



Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Verbiest, T; Bouffler, S; L.Nutt, S; Badie, C

Title:

PU.1 downregulation in murine radiation-induced acute myeloid leukaemia (AML): From molecular mechanism to human AML

Date:

2014-07-10

Citation:

Verbiest, T., Bouffler, S., L.Nutt, S. & Badie, C. (2014). PU.1 downregulation in murine radiation-induced acute myeloid leukaemia (AML): From molecular mechanism to human AML. *Carcinogenesis*, 36 (4), pp.413-419. <https://doi.org/10.1093/carcin/bgv016>.

Persistent Link:

<https://hdl.handle.net/11343/263694>

License:

[CC BY-NC](#)

## REVIEW

# PU.1 downregulation in murine radiation-induced acute myeloid leukaemia (AML): from molecular mechanism to human AML

Tom Verbiest<sup>1,2</sup>, Simon Bouffler<sup>1</sup>, Stephen L.Nutt<sup>3,4</sup> and Christophe Badie<sup>1,\*</sup>

<sup>1</sup>Biological Effects Department, Centre for Radiation, Chemical and Environmental Hazards, Public Health England, Didcot OX11 0RQ, UK, <sup>2</sup>CRUK & MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford OX3 7DQ, UK, <sup>3</sup>Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia and <sup>4</sup>Department of Medical Biology, University of Melbourne, Parkville, Victoria 3010, Australia

\*To whom correspondence should be addressed. Tel: +441235825088; Fax: +441235833891 Email: [christophe.badie@phe.gov.uk](mailto:christophe.badie@phe.gov.uk)

## Abstract

The transcription factor PU.1, encoded by the murine *Sfpi1* gene (*SPI1* in humans), is a member of the Ets transcription factor family and plays a vital role in commitment and maturation of the myeloid and lymphoid lineages. Murine studies directly link primary acute myeloid leukaemia (AML) and decreased PU.1 expression in specifically modified strains. Similarly, a radiation-induced chromosome 2 deletion and subsequent *Sfpi1* point mutation in the remaining allele lead to murine radiation-induced AML. Consistent with murine data, heterozygous deletion of the *SPI1* locus and mutation of the –14 kb *SPI1* upstream regulatory element were described previously in human primary AML, although they are rare events. Other mechanisms linked to PU.1 downregulation in human AML include *TP53* deletion, *FLT3*-ITD mutation and the recurrent *AML1*-*ETO* [t(8;21)] and *PML*-*RARA* [t(15;17)] translocations. This review provides an up-to-date overview on our current understanding of the involvement of PU.1 in the initiation and development of radiation-induced AML, together with recommendations for future murine and human studies.

## Introduction

Just over a decade following the discovery of X-rays by Wilhelm Roentgen in 1895, the first cases of leukaemia were suspected in medical radiation workers (1). Over the past 70 years, a variety of epidemiological studies provided support for an increased leukaemia incidence after both high and low dose radiation exposure (radiotherapy and CT-scan, respectively). The continuing epidemiological survey of the Japanese atomic bomb survivors estimates that ~50% of all leukaemia cases observed in survivors exposed to >5 mGy, are associated with radiation exposure. Remarkably, over the last 15 years of this follow-up, acute myeloid leukaemia (AML) became the pre-dominant radiation-associated leukaemia, accounting for ~80% of excess leukaemia cases (2). Although a clear association between radiation exposure and leukaemia development is acknowledged, today, 80 years since two-time Nobel Prize winner Marie Curie died of radiation-induced leukaemia, the underlying mechanisms remain unidentified. Elucidating the

cytogenetic and molecular alterations in murine radiation-induced AML (rAML) should provide further insight in the genetic basis of human radiation leukaemogenesis. Here we provide an overview on our current understanding of the involvement of the haematopoietic transcription factor PU.1 in the initiation and development of murine rAML, and seek to further define its involvement in human primary and therapy-related AML (tAML).

## Murine modelling of rAML

Since 99% of human protein-coding genes are also present in mice and given the power of murine genetic interventions (including gene knock-in/-down), the use of murine models remains the most representative and ethical option available to study the cytogenetic and molecular mechanisms underlying radiation leukaemogenesis. The CBA mouse strain is regarded

Received: November 18, 2014; Revised: February 4, 2015; Accepted: February 24, 2015

© The Author 2015. Published by Oxford University Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

**Abbreviations:**

|      |                                   |
|------|-----------------------------------|
| AML  | acute myeloid leukaemia           |
| CMP  | common myeloid progenitor         |
| GMP  | granulocyte/macrophage progenitor |
| HSC  | haematopoietic stem cell          |
| MDR  | minimal deleted region            |
| rAML | radiation-induced AML             |
| tAML | therapy-related AML               |
| URE  | upstream regulatory element       |

as the primary model in the study of rAML due to the very low background leukaemia incidence (<1/1000), the reproducible rAML induction in ~20% of mice exposed to a single acute 3 Gy X-irradiation, and the histopathological similarities with human myeloid leukaemia (3). The CBA inbred strain originates from the cross of a Bagg albino female and a DBA male. Three rAML susceptible CBA substrains [CBA/H, MRC Harwell (UK); CBA/Ca, Institute of Animal Genetics (UK); CBA/Cne, ENEA Casaccia Laboratory (Italy)] have been extensively used in the study of radiation leukaemogenesis (4). Beside the CBA strain, three further mouse strains were described previously as moderately susceptible to rAML: the RF, SJL/J, and the C3H/He strains. For the above-mentioned reasons, we will mainly focus on the CBA leukaemogenesis model and its relevance to rAML in humans.

