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Spotlight

Base-editing screens illuminate variant effects in human hematopoiesis

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In a recent issue of *Cell*, Martin-Rufino et al. develop a strategy for performing high-throughput base-editing CRISPR screens coupled with single-cell readouts in the context of human hematopoiesis. Through a series of proof-of-principle experiments, the authors demonstrate the potential of base-editing screens for the study and treatment of hematological disorders.

In the past decade, CRISPR-Cas9 technology has revolutionized biological research and opened novel therapeutic avenues in human disease by enabling targeted genome editing. Genome editing by CRISPR-Cas9 relies on the induction of DNA double-strand breaks (DSBs) within genomic loci of interest. Repair of DSBs through error-prone non-homologous end-joining (NHEJ) results in sequence disruption, which can be exploited to abolish gene function in a targeted manner.[1] Alternatively, DSBs can be repaired by homology-directed repair (HDR) to introduce precise genomic edits. While highly accurate, HDR is inefficient and requires the use of exogenous DNA donor templates.[1] The recent development of CRISPR-mediated base editing has allowed the introduction of targeted single nucleotide changes independent of DSBs and DNA donor templates. Classical base editors include adenine (ABEs) and cytosine (CBEs) base editors, which induce A-to-G and C-to-T transitions, respectively.[2] Recent studies have successfully employed CRISPR base editors for the systematic screening of variants on a high-throughput scale.[3,4] Distinct from high-throughput approaches that employ standard CRISPR-Cas9 technology, which enable the identification of loss-of-function gene mutations, base-editing screens also allow the identification of gain- and separation-of-function mutants. Additionally, they facilitate the discovery of new domains, the classification of clinically relevant variants of uncertain significance (VUSs), and the identification of drug-target interactions. While base-editing screens have been successfully conducted in both immortalized and cancer cell lines,[3,4] their application in primary cells has remained underdeveloped. Furthermore, these approaches have not been utilized to investigate the function of variants across the diverse spectrum of cell states and types within specific tissues of interest.

In a recent issue of *Cell*, Martin-Rufino et al.[5] applied CRISPR base-editing screening technologies to the study of nucleotide variants in primary hematopoietic stem and progenitor cells (HSPCs) and across distinct hematopoietic lineages. Given the well-established protocols for the isolation of HSPCs and the ability of HSPCs to reconstitute all blood cell types, these cells are widely employed in targeted gene and cell therapies. Multiple clinical trials are examining the utility of CRISPR-Cas9 in treating several hematological disorders, including blood cancers and hemoglobinopathies.[6] In their study, the authors employed base editors to conduct pooled CRISPR screens in HSPCs (Figure 1). Additionally, the authors coupled CRISPR-based variant screening with single-cell transcriptomics and genotyping to gain a better understanding of the phenotypes given by nucleotide variants over the course of hematopoietic differentiation and determine base-editing outcomes with high precision. The authors termed the use of single-cell RNA sequencing (RNA-seq) in combination with base-editing Perturb(BE)-seq based on its conceptual similarity to other Perturb-seq approaches previously applied to CRISPR-Cas9 knockout or CRISPR interference (CRISPRi) screens.[7] Through their work, Martin-Rufino et al.[5] demonstrate that systematic base-editing screens can not only provide insights into molecular mechanisms of hematopoiesis but also advance therapeutic applications, as discussed below.

Chimeric antigen receptor (CAR) T cell therapy has emerged as a highly promising approach for targeted cancer treatment. In this therapeutic strategy, patient T cells are genetically engineered to express CARs that recognize antigens on the surface of cancer cells, promoting their killing. CD33 is a prime target antigen for CAR T cells expressed on the surface of acute myeloid leukemia (AML) cells. However, effective CAR T cell therapy is limited because CD33 is also expressed on the surface of normal hematopoietic cells. This issue can be circumvented by infusing patients with HSPCs in which CD33 has been disrupted. Gene inactivation can be obtained by using CRISPR-Cas9 knockout approaches or by introducing nonsense or splice mutations into gene open reading frames using base editors.[1] Through base-editing screens, Martin-Rufino et al.[5] identified potent single guide RNAs (sgRNAs) creating splice mutations that abolished CD33 expression in HSPCs. The authors then showed that base-edited HSPCs readily engrafted in immunodeficient mice and supported the reconstitution of all hematopoietic lineages with a long-term reduction in CD33 expression. While CRISPR-Cas9-mediated genetic knockout of CD33 in HSPCs

