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Short communication

Design and characterization of a salicylic acid-inducible gene expression system for Jurkat cells

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ABSTRACT

With continued progress in cell and gene therapies, there is an immediate need for exogenously tunable gene expression systems with safe and predictable behavior in specific human cell types. Here, we demonstrate the ability of the salicylic acid (SA)-inducible MarR repressor protein from *Escherichia coli* to regulate target gene expression in a human T lymphocyte cell line. Two lentiviral vectors, one encoding an enhanced green fluorescent protein (EGFP) reporter cassette and the other a repressor cassette, were sequentially transduced into Jurkat cells, using fluorescence-activated cell sorting (FACS) to isolate stable Jurkat progeny. As a result, EGFP expression was repressed by MarR and was inducible upon the addition of SA (~1.3 fold). This represents the first example of functional expression of bacterial MarR in mammalian cells, and opens the possibility for further development of regulated, SA-tunable gene expression system for T-cells.

1. Introduction and results

Gene regulatory systems that offer reliable and predictable control over gene expression in mammalian cells have broad applications that include basic biological discovery, epigenetic remodeling, and performing therapeutic tasks (Doshi et al., 2020; Kallunki et al., 2019; Lebar and Jerala, 2018). We are interested in drug-inducible gene expression systems for use in T-cells. The potential of engineered T-cells has been recognized for the treatment of many hematologic malignancies (Holstein and Lunning, 2020), yet their therapeutic impact is limited by the risk of life-threatening complications such as cytokine release syndrome (CRS) and CAR T-cell-related encephalopathy syndrome (CRES) (Neelapu et al., 2018). In this context, a handful of drug-inducible systems have been developed to enable either controlled elimination or controlled activation of engineered T-cells and thus, increase the safety of these T-cells in therapy (Diaconu et al., 2017; Zhou et al., 2015; Bonini et al., 1997; and Chakravarti et al., 2019). Two recent studies describe Rimiducid-inducible expression of caspase-9 (iC9) in engineered "iC9. CD19. CAR-T" cells, aiding in dose-dependent ablation of the engineered CAR-T cells to prevent toxic events (like CRS), without complete elimination of these engineered T-cells (Diaconu, and Zhou et al., 2017, 2015). Loss of sensitivity to Rimiducid in engineered cells in vivo can potentially block the elimination of activated T-cells and undermine the safety of this system (Bonini et al., 1997). Several tetracyclines-inducible (TetR-based) platforms have also been developed to regulate target genes in the model human T-cell line, Jurkat cells (Rad et al., 2020, Andrea et al., 2020, and Gu et al., 2018). However, antibiotics, including tetracyclines, may elicit changes in cell behavior (Ahler et al., 2013; Kuzin et al., 2001; Michaels et al., 2019), and their continuous administration can lead to unwanted antibiotic-resistant microorganisms (Gu et al., 2018). Food and cosmetic additives are an attractive class of small-molecule inducers with favorable safety profiles. Recently, Bai et al. designed a menthol-inducible system that increased SEAP secretion in Jurkat cells (3.7-fold) (Bai et al., 2019). This system however relies on the nuclear factor of activated T cells (NFAT) and is thus prone to cross-talk upon activation of T cells, leading to SEAP expression even in the absence of menthol. Engineered transcriptional systems that are of non-human origin, and thus not prone to cross-talk by endogenous signals, are valuable for synthetic cellular and gene therapy applications. Furthermore, simultaneous, independent, and inducible expression of multiple genes likely requires implementing multiple orthogonal modes of control (Stanton et al., 2014). It is therefore

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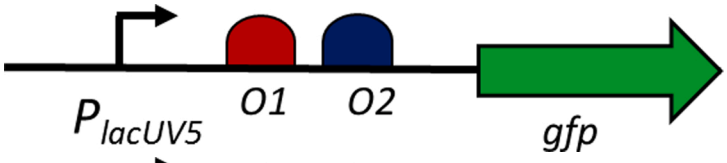
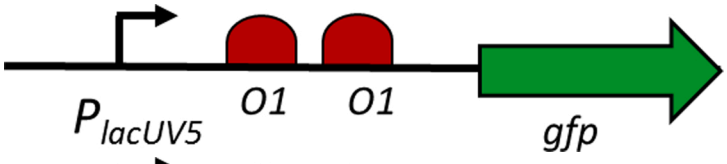
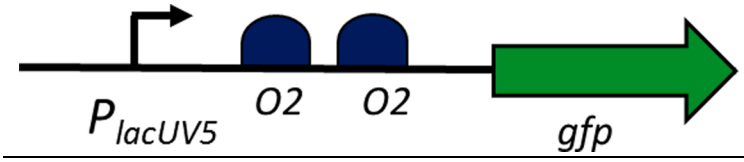
Table 1
Different combinations of *marO1* (O1) and *marO2* (O2) sites downstream of the lacUV5 promoter demonstrate MarR-dependent repression and SA-inducible GFP expression in *E. coli*. "Background GFP" represents fluorescence (RFU/OD_{595}) measured in the absence of inducer. "Fold-induced GFP expression" is (normalized) fluorescence in the presence of SA divided by background GFP. Data points are average of three values ± SD.