### Cytogenetic aberrations following X-ray exposure

Twenty-four hours following a single acute exposure to 3 Gy whole-body X-irradiation (the optimal AML induction dose), all CBA/H mice exhibit bone marrow cells carrying chromosome 2 aberrations as assessed by G-banding, at an approximately 2-fold greater frequency in chromosome 2 compared with chromosomes 1 and 3 (5). Conversely, chromosomal aberration frequencies in chromosomes 1, 2 and 3 of non-susceptible mouse strains (such as C57BL/6 and AKR) are statistically identical (6). Over time, an increasing number of mice show clonal expansion of bone marrow cells with chromosome 2 aberrations. After 12–18 months, ~20% of the irradiated mice develop rAML (5). Leukaemic mice present in most cases with hepato-/splenomegaly and myeloblastic infiltration of the bone marrow, liver and spleen (3).

The first experimental murine rAML cases were described in the early 1930s. However, it was not until 1977 that a partial deletion of one chromosome 2 homologue was reported as the characteristic molecular finding in murine rAML cells (>80% of the cases), irrespective of the radiation quality (high or low linear energy transfer) (7–10). Almost 20 years later, the loss of a putative tumour suppressor gene in the minimal deleted region (MDR) on chromosome 2 was postulated (11), followed a few years later by the identification of the *Sfpi1* gene which encodes the transcription factor PU.1 (12). Fe-ion-irradiated mice initially showed two to three times more *Sfpi1* deleted cells than X- or  $\gamma$ -irradiated mice, but by 1-month post-irradiation, similar numbers were observed in both groups, indicating that although Fe-ions induce *Sfpi1* deletions more effectively, they also cause more damage to the cells than sparsely ionising radiation and eventually result in increased cell killing. rAML-resistant C57BL/6 mice had significantly fewer *Sfpi1* deleted cells compared with rAML-susceptible mice, and the deleted cells did not persist in the C57BL/6 mice, irrespective of the radiation quality (13). Genetic background therefore seems to play an important role in radiation-induced leukaemogenesis.

Aside of a consistent partial chromosome 2 deletion, secondary chromosomal aberrations were reported in >30% of murine

rAMLs: (i) loss/gain of the Y chromosome (7,8), (ii) loss/gain of chromosome 6 (7,8) and (iii) allelic loss on chromosome 4 (*Lyr2/TLRS5*) (7,14). Most chromosome 2 deletions are extensive (>20 cM, representing 33–66% of chromosome 2), while most chromosome 4 deletions are relatively small (<10 cM; involving <10% of chromosome 4) (14).

### *Sfpi1* exon 5 point mutation

Interestingly, in ~85% of murine rAML cases the remaining *Sfpi1* copy exhibits point mutations confined to a single CGC sequence in exon 5, the DNA binding domain of the protein [Table 1; *Sfpi1* DNA sequence from the Mouse Genome Informatics (MGI) entry MGI:98282]. The amino acid residue arginine 235 (R235) is replaced with cysteine (R235C; 62%), histidine (R235H; 9%), serine (R235S; 8%) or leucine (R235L; 2%). The first cytosine of the CGC sequence is often methylated (10,16). Spontaneous deamination of 5-methylcytosine leads to a C to T transition via uracil formation and the replacement of thymine in one of the two daughter strands during the next replication round. Similarly, the mutation spectrum of 24 human primary AML cases reported mainly transitions, with C to T transition as the most common single nucleotide mutation (19). To be noted, TET2, an important epigenetic regulator, which oxidates 5-methylcytosine, is often found mutated in human primary AML (20). We have recently sequenced exon 5 in a large murine rAML panel (>100 cases). Remarkably, no mutations could be found outside codon 235 (unpublished observations). It is surprising that no additional passenger mutations are captured between the occurrence of the initiating event (chromosome 2 deletion) and the second hit (R235 point mutation), as the latency for murine rAML is over a year.

Recently, the R235 point mutation was reported as not being a major rate limiting step in murine rAML (21). There are data suggesting that this point mutation probably leads to complete abolition of PU.1 activity (22), and that mice carrying a deletion of the other allele might develop AML relatively soon after the mutation is acquired. To verify this observation, it will be important to investigate AML frequency and latency in mice engineered to carry a R235 point mutation (see Future directions). It was also postulated that hemizygosity for one of the numerous genes located in the MDR could facilitate radiation leukaemogenesis (21). For example, the *DDB2* gene is present in the MDR and is involved in nucleotide excision repair. Nevertheless, this repair pathway is associated with the resolution of bulky adducts and pyrimidine dimerization, and not with abasic sites and damaged bases which are corrected by the base excision repair pathway, and its role in point mutagenesis has yet to be clarified.

The two-hit model of rAML involves a first irreversible mutational hit (interstitial deletion of one *Sfpi1* gene on chromosome 2) creating intermediate cells that have a growth advantage but which are not fully malignant. Subsequently, intermediate cells acquire a second mutational hit (point mutation in the remaining *Sfpi1* copy), resulting in the formation of a malignant cell which undergoes further clonal expansion and eventually develops into cancer (23). This two-hit model is supported by the finding that *Sfpi1* mutations are only observed in rAMLs with a partially deleted chromosome 2 homologue (9,10). Whereas 87% of (CBA/H  $\times$  SJL) F1 rAMLs (i.e. 2 rAML-susceptible genomes) harbour *Sfpi1* point mutations (16), only 67% of (CBA/H  $\times$  C57BL) F1 rAMLs [i.e. one rAML-susceptible genome (CBA/H) and one rAML-resistant genome (C57BL)] harbour this point mutation (18). This percentage decreases even further when backcrossing

