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Mll fusions generated by Cre-*loxP*-mediated *de novo* translocations can induce lineage reassignment in tumorigenesis

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Chromosomal translocations are primary events in tumorigenesis. Those involving the mixed lineage leukaemia (*MLL*) gene are found in various guises and it is unclear whether MLL fusions can affect haematopoietic differentiation. We have used a model in which chromosomal translocations are generated in mice *de novo* by Cre-*loxP*-mediated recombination (translocator mice) to compare the functionally relevant haematopoietic cell contexts for Mll fusions, namely pluripotent stem cells, semicommitted progenitors or committed cells. Translocations between *Mll* and *Enl* or *Af9* cause myeloid neoplasias, initiating in pluripotent stem cells or multipotent myeloid progenitors. However, while *Mll-Enl* translocations can also cause leukaemia from T-cell progenitors, no tumours arose with *Mll-Af9* translocations in the T-cell compartment. Furthermore, *Mll-Enl* translocations in T-cell progenitors can cause lineage reassignment into myeloid tumours. Therefore, a permissive cellular environment is required for oncogenicity of *Mll*-associated translocations and Mll fusions can influence haematopoietic lineage commitment.

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Introduction

Cancer generally arises from a single cell that acquires a somatic mutation in a gene capable of eliciting a cell division property and/or immortalisation (Vogelstein and Kinzler, 2004). This initiating cell also gains the characteristic of self-renewal but may or may not be the cell that has the self-maintenance capacity of the putative cancer stem cell (Reya *et al*, 2001). Chromosomal translocations are primary events in cancer, and create an immortal lineage that provides cells for secondary mutations to give frank malignancy. Leukaemia and lymphomas arise within a hierarchy of progenitor, committed or terminally differentiated cells. These are exemplified, respectively, by chronic myeloid leukaemia (CML), Burkitt's lymphoma and multiple myeloma. Among the somatic mutations that characterise these tumours, recurrent chromosomal translocations are generally found at presentation of disease. Moreover, chromosomal translocations are often the sole cytogenetic chromosomal abnormality at such time (see http://cgap.nci.nih.gov/Chromosomes/Mitelman). Important studies of concordant leukaemias in monozygotic twins have shown the presence of identical *MLL-AF4* (mixed lineage leukaemia) chromosomal translocations but with different immunoglobulin gene rearrangements, demonstrating the *in utero* origin of this leukaemic precursor cell (Mori *et al*, 2002; Greaves *et al*, 2003).

Chromosomal translocations involving the *MLL* gene typify gene fusion. *MLL* is involved in multifarious chromosomal translocations in human acute leukaemias with adverse prognosis. The *MLL* gene is located on human chromosome 11, band q23, and is associated with translocations, inversions and duplications of 11q23 (see Daser and Rabbitts, 2004). *MLL* is a homologue of the *Drosophila Trx* gene and *Hox* genes are necessary for Mll-fusion-induced leukaemia in mice (Ayton and Cleary, 2003; Kumar *et al*, 2004). Thus, presumably MLL-fusion proteins confer deregulation on *HOX* genes, in turn as a consequence of chromosomal abnormalities of chromosome 11q23. MLL protein can bind directly to *HOXA9* and *HOXC8* promoters (Milne *et al*, 2002; Nakamura *et al*, 2002) and can act as a histone methyltransferase (methylating Lys4 of histone H3) (Sobulo *et al*, 1997; Birke *et al*, 2002; Milne *et al*, 2002; Xia *et al*, 2003; Hess, 2004; Yokoyama *et al*, 2004).

MLL-associated translocations represent a cross-section of possible associations found in acute leukaemias. There are more than 30 different known chromosome partners involving *MLL*, each appearing to result in a fusion of *MLL* with a gene from a different chromosomal region (see Daser and Rabbitts, 2004). A notable feature of *MLL* translocation leukaemias is that the main fusion partners (i.e. *AF4*, *AF9*, *ENL*, *ELL*) are found in tumours of either lymphoid lineage (*AF4*) or myeloid lineage (*AF9* or *ELL*) or of both lineages (*ENL*). It is not known if MLL fusions are simply oncogenic in any haematopoietic cell, either progenitor or committed cells. Furthermore, it is still unclear if an MLL-fusion protein has an instructive property on a cell, driving differentiation into specific haematopoietic lineages. Mouse models have begun to address these issues. Studies of retroviral transduction of bone marrow stem cells with viruses expressing *MLL* fusions (Ayton and Cleary, 2001; So *et al*, 2003; Zeisig *et al*, 2003), gene targeting knock-in models expressing *Mll-AF9* fusion (Corral *et al*, 1996; Dobson *et al*, 1999) or a translocator mouse model, in which *de novo* translocations of *Mll-Enl* occur (Forster *et al*, 2003), show that haematopoietic stem cells (HSC) are targets for Mll fusions. In addition, retroviral

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transduction of semicommitted, common myeloid progenitors (CMP) (Miyamoto *et al*, 2002) has shown that similar leukaemias result from these progenitors as from transduced bone marrow HSC (Cozzio *et al*, 2003). Recently, an *Mll-CBP* conditional knock-in model indicated targeting of granulocyte–macrophage progenitors (Wang *et al*, 2005).

In the translocator mouse model (Buchholz *et al*, 2000; Collins *et al*, 2000), chromosomal translocations are made *de novo* in a cell-specific manner, using Cre-*loxP* recombination *in vivo* (Smith *et al*, 1995; van Deursen *et al*, 1995). Chromosomal translocations can thus be generated as primary genetic changes, initially in the absence of any other changes and at predetermined stages of haematopoiesis. Using *Lmo2-Cre* to express Cre recombinase, chromosomal translocations were made in pluripotent stem cells, and *Mll-Enl* fusion resulted in myeloid leukaemia (Forster *et al*, 2003). We have now applied the translocator approach using an *Lck-Cre* allele that specifically causes expression of Cre recombinase in T cells and their progenitors (McCormack *et al*, 2003; Codrington *et al*, 2005). We find that *Mll-Enl* fusions can be oncogenic in the lymphoid lineage as in human leukaemias. However, when *Mll-Af9* translocations are induced in similar T-cell populations via *Lck-Cre* expression, no leukaemias arose, although *Mll-Af9* translocations in uncommitted progenitors do induce myeloid leukaemias. Therefore, Mll fusions are not necessarily oncogenic in any haematopoietic cell. Furthermore, the Mll-associated leukaemic precursor cell expressing the *Mll-Enl* fusion can undergo lineage reassignment from the lymphoid lineage into myeloid lineage.

Results

Mll-Enl and Mll-Af9 fusions cause myeloid neoplasias in translocator mice

Mice have been described (Collins *et al*, 2000; Forster *et al*, 2003) carrying *Mll*, *Enl* or *Af9* genes with *loxP* sites within the appropriate introns for Cre-mediated chromosomal translocations equivalent to those found in human leukaemias (the *loxP* sites were introduced by homologous recombination in embryonic stem (ES) cells and these targeted ES cells were used to generate mice). These mice were designated translocator mice (Forster *et al*, 2003). The *Mll*, *Enl* or *Af9* alleles are diagrammatically shown in Figure 1A, indicating the intronic location of the *loxP* sites. Cre recombinase can recognise these sites and cause interchromosomal translocations resulting in translocation t(9;4) (*Mll-Af9*) or t(9;17) (*Mll-Enl*) (Figure 1A).

