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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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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.

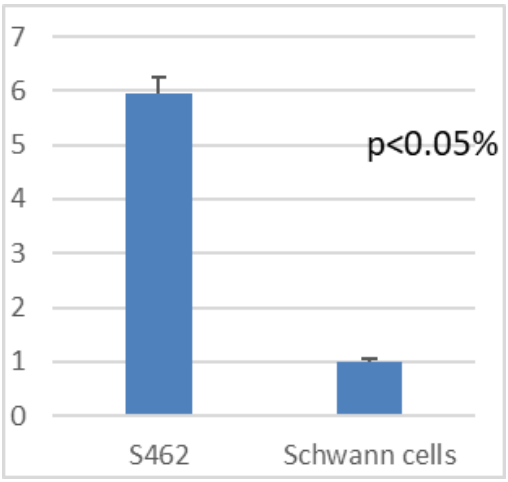
2-Keywords

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

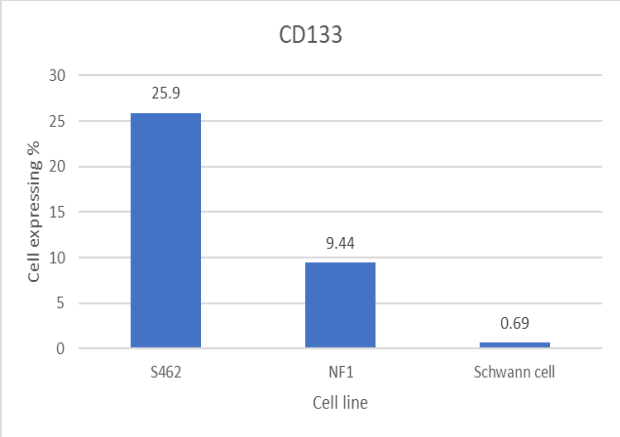
Specimen	Attune Live Cell Count cell/ul	Attune Live Cell Count cell/ml	To Be Stained	Volume in ml for 10^6	Note
NF1 S2	2.29E+03	2.29E+06	1.00E+06	0.437	Stained 10^6 cells per tube.
Schwann Cell S2	4.19E+03	4.19E+06	1.00E+06	0.239	Stained 10^6 cells per tube.

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

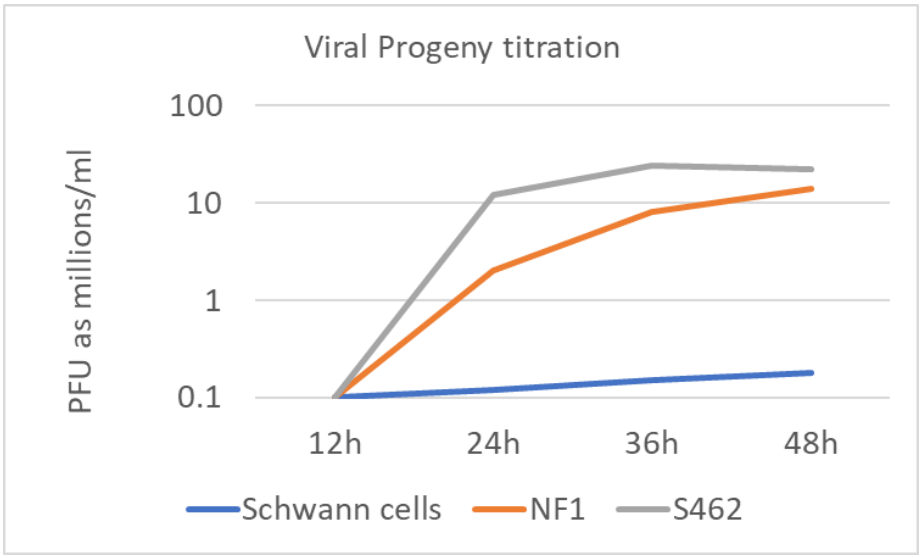
Method:
1. Received 2 conical tube of cells.
2. Counted cells on the Attune, used PI to discriminate live cells from dead cells. Cells were found to be >96% live.
3. Aliquoted out the antibody as in the table above. Prepared an unstained control, as ell.
4. Add 10^6 cells in 100 ul of wash buffer (PBS + 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.

[

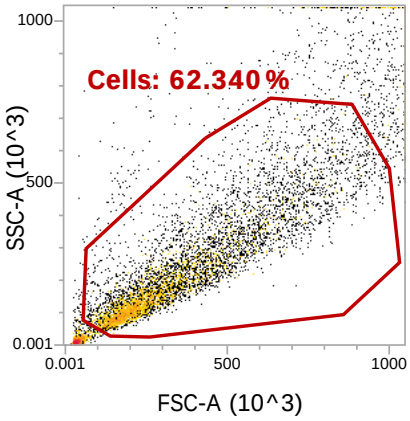
Cell line	Cell Expressing CD133%
S462	25.9
NF1 (Series 1)	20.8
Schwann cell (Series 1)	9.2
NF1 (Series 2)	9.5
Schwann cell (Series 2)	0.6



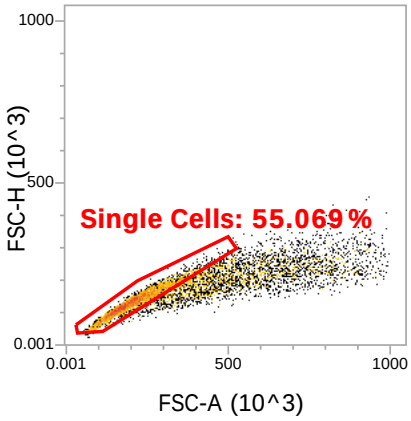
In order to do the titration assay cells were grown in eight-well slide chambers (Falcon) and infected with HSV-1 at a multiplicity of infection (MOI) of 0.5 plaque-forming units (p.f.u.) per cell, or mock-infected. Twenty hours after infection, the cells were fixed in acetone (100%) for 10 min and then 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.



