Optimal Force-tuned Synthetic Receptor and its Circuit for Magneto-mechanical Genetic Immunotherapy

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Abstract

As a promising approach to cancer treatment, chimeric antigen receptor (CAR) T-cell immunotherapy has been extensively studied over the past few decades. CAR-T therapy involves the engineering of T-cells to express CARs that specifically bind to tumor antigens, allowing precise targeting and elimination. However, current CAR-T therapy has several limitations including safety concerns, low efficacy in treating solid tumors, and CAR T-cell exhaustion. Among all, safety issues caused by on-target, off-tumor toxicity may pose life-threatening risks. Therefore, tuned spatiotemporal control of CAR expression has been prioritized for safer treatments. Here, we present a tension-tuned synNotch with optimal cleavage force for magneto-mechanical genetic(MMG) CAR expression control system. Furthermore, we demonstrated a gene circuit that reduces the leakage by over 50%. Comprehensively, this project allows precise control of CAR expression and its extension for immunotherapy holds high therapeutic value.

Introduction

Previously, the team has developed a magnetic nanoparticle cluster(MNC) to mechanically cleave synNotch when a rotating magnetic field (RMF) is applied, enabling a remote control of CAR expression. Unfortunately, due to the size of the MNC, the binding incidence of the MNC to the synNotch applied sufficient force to induce cleavage even without the application of RMF, hindering the precise regulation of CAR expression. To overcome the issue of non-specific activation, we screened multiple synNotch variants with differing cleavage forces to optimize the CAR expression control system.

In addition, previous flow cytometry analysis revealed a significant leakage of the current GAL4-UAS circuit, 5XUAS-CMV_{mini}-CAR-P2A-GFP. We hypothesized that the minimal-CMV domain, a sequence derived from a strong promoter, caused the leaks. Hence, the minimal-CMV domain was switched to a relatively weaker promoter sequence, a TATA box, through cloning.

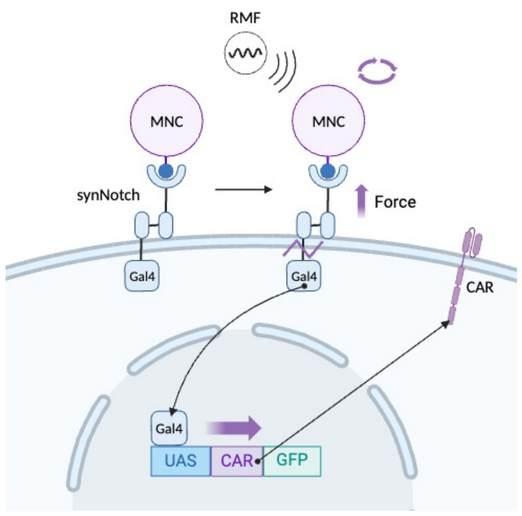

Figure 1: Overall scheme of MMG CAR expression control system

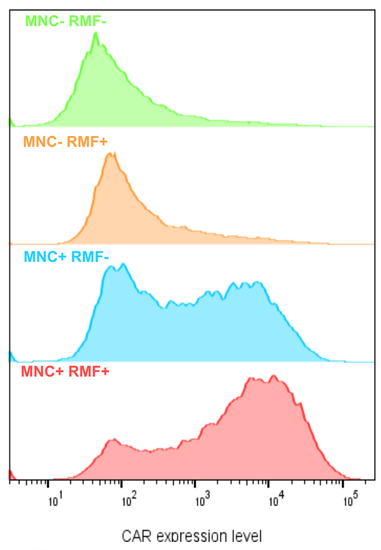

Figure 2: Flow cytometry results from a previous study showing a significant amount of non-specific activations and leaks