The effects of targeting the Mll chromosomal translocations to haematopoietic cells of differing maturities, and comparative assessment of chromosomal translocations between *Mll* and different fusion partners such as *Enl* or *Af9* genes, were analysed with these *Mll; Enl* or *Mll; Af9* translocators. We used a strain of mice with *Cre* recombinase expression under the control of the *Lck* promoter (Wildin *et al*, 1995), which restricts *Cre* expression to the T-cell lineage (McCormack *et al*, 2003; Codrington *et al*, 2005), or the *Lmo2* promoter, which works in HSC (Yamada *et al*, 1998; McCormack *et al*, 2003; Schlaeger *et al*, 2004). Cohorts of 20 mice were established with *Mll* plus *Enl* or *Mll* plus *Af9 loxP* alleles and carrying the *Lck-Cre* transgene (designated, respectively, *Mll; Enl; Lck-Cre* or *Mll; Af9; Lck-Cre* mice). In addition, a cohort of *Mll; Af9; Lmo2-Cre* mice was studied.

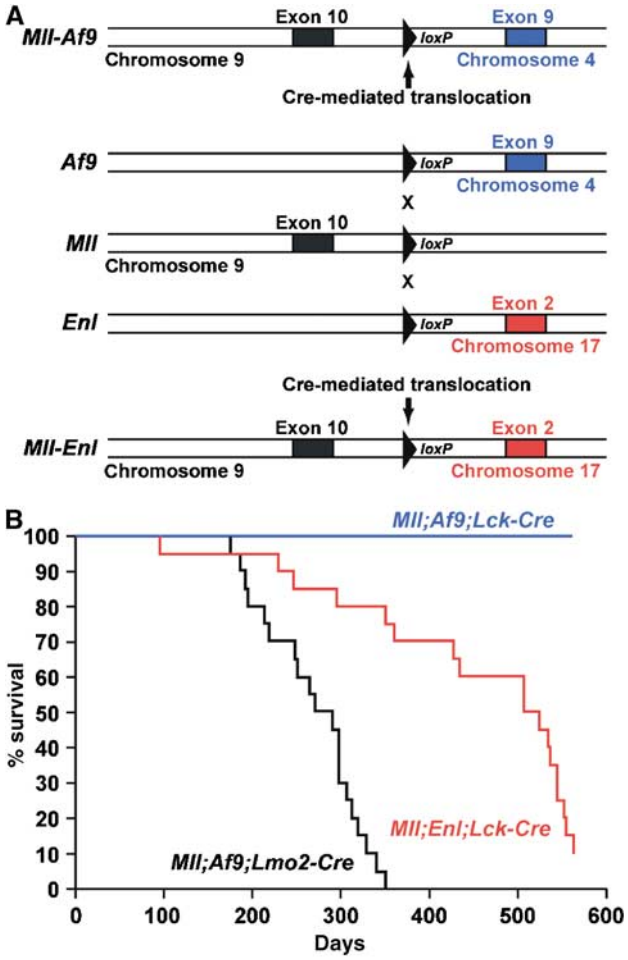

Figure 1 Incidence of leukaemias in *Mll* translocator mice. (**A**) Diagram of *Mll*, *Enl* and *Af9* targeted alleles in translocator mice (Collins *et al*, 2000; Forster *et al*, 2003). *LoxP* sites were introduced, by homologous recombination in ES cells, into introns of mouse *Mll*, *Enl* or *Af9*, corresponding to the human introns where chromosomal translocations are typically found. Specific cells in mice carrying *loxP* sites in both *Mll* and *Enl* or *Mll* and *Af9* genes can undergo chromosomal translocations, mediated by Cre recombinase, to create fusion genes analogous to those of human leukaemias, as indicated. (**B**) Survival curves for translocator mice. Cohorts of 20 translocator mice expressing Cre under the control of *Lck* or *Lmo2* promoters (McCormack *et al*, 2003) were analysed over a period of about 1.5 years.

Comparable control groups with only the *Lck-Cre* allele, the *Mll* plus *Enl loxP* or *Mll* plus *Af9 loxP* alleles were also studied. All mice were monitored for possible adverse effects. Out of the 20 *Mll; Enl; Lck-Cre* mice, 18 developed haematopoietic neoplasms within 18 months (Figure 1B) and all the mice in the *Mll; Af9; Lmo2-Cre* cohort developed haematological malignancies. No haematological malignancies were observed in the mice of the *Mll; Af9; Lck-Cre* cohort.

Mll; Enl; Lck-Cre translocators develop either lymphoid or myeloid tumours

Haematopoietic neoplasias in the *Mll-Enl* translocators were dependent on *Lck-Cre* and in *Mll-Af9* translocators on *Lmo2-Cre*, supporting a mandatory role for chromosomal translocations in neoplasia in these mice. The presence of chromosomal translocations in the cells characterising the

haematological malignancies was verified by either genomic DNA filter hybridisation to detect the chromosomal fusion point or by fluorescence *in situ* hybridisation (FISH) (Figure 2). Exemplification is presented in Figure 2, showing filter hybridisation of *Mll; Enl; Lck-Cre* or *Mll; Af9; Lmo2-Cre* translocator DNA (tumour, thymus and liver biopsy) with 5′ *Mll*, 3′ *Mll* (Figure 2A and B), 3′ *Enl* probes (Figure 2C) or a 3′ Af9 probe (Figure 2D). The translocated alleles were observed in all three tissues examined, even in the liver where some sections have high numbers of cells with the chromosomal translocation. An independent assessment of the presence of chromosomal translocations in the tumour cells was obtained by FISH. Metaphase spreads from spleen cells of tumour mice were hybridised with whole chromosome paints for chromosome 9 (*Mll*, green) and chromosome 17 (*Enl*, red) (Figure 2C) or chromosome 4 (Af9, red; Figure 2D). In addition, expression of the *Mll*-fusion genes was confirmed by RT–PCR analysis of RNA prepared from cell lines established from tumours (see below). We could detect the relevant *Mll*-fusion mRNA in RNA prepared from *Mll-AF9* knock-in, *Mll; Enl; Lmo2-Cre* and *Mll; Af9; Lmo2-Cre* translocator mice (Figure 2E). The cells present in the tumours, therefore, express *Mll*-fusion mRNA, carry reciprocal chromosomal translocations and the malignancies are caused

