UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effect of the JAK2/STAT3 inhibitor TG101209 on the proliferation of tumor neurosphere cells derived from glioblastoma patients

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requirements for the degree Master of Science
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by
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The thesis of Rohit Saklecha is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

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I dedicate this thesis to the members of the Kesari Lab and my parents for their support and confidence during my graduate year.

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This thesis was co-authored by Rajesh Mukthavaram, this thesis in full may appear in future publications from the Kesari Lab. I was the primary investigator and co-author of this material.

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ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California, San Diego, 2013

Professor Santosh Kesari, Chair

Professor Milton Saier, Co-Chair

Janus kinase 2 (JAK2) is a signal transducer and activator of transcription (STAT3) pathway is known to be involved in the development and progression of different types of cancers, including gliomas. STAT3 is activated by the phosphorylation of tyrosine 705, after which it is dimerizes and translocates in to the nucleus of cancer cells. The STAT3 signaling cascade promotes several oncogenic signaling pathways,

and recent studies reveal a correlation between STAT3 signaling and glioblastoma multiforme stem-like cell (GBM-SC) expansion. In the present study, we focused on a novel small molecule inhibitor TG101209, and examined its capacity to suppress STAT3 phosphorylation and the growth of human GBM neurospheres in vitro and in vivo. TG101209 potently inhibited STAT3 phosphorylation and inhibited cell proliferation (IC50 1-10 μM), down regulated Bcl-Xl and induced PARP cleavage to induce apoptosis in GBM neurospheres. We conclude that TG101209 potently inhibits STAT3, and may have therapeutic potential for GBM expressing JAK2.

I:

Introduction

Glioblastoma multiforme (GBM) the highest-grade glioma (grade IV in WHO classification) is the most common brain tumor in adults and a leading cause of cancer death in adults and children [1] [2] [3]. The current standard of care for patients with GBM includes tumor resection followed by adjuvant radiation therapy (RT) and chemotherapy (CT) [4] [5]. A survival benefit has been reported for GBM patients treated with Temozolomide (TMZ) combined with RT to yield a mean survival time of <2 years [6] [7]. Because of its infiltrating characteristics, the complete resection of GBM remains impossible and tumor recurrence occurs invariably at the primary tumor site. Recurrence seems to be due to the resistance of the initial tumor, but recent data suggests that it may be linked to the presence of a subpopulation of therapy resistant cells with stem cell like characteristics. These cells, called ‘‘tumor-initiating stem cells’’ (TISC) or ‘‘cancer stem cells’’ (CSC), exhibit normal stem cell properties including, i) the capability for self-renewal; ii) multi-lineage differentiation; and iii) the ability to regenerate GMB tumors histologically similar to the original tumors in xenografts [8] [9] [10] [11] [12] [13] [14]. Based on these observations, it is important to develop therapeutic agents for GMB-SC and affect cell proliferation and resistance to chemo-radiation [15]. Therefore, a new therapeutic approach targeting GBM stem-like cells is required to overcome recurrence and resistance to standard radio-chemotherapy protocols.

The alteration of several signaling pathways including receptor tyrosine kinase [16], serine/treonine-specific protein kinase [17] [18] , Wnt [19] and Notch and Hedgehog [20] pathways is involved in the progression of GBM. Importantly, constitutive activation of the Janus kinase (JAK)/signal transducer and activator of the transcription factor (STAT) promotes tumor cell proliferation and the inhibition of

apoptosis [21]. The STAT protein family is a group of transcription factors that plays important roles in relaying signals from growth factors and cytokines to downstream signaling targets and effector proteins, and is involved in oncogenesis [22] [23]. In GBM cells, The STAT3 signaling cascade becomes activated through multiple signaling pathways, which upregulate the transcription of several genes that control tumor cell survival, resistance to apoptosis, cell cycle progression and angiogenesis [24]. STAT3 is activated by the phosphorylation of tyrosine 705 (which is often accompanied by phosphorylation of serine727) in cells stimulated with a variety of cytokines (IL-6, IL-11) and growth factors (EGF, TGF-a, PDGF and HGF) [25] [26]. Targets of STAT3 include Bcl-2, Bcl-xL, c-myc, survivin, cyclin D1, vascular endothelial growth factor (VEGF) [27] [28] [29].

The connection between STAT3 signaling and GBM-SC development has been investigated via genetic knockdown of STAT3 using short hairpin RNA inhibited the proliferation and the formation of neurospheres by GBM-SC, indicating that STAT3 can regulate GBM-SC growth and self-renewal [30] [31]. In the context of this, small molecules have been explored and further reveal antitumor effects through the regulation of GBM-SC.

Considering that STAT3 is a good target, we used a JAK2/STAT3 inhibitor TG101209 for our study. Previous studies identified TG101209 as a potent inhibitor of the JAK2/STAT3 pathway, which exhibited a potent anti tumor effect in multiple myeloma cell lines and suppress growth of hematopoietic colonies from primary progenitor cells and displayed enhanced radiosensitivity of lung cancer cells *in vitro* and tumor growth delay *in vivo* [32] [33]. The present study examined the mechanism of

cell killing caused by TG101209 in GBM tumorspheres derived from patient GBM stem-like cells. These tumorsphere lines reproduce the genetic, phenotypic and invasive characteristics of human GBM. TG101209 was found to be a potent inhibitor of STAT3 phosphorylation, which inhibited the proliferation in a range of human GBM cell lines, including the stem cell, derived GBM tumorspheres. We found that the mechanism of cell death in the tumorspheres was the induction of PARP cleavage mediated apoptosis.

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II:

Results

Establishment of primary GBM-SC lines from GBM patients.

