with experience yield went from 5 x 10^6 to 15 x 10^6 WBMCs/femur), deteriorating antibody binding efficiency with time, and loss of bead magnetism. These complications often resulted in a Lin' fraction consisting of >10% of input bone marrow cells, although usually these problems were evident prior to Lin' cell counting and were evidenced by distinctly coloured pellets. Under the experimental conditions designed in this study, the Lin' pellet is straw coloured. Loss of bead magnetism results in a light brown pellet (colour of the beads) while inefficient anti-RBMC antibody (Ter119) results in a red pellet (presence of RBMC). In this study, recovery of Lin' cells comprising >3% of the total WBMC input were excluded.

### 3.3.2.1 Phenotypically defined Lin' cells

Lin', Sca-1**, c-Kit++ bone marrow cells represent the whole stem cell compartment (section 1.5.2.5). Lin', Sca-1**, c-Kit++ Flk-2' bone marrow cells represent the LT-HSC and Lin', Sca-1**, c-Kit++ Flk-2++ bone marrow cells the ST-HSC (Christensen & Weissman, 2001). This FACS study was limited to 2 antibody combinations: (1) FITC-labelled anti Sca-1 and PE-labelled anti c-Kit antibodies to analyse the total bone marrow stem cell compartment, and (2) FITC-labelled anti Sca-1 and PE-labelled anti Flk-2 which includes the ST-HSCs thereby enabling the analysis of the more mature HSCs.

FACS analysis of CBA/H and C57BL/6 Lin' bone marrow cells equally stained with either of these two fluorochrome labelled antibody combinations, identified reproducible strain-specific differences in a specific cell population that was subsequently gated and designated Region 1 (R1; figure 3.6). Region 1 was gated based on visual identification of cells. High intensity (antigen++) staining cells were
gated above the $10^2 \times 10^2$ log scale and low intensity (antigen$^+$) staining cells between the $10^2 \times 10^2$ log scale and the unstained cells (figure 3.3). Subsequent statistical analysis of lower equal intensity staining cells Lin', Sca-1$^+$, c-Kit$^+$ (table 3.3G) and Lin', Sca-1$^+$, Flk-2$^+$ (table 3.4G) has indicated no statistically significant differences between the two mouse strains ($p = 0.8541$ and $p = 0.4684$ respectively).

**Figure 3.6 – Gating of high intensity, double stained Lin' bone marrow cells**

Lin' bone marrow cells showing high intensity staining for both flourochrome labelled antibodies were identified in the top right hand quadrant. This cell population was gated into a region designated as Region 1 (R1). In all analyses, FITC-labelled antibody staining is located along the y axis and PE-labelled antibody staining on the x axis. The lower region of the 'high intensity' staining cells (antigen $^{++}$) is defined by the $10^2 \times 10^2$ log scale (figure 3.3). Figure 3.3 indicates the staining intensity of all previously identified quadrants.

As shown in figure 3.7, CBA/H Lin' bone marrow cells are enriched in a 'tail' population of equally stained Lin', Sca-1$^{++}$, c-Kit$^{++}$ cells (figure 3.7A; solid arrow) and Lin', Sca-1$^{++}$, Flk-2$^{++}$ cells (figure 3.7C; solid arrow) compared to C57BL/6 Lin' bone marrow cells (figure 3.7B and 3.7D respectively). Occasionally both CBA/H and C57BL/6 Lin' bone marrow cells demonstrated the presence of a small number of higher intensity staining c-Kit cells i.e. Lin', Sca-1$^{++}$, c-Kit$^{+++}$ cells (figure 3.7B; asterisk).

Ly6A/E (Sca-1 staining) haplotype differences between the two stains were identified and this is clearly indicated in figure 3.7C and D (broken arrow) although not so obvious in figure 3.7A and B (broken arrow).
Figure 3.7 – FACS contour cytograms obtained from staining Lin- bone marrow cells with fluorescently labelled antibodies.

Lin- bone marrow cells were stained with fluorescently labelled antibodies to Sca-1, c-Kit and Flk-2. Contour plots were used to emphasize the differences between the CBA/H (A & C) and C57BL/6 (B & D) mouse strains. A & B represent Sca-1 and c-Kit staining and C & D Sca-1 and Flk-2 staining. The solid arrows indicate the discrete R1 region (figure 3.6) which in A & B contains equally staining Lin-, Sca-1**, c-Kit** bone marrow cells and in C & D represent equally staining Lin-, Sca-1**, Flk-2** bone marrow cells. The broken arrow represents the Lin-, Sca-1** bone marrow cells and the asterisk (*) Lin-, Sca-1**, c-Kit*** bone marrow cells. In all cytograms 5000 bone marrow cells were analysed. Percentages for the lower left hand regions are A (62.12%), B (75.92%), C (93.34%), D (96.02%). The percentages for the gated regions are A (3.72%), B (0.32%), C (2.12%), D (0.51%).
For quantitation, cytograms were subdivided into 10 regions according to the intensity of staining (figure 3.6). Previous counting of Lin' cells had identified two cell populations (WBMC and contaminating RBMC). The number of stained cells in each region was estimated as a percentage of the Lin' WBMCs only and therefore corrected against the other cell population (section 3.2.2). The small cell population (3-6μm) comprises of 1.43% (n=35; ± 0.24 s.e.m) of the total RBMC and may represent inefficient removal of mature RBMC or magnetic beads. Comparison of bone marrow cells stained with FITC-labelled anti Sca-1 and PE-labelled anti c-Kit (figure 3.5C) with similarly stained Lin- bone marrow cells (figure 3.7A) demonstrates that immuno-magnetic sorting has removed most RBMCs and enriched for Lin' WBMCs.

Table 3.3 compares frequencies of CBA/H and C57BL/6 Lin' WBMCs stained with FITC-labelled anti Sca-1 and PE-labelled anti c-Kit. CBA/H bone marrow exhibited approximately three times as many gated Lin', Sca-1++, c-Kit++ cells (table 3.3F) than C57BL/6 bone marrow (p=0.0054). The comparison of Lin' CBA/H and C57BL/6 bone marrow cells also gave a statistically significant differences in Sca-1 staining cells (table 3.3C; p=0.04) and this difference was enhanced by restricting the analysis to high intensity staining cells (table 3.3B; p= <0.001). Strain differences in Sca-1 staining cells were expected, due to the Ly6A/E (Sca-1) haplotype of the different mouse strains. CBA/H bone marrow exhibited more high intensity stained Lin', c-Kit++ cells (table 3.3D, p= 0.019) although this statistical significant difference is not maintained when all c-Kit staining cells (c-Kit+/+) were analysed (3.3E).

Table 3.4 compares Lin' WBMCs stained with FITC-labelled anti Sca-1 and PE-labelled anti Flk-2. CBA/H bone marrow exhibited approximately twice as many Lin', Sca-1++, Flk-2++ cells (table 3.4F; p= 0.005) than C57BL/6 bone marrow. A statistically significant difference between the frequency of high intensity stained Lin', Sca-1++ bone marrow cells (table 3.4B; p= <0.001) was detected but this difference was not maintained when all Sca-1 staining cells (Sca-1+/+) were analysed (table 3.4C; p= 0.173). Statistical differences between the Flk-2 staining cells was also restricted to the high intensity Lin', Flk-2++ bone marrow cells (table 3.4D; p=<0.001).

Assessment of Sca-1 staining alone using the designated quadrats (table 3.3B/C; table 3.4B/C) demonstrated that Sca-1 staining corresponded to the Sca-1 haplotypes –
C57BL/6 Lin− bone marrow cells exhibiting higher Sca-1 staining than CBA/H Lin− bone marrow cells. The comparison of double stained cells suggested that not all sub-populations of Sca-1 staining cells conform to the Sca-1 haplotype. Indeed, some sub-populations of double stained cells demonstrate that CBA/H Lin− bone marrow cells and C57BL/6 Lin− bone marrow cells have similar numbers of some cell populations - Lin−, Sca-1+, c-Kit+ cells (table 3.3G; \( p=0.8541 \)) and Lin−, Sca-1+, Flk-2+ cells (table 3.4G; \( p=0.4684 \)) while CBA/H Lin− bone marrow cells have more Lin−, Sca-1++, c-Kit++ (table 3.3F; \( p=0.054 \)) and Lin−, Sca-1++, Flk-2++ bone marrow cells (table 3.4F; \( p=0.005 \)) than C57BL/6 Lin− bone marrow cells. This suggests that heterogeneity exists within the Sca-1 staining population.
Table 3.3 – Regional comparison of Lin’ bone marrow cells stained with FITC-labelled anti Sca-1 and PE-labelled anti c-Kit.

<table>
<thead>
<tr>
<th>Cytogram Region</th>
<th>Cell surface antigen</th>
<th>% FACS cells expressing cell surface antigen (± s.e.m)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CBA/H</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>A</td>
<td>Lin’ WBMC (x10⁶/2 femur)</td>
<td>0.576 ± 0.06 (n=17)*</td>
<td>0.727 ± 0.07 (n=18)*</td>
</tr>
<tr>
<td>B</td>
<td>Lin’, Sca-1** (% Lin’ WBMC)</td>
<td>2.85 ± 0.33 (n=21)</td>
<td>6.25 ± 0.36 (n=18)</td>
</tr>
<tr>
<td>C</td>
<td>Lin’, Sca-1**/ (%) Lin’ WBMC)</td>
<td>17.43 ± 1.45 (n=21)</td>
<td>20.99 ± 1.44 (n=18)</td>
</tr>
<tr>
<td>D</td>
<td>Lin’, c-Kit** (% Lin’ WBMC)</td>
<td>34.07 ± 2.57 (n=21)</td>
<td>26.40 ± 1.3 (n=18)</td>
</tr>
<tr>
<td>E</td>
<td>Lin’, c-Kit**/ (%) Lin’ WBMC)</td>
<td>46.64 ± 3.64 (n=21)</td>
<td>41.03 ± 2.48 (n=18)</td>
</tr>
<tr>
<td>F</td>
<td>Lin’, Sca-1**/c-Kit**+ (%) Lin’ WBMC)</td>
<td>1.89 ± 0.15 (n=21)</td>
<td>0.547 ± 0.03 (n=18)</td>
</tr>
<tr>
<td>G</td>
<td>Lin’, Sca-1**,c-Kit* (%) Lin’ WBMC)</td>
<td>4.23 ± 0.52 (n=21)</td>
<td>4.08 ± 0.46 (n=18)</td>
</tr>
<tr>
<td>H</td>
<td>Lin’, Sca-1**,c-Kit (%) Lin’ WBMC)</td>
<td>61.02 ± 1.81 (n=21)</td>
<td>61.89 ± 2.05 (n=18)</td>
</tr>
</tbody>
</table>

Table 3.3 summarises the data for Lin’ cells stained with anti Sca-1 and anti c-kit. With the exception of Lin’ WBMC which represents the absolute number of immature white cells in 2 femurs and was obtained as an absolute value using a Scharfe system casey® 1 cell counter (*; x 10⁶), the number of stained cells in each region is estimated as a percentage of Lin’ WBMC. Statistical analysis uses the Mann-Whitney U-test.

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Table 3.4 – Regional comparison of Lin’ bone marrow cells stained with FITC-labelled anti Sca-1 and PE-labelled anti Flk-2.

<table>
<thead>
<tr>
<th>Cytogram Region</th>
<th>Cell surface antigen</th>
<th>% FACS cells expressing cell surface antigen (± s.e.m)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CBA/H</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>A</td>
<td>Lin’ WBMC (x10⁶/2 femur)</td>
<td>0.576 ± 0.06 (n=17)*</td>
<td>0.727 ± 0.07 (n=18)*</td>
</tr>
<tr>
<td>B</td>
<td>Lin’, Sca-1** (% Lin’ WBMC)</td>
<td>3.32 ± 0.34 (n=22)</td>
<td>6.08 ± 0.41 (n=17)</td>
</tr>
<tr>
<td>C</td>
<td>Lin’, Sca-1** (% Lin’ WBMC)</td>
<td>15.57 ± 1.54 (n=22)</td>
<td>17.07 ± 1.17 (n=17)</td>
</tr>
<tr>
<td>D</td>
<td>Lin’, Flk-2** (% Lin’ WBMC)</td>
<td>4.41 ± 0.59 (n=22)</td>
<td>3.00 ± 0.63 (n=17)</td>
</tr>
<tr>
<td>E</td>
<td>Lin’, Flk-2** (% Lin’ WBMC)</td>
<td>15.88 ± 1.76 (n=22)</td>
<td>14.7 ± 2.0 (n=17)</td>
</tr>
<tr>
<td>F</td>
<td>Lin’, Sca-1** Flk-2** (% Lin’ WBMC)</td>
<td>1.28 ± 0.05 (n=22)</td>
<td>0.53 ± 0.03 (n=17)</td>
</tr>
<tr>
<td>G</td>
<td>Lin’, Sca-1*, Flk-2* (% Lin’ WBMC)</td>
<td>4.90 ± 0.82 (n=21)</td>
<td>4.65 ± 0.46 (n=18)</td>
</tr>
<tr>
<td>H</td>
<td>Lin’, Sca-1*, Flk-2* (% Lin’ WBMC)</td>
<td>82.14 ± 1.81 (n=21)</td>
<td>80.42 ± 1.56 (n=18)</td>
</tr>
</tbody>
</table>

Table 3.4 summarises the data for Lin’ cells stained with anti Sca-1 and anti Flk-2. With the exception of Lin’ WBMC which represents the absolute number of immature white cells in 2 femurs and was obtained as an absolute value using a Schärfe system casey® 1 cell counter (*; x 10⁶), the number of stained cells in each region is estimated as a percentage of Lin’ WBMC. Statistical analysis uses the Mann-Whitney U-test.
Individual analysis of cells stained with each fluorescently stained antibody showed that the most statistically significant differences were identified in the more intensely stained cells (antigen\textsuperscript{++}). These results were highly reproducible both between strains and in the case of FITC-labelled anti Sca-1, in both staining protocols. For example, Sca-1\textsuperscript{++} cells were assessed independently using Sca-1/c-Kit and Sca-1/Flk-2 staining. Statistical analysis of the frequency of Lin\textsuperscript{−}, Sca-1\textsuperscript{++} cells from: (1) FITC-labelled anti Sca-1 and PE-labelled anti c-Kit, and (2) FITC-labelled anti Sca-1 and PE-labelled anti Flk-2 Lin\textsuperscript{−} cells clearly shows that the comparable Sca-1 staining is obtained regardless of the PE-labelled antibody (table 3.5). Together with other strain specific differences, this is strong supportive evidence that these results are not an experimental artefact.

Table 3.5 – Comparison of Lin\textsuperscript{−}, Sca-1\textsuperscript{++} bone marrow cells obtained using different PE-labelled antibodies.

<table>
<thead>
<tr>
<th></th>
<th>FITC-labelled anti Sca-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CBA/H</td>
</tr>
<tr>
<td>PE-labelled anti c-Kit</td>
<td>2.85 ± 0.33 (n= 21)</td>
</tr>
<tr>
<td>PE-labelled anti Flk-2</td>
<td>3.32 ± 0.34 (n= 22)</td>
</tr>
<tr>
<td>\textit{p} value</td>
<td>\textit{p} = 0.196</td>
</tr>
</tbody>
</table>

Each analyses used FITC-labelled Sca-1 thereby enabling a comparison between the frequency of Sca-1\textsuperscript{++} cells obtained by co-staining with PE-labelled c-Kit and also with PE-labelled Flk-2. Statistical analysis used the Mann Whitney \textit{U}-test. No statistical difference indicates that the Sca-1 staining of cells is independent of the second antibody.

In summary, CBA/H mice have more Lin\textsuperscript{−}, Sca-1\textsuperscript{++}, c-Kit\textsuperscript{++} and Lin\textsuperscript{−}, Sca-1\textsuperscript{++}, Flk-2\textsuperscript{++} bone marrow cells than C57BL/6 mice – strong evidence that CBA/H bone marrow contains more HSCs (LT-HSC and ST-HSC) than C57BL/6 bone marrow. Single staining differences were also identified with Sca-1 staining being consistent with the Ly6A/E (Sca-1) haplotypes previously discussed (section 1.5.2.2.1) and work is currently underway to map QTLs that determine these phenotypical differences.
3.4 Discussion

The definition of HSCs has primarily depended on phenotypic isolation of cells and assessing their functional ability to rescue lethally irradiated recipients. This study defined HSCs as Lin', Sca-1++, c-Kit++ bone marrow cells. Sca-1 is accepted as a HSC marker and has been used to identify these cells in numerous studies (Okada et al., 1992; Spangrude & Brooks, 1992; Philips et al., 1992; Uchida & Weissman, 1992; Osawa et al., 1996; Christensen & Weissman, 1999, Henchaerts et al., 2002). Co-expression of other cell surface antigens are used to sub-classify HSCs including c-Kit (Okada et al., 1992), Thy1.1 (Spangrude & Brooks, 1992) and c-Kit, Thy1.1 and Flk-2 (Christensen & Weissman, 1999). Although Lin', Sca-1++, c-Kit++ bone marrow cells are highly enriched for LT-HSC, not all cells within this population have the ability to rescue lethally irradiated mice. Only 21% of lethally irradiated animals maintained haemopoiesis following transplantation of a single Lin', CD34lo Sca-1+, c-Kit+ cell (Osawa et al., 1996) suggesting that either some cells within this population are not LT-HSCs or some cells lose their LT-HSC capacity following experimental manipulation.

Various techniques have been used to measure HSC numbers and as a consequence large inconsistencies exist in establishing the bone marrow HSC number in inbred mouse strains. In vitro studies of day 35 CAFC (cobblestone area-forming cell) numbers reported HSC frequencies of inbred strains to be AKR/J > DBA/2 > A/J > 129/Sv > C57BL/6 > BALB/c > C3H/HeJ > CBA/J (de Hann & Van Zant, 1997B). Studies using the LTC-IC (Long term culture initiating cell) assay supported that DBA/2 had more HSC than C57BL/6 but indicated that C57BL/6 had less HSC than Balb/c (Müller-sieburg & Riblet, 1996). In contradiction, Henchaerts et al (2002; 2004) defined their HSC population as Lin', Sca-1++, c-Kit++ and found that C57BL/6 mice had more ‘HSCs’ than DBA/2 mice.

Conflicting data for HSC numbers suggests that the different assays are not measuring the same HSC cell population and this is supported by the identification of distinct quantitative trait loci (QLT) for these individual cell types in inbred mice strains (de Hann & van Zant, 1997B; Müller-Sieburg & Riblet, 1996; Morrison et al., 2002). Alternatively, the reported differences could be a function of in vivo vs in vitro assays. In vivo studies use the natural host and therefore the longevity of in vivo
transplantation studies is months and the HSC is identified by its ability to 'home' to the bone marrow and produce viable lymphomyeloid progeny. In vitro assays are performed in an artificial environment – longevity is expressed in weeks, the source of substrate and regulatory factors is the feeder layer and HSCs are identified retrospectively by the presence of colony forming cells (Coulombel, 2004).

Fluorescence activating cell scanning is a direct method for measuring HSCs and reduces the variability associated with in vitro clonogenic assays. Confirmation that region 1 (R1) in this study contains the HSCs is essential. Two options for authenticating these cells are either (1) functionally i.e transplanting cells into lethally irradiated animals or (2) genetically by looking for genetic association. Various studies have mapped bone marrow HSC frequency QTLs. Studies of recombinant inbred mice implicated three loci – two on chromosome 1 and one on chromosome 11 – in the frequency of LTC-IC although these were not statistically significant (Müller-Sieburg & Riblet, 1996). Similar studies have mapped day 35 CAFC frequency to chromosome 18 (de Hann & van Zant, 1997B). The frequency of FACS sorted Lin⁺, Sca-1⁺⁺, c-Kit⁺ bone marrow cells have been mapped to chromosomes 2, 4, 7, 12, 14 and 16 (Henckaerts et al., 2002; 2004) and Lin⁺, Thy-1.1low, Sca-1⁺, c-Kit⁺ cells to chromosome 17 (Morrison et al., 2002).

During the course of this study, I helped set up an F2 genetic linkage study in a collaborative team effort which used the protocol I had previously established. Genetic analysis of the frequency of gated Lin⁺, Sca-1⁺⁺, c-kit⁺⁺, cells in (CBA/H x C57BL/6) intercross mice was carried out by A Zanker and M Jawad. Results implicated 2 QLTs associated with this distinct population – chromosome 1 (~65cM; LOD 3.0) and chromosome 18 (~16cM; LOD 3.6) (M Plumb, manuscript submitted). Both these chromosomes have been implicated in the frequency of long-term repopulating HSCs as measured using in vitro studies. The chromosome 18 locus mapped to approximately the same location as the day 35 CAFC (de Hann & van Zant, 1997) and the locus on chromosome 1 mapped to the stem cell frequency regulator 2 (Scfr2) (Müller-Sieburg & Riblet, 1996). The stem cell frequency regulator 1 (Scfr1) locus reported (albeit not statistically significant) by Müller-Sieburg & Riblet (1996), and detected in the r-AML affected CBA/H x (CBA/H x C57BL/6) backcross mice (Boulton et al., 2003), was not revealed. One possible explanation being that the
heterogeneity within the Lin⁺, Sca-1++, c-kit++ cells (as demonstrated functionally by Osawa et al., 1996), may mask the distal chromosome 1 Scfrl locus. However, our genetic linkage evidence from the (CBA/H x C57BL/6)F1 intercross study indicates that the Lin⁺, Sca-1++, c-kit++ bone marrow population gated in R1 contains day 35 CAFCs and LTC-IC. Other cell populations may be so small that linkage (Scfrl) is masked by the heterogeneity of the sample.

The home office project licence did not permit murine transplantation studies and therefore FACS sorting and transplantation into lethally irradiated mice was not possible. Chapter 4 monitors the cell death and recovery of bone marrow cells following exposure to a single acute leukaemogenic dose of 3 Gy X-rays. While many cells are decimated by X-ray exposure (WBMCs; Mac-1++ WBMCs; Lin⁺ WBMCs and Lin⁺, Sca-1⁺, c-Kit++ WBMCs), Lin⁺, Sca-1++, c-kit++ and Lin⁺, Sca-1++, Flk-2++ bone marrow cell populations are amplified – supportive evidence that these cells contain the HSC populations that drive bone marrow recovery.

