• A Case Study •

Long hybrid ssDNA HDR templates enable high yield non-viral cell therapy manufacturing

In collaboration with Brian Shy | Marson Laboratory,
Gladstone-UCSF Institute of Genomic Immunology

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Highlight:

Single-stranded DNA (ssDNA or ssODN) is proven to be a reliable CRISPR homology directed repair (HDR) template for creating gene knock-in with high editing efficiency, minimal toxicity, and reduced off-target integration.

The newly designed hybrid ssDNA HDR templates (HDRTs) that incorporate Cas9 target sequences (ssCTS) enabled high knock-in efficiencies (>60%) across a variety of target loci, knock-in constructs, and primary human cell types.

At a clinical scale for non-viral CAR-T cell manufacturing, ssDNA HDRTs enabled high knock-in efficiencies of ~46%.

Introduction:

The CRISPR/Cas9 genome editing toolkit has been widely used and adapted for diverse applications due to its simplicity and versatility. Its competence to offer precise and efficient genome editing makes it a powerful research tool for both knocking out endogenous TCR and inserting functional chimeric antigen receptor (CAR) or T cell receptor (TCR) for developing next-generation CAR/TCR-T cells.

Both viral and non-viral methods for delivery of CRISPR/Cas9 gene-editing tools and payload templates have been recently developed. Viruses have been traditionally used for delivering knock-in gene templates into cells with high efficiency, yet issues including high immunogenicity, increased risk of insertional mutagenesis, high cost and long lead time for manufacturing have raised concerns among researchers. Non-viral delivery of CRISPR/Cas9 editing tools and payload template DNA holds advantages by enabling site-specific transgene insertion via homology-directed repair (HDR) with minimized toxicity and reduced regulatory concerns. More and more researchers are trying to improve the efficiency of non-viral methods to develop a safer and more effective approach for gene and cell therapy[1].

Dr. Nguyen and Roth et al. previously reported that Cas9 target sequences (CTS) could be introduced into double-stranded DNA (dsDNA) HDR templates (HDRTs) to improve knock-in efficiency. In this approach, CTS allow the co-electroporated ribonucleoprotein (RNPs) to bind the HDRTs, facilitating their delivery into the nucleus and increasing insertion efficiency by bringing knock-in templates close to CRISPR RNP cutting sites[2]. Yet the yields and efficiencies were limited by toxicity at higher dsDNA concentrations.

In this case study, Dr. Shy et al. developed a hybrid single-stranded DNA ssDNA HDRTs with CTS (ssCTS), which boosted knock-in efficiency by >5-fold and the live cell yields by >7-fold. This strategy takes advantage of ssCTS's low toxicity at higher concentrations compared to dsDNA HDRTs with CTS (dsCTS). Due to the challenge in the production of long ssDNA sequences, Dr. Shy et al. collaborated with GenScript and were able to perform therapeutic gene editing on primary T cells at a clinical scale[3].

References:

1. ***Ehsan Razeghian et al.*** A deep insight into CRISPR/Cas9 application in CAR-T cell-based tumor immunotherapies. Stem Cell Research & Therapy, 428 (2021) .
https://doi.org/10.1186/s13287-021-02510-7.

2. ***Nguyen, D. N. et al.*** Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency. Nature biotechnology 38, 44-49, doi:10.1038/s41587-019-0325-6 (2020).
https://doi.org/10.1038/s41587-019-0325-6.

3. ***Brian Shy et al.***, Hybrid ssDNA repair templates enable high yield genome engineering in primary cells for disease modeling and cell therapy manufacturing. bioRxiv, Sep 2021.
https://doi.org/10.1101/2021.09.02.458799.

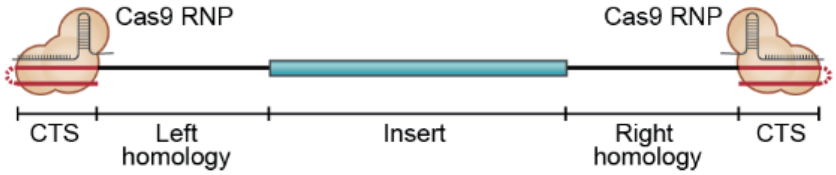

Figure 1.

Diagram of hybrid ssDNA HDRT designs incorporating Cas9 target sites (CTS).

