Achieving CAR-T cell generation in a single intervention using a multiplex base editing system: harnessing the modularity of the Pin-point™ base editing platform.

Robert Blassberg, Bronwyn Joubert, Jennifer Harbottle, Olga Mielczarek, Jesse Stombaugh, Lucinda Lee, Pablo Perez-Duran, John Lambourne, Kevin Hemphill, Žaklina Strezoska, Anja van Brabant Smith, Immacolata Porreca
Email address: baseediting@horizondiscovery.com

Abstract

The negative consequences of multiplex gene editing using traditional CRISPR-Cas and the advantages offered by base editing are well described in the literature. However, even multi-gene knock-out does not provide the ultimate cell or therapy in a single intervention. Herein we describe the exploitation of the aptamer-based recruitment mechanism of our Pin-point base editing platform to achieve simultaneous multi-gene knockout and CAR insertion to create CAR-T cells in a single reaction. We also show the advantage of this platform in iPSCs, which are known to be sensitive to gene editing.

The Pin-point base editing system is a modular technology where the CRIPSR-Cas and the deaminase modules are delivered to the target cells as individual components. The assembly of the base editing machinery at the target locus relies on the interaction between an aptamer binding protein fused to the deaminase and an RNA aptamer located on the gRNA. The modularity and aptamer-dependent nature of the Pin-point system allows for high flexibility in the customization of each individual component to address specific editing needs and enables complex genetic modifications. Additionally, by swapping aptamers and their complementary aptamer binding proteins, different effector molecules could be specifically directed to different genomic loci allowing a suite of independent operations while utilising a single Cas module and avoiding gRNA crosstalk. And by removing the aptamer and therefore the deaminase recruitment, the CRISPR-Cas component can be used to allow for gene insertion at the DNA target site. This unique modularity provides unparalleled editing flexibility and significantly increases the complexity of simultaneous edits that are achievable. By using the same CRISPR-Cas, it also minimizes the size of the payload that needs to be delivered.

Here we provide an example of one configuration of the Pin-point technology for the streamlined generation of allogeneic CAR-T cells and hypoimmunogenic iPSCs. By delivering Rat APOBEC1 fused to the MS2 coat protein (MCP), SpCas9 nickase and gene specific gRNAs containing the bacteriophage MS2 RNA aptamer, we achieve high knockout efficiency and editing purity at multiple sites simultaneously. At the same time, aptamer-less gRNAs were used to introduce targeted transgene knock-in in both human primary T cells and iPSCs. Site-specific knock-in and multiplex gene knockout are achieved within a single intervention and without the requirement for additional sequence-targeting enzymes for the streamlined generation of allogeneic cell models.

We also report examples of reconfiguration of the Pin-point system with alternative Cas enzymes.

1 Pin-point base editing platform

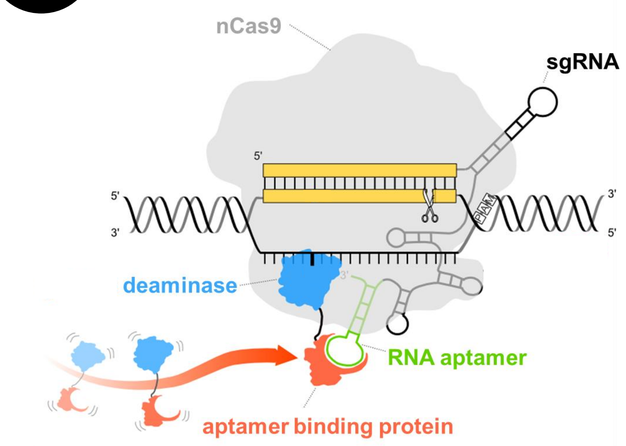

Figure 1: Schematic of the modular Pin-point base editor system*. A Cas nickase (e.g.nCas9) is recruited to the DNA target site via a single guide RNA (sgRNA) encoding an aptamer in the scaffold region. The aptamer recruits a DNA-modifying deaminase to the DNA target site via an aptamer binding protein. The three independent components of the system can be configured according to editing requirements and delivered to cells either as mRNA and synthetic sgRNA or packaged in viral particles.*

