Durable, Multiplexed Cell Engineering Using Zinc Finger–Guided Transcriptional Regulators Delivered via a Single Viral Particle

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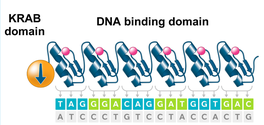
Abstract

Introduction: The success of engineered cell therapies, such as CAR-T cells (chimeric antigen receptor T cells), and the continued development of genomic engineering tools point to a future of highly customized cell therapies with improved therapeutic potential and minimal adverse effects. However, most genome engineering tools, such as nucleases and base editors, are associated with shortcomings that include off-target mutations, double-strand breaks, and limited editing options, only creating knockouts or a limited range of single-base changes. When nucleases are multiplexed, chromosomal translocations become a significant issue. Indeed, current efforts to develop multiplexed edited CAR-T cell therapies have encountered these very issues (e.g. Stadtmauer et al., 2020). Furthermore, the efficiency of generating multiple independent modifications decreases significantly as the number of modifications increases.

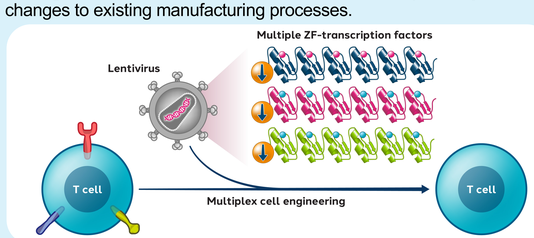
Methods: Here we describe a platform capable of efficient, durable, multiplexed, epigenetic cell engineering using zinc finger (ZF) guided transcriptional regulators delivered via a single viral particle. We have designed highly specific independent ZF-transcription factors (ZF-TFs) to up-regulate or down-regulate target genes of choice.

ZF-Repressors: ZF-TFs using a repressor as functional domain



- **Human origin**
  ZFP & KRAB come from human genes
- **High potency**
  2 targets per cell
- **Compact**
  <900 bp – easily packaged into LV or AAV
- **Precise control**
  Achieve specified gene regulation level

The degree of regulation is tunable, offering the possibility of partial knockdown or variable overexpression. Their compact size allows multiple ZF-TFs to be combined in a single viral construct to achieve highly efficient multigene modulation in a single transduction event and without the need for double-strand breaks. Delivery by lentivirus leverages a well-established method and does not require major changes to existing manufacturing processes.

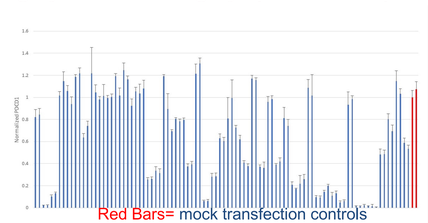


Results: As proof of concept for this novel platform, we engineered primary human T cells using multiple ZF-TFs encoded in a single lentivirus with and without a CAR, to repress expression of several allogeneic engineering targets or checkpoint inhibitors. We demonstrate that ZF-TFs act with high efficiency and specificity on target genes of choice at both the RNA and the protein level.

Conclusions: Multiplexed, epigenetic cell engineering using ZF-guided transcription factors offers a significantly improved approach to engineered cell therapies. It could be used in many cell types and has the potential to be deployed in both ex vivo and in vivo applications.

Results

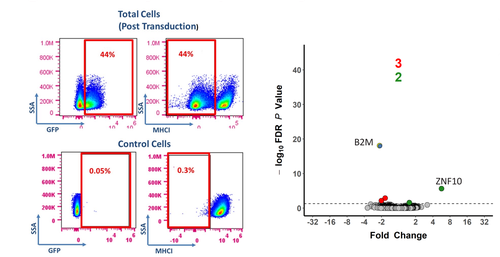
Highly active and highly specific ZF-repressors were identified by transient transfection of T cells

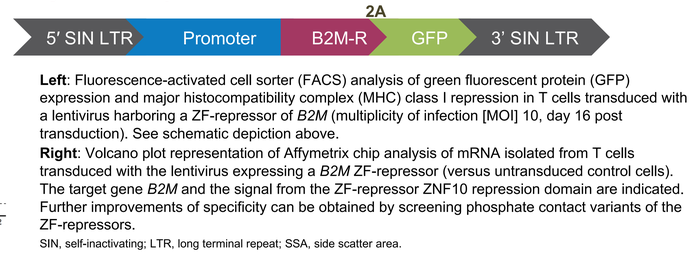


Representative data from a transient transfection of ZF-TF mRNAs targeting the *PDCD1* locus into T cells.

Left: *PDCD1* mRNA was analyzed 2 days after transfection and normalized using housekeeping gene controls. Several ZF-TFs drive near-complete mRNA repression.
Middle: Volcano plot representation of *P* value and fold change in expression of an Affymetrix chip analysis of T cells transiently transfected with a ZF-repressor of *PDCD1*. mRNA was harvested 1 day post transfection and compared to mRNA isolated from mock transfected cells. The target gene *PDCD1* and the signal from the ZF-repressor ZNF10 repression domain are shown.
Right: Schematic representation of *PDCD1* mRNA knockdown versus the ZF-TF binding site around the *PDCD1* start site of transcription. Triangles represent binding sites for ZF-repressors; darker colors indicate stronger repression.
FDR, false discovery rate.

