APPLICATION NOTE

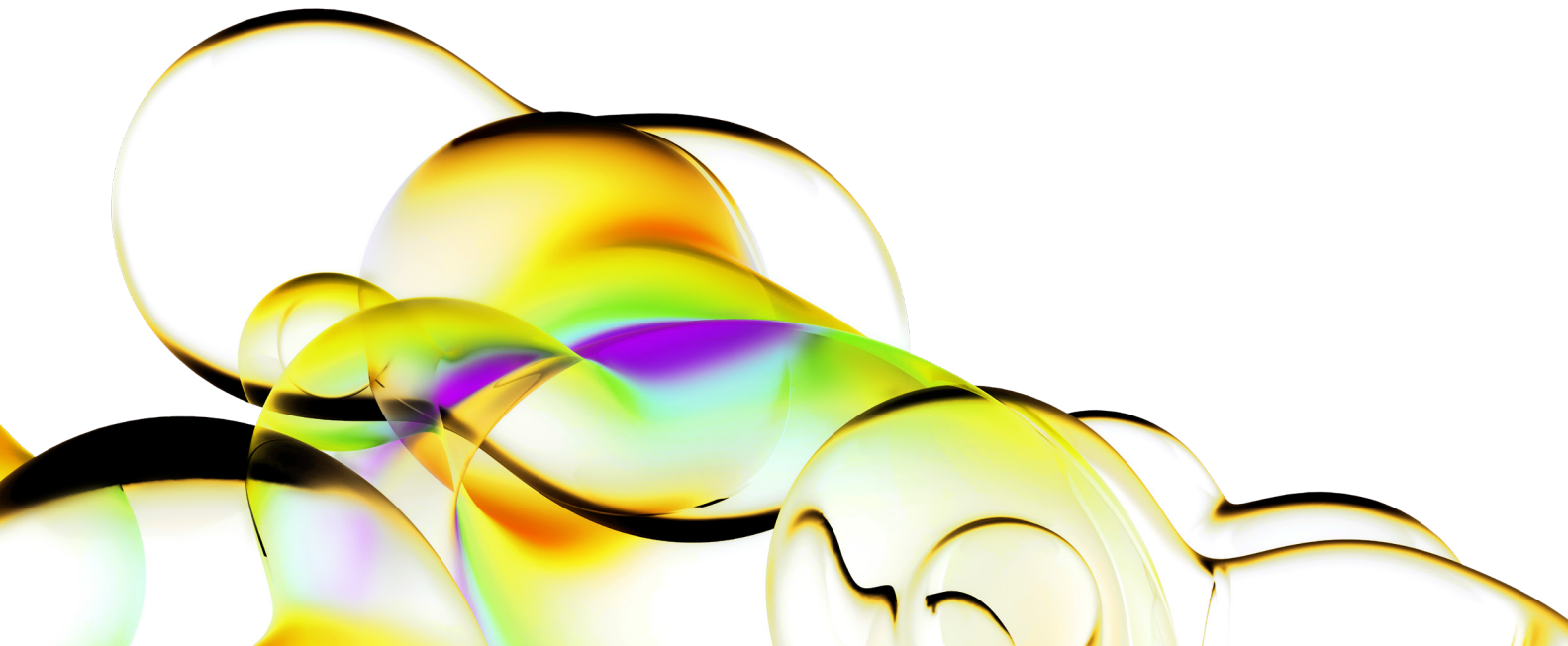
Guidance for using unmodified versus 5-methoxyuridine (5moU) modified mRNAs with chemically synthesized sgRNAs.

Introduction

Optimized stem and immune cell editing with the Pin-point™ base editing platform.

The use of CRISPR-Cas9 based genome editing technologies has transformed the way we engineer cell and gene therapies. These technologies have rapidly evolved, resulting in next-generation systems such as the aptamer-mediated Pin-point™ base editing platform[1]. The Pin-point platform consists of three components: a CRISPR-Cas enzyme that is recruited to a DNA target site, a deaminase fused to an aptamer binding protein, and a synthetic single guide RNA (sgRNA) with an appended aptameric scaffold that simultaneously recruits both the Cas enzyme and the deaminase to a target site of interest (Figure 1).

