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PRINCIPAL INVESTIGATOR: Faris Farassati, PhD

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Faris Farassati, PhD

E-Mail: faris.farassati@va.gov

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Midwest Veterans' Biomedical Research Foundation
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Kansas City, MO 64128-2226

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14. ABSTRACT

Although monoclonal in origin, most tumors appear to contain heterogeneous populations of cancer cells. One possible explanation of this tumor heterogeneity is that human tumors are not merely monoclonal expansions of a single transformed cell, but rather caricatures of normal tissues, and their growth is sustained by cancer stem cells (CSCs). This conceptual shift has important implications, not only for understanding tumor biology but also for developing and evaluating effective anticancer therapies. These CSCs are thought to be more resistant to apoptosis, to survive therapy and to eventually give rise to second-line tumors, which are harder to eliminate by the first-line therapy. In this proposal, we are introducing our data in detection of CSCs in malignant peripheral nerve sheath tumors (MPNSTs) for the first time, and explain our plans for targeting these cells with a novel oncolytic virus developed in our lab. We have identified a sub-population of cells in primary human MPNST cells which are positive for CD133 (a well-known marker for CSCs) and other stem cell markers. The SS2 virus developed in our lab is capable of targeting CD133+ cells and we intend to investigate the effects of this virus as a novel therapeutic tool against MPNST. Oncolytic viruses are a novel of anti-cancer agents in advanced clinical trials which have obtained FDA approval for treatment of melanoma. We now intend to test the virus developed in our lab in the therapy of MPNST.

15. SUBJECT TERMS

MPNST, CD133, HSV-1, cancer stem cells, NF1

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Progress report:

Principal Investigator: **Faris Farassati**

[1574289-1] **Cancer Stem Cells in MPNST: A Translational Concept?**

1-Introduction

Most tumors appear to contain heterogeneous populations of cancer cells and cancer stem cells (CSC). These CSCs are more resistant to apoptosis, survive chemotherapy and eventually give rise to secondary tumors, which are harder to eliminate after the first-line of treatment. The reasons for resilience of CSCs to therapy are not well known at present. Understanding the biology of tumors and their CSCs will aid in developing novel anti-cancer therapies. We set course to investigate if cells positive for CD133 (an important CSC marker) exist in the culture of primary human in malignant peripheral nerve sheath tumors (MPNST) cells and if their elimination by an oncolytic virus can result in significant tumor regression.

We developed the first mutated version of herpes simplex virus-1(HSV-1) that is transcriptionally targeted against CD133+ cells (SS2). CD133 is identified as one of the most important biomarkers for cancer stem cells involved in the biology of a multitude of human cancers, including those of the liver, brain, colon, skin and pancreas. By programming an oncolytic virus to infect and efficiently destroy the fraction of tumor cells that act as the stem cell backbone of the tumor, will result in a robust regression in tumor growth.

In this proposal, we introduce our data in detection of CSCs MPNSTs and explain our plans for targeting these cells as a novel therapeutic strategy.

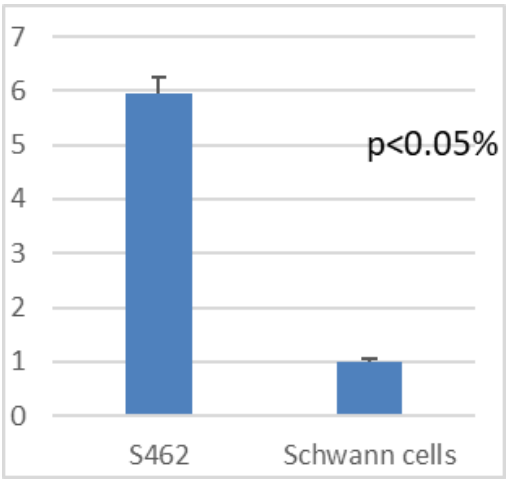
2-Keywords

MPNST, CD133, HSV-1, cancer stem cells, NF1

3. Accomplishments

Specific Aim 1: Specific Aim 1: To study the effects of SS2 virus on primary MPNST cells along with their non-malignant counterpart (normal primary human Schwann cells) (months 1-12):

Subtask 1: Evaluation of promoter activity of CD133 expression in primary MPNST cells and normal HSCs.
Methods: Dual luciferase reporter assay.
Percentage accomplished:100%
Luciferase Assay
Dual-Luciferase® Reporter Assay System (Promega, WI) was used for the promoter assay. The CD133 promoter (0.9 kb) was cloned into reporter vector pGL4.10 directly upstream of the firefly luciferase gene. The pGL4.75 plasmid, expressing the Renilla luciferase using cytomegalovirus promoter (CMV) promoter, was used as internal normalization control. The assay was performed following the manufacture's instruction. (Promega). Briefly, 1μg experimental plasmid and 1ng control plasmid were co-transfected into 1-2x10^5cells, which were cultured at 24 well plate, using lipofectamine® 2000 reagent (Lifetechnologies, CA). Cell lysates were prepared 24 hours post transfection using Passive Lysis Buffer (Promega). Cell debris was removed via centrifugation and Luciferase Assay Reagent was added to the supernatant. Bioluminescence was measured and compared using a GloMax illuminometer (Promega). The results shown here portrays 6 folds more activity in S462 compared to Schwann cells.



Subtask 2: Evaluation of generation of SS2 viral proteins in primary MPNST cells and normal HSCs. Methods: Titration assay, immune-histochemistry and western blotting.
Percentage accomplished: 75%

The expression profile of CD133 in cultured cells were analyzed by flow cytometry. About 1-10^6 cells were incubated with 100 L of 1% BSA in PBS containing 1 μg of CD16/CD32 for 30 mins on ice to block unspecific Fc intersection, then labeled with PE-conjugated anti-CD133 for 1 h. labeled cells were resuspended in PBS with 1% FBS, and analyzed by flow cytometer.
Unstained cells served as negative controls. The relative percentages of cell expressing CD133 shows that 25.9 % of S462 cells are CD133+. Two series of NF1 and Schwann cells were tested and different results are obtained by flow cytometry analysis. The first NF1 and Schwann cell lines analysis shows that 20.5 % of NF1 Cells and 9.2 % of Schwann cells are CD133+.
The second NF1 and Schwann cell lines flow cytometry results indicates that a very low percentage of CD133 are present in Schwann cells (0.69%), while NF1 cells present a relatively higher percentage of CD133 (9.5 %). An interesting observation was from both series of NF1 and Schwann cell lines which were a pair meaning that schwann cell was derived from a patient with NF1 tumor, as NF1 cell lines were significantly higher in CD133 percentage.

Protocol for CD133 expression analysis

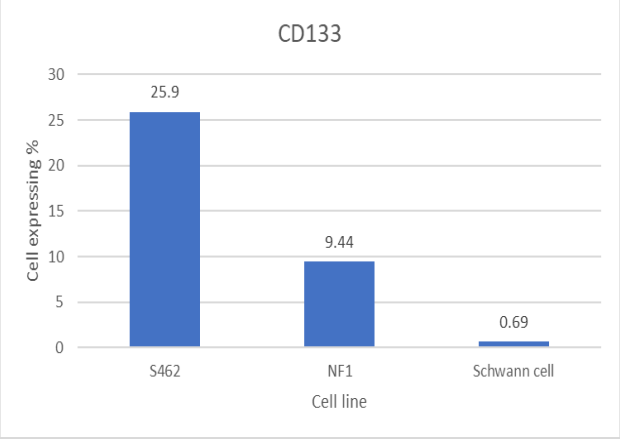
Specimen	Attune Live Cell Count cell/ul	Attune Live Cell Count cell/ml	To Be Stained	Volume in ml for 10^6	Note
NF1 S2	2.29E+03	2.29E+06	1.00E+06	0.437	Stained 10^6 cells per tube.
Schwann Cell S2	4.19E+03	4.19E+06	1.00E+06	0.239	Stained 10^6 cells per tube.

