AEG-1 alters methylation status of IGFBP7 promoter via activation of the Wnt/β-catenin signaling pathway in HCC

\*\*will add figures and in-text references – did not add to this draft due to lack of time\*\*

1. **Introduction**

Hepatocellular carcinoma (HCC) is a common malignancy of the liver, where it develops due to mutations that cause the cells to increase in replication and sidestep apoptosis. In regard to treatments for HCC, surgery can be a major option, however less than one-third of patients can qualify as surgical candidates’ due to various limiting factors. So, in many cases there is not an effective therapy to different HCC cases. However, insulin-like growth factor binding protein 7 (IGFBP7) is a secreted protein consisting of two growth factors known as IGF-I and IGF-II along with their corresponding receptors. IGFBP7 has been known to play major roles in growth, differentiation, and proliferation and it displays potential tumor suppressive activity and downregulation as the tumor progresses. Although this possible tumor suppressor exists, the malignancy of HCC continues due to the presence of astrocyte elevated gene-1 (AEG1) which acts as an oncogene and helps with progression and development of HCC.

AEG1 is known as a mediator of tumor malignancy and has been regarded as a key conversing point in the network of oncogenic signaling pathways. Previous studies demonstrate that AEG1 was significantly overexpressed in various malignant cells and plays a major role in tumorigenesis, proliferation, invasion and metastasis. The oncogene is located in 8q22 and this region is intensified among multiple cancers, including HCC; also, nearly 90% of HCC patients display the overexpression of this oncogene. Further studies have made the connection between IGFBP7 and AEG1, where IGFBP7 is one of the most downregulated genes via overexpression of AEG1. The overexpression of AEG1 can be accounted for due to a number of pathways, including MAPK, P13K/Akt/mTOR pathway, NF –kB pathway, and the Wnt/β-catenin signaling pathway. These pathways can be used in different types of HCC, in this case, the Wnt signaling pathway will be focused on due to its close association with various tumor developments and its roles in proliferation and invasion. Once this pathway is activated by AEG1, an oncogenic transcription factor, c-Myc is induced, causing for increased hepatocarcinogenesis.

I.A. Wnt/β-catenin Signaling Pathway

The Wnt signaling pathway affects the activated site of multiple effector molecules downstream. β-catenin is the main downstream effector of this signaling pathway. When the pathway is activated, the β-catenin collects in the cytosol at high levels, binds to T-cell factor/lymphoid enhancer factors, and shifted to the nucleus. By shuttling to the nucleus, target genes including MMP9, cyclin D1, and c-Myc, are expressed. Further, the high level of β-catenin in the nucleus acts as an indicator of an active Wnt signaling pathway.

**The purpose of this experiment is to show how AEG1 alters the methylation status of IGFBP7 promoter in human HCC via activation of the Wnt/ β-catenin signaling pathway.**

1. **Experiment**

In order to determine if AEG1 will activate the Wnt/ β-catenin signaling pathway while downregulating IGFBP7 in human HCC, an *in vitro* experiment focused on AEG1 silencing affects will be performed. This experiment will, in many ways, be modeled after Li et al. and Yoo et al. with their experiment to show how AEG1 promotes proliferation of breast cancer via activation of the Wnt/ β-catenin signaling pathway. HCC cell lines will be grown, maintained, and separated into two different experimental groups. The first group will be the Lenti-AEG, consisting of Lenti-AEG1-infected cells, and the second group will be the Lenti-control-infected cells. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting in these various groups detected the relative mRNA and protein levels of axin and adenomatous polyposis coli protein (APC). Axin is known as an inhibitor of the Wnt signaling pathway and APC is known as a tumor suppressor gene. With the information gained from these studies, it can be determined if AEG1 alters the methylation of IGFBP7 via the Wnt/β-catenin signaling pathway.

