Virginia Commonwealth University
VCU Scholars Compass

Theses and Dissertations

Graduate School

2011

WILMS' TUMOR-1 (WT1) PROTEIN EXPRESSION IN GLIOMA CELLS ACTUATES CELLULAR INVASIVENESS-IDENTIFYING ITS TARGET GENES

Archana Chidambaram
Virginia Commonwealth University

Follow this and additional works at: http://scholarscompass.vcu.edu/etd

Part of the Nervous System Commons

© The Author

Downloaded from

http://scholarscompass.vcu.edu/etd/2454

WILMS' TUMOR-1 (WT1) PROTEIN EXPRESSION IN GLIOMA CELLS ACTUATES
CELLULAR INVASIVENESS- IDENTIFYING ITS TARGET GENES.

A dissertation submitted in partial fulfillment of the requirements for the degree of PhD at
Virginia Commonwealth University.

by

ARCHANA CHIDAMBARAM

M.B., B.S., Seth G.S. Medical College & K.E.M. Hospital, 2003.

Director: William C. Broaddus, MD, PhD

Hord professor of Neurosurgery

DepartMent of neurosurgery

Virginia Commonwealth University

Richmond, Virginia

May, 2011.

Acknowledgement

I would like to express my most sincere gratitude to the following people, without whose help, this work would have never been possible. Dr. William Broaddus, my advisor, has been a mentor in the truest sense of the word. Every time I came away from a meeting with Dr. Broaddus, my head was abuzz with new ideas! His unfailing support and meticulous attention to detail combined with his trademark confidence, have all contributed towards making this a great learning experience. Dr. Helen Fillmore and Dr. Timothy Van Meter both clarified several technical difficulties that had me confounded on numerous occasions. I would especially like to thank them for the spirit of camaraderie and the passion for science they infused in each member of the lab. I would like to acknowledge Dr. Martin Graf for his valuable help with the flow cytometry experiments- a technique of which I'm grateful to have at least a working knowledge. I would also like to thank Dr. Lynne Elmore- who served on my graduate committee- for the insightful input she always provided. I am extremely grateful to the department of Anatomy and Neurobiology- especially Dr. John Bigbee, Dr. George Leichnetz and Dr. John Povlishock. They accepted me into the program under rather extenuating circumstances, and I have striven to justify their expectations. Dr. Bigbee was also an esteemed member of my graduate committee and his attention to detail has helped me hone my presentation skills tremendously. I also owe a special thanks to the department of Neurosurgery for all their support. In this regard, I sincerely acknowledge the generosity of Mrs. and Mr. Fenton N. Hord who established the Hord

foundation at VCU to fund brain tumor research in memory of their beloved son who succumbed to this dreadful disease in 1991. They (and countless other such families) have provided me with the constant inspirational reminder that our work serves a higher purpose- one that is much more important than publishing papers or gaining fame.

Dr. Catherine Dumur and Tana Blevins (department of Pathology) contributed immensely to the progress of this project through their assistance with the microarray studies. Dr. Dumur's kind patience in answering my numerous questions pertaining to this rather complicated technique is much appreciated. I would also like to sincerely acknowledge Ruth Carvalho and the rest of the Nucleic Acid Research Facilities for tolerating my overwhelming requests with admirable fortitude!

A large chunk of my gratitude and warm appreciation is owed to all my former lab members- each of whom has had an impact on my professional and personal development. Aaron and Mike, the pioneers of the "WT1 project", were among the first to give me an idea of what path to follow. For their guidance in my most formative years in the lab, for their extremely well laid-out protocols, and for their continued support, wisdom and encouragement, I am eternally indebted. Dr. Justin McClain, Dr. Nicholas Pullen, Dr. Anil Kumar, Oran Kremen and Matthew Parry were the other lab "veterans" when I first came in. Time and again, I have relied on them- to understand concepts, to borrow from their inexhaustible database of knowledge, resolve technical queries and even for assistance with sacrificing mice (thanks again, Nick!)! I'd like to

Table of Contents

List of Tables

List of Figures

List of Common Abbreviations

ARF- alternate reading frame

BM- basement membrane

CK II- casein kinase II

CNS- central nervous system

CTE- constitutive transport element

CDK4- cyclin dependent kinase-4

MERTK- c-mer proto-oncogene tyrosine kinase

DAF- decay accelerating factor

DSRCT- Desmoplastic small round cell tumor

DPYSL3- Dihydropyrimidinase-like 3

Egr-1- early growth response-1

Cdh-1- E-cadherin

EPAS1- Endothelial PAS domain protein 1

EGFR- epidermal growth factor receptor

EMT- epithelial-mesenchymal transition

ECM- extracellular matrix

FAM57A- family with sequence similarity 57, member A

FGFR- fibroblast growth factor receptor

FLAIR- Fluid attenuation inversion recovery

FDG-PET- F-18 fluorodeoxyglucose- positron emission tomography scanning

GABA- gamma-aminobutyric acid

GABRA1- GABA A receptor

GBM- glioblastoma multiforme

GAG- glycosaminoglycan

GPCR- G-protein coupled receptor

INPP5A- Inositol polyphosphate-5-phosphatase, 40kDa

IGF-1R- Insulin- like growth factor-1 receptor

IDH1- isocitrate dehydrogenase

LZTS-1 - Leucine zipper, putative tumor suppressor 1

LOH- loss of heterozygosity

MRI- magnetic resonance imaging

MET- mesenchymal-epithelial transition

MDM2- murine double mutant-2

NES- nestin

NF1- neurofibromin-1

NEFL- neurofilament, light polypeptide

NHA- normal human astrocyte

NKX2-2- NK2 transcription factor

OLIG-2- oligodendrocyte transcription factor 2

PTEN- phosphatase and tensin homolog

PIP2- Phosphatidylinositol-4,5- Bisphosphate

PI3K- phosphatidyl inositol 3 kinase

PIP3- Phosphatidylinositol- 3,4,5- Triphosphate

PLC-γ- phospholipase-γ

PDGF- platelet-derived growth factor

PDGFR-α- platelet-derived growth factor receptor- alpha

PH- pleckstrin homology

Par-4- prostate apoptotic response-4

PK (A/C)- protein kinase (A/C)

RT- radiation therapy

Ras-GAP- Ras- GTPase activating protein

ROS- reactive oxygen species

RLU- Relative luminescence units

RT-PCR- Reverse Transcription- Polymerase Chain Reaction

RNP- ribonucleoprotein

RTK- receptor tyrosine kinase

RB- retinoblastoma

pRb- retinoblastoma protein

SF/HGF- Scatter Factor / Hepatocyte Growth Factor

siRNA- silent interfering RNA

Snai1- Snail-1

SLC12A5- solute carrier family 12 (potassium/chloride transporter), member 5

SHH- sonic hedgehog

SD- stable disease

SYT1- synaptogamin 1

TMZ- temozolamide

TCGA- The Cancer Genomic Atlas

TYMS- thymidylate synthetase

TIMP3- TIMP metallopeptidase inhibitor 3

TM7- transmembrane-7

MAFF- v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)

WT1- Wilms' Tumor-1

WIPI-1- WD repeat domain, phosphoinositide interacting 1

Abstract

WILMS' TUMOR-1 (WT1) PROTEIN EXPRESSION IN GLIOMA CELLS ACTUATES

CELLULAR INVASIVENESS: IDENTIFYING ITS TARGET GENES

.

By Archana Chidambaram, M.B.B.S

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: William C. Broaddus, M.D., Ph.D
Hord Professor
Department of Neurosurgery, Department of Anatomy and Neurobiology

Previous studies in our laboratory demonstrated the expression of WT1 in a significant

number of glioma cells and established its role in promoting tumor cell proliferation. Here, we

noted the effect(s) of manipulating WT1 levels on the expression levels of genes that were previously shown to be regulated by WT1. We found no correlation between the expression levels of WT1 and PDGF-A, Snai1 and E-cadherin and a consistent inverse correlation between WT1 and IGF-1R expression in U251-MG cells. To ascertain whether the increased IGF-1R levels resulting from WT1 silencing could account for decreased cellular proliferation, we utilized siRNA mediated knockdown of IGF-1R and found a modest decrease in cellular proliferation instead of an expected increase.

Gene expression profiling in U251-MG cells was then used to identify candidate target genes for WT1. Several genes whose levels directly correlated with WT1 were observed to have putative or established oncogenic role(s) in glioma cells or other malignancies. Among the genes that correlated inversely, a tumor-suppressor role was attributed to some. Real time RT-PCR helped to substantiate these microarray findings in U251-MG cells.

We also characterized the expression and function of WT1 in U1242-MG and GBM6 cells. Interestingly, in these cells WT1 facilitated cell invasiveness but had no discernible influence on cellular proliferation. The expressions of the candidate WT1 target genes were also determined in these 2 cell lines. At least 3 genes were consistently down-regulated with WT1 silencing in the three cell lines- INPP5A, CD97, and TYMS. To determine whether CD97 assisted WT1 in facilitating cellular invasion, we silenced CD97 expression using siRNA and noted a significant decrease in the cells' ability to invade through Matrigel-coated filters. We propose that WT1

profoundly impacts the glioma cells' invasive ability, and this function is mediated by CD97 alone or in conjunction with other pro-invasive molecules.

Our findings argue for the oncogenic role of WT1 in the specific context of glioma cells. They also point to a role for the novel pro-invasive protein- CD97- in glioma cells. Further studies are necessary to confirm the mechanism by which CD97 promotes invasion as well as to explore its potential as a diagnostic and/or therapeutic target.

Chapter 1: Introduction

Tumors of the central nervous system (CNS), as with all other organ systems, can be either benign or malignant. That is where the similarity ends though, because the consequences of even the best-behaved growth in these parts can be extremely morbid, or even fatal, depending on its exact location. The utterly non-expansive confines within which the brain and the spinal cord are housed, the extreme vulnerability of the neurons to ischemic damage and the risk of damage to vital functions entailed in the surgical removal of *any* tumor lodged in and around the organs of the CNS are all factors that set these tumors apart from all others.

According to the 2007 WHO classification [1], tumors of the CNS can be broadly classified into different categories. These include tumors of the neuroepithelial tissue (astrocytomas, oligo-dendroglioma/ -astrocytomas, ependymal tumors and so on), cranial and paraspinal nerve tumors (for example. Schwannomas, neurofibromas and so on), tumors of the meninges, lymphomas and hematopoietic tumors, sellar tumors, germ cell tumors and the metastatic tumors.

The biological behavior of these tumors can be predicted by grading the neoplasms based on their histological appearance. Tumor grading- which is similar across all CNS tumor entities- also helps determine the appropriate therapeutic approach- especially in deciding whether to include or exclude adjuvant radiation and specific chemotherapy protocols[1].

According to the 2007 WHO classification system, Grade I uniformly refers to tumors that have a low proliferative potential and that can be cured by surgical resection alone. Neoplasms that are designated Grade II also have low proliferative rates, but possess the ability to infiltrate surrounding brain parenchyma. Hence, surgery alone cannot effect a complete cure

and Grade II tumors often tend to recur. Such recurrences are usually more aggressive, both in their behavior and in their resistance to therapy. Grade III neoplasms differ from the lower grades in the high proliferative capacity that the tumor cells possess. There is a significant increase in the number of mitotic figures and nuclear atypia seen on microscopic examination of these tumor specimens. Patients with Grade III CNS tumors often receive adjuvant radiation and/ or chemotherapy. The highest grade of malignancy- Grade IV- is assigned to tumors with a rapidly deteriorating course and an inevitably fatal outcome. Microscopically, these tumors are associated with a significantly high level of abnormal cells and mitotic figures, and areas of necrosis. Examples of Grade IV CNS neoplasms include Glioblastoma Multiforme (GBM), most embryonal neoplasms and some sarcomas. Such aggressive tumors are characterized by widespread tissue infiltration and a propensity for craniospinal dissemination[1].

Gliomas (derived from mature glia or their less differentiated precursors) mostly infiltrate the surrounding parenchyma diffusely. The WHO classifies these tumors broadly into one of 3 categories: Astrocytomas, Oligodendrogliomas and Oligoastrocytomas. Differentiating between astrocytic and oligodendroglial tumors on histologic examination is somewhat possible by taking into account the cell shape, cytoplasmic appearance and the nuclear characteristics[2]. Astrocytomas are characterized by cells with elongated nuclei which are hyperchromatic and irregular. Prominent nucleoli and perinuclear halos are not typical. Some astrocytomas are characterized by prominent pink cytoplasm (e.g. gemistocytic or granular cell astrocytoma) while others have minimal fibrillary cytoplasm (e.g. fibrillary and small cell astrocytoma) [2].

Tumor cells in oligodendrogliomas, on the other hand, have nuclei that are usually round, regular and show little inter-cellular pleomorphism. The phenomenon of perinuclear cytoplasmic clearing- which gives rise to a "scrambled egg" appearance- although helpful is a rather

inconsistent finding[2]. Some of the non-specific features that also help to form a diagnosis of oligodendroglioma include cortical involvement, microcalcifications, delicate branching capillaries and microcysts[2].

Astrocytomas are the most common primary neoplasms of the CNS from among these (Ref: CBTRUS), and also the most intensely studied. The WHO uses the 3-tiered system to grade these tumors from Grade II through Grade IV. Based largely on the grading criteria established by the St. Anne/ Mayo system, the WHO classification differs from this system only in its non-recognition of Grade I gliomas, which are non- infiltrative and lack nuclear atypia, mitoses, endothelial proliferation and necrosis and therefore, are associated with a very low likelihood of recurrence[2]. The characteristic features that help differentiate Grade II through Grade IV/ GBM are outlined in the table as shown below:

Table 1: Features that help differentiate the different grades of gliomas.

Basis	**Grade II**	**Grade III/ AA**	**Grade IV/ GBM**
1. Infiltrative capacity	+	+	+
2. Nuclear atypia & pleomorphism	+	++	+++
3. Mitotic figures	+ (0-1)	++ (>1)	++(+)
4. Hyper-cellularity	+	++	+++
5. Endothelial proliferation	-	-	+
6. Necrosis	-	-	+

Genetic subtypes of GBM based on genomic analysis

Despite the similar clinical course and histological appearance amongst GBMs, there exists a remarkable heterogeneity at the genetic level. The study of the pathogenesis of astrocytomas is broadly based on two themes[3]- to identify the cell of origin of these tumors (that is, whether they arise from de-differentiation of mature astrocytes or from the transformation of astrocytic precursor cells) and to uncover the specific genetic abnormalities in different phases or grades of these astrocytic neoplasms. While nearly a century's worth of experiments has not yet generated an unequivocal answer to the first question, distinct molecular signaling pathway abnormalities have been delineated, that have helped us understand how these tumors progress from lower-to higher- grades, or how highly malignant astrocytomas are spontaneously generated. In 2009, The Cancer Genome Atlas Network (TCGA) undertook a massive venture, studying 206 human GBM samples with respect to their DNA copy number, gene expression, DNA methylation, and nucleotide sequence aberrations [4]. These investigations revealed that the frequency of somatic alterations, based solely on the copy number data, of the retinoblastoma (RB), TP53 and receptor-tyrosine kinase (RTK) pathways was 66%, 70% and 59% respectively and these values were greater when sequencing data were taken into account. Moreover, the alterations of components *within* each pathway trended towards statistically significant mutual exclusivity. This suggests that deregulation of one component within the pathway diminishes the selective pressure for additional ones. However, any one sample was found to have at least one aberrant gene from each of the three pathways, and 74% harbored abnormalities involving all three pathways.

The RB pathway: The retinoblastoma protein (pRb) is active in its hypo-phosphorylated state [5] and acts to inhibit cell cycle progression through its inhibitive regulatory effect on the E2F

family of transcription factors [6] which have been established to push cells through the G1 phase of the cell cycle into the S-phase [7]. Cyclin dependent kinase 4/6 phosphorylates pRb [8] and inactivates it, thereby lifting the hold on cell-cycle progression. The cyclin-dependent kinase inhibitor - p16^{INK4A} also known as CDKN2A- binds all available cdk4/6 and causes Cyclin-D1 to detach from the cdk4/6 complex [5]. Thus unprotected, Cyclin-D undergoes degradation and is no longer able to participate in cell cycle regulation [5].

As per the TCGA studies [4], 77% of the 206 samples examined had mutations belonging to the Rb pathway, and of these, the most common event was deletion of the CDKN2A/CDKN2B locus on chromosome 9p21 (55% and 53%), followed by amplification of the CDK4 locus (14%). It was also found that the 9 samples that contained RB1 nucleotide substitutions lacked the above-mentioned mutations in the upstream signaling components.

TP53 pathway: The p53 protein has long been hailed as the "guardian of the genome", owing to its contribution towards maintaining genomic stability [9, 10] in addition to its myriad other extensively documented functions as a tumor suppressor (reviewed in [10]). The p53 protein can prevent tumor formation or progression by causing cell-cycle arrest, inhibiting cell growth, eliminating the generation of reactive oxygen species (ROS) on account of its antioxidant behavior, promoting the removal of permanently damaged cells through death or senescence and by promoting apoptosis or autophagy. The exact molecular targets that enable it to influence each of these functions are extensively detailed elsewhere [10, 11]. One transcriptional target of p53 is the murine double mutant-2 (MDM2) [11]. MDM2, when transcriptionally activated serves to inhibit the actions of p53 in two ways [11]- it binds and covers the transactivational domain of the latter protein, thereby thwarting its transcriptional activity. Additionally, it also targets p53 for degradation either by a mechanism involving p300 (which in its turn is inhibited by Arf) or

5

by phosphorylating p53 at its N-terminal end. The CDKN2A locus on chromosome 9p21 mentioned earlier encodes two different proteins. We have already described briefly, the functions of one of these- p^{16INK4A} , which plays an important role in ensuring the functionality of pRb. The second product- named ARF (or alternate reading frame) differs from this protein in one exon [12]. This protein also serves to stabilize p53 protein by binding MDM2, an event that leads to decreased degradation of p53 [12].

Based on the findings from the TCGA studies [4], the most common aberrations seen in the TP53 signaling pathway included ARF deletions (55%), MDM2 (11%) and MDM4 (4%) amplifications, in addition to mutations seen with the p53 gene itself (35%). Previous studies had reported that in grades II and III astrocytomas, p53 mutations correlated with an allelic loss of 17p [3]. Interestingly, loss of heterozygosity (LOH) at 17p was associated with an increase in mRNA levels of PDGF-Rα [3], although these studies also showed that the latter abnormality could exist without p53 mutations, suggesting that while p53 mutation might be an early event in tumor progression, it was not the initial event. Another interesting study [13] demonstrated that the acquisition of p53 mutations correlated with progression of brain tumors from low-grade to higher grades, and these mutations were shown to be propagated in a clonal fashion, establishing the importance of this "master watchman" protein in halting malignant progression.

RTK pathway: Receptor tyrosine kinases are cell surface receptors; the ones that are important in glial neoplasms include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGF-R) and fibroblast growth factor receptor (FGF-R). These receptors bind their corresponding growth-factor ligands on the extracellular N-terminal side, and are consequently activated by a process known as *auto-phosphorylation*. Phosphorylation of the receptor serves two purposes [14]. It results in activation of the intracellular kinase domain, which can then

phosphorylate the downstream substrates. Additionally, it creates docking sites for these signaling effector molecules involved in transducing the receptor's signal. These downstream effectors of signal transduction are mostly proteins that possess domains (like the SH2 and PTB domains) which recognize and bind the phosphorylated tyrosine residues on the activated receptor [14-16]. Most of the transducers are proteins with inherent or associated catalytic activity, for example- Phosphatidylinositol-3-kinase (PI3-kinase), Phospholipase C- γ (PLC- γ), Src family of tyrosine kinases, and the Ras- GTP-ase activating protein (Ras-GAP) among others. Other proteins that bind the activated receptor, but are devoid of any catalytic activity are the adaptor proteins- such as Grb2, Grb3, Nck, Shc, Crk etc. All these signaling pathway components participate in a cascade of reactions that eventually result in cell transformation, enhanced survival and proliferative abilities, invasiveness and tumor angiogenesis.

Studies have proven that the PI3-kinase/AKT (Protein Kinase B) pathway is one of the most important ones mediating cellular transformation following receptor tyrosine kinase stimulation (reviewed in 17). In fact, it has been established that several growth factor signaling pathways converge onto this particular enzyme. The Class I family of PI3-kinases, like the others, has two subunits- a p85 regulatory subunit and a p110 catalytic subunit. The enzyme binds phosphorylated tyrosine residues via the SH2 domain on the *p85* subunit, which leads to stimulation of the kinase activity of the p110 catalytic subunit [18]. It is this activation that leads to further phosphorylation of the downstream effectors.

PI3-kinase, which is a lipid kinase, acts upon Phosphatidylinositol-4,5- Bisphosphate (PIP2), which is then phosphorylated (and thereby activated) to Phosphatidylinositol- 3,4,5- Triphosphate (PIP3). PIP3 now recruits AKT to the cell membrane via its pleckstrin homology (PH) domain. Complete AKT phosphorylation is mandatory for its activation. Once that has

occurred, active AKT can either act upon cytoplasmic targets or translocate to the nucleus and interact with nuclear effectors. One of its many substrates include BAD- a pro-apoptotic protein, and the net result is a decrease in its activity. Unopposed action of the anti-apoptotic proteins [19], such as Bcl-2 and Bcl-Xl results in cell survival and consequently, proliferation.

Tumor suppressor proteins such as neurofibromin 1(NF1) and phosphatase and tensin homolog (PTEN) act to keep the proteins that function downstream of RTKs and, therefore, the cell's survival machinery under control. NF1 has a functional Ras-GAP domain and can therefore negatively regulate Ras activity [17]. PTEN negatively impacts the PI3K pathway by dephosphorylating PIP3 to PIP2 [17], thus blocking the pathway from proceeding further downstream.

In astrocytomas, abnormal functioning of the growth factor receptors can arise in several ways- increased expression of ligand with consequently increased frequency of receptor-ligand complex formation, increased receptor expression or constitutively activated receptors that circumvent the need for ligand binding interactions. Mutations involving the effector molecules that lie downstream of the receptors are also common.

The TCGA studies confirmed previous findings that the variant III deletion of the extracellular domain (vIII mutant) and carboxy-terminal deletions with correspondingly altered transcripts were the most common EGFR mutations[4]. In general, EGFR gene alterations were seen in 45% of the cases. Further. 22 out of the 91 GBM specimens studied had focal EGFR amplifications, 16 had point mutations and focal amplifications, and 3 cases had only EGFR point mutations without amplifications. Other studies have reported frequent over-expression and amplification of PGFR-α and β in GBM specimens [3]. FGF-Rs were interesting in their pattern

of expression, as studies have shown that while low-grade astrocytomas express FGF-R2, they switch to FGF-R1 as they progress to high-grade tumors (reviewed in 3), although the functional significance of this was not yet determined.

The PI3K pathway was found to be dysregulated by activating missense mutations in the p110 encoding gene- *PI3KCA*, resulting in an activated catalytic subunit of PI3K. The contribution of NF1 mutations to sporadic GBM has been definitively confirmed by the TCGA's findings of somatic inactivating mutations or deletions of the NF1 gene in at least 47 of the 206 cases [4]. PTEN mutations frequently involve the phosphatase domains, and germline mutations in the NF1 and PTEN genes (resulting in Neurofibromatosis 1 and Cowden disease, respectively) each render the individual susceptible to sporadic glioblastoma [17].

Based on these gene expression studies analyzing the molecular make-up across hundreds of GBM tumor specimens, 4 different classes are known, each of which has a predominant pattern of abnormal genetic alteration(s) [20]. These four classes and their accompanying abnormal genetic markers are as follows:

1. Classical subtype: Chromosome 7 amplification and chromosome 10 loss are seen in 100% of tumors belonging to this sub-type with high level EGFR amplification seen in 97% of the Classical sub-type. Over 50% of the tumor samples belonging to this sub-type demonstrated point or vIII mutations in EGFR. Along with the EGFR amplification, *p53* mutations were found lacking. Neural precursor and stem cell marker, nestin (*NES)*, as also Notch and Sonic Hedgehog (SHH) pathways were found to be highly up-regulated in these specimens.

2. Mesenchymal subtype: Tumor samples belonging to this sub-type displayed a high frequency of mutations of the *NF-1* gene. They were also found to display a combination of increased activity of mesenchymal and astrocytic markers- CD44 and c-mer proto-oncogene tyrosine kinase (MERTK) - suggestive of the process of epithelial-mesenchymal transition (EMT) which has been associated with trans-differentiated and de-differentiated tumors.

