MLL gene fusions in human leukaemias: in vivo modelling to recapitulate these primary tumourigenic events

S. Rodriguez-Perales · F. Cano · M. N. Lobato ·
T. H. Rabbitts

Abstract Recurrent reciprocal chromosomal translocations are frequently found in leukaemias and sarcomas as initiating events in these cancers. Mouse models of chromosomal translocations are not only important for the elucidation of the mechanism of these factors underlying the disease but are also important pre-clinical models for assessing new drug combinations, developing new rational therapeutic strategies based on new drugs and testing novel macromolecular drugs. We describe three technologies for creating chromosomal translocation mimics in mice, applied specifically to understand how the MLL-fusions contribute to leukaemia. An important finding of this work is that the lineage of the tumours can be controlled by the MLL-protein fusion. The translocation mimic methods can be applied to any human reciprocal chromosomal translocation.

Keywords Leukaemia · MLL ·
Chromosomal translocations · Cre recombinase ·
Gene fusion

S. Rodriguez-Perales · F. Cano · M. N. Lobato ·
T. H. Rabbitts (✉)
Leeds Institute of Molecular Medicine,
Section of Experimental Therapeutics,
St. James's University Hospital,
Wellcome Trust Brenner Building,
Leeds LS9 7TF, UK
e-mail: thr@leeds.ac.uk

S. Rodriguez-Perales · F. Cano · M. N. Lobato ·
T. H. Rabbitts
MRC Laboratory of Molecular Biology,
Hills Road, Cambridge CB2 2QH, UK

1 Introduction

Among the most common chromosomal breakpoint region in human leukaemias is the chromosome band 11q23 [1, 2]. The analysis of this region led to the identification of the *MLL* gene (also called *HRX, HTRX, TRX1* or *ALL-1*) [2]. *MLL* gene abnormalities occur in both childhood (~10% of all paediatric leukaemias) and adult leukaemias (~5% of acute leukaemias) [3]. Around 5–10% of all *MLL*-associated leukaemias are therapy related, either following treatment with topoisomerase II targeting drugs or following other treatments including radiotherapy [4]. The many MLL fusion partners that have been identified to date (more than 60) [5] represent a structurally heterogeneous group of proteins, and where the most frequent partners are AF4, AF9 and ENL resulting from chromosomal translocations t(4;11), t(9;11) or t(11,19), respectively.

2 The *MLL* gene and protein

MLL encodes a large protein of around 430 kDa (illustrated in Fig. 1a) with many interaction domains resulting in the formation of a multi-protein super-complex involved in transcription regulation. MLL has three AT hook domains near the N terminal region that mediate binding to the minor groove of the DNA helix in AT-rich DNA [6]. These are followed by two nuclear localization signals (SNL1 and SNL2) and a large transcriptional repression domain. This repression domain, in turn, has three structurally different subdomains forming two mechanistically different repression domains: RD1 and RD2. RD1 contains the DNMT1 DNA methyltransferase homology domain, which recruits the polycomb repressor proteins HPC2 and BMI-1, and the corepressor CTBP [7].

