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REVIEW

Murine models based on acute myeloid leukemia-initiating stem cells xenografting

Cristina Mambet, Mihaela Chivu-Economescu, Lilia Matei, Laura Georgiana Necula, Denisa Laura Dragu, Coralia Bleotu, Carmen Cristina Diaconu

Cristina Mambet, Mihaela Chivu-Economescu, Lilia Matei, Laura Georgiana Necula, Denisa Laura Dragu, Coralia Bleotu, Carmen Cristina Diaconu, Cellular and Molecular Pathology Department, Stefan S. Nicolau Institute of Virology, Bucharest 030304, Romania

Laura Georgiana Necula, Nicolae Cajal Institute, Titu Maiorescu University, Bucharest 040441, Romania

ORCID number: Cristina Mambet (0000-0001-8495-7704); Mihaela Chivu-Economescu (0000-0001-7512-4700); Lilia Matei (0000-0003-4479-655X); Laura Georgiana Necula (0000-0001-6531-7939); Denisa Laura Dragu (0000-0002-5868-2636); Coralia Bleotu (0000-0002-9031-338X); Carmen Cristina Diaconu (0000-0002-2259-1425)

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Correspondence to: Mihaela Chivu-Economescu, PhD, Research Assistant Professor, Research Scientist, Senior Researcher, Cellular and Molecular Pathology Department, Stefan S. Nicolau Institute of Virology, 285 Mihai Bravu Ave, Bucharest 030304, Romania. mihaela.chivu@gmail.com

Telephone: +40-21-3242590
Fax: +40-21-3242590

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Abstract

Acute myeloid leukemia (AML) is an aggressive malignant disease defined by abnormal expansion of myeloid blasts. Despite recent advances in understanding AML pathogenesis and identifying their molecular subtypes based on somatic mutations, AML is still characterized by poor outcomes, with a 5-year survival rate of only 30%-40%, the majority of the patients dying due to AML relapse. Leukemia stem cells (LSC) are considered to be at the root of chemotherapeutic resistance and AML relapse. Although numerous studies have tried to better characterize LSCs in terms of surface and molecular markers, a specific marker of LSC has not been found, and still the most universally accepted phenotypic signature remains the surface antigens CD34+CD38- that is shared with normal hematopoietic stem cells. Animal models provides the means to investigate the factors responsible for leukemic transformation, the intrinsic differences between secondary post-myeloproliferative neoplasm AML and *de novo* AML, especially the signaling pathways involved in inflammation and hematopoiesis. However, AML proved to be one of the hematological malignancies that is difficult to engraft even in the most immunodeficient mice strains, and numerous ongoing attempts are focused to develop "humanized mice" that can support the engraftment of LSC. This present review is aiming to in-

troduce the field of AML pathogenesis and the concept of LSC, to present the current knowledge on leukemic blasts surface markers and recent attempts to develop best AML animal models.

Key words: Acute myeloid leukemia; Leukemia-initiating stem cells; Antigen markers; Murine models; Xenografts

Core tip: The review is aiming to introduce the field of acute myeloid leukemia (AML) pathogenesis, the concept of leukemic stem cells, and also to present the current attempts to develop best AML animal models as means to investigate the factors responsible for leukemic transformation. Due to difficulties in engraftment of less aggressive AML samples, it is currently being attempted to develop humanized mice by introducing supporting human stromal cells as a source of proper cytokines, in a challenge to mimic an appropriate bone marrow niche able to support leukemic stem cells engraftment.

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INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive cancer characterized by unrestricted proliferation of functionally immature myeloid cells. High heterogeneity and variable expansion capacity of multiple clones within each patient[1,2], clinical and molecular differences between de novo and secondary AML, complicate even more treatment choices and make targeted therapy a goal yet to far to reach without using models that are able to simplify the multitude of mechanisms that might be involved in leukemogenesis.

