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Sirna Therapy In Glioblastoma Stem Cells: Identification Of Target Genes And Potential Therapeutic Implications.

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siRNA therapy in glioblastoma stem cells: identification of target genes and potential therapeutic implications.

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SIRNA THERAPY IN GLIOBLASTOMA STEM CELLS: IDENTIFICATION OF TARGET GENES AND POTENTIAL THERAPEUTIC IMPLICATIONS. Benjamin T. Himes, Jiangbing Zhou, Toral Patel, Marie-Aude Guie, Michael Wyler, Joseph M. Piepmeier, W. Mark Saltzman, Department of Biomedical Engineering, Yale University, New Haven, CT.

Glioblastoma multiforme (GBM), the most common primary brain malignancy, carries a grim prognosis; survival statistics have scarcely improved in decades. Even with the development of temozolomide, the current front-line chemotherapeutic agent for GBM, improvement in long-term survival has been minimal, with recurrence virtually assured. One explanation for the persistence of this disease is the presence of a stem-like cell population within GBM (glioblastoma stem cells, or GSCs). These cells are capable of self-renewal, tumor initiation, and are resistant to chemotherapy. We hypothesized that derangement in the expression of genes critical for the maintenance of GSCs could eliminate these cells outright, or induce sufficient cell differentiation to sensitize them to existing chemotherapeutic agents. To this end we performed a genome-wide small interfering RNA (siRNA) screen in search of genes that, when reduced in expression, cause GSC cell death or induce differentiation as measured by changes in nestin expression or cell morphology. Our screening yielded a number of candidate siRNAs: their efficacy in reducing cell viability was demonstrated across a number of genetically distinct GSC cell lines. We further identified two siRNAs, targeting ubiquitin C (UBC) and disheveled 2 (DVL2), respectively, that significantly sensitize GSCs to the effects of temozolomide ($p<0.05$). A similar but not significant effect was also observed in combination treatment with siRNA and either paclitaxel or doxorubicin. We conclude from these observations that siRNA-mediated gene knockdown presents a promising avenue in the development of novel treatments for GBM by taking into account the unique biologic attributes of the therapeutically problematic GSC population.

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Introduction

Glioblastoma multiforme

With approximately 15,000 new diagnoses in the United States annually, glioblastoma multiforme (GBM) represents the most common adult brain malignancy (1). With this diagnosis comes an exceedingly poor prognosis. Surgical resection of as large a volume of tumor as possible, combined additional treatment with radiosurgery and temozolomide chemotherapy, modestly improve survival, but overall 5-year survival stands under 4% (2, 3). Median survival statistics are similarly grim, with most patients living just beyond a year following diagnosis (3, 4). While predominately found in older patients (median age at diagnosis is 65), GBM occurs in all adult age groups, and risk factors are poorly defined, with little evidence for family history or environmental exposures playing a role in disease development (1, 5). Disease recurrence is all but assured given the infiltrative nature of GBM, which penetrates the surrounding brain parenchyma and prevents total resection (3).

It is with this knowledge in hand, statistics largely unchanged in recent decades, that researchers have sought to develop new treatments for GBM. The current standard of care involves surgical resection of the tumor, followed by radiation and chemotherapy with temozolomide (4). There has been some progress in the development of novel therapeutic strategies in GBM, including local delivery of chemotherapeutic agents using polymer systems, but the effects have been very modest (6, 7). Substantial efforts have also been put into developing targeted therapies for GBM, by attempting to take advantage of ligand receptors, such as those for epidermal and platelet-derived growth factors (EGF and PDGF, respectively), that are often over-expressed in GBM (4).

Recent research has also attempted to identify new avenues of therapeutic development by better defining the truly heterogeneous group of tumors that are classified as glioblastoma. Common genetic alterations include the aforementioned amplifications in EGF and PDGF expression, loss of heterozygosity in the chromosome q10 region, and mutations in the p53, PTEN, and p16 tumor-suppressor genes (8). The clinical relevance of subdividing what was once thought of as a single disease is described in a recent landmark paper by Veerhak et al, who utilized the Cancer Genome Atlas to identify four subtypes of GBM: classical, mesenchymal, proneural, and neural subtypes. Their study showed that current treatment modalities were preferentially beneficial in classical and mesenchymal subtypes, while patients with the proneural subtype tended to survive longer (9). The essence of their work and others is that it is futile to think of GBM as a single disease entity, rather a group of phenotypically similar but genetically distinct tumors, and that therapies must be developed with this diversity in mind if they are to be beneficial to patients.

Glioblastoma stem cells

Expanding upon the theme of the heterogeneity of GBM, in developing new therapies, it is important to attend to differences in gene expression and cellular behavior between tumors, as well as to those within a given tumor. It is clear at the most superficial level that GBM can scarcely be called a uniform mass. Its classical appearance on MRI is that of a 'ring-enhancing' lesion: an infiltrative tumor edge surrounding a necrotic core (10). It is also fundamentally clear that not all cells within a GBM are phenotypically the same, or at the very least are capable of changing under selective pressures, as evidenced by the recurrence of tumor following chemotherapy and radiation. It is this recurrence following therapy that necessitates therapeutic targeting of different cell populations within a given GBM.

In particular there is a subpopulation of tumor cells that exhibit stem cell-like properties. Initially described in glioblastoma in 2003 by Singh et al, these cells exhibit the capacity for self-renewal, differentiation, and tumor initiation (11, 12). Such cells have been described in a number of different cancers, and represent a possible reservoir of malignancy in the tumor, capable of triggering rapid disease recurrence in the wake of therapy (13). A number of potential markers have been put forth as being specific for a GBM stem cell (GSC), most notably the membrane glycoprotein CD133, but the ultimate definition of a cancer stem cell is a functional one, and the use of a single marker likely excludes a number of cells capable of exhibiting stem cell-like behavior (11, 12). There is significant controversy surrounding the definition of cancer stem cells, with delineation between the cell of origin for a particular tumor and all cells capable of differentiation, self-renewal, and tumor initiation (14). For the purposes of this discussion, the latter definition is of more interest, since it is cells exhibiting this pattern of behavior that are likely responsible for treatment failures.

GSCs and their counterparts in other forms of cancer present unique therapeutic challenges. Their capacity for tumor initiation implies that should even a single cell escape treatment, recurrence is all but assured. Cancer stem cells asymmetrically divide, giving rise to a renewed stem cell and a transient amplifying cell that rapidly divides and gives rise to the bulk of tumor growth (15). This transient amplifying cell is readily targeted by traditional treatment modalities that rely on crippling cell division, such as radiation therapy or alkylating chemotherapeutics. However, the cancer stem cell population itself remains largely quiescent, thus limiting its sensitivity to these types of therapy (16, 17). Additionally, in some cancers, the stem cell population shows increased activity of ATP-binding cassette

(ABC) transporter proteins, a transport mechanism that actively extrudes chemotherapeutic agents from the cells (18). There is also evidence in that glioblastoma stem cells are inherently resistant to temozolomide therapy (19). Increased DNA damage repair and *Wnt* signaling pathway activity have also been demonstrated in multiple cancer stem cell populations, and also present a likely mechanism for chemoresistance (18). Further compounding matters in the realm of GBM is the presence of a highly necrotic and hypoxic tissue environment, which not only promotes the cancer stem cell phenotype, but also impedes delivery of any therapeutic agents to the tumor cells (18, 20).

These unique characteristics of GSCs necessitate novel therapeutic strategies in order to effectively combat tumor recurrence. Attempts to treat GSCs can be broadly characterized into two groups. The most straightforward strategy is to find treatments to which GSCs are uniquely susceptible, a drug particularly toxic to GSCs or an oncolytic virus capable of binding to specific GSC surface ligands, for example. A second strategy is to explore agents that may sensitize GSCs to existing therapeutic interventions. Such an approach is attractive for several reasons. Firstly, the bulk tumor must still be treated in addition to the GSC population; so traditional chemotherapeutics/radiation therapy will remain a mainstay of treatment. Secondly, the capacity of GSCs for differentiation is well documented. These cells, as part of the natural history of the disease, divide into transient amplifying cells that are highly susceptible to existing therapies, so the biologic machinery for such a transition is clearly not only in place, but readily utilized by these cells. One needs only the proper tools to nudge the GSCs along this path.

RNA interference

The pursuit of such a therapeutic avenue necessitates the efficient and specific manipulation of the expression profile of GSCs in order to increase their susceptibility to chemotherapeutic agents. Through the use of small interfering RNAs (siRNAs), such fine control is achievable. siRNA takes advantage of the endogenous RNA interference machinery of the cell in order to specifically reduce the expression of a target gene at the messenger RNA level.

RNA interference (RNAi) was originally described by Fire et al in *C. elegans*, in which the authors discovered that the administration of double-stranded RNA (dsRNA) resulted in reduction in the levels of the mRNA of their gene of interest (21). An enzyme complex in the cell, termed Dicer, recognizes the dsRNA and cleaves mRNA corresponding to the antisense strand of the dsRNA through the formation of RNA-induced silencing complexes (RISCs) (22). RISCs also play a role in direct inhibition of translation and chromatin modification, further augmenting their gene-silencing role (22).

This advance in the basic understanding of cell biology led logically to the development of RNAi as a tool for manipulating gene expression. Administration of as specific dsRNA could allow for specific knockdown of a target mRNA, and allow for study of the effects of selective knockdown of a given gene. siRNA was developed following the discovery that dsRNA oligonucleotides of no longer than 21-22 base pairs were needed in order to achieve effective and specific knockdown of target mRNAs (23). This not only allowed for more efficient and economical synthesis, but also more effective transfection of cells and a reduction in the cellular interferon response that often accompanied administration of longer dsRNA molecules (this is likely because siRNA loads directly into

RISCs, and does not load into the Dicer complex, as do longer dsRNAs) (23). As a potential therapeutic platform, siRNA is extremely attractive in that it allows for exquisitely specific control of gene expression. However, this specificity is also a limitation in that therapeutic administration of siRNA would likely only allow for the selective knockdown of a single candidate genes (possibly more if multiple siRNAs are co-administered, but such in such as scenario it is challenging to ensure equal delivery of siRNAs to the target cells). The likelihood of single gene therapy as a stand-alone therapeutic in GBM treatment is extremely unlikely. As mentioned earlier, however, the potential to sensitize GSCs to existing therapeutics is a promising avenue of inquiry, and one for which siRNA is ideally suited.

