Development of a Novel SMARTvector™ Multiplex shRNA Platform for Safer Cell Therapy Engineering

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1 Abstract

Chimeric antigen receptor (CAR) T cell therapy represents a new era of cell-based immunotherapies, allowing for targeted killing of cancer, often blood cancers, expressing a specific antigen. Despite the success of several CAR T cell therapies achieving FDA approval, these therapies often suffer from T cell exhaustion, which can present as reduced cytolytic activity, increased expression of inhibitory receptors, and reduced proliferative capacity. These outcomes render the treatment less potent, pointing towards a need to develop more advanced and tunable methods of modulating CAR T cells to be more robust and reliable. Gene editing approaches such as CRISPR-Cas9 have successfully been used for disruption of genes such as PDCD1, an inhibitory receptor involved in CAR T cell dysfunction, to enhance CAR T cell performance *in vitro*. Despite the efficiency of CRISPR-Cas9, the number of targets that can be edited in a single sample is partially limited by increased cytotoxicity associated with DNA double-strand breaks (DSBs), thus limiting the therapeutic applicability. Here we present the SMARTvector lentiviral shRNA technology, a method for simultaneous multiplex gene knockdown in immune cells from delivery of a single expression vector. We show that up to eight shRNAs and an anti-CD19 CAR can be expressed after a single lentiviral transduction, thus providing a safer alternative of targeting multiple genes while maximizing CAR T cell engineering.

We developed the SMARTvector multiplex shRNA technology with multiple repeats of a novel patented microRNA scaffold, flanking artificial DNA sequences that target mRNA transcript(s) of interest for efficient gene knockdown. The vector design includes a tunable promoter and selection markers for specific targeted development. Here, an array of multiple shRNAs was generated in a single expression vector with efficacy measured by RT-qPCR to assess relative mRNA transcript knockdown or flow cytometry for assessment of functional protein knockdown.

We have demonstrated that multiple shRNAs can be expressed from a single expression vector, resulting in sustained expression and efficient gene knockdown in primary T cells and iPSCs. In addition to knockdown efficiency, we have engineered a vector with optimal linker length between each microRNA-based shRNA encoding region and evaluated position related impacts of each shRNA on the multiplexed cassette. We demonstrate that the SMARTvector multiplex shRNA technology is highly modular and adaptable, making it primed for use in a wide range of cell therapy applications, such as generation of allogenic CAR T cell therapies.

2 shRNAs use the endogenous eukaryotic pathway for micro-RNA based gene regulation

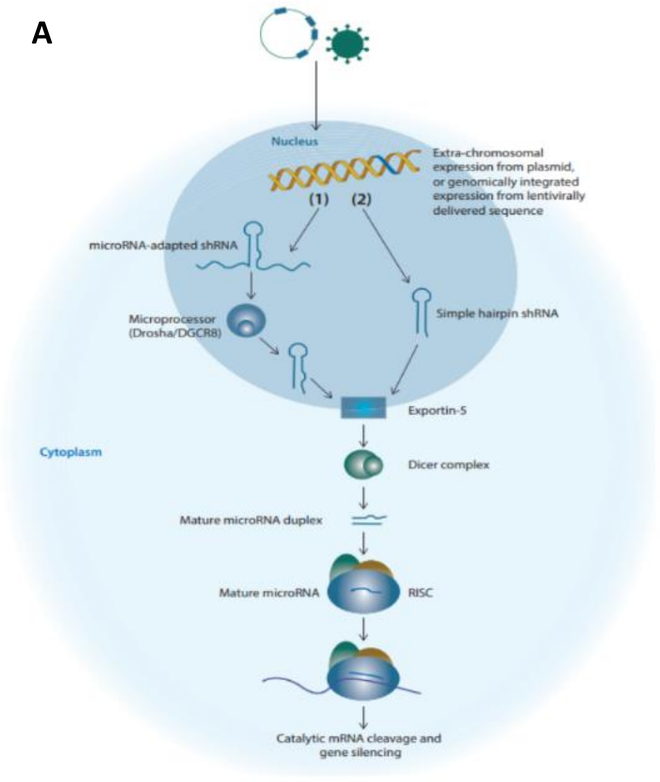

Figure 1 A) shRNA approaches include the introduction of plasmid-based or viral-based vectors expressing the silencing sequences embedded in a microRNA-adapted scaffold (1) or simple hairpin shRNA (2). Expressed shRNAs (blue) enter the endogenous pathway at an early stage and are efficiently processed into potent silencing molecules using the endogenous microRNA mechanism, which leads to target mRNA cleavage (purple) and gene knockdown.