Experimental Methods

1. Screening of synNotch Variants with Differing Cleavage Force

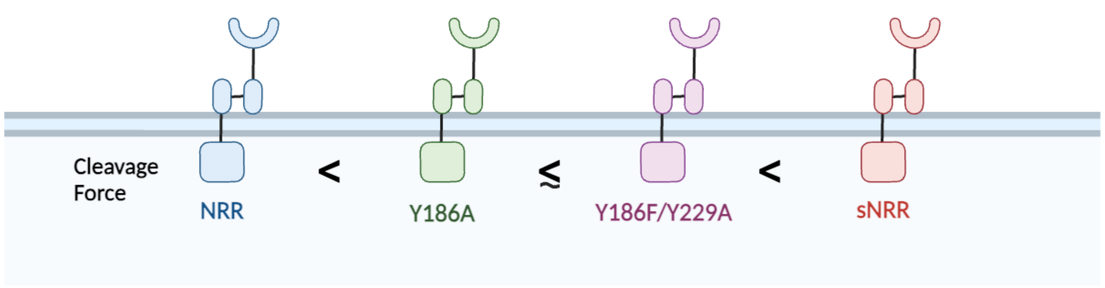

Figure 3: Diagram depicting the order of relative cleavage forces among synNotch variants

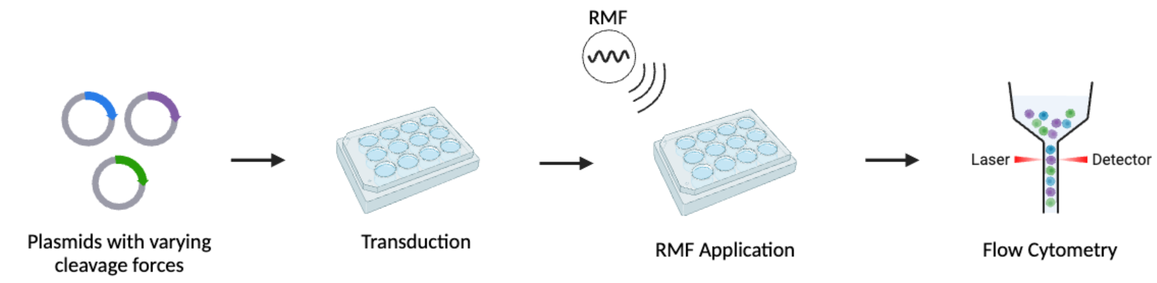

Figure 4: Screening method of synNotch variants

The ordered synNotch plasmids were transfected to LentiX-293T by Lipofectamine 3000. Then, the lentiviruses were transduced to Jurkat cells.
The cells were divided into three groups: negative control group, MNC only group, and MNC + RMF group.
The flow cytometry analysis was performed to verify the expression of CAR.

2. Reducing the Leakage of the Current GAL4-UAS Circuit

Figure 5: Gene diagrams of before(left) and after(right) InFusion cloning

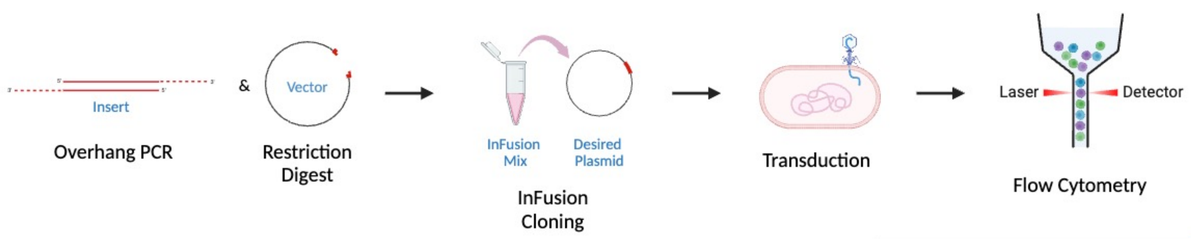

Figure 6: Replacement method of the minimal-CMV domain in the current GAL4-UAS circuit with a TATA box

The overhang PCR was performed on the insert sequences to generate ends that are complementary to the ends of the vector, which had been restriction digested.
Through InFusion cloning, the desired plasmid were obtained and verified by sequencing. The obtained plasmid was lentivirally transduced into Jurkat cells.
The leaks of the cells with the original gene circuit and the switched gene circuit were compared and analyzed via flow cytometry.