Antibody/Stain	Concentration in µg/ml	Best Signal to Noise Amount	µl Needed for Best S/N
Invitrogen CD133 APC	25	0.125	5

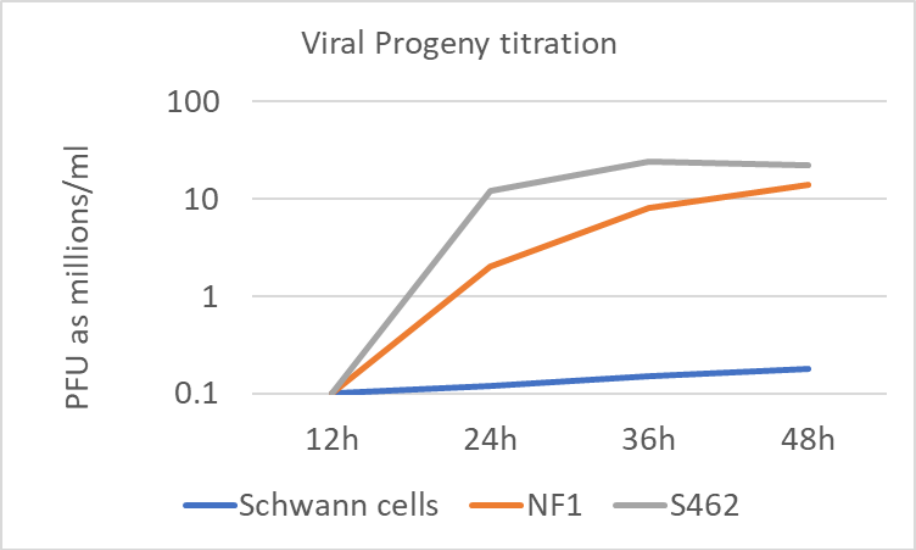
Method:
1. Received 2 conical tube of cells.
2. Counted cells on the Attune, used PI to discriminate live cells from dead cells. Cells were found to be >96% live.
3. Aliquoted out the antibody as in the table above. Prepared an unstained control, as ell.
4. Add 10^6 cells in 100 ul of wash buffer (PBS + 2% FCS).
5. Incubate on ice for 30 minutes, wash with 3 ml of wash buffer, spin down and resuspend in 350 µl/tube.
6. Run samples, collect 10k cells/tube.

[

Cell line	Cell Expressing CD133 %
S462	25.9
NF1 (Series 1)	20.8
Schwann cell (Series 1)	9.2
NF1 (Series 2)	9.5
Schwann cell (Series 2)	0.6



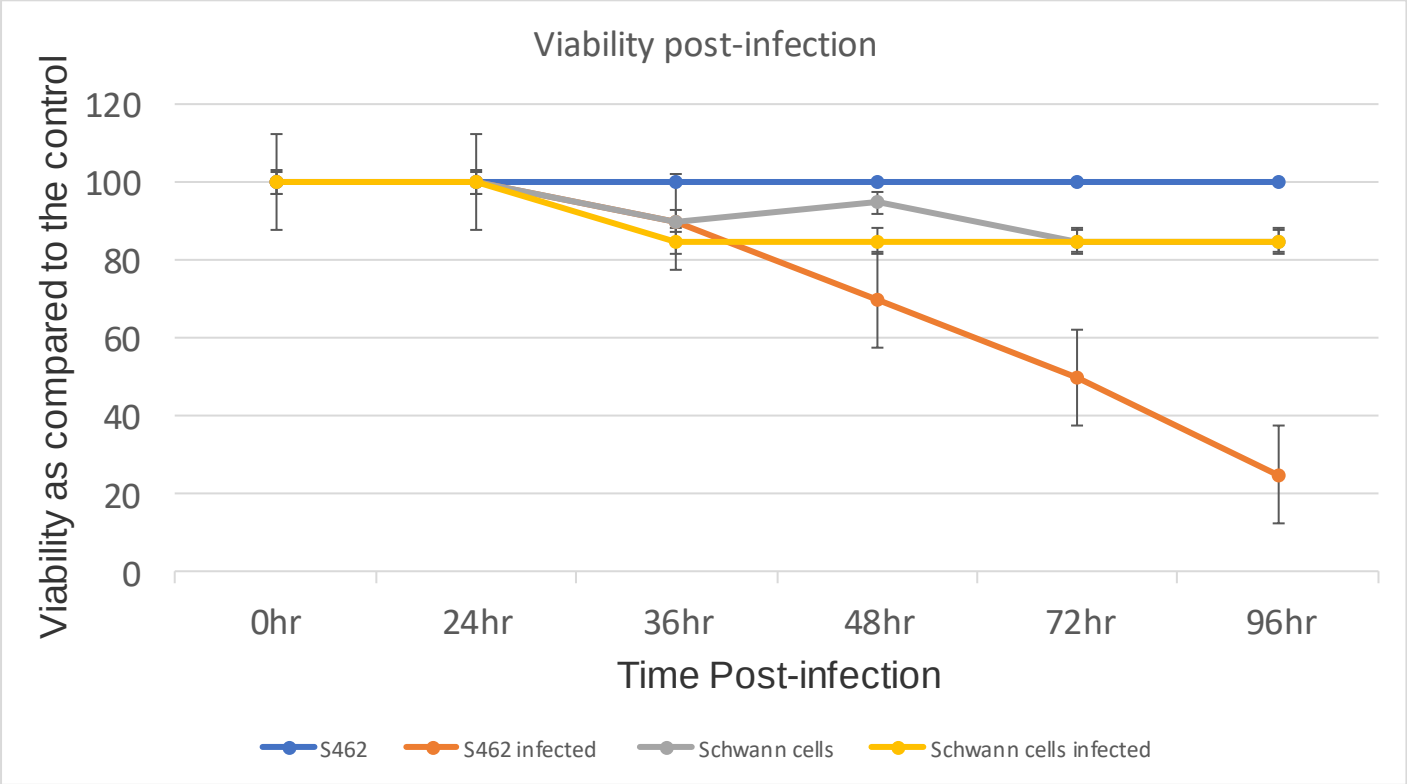
In order to do the titration assay cells were grown in eight-well slide chambers (Falcon) and infected with HSV-1 at a multiplicity of infection (MOI) of 0.5 plaque-forming units (p.f.u.) per cell, or mock-infected. Twenty hours after infection, the cells were fixed in acetone (100%) for 10 min and then left at room temperature to dry. The fixed and dried cells were incubated with a fluorescein-labelled mouse monoclonal antibody to the HSV-1 gC antigen (SyvaMicrotrak from Behring) for 30 min at 37 °C. The slides were then washed with distilled water, dried and mounted in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope on which a Carl Zeiss camera was mounted. The viral progeny production seems to be extremely higher in 462 cells.



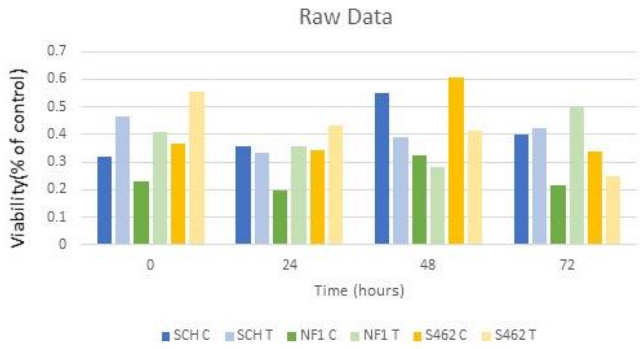
Subtask 3: Evaluation of the effects of SS2 on proliferation, invasiveness and colony formation in primary MPNST cells. *Methods: WST-1, Matrigel-invasion and colony formation assays.*
Percentage completed: 50%

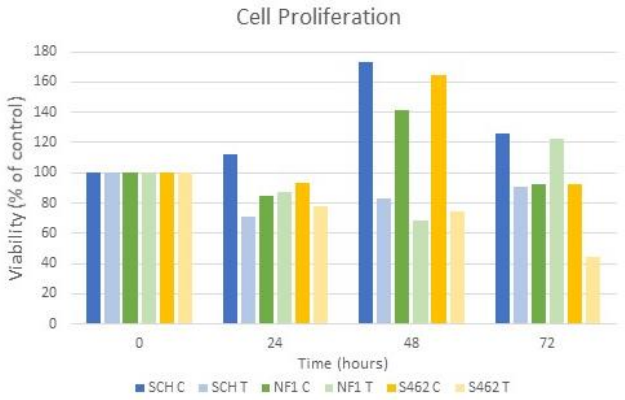
WST-1 Proliferation assay

One thousand cells were plated onto 96 well plates (BD Biosciences) and incubated overnight in 100 µl of DMEM. The next day, designated wells were trypsinized and cells counted. Wells of interest were infected starting at multiplicity of infection of 3 (MOI~3). Each 24 hours up to 144 hours, 49 µl XTT reagent (Cell Signaling) and 1 µl electron coupling solution were added to three control and three infected wells and incubated for one hour. Colorimetric viability was assessed by spectrophotometer at 450nM. The results show a significant decrease in the viability of S462 cells while Schwann are much less affected. Viability of S462 cells is significantly decreased at 48-96 hours post-infection.



