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Genetically engineered mouse models of human B-cell precursor leukemias

Julia Hauer[1,*], Arndt Borkhardt[1], Isidro Sánchez-García[2], and César Cobaleda[3,*]

[1]Department of Pediatric Oncology; Hematology and Clinical Immunology; Heinrich-Heine University Dusseldorf; Medical Faculty; Dusseldorf, Germany; [2]Experimental Therapeutics and Translational Oncology Program; Instituto de Biología Molecular y Celular del Cáncer; CSIC/ Universidad de Salamanca; Salamanca, Spain; Institute of Biomedical Research of Salamanca (IBSAL); Salamanca, Spain; [3]Centro de Biología Molecular Severo Ochoa; CSIC/Universidad Autónoma de Madrid; Campus de Cantoblanco; Madrid, Spain

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Abbreviations: CLP, common lymphoid progenitor; GEMM, genetically engineered mouse model; LIC, leukemia-initiating cell; pB-ALL, preB-Acute lymphoblastic leukemia; ROS, reactive oxygen species.

B-cell precursor acute lymphoblastic leukemias (pB-ALLs) are the most frequent type of malignancies of the childhood, and also affect an important proportion of adult patients. In spite of their apparent homogeneity, pB-ALL comprises a group of diseases very different both clinically and pathologically, and with very diverse outcomes as a consequence of their biology, and underlying molecular alterations. Their understanding (as a prerequisite for their cure) will require a sustained multidisciplinary effort from professionals coming from many different fields. Among all the available tools for pB-ALL research, the use of animal models stands, as of today, as the most powerful approach, not only for the understanding of the origin and evolution of the disease, but also for the development of new therapies. In this review we go over the most relevant (historically, technically or biologically) genetically engineered mouse models (GEMMs) of human pB-ALLs that have been generated over the last 20 years. Our final aim is to outline the most relevant guidelines that should be followed to generate an "ideal" animal model that could become a standard for the study of human pB-ALL leukemia, and which could be shared among research groups and drug development companies in order to unify criteria for studies like drug testing, analysis of the influence of environmental risk factors, or studying the role of both low-penetrance mutations and cancer susceptibility alterations.

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pB-ALL is a clonal malignant disease clinically characterized by a massive accumulation of immature $CD10^+$, $CD19^+$ lymphocytes that interferes with normal haematopoietic development. B-lineage leukemias are the most frequent ones within the lymphoid leukemia groups in both children (where >80% ALLs are pB-ALLs) and adults (>75%).[1] The origin of leukemia in humans is often impossible to determine, due to the advanced tumoral stages in the children when they enter the clinic. Our knowledge about the etiology of leukemia is consequently mainly derived from animal models that recapitulate human disease. In spite of many attempts over the last 2 decades, animal models faithfully recapitulating all the aspects of human pB-ALL are still an essential unmet need. These models should allow us to dissect the processes that lead to human pB-ALL, and therefore, should also shed light on the unsolved questions about the role of environmental factors in pB-ALL development (**Fig. 1**). But the question therefore is: can we mimic/reproduce human pB-ALL disease in the mouse? The final objective should be to be able of mimicking in the mouse the entire molecular, cellular, histological and organic characteristics of human pB-ALL, including its initiation, progression, evolution, response to therapy and eventual cure or relapse. The models should present similar histological features to those seen in human cancer, should progress through the same stages, present the same systemic effects that leukemia induces it the human patients, should involve the same genetic pathways implicated in human tumor initiation and progression, and must have the same response to current therapeutic approaches. However, all pB-ALL models generated so far have diverse shortcomings in their capacity of mimicking the human disease.

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*Correspondence to: Julia Hauer; Email: julia.hauer@med.uni-duesseldorf.de; Cesar Cobaleda; Email: cesar.cobaleda@csic.es
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pB-ALL non-murine versus murine models

The haematopoietic system has been well-characterized in many common experimental models, and this is the case of 2 of the most relevant ones, zebrafish (*Danio rerio*) and *Drosophila*.[2,3] *Drosophila* is, in itself, a tremendously powerful system for the study of genetic and molecular interactions among key player genes in leukemia but its possibilities of mimicking full human leukemia are very limited, particularly since true malignancies do not really develop in the fly. The zebrafish also offers important advantages for the investigation of developmental hematopoiesis and of the molecular mechanisms of leukemogenesis.[4] In general, zebrafish models have been very useful for the identification of secondary hits partners in forward genetics mutagenesis screens. However, with the advent of genomic technologies and the