Specific Aims

Specific Aim I: Identify genes responsible for proliferation and self-renewal in glioblastoma stem cells that are sensitive to siRNA knockdown.

Given the above discussion, there is an obvious alignment between the goal of developing specific therapies for GSCs and the technology of siRNA-mediated RNAi. If it is possible to identify those genes critical for the function of GSCs, either those important for maintenance of their self-renewal properties or for continued proliferation and viability, then novel therapeutic targets will present themselves. To this end we performed a genome-wide siRNA library screen in order to identify candidate siRNAs. Criteria for evaluation included reduction in cell number, reduction in nestin expression, and changes in cell morphology. The former was chosen as a marker of cell proliferation and viability, while the latter two were selected as indicators of differentiation.

Specific Aim II: Determine the effect of combination therapy with siRNA and chemotherapeutic agents on the viability of GSCs.

As previously discussed, one of the most promising avenues for developing siRNA as a therapeutic tool in GBM is through its potential to sensitize GSCs to conventional chemotherapeutic agents. The likelihood of a stand-alone siRNA therapy making a meaningful impact in the treatment of GBM is low given the intricacy of the genetic derangements in the disease. However, there is an extensive literature regarding adult and embryonic stem cells regarding genes critical for maintenance of the stem cell phenotype (24-26). In addition to genes critical for maintenance of stem cell functionality, there may be additional genes that, when disrupted, impair GSC homeostasis sufficient to impede the normal mechanisms of chemotherapeutic resistance. To examine this possibility we studied the effect of pretreatment of a number of GSC cell lines with candidate siRNAs, followed by treatment with a number of chemotherapeutic agents including temozolomide, the standard agent in GBM therapy.

Materials and Methods

GSC spheroid culture

Glioblastoma stem cell enriched spheroids were isolated from primary tumor samples via dissociation and culture in serum-free media as described by Lee et al (27). Briefly, cells were cultured as spheroids in suspension using. Neurobasal-A media (Invitrogen) supplemented with B27 nutritional supplement (Gibco), basic fibroblast growth factor (bFGF), and epidermal growth factor. (EGF). Cell culture work performed by B Himes, J Zhou, and T Patel.

High-throughput screening

Transfections using a library of ~20,000 siRNAs (Dharmacon siGENOME library, sequences available in Supplementary Table 2) were performed in triplicate on the GS5 GSC

cell line at the Yale High Throughput Screening center, using pooled siRNAs targeting specific genes (4 per gene) and Lipofectamine RNAiMAX transfection system (Invitrogen). Lipofectamine RNAiMAX reagent was diluted in OPTI-MEM serum-free media (Gibco) and added to 384-well plates containing pooled siRNAs. After a twenty-minute incubation, GS5 cells were added to the transfection plates following mechanical spheroid dissociation.

Three days following transfection, cells were formalin fixed and stained. Genes for secondary screening were selected based upon changes in morphology (expressed as increased cell length as determined by measurement algorithm developed by M Wyler), nestin (a stem cell marker) expression, or cell number (assessed via Hoechst 3342 nuclear staining). Secondary screening was performed in duplicate using 4 individual siRNAs targeted toward 100 genes selected based upon these three criteria. Transfections performed by B Himes, T Patel, and J Zhou. Plate reading and staining performed by M Wyler. Statistical analysis performed by M Guie. Gene selection for secondary screen was performed by B Himes and J Zhou.

Cell viability assessment

AlamarBlue colorimetric assays were performed 4 days following siRNA transfection using Lipofectamine RNAiMAX (96-well plate, 5000 cells/well). Cells were transfected at siRNA concentrations of 80nM and Lipofectamine concentrations of 0.3μL/well. Cells were incubated for 24hrs with AlamarBlue reagent (Invitrogen) prior to fluorescence reading with Molecular Devices Spectramax spectrophotometer. All assays performed by B Himes.

Chemotherapeutic Combination Studies

Transfection conditions were the same as those described above. Temozolomide (Sigma), paclitaxel (ARC), or doxorubicin (MP biomedical) was administered 24 hrs post-

transfection diluted in DMSO (DMSO concentration 0.5% of total media volume). AlamarBlue reagent was added 3 days post drug treatment. Cells were incubated for 24 hrs with AlamarBlue reagent prior to fluorescence reading. Vehicle treatments consisted of DMSO only to a concentration of 0.5% of total media volume. Studies performed by B Himes.

Real-Time PCR

Knockdown confirmation studies were performed using real-time quantitative PCR. Two to three days following transfection with siRNA, total cellular mRNA was harvested using Qiagen RNeasy Mini Kit according to manufacturers instructions. First-strand cDNA synthesis was accomplished using the iScript cDNA synthesis kit (Bio-Rad). Quantification and quality control of both harvested RNA and cDNA were performed using NanoDrop ND-2000 spectrophotometer (Thermo Scientific). PCR primers were designed and ordered using PrimeTime qPCR web-based assay design software (Integrated DNA technologies). Detection of amplification products was performed using iQ SYBR Green Supermix and collected with a MyiQ detection and amplification system (Bio-Rad). Analysis of amplification was performed using the ΔΔCt method to calculate fold-change between experimental and control siRNA treated groups following data normalization to amplification of GAPDH control. Primer sequences available in Supplementary Table 3. All studies performed by B Himes.

Immunofluorescence

Eight-well chamber slides (Lab-Tek) were coated for a minimum of 3 hours with poly-L-ornithine (PLO) to induce cell adherence. Cells were plated at time of transfection with Lipofectamine RNAiMAX (Invitrogen) at a density of 10,000 cells/well. Fixation and

staining were performed three days post-transfection. GFAP staining was performed using rabbit polyclonal antibody (Dako) and goat anti-rabbit AlexaFluor 546 secondary antibody (Invitrogen). Images were acquired using an Olympus IX71 microscope. Image analysis and quantification performed using ImageJ software. Briefly, images were imported in .tif format and normalized by setting a baseline signal threshold to remove background signal. ImageJ software then was used to calculate the integrated density of the image (sum total of all normalized pixel intensities). This data was then divided by the number of cells per image to account for changes in cell number across fields of view.

Results

Genome-wide screening

In order to identify genes important for GSC maintenance and viability that are potentially susceptible to siRNA-mediated therapy, we performed a genome-wide screen using the Dharmacon siGenome library. This library consists of approximately 18,000 siRNAs spread over fifty-eight 384-well plates, with 4 siRNAs specific to each gene. The primary screen was performed in triplicate using the GS5 GSC line. Cells were transfected with siRNA from the library and plated on PLO-coated 384-well plates. Three days post-transfection, cells were formalin fixed and stained in order to quantify changes in cell number, changes in nestin expression, and changes in cell morphology. Results were sorted based on degree of reduction in cell number relative to control wells, statistically significant reduction in nesting expression according to a robust z-score (RZ), and a significant increase in the percentage of lengthy cells, also determined by a robust Z score. The data were highly reproducible across each of the three replicates (see Figure 1). Control siRNAs included a nestin siRNA as a positive control and a RISC-free siRNA as a negative control (a

proprietary siRNA that is not processed by the RISC complex, minimizing the off-target effects seen with scramble siRNAs (28)).

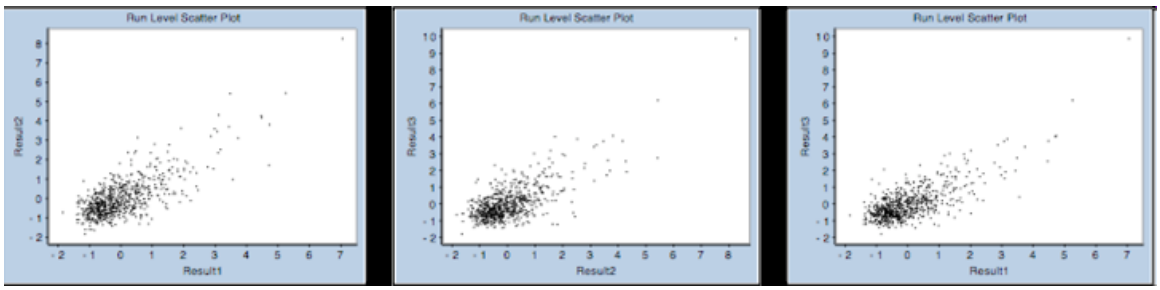


Figure 1. High-throughput siRNA screening is reproducible. Plots of signal intensity from each of three replicate siRNA plates plotted against one another. Note the strong correlation across plates.

From this initial dataset, genes were selected for confirmation screening, wherein transfections were repeated using a single siRNA per well rather than pooled siRNAs targeting a specific gene. Transfection and staining was performed in an identical manner to the primary screen, again in triplicate using the GS5 cell line, with four siRNAs for each gene. Genes selected for confirmation screening were chosen due to a substantial decrease in cell number (greater than 50% reduction in cell number in at least 2/3 replicates), a low cell width: length ratio significantly different than that of the negative control cells (RZ>7), or a significant reduction in nestin expression (RZ<-2). Due to limitations with the scope of the pilot study through the Yale High-Throughput Center, only one hundred genes could be selected for confirmation screening, and as such significant weight was given to the likely biologic activity of the candidate siRNAs, and an extensive search of the literature was undertaken to investigate the applicability of the candidate genes in GBM pathogenesis, GSC maintenance, or in other cancer or stem cell biology. The full list candidate genes selected can be found at the end of this manuscript (supplementary table 1).

Genes evaluated on cell length criteria were termed a confirmed hit if one siRNA in each of the three replicates produced a decrease in cell width: length ratio with a RZ score of greater than 2. Genes evaluated on cell viability metrics were deemed hits if a siRNA resulted in at least a 30% decrease in cell number across all replicates. Those genes evaluated on the criteria of nestin expression were confirmed as hits if one siRNA in each of the three replicates reduced nestin expression relative to control by at least 30%. Of the one hundred genes screened, there were seven confirmed hits based on reduction in nestin expression, six were confirmed based on reduction in cell viability, and fifteen were confirmed based on increased cell length criteria (Tables 1, 2, and 3).