Seven GBM-SC lines (GBM4, GBM8, SK1035, SK987, SK892) established from GBM patient-derived tumors were successfully grown as neurospheres in neural stem cell medium, as shown by representative images of GBM4, GBM8, SK987 and SK892 lines are shown in Figure 1.

Expression and constitutive activation of STAT3 in glioma cell lines and human tumor neurosphere lines.

To determine whether the STAT3 pathway was activated in the malignant glioma cells, we used immunodetection for phosphorylated STAT3 at Tyr705 in patient derived GBM lines, A172, U87 and U251 cell lines. Western blot analysis with anti-phosphospecific STAT3 (Tyr705) and anit-STAT3 showed constitutive phosphorylation of STAT3 in all cell lines except SK892 (Fig 2A,B)

Cell viability assay.

We examined the effect of JAK2 inhibitor TG101209 on cell proliferation in five different GBM cells lines in vitro. Treatment with TG101209 up to 40 μM for 72 h exhibited a similar inhibitory effect on GBM4, GBM8, SK1035, SK987 stem cells and A172 cell lines with IC_{50} values of 1-2 μM, whereas in U87 and U251 cell lines the IC_{50} values were between 5-8 μM. However, in SK892 cells proliferation inhibitory effect was low (IC50 ~25 μM). Taken together, these results indicate that TG101209 can be used to selectively target GBM cells (Fig 3A-E).

TG101209 down-regulated STAT3 phosphorylation in cancer cells:

To gain mechanistic insight into inhibition of the STAT3 pathway we used GBM4 and GBM8 cells. First we measured phosphorylated STAT3 levels after incubation with different concentrations of TG101209 for 16 h. In both cell types, treatment with 10, 2 and 0.1 μM TG101209 showed a dose-dependent inhibition of phosphorylation, and 10 and 2 μM TG101209 rendered STAT3 phosphorylation undetectable (Figure 4A).

TG101209 induced apoptosis by down regulating pro-survival protein and inducing PARP cleavage:

To determine whether TG101209 induces apoptosis in GBM4 and GBM8 patient derived cancer stem-like cell tumorsphere lines, we performed a western blot analysis for indicators of apoptosis. The induction of apoptosis by TG101209 was evidenced by cleaved poly-ADP ribose polymerase (PARP) PARP in the tumorsphere cells. The sub-G1 population, which is comprised of apoptotic cells, increased in a dose-dependent manner 16h after treatment with TG101209.

Many studies have reported that STAT3 induces apoptosis by inhibiting the expression of pro-survival or anti-apoptotic proteins or oncogenes in various cancer cell types. Here for GBM tumorspheres we performed western blotting for a prosurvival protein that is a target of STAT3. The expression of Bcl-Xl in both GBM4 and GBM8 cell lines decreased 16 h after treatment with TG101209, but we did not see dose dependent down regulation (Fig 4A). Further, DNA content analysis by flow cytometry showed reduced PI staining of apoptotic cells after treatment with TG101209 secondary to DNA fragmentation and loss of nuclear DNA content (Figure 4B).

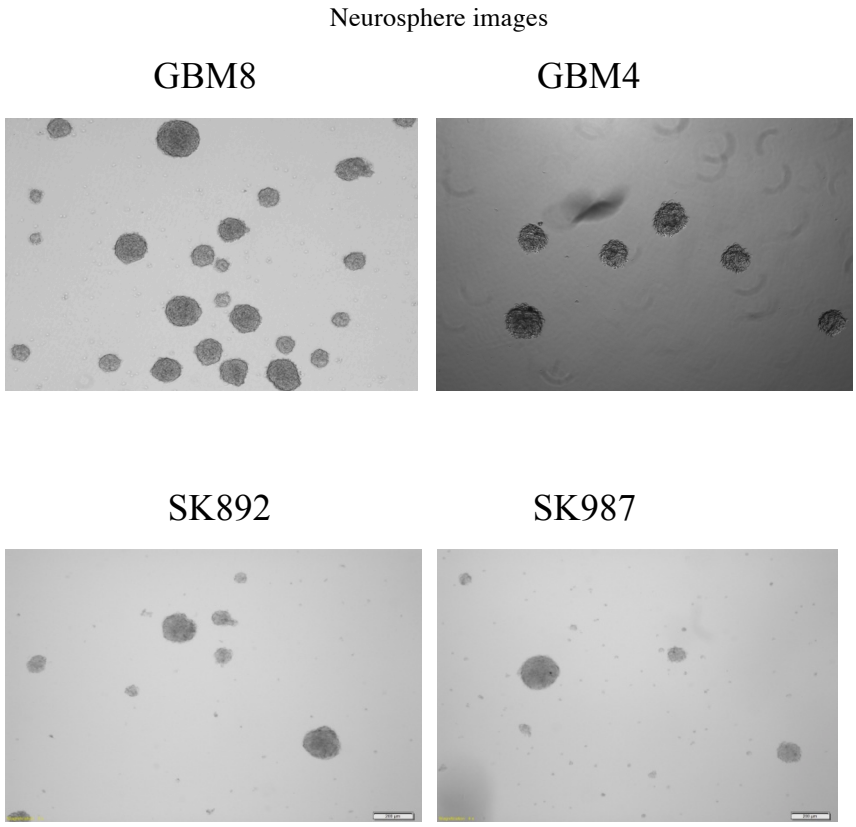

Figure 1. Morphological features of GBM lines. Representative Images of the neurospheres Isolated from human glioblastoma tumors.

