Radiation leukaemogenesis at low doses

DE-FG02-05 ER 63947

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1. Introduction

There is ample epidemiological evidence indicating that ionising radiation is carcinogenic in the higher dose range. This evidence, however, weakens and carries increasing uncertainties at doses below 100-200 mSv. At these low dose levels the form of the dose-response curve for radiation-induced cancer cannot be determined reliably or directly from studies of human populations. Therefore animal, cellular and other experimental systems must be employed to provide supporting evidence on which to base judgements of risk at low doses. Currently in radiological protection a linear non-threshold (LNT) extrapolation of risk estimates derived from human epidemiological studies is used to estimate risks in the dose range of interest for protection purposes.

Myeloid leukaemias feature prominently among the cancers associated with human exposures to ionising radiation (eg UNSCEAR 2006; IARC 2000). Good animal models of radiation-induced acute myeloid leukaemia (AML) are available including strains such as CBA, RFM and SJL (eg Major and Mole 1978; Ullrich et al 1976; Resnitzky et al 1985). Early mechanistic studies using cytogenetic methods in these mouse models established that the majority of radiation-induced AMLs carried substantial interstitial deletions in one copy of chromosome (chr) 2 (eg Hayata et al 1983; Trakhtenbrot et al 1988; Breckon et al 1991; Rithidech et al 1993; Bouffler et al 1996). Chr2 aberrations are known to occur in bone marrow cells as early as 24 hours after *in vivo* irradiation (Bouffler et al 1997). Subsequent molecular mapping studies defined a distinct region of chr2 that is commonly lost in AMLs (Clark et al 1996; Silver et al 1999). Further, more detailed, analysis identified point mutations at a specific region of the *Sfpi1/PU.1* haemopoietic transcription factor gene which lies in the commonly deleted region of chr2 (Cook et al 2004; Suraweera et al 2005). These lines of evidence strongly implicate the *Sfpi1/PU.1* gene as a tumour suppressor gene, dysregulation of which leads to myeloid leukaemia.

The main focus of this project was to utilise the CBA mouse model of radiation leukaemogenesis to explore mechanisms of low dose and low dose-rate leukaemogenesis. A series of mechanistic investigations were undertaken, the central aim of which was to identify the events that convert normal cells into myeloid leukaemia cells and explore the dose-response relationships for these. Much of the work centred on the *Sfpi1/PU.1* gene and its role in leukaemogenesis. Specific studies considered the dose-response and time-course relationships for loss of the gene, the functional consequences of *Sfpi1/PU.1* loss and mutation on transcriptional programmes and developing an *in vivo* reporter gene system for radiation-induced alterations to PU.1 expression. Additional work sought further genetic changes associated with radiation-induced AMLs and a better characterisation of the cell of origin or 'target cell' for radiation-induced AML. All the information gathered is of potential use in developing biologically realistic mathematical models for low dose cancer risk projection.

This report provides a complete summary of the work undertaken and results obtained under US Department of Energy grant DF-FG02-05 ER 63947, *Radiation leukaemogenesis at low doses*.

2. Results

2.1 An assay for Sfpi1/PU.1 loss

A major part of this project required the establishment and optimisation of methods for analysing radiation-induced AMLs and early events in leukaemogenesis. In light of the evidence for the involvement of *Sfpi1/PU.1* that was emerging at the time that this grant got underway, we first developed an assay to assess quantitatively *Sfpi1/PU.1* loss in single cells at any time after radiation exposure of mice *in vivo* from 24 hours until presentation of AMLs at a mean latent period of ~18 months.

A fluorescence *in situ* hybridisation (FISH) approach was adopted that allowed quantification of *Sfpi1/PU.1* losses in metaphase preparations from AMLs and *in vivo* irradiated bone marrow cells. This assay drew on knowledge obtained through earlier work mapping the commonly deleted region of chr2 in AMLs (Silver et al 1999; Suraweera et al 2005). FISH assays require careful control and hence considerable effort was placed on ensuring that internal control and experimental probes were working optimally. The standard protocol, described in Peng et al (2009a) involved concurrent hybridisation of three bacterial artificial chromosome (BAC) probes isolated from the Research Genetics mouse BAC library release II – the *Sfpi1/PU.1* carrying clone 253H22; a clone locating close to the centromere of chr2 containing D2Mit31, 49N22; and a clone locating close to the telomeric end of chr2 containing D2Mit74. The *Sfpi1/PU.1* probe was labelled by nick translation with Spectrum Orange (Abbott Molecular Inc.) while the centromeric and telomeric BACs were labelled with Spectrum Green. Following a standard FISH protocol metaphases were examined by fluorescence microscopy. Only cells carrying a full set of control probe hybridisation signals (ie the centromeric and telomeric chr2 probe signals) were considered for assessment of *Sfpi1/PU.1* loss. Figure 1 shows examples of control and *Sfpi1/PU.1* deleted cells.

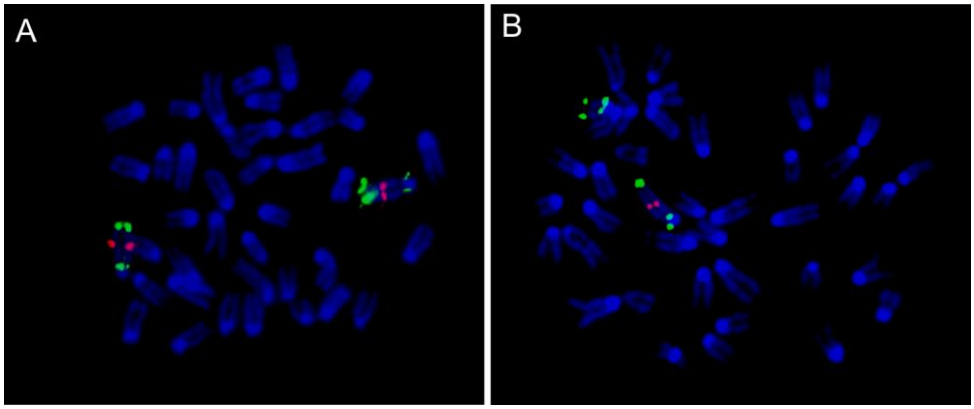

Figure 1. BAC-FISH image of a normal spleen cell metaphase spread (A) and spread from the SA2 cell line (B). Sfpi1/PU.1 labelled with spectrum-orange probes in both figures. In spleen cells both copies of Sfpi1/PU.1 can clearly be seen, whereas in SA2 only one copy is present. Two spectrum-green labelled markers for centromere and telomere regions of chromosome 2 are used as controls.

This assay was applied to assess early point-irradiation *Sfpi1/PU.1* losses in bone marrow and spleen and also to characterise radiation-induced AML cell lines.

2.2 *In vivo* dose-response for Sfpi1/PU.1 loss

An experiment exposing AML-sensitive CBA mice to acute x-ray doses down to 0.1 Gy was undertaken to determine the frequency of *Sfpi1/PU.1* loss in the lower dose range. Using 8 week old CBA/Ca mice x-irradiated with 0.1, 0.5, 1 and 2 Gy and sacrificed 24 hours and 1 month after exposure, the shape of the dose-response relationship for direct *Sfpi1/PU.1* deletion was investigated. Both spleen and bone marrow cells have been harvested from sacrificed animals, and used in BAC FISH analyses. The shape of the dose response for bone marrow at 24 hours

and 1 month, and for spleen cells at 1 month are similar, showing a linear relationship between increasing exposure and *Sfpi1/PU.1* loss (Figures 2-5). The incidence of *Sfpi1/PU.1* loss at 1 month is slightly higher than at the 24 hour time point. The data suggest that bone marrow cells may be more sensitive than spleen cells at the 24 hour time, with spleen *Sfpi1/PU.1* loss only getting above background levels after 1 or 2 Gy doses. There is a background rate of approximately 1.5% *Sfpi1/PU.1* loss in un-irradiated cells in this experiment, a little higher than expected, but this is reproducible.

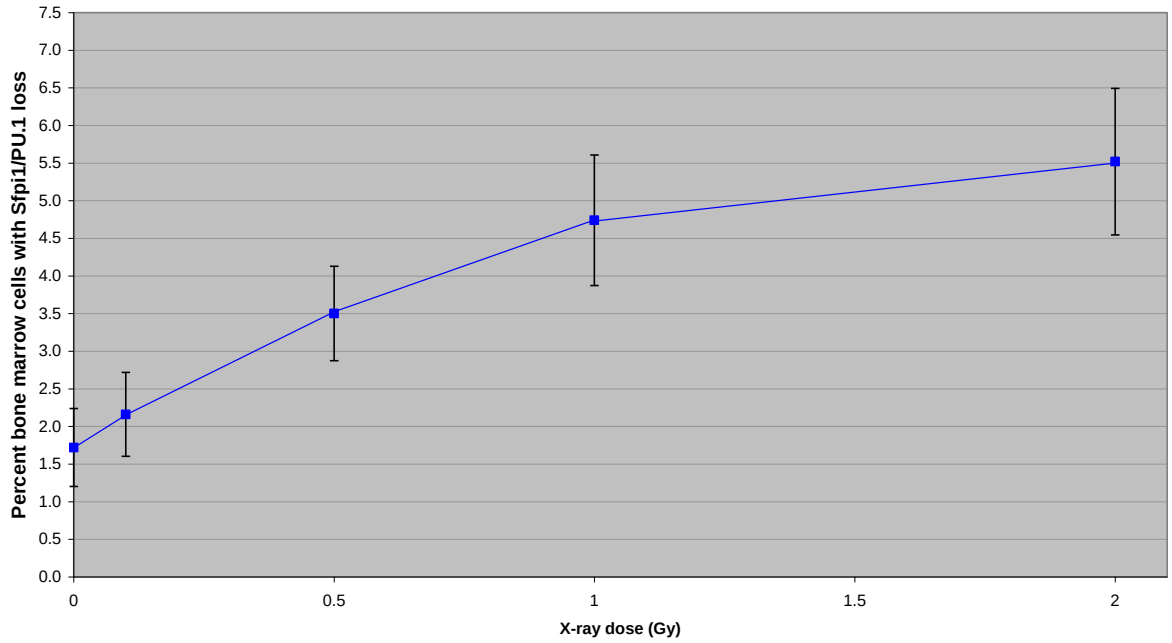

Figure2 . Dose response for loss of Sfpi1/PU.1 in bone marrow 24 hours after in vivo x-irradiation of CBA/H mice. Means ± Poisson standard error.