In the last decades, murine models become very important tools in the field of preclinical research in oncology, hematology, and immunology, providing a platform for study of tumor biology and for *in vivo* evaluation of drugs in patient-derived xenograft tumors (PDX). Nowadays, a large variety of immunodeficient mice strains have emerged, able to support the xenografting and development of a complex human hemato-lymphoid system. The most difficult to reproduce is the immune system and the bone marrow (BM) microenvironment, mostly because of the differences between the signaling molecules responsible for the maturation of different hematopoietic cell populations[3]. Although the field of animal models has experienced a recent exponential growth through the development of IL2rg^{null} immunodeficiency mice, AML remains one of the hematologic malignancies difficult to engraft into the existing strains of mice due to the lack of a proper BM niche and absence of specific human growth factors and supporting stromal cells[4]. As a result several attempts were made to develop "humanized mice" that can better support myeloid leukemia-initiating stem cells xenografting.

This review is aiming to introduce AML pathogenesis and the concept of leukemic stem cells and the current most advanced strategies to overcome challenges in obtaining AML murine models.

AML PATHOGENESIS AND THE CONCEPT OF LEUKEMIC STEM CELLS

AML is a heterogeneous hematopoietic malignancy defined by clonal expansion of abnormally differentiated or undifferentiated myeloid progenitors (blasts) that accumulate in the BM and impair hematopoiesis, leading to multi-lineage cytopenias[5,6]. Blasts can also migrate from BM into peripheral blood and infiltrate other tissues[5].

AML can be divided in 3 categories taking into account their clinical ontogeny: Secondary AML (s-AML) occurred after leukemic transformation of a pre-existing myelodysplastic syndrome or myeloproliferative neoplasm, therapy-related AML (t-AML) developed in patients that received leukemogenic chemotherapy for antecedent non-myeloid malignancies and *de novo* AML generated in the absence of a previous stem cell disorder or a therapeutic exposure to cytotoxic drugs[7].

Despite recent progress in understanding AML pathogenesis and recognizing molecular subtypes of AML that have prognostic impact, AML is still characterized by poor outcomes, with a 5-year survival rate of only 30%-40%. The dismal prognosis is mainly related to high rate of relapse and refractory disease[2,8]. Patients with s-AML and t-AML display even a much worse prognosis, the median overall survival rate being 7 months. Notably, somatic mutations in *SRSF2, SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, or *STAG2* proved to be highly specific for s-AML. They are acquired early in leukemogenesis and tend to persist during clonal remissions[7].

Increasing evidence support the concept that a minor population of stem cells, named leukemia stem cells (LSCs), is responsible for leukemia initiation, disease progression and relapse, as well as drug resistance[9]. AML was among the first diseases in which the existence of cancer stem cells was documented using xenograft animal models[10].

LSCs are derived from transformed hematopoietic stem cells (HSCs) or downstream committed progenitors[11]. They are able to initiate the disease after transplantation into immunodeficient mice and are characterized by both unlimited self-renewal potential inducing disease in serial transplantation and capacity to

partially differentiate into non-LSC blasts that lack self-renewal properties and possibility of engraftment[12]. Although LSCs and non-LSC blasts harbor a common set of mutations there are epigenetic differences between them. A predominant hypo-methylation of *HOXA* gene cluster that has been involved in leukemogenesis represents a main feature of LSCs[13].

It is thought that, similarly to normal hematopoietic system, AML displays a hierarchical organization with LSCs on the top, being able to generate the whole population of AML blast cells[10]. Signaling pathways that control self-renewal of HSCs, such as Wnt/β-catenin, PI3K/Akt/mTOR, or Hedgehog, are also involved in LSC survival and expansion and can serve as therapeutic targets to facilitate eradication of LSCs[8,11]. Moreover, LSCs might escape apoptosis through up-regulation of NF-κB or downregulation of Fas/CD95. Additionally, CXCL12-CXCR4 axis promotes retaining of LSCs within the protective BM microenvironment[11].