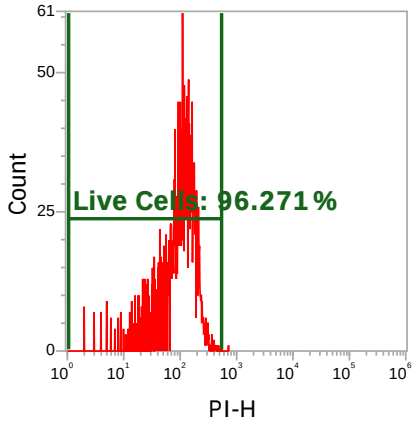
All Events - NF1 S2



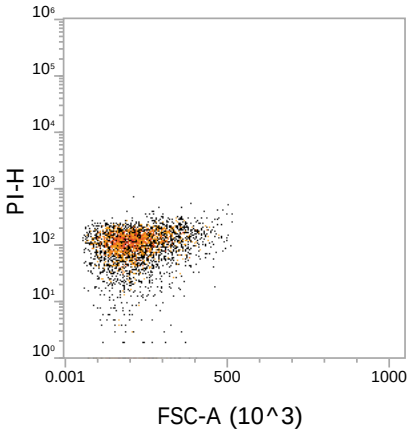
Cells - NF1 S2



Single Cells - NF1 S2



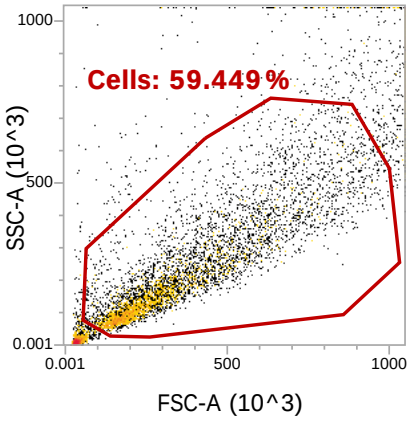
Single Cells - NF1 S2



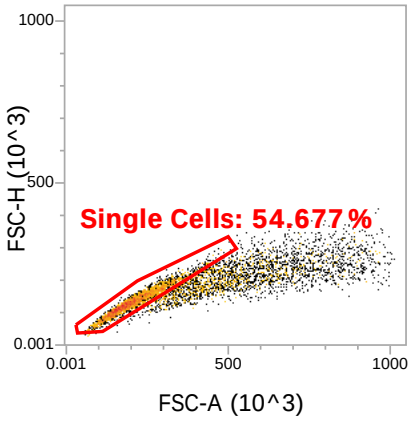
Experiment: **20 Feb 25 Count**
Group: **Group**
Sample: **NF1 S2**
Time Recorded: **11:41:04**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	10,000	100.000	100.000
Cells	Cells	FSC-A	SSC-A	6,234	62.340	62.340
Single Cells	Single Cells	FSC-A	FSC-H	3,433	34.330	55.069
Live Cells	Live Cells	BL2-H		3,305	33.050	96.271

