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THE EFFECT OF SILENCING THE WILMS’ TUMOR 1 GENE ON THE RADIATION SENSITIVITY OF GLIOBLASTOMA CELLS

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THE EFFECT OF SILENCING THE WILMS' TUMOR 1 GENE ON THE RADIATION SENSITIVITY OF GLIOBLASTOMA CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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ABSTRACT

THE EFFECT OF SILENCING THE WILMS' TUMOR 1 GENE ON THE RADIATION SENSITIVITY OF GLIOBLASTOMA CELLS

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Glioblastomas are among the most devastating of human cancers with a median survival of only 9-12 months. This type of brain tumor is incurable, largely due its remarkable proliferative capacity and resistance to current treatments. High levels of the Wilms' Tumor 1 (WT1) gene have been identified in glioblastomas, suggesting an oncogenic function. Moreover, known WT1 target genes have been implicated in resistance to radiation. To determine the role of WT1 in radiation resistance, two glioblastoma cell lines expressing WT1 were treated with siRNAs to silence this gene. Confirmation of WT1 knockdown was achieved through real-time PCR and Western blot. After treatment with siRNA, cells were irradiated, and cell survival was assessed using a luminescent ATP assay and clonogenic survival assay. We demonstrate that treatment with WT1 siRNA increased the radiation sensitivity in both cell lines. These findings suggest that WT1 functions to protect glioblastoma cells from radiation-induced death.
CHAPTER 1

INTRODUCTION
1.1 Brain Tumors

1.1.1 Overview of Brain Tumors

The term primary brain tumor refers to a cluster of abnormally growing cells that originate from normal cells in the central nervous system (CNS), including both intracranial tissue and meninges. It is thought that these tumors occur as a result of genetic mutations which allow these cells to grow rapidly and invade surrounding tissue. Although these tumors very rarely metastasize outside the CNS, they are able to evade normal regulatory growth mechanisms and can cause severe damage. Patients with brain tumors typically present with four main types of symptoms\(^1\), the first being partial or generalized seizures. Second, the bulk of the tumor results in raised intracranial pressure and leakage of the blood-tumor and blood-brain barrier, causing vasogenic edema. This increased pressure can result in symptoms of headaches, nausea, vomiting, drowsiness, and visual abnormalities. Third, neurological deficits manifest depending on the location of the tumor. Lastly, patients may have cognitive dysfunction that varies with tumor type and grade. While the cause of brain tumors has yet to be identified, it is known that genetic inheritance occurs infrequently. Environmental factors such as the use of cellular phones, diet, and tobacco intake are currently being investigated as potential neurocarcinogens, although none have demonstrated strong associations with brain tumor incidence\(^2\).

Primary malignant and benign CNS tumors combined occur at a rate of 14.8 cases per 100,000 person-years\(^3\); that is, for every 100,000 people in one
year, 14.8 individuals will be diagnosed with a CNS tumor. Malignant brain
tumors are among the deadliest of cancers and account for approximately 2% of
all cancers in adults\(^2\). These tumors occur at a rate of 6.4 cases per 100,000
person-years\(^3\) and affect more men than women. Gliomas are brain tumors that
originate from glial cells which normally provide support and nutrients to neurons
in the CNS; these include astrocytes and oligodendrocytes. Furthermore,
gliomas constitute 40% of all CNS tumors and 78-86% of all malignant brain
tumors\(^1\)\(^2\)\(^2\). Thus, the focus of this paper will be on gliomas, specifically
glioblastoma multiforme (GBM), which accounts for over half of gliomas.
Unfortunately, many gliomas are incurable and patient survival is poor in
comparison to other cancers; prognostic factors include age, histologic subtype,
tumor grade, and presenting symptoms\(^1\)\(^2\)\(^4\). In addition, the Karnofsky
Performance Scale (0-100) rates patients' overall functioning ability; generally, a
higher score indicates a better prognosis.

The majority of gliomas are supratentorial; that is, they are commonly
located in the frontal, temporal, parietal, and occipital lobes of the brain\(^1\)\(^2\). As a
result, patients present with symptoms such as motor or sensory deficits,
hemianopsia, aphasia, or a combination of these\(^1\). In the United States, gliomas
occur at a rate of 5.9 per 100,000 person-years\(^3\) and can be further divided into
different categories. The World Health Organization (WHO) classification and
grading system is the most widely used scheme to categorize gliomas and is
based on the cell type the glioma was derived from. The three main types of
gliomas are astrocytomas (of astrocytic origin), oligodendrogliomas (of
oligodendrocytic origin), and mixed oligoastrocytomas which display features of both astrocytes and oligodendrocytes. Each of these types of gliomas can be distinguished by histological examination and biological markers. WHO grading is dependent on four main features: 1) nuclear atypia, 2) mitoses, 3) microvascular proliferation, and 4) necrosis\(^1\). Astrocytomas are graded on a scale of I-IV in order of increasing malignancy; these include pilocytic astrocytomas (WHO grade I), diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III), and glioblastoma multiforme (WHO grade IV). Oligodendrogliomas and mixed oligoastrocytomas only incorporate two grades: low grade (WHO grade II) and anaplastic (WHO grade III). It is believed that highly malignant (WHO grade IV) variants of the latter two types of tumor do occur, but are indistinguishable from the astrocytic variant of glioblastoma.

WHO grades I and II are considered low grade gliomas; the median survival of these patients ranges from 12-128 months. These tumors mostly affect young adults and are commonly located in the frontal, temporal, and insular lobes of the brain. Up to 80% of patients with a grade I or II glioma present clinically with partial or generalized seizures\(^1\); fortunately, magnetic resonance imaging (MRI) allows clinicians to diagnose and manage this disease in its early stages more often. While grade I gliomas can be surgically curable if completely resected, the majority of low grade tumors are grade II and will eventually undergo malignant transformation. WHO grades III and IV gliomas are considered high grade or malignant; these include anaplastic astrocytoma (grade III) and glioblastoma (grade IV), which unfortunately are the most
common types of glioma. The median survival of patients with anaplastic astrocytoma is 2-3 years, while the median survival of patients with glioblastoma is 9-12 months, despite the most aggressive treatment efforts\textsuperscript{1,5,6}.

Of the 18,000 patients diagnosed each year with malignant primary brain tumors in the US, over half are diagnosed with a GBM\textsuperscript{3,6}, which occurs four times more frequently that anaplastic astrocytoma. Because GBMs are the most common malignant primary brain tumor in adults and are the most difficult to treat, the remainder of this paper will focus on this particular type of glioma. Glioblastomas are among the most devastating of human cancers; they are highly aggressive, invasive, and cause extensive neurological damage. The mean age at onset is about 53 years of age\textsuperscript{3}, and regardless of maximum therapy, the majority of patients die within two years of diagnosis\textsuperscript{6}.

Glioblastoma is clinically characterized by rapid proliferation, diffuse invasion, angiogenesis, and cellular necrosis\textsuperscript{5}. There are two pathways that lead to GBM\textsuperscript{1,5,6}; a primary, or de novo GBM is typically observed in older patients without any evidence of prior clinical disease, whereas a secondary GBM usually presents in younger patients with evidence or prior history of a low grade astrocytoma. Within 5-10 years of initial diagnosis, the low grade astrocytoma usually transforms into a GBM, despite prior therapy. Although primary and secondary GBMs are clinically indistinguishable, each pathway most likely involves a different multistep pathogenesis with several genetic alterations. For example, primary GBMs exhibit amplification and overexpression of the epidermal growth factor receptor (EGFR)\textsuperscript{7}, loss of chromosome 10, amplification
and overexpression of the murine double minute 2 (MDM2) gene, and deletion or mutation of the phosphatase and tensin homolog from chromosome 10 (PTEN)\textsuperscript{1;5;6;8}. In contrast, secondary GBMs are associated with inactivation of p53 and overexpression of platelet derived growth factor (PDGF) receptors and ligands\textsuperscript{1;5-8}.

### 1.1.2 Treatment

At present, glioblastomas are unfortunately incurable and progression is inevitable despite treatment. Therefore, the goal of most treatments used today are to alleviate symptoms, increase survival time, and attempt to maintain the best possible quality of life for patients. The current standard therapy for GBM includes surgery where possible, radiation therapy, and adjuvant chemotherapy, each of which will be discussed in the following section.

**Surgical:** Because recurrence occurs within 2-3cm of the original tumor in up to 80% of patients\textsuperscript{1}, surgery aims to achieve maximum resection of the tumor to alleviate symptoms, as previously discussed, and to determine the histological type of the tumor, while preserving the greatest amount of normal tissue. Furthermore, since many tumors cause death through raised intracranial pressure, surgical resection may delay this occurrence. Several studies have shown that a more extensive resection is associated with longer survival for patients, and several advances have been made to maximize the extent of resection\textsuperscript{4}. The first is image-guided surgery, which incorporates preoperative imaging data to target the location and extent of resection; these images, however, cannot account for movements of the brain during the operation and
therefore cannot accurately detect residual tumor at the end of the procedure. Intraoperative imaging involving CT, ultrasound, and MRI can help correct for this and may improve accuracy, although these techniques are expensive and limited. Awake craniotomy is a third approach that is used to maximize the resection margin by monitoring the patient’s neurological function while removing the tumor; this is done when the tumor is located in eloquent areas such as the motor strip and speech areas. Lastly, fluorescence-guided surgery can maximize the resection margin as well. A phase III randomized trial was recently completed by a group in Germany, who reported that the use of this technique allowed for a higher rate of complete resection in addition to a higher 6 month progression-free survival in comparison to the control group\(^9\). In this procedure, patients are preoperatively injected with a compound that is taken up by glioma cells and converted into a fluorescing substance; this substance can then be visualized by an operating microscope that is modified to incorporate the appropriate illumination.

