Gene Engineering of multi-antigen-sensing SynNotch TGF-β trap circuit against solid tumor microenvironments applied to CAR-T cell immunotherapy

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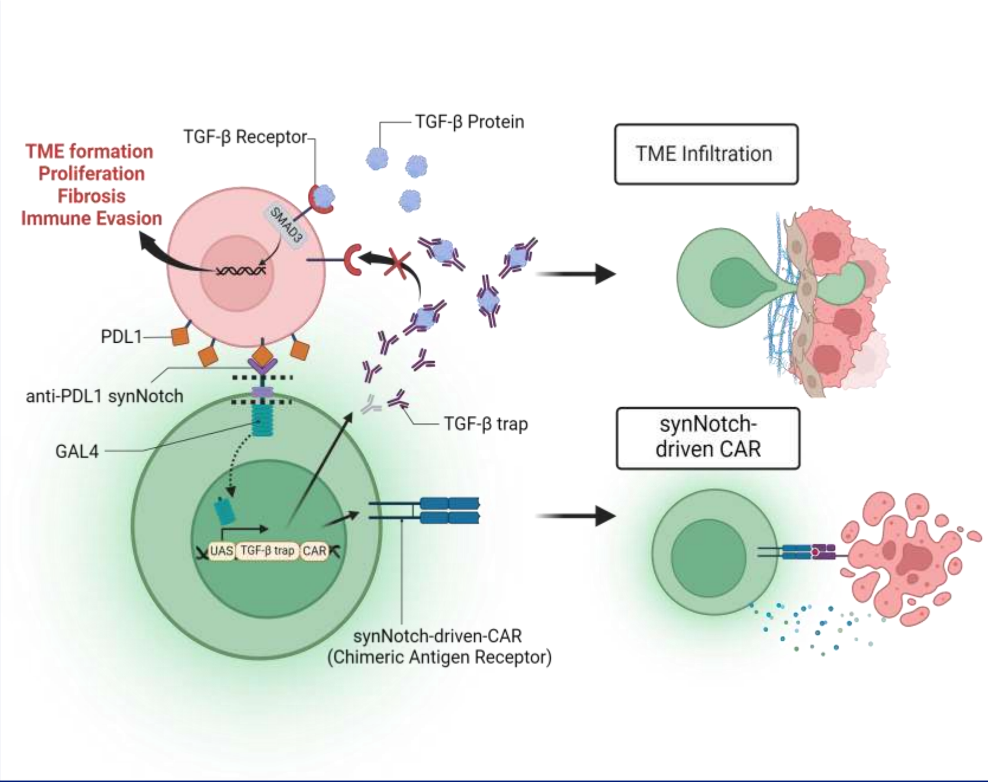
Introduction

Figure 1 I Schematic of overcoming solid TME by Synnotch signaling.



Effectively targeting tumors and cancers has been the endeavor of many scientists. Of the many attempts to cure tumors, chimeric antigen receptor (CAR) T cell therapy has been a revolutionary pillar in cancer therapy. However, CAR-T cell therapy is only effective in hematological cancers such as lymphomas and leukemias and is rendered ineffective in treating solid tumors due to the characteristic of solid tumors creating a TME (tumor microenvironment). To improve CAR-T cell therapy in targeting solid tumors, we constructed the synNotch (synthetic Notch) receptor and incorporated it with the TGF-Beta trap (a molecule that sequesters TGF-Beta protein, inhibiting TGF-Beta receptors) in effort to nullify the TME. To verify whether the system operates effectively, several experiments (tBFP reporter assay, Luciferase assay) were conducted.

Experimental Methods

Figure 2 I Lentiviral transduction and confirmation of synNotch and tBFP reporter gene expression