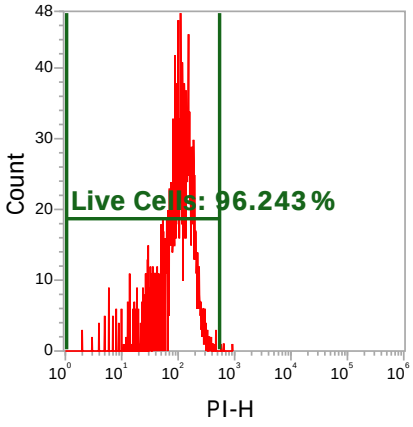
All Events - NF1 S2 + PI



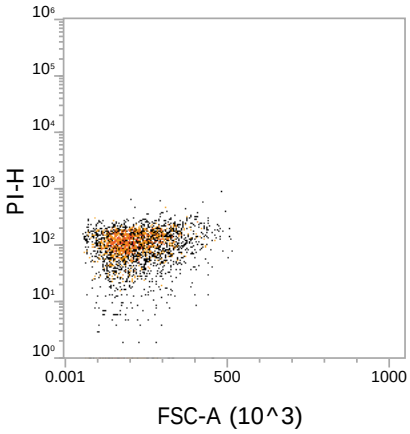
Cells - NF1 S2 + PI



Single Cells - NF1 S2 + PI



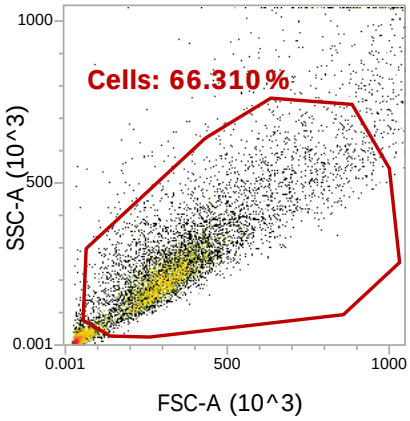
Single Cells - NF1 S2 + PI



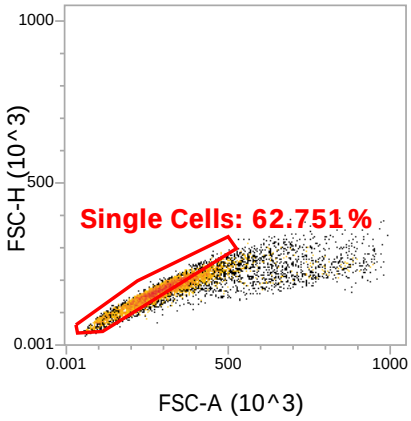
Experiment: **20 Feb 25 Count**
Group: **Group**
Sample: **NF1 S2 + PI**
Time Recorded: **11:45:34**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	9,334	100.000	100.000
Cells	Cells	FSC-A	SSC-A	5,549	59.449	59.449
Single Cells	Single Cells	FSC-A	FSC-H	3,034	32.505	54.677
Live Cells	Live Cells	BL2-H		2,920	31.283	96.243

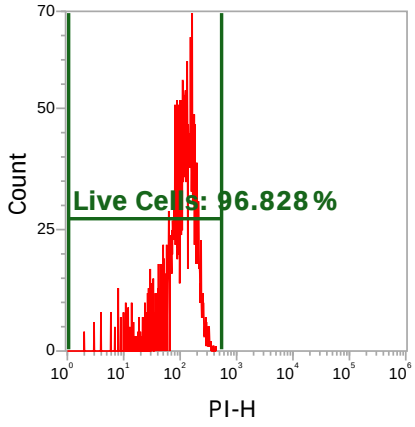
All Events - Schwann Cell S2



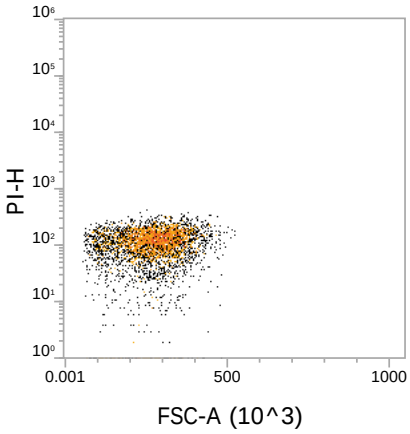
Cells - Schwann Cell S2



Single Cells - Schwann Cell S2



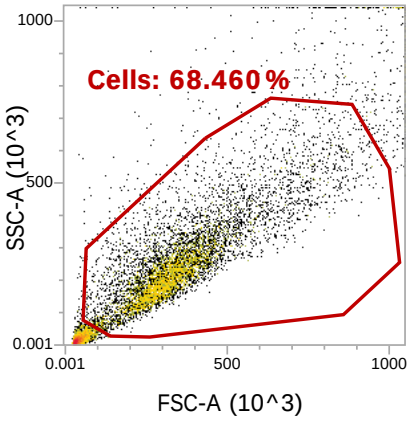
Single Cells - Schwann Cell S2



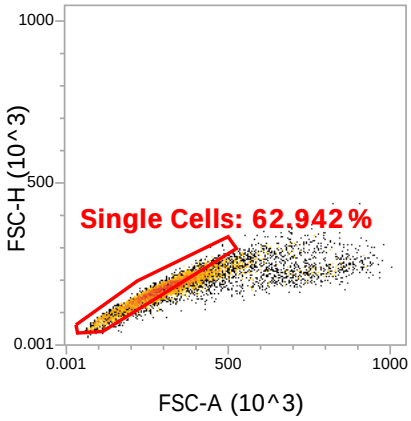
Experiment: **20 Feb 25 Count**
Group: **Group**
Sample: **Schwann Cell S2**
Time Recorded: **11:48:48**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	10,000	100.000	100.000
Cells	Cells	FSC-A	SSC-A	6,631	66.310	66.310
Single Cells	Single Cells	FSC-A	FSC-H	4,161	41.610	62.751
Live Cells	Live Cells	BL2-H		4,029	40.290	96.828

