Title: Utility of CRISPR/Cas9 systems in hematology research

Author names and affiliations.

Daniel Lucas[1], Heather A. O'Leary[2], Benjamin L. Ebert[3,4], Chad A. Cowan[5,6,7], Cedric S. Tremblay[8]

[1] Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, USA.

[2] Department of Microbiology/Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA.

[3] Department of Medical Oncology, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Boston, MA 02115, USA.

[4] Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA.

[5] Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Cambridge, MA 02138, USA.

[6] Broad Institute, Cambridge, MA 02142, USA.

[7] Center for Regenerative Medicine, Massachusetts General Hospital, Boston, MA 02114, USA.

[8] Australian Centre for Blood Diseases, Monash University, Melbourne, Victoria, Australia.

Corresponding author. Heather A. O'Leary, Ph.D. haoleary@iupui.edu

Or Cedric Tremblay, Ph.D. cedric.tremblay@monash.edu

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Utility of CRISPR/Cas9 systems in hematology research

Daniel Lucas[1], Heather A. O'Leary[2], Benjamin L. Ebert[3,4], Chad A. Cowan[5,6,7], Cedric S. Tremblay[8]

[1] Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, USA.
[2] Department of Microbiology/Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA.
[3] Department of Medical Oncology, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Boston, MA 02115, USA.
[4] Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA.
[5] Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Cambridge, MA 02138, USA.
[6] Broad Institute, Cambridge, MA 02142, USA.
[7] Center for Regenerative Medicine, Massachusetts General Hospital, Boston, MA 02114, USA.
[8] Australian Centre for Blood Diseases, Monash University, Melbourne, Victoria, Australia.

Abstract:
Since the end of the 20th century, the development of novel approaches have emerged to manipulate experimental models of hematological disorders, so they would more accurately mirror what is observed in the clinic. Despite these technological advances, the characterization of crucial genes for benign or malignant hematological disorders remains challenging, mainly because of the dynamic nature of the hematopoietic system and the genetic heterogeneity of these disorders. To overcome this limitation, genome editing technologies have been developed to specifically manipulate the genome via deletion, insertion or modification of targeted loci. These technologies have swiftly progressed, allowing their common use to investigate genetic function in experimental hematology. Amongst them, homologous recombination (HR)-mediated targeting technologies have facilitated the manipulation of specific loci by generating knockout and knock-in models. Despite promoting significant advances in the understanding of the molecular mechanisms involved in hematology, these inefficient, time-consuming and labor-intensive approaches did not permit the development of cellular or animal models recapitulating the complexity of hematological disorders. In October 2016, Dr. Ben Ebert and Dr. Chad Cowan shared their knowledge and experiences with the utilization of CRISPR for models of myeloid malignancy, disease, and novel therapeutics. Here we provide an overview of the topics they covered including insights into the novel applications of the technique as well as its strengths and limitations.

Introduction:

Over the past decade, novel genome-editing approaches targeting DNA double-stranded breaks (DSBs) were developed to stimulate DNA repair pathways, namely non-homologous end-joining (NHEJ) and site-specific HR. The introduction of DSB stimulates the recruitment of the HR machinery at a specific loci. This crucial process involves different targeted nucleases with distinguishing characteristics, such as DNA recognition and endonucleic DNA cleavage capacities. The discovery these targeted nucleases and the subsequent development of engineered nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Short Palindrome Repeats and their associated Cas proteins (CRISPR/Cas9), has enabled the development of novel site-specific gene editing technologies (1). However, these technologies generated random insertions or deletions (indels) following the stimulation of error-prone NHEJ in most cases, leading to frameshift mutations. When available, the use of an exogenous donor DNA template surrounding the site-specific DSBs allowed to bypass this significant hurdle by involving high-fidelity HR-mediated repair. Although ZFNs and TALENs have significantly improved our capacity to perform site-specific genome editing, their widespread application in hematology has been restricted by elaborate procedures, high cost and low specificity. On the other hand, the noticeable efficiency of the CRISPR/Cas9 system offers the greatest flexibility for genome editing by overcoming the limitations of earlier targeted nuclease-mediated methods.

In that regards, the development of the CRISPR/Cas9 system may represent a milestone for medical research as it allows researchers to effectively develop complex experimental models of benign and malignant hematological disorders. Here we highlight the implications of this powerful system by summarizing the ISEH webinar presented on October 26th, 2016 by Benjamin Ebert and Chad Cowan, entitled "*Utility of CRISPR/Cas9 systems in hematology research*", which was moderated by Eirini Papapetrou (2).