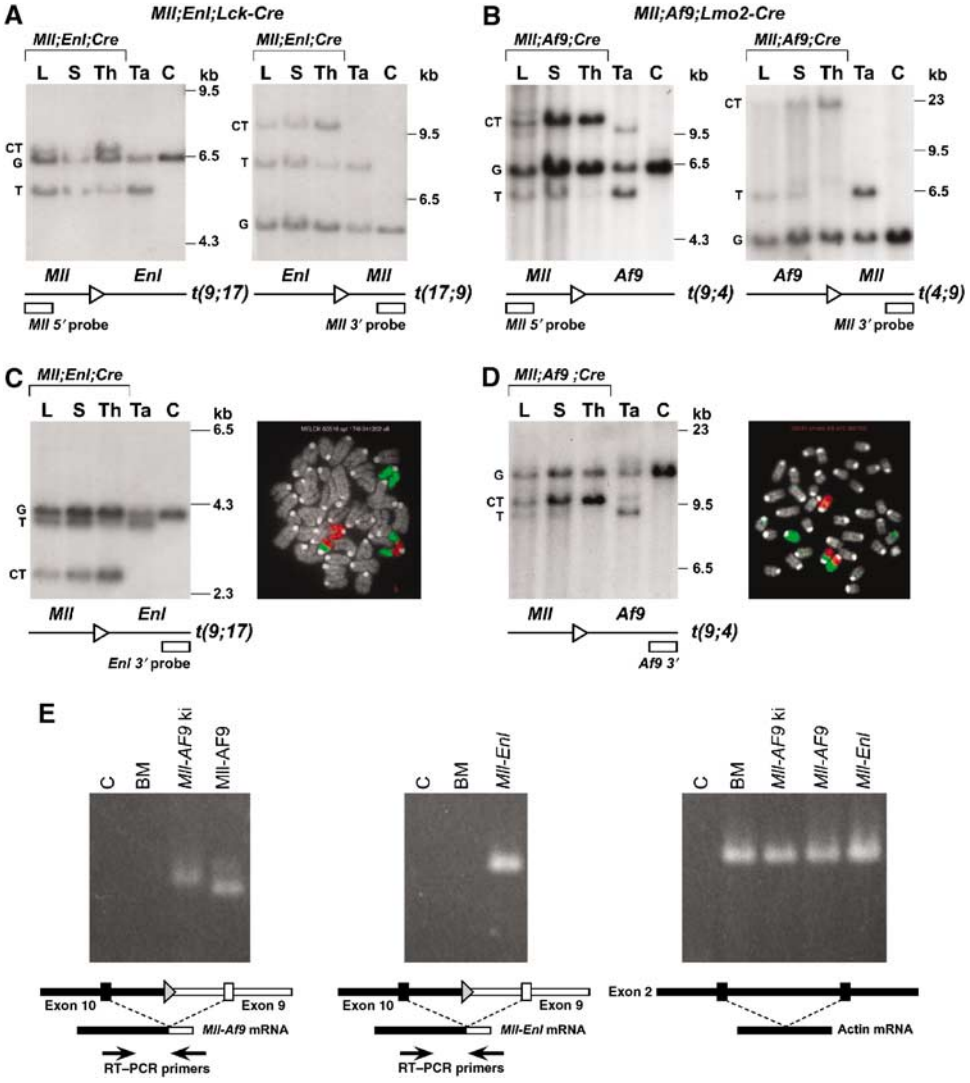

Figure 2 Haematological malignancies in *Mll* translocators have chromosomal translocations. *Mll; Enl; Lck-Cre* (**A**, **C**) or *Mll; Af9; Lmo2-Cre* (**B**, **D**) tumours were examined for the presence of chromosomal translocations by filter hybridisation and by FISH. For the former, DNA samples are designated as follows: C: control C57BL/6 tail or CCB ES DNA; Ta: tail; Th: thymus; S: spleen; L: liver. For each panel, the spleen, liver and thymus DNAs were prepared from *Mll; Enl; Lck-Cre* or *Mll; Af9; Lmo2-Cre* mice (overlined samples). Hybridising bands correspond to targeted (T), germline (G) or chromosomal translocation (CT) alleles. Hybridisation probes from either side of the translocation junction, namely 5′ and 3′ *Mll* probes and a 3′ *Enl* probe (A and C, respectively) or 5′ and 3′ *Mll* probes and a 3′ *Af9* probe (B and D, respectively), were used to detect rearrangements. For FISH analysis (C, D), metaphase spreads were made with cells from the spleen and painted with whole chromosome paints for chromosome 9 (green) and chromosome 4 (*Af9*) or 17 (*Enl*) (red). Painted chromosomes from an *Mll; Enl; Lck-Cre* (C) or *Mll; Af9; Lmo2-Cre* spleen (D) are shown. (**E**) RT–PCR analysis of *Mll*-fusion mRNA. Cell lines were established from *Mll-AF9* knock-in mice (Corral *et al*, 1996; Dobson *et al*, 1999), *Mll; Enl; Lmo2-Cre* translocators (Forster *et al*, 2003) and *Mll; Af9; Lmo2-Cre* translocators (this paper). RNA was made and converted to cDNA for RT–PCR amplification with primers spanning junctions of *Mll* and *Enl* or *Af9* RNA sequences as indicated. Total bone marrow RNA from a wild-type mouse was used as a negative control for cDNA lacking Mll-fusion sequences and RT–PCR with *actin* primers was used for quality control of cDNA. C: no template (H_2O) control; BM: bone marrow; ki: knock-in.

by the Mll-fusion protein since they are dependent on the presence of *Mll-loxP*, *Enl-loxP* or *Af9-loxP* plus the *Cre*-expressing alleles.

Post-mortem analysis revealed splenomegaly typifying the haematological malignancy in both the *Mll; Enl; Lck-Cre* and *Mll; Af9; Lmo2-Cre* translocators, due to an almost total repopulation of the spleen with malignant cells (Figure 3) represented by loss of distinct white and red pulp architecture. In *Mll; Af9; Lmo2-Cre* translocators, perivascular deposits of these same cells are evident with associated infiltration of large areas of the liver (a similar picture was found with kidney and lung, data not shown) and involvement of lymph nodes. These features, together with myeloid surface marker expression on the neoplastic cells (Figure 4 and Supplementary Table 1B), suggest that these mice consistently develop myeloid lineage malignancies. Blood smears show circulating leukaemic cells of various maturities, including a large proportion of well-differentiated cells. By the Bethesda classification of nonlymphoid tumours (Kogan *et al*, 2002), these mice have myeloproliferative (MPD)-like myeloid leukaemia. Blood smears of the *Mll; Enl; Lck-Cre* translocator mice showed little evidence of circulating leukaemic cells (Figure 3), unlike the *Mll; Enl; Lmo2-Cre* translocators previously described (Forster *et al*, 2003) and the *Mll; Af9; Lmo2-Cre* translocators described here (Figures 3 and 4), both of which have a similar MPD-like myeloid leukaemia. This distinction may reflect differences in the leukaemic precursors in the translocator models since the chromosomal translocations are caused by Cre expression controlled by different *Cre* alleles.

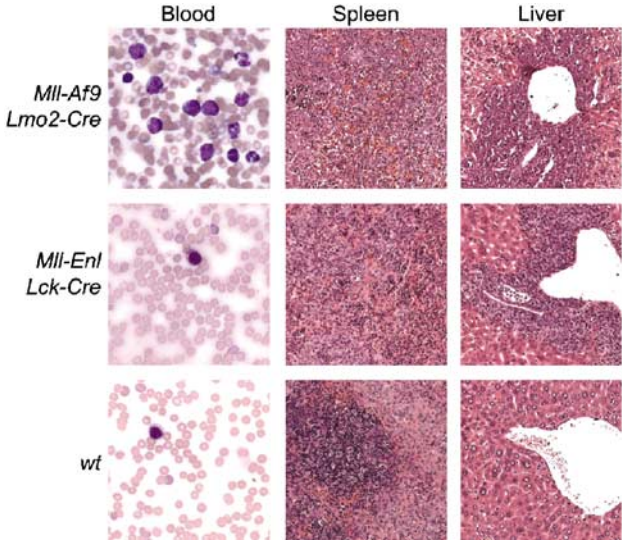

Figure 3 Histopathology of tumours from *Mll-Enl* and *Mll-Af9* translocator mice. Tissues were fixed in 10% formalin and wax embedded prior to generation of histological sections. The sections were stained with H&E and photographed at ×40 magnification. May–Grünwald–Giemsa (MGG)-stained blood films and sections shown are from an *Mll; Af9; Lmo2-Cre* translocator mouse (top row), from an *Mll; Enl; Lck-Cre* translocator mouse (middle row) or from a C57BL/6 wild-type mouse (bottom row).