Construct (schematic)	Background GFP expression	Fold-induced GFP expression with 2 mM SA
	69 ± 1	10 ± 1
	149 ± 5	10 ± 1
	143 ± 6	9 ± 0.4

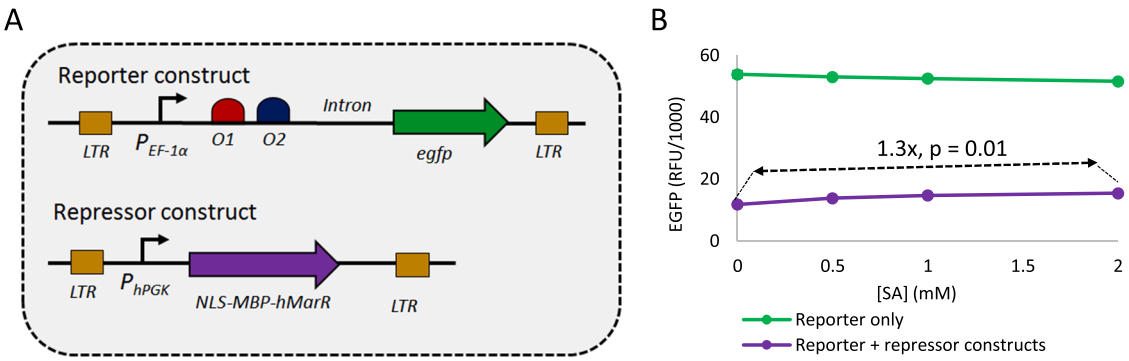


Fig. 1. SA-inducible EGFP expression in human Jurkat cell lines. A) Gene constructs used for SA-inducible gene expression in Jurkat cells. Reporter and the repressor proteins were expressed from two separate third-generation lentiviral backbone plasmids. B) EGFP fluorescence (RFU) in Jurkat cells carrying either reporter alone, or the reporter + repressor construct, was measured at 24 h post-induction in SA-containing media. All data points are the average of three values and error bars represent the SD. The reported p-value was determined using a two-tailed, Student's t-test between indicated datasets. Cells expressing the reporter + repressor constructs showed a 1.3-fold increase in EGFP fluorescence in the presence vs. absence of 2 mM SA.

important to develop additional gene regulation systems for mammalian cells to expand the synthetic biology toolbox and potentially address limitations of existing tools for clinical applications.

Salicylic acid (SA) is a naturally occurring, bioactive compound and hormone involved in plant immune responses (Malakar, 2017). SA and related derivatives such as acetylated SA (Aspirin) are known for their analgesic, anti-pyretic, and/or anti-inflammatory effects in humans (Gaziano and Greenland, 2014). Salicylates are inexpensive, easily available over the counter, and safe when used at therapeutic doses (1.1–2.2 mM) (Arif and Aggarwal, 2020). Here we describe the design of a SA-inducible transgene expression system directly in Jurkat cells, using lentiviral transduction. Our SA-inducible gene switch is based on the *Escherichia coli* "multiple antibiotic resistance repressor protein" (MarR). In *E. coli*, MarR represses transcription by binding (as a homo-dimer) DNA operators *marO1* and *marO2* (Duval et al., 2013). Upon binding inducer (SA and a narrow range of other phenolic compounds (Alekshun and Levy, 1999a, 1999b)), MarR interaction with operators is relieved, allowing for transcriptional activation of downstream genes (Duval et al., 2013).

marO1 and *marO2* contain the same palindromic repeat (5′-TTGCC-3′) but have different flanking sequences (Fig. S1). To investigate the optimal placement of operator sites, we first designed different MarR-regulated GFP expression plasmids. Each construct contained the lacUV5 promoter followed by one of three different combinations of operators: *marO1-marO2, marO1-marO1,* or *marO2-marO2*. We measured the extent of fluorescence repression with and without SA in *E. coli* (strain JW5248–1). While similar levels of GFP fluorescence were observed for all three constructs, the *marO1-marO2* combination showed tightest repression (lowest background GFP fluorescence in the absence of inducer), as shown in Table 1 and Table S2. We therefore chose the *marO1-marO2* sequence of operators for engineering an SA-inducible promoter for Jurkat cells.