3. Proneural subtype: Tumors belonging to this sub-type are characterized by alterations of *PDGFRA* and point mutations in isocitrate dehydrogenase 1 (*IDH-1)* [20, 21]. PDGFRA alterations take the form of focal amplification as well as high levels of PDGFRA expression. *TP53* mutations and loss of heterozygosity (LOH) were also seen to occur frequently in these tumors, while chromosome 7 amplification and chromosome 10 loss were infrequent events. This group of tumors also demonstrates an up-regulation of genes that are involved in oligodendrocytic development such as oligodendrocyte transcription factor 2 (*OLIG2)*, *PDGFRA* and NK2 transcription factor (*NKX2-2)*. High levels of *OLIG2* have been shown to down-regulate the tumor suppressor p21, a finding that is confirmed in proneural tumors.

4. Neural subtype: Expression of neuronal markers like neurofilament, light polypeptide (*NEFL)*, gamma-aminobutyric acid (GABA) A receptor (*GABRA1)*, synaptogamin 1 (SYT1) and solute carrier family 12 (potassium/chloride transporter), member 5 (*SLC12A5*) characterize tumors belonging to this group. The Gene Ontology (GO) categories associated with this sub-group included neuron projection and synaptic and axon transmission.

This information was then used in the study to compare against data from the brain transcriptosome database presented by Cahoy et al. which classifies genes into different sets associated with neurons, oligodendrocytes, astrocytes and cultured astroglial cells[20]. This comparison showed that genes associated with the proneural subtype were strikingly enriched with the oligodendrocytic signature, and not astrocytic. The classical group, on the other hand, is strongly related to the murine astrocytic signature. The neural sub-type was associated with genes involved in astrocytic and oligodendrocytic differentiation and also those expressed by neurons. The mesenchymal group was associated with cultured astroglial gene signature. Finally, it was noticed that while WHO grade III tumors and certain GBMs occurring in young patients mainly comprised proneural sub-type, classical and mesenchymal tumors were all GBMs [21]

Glioma Cell Invasiveness

As mentioned above, the property of invasiveness is the first malignant characteristic that glioma cells acquire, and it also constitutes the chief confounding factor in therapy (see below). The earliest descriptions of glioma cell invasion are credited to Hans-Joachim Scherer, a neuropathologist who, while a political refugee in Belgium in the aftermath of WWII, published numerous papers that dealt extensively with the behavioral patterns of malignant gliomas [22]. In his studies[23], he designated those structures that were inherent to the glioma tumor and did not *depend on pre-existing tissue* (for example, canaliculi and papillary formations) as "proper structures", whereas those that did depend on pre-existing tissue, were called "secondary structures" (including perivascular-, surface-, perineuronal-

and intrafascicular-growth). Finally, he described the formations wrought by tumor cell interactions with the proliferating mesenchymal tumor tissue as "tertiary structures".

During normal embryonic development, neural stem cells and glial progenitor cells extensively proliferate and travel great distances to reach their eventual destination. These activities are considerably diminished as the organism reaches maturity. The migratory pattern of glioma cells has been likened, interestingly, to that of their stem cell predecessors. Malignant gliomas, while similar to other malignant cells in their *tendency* to infiltrate, are nevertheless different. One reason for this difference is the limited volume of extracellular space available, owing to the complex mesh formed by the extensive axonal-, dendritic- and glial branching. However, it has been observed that in infiltrative gliomas (which includes Grades II, III and IV gliomas), this extracellular space increases in volume and becomes more irregular as extracellular matrix (ECM) components unusual for this space are seen to accumulate [23]. Secondly, in most other malignant tumors, the cells move in and out of blood vessels and lymphatics, and travel via the bloodstream/lymph respectively to distant sites in the body. However, while glioma cells can penetrate the glial-derived portion of the blood vessels' basement membrane (BM), they generally do not disrupt the endothelial portion of the BM, resulting in an inability of these cells to gain access to the vascular lumen [24]. Thus, metastasis outside the brain by primary brain tumors is a relatively rare occurrence.

Within the confines of the brain parenchyma, however, these malignant cells quite literally know no boundaries. In this regard, they have often been likened to "guerilla warriors" [23, 24] as these cells invade alone or in small groups, and abuse the "pre-existent supply lines". The ECM component of the brain also includes material other than the vascular BM, and comprises such molecules as glycosaminoglycans (GAGs) and a few

structural ECM glycoproteins. The GAGs are of 4 kinds in the brain- hyaluronic acid, chondroitin sulfate, keratan sulfate and heparan sulfate [24]. Although it isn't clear yet as to how glioma cells directly interact with these GAGs in their path through the ECM, it is presumed that specific cell-substrate interactions must be at work, to facilitate the spread of these cells through the corpus callosum, the anterior commissure or the optic radiation, which are all very frequent routes of glioma cell invasion. Besides ECM components, the neoplastic cells may also utilize the surfaces of neighboring neuronal and glial cells as a scaffold for diffusely infiltrating the parenchyma, and it has been confirmed that myelin is among the most permissive substrates for glioma cells for adhesion and migration. This may account for the common histopathological finding of glioma cells' preferential migration in white matter tracts.

It has been estimated that these tumor cells can move to a distance of 4.0 ± 7.0 cm from the parent tumor [24]. Using time-lapse video microscopy it was found that glioma cells when grown in monolayer cell cultures, migrate at different rates depending on the substrate(s) they are exposed to, while other experiments have shown that the brain's ECM components- particularly laminin, tenascin, collagen IV and hyaluronic acid- are preferred substrates for tumor cell adhesion and migration [24].

The invasion of the neoplastic cells through the surrounding normal tissue is a complex process. In gliomas, this process can be broken down into 3 main steps [24]: A. Adherence of the tumor cells to the ECM via cell adhesion receptors (Integrins, CD44). B. Degradation of the local ECM by proteases (such as matrix metalloproteases) and C. Sending out processes into the newly cleared space followed shortly thereafter by migration of the cell itself (involving actin cytoskeleton rearrangements).

Angiogenesis has also been postulated to contribute significantly to the process of proliferation and migration of glioma cells. In fact, it has been noted that the processes of tumor cell invasion and neovascularization are effected by similar signaling pathways, and mechanisms such as adhesion, proteolysis and migration are also common to both [24]. For example, the expression of Scatter Factor (SF)/ Hepatocyte Growth Factor (HGF) has been observed to strongly influence glioma cell migration as well as endothelial cell migration in vitro. Moreover, the endothelial cells recruited by glioma cells to vascularize the growing tumor are a source of proteases that assist with invasion. In turn, the invading tumor cells utilize the newly formed vessels as guiding structures as they invade the host.

Clinical features of patients with gliomas and current standard of care:

The signs and symptoms with which a patient presents can be either non-specific (such as due to increased intracranial pressure) or specific (secondary to the tumoral location). Symptoms and signs arising out of an elevation in the increased intracranial pressure typically include headache, vomiting, nuchal rigidity, papilledema and sixth nerve palsy. Headache is usually worse in the morning (presumably due to mild CO_2 retention during sleep leading to cerebral vasodilatation) and with certain postural positions. It may be relieved by vomiting. It is noteworthy that the headache, while prolonged and eventually constant, is rarely as severe in intensity as that seen in migraine or subarachnoid hemorrhage. These non-specific signs and symptoms are more common in the higher-grade gliomas as compared to the low grade tumors.

Clinical features owing to the location of the tumor arise due to the irritative or destructive nature of the tumor. The symptoms and signs produced by these masses can

provide valuable clues as to their location. For example, a glioma situated in the temporal lobe or near the motor cortex is more likely to cause seizures. Tumors situated in the fronto-temporal cortex can cause a person to present with mental apathy or memory loss and personality disturbances, while those located in the fronto-parietal cortex can result in hemiparesis and/ or sensory loss.

MRI imaging- including T-1 weighted images (± gadolinium contrast) and T-2 weighted images- is the investigative method of choice, as it enables localization of the tumor and also aids in assessing the extent of infiltration into surrounding tissue and the tumor's contrast enhancing ability. GBMs usually show up as hypo-intense to intense on T-1 images and, if intravenous contrast has been injected, a ring-like pattern of enhancement is seen, suggestive of leaky vessels typical of high grade malignant gliomas [25]. On T-2 weighted and FLAIR images, in contrast, these masses show up as hyper-intense compared to normal brain tissue. Some of the more advanced tools that may provide further information about the tumor's biochemistry, physiology and metabolic rate include MR spectroscopy, MR perfusion and F-18 fluorodeoxyglucose- positron emission tomography scanning (FDG-PET). These can also help once therapy has begun, to distinguish areas of radiation necrosis from tumor recurrence [25], which appear very similar on FLAIR images.

The current standard of care of treatment for gliomas (Grade III & IV) often involves a combination of Surgery, Radiation therapy (RT) and Chemotherapy. Surgery enables debulking of the tumor which facilitates a lowering of the intracranial pressure and provides tissue that can be examined microscopically to form a definitive diagnosis. However, the median survival time with surgery alone is 3-6 months [26]. While surgical resection removes much of the malignant tissue and provides rapid symptomatic relief, a sizeable number of

residual tumor cells remain that have infiltrated the brain parenchyma and cannot be visualized. This accounts for the frequent recurrence and dismal survival time seen in these patients. In order to eliminate these cells, and prevent further recurrences, the patient is subjected to external beam fractionated radiotherapy as well as chemotherapy. Currently, the most widely used drug for the latter course of therapy is temozolamide (TMZ). It has been demonstrated that the 2-year survival rate for patients with GBM has gone from 10% to 40% from 2000 to 2010, since the practice of administering TMZ concomitantly with radiation has been adopted [27]. Despite the multimodal approach to therapy the median survival of patients with GBM treated with RT and TMZ is only marginally better than that in patients treated with RT alone (14.6 months versus 12.1 months, respectively) [26]. Hence, intensive research is being conducted- at the bench and at the bedside- to identify new targets and better drug delivery techniques. These strategies are predominantly aimed at identifying proteins (growth factors/ growth factor receptors/cytokines/transcription factors and so on.) that are aberrantly expressed in tumor cells versus their normal counterparts, and restoring them to their normal levels.

One such protein that has been found to be highly expressed by glioma cells is a zinc finger transcription factor- Wilms' Tumor-1 (WT1) [28]. While WT1 plays a major role during the development of several different organ systems, its expression has been found to be down-regulated in most of the adult tissues- including adult human astrocytes [29]. Previous studies in our lab have shown that although absent in normal human astrocytes, WT1 is expressed in a significant number of glioma cells from primary tumor specimens and GBM cell lines [28]. The level of expression varied directly with the tumor grade, and down-regulating the protein was found to decrease cell viability in vitro and in vivo, while

increasing cellular sensitivity to chemo- and radiation therapy [30-32]. Given its transcription factor status, WT1 might mediate these effects by promoting and maintaining the malignant phenotype through the regulation of other genes. Little is known about the exact role of this protein in the pathogenesis of brain tumors: an understanding of the basic structure and functions of WT1 is therefore essential in order to better understand the possible mechanism(s) by which it can facilitate tumorigenesis.

Wilms' Tumor-1:

The Wilm's Tumor- 1 protein (WT1) is a transcription factor belonging to the zinc-finger family. The gene encoding this protein- *wt1*- was first cloned in 1990 [33] and has been localized to the chromosome 11p13 [34, 35]. The mRNA transcript consists of ten exons [36], with the two most common splicing site variations involving the inclusion or exclusion of exon 5 and/or exon 9 (Fig.). Exon 5 codes for an in-frame 17-amino- acid peptide and exon 9 codes for a tripeptide consisting of lysine, threonine, and serine (KTS). This tripeptide sequence is inserted in-frame between the 3rd and 4th zinc fingers [37]. It has been demonstrated that presence of both splice inserts (+Exon 5/+KTS) is the most common variant and the absence of both (-/-) is the least [36]. Further, the ratio of +KTS to −KTS isoforms, under normal physiological conditions, has been found to be 2:1 [34].
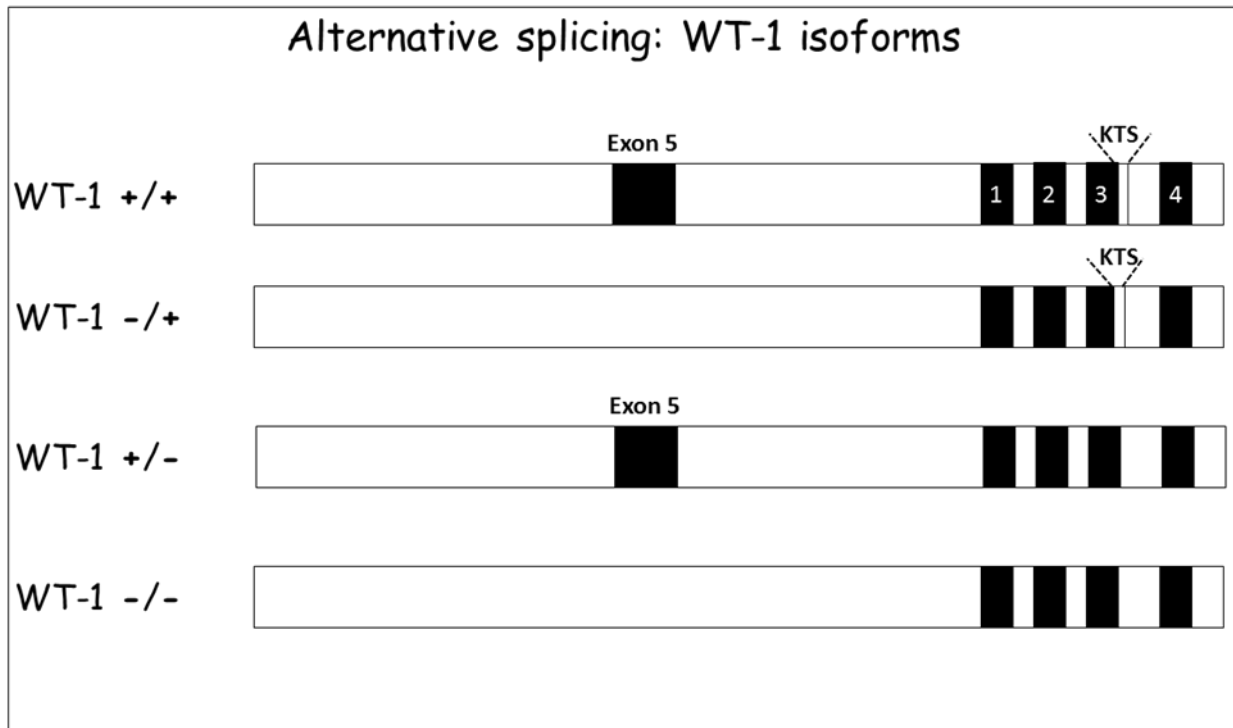
Fig. 1-1: Schematic representation of the four most commonly studied isoforms of WT1. The inclusion or exclusion of exon 5 and KTS tripeptide give rise to these four isoforms. Numbers 1-4 represent the zinc fingers that bind DNA.

Other isoforms arise from utilizing alternate start sites that may be upstream of the regular start site or within the exons. Certain exons are subject to RNA editing [38] giving rise to additional differences in the protein product. Recently, another isoform of WT1, called Alternative WT1 (AWT1) was discovered, which was found to be encoded from within the first intron and consequently has an alternative exon 1 sequence [34, 38]; this protein shows exon 5/exon 9 splice variants similar to its regular counterpart. Another isoform has been isolated in different cancer cells, and its start site lies at the end of the fifth intronic sequence [38]. This diversity in coding start sites, alternative splicing and mRNA editing contributes to the existence

of nearly 36 isoforms [38] known today, and does not preclude the possibility of more such variants being discovered in the future.

The net result is the production of the WT1 protein, a 52-54 kDa protein [36], that serves as an important regulator of several growth and developmental processes. Early experiments using WT1 knockout mouse models showed that a congenital absence of WT1 protein led to death (by apoptosis) of the cells constituting the metanephric blastema, and renal agenesis by E12 [39, 40]. However, the fatality associated with WT1 gene knockout in these mouse embryos appears to have resulted from severe cardiovascular anomalies [24]. Eliminating expression of the protein *after* E13 in these animals resulted in a disruption of differentiation of the metanephric mesenchyme, and not apoptotic cell death. Hence, it has been argued that the role of WT1 in promoting proliferation versus facilitating differentiation would depend, among other things, on the differentiation status of the cells involved.

Structurally, WT1 protein consists of an amino terminal that is rich in proline and glutamine, and a carboxy terminal that has four adjacent Krupple-like residues, two each of cysteine and histidine [34, 35]. These residues chelate zinc, thereby forming a "zinc finger," which then fits neatly into the groove of the DNA helix. The zinc fingers of the WT1 are designated I through IV. The N-terminal mediates interactions of the protein with RNA and other proteins. It is critical for the transcriptional regulatory activities of the protein as proven by deletion studies [34]. The AWT1 lacks the first 147 amino acids due to an alternative start site. Consequently, it lacks a functional repression domain[34]. Madden et al. [41] showed that the transcriptional repression site (R) was located between amino acid residues 70 to 180, while the transcriptional activation domain (A) was juxtaposed, located between amino acid residues 180 to 250. Later, it was shown that this R domain could directly suppress the function of the A domain- by self-

association or through interaction with a nuclear protein, and this inhibition was not cell-type specific [42].

The zinc fingers have important interactions with proteins- p53, prostate apoptosis response factor-4 (Par-4), E1b, Ciao1 and U2AF65 [34, 42], which can modify the function of the WT1. Zinc fingers I, II and III also contain nuclear localization signals [36]. The key purpose of these sites though, is to serve as the DNA binding motifs of which they recognize a variety. These include GC- rich sites that resemble the Egr-1 consensus sequences, with which zinc fingers II-IV share ~61% homology (5'-GCGGGGGCG-3') [36], a high-affinity WTE site (5'-GCGTGGGAGT-3') [43], or $(TCC)_n$ motif [44].

Studies have revealed that +KTS isoforms- which account for most of the WT1 present in cells- could not bind the established consensus sites for WT1 with as much ease as the −KTS isoform [45-47]. The insertion of the KTS tripeptide between the 3rd and 4th zinc fingers increases the flexibility of the linker region between them, which de-stabilizes the DNA-protein complex, and in turn leads to a decreased affinity of this particular isoform for DNA binding sequences [45, 46]. The alternative splicing event of WT1, to include or exclude KTS, is evolutionarily conserved [48], and its consequences led scientists to deliberate over its significance. Their search for an answer led to the discovery of an additional role for WT1- namely post-transcriptional regulation.

The concept of a transcription factor that can also regulate post-transcriptional processes, although unusual, has been acknowledged [49-52]. The earliest findings pointing to a role for WT1 in post-transcriptional regulation established that + and - KTS isoforms of WT1 differentially localized with splicing factors and transcription factors, which were RNase and DNase sensitive, respectively [53, 54]. The RNA recognition motif was then located in the protein sequence [55], and

subsequent studies demonstrated that WT1 co-localized with several splicing factors- such as U2AF65 and WTAP among others [56-58]. The presence of WT1 in nuclear poly (A)$^{+}$ ribonucleprotein (RNP) particles [54] and, more recently, RNP particles in acute myeloid leukemia cells[59], its ability to shuttle between the nucleus and cytoplasm and localization of the protein in functional polysomes [60] all substantiated its newly proposed role. That WT1 protein also bound mRNA transcripts was demonstrated both *in vitro* and *in vivo* [48, 49, 61] and the zinc fingers were found to play a role in this RNA binding interaction [49, 61]. Significantly, a protein that binds the poly-A tail of a mRNA transcript can increase the stability of the latter by protecting it from rapid destruction [62]. Recent experiments have also demonstrated that WT1+KTS, in association with the Constitutive Transport Element (CTE), can promote the translation of unspliced transcripts with retained introns [63].

WT1 in malignancies

In the developing embryo, WT1 is expressed in the kidney (condensing metanephric blastema and podocytes), brain (area postrema), spinal cord (ventral horn motor neurons), spleen, the gonadal ridge mesothelium and the mesothelial lining of all organs [64]. In the adult, WT1 is normally expressed in the glomerular epithelium of the kidney, uterine decidualcells, granulosa cells of the ovary and Sertoli cells of the testes [64].The importance of WT1 was first discovered by virtue of its absence leading to the development of Wilms' tumor or nephroblastoma, a pediatric renal malignancy that was first described by Karl Max Wilms in 1899 [34-36]. This childhood tumor was thought to arise from mesenchymal blastema cells that undergo abnormal proliferation instead of differentiating into metanephric structures. It was, therefore, naturally

postulated that the WT1 functions as a tumor suppressor in this context, and most studies assiduously set about proving this point.

It was subsequently discovered that most Wilms' tumors also contained wildtype WT1 [43], as opposed to earlier findings. WT1 expression is seen to be activated in a variety of other tissue types as the cells undergo malignant transformation. These tissues can be of epithelial (carcinomas of the gastrointestinal tract, lung or hepatocellular carcinoma), mesenchymal (bone- and soft tissue sarcomas, desmoplastic small round cell tumors or DSRCTs), ectodermal (e.g. gliomas, melanomas) or hematopoietic (leukemias and others) origin [28, 59, 65-78]. Using immunohistochemical staining across a broad spectrum of tumor specimens, it was observed that the protein localized in different sub-cellular compartments depending on the tumor type [79]. In tumor specimens isolated from patients with GBM, some soft tissue sarcomas, osteosarcomas, and malignant melanomas of the skin, WT1 was found predominantly in the cytoplasm. However, in certain other malignancies, such as ovarian tumors and desmoplastic small round cell tumors, WT1 was found in the intranuclear compartment.

Ye at al. [80] demonstrated that in the region of its DNA-binding domain- the zinc fingers, WT1 could be phosphorylated *in vitro* by a variety of purified kinases including cAMP dependent kinases such as Protein Kinase A (PKA), Protein Kinase C (PKC) and Casein Kinase II (CKII) as well as by those obtained from nuclear extracts. Interestingly, they showed that phosphorylation of WT1 resulted in an impairment of its ability to bind DNA but not mRNA, and activating PKA significantly influenced the sub-cellular compartmental localization of WT1 by trapping the protein in the cytoplasm. In the particular case of brain tumors, the intra-cytoplasmic localization of the protein [51, 79, 81] might enable it to interact with the RNA, stabilize

it, and alter its processing and translation, leading to elevated levels or a mutant form of the protein. This could be one way in which WT1 can function as an oncogenic protein in gliomas.

Moreover, the cytoplasmic localization of WT1 has been linked with its binding to actin filaments via the zinc finger domain [82]. Interfering with actin depolymerization resulted in an abolition of nucleo-cytoplasmic shuttling of WT1, moved it off the cytoplasmic polysome fraction and impaired its ability to bind DNA and RNA. The study concluded that WT1 might function as an adaptor molecule, hoisting a specific subset of mRNAs on to the actin cytoskeleton for transporting to the target location. In a separate study using stably transduced TYK (ovarian carcinoma) cells, 6 other carcinomatous cell lines and one fibrosarcoma cell line with the different WT1 isoforms mentioned above, it was noted that in 5 out of the 8 cell lines, the expression of WT1(-/-) isoform induced morphological changes[83]. Further studies in TYK cells showed that these morphological changes were associated with decreased cell-substrate adhesion, increased cellular invasion, decreased levels of α-actinin 1 and cofilin expression, and an increase in gelsolin expression, i.e. an alteration in cytoskeletal dynamics. On a similar note, WT1 was found to be expressed in a significantly higher number of vertically growing melanoma cells compared to radially spreading ones, again arguing for a possible role of WT1 in facilitating tumor cell invasion [76].

WT1 has also been found to be expressed in the endothelial cells of tumors, consistent with its role during development where it significantly contributes to the normal vascularization of the embryonic heart [84]. 113 tumor specimens from different tissue types were analyzed and WT1 protein was found to be expressed in the vascular endothelial cells of 108. WT1 was also shown to increase the proliferation and migration of endothelial cells by regulating the levels of the Ets-1 transcription factor in these endothelial cells [85].

An important aspect of studying WT1 biology involves ascertaining its target genes. This list and the regulatory effect usually vary from one cell type to another. For example, while WT1 was found to activate the transcription of E-cadherin (*Cdh-1*) in stably transfected mesenchymal fibroblasts- the NIH3T3 cells [102], in epicardial cell extracts from mouse embryos, WT1 was found to *repress Cdh-1* expression [86]. This cell-specific effect may arise from the role of WT1 in regulating a balance between mesenchymal- epithelial transition (MET) or epithelial-mesenchyme transition (EMT) [87]. In developing kidney cells, it has been postulated that WT1 expression facilitates cell differentiation and their exit from the cell cycle (MET). In other tissue types, such as the Sertoli cells and neuronal (+KTS isoforms especially) and vascular progenitor cells, WT1 appears to *protect* cells from apoptosis and facilitates cellular proliferation; in the developing heart, in particular, it has been found to push towards EMT- a state also seen in tumorigenesis.