 II.A. Cell Culture and Colony-Formation

 Primary rat hepatocytes were isolated and cultured. SNU-423 HCC cells acquired from ATCC will be obtained and used for this experiment. The viability of these cells will be determined via standard MTT assays. MTT assay can be done in order to act as an indicator of cellular metabolic activity. The assay depends on the reduction of MTT, a yellow water-soluble tetrazolium dye via mitochondrial dehydrogenases, into purple formazan crystals. The product of formazan crystals can be measured spectrophotometrically at 550nm after its dissolution in DMSO. DMSO gives an approximation of the extent of toxicity to the cells. The HCC cell lines will be supplemented with 10% fetal bovine serum (FBS) due to its high content of growth factors, along with antibodies, 100 IU/mL penicillin, and 100mg/mL streptomycin, in 5% CO2 at 37˚C. The HCC cells were divided into the two experimental groups mentioned above of the Lenti-AEG1 group and the Lenti-control group. The AEG1 will be targeted by a sequence of short hairpin (shRNA) that contains the lenti-viral vector. The sequence will be known as “5’-AACTTACAACCGCATCATT-3’. \*\*will insert figure describing cell culture set-up\*\*

 II.B. RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

 Total RNA was extracted from the HCC cells using E.Z.N.A., which allows for the isolation of both genomic DNA and total RNA from the same cells. After doing so, mRNA was reversely transcribed into cDNA, which is DNA synthesized from a single stranded RNA like mRNA or miRNA. RT-qPCR was completed using SYBR Premix Ex Taq II; the following PCR machine will be used due to its high level of specificity. The thermocycling conditions will be set as noted: 95˚C for 30 seconds, then 39 cycles at 95˚C for 5 seconds, and 69˚C for 34 seconds. The experiment will utilize GADPH as the reference gene because it often brings good results and proves to be the most stable among genes with little to no variation between tissues. Multiple primers will be used, including AEG1 forward, AEG1 reverse, APC forward, APC reverse, axin forward, axin reverse, GADPH forward, and GADPH reverse. This experiment will be repeated for 3 trials in order to verify the results and to exclude any random errors.

 II.C. Western Blotting

 HCC cells will be washed using ice-cold Phosphate buffered saline (PBS). PBS is a balanced salt solution and is commonly used in this method in order to maintain a constant pH and osmolarity of the cells. The cells will then be lysed using ice-cold RIPA buffer, phosphatase inhibitor sodium fluoride (NaF), and sodium vanadate (NaVO3). NaF will be used in order to inactivate endogenous phosphatases and to protect protein phosphorylation. Following cell lysis, the proteins in the cell will undergo separation by 5-10% SDS-PAGE. SDS is a detergent that denatures proteins by altering their tertiary structure via unfolding them, after their binding of one SDS per two amino acids. This process charges the protein, causing for the protein to move down the polyacrylamide gel after an electric current is induced. And so, smaller proteins will move farther down the gel, towards the anode, whereas larger proteins will not move very far down the gel and remain towards the cathode. The following process is known as electrophoresis.

The proteins will then be moved onto a PVDF membrane. PVDF membranes are highly hydrophobic, so they will be given either methanol or ethanol prior to submersion in the transfer buffer. Then the polyacrylamide gel will be placed against the membrane between two sheets of porous polyethylene facing toward the cell, creating a transfer stack. The porous polyethylene is very rigid and can facilitate in handling, making it a better choice in comparison to the alternative of filter paper. Again, the use of an electric current will cause for the protein to transfer over to the membrane because of the negative charges of the proteins.

The membranes will then be blocked 1xTBST with 5% skim milk for 1 hour and will be incubated with their corresponding antibodies overnight at 4˚C. In this experiment, the primary antibodies include Rabbit anti-AEG1, rabbit anti-c-Myc, rabbit anti-axin, rabbit anti-APC, and mouse anti-β-catenin. After incubation with the primary antibodies, the membranes were washed with TBST 3 times and then incubated with the secondary antibody for 2 hours at room temperature. The secondary antibody will be used to probe the primary antibodies. In this experiment, the secondary antibody is a goat anti-rabbit IgG-horseradish peroxidase or a goat anti-mouse IgG horseradish peroxidase. Following the 3 washes of TBST, each protein will be detected using ECL Western Blotting. ECL western blotting substrate is an enhanced luminol-based chemiluminescent substrate used for the detection of horseradish peroxidase (HRP). The substrate can detect antigen via imaging and visualize the presence of HRP. The experiment will be repeated three times. \*\*will insert western-blot figure using HCC cells\*\*