A total of 90% of the *Mll; Enl; Lck-Cre* translocator mice developed haematological malignancy in the 18-month period of the experimental analysis. The haematological malignancy in these translocators was analysed by surface protein expression profile using FACS analysis to determine the major cell types from spleens of mice with splenomegaly. Figure 4 and Supplementary Table 1 embody findings with the *Mll; Enl; Lck-Cre* translocators compared with *Mll; Af9; Lmo2-Cre* translocator mice. Unlike the uniform finding of MPD-like myeloid leukaemia in *Mll; Af9; Lmo2-Cre* translocator mice described here and *Mll; Enl; Lmo2-Cre* mice (Forster *et al*, 2003), the neoplasias found in *Mll; Enl; Lck-Cre* mice were either of the T-cell or the myeloid lineage. Examples are shown in Figure 4, where a Mac-1/Gr-1 double positive

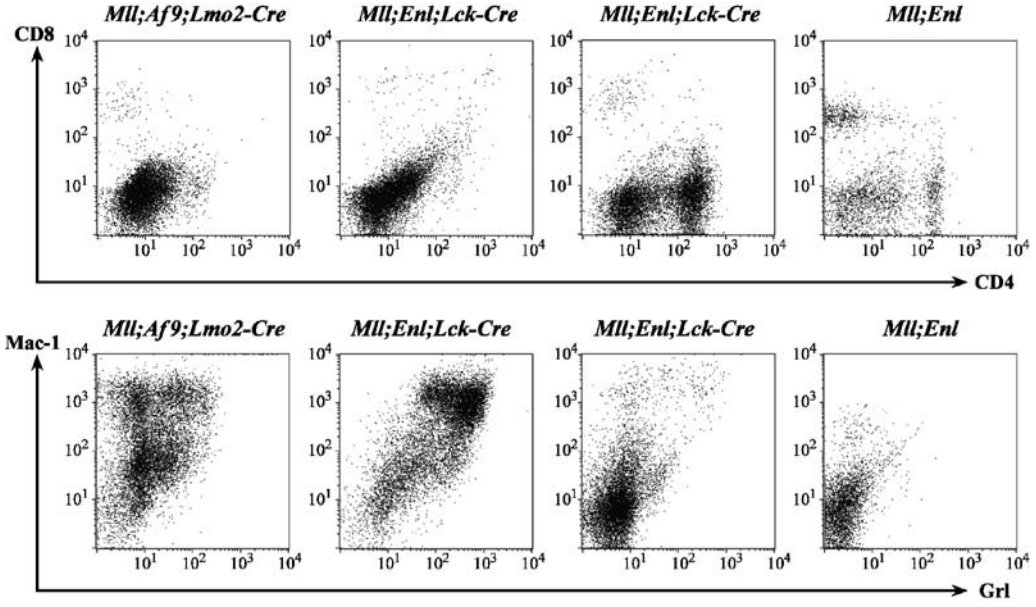

Figure 4 Cell surface profiles of leukaemias vary in different translocator mice. Single-cell suspensions from splenic tumours of *Mll; Enl; Lck-Cre* or *Mll; Af9; Lmo2-Cre* translocator mice were compared by flow cytometry with splenic leucocytes from a control (*Mll; Enl*) mouse lacking the *Cre* allele. Surface proteins were labelled with fluorescent antibodies detecting CD8 plus CD4 (T-cell markers) or Mac-1 plus Gr1 (myeloid markers).

myeloid tumour is compared with a CD4-positive, CD8-negative T-cell tumour. Data for 18 tumours in the *Mll; Enl; Lck-Cre* cohort are shown in Supplementary Table 1A. In addition to the cell surface marker phenotype, examination of histopathology defined the tumours as T-cell lineage. The characteristics of these T-cell tumours (including T-cell receptor *Tcrb* gene rearrangements; see Figure 5) were splenomegaly, lymphadenopathy without enlargement of thymus and expression of T-cell receptor-associated CD4 and/or CD8 molecules. This classifies the tumours as mature T-cell neoplasms, specifically small T-cell lymphoma (STL) according to the Bethesda classification (Morse *et al*, 2002).

While some *Mll; Enl; Lck-Cre* mice developed STL, others developed tumours expressing myeloid markers (Figure 4 and Supplementary Table 1), which showed few, poorly differentiated cells in blood, myeloid cell histology in spleen and had infiltrates of neoplastic cells in the liver and kidneys. These criteria demonstrated the presence of myeloid leukaemia without maturation in these mice, by the Bethesda classification of nonlymphoid neoplasms (Kogan *et al*, 2002).

All Mll; Enl; Lck-Cre tumours have a footprint of Rag V-D-J recombinase activity

The finding of T-cell or myeloid tumours in *Mll; Enl; Lck-Cre* mice is intriguing because of the specific expression of the *Lck-Cre* transgene in T cells (McCormack *et al*, 2003). This implies that both the STL and the myeloid neoplasias in the *Mll; Enl; Lck-Cre* translocator mice have their origin within cells of the lymphoid lineage. The precursors of the STLs were presumably committed to T-cell differentiation, whereas the myeloid leukaemias could have arisen from early lymphoid progenitor cells retaining various differentiation options, allowing reassignment of cellular commitment, as suggested by early studies of human acute myeloid leukaemia (AML) (Boehm *et al*, 1987a, b; Adriaansen *et al*, 1991). The corollary of *Lck-Cre* expression, which initiates in immature thymocytes, is *Mll; Enl; Lck-Cre* translocators should undergo

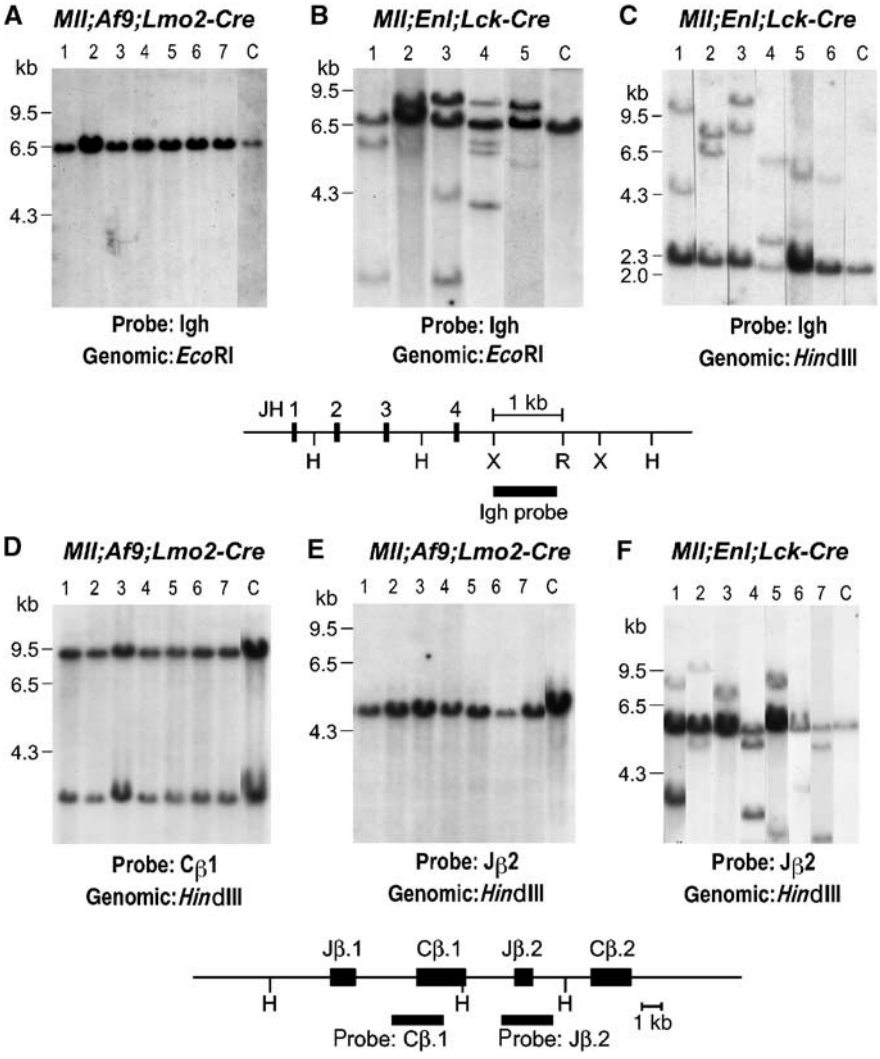

Figure 5 Rearranged antigen receptor genes in tumours from *Mll; Enl; Lck-Cre* translocator mice. Genomic DNA from splenic tumours of *Mll; Af9; Lmo2-Cre* (**A, D, E**) or *Mll; Enl; Lck-Cre* (**B, C, F**) translocator mice were analysed by filter hybridisation using an *Igh*, *Tcr Cβ1* or *Tcr Jβ2* probe. For hybridisation of the DNAs with the *Igh* probes, digestions were either with *Eco*RI (A, B), which will detect all joining events to the whole Ig JH region, or with *Hin*dIII (C). Hybridisation of TCR probes was with *Hin*dIII-digested DNA. V-D-J or D-J joins were detected with *Cβ1* or *Jβ2* probes. Partial restriction maps of the *JH* or *Cβ1–Jβ2* genomic regions are shown, indicating the location of the probe fragments. H: *Hin*dIII; X: *Xba*I; R: *Eco*RI.