**Table 1.** Frequency of *Sfpi1* R235 point mutations in murine rAML

| Genetic background                        | rAML-susceptible genomes | Radiation quality | Dose   | WT R235     | R235C       | R235H      | R235L     | R235S      | Reference |
|---|--------------------------|-------------------|--------|-------------|-------------|------------|-----------|------------|-----------|
|   |                          |                   |        | CGC         | TGC         | CAC        | CTC       | AGC        |           |
| CBA/H                                     | 2                        | X-ray             | 3 Gy   | 17% (1/6)   | nd          | nd         | nd        | nd         | (15)      |
| C3H/He                                    | 2                        | X-ray             | 3 Gy   | 19% (5/27)  | 67% (18/27) | 0          | 0         | 15% (4/27) | (9)       |
|   | 2                        | $\gamma$ -ray     | 3 Gy   | 22% (4/18)  | 50% (9/18)  | 11% (2/18) | 0         | 17% (3/18) |           |
| (CBA/H $\times$ SJL) F1                   | 2                        | $\gamma$ -ray     | 3 Gy   | 13% (5/39)  | 54% (21/39) | 18% (7/39) | 5% (2/39) | 3% (1/39)  | (16)      |
| CBA/CaJ                                   | 2                        | $\gamma$ -ray     | 2 Gy   | 0           | 100% (7/7)  | 0          | 0         | 0          | (10)      |
|   |                          |                   | 3 Gy   | 14% (1/7)   | 86% (6/7)   | 0          | 0         | 0          |           |
| CBA/H                                     | 2                        | neutron           | 0.4 Gy | 44% (4/9)   | 56% (5/9)   | 0          | 0         | 0          | (10)      |
| (CBA/H $\times$ C57BL/6) $\times$ CBA/H   | 1.5                      | X-ray             | 3 Gy   | 14% (4/29)  | 66% (19/29) | 7% (2/29)  | 3% (1/29) | 10% (3/29) | (17)      |
| (CBA/H $\times$ C57BL/6) F1               | 1                        | X-ray             | 3 Gy   | 18% (3/17)  | nd          | nd         | nd        | nd         | (15)      |
| (CBA/H $\times$ C57BL/Lia) F1             | 1                        | X-ray             | 3 Gy   | 33% (15/45) | 49% (22/45) | 2% (1/45)  | 0         | 16% (7/45) | (18)      |
| (C3H/He $\times$ C57BL/6) F1              |                          |                   |        |             |             |            |           |            |           |
| (CBA/H $\times$ C57BL/6) $\times$ C57BL/6 | 0.5                      | X-ray             | 3 Gy   | 89% (17/19) | 0           | 0          | 0         | 11% (2/19) | (17)      |

nd, not determined.

(CBA/H  $\times$  C57BL) F1 mice to C57BL (11%). Intriguingly, none of these 11% *Sfpi1* point-mutated rAMLs exhibited the most common C to T transition (17). The type of R235 point mutation appears to differentially affect PU.1 mRNA levels in rAML cell lines. Whereas mRNA levels in R235S AMLs are similar to those observed in normal bone marrow, mRNA levels are 4-fold higher in R235C AMLs. Interestingly, PU.1 mRNA levels did not correspond to protein levels (no detectable protein levels in R235C and R235H AMLs). This discrepancy might be explained by a greater stability of the R235S protein, a differential regulation of mRNA translation, or be related to protein phosphorylation as serine 235 has been identified as a strong novel candidate phosphorylation site (24). Furthermore, the mutations were assessed as being deleterious to protein function by a range of bioinformatics approaches. In contrast, several PU.1 target genes (including *Flt3*, *GATA-1* and *c-Jun*) were downregulated, irrespective of the type of R235 mutation (24). While the fact that these are single base substitutions suggests that they are of spontaneous origin (13), it remains unknown at what time after irradiation these point mutations occur.

## Target cells for rAML development

Little is currently known about the characteristics of the 'cell at risk' in radiation leukaemogenesis and it remains unknown whether the mutations leading to the generation of leukaemic stem cells occur in haematopoietic stem cells (HSCs) or progenitor cells. Adult HSCs reside primarily in the  $G_0$  phase of the cell cycle (25). Haematopoietic reconstitution following radiation exposure requires the release of the surviving HSCs from their quiescent state into  $G_1$ . Approximately 60% of surviving HSCs actively cycle for >10 months, with the number of cell divisions per surviving HSC reported 10 times as high compared with unexposed mice (26). Upon entry in  $G_1$ , DNA damage is primarily repaired by error-prone non-homologous end-joining, potentially promoting the formation of *de novo* mutations following DNA damage (25). Similarly, the enhanced replicative stress contributes to premature HSC ageing and induction of HSC premature senescence in a reactive oxygen species-dependent manner (27), hereby decreasing their DNA repair capacity and rendering them more prone to spontaneous mutations. This hypothesis is further supported by the finding of a common myeloid progenitor (CMP)-like leukaemic stem cell (*Lin*<sup>-</sup> *Sca-1*<sup>-</sup> *c-Kit*<sup>+</sup>

*CD34*<sup>+</sup>) in a murine rAML model where it was postulated that, amid continuous cycling to reconstitute the CMP population, chromosome 2 deleted HSCs acquired additional aberrations, including point mutations of the remaining *Sfpi1* allele (28).

The increased mutation rate with age might also play a role in the acquisition of secondary point mutations, hereby explaining the relatively long latency between exposure and rAML presentation (10). In addition, PU.1 directly regulates the HSC cell cycle machinery by inhibition of cell cycle activators and induction of cell cycle inhibitors: loss of PU.1 autoregulation dysregulated the balanced cell cycle regulation, leading to excessive proliferation (29), thereby increasing the acquisition of point mutations, and eventually resulting in either exhaustion of the HSC pool or leukaemia.

Recent advances in genetic engineering of mouse models allow tracking of cytogenetic alterations in living cells, from the time of irradiation and throughout tumour ontogenesis. In 2005, the Nutt laboratory generated a *Sfpi1*<sup>GFP</sup> reporter strain containing an internal ribosome entry site GFP cassette adjacent to the *Sfpi1* gene within the radiation-induced MDR (30). Therefore, GFP fluorescence levels decrease (CBA/H *Sfpi1*<sup>GFP/GFP</sup>) or disappear (CBA/H *Sfpi1*<sup>GFP/+</sup>) following a radiation-induced chromosome 2 deletion, allowing this leukaemia initiating event to be detected by flow cytometry in live leukaemic cells (31). Using fluorescent in situ hybridisation, we previously demonstrated that partial chromosome 2 deletions are present in the HSC-enriched LSK subpopulation, but are not observed in the more differentiated lineage-negative cells (32).