The transcriptional activity of WT1 has also been found to depend on its expression levels, the predominant isoforms (+KTS versus –KTS) and the relationship of the transcriptional start site to the WT1 DNA binding site (upstream, downstream or both) [34], as was noted with its effect on *PDGF-A*. The PDGF-A promoter was found to have a pair of Egr-1 consensus sequences upstream and a $(TCC)_n$ site downstream of the start site [44], and it was established that transcription of PDGF-A could be activated or repressed, depending upon whether binding of WT1 occurred on one or both sides of the start site, respectively [88-90].

Thus, it is clear that there are several complex issues that need to be addressed to get a clearer understanding of the role that WT1 might play in tumorigenesis, and, more specifically in gliomagenesis. One approach would be to examine its role(s) and target genes in a very tissue- and cell-type specific manner, keeping in mind its ability to interact with other proteins such as

p53 [91], which could also alter its regulatory ability. Moreover, it is rational to study the effects of WT1 in cell-based model systems by altering the levels of endogenously expressed WT1 rather than inducing its expression in WT1 null cells, since the role of WT1 is very isoform- and concentration- sensitive as mentioned previously.

WT1 has also been found to be a potential cancer antigen target on account of two features [92, 93]. The first is based on its oncogenic function in promoting and maintaining the transformed phenotype of malignant cells which ensures its constant expression, and prohibits the escape of tumors from immunological surveillance by down-regulating WT1 expression. Moreover, WT1 is expressed by a variety of malignancies. Hence, it could potentially be used in antigen-directed immunotherapy in several different malignancies. A study conducted by the NCI examined the potential of 75 antigenic molecules to determine which of these were candidate antigens for immunotherapy [94]. Different criteria were utilized in this analysis in the following descending order of importance: (a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location of antigen expression. The results of the study showed that while there was no *ideal* candidate antigen, WT1 was found to be the most promising cancer antigen [94, 95].

It has been pointed as a shortcoming of immunotherapy that, in comparison to chemotherapy, the response rate (which is given by the percentage of complete response plus partial response) is very low [95]. However, in defense of the immune-therapeutic strategy, the mechanism of action is very different from that in chemotherapy in that immunotherapy utilizes the body's inherent defense mechanism to kill tumor cells, which gives rise to a latent period

during which the tumor continues to grow. Thus, it contrasts sharply with the rapid cytolytic effects seen with chemotherapeutic drugs. The advantage with immunotherapy, however, is its low risk of toxicity, enabling repeated vaccinations. This results in disease stabilization or tumor shrinkage after an initial growth phase (late response) or long-term disease stabilization with good quality of life. Hence, it has been suggested that stable disease (SD) should be given more importance when evaluating response to a cancer-antigen directed vaccine, especially if the SD index persists long-term, as was observed to be the case with the use of WT1-specific vaccine in patients with GBM [93]. More specifically, the disease control rate (calculated by the number of patients with complete response, partial response and stable disease) in the 3-month trial period was found to be 57.1% [96]. Current studies in this context aim at determining whether combining WT1 peptide vaccination with the current chemotherapeutic drug of choice- TMZ- is likely to be more efficacious than either treatment alone.

Conclusion

It appears that the WT1 protein has a very complex behavioral pattern. Rather than classifying it as a tumor suppressor or oncogene, it should be viewed as a regulator of several key processes including proliferation, cell migration, angiogenesis and differentiation. Its exact function depends on the cell context and cell differentiation status, among other things. In glioma cells, it is undoubtedly a *promoter* of the malignant phenotype, and a thorough examination of its role and its target gene repertoire is, therefore, of utmost importance. This would enable the development of a multi-target therapeutic approach to deliver a stronger blow while simultaneously circumventing issues such as tumor cells resorting to alternate survival pathways.

Chapter 2: WT1 expression in U1242-MG and GBM-6 cell lines promotes cellular invasiveness.

Abstract

WT1 expression was previously found in our laboratory to be up-regulated in a significant number of glioma cell lines and primary tumor specimens, and this transcription factor was noted to significantly impact cellular invasiveness and cellular proliferation (in vitro and in vivo) in U251-MG cells. This study aimed to analyze its expression in two hitherto unexamined cell lines- U1242-MG and GBM-6- both of which are noted for their invasive potential [99,100]. Using Real Time RT-PCR and Western blotting, we found that WT1 was expressed in both cell lines. Moreover, silencing WT1 expression in these cell lines was associated with a remarkable decrease in their invasiveness in vitro but the proliferative rates of the siRNA-treated cells were similar to their control counterparts. We also sought to examine the effects of WT1 silencing on the expression of some of the previously established target genes- *PDGF-A*, *Snai1*, *E-cadherin* and *IGF-1R*, to account for the functional effects of WT1. While no consistent effect was noted upon manipulating WT1 levels on the expression of *PDGF-A*, *Snai1* and *E-cadherin*, the amount of WT1 inversely correlated with IGF-1R expression in U251-MG cells at the RNA and protein levels. To ascertain whether the increased IGF-1R expression could account for increased cell death seen with WT1 silencing, we performed cell viability assays on cells treated with IGF-1R siRNA. Our results show a trend towards a *decrease* in cell viability upon IGF-1R silencing, suggesting that the decreased cell proliferation caused by WT1 down-regulation in U251-MG cells does not involve IGF-1R mediated paraptosis. We conclude that the

target genes previously established for WT1 are not upheld in the glioma cell lines studied, and further studies are necessary to elucidate its downstream targets in the specific context of glioma cells.

Introduction

Malignant gliomas include Grade III and Grade IV glial tumors and constitute an extremely formidable group of malignant neoplasms that are highly aggressive and resistant to most conventional modes of therapy. Studies are in progress to identify molecules that can be targeted to achieve a better therapeutic outcome. One such intracellular protein that has been implicated for its potential role in the neoplastic progress is the zinc finger transcription factor-Wilms' Tumor-1 (WT1).

WT1 was thought to function as a tumor-suppressor for several years following its initial discovery, and it has been well established as a co-ordinator of several key processes during embryonic development [36, 64]. Most of its functional studies were conducted in NIH3T3 and HEK293 cells [41, 89, 95], wherein reporter assays mostly showed a repressive effect on the transcriptional regulation of most oncogenic proteins. This was followed by a diametrically opposing change towards ascribing an oncogenic role to WT1, as its unusual expression in malignant cells of different tissue types became increasingly evident [59, 65-78]. In most of these tumors, it was noted that down-regulating the endogenous expression of WT1 led to a reversal of the malignant phenotype of the tumor cells [97, 98]. Moreover, it was also demonstrated that not only did WT1 regulate the expression of its targets at the transcriptional level, but it could also exert its influence beyond transcription [53, 54]. At present, researchers studying the intriguing facets of this very complex protein have arrived at the conclusion that interpretation of the roles

and functions of WT1 should be viewed strictly in the context of the the specific features of the cells under investigation, including cell type, cell differentiation status, DNA binding sites available and the isoforms of WT1.

We have previously demonstrated that the aberrant expression of WT1 in glioma cells contributes to enhanced cell proliferation *in vitro* and *in vivo* and resistance to radiation- and chemotherapy [28, 30-32]. Our findings on the effect of WT1 on cell proliferation are in agreement with other studies involving glioma cells as well as other tumors. While it has been shown that immunologically targeting WT1 in patients with gliomas has had promising results [96], aspects of its functional biology in these tumors remains largely unknown. Here, we show for the first time that WT1 is expressed in two highly invasive glioma cell lines- U1242-MG and GBM-6. In these cells, WT1 was found to dramatically enhance cell invasion but not proliferation. Our rationale for selecting these two cell lines was based on previous studies by other groups that have established these cells as closely mimicking the pathology of GBM when implanted orthotopically in athymic mice [99, 100].

In this study, we also sought to identify potential molecular targets for WT1 in facilitating these malignant phenotypic features of glial neoplasms. To do this, we examined the effect of manipulating WT1 on four of its previously established target genes- IGF-1R [101], PDGF-A [88-90], Snai1 and E-cadherin [86, 102]. The latter three candidate proteins were chosen mainly for their facilitation of the invasive process (PDGF-A and Snai1 [14, 103, 104]) or for inhibiting it (E-cadherin [104, 105]). The up-regulation of IGF-1R following WT1 silencing in T98G [30] cells was previously postulated to contribute to a non-apoptotic form of cell death called paraptosis [30, 106]. We set out to ascertain the validity of this hypothesis by first confirming the inverse correlation between these two proteins in U251-MG cells, followed by siRNA

mediated IGF-1R silencing to determine whether it led to enhanced cell proliferation. Our findings show that WT1 is consistently and inversely correlated with IGF-1R expression in U251-MG cells. This inverse correlation, however, was not found to explain the effect of WT1 on promoting cellular proliferation. We also noted a lack of consistent correlation between the expression levels of WT1 and the pro-invasive proteins that we examined- PDGF-A, Snai1 and E-cadherin in the three cell lines tested.

Materials & Methods

Cell culture: Human glioma cell line, U251-MG, was obtained from the ATCC, U1242-MG and GBM-6 cells were kindly gifted by Dr. James Van Brocklyn and Dr. C. David James/Dr. Paul Dent respectively. All cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, glutamine, nonessential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2.

siRNA transfections: Cells were plated in 6-well plates at a density of 1.5-2 x 10^5 cells per well. 24 hours after plating, the cells were transfected as described previously [32]. Briefly, transfections were carried out using the pre-designed siGENOME SMARTpool (Dharmacon) containing 4 siRNA sequences directed against WT1 and/ or IGF-1R. A pool of 4 non-targeting siRNA sequences (siCONTROL Non-Targeting Pool, Dharmacon) was used as a control and will be referred to as siScramble (Scr). The final concentrations of Scr control and anti-WT1 siRNA were 100nM, and for siRNA directed against IGF-1R, the final concentration was 50nM. These siRNA concentrations were determined by a series of optimization experiments. 24 hours after transfection, 10% DMEM supplemented with 1% penicillin/streptomycin was added. 48 hours after transfection, the cells were harvested with trypsin, and following re-suspension of the

cell pellet in 10% DMEM, the cells were re-plated for RNA extraction for qRT-PCR, protein extraction for Western Blotting and for cell proliferation assays and/ or invasion assays.

Plasmid construction and transient transfection: WT1 expression plasmids (in pcDNA3, Invitrogen) containing either WT1 (+/+) or WT1 (-/+) isoforms were kindly provided by Dr. Charles T. Roberts, Jr. and have been described previously [32]. Cells were plated in triplicate at a density of 2 x 10^5 in six well plates and allowed to attach overnight in DMEM without penicillin/streptomycin. Cells were transfected with a mixture of 3μl Lipofectamine 2000 (Invitrogen) and 1.0μg plasmid diluted in 500μ1 DMEM without fetal bovine serum or penicillin-streptomycin and incubated for 4 hours at 37^0 C. After 4 hours, cells were supplemented with 1.5 ml DMEM with 10% fetal bovine serum without penicillin-streptomycin. Twenty four hours after transfection, the conditioned media was removed and replaced with 2.0 ml fresh DMEM with 10% fetal bovine serum without penicillin/streptomycin. Forty eight hours after transfection, cells were selected in DMEM containing 600μg/ml Geneticin (Invitrogen). Selection media was changed every 4 days.

RNA Extraction: Total RNA was extracted using Trizol (Invitrogen) as per the manufacturer's protocol. Following RNA extraction, the RNA content was quantified using the spectrophotometer, and roughly 12μg of RNA per sample were subjected to DNase treatment using RQ1 DNase (Promega). Samples were then analyzed using Real Time RT-PCR. Synthesis of probes and primers for WT1 was carried out at the VCU Nucleic Acid Research Facilities. The Taqman primer mixes for the target genes were purchased from Applied Biosystems. All Real Time PCR reactions were performed at the VCU Nucleic Acid Research Facilities.

Western Blotting: Protein was extracted from cell lines using SDS buffer (50 mM Tris-C1, 1% SDS, 10% glycerol) supplemented with protease inhibitors. The concentration of protein was determined by DC protein assay (Bio Rad). For protein analysis of cells transfected with WTl, 10µg of total protein lysate were separated by SDS-PAGE and transferred to nitrocellulose membrane as per manufacturers' protocol (Invitrogen). The membrane was then blocked with 5% nonfat milk solution for 1 hour at room temperature. Mouse anti-WT1 monoclonal antibody (1:200, Clone: 6F-H2, Dako), rabbit anti-IGF-1R polyclonal antibody (1:1000, Cell Signaling) were diluted in blocking buffer and the blots were incubated with the respective primary antibodies overnight at 4^0 C. The membranes were then washed six times in tris buffered saline containing 0.05% Tween-20 before and after a 1-hour incubation at room temperature with horseradish peroxidase-conjugated anti-mouse (1:2000) and anti-rabbit (1: 3000) secondary antibodies directed against anti-WT1 and anti-IGF-1R, respectively. Anti-Cyclophilin A monoclonal antibody (1: 30000, Upstate Biotechnology) or Anti-beta actin monoclonal antibody (1:5000, Sigma) was used as a control for protein loading. Blots immunostained for WT1 were developed using Pierce Supersignal West Dura Substrate and those for IGF-1R were developed using the ECL™ Western Blotting Detection Reagents (Amersham Biosciences).

Cell proliferation Assay: Cellular proliferation was measured at specified time-points after transfection using the CellTiter-Glo® Luminescent Cell Viability assay (Promega). Cells were plated in 3-5 replicates per treatment group in opaque 96-well plates 48 hours after *transfection.* These cells were lysed for 15 minutes in Cell-titer Glo ATP viability assay reagents as per the protocol at specified time points following transfection. Relative luminescence was then detected on a Lumistar luminescence plate reader (BMG Technologies). Each experiment was performed in triplicate. Validation studies in our laboratory demonstrate that this assay provides highly

reproducible results with strong linear correlation ($R^2 > 0.98$) to cell counting with the Trypan exclusion technique, and with high throughput capacity (data not shown).

Invasion assay: Matrigel (BD Biosciences), previously aliquoted was thawed at 4^0 C on ice overnight before use. 48 hours post-transfection, cells were plated in triplicate on Matrigel-coated 8.0µm Pore Polycarbonate Membrane Inserts of 6.5mm Transwell® plates (Corning-3422) at a density of 5000 cells/500µL. 96 hours following transfection, media was removed from the filters and the lower chambers, the upper part of each filter was scraped with cotton-tipped applicators to remove the cells that had not invaded, and each lower chamber was rinsed with PBS. 200µL of trypsin-EDTA (Invitrogen) was added to each chamber to harvest the cells, and 800µL of 10% DMEM was used to neutralize the enzyme. The lower part of each filter was rinsed with media, in order to collect cells that had invaded through the Matrigel-coated insert, but not adhered to the base of the lower chamber. After centrifugation at 1000 rpm for 5 minutes to collect the cells, the supernatant was removed from each tube, and fresh media was added to a volume of 500µl. The entire volume was then plated (100µl/well) into an opaque 96-well plate for assaying the ATP content as described above. Each experiment was conducted in triplicate.

Statistical Analysis: All comparisons were made between two groups- cells treated with non-targeting siRNA (scr) and those treated with siRNA directed against WT1 or IGF-1R. Each experiment was performed at least three times. Results were analyzed using Student's T-test (two-tailed, paired). Significance level was set at p value <0.05. Error bars were calculated using standard deviation measurements.

WT1 is expressed in GBM-6 and U1242-MG cell lines. Using western blotting, we detected WT1 expression in these cell lines (Fig.2-1). Cell extracts from the prostatic carcinoma cell line (PC3) was used as a positive control for WT1. Both GBM6 and U1242-MG show the characteristic WT1 double band, suggesting the possibility of the + and − exon 5 variants that differ by 17 amino acids, at the predicted molecular weight level (52-54 kDa).

Figure 2-1: WT1 expression in GBM-6 and U1242-MG cells. Using western blotting technique, WT1 protein was detected in these highly invasive cell lines. 20 µg of protein was loaded in each lane. Cell lysates in Lane A: PC3 (control), lane B: GBM-6, lane C: U1242-MG.
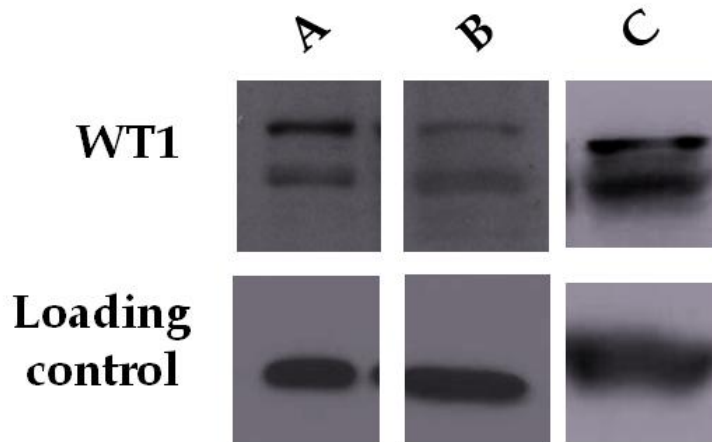
Silencing WT1 inhibits cellular invasiveness in GBM-6 and U1242-MG cells in vitro. Using siRNA directed against WT1, we confirmed efficient silencing of this transcription factor by Real Time RT-PCR (Fig.2-2A). On the third day after transfection, we found that WT1 level in GBM-6 cells that had been treated with si- WT1 was 14.44% (S.D.: ± 2.89) of that seen in its control counterpart that had been treated with non-targeting siRNA (n=3, **p≤0.001*). In U1242-MG cells, there was less knockdown (50.47% [S.D.: ± 3.49] of control, which was nevertheless highly significant (n=3, **p≤0.001*).

We observed that these decreases in cellular content of WT1 remarkably depressed the invasive capacity of these cells (Fig.2-2B). In GBM-6 cells, there was nearly a 95% decrease (S.D.: ± 22.65) in the ability of the siWT1-treated cells to invade through the Matrigel-coated filter (n=3, *p≤0.05*) compared to the control cells, while in U1242-MG cells, silencing WT1 caused a decrease in cellular invasiveness to 20.62% (S.D.: ± 1.12) of the control cells (n=3 **p≤0.001*). These differences in the ability to invade could potentially be accounted for by decreased proliferative rates in the siRNA transfected cells versus control cells. Hence, we performed cell viability assays on these same groups of cells. Our results showed that the growth rates of control and treated cells were not significantly different across three independent experiments (Fig.2-2C). Also, based on our previous experiments we found no statistically significant differences between untreated and non-targeting siRNA (scr) treated cell groups. Hence, we show only comparisons between the scr and targeting siRNA treated groups.

Figure 2-2: WT1 silencing in GBM-6 and U1242-MG cells causes decrease in cellular invasiveness. A. RNA was extracted at day 3 post-transfection. WT1 silencing was confirmed using Real Time RT-PCR (n=3, $p \leq 0.001$). WT1 expression in siWT1 treated cells is first normalized to the loading control and expressed as a percentage of their control counterparts. B.WT1 down-regulation was associated with a decrease in GBM-6 and U1242-MG cellular invasiveness at day 4 post-transfection (n=3, *$p < 0.05$, **$p < 0.001$; RLU= relative luminescence units). Cell invasiveness in WT1 silenced cells is expressed by calculating the ATP content in the si groups versus scr groups and then expressed as a percentage of that seen in control cells. C. GBM-6 and U1242-MG cells that were treated with siRNA directed against WT1 exhibited proliferative rates similar to the cells that were treated with non-targeting siRNA (n=3; RLU= relative luminescence units).

A
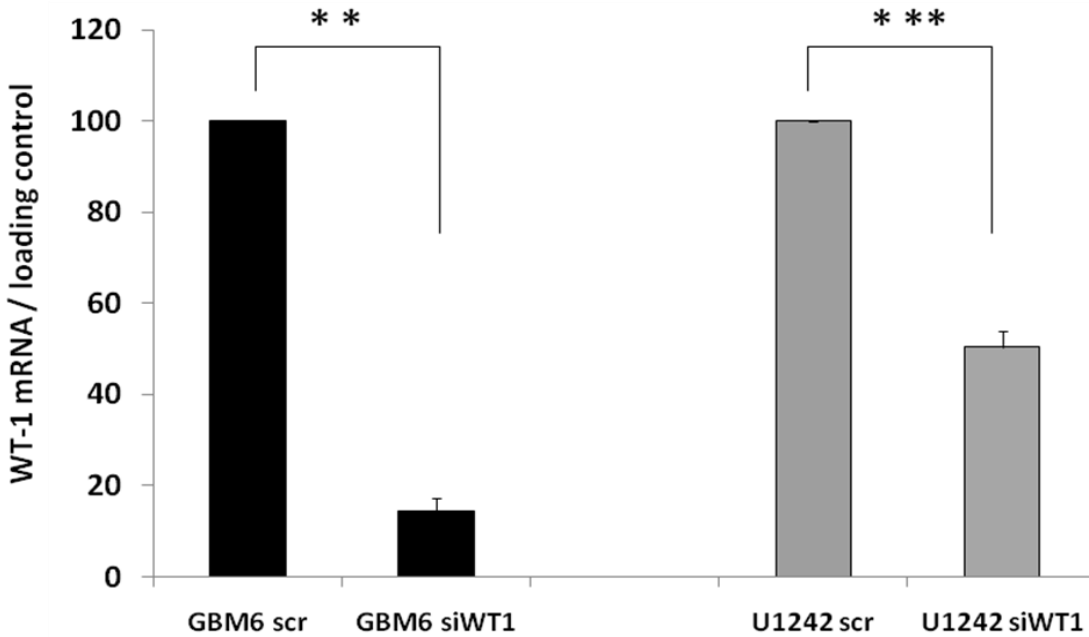
B
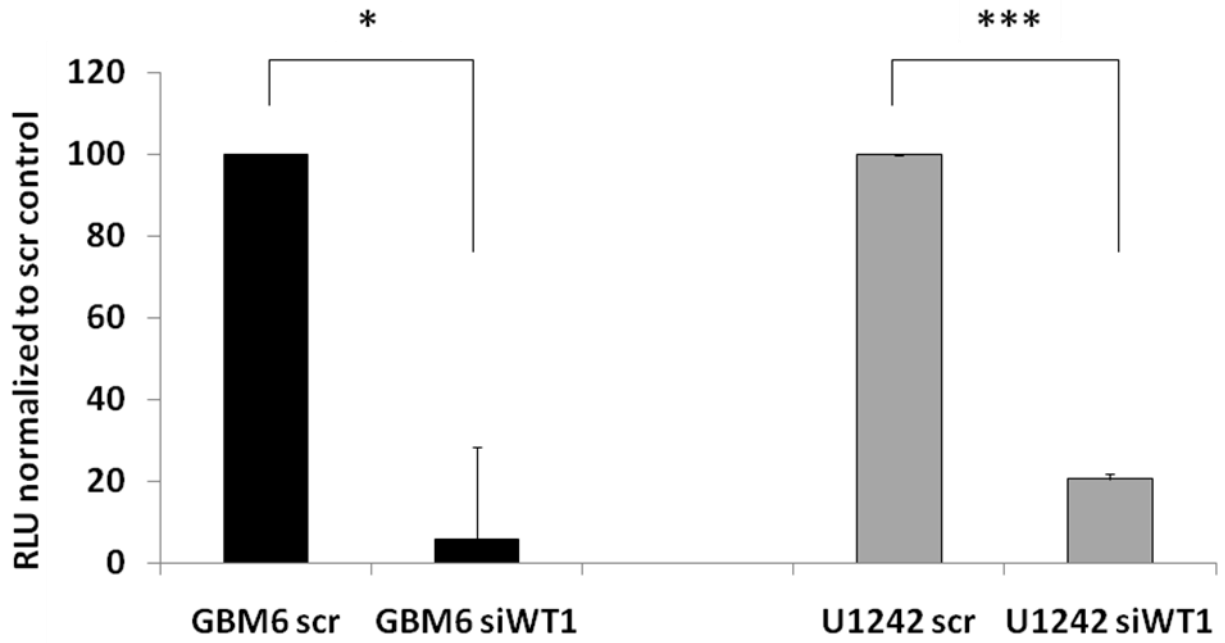
C
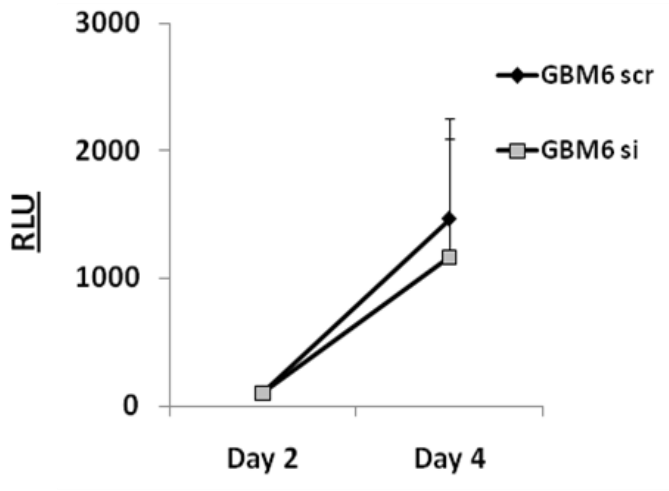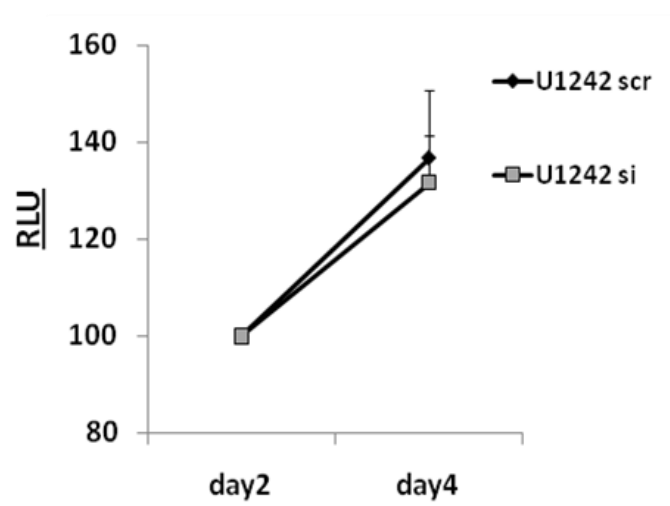
GBM-6 cells   U1242-MG cells

WT1 expression in U251MG cells correlates inversely with IGF-1R levels, but not with PDGF-A. We have previously shown a significant decrease in cellular proliferation *in vitro* and *in vivo* with WT1 silencing [31]. Our current results in U1242-MG and GBM-6 cells also confirm our previous findings in U251-MG cells that WT1 plays a major role in regulating cellular invasiveness. Based on these findings, we chose PDGF-A, Snai1, E-cadherin and IGF-1R to determine if WT1 might mediate these effects on invasiveness and proliferation by regulating the expression(s) of any of these genes. We could find no consistent effect upon manipulating WT1 expression on the level of PDGF-A, Snai1 or E-cadherin (data not shown).