Dr. Shy et al. designed a hybrid ssDNA HDR template (HDRT) structure comprising small stretches of dsDNA incorporating the Cas9 target sites (CTS), through annealed complementary oligonucleotides, in addition to the homology arms on each end of the gene insert. The added CTS can interact with Cas9 ribonucleoproteins (RNPs) to shuttle the template to the nucleus and enhance HDR efficiency.

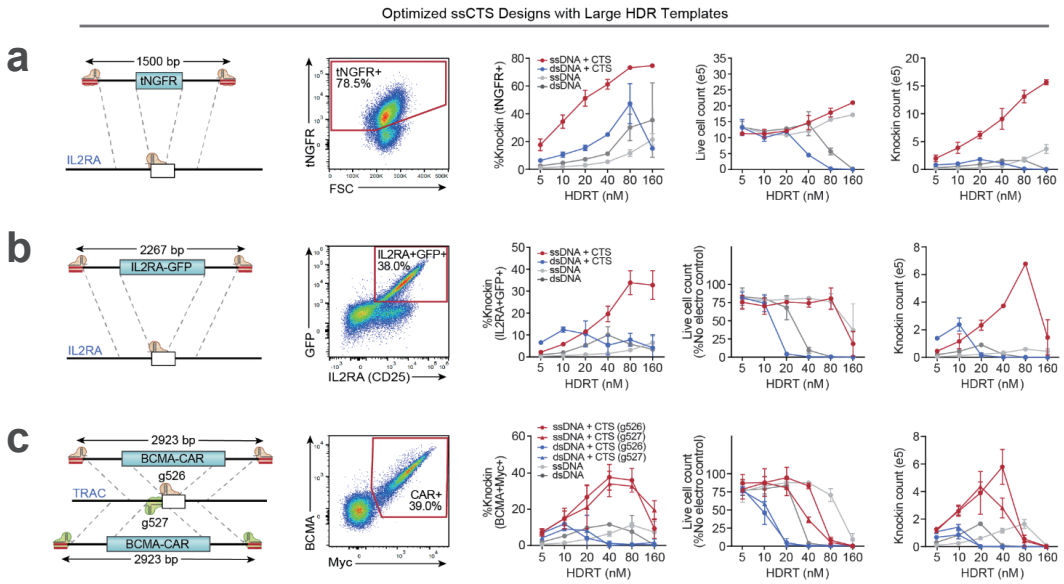

Figure 2.

Development of large ssCTS templates for different gene knock-in across different target loci for high-efficiency and low-toxicity HDR in primary human T cells.

The data showed knock-in strategy, gating, knock-in efficiency, live cell counts, and knock-in cell counts for large ssCTS templates including

- **(a)** tNGFR knock-in at the IL2RA locus,
- **(b)** IL2RA-GFP fusion protein knock-in at the IL2RA locus, and
- **(c)** two different HDRTs inserting a BCMA-CAR construct at TRAC locus via two different gRNAs (g526 and g527).

The use of ssCTS templates achieved 78.5% knock-in efficiency with a ~1.5kb tNGFR construct, and a 38% knock-in efficiency with a ~2.3kb IL2RA-GFP construct targeting the IL2RA locus; and a 39% knock-in efficiency with a ~2.9kb BCMA-specific CAR construct targeting the TRAC locus at concentrations compatible with high yields of live knock-in cells.

The use of ssCTS with large ssDNA constructs outperformed all the other templates with higher knock-in efficiencies and lower toxicity, including dsCTS templates.