Experiment Number 2:
This experiment was conducted with MOI~1 of the virus with time frames up to 72 hours. Once again a reduction in the viability of malignant cells was observed while Schwann cells seemed to be resistant.





Subtask 4: Evaluation of the effects of SS2 virus on invasiveness and sphere formation capability of CD133+ MPNST cells.
Method: Colony formation assay.
We have completed these two experiments finishing all in-vitro work needed for this proposal. Results and explanations are attached here.

Effects of SS2 Virus on MPNST Cells

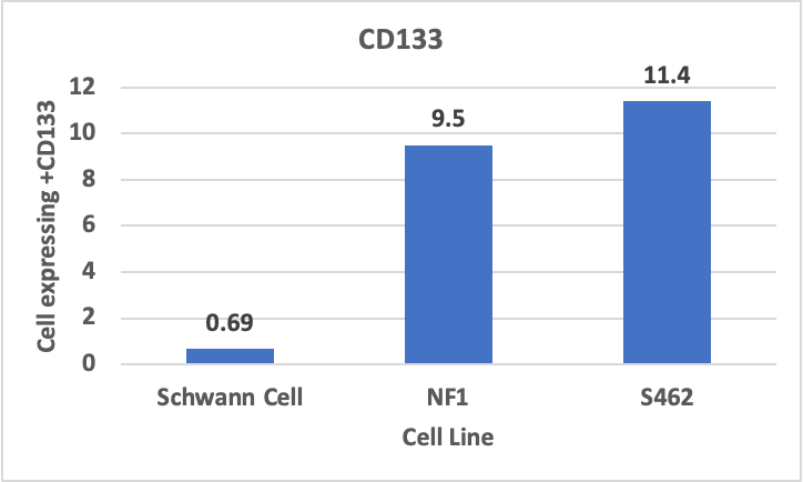
by Adriana Herdoiza & Riley Demo

CSCs in MPNST

- Background
  - Malignant peripheral nerve sheath tumors (MPNSTs) are malicious sarcomas that are commonly found in individuals diagnosed with neurofibromatosis type 1 (NF1)
  - MPNST stemming from NF1 is largely untreatable
  - It is theorized that cancer stem cells (CSCs) are responsible for the proliferation and sustainability of most tumors including MPNSTs
  - These CSCs are known to be more resistant to programmed cell death and current therapies; can even create second line tumors, which are harder to treat
  - An important CSC marker is called CD133; in this research, we are utilizing a mutated version of oncolytic virus herpes simplex virus-1 (HSV-1), named Signal-Smart 2 (SS2), that transcriptionally targets cells abundant in CD133
  - It is proposed that if CD133+ positive MPNST cells are destroyed, as a result tumor growth will decrease greatly
  - When CD133 promoter is active in cells, the SS2 virus replicates and destroys the cell

Flow Cytometry

- The expression profile of CD133 in cultured cells were analyzed by flow cytometry. About 1-10^6 cells were incubated with 100 L of 1% BSA in PBS containing 1 mg of CD16/CD32 for 30 mins on ice to block nonspecific Fc intersection, then labeled with PE-conjugated anti-CD133 for 1 h. labeled cells were resuspended in PBS with 1% FBS, and analyzed by flow cytometer
- Unstained cells served as negative controls
- Schwann cell, NF1 and S462 cell lines' flow cytometry results indicates that a very low percentage of CD133 are present in Schwann cells (0.69%), while NF1 and S462 cells present a relatively higher percentage of CD133 (9.5 and 11.4 %, respectively)
- An interesting observation was from NF1 and Schwann cell lines which were a pair meaning that schwann cell was derived from a patient with NF1 tumor, as NF1 cell lines were significantly higher in CD133 percentage

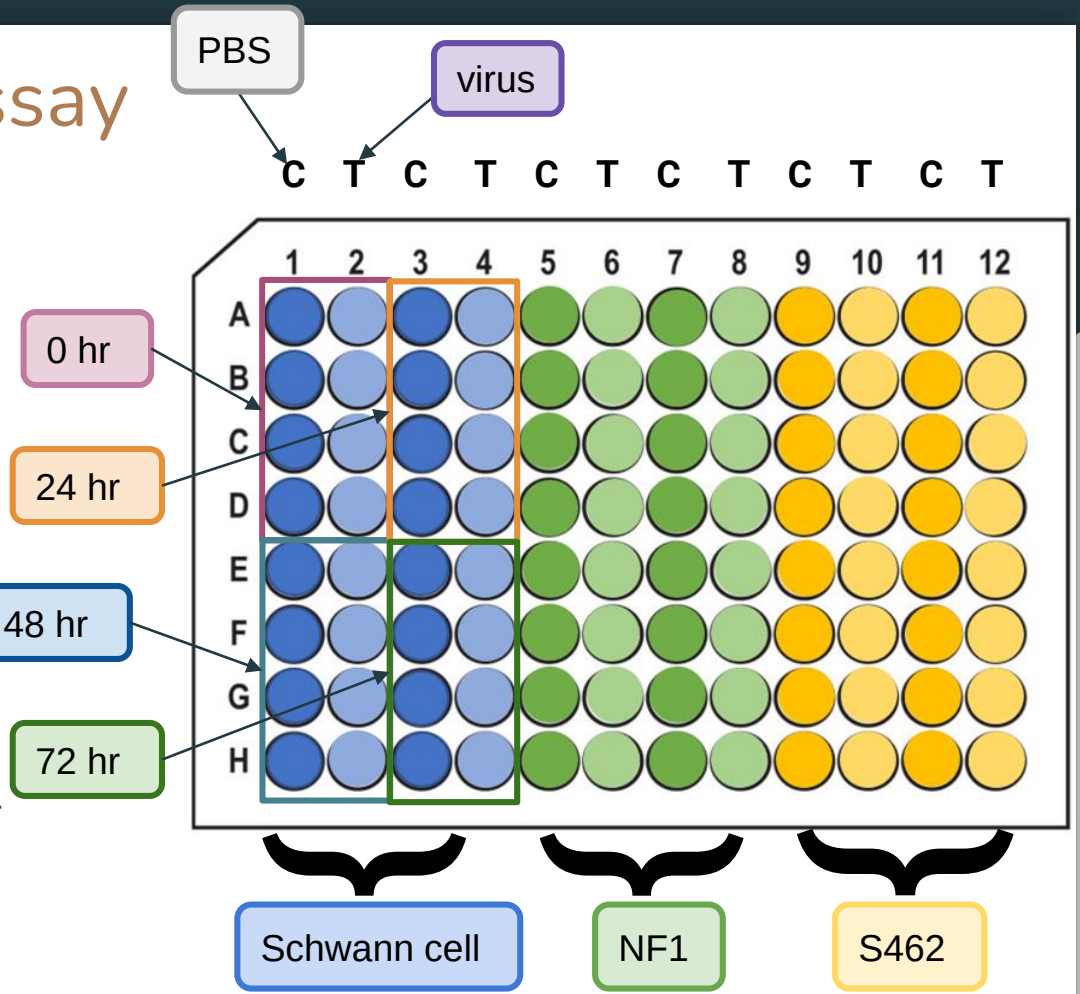


Cell Proliferation Assay

- Background
  - This assay was used for the measurement of cell proliferation in response to the introduction of oncolytic herpes simplex virus-1 (HSV-1)
- Kit Components
  - WST-1 Reagent (lyophilized) - 1 vial
  - Electro Coupling Solution (ECS) - 5mL
- Reagent Preparation
  - Dissolve the lyophilized WST-1 reagent with 5mL of the Electro Coupling Solution (ECS)
- Cell Lines
  - Schwann cell
  - NF1
  - S462

