Inducible and constitutive T-cell-specific promoters to improve safety and efficacy of *in vitro* and *in vivo* T-cell therapies

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OVERVIEW

Promoter choice is a critical and often underappreciated determinant of CAR-T safety and efficacy. Off-target expression introduces safety liabilities; insufficient on-target strength limits therapeutic activity.

Using our unique SuRE™ platform, which functionally screening the full genome at scale, rather than relying on a limited set of established elements or solely on computational predictions, we developed constitutive (Tpromo) and inducible (hITA) T-cell-specific promoters that outperform existing standards in both specificity and activity.

These promoters are now available for evaluation and licensing, and are already under evaluation at multiple leading cell therapy developers.

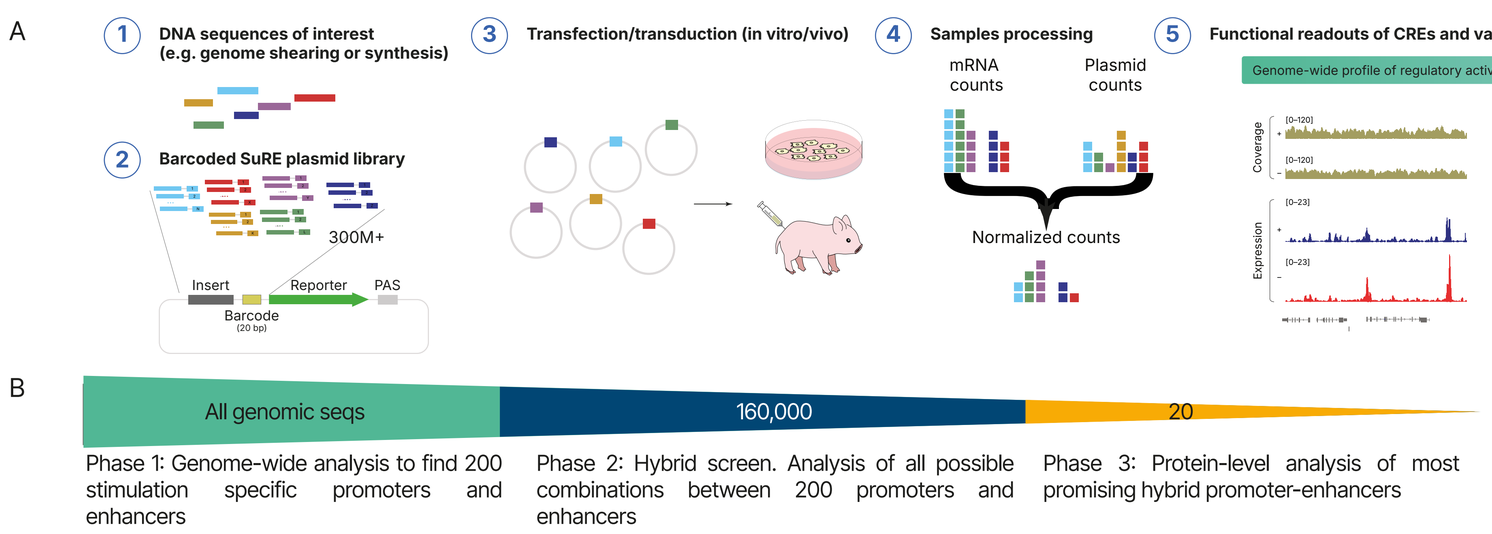

Figure 1. Genome-wide SuRE™. (A) Schematic representation of our genome-wide SuRE platform to discover promoters and enhancers and (B) a workflow how SuRE is being used to develop novel promoters through (phase 1) genome-wide screen in on- and off-target cell types; (phase 2) SuRE screening of combined hybrid elements and (phase 3) the protein-level validation of promoters in relevant cell types, e.g. primary T cells.