3 SMARTvector Scaffold Design

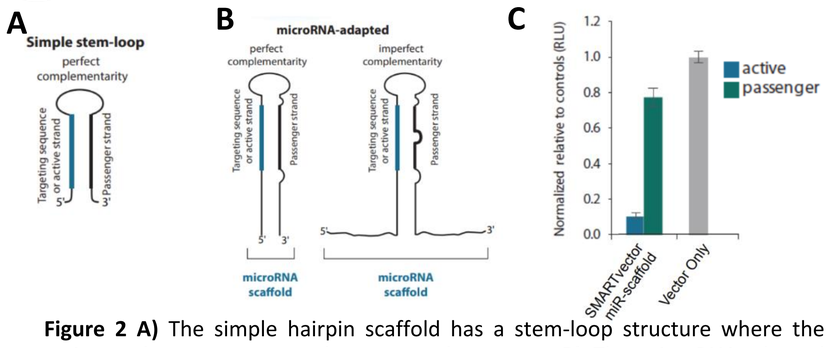

Figure 2 A) The simple hairpin scaffold has a stem-loop structure where the active and passenger strands are complementary. **B)** The microRNA-adapted scaffold has a longer stem-loop structure based on naturally occurring microRNAs. The active and passenger strands have perfect or imperfect complementarity (e.g., mismatches, G:U wobbles), and may also contain essential single-strand sequence, flanking sequence, upstream or downstream of the stem-loop. **C)** The ideal microRNA scaffold (miR-scaffold) has high knockdown efficiency of the active strand and low knockdown efficiency of the passenger strand.

4 Efficient Proof of Concept Knockdown Using a Multiplex shRNA System

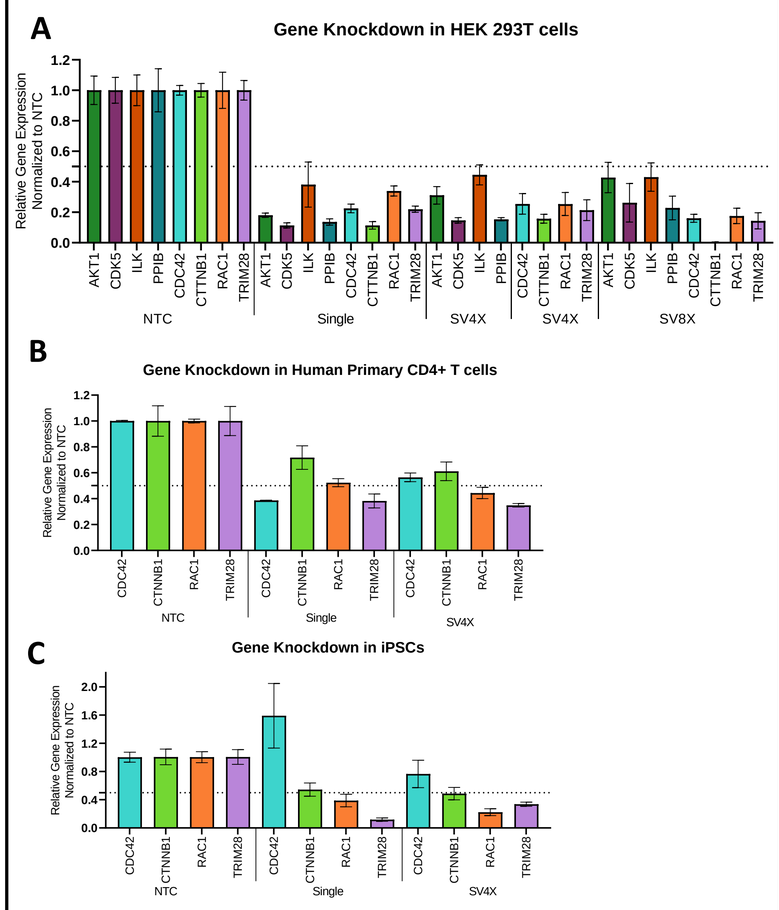

Figure 3) With the SMARTvector multiplex technology, several genes can be downregulated using a single lentiviral construct. **A)** The expression of four (SV4X) or eight (SV8X) different shRNAs leads to gene knocked down in 293T cells using the multiplex SMARTvector design. **B and C)** The expression of up to four (SV4X) shRNAs inhibits the expression of multiple genes in primary T cells **(B)** and induced pluripotent stem cells (iPSCs) **(C)**. Knockdown using the multiplex system reaches comparable levels when compared to single-plex knockdown in all examined cell types.