chromosomal translocation around the time of V-D-J recombinase gene expression (*Rag1* and *Rag2*). Thus, Rag proteins may have caused antigen receptor gene rearrangement leaving a 'footprint' at the DNA level reflected by new 'rearranged' restriction fragments for T-cell receptor (*Tcr*) or immunoglobulin H chain (*Igh*) genes.

We examined the genomic DNA of the *Mll; Enl; Lck-Cre* translocator splenic tumours with *Tcr* β chain and *Igh* probes. All except one (tumour 11, which showed a myeloid surface marker pattern of splenic tumour cells) showed rearrangement of the *Tcr* β chain and/or *Igh* probes (Figure 5 and Supplementary Table 1A). Most exhibited bi-allelic rearrangements, and some showed four rearranged bands implying that the tumour is either not clonal or comprises two distinct populations of cells (e.g. Figure 5B, lane 4). This is not a random rearrangement phenomenon as neither the myeloid lineage tumours from *Mll; Enl; Lmo2-Cre* mice (F Cano and TH Rabbitts, unpublished) nor splenic tumours from the *Mll; Af9; Lmo2-Cre* translocators described here showed *Tcr* β chain or *Igh* gene alterations (14 *Mll; Af9; Lmo2-Cre* tumours were compared and seven are shown for reference in Figure 5A, D and E). Thus, the cells that constitute all the *Mll; Enl; Lck-Cre* translocator splenic tumours have passed through a differentiation stage during which the *Rag* recombinase genes were expressed and the Rag proteins were actively performing their function of V-D-J recombination in these precursors. This suggests an intermediate stage of T-lymphoid differentiation in all these tumours, even those that present with a myeloid lineage phenotype.

Cell-specific effects of Mll-Af9 chromosomal translocations

Our observations with *Mll-Enl* translocator mice showed that haematopoietic malignancies ensue if *de novo* chromosomal translocations are induced by Cre expression from the *Lmo2-Cre* allele (Forster *et al*, 2003) or from the *Lck-Cre* allele (this paper). We previously found that mice expressing an *Mll-AF9* fusion knock-in allele developed haematological malignancies with a myeloid phenotype (Corral *et al*, 1996; Dobson *et al*, 1999). We have compared the effects of *Mll; Af9* translocator mice made with *Lck-Cre* or *Lmo2–Cre* alleles. A cohort of *Mll; Af9; Lck-Cre* translocators was studied for an 18-month period. Surprisingly, no signs of ill health or postmortem abnormalities were found in these mice at the 18-month point. This contrasts with malignancies in the cohort of *Mll; Af9; Lmo2-Cre* translocators, which all succumbed to leukaemia within about 1 year (Figure 1B). These latter mice have a similar pathology to the *Mll; Enl; Lmo2-Cre* translocators, showing MPD-like myeloid leukaemia. The tumour cells carry reciprocal chromosomal translocations as judged by filter hybridisation with 5′ or 3′ *Mll* DNA probes (Figure 2B) or with a 3′ Af9 probe (Figure 2D). In addition, FISH analysis of spleen cells from the tumour-bearing mice showed the reciprocal chromosome (Figure 2D) and the typically diploid state of the tumours.

Thus, while *Mll; Af9; Lmo2-Cre* translocators can develop leukaemias, *Mll; Af9*; *Lck-Cre* do not. It is possible that chromosome territory within mouse lymphoid cells specifically precludes chromosomal translocations between the *Mll* and *Af9* genes. We investigated the presence of *Mll* and *Af9* interchromosomal translocations in asymptomatic mice using a genomic PCR assay dependent on the ability to amplify a product if *Mll* and *Af9* sequences were abutted following translocation (Figure 6). Pups were identified from 8-day postnatal litters of *Mll; Enl; Lck-Cre*, *Mll; Af9; Lmo2-Cre* or *Mll; Af9; Lck-Cre* genotype. Thymus and bone marrow DNA was prepared and used in nested PCR analysis for the presence of chromosomal translocations. In each case, five pups of appropriate genotypes were used. Examination of DNA from *Mll; Enl; Lck-Cre* pups showed that one of the five had detectable *Mll-Enl* translocations in both thymus and bone marrow DNA (Figure 6A). By contrast, all five *Mll; Af9; Lck-Cre* pups showed chromosomal translocations in thymic DNA but none had any detectable in bone marrow (Figure 6C). This striking difference is despite the fact that *Mll; Af9; Lck-Cre* translocators do not develop leukaemias whereas essentially all *Mll; Enl; Lck-Cre* do. Finally, all the *Mll; Af9; Lmo2-Cre* pups had translocations in both thymus and bone marrow (Figure 6B) consistent with the rapid leukaemogenesis in these mice. Therefore, despite the absence of tumorigenesis, *Mll-Af9* chromosomal translocations do occur in *Lck-Cre*-expressing cells and furthermore, they occur in T cells very early in the life of the *Mll; Af9; Lck-Cre* mice.

The expression of *Mll-Af9* fusion mRNA was demonstrated by RT–PCR with RNA-cDNA made from *Mll; Af9; Lck-Cre* mice at 3 or 6 months of age (Figure 6D). We found that RNA from the spleen, thymus and bone marrow, even at 6 months, contained *Mll-Af9* transcripts, whereas other tissues such as the kidney did not. Thus, sustained expression of the chromosomal translocation occurs in *Mll; Af9; Lck-Cre* mice. These results suggest that Mll-Af9 is inert in committed T cells.

Mll-Af9 and Mll-Enl translocator myeloid tumours are transplantable

The ability of the Mll-fusion-dependent tumours to undergo independent growth was assessed by establishment of tissue culture lines from *Mll-AF9* knock-in mice (Corral *et al*, 1996; Dobson *et al*, 1999) and *Mll; Enl; Lmo2-Cre* translocators (Collins *et al*, 2000; Forster *et al*, 2003). We have been unable to make lines from *Mll; Enl; Lck-Cre* translocators, irrespective of the lymphoid or myeloid phenotype. The tumorigenicity of the other lines was tested in a nude mouse assay in which cells were inoculated subcutaneously and tumour growth was assessed. All the lines developed into tumours in a high proportion of recipients, with latencies less than 3 weeks (Table I). In addition, the retention of either the *Mll-AF9* knock-in allele or the *Mll-Af9* or *Mll-Enl* chromosomal translocation was confirmed by filter hybridisation (data not shown).