However, in U251-MG cells, silencing WT1 was associated with an increase in IGF-1R protein expression (Fig. 2-3A). We transduced U251-MG cells with plasmids containing WT1 (+/+) and (-/+) isoforms as these are the most predominant isoforms seen in glioma cells. The increase in WT1 expression as a result of this manipulation was associated with a decrease in IGF-1R expression at the RNA level as shown in Fig. 2-3B (n=3). Over-expressing WT1 (+/+) resulted in a marked increase in WT1 protein expression and was accompanied by a reduction of approximately 50% (S.D.: ± 13.35) in IGF-1R protein expression while only a small increase in WT1 protein expression with WT1 (-/+) transfection paradoxically increased IGF-1R protein levels (Fig.2-3C). Notably, silencing WT1 expression in U1242-MG and GBM-6 cells had no effect on IGF-1R expression levels (data not shown). Thus, it appears that in U251-MG cells, and as per previous studies in T98G cells [30], WT1 expression correlates inversely with IGF-1R.

Figure 2-3: WT1 levels vary inversely with expression of IGF-1R in U251MG cells. A. WT1 silencing was associated with increased IGF1R expression at the protein level (representative blot). 20 μg protein was loaded in each lane. Cyclophilin was used as a control to ensure equal protein loading. B. WT1 over-expression was carried out using WT1 (+/+) and WT1 (-/+) isoform containing plasmids. Cells transduced with an empty plasmid served as Vector Control (VC). IGF-1R expression was specifically and consistently low in cells that had been transfected with WT1 (+/+) plasmid at the RNA (n=3, p=0.05) and (Fig.2-3C) protein levels. U251-MG cells transfected with WT1 (-/+) did not exhibit a decrease in IGF-1R protein level, despite a decrease in IGF-1R mRNA levels. Representative blot is shown. All experiments were replicated $\geq$ 3 times with similar results.

A
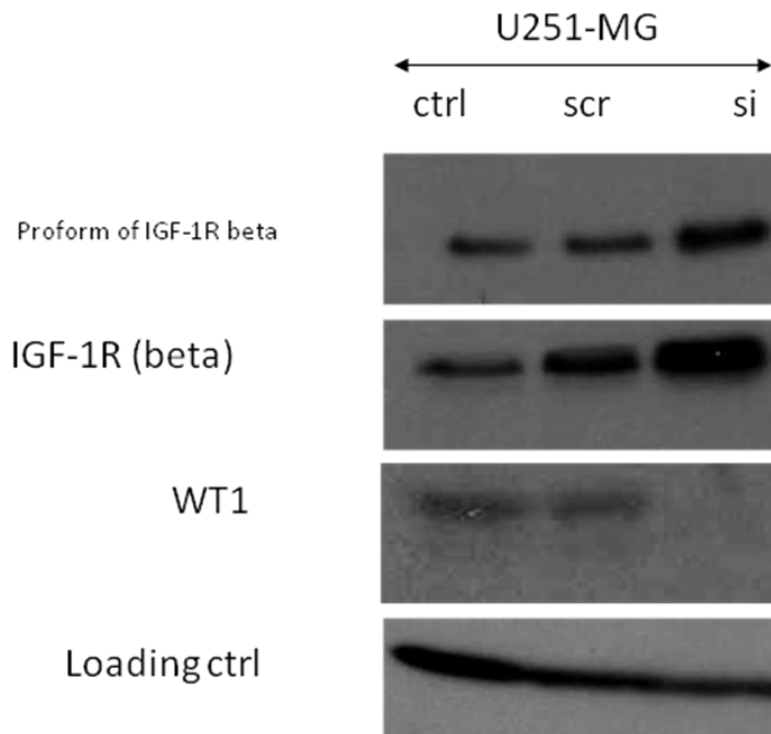
B
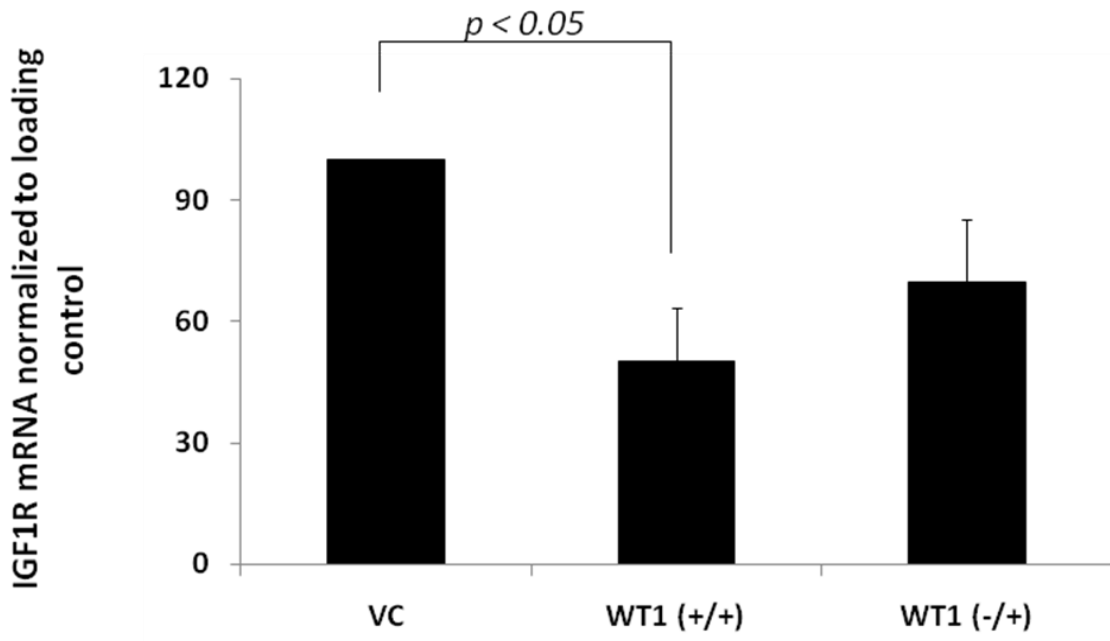
C
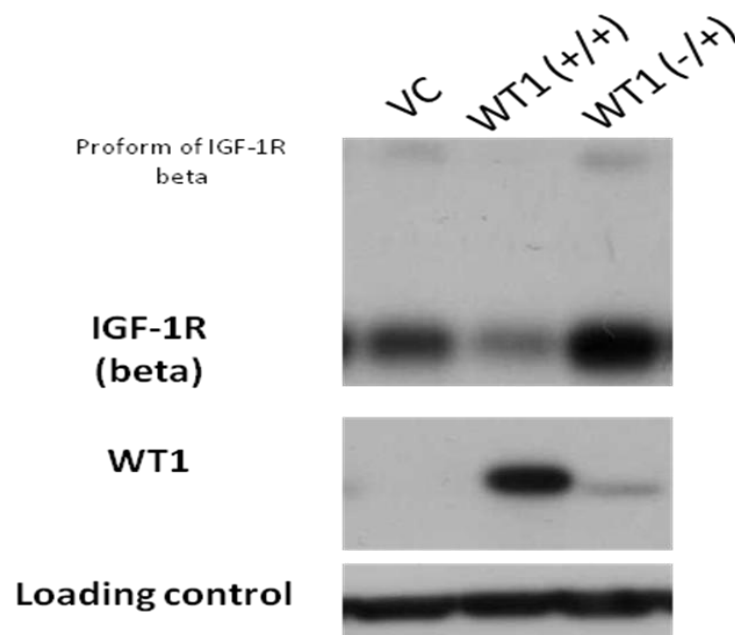
IGF1R silencing decreases cellular proliferation in U251-MG cells. The increase in IGF1R expression seen as a result of WT1 silencing could potentially decrease the ability of these cells to proliferate by means of a non-apoptotic cell death called paraptosis [106]. Conversely, it could be a compensatory response by the cells to escape death due to eliminating the effects of WT1. In order to ascertain which of these two scenarios was more likely, U251-MG cells were transfected with siRNA against IGF-1R. Initial optimization experiments revealed that using a concentration of siRNA of 50nM delivered a 69% decrease in IGF-1R protein even at day 5 following transfection (Fig.2-4A). This dose was then used to determine the optimum time period of IGF-1R knockdown, and we found that this was at day 2 and day 3 following transfection (Fig. 2-4B). On the 2nd post-transfection day, the IGF-1R mRNA level in si cells was about 42.7% the level in scr controls (n=3, p< 0.01) and 51.42% of the level in untreated control cells (n=3, p< 0.5). Cell proliferation assays were carried out until day 5 post-transfection. At this time point, we observed that the IGF-1R mRNA level in siRNA treated cells was still decreased to 58.93% and 71.79% of the levels in the scr and control cells respectively (n=3, p > 0.05). While there was no difference in proliferative abilities between the three experimental groups up to day 4 despite maximal RNA knockdown at days 2 and 3 post-transfection, silencing IGF-1R expression was generally found to lead to a decrease in proliferative rates (Fig.2-4C). However, this was not observed to be statistically significant between the non-targeting siRNA and IGF-1R siRNA treated groups, but only between the untreated and IGF-1R siRNA treated groups (n=3, *p<0.05*).

Fig.2-4: IGF-1R silencing in U251-MG cells decreases cellular proliferation. A. Western Blot densitometry results showing the effect of IGF-1R knockdown at day 5 post-transfection. Optimization experiments were conducted with different concentrations of siIGF-1R: 5-, 10-, 50- and 100 nM (n=1). Maximal knockdown efficacy at this time-point was seen with [siIGF-1R] = 50nM. B. Real Time RT-PCR data showing maximal IGF-1R levels suppression between scr and si groups on Days 2 and 3 post-transfection, and between untreated (ctrl), non-targeting siRNA treated (scr) and siWT1 treated (si) groups on Day 3 post-transfection (n=3, * $p < 0.05$, ** $p < 0.01$). C. Cell proliferation assay demonstrating a significant but small decrease in cell viability in siIGF-1R treated cells as compared to the untreated cells at day 5 post-transfection (n=3, * $p < 0.04$; RLU= relative luminescence units). No statistical significance was observed between scrambled RNA and siRNA treatment groups.

A
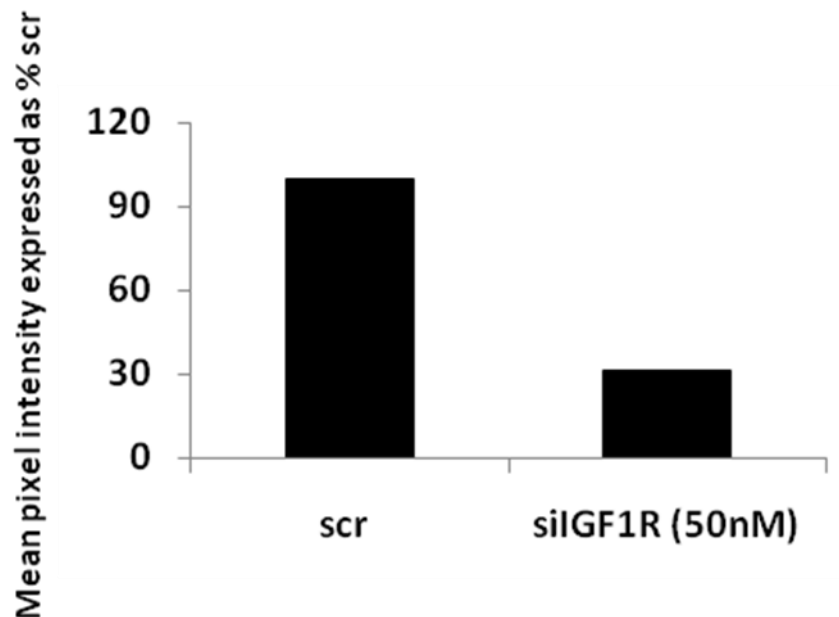
B
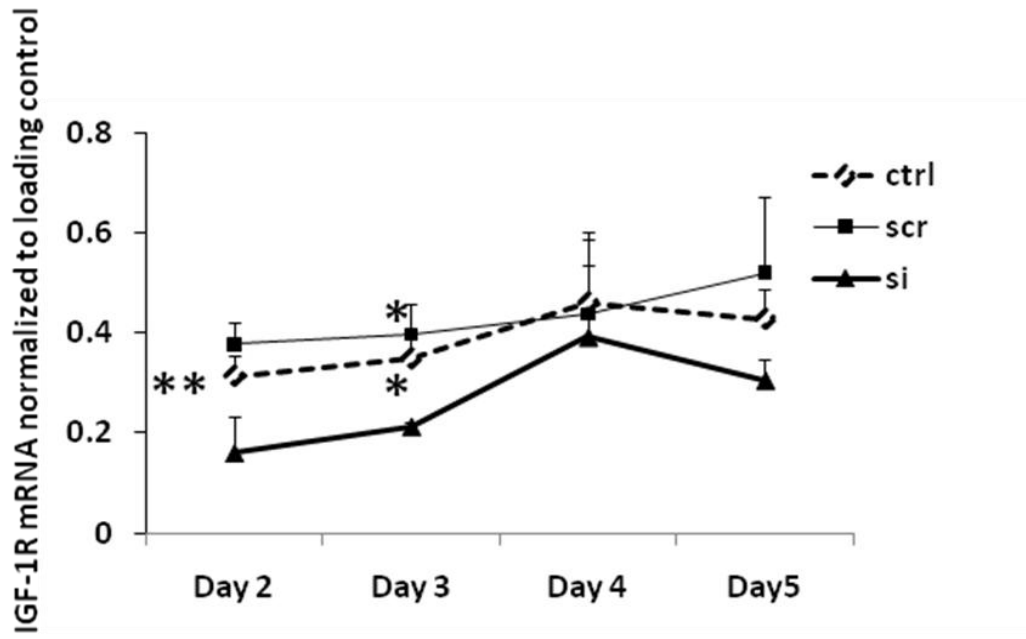
C
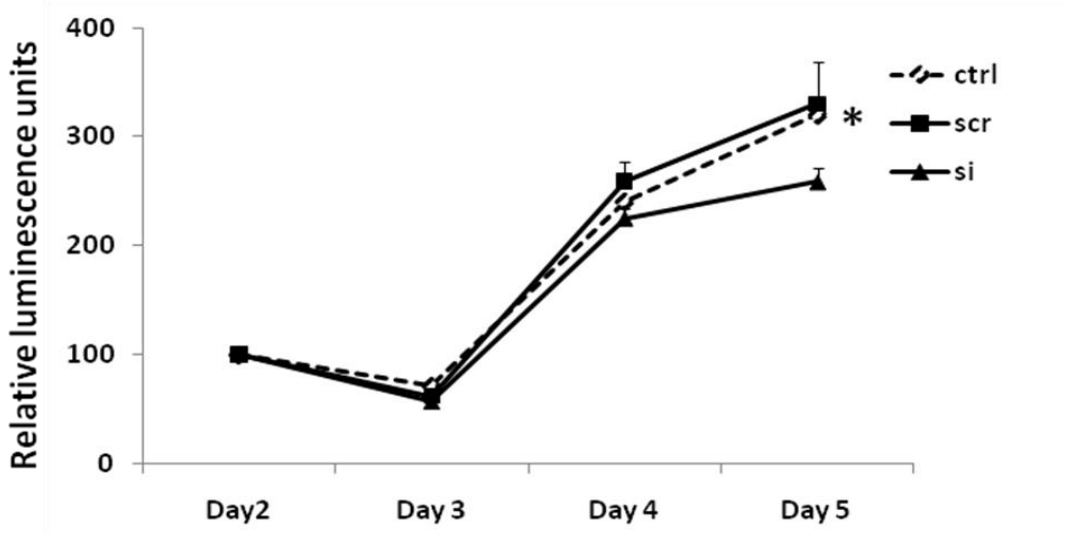
Discussion

We have previously shown that WT1, a zinc finger transcription factor, is expressed in a significant number of glioma cells, and impacted tumor cell proliferation in U251-MG and T-98G, invasiveness, and resistance to radiation- and chemotherapy [28, 30-32]. In this study, we have shown that WT1 is expressed in two additional glioma cell lines- U1242-MG and GBM-6 and in these cells it is seen powerfully affect their invasive abilities. A decrease in the endogenous expression of WT1 decreases the invasive capacity of glioma cells, despite equivalent proliferative rates. These differences in invasiveness appear *not* to involve the PDGF-A or Snai1 signaling pathways or down-regulation of E-cadherin. We also demonstrate here that in U251-MG cells, WT1 level is inversely correlated with that of IGF-1R, similar to our previous findings in T98-G cells [30]. IGF-1R silencing, however, resulted in a trend towards *decreased* cell viability, suggesting that its up-regulation with WT1 silencing might represent activation of an alternate survival pathway.

In malignant cells, contrary to what was believed earlier, recent evidence has leaned more in favor of a putative oncogenic role(s) ascribable to WT1 [30, 31, 65-79, 85]. It is worth reiterating that the functions of this highly complex protein are variable and should therefore be interpreted strictly based on the cell context in which they are studied. It has been established that the ability of WT1 to promote proliferation versus differentiation also depends upon the differentiation status of the cells involved. For example, its ability to induce mesenchymal-to-epithelial transformation (MET) leads to an exit from the cell cycle of developing cells in the kidney and their consequent differentiation to generate nephrons [38]. However, in other tissue types- such as neuronal and vascular progenitor cells- WT1 protects cells from apoptosis, and maintains cells in the proliferative, undifferentiated state [38]. Further, we postulate that the ability of WT1 to

influence proliferation in glioma cells might also depend upon other factors, such as the PTEN status and/ or its interactions with other proteins. It was interesting to note that in U251-MG and T98G cells, where WT1 significantly impacted cellular proliferation, the PTEN status was either null or mutated [107, 108]. In U1242-MG and GBM-6 cells, on the other hand, WT1 was found to influence migratory properties and not proliferation. Both these cell lines have wildtype PTEN [109, 110]. There is no documentation at present regarding the influence of PTEN status on the ability of WT1 to regulate cell proliferation. Interestingly, there is evidence that the presence of wildtype PTEN acts as a deterrent in the cellular invasiveness process by mechanisms that are independent of its effect on cellular proliferation [3]. Yet, despite the presence of wildtype PTEN in U1242-MG and GBM-6 cells, there appears to be no inhibition of invasion, in the presence of WT1. Thus, a complex interplay exists between proteins belonging to different intracellular signaling pathways in a malignant cell, and it would be interesting to determine if an interaction between WT1 and PTEN contribute towards the functional effects of each in glioma cells.

There have been several reports implicating WT1 in fostering invasiveness of different types of tumor cells [76, 82, 83, 111, 112]. More recently, a new binding partner for WT1 has been revealed in actin- a key component of the cytoskeleton that dictates such important processes as changes in cell shape, movement and cell division [82]. It is clear from our results that in glioma cells, WT1 co-ordinates cellular motility. The earliest studies that sought to determine WT1's target genes highlighted *PDGF-A* as being both- transcriptionally activated as well as repressed by WT1, depending upon the cell context and the locations of the binding sites for WT1 on its promoter [88-90]. Considerable data exists demonstrating the importance of the PDGF-signaling pathway in glial neoplasms (reviewed in 15). However, in our experimental models, we failed to

find a consistent correlation between WT1 and PDGF-A expression levels, and therefore rule out this interaction as an explanation to the former's impact on cell migration.

Snai1 and E-cadherin are both well established regulators of the crucial process of epithelial-mesenchymal transition (EMT) (reviewed in 113). Snai1 down-regulates the expression of E-cadherin and pushes the cell towards a more undifferentiated phenotype, with a consequent decrease in inter-cellular adhesive forces (also a consequence of decreased E-cadherin levels) and increase in cellular motility [33, 103-105, 114]. In vascular progenitor cells, *Snai1* was shown to be transcriptionally activated by WT1, while *E-cadherin* was shown to be repressed [86]. However, altered expression of these genes could not be shown to explain the enhanced cell motility conferred upon glioma cells by WT1.

IGF-1R is known to be a key factor in promoting cellular transformation. However, there have been reports ascribing a unique role to this growth factor receptor, in promoting a non-apoptotic form of programmed cell death called paraptosis [106]. Similar to the findings described in that study, we have previously shown that in T98G cells treated with WT1 siRNA, there were no discernible differences in markers for apoptosis or autophagy, but the cell viability was, nevertheless, markedly decreased, along with inverted light microscopy features of prominent cytoplasmic pleomorphism and vacuolization [30]. These changes were associated with an up-regulation in IGF-1R expression in this cell line, as was seen with the U251-MG cells. Surprisingly however, when we silenced IGF-1R expression using siRNA in U251-MG cells, the cells displayed a decrease in their proliferative capacity. This suggested that the up-regulation of IGF-1R with WT1 silencing was not a satisfactory mechanism to explain the decrease in cell viability, but, in fact, might point to activation of an alternate survival pathway that these malignant cells are notorious for. Interestingly, the maximal knockdown of IGF-1R mRNA was

observed at days 2- and 3 post- transfection, but cell proliferation rates were noticeably low only at day 5 post-transfection at which time point the protein levels of IGF-1R were also low. This observed delay in the effect on cell proliferation might be accounted for by pre-existing functional IGF-1R protein that continues to promote the proliferative potential of tumor cells in the immediate post-transfection period. This can be roughly evaluated by assessing the levels of phosphor-IGF1R- the activated form of the receptor protein.

To summarize, we have demonstrated a novel role for WT1 in enabling glioma cells to invade, *in vitro*. The target gene/s that help(s) it achieve this process, as well as those that enable it to promote cellular proliferation, remain(s) to be discovered and form the focus of our current and future studies. Considering that it is the property of cellular invasiveness that makes these tumors difficult to treat, the need to identify the molecules mediating this process can easily be recognized as imperative. A more in-depth examination of its target gene(s) and their contributions will undoubtedly facilitate the development of a multi-targeted therapeutic strategy, which might be our only hope in effecting a cure for this deadly disease.

Chapter 3: Candidate target genes for WT1 in glioma cells- Possible involvement of CD97 in mediating its effects on glioma cell invasiveness.