Cell Proliferation Assay

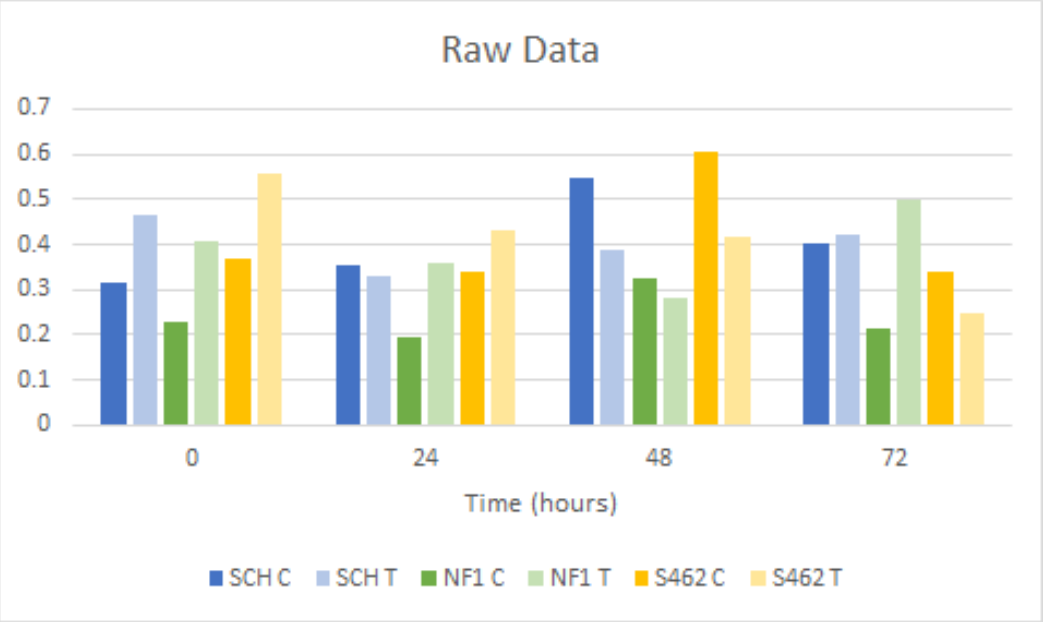
- Steps
  - Cells were cultured in a 96-well plate
  - PBS was added to the control while virus was added to test
  - At each time period, WST-1/ECS solution was added to each respective well in that section
  - The stain allowed the absorbance of the cells to be read using a spectrophotometer
  - The wavelength was set to 440-480nm



Cell Proliferation Assay

- Raw Data

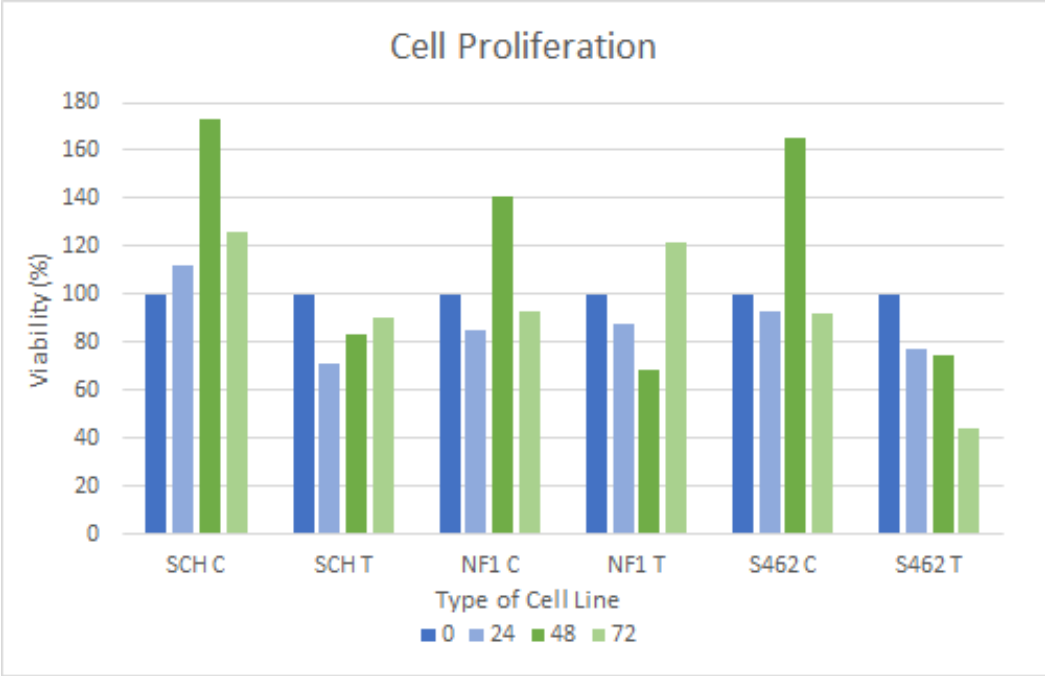
	0	24	48	72
SCH C	0.31775	0.35575	0.54925	0.4005
SCH T	0.4655	0.332	0.388	0.42175
NF1 C	0.2305	0.19575	0.325	0.21375
NF1 T	0.40775	0.35675	0.27975	0.498
S462 C	0.367	0.342	0.60525	0.3395
S462 T	0.556	0.4315	0.41475	0.24775



Cell Proliferation Assay

- Results

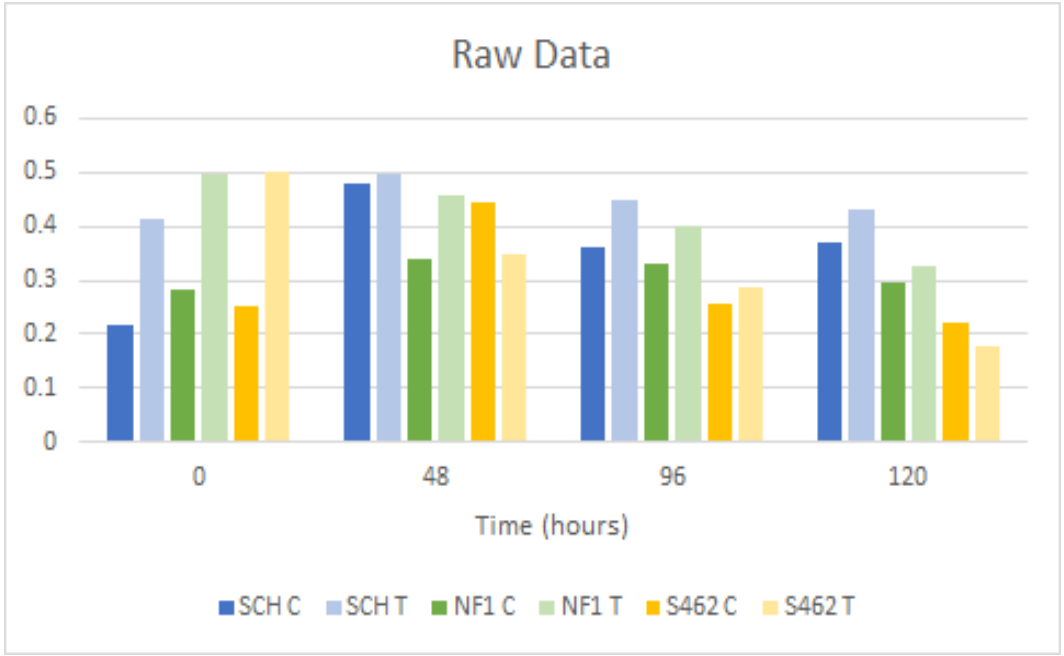
	0	24	48	72
SCH C	100	112	172.9	126
SCH T	100	71.3	83.4	90.6
NF1 C	100	84.9	141	92.7
NF1 T	100	87.5	68.6	122.1
S462 C	100	93.2	164.9	92.5
S462 T	100	77.6	74.6	44.6