Discussion

The translocator model recapitulates analogous events in human cancers

The translocator mouse model (Smith *et al*, 1995; van Deursen *et al*, 1995; Buchholz *et al*, 2000; Collins *et al*, 2000) can recapitulate chromosomal translocations found in human tumours by allowing cell-specific *de novo* chromosomal translocations to take place. This means that reciprocal translocations can be engineered *in vivo* for the first time. We have shown that *Mll*-associated translocations can be induced by Cre recombinase between two pairs of

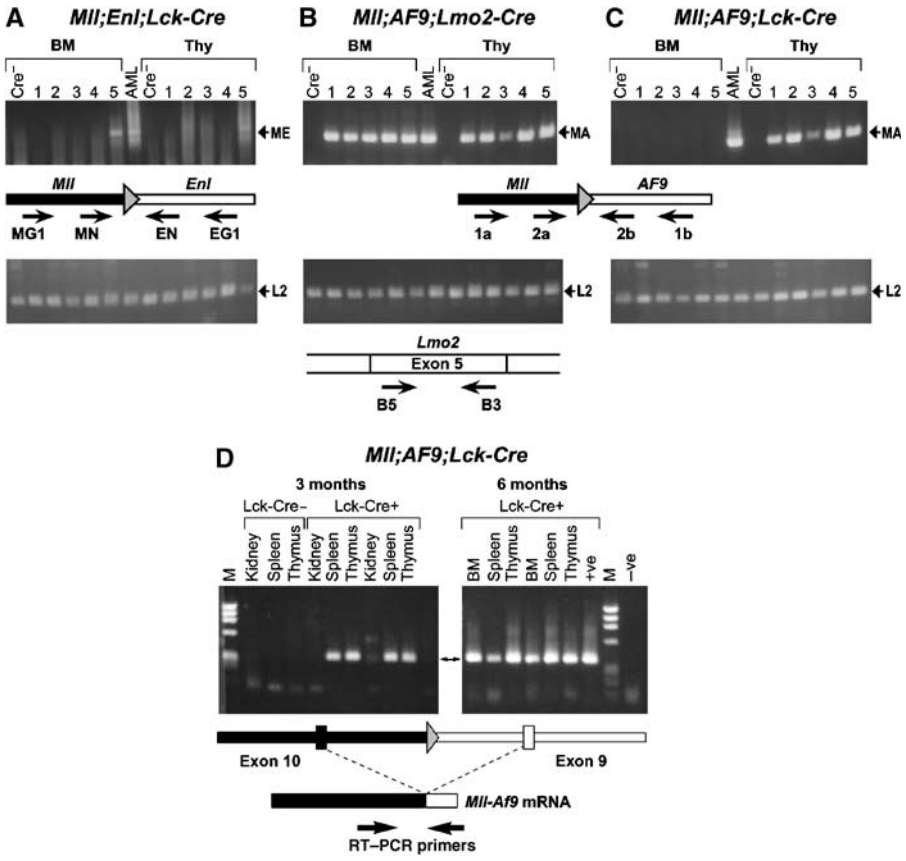

Figure 6 Chromosomal translocations are present in *Mll; Af9; Lck-Cre* translocators by postnatal day 8 and *Mll-Af9* expressed at 6 months. (**A–C**) Bone marrow (BM) and thymus (Thy) genomic DNA was prepared from 8-day-old pups (five littermates for each genotype) from *Mll; Af9; Lck-Cre* or *Mll; Enl; Lck-Cre* translocators. PCR was performed for 70 cycles using nested primers to amplify either (A) *Mll* plus *Enl* (MG1 + EG1, followed by MN + EN; Forster *et al*, 2003) or (B, C) *Mll* plus *Af9* primers (1a + 1b, followed by 2a + 2b; Collins *et al*, 2000). Positive controls were performed on DNA from *Mll; Af9 or Mll; Enl* translocator splenic tumours (indicated as AML) using 35 cycles of amplification, and negative controls were performed, using 70 cycles of amplification, on DNA prepared from spleens of P8 litter mates of *Mll; Af9* or *Mll; Enl* mice without the *Cre* gene (indicated as Cre−). Quality control of DNA was determined by PCR amplification of the *Lmo2* gene with primers B5 and B3 (McCormack *et al*, 2003) using 35 cycles of amplification. Primer locations are diagrammatically shown. Bands corresponding to amplified products are indicated as follows: ME: *Mll-Enl* junction; MA: *Mll-Af9* junction; L2: *Lmo2* gene product. (**D**) Litters of *Mll; Af9; Lck-Cre* mice, including *Mll; Af9* controls lacking *Cre* gene, were established and at 3 or 6 months, RNA was prepared from the spleen, thymus, kidney and bone marrow (latter at 6 months only). RT–PCR was carried out with primers from exon 10 of *Mll* or exon 9 of *Af9* to detect RNA products of the *Mll-Af9* translocated fusion allele. The double-headed arrow indicates the gel band corresponding to the product of this translocated allele. *Mll-Af9* fusion mRNA transcript was present in the spleen and thymus at 3 months and in addition detected in these tissues and in bone marrow at 6 months of translocator mice. M: ϕX174 *Hae*III-digested DNA markers; + ve: RT–PCR product from an Mll-Af9 leukaemia produced in *Mll; Af9; Lmo2-Cre* mice; −ve: RT–PCR in the absence of template; BM: bone marrow.

chromosomes (i.e. between chromosomes 9 and 4 or between chromosomes 9 and 17) in similar cell types, as well as in distinct lineages. Chromosomes 4 and 17 therefore seem equally accessible to translocate with chromosome 9, indicating that chromosome territory might not impinge too heavily on the ability of translocation between different *Mll* partners. In addition to mimicking the ability of *MLL* to fuse with various partners and cause leukaemia, the outcome of the mouse *Mll-Enl* and *Mll-Af9* chromosomal translocations parallels the tumour phenotypes found in humans, where the former is seen in T-cell tumours or in myeloid tumours whereas *MLL-AF9* is almost exclusively seen in myeloid tumours.

Leukaemic precursor cells in Mll-associated leukaemias

Chromosomal translocations are primary events in haematopoietic tumours (Rowley, 1999) and the putative self-renewing leukaemic stem cell (LSC) must carry the chromosomal translocation. The oncogenicity of *Mll*-fusion proteins in

Table I Translocator tumours are transplantable

Genotype	Cell line	Cell surface phenotype	No. of tumours	Average time for growth (days)
Mll-AF9 knock-in	#1	Mac^+; $Gr1^-$	8/10	18
	#2	Mac^+; $Gr1^-$	9/10	17
	#3	Mac^+; $Gr1^-$	9/10	14
Mll; Enl; Lmo2-Cre	#1	Mac^+; $Gr1^{low}$	11/14	18
	#2	Mac^+; $Gr1^{low}$	11/14	18
	#3	Mac^+; $Gr1^{low}$	11/14	13

Cell lines from either *Mll-AF9* knock-in or *Mll-Enl* translocator mice were cultured in the presence of growth factors (see Materials and methods) and $1–5 \times 10^6$ cells were injected into the flanks of 6- to 8-week-old female MF1 nude mice. Tumours were resected at approximately 1.5 cm size and post-tumour growth evaluation was conducted for genotype, presence of the relevant translocation alleles and surface marker expression. Number (No.) of tumours is expressed as fraction of tumours/number of injection sites.
All lines were negative with antibodies recognising Sca1, C-kit, Ter119, CD4, CD8, Thy1, CD25 and B220.

different cells of haematopoiesis has been evaluated using knock-in alleles (Corral *et al*, 1996; Dobson *et al*, 1999, 2000; Wang *et al*, 2005), or retroviral delivery of MLL fusions to HSC (Slany *et al*, 1998; Lavau *et al*, 2000; Deguchi *et al*, 2003; So *et al*, 2003; Zeisig *et al*, 2003) or translocator mice (Forster *et al*, 2003). These demonstrate that tumours develop with a differentiated phenotype. Furthermore, transduction of *Mll-Enl*-expressing retroviruses into purified GMP progenitor cells showed that leukaemias could also develop from these (Cozzio *et al*, 2003).