**Radiation therapy:** Several studies have shown that radiation therapy significantly improves survival time\(^1\). As part of standard treatment for GBMs, adjuvant radiation therapy is given to a localized and clearly defined treatment field. It is generally administered in a fractionated fashion; that is, a 1.8-2Gy dose is administered repeatedly over a period of about 6 weeks to deliver a total radiation dose of 58-60Gy. Another form of radiation therapy is brachytherapy, in which a radiation source is implanted into the tumor bed at the time of resection to deliver a high dose to the resection margin with minimal exposure to distant
sites. Although various sources have been investigated, the most commonly used radioactive substance is Iodine-125. While the addition of radiation therapy to surgery significantly increases average survival in these patients, long-term survival remains extremely rare because glioblastomas are relatively resistant to radiation.

**Medical:** The role of adjuvant chemotherapy for the treatment of GBM is highly debatable. Over 30 years of clinical trials have investigated the efficacy and benefit of chemotherapy in the treatment of malignant gliomas. Unfortunately, the results have been rather disappointing. The most investigated agents are nitrosoureas, but their efficacy is quite controversial. While some studies report a significant increase in 18-month survival, other studies report that adjuvant chemotherapy with nitrosoureas have no effect on survival. Lonardi, et al summarized past clinical trials that have investigated various compounds in adjuvant chemotherapy; the most significant increase in survival was seen in patients who were treated with both temozolomide and radiation therapy. This phase III randomized trial conducted by the European Organization for Research and Treatment of Cancer reported that patients newly diagnosed with GBM demonstrated significantly increased 2-year survival when treated with temozolomide and radiation therapy versus radiation therapy alone; median progression-free survival increased from 5 to 6.9 months, while median overall survival increased from 12.1 to 14.6 months. Another clinical trial demonstrated the promise of using carmustine (a nitrosourea) wafers implanted into the tumor bed at the time of resection; unfortunately, the wafers became
unavailable and completion of the study could not be accomplished\textsuperscript{12}. Despite these encouraging advancements, the fact remains that long-term outcome is still relatively unaffected and resistance to chemotherapy is very common.

**Genetic and Molecular:** Because glioblastomas are resistant to both radiation therapy and chemotherapy, the biology of these tumors is becoming increasingly important. An understanding of the mechanisms by which these tumors proliferate or evade current treatment strategies is essential to either block their growth or render them susceptible to adjuvant therapy. Thus, newer strategies are under investigation, which aim to target the molecular and genetic features of GBMs that aid their growth. For example, studies are being conducted to assess the effects of inhibiting O6-methylguanine-DNA-methyltransferase, the enzyme responsible for resistance to chemotherapy with DNA alkylating agents such as carmustine and temozolomide. Additional studies are looking at the inhibition of signaling pathways responsible for progression through the cell cycle, stimulation of apoptosis, and inhibition of angiogenesis to block the blood supply source to the tumor.

More specifically, small molecule inhibitors that block tyrosine kinase receptors, such as EGFR and PDGFR, are being tested for their efficacy in improving patient survival\textsuperscript{6,13}; no significant results have currently been reported. It has been shown that an overactive EGFR pathway is associated with resistance to radiation therapy\textsuperscript{14-16}, so the use of these inhibitors in conjunction with radiation therapy or chemotherapy may increase the effectiveness of current treatments. In fact, recent clinical trials have shown that combined use of the
EGFR antibody and radiation therapy improved tumor control and survival in patients with head and neck cancer versus radiation therapy alone\textsuperscript{17,18}. In addition, the EGFR antibody in combination with both radiation therapy and temozolomide to treat glioblastomas is currently in a Phase I/II trial in Germany\textsuperscript{19}. More downstream targets are also under investigation, such as the PI3K/AKT pathway which is activated by EGFR and PDGFR. The PI3K/AKT pathway has recently been associated with greater resistance to radiation therapy in gliomas\textsuperscript{20}. The PTEN gene product normally inhibits this pathway, but as previously mentioned, PTEN is either deleted or mutated in 40-50% of GBMs. Thus, current clinical trials are also studying the efficacy of inhibiting the PI3K/AKT pathway\textsuperscript{8}. Hence, a better understanding of the molecular characteristics of glioblastomas is very important to develop advancements in therapeutic regimens. It is to be hoped that these targeted therapies in combination with current standard care will improve patient management and may eventually lead to a cure for this deadly disease.

\textbf{1.1.3 Molecular Biology of Brain Tumors Pertaining to Radiation}

One of the main reasons radiation therapy cannot cure glioblastomas is because it is impossible to deliver a high enough dose to the treatment field that will eradicate all clonogenic tumor cells without significantly affecting the surrounding normal tissue. It is currently believed that factors which affect radiation sensitivity include distribution of cells throughout the cell cycle, the number of dividing cells, the number of cells with clonogenic capacity to reestablish uncontrolled growth, the ability of cells to repair radiation-induced
DNA damage, and the ability of cells to undergo programmed cell death when necessary. These factors will be explored in the following section with a particular emphasis on how tumor cells are thought to evade destruction.

**Cell Cycle:** The cell cycle is a determined set of events that culminates in the division of one cell into two identical daughter cells. There are 4 phases of the cell cycle: 1) the G1 phase is a period of growth 2) DNA synthesis occurs in the S phase 3) the G2 phase is when cells continue to grow and prepare for cellular division 4) the M phase is the period in which cells undergo mitosis. Cells can also exit the G1 phase and enter G0, the quiescent or resting phase; non-dividing cells remain in the G0 phase and do not progress through other phases of the cell cycle. Between each phase, there are checkpoints that serve to regulate proper progression into the next phase to ensure the production of two healthy daughter cells.

The G1 checkpoint ensures that cells are the proper size and have accurate copies of DNA before they can enter the S phase; tumor cells with a defective G1 checkpoint are more resistant to radiation compared to normal cells\(^\text{21}\). The S phase checkpoint ensures that DNA has been replicated; radiation inhibits both the initiation and elongation stages of DNA replication\(^\text{21}\). The G2 checkpoint also ensures that DNA has been replicated accurately before cells are allowed to enter the mitosis phase. The DNA damage checkpoint detects damaged DNA and causes cells to arrest in G1, slow down progression in S, and arrest in G2; it also induces the transcription of repair genes to mend the damaged DNA\(^\text{21}\). Progression from G1 to S and G2 to M are mediated by a
group of enzymes called cyclin-dependent kinases (CDKs); these enzymes are activated by cyclins, whose expression is highly regulated within the cell. Radiation induces nuclear DNA damage, resulting in single- and double-strand DNA breaks\textsuperscript{21-23}. This causes cells to arrest in G1, S, and more commonly, in the G2 phase\textsuperscript{24,25}. Cells in the M phase or in the G1 to S phase transition are reported to be more sensitive to radiation\textsuperscript{24}. When a cell sustains damage due to ionizing radiation, it will enter one (or possibly both) of two pathways: survival and repair, or apoptosis. Commitment to either pathway depends on the extent of damage, as well as genetic and environmental factors. Minor or moderate DNA damage will induce cell cycle arrest, followed by repair and resumption of proliferation. Major DNA damage will also induce cell cycle arrest and an attempt to repair damages; if repair is successful, the cell will resume proliferation, but if not, the cell will undergo delayed apoptosis. Irreparable DNA damage will cause the cell to undergo early apoptosis.

**Repair mechanisms:** When a cell incurs DNA damage, particularly a double-strand break, the entire genome becomes compromised. If the proper repairs are not made, all the genes distal to the break will be lost as the cell divides, which can lead to cell death. Furthermore, if the repair is not accurate and cells continue to proliferate, genetic mutations can be introduced which may cause severe damage to the organ and its surrounding tissue. DNA damage recognition and repair pathways have therefore evolved to prevent such consequences. These mechanisms are responsible for ensuring that damaged
cells arrest growth at any of the cell cycle checkpoints until repair is efficiently and accurately completed.

