Functional genomic analysis of glioblastoma multiforme through short interfering RNA screening: a paradigm for therapeutic development

NIKHIL G. THAKER, B.S.,¹ FANG ZHANG, PH.D.,²⁻⁴ PETER R. McDONALD, PH.D.,²⁻⁴
TONG YING SHUN, PH.D.,²⁻⁴ JOHN S. LAZO, PH.D.,²⁻⁴ AND IAN F. POLLACK, M.D.⁵

¹University of Medicine and Dentistry of New Jersey–New Jersey Medical School, Newark, New Jersey; Departments of ²Pharmacology and ³Chemical Biology, ⁴Drug Discovery Institute, University of Pittsburgh, Pittsburgh, Pennsylvania; and ⁵Department of Neurological Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania

Glioblastoma multiforme (GBM) is a high-grade brain malignancy arising from astrocytes. Despite aggressive surgical approaches, optimized radiation therapy regimens, and the application of cytotoxic chemotherapies, the median survival of patients with GBM from time of diagnosis remains less than 15 months, having changed little in decades. Approaches that target genes and biological pathways responsible for tumorigenesis or potentiate the activity of current therapeutic modalities could improve treatment efficacy. In this regard, several genomic and proteomic strategies promise to impact significantly on the drug discovery process. High-throughput genome-wide screening with short interfering RNA (siRNA) is one strategy for systematically exploring possible therapeutically relevant targets in GBM. Statistical methods and protein-protein interaction network databases can also be applied to the screening data to explore the genes and pathways that underlie the pathological basis and development of GBM. In this study, we highlight several genome-wide siRNA screens and implement these experimental concepts in the T98G GBM cell line to uncover the genes and pathways that regulate GBM cell death and survival. These studies will ultimately influence the development of a new avenue of neurosurgical therapy by placing the drug discovery process in the context of the entire biological system. (DOI: 10.3171/2009.10.FOCUS09210)

KEY WORDS • **RNAi** • **glioblastoma multiforme** •
short interfering RNA • **genome-wide screening** • **protein-protein interaction network**

GLIOMASTOMA multiforme is the most common form of primary human brain tumor,³⁰ and despite major advances in the management and treatment of these tumors, the prognosis remains dismal. Resistance of GBM to conventional chemotherapy and radiation therapy has necessitated a search for more effective therapies, which are beginning to encompass modern molecular biology and drug discovery techniques to identify and target the specific molecular genetic aberrations that underlie the pathogenesis of GBM.⁵⁸ However, the results of first-generation clinical trials with molecularly targeted agents have generally been disappointing, owing to tumor heterogeneity and an incomplete understanding of the interconnecting molecular pathways that promote and maintain tumor growth.

Therapeutic strategies that target genes and biological

pathways responsible for the development of tumors or potentiation of current therapies could improve patient outcomes. Accordingly, several genomic and proteomic methodologies promise to expand the current drug discovery process. High-throughput screening with siRNA is one strategy for systematically exploring the possible therapeutically relevant targets in cancers, such as GBM. Short interfering RNA are 20–25 nucleotide-long double-stranded RNA molecules that can selectively silence specific genes through sequence-specific mRNA transcript degradation.^{55,57} The availability of siRNA libraries and automated liquid handling platforms have spawned an evolution in genome-wide investigations of loss-of-function phenotypes.^{25,30,52,75,77} However, this genomics approach has not yet been implemented in neurooncology and will require an analysis of biological pathways as a central reference point to provide a global perspective on the development, function, and pathological basis of neurosurgical disease.

In this study, we describe the drug discovery process, considerations and applications of genome-wide siRNA screening, and the integration of high-content statistical

Abbreviations used in this paper: GBM = glioblastoma multiforme; IPA = Ingenuity Pathways Analysis; KEGG = Kyoto Encyclopedia of Genes and Genomes; MAD = median of the absolute deviation; RISC = RNA-induced silencing complex; RNAi = RNA interference; siRNA = short interfering RNA; TNF = tumor necrosis factor; V-ATPase = vacuolar type H⁺-ATPase.

FIG. 1. The mechanism of RNAi. Long double-stranded RNA molecules are cleaved by the RNase-III-like enzyme Dicer into siRNA molecules 20–25 base pairs long with 3' base pair overhangs. Synthesized siRNA molecules may be directly transfected into cells and do not undergo processing with Dicer. The antisense strand of this siRNA molecule is then incorporated in the RISC complex. This sequence binds to a complementary sequence on an mRNA, and an RNase within the RISC complex cleaves and destroys the mRNA by endonucleolytic cleavage, resulting in silencing of gene expression and reduction of protein levels (Kittler and Buchholz; Martinez et al.).

methods and protein-protein interaction network databases. We then highlight several high-throughput genome-wide siRNA screens in a spectrum of disease models and use these experimental concepts to implement a high-throughput siRNA screen in the T98G GBM cell line to uncover the genes and pathways that modulate GBM cell death and survival.