Although r-AML is a HSC malignancy, analyses of the 'Leukaemic stem cell' (LSC) suggest that r-AML may potentially originate from the most immature stem cell to a progenitor cell that has reacquired self-renewal capacity (section 1.4.4). Consequently, although this study concentrates primarily on the stem cell and progenitor compartments as a whole (Lin⁺, Sca-1++, c-kit++), the number of more mature stem cells (Lin⁺, Sca-1++, Flk-2+++) were also analysed. Flk-2 expression has superseded Thy1.1 expression in the identification of long term HSC (LT-HSC) and short term HSC (ST-HSC). Lin⁺, Sca-1++, c-Kit++ Flk-2++ bone marrow cells have short term repopulating abilities and Lin⁺, Sca-1++, c-Kit++ Flk-2+ bone marrow cells have long term repopulating abilities (Christensen & Weissman, 2001). Removing Flk-2++ bone marrow cells prior to Sca-1 and c-Kit staining was considered and preliminary studies undertaken. However these studies were not pursued due to time and financial constraints, and evidence that the LT-HSC is not necessarily the target cell in r-AML (reviewed Hope et al., 2003; Warner et al., 2004).

In summary, genetic linkage studies of r-AMLs affected CBA/H x (CBA/H x C57BL/6) backcross mice following exposure to a single acute dose of 3Gy X-rays has implicated a r-AML susceptibility locus on Chromosome 1 (Boulton et al., 2003) that
coincides with the location of the stem cell frequency regulator 1 locus (Müller-sieberg & Riblet., 1996). As 'target size' is one potential factor in leukaemogenesis, then differences in HSC number between r-AML susceptible CBA/H mice and r-AML resistant C57BL/6 mice may be an important factor in disease incidence. A discrete population of cells designated Lin', Sca-1++, c-kit++ represent the HSC population and evidence that this population contains the HSCs is gained from the genetic analysis implicating the day 35 CAFC locus and Scfr2 locus (M Plumb personal communication) and also functional response of these cells post irradiation (chapter 4). In this study CBA/H mice have more Lin', Sca-1++, c-kit++ and Lin', Sca-1++, Flk-2++ cells than C57BL/6 mice, and using this criteria CBA/H mice have more HSCs and therefore more 'target cells' than C57BL/6 mice.
Chapter 4: Cell death and recovery following exposure to an in vivo acute leukaemogenic dose of X-rays.

4.1 Introduction

The target cell in myeloid leukaemogenesis is the haemopoietic stem cell (reviewed Bell & Van Zant, 2004) and HSC number is theoretically one risk factor in AML (chapter 3). As murine r-AML is a radiation induced disease, target size (risk) is actually the number of bone marrow HSCs that survive radiation exposure rather than the pre-irradiation HSC number. This chapter considers the impact of the leukaemogenic dose of 3 Gy X-rays on bone marrow cellularity as discussed in chapter 3. Thus, this is a detailed analysis of bone marrow cell death and recovery in the r-AML susceptible CBA/H and r-AML resistant C57BL/6 inbred mouse strains.

4.2 Experimental Design

Eight to 12 week old male CBA/H and C57BL/6 mice were exposed to a single acute dose of 3 Gy X-rays and culled at various time points ranging from 1-196 days post exposure. Bone marrow was harvested at each time point and the number of white bone marrow cells (WBMC) and red bone marrow cells (RBMC) measured using a Schärfe System Casy® 1 cell counter. Flow cytometry (Becton Dickinson FACScan) was used to quantitate phenotypically defined haemopoietic cells using the protocol established in chapter 3.

Two aliquots of total bone marrow were stained: (1) FITC-labelled anti Mac-1 and PE-labelled anti Flk-2, and (2) FITC-labelled anti Sca-1 and PE-labelled anti c-Kit. Antibody mediated immuno-magnetic separation was used to obtain Lin' cells which were also divided into two aliquots and stained with: (1) FITC-labelled anti Sca-1 and PE-labelled anti c-Kit; and (2) FITC-labelled anti Sca-1 and PE-labelled anti Flk-2.

At each time point, RNA was extracted from the excess harvested bone marrow and the spleen. Northern blots were generated and hybridised with a number of available cDNA probes either associated with haemopoietic differentiation (chapter 4) or with putative candidate genes located within the Chromosome 1 r-AML susceptibility locus (chapter 5).
4.3 Experimental Variables

4.3.1 Age

Murine r-AML arises 6-24 months following a single acute dose of 3Gy X-rays with a mean latency of 18 months (Major & Mole, 1978; Major, 1979; Wright & Lorimore, 1990; Plumb et al., 1998). The initial time frame for this study was 1 day post irradiation to establish the immediate effect, to day 196 as this includes the pre-leukaemic phase. All mice were 8-12 weeks old at exposure and consequently the sacrificial age range was 8-40 weeks old. In vitro studies had reported a 2-3 fold increase in the frequency of day 35 CAFCs with age (de Hann et al., 1997A; 1999) and therefore to eliminate age related changes in this study unexposed CBA/H mice were analysed at different ages (table 4.1).

Table 4.1 – Age comparison of CBA/H mice.

<table>
<thead>
<tr>
<th>Bone marrow cell parameter</th>
<th>8-12 Weeks</th>
<th>18 Weeks</th>
<th>26-33 Weeks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBMC (x10^6 /2 femur)</td>
<td>31.2 ± 1.32 (n=20)</td>
<td>29.33 ± 1.55 (n=9)</td>
<td>30.9 ± 1.46 (n=11)</td>
<td>p=0.75</td>
</tr>
<tr>
<td>RBMC (x10^6 /2 femur)</td>
<td>24.2 ± 0.93 (n=20)</td>
<td>24.8 ± 1.37 (n=9)</td>
<td>30.5 ± 1.56 (n=11)</td>
<td>P=0.0134</td>
</tr>
<tr>
<td>WBMC/RBMC Ratio (2 femur)</td>
<td>1.3 ± 0.04 (n=20)</td>
<td>1.191 ± 0.046 (n=9)</td>
<td>1.026 ± 0.047 (n=11)</td>
<td>P=0.0022</td>
</tr>
<tr>
<td>Lin' (x10^6 /2 femur)</td>
<td>0.592 ± 0.262 (n=17)</td>
<td>0.572 ± 0.07 (n=7)</td>
<td>0.613 ± 0.265 (n=5)</td>
<td>p=0.686</td>
</tr>
<tr>
<td>Lin', Sca-1++, c-Kit++ (as % of Lin' cells)</td>
<td>1.2 ± 0.24 (n=20)</td>
<td>0.899 ± 0.225 (n=9)</td>
<td>4.74 ± 0.81 (n=5)</td>
<td>P=0.0033</td>
</tr>
<tr>
<td>Lin', Sca-1++, Flk-2++ (as % of Lin' cells)</td>
<td>1.25 ± 0.27 (n=21)</td>
<td>0.738 ± 0.177 (n=9)</td>
<td>3.75 ± 0.37 (n=5)</td>
<td>p=0.004</td>
</tr>
</tbody>
</table>

Three groups of mice were studied. 8-12 weeks is the age of the mice pre irradiation, 18 weeks is the maximum age of a mouse on a 6 week time course and 26-33 weeks are arbitrary time points to represent a longer time course. All bone marrow cell parameters are discussed in chapter 3 –WBMC (white bone marrow cells), RBMC (red bone marrow cells) and Lin’ cells (Lin- white cells) are absolute numbers obtained by Schärfe system casey® 1 cell counter; Lin’, Sca-1++, c-Kit++ cells and Lin’, Sca-1++, Flk-2++ cells are both represented as a percentage of the Lin’ white cell count. Results show standard error of the mean (s.e.m) and were statistically compared using the Kruskal-Wallis non parametric test.
Statistical analysis was performed using the Kruskal-Wallis test. This is a non-parametric test which ranks values from all three populations and considers them as a single population, is suitable for data sets with more than two groups (Sokal & Rohlf, 1994). When all three age groups were compared to each other, the number of total bone marrow white cells (WBMC) and Lin' WBMCs did not change with age ($p=0.75$ and $p=0.686$). However, there was a statistically significant difference in the frequency of Lin', Sca-1++, c-Kit++ cells ($p=0.0033$); Lin', Sca-1++, Flk-2++ cells ($p=0.004$) and RBMC ($p=0.0134$) or more accurately, the WBMC/RBMC ($p=0.0022$) consistent with the published data (de Hann et al., 1997A; 1999).

To establish whether the statistical differences were due to the longer time point (26-33 weeks), the two earlier time points (8-12 weeks and 18 weeks) were compared. Statistical analysis used the Mann-Whitney $U$-test as there are only two samples. No statistical significant difference was identified indicating that the statistical difference is caused by the older 26-33 week age group.

Table 4.2 – Comparison of CBA/H mice up to 18 weeks of age.

<table>
<thead>
<tr>
<th>Bone marrow cell parameter</th>
<th>8-12 Weeks</th>
<th>18 Weeks</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBMC ($x10^6/2$ femur)</td>
<td>$31.2 \pm 1.32$</td>
<td>$29.33 \pm 1.55$</td>
<td>$p=0.4089$</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=9)</td>
<td></td>
</tr>
<tr>
<td>RBMC ($x10^6/2$ femur)</td>
<td>$24.2 \pm 0.93$</td>
<td>$24.8 \pm 1.37$</td>
<td>$p=0.7199$</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=9)</td>
<td></td>
</tr>
<tr>
<td>WBMC/RBMC Ratio (2 femur)</td>
<td>$1.3 \pm 0.04$</td>
<td>$1.191 \pm 0.046$</td>
<td>$p=0.1350$</td>
</tr>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=9)</td>
<td></td>
</tr>
<tr>
<td>Lin' ($x10^6/2$ femur)</td>
<td>$0.592 \pm 0.262$</td>
<td>$0.572 \pm 0.07$</td>
<td>$p=0.8519$</td>
</tr>
<tr>
<td></td>
<td>(n=17)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>Lin', Sca-1++, c-Kit++ (as</td>
<td>$1.2 \pm 0.24$</td>
<td>$0.899 \pm 0.225$</td>
<td>$p=0.4727$</td>
</tr>
<tr>
<td>% of Lin' WBMCs)</td>
<td>(n=20)</td>
<td>(n=9)</td>
<td></td>
</tr>
<tr>
<td>Lin', Sca-1++, Flk-2++ (as</td>
<td>$1.25 \pm 0.27$</td>
<td>$0.738 \pm 0.177$</td>
<td>$p=0.2466$</td>
</tr>
<tr>
<td>% of Lin' WBMCs)</td>
<td>(n=21)</td>
<td>(n=9)</td>
<td></td>
</tr>
</tbody>
</table>

Two mice age groups were compared. 8-12 weeks represents the pre irradiation age and 18 weeks the maximum age of a mouse during a 6 week time course. Results show standard error of the mean (s.e.m) and use the Mann-Whitney $U$-test statistic.
Thus, age-related differences in bone marrow cellularity become more significant after 18 weeks of age. As we irradiated mice at 8-12 weeks old, to avoid age-related differences, a maximum culling age of 18 weeks was chosen and thus restricted the time course study to 6 weeks post irradiation. Data obtained from longer irradiation time points i.e. post irradiation days 70, 96, 147 and 196 were not informative due to insufficient numbers of age matched controls.

4.3.2 Modification of the flow cytometry method

Chapter 2 section 2.7 describes the basic biotinylated antibody cocktail that was used for the immuno-magnetic separation of bone marrow cells containing ~10 million WBMCs and ~8 million RBMCs. X-ray exposure kills ~80% of WBMCs while the RBMC count increases by up to 2 fold. To compensate for the increased RBMC count, and to ensure all RBMCs were removed, extra biotin conjugated rat anti-mouse Ter119 antibody (red cell antibody) was added in the cocktail. 16μl of Ter119 (0.5mg/ml) is sufficient to remove ~ 8 million RBMCs and this volume was increased as required.

4.4 Results

4.4.1 Cell death and recovery of murine bone marrow following an in vivo exposure to a single acute dose of 3 Gy X-rays.

4.4.1.1 Total bone marrow - cellularity

Total bone marrow cellularity was analysed by counting the number of murine femoral bone marrow white cells (WBMC) and red cells (RBMC) using a Schärfe System Casy® 1 cell counter and by examining bone marrow smears. Figure 4.1 and 4.2 show the effect of 3 Gy X-ray exposure on the number of white cells in the murine femoral bone marrow and this data is summarised in figure 4.3A. Two days post irradiation, ~80% of WBMC have been killed in both mouse strains. Cell death is followed by the gradual recovery in the WBMC count to pre-irradiation levels by day 10-14. This is consistent with a previous study which reported recovery rates of murine femoral nucleated cell counts to be within 2-3 weeks (Testa \textit{et al.}, 1985).

Irradiation results in a >2 fold increase in the absolute number of RBMCs at day 2 (figures 4.1 and 4.3B). There may be differences between the two inbred mice strains
at early time points (figure 4.3B; day 1-4), but these RBMC differences can be explained by inter-strain variation in the number of RBMC (table 3.1). Control CBA/H mice have on average ~25% more RBMC ($24.5 \times 10^6 /2$ femurs) than C57BL/6 mice ($19.4 \times 10^6 /2$ femurs). The absolute RBMC counts at day 2 for two femurs are $51.8 \times 10^6 \pm 0.19$ (n=27) for CBA/H and $51.6 \times 10^6 \pm 0.2$ (n=13) for C57BL/6. This corresponds to the RBMC value of 210% for CBA/H and 260% for C57BL/6 compared to the control values (4.3B; day 2) and suggests that the RBMC increase is similar in both strains. The RBMC count returns to pre-irradiation control levels by ~day 10 post irradiation.

The WBMC and RBMC data was analysed as a ratio (figure 4.3C) because it is independent of ability to harvest bone marrow. No statistically significant difference was seen in the recovery kinetics of WBMC ($p=0.06$) or the WBMC/RBMC ratio ($p=0.589$) in the mouse strains.

**Figure 4.1 – Schärfe System Casy® 1 cell counter bone marrow profiles over a period of six days post exposure to an in vivo single acute dose of 3 Gy X-rays.**

Red bone marrow cells (RBMC) and white bone marrow cells (WBMC) are identified as two distinct populations defined by their cell size. X-axis values are consistent showing size in μM (0-15μM) while the cell count on the y-axis differs. The data emphasises the increase in RBMC (3-6μM) at day 2 and the gradual return of WBMC (>6μM) and RBMCs towards control levels (day 6).
Figure 4.2 – Jenner: Giemsa staining of CBA/H bone marrow smears over a period 10 days post exposure to an *in vivo* single acute dose of 3 Gy X-rays.

<table>
<thead>
<tr>
<th>Control CBA/H</th>
<th>Day 2 Post Irradiation</th>
<th>Day 4 Post Irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Control CBA/H" /></td>
<td><img src="image2" alt="Day 2 Post Irradiation" /></td>
<td><img src="image3" alt="Day 4 Post Irradiation" /></td>
</tr>
<tr>
<td>Day 6 Post Irradiation</td>
<td>Day 10 Post Irradiation</td>
<td></td>
</tr>
<tr>
<td><img src="image4" alt="Day 6 Post Irradiation" /></td>
<td><img src="image5" alt="Day 10 Post Irradiation" /></td>
<td></td>
</tr>
</tbody>
</table>

Bone marrow slides were stained with Jenners and Giemsa stains. Healthy bone marrow (control CBA/H) shows uniform cellularity with the presence of various cell types including metamyelocytes (solid arrow). Two days post irradiation, few bone marrow white cells (WBMC) remain and the bone marrow cavity is filled with mature red blood cells (broken arrow). Cellularity gradually recovers over a period of 10 days.
The effect of a single acute dose of 3 Gy X-rays on the number of bone marrow white cells (WBMC), bone marrow red cells (RBMC) and WBMC/RBMC ratio over a period of 42 days is illustrated in figures 4.3A, 4.3B and 4.3C respectively. All cells are measured using a Schärfe system casy® 1 machine. The number of cells as a percentage of the control value is plotted against time post irradiation with the dashed line indicating 100% i.e. control levels. Ranges (s.e.m) are shown and the number above each time point indicates the number of individual mice analysed for each time point. Total numbers of control mice analysed were 36 (WBMC), 42 (RBMC) and 56 (WBMC/RBMC ratio). In all graphs, CBA/H data is represented as red and C57BL/6 data as blue.
4.4.1.2 Total bone marrow – phenotypically defined cells

Fluorescence staining using FITC and PE labelled antibodies enables specific phenotypically defined populations to be analysed. Antibodies against cell surface antigens analysed by this study were FITC-labelled anti Mac-1, FITC-labelled anti Sca-1, PE-labelled anti c-Kit and PE-labelled anti Flk-2. Of the available antibodies, the antibody that stained the highest percentage of total WBMC cells in control mice was FITC-labelled anti Mac-1 which is a myeloid marker (chapter 3; table 3.2). FITC-labelled anti Mac-1 was therefore used to monitor the recovery of myeloid cells in total bone marrow.

FACS analysis (figure 4.4) illustrates the dramatic changes in the bone marrow cells stained with FITC-labelled anti Mac-1 and PE-labelled anti Flk-2 over the initial 6 days post irradiation. Two days post irradiation, the number of Mac-1 staining cells (broken arrow) is dramatically reduced and a distinct population of cells that stain for neither Mac-1 nor Flk-2 (Mac-1/Flk-2) predominates (solid arrow). Morphological analysis of bone marrow smears post irradiation (figure 4.2) and Schärfe System Casy® 1 cell counter profiles (figure 4.1) identifies the Mac-1/Flk-2 cells as red blood cells (<6μM; figure 4.1).

Although exposure to ionising radiation decimates the WBMC population (figure 4.3A), the FACS cytograms in figure 4.4 shows the presence of Mac-1 staining cells two days post irradiation. Mac-1 is expressed on granulocytes, macrophages, myeloid-derived dendritic cells, natural killer cells and B-1 cells in the peritoneal and pleural cavities (BD Biosciences). This cell population may represent an influx of macrophages resulting from increased vascular permeability. These cells are actively phagocytic and digest cell debris, dead and injured cells (Kuby, 1997). Positive identification of this cell population however requires further characterisation of other cell surface antigens.
Figure 4.4 – Cytogram representation of changes in Mac-1 and Flk-2 staining of total bone marrow cells over a period of 6 days post exposure to an in vivo acute dose of 3 Gy X-rays.

FACS cytograms of CBA/H bone marrow cells stained with FITC-labelled anti Mac-1 and PE-labelled anti Flk-2 over a period of 6 days post irradiation. The solid arrow indicates a discrete unstained population of cells 2 days post irradiation which represents red cells (figures 4.1-3). The broken arrow represents the recovering Mac-1** staining population.

In all cytograms 5000 bone marrow cells were analysed. Percentages for the lower left hand regions are Control (81.69%), Day 2 (90.16%), Day 4 (80.41%), Day 6 (74.52%).
Analysis of the Mac-1 staining WBMC is complicated by the presence of RBMC because FACS analysis works by measuring the fluorescence of a specific number of cells. A large population of one particular cell type (in this case red cells), will mask other cell populations. While Figure 4.4 shows the staining of Mac-1** cells relative to total bone marrow, figure 4.5 illustrates the staining of Mac-1** cells relative to the WBMC count only, as a function of time post irradiation using the formula discussed in chapter 3. Two days post irradiation, 50% of CBA/H Mac-1** WBMCs and 65% of C57BL/6 Mac-1** WBMCs have been killed, indicating that these cells are relatively radio-resistant compared to total WBMC (figure 4.3A) which demonstrated 80% cell death. Both mouse strains show a gradual recovery of Mac-1** bone marrow cells, returning to control levels by day 14-36 post irradiation. These results were reproducible suggesting the variation seen in figure 4.5 may indeed be mouse strain specific. Data presented in figure 4.1A demonstrates that WBMC number recovers by day 10 in both mice strains, thus suggesting individual cell types within the WBMC population may have different recovery rates.

Figure 4.5 – Cell death and recovery of Mac-1** staining WBMC following exposure to an in vivo single acute dose of 3 Gy X-rays.

The effect of a single acute dose of 3 Gy X-rays on Mac-1** staining WBMC cells. The number of Mac-1** staining WBMC as a percentage of the control value is plotted against time post irradiation with the dashed line indicating 100% i.e. control levels. Ranges (s.e.m) are shown and the number of individual mice analysed for each time point is day 2 (10), day 3 (8), day 6 (20), day 10 (12), day 14 (14), day 21 (8), day 36 (8), day 42 (7). CBA/H data is represented in red and C57BL/6 data in blue.
4.4.1.3 Immature bone marrow cells

To enable WBMC recovery within 10 days post irradiation, at least some of the cells that survive irradiation exposure must have the ability to self-renew, amplify and differentiate and so drive recovery and reconstitute the bone marrow. Obvious candidates are haemopoietic stem cells and progenitor cells which have the proliferative capacity and/or self-renewal ability required to produce a large number of more mature (Lin⁺) bone marrow cells (Bell & Van Zant, 2004; Warner et al., 2004).

In Chapter 3, antibody mediated immuno-magnetic separation was used to enrich for an immature bone marrow stem cell/progenitor cell population (Lin⁻). Figure 4.6 illustrates the effect of an acute dose of 3 Gy X-rays on the immature Lin⁻ cell population. Approximately 82-88% of Lin⁻ cells are killed within 2 days following an in vivo dose of 3Gy X-rays, indicating that these cells are more radio-sensitive than total WBMC (figure 4.3A; 80% kill) or Mac-1⁺⁺ white cells (figure 4.5; 50-65% kill). This cell death is followed by a steady recovery in both strains to 100% at ~ 36 days post irradiation although longer time points suggest that long term recovery is not 100%. Incomplete recovery of CFU-S has previously been reported following a single acute dose of X-rays (reviewed Testa et al., 1985) with recovery restricted to ~50%.
Figure 4.6 — Cell death and recovery of immature haemopoietic bone marrow cells (Lin') exposed to an in vivo single acute dose of 3Gy X-rays.