With a Pin-point configuration consisting of a Cas9 nickase (modified to cut just a single strand of DNA) and a Rat APOBEC cytidine deaminase, C:G base pairs can be efficiently converted to T:A bases. By precisely introducing a mutation to a splice donor or acceptor site, or to generate a premature stop codon, base editing becomes a powerful tool for generating gene knockouts while avoiding the occurrence of DNA double-strand breaks (DSBs). The use of technologies such as base editing that reduce the cytotoxic risks associated with DSB generation results in a much more favorable safety profile compared to gene editing with wild-type Cas9[2].



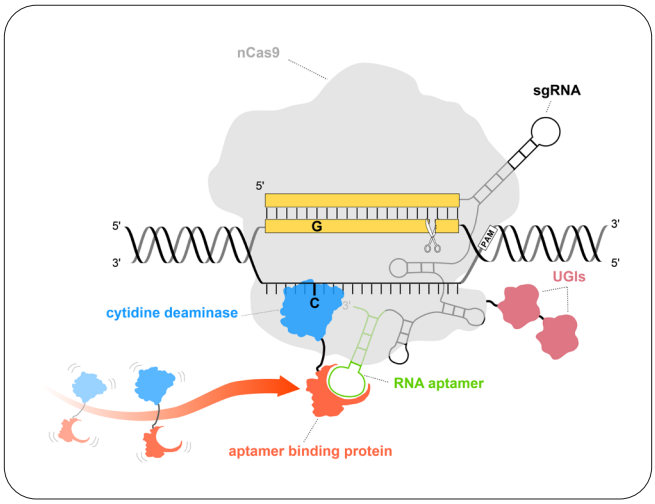


Figure 1: Illustration of the Pin-point base editing platform, shown with a nickase Cas9 and cytidine deaminase configuration.

To deliver the three Pin-point platform components into primary human cells for efficient base editing, the use of mRNA and synthetic sgRNA reagents is recommended (Figure 2). By comparison, the use of plasmid DNA requires entry into the nucleus for efficient transcription and subsequent translation of the Cas and deaminase components, while delivery of mRNA for these components results in immediate expression in the cytoplasm. Additionally, the use of DNA-free reagents allows for a more transient expression of the Cas, deaminase, and sgRNA in the cell compared to expressed plasmid DNA or viral based delivery. This enables high on-target editing to take place prior to the components being degraded, thus reducing the potenital of off-target activity and improving specificity as a result of reduced length of expression in the cell.[3]

However, several considerations must be made when delivering exogenous RNA into the cell. Foreign RNAs have been shown to induce inflammatory immune responses that can often differ in magnitude and impact in different cell types. To combat elicitation of this inflammatory immune response, different measures can be taken. One prominent approach is incorporating the use of chemical modifications. For *in vitro* transcribed mRNA, substitution of uridine triphosphates with 5-methoxyuridine (5moU) modified triphosphates has been shown to reduce the immune response and consequently increase activity of CRISPR-Cas based gene and base editors.[4,5] These modifications have also been shown to impact translation efficiency[6], potentially affecting efficacy and targeted use in different cell types. Additionally, incorporation of site-specific chemical modifications in the sgRNA for such systems has been demonstrated to affect activity likely through increased stability and enhanced protection from exonuclease activity.[7]

Here, we demonstate the use of unmodified and 5moU modified mRNAs for Pin-point nCas9 and Rat APOBEC mRNAs, paired with chemically synthesized Pin-point sgRNAs, to achieve optimal editing in primary human T cells, induced pluriopotent stem cells (iPSCs), and hematopoietic stem and progenitor cells (HSPCs).