It is currently believed that at least three pathways exist to repair double-strand breaks\textsuperscript{26}. The first pathway is based on homologous recombination with an intact copy of the damaged sequence being either the homologous chromosome or a sister chromatid\textsuperscript{27}. This strand serves as a template for synthesis of a new complement strand, and is highly accurate in most cases\textsuperscript{28}. The other two pathways incorporate nonhomologous end-joining (NHEJ), in which there is no intact homologous copy to serve as a template; each of these pathways is mediated by a different complex of proteins. The predominant NHEJ pathway is probably the Ku-dependent pathway which joins the ends of partially complementary 3' overhangs, followed by polymerization of missing sequences\textsuperscript{26}. The other NHEJ pathway involves the separation of ends by a helicase or resection by an exonuclease to expose short homologous DNA sequences called microhomologies. These microhomologies then anneal and the resulting double-strand DNA is trimmed and ligated to form the final product\textsuperscript{26}. NHEJ is the most common double-strand break repair mechanism in mammalian cells; unfortunately, the lack of an intact copy also makes it more error-prone\textsuperscript{26}. While defects in double-strand break repair machinery can promote the likelihood of malignant transformation, it can also be used in targeted therapies for cancers; if repair of the DNA can somehow be prevented altogether, the cell will be forced to undergo apoptosis, thus enhancing the effects of radiation and/or chemotherapy.
**Apoptosis:** Current knowledge in cell biology suggests that there are 3 mechanisms of cell death; the first is necrosis, a disordered event that results in inflammation of the surrounding tissue. The second is autophagy, in which cellular components are sequestered and delivered to the lysosome for degradation. The third form is apoptosis, a highly ordered and evolutionarily conserved mechanism, in which cells undergo programmed cell death when damages incurred by the cell are irreparable. Apoptosis is the major mechanism by which radiation kills cells\(^{25,29}\); in respects to radiation, two types of apoptosis have recently been termed. In premitotic apoptosis, cells commit suicide before reaching the mitotic phase; this type of apoptosis is usually induced by high dose radiation, mainly occurs in the S phase, and involves rapid or early apoptosis\(^{30}\). In postmitotic apoptosis, cells commit suicide after having gone through one or a few cycles of mitosis; it is usually induced by low dose radiation, occurs mostly in the G1 phase, and involves delayed apoptosis\(^{30}\). Postmitotic apoptosis is also characterized by the loss of clonogenic cell survival, and death most likely results from reaching a critical level of genomic instability.

Apoptotic cells generally display distinct morphological and biochemical features such as DNA fragmentation, chromatin condensation, membrane blebbing, and cytoplasm shrinkage. The culmination of these events results in the formation of apoptotic bodies that are phagocytosed by the appropriate cells to prevent inflammation and damage to surrounding tissue\(^{29}\). Apoptotic events are mediated by a family of cysteine proteases called caspases that are synthesized as procaspases in the cell; an apoptotic signal will trigger the
conversion of these zymogens into active caspases that execute cellular suicide. There are two major apoptotic pathways\textsuperscript{29,31-33}, one of which is mitochondria-initiated and occurs through caspase-9\textsuperscript{31,34,35}. DNA damage triggers the release of cytochrome c from the mitochondria; this molecule then interacts with Apaf1 to form a complex that binds to and activates caspase-9. Caspase-9 then activates downstream targets to execute cell death. The release of cytochrome c is regulated by anti-apoptotic and pro-apoptotic members of the Bcl-2 family of proteins. The other pathway is death-receptor-mediated and requires caspase-8\textsuperscript{31,34,35}. A ligand such as Fas or Tumor Necrosis Factor α (TNFα) binds to the death receptor, inducing a conformational change that recruits another molecule, Fas Associated Death Domain (FADD). FADD then binds to and cleaves procaspase-8 into an active enzyme that will activate downstream targets to achieve cell death.

The p53 protein is a major mediator of apoptosis, although programmed cell death can occur either in a p53-dependent or p53-independent fashion. Recent evidence suggests that radiation induces activation of caspase-8 in glioma cells lacking functional p53, mediating a p53-independent apoptosis pathway\textsuperscript{31}. Apoptosis is also mediated by the molecule ceramide\textsuperscript{22,36}; exposure to radiation induces the enzyme sphingomyelinase to hydrolyze plasma membrane-derived sphingomyelin, producing ceramide. This will also lead to apoptosis, although the exact pathway has yet to be elucidated.

**Glioblastoma and radiation:** Despite all these complex intrinsic mechanisms, glioblastoma cells still remain resistant to radiation therapy. P53
does mediate growth arrest in radiated tumor cells; however, small populations of clonogenic cells are able to re-establish uncontrolled growth and lead to tumor recurrence\textsuperscript{29,31}. Not surprisingly, molecules that regulate apoptosis or cell cycle progression and proliferation are thought to play a role in radiation sensitivity. For example, the molecule survivin has been proposed as a key factor in protecting glioblastoma cells from radiation\textsuperscript{29,31,37,38}; this molecule is a member of the family of inhibitor of apoptosis proteins and is involved in modulation of apoptosis as well as regulation of cell growth\textsuperscript{39,40}. Survivin suppresses caspase-mediated apoptosis by directly binding to and inhibiting caspase-9\textsuperscript{40}. Furthermore, increased levels of survivin have been correlated with a worse prognosis and decreased survival times in patients with glioblastoma, in addition to reduced apoptotic ability of the tumors\textsuperscript{41}. Other studies demonstrate that survivin plays a role in cell division to control both microtubule stability and assembly of normal mitotic spindle, which may facilitate evasion from cell cycle checkpoints\textsuperscript{42}.

Bcl-2 family members have also been shown to play a role in radiation resistance in glioblastoma. Glioma cell lines with lower BAX expression (a pro-apoptotic member) were more resistant to radiation than those with higher expression; conversely, glioma cell lines with increased Bcl-X expression (an anti-apoptotic member) were also more resistant to radiation than those with lower expression\textsuperscript{43}. Other groups are investigating the role of vascular endothelial growth factor (VEGF), which has been demonstrated to increase after radiation in glioblastoma cell lines\textsuperscript{44}; this may implicate a role for angiogenesis in
resistance to radiation. And, as previously mentioned, various studies have demonstrated that overexpression of EGFR or overly active EGFR pathways are also associated with radiation resistance\textsuperscript{14-16}.

1.2 Wilms’ Tumor 1

1.2.1 Overview and Characterization

Wilms’ tumor, also commonly known as nephroblastoma, is a malignant pediatric kidney tumor that was first described by Max Wilms in 1899. 1 in 10,000 children are affected by this tumor, and it accounts for 7.5\% of all childhood tumors\textsuperscript{45}. The observation of karyotype abnormalities in predisposed children and molecular genetic examinations of Wilms’ tumor specimens led to the identification of the Wilms’ tumor 1 gene (WT1)\textsuperscript{46}, located at chromosome locus 11p13; it spans about 50kbp, contains 10 exons, and produces mRNAs of about 3kbp\textsuperscript{47,48}. Originally classified as a tumor suppressor, extensive research has demonstrated that this protein is highly complex with a number of possible functions and is associated with over 20 target genes, a few of which will be discussed further. Numerous studies have implicated that the WT1 protein functions in a variety of cellular processes including proliferation, differentiation, and apoptosis\textsuperscript{45,49}.

The N-terminus is rich in proline and glutamine, suggesting functions as a transcription factor, while the C terminus contains 4 Cys2-His2 zinc fingers\textsuperscript{47}. The zinc fingers are thought to be a possible DNA binding domain since it shares remarkable homology with the early growth response 1 (EGR-1) transcription factor. Exons 5 and 9 of the message are alternatively spliced, which result in 4
different isoforms\textsuperscript{48,50}. Exon 5 consists of a stretch of 17 amino acids and is located between the N-terminus and the zinc fingers. Exon 9 incorporates the three amino acids, lysine, threonine, and serine (KTS) between the third and fourth zinc fingers. The (+/+) isoform includes both exons, while the (-/-) isoform lacks both exons. The (-/+), isoform lacks exon 5, but includes exon 9, and conversely the (+/-) isoform includes exon 5, but lacks exon 9. WT1 (+/+) is the most prevalent isoform in humans and the (-/-) isoform is the least common\textsuperscript{50}. The WT1 proteins have molecular masses of 52 and 54kD; the (-/-) isoform is 52kD, and the (+/+), is 54kD\textsuperscript{51}. Additional mRNA editing produces a total of eight different mRNA isoforms\textsuperscript{52}, while differences in translation initiation produce 24 different protein isoforms\textsuperscript{53,54}. Thus begins the enormous complexity of this molecule, suggesting a large potential of different functions.

1.2.2 Potential Functions

\textbf{Regulator of transcription:} A number of studies have been conducted to study the role of WT1 as a possible transcription factor. Results have shown that WT1 can either activate or repress a number of genes, but that its transcription-regulating activity depends on the choice of cell system, expression vector, or the topology of the reporter construct\textsuperscript{49}. For example, it has been demonstrated that the WT1 (-/+), isoform functions to suppress the p-153 PDGF promoter, whereas it activates the shorter p-60 PDGF promoter\textsuperscript{55}. It is currently unknown what role the splice variants play in regulation of transcription, but recent data suggest that the splice variants may differentially regulate the same target genes. The WT1 proteins that both include exon 9 (+KTS) and lack exon 9
(-KTS) have been shown to bind overlapping DNA sequences in the promoters of the IGF-II gene, as well as the genes encoding the PDGF-A chain, Wilms' tumor, and PAX-2. Furthermore, it has also been observed that the WT1 (+/-) isoform suppresses transcription of a modified PDGF-A promoter construct while the WT1 (-/-) isoform activates its transcription.

