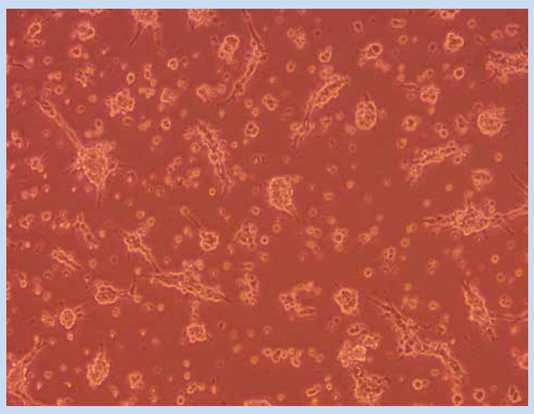
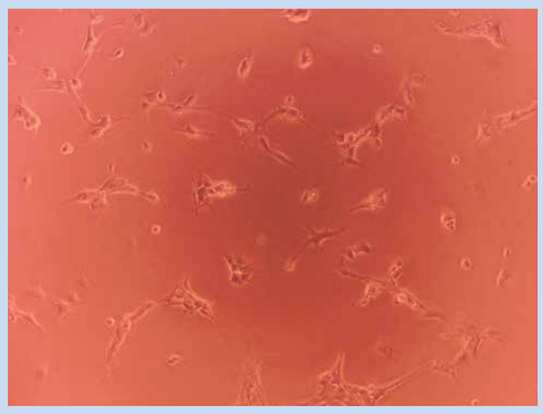
Main Panel

Investigating the Effect of Fibulin-2 on NF-κB Pathway Activity and Proliferation of Pediatric Gliomas



Microscopic view of PBT22, in well before addition of fibulin-2 treatment and western blotting

Microscopic view of PBT22, 72 hours after addition of fibulin-2, to be tested as a possible tumor suppressor



Methods

PBT: Pediatric Brain Tumor, DIPG (Diffuse Intrinsic Pontine Glioma

Four neural cell lines:
- PBT 22 (H3.3 K27M)
- PBT 24 (H3.1 K27M)
- PBT 27 (H3 wt)
- CB 660

CB660: Normal neural stem cell

Cell Titer-Glo Viability Assay

Treatments:
- rhFibulin 2 (#9559-FB)
- rhFibulin 3 (#8416-FB)
- TNFα (Cat #300-01A)

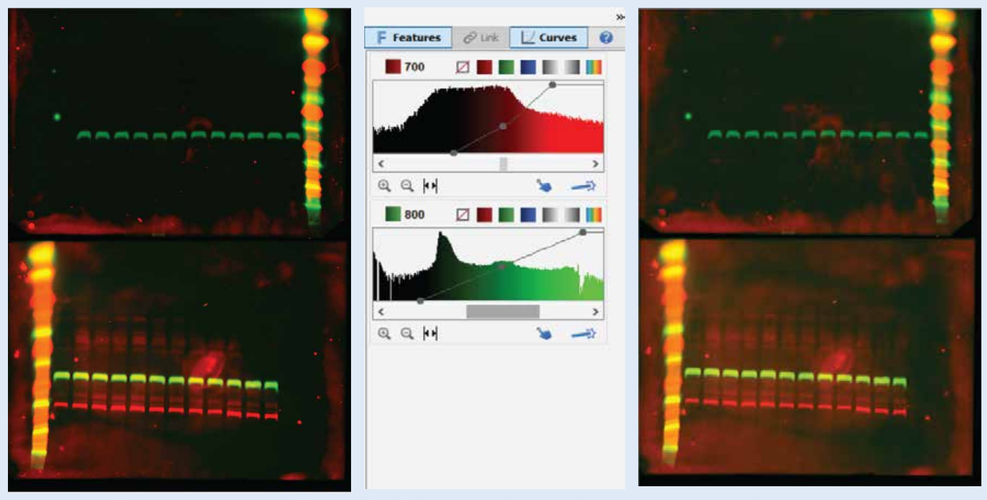
Western Blotting

72 Hours — Term of Assay — 24 Hours

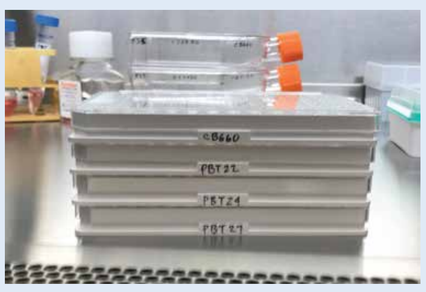
Record Cell Proliferation

Calculate NF-κB Activity

Antibody Treatments:
- p65 (Cat#8242s)
- phospho-p65 (Cat#3033s)
- Beta-actin (Cat#3700s)