## PU.1 and haematopoietic cell fate

Beyond its role as a tumour suppressor, PU.1 plays a vital role in commitment and maturation of the lymphoid and myeloid lineages. Analysis of *Sfpi1*<sup>GFP</sup> reporter mouse strains has revealed a dynamic regulation of PU.1 expression in adult haematopoietic cell lineages. All HSCs and common lymphoid progenitors express high levels of PU.1, with expression being reduced to a characteristic low level in early B-cell development. In contrast, two distinct PU.1 expressing subpopulations were identified within the CMPs. PU.1<sup>high</sup> CMPs were characterised as precursors of granulocyte/macrophage progenitors (GMPs), while the PU.1<sup>low</sup> CMPs were reported immediate precursors of the megakaryocyte/erythrocyte progenitors, confirming the hypothesis that downregulated PU.1 expression in CMPs restricts

their differentiation to the erythroid and megakaryocytic lineages (30). Small changes in PU.1 expression are also proposed to impact on cell fate decisions of the GMP through alterations in the PU.1 to CEBPA ratio: relatively high PU.1 commits GMPs towards a monocyte/macrophage fate, while increased CEBPA results in commitment towards granulocytes (33). Similarly, lymphomyeloid progenitors lengthen their cell cycle to allow PU.1 to accumulate to higher levels, which is functionally important for macrophage differentiation (34).

The proximal promoter of *Sfpi1* contains, beside Oct-1, Sp1 and GATA-1 binding sites, binding sites for PU.1, suggesting autoregulation of its expression (35). Additional regulation is under control of a conserved distal regulatory enhancer (upstream regulatory element, URE) located -14kb from the transcriptional start site (36). Targeted deletion of the URE in mice (*Sfpi1<sup>ure/ure</sup>*) reduced the PU.1 expression to ~20% of the wild-type level, leading to impaired maturation of myeloid progenitors (37). Molecular analysis of the URE revealed binding sites for various transcription factors, including Elf-1, NF-κB and PU.1 itself (29,36,38).

In contrast to *Sfpi1<sup>ure/ure</sup>* mice, *Sfpi1<sup>-/-</sup>* embryos (expressing 0% of the wild-type PU.1 protein) die at day 18 of gestation, displaying a multilineage defect in the maturation of erythroblasts and the development of progenitors for granulocytes, monocytes, and B- and T-lymphocytes (39). When transplanted in lethally irradiated mice, embryonic stem cells of *Sfpi1<sup>-/-</sup>* mice contribute to the formation of erythrocytes, but not to cells of myeloid and lymphoid origin (40). Six months following transplantation of *Sfpi1<sup>-/-</sup>* foetal HSCs into lethally irradiated mice, haematopoiesis disappears completely, indicating the essential role of *Sfpi1* in HSC self-renewal. A second *Sfpi1*-deficient mouse model generated by the insertion of a Neomycin resistance cassette into exon 5, survives up to two weeks post-birth and is likely to be a severely hypomorphic allele (41). This conclusion is supported by the similar early lethality of two further PU.1-knock-down mouse models (*Sfpi1<sup>BlaC/BlaC</sup>* and *Sfpi1<sup>BN/BN</sup>* mice, expressing 2% and 20% of the wild-type PU.1 protein, respectively) generated by inserting a LacZ expression cassette into exon 1 of *Sfpi1* (42).

Conditional deletion models have now overcome the limitations of the early lethality of *Sfpi1*-deficient mice. Inactivation of PU.1 in adult HSCs resulted in severely perturbed haematopoiesis, characterized by excess granulopoiesis and impaired production of other myeloid cells and lymphocytes as well as decreased ability to compete with wild-type HSCs (43). Whereas a *Sfpi1*-knockout in myeloid progenitors inhibited their maturation but not their proliferation, neither maturation nor proliferation of B-cells were altered by a knockout in lymphoid progenitors (44).

## Gene regulation by PU.1

Recent studies have identified tens of thousands of PU.1 binding sites in bone marrow derived macrophages. Most of the sites are located in introns and distant intergenic regions, and possess chromatin signatures of enhancers. PU.1 also binds to 80% of promoters associated with actively transcribed genes. Ectopic binding of PU.1 in those studies has an instructive role in nucleosome remodelling, changing chromatin marks and establishing functional enhancers (45,46). In this sense, PU.1 is considered to have a 'pioneer' role in establishing competence for genes to be regulated, in a positive or negative fashion, depending on the cross-talk with other transcription factors and co-factors. Prominent among the many known PU.1-regulated genes, are those encoding cytokine receptors and integrins (47,48).

PU.1 has long been recognized as a dose-dependent regulator of haematopoietic cell fate determination, however surprisingly few dose dependent target genes have been reported to date. Examples of dose-sensitive genes include *Flt3*, whose expression is reduced

in *Sfpi1<sup>+/-</sup>* progenitors (49), and *Sfpi1<sup>BlaC/BlaC</sup>* and *Sfpi1<sup>BN/BN</sup>* mice that show a dose-dependent suppression of various T-cell and natural killer cell genes in interleukin-3 dependent myeloid cell lines (42). A direct comparison of gene expression in adult HSCs derived from *Sfpi1<sup>ure/ure</sup>* and *Sfpi1<sup>-/-</sup>* [generated from a PU.1 conditional knockout strain (22)] mice would greatly add to our understanding of how PU.1 functions in a dose-dependent manner.