1. **Discussion**

From this experiment, the level of AEG1 mRNA expression could be higher or lower dependent on which experimental group it was placed in. In the Lenti-AEG1 group, the expression would be significantly lower compared to the Lenti-control group. Further, the effect of AEG1 silencing on the activation of the Wnt/ β-catenin signaling pathway can be assessed. The mRNA levels of axin and APC in the Lenti-AEG1 group were significantly higher compared to the Lenti-control group. This would suggest AEG1 silencing inhibits the activation of the Wnt/ β-catenin signaling pathway. Looking back to the IGFBP7 as mentioned previously, it is one of the most downregulated genes from the overexpression of AEG1. The purpose of this experiment was to show that AEG1 activates the Wnt/ β-catenin signaling pathway in HCC, thus causing for altered methylation of IGFBP7.

 If the RT-qPCR data of mRNA levels among APC and axin shows the Lenti-AEG1 cells to be lower in expression than the Lenti-control cells, then this would demonstrate how AEG1’s overexpression causes tumorigenesis. Further, if the western blot analysis shows the Lenti-AEG1 cells to be darker than the control cells, then this would demonstrate the effect of AEG1 silencing on the activation of the Wnt/ β-catenin signaling pathway. And from the following results showing AEG1 does decrease expression, the connection to IGFBP7 can be made, where AEG1 overexpression will decrease its expression by default. If the following results are achieved, then this would show this experiment was effective in restructuring the work of Li et al. and Yoo et al.

 However, there are other possible, opposing outcomes to this experiment. If AEG1 mRNA expression in the experimental group increases or stays the same, this would suggest IGFBP7 is not downregulated via AEG1, but by another factor. Also, this would suggest AEG1 silencing does not inhibit the Wnt/ β-catenin signaling pathway because the mRNA levels of APC and axin in the experimental group would be lower than the control group cells. More likely, AEG1 hypermethylation does affect the status of IGFBP7, but a different signaling pathway could be more plausible versus the Wnt/ β-catenin signaling pathway.