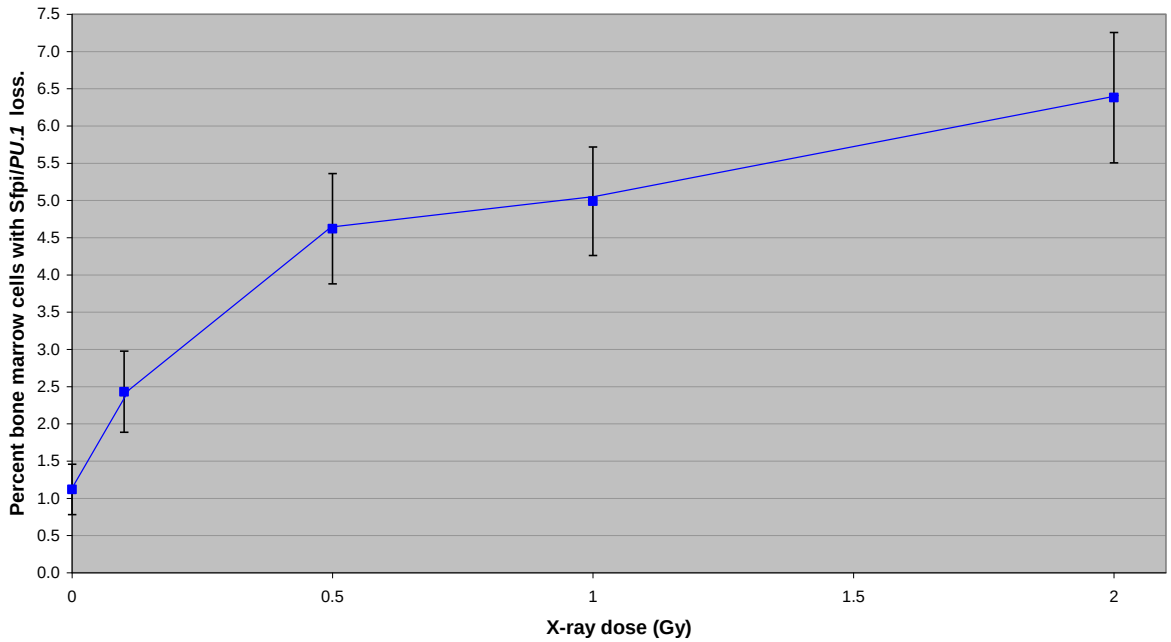

Figure 3. Dose response for loss of Sfpi1/PU.1 in bone marrow 1 month after in vivo x-irradiation of CBA/H mice. Means ± Poisson standard error.

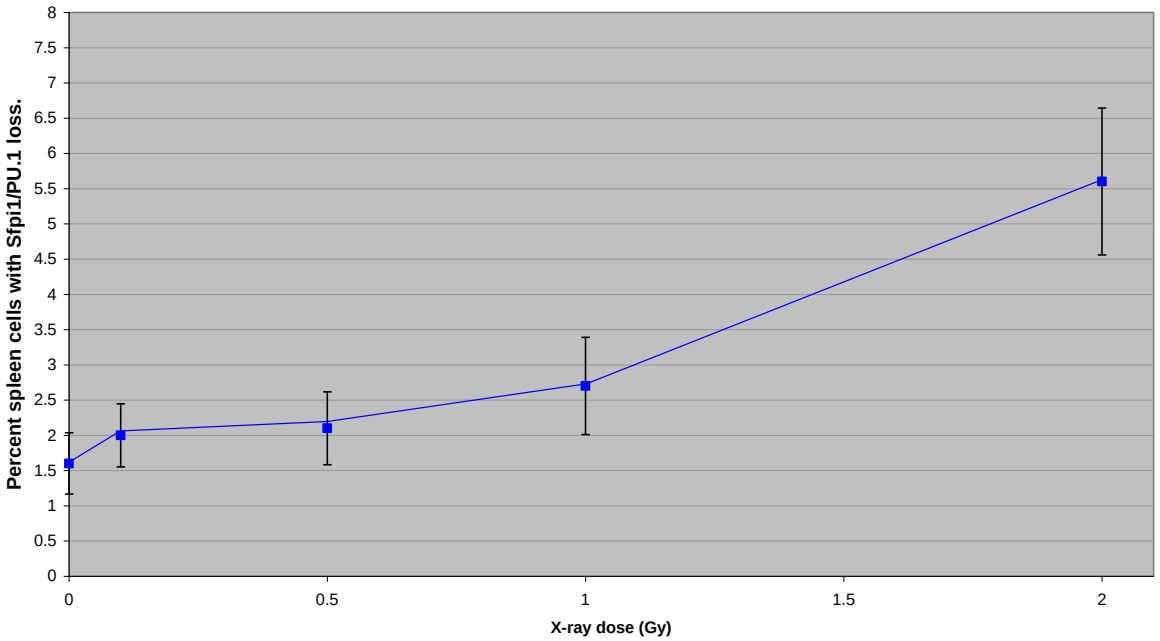

Figure 4. Dose response for loss of Sfpi1/PU.1 in spleen 24 hours after in vivo x-irradiation of CBA/H mice. Means ± Poisson standard error.

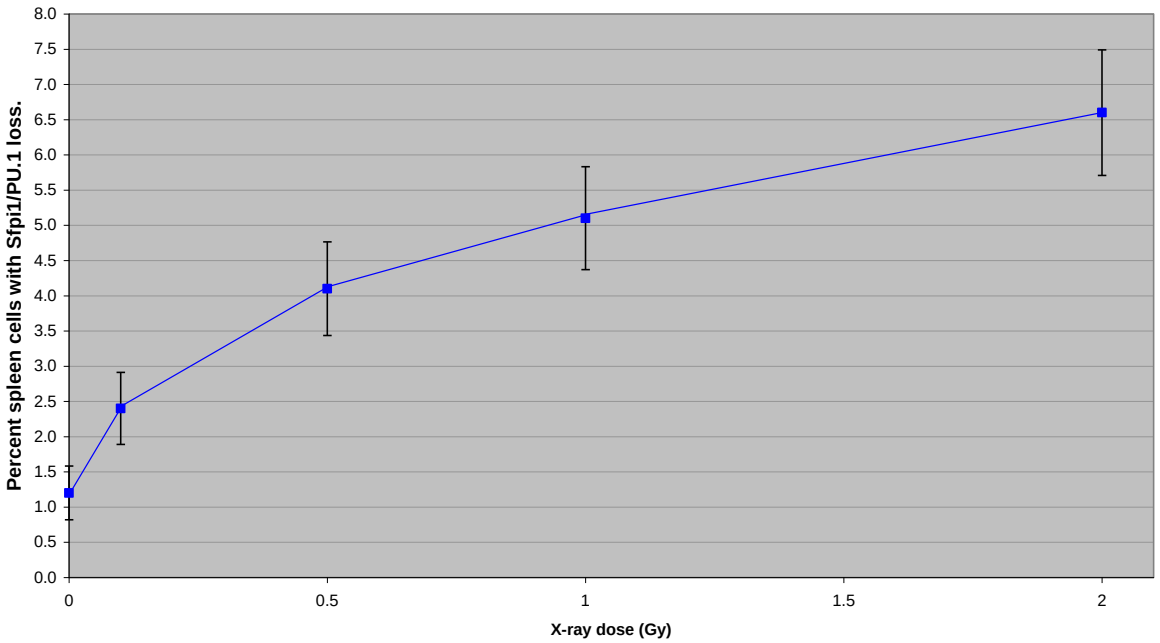

Figure 5. Dose response for loss of Sfpi1/PU.1 in spleen 1 month after in vivo x-irradiation of CBA/H mice. Means ± Poisson standard error.

