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Transcriptional Analysis of Reciprocal Tumor-Microenvironment Interactions in Glioblastoma

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Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Molecular Genetics and Genomics

Dissertation Examination Committee:
Robi Mitra, Chair
Joshua Rubin, Co-Chair
Patrick Jay
Albert Kim
Elaine Mardis
Gary Stormo

Transcriptional Analysis of Reciprocal Tumor-Microenvironment Interactions in Glioblastoma

By

Michael Dale Brooks

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
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requirements for the degree
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Dedication

This thesis is dedicated to my grandmother, Pauline Mounce, who bought me my first computer when I went off to undergrad, setting in motion my love of computers and who recently started her own battle with cancer, thereby reminding us all of why we do what we do.

ABSTRACT OF THE DISSERTATION

Transcriptional Analysis of Reciprocal Tumor:Microenvironment Interactions in Glioblastoma

by

Michael Dale Brooks

Doctor of Philosophy in Molecular Genetics and Genomics

Washington University in St. Louis, 2014

Professor Robi Mitra, Chair
Professor Joshua Rubin, Co-chair

In the last twenty years both computational biology and cancer biology have made great strides and in the last 5 years the merger of the two has helped to revolutionize our knowledge of personalized targeted therapy and the diversity of cancer. In cancer, cell-to-cell interactions between tumor cells and their microenvironment are critical determinants of tumor tissue biology and therapeutic responses. Interactions between glioblastoma (GBM) cells and endothelial cells (ECs) establish a purported stem cell niche. We hypothesized that genes that mediate these interactions would be important, particularly as therapeutic targets. Using a novel computational approach to deconvoluting expression data from mixed physical coculture of GBM cells and ECs, we identified a previously undescribed upregulation of the cAMP specific phosphodiesterase PDE7B in GBM cells in response to ECs. We further found that elevated PDE7B expression occurs in most GBM cases and has a negative effect on survival. PDE7B overexpression resulted in the expansion of a stem-like cell subpopulation, increased tumor aggressiveness, and increased growth in an intracranial GBM model. This deconvolution algorithm provides a new tool for cancer biology, particularly when looking at cell-to-cell interactions, and these results identify PDE7B as a therapeutic target in GBM.

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Chapter 1: Introduction – Research at the interface of computational biology and cancer biology

History of Cancer

To best understand where the field of cancer biology is currently, it is important to know where the field started. Cancer, though not called that at the time, has been known since the time of the ancient Egyptians. Studies on mummies have found evidence of a number of tumors (1) and the Edwin Smith papyrus appears to be the first document describing cancer (2). It wasn't until one thousand years later when Hippocrates (c. 460 BC – c. 370 BC), arguably the founding father of modern medicine, gave it the name "carcinoma" after the Greek word for crab because of the finger like projections from the main tumor. This was later translated to Latin as "cancer" (3).

Throughout history, the explanation for the cause of cancer has changed drastically. Original theories were archaic by today's standards and revolved around the theory that the body had 4 humors, or liquids required for life, and that cancer was caused by an excess of the "black bile" humor (4). As time passed, future theories maintained the idea of a fluid as the cause of cancer, but were at least based on some understanding of human anatomy. Under this hypothesis, lymph fluid in the body was a waste product excreted from the blood, and as this collected and grew, it formed tumors (4). It wasn't until 1838 that Johannes Muller (1801-1858) finally proposed that cancer was, in fact, made up of cells (5,6). It was during this time that Muller, along with Rudolf Virchow (1821-1902), the father of modern pathology who used microscopy to start correlating cellular tumor patterns with prognosis (7) (8), ushered in the cellular age of cancer biology.

Even with the knowledge that cancer was a disease of cells, the mechanism of cancer formation eluded scientists into the beginning of the twentieth century. It was Theodor Boveri (1862-1915) who, while experimenting on chromosomes in sea urchins, proposed that cancer was a result of

scrambled chromosomes causing a single cell to grow into a tumor (9). Around the same time,

Peyton Rous (1879-1970) discovered that cancer could be transmitted from chicken to chicken

through the application of a cell-free filtrate (10). This was later shown to be caused by a virus,

now known as the Rous sarcoma virus (RSV). Interestingly, it would be more than 60 years

before Harold Varmus and J. Michael Bishop discovered the human origin of the viral proto-

oncogene c-Src (11) which is related to v-Src in RSV. These were both critical developments

toward the idea that cancer was a cell intrinsic disease, driven by somatic mutations conferring a

proliferation advantage.

Cancer as a cell instrinsic disease of somatic mutations

Perhaps themost important hypothesis concerning somatically driven cancer is the two-hit

hypothesis first put forth by Carl Nordling in 1953 (12) and further refined by Peter Armitage

and Richard Doll in 1954 (13) and 1957 (14) and formalized by Alfred Knudson in 1971 (15).

During the 1950s there was a lot of work on mathematical modeling of cancer progression as a

function of age (16,17), but Carl Nordling made the most significant contributions. His model,

based on cancer prevalence as a function of age, predicted, with amazing accuracy, that there

would be approximately 6 mutations required for any particular cell to become cancerous.

Nordling studied a number of tumor types to come to this conclusion, but noted that the math did

not apply to pediatric cancers, which while rare, still occurred far more often than his 6th power

law relationship would predict (12).   Doll and Armitage would expand upon the higher power

law model in their 1954 paper but in 1957 would propose a simpler two-stage theory. Their

main conclusion was that only two hits were required for carcinogenesis. An early hit which

would be induced by exposure to a carcinogen then followed by a long linear latent period of further mutations, finally resulting in tumorigenesis (14). It wouldn't be until 1971, when Alfred Knudson used the familial predisposition syndrome of retinoblastoma to rigorously define the two-hit hypothesis (15). These studies firmly cemented the idea that cancer was primarily the result of somatic mutations driving a proliferation advantage, but what genes these mutations were in, or what effect they were having, was only just beginning to be discovered in 1971.

The idea of dividing cancer's causative mutations up into two classes, now called oncogenes and tumor suppressors, far outdated anyone having any idea what they actually were. In fact the original idea probably dates as far back as Theodor Boveri's 1902 paper showing that abberant chromosomes could cause tumorigenesis (9). In that paper he introduces the terms "hemming-seinrichtungen", "teilungshemmende chromosomen", and "teilungsforedernde chromosomen", roughly "Cell cycle checkpoints", "Tumor suppressor genes", and "Oncogenes" respectively (18) (9). The idea that activating mutations, oncogenes, were responsible for tumorigenesis began with the discovery of the Rous Sarcoma Virus (10) and further studies of other viruses responsible for causing tumors only strengthened the case (19) (20). Examples of tumor suppressors, on the other hand, were hard to come by. Though in 1971, Henry Harris published results of a study that made the case for the importance of tumor suppressors. Harris showed that, using the recently described technique of cell fusion, normal mouse cells were dominant to malignant mouse cells (21). This argues against an activated oncogene being the primary cause of malignancy and argues for the required loss of both copies of some sort of tumor suppressing gene in the malignant cells for which function was restored once the normal cell fused with the cancerous one. A formal model for loss of both copies of tumor suppressor genes was officially

proposed in 1973 by David Comings (22), but it was Cavenee et al in 1983 who not only mapped

the retinoblastoma (RB) gene to chromosome 13 but showed that both the familial and sporadic

mutations mapped to the same location and caused homozygous loss of the gene (23). A large

number of independent groups worked towards refining the map and cloning of the RB gene

over the next few years (24) (25) (26) leading to the final complementation experiment where a

clone of RB was used to rescue retinoblastoma cells (27). Of course, the other side of the tumor

suppressor story is p53, which has now been shown to be mutated in a very large percentage of

cancers (28). Originally though, it was thought that p53 was an oncogene. Most of the evidence

for this came from mistakenly using a mutant cDNA (now shown to be a dominant negative form

of the protein) for the overexpression experiments instead of a wild type version (29) (30). This

was corrected only a few years later, in 1989, when Bert Vogelstein's group showed,

conclusively, that p53 was a tumor suppressor mutated in a large percentage of colorectal

cancers (31).

Moving past cell intrinsic cancer research into cancer as a tissue disease

While the work done with the idea of cancer mainly being a cell intrinsic proliferation disease

allowed for huge leaps in advancing the field, and is still important for targeted therapies (32)

(33), cancer research has slowly started to change its approach, where it is now appreciated that

cancer is a disease of tissues, where complex interactions between tumor cells with growth

advantages and the local tumor microenvironment synergize to produce highly malignant tumors.

However, this change didn't occur overnight. In 1975 an elegant experiment showed that mutation alone could not drive tumorigenesis. Beatrice Mintz and Karl Illmensee demonstrated this by taking malignant teratocarcinoma (a germ cell tumor) and injecting them into normal blastocysts of mice. They saw incorporation of the tumor cells into the developing mice, including a gene for a particular coat color, Steel, that was not present beforehand. This coat color could even be passed down to progeny (34). Later work would show that not all tumorigenic lines behave in this way (35) (36), but it was a landmark study showing how important the microenvironment could be and that proper cell:cell interactions could block tumor growth.

However it was the work of Mina Bissell that really showed just how important disorganization (i.e. inflammation) in the local microenvironment could be in driving tumorigenesis and propelled the idea to the forefront of cancer research. Work by Peyton Rous (10) had shown that cancer could be caused and propagated by a virus gathered from sarcomas in chickens and then injected into other chickens. In 1984, Mina Bissell and David Dolberg showed, in experiments that paralleled the work of Mintz and Illmensee, that introducing the virus into chicken embryos caused no tumorigenesis in the developed chickens, even though the virus was spread throughout, and active v-src was found in almost all cells. Furthermore they noted that while the adult chickens showed no signs of tumorigenesis, as soon as cells were removed and cultured without the proper cell:cell interactions they quickly showed signs of transformation (37). The question became how did a virus that could cause near universal tumorigenesis lose its power when introduced early? It turns out that the key difference was that the previous adult RSV experiments had been done by introducing the virus by subcutaneous or intramuscular injection,

which caused local wounding. In a follow up study in 1985, Bissell and colleagues showed that it was this local wounding that was required for tumorigenesis. In fact once the virus was established, secondary wounding in other locations would cause tumor formation (38). A few years later Bissell and colleagues would show the mechanism of this phenomenon was through transforming growth factor beta 1 (TGF-β) signaling, and that physical wounding was not required and adding recombinant TGF-β was sufficient to induce tumorigenesis (39). This was a remarkable set of experiments showing that a cell intrinsic proliferation advantage was not sufficient for tumorigenesis and that the microenvironment was playing a much larger role than previously appreciated.

Cancer stem cells (CSCs) are a relatively new, and controversial, addition to the study of the tumor microenvironment. The current definition of CSCs are cells which constitute a small subpopulation of the total cells in a tumor, have an increased tumorigenic capacity compared to the rest of the tumor cells, are able to self renew, and can reconstitute the various cell types found in the original tumor. Part of the controversy about CSCs comes not only from trying to find a consensus on what defines their function, but also of semantics as far as what to call them. Some authors prefer cancer stem cells while some prefer tumor initiating cells to further distance them from normal stem cells. Even with the controversies, the literature keeps pouring in to support the idea that there is certainly a subpopulation of tumor cells with increased tumorigenicity (reviewed in (40) (41) (42)) and understanding them is an extremely important step going forward towards improving cancer treatments.

The field of cancer stem cells started, for the most part, with an acute myeloid leukemia (AML) paper from 1997 (43), though there was other evidence of cells with increased tumorigenicity going back as far as 1937 (44). The AML paper by Dominique Bonnet and John Dick showed that taking AML cells and cell sorting them to purify the $CD34^+CD38^-$ population could produce a subpopulation of cells which were both more tumorigenic and could recapitulate the limited proliferative leukemic blast cell populations. While the paper itself did not use the term cancer stem cell, their direct comparison to the normal stem cell of the hematopoietic lineage that is also $CD34^+CD38^-$ and their hypothesis that it was direct somatic event in the normal population of hematopoietic stem cells which lead to the hypothesis that they were cancerous stem cells.

While work continued on cancer stem cells in blood cancers, it took another 6 years before evidence of cancer stem cells were found in solid tumors. In 2003, cancer stem cells were found in breast cancer (45), followed shortly by identifying them in brain tumors (46). Different sets of cell surface markers are used for different tumors, though there is some overlap. While leukemic CSCs are traditionally isolated with $CD34^+CD38^-$, for breast cancer Al-Hajj et al isolated $CD44^+CD24^-$ populations (45) and Singh et al isolated $CD133^+$ brain tumor cells for their CSCs (46). The choice of these cell surface antigens is important because while traditionally they have just been seen solely as a marker to identify a subpopulation of cells, recently these proteins have been shown to have important functions, particularly in the microenvironment. For example, CD44 is a cell surface glycoprotein which functions as the receptor for hyaluronic acid (47) (an important component of the extracellular matrix) and has been implicated in breast cancer invasion (48)and functions to help mesenchymal stem cells (MSCs) transition to tumor associated fibroblasts (49). CD133 has recently been shown to induce tumorigenic properties in

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HEK293 cells (50), increase drug resistance in glioma cells (51), increase proliferation of glioma cells through ERK pathway activation (52), and even increase epithelial-mesenchymal transition (EMT) rates in head and neck cancers (53).

Not only is the field of cancer stem cells extremely complicated with many unanswered questions, but cancer stem cells are only the tip of the iceberg as far as the tumor microenvironment goes. The cellular constituents of the tumor microenvironment can vary greatly depending on the tumor type but in glioblastoma include: pericytes (54) (55), endothelial cells (56), microglia (57), astrocytes (58) (59), cancer stem cells (45), and tumor cells. The specialized glioblastoma tumor microenvironment known as the perivascular niche is extensively reviewed in Chapter 1, but the importance here is to note how complex a tumor microenvironment can be, and how, with the era of computational biology and bioinformatics upon us, these tools can be applied to the tumor microenvironment in a high-throughput fashion to expedite our understanding of the functions of a microenvironment to develop improved cancer treatments.

History of genomics and bioinformatics

While genomics refers to the specific study of genomes, bioinformatics refers to the storage and manipulation of biological sequencing data. Both fields are relatively new, having only been around for the past 30 years or so. It was 1977 when the first complete genome, the bacteriophage ΦX174, was published (60). The term genomics wasn't coined until 1986 by Tom Roderick (61) and while the term bioinformatics actually predates genomics (62), during these

earlier days it wasn't particularly applied to DNA based sequencing data since there was not enough data being generated to require heavy computational analysis tools.

The early focus of the field of genomics was on improving the technology of generating sequence data. In fact, while the sequence of ΦX174 was generated with the "plus and minus" method worked out by Fredrick Sanger, he quickly published an improved method of sequencing with dideoxy-terminating nucleotides that same year (63). This work would later win Sanger, along with Paul Berg and Walter Gilbert, the Nobel Prize in Chemistry in 1980. Sanger's dideoxy sequencing would be quickly become the gold standard, and is still used today for some applications, but at the time was still a laborious process. After running the sequencing reactions, they would be loaded onto a polyacrylamide gel, ran out for a very long time (~14 hours), and then annotated by hand to determine the sequence (63). As more genes and genomes were sequenced, and as scientists looked ahead towards sequencing larger genomes, it became clear that running every reaction out on a gel and hand annotating the sequence would be impossible. The next big leap in technology, and perhaps the most critical of all for the genomic revolution, came in 1986 when Leroy Hood and collaborators at Applied Biosystems developed and commercialized the automated DNA sequencing machine. Hood improved Sanger's method by using dye-labeled, instead of radioactively, dideoxynucleotides and instead of running out the reactions in a large polyacrylamide gel, eventually ran the reactions in capillary tubes filled with a denaturing polymer. This allowed each passing fluorescent band to be detected by laser and automatically recorded and greatly improved the throughput of sequencing projects enabling the ambitious Human Genome Project (HGP) to be feasible.

The human genome project was officially launched in 1990 with funding from the US

Department of Energy (DOE) and the National Institutes of Health (NIH) along with number of

international collaborators. Projected to be a 15 year project costing 3 billion US dollars, it came

in slightly under budget and nearly two years early, with a draft being released in 2001 (64) and

the project being declared finished in 2003 (65) (66), though there have been continual smaller

updates since then. The HGP was designed as a hierarchical shotgun project, with the original 3

billion basepair genome being broken up into 150-200,000 bp bacterial artificial chromosomes

(BACs). It took about 20,000 BACs to adequately cover the whole genome. These BACs were

then subdivided into subclones, each containing about 2000bp. These were fully sequenced with

Sanger sequencing, reassembled into the full length BACs and finally into full chromosomes

(64).

It was during The Human Genome Project that the power of bioinformatics became an important

cog in the machine of sequence generation. A given sequencing center could be generating

millions of basepairs per day and this data needed to be stored and analyzed. Programs such as

phred, which took the raw Sanger sequencing traces and turned them into sequence data and

quality scores (67) (68), and phrap, which assembled the sequences into contigs, were important

tools used in the data analysis pipeline. It's important to note though, that while bioinformatics

was becoming more important, it was not the rate limiting step, which was still sequence

generation (69).