Cell Viability Confirmed Hits				
Name	Aliases	Description	Entrez Gene ID	References
CCNB1	CCNB	cyclin B1	891	Horvath (29)
DVL2		dishevelled, dsh homolog 2 (Drosophila)	1856	Pulvirenti (30)
RPS6	RP11-513M16.6, S6	ribosomal protein S6	6194	Mayer (31)
SIK2	LOH11CR1I, QIK, SNF1LK2	salt-inducible kinase 2	23235	Bright (32)
UBC	HMG20	ubiquitin C	7316	Chen (33)
WEE1	FLJ16446; DKFZp686I18166	Ser/Thr kinase	7465	Mir (34)

Table 1. Confirmed cell viability hits. Genes with a reduction in cell viability of at least 30% in at least one instance across all three replicates. Aliases and descriptions taken from Entrez Gene available at http://www.ncbi.nlm.nih.gov/gene/.

Nestin Reduction Confirmed Hits				
Name	Aliases	Description	Entrez Gene ID	References
AKT1	AKT, PKB, PKB-ALPHA, PRKBA, RAC,	v-akt murine thymoma viral oncogene homolog 1	207	Eyler (35)

	RAC-ALPHA			
DUB3	DUB3; USP17L2	ubiquitin specific peptidase 17-like 2	377630	Pereg (36)
H-PLK	HPF9; H-plk; MGC22613; ZNF117	zinc finger protein 117	51351	
HSPA6		heat shock 70kDa protein 6 (HSP70B')	3310	Gama Fisher (37)
MRLC2	MLC-B; MRLC2; MYL12B	myosin, light chain 12B, regulatory	103910	
TKTL1	TKR; TKT2; TKTL1	transketolase-like 1	8277	Yuan (38)

Table 2. Confirmed nestin reduction hits. Genes with a reduction in nestin expression of at least 30% in at least one instance across all three replicates. As above, aliases and descriptions taken from Entrez Gene.

Cell Length Confirmed Hits				
Name	Aliases	Description	Entrez Gene ID	References
C2ORF28	p18; APR3; APR-3; APR--3; PRO240; HSPC013	Apoptosis-related protein 3-multiple splice sites	51374	Yu et al (39)
COMMD1	MURR1; C2orf5; MGC27155;	copper metabolism (Murr1) domain containing 1	150684	Zoubeidi (40)
CRYAA	CRYA1; HSPB4; CRYAA	crystallin, alpha A	1409	Goplen (41)
DDB1	XPE; DDBA; XAP1; XPCE; XPE-BF; UV-DDB1; DDB1	damage-specific DNA binding protein 1, 127kDa	1642	Jiang (42)
EDG5	H218; LPB2; S1P2; AGR16; EDG-5; Gpcr13; S1PR2	sphingosine-1-phosphate receptor 2	9294	Estrada (43)
ETS2	ETS2IT1	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	2114	Valter (44)
MAP3K2	MEKK2, MEKK2B	mitogen-activated protein kinase kinase kinase 2	10746	Xia (45)
MRLC2	MLC-B; MYL12B	myosin, light chain 12B, regulatory	103910	
POU4F1	Brn-3, Brn-3.0, Brn3, Brn3.0, Brn3a,	POU class 4 homeobox 1	5457	

	E130119J07Rik			
RARG	RARC; NR1B3	retinoic acid receptor, gamma	5916	Das (46)
RHOA	ARHA; ARH12; RHO12; RHOH12; RHOA	ras homolog gene family, member A	387	Hoshino (47)
TEF	KIAA1655; TEF	thyrotrophic embryonic factor	7008	
UNQ739	UNQ739/PRO1434, PSST739, VWC2	von Willebrand factor C domain containing 2	375567	
WFDC11	WAP11; MGC71905; WFDC11	WAP four-disulfide core domain 11	259239	
ZNF513	RP58; FLJ32203; HMFT0656	zinc finger protein 513	130557	

Table 3. Confirmed cell length hits. Genes with corresponding siRNAs inducing a significant increase in cell length with a RZ score greater than 2 for at least one siRNA in all replicates. As above, alias and description information taken from Entrez Gene database.

As noted in the above tables, a number of these confirmed target genes have known roles in glioblastoma pathogenesis or are prominently involved in other cancers. Some of the confirmed genes, such as DVL2, have even been shown to be important in the maintenance of GSCs (30). This documented biologic relevance provides encouragement that some of the identified genes may have therapeutic applications, and the nature of the screen is indicative of their relative susceptibility to treatment with siRNA.

However, there are limitations to this screening approach and the data generated from this study. Notably, the primary screen and subsequent confirmation studies were only performed on the GS5 cell line. Given that siRNA therapy only targets a single gene at a time, it is essential to confirm the efficacy of knocking down the expression of a given target gene in multiple genetically diverse GSC populations. While there is undoubtedly tumor heterogeneity that needs to be taken into account as targeted therapies such as siRNA

platforms are developed, it is also critical to develop therapies that have as broad applicability as feasible. There is also some concern with the use of nestin as a marker for GSC maintenance and its absence as a marker for differentiation. While there is a wide literature on the use of nestin as a stem cell marker, the short timescale of this screen coupled with the often subtle changes observed in nestin intensity may call its utility here into question. Finally, many of the effects observed overall in the screen were relatively small, albeit reproducible. This is not surprising when one considers that siRNA treatment reduces the expression of a single target gene, and in a population of cells that are typically quiescent, there is likely a robust ability to overcome such modest derangements. Yet, the fact that these effects are reproducible and statistically significant points to a real and measurable change in cell behavior. If the siRNA itself does not provide a stand-alone therapeutic option, then perhaps it can be used to enhance the utility of other therapeutic agents that have hitherto been ineffective in treating GSCs.

Treatment across cell lines

Moving forward in our studies, we focused our efforts on a more narrow group of genes than those confirmed by the initial screen. We determined that those genes that have a direct effect on GSC viability were the best candidate targets moving forward, as knockdown of these genes produced the most dramatic effect of the three criteria measured (cell death), and also the most easily quantifiable one. Five genes were selected for further study: UBC, DVL2, CCNB1, WEE1, and TPX2. The latter gene, an Aurora kinase-associated protein, did not meet the criteria used to define a hit on the cell viability confirmation screen because it did not induce sufficient cell death in one of the three replicates. However, because of its implications in a number of oncogenic pathways, and because in the other replicate plates,

three and two of the four siRNAs targeting TPX2 resulted in cell death meeting the criteria for a hit, we decided it merited further study (48).

To examine the effects of siRNA knockdown of these candidate genes on multiple cell lines, cells from the GS5, PS11, PS16, and PS32 lines were treated with siRNAs targeting TPX2 and UBC. Previous work in the laboratory to categorize these cell lines has shown that they express dramatically different levels of key genes in glioblastoma pathogenesis, such as NF1, EGFR, and PDGFRA (Michael Fu, unpublished data, see Supplementary Figure 1.). AlamarBlue assays performed following transfection with siRNA demonstrated a significant reduction in cell viability with siUBC treatment across all cell lines (Figure 2). A reduction in cell viability across all lines was also observed with siTPX2 treatment, but it did not reach statistical significance.

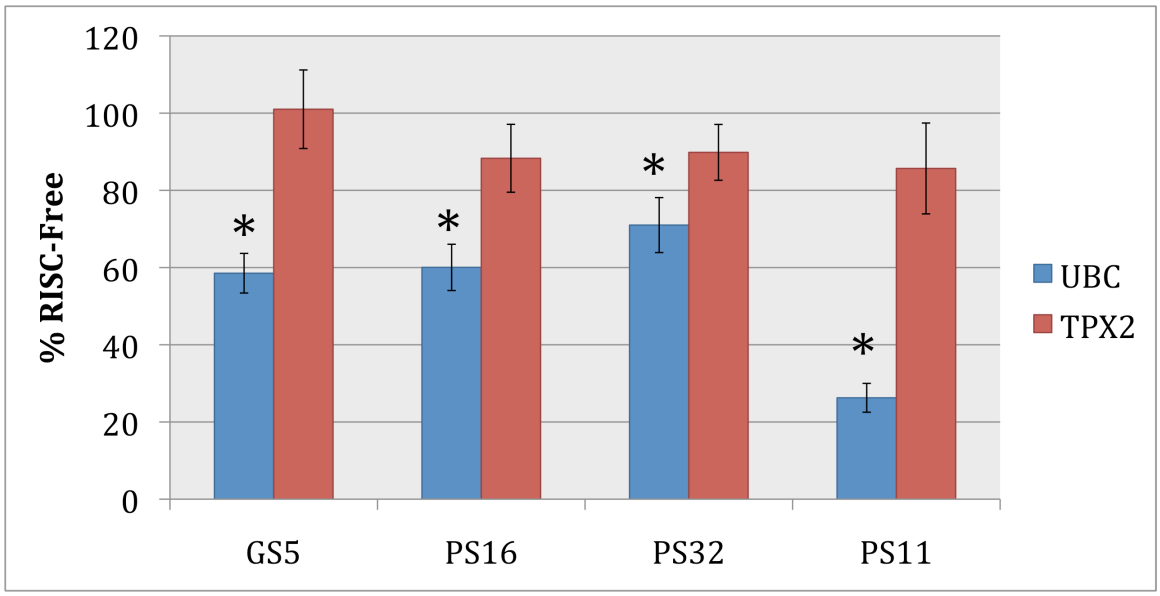


Figure 2. UBC knockdown reduces cell viability across multiple cell lines. AlamarBlue data (assay on d4 post-transfection, n=3 biological replicates with three technical replicates each) demonstrates a significant reduction in cell-viability relative to RISC-free siRNA-treated control. * = $p<0.05$ by t-test. Error bars = SEM.