P Stat 3 expression in GBM lines

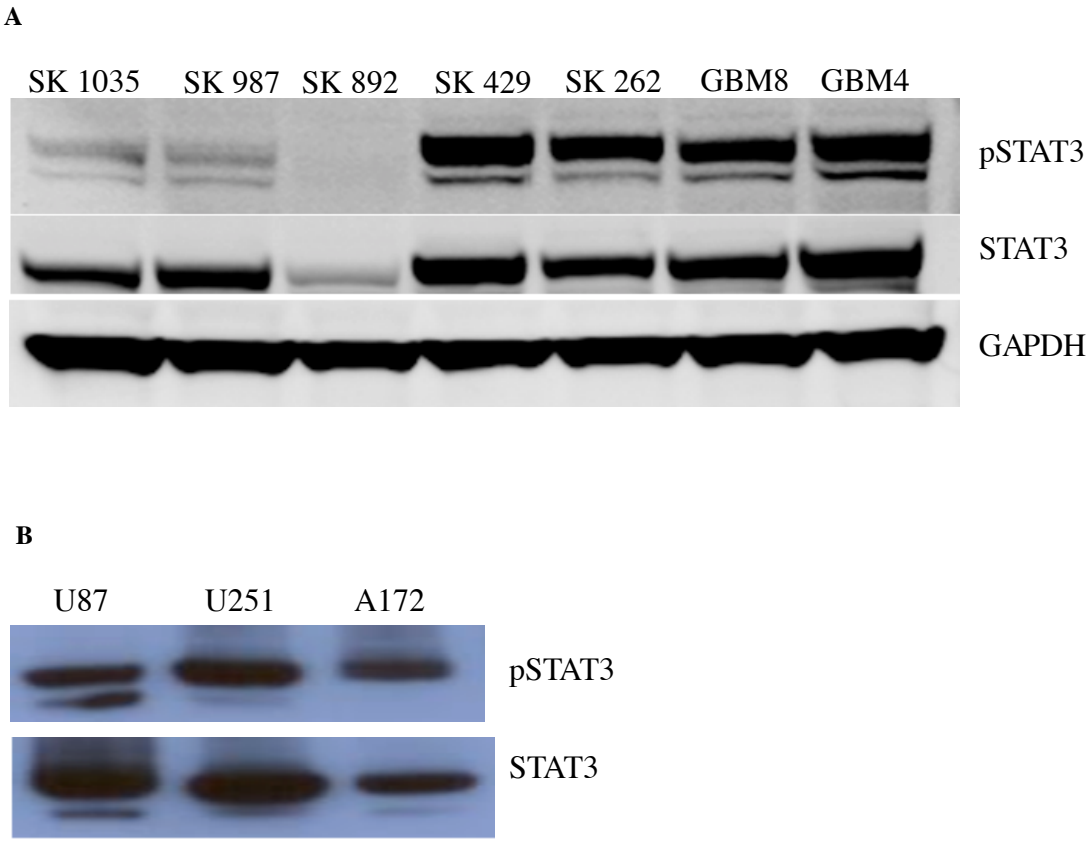

Figure 2: STAT3 phosphorylation status in glioblastoma cell lines. A. STAT3 phosphorylation in patient derived neurospheres cell lines B. Adherent cell lines.