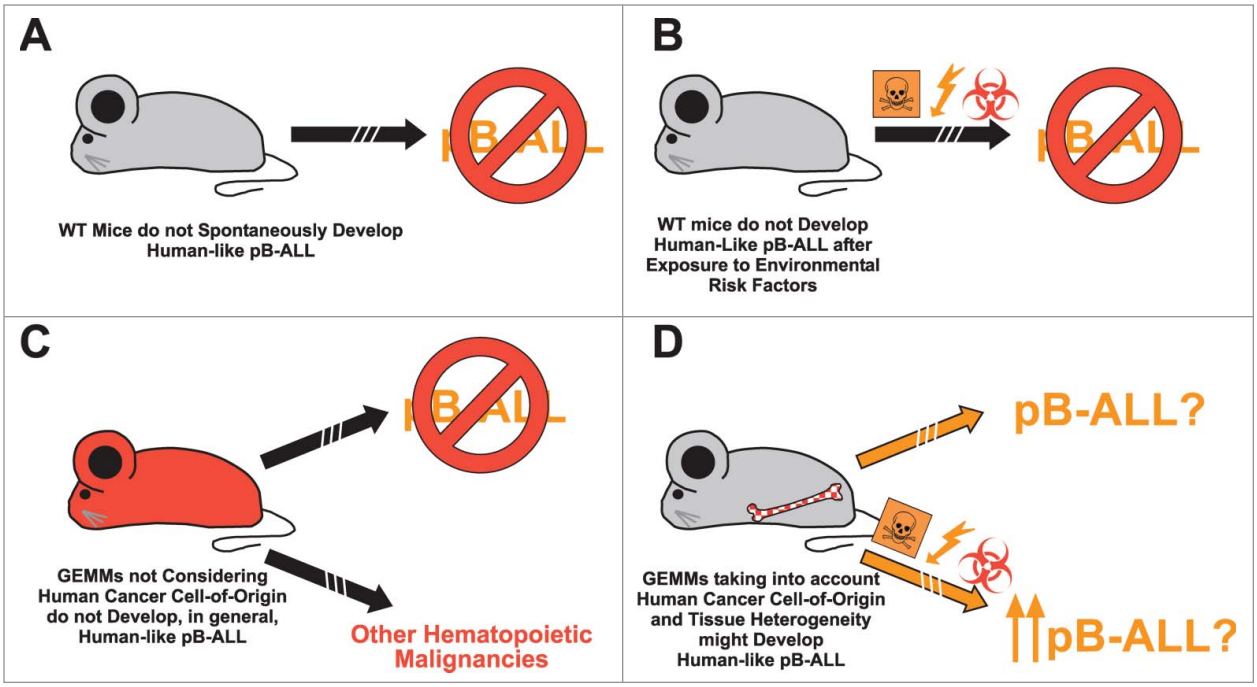

Figure 1. Desired characteristics for an ideal pB-ALL GEMM. (**A**) WT mice do not have any tendency to spontaneously develop pB-ALL. (**B**) When exposed to environmental agents (to test for their capacity of inducing pB-ALL), WT mice do not develop human-like pB-ALLs (except for ionizing radiations, whose molecular mechanisms of leukemogenicity differ from spontaneous human pB-ALLs). (**C**) Conventional GEMMs generated so far without taking into account the nature of the human pB-ALL cell-of-origin fail to generate human-like pB-ALL, although they consistently give rise to different types of haematopoietic malignancies. (**D**) One way for the successful generation of GEMMs developing human-like pB-ALL would be to take into account the nature of the leukemic cell-of-origin in humans (most likely in the bone marrow), and to consider that leukemias arise from a few cells in the context of a normal tissue. These mice might develop human-like pB-ALL, and the incidence, onset or pathogenicity of the disease should increase in the presence of any real leukemia-inducing environmental agent.

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capacity of sequencing the full genome of cancer patients, the discovery of the mutations participating in the disease at all its stages, directly in the human, has become much easier. In summary, even taking into account the many similarities of zebrafish haematopoietic system with that from mammals, still the differences are relevant enough so as to prevent us from considering this model as a suitable model for reproducing all the aspects of human disease. As a way of an example, one of the main problems is the fact that zebrafish has a duplicated genome. Since chromosomal gains and losses are very important events in leukemic development, this biological fact makes zebrafish less adequate for ALL modeling.

Xenotransplants/retroviral transduction models vs. GEMMs

Until recently, the study of the origin of leukemia has been primarily addressed by transplantation of purified subpopulations (putative leukemia-initiating cells, LICs) of human pB-ALL cells into immunodeficient mice. From a leukemia developmental point of view, this technological approach has been extremely useful in demonstrating the hierarchical structure of many human pB-ALL types.[5-10] However, these models are very difficult to standardize between laboratories, thus precluding the good reproducibility of the results.[11] Other disadvantages, of especial relevance for the study of the role of the stroma in tumoral evolution, are that recipient mice should be irradiated with gamma radiation before the injection of transduced cells (2 to 9 Gy), and also that the human leukemia develops into otherwise normal/immunocompetent human children. This methodological need also clearly precludes the use of these approaches for the study of the effects of several environmental factors (notably electromagnetic fields) in childhood pB-ALL.

Given the mentioned limitations of the xenografts of human samples to understand leukemia biology/etiology, other possible approach that has been employed for the modeling of pB-ALL is the introduction (usually by viral transduction) of the human translocation genes in either human or mouse blood progenitors (or, in a less optimal setup, cell lines) and then to re-inject them into mice and observe their development. This approach has been instrumental for defining many biological properties of oncogenes, and for the development of gene therapy approaches. Nevertheless, all these virus-based models present limitations of inter-laboratory reproducibility and homogeneity, mainly due to 3 reasons: it is an ex vivo based method that does not mimic human cancer origin, the viral integration is potentially mutagenic by itself, and the recipient mice should also be irradiated with gamma radiation before the injection of transduced cells. Therefore, although very useful for basic research, their potential for understanding the etiology of human pB-ALL leukemias and for large-scale inter-laboratory approaches is limited.

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Genetically engineered mouse models of pB-ALLs

Therefore, in order to accurately reproduce all the aspects intervening in pB-ALL development, it is necessary to perform experiments in an in vivo setting in which the neoplasm emerges in the appropriate microenvironment. The complexity of modeling cancer in mice has increased to the extent that nowadays one can induce, study and manipulate the leukemic process in a manner impossible to perform in human patients or with human-derived samples. However, from a historical perspective, the creation of every single GEMM has been clearly influenced at each time point by the existing knowledge and level of characterization of human pB-ALL through the appearance of new technological approaches (PCR, advanced flow cytometry, fluorescence in situ hybridization, etc.).