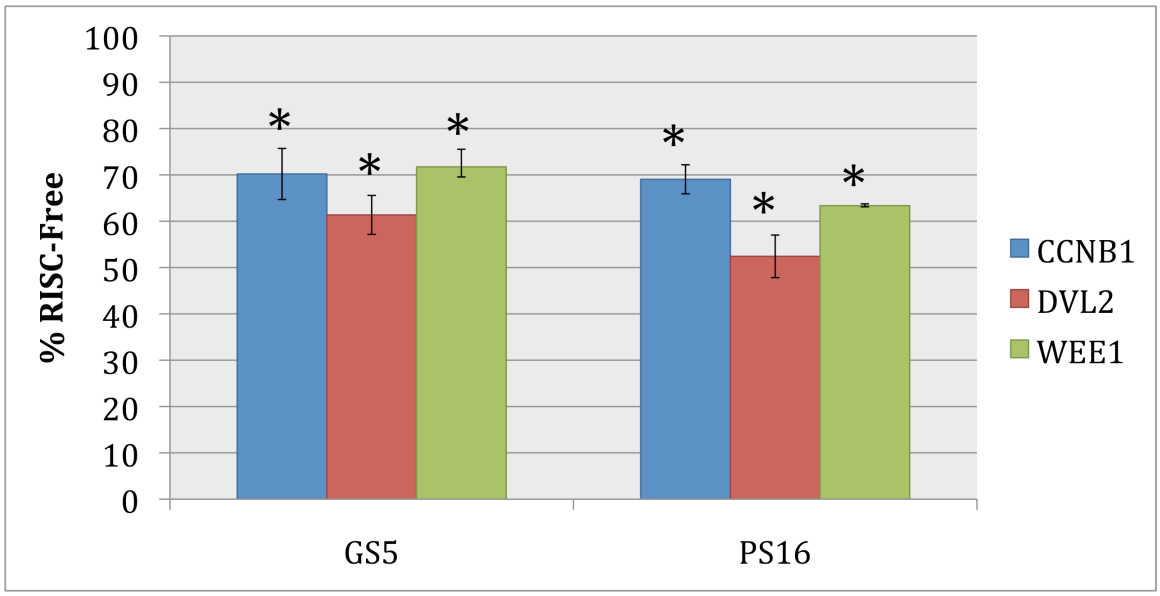


Figure 3. Knockdown of siCCNB1, DVL2, and WEE1 produces a significant reduction in cell viability across cell lines. AlamarBlue data (assay on d4 post-transfection, n=3 biological replicates with three technical replicates each) demonstrates a significant reduction in cell-viability relative to RISC-free siRNA-treated control. * = $p<0.05$ by t-test. Error bars = SEM.

Similar studies were performed using siCCNB1, siDVL2, and siWEE1. These studies were only carried out on the GS5 and PS16 cell lines however, due to the higher reliability of the lipid-based transfections in these cell lines. In these studies, all siRNAs showed a significant reduction in cell viability relative to treatment with control siRNA in both cell lines. This effect was not as substantial as that observed in with siUBC, however (Figure 3).

In order to confirm that the siRNAs under study actually reduce expression of the target genes in question, the levels of target gene mRNA following siRNA transfection were assessed. To this end we performed real-time quantitative PCR to assess target gene mRNA levels 48 hours following transfection (Figure 4). These studies demonstrated substantial knockdown of expression of all candidate genes, most notably UBC. Knockdown of WEE1

and CCNB1 were less than ideal, with less than 50% knockdown in expression, though given the observed efficacy in cell viability reduction this seems more likely due to suboptimal transfection efficiency. Overall however, these results support that the observed reduction in cell viability detailed in Figures 2 and 3 is due to reduced expression of the desired target gene.

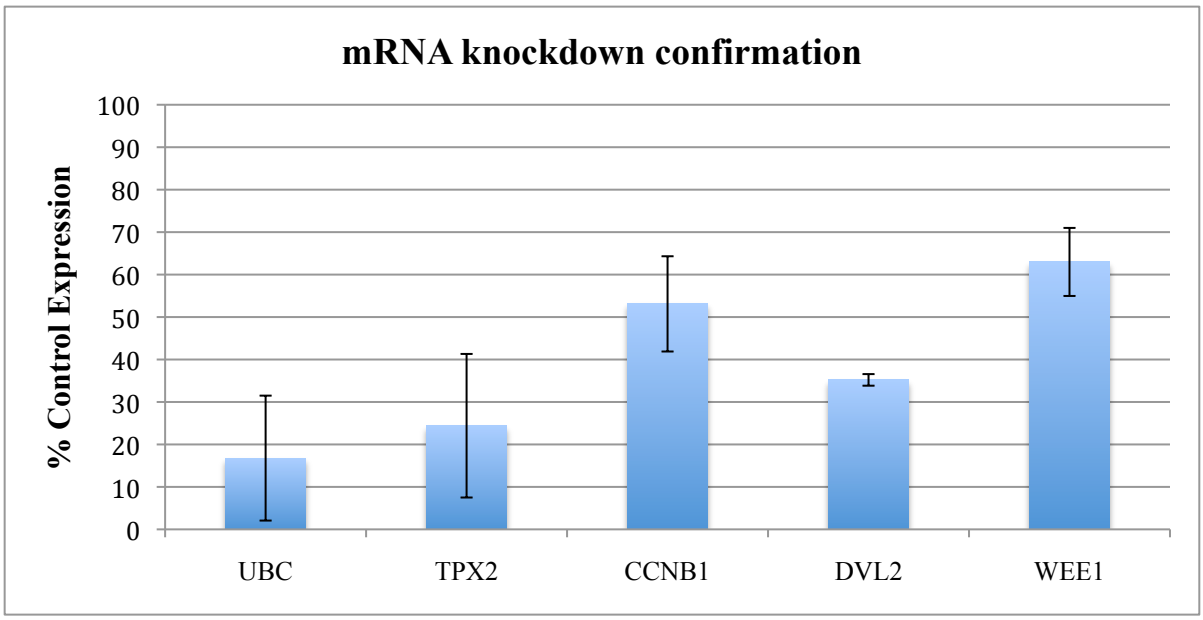


Figure 4. Confirmation of target gene mRNA knockdown. Quantitative real-time PCR results for mRNA expression of target genes after treatment with a given siRNA relative to RISC-free siRNA-treated controls. Data is an average of three technical replicates each of GS5 and PS16 cell lines. Error bars = SEM.

Chemotherapeutic studies

The data demonstrate a consistent effect of siRNA treatment across multiple cell lines in reducing cell viability (Figures 2 through 4). However, this effect, with few exceptions (such as siUBC treatment of the PS11 cell line) was modest. This is not surprising. While there are some tumors in which a given genetic target is critical for cancer cell maintenance, gene expression patterns within a given glioblastoma, as well as across different tumors, are

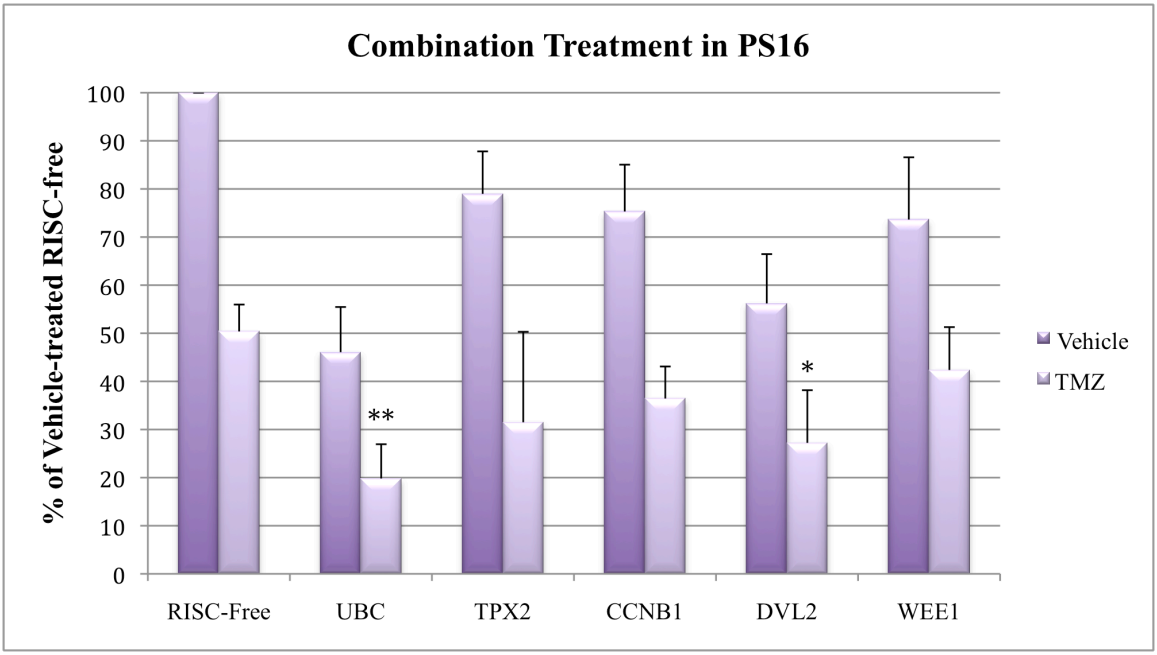
typically heterogeneous (49, 50). It is highly unlikely, given the variety of genetic derangements in GBM and the relative quiescence of the GSC population that modulation in expression of a single gene would be able to eliminate this problematic group of cells across a number of different cell lines.

However, the observed effects indicate a reproducible, albeit modest, response to siRNA therapy in these cells. If the siRNA therapy alone is insufficient to kill this cell population, we reasoned that it may be sufficient to sensitize GSCs to traditional chemotherapeutic agents either by induction of differentiation or through disruption of cellular homeostasis. As mentioned earlier, GSCs are typically resistant to temozolomide, the current chemotherapeutic standard of care in GBM (19, 51). The ability of a single factor to induce differentiation of adult or embryonic stem cells, differentiated cells, or cancer stem cells is well documented (52-54).

To determine the impact of siRNA therapy on GSC response to chemotherapeutic agents, we transected cells with candidate siRNAs, and then treated cells with temozolomide at a concentration of 500μM for 24hr after transfection (this large concentration of temozolomide was used in order to attain a reliable 50% reduction in cell viability by AlamarBlue assay). Cell viability was assessed by colorimetric assay three days following drug treatment. Studies were conducted using the GS5 and PS16 cell lines.

A dramatic decrease in cell viability was observed in the siUBC-treated following addition of temozolomide (TMZ) treatment, with reductions in florescence of over 80% relative to vehicle and RISC-free treated control (Figure 5, top and bottom). This observation reached statistical significance relative to treatment with temozolomide and RISC-free siRNA in both cell lines tested. A similar additive effect was seen across all siRNA+TMZ

combinations, but the only other to reach statistical significance was siDVL2+TMZ in the PS16 cell line. A notable decrease in cell viability was also observed in the siTPX2+TMZ and siDVL2+TMZ treated GS5 cells, and in the siCCNB1+TMZ treated PS16 cells, but these changes did not reach statistical significance when compared to siRISC-free+TMZ treated cells (in all cases p~0.1).



