Linking Diet and Cancer: Arachidonic Acid Augments Canonical Wnt Signaling to Enhance Stemness

Vyom Shah

Jericho High School

2020

Abstract

Cancer stem cells promote resistance to current chemotherapeutics through the promotion of a tumor microenvironment and cellular heterogeneity. Dietary factors could propagate cancer stem cells through stemness. As the mechanisms linking dietary factors and stemness remains elusive, this study investigated the role of arachidonic acid (AA) in the perpetuation of intestinal cell stemness. Annotated-cluster, differentiation lineage, gene-level and differential expression analysis of single-cell RNA sequencing data elucidated AA's impact. AA decreased crypt domain frequency ($p<.001$) and enlarged organoids ($p<.001$) suggesting decreased differentiation and increased inflammation, proliferation, and cell growth, indicating stemness was promoted. Annotated cluster analysis revealed AA increased stem cell frequencies ($p<.001$). A lack of cluster relapse in differentiation lineages reveals AA promotes stemness exclusively through symmetric division, not dedifferentiation. Gene-level analysis revealed AA and metabolite, PGE2, increased β-catenin ($p<0.001$) and β-catenin target gene ($p<0.001$) expression. As expression was greater in PGE2 than AA($p<0.001$), this suggests AA promotes stemness through PGE2 induced canonical Wnt signaling. Differential expression and gene-level analysis revealed S100A6 expression was upregulated two-fold with AA ($p<0.0001$) and six-fold with PGE2 ($p<0.0001$) suggesting AA metabolite, PGE2, recruits S100A6 to promote β catenin. The correlation between S100A6 and canonical Wnt signaling presents a potential therapeutic target for cancer stem cells in colorectal cancer. CUT&RUN analysis identified AA increases promoter length of S100A6 suggesting epigenetic upregulation. Future investigations involve identification of the specific molecular interaction between AA induced S100A6 and Canonical Wnt signaling.

Introduction

Despite studies correlating obesity and colorectal cancer incidence, little is known regarding the adaptation of intestinal cells to pro-obesity diets in cancer incidence [1]. Induced through a combination of poor diet and a lack of physical activity, obesity impacts 93 million US adults [2, 3]. The correlation between obesity and colorectal cancer incidence is troubling as colorectal cancer is the leader in cancer incidence and mortality in young adults [4, 5]. Understanding the molecular mechanisms linking obesity to colorectal cancer incidence must be understood in order to identify potential therapeutics.

Obesity is characterized by an influx of fatty acid concentrations [6, 7]. 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 [8]. Arachidonic acid (AA), is an omega-6 cis polyunsaturated fatty acid that is found in red and lean meats [9]. AA is most notable for its role in the production of metabolites, such as prostaglandins, that are correlated with colorectal cancer growth and progression [10]. One particularly interesting metabolite of AA is PGE2 which has been associated with oncogenic signaling in the intestinal epithelium [10].

Nutrient absorption primarily occurs in the intestinal epithelium. The intestinal epithelium is composed of extrusions called villus and invaginations known as crypts [11]. In order to sustain environmental stress, the epithelial layer is replenished frequently [12]. Intestinal regeneration occurs along the crypt/villus axis and is facilitated through intestinal stem cells

which reside at the base of the crypt and can be identified through the marker expression of LGR5 [13].

Intestinal stem cells instantiate differentiation through the production of transit-amplifying precursor cells that produces a multitude of cell types, such as Enterocytes, Enteroendocrine, Goblet and Paneth cells [14]. Differentiation of a cell is tightly regulated through the intestinal stem niche which can be described as an environment that regulates the self-renewing, multipotent intestinal stem cell population and the generation and positioning of differentiated subtypes [15]. Stem cell counts are regulated through interactions between the intestinal stem cell niche, distinct stromal cell populations, and the extracellular matrix [16]. In the context of signaling, the subepithelial mesenchyme produces Wnt and EGF ligands such as R Spondin, Noggin, gremlin 1/2 and chordin-like 1. Paneth cells also stimulate Notch, EGF, and Wnt3 ligands [17]. The promotion of these pro stem signals to induce basic stem cell properties can be described as stemness [18].

In the context of cancer, stemness is promoted in cancer stem cells to drive tumor growth and progression through self-renewal, metastasis and cell growth [19]. Cancer stem cells present prowess in their organizational abilities through increased nutrient availability and immune evasion: thereby promoting an ideal tumor microenvironment[20]. The promotion of stemness in cancer stem cells increases resistance to stress and chemotherapeutics by promoting heterogeneous cell populations [21]. Understanding regulatory processes of cancer stem cells can help to improve the efficiency of current therapeutics.

The β catenin-dependent (canonical) Wnt signaling pathway is a key regulator of intestinal stemness [22]. In colorectal cancer, canonical Wnt signaling stimulates stemness through symmetric division of stem cells and de-differentiation of terminally differentiated cells.