Post-transcriptional regulation: There is also evidence to suggest that WT1 is involved in the post-transcriptional processing of RNA. An evolutionarily conserved RNA recognition motif in the N-terminus of all WT1 isoforms has been identified; Caricasole et al. showed that the zinc fingers of both the (+KTS) and (-KTS) proteins are involved in binding to exon 2 of the IGF-II transcript. In addition, Larsson et al. used nuclear staining to demonstrate that the (+KTS) proteins localize in the nucleus in a "speckled" pattern similar to that of clusters of interchromatin granules that contain components of spliceosomes. Furthermore, Davies et al. showed that WT1 not only associates directly with the splicing protein U2AF65, but that it can also incorporate into spliceosomes in an in vitro splicing assay; these data suggest a potential role of WT1 in splicing events. Thus, WT1 can exert powerful effects in its ability to regulate gene expression at various different levels.

Influence on apoptosis: WT1 has been shown to be capable of both inducing and inhibiting apoptosis. For example, expression of the 4 splice variants have been revealed to induce apoptosis in 2 osteosarcoma cell lines, while the (-KTS) isoform has been demonstrated to induce apoptosis in 2 hepatoma cell lines. In contrast, WT1 can bind to the p53 protein through its
zinc fingers and effectively suppress p53-mediated apoptosis without affecting p53-mediated cell cycle arrest. Moreover, WT1 has been shown to transcriptionally activate the Bcl-2 gene which encodes an anti-apoptotic protein; chemotherapy agents that exert their effects by inducing apoptosis are rendered ineffective in these cells expressing WT1 and consequent upregulation of Bcl-2. While this paradoxical effect may seem baffling, such results have been used to support the concept that the activity of WT1 depends on cell type, expression vector, and topology of the reporter construct.

**Tumor suppressor or oncogene:** As previously mentioned, WT1 was originally classified as a tumor suppressor; it is now currently debated whether WT1 acts as a tumor suppressor or as an oncogene. Unlike most tumor suppressors, WT1 is not ubiquitously expressed throughout the body, nor is it commonly mutated in various cancers. In fact, a homozygous WT1 mutation is found in only 5-10% of Wilms' tumors. Furthermore, WT1 has been found to be overexpressed in a myriad of tumors including lung cancers, colorectal adenocarcinoma, thyroid cancers, pancreatic ductal adenocarcinoma, leukemias, and breast cancer, to name a few. Additionally, high levels of WT1 have been correlated with a worse prognosis in patients with breast cancer and acute leukemia. These data, in accordance with its ability to suppress apoptosis, argue that WT1 functions more as an oncogene than as a tumor suppressor.
1.2.3 Role in Brain Tumor Biology

In addition to the cancers mentioned above, the expression of WT1 has also been demonstrated in gliomas; using RT-PCR, Dennis et al identified 2/5 low grade gliomas, 2/7 anaplastic astrocytomas, and 7/9 glioblastomas\textsuperscript{79} to express WT1 with no mutation of the zinc fingers. Another group used immunohistochemical staining to demonstrate that 48/51 glioblastoma samples were positive for WT1 protein, and that high levels of WT1 mRNA were also expressed in the GBM samples\textsuperscript{80}. Moreover, WT1 was found to be expressed in 5/6 low grade astrocytic tumors and 18/18 high grade gliomas, and WT1 expression in the high grade gliomas was significantly elevated in comparison to expression in low grade specimens; WT1 protein was not detected in normal glial cells contained in these tumor specimens\textsuperscript{81}. This same group also tested for mutations in the glioblastoma samples, none of which were found. Additionally, this group treated glioblastoma cell lines with antisense WT1 oligomers, which significantly inhibited their growth in comparison to the control group treated with random oligomers, although confirmation of WT1 knockdown was not made. Our laboratory has also identified WT1 mRNA expression in 5/9 GBM cell lines, 13/16 low grade gliomas, and 44/50 GBM specimens. These data suggest that WT1 acts as an oncogene in GBM, although its exact role is still unknown.
1.2.4 WT1 and Radiation Sensitivity

Among the multitude of WT1 target genes is EGFR. While it activates EGFR in the PC12 rat pheochromocytoma cell line\textsuperscript{82}, it also represses EGFR in osteosarcoma cell lines to induce apoptosis\textsuperscript{63}; this paradox, however, may be attributed to cell-type dependent functions. Nonetheless, since EGFR is found to be overexpressed in glioblastoma\textsuperscript{7} and overexpression of EGFR has been associated with the resistance of glioblastoma cells to radiation\textsuperscript{14-16}, it is possible that WT1 may be responsible for this resistance due to its regulatory functions on EGFR. Moreover, the PI3K/AKT pathway, activated by EGFR and PDGFR, has also been associated with increased resistance to radiation\textsuperscript{20}, and both EGFR and PDGFR have been shown to be regulated by WT1\textsuperscript{55,63,82}; these data further signify a potential role of WT1 in radiation resistance. Additionally, the Bcl-2 family members have been associated with radiation resistance in GBMs\textsuperscript{43}; since WT1 regulates Bcl-2\textsuperscript{67,68}, WT1 may be the upstream signal that is aiding glioblastoma cells to evade destruction. Lastly, WT1 has been shown to bind p53\textsuperscript{65,66} and to suppress p53-mediated apoptosis\textsuperscript{66}, which may also account for the observed resistance. Whatever the case, little is known about how glioblastoma cells evade eradication by radiation, and WT1 may provide clues that will help solve this mystery.
1.3 Hypothesis and Specific Aims

There is convincing evidence that WT1 plays a role in glioblastoma; because the target genes of WT1 have been implicated as responsible for radiation resistance in glioblastoma cells, we hypothesized that WT1 functions to promote cell survival, despite severe radiation-induced damage.

We aimed to characterize the effects of radiation on glioblastoma cell lines expressing WT1, and to determine the effect of silencing WT1 on radiation sensitivity. We used siRNAs to knockdown WT1 in 2 glioblastoma cell lines and confirmed knockdown by real-time polymerase chain reaction (PCR) and Western blot. We then irradiated these cells with a Cesium-137 source and determined cell proliferation by an ATP cell viability assay and the clonogenic cell survival assay. We hypothesized that silencing WT1 with RNA interference would enhance the radiation sensitivity of glioblastoma cells.
CHAPTER 2

MATERIALS AND METHODS
2.1 Cell Culture

Human glioblastoma derived cell lines (Ln18 and T98G) were obtained from American Type Culture Collection and cultured in 1x Dulbecco’s Modified Eagle Medium (Invitrogen) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS from Biomed), 1% L-glutamine (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 1% non-essential amino acids (Invitrogen). Both cell lines were maintained in a humidified incubator at 37°C with a 5% carbon dioxide concentration.

2.2 siRNA Transfection

Cells were plated at a density of 250,000 cells in 2mL complete media per well in 6 well plates. The following day, each well was washed with 2mL 1x Opti-MEM Reduced Serum Medium (Invitrogen) supplemented with 1% penicillin/streptomycin. 800uL of Opti-MEM was then added to each well, and each well was transfected with a final concentration of 100nM siRNA using Oligofectamine Reagent (Invitrogen). For each well, 4.7uL of Oligofectamine Reagent was combined with 12.8uL of Opti-MEM and incubated at room temperature for 10 minutes. In a separate tube, 100nM siRNA (Dharmacon) was added to 204uL Opti-MEM; the Oligofectamine/Opti-MEM solution was then added to the siRNA/Opti-MEM solution, mixed, and incubated at room temperature for 20 minutes. 200uL of the final solution was then added to each well and incubated at 37°C for 4 hours. 500uL of a 3x FBS/Opti-MEM solution was then added to each well.
siRNAs targeting WT1 consisted of a pool of 4 siRNAs synthesized by Dharmaco; the sequences are as follows:

1) sense: 5'-CUACAGCAGUGACAAUUUAU-3'
    antisense: 5'-UAAAUUGUCACUGCUAGUACUU-3'

2) sense: 5'-GGAAUCAGAUGAAGCUAGGUU-3'
    antisense: 5'-CCUAAGUUCAUCUGAUUCCUU-3'

3) sense: 5'-GGACUGUGAAGAUCUGUUU-3'
    antisense: 5'-AAACCUUCGUUACACAGUCCUU-3'

4) sense: 5'-UACCCAGGCUGCAUAAGAUU-3'
    antisense: 5'-UCUUAUUGCAGCCUGGGUAAU-3'

siCONTROL Non-Targeting Pool (Dharmacon) was used as a control and will be referred to as siScramble; this pool consists of 4 non-targeting siRNAs. 24 hours after transfection, cells were washed with 1x PBS and detached with 1mL of 1x Trypsin-EDTA (Invitrogen), neutralized with 4mL complete media, and spun at 1000rpm for 5 minutes at 24°C. Cells were then resuspended in fresh media, counted via Trypan Blue, and re-plated into corresponding plates for the clonogenic cell survival assay and the luminescent cell viability assay as described below.