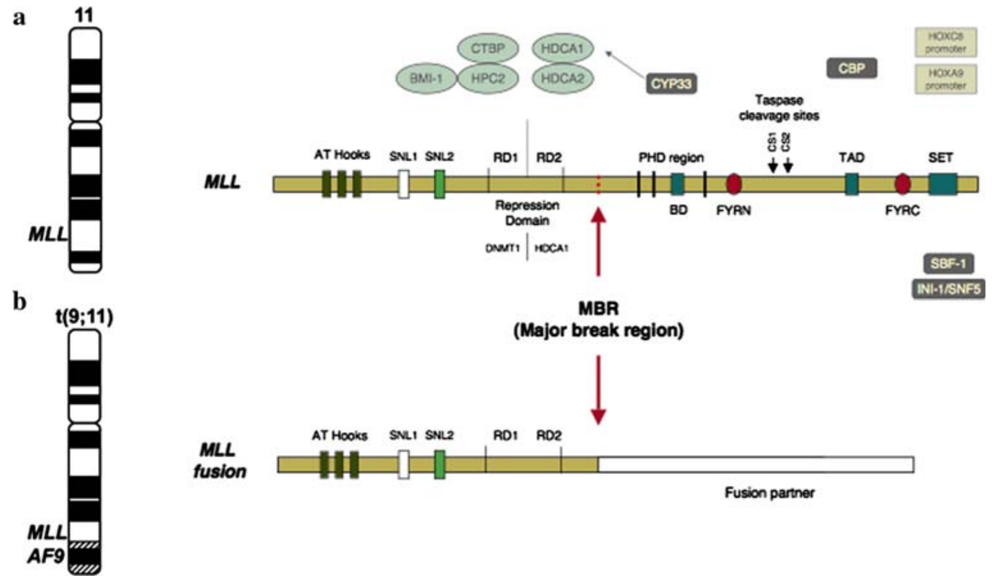

Fig. 1 Diagram of the normal and fusion MLL protein. **a** The *MLL* gene on human chromosome 11, band q23 encodes a very large protein with many regions of homology to other proteins. The frequent chromosomal translocation into this gene results in breakage of *MLL* and fusion with one of over 60 possible partners. **b** In most cases the break within *MLL* results in loss of coding from the PHD region and the COOH-terminal part of MLL. CS1, CS2: "conserved cleavage sites" for taspase. *SNL* signal for nuclear localization, *RD* repression domains

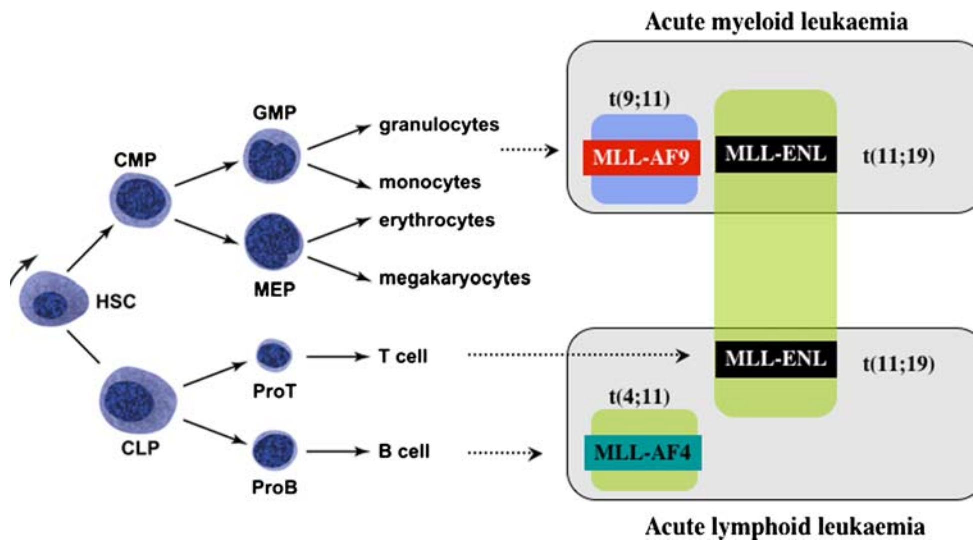

Fig. 2 Cellular tropism of the major MLL fusion proteins. The main cell types of the haematopoietic pathway and where the three most frequently occurring fusions of MLL are found viz. MLL–AF4, MLL–ENL and MLL–AF9. The MLL–AF4 fusion (restricted to leukaemias in infants or children) is found only in pro-B cell leukaemias, whereas MLL–AF9 is found almost exclusively in the myeloid lineage. MLL–ENL fusions, however, are found in either myeloid or lymphoid lineage tumours, the latter being in the T cell lineage. *CLP* common lymphoid progenitor, *CMP* common myeloid progenitor, *GMP* granulocyte–monocyte precursor, *HSC* haematopoietic stem cell, *MEP* megakaryocyte–erythrocyte precursor