Inhibition of the β catenin destruction complex increases the nuclear accumulation and T-cell factor/lymphoid enhancer-binding factor binding of ß catenin to enhance the pro-stem effects induced by β catenin targets [23]. Beyaz and colleagues determined that a high fat diet can stimulate LGR5 induced APC loss and upregulate PPARδ [24]. However, the mechanism by which a high fat diet promoted β catenin induced stemness has yet to be identified. This study strives to identify the effect of AA treatment on stemness and elucidate the molecular mechanism on how a high fat diet can promote stemness. Understanding the mechanisms regulating stemness will aid in the development in methods to mediate chemoresistance of intestinal cancer stem cells and develop novel therapeutics for colorectal cancer.

Methods

Cell culture:

Crypt intestinal epithelial organoids were gifted from the Beyaz Laboratory (CSHL, NY). Organoids were maintained using Advanced DMEM (Gibco 12491023) supplemented with .5M N2 (Gibco California 17502048) and .05M B27 (Gibco California 17504044). Experimental group organoids received control media supplemented with AA (Sigma Aldrich USA, 10931) at a variable concentration (10µM, 50µM, 100µM, and 200µM) or PGE2 (Sigma Aldrich USA, P0409) in the media. Organoids were cultured in 24 well plates (Corning CLS3526) and embedded in 25-30 µl of growth factor reduced Matrigel (Corning 356231). Matrigel droplets rested in wells for 30 minutes at room temperature before 800 µl of media was administered to each well. Organoids were maintained in a closed environment at 37 °C and 5% CO_2. Media was changed every three days. Crypt domains and organoid size were identified using ImageJ to quantify stemness and growth on day 5 of the cell culture.

Single-cell RNA (scRNA) capture and library preparation:

Cells from organoids in control, AA and PGE2 groups, were isolated using Sony SH800 Cell Sorter and placed into 96 well plates (Corning 3763) containing 5 µl of control media. Cells were then lysed using 5 µl of Trizol (Zymo Research California R2050-1-5u) at 37 °C for 1 min.

scRNA Seq libraries were produced using Single Cell/Low Input RNA Library Prep Kit for Illumina (NEBNext E6420S), according to manufacturer's instructions. Samples were sequenced on Illumina NextSeq500 sequencer.

Single-cell RNA seq Analysis:

Cell ranger version 3.1 (10X Genomics) was used for transcriptome alignment, demultiplexing and UMI-collapsing. Cells with reads less than 1000 counts, expressing less than 30 genes, and mitochondrial gene count greater than 10% were identified as stressed and were excluded from the dataset as these cells represented increased. Normalization involved size factor calculation and expression value calculation to account for count size variability. Size factors were calculated through compute SumFactors() in the scran package using default parameters[25]. Log2(TPM) values were calculated as described by Haber *et* al. Batch correction was conducted using the combat() function in the Scanpy package[26]. Cluster analysis and annotation, pseudo-time differentiation trajectory analysis, gene level and differential expression analysis were conducted as described by [27]. Single cell

CUT&RUN procedures and analysis were conducted as described by [].

Results and Discussion

Arachidonic acid promotes stemness

To determine if AA stimulates stemness, intestinal organoids were treated with AA and the frequency of crypt domains and organoid size quantified. AA treated organoids presented a circular, cystic phenotype (Figure 1A). AA treatment of 50 µM or above induces a decrease in crypt domains per organoids (Figure 1B). In Figure 1C, AA treatment increases organoid size 4-fold compared to control.