2.3 Irradiation

48 hours after siRNA transfection, cells were treated with γ-irradiation using a Cesium-source Mark I Irradiator (Cs-137, 4.149Gy/min) at indicated doses. This time point was chosen to allow cells to recover from the possible stress sustained from re-plating.
2.4 Clonogenic Cell Survival Assay

Following siRNA transfection as described above, Ln18 cells were harvested and re-plated into 6 well plates at a density of 500 cells in 3mL of complete media per well; each treatment group was plated in triplicate. Plates were then irradiated 48 hours after transfection at indicated doses; 8 days after irradiation, the media was removed and each well was washed with 1x room temperature PBS. 600uL of methanol was then added to each well to fix the cells and plates were incubated at room temperature for 30 minutes. The methanol was then removed and 1mL of Giemsa Stain, diluted 1:2 in nanopure water, was added to each well and incubated for 1 hour. The Giemsa Stain was then removed and each well was washed 3 times with nanopure water. Colonies consisting ≥ 50 cells were counted.

2.5 Luminescent Cell Viability Assay

Experiments were performed using a semi-automated ATP assay in 96 well plates to determine the number of viable cells after treatment. This assay quantifies the amount of ATP present, which is an indicator of metabolically active cells. The luciferase reaction is used to generate a luminescent signal that is proportional to the amount of ATP present, which is proportional to the number of viable cells in culture. In addition to citing a number of studies that support the use of ATP bioluminescence to measure viable cell number, Promega has also validated this technique by demonstrating a linear relationship between the number of cells in culture and the luminescence measured with their product. Similar studies with this assay in our laboratory, including with the cell lines
utilized for these studies, have confirmed an excellent linear correlation between relative luminescence units (RLU) produced and viable cell number, as quantified by Trypan blue exclusion (data not shown).

Following siRNA transfection, both Ln18 and T98 cells were harvested and re-plated into white 96 well plates with clear bottoms at a density of 1000 cells in 100uL of complete media per well; each treatment group was plated in 10 replicates. Plates were then irradiated 48 hours after siRNA transfection at indicated doses; 5 days after irradiation, CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was performed according to the protocol supplied by the manufacturer. After the buffer, substrate, and plates were equilibrated to room temperature, the buffer was added to the substrate and inverted several times to ensure thorough mixing. 100uL of this solution was added to each well, and the plate was placed on an orbital shaker for 2 minutes to induce cell lysis. The plate was then incubated at room temperature for 10 minutes to allow stabilization of the signal, and subsequently read in the Lumi Star (BMG) Luminometer. Replicates were averaged and plotted as either Average RLU or as percent survival. Percent survival was calculated by taking each irradiated replicate of a treatment group and dividing it by the average of the corresponding non-irradiated treatment group; the average of these values was plotted.
2.6 RNA Isolation and Real-time PCR

RNA was extracted from both cell lines using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Cells were plated in 6 well plates at a density of 250,000 cells in 2mL of complete media per well. Each treatment group was transfected in triplicate the following day with Oligofectamine only or siRNA (in Oligofectamine reagent). 48 hours after transfection, the media was removed; the wells were washed with room temperature 1x PBS, and 1mL of TRIzol reagent was added directly to each well and incubated for 5 minutes at room temperature. The TRIzol/cell solution was then transferred to a 1.5mL RNase-free Eppendorf tube and 200uL of chloroform was added to each tube. The tubes were shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes and then spun at 12,000g for 15 minutes at 4°C. The aqueous phase containing the RNA was then removed and transferred into a new tube. 500uL isopropyl alcohol was added to each tube, and the tubes were inverted several times to mix the solution and incubated at room temperature for 10 minutes. The tubes were then spun at 12,000g for 10 minutes at 4°C. The supernatant was discarded, and 1mL of 75% ethanol was added to each tube. The tubes were vortexed briefly and subsequently spun at 7500g for 5 minutes at 4°C. The supernatant was again discarded, and the pellets were air-dried to allow evaporation of the ethanol. 30uL of DEPC water was then added to each tube and incubated in a 55°C water bath for 10 minutes to resuspend the pellet.

RNA was quantified by a spectrophotometer, by measuring the absorbance of the sample at the 260nm wavelength. Prior to real-time PCR, the
RNA samples were treated with DNase (Promega). In an RNase-free Eppendorf tube, 1ug of RNA was combined with 1uL RQ1 10x reaction buffer and 1uL of RQ1 RNase-free DNase. Samples were then incubated in a 37°C water bath for 30 minutes. 1uL of Stop Solution (20mM EGTA) was then added to each sample, and the samples were incubated in a 65°C water bath for 10 minutes.

Real-time PCR was performed using the ABI Prism® 7900 Sequence Detection System in the Molecular Core of the Virginia Commonwealth University-Massey Cancer Center Nucleic Acids Research Facilities. 10uL reactions were prepared in 384 well plates from TaqMan® One Step RT-PCR Master Mix Reagents Kit (ABI), RNase-free water, 0.9uM each of the forward and reverse primers, 0.3uM of the TaqMan probe, and 20ng of RNA. All samples were tested in triplicate under the following cycling conditions: 48°C /30min; 95°C /10min; and 40 cycles of 95°C /15sec and 60°C /1min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). The probes and primers were designed using the Primer Express® 2.0 version. Probes were labeled in the 5'end with FAM (6-carboxyfluoresceine) and in the 3'end with TAMRA (6-carboxytetramethylrhodamine). Ribosomal RNA (18s rRNA) from the Pre-developed TaqMan® Assay Reagents (ABI) was used as an endogenous control. Primers and probe sequences for WT1 are as follows:

Forward Primer: 5'-AGGACTGTGAACGAAGGTTTTCTC-3'
Reverse Primer: 5'-GACAAGTTTTACACTGGAATGGTTTC-3'
Probe: 5'-CACCTGTATGTCTCCTTTGGTGTCTTTTGAGCT-3'
2.7 Protein Extraction and Western Blotting

Cells were plated at a density of 250,000 cells in 2mL complete media per well in 6 well plates and allowed to attach overnight. Cell were then transfected in replicates of 6 the following day as described above; at indicated times following siRNA transfection, the 6 replicates were combined into one tube and protein was extracted using SDS buffer (50mM Tris-Cl, 1% SDS, 10% glycerol) supplemented with protease inhibitors. Protein concentrations were determined using the BioRad DC Protein Assay Kit. 100ug of protein was loaded into a 10% Bis-Tris gel and separated by SDS-PAGE. The protein was then transferred onto a nitrocellulose membrane, and the membrane was blocked with a 5% nonfat milk solution. Blots were then incubated with mouse anti-WT1 monoclonal antibody (Dako), diluted 1:200, and washed in Tris buffered saline containing 0.05% Tween-20 before and after incubation with anti-mouse secondary antibody conjugated to horseradish peroxidase, diluted 1:1000. Blots were then developed using the ECL Western Blotting Detection Reagents (Amersham). Rabbit anti-cyclophilin A monoclonal antibody (Upstate) was used to control for protein loading.

Protein bands were quantified by densitometry using the Scanning Imager PDS1 and ImageQuant software. The relative optical density (ROD) of each WT1 protein band was measured and divided by the ROD of the corresponding cyclophilin A band. Total protein was reported as the sum of the ROD values of the two isoforms.
2.8 Statistical Analysis

An Analysis of Variance (ANOVA) followed by Tukey’s HSD Post Hoc test was performed to determine differences between the four treatment groups (untreated, Oligofectamine control, siScramble, siWT1). A separate ANOVA value is reported for each of the three groups analyzed (control cells, irradiated cells, percent survival); this analysis was performed on all experiments requiring comparisons of more than two groups. Analysis of data comparing only two groups was performed using Student’s t-Test.
3.1 Characterization of Irradiation Effects

To determine the effects of irradiation on the glioblastoma cell lines, Ln18 and T98, we performed an irradiation (IR) dose response using the luminescent cell viability assay based on ATP content (Figure 1a, 1b) and the cell clonogenic survival assay (Figure 1c). We determined the ED50 to be 5Gy for Ln18 cells and 7Gy for T98 cells using the luminescent cell viability assay, and 2Gy for Ln18 cells using the clonogenic cell survival assay. These doses were then used to study the effects of WT1 on the radiation sensitivity of these cell lines. Additionally, time courses were performed to determine the optimal number of days after irradiation to perform the luminescent cell viability assay (Figure 2a - 2d). Percent survival for each time point was calculated by dividing the relative luminescence units (RLU) of each irradiated replicate by the average RLU of non-irradiated controls; each time point had its own control. Following radiation exposure, cell viability decreased to a minimum value at 5 days, after which viability progressively increased, due to recovery and re-growth of the cell population. Thus, the optimal time to monitor cell viability appeared to be 5 days post irradiation for both cell lines.
Figure 1. Irradiation Dose Response: Ln18 or T98 cells were plated into 96 well plates (1000 cells per well) and incubated for 24 hours prior to radiation exposure. After carrying out radiation exposure, the plates were incubated for 5 days, following which they were subjected to the luminescent cell viability assay for ATP content. The ED50 was determined to be 5Gy in Ln18 cells (1a) and 7Gy in T98 cells (1b). Ln18 cells were also assessed for clonogenic survival; cells were plated in 6 well plates (500 cells per well) and incubated for 24 hours prior to radiation exposure. After incubation for 8 days post irradiation, cells were analyzed. Using the clonogenic cell survival assay, the ED50 was determined to be 2Gy in Ln18 cells (1c). Results shown for the luminescent cell viability assay are means of 10 replicates, while results shown for the clonogenic survival assay are means of triplicates, both from a representative experiment. Error bars represent the standard deviations. All labeled plots were statistically significantly different from the non-irradiated control by Student’s t-Test (all p values < 0.001).
1a