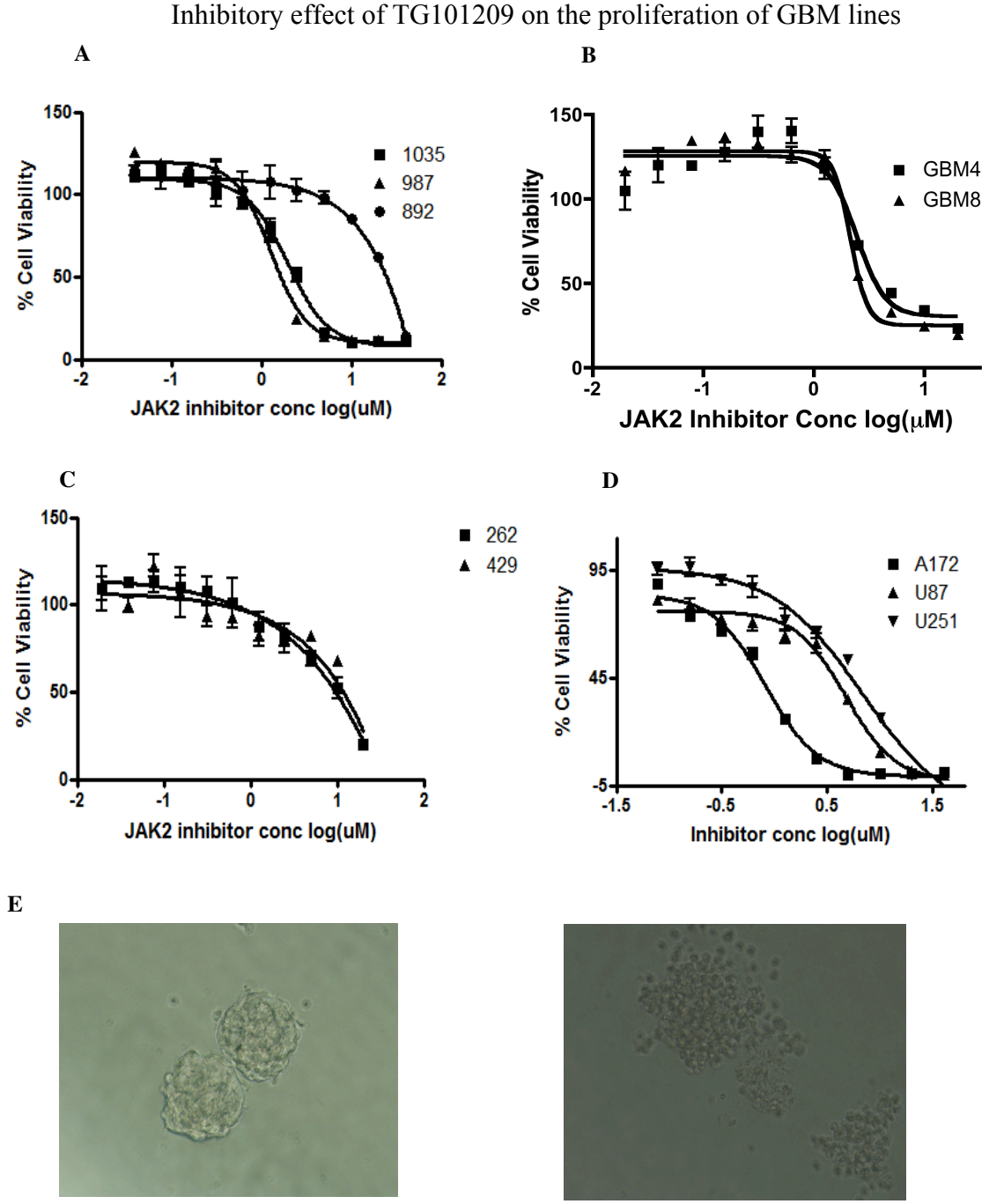

Figure 3. GBM-SC and established cells were seeded into each well of a 96-well micro-culture plate and various compounds diluted with TG101209 (40-0.075 μM) were added. Each point shows the mean for three samples. A (SK1035, SK987 and SK892), B (GBM4 and GBM8), C (SK262, SK429) are IC50 curves for patient derived cell lines. D is IC50 curve of U87, A172 and U251. E is representative images of GBM8 cells treated with 10 μM TG101209 and control.

TG101209 effectively inactivates STAT3

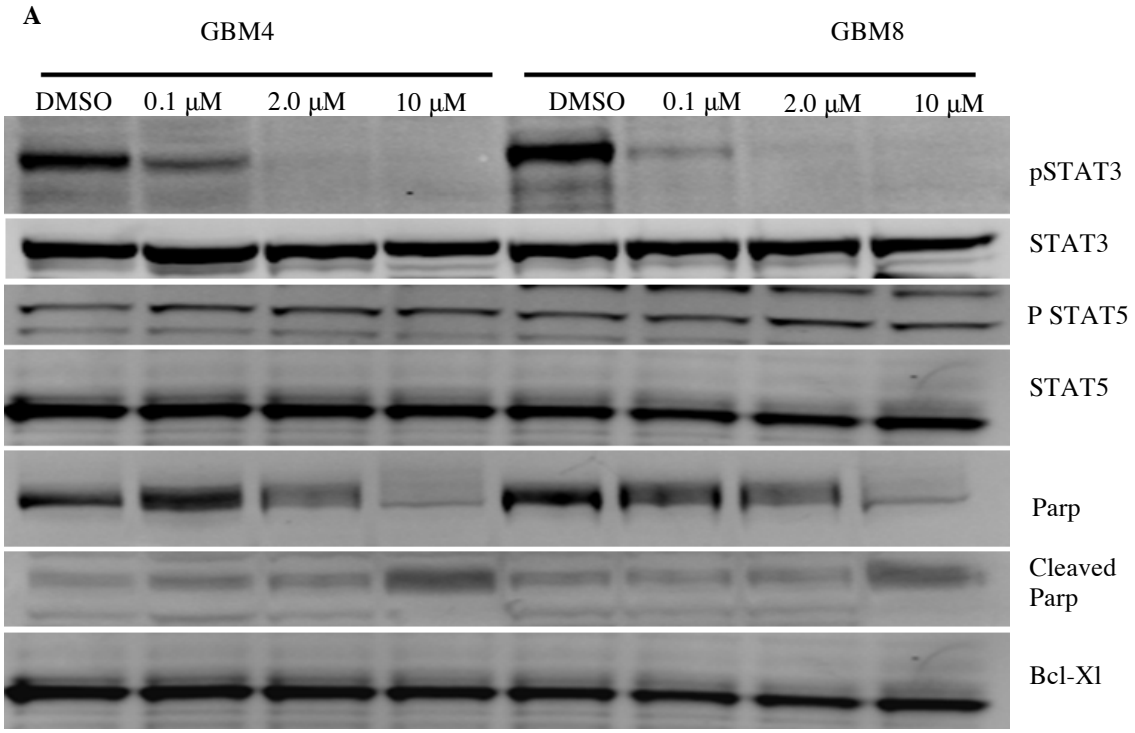

Figure 4. TG101209 effectively inactivates STAT3, down regulates Bcl-Xl and induces PARP cleavage-mediated apoptosis. (A).

Western blots of phosphorylated STAT3 at Tyr705 and total STAT3, Bcl-Xl and Parp in GBM4 and GBM8 cells treated with different concentrations of TG101209 and harvested at 16h (B). Cells were treated with the 2 μM concentrations of TG101209 for 16 h and then subjected to flow cytometry for cell cycle analysis. (i) GBM4 cells treated with DMSO (ii) GBM4 cells treated with 2 uM of TG101209. (iii) GBM8 cells treated with DMSO (iv) GBM8 cells treated with 2uM of TG101209.Percent of cells in sub G1 phase was indicated.

