# The anticoagulant Protein S inhibits pancreatic cancer cell lines and spheroid formation

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## Introduction

### Background

Pancreatic cancer is a relatively rare cancer but is the third leading cause of cancer related deaths in the United States (1). Pancreatic cancer is characterized by an increased risk of venous thromboembolism (2). Previous data from Dr. Majumder's laboratory has shown that Protein S (PS), a vitamin K dependent natural anticoagulant inhibits the growth of PANC-1 cells, a human pancreatic cancer cell line. Interestingly, Dr. Majumder's lab has also shown that another vitamin K dependent protein, growth arrest specific 6 (GAS6) acts as a promoter of PANC-1 growth (2). 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 its growth (2).

### Significance

Given the aggressive nature of pancreatic cancer and 5-year survival rate of ~10%, understanding more about the genetic and biochemical nature of pancreatic cancer cells is of critical importance for new therapeutic development.

### Hypothesis

We hypothesize that PS binds TAM receptors to downregulate PANC-1 growth. Our objective is to create a PANC-1 cell line with GAS6 knock out and compare it side by side 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 play a key role in developing therapeutic resistance in pancreatic cancer.

### Experimental Approach

***CRISPR-Cas9 Mediated Knockdown of GAS6 in PANC-1 Cells***

To make PANC-1 cells with knocked down GAS6, PANC-1 cells were cultured and Crispr-Cas9 was used to knockout the GAS6 gene, as shown in **Figure 1.** The results were confirmed with Western blot.

***Stable PS Overexpression in PANC-1 Cells***

As shown in **Figure 2**, 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. The results were confirmed with Western blot.

***The Effect of Exogenous PS on PANC-1 Spheroids***

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.

## CRISPR-Cas9 Mediated Knockdown of GAS6 in PANC-1 Cells

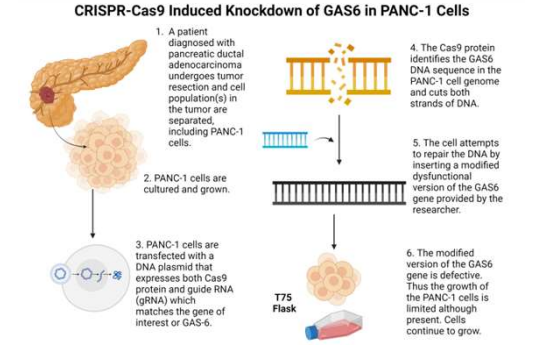



**Figure 1.** Flow chart showing GAS6 knock out in PANC-1 cells using CRISPR-Cas9. Results are confirmed via Western blot results.

## Stable PS Overexpression in PANC-1 Cells

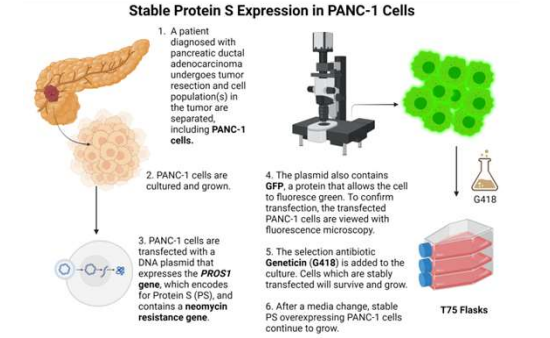
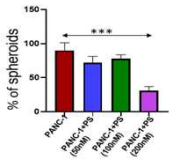



**Figure 2.** Flow chart showing PANC-1 cell line overexpressing stable Protein S. Results are confirmed via Western blot results.

## The Effect of Exogenous PS on PANC-1 Spheroids

**Figure 3.** The spheroid formation assay showed that PS at 200 nM significantly suppresses PANC-1 cancer stem cells (CSCs). The concentration of PS is indicated under each bar according to the x-axis legend. Statistical significance upon one-way ANOVA with Bonferroni correction for multiple comparisons was done and is shown in the histogram with *** = p<0.001.




## Results

- Western blot results which showed absent levels of GAS6 protein in the GAS6 Knock-down PANC-1 cells. These results indicate successful transfection.
- Western blot results showed elevated levels of PS expressed in the PS-overexpressing PANC-1 cells and positive fluorescence of GFP was noted on fluorescence microscopy. These results indicated successful transfection.
- The spheroid formation assay showed that PS at 200 nM significantly suppresses PANC-1 cancer stem cells (CSCs).

## Conclusion

- Successful modulation of PANC-1 cells has been accomplished by knocking out GAS-6 gene and upregulating expression of Protein S.
- Protein S suppresses PANC-1 cancer cell growth.
- There is initial evidence as documented by spheroid formation assay to further investigate the use of Protein S not only to block bulk tumor growth but concurrently to suppress CSCs.

## References

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