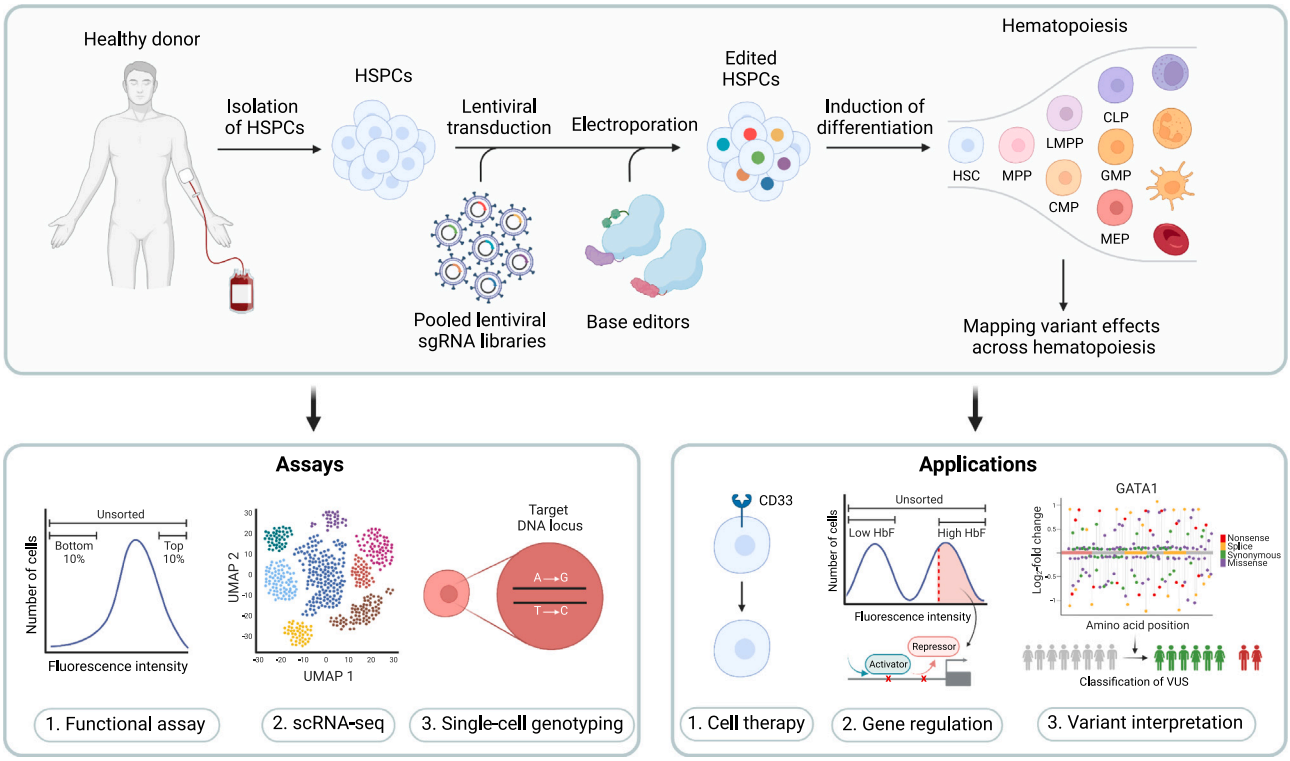

Figure 1. High-throughput base-editing screens in human hematopoiesis

Schematic of the protocol employed for variant screening in primary hematopoietic stem and progenitor cells (HSPCs) and different hematopoietic lineages (top). HSPCs isolated from healthy donors are transduced with a lentiviral sgRNA library targeting the gene(s) of interest, followed by electroporation of base editors. Then, HSPC maintenance media can be substituted with differentiation media to promote hematopoiesis. Hematopoietic stem cells (HSCs) represent one of the subgroups within HSPCs that possess self-renewal and multilineage differentiation capacity. MPP (multipotent progenitor), LMPP (lymphoid-primed multipotential progenitor), CLP (common lymphoid progenitor), CMP (common myeloid progenitor), GMP (granulocyte–monocyte progenitors), and MEP (megakaryocyte–erythrocyte progenitors) are also shown. The effect of nucleotide variants in hematopoiesis is determined using (1) functional assays, such as FACS; (2) single-cell RNA sequencing (scRNA-seq); and (3) single-cell genotyping (bottom left). Findings of base-editing screens in hematopoiesis (1) can advance cell therapies by improving cell engineering, (2) allow the modulation of gene expression through the editing of gene regulatory elements, and (3) facilitate the interpretation of the effects of nucleotide variants on gene function, including the classification of VUSs (bottom right). Figure created using BioRender (https://biorender.com).

is being currently investigated in clinical trials to augment CAR T cell therapy of AML (NCT04849910), Martin-Rufino et al.[5] showed that base editing could yield similar results without the induction of DSBs. This circumvents both the risk of undesirable genomic instability and p53 pathway activation caused by DSBs, which affects the reconstitution potential of HSPCs.[6] In line with the promise of base editing for cancer therapy, a recent study reported that CAR T cells engineered with base editors led to durable cancer remission in patients with T cell acute lymphoblastic leukemia.[8] Together, the findings of Martin-Rufino et al.[5] demonstrate the potential of base-editing screens in improving cell-based therapies for hematological malignancies (Figure 1).