## PU.1 and myeloid leukaemia

In keeping with the role of PU.1 as a suppressor of rAML, *Sfpi1<sup>ure/ure</sup>* mice or those resulting from the conditional deletion of the DNA binding Ets domain (exon 5) develop AML (22,37). For both models, ~95% of the mice develop AML in 8 months, a significant shortening compared with the 12–18 months latency period following radiation exposure. Leukaemia is observed even earlier (within 3 months) in (*PU.1<sup>ure/ure</sup> × p53<sup>-/-</sup>*) F1 mice, only expressing 10% of wild-type PU.1 levels. The highly aggressive AML phenotype is attributed to further upregulation of MYB and miR-155 upon p53 loss, resulting in additional downregulation of PU.1 (50). These data clearly indicate that the transcription factor PU.1 acts as a tumour suppressor. However, *Sfpi1* can also act as an oncogene in other contexts, since PU.1 overexpression inhibits erythroid lineage maturation, eventually resulting in erythroleukaemia (51).

## Relationship between murine rAML and human (t)AML

tAML arises following exposure to chemo- and/or radiotherapy with an average latency period of 5 years, and is more frequently accompanied by unbalanced cytogenetic abnormalities compared with primary AML (75% versus 51%, respectively). The cytogenetic abnormalities 5q-/-5 and 7q-/-7 are often overrepresented in tAML. Among patients with a normal karyotype, a similar incidence of molecular abnormalities is observed in both primary AML and tAML (52). A Danish tAML study predominantly observed *FLT3* mutations in patients who previously received radiotherapy and in those patients with a normal karyotype (53). The most common aberrations were chromosome 5 and 7 deletions (50–60% of tAMLs), followed by *FLT3*-ITD and *TP53* point mutations in 20% of tAMLs. Interestingly, the genetic defects 5q-/-5 and 7q-/-7 were positively associated with *TP53* and *AML1* point mutations, respectively (53). It is important to note that a comparison with murine rAML data is difficult as neither study distinguishes between tAML patients who received radiation therapy alone, chemotherapy alone or a combination of both. To overcome this limitation, we are currently analysing historical samples of tAML patients who received radiation therapy alone and which should provide a better understanding of human tAML development following radiation therapy. Similarly, FISH analysis of Japanese AML patients revealed that those patients heavily exposed (>1 Gy to the bone marrow) during the Hiroshima atomic bombings had a higher incidence of chromosomes 5, 7, 13 and 20 deletions, compared with non-exposed Japanese AML patients (54).

Recently, we reported the presence of *Flt3*-ITD mutations in murine rAML (3/30 rAMLs) (15). Remarkably, they were only found in rAML with an intact chromosome 2 and no *Sfpi1* point mutation, suggesting the involvement of *Flt3*-ITD and *Sfpi1* are mutually exclusive and which is in agreement with published data from the Danish tAML study (53). The observed murine *Flt3*-ITD mutations were similar to those seen in human primary AMLs (10–30bp insertion into the tyrosine-rich juxtamembrane domain). Trisomy 8 is also observed in human primary and tAML (chromosome 8 is homologous to the murine chromosome 15). Half of the AML cases observed in *PU.1<sup>ure/ure</sup>* mice (4/7 AMLs) gained a chromosome 15 copy (37). The smallest region gained

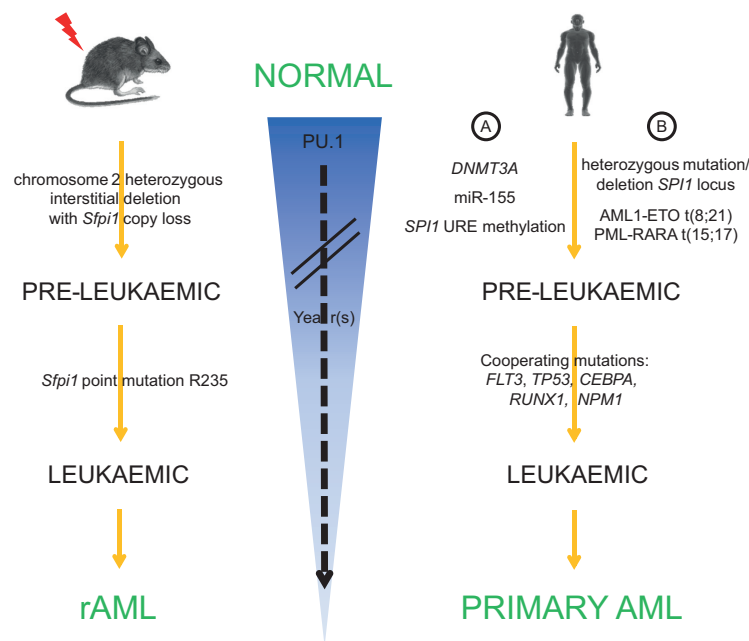
contains the proto-oncogene *c-Myc*. Interestingly, *c-Myc* expression was significantly elevated in PU.1-knockdown mice with AML (37). Similarly, *c-Myc* expression levels were significantly elevated in murine rAMLs with a partial chromosome 2 deletion, compared with rAMLs with an intact chromosome 2 (9).

Consistent with murine data, heterozygous mutations and deletions of the *SPI1* locus (the human analogue of *Sfpi1*) have been described in human primary AML, although it is a rare event (55). Heterozygous *SPI1* mutations were reported in 7% of a Japanese AML study (9/126 AMLs, with half of the cases comprising point mutations and the other half deletions). Mutated *SPI1* led to a reduced function of the transcription factor and the median survival of patients with *SPI1* mutations was significantly shorter than patients without *SPI1* mutations. These mutations were predominantly found in undifferentiated and in (myelo) monocytic leukaemia subtypes, which is in line with the physiologic role of PU.1. Interestingly, they were not observed in leukaemic patients harbouring the chromosome translocations most commonly observed in primary AML, such as AML1-ETO and PML-RARA (55). In contrast with the results of the Japanese AML study, investigations of North-American and European cohorts reported *SPI1* mutations in <1% of primary AML patients (56).