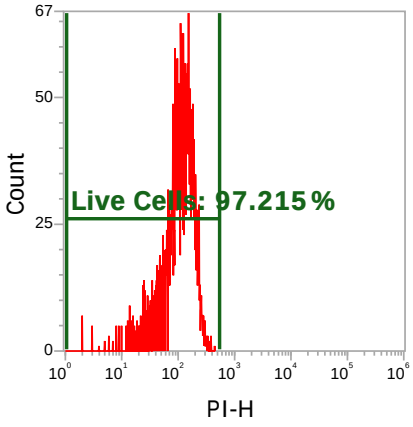
All Events - Schwann Cell S2 + PI



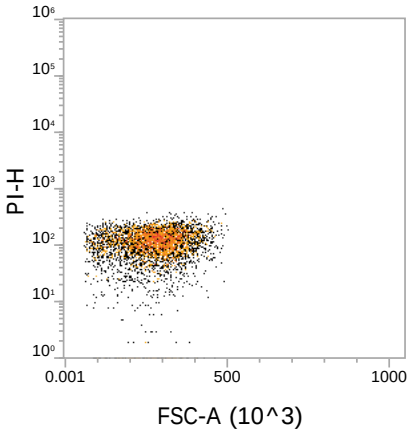
Cells - Schwann Cell S2 + PI



Single Cells - Schwann Cell S2 + PI



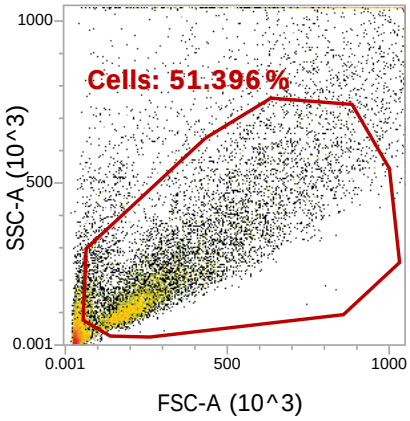
Single Cells - Schwann Cell S2 + PI



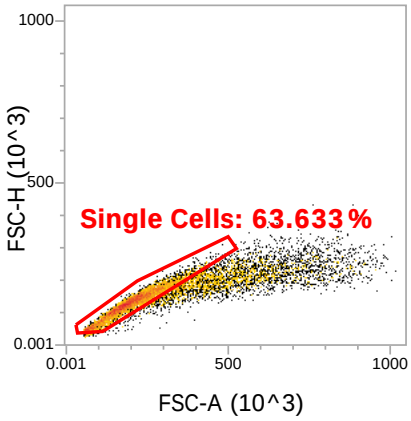
Experiment: **20 Feb 25 Count**
Group: **Group**
Sample: **Schwann Cell S2 + PI**
Time Recorded: **11:51:19**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	10,000	100.000	100.000
Cells	Cells	FSC-A	SSC-A	6,846	68.460	68.460
Single Cells	Single Cells	FSC-A	FSC-H	4,309	43.090	62.942
Live Cells	Live Cells	BL2-H		4,189	41.890	97.215

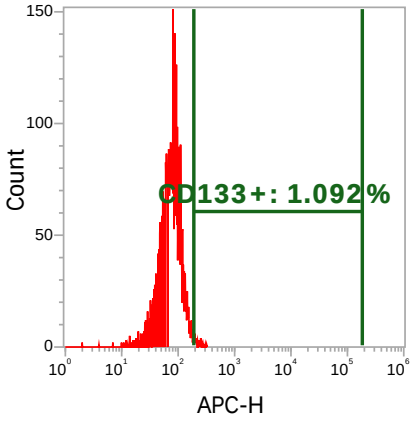
All Events - NF1 S2 Unstained



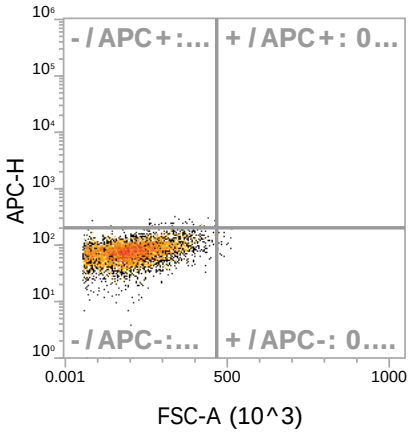
Cells - NF1 S2 Unstained



Single Cells - NF1 S2 Unstained



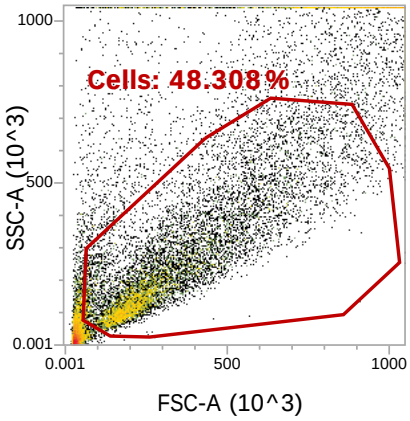
Single Cells - NF1 S2 Unstained



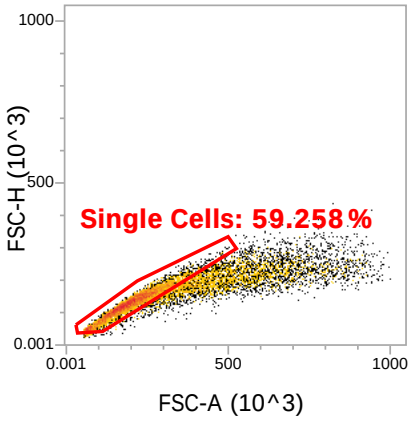
Experiment: **20 Feb 25 NF1 S2 CD133**
Group: **Group**
Sample: **NF1 S2 Unstained**
Time Recorded: **13:33:40**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	17,083	100.000	100.000
Cells	Cells	FSC-A	SSC-A	8,780	51.396	51.396
Single Cells	Single Cells	FSC-A	FSC-H	5,587	32.705	63.633
CD133+	CD133+	RL1-H		61	0.357	1.092
-/APC+	-/APC+	FSC-A	RL1-H	59	0.345	1.056
+/APC+	+/APC+	FSC-A	RL1-H	2	0.012	0.036
-/APC-	-/APC-	FSC-A	RL1-H	5,513	32.272	98.675
+/APC-	+/APC-	FSC-A	RL1-H	13	0.076	0.233