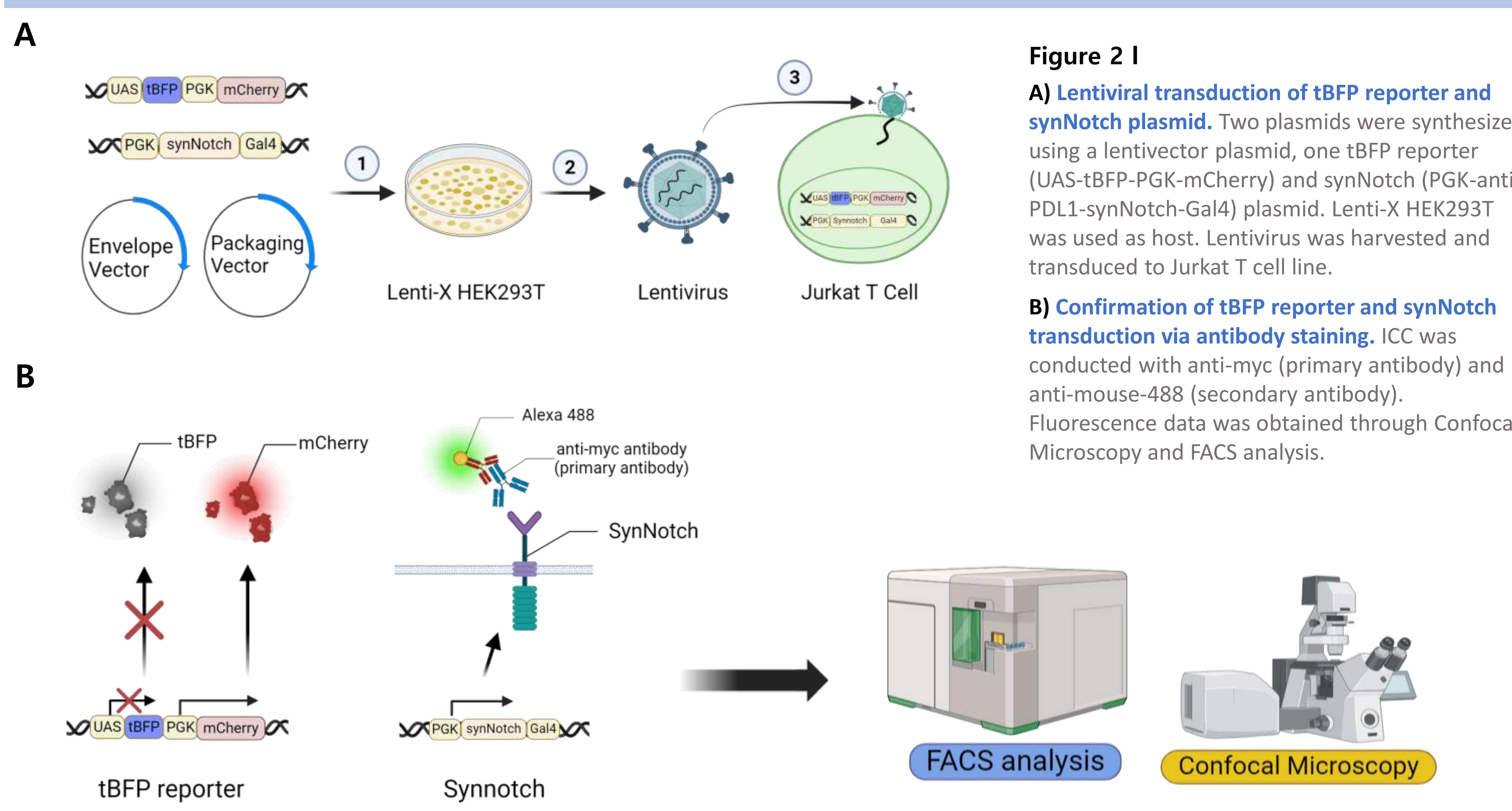
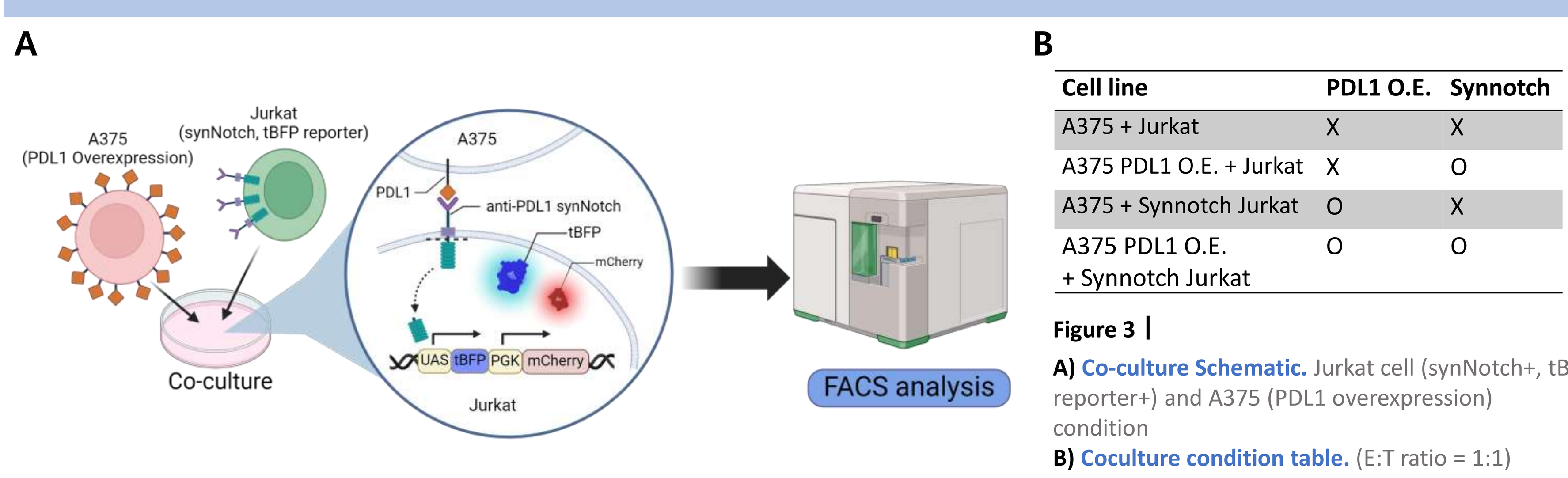