Drug Discovery and the Druggable Genome

The identification and validation of novel drug targets has remained difficult and costly, despite advances in molecular biology, molecularly targeted therapies, and high-throughput screening technologies. Techniques that target gene expression can facilitate the drug discovery process by replicating the potential effects of pharmacologically blocking a given protein, thereby providing insights for the drug development process. In recent years, siRNA has become a powerful tool for assessing the loss-of-function

FIG. 2. Commonly targeted gene families of the druggable genome. These gene families represent the genes that are targeted by RNAi screening libraries. This pie chart is approximately based on the Ambion Silencer Druggable Genome siRNA Library version 1.0. GO = gene ontology; GPCR = G-protein coupled receptor.

phenotype associated with protein knockdown within the cell. In the RNAi mechanism, gene expression is silenced through sequence-specific mRNA transcript degradation modulated by sequence complementarity within the RNA-induced silencing complex (RISC)⁴ (Fig. 1). Short interfering RNA technology has also facilitated multiple steps of the drug discovery process, which includes target identification, target validation, compound screening, lead optimization, and clinical applications.

Druggable Genome

The drug discovery process may be enhanced through an assessment of the genes and proteins that represent opportunities for therapeutic intervention.²⁴ Whole-genome sequencing has facilitated the functional annotation of a list of prospective drug targets, and “in silico” experimental molecular techniques have further allowed refinement of the list of molecules that can be targeted with drugs or drug-like molecules.^{24,56} Analysis of this so-called “druggable genome” provides a basis for tailoring drug discovery efforts to focus on a high-yield subset of genes, and RNAi libraries have been developed to consist of molecules that specifically target these druggable proteins⁴⁸ (Fig. 2). Given the high attrition rate with conventional drug development strategies, this approach promises to rapidly assess and prioritize the most therapeutically promising targets.

Implementing siRNA Screens

Genome-wide screening has significantly contributed to our understanding of biology; these studies have examined signaling pathways, disease-associated genes, and genes involved in viability, secretion, chromosome segregation, neuron development, and neuron outgrowth.^{11,18–20,26,36,38,40,42,45,60,65,67,69,70,74,75} Short interfering

TABLE 1: Summary of genome-wide RNAi screens in mammalian model systems

Authors & Year	Model	Summary
Whitehurst et al., 2007	non-small cell lung cancer	paclitaxel chemosensitivity
Giroux et al., 2006	pancreatic adenocarcinoma	spontaneous apoptosis & gemcitabine chemosensitivity
Morgan-Lappe et al., 2006	renal & pancreatic carcinoma	chemosensitivity to Akt inhibition
Tu et al., 2009	adipocytes	insulin signaling pathway constituents that modulate insulin resistance
Ganesan et al., 2008	melanocytes	genes & pathways that modulate melanogenesis
Tai et al., 2009	hepatitis C virus	cellular cofactors of hepatitis C virus replication
Gobeil et al., 2008	melanoma	identification of melanoma metastasis suppressor genes by shRNA screen
Leal et al., 2008	mouse embryonic fibroblast	downregulation of S-adenosylhomocysteine contributes to tumorigenesis
Tang et al., 2008	cervical & colorectal cancer	TCF transcription factors identified in Wnt pathway activation
Sepp et al., 2008	embryonic cerebral cortical neurons	identifying neural outgrowth genes
Loh et al., 2007	neuroblastoma	kinase cluster required for neurite outgrowth & retraction
Hu et al., 2009	mouse embryonic stem cells	transcriptional modules required for self-renewal
Turner et al., 2008	breast & cervical cancer	genes mediating sensitivity to PARP inhibition
MacKeigan et al., 2005	cervical cancer	survival kinases & phosphatases
Collins et al., 2006	ovarian carcinoma	MAP4K4 identified as a promigratory kinase
Westbrook et al., 2005	human mammary epithelial cells & colorectal cancer	identification of a previously unrecognized tumor suppressor REST
Luo et al., 2009	Ras mutant cells	identification of PLK1 & the proteasome as synthetic lethal targets in Ras mutant cells

RNA screening offers an unbiased, systematic strategy for uncovering the biological genes or pathways that underlie disease processes, allowing a deeper understanding of the poorly described molecular mechanisms governing various cellular processes or diseases.