There are two main ways that the HGP had a profound impact on science; direct research on the information gathered and using the sequence as a scaffold to build the next step in sequencing technology development. A number of very important projects grew out of this data, including 1,000 Genomes (70) and HapMap (71) (72), but the ENCODE (Encyclopedia of DNA Elements) project was the largest of these collaborative efforts. This project produced a vast array of data spread out over a number of different journals (73) (74) (75) (76) (77) and continues to this day. While the lasting impact and legacy of the ENCODE project is yet to be determined, the importance of using the reference genome as a scaffold to build next technologies off of is already firmed established. *De novo* sequencing of the human genome required the long reads provided by Sanger sequencing to have any chance of assembling the data back into progressively larger pieces. Once the entire genome was complete though, it made way for a wave of new technologies that sacrificed read length for massive increases in throughput (78).

This era of "next-generation" (next-gen) sequencing started around 2005 when the 454 Sequencing company released their GS20 sequencer. Their technology aimed to greatly increase the throughput of sequencing compared to Sanger sequencing by running reactions in a highly multiplexed microtiter plate and recording the introduction of specific nucleotides by the release of pyrophosphate (79). This 454 sequencing still had read numbers on the low end (compared to other soon to come technologies) but did have read lengths comparable to traditional Sanger sequencing. This allowed the technology to specialize in high throughput bacterial sequencing where assembling the genome back together de novo without a scaffold was still required.

Another important technological milestone of next-gen sequencing was Polony Sequencing, a technology developed by Robi Mitra, Jay Shendure, and George Church (80). The original technology, published in 2003, focused on forming colonies of clonal DNA by PCR which were immobilized in polyacrylamide on a microscope slide. Once created, the Polony could be sequenced by synthesis with fluorescent nucleotides. While not as high-throughput as some of the other technologies, Polony Sequencing had the advantage of having a lower cost of entry, since a lab could use an existing fluorescent microscope for imaging. The technology was further developed and improved by Jay Shendure to fully sequence a bacterial genome (81), and eventually licensed to Dover Systems to produce a self-contained Polony Sequencing machine called the Polonator that would serve as a low-cost entry-level sequencing system.

In 2006, Solexa (later purchased by Illumina and generally refered to as "Illumina Sequencing") released their first next-gen sequencing machine the Genome Analyzer. This technology shares some similarities to Polony Sequencing, in that PCR is used to form distinct areas of clonal molecules. While the colonies in Polony Sequencing were supported in a polyacrylamide gel, Illumina uses a solid surface as the base for a reaction called Bridge PCR, where the primers for amplification are attached directly to glass flow cells, and the reagents to run the PCR can be flowed directly into the chamber. This allowed very high densities of spots of clonal DNA, called "clusters" (82). Through the years this technology has constantly improved with the introduction of various new machines with improvements to the cameras, flow cells, and reagents leading to continued increases in data generation, pushing the cost of sequencing lower and lower towards the goal of a $1,000 genome.

Cancer sequencing projects

As the cost of sequencing became cheaper, scientists were constantly searching out new ways to use the technology. Applying next-gen sequencing to cancer biology was one of early favorites. It was already known how important specific mutations could be in driving cancer, but it was also clear that a great deal was unknown about the distribution of mutations in cancer as a whole, and that by sequencing enough individual patient's tumor and matched normal DNA it would start to paint a picture of what stayed constant between different patients and what was different.

In 2008 the first whole genome cancer sequencing paper was published by Ley and Mardis et al (83). There they took a single acute myeloid leukemia (AML) patient showing a cytogenetically normal karyotype and thoroughly sequenced both the tumor and matched normal DNA. Analysis of the sequencing results showed that the tumor DNA contained an overwhelming ~2.6 million single nucleotide varients (SNVs), but by subtracted out those that were also in common with the matched normal DNA (and therefore unlikely to be causative), and limiting the analysis to only SNVs in coding regions of known genes, the final results showed 10 mutations that were potentially causative, 8 novel mutations in a variety of genes, and two previously known mutations. This paper was critical in providing a foundation for the methodology in future cancer sequencing projects. It showed the critical importance of sequencing matched normal DNA and gave a starting point for other groups to start sequencing more AML patients, and other tumor types.

Since 2008, there have been hundreds of papers publishing cancer genomes from groups across the world. A small list of the published cancer types include: AML (84), ALL (85), breast (86), prostate (87), liver, brain (88) (89), lung (90), pancreatic (91), colon (92), melanoma (93), and the list could go on. The first few papers in 2008 focused on one or two patients but as sequencing became cheaper, it became possible, and necessary, to include more and more samples to get a better idea of what the mutational landscape looked like across patients. To prevent redundancy and facilitate the sharing of data an organizational system was needed. To accomplish this, a number of consortiums, which either previously existed or were created, were used to keep the various research groups on the same page. These included the International Cancer Genome Consortium (ICGC) (94), The Cancer Genome Atlas (TCGA) (95), and the Cancer Genome Project (96). These projects have been instrumental in organizing the joint effort of multiple research groups and providing databases to deposit data and make it available to other researchers.

Heterogeneity has always been an important concern in cancer sequencing projects and while they have revealed the huge degree of heterogeneity in some tumors (97) (98), they have also struggled with how to deal with that heterogeneity from a technical standpoint. There are two types of heterogeneity in tumor sequencing. The first is heterogeneity in the cancer cells themselves caused by evolution of the tumor as it grows. The initial causative mutations are always present, but as the tumor grows, further mutations are gathered, and these various subclones go on to populate different percentages of the final tumor. The second source of heterogeneity is particularly relevant in solid tumors where normal non-tumorous cells can account for a large percentage of the local tumor microenvironment.

At first, to simplify the experiment and analysis, both types of heterogeneity were minimized as much as possible. The initial Ley and Mardis et al paper was concerned with contamination of normal DNA in their tumor DNA and therefore used the fact that an initial bone marrow sample contained nearly 100% myeloblasts to help select their patient. Their analysis also showed a pretty low level of intratumoral heterogeneity. Of the mutations discovered, all eight were in almost all of the cells sequenced, both at initial presentation and relapse (83). On the other hand, recently some studies have started to embraced having mixed populations of tumors cells (though still treating normal cells as contamination and avoiding them) to look at intratumoral heterogeneity and evolution. These studies have developed new computational tools (99) as well as designing experiments to detect the heterogeneity (100) (101). Unfortunately, heterogeneity of the tumor microenvironment has still not been addressed to the same level. There is a lack of knowledge about how tumors cells change as a function of their interaction with the microenvironment and what mediates that interaction.

Can whole genome transcriptional analysis be applied to tumor:microenvironment interactions?

Therefore the question of my thesis revolves around whether computational tools could be applied to tumor:microenvironment interactions. Using glioblastoma as a model system, we wondered whether two cell types from the local microenvironment, endothelial cells and tumors cells, could be grown together to ask what reciprocal interactions are induced in each cell type, and whether any of these differentially expressed genes would make good targets for future therapeutically targets.

Summary of contribution of thesis to the field

Cancer biology is in progress of a transition from looking at cancer as mostly a disease of somatic mutations leading to proliferation advantages to a whole tissue disease with many different cell types contributing to tumorigenesis. Not only are new tools required for looking at complex cellular interactions, but these tools need to provide biologically relevant results. This thesis looks to not only provide a method to look at the global expression profile of the reciprocal interactions between two cell types, but takes the example of glioblastoma cells interacting with endothelial cells and follows up on a top candidate gene, PDE7B, and begins to elucidate its importance in the GBM perivascular niche.

Contribution to co-authored work presented in this thesis

In accordance with university policies, any co-authored work presented is to be accompanied by an explanation of the contribution by the thesis author, Michael Brooks, to those co-authored works. Chapter one of this thesis is a review on the glioblastoma perivascular niche previously published in Current Pathobiology Reports under the title "Hitting Them Where They Live: Targeting the Glioblastoma Perivascular Stem Cell Niche". I was first author on this review and, along with Dr. Rubin, we decided on the topics to be covered. I also wrote the sections on cell adhesion signaling, created the figure, and performed final edits of the paper before submission.

Chapter three of this thesis is a primary research paper on the functions of PDE7B in the glioblastoma perivascular niche currently in submission. I conceived and designed the

experiments along with Dr. Mitra and Dr. Rubin. I performed all of the experiments except the

tissue microarray. I also analyzed the data and constructed all figures. I wrote the manuscript

with edits and revisions from Dr. Mitra and Dr. Rubin.

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Chapter 2: Hitting Them Where They Live: Targeting the Glioblastoma Perivascular Stem Cell Niche

Authors:
Michael D. Brooks, Rajarshi Sengupta, Steven C. Snyder, & Joshua B. Rubin

Department of Pediatrics, Washington University School of Medicine, 660 South Euclid Ave. St Louis, MO 63110

Abstract

Glioblastoma growth potential and resistance to therapy is currently largely attributed to a subset of tumor cells with stem-like properties. If correct, this means that cure will not be possible without eradication of the stem cell fraction and abrogation of those mechanisms through which stem cell activity is induced and maintained. Glioblastoma stem cell functions appear to be non-cell autonomous and the consequence of tumor cell residence within specialized domains such as the perivascular stem cell niche. In this review we consider the multiple cellular constituents of the perivascular niche, the molecular mechanisms that support niche structure and function and the implications of the perivascular localization of stem cells for anti-angiogenic approaches to cure.

Introduction

As initially conceived by Judah Folkman, tumor growth is indeed "angiogenesis dependent" (1). While visionary, this revolutionary statement was limited by knowledge current in 1971 to a declaration regarding the necessity of a blood supply for nutrients and oxygen. Dr. Folkman and his contemporaries could not have imagined that angiogenesis was also a process that creates a specialized domain for the support, expansion and spread of a subpopulation of tumor cells with stem cell like properties (cancer stem cells (CSCs)). This specialized space, the perivascular domain or niche (PVN) is an exquisite collaboration between tumor cells, endothelial cells, pericytes and tissue specific components, for the maintenance of the tumor stem cell population.

In light of this greater appreciation for the importance of angiogenesis to tumor persistence and progression, targeting angiogenesis for cancer therapy would seem to have even greater potential than originally conceived. Not only can it disrupt blood supply and oxygen delivery, it can abrogate the formation of niche space and thereby terminate the potential for tumor growth. However, clinical experience with anti-angiogenic therapy that targets the single most potent angiogenic factor, vascular endothelial cell growth factor (VEGF), or its receptors has taught us that there are multiple mechanisms by which tumors stimulate angiogenesis and resist anti-VEGF therapies. These mechanisms are diverse and involve additional soluble angiogenic factors, changes in the cellular constituents of the vascular unit, and even transdifferentiation of tumor cells into endothelial cells. This experience suggests that targeting the structure of the niche by simply trying to block its formation may not be practical. Instead, alternatively targeting niche function may have superior therapeutic effect without stimulating resistance mechanisms. In order to succeed in this endeavor it is imperative to understand the functions of the niche and the mechanisms that serve those functions. In this review we will examine the cellular components of the brain tumor stem cell niche and core modes of intercellular communication that support its coordinated activities.

Functions of the Perivascular Niche

Experience with culturing brain tumor stem cells suggests that the stem cell state is an unstable one and that in the absence of appropriate signals these cells will undergo spontaneous differentiation. Thus we can conclude that the functions of the niche include blocking differentiation in order to maintain the stem cell phenotype. Consistent with this, when brain

tumor stem cells are grown *in vitro* in the presence of endothelial cells, there is a measureable

increase in self-renewal capacity and quaternary tumor sphere formation (2-5). Moreover,

treatment of xenograft brain tumor models with anti-angiogenic agents alone or in combination

with cytoxic chemotherapy results in decreases in the population of self-renewing CD133+,

Nestin+ cancer stem cells (3,6).

In addition to maintaining the cancer stem cell population, the PVN also promotes tumor cell

proliferation (2,3). Primary glioblastoma multiforme (GBM) cells grown in the presence of

human brain microvascular endothelial cells (HBMECs) exhibit increased growth *in vivo* and *in

vitro* compared to GBM cells alone, and like the normal neural stem cell niche this is due at least

in part to the actions of endothelial cell-derived CXCL12 (7,8). In addition, GBM-associated

endothelial cells express sonic hedgehog (SHH, (9)), which can also stimulate tumor cell

proliferation (10).

Importantly, the PVN can provide sanctuary and protect GBM from the actions of both radiation

and chemotherapy. The backbone of malignant brain tumor treatment is DNA damaging agents

like radiation therapy and alkylator chemotherapy. The efficacy of these regimens is highly

dependent upon mitotic activity in target cells and a fraction of the tumor stem cells are found in

a slow-cycling or quiescent state, which would render them resistant to DNA damaging agents

(11,12). In addition, the efficacy of DNA damaging agents is sensitive to changes in DNA repair

capacity. Within the PVN there is a measureable increase in DNA repair capacity, possibly

through the actions of microenvironment-derived TGF-β (13). This would also mitigate against

the impact of DNA damaging agents (14,15). Moreover, tumor stem cells exhibit increased

expression of multidrug resistance transporters (such as ABC and MDR transporters), which are responsible for the efflux of chemotherapeutics out of cells and thus limit the exposure of tumor cells within the PVN to DNA damaging agents (16,17). This property has been used to identify GBM stem cells as the Hoechst stain negative side-population of tumor cells on FACS analysis (18). Finally, GBM stem cells avoid immune detection and suppress immune activity through diminished expression of MHC (19) and secretion of immunosuppressive cytokines that block T cell proliferation and activation (20), an effect that is augmented by hypoxia (21).

The peri-endothelial space also provides an important conduit for infiltrative spread of GBM throughout the brain. In 1938, Scherer described the movement of GBM cells away from the primary tumor mass along the perivascular space (22), and dispersal of GBM along through this space may be a critical component of tumor recurrence after gross total resections and tumor bed irradiation. The basis for this pattern of GBM cell movement may be due to chemotactic effects of high levels of CXCL12 found within the PVN (7,23) and CXCL12's effects on expression of cathepsins and matrix metalloproteinases (MMP) (24).

Origins of the Perivascular brain Tumor Stem Cell Niche
Multiple mechanisms have been proposed through which brain tumor cells might forge stem cell supportive interactions with endothelial cells, including: co-opting existing blood vessels and stimulating angiogenesis. Surprisingly, however, in three recent papers (25) (26) (27) it was shown that GBM stem cells themselves can transdifferentiate into endothelial cells. Up to 60% of the tumor-associated endothelial cells shared genetic background with tumor cells, and a subset of the CD133 positive brain tumor stem cell fraction were also positive for vascular

endothelial-cadherin (CD144). Similar transdifferentiation of normal neural stem cells into endothelial cells has also been described (28) and may represent a broadly important phenomenon. The frequency of GBM-derived endothelial cells in patient specimens remains to be fully determined and the potential for these GBM-derived endothelial cells to provide structural niche space and regulatory control of niche function remains to be defined.

Components of the brain tumor stem cell niche

Development of the tumor PVN involves recruitment of a multiple cell types to the niche. We are only starting to understand the complex cellular architecture of the niche and the significance of each cell type to the functions of this microdomain. The brain tumor PVN is comprised of vascular endothelial cells, pericytes and astrocytes as well as macrophages/microglia. Understanding the molecular mechanisms by which each of these cell types interact with each other and with the CSCs will help us therapeutically target those interactions within the PVN and block tumor progression.

Endothelial cells – In adult neurogenic niche, CSCs are often localized along the tumor vasculature (29). Glioma stem cells, which are frequently identified by their expression of surface markers such as CD133 (30), constitute a small fraction of the total tumor population. They appear to preferentially align themselves in the peri-endothelial space, compared to their non-stem cell counterparts, and their fractional abundance within total tumor cell numbers is strongly and positively correlated with tumor grade (31,32). A repertoire of soluble and cell-

surface molecules have been identified, which through paracrine and/or autocrine mechanisms mediate reciprocal cross talk between the endothelium and tumor cells in GBM. We recently reported that brain endothelial cell derived CXCL12 chemoattracts and supports proliferation of primary human GBM cells (7). Signaling pathways such as Notch, sonic hedgehog (SHH), VEGF, hepatocyte growth factor (HGF), pigment epithelium-derived factor (PEDF) and nitric oxide (NO), many of which are also important for neural stem cell proliferation, have been implicated in the inter-cellular communication between endothelial and tumor stem cells within the PVN (2,4,15,33-37). It is interesting to note that a major distinction between tumor cells and normal neural cells, is that the tumor stem cell population can be replenished from the non-stem cell fraction, a phenomenon that is not observed for normal neural cells (38). Based on the frequent localization of tumor stem cells to the PVN, as well as the observation that pathways critical for stem cell survival are active within this niche, the PVN may function to chemoattract tumor cells, promote their transition to a "stem" like phenotype and support their maintenance and proliferation.

Pericytes – Pericytes are mesenchymal cells that are usually embedded in the vascular basement membrane where they surround and stabilize the newly formed vasculature. Several reports have indicated that pericytes are an integral part of the tumor PVN and regulate proliferation, invasion and angiogenesis through their interactions with endothelial cells. Studies in a variety of cancers including melanomas, pancreatic cancer, lung adenocarcinoma and GBM have identified different signaling pathways such as platelet-derived growth factor-β) (PDGF-β), epidermal growth factor (EGF), hypoxia-inducible factor- 1α (HIF-1α) and CXCL12 that are involved in the recruitment of pericytes to the tumor vessels (39). Reciprocal signaling between endothelial

cells and adjacent pericytes through soluble, as well as membrane bound, factors such as PDGF, angiopoetin-Tie2, and angiotensin can actively regulate angiogenesis (40). In contrast to normal pericytes, tumor pericytes are loosely associated with the endothelial cells leading to leaky vasculature suggesting that normalization of the tumor vessels may have therapeutic relevance. The limited success of anti-VEGF therapy in GBM and other tumors has led to the proposal that double targeting of pericytes and endothelial cells might be productive of greater therapeutic effect (41). However, the failure of endothelial targeting in the absence of pericytes in certain tumor models suggests that the role of pericytes in the PVN needs further investigation (42).