The existence of a preleukemic stage in AML was proven by isolating from leukemia patients a population of HSCs that was found to bear some, but not all, of the mutations identified in the downstream leukemia. These preleukemic HSCs, that can be distinguished from LSCs by the surface antigen markers, TIM3 and CD99, are capable to generate bi-lineage engraftment in NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl} (NSG) mice and the derived lymphoid and myeloid progeny display the same preleukemia mutations[14]. According to the currently proposed model of preleukemic clonal evolution, the first leukemia-related mutation has to occur in a cell that possesses self-renewal capacity or, alternatively, must confer self-renewal properties to a more differentiated progenitor[15]. By single-cell analysis it was shown that during the process of leukemogenesis, the preleukemic HSCs gradually acquire somatic mutations in a nonrandom pattern[16]. Thus, in the early phases of AML evolution there is enrichment for mutations in epigenetic modifiers such as *TET2*, *DNMT3A, IDH1/2*, and *ASXL1*. On the other hand, mutations in genes involved in signaling pathways and proliferation, such as *FLT3* and *KRAS* occur in later stages. Other leukemogenic mutations in genes like *NPM1*, *CEBPA*, and *WT1* can be found in preleukemic phase as well as in later stages[15,16].

The preleukemic HSCs that eventually give rise to AML persist in patient samples at diagnosis and are resistant to current chemotherapy, thus representing a source of disease recurrence[17].

LEUKEMIA-INITIATING STEM CELLS AND BLASTS

The identification of LSCs in AML plays an important role in disease diagnosis, prognosis and AML therapy monitoring, and also represents an important step in development of targeted therapy and drug discovery[9]. Although initial studies suggested that LSCs were CD34+CD38- and did not expressed other lineage markers[18-21], later studies proved that the LSC phenotype was more complex and heterogeneous[22,23]. At present, it is established that LSCs are characterized by increased or decreased expression of surface markers of normal myeloid precursors (CD34, CD38, CD33, CD13, CD117, and CD123), asynchronous expression of antigens determined by AML morphological subtype and by the LSC stage of differentiation (CD4, CD11b, CD14, CD15, CD36, CD61, CD64, CD71, *etc*.), as well as by aberrant expression of lymphoid antigens (cross-lineage expression) (CD2, CD5, CD7, CD19, CD22, CD56, Tim3, *etc*.)[24-30]. LSCs reside mainly in CD34+CD38- population, but may be present also in other cellular fractions, usually CD34+CD38+, and in some cases, in CD34- population[12,31]. Additional markers, more specific for the advanced characterization of cellular subpopulations in AML, include: CD90[32], CD96[33], CD123[34,35], CD47[36], CD44, C-type lectin-like molecule-1 (CLL1)[37], aldehyde dehydrogenase, *etc*[38]. Currently, standard diagnosis and sub-classification of AML integrate the study of cell morphology, genetics/cytogenetics and multi-parametric immuno-phenotyping. The antibody panels for surface markers used for sub-classification of each AML group are showed in Figure 1.

Methods commonly used to assess HSC properties are colony-forming cell (CFC) assay, long term culture (LTC), flow cytometry and competitive repopulation.

The CFC assay is an important tool used to evaluate the ability of the progenitor cells to proliferate and differentiate into multiple lineages. In order to produce colonies, cells are cultured in a semisolid medium, in the presence of appropriate cytokines for 7-14 d. Colonies are counted and characterized according to morphologic and phenotypic criteria. Although this short-term colony assay can determine the frequencies of hematopoietic progenitor cells in analyzed populations, still, it is not able to detect more immature progenitors or HSCs/LSCs. To overcome this limitation, the cells can be cultured for 5-8 wk on a stromal feeder layer that can provide a substrate and a source of cytokines and growth factors, in effort to mimic the *in vivo* niche conditions[39]. The long-term culture-initiating cells (LTC-ICs) can be evaluated by their capacity to generate CFCs in culture supernatant after 5 wk. This period allows CFCs present in the inoculum to terminally differentiate and the remaining CFCs may represent the progeny of LTC-ICs. Subsequent limiting dilution tests can be performed to determine the LTC-IC frequency[40]. Although this method facilitates the detection of more immature progenitor cells, it is time consuming and the presence of stromal cells can induce procedure variations and different outcomes[41,42].