CRISPR base-editing screens can also be applied to the functional characterization of non-coding genetic variants, which can influence gene expression and impact disease. Upregulation of the expression of fetal hemoglobin (HbF) has been shown to ameliorate symptoms in individuals suffering from sickle cell anemia and β-thalassemia.[9] Previous work in immortalized human erythroblast cells identified non-coding *cis*-regulatory elements that govern heritable changes in HbF expression and could potentially be used to upregulate HbF levels in patients suffering from hemoglobinopathies.[10] In the present study, Martin-Rufino et al.[5] combined Perturb(BE)-seq, fluorescence-activated cell sorting (FACS)-based functional approaches, and single-cell genotyping in HSPCs to identify variants in the HbF promoter that directly enhance HbF transcription in a therapeutically relevant context and with greater sensitivity. Specifically, the authors uncovered variants that upregulate HbF expression by either disrupting transcriptional repressor binding sites or creating *de novo* transcription factor (TF) binding sites. These findings reveal new avenues to therapeutically enhance HbF levels in patients without altering TF expression, which could otherwise have undesirable pleiotropic effects within a cell's transcriptional landscape (Figure 1).

By using Perturb(BE)-seq, Martin-Rufino et al.[5] also interrogated functional nucleotide variants of *GATA1*, a master transcriptional regulator of hematopoiesis. Mutations in this TF are associated with congenital anemias, thrombocytopenia, and myeloid malignancies. Pertub(BE)-seq performed on HSPCs under conditions of erythroid differentiation allowed Martin-Rufino et al.[5] to track the

impact of *GATA1* mutations at the level of single cells as they undergo hematopoiesis. This screen reliably recapitulated known pathogenic GATA1 mutations as well as deleterious mutations in conserved amino acid residues putatively involved in disease. Additionally, single-cell transcriptomic analyses allowed the authors to stratify mutation-causing sgRNAs based on their contribution to erythroid differentiation. Finally, Perturb(BE)-seq enabled profiling of the differential downstream erythropoietic gene regulation as a consequence of *GATA1* mutations. Collectively, these observations recapitulate GATA1's central role in orchestrating erythroid differentiation and serve as a roadmap for the interpretation of the function of gene mutations in base-editing screens (Figure 1).

VUSs represent a unique challenge arising from high-throughput sequencing of clinical samples. While deep mutational scanning approaches have enabled the interrogation of VUSs through the phenotypic assessment of variant DNA libraries, the necessity for exogenous transfection of the genomic elements being evaluated poses challenges when it comes to interpreting the function of variants.[11] HDR-based screening approaches, also known as saturation genome editing, have enabled accurate VUS characterization in their endogenous genomic context but are subject to limitations in editing efficiency and number of targetable genomic sites.[11,12] While less accurate than HDR-based screens, base-editing screens offer an easily scalable option to simultaneously query multiple clinically relevant variants throughout the genome.[12] In their work, Martin-Rufino et al.[5] showed that the functional variant data yielded by Perturb(BE)-seq in HSPCs undergoing erythropoietic differentiation can be utilized to assess the causal role of VUSs detected in patients. Specifically, Perturb(BE)-seq successfully identified a previously uncharacterized pathogenic variant of *GATA1* detected in a patient displaying erythroid hypoplasia and dyserythropoiesis (Figure 1).

In conclusion, through their work, Martin-Rufino et al.[5] provide a blueprint for the interrogation of functional variants in primary cells during hematopoiesis. The approach described by the authors improves upon previous functional base-editing screens by adding the use of single-cell genotyping and RNA-seq to assess diverse outcomes of base editing.[3,4] This allows the authors to flexibly assess the impact of nucleotide variants in the context of actively differentiating primary cells. In future applications, similar strategies could be extended not only to cell types from multiple tissues but also to patient-derived primary cells, unveiling complex variant effects specific to distinct cell types and genetic backgrounds, while also uncovering mutations that confer therapeutic benefit. Recent advances in genome editing and single-cell sequencing technologies are bound to contribute to the rapid evolution of Perturb(BE)-seq. For example, genome-editing technologies offering greater flexibility of editing outcomes, such as near-PAMless base editors, C-to-G and adenine transversion base editors, and prime editors, will greatly enhance the breadth of variants that can be interrogated.[1,13] Furthermore, coupling single-cell genotyping to transcriptomics will enable one-to-one correlations between editing outcomes and associated effects. These approaches will circumvent the limitations of previous strategies that rely on inferring editing outcomes based on sgRNA presence, greatly enhancing the confidence with which functional causalities are ascribed to mutations in base-editing screens.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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