Figure 4.6 compares the effect of a single acute dose of 3 Gy X-rays on the Lin' bone marrow cells. Lin' cells were counted using a Schäfer System Casy® 1 counter and plotted as a percentage of the control value against time post irradiation. The dashed line indicates 100% i.e. control levels. Ranges (s.e.m) are shown and the number above each time point indicates the number of individual mice analysed for each time point. 17 CBA/H and 18 C57BL/6 control mice were analysed. CBA/H data is represented in red and C57BL/6 data in blue.
Figure 4.7 – Cytogram representation of changes in Sca-1 and c-Kit staining of Lin⁻ bone marrow cells over a period of six days post exposure to an in vivo single acute dose of 3 Gy X-rays.

Lin⁻ bone marrow cells were stained with FITC-labelled anti Sca-1 and PE-labelled anti c-Kit. Data is summarised in figure 4.8 but the most obvious cell death and recovery is indicated by the solid arrow which represents a discrete Lin⁻, Sca-1⁻,c-Kit++ staining cell population.

In all cytograms 5000 bone marrow cells were analysed. Percentages for the lower left hand regions are Control (62.12%), Day 2 (85.12%), Day 4 (75.89%), Day 6 (74.29%). Percentages for the gated regions are Control (3.72%), Day 2 (6.02%), Day 4 (1.42%), Day 6 (2.40%).
Cell surface antigens were exploited to phenotypically identify the whole HSC compartment (Lin', Sca-1++,c-Kit++ cells), the more mature HSCs (Lin', Sca-1++,Flk-2++ cells) and to monitor changes in the cytogram regions discussed in chapter 3.

FACS analysis (figure 4.7) illustrates the changes in the CBA/H Lin' bone marrow cells stained with FITC-labelled anti Sca-1 and PE-labelled anti c-Kit over the initial 6 days post irradiation. In contrast to the cell death seen in WBMC (figure 4.3A) and Lin' cells (figure 4.6), 0-4 days post irradiation there is a 100% increase in the number of Lin', Sca-1++, c-Kit++ and Lin', Sca-1++, Flk-2++ cells compared to controls (figure 4.7). Figure 4.8 demonstrates that there appears to be two phases of cell amplification. The first amplification is 1-4 days post irradiation and the second amplification is 4-36 days post irradiation (figure 4.8A/B). Both mouse strains show large fluctuations (in mean value and standard error of the mean) over the 42 day time course. Statistical analysis indicated that although there were differences between the two strains, the general recovery was not statistically different. Differences probably reflect individual variation and experimental error rather than inter strain differences.

To determine whether amplification was specific to Lin', Sca-1++, c-Kit++ and Lin', Sca-1++, Flk-2++ bone marrow cells, single staining of Sca-1++/+ , c-Kit++/+ and Flk-2++/+ bone marrow cells were quantitated as a function of time post irradiation. Figure 4.7 (solid arrows) illustrates death and recovery of Lin', Sca-1', c-Kit++ bone marrow cells which are more amenable to visual representation because there are more Lin' cells that stain with c-Kit than Sca-1 (chapter 3 table 3.3). Two days post irradiation the c-Kit++ staining cell population has disappeared but gradually recovers with similar kinetics to total bone marrow (figure 4.3A). ~40% of Lin', c-Kit++ bone marrow cells are killed (figure 4.9C) and this number is a combination of the decimation of Lin', Sca-1', c-Kit++ (lineage committed progenitors) and amplification of the Lin', Sca-1++, c-Kit++ bone marrow cells (HSCs). Lin', Sca-1++ and Lin', Flk-2++ staining cells are amplified post irradiation (figures 4.9A/B) and this is consistent with the amplification of Lin', Sca-1++, c-Kit++ and of Lin', Sca-1++, Flk-2++ bone marrow cells (figures 4.8A/B). Sca-1++ and Flk-2++ staining cells constitute a small percentage of Lin' WBMC (Tables 3.3; 3.4) and therefore the increase in the frequency of Lin', Sca-1++, c-Kit++ and of Lin', Sca-1++, Flk-2++ bone marrow cells is sufficient to manifest as an overall increase in Lin', Sca-1++ bone marrow cells and Lin', Flk-2++ bone marrow cells.
Figure 4.8 – Cell death and recovery of double stained Lin', Sca-1++, c-Kit++ and Lin', Sca-1++, Flk-2++ bone marrow cells following exposure to an *in vivo* single acute dose of 3 Gy X-rays.

A  B

500 400
Lin', Sca-1++, c-Kit++, % Control.

300 200

100

Figure 4.8 compares the effect of a single acute dose of 3 Gy X-rays Lin', Sca-1++, c-Kit++ cells (4.8A) and Lin', Sca-1++, Flk-2++ cells (4.8B) over a time period of 42 days (6 weeks). Each population is plotted as a percentage of the control value against time post irradiation. The dashed line indicates 100% i.e. control levels. Ranges (s.e.m) are shown and the number above each time point indicates the number of individual mice analysed for each time point. The number of control mice used were Lin', Sca-1++, c-Kit++ cells (23 CBA/H; 18 C57BL/6) and Lin', Sca-1++, Flk-2++ cells (23 CBA/H; 17 C57BL/6). CBA/H data is represented in red and C57BL/6 in blue.
Figure 4.9 – Cell death and recovery of single stained Lin', Sca-1'', Lin', Flk-2'' and Lin', c-Kit'' bone marrow cells following exposure to an in vivo single acute dose of 3 Gy X-rays.

Figure 4.9 illustrates the effect of a single acute dose of 3 Gy X-rays on Sca-1'' (4.9A), Flk-2'' (4.9B) and c-Kit'' (4.9C) Lin' bone marrow cells over a period of 42 days (6 weeks). Each population is plotted as a percentage of the control value against time post irradiation. The dashed line indicates 100% i.e. control levels. Ranges (s.e.m) are shown and the number above each time point indicates the number of individual mice analysed for each time point. CBA/H and C57BL/6 mice data was pooled (controls n=39).
4.4.1.4 Summary of Results

Analysis of cell death and recovery of murine bone marrow post exposure to an in vivo leukaemogenic dose of 3 Gy X-rays has shown:

- A massive (50-90%) reduction in the number of total WBMC, Lin⁻ WBMC, Myeloid WBMC (Mac-1⁺⁺) and Lin⁻, Sca-1⁻, c-Kit⁺⁺ WBMCs (lineage committed progenitors) post irradiation exposure.
- Amplification of Lin⁻, Sca-1⁺⁺, c-Kit⁺⁺ (whole HSC compartment) and Lin⁻, Sca-1⁺⁺, Flk-2⁺⁺ bone marrow cells (ST-HSC).
- An increase in the number of total bone marrow Red cells (RBMC) in the bone marrow to compensate for the reduced WBMC. This possibly reflects haemorrhage or increased vascular permeability.
- Little evidence of a significant difference in cell death and recovery between CBA/H and C57BL/6 mouse strains suggesting that radiosensitivity is probably not a major genetic factor.

4.4.2 mRNA levels in murine bone marrow and spleen following an in vivo exposure to a single acute dose of 3 Gy X-rays.

Flow cytometry enabled the recovery of bone marrow myeloid cells to be monitored using Mac-1 staining (section 4.4.1.2). Changes observed in bone marrow composition during cell death and recovery should be detected at the mRNA level as cellularity changes with time post irradiation. Therefore, to identify changes in mRNA levels following exposure to a single acute dose of 3 Gy X-rays, spleen and bone marrow RNA was extracted for each time point analysed by FACS. RNA was quantified using a spectrophotometer and used to make a series of Northern blots (~10μg/blot). Northern blots were hybridised with lineage specific/restricted cDNA probes including myeloid specific lineage enzymes MPO (Myeloperoxidase) and LysM (Lysozyme M), B-lymphocyte restricted markers Pax5 and CD19 and red cell marker HBB-B1 (Haemoglobin beta adult major chain). The two tissues analysed were bone marrow which is the site of haemopoiesis and the spleen which is a site of leukaemic infiltration in murine r-AML (Major, 1979).
4.4.2.1 **Standardisation of Northern blot analysis**

A common problem of quantifying mRNA levels using northern blot analysis is over or under loading of RNA samples. As mRNA normalisation is essential for mRNA quantification, all blots were probed with two ‘ubiquitously’ expressed ‘housekeeping’ genes — *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *glutathione peroxidase* (*GPX*). Internal standards (loading controls) ideally should be unaffected by experimental conditions, and be homogenously expressed in cell lines from different tissue types and during all stages of development (Bustin *et al.*, 2000).

All Northern blots probed with *GAPDH* and *GPX* cDNA exhibited highly variable mRNA levels (figure 4.10) and consequently loading was also monitored using ethidium bromide (EtBr) staining of 28S ribosomal RNA prior to transferring the RNA gel to the membrane. This semi-quantitative method involved measuring the staining intensity of the 28S rRNA band under fluorescence and comparing it to control tissue using the Syngene GeneTools computer package. Significant discrepancies were identified between normalisation using EtBr staining and *GAPDH* or *GPX* cDNA probes. Figure 4.10 illustrates B-cell leukaemias hybridised with *GAPDH* and *GPX* cDNA. Compared to control spleen, lane 3 exhibits ~6 fold overloading when corrected by *GAPDH*, ~4 fold overloading when corrected by *GPX* and ~1.5 fold overloading when corrected by EtBr staining. Probing Northern blots with *GAPDH* cDNA suggests that some B cell malignancies were overloaded by ~10 fold (figure 4.10, lanes 16 and 20) and some r-AML samples were ~14-16 fold overloaded (data not shown). Examination of 28S rRNA EtBr staining demonstrated that this was clearly not the case, suggesting that *GAPDH* and *GPX* were not good loading controls for murine radiation induced malignancies.
Figure 4.10 – Standardisation of a B cell leukaemic Northern blot.

Northern blot of B cell malignancies (3-20) stained with ethidium bromide (EtBr) prior to transfer to membrane and probed with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Glutathione peroxidase (GPX) cDNAs. Position 1 and 2 contain CBA/H spleen and bone marrow RNA respectively. All results in the above histogram were compared to CBA/H spleen.

Figure 4.11 – Standardisation of an irradiated bone marrow Northern blot.

Representative Northern blot analysis of bone marrow RNA in a 6 week time course following exposure to 3 Gy X-rays. The blot was stained with ethidium bromide (EtBr) prior to transfer to membrane and probed with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Glutathione peroxidase (GPX). All results are compared to unirradiated bone marrow which is represented by the '0' time point.
Northern blots generated from bone marrow RNA and spleen RNA extracted during the six week time course, were also probed with GAPDH and GPX cDNA and compared to control bone marrow. GAPDH mRNA levels increase post irradiation with levels up to 7 fold 6-10 days post irradiation (figure 4.11) and a similar increase is seen with spleen RNA (data not shown). In bone marrow however, GPX mRNA levels are comparable to EtBr staining suggesting that this may be a suitable loading control for this tissue and indicating that loading anomalies are restricted to the radiation induced leukaemias.

As internal standards should show homogenous expression, GAPDH and GPX cDNAs were used to probe bone marrow, blood, spleen, kidney, brain and liver (figure 4.12). The levels of GAPDH mRNA were brain>>kidney = liver>>bone marrow = spleen with no mRNA detected in blood. The levels of GPX mRNA were bone marrow = blood >> liver >spleen = kidney with no mRNA detected in the brain. GAPDH and GPX mRNA levels are not homogenously expressed in complex tissues in vivo and therefore not suitable for use as loading controls.

**Figure 4.12 – Mouse tissues probed with 'housekeeping' cDNAs**

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Glutathione peroxidase (GPX) mRNA levels detected in different mouse tissues taken from a male CBA/H mouse.

1 = Bone Marrow; 2 = Blood; 3 = Spleen; 4 = Kidney; 5 = Brain; 6 = Liver.

### 4.4.2.1.1 Alternative normalisation strategies.

Various studies have reported changes in GAPDH mRNA levels as a result of experimental conditions. These include exposure to mitogenic agents (Savonet et al., 1997) and γ-irradiation (Mayer et al., 2002). The choice of internal standard is further complicated by tissue complexity, fluctuation between and within individuals, and aberrant levels resulting from disease - GAPDH is up regulated in cancer (reviewed Bustin et al., 2000). The use of GAPDH or GPX as loading controls for murine radiation induced leukaemias appears inappropriate in the context of RNA quantification. Although GPX mRNA levels were less affected by the experimental
conditions and tissues and therefore potentially an acceptable loading control for the bone marrow and spleen time course, ethidium bromide staining of 28S ribosomal RNA was used as a loading control for all subsequent blots to maintain consistency throughout this thesis.

Alternative normalisation probes that could have been used in this thesis include β-actin and hypoxanthine-guanine phosphoribosyl transferase (HPRT). β-actin mRNA levels have, however, also been reported to be affected by mitogenic treatment (Savonet et al., 1997) and transcription levels vary in leukaemic patient tumour samples (reviewed Huggett et al., 2005). HPRT mRNA levels are constitutively expressed in most human tissues but elevated in certain parts of the central nervous system (reviewed Huggett et al., 2005). In summary, all ‘historic’ normalisation probes have been reported to be affected by experimental conditions. Northern blot normalisation however, is not restricted to hybridisation probes. Staining of the gel prior to blotting with acridine orange staining is considered to be a reliable method as it does not interfere with hybridization. Staining is not restricted to pre hybridisation and filters can be stained with methylene blue. This is a sensitive technique but prevents further hybridisation of the filter (Savonet et al., 1997).

Alternative techniques for quantifying mRNA are in situ hybridisation, RNAse protection assays, real-time reverse transcription PCR (real time RT-PCR) and cDNA microarrays. Of these techniques, real time RT-PCR is the most sensitive and flexible of the quantification methods and is a commonly used procedure. The cheapest option for real time RT-PCR is DNA dye-binding assays such as the detection fluorescent dye (SYBR Green) binding to DNA. SYBR Green is more fluorescent that ethidium bromide and fluorescence is enhanced as the dye binds to DNA (reviewed Bustin et al., 2000).

As with Northern blot analysis, normalisation is a problem in real-time RT-PCR. Real time RT-PCR amplifies both the target RNA and control RNA simultaneously. The control RNA then serves as an internal reference against which the target RNA can be normalised. Strategies proposed for normalisation in real time RT-PCR include sample size, RNA quantification, genomic DNA, reference genes and artificial molecules (reviewed Huggett et al., 2005). Commonly used reference genes include
GAPDH, Hypoxanthine-guanine phosphoribosyl transferase (HPRT) and β-actin which as stated above have been shown to be influenced by experimental conditions and the use of multiple reference genes has been suggested (reviewed Huggett et al., 2005). An alternative suggestion is the use of fluorescent nucleic acid stains i.e RiboGreen RNA quantitation (reviewed Bustin et al., 2000).

4.4.2.2 Bone marrow and Spleen mRNA levels post irradiation.

4.4.2.2.1 Bone marrow

Figure 4.13B illustrates the mRNA levels of myeloid differentiation markers (MPO and LysM) in control and irradiated bone marrow at various times post irradiation. MPO is an enzyme located in the azurophilic granules associated with immature myeloid cells (Lübbert et al., 1991). There is a gradual increase in the level of MPO mRNA between days 0-4 reaching a maximum 5 fold increase. The FITC-labelled anti Mac-1 antibody (figure 4.13A; section 4.4.1.2) reacts with the αM chain of Mac-1 which is expressed on granulocytes, macrophages, myeloid-derived dendritic cells and natural killer cells (BD Biosciences Pharmingen). The Mac-1++ cell population analysed by FACS should include MPO expressing cells. The cell death and recovery of the Mac-1++ cell population does not correlate with MPO mRNA levels - Mac-1++ cell number decline post irradiation but increased levels of MPO mRNA are detected. MPO expression may therefore reflect a subpopulation of Mac-1++ cells responsible for removing damaged or dead cells by phagocytosis. LysM is expressed by macrophages and therefore the ~3 fold increase in LysM mRNA levels at day 1, day 6, and day 21 possibly represents waves of apoptotic activity and mobilisation of macrophages for phagocytosis (Kuby, 1997). Although analysed, Pax5 and CD19 mRNA was not detected in unirradiated or irradiated bone marrow (data not shown).

HBB-B1 (Haemoglobin beta adult major chain) is a red cell marker and therefore HBB-B1 mRNA levels should reflect RBC cellularity in the bone marrow. Post irradiation there is a ~2 fold increase in the number of RBMCs, returning to control levels at ~ day 10 (figure 4.14A). HBB-B1 mRNA levels increase ~6 fold within the first day before returning to normal control levels (figure 4.14B). HBB-B1 mRNA levels to not correlate with the changes detected in RBMC number and therefore HBB-B1 probably is not a good marker for the mature red cells observed by cell counting and blood smear examination (Figures 4.1; 4.2).
Figure 4.13 – Cell death and recovery of myeloid cells post exposure of inbred mice to an *in vivo* single acute dose of 3 Gy X-rays.

Figure 4.13A – Cell death and recovery of Mac-1*+* staining WBMC

The effect of a single acute dose of 3 Gy X-rays on Mac-1*+* staining WBMC cells. The bone marrow recovery of both mice strains are compared. The number of cells as a percentage of the control value is plotted against time post irradiation with the dashed line indicating 100% i.e. control levels. For full description see section 4.4.1.2.

Figure 4.13B – Post irradiation bone marrow mRNA levels - Myeloid Markers.

Changes in mRNA levels detected in total bone marrow post exposure to a single dose of 3 Gy X-rays. Histogram representation is shown as a function of control bone marrow (column ‘0’) and uses ethidium bromide (EtBr) staining for normalisation (*GPX* = ‘housekeeping’ *Glutathione Peroxidase*). The same data is shown in the autoradiography profiles. Two lineage specific markers are used – *MPO* (Myeloperoxidase) and *LysM* (Lysozyme M).
Figure 4.14 – Cell death and recovery of red bone marrow cells (RBMC) post exposure of inbred mice to an *in vivo* single acute dose of 3 Gy X-rays.

**Figure 4.14A – Cell death and recovery of RBMC**

The effect of a single acute dose of 3 Gy X-rays on the number of red bone marrow cells (RBMC) as measured using a Schärfe system easy® 1 counter. The number of cells as a percentage of the control value is plotted against time post irradiation with the dashed line indicating 100% i.e. control levels. For full description see section 1.4.1.1.

**Figure 4.14B – Post irradiation bone marrow *HBB-B1* mRNA levels**

Changes in *HBB-B1* mRNA levels detected in total bone marrow post exposure to a single dose of 3 Gy X-rays. Histogram representation is shown as a function of control bone marrow (column '0') and uses ethidium bromide (EtBr) staining for normalisation. The same data is shown in the autoradiography profiles. *GPX = Glutathione Peroxidase.*
Figure 4.15 – Spleen mRNA levels post exposure of inbred mice to an *in vivo* whole body single dose of 3 Gy X-rays, probed with B lymphoid differentiation markers.

Changes in mRNA levels in splenic tissue extracted from mice that had been subjected to an acute dose of 3Gy X-rays. This time course shows the changes identified in *Pax5* and *CD19* expression over a period of 6 weeks. Column ‘0’ represents unexposed spleen and all subsequent time points are expressed relative to this control tissue. The histogram shows the mRNA expression relative to control spleen and standardised using ethidium bromide staining. The same data is shown in the autoradiography profiles.

4.4.2.2.2 Spleen

Figure 4.15 illustrates the mRNA levels of B lymphoid differentiation markers in spleen. The *Pax5* gene encodes the B cell specific activator protein (BSAP) which regulates several B cell-specific genes including *CD19* (Nutt *et al.*, 1997A; 1998) and consequently, *CD19* expression tends to correlate with that of *Pax5* during B-lymphopoiesis (chapter 6). *Pax5* expression is required throughout the whole of B cell lymphopoiesis until plasma cell differentiation (Lin *et al.*, 2003). *Pax5* and *CD19* mRNA levels are reduced following irradiation and do not recover during the 6 week time period (Histogram; Figure 4.15B). Accurate quantitation of *Pax5* mRNA levels is difficult because the levels detected by Northern blot analysis are very low and therefore possibly over-estimated in the histogram. The autoradiography profiles demonstrate that post irradiation, the *CD19* mRNA levels do not correlate with the *Pax5* mRNA levels. This may reflect the preferential/efficient elimination of
immature *Pax5*⁺ B cells over *Pax5*, *CD19*⁺ B cells. B cells are highly radiosensitive during immunoglobulin rearrangement (reviewed Hoeijmakers, 2001), a process associated with *Pax5* expression.

### 4.4.2.3 Conclusions

Quantification of Northern blots generated from irradiated tissue is complicated by the lack of suitable loading controls. Ethidium bromide fluorescence staining of the 28S rRNA band is only semi-quantitative and as it is obtained directly from the gel does not control for the transfer of RNA to the membrane, the integrity of the blot itself or the phosphoimager quantification. Despite these problems, Northern blot analysis has supplied some interesting data on the recovery of haemopoietic cells post irradiation.

### 4.5 Discussion

Exposure of total bone marrow to a leukaemogenic dose of 3 Gy X-rays *in vivo* has a dramatic effect on the cell populations analysed. Decimation of the white cell population (both WBMC and Lin⁻ white cells) is seen accompanied by increase in the number of bone marrow red cells (RBMC) located within the femur shaft. This increase in the number of RBMC can not represent RBC production because of the devastating effect of irradiation exposure on bone marrow haemopoiesis and possibly reflects either haemorrhage or increased vascular permeability. Quantitatively, WBMC cell recovery is seen by ~ day 10 although FACS analysis of phenotypically defined cells, the Lin⁻ white cell count and mRNA levels indicate that the cellular composition may not return to normal by 6 weeks post irradiation. The haemopoietic system has however achieved viability, despite the loss of ~80% WBMC, and the initial haemopoietic recovery is sufficient to ensure that the survival of the animal is not compromised.