Astrocytes - In the normal brain, astrocytes provide structural support to the brain vasculature and maintain blood brain barrier (BBB) integrity through end processes that interact with the vascular endothelial cells (43). In the normal adult neurogenic niche, astrocytes induce stem cell proliferation through the activation of purinegic receptors on stem cells while negatively regulating neurogenesis through the Notch pathway (29,44). Gliomas induce changes in proteins expressed in astrocytic endfeet leading to a loss of astrocytic regulation of endothelial functions and dysregulation of the BBB (45). Gliomas often contain pathology-associated or reactive astrocytes, which may mediate tumor cell invasion via activation of MMPs. Astrocyte elevated gene (AEG-1), initially isolated in fetal astrocytes is often implicated in metastatic progression and invasion of gliomas (40). In a PDGF-induced glioma model, SHH expressing reactive astrocytes were identified in close association with nestin expressing tumor cells (9). Glioma stem cells have been shown to express the SHH receptor patched (PTC) and inhibition of the pathway leads to the disruption of stem-like and tumorigenic properties suggesting that SHH producing microenvironment may act as a stem cell niche.

Macrophage/microglia – Tumor associated macrophages/microglia (TAM/Ms) may constitute up to 5-30% of the tumor cell population. They are frequently localized adjacent to tumor stem cells in the PVN (46). Chemokines such as macrophage chemotactic protein (MCP)-1 and 3 as well as cytokines including colony stimulating factor (CSF)-1, granulocyte colony stimulating factor (G-CSF), and HGF have been implicated in the chemo-attraction of the macrophages to the PVN and CSCs (40). Reciprocal interactions between the glioma cells and macrophages facilitate an immune suppressive but tumor supportive phenotype for macrophages that promote tumor growth and invasion through activation of MMPs. Glioma CSCs have been shown to inhibit macrophage/microglia phagocytosis, induce secretion of immune-suppressive cytokines such as IL-10 and transforming growth factor (TGF)-β1 and enhance macrophage/microglia induced T-cell proliferation via STAT-3 pathway (47,48). Recent studies have demonstrated that TAM/Ms can enhance angiogenesis, as well as the proliferation and invasiveness of glioma CSCs via release of TGF-β1, which induces expression of MMP-9 by glioma CSCs (49).

Extra-cellular matrix – In addition to the cellular milieu cancer stem cells like neural stem cells also interact with the extracellular matrix components within the PVN (50), especially laminin (51). The composition of laminins has been correlated with tumor grade and patient survival in gliomas (52). Furthermore, the laminin receptor integrin $\alpha_6 \beta_1$ has been shown to promote endothelial cell growth in GBM, which may indirectly modulate tumor stem cell survival (53). The role of other ECM components in modulating CSCs and tumorigenesis needs further investigation.

Ependymal Cells – While the tumor PVN and normal neural stem cell niche share many features, there are also distinct differences on both the cellular and molecular levels. For example, ependymal cells are a critical component of the SVZ stem cell niche, and their cell number within the neurogenic zone correlates with stem cell number and neurogenesis (54) (55) (56). Among the identified mechanisms by which ependymal cells regulate stem cell function is the negative regulation of BMP signaling through expression of LRP2 (55). Recently, molecular profiles of the cellular constituents of the niche have been published and provide several additional intriguing candidate mediators of ependymal effects on stem cell function (56). Whether ependymal cells are similarly involved in the brain tumor PVN is unknown at this time, though the deeper parenchymal location of most GBM associated niches would suggest that ependymal cell involvement is unlikely. This raises the interesting question of what, if any, impact this has on the regulation of stem cell activity within the tumor PVN.

Cell adhesion signaling in the PVN

Many important pathways serve the functions of the niche, and most of these have been expertly reviewed elsewhere. Therefore, we will focus on a less frequently discussed aspect of the PVN for which potential therapeutics exist, cellular adhesion signaling including: integrins and cadherins, and how these molecules influence both cell to cell and cell to ECM interactions within the PVN.

Integrins in the niche

Integrins are essential transmembrane proteins that both anchor cells to the extracellular matrix and transmit extracellular signals across the cell membrane in response to ligation by extracellular matrix components like laminin, fibronectin vitronectin, collagen, thrombospondin and osteopotin as well as other factors such as FGF. There are currently 24 known heterodimeric integrins, comprised of one of 18 alpha subunits and 8 beta subunits. While integrins lack intrinsic kinase activity they transmit signals by forming multimeric complexes called focal adhesions with other signaling proteins such as focal adhesion kinase (FAK) (57) and adaptor proteins like p130CAS (58). Unbound integrins can transmit pro-apoptotic signals (59) while complexed integrins activate core growth and migratory pathways such as the MAPK, PI3K, NF-kB and Src pathways (60). These activities regulate cell:cell interactions between tumor cells and endothelial cells as well as between non-tumor stromal elements of the PVN such as perictytes and endothelial cells. In this fashion, integrins regulate the three-dimensional structure and function of the stem cell niche.

Importantly, the only gene in common between expression profiling analyses of multiple stem and progenitor cell populations is the laminin receptor integrin α_6 (61-63). Integrin α_6 is also highly expressed by GBM stem cells where it appears to be required for self-renewal activity (64). Consistent with the importance of laminin and laminin receptors to the functions of the neural stem cell niche, expression of integrin β_1, one of two dimerization partners for integrin α_6, exhibits restricted expression to proliferative cells within the normal subepenbdymal neural stem

cell niche (65). Moreover, surface localization of integrin β_1 is enhanced by Galectin 1 (66), an adhesion molecule that is expressed in normal neural stem cells where it is known to regulate proliferation (67) (68), as well as in GBM where it additionally promotes invasion (69).

Integrin β_1 can function in a signaling axis together with the chemokine receptor CXCR4 (70). As both Integrin β_1 and CXCR4 are highly expressed within the PVN, their crosstalk might regulate GBM stem cell functions. The impact of integrins on stem cell biology may relate to their modulation of key stem cell pathways like the Wnt (71), SHH (10) and Notch (72) pathways.

Malignant transformation is associated with changes in integrin expression in a tumor specific fashion. Increased $\alpha_v\beta_3$ and $\alpha_v\beta_5$ is found in glioblastoma and associated with increased invasion, especially at the margins of the tumor (73). Interestingly, $\alpha_v\beta_8$ is also expressed in GBM and levels of $\alpha_v\beta_8$ expression correlate with two important growth phenotypes of GBM: angiogenic and infiltrative. GBM cells with high levels of $\alpha_v\beta_8$ expression exhibit correspondingly high levels of TGF-β pathway activation and an invasive pattern of growth (74). In contrast, GBM cells with low levels of $\alpha_v\beta_8$ expression exhibit correspondingly low levels of TGF-β pathway activation and an angiogenic pattern of growth. This is relevant to the present discussion, as a shift from an angiogenic to infiltrative pattern of growth has been observed in GBM treated with anti-angiogenic therapies, which alter PVN structure and function (75-77).

While the molecular basis for changes in integrin expression remains to be fully defined, components of the PVN including TGF-β and CXCL12/CXCR4 regulate integrin expression in

various tumor types. In GBM, both TGF-β1 and TGF-β2 can increase expression of $\alpha_v\beta_3$ in tumor cells and increase their migratory activity (78). The chemokine CXCL12 and its receptor CXCR4 are important components of the PVN where they recruit brain tumor cells and stimulate brain tumor cell proliferation (7). Recently it was shown that CXCL12 signaling in prostate cancer affects the expression of two different integrins; $\alpha_v\beta_3$ (79) and $\alpha_5\beta_3$ (80) both of which are correlated with tumor progression (73).

Cadherins in the niche

Cadherins are calcium-dependent cell adhesion molecules that mediate cell:cell interactions critical for the maintenance of normal tissue structure including the neural stem cell niche (81). The cadherin superfamily contains multiple members within several subfamiles in which individual members mediate primarily homotypic interactions to form adherens junctions that serve to segregate different cells into homogeneous populations or functional units within tissues. A number of regulators of fate and function are concentrated in adherens junctions in the central nervous system including: β-catenin (82), protein kinase C (83), cdc42 (84) and Numb (85). Consequently, dynamic regulation of cadherin expression or cadherin-switching controls cell migration, fate and function during normal development and oncogenesis. In the normal neural stem cell niche, N-cadherin expression is required to maintain the progenitor state while loss leads to delamination and differentiation of newly generated neurons (86). Much attention has been focused on the regulation of cadherin expression in cancer as dramatic changes in cadherins accompany Epithelial-Mesenchymal Transition (EMT), a critical step in malignant progression.

Alterations in GBM cadherin expression are also documented to accompany alterations in growth. The switch from angiogenic to infiltrative pattern of growth seen with VEGF pathway antagonism is accompanied not only by changes in integrin expression but also by a T to N cadherin switch (87). Similarly, Cadherin 11, a marker of mesenchymal subtype of GBM, enhances GBM cell migration and appears to be required for tumor growth *in vivo* (88). Possibly most exciting with regard to cadherins and GBM stem cell activity and the PVN is the observation that expression of E-Cadherin in GBM patient specimens is associated with poor prognosis (89) and that a subset of E-cadherin expressing CD133 positive GBM stem cells appears to have the capacity for transdifferentiation into endothelial cells (25,27).

Cadherin expression is regulated by several transcription factors including FoxP2 and 4 (86), Twist (90) and Snail (91). In cancer, it appears that cadherin expression is also regulated by cytokines like IL-8 (92). Increased IL-8 expression is associated with EMT in breast cancer (93) and positively correlated with astrocytoma grade (94). Importantly, IL8 is expressed at high levels by tumor associated endothelial cells (95) and thus is likely to be active within the PVN.

Finally, interactions between cadherins and integrins have been recently observed in GBM stem cells (96). These interactions appear to regulate intracellular signaling and migration. Moreover, co-regulation of N-cadherin and integrin β1 by the receptor tyrosine kinase Tie2 is required for the adhesion of GBM cells to the endothelium as occurs within the PVN (97).

Targeting the Niche

The identification of brain tumor stem cells and their perivascular niche has energized efforts to develop stem cell directed therapies. Targeting stem cell activity can theoretically be achieved by: 1) targeting the stem cells themselves, 2) by targeting PVN formation or, 3) by targeting PVN function.

Abrogation of PVN formation through anti-angiogenic therapy is a potentially powerful approach to stopping tumor progression. VEGF antagonism is well tolerated and has efficacy but alone, or in combination with irinotecan, it does not have a lasting effect on survival. Multiple mechanisms can drive tumor progression in the setting of VEGF antagonism (98). The mechanisms of resistance to VEGF antagonism are diverse and instructive when considering how to block PVN formation. In response to bevacizumab there are increases in expression of pro-angiogenic factors like FGFs 1 and 2 and CXCL12 (99,100), as well as increased
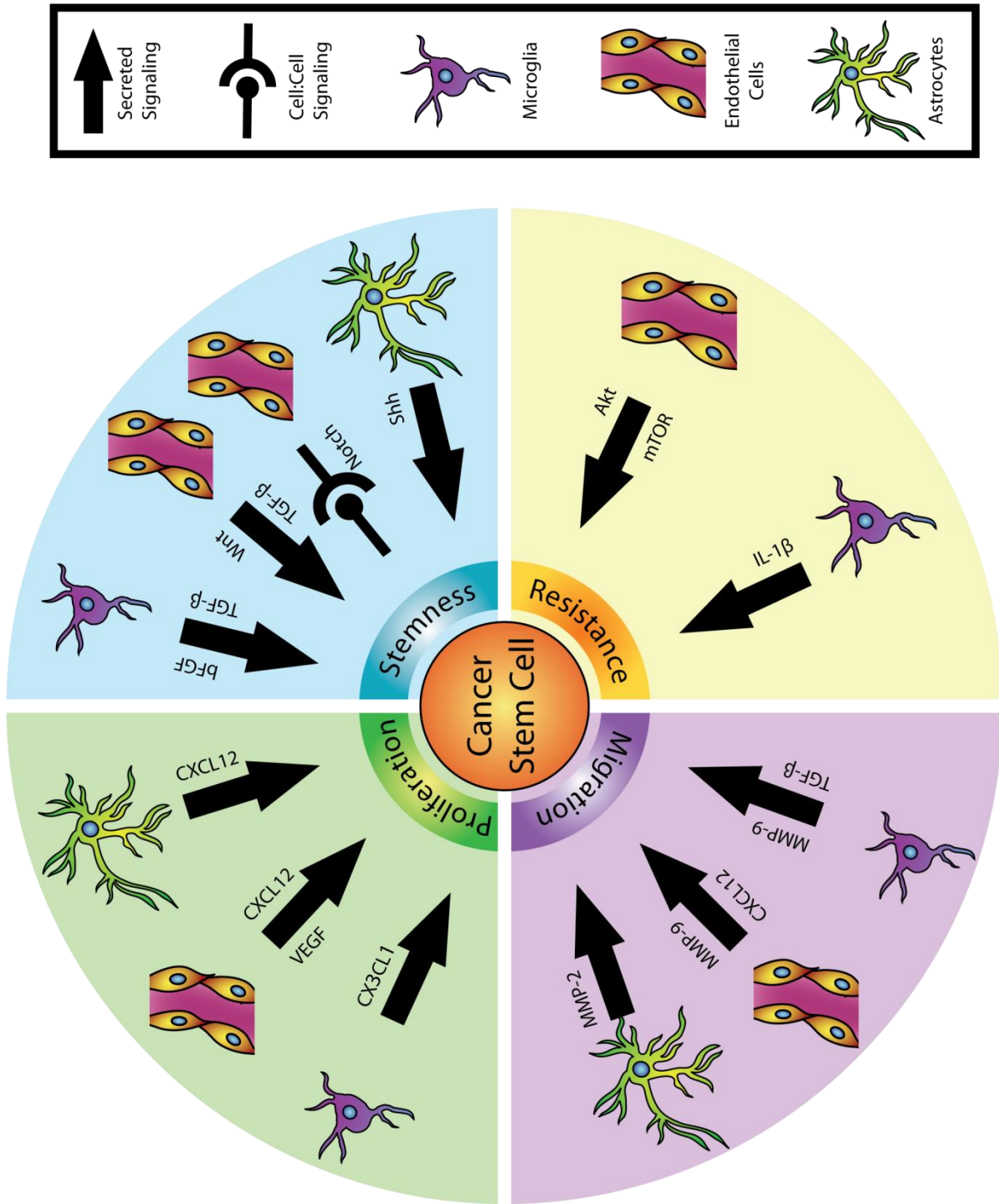
Figure 2-1. The functions of the PVN to maintain the stem cell population may be divided into four categories: Induction/Maintenance of Stemness, Proliferation, Resistance, and Migration/Invasion. Shown are key pathways utilized by each cellular component of the niche to communicate with the CSCs.

recruitment of pro-angiogenic bone-marrow-derived cells (101-103). In addition, transdifferentiation of glioma stem cells into endothelial-like cells may also contribute to VEGF-independent angiogenesis (26,104,105). Finally, avastin therapy may produce a shift in the growth pattern of GBM from angiogenic to infiltrative in which new niche formation may be induced in co-opted existing blood vessels (75-77). Thus resistance to VEGF antagonism involves a complex mix of responses that suggests it may be difficult to completely block the formation of new GBM stem cell niches.

While the logic of targeting stem cells themselves is robust, recent work has demonstrated that the stem cell population is heterogeneous and may not be a discrete subpopulation. Instead, stem cells may exist as a component of a dynamic steady state involving a number of tumor cell phenotypes in which transitions occur between stem cell and non-tumor cell states, including into endothelial cells (38,106). Therefore targeting the stem cell state may also prove to have limitations with regard to abrogating stem cell activity.

Thus it may be more important to target the mechanisms that favor transitions to the stem cell state and thereby prevent the replenishment of tumor-initiating capacity from non-tumor stem cells. This may also have the added advantage of targeting functions of non-neoplastic cells within the niche, i.e., astrocytes, endothelial cells, pericytes, which may have more limited capacity for resistance.

As described above, homeostasis within the stem cell niche is maintained through the choreographed activities of a small network of cell types (Figure 1). While the cellular diversity

and molecular mechanisms that serve the niche provides many opportunities for targeted approaches to GBM therapy, targeting adhesion molecules may have the potential advantage of blocking the ability of the component cell types to band together and perform their coordinated functions. Over the past several years a number agents that target cadherins and integrins have been evaluated in cancer clinical trials including for GBM (107,108). In general these have been well tolerated. In fact, in several cases maximal tolerated doses were not defined. In addition there are early indications of efficacy that have been attributed to anti-angiogenic effects as well as to direct anti-tumor cell effects. Not fully evaluated is whether a component of the anti-tumor effects are the result of reduced stem cell activity, and whether a more complete appreciation for this potential target of adhesion molecule therapeutics might support refined efforts to abrogate stem cell niche function. Particularly important might be the combination of adhesion molecule directed therapies with cytotoxic agents and anti-angiogenics. The efficacy of these approaches is currently being evaluated for GBM.

