CRISPRi Screening in Cancer Spheroids

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BME 402 Capstone Design

PROBLEM STATEMENT

- In 2024, the United States reported 2.2 million new cancer cases and 736,790 deaths [1]
- 2D models have limited biological relevance
- Spheroids must be compatible with a genome-wide CRISPR interference (CRISPRi) screen to find tumor factors that regulate genome stability
- Utilize real-time quantitative PCR (RT-qPCR) to measure SOX2 expression levels
- Optimize a γH2AX stain protocol to identify sources of DNA double-strand breaks (DSBs)

INTRODUCTION

Background

- Spheroids mimic cell-cell interactions, nutrient gradients, and drug responses better than 2D models [2]
- SOX2 promotes tumor progression by maintaining the stem-like properties of cancer cells and drug resistance [3]
- Etoposide is a antineoplastic agent that induces DSBs [5]
- γH2AX recruits DNA repair proteins and facilitates genomic stability [5]
- CRISPRi uses dCas9 fused to a transcriptional repressor to block the transcription of specific genes [7]

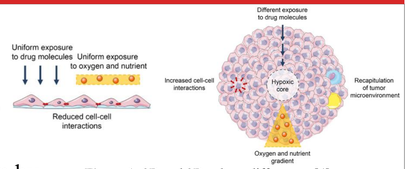

Figure 1: 2D and 3D culture differences [4].

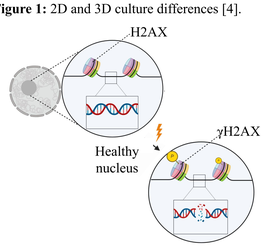

Figure 2: H2AX phosphorylation into γH2AX during DNA breaks [6].

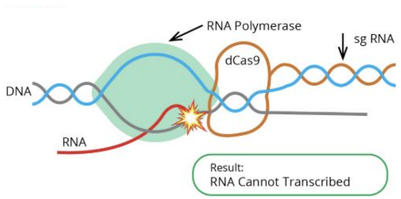

Figure 3: CRISPRi utilizing dCas9 to silence gene expression by preventing transcription [7].

Design Criteria

- Select suitable cancer cell line
- Select and optimize spheroid formation protocol
- Increase 3D culture scale
- Adhere to Biosafety Level 2 standards
- Budget of $1000

PREVIOUS PROGRESS

WT A549

- Non-small cell lung cancer (NSCLC)
- Adherent [8]
- 50 µm cell diameter [8]
- Doubling time: 22 hours
- Confluency 5,000,000 cells/10 mL

PolyHEMA stock solution

- 1.3 g poly-HEMA [9]
- 33 mL 99% ethanol [9]

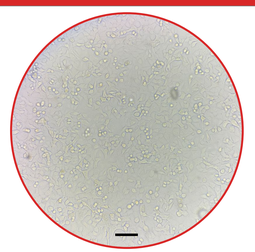

Figure 4: 20x Brightfield image of WT A549 Passage 14 cells taken at 147% confluency. Scale bar = 100 µm.

SPHEROID DESIGN PROCESS

- Seed cells on polyHEMA-coated wells in full DMEM at varying cell densities (CD) (50k and 75k cells/cm^2) and methylcellulose concentration (MC) (0.75%, 1.0%, 1.25%)
- Select condition with optimal spheroid size, spheroid count, and cell viability
- Protocol adaptable for 6-, 24-, and 96-well plates

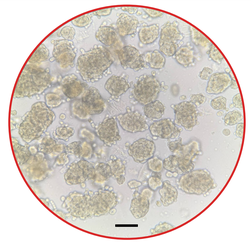

Figure 5: 20x Brightfield image of WT A549 Spheroids at day 5. Scale bar = 100 µm.

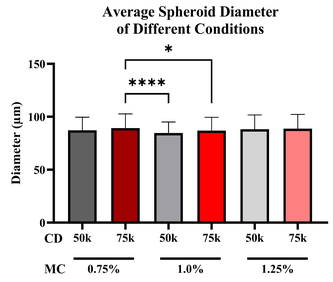

Figure 6: Average spheroid size for each condition (n=4). Condition 75k cells/cm^2, 0.75% methylcellulose yields largest spheroids.