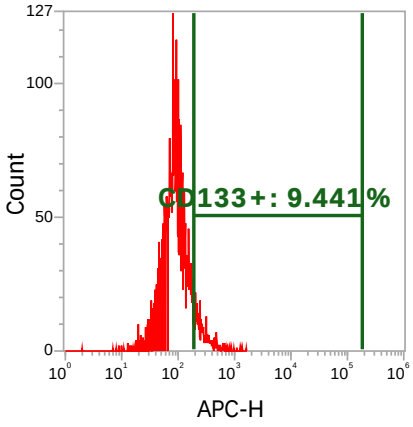
All Events - NF1 S2 + CD133



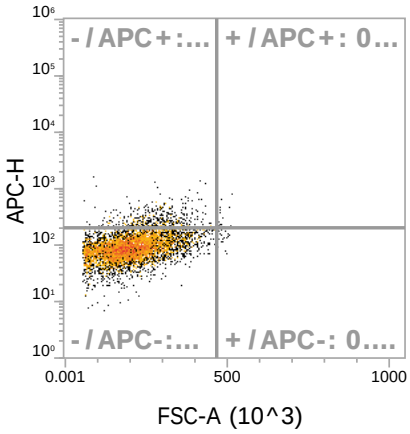
Cells - NF1 S2 + CD133



Single Cells - NF1 S2 + CD133



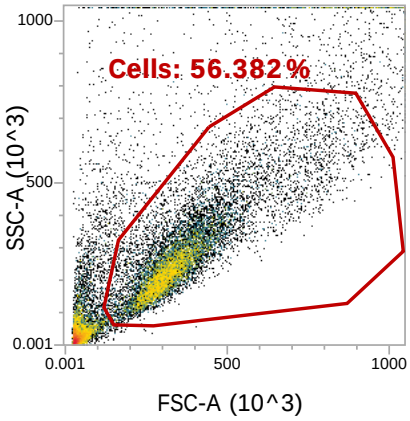
Single Cells - NF1 S2 + CD133



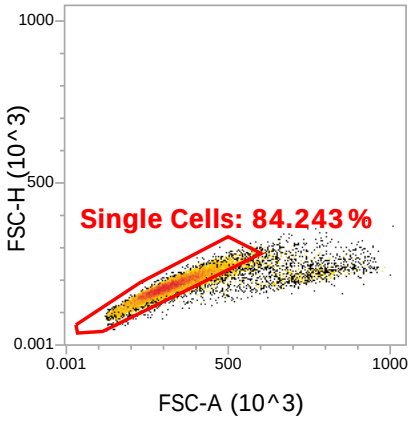
Experiment: **20 Feb 25 NF1 S2 CD133**
Group: **Group**
Sample: **NF1 S2 + CD133**
Time Recorded: **13:36:53**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	20,425	100.000	100.000
Cells	Cells	FSC-A	SSC-A	9,867	48.308	48.308
Single Cells	Single Cells	FSC-A	FSC-H	5,847	28.627	59.258
CD133+	CD133+	RL1-H		552	2.703	9.441
-/APC+	-/APC+	FSC-A	RL1-H	537	2.629	9.184
+/APC+	+/APC+	FSC-A	RL1-H	15	0.073	0.257
-/APC-	-/APC-	FSC-A	RL1-H	5,280	25.851	90.303
+/APC-	+/APC-	FSC-A	RL1-H	15	0.073	0.257

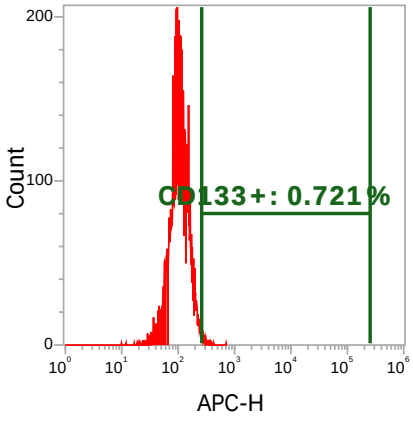
All Events - Schwann Cell S2 Unstained



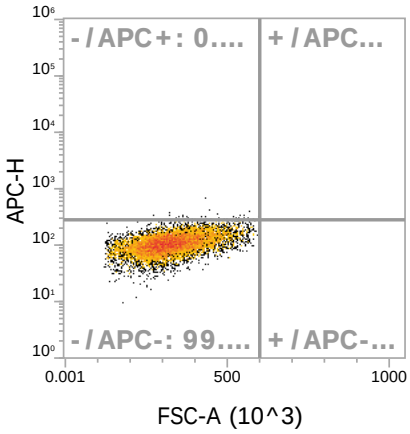
Cells - Schwann Cell S2 Unstained



Single Cells - Schwann Cell S2 Unstained



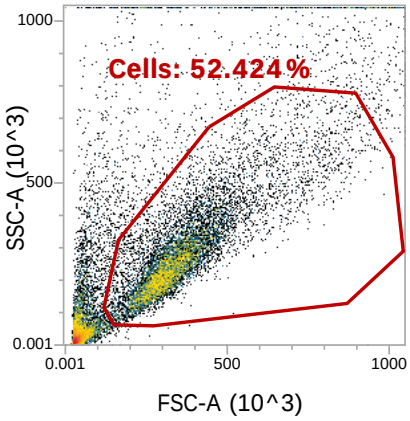
Single Cells - Schwann Cell S2 Unstained



