A. Rationale:

Stemness is described as the promotion of molecular mechanisms that stimulate basic stem cell properties such as self-renewal [1]. In the context of cancer, stemness is promoted in cancer stem cells (CSCs), a subpopulation of tumors that drive tumor growth and progression through self-renewal, metastasization, and cell growth [2]. CSCs also promote an ideal tumor microenvironment by increasing nutrient availability and immune evasion [3]. The promotion of stemness in CSCs increases resistance to stress and chemotherapeutics by promoting heterogeneous cell populations [4]. As CSCs are a limitation to current therapeutics, studies must be conducted to target these cells.

In colorectal cancer, CSCs compose 2.5% of the tumor and play a critical role in progression, metastasis, and relapse [5]. Colorectal cancer incidence is correlated with body mass index(BMI) and obesity [6]. In environments favorable to malignant cells, fatty acids stimulate malignant phenotypes such as growth, inflammation, proliferation, and migration [8]. Studies suggest fatty acids promote malignant phenotypes by generating an ideal microenvironment, facilitating membrane biogenesis, stimulating energy metabolism, and activating inflammatory pathways [7]. Arachidonic acid (AA), is an omega-6 cis polyunsaturated fatty acid that is found in red and lean meats [8]. AA is most notable for its role in the production of metabolites, such as prostaglandins, that are correlated with colorectal cancer growth and progression [9]. Specifically PGE2 has been associated with NF-kB, MAPK, Notch, and Sonic hedgehog signaling [10]. As the intestinal stem cells respond to dietary signals by adjusting daughter stem cell production, a link between diet and colorectal cancer incidence was

suggested. Understanding the mechanisms linking diet to oncogenic stemness can help to target the niche cell type and promote the effectiveness of current chemotherapeutics.

B. Research Questions

How do dietary fatty acids enhance intestinal stemness?

How does fatty acid metabolism contribute to intestinal stemness?

How can diet alter mechanisms to enhance stem niche?

B. Hypotheses

AA treatment is predicted to enhance intestinal epithelial stemness through promotion of the Canonical Wnt signaling cascade.

B. Engineering Goals

Determine the effect of AA treatment on intestinal cells on a cellular and molecular level

- Identify AA treatment induced phenotypic alterations suggesting stemness
- Identify AA induced transcriptomic alterations that explain promoted stemness
- Identify epigenetic mechanisms that elucidate transcriptomic alterations

B. Expected Outcomes

With AA treatment is expected to induce a cystic or bloated phenotype in organoids. Annotated cluster and gene level analysis of single cell RNA sequencing data is expected to reveal an increase in the number of stem cells and stem marker gene expression. The role of Canonical Wnt signaling in the regulation of stem cell counts in the intestinal epithelium is suggestive of its role in AA-induced stemness [11]. AA likely promotes stemness through its metabolites; as a result, gene level analysis of ß catenin and ß catenin target gene expression

would be maximised. Differential expression analysis will then be conducted in order to identify what genes are most significantly altered with treatment. These genes would present potential mechanisms for linking AA to canonical wnt signaling. The objective of this experiment is to clarify a mechanism linking AA to intestinal stemness.

C. Procedure/Risk and Safety/Data Analysis

Procedure

1. Organoid culture
    a. Cultured intestinal epithelial Mus Musculus organoids will be gifted from the Beyaz Laboratory (CSHL, NY).
    b. Organoids will be maintained using Advanced DMEM (Gibco 12491023) supplemented with .5M N2 (Gibco California 17502048) and .05M B27 (Gibco California 17504044).
    c. Experimental group organoids will receive media further supplemented with 100 µl of AA (Sigma Aldrich USA, 10931) at a variable concentration (10µM, 50µM, 100µM, and 200µM) or 100 µl of 50µM PGE2 (Sigma Aldrich USA, P0409)
    d. Organoids will be cultured in 6 24-well plates (Corning CLS3526) and embedded in 25-30 µl of growth factor reduced Matrigel (Corning 356231). Matrigel droplets will rest in wells for 30 minutes at room temperature before 800 µl of adjusted media is administered to each well. Organoids will be maintained in an incubator at 37 °C and 5% CO_2.
    e. Media will be changed every three days.

f. Crypt domains and organoid size will be identified using ImageJ to quantify stemness and growth on day 5 of the cell culture.

g. All experimentation will be conducted under a sterilized BSL 2 biosafety cabinet in order to reduce contamination. Prior to the introduction of new equipment to BSL 2 biosafety cabinet, items will be thoroughly sterilized using a 70% ethanol solution. Protective equipment will be worn.

h. Once cell culture is completed, cell culture will be treated with 1L of 10000ppm hypochlorite for 2 hours. Plates will be then placed in biohazard disposal bins while awaiting biosafety pickup.