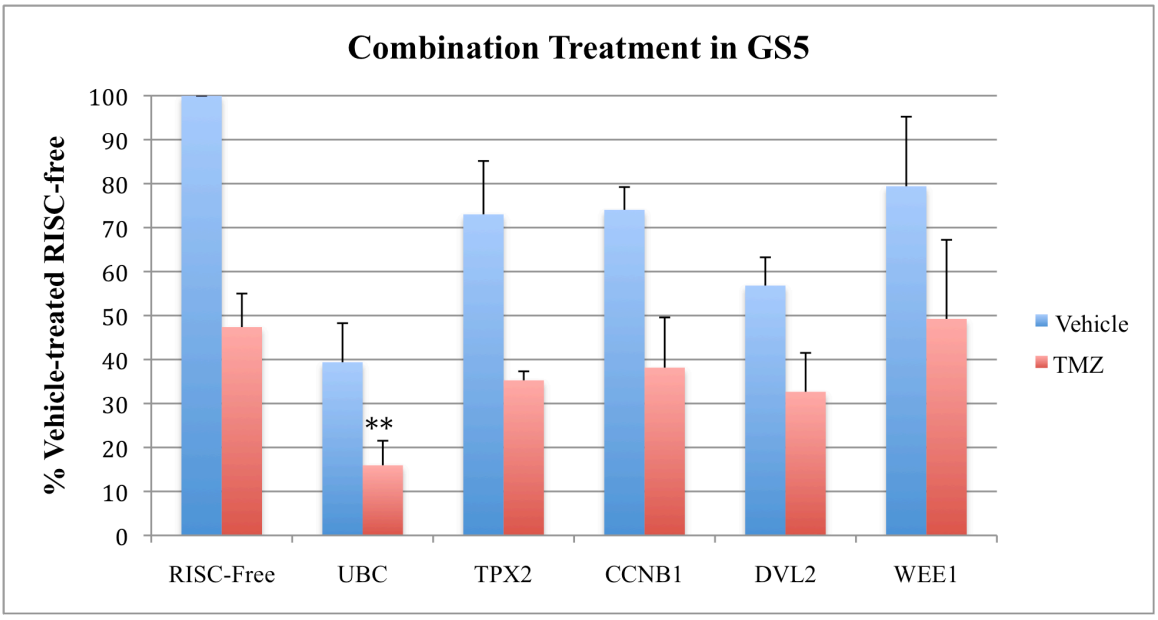


Figure 5. siRNA treatment augments the effect of temozolomide (TMZ) on GSCs. AlamarBlue assays following treatment with siRNA and subsequent temozolomide treatment at 500μM in the PS16 (top) and GS5 (bottom) cell lines. * = $p<0.05$ ** = $p<0.01$. Error bars = SEM.

The above studies demonstrate that when used in combination with temozolomide, siRNA therapy directed toward a number of our selected candidate genes, particularly UBC, can have a dramatically larger effect on GSC viability than siRNA or chemotherapy alone. This is in line with the hypothesis that siRNA can be used to sensitize GSCs to chemotherapeutic agents. It is important to note that the concentrations of temozolomide used in these experiments are substantially higher than those used in clinical practice; this was done in order to achieve a reliable dose response.

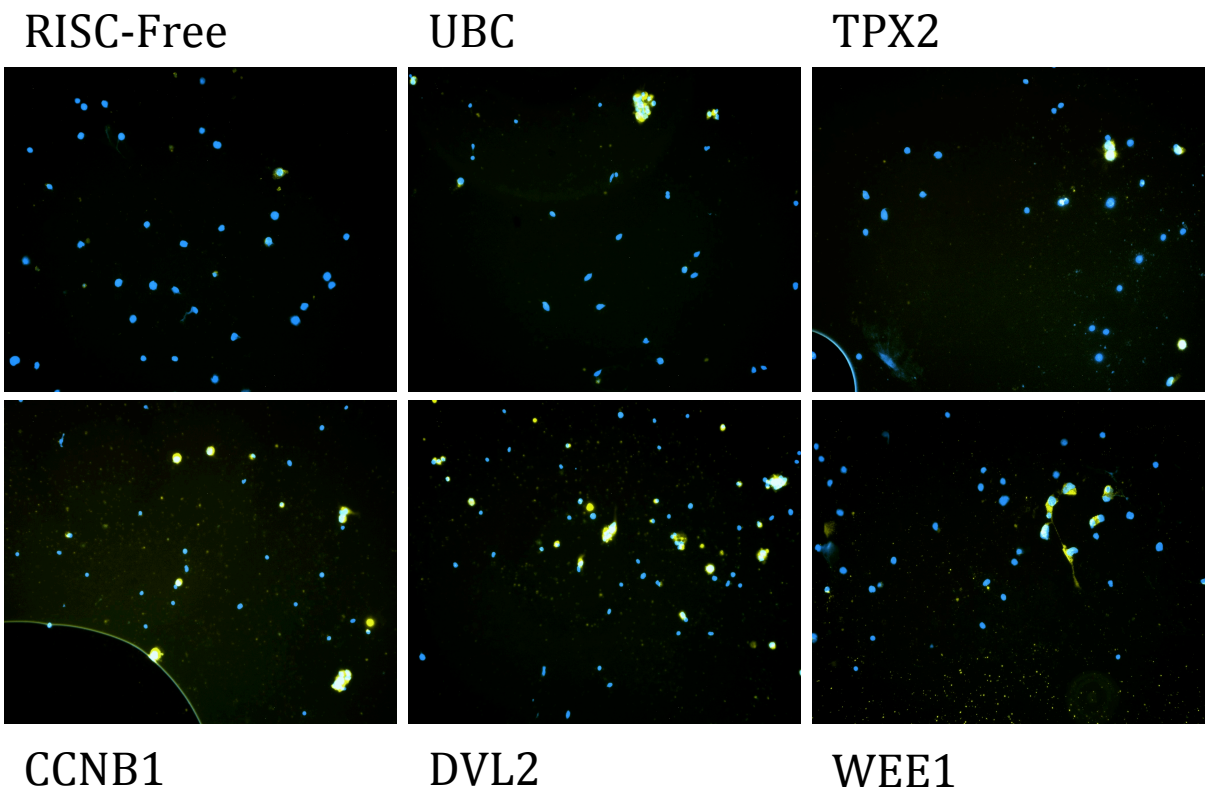
GSC differentiation

In the primary siRNA library screen described earlier, genes were selected based on markers of differentiation (nestin expression and increased cell length) in addition to reduction in cell number. The latter category was chosen out of an interest in determining those genes most directly responsible for GSC maintenance and critical for their survival, as well as due to its ease of quantitation. However, in exploring the reasons behind the above described effect of increased sensitivity to temozolomide, it is important to consider the possibility that these genes important for viability may also be important for maintaining the stem cell properties of GSCSs, or that the reduction in their expression causes sufficient derangement in GSC homeostasis so as to force differentiation. In order to assess this possibility, we performed immunofluorescent staining of GS5 cells following transfection

with the previously examined genes identified based on impact on cell viability. Three days after transfection, cells were fixed and stained for glial fibrillary acidic protein (GFAP), a marker of astrocytic differentiation (Figure 6) (55, 56). GSCs typically express some level of GFAP, but GSCs often express less than bulk tumor or normal glial cells (27, 57). Increased staining for GFAP was observed in most treatment conditions, particularly in those cells transfected with siDVL2 and siCCNB1. A more modest increase in GFAP staining was observed in those treated with siUBC, siTPX2, and siWEE1.

In an effort to quantify the expression of GFAP in these studies, we utilized the ImageJ software package to analyze levels of fluorescence. As most cells in all studies stained at least weakly for GFAP (very weakly in the case of the RISC-free treated cells which was expected based on the literature (11)), we chose to quantify the overall GFAP-coupled fluorescence as it related to the number of cells in a given field of view (Figure 6, bottom). By setting a threshold level of fluorescent detection, we controlled for background fluorescence and used to software to measure the total signal intensity of GFAP-couple fluorescence in a particular field of view. To control for variations in cell number, this total fluorescence was divided by the number of cells the field.

The data generated by this method largely corroborate the above observations based on gross visual inspection. All therapeutic conditions showed a higher level of GFAP fluorescence than the siRISC-free treated GSCs. DVL2 in particular showed a high level of fluorescence with relatively little variability across fields of view. Expression in other experimental conditions, notably that of the siWEE1-treated cells, was much more variable, though clearly greater than control levels.



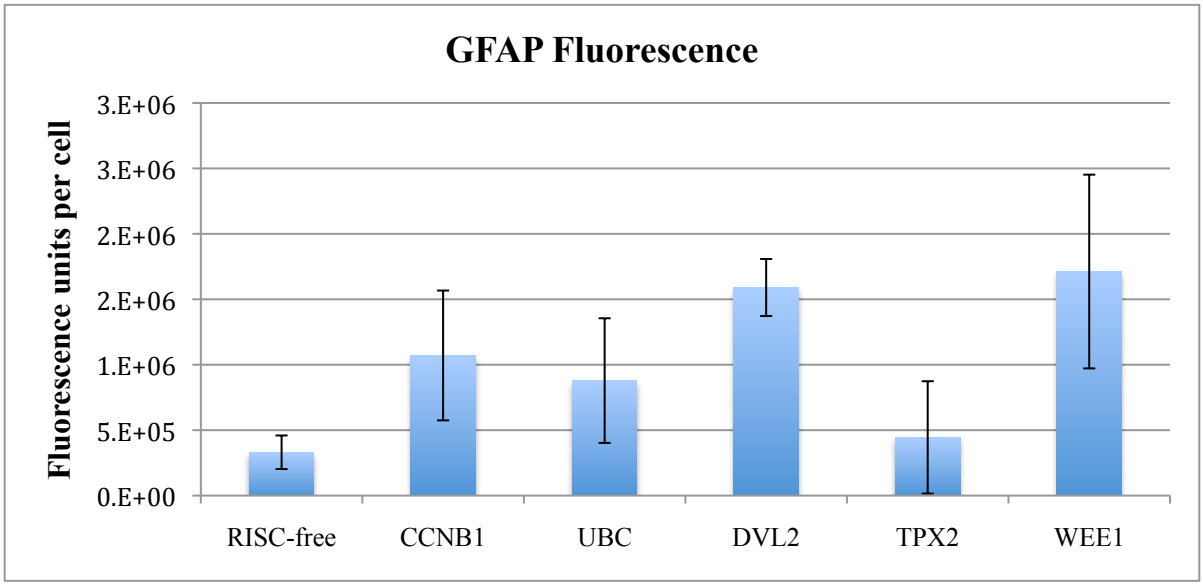


Figure 6. Changes in GFAP staining of PS16 GSCs following siRNA transfection. Top: Immunofluorescent staining for GFAP (AlexaFluor 546, green) or DAPI nuclear stain (blue). Bottom: Quantification of GFAP fluorescence following siRNA transfection and staining expressed in fluorescence units per cell. Error bars = Standard deviation.