The essential first step in the cancer drug discovery process is the identification of novel drug targets. Recent target identification has relied on characterization of genomic mutational spectra or proteomic expression signatures for correlation of target identification or tumor response to therapies.^{1,50} In this context, siRNA screening represents a complementary hypothesis-generating approach by defining the consequences of blocking a given target. The events that underlie tumorigenesis, tumor progression, and tumor response to conventional therapies each represent excellent targets for selective killing of cancer cells versus normal cells that may be delineated on an siRNA-based screen, although the potential applications of these approaches in neurooncology and neurosurgery may have relevance from both a therapeutic and mechanistic perspective.^{38,60} Recent siRNA screens in cancer and disease models have focused on: 1) reversing the cancer phenotype, 2) identifying synthetic lethal targets, 3) developing synergistic drug combinations, and 4) clarifying underlying mechanisms of biological processes (Table 1).

Reversing the Cancer Phenotype

The cancer phenotype is associated with several well-defined hallmarks, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, and genome instability.^{22,30} Notably, invasion and metastatic spread of cancer are complex biological processes that are directly

involved in the pathological process.¹¹ In an siRNA screen targeting 5234 human genes in an ovarian carcinoma cell line, the authors identified the potential therapeutic utility of targeting mitogen-activated protein kinase pathway in cancer progression.¹¹ This effect was also reproduced with a small-molecule inhibitor of c-Jun N-terminal kinase (JNK). In another genome-wide RNAi study, the authors described the role of *Growth arrest-specific 1* as a novel tumor suppressor gene that effectively suppressed melanoma metastasis.²⁰ Furthermore, another RNAi screen identified a novel tumor suppressor gene *REST/NRSF*, which is a transcriptional repressor of neuronal gene expression, in human mammary epithelial cells and observed its associated frequent deletion in colorectal cancer cells.⁷⁴ These studies demonstrate the ability of RNAi screening to identify novel targets that may represent cruxes of the cancer phenotype, and the identified proteins may represent nodes of chemosensitivity in various disease models.

Synthetic Lethality

Of utmost importance to clinicians is the development of novel combination therapies that can be swiftly translated into clinical application. Modern drug discovery aims to create novel drug combinations that will selectively kill cancer cells while leaving normal cells unharmed. However, this process has been difficult, owing to exploitation of normal enzyme functionality by oncogenes and the inability to pharmacologically target tumor-suppressor genes that have low or absent activity.²⁸ Synthetic lethality holds promise to evade some of these difficulties, and RNAi screening can be used to identify these synthetic lethal relationships using high-throughput technologies. Two genes are considered synthetic lethal if mutation of either is compatible with cell viability but

FIG. 3. Schematic diagram depicting abrogation of protein function in single, parallel, converging, and diverging pathways. **A:** Abrogation of enzymatic or protein function in a single, essential pathway can occur upstream or downstream in the pathway. **B:** Abrogation of 2 proteins simultaneously in a parallel pathway, a converging pathway, or a diverging pathway can be assessed with synthetic lethal or chemosensitizer screening assays.

mutation of both leads to cell death.³¹ Once this screening tool has identified such genes, anticancer therapies can be developed to target the molecular pathways. For instance, mutation of 2 essential components on 1 linear pathway, such as Proteins 1 and 3 in Fig. 3A, or mutation of 2 components of parallel, converging, or diverging pathways, such as Proteins 3 and 4 in Fig. 3B, may be synthetically lethal.

A well-known synthetic lethal relationship has been demonstrated by the inhibition of poly (ADP-ribose)-polymerase-1 (PARP1) in breast cancer cells deficient in *BRCA1*, *BRCA2*, or other components of the homologous recombination pathway, while normal cells remain unaffected.^{8,15,16,59,70} In normal cells, both the homologous recombination and base-excision repair pathways repair damaged DNA; PARP1 is an enzyme required for base-excision repair, which is a pathway that repairs single-strand breaks; *BRCA1* and *BRCA2*, which are tumor-suppressor genes, are required for DNA double-strand break repair by homologous recombination, and mutations in *BRCA1* and *BRCA2* predispose to breast and ovarian carcinomas. Loss of PARP1 increases DNA damage repair through the homologous recombination pathway; thus, abrogation of the homologous recombination pathway concomitant with base-excision repair pathway inhibition could lead to significant cell death. Indeed, recent studies have shown that *BRCA1* or *BRCA2* mutation or absence sensitizes cells to inhibition of the PARP1 enzyme,¹⁵ and patients with hereditary breast or ovarian cancers may be excellent candidates for treatment with PARP1 inhibitors.

