A PLATFORM FOR RAPID AND EFFICIENT ENGINEERING OF MULTI-EDITED CLONAL IPSC LINES FOR ALLOGENEIC T CELL THERAPIES

Notch THERAPEUTICS

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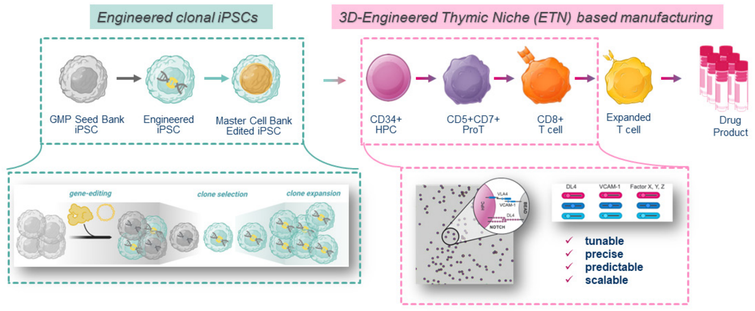
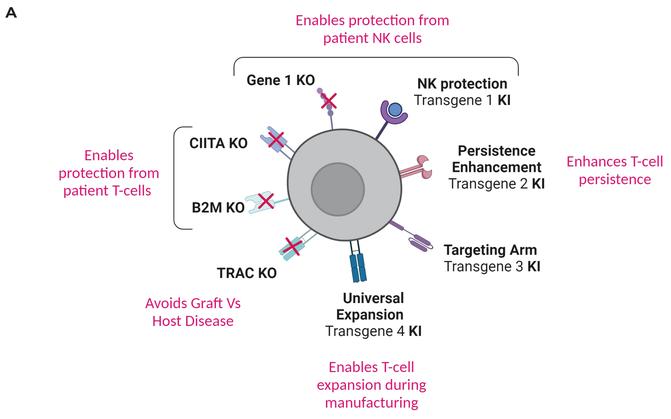
OVERVIEW

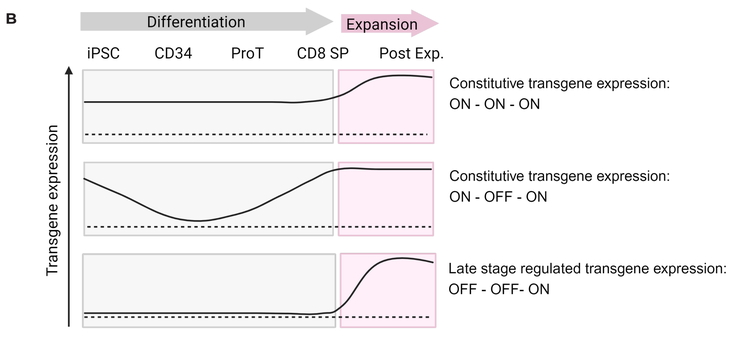
Notch's end to end capabilities for iPSC-derived therapeutics



- Notch has streamlined the iPSC engineering process, which entails genome editing with MAD7, single-cell cloning using high-efficiency dispenser (VIPS™), screening, and expansion
- This workflow yields genetically stable homogeneous iPSCs with validated characteristics and modifications
- Implemented and developed various complementary analytical techniques to analyze edits and ensure genomic stability to expedite decision making and banking of clonal lines
- Notch's 3D ETN platform for scalable T-cell manufacturing is built on our discovery that immobilized DLL4/VCAM supports T-cell differentiation *in vitro*[1]

Notch's cell product design





Notch's iPSC-derived CAR T cell product is composed of multiple knock-outs (KOs) and knock-ins (KIs) that include allogeneic edits for protection of the product from patient's immune cells.

To enable appropriate differentiation and maximize functional activity, the targeting arm, universal expansion gene and NK protection gene must exhibit: 1) specific expression dynamics during differentiation; and 2) sufficient expression levels in final differentiated product.

1 Shukla et al (2017) Progenitor T-cell differentiation from hematopoietic stem cells using Delta-like-4 and VCAM-1. Nature methods 14 531-538.