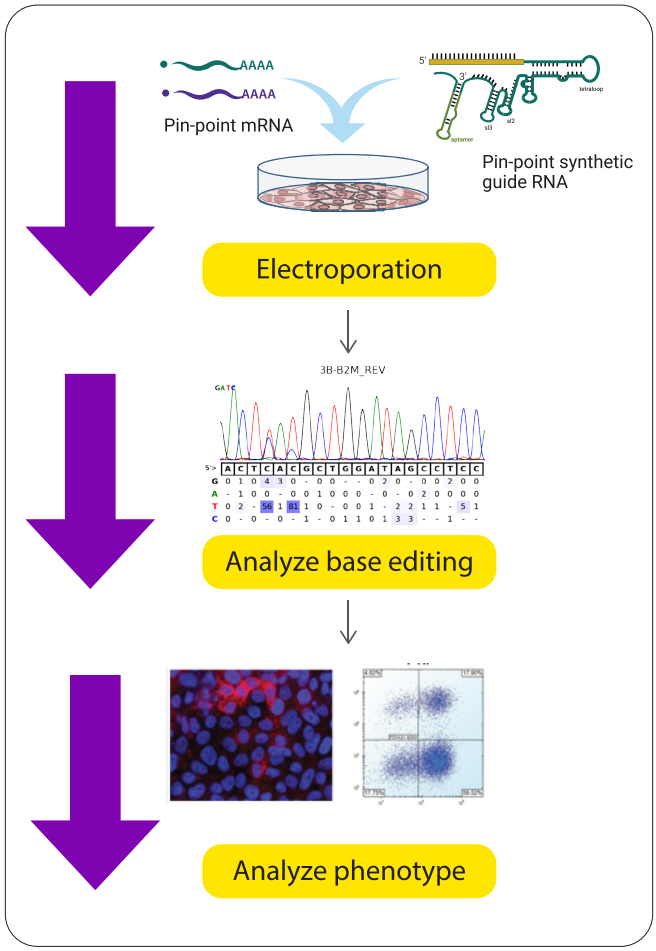


Figure 2: Delivery of Pin-point base editing platform components, including *in vitro* transcribed mRNAs and chemically synthesized sgRNAs into primary cells.

Results

Multiplex editing in primary human T cells

To demonstrate the ability of the Pin-point platform to edit multiple loci simultaneously in primary T cells, synthetic sgRNAs were designed to cause protein knockout through C to T conversion in four target genes: a chain of the major histocompatibility complex class 1 (MHC-1 chain), cluster of differentiation 52 (CD52), T cell receptor alpha constant (TRAC), and programmed cell death protein 1 (PCDC1). All four sgRNAs were simultaneously delivered along with either unmodified or 5moU modified Pin-point mRNAs. Resulting assessment of base editing (C to T conversion) at the DNA level, as well as functional protein knockout of all four targets: MHC-1 chain, CD52, PD1, and TCR, showed increased efficiency when using unmodified mRNAs. This suggested a recommended use of unmodified Pin-point mRNAs when delivering via electroporation in primary T cells for optimal base editing efficiency. However, total cell count following electroporation was reduced in samples electroporated with the unmodified mRNAs, indicating that 5moU modified mRNAs could be the preferred format in specific applications with cell yield as the driving influence (Figure 3).