2. Library Preparation

a. Cells from organoids in control, AA and PGE2 groups,(two plates per group) will be isolated using Sony SH800 Cell Sorter and placed into 96 well plates (Corning 3763) containing 5 µl of control media.

b. Cells will be lysed using 5 µl of 1M TRIzol (Zymo Research California R2050-1-5u) at 37 °C for 1 min.

c. scRNA Seq libraries will be produced using Single Cell/Low Input RNA Library Prep Kit for Illumina (NEBNext E6420S), manufacturer's instructions will be followed [12].

i. Reverse Transcription: add 2 µl of RT primer mixture to 7 µl of RNA. To anneal primers, incubate sample for 5 minutes at 70°C in a thermocycler with the heated lid set to 105°C. Mix 5 µl of reverse transcription buffer, 3

µl of nuclease free water, 2 µl of enzyme mix, 1 µl of template switch oligo. Centrifuge and add to annealed RNA.

ii. cDNA amplification: mix 50 µl cDNA PCR Master Mix, 2 µl cDNA PCR Primer, 0.5 µl lysis buffer (10x), 27.5 µl nuclease-free water. Pipette sampl up and down to mix. Cycle PCR 17-18 times based upon RNA quantities. Cleanup amplified cDNA by adding to Solid Phase Reversible Immobilization beads.

iii. cDNA Fragmentation will conducted using FS Enzyme Mix. In a thermocycler, with the heated lid set to 75°C, run the following program: 25 minutes at 37°C, 30 minutes at 65°C, hold at 4°C.

iv. Adapter ligation: Dilute adaptor for Illumina by 25-fold (0.6 µM) using adaptor dilution buffer. Mix the Ultra II Ligation Master Mix by pipetting up and down several times. Incorporate 30 µl Ultra II Ligation Master Mix, 1 µl of Ligation enhancer, 2.5 µl for Illumina (diluted 1:25). Incubate at 20°C for 15 minutes in a thermocycler with the heated lid off. Add 3 µl of USER® Enzyme to the ligation mixture. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

v. PCR enrichment: Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. Place the tube on a thermocycler and perform PCR amplification.

vi. Libraries are stored in -20 °C freezers until sequencing.

d. Libraries will be sequenced on an Illumina NextSeq500 sequencer by CSHL Core Facilities and gifted to student.

3. Single cell RNA sequencing preprocessing
    a. Cell ranger version 3.1 (10X Genomics) will be used for transcriptome alignment, demultiplexing and UMI-collapsing.
    b. Cells with reads less than 1000 counts, expressing less than 30 genes, and mitochondrial gene count greater than 10% will be identified as stressed and excluded from the dataset.
    c. Normalization
        i. Size factors will be calculated through compute SumFactors() in the Scran package using default parameters [13].
        ii. Log2(TPM) values will be calculated as described by [14].
        iii. Batch correction will be conducted using the combat() function in the Scanpy package [15].
4. Sc RNA seq Analysis
    a. Annotated cluster analysis and annotation, pseudo-time differentiation trajectory analysis, gene level and differential expression analysis will be conducted as described by [16] with few alterations to workflow. Analysis will be conducted on Jupyter lab version 1.0.4.
        i. For annotated cluster analysis, louvain clustering will be conducted at a resolution of .6 rather than .5 using scanpy package [15].

1. Annotate clusters based off average expression of cell type marker genes per cluster.
2. Cell type marker genes described by [14].

ii. Psuedotime analysis

1. Identify cell of origin through the function in the numpy package [17]
    a. Use the isin() function to specify the trajectory begins in the stem cluster.
    b. Use the argmin() and arrange() functions to identify the stem cell with the highest DC3 value to act as root for the diffusion pseudotime.
    c. Calculate differentiation lineages using diff plot function in the scanpy package [15].
2. Utilize diffmap to plot differentiation lineages and pseudotime progression.

iii. Gene level analysis

1. Create new gene dictionary to incorporate 96 ß catenin target genes identified through literature review [18-21].
2. Calculate mean expression using the mean() function
3. Plots will be made using barplot() function in seaborn package [22]. Specify X axis to be experimental treatment and Y value to be average gene expression. Error bars represent standard error.

iv. Differential expression analysis

1. Conducted analysis on all cells, will not subset to specific cluster.
2. Using the deseq2 and MAST package, calculate log2 fold change and significance of fold change [23-24].
3. Use seaborn package to form volcano plot [22].

5. Cleavage Under Targets and Release Using Nuclease (CUT&RUN) procedures and analysis will be conducted as described by [25].
    a. While working with Digitonin, utilize a fume hood and avoid direct contact with hazardous substance. Wear protective clothing and face protection while using Digitonin.