RESULTS

Constitutive, T-cell specific promoters for CAR expression in both *in vivo* and *in vitro* CAR-T

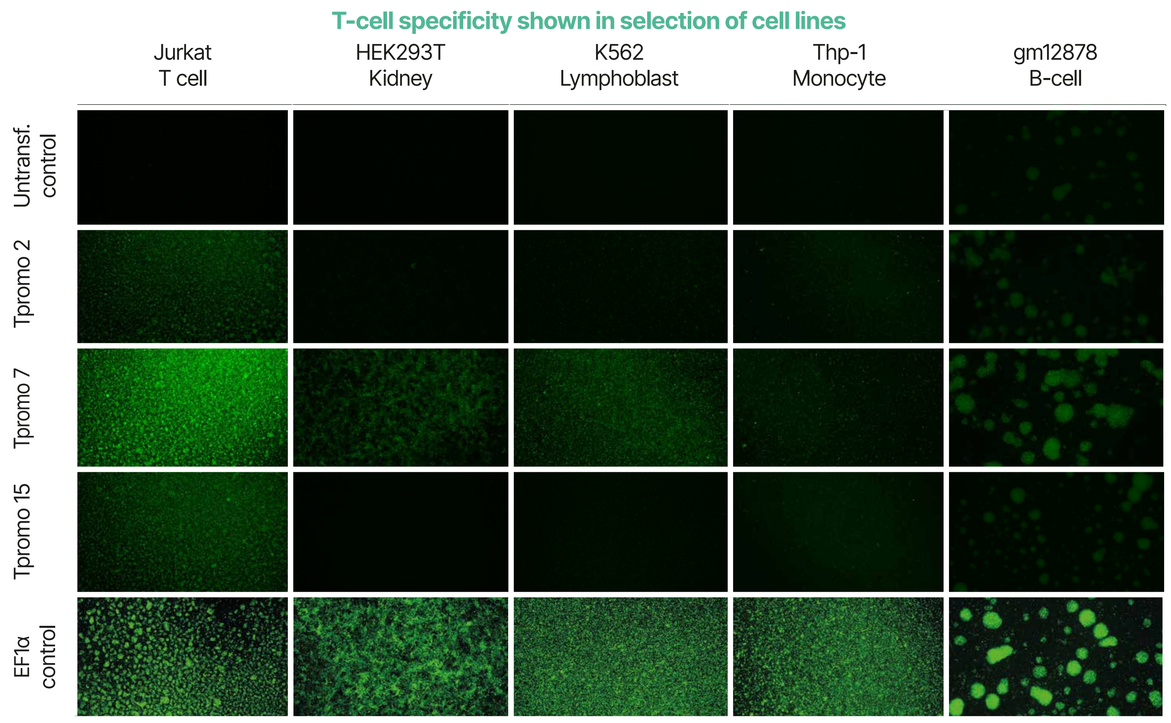

Figure 2. GFP fluorescence driven by T cell promoters in Jurkat (on-target) T cells and off-target cell lines. Three ~900 bp Annogen promoters were compared to the endogenous EF1α promoter and an untransfected control. Promoters were derived from genome-wide SuRE screens in >17 cell lines, selected for T cell specificity. Exposure times are consistent per cell type; all conditions assessed 7 days post lentiviral transduction.

First-generation T-promo candidates achieve up to 33% of EF1α expression level in primary T cells

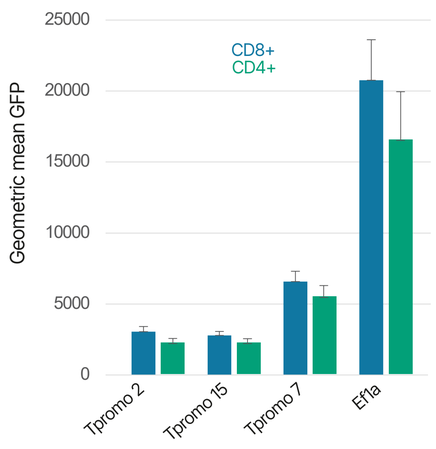

Figure 3. Geometric means of GFP signal in PBMC derived T cell populations. Cells were transduced 1 day after TransAct stimulation and 7 days after transduction data was collected by FACS. Bars represent the average of 7 donors in 2 independent experiments. the candidates are not affected by CD3/CD28 stimulation (data not shown).