B

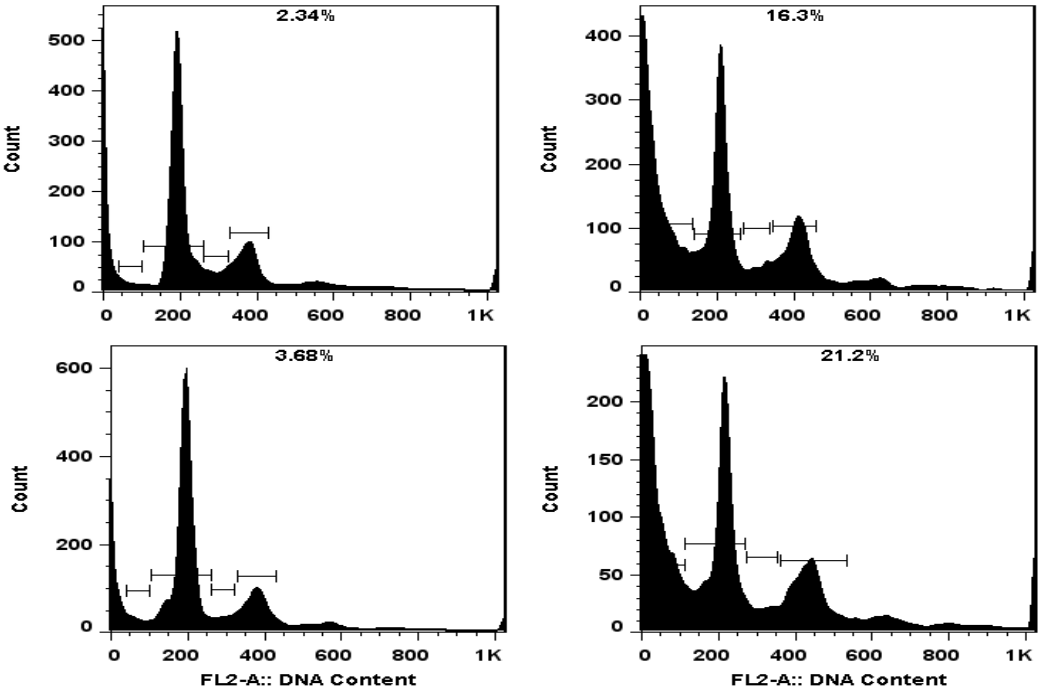

Figure 4. Continued

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III:

Discussion

Glioblastoma multiforme (GBM) is the common, most malignant and aggressive brain cancers in adults. Despite recent therapeutic advances, less than one-third of GBM patients survive more than 2 years [5] [7]. The main therapeutic challenges are recurrence and treatment resistance. The constitutive activation of STAT3 is frequently detected in various cancer type along with primary human GBM cells and established GBM cell lines [29] [33] [34] [35]. STAT3 activation in GBM is associated with invasion, survival, and growth of GBM both in vitro and in vivo [24] [36]. The precise oncogenic or suppressive role played by STAT3 was reported to depend on the genetic background of the target tumor [24]. However in the present study, we utilized a tumorsphere model that exhibited genetic and phenotypic diversity, and demonstrated that TG101209 efficiently inhibited STAT3 phosphorylation, which resulted in the induction of apoptosis.

Since GBM-stem cells were identified as tumor-initiating and resistant cells, a focus has developed on targeting this tumor compartment. A substantial body of literature has emerged concerning specific features in terms of GBM stem cell isolation and expansion as tumorsphere cultures *in vitro,* the capacity for extended self renewal, multi lineage differentiation and the ability to reproduce the histology of human GBM tumors in xenografts [8] [9] [10]. Our Isolated GBM stem cells exhibited all these key features (Figure1).

STAT3 is of interest as it is expressed in stem cells, and regulates numerous physiological and oncogenic signaling pathways affecting target gene expression. STAT3 is constitutively activated in various types of cancers including hematological and solid cancers. STAT3-SH2 dimerization and phosphorylation promotes cell proliferation and induces anti-apoptotic activity. STAT3 was activated in our isolated GBM stem like cells

but not in the non-malignant SK892 cell line, which indicates selectivity (Fig 2A). Experimental approaches for blocking STAT3 signaling using small interfering RNA (siRNA), small hairpin RNAs (shRNA) and STAT3 antisense molecules have successfully inhibited cell proliferation *in vitro* and tumor growth *in vivo* [31] [37]. The constitutive activation of the STAT3 pathway is linked to tumor promotion and maintenance. With regard to GBM-Stem cell signaling, the interaction between STAT3 and other pathways including EGFR, Notch, Wnt, Hedgehog, Akt, mTOR, olig2, PKC, MAPK, NF-κB and BMP4 was shown to regulate self-renewal [38]. We observed in vitro activity of TG101209 in a variety of patient derived cells and established GBM cell lines. The TG101209 inhibited cell proliferation in patient derived cell lines with an IC_{50} of 1-2 μM except in SK892, a cell line with no constitutively activated Stat3 signaling. In established cell lines the IC_{50} was in between 2-10 μM (Fig 3).

Interestingly, STAT3 is involved in both stem cell maintenance and astrocytic differentiation, which could be important therapeutically and therefore represent a treatment opportunity for JAK-STAT inhibitors such as TG1010209. An advantage of targeting STAT3 in cancer stem-like cells is that the inhibition of this pathway affects multiple downstream molecules, thus enhancing antitumor effects. For example, STAT3 plays an important role in oncogenesis by upregulating the transcription of several genes that control tumor cell survival, resistance to apoptosis, cell cycle progression and angiogenesis. Target genes of STAT3 include Bcl-2 [39], Bcl-XL [29], c-myc [40] and Mcl-1 [41], and genes encoding cyclin D1[29] and vascular endothelial growth factor [42]. It was previously shown in multiple myeloma that TG101209 down regulation of pJAK2, pSTAT3 and Bcl-xl levels correlated with up-regulation of pErk and pAkt levels,

indicating cross talk between these signaling pathways. In line with these findings, we extended the findings in the literature to a relevant GBM model, and demonstrated that TG101209 down regulated the antiapoptotic protein (Bcl-XL) and stimulated PARP cleavage to induce apoptosis. Moreover, flow cytometric analysis for DNA content of cells treated with TG101209 showed partial loss of DNA due to the activation of endogenous nucleases and diffusion of low-molecular weight DNA outside the cells.