The data on radiation-induced loss of Sfpi1/PU.1 from bone marrow were combined for publication with data from our collaborating colleagues at Colorado State University on gamma radiation and iron ion radiation (Peng et al 2009a,b). Together these studies have demonstrated that radiation-induced loss of *Sfpi1/PU.1* can be detected at 24 hours after *in vivo* irradiation and therefore must be directly induced radiation events. Loss is detectable at 0.1 Gy and dose-responses are linear or near linear in form. From these experiments it was concluded:

- *Sfpi1/PU.1* losses are directly induced by *in vivo* irradiation of bone marrow and spleen tissues.
- Dose-response data suggest that the relationship between absorbed dose and *Sfpi1/PU.1* loss has a tendency to saturate at higher doses.
- No threshold of ≥0.1 Gy exists for *Sfpi1/PU.1* deletion, an initiating event for radiation AML.

2.3 *In vivo* time course for Sfpi1/PU.1 loss

The time course of *Sfpi1/PU.1* losses was followed in spleen and bone marrow over an 18 month period after 3 Gy *in vivo* x-irradiation of AML sensitive CBA/H mice and AML resistant C57 BL/6 mice. The time course data up to 3 months following irradiation were included in Peng et al (2009a). Figures 6 and 7 provide the full time course data for both strains and tissues.

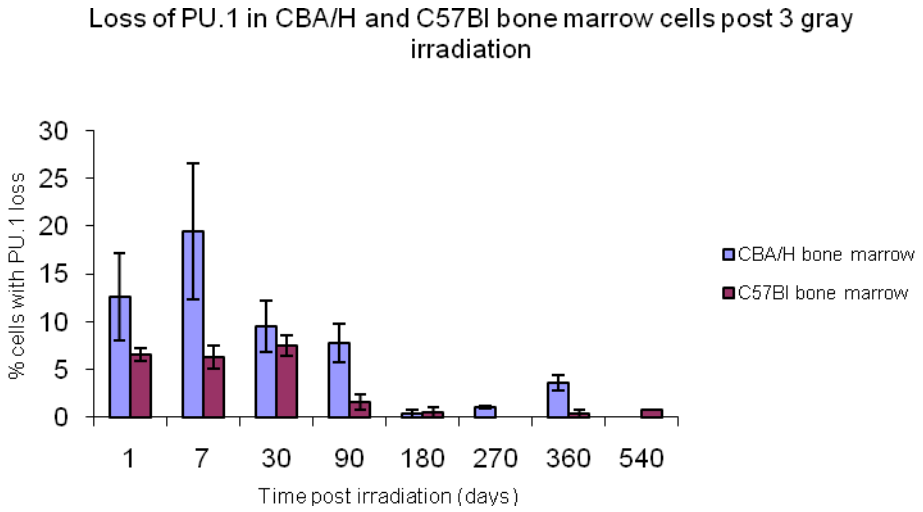

Figure 6. Time course of Sfpi1/PU.1 losses in total bone marrow cell populations following 3 Gy in vivo x-irradiation.

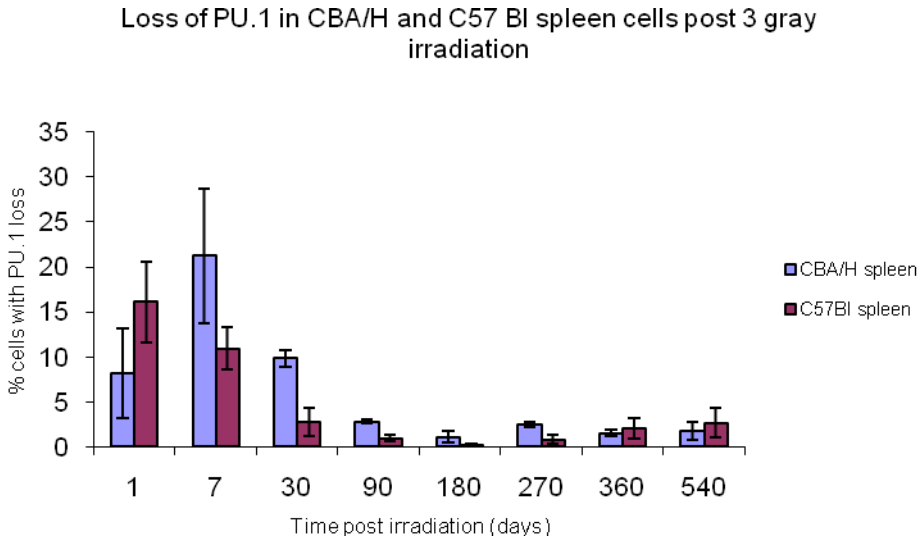

Figure 7. Time course of Sfpi1/PU.1 losses in spleen cell populations following 3 Gy in vivo x-irradiation.

These experiments indicate that *Sfpi1/PU.1* losses are generally higher in CBA/H mice than C57BL/6 at early times in both bone marrow and spleen. There is a more rapid reduction in cells carrying gene losses in C57BL/6 than in CBA. However in both strains Sfpi1/PU.1 losses are at levels at or close to background, spontaneous levels by 6 months (180 days). These findings specific for Sfpi1/PU.1 loss are in contrast to earlier conventional cytogenetic analyses that indicated that chr2 aberrations in bone marrow remained at a constant level in proliferating marrow cells between 1 month and 9 months after irradiation (Bouffler et al 1997). The CBA bone marrow data are not entirely consistent with published results for γ-irradiated CBA/Ca animals (Peng et al 2009a). Nevertheless CBA/H marrow cells do show an elevation in *Sfpi1/PU.1* deleted cells at 1 year (360 days). This again drops at 540 days, perhaps due to some mice with relatively high *Sfpi1/PU.1* loss frequency presenting with AML.

Further work and new approaches will be required to build a complete understanding of the dynamics of AML development in Sfpi1/PU.1 deleted cells *in vivo*. However some key conclusions may be drawn:

- CBA/H bone marrow cells present with a higher frequency of *Sfpi1/PU.1* losses than C57BL/6 at all times up to 3 months post *in vivo* whole body irradiation.
- *Sfpi1/PU.1* deleted cells tend to persist longer in CBA/H than C57BL/6 animals.
- Later elevations in Sfpi1/PU.1 loss frequency may signal AML development but is not sufficient to promote AML development in the C57BL/6 background.

2.4 Characterisation of cell lines – cell surface phenotype

The data presented in 2.2 and 2.3 demonstrated that early post-irradiation pre-leukaemic losses of *Sfpi1/PU.1* could be detected in bulk bone marrow and spleen cells. These experiments could not however determine the haemopoietic cell type in which losses occurred. Identification and quantification of the population of cells at risk or cells of origin for specific radiation induced tumours is critically important for disease modelling studies.

To characterise the cell of origin for radiation-induced AMLs we undertook a study of six AML cell lines. The cell surface phenotype was determined by flow cytometric evaluation of staining of the cell lines by immunofluorescence using primary antibodies against the following surface antigens

- Ly6c – *myeloid lineage marker*
- Gr.1, Mac-1 – *neutrophil and macrophage/monocyte lineage markers*
- CD3 – *T lymphocyte lineage marker*
- B220/CD45 – *B lymphocyte lineage marker*
- C-Kit, CD31 – *haemopoietic stem cell markers*
- Sca-1 – *multipotential haemopoietic stem cell marker*

The cell lines had differing mouse strain origins as shown in Table 1 and were obtained from Drs Kazuko Yoshida (8016, 8709, 7926), Emmy Meijne (MLP3, 3124) and Andrew Riches (SA2).

Table 1 *Cell surface marker phenotype of 6 radiation-induced cell lines. – No expression, + - weakly positive expression, ++ - medium level expression, +++ strongly positive expression*

	Cell line/Strain of origin					
	8016	8709	7926	3124	SA2	MLP3
	C3H	C3H	C3H	CBAxC57 F1	CBA	CBA
Ly6C	++	++	++	++	++	++
Gr.1	-	++	+	++	-	++
Mac-1	-	++	+	+	-	-
CD3	-	-	-	-	-	-
B220/CD45	-	-	-	-	-	-
c-Kit	++	++	++	+	++	+
CD31	++	+++	++	+++	+++	+++
Sca-1	-	++	-	-	-	-

Table 1 shows that all cell lines were negative for T- and B- cell markers, positive for myeloid markers and positive for haemopoietic stem cell markers. This surface phenotype is closely similar to that of the *common myeloid progenitor* (CMP) (Akashi et al 2000)

It is concluded that:

- radiation-induced AMLs arise in a common myeloid progenitor-like cell.

These data have now been published (Brown et al 2010). This result provides a means by which the size of target cell population could be assessed in AML sensitive and resistant mouse strains.

2.5 Sfpi1/PU.1 gene, transcript and protein status of cell lines

The panel of cell lines was also used to examine the relationship between *Sfpi1/PU.1* copy number/mutation status and protein levels and further to explore the consequences of the *Sfpi1/PU.1* changes on the transcription.

Sfpi1/PU.1 losses were determined by the FISH assay described in 2.1. Mutations in exon 5 of the gene were determined by DNA sequence analysis as described by Suraweera et al (2005).

All cell lines were found to be hemizygous for *Sfpi1/PU.1* except for MLP3 which was nullizygous. The deletion of both *Sfpi1/PU.1* copies in MLP3 had been noted previously by Southern blot analysis (Silver et al 1999). DNA sequence analysis showed the five hemizygous cell lines to carry mutations affecting the argentine 235 (R235) residue of the encoded protein. Three mutations to serine (R235S, 7926, 3124, SA2), one to histidine (R235H, 8709) and one to cysteine (R235C, 8016) were observed. These mutations have been described in primary and *in vivo* transplanted AMLs but the R235C is most commonly observed in primary AMLs (Cook et al 2004; Suraweera et al 2005). Therefore *Sfpi1/PU.1* mutation does not absolutely determine the ability to adapt to culture but those carrying R235S appear to do so more readily.