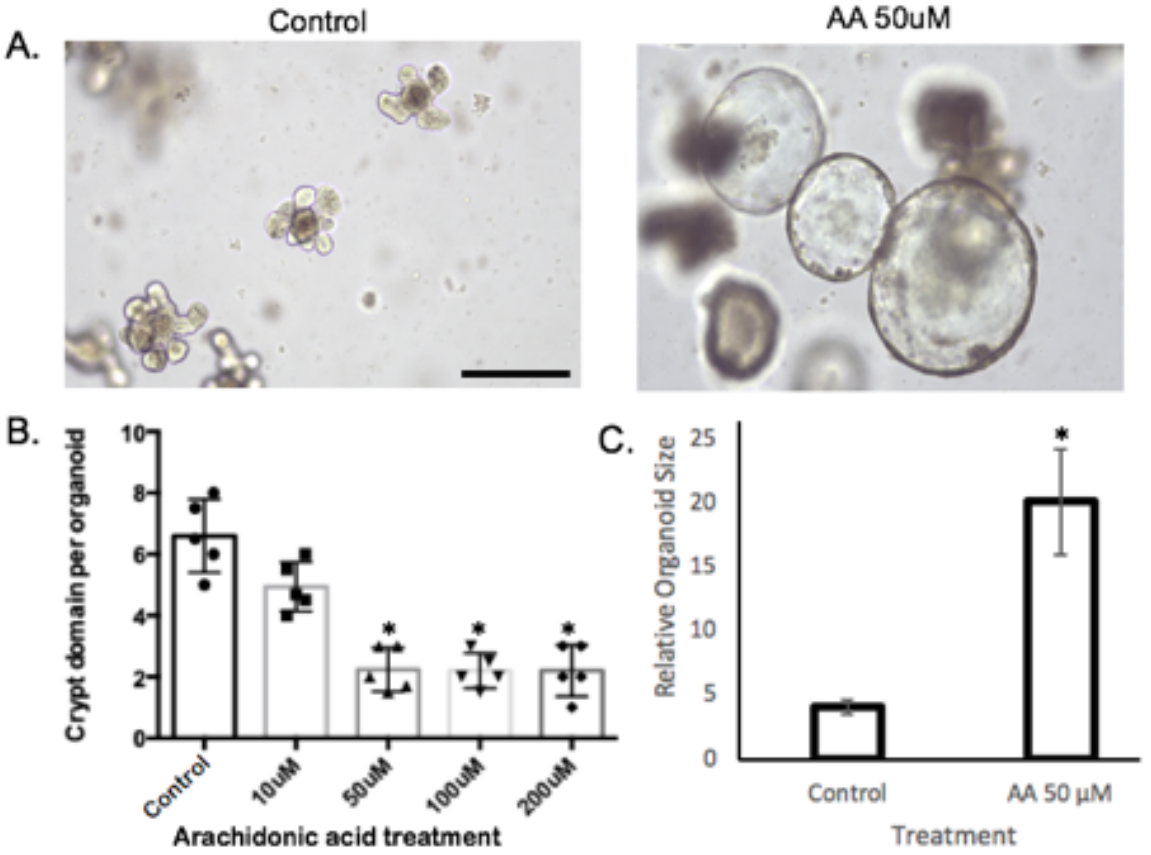

Figure 1. **Arachidonic acid (AA) treatment promotes growth and decreases crypt domains in intestinal organoids.** Intestinal organoids of control and AA treated groups. (B) Crypt domain frequency decreases with AA treatment. (C) AA treatment increased organoid size. B, C: (n=5). * = p<.001 determined by a one-sided student t-test. Scale bars measure 100µm.

AA treatment results in irregular organoid phenotype (Figure 1A) which prompts further analysis. As crypt domains are a metric for stemness, the decrease in crypt domains as shown in

figure 1B suggests AA treatment produced cystic organoids and promoted stemness [29]. In previous studies, the cystic phenotype in intestinal epithelial cells has been correlated with the upregulation of the canonical Wnt signaling pathway, [29]. In figure 1C, the increased organoids size with AA treatment suggests inflammation, growth, or proliferation of organoids was stimulated, similar to the effects of stem cells[29]. Figure 1 displayed that AA treatment promoted an irregular cystic phenotype in intestinal epithelial cells indicating stemness was promoted.

AA promotes stemness through symmetric division, not dedifferentiation

To further elucidate the phenotypic link between AA and Stemness, annotated cluster, gene level, and pseudo-time trajectory analysis was conducted. Figure 2A displays number of cells per cell type for each experimental variable; in the AA treated group, the proportion of stem cells is substantially greater than that of the control. Figure 2B displays, AA induced an increased in stem markers while decreasing the expression of differentiated markers. In Figure 2C, the differentiation trajectories branch from the stem cluster without cluster relapse over pseudo-time, indicating no de-differentiation occurred.

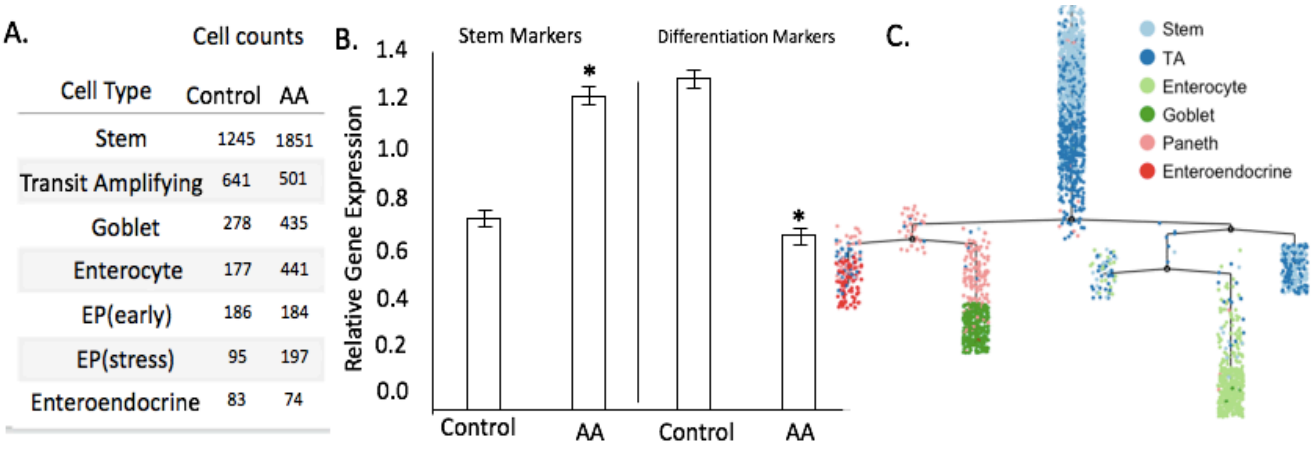

Figure 2. **Single cell RNA sequencing (SCRNA seq) analysis reveals AA promotes stem cells frequency while maintaining differentiation trajectories.** (A) stem cell frequencies. (B) expression of cell type markers. (c) Differential trajectory analysis. * = p<.001 determined by a one-sided student t-test.