Conclusions

Our results demonstrate that TG101209 potently inhibits both STAT3 phosphorylation and STAT3 downstream target gene expression, and induces apoptosis in established GBM lines and primary patient derived cancer stem cell based GBM tumorspheres. These findings justify and provide an initial basis for future translational and mechanistic studies of TG101209 as a GBM therapeutic.

This thesis was co-authored by Rajesh Mukthavaram, this thesis in full may appear in future publications from the Kesari Lab. I was the primary investigator and co-author of this material.

IV:

Materials and Methods

Materials

JAK-2 inhibitor TG101209 obtained from Targegen (now Sanofi), Alamar Blue purchased from AbD Serotech. Anti-pY-STAT3 and anti-STAT3 were purchased from cell signaling. Horseradish peroxidase-linked anti-rabbit or mouse IgG were from Jackson ImmunoResearch (West Grove, PA, USA). Odyssey® Blocking Buffer and IRDye 680 were obtained from Odyssey.

Establishment of primary cancer stem cell lines from GBM patients:

GBM tumor samples were obtained from surgically resected materials. The UCSD Moores Cancer Center Institutional Review Board approved translational research using tumor tissues from GBM patients. All patients gave written informed consent. Tumor samples were washed 2-3 times with 5-10ml of PBS/NSC basal medium to remove blood and debris. The PBS/ Medium was removed and the GBM tumor tissue placed in a Petri dish. The tumor tissue was cut into small pieces and minced for 1-3 minutes with a No. 10 scalpel blade to increase the surface area for trypsinization. The minced tissue was enzymatically dissociated by using 3-5ml of pre-warmed accutase in for 10-15 minutes at a 37°C water bath. A pipette transferred the minced tumor tissue with trypsin into a 15ml Falcon tube. An equal volume of NSC medium was added to stop the enzymatic reaction after the incubation period. Pipetting the suspension up and down several times ensured enzyme inactivation. Then, centrifuging at 190g for 6min pelleted the suspension. After discarding the supernatant the tissue was resuspended in 1ml of sterile NSC basal medium. Clumps were dissociated by gently pipetting up and down (3-7 times) until a smooth milky single cell suspension was achieved. The number

of pipetting steps directly depended on the size of particles in the minced tissue. Lengthy and vigorous mechanical dissociation was avoided as it could have resulted in cell death and a reduction in sphere formation. To remove un-dissociated pieces and debris, 10-15 ml of basal medium was added to the tube and the cell suspension filtered through a 40 micron cell strainer into a 50ml tube. The filtered suspension was centrifuged at 190g for 6min, and the supernatant discarded. Pelletted cells were then resuspended in 1-2ml of complete NSC medium for cell counting.

10 μL of the cell suspension was added to 90 μL of 0.04% Trypan blue in a 1ml eppendorf tube was pipetted up and down to mix the suspension. 10 μL of the cells/trypan blue mixture was transferred to hemocytometer in order to count cell density. Cells were plated in NSC medium supplemented with 20ng/ml EGF, 10ng/ml bFGF and heparin (2ng/ml) in appropriate tissue culture vessels. 5 and 15 mL of medium is used for T25 and T75, respectively. Antibiotics were added to the medium at a concentration of 1:100 in case of contamination. The flask mixture was incubated at 37°C and 5% CO_2.

Passaging and expansion of GBM derived spheres:

We cultured five patient derived neurosphere cell lines GBM4, GBM8, SK1035, SK987, SK892, SK429 and SK262. When the neurospheres reached an average size of 150-200 μm in diameter, subculture was initiated. The content of each flask was removed and placed in an appropriately sized sterile tissue culture tube, and centrifuged at 190g for 6 min at room temperature. The supernatant was removed and the pellet dissociated to create a single cell suspension using a commercially available dissociation kit. The cell suspension was centrifuged at 190g for 6 min. Then, the supernatant was aspirated and

the cells resuspended in 1 ml of NSC medium, incubated at 37° C in a humidified incubator with 5% CO_2.

Cell Culture

Human malignant glioma U87, U251 and A172 cells and patient derived tumor neurospheres: GBM 4 and 8, were used. U87, U251 and A172 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_2–95% air. Patient derived tumor neurospheres were cultured in stem cell medium supplemented with human recombinant EGF (20 ng/ml), human bFGF (10 ng/ml) and heparin (2 ng/ml).

Cell viability assay

The cytotoxic effect of TG101209 was determined using the Cell Proliferation Reagent alamar blue assay (AbD sciences). Cells (2×10^3 cells/well, 100 µl) in 96-well flat-bottomed plates, via overnight incubation at 37°C and in 5% CO_2–95% air. After exposure to the JAK2 inhibitor (TG101209) at concentrations between 0.1 and 40 µM, for 72 h, the cells were incubated with alamar blue for 4-12 h, and fluorescence measured using a Tecan plate reader.