RD2 mediates repression through recruitment of histone deacetylases HDAC1 and HDAC2, which can also interact with part of RD1 [7]. A transcriptional activation domain (TAD) binds directly to the co-activator CBP (CREB-binding protein) [8]. CBP is an acetyltransferase, possibly acetylating H3 and H4 in connection with MLL binding to promoters of HOX genes [9, 10]. There is an SET domain near the C-terminus of MLL, which is a histone methyltransferase at lys 4 (H3-K4) [9, 10]. The H3-K4 methylation status correlates with an active state of transcription, so it is probably the *HOX* gene promoters are H3-K4 methylated through the MLL SET domain, playing a direct role in the transcription of *HOX* genes. Chromosomal abnormalities that affect *MLL* are clustered in a major break region (MBR) localised just after the repression domain. The transcriptional repression domain is retained in all MLL fusions and is required for transformation by the fusion proteins [8] (Fig. 1b). The chromosomal translocation of MLL invariably results in the replacement of the C-terminal domains with a fusion partner affecting the ability to recruit proteins to the MLL multi-protein complex [10].

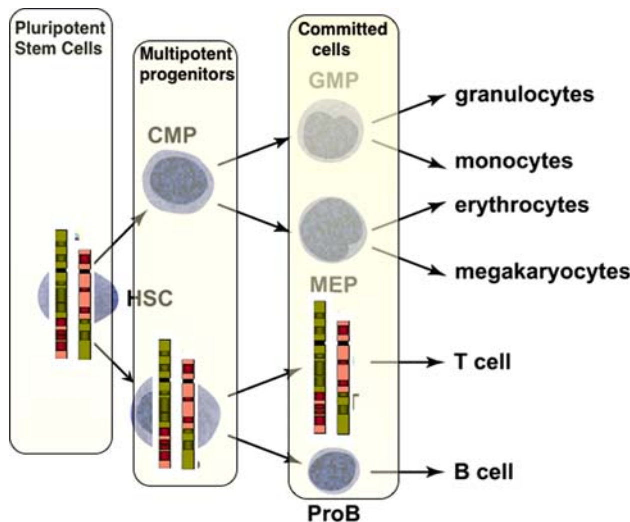

Fig. 3 Objective of *de novo* translocation models of *MLL*-translocations. The rationale behind conditional models that either create translocations *de novo* or make fusion protein production happen in specific haematopoietic cells is to assess the effects of these fusions in either pluripotent (self-renewing haematopoietic stem cells, HSC), multi-potent progenitors (e.g. common lymphocyte progenitor, CLP) or lineage restricted cells (e.g. T cells and their specific progenitors). The same approaches would be possible with activation of any oncogene. In the MLL-model systems described in this paper, we achieved the formation of the translocations or the mimics in HSCs using *Lmo2–Cre*, in CLP using *Rag–Cre* and in T cell lineage using *Lck–Cre* [21]