These results indicate some level of increased glial differentiation in GSCs treated with these siRNAs, pointing to a potential mechanism for the observed increase in sensitivity to temozolomide treatment. Direct toxicity to GSCs is likely of equal if not greater importance than induced differentiation in the case of several of these siRNAs, however, as transfection with siUBC, which resulted in the greatest decrease in cell viability, caused only a small increase in GFAP staining. Transfection with siDVL2, however, resulted in both a substantial decrease in cell viability and the most dramatic increase in GFAP expression observed, indicating a possible link between the two in this instance.

Discussion

In an effort to identify novel therapeutic targets for glioblastoma, we performed a genome-wide screen using a library of candidate siRNAs, examining their effects on glioblastoma stem cells. We identified a number of genes important for GSC proliferation and maintenance by measuring changes in cell number, nestin expression, and cell morphology. We demonstrated the efficacy of candidate genes identified in this screen in reducing GSC viability in several genetically distinct GSC cell lines. Further, we showed that several of these candidate siRNAs, most notably siUBC, significantly and substantially increase the sensitivity of GSCs to treatment with temozolomide, raising the possibility of a role for siRNA as an adjunct therapeutic for treating GBM. Finally, we explored the mechanism of this increased sensitivity, demonstrating that in addition to the impediment to cell proliferation observed on the initial screen, transfection with number of the candidate siRNAs leads to GSC differentiation as measured by increased GFAP expression. These data all support the potential use of siRNA, particularly siUBC as identified in these studies, as a

worthwhile therapeutic avenue to pursue in the treatment of the therapeutically challenging GSC population.

Biologic relevance

In any study involving a genome-wide screening approach, it is important to proceed with an eye to the biologic functionality of the candidate genes evaluated in order to make sense of what can be a daunting amount of data. As demonstrated in Table 1, 2, and 3, a number of the genes identified in both the primary and confirmation screens have direct biologic relevance to cancer or stem cell biology in general, or to glioblastoma in particular.

The ubiquitin gene encodes a protein critical for the maintenance of protein homeostasis in cells, targeting proteins for degradation by the proteosome complex (58). Given the role of the protein in such fundamental process, ubiquitin is a key component of many cellular processes, including cell cycle, where it triggers the degradation of a number of cyclin checkpoint proteins, as well as their inhibitors (33, 59). This implies an important role of the proteosome and its regulatory pathways in the cell cycle regulation of both normal and malignant cells, making it a potentially attractive therapeutic target. Proteosome activity is increased in a number of cancers, including breast cancer and melanoma, and increased proteosomal activity is thought to allow cellular avoidance of apoptosis (33, 60-62). In fact, inhibition of the proteosome and has been shown not only to increase apoptosis, but bortezomib, a proteosome inhibitor, has even been shown to aid in overcoming chemoresistance (33).

DVL2, part of the Wnt signaling pathway, has recently been described as playing a significant role in glioblastoma in a manner consistent with that observed in our studies (30). Pulvirenti and colleagues, using bulk tumor glioblastoma lines, as well as primary GBM

samples, demonstrate that DVL2 is frequently highly expressed in these tumors, and that inhibition of DVL2 expression through siRNA knockdown inhibits cell proliferation. They also show that DVL2 knockdown inhibits tumor formation in a mouse model, and, interestingly, lead to increased differentiation of tumor cells (30). Derangements in Wnt pathway expression, including DVL2, while not extensively described in glioblastoma, are a common area of study in other cancers, including colorectal and non-small cell lung cancer (63, 64).

Despite a less dramatic observed effect on GSCs in our studies, TPX2 also remains an interesting candidate gene. A microtubule-associated gene, TPX2 has garnered some interest as a potential therapeutic target as it regulates the protein Aurora, which is critical for chromosomal segregation in mitosis (65). Very recently, however, Guvenc and colleagues described inhibition of the Survivin-Ran protein complex, for which TPX2 is an effector protein, as importance for GSCs survival (66). They describe inhibition of GSC spheroid growth as well as cell death through a caspase-mediated mechanism. Though they did not investigate TPX2 itself as a therapeutic target in this study, they demonstrate a key pathway of which it is a part to be an intriguing therapeutic option, specifically in treating GSCs.

Similar biologic evidence can be found for many of the other candidate genes identified as listed in the earlier tables and in the supplementary table below. CCBN1 is a protein critical for cell cycle that has long been the subject of investigation in oncology (67). Similarly, WEE1 has been the focus of study given its role in cell cycle progression (68). In this way we feel that the siRNA screening approach accomplished our goal to identify novel GSC therapeutic targets that are biologically relevant and have the potential to be therapeutically meaningful.

Future directions

Given the large number of candidate genes identified through the genome-wide screen, a great deal of study remains to be done in assessing the importance of the genes identified. Given the possible role of differentiation in sensitizing GSCs to chemotherapy, even in those cells identified because of their toxicity to GSCs, it is essential to revisit those genes identified based on changes in nestin expression and increased cell length in order to examine the effects of chemotherapy following treatment with these siRNAs. However, those genes identified based on toxicity discussed above, if they truly induce differentiation in addition to being toxic to GSCs, will likely prove the best therapeutic candidates.

We have begun exploring the potential for siRNA combination therapy by performing simultaneous transfection with multiple candidate siRNAs. A central problem with siRNA treatment in cancer therapy is the idea that siRNA can only reduce the expression of a single specific gene. While it is this exquisite specificity that makes siRNA such an attractive research tool, it is a limitation in treating a disease as genetically complex as GBM. By combining siRNAs that act on genes in different critical pathways, we hope to explore the potential for siRNA as a stand-alone therapy if the correct cocktail of siRNAs is utilized. We have begun some preliminary studies to examine the effects of using those siRNAs identified as reducing cell viability in combination with one another, and have also begun to examine the use of siRNAs identified based on induction of differentiation in combination with the most toxic siRNA identified, siUBC (See Supplementary Figures 2 and 3).

Studies are also underway to examine the effects of different chemotherapeutic agents in conjunction with siRNA therapy. While temozolomide represents the standard of care in GBM, siRNAs that are ineffective in sensitizing GSCs to temozolomide therapy might be

more effective if a different agent, such as paclitaxel or doxorubicin, is used. We have begun to examine the effects of paclitaxel and doxorubicin treatment in GSCs in conjunction with our siRNA therapy, and early results show similar effects to that observed with temozolomide (Figure 7.)

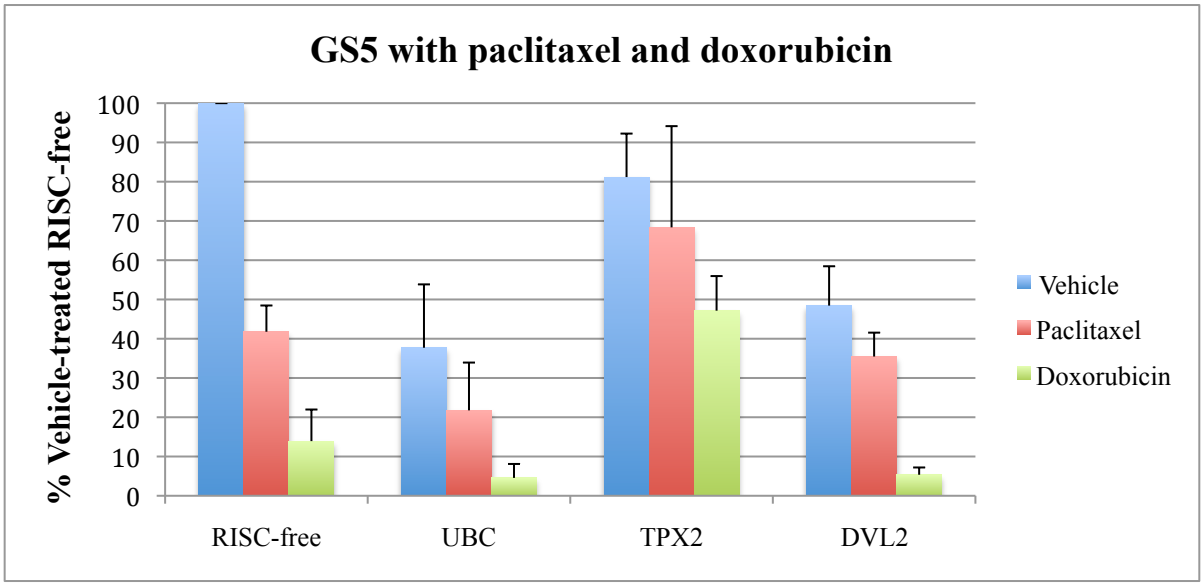


Figure 7. Paclitaxel and doxorubin in combination with siRNA therapy in GS5 cells. AlamarBlue assays demonstrate reduced cell viability with siRNA therapy followed by treatment with either 250nM paclitaxel of 24nM doxorubicin. Interpretation of this data is challenging as these drugs are much more toxic to GSCs than temozolomide, making it difficult to assess any additional impact of siRNA treatment. n = 3 for RISC-free, UBC, and TPX2; n = 2 for DVL2. Error bars = standard error.

Ultimately, however, the utility of siRNA therapy, both alone and in conjunction with chemotherapy, must be demonstrated in an *in vivo* model in order to better assess the therapeutic viability of these candidate genes. GSCs form tumors in a xenograft model; the challenge lies in effective delivery of siRNA to the tumor, where endogenous nucleases, cellular uptake, and migration to the cytoplasm all present challenges (69).

Clinical applicability

A number of proposed solutions have arisen in order to combat the problem of siRNA delivery, which must be overcome to translate therapeutics to clinical practice. Modification of the RNA molecule itself in order to make it more resistant to nuclease degradation is one widely used method. 2'-O-methylation of the sugar backbone at several nucleotides within the siRNA molecule allow for not only increased resistance to RNAse activity, but also reduced inflammatory response and some limitation of off-target effects (70). Liposomal delivery systems have also shown some promise in delivering siRNA to target tissues intact (71). Additionally conjugation to antibodies and other protein moieties has shown some potential for improved targeted delivery (71, 72). All of these technologies, however, have failings in one or more areas that impede their ability to become an effective solution for the problem of therapeutic siRNA delivery. Structural reinforcement of the siRNA may improve resistance to degradation, but it does little to effectively target siRNA delivery or assure entrance into the cell cytoplasm. Antibody conjugation allows for targeted delivery, but is limited in the degree to which it can be modified for effective diffusion through tissues and are limited to recognizing a single receptor group. Liposomal constructs may have limitations to the extent to which they can achieve specific delivery, and do not provide a means of administering controlled release (73).