Transcription of *Sfpi1/PU.1* was determined by quantitative RT PCR using in-house designed Taqman-like primers/probes (Table 2). Expression was compared to that of *HPRT*, the full method is given in Brown et al (2010).

Table 2 QRTPCR primers/probes

Gene	Forward primer	Reverse primer	Probe
HPRT	tggtgaaaaggacctctcgaa	tcaagggcatatccaacaaca	tgttggatacaggccagacttt gttgga[5^1]FAM[3^1]BHQ-1
Sfpi1/PU.1	agaagctgatggcttggagc	gcgaatctttttcttgctgcc	tgggccaggtcttctgcacgg [5^1]HEX[3^1]BHQ-1

Figure 8 shows *Sfpi1/PU.1* expression levels normalised to *HPRT* expression levels and *Sfpi1/PU.1* expression levels in the cell lines normalised to that in total bone marrow. Expression of the gene in the R235C line 8016 was approximately four fold higher than in bone marrow. R235S lines had similar levels of transcript to bone marrow while the R235H mutant (8709) had reduced transcript levels. No transcript was detected in MLP3. This suggests that the mutation types differentially affect mRNA transcript levels.

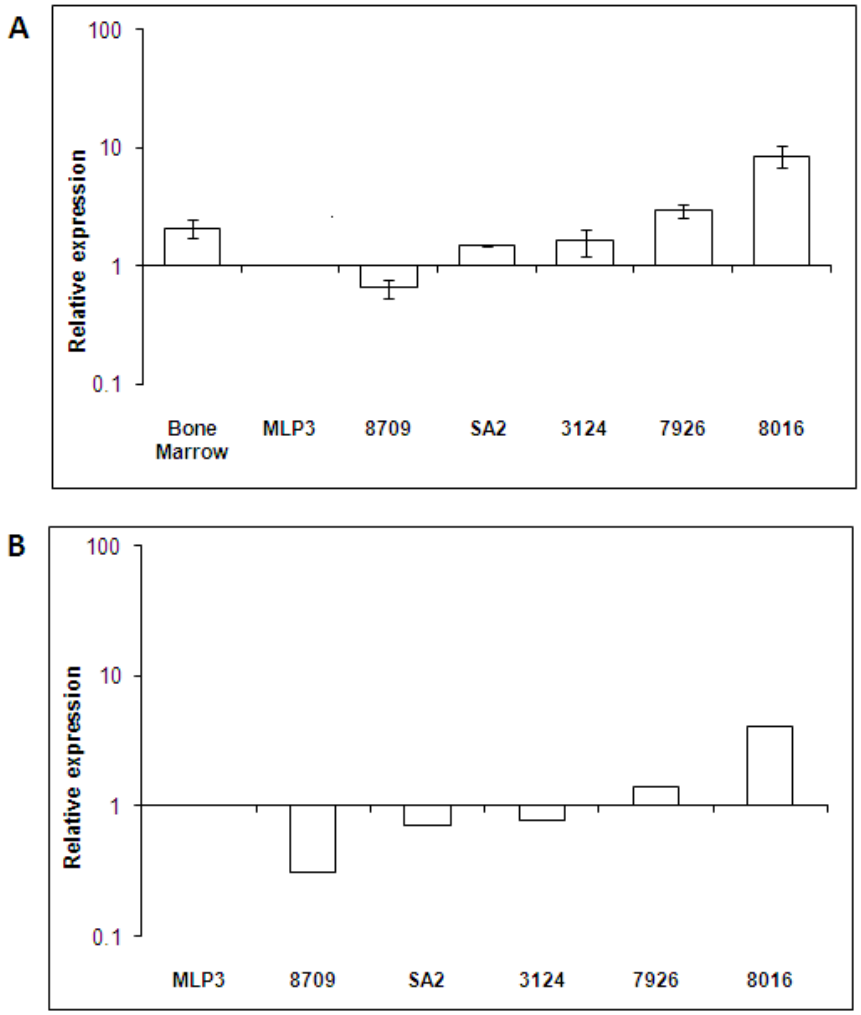

Figure 8. Expression of Sfpi1/PU.1 in AML cell lines and controls relative to the control gene HPRT, as determined by ΔCt (A) and ΔΔCt (B) respectively. Standard error bars are included in (A).

Two methods were used to examine protein expression, immunocytochemistry and Western blotting. Identifying suitable antibodies for each application proved to be extremely problematic. After testing many commercially available and custom produced antibodies one was found suitable for both applications in mouse (Cell Signalling Technology). Full methods are described in Brown et al (2010). Peroxide based immunocytochemical detection of Sfpi1/PU.1 in cytospin preparations of the cell lines is illustrated in Figure 9.

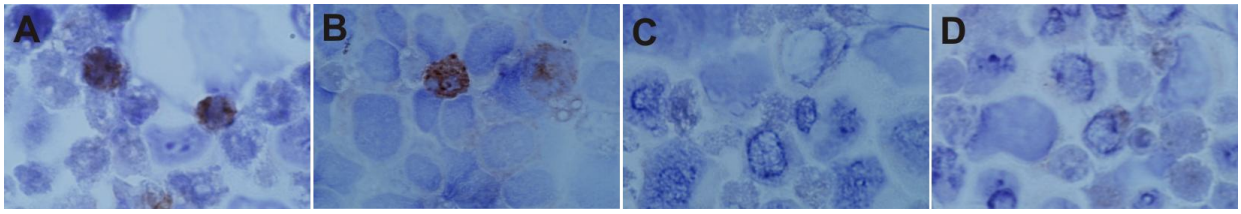

Figure 9. Immunocytochemical analysis of Sfpi1/PU.1 expression. (A) 3124, (B) SA2, (C) 8709, and (D) MLP3. Staining was detected in 3124 and SA2 with very faint expression in 7926 (not shown) cell lines. No expression could be detected in 8709, 8016 (not shown) or MLP3.

Protein was detected in the R235S lines 3724, SA2 and 7926 but no positive staining was observed in 8709, 8016 or MLP3. Significant cell-to-cell variation in expression was noted in all of the positive cell lines and this may relate to a low level of ongoing differentiation that affects Sfpi1/PU.1 levels in the cell lines. Western blotting confirmed the immunocytochemistry. Several controls were included in the Western analysis. Negative controls were thymus and U20S cells transfected with a gfp construct. Positive controls were THP1, a human monocytic leukaemia cell line, bone marrow, spleen and U20S cells transfected with the gfp construct and/or a *Sfpi1/PU.1* cDNA carrying plasmid. Results can be seen in Figure 10.

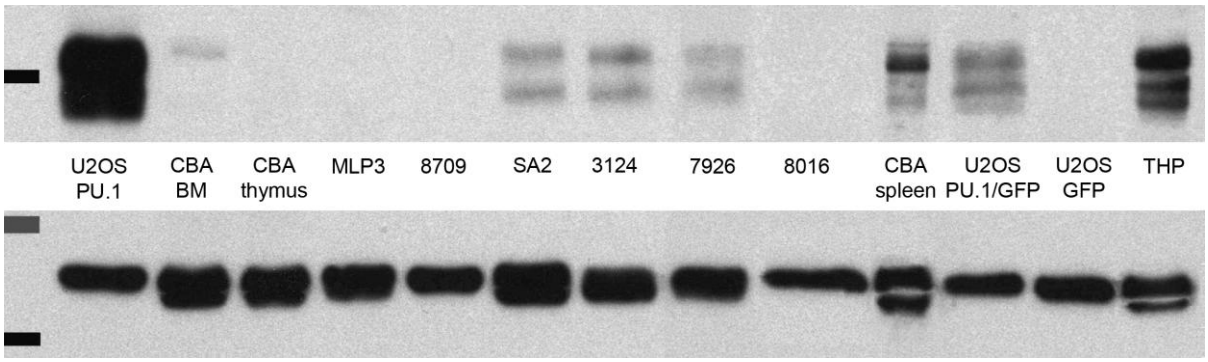

Figure 10. Western blot analysis of Sfpi1/PU.1 (top) expression in all 6 AML cell lines and controls, as indicated by size marker at 37 kDa. Two clear protein bands can be seen in cell lines, at approximately 42 and 35 kDa. The lower blot shows a reprobe of the membrane using β-actin as a loading control, with size markers indicated at 50 and 37 kDa. 7.5 µg protein loaded in all cases, except for two samples, U2OS PU.1 + GFP positive control (lane 11) and U2OS negative control (lane 12), for which 5 µg was loaded. Positive controls – lane 1 (U2OS transfected with PU./1 reactor), lane 2 (CBA total bone marrow), lane 10 (CBA spleen), lane 11 (U2OS transfected with PU.1 vector and GFP vector), lane 13 (human THP1 monocytic leukaemia cell line). Negative controls – lane 2 (CBA thymus), lane 12 (U2OS transfected with GFP vector only).