The increase in stem cells and stem markers suggests an increase in stem promoting signals to stimulate higher stem cell frequencies, suggesting stemness was promoted. Once established that AA propagates cancer stem cells. The number of stem cells can increase by either de-differentiation of terminally differentiated cells through promotion of stem signals or symmetric division of stem cells. Since both methods of promoting stemness involve different pathologies, identifying which method is promoted can aid in identifying the mechanisms that link AA to stemness [30-31]. Trajectory lineages represent the estimated progression of differentiation [32]. In de-differentiation, lineages would have terminally differentiated cells revert to a more primitive state [32]. As the lineages in figure 2C fails to show these trends, AA does not promote stemness through de-differentiation. Figures 2A and 2B shows the AA group to contain more stem cells and stem markers increase their expression, suggesting AA stimulates an increased number of stem cells. As the number of stem cells does not increase due to de-differentiation, the number of stem cells must increase through symmetric division. Figure 2 indicates AA promotes stemness through symmetric division

AA correlates with Canonical Wnt Signaling

To understand the changes in gene expression that drive induced stemness, gene level analysis was conducted. As canonical Wnt signaling drives intestinal stemness, the effect of AA and metabolite, PGE2, on canonical Wnt was elucidated. Analysis revealed a positive trend in β catenin expression (figure 3A) and the average expression of 96 β catenin target genes (figure 3B) where AA has significantly higher expression than control($p<.001$) and PGE2 significantly

increasing expression from AA and Control (p<.001).

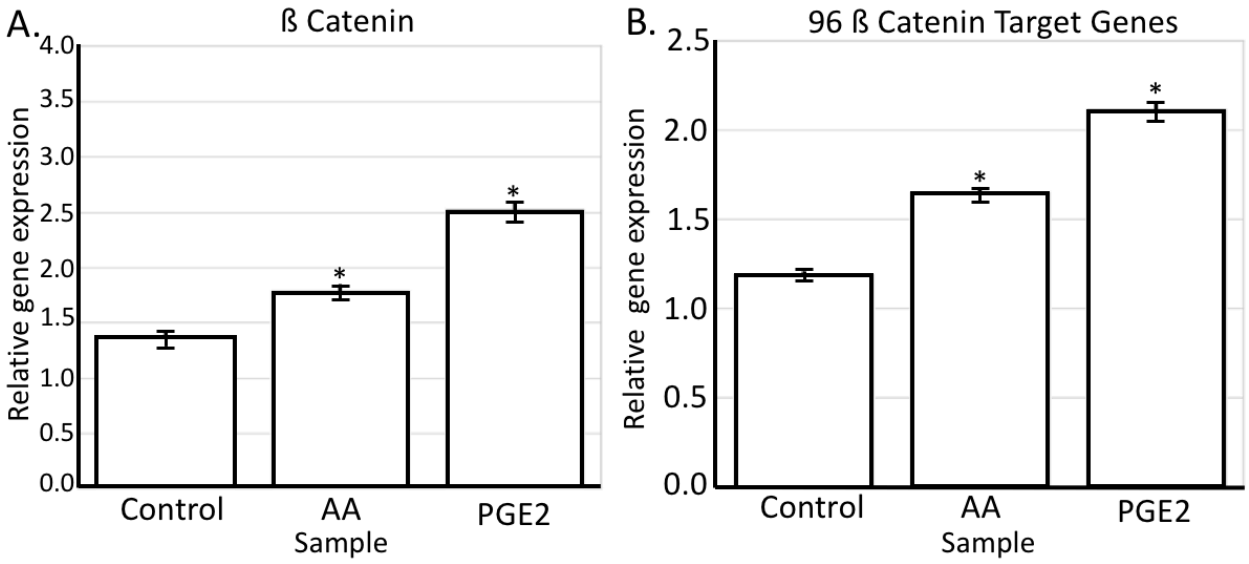

Figure 3. **Gene level analysis of ß Catenin and targets.** Relative of expression of (A) Ctnnb1 and (B) 96 ß Catenin targets as described by Beyaz et al. Significance determined through a Wald test, * =(p<.001).