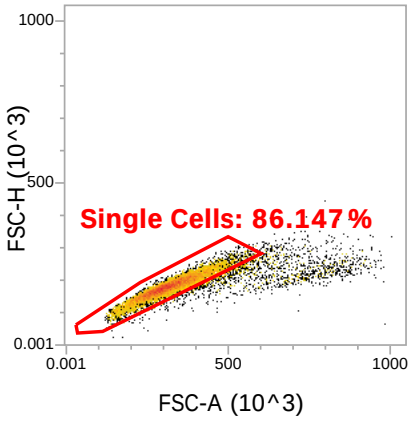
Experiment: **20 Feb 25 Schwann Cells S2 CD133(1)**
Group: **Group**
Sample: **Schwann Cell S2 Unstained**
Time Recorded: **13:45:59**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	19,563	100.000	100.000
Cells	Cells	FSC-A	SSC-A	11,030	56.382	56.382
Single Cells	Single Cells	FSC-A	FSC-H	9,292	47.498	84.243
CD133+	CD133+	RL1-H		67	0.342	0.721
-/APC+	-/APC+	FSC-A	RL1-H	67	0.342	0.721
+/APC+	+/APC+	FSC-A	RL1-H	0	0.000	0.000
-/APC-	-/APC-	FSC-A	RL1-H	9,225	47.155	99.279
+/APC-	+/APC-	FSC-A	RL1-H	0	0.000	0.000

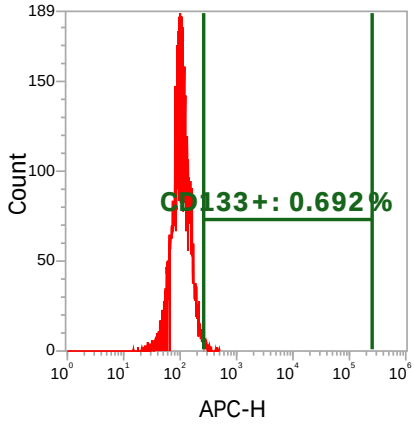
All Events - Schwann Cell S2 + CD133



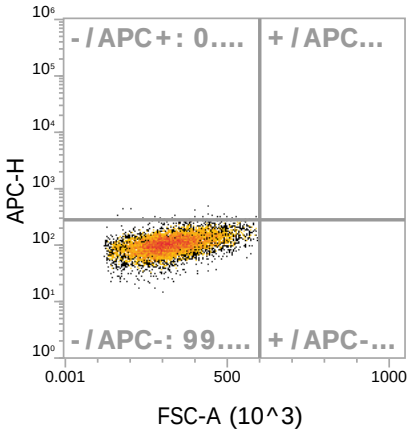
Cells - Schwann Cell S2 + CD133



Single Cells - Schwann Cell S2 + CD133



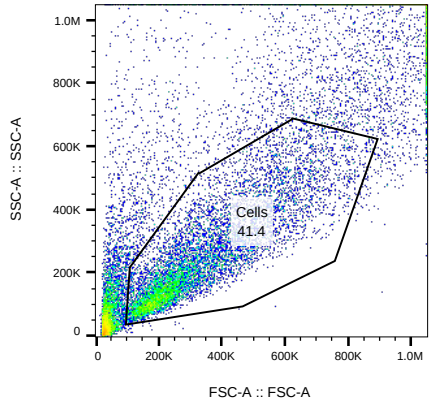
Single Cells - Schwann Cell S2 + CD133

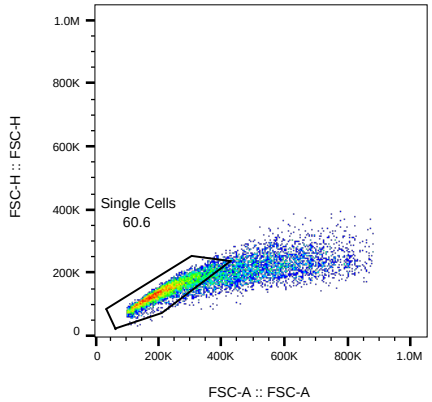


Experiment: **20 Feb 25 Schwann Cells S2 CD133(1)**
Group: **Group**
Sample: **Schwann Cell S2 + CD133**
Time Recorded: **13:51:21**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	18,562	100.000	100.000
Cells	Cells	FSC-A	SSC-A	9,731	52.424	52.424
Single Cells	Single Cells	FSC-A	FSC-H	8,383	45.162	86.147
CD133+	CD133+	RL1-H		58	0.312	0.692
-/APC+	-/APC+	FSC-A	RL1-H	58	0.312	0.692
+/APC+	+/APC+	FSC-A	RL1-H	0	0.000	0.000
-/APC-	-/APC-	FSC-A	RL1-H	8,325	44.850	99.308
+/APC-	+/APC-	FSC-A	RL1-H	0	0.000	0.000