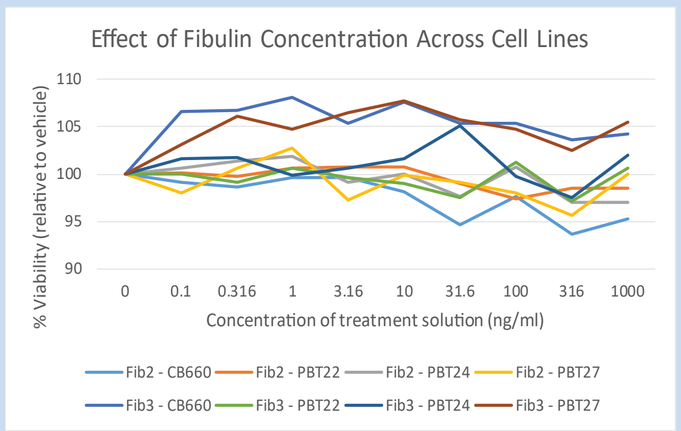
Original western blot (left) read with Li-Cor Odyssey imager, and enhanced using ImageStudio (right) to analyze beta-actin bands (green), p65 bands (red at bottom), and phospho-p65 bands (faint-red at top)

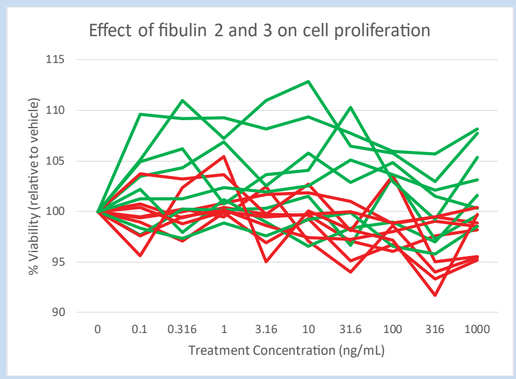


Wells plated with cell lines and different concentrations of treatment solutions (top), and cell titer-glo reagent added after 72 hours (right)

Cell Proliferation Data

Averages	Vehicle	0.1	0.316	1	3.16	10	31.6	100	316	1000
Fib2 - CB660	100	99.19209	98.63804	99.65491	99.66946	98.18672	94.58693	97.6535	93.67684	95.31925
Fib2 - PBT22	100	100.0728	99.71076	100.6519	100.715	100.7625	99.05609	97.40051	98.52801	98.55165
Fib2 - PBT24	100	100.6762	101.3523	101.8988	99.15864	100.0552	97.69093	100.7865	97.05712	97.02429
Fib2 - PBT27	100	98.01677	100.5878	102.7073	97.24909	99.92468	99.09525	98.01376	95.57984	100.056
Fib3 - CB660	100	106.5621	106.7466	108.0551	105.3548	107.5822	105.334	105.3841	103.6136	104.2174
Fib3 - PBT22	100	99.9475	99.06898	100.6542	99.66505	99.03458	97.52649	101.2083	97.11797	100.5915
Fib3 - PBT24	100	101.6285	101.772	99.82194	100.6248	101.6616	105.0846	99.77639	97.51124	101.9375
Fib3 - PBT27	100	103.1678	106.1181	104.7955	106.4336	107.7008	105.7702	104.7111	102.5441	105.4329





Fibulin-2 Fibulin-3

Average across lines	0	0.1	0.316	1	3.16	10	31.6	100	316	1000
Fibulin-2	100	99.48946	100.0722	101.2282	99.19805	99.73229	97.6073	98.46356	96.21045	97.73779
Fibulin-3	100	102.8265	103.4264	103.3317	103.0195	103.9948	103.4288	102.77	100.1967	103.0448
StdDev Fib2	0	1.000729	1.011422	1.1667	1.257319	0.94756	1.833208	1.358728	1.796161	1.760312
StdDev Fib3	0	2.439014	3.162022	3.314367	2.9196	3.763368	3.416528	2.34532	2.910141	1.892275

