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Editorial
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The "Never-Ending" Mouse Models for MLL-Rearranged Acute Leukemia Are Still Teaching Us

Katrin Ottersbach[1], Alejandra Sanjuan-Pla[2], Raúl Torres-Ruíz[3], Clara Bueno[3], Talia Velasco-Hernández[3], Pablo Menendez[3,4,5]

Correspondence: Pablo Menendez (pmenendez@carrerasresearch.org), Katrin Ottersbach (katrin.ottersbach@ed.ac.uk).

The mixed lineage leukemia (*MLL*, also known as *KMT2A*) gene is frequently rearranged in human acute leukemia. Chromosomal rearrangements involving *MLL* are biologically and molecularly very intriguing because of the unique ability of *MLL* to "break and fuse" with more than 135 fusion partners, as recently reported by the 2017 *MLL* Recombinome Consortium.[1] *MLL* fusions are commonly associated with poor disease outcome in infant, pediatric, adult, and therapy-induced acute leukemias. The contribution of *MLL* fusions to leukemia initiation and evolution, therapy resistance and relapse is still under active investigation. In this issue of *HemaSphere*, Stavropoulou et al[2] report a novel inducible transgenic mouse model of *MLL-ENL*-driven mixed lineage acute leukemia which reveals that the cell-of-origin and the fusion gene expression level are both critical determinants for *MLL-ENL*-driven acute leukemia. Here, we revisit the main advantages and pitfalls for current mouse models for *MLL-AF4*, *MLL-ENL*, and *MLL-AF9*, the commonest *MLL* translocations found in human acute lymphoid and myeloid acute leukemia.

The large variety of mixed lineage leukemia (*MLL*) gene fusions (affecting 11q23) found in acute leukemia indicates that the *MLL* gene is a hotspot genomic region for chromosomal translocations.[1,3] Longitudinal genomic studies reveal large tumor-mutational heterogeneity for secondary driver mutations[4] but not for *MLL* fusions, which are clonal and present in all leukemic cells, thus representing early initiating leukemogenic events.[5,6] *MLL*-rearranged leukemias represent a major subgroup of acute leukemias in infants and pediatric patients but also affect adults (de novo or therapy-related acute leukemia). *MLL* rearrangements are usually found both in B-cell acute lymphoblastic leukemia (B-ALL) and acute myeloid leukemia (AML) as well as in biphenotypic acute leukemias in which *MLL* fusions are a hallmark pathogenic event.[7] Although there are several clinical and biological factors influencing the long-term prognostic value of *MLL* rearrangements, the current molecular diagnostic criteria place acute leukemias with 11q23 rearrangements as intermediate/high-risk patients.

Several reasons have contributed to a very dynamic research over the last 10 to 15 years on modeling the leukemogenic impact of MLL fusions. Among these are the unfavorable clinical outcome of these patients, the relatively high frequency of *MLL* leukemias in children, the prenatal origin of MLL rearrangements in utero during fetal hematopoietic development and the impressively large number of distinct *MLL* partners eventually contributing to the same (or similar) phenotype. A wide array of transgenic mouse models have been generated for studying the leukemogenic mechanisms of MLL fusions, with special interest in the commonest MLL fusions: *MLL-AF4*, *MLL-ENL*, and *MLL-AF9* resulting from the balanced translocations *t*(4;11), *t*(11;19), and *t*(9;11), respectively. These available mouse models have proven very useful to further our understanding about the leukemogenic role of *MLL* fusions; however, they are all somehow subjected to disadvantages which prevent them to faithfully reproduce all the disease phenotypic and latency features. The different experimental strategies, molecular approaches, inducible systems and target cells certainly contributed to the current "controversial" state-of-the-art. Aspects such as the cell-of-origin in which the translocation is specifically induced, the timing and level of *MLL* fusion expression, the interaction with the bone marrow microenvironment, and the differences between transgenic approaches contribute to the existing diversity of *MLL* mouse models. The CRISPR/Cas9 system has revolutionized the way to approach functional genomics.[8,9] We envision that the use of more accurate models generated by genome engineering techniques in the appropriate human and mouse target cells will soon transform the field of *MLL* leukemia biology.