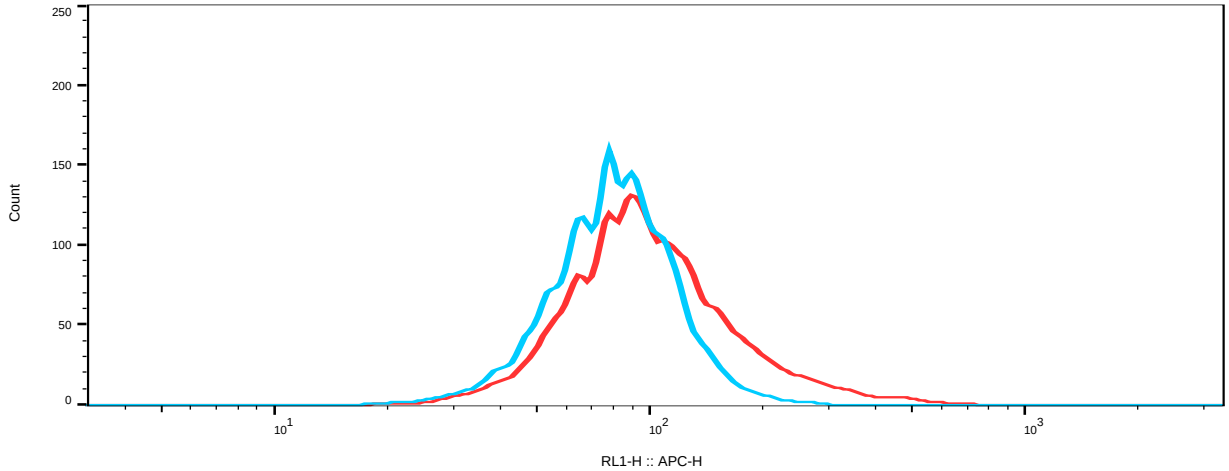
	Sample Name	Count	$DATE
■	NF1 S2 + CD133.fcs	20425	25-Feb-2020





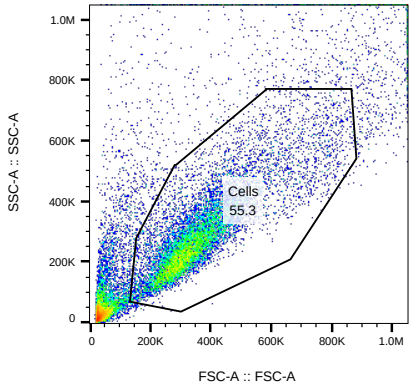
NF1 S2 + CD133.fcs
Ungated
20425

NF1 S2 + CD133.fcs
Cells
8454

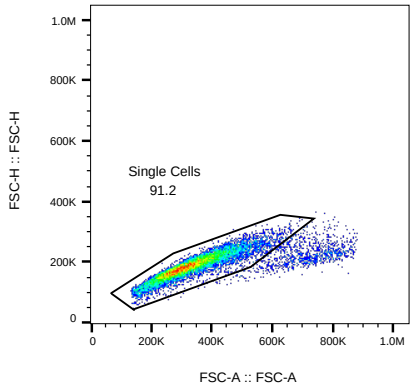


	Sample Name	Subset Name	Count	$DATE	Median : RL1-H	Mean : RL1-H
□	NF1 S2 + PI.fcs	Single Cells	4850	25-Feb-2020	80.1	85.4
□	NF1 S2 + CD133.fcs	Single Cells	5125	25-Feb-2020	96.0	117

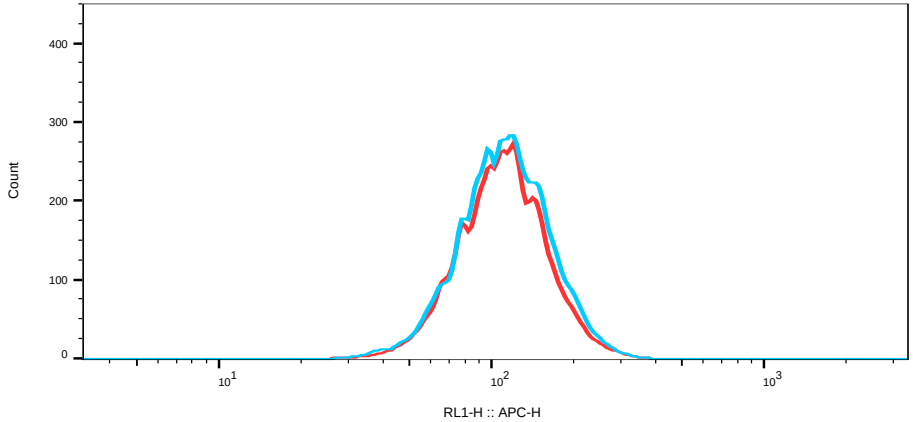
	Sample Name	Count	$DATE
■	Schwann Cell S2.fcs	19563	25-Feb-2020