Synthetic nanoparticles hold promise in solving the delivery problems surrounding siRNA delivery. Nanoparticles are constructed with biodegradable and biocompatible materials, can be coated with materials to increase bioavailability and provide targeted delivery, can be fabricated in different formulations and sizes for controlled release, and can be loaded with agents to aid in the efficacy of siRNA: these properties have the potential to

solve the major delivery challenges surrounding siRNA (70). The use of biodegradable or biocompatible materials increases the safety profile and allows for controlled release of loaded material, and the ability to coat the particles with agents like polyethylene glycol (PEG) to increase the hydrophilic nature of the molecules improves their penetration into target tissues (74, 75). Recently, Woodrow et al. and Zhou et al. have demonstrated the use of poly(lactic-co-glycolic acid) (PLGA) nanoparticles that can be readily modified to allow for effective siRNA delivery (76, 77). PLGA, known to be biodegradable, is also readily modified to adjust the rate of release of loaded agent (78). These particles make use of conjugated peptides such as iRGD, which has been shown to enable particles to home to a target tumor when administered systemically (79). They may also be loaded with agents to enhance endosomal survival and escape, and to increase the potency of siRNA (80, 81). With all of these finely tunable elements, these nanoparticles hold great potential as a powerful delivery vehicle for siRNA-based therapy.

In sum, with advances in delivery technology, siRNA is rapidly becoming a feasible means by which one can realistically hope to treat disease. The question becomes, then, of choosing the delivery system and candidate siRNAs most appropriate for the disease process in question. Much recent work in the Saltzman lab has centered on the development of an effective nanoparticle delivery system for siRNA, as well as studying convection-enhanced delivery of these nanoparticles intracranially. For these reason we embarked on this genome-wide screen to identify potential therapeutic targets with which to take advantage of this platform. Now that a number of interesting candidate genes have been identified, it is time to marry the platform and the payload, and attempt to develop a novel therapy for glioblastoma.

Supplementary Table 1. Genes selected for confirmation screening.

Name	Description	Entrez Gene ID	References
ACVRL1	activin A receptor type II-like 1	94	Dieterich (82)
AD-003	methyltransferase like 11A	28989	Tooley (83)
AKT1	v-akt murine thymoma viral oncogene homolog 1	207	Eyler (35)
ALDOA	aldolase A, fructose-bisphosphate	226	
ARRB1	arrestin, beta 1	408	Bonnas (84)
ARRB2	ARB2, ARR2, BARR2	409	
AURKB	AIK2, AIM-1, AIM1, ARK2, AurB, IPL1, PPP1R48, STK12, STK5	9212	Vital (85)
BLOC1S1	biogenesis of lysosomal organelles complex-1, subunit 1	2647	
BMP4	bone morphogenetic protein 4	652	Piccirillo (86)
C1ORF40	shisa homolog 4 (Xenopus laevis)	149345	Furuchima (87)
C20ORF32	Cas scaffolding protein family member 4	57091	Singh (88)
C2ORF28	Apoptosis-related protein 3- multiple splice sites	51374	Yu (39)
CARD9	caspase recruitment domain family,	64170	Adrian (89)

	member 9		
CASP8AP2	caspase 8 associated protein 2	9994	Lee (90)
CCNB1	cyclin B1	891	Horvath (29)
CCNB1IP1	cyclin B1 interacting protein 1, E3 ubiquitin protein ligase	57820	
CNTNAP5	contactin associated protein-like 5	129684	
COMMD1	copper metabolism (Murr1) domain containing 1	150684	Zoubeidi (40)
COMMD3	COMM domain containing 3	23412	Cai (91)
CRYAA	crystallin, alpha A	1409	Goplen (41)
CSNK1A1	casein kinase 1, alpha 1	1452	Bak (92)
CSPG3	neurocan	13004	Sajad (93)
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	1463	Chiba (94)
DDB1	damage-specific DNA binding protein 1, 127kDa	1642	Jiang (42)
DISP2	dispatched homolog 2 (Drosophila)	85455	Vestergaard (95)
DKFZP564O243	ABHD14A abhydrolase domain containing 14A	25864	
DPP4	dipeptidyl-peptidase 4	1803	Yang (96)
DUB3	ubiquitin specific peptidase 17-like 2	377630	Pereg (36)
DUSP8	dual specificity	1850	

	phosphatase 8		
DVL2	dishevelled, dsh homolog 2 (Drosophila)	1856	Pulvirenti (30)
EDG5	sphingosine-1-phosphate receptor 2	9294	Estrada (43)
ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)	2004	Wasylyk (97)
EPAS1	endothelial PAS domain protein 1	2034	Liang (98)
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	2114	Valter (44)
EYA1	eyes absent homolog 1 (Drosophila)	2138	Drake (99)
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	2354	Kesari (100)
FRAP1	mechanistic target of rapamycin (serine/threonine kinase)	2475	Sunayama (101)
GNG2	guanine nucleotide binding protein (G protein), gamma 2	54331	
GSH-2	GS homeobox 2, role in neural development	170825	Waclaw (102)
H-PLK	zinc finger protein 117	51351	
HBM	hemoglobin, mu	3042	
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	3091	Yoshida (103)

HOXB3	homeobox B3	3213	Bodey (104)
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	7184	See (105)
HSPA5BP1	transmembrane protein 132A	54972	Oh-Hashi (106)
HSPA6	heat shock 70kDa protein 6 (HSP70B')	3310	Gama Fisher (37)
IRAK2	interleukin-1 receptor-associated kinase 2	3656	Muzio (107)
KIF15	kinesin family member 15	56992	Rath (108)
KIF3A	kinesin family member 3A	11127	Rath (108)
MAP3K2	mitogen-activated protein kinase kinase kinase 2	10746	Xia (45)
MAP3K6	mitogen-activated protein kinase kinase kinase 6	9064	Sirotkin (109)
MAPK12	mitogen-activated protein kinase 12	6300	
MAPK13	mitogen-activated protein kinase 13	5603	
MGC54289	DNA-damage regulated autophagy modulator 2	128338	Park (110)
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	4316	Rome (111)
MRLC2	myosin, light chain 12B, regulatory	103910	
NOTCH3	notch 3	4854	Kanamori (112)
NRN1	neuritin 1	51299	Le Jan (113)

PAX2	paired box 2	5076	Ozcan (114)
PDK1	pyruvate dehydrogenase kinase, isozyme 1	5163	Lee (115)
PDLIM7	PDZ and LIM domain 7 (enigma)	9260	
PHYHIPL	phytanoyl-CoA 2-hydroxylase interacting protein-like	84457	
POU4F1	POU class 4 homeobox 1	5457	
PRAF2	association with glioma	11230	Bosics (116)
PRB1	proline-rich protein BstNI subfamily 1	5542	Collins (117)
PRES	prestin (motor protein) - possible role in cell length	375611	
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1	5682	Wernike (118)
RARG	retinoic acid receptor, gamma	5916	See (119)
RHOA	ras homolog gene family, member A	387	Jin (47)
RHOC	ras homolog family member C	389	Sasayama (120)
RPS27A	ribosomal protein S27a	6233	Noerholm (121)
RPS6	ribosomal protein S6	6194	Mayer (31)
SATB1	SATB homeobox 1	6304	Chu (122)
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member	5055	Motaln (123)

	2		
SFRS3	serine/arginine-rich splicing factor 3	6428	Jia (124)
SIK2	salt-inducible kinase 2	23235	Bright (32)
SMO	smoothened, frizzled family receptor	6608	Shahi (125)
SNAI2	snail homolog 2 (Drosophila)	6591	Yang (126)
TEF	thyrotrophic embryonic factor	7008	
TF	transferrin	7018	Martell (127)
TFAP2BL1	transcription factor AP-2 delta (activating enhancer binding protein 2 delta)	83741	
TGFB2	transforming growth factor, beta 2	7042	Schneider (128)
THAP5	THAP domain containing 5	168451	Ola (129)
TKTL1	transketolase-like 1	8277	Yuan (38)
TNEM23	sphingomyelin synthase 1, possible role in apoptosis	259230	Lafont (130)
TP53	tumor protein p53	7157	Zheng (131)
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	22974	Asteriti (48)
TRAF3	TNF receptor-associated factor 3	7187	Xie (132)
TSC2	tuberous sclerosis 2	7249	Mieulet (133)
TSC	tuberous sclerosis 1	7248	
UBB	ubiquitin B	7314	Chen (33)

UBC	ubiquitin C	7316	Chen (33)
UNQ739	von Willebrand factor C domain containing 2	375567	
WEE1	Ser/Thr protein kinase.	7465	Mir (34)
WFDC11	WAP four-disulfide core domain 11	259239	
WNT9B	wingless-type MMTV integration site family, member 9B	7484	
YAP	Yes-associated protein 1	10413	Hélias-Rodzewicz (134)
ZDHHC18	zinc-finger	84243	
ZNF322A	zinc finger protein 322A	79692	
ZNF513	zinc finger protein 513	130557	

Supplementary Table 2. siRNA sequences from the Dharmacon siGENOME library.