Figure 2 I

A) **Lentiviral transduction of tBFP reporter and synNotch plasmid.** Two plasmids were synthesized using a lentivector plasmid, one tBFP reporter (UAS-tBFP-PGK-mCherry) and synNotch (PGK-anti-PDL1-synNotch-Gal4) plasmid. Lenti-X HEK293T was used as host. Lentivirus was harvested and transduced to Jurkat T cell line.

B) **Confirmation of tBFP reporter and synNotch transduction via antibody staining.** ICC was conducted with anti-myc (primary antibody) and anti-mouse-488 (secondary antibody). Fluorescence data was obtained through Confocal Microscopy and FACS analysis.

Figure 3 I Co-culture for confirming selective binding of synNotch to PDL1 overexpressing A375 cells.



B

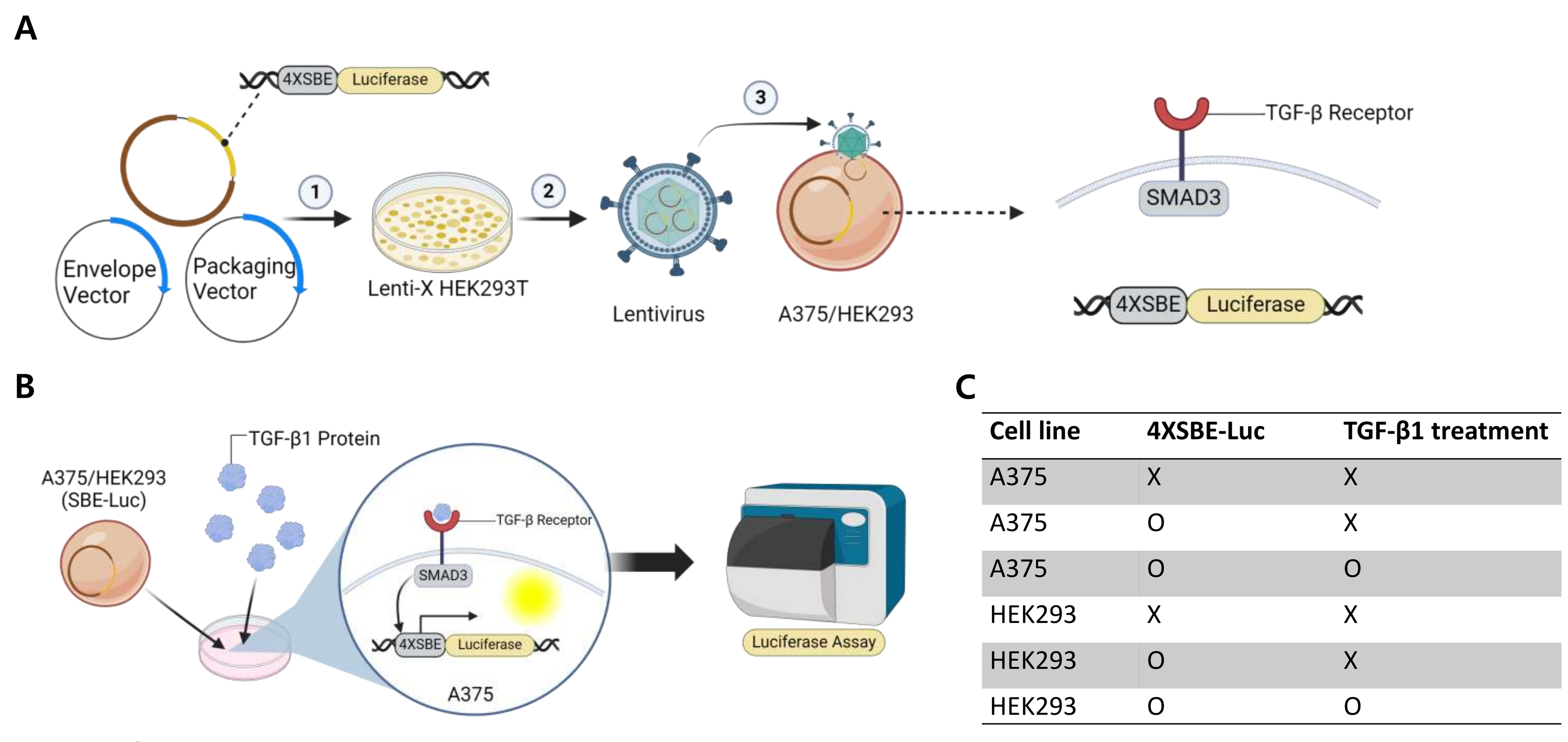
Cell line	PDL1 O.E.	Synnotch
A375 + Jurkat	X	X
A375 PDL1 O.E. + Jurkat	X	O
A375 + Synnotch Jurkat	O	X
A375 PDL1 O.E. + Synnotch Jurkat	O	O