Funding/support: None.
KO, AS-P, TV-H, and PM contributed equally to this work.
The authors have indicated they have no potential conflicts of interest to disclose.
Author contributions: All authors contributed to analysis of the literature and writing of the manuscript.
[1]*MRC Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, UK*
[2]*Hematology Research Group, IIS La Fe, Valencia, Spain*
[3]*Josep Carreras Leukemia Research Institute, Department of Biomedicine, School of Medicine, University of Barcelona, Barcelona, Spain*
[4]*Centro de Investigación Biomédica en Red de Cáncer (CIBER-ONC), Barcelona, ISCIII, Spain*
[5]*Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain.*

HemaSphere (2018) 2:4(e57)
Received: 17 May 2018 / Accepted: 19 May 2018
Citation: Ottersbach K, Sanjuan-Pla A, Torres-Ruíz R, Bueno C, Velasco-Hernández T, Menendez P. The "Never-Ending" Mouse Models for MLL-Rearranged Acute Leukemia Are Still Teaching Us. *HemaSphere*, 2018;2:4. http://dx.doi.org/10.1097/HS9.0000000000000057

MLL-AF4/*t*(4;11) mouse models

The translocation between chromosomes 4 and 11, *t*(4;11), which fuses *MLL* to the *AF4* gene, is the most common genetic-chromosomal alteration found in infant leukemia and is associated with a particularly dismal prognosis.[6,10] It mostly manifests itself as B-ALL; however, as other *MLL*-rearranged leukemias, can also appear biphenotypic, with patient blast cells coexpressing lymphoid and myeloid markers. Importantly, it has the capacity to undergo lineage switching, from B-ALL to AML, following either conventional chemotherapy-based treatment or immunotherapy with CD19-specific chimeric antigen receptor-modified T cells (CAR-T cells).[11] Understanding the lineage preference and plasticity of *MLL*-rearranged leukemias and how this is influenced by the properties of the cell-of-origin and the specific *MLL* fusion is thus of utmost importance for the design of successful treatment strategies.

Recent sequencing studies have revealed *MLL-AF4*+ infant B-ALL to have one of the most silent mutational landscapes with no other recurrent genetic abnormalities apart from the initiating *t*(4;11) translocation.[12] Despite this seemingly genetic simplicity, it has proven to be extremely difficult to model MLL-AF4+ leukemia in mice. The first attempt involved a straight knock-in of the human *AF4* gene into the mouse *MLL* locus; however, despite considerable embryonic lethality, the surviving mice developed hematological malignancies only after a very long latency, and without an acute leukemia phenotype, eventually succumbing to lymphoid and myeloid hyperplasias and, most commonly, B cell lymphomas.[13] An alternative model was based on the invertor technology and allowed cell lineage-specific expression of *MLL-AF4* via Cre recombinase-mediated inversion of human *AF4* within the mouse *MLL* locus, creating an *MLL-AF4* fusion.[14] Interestingly, targeting *MLL-AF4* expression to the T cell and the B cell lineage produced a B cell malignancy in both cases, thus demonstrating a clear B lymphoid bias; however, disease was once again a more mature B lymphoma that developed after a long latency. Using the same mouse model, but initiating *MLL-AF4* expression already in the first definitive hematopoietic cells generated during development, thus more closely recreating conditions in the infant disease, Barrett et al.[15] were able to describe the preleukemic prenatal stages and the lymphoid-primed multipotent progenitor (LMPP) as the likely cell-of-origin, which was also highlighted in the present study by Stavropoulou et al[2] as a potential cell-of-origin for *MLL-ENL*. However, embryonic expression of *MLL-AF4* in this model did not shorten disease latency and did not result in acute leukemia development. An acute leukemia phenotype with a much shorter latency was achieved in the conditional Mx1-Cre-induced model generated in the Armstrong lab.[16] Around 30% of the mice developed AML, while approximately 40% succumbed to ALL, albeit with a slightly more mature pre-B phenotype. Using this model, the authors were able to highlight H3K79 methylation as a hallmark for MLL-AF4+ leukemia.

Despite the valuable knowledge gained from these genetic models, their failure to faithfully recapitulate the human disease suggests that they are missing crucial elements. B-ALL has generally been challenging to model in murine models. One possible explanation may be that there are fundamental differences in lymphoid development between mice and humans. This notion has recently received a lot of support in a study where mouse progenitor cells transduced with a human–mouse *MLL-Af4* construct induced AML upon transplantation, while human progenitors transduced with the same construct were able to initiate pro-B ALL.[17] Other crucial factors may include contributing immune stimuli which is somewhat supported by the stronger phenotype observed in the model that relies on polyI:C injections for fusion gene expression,[16] which is known to induce an inflammatory response. The stromal microenvironment may also play an important role in leukemia initiation as indicated by the detection of the *t*(4;11) translocation and fusion transcript in a subset of stromal cells from leukemia patients.[18] A model like the one published by Stavropoulou[2] would lend itself particularly well to addressing this question as the timing and level of fusion gene expression can be tightly regulated in any cell type.