This synthetically lethal relationship was deduced by leveraging current understanding of cell biology and the known molecular genetic alterations within cancer. However, additional synthetically lethal combinations may not be readily deduced through a rational mechanistic understanding of cancer cell biology, and an unbiased screening method is needed to systematically detect novel rela-

tionships. In a recent genome-wide RNAi study, Turner et al.⁷⁰ identified targets that modulated the sensitivity of breast cancer cells to the effects of PARP1 inhibition. Silencing of several kinases strongly sensitized PARP1 inhibition, and these targets included cyclin-dependent kinase 5 (CDK5), MAPK12, PLK3, PNKP, STK22c, and STK36. The presence of CDK5, which is required for DNA-damage checkpoint activation, suggests that normal checkpoint function may be essential for DNA repair when the PARP1 enzyme is inhibited.^{28,70} Genome-wide RNAi screens, therefore, offer a unique opportunity to implement a systematic, rapid, and unbiased method to uncover novel synthetic lethal relationships.

Synergistic Drug Combinations

Despite promising in vitro and in vivo data, intrinsic or acquired resistance to conventional therapies has been a major therapeutic obstacle. Tumor heterogeneity, redundancy and parallel processing of intracellular signaling pathways, inactivating metabolism, mutation within a specific targeted pathway, loss of negative inhibition, mutations leading to constitutive activation, and limited drug delivery are the most common resistance mechanisms.⁵¹ Given these therapeutic challenges and the multiple mutations leading to tumorigenesis, tumor cells will need to be targeted with several agents simultaneously to ensure a cure or long-term survival. Combination therapies that target multiple signaling pathways or different constituents in the same pathway (Fig. 3) may overcome resistance mechanisms and widen the therapeutic window, ultimately enhancing the effect on tumor cells without increasing toxicity for normal cells. However, therapeutic combinations are limitless, and a strategy is necessary to select only the most effective and synergistic of combinations.

Investigators have used RNAi screens to identify targets that chemosensitize cancer cells to conventional treatments or molecularly targeted therapies^{19,42,45,75} (Table

Fig. 4. Functional genomic analysis through genome-wide siRNA screening. This flow diagram depicts the chronology of events in the implementation and functional genomic analysis of a genome-wide siRNA screen. After statistical analysis with one or several methods, screening “hits” are uploaded to the web-based protein-protein interaction network IPA. Gene ontology enrichment is conducted with the Fisher exact test at $\alpha = 0.05$ by comparing the “hits” to a list of ontology categories. Pathway enrichment is similarly conducted by comparing “hits” to the KEGG list of pathways.

1). In the most well-defined of these studies, Whitehurst et al.⁷⁵ identified gene loci that chemosensitized non-small cell lung cancer cells to the microtubule stabilizer paclitaxel. As a proof of principle, the authors identified several proteasome components as chemosensitizing targets, and proteasome inhibitors have been shown to enhance paclitaxel-induced apoptosis in several cancers.^{41,49} The authors also reported that exposure to the V-ATPase inhibitor salicylhalamide A combined with low concentrations of paclitaxel achieved a synergistic decrease in cell viability. Such proof-of-principle synergism studies are also being applied to neurooncology models, which will be described below.

Mechanistic Clarification of Biological Processes

Systematic phenotyping by means of RNAi and other approaches will also provide novel perspectives on a gene’s or pathway’s function in the context of the genome. Function and development of the brain require the coordinated action of numerous genes, but we currently understand the functions of only a small fraction of them.⁶⁰ The integration of phenotypic information from genomic and proteomic data sets has revealed many important cellular processes at an unprecedented resolution.¹⁷ There are several such studies in neuroscience that have provided insight into the development and function of the nervous system, including neural outgrowth⁶⁰ and identification of kinase clusters that are required for neurite outgrowth and retraction³⁸ (Table 1).

Utilizing an RNAi screening approach, a recent study identified a pathogenic link between the endocytic pathway and neuronal dysfunction in synucleinopathies, such as Parkinson disease.³⁴ Other RNAi studies have focused on dissecting various cell processes, such as insulin signaling, melanogenesis, Wnt pathway signaling, and self-renewal (Table 1), and this screening strategy holds promise for the mechanistic clarification behind brain

development, function, and the pathological basis of diseases, such as GBM.