The number and type of genetic alterations associated with leukemia is varied but limited. Leaving aside chromosomal aneuploidies, these alterations can be roughly grouped into gain-of-function mutations, usually resulting from chromosomal translocations and assumed to be the first leukemogenic hits, and loss-of-function mutations that are typically considered second hits (but not always). In the next sections we will revise the most relevant (historically, technically or biologically) mouse models of precursor B-ALLs, and we will not consider other B cell malignancies.[12,13] For each of the most relevant pB-ALL-associated genetic alterations we will first describe constitutive one-hit models, 2-hit models, followed by conditional and/or inducible models (indistinctly one or 2-hit models).

***BCR-ABL*$^+$ GEMMs**

Since the discovery that chimeric fusion proteins are tightly associated with specific leukemic types in human patients, gain-of-function mouse models started to be generated trying to reproduce human disease by ectopically expressing the oncogenic proteins in the mouse. At the beginning, these models were generated by classical transgenesis. In 1990, Heisterkamp et al.[14] demonstrated the oncogenic capacity of the BCR-ABLp190 fusion protein by expressing it under the control of the methalothionein promoter in the mouse, and showing that the resulting mice died of acute leukemia (myeloid or lymphoid) in a few weeks. With this model it was also already noted that, even when using a transgene expressed in many different cell types, the phenotype of the disease was specifically restricted to the haematopoietic system, as dictated by the oncogene.[15] Although some reports have described differences in the phenotype resulting from the transgenic expression of the different BCR-ABL forms,[16] given the fact that all the *BCR-ABL* oncogenes, in spite of their differences, are kinases, there is a significant extent of overlapping aberrant phenotypes associated to their overexpression in different transgenic settings. For example, *BCR-ABLp210* expressed under the control of the mouse *bcr* locus is embryonic lethal,[17] while under the methallothionein promoter, it causes T-cell leukemia.[18] The *bcr-ABLp190* oncogene made by homologous recombination into the mouse *bcr* locus produced acute leukemias in the chimeras of a B-cell precursor immunophenotype, mimicking the human pathology.[19] Also using this model, it was shown that the endogenous bcr locus was not required for the disease to develop. However, the chimeras did not transmit and the constitutive expression of *BCR-ABLp190* in all the cells of F1 mice led to embryonic lethality, similarly as previously mentioned for the p210 form.

In this context, the additional introduction of second hits (either gains or losses of function) selected on basis of their known involvement in human pB-ALL or in normal B cell development, has allowed confirming, in many cases, the molecular mechanisms of leukemic evolution. For example, one of the most common secondary alterations in human *BCR-ABL*$^+$ pB-ALLs is the deletion of the *IKZF1* (*IKAROS*) gene.[20-22] Crossing transgenic *BCR-ABL* mice[14] with mice carrying an hypomorphic *Ikzf1* allele[23] showed that impaired Ikaros function indeed accelerates leukemic progression and tumor onset by circumventing the need to accumulate other additional genetic hits that might cooperate with the *BCR-ABL* oncogene in pB-ALL.[24]

A particular experimental approach standing in between GEMMs and viral transduction involves the use of bone marrow cells from genetically modified mice as a target for viral transduction. Generally, the target cells used are derived from a mouse carrying the "second hit" (usually a loss-of-function allele, versus WT control cells) and they are transduced with the "first hit" oncogene. This approach has been used, for example, to study the role of the $p19^{ARF}$ locus in the phenotype of *BCR-ABL*$^+$ pB-ALLs.[25,26] Retroviral transduction of *BCR-ABLp185* into $Arf^{-/-}$ mouse bone marrow cells rapidly generates polyclonal populations of continuously self-renewing pre-B cells, virtually all of which have leukemic potential.[26] Also, in the presence of WT $p19^{ARF}$, the only cells that can be transformed by BCR-ABL expression are haematopoietic stem cells (HSCs), while in an $Arf^{-/-}$ background, also common lymphoid progenitors (CLPs) and early B lymphocyte precursors can generate leukemic stem cells.[25] This mouse model was afterwards used to demonstrate that NFAT inhibition with cyclosporine-A enabled leukemia cell eradication by the BCR-ABL inhibitor dasatinib, and significantly improved mice survival.[27] Similar experimental approaches have been used with *AID* (Activation-Induced Cytidine Deaminase) knockout cells,[28] showing that AID accelerates clonal evolution in *BCR-ABL*$^+$ pB-ALL, or with $C/EBP\beta^{-/-}$ cells, demonstrating that C/EBPβ is involved in BCR-ABL-mediated myeloid expansion.[29]

These transduction-based approaches, although very useful in basic research aimed at understanding the molecular mechanisms underlying the synergy and leukemogenic effects of ALL collaborating lesions, have the same drawbacks previously mentioned for viral transduction, and henceforth we will not discuss them in more detail in the remaining sections.

Another possibility of introducing the second hit, instead of using a candidate-based approach, comes from the combination of transgenic lines expressing the "first hit" with some forward genetics approach in which genetic alterations are induced that interact with the first oncogenic hit to change some aspect of the induced disease (e.g., latency, incidence, aggressiveness or other phenotype). Afterwards, these alterations can be identified and their relevance for human disease can be evaluated. In the context of transgenic *BCR-ABLp210* mice,[30] Miyazaki et al.[31]

backcrossed them to BXH2 mice, a recombinant inbred mouse strain that develop leukemia mainly due to a horizontally transmitted replication-competent retrovirus. Some mice developed acute lymphoblastic leukemias and this was due to aberrant expression of the *Zfp423* (*Zinc finger protein 423*, also known as *Early B-cell factor-associated zinc finger protein, Ebfaz*) gene, showing that *Zfp423* possesses a cooperative oncogenicity with *BCR-ABLp210* in vivo, accelerating disease onset, increasing aggressiveness and changing the tumoral phenotype. Zfp423 binds to and interferes with the activity of Ebf1, a master regulator or B cell differentiation from the earliest stages, therefore suggesting a mechanism of action for the cooperative oncogenicity.[31] However, the fact that one has to introduce second cooperating hits that naturally happen during human pB-ALL development may imply that the model does not fully mimic human pB-ALL.