Despite the silent mutational landscape, activating mutations in the RAS pathway have been commonly observed in MLL-AF4 + patients; however, these were subclonal and often disappeared at relapse.[12] Accordingly, an activating KRAS mutation proved unable to initiate leukemia in a lentiviral *MLL-AF4* transplant model, but was shown to enhance engraftment and extramedullary hematopoiesis.[21] The current *MLL-ENL* study by Stavropoulou also detected an activating KRAS signature in the leukemia-propagating population suggesting that RAS pathway activation, despite not being essential, is nevertheless an important contributing factor. What sets MLL-AF4 apart from other *MLL*-rearranged leukemias is a possible role for the reciprocal fusion, *AF4-MLL*. It is expressed in a large proportion of patients, but not all, arguing against an essential function, which is supported by a recent study in which it was shown to enhance engraftment, but was unable to initiate disease.[22] While there have been some important advances and discoveries recently, a genetic mouse model for MLL-AF4+ infant B-ALL in which all stages from prenatal initiation can be studied via disruption of normal fetal hematopoiesis to full-blown early onset pro-B ALL in vivo has not yet been generated. Uncovering the missing elements may highlight important therapeutic targets. Table 1 summarizes current mouse models available for *MLL-AF4*+ acute leukemia.

MLL-ENL/*t*(11;19) mouse models

Mixed-lineage leukemia-eleven-nineteen-leukemia translocation, known as *t*(11;19)/*MLL-ENL* is found in both adult and pediatric B and T-ALL and also in adult AML, in this case being associated with favorable or intermediate prognosis. *MLL-ENL* is more common in B-ALL than AML and in contrast to *MLL-AF4* and *MLL-AF9*, it is the only 11q23 abnormality found in T-ALL. An important feature of *MLL-ENL* is the ability to cause lineage reassignment and switch between AML and ALL by reprogramming the transcriptome of MLL-ENL+ cells.[6,23]

To address how *MLL-ENL* specifies leukemia phenotype and outcome, different in vivo mouse models have been described. The *MLL-ENL* translocator mice which carries the chromosomal rearrangement after Cre-loxP-mediated recombination, was crossed with different lineage-specific Cre lines to express *MLL-ENL* in different compartments, such as HSC (*Lmo2-Cre*), B/T progenitors (*Rag1-Cre*), T cells (*Lck-Cre*), and B cells (*CD19-Cre*). These translocator models evidenced that targeted cells influence leukemic development and not all compartments could initiate leukemia.[23,24] For example, *MLL-ENL* expression in B cells did not result in a malignant phenotype.[25] Later on, studies using tamoxifen or doxycycline-inducible expression of *MLL-ENL* (iMLL-ENL) in distinct hematopoietic populations were performed to fine-tune the dosage and restrict the window

Table 1

Summary of MLL-AF4 Mouse Models

Strategy	Cre Line	Disease Phenotype (LIC)	Average Latency	Refs.
Mll-AF4 constitutive knock-in	NA	Lymphoid and myeloid hyperplasia B-cell lymphoma (most common) MPD-like myeloid leukemia Erythroid leukemia	520 d	Chen et al[13]
Mll-AF4 conditional invertor	• *Lmo2-Cre (HSC)*	Embryonic lethal	N/A	Metzler et al[14]
	• *Rag1-Cre* (B, T cells)	B-cell lymphoma	317–466 d	
	• *Lck-Cre* (T cells)	B-cell lymphoma	416–472 d	
	• *CD19-Cre* (B cells)	B-cell lymphoma	460–475 d	
	• *Vav-Cre* (all definitive hematopoietic cells)	B cell lymphoma (most common), T cell lymphoma	556 d	Barrett et al[15]
	• *VE-Cadherin-Cre* (hemogenic endothelium + all definitive hematopoietic cells)	B cell lymphoma (most common), T cell lymphoma, lymphoproliferative disorder	437 d	
Mll-AF4 conditional knock-in	*Mx1-Cre*	Pre-B ALL or AML	152 d 144 d	Krivtsov et al[16]
MLL-AF4 transgenic	NA	Pro-B ALL or lymphoma	170 d	Tamai and Inokuchi[20]

ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia, HSC = hematopoietic stem cells, MPD = myeloproliferative disorder, NA = not applicable.