Cell Proliferation Assay (Trial 2)

- Raw Data

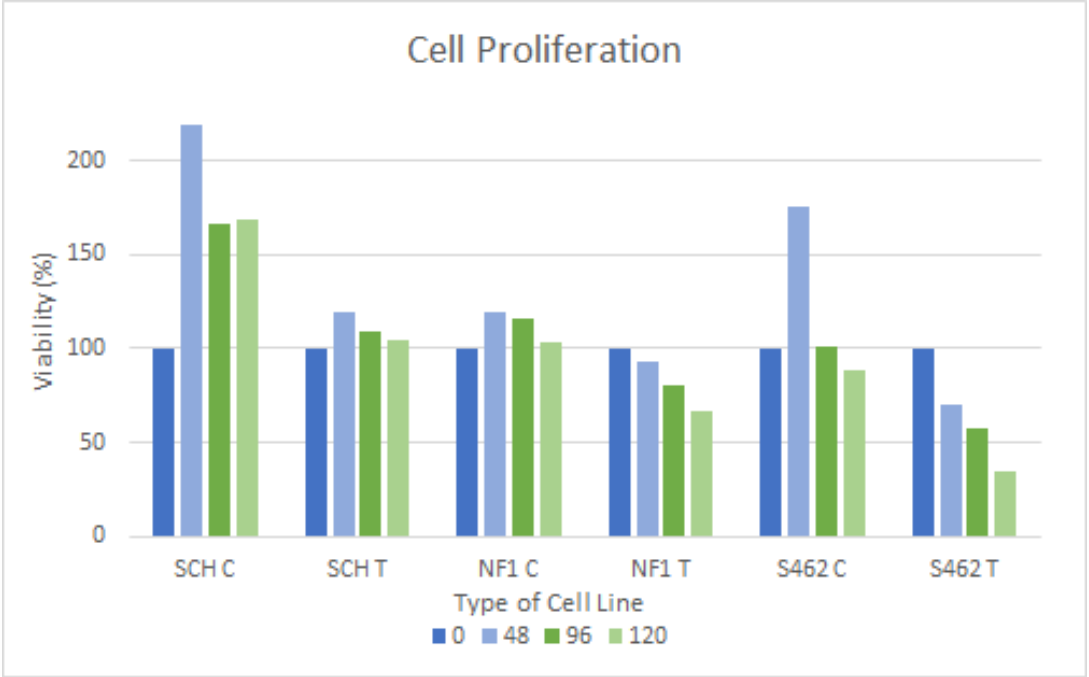
	0	48	96	120
SCH C	0.2185	0.47875	0.3635	0.369
SCH T	0.4135	0.49575	0.449	0.433
NF1 C	0.284	0.339	0.33	0.294
NF1 T	0.4955	0.45925	0.3995	0.3285
S462 C	0.2525	0.44325	0.2565	0.223
S462 T	0.49925	0.349	0.2855	0.17575



Cell Proliferation Assay (Trial 2)

- Results

	0	48	96	120
SCH C	100	219.1	166.4	168.9
SCH T	100	119.9	108.6	104.7
NF1 C	100	119.4	116.2	103.5
NF1 T	100	92.7	80.6	66.3
S462 C	100	175.5	101.6	88.3
S462 T	100	69.9	57.2	35.2



Cell Proliferation Assay

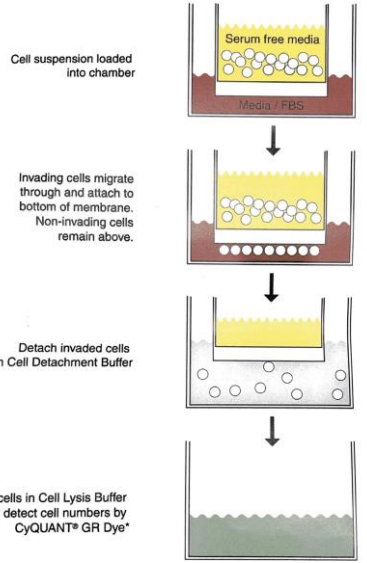
- Conclusion
  - The application of the herpes virus on cancerous cells (NF1, S462) resulted in a greater decrease of population than the healthy cells (Schwann)
  - The virus is targeting the CD133 markers of the cancer cells, which are present at a much higher number than Schwann
  - A few inconsistencies due to error, more trials of this experiment would be beneficial

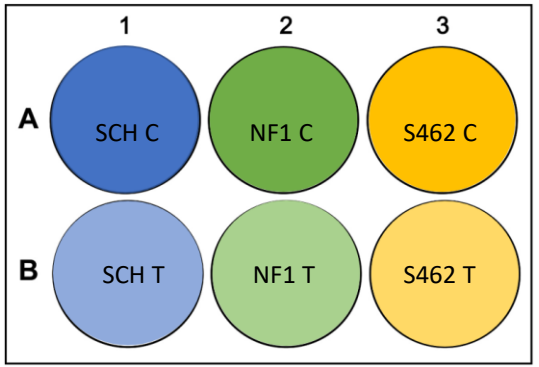
Cell Invasion Assay

- Background
  - When cancer cells invade healthy cells through the extracellular matrix (ECM), they adhere and spread along the blood vessel wall
  - Proteolytic enzymes dissolve small holes in the basement membrane around blood vessels to allow the invasion of cancer cells
  - In this experiment, invaded cells on the bottom of the insert membrane are dissociated utilizing the Cell Detachment Buffer; these cells are then lysed and detected by CyQuant GR dye which exhibits strong fluorescence enhancement when bound to cellular nucleic acids
- Kit Components
  - Cell Detachment Solution - 16mL
  - 4X Cell Lysis Buffer - 16mL
  - CyQuant GR Dye - 75µL
- Cell Lines
  - Schwann cell
  - NF1
  - S462

Cell Invasion Assay

- Steps
  - Serum-free media was added to the inserts to rehydrate the ECM layer
  - The media inserts were removed and cell suspension was prepared and added to each insert
  - Serum-free media was added to the lower chamber and the plate was incubated for 24 hours
  - The cells/media were removed and the invasion chamber inserted into a clean well containing Cell Detachment Solution and incubated for 30 minutes
  - The cells were dislodged and the insert was removed from the well
  - Lysis Buffer/Dye Solution was prepared and added to each well containing cell detachment solution with the cells that invaded through the ECMatrix-coated membrane, then incubated for 15 minutes
  - The mixture was transferred to a 96-well plate and read with a fluorescence plate reader using a 480/520 nm filter set