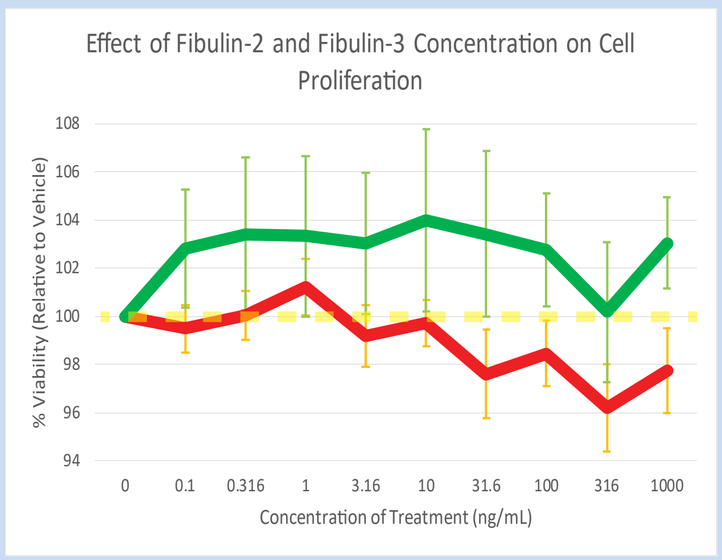
NF-κB Signaling Data

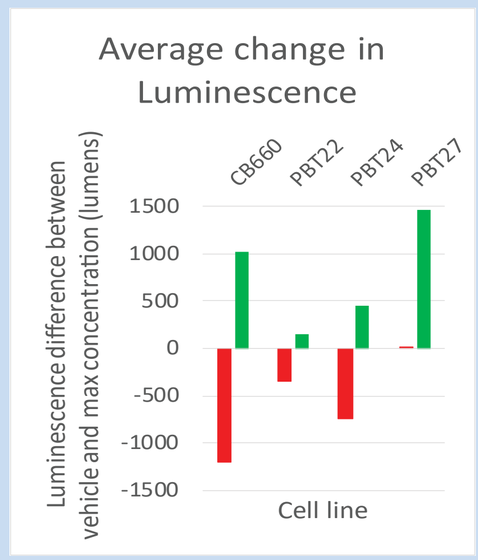
Well	Description	B-Actin	p65 Total	B-Actin2	P-p65
1	Water Hi	19958.23	319.82	1805.26	13.66
2	Water Med	20508.46	557.76	1853.65	9.34
3	Water Lo	18782.64	653.91	2015.12	29.47
4	Fibulin 2 Hi	18119.73	688.80	1692.79	1.47
5	Fibulin 2 Med	20333.76	790.85	1854.73	17.36
6	Fibulin 2 Lo	15387.85	570.37	1612.45	13.75
7	Fibulin 3 Hi	14936.65	545.57	1676.45	51.10
8	Fibulin 3 Med	13130.71	303.19	1227.70	18.32
9	Fibulin 3 Lo	14032.34	276.32	1313.38	18.43
10	TNFa Hi	13659.31	629.15	1745.36	4.12
11	TNFa Med	15451.98	391.08	1719.38	28.00
12	TNFa Lo	12725.36	531.90	1713.71	35.43

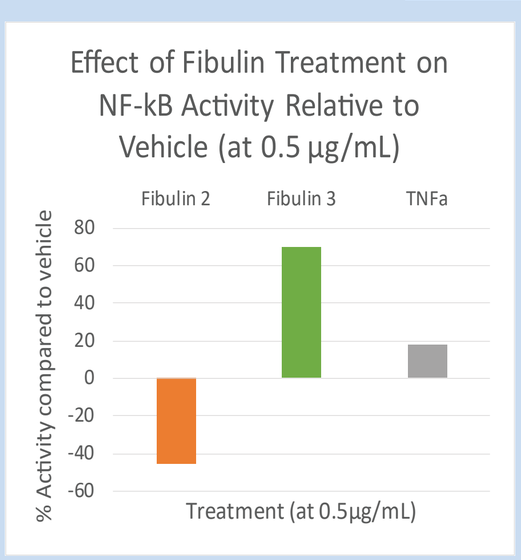
Intensities were recorded for gel 1 and gel 2 (two columns each) using Li-Cor Odyssey imager and ImageStudio. Lo, Med, and Hi represent different concentrations (Lo is 0.5µg/mL, Med is 1 µg/mL, and Hi is 3 µg/mL) of each treatment.