In studies of *MLL-AF4* chromosomal translocations in monozygotic twins, leukaemias were found that arose with clonal origin *in utero* but with different immunoglobulin rearrangements in the leukaemias at presentation (Greaves and Wiemels, 2003). This provides strong evidence that the *MLL-AF4* chromosomal translocations were primary in the LSC and that MLL-AF4 can exert an effect on pluripotent HSC. However, the fusion protein may also have molecular consequences for later cells, which are no longer pluripotent. We show in our translocator model that the primary effects of Mll-Enl fusion can occur in noncommitted cells or cells that are partially or fully committed to the T-lymphocyte lineage. Thus, Mll fusions do not need the environment of noncommitted cell to be oncogenic, suggesting that *MLL* translocation-associated leukaemia in man is not a mandatory stem cell leukaemia.

Mll-fusion proteins require a permissive environment for oncogenicity

The Mll-Af9 fusion appears to be unable to support tumorigenesis in T-lymphopoiesis whereas it is a potent inducer of myeloid leukaemias. The absence of Mll; Af9; Lck-Cre-induced leukaemias is not due to the lack of translocations or expression of *Mll-Af9* fusion mRNA in *Mll; Af9; Lck-Cre* nonleukaemic mice (Figure 6). Therefore, Mll-Af9 is inert in the multipotent progenitors destined to produce the T-cell lineage. This 'inertia' (depicted in Figure 7) may be due to the nonresponsiveness of target genes to lineage activation by Mll-Af9, or secondary mutations necessary for Mll-Af9 complementation do not typically occur in T cells or cofactors needed for Mll-Af9 function do not exist in T cells. The first possibility raises intriguing questions about the nature of MLL-fusion protein target genes, namely the possibility of common and specific target genes for MLL fusions in haematopoietic cells. If all MLL fusions have common effects on the transcriptome (e.g. control of *HOX* gene expression), we should anticipate the same consequence of having *Mll-Enl* and *Mll-Af9* translocations in T cells. Our data therefore suggest separation of MLL-fusion protein function into distinct parts, one being related to cell phenotype control (instructive function and controlling putative Mll-fusion-specific target genes) and another to development of oncogenic phenotype.

The Lck-Cre expression causes Mll-Af9 translocations but not leukaemia

We have shown that *Mll-Enl* is an effective oncogene when chromosomal translocations are caused by Cre recombinase made from an *Lck-Cre* allele. Virtually all the *Mll; Enl; Lck-Cre* mice developed leukaemia (Figure 1) but the phenotypes of these tumours were disparate, only a proportion being T-cell neoplasias and the majority myeloid tumours. Crucially,

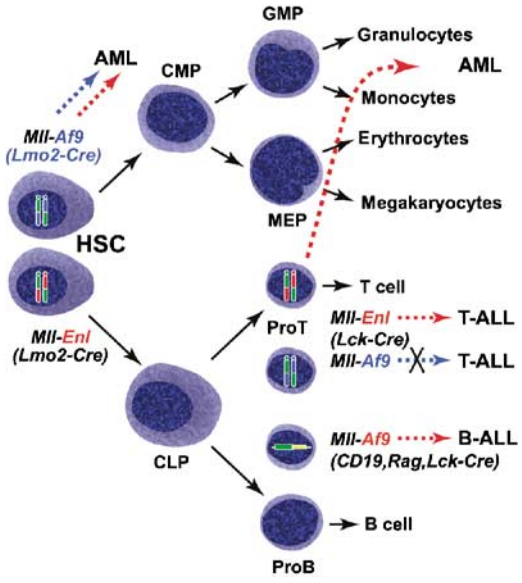

Figure 7 Summary of Mll fusions in haematopoiesis and tumorigenesis. When chromosomal translocations are generated *de novo* in *Mll-Af9* or *Mll-Enl* translocator mice, haematopoietic malignancies arise if translocations are mediated via Cre expression with either *Lmo2-Cre* (*Mll-Af9* or *Mll-Enl*) or *Lck-Cre* (*Mll-Enl* only) alleles. No tumours arose when *Mll-Af9* chromosomal translocations were induced by *Lck-Cre* expression, even though both chromosomal translocations and *Mll-Af9* fusion mRNA could be detected. Thus, when *Mll* translocations are induced in the pluripotent stem cells or in the multipotent myeloid progenitors using *Lmo2-Cre*, myeloid malignancies arise (this paper and Forster *et al*, 2003). A more complex situation was observed when the chromosomal translocations between *Mll* and *Enl* genes were induced by *Lck-Cre*-controlled expression of Cre recombinase. In this case, either T-cell malignancies or myeloid leukaemias appeared; however, in both types, the leukaemia progenitors had arisen from cells that had expressed functional Rag V-D-J recombinase. Thus, the myeloid malignancies of the *Mll; Enl; Lck-Cre* translocator mice appear to arise from cells that have undergone a lineage reassignment from a semicommitted, Rag+ lymphoid cell to a myeloid cell. This effect may occur by reprogramming the transcriptome of cells with the chromosomal translocations to realigning differentiation into myeloid from lymphoid status. HSC: haematopoietic stem cells; CMP: common myeloid progenitors; GMP: granulocyte–monocyte precursors; MEP: megakaryocyte–erythrocyte precursors; CLP: common lymphoid progenitors.

irrespective of the cell lineage, the tumours had detectable rearrangements of immunoglobulin and/or T-cell receptor genes (95% of tumours). This footprint of *Rag* gene expression means that the precursors of all these tumours have been through a stage during which Rag recombination takes place, which is a feature of lymphoid development (Gellert, 2002). This critical finding places the initiating chromosomal translocation event for the myeloid tumours of *Mll; Enl; Lck-Cre* mice within cells after the common lymphocyte precursor stage, implying that these are noncommitted lymphoid progenitors (see below).

An additional fundamental issue is the specificity of the *Lck-Cre* allele for *Cre* expression in the T-cell lineage as misexpression in progenitors might explain the myeloid tumours in *Mll; Enl; Lck-Cre* translocators. Several lines of evidence demonstrate that *Lck-Cre* is T-cell specific. Only those cells designated as being within the T-cell pathway (from DN1 cells onwards, which is just a stage before endogenous Lck activity is apparent; Wolfer *et al*, 2001,

2002) showed Cre-dependent deletion of a floxed chromosomal segment of the *Lmo2* gene as a reporter (McCormack *et al*, 2003) or activation of the *lacZ* gene in the *ROSA* locus with the *Lck-Cre*-controlled Cre (Codrington *et al*, 2005). There was no evidence of *Lck-Cre* activity in progenitors or in purified Mac-1- or Gr-1-positive cells (using a radiolabelled hybridisation assay for sensitive detection of deletion of a *loxP*-flanked reporter gene (McCormack *et al*, 2003) and the other direct detection of β-gal-positive cells (Codrington *et al*, 2005)). The analysis has been extended by the use of the *ROSA-YFP* reporter line (Srinivas *et al*, 2001) in which no progenitor or myeloid cells were found coexpressing relevant surface markers and YFP (markers were CD34, Sca1, Ter119, Mac-1 or Gr-1) (LF Drynan & TH Rabbitts, unpublished). Further, no Cre activity could be detected after selection of cells expressing B-cell markers (CD19, B220, IgM, IgD or IgG).