1. **References**
2. Chen, D., Yoo, B.K. et al. 2011. Insulin-like Growth Factor-Binding Protein-7 Functions as a Potential Tumor Suppressor in Hepatocellular Carcinoma. Human Cancer Biology. Pgs. 6693-6701. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3207018/>
3. Yoo, B.K., Emdad, L. et al. 2009. Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. The Journal of Clinical Investigaation. Volume 119. Pgs. 465-477. <https://www.ncbi.nlm.nih.gov/pubmed/19221438>
4. Li, M, Dai, Y., Wang, L., and Li, L. 2017. Astrocyte elevated gene-1 promotes the proliferation and invasion of breast cancer cells by activating the Wnt/β-catenin signaling pathway. Oncology Letters. Volume 13. Pgs. 2385-2390. <https://www.ncbi.nlm.nih.gov/pubmed/28454408>
5. Jiang, D., Cho, W., Li, Z., Xi, X., Qu, Y., Jiang, Z., Guo, L., Xu, G. 2017. MiR-758-3p suppresses proliferation, migration and invasion of hepatocellular carcinoma cells via targeting MDM2 and mTOR. Biomedicine and Pharmacotherapy. Volume 96. Pgs. 535-544. <https://www.ncbi.nlm.nih.gov/pubmed/29032337>
6. Imura, S., Yamada, S., Saito, Y., Iwahashi, S., Arakawa, Y., Ikemoto, T., Morine, Y., Utsunomiya, T., Shimada, M. 2017. miR-223 and Stathmin-1 Expression in Non-tumor Liver Tissue of Patients with Hepatocellular Carcinoma. Anticancer Research. Volume 37. Pgs. 5877-5883. <https://www.ncbi.nlm.nih.gov/pubmed/28982915>
7. Akiel, M., Guo, C. et al. 2017. IGFBP7 deletion promotes hepatocellular carcinoma. Cancer Research, Volume 77(15), Pgs. 4014–4025. <https://www.ncbi.nlm.nih.gov/pubmed/28619711>
8. Akiel, M., Rajasekaran, D., Gredler, R., Siddiq, A., Srivastava, J., Robertson, C., Jariwala, N., Fisher, PB., Sarkar, D. 2014. Emerging role of insulin-like growth factor-binding protein 7 in hepatocellular carcinoma. Journal of Hepatocellular Carcinoma. Pgs. 9-19. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4918263/>
9. Yoo, BK., Santhekadur, PK., Chen, D., Emdad, L., Bhutia, S., Pannell, L., Fisher, PB., Sarkar, D. 2012. Increased RNA-Induced Silencing Complex (RISC) Activity Contributes to Hepatocellular Carcinoma. Volume 53. Pgs.1538-1548. <https://www.ncbi.nlm.nih.gov/pubmed/21520169>
10. Kamel, RR., Amr, KS., Afify, M., Elhosary, YA., Hegazy, AE., Fahim, HH., Ezzat, WM. 2016. Relation between microRNAs and Apoptosis in Hepatocellular Carcinoma. Macedonian Journal of Medical Sciences. Volume 4. Pgs. 31-37. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4884247/>
11. Pradhan, AK., Emdad, L., Das, SK., Sarkar, D., Fisher, PB. 2017. The Enigma of miRNA Regulation in Cancer. Advances in Cancer Research. Volume 135. Pgs. 25-52.
12. Tomimaru, Y., Eguchi, H. et al. 2012. IGFBP7 downregulation is associated with tumor progression and clinical outcome in hepatocellular carcinoma. International Journal of Cancer. Volume 130. Pgs.319-327. <https://www.ncbi.nlm.nih.gov/pubmed/21328580>
13. Sullivan, L., Murphy, TM., Barrett, C., Loftus, B., Thornhill, J., Lawler, M., Hollywood, D., Lynch, T., Perry, AS. 2012. IGFBP7 Promoter Methylation and Gene Expression Analysis in Prostate Cancer. The Journal of Urology. Volume 188. Pgs. 1354-1360. <https://www.ncbi.nlm.nih.gov/pubmed/22906661>
14. Strzalka-Mrozik, B., Kimsa-Furdzik, M., Kabiesz, A., Michalska-Malecka, K., Nita, M., Mazurek, U. 2017. Gene expression levels of the insulin-like growth factor family in patients with AMD before and after ranibizumab intravitreal injections. Clinical Interventions in Aging. Volume 12. Pgs. 1401-1408. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5592959/pdf/cia-12-1401.pdf>
15. Okamura, J., Huang, Y., Moon, D., Chang, X., Kim, MS. 2012. Downregulation of insulin-like growth factor-binding protein 7 in cisplatin-resistant non-small cell lung cancer. Cancer Biology and Therapy. Volume 13. Pgs. 148-155. <https://www.ncbi.nlm.nih.gov/pubmed/22277373>
16. Chen, D., Siddiq, A. et al. 2013. Insulin-like Growth Factor-binding Protein-7 (IGFBP7); A Promising Gene Therapeutic for Hepatocellular Carcinoma (HCC). Molecular Therapy. Volume. Pgs. 758-766. <https://www.ncbi.nlm.nih.gov/pubmed/23319057>
17. Chen, D., Yoo, BK., Santhekadur, PK. Gredler, R., Bhutia, SK., Fuller, C., Su, ZZ., Fisher, PB., Sarkar, D. 2011. Insulin-like growth factor binding protein-7 (IGFBP7) functions as a potential tumor suppressor in hepatocellular carcinoma (HCC). Clinical Cancer Research.