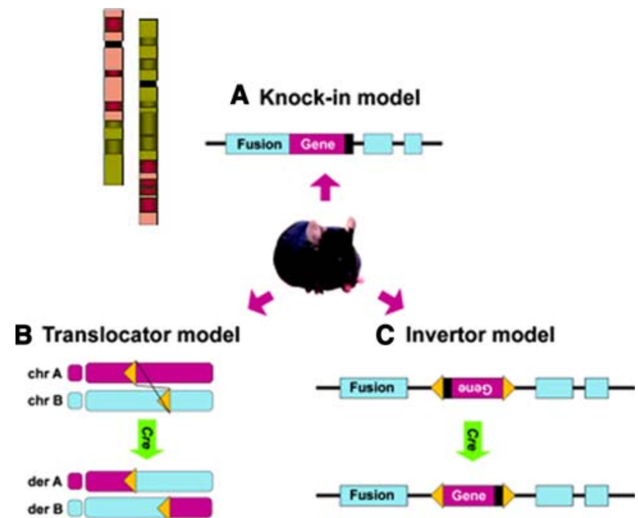

Fig. 4 *Mll*-associated chromosomal translocation mimics in mice. *A* The knock-in model results from gene targeting by homologous recombination in ES cells. The targeting vector carries a cDNA segment, (indicated in *magenta*) with a translation stop codon and a polyA site that is knocked-in to fuse with the appropriate exon of a target gene (exons indicated in *blue*), forming a fusion gene. The first example of the knock-in method was the creation of an *Mll–AF9* fusion [13]. *B* The translocator model is a conditional model that facilitates the creation of *de novo* reciprocal chromosomal translocations between genes on different chromosomes using a Cre–*loxP* recombination system. In the figure, the *loxP* sequences (shown as *yellow triangles*) are introduced by homologous recombination into introns of two target genes, here illustrated as chromosome A (*chr A*) and B (*chr B*). Inter-chromosomal translocations occur when *Cre* is activated in a cell-specific manner. *C* The invertor model is an alternative, fully conditional method for generating chromosomal translocations in those cases in which the translocator model cannot be applied due to incompatible gene orientation. In this approach, an inverted cDNA cassette, flanked by *loxP* sites (floxed) is knocked-in to an intron of the target allele and brought it into the correct transcriptional orientation by Cre–*loxP* recombination. *chr* chromosome, *der* derivative chromosome

3 The major *MLL*-fusions are found in restricted leukaemia sub-types

As with other haematopoietic and sarcomas malignancies, the recurrent chromosomal translocations that affect the *MLL* gene display a tropism with respect to the cell in which they are found. The most frequently occurring fusion partners are *AF4*, *ENL* and *AF9*, and these different fusions distinguish distinct types of leukaemias (Fig. 2). Whilst the *MLL–AF9* fusion is almost exclusively found in myeloid leukaemias, the *MLL–AF4* fusion is restricted to pro-B cell lymphoid leukaemias [11] and others, such as *MLL–ENL*, can be found in myeloid and lymphoid leukaemias [2]. Interestingly, *MLL–AF4* fusion is usually only found in infant or childhood leukaemia.

4 Chromosomal translocation mimic technology

In order to understand the role of the different MLL-fusions in leukaemogenesis and to determine their possible role in lineage determination, we have used homologous recombination in embryonic stem (ES) cells to create fusion genes on one *Mll* allele or used conditional translocation mimics to generate *de novo* translocations or mimics [12]. The rationale was to build technologies that would allow cell-specific expression of *Mll*-fusions, using Cre–*loxP* mediated recombination (Fig. 3).

Our first development was a method known as knock-in, exemplified by the fusion of the human *AF9* cDNA into the mouse *Mll* gene to produce the *Mll–AF9* fusion [13, 14] (Fig. 4, A). This showed that the Mll–AF9 fusion causes myeloid leukaemias (both in chimaeric and heterozygous mice) [13, 14]. However, this knock-in model lacked cell specificity because the targeted *Mll–AF9* allele was expressed in all cells where the *Mll* promoter is active. Also, fusion proteins can result in embryonic lethality, such as our finding with Mll–AF4 [12]. To avoid these limitations, we developed conditional systems allowing the controlled expression of translocation alleles in different cell types and at different cellular differentiation stages (Fig. 3).

A conditional mouse model was developed using Cre recombinase to mediate inter-chromosomal translocations

called the translocator model [15, 16]. In this technology, *loxP* sites were introduced, by homologous recombination, into the appropriate introns of the two genes between to make a translocation (Fig. 4, B) and *Cre* expression is used to induce inter-chromosomal recombination between the *LoxP* sites and thus make a *de novo* reciprocal chromosomal translocation in mice. These translocations occur at low frequency in mice [17], and thus cancers arise from single cell origin. The translocator model is the ideal method to reproduce the reciprocal translocations found in human cancers (Fig. 4, B). This translocator approach can in principle be applied to recapitulate any human reciprocal translocation; the strategy requires that the genes have the same transcription orientation with respect to the centromere in the mouse chromosomes. If they do not, any translocation that does occur will give dicentric and acentric derivative chromosomes and a non-viable cell.

A conditional knock-in model is required in those latter cases. One approach is the *loxP*-STOP method [18] in which transcription of a knock-in allele is inhibited by transcription stop site flanked by *loxP* sites, so that Cre-dependent deletion of the transcription stop can precede activation of transcription of the knock-in gene. However, in some cases such as *Mll–AF4* [12], there is transcription read-through of the transcription stop site. In view of this, we developed a fully conditional knock-in method called the invertor method [19] (Fig. 4, C). The invertor technology is based on the knocking-in of a floxed cDNA cassette into the appropriate intron of a target gene but in the opposite orientation for transcription/splicing. Expression of *Cre* induces the inversion of the floxed cassette and the formation of the fusion gene in a cell-specific manner, recapitulating the translocation associated with human cancer.