A critical biological control that provides a very powerful assessment of the specific association of Cre activity with T-cell lineage precursors or mature T cells is our observation that no tumours arose in translocator mice with *Mll-Af9* translocations induced by *Lck-Cre*. Our previous work with *Mll-AF9* knock-in mice (Corral *et al*, 1996; Dobson *et al*, 1999), and with *Mll-Af9* translocators in this paper, shows that this fusion creates a very potent myeloid oncogenic protein. In addition, we show that *Mll-Af9* chromosomal translocations occur in all thymuses of *Mll;Af9; Lck-Cre* mice tested as early as 8 days after birth and the fusion gene is expressed at least to 6 months of age (Figure 6). This shows that these translocator T cells can undergo translocation and there is no obvious clonal deletion of the cells carrying the expressed *Mll-Af9* fusion allele. If the myeloid tumours in the *Mll; Enl; Lck-Cre* arose because the *Lck-Cre* allele was expressing at some level in myeloid progenitors or in some occasional progenitor cell, we would expect myeloid tumours in the *Mll; Af9; Lck-Cre* translocators. A final piece of evidence of a T-cell progenitor origin for the myeloid tumours in *Mll; Enl; Lck-Cre* translocators is that they have *Tcr/Igh* gene rearrangements, unlike the myeloid tumours arising in progenitors such as those made in *Mll; Af9; Lmo2-Cre* or *Mll; Enl; Lmo2-Cre* translocator mice. We conclude that the *Lck-Cre* allele is specific for the T-cell lineage and that the myeloid tumours originate in cells after the CLP stage in the *Mll; Enl; Lck-Cre* translocator mice.

Mll-Enl can cause lineage reassignment

We have found that either T-cell or myeloid type leukaemias arise from *Mll-Enl* chromosomal translocations mediated by the T-cell-specific Cre recombinase made from our *Lck-Cre* transgenic mouse (McCormack *et al*, 2003). These findings suggest that expression of Mll-Enl in immature T-cell precursors elicits reprogramming of the cells into the myeloid lineage. A process of lineage reassignment in human leukaemias has already been indicated by previous observations that AML often have rearranged antigen receptor genes (Boehm *et al*, 1987a, b; Adriaansen *et al*, 1991). Direct experimental support for reprogramming of lymphoid precursors into myeloid cells has been provided by ectopic expression of v-*raf* (Klinken *et al*, 1988) or CEB/P (Xie *et al*, 2004), indicating that aberrant gene expression can force cells to respond to different developmental programmes. These data indicate that the boundaries in haematopoietic lineage assignment may be blurred, as strict development along individual pathways is subvertable (Klinken *et al*, 1988; Xie *et al*, 2004). The ability of Mll-Enl to reassign lymphoid cells to myeloid lineage reflects a pathogenic subversion of lineage commitment and supports an instructive function to this (and possibly other) MLL fusion, in accordance with the master gene model for the effects of oncogene activation following chromosomal translocations in acute leukaemias (Rabbitts, 1991). The model that emerges is of a noncommitted T-cell progenitor that would normally have only one fate, namely to develop into a mature thymocyte (i.e. after Rag recombinase expression). If an *Mll-Enl* translocation occurs in this early, noncommitted T-cell progenitor, the influence of the Mll-Enl fusion is reassignment of lineage into the myeloid pathway. Conversely, if the translocations occur after the cell has progressed sufficiently far along the T-cell differentiation pathway, the neoplastic cell can only present as a T-cell leukaemia. The molecular events by which this lineage reassignment occurs will be interesting, as it should identify molecular targets of instructive MLL-fusion protein function distinct from other putative common functions.

Materials and methods

Analysis of translocator mouse strains

Mouse lines carrying *loxP* sites in either an *Mll* intron, an *Af9* intron or an *Enl* intron have been described (Collins *et al*, 2000; Forster *et al*, 2003). The general structure of these alleles is summarised in Figure 1A. Mice expressing Cre recombinase either under the control of the *Lmo2* or the *Lck* promoter (McCormack *et al*, 2003) were bred with those with *Mll*, *Enl* or *Af9 loxP* alleles to generate cohorts of 20 mice for each genotype. Mice carrying *Mll* and *Enl* or *Af9* alleles with *loxP* sites with or without a *Cre* allele are designated respectively (*Mll; Enl; Lmo2-Cre*), (*Mll; Enl; Lck-Cre*) or (*Mll; Enl*) or (*Mll; Af9; Lmo2-Cre*), (*Mll; Af9; Lck-Cre*) or (*Mll; Af9*). At the signs of ill health, post mortem was carried out and tissues were removed as needed. Histological sections (4 μm in thickness) were made from fixed tissue using wax embedding and were stained with haematoxylin and eosin (H&E). Flow cytometry was conducted using a FACScaliber and surface proteins were detected with fluorescent antibodies (BD-Pharmingen).

Detection of chromosomal translocations was carried out using Southern filter hybridisation or FISH as described (LeFranc *et al*, 1986; Forster *et al*, 2003). Filters were incubated for 24 h with ^{32}P-labelled probes at 65°C in a 3 × SSC buffer and washed at 65°C with 0.1 × SCC and 0.1% SDS. Hybridisation signal was detected with pre-fogged X-ray films at −70°C with intensifying screens. FISH analysis was carried out with metaphase spreads of spleen cells and metaphase spreads were painted with whole chromosome paints for chromosome 9 (green, *Mll* chromosome), 17 (red, *Enl* chromosome) or 4 (red, *Af9* chromosome) (Cambio).

Antigen receptor gene rearrangements were detected by Southern filter hybridisation. Splenic tumour genomic DNA or CCB ES cell DNA was restriction digested, fractionated and transferred to nylon membranes followed by hybridisation to either a mouse *Igh* probe (Neuberger and Williams, 1986) or T-cell receptor probes (*Cβ1* or *Jβ2*) (Malissen *et al*, 1984). DNA was cleaved with either *Hin*dIII or *Eco*RI for *Igh* gene status or *Hin*dIII for *Tcr* gene status.

Detection of Mll chromosomal translocations and mRNA in nonleukaemic mice

Genomic PCR was carried out using nested primers and 0.5 μg genomic DNA per initial 25 μl PCR reactions. A 1 μl portion of this reaction was used with nested primers, again in 25 μl reaction. PCR reactions were initially denatured at 95°C for 2 min followed by 35 cycles comprising 95°C for 1 min, 65°C for 1 min and 72°C for 1 min with a final 72°C step for 10 min. The PCR primers flanking the *Mll-Enl* translocation junction were MG1 + EG1 followed by nested primers MN + EN (Forster *et al*, 2003). Primers flanking the *Mll-Af9*

translocation junction were 1a + 1b followed by nested primers 2a + 2b (Collins *et al*, 2000). As an internal control for DNA quality, the *Lmo2* primers B5 + B3 were used to amplify an *Lmo2* gene segment (McCormack *et al*, 2003). For inter-exon RT–PCR on cDNA, MEX7F + F9B2L were used corresponding to sequences present in exon 10 of *Mll* and exon 9 of *Af9*. RT–PCR primers for amplification of *Mll-Enl* mRNA were as described (Forster *et al*, 2003).

Cell lines

Bone marrows were cultured in DMEM media in 10% FCS plus growth factors (1 μl/ml IL7 (Roche), 2 μl/ml IL2 (Roche), 0.5 μl/ml IL6 (Roche), 5% IL3 (WEHI supernatant) and 1 μl/ml GMSCF (Boehringer) at 37°C under 5% CO_2. For tumorigenicity assays, $1–5 \times 10^6$ cells were injected subcutaneously into flanks of 6- to 8-week-old female MF1 nude mice. Tumour growth was terminated at ~1.5 cm diameter. Tumours were resected, single-cell suspensions were made, followed by flow cytometry and genomic DNA filter hybridisation analysis to confirm concordance between the cultured cell lines and the tumours.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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