Engineering a Gene Circuit-Enabled Cell Therapy with a Tamoxifen Regulated Safety Switch for Inducible Cell Death in Human Pluripotent Stem Cells and their Derivatives

Chew-Li Soh[1], Rebecca Cottman[2], Conor McAuliffe[1], Vanessa Johnson[2], Michelle Hung[2], Yin Yin Chong[2], Noah Jacobs-Rebhun[1], Cloe Grace[1], Jeffrey Levin[1], Tackla Winston[1], Mark Ebel[1], Eren Chu[2], Assen Roguev[2], Russell Gordley[2], Tim Lu[2], Mark Tomishima[1]

[1]BlueRock Therapeutics, New York, NY, USA

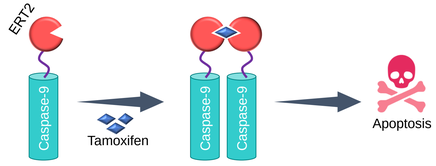
[2]Senti Biosciences, Inc., South San Francisco, CA, USA

INTRODUCTION

Therapeutic cell products derived from human pluripotent stem cells (hPSCs) can be used to replace lost cells and therefore restore function and reverse disease for an array of clinically intractable conditions. While cell therapies have the potential to change the practice of medicine, all such “living” drugs carry potential risks. One safeguard against these risks is the implementation of a safety switch that can ablate transplanted cells from a patient if desired.

We designed a novel gene circuit which functions as a Safety Switch regulated by tamoxifen, an FDA approved drug with extensive clinical history and ability to cross the blood-brain-barrier. The gene circuit-engineered Safety Switch is composed of a small molecule (SM) binding domain (ERT2) fused to Caspase-9 (Casp9) which dimerizes in the presence of tamoxifen metabolites, initiating the apoptotic pathway and cell death.

A tamoxifen-regulated Safety Switch

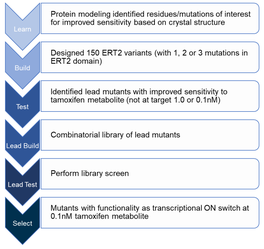


ERT2 MUTANT IDENTIFICATION & SAFETY SWITCH SCREEN

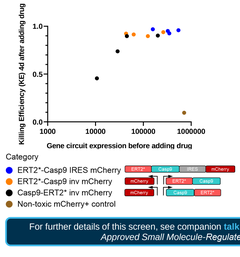
To ensure the Safety Switch would be translatable to the clinic, we aimed to ensure the ERT2 SM binding domain would dimerize at concentrations of tamoxifen metabolites present in the brain at FDA-approved doses of tamoxifen. To address this, we computationally identified mutations within the SM binding region of ERT2 to build a large combinatorial library. Screening of the combinatorial library produced hundreds of ERT2 mutants that were further evaluated for improved drug sensitivity in the context of a synthetic transcription factor and activation of an mCherry reporter gene.

Four representative engineered ERT2 mutants demonstrating improved sensitivity to tamoxifen metabolites (4-OHT and Endoxifen) that were identified from the transcription factor/reporter screen were next tested in the context of the Safety Switch. All four engineered ERT2-Casp9 candidates showed greater than 95% killing efficiency after the addition of 1μM 4-OHT in HEK293T cells. Effective killing was also observed after combination treatment with therapeutically translatable doses of tamoxifen metabolites (0.4nM 4-OHT and 2.5nM Endoxifen).