Table 2-1   Clinical trials of cilengitide for high grade gliomas (HGG)

Trial Number	Details	Status
NCT01165333	Phase I evaluation of increasing doses of cilengitide with irradiation for newly diagnosed diffuse intrinsic pontine glioma in individuals 6 months to 21 years of age	R
NCT01517776	Phase II evaluation of cilengitide with oral metronomic temozolomide for individuals ≥ 3 and <18 years old with progressive or refractory HGG	R
NCT00679354	Phase II evaluation of cilengitide in individuals <21 years old with recurrent or progressive HGG	S
NCT00063973	Phase I evaluation of escalating doses of cilengitide in individuals <21 years old with recurrent, progressive or refractory CNS tumors including HGG	C
NCT00813943	Phase II evaluation of cilengitide with standard radiation and temozolomide in individuals >18 years old with newly diagnosed GBM and unmethylated MGMT gene promoter	A
NCT00689221	Phase III evaluation of cilengitide with standard radiation and temozolomide versus standard therapy alone in individuals >18 years old with newly diagnosed GBM and methylated MGMT gene promoter	A
NCT01558687	Phase I evaluation of cilengitide with standard radiation and temozolomide in individuals >18, <70 years old yrs old with newly diagnosed GBM. Evaluations also include measurements of vascular function	R
NCT00979862	Phase I evaluation of cilengitide with cediranib maleate in individuals >18 years old with progressive or recurrent GBM	A
NCT01122888	Biomarker study of cilengitide with sunitinib in individuals >18 years old with progressive or recurrent GBM and other solid tumors	R
NCT01124240	Phase II evaluation of cilengitide with standard radiation and chemotherapy followed by temozolomide and procarbazine in individuals >18 years old with newly diagnosed GBM and unmethylated MGMT gene promoter	R
NCT00112866	Phase II evaluation of cilengitide in individuals >18 years old with progressive or recurrent GBM undergoing surgery. Evaluations will include tissue correlates of cilengitide effects on integrin expression	C
NCT00085254	Phase I/II evaluation of cilengitide with standard radiation and chemotherapy in individuals >18 years old with newly diagnosed GBM	C
NCT00093964	Phase II evaluation of cilengitide in individuals >18 years old with progressive or recurrent GBM	C
NCT00006093	Phase I/II evaluation of cilengitide in individuals >18 years old with progressive or recurrent HGG	

R recruiting, *S* suspended, *C* completed, *A* active but not recruiting

While Cilengitide, an integrin α_V antagonist has progressed furthest in clinical trial for GBM (**Table 1**), the N cadherin targeting agent ADH-1 or drugs with the potential to target integrin β_1 (PF-04605412, M200) may deserve special attention for their potential to disrupt the GBM stem cell niche.

Conclusions

The complexity of the cancer stem cell niche creates many potential obstacles to the successful inhibition of stem cell activity. Understanding the mechanisms that support niche formation and function may expose the Achilles heel(s) of the PVN and the key to GBM cure. Particularly important will be considerations of treatment regimens that can both target niche formation and the mechanisms of stem cell induction and maintenance. In this regard, therapies that target adhesion molecules may have the advantage of dual function, with the potential to block both niche structure and function.

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Chapter 3 – Methods of Transcriptional Analysis

Portions of this chapter were previously published or will be published shortly. The sections containing this data are noted in the text.

The Importance of Studying Transcription

The central dogma of biology is that DNA is transcribed into RNA which is turned into proteins (1,2). The ultimate goal of a cell wanting to change one of its properties is a change in protein level, but monitoring protein level changes is sometimes difficult, especially when looking at global changes across all proteins. Changes in RNA levels can also occur on a much shorter timescale than changes in protein levels. Therefore, looking at transcription levels is a popular option for determining which pathways are being altered in a cell upon some sort of stimulus or perturbation.

Tools for Studying Transcription

The Northern blot was one of the earliest forms of transcriptional analysis. It was described in 1977 (3), a few years after the Southern blot for analyzing DNA was pioneered (4). A Northern blot is a low-throughput technique where RNA from a sample is traditionally ran on an agarose gel with formaldehyde to denature secondary structure. Once the RNA has been completely run out on the gel, it is transferred to a nitrocellulose membrane where any particular gene of interest can be detected and quantified by using either a radioactively or fluorescently labeled anti-sense probe. While Northern blots are not used a great deal anymore, they do have the advantage of being able to detect relatively small changes because the large number of cell's worth of RNA which is loaded onto a single blot. One of the major disadvantages is that there are a lot of processing steps that go into setting up a Northern blot, and that provides numerous opportunities to introduce contaminating RNases which can degrade RNA (5).

Another technique for transcriptional analysis is quantitative reverse transcription PCR (qRT-PCR). In this technique, a PCR reaction is set up with primers designed to amplify a small region of a gene of interest. During the PCR step, amount of the PCR product is detected by a number of different techniques including intercalating dyes, TaqMan probes (6), Scorpion probes (7), molecular beacons (8), and others, but intercalating dyes and TaqMan probes remain the most commonly used.

For all of these methods, the first step is to reverse transcribe the RNA into cDNA. This stabilizes the signal by turning it into the more stable cDNA and makes it easier to perform PCR. The specific details of how this cDNA is quantified in the PCR reaction are where the various methods start to differ. One of the most common methods involves addition of a general intercalating dye that will increase in fluorescence as the amount of double stranded DNA increases. Ethidium bromide was originally used (9) but today Sybr Green I (10) has become the standard, though there are a number of other different dyes, with different properties, available (11). This method requires a simple reaction mix, requiring just the intercalating dye, primers for the gene of interest, and PCR reaction mix. Each cycle creates more PCR products and at the end of each of these cycles the detector on the specialized thermocycler detects and records the amount of fluorescence from the intercalating dye. Intercalating dyes also allow for a final step called a melt curve. One downside of a general intercalating dye is that there is little specificity, so primer dimers and non-specific mispriming products will produce just as much signal as true products, and melt curves are a good way to distinguish true signal from false signal. At the end of the PCR reaction, a starting temperature is picked which is lower than any of the likely products formed, approximately 50 degrees C. This temperature is slowly increased, usually in

0.1 degree increments, and after each increase, the fluorescence is recorded. Each product in the reaction, primer dimer, real product, etc, will have a specific melting temperature based on their length and base composition. When the temperature is increased to that point, the product will melt and the fluorescence will rapidly decrease as the amount of double stranded DNA to intercalate into melts. Therefore if time/temperature is plotted against change in fluorescence, each product will produce a specific peak in the melt curve that can reliably be seen from reaction to reaction. Therefore ideally at the end of a run, a melt curve will have only one peak that corresponds to the desired PCR product, and if other peaks are present, it can indicate contamination or high levels of primer dimer.

Another very common method called Taqman (6) provides a higher degree of specificity but is more complicated and expensive. This method involves two normal PCR primers, similar to the previous method, but incorporates a third primer, which binds internally to the forward and reverse primers. This method has no intercalating dye but this internal primer is labeled with one of any number of fluorophores. To prevent constant fluorescence it is also labeled with one or more quencher molecules which prevent the fluorophore from actively producing light. However, during the PCR cycles, this internal primer will anneal to its complementary region in the middle of the PCR product and as the polymerase comes along, it degrades this internal primer, releasing the nucleotide with the fluorophore into solution, preventing it from being quenched, and thereby producing more and more fluorescence each round, which is again detected and recorded at the end of every PCR cycle. While there is no corresponding melt curve to determine the sensitivity of the reaction, the internal primer provides an extra level of

specificity since neither primer dimers nor mispriming products will contain the sequence complementary to the internal primer.

Overall, qRT-PCR has a number of advantages over Northern blots. It contains a high dynamic range, has high sensitivity, can be easily multiplexed, and requires less manipulation of the RNA and therefore provides less opportunity for RNA degradation (12).

With the advent of next-generation sequencing, another option for transcriptional analysis has been developed called RNA-sequencing (RNA-Seq). In this method, RNA is once again turned into cDNA, but instead of assessing gene expression with PCR for a specific gene, this cDNA is turned into a next-gen library and the number of reads that map back to a particular gene are used as a proxy for expression from that gene. This allows for detection of gene expression globally and without any *a priori* knowledge of gene sequence or expression. The first publication utilizing this approach was from Barbara Wold's group (13) in 2008. RNA-Seq provides a large number of advantages over other techniques. Foremost, not only does it provide quantification of gene expression, but it can also provide quantification and analysis of many other forms of transcription such as alternative splice forms, allelic expression, RNA editing, and SNPs present in coding sequences. It also has, in theory at least, infinite dynamic range. For example, microarrays, which have a large but not infinite dynamic range, will be unable to detect genes with very low levels of transcription without having high transcription genes saturated. RNA-Seq has the ability to detect genes with a single copy per cell up to genes with hundreds of thousands of copies per cell. Though this requires sequencing to a very high depth to detect the very, very lowly expressed genes, but most of the time the reads will be from those very highly

expressed genes, therefore wasting computational time and money. One of the disadvantages of

the original RNA-Seq was the requirement of starting with a large amount (25ug) or RNA. The

main reason for this high starting amount was that the protocol required polyA selection of

mRNA to avoid sequencing of ribosomal RNA that makes up a very large percentage of total

RNA but would be relatively uninformative.   This did have the side effect of making detection

of non-polyadenylated RNA species difficult. Through continued technology development these

disadvantages have been overcome for the most part. Currently there are commercial kits and

special library preparation methods which allow RNA-Seq all the way down to single cells (14)

(15) (16) and there are library preparation and analysis methods to detect many of the various

kinds of RNA present in a variety of organisms (17) (18).

Methods of Microarray Analysis

Another major category of transcriptional analysis tool is a microarray. While the term

microarray refers to any array of probes put down onto a solid surface where each probe

represents a different gene or protein which can then all be assayed all at the same time, the

pertinent to this discussion is the expression microarray, designed to assess the levels of mRNA

genome-wide. One of the first expression arrays was a set of 4,096 randomly cloned human

colon cancer cDNAs cloned into *E. coli*, spotted onto nitrocellulose and probed with radiolabeled

RNA from other colon cancer related samples (19). Since then there has been a great deal of

technology development, and two main commercial products have come to dominate the field,

Affymetrix Genechips and Illumina BeadArrays. While being based on different technologies,

both provide the same function, providing quantification of almost all the genes currently known

for a variety of model organisms including human, mouse and rat.

Once the microarray has been performed and the data generated, the first step of microarray

analysis is to take the raw data output and background subtract and normalize it. In the early

days of cDNA microarrays this would quantifying negative control background spots and

subtracting that from all of the other spots. These days each commercial product has separate

procedures for performing the background subtraction. For example, Illumina uses a set of

around 1,000 beads which contain randomized sequences which should have no targets in their

designed transcriptome to serve as background beads. They also have a local background

subtraction step (20). Conveniently these steps are able to be performed automatically by the

Illumina software upon initial data generation, though commonly available plug ins are also

available for R to reanalyze the data completely from raw data (21) (22).

The next step in microarray analysis is normalization. The goal of normalizing microarray data

is to remove as much of the systematic variation as possible while leaving biologic variation.

This is critical when trying to analyze data that was generated on different chips, at different

times, or by different research groups. Normalization makes 3 main assumptions; 1) the majority

of genes are not differentially expressed, 2) any systematic bias that is present is evenly

distributed across the chip or beads, and 3) the normalization will only remove the systematic

bias without affecting biological variance (23). There are many different algorithms to

normalize data and, while each has their own slight advantages and disadvantages, there are a

few main ones used more often (24).

Another step in microarray data analysis is transformation. The most common form of data transformation is a $\log_2$ transformation. This has two main benefits. First, it reduces the variance of large values. This is important because microarray data can sometimes have a large difference between the highest and lowest expressed genes on the array, and these highly expressed genes will just naturally have a much larger variance than the smaller ones. By taking the $\log_2$ of all of the data, it helps to make comparisons between high and low expressed genes easier. Secondly it helps to facilitate treating up and down regulated genes similarly. Meaning that if you calculate a fold change for un-transformed data, by dividing condition #1 by condition #2, the genes that were higher expressed in condition #1 will all now have a value above 1 for their fold change, but genes with a higher value in condition #2 will have a value between 0 and 1, which would then further need to have the negative reciprocal taken to have comparable fold changes. On the other hand, if the data is already log2 transformed, calculating the fold change only requires subtracting condition #2 from condition #1, and the fold change is already correct for both situations.

Once the data is background subtracted, normalized, and transformed, the question becomes how to tell which genes are differentially expressed between the various samples or conditions. In the early days of microarrays, analysis could consist of as little as determining the fold change for each gene between samples or conditions, and considering anything over a two-fold change as differentially expressed (19). Of course this sort of analysis had no real statistical basis and created many false positives so researchers moved towards something with more statistical rigor. These days there are two main statistical tests done for microarrays. The easiest is the Student's

t-test. For each gene, an individual t-test is run for each comparison of interest. This creates an independent p-value for each gene and each comparison.

If microarrays are done on multiple samples, and it is important to test whether there is a difference between any of those samples without performing all possible pairwise comparisons (and therefore having many more tests to correct against), then the second main statistical test is performed instead; the analysis of variance, or ANOVA test. This will determine if the gene is differentially expressed across any of the samples.

No matter the statistical test chosen, for an average microarray, the analysis would have consisted of many thousands of statistical tests. A normal p-value cutoff would be 0.05, and that would set the limit that 5% of the results are false positives. The problem then becomes that if there were 40,000 genes, and therefore that many statistical tests, there will be 2,000 false positives. This creates quite a problem, and therefore needs to be accounted for by what is called multiple hypothesis (MH) correction. There are a number of different methods for this including Bonferroni correction (25) or Benjamini-Hochberg (26). The Bonferroni correction is one of the simplest but most stringent MH corrections. It aims to completely reduce type I errors (false positives) but in doing so also has a high rate of type II errors (false negatives). It is implemented by multiplying each p-value by the number of tests performed and then asking if these new corrected p-values still fall below some cutoff, traditionally 0.05. One of the other less stringent MH corrections is Benjamini-Hochberg. In this correction, type I errors are not completely reduced, they are kept at some threshold level, for example 5%, but this also prevents many of the type II errors that a more stringent correction would make (26). Benjamini-

Hochberg also has a more complicated implementation. The results are ranked by p-value from lowest to highest (lowest being rank = 1), and each p-value is multiplied by the total number of genes in the list divided by its rank in the list of p-values.

Once the statistical tests have been run, and multiple hypothesis correction applied, the end result is a list of genes which are differentially expressed, but going through a list and just picking random genes and testing them further is a long and expensive process. Therefore, researchers have found ways to determine if there are patterns in these genes and make educated guesses about which ones to test first. The technique is called pathway analysis and there are a number of programs and algorithms designed for it including GO Onotology from the original consortium (27), KEGG pathway analysis (28), and DAVID analysis tools (29). The overall idea is that given a short list of differentially expressed genes, these programs take the background list of all of the possible genes which could have been differentially expressed, and a list of associations that each gene has to one or more biological processes, and performs a Fisher's exact test to determine if there is an enrichment of any of these biological processes in the short differentially expressed gene list compared to the background gene list (30). This test will output p-values and multiple hypothesis corrected p-values, for each association. These lists of enriched biological processes can then be compared to the experimental design for validation of the system, and prioritization of the genes found to be differentially expressed for further experimentation.

Having developed an expertise in microarray analysis, I have used these skills to assist in a number of other collaborations to analyze microarray data in a variety of systems. The following is a description of those systems, and the analysis performed.

"Conditional Mutagenesis of Gata6 in SF1-Positive Cells Causes Gonadal-Like Differentiation in the Adrenal Cortex of Mice" by Pihlajoki M, Gretzinger E, Cochran R, Kyronlahti A, Schrade A, Hiller T, Sullivan L, Shoykhet M, Schoeller EL, Brooks MD, Heikinheimo M, Wilson DB.

This study (31) focused on the effect of Gata6 in the adrenal cortex of mice. Gata6 is a known transcription factor in adrenocortical development (32). Since complete knockout of Gata6 is embryonic lethal (33), a conditional knockout (cKO) of Gata6 was created using Cre-LoxP recombination being driven by SF1-cre. SF1 is another transcription factor important in adrenal steroidogenesis and is highly expressed in fetal and adult adrenal cortex (34). Therefore Gata6 is specifically knocked out in adrenocortical cells in the mouse brain.

Gata6 cKO mice, while viable and fertile, have a number of defects, all of which are thoroughly described in the paper. Briefly though, these mice showed smaller adrenal cortex sizes compared to wild type mice and an upregulation of gonadal-like markers. Also, the x-zone, a transient area of cells which disappears in pubescent male mice and during pregnancy in female mice, was shown to be significantly reduced in Gata6 cKO mice.