Recovery must be dependent on cells that not only survive irradiation exposure, but also have the ability to generate replacement cells. These cells must be located within the discrete populations of Lin⁻, Sca-1⁺⁺, c-Kit⁺⁺ and Lin⁻, Sca-1⁺⁺, Flk-2⁺⁺ cells as these cells dramatically increase in numbers 0-4 days post irradiation indicating a strong proliferative response and the potential to drive the recovery of mature Lin⁺ cells. All other white cell populations analysed – WBMC, Mac-1⁺⁺ WBMC, Lin⁻ white cells and Lin⁻, Sca-1⁻, c-Kit⁺⁺ white cells – are reduced during this time frame,
although Northern blot analysis of mRNA levels suggests that certain subpopulations of haemopoietic cells display a degree of radio-resistance. Many HSC studies rely on phenotypically defined Lin⁺, Sca-1⁺⁺, c-Kit⁺⁺ bone marrow cells being isolated by fluorescence activated cell sorting and functionally confirmed as haemopoietic stem cells by subsequent transplantation into lethally irradiated mice (Ikuta & Weissman, 1992; Okada et al., 1992; Osawa et al., 1996; Christensen & Weissman, 2001). The data presented in this thesis is obtained by the in vivo irradiation of inbred mice and the analysis of the Lin⁺, Sca-1⁺⁺, c-Kit⁺⁺ bone marrow cells at specific time points post irradiation. This study is therefore equivalent to or a surrogate in vivo functional assay. The advantage of an in vivo functional assay is that it reduces cell manipulation and may explain why many studies do not detect an increases in Lin⁺, Sca-1⁺⁺, c-Kit⁺⁺ and Lin, Sca-1⁺⁺, flk-2⁺⁺ cell number. Studies have report that HSC numbers do not recover to pre-irradiation levels (Down et al., 1995; Testa et al., 1995), possibly reflecting loss of HSC activity during in vitro growth, unlike this study where ~100% recovery is detected at day 42.

Phenotypical and functional identification of the mouse HSC is further supported by genetic linkage analysis. As discussed in Chapter 3, a collaborative group genetic linkage analysis of the Lin⁺, Sca-1⁺⁺, c-kit⁺⁺, population in (CBA/H x C57BL/6) intercross mice was carried out by A Zanker and M Jawad. Two QTLs previously implicated in determining the frequency of long-term repopulating HSCs using in vitro studies were identified. A chromosome 18 QTL mapped to approximately the same location as the day 35 CAFC QTL (de Hann & van Zant, 1997) and a chromosome 1 QTL mapped to the stem cell frequency regulator 2 (Scfr2) using LTC-ICs (Müller-Sieburg & Riblet, 1996). This data supports the hypothesis that the Lin⁺, Sca-1⁺⁺, c-Kit⁺⁺ bone marrow cells contains the HSCs.

r-AML is a disease of the haemopoietic stem cell and the preferred model for the development of this malignancy is that the initiating mutation is in the HSC and that further mutations dictate the disease type (reviewed Blair & Pamphilon, 2003; Hope et al., 2003; Warner et al., 2004). Subpopulations of HSCs are selectively resistant to radiation damage based on their different proliferative capacities and cell cycle status (reviewed Daniak, 2002). Under steady state conditions, cell cycle arrest enables efficient DNA damage repair thus preventing replication of cells containing DNA
damage (reviewed Bernstein et al., 2002). Bone marrow is one of the most radiosensitive tissues (Stov, 1999; Andreassen et al., 2002) and this may explain why radiation specifically induces AML, a HSC malignancy, as opposed to other malignancies. Extensive and immediate amplification of HSCs post irradiation to reconstitute the bone marrow may result in insufficient ability, or time, to repair radiation induced DNA damage and therefore mutations in the haemopoietic cells become fixed by replication.

Cell death and recovery patterns are similar between the r-AML susceptibility and r-AML resistant strains. Chapter 3 demonstrated that r-AML susceptible CBA/H mice had 3 times as many Lin', Sca-1++, c-Kit++ and twice as many Lin', Sca-1++, Flk-2++ bone marrow cells than r-AML resistant C57BL/6 mice. The similar recovery kinetics identified in this study indicates that the difference between the two strains is retained post irradiation with the CBA/H mice still having more Lin', Sca-1++, c-Kit++ and Lin', Sca-1++, Flk-2++ bone marrow cells than C57BL/6 mice and therefore a larger ‘target population’ for mutations. Target size therefore can not be excluded as a risk factor in radiation induced leukaemia.
5 Chapter 5: Candidate r-AML susceptibility genes on distal Chromosome 1.

5.1 Introduction

The CBA/H r-AML susceptibility locus identified on chromosome 1 covers a relatively large interval (92.3 cM to 100 cM) and consequently contains many genes any of which could potentially be relevant to the increased risk of r-AML (Boulton et al., 2003). Figure 5.1 represents only high priority candidate genes obtained from the 2000 chromosome 1 committee report, chosen because of their potential association or role in haemopoiesis and/or in radiation leukaemogenesis.

Figure 5.1 – CBA/H r-AML susceptibility region implicated on Chromosome 1

<table>
<thead>
<tr>
<th>cM</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>87.9</td>
<td>D1Mit15 p=0.047</td>
</tr>
<tr>
<td>90</td>
<td></td>
</tr>
<tr>
<td>92.3</td>
<td>D1Mit111 p=0.0012</td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>100.5</td>
<td>D1Mit150 p=0.0002</td>
</tr>
<tr>
<td>113</td>
<td></td>
</tr>
</tbody>
</table>

Genes located within distal chromosome 1 were identified using the 2000 chromosome committee reports (http://www.informatics.jax.org). The r-AML susceptibility locus is represented by a dashed line (Boulton et al., 2003) and the stem cell frequency regulator locus by a thin solid line (Müller-Sieburg & Riblet, 1996). Units are in centiMorgans. Microsatellite p values were obtained from Boulton et al., 2003 and high priority genes are shown.
This study focuses on three genes (*Hlx*, *Adprt1* and *Ephx1*) located close to the most statistically significant microsatellite marker D1Mit150 (100 cM; figure 5.1). *Hlx* (H2.0 like homeobox) has a potential role in establishing haemopoiesis during development and/or differentiation (section 1.3.1.1). Aberrant expression of other hox genes have been implicated in haematological malignancies (reviewed Owen & Hawley, 2002) and hox proteins have been implicated in HSC self-renewal (reviewed Bell & Van Zant, 2004). *Hlx* is therefore a good candidate for the stem cell frequency regulator. The two other genes are *Adprt1* (Poly (ADP-ribo) polymerase 1) and *Ephx1* (microsomal epoxide hydrolase-1). *Adprt1* has an important role in DNA repair and has a protective role against cancer (section 1.2.3.1.1) while *Ephx1* is functionally a less promising candidate because its primary role is in epoxide substrate detoxification and bioactivation. *Ephx1* however is known to have a genetic polymorphism in mouse which results in a functional difference (Lyman & Poland, 1980) and *Ephx1* polymorphisms have been implicated in human cancers including -7/del(7q) and t(8;21) acute myeloid leukaemia (Lebailly et al., 2002).

**5.2 Experimental Design**

As CBA/H mice are susceptible to r-AML and C57BL/6 are resistant, there must either be a functional difference or a difference in expression levels of the r-AML susceptibility gene products in the inbred mice that confer a selective advantage or disadvantage during leukaemogenesis. CBA/H and C57BL/6 candidate gene cDNAs were therefore generated by RT-PCR and sequenced to screen for polymorphisms/variant alleles. Northern blot analysis was used to establish differential expression of the putative genes in the normal tissues of the two mouse strains.

As genetic differences may manifest themselves in response to radiation exposure, the levels of candidate gene mRNA following exposure to a single acute dose of 3 Gy X-rays were also assessed in spleen and bone marrow RNA extracted from CBA/H and C57BL/6 inbred mice in a six week post irradiation time course. Leukaemic spleen RNA extracted from long term exposed CBA/H x (CBA/H x C57BL/6)F1 backcross mice was also analysed. Three major haemopoietic malignancies arise following exposure of these backcross mice to a single acute dose of 3 Gy X-rays – Thymic
lymphomas, r-AML and B-cell leukaemias (BCL) (Boulton et al., 2002; Cleary et al., 2001; section 1.2.2).

5.3 Results

5.3.1 Variant alleles in CBA/H and C57BL/6 mice

To determine whether functional differences in the putative r-AML susceptibility gene products were a consequence of polymorphic variations in the gene coding region, bone marrow and spleen cDNAs from both inbred mouse strains were generated by RT-PCR, cloned and sequenced in both directions.

5.3.1.1 Hlx (H2.0 like homeobox)

A 2265bp mRNA transcript (NM_008250) encodes the 476 amino acid Hlx protein. RT-PCR and cloning were used to generate a 2036 bp Hlx cDNA. Multiple primers were then used to sequence the cDNA and although no polymorphisms were detected, alternative splicing was identified. Normal splicing generates a full length mRNA transcript encoding the Hlx protein (figure 5.2A). Alternative splicing was detected in both CBA/H and C57BL/6 spleen and bone marrow - CBA/H spleen (7%; 2/28 colonies), CBA/H bone marrow (7%; 2/27 colonies), C57BL/6 spleen (10%, 3/29 colonies) and C57BL/6 bone marrow (4%; 1/24 colonies). Figure 5.2B illustrates the Hlx splice variants identified. Lanes 1 and 6 represent the DNA sequence and the full-length Hlx RNA sequences respectively. Exon/intron boundaries are integral to RNA splicing with the critical sequence being ‘ag’ – ‘gt’. Introns almost always start with a splice donor site ‘gt’ (guanine and thymine) and ends with a splice acceptor site ‘ag’ (adenine and guanine). In 7/8 cases, loss of exon II was associated with the retention of an adenine and guanine base (lane 2; ‘ag’). In 1/8 cases, the loss of exon II and retention of ‘ag’ was also associated with the retention of a ‘gt’ (lane 3; C57BL/6 spleen). Two CBA/H spleen Hlx cDNAs demonstrated normal splicing but either extra bases (lane 4) or loss of one base (lane 5). All sequence changes identified caused a frame shift, a premature stop codon and the loss of the homeobox motif. No significant difference was detected in the sequence or number of variant mRNAs between the two mouse strains or tissues.
Figure 5.2 – Evidence of alternative splicing in Hlx.

Figure 5.2A – Normal Splicing of Hlx.

Normal splicing generates a full length mRNA transcript encoding the Hlx protein. It contains a homeobox (180 bp conserved sequence motif) and a hep (H2.0 engrailed paired) motif (responsible for interactions with PBX homeoproteins). Downstream of the homeobox is the proposed transactivation domain (Allen et al., 1991).

Figure 5.2B – Alternative splicing and sequence anomalies in Hlx cDNA

Figure 5.2B illustrates the sequences immediately around the splice donor and acceptor sites. Intronic sequence is represented in black and sequence of 3 of the 4 exons displayed in colour. Lane 1 represents the DNA sequence and lane 6 the full length RNA sequences. Lanes 2 and 3 represent splicing of exon II and the retention of 2 and 4 extra bases respectively. Lane 4 and 5 retain exon II but extra/loss of bases. The consequence of lanes 2-5 are frame shift, premature stop codons and loss of the homeobox motif.
5.3.1.2 Ephx1 (microsomal epoxide hydrolase-1)

Ephx1 is a 455 amino acid monomeric protein coded by a 1708 bp mRNA transcript (NM_010145). 1284 bp cDNAs were generated from spleen by RT-PCR and cloned (5 colonies/strain; both directions). Two homozygous polymorphisms within the protein coding region were identified - a T to C transition at 596 bp (Figure 5.3) and a C to T transition at 1086 bp (data not shown). This is consistent with the previous mouse strain sub-classification of CBA/J and CBA/CaJ (Ephx1\textsuperscript{d}) and C57BL/6 (Ephx1\textsuperscript{b}) (Lyman & Poland, 1980). The 596 bp transition does not alter the encoded aspartate amino acid. The 1086 bp transition substitutes an arginine for a cysteine at codon 338 and has been previously reported to alter murine Ephx1 enzymatic function through modifying protein stability (Hartsfield & Everett, 2000).

Figure 5.3- Ephx1 cDNA polymorphism

![Polymorphism](image)

Sequencing was carried out on an Applied Biosystems Model 377 DNA sequencing system, with the ABI PRISM BigDye\textsuperscript{TM} Terminator Cycle Sequencing Ready Reaction Kit and analysed using Factura\textsuperscript{TM} Release 1.2.0. Figure 5.3 demonstrates one of the documented polymorphisms. CBA/H has a cytosine at 596bp and C57BL/6 has a thymine as indicated by the asterisks. Unlike the 1086bp transition, this polymorphism is not functional as it does not change the amino acid sequence as the amino acid remains as an Aspartate.

5.3.1.3 Adprt1(Poly (ADP-ribo) polymerase 1)

A 3845 bp Adprt1 mRNA transcript (NM_007415) codes for a 1014 amino acid protein (Parp1). A 1209 bp Adprt1 cDNA was generated from spleen by RT-PCR and cloning. Sequencing was problematic and although sufficient bases were identified to confirm Adprt1 cDNA had been cloned, the presence of polymorphisms can not be excluded.
5.3.2 Irradiated CBA/H and C57BL/6 inbred mouse strains.

To establish whether genetic differences were at the level of response to radiation exposure, bone marrow and spleen RNA was extracted over a six week time course following exposure of CBA/H and C57BL/6 mice to a single acute dose of 3 Gy X-rays. Northern blots were generated and hybridised with \textit{Hlx}, \textit{Ephx1} and \textit{Adprt1} cDNA probes. Chapter 4 demonstrated that bone marrow cellular composition is altered by radiation exposure, and that both bone marrow and spleen exhibit changing mRNA levels of haemopoietic cell markers. \textit{Ephx1} and \textit{Adprt1} are stress response genes and therefore changes in mRNA levels were expected. \textit{Hlx} may have a differentiation role in haemopoiesis and therefore changes in \textit{Hlx} mRNA levels may reflect the changes in bone marrow cellularity.

\textit{Hlx} mRNA is detected in bone marrow (5.4A) and spleen (5.4B). 2-3 days post irradiation, CBA/H \textit{Hlx} mRNA levels in both tissues are comparable to control levels although there is a reduction in bone marrow levels over the following few weeks. C57BL/6 \textit{Hlx} spleen mRNA levels are relatively constant (<2 fold increase) although there is a transient increase in \textit{Hlx} mRNA levels in the C57BL/6 bone marrow at ~ day 6 and again at day 36.

\textit{Ephx1} mRNA was not detected in unirradiated bone marrow (figure 5.5A) and although \textit{Ephx1} cDNA was probed against the bone marrow time course, radiation exposure had not induced \textit{Ephx1} mRNA levels. ~3/4 days post irradiation, splenic \textit{Ephx1} mRNA levels increase (~3 fold) in both CBA/H and C57BL/6 mouse strains (figure 5.4B). After this time point, the \textit{Ephx1} mRNA levels correspond to the ethidium bromide loading control. Bone marrow and spleen \textit{Adprt1} mRNA levels also mirror the ethidium bromide staining in both mouse strains and correlate with mRNA levels of another DNA damage repair gene \textit{DDB1} (figure 5.4A/B).

Northern blot analysis is a relatively insensitive technique which can reliably detect only substantial differences in mRNA levels. This insensitivity is compounded by problems in quantification - this study is semi-quantitative because of the effect of radiation on reportedly ubiquitously expressed normalisation controls in complex tissues \textit{in vivo} (section 4.4.2.1). Overall, no major differences were observed between CBA/H and C57BL/6 mice for any of the candidate genes. It is however, not possible.
to exclude differences in specific cells within a tissue, and/or differences at the protein level.

Figure 5.4 – Bone marrow and spleen mRNA levels post exposure of inbred mice to an in vivo whole body single acute dose of 3Gy X-rays.

CBA/H and C57BL/6 mice were exposed to an in vivo dose of 3 Gy X-rays, sacrificed at specific time points post irradiation, and bone marrow and spleen RNA extracted. To ensure sufficient total cellular RNA (~20 µg) was obtained, tissue from 3-4 mice were pooled for each time point. Northern blots were prepared, blotted and probed with Hlx, Ephx1, and Adprt1 cDNAs. The numbers 0 to 42 represent the number of days post irradiation and cumulate in a six week time course as defined in chapter 4. '0' represent unirradiated control tissue. Ethidium bromide staining of 28S ribosomal RNA prior to transferring the RNA gel to the membrane was used to assess loading.

5.3.3 Leukaemic mice

Northern blots were generated from leukaemic spleen RNA extracted from animals diagnosed with r-AML and BCL, and these were probed with all three candidate gene cDNAs to discover whether leukaemic mRNA levels might suggest a role of these genes in leukaemogenesis (figure 5.5).
Figure 5.5 – Northern blot analysis of radiation induced malignancies probed with chromosome 1 putative candidate genes.

Representative Northern blots containing total cellular RNA (~20µg) prepared from CBA/H control spleen (Sp) and bone marrow (BM), and spleen leukaemic RNA (lanes 3-20) probed with the 3 candidate genes – Hlx, Ephx1 and Adprtl cDNA probes. Two sets of blots were prepared – B cell leukaemia (A) and r-AMLs (B). Ethidium bromide staining of 28S ribosomal RNA prior to transferring the RNA gel to the membrane was used to assess loading.

Northern blots were quantified using a Phosphoimager (section 2.5.6) and RNA loading monitored using ethidium bromide staining of 28S ribosomal RNA prior to transferring the RNA gel to the membrane. As r-AML is a bone marrow disease, mRNA levels were compared to control bone marrow. In contrast, progenitor cell BCL mRNA levels were compared to control spleen. 71% (45/63) of r-AMLs exhibited increased levels (~ 4 fold) of Hlx mRNA compared to control bone marrow, and 25% (16/63) showed comparable levels (figure 5.5B). This is consistent with the known expression of Hlx in myeloid-macrophage lineage cells (Allen et al., 1991). 84% (21/25) of BCLs however demonstrated reduced (~2 fold) Hlx mRNA levels compared to both control spleen (figure 5.5A). Although Hlx expression has been detected in early B-cell development (Allen et al., 1991), BCLs are characterised by
the presence of heavy chain gene rearrangements indicating that these cells have progressed to the pre-B stage of lymphopoiesis. Low Hlx mRNA levels may therefore purely reflect the B cell leukaemic differentiation block.

Most BCLs (n=14/18) exhibited reduced (~5 fold) Ephx1 mRNA levels compared to control spleen (figure 5.5A). 10/18 r-AMLs exhibited increased (~2 fold) Ephx1 mRNA levels (figure 5.5B) compared to control bone marrow. Analysis of control tissue (figure 5.5A/B, lanes 1 and 2; figure 5.4B) indicates that Ephx1 mRNA is present in murine spleen but not in murine bone marrow. The low levels detected in the leukaemic spleens probably reflect the infiltration of malignant haemopoietic cells into splenic tissue.

Northern blots of BCL leukaemic spleen hybridised with Adprt1 cDNA (figure 5.5A) showed that 50% (9/18) had comparable mRNA levels to spleen; 28% (5/18) had reduced Adprt1 mRNA levels (~ 2 fold) and 22% (4/18) had increased (~2-4 fold) Adprt1 mRNA levels. Similar analysis of r-AML leukaemic spleen showed 44% (16/36) had comparable levels to control bone marrow and 56% (20/36) had increased (~2-3 fold) Adprt1 mRNA levels. Although some leukaemias demonstrate very high Adprt1 mRNA levels (figure 5.5A, lane 5 & 18) subsequent normalisation using ethidium bromide staining of 28S RNA as a loading control suggests the increase is only ~3 fold – emphasising the importance of standardisation in quantification of Northern blots. Although Adprt1 has an essential role in DNA repair (section 1.2.3.1.1), it is also involved in various other cellular processes including cell proliferation and gene transcription regulation of certain differentiation genes (Tong et al., 2001). The increased Adprt1 mRNA levels detected in this study may reflect the proliferative status of leukaemic cells.

5.3.4 Gene expression - mRNA levels in control tissues
To determine whether variation in mRNA levels (transcription and/or stability) could account for the inter mouse strain difference in susceptibility to r-AML, mRNA levels were assessed in murine haemopoietic (spleen and bone marrow) and non-haemopoietic organs (kidney and liver) by Northern blot analysis. Figure 5.6
illustrates mRNA levels for each candidate gene. mRNA levels were compared to CBA/H spleen and normalised by ethidium bromide staining.

5.3.4.1 Hlx (H2.0 like homeobox)
Bone marrow exhibited the highest expression of Hlx mRNA (~2 fold CBA/H spleen; figure 5.5A; lanes 1 & 2) reflecting the prevalence of myeloid lineage cells in this tissue. Hlx mRNA detected in the spleen may reflect the spleen’s role as the site of B lymphopoiesis in the mouse and the reported expression of Hlx at the pre-B stage of lymphopoiesis (Allen et al., 1991). Kidney Hlx mRNA levels are comparable to spleen Hlx mRNA levels while the liver exhibited trace levels of Hlx (figure 5.6A; raw data not shown). No significant differences in Hlx mRNA levels were detected in the CBA/H and C57BL/6 mouse tissues analysed.

5.3.4.2 Ephx1 (microsomal epoxide hydrolase-1)
Differences in Ephx1 mRNA levels were identified between both tissues and strains. Both CBA/H spleen and C57BL/6 spleen exhibited similar levels of Ephx1 mRNA, as did CBA/H and C57BL/6 bone marrow. There is however ~10 fold less Ephx1 mRNA in bone marrow than in spleen (figure 5.6B; figure 5.5B lane 1 & 2). The highest Ephx1 mRNA levels were in the liver which is consistent with the detoxification properties of this tissue (Sherwood, 1997) and the CBA/H liver exhibited ~2 fold less Ephx1 mRNA than C57BL/6 liver. The largest inter-strain variation was identified in the kidney with ~3 fold less Ephx1 mRNA in the CBA/H kidney that the C57BL/6 kidney.

5.3.4.3 Adprt1 (Poly (ADP)-ribo) polymerase 1)
No significant difference was detected in Adprt1 mRNA levels between the two strains. Adprt1 mRNA levels were restricted to the CBA/H and C57BL/6 spleen (figure 5.5A, lane 1) with all other tissues exhibiting negligible levels (figure 5.6C).
Figure 5.6 – Histograms of Hlx, Ephxl, and Adprt1 mRNA levels in control tissues.