Results

Confocal Imaging of Jurkat Cells Post Transduction

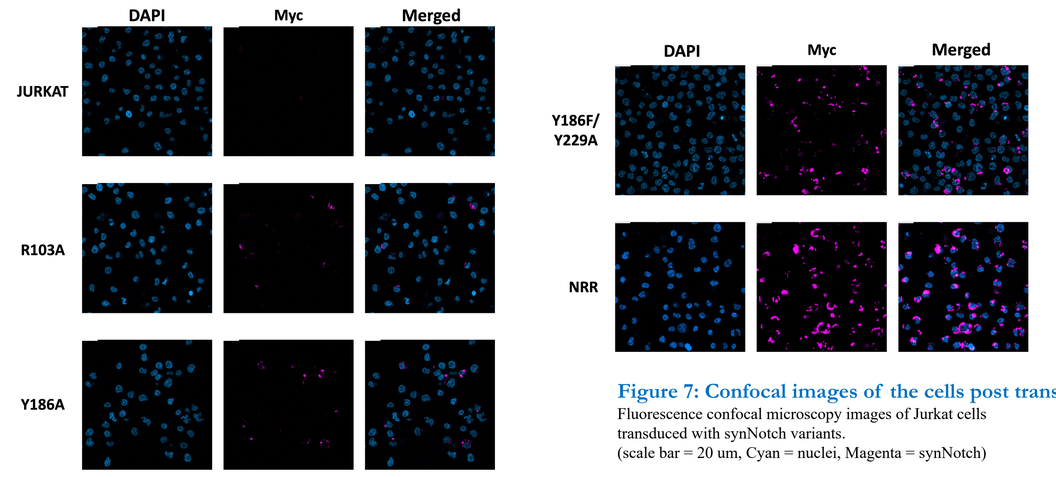

Figure 7: Confocal images of the cells post transduction

Fluorescence confocal microscopy images of Jurkat cells transduced with synNotch variants.
(scale bar = 20 um, Cyan = nuclei, Magenta = synNotch)

Screening of synNotch Variants to Minimize Non-specific Activation

Fig. 8(A)

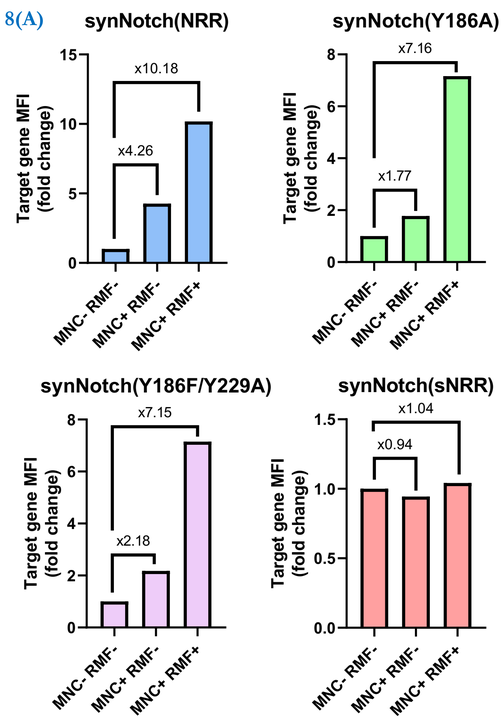

Fig. 8(B)

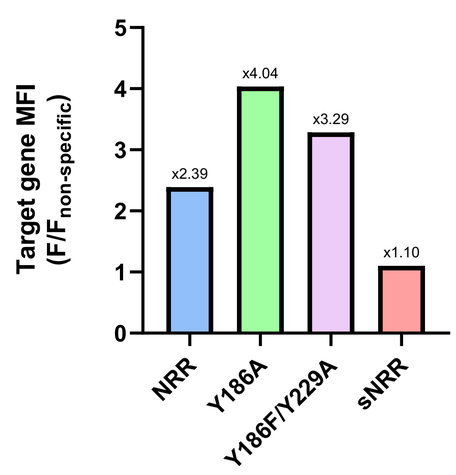

Figure 8: Fold change analysis of mean fluorescence intensities

(A) Comparing non-specific activation of synNotch variants. Jurkat cells were lentivirally transduced to express synNotch variants and their downstream genes.
(B) Comparison of magneto-mechanical activation over non-specific activation.
The binding incidence of MNC to synNotch leads to non-specific activation.
The higher the cleavage force threshold is, the more reduced the non-specific activation.