To determine which genes were dysregulated by the cKO of Gata6, a series of microarrays were performed on RNA isolated from whole adrenal glands of 2 month old virgin females cKO mice (n=3) and control (n=3) mice. Illumina Mouse6v2 were performed by the Washington

University in St. Louis's Genome Technology Access Core (GTAC) according to standard protocols. Background subtracted data was quantile normalized and imported into Excel for further analysis. Once into Excel, the probes with no expression (as determined by the Illumina generated Detection p-value > 0.05) in all 6 datasets were filtered out, taking the dataset from 45,282 down to 20,073. Next, genes with a fold change less than 2 fold between cKO and wild type (in either direction) were filtered out, leaving only 1,985 probes. These remaining genes then had multiple hypothesis correction applied using the Benjamini-Hochberg method. The top twenty up and down regulated genes are shown in Table 3-1.

One of the genes found to be differentially expressed during the analysis was the downregulation of Pik3c2g, a member of the Akt/PI3K signaling pathway, in the cKO mice. Further analysis showed the Pik3c2g was a specific marker of the X-zone by showing that Pik3c2g was present in

Gene Name	Fold (Control / cKO)		Gene Name	Fold (Control / cKO)
Pik3c2g	-38.32		Adh7	51.04
Arl3	-25.43		Cox7a2l	35.84
Omp	-22.49		Ibsp	13.71
Fetub	-11.12		Megf10	9.32
Akr1c18	-9.47		Ddit4l	6.90
Smpdl3b	-5.61		Scara3	5.73
Tm4sf4	-5.40		Hmgcs2	5.51
Ly6d	-5.12		Aqp5	5.08
Acoxl	-4.68		Cdon	4.89
Kng1	-4.58		Mia1	4.22
Oxtr	-4.48		Kcnip3	4.07
Rgl1	-4.34		9430052C07Rik	4.05
Kng2	-4.09		Gli2	4.03
Fam20b	-3.53		Cpne4	3.85
Pign	-3.20		Enpp2	3.84
C77370	-2.97		BC064033	3.82
Dysfip1	-2.96		Lum	3.78
Cyp2f2	-2.90		Ccl21c	3.75
Masp1	-2.82		Shisa2	3.72
Gsg1l	-2.69		Rgs7bp	3.64

Table 3-1. The top 20 up and down regulated genes in Gata6 cKO mice. Fold change is control over cKO value, therefore negative values were genes with lower expression in cKO mice.

virgin females and gonadectomized males and females, but not present in post-puberty males or previously pregnant females. To further verify that Pik3c2g was specific to the X-zone, laser capture microdissection was used to gather tissue from different areas of from virgin female and control mice adrenal glands showing specific Pik3c2g expression in X-zone cells.

These microarrays helped to identify Pik3c2g as a novel marker of the X-zone in mouse adrenal glands. While the exact function of Pik3c2g is yet unknown, it appears to specifically phosphorylate phosphatidylinositol (PI) and PI4P, but does not phosphorylate PI(4,5)P2 (35). The overall studies in the paper reveal Gata6 as an important mediator of the balance between adrenal and gonadal pathways in stem/progenitor cells in the adrenal gland and that Pik3c2g is an important downstream gene in the pathway and that further studies will shed additional information about its functional role.

"Cell Intrinsic Sexual Dimorphism in Stem Cell Induction and Transformation Underlies Male Predominance in Glioblastoma" by Sun T, Warrington NM, Luo J, Brooks MD, Dahiya S, Sengupta R, & Rubin J.

Currently under revision at the Journal of Clinical Investigation.

This study (currently in review at Journal of Clinical Investigation) aims to better characterize the phenomenon of males being more susceptible to brain tumor formation than females. When analyzed across a number of brain tumor types including glioblastoma (GBM), medulloblastoma, oligodendroglioma, ependymoma, and pilocytic astrocytomas, males invariably showed higher rates of tumor formation than females (36).   Since glioblastoma is the most common adult malignant primary brain tumor, and has one of the worst prognoses (37), a great deal of work has gone in to understanding the basic tumor biology behind it. Further research on the transcriptional signatures of GBM tumors showed four distinct molecular subtypes; classic, proneural, neural, and mesenchymal (38). In this study, data from the TCGA database (38) and

additional data from recent studies containing a total of 426 GBM samples (GSE16011 (39) and GSE13041 (40)) were analyzed for tumor formation rate between males and females. When broken down into these four molecular subtypes, the mesenchymal subtype had the greatest sexual disparity (Table 3-2). It has also been shown that the mesenchymal subtype has frequently been associated with loss of neurofibromin 1 (NF1), p53, and PTEN (38). In a mouse model of glioblastoma, NF1+/-;p53+/- mice have an increased susceptibility of tumor formation (41) and male mice show higher tumor formation than female mice (1.38:1) when the chromosome containing the mutant NF1 and p53 is maternal in origin (42) (43). Based on this data, NF1 and p53 were prioritized for a stepwise transformation model to determine if loss of these two genes would reveal a cell intrinsic difference between male and female astrocytes which could account for difference in tumor formation rates.

Table 3-2

Molecular Subtype	Male	Female	Male Proportion	p-value*
TCGA				
Classical	18	20	0.47 (0.32,0.63)	0.8711
Mesenchymal	38	18	0.68 (0.55,0.79)	0.0111
Neural	19	7	0.73 (0.54,0.87)	0.0310
Proneural	33	20	0.62 (0.49,0.74)	0.0993
GSE16011#				
Classical	23	16	0.59 (0.43,0.73)	0.3367
Mesenchymal	39	14	0.74 (0.60,0.84)	0.0010
Neural	10	5	0.77 (0.49,0.93)	0.0960
Proneural	36	18	0.67 (0.53,0.78)	0.0207
GSE13041#				
Classical	39	26	0.6 (0.48,0.71)	0.1366
Mesenchymal	56	32	0.64 (0.53,0.73)	0.0142
Neural	29	15	0.66 (0.51,0.78)	0.0500
Proneural	43	27	0.61 (0.50,0.72)	0.0730

*p value calculated by two-sided one sample z- test for proportion (H0: p=0.5 vs. Ha: p ≠ 0.5)
Subtype assignment for subjects in these cohorts was based on k-nearest neighbor method (k=9) using TCGA prototype data. We have varied k in the range of 8~20 and the results are similar.

For the first stage of the model, male and female wild type astrocytes were isolated from p1/p2 mouse pups. While a number of different cells in the brain, including astrocytes, have been shown to have the potential to be the initiating cell for glioblastoma (44,45), astrocytes were chosen because of their abundance in the developing mouse brain and ability to grow in vitro. For the next step of the step-wise transformation, astrocytes were gathered from either male or female mouse pups where NF1 had been homozygously deleted using a Cre-Lox system (46) (47). Comparisons between male and

female NF$^{-/-}$ astrocytes from this step showed no difference in growth rates and no signs of transformation. For the third step in transformation, the male and female NF1$^{-/-}$ astrocytes were infected with a dominant negative form of p53 (DNp53) (48). While the addition of DNp53 did slightly increase the growth rate of the female astrocytes, it drastically increased the growth rate of the male astrocytes allowing the male NF1$^{-/-}$;DNp53 cells to grow much faster than their female counterparts. However, neither line had been transformed yet as they did not form colonies in a soft agar assay. It wasn't until a final EGF treatment, which has been shown to inactivate the retinoblastoma (Rb) pathway, a key oncogenic event in most cancers (49) (50), that male cells were transformed and formed colonies in soft agar yet female cells still showed no signs of colony formation.

Further analysis of these lines showed some striking observations including male NF1$^{-/-}$;DNp53 cells having a greater in vivo tumorigenicity compared to female cells (independent of the sex of the local microenvironment) and male cells showing higher levels of stemness in an extreme limiting dilution assay (ELDA). However the mechanism for these changes was unknown, so the question became; "What transcriptional changes were occurring during each of these stepwise events?" To answer this, RNA was gathered from both male and female cells from each of the four lines: wild type, NF1$^{-/-}$, NF1$^{-/-}$;DNp53, and NF1$^{-/-}$;DNp53+EGF. Samples were generated in triplicate, and the RNA submitted for Illumina microarrays. The microarrays were performed at Washington University in St. Louis's GTAC core following standard protocols. Quantile normalized, background subtracted data was imported into Excel and as previously described, probes with no expression were filtered out, and only probes showing greater than two fold up or down-regulation and a p-value less than 0.05 for any particular comparison were kept.

Male and female wild type astrocytes were very similar to each other, only having 13 genes with expression differences and those included a reasonable number of genes specific to either the X or Y chromosomes. When male NF1$^{-/-}$ astrocytes were compared to female NF1$^{-/-}$ astrocytes the number of differentially expressed genes slightly increased to 18. The addition of DNp53 had the greatest effect, increasing the number of differentially expressed genes between NF1$^{-/-}$;DNp53 males and NF1$^{-/-}$;DNp53 females to 606 while the further addition of EGF caused there to be 527 gene changes. The top differentially expressed genes in each group were selected and a heatmap was generated to show the stepwise progression of the transcriptional changes (Figure 3-1).

Figure 3-1
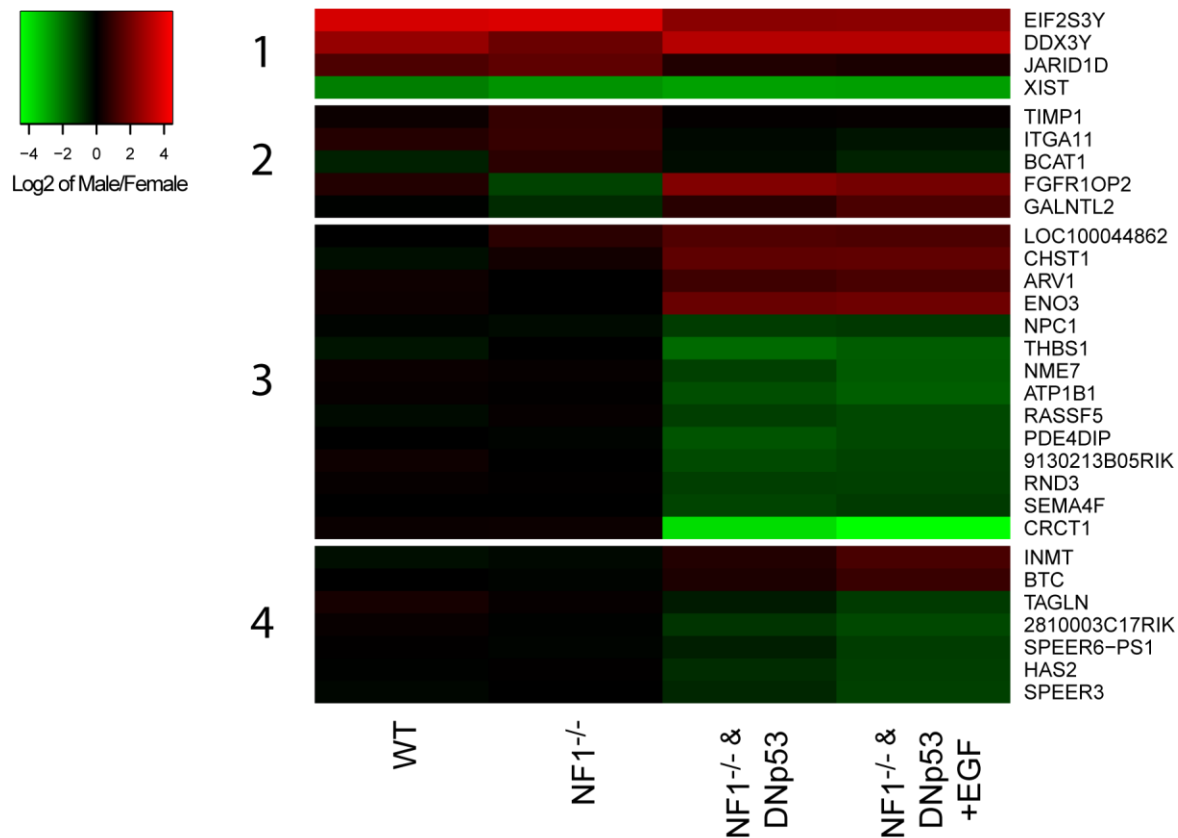
The top differentially expressed genes from each group were selected. Group 1 were genes differentially expressed between wild type male and female astrocytes, while group 4 was the top genes differentially expressed between male and female NF-/-;DNp53+EGF cells.

Further analysis and experiments on these changes detected during the stepwise transformation of male and female astrocytes will hopefully provide valuable clues as to the molecular mechanism behind males developing more brain tumors than females.

The Future of Transcriptional Analysis

One newly developed method for transcriptional analysis is called translating ribosome affinity purification (TRAP) (51) (52) (53). It is designed to label a specific cell and then isolate RNA from that cell out of a complex cell population to determine any gene expression changes. The idea is to fuse a fluorescent protein, usually GFP, to a ribosomal translating protein and then insert that into your cell of interest. This GFP-ribosomal protein will be incorporated into the ribosomes of the cell, and begin translating other proteins. Then a complex mixture of cells (including the cell with the TRAP protein) can be gathered and lysed where the polysomes (complexes of ribosomes and mRNAs being translated) can be purified with magnetic beads coated in antibodies against GFP. The magnetic beads are then separated and immediately put into Trizol for RNA extraction (51). This is a great system for complex cellular systems, assuming you can isolate and manipulate the cells and then recapitulate that system. This system would be particularly helpful for other tumor-microenvironmental interaction studies.

While Illumina sequencing was once called next-generation sequencing because of how advanced it was, it is now fully entrenched as a technology, and we are coming up on the next generation of sequencing machines. While there are a number of technologies being developed, one of the most promising is what is referred to as nanopore sequencing (54) (55) (56). Here

pores in a membrane have single molecules of DNA threaded through them, and electrical

conductivity is measured to determine what base is traveling through the pore at any given time.

How the DNA is threaded through, and how its speed is controlled all questions up in the air and

being worked on currently. One major advantage of this technology is that unamplified, native

molecules, including RNA and methylated DNA, will be able to be sequenced without complex

library preparation (57). Since RNA-Seq library preparation can introduce a number of biases,

being able to directly sequence RNA could be a significant improvement in transcriptional

quantification.

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Chapter 4 : Discovery of PDE7B as a novel effector of GBM growth by computational deconvolution of an in vitro physical coculture system.

This chapter is currently being revised and will be published shortly.

Authors:
Michael D Brooks[1,7], Erin Jackson[2,3] , Nicole M Warrington[1], Sara Taylor[4], Diane D Mao[4], Jeffrey R Leonard[4], Albert H Kim[4], David Piwnica-Worms[2,3,5,6], Robi D Mitra*[7] & Joshua B Rubin*[1,8]

*These authors contributed equally to this work
[1]Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri
[2]BRIGHT Institute, Washington University School of Medicine, St. Louis, Missouri
[3]Molecular Imaging Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri
[4]Department of Neurosurgery, Washington University School of Medicine, St. Louis
[5]Department of Cell Biology & Physiology, Washington University School of Medicine, St. Louis, Missouri
[6]Department of Cancer Systems Imaging, University of Texas MD Anderson Cancer Center, Houston, TX
[7]Department of Genetics and Center for Genome Sciences and Systems Biology, Washington University School of Medicine in St. Louis &
[8]Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri, United States of America

Targeting the tumor promoting interactions that occur between tumors cells and stromal elements within the perivascular space is gaining traction for glioblastoma. The scope of molecular targets and pathways is continually being updated. In this manuscript we identify phosphodiesterase 7B (PDE7B) as a mediator of glioblastoma growth whose expression is induced in GBM through functional interactions that occur between GBM and endothelial cells. PDE7B was discovered using a novel method for studying reciprocal interactions between glioblastoma and endothelial cells that involves computational deconvolution of their coincident transcriptional profiles. The translational relevance of PDE7B was evident in analyses of RNA and protein expression in primary glioblastoma specimens, survival analysis of clinical data, and the effect of PDE7B overexpression on the intracranial growth of U87 xenografts. These new findings indicate that PDE7B should be further evaluated as a prognostic marker and as a molecular target in glioblastoma.

Abstract

Cell-to-cell interactions between tumor cells and their microenvironment are critical determinants of tumor tissue biology and therapeutic responses. Interactions between glioblastoma (GBM) cells and endothelial cells (ECs) establish a purported stem cell niche. We hypothesized that genes that mediate these interactions would be important, particularly as therapeutic targets. Using a novel computational approach to deconvoluting expression data from mixed physical coculture of GBM cells and ECs, we identified a previously undescribed upregulation of the cAMP specific phosphodiesterase PDE7B in GBM cells in response to ECs. We further found that elevated PDE7B expression occurs in most GBM cases and has a negative effect on survival. PDE7B overexpression resulted in the expansion of a stem-like cell subpopulation, increased tumor aggressiveness, and increased growth in an intracranial GBM

model. This deconvolution algorithm provides a new tool for cancer biology, and these results identify

PDE7B as a therapeutic target in GBM.

Introduction

Frequently, studies of tumor biology have focused on the intrinsic properties of cancer cells, such as their

growth rate, signaling cascades, or DNA repair capacity, without fully accounting for how the

microenvironment can influence these functions. Tumor progression however, is a consequence of

collaboration between the genomic lesions present in the tumors cells and alterations in the tumor

microenvironment (1). The tumor microenvironment is highly heterogeneous (2) with varying cellular

constituents within multiple tumor microdomains such as the leading edge of invasion and the peri-

necrotic spaces as well as the perivascular space. Within each of these microdomains, genetically

identical tumor cells may exhibit different patterns of gene and protein expression resulting in regions of

distinct cellular phenotypes being simultaneously present within the same tumor. This intratumor

heterogeneity creates an experimental challenge in studying cancer biology (3).