Detectable Sfpi1/PU.1 protein was present only in the R235S carrying cell lines and in these two protein species (42 and 35 kDa) were present. Multiple forms of PU.1 have been described previously, eg Carey et al (1996) reported three forms in B-lymphocytes (42, 35 and 30 kDa). These forms are likely to represent a selection of the five phosphoserine isoforms described (Mao et al 1996).

The predicted impact of amino acid substitutions on protein function can be assessed by an increasing number of computer algorithms that assess the evolutionary conservation of motifs and/or changes to known functional motifs in proteins. SIFT (Ng and Henikoff 2001), Polyphen (Ramensky et al 2002), PANTHER (Brunham et al 2005), PhD-SNP (Capnotti et al 2006) and SNAP (Bromberg and Rost 2007) all predicted that the R235S, R235C and R235H mutations would significantly affect protein function (Table 3).

Table 3. Evaluation by various algorithms of disease-causing properties of amino acid variants R235S, R235C and R235H. Each of the algorithms reports results differently. In Panther scores < -3 are reported as 'disease causing', ie likely to affect protein function. PhD SNP reports alterations as 'neutral' or 'disease' with an increasing scale of severity. Polyphen scores changes that are 'probably damaging' to protein function on an increasing scale with values >2 being probably damaging. In SIFT scores are reported to two decimal places with those <0.05 reported as affecting protein function. SNAP reports changes likely to affect protein function as 'non-neutral' with an increasing numerical scale of likely severity.

	PANTHER	PhD-SNP	Polyphen	SIFT	SNAP
R235H	-10.80 Disease	Disease 3	PRD[1] 2.24	APF[2] 0.00	Non-neutral 6
R235C	-10.96 Disease	Disease 3	PRD 2.93	APF 0.00	Non-neutral 6
R235S	-10.60 Disease	Disease 1	PRD 2.46	APF 0.00	Non-neutral 6

[1] PRD, probably damaging; [2] APF, affect protein function

Clearly the R235S mutation creates a potential additional protein phosphorylation site. The algorithms NetPhos 2.0 (Miller and Blom 2009) and Scansite (Obenauer et al 2003) made strongly positive predictions for the phosphorylation of R235S. For example NetPhos 2.0 assigned R235S a score of 0.928, one of eight serine residues with strongly positive scores in the range 0.910-0.997.

To conclude, the analyses of Sfpi1/PU.1 in the cell lines has revealed:

- hemizygous or complete loss of *Sfpi1/PU.1* appears compatible with adaptation to *in vitro* culture
- all R235 mutants can adapt to culture but R235S mutants appear to adapt to *in vitro* culture more easily
- the R235 mutation types differentially affect mRNA transcript levels, although it should be noted that additional levels of control on *Sfpi1/PU.1* transcription are known and are likely to play a role
- there is not a simple relationship between transcript level and protein levels but the R235S protein is more abundant than the R235H or R235C mutants.
- multiple species of Sfpi1/PU.1 are present and the R235S protein is predicted to exist in high and low phosphoserine forms and the R235S site is likely to be phosphorylated.

2.6 Transcription of selected Sfpi1/PU.1 regulated genes in cell lines

The cell lines also provide an opportunity to explore the functional consequences of the Sfpi1/PU.1 mutation/expression patterns of genes regulated by the transcription factor. The expression of a selection of Sfpi1/PU.1 regulated genes was examined by RT-PCR using Sybar-green fluorescence incorporation. The selected genes and primer sequences used are shown in Table 4. Full methods are given in Brown et al (2010). Many genes are regulated by Sfpi1/PU.1, including those coding for DNA binding proteins, cell-cycle regulators and tyrosine kinases. We

chose a range of these genes based on their interaction with or regulation by Sfpi1/PU.1. Gata-1 and Gata-2 are sequence-specific DNA binding proteins interacting directly with Sfpi1/PU.1. Gata-1 is essential in lineage commitment of common myeloid progenitors and binding with Sfpi1/PU.1 results in mutual inhibition of transcriptional activity (Liew et al 2006 [29]). c-Jun is a component of the AP-1 transcription factor complex involved in monocyte differentiation brought about by its interaction, binding and co-activation of Sfpi1/PU.1 (Behre et al 1999 [30]). The association of the ETS domain of Sfpi1/PU.1 with the basic domain of c-Jun enables an interaction with macrophage colony stimulating factor receptor, resulting in cellular differentiation. Cdk6 regulates murine erythroleukaemia cells during terminal differentiation with a down-regulation in Cdk6 being required to reinitiate differentiation. Activation of *Sfpi1/PU.1* expression leads to a rapid induction of *Cdk6* mRNA in murine erythroleukaemia cells (Matushansky et al 2003 [31]). *Flt3*, a member of the receptor tryrosine kinase family, is the most commonly mutated gene in human AML. It is expressed in normal human bone marrow, in early progenitor cells, and at high levels in AML cells (Rosnet et al 1996 [32]). There is a reverse correlation between *Flt3* and *Sfpi1/PU.1* expression in cells, in which over-expression of Sfpi1/PU.1 represses *Flt3* promoter activity in AML patient samples (Inomata et al 2006 [33]), with higher levels of protein being detected than in normal bone marrow (Carrow et al 1996 [34]).

Table 4 Sfpi1/PU.1 regulated genes examined and primer sequences utilised

Gene	Forward primer	Reverse primer
Gata-1	caaacgaccactacaacac	ggttcctcgtctggattc
Gata-2	tgtgggctctaccacaagatgaatgg	tgacaatttgcacaacaggtgccc
c-jun	gagcatgacttgaacctg	acgtgagaaggtccgagtt
Flt3	tctctcaagcctcatttcct	actctcctggcttgttctt
Cdk6	accagcagtggacagataa	gactggagcaggacttat

Figure 11 shows that Gata-1 expression was severely reduced in all cell lines compared to normal bone marrow irrespective of *Sfpi1/PU.1* copy number, mutation status and expression level. *Gata-2* expression was also modestly reduced in general (Figure 11b). *c-Jun* and *Flt3* expression (11.c,d) also tended to be reduced except in 8016 where a slightly higher level of expression was detected. Expression of *Cdk6*, a gene that is reported to be upregulated in response to *Sfpi1/PU.1* in murine erythroleukaemias (Matushansky et al 2003) was, surprisingly elevated in the cell lines but there was no simple relationship between *Cdk6* mRNA and Sfpi1/PU.1 status.

Expression of the haemopoietic transcription factor *Gata-1* is severely reduced in all lines, *Gata-2* expression also tends to be reduced, likewise *c-Jun* and *Flt3* expression are in general reduced. It should be noted that mRNA from unfractionated normal bone marrow was used as the reference RNA for these comparisons. Some of the changes in the cell lines, for example the low Gata-1 expression, are likely to be attributable to the lineage origin of the AML cell lines. There is no clear mutation-dependent differences in the expression of these genes. By contrast *Cdk6* expression is uniformly elevated 1.5 – 4.5 fold. The expression of *Cdk6* is induced in mouse erythroleukaemias as a consequence of upregulated *Sfpi1/PU.1* (Matushansky et al 2003; Choe et al 2010). The results here indicate that there is not a simple relationship between Sfpi1/PU.1 levels and *Cdk6* expression. Clearly other factors regulate *Cdk6* expression in addition to Sfpi1/PU.1. It is of course also the case that the regulation of all the transcripts examined will be complex and affected by multiple factors.