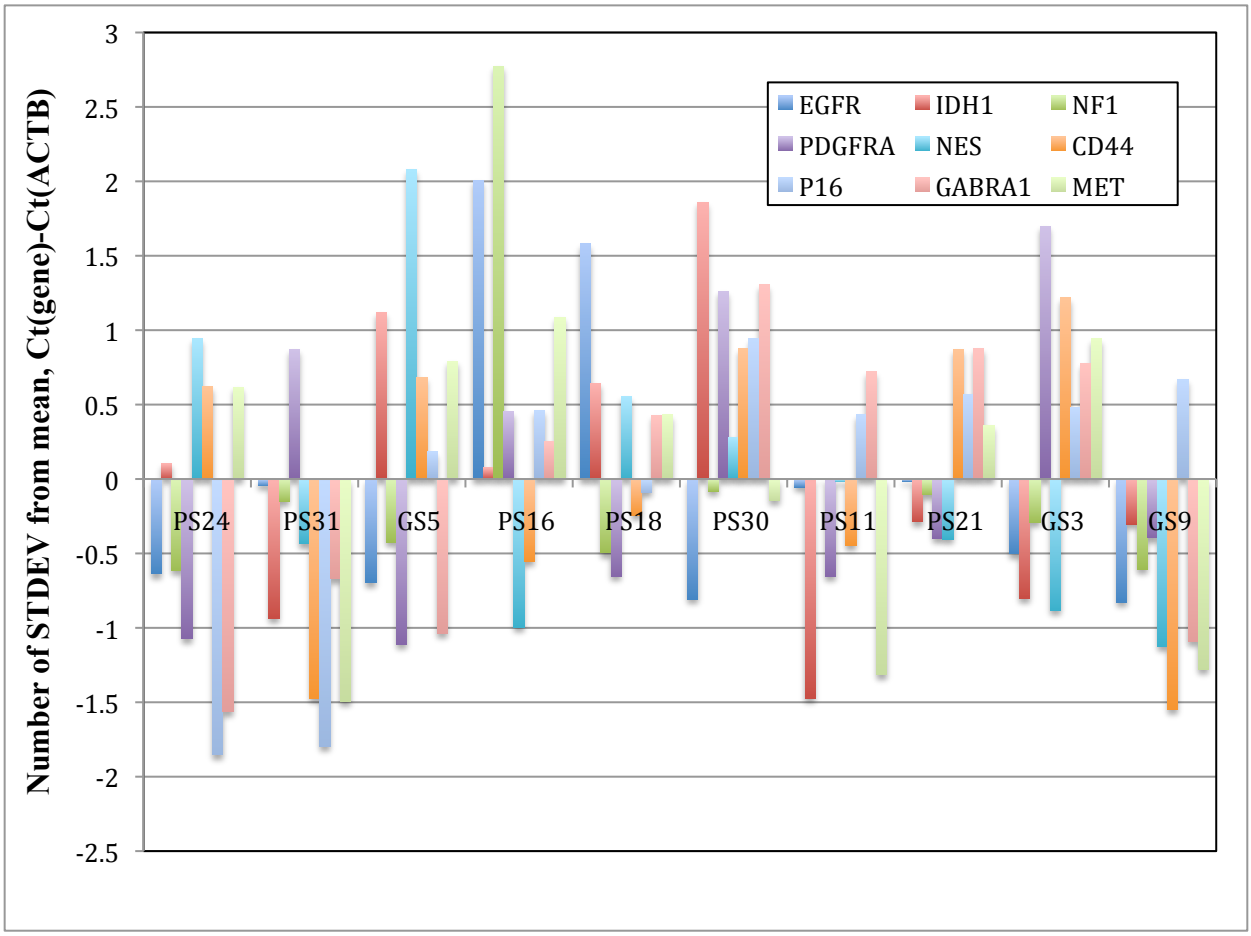
Target Gene	Sequence
UBC	GUGAAGACCCUGACUGGUA
TPX2	GGACGAACCGGUAGUGAUA
DVL2	UGUGAGAGCUACCUAGUCA
CCNB1	GAAAUGUACCCUCCAGAAA
WEE1	GGGAAUUUGAUGUGCGACA

Supplementary Table 3. Real-time qPCR primers.

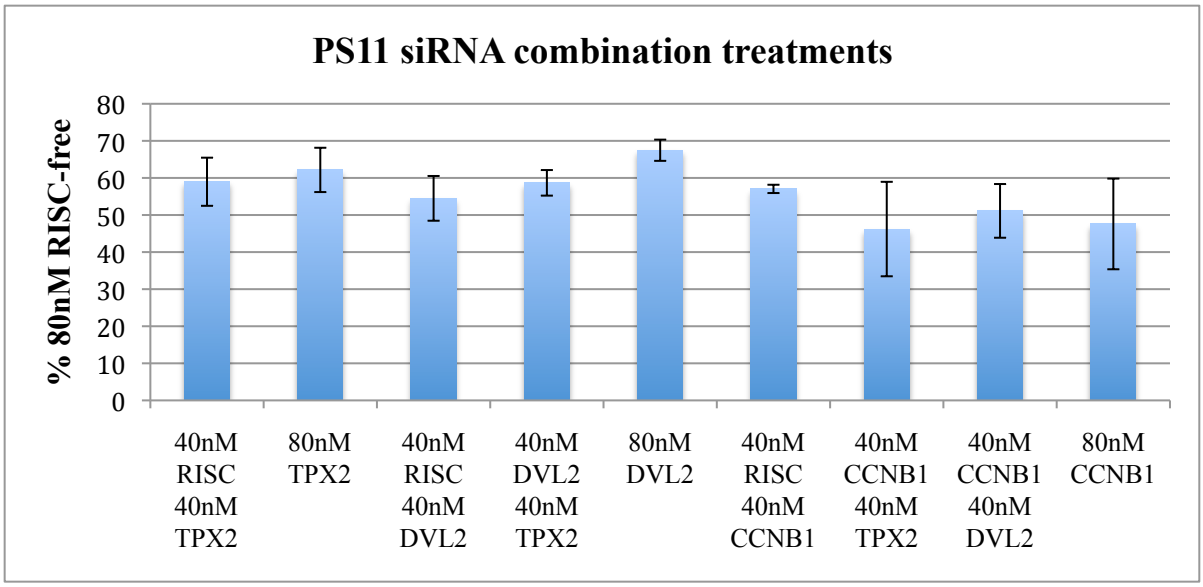
Gene	5'->3' Sequence	Tm (°C)	%GC
UBC			
Forward	ACGCACCCTGTCTGACTACAACAT	60	50

Reverse	AGGGATGCCTTCCTTGTCTTGGAT	60	50
TPX-2			
Forward	AAGAAGCCAGAGGAAGAAGGCAGT	60	50
Reverse	AGAAACTTCTGCTTTGCAGGTGGC	60	50
RHOA			
Forward	AGGTAGAGTTGGCTTTGTGGGACA	59.9	50
Reverse	TATTCCCAACCAGGATGATGGGCA	60	50
CCNB1			
Forward	TGTGGATGCAGAAGATGGAGCTGA	59.9	50
Reverse	TTGGTCTGACTGCTTGCTCTTCCT	60	50
DVL-2			
Forward	ATGTGGCTCAAGATCACCATCCCT	59.9	50
Reverse	TCTTGTTGACGGTGTGTCGGATCA	60	50
WEE1			
Forward	AAACAGCCCTTGGTTTGGCCTATG	59.8	50
Reverse	TATAACCTGGGAAGCGCTGTGGAA	59.9	50
HSPA5BP1			
Forward	ATGGTGTGGGAAATCCTGGTGTCT	60.1	50
Reverse	TGTATTCACCAGCTCCTCAGCCTT	59.8	50
C20ORF32			
Forward	ACCGCATCCTGCTTGAAACAAAGG	60.1	50
Reverse	GGCAATGACAATGGAGGCAAACCT	60	50
GAPDH			
Forward	TCGACAGTCAGCCGCATCTTCTTT	60.3	50
Reverse	ACCAAATCCGTTGACTCCGACCTT	60.1	50
PLK			

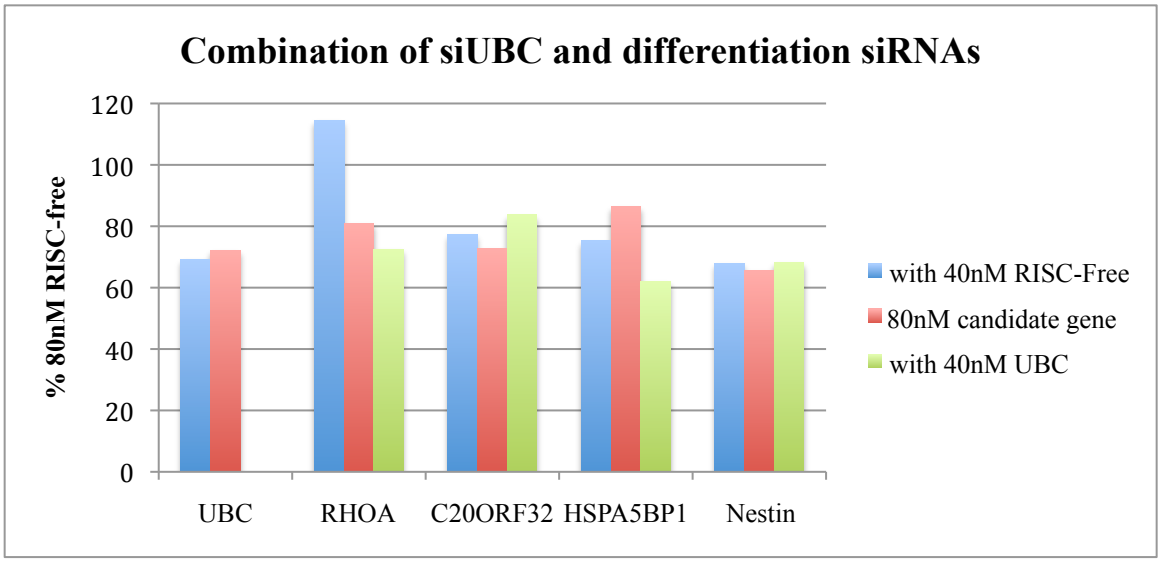
Forward	TGTACATGTTCGGGTGTGGGTTCT	60	50
Reverse	AAGCCAAGGAAAGGACAGTTCCGA	60.3	50



Supplementary Figure 1. Differential expression of genes across GSC lines. Real-time PCR data studies performed by Michael Fu demonstrate broad variability in the expression of key genes in GBM pathogenesis across numerous GSC cell lines. Note the positions of the GS5 and PS16 lines. Results shown as standard deviations from mean expression across all cell lines, normalized to β-actin expression.



Supplementary Figure 2. siRNA combination treatment in PS11. Simultaneous treatment with two different siRNAs at a concentration of 40nM as compared to 80nM single RNA doses. Data is preliminary and few substantial effects seen, though there is perhaps a marginally larger effect in the siCCNB1/siTPX2 combination than in either alone, though it is highly variable. Data are results of AlamarBlue assays performed in the manner described above. n=2 biological replicates with 3 technical replicates each. Error bars = standard error.



Supplementary Figure 3. Differentation-inducing siRNA combination with siUBC in GS5. Preliminary data demonstrating the effect of the combination of genes identified as inducing GSC differentiation (see Tables 2 and 3) with siUBC, the siRNA most consistently effective in reducing cell viability. Above data are the results of one AlamarBlue assay conducted in the manner described above.

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