Several cancers are reported to display significant intratumor heterogeneity including glioblastoma

multiforme (GBM), the most common malignant primary brain tumor in adults. While the study of peri-

necrotic and invasive edge biology in GBM has generated insights into the metabolism of cancer cells in

hypoxia (4), Notch signaling (5), and the importance of matrix metalloproteinases (MMPs) (6), it is the

focus on the biology of the perivascular niche (PVN) that has yielded the greatest body of information.

The PVN is home to a subpopulation of tumor cells with stem cell-like properties. The GBM PVN

contains GBM cancer stem cells (CSCs), endothelial cells, pericytes (7), astrocytes (8), and microglia (9).

While multiple pathways have been identified as essential for the specialized function of the PVN (10,11)

how this specialized domain is established remains largely unknown. It is clear that endothelial cells

within the GBM PVN are distinct from endothelial cells in the normal brain and that tumor cells within

the perivascular space are distinct from bulk tumor cells (12). Identifying the reciprocal interactions that

induce these changes in the component cell types of the PVN will be essential for understanding and

effectively targeting its function.

Previously, we reported an *in vitro* model of the GBM PVN comprised of primary cultures of human

brain microvascular endothelial cells (HBMECs) in Matrigel and either an established GBM cell line

(U87-MG) or primary GBM cells (13). Functional studies using this system revealed that expression of

the chemokine CXCL12 by HBMECs promoted localization of GBM cells to the peri-endothelial cell

space and triggered their expansion. These studies demonstrated the utility of an *in vitro* co-culture

system for modeling GBM-PVM interactions. Here we sought to use this system to identify the pathways

that are modulated by interactions between endothelial and GBM cells.

Prior efforts to identify the targets in cell:cell interactions have frequently relied on conditioned media

experiments (14,15) in which a target cell type of interest (e.g. U87-MG) is grown in media conditioned

by "controller/inducer" cells (e.g. HBMECs). One drawback to this approach is that changes induced by

physical cell:cell interactions or reciprocally induced interactions (feedback loops) cannot be detected. In

an alternative approach, two cell types have been labeled with two different fluorescent proteins, so that

they can be grown together, separated by Fluorescent Activated Cell Sorting (FACS), and then analyzed

independently (16). In principle, this method has the potential to capture the effects of both secreted and

direct interactions between cell types; however, in practice, separating cells from matrix containing three-

dimensional cocultures quickly and efficiently without disrupting the very signal that is being interrogated

is difficult, especially when analyzing changes in mRNA abundance.

Thus, for global gene expression analysis, the ideal system would allow for the immediate isolation of RNA during the experiment, without the need for extra processing of cells. However, the immediate purification of RNA mixes the RNA species from both cell types, making the measurement of gene expression changes within each cell type a challenging task. Therefore, we developed a unique computational approach to deconvolve the combined transcriptional signal and accurately measure gene expression changes that occur when endothelial cells and GBM cells interact. Using this method, we identified a small number of key target pathways involved in PVN function and discovered a new role for the cAMP-specific phosphodiesterase (PDE), PDE7B, which is induced in GBM cells by a contact-mediated interaction with endothelial cells. PDE7B is overexpressed in primary GBM specimens, has prognostic significance in human disease and when overexpressed in GBM cell lines, results in increases in the stem-like cell population *in vitro*, and drives tumorigenicity *in vivo*. Together these studies validate this new approach for discovery of pathways that mediate the critical phenotypic variation that occurs when tumor cells interact with stromal elements within tumor microdomains.

Results

Global expression profiling identifies genes regulated by GBM cell:endothelial cell interactions

We previously demonstrated that the physical co-culture of primary human brain microvascular endothelial cells (HBMECs) and either primary human GBM cells or the U87 GBM cell line resulted in endothelial cell-dependent growth of the GBM cells (13). To elucidate how a cooperative interaction between tumor cells and their microenvironment might drive GBM growth, we performed gene expression profiling with Illumina BeadArray microarrays using RNA isolated from the mixed cell population after 48 hours of culture and RNA isolated from parallel monocultures of HBMECs and U87
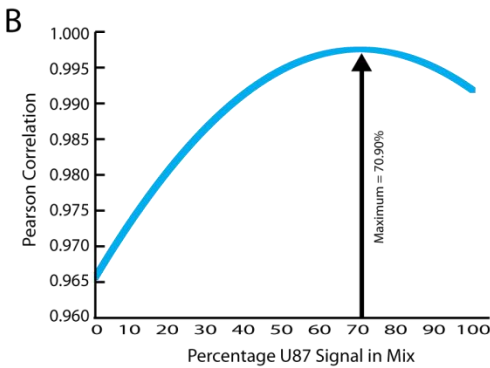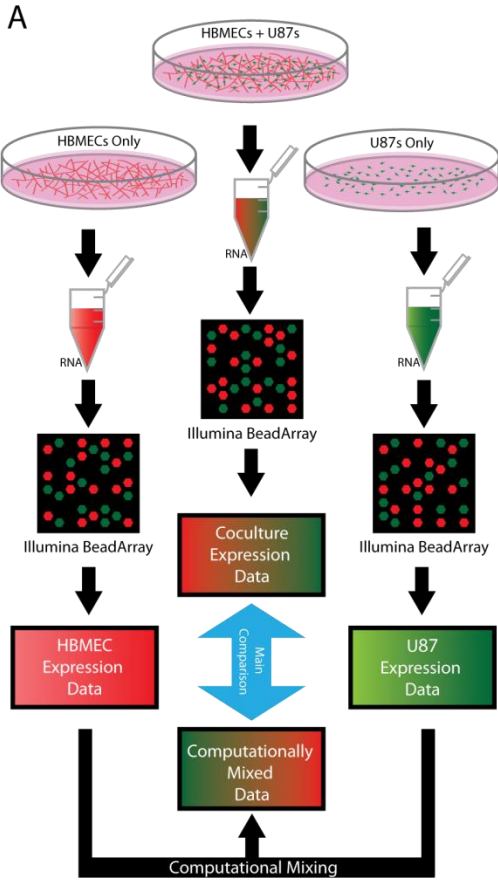
A

B

Figure 4-1: Sample preparation and computational deconvolution of the mixed culture RNA signal. (A) Flow chart of sample preparation from culture to gathering RNA and computational analysis. (B) The percentage of each monoculture to use for the control dataset was determined by creating one thousand test datasets, each with a different amount of U87 or HBMEC signal, and then calculating the Pearson Correlation between each of these datasets and the measured coculture dataset.

cells (Figure 4-1A). Because the isolated RNA was derived from a mixed population of cells, changes in gene expression that were detected could be attributed to either changes in the relative numbers of HBMECs and U87 cells over the 48-hour co-culture time period, or to gene expression changes caused by functional interactions between the two cell types. As we were interested only in the latter, it became essential to develop computational methods to distinguish between these sources of changes in expression levels.

Thus we developed an approach that uses gene expression data to precisely determine the ratio of HBMEC and U87 cells within a mixed culture. Our strategy exploits the fact that the expression levels of most genes in either cell type are likely to be unchanged upon co-culture, and only a small percentage of genes altered by their interaction. Using expression profiling data obtained separately from HBMEC and U87 monocultures, we computed the expected gene expression profiles for every possible mixture of the two cell types, from a ratio of 0.1% HBMEC/99.9% U87 to a ratio of 99.9% HBMEC/0.1%U87. We then used these computed profiles to determine the ratio of GBM and HBMECs in the actual co-culture experiment. To do so, we

calculated the Pearson correlation between the experimentally measured co-culture expression data and the full series of computational generated expression profiles. The synthetic profile with the highest correlation provided the closest approximation to the actual ratio of U87 cells and HBMECs in the co-culture (Figure 4-1B). Once this ratio was determined, we normalized the microarray data to account for changes in the amount of each cell type. This calculation was performed for three independent sets of co-culture data. From these combined gene expression data sets, we could then further identify statistically significant outliers, which represented candidate genes whose expression was either increased or decreased as a consequence of functional interactions between HBMECs and U87 cells.

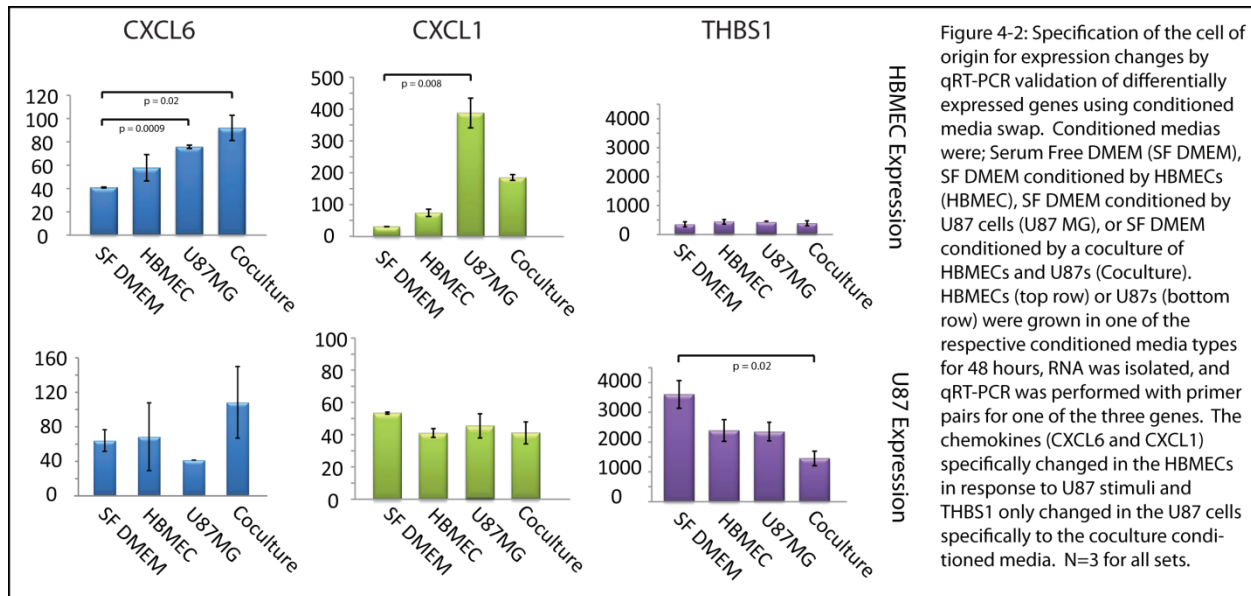| Table 1: Genes differentially expressed by coculture of U87 and HBMECs.						
Symbol	Corrected p-value	Fold Change		Symbol	Corrected p-value	Fold Change
PRRX2	3.25E-04	4.43*		CD36	3.25E-04	-2.37
CTHRC1	0.013	2.06		MBP	7.72E-03	-2.95
FILIP1L	0.015	2.86		LOC100131643	8.21E-03	-2.85
C8orf4	0.015	2.04		KIAA1199	0.0082	-2.64
PDE7B	0.020	2.20		DLX5	0.0082	-2.42
SOBP	0.020	2.19		MBTD1	0.0084	-2.15
MTRR	0.022	8.83		KIAA0692	0.015	-9.23
ICAM1	0.022	4.56		GREM1	0.020	-2.00
CXCL1	0.022	4.51		MIR204	0.024	-18.27
A4GALT	0.022	3.84		ZFAT	0.024	-2.52
MSI2	0.022	2.49		ZXDA	0.024	-2.23
MXRA5	0.022	2.33		MIR2117	0.026	-3.34
FAM179A	0.022	2.24		ZMYM3	0.028	-20.38
FZD9	0.026	12.33		KLK11	0.029	-3.40
MED15	0.026	2.24		DIRC1	0.032	-2.10
LOC650566	0.028	2.73		HS3ST2	0.032	-2.27
FNDC5	0.029	27.45		THBS1	0.033	-2.58
CDKN2C	0.032	22.06		C9orf96	0.033	-2.29
CCL20	0.034	2.68		EGR3	0.034	-3.00
PNLIPRP3	0.044	2.25		LOC285501	0.034	-2.47
SLCO2A1	0.046	10.51		RCAN2	0.040	-3.04
LOC729666	0.047	3.22		CD80	0.048	-2.30
C9orf150	0.048	2.20				
* All genes with a Benjamini-Hochberg corrected p-value < 0.05 and greater than a two-fold change (Computational Dataset / Coculture Dataset) shown.						

In this manner, 45 genes were identified as significantly (after Benjamini-Hochberg multiple hypothesis correction) upregulated or downregulated by greater than a 2-fold as the result of functional interactions between HBMECs and U87 cells (Table 4-1). Consistent with known effects of GBM cells on endothelial cells, this list of genes conta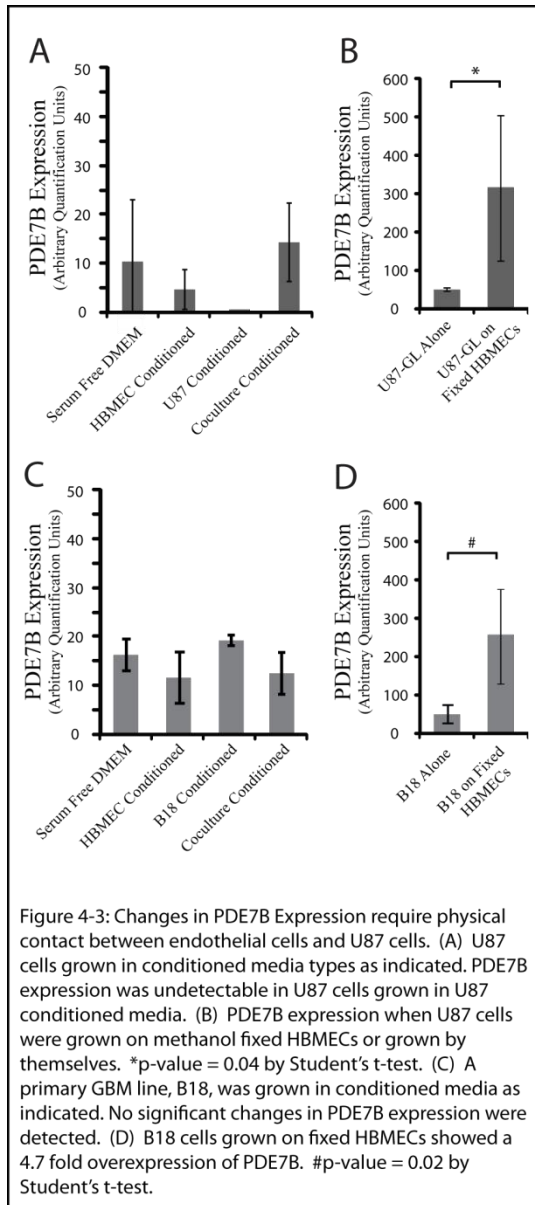ined several regulators of angiogenesis such as Thrombospondin-1 (17), ICAM1 (18), and CXCL1 (19). Also notable is the upregulation of WNT signaling genes CTHRC1 (20) and Frizzled-9 (21). WNT signaling is known to be involved in maintaining stemness and cell proliferation (22).

Expression profiling of the mixed co-culture directly identified genes whose expression changed as a result of cell:cell interactions, but did not identify in which cells these changes were occurring or whether the changes were in response to the actions of secreted factors or cell-cell contact. To establish requirements for contact and to identify the target cell for expression changes, we performed monoculture experiments in which endothelial cells and U87 cells were treated with either basal media, media conditioned by U87 or endothelial cell monocultures, or by U87-endothelial cell co-cultures. We

Figure 4-2: Specification of the cell of origin for expression changes by qRT-PCR validation of differentially expressed genes using conditioned media swap. Conditioned medias were; Serum Free DMEM (SF DMEM), SF DMEM conditioned by HBMECs (HBMEC), SF DMEM conditioned by U87 cells (U87 MG), or SF DMEM conditioned by a coculture of HBMECs and U87s (Coculture). HBMECs (top row) or U87s (bottom row) were grown in one of the respective conditioned media types for 48 hours, RNA was isolated, and qRT-PCR was performed with primer pairs for one of the three genes. The chemokines (CXCL6 and CXCL1) specifically changed in the HBMECs in response to U87 stimuli and THBS1 only changed in the U87 cells specifically to the coculture conditioned media. N=3 for all sets.

validated the approach with three differentially expressed genes: CXCL6, CXCL1, and THBS1. For all four genes, conditioned media treatments recapitulated the gene expression changes predicted from the microarray results indicating that their regulation was through the actions of secreted factors (Figure 4-2). CXCL6, IL8, and CXCL1 all were upregulated in HBMECs in response to factors secreted by U87 cells. In contrast, THBS1 was strongly downregulated in U87 cells by media conditioned by HBMECs. These results provided validation for our computational and experimental approach and indicated that conditioned media experiments in combination with qRT-PCRs could distinguish the regulator and target cell type for most gene expression changes that occurred through secreted GBM and endothelial cell interactions.