2 Efficient multi gene editing in T cells and iPSC

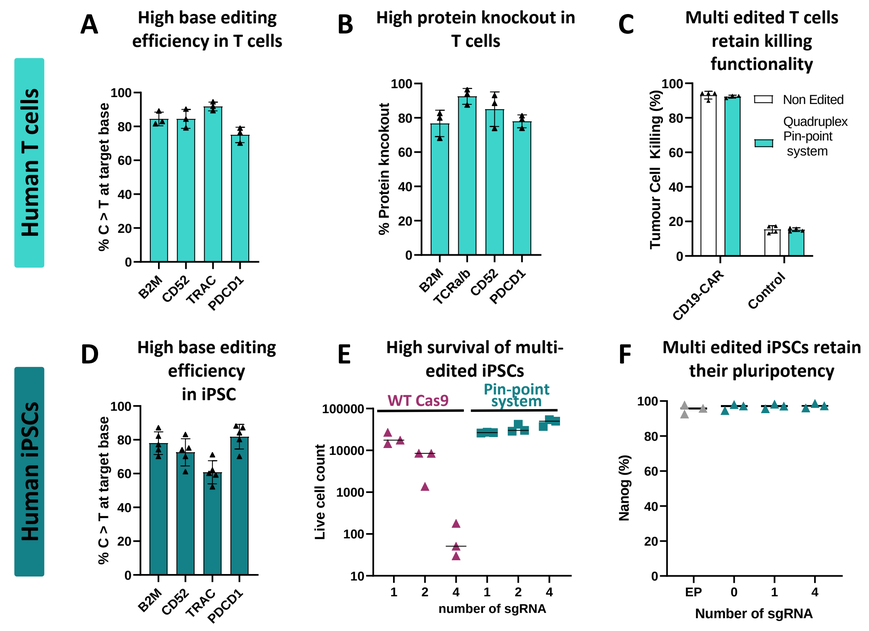

Figure 2: Pin-point platform facilitates robust, high efficiency multi-gene editing in human T cells and iPSCs *A) Four genes are edited simultaneously in T cells after delivery of Pin-point mRNAs (nSpCas9 and rAPOBEC1) and synthetic aptameric sgRNAs. Percentages of C to T conversion are reported. B) Protein knockout of 4 targets analysed by flow cytometry. C) In vitro tumour cell killing assay performed with either empty (control) or CD19-CAR expressing lentiviral vector transduced cells and mock electroporated (non edited) or Pin-point edited T cells (Pin-point Quadruplex). n = 3 T cell donors for A-B, n = 2 T cell donors for C. D) Four genes are edited simultaneously in iPSCs after delivery of Pin-point mRNAs (nSpCas9 and rAPOBEC1) and synthetic aptameric sgRNAs. Percentages of C to T conversion are reported. E) Viability of iPSCs edited with single or multiple sgRNAs using the Pin-point system or Cas9 analysed by flow cytometry 48hrs post electroporation. Viability was normalized to a no sgRNA control. C) Expression of the pluripotency marker Nanog in cells edited with the Pin-point base editing system analysed by flow cytometry 4 days post electroporation. Data shown in D, E, F are from two independent experiments with 2 iPSC lines.*

3 Enhanced safety of multiplex editing

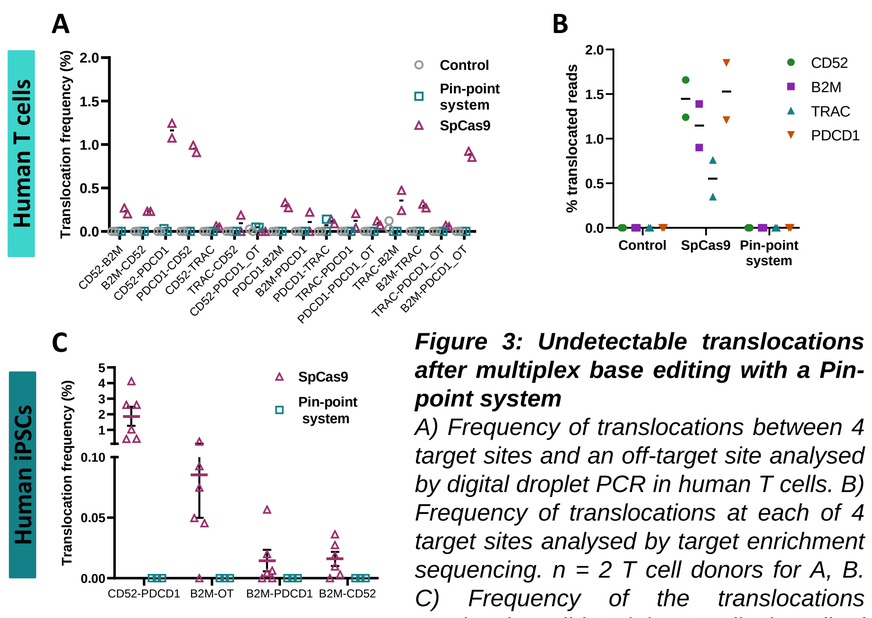

Figure 3: Undetectable translocations after multiplex base editing with a Pin-point system