Figure 3A suggests AA enhances the expression of β catenin. However, AA not only enhances the expression of β catenin but only enhances its protein activity as 96 target genes induced through TCF /LEF transcriptional activation [33]. Therefore, figures 3A and 3B suggests AA enhances nuclear accumulation and TCF/LEF ß catenin binding to stimulate canonical Wnt signaling[]. However, as the metabolite of AA, PGE2, upregulates β catenin and its target genes more significantly, this indicates that AA recruits its metabolites in order to enhance stemness. Previous studies suggest increased PGE2 concentrations augments canonical Wnt activity through regulation of the ß catenin destruction complex []. Figure 3 indicates AA recruits its metabolites in order to enhance intestinal stemness through Canonical Wnt Signaling.

S100 Family Upregulated with AA Treatment

To elucidate the molecular link between PGE2 and β catenin, differential expression and gene level analysis was conducted on the scRNA seq data. Figure 4A displays a volcano plot comparing significance to $\log_2$ fold change: S100 Calcium Binding Protein A6(S100A6) was among the most significantly upregulated genes. Figure 4B displays a gene level analysis of S100A6 where AA increased expression two-fold(P<.0001) and PGE2 promoted increased expression six-fold(P<.0001).

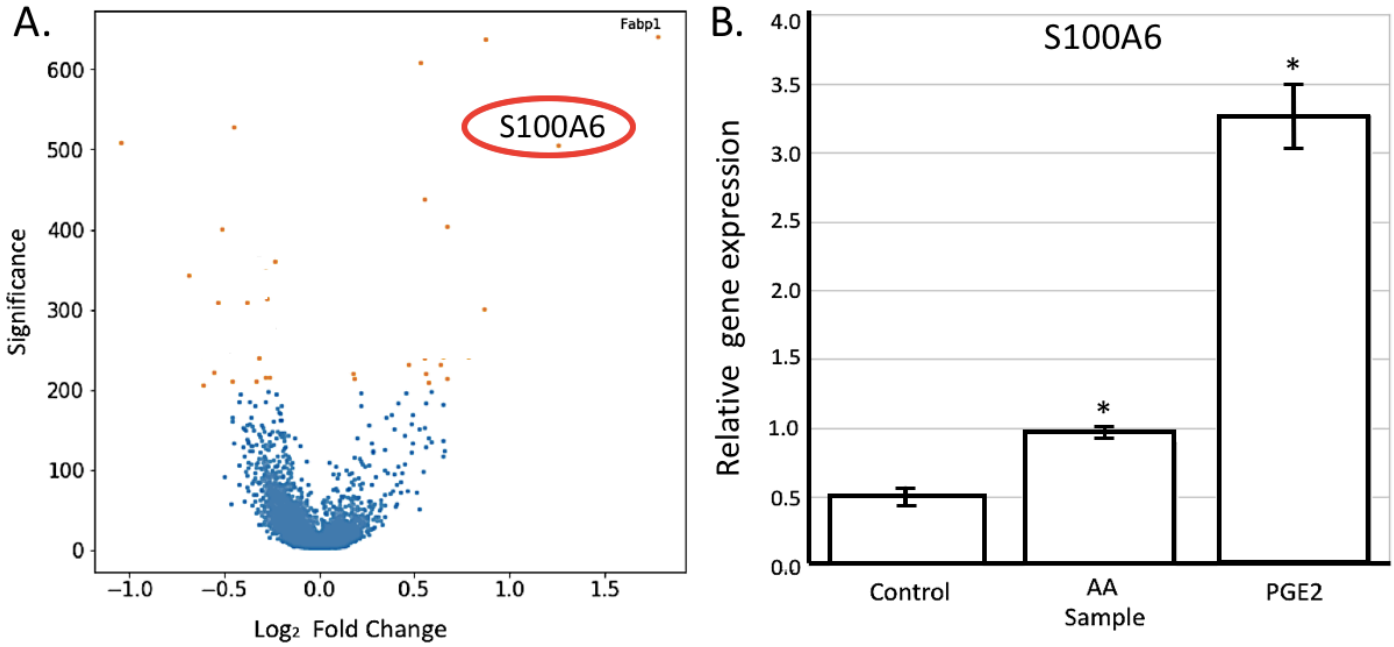

Figure 4. **Differential expression analysis of SCRNA seq data.** (A) Differential expression results of AA gene expression when compared to control. (B) S100A6 expression in three experimental conditions. Significance determined through a Wald test * = (p<.0001).

S100A6 regulates the dynamics of cytoskeleton constituents, cell growth and differentiation, and calcium homeostasis [34]. In the dietary tract, calcium binds with fatty acids to form insoluble soaps, to decrease lipid absorption [35]. This suggests, S100A6 expression is increased as a negative regulatory response to AA treatment. Specifically, S100A6 has been shown to upregulate β catenin expression in endometrial and pancreatic cells through interaction with the β catenin destruction complex constituent SIAH1[36]. The role of S100A6 in the

canonical Wnt signaling pathway suggests AA treatment might promote stemness through the S100A6 dependent activation of the canonical Wnt pathway. Previous studies suggest the dysregulation of S100 proteins promotes malignant transformations in human pancreatic cancer, breast cancer, hepatocellular carcinoma, lung cancer, prostate cancer, and colorectal cancer [34]. Most significantly, the positive trend observed in figure 4B suggests that AA recruits PGE2 to upregulate Canonical Wnt signaling through S100A6. Figure 4. suggests AA promotes stemness through PGE2 recruitment of S100A6 to activate Canonical Wnt signaling.

