Cas-CLOVER™: A High-Fidelity Genome Editing System for Safe and Efficient Modification of Cells for Immunotherapy

Xianghong Li, Xinxin Wang, Min Tong, Yening Tan, Julian D. Down, Devon J. Shedlock, Eric M. Ostertag

Poseida Therapeutics, Inc. 4242 Campus Point Court, Suite 700, San Diego, CA, 92121

POSEIDA THERAPEUTICS

Abstract

The discovery of the CRISPR/Cas9 system has created a new era for genome editing. While it is a simple, versatile and precise method among many current gene editing tools, its clinical application has been hampered by high off-target activity, which may result in unwanted and potentially detrimental mutations in the genome of edited cells. We developed a novel fully dimeric Clo51-dCas9 genome editing technology (Cas-CLOVER™) to surmount these concerns, as well as to deliver efficient and high-fidelity gene editing of human cells that can be safely used for adoptive immunotherapies in the clinic.

As opposed to the use of a single guide RNA (gRNA) for sequence-specific guidance of CRISPR/Cas9 binding and cutting, the Cas-CLOVER™ system utilizes a dual gRNA-guided nuclease, in which each half-site subunit of the enzyme contains a fusion protein of a catalytically inactive Cas9 (dCas9) and the Type IIS restriction endonuclease, Clo51. Like FokI that has been extensively used in the applications of TALEN and zinc figure nucleases (ZFNs), Clo51 activity is contingent upon formation of a dimer, and thus DNA cleavage is strictly dependent on the simultaneous on-target binding of two distinct gRNA-guided endonucleases within a specific proximity. While high cutting efficiency by the enzyme was observed when both half-site gRNAs are co-delivered to cells, no on-target disruption was observed in primary human T cells when either half-site gRNA was delivered individually. In addition, direct comparison of wild-type (WT) CRISPR/Cas9 and Cas-CLOVER™ in T cells show that, at the same gene locus, both systems edit the genome with high efficiency.

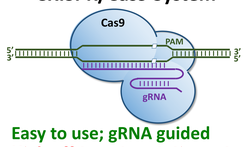
We tested the efficiency of the Cas-CLOVER™ system by targeting several key cell surface markers in primary human T cells known to be critical in mediating cellular graft response. We designed a number of gRNA pairs specific for the human T cell receptor α chain (TCRα), β chain (TCRβ) and β-2 microglobulin (β2M), as well as several surface inhibitory receptors. Editing efficiencies were measured in both activated and resting T cells, the latter presenting the greatest challenge to other gene editing systems, such as TALEN, presumably due to more compact chromatin. The Cas-CLOVER™ system showed high gene disruption efficiencies in resting T cells, with 84% knockout efficiencies for TCRα, 91% for TCRβ, 62% for β2M, and 40-60% for the surface inhibitory receptors PD-1, CTLA-4, Tim3, Lag-3, and TGFBRII.

Next, to determine the safety of Cas-CLOVER™ gene editing, possible off-target effects were interrogated in gene-edited T cells using next-generation target deep sequencing (NGS). TCRα, β2M or PD-1 gene-edited T cells, using multiple Cas-CLOVER™ guide RNA pairs for each target, were DNA sequenced at the top predicted off-target sites. No off-target editing above background was observed among all predicted off-target sites that were sequenced. We further showed that these editing efficiencies were maintained over a wide range of gap distances between the DNA-binding sites of the two guide RNA pairs, as evidenced by gRNAs targeting both indicator plasmids and the TCRα loci in the genome of the Jurkat T cell line.

In conclusion, the Cas-CLOVER™ system represents a novel advanced gene editing system exhibiting high specificity and efficiency in both proliferating and resting T cells, thereby overcoming significant limitations of the CRISPR/Cas9 and TALEN systems. The capability of editing genomic DNA with higher fidelity provides greater safety for gene modification in various clinical applications. Furthermore, the ability to edit resting T cells may prove crucial for the creation of allogeneic products with desirable phenotypes as activating the T cells can cause differentiation and loss of efficacy.