5 The knock-in model of *Mll–AF9* shows propensity to myeloid lineage tumours

The *Mll–AF9* gene fusion model (using the knock-in approach to translocation mimics, Fig. 4, A) produced mice that developed myeloid leukaemias in about 6 months [13, 14]. The power of the technology was shown by both chimaeric and heterozygous mice carrying the gene fusion, as they developed leukaemias with the same latency period; and these neoplasias were almost exclusively acute myeloid leukaemias [13]. There was also detectable proliferation of myeloid cells in bone marrow by as little as 6 days after birth, probably resulting in early accumulation of myeloid precursors which acquire secondary mutations that cooperate in the appearance of overt cancer.

The knock-in approach was carried further by fusing a segment comprising the *lacZ* gene (encoding beta-galactosidase, βgal) in *Mll* exon 10 [14]. The surprising outcome was the observation of leukaemias (AML) in the mice expressing Mll–βgal fusion as beta-galactosidase had not been previously reported to be oncogenic. We proposed that the Mll–βgal protein was present as a dimer of dimers (via the beta-galactosidase tetrameric interaction) and therefore, that dimerisation was necessary for MLL-fusion mediated oncogenecity [14].

6 *De novo* chromosomal translocations in mice

We developed a system for *de novo* translocation production designated translocator to overcome problems imposed by the knock-in approach. Mice were created with the *loxP* recombination sites introduced into *Mll* intron 10 [15], *Af9* intron 9 [15] or *Enl* intron 1 [16] and crossed with Cre-expressing mice. In one set of experiments, the promoter chosen to drive Cre recombinase expression was *Lmo2*, which is expressed in pluripotent stem cells/multipotent myeloid progenitor. Only myeloid neoplasias occurred when either *Mll–Enl* or *Mll–Af9* translocations was initiated in these progenitors [16, 20].

In a separate analysis, the *Lck* promoter was used to control *Cre* expression (expression being within the T cell lineage and its progenitors [21]) and produced a different outcome. No tumours appeared in the *Mll–Af9* translocator model even though translocations could be found in the T cells, and fusion mRNA could be detected [20]. Thus, Mll–Af9 fusion requires the correct cellular context to be oncogenic, and it can be inferred that MLL translocations are not necessarily oncogenic in any haematopoietic cell type. The translocator technology was also used to reproduce the *MLL–ENL* reciprocal translocation, t(11;19), associated with both myeloid and lymphoid human leukaemias [20]. Unlike *Mll–Af9* translocator mice, *Mll–Enl* translocators using *Lck–Cre* presented with haematological malignancies either in the myeloid lineage or the T cell lineage. However, no tumours formed when the *Mll–Enl* translocations occurred in B cell [17]. This mirrors the situation found in patients with leukaemias carrying the *MLL–ENL* fusion. The myeloid tumours in the Mll–Enl translocators had undergone Rag-mediated V-D-J joining characteristic of lymphoid lineage development (unlike myeloid tumours from the translocators using *Lmo2–Cre*), and we conclude that the myeloid tumours caused by the *Mll–Enl* translocation mediated by the *Lck–Cre* transgene are due to lineage reassignment from non-committed T cell progenitors, which had already undergone *Rag* gene expression and Rag-mediated *TCR* gene rearrangement, to the myeloid lineage (see Fig. 5).