A) Frequency of translocations between 4 target sites and an off-target site analysed by digital droplet PCR in human T cells. B) Frequency of translocations at each of 4 target sites analysed by target enrichment sequencing. n = 2 T cell donors for A, B. C) Frequency of the translocations previously validated in T cells in edited iPSCs analysed by digital droplet PCR. n = 2 iPSC lines.

4 A solution for complex engineering: simultaneous knock-in and multiple knockout

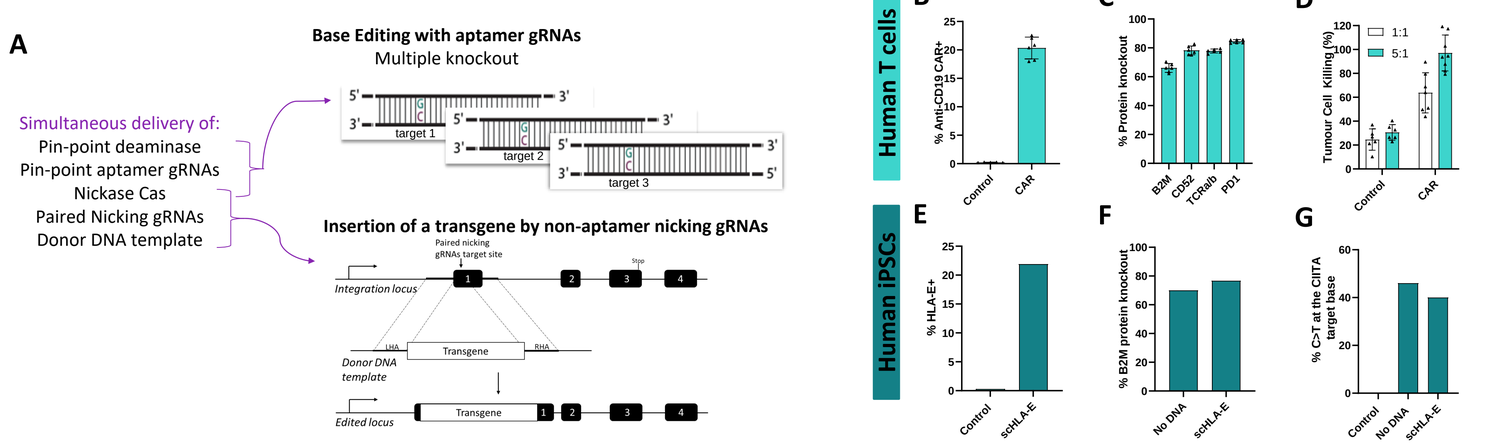

Figure 4: Pin-point system enables site-specific integration and base editing at target sites in one step in T cells and iPSCs. *A) Schematic of the simultaneous knock-in and knock-out strategy: by co-delivering rAPOBEC1-MCP, nSpCas9, Pin-point gRNAs containing the MS2 RNA aptamer, a pair of aptamer less gRNAs targeting the integration site and a homology-directed donor DNA, base conversion and high knockout efficiency at the sites where the deaminase is recruited and simultaneously targeted transgene knock-in at the integration site are achieved. B-D) CD19-CAR delivered via AAV was integrated in the TRAC locus and simultaneously B2M, CD52 and PD1 were knocked-out by base editing in T cells. B) Percentage of CAR positive T cells in the edited population. C) Protein knockout of 4 targets in T cells. D) In vitro tumour cell killing assay performed with edited CAR-T cells. n = 2 T cell donors. E-G) HLA-E delivered via plasmid was integrated in the B2M locus and simultaneously CIITA was knocked-out by base editing in iPSCs. E) Percentage of HLA-E positive iPSCs in the edited population. F) B2M knockout in the edited population. G) Percentage of C to T editing at the CIITA locus.*