Abstract

WT1 is a zinc finger transcription factor with well-established roles in the development of different organ systems during embryonic development. Its expression is absent in normal adult astrocytes, but is switched back on in astrocytomas, and the more aggressive tumors exhibit increasing levels of expression. While in U251-MG and T-98G glioma cell lines, this protein has been demonstrated to influence tumor cell proliferation, it has also been found to play a very prominent role in mediating cell invasiveness as we have shown previously. Although its functional effects have been studied to some extent and this knowledge has been employed in devising therapeutic strategies to immunologically target WT1, little is known yet about the molecular allies that help it mediate these oncogenic functions in the specific context of glioma cells. Here, we present for the first time in glioma cells, the identities of some of these proteins. Using gene expression analysis, we found that silencing the endogenous expression of WT1 in U251-MG cells was associated with the down-regulation of several genes that have putative oncogenic functions, and the up-regulation of some proteins considered to have tumor-suppressor effects. When compared to normal human astrocytes, the expression levels of most of the candidate target genes that directly correlated with WT1 were higher in glioma cells. We validated the microarray findings at the RNA level using Real Time RT-PCR, and also confirmed these correlations in two other glioma cell lines- U1242-MG and GBM-6- which are well known for their high invasive capacities [99,100]. One of the genes that was found to be consistently and significantly decreased upon WT1 silencing in all three cell lines studied was

CD97. We sought to ascertain whether this protein, which has been implicated in facilitating cell invasiveness and even angiogenesis in gastrointestinal carcinomas and other tumors, has any effect in mediating the pro-invasive effects of WT1. Our invasion assay results *in vitro* showed that cells that had been treated with siRNA directed against CD97 displayed a markedly lower propensity to invade as compared to their control counterparts. We conclude that CD97 has a very important role to play in dictating one of the most characteristic features of glial tumor cells, and further studies are imperative to clarify the extent of its impact.

Introduction

The Wilms' Tumor-1 (WT1) protein was originally believed to function as a tumor-suppressor. Early studies showed that the protein repressed the transcription of several oncogenes such as *PDGF-A*, *TGF-beta*, *EGFR*, *IGF-1R* and so on, and increased the expression of genes that prevented malignant transformation such as *E-cadherin*, and pro-apoptotic molecules (reviewed in 34). This impression, however, began to undergo a remarkable transformation, as it was increasingly noticed that the ability of WT1 to transcriptionally activate or repress a putative target depended largely on the cell type, the isoforms studied, the number of WT1 binding sites present on the target promoter and other factors. The factor that most put into serious doubt WT1's tumor-suppressor status, however, was the escalating incidence of its discovery in malignant cells from several different tissue types (reviewed in 115). A key regulator of various developmental processes, WT1 expression was known to be switched off as tissues developed into the normal adult stage [36, 38-40]. However, wildtype WT1 was found to be expressed in leukemias and other hematopoietic malignancies, lung and breast cancers, gastrointestinal cancers, sarcomas, tumors in the head and neck, reproductive organ neoplasms, and gliomas (reviewed in 115). In most or all of these tumors, WT1 was not merely expressed,

but appeared to play a decisive role in promoting proliferation, invasion and/ or angiogenesis [30, 31, 65-80, 82-85].

We have previously demonstrated aberrant WT1 expression in a significant number of glioma cell lines and tumor specimens [28]. The predominant WT1 isoforms expressed in these cells was discovered to be the one containing exon 5 *and* the tripeptide KTS between zinc fingers 3 & 4 (WT1 +/+). Our studies showed that glioma cells that had endogenous WT1 expression relied heavily on the protein for their growth and motility. WT1 also conferred upon these cells resistance to radiation therapy and some chemotherapeutic agents [30, 32]. The question then arose from these observations: How does WT1 mediate these effects in glial neoplasms?

Given its structural identity- a zinc finger transcription factor- and its functional history, it seemed logical to hypothesize that WT1 might regulate the transcription (or post-transcriptional expression) of other genes, which might then directly cause the above-mentioned effects. However, when we looked for possible correlations between WT1 and its previously established targets- PDGF-A and IGF-1R, we found that in glioma cells WT1 had no consistent effect on PDGF-A, while its inverse correlation with IGF-1R levels appeared *not* to be related to a regulatory mechanism. Therefore, in order to identify which genes might vary in glioma cells in their expression patterns as a result of WT1-mediated regulation, we utilized the gene expression profiling technique to characterize differential gene expression. This method enables rapid screening of the entire genome to find all the possible candidate genes that are differentially expressed as a result of any manipulations. Our analyses revealed the identity of some such presumed or established oncogenes whose levels paralleled that of WT1 in glioma cells- *PDGF-D*, *TYMS*, *INPP5A*, *CD97* and *FAM57A*. Genes that were increased as a result of WT1 suppression, on the other hand, included putative tumor suppressors- such as *LZTS1*,

TIMP3, *MAFF*, *WIPI-1* and so on. We selected *CD97* for further investigations, based on its consistent and direct correlation with WT1 across the GBM cell lines examined and because of its putative roles in facilitating cell invasiveness and neo-angiogenesis in gastrointestinal and thyroid tumors. This is the first time that expression of this protein has been reported in glial neoplastic cells.

CD97 is a cell surface receptor that belongs to the Adhesion-G-Protein Coupled Receptor (GPCR) family and is therefore characterized by heptahelical hydrophobic segments that form the seven-transmembrane spanning domain (TM7), an extracellular N-terminus containing epidermal growth factor (EGF)- like structural domains (comprising the α-subunit) and an intracellular C-terminus [116]. Through alternative splicing of the mRNA transcript, three isoforms of the α-subunit of CD97 can be generated, that differ in the number of EGF repeats they contain- these isoforms are hCD97 (EGF 1,2,5), hCD97 (EGF 1,2,3,5) and hCD97 (EGF 1,2,3,4,5) [117]. Also present on the nucleotide sequence of the extracellular segment, after the EGF-like repeats and before the first membrane-spanning sequence at position 318 is an RGD motif. This motif has been established to act as a binding site for several classes of integrins, which are well known for their role in mediating attachment to the extracellular matrix and to other cells [117]. The ligands that have been found to bind CD97 include CD55/ Decay Accelerating Factor (DAF) (involved in protection from complement mediated attack and lysis), Chondroitin Sulfate (a glycosaminoglycan that affects cell attachment), and $\alpha_5\beta_1$ integrin [118]. Interestingly, the intracellular signaling mechanism(s) by which CD97 and other EGF-TM7 family members might act is/are yet unclear [119].

CD97 expression has been reported mainly in leukocytes and in this group, it has been found predominantly in myeloid cells. Besides these, its expression has also been reported in

smooth muscle cells and in epithelial tumors [118]. Its expression in thyroid carcinoma cells has been linked with promoting de-differentiation [120], while in colorectal and gastric carcinomas and fibrosarcomas, its expression has been alleged to promote cellular invasiveness [119]. Our results corroborated the findings from these earlier studies and we confirm that in glioma cells, too, CD97 appears to influence cellular invasiveness. Future studies will be directed at confirming whether WT1 regulates expression of this protein at the transcriptional and/or at the post-transcriptional level(s).

Materials & Methods

Cell culture and reagents: Normal human astrocyte RNA (Cat.No.: 1805) was procured from ScienCell Research Laboratories. Cell lines used are described on page 31.

siRNA transfections: The procedure for siRNA transfection has also been described on page 31. The final concentration of non-targeting siRNA control, anti-WT1 and anti-CD97 siRNA was 100nM and determined by performing optimization experiments. 48 hours after transfection, the cells were harvested with trypsin, and following re-suspension of the cell pellet in 10% DMEM, the cells were re-plated for RNA extraction for qRT-PCR and microarray analyses, protein extraction for western blotting and for cell proliferation assays and/ or invasion assays.

RNA extraction: The technique for RNA extraction and processing was similar to that described on page 32.

Western blotting: Western blotting using cell lysates was performed exactly as described on page 33. Rabbit anti-CD97 polyclonal antibody (Abcam, 1:200) were diluted in blocking buffer and the blots were incubated with the respective primary antibodies overnight at 4^0 C. After this, the membranes were washed six times in Tris buffered saline containing 0.05% Tween-20 before

52

and after a one hour incubation at room temperature with horseradish peroxidase-conjugated anti-rabbit (1: 3000) secondary antibodies directed against anti-CD97 respectively. Anti-Cyclophilin A monoclonal antibody (1: 30000, Upstate Biotechnology was used as a control for protein loading. Blots were developed using Pierce Supersignal West Dura Substrate.

Cell proliferation assay: This assay has been described on page 33.

Invasion assay: Technique described on page 34.

Gene Expression Profiling: The quality of total RNA sample as well as cDNA and cRNA synthesis products was assessed by running 1 µL of every sample in RNA 6000 Nano or DNA 7500 LabChips on the 2100 Bioanalyzer (Agilent, Palo Alto, CA), following the manufacturer's protocol. Quality control criteria included cDNA and cRNA synthesis products within median lengths of 2.0 and 3.0 kb, respectively, and 3'/5' ratios close to 1.00 for both housekeeping genes, *GAPDH* and β-actin [121]. The microarray reactions were performed using the Affymetrix GeneChip Standard protocol. The "significance-score" algorithm (S-score) developed by Dr Li Zhang was used to produce a score for the comparisons of the expression summaries between cell groups [122]. The Minimum Information About a Microarray Gene Experiment guidelines have been met with, and the microarray raw data has been deposited with the National Center for Biotechnology Institute Gene Expression Omnibus (GEO), accession number GSE22578.

Promoter analyses: The UCSC genome browser was used to and nucleotide sequences that lay 1000 base-pairs upstream and down-stream of the start site were selected. These regions were entered into the MatInspector software and searched for the presence of potential binding sites for WT1. The following criteria were used to select sequences of interest: a. Sequences

belonging specifically to the WT1 matrix b. Sequences that had a matrix similarity of ≥ 0.9 and c. Sequences that lay on the positive strand.

Statistical analysis: All comparisons were between two cell groups- cells treated with non-targeting siRNA or untreated cells and cells treated with siRNA directed against WT1 or CD97. Each experiment was replicated at least three times. Student's T-test (two-tailed, paired) was used to determine statistical significance. Error bars were calculated using standard deviation measurements.

Results

Gene Expression Profiling reveals putative target genes for WT1 in glioma cells. In order to ascertain the identity of the potential target genes for WT1 in the particular context of glioma cells, we chose to perform the gene expression profiling experiments, which have often been reliably used for this purpose. U251 cells were transfected with siRNA against WT1 and after confirming WT1 knockdown with Real Time RT-PCR, the microarray reactions were carried out using the standard Affymetrix GeneChip Standard protocol. Quality control assays were performed and RNA that met with the criteria that have been described in detail from previous studies in our laboratory [121, 123] was utilized for microarray analyses. Fig. 3-1 shows the supervised cluster analyses from three independent experiments. It is immediately evident from the dendrogram above the heat map that the control and scr cell groups cluster together and separately from cells that were treated with siWT1. To the right side of the heat map is a list of all the genes whose levels were found to be altered with this manipulation. WT1 (highlighted in yellow) appears prominently down-regulated as a result of our experimental treatment and speaks for successful down-regulation using siRNA knockdown. Also, there is a high level of

consistency in the patterns of up- or down- regulation of the selected genes, among the three replicates for each group of cells. Analyses of the data obtained in the 3 sets of experiments are summarized in Table 2, revealing a total of 27 genes that were down-regulated and 11 genes that were up-regulated, in response to WT1 silencing. These genes passed two separate levels of stringency for being declared significantly altered- the S-score which establishes significance at the univariate level and the Benjamini-Hochberg correction which corrects for multiple comparisons [122].

The genes that were down-regulated with WT1 silencing, implying a direct correlation, included several molecules (identified by searching the available literature) that have either putative or established oncogenic roles in gliomas and/or other malignancies. These included *INPP5A*, *TYMS*, *PDGF-D*, *CD97*, *EPAS1*, *FAM57A* and *HSPC111* (marked by asterisks and bold lettering, Table 2). Conversely, the genes that inversely correlated with WT1 levels included some prominent tumor-suppressors such as *SSAT1*, *LZTS1*, *WIPI1*, *TIMP3*, and *DPYSL3* (marked by asterisks and bold lettering, Table3). Table 4 summarizes the information that is currently available regarding the role(s) played by each of these genes.

Fig.3-1: Supervised Cluster Analysis. Two-dimensional hierarchical clustering of samples and genes using Pearson (centered) correlation and average linkage. Three independent transfection experiments were performed for each microarray analysis. The dendrogram demonstrates that control and scr groups of cells cluster together and separately from the siWT1 treated cells. Each row in the heat map below the dendrogram shows the relative expression for that specific gene in the 9 individual samples (columns). The color scale at the bottom of the heat map represents the relative gene expression levels (0-fold to 3-fold) and the red and green areas correspond to over-expression and under-expression, respectively. The genes that are differentially altered by silencing WT1 expression are listed to the left of the heat map. WT1 is highlighted in yellow to distinguish it from the other genes on the list.
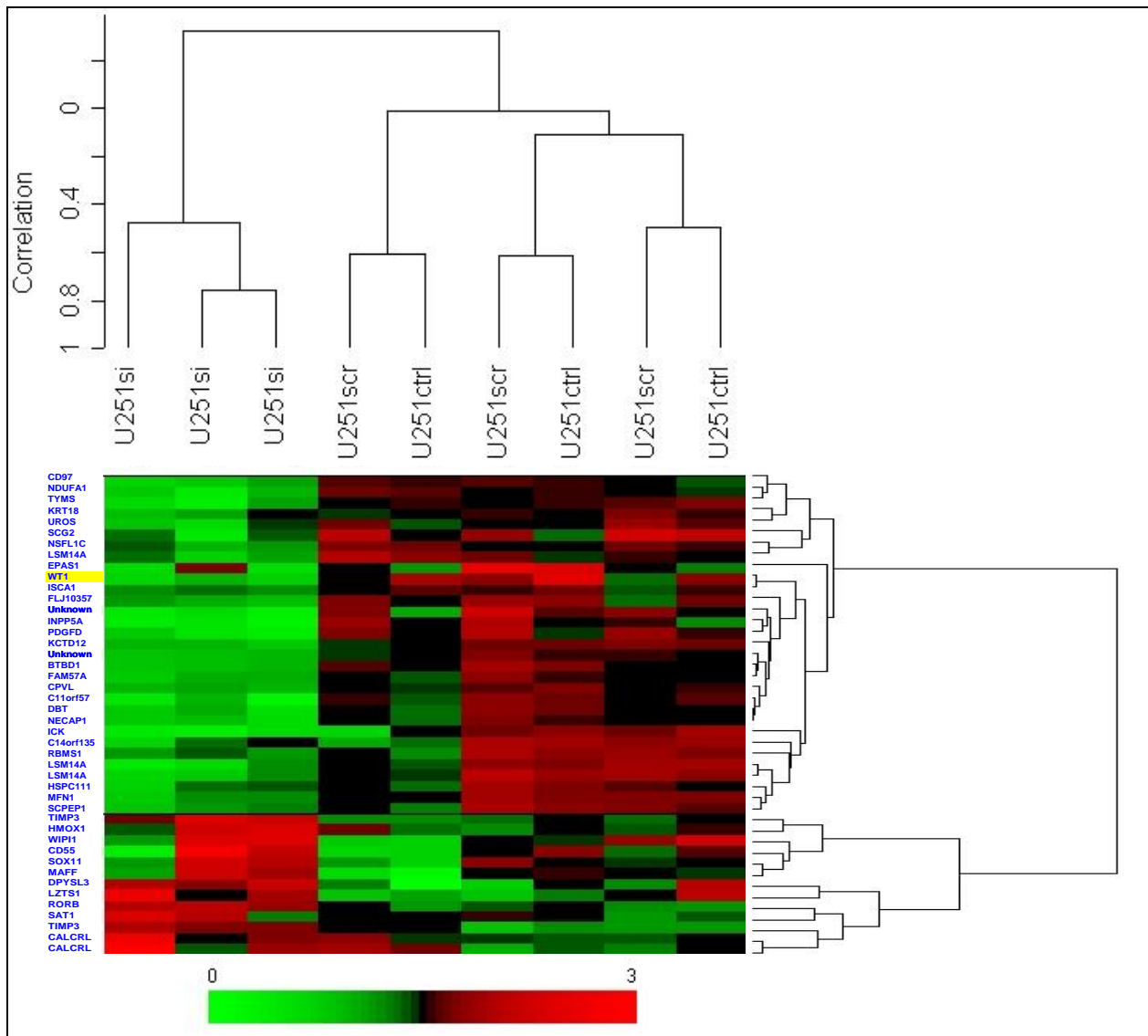
Table 2: Gene expression profiling demonstrating the fold change of genes that are significantly down-regulated in WT1-silenced U251MG cells. Total down-regulated genes= 27 (including WT1- not shown in table). Genes that have putative or established roles in suppressing cancers are highlighted with both asterisks (*) and green bold lettering. A total of three independent microarray experiments were conducted.

| Gene Title	Gene symbol	Fold change si Vs ctrl	Fold change si Vs scr
* intestinal cell (MAK-like) kinase	ICK	0.189	0.287
* inositol polyphosphate-5-phosphatase, 40kDa	INPP5A	0.25	0.1649
chromosome 11 open reading frame 57	C11orf57	0.25	0.25
* thymidylate synthetase	TYMS	0.26	0.287
* platelet-derived growth factor-D	PDGF-D	0.26	0.176
LSM14A, SCD6 homolog A (S. cerevisiae)	LSM14A	0.287	0.26
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	NDUFA1	0.287	0.287
dihydrolipoamide branched chain transacylase E2	DBT	0.33	0.307
potassium channel tetramerisation domain containing 12	KCTD12	0.33	0.33
* CD97 molecule	CD97	0.33	0.307
NECAP endocytosis associated 1	NECAP1	0.33	0.307
BTB (POZ) domain containing 1	BTBD1	0.33	0.307
* endothelial PAS domain protein 1	EPAS1	0.353	0.287
hypothetical protein FLJ10357	FLJ10357	0.353	0.353
secretogranin II (chromogranin C)	SCG2	0.353	0.233
mitofusin 1	MFN2	0.353	0.33
carboxypeptidase, vitellogenic-like	CPVL	0.353	0.3789
LSM14A, SCD6 homolog A (S. cerevisiae)	LSM14A	0.353	0.33
chromosome 14 open reading frame 13	C14orf135	0.3789	0.3789
uroporphyrinogen III synthase (congenital erythropoietic porphyria)	UROS	0.3789	0.33
* family with sequence similarity 57, member A	FAM57A	0.3789	0.353
serine carboxypeptidase 1	SCPEP1	0.3789	0.33
NSFL1 (p97) cofactor (p47)	NSFL1C	0.406	0.3789
* hypothetical protein HSPC111	HSPC111	0.406	0.353
iron-sulfur cluster assembly 1 homolog (S. cerevisiae)	ISCA1	0.406	0.435
* keratin 18	KRT18	0.406	0.3789
RNA binding motif, single stranded interacting protein 1	RBMS1	0.406	0.353

Table 3: Gene expression profiling demonstrating the fold change of genes that are significantly up-regulated in WT1-silenced U251MG cells. Total up-regulated genes = 11. Genes that have putative or established roles in suppressing cancers are highlighted with both asterisks (*) and red bold lettering. A total of three independent microarray experiments were conducted.

Gene title	Gene symbol	Fold change si Vs ctrl	Fold change si Vs scr
CD55 molecule, decay accelerating factor for complement (Cromer blood group)	CD55	2.46	2.828
* spermidine/spermine N1-acetyltransferase 1	SAT1	2.639	2.639
* WD repeat domain, phosphoinositide interacting 1	WIPI1	2.639	2.828
calcitonin receptor-like	CALCRL	2.639	3.03
calcitonin receptor-like	CALCRL	2.639	2.639
* leucine zipper, putative tumor suppressor 1	LZTS1	2.828	3.73
* TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	2.828	3.03
heme oxygenase (decycling) 1	HMOX1	2.828	2.828
* SRY (sex determining region Y)-box 11	SOX11	2.828	2.46
* v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	3.03	2.828
RAR-related orphan receptor B	RORB	3.24	2.828
TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	3.24	3.48
* dihydropyrimidinase-like 3	DPYSL3	3.48	3.73

Table 4: Functions of candidate target genes identified using microarray. A literature search was conducted to determine the functions of all the genes that were revealed to be dys-regulated by WT1 silencing. Of the 27 down-regulated genes (green), 9 were found to possess an oncogenic function. Conversely, of the 11 up-regulated genes (red), 6 were found to be associated with tumor-suppressor activity. Corresponding references are listed in the table below in parentheses.

Gene symbol	Function(s)	Reference
ICK	Promotes proliferation, G1 cell cycle progression in intestinal epithelial cells	[124]
INPP5A	Potential role in promoting radio-resistance of U343-MG cells.	[125]
TYMS	High proliferative rate in cells; translational repression of p53; resistance to chemotherapeutic drugs	[126-128]
PDGF-D	Promotes proliferation, migration, invasion, angiogenesis	[129]
CD97	Promotes cellular invasion, angiogenesis	[118, 120, 130]
EPAS1	VEGF up-regulation → angiogenesis	[131]
FAM57A	Promotes proliferation of lung cancer cells	[132]
HSPC111	Estrogen and c-myc target; associated with adverse outcome in breast cancer cells	[133]
KRT18	Promotes invasion (melanoma and breast cancer)	[134]
SAT-1	Promotes cell death	[135]
WIPI-1	Involved in autophagy	[136]
LZTS1	Inhibition of cell cycle progression	[137, 138]
TIMP3	Promotes apoptosis; inhibition of invasiveness.	[139-141]
MAFF	Transcriptional transactivator involved in transformation	[142]
DPYSL3	Inhibits invasiveness of prostatic carcinoma cells; inhibits neurite outgrowth	[143, 144]

Candidate genes identified as correlating directly or indirectly with WT1 expression levels compared among normal human astrocytes and GBM cells. We then examined Normal Human Astrocytic (NHA) RNA to determine whether these candidate targets were expressed therein. As shown in Fig. 3-2A and corroborating evidence from previous studies, WT1 was not detectable in adult non-malignant human astrocytes. Our results (Fig.3-2A) also show that the genes that correlate directly with WT1 levels are expressed at extremely low levels or are undetectable in the normal adult cells. No expression could be determined for *INPP5A* or *PDGF-D* either, and the expression levels for *TYMS*, *CD97* and *FAM57A* (normalized to beta-actin) were 0.05, 0.075 and 0.049 (n=1), respectively. However, glioma cells U251-MG, U1242-MG and GBM-6 have much higher levels of all these genes. For each cell line (as for the NHA RNA), target gene values were normalized to the corresponding value of beta actin for that cell line. These normalized values in U251-MG cells (n=3) were observed to be: WT1: 0.058 ($\pm$ 0.058), INPP5A: 1.835 ($\pm$ 0.488), TYMS: 1.042 ($\pm$ 0.06), PDGF-D: 13.13 ($\pm$ 4.187), CD97: 0.792 ($\pm$ 0.2), FAM57A: 0.577 ($\pm$ 0.11). For U1242-MG cells (n=3), the normalized values were as follows: WT1: 0.253 (S.D.: $\pm$ 0.196), INPP5A: 1.88 ($\pm$ 0.0.239), TYMS: 0.61 ($\pm$ 0.96), PDGF-D: 0.074 ($\pm$ 0.019), CD97: 0.564 ($\pm$ 0.0.075). Finally, for GBM-6 cells (n=3), the normalized values for WT1 and candidate gene expression levels were as follows: WT1: 0.504 ($\pm$ 0.051), INPP5A: 1.12 ($\pm$ 0.917), TYMS: 1.707 ($\pm$ 0.284), PDGF-D: 2.808 ($\pm$ 0.733), CD97: 1.942 ($\pm$ 0.946), FAM57A: 2.631 ($\pm$ 1.441). The genes that correlated inversely with WT1 as per the microarray findings were somewhat variable in their expression patterns in the 4 different cell types studied (Fig. 3-2B). Only the expression of TIMP-3 was found to be high in the normal state and suppressed in tumor cells (0% of NHA content in U251-and U1242-MG cells, and 10% of NHA levels in GBM-6 cells). This is consistent with the hypothesis that in the process of

malignant transformation of cells, TIMP3 expression is down-regulated in association with the up-regulation of WT1. However, the experiment will have to be repeated multiple times to confirm these findings. This relation was also evident, though less dramatic, with the expression level of *MAFF*, which was decreased to roughly 77% and 85% of the NHA level in U251-MG and GBM-6 cells respectively, while its expression in U1242-MG cells couldn't be determined. From among the other genes whose levels were inversely correlated with that of WT1 as per the microarray analyses, we found that *WIPI* was down-regulated in U251-MG cells to 48% of the level seen in NHA, but remarkably up-regulated in U1242-MG cells. In GBM-6 cells, expression of this gene could not be determined at all.