GBM PDE7B expression is regulated by physical contact with HBMECs

Figure 4-3: Changes in PDE7B Expression require physical contact between endothelial cells and U87 cells. (A) U87 cells grown in conditioned media types as indicated. PDE7B expression was undetectable in U87 cells grown in U87 conditioned media. (B) PDE7B expression when U87 cells were grown on methanol fixed HBMECs or grown by themselves. *p-value = 0.04 by Student's t-test. (C) A primary GBM line, B18, was grown in conditioned media as indicated. No significant changes in PDE7B expression were detected. (D) B18 cells grown on fixed HBMECs showed a 4.7 fold overexpression of PDE7B. #p-value = 0.02 by Student's t-test.

Among the genes upregulated through interactions between HBMECs and U87 cells was the cAMP specific phosphodiesterase PDE7B (23). This transcript was of interest as we previously demonstrated that another cAMP-specific phosphodiesterase PDE4A1 had potent pro-tumorigenic effects in both low and high-grade glioma models (24) (25). Thus we hypothesized that if PDE7B were upregulated in GBM cells, it would also stimulate GBM growth. We first sought to determine whether PDE7B regulation occurred through the actions of secreted factors or whether it required physical contact between HBEMCs and U87 cells. When we performed the conditioned media screen for secreted factor effects on expression we saw very low levels of PDE7B that were unaffected by the conditioned media (Figure 4-3A). Therefore we suspected that PDE7B was being regulated by cell:cell contact. To directly test this hypothesis, HBMECs were grown to ~80% confluence, then methanol fixed and washed. These fixed cells, and their associated matrix, maintain relevant epitopes for cell:cell and cell:matrix interactions but are devoid of secreted factors and RNA (26). U87 cells were then cultured on the fixed HBMECs for 48 hours prior to RNA extraction and qRT-PCR analysis for

PDE7B. Under these conditions, PDE7B was specifically upregulated in the U87 cells, indicating that U87 cells were the target cell for gene expression changes detected in the co-culture expression profiling and, in contrast to the regulators of angiogenesis previously evaluated, changes in PDE7B occur only through physical cell:cell contact with HBMEC cells (Figure 4-3B). To verify that HBMEC-induced changes in PDE7B were not confined to U87 cells and to increase the relevance of these findings to GBM, we performed an identical evaluation with a primary low passage GBM cell line B18. Consistent with the results obtained with U87 cells, PDE7B was upregulated in B18 cells, but only through direct physical contact (Figure 4-3C and 4-3D).

PDE7B is frequently upregulated in GBM and has prognostic significance

If endothelial cell induction is an important mechanism for the regulation of PDE7B expression in GBM, then this should be apparent at the mRNA and protein level in primary GBM specimens. We obtained RNA from 22 primary GBM specimens from the Siteman Cancer Center Tissue Procurement Core, generated cDNA, performed qRT-PCR for PDE7B on these samples, and compared the expression level to that in normal human astrocytes. We saw a strong upregulation of PDE7B in 18 of the 22 samples (Figure 4-4A), indicating that upregulation of PDE7B mRNA commonly occurs in GBM. Next, we analyzed protein expression by performing immunohistochemical (IHC) staining for PDE7B on a tissue microarray containing duplicate cores of 35 different astrocytomas. These astrocytomas ranged from grade 1 to grade 4. PDE7B was expressed in the majority of tumor specimens, variably involving endothelial cells, perivascular cells, and non-perivascular tumor cells (Figure 4-4C, Supplemental Table 4-S1). When low grade tumors (Grades 1 and 2) were compared to high grade tumors (Grades 3 and 4), high grade tumors had significantly more staining than low grade (high grade average = 2.25, low grade

average = 1.27, p-value = 0.01 by Student's t-test, Figure 4-S1). These results establish that PDE7B is expressed in GBM at both the mRNA and protein level and is found within the perivascular space.
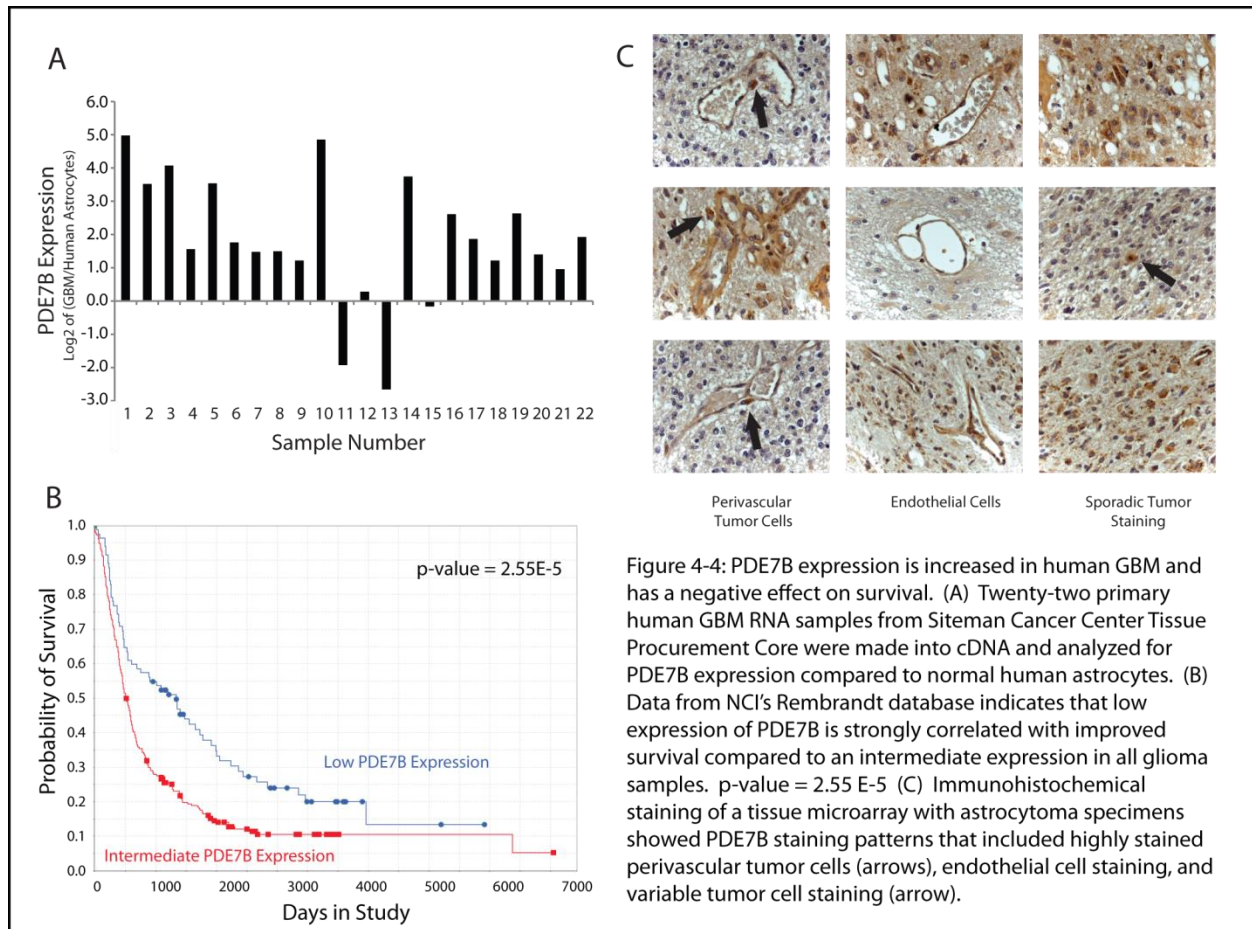

Figure 4-4: PDE7B expression is increased in human GBM and has a negative effect on survival. (A) Twenty-two primary human GBM RNA samples from Siteman Cancer Center Tissue Procurement Core were made into cDNA and analyzed for PDE7B expression compared to normal human astrocytes. (B) Data from NCI's Rembrandt database indicates that low expression of PDE7B is strongly correlated with improved survival compared to an intermediate expression in all glioma samples. p-value = 2.55 E-5 (C) Immunohistochemical staining of a tissue microarray with astrocytoma specimens showed PDE7B staining patterns that included highly stained perivascular tumor cells (arrows), endothelial cell staining, and variable tumor cell staining (arrow).

To determine whether there is any prognostic significance to PDE7B expression, we examined the National Cancer Institute's (NCI) Rembrandt Database for astrocytomas. To increase the power of our analysis, we evaluated data from patients with all grades of astrocytomas. In this population, increased PDE7B expression was correlated with decreased survival (p-value < 2.55E-5). Patients with low PDE7B expression survived significantly longer than those with intermediate PDE7B expression (Figure 4-4B). This survival analysis could either reflect a correlation between PDE7B expression and astrocytoma grade and/or an independent effect of PDE7B expression within any or all grades of astrocytoma. Together, these results are suggestive of an important role for PDE7B in glioma biology.

Overexpression of PDE7B stimulates in vitro stemness and in vivo GBM growth and tumorigenicity

To directly test whether the level of PDE7B expression affects GBM biology, we cloned and

overexpressed PDE7B in U87-GL cells. We desired to have a catalytically inactive form of PDE7B as a

control. Catalytically inactive forms of other cAMP specific phosphodiesterases have been generated by

mutating an essential amino acid in the catalytic site (24,27). Using comparative genomics we aligned the

sequences of these phosphodiesterases with PDE7B, identified the same essential histidine and generated

an analogous catalytically inactive histidine to glutamine (H217Q) mutant. Both the wild type and mutant

forms of PDE7B were stably introduced into U87-GL cells using PiggyBac transposase and hygromycin

selection. Verification of the overexpression was determined by qRT-PCR (Supplemental Figure 4-S2A),

and Western blot analysis (Supplemental Figure 4-S2C). The activity of wild type and mutant PDE7B on

cAMP levels was verified by cAMP ELISA. Overexpression of wild type PDE7B, but not catalytically

inactive PDE7B, resulted in significantly lower levels of cAMP (Supplemental Figure 4-S2B).

Since we had previously found that the overexpression of another phosphodiesterase, PDE4A1,

suppressed intracellular cAMP levels and stimulated growth in multiple brain tumor cell lines (25) (24),

we hypothesized that the overexpression of PDE7B might have similar effects. Interactions between

endothelial cells and GBM cells are known to regulate tumor growth through maintenance of the GBM

stem-like cell population. To determine whether the induction of PDE7B in GBM cells through an

interaction with endothelial cells might influence stem-like cell behavior, we first measured the fraction

of U87 cells with stem-like cell function in extreme limiting dilution assays (ELDAs). Briefly, replicate

cultures were established with a series of extremely low cell numbers in sphere formation media, and

allowed to grow for 3 weeks. Under these conditions, only cultures that contain a stem-like cell are

capable of forming a colony. The fraction of wells with colonies as a function of cells plated provides a measure of the frequency of cells with stem-like properties (Supplemental Figure 4-S3A). When analyzed, the U87-GL+PDE7B(WT) cell line had a significant increase in the percentage of stem-like cells compared to the catalytically inactive control line (p-value = 0.03, 11.1% vs 7.0% respectively, Supplemental Figure 4-S3B).
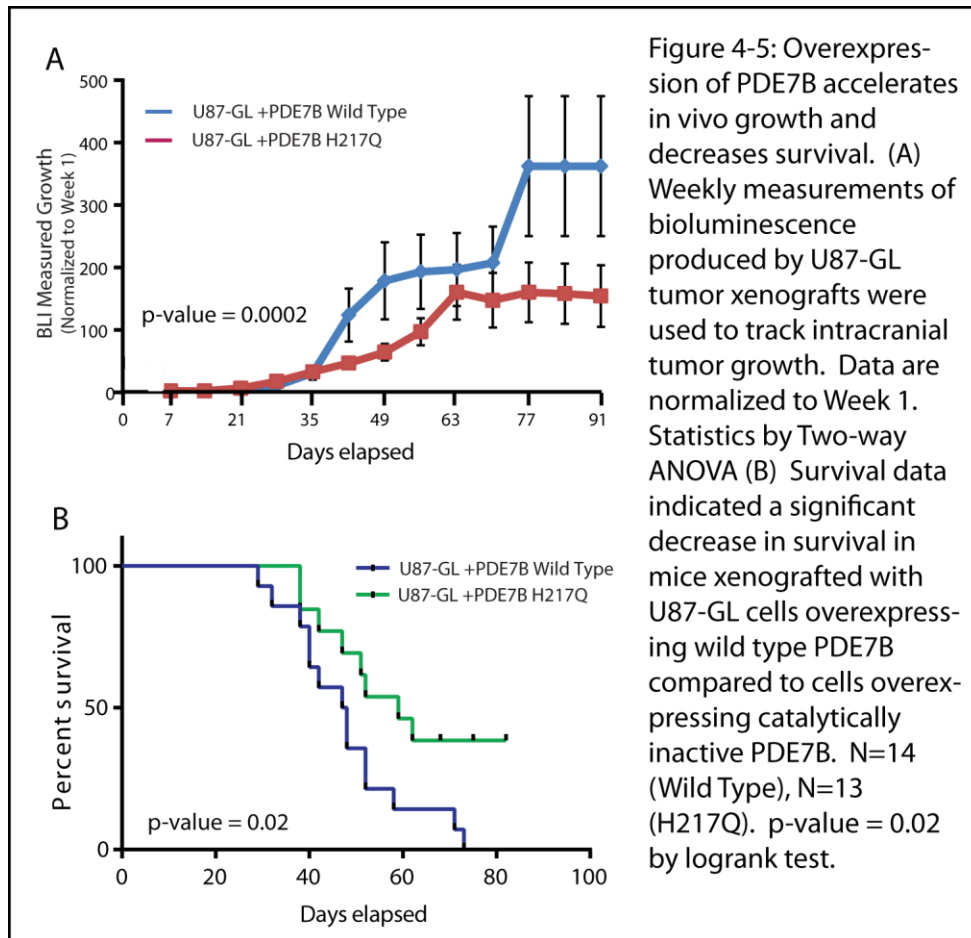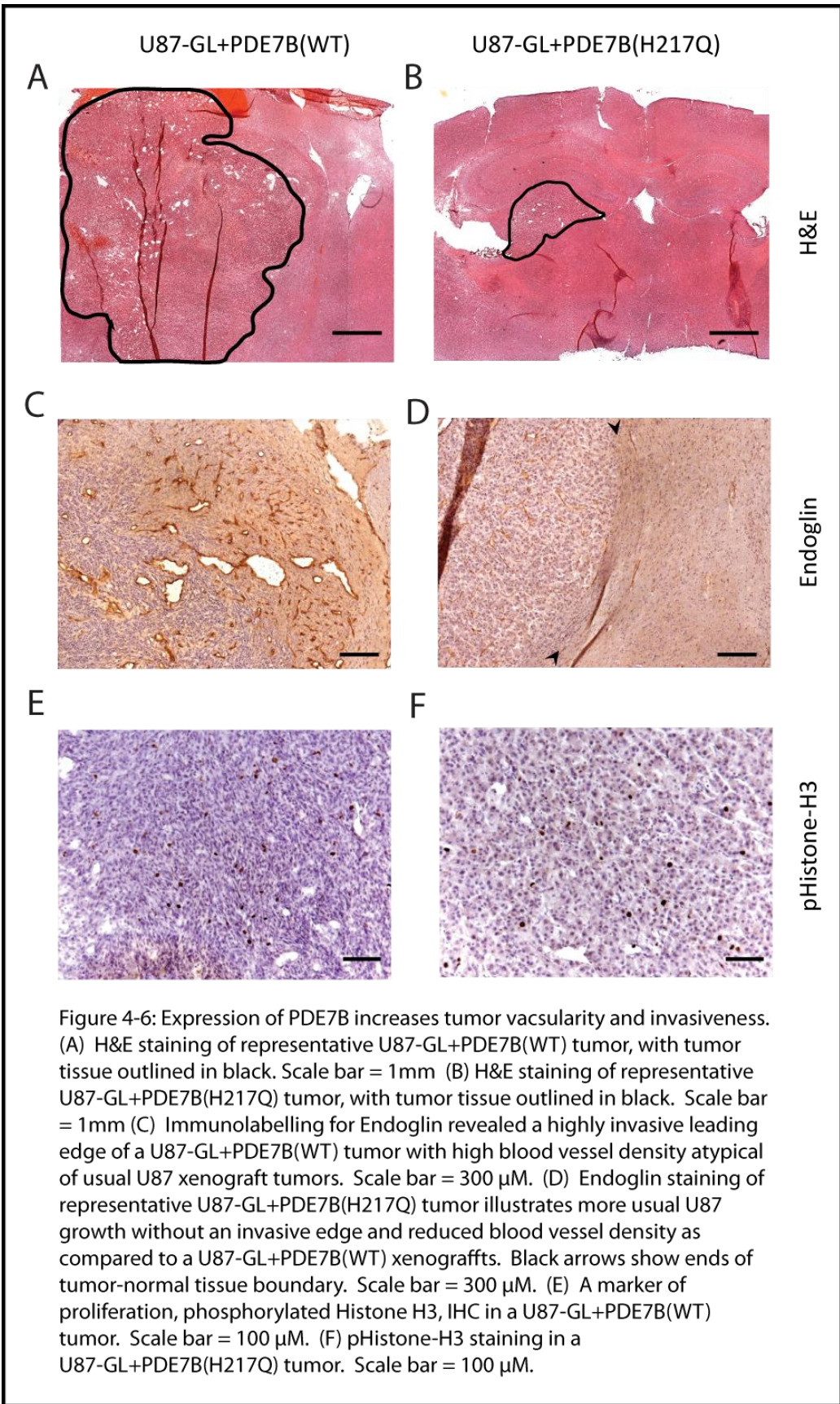

Figure 4-5: Overexpression of PDE7B accelerates in vivo growth and decreases survival. (A) Weekly measurements of bioluminescence produced by U87-GL tumor xenografts were used to track intracranial tumor growth. Data are normalized to Week 1. Statistics by Two-way ANOVA (B) Survival data indicated a significant decrease in survival in mice xenografted with U87-GL cells overexpressing wild type PDE7B compared to cells overexpressing catalytically inactive PDE7B. N=14 (Wild Type), N=13 (H217Q). p-value = 0.02 by logrank test.