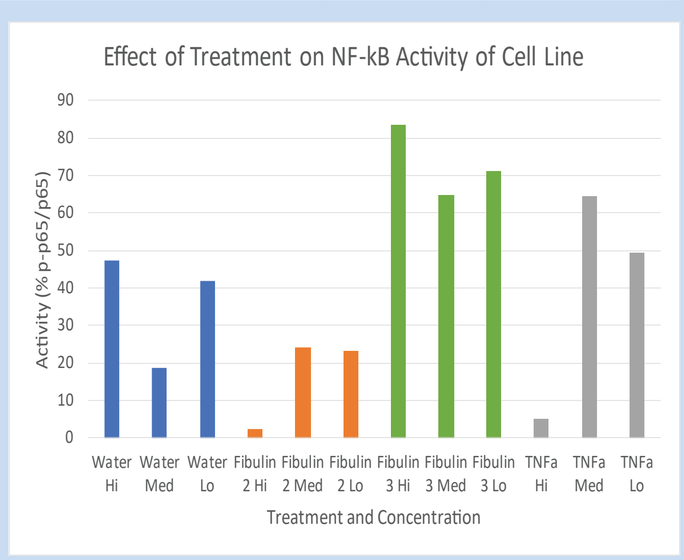
The expression values were normalized to beta-actin, and the normalized phospho - p65 was divided by p65 to determine pathway activity (pp65 is the phosphorylated product of p65, and is a product of this signaling pathway).

Normalized Values (% of actin)	p65	pp65	NF-kB Activity (%)
Water Hi	1.6024677	0.7564697	47.2065486
Water Med	2.7196474	0.5036504	18.5189599
Water Lo	3.4814808	1.4625764	42.0101803
Fibulin 2 Hi	3.8013649	0.0867651	2.2824734
Fibulin 2 Med	3.8893328	0.9357405	24.0591525
Fibulin 2 Lo	3.7066076	0.8529813	23.0124508
Fibulin 3 Hi	3.6525354	3.0479603	83.4477978
Fibulin 3 Med	2.3089958	1.4922476	64.6275586
Fibulin 3 Lo	1.9691398	1.4035203	71.2758058
TNFa Hi	4.6060339	0.2361171	5.1262556
TNFa Med	2.5309509	1.6287199	64.3520953
TNFa Lo	4.1798609	2.0674202	49.4614600









Left Side Panel

Introduction

The survival rate for patients with **gliomas** (the most common type of brain tumor) is 5.6%. Pediatric gliomas specifically are the leading cause of cancer-related death among children, surpassing leukemia. There have only been 4 FDA-approved drugs developed for treatment in the last 30 years, and none of these have been approved for pediatric gliomas. Gliomas are primarily driven by a small number of major signaling hubs, like the **NF-κB pathway**, known to play a key role in glioma growth and invasion, via enhancement of cell proliferation. Additionally, recent studies have highlighted the importance of **fibulins** in the development of various cancers (Yi et al., 2007). Fibulins are a family of **extracellular matrix (ECM) glycoproteins**, which are integral for cell structure and adhesion (Argraves et al., 2003). Fibulin-3 has recently been shown to activate NF-κB signaling in gliomas, and promote cancerous growth (Nandhu et al., 2017). However, no research has been done on the effect of other fibulins, like **fibulin-2**, and no estracellular matrix research has been done on **pediatric brain tumors**. This project seeks to investigate the role of fibulin-2, a protein that has been shown to be found in lesser concentrations in higher grades of gliomas (Ren et al., 2016). The effect of fibulin-2 on the tumor-driving NF-κB pathway will also be investigated, in addition to its effects on cancer cell proliferation. This research seeks to expand the field of fibulin research to include pediatric brain tumors, and advance research into novel glioma treatments based on ECM (extracellular matrix) targeting approaches to glioma inhibition, by targeting structures used by gliomas for cell structure and adhesion.

Research Question

How does the presence of fibulin-2 affect the NF-κB pathway activity and the proliferation of pediatric glioma cells?