These data show that the Mll–Enl fusion protein defines the lineage of leukaemia (Fig. 5). Our current model is that

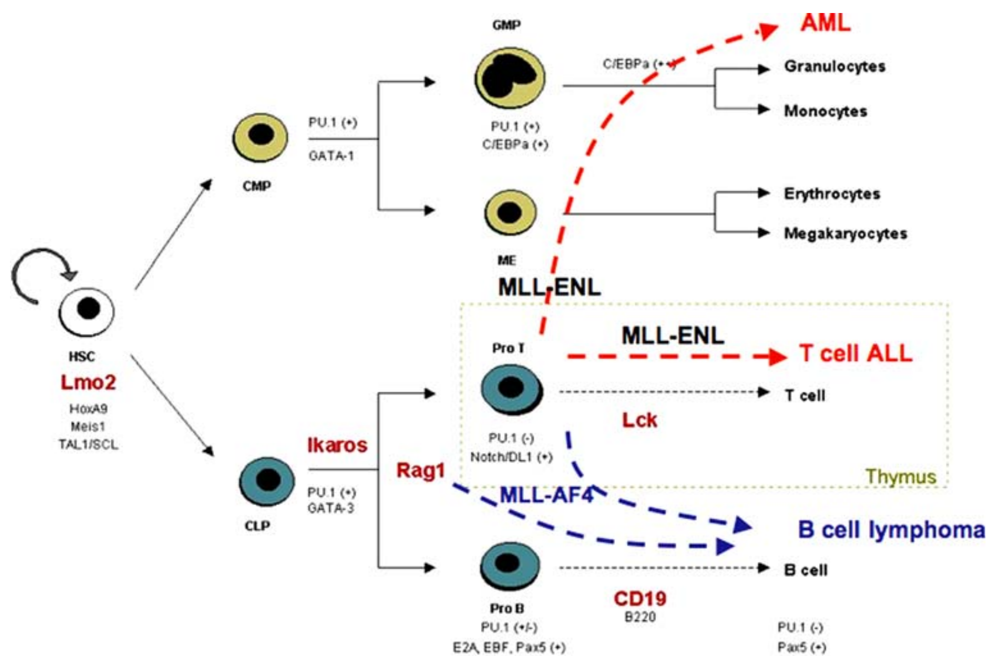

Fig. 5 Lineage reassignment induced by *Mll–Enl* translocation in non-committed T cells. *Mll–Enl* chromosomal translocations have a different outcome depending on the cell type in which they are induced. When a translocation is generated in a pluripotent stem cell or in a multi-potent myeloid progenitor (using Cre recombinase expressed under *Lmo2* promoter control) mice develop acute myeloid leukaemia as MPD-like myeloid leukaemia. However, when the Cre expression is mediated by the *Lck* promoter, which mediates Cre expression in T cells and their progenitors, the *Mll–Enl* translocation results not only in T cell malignancies but also in myeloid leukaemias [20]. The hypothesis is that if the translocation occurs in an early non-committed T cell, the *Mll–Enl* fusion is able to re-programme lymphoid differentiation into the myeloid lineage as the progenitor retains differentiation options at this stage. Conversely, if the translocation occurs in a fully committed cell, the developmental fate of the cell is not affected but nevertheless transforming into a malignant cell. *AML* acute myeloid leukaemia, *CLP* common lymphoid progenitor, *CMP* common myeloid progenitor, *GMP* granulocyte–monocyte precursor, *HSC* hematopoietic stem cell, *MEP* megakaryocyte–erythrocyte precursor, *T-ALL* T-acute lymphoid leukaemia

a translocation between *Mll* and *Enl* in a translocator mouse within a permissive T cell progenitor at a non-committed stage of differentiation, (i.e. in cells that still have some trans-differentiation ability) allows lineage reassignment of the developmental pathway from T cell to myeloid cell lineage. However, if the translocation event happens in a more differentiated T cell, it has no further developmental options and a T cell leukaemia that arises. Finally, it is clear that *Mll–Enl* translocation-associated leukaemia initiating cells can be lineage-committed cells or pluripotent haematopoietic stem cells.

