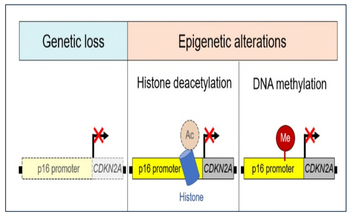
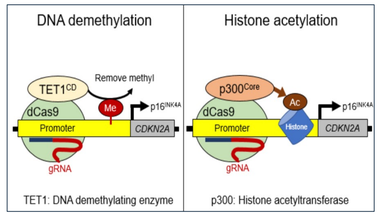
CRISPR-Bac Activation of CDKN2A to Inhibit Skin Cancer

Anh Huynh[1], Takuma Uo[2], Masaoki Kawasumi[2]
[1]University of Washington School of Medicine, Wyoming WWAMI; [2]University of Washington Department of Dermatology

BACKGROUND

Biallelic inactivation of the *CDKN2A* gene and loss of the encoded protein, $p16^{INK4A}$, is a key event involved in the transition to invasive behavior in skin carcinogenesis. Aberrant hypermethylation of CpG islands in promoter of $p16^{INK4A}$ and histone modification are common mechanisms for *CDKN2A* inactivation. We hypothesized that reactivation of *CDKN2A* gene would restore $p16^{INK4A}$ expression and inhibit growth of skin cancer cells.





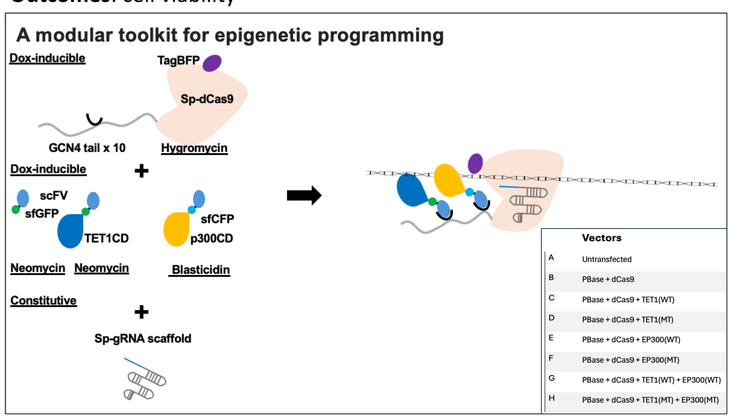
METHODS

Study Design: CRISPR-Bac expression system

- PiggyBac cargo vector contained 1) a doxycycline-inducible dCas9, 2) TET1 (WT or mutant) or EP300 (WT or mutant), 3) antibiotic resistance gene, and 4) fluorescent molecule
- 48 hrs after transfection, cells were treated with antibiotics to select for cells containing the vector
- Stable cell lines were treated with doxycycline to induce expression of TET1, EP300 or both
- 24 hrs after doxycycline, cells were examined with fluorescent microscope
- Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8)
- All steps were optimized using SK-MEL2 cells

Cell line: SK-MEL2 (a human melanoma cell line)

Outcomes: cell viability



RESULTS

Dose Response Curve for Antibiotic Selection of SK-MEL2

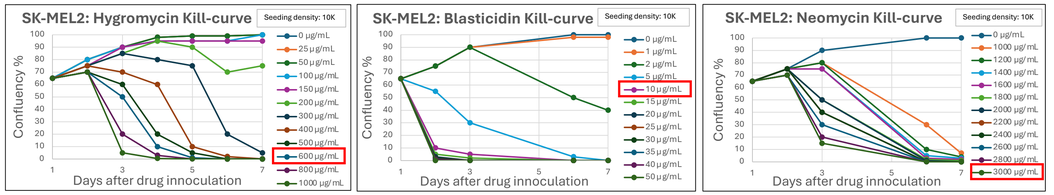


Figure 1: Optimization of antibiotic dose needed to kill all the untransfected cells. Doses tested on 2.5K, 5K and 10K seeding densities (10K curves shown).

Transfection Optimization

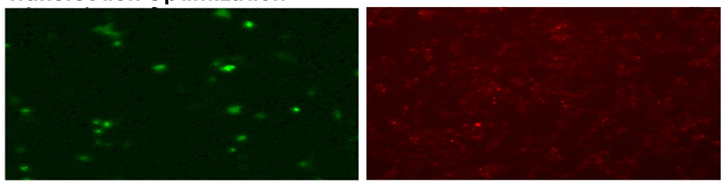


Figure 2: Fluorescent microscopy images of best transfection conditions for SK-MEL2 cells. Cell density, transfection reagent (FuGENE):DNA ratio and amount of transfection reagent used were all optimized.
Green depicts optimized results for DNA plasmid; Red depicts optimized results for sgRNA (scrambled control)

CCK-8 Cell Proliferation Assay Optimization

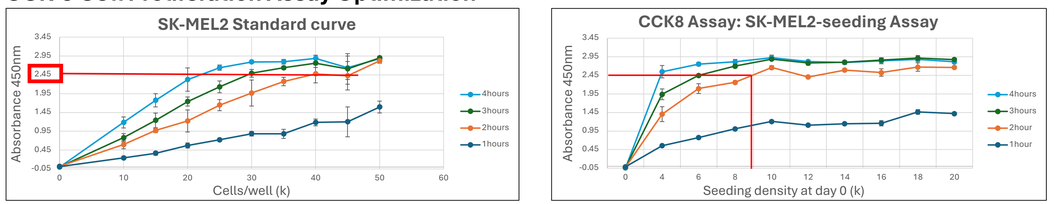


Figure 3: CCK-8 assay was optimized using untransfected SK-MEL2 cells. A) Optimal reagent incubation time was 2 hrs. B) Optimal density was 9K per well.