The use of inducible systems in which one can control the timing of the action of the oncogene has enabled a deeper understanding of the etiopathogenesis of pB-ALL. For example, induction of the expression of *BCR-ABLp210* under the control of a tetracycline-controllable promoter led to the development of lethal acute pre-B cell leukemia in 100% of the mice,[32] and the pathology was reversible upon discontinuation of oncogene expression. However, it has to be noted that the p210 form of BCR-ABL is only associated with B-ALLs in humans in the content of a blast crisis which, conversely, stops responding to BCR-ABL inhibition by Imatinib. In other study, using the pB-ALL-associated *BCR-ABLp190* form in a tetracycline controllable system, it has been shown that inactivation of the oncogene expression, however, does not stop tumor growth once initiated.[33] These inducible transgenic mice suffer from pB-ALL that cannot be rescued by oncogene inactivation or by Imatinib treatment, similarly to what happens in humans, suggesting that the induction of tumorigenesis does not only depends on the oncogene, but also largely on its pattern of expression, and pointing toward a setting in which the oncogene imposes a leukemic stem cell-specific epigenetic context in which oncogene inactivation cannot lead anymore to a reversion of the phenotype.[33] The discrepancies between the results arising from models that are not so dissimilar in their design also highlight and exemplify the importance of defining stringent phenotypic requirements before concluding that a given mouse model actually recapitulates one of several aspects of the pathology in humans.

The use of inducible systems allows a tighter control of the conditions required for the disease to originate or progress, and permits the testing of the role of other variables in pathogenesis. One of these variables is aging. Using the aforementioned inducible *BCR-ABLp190* transgene, it has recently been shown that cells from old mice develop leukemias at a much faster rate than cells from young animals, when transplanted into recipients of the same age (4 weeks), therefore suggesting that an increase in the age at which transformation occurs has a direct impact in the malignant potential of leukemic cells.[34]

E2A-PBX1$^+$ or *E2A-HLF*$^+$ GEMMs

Many of the translocations associated to pB-ALL involve transcription factors whose function becomes deregulated as a result of the generation of a chimeric protein. For example, the essential regulator of B cell development *E2A* is involved in 2 translocations[35,36] which are always associated with pB-ALL in humans. One of them is the translocation t(1;19)(q23;p13.3) originating the *E2A-PBX1* fusion gene, mainly present in childhood leukemias, but also in adult ones, with very different prognoses, since *E2A-PBX1*$^+$ childhood pB-ALLs have a favorable outcome, while adult ones have a much poorer evolution. Many in vitro experiments have shown that E2A-PBX1 is capable of transforming cells, but the in vivo experiments in mice have yielded inconsistent results. Transgenic expression from an immunoglobulin promoter/enhancer led to the generation of T-lymphoblastic lymphomas by 5 months of age,[37] most likely due to the today well-known expression of these regulatory elements in T cell development. However, the numbers of B cells in the bone marrow of these mice were reduced by 60% to 80%, therefore suggesting a toxic effect of the oncogene in this compartment. This model was also the basis to study the role of PBX1 homeodomain in oncogenesis, and a mutant form of E2A-PBX1 lacking this domain proved to have similar oncogenic effects compared to the full-length oncogene.[38]

The aforementioned *E2A-PBX1* transgenic mouse model has been used in combination with forward genetic analyses to identify genes that could potentially collaborate in leukemogenesis. Retroviral insertional mutagenesis using Moloney Murine Leukemia Virus (M-MuLV, MMLV) was employed to identify collaborating oncogenes. This approach allowed the identification of mutations in *Pim1* (ref. 39) and *Notch1* (ref. 40) as collaborating in the development of thymic lymphomas.

Other transgenic model, based on similar lymphoid-specific promoter/enhancer regulatory elements, also developed mainly T-cell neoplasias and, with less frequency, B-ALLs with a longer latency.[41] To avoid the interference of the T-cell disease and to enhance the occurrence of B-cell leukemias, these *E2A-PBX1* transgenic animals were crossed into a *CD3*$\varepsilon^{-/-}$ background, which lacks T cells. The resulting animals were subjected to retroviral insertional mutagenesis using MMLV to reveal collaborating genes. The identification of relevant targets showed that the *Hoxa* gene cluster is preferentially targeted in the *E2A–PBX1*-induced tumors, therefore pointing toward a functional collaboration between these oncogenes in pB-ALL.[41] Further studies crossing *E2A-PBX1* transgenic mice with specific transgenes expressing genes from the *Hoxa* cluster showed oncogene collaboration also in T cell leukemias,[42] although this oncogene has not ever been found to be associated with T-ALL in humans.