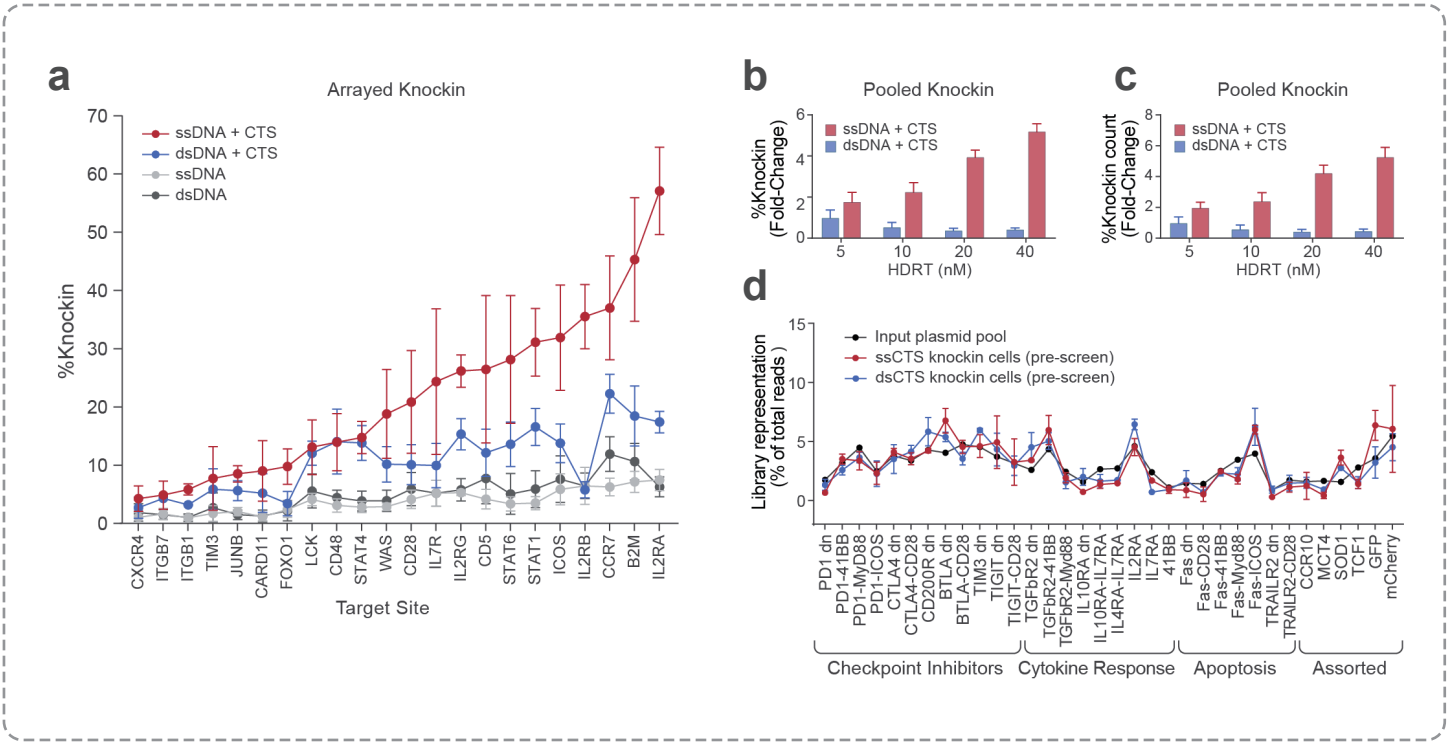

Figure 3.

Evaluation of performance of ssCTS templates across a broad array of genomic loci and knock-in constructs.

- **(a)** Knock-in efficiencies for constructs targeting a tNGFR marker to 22 different genome loci.

The ssCTS constructs outperformed alternative HDRT variations for both knock-in efficiency (up to 5-fold increase) with only a few exceptions that appeared equivalent to dsCTS constructs.

- **(b-d)** Evaluation of performance with a pooled library of knock-in constructs targeting an NY-ESO-1 specific TCR and additional gene products to the endogenous TRAC locus.

Knock-in efficiency and absolute knock-in counts were both increased by >5-fold in comparison to optimal dsCTS concentrations, significantly increasing coverage for each individual construct while retaining consistent representation of the initial library in the final knock-in population.