Drug selection on Untransfected and Transfected SK-MEL2 Cell Lines

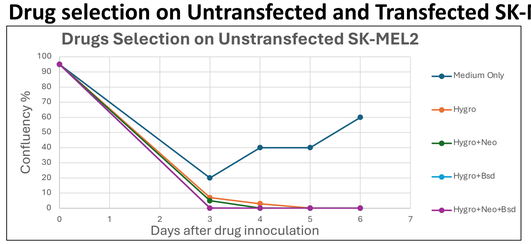


Figure 4a: Confluency of unstranfected SK-MEL2 cells reached 0% after 6 days of antibiotic inoculation. Concentration of drugs used are 600 µg/mL Hygromycin, 10µg/mL Blasticidin, and 3000 µg/mL of Neomycin

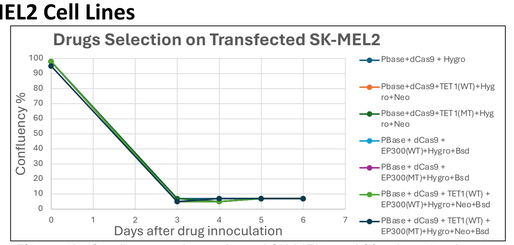


Figure 4b: Confluency of transfected SK-MEL2 stabilized at 7% after 6 days of antibiotic inoculation. Concentration of antibiotic used are 600 µg/mL Hygromycin, 10µg/mL Blasticidin, and 3000 µg/mL of Neomycin

Doxycycline Induction:

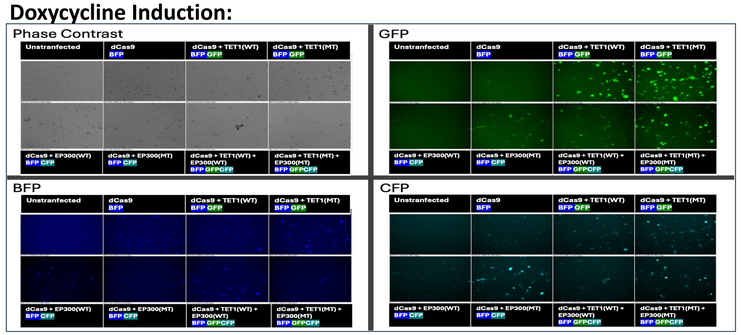


Figure 5: Cells were grown to similar confluency and then treated with Doxycycline. Fluorescent microscopy images were taken 24 hrs post-induction. Expression of vectors was confirmed by positive fluorescence. Expression of wild type TET1 (WT) and EP300 (WT) reduced cell number compared to expression of mutant TET1 (MT) and EP300 (MT).

DISCUSSION

Antibiotic selection optimization:

- 600 µg/mL Hygromycin and 10µg/mL Blasticidin kill 100% of cells at all three cell densities (2.5K, 5K, 10K) after 6 days.
- 3000 µg/mL of Neomycin kills 99.99% of cells at all three cell densities after 6 days.

Transfection optimization:

- **FuGENE4K for DNA plasmid**: 20K cells/well cell density, FuGENE:DNA ratio of 4:1, 5 µL of transfection mixture
- **FuGENE SI for sgRNA**: 20K cell density, 5pmol of siRNA, 0.3 µL of FuGENE SI

CCK-8 Cell Proliferation Assay optimization:

- Linear range best observed with maximum absorbance at 2.45, cell density of 9K cells/well on 96-well plate, and 2-hour incubation time with CCK-8 Reagent

Selection of Transfected Cells

- Most cells died following exposure to selective antibiotics; confluency stabilized at 7% at day 6.

Doxycycline Induction:

- Fluorescent images taken 24 hrs post-induction suggest that TET1 and EP300 expression reduce the number of SK-MEL2 cells
- Greater reduction in cell numbers seen with combined expression of TET1 and EP300

Limitations:

- Expression of TET1(WT) and EP300(WT) appears to kill cells and makes proliferation assays difficult to perform.
- Studies need to be repeated

Additional Experiments to Consider:

- Methylated DNA Immunoprecipitation-quantitative PCR (MeDIP-qPCR) assay to access DNA methylation status
- Chromatin Immunoprecipitation (ChIP)-qPCR assay with H3K27ac antibody to access histone acetylation level
- Wound Scratch Assay to measure cell migration

CONCLUSIONS

In this project, we developed and optimized a novel framework to induce expression of p16 in the human melanoma cell line SK-MEL2 to investigate its effects on metastatic phenotypes and potential as a therapeutic option for skin cancer. We were able to successfully establish 6 genetically distinct cell lines carrying the novel system of epigenomic editing with expression of TET1, EP300, and TET1+EP300. Future research direction can be focusing on investigating the application of this framework for prevention or treatment of genetic syndromes and cancers resulted from epigenetic silencing of p16 expression such as Burkitt's lymphoma, HPV-negative head and neck small cell carcinoma, ovarian and prostate cancer.