Fig. 3-2: Aberrant expression of candidate target genes in glioma cell lines. RNA extracted from NHA (n=1), and the GBM cell lines (n=3) - U251-MG, U1242-MG and GBM-6 were analyzed using Real Time RT-PCR for WT1 and the candidate target genes identified using the microarray. All values are shown after normalizing by the corresponding beta actin value. A. Genes that directly correlated with WT1 as per the microrarray findings. B. Genes that were found on gene expression profiling to correlate inversely with WT1 levels.
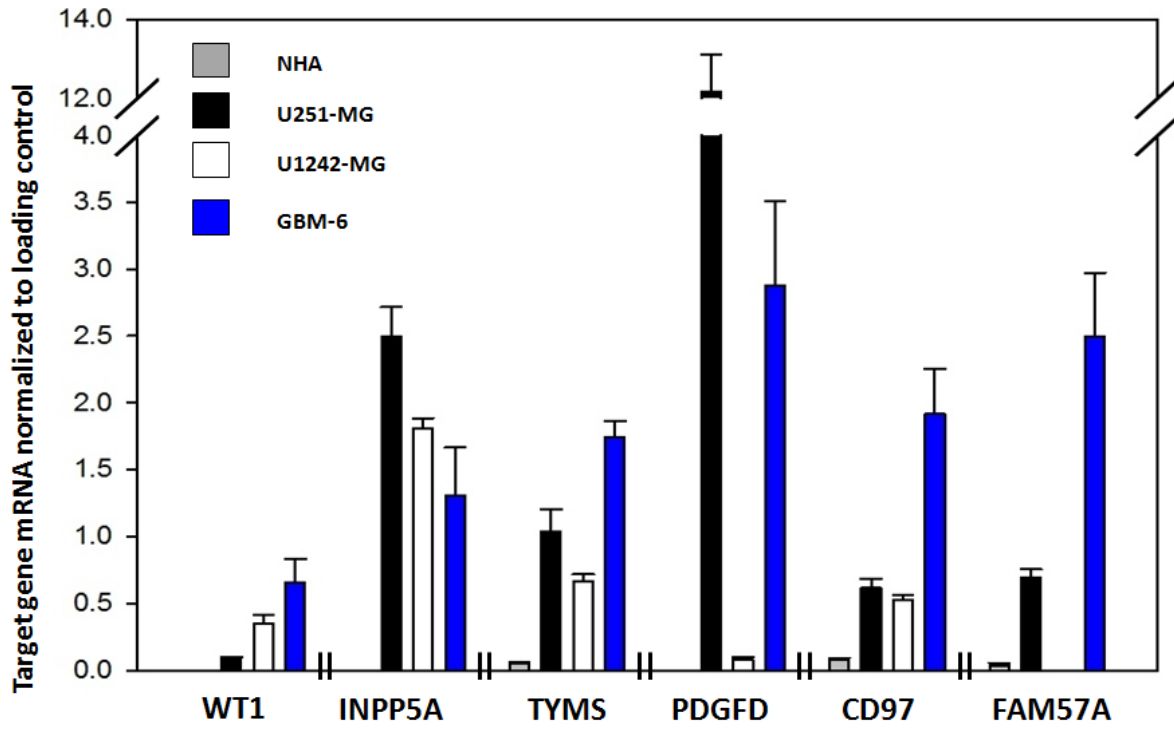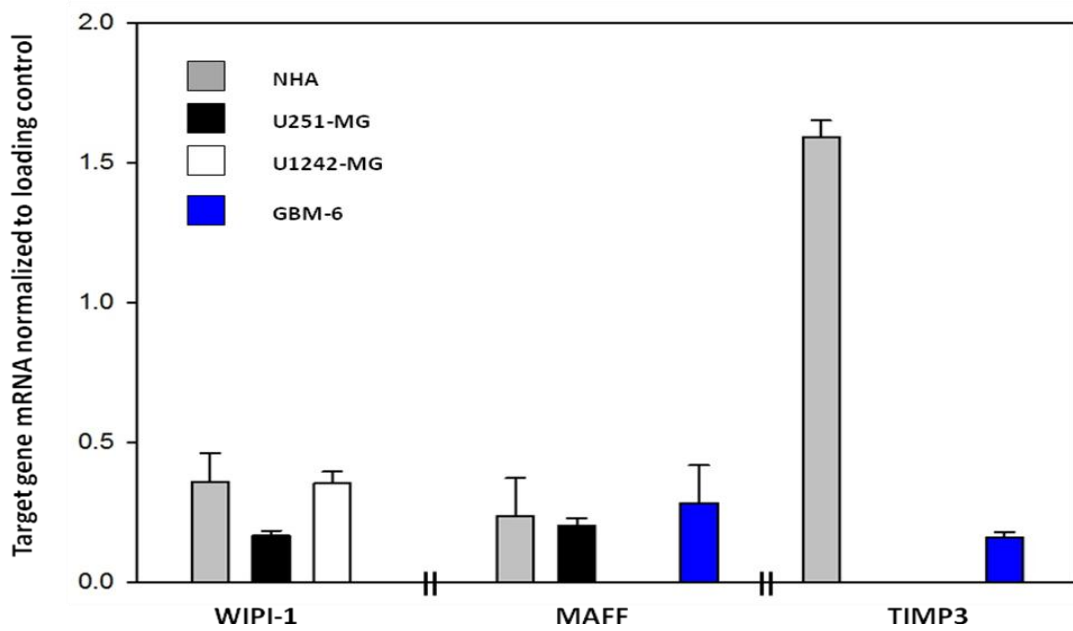
Microarray findings were validated using Real Time RT-PCR. We used qRT-PCR to

validate our microarray findings, because it was quicker, infinitely more sensitive and would

provide information pertaining to the gene expression levels, which is of particular interest to us

since we are assessing the potential transcriptional targets of WT1. For our validation

experiments, we selected from among all the targets identified by microarray, only those that had

been implicated as having a relevant role in oncogenesis or tumor-suppression (Table 4).

Furthermore, certain genes such as *ICK*, *KRT18* and *TIMP3* are not represented in the graph

owing to lack of reproducible results. The $2^{-\Delta\Delta CT}$ method was used to calculate fold changes in

the expression levels of the candidate genes against beta-actin. The values thus obtained for the

si groups of cells were then compared to the corresponding values in the scr groups of cells.

Figure 3-3 represents a comparison of the fold change of each candidate gene obtained from the

qRT-PCR analyses (bar graph) against the corresponding value derived from the microarray

analyses (line-graph). We successfully substantiated the microarray results with qRT-PCR by

showing that with both sets of genes there is a consistent trend towards down- or up-regulation of

their expression levels. In case of the genes that were down-regulated with WT1 silencing, the

magnitude of the fold change values using qRT-PCR (0.25-0.53 fold-change [S.D.: ± 0.04-

±0.22]) is seen to be close to or only slightly different from the values obtained through the

microarray experiments (0.16-0.35). With the inversely correlated genes, the fold-change values

are much higher in the microarray results (2.6-3.7 fold differences) as compared to the qRT-PCR

results (0.9-1.9 fold differences [S.D.: ±0.21- ±1.07]).

Fig. 3-3: Validation of microarray results. U251 cells were transfected with Non-targeting siRNA (Scr) or WT1 targeting siRNA (si). RNA samples extracted 48 hours following transfection were analyzed by Real Time RT-PCR; n= 3, *p≤ 0.05; **p≤ 0.01. Bar graph: Fold-change values calculated from qRT-PCR experiments. Line-graph: Fold-change values calculated using microarray analyses.
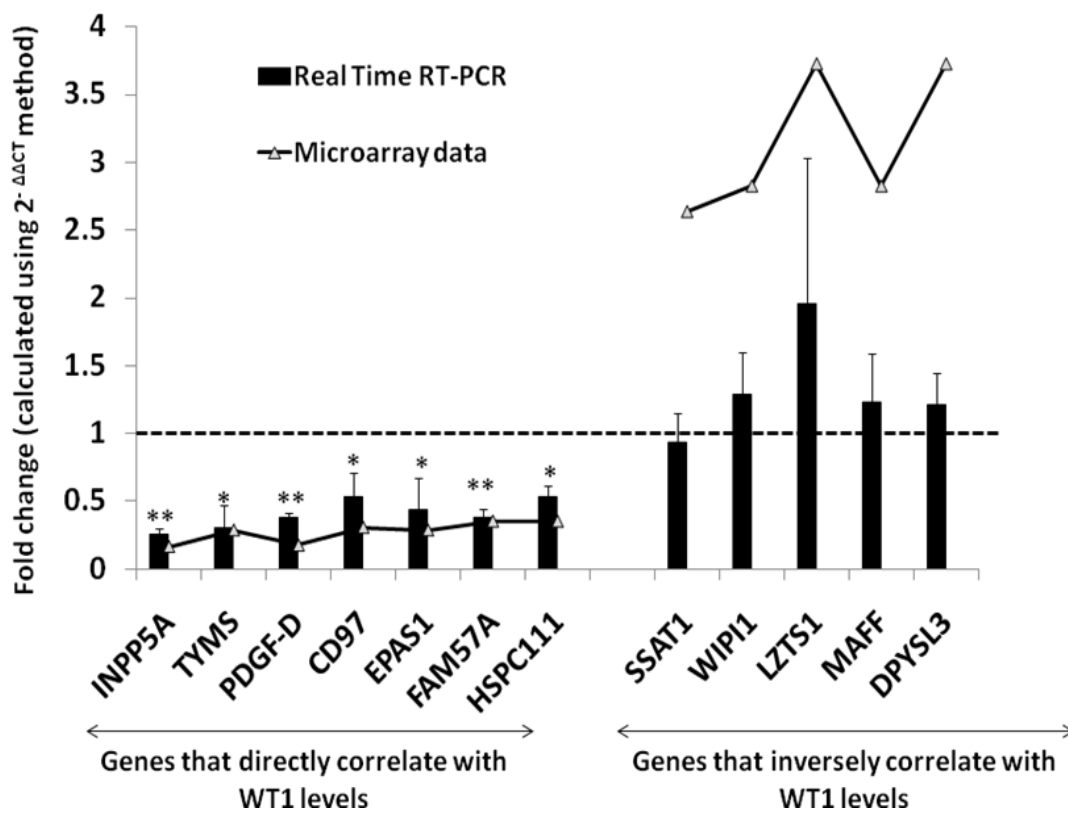
Putative target genes identified by microarray confirmed in U1242-MG and GBM-6 cell lines. We subsequently investigated if these genes that were dysregulated in U251-MG cells were replicated in GBM cell lines- U1242-MG and GBM-6. Cells from both these cell lines were subjected to siRNA transfections targeting WT1 and non-targeting siRNA (scr) was used to generate appropriate controls. RNA samples from these groups were analyzed using qRT-PCR, and it was seen that from among the genes whose levels paralleled that of WT1, *INPP5A*, *CD97*, and *TYMS* were down-regulated in the si groups in both U1242-MG and GBM-6 cells as compared to scr groups (Fig. 3-4). In U1242-MG cells (Fig. 3-4B, n=3, *$p \leq 0.05$, ** $p \leq 0.01$), the extent of knockdown of these genes (as a percent of scr control) was as follows: INPP5A- 65.5% (S.D.: ± 0.11), CD97- 30.6% (S.D.: ± 0.09) and TYMS- 47.7% (S.D.: ± 0.04). In GBM-6 cells (n=3, *$p \leq 0.05$, ** $p \leq 0.01$), the extent of knockdown was as follows (Fig. 3-4A): INPP5A- 51.77% (S.D.: ± 0.26), CD97- 49.6% (S.D.: ± 0.14) and TYMS- 52.4% (S.D.: ± 0.16). Moreover, in GBM-6 cells, the levels of *PDGF-D* and *FAM57A* in the siWT1 treated groups of cells were significantly decreased to 16.2% and 44.1% of their scr counterparts respectively (S.D.: ± 0.06 and S.D.: ± 0.23 respectively). Likewise, in U1242-MG cells (Fig.3-4B), the level of *EPAS1*, a potent angiogenesis facilitator, was also decreased with WT1 silencing. siWT1 treated cells had 77.5% of EPAS1 expression compared to their corresponding control values, although statistical significance could not be established. With the genes that were demonstrated to be up-regulated by the microarray analyses with WT1 silencing, we found a trend towards an increase in *DPYSL* and *MAFF* (GBM-6) and *WIPI-1* (U1242-MG), but the high variability across the three independent experiments precluded the establishment of statistical significance.

Fig.3-4: Confirmation of altered regulation of target genes across different glioma cell lines transfected with siWT1. A. GBM-6 cells (n= 3, *$p \leq 0.05$, ** $p < 0.01$) B. U1242-MG cells (n= 3, *$p \leq 0.05$, ** $p < 0.01$). X- axis: Target genes Y-axis: Fold change in si cells (value of scr set at 1). §§- Genes that were not found to correlate/ be expressed.A

GBM-6

B

U1242-MG
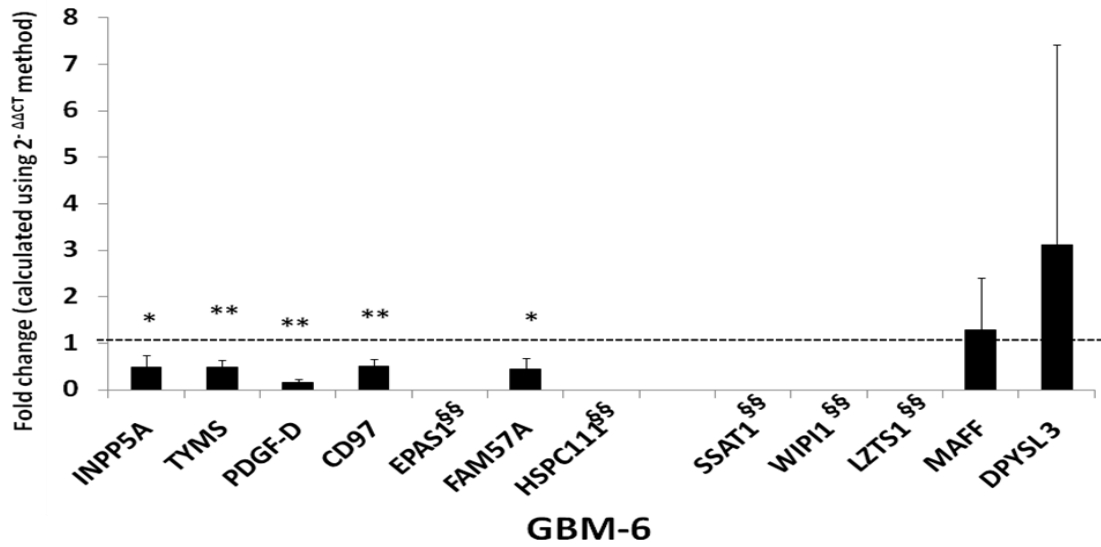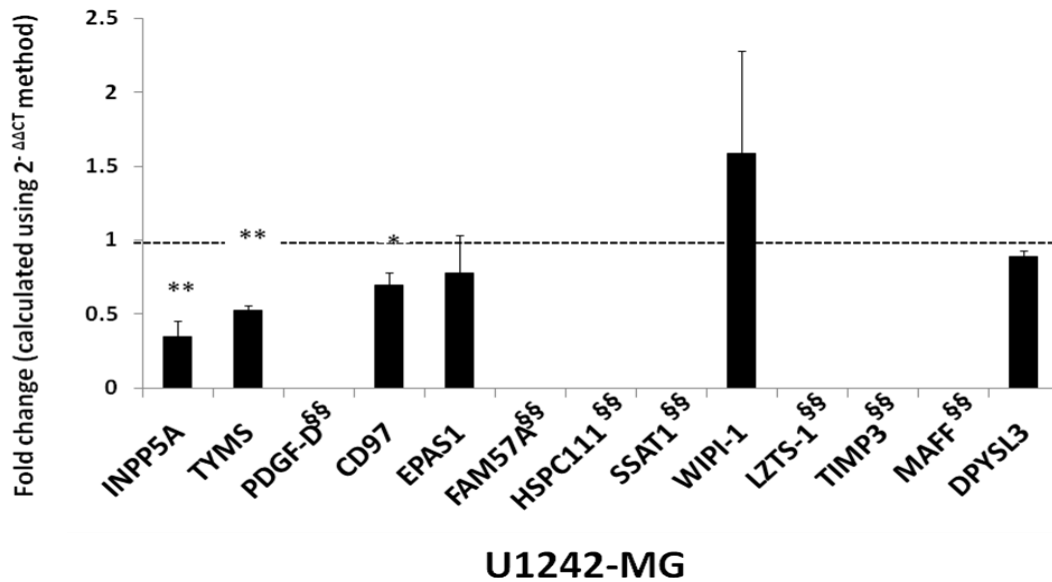
CD97 expression in glioma cells is associated with cellular invasiveness. Based on our microarray findings and our confirmatory findings using Real Time PCR, we conducted promoter analyses to look for potential binding sites in the promoter regions of the genes that appeared to be differentially regulated by WT1 (3-5). With the exception of PDGF-D (which had binding sites for Egr-1, but not specifically WT1), all the remaining candidate target genes were found to have at least one potential WT1 binding site. This information coupled with the finding of consistent CD97 down-regulation in all three GBM cell lines that had been treated with WT1 siRNA, led us to further examine the role of CD97 in these cells with respect to its expression levels and consequent effects on cellular invasiveness.

We first ascertained the expression of CD97 at the protein level using western blot analysis which revealed that it was expressed in all three cell lines examined (Fig. 3-6). Qualitatively, it appears that expression level is highest in U251-MG cells, intermediate in GBM-6 and least in U1242-MG cells. Subsequently, we down-regulated CD97 levels in all these three cell lines and noted the biological effects of this manipulation. As shown in Figure 3-7A, real time RT-PCR analyses showed that we were able to achieve a significant knockdown in all the three cell lines even at Day 4 post-transfection (n=3, *p<0.05*). In U251-MG cells, there was a nearly 50% decrease in CD97 mRNA levels at this time point while in GBM-6, there was a nearly 80% decrease. In U1242-MG cells, there was a less remarkable knockdown (20% decrease compared to the control cells), which was nevertheless statistically significant. When plated on Matrigel-coated filters of transwell plates, we found that these decreases in CD97 mRNA levels were associated with significantly lower cellular invasive capacities (Fig. 3-7B). In U251-MG cells treated with siRNA against CD97, the cellular invasive potential was decreased to roughly 53.85% of its control counterpart. In U1242-MG cells, even a modest decrease in

CD97 RNA levels was associated with a striking decrease in invasiveness (25.5% of control) while in GBM-6 cells, the 80% decrease in CD97 mRNA levels caused a nearly 50% decrease in invasive capacity.

Table 5 summarizes our findings of the effect of WT1 and CD97 silencing on the levels of CD97 mRNA and how each correlates with changes in cell invasiveness. In the U251-MG cells, silencing WT1 correlated with a decrease in CD97 mRNA levels to about 52.9% of the level in control cells (shown in Fig. 3-3), and we have previously shown that decreasing WT1 was associated with a decrease in cellular invasiveness in these cell lines to about 40% of the control cells [31]. In U1242-MG and GBM-6 cells, WT1 silencing resulted in a decrease in CD97 levels to about 69.45% (S.D.: ± 0.09) and 51% (S.D.: ± 0.14), respectively, of the level in their respective controls (Fig. 3-4A & B). These values correlated with a decline in the cellular invasive property to about 20.6% (S.D.: ± 1.12) and 5.9% (S.D.: ± 22.65) respectively in U1242-MG and GBM-6 cells (Fig.2-2B). CD97 silencing in U251-MG, U1242-MG and GBM-6 cells (Fig.3-7) resulted in a decrease in cell invasiveness to 53.85% (S.D.: ± 14.64), 25.5% (S.D.: ± 21.38) and 49.82% (S.D.: ± 11.71), respectively. Thus, it is clear that the expression of CD97 in glioma cells is of great functional significance. We also examined the effects (if any) of knocking down CD97 on cellular proliferation, even though such a role has not frequently been attributed to this molecule. Our findings showed that there were no significant differences between the proliferative rates of the control versus experimental groups across all the three cell lines (data not shown).

Fig.3-5: Promoter analyses of candidate target genes selected on the basis of their putative roles as oncogenes or tumor-suppressors. The UCSC genome browser was used to search for promoter sequences for each gene using the search criteria of 1000 base-pairs upstream and downstream of the start site. Sequences were then entered into the MatInspector software, and a search was conducted for potential binding sites specifically for the WT1 matrix within the Egr-1 family of transcription factors. From the results generated, sequences that fulfilled the following criteria were selected- a) members of the WT1 matrix, b) matrix similarity of ≥ 0.9 and c) sequences that were on the positive strand. Sequences with green numbering belong to genes that are down-regulated with WT1 silencing, while those with red numbering are for genes that correlate inversely with WT1 levels. The underlined areas represent the potential binding sites. The 4-capitalised nucleotide sequences represent the core- The "core sequence" of a matrix is defined as the (usually 4) highest conserved positions of the matrix. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence.

CD97:   -399   GCCAACTCTGGCATGCCAAAGCTCTGGGCACAGGGCGAGTCCCAC**CAGCG**

TGGGcgtccaga**GA**GCTATTTCTGACAAGACCCCTTTGCGCCTGCCC-305

+501   ATAACGCATAGAAGGAC**ATGGGCGGGGGTGGGGA**AGCTAGAAGCCAGGAC+550

TYMS: -49   CTC**TAGAGCGGGGGCCGCCG**CGACCCCGCCGAGCAGGAAGAGGCGGAGCG-0

INPP5A: -599   CGCCCGGCACGCGGCTGGACGACTGCGG**CCGCGCGGGGGCTGGAA**CATCC-550

-450   AAGGGCGGAGTCTGTGCGCAGCCG**CGGCGCGGGGGGCGGGG**CGGGGGCGC-400

-449   AAGGGCGGAGTCTGTGCGCAGCCGCGGCGCGGGGGG**CGGGGCGGG**GGCGC

GGCGTTCTTGCGGGCCTGACTGACAGGAGCGCCCTCCGGGAGCGACCAGT -350

EPAS-1:   -249   AAGTGCGGAGGCAGGAGGCGGGGCG**CAGCGCGGGGGCAGGAG**GCGGGCCA-200

-150   GAGCCGCCCGGAGCT**CGGCGCGGGCGGGGCCT**GCCGGCGCGTGCCCGCCC-100

+651   GAGGCTCGGTTTGGAGGG**CTGTGAGGGGGAGAGCA**TGTGCCCGGTTTGGG+700

+1000   GCCCAAGTGGGCAAAG**GGGGGCGGGAGGCGGAG**AGCGGCTCAGGGACACG+1050

FAM57A: +301   TTCCGAAGCCCCTCCGCGTCCTCCCCTGGCGGGAGCGGGGC**CGGGGCGGG**

CGGGAATGGCCGATGAGCCTCCGGAGCCCGCTCCCCACATCTGCCTGCGG+400

WIPI-1: +191CTGCCAG**GGAGGTGGGGGACAGAG**TGTCAGGACCCAGGGGTGTCGTCCTGAGAGCAGCTT+250

LZTS-1:   +851CCGCTTCTCTCCCCGGGAG**ACTCGTGGGGGTGGGTG**CGGTCTTCTCATTT+900

+851CCGCTTCTCTCCCCGGGAGACTCGT**GGGGGTGGG**TGCGGTCT**T**TCTCATTT+900

MAFF: +351GCGCC**TGGGGTGGGCGCCCGGG**CCCGATCCGTCGGGGCTCCCGGAGCTCC +400

+351GCCGAGGACGGGGCCGAGGCCAGCTCGG**GGGAGTGGGGGTGGAGA**CGGGG +351

+501CCCCTTCCCG**GGGCGCGGGCGGGTAGG**CGGCGGCGCCGGGAGGAAGGGGC +550

+551AGCGCAGGCCGGCGATCTCCGCGTCCGCCCGGC**GGGGGCGGGGGCGCTGT** +600

+651GTGCGTGCGCGGTTTGTTTACGTCC**GAGGGCGGGCGTGTGTT**CCCTGGCG +700

DPYSL3:   -149 CAAAACCCGGGCGTGAACGCGGGTGGAGGAGGGT**GTGCGTGGGGGGGCGG** GGGTGAAAGG -90

-149 CAAAACCCGGGCGTGAACGCGGGTGGAGGAGGGTGTGCGTGG**GGGGGCGGG**GGTGAAAGG -90

-49 GGGAGC**CCGGGAGGGGGGCGGAG**GCGGGCGGAGGCGGCGGCGCGGCCAGC-0

+41AGGA**AGGCGCGGCGAGCCGG**AGCAGAAGAAGGAGGGAGGGAGCCAGCCGCTGCAG

CCAC+100 *(– INTRA-EXONAL BINDING SITE)*

73

Fig.3-6: Western Blot showing CD97 protein expression (≈92 kDA) in U251-MG, U1242-MG

and GBM-6 glioma cells. Cell lysates were prepared using 1% SDS-lysis buffer. 10 µg protein

was loaded in each lane. Rabbit polyclonal Ab (Abcam) was used to detect CD97 (1: 200).