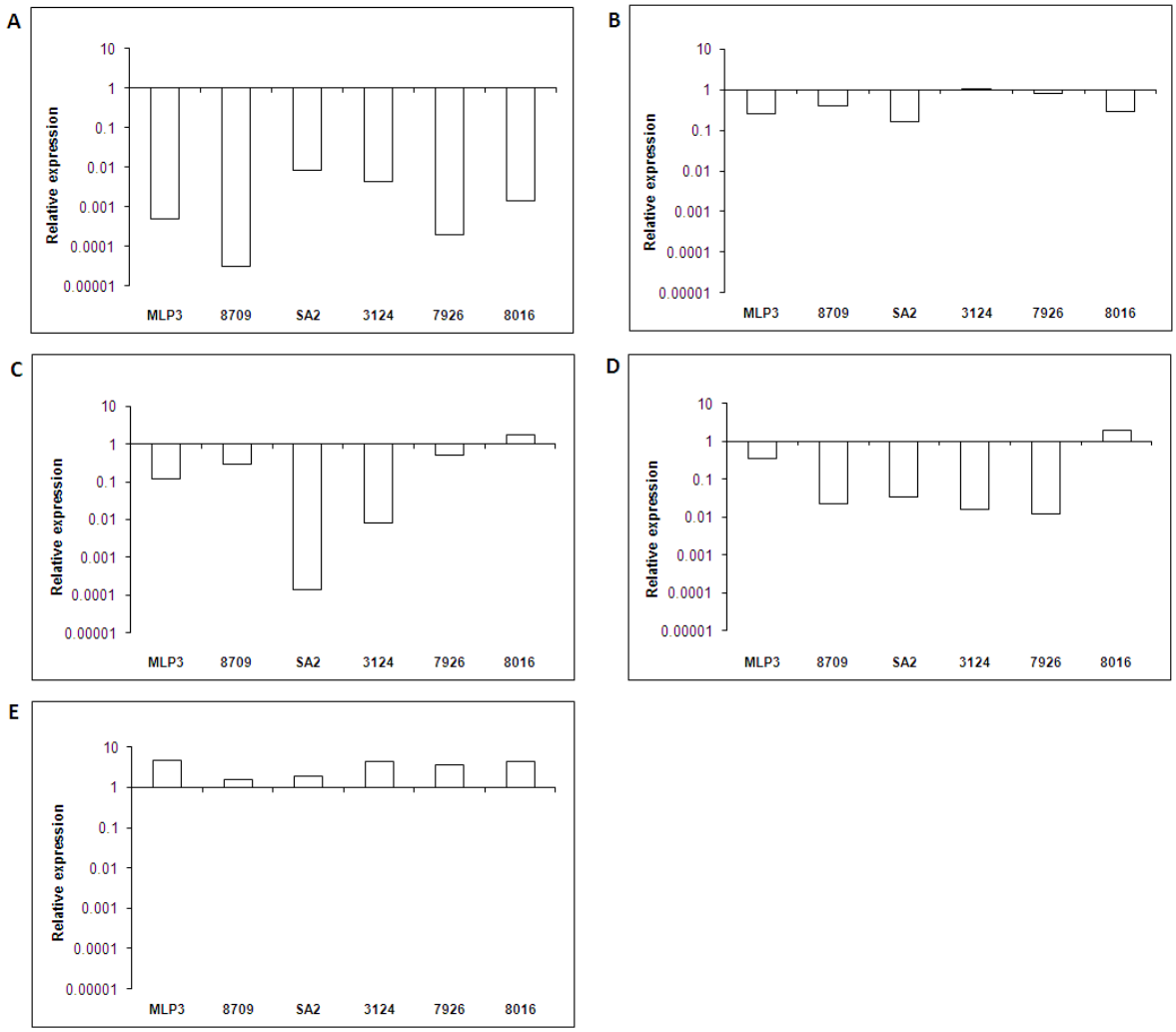

Figure 11. Expression of Gata-1 (A), Gata-2 (B), c-Jun (C), Flt3 (D) and Cdk6 (E) by Q-PCR relative to HPRT, normalised to CBA bone marrow (ΔΔCt method) in AML cell lines.

This transcriptional analysis suggests:

- AML-associated changes in *Sfpi1/PU.1* affect the differentiation and proliferative status of cells
- *Sfpi1/PU.1* mutation type does not significantly affect the functional consequences of the AML-associated mutations
- The relationship between *Sfpi1/PU.1* and *Flt3* expression provides a potential bridge between the mouse model and human myeloid leukaemogenesis.

2.7 A novel reporter gene system for radiation-induced changes to Sfpi1/PU.1

A long term aspiration of our radiation leukaemogenesis research is to be able to track radiation-induced events important in the development of AML in real time *in vivo*. A combination of *in vivo* imaging techniques with appropriate reporter gene constructs should facilitate such studies. We obtained mice carrying a green fluorescent protein (gfp) gene driven by the endogenous *Sfpi1/PU.1* promoter from Professor Steven Nutt (Nutt et al 2005). This strain expresses gfp in proportion to *Sfpi1/PU.1* expression. The reporter construct was bred onto the CBA/H and C57BL/6 genetic backgrounds by successive backcrossing over 5 or more generations.

The use of flow cytometry has proven that the majority of bone marrow cells expressing GFP also express *Sfpi1/PU.1* in CBA mice. Cells from the bone marrow of GFP positive CBA mice were harvested and immuno-stained with *Sfpi1/PU.1* antibody (Figures 12,13). Approximately 59% of the total bone marrow is GFP positive (this figure is true for both the *Sfpi1/PU.1* stained and unstained control samples).

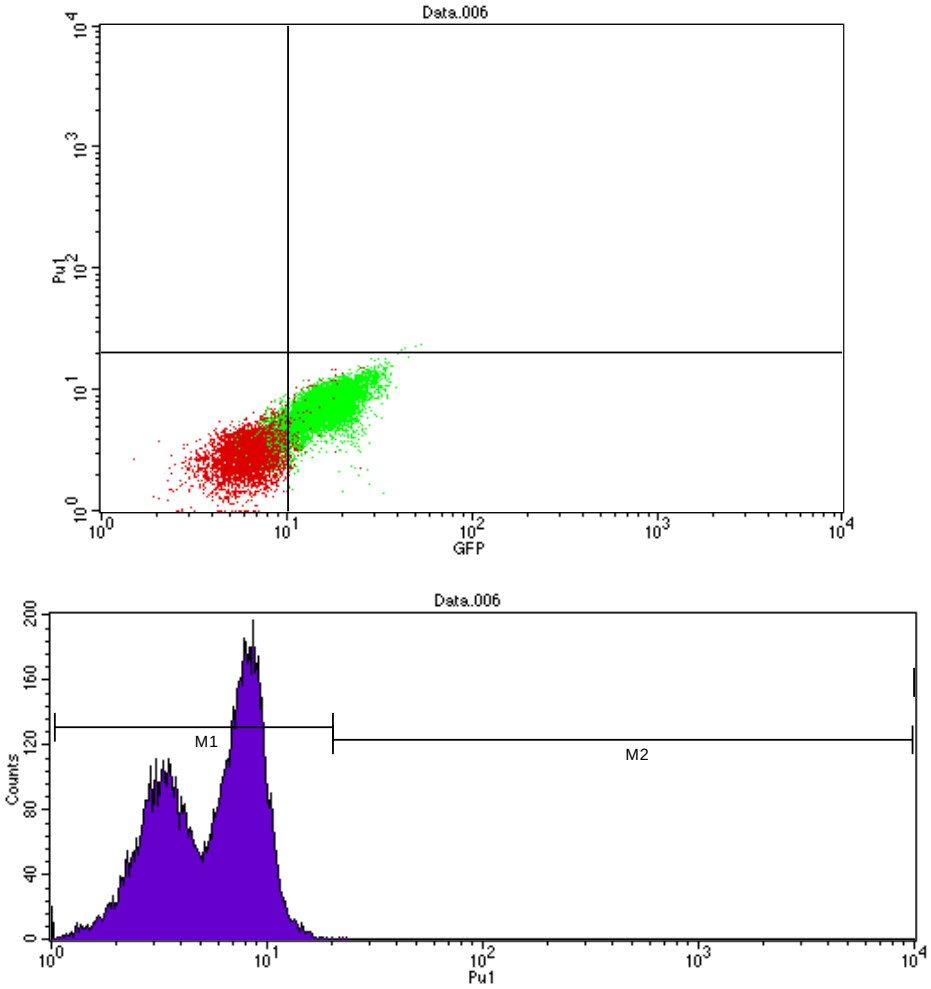

Figure 12. Gfp expression detected by flow cytometry in bone marrow cells from CBA mice carrying a gfp gene driven by the Sfpi1/PU.1 promotor – control with no anti-Sfpi1/PU.1 antibody staining. Two populations of cells can be distinguished on the basis of gfp expression (green and red cell populations in the top plot). There is no significant expression of Sfpi1/PU.1 (lower plot, compare with figure 13 below), with approximately 59% of cells being positive for gfp. The double peak in PU.1 expression below 10^1 is likely attributable to' cross talk' of gfp fluorescence into the PU.1 channel