Competitive repopulation represents the best method to assess the functional abilities of immature progenitor/stem cells by serial transplants in immunocompromised mice. This method is based on the ability

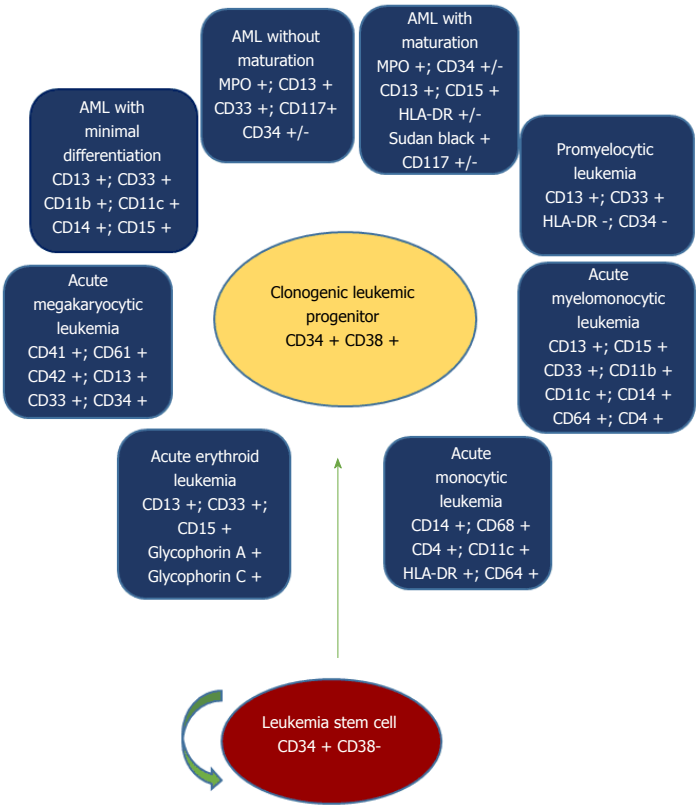

Figure 1 Advanced characterization of leukemic stem cell, clonogenic leukemic progenitors and various cellular subpopulations in acute myeloid leukemia. AML: Acute myeloid leukemia.

of cells that are investigated to compete with non-manipulated standard cells to repopulate the BM of an irradiated recipient[43].

MURINE MODELS - WHICH ARE THE BEST CHOICE?

Animal models are used as replacement for human biological niches due to ethical restrictions in the use of human tissue samples from donors. Moreover, animal models accurately recapitulate human disease and have been an important tool in advancing the understanding of human pathology, and development of pre-clinical therapy. Small animals, such as mice and rats, are often used as a model for various diseases because of their ease in breeding, maintenance, and manipulation. In spite of these many advantages, there are limitations due to the disparities between the murine and human biological systems. Human immune system and the BM microenvironment are the most difficult to be reproduced in mouse models because of the differences in the signaling molecules responsible for the maturation of various hematopoietic cell populations[3]. As a result, many malignant hematopoietic and other hematologic disorders do not successfully engraft in conventional mice models.

AML is one of these hematologic malignancies that fail to properly graft into the existing strains of mice due to the lack of a proper BM niche, homing elements, absence of specific human growth factors and supporting stromal cells[4]. As a result, several attempts have been made to develop murine models that reproduce with fidelity human hematopoiesis, particularly the development of the myeloid line.

Early attempts to increase the support for myelopoiesis involved the use of mice injected with IL-3, GM-CSF, SCF[44], mice producing human TPO[45] or MISTRG mice strain which produces human tumor necrosis factor and IL-6[46]. These confirmed that the introduction of human genes into mice led to the production of functional proteins capable of supporting engraftment and proliferation of human grafts.

The following attempts were aimed to develop next-generation mouse models genetically engineered to support myeloid differentiation from human HSC. Thus, it was necessary to act at three major levels in order to induce tolerance in the murine host, provide a supportive niche, and support hematopoiesis/proliferation with appropriate growth factors and cytokines.

The first request was fulfilled by the development of mice that lacked the adaptive and innate immune compartment like NSG and NODShi.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug} mice strains. These strains were developed on NOD *scid* immunodeficient mice by modifying them to bear mutations in the IL-2 receptor gamma chain gene th-

at induced either the absence or the presence of an nonfunctional truncated form of the receptor subunit. The gamma chain subunit is a major component of the IL2, IL4, IL7, IL9, IL15 and IL21 receptors, and is indispensable for binding and signaling of these cytokines[47,48].