Analyzing RNAi Screens

With the large amount of data generated from siRNA screening, there is a clear need for data reduction methods, which would allow prioritization of targets and determination of the gene expression products most significantly affected by siRNAs in the disease model.⁹ Screening typically relies on sophisticated automation, appropriate controls, and state-of-the-art detection technologies to organize and analyze thousands of test samples.^{5,7} Unfortunately, siRNA screening is associated with “off-target” effects, which can affect the analysis and final results. Data output, therefore, requires sophisticated and rigorous analysis methods to reduce the number of high-confidence “hits.” Statistical analyses and the use of protein-protein interaction network databases are 2 such methods that can facilitate the data analysis process. An overview of several such data analysis methodologies is depicted in Fig. 4.

Statistical Analysis

Although a comprehensive review of statistical methods for high-throughput screening “hit” selection is beyond the scope of this review, we present the most popular methods with accompanying references for further review. As with any high-throughput methodology, the output varies due to: 1) systematic variation, or 2) unsystematic, random influences. Systematic effects that are not adjusted for can bias the final results of the screen, creating false-positive and false-negative results. The level of random “noise” can also similarly confound the results of a study and therefore also needs to be accounted for in the “hit” selection process.

Most high-throughput screens are conducted in 384-

well plate formats, and most well-designed experiments will include an in-plate positive control (a control that will yield a positive result; for example, cell death in a toxicity assay) and a negative control (a control that will yield a negative result; for example, minimal cell death in a toxicity assay). These controls are used to normalize the wells with targeting siRNAs. Although this methodology is frequently used and is the traditional way that biologists view changes in biological activity, controls-based methods have several potential problems. These include positional variability based on the location of the well on the plate; systematic biases among the controls; variability between control wells; and outliers due to measurement problems.^{7,12}

With these issues in mind, some investigators are utilizing non-controls-based normalization, such as normalizing to the median of all values on a plate. The median, unlike the mean, is not affected by outliers. Because it is the outliers on a plate that may be of greatest interest (that is, those wells that had the greatest amount of cancer cell death), this method would help distinguish outliers from the majority of the screening plate. Several popular analysis methods include Z-scores,⁷⁶ viability ratios and Benjamini-Hochberg correction,⁷⁵ median of the absolute deviation (MAD) method,^{10,23,29} other non-controls-based normalization methods,^{7,12} and orthogonal analysis methods based on a combination of these statistical methods.⁷⁵ We have uniquely developed and applied the MAD method for the detection and removal of outlier data points from high-throughput screening data and further describe our techniques below.

Protein-Protein Interaction Network Analysis

Despite many decades of experiments and many thousands of data points, the cellular and molecular functions of the cancer genome or proteome have not yet been systematized. Because of the complexity of the cellular network, biologists have preferred to consider parts of it by subdividing it into biological pathways that comprise sets of molecules involved in a particular function or process.² Pathways can therefore serve as a scaffold for assessing the impact of single molecules on the network of cellular proteins. For instance, proteins that connect to numerous molecules within interaction networks are more likely to produce a cytotoxic effect when deleted (knocked down), whereas proteins that may be part of parallel or redundant biological pathways are less likely to cause lethality when deleted (Fig. 3). Several commercially available protein-protein interaction network databases^{13,14,68} have focused on the pathways relevant to human disease, and these interaction analyses can be used in the target identification and validation phases of the drug discovery process.

Protein-protein interaction network analysis can uncover the underlying enriched (over-represented) gene functions and pathways that may not be readily apparent otherwise. Subsequent gene ontology and pathway enrichment analyses are then used to narrow down the list of “hits” to uncover biological functions that are most significantly affected by siRNAs²¹ (Fig. 4). To allow functional enrichment according to existing functional annotation systems, the Fisher exact test is adopted to mea-

sure gene enrichment of annotation classifications such as Gene Ontology terms^{21,63} or pathways from the KEGG database.³² For each annotation term, the Fisher exact probability describes the probability of sampling without replacement from a finite population consisting of 2 types of elements, and an analogous approach is implemented for the pathway enrichment of siRNA targets. Such an analysis can now be performed using open-access interaction network analyses.²⁷

Several studies have implemented this analysis on gene expression data and RNAi screening data.^{6,46,69,72} For instance, Bredel et al.⁶ identified 3 novel MYC-interacting genes in human gliomas through functional network analysis of gene expression data with Ingenuity Pathways Analysis (IPA); Mori et al.⁴⁶ reported that gene profiling and pathway analysis helped to elucidate the molecular mechanisms involved in neuroendocrine transdifferentiation of prostate cancer cells; and Tu et al.⁶⁹ used an siRNA screening approach to identify a reliable set of components/modulators of the insulin signaling pathway. Thus, functional genomic analyses that leverage multiple types of information have begun to show promise in uncovering important biology not apparent from standard analysis methods.