RESULTS

Section I: MAD7-based editing platform enables high KO and KI efficiencies in iPSCs and rapid production of multiplex edited clonal iPSC lines

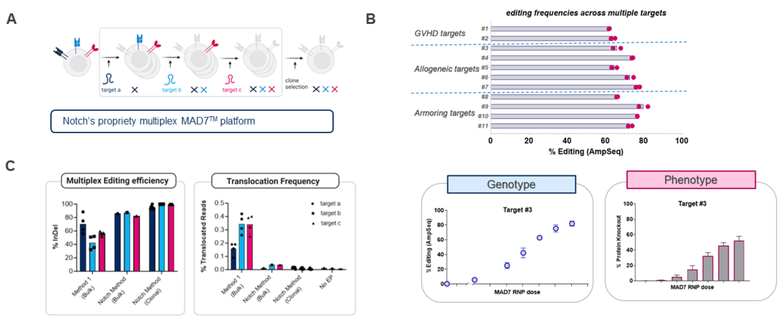


Figure 1: (A) Notch's multiplex gene editing platform uses MAD7 nuclease that belongs to the Class 2 type V-A CRISPR-Cas family, which recognizes thymidine-rich PAM 'YTTV' and creates double stranded staggered breaks. **(B)** Bulk editing efficiencies in Notch's GMP compliant iPSC line using proprietary editing protocol targeting clinically relevant genes. Our high-throughput gRNA screening workflow incorporates viability assessment and indel detection through Synthego's Interference of CRISPR edits (ICE) tool followed by deep analysis via targeted amplicon sequencing (left). Phenotypic validation of knock-outs in primary T cells (right) **(C)** Our multiplex editing methodology achieves significantly higher editing efficiency (left chart) and significantly reduced On target translocation rates (middle chart) compared to other multiplex approaches

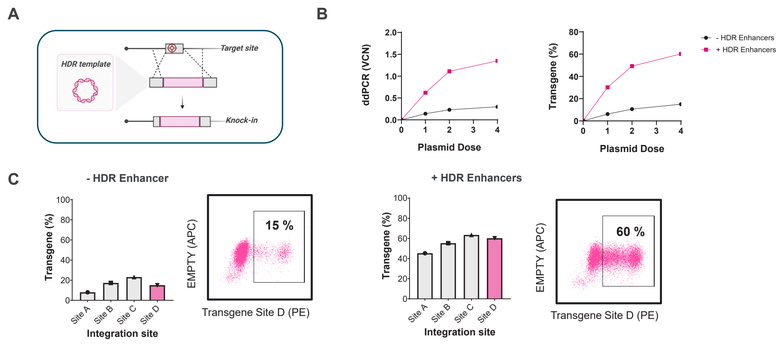
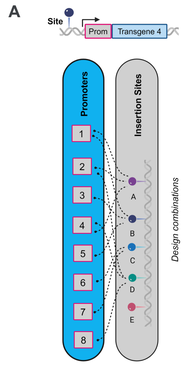


Figure 2 (A) Transgene knock-in by co-transfection of a non-viral (DNA) template with MAD7-gRNA complex for homology-directed repair at the target site. Using plasmid as HDR template and a combination of chemical HDR enhancers we achieve 3-5 fold increase in integration to 40-60% across different plasmid doses **(B)** and across different clinical target sites **(C)**.

Section II: Promoter-integration site screening identifies desired transgene expression profiles during iPSC differentiation



B

Integration site	Promoter	iPSC Bulk	iPSC Clones	CD34+	CD5+ CD7+	CD8+ SP	Post-expansion
A	1	✓	✓	x	x	x	x
	2	✓	✓	✓	✓	✓	✓
	3	✓	✓				
	4	✓					
	5	x					
	6	x					
	7	x					
B	1	✓	x				
	2	✓	✓	✓	✓	✓	✓
	3	✓	✓	✓	✓	x	✓
	4	✓	✓	✓	✓	✓	✓
	6	x					
C	2	✓	✓	✓	✓	✓	✓
	4	x					
D	2	✓	x				
	4	✓	x				
E	8	✓	✓	✓	✓	✓	✓

Figure 3: A) Up to 8 different promoters have been screened to express Universal Expansion (Transgene 4) from five different integration sites (A,B,C,D,E). **B)** iPSCs have been analyzed in bulk, 5-7 days after transfection, in terms of integration efficiency at the integration site using ddPCR assay (SSI VCN) and transgene expression (%) measured by Flow Cytometry. After single cell printing, bi-allelic clones have been selected and analyzed for transgene expression. Clonal iPSC lines with sufficient transgene expression were differentiated using 3D-ETN differentiation platform resulting in identification of 5 promoter-integration site combinations that drive high expression of Universal Expansion Gene throughout differentiation and after expansion.

