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ANTI-EGFR^{VIII} ANTIBODIES AS A THERAPEUTIC AND DIAGNOSTIC CHALLENGE

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INTRODUCTION

Glioblastoma (GB) is a rapidly developing malignant brain tumor expressing the epidermal growth factor receptor variant III (EGFR^{vIII}). The mutated receptor may be a unique target of CAR-T (chimeric antigen receptors T cell therapy). Due to loss of 267 amino acids in the extracellular domain [1], EGFR^{vIII} has a unique epitope for the L8A4 antibody (able to bind all EGFR^{vIII} conformers), which single-chain variable fragments (scFv) could be used in construct of CAR anti-EGFR^{vIII} in modified T cells.
CAR-T *in vivo* may result in serious side effects such as cytokine release syndrome (CRS). In the course of CRS, a rapid multiplication of CAR-T lymphocytes introduced into the oncological patient's body and sudden release of pro-inflammatory cytokines lead to extensive cytotoxicity and neurotoxicity. In would be desirable to develop a strategy to regulate the occurrence of CRS cytotoxic effects after administration of modified CAR-T cells to patient's body [2].
The activities undertaken as part of the doctoral thesis in the academic year 2022-2023 are aimed at developing a system regulating the expression of chimeric antigen receptor anti-EGFR^{vIII} in modified T cells using chemical compounds naturally occurring in the patient's body (non-xenobiotic). The antagonist will block binding of the transcription factor (TF) present in eukaryotic cells to the inserted anti-EGFR^{vIII} CAR promoter in order to silence CAR expression, and the agonist will activate transcription factor to induce expression of the introduced CAR **(Fig. 1)**.

METHODS

In the first step, the transgene regulation system were tested. For this purpose, vectors encoding reporter genes in place of the CAR sequence were obtained. Plasmid vectors containing sequences of a transcription factor binding site, antibiotic resistance genes in bacterial and eukaryotic cells, and reporter genes – a gene encoding luciferase (Luc) and green fluorescent protein (GFP) were designed. Plasmids were obtained by genetic engineering methods. The correctness of plasmids was confirmed using restriction enzymes, electrophoretic separation and Sanger sequencing. Then, the viral vectors (encoding the transcription factor binding site, drug resistance, Luc, GFP) were obtained. The vectors were added to the culture of HEK-293T adherent cells (showing the highest expression of a given transcription factor among the analyzed cell lines). An antibiotic selection was performed. The cells were expanded and treated with an agonist and an antagonist inducing or blocking transcription factor binding to the promoter of Luc gene. The effects of the agonist/antagonist were verified by analyzing the level of luminescence **(Fig. 2)**.

RESULTS

The results of analyses are presented in **Fig. 3** and **Fig. 4.**
AG1 and AG2 induced a significant increase of luminescence in HEK-293T cells with introduced transcription factor TF1 and TF2 binding sites, respectively (statistically significant results). The addition of ANT1 or ANT2 antagonists and combinations of AG1+ANT1 or AG2+ANT2 reduced the level of luminescence.

CONCLUSION

In conclusion, the application of proposed regulation systems may prove to be an effective strategy to control the expression of chimeric antigen receptor in CAR-T cells *via* the appropriate agonists/antagonists of transcription factors binding the transgene promoter.
Further analyses need to be performed in order to obtain target vectors coding anti-EGFR^{vIII} CAR (not the reporter genes) together with CAR regulation system in T cells. The modified cells must be analyzed for the ability to eliminate the population of EGFR^{vIII}-positive cells.