In development: Second generation promoters driving higher expression with improved specificity

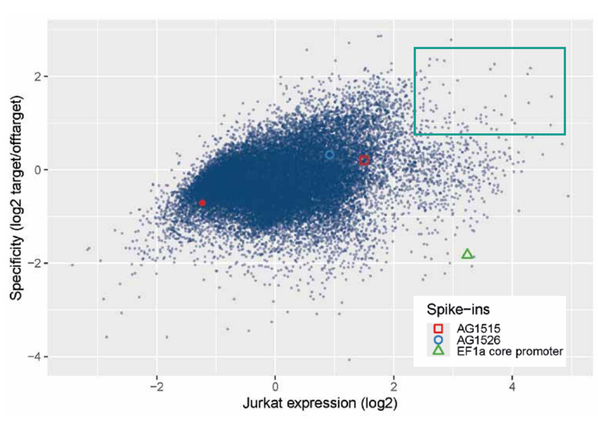

Figure 4. Hybrid-stage screening and selection of novel T-cell specific promoters. Novel elements, either hybrids (~600bp) or singletons (~300bp) in size derived both from optimizations and ML-based predictions were assessed for expression in T-cells (Jurkats) and specificity against HEK293T, GM12878, K562, HepG2, and THP-1. Highlighted elements (green box) demonstrate expression levels similar to EF1α while driving highly specific expression in Jurkat T cells and not in HEK293T.

T-cell activation-inducible (hITA) promoters for the expression of cytokines to enhance and sustain CAR-T cell function

High induction in primary PBMC-derived T-cells

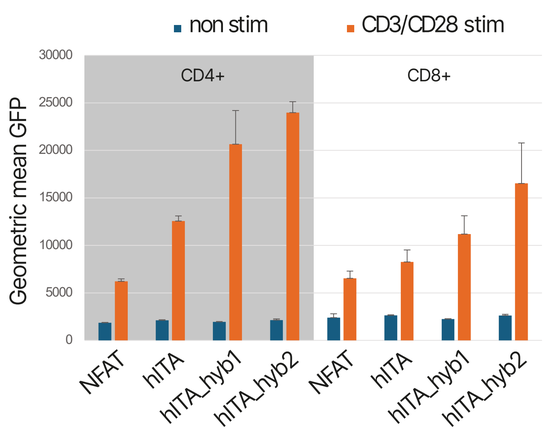

Figure 5. Geometric means of GFP signal in PBMC derived T cell populations. Transduction were done one day after activation. Cells were re-stimulated with CD3/CD28 7 days after transduction and data was collected 18 hours later by FACS. Bars represent the average of 7 donors across 2 experiments.

11 days after transduction, background expression of hITA constructs as low as untransduced

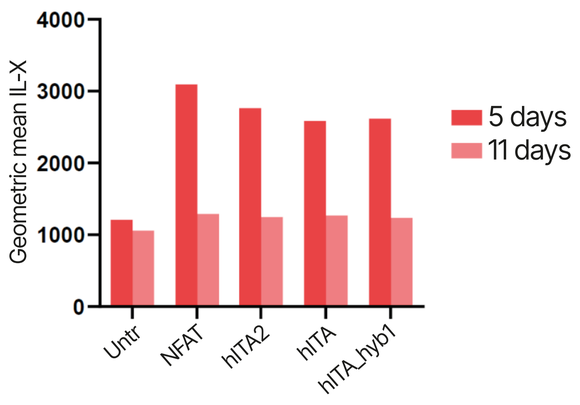

Figure 6. Expression of secreted cytokine in iPSC-derived T-cells. iPSC-derived T-cells were transduced with lentiviral particles expressing cytokine IL-X driven by the indicated promoters and IL-X levels were assayed 5 and 11 days post-transduction

hITA promoters vastly improve T-cell induced expression of secreted cytokine to functionally relevant levels