Section III: *Selected cell product designs* show expected transgene expression, phenotype & *in vitro* function

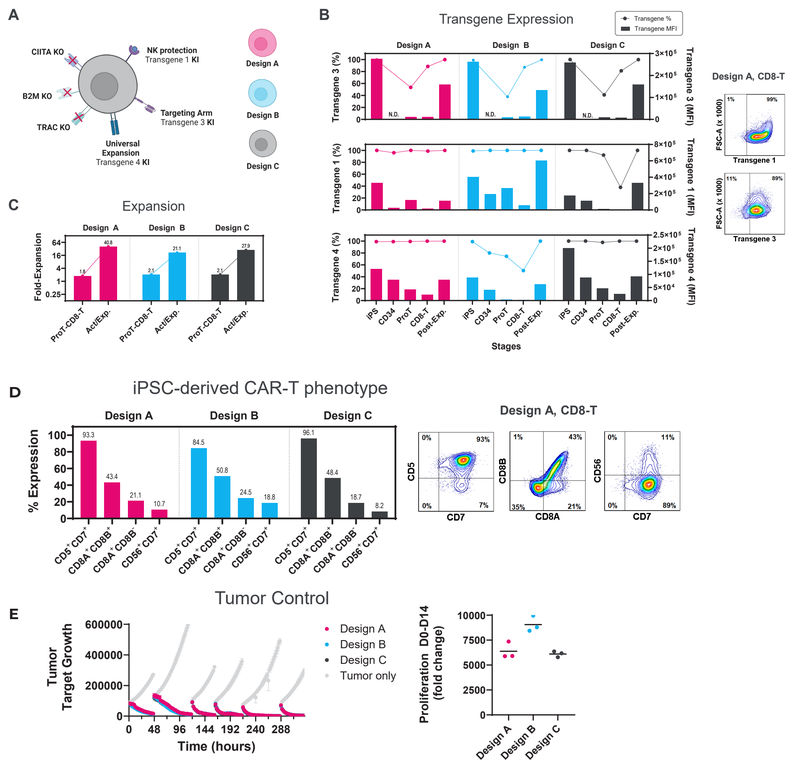
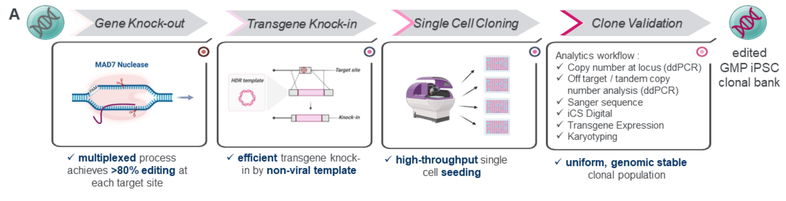


Figure 4: (A) Using identified promoter-integration site combinations from Fig.3, clonal iPSC lines with three different combination designs (A,B,C) have been engineered. All designs contain bi-allelic knock-outs for TRAC, CIITA and B2M and integration of targeting arm at TRAC locus. Integration site and promoter of NK protection gene (Transgene 1) and universal expansion gene (Transgene 4) varies in design A, B, C. **B)** Transgene expression profiles throughout differentiation for each transgene within each combination design with representative Flow scatter plot of design A at end stage **C)** Fold expansion throughout differentiation and during post-activation D) CD8-Tphenotype break-down of each design with representative scatter plots of design A. . **E)** Serial *in vitro* tumor killing assay with five rounds of tumor rechallenge over the course of 14 days.

SUMMARY



- Notch's gene editing platform for clonal iPSC production for knock-out and targeted transgene integration (knock-in) is compliant with GMP conditions and allows rapid engineering of complex clonal iPSC-derived therapeutics.
- Rapid generation of clonal iPSC lines with a library of promoter and integration sites design in iPSC and during iPSC differentiation
- Multiplex edited clonal iPSC lines with up to 6 edits with different cell product designs have been successfully cloned and differentiated showing different fold-expansion characteristics, desiredphenotype and the ability to control tumor *in vitro* over multiple rounds of stimulation.