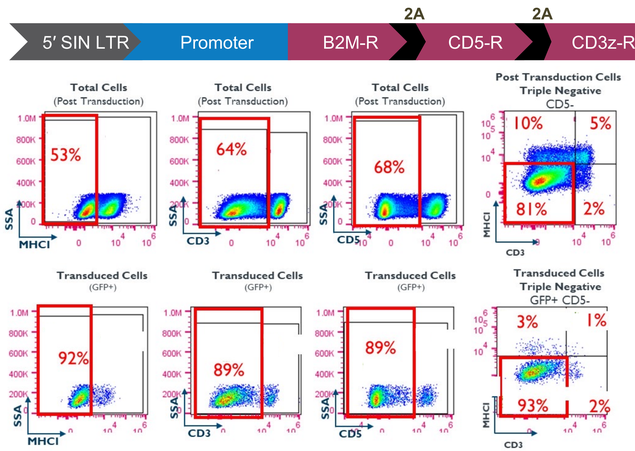
High efficiency and specificity after lentivirus delivery of a ZF-repressor into T cells





Left: Fluorescence-activated cell sorter (FACS) analysis of green fluorescent protein (GFP) expression and major histocompatibility complex (MHC) class I repression in T cells transduced with a lentivirus harboring a ZF-repressor of *B2M* (multiplicity of infection [MOI] 10, day 16 post transduction). See schematic depiction above.
Right: Volcano plot representation of Affymetrix chip analysis of mRNA isolated from T cells transduced with the lentivirus expressing a *B2M* ZF-repressor (versus untransduced control cells). The target gene *B2M* and the signal from the ZF-repressor ZNF10 repression domain are indicated. Further improvements of specificity can be obtained by screening phosphate contact variants of the ZF-repressors.
SIN, self-inactivating; LTR, long terminal repeat; SSA, side scatter area.

Efficient triple-gene repression after T cell transduction with a single lentivirus carrying 3 ZF-repressors

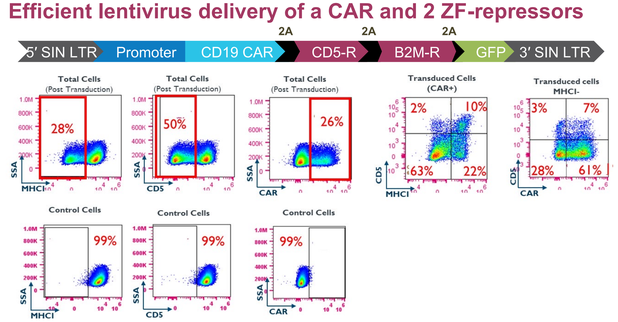


~50% triple repression post transduction

~80% triple repression in transduced cells

Top: Schematic representation of a lentivirus harboring 3 ZF-repressors.
Bottom: FACS analysis of lentivirus-transduced T cells 9 days post transduction (MOI 10).
The top row shows analysis of the cell pool, the bottom row analysis of transduced cells, using the GFP marker. The percentage of triple-repressed cells was calculated based on gating on CD5-negative cells and plotting CD3-negative cells and MHCI-negative cells. Untransduced T cells were ≥98% positive for target gene expression by FACS analysis (not shown).
MHCI, major histocompatibility complex class I

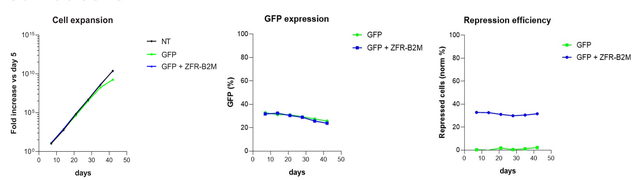
Efficient lentivirus delivery of a CAR and 2 ZF-repressors



FACS analysis of T cells transduced with a lentivirus harboring a CAR and 2 ZF- repressors (see schematic depiction) 5 days post transduction (MOI 10).

Top row: Analysis of the lentivirus transfected cell pool. **Bottom row:** Analysis of untransduced control T cells. **Top row, right two panels:** Target gene repression in transduced T cells was assessed by gating on CAR+ cells, and dual repression by gating on MHCI-negative cells.

Long term repression after lentivirus transduction of Jurkat cells



Analysis of long-term transgene expression and ZF-repressor–mediated target gene repression in Jurkat cells transduced with the *B2M* ZF-repressor-2a-GFP lentivirus.

Left: Exponential expansion of lentivirus-transduced Jurkat cells over 42 days.
Middle: Percentage of GFP-positive cells in Jurkat cells transduced with the *B2M* ZF-repressor-2a-GFP lentivirus and a GFP-only control lentivirus over time.
Right: Percentage of MHC class I–negative cells over time.

Summary and Conclusions

- We identified highly active and specific ZF-repressors for several therapeutically relevant target genes.
- The ZF-repressors were successfully introduced into lentiviruses, and efficient repression of up to three target genes was observed.
- Stable long-term target gene repression was observed.
- Functional tests in appropriate in vitro and mouse model systems are underway.
- The ZF-repressor platform is an efficient alternative, or complement, to nuclease editing approaches in T cells, dramatically expanding the options for the generation of optimized T cell products.

References

1. Stadtmauer et al., CRISPR-engineered T cells in patients with refractory cancer, *Science* (2020)
2. Zeitler et al., Persistent repression of tau in the brain using engineered zinc finger protein transcription factors, *Science Advances* (2021)

Author Disclosures

All authors are or were employees of Sangamo Therapeutics.

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