Fig.3-7: CD97 silencing suppresses cellular invasiveness in glioma cells. A. Using siRNA directed against the CD97 receptor, we confirmed a significant knockdown in CD97 RNA in the tumor cells on day 4 post-transfection by Real Time RT-PCR in U251-MG, U1242-MG and GBM-6 cells (n=3, *p<0.05*). B. Treatment of U251-MG, U1242-MG and GBM-6 cells with siRNA against CD97 resulted in a significant decrease in their ability to invade through the Matrigel-coated filters of transwell plates, as compared to cells that were treated with non-targeting siRNA (n=3; RLU= Relative luminescence units). We confirmed that the decreased expression of CD97 did not result in decreased proliferation rates across the three cell lines.

A
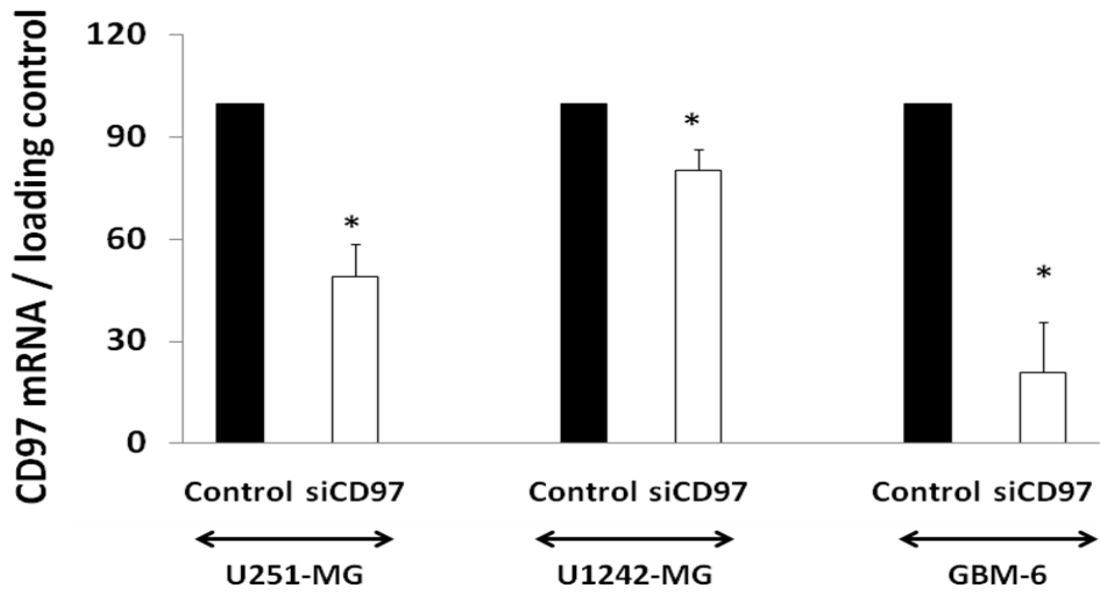
B

Table 5: Effect of WT1 and CD97 silencing on CD97 mRNA levels- Correlating these results with changes in cellular invasiveness.

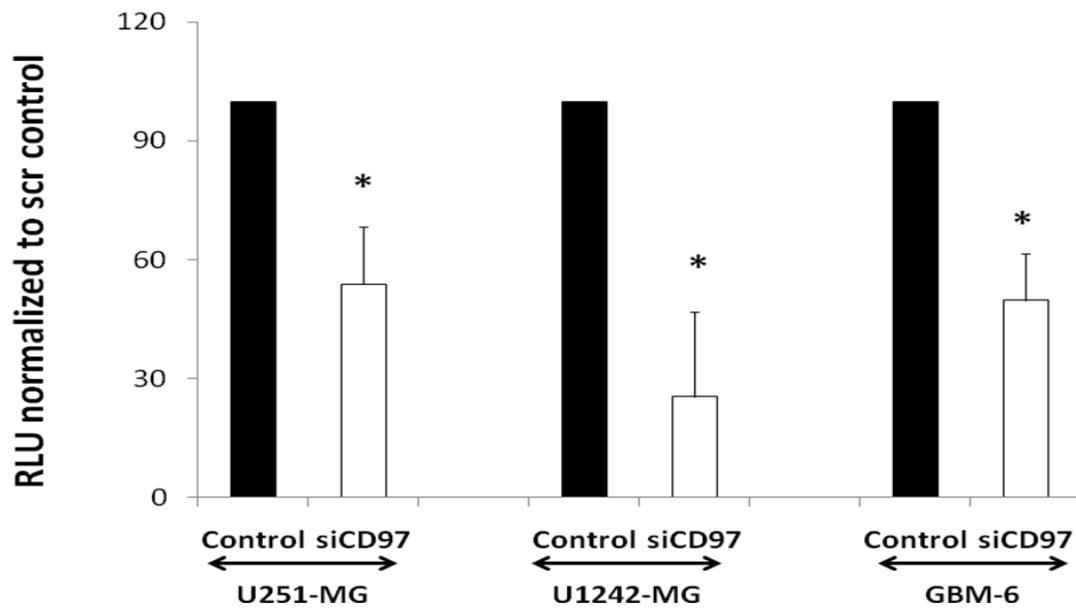| Cell Line	Following transfection with siWT1		Following transfection with siCD97	
	CD97 mRNA level (% control)	Cell invasiveness (% control)	CD97 mRNA level (% control)	Cell invasiveness (% control)
U251-MG	52.9%	40% [31]	49.16%	53.8%
GBM-6	51%	5.9%	20.87%	49.82%
U1242-MG	69.45%	20.6%	80.42%	25.5%

Discussion

We have identified, using gene expression profiling, some of the genes that correlate directly or inversely with WT1 levels in the specific context of astrocytomas. Of the 27 genes whose expression patterns mirrored that of WT1, 7 candidates were selected for having established or putative oncogenic functions, based on published literature. Conversely, of the 11 genes whose levels were up-regulated with WT1 silencing, 5 had been implicated in the suppression of tumorigenesis in some way. We have validated the microarray findings for these genes in U251-MG cells with Real Time RT-PCR and have also confirmed these expression patterns in two other GBM cell lines. We have also demonstrated here that most of the genes that correlate directly with WT1 levels are not expressed in normal human astrocytes, but are highly expressed in GBM cells. This observation strongly implicates these genes in the process of malignant transformation, necessitating further investigations into their precise contributions to

77

this process. Our promoter studies revealed potential binding sites for WT1 in the promoter regions of all but one of the putative target genes. In the lone exception- *PDGF-D*- the promoter region did however show potential binding sites for the Egr-1 family of transcription factors. It has been established in previous studies [36, 43, 44] that WT1 shares the consensus sequences recognized and bound by the Egr-1 family of proteins. CD97 was uniformly down-regulated in all three cell lines with WT1 silencing and displayed sites on its promoter that could be theoretically bound by WT1. Moreover, it has been well established as a facilitator of tumor cell invasiveness in other malignancies. Given these considerations, we chose to further examine the role of this protein in gliomas. This novel undertaking revealed that in glioma cells, as in gastrointestinal malignancies and fibrosarcoma cell line HT-1080 [119], the CD97 receptor appears to foster cellular invasiveness, and decreasing its endogenous expression caused significant and striking decreases in the ability of the cells to invade through Matrigel.

The complexity of the WT1 protein has for long posed a formidable challenge to those that have ventured to study its varied functions. Its vastly differing roles in different cell types make it difficult to categorize this protein as a tumor-suppressor or an oncogene. In certain cells like the progenitor cells of the kidney, WT1 facilitates their exit from the cell cycle with subsequent differentiation [87]. In the neural and vascular progenitor cells however, it has the exact opposite effect- facilitating proliferation and preventing differentiation [87]. Hence, any conclusions about its role(s) must be made strictly in the context of the cell-type examined. This was the basis for our decision to choose the microarray technique to study differences in gene expression arising out of manipulating the levels of WT1 in cells that expressed it endogenously. Moreover, silencing the endogenous expression of the protein to look for the resultant differences was a more appropriate model rather than over-expressing WT1 in a WT1 null cell

line, due to the myriad variables (WT1 isoforms, concentrations etc) associated with the latter technique; the former method was therefore selected in order to more faithfully assess the possible functions of WT1.

Identifying the target genes via which WT1 might influence its role in the neoplastic transformation of glial cells was of paramount importance for many reasons. First, as a regulator of transcriptional and post-transcriptional processes, WT1 has the ability to co-ordinate all the key aspects of glioma biology- proliferation, invasiveness and angiogenesis, by regulating the expression of the molecules that mediate any or all of these processes. Moreover, given the rapid progress that is currently being made in the field of immunologically targeting WT1, it is evident that WT1 is a force to be reckoned with in gliomas, and our findings also help to fill in the missing links in understanding how it functions. Finally, knowing the identity of its associates can help in devising therapeutic strategies that target one or more of these components. Since these components belong to different intracellular signalling pathways, targeting them *ensemble* would shut down any possible "escape routes" that malignant cells are notorious for taking.

The identification of at least 7 candidate oncogenes that were suppressed and 5 putative tumor-suppressors that were up-regulated by WT1 silencing confirmed our hypothesis that in gliomas, WT1 has more of an oncogenic function. What was more interesting was that among the genes that varied directly with WT1 levels, only *TYMS*, *CD97* and *FAM57A* were expressed in normal human astrocytic RNA and even then, their expressions were in the order of a 100ths of a fraction. These same genes were expressed between 10-100 times or more in untreated GBM cells, thus making a powerful argument in favor of their roles in promoting malignant transformation.

Our study has also revealed a novel protein- CD97- that is aberrantly over-expressed in GBM cells. This protein has well-documented roles in mediating tumor cell invasion and even angiogenesis [118, 120, 130]. In various gastrointestinal malignancies, an increased expression of CD97 has been demonstrated at the invading front of the tumor [145, 146]. Likewise, we have shown here that in glial neoplastic cells, too, it has a significant role in conferring invasiveness upon the cells. From our findings, it appears that in U1242-MG cells the extent to which WT1 decreases cellular invasiveness can be roughly accounted for by CD97 alone. In U251-MG and GBM-6 cells, on the other hand, WT1 silencing causes a much more pronounced decrease in cellular invasiveness than that seen with CD97 silencing alone. In both these cell lines, PDGF-D was also seen to be significantly decreased with lowered expression of WT1. It is well established that PDGF-D is a strong mitogenic and chemo-attractant molecule and it can initiate several malignant features like proliferation, invasion and angiogenesis in brain tumors (reviewed in Ref 129). Examining in more detail the relationship of this growth factor to WT1 undoubtedly merits further attention.

Although our promoter studies suggest putative binding sites for WT1 on the CD97 promoter, definitive evidence is still required, preferably using the Chromatin Immunoprecipitation (ChIP) Assay or even Luciferase Reporter assay techniques. For the former, a reliable antibody to immunoprecipitate WT1 is essential, and has been a confounding factor in our attempts to study this.

The ability to localize the invading neoplastic cells in situ has for a long time been a challenge in the treatment of brain tumors. With the identification of CD97 expression and function in glioma cells, determining its ability to serve as a reliable marker of invasiveness using immunohistochemical staining of glioma tumor specimens opens up a potentially

interesting line of investigation. Another option would be to devise a therapeutic strategy aimed at targeting the CD97 receptor, which would be relatively safe, since it is virtually absent in the normal human astrocytes. Further investigations are also necessary to determine the effect of targeting the CD97 receptor on angiogenesis, as it is also known to affect this process.

Our study thus opens up entirely new avenues in the field of glioma biology. Hitherto unknown targets have been uncovered, and more in-depth analyses of these molecules should hopefully take us several steps further in our attempts to conquer the fatal disease that constitutes GBM.

Chapter 4: General discussion and future directions.

In the preceding chapters, we have shown that the developmental regulator Wilms'
Tumor-1 (WT1) has a significant contribution to the invasive property that typifies tumor cells in
malignant gliomas. WT1 was also shown to promote tumor cell growth in vitro and in vivo, by
studies in our laboratory and others [28, 30-32]. Given its zinc-finger transcription status, we
embarked on a mission to uncover the target genes via which these effects are mediated.
Previously established pro-invasive target gene candidates such as PDGF-A [88, 89], Snail-1 [86]
and E-cadherin [86] failed to demonstrate a consistent correlation with WT1 expression levels.
Expression levels of the tyrosine-kinase receptor IGF-1R were analyzed in relation to WT1, to
account for differences in proliferative rates between cells that had WT1 expression versus those
in which endogenous WT1 expression was abolished or increased. Our hypothesis was that IGF-
1R, when up-regulated, might mediate a non-apoptotic form of cell death called paraptosis [101,
106]. We observed consistent up-regulation of IGF-1R with WT1 silencing in U251-MG and T-
98G cells [31], and a down-regulation of IGF-1R protein and RNA upon over-expressing the WT1
(+/+) isoform in U251-MG cells. However, siRNA-mediated silencing of IGF-1R in U251-MG
cells was associated with a decrease in cell proliferation, thereby indicating that the IGF-1R up-
regulation consequent to WT1 silencing was more likely due to activation of an alternate survival
pathway by the tumor cells, rather than the mechanism by which WT1-mediated cell-death.

These findings support the theory that the transcriptional targets for WT1 vary depending upon
the cell type and differentiation status, the isoforms of WT1 present in the cells under
investigation, and the level of WT1 expression. Hence, in order to ascertain potential targets for
WT1 in gliomas, we chose to evaluate changes in global gene expression levels following

suppression of endogenous WT1, using microarray analyses. Our results from three independent microarray experiments revealed the identities of some candidate target genes that were consistently down- or up-regulated with WT1 silencing, implying a direct or indirect correlation respectively, with this protein. Based on literature search results, the altered regulation of these genes, we observed, could not only explain WT1's effects on proliferation and invasion, but also uncovered a potential role in angiogenesis (Fig.4-1).

Fig.4-1: WT1 regulates different aspects of the malignant phenotype that characterizes glioma cells- cell proliferation, invasion and angiogenesis. The potential target genes that enable it to effect these processes (+), or those that inhibit the process (-) and correlate inversely with WT1 levels are also listed. Genes highlighted in bold are those that are common among three different glioma cell lines examined.
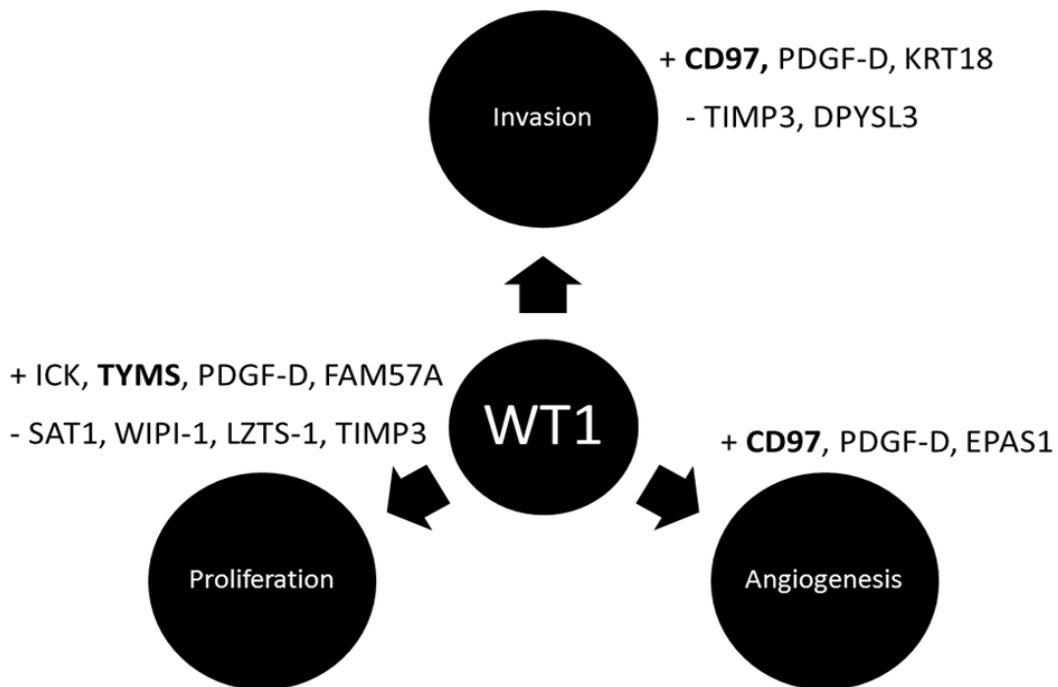
An involvement in angiogenesis will not be surprising given that the fatality in WT1 null mice is a consequence of severe cardiovascular abnormalities [84]. Moreover, in glioma tumor specimens, WT1 expression has been discovered in the endothelial cells of tumor vessels [85], and while it has also been shown to regulate the expression of pro-angiogenic molecules like Ets-1 [85] in tumors, its own transcription is potentially activated by hypoxia-inducible factor-1 (HIF-1) with oxygen restriction [84]. WT1 is thus well-linked to the process of blood vessel formation and it would be interesting to study if the expression in vascular endothelial cells in tumors is a coincidental finding, or if it actually contributes to the process. Our laboratory has established an in vitro 3-dimensional model to study angiogenesis, [147] which would well serve this purpose. Further, this model could also be used to ascertain which of the pro-angiogenic target genes identified- *CD97, PDGF-D* and *EPAS1*- contribute to this process in glioma cells.

Based on our findings, it is evident that WT1 regulates the invasiveness of glial neoplastic cells, possibly via altering the expression of CD97. While definitive evidence for CD97 regulation by WT1 would only be obtained by demonstrating binding of the latter to the *CD97* promoter, there is substantial proof to validate this hypothesis. For one, the expression patterns of both WT1 and CD97 are closely related across all the cell lines studied- both proteins are expressed at much higher levels in GBM cells as opposed to normal human astrocytes and suppressing the endogenous expression of WT1 significantly and consistently down-regulates CD97 expression. Further, both proteins notably affect the invasiveness of the neoplastic cells. In U251-MG and GBM-6 cells, down-regulating WT1 has a much greater impact on decreasing cellular invasiveness than that seen with CD97 suppression alone, and pro-invasive molecules like PDGF-D could be additionally implicated. In the highly invasive GBM cell line- U1242-

MG, however, even a modest decrease in CD97 level dramatically impairs the cells' ability to invade. The absence of significant dysregulation of *Snai1*, *E-cadherin*, *PDGF-D*, *KRT18* or *DPYSL3* in these cells gives rise to the hypothesis that U1242-MG cells are heavily dependent on the CD97 receptor expression levels to retain their invasiveness.

Invasion of the neoplastic cells into surrounding normal brain parenchyma involves inter-cellular interactions and interactions occurring between tumor cells and the surrounding extracellular matrix, facilitated by adhesion molecules, chemo-attractants, and perhaps most importantly- by matrix proteases produced by tumor cells [148]. In vivo assays and organotypic brain slice models are considered superior to in vitro assays for invasion, since they provide an environment that facilitates this study of how the invading neoplastic cells interact with the unique extracellular matrix in the brain. However, a key element in promoting cellular invasiveness is the inherent motility acquired by malignant cells [148], which is a property that can be examined in great detail using an in vitro invasion assay. In this system, it is also possible to determine and manipulate the properties that confer this enhanced motility upon the transformed cell. In our case, for example, manipulating the expression level of WT1 or CD97 generates an excellent system by which to study the exact contribution(s) of these proteins to cellular invasion.

The microarray analyses identified several pro-proliferative proteins that varied directly with WT1 levels, such as ICK, TYMS, PDGF-D and FAM57A, and several pro-apoptotic molecules that varied inversely, like SAT-1, LZTS-1 and TIMP-3. WIPI-1 which also inversely correlated with WT1 plays a role in autophagy [136]- a non-apoptotic form of cell death. It is interesting to note that among the putative oncogenic targets, only PDGF-D has been studied for its contribution to gliomagenesis. Information pertaining to the other molecules is mostly from

studies conducted in gastrointestinal carcinomas or lung malignancies [118, 120, 124, 126-133]. Thus, the aberrant dysregulation of several genes with hitherto unknown functions in gliomas has been presented for the first time in these studies.

Future directions

We have validated the microarray findings at the mRNA level for all the candidate genes in three different cell lines. It is important to demonstrate that these genes are similarly expressed at the protein level, as well, using western blotting, in order to rule out any post-transcriptional regulatory differences. Further analyzing the exact role and contributions of each protein to glioma is beyond the scope of this study. However, such investigations would undoubtedly broaden our current understanding of this disease.

Our findings from the in vitro invasion assays following WT1 and CD97 suppression can be supplemented by orthotopically implanting cells in which WT1 has been silenced, and detecting changes in CD97 expression at the invasion front, using immunohistochemistry. Further, the invasive patterns of the tumor cells can be visualized histologically or using MRI. Brain slice models can be similarly utilized in place of or in addition to the above-mentioned system. The U1242-MG xenograft model has been established to closely mimic the key features seen in GBM with features of extensive infiltration into the brain parenchyma, perivascular and subpial spread of cells, microvascular proliferation with typical neovascularization and even necrosis [99]. Moreover, based on our findings from in vitro experiments this cell line demonstrates a high sensitivity to CD97 expression levels. Thus, it would serve as a good model to test our findings using these experimental model systems.

Finally, demonstration of WT1 binding to CD97 promoter using chromatin immunoprecipitation assay or luciferase reporter assay is also vital to confirm transcriptional regulation of the latter protein by WT1.

Literature cited

Literature cited

1. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system *Acta Neuropathol*. 2007 Aug;114(2):97-109.

2. Brat DJ, Prayson RA, Ryken TC, Olson JJ. Diagnosis of malignant glioma: Role of neuropathology *J Neurooncol*. 2008;89:287-311.

3. Konopka G, Bonni A. Signaling pathways regulating gliomagenesis *Curr Mol Med*. 2003;3:73-84.

4. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways *Nature*. 2008;455:1061-1068.

5. Weinberg RA. The retinoblastoma protein and cell cycle control *Cell*. 1995;81:323-330.

6. Wu CL, Zukerberg LR, Ngwu C, Harlow E, Lees JA. In vivo association of E2F and DP family proteins *Mol Cell Biol*. 1995;15:2536-2546.

7. Nevins JR. E2F: A link between the rb tumor suppressor protein and viral oncoproteins *Science*. 1992;258:424-429.

8. Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4 *Genes Dev*. 1993;7:331-342.

9. Menendez D, Inga A, Resnick MA. Potentiating the p53 network *Discov Med*. 2010;10:94-100.

10. Vousden KH, Prives C. Blinded by the light: The growing complexity of p53 *Cell*. 2009;137:413-431.

11. el-Deiry WS. Regulation of p53 downstream genes *Semin Cancer Biol*. 1998;8:345-357.

12. Stott FJ, Bates S, James MC, et al. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2 *EMBO J*. 1998;17:5001-5014.

13. Sidransky D, Mikkelsen T, Schwechheimer K, Rosenblum ML, Cavanee W, Vogelstein B. Clonal expansion of p53 mutant cells is associated with brain tumour progression *Nature*. 1992;355:846-847.

14. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor *Physiol Rev*. 1999;79:1283-1316.

15. Feldkamp MM, Lau N, Guha A. Signal transduction pathways and their relevance in human astrocytomas *J Neurooncol*. 1997;35:223-248.

16. Claesson-Welsh L. Mechanism of action of platelet-derived growth factor *Int J Biochem Cell Biol*. 1996;28:373-385.

17. Lim SK, Llaguno SR, McKay RM, Parada LF. Glioblastoma multiforme: A perspective on recent findings in human cancer and mouse models *BMB Rep*. 2011;44:158-164.

18. Yu JC, Gutkind JS, Mahadevan D, et al. Biological function of PDGF-induced PI-3 kinase activity: Its role in alpha PDGF receptor-mediated mitogenic signaling *J Cell Biol*. 1994;127:479-487.

19. Newton HB. Molecular neuro-oncology and development of targeted therapeutic strategies for brain tumors. part 2: PI3K/Akt/PTEN, mTOR, SHH/PTCH and angiogenesis *Expert Rev Anticancer Ther*. 2004;4:105-128.

20. Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1 *Cancer Cell*. 2010;17:98-110.

21. Jones TS, Holland EC. Molecular pathogenesis of malignant glial tumors *Toxicol Pathol*. 2011;39:158-166.

22. Peiffer J, Kleihues P. Hans-joachim scherer (1906-1945), pioneer in glioma research *Brain Pathol*. 1999;9:241-245.

23. Claes A, Idema AJ, Wesseling P. Diffuse glioma growth: A guerilla war *Acta Neuropathol*. 2007;114:443-458.

24. Goldbrunner RH, Bernstein JJ, Tonn JC. Cell-extracellular matrix interaction in glioma invasion *Acta Neurochir (Wien)*. 1999;141:295-305; discussion 304-5.

25. Adamson C, Kanu OO, Mehta AI, et al. Glioblastoma multiforme: A review of where we have been and where we are going *Expert Opin Investig Drugs*. 2009;18:1061-1083.

26. Mirimanoff RO. The evolution of chemoradiation for glioblastoma: A modern success story *Curr Oncol Rep*. 2006;8:50-53.