Four primary AML patients presented with a mutated NF- $\kappa$ B binding site in the URE of *SPI1* (38), thereby suppressing PU.1 mRNA expression. Another primary AML case harboured a deletion spanning at least 40kb of the *SPI1* locus, including the URE region but no *CEBPA*, *FLT3* and *NPM1* mutations were detected. The PU.1 mRNA level was significantly lower compared with other AML-M2 patients and 10 times lower compared with unsorted bone marrow cells obtained from healthy individuals (56). Such minor deletions would escape detection by conventional sequencing techniques and may therefore further clarify the conflicting reports on the presence of *SPI1* mutations in human primary

AML patients. As the mutations in the PU.1 DNA binding domain observed from human primary AML (55) do not correspond to the residues mutated in murine rAML (16), it was speculated that *SPI1* point mutations might be more often associated with tAML. However a small human tAML study (24 AML patients who had received therapy for a prior malignancy) did not report any *SPI1* mutations in the coding, untranslated or promoter regions (18). To the authors' knowledge, there are no studies reporting on *SPI1* mutations in tAML patients who received radiation therapy alone.

Although *SPI1* seems only infrequently mutated in human primary and tAML, several reports have highlighted downregulated *SPI1* gene/PU.1 mRNA expression in primary AML patients. Approximately half of primary AML patients (9/20 AMLs) had lower *SPI1* gene expression, compared with *SPI1* expression in HSCs from healthy donors (57). Similarly, a Czech primary AML study reported decreased PU.1 mRNA levels in the majority of AML patients (27/36 AMLs) (50), which corresponds with our previous finding of decreased PU.1 mRNA levels in murine rAMLs with a wild-type *SPI1* allele (31). Interestingly, *TP53* deletions were only observed in human primary AMLs with low PU.1 levels, and concomitantly with elevated *MYB* and *miR-155* levels, directly linking *TP53* inactivation and a dysregulated *MYB*/*miR-155*/PU.1 pathway in human AML (50). Transduction of human primary AML blasts with a lentivector encoding PU.1 increased the myelomonocytic marker expression and decreased proliferation rates together with increasing apoptosis, changes characteristic of restored differentiation in these myeloid leukaemic blasts (58). Similarly, all *trans* retinoic acid therapy leads to *in vivo* differentiation of acute promyelocytic leukaemia blasts through increased PU.1 activity (59), resulting in complete remission in almost all leukaemic patients. Various other mechanisms seem involved in PU.1 downregulation in human primary AML (Figure 1). Firstly, the formation of fusion proteins



**Figure 1.** Involvement of PU.1 in murine rAML and human primary AML. In mice, exposure to ionising radiation induces a first mutational hit in haematopoietic cells (interstitial deletion of one *Sfpi1* gene on chromosome 2), creating pre-leukaemic cells that have a growth advantage but which are not fully malignant. Subsequently, these cells acquire a second mutational hit (point mutation in the remaining *Sfpi1* copy), resulting in the formation of a leukaemic cell which undergoes further clonal expansion and eventually leads to the development of rAML. In humans, no data is currently available on the involvement of PU.1 in tAML. However, various epigenetic (A) and genetic (B) modifications, contributing towards the formation of pre-leukaemic cells, have been linked with downregulation of PU.1 in human primary AML. These pre-leukaemic cells harbour some, but not all, of the mutations found in leukaemic cells (60). Numerous pre-leukaemic and leukaemic mutations contribute towards PU.1 downregulation in human primary AML, including the top three genes found mutated in human primary AML [*NPM1*, *FLT3*, and *DNMT3A* (17)]. Although the molecular targets seem to differ between species, it appears that a common PU.1 downregulation pathway is affected in murine rAML and human primary AML. Future studies investigating tAML patients receiving radiotherapy alone should confirm whether PU.1 is also commonly downregulated in human tAML. Blue intensity indicates PU.1 expression/activity levels (dark equals high levels).

following chromosome translocations, including AML1-ETO in t(8;21) (61,62) and PML-RARA in t(15;17) (62,63), confers reduced PU.1 expression. Similarly, internal tandem duplications in the FLT3 receptor tyrosine kinase suppress the PU.1 expression and function (62,64). Finally, with four of the top eight genes found mutated in human primary AML being epigenetic regulators, epigenetic silencing is most likely involved as well (20). This hypothesis is further supported by the findings that miR-155, a known regulator of PU.1 mRNA, is overexpressed in bone marrow samples of some primary AML subtypes (65), and that in high-risk myelodysplastic patients PU.1 downregulation is associated with DNA methylation of the PU.1 URE (66).

## Future directions and conclusion

To begin to define how PU.1 functions as a tumour suppressor in rAML, we have recently generated a *Sfpi1* point mutation mouse model. The *Sfpi1* point mutation (*Sfpi1<sup>pm</sup>*) comprises a C to T substitution in the PU.1 DNA binding domain (R235) and is the most common base transition observed in murine rAML. Preliminary results show that *Sfpi1<sup>pm/pm</sup>* mice die during late gestation, indicating the importance of the DNA binding domain in PU.1 functioning (unpublished observations). In-depth analysis is on-going to investigate the impact of this point mutation on haematopoiesis and (radiation) leukaemogenesis. It will be of great interest to see if the presence of the point mutation alters the AML frequency and latency. At the same time, we have developed a mouse model in which the radiation-induced chromosome 2 deletion and concomitant PU.1 loss can be detected early after exposure in live haematopoietic progenitor cells (i.e. knock-in of the mCherry reporter gene in the MDR). Both mouse models should help to characterise the cells at risk and to elucidate the molecular mechanisms underlying radiation leukaemogenesis, and assess how applicable they are to human primary and tAML. Furthermore, gene expression analysis in pre-leukaemic and AML populations of PU.1 knock-down models would aid deciphering the specific changes leading to leukaemia. Consequently, analysis of global PU.1 DNA binding via ChIP-sequencing in normal haematopoietic progenitors and AML cells in murine, and most importantly patient material, would further clarify the impact of reduced PU.1 activity on target gene regulation.