Ln18 cells - IR Dose Response by ATP Assay

<table>
<thead>
<tr>
<th>IR Dose</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00</td>
</tr>
<tr>
<td>2Gy</td>
<td>80.00</td>
</tr>
<tr>
<td>4Gy</td>
<td>60.00</td>
</tr>
<tr>
<td>6Gy</td>
<td>40.00</td>
</tr>
<tr>
<td>8Gy</td>
<td>20.00</td>
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<tr>
<td>16Gy</td>
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1b

T98 cells - IR Dose Response by ATP Assay

<table>
<thead>
<tr>
<th>IR Dose</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
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<tr>
<td>2Gy</td>
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<td>4Gy</td>
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<td>6Gy</td>
<td>40.00</td>
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<tr>
<td>8Gy</td>
<td>20.00</td>
</tr>
<tr>
<td>16Gy</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Ln18 cells - IR Dose Response by Clongenic Survival Assay

Surviving Fraction

IR Dose

Control  1Gy  2Gy  4Gy  6Gy  8Gy
Figure 2. Luminescent Cell Viability Time Course: Ln18 or T98 cells were plated into parallel 96 well plates for each time point (1000 cells per well) and incubated for 24 hours prior to radiation exposure. At the indicated time points, plates were subjected to the luminescent cell viability assay. Luminescence due to cellular ATP content is expressed as RLU; average RLU over time is shown for both cell lines (2a, 2c). Labeled plots for each time point indicate a statistically significant decrease in cell viability of irradiated cells compared to control cells for that particular time point by Student's t-Test (all p values < 0.001). Percent survival is also shown for both cell lines; the ED50 is most effectively obtained 5 days post irradiation in both Ln18 cells irradiated at 5Gy (2b), as well as in T98 cells irradiated at 7Gy (2d). Labeled percent survival plots indicate a statistically significant difference relative to Day 5 by Student's t-Test (all p values < 0.001). Results shown are the means of 10 replicates from a representative experiment. Error bars represent the standard deviations.
2a

Ln18 cells - Average RLU Over Time

Control  5Gy

Avg RLU

140000.00
120000.00
100000.00
80000.00
60000.00
40000.00
20000.00
10000.00
0.00

Day 1  Day 3  Day 5  Day 7  Day 9

Days post IR

2b

Ln18 cells - % Survival Over Time

% Survival

120.00
110.00
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00

Day 1  Day 3  Day 5  Day 7  Day 9

Days Post IR
2c

T98 cells - Average RLU Over Time

![Bar chart showing average RLU over time for Control and 7Gy conditions.](chart)

<table>
<thead>
<tr>
<th>Days Post IR</th>
<th>RLU</th>
</tr>
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<tbody>
<tr>
<td>Day 1</td>
<td>140000.00</td>
</tr>
<tr>
<td>Day 3</td>
<td>7~4</td>
</tr>
<tr>
<td>Day 5</td>
<td>**</td>
</tr>
<tr>
<td>Day 7</td>
<td>†</td>
</tr>
<tr>
<td>Day 9</td>
<td>++‡</td>
</tr>
</tbody>
</table>

2d

T98 cells - % Survival Over Time

![Bar chart showing % survival over time for Control and 7Gy conditions.](chart)

<table>
<thead>
<tr>
<th>Days Post IR</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>*</td>
</tr>
<tr>
<td>Day 3</td>
<td>*</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>*</td>
</tr>
<tr>
<td>Day 9</td>
<td>*</td>
</tr>
</tbody>
</table>
3.2 Confirmation of siRNA efficiency

Confirmation of WT1 knockdown was achieved through real-time PCR and Western Blot. Figure 3 depicts the amount of WT1 mRNA in each of the treatment groups 48 hours after siRNA transfection; WT1 mRNA levels were normalized to 18s rRNA. There is some non-specific effect observed with siScramble in Ln18 cells (Figure 3a), as WT1 mRNA levels are decreased after treatment with siScramble; this is not observed in T98 cells (Figure 3b). Treatment with siWT1, however, significantly decreases the WT1 mRNA levels in both cell lines. In Ln18 cells, the addition of siWT1 results in decreases of 84%, 87%, and 70% in WT1 mRNA levels compared to untreated, Oligofectamine, and siScramble controls, respectively. In T98 cells, siRNA against WT1 produces decreases of 84%, 84%, and 86% compared to untreated, Oligofectamine, and siScramble controls, respectively. Of note, WT1 mRNA levels are significantly higher in Ln18 cells, as compared to T98 cells.

Figures 4 and 5 illustrate WT1 protein expression at various times after siRNA transfection in Ln18 and T98 cells, respectively. The doublet consists of two WT1 isoforms: WT1 (+/+ ) at 54kD and WT1 (-/- ) at 52kD. At 24 hours after transfection, there is decreased WT1 protein expression with treatment of siWT1, however residual protein is still observed in both cell lines (Figures 4a and 5a). At 48 hours after transfection, there is a marked decrease with minimal residual protein in Ln18 cells (Figure 4b), while expression of WT1 is almost absent in T98 cells (Figure 5b). At 96 hours after transfection, there is virtually no protein expression in both cell lines (Figures 4c and 5c). Densitometry measurements
(Table 1) reveal a total WT1 protein expression in Ln18 cells at 24 hours after siWT1 treatment that represents a decrease of 37%, 42%, and 30% relative to untreated, Oligofectamine, and siScramble controls, respectively. At 48 hours after siWT1 treatment, the total WT1 protein expression is decreased 68%, 81%, and 81% respectively, while at 96 hours, siWT1 treatment results in greater than 99.9% decrease in total WT1 protein expression.

In T98 cells, a decrease in total WT1 protein expression of 72%, 73%, and 60% relative to the respective controls is observed at 24 hours. At 48 hours, the decrease is 99%, 98%, and 98% of the respective controls, while at 96 hours expression of WT1 protein is completely absent. In accordance with the real-time PCR data, there is evidence of some non-specific effect with treatment of siScramble, particularly at 96 hours in Ln18 cells; however, there is a significantly greater and marked loss of WT1 protein with siWT1 treatment.
**Figure 3. WT1 mRNA Levels:** Ln18 (3a) and T98 (3b) cells were treated in triplicate and RNA was harvested 48 hours after siRNA transfection. Aliquots of the RNA extracts were then subjected to real-time PCR for WT1 mRNA quantification. mRNA levels were normalized by dividing the quantity of WT1 by that of 18s. The averages of the triplicate values are plotted; error bars represent standard deviations. In Ln18 cells, labeled plots indicate a statistically significant difference relative to the siWT1 treatment group by Tukey's HSD Post Hoc Test (all p values < 0.01). In T98 cells, labeled plots also indicate a statistically significant difference relative to the siWT1 treatment group by Tukey's HSD Post Hoc Test (* indicates p values < 0.01, ** indicates p < 0.05).
3a