Arachidonic acid epigenetically enhances canonical Wnt Signaling

As environmental factors have been implicated with altering the transcriptome through the epigenome, a Cleavage Under Target & Release Using Nuclease (CUT&RUN) assay was conducted to reveal the S100A6 dependent mechanism between AA and canonical Wnt signaling. Figure 6A. displays count alignment distributions over the genome and log expression values for the S100A6 promoter region. AA yields increased count binding and upregulates log expression for the S100A6 promoter three-fold. Figure 6B. indicates that AA group held increased count alignment and upregulated the log expression of the ß catenin promoter regions approximately two-fold.

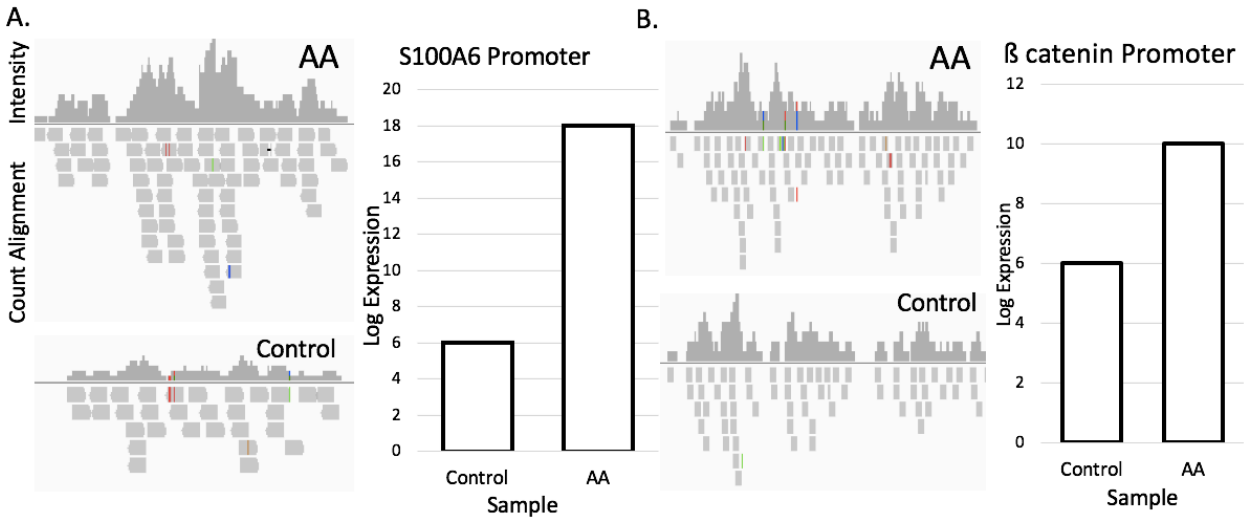

Figure 6. Command line CUT&RUN analysis of S100A6 and ß Catenin promoter regions. Relative log expression and count distribution over promoter region for genes (A) S100A6 and (B) Ctnnb1 or ß catenin. Image created by student

Increased count alignment in the AA group suggests localized histone acetylation near the S100A6 and CTNNB1 genes. Histone acetylation increases the negative charge of the histones which repels negative charged DNA, revealing the S100A6 promoter region [37]. As log expression of promoter regions are upregulated, this suggests a histone acetylation. Acetylation induced promoter sizes increases the number of DNA fragments possible for the promoter region; there are more counts available for genome alignment [37]. Taken together, Figure 6. suggests AA enhances S100A6 dependent canonical Wnt signaling through acetylation of S100A6 and ß catenin promoters.

Conclusion

This study has been demonstrated that AA stimulates stemness by activating the epigenetically upregulating canonical Wnt signaling through S100A6. The promotion of stem signals has been linked to a greater frequency to cancer stem cells [16]. Cancer stem cells are the driving factor for the inefficiency of current therapeutics [38]. Cancer stem cells promote cellular heterogeneity which can provide resistance to chemotherapeutics and microenvironment induced stress [18]. Diet induced canonical Wnt signaling is likely the cause for high correlations between obesity and colorectal cancer incidence as AA epigenetically enhances stemness. The enhancement of stemness in APC mutated cells potentiates tumorigenesis[24]. If the mechanisms underlying diet induced stemness are mediated, the malignant consequences of cancer stem cells would be attenuated.

The S100 protein family offers an ideal method to reduce the abundance of cancer stem cells. The structure of S100 proteins is essentially two subunits, each with 4 alpha helices [39]. In between these two subunits offers an ideal position for an allosteric inhibitor [40-42]. Previous studies have suggested that the deletion of genes associated with the S100 family has minimal effects on normal physiology [43].