5 Multiplex shRNA Modulation of Primary T cell Surface Proteins

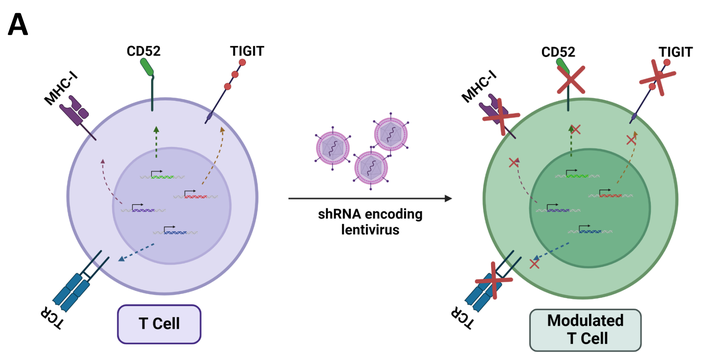

Figure 5 A) Schematic of T cell modulation by the SMARTvector multiplex technology.

6 Efficient Knockdown Using a Multiplex shRNA System in CD8+ T cells

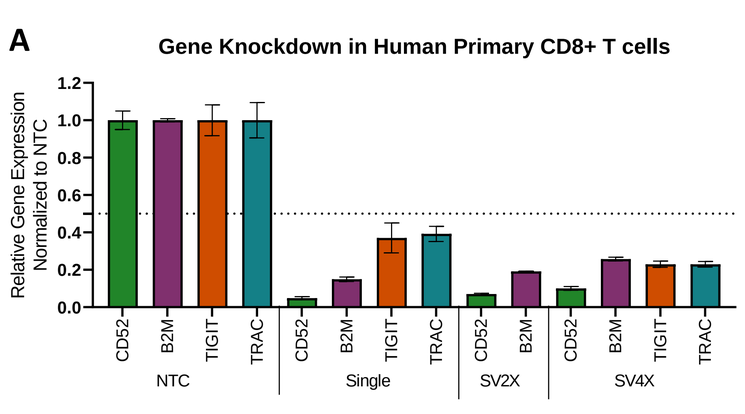

Figure 6 A) The SMARTvector multiplex technology was demonstrated in CD8+ T cells for knockdown efficiency of vectors expressing a single, two (SV2X) or four (SV4X) shRNAs. For these experiments, the therapeutically relevant targets CD52, B2M, TIGIT, and TRAC were cloned into the respective SMARTvector backbones. Gene knockdown efficiency is maintained in both the SV2X and SV4X vectors, when compared to the single-plex system.