Schwann Cell S2.fcs
Ungated
19563

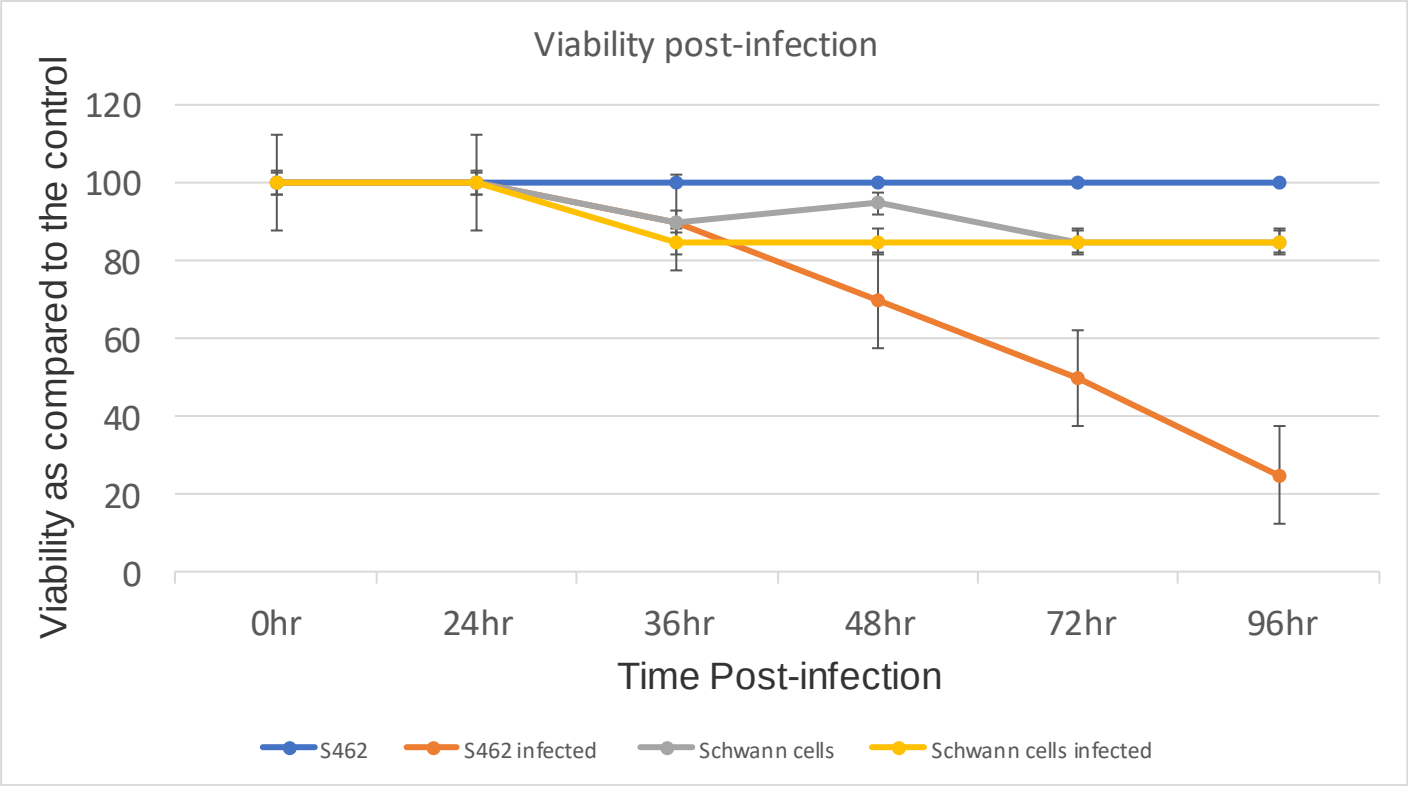


Schwann Cell S2.fcs
Cells
10820

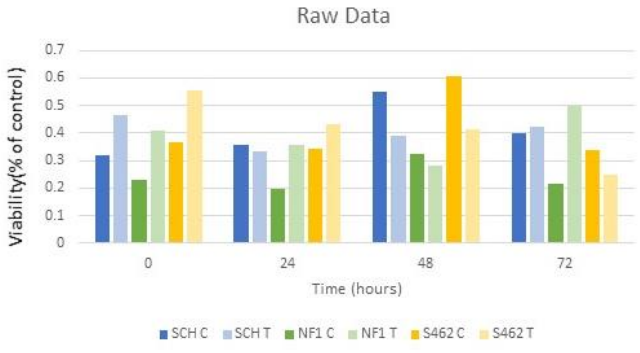


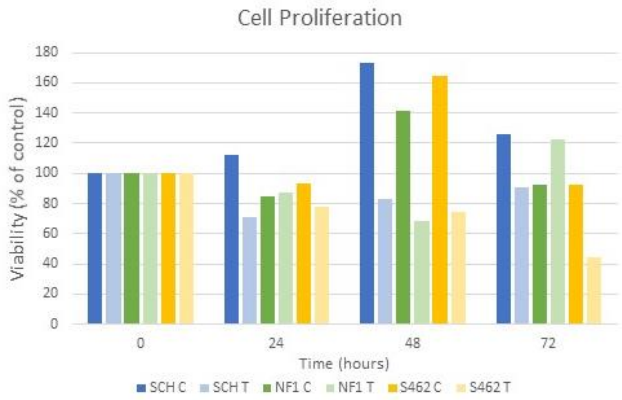
	Sample Name	Subset Name	Count	$DATE	Median : RL1-H	Mean : RL1-H
□	Schwann Cell S2.fcs	Single Cells	9866	25-Feb-2020	112	120
□	Schwann Cell S2 + CD133.fcs	Single Cells	8875	25-Feb-2020	110	117

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 sphere formation capability of CD133+ MPNST cells. Method: Colony formation assay.
At this time our efforts to have colonies of CD133+ cells have not been successful. We will continue our efforts once the stay-home-order has been removed from our state and staff return.

We are now preparing for completion of the invasion assay and colony formation assay. Considering the importance of using low passage number cells, we are re-ordering the cells and growing them to complete these tasks.

Specific Aim 2: We will investigate the effects of SS2 virus in treatment of MPNST in orthotopic xenograft model for MPNST (months 12-24):
Being pursued after re-opening of the KUMC animal research center. We are currently preparing for training of our staff.

3.1-Future directions:

We are now in the process of completing the invasion assay and colony formation assay.

In parallel to continuing the in-vitro experiments, we will pursue 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 for training of our staff.

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. We also suffered from shut downs due to Covid-19.

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. *OncoTarget and Therapy*, In Press, 2020.

7-Participants & Other Collaborating Organizations

Mahsa Hosseini, PhD

8-Special Reporting Requirements: N/A

9-Appendices; N/A