of protein expression.[26,27] When different populations from *Col1a1*-tetO-*MLL-ENL* mice were isolated and transplanted into DOX-treated mice, AML leukemia developed from multiple progenitors (GMLP, pGM, committed myeloid progenitors (GMP), and common lymphoid progenitors (CLP)) but not from HSC, MPP or PreMeg/E.[27,28] Now, a novel inducible *MLL-ENL* mouse model reported that hematopoietic stem and early multipotent precursor cells (LT-HSC, LMPP, MMP, and CMP) rather than GMP could act as cell-of-origin and give rise to a biphenotypic leukemia.[2]

Given that *MLL-ENL*-initiated ALL was never observed in mice, Ugale et al investigated the impact of inducible MLL-ENL expression in lymphoid progenitors. They hypothesized that *MLL-ENL* fails to initiate ALL owing to either fundamental differences in lymphoid development between species or the requirement of additional cooperating mutations in MLL-ENL+ cells. When different B and T cell developmental stages were isolated from iMLL-ENL mice and transplanted into DOX-treated recipients, only T-cell DN1 progenitors and B-cell progenitors (BLP) gave rise to AML, likely due to their latent myeloid potential. If *MLL-ENL* fusion was coexpressed with an active KRAS mutant form ($KRAS^{G12D}$), appearance of GMLP-initiated AML leukemia was accelerated. Experiments addressing the impact of sequential acquisition of oncogenic hits revealed that the mutation order determines leukemia phenotype. Thus, a T-ALL was observed when $KRAS^{G12D}$ preceded MLL-ENL whereas a myeloid leukemia was more common when *MLL-ENL* preceded $KRAS^{G12D}$.[27]

In spite of these studies, the nature of the leukemia initiating cell (LIC) still remains controversial and not all *MLL-ENL*-associated leukemia phenotypes observed in humans could be recapitulated in mice. Differences in *MLL-ENL* expression levels between the knock-in approaches could explain these discrepancies since *MLL-ENL* leukemogenic capacity requires fusion expression levels above those of the endogenous *MLL1* gene.[2] It is likely that both lineage potential of the target cells and *MLL-ENL* expression levels are key determinants for establishing leukemia phenotype. Additionally, factors influencing the lineage choice in LIC cells could exist and be elusive for *MLL*-rearranged leukemias. Nonetheless, these mouse models proved useful for initial drug screening studies as reported in the Ara-C induced leukemia remission in *MLL-ENL*/Lmo2-Cre translocator mice.[25] Their refinement will contribute to better in vivo *MLL* mouse models for preclinical drug testing. Finally, despite a silent genetic landscape in infant *MLL*-rearranged B-ALL in which only mutations in PI3K-RAS signaling pathways were found[12] a whole-genome mutational landscape of MLL-ENL+ leukemias has not been analyzed in a patient cohort sufficiently large as to reveal recurrent cooperating mutations that could be functionally explored in these mouse models. Table 2 summarizes current mouse models available for *MLL-ENL*+ acute leukemia.

MLL-AF9/*t*(9;11) mouse models

Translocation *t*(9;11) results in the expression of MLL-AF9 fusion protein found in both B-ALL and AML in infants and children, and AML in adults.[29] MLL-AF9+ leukemia is associated with extramedullary tumor infiltration, frequent relapses and variable prognosis depending on the age of the patient and phenotype of the leukemia, being intermediate risk (childhood) or intermediate-high (adulthood) prognosis in AMLs, and overall poor prognosis for childhood B-ALL.[30] *MLL-AF9*-induced leukemia has been easier to model in vivo in comparison to other *MLL*-rearranged leukemias, mimicking phenotype and latency of the human disease quite accurately, which has allowed an extensive research of the biology of this disease. Besides retroviral models where the fusion oncogene is introduced into the target cells by viral vectors with an uncontrolled expression-integration, numerous mouse models have been developed to recreate a more physiological initiation of the disease.