To test the MarR-regulated SA-inducible (SA_{ON}) system in Jurkat cells, we developed a two-component transcription repression circuit inspired by the well-characterized TetR-based mammalian expression system (Nevozhay et al., 2013). The first component consisted of a lentiviral vector reporter construct, containing the *marO1-marO2* sequence downstream of the strong human elongation factor 1 alpha promoter (EF-1α), controlling expression of the gene encoding EGFP (*egfp*) (Fig. 1A and Fig. S1). For the second component, functional MarR expression in Jurkat cells was accomplished by placing an NLS-MBP-hMarR construct (encoding human codon-optimized MarR fused to the simian virus 40 (SV40) NLS tag and a maltose binding protein (MBP) domain, both at the N-terminus) under control of the

human phosphoglycerate kinase-1 (PGK-1) promoter (Fig. 1A), on a second lentiviral vector. The MBP domain was used as it has been shown to enhance functional, heterologous protein expression in mammalian cells (Reuten et al., 2016). A polyclonal population of Jurkat cells stably carrying these repressor / EGFP expression constructs was thus generated via a sequential, two-step lentiviral transduction approach (see Methods in supplementary file).

The gene expression profiles of cell sub-clones bearing either the single reporter or double reporter + repressor constructs were assessed after incubation for 24 h at various levels of SA, up to the maximum physiologically tolerated concentration (0, 0.5 mM, 1 mM, and 2 mM) (Arif and Aggarwal, 2020). The single reporter construct showed robust constitutive expression of EGFP irrespective of the SA levels, illustrating functional EF-1α promoter in Jurkat cells (Fig. 1B, green line and Table S3). Conversely, cells with the reporter + repressor construct showed significantly diminished expression of EGFP in the absence of inducer, demonstrating transcriptional repression of EF-1α promoter by MarR in Jurkat cells (Fig. 1B, purple line and Table S3). Upon addition of progressively higher concentrations of SA, EGFP expression in cells bearing the reporter + repressor construct increases accordingly, resulting in overall 1.3-fold induction ($p\text{-value} = 0.01$, $n = 3$) of expression between 0 and 2 mM SA (Fig. 1B and Fig. S2). While fluorescence did not plateau at 2 mM SA, and fold-induction may be greater at higher SA concentrations, changes in cell morphology and rapidly decreased cell viability in the presence of > 2 mM SA precludes such determination. While the dynamic range is low, this is the first demonstration of an SA-inducible system directly used in human T cells. MarR-based regulation in Jurkat and other mammalian cells has the potential for a variety of improvements, for example through designing and implementing MarR variants having greater sensitivity to SA, further optimization of promoters, introduction of transcriptional and translational enhancers, and designing fusions with transcriptional silencing or activation domains (Nevozhay et al., 2013).

2. Discussion

In recent years, various synthetic gene regulation systems providing tunable control over transcription of target genes have been designed. Developing such systems with high specificity (orthogonality and minimal off-target effects) and tight regulation (low background and wide dynamic range) is essential to most applications. Transferring gene regulation tools to mammalian cells is met with the challenges of functional and adequate heterologous expression. In often 'problematic' primary or immune cells this challenge is further compounded by difficulties associated with preserving gene regulatory capabilities of heterologous regulatory elements (Bai et al., 2019). Designing expression systems directly in T-cells avoids potential pitfalls encountered when these are first developed in an alternate cell line, prior to transferring to T-cells. Here, the *E. coli* MarR protein was shown to retain repressive function and provide some level of target gene expression regulation in Jurkat cells, by controlling the availability of SA. While fold-inducibility with the current construct is low, this is likely attributed to insufficient relief of repression, which in turn is constrained by SA toxicity. This may be overcome by engineering a MarR variant that is more sensitive to SA, or testing the use of alternate, less-toxic inducers known to interfere directly with MarR function (Alekshun and Levy, 1999a, 1999b). Alternative designs can also be adopted to improve the dynamic range, for example by promoter optimization, by fusion of a trans-silencing domain (e.g. KRAB) to the repressor construct, or by implementing negative feedback (Nevozhay et al., 2009).

CRediT authorship contribution statement

Aarti Doshi: Investigation, Formal analysis, Visualization, Writing – original draft. **Irfan Bandey:** Investigation. **Dmitry Nevozhay:** Writing – review & editing, Visualization. **Navin Varadarajan:** Supervision, Resources, Funding acquisition. **Patrick C. Cirino:** Supervision, Resources, Writing – review & editing, Visualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2022.01.003.

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