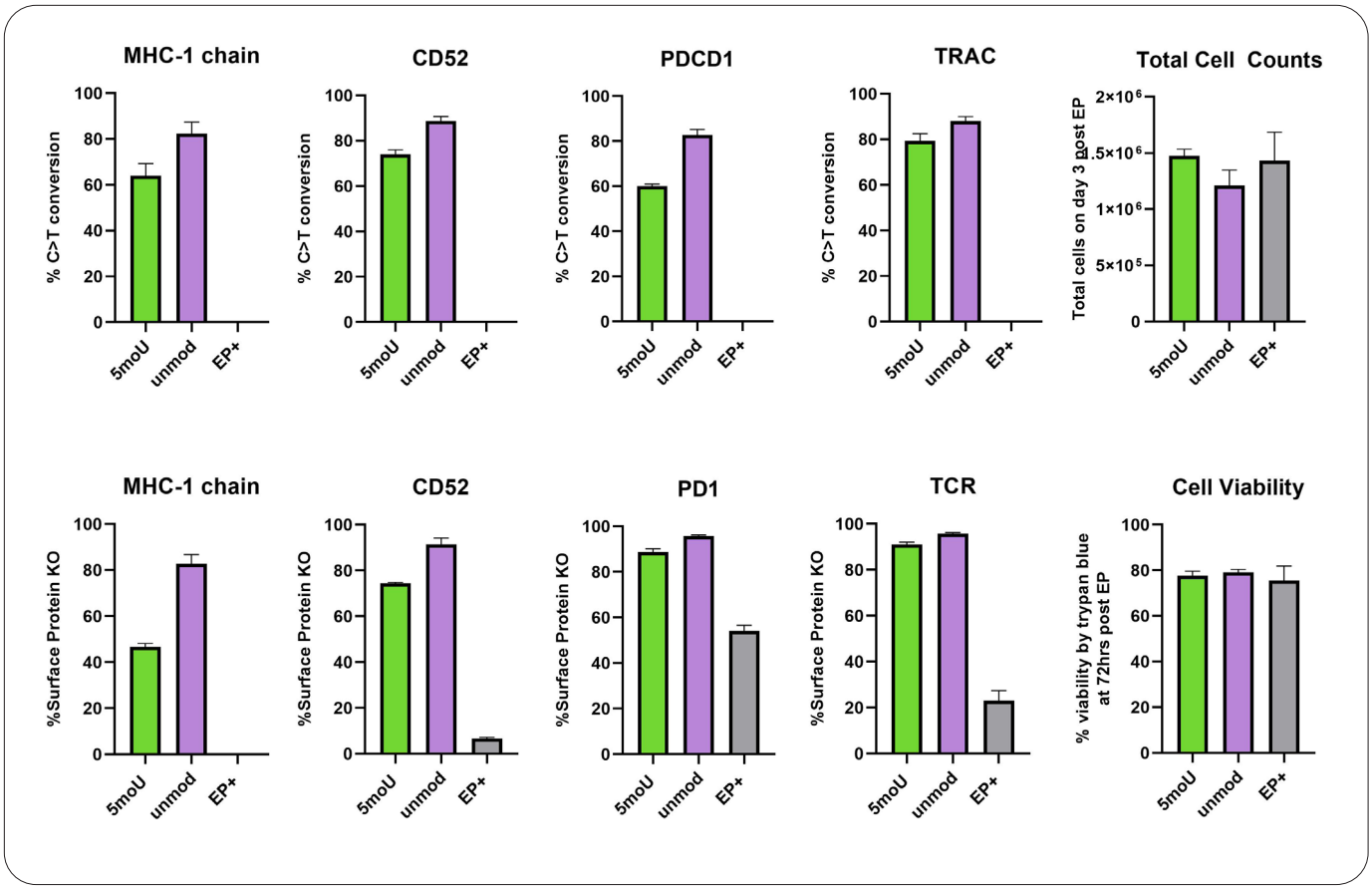


Figure 3: Multiplex base editing and assessment of viability in primary human T cells following delivery of Pin-point sgRNAs and either unmodified or 5moU modified Pin-point mRNAs. Electroporation only controls indicated as EP+.

Multiplex editing in iPSCs

Human iPSCs have the potential for broad application in cell therapy development due to their ability to differentiate into all somatic cell types. Using the same four sgRNAs targeting MHC-1 chain, CD52, PDCD1, and TRAC as for the T cell application, we further demonstrated efficient base editing in iPSCs. Here, the effect of using unmodified Pin-point mRNAs is much more striking with nearly double the level of base editing compared to the 5moU mRNAs.