Western blotting

Untreated and JAK2 inhibitor treated human glioma cells were harvested by disassociation, washed with ice-cold PBS, and lyzed in NP40 buffer containing 50 mM

Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.1% sodium deoxycholate, protease inhibitor (complete mini, Roche Scientific) and phosphatase inhibitor (Roche Scientific). Protein quantification was performed with a BCA protein assay from Pierce (Fisher Scientific). Protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with a solution consisting of 5% milk powder in 0.05% Tween 20-PBS (TBS). The membranes were treated with anti-STAT3, antiphospho-STAT3, antiphospho-JAK-2, antiphospho-STAT5 and anti-PARP antibodies (Cell Signaling), anti-Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH antibody (Genetex); then subjected to Western blotting using an ECL (enhanced chemiluminescence). For blots read with Licor the methods change after the gel is transferred to the nitrocellulose membrane. The membranes were blocked with the Odyssey Blocking Buffer for an hour. Then the membranes were treated with the primary antibodies listed above for a minimum 12 hours. After which the membranes were washes with distilled water, and incubated in Blocking buffer and IRDye 680. After 1 hour incubation in the dark the membranes were read on an Odyssey Licor instrument.

Cell cycle analysis

For cell cycle analysis, GBM4 and GBM8 cells were plated at 200,000 cells/well and incubated for 16 hours with 2 μM of TG101209 and DMSO. Cells were dissociated, fixed in ice cold methanol. Cell were incubated at RT in for 30 min PBS containing 50ug/ml RNAse A. Cellular DNA was stained with 250 μl of PI (10 μg/ml), followed by flow cytometric analysis (FACS Calibur flow cytometer, BD Biosciences).

This thesis was co-authored by Rajesh Mukthavaram, this thesis in full may appear in future publications from the Kesari Lab. I was the primary investigator and co-author of this material.

References

1. Wen PY, Kesari S: **Malignant gliomas in adults**. *N Engl J Med* 2008, **359**(5):492-507.

2. Johnson JH, Phillips PC: **Malignant gliomas in children**. *Cancer Invest* 1996, **14**(6):609-621.

3. Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schuler D, Probst-Hensch NM, Maiorka PC, Baweza N, Pisani P, Yonekawa Y, Yasargil MG, Lutolf UM, Kleihues P: **Genetic pathways to glioblastoma: A population-based study**. *Cancer Research* 2004, **64**(19):6892-6899.

4. Reardon DA, Rich JN, Friedman HS, Bigner DD: **Recent advances in the treatment of malignant astrocytoma**. *Journal of Clinical Oncology* 2006, **24**(8):1253-1265.

5. Maciej M Mrugala SK, Naren Ramakrishna, Patrick Y Wen: **Therapy for recurrent malignant glioma in adults. Expert review of anticancer therapy**. 2004, **4**:759-782.

6. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Carncross JG, Eisenhauer E, Mirimanoff RO; European Organization for Research and Treatment of Cancer Grain Tumor and Radiotherapy Groups; National Cancer Institue of Canada Clinical Trials Group:**Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma**. *New Engl J Med* 2005, **352**(10):987-996.

7. Grossman SA, Ye XB, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M, Fisher J, Consortium NC: **Survival of Patients with Newly Diagnosed Glioblastoma Treated with Radiation and Temozolomide in Research Studies in the United States**. *Clinical Cancer Research* 2010, **16**(8):2443-2449.

8. Dalerba P, Cho RW, Clarke MF: **Cancer stem cells: Models and concepts**. *Annual Review of Medicine* 2007, **58**:267-284.

9. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB: **Identification of a cancer stem cell in human brain tumors**. *Cancer Research* 2003, **63**(18):5821-5828.

10. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB: **Identification of human brain tumour initiating cells**. *Nature* 2004, **432**(7015):396-401.

11. Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, Fiocco R, Foroni C, Dimeco F, Vescovi A: **Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma**. *Cancer Research* 2004, **64**(19):7011-702

12. Yuan XP, Curtin J, Xiong YZ, Liu GT, Waschsmann-Hogiu S, Farkas DL, Black KL, Yu JS: **Isolation of cancer stem cells from adult glioblastoma multiforme**. *Oncogene* 2004, **23**(58):9392-9400.

13. Wakimoto H, Kesari S, Farrell CJ, Curry WT, Jr., Zaupa C, Aghi M, Kuroda T, Stemmer-Rachamimov A, Shah K, Liu TC Jeyaretna DS, Debasitis J, Pruszak J, Martuza RL, Rabkin SD:**Human glioblastoma-derived cancer stem cells: establishment of invasive glioma models and treatment with oncolytic herpes simplex virus vectors**. *Cancer Res* 2009, **69**(8):3472-3481.

14. Lee J KS, Kotliarov Y, Li A, Su Q, Donin NM, Pastorino S, Purow BW, Christopher N, Zang W, Park JK, Fine HA: **Tumor stem cells derived from glioblastomas cultures in bFGF and EGF more closely mirror the phenotype and genotype of primary tumore than do serum-cultured cell lines**. *Cancer Cells* 2006, **9**:391-403.

15. Dietrich J, Imitola J, Kesari S: **Mechanisms of Disease: the role of stem cells in the biology and treatment of gliomas**. *Nat Clin Pract Oncol* 2008, **5**(7):393-404.

16. Brennan C MH, Hambardzumyan D, Ozawa T, Tandon A, Pedraza A, Holland E: **Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations**. *Plos One* 2009, **4**:e7752.

17. Cheng CK FQ, Weiss WA: **PI3K signaling in glioma-animal models and therapeutic challenges**. *Brain Pathology* 2009, **19**:112-120.

18. Demuth T, Reavie LB, Rennert JL, Nakada M, Nakada S, Hoelzinger DB, Beaudry CE, Henrichs AN, Anderson EM, Berens ME: **MAP-ing glioma invasion: mitogen-activated protein kinase kinase 3 and p38 drive glioma invasion and progression and predict patient survival**. *Mol Cancer Ther* 2007, **6**(4):1212-1222.