Improving the GAL4-UAS Circuit for Precise Gene Expression Control

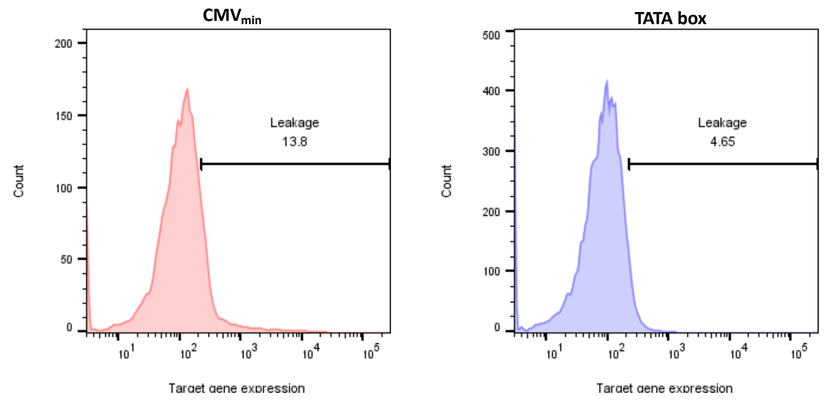

Figure 9: Leakage analysis of GAL4-UAS based minimal-CMV and TATA box circuits

Jurkat cells were lentivirally transduced to express synNotch(NRR) and two different downstream genes, (A) 5XUAS-CMV_{mini}-CAR-P2A-GFP and
(B) 5XUAS-TATA-CAR-P2A-GFP. Target gene expression level was quantified by GFP signal. TATA box-based gene circuit showed a significant leakage decrease.

Conclusion & Further Study

In this study, two separate experiments were conducted, both aiming to improve the current MMG CAR expression control system. While the first experiment focused on screening the synNotch variants with optimal cleavage force to reduce non-specific activations, the second experiment centered on minimizing the leaks by replacing the minimal-CMV domain of the gene circuit into a TATA box.

Firstly, the flow cytometry analysis of synNotch variants yielded promising results for MMG-based immunotherapy (Fig.8). Prior to that, the successful transductions of synNotch variants to the Jurkat cells were confirmed through confocal fluorescence imaging (Fig. 7). Intriguingly, there was a significant difference in the amount of synNotch that was expressed between the synNotch variants and NRR. This may be due to the difference in the promoter and signal peptide usage in the purchased synNotch variants' plasmids. The flow cytometry analysis revealed that NRR exhibited the highest expression level when both MNC and RMF were applied (Fig. 8A). However, for the sake of higher efficacy and safety, precise control over the customized output is essential. Therefore, we choose Y186A variant as the most promising candidate as it demonstrated 4.04-fold increase compared to non-specific activation induced by MNC labeling without RMF application.

Secondly, the replacement of minimal-CMV domain into a TATA box decreased leaks by over 50% (~66%) in the absence of both MNC and RMF. As the promoter was altered to be weaker, consequently, the CAR expression when both MNC and RMF are applied may significantly reduce as well. Thus, further analysis of the gene circuits under the application of both MNC and RMF is necessary to determine whether such a trade-off is justifiable.

Further studies must be conducted to optimize MMG CAR expression control system and to address safety concerns. First and foremost, additional screenings should be done after the replacement of the synNotch variants' EF-1α promoter to a PGK promoter and the insertion of appropriate signal peptides at the N' terminus. In vivo studies using a mouse model are essential, along with assessments to assay the efficacy and superiority of such a control system in eliminating tumors. Moreover, the system's ability to mitigate on-target, off-tumor toxicity should be compared to conventional CAR-T therapy lacking such control mechanisms.

Reference

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