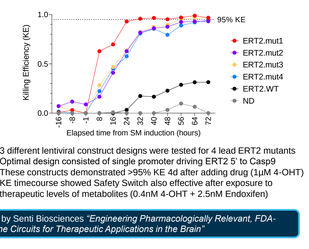
Computational modeling and screening to identify ERT2 mutations with improved sensitivity to tamoxifen metabolites



Different expression formats of mCherry



KE comparison of ERT2.muts v ERT2.wt using the optimal design at 0.4nM 4-OHT & 2.5nM Endoxifen



- 3 different lentiviral construct designs were tested for 4 lead ERT2 mutants
- Optimal design consisted of single promoter driving ERT2 5’ to Casp9
- These constructs demonstrated >95% KE 4d after adding drug (1μM 4-OHT)
- KE timecourse showed Safety Switch also effective after exposure to therapeutic levels of metabolites (0.4nM 4-OHT + 2.5nM Endoxifen)

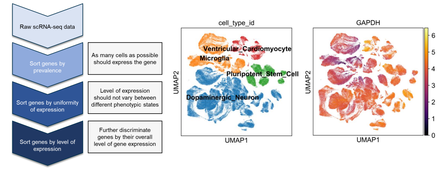
For further details of this screen, see companion talk #150 by Senti Biosciences *“Engineering Pharmacologically Relevant, FDA-Approved Small Molecule-Regulated Gene Circuits for Therapeutic Applications in the Brain”*

METHOD FOR hPSC ENGINEERING

To maximize the expression of the Safety Switch gene circuit, we engineered it into hPSCs using the Sustained Transgene Expression Loci (STEL) platform for robust, stable and ubiquitous expression of biological cargo. Long-term transgene expression within a cell remains a challenge because an introduced transgene may be subject to DNA methylation and histone modifications during prolonged culture and differentiation that causes transgene silencing. The STEL search identified transgene integration sites that permitted sustained transgene expression in hPSCs and their derivatives.

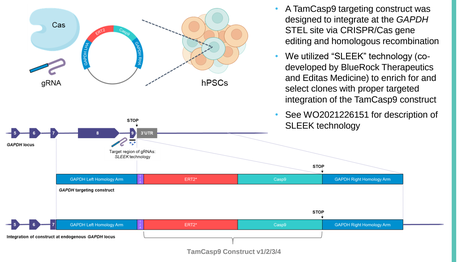
We selected the STEL site, *GAPDH*, for expression of the TamCasp9 Safety Switch. Briefly, we used CRISPR/Cas to engineer the four lead ERT2-Casp9 candidates in-frame after the coding sequence of the endogenous *GAPDH* gene via a 2A peptide. *GAPDH* remains active in a cell, thus allowing expression of the Safety Switch to be constitutive and sustained in hPSCs and their derivatives.

scRNA-seq to identify Sustained Transgene Expression Loci (STEL) for stable long-term transgene expression in hPSCs and their derivatives



- scRNA-seq dataset was collected from undifferentiated hPSCs, and differentiated dopaminergic neurons, ventricular cardiomyocytes and microglia at various timepoints
- Ranked genes by highest prevalence, uniformity of expression and highest level of expression
- Identified several genes termed “STEL” that demonstrated sustained and robust expression in different cell types and during the course of differentiation
- This included the essential gene *GAPDH*
- See WO2021072329 for description of STEL platform

Expression of the TamCasp9 Safety Switch gene circuit using STEL

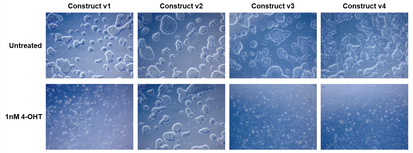


- A TamCasp9 targeting construct was designed to integrate at the *GAPDH* STEL site via CRISPR/Cas gene editing and homologous recombination
- We utilized “SLEEK” technology (co-developed by BlueRock Therapeutics and Editas Medicine) to enrich for and select clones with proper targeted integration of the TamCasp9 construct
- See WO2021226151 for description of SLEEK technology

SAFETY SWITCH ACTIVATION IN hPSCs

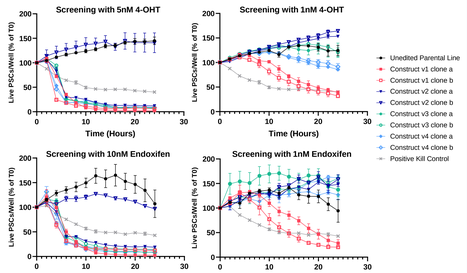
Clonally derived TamCasp9 hPSC lines demonstrated induced apoptosis after administration of the tamoxifen metabolites, 4-OHT and Endoxifen, at nanomolar concentrations. Differential sensitivity in Safety Switch activation was observed among TamCasp9 constructs comprised of different ERT2 mutants.