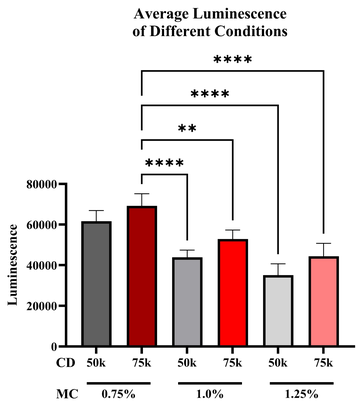

Figure 7: Average luminescence from CellTiter-Glo® Luminescent Cell Viability Assay for each condition (n=4). Higher luminescence indicates higher ATP activity, which is associated with higher cell viability. Condition 75k cells/cm^2, 0.75% methylcellulose yields highest viability.

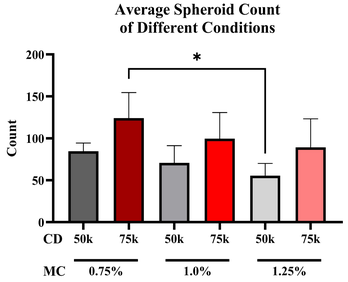

Figure 8: Average spheroid count for each condition (n=4). Condition 75k cells/cm^2, 0.75% methylcellulose yields highest spheroid count.

- All data are presented as mean ± STD
- *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
- Comparison with selected condition if not shown is not statistically significant (ns)

ACCUTASE DISSOCIATION

- Gently pipette up and down near well walls to mechanically break up spheroids
- Wash each well with PBS
- Add PBS to have 1:3 ratio to media [2]
- Pellet spheroids at 800g for 15 min at 22°C [2]
- Aspirate supernatant with pasteur pipette on vacuum tube add Accutase to pellet [2]
- DMEM 3:1 ratio to Accutase to neutralize enzyme

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RT-qPCR

- TaqMan method, QuantStudio 6 Pro for analysis
- GAPDH Ct values not significantly different on 2D vs. 3D
- No SOX2 amplification on 2D or 3D

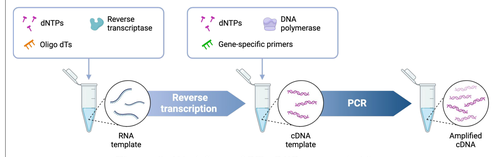

Figure 9: Workflow of RT-qPCR protocol [10].

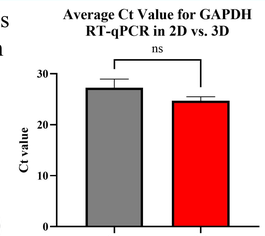

Figure 10: Ct values for GAPDH RT-qPCR for 2D vs. 3D (n=11). Data are presented as mean ± STD. ns = not significant.

γH2AX STAIN

Protocol

- Form spheroids (3D) in 6-well plate, incubate for >96 hrs
- Seed cells (2D) on 6-well plate
- Add Etoposide 3 wells per condition
- Dissociate spheroids

Analysis

- Read fluorescence on cytoFLEX
- Same stain sensitivity for all conditions

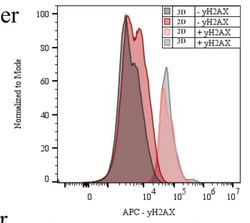

Figure 11: Single-tube APC value comparison for 2D and 3D.

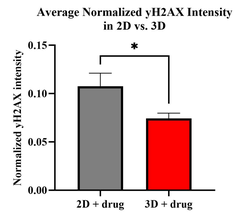

Figure 12: Average normalized APC intensity value comparison for 2D drug and 3D drug (n=3). Data are presented as mean ± STD. * p<0.05.

DISCUSSION/CONCLUSIONS & FUTURE WORK

Discussion/Conclusions

- Selected 75k cells/cm² and 0.75% Methylcellulose concentration as optimized parameters for spheroid formation
- Established accutase dissociation protocol reflective of spheroid doubling time (~1.5x every 5 days)
- GAPDH Ct values (<29) indicated high amounts of target sequence [11]
- Etoposide concentration was insufficient to induce DNA damage
- Relatively similar γH2AX intensities for 2D and 3D confers that staining protocol was successful

Future Work

- Troubleshoot SOX2 RT-qPCR assay for A549
- Repeat γH2AX staining with higher etoposide concentration, different chemotherapy drug, or/and seed at a lower passage number
- Perform γH2AX staining with A549 CRISPRi cell line to prepare for genome-wide screen

ACKNOWLEDGMENTS

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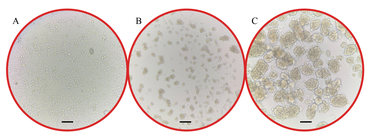
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