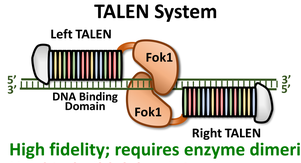
Introduction

CRISPR/Cas9 System



- Easy to use; gRNA guided
- High off-target mutation rate

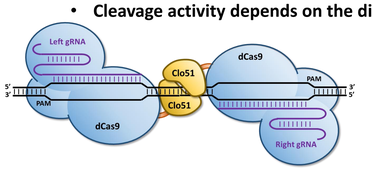
TALEN System



- High fidelity; requires enzyme dimerization
- Hard to build: labor intensive

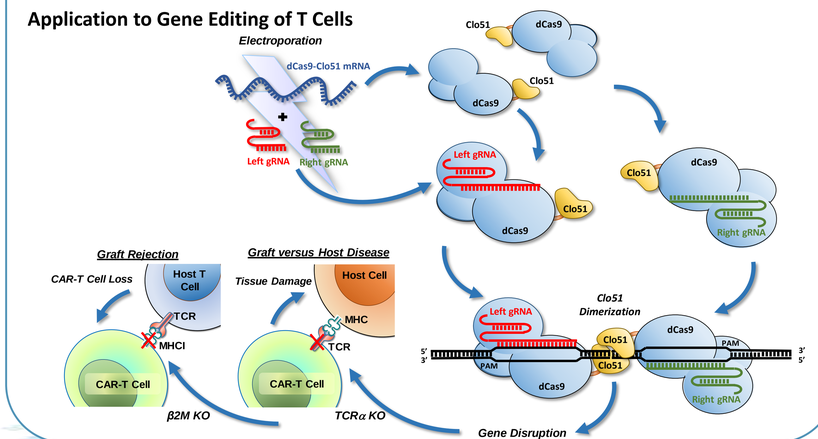
Cas-CLOVER™ Gene Editing System

- dCas9 is mutated and unable to cut DNA; serves as a DNA binding protein only
- Cleavage activity depends on the dimerization of an "obligate dimer" of Clo51



- Ease of use; double gRNA-guided
- Highly efficient; dCas9-guided RNA recognition
- High fidelity; cuts only when Clo51 nuclease dimerizes

Application to Gene Editing of T Cells



Methods & Results

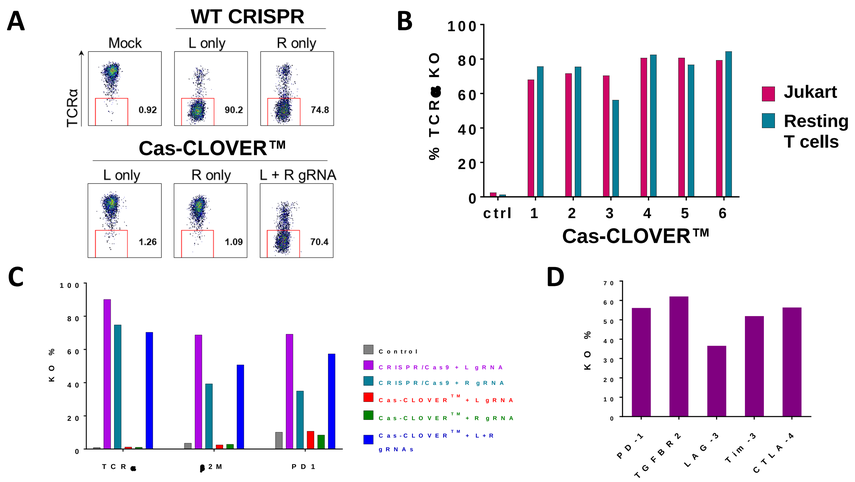


Figure 1: Cas-CLOVER™ gene editing is highly restricted and only functions when the paired gRNAs co-exist with comparable gene editing efficiency in resting T cells to WT CRISPR.

(A) Side-by-side comparison of WT CRISPR and Cas-CLOVER™ disruption of surface TCR expression in resting human T cells as assessed by flow cytometry using single or paired left (L) + right (R) TCRα-specific gRNAs. In contrast to WT CRISPR, Cas-CLOVER™ requires on-site targeting of both L and R of the paired gRNAs. **(B)** Comparable disruption of TCR surface expression in proliferating Jurkat T cells (magenta bars) and non-dividing resting T cells (aqua bars) by Cas-CLOVER™ targeting of TCRα and measured by FACS. **(C)** Comparison of WT CRISPR and Cas-CLOVER™ editing efficiency of TCRα, β2M and PD1 targets with single gRNA or paired gRNAs. **(D)** Knockout efficiency of inhibitory targets in resting human T cells.

