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"Formation of a GAS6 Knockdown PANC-1 cell line, Protein S overexpressing PANC-1 cell line, and the impact of exogenous Protein S on PANC-1 spheroid formation"

Objective: Pancreatic cancer is a relatively rare cancer but is the third leading cause of cancer related deaths in the United States. Pancreatic cancer is characterized by an increased risk of venous thromboembolism. Previous data from Dr. Majumder's laboratory has shown that a natural anticoagulant, Protein S (PS) inhibits the growth of PANC-1 cells, a human pancreatic cancer cell line. Interestingly, the gene that encodes growth arrest specific protein 6 (GAS6) acts as an inducer to spur PANC-1 growth. Protein S and Gas6 both act as ligands to TAM receptors (Tyro3, Axl, and Mer), a group of tyrosine kinase receptors. Our data suggests that elevated levels of PS inhibit PANC-1 cell growth and GAS6 gene knock out in PANC-1 cells slows growth. We hypothesize that PS binds TAM receptors to downregulate PANC-1 growth. Our objective is to create a PANC-1 cell line with GAS6 knocked down and to be compared to a PANC-1 cell line overexpressing PS. Another objective is to determine if PS, which is given exogenously to PANC-1 spheroids, limits spheroid formation and growth. Notably, spheroid formation assay is a surrogate assay to address cancer stem cells (CSCs) which plays a key role in developing therapeutic resistance in pancreatic cancer.

Method: To create PANC-1 cells with GAS6 knocked down, PANC-1 cells were cultured and Crispr-Cas9 was used to knockout the GAS6 gene and confirmed with Western Blot results. To create the PANC-1 cells with PS overexpression, vectors containing a neomycin selection gene and the *PROS1* gene, which encodes Protein S, were transformed in DH5α *E. coli*. The plasmids were harvested and 5 ug of plasmid DNA with Lipofectamine 2000 was used to transfect $1x10^6$ PANC-1 cells. Western blot of the PS overexpressing samples with GAPDH as a control was used, in addition to visual confirmation with fluorescence microscopy, to confirm successful transfection. Two doses of 800 ug/mL of Geneticin (G418), a selection antibiotic, were given 48 hours apart and a third dose of G418 was given at 400 ug/mL after 4 days. To form the PANC-1 spheroids, a 0.24% methylcellulose media solution, or 3D cell culture media, was made. Stock solutions of 3D media and the indicated concentrations of PS at 0 nM (control), 50 nM, 100 nM, and 200 nM were used to suspend the 10,000 cells/well in two 6 well, ultra-low attachment plates in triplicate. A repeat dose of each PS concentration was given 48 hours after the initial dose, and the spheroids were harvested 48 hours after the second dose of PS. The spheroids were washed with PBS and diluted into a 96 well plate. The wells were imaged 12 hours later, and spheroid formation was analyzed with ImageJ software.

Results: The GAS6 knockdown PANC-1 cell line was made. A Western blot will be completed soon to confirm the absence of GAS6. The PS overexpressing cells were successfully created. Both cell lines will be used in the future to compare growth inhibition. The spheroid formation assay showed that PS at 200 nM significantly suppresses PANC-1 cancer stem cells (CSCs).

Conclusion: Our data show a critical function of Protein S in suppressing PANC-1 cancer cell growth. Moreover, our data provides initial evidence to further investigate the use of PS not only to block bulk tumor growth but concurrently to suppress CSCs.