Druggable Genome-Wide siRNA Screening in GBM: Working Examples

Cancer cell survival depends on the balance of signaling through survival and apoptotic pathways.⁶⁶ An increase in survival signaling, through increased survival factors or decreased apoptotic signaling, could confer a proliferative advantage, which may ultimately enhance chemoresistance. Conversely, uncovering the genes or pathways that are most essential for cancer cell survival may enhance the drug discovery process by identifying promising drug targets. To identify the genes and pathways that represent these chemoresistance and chemosensitivity nodes in GBM, we conducted several druggable genome-wide siRNA screens to identify the gene nodes and pathways that modulate GBM cell death and survival.

Methods

We used a high-throughput siRNA screen with 16,560 siRNAs targeting 5520 unique human genes in the T98G GBM cell line. We selected the T98G cell line because it is a widely available, GBM-derived, human cell line, with a well-characterized radioresistance and chemoresistance profile,^{61,62,73} and it provides an in vitro surrogate for the identification and subsequent validation of novel therapeutic drug targets for GBM. We measured cell viability at 96 hours after siRNA transfection with a resazurin fluorescent dye assay and normalized the targeting siRNA wells to in-plate positive and negative controls. Viability ratios were calculated by normalizing cell viabilities to the overall median cell viability of all 5520 genes when averaged over the screening replicates.

For the siRNA-only screen, we selected the viability ratios that were 3 SDs above the median after statistical analysis with the MAD method (see *Statistical Analysis*

TABLE 2: Protective genes in the T98G GBM cell line

Gene Symbol	Gene Accession	Full Gene Name	Viability Ratio
TRPC4AP	NM_199368	transient receptor potential cation channel, subfamily C, member 4 associated protein	1.544873
BTN3A1	NM_194441	butyrophilin, subfamily 3, member A1	1.520764
SSTR2	NM_001050	somatostatin receptor 2	1.514676
RHOV	NM_133639	ras homolog gene family, member V	1.514118
PDPR	NM_017990	PDPR	1.508784
RARRES2	NM_002889	retinoic acid receptor responder (tazarotene induced) 2	1.498987
FLJ10858	NM_018248	nei endonuclease VIII-like 3 (E. coli)	1.497508
NR2F2	NM_021005	nuclear receptor subfamily 2, group F, member 2	1.493387
TAS2R39	NM_176881	taste receptor, type 2, member 39	1.49172
NR4A2	NM_173172	nuclear receptor subfamily 4, group A, member 2	1.491645
COX4I1	NM_001861	cytochrome c oxidase subunit IV isoform 1	1.488063
TPRA40	NM_016372	G protein-coupled receptor 175	1.485445
ADAM18	NM_014237	a disintegrin and metalloproteinase domain 18	1.483975
PGAM1	NM_002629	phosphoglycerate mutase 1 (brain)	1.480993
RHOBTB3	NM_014899	Rho-related BTB domain containing 3	1.477528
GNAL	NM_182978	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	1.469045

above) and classified this set of genes by shared molecular and biological functions using the protein analysis through evolutionary relationships classification system (PANTHER).⁴⁴ We then uploaded these lists of “hits” to a web-based application for analysis of biological functions, disease categories, toxicological categories, canonical signaling pathways, drug inhibitors, and pathway and gene ontology enrichment analysis.⁶⁸ The Fisher exact test was used with $\alpha = 0.05$ to calculate the probability that each function and pathway classification assigned to the set of survival genes was due to chance. This procedure is detailed in Fig. 4.

Results and Discussion

We identified 16 targeting siRNA reactions that resulted in a significant increase in cell viability (Table 2). Knockdown of these genes appear to enhance T98G GBM cell survival, suggesting that these genes may be functionally associated with cell death pathways. Interestingly, the products of these genes function as nuclear hormone receptors, GTPases, G-protein coupled receptors, oxidases, and mutases, while several genes were unclassified. Utilizing a knowledge-based interaction network, we found that these genes have been implicated in various biological processes including neurological disease, genetic disease, cellular movement, nervous system development and function, and cell signaling. Nine of 16 genes were reportedly overexpressed in primary or secondary glioma,⁵⁴ which provided further clinical evidence of the importance of these nodes in glioma cells. The most statistically significant protein-protein interaction network consisted of genes implicated in gene expression, cell death, and endocrine system disorders and was centered around beta-estradiol, TNF, and the NF- κ B complex (Fig. 5). Recent studies have described the role

of the NF- κ B pathway in resistance to TNF-mediated cell death in human glioma, and its role in inflammation, tumor growth, immunity, and an invasive phenotype.^{53,64} Thus, our results highlight several cellular factors and complexes that may be implicated in GBM cell death pathways.