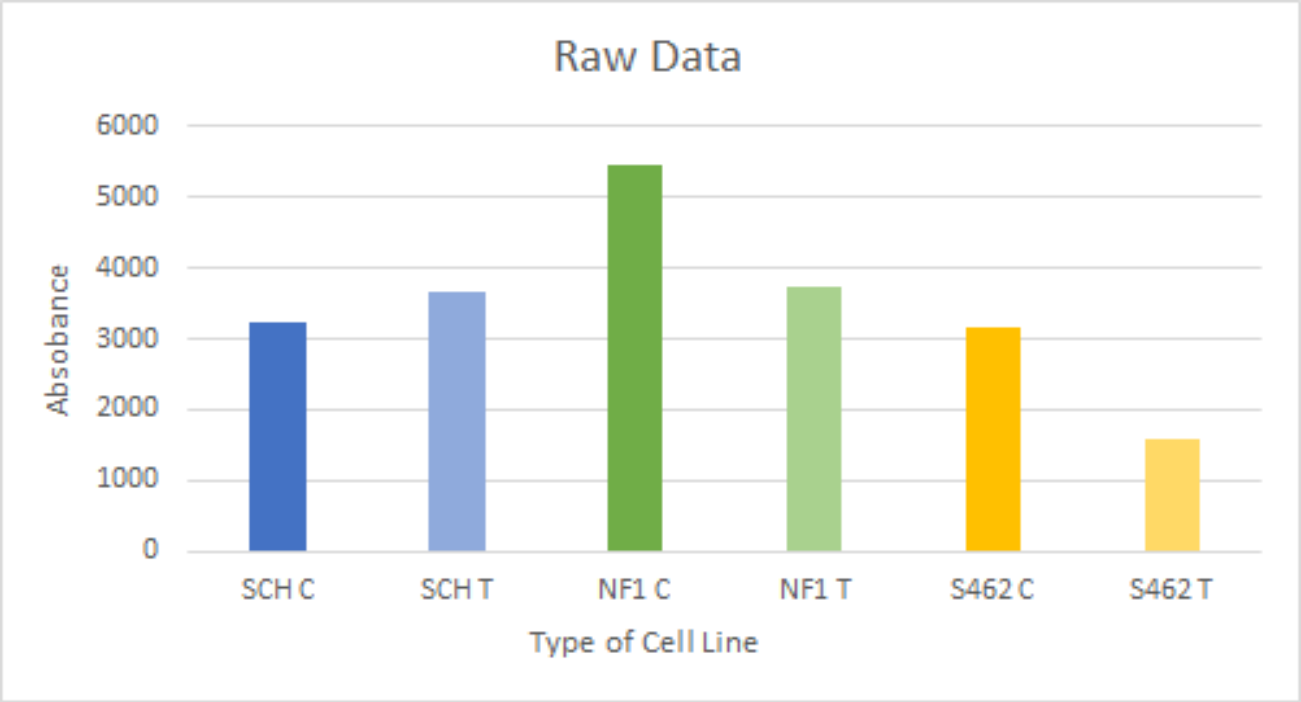




Cell Invasion Assay

- Raw Data

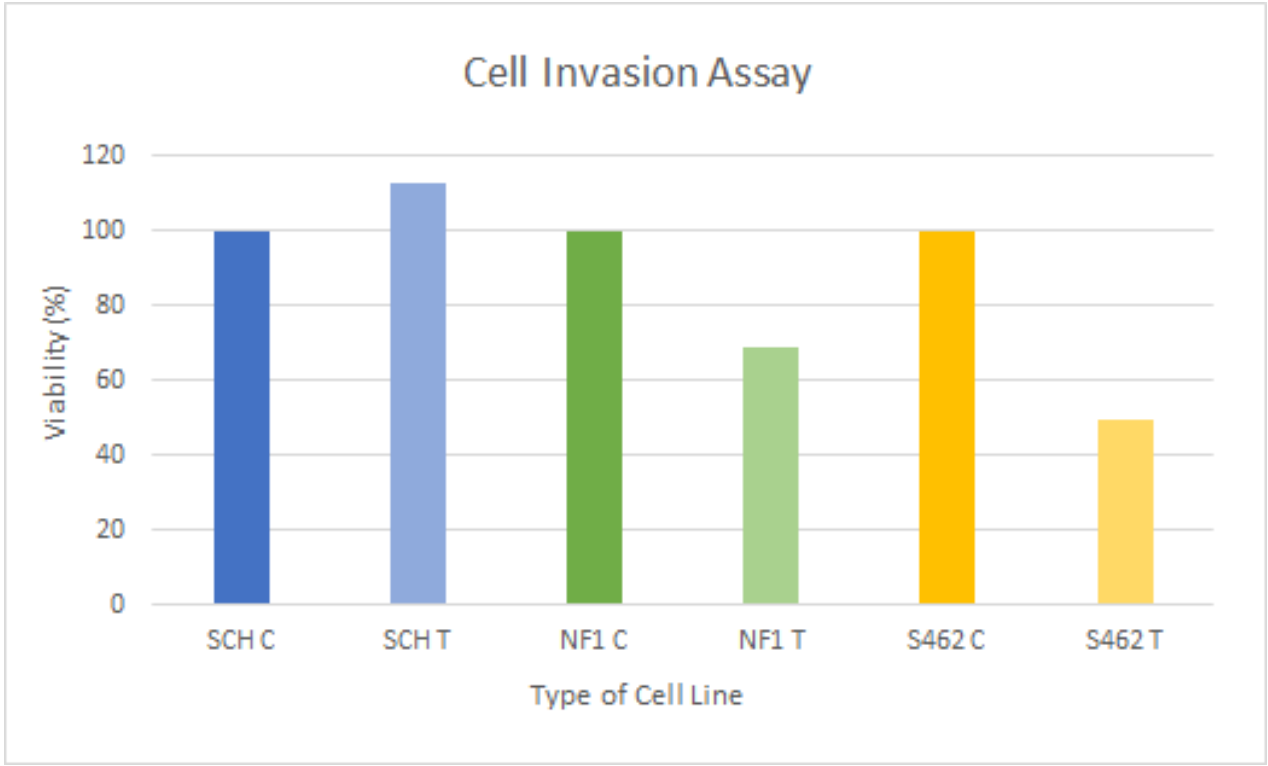
	Absorbance
SCH C	3223
SCH T	3645
NF1 C	5441
NF1 T	3731
S462 C	3161
S462 T	1571



Cell Invasion Assay

- Results

	Viability
SCH C	100
SCH T	113.1
NF1 C	100
NF1 T	68.6
S462 C	100
S462 T	49.7



Cell Invasion Assay

- Conclusion
  - The cells that are alive can pass through the membrane; the cells that are dead cannot
  - The cancer cells (NF1, S462) showed a decrease in invasion and lesser viability rate than the healthy cells (Schwann)
  - Similarly to the proliferation assay, the herpes virus killed the cancer cells at higher rates and prevented invasion
    - Schwann could still pass through the membrane even after the virus was added

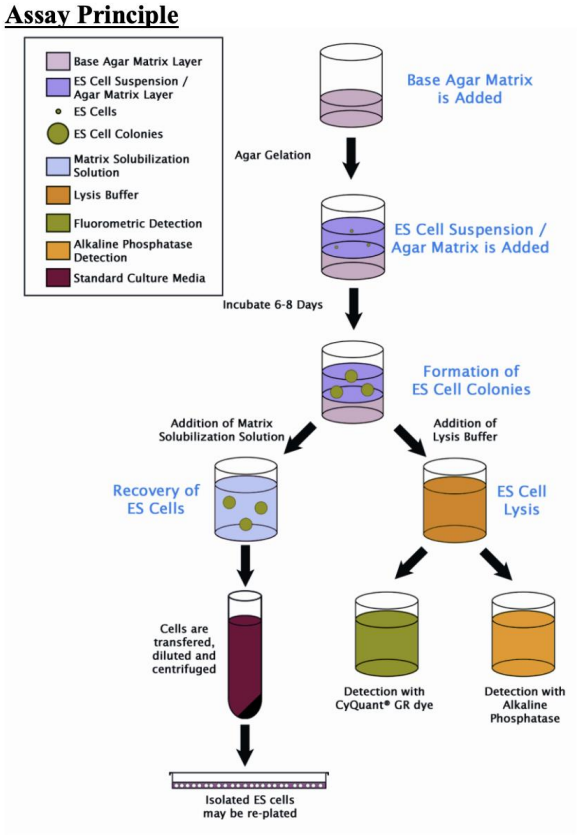
Stem Cell Colony Formation Assay

- Background
  - A cell colony formation assay is commonly used to analyse anchorage-independent growth, measuring proliferation of cells in a semi-solid media.
  - Analysis of the colony formation assay is important in testing proliferation of a single cell into colony formation
  - The reason for using this kit was to turned the 3-4 week process into a 6-8 day process and extrapolate the results in more meaningful ways.
  - Independent variable in this experiment was presence of the Herpes Simplex Virus-1, named Signal-Smart 2 (SS2)
  - Dependent variable: When CyQuant® GR Dye was put in a fluorescent plate reader and absorbance was measured. Alternatively, Alkaline phosphatase activity colorimetrically was measured to assess the presence of stem cell growth as it is a biomarker.
- Kit Includes:
  - 10X CytoSelect™ Agar Matrix Solution: One sterile bottle – 10.0 mL
  - CytoSelect™ Matrix Diluent): One sterile bottle – 4.0 mL
  - 3. 5X DMEM Solution : Three sterile tubes – 1.5 mL each
  - 10X Matrix Solubilization Solution: One sterile tube – 1.8 mL 5.
  - CyQuant® GR Dye: One tube – 75 µL 6.
  - Lysis Buffer: One bottle – 20.0 mL 7.
  - StemTAG™ AP Activity Assay Substrate
  - One amber bottle – 5.0 mL 8. AP Stop Solution:
  - One bottle – 20.0 mL 9. AP Activity Assay Standard
  - One tube – 1.0 mL of 5 mM p-Nitrophenol
- Materials Not Supplied
  - Stem Cells and Culture Medium
  - 37°C Incubator, 5%CO2 atmosphere
  - Light Microscope
  - 96-well fluorometer
  - 96-well sterile microplate (flat bottom)
  - 37°C and boiling water baths