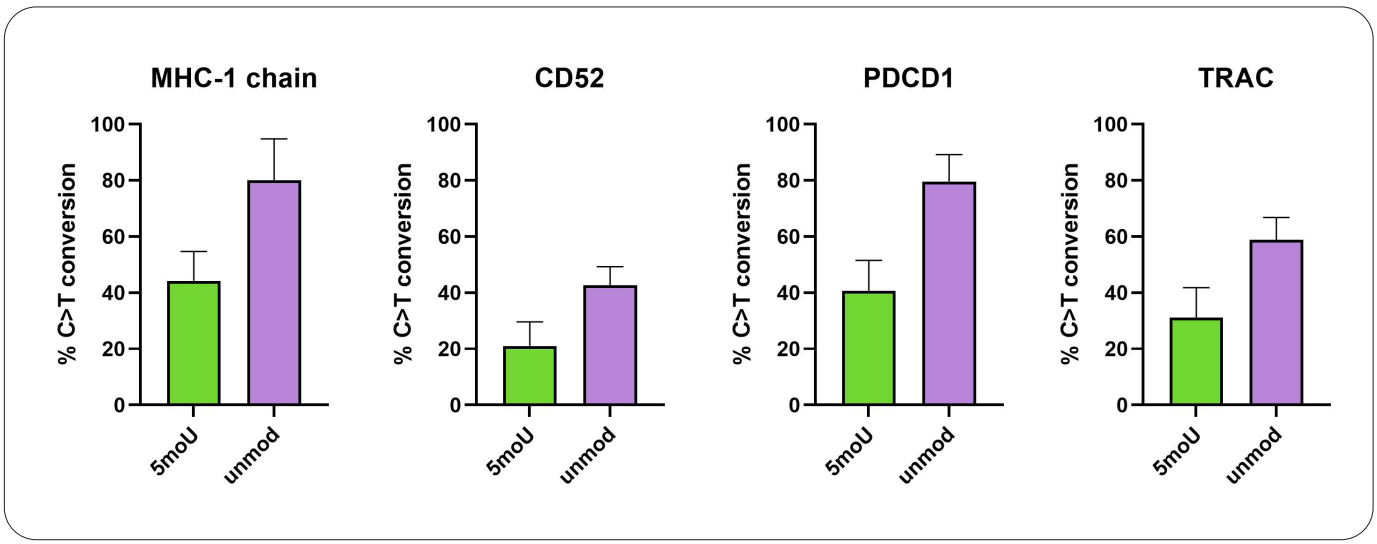


Figure 4: Multiplex base editing in iPSCs following delivery of Pin-point sgRNAs and either unmodified or 5moU modified Pin-point mRNAs.

Editing in HSPCs

HSPCs from healthy donors have been used as a stem cell therapy for many years, but the advent of gene editing technologies launches a myriad of new therapeutic opportunities. In autologous stem cell therapies a patient's own HSPCs can be engineered to express a healthy copy of the mutated gene and then transfused back into the patient to offer a life-long cure to diseases previously considered untreatable. Here we demonstrate efficient base editing of MHC-1 chain as well as two therapeutically relevant targets while maintaining high cell viability and yield. In HSPCs the use of 5moU modified Pin-point mRNAs results in double the editing efficiency compared to unmodified mRNAs across all 3 targets, as well as offering improved cell viability and yield.