In our recent work, we implemented an siRNA screen to uncover the core genes and pathways that are essential for GBM cell survival (that is, where siRNA-induced protein knockdown induced cell death).⁶⁸ Interestingly, several identified genes were components of the proteasome complex, suggesting that these components may be essential for cell survival or proteasome structure or function or may have the most rapid protein turnover. Further mechanistic validation has shown that disruption of these components may actually induce instability of the proteasome complex by accumulating intermediate forms, which could contribute to loss of cell viability. Furthermore, using the protein-protein interaction network database, we identified clusters of cellular processes that included protein ubiquitination, purine and pyrimidine metabolism, nucleotide excision repair, and NF- κ B signaling, among others. Overall, these findings regarding the significance of the proteasome complex in GBM cell survival represent an unpredicted observation that would not have been obtained without an siRNA-based screening approach.

However, because GBM is a notoriously heterogeneous tumor (with respect to cells from different anatomical regions of a patient’s tumor as well as between patients), identifying targets in a single cell line, such as the T98G GBM cell line, may not represent drug targets that are effective and consistent outside of this cell line. While our preliminary investigation with this single cell line is proof of principle for this screening approach, we

Fig. 5. Mapping of protective genes onto a protein-protein interaction network. Functional analysis of protective genes was performed with IPA. The genes are represented as *nodes*, and *edges* connecting 2 *nodes* represent a biological relationship that is supported by at least 1 published reference or the IPA knowledge base. *Shaded nodes* represent protective genes. This protein-protein interaction network consisted of genes implicated in gene expression, cell death, and endocrine system disorders and was centered around beta-estradiol, TNF, and the NF- κ B complex.

additionally applied this genetic tool to a panel of glioma cell lines and nonglioma cancer cell lines to determine if this approach yielded consistent groups of genomic targets within and between cancer cell lines despite the well-characterized molecular heterogeneity. For instance, the glioma cell lines T98G, U373, U87, LN-Z308, LN-Z428, and A172; breast adenocarcinoma cell line MCF7; and lung adenocarcinoma epithelial cell line A549 were transfected with PSMB4 siRNA, and cell viability was measured at 96 hours. The A549 and A172 cell lines were most sensitive, while LN-Z308 and LN-Z428 were most resistant to cell death. Growth inhibition for all cell lines was significantly different from that in control cells ($p < 0.05$).⁶⁸ We also reproduced these cytotoxic effects using the small-molecule proteasome inhibitor MG-132.⁶⁸ Despite the heterogeneity that exists between and within tumors, screening in a single cell line can yield generalizable results, although they must be ultimately corroborated with a focused secondary screen in other cell lines or primary tumor-derived cell lines. Overall, genome-wide screening in a range of cancer cell lines will continue to provide insight into the similarities and differences in the molecular mechanisms that regulate specific cellular processes.

These studies provide examples of the power and utility of systematic and unbiased functional genomic analysis tools for the identification of novel chemotherapeutic treatment strategies for GBM, and targeting these genes and pathways may provide promising avenues for drug development.

Conclusions and Future Directions

Recent advances in genomics, such as genome-wide sequencing and the discovery of RNAi, have enabled a detailed study of the integrative nature of cellular signaling and protein-protein interactions. Genome-wide siRNA screening can be used to systematically interrogate the loss-of-function phenotypes associated with protein knock-down and can provide insight into previously unknown gene or pathway functions. Until now, this functional genomics approach has not been applied to the study of neurooncological diseases, such as GBM. In this study, we highlighted several genome-wide siRNA screens conducted in various disease models (Table 1) and then implemented a high-throughput screen to test the protective and synergistic effects of the knockdown of 5520 druggable human genes in the T98G GBM cell line. These studies have yielded a global view of the genes that are implicated in GBM cell survival, chemoresistance, and chemosensitization to various chemotherapeutic agents.