A

mRNA level relative to control CBA/H spleen

Hlx mRNA levels

B

mRNA level relative to control CBA/H spleen

Ephxl mRNA levels

C

mRNA level relative to control CBA/H spleen

Adprt1 mRNA levels

Figures 5.4A, 5.4B and 5.4C represent histograms of the tissue specific mRNA levels of Hlx, Ephxl and Adprt1 respectively. In each case, mRNA levels are all compared to the CBA/H splenic mRNA levels thereby enabling comparison of both tissues within a strain and also between strains. Blots were normalised against ethidium bromide staining of the 28S ribosomal RNA.
5.4 Discussion

Although r-AML and BCL arise in X-irradiated mice, genetic linkage on chromosome 1 was only detected in r-AMLs. The CBA/H r-AML susceptibility locus associates inheriting two CBA/H alleles in this region with an increased risk of r-AML (Boulton et al., 2003). r-AML susceptibility must be due to strain specific variant alleles - either inherent or as a consequence of the radiation exposure – that may confer a selective advantage or disadvantage to the development of r-AML. Three genes located close to the statistically significant microsatellite marker DlMit150 (100cM) were analysed. Two of these genes are involved in response to physiological stress (Adprl and Ephx1) and one is a good candidate for the stem cell frequency regulator (Hlx).

Sequencing CBA/H and C57BL/6 bone marrow and spleen candidate gene cDNAs detected two possible mechanisms that could generate functional differences in the gene products – a protein coding polymorphism in Ephx1 and alternative splicing in Hlx.

Ephx1 catalyzes the hydrolysis of epoxides and/or epoxide intermediates. While hydrolysis of endogenous substrates i.e. steroid epoxides generates a less reactive water soluble dihydrodiol which can be excreted from the body, hydrolysis of xenobiotic substrates i.e. tobacco carcinogens and benzene produces highly mutagenic and carcinogenic diol epoxides (Fretland & Omiecinski, 2000). The Ephx1 enzyme therefore can function as a bioactivator or detoxifier depending on the nature of the substrate.

Human and murine functional Ephx1 polymorphisms have been detected (Hassett et al., 1994; Lyman & Poland, 1980). Lyman and Poland (1980) reported that mouse strains could be sub-classified into Ephx1<sup>a</sup> and Ephx1<sup>b</sup> mice based on Ephx1 enzyme activity after heating liver microsomes at 62°C for 30 minutes. Liver microsomes from Ephx1<sup>a</sup> mice demonstrated reduced Ephx1 enzyme activity (&lt;3% of initial value) while liver microsomes from Ephx1<sup>b</sup> mice demonstrated only a slight decrease in activity. Changes in heat stability were predicted to be caused by changes in protein stability (Lyman & Poland, 1980). Sequence analysis of the two Ephx1 sub-classifications identified a single base change C to T transition in the cDNA coding
sequences that changed an arginine to a cytosine at residue 338 (Hartsfield & Everett, 2000).

Hassett et al. (1994) identified two functional polymorphisms of human epoxide hydrolase 1 (HYL1) - the HYL1*2 allelic polymorphism (substitution of histidine for tyrosine at residue 113 exon 3) exhibited a 39% decrease in HYL1 protein and the HYL1*3 allelic polymorphism (substitution of arginine for histidine at residue 139 exon 4) exhibited a 25% increase in HYL1 protein. Individuals that are homozygous for the HYL1*3 polymorphism have a significantly increased risk of lung cancer (Cajas-Salazar et al., 2003) while there is an increase risk of colon and liver cancer in individuals with the HYL1*2 polymorphism (reviewed Bauer et al., 2003).

Lebailly et al. (2002) reported that HYL1 polymorphisms can be associated with acute myeloid leukaemia (AML). An increased risk of AML is identified in male patients which have the HYL1*3 polymorphism but only in conjunction with the t(8;21) or -7/del(7q) karyotype. HYL1*2 heterozygous individuals are associated with decreased risk in AML. The r-AML susceptible mouse strain in this thesis is the CBA/H mouse which has the Ephx1d polymorphism (<3% enzyme activity post heat exposure) (Lyman & Poland, 1980). This is not consistent with the Lebailly et al., (2002) human data where increased risk of AML is associated with the HYL1*3 polymorphism (25% increase in HYL1 enzyme activity) and where a low level of HYL1 enzyme activity is protective.

Ephx1 knockout mice have no unusual phenotype indicating that in mice, microsomal epoxide hydrolase is not essential for reproduction and physiological homeostasis (Miyata et al., 1999). Ephx1 is required for the production of the diol-epoxide of 7,12-dimethylbenz[a]anthracene (DMBA) which is highly mutagenic and therefore Ephx1 knockout mice are therefore resistant to DMBA induced carcinogenesis (Miyata et al., 1999). Male 129/Sv mice develop significant haemotoxicity and genotoxicity on exposure to Benzene while Ephx1 knockout male mice are unresponsive. However, there was little response to Benzene in either female 129/Sv or Ephx1 knockout female mice (Bauer et al., 2003). Gender based differences were also seen in humans (Lebailly et al., 2002) although susceptibility to r-AML is not sex linked in the mice studied in this thesis (Boulton et al., 2001).
Previous studies have concentrated on the bioactivation or detoxification role of Ephx1 whereby the initiating substrate i.e benzene is known. The detection of a functional Ephx1 polymorphism in r-AML susceptible CBA/H and r-AML resistant C57BL/6 mice is promising but the role of Ephx1 in radiation is unclear. Ephx1 is capable of mediating the sodium-dependent uptake of bile acids (which have a central role in cholesterol metabolism) and decreased expression of Ephx1 protein results in highly elevated serum bile acid levels (reviewed Zhu et al., 2004B). Ionising radiation has been shown to alter hepatic cholesterol metabolism in the Syrian hamster (Feurgard et al., 1999) and bile acid profiles in pigs (Scanff et al., 1999).

Although Ephx1 may have a role in r-AML through bile acid uptake, an alternative mechanism could be through expression of transcription factors especially those aberrantly expressed in leukaemia. GATA-4 stimulates EPHX1 promoter and has a critical role in regulating EPHX1 expression (Zhu et al., 2004B). Over expression of other GATA genes (GATA-3 and GATA-6) were also reported by this group to stimulate the EPHX1 promoter, suggesting a possible role of the GATA genes transcribed during haemopoiesis in r-AML. C/EBPα-dependent EPHX1 activation has also been described (Zhu et al, 2004A).

Alternative splicing enables a single primary transcript to generate multiple mature mRNAs from a single gene. This produces protein diversity often including proteins with antagonistic dominant negative functions (reviewed Brinkman, 2004). Sequencing of Hlx cDNA identified alternative splicing in 4-10% of colonies with skipping of exon 2 creating a frame shift, a premature termination codon and loss of the homeobox motif. The low level of alternative splicing detected in this study may represent RT-PCR artefact, although other studies have confirmed alternative splicing of homebox genes is often associated with loss of the homeodomain (Lawrence & Largman, 1992). A premature termination codon is frequently associated with unstable mRNA which is rapidly degraded in vivo by nonsense-mediated mRNA decay (Strachan & Read, 1999). Alternatively, viable splice variants may produce truncated polypeptides with distinct DNA binding and transcriptional regulatory activities which can compete with wild type protein. Imbalance of wild type and variant splice variants in some genes e.g CD44, and Wilms’ tumor 1 (WT1) has been implicated in
malignancy (reviewed Brinkman, 2004). *Hlx* alternative splicing was detected in both CBA/H and C57BL/6 mice, but bone marrow and spleen are complex tissues and therefore mouse-strain differences in specific cells cannot be discounted. There appears to be no evidence of a human alternate splice in HLX1 (M. Jawad, personal communication).

All three candidate gene mRNAs were assessed in r-AML and BCL leukaemic spleen RNAs, and in bone marrow and spleen RNA following 3 Gy X-ray exposure. *Hlx* mRNA levels were increased in r-AMLs (71%) and decreased in BCLs (84%) consistent with the differentiation expression of *Hlx* in myeloid-macrophage lineage (Allen et al., 1991) and the progression of BCLs beyond the early B stage of lymphopoiesis as indicated by IgH gene rearrangements. The detection of *Hlx* mRNA levels comparable to control tissues 2-3 days post irradiation indicates that these cells are relatively radio-resistant or are amplified. This is consistent (albeit not as obvious) with the amplification of Lin−, Sca-1++, c-Kit++ and Lin−, Sca-1++, Flk-2++ bone marrow cells (chapter 4). High levels of Hlx protein promotes myelomonocytic differentiation (Allen et al., 1991; Allen & Adams, 1993) and impairs lymphoid cell development (Allen et al., 1995).

*Ephxl* and *Adprt1* are stress response genes and therefore changes in mRNA levels were expected. The levels of *Adprt1* mRNA varied dramatically between different BCLs and r-AMLs. Parp1 (the protein encoded by *Adprt1*) has an important role in DNA damage repair and responds within seconds of radiation exposure (reviewed Chalmers et al., 2004). It also is involved in a plethora of cellular processes including DNA replication, DNA repair, recombination, cell proliferation, cell death and gene transcription (Tong et al., 2001). The increased *Adprt1* mRNA levels may therefore reflect cell proliferation, aberrant gene transcription and ongoing DNA damage while reduced mRNA levels represent the replacement of normal splenic tissue with leukaemic cells. *Ephxl* mRNA was not induced in irradiated bone marrow but a transient increase in spleen *Ephxl* mRNA levels was observed in both strains ~3/4 days post irradiation.

Of the three putative candidate genes analysed in this study, two (*Hlx* and *Adprt1*) exhibit no obvious strain specific differences in tissue specific mRNA levels or
response to radiation exposure. The most promising candidate gene remains Ephx1 which exhibit a functional polymorphism, strain-specific differences in tissue specific Ephx1 mRNA levels and exhibits increased mRNA levels in r-AMLs but reduced levels in BCLs – all the necessary criteria for the r-AML susceptibility candidate gene. Although Ephx1 genetic polymorphisms are associated with several types of human cancer, it is in the context of xenobiotic substrate detoxification or bioactivation. Further analysis is necessary to establish or discount a potential role of Ephx1 in susceptibility to murine radiation induced AML.
6 Chapter 6: **Pax5 inactivation in radiation-induced leukaemias and lymphomas**

6.1 Introduction

Chromosome 4 LOH has been detected in >95% of informative L-ML (early Pre B Lympho-myeloid leukaemias), 53% of informative r-AML and 92% of informative thymic lymphomas that arose in X-irradiated CBA/H x (CBA/H x C57BL/6)F1 backcross mice. The preferential loss of the maternally transmitted CBA/H allele was observed in 88% of informative malignancies and suggests imprinting may be involved (Cleary et al., 2001; Boulton et al., 2002). A 3.4cM minimally deleted region (MDR) was mapped between D4Mit286 (14.5cM; 42076kb) and D4Mit214 (17.9cM; 44504kb) and this study aimed to identify putative tumour suppressor genes located within this MDR.

<table>
<thead>
<tr>
<th>Mouse (Chr 4) kb</th>
<th>Human (Chr 9) kb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fancg</strong> Fanconi Anaemia complementation group G</td>
<td>(41850) (35064)</td>
</tr>
<tr>
<td><strong>D4Mit286</strong></td>
<td></td>
</tr>
<tr>
<td><strong>CD72</strong> Immune response receptor</td>
<td>(42295) (35600)</td>
</tr>
<tr>
<td>Tesk1 Testes-specific protein kinase</td>
<td>(42280) (35595)</td>
</tr>
<tr>
<td>Car9 Carbonic anhydrase</td>
<td>(42350) (35571)</td>
</tr>
<tr>
<td>Tpm2 Tropomyosin</td>
<td>(35680)</td>
</tr>
<tr>
<td>Tln Talin</td>
<td>(42390) (35687)</td>
</tr>
<tr>
<td><strong>Creb3</strong> cAMP response</td>
<td>(42480) (35722)</td>
</tr>
<tr>
<td>Gba2 Glucosidase beta 2</td>
<td>(42418) (35727)</td>
</tr>
<tr>
<td>Serf2 Spinal muscular atrophy modifier</td>
<td>(42550)</td>
</tr>
<tr>
<td><strong>Reck</strong> Reversion-inducing cysteine rich protein</td>
<td>(42750) (36027)</td>
</tr>
<tr>
<td>Glipr2 GLI pathogenesis-related</td>
<td>(42812) (36127)</td>
</tr>
<tr>
<td>Cita Clathrin</td>
<td>(42868) (36181)</td>
</tr>
<tr>
<td>Uael UDP-N-acetylglucosamine-2-epimerase</td>
<td>(42925)</td>
</tr>
<tr>
<td>Melk Maternal embryonic leucine zipper kinase</td>
<td>(43175) (36563)</td>
</tr>
<tr>
<td><strong>Pax5</strong> Pax5</td>
<td>(44450) (36828)</td>
</tr>
<tr>
<td><strong>D4Mit214</strong></td>
<td>(44504)</td>
</tr>
<tr>
<td><strong>TgfβR1</strong> Transforming growth factor beta receptor 1</td>
<td>(46240) (98947)</td>
</tr>
</tbody>
</table>

This list of putative candidate genes was obtained from the chromosome 4 contig in 2001 (Mouse Chr 4) supported with information from the RH map of the D4mit286-D4Mit214 interval and the syntenic region on human chromosome 9 (Human Chr 9).
Numerous known (figure 6.1) and unknown genes (data not shown) are located within the MDR as well as a recessive maternally transmitted genetic locus (Lyr2) which confers a resistance to spontaneous and/or radiation induced murine pre-B and T cell lymphoma. This locus was mapped using SL/Kh mice which are genetically predisposed to spontaneous pre-B lymphomas and SL/Ni mice which are lymphoma-resistant. The incidence of acute pre-B lymphomas was reduced in F1 crosses with a maternal SL/Ni indicating that the maternal SL/Ni allele promoted lymphoma-resistance (reviewed Cleary et al., 2001). Also located within in this region are Testis-specific protein kinase 1 (Teskl) and Maternal embryonic leucine zipper kinase (Melk). Teskl mRNA is predominantly expressed in testicular germ cells and changes in expression levels appear during spermatogenesis. Melk is also expressed in specific patterns during mammalian embryogenesis with predominant expression during oocyte maturation (www.informatics.jax.org).

The presence of Lyr2, Melk and Teskl is evidence that the whole region may be imprinted (section 1.1.3.1) and is consistent with the preferential loss of the maternally transmitted CBA/H allele in the radiation induced leukaemias/lymphomas. Genes within and immediately outside this region were prioritised based on their potential role in haemopoiesis and/or leukaemogenesis. Genes were defined as low priority based on their function e.g. cytoskeleton organization (Tpm2, Tlnl, Serf2), enzyme/kinase activity (Car9, Clta) or on their tissue specific expression (Teskl, Melk). High priority genes are highlighted in figure 6.1 and included CD72 (regulatory role in B cell development), Reck (metastasis), Creb3 (DNA binding) and transforming growth factor receptor TgfβR1.

This study concentrated on two candidate genes – Fancg (Fanconi anaemia complementation group G) and Pax5. Although Fancg is located outside the MDR (figure 6.1), genetic maps were often not totally reliable and subject to frequent changes. Fancg is a good candidate as it has an important role in the cellular response to DNA damage and individuals with Fanconi Anaemia are predisposed to Acute Myeloid Leukaemia (section 1.1.2). Pax5 is located within the MDR but as it is essential for B cell differentiation (section 1.3.3), a role in r-AML is less obvious. However, the independent regulation of the two murine Pax5 alleles (Nutt et al.,
1999B) may be consistent with the maternal effects observed in murine haemopoietic malignancies.

6.2 Experimental Design

Tumour suppressor gene inactivation in cancer requires '2 hits' i.e. both alleles need to be inactivated (section 1.1.1). If one allele is lost by deletion (LOH) then the other allele can be inactivated by either another deletion, a mutation or silenced by gene promoter methylation. Northern and Southern blot analyses were used to screen for obvious genetic and epigenetic changes of Fancg and Pax5 genes. Southern blot analysis can detect homozygous deletions, gene rearrangements and DNA methylation while Northern blot analysis identifies changes in mRNA levels with reduced expression being associated with homozygous deletions or gene promoter methylation.

The experimental strategy use in this study was influenced by the observed preferential loss of the maternally transmitted CBA/H allele in the leukaemias and lymphomas. Three possible explanations for this preferential loss are (1) imprinting, (2) variant alleles or (3) chance due to mouse breeding protocol. Imprinting of the putative chromosome 4 TSG implies that inactivation in the leukaemogenic process only requires a single 'hit' i.e. loss of the maternal transcriptionally active CBA/H allele. Confirmation of transcription of the maternally transmitted allele requires the identification of a polymorphism (SNP) that can distinguish between the CBA/H and C57BL/6 mRNAs. An appropriate polymorphism could then be used in a controlled breeding program whereby the genetic background and gender of each parent is known thus enabling confirmation of maternal expression.

Losing the CBA/H allele may confer a greater selective advantage during leukaemogenesis. This scenario would require genetic differences between the CBA/H and C57BL/6 alleles – either a mouse strain specific DNA polymorphism that results in a protein coding variant, or sequence difference(s) in the promoter region that alters expression, or alternatively, differential expression of the putative gene in normal tissues between strains. However it is also possible that preferential loss of the maternal allele is coincidental.
6.3 Results

6.3.1 Gene sequencing and mRNA levels in inbred mice.

To determine whether differences in the protein coding region of\textit{ Fancg} or \textit{Pax5} genes accounted for the preferential loss of the CBA/H allele in leukaemias and lymphomas, cDNAs from both inbred mouse strain spleens were generated by RT-PCR and sequenced. 1136/1923 bases of \textit{Fancg} mRNA (NM\textunderscore 053081) (CBA/H colonies n=3; C57BL/6 colonies n=5) and 957/1208 bases of \textit{Pax5} mRNA (NM\textunderscore 008782) (CBA/H colonies n=3; C57BL/6 colonies n=4) were sequenced in both directions. In all cases the sequencing included the complete protein coding region. No mouse-strain specific polymorphisms were detected indicating that a protein coding sequence change was not responsible for the preferential loss of the CBA/H allele. Furthermore as no polymorphism was detected it was not possible to confirm the parental origin of the remaining allele in the malignancies or maternal expression.

To determine whether strain specific polymorphisms in the \textit{Pax5} gene promoter region could alter \textit{Pax5} gene expression, 505 bases of \textit{Pax5} gene promoter DNA were sequenced (figure 6.2; solid black arrows; -400 to +105 bp) in both inbred mouse strains. Three CBA/H colonies and 3 C57BL/6 colonies were sequenced in both directions and no polymorphism detected. As no polymorphisms were detected in either the \textit{Pax5} coding region or the promoter region, the loss of the transcriptionally active allele could still not be confirmed. Attempts to identify intronic polymorphisms were undertaken with the aim of using intronic SNPs to probe nuclear RNA (pre-splicing). 700 bases within intron 4 were analysed and no again polymorphisms were detected.

To establish whether inter strain differences existed in tissue mRNA levels between CBA/H and C57BL/6 mice, \textit{Fancg} and \textit{Pax5} mRNA levels were assessed in murine haemopoietic (spleen and bone marrow) and non-haemopoietic organs (kidney and liver) by Northern blot analysis (figure 6.3A). \textit{Fancg} mRNA was minimal in all tissues analysed (figure 6.3C) indicating that \textit{Fancg} mRNA is not present in these tissues. The highest \textit{Pax5} mRNA levels were detected in the spleen with both inbred mice strains exhibiting comparable levels (figure 6.3B).
As no coding sequence polymorphisms or differences in tissue mRNA levels were detected between the two inbred mice strains, preferential loss of the CBA/H allele identified in murine leukaemias is probably not due to 'genetic' differences between the two strains and therefore possibly due to chance.
Figure 6.2 – *Pax5* gene promoter sequence

```
TGCTACAAGGTCTCTTGCCCGAAGGCGAATGCTAATTTAGGGCTTGAAAGGTG

TCACAGATTGGTTCCCTGCTCGGAGGTCGCTACTATGCTTTCTGCTTGCCGGCTG

CAGAAGGAGGCTTTCATAGCTAGCTACGACCTTTCCGGTAAACTCTTGCCGCTG

AAAGCCAGACGGATAGGCTGAAATATCGAAATGGATTTAGAGAAAAATTACCCGACTC
CTCGGACCATCAGGACAGGTAGGAACACGCAGAATCTCGTACTCTCTTG

Kev
```

**Key**
- Primers: *Pax5* promoter probe (solid black arrows), C39F1/R1 (green arrows), C39F2/R2 (blue arrows), C39F3/R3 (pink arrows).
- Sequence: CpG island (blue), mRNA (yellow), *Hpall* sites (red, underlined).
- Broken arrow: Transcription initiation site (Busslinger *et al.*, 1996).
- Black, underline sequence: TATAAA box.
Figure 6.3 – Tissue specific mRNA levels

Figure 6.3A – Northern blot analysis of tissue specific Pax5 mRNA levels

Pax5 mRNA levels were measured in CBA/H and C57BL/6 mouse tissues. Lanes 1-4 contain RNA from CBA/H tissues (1 = spleen; 2 = bone marrow, 3 = kidney, 4 = liver) and lanes 5-8 RNA from C57BL/6 tissues (5 = spleen; 6 = bone marrow, 7 = kidney, 8 = liver). This blot demonstrates that Pax5 mRNA is restricted to the spleen and was repeated on one further occasion giving comparable results. Pax5 mRNA levels for spleen and bone marrow are consistent with the control levels observed in the radiation induced malignancies (figure 6.4). GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

Figure 6.3B – Histograms of the tissue specific Pax5 mRNA levels.

CBA/H and C57BL/6 Pax5 mRNA levels were compared relative to control CBA/H spleen. Comparable Pax5 mRNA levels were detected within tissues between strains. Standardisation utilised Ethidium Bromide staining of the gel prior to membrane transfer and hybridisation. mRNA levels were quantified using phosphor screen phosphoimager.

Figure 6.3C – Northern blot analysis of tissue specific Fancg mRNA levels.

Fancg mRNA levels were measured in CBA/H and C57BL/6 mouse tissues. Lanes 1-4 contain RNA from CBA/H tissues (1 = spleen; 2 = bone marrow, 3 = kidney, 4 = liver) and lanes 5-8 RNA from C57BL/6 tissues (5 = spleen; 6 = bone marrow, 7 = kidney, 8 = liver). Lanes 9 and 10 contain splenic r-AML RNA as positive controls. GAPDH = Glyceraldehyde-3-phosphate dehydrogenase. Comparable results were obtained from similar blots.
6.3.2 *Pax5* mRNA levels in Leukaemias and Thymic lymphomas.