The second condition, involved the ablation of mouse cells to create open niches for human transplanted cells. These were achieved through irradiation or depletion of mouse stroma *via* introducing mutations in *c-Kit* gene encoding for SCF receptor. SCF plays an important role in the maintenance and differentiation of HSCs[49]. The c-kit mutated mice strain, known as NODB6.SCID Il2rγ$^{-/-}$ Kit (W41/W41) (NBSGW) mice, supports engraftment studies with human HSCs without prior irradiation. McIntosh *et al*[49] showed that in peripheral blood, the median human CD45+ count in non-irradiated NBSGW mice was similar to the count in irradiated NSG. In BM a significant increase in CD45+ was observed in non-irradiated NBSGW (97%) compared to non-irradiated NSG (30%).

The third constraint regarding the need for supportive myeloid cytokines was overcome using animal models with transgenic expression of hSCF, hGM-CSF and hIL-3 on the NOD SCID background resulting the NSG-SGM3 mouse strain [also known as NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}Tg(CMV-IL3,CSF2,KITLG)/1Eav)]. Wunderlich *et al*[4] reported the development and use of NSG-SGM3 mouse for the engraftment of normal CD34+ and AML xenografts. The results showed an improvement in the expansion of normal human myeloid cells, as well as an enhanced engraftment of primary human AML samples. They injected five samples of primary AML in sub-lethally irradiated NGS and NSG-SGM3 mice. Only three samples from five engrafted in NSG mice, compared to five samples in NSG-SGM3 mice, showing variability in the engraftment potential across the AML samples, and that NSG-SGM3 strain was a better host for some subsets of AML, relative to NSG mice. Moreover, three of five samples with primary AML had higher BM engraftment level in NSG-SGM3[4]. Similar results regarding variable engraftment potential in immunodeficient mice strains were obtained by Klco *et al*[50] They injected blasts from six AML samples in tail vein of non-irradiated NSG and NSG-SGM3. The results showed that four samples had higher BM engraftment and CD34+ expression level in NSG-SGM3 than NSG mice.

Finally, we can conclude that next-generation humanized mouse models are able to support myeloid blast development and differentiation.

XENOGRAFT MOUSE MODELS USED FOR ACUTE MYELOID LEUKEMIA

Cell line derived xenografts

AML represents a heterogeneous disease including several subtypes which are characterized by specific fusion oncogenes as a result of chromosome abnormalities. The fusion oncogenes in AML are associated with different clinical and laboratory characteristics, highlighting the different ways of malignant transformation in this disease. A study focused on the evaluation of four important AML fusion oncogenes reported that MLL-AF9 and NUP98-HOXA9 had very similar effects *in vitro* on primary human CD34+ cells, resulting in erythroid hyperplasia and an obvious blockage in erythroid and myeloid maturation while AML1-ETO and PML-RARA produced only modest effects on myeloid and erythroid differentiation. Moreover, MLL-AF9, NUP98-HOXA9 and AML1-ETO fusion oncogenes generated a significant increase in long-term proliferation and self-renewal of CD34+ cells. The characterization of gene profiles determined by AML fusion oncogenes can be considered an important tool for the discovery of new potential drug targets. In this study, two different time patterns of gene deregulation as result of fusion of these oncogenes were observed: MLL-AF9 and NUP98-HOXA9, caused gene deregulation 3 d after transduction, while gene deregulation by AML1-ETO and PML-RARA appeared within 6 h. Interestingly, p53 inhibitor MDM2 was upregulated by AML1-ETO at 6 h suggesting that MDM2 upregulation was involved in cell transformation, being related to AML1-ETO[51].