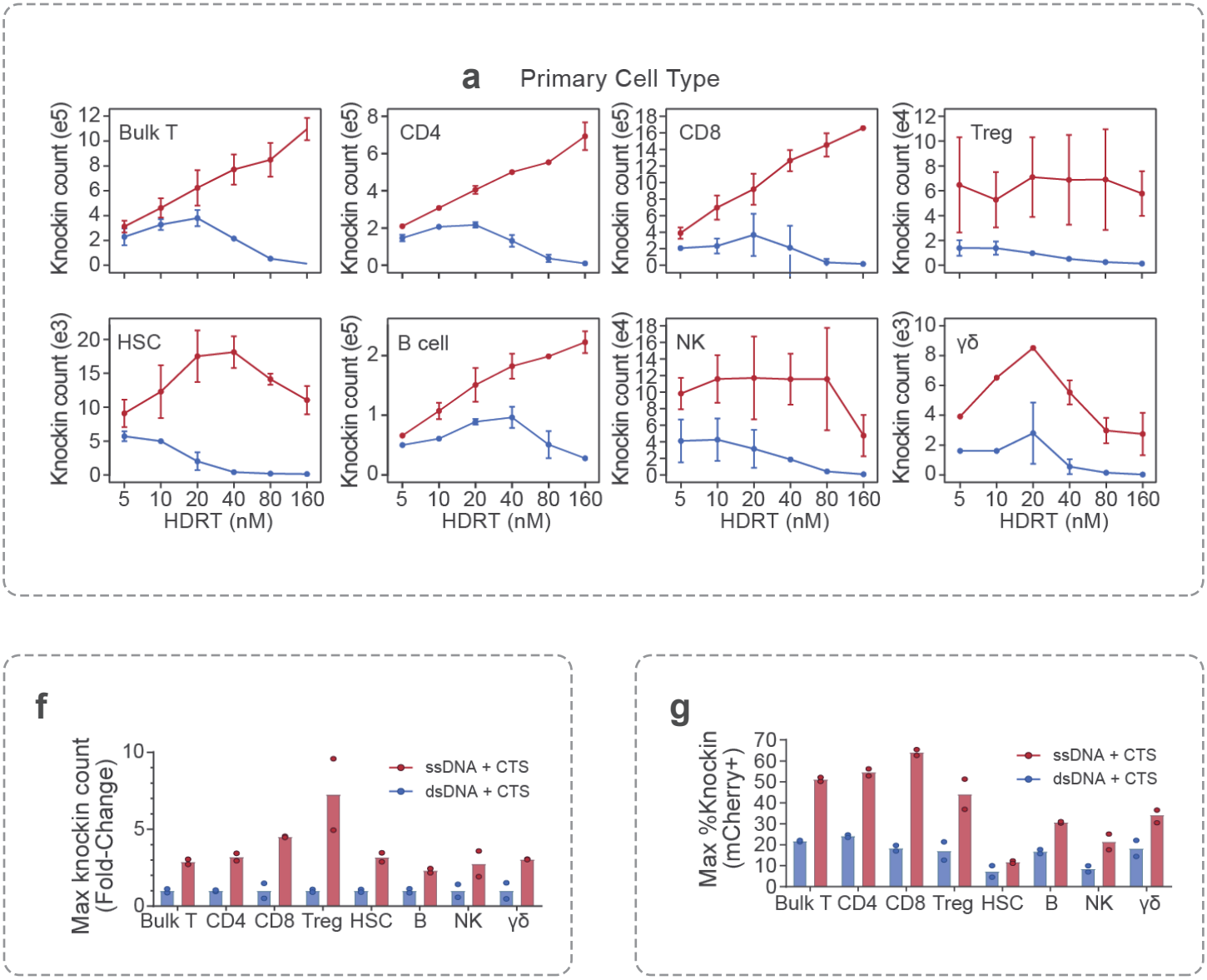

Figure 4.

Evaluation of performance of ssCTS templates across a variety of clinically relevant primary cell types including CD4+ T cells, CD8+ T cells, regulatory T cells (Treg), NK cells, B cells, hematopoietic stem cells (HSC) and gamma-delta T cells using an mCherry knock-in construct targeting the clathrin light chain A (CLTA) gene.

The results showed that the ssCTS templates demonstrated significantly lower toxicity and higher knock-in efficiency and absolute knock-in cell counts in all evaluated cell types, compared with dsCTS templates.