5 Compatibility with numerous nucleases and deaminase

	Type II			Type V						
	A	B	C	D	E	F	G	H	I	J
Enzyme activity	nickase	nickase	nickase	deactivated	deactivated	deactivated	deactivated	deactivated	deactivated	deactivated
Demonstrated nuclease activity in mammalian cells	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Demonstrated with the Pin-point system	✓	✓	In progress	✓	In progress	✓	In progress	✓	✓	In progress
sgRNA optimized	✓	✓		In progress		✓		✓	✓	
Confirmed at multiple targets (2+)	✓	✓				✓		✓	✓	
Demonstrated in multiple cell types (2+)	✓	In progress				✓		✓		
Demonstrated with multiple deaminases (2+)	✓							✓	✓	

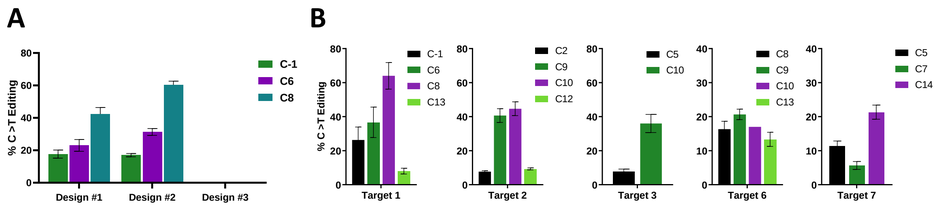

Figure 5: Evaluation of a smaller nickase Type II enzyme with the Pin-point platform. *A) Three different gRNA designs have been tested with the alternative enzyme at one target site in HEK293T cells by plasmid delivery of the Pin-point system components. Sanger sequencing analysis shows activity of the alternative nickase Type II enzyme with 2 of the 3 gRNA designs. B) The activity of the alternative Type II enzyme was validated at multiple target sites in HEK293T cells. This configuration of the Pin-point system allows to target Cs outside the standard editing window with the spCas9 configuration (i.e. C10-C14).*

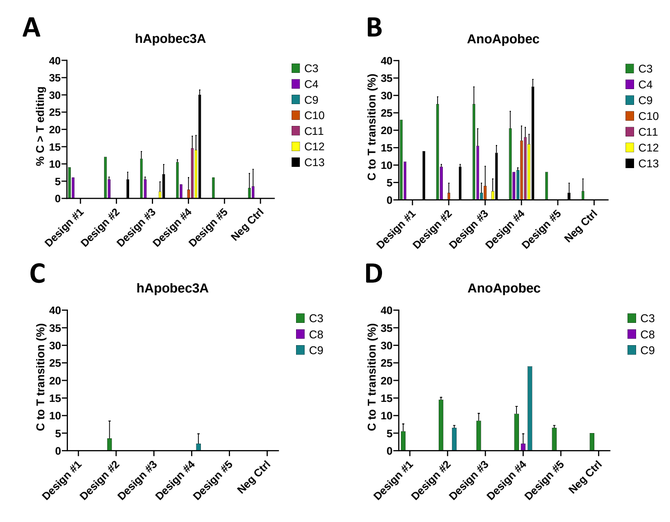

Figure 6: The Pin-point platform configured with the compact dead Type V effector protein CasMINI. *Five different gRNA designs have been tested with the Type V enzyme with two different cytidine deaminases in HEK293T cells by plasmid delivery of the Pin-point system components. Sanger sequencing analysis of editing activity on two sites, VEGFA-1 (A, B) and VEGFA-2 (C, D) shows good levels of editing with specific gRNA designs. Higher activity across all the designs and the two sites is observed with the AnoApobec deaminase.*

Conclusions

- **We applied multiplex base editing using the Pin-point system to the development of engineered CAR-T cells and hypoimmunogenic iPSCs.**
- **The Pin-point base editing system achieved greater than 70% knockout efficiency without any selection or enrichment and high purity at therapeutically relevant target sites in T cells and iPSCs.**
- **Multiplex gene editing with the Pin-point platform substantially reduces chromosomal translocations compared to Cas9-mediated knockout.**
- **The Pin-point platform enables simultaneous site-specific transgene knock-in and multi-gene knockout by base edit in T cells and iPSCs.**
- **We observed greatly improved viability of iPSCs following multi-gene editing with the Pin-point system compared to Cas9.**

References

- Collantes et al. The CRISPR Journal. Feb 2021.58–68
- Porreca et al. bioRxiv 2023.06.20.545315
- dCasMINI-app-note (horizondiscovery.com/en/gene-editing/pin-point-base-editing-platform)
- base-editing-modularity-app-note (horizondiscovery.com/en/gene-editing/pin-point-base-editing-platform)