Figure 3 I

A) **Co-culture Schematic.** Jurkat cell (synNotch+, tBFP reporter+) and A375 (PDL1 overexpression) condition

B) **Coculture condition table.** (E:T ratio = 1:1)

Figure 4 I Lentiviral transduction/transfection of SBE-Luc reporter to confirm TGF- β1 induced TGF-Beta SMAD signaling via luciferase assay.



C

Cell line	4XSBE-Luc	TGF-β1 treatment
A375	X	X
A375	O	X
A375	O	O
HEK293	X	X
HEK293	O	X
HEK293	O	O

Figure 4 I

A) **SBE-Luc reporter plasmid preparation / lentiviral transduction of luciferase reporter plasmid.** SBE-Luc reporter was synthesized by infusion cloning with restriction enzyme (EcoR1 and Not1). SBE-Luc reporter was tranduced and tranfected to quantitively measure TGF-Beta signaling via luciferase assay. Transduction was executed with lentiviral transduction and transfection was executed with lipofectamine.

B) **TGF-β1 treatment / Luciferase assay.** TGF-beta1 protein treatment with A375/HEK293 was conducted to verify luciferase reporter function. Luciferin added upon cell lysate to measure photon emission.

C) **Coculture condition table** to confirm function of luciferase reporter as a downstream signal of TGF-Beta signaling.

Reference

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Results

Figure 5 I Jurkat T-cell expresses each fluorescence component after lentiviral transduction