Future research should strive to identify specific molecular interactions between PGE2, S100A6 and β catenin through inhibition analysis. Understanding how AA metabolites can epigenetically promote stemness is suggestive of a more diverse role of fatty acids in promoting stemness. Through the utilization of the mechanisms proposed in this study, new therapeutics can be developed that have a minimal effect on normal physiology and mitigate the effects of cancer stem cells, thereby solving the current limitations of chemotherapeutics.

References

[1] "Obesity and Cancer Fact Sheet," National Cancer Institute. [Online]. Available: https://www.cancer.gov/about-cancer/causes-prevention/risk/obesity/obesity-fact-sheet. [Accessed: 02-Oct-2019].

[2] Q. As, "Obesity, Diabetes and Cardiovascular Diseases," Journal of Human Anatomy, vol. 2, no. 2, 2018.

[3] N. F. Aykan, M. Artac, and T. Özatli, "Body Mass Index and Colorectal Cancer," *Body-mass Index and Health*, 2019.

[4] S. Moran-Ramos, A. R. Tovar, and N. Torres, "Diet: Friend or Foe of Enteroendocrine Cells: How It Interacts with Enteroendocrine Cells," *Advances in Nutrition*, vol. 3, no. 1, pp. 8–20, Jan. 2012.

[5] A. Bhandari, M. Woodhouse, and S. Gupta, "Colorectal cancer is a leading cause of cancer incidence and mortality among adults younger than 50 years in the USA: a SEER-based analysis with comparison to other young-onset cancers," *Journal of Investigative Medicine*, vol. 65, no. 2, pp. 311–315, 2016.

[6] A. Tarasiuk, P. Mosińska, and J. Fichna, "The mechanisms linking obesity to colon cancer: An overview," *Obesity Research & Clinical Practice*, vol. 12, no. 3, pp. 251–259, 2018.

[7] B. D. Hegarty, G. J. Cooney, E. W. Kraegen, and S. M. Furler, "Increased Efficiency of Fatty Acid Uptake Contributes to Lipid Accumulation in Skeletal Muscle of High Fat-Fed Insulin-Resistant Rats," *Diabetes*, vol. 51, no. 5, pp. 1477–1484, Jan. 2002.

[8] T. Wallin *et al.,* "Facilitation of fatty acid uptake by CD36 in insulin-producing cells reduces fatty-acid-induced insulin secretion and glucose regulation of fatty acid oxidation," *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, vol. 1801, no. 2, pp. 191–197, 2010.

[9] E. Currie, A. Schulze, R. Zechner, T. C. Walther, and R. V. Farese, "Cellular Fatty Acid Metabolism and Cancer," *Cell Metabolism*, vol. 18, no. 2, pp. 153–161, 2013.

[10] J. Seah *et* al.,"Consumption of Red Meat, but Not Cooking Oils High in Polyunsaturated Fat, Is Associated with Higher Arachidonic Acid Status in Singapore Chinese Adults," *Nutrients*, vol. 9, no. 2, p. 101, 2017.

[11] Clevers H. The intestinal crypt, a prototype stem cell compartment. Cell 154: 274–284, 2013

[12] Trentin A, Glavieux-Pardanaud C, Le Douarin NM, Dupin E. 2004. Self-renewal capacity is a widespread property of various types of neural crest precursor cells. Proc. Natl Acad. Sci. USA 101, 4495–4500. (10.1073/pnas.0400629101)

[13] Barker N, et al. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007. (10.1038/nature06196)

[14] Cheng H. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. IV. Paneth cells. Am J Anat 141: 521–535, 1974

[15] Shyh-Chang N, Daley GQ, Cantley LC. 2013. Stem cell metabolism in tissue development and aging. Development 140, 2535–2547. (10.1242/dev.091777)

[16] Takeda, N. et al. Interconversion between intestinal stem cell populations in distinct niches. Science 334, 1420–1424 (2011).

[17] Lei NY, Jabaji Z, Wang J, Joshi VS, Brinkley GJ, Khalil H, Wang F, Jaroszewicz A, Pellegrini M, Li L, Lewis M, Stelzner M, Dunn JC, Martin MG (2014) Intestinal