The first attempt to recreate *MLL-AF9* translocation in mice was performed by the Rabbitts' lab using a targeting vector encoding for *Mll* (exon 8)-*AF9* (human sequence) fusion was inserted by homologous recombination into mouse ES cells in the endogenous *Mll* gene, thus being expressed at physiological levels.[31] Extensive characterization of the chimeric and heterozygous mice[32] showed that they recapitulate a human AML disease with the similar expansion of immature myeloid cell populations, macroscopically symptoms, and organ infiltration. Interestingly, 2 out of 24 chimeric mice developed B-ALL, similar to the proportion (~10%) of MLL-AF9+ B-ALL described for

Table 2

Summary of MLL-ENL Mouse Models

Strategy	Cre Line	Disease Phenotype (LIC)	Average Latency	Refs.
Mll-Enl translocator model by LoxP/Cre-mediated recombination	• *Lmo2-Cre (HSC)*	Myeloproliferative-disease-like myeloid leukemia	120 d	Forster et al[24]
	• *Lck-Cre* (T cells)	Either lymphoid or myeloid neoplasia	550 d	Drynan et al[23]
	• *Lmo2-Cre* (HSC)	Myeloid leukemia		
	• *Lck-Cre* (T cells)	AML and ALL	120–180 d	Cano et al[25]
	Rag1-Cre (B, T cells)	Myeloid-like leukemia	170 d	
	• *CD19-Cre* (B cells)	No phenotype	550 d	
Tamoxifen-inducible MLL-ENL-ERTm inserted at the endogenous locus	NA	Long latency MPD with progression to AML upon DDR inhibition	229–140 d (primary recipients) 165–140 d (secondary recipients)	Takacova et al[26]
	NA	AML when expressed from progenitors, but not from HSC	EFS: 5–30 wk depending on targeted cell transplanted (GMLP < pGM < CLP < GMP)	Ugale et al[27]
	NA	AML (T progenitors: DN1–DN3)	DN1: 9–14 wk post-transplant	Ugale et al[28]
DOX-inducible MLL-ENL inserted at the *Col1a1* locus under tetracycline-regulated control (Col1a1-tetO-MLL/ENL)	NA	AML (BLP and Hardy fractions B–F)	BLP: 20 wk post-transplant	
	$KRAS^{G12D}$ upon in vitro Tat-Cre recombination	AML (GMLP with simultaneous hits)	Only MLL-ENL: 100 d MLL-ENL × $KRAS^{G12D}$: 31 d	
		T-ALL (GMLP with sequential hits: $KRAS^{G12D}$ first and MLL-ENL later)	Only $KRAS^{G12D}$: 143 d MLL × $KRAS^{G12D}$: 111 days	
DOX-inducible MLL-ENL inserted at the *Hprt* locus under tetracycline-regulated control (iMLL-ENL)	NA		72 d (primary recipients) 15 d (secondary recipients)	Stavropoulou et al[2]
		Biphenotypic mixed lineage leukemia	LT-HSC: 170 d post-transplants CMP: 120 d post-transplants MPP: 61 d post-transplants LMPP: 54 d post-transplants	

ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia, BLP = B-cell progenitors, CLP = common lymphoid progenitor, CMP = committed myeloid progenitors, DDR = DNA damage response, DOX = doxycycline, EFS = event-free survival, ERTm = ligand-binding domain of estrogen receptor, GMLP = granulocyte–monocyte–lymphoid progenitor, GMP = granulocyte and macrophage progenitor, LT-HSC = long-term hematopoietic stem cell, LIC = leukemia-initiating cells, LMPP = lymphoid-primed multipotent progenitor, MPD = myeloproliferative disorder, NA = not applicable, pGM = pregranulocyte–monocyte progenitor.

patients with a *t*(9,11).[29] Using the same model, Kersey's lab assessed the potential of endogenous *Mll-AF9* to transform phenotypically defined populations (HSC, CLP, CMP, and GMP) and to initiate leukemia.[33] They showed that both HSCs and CMPs could be immortalized in vitro and transformed in vivo by *MLL-AF9* so AML was initiated even when a low number of MLL-AF9+ cells were transplanted. In contrast, committed myeloid progenitors (CMP) were somehow refractory to *MLL-AF9* transformation and a large number of MLL-AF9+ cells had to be transplanted for leukemia initiation. GMP progenitors could not be immortalized by *MLL-AF9* so far, indicating they are not target cells for such a fusion. In addition, the Rabbitts' lab developed a conditional knock-in mouse model to address which is the cell-of-origin of this disease.[34] This model consisted in a translocator mice where a loxP sequence was included at the desired breaking point in the sequences of both *Mll* and *Af9* genes, promoting their recombination upon Cre expression. To specifically promote the recombination in particular cell types, this translocator model was bred with specific Cre-models (*Lmo-Cre*, expressing Cre enzyme in the HSC compartment and *Lck-Cre* expressing Cre in the T-cell compartment).[23] Contrarily to the *Mll-Enl* model described above, the *Mll-Af9* model was unable to recapitulate hematological malignancies when *Mll-Af9* was expressed in the T cell compartment. However, when expressed into more primitive cells/HSCs, a myeloproliferative disorder (MPD)-like myeloid leukemia was observed, underlying the importance of the cell-of-origin for the oncogenic fusion to drive a specific leukemia development/phenotype.