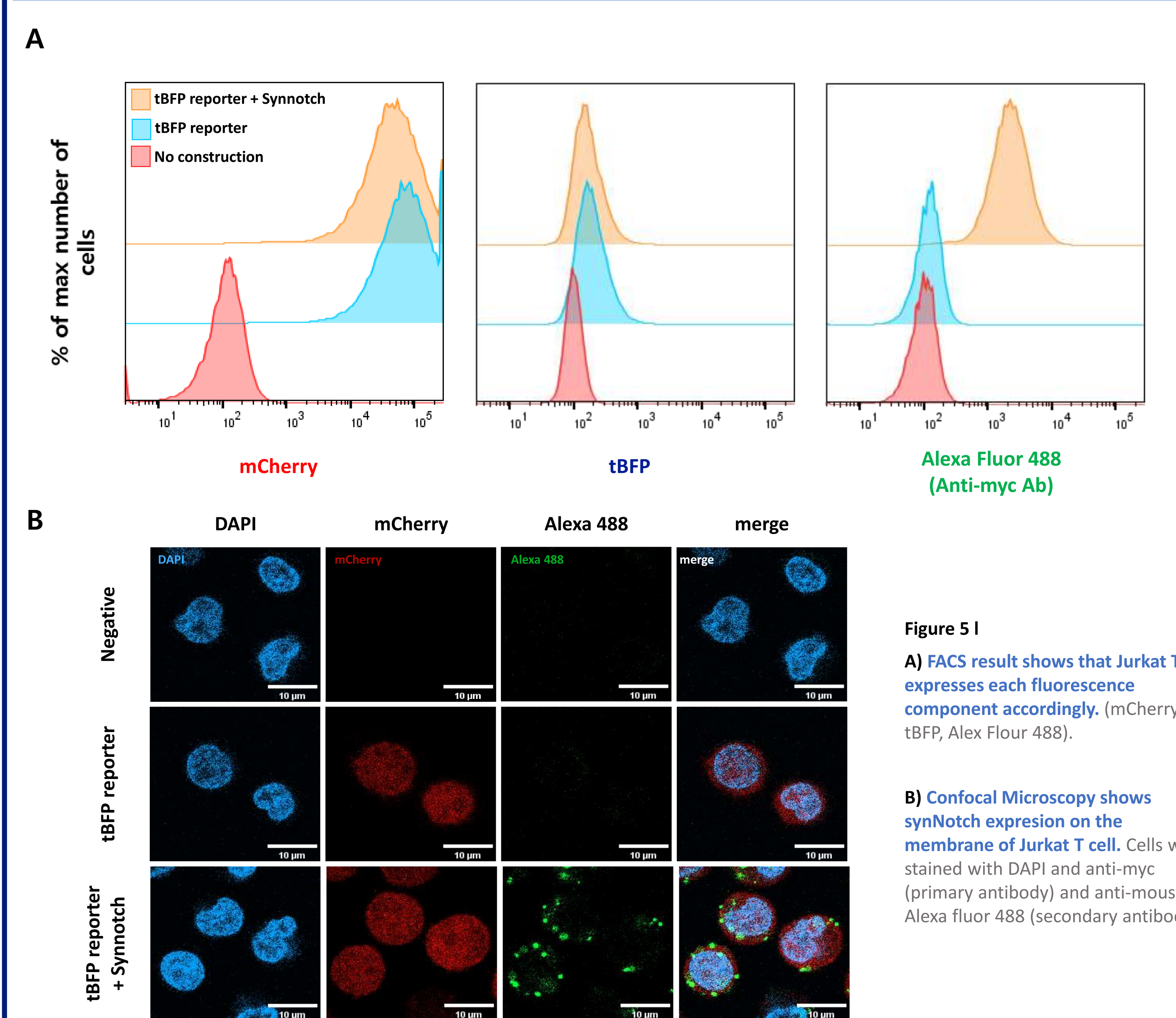

Figure 5 I

A) **FACS result shows that Jurkat T cell expresses each fluorescence component accordingly.** (mCherry, tBFP, Alex Flour 488).

B) **Confocal Microscopy shows synNotch expresion on the membrane of Jurkat T cell.** Cells were stained with DAPI and anti-myc (primary antibody) and anti-mouse Alexa fluor 488 (secondary antibody).

Figure 6 I Jurkat T-cell expresses tBFP selectively when co-cultured with PDL1 overexpressing A375 cells.