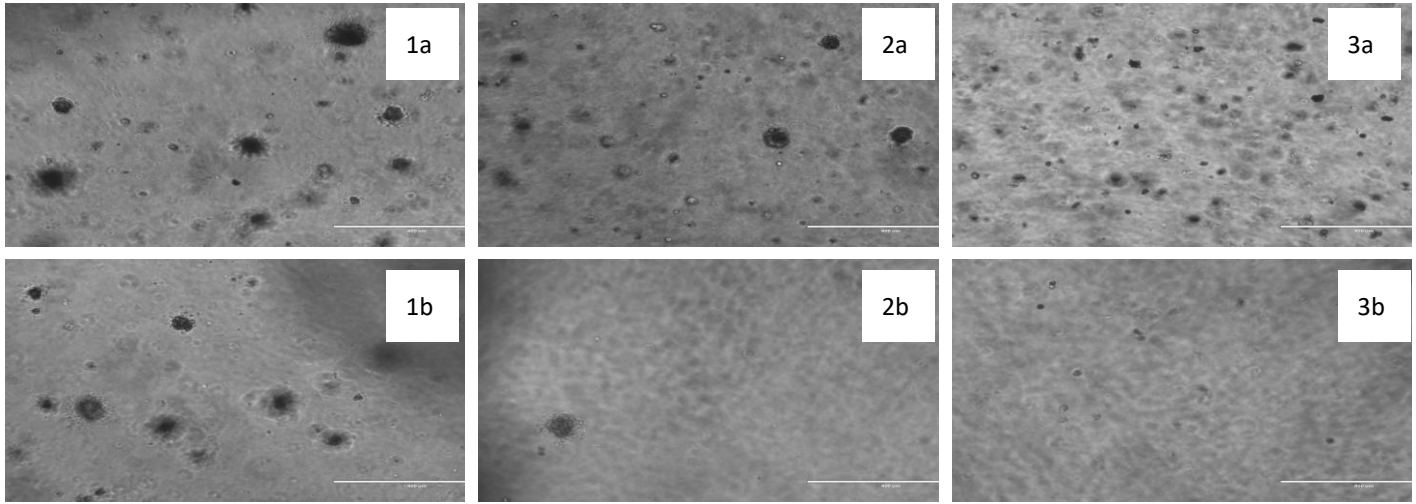
Stem Cell Colony Formation Assay

- Steps

1. Preparation of Reagents
2. Preparation of Base Agar Matrix Layer
3. Addition of Cell Suspension/Agar Matrix Layer
4. Quantitation or Anchorage-Independent Growth
5. Quantitation of Alkaline Phosphatase Activity
6. Cell Recover and Re-plating (if necessary)



Cell Colony Formation

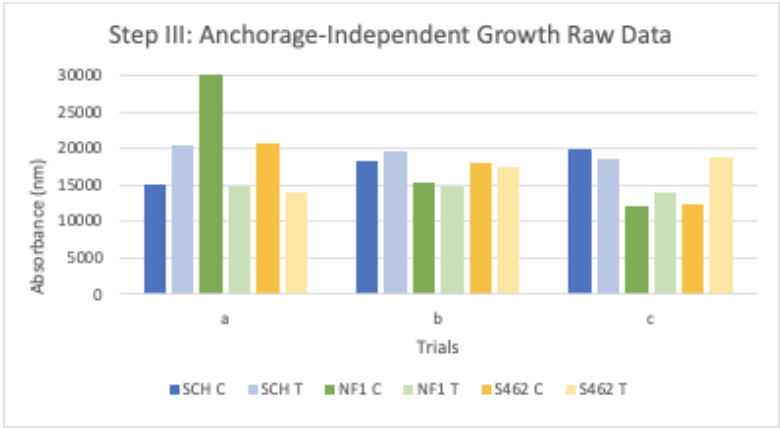


- Colony formation of Schwann cells, NF1 and S462 have been investigated after 6 days in agar in presence and absence of viruses.
- Pictures 1a (Schwann cells) and 1b (Schwann cells+virus) show the low effect of virus on colony formation in Schwann cells. Pictures 2a (NF1 cells), 2b (NF1+virus), 3a (S462 cells) and 3b (S462+virus) show that presence of virus and a higher significant effect on colony formation in these cell lines.

Stem Cell Colony Formation Assay: Step III

- Raw Data: Quantitation of Anchorage-Independent Growth via CyQuant® GR dye.

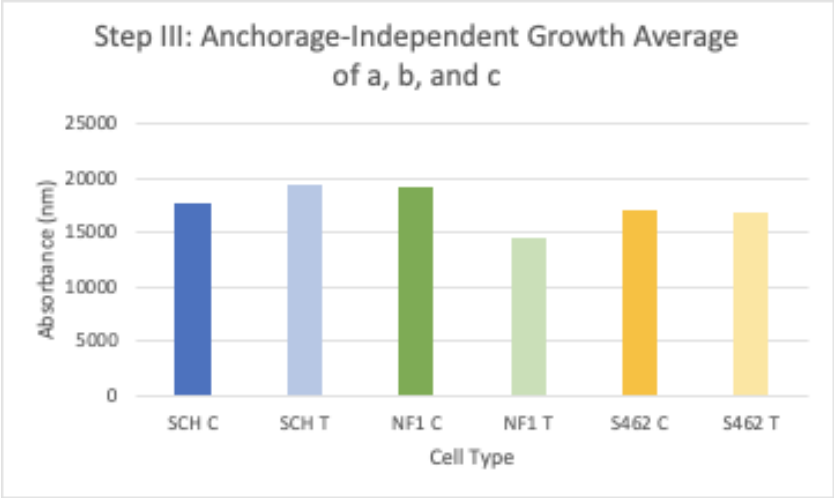
	a	b	c	AVG	SD
SCH C	15157 nm	18249 nm	19968 nm	17791 nm	2437.9
SCH T	20373 nm	19554 nm	18460 nm	19462 nm	959.7
NF1 C	30087 nm	15447 nm	12069 nm	19201 nm	9577.7
NF1 T	14764 nm	14836 nm	14043 nm	14547 nm	438.5
S462 C	20741 nm	17902 nm	12277 nm	16973 nm	4307.7
S462 T	14061 nm	17609 nm	18939 nm	16869 nm	2521.6



Stem Cell Colony Formation Assay: Step III

- Results: T-Test for Quantitation of Anchorage-Independent Growth via CyQuant® GR dye.