Ln18 cells - WT1 mRNA Levels

3b

T98 cells - WT1 mRNA Levels
Figure 4. WT1 Protein Expression in Ln18 Cells at: 24 hours (4a), 48 hours (4b), and 96 hours (4c) after siRNA transfection. Cells were plated in 6 well plates (250,000 cells per well) and transfected the following day. A total of 6 wells were transfected for each treatment group to ensure sufficient protein. Protein was extracted at indicated times, separated, and transferred to a nitrocellulose membrane. Blots were incubated with mouse anti-WT1 monoclonal antibody diluted 1:200. An antibody against cyclophilin A (CypA) was used to control for protein loading. Densitometric analysis of these results is presented in Table 1.
Figure 5. WT1 Protein Expression in T98 Cells at: 24 hours (5a), 48 hours 
(5b), and 96 hours (5c) after siRNA transfection. Cells were plated in 6 well 
plates (250,000 cells per well) and transfected the following day. A total of 6 
wells were transfected for each treatment group to ensure sufficient protein. 
Protein was extracted at indicated times, separated, and transferred to a 
nitrocellulose membrane. Blots were incubated with mouse anti-WT1 
monoclonal antibody diluted 1:200. An antibody against cyclophilin A (CypA) 
was used to control for protein loading. Densitometric analysis of these results is 
also presented in Table 1.
Table 1. Relative Optical Density (ROD) Values: ROD values were obtained for Ln18 (1a) and T98 (1b) by densitometry measurements. Normalized values were obtained by dividing the WT1 ROD by cyclophilin A (CypA) ROD, while total protein values reflect the sum of the normalized 54kD and 52kD values.
<table>
<thead>
<tr>
<th>Ln18 cells after 24hrs</th>
<th>WT1 Untreated</th>
<th>WT1 Oligofect</th>
<th>WT1 siScram</th>
<th>WT1 siWT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalized 54kD (ROD)</td>
<td>0.358</td>
<td>0.349</td>
<td>0.275</td>
<td>0.187</td>
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<tr>
<td>Normalized 52kD (ROD)</td>
<td>0.206</td>
<td>0.262</td>
<td>0.228</td>
<td>0.166</td>
</tr>
<tr>
<td>Normalized total WT1 (ROD)</td>
<td>0.564</td>
<td>0.611</td>
<td>0.503</td>
<td>0.353</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ln18 cells after 48hrs</th>
<th>WT1 Untreated</th>
<th>WT1 Oligofect</th>
<th>WT1 siScram</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Normalized 54kD (ROD)</td>
<td>0.169</td>
<td>0.290</td>
<td>0.313</td>
<td>0.062</td>
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<tr>
<td>Normalized 52kD (ROD)</td>
<td>0.118</td>
<td>0.187</td>
<td>0.170</td>
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<th>WT1 Oligofect</th>
<th>WT1 siScram</th>
<th>WT1 siWT1</th>
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<td>T98 cells after 24hrs</td>
<td>WT1 Untreated</td>
<td>WT1 Oligofect</td>
<td>WT1 siScram</td>
<td>WT1 siWT1</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------</td>
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<td>-----------</td>
</tr>
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<td>0.237</td>
<td>0.040</td>
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<th>WT1 siWT1</th>
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<td>0.178</td>
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<th>T98 cells after 96hrs</th>
<th>WT1 Untreated</th>
<th>WT1 Oligofect</th>
<th>WT1 siScram</th>
<th>WT1 siWT1</th>
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<td>0.678</td>
<td>0.521</td>
<td>0.487</td>
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3.3 Cell Viability and Proliferation

Ln18 and T98 cells were plated in 6 well plates (250,000 cells per well) and transfected the following day with Oligofectamine only, siScramble, or siWT1. 24 hours after transfection, cells were harvested and re-plated into 96 well plates for the luminescent cell viability assay (1000 cells per well) and 6 well plates for the clonogenic cell survival assay (500 cells per well). 48 hours after siRNA transfection, cells were irradiated at indicated doses. Figure 6a depicts the viability of Ln18 cells after treatment with Oligofectamine only, siScramble, or siWT1 in both non-irradiated, control cells and cells irradiated at 5Gy. Statistical significance is achieved within both the control group and irradiated groups (ANOVA p < 0.0001). There is a statistically significant decrease in Ln18 cell viability after treatment with siWT1 relative to the untreated group in irradiated cells only (p < 0.01). Treatment with siWT1 also results in decreased cell viability in both control and irradiated cells when compared to Oligofectamine control and irradiated cells (p < 0.05 between control cells and p < 0.01 between irradiated cells); no significant difference, however, is observed when compared to siScramble in either control or irradiated cells. Figure 6b illustrates the percent survival of irradiated Ln18 cells relative to control Ln18 cells. Statistical significance is again achieved (ANOVA p < 0.0001); treatment with siWT1 significantly decreases percent survival relative to untreated and Oligofectamine treatment groups (both p values < 0.01), although no significant difference in percent survival is observed between siScramble and siWT1.
Figure 7a depicts the viability of T98 cells after identical treatments in both control cells and cells irradiated at 7Gy. Statistical significance is achieved within both the control and irradiated groups (ANOVA p < 0.0001). Treatment with siWT1 results in a significant decrease in T98 cell viability relative to all treatment groups in both control and irradiated cells (all p values < 0.01). Figure 7b illustrates the percent survival of irradiated T98 cells relative to control T98 cells for each treatment group. Statistical significance is again achieved (ANOVA p < 0.0001); treatment with siWT1 results in a significant decrease in percent survival relative to all treatment groups (all p values < 0.01).

The ability of cells to reproduce is also an indicator of cell survival. The clonogenic cell survival assay measures the ability of cells to proliferate and form colonies, and is thus a measure of both cell viability and proliferation. Figure 8a illustrates the number of Ln18 colonies formed after treatment in both control and irradiated cells; statistical significance is demonstrated within both groups (ANOVA p < 0.0001). The number of colonies formed after treatment with siWT1 is significantly decreased compared to all other treatment groups in both control and irradiated cells (all p values < 0.01). Figure 8b illustrates the surviving fraction of Ln18 colonies for each treatment group. Statistical significance is also achieved (ANOVA p < 0.0001); treatment with siWT1 significantly decreases the surviving fraction compared to untreated and Oligofectamine, although no difference in survival is observed between siScramble and siWT1. No data for clonogenic survival in T98 cells are shown because these cells would not grow under the conditions of the assay.
Figure 6. Luminescent Cell Viability of Ln18 Cells: Ln18 cells were treated with Oligofectamine, siScramble, or siWT1 and irradiated 48 hours after transfection. Following a 5-day incubation period, cells were subjected to the luminescent cell viability assay. The average of 10 replicates is plotted as Average RLU (6a) or as percent survival, comparing irradiated cells of each treatment group to its corresponding non-irradiated controls (6b). Error bars represent standard deviations. Both graphs are representative of 3 separate, independent experiments. Plots labeled with a * reflect p values < 0.01 when compared to each other, while plots labeled with a ** reflect a p value < 0.05 relative to each other by Tukey's HSD Post Hoc Test. Other comparisons were statistically significant but are not shown for purposes of clarity.
Figure 7. Luminescent Cell Viability of T98 Cells: T98 cells were treated with Oligofectamine, siScramble, or siWT1 and irradiated 48 hours after transfection. Following a 5-day incubation period, cells were subjected to the luminescent cell viability assay. The average of 10 replicates is plotted as Average RLU (7a) or as percent survival, comparing irradiated cells of each treatment group to its corresponding non-irradiated controls (7b). Error bars represent standard deviations. Both graphs are representative of 3 separate, independent experiments. Plots labeled with * are statistically different from each other, plots labeled with ** are statistically different from each other, plots labeled with ‡ are statistically different from each other, and plots labeled with ‡‡ are statistically different from each other. All p values are < 0.01 by Tukey's HSD Post Hoc Test. Other statistical differences are not shown for purposes of clarity.
7a T98 cells – Cell Viability by ATP assay

- Untreated
- Oligofectamine
- siScramble
- siWT1

7b T98 cells – Cell Viability by ATP assay

- Untreated
- Oligofectamine
- siScramble
- siWT1
Figure 8. Clonogenic Cell Survival of Ln18 Cells: Ln18 cells were treated with Oligofectamine, siScramble, or siWT1 and irradiated 48 hours after transfection. 8 days after radiation exposure, cells were analyzed for clonogenic survival. The number of colonies is either plotted as the average of nine replicates (8a) or as a surviving fraction relative to non-irradiated controls (8b). Error bars represent standard deviations. Both graphs represent the combination of 3 separate, independent experiments. Plots labeled with * are statistically different from each other, plots labeled with ** are statistically different from each other, plots labeled with † are statistically different from each other, and plots labeled with ‡ are statistically different from each other. All p values are < 0.01 by Tukey's HSD Post Hoc Test. Other statistical differences are not shown for purposes of clarity.
8a Ln18 cells – Clonogenic Cell Survival

- Control
- 2Gy

Avg # Colonies Formed

- Untreated
- Oligofectamine
- siScramble
- siWT1

8b Ln18 cells – Clonogenic Cell Survival

Surviving Fraction

- Untreated
- Oligofectamine
- siScramble
- siWT1
CHAPTER 4

DISCUSSION
To date, there are no known papers that have investigated a role for WT1 in the radiation sensitivity of glioblastoma cells or any other cell type. Studies have shown, however, that WT1 is expressed in the majority of high grade gliomas\textsuperscript{79-81} and not in normal glial cells\textsuperscript{81}. While previous studies demonstrate that WT1's target genes do function to confer radiation resistance in glioblastoma cells\textsuperscript{14-16,20,43,83}, there can only be speculation that WT1 may also be responsible for the observed resistance. After characterizing the effects of gamma-induced irradiation on two glioblastoma cell lines expressing WT1 and confirming siRNA efficiency, we determined that WT1 functions to both promote cell proliferation as well as to aid glioblastoma cells in evading irradiation-induced death.