The putative candidate TSG is predicted to be inactivated during the mouse leukaemogenic process. Northern blot analysis of murine leukaemias (spleen RNA) and thymic lymphomas (thymus RNA) was therefore employed to establish whether *Fancg* and *Pax5* mRNA levels were low (i.e. silenced). Although all blots were probed with housekeeping genes *Glyceraldehyde-3-phosphate dehydrogenase* and *Glutathione peroxidase*, ethidium bromide staining was used to compensate for loading due to anomalies discussed in chapter 4, section 4.4.2.1.

Figure 6.4A is representative of mRNA levels detected in B cell related leukaemias (BCL). All BCLs had previously been defined as B cell malignancies based on the presence of IgH immunoglobulin heavy chain gene rearrangements (analysed by E.Boulton and H.Cleary) and included pure B cell leukaemias and mixed lineage lympho-myeloid leukaemias (L-ML) as defined by increased MPO mRNA levels (eg figure 6.4A lanes 4,10,11,13,15,18). Lane 1 and 2 are control RNA from CBA/H spleen and bone marrow respectively. Lanes 3-20 contain splenic RNA from animals diagnosed with BCL. Compared to control spleen, increased *Fancg* mRNA levels were detected in 22/31 BCLs and comparable levels in 9/31. 27/43 BCLs exhibited reduced *Pax5* mRNA levels and 16/43 had levels comparable to control spleen. This suggests that *Pax5* mRNA was not expressed in 63% of BCLs, whereas *Fancg* mRNA was increased in 71% of BCLs.

Although low *Pax5* mRNA levels in BCL is evidence of the silencing predicted for an imprinted tumour suppressor gene, 37% of BCLs exhibited significant *Pax5* mRNA levels. In all animals analysed, the source of leukaemic tissue is the spleen. The *Pax5* mRNA levels detected by Northern blot may be due to *Pax5* expressing leukaemic cells or reflect the presence of normal B cells.

The *Pax5* gene encodes BSAP which regulates several B cell-specific genes including *CD19* (Nutt et al., 1997A; 1998). *Pax5* gene expression therefore co-exists with *CD19* gene expression and with one exception (figure 6.4A lane 18), *Pax5* mRNA and *CD19* mRNA levels are comparable within a sample. To determine whether a mutation in *Pax5* accounted for the non expression of *CD19* in the one exception, *Pax5* cDNA from this BCL was generated from RT-PCR and sequenced. No mutations were
detected indicating that the down regulation of CD19 mRNA was not a consequence of a Pax5 gene mutation and was probably caused by another CD19 gene regulation factor.

Figure 6.4B shows a similar Northern analysis of r-AML (lanes 3-20) compared to CBA/H spleen (lane 1) and CBA/H bone marrow (lane 2). Although the source of r-AML leukaemic tissue is spleen, AML is a bone marrow cell leukaemia and therefore mRNA levels were compared to control spleen and bone marrow. 15/18 r-AMLs exhibit significant increased Fancg mRNA levels compared to both control tissues. 35/36 r-AMLs demonstrate reduced Pax5 mRNA levels and 1/36 comparable levels to control spleen. 33/36 r-AMLs exhibited comparable and 3/36 increased Pax5 mRNA levels compared to control bone marrow. As Pax5 gene expression is required for B lymphopoiesis, Pax5 would not be expected to be expressed in a myeloid malignancy. The 8% of r-AMLs that exhibited increased Pax5 mRNA levels compared to normal bone marrow show comparable levels to spleen, and may therefore reflect low infiltration of myeloid cells into the spleen.

Evidence for normal B cell infiltration into haemopoietic malignant tissues is supplied by similar analyses of thymic lymphomas (TL). A diagnosis of TL is based on an enlarged thymus with further classification based on expression of T cell markers and the presence of T-cell receptor beta (TCRβ) and IgH gene rearrangements. The thymus is the site of T cell maturation. Pax5 blocks T cell development by repressing Notch1 (section 1.3.2.2) and therefore Pax5 is not present in the thymus (figure 6.4C lane 2) unless from leukaemic or infiltrating B cells. Immunogenotyping for both IgH and TCRβ gene rearrangements revealed 4 different disease groups - single lineage B (IgH^R, TCRβ^G), single lineage T (IgH^G, TCRβ^R) lymphomas, mixed lineage B + T (IgH^R, TCRβ^R) lymphomas and uninformative IgH^G, TCRβ^G lymphomas (Boulton et al., 2002). The TCRβ and IgH rearrangements defining the individual malignancies analysed in this study were analysed by E.Boulton and H.Cleary and are represented along the bottom of figure 6.4C.

14/18 TLs exhibit increased Fancg mRNA levels with 4/18 comparable to control thymus and 11/18 TLs demonstrated increased Pax5 mRNA levels. Single lineage B (TCRβ^G and IgH^R) and mixed lineage B+T (IgH^R, TCRβ^R) TLs indicate a B cell
malignancy and therefore might express Pax5 and CD19 (figure 6.4C, lanes 4, 8, 9 and 13). Figure 6.4C however, contains one example of a B + T TL which does not show Pax5 mRNA (lane 20). Leukaemic cells with a germline IgH<sub>G</sub> gene rearrangement would be predicted not to express Pax5 and CD19. Pax5 and CD19 mRNA levels are detected in several TLs (lanes 3, 5, 7, 11, 12, 17,18; figure 6.4C), none of which show IgH rearrangements. Non-clonal Pax5 expressing cells must be present in the TLs thus indicating normal B cell infiltration and raising the possibility that normal B cell infiltration also occurs in the spleen.

In summary, murine leukaemias and lymphomas exhibit increased Fancg mRNA levels. Increased Fancg mRNA levels have been associated with proliferating and differentiating tissues (van de Vrugt, 2002). It has been reported that the highest Fancg expression in bone marrow is within the CD34<sup>+</sup> haemopoietic stem cell compartment (>2 fold higher than in total or Lin<sup>-</sup> bone marrow) (Aubé et al., 2003). Pax5 mRNA levels are reduced in many but not all leukaemias and lymphomas, and evidence from thymic lymphomas suggests that Pax5 mRNA levels detected in leukaemia/lymphoma may be complicated by the presence of normal B cells in some cases.

Based on the hypothesis that the putative candidate gene was predicted to be inactivated during the mouse leukaemogenic process, Fancg was discounted and Pax5 retained as a potential tumour suppressor gene. However, subsequent evidence of haploinsufficiency in murine r-AML (Cook et al., 2004) and the effect of transcription factor concentrations on leukaemic stem cells (Rosenbauer et al., 2005) suggest that discounting Fancg based on mRNA levels may have been premature.
Figure 6.4 – Northern blot analysis of Radiation induced Malignancies.

Figure 6.4A – B cell related leukaemias (BCL)

Lane 1 (CBA/H control spleen), lane 2 (CBA/H control bone marrow) and lanes 3-20 (BCL leukaemic spleen RNA). The BCLs analysed later in this chapter are B1 (lane 19), B2 (lane 5), B3 (lane 14) and B4 (lane 20). BCLs were previously diagnosed based on the presence of IgH rearrangements (IgH<sup>B</sup>) and/or detection of VpreB1 mRNA. Many BCLs exhibited myeloid markers -MPO (figure 6.3A) and LysM. CD19 = B cell marker and Sca-1 = HSC/progenitor marker.

Figure 6.4B – Radiation induced Acute Myeloid Leukaemia (r-AML)

Lane 1 (CBA/H control spleen), lane 2 (CBA/H control bone marrow) and lanes 3-20 (r-AML leukaemic spleen RNA). The r-AMLs analysed later in this chapter are A2 (lane 3) and A1 (lane 15).

Figure 6.4C – Thymic Lymphomas (TL)

CBA/H control spleen and Thymus is located in lane 1 and 2 respectively. Lanes 3-20 contain Thymus RNA from animals diagnosed with TL. TCR and IgH gene rearrangements are shown. R indicates the presence of rearrangements while G represents a germline configuration.
6.3.3 *Pax5* gene rearrangements/homozygous deletions in leukaemias and thymic lymphomas.

To determine whether the low levels of *Pax5* mRNA detected in leukaemias and lymphomas were due to homozygous gene deletions or rearrangements, Southern blots generated from restriction enzyme digested leukaemic spleen genomic DNA were hybridised with *Fancg* and *Pax5* probes. *EcoR1* restriction digested Southern blot analysis of 60 BCLs and 18 r-AMLs hybridised with *Fancg* demonstrated no evidence of *Fancg* homozygous deletions or gene rearrangements (figure 6.5C). These blots were unsuitable for identifying homozygous deletions or gene rearrangements in *Pax5* because of the low incidence of *EcoR1* restriction sites in this gene and therefore *HindIII* and *PstI* restriction digested DNA was used. 4/60 B-cell malignancies showed potential 'partial homozygous deletions' (figure 6.5A and data not shown) and these were confirmed on two further occasions. However, these potential ‘deletions’ may represent incomplete restriction enzyme digestion and confirmation would require the sequencing of the exon 3 region in these leukaemias. No homozygous deletions were identified in the r-AMLs (n=18) hybridised with *Pax5* or *Pax5* gene promoter probes. 1/32 B cell malignancies probed with the *Pax5* gene promoter showed abnormal banding (figure 6.5B) supplying further evidence of gene aberrations or alternatively representing a polymorphism that creates an extra *HindIII* or *PstI* restriction site – again DNA sequencing is required for confirmation. However, if the genetic alterations are confirmed in these leukaemias, they are rare events and therefore not the main mechanism of gene silencing.
Figure 6.5 - *HindIII* and *PstI* digested genomic DNA Southern blot hybridised with (A) *Pax5* gene and (B) *Pax5* gene promoter.

*HindIII* and *PstI* digested genomic DNA (~15µg) resolved by 1%(w/v) agarose gel electrophoresis, blotted and probed with (A) *Pax5* gene and (B) *Pax5* gene promoter. Lanes 4-20 contain B cell leukaemic spleen DNA, lane 1 contains 1Kb ladder, and lane 3 contains control spleen (CBA/H). Figure 6.4B represents lane 9-15 of figure 6.4A probed with *Pax5* gene promoter cDNA. Arrows indicate examples of suspected partial homozygous deletions.

Figure 6.5C - *EcoR1* digested genomic DNA Southern blot hybridised with *Fancg*

*EcoR1* digested genomic DNA (~15µg) resolved by 1%(w/v) agarose gel electrophoresis, blotted and hybridised with the *Fancg* probe. Lanes 2-14 contain B cell leukaemic spleen DNA, lane 1 contains control spleen (CBA/H). No homozygous deletions were detected. Numbers down the right hand side represent fragment sizes in bp and indicate the corresponding exon.
6.3.3.1 *Fancg* and *Pax5* – are they still good candidates?

Initial results together with the location of *Fancg* outside the MDR, was taken as evidence that *Fancg* is not the TSG. In contrast, reduced *Pax5* mRNA levels in the BCLs in conjunction with the possible partial *Pax5* homozygous deletions identified by Southern blot analysis, maintains *Pax5* as a good candidate gene. The potential *Pax5* gene 'silencing' observed was rarely due to homozygous deletions and an alternative obvious mechanism was gene promoter methylation.

6.3.4 *Pax5* – Promoter methylation in normal tissue and haemopoietic malignancies.

The next phase of this study examined *Pax5* gene promoter methylation as a possible silencing mechanism. This strategy was chosen because; (1) this region of chromosome 4 is associated with the maternally transmitted genetic locus (*Lyr2*) and maternal embryonic leucine zipper kinase gene (*Melk*); (2) preferential loss of the maternally transmitted CBA/H allele is observed in radiation induced leukaemogenesis (*Cleary et al.*, 2001; *Boulton et al.*, 2002); and (3) *Pax5* alleles are independently regulated (*Nutt et al.*, 1999B). As it is possible that methylation in leukaemias is not *de novo*, control and malignant tissues were analysed.

6.3.5 Promoter Methylation

*Pax5* has two distinct promoters, which through alternative splicing, results in two BSAP proteins with distinct N termini (*Busslinger et al.*, 1996). Both *Pax5* mRNAs share common exons (2-10) but have a different exon 1. *Pax5* 1α is a TATA containing promoter located ~12.4 Kb upstream of exon 2 whereas *Pax5* 1β is TATA less, located ~5.7 Kb upstream of exon 2. This study concentrates on the *Pax5* 1α promoter (hereafter referred to as *Pax5* promoter) because it contains a CpG Island, drives *Pax5* expression in B lymphocytes and it is the *Pax5* 1α promoter which is silenced by methylation in Myeloma cells lines (*Danbara et al.*, 2001). It has been reported however that both *Pax5* gene promoters are methylated in human cancers (*Palmisano et al.*, 2003).
6.3.5.1 Restriction Digestion with methyl-sensitive \textit{HpaII}

The \textit{Pax5} gene promoter has 5 CCGG \textit{HpaII} restriction sites (figure 6.2 and 6.6A). To analyse methylation at these \textit{HpaII} sites, genomic DNA was first digested with \textit{EcoRI} as this generated a 2.187 Kb \textit{EcoRI} genomic restriction fragment that contains the \textit{Pax5} gene promoter (-1 to -798 bp), exon 1 (613 bp) and part of intron 1 (776 bp). Also present is a CpG island (39 CpGs) which is located between the promoter and exon 1 (-155 to +339 bp) and contains 3 of the 5 \textit{HpaII} sites (figure 6.2). \textit{HpaII} is a methylation sensitive enzyme which cuts the CCGG DNA sequence only if the internal cytosine is unmethylated. Figure 6.6A demonstrates the possible combinations of restriction fragments sizes that can be detected using the \textit{Pax5} gene promoter probe depending on the methylation status of each \textit{HpaII} site.
Figure 6.6 – Methyl-sensitive Southern blot of BCLs.

Figure 6.6A – EcoR1/HpaII digestion of the Pax5 gene promoter region.

A, B, C, D, and E are the 5 HpaII sites located within the EcoR1 generated boundary. Included are restriction fragment sizes that could be detected using the Pax5 gene promoter probe (chapter 2; table 2.4) depending on which HpaII sites (if any) are methylated.

Figure 6.6B – Methyl-sensitive Southern blot analysis of B cell leukaemias

Restriction digested genomic DNA (~15µg) was resolved by 1.8% (w/v) agarose gel electrophoresis, blotted and hybridised with the Pax5 gene promoter probe. Lane 1 contains 1KB DNA ladder enabling band sizing (sizes in base pairs down left hand column). Lane 3 and 4 contains CBA/H genomic spleen DNA which has been digested with EcoR1 only and HpaII only respectively. Lanes 5-20 represent BCL leukaemic spleen DNA digested with both EcoR1 and HpaII. Arrows indicate the presence of faint bands. Base pair sizes down the right hand column represent restriction fragments sizes. Comparing fragment sizes to figure 6.6A indicates the HpaII methylation patterns within a sample. Arrows indicate the presence of faint bands. Two BCLs analysed by bisulphite treatment later in this chapter are B1 (lane 10) and B3 (lane 16).
Figure 6.7 – Methyl-sensitive Southern blot of r-AMLs.

Figure 6.7A – EcoR1/HpaII digestion of the Pax5 gene promoter region.

A, B, C, D and E are the 5 HpaII sites located within the EcoR1 generated boundary. Included are restriction fragment sizes that could be detected using the Pax5 gene promoter probe (chapter 2; table 2.4) depending on which HpaII sites (if any) are methylated. This is a repeat of 6.6A.

Figure 6.7B – Methyl-sensitive Southern blot analysis of r-AMLs

Restriction digested genomic DNA (~15μg) was resolved by 1.8% (w/v) agarose gel electrophoresis, blotted and hybridised with the Pax5 gene promoter probe. Base pair sizes down the left hand column represent the 1 Kb ladder molecular weight marker. Lane 15 contains CBA/H genomic spleen DNA which has been digested with EcoR1 only. Lanes 1-14 represent r-AML leukaemic spleen DNA digested with both EcoR1 and HpaII. Base pair sizes down the right hand column represent restriction fragments sizes.
Southern blot analysis of EcoR1 and HpaII digested control CBA/H and C57BL/6 DNA (spleen, bone marrow and kidney) hybridised with the 505 bp Pax5 promoter genomic probe (-400 to +105 bp; figure 6.2 & 6.6A) consistently exhibited two bands (458 bp and 197 bp), representing restriction digestion at unmethylated HpaII sites A/B, C, D & E (figure 6.6A). A faint 660 bp band was detected in all control tissues (e.g. figure 6.6B, lane 4) indicating that a small sub-population of normal cells are methylated at HpaII sites C and D (figure 6.6A). As illustrated in figures 6.6B and 6.7B, EcoR1 and HpaII restriction digested southern blot analysis detected methylation of one or more of the HpaII sites in 20/47 (43%) of B cell leukaemias (example figure 6.6B), 30/36 (83%) of r-AML (example figure 6.7B) and 11/18 (61%) of thymic lymphomas (data not shown). These malignancies exhibited various methylation patterns. Restriction fragments of different sizes were identifiable in both BCLs (figure 6.6B, e.g. lane 15 and 16) and r-AMLs (figure 6.7B, e.g. lanes 5 and 6). Often multiple sized restriction fragments are present within a sample, and although this was prevalent in r-AMLs (figure 6.7B) and thymic lymphomas (data not shown) this was less common in BCLs (figure 6.6B). The extent of HpaII methylation also varies within a sample as demonstrated by equal intensity bands (figure 6.7B, lane 10, top 2 bands) and unequal intensity restriction fragments (figure 6.6B, lane 11, top 2 bands).

The presence of multiple 'partially' digested HpaII fragments and fully digested 458 bp and 197 bp fragments within a sample suggests that either restriction enzyme digestion is incomplete or that methylation is subclonal within clonal leukaemias/lymphomas. All Southern blots were hybridised with a Mitochondrial DNA (mtDNA) probe as designed by Walsh & Bestor (1999). All CCGG sites in Mitochondrial DNA are unmethylated and therefore are cut by HpaII restriction enzyme digestion. As illustrated in figure 6.8, the mtDNA probe will detect a >6Kb restriction fragment following digestion with EcoR1 alone, a 1737 bp restriction fragment following digestion with HpaII alone and a 1617 bp restriction following complete restriction digestion of DNA with both restriction enzymes. Most leukaemic blots exhibited complete digestion suggesting that leukaemic methylation is subclonal.
Figure 6.8—Methyl-sensitive Southern blot of BCLs hybridised with a mitochondrial DNA probe.

A) Restriction digestion fragment sizes that could be detected using the MtDNA probe (chapter 2; table 2.4). Mitochondrial DNA is unmethylated and therefore complete digestion results in: 1737 bp fragment (HpaII alone), >6Kb (EcoRl alone) and 1617bp (both EcoRl and HpaII digest).

B) Representative of an EcoRl and HpaII digested southern blot (previously hybridised with the Pax5 gene promoter probe), hybridised with mitochondrial DNA (mtDNA). Lane 1 contains 1KB ladder enabling sizing. Lanes 3-20 represent EcoRl and HpaII digested BCL spleen DNA. Restriction fragment sizes detected by complete digestion were compared against the 3 fragment sizes presented above. Lane 3, 13 and 15 all have the 1737 bp (black arrows) and 1617 bp restriction fragments present, suggesting that while complete digestion with HpaII occurred, there is incomplete digestion of EcoRl.
In summary, *Pax5* gene promoter methylation maybe responsible for gene silencing in r-AMLs, BCLs and TLs. *Pax5* gene promoter methylation is dynamic with different sized restriction fragments within a sample indicating methylation is subclonal. Ongoing subclonal methylation was detected in clonal malignant tissue suggesting that subclonal methylation may confer a selective advantage during leukaemogenesis *in vivo*. Methylation was detected in both haemopoietic control tissues (spleen and bone marrow) and non haemopoietic control tissue (kidney) suggesting that *Pax5* gene promoter methylation is not specific to haemopoietic differentiation. Analysis of the methylation status of individual genomes requires a more sensitive technique than Southern blot analysis and this study used bisulphite treatment, cloning of the PCR product and DNA sequencing to sample individual genomes.

### 6.3.5.2 Bisulphite Sequencing

Although restriction enzyme digestion followed by Southern blot analysis enables a large number of samples to be screened, it is a relatively insensitive method that tends to underestimate methylation because analysis is restricted to CpG dinucleotides located within the CCGG *HpaII* restriction site. Bisulphite DNA sequencing enables the precise analysis of all CpG dinucleotides and because the method used in this study is PCR based, small quantities of DNA extracted from Lin` bone marrow cells were also analysed. Lin` bone marrow cells are enriched for haemopoietic stem and progenitor cells and consequently are more representative of the r-AML target stem cell (chapter 3). Complete bisulphite treatment was confirmed using the conversion of cytosines that are not CpG dinucleotides to thymines. In all cases 99-100% of non CpG dinucleotide cytosines were converted to thymines (data not shown) confirming that the bisulphite treatment had worked.
Three regions were analysed by bisulphite sequencing - Region 1 (222 bp), Region 2 (222 bp) and Region 3 (185 bp). Both region 2 and 3 are located within the CpG island with region 2 crossing the transcription initiating site. A, B, C, D and E represent the 5 HpaII sites analysed by methyl-sensitive restriction southern blot analysis. The location of these HpaII sites relative to the start codon are A (-554 bp), B (-531 bp), C (-72 bp), D (-68 bp) and E (+129 bp).

Figure 6.9 represents a schematic diagram of the region of the Pax5 gene promoter analysed in this study. The Pax5 gene promoter region was divided into 3 areas chosen to cover the HpaII sites analysed by Southern blot.

- Region 1 (222 bp; 5 CpG dinucleotides; 2 HpaII sites).
- Region 2 (222 bp; 15 CpG dinucleotides; 2 HpaII sites).
- Region 3 (185 bp; 15 CpG dinucleotides; 1 HpaII site).

Region 1 is located 385 bp upstream from the transcription initiation site and Regions 2 and 3 are located within the CpG island (figures 6.2 and 6.9).