Dr Benjamin Ebert: CRISPR models of myeloid malignancy

Dr. Benjamin Ebert presented recent work from his lab where they have used CRISPR/Cas9 technology to model myeloid malignancies *in vivo*. Most myelodyplastic syndrome (MDS)/ acute myeloid leukemia (AML) patients have, on average, three to five mutations that contribute to disease progression (3). Further, these mutations are not uniformly distributed through the hematopoietic cells of the patient. It is now clear that initiating mutations cause the expansion of preleukemic clones that then acquire additional mutations that lead MDS and ultimately AML. Leukemic and preleukemic clones with different mutations coexist *in vivo* and chemotherapy might eliminate some clones without affecting others. Modeling these complex behaviors using transgenic mouse models is thus not feasible. The Ebert research group has recognized that there is a need for better models that reflect the genetic complexity and cellular heterogeneity observed in cancer patients. The ideal model should target hematopoietic stem and progenitor cells (HSPCs), work *in vivo*, and mimic the genetic complexity of human disease. The model should also be easily customizable and serially transplantable and amenable to pharmacological testing. To accomplish this the Ebert laboratory has used CRISPR/Cas9 to model MDS/AML in murine and human HSPCs. Their approach is to use CRISPR/Cas9 to introduce insertions or deletions in multiple alleles in a single HSPC. For achieving this, they co-infected HSPCs with a lentiviral vector carrying a single RNA guide, the Cas9 cDNA and an eGFP reporter concomitantly with multiple vectors each expressing a single RNA guide and a red fluorescent protein (RFP) reporter (4). Using this strategy they were able to simultaneously, and biallelically, inactivate *Dnmt3a*, *Ezh2*, *Smc4* and *Nf1* (eight different alleles) in a single HSPC leading to leukemia after transplantation in mice (4). The Ebert group has also used CRISPR/Cas9 to model genetic progression in mice by starting with *Tet2*$^{-/-}$ HSPCs, followed by the introduction of secondary mutations in six different alleles. They subsequently assessed the effect of these mutations in a primary transplant, from which mutated HSPCs were harvested and two additional mutations were introduced for testing in secondary transplants. These experiments confirm the feasibility of using CRISPR/Cas9 to rapidly model multiple mutations and leukemic progression in a much faster fashion than using conventional gene targeting and breeding strategies in mice. The other significant advantage is that CRISPR/Cas9 can also be used in human HSPCs. The Ebert research group has used these engineered human HSPCs carrying mutations that were predicted to confer sensitivity to hypomethylating agents (e.g in the *Tet2* locus) and demonstrated that these mutant HSPCs are more vulnerable to azacitidine *in vivo* (5). These experiments indicate that these engineered HSPCs can be used to test new drugs for complex hematological malignancies.

Dr Chad Cowan: Genome editing – from modeling disease to novel therapeutics

Dr. Chad Cowan discussed the use of CRISPR/Cas9 for genome editing to create novel disease/animal models and for potential therapeutic use to bridge biological discovery to clinical therapy. Initial comparison studies of genome editing via CRISPR/Cas9 (an RNA guided endonuclease) versus the use of TALENs (DNA binding motif based endonuclease) showed that CRISPR/Cas9 had higher overall efficiency (50-80%), as well as an increase in homozygous knock outs and knock in detection, and no off target events where the TALENs had 0-30% efficiency, diminished knock out and knock in potential, and 1 on target translocation detected (6-8). Therefore, his group uses the CRISPR/Cas9 system because it allows them to efficiently create clinically relevant, pluripotent, isogenic models (that can be differentiated into hepatocytes or other cells of interest) with side by side mutational analysis without the need for patient recruitment or quality control of multiple induced pluripotent stem cell (iPS) lines. Dr. Cowan's group has utilized these models for high throughput screens to detect genes associated with lipid metabolism (such as *A1CF*) as well as for investigations of the therapeutic potential of CRISPR in the context of HIV. To investigate the therapeutic potential the CRISPR/Cas9 system, they engineered a novel doubled guided approach to enhance efficiency of the CCR5 (the co-receptor for HIV) knockout in CD34+ HSPCs from mobilized peripheral blood. This strategy resulted in homozygous bi-allelic editing (40-50% efficiency) with no change in the *in vitro* or *in vivo* multi-lineage potential of the cells. To confirm the safety of this strategy Target Capture Sequencing was done to allow for very deep sequencing at specific sites, especially those that are similar to the guide RNA, as these sites are most likely to have mutations in the CRISPR/Cas9 system. Only 1 statistically significant mutation was detected, which could have been eliminated with utilization of different software, highlighting that good guide design is absolutely critical when using the CRISPR/Cas9 system. Overall, the Cowan laboratory has shown that CRISPR/Cas9 system enables the creation of clinically relevant disease models (utilizing cell types that have classically had low rates

of efficiency) with very low occurrence off target mutations making it an extremely useful tool for biological discovery and potentially clinical therapeutics.

Summary

Altogether, these studies describe the few of the possible applications of the CRISPR/Cas9 system to effectively develop complex experimental models of benign and malignant hematological disorders. As such, this powerful tool may represent a milestone for medical research as it renders possible to model more accurately complex multigenic and heterogeneous diseases. The research programs led by Dr. Ebert and Dr. Cowan brilliantly highlight a few of the various possibilities that offer the CRISPR/Cas9 system, such as the development of cell-based clinically relevant drug screens for complex hematological malignancies. The CRISPR/Cas9 system has already triggered a technical revolution in medical research, and we can expect that its wonderful possibilities will soon be translated into the clinic.

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Highlights for Utility of CRISPR/Cas9 systems in hematology research:

- CRISPR/Cas9 overcomes limitations of earlier targeted nuclease-mediated methods.
- CRISPR/Cas9 allows for insertions/deletions in multiple alleles in a single HSPC.
- Genome editing provides models to bridge biological discovery to clinical therapy.
- CRISPR/Cas9 allows for generation of isogenic models with mutational analysis.

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