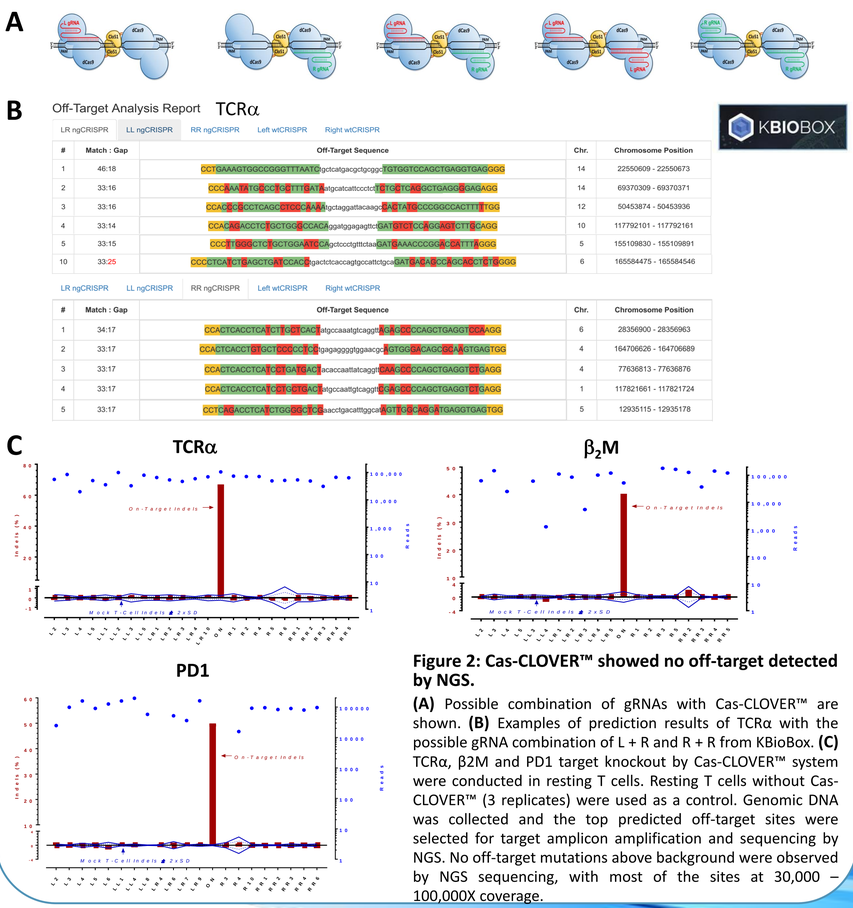


Figure 2: Cas-CLOVER™ showed no off-target detected by NGS.

(A) Possible combination of gRNAs with Cas-CLOVER™ are shown. **(B)** Examples of prediction results of TCRα with the possible gRNA combination of L + R and R + R from KBioBox. **(C)** TCRα, β2M and PD1 target knockout by Cas-CLOVER™ system were conducted in resting T cells. Resting T cells without Cas-CLOVER™ (3 replicates) were used as a control. Genomic DNA was collected and the top predicted off-target sites were selected for target amplicon amplification and sequencing by NGS. No off-target mutations above background were observed by NGS sequencing, with most of the sites at 30,000 – 100,000X coverage.

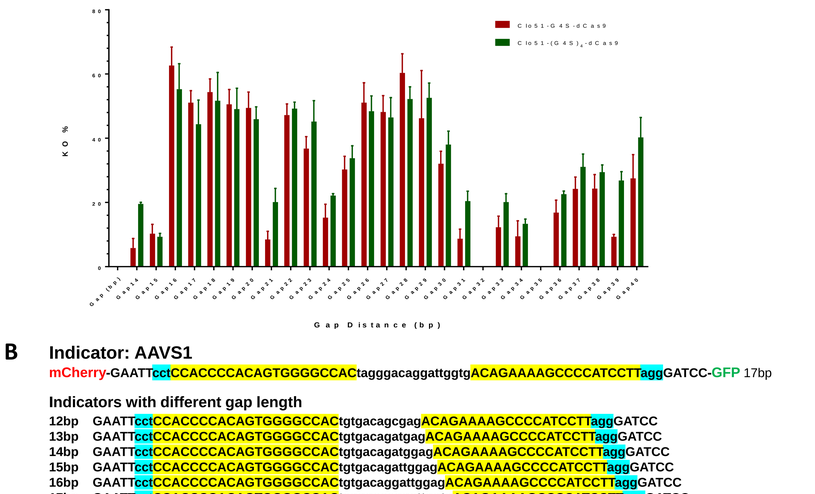


Figure 3: Cas-CLOVER™ works over a range of spacer length.

(A) gRNA pairs designed with different spacer lengths in a human T cell surface marker target gene were used to test Cas-CLOVER™ in Jurkat cells. The cell surface marker was stained and flow cytometry was used to determine relative Cas-CLOVER™ activity with varied spacer lengths (bps) between L and R gRNAs. These data indicated that Cas-CLOVER™ is highly restricted by spacer length, in which optimal activity occurred in a specific range. **(B)** Design for a cutting indicator assay targeting the AAVS1 locus in the human genome. A stop codon is included in the spacer; gene editing events may cause a frame shift and gives the mChe-GFP fusion protein expression. **(C)** The indicator assay performed in HEK293 cells was used to determine relative Cas-CLOVER™ activity upon varied spacer lengths (bps) between L and R gRNAs specific for a single target gene. These data indicated that Cas-CLOVER™ is highly restricted by spacer length, in which optimal activity occurred in a specific range.

Conclusions

- Cas-CLOVER™ is an advanced gene editing system exhibiting high efficiency cutting and indel formation in primary T cells
- Cas-CLOVER™ nuclease activity is strictly controlled by on-site target binding of both gRNAs within a specific spacer length and no off-target editing was detected by deep sequencing analysis
- Cas-CLOVER™ has high activities in both rapidly proliferating and non-dividing resting T cells
- Cas-CLOVER™ was highly efficient at disrupting genes which are critical for alloreactivity in resting T cells
- In combination with the piggyBac™ gene delivery system, Cas-CLOVER™ gene editing has great promise for the development of non-viral allogeneic CAR-T cell therapy

1. **This poster, among others describing Poseida CAR-T products and technologies, is available at Poseida.com/publications/**
2. **Multiple positions are currently available and listed at Poseida.com/Career/**