Also involving the *E2A* gene, the E2A-HLF fusion protein arises as a result of the translocation t(17;19)(q22; p13.3) and, although it is present with a very low frequency in general, its prognosis is highly unfavorable. Transgenic models in which the *E2A-HLF* expression is driven by the immunoglobulin enhancer resulted in interference with the differentiation of the lymphoid lineages, causing T-cell apoptosis, B-cell maturation arrest, and development of long-latency T-ALLs. Tumors were monoclonal, suggesting the need for secondary genetic events, and presented the phenotype of developmentally blocked T cells or, infrequently, B-cell progenitors.[43,44] These results and those from the

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E2A-PBX1 mice have been interpreted as due to a dominant-negative effect of the E2A fusion proteins with the normal dimerization dynamics of the endogenous E2A proteins and Id proteins, required for normal lymphocyte differentiation.[45]

Since the previously mentioned methods of ectopic expression did not fully recapitulate the human pathology related to E2A-HLF, more sophisticated approaches have been undertaken. A conditional knock-in model was generated in which the *E2A-HLF* cDNA was placed into the *E2a* locus, mimicking the human situation.[46] The expression of the oncogene was blocked by a *Neo* cassete flanked by loxP sites, so that it could be activated by using (in this case) the interferon-inducible Mx-Cre transgenic line.[47] However, no disease was developed in the mice during a long observation period, therefore indicating either the need for secondary alterations which naturally do not occur in mice, or that the way these leukemias are initiated in humans follows a different pattern that the one used to model the disease in mice. To provide for the secondary hits, retroviral insertional mutagenesis was used, which resulted in the induction of acute leukemias, some of them of the B cell type, that also presented with features like the previously described coagulopathic property of translocation t(17;19)-positive leukemic cells.[47] Investigation of the retrovirally overexpressed genes involved in this process led to the identification of several genes, among them *Gfi1*, *Ikaros* and *zinc-finger protein 521* (*Zfp521*), which was afterwards found to be also overexpressed in human leukemic *E2A-HLF*$^+$ cell lines. *Zfp521* is homologous to the previously mentioned *Zfp423* gene, and can also interfere with the activity of the master regulator or B cell differentiation Ebf1, therefore suggesting a mechanism of action for the cooperative oncogenicity.

TEL-AML1$^+$ GEMMs

The translocation t(12;21)(p13;q22) (*TEL-AML1*, *RUNX1-ETV6*) accounts by itself for 25% of the cases of childhood pB-ALL, and it is specifically linked to pB-ALL in humans. Andreasson et al. expressed the *TEL-AML1* fusion gene under the control of the immunoglobulin heavy chain enhancer/promoter, but these mice did not develop any hematologic disorder of any kind.[48] There is no follow-up of this mouse model in the literature, but a very similar transgenic strategy has been used recently,[49] showing again a very mild phenotype, with only a minor increase in early B cell progenitor percentages and lacking any malignant phenotype. A recently generated *CD19-TEL-AML1* BAC-transgenic mouse does not have any detectable haematopoietic anomaly, although ROS levels in B cells are elevated, therefore suggesting that the expression of the oncogene might trigger mutagenesis through the enhanced production of reactive oxygen species (ROS).[50]

In one of the latest studies,[51] a knock-in was generated in such a way that a copy of the *AML1* gene was introduced in a conditional manner into the endogenous *Tel* locus of the mouse. Using this model, Schindler et al. could show that TEL-AML1 can induce a limited expansion of HSCs. However, once again, these mice did not develop pB-ALL and, only with the additional treatment of the mice with the powerful mutagen ENU, they develop T cell malignancies, which is in sharp contrast to what can be seen in human patients.[51] A similar knock-in approach has been recently employed by van der Weyden et al.[52] They introduced, in a constitutive manner, the *AML1* cDNA into the mouse *Tel* locus, but they also introduced downstream of it an Internal Ribosomal Entry Site (IRES) followed by the cDNA of a hyperactive variant of the *Sleeping Beauty* transposase. In order to model the second hit believed to occur in human pB-ALL patients, they crossed these mice with mice carrying the *T2Onc* transposon array.[53] These mice are, so far, the only TEL-AML1 model that has been reported to generate (albeit with a low frequency, 13 out of 90) pB-ALL.[52] In this study, several of the genes or the chromosomal locations altered by the transposase activity in the pB-ALLs arising in the mice were coincident with genes known to be involved in human pB-ALLs, therefore showing the validity of this kind of approaches. However, these mice also present with other leukemias, with even higher frequencies (AML, 34 out of 90 mice; and T-ALL, 21 out of 90) which, again, do not recapitulate the high specificity of TEL-AML1 for CD10$^+$, CD19$^+$ human leukemias.

Once more, the lack of accuracy in recapitulating the human disease shows that there are aspects of the leukemia biology that are still being overlooked when attempting to model the disease in mice. In the case of human patients, it was described more than 10 y ago that the frequency of newborns carrying the *TEL-AML1* translocation in their blood is higher than the overall incidence rate of pB-ALL.[54] This finding, although it remains controversial [55-58] would imply that preleukemic clones are frequent, but they are normally controlled or eliminated by natural processes, and only a percentage of them finally give rise to an overt leukemia. In this context, one would also expect that the penetrance of pB-ALL in *TEL-AML1*$^+$ GEMMs should be very low, even if one could exactly mimic target cell, oncogene levels, etc., like they occur in humans (which is most likely not the case, as we are seeing). From this point of view, the *TEL-AML1* translocation (and possibly other pB-ALL-related ones) could be almost considered like an increased susceptibility allele with a moderate final influence in the appearance of the disease. Accepting this, the nature and incidence of the second hit(s) (either endogenous or due to the exposure to environmental factors) becomes the essential leukemia-unleashing event.[59,60] There are many possible candidate alterations that could play a role, but their cause is still to be determined: exogenous or endogenous exposures, genetic susceptibility, and chance can all have a role.[61] Still, an appropriate first-hit *TEL-AML1* animal model is missing and it will be of essential importance to test for all the possible contributing secondary causes, but the nature of the disease, as discussed, makes it difficult to exclude potential contributing causes on basis of negative results from animal models. In general, in light of these recent discoveries, a common problem of all the mouse models discussed in this review is that the design of the models considers that the oncogene is expressed in all B-cells since the beginning, a situation that does not happen in humans, where the preleukemic clone can reside within the BM HSCs without peripheral contribution for years.