Inducible transgenic models allow for temporal control of transgene expression. An inducible *MLL-AF9* (human sequence) model was developed previously in the Schwaller's lab,[19] similar to the iMLL-ENL model reported in this issue of *HemaSphere*.[2] In the iMLL-AF9 study, authors recreated AML in mice upon doxycycline administration, showing that the leukemic cells become oncogene-addicted, since the disease regressed after doxycycline removal, demonstrating that *MLL-AF9* is necessary for AML maintenance. They also investigated the cell-of-origin of *MLL-AF9*-induced AML using purified LT-HSCs and GMPs populations. Both populations gave rise to AML after doxycycline induction but with different latencies. Resulting AML showed a primitive progenitor phenotype, cytotoxic drug resistance and a stemness and migration gene signature. They also observed a subtype of LT-HSCs-derived AML (LT-HSCs-early-AML), arising from a more immature HSC phenotype, enriched for LICs and more aggressive than LT-HSCs-late- and GMP-derived AML.

Table 3

Summary of MLL-AF9 Mouse Models

Strategy	Cre Line	Disease Phenotype (LIC)	Average Latency	Refs.
Mll-AF9 constitutive knock-in	NA	AML (chimeras + heterozygous mice) ALL (8% in chimeras)	7 mo (chimeras) 5 mo (heterozygous mice) HSC: 165 d (100 cells) CLP: 198 d (100 cells)	Corral et al[31] Dobson et al[32] Chen et al[33]
	NA	AML	CMP: 181 d (2500 cells) GMP: No disease (2500 cells)	
Mll-Af9 translocator model by LoxP/Cre-mediated recombination	• Lmo2-Cre (HSC)	MPD-like myeloid leukemia	300 d	Collins et al[34]
	• Lck-Cre (T cells)	No hematological malignancy	No overt disease after 550 d 118 d (transgenic mouse) 73 d (primary recipients)	Drynan et al[23] Stavropoulou et al[19]
DOX-inducible MLL-AF9 inserted at the *Hprt* locus under tetracycline-regulated control (iMLL-AF9)	NA	AML	32 d (secondary recipients) LT-HSC: 43 d (1000 cells) GMP: 109 d (2500 cells)	

ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia, CLP = common lymphoid progenitor, CMP = common myeloid progenitor, DOX = doxycycline, GMP = granulocyte and macrophage progenitor, HSC = hematopoietic stem cell, LIC = leukemia initiating cells, LoxP = LoxP site, LT-HSC = long-term hematopoietic stem cell, MPD = myeloproliferative disorder, NA = not applicable.

In conclusion, *MLL-AF9* mouse models have been able to recapitulate the main features including phenotype and latency of human ALL and AML using different mouse models expressing either a chimeric (mouse–human) or a human version of the fusion gene. Humanized models based on retro or lentiviral gene delivery into human HSC followed by xenotransplantation into immunosuppressed mice have also been developed in order to better mimic the biology of the human MLL-AF9+ leukemia, but similar transgene expression levels caveats were reported. Cutting-edge genome editing (TALEN and CRISPR/Cas9) strategies are currently been explored as more accurate mechanisms to recreate the allele-specific exact translocation process, with the advantages of reproducing endogenous expression levels and also reciprocal translocations.[35] These models will, without hesitation, continue providing more precise experimental tools for modeling MLL-rearranged leukemia. Table 3 summarizes current mouse models available for *MLL-AF9*+ acute leukemia.

Acknowledgments

PM laboratory is supported by the European Research Council (CoG-2014-646903, PoC-2018-811220), the Spanish Ministry of Economy and Competitiveness, the Asociación Española Contra el Cancer, the ISCIII, the Obra Social La Caixa-Fundaciò Josep Carreras, and the Generalitat de Catalunya (SGR330 and SLT002/16/00299). PM is an investigator of the Spanish Cell Therapy cooperative network (TERCEL). TV is supported by a Marie Curie individual European fellowship (H2020-MSCA-IF-2017-792923). RTR is supported by a postdoctoral fellowship from the AECC scientific foundation. ASP laboratory is supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2015-17534 and SAF-2017-82171). KO laboratory is supported by Cancer Research UK.

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