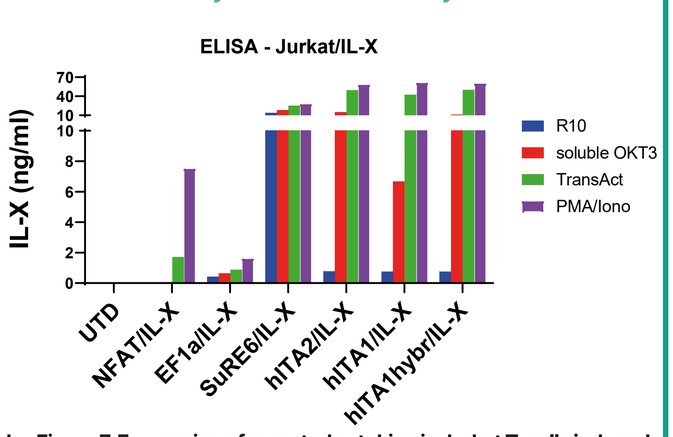

Figure 7. Expression of secreted cytokine in Jurkat T-cells induced by various T-cell stimuli. (B) Expression of secreted IL-X was tested in Jurkat (T cell, on-target) cells by activating with soluble anti-CD3 antibody (OKT3), TransAct (polymeric nanomatrix conjugated to humanized CD3 and CD28 agonists) or PMA/Ionomycin. UTD, NFAT and EF1a are controls, SuRE6 is a ubiquitous strong promoter. hITA2, hITA1 and hITA1hybrid are different promoters queried.*

Improved cytokine-induced killing in CAR-T cells compared to reference promoters

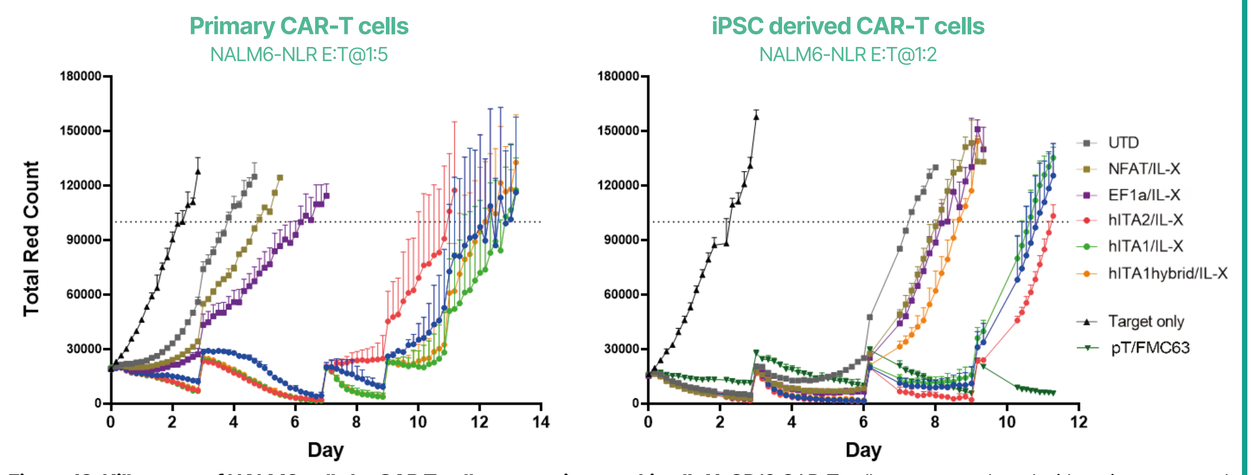

Figure 10. Kill curves of NALM6 cells by CAR T cells expressing cytokine IL-X. CD19 CAR-T cells were transduced with various secreted IL-X lentiviral constructs where expression was controlled by an NFAT promoter, EF1a-long promoter or the different hITA and SuRE6 promoters. Cells were tested in a repeat killing assay with CD19 target cells (Nalm6). The hITA and SuRE6 promoters all provided cytokine support to enhance repeat killing by these cells, suggesting that the increased expression seen in Jurkat (T cell, on-target) cells is meeting a threshold for functional activity in CAR-T cells. *

*Results generated by an independent, undisclosed clinical-stage biotech company.

SUMMARY AND OUTLOOK

- Annogen enables unmatched therapy safety and effectiveness through bespoke promoter development, enabled by our SuRE™ platform
- We generated and validated promoters for use in CAR-T therapies, outperforming industry-standards:
  - Constitutive: With low expression in HEK293T cells and up to 35% of EF1a expression levels in primary T-cells (CD4+ and CD8+).
  - Inducible: Very high induction upon T-cell activation with minimal background, improving CAR-T cell potency and persistence.
- Our existing candidates are currently being optimized (e.g. size reduction and strength), while additional candidates are currently being developed.
- **These promoters are readily available for evaluation and licensing.**

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