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***MLL*-involving translocations-based GEMMs**

Among pediatric ALLs, a special case is that of infant leukemias associated with translocations involving the *MLL* gene. They occur typically in the first year of age, and they have a very bad prognosis. The rearrangements have been shown to take place in utero. More than 50 different translocations have been identified in which *MLL* is involved, and the most frequent ones in pB-ALL (80% of the cases in which the MLL gene is involved in pB-ALLs) are those affecting the genes *AF4*, *AF9*, *ENL*, *AF10* and *AF6* (ref. 62). Contrary to what we have described until now, in *MLL*-related ALLs, additional genetic alterations are uncommon (although alterations in *FLT3* expression are frequently seen in *MLL*-based leukemias[63]), suggesting that all the changes necessary for leukemogenesis are directly or indirectly driven by the oncogene. This could be due to the particular nature of MLL as a methyltransferase involved in the regulation of *Hox* genes, and in establishing histone methylation patterns that regulate gene expression.[64]

Knock-in mice chimeras carrying ES cells in which the *AF9* cDNA was introduced into the mouse *Mll* locus developed leukemias (mainly AML, but also B-ALL), but the long latency seems to support the need for additional genetic hits in this model.[65,66] Using this model, the different kinetics of leukemia generation from fetal liver (FL) or bone marrow (BM) cells was studied, showing that there is a significant delay in the onset on leukemia from transplanted transgenic FL cells when compared with BM cells, suggesting that, by the time the cells have matured to the BM stem/progenitor stage, secondary hit(s) have already taken place.[67]

From a mechanistic point of view, one of the most sophisticated and elegant mouse models of chromosomal translocations generated so far involved the induction of such translocations between the endogenous *Mll* and *AF9* loci of the mice in vivo.[68] This same approach was afterwards applied to mimic in the mouse the translocation t(11;19)(q23;p13.3) involving the *Mll* and *Enl* loci.[69] LoxP sites were introduced in the respective loci in chromosomes 9 and 17 (*Mll* and *Enl* chromosomes, respectively), and recombination was achieved by using the pan-haematopoietic *Lmo2-Cre* recombinase.[70] These mice developed a "myeloproliferative-disease-like myeloid leukemia" in less than 6-months' time, therefore directly implicating Mll-Enl in leukemic development. More closely concerning human pediatric pB-ALLs, a constitutive mouse model carrying a knock-in of *AF4* into the *Mll* locus developed mixed lymphoid and myeloid hyperplasias and B-cell lymphomas, with a long latency.[71] Also in this article, *MLL-AF9* knock-ins were generated (same ones as described above) that developed myeloid malignancies.

Another experimental and very innovative approach led to the generation of conditional *Mll-AF4* mice by using the so-called "invertor" technology, in which the *AF4* cassette was knocked-in into the *Mll* locus in an inverted orientation and flanked by lox-P sites. The action of the Cre led to the inversion of the cassette and the subsequent generation of the chimeric *Mll-AF4* oncogene.[72] Using different, lymphoid specific, Cre recombinases, these mice developed B cell neoplasias of a mature phenotype (diffuse large B cell lymphomas), instead of the pro-B-ALLs usually associated with *MLL-AF4*$^+$ infant leukemia. It is reported that constitutive expression of the fusion oncogene is embryonic lethal. However, one aspect that is clear from all these models is that, even if the knock-in expresses the oncogene all throughout the organism, only haematopoietic malignancies develop in the mice, indicating that the *MLL* fusions are only tumorigenic in this compartment. The authors conclude that, according to these data, *Mll-AF4* is oncogenic in committed cells of the lymphoid lineage and does not have to be expressed from the HSCs. However, one important question that remains is what the phenotype would be if the expression of the Cre had been targeted to more primitive compartments.[72] Another model of infant *MLL-AF4*-associated human pro-B-ALLs was developed by Krivtsov et al.[73] using, interestingly, a similar strategy but a different Cre-line. They used a conditional knock-in model of *AF4* into the mouse *Mll* locus, preceded by a loxP-flanked STOP cassette, in combination with an interferon-inducible, pan-haematopoietic *Mx1-Cre* line.[47] Fourteen out of 22 mice developed a disease consistent with acute leukemia, and unsupervised hierarchical clustering analysis of microarray profiling demonstrated that these ALLs were similar to pre-B cells. Furthermore, recent studies suggest that MLL fusion proteins control gene expression by recruiting the histone H3 lysine79 (H3K79) methyltransferase DOT1L[74,75] and, comparing leukemia cells from the *Mll-AF4*$^+$ mice with human ones, it has been shown[73] that H3K79 methylation is similarly enhanced at many loci, and this elevation is correlated with enhanced gene expression. Also, suppression of H3K79 methylation leads to inhibition of gene expression in *MLL-AF4*$^+$ cells, therefore suggesting that inhibition of DOT1L may be a therapeutic possibility in this type of ALLs.[76]

This aforementioned mouse model is a good example of how the combination of an accurate molecular copy of the genetic lesion taking place in humans, together with its activation in the right cellular compartment, can lead to a much improved model of human leukemia. This question of what is the right target cell for the action of the oncogene (which we will discuss in a more general way at the end) is well illustrated with other examples, also concerning mouse models of *MLL*-associated translocations. Although some of these models are based on retroviral transduction, we will discuss some of the findings obtained in this particular context. Using retroviral transduction of *MLL-ENL* in defined mouse haematopoietic subpopulations, it was found that the oncogene could induce the leukemic phenotype in both self-renewing stem cells and short-lived myeloid progenitors.[77] Also, it has been described that retrovirally transduced *MLL-AF9* can transform both early haematopoietic progenitors[78] and committed myeloid progenitors.[79] However, when studying the role of *Mll-AF9* in knock-in mice, it was found that there are high expression levels of the oncogene and its downstream target genes in HSCs compared to committed progenitor cells.[80] This has direct consequences in the leukemogenic capacity, since there seems to be a direct relationship between oncogene dosage and the cellular susceptibility to transformation in committed progenitor cells, which can be transformed by retroviral-driven oncogene but not by the endogenously controlled one.[80] Using a conditional transgenic model of *MLL-ENL*, it has recently been