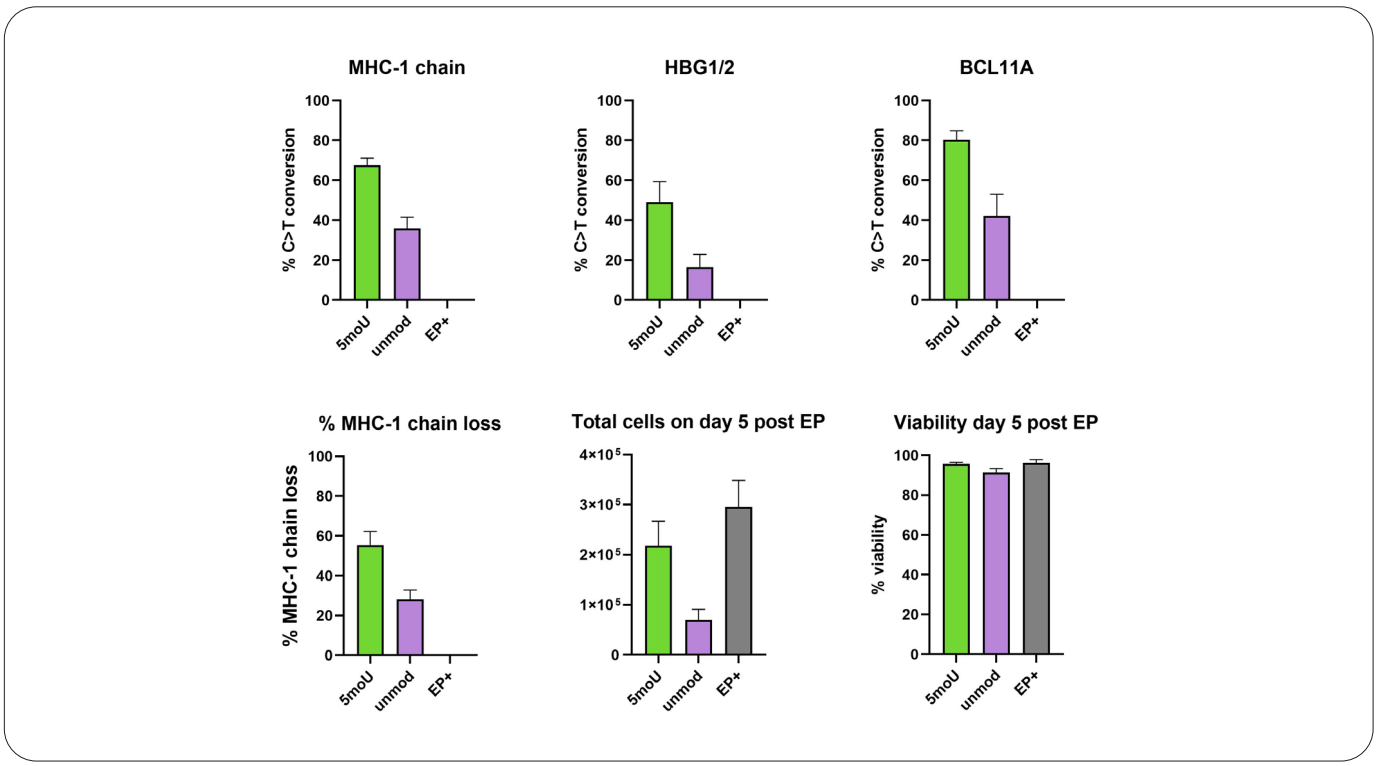


Figure 5: Base editing and assessment of viability in HSPCs following delivery of Pin-point sgRNAs and either unmodified or 5moU modified Pin-point mRNAs

Conclusion

Delivery of chemically synthesized Pin-point sgRNAs and *in vitro* transcribed Pin-point mRNAs results in highly efficient base editing in primary human immune and progenitor cells when the most suitable mRNA modification is selected. Here, we demonstrate specific use cases of selecting mRNA that is either unmodified or fully substituted with 5moU modifications when working with T cells, iPSCs, and HSPCs. Due to the specialized nature and independent immune responses elicited in different primary human cell types, it is important that each use case and additional cell type is experimentally validated to ensure optimal efficiency.

Materials and methods

Primary human T cell isolation and culture and electroporation

Primary human T cells were cultured and electroporated as reported in Porreca et al[2]. Briefly, CD3+ T cells were isolated from fresh whole peripheral blood (CPD Blood bags, Cambridge Bioscience). T cells were isolated by immunomagnetic negative selection using the EasySep™ Human T Cell Isolation Kit (STEMCELL Technologies). T cells were cultured in Immunocult™-XF T cell expansion medium (STEMCELL Technologies), supplemented with Penicillin- Streptomycin (Gibco) and IL-2 (100 IU/mL; STEMCELL Technologies), and activated with DynaBeads™ Human T-Activator CD3/28 (Gibco) at a 1:1 bead to cell ratio for 48 hours prior to electroporation. After removing DynaBeads particles from culture medium, the activated T cells were counted, pelleted by centrifugation, and resuspended in the total volume of R buffer needed for all conditions. Activated T cells were electroporated with sgRNA at 2 µM and 1.6 µg of of Pin-point nCas9-UGI-UGI and 0.2 µg of Pin-point rApobec1 (Horizon Discovery, a subsidiary of Revvity Inc, and TriLink Biotechnologies®) using the Neon™ Electroporation System (Invitrogen). Post-electroporation, T cells were cultured in antibiotic free ImmunoCult™-XV T cell expansion medium (STEMCELL Technologies) supplemented with IL-2 (100 IU/ml; STEMCELL Technologies), IL-7 (100 IU/ml; Peprotech, New Jersey, USA) and IL-15 (100 IU/ml; Peprotech, New Jersey, USA) and incubated at 37 °C, 5% CO2 for 3-7 days.

iPSC culture and electroporation

WTC-11 (Corriell Institute, www.AJSC.us/ISSN:2160-4150/AJSC1304002) and NH50191 (NINDS Repository) lines were cultured on Geltrex® basement membrane matrix (ThermoFisher). The Gibco iPSC line (A18945, doi:10.1371/journal.pone.0018293) was cultured on Vitronectin XF (STEMCELL Technologies). All lines were maintained in mTeSR™-PLUS medium (STEMCELL Technologies) Y-27632 (STEMCELL Technologies). Electroporation was performed with the Amaxa™ 4D-Nucleofector (Lonza) and in either 20 µL or 100 µL Amaxa NucleoCuvette® Cartridges (Lonza) at a density of 2e5 or 1e6 cells per cuvette, respectively. Pelleted iPSCs were resuspended in P3 Primary Cell Nucleofector® Solution (Lonza), and electroporated with 40 pmol sgRNA and 2.56 µg Pin-point nCas9-UGI-UGI and 0.74 µg of Pin-point rApobec1 mRNAs (Agilent, Horizon Discovery, a subsidiary of Revvity Inc, and TriLink Biotechnologies®) using the Amaxa program CM138. After electroporation, cells were recovered in mTeSR™-PLUS medium (STEMCELL Technologies) and Y-27632 (STEMCELL Technologies).