19. Pu P, Zhang Z, Kang C, Jiang R, Jia Z, Wang G, Jiang H: **Downregulation of Wnt2 and beta-catenin by siRNA suppresses malignant glioma cell growth**. *Cancer gene therapy* 2009, **16**(4):351-361.

20. Purow BW, Haque RM, Noel MW, Su Q, Burdick MJ, Lee J, Sundaresan T, Pastorino S, Park JK, Mikolaenko I, Maric D, Eberhart CG, Fine HA: **Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation**. *Cancer Res* 2005, **65**(6):2353-2363

21. Ana P. Costa-Pereira NAB, Michael J. Seckl: **Dysregulation of janus kinases and signal transducers and acticatos of transcription in caner**. *Cancer Research* 2011, **1**(6):806-816.

22. Levy DE, Darnell JE, Jr.: **Stats: transcriptional control and biological impact**. *Nature reviews Molecular cell biology* 2002, **3**(9):651-662.

23. Zhong Z, Wen Z, Darnell JE, Jr.: **Stat3 and Stat4: members of the family of signal transducers and activators of transcription**. *Proc Natl Acad Sci U S A* 1994, **91**(11):4806-4810.

24. de la Iglesia N, Konopka G, Lim KL, Nutt CL, Bromberg JF, Frank DA, Mischel PS, Louis DN, Bonni A: **Deregulation of a STAT3-interleukin 8 signaling pathway promotes human glioblastoma cell proliferation and invasiveness**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2008, **28**(23):5870-5878.

25. Lichtor T, Libermann TA: **Coexpression of interleukin-1 beta and interleukin-6 in human brain tumors**. *Neurosurgery* 1994, **34**(4):669-672; discussion 672-663.

26. Darnell JE, Jr., Kerr IM, Stark GR: **Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins**. *Science* 1994, **264**(5164):1415-1421.

27. Jing N, Tweardy DJ: **Targeting Stat3 in cancer therapy**. *Anti-cancer drugs* 2005, **16**(6):601-607.

28. Rahaman SO HP, Chernova O, Barnett GH, Vogelbaum MA, Haque SJ **Inhibition of Constituively active STAT3 supresses proliferation and induces apoptosis in glioblastoma multiforme cells**. *Oncogene* 2002, **21**:8404-8413.

29. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr.: **Stat3 as an oncogene**. *Cell* 1999, **98**(3):295-303.

30. Sherry MM, Reeves A, Wu JK, Cochran BH: **STAT3 is required for proliferation and maintenance of multipotency in glioblastoma stem cells**. *Stem cells* 2009, **27**(10):2383-2392.

31. Li GH, Wei H, Lv SQ, Ji H, Wang DL: **Knockdown of STAT3 expression by RNAi suppresses growth and induces apoptosis and differentiation in glioblastoma stem cells**. *International journal of oncology* 2010, **37**(1):103-110.

32. Sun Y, Moretti L, Giacalone NJ, Schleicher S, Speirs CK, Carbone DP, Lu B: **Inhibition of JAK2 signaling by TG101209 enhances radiotherapy in lung cancer models**. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* 2011, **6**(4):699-706.

33. Bowman T, Garcia R, Turkson J, Jove R: **STATs in oncogenesis**. *Oncogene* 2000, **19**(21):2474-2488.

34. Bromberg J: **Stat proteins and oncogenesis**. *J Clin Invest* 2002, **109**(9):1139-1142.

35. Bromberg JF: **Activation of STAT proteins and growth control**. *BioEssays : news and reviews in molecular, cellular and developmental biology* 2001, **23**(2):161-169.

36. Owen D. Stechishin HAL, Yibing Ruan, Michael D. Blough, Stephanie A. Nguyen, John J. Kelly, J. Gregory Cairncross, Samuel Weiss: **On-target JAK2/STAT3 inhibition slows disease progression in orthotopic xenografts of human glioblastoma brain tumor stem cells.** *Nuero-Oncology* 2013, **15**(2):198-207.

37. Zhixia Sun HJ, Baofeng Guo, Xiangbo Kong, Hui Wang, Ling Zhang.: **A DNA-based small interfering RNA targeting Stat3 inhibits the growth of renal carcinoma 786-O cells.** *Gene Theruputics Molecular Biology*, **14**:1-8.

38. Mitsutoshi Nakada DK, Takuya Watanabe, Yutaka Hayashi, Lei Teng,Ilya V. Pyko, Jun-Ichiro Hamada.: **Aberrant Signaling Pathways in Glioma**. *Cancers* 2011, **3**(324-3278).

39. Bhattacharya S, Ray RM, Johnson LR: **STAT3-mediated transcription of Bcl-2, Mcl-1 and c-IAP2 prevents apoptosis in polyamine-depleted cells**. *The Biochemical journal* 2005, **392**(Pt 2):335-344.

40. Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, Mizuno K, Hibi M, Hirano T: **STAT3 is required for the gp130-mediated full activation of the c-myc gene**. *The Journal of experimental medicine* 1999, **189**(1):63-73.

41. Epling-Burnette PK, Liu JH, Catlett-Falcone R, Turkson J, Oshiro M, Kothapalli R, Li Y, Wang JM, Yang-Yen HF, Karras J, Love R, Loughran TP Jr.: **Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression**. *J Clin Invest* 2001, **107**(3):351-362.

42. Guilian Niu KLW, Mei Huang, Lanxi Song, Eric Haura, James Turkson, Shumin Zhang, Tianhong Wang, Dominic Sinibaldi, Domenico Coppola, Richard Heller, Lee M Ellis, James Karras, Jacqueline Bromberg, Drew Pardoll, Richard Jove, Hua Yu. : **Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis.** *Oncogene* 2002, **21**:2000-2008.