7 Multiplex shRNA Knockdown of Surface Protein in CD8+ T cells

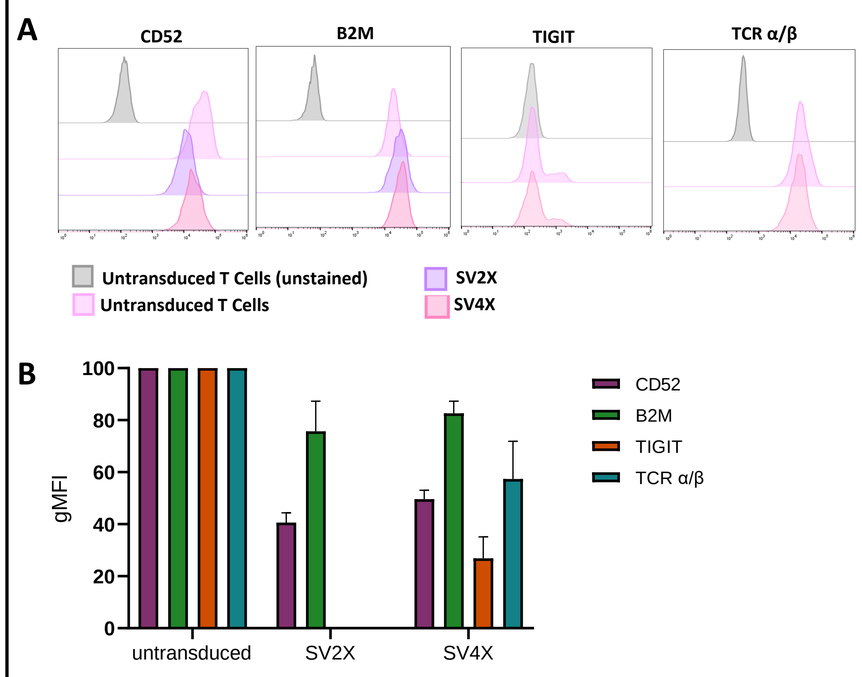

Figure 7) Primary CD8+ T cells were transduced with the SV2X and SV4X SMARTvector multiplex vectors and evaluated for cell surface protein loss compared to untransduced cells. **A)** Representative histogram plots for each target and **B)** cell surface expression relative to untransduced cells for each target (gMFI).

8 CAR Integration for Cell Therapy Engineering

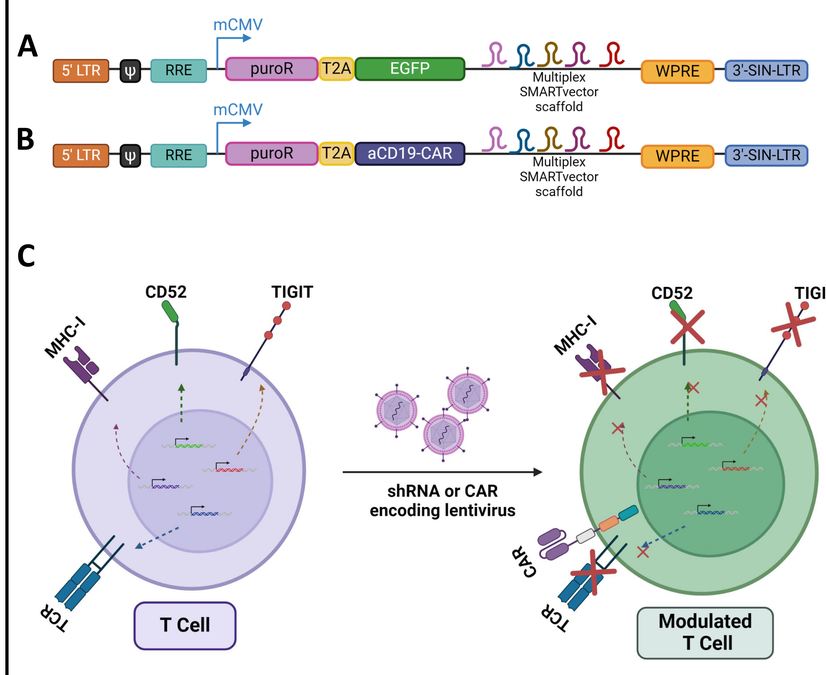

Figure 8 A) The original SMARTvector multiplex design included an Enhanced Green Fluorescent Protein (EGFP) preceding the multiplex SMARTvector scaffold. **B)** A new multiplex vector was designed with an anti-CD19-CAR (aCD19-CAR) cloned into the previous EGFP position. **C)** With a single lentiviral transduction, human primary T cells can be modulated for knockdown of up to four targets while simultaneously integrating a CAR.

9 Summary

- The SMARTvector technology contains a selected microRNA scaffold for highly efficient processing via the endogenous RNAi pathway, resulting in reduced off-targets, due to both preferential loading of the active strand and efficient processing through the optimized microRNA-adapted scaffold.
- The SMARTvector technology can be expanded to enable efficient multiplex shRNA expression and gene knockdown in a variety of cell types, including HEK 293T, CD4+ and CD8+ T cells, and iPSCs.
- The SMARTvector multiplex technology translates to a reduction in cell surface protein expression in CD8+ T cells.
- A CAR can be integrated into the SMARTvector multiplex vector, making the generation of less exhausted CAR-T cells possible by knocking down therapeutically relevant targets.