7 Conditional invertor alleles for *MLL* translocation mimics

The translocator model is a recapitulation of human chromosomal translocations and should be applicable to a range of translocations. The success with this system depends on the compatibility of the transcription orientation of the two genes on the mouse chromosomes. If the relative transcriptional orientation on each respective chromosome arm is not the same, the mouse translocator model is not applicable. A different conditional translocation mimic, the “invertor” model, was developed for these situations (Fig. 4, C). This method was applied to the *EWS–ERG* fusion gene (commonly found in Ewing’s sarcoma) using *Rag1–Cre* in the mouse model [22]. The prevailing dogma was that EWS-fusions are activated in primitive stem cells of mesenchymal or haematopoietic lineages. Our results with the *Ews–ERG* invertor showed that T cell lymphomas arise when the Ews–Erg invertor was activated by *Rag1–Cre*, a gene active in both T and B cell progenitors. Therefore, we conclude that the tumour initiating cells for the Ews–ERG-dependent lymphomas are committed cells of the T cell lineage and not multi-potent progenitors.

An *Mll–AF4* invertor model was also made by knocking-in the *AF4* cDNA invertor cassette in *Mll* intron 10 [23]. The conditional inversion of *AF4* was achieved by mating the invertor mice with those expressing Cre recombinase under the control of either *Rag1, Lck* or *CD19* promoters. These lines of mice all developed B cell tumours, but the phenotypes of the tumours were not the immature pro-B cell leukaemias characteristic of the

MLL–AF4 childhood leukaemia, but were mature diffuse large B cell lymphomas. It was puzzling why *Mll–AF4*, *Rag1–Cre* mice, expressing Cre in T and B cell progenitors gave rise to mature B cell tumour phenotype as indeed was the case with *Mll–AF4*, *Lck–Cre* mice, that have T cell expression. This suggests that the Mll–AF4 fusion protein has an instructive role in stimulating differentiation into B cells, from either non-committed, Rag-expressing progenitors or from non-committed T cell progenitors that can still undergo trans-lineage differentiation.

8 Conclusions about *MLL*-translocation mimics

We have developed mouse models for the generation of chromosomal translocations as primary changes in cancer, using conditional alleles activated at pre-determined stages of haematopoiesis.

Our studies using Mll-fusions showed

1. MLL fusions are not necessarily oncogenic in any haematopoietic cells but some fusions do not need to occur in pluripotent stem cells to cause leukaemia
2. The late onset of overt leukaemia in most cases suggests the necessity of secondary additional mutations (i.e the translocation is necessary but not sufficient)
3. Translocation products can function in committed cells as long as these are permissive to generate a tumour with that fusion
4. Some Mll translocation products can be responsible for determining the lineage of the cancer. We illustrated this by the observed lineage reassignment of T cell progenitors to myeloid tumours by Mll–Enl and lineage reassignment of T cell progenitors to B cell tumours by Mll–AF4.

9 Generality of the translocator method to model human chromosomal translocations in mice

The creation of genetically engineered mice that can mimic human cancers has been made possible by a number of significant developments in gene targeting technologies. The translocator model is the ideal system for recreating *de novo* chromosomal translocations as long as the genes involved in the translocation are not in opposite orientations in the mouse. The insertion of a *loxP* sequence in each chromosome is the only requirement to produce inter-chromosomal rearrangements under Cre-recombinase expression. With this translocator method, we have shown that *Mll*-associated translocations can be mediated by Cre recombinase between two pairs of chromosomes where a *loxP* site had been inserted, thus recreating *de novo* chromosomal translocations, equivalent to those found in human tumours [15, 16].

In principle, the translocator method can be applied to model any human translocation in which the mouse genes are evolutionarily maintained in the appropriate orientation. Our findings with the *Mll* translocations show that these inter-chromosomal events can be induced between the *Mll* chromosome (chr. 9) and any of two non-homologous chromosomes tested (chromosome 4 for *Af9*, 17 for *Enl*). However, the generation of the translocation and the transcription of the translocation gene can be insufficient for tumourigenesis because *Mll–Enl* fusion in B cells has no discernable pathological outcome. Our results suggest that in principle any translocation could be generated by the translocator method. However, the development of the disease depends entirely on the biological consequences of the resulting fusion protein. The translocator model is therefore a very good tool to study in vivo the effect of different translocations in different cellular environments. The advantage of the translocator system is that a restricted number of cells gain the translocation, and in future versions of this method, it should be possible to incorporate further variants of inducibility and reporter genes allowing both the tracking of the cells of origin and the monitoring development of tumours as they occur.

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