Wei *et al*[52] evaluated the *in vitro* and *in vivo* effects of *MLL-AF9* gene fusion in human CD34+ cord blood cells using retroviral vectors. Thus, MA9 transduced cells became immortal and doubled in number every 2-3 d. The expression of CD33, CD11b, CD13, CD14 and CD15 suggested a myelo-monocytic lineage. Moreover, long-term cultured MA9 cells failed to differentiate towards the erythroid or B lymphoid lineages, remaining cytokine and FLT3L dependent for growth. In non-obese diabetic/severe combined immunodeficient [NOD/SCID (NS)], NS-β2M-/- (NS-B2M) and NS mice, MA9 cells induced acute myeloid, lymphoid, or mixed-lineage leukemia with blast cells present in the peripheral blood, BM, spleen and liver. Gene expression profile of MLL-AF9 transduced cells was similar to human AML with 11q23 translocations, Rac signaling pathway being the most affected pathway and a promising therapeutic target in MLL-rearranged AML[52].

Another AML subtype with a particularly poor outcome is characterized by the t(6;9)(p22;q34) chromosome rearrangement which generates DEK-NUP214 chimeric gene. Qin *et al*[53] developed an AML model harboring DEK-NUP214, using CD34+ human hematopoietic progenitor cells and MO7e cell lines xenografted into immunocompromised mice that expressed human myeloid cell growth factors. The M07e human megakaryoblastic leukemia cell line was strictly dependent on either IL-3 or GM-CSF for survival; retroviral expression of this fusion gene in IL-3 dependent M07e cell line induced a cytokine independence and increased colony formation ability in soft-agar. DEK-NUP214 expression also modified the differentiation of human cord blood CD34+ progenitor

cells, which expressed myeloid lineage markers (CD13+), with small subsets showing T- (CD3+) and B- (CD19+) cell linage markers. The obtained results suggested that DEK-NUP214 was involved in leukemic transformation and differentiation of myeloid cells. In this study, CD34+ progenitor cells obtained from three different umbilical cord blood samples and transduced with chimeric DEK-NUP214 were engrafted in NSG-SGM3 mice strain. Interestingly, two months after transplantation, almost 20% of peripheral blood cells from the transplanted mice displayed a human-specific CD45 immuno-phenotype with CD45+CD13+CD34+CD38+ cells. The analysis of peripheral blood smears also showed the typical human AML cell morphology with a larger nucleus and reduced cytoplasmic ratio. Therefore, the study demonstrated that DEK-NUP214 could transform human CD34+ progenitor cells and induced human AML *in vivo*. Gene profiling of this model revealed that several genes of HOX family (*HOXA9, 10, B3, B4* and *PBX3*) were highly upregulated. In this AML model pathways involving KRAS, BRCA1 and ALK were significantly dysregulated[53].

Similar results were obtained in case of t(8;13)-(p11;q12) chromosome translocation which led to ZMYM2-FGFR1 chimeric kinase, characteristic for another AML subtype. Human CD34+ cells harboring ZMYM2-FGFR1 transplanted into immune-compromised mice developed myeloproliferative disease that progressed to AML. Mice displayed hepatosplenomegaly, hypercellular BM and a CD45 + CD34 + CD13+ immunophenotype[54].

Preclinical cancer research remains essential for the discovery and the development of new therapies in case of the most advanced cancers. Various cancer cell lines have been developed and used for the study of cancer but with a great disadvantage that they do not really reflect the behavior of the original cancer cells, due to the artificial nature of their culture conditions.

Patient derived xenografts - patient stem-cells derived xenografts

PDX models established by transplanting patient cancer cells into immunocompromised mice represent an important tool in cancer research. They have a great potential to offer important information on cancer biology and to guide the therapeutic approach. Unlike cell lines derived from primary tumors that might have lost their original characteristics due to a prolonged *in vitro* growth, PDX mouse models seemed to be able to overcome this issue[55,56]. Many studies demonstrated that PDX models kept the most important features of the original tumor including histology, genomic pattern, cellular heterogeneity, and more important, drug responsiveness or personalized drug selection[57].

The development of PDX models of AML allows us to monitor *in vivo* the progression of the disease and to evaluate the efficacy of an experimental treatment on tumor growth using imaging techniques[58].