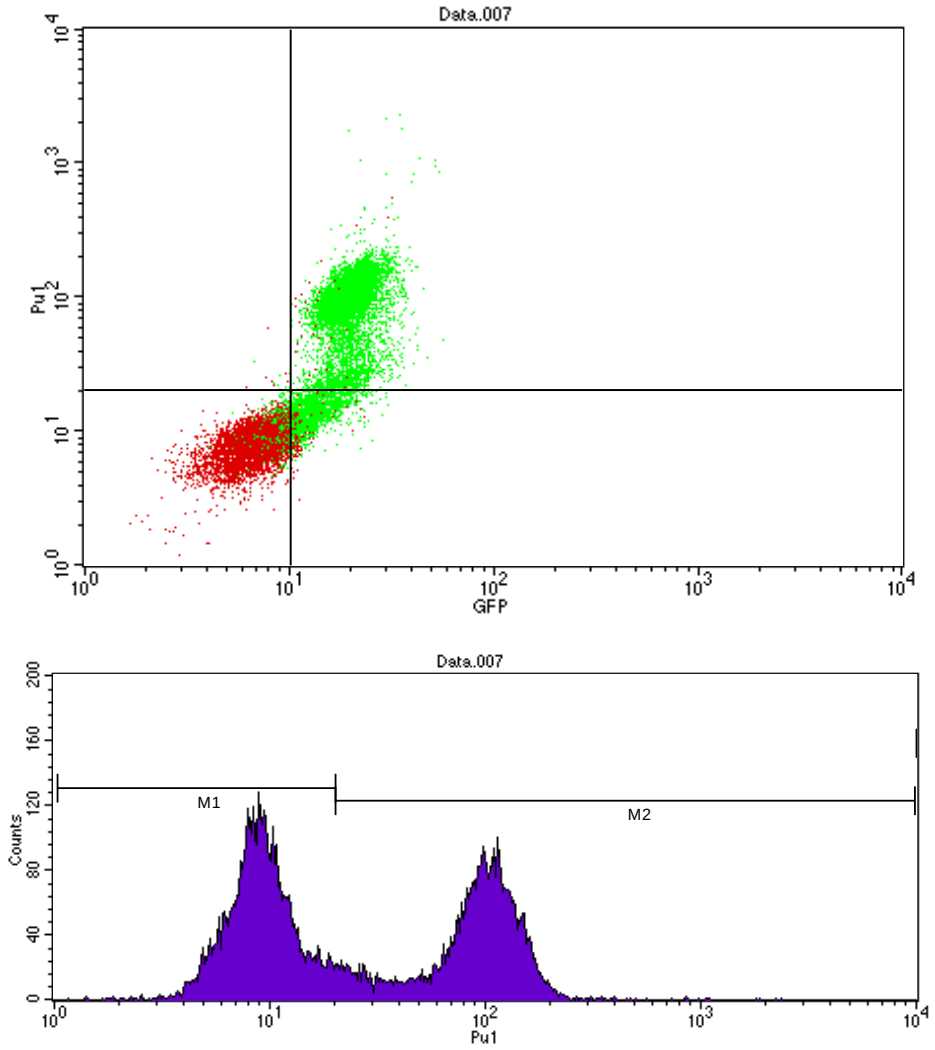

Figure 13. Gfp and Sfpi1/PU.1 expression detected by flow cytometry in bone marrow cells from CBA mice carrying a gfp gene driven by the Sfpi1/PU.1 promotor. Two populations of cells can be distinguished on the basis of Sfpi1/PU.1 staining. Sfpi1/PU.1 expression can be seen in approximately 82% of gfp positive cells.

Having established these strains a number of studies are being carried out including AML induction studies in mice carrying the gfp reporter in both a heterozygous and homozygous state. Groups of 50 mice carrying the *Sfpi1/PU.1-gfp* reporter in homozygous and heterozygous states on the CBA/H background have been exposed to 3 Gy x-irradiation. At the current time (12 months after irradiation) three AMLs have been diagnosed in heterozygous reporter carrying mice. Two of these appear gfp positive by flow cytometric analysis while one is gfp negative. No AML cases have been seen in homozygous reporter carrying mice at 10 months after exposure. Small groups of heterozygous and homozygous reporter carrying mice on a C57BL/6 background (AML resistant) have been irradiated also. Full analysis will include investigation of *Sfpi1/PU.1* losses and point mutations, and gene expression studies.

Currently we are also following up mice which were lethally irradiated and rescued by injection of haematopoietic stem cells/early progenitors (LSK: Lin-, c-Kit+ and Sca+) obtained by magnetic and flow-cytometric sorting. These cells were exposed to radiation *in vivo* or *in vitro* to study the role of the microenvironment in AML induction. Cells were sorted according to their level of gfp expression (as a surrogate of PU.1 expression and PU.1 copy number). These mice are currently being closely monitored for AML presentation. Retrospectively we will be able to tell which cells have been injected and further characterise them (aliquots of all the injected cells have been kept frozen).

In order to analyse independent radiation-induced chromosome 2 deletions, mice will be used to 'sort' (equivalent to a biological cell sorter) GFP-expressing hematopoietic progenitors. Briefly, sham- or 3 Gy irradiated (*in vitro* or *in vivo*) bone marrow cells from male CBA/H (carrying the gfp reporter gene in the homozygous state) will be injected in lethally irradiated recipient mice. Individual spleen colonies (CFU-S) will be dissected and analysed by flow-cytometry and FISH for PU.1 loss/level of expression. A percentage of colonies formed from individual progenitors should potentially carry chromosome 2 (PU.1) deletions and be detected by a decrease of gfp expression.

The full results of these long term experiments will only become available outside the finish date for the current project but will continue with support from HPA.

Nevertheless, these experiments are seen to be critical for the longer term development of this mouse model. As well as providing a first approach to monitoring leukaemia-associated events in living cells, two important outcomes may follow.

Firstly, the experiments will provide a proof-of-principle for the approach of using reporter genes. Full exploitation of *in vivo* imaging capacity will require the development of new reporters using reporter tags with tissue transmission properties superior to those of gfp. Longer wavelength fluorochromes would appear to be promising in this respect.

Secondly, the ability to distinguish cells by virtue of the gfp marker will allow the development of protocols to investigate the role of microenvironmental factors on AML development. The majority of our work to date has focused on target cell analysis. The combination of reporter constructs, surface marker analysis and transplantation protocols provides an opportunity to extend the analysis to investigate the effects of the irradiated microenvironment on leukaemogenesis. An important aspect of such work is the development of clonal assays capable of unequivocal determination of *gfp-Sfpi1/PU.1* losses. An outline of such a method is given in figure 14 below.

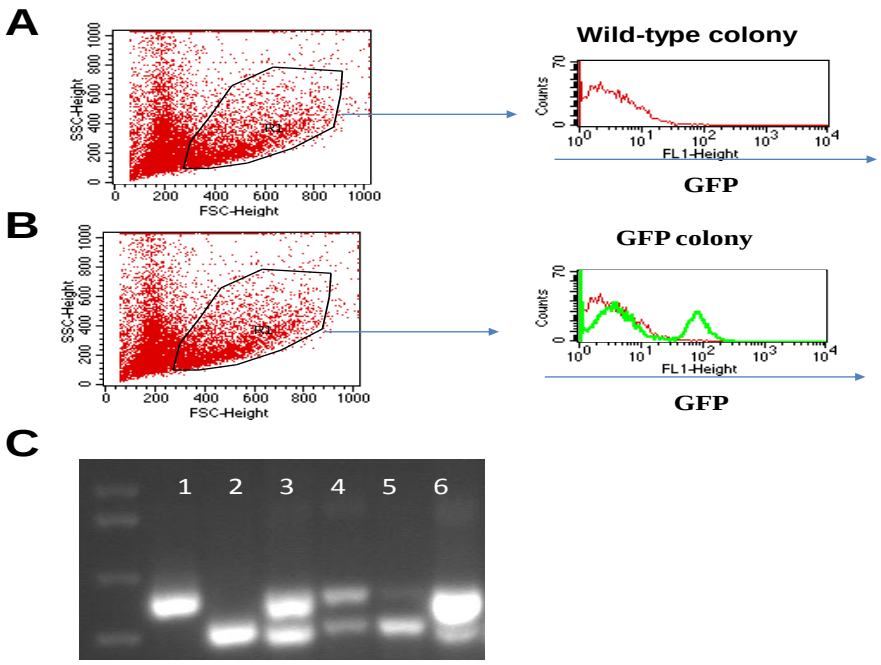

Figure 14. Protocol to screen live cells from colonies derived from GFP expressing mouse haematopoietic progenitors in order to detect those carrying a chromosome 2 deletions.

In this protocol, un-irradiated bone marrow (BM) cells are injected into lethally irradiated recipient mice (8.5 Gy, whole body irradiation). 12 days later, mice are sacrificed and single spleen colonies are dissected (1 to 4 per spleen). After disaggregation, cells are analysed by FACS showing a unique peak of non-GFP expressing cells (fig. 14A). The non gated cells are mostly dead cells and red blood cells. In the gated cells population, cells with lower side scatter are lymphocytes and cells with higher side scatter are from the myeloid lineage (i.e. monocytes and granulocytes). Following injection of GFP expressing BM cells, a different profile constituted of two clearly distinct peaks can be observed 12 days later; an overlay histogram showing ,in red the wild-type control colony and in green, the GFP positive colony is presented in (fig. 14B). The GFP positive peak corresponds to cells derived from a donor haematopoietic progenitor (GFP expressing cell). The other peak is composed of some contaminating spleen cells from the recipient mouse (GFP negative) and differentiated cells from the colony expressing less PU.1, therefore less GFP. The different sub-populations constituting the colony can be characterised using a panel of antibodies specific for some cell surface markers (e.g. CD3-B220-Mac1 and Gr1 for respectively T-cells, B-cells, monocyte and granulocyte identification).
BM cells from GFP heterozygote mice can also be exposed to a range of X-ray doses (for example 3 Gy) *in vivo* in the donor mouse or *in vitro* directly after isolation). The dissected colonies can be analysed by FACS (GFP expression analysis), PCR for confirmation (PU.1 loss screening, see fig. 14C (lane 1: wild-type, lane 2: GFP homozygote, lane 3: GFP heterozygote, lane 4: cells without copy loss, lane 5: cells with wild-type copy loss, lane 6: cells with GFP copy loss), FISH and an aliquot of live cells may be frozen for further studies.

2.8 Additional pathways of radiation leukaemogenesis

The cell line analysis of transcription (see section 2.6) pointed to a potential link between radiation-induced *Sfpi1/PU.1* mutations and *Flt3* expression. *Flt3* is the gene most commonly mutated in human acute myeloid leukaemia (eg Meshinchi and Appelbaum 2009). In human AML the most common mutation is the internal tandem duplication (ITD) which leads to ligand-autonomous activation of this receptor tyrosine kinase.