Testing Safety Switch activation in clonally derived *GAPDH::ERT2-Casp9* (TamCasp9) hPSC lines



- Brightfield images showing clonally derived hPSC cultures containing either TamCasp9 Construct v1-4 after 48 hours of treatment with 1nM 4-OHT or left untreated for the same duration

TamCasp9 hPSC lines demonstrate induced apoptosis with different sensitivities after drug treatment

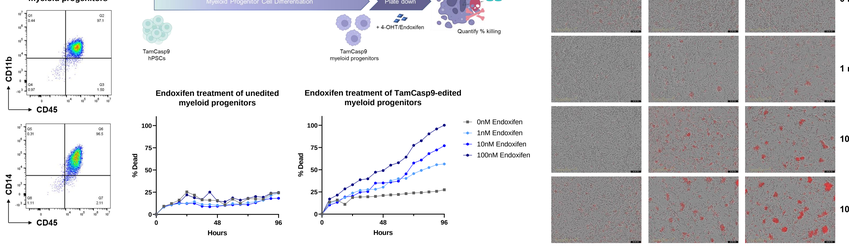


- Graphs demonstrating apoptotic efficacy after tamoxifen metabolite treatment of hPSCs targeted with 4 different lead TamCasp9 constructs. Two different clones for each construct were tested. Clones were compared to an unedited parental control line and a line edited with Construct v1 that had been treated with 0.4 mg/mL hygromycin as a positive kill control. Most clones treated with 5nM 4-OHT or 10nM Endoxifen display apoptotic efficacy, with differential sensitivity observed at 1nM.

SAFETY SWITCH ACTIVATION IN MYELOID PROGENITORS

To validate conservation of Safety Switch function post-differentiation, hPSCs engineered with the TamCasp9 Construct v1 Safety Switch were differentiated into myeloid progenitor cells and shown to have induced apoptosis following treatment with Endoxifen.

The Safety Switch can be activated following differentiation of TamCasp9-edited hPSCs to the myeloid lineage



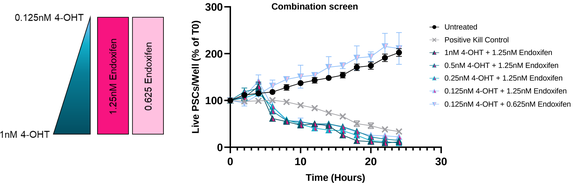
- Graphs and brightfield images showing activation of the Safety Switch following efficient differentiation of TamCasp9 hPSCs into myeloid progenitor cells and addition of Endoxifen. Cells were stained with AO/PI, with dead cells appearing red after uptake of Propidium Iodide. A dose response can be seen with higher concentrations of Endoxifen, while cells that do not contain the Safety Switch remain unaffected.

CONCLUSIONS & FUTURE WORK

We are currently determining Safety Switch sensitivity by performing titrations with 4-OHT and Endoxifen, alone and in combination in myeloid progenitor cells. Synergistic activation of the Safety Switch in hPSCs was able to further lower the minimally effective dosage of both metabolites to the level expected to be present in the brain at FDA-approved doses of tamoxifen administration, and we expect a similar effect for differentiated cells.

Overall, we have demonstrated that hPSCs harboring a novel TamCasp9 gene circuit-engineered Safety Switch mechanism expressed robustly from the *GAPDH* locus can allow hPSCs and differentiated cells to be removed with pharmacologically relevant concentrations of tamoxifen metabolites.

4-OHT/Endoxifen combination treatment in hPSCs lowers the minimally effective dosage of both metabolites



- Graph depicting apoptotic efficacy after treatment of TamCasp9 Construct v1 clone b hPSCs with combinations of 4-OHT and Endoxifen
- 4-OHT/Endoxifen combination treatment will next be tested in TamCasp9-edited myeloid progenitor cells