subepithelial myofibroblasts support the growth of intestinal epithelial stem cells. PLoS ONE 9: e84651
[18] D. Melton, "'Stemness,'" Essentials of Stem Cell Biology, pp. 7–17, 2014.
[19] M. C. Cabrera, "Cancer stem cell plasticity and tumor hierarchy," *World Journal of Stem Cells*, vol. 7, no. 1, p. 27, 2015.
[20] T. Reya and H. Clevers, "Wnt signalling in stem cells and cancer," *Nature*, vol. 434, no. 7035, pp. 843–850, 2005.
[21] T. Isobe *et al*., "miR-142 regulates the tumorigenicity of human breast cancer stem cells through the canonical WNT signaling pathway," *eLife*, vol. 3, 2014.
[22] W.-L. Hwang *et al*., "MicroRNA-146a directs the symmetric division of Snail-dominant colorectal cancer stem cells," *Nature Cell Biology*, vol. 16, no. 3, pp. 268–280, 2014.
[23] S. Schwitalla *et al*., "Intestinal Tumorigenesis Initiated by Dedifferentiation and Acquisition of Stem-Cell-like Properties," *Cell*, vol. 152, no. 1-2, pp. 25–38, 2013.
[24] S. Beyaz *et al*., "High-fat diet enhances stemness and tumorigenicity of intestinal progenitors," *Nature*, vol. 531, no. 7592, pp. 53–58, 2016.
[25] A. T. Lun, D. J. Mccarthy, and J. C. Marioni, "A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor," *F1000Research*, vol. 5, p. 2122, 2016.
[26] F. A. Wolf, P. Angerer, and F. J. Theis, "SCANPY: large-scale single-cell gene expression data analysis," *Genome Biology*, vol. 19, no. 1, Jun. 2018.
[27] M. D. Luecken and F. J. Theis, "Current best practices in single-cell RNA-seq analysis: a tutorial," *Molecular Systems Biology*, vol. 15, no. 6, 2019.
[28] Meers M, Bryson T, Henikoff S (2019),A streamlined protocol and analysis pipeline for CUT&RUN chromatin profiling. bioRxiv 569129; 10.1101/569129
[29] T. Zhang, Y. Lee, Y. Rui, T. Cheng, X. Jiang, and G. Li, "Bone marrow-derived mesenchymal stem cells promote growth and angiogenesis of breast and prostate tumors," Stem Cell Research & Therapy, vol. 4, no. 3, p. 70, 2013.
[30] L. Shahriyari and N. L. Komarova, "Symmetric vs. Asymmetric Stem Cell Divisions: An Adaptation against Cancer?," PLoS ONE, vol. 8, no. 10, 2013.
[31] D. Sánchez-Taltavull and T. Alarcón, "Robustness of differentiation cascades with symmetric stem cell division," Journal of The Royal Society Interface, vol. 11, no. 95, p. 20140264, Jun. 2014.
[32] S. C. Bendall *et al.*, "Single-Cell Trajectory Detection Uncovers Progression and Regulatory Coordination in Human B Cell Development," Cell, vol. 157, no. 3, pp. 714–725, 2014.
[33] K. S. Carmon, X. Gong, Q. Lin, A. Thomas, and Q. Liu, "R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/ -catenin signaling," Proceedings of the National Academy of Sciences, vol. 108, no. 28, pp. 11452–11457, 2011.
[34] X.-H. Wang *et al.*, "S100A6 Overexpression Is Associated with Poor Prognosis and Is Epigenetically Up-Regulated in Gastric Cancer," *The American Journal of Pathology*, vol. 177, no. 2, pp. 586–597, 2010.
[35] H. Alomaim *et al.*, "Dietary calcium affects body composition and lipid metabolism in rats," *Plos One*, vol. 14, no. 1, Oct. 2019.

[36] X. Chen, X. Liu, H. Lang, S. Zhang, Y. Luo, and J. Zhang, “S100 Calcium-Binding Protein A6 Promotes Epithelial-Mesenchymal Transition through β-Catenin in Pancreatic Cancer Cell Line,” *Plos One*, vol. 10, no. 3, 2015.
[37] Goudarzi A., Zhang D., Huang H., Barral S., Kwon O.K., Qi S.K. et al. (2016) Dynamic competing histone H4 K5K8 acetylation and butyrylation are hallmarks of highly active gene promoters. Mol. Cell 62, 169–180
[38] T. Nunes *et al*., “Targeting Cancer Stem Cells to Overcome Chemoresistance,” International Journal of Molecular Sciences, vol. 19, no. 12, p. 4036, 2018.
[39] R. Donato *et al.*, “Functions of S100 Proteins,” Current Molecular Medicine, vol. 13, no. 1, pp. 24–57, Jan. 2012.
[40] T. Arumugam *et al.*, “Designing and Developing S100P Inhibitor 5-Methyl Cromolyn for Pancreatic Cancer Therapy,” Molecular Cancer Therapeutics, vol. 12, no. 5, pp. 654–662, Sep. 2013.
[41] T. R. K. Reddy, C. Li, P. M. Fischer, and L. V. Dekker, “Three-Dimensional Pharmacophore Design and Biochemical Screening Identifies Substituted 1,2,4-Triazoles as Inhibitors of the Annexin A2-S100A10 Protein Interaction,” ChemMedChem, vol. 7, no. 8, pp. 1435–1446, 2012.
[42] P. Björk *et al.*, “Common Interactions between S100A4 and S100A9 Defined by a Novel Chemical Probe,” PLoS ONE, vol. 8, no. 5, Aug. 2013.
[43] A. R. Bresnick, D. J. Weber, and D. B. Zimmer, “S100 proteins in cancer,” Nature Reviews Cancer, vol. 15, no. 2, pp. 96–109, 2015.