Overall, although PU.1 is rarely directly mutated in human primary and tAML, it appears to be commonly downregulated through a series of indirect mechanisms. The identification of biomarkers for PU.1 downregulation would greatly aid our understanding of how this event facilitates leukaemogenesis. Therefore, we suggest that, in future studies, the levels of PU.1 expression in human primary and tAML are carefully monitored.

## Funding

European Union FP7 EURATOM DoReMi network of excellence (249689 to C.B. and S.B.), Risk, Stem Cells and Tissue Kinetics—Ionising Radiation (323267 to C.B. and S.B.), an Australian Research Council Future Fellowship (S.L.N.), Victorian State Government Operational Infrastructure Support, Australian Government NHMRC IRIIS (S.L.N.) and the National Institute for Health Research Centre for Health Protection Research.

## Acknowledgements

The authors alone are responsible for the content and writing of the article. This report is work commissioned by the National Institute for Health Research. The views expressed in this publication are those of the authors and not necessary those

of the NHS, the National Institute for Health Research or the Department of Health.

*Conflict of Interest Statement:* None declared.

## References

- Upton, A.C. (1986) Historical perspectives on radiation carcinogenesis. In Upton, A.C., Albert, R.E., Burns, F.J. and Shore, R.E. (eds) *Radiation Carcinogenesis*. Elsevier, New York, pp. 1–10.
- Hsu, W.L. et al. (2013) The incidence of leukemia, lymphoma and multiple myeloma among atomic bomb survivors: 1950–2001. *Radiat. Res.*, 179, 361–382.
- Major, I.R. (1979) Induction of myeloid leukaemia by whole-body single exposure of CBA male mice to x-rays. *Br. J. Cancer*, 40, 903–913.
- Rithidech, K.N. et al. (1999) Advantages of the CBA mouse in leukemogenesis research. *Blood Cells Mol. Dis.*, 25, 38–45.
- Bouffler, S.D. et al. (1997) Chromosome 2 hypersensitivity and clonal development in murine radiation acute myeloid leukaemia. *Int. J. Radiat. Biol.*, 72, 181–189.
- Darakhshan, F. et al. (2006) Evidence for complex multigenic inheritance of radiation AML susceptibility in mice revealed using a surrogate phenotypic assay. *Carcinogenesis*, 27, 311–318.
- Rithidech, K.N. et al. (1993) A specific chromosomal deletion in murine leukemic cells induced by radiation with different qualities. *Exp. Hematol.*, 21, 427–431.
- Bouffler, S.D. et al. (1996) Chromosomal abnormalities in neutron-induced acute myeloid leukemias in CBA/H mice. *Radiat. Res.*, 146, 349–352.
- Hirouchi, T. et al. (2008) Upregulation of c-myc gene accompanied by PU.1 deficiency in radiation-induced acute myeloid leukemia in mice. *Exp. Hematol.*, 36, 871–885.
- Steffen, L.S. et al. (2013) Molecular characterisation of murine acute myeloid leukaemia induced by 56Fe ion and 137Cs gamma ray irradiation. *Mutagenesis*, 28, 71–79.
- Alexander, B.J. et al. (1995) Gene deletion explains both *in vivo* and *in vitro* generated chromosome 2 aberrations associated with murine myeloid leukemia. *Leukemia*, 9, 2009–2015.
- Silver, A. et al. (1999) Molecular mapping of chromosome 2 deletions in murine radiation-induced AML localizes a putative tumor suppressor gene to a 1.0 cM region homologous to human chromosome segment 11p11-12. *Genes Chromosomes Cancer*, 24, 95–104.
- Peng, Y. et al. (2009) Radiation leukemogenesis in mice: loss of PU.1 on chromosome 2 in CBA and C57BL/6 mice after irradiation with 1 GeV/nucleon 56Fe ions, X rays or gamma rays. Part I. Experimental observations. *Radiat. Res.*, 171, 474–483.
- Cleary, H. et al. (2001) Allelic loss on chromosome 4 (*Lyr2*/TLRS5) is associated with myeloid, B-lympho-myeloid, and lymphoid (B and T) mouse radiation-induced leukemias. *Blood*, 98, 1549–1554.
- Finnon, R. et al. (2012) Flt3-ITD mutations in a mouse model of radiation-induced acute myeloid leukaemia. *Leukemia*, 26, 1445–1446.
- Cook, W.D. et al. (2004) PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain. *Blood*, 104, 3437–3444.
- Jawad, M. et al. (2006) Evidence for clustered tumour suppressor gene loci on mouse chromosomes 2 and 4 in radiation-induced acute myeloid leukaemia. *Int. J. Radiat. Biol.*, 82, 383–391.
- Suraweera, N. et al. (2005) Mutations of the PU.1 Ets domain are specifically associated with murine radiation-induced, but not human therapy-related, acute myeloid leukaemia. *Oncogene*, 24, 3678–3683.
- Welch, J.S. et al. (2012) The origin and evolution of mutations in acute myeloid leukemia. *Cell*, 150, 264–278.
- Cancer Genome Atlas Research Network. (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N. Engl. J. Med.*, 368, 2059–2074.
- Genik, P.C. et al. (2014) Leukemogenesis in heterozygous PU.1 knockout mice. *Radiat. Res.*, 182, 310–315.
- Metcalfe, D. et al. (2006) Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. *Proc. Natl. Acad. Sci. U. S. A.*, 103, 1486–1491.