There are two ways of interpreting the data presented, depending on what is considered the appropriate control for siRNA treatment. The use of siRNAs to silence target genes is a relatively recent advancement made in molecular biology. As exciting and extremely useful as this tool is, the lack of valuable controls is a current topic of debate. The two major controls used in these experiments were Oligofectamine as a vehicle control and the pool of non-targeting siRNAs (with Oligofectamine) purchased from Dharmacon. While the scrambled siRNAs seem like the more useful control, members of the 2003 Horizon meeting on RNA expressed concern that these siRNAs are often too unrelated to the target gene to be a truly informative control\textsuperscript{84}. Furthermore, there is apparently a fine line between siRNAs and miRNAs (micro RNAs). Like siRNAs, miRNAs also inhibit translation through a pathway closely related to siRNAs\textsuperscript{84}. Consequently, while these siRNAs are designed to be non-targeting,
they may in fact affect the levels of the target gene or other genes through an miRNA-like mechanism. These unanticipated effects may thus cause this control to be of limited value. With this in mind, let us now turn to the data presented.

If siScramble is used as the control, the Ln18 ATP data suggest that WT1 has no effect on the radiation sensitivity of these cells, as no significant difference is seen between the two treatment groups. The Ln18 clonogenic data, however, does implicate a small but significant role for WT1. The data demonstrate that silencing WT1 increases the radiation sensitivity of Ln18 cells, although no difference in cell survival is observed; this suggests that WT1 promotes cell survival, but that the effect of silencing WT1 only adds to the effects of irradiation, rather than enhancing this mode of destruction. The contradictory results between the ATP data and the clonogenic data may be the result of two different endpoints. While the ATP assay reflects the number of metabolically active cells, the clonogenic cell survival assay reflects only the number of cells with reproductive capacity; thus the resulting survival after treatment with siWT1 is higher using the ATP assay compared to the clonogenic assay. In evaluating the two sets of data, perhaps what is most clinically relevant in glioblastoma is the loss of reproductive capacity; since neurological deficits and ultimate death are the result of tumor cells proliferating and invading surrounding normal tissue, inhibition of growth is the more ideal outcome.

If however, Oligofectamine is considered the control in these experiments, WT1 plays more than a minor role in protecting Ln18 cells from irradiation; in fact, silencing WT1 considerably enhances the effects of irradiation in these cells,
as significant differences are observed in cell viability, clonogenic capacity, and percent survival. An argument can be made that Oligofectamine is perhaps the more valuable control in this cell line, since the levels of WT1 are altered by treatment with siScramble, as seen with both real-time PCR and Western blot data. While treatment with siWT1 produces a greater deficit, decreases in the WT1 levels with addition of the scrambled siRNAs may contribute to the cytotoxic effects observed.

The role of WT1 in radiation sensitivity is further confirmed in experiments with T98 cells; silencing WT1 in these cells enhances their radiation sensitivity, as significant differences in both cell viability and percent survival are seen with treatment of siWT1, regardless of the control used. The effect, however, is more pronounced when Oligofectamine is considered the control. In addition, while very minimal decreases of WT1 protein levels are observed with siScramble in Western blot analysis, it does not seem to have significant cytotoxic effects. Unfortunately, clonogenic survival could not be assessed with T98 cells due to poor colony formation by untreated control cells; however, because the Ln18 data suggest that the clonogenic cell survival assay may be a more sensitive test, it does not seem likely that conclusions drawn from the T98 data presented would be significantly affected. One way to confirm this would be to consider an alternative test such as the colony formation in soft agar assay. Although normal cells undergo anchorage-dependent growth, T98 cells have undergone malignant transformation, which may permit them to grow in the semi-solid media and allow
for parallel clonogenic testing. These data would be valuable to further confirm that treatment with siWT1 enhances the radiation sensitivity of T98 cells.

While we have shown that silencing WT1 does enhance radiation sensitivity, the magnitude of its effects is short of remarkable; this may be due to the time chosen to irradiate the cells. While there is significant knockdown at 48 hours after siRNA transfection in both cell lines, WT1 protein deficits are most pronounced at 96 hours after transfection; there is very minimal residual WT1 protein in Ln18 cells and a complete lack of WT1 protein in T98 cells at 96 hours. In these experiments, cells were irradiated at 48 hours after transfection to minimize the amount of stress induced. Cells were transfected in 6 well plates because verification of knockdown was not possible in the 96 well plates required for the luminescent ATP assay. Thus re-plating was necessary and performed 24 hours after siRNA transfection; cells were irradiated 24 hours later to allow for recovery from re-plating stresses. Perhaps it would be of value to investigate multiple time points to perform the irradiation; since protein levels are lowest at 96 hours, irradiating the cells at this time point may produce a more striking effect. Of interest, while untreated Ln18 cells express significantly more WT1 than untreated T98 cells, Ln18 cells contain almost five times more WT1 mRNA levels (relative to 18s RNA) than T98 cells, even after treatment with siWT1. This may account for the more marked effect seen in the T98 experiments.

In regards to the different controls used, it seems that each cell line should utilize a different control. Significant reductions in WT1 levels are observed in Ln18 cells treated with siScramble. While treatment with siWT1 results in a
greater deficit, WT1 mRNA levels are reduced to half the amount expressed in untreated cells after treatment with siScramble. This suggests that siScramble may be working through other mechanisms, possibly through an miRNA-like mechanism to inhibit translation of WT1. Evidence of this is also observed in Western blot analysis, particularly at the 96 hour time point. This may contribute to the lack of statistical differences observed between the siScramble and siWT1 treatment groups. While the possibility of off-target effects cannot be entirely excluded, it does not appear to be a likely event since no statistical differences in cell viability are observed between non-irradiated cells treated with siScramble and siWT1. Therefore, Oligofectamine appears to be the more appropriate control in Ln18 cells since WT1 levels are not significantly altered.

Alternative controls for future studies with Ln18 cells may include just one non-targeting siRNA, rather than the pool of four used in these experiments; this could minimize effects on WT1 levels. Additionally, siCONTROL RISC-free siRNA (Dharmacon) has been developed to impair uptake and processing by the RNA-induced silencing complex (RISC), which is the mechanism by which siRNAs target mRNAs for degradation; this may also be a valuable control, although both alternatives mentioned should be validated before use. In contrast to Ln18 cells, siScramble does not significantly alter WT1 mRNA or protein levels in T98 cells; thus, siScramble is the more appropriate control for these cells. While cellular context may account for the differences in controls between these two cell lines, it is probably best to determine an appropriate control that can be used in both cell lines.
Overall, our findings that silencing WT1 does enhance radiation sensitivity are not surprising, given that WT1 has also been linked to chemotherapy resistance; this has been reported in the literature as well as in studies done by members of our laboratory. These two therapies work through similar mechanisms, in that they both induce DNA damage. Both WT1 and the multidrug resistance 1 (MDR1) gene products were detected in patients with acute myeloid leukemia; moreover, high levels of WT1 were significantly associated with high levels of MDR1\textsuperscript{83}. Consistent with these findings, correlation between upregulation of WT1 and MDR1-mediated vincristine resistance in leukemia/lymphoma cells lines has also been reported\textsuperscript{85}. In addition, transcriptional activation of the Bcl-2 promoter by WT1 has been associated with resistance to three chemotherapy agents\textsuperscript{68}, as previously described. While WT1 has been implicated in chemoresistance, no studies have reported this function in gliomas.

WT1 has also been correlated with other features that contribute to the malignancy of glioblastomas. One such characteristic is the ability of GBM cells to invade surrounding normal brain tissue. A recent study demonstrated that constitutive expression of the WT1 (-/-) isoform was associated with enhanced cell migration and invasion in vitro using ovarian cancer cells\textsuperscript{86}. This novel study indicated that WT1 regulates cytoskeletal components to achieve these oncogenic functions. Invasion, however, is not limited to high grade gliomas; low-grade gliomas are also capable of invading surrounding normal brain. Additionally, since WT1 correlates more strongly with high grade gliomas, this
raises questions as to whether WT1 plays a significant role in the invasion of glioblastomas. Another malignant trait of GBM is genomic instability. Because WT1 has been shown to inhibit apoptosis by suppression of p53 as well as by activation of Bcl-2, it is possible that WT1 promotes genomic instability in glioblastomas by preventing cellular suicide in cells that have sustained significant DNA damage. These functions, however, have yet to be validated in GBMs.

**Future Directions:** With regards to radiation resistance, it would be valuable to examine whether different time points after radiation exposure would provide more pronounced effects. Furthermore, a single irradiation dose based on the ED50 was chosen for these experiments. The rationale behind using the ED50 was to utilize a dose that would have obvious effects in order to determine whether treatment with siWT1 would alter the radiation sensitivity of these cell lines. Either too low or too high of a dose would make detection of any effects rather difficult. It may be of interest for future studies to investigate the effects of using a slightly higher dose with multiple radiation exposures, as is done clinically with fractionated doses. It is possible that the effects of silencing WT1 may show a greater effect using this approach and would be more relevant to patient care if proven. In addition, experiments that utilize siRNAs to silence genes should first identify a control that does not significantly alter levels of the target gene. To better understand the function of WT1 in glioblastoma, future studies should address the effects of WT1 on its known target genes, specifically in GBMs. Additional investigation could validate WT1 activation/upregulation of the
previously discussed pro-survival genes (ie EGFR, PDGFR, Bcl-2) or the suppression of apoptotic genes like p53 in glioblastomas; this would further elucidate its probable function as an oncogene in this cell type.
LITERATURE CITED
Literature Cited