6.3.5.2.1 Control samples

For each inbred mouse strain, spleen, bone marrow and the immature progenitor/HSC Lin' cells (chapter 3) were analysed. Data was evaluated using two approaches - the number of individual CpG dinucleotides methylated within an individual colony, and the total number of methylated CpG dinucleotides as a percentage of all CpGs sequenced. Table 6.1 summarises this data and illustrates that Pax5 gene promoter methylation was detectable in all control tissues from both inbred CBA/H and
C57BL/6 mice. The colony methylation patterns detected within a region were highly heterogeneous. This enabled the sampled colonies to be divided into two groups: (1) colonies containing little or no methylation; and (2) colonies that are heavily methylated.

As illustrated in table 6.1, only a small proportion of control colonies are heavily methylated. The data infers that Lin' bone marrow cells (both strains) and CBA/H bone marrow cells exhibit more heavily methylated colonies than spleen (both strains) and C57BL/6 bone marrow cells. The presence of promoter methylation in control samples suggests that at least one or maybe both Pax5 alleles are normally silenced in some cells. If one of these cells is the target cell in leukaemogenesis, then de novo methylation is not necessary during the leukaemogenic process.
Table 6.1 – Methylation status of \textit{Pax5} gene promoter regions 1, 2 and 3 in control mouse tissues.

<table>
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<tr>
<th>Region 1</th>
<th>Spleen Colonies</th>
<th>Bone Marrow Colonies</th>
<th>Lin' Colonies</th>
</tr>
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<td>Me CpG/Colony</td>
<td>CBA/H</td>
<td>C57BL/6</td>
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<td>C57BL/6</td>
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<td>14</td>
</tr>
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</tr>
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<td>2</td>
<td>2</td>
<td>0</td>
</tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
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<tr>
<td></td>
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<td>Total for tissue</td>
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<th>Lin' Colonies</th>
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<td>C57BL/6</td>
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<th>Bone Marrow Colonies</th>
<th>Lin' Compartment Colonies</th>
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</thead>
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<td>Me CpG/Colony</td>
<td>CBA/H</td>
<td>C57BL/6</td>
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<td></td>
<td></td>
<td>CBA/H</td>
<td>C57BL/6</td>
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<td>7</td>
<td>11</td>
<td>16</td>
</tr>
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<td>2</td>
<td>1</td>
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</tr>
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<td>3-8</td>
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</tr>
<tr>
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<td>Total % Me CpG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(2/89)</td>
<td>(1/122)</td>
</tr>
<tr>
<td>Total for tissue</td>
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<td>1.4%</td>
<td>15%</td>
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Table 6.1 summarises the control bisulphite sequence data. Each table represents one of three \textit{Pax5} gene promoter regions analysed and illustrated in figure 6.9. The top part of each table concentrates on the number of methylated CpG dinucleotides in each colony. For example in spleen CBA/H region 1, 16 colonies exhibited no methylation; 4 colonies exhibited 1 methylated CpG; 2 colonies exhibited 2 methylated CpGs, 2 colonies exhibited 3 methylated CpGs and no colonies exhibited 4 methylated CpGs. The bottom part of each table considers the number of methylated CpG dinucleotides as a percentage of the total number of CpG dinucleotides sequenced. The final percentage pools both the CBA/H and C57BL/6 mouse data for each tissue.
6.3.5.2.2 Leukaemias

Two r-AMLs (A1 and A2) and 4 BCLs (B1-B4) were chosen for analysis based on Pax5 mRNA levels detected by Northern blot, and Pax5 gene promoter methylation status assessed by methyl-sensitive Southern blot. Table 6.2 illustrates the different methylation patterns observed in individual sampled colonies across the three regions in the 6 leukaemias. Both r-AMLs (A1 and A2) demonstrate the same methylation pattern. No methylation was detected across regions 1 and 2 while interestingly, the pattern of methylation detected across region 3 was identical in both leukaemias with the same CpG dinucleotide being unmethylated (table 6.2, region 3) thus suggesting that methylation is clonal although the number of colonies sampled is low. The significance of the unmethylated CpG (+76bp) is unclear although it is possible that it has been protected from methylation by the binding of a transcription factor. BCL B1 also showed a distinct pattern of methylation in region 3 (table 6.2) with one CpG dinucleotide consistently remaining unmethylated (+117 bp). This CpG dinucleotide is different from the unmethylated CpG dinucleotide identified in the r-AMLs.

As illustrated in table 6.2, considerable heterogeneity was observed between the BCLs. B1 and B3 exhibited methylation across all three regions while methylation was restricted in B2 (present only in region 3) and B4 (present in regions 2 and 3). Methylation pattern heterogeneity supplies supportive evidence for subclonal methylation of different HpaII sites across the Pax5 gene promoter detected by Southern blot analysis. Methylation analysis in collaboration with LOH studies infer that subclonal methylation can exist within clonal malignancies and accounts for the unmethylated 458bp and 197bp restriction fragments that are nearly always detected by Southern blot (figures 6.6B;6.7B).

Detection of LOH in murine leukaemias/lymphomas requires; (1) a clonal malignancy and (2) <20% contaminating normal cells. To estimate the proportion of methylated colonies, sampled colonies were divided into either lightly (L- Me CpG; <50% CpG dinucleotides methylated) or heavily methylated (H- Me CpG; >50% CpG dinucleotides methylated). Table 6.3 summarises the heavily methylated data presented in table 6.2 and includes the control tissue data for comparison. Compared to controls, heavy methylation was restricted to B1 in regions 1 and 2 but was detected in all the leukaemias in region 3. Evidence of bias in the bisulphite sequencing technique, and
the small number of colonies sampled, suggests that detailed statistical analyses are potentially suspect and consequently unwarranted. For example, in the control tissues there are marginally statistical differences between inbred strains, but a much larger significant difference between tissues. The $p$ values make no contribution to the interpretation of the data which is based on the biology of the system. Nevertheless, a very crude statistical analysis was used to illustrate differences between the pooled control (to increase sample size) and leukaemic data. The $\chi^2$ test of independence was used to compare the pooled control $H^{-}\text{MeCpG}/L^{-}\text{MeCpG}$ ratio to the $H^{-}\text{MeCpG}/L^{-}\text{MeCpG}$ ratio in the individual leukaemias. Statistically significant differences ($p=0.05$) were obtained for

1. A2 – region 3 ($p = 0.0002$).
2. B1 – region 1 ($p=0.004$), region 2 ($p=0.0003$) and region 3 ($p=0.0006$).
Table 6.2 – *Pax5* promoter methylation patterns in radiation induced malignancies.

<table>
<thead>
<tr>
<th>Region 1</th>
<th>r-AML</th>
<th>B-related Leukaemia</th>
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<td>Number of colonies</td>
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**Position of *HpaII* sites A & B**

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**Position of *HpaII* sites C & D**

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</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
</tr>
<tr>
<td>Number of colonies</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫⚫</td>
<td>0</td>
<td>0</td>
</tr>
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<tr>
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<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

**Position of *HpaII* site E**

In all cases, the presence of an open dot (⚪) represents an unmethylated CpG site while the presence of a closed dot (⚫) represents a methylated CpG sites. Sites where sequencing was unsuccessful are represented by a dot with a line through it (Φ). All leukaemic data analysed is from spleen tissue.

Again all data has been split into the 3 regions (figure 6.9). Each table shows the number of colonies analysed per leukaemia and the pattern of methylation in each colonies. * represents the position of the *HpaII* sites.
Table 6.3 – Heavily Methylated (H- M<sub>e</sub>CpG) colonies.

<table>
<thead>
<tr>
<th>Heavily methylated colonies (% number of colonies sampled)</th>
<th>Region 1 (2-4 M&lt;sub&gt;e&lt;/sub&gt;CpG)</th>
<th>Region 2 (&gt;4 M&lt;sub&gt;e&lt;/sub&gt;CpG)</th>
<th>Region 3 (&gt;9 M&lt;sub&gt;e&lt;/sub&gt;CpG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r-AML A1 (Pax5&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>0% (n=8)</td>
<td>0% (n=13)</td>
<td>25% (n=12)</td>
</tr>
<tr>
<td>A2 (Pax5&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>0% (n=8)</td>
<td>0% (n=11)</td>
<td>60% (n=10)</td>
</tr>
<tr>
<td>BCL B1 (Pax5&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>45% (n=22)</td>
<td>29% (n=14)</td>
<td>44% (n=16)</td>
</tr>
<tr>
<td>B2 (Pax5&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>0% (n=10)</td>
<td>0% (n=14)</td>
<td>75% (n=12)</td>
</tr>
<tr>
<td>B3 (Pax5&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>9% (n=11)</td>
<td>8% (n=12)</td>
<td>18% (n=11)</td>
</tr>
<tr>
<td>B4 (Pax5&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>0% (n=12)</td>
<td>0% (n=12)</td>
<td>25% (n=12)</td>
</tr>
<tr>
<td>Total Bone Marrow</td>
<td>0% (n=43)</td>
<td>0% (n=38)</td>
<td>12% (n=51)</td>
</tr>
<tr>
<td>Lin&lt;sup&gt;−&lt;/sup&gt; Bone Marrow</td>
<td>60% (n=42)</td>
<td>0% (n=42)</td>
<td>5% (n=39)</td>
</tr>
<tr>
<td>Pooled tissues</td>
<td>23% (n=135)</td>
<td>0% (n=95)</td>
<td>7% (n=111)</td>
</tr>
</tbody>
</table>

The heavily methylated colonies are considered as a percentage of the total number of colonies sampled. Neither r-AML (A1 and A2) nor two of the BCLs (B1 and B2) express Pax5 as represented by Pax5<sup>−</sup>. The two remaining BCLs (B3 and B4) express Pax5 (Pax5<sup>+</sup>). The control data is from table 6.1. 'n' = total number of colonies sequenced.

6.3.6 Southern blot analysis vs Bisulphite Sequencing.

Leukaemias analysed for Pax5 gene promoter methylation were initially chosen based on presence or absence of Pax5 mRNA by Northern blot analysis. Numerous analyses had previously been undertaken on these malignancies including LOH 2 and 4 studies (Cleary et al., 1999; 2001; Boulton et al., 2002). LOH studies are informative for mice that either exhibit LOH or are heterozygous (HET) but not for those who inherited homozygous (HOM) alleles. Furthermore, LOH require <20% contaminating cells. Detailed analyses of the 6 leukaemias analysed by bisulphite sequencing are:
6.3.6.1  **BCL B1: Pax5, LOH4**

Three restriction fragments were detected by Southern blot analysis of EcoRI and HpaII digested B1 genomic DNA – 2187bp, 458bp and 197bp (figure 6.6B lane 10). The 2187bp restriction fragment represents full methylation of all 5 HpaII sites while the 458bp and 197bp restriction fragments are the results of all 5 HpaII sites being unmethylated. The presence of unmethylated HpaII sites may represent normal B cells (DNA was extracted from a complex tissue and not a leukaemic cell line) or may reflect the fluid subclonal Pax5 gene promoter methylation. Bisulphite sequencing data revealed that methylation was present across all three regions (each of which included at least 1 HpaII site) with percentages of colonies methylated ranging from 29-44%. Methylation across all three regions is consistent with full methylation of the Pax5 gene promoter as detected by Southern blot analysis but not consistent with the detection of LOH which requires >80% clonality. B1 exhibits LOH4 and no Pax5 mRNA. This suggests that one allele has been lost by deletion, with ongoing subclonal promoter methylation accounting for the silencing of the second allele in most cells but not all leukaemic cells thereby suggesting a selective advantage.

6.3.6.2  **BCL B2: Pax5, HET4**

Bisulphite sequencing revealed that Pax5 gene promoter methylation was present but exclusive to region 3. Unfortunately, insufficient leukaemic spleen DNA was available for methyl-sensitive Southern blot analysis although based on bisulphite sequencing, two restriction fragments would be expected, representing 75% methylation in region 3 (1457bp) and 0% methylation in regions 1 and 2 (458bp). Pax5 mRNA and LOH4 were not detected in this leukaemia. Data suggest that Pax5 alleles must be silenced to prevent Pax5 expression, possibly by bi-allelic methylation. Alternatively, small deletions may not have been detected in the LOH studies or deletions are masked by the presence of >20% normal cells.

6.3.6.3  **BCL B3: Pax5+, LOH4**

Four restriction fragments were detected by Southern blot analysis of EcoRI and HpaII digested B3 genomic DNA – a weak partial HpaII fragment (1457bp), a strong partial HpaII fragment (725bp) and two fully digested unmethylated fragments (458bp and 197bp) (figure 6.6B lane 16). The intensity of the 725bp and 458bp fragments are equal. The strong 725bp fragment corresponds with methylation of sites A/B and the
458bp fragment corresponds with unmethylated sites A/B and C/D. The weak 1457bp band reflects methylation of site E while the weak 197bp band indicates site E is unmethylated. Methylation of the \textit{HpaII} sites is heterogeneous and this is qualitatively but not quantitatively supported by bisulphite sequencing. A low frequency of methylation across all \textit{HpaII} sites was observed by bisulphite sequencing and, compared to the intensities of the southern bands (725bp and 458bp) this suggests that bisulphite sequencing has underestimated methylation in region 1 and possible bias in region 3. This leukaemia exhibited LOH4 (i.e. the spleen contained >80% leukaemic cells) and \textit{Pax5} mRNA was detected by Northern blot. \textit{Pax5} mRNA may reflect the presence a small number of normal B cells or indicate that not all patterns of methylation are sufficient to switch off the \textit{Pax5} gene.

\textbf{6.3.6.4 BCL B4: Pax5$^+$, HET4}

Southern blot analysis of \textit{EcoRI} and \textit{HpaII} digested B4 genomic DNA showed no evidence of \textit{HpaII} site methylation (data not shown) and this is consistent with the expression of \textit{Pax5} mRNA in this leukaemia. Low levels of heavily methylated colonies (25\%) were identified by bisulphite sequencing in region 3. This level of methylation is not statistically significantly different from the control tissues and may be a consequence of colony sampling.

\textbf{6.3.6.5 r-AML A1: Pax5$^+$, HET4, HOM2}

Three restriction fragments were detected by Southern blot analysis of \textit{EcoRI} and \textit{HpaII} digested A1 genomic DNA – a strong partial \textit{HpaII} fragment (1457bp), and two weaker partial \textit{HpaII} fragments (725bp and 927bp) (data not show). These fragments are strong evidence of heterogeneous and sub-clonal methylation of \textit{HpaII} sites as fragments 1457 and 725bp are consistent with methylated sites A/B and E and unmethylated C/D sites, while fragment 927bp is consistent with methylated A/B, C/D and unmethylated E sites. The bisulphite sequencing data again only partially supports the Southern data as methylation is only observed in region 3 which contains \textit{HpaII} site E. \textit{Pax5} mRNA was not detected in this leukaemia and A1 exhibits heterozygous 4. This raises the possibility that either both alleles are silenced by bi-allelic methylation, small deletions were not detected or >20\% normal cells are present.
6.3.6.6 r-AML A2: \textit{Pax5}, HOM4, LOH2

\textit{EcoR}1 and \textit{Hpa}II digested Southern blot analysis of A2 genomic DNA revealed three restriction fragment bands – a strong 1457bp partial \textit{Hpa}II restriction fragment in addition to fully digested 458bp and 197bp restriction fragments. Fragment 1457bp represents methylation only at site E and this is confirmed by the exclusive heavy (60\%) methylation revealed by bisulphite sequencing at region 3 (table 6.3). Although A2 is homozygous on chromosome 4 and consequently uninformative as to whether one allele is lost by deletion, it exhibits LOH2 indicating a clonal malignancy (>80\% leukaemic cells). The mechanisms for silencing \textit{Pax5} in this leukaemia include deletion and promoter methylation, or bi-allelic promoter methylation.

Five of the six chosen leukaemias were analysed by both methyl-sensitive Southern blot analysis and bisulphite sequencing and in 3/5 cases (B1, B4 and A2), Southern blot analysis is supported by the bisulphite sequencing data. Methyl-sensitive Southern blot analysis is the easiest, less labour intensive and apparently most robust method for assessing the \textit{Pax5} gene promoter region giving a genome average and covering the whole promoter region. As this study suggests that \textit{Pax5} gene promoter methylation in the leukaemias is subclonal and fluid, Southern blot analysis is probably more efficient in detecting methylation although information is restricted to the \textit{Hpa}II sites.

Bisulphite sequencing was not supportive of Southern blot analysis in 2/5 cases. Bisulphite sequencing gives a detailed ‘snapshot’ of one small portion of the \textit{Pax5} gene promoter per genome but can not supply any information concerning the methylation status of the other regions. Data in this study is strongly affected by the relatively small number of genomes sampled and potential bias of PCR amplification of bisulphite treated DNA and is therefore only semi-quantitative. These limitations may account for the underestimation of \textit{Pax5} gene promoter methylation by bisulphite sequencing compared to Southern blot analysis in B3 and A1.

6.3.7 DNA Methyltransferase mRNA levels

To determine whether aberrant \textit{Dnmt} expression coincides with aberrant \textit{Pax5} gene promoter methylation, Northern blots of splenic RNA extracted from mice diagnosed
with BCL or r-AML were hybridised with cDNA probes from three DNA methyltransferases – *Dnmt1, Dnmt3a* and *Dnmt3b* (figure 6.10). 94% (17/18) of the leukaemias exhibited increased *Dnmt1* mRNA levels and 39% (7/18) of BCL and 67% (12/18) of r-AMLs increased *Dnmt3b* mRNA levels (figure 6.10 and data not shown). Both malignancies were analysed for *Dnmt3a* mRNA and none was detected. Increased *Dnmt3a* mRNA levels (4.4 fold control values) have been reported in *de novo* AML (Mizuno et al., 2001) and our failure to detect *Dnmt3a* mRNA may simply represent the insensitive technique of northern blot analysis compared to competitive PCR.

Mizuno et al (2001) also reported a direct correlation between increased *Dnmt1* and *Dnmt3b* mRNA levels and methylation of the p15*INK4B* gene promoter, suggesting a potential role of DNA methyltransferases in aberrant regional hypermethylation. Increased *Dnmt* mRNA levels may however simply reflect the proliferative status of leukaemic cells (Kimura et al., 2003). As Northern blots in this study were not hybridised with a proliferation marker, this could not be confirmed or discounted but suggests that the *Dnmt* mRNA levels obtained in this study should not be overinterpreted.

**Figure 6.10 – mRNA levels of DNA Methyltransferases in BCLs**

![Figure 6.10](image)

Figure 6.10 is representative of a Northern blot containing total cellular RNA (20μg) prepared from spleen (lane 1), bone marrow (lane 2) and BCLs (lane 3-20) probed with *Dnmt1* and *Dnmt3b*. Included are those BCLs assessed by bisulfite treatment (lane 5 = B2; lane 14 = B3; lane 19 = B1; lane 20 = B4) all of which demonstrated increased *Dnmt1* and *Dnmt3B* mRNA levels relative to ethidium bromide (EtBr) staining.

6.3.8 Conclusions

*Pax5* mRNA levels are reduced in 63% BCLs and 92% r-AMLs (*Pax5* negative) compared to control tissue. *Pax5* gene promoter methylation is detected in 43% BCL and 83% r-AMLs by *EcoR1* and *HpaII* restriction digested DNA Southern blot analysis and evidence of subclonal methylation in clonal malignancies is strongly
supported by the r-AML data (figure 6.7B). Methylation of individual CpG
dinucleotides appears to be random suggesting that Pax5 is silenced by various
combinations of methylated promoter/exon 1 CpG dinucleotides, and that selective
pressure ensures sufficient CpGs are methylated in each leukaemic cell genome to
silence the Pax5 gene.

Closer examination of 6 leukaemias by bisulphite sequencing and comparison of
leukaemic data to control data (Pax5 gene promoter methylation is also detected in
normal tissue) demonstrated the methylation of colonies from Pax5 negative BCLs
(B1 and B2) were significantly different to that of the control colonies. Although low
levels of Pax5 gene promoter methylation was detected in one of the Pax5 positive
BCLs (B4), it is comparable to the control levels and consistent with the absence of
HpaII site methylation in Southern blots and the significant levels of Pax5 mRNA
detected by Northern analysis. The presence of Pax5 gene promoter methylation in the
other Pax5 positive BCL (B3) is unclear and highlights the limitations of the two
chosen techniques. The detection of Pax5 mRNA by Northern blot in conjunction
with Pax5 gene promoter methylation in B3 could reflect the presence of normal B
cells or indicate that in this leukaemia insufficient CpGs are methylated to prevent
Pax5 expression. Alternatively it is possible that as Pax5 mRNA was detected in 37%
of BCLs and 57% BCLs did not demonstrate Pax5 promoter methylation by Southern
blot analysis, that Pax5 does not have a role in all BCLs.

6.4 Discussion

Chromosome 4 LOH observed in mouse leukaemias and lymphomas implicates
tumour suppressor gene (TSG) inactivation in maturation arrest and malignant
progression. Two putative candidate TSGs assessed in this study were Fancg which
was subsequently (and possibly prematurely) excluded, and Pax5. Reduced expression
of Pax5 as indicated by Northern blot analysis implicated homozygous deletions or
gene promoter methylation as mechanisms for silencing the gene. Possible
homozygous deletions were detected but occurred rarely and require confirmation.
Strong evidence of Pax5 gene promoter methylation was indicated by Southern blot
and/or Bisulphite sequencing. Pax5 gene promoter methylation had previously been
detected in murine Myeloma cell lines (Danbara et al., 2001) and some human Myeloma cell lines do not express Pax5 mRNA (Mahmoud et al., 1996).

Surprisingly, low levels of Pax5 gene promoter methylation were detected in control tissues (spleen, bone marrow, HSC/early progenitor Lin- fraction and Kidney). During B-lymphopoiesis, the two Pax5 alleles are independently regulated. This allele specific regulation is stochastic, reversible and independent of parental origin. Mono-allelic expression predominates in early B-lymphoid progenitors and mature B cells while transient bi-allelic transcription predominates in pre-B and immature B cells (Nutt et al., 1999B). One possible mechanism for this independent regulation of the alleles is promoter methylation and this potentially could account for the methylation seen in our control genomes. The detection of only low levels of Pax5 gene promoter methylation in control tissues, however, indicates that methylation is not employed to silence Pax5 gene transcription during non-B cell terminal differentiation in vivo. A previous study (assessing across our region 2) demonstrated that independent regulation of the Pax5 alleles does not involve Pax5 gene promoter methylation (Danbara et al., 2001).