Hypotheses

1. The presence of fibulin-2 will lead to **decreased** cell proliferation, and higher rates of cell death/inactivation, based on previous studies that have shown higher levels of FBLN2 gene expression in higher grades of adult gliomas (Ren et al., 2016).
2. The presence of fibulin-2 will also lead to **decreased** NF-κB pathway activity, because fibulin-3 has been shown to increase activity, which has been linked to increased cell growth and proliferation (Nandhu et al., 2017).

Bibliography

Argraves, W. S., Greene, L. M., Cooley, M. A., & Gallagher, W. M. (2003). Fibulins: physiological and disease perspectives. EMBO reports, 4(12), 1127–1131. https://doi.org/10.1038/sj.embor.7400033

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Yi, C. H., Smith, D. J., West, W. W., & Hollingsworth, M. A. (2007). Loss of fibulin-2 expression is associated with breast cancer progression. The American journal of pathology, 170(5), 1535–1545. https://doi.org/10.2353/ajpath.2007.060478

Right Side Panel

Data Analysis

Analysis of Western Blots led to identification of relative activity values after each treatment, at three different concentrations. Two out of the 12 generated data points were clearly outliers -- the highest concentrations of both TNF-alpha and Fibulin 2, and were removed from analysis. Overall, At 0.5 µg/mL, addition of fibulin-2 led to a 45.2% **decrease** in NF-κB activity, and addition of fibulin-3, the positive fibulin control, led to a 69.7% increase, as predicted. Therefore, the observed decrease in activity after addition of fibulin-2 is **significant**.

Cell proliferation data from the viability assay was also analyzed, and at the highest concentration (1000ng/mL), fibulin-2 led to a 2.3% **decrease** in cell proliferation, and fibulin-3 led to a 3.0% increase in cell proliferation, as predicted for a positive control. Raw luminescence values were compared between fibulin-2 at 1000ng/mL and the control (vehicle). Fibulin-2 readings had a standard deviation of **1.898%**. The decrease in cell proliferation as a result of fibulin-2 treatment was tested using a **two tailed, paired t-test, with 7 degrees of freedom**. There was a **statistically significant difference** in the luminescence values of fibulin-2 vs control, with a **p-value of .0183** and t-value of 3.06, well above the expected 2.36 at an alpha-value of 0.05. Furthermore, there was a **very statistically significant difference** between the viabilities of fibulin 2 and 3, with a **p-value of 0.0055**, verified by a 2-tailed paired t-test of normalized percentage values. Overall, this experiment **successfully rejected the null hypothesis.**

	Viability (Lumens)		
Cell line	Vehicle	Fib2	Diff
CB660	25494	24268	-1226
	25678	24509	-1169
PBT22	24372	23935	-437
	24283	24015	-268
PBT24	24936	23818	-1118
	25614	25238	-376
PBT27	28516	28426	-90
	28534	28656	122
Mean	25928.38	25358.13	-570.25
SD	1579.148	1884.572	493.1067
p-value	0.018332154		

Conclusions

This research successfully verified all parts of the hypothesis and **answered the research question, rejecting the null hypothesis an overall p-value of 0.0183.**

Fibulin-2 was shown to lead to **decreased cell proliferation**, which supports the hypothesis that it acts as a **tumor suppressor** in pediatric gliomas. This was verified through the use of a positive ECM-protein control, fibulin-3, which has been previously demonstrated to increase proliferation in adult gliomas.

Fibulin-2 was also shown to **decrease the activity of the NF-κB signaling pathway**, which is a possible mechanism by which fibulin-2 decreases glioma cell proliferation. This was verified through the use of a positive pathway control, TNF-alpha, which is known to increase NF-κB pathway activity.

Future Directions

This novel research has successfully demonstrated the role ECM (Extracellular Matrix) proteins, especially fibulin-2 can have in cell proliferation with pediatric as well as adult gliomas, and shows that **targeting of ECM proteins is a possible, novel method for treatment of pediatric gliomas.** To further our understanding of the mechanisms of Fibulin-2, more specific assays must be run, including a **cell adhesion assay.**

Another possible future direction is drug viability tests involving **Simvastatin**, a cardiac drug that is proven to inhibit fibulin-3 and promote fibulin-2, as **a possible and novel method of treatment for gliomas.**