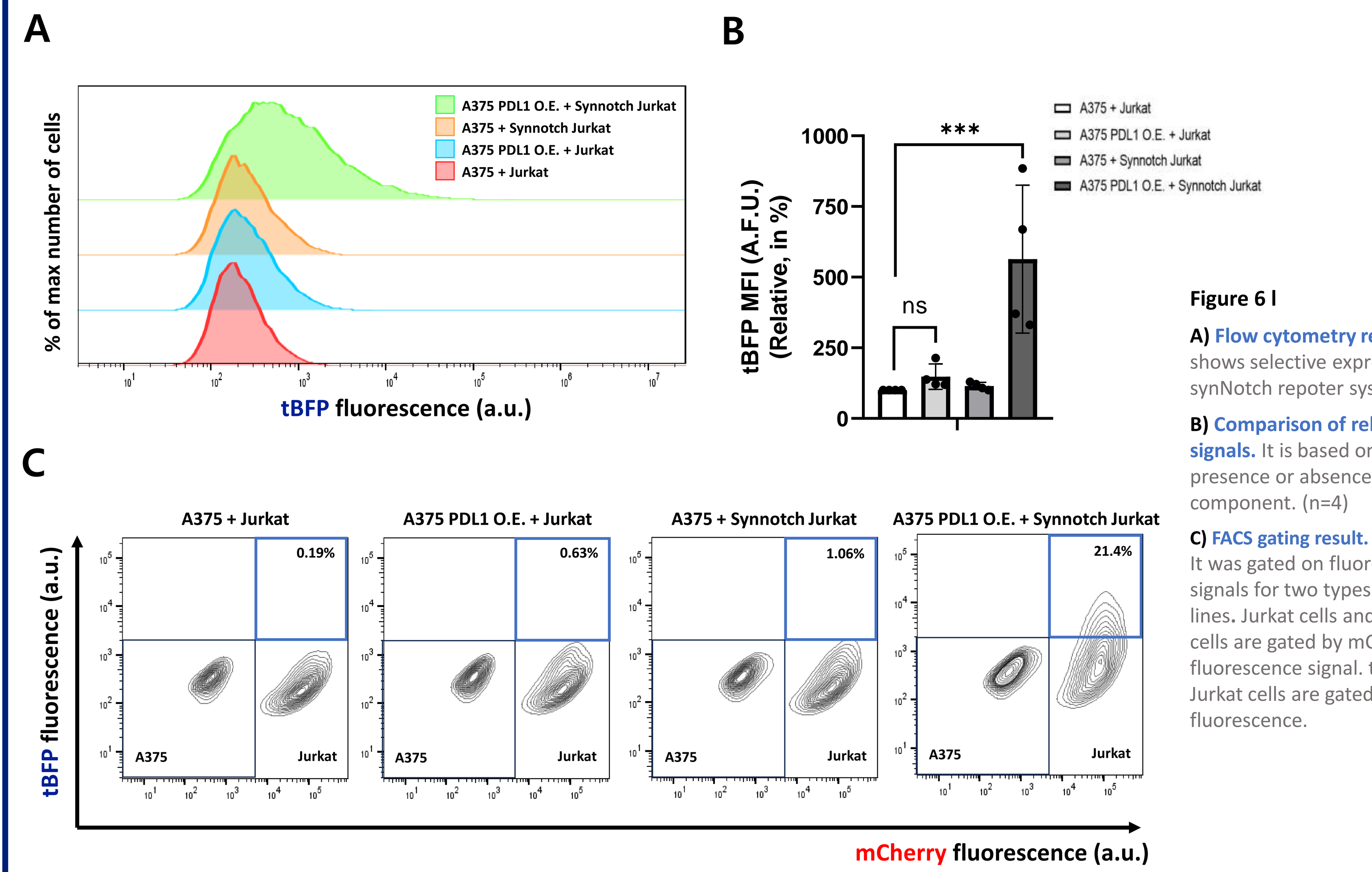

Figure 6 I

A) **Flow cytometry result.** shows selective expression of synNotch reporter system.

B) **Comparison of relative tBFP signals.** It is based on the presence or absence of each component. (n=4)

C) **FACS gating result.** It was gated on fluorescent signals for two types of cell lines. Jurkat cells and A375 cells are gated by mCherry fluorescence signal. tBFP+ Jurkat cells are gated by tBFP fluorescence.

Figure 7 I Luciferase activity increases with TGF- β1 treatment.