HSPC culture and electroporation

CD34+ cells (Hemacare, #M34C-GCSF-1) were cultured overnight in StemSpan SFEM II medium (Stemcell technologies #09655) supplemented with StemSpan CD34+ expansion supplement (Stemcell Technologies #02691). Pelleted cells were resuspended in buffer R and mixed with 2.79 µg Pin-point nCas9-UGI-UGI mRNA, 1.95 µg Pin-point rApobec1 mRNA, and 6.25 µM sgRNA, then electroporated using the Neon™ Electroporation System (Invitrogen), 50’000 cells per 10 uL electroporation, 1400V, 10ms, 3 pulses. After a further overnight incubation in StemSpan SFEM II medium supplemented with StemSpan CD34+ expansion supplement, medium was replaced with StemSpan SFEM II medium supplemented with human AB serum (3%, Sigma #H4522) and StemSpan Erythroid Expansion Supplement (Stemcell Technologies #02692). Editing efficiency, cell viability and yield were assessed 5 days after electroporation.

Cell lysis, PCR, and base editing analysis

For genomic DNA preparations, cells were resuspended (T cells) or lysed (iPSCs and HSPCs) in 40 µL of lysis buffer (direct PCR lysis reagent; Viagen #732-3260) and incubated at 55 °C for 30 minutes, followed by 95°C for an additional 30 minutes. Lysates were used to generate PCR amplicons spanning the region containing the base editing site(s). PCR amplicons between 400-1000 bp in length were generated and sequenced by Sanger sequencing. Base editing efficiencies were calculated from Sanger sequencing reads and displayed as % C to T editing, using the Chimera™ analysis tool, an adaptation of the open-source tool BEAT. Chimera first determines the background noise to define the expected variability in a sample, using a geometric mean with outliers capped to the median value. Following this, Chimera subtracts the background noise to determine the editing efficiency of the base editor over the span of the input guide sequence.

T cell flow cytometry

Prior to flow cytometric analysis, T cells were stimulated to induce expression of PD-1 (PDCD1). T cells were split into two groups, stimulated and unstimulated. T cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich) and ionomycin (250 ng/mL; Millipore) in the presence of IL-2 (100 IU/mL; STEMCELL Technologies) for 48 h prior flow cytometry analysis to induce the expression of PD-1, while unstimulated cells were treated with an equal volume of non-stimulating media containing IL-2 (100 IU/mL; STEMCELL Technologies). After 48 h, T cells were stained with the following fluorophore conjugated anti-human antibodies: TCR alpha/beta-BV785 (BioLegend #306742), MHC-1 chain (BioLegend # 316304), CD52-PE (BD Biosciences #562945), and PD1-APC (BioLegend #329908). Fluorescence minus one (FMO) controls were included for accurate gating strategy. DAPI was used to stain for live cells. Cells were acquired on an IntelliCyte® IQue PLUS or Sartorius iQue3 flow cytometer using iQue ForeCyt® Enterprise Client Edition 9.0 (R3) Software. Single, live, cells negative for each specific marker were gated. The level of PD-1 knockout was evaluated in cells stimulated with PMA/ionomycin, while the levels of TRAC, MHC-1 chain and CD52 was evaluated in non-stimulated cells. T cells were stained with DAPI to measure percent cell viability and live cell counts at 72 h post electroporation.

HSPC flow cytometry and viability

From each well, 50 µL cell suspension was collected and stained with antibody conjugated with APC (Biolegend #316312) and DAPI to assess viability. Cells were acquired on an IntelliCyte IQue PLUS or Sartorius iQue3 flow cytometer using iQue ForeCyt Enterprise Client Edition 9.0 (R3) Software. Single live cells were gated and enumerated. Viable cell concentration was calculated by acquiring the entire sample and dividing by 50 µL. Total cells was calculated by multiplying the viable cell concentration by the volume. For percentage MHC-1 chain loss, MHC-1 chain negative cells were gated from the single live population.

References

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