Short interfering RNA screening can be used to develop novel avenues of neurosurgical and neurooncological therapies. The aim of proteomics, which can be described as the study of the role of each gene product in its cellular context, in drug discovery is to identify potential novel drug targets and to achieve a comprehensive description of complex molecular mechanisms.³ Once we have identified an siRNA molecule that confers a phenotype of interest (for example, when protein knockdown of gene X results in cell death), we can focus our efforts on

the development of a small-molecule inhibitor that can phenocopy the effect of the siRNA. Utilizing this new target for lead optimization, we can then streamline the development of novel monotherapies and combination therapies. Small interfering RNA screening can further impact neurosurgical treatment by identifying promising drug targets, uncovering side-effect profiles of novel and old therapies, allowing rapid assessment of promising drug targets, catalyzing swifter movement through the validation phase of the drug discovery process, identifying novel combination therapies through synthetic lethal and synergism screens, and furthering development of a systems biology understanding of the molecular mechanisms underlying neurological diseases and drug action.

Overall, these genetic studies can be directed toward the therapeutic targeting of essentially any cellular process. This screening approach is not only limited to cell viability assays, as have been described in this work, but can also be used to target various facets of tumor biology, such as tumor microenvironment, tumor invasion, factors that enhance growth in hypoxic conditions, and angiogenesis in a wide range of cell types. This screening tool can also be used to determine the effects of multimodal therapies such as chemo- and radiotherapies on cancer survival. As high-throughput screening technology improves, a more complex assay-end point can be used. For instance, instead of measuring cell viability at the end of the assay, we can measure modulation of angiogenesis using cell-culture and animal models,³⁹ cell invasion using migration assays,⁷¹ and microtubule destabilization using high-content confocal microscopy.⁴⁷ Through these studies, this genetic tool will provide further insight into the mechanisms behind cellular processes and gene functions.

On a systems biology level, gene-expression signatures can now be accurately compared, essentially independent of the platform on which they were generated.³⁵ The future of high-throughput siRNA screening technology will include integration with DNA microarrays, protein-protein interaction data, and tools like the ConnectivityMap³⁵ to provide molecular clarification of novel loss-of-function phenotypes in various cell-based systems. It is also possible that high-throughput proteomic profiling could be combined with siRNA and small-molecule experiments to further inform drug development. However, current cellular networks are incomplete, since only well-studied proteins and interactions are represented (that is, typical nuclear or cytoplasmic proteins). As cancer and disease models become more sophisticated and comprehensive, it will also become important to define standards for communicating genomic profiles across diverse experimental systems. The RNAi technology is allowing the rapid development and implementation of genome-wide screens for disease processes and functions³⁷ and will influence the development and understanding of new avenues of neurosurgical therapy.

This genome-wide screening strategy will also have multiple impacts on neurooncological treatments within the clinical setting. For instance, functional genomic profiling of a patient's tumor will enable a more individualized and targeted therapeutic design based on the molec-

ular genetic aberrations unique to the tumor's genome. As RNAi transfection technology in primary cells improves, this screening approach will be directly conducted in patient-derived tumor cells, which will in turn allow selection of single-agent or multiagent molecularly targeted therapies that target the biological weaknesses specific to the patient's tumor.⁵¹ Additionally, this screening strategy will be used to identify the most promising in vitro drug combinations for future clinical trials. By implementing an unbiased, systematic interrogation of the druggable genome, this screening approach will identify novel drug combinations that would not have been identified based on current mechanistic knowledge and will thereby create a list of high-confidence drug combinations for future clinical trials. This screening strategy will also facilitate patient stratification in clinical trials based on the functional significance of specific mutations. Based on these rapidly evolving future screening applications, this screening strategy will significantly impact the clinical approach to individualized genome-based therapies.

Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: NG Thaker, PR McDonald, JS Lazo, IF Pollack. Acquisition of data: NG Thaker, F Zhang. Analysis and interpretation of data: NG Thaker, F Zhang, TY Shun, JS Lazo, IF Pollack. Drafting the article: NG Thaker, IF Pollack. Critically revising the article: NG Thaker, F Zhang, JS Lazo, IF Pollack. Reviewed final version of manuscript and approved it for submission: NG Thaker, F Zhang, JS Lazo. Statistical analysis: TY Shun. Administrative/technical/material support: JS Lazo, IF Pollack. Study supervision: JS Lazo, Ian F Pollack.

This work was supported in part by National Institutes of Health Grants P01 NS40923, P01 CA78039, A168021 to Drs. Lazo and Pollack; and by the Doris Duke Charitable Foundation (N. G. Thaker, fellowship).

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Manuscript submitted September 12, 2009.

Accepted October 27, 2009.

Address correspondence to: Nikhil G. Thaker, B.S., 601 Riverside Avenue, Apartment 424, Lyndhurst, New Jersey, 07071. email: thakerng@umdnj.edu.