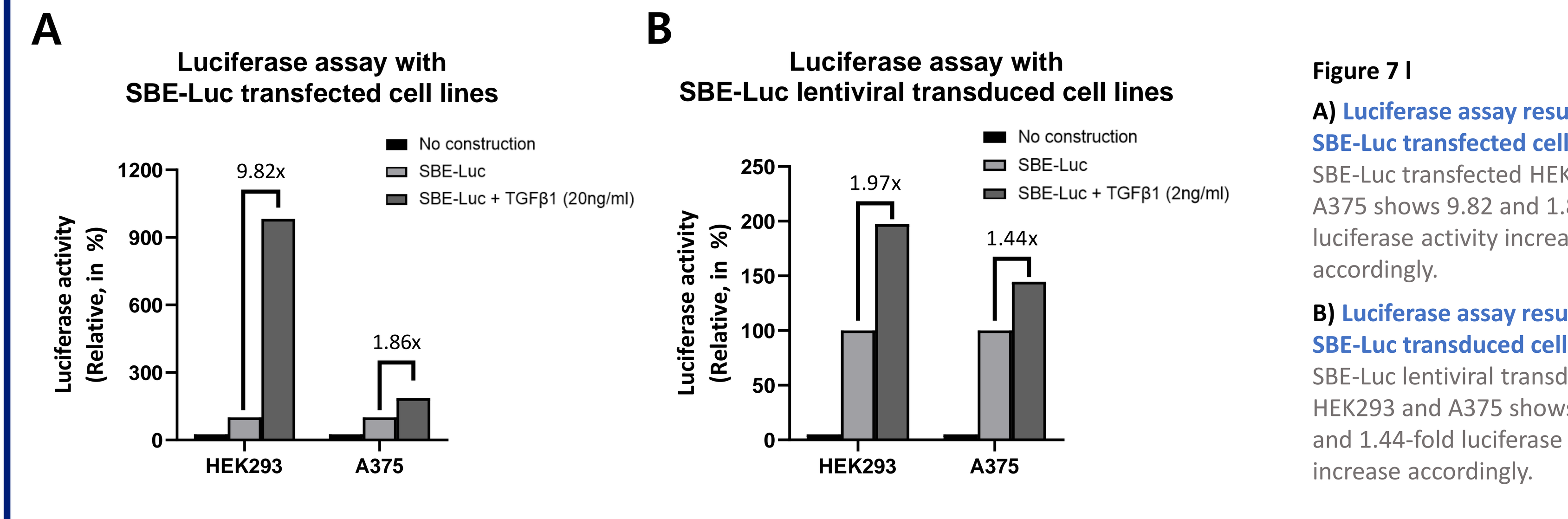

Figure 7 I

A) **Luciferase assay result of SBE-Luc transfected cell lines.** SBE-Luc transfected HEK293 and A375 shows 9.82 and 1.86-fold luciferase activity increase accordingly.

B) **Luciferase assay result of SBE-Luc transduced cell lines.** SBE-Luc lentiviral transduced HEK293 and A375 shows 1.97 and 1.44-fold luciferase activity increase accordingly.

Conclusion & Further Study

Firstly, to confirm the transduction and expression of the synNotch receptor, we conducted flowcytometry analysis confirming that fluorescence components were expressed accordingly (Fig 5 A). The confocal microscopy showed Alexa Floura 488 on the cell membrane of our Synnotch+ Jurkat cell, which suggests that the synNotch receptor is well expressed (Fig. 5 B). After confirming the expression, we experimented on the selective binding of our synNotch receptor to cancer cells. FACS analysis after coculture with each cell lines demonstrated that tBFP signal for the "A375 PDL1 O.E. + Synnotch Jurkat" showed a 9-fold increase than the negative control "A375 + Jurkat" (Fig. 6 A-C). This suggests that the anti-PDL1 synNotch is selectively turned on in an environment where PDL1 is overexpressed, a characteristic of tumor cells *in vivo*.

To verify if TGF-Beta1 protein induces TGF-Beta signaling, we conducted a luciferase assay using our transduced/transfected SBE-Luc A375/HEK293 cell line. The transduced/transfected cells showed an increase in luciferase activity as expected (Fig. 7 A-B). The increase was observed in a concentration, gene insertion, and cell line-dependent manner. The difference in fold increase in transfected and transduced cells can be due to the low efficiency of transduction. Difference in fold increase in HEK293 and A375 cells can be attributed to the fact that HEK293 cells naturally express more of TGF-Beta receptors than A375 melanoma cell lines, thus having more TGF-Beta signaling.

To validate if the TGF-Beta trap protein can be incorporated into the synNotch system to nullify the TME, we are planning to incorporate the TGF-Beta trap as a downstream signal for our anti-PDL1 synNotch instead of the tBFP reporter. Currently, the plasmid necessary for this experiment has been synthesized and coculture has been executed. However, optimization is required at the moment. In good hope, we can hypothesize that this system, if viable and functional, can aid the CAR T-cell therapy regarding solid tumors that have TMEs as an obstacle to overcome. To verify this system and hypothesis, more in vivo and in vitro experiments are necessary along with necessary optimization protocols.