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1. *Rutkowska A, Stoczyńska-Fidelus E, Janik K, Włodarczyk A, Rieske P. EGFR^{vIII}: An Oncogene with Ambiguous Role. J Oncol. 2019;2019:1092587. Published 2019 Dec 16. doi:10.1155/2019/1092587*
2. *Kierasińska A, Ciunowicz D, Węgierska M, Stoczyńska-Fidelus E, Rieske P. CAR-T therapy in oncology and other fields of medicine. Alergologia Polska - Polish Journal of Allergology. 2021;8(2):77-91. doi:10.5114/pja.2021.106685.*

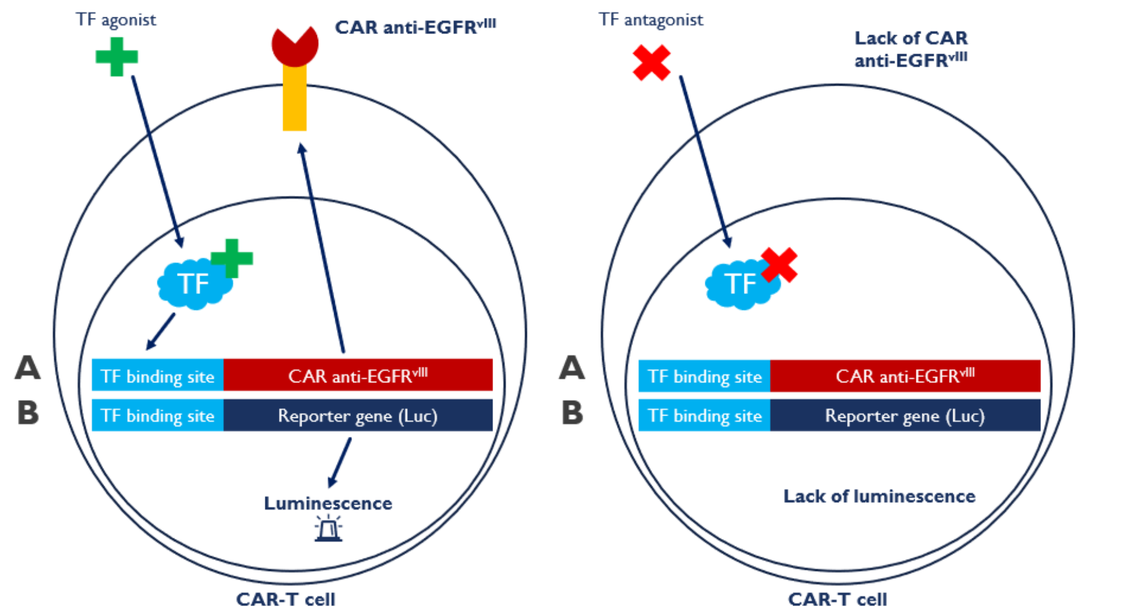

Fig. 1. Model of transgene regulation system. **(A)** Regulation of CAR anti-EGFR^{vIII} expression. **(B)** Regulation of Luciferase (Luc) reporter gene expression. *TF – transcription factor; CAR – chimeric antigen receptor; Luc – luciferase*

1. Production of plasmid vectors encoding transcription factors (TF1 and TF2) binding sites, antibiotic resistance, reporter genes – GFP and Luc.

2. Production of lentiviral vectors encoding TF1/TF2 binding sites, antibiotic resistance, GFP, Luc.

3. Introduction of viral vectors to HEK-293T adherent cells.

4. 24-hour incubation of HEK-293T wild-type (control) and HEK-293T cells with introduced TF1 binding site to agonist (AG1; 0.25 μM) and antagonist (ANT1; 10 μM) of TF1.

5. 72-hour exposure of HEK-293T with introduced TF2 binding site to agonist (AG2; 1 μM) and antagonist (ANT2; 1 μM) of TF2.

6. Cell culture on 96-well plates, 10-minutes incubation with luciferin (1:200) and luminescence measurement.

Fig. 2. Procedures conducted to analyze the effectiveness of transgene regulation system.