Studies of breast cancer cell lines have demonstrated that normal breast cells not only have methylated CpG islands but that this pre-existing methylation promotes de novo methylation (Huang et al., 1999). It is proposed that methylation ‘spreads’ from within the CpG island from individual methylated CpGs and potentially this may produce the ‘partial’ methylation detected by Southern blot analysis.

Although not evident with Pax5, DNA methylation of some genes is important during haemopoietic differentiation. The role of epigenetic mechanisms in haemopoiesis is illustrated by the epigenetic silencing of c-fms during B lymphopoiesis. c-fms is up-regulated during macrophage differentiation and silencing of c-fms mRNA in B lymphocytes is dependent on the presence of Pax5. Throughout B lymphopoiesis, c-fms chromatin is active but silent because transcription factors can not stably bind to the c-fms promoter or intron regulatory element (FIRE) (Tagoh et al., 2004). As c-fms is not completely assembled into silent chromatin in mature B cells, c-fms reactivation can occur under certain conditions i.e. loss of Pax5 or over expression of C/EBP (Mikkola et al., 2002; Xie et al., 2004). Although c-fms silencing uses chromatin
configuration and transcription factor binding, DNA methylation is present. The \textit{c-fms} promoter and FIRE are unmethylated in macrophages and B cells, but methylated in T cells and fibroblasts. There is also a second conserved regulatory region between the promoter and FIRE which is methylated in B and T cells (Tagoh \textit{et al.}, 2004).

Immunogenotyping for IgH and TCR\textsubscript{\beta} rearrangements and analysis of lineage-specific/restricted markers identified mixed lineage leukaemias and lymphomas in irradiated CBA/H x (CBA/H x C57BL/6)F\textsubscript{1} backcross mice (Cleary \textit{et al.}, 2001; Boulton \textit{et al.}, 2002). Mixed lineage malignancies suggest the presence of a weak differentiation block and the ability of haemopoietic cells to differentiate down alternative lineage pathways. \textit{Pax5 (-/-)} pro B cells have extensive self-renewal properties and the capacity to oscillate down different lineages (section 1.3.3.2). Unlike HSCs, \textit{Pax5 (-/-)} pro B cells can not reconstitute the haemopoietic system for life because they differentiate into other lineages with different efficiencies (Schaniel \textit{et al.}, 2002) – differentiating along T lymphopoiesis more effectively than myelopoiesis (reviewed Heavey \textit{et al.}, 2003). The B lineage commitment function of \textit{Pax5 (+/+)} B cells is dominant over many differentiation genes including \textit{C/EBP\alpha} (granulocytic development), \textit{GATA3} (early T cell development), \textit{Notch1} (T cell development) and \textit{c-fms} (macrophage development) (Heavey \textit{et al.}, 2003; Souabni \textit{et al.}, 2002; Tagoh \textit{et al.}, 2004). Hypothetically, the manipulation of \textit{Pax5 (-/-)} pro B cells into other cell lineages following exposure to specific transcription factors or environmental conditions could generate the mixed lineage leukaemias and lymphomas identified in the irradiated backcross mice.

Although \textit{Pax5} is a strong candidate for B-related malignancies, its role in r-AML is less obvious. r-AMLs are strongly associated with chromosome 2 deletions (section 1.2.4.1) and consequently chromosome 4 allelic loss may represent a secondary event that confers a selective advantage for malignant development. >92\% of the r-AMLs in this study exhibited no \textit{Pax5} mRNA levels as predicted for a myeloid malignancy. It is possible that \textit{Pax5} inactivation creates a selective advantage by promoting the myeloid lineage, or that \textit{Pax5} inactivation is independent of r-AML development and that there is another as yet unidentified gene located within the MDR conferring a selective advantage in r-AML.
Further characterisation of our leukaemic mice have identified the \textit{PU.1} mutation described by Cook \textit{et al.}, (2004) (M.Plumb personal communication) in the same r-AMLs that show \textit{Pax5} gene promoter methylation. This is evidence of a double differentiation block in the r-AMLs – the \textit{PU.1} mutation/LOH2 blocks the myeloid route while \textit{Pax5} promoter methylation and/or LOH4 blocks the B lymphoid route. If \textit{Pax5} (-/-) pro B cells are more efficient at differentiating down the T cell route rather than the myeloid route (reviewed Heavey \textit{et al.}, 2003) then it is not unreasonable to predict a T cell differentiation block in the r-AML.

One obvious candidate is \textit{Notch1} which has an important function in the T versus B lineage decision (Radtke \textit{et al.}, 2004; section 1.3.2.2). \textit{Pax5} represses \textit{Notch1} and therefore inactivation of \textit{Pax5} can lead to constitutive \textit{Notch1} signalling which is associated with T cell leukaemias/lymphomas (Zweidler-McKay & Pear, 2004) and may possibly account for the T cell lymphomas (TCR$\beta^R$,$\text{IgH}^G$) detected in our irradiated mice. Inhibition of \textit{Notch1} enables B cell development in the thymus possibly reflected in the B cell lymphomas (TCR$\beta^G$,$\text{IgH}^R$) detected in our irradiated mice.

In summary, \textit{Pax5} gene inactivation was detected in all radiation induced malignancies (r-AML, BCL and Thymic lymphoma) that arose in irradiated CBA/H x (CBA/H x C57BL/6)F1 backcross mice with silencing being caused by LOH, \textit{Pax5} gene promoter methylation and potentially partial homozygous deletions. \textit{Pax5} inactivation is not exclusive to a specific haemopoietic malignancy, and is neither necessary nor sufficient to cause that malignancy. \textit{Pax5} silencing may simply reflect a selective advantage and other additional events are required for malignant transformation.
7 Chapter 7: Discussion.

The work described in this thesis represents the second phase of an ongoing study to identify factors that confer a susceptibility to radiation induced AML in the CBA/H mouse. LOH studies and a genome wide genetic linkage analysis of r-AML affected CBA/H x (CBA/H x C57BL/6)F1 backcross mice identified chromosomal deletions on chromosome 2 and 4 (Cleary et al., 1999; 2001), and detected 2 potential r-AML susceptibility loci (Boulton et al., 2003). One of the suggestive CBA/H r-AML susceptibility loci is on chromosome 1 CBA/H and the other on chromosome 6 (Boulton et al., 2003). LOH studies on two further malignancies that arose in this irradiated backcross identified LOH4 in early pre-B lympho-myeloid leukaemia (L-ML) and LOH4, LOH11 and LOH14 in thymic lymphoma (Cleary et al., 1999; 2001; Boulton et al., 2002). This thesis describes experiments designed to identify TSGs on chromosome 4 and r-AML susceptibility genes on distal chromosome 1.

7.1 Summary of findings.

This project provides evidence that:

• Lin', Sca-1**, c-Kit+ + bone marrow cells contain haemopoietic stem cells and progenitor cells. r-AML susceptible CBA/H mice have more Lin', Sca-1**, c-Kit+ + and Lin', Sca-1++, Flk-2++ bone marrow cells than r-AML resistant C57BL/6 mice. As r-AML is a HSC malignancy and r-AML susceptible mice have more HSCs, then this supports the hypothesis that target size is one risk factor in leukaemogenesis.

• Bone marrow cell death and recovery was also compared in both inbred mouse strains post exposure to a 3 Gy leukaemogenic dose of X-rays. The strain-specific differences in the number of Lin', Sca-1++, c-Kit++ bone marrow cells and Lin', Sca-1++, Flk-2++ bone marrow cells is retained up to 42 days post irradiation. Therefore differential radiosensitivity is excluded as a confounding factor.

• Hlx and Adrpt1 were not established as chromosome 1 r-AML susceptibility candidate genes. Ephx1 is a promising candidate, but the role of Ephx1 in radiation leukaemogenesis is unclear.

• Pax5 is inactivated in radiation induced malignancies by deletion (LOH4) and/or Pax5 gene promoter methylation.

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7.2 Allelic loss on chromosome 4.

LOH4 was detected in 92% of informative thymic lymphomas (TL), >95% of informative early pre-B lympho-myeloid leukaemias (L-ML) and 53% of informative r-AMLs that arose in X-irradiated CBA/H x (CBA/H x C57BL/6)F1 backcross mice (Cleary et al., 2001; Boulton et al., 2002). A 3.4cM minimally deleted region was identified (Cleary et al., 2001) and it was postulated that the loss of a tumour suppressor gene (TSG) within this region promoted leukaemogenesis. This study shows that the chromosome 4 Pax5 gene is silenced in these malignancies by gene promoter methylation and/or chromosomal deletion. Allelic loss on chromosome 4 and Pax5 gene promoter methylation leads to Pax5 deficiency which has different effects in different haemopoietic malignancies.

7.2.1 Mixed lineage leukaemias and lymphomas

Figure 7.1 is a proposed model of mixed lineage leukaemogenesis in the mouse, highlighting the potential role of Pax5 inactivation in malignant transformation of the target cell - the B cell progenitor (BP).

Figure 7.1 - Fate of an irradiated B cell progenitor.

The presumed target cell is the B cell progenitor (BP) which exhibits clonal immunoglobulin rearrangements (IgH<sup>R</sup>). Pax5 inactivation (deletion and/or promoter methylation) enables the BP cell to progress down any cell line (except B lineage) dependent on the location of the BP cell (spleen or thymus) and exposure to other transcription factors. It is proposed that a BP cell that transforms in the spleen acquires myeloid characteristics because there is no Pax5 mediated repression of myeloid transcription factors. This manifests as an early pre B lympho-myeloid leukaemia (LML). MPO (Myeloperoxidase) and LysM (Lysozyme M) are myeloid markers. Transformation of the BP cell in the thymus generates thymic lymphomas - BTTL (mixed B+T cell thymic lymphoma) and BTL (single lineage B cell thymic lymphoma) presumably dependent on the thymus environment and combination/expression of transcription factors. LOH11 and LOH14 have been detected in thymic lymphomas indicating that other TSGs are probably involved.
B cell malignancies exhibit clonal heavy chain rearrangements (IgH\textsuperscript{R}) indicating that prior to exposure, the target cell had progressed beyond the initial somatic recombination of the IgH gene. \textit{Pax5} is a critical B-lineage transcription factor (Busslinger, 2004) and \textit{Pax5} (-/-) early pro B cells are capable of oscillating down other cell lineages (Nutt \textit{et al.}, 1999A; Graf, 2002; Schaniel \textit{et al.}, 2002; Rolink \textit{et al.}, 2002). Differentiation of \textit{Pax5} (-/-) early pro B cells is dependent on exposure to other transcription factors and/or environments, and therefore our model postulates that the mouse mixed lineage leukaemias and lymphomas are Pax5 deficient and therefore oscillate down different cell lineages in response to environment – Spleen (lympho-myeloid leukaemias) or Thymus (B+T thymic lymphomas).

\textit{Pax5} blocks the survival and expansion of early myeloid cells by suppressing their response to myeloid growth factors (Chaing & Monroe, 1999; 2001). The inactivation of the \textit{Pax5} gene therefore promotes survival/expansion of early myeloid cells which will express myeloid markers. This is consistent with the detection of \textit{MPO} and \textit{LysM} mRNA in the L-MLs (chapter 6, figure 6.4A). \textit{Pax5} (-/-) early pro B cells differentiate into T cells more efficiently than myeloid cells (reviewed Heavey \textit{et al.}, 2003) although differentiation into T cells does require the thymus environment. An irradiated B cell progenitor that migrates to the thymus may therefore transform into a B+T thymic lymphoma (BTTL) or a single lineage B cell lymphoma (BTL).

\textit{Pax5} is a negative regulator of \textit{Notchl} (Souabni \textit{et al.}, 2002). Like most differentiation genes, \textit{Notchl} gene expression changes during differentiation. \textit{Notchl} signalling is required for T cell commitment but must be down regulated for CD4\textsuperscript{+} or CD8\textsuperscript{+} cell maturation (Zweidler-McKay & Pear, 2004). The migration of a B cell progenitor into the thymus under conditions of normal/enforced Notchl expression may explain the B+T thymic lymphomas (figure 7.1) which exhibit both IgH rearrangements (from the target cell pre irradiation) and TCR\textbeta rearrangements - Notchl regulates T cell V\textbeta-DJ\textbeta rearrangements (Höflinger \textit{et al.}, 2004). The identification of \textit{Pax5} mRNA in B thymic lymphomas (either as a consequence of \textit{Pax5} expressing leukaemic cells or infiltrating normal B cells) is not however consistent with enforced Notchl expression. Notchl is essential for suppression of B cell development in the thymus (Schebesta \textit{et al.}, 2002A) and \textit{Pax5} negatively
regulates Notchl. Enforced Notchl expression should therefore prevent B cell
development in the thymus and also down regulate Pax5 by inhibiting E2A function
(Harper et al., 2003). The colonisation of B cells in the thymus may represent
repression of Notchl by Pax5 or a separate Notchl block. Interestingly, it has been
proposed that Ikaros inhibition facilitates T cell transformation by Notchl (Zweidler-
McKay & Pear, 2004). Homozygous deletions and point mutations of the Ikaros gene
had previously been identified in radiation induced mouse thymic lymphomas (Okano
et al., 1999).

7.2.2 r-AML

Although LOH4 and Pax5 gene promoter methylation is detected in r-AML, L-ML
and TLs, chromosome 2 deletions are characteristic of mouse r-AMLs. LOH2 can
therefore be use to genetically separate lymphoid (B and T cell) and myeloid (r-AML)
malignancies as >95% of mouse r-AMLs and <5% BCLs and TLs exhibit chromosome
2 deletions. These chromosome 2 deletions are large (>20cM), often comprising of
33-66% of the 120 Mb chromosome (Hayata et al., 1983; Alexander et al., 1995;
Cleary et al., 1999) while LOH4 deletions are small (<10cM; Cleary et al., 2001;
Boulton et al., 2002). Site specific exposure of hybrid CHO cells (contain a single
copy of human chromosome 11) has demonstrated that directing 8 α-particles through
the nucleus causes large ~120 Mb deletions (86% chromosome 11) while small ~2 Mb
deletions arise when 8 α-particles are directed through the cytoplasm (Wu et al., 1999).
Chromosome 2 deletions may be an initiating event in mouse myeloid
leukaemogenesis while chromosome 4 deletions therefore represent a recurrent
secondary event arising from the indirect/delayed effect of X-ray exposure.

Figure 7.2 is a proposed model for murine r-AML. Recently PU.1 (chromomome 2)
has been implicated in mouse r-AML (Cook et al., 2004). Unexpectedly, deletion of
one PU.1 allele and mutation of the second PU.1 allele does not result in PU.1
silencing, and therefore is not consistent with PU.1 being a TSG. The mutant PU.1
demonstrates limited differentiation ability (~20% of wild type) which is speculated to
be sufficient to enable differentiation of the HSC to myeloid progenitor, but not
sufficient to enable terminal myeloid differentiation. PU.1 suppresses myeloid
leukaemia by promoting maturation, and mutant PU.1 promotes r-AML by blocking
this maturation process (Cook et al., 2004). Irradiation initiates LOH2 deletion and PU.1 mutations creating a 'pre-leukaemic' stem cell. The minimal PU.1 level promotes the differentiation of the HSC to the myeloid progenitor where secondary events characterise the leukaemia (Warner et al., 2004). If Pax5 inactivation in r-AMLs represents a secondary event/block preventing progression down the B cell route, then further blocks (i.e. T cell block) may also exist.

Figure 7.2 - Fate of an irradiated Haemopoietic Stem Cell

The haemopoietic stem cell (HSC) is presumed to be the pre-leukaemic cell in r-AML. Cook et al., (2004) demonstrated that LOH2 and PU.1 mutations generated a mutant PU.1 protein that had sufficient PU.1 expression to enable differentiation of the HSC to the myeloid progenitor (MP) and subsequently promoting r-AML by preventing myeloid differentiation. This model proposes that Pax5 inactivation may represent a second block possibly preventing progression down the B cell route. Hypothetically other lineage blocks may exist.

Genetic consequences of radiation induced DNA damage are chromosomal deletions and genetic instability. DNA damage and accumulating genetic aberrations, together with genetic susceptibility and surrounding microenvironment, may enable certain cells to acquire a selective advantage. Pax5 inactivation creates a 'leaky' differentiation block and it is proposed that this 'leaky' block aids the development of diverse range of haemopoietic malignancies. Although Pax5 inactivation may be insufficient to initiate malignant progression, Pax5 inactivation itself may contribute to the leukaemogenic process by providing a selective advantage within a leukaemia or lymphoma.
7.3 r-AML susceptibility locus on distal chromosome 1.

A genome wide genetic linkage analysis of r-AML affected X-irradiated CBA/H x (CBA/H x C57BL/6)F1 backcross mice identified a locus on distal chromosome 1 which overlaps with a genetically determined QTL that modulates the mouse bone marrow stem cell frequency (Müller-Sieburg & Riblet, 1996). One factor in cancer risk is target cell number. There are two major hypotheses as to which target cell in normal haemopoiesis acquires sufficient transforming mutations to generate leukaemia. One model postulates that all cells throughout the stem cell/progenitor compartment are susceptible to transformation, and that the malignancy reflects the particular differentiation stage of the target cell. The second (favoured) hypothesis restricts transformation events to the HSC and the subsequent malignancy is dependent upon specific transformation events and their effect on differentiation (Hope et al., 2003; Passegué et al., 2003).

In this study, the whole HSC compartment was defined by FACS as Lin', Sca-1++, c-Kit++ bone marrow cells and ST-HSCs included within the Lin', Sca-1++, Flk-2++ bone marrow cells analysed. Genetic analysis of the Lin', Sca-1++, c-Kit++ bone marrow cells equated these cells with the day 35 CAFC and LTC-IC (M Plumb, personal communication). Subsequent analysis of the cell death and recovery of this cell population post irradiation inferred that HSCs were located within the Lin', Sca-1++, c-Kit++ bone marrow cell population as they were the only white bone marrow cell population that demonstrated the amplification post irradiation that could drive the recovery of the bone marrow cellularity. The r-AML susceptibility CBA/H mouse strain on average had more Lin', Sca-1++, c-Kit++ and Lin', Sca-1++, Flk-2++ bone marrow cells than the r-AML resistant C57BL/6 mouse strain indicating that CBA/H mice have a greater target size for leukaemogenesis, and cell death and recovery analyses demonstrated that the difference was maintained post irradiation.

The stem cell amplification and proliferation time frame of 0-4 days post irradiation in this study coincides with radiation-induced folate depletion. 5 days post exposure to a single dose of 7Gy (0.79Gy/min) liver folate is depleted by ~47% (Kesavan et al., 2003). Folate dependent reactions include the synthesis of thymidine triphosphate (dTTP) from its precursor deoxyuridine monophosphate (dUMP) and synthesis of S-
adenosyl methionine required for DNA methylation (Hoffbrand & Pettit, 1993). dTTP is essential for DNA synthesis and folate deficiency thus causes uracil (dUTP) incorporation into DNA. Excising uracil from DNA forms single strand DNA breaks and increases mutation frequency (Blount et al., 1997). Insufficient time or resources to correct DNA damage prior to fixation and/or DNA hypomethylation may predispose the HSC to malignant transformation and subsequently result in r-AML.

Unlike LOH2 which is essential to r-AML development, inheriting two CBA/H alleles in the chromosome 1 r-AML susceptibility region is not sufficient or necessary for r-AML and therefore contributes to risk rather than initiates the disease (Boulton et al., 2003). Three candidate genes were looked at in detail. Inter strain functional and expression differences were only detected in Ephx1, a gene whose role in radiation leukaemogenesis remains unclear. All three putative candidate genes exhibited increased mRNA levels in r-AMLs. Both Adprt1 and Hlx have a potential role in myeloid leukaemogenesis. The Adprt1 encodes PARP1 which controls a number of genes including Yin Yang 1 (YY1) whose deregulation is proposed to interfere with normal myeloid differentiation (Erkeland et al., 2003). Hlx is expressed in myeloid-macrophage lineage cells and therefore increased mRNA levels of Hlx may reflect the cell type present or the attempted promotion of myeloid differentiation (Allen et al., 1991; Allen & Adams, 1993). Other Hox genes however have been associated with HSC self-renewal properties with over expression of Hox genes causing selective expansion of the HSC population (Bell & Van Zant, 2004). Aberrant expression of homeobox genes may be insufficient for carcinogenesis, but are proposed to ‘tip the balance’ (Abate-Shen, 2002) and therefore provide a selective advantage such as that proposed with homeobox gene Pax5.
7.4 Further Work

- Thymic lymphomas that arose in these X-irradiated backcross mice will be used to confirm the LOH on chromosome 14 and 11 and refine the minimally deleted regions. This should confirm the exclusion/inclusion of Ikaros as a candidate gene on chromosome 11.
- Examine the proposed role of Notch1 and Ikaros in radiation leukaemogenesis.
- FACS analysis of both total bone marrow and Lin' bone marrow cells has shown that strain specific differences exist between CBA/H and C57BL/6 inbred mouse strains. Breeding (CBA/H x C57BL/6)F2 mice, quantification of Mac-1, c-Kit, and Flk-2 staining intensity, genome wide genetic linkage screen should establish whether strain specific haplotypes can explain the identified staining differences. Identical analysis of Lin’, Sca-1++, Flk-2++ bone marrow cells and bone marrow red count (RBMC) will be used to establish ST-HSC and RBMC QTLs.
- FACS and genetic linkage analysis of Lin’, Sca-1++ , c-Kit++, Flk2' bone marrow cells to establish if the LT-HSC QTLs correspond to the stem cell frequency regulator 1 locus identified in the r-AML susceptible mice.
- A review of the literature has identified a potential Pax5 polymorphism in the G(A) repeat region of the Pax5 gene promoter (Meijne et al., 2001) which may be able to confirm or discount the loss of the maternally transmitted CBA/H allele.
- Further characterisation of the role of Ephx1 in radiation induced leukaemia including bile uptake, cholesterol metabolism and the role of transcription factors i.e. GATAs and C/EBPα.
8 Chapter 8: References


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