27. Holdhoff M, Grossman SA. Controversies in the adjuvant therapy of high-grade gliomas *Oncologist*. 2011;16:351-358.

28. Clark AJ, Dos Santos WG, McCready J, et al. Wilms tumor 1 expression in malignant gliomas and correlation of +KTS isoforms with p53 status *J Neurosurg*. 2007;107:586-592.

29. Schittenhelm J, Mittelbronn M, Nguyen TD, Meyermann R, Beschorner R. WT1 expression distinguishes astrocytic tumor cells from normal and reactive astrocytes *Brain Pathol*. 2008;18:344-353.

30. Chen MY, Clark AJ, Chan DC, et al. Wilms' tumor 1 silencing decreases the viability and chemoresistance of glioblastoma cells in vitro: A potential role for IGF-1R de-repression *J Neurooncol*. 2010.

31. Clark AJ, Ware JL, Chen MY, et al. Effect of WT1 gene silencing on the tumorigenicity of human glioblastoma multiforme cells *J Neurosurg*. 2010;112:18-25.

32. Clark AJ, Chan DC, Chen MY, et al. Down-regulation of wilms' tumor 1 expression in glioblastoma cells increases radiosensitivity independently of p53 *J Neurooncol*. 2007;83:163-172.

33. Call KM, Glaser T, Ito CY, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 wilms' tumor locus *Cell*. 1990;60:509-520.

34. Yang L, Han Y, Suarez Saiz F, Minden MD. A tumor suppressor and oncogene: The WT1 story *Leukemia*. 2007;21:868-876.

35. Bardeesy N, Pelletier J. Overlapping RNA and DNA binding domains of the wt1 tumor suppressor gene product *Nucleic Acids Res*. 1998;26:1784-1792.

36. Scharnhorst V, van der Eb AJ, Jochemsen AG. WT1 proteins: Functions in growth and differentiation *Gene*. 2001;273:141-161.

37. Hamilton TB, Barilla KC, Romaniuk PJ. High affinity binding sites for the wilms' tumour suppressor protein WT1 *Nucleic Acids Res*. 1995;23:277-284.

38. Hohenstein P, Hastie ND. The many facets of the wilms' tumour gene, WT1 *Hum Mol Genet*. 2006;15 Spec No 2:R196-201.

39. Kreidberg JA, Sariola H, Loring JM, et al. WT-1 is required for early kidney development *Cell*. 1993;74:679-691.

40. Huff V. Wilms' tumours: About tumour suppressor genes, an oncogene and a chameleon gene *Nat Rev Cancer*. 2011;11:111-121.

41. Madden SL, Cook DM, Rauscher FJ,3rd. A structure-function analysis of transcriptional repression mediated by the WT1, wilms' tumor suppressor protein *Oncogene*. 1993;8:1713-1720.

42. McKay LM, Carpenter B, Roberts SG. Regulation of the wilms' tumour suppressor protein transcriptional activation domain *Oncogene*. 1999;18:6546-6554.

43. Mayo MW, Wang CY, Drouin SS, et al. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene *EMBO J*. 1999;18:3990-4003.

44. Wang ZY, Qiu QQ, Enger KT, Deuel TF. A second transcriptionally active DNA-binding site for the wilms tumor gene product, WT1 *Proc Natl Acad Sci U S A*. 1993;90:8896-8900.

45. Laity JH, Dyson HJ, Wright PE. Molecular basis for modulation of biological function by alternate splicing of the wilms' tumor suppressor protein *Proc Natl Acad Sci U S A*. 2000;97:11932-11935.

46. Laity JH, Chung J, Dyson HJ, Wright PE. Alternative splicing of wilms' tumor suppressor protein modulates DNA binding activity through isoform-specific DNA-induced conformational changes *Biochemistry*. 2000;39:5341-5348.

47. Hewitt SM, Fraizer GC, Wu YJ, Rauscher FJ,3rd, Saunders GF. Differential function of wilms' tumor gene WT1 splice isoforms in transcriptional regulation *J Biol Chem*. 1996;271:8588-8592.

48. Ladomery M, Sommerville J, Woolner S, Slight J, Hastie N. Expression in xenopus oocytes shows that WT1 binds transcripts in vivo, with a central role for zinc finger one *J Cell Sci*. 2003;116:1539-1549.

49. Caricasole A, Duarte A, Larsson SH, et al. RNA binding by the wilms tumor suppressor zinc finger proteins *Proc Natl Acad Sci U S A*. 1996;93:7562-7566.

50. Drummond IA, Rupprecht HD, Rohwer-Nutter P, et al. DNA recognition by splicing variants of the wilms' tumor suppressor, WT1 *Mol Cell Biol*. 1994;14:3800-3809.

51. Morrison AA, Viney RL, Ladomery MR. The post-transcriptional roles of WT1, a multifunctional zinc-finger protein *Biochim Biophys Acta*. 2008;1785:55-62.

52. Wilkinson MF, Shyu AB. Multifunctional regulatory proteins that control gene expression in both the nucleus and the cytoplasm *Bioessays*. 2001;23:775-787.

53. Larsson SH, Charlieu JP, Miyagawa K, et al. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing *Cell*. 1995;81:391-401.

54. Ladomery MR, Slight J, Mc Ghee S, Hastie ND. Presence of WT1, the wilm's tumor suppressor gene product, in nuclear poly(A)(+) ribonucleoprotein *J Biol Chem*. 1999;274:36520-36526.

55. Kennedy D, Ramsdale T, Mattick J, Little M. An RNA recognition motif in wilms' tumour protein (WT1) revealed by structural modelling *Nat Genet*. 1996;12:329-331.

56. Davies RC, Calvio C, Bratt E, Larsson SH, Lamond AI, Hastie ND. WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes *Genes Dev*. 1998;12:3217-3225.

57. Little NA, Hastie ND, Davies RC. Identification of WTAP, a novel wilms' tumour 1-associating protein *Hum Mol Genet*. 2000;9:2231-2239.

58. Markus MA, Heinrich B, Raitskin O, et al. WT1 interacts with the splicing protein RBM4 and regulates its ability to modulate alternative splicing in vivo *Exp Cell Res*. 2006;312:3379-3388.

59. Miwa H, Beran M, Saunders GF. Expression of the wilms' tumor gene (WT1) in human leukemias *Leukemia*. 1992;6:405-409.

60. Niksic M, Slight J, Sanford JR, Caceres JF, Hastie ND. The wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes *Hum Mol Genet*. 2004;13:463-471.

61. Morrison AA, Venables JP, Dellaire G, Ladomery MR. The wilms tumour suppressor protein WT1 (+KTS isoform) binds alpha-actinin 1 mRNA via its zinc-finger domain *Biochem Cell Biol*. 2006;84:789-798.

62. Ross J. mRNA stability in mammalian cells *Microbiol Rev*. 1995;59:423-450.

63. Bor YC, Swartz J, Morrison A, Rekosh D, Ladomery M, Hammarskjold ML. The wilms' tumor 1 (WT1) gene (+KTS isoform) functions with a CTE to enhance translation from an unspliced RNA with a retained intron *Genes Dev*. 2006;20:1597-1608.

64. Rauscher FJ,3rd. The WT1 wilms tumor gene product: A developmentally regulated transcription factor in the kidney that functions as a tumor suppressor *FASEB J*. 1993;7:896-903.

65. Miyagi T, Ahuja H, Kubota T, Kubonishi I, Koeffler HP, Miyoshi I. Expression of the candidate wilm's tumor gene, WT1, in human leukemia cells *Leukemia*. 1993;7:970-977.

66. Tamaki H, Ogawa H, Ohyashiki K, et al. The wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes *Leukemia*. 1999;13:393-399.

67. Oji Y, Ogawa H, Tamaki H, et al. Expression of the wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth *Jpn J Cancer Res*. 1999;90:194-204.

68. Oji Y, Inohara H, Nakazawa M, et al. Overexpression of the wilms' tumor gene WT1 in head and neck squamous cell carcinoma *Cancer Sci*. 2003;94:523-529.

69. Oji Y, Miyoshi Y, Koga S, et al. Overexpression of the wilms' tumor gene WT1 in primary thyroid cancer *Cancer Sci*. 2003;94:606-611.

70. Oji Y, Yano M, Nakano Y, et al. Overexpression of the wilms' tumor gene WT1 in esophageal cancer *Anticancer Res*. 2004;24:3103-3108.

71. Oji Y, Miyoshi S, Maeda H, et al. Overexpression of the wilms' tumor gene WT1 in de novo lung cancers *Int J Cancer*. 2002;100:297-303.

72. Loeb DM, Evron E, Patel CB, et al. Wilms' tumor suppressor gene (WT1) is expressed in primary breast tumors despite tumor-specific promoter methylation *Cancer Res*. 2001;61:921-925.

73. Oji Y, Nakamori S, Fujikawa M, et al. Overexpression of the wilms' tumor gene WT1 in pancreatic ductal adenocarcinoma *Cancer Sci*. 2004;95:583-587.

74. Oji Y, Yamamoto H, Nomura M, et al. Overexpression of the wilms' tumor gene WT1 in colorectal adenocarcinoma *Cancer Sci*. 2003;94:712-717.

75. Ueda T, Oji Y, Naka N, et al. Overexpression of the wilms' tumor gene WT1 in human bone and soft-tissue sarcomas *Cancer Sci*. 2003;94:271-276.

76. Garrido-Ruiz MC, Rodriguez-Pinilla SM, Perez-Gomez B, Rodriguez-Peralto JL. WT 1 expression in nevi and melanomas: A marker of melanocytic invasion into the dermis *J Cutan Pathol*. 2010;37:542-548.

77. Perugorria MJ, Castillo J, Latasa MU, et al. Wilms' tumor 1 gene expression in hepatocellular carcinoma promotes cell dedifferentiation and resistance to chemotherapy *Cancer Res*. 2009;69:1358-1367.

78. Gerald WL, Haber DA. The EWS-WT1 gene fusion in desmoplastic small round cell tumor *Semin Cancer Biol*. 2005;15:197-205.

79. Nakatsuka S, Oji Y, Horiuchi T, et al. Immunohistochemical detection of WT1 protein in a variety of cancer cells *Mod Pathol*. 2006;19:804-814.

80. Ye Y, Raychaudhuri B, Gurney A, Campbell CE, Williams BR. Regulation of WT1 by phosphorylation: Inhibition of DNA binding, alteration of transcriptional activity and cellular translocation *EMBO J*. 1996;15:5606-5615.

81. Oji Y, Suzuki T, Nakano Y, et al. Overexpression of the wilms' tumor gene W T1 in primary astrocytic tumors *Cancer Sci*. 2004;95:822-827.

82. Dudnakova T, Spraggon L, Slight J, Hastie N. Actin: A novel interaction partner of WT1 influencing its cell dynamic properties *Oncogene*. 2010;29:1085-1092.

83. Jomgeow T, Oji Y, Tsuji N, et al. Wilms' tumor gene WT1 17AA(-)/KTS(-) isoform induces morphological changes and promotes cell migration and invasion in vitro *Cancer Sci*. 2006;97:259-270.

84. Scholz H, Wagner KD, Wagner N. Role of the wilms' tumour transcription factor, Wt1, in blood vessel formation *Pflugers Arch*. 2009;458:315-323.

85. Wagner N, Michiels JF, Schedl A, Wagner KD. The wilms' tumour suppressor WT1 is involved in endothelial cell proliferation and migration: Expression in tumour vessels in vivo *Oncogene*. 2008;27:3662-3672.

86. Martinez-Estrada OM, Lettice LA, Essafi A, et al. Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of snail and E-cadherin *Nat Genet*. 2010;42:89-93.

87. Hohenstein P, Hastie ND. The many facets of the wilms' tumour gene, WT1 *Hum Mol Genet*. 2006;15 Spec No 2:R196-201.

88. Gashler AL, Bonthron DT, Madden SL, Rauscher FJ,3rd, Collins T, Sukhatme VP. Human platelet-derived growth factor A chain is transcriptionally repressed by the wilms tumor suppressor WT1 *Proc Natl Acad Sci U S A*. 1992;89:10984-10988.

89. Wang ZY, Madden SL, Deuel TF, Rauscher FJ,3rd. The wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene *J Biol Chem*. 1992;267:21999-22002.

90. Wang ZY, Qiu QQ, Deuel TF. The wilms' tumor gene product WT1 activates or suppresses transcription through separate functional domains *J Biol Chem*. 1993;268:9172-9175.

91. Maheswaran S, Park S, Bernard A, et al. Physical and functional interaction between WT1 and p53 proteins *Proc Natl Acad Sci U S A*. 1993;90:5100-5104.

92. Oka Y, Tsuboi A, Oji Y, Kawase I, Sugiyama H. WT1 peptide vaccine for the treatment of cancer *Curr Opin Immunol*. 2008;20:211-220.

93. Oka Y, Tsuboi A, Fujiki F, et al. WT1 peptide vaccine as a paradigm for "cancer antigen-derived peptide"-based immunotherapy for malignancies: Successful induction of anti-cancer effect by vaccination with a single kind of WT1 peptide *Anticancer Agents Med Chem*. 2009;9:787-797.

94. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of cancer antigens: A national cancer institute pilot project for the acceleration of translational research *Clin Cancer Res*. 2009;15:5323-5337.

95. Oka Y, Sugiyama H. WT1 peptide vaccine, one of the most promising cancer vaccines: Its present status and the future prospects *Immunotherapy*. 2010;2:591-594.

96. Izumoto S, Tsuboi A, Oka Y, et al. Phase II clinical trial of wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme *J Neurosurg*. 2008;108:963-971.

97. Glienke W, Maute L, Koehl U, Esser R, Milz E, Bergmann L. Effective treatment of leukemic cell lines with wt1 siRNA *Leukemia*. 2007;21:2164-2170.

98. Mi Y, Wang L, Bian S, Meng Q, Chen G, Wang J. Effect of WT1 gene expression on cell growth and proliferation in myeloid leukemia cell lines *Chin Med J (Engl)*. 1999;112:705-708.

99. Zhao Y, Xiao A, diPierro CG, et al. An extensive invasive intracranial human glioblastoma xenograft model: Role of high level matrix metalloproteinase 9 *Am J Pathol*. 2010;176:3032-3049.

100. Giannini C, Sarkaria JN, Saito A, et al. Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme *Neuro Oncol*. 2005;7:164-176.

101. Werner H, Roberts CT,Jr, Rauscher FJ,3rd, LeRoith D. Regulation of insulin-like growth factor I receptor gene expression by the wilms' tumor suppressor WT1 *J Mol Neurosci*. 1996;7:111-123.

102. Hosono S, Gross I, English MA, Hajra KM, Fearon ER, Licht JD. E-cadherin is a WT1 target gene *J Biol Chem*. 2000;275:10943-10953.

103. Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer *Proc Jpn Acad Ser B Phys Biol Sci*. 2009;85:314-323.

104. Pena C, Garcia JM, Larriba MJ, et al. SNAI1 expression in colon cancer related with CDH1 and VDR downregulation in normal adjacent tissue *Oncogene*. 2009;28:4375-4385.

105. Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer *Cancer Metastasis Rev*. 2009;28:151-166.

106. Liu Y, Lehar S, Corvi C, Payne G, O'Connor R. Expression of the insulin-like growth factor I receptor C terminus as a myristylated protein leads to induction of apoptosis in tumor cells *Cancer Res*. 1998;58:570-576.

107. Levitt RJ, Georgescu MM, Pollak M. PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2 *Biochem Biophys Res Commun*. 2005;336:1056-1061.

108. Izumoto S, Ohnishi T, Kanemura H, et al. PTEN mutations in malignant gliomas and their relation with meningeal gliomatosis *J Neurooncol*. 2001;53:21-26.

109. Li Y, Guessous F, Kwon S, et al. PTEN has tumor-promoting properties in the setting of gain-of-function p53 mutations *Cancer Res*. 2008;68:1723-1731.

110. Sarkaria JN, Yang L, Grogan PT, et al. Identification of molecular characteristics correlated with glioblastoma sensitivity to EGFR kinase inhibition through use of an intracranial xenograft test panel *Mol Cancer Ther*. 2007;6:1167-1174.

111. Xu Z, Wang W, Deng CX, Man YG. Aberrant p63 and WT-1 expression in myoepithelial cells of pregnancy-associated breast cancer: Implications for tumor aggressiveness and invasiveness *Int J Biol Sci*. 2009;5:82-96.

112. Barbolina MV, Adley BP, Shea LD, Stack MS. Wilms tumor gene protein 1 is associated with ovarian cancer metastasis and modulates cell invasion *Cancer*. 2008;112:1632-1641.

113. Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis *Cancer Res*. 2006;66:8319-8326.

114. Olmeda D, Moreno-Bueno G, Flores JM, Fabra A, Portillo F, Cano A. SNAI1 is required for tumor growth and lymph node metastasis of human breast carcinoma MDA-MB-231 cells *Cancer Res*. 2007;67:11721-11731.

115. Sugiyama H. WT1 (wilms' tumor gene 1): Biology and cancer immunotherapy *Jpn J Clin Oncol*. 2010;40:377-387.

116. Yona S, Lin HH, Siu WO, Gordon S, Stacey M. Adhesion-GPCRs: Emerging roles for novel receptors *Trends Biochem Sci*. 2008;33:491-500.

117. Gray JX, Haino M, Roth MJ, et al. CD97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation *J Immunol*. 1996;157:5438-5447.

118. Veninga H, Becker S, Hoek RM, et al. Analysis of CD97 expression and manipulation: Antibody treatment but not gene targeting curtails granulocyte migration *J Immunol*. 2008;181:6574-6583.

119. Kwakkenbos MJ, Kop EN, Stacey M, et al. The EGF-TM7 family: A postgenomic view *Immunogenetics*. 2004;55:655-666.

120. Aust G, Eichler W, Laue S, et al. CD97: A dedifferentiation marker in human thyroid carcinomas *Cancer Res*. 1997;57:1798-1806.

121. Dumur CI, Nasim S, Best AM, et al. Evaluation of quality-control criteria for microarray gene expression analysis *Clin Chem*. 2004;50:1994-2002.

122. Zhang L, Wang L, Ravindranathan A, Miles MF. A new algorithm for analysis of oligonucleotide arrays: Application to expression profiling in mouse brain regions *J Mol Biol*. 2002;317:225-235.

123. Van Meter T, Dumur C, Hafez N, Garrett C, Fillmore H, Broaddus WC. Microarray analysis of MRI-defined tissue samples in glioblastoma reveals differences in regional expression of therapeutic targets *Diagn Mol Pathol*. 2006;15:195-205.

124. Fu Z, Kim J, Vidrich A, Sturgill TW, Cohn SM. Intestinal cell kinase, a MAP kinase-related kinase, regulates proliferation and G1 cell cycle progression of intestinal epithelial cells *Am J Physiol Gastrointest Liver Physiol*. 2009;297:G632-40.

125. Bassi C, Mello SS, Cardoso RS, et al. Transcriptional changes in U343 MG-a glioblastoma cell line exposed to ionizing radiation *Hum Exp Toxicol*. 2008;27:919-929.

126. Liu J, Schmitz JC, Lin X, et al. Thymidylate synthase as a translational regulator of cellular gene expression *Biochim Biophys Acta*. 2002;1587:174-182.

127. Lenz HJ, Hayashi K, Salonga D, et al. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: An analysis of response and survival *Clin Cancer Res*. 1998;4:1243-1250.

128. Ayusawa D, Shimizu K, Koyama H, Kaneda S, Takeishi K, Seno T. Cell-cycle-directed regulation of thymidylate synthase messenger RNA in human diploid fibroblasts stimulated to proliferate *J Mol Biol*. 1986;190:559-567.

129. Wang Z, Ahmad A, Li Y, et al. Emerging roles of PDGF-D signaling pathway in tumor development and progression *Biochim Biophys Acta*. 2010;1806:122-130.

130. Wang T, Ward Y, Tian L, et al. CD97, an adhesion receptor on inflammatory cells, stimulates angiogenesis through binding integrin counterreceptors on endothelial cells *Blood*. 2005;105:2836-2844.

131. Liang Y, Li XY, Rebar EJ, et al. Activation of vascular endothelial growth factor A transcription in tumorigenic glioblastoma cell lines by an enhancer with cell type-specific DNase I accessibility *J Biol Chem*. 2002;277:20087-20094.

132. Li Z, Shao S, Xie S, Jiao F, Ma Y, Shi S. Silencing of CT120 by antisense oligonucleotides could inhibit the lung cancer cells growth *Ir J Med Sci*. 2010;179:217-223.

133. Butt AJ, Sergio CM, Inman CK, et al. The estrogen and c-myc target gene HSPC111 is over-expressed in breast cancer and associated with poor patient outcome *Breast Cancer Res*. 2008;10:R28.

134. Hendrix MJ, Seftor EA, Chu YW, et al. Coexpression of vimentin and keratins by human melanoma tumor cells: Correlation with invasive and metastatic potential *J Natl Cancer Inst*. 1992;84:165-174.

135. Allen WL, McLean EG, Boyer J, et al. The role of spermidine/spermine N1-acetyltransferase in determining response to chemotherapeutic agents in colorectal cancer cells *Mol Cancer Ther*. 2007;6:128-137.

136. Tanida I. Autophagy basics *Microbiol Immunol*. 2011;55:1-11.

137. Vecchione A, Baldassarre G, Ishii H, et al. Fez1/Lzts1 absence impairs Cdk1/Cdc25C interaction during mitosis and predisposes mice to cancer development *Cancer Cell*. 2007;11:275-289.

138. Ishii H, Vecchione A, Murakumo Y, et al. FEZ1/LZTS1 gene at 8p22 suppresses cancer cell growth and regulates mitosis *Proc Natl Acad Sci U S A*. 2001;98:10374-10379.

139. Lam P, Sian Lim K, Mei Wang S, Hui KM. A microarray study to characterize the molecular mechanism of TIMP-3-mediated tumor rejection *Mol Ther*. 2005;12:144-152.

140. Gabriely G, Wurdinger T, Kesari S, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators *Mol Cell Biol*. 2008;28:5369-5380.

141. Anania MC, Sensi M, Radaelli E, et al. TIMP3 regulates migration, invasion and in vivo tumorigenicity of thyroid tumor cells *Oncogene*. 2011.

142. Kataoka K, Yoshitomo-Nakagawa K, Shioda S, Nishizawa M. A set of hox proteins interact with the maf oncoprotein to inhibit its DNA binding, transactivation, and transforming activities *J Biol Chem*. 2001;276:819-826.

143. Gao X, Pang J, Li LY, et al. Expression profiling identifies new function of collapsin response mediator protein 4 as a metastasis-suppressor in prostate cancer *Oncogene*. 2010;29:4555-4566.

144. Duplan L, Bernard N, Casseron W, et al. Collapsin response mediator protein 4a (CRMP4a) is upregulated in motoneurons of mutant SOD1 mice and can trigger motoneuron axonal degeneration and cell death *J Neurosci*. 2010;30:785-796.

145. Han SL, Xu C, Wu XL, Li JL, Liu Z, Zeng QQ. The impact of expressions of CD97 and its ligand CD55 at the invasion front on prognosis of rectal adenocarcinoma *Int J Colorectal Dis*. 2010;25:695-702.

146. Liu Y, Chen L, Peng S, et al. The expression of CD97EGF and its ligand CD55 on marginal epithelium is related to higher stage and depth of tumor invasion of gastric carcinomas *Oncol Rep*. 2005;14:1413-1420.

147. Chen Z, Htay A, Dos Santos W, et al. In vitro angiogenesis by human umbilical vein endothelial cells (HUVEC) induced by three-dimensional co-culture with glioblastoma cells *J Neurooncol*. 2009;92:121-128.

148. Chicoine MR, Silbergeld DL. The in vitro motility of human gliomas increases with increasing grade of malignancy *Cancer*. 1995;75:2904-2909.

VITA

Archana Chidambaram was born on September 10, 1979 in Mumbai, Maharashtra, India. Archana is currently a citizen of India. She graduated from Fr. Agnel Multipurpose School, Mumbai. She attended Seth G.S. Medical College in Mumbai and graduated with an M.B.B.S. degree in 2003. After completing her medical education, Archana worked as a physician in department of Psychiatry and E.N. & T departments at Bhabha Atomic Research Center Hospital in Mumbai. She then joined the Department of Anatomy and Neurobiology at Virginia Commonwealth University in Richmond, Virginia in 2006. During this period, she presented her work at several national conferences and served as a teaching assistant for neuroanatomy and histology. Archana completed her doctoral work in Anatomy and Neurobiology at Virginia Commonwealth University, Richmond, Virginia in May, 2011 and will commence her residency training in Internal Medicine at the UMDNJ/ RWJ/ Cooper University hospital, Camden, NJ from June 2011.