Risk and Safety

1. Human participants: not applicable
2. Vertebrate animals: not applicable
3. Potentially hazardous biological agents research
    a. Beyaz et al. (2019) extracted live tissue cultures from intestinal crypts found in the intestine of wildtype C57 black 6 mice for their study titled: "Dietary and epigenetic regulation of cancer immunity". These cells will be cultured into 3D organoid primary cell cultures and gifted to student researcher. T. Broda in Generation of human antral and fundic gastric organoids from pluripotent stem cells, classifies culturing primary organoid cultures as BSL 2[26].

b. Safety precautions and disposal methods

i. Safety precautions

All experimentation will be conducted under a sterilized BSL 2 biosafety cabinet in order to reduce contamination. Prior to the introduction of new equipment to experimentation environment, items will be thoroughly sterilized using a 70% ethanol solution. Skin will be thoroughly washed after handling cells. While working with live tissue cultures: do not eat, drink or smoke. Wear protective gloves, clothing, and eyewear during experimentation [27].

ii. Methods of disposal

Prior to cell culture disposal treat with 10000ppm of hypochlorite for 2 hours. Plates will be then placed in biohazard disposal bins while awaiting biosafety pickup.

4. Hazardous chemical, activities and devices

a. 100 µl of 50µM PGE2 (Sigma Aldrich USA, P0409)

i. Risk Assessment process: PGE2 has acute oral toxicity and reproductive toxicity. Do not ingest and avoid consumption near PGE2.

ii. Supervision: during use of PGE2 student will be supervised by qualified scientist.

iii. Wash skin thoroughly after handling. Do not eat, drink or smoke when using hazardous materials. Wear protective clothing and face protection while using PGE2. If swallowed, contact poison control and rinse mouth. If exposed or concerned: get medical attention.

iv. PGE2 and container will be disposed in specified hazardous material bins prior to proper biosafety pickup.

b. 500 µl of 1M TRIzol (Zymo Research California R2050-1-5u)

i. TRIzol is toxic if touched, inhaled or swallowed. May cause organ damage or genetic defects if exposed repeatedly.

ii. Supervision: during use of TRIzol student will be supervised by qualified scientist.

iii. Wash skin thoroughly after handling. Do not eat, drink or smoke when using TRIzol. Wear protective clothing and face protection while using TRIzol. If swallowed contact poison control and rinse mouth. If exposed or concerned: get medical attention. Do not breathe fumes emitted by TRIzol. Handle materials in a vacuum fume hood.

iv. TRIzol and container will be disposed in specified hazardous material bins prior to proper CSHL biosafety pickup.

c. 1L of 10000ppm sodium hypochlorite (Fisher Chemical, 7681-52-9,7732-18-5)

i. sodium hypochlorite causes severe skin burns and extreme eye damage. sodium hypochlorite yields extreme single exposure respiratory toxicity. Do not ingest and avoid consumption near sodium hypochlorite. Avoid direct inhalation of sodium hypochlorite.

ii. Supervision: during use of sodium hypochlorite student will be supervised by qualified scientist.

iii. Wash skin thoroughly after handling. Do not eat, drink or smoke when using hazardous materials. Wear protective clothing and face protection while using sodium hypochlorite. If swallowed, contact poison control and rinse mouth. If exposed or concerned: get medical attention.

iv. sodium hypochlorite and container will be disposed in specified hazardous material bins prior to proper CSHL biosafety pickup.

d. 2 ml of 5% Digitonin (Abchem Boston, ab141501)

i. Digitonin is toxic if swallowed, inhaled or touched.

ii. Supervision: during use of Digitonin student will be supervised by qualified scientist.

iii. Wash skin thoroughly after handling. Do not eat, drink or smoke when using Digitonin. Wear protective clothing and face protection while using Digitonin. If swallowed contact poison control and rinse mouth. If exposed or concerned: get medical attention. Do not breathe fumes emitted by Digitonin. Handle materials in a vacuum fume hood.

iv. Digitonin and container will be disposed in specified hazardous material bins prior to proper CSHL biosafety pickup.

Data analysis

The Deseq2 package will be calculated significance of Log2 changes through a wald test. Significance testing for gene level analysis and phenotypic alterations will be conducted via a one sided student T-test. Alpha value will be .01. Graphs will be made in Jupyter lab using the seaborn package, the Barplot(), regplot(), and pivot_table(). Pseudotime trajectory diffusion

maps will be produced through diffmap() function in Scanpy package[15]. CUT&RUN .bam files will be analyzed using the integrative genomics viewer program [25].

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E. Addendum

There were no additions to research plan post SRC approval.