	T-Test Value	P-value	significant
SCH	-1.10465	0.331286	no
NF1	0.84064	0.447863	no
S462	0.03597	0.973028	no

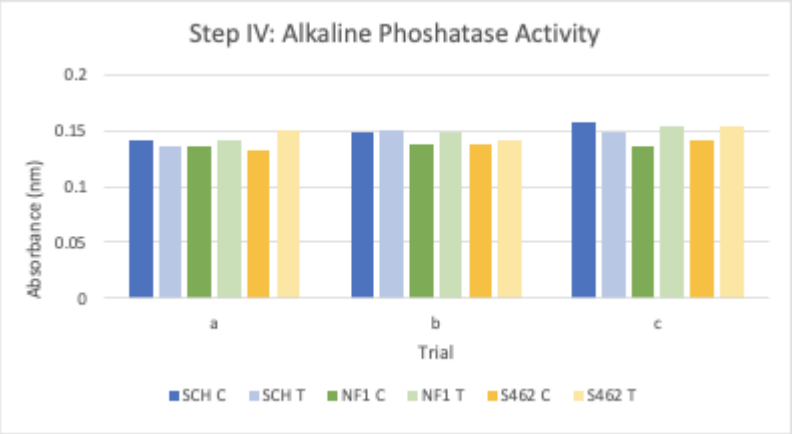


Averages of a, b, and c as a visual comparison

Stem Cell Colony Formation Assay: Step IV

- Raw Data: Quantitation of Anchorage-Independent Growth via Alkaline Phosphatase Activity

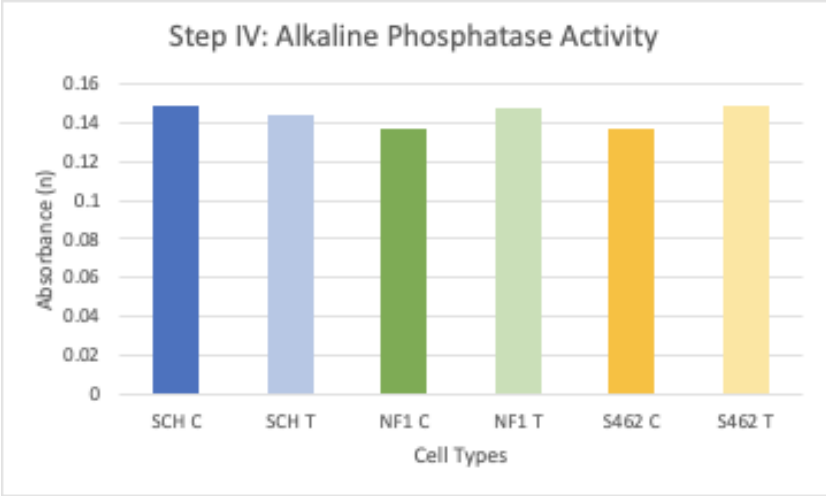
	a	b	c	AVG
SCH C	0.141 nm	0.148 nm	0.157 nm	0.149
SCH T	0.135 nm	0.15 nm	0.148 nm	0.143
NF1 C	0.136 nm	0.138 nm	0.135 nm	0.136
NF1 T	0.142 nm	0.148 nm	0.154 nm	0.148
S462 C	0.133 nm	0.137 nm	0.142 nm	0.137
S462 T	0.15 nm	0.142 nm	0.154 nm	0.149



Stem Cell Colony Formation Assay: Step IV

Results: T-Test for Quantitation of Anchorage-Independent Growth via Alkaline Phosphatase Activity

	T-Test Value	P-value	significant
SCH	0.6566	0.547294	no
NF1	-3.26377	0.030971	yes
S462	-2.58497	0.061006	no

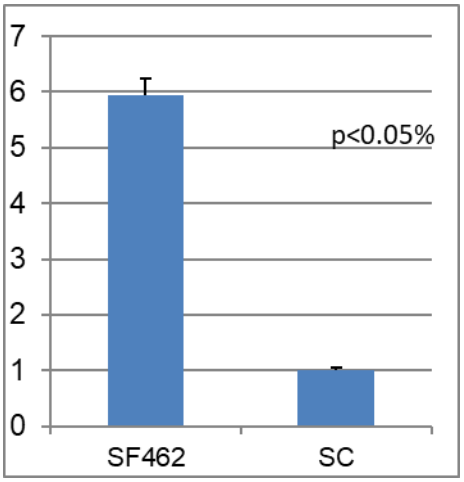


Averages of a, b, and c as a visual comparison

Errors and Conclusion: Colony Formation Assay

- Inconsistency of data across both control and test
- Qualitative results: misnomer? Inconsistency with quantitative results
- No significant difference when t-test was performed and analyzed
- Overall: we can not conclude that the SS2 had a significant difference on cell colony formation in qualitative results as the results were too inconsistent for appropriate analysis
- Errors: Human? Quantitative? Hour Wait Analysis?
- My Conclusion: Due to lack of trial and too many inconsistencies, if we need these results for further study of the SS2 virus in vivo, we need to redo this test and analyse more data to hold scientific integrity

Additional experiment accomplished:
Promoter assay for CD133:
Luciferase Assay
A 1.8-kb fragment containing the CD133 promoter was amplified by nested PCR from human genomic DNA isolated from cultured Hep3B2 cells. The first round of PCR was performed using forward primer 5′-aaactgtctttcctggcttc-3′ and reverse 5′-ttccttaaacatactcaccg-3′. The nested PCR was performed using forward primers 5′-tgaaccggtcctgcaagcggcacatcagag-3′ (*AgeI* site, underlined, was added to this primer) and reverse primer 5′-tgaactagtgcgttagcatcgctttaattcag-3′ (the *SpeI* site, underlined, was added to this primer). The PCR fragment containing the CD133 promoter was cloned into reporter vector pGL4.10 directly upstream of the firefly luciferase gene. The pGL4.75 plasmid, expressing the Renilla luciferase using cytomegalovirus promoter (CMV) promoter, was used as internal normalization control. The assay was performed following the manufacture's instruction. (Promega). Briefly, 1μg experimental plasmid and 1ng control plasmid were co-transfected into 1-2x10^5cells, which were cultured at 24 well plate, using lipofectamine® 2000 reagent (Lifetechnologies, CA). Cell lysates were prepared 24 hours post transfection using Passive Lysis Buffer (Promega). Cell debris was removed via centrifugation and Luciferase Assay Reagent was added to the supernatant. Bioluminescence was measured and compared using a GloMax illuminometer (Promega).



Specific Aim 2: We will investigate the effects of SS2 virus in treatment of MPNST in orthotopic xenograft model for MPNST (months 12-24):

Our protocol for animal research is now approved. There were a long delay in this due to the pandemic and expiration of our previous protocol. We are now about to start ordering animals. The animal protocol is attached which does not change any of the specific aims of the project.

3.1-Future directions:

We are pursuing establishing the xenograft model for MPNST and treating the tumors with the CD133 virus. In order to get a more comprehensive set of data, we are planning to use two MPNST cell lines for this specific aim. We are currently preparing ordering animals per the protocol attached.

4-Impact

To the best of our knowledge, this is the first time that a virus is being developed to transcriptionally target CD133+ CSCs in a cell-specific manner. If proven to be successful, such strategy can significantly diminish the growth, regeneration and maintenance capabilities of MPNST. Therefore,

the virus proposed here could be used as a gene therapy tool against MPNST. Also, once the overall strategy in targeting CD133 cells is proven to be efficient in causing tumor regression, it would open a new horizon towards developing other strategies such as drug therapy against CD133.
From clinical standpoint, different members of oncolytic viruses have entered clinical trials and therefore such therapeutic strategy is highly relevant to the clinic.

5-Changes/Problems

We faced some issues with hiring the correct personnel due to the government shutdown in 2019. Later, we had problems with viability of cells sent to us by ATCC which was reported back and replaced with new batches. At this point, we face a level of inconsistency in the CD133 expression in tested cells. We are now ordering our 4th badge of cells and attempting to repeat the experiments along with performing other in-vitro assays necessary

6-Products

1-Targeting Cancer Stem Cells by Oncolytic Viruses and Nano-Mediated Delivery
Mahsa Hosseini, Fatemeh S **Farassati**, Faris **Farassati**
Onco Targets Ther. 2020; 13: 9349–9350. Published online 2020 Sep 22. doi: 10.2147/OTT.S279639
PMCID: PMC7519821

2-MPNST stem cells and the possibility of targeting them: M. Hosseini, F. Farassati. *Oncolytic Virotherapy*, In Press, 2021.

7-Participants & Other Collaborating Organizations

Mahsa Hosseini, PhD

8-Special Reporting Requirements: N/A

9-Appendices; Please see attached slides.