A first full study on the engraftment ability of a large cohort of AML samples in immunodeficient animal models was published by Kennedy *et al*[59] who transplanted BM or peripheral blood cells from 307 AML patients intra-femorally into sublethally irradiated NOD. SCID mice pre-treated with an anti-CD122 antibody. AML xenografts were obtained in 44% of cases, leukemic engraftment being associated with a higher white cell count in peripheral blood (mean of 92 x 10^9/L in engrafters *vs* 67 x 10^9/L in non-engrafters, $P = 0.01$). Moreover, results showed that complete remission was achieved in only 51% of patients whose diagnostic samples established AML xenografts, compared to 80% of non-engrafting samples ($P < 0.0001$). As a conclusion, AML xenografting was successful when using samples from AML patients with aggressive disease and with a poor response to standard induction therapy.

OVERCOMING CHALLENGES IN PATIENT-DERIVED XENOGRAFTS OF AML

Recent experiments are trying to improve mouse experimental models of AML, aiming to engraft with a higher success rate even less aggressive leukemia samples. Most of them are based on immunodeficient mice with humanized microenvironment created by injection of human mesenchymal stem cell (MSC) that provide a better niche for leukemic blast engraftment.

An interesting approach was that of Reinisch *et al*[60], who used human MSC grafts injected subcutaneously in NSG mice to form a humanized microenvironment named "ossicles", in which they subsequently injected (8 wk later) human HSCs and AML blasts. The final aim was to analyze the engraftment capacity the resulting niche. After 6-10 wk, the "ossicles" showed human BM–like functions and morphology and allowed enhanced engraftment of primary patient-derived AML.

A further attempt employed humanized niches based on genetically modified MSCs to express huIL-3 and TPO. Carretta *et al*[61] implanted subcutaneously these human MSCs in ceramic scaffolds or Matrigel in NGS mice, and 6 to 8 wk later transplanted CD34+ enriched AML blasts in the ectopically engineered BM niches. The engraftment capacity was then compared with the one from non-engineered MSC niches. The results showed that leukemic blasts efficiently engrafted in both models with no significant differences. An unexpected result was that CD33+-sorted myeloid clones from the animal model failed to self-renew in secondary recipients, probably due to overexpressed IL-3 and TPO cytokines from modified microenvironment that might have affected a proper self-renewal of myeloid blasts.

An important challenge for obtaining mouse models valuable as preclinical models is the capability of PDX cells to authentically mimic the heterogeneity of the initial disease. The xenograft mouse model of AML has been used mainly to study primary transplantation and further serial experiments were performed to

verify self-renewal competence or stability of gene expression profiles of engrafted cells. However, this model was rarely employed to investigate deeply AML biology or therapy[62]. Most of the published results revealed that PDX cells resembled the primary samples in terms of gene expression profiles but sub-clonal profiles were often not reflecting the primary sample. Another important drawback was the inability of the most proposed models to sensitively and repetitively monitor disease progression or drug effects. These were determined at single time points by invasive procedures or post mortem. However, researchers tried to overcome these challenges by proposing a better control of PDX cells. This control aimed to check the pattern of alterations in mutational or antigen expression possibly occurred during engraftment. For better monitoring disease progression or drug effects, recombinant luciferase enabled bioluminescence *in vivo* imaging has been proposed to facilitate *in vivo* monitoring of PDX AML cells as a quantitative, sensitive, reliable method for quantifying leukemia initiating cells[62].

CONCLUSION

Mouse models were of tremendous importance for understanding the molecular etiology of leukemia, proven to be valuable tools to facilitate preclinical *in vivo* studies.

Most of the studies verified that PDX models kept the most important features of the original tumor. However, mouse models should be controlled more carefully before and after xenotransplantation, especially in serial transplantation experiments, in order to ensure that the heterogeneity of the original sample is conserved and genetic drift is not modifying genetic, phenotypic or functional characteristics of the original disease.

Prospectively, advancements allowing repetitive, reliable, sensitive and fast studies, able to evaluate the efficacy of an experimental treatment in well genetically defined and heterogeneous subgroups of AML, will represent valuable tools to improve the individualized xenograft mouse model of AML and drastically reduce the number of mice to be used in these kind of experiments.

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