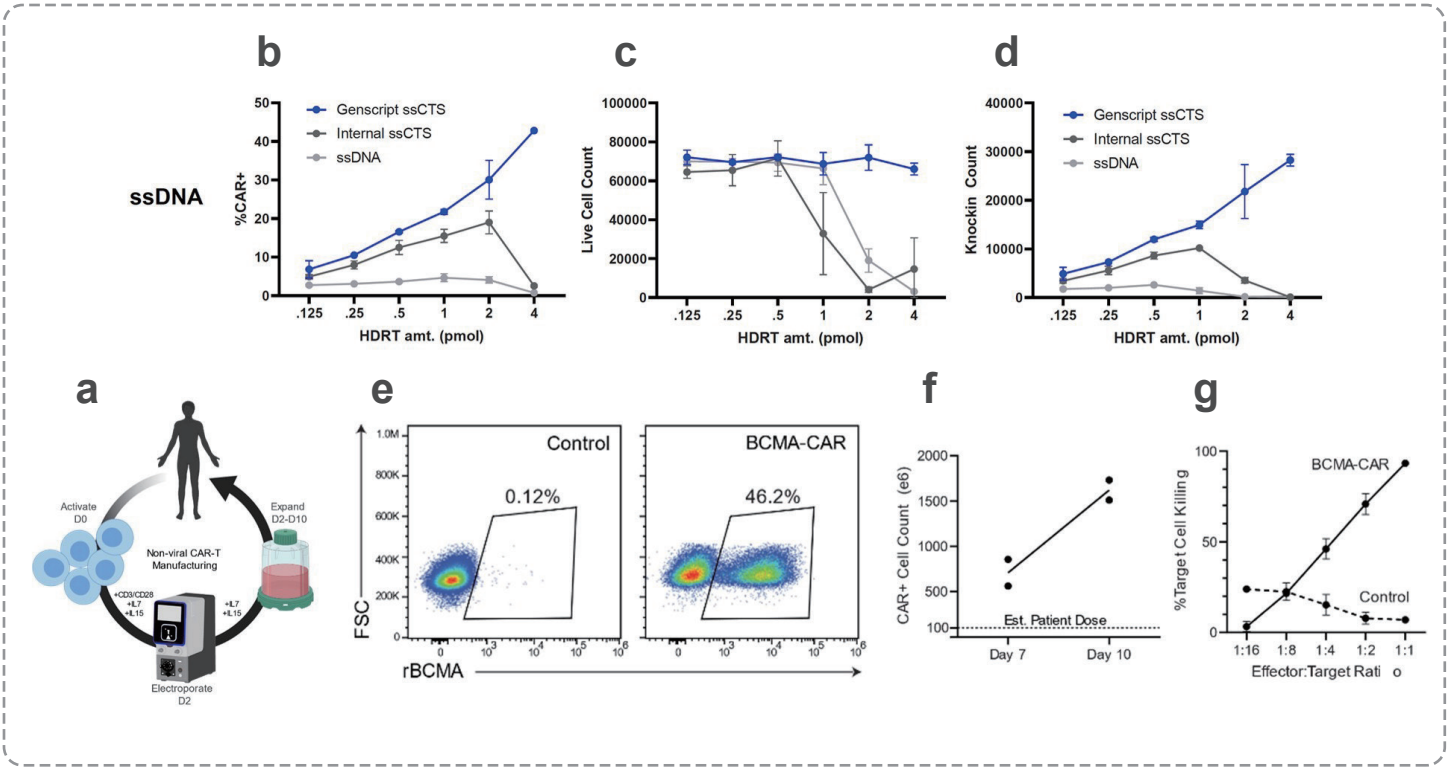

Figure 5.

GenScript synthesized ssCTS for non-viral CAR-T cell manufacturing at clinical scale.

- **(a)** Diagram of non-viral CAR-T cell manufacturing process.
- **(b-d)** The results showed that GenScript's ssCTS consistently outperformed in-house generated ssCTS or ssDNA without CTS design, showing lower levels of toxicity and higher knock-in efficiencies.
- **(e)** The knock-in efficiency of GenScript's ssCTS via electroporation without any enhancer can reach ~ 46.2% at a clinical scale.
- **(f-g)** Knock-in positive live cell count generated by GenScript's ssCTS was above estimated patient dose (100*10e6). In vitro assays demonstrated efficient killing of BCMA-CAR cells.

Conclusion:

Dr. Shy from Marson's lab, Gladstone-UCSF, developed hybrid ssDNA HDR templates that incorporate Cas9 target sequences (ssCTS) as novel payloads for non-viral approach to genome editing at a clinical scale.

To take the finding to the next step, large ssDNA sequence payloads with extremely high purity and efficiency are essential for the success of future clinical studies. GenScript, a leading gene synthesis provider with 19+ years of experience, offershigh quality, sequence verified GenExact™ ssDNA and GenWand™ dsDNA templates to help scientists in the gene and cell therapy research field.

Learn more: https://www.genscript.com/homology-directed-repair-knock-in-templates.html

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