shown that *MLL-ENL*, when expressed at a level similar to that of the endogenous *Mll* locus, can selectively induce the leukemic transformation of a restricted subpopulation of HSCs, and that this effect is achieved through the upregulation of the *Promyelocytic leukemia zinc finger* (*Plzf*) gene.[81] Something similar had been shown before for *Evi1* (ref. 82) and also, importantly in the case of *MLL* fusion genes, for *Hox* genes like *Hoxa9* (ref. 83). These findings highlight the importance that expression levels and windows might have in the generation of a reliable model that faithfully recapitulates human disease. Finally, it has also been shown, using *Mll-AF9* knock-in mice, that there is a stepwise progression in the pathogenesis of the ALL, from prenatal to postnatal stages, in such a way that there are significant differences between prenatal and postnatal myeloid cells, and suggesting that there are several steps that precede the development of overt leukemia in the adult mouse.[84]

Loss-of-function-based pB-ALL GEMMs

We have revised the most representative genetic alterations classically associated with pB-ALL. However, there are others that, although accounting only for a small number of cases, are of biological relevance because of the mechanistic information that they provide us regarding the biology of leukemia. On the other hand, many of them have not yet been modeled in genetically engineered mice, and only, if at all, in retroviral transduction settings.

The B cell master regulator *PAX5* is frequently inactivated by mutation in pB-ALLs,[85] and it is also involved in translocations fusing it to *TEL*, *FOXP1*, *ZFP512* or to *ELN*.[86] Although it is presumed that the chimeric proteins resulting from these translocations involving *PAX5* act as dominant-negative forms, interfering with the normal B-cell developmental program,[87] the truth is that, as of today, there are not GEMMs available to study in detail their role in vivo. A conditional loss-of-function model of *Pax5* has shown that its loss in committed B cells in vivo leads to the development of tumors with the properties of progenitor cell lymphomas, with a gene expression pattern similar to that of uncommitted $Pax5^{-/-}$ pro-B cells, although they carry immunoglobulin heavy-chain and light chain gene rearrangements, indicating that they must originate by dedifferentiation from immature or mature B cells.[88] Also, new studies demonstrate that germline hypomorphic mutations of *PAX5* are associated with susceptibility to pB-ALL, implicating *PAX5* mutations in familial leukemia predisposition syndromes.[89,90] The combination of heterozygous or homozygous (conditional) loss-of-function alleles of *Pax5* with gain-of-function translocation models will likely lead to acceleration or an increased penetrance of the disease.

This effect has already been detected in the context of mutations in the *Ikaros* (*Ikfz1*) gene, as we have previously mentioned in the context of *BCR-ABL* translocation models.[24] The *IKAROS* mutations that can be found in human patients cover the range from haploinsuficiency to null mutations, including mutations with a dominant negative effect (due to the dimerization required by IKAROS and its related proteins AIOLOS, HELIOS or EOS),[91] and they are not randomly distributed among the different ALLs, suggesting, for example, that the selective pressure necessary for the acquisition of strong *IKAROS* mutations is higher in $BCR\text{-}ABL^{+}$ positive pB-ALLs. *IKAROS* mutations are in the majority of the cases second hits,[20] but also specific inherited polymorphisms of *IKZF1* predispose to pB-ALL (reviewed in ref. 91). The roles of *Ikaros* in lymphoid development are manifold, so modulations of its activity by mutations or among different allelic variants will most probably have different impacts in leukemic development and progression. This also makes it difficult to choose a specific lack-of-function Ikaros general mouse model that could be of general applicability for the study of its role in ALLs.

Similarly to the 2 examples that we have described, there are many other genes that have been found to be associated with pB-ALL in humans with a small frequency (individually), either as first or second hits. In general, the interference with genes involved in B cell development or function is related with alterations in B cell differentiation and hence, is susceptible to participate in leukemic evolution. Along these lines, genes involved are *EBF1*, *RAG1* and *RAG2* genes, signal transduction genes like *JAK/STAT* or *SLP65/BLNK*, cytokine receptor genes like *IL7R*, *FLT3*, *TSLP/CRLF2*. Also of course, like in most other cancers, genes involved in cell proliferation/cell cycle like *CDKN2A/CDKN2B*, *RB1*, *TP53*, or in control of cell death like *BCL2*, or *BTG1*, are also participating in many cases of human B-ALL (reviewed in [61,92,93]).

Loss-of-function mouse models affecting these genes and related ones exist, for the majority of them. Many of them present with characteristics resembling, in a variable degree, human ALLs. For example, indirect interference with the function of Ebf1 leads to the development of ALLs in a mouse model of retrovirally-induced mutagenesis.[94] *Rag1* deficiency contributes to leukemogenesis in mouse when it is combined with secondary oncogene activation, like *p53* (ref. 95) or tumor suppressor gene inactivation, like *p19ARF*[96]. The combined lack of *Rag1* and *p19Arf* gives rise to leukemias characterized by the appearance of a new subset of $Sca1^{+}CD19^{+}$ B-cell-precursor cells containing leukemia-initiating cells, characterized by increased expression of *Notch1*. Using $Stat5^{fl/fl}$ *Mx1Cre* mice, it has been shown that the STAT5 signaling pathway is required for the maintenance of the leukemic state in $BCR\text{-}ABL^{+}$ leukemias.[97] It has also been shown that haploinsufficiency of either *Pax5* or *Ebf1* synergized with a constitutively active form of *Stat5b* to rapidly induce ALL in 100% of the mice.[98] Also, in AML mouse models it has been demonstrated that Jak inhibitors suppress the leukemic phenotype.[99] $Slp65^{-/-}$ mice spontaneously develop pre-B cell leukemia, whose proliferation is believed to be driven by constitutive *Jak3/Stat5* signaling.[100]

