

Modeling mixed-lineage-rearranged leukemia initiation in CD34⁺ cells: a "CRISPR" solution

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In this issue of *haematologica*, Reimer *et al.*¹ present an improved strategy based on genome engineering, viral vector transduction, and the use of CD34⁺ human hematopoietic stem and progenitor cells (HPSCs) to recreate a human leukemic chromosomal rearrangement, t(11;19)/*MLL-ENL*, in its natural genomic environment. This model provides new clues as to the complex molecular mechanisms of mixed-lineage-rearranged (MLLr) leukemia and opens up new avenues for the genomic reconstruction to study leukemia initiation and evolution.

A common and disease/lineage-specific molecular signature of leukemia involves the generation of recurrent reciprocal chromosomal translocations, which are considered to be the oncogenic initiating drivers.² Chromosomal genomic rearrangements are complex and implicate illegitimate recombination or juxtaposition of normally separated genes during DNA replication, and results in oncogene activation or, more commonly in leukemia, the generation of novel fusion genes.³ Our current understanding on how the nature of the target cell and the spatial organization of chromosomes in the nucleus contribute to chromosomal rearrangements (i.e. translocations) is very limited. Questions about the nature of the target cell in which the translocation arises and initiates leukemia can not really be studied with primary patient samples because all molecular insults are in place at the time of disease presentation.^{4,6} As an alternative, patient-derived cell lines have been widely exploited to study the contribution of translocations to leukemogenesis; however, associated problems can arise when using this material. Human cancer cell lines are generated from primary cells once the full transformation events have taken place, and this can present challenges for distinguishing between driver and passenger events. Moreover, serial passage of cell lines can cause genotypic variation, and even heterogeneity in cultures, resulting in a loss of information on the leukemia initiation and the different steps of progression.⁷ These caveats aside, cell lines are powerful tools for ascertaining and characterizing the cancer gene, and have over recent decades increased our general understanding of the molecular pathophysiology of chromosomal rearranged leukemia.⁸ Beyond *in vitro* studies, genetically modified animal and cellular models constitute invaluable tools for cancer investigation, but they also have limitations, due in part to the manner in which they are generated. Most of the extensively used leukemia models are generated using viral vector-based approaches (primarily recombinant retroviruses and lentiviruses), randomly integrated plasmid DNA or, in a more refined manner, by generating "knock-in" fusion genes.^{5,6,9,10} Some of the major concerns regarding these methodologies are: i) the high potential mutagenic rate associated with uncontrolled

cassette integration that could lead to a growth advantage and variegated cell lines (for plasmid DNA and integrative recombinant virus approaches); ii) an exogenous strong transgene expression controlled by promoters that function in a broad range of cells; or iii) the expression of just one of the fusion genes generated by a chromosomal translocation and the retention of the two wild-type alleles (for the "knock-in" strategy) which is not seen in patients.¹¹

These concerns guide the search for more faithful models capable of recapitulating the initial genetic events associated with the leukemogenic process with the least alteration in the genomic architecture. Until recently, the most effective way to replicate these events was based on the generation of chromosomal translocations using translocator technology. This approach involves the use of the *Cre/loxP* site-specific system *via* prior engineering in the mouse genome.¹² While some leukemia animal and cellular models have been successfully developed using this strategy, its use in human cells is extremely inefficient. More recently, the explosion of new genome editing technologies, particularly the CRISPR/Cas9 system, has permitted the efficient recreation of *de novo* cancer-associated translocations *in vitro* in mouse and human cells,¹³ and *in vivo* in mouse models.¹⁴ This is important because it has been demonstrated that, although conditional models or viral vector expression systems can efficiently generate chromosomal translocations, the engineered cells do not always initiate a leukemic-like phenotype in mouse xenograft models.

In the present article, Reimer *et al.*¹ combine the undemanding aspects of lentiviral generation and delivery with the advantages of chromosomal translocation generation by the CRISPR system. The authors engineered an advanced lentiviral CRISPR/Cas9 vector for efficient transduction of human CD34⁺ HPSCs. This new lentiviral vector permits the induction of double strand breaks (DSBs) in the *MLL* and *ENL* intronic regions with very high efficiency (78-83%), favoring the generation of the t(11;19) rearrangement. As a consequence of the translocation, two derivative chromosomes are generated, der(11) and der(19), leading to the expression of both fusion transcripts and the concomitant loss of one copy of the wild-type *MLL* and *ENL* genes. The authors describe a transient outgrowth advantage in long-term cultures of the primary human CD34⁺ HSPCs t(11;19)⁺, but more significantly, they demonstrated that when these cells were injected into primary immunodeficient recipients, the *in vivo* environment favored oncogenic transformation, initiating a monocytic leukemia-like disease. It is important to emphasize that whereas this transformative process does

not fully recapitulate the human leukemic phenotype in primary recipients, secondary recipients developed acute lymphoblastic leukemia, albeit with incomplete penetrance. Based on these findings, the authors conclude that “environmental cues not only contribute to the disease phenotype, but also to t(11;19)/MLL-ENL-mediated oncogenic transformation”.¹ This result resembles the effect of chemotherapy in patients, but it will need to be confirmed in future studies.

While the results described in this study represent a great improvement in the field, some questions remain and some issues have still not been fully clarified. For example, although the authors demonstrated an extremely high DSB induction efficiency, the translocation rate remained very low (0.2% or 1.6×10^{-3}), even when compared with other publications.^{13,15,16} This could be due to the genome architecture of the leukemia-initiating cell, the proximity between involved loci or the presence of repetitive elements.¹⁷ In addition, although it is a more advantageous model due to the conservation of all endogenous regulatory elements (promoters, enhancers, miRNA binding sites and rearranged genome architecture) and the possibility to study the initial steps of the leukemic process, the approach is grounded on the use of random integrative lentiviruses, which could lead to mutagenic effects and growth advantages associated with their integration pattern. Moreover, the continuous expression of the CRISPR/Cas9 components may increase the likelihood of undesirable off-target effects over time. Regarding this latter issue, two alternative approaches have recently been described that could further improve the use of the CRISPR system in the generation of leukemic models. The first one relies on the use of a “hit-and-run” protein-based Cas9 system that circumvents the lentiviral integration concerns and lessens the chances of off-target effects while permitting the generation of human chromosomal translocations in a wide variety of primary stem cells with higher efficiencies.¹⁶ The second approach takes advantage of the classical “knock-in” model, but with application to human cells with conditional allele expression and resistance selection cassettes in combination with the CRISPR/Cas9 system.¹⁵ Because of their inducible nature, both approaches could widen our knowledge of the first steps leading to the establishment of cancer and to its progression.

The CRISPR/Cas9 system has revolutionized functional biology, biotechnology, and genomic medicine. The present article by Reimer *et al.*¹ illustrates how the use of more accurate models generated by genome engineering techniques in human CD34⁺ HPSCs can transform the field of basic leukemia biology. A deeper knowledge of the CRISPR approach and the development of new applications should open new horizons for the study of the molecular and cellular processes of cancer, and will make it easier to reproduce the complex cancer genome and epigenome, allowing a more rigorous molecular analysis of the molecular mechanisms involved in tumor progression and the identification of oncogenes and tumor suppressor genes. Specifically, further developments to isolate the minor fraction of *bona fide* genome-edited CD34⁺

clones (via antibiotic selection, reporter expression, etc.) would open up fascinating new avenues in the study of leukemia and cancer modeling.

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