23. Dekkers, F. et al. (2011) A two-mutation model of radiation-induced acute myeloid leukemia using historical mouse data. *Radiat. Environ. Biophys.*, 50, 37–45.
24. Brown, N.L. et al. (2011) Sfp1/PU.1 mutations in mouse radiation-induced acute myeloid leukaemias affect mRNA and protein abundance and associate with disrupted transcription. *Leuk. Res.*, 35, 126–132.
25. Mohrin, M. et al. (2010) Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. *Cell Stem Cell*, 7, 174–185.
26. Ban, N. et al. (2009) Implication of replicative stress-related stem cell ageing in radiation-induced murine leukaemia. *Br. J. Cancer*, 101, 363–371.
27. Shao, L. et al. (2014) Total body irradiation causes long-term mouse BM injury via induction of HSC premature senescence in an Ink4a- and Arf-independent manner. *Blood*, 123, 3105–3115.
28. Hirouchi, T. et al. (2011) Cell surface marker phenotypes and gene expression profiles of murine radiation-induced acute myeloid leukemia stem cells are similar to those of common myeloid progenitors. *Radiat. Res.*, 176, 311–322.
29. Staber, P.B. et al. (2013) Sustained PU.1 levels balance cell-cycle regulators to prevent exhaustion of adult hematopoietic stem cells. *Mol. Cell*, 49, 934–946.
30. Nutt, S.L. et al. (2005) Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *J. Exp. Med.*, 201, 221–231.
31. Olme, C.H. et al. (2013) Live cell detection of chromosome 2 deletion and Sfp1/PU.1 loss in radiation-induced mouse acute myeloid leukaemia. *Leuk. Res.*, 37, 1374–1382.
32. Olme, C.H. et al. (2013) Frequency of acute myeloid leukaemia-associated mouse chromosome 2 deletions in X-ray exposed immature haematopoietic progenitors and stem cells. *Mutat. Res.*, 756, 119–126.
33. Dahl, R. et al. (2003) Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat. Immunol.*, 4, 1029–1036.
34. Kueh, H.Y. et al. (2013) Positive feedback between PU.1 and the cell cycle controls myeloid differentiation. *Science*, 341, 670–673.
35. Chen, H. et al. (1995) PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene*, 11, 1549–1560.
36. Okuno, Y. et al. (2005) Potential autoregulation of transcription factor PU.1 by an upstream regulatory element. *Mol. Cell. Biol.*, 25, 2832–2845.
37. Rosenbauer, F. et al. (2004) Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat. Genet.*, 36, 624–630.
38. Bonadies, N. et al. (2010) PU.1 is regulated by NF-kappaB through a novel binding site in a 17kb upstream enhancer element. *Oncogene*, 29, 1062–1072.
39. Scott, E.W. et al. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*, 265, 1573–1577.
40. Scott, E.W. et al. (1997) PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity*, 6, 437–447.
41. McKercher, S.R. et al. (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.*, 15, 5647–5658.
42. Kamath, M.B. et al. (2008) Dose-dependent repression of T-cell and natural killer cell genes by PU.1 enforces myeloid and B-cell identity. *Leukemia*, 22, 1214–1225.
43. Dakic, A. et al. (2005) PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. *J. Exp. Med.*, 201, 1487–1502.
44. Iwasaki, H. et al. (2005) Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood*, 106, 1590–1600.
45. Heinz, S. et al. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell*, 38, 576–589.
46. Ghisletti, S. et al. (2010) Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity*, 32, 317–328.
47. Dakic, A. et al. (2007) Is PU.1 a dosage-sensitive regulator of haemopoietic lineage commitment and leukaemogenesis? *Trends Immunol.*, 28, 108–114.
48. Turkistany, S.A. et al. (2011) The transcription factor PU.1 is a critical regulator of cellular communication in the immune system. *Arch. Immunol. Ther. Exp. (Warsz.)*, 59, 431–440.
49. Carotta, S. et al. (2010) The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity*, 32, 628–641.
50. Basova, P. et al. (2014) Aggressive acute myeloid leukemia in PU.1/p53 double-mutant mice. *Oncogene*, 33, 4735–4745.
51. Moreau-Gachelin, F. et al. (1988) Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature*, 331, 277–280.
52. Kayser, S. et al. (2011) The impact of therapy-related acute myeloid leukemia (AML) on outcome in 2853 adult patients with newly diagnosed AML. *Blood*, 117, 2137–2145.
53. Pedersen-Bjergaard, J. et al. (2008) Genetics of therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia*, 22, 240–248.
54. Nakanishi, M. et al. (1999) Chromosomal instability in acute myelocytic leukemia and myelodysplastic syndrome patients among atomic bomb survivors. *J. Radiat. Res.*, 40, 159–167.
55. Mueller, B.U. et al. (2002) Heterozygous PU.1 mutations are associated with acute myeloid leukemia. *Blood*, 100, 998–1007.
56. Bonadies, N. et al. (2010) Heterozygous deletion of the PU.1 locus in human AML. *Blood*, 115, 331–334.
57. Steidl, U. et al. (2006) Essential role of Jun family transcription factors in PU.1 knockdown-induced leukemic stem cells. *Nat. Genet.*, 38, 1269–1277.
58. Durruel, S. et al. (2007) Lentiviral PU.1 overexpression restores differentiation in myeloid leukemic blasts. *Leukemia*, 21, 1050–1059.
59. Mueller, B.U. et al. (2006) ATRA resolves the differentiation block in t(15;17) acute myeloid leukemia by restoring PU.1 expression. *Blood*, 107, 3330–3338.
60. Corces-Zimmerman, M.R. et al. (2014) Pre-leukemic evolution of hematopoietic stem cells: the importance of early mutations in leukemogenesis. *Leukemia*, 28, 2276–2282.
61. Vangala, R.K. et al. (2003) The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. *Blood*, 101, 270–277.
62. Staber, P.B. et al. (2014) The Runx-PU.1 pathway preserves normal and AML/ETO9a leukemic stem cells. *Blood*, 124, 2391–2399.
63. Walter, M.J. et al. (2005) Reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARalpha. *Proc. Natl. Acad. Sci. U. S. A.*, 102, 12513–12518.
64. Mizuki, M. et al. (2003) Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. *Blood*, 101, 3164–3173.
65. O'Connell, R.M. et al. (2008) Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.*, 205, 585–594.
66. Curik, N. et al. (2012) 5-azacitidine in aggressive myelodysplastic syndromes regulates chromatin structure at PU.1 gene and cell differentiation capacity. *Leukemia*, 26, 1804–1811.