Chromosomal rearrangements affecting cytokine receptors are becoming a subclass in themselves, especially the ones affecting *TSLP/CRLF2*, leading to its increased expression, very often associated with mutations activating *JAK1* or *JAK2*. Not suitable mouse models are yet available to try and reproduce in the mice their effect in human B-ALL biology. The role of *FLT3* deregulation in *MLL*-leukemias is well-ascertained, and it has been shown in a retrovirally transduced mouse model that Flt3 inhibition might be of therapeutic value in this type of leukemias.[63]

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Many GEMMs exist already for both the loss- and gain-of-function of genes involved in the control of cell proliferation, cell cycle, apoptosis, etc. Therefore, the individual contributions of each of these genes to leukemia can be evaluated, either alone or in combination with other models, in a relatively simpler way using the already existing models.

Finally, it is also important to mention that there are no good models available of high hyperdiploid pB-ALL, because the modeling of leukemias characterized by the gain of many whole chromosomes is technically very difficult with today's tools. However, such models would be highly desirable as this genetic subgroup is very frequent, exceeding even that of *TEL-AML1*$^{+}$ pB-ALL in children.

Conclusions and future directions

The GEMMs of pB-ALL generated so far have provided an enormous amount of extremely valuable biological information, have helped to demonstrate the oncogenic nature of the different genetic lesions present in human patients and have provided the ground for the development of new targeted therapies. However, in scientific research, there is always a constant evolution and incorporation of new concepts. The most recent findings in the field of leukemia and in cancer in general, are revealing the existence of complex intratumoral heterogeneity, intricate internal clonal progression, hierarchical, stem cell-based, structure of the leukemic population and novel roles of oncogene function beyond the deregulation of cellular proliferation and survival.[101-103] All these aspects must be now taken into account in our attempts to generate suitable animal models of leukemia that can faithfully recapitulate these characteristics of the disease in humans (**Fig. 1**).

The main focus while attempting to model cancer has been put in the oncogene, while the aspect of the cellular context in which the oncogene is exerting its action was generally taken for granted, since it was normally assumed that the cancer cell-of-origin correlated with the closest non-pathological relative of the main cellular type composing the mass of human tumors. However, from the restricted capacity of the existing GEMMs to accurately model all the aspects of the human disease, it is clear that this aspect needs to be incorporated in the generation of new mouse models (**Fig. 1C and D**). Only when acting in the right type of cell can the oncogene originate the right leukemic pathology. This cell-of-origin usually does not have to present any phenotypic similarity with the cells forming the main tumoral mass.[103] One clear example can be found in chronic myelogenous leukemia, in which the translocation creating the *BCRABLp210* oncogene appears and exerts its first oncogenic effects in HSCs, in spite of the fact that the disease is characterized by the accumulation of differentiated granulocytic cells.[104] An additional aspect is that the initiating genetic lesion(s) found in human leukemias seems to take place only during certain specific periods, and to be restricted to a limited number of cells because they can reprogram the right target cell into a specific tumoral cell fate,[103,105,106] implying that oncogenes do not have a homogeneous mode of action all throughout the tumoral cell population, and at all the stages of cancer development.

From all these evidences we can conclude that mouse models in which the initiating oncogenic alteration(s) is not directed to the right cell-of-origin are unlikely to accurately recapitulate the etiology of the human disease, and will originate an inaccurate model of human leukemia (**Fig. 1C, D**). However, even targeting the oncogene expression to the stem/progenitor compartment is still not completely analogous to what happens in humans, since in humans the preleukemic clone carrying the translocation is restricted to the BM during the preclinical stage. This is however, not the case in most currently existing mouse models, in which the transgenic lesion, once activated, is transmitted (and, in the majority of the cases, expressed) to all the descendants of the targeted cell during the preclinical phase. Therefore, an essential need is to limit oncogene expression to the right developmental compartment, avoiding "off-target" windows of expression, both before and after the right moment in which these lesions appear and cause the leukemogenic effects in humans. Ideally, initially one could think of knock-in approaches as the ones having the major chances or recapitulating the correct pattern of oncogene expression. However, this could also cause undesirable variations in the levels of expression between the different developmental compartments that, in turn, lead to differences in the leukemogenic capacity, since the relationship between oncogene dosage and cellular susceptibility to transformation is different between progenitors and HSCs.[80] So, although it might seem counterintuitive, it could happen that the right pattern of expression might in some cases be better provided by other promoters, rather than by the endogenous mouse regulatory sequences. Once more, the search for these regulatory elements must be largely empirical, but will undoubtedly be profitable.

In summary, pB-ALL is the subject of intense research around the world, and the different GEMMs generated so far have been extremely useful to better understand many aspects of leukemic development. However, many questions about the etiology of the human disease remain unanswered. How exactly does pB-ALL start and how does it evolve? Mouse models of pB-ALL in which precise control of the timing of the oncogene action is possible will be instrumental to address these and other questions to understand the complexity of pB-ALL in order to know how to eradicate the preleukemic clone and/or to inactivate its conversion into a full malignant leukemia. Similarly, assessing the ability of any candidate therapy to destroy these cells would be crucial to predict its efficacy. Here again the right mouse models will be pivotal tools to achieve this aim.

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