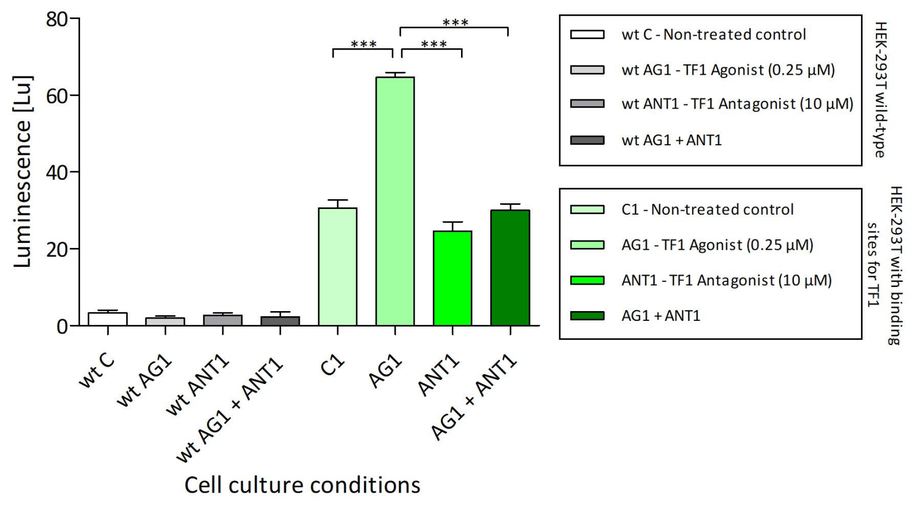

Fig. 3. Analysis of the luminescence level after 24-hour incubation of HEK-293T wild-type and HEK-293T cells with introduced TF1 binding sites with the transcription factor AG1 agonist and/or ANT1 antagonist. *wt C, C1 – control (cells not incubated with TF1 agonist/antagonist); Statistical significance: $p < 0.05$, "*"; $p < 0.01$, "**"; $p < 0.001$, "***".*

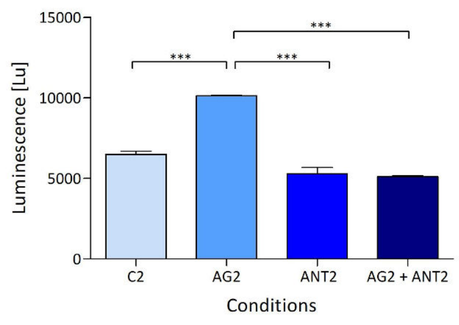

Fig. 4. Result of the luminescence level after 72-hour incubation of HEK-293T with introduced TF2 binding sites with the transcription factor AG2 agonist and/or the ANT2 antagonist. *C2 – control (cells not incubated with TF2 agonist/antagonist), AG2 – TF2 agonist (1 mM), ANT2 – TF2 antagonist (1 mM); Statistical significance: $p < 0.05$, "*"; $p < 0.01$, "**"; $p < 0.001$, "***".*

Scientific achievements and other activity directly related to implementation of doctoral dissertation

Publications:

1. *Włodarczyk, A., Tręda, C., Rutkowska, A., Grot, D., Dobrewa, W., **Kierasińska, A.,** Węgierska, M., Wasiak, T., Strózik, T., Rieske, P., & Stoczyńska-Fidelus, E. (2022). Phenotypical Flexibility of the EGFRvIII-Positive Glioblastoma Cell Line and the Multidirectional Influence of TGFß and EGF on These Cells-EGFRvIII Appears as a Weak Oncogene. International journal of molecular sciences, 23(20), 12129. https://doi.org/10.3390/ijms232012129*
2. *Tręda, C., Włodarczyk, A., Pacholczyk, M., Rutkowska, A., Stoczyńska-Fidelus, E., **Kierasińska, A.,** & Rieske, P. (2023). Increased EGFRvIII Epitope Accessibility after Tyrosine Kinase Inhibitor Treatment of Glioblastoma Cells Creates More Opportunities for Immunotherapy. International journal of molecular sciences, 24(5), 4350. https://doi.org/10.3390/ijms24054350*