A simple PCR based assay method for *Flt3*-ITD mutation detection was provided by Dr Michael Rosemann (Helmholz Institute, Munich). Primary and *in vivo* transplanted AMLs have been screened for Flt3-ITD mutations. No Flt3-ITD mutations were observed in del2, *Sfpi1/PU.1*$^{+/-}$ AMLs. However, in three of seven *Sfpi1/PU.1*$^{+/+}$ AMLs Flt3-ITD mutations were detected, either in a homozygous or heterozygous state (Finnon et al 2010). This is seen as an important finding that provides stronger linkage between the CBA mouse radiation AML model and human AML. It should be noted also that some human AML associated translocations act, at least in part through disruption of Sfpi1/PU.1 regulation (eg Vangala et al 2003; Walter et al 2005). Also mutations in the PU.1 gene or its regulatory elements have been described in human AML (Mueller et al 2002; Bonadies et al 2010a; Bonadies et al 2010b).

These new findings of Flt3 mutations in radiation-induced AMLs allow a more complete classification of pathways of radiation leukaemogenesis (Figure 15).

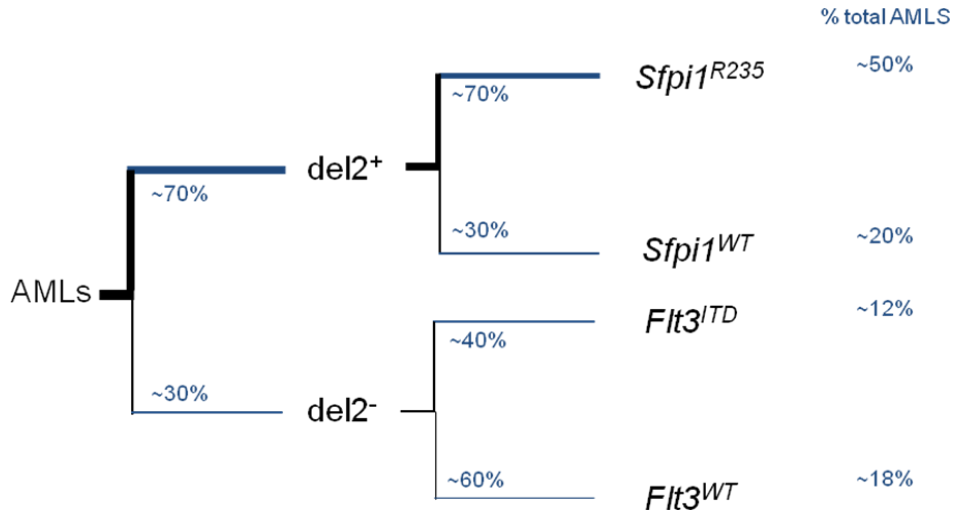

Figure 15. Classifcation of AMLs based on Sfpi1/PU.1 and Flt3 status.
From this study it is concluded that:

- *Flt3* mutations sometimes drive radiation leukaemogenesis
- multiple pathways of radiation leukaemogenesis exist.

2.9 Array comparative genomic hybridisation assessment of AML-associated losses

Early conventional cytogenetic analysis identified some potential additional chromosomal changes commonly associated with AML (eg Bouffler et al 1996a,b, 1997). There is only limited resolution possible with conventional cytogenetics. Studies with Dr Wei-Wen Cai (Baylor Collgege of Medicine) tried to identify additional regions of DNA sequence with gain or loss in both chr2 deleted (del2) AMLs (24) and non-del2 AMLs (2) by means of BAC array comparative genomic hybridisation (CGH). We previously identified a common region of amplification on chr6 in del2 AMLs and several other candidate regions of loss/gain in non-del2 AMLs including loses on chrs 12, 16 and 18, and amplification of a chr15 region. The chr18 region is of particular interest because it includes the interval that has been implicated as harbouring an AML susceptibility factor, *Rbbp8* (Darakhshan et al 2006) (Figure 16). To follow up on the chr18 region, loss of heterozygosity (LOH) studies have been utilised. Efforts focused on determining whether the interval harbouring an AML susceptibility factor on chr18 shows any loss events in CBA mice. A panel of 6 primary AML samples composed of 4 non-del2 AML with a matched brain control, and 2 del2 AML samples were chosen for use. Four primer sets located within 2cM of *Rbbp8* and 5 other primer sets located at 6, 8.7, 12, 16 and 18 cM were used. Results showed no loss or gain events in the AML panel (in either the del2 or non-del2 AMLs). From this it can be concluded that is very difficult to be confident of loss or gain of chromosome segments based on CGH data alone. No evidence for somatic changes to *Rbbp8* or other chr18 regions was obtained by PCR-based LOH analysis.

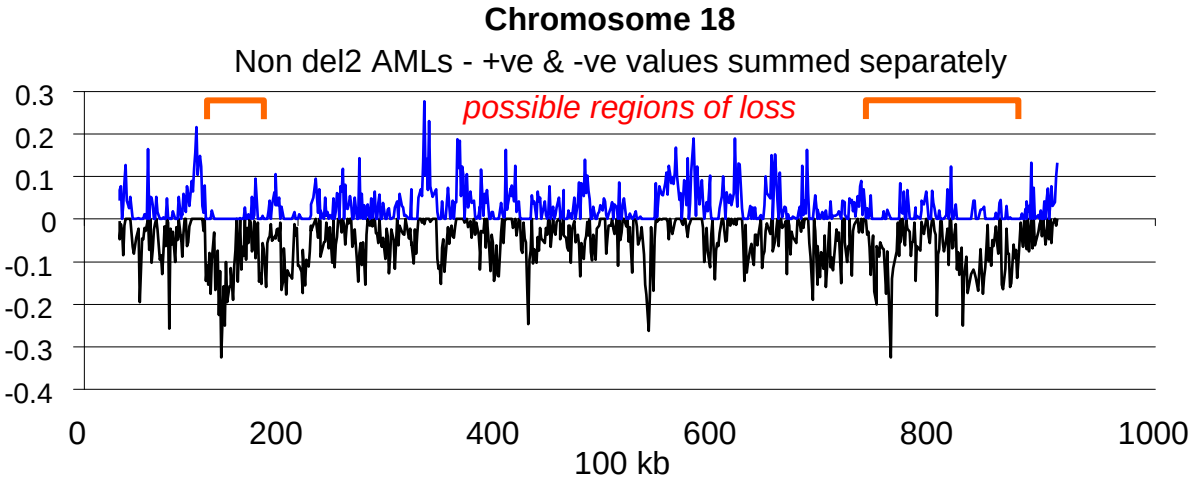

Figure 16. BAC array CGH analysis of chr 18 in two non-del2 AMLs. Positive and negative signals for each BAC clone were individually summed and are plotted. Possible regions of loss on chr 18 are indicated. Data supplied by Dr Wei-Wen Cai of Baylor College of Medicine.

2.10 Microsatellite instability

While not formally part of the work carried out under this contract recent studies have suggested that microsatellite instability (MSI) may contribute to leukaemogenesis after exposures to higher LET radiations (neutrons). While it is clear that MSI is not required for leukaemogenesis, the presence of MSI may suggest that high LET exposures have been involved (Haines et al, 2010)

From this data it is concluded that:

- MSI is not required for radiation leukaemogenesis
- MSI may contribute to leukaemogenesis after high LET exposures
- MSI may be a marker of the involvement of high LET exposure in tumorigenesis.

3. Conclusions

Overall, this project has enabled a much deeper understanding of the processes that contribute to radiation-induced acute myeloid leukaemogenesis in the mouse. The data obtained will be used to continue to build a whole organism systems level understanding of leukaemogenesis in this model. This system may therefore be considered to be critical for a proof of principle test of mechanistic/systems level approaches to modelling cancer and estimating cancer risk. Several promising future research directions have been identified including consideration of micro-environmental effects as well as target cell phenomena.

4. Publications from this grant

Brown NL, Finnon R, Bulman RA, Finnon P, Moody J, Bouffler SD, Badie C (2010). *Sfpi1/PU.1* mutations in mouse radiation-induced AMLs affect RNA and protein abundance and associate with disrupted transcription. *Leukaemia Res.* 2010, Jul 15 [Epub ahead of print].

Finnon R, Moody J, Brown N, Badie C, Huiskamp R, Meijne E, Sutmuller M, Rosemann M, Bouffler S (2010). Flt3-ITD mutations detected in a mouse model of radiation-induced acute myeloid leukaemia (AML) identified a direct mechanistic link to human AML. *Manuscript submitted.*

Peng Y, Brown N, Finnon R, Warner CL, Liu X, Genik PC, Callan MA, Ray FA, Borak TB, Badie C, Bouffler SD, Ullrich RL, Bedford JS, Weil MM (2009a). Radiation leukaemogenesis in mice: loss of PU.1 on chromosome 2 in CBA and C57BL/6 mice after irradiation with 1 GeV/nucleon ^{56}Fe ions, x-rays or γ rays. Part 1. Experimental observations. *Radiat. Res.* **171**: 474-483.

Peng Y, Borak TB, Bouffler SD, Ullrich RL, Weil MM, Bedford JS (2009b). Radiation leukaemogenesis in mice: loss of PU.1 on chromosome 2 in CBA and C57BL/6 mice after irradiation with 1 GeV/nucleon ^{56}Fe ions, x-rays and γ rays. Part II. Theoretical considerations based on microdosimetry and the initial induction of chromosome aberrations. *Radiat. Res.* 171: 484-493.

5. Staff contributing to this work

Principle investigator: Simon Bouffler
Co-investigators: Natalie Brown (DoE funded), Christophe Badie, Rosemary Finnon, Paul Finnon, John Moody (to 2007 only).

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