In vitro expansion of the stem-like cell population by overexpression of PDE7B, together with the negative prognostic effect of PDE7B expression in human disease suggested that overexpression of PDE7B might have a pro-tumorigenic effect in vivo. To investigate this possibility, we generated intracranial xenografts in NCR nude mice with 50,000 U87-GL+PDE7B(WT) or U87-GL+PDE7B(H217Q) cells engineered to also express firefly luciferase (24). Tumors were generated in three separate cohorts of mice with 5 mice per group in each cohort. Tumor growth was quantified with weekly bioluminescence imaging (BLI) and survival was measured. Tumors expressing wild type PDE7B

U87-GL+PDE7B(WT)   U87-GL+PDE7B(H217Q)

A   B   H&E

C   D   Endoglin

E   F   pHistone-H3

Figure 4-6: Expression of PDE7B increases tumor vacsularity and invasiveness. (A) H&E staining of representative U87-GL+PDE7B(WT) tumor, with tumor tissue outlined in black. Scale bar = 1mm (B) H&E staining of representative U87-GL+PDE7B(H217Q) tumor, with tumor tissue outlined in black. Scale bar = 1mm (C) Immunolabelling for Endoglin revealed a highly invasive leading edge of a U87-GL+PDE7B(WT) tumor with high blood vessel density atypical of usual U87 xenograft tumors. Scale bar = 300 μM. (D) Endoglin staining of representative U87-GL+PDE7B(H217Q) tumor illustrates more usual U87 growth without an invasive edge and reduced blood vessel density as compared to a U87-GL+PDE7B(WT) xenograffts. Black arrows show ends of tumor-normal tissue boundary. Scale bar = 300 μM. (E) A marker of proliferation, phosphorylated Histone H3, IHC in a U87-GL+PDE7B(WT) tumor. Scale bar = 100 μM. (F) pHistone-H3 staining in a U87-GL+PDE7B(H217Q) tumor. Scale bar = 100 μM.

exhibited a significantly greater rate of growth than those expressing mutant PDE7B (Figure 4-5A) and two-way ANOVA showed a statistically significant difference with a p-value of 0.002. The greater rate of growth resulted in significantly decreased survival as determined by log-rank testing of Kaplan-Meier survival (Figure 4-5B).

Consistent with the BLI and survival data, histological analysis revealed that tumor size was consistently greater in the PDE7B WT tumor group (Figure 6A) than the PDE7B H217Q tumor group (Figure 6B). Strikingly, PDE7B WT tumors exhibited a more aggressive tumor phenotype than is normally seen with U87 xenografts (28). This was most evident in the extensive infiltration and neovascularization (Figure 6C) as compared to the PDE7B H217Q tumors (Figure 6D). Proliferation was evaluated by phosphorylated Histone H3 staining. Overall cell density was higher in the tumors overexpressing PDE7B WT compared to those overexpressing PDE7B H217Q, and phosphorylated Histone H3 staining correlated with cell density (Figure 6E & F).

Discussion

The complexity of the tumor microenvironment presents obstacles to progress in both understanding molecular oncology and in developing therapeutics to effectively target contextualized tumor biology. Advancement may depend upon new model systems in which the multi-faceted microenvironment can be deconvoluted in order to assess the contributions that specific subsets of cells, matrix, and three-dimensional structures make to tumor tissue biology. Model systems like these should support discovery of genes and pathways that mediate the important reciprocal interactions that occur between tumor cells and their microenvironment. We were interested in understanding the interactions between GBM and endothelial cells as these are purported to be essential for tumorigenesis (10), tumor progression (29) and recurrence after standard treatment (30). Direct global expression profiling of co-cultured GBM and endothelial cells, enabled by our computational deconvolution algorithm, revealed a number of pathways that were induced or suppressed by the interaction of these cells. Among them was upregulation of the cAMP-specific phosphodiesterase, PDE7B, in GBM cells.

A major advantage of this computational approach is the ability to capture changes in gene expression that occur as a consequence of the actions of secreted factors and/or physical cell:cell contact. Neither conditioned media swap nor trans-well experiments can detect changes mediated by cell:cell contact. Moreover, these commonly applied techniques cannot measure changes in gene expression that occur through reciprocal interactions between cells. These types of interactions have been shown to be critical in the tumor microenvironment (31). Therefore, the inability to detect them is a significant limitation to the use of conditioned media swap and trans-well experiments alone for assessing paracrine effects in tumor biology. Computational deconvolution supports the immediate isolation of RNA from the co-cultures, which prevents the degradation of the signal that can result when additional processing time is required for cell sorting.

This computational approach has its own limitations. First, the detected gene expression changes are a mixture of the signal from both of the constituent cell types. Therefore, for each gene, further studies are required to determine in which of the cell types the change occurred, and whether this change was a function of secreted factors or cell:cell contact. Furthermore, reciprocal gene induction and suppression could theoretically result in no apparent change in signal and a diminution of the model's sensitivity. We suspect that processes like these contribute to the marginally weaker significance we detected for changes in gene expression as compared to many other global expression studies. However, we were able to identify 45 genes whose expression was significantly altered. After multiple hypothesis correction, validation by qRT-PCR and *in vivo* experiments we did not uncover any false positives, reassuring us that the analysis provided a high quality list of changes in gene expression as a consequence of functional interactions between GBM and endothelial cells. Currently, the system serves as a proof of principle and evaluates a single set of interactions between two cell types from the tumor microenvironment. Further

work will be necessary to systematically add other tumor microenvironmental cells types and determine the additional effects on gene expression.

Of the genes discovered by our assay, PDE7B was chosen for further studies. This focus was supported by our previous work demonstrating that PDE4A1 and cAMP suppression can contribute to tumorigenesis (24) and drive intracranial brain tumor xenograft growth (32) (25). Low levels of cAMP have been correlated with malignant brain tumors for many years (33), but the mechanisms that control these abnormal cAMP levels and their cancer relevant targets are often undefined or remain poorly understood. The cyclic AMP pathway is integral to the regulation of numerous cellular processes including growth and differentiation (34,35). While the bulk of cAMP signaling is transmitted by just two mediators, protein kinase A (PKA) and exchange protein activated by cAMP (EPAC), exquisite specificity in cAMP signaling is achieved through subcellular localization and activation (36). A critical component of this specification is the establishment of defined intracellular pools of differing cAMP levels as a result of highly localized and regulated cAMP degradation through PDEs.

Humans have 11 families of PDEs, three of which are cAMP specific (PDE4, PDE7, PDE8), and each of these families can have multiple gene homologs throughout the genome, and each of these genes can have multiple splicing isoforms (36,37). The canonical PDE structure includes a common catalytic domain, an amino terminal subcellular localization motif and regulatory kinase sites and protein-protein interacting domains. Consequently, PDE expression and activation result in the generation of highly specific gradients of intracellular cAMP (38) and the formation of multiprotein signaling complexes that carry out localized functions (39,40). This process has been most extensively illustrated for the PDE4 family of cAMP-specific PDEs (41,42).

While we have yet to define how PDE7B promotes GBM tumorigenecity, there are some likely mechanisms. Cyclic AMP is a recently established regulator of neural stem cell proliferation, differentiation, and survival (43) and it is known that endothelial cells in the PVN support GBM CSCs and therefore the induction of PDE7B expression in tumor cells localized to the perivascular space may function to promote CSC function. The detection of a modest, but significant, increase in stemness, as measured by our *in vitro* limiting dilution assay, in U87 cells overexpressing wild type PDE7B is consistent with this hypothesis. This would also be in line with our observation that PDE7B WT intracranial tumors showed signs of increased invasion and aggressiveness compared to the PDE7B H217Q tumors. The increase in blood vessel density in wild type tumors also suggests that tumor cell PDE7B might function as part of a positive feedback loop for promoting angiogenesis.

Finally, the effect of PDE7B on outcome in GBM and its ability to drive intracranial tumor growth in the U87 model suggests that inhibition of PDE7B should be evaluated as a novel therapeutic for GBM. We previously demonstrated that targeted inhibition of an alternate cAMP-specific phosphodiesterase PDE4 had significant anti-brain tumor activity, when tested in a spontaneous model of low-grade glioma and in intracranial xenograft models of GBM and medulloblastoma (24) (25). Together with the current findings, these results provide a strong rationale for cAMP elevation in the treatment of brain tumors. While there have not been any studies of specific PDE7B inhibitors in the context of cancer treatment, there has been work done evaluating PDE7B inhibition for the treatment of autoimmune disorders (44,45). Therefore future work should focus on taking specific inhibitors of PDE7B from the autoimmune studies and determining their efficacy in blocking glioma growth *in vitro* and *in vivo* with the hope of translating these findings to the clinic as quickly as possible.

Materials and Methods

All animals were used in accordance with an established Animal Studies Protocol approved by Animal Studies Committees of the Washington University School of Medicine.

Cell culture and reagents:

U87MG cells (Catalog #HTB-14) authenticated by STR were obtained from ATCC, grown in MEM alpha (Life Technologies Cat# 12561-049) with 10% fetal bovine serum (Sigma-Aldrich Cat# F4135-500ML), and used within 4 months of passage zero. Cells were then infected with a lentivirus expressing a fusion protein of eGFP and Firefly Luciferase (GL) to allow the cells to be visualized by fluorescence and quantified by bioluminescent imaging (24). HBMECs were obtained from ScienCell (Catalog #1000), grown in complete endothelial media EGM-2MV (Lonza Cat# CC-3202), and all experiments were performed between passage 6 and 10. B18 GBM line was created following an IRB-approved protocol as previously described (46). Briefly, primary GBM tumor tissue was cleaned manually of RBCs, mechanically dissociated with forceps and scalpel, and dissociated with Accutase (Sigma Cat# A6964-100ML). Cells were then spun down, triturated gently, and plated on PLO (Sigma Cat# P4957) and Laminin (Sigma Cat# L2020) coated Primaria plates (BD Biosciences Cat# 353824). Cells were used for experiments after the fifth passage. Media is RHB-A with EGF and FGF.

Fixed cell cocultures:

Cells were grown to 80% confluence in 6-well plates. The media was then removed and replaced with ice cold 100% methanol, placed at -20C for 20 minutes, and then washed three times with PBS before 150,000 glioblastoma cells were placed on top in their respective media.

<u>*In vitro* coculture</u>:

150,000 HBMECs were plated on Matrigel (BD Biosciences Cat# 354234) using the thin gel protocol and in EGM-2MV in 6-well plates and allowed to form tubules for 24 hours. 150,000 U87-GL cells were plated on top of the HBMEC tubules, grown together for another 48 hours, and then RNA was harvested with Trizol (Invitrogen #15596-026).

<u>Microarray analysis</u>

Starting with the quartile normalized Illumina Beadarray data from the standard Illumina analysis, a perl script loaded the data for three biological replicates of U87 monoculture, HBMEC monoculture, and U87 plus HBMEC coculture. Data was filtered for genes that had expression p-values > 0.05 (as deteremined by the Illumina software). For each test computational dataset to be created (0.1% U87/99.9% HBMEC for example), and for each gene in the dataset, the value for biological replicate 1 of the U87 data was multiplied by 0.001 (for example) and added to the value for biological replicate 1 of the HBMEC data after it was multiplied by 0.999. This was repeated for the other two biological replicates to give 3 new computationally derived replicates and then repeated for all of the genes. After each of these computational datasets was complete, a Pearson correlation was calculated between of all the genes for biological replicate 1 of the coculture dataset versus all the genes of the computationally derived biological replicate 1. This was repeated for all three replicates, and then these three Pearson correlations were averaged, and then recorded. Figure 1B is a plot of all the different computationally derived datasets versus the corresponding average Pearson correlations.

Once the computational dataset was created, genes without any expression (average of the three biological replicates < 30) in any of the samples, were removed from the analysis leaving 14,712 genes.

Next, a Student's t-test was performed between the 3 computational replicates and the three coculture replicates. Further filtering was performed to leave only those genes which had a fold change greater than 2 fold (up or down) between the computational dataset and the coculture dataset. Benjamini-Hochberg multiple hypothesis correction was then performed, or which 45 genes remained statistically significant.

qRT-PCR:

qRT-PCR primers were designed in Primer 3 using previously described settings (47). Primers were: GAPDH(F-TGTAGTTGAGGTCAATGAAGGG, R-ACATCGCTCAGACACCATG), CXCL1(F-GCACTGCTGCTCCTGCTCCTGGTAG, R- CGCCCATTCTTGAGTGTGGCTATGA), IL8(F-GACCACACTGCGCCAACACAGAAAT, R- CCAGTTTTCCTTGGGGTCCAGACAG), THBS1(F-CTGAGTTGGACGTCCCCATCCAAAG, R- CCACGTTGTTGTCAAGGGTGAGGAG), CXCL6(F-TGCACTTGTTTACGCGTTACGCTGAG, R- TTCCGGGTCCAGACAAACTTGCTTC), and PDE7B(F-CTGTTAAGTAGGCGGAAGTCAA, R-CGATCAGAATGCCAAATGTGTT). Thermo-Fisher qRT-PCR master mix (Thermo-Fisher Cat # AB-1166/B) was used according to manufacturer's directions. Data was acquired on a BioRad CFX96 qPCR machine (Cat # 184-5096) and analysis was done in Microsoft Excel using the $\Delta\Delta C$ method.

Tissue sections and staining:

Immunohistochemistry was done as previously described (24). Antibodies used: phosphorylated Histone H3 1:500 (Cell Signaling #9701), Endoglin 1:250 (R&D Systems #AF1320), cleaved caspase 3 1:200 (Cell Signaling #9664).

Creation of overexpression cell lines:

Wild Type PDE7B or a catalytically inactive version were cloned into a vector with CMV driving cassette expression, hygomycin as a selectable marker, and flanked with PiggyBac Transposase LTRs. These plasmids were cotransfected into an existing U87-GL line with a plasmid transiently expressing PiggyBac Transposase to create stable lines as described previously (48).

Site directed mutagenesis:

The catalytically inactive version of PDE7B was created by starting with the already cloned wild type version of PDE7B and using a site directed mutagenesis kit (Agilent Cat # 200521), as directed, with primers (F- GCTGGTTCACCCCTGGCTGGTCCACATCGTGTGC) and (R- GCACACGATGTGGACCAGCCAGGGGTGAACCAGC).

Tissue microarray:

A tissue microarray for astrocytomas was purchased from US Biomax (Cat # BS17016a). Citric acid antigen retrieval was performed on the slide then blocked with 2% BSA/10% donkey serum in PBST for two hours. PDE7B primary antibody from ThermoScientific (Cat# PA5-32976), or a no primary control, was used at 1:50 overnight at room temperature, followed by Biotin labeled anti-Swine secondary (1 hour) and Streptavidin-HRP tertiary (1 hour). Slides were developed in DAB and counterstained with hematoxylin.

Extreme limiting dilution assays:

Extreme limiting dilution assays were performed as described previously (49). Briefly, for each of 5 biological replicates U87-GL+PDE7B(WT) or U87-GL+PDE7B(H217Q) cells were plated at 81, 27, 9, 3, or 1 cells per well (in 12 technical replicate wells each) in tumorsphere media in 96 well plates, grown for 3 weeks, and then counted.

Generation of intracranial xenografts:

Intracranial xenografts were generated as described previously (32). Briefly, homozygous NCR nude mice were positioned in a stereotactic frame (Stoelting) and 50,000 cells in 5□l PBS were injected through a 27 gauge needle over 1 min at 3 mm below the dura mater.

Bioluminescence imaging:

NCR nude mice bearing intracranial xenografts of U87-GL overexpressing Wild Type PDE7B or catalytically inactive control PDE7B-H217Q were injected with D-luciferin (150 µg/g; Biosynth) as previously described (24). After anesthesia using 2.5% isoflurane, mice were imaged with a charge-coupled device camera-based bioluminescence imaging system (IVIS 50; Caliper Perkin-Elmer). Images were processed using Living Image and IgorPro Software (Version 2.50) as described. Raw data were expressed as total photon flux (photons/s).

cAMP measurement

cAMP was measured by competitive immunoassay using a Correlated Enzyme Immunoassay Kit (Assay Designs) according to the manufacturer's instructions and as previously described (50). cAMP values were normalized to protein for each sample individually.

Supplemental Table 4-S1:

| Sex	Age	Pathology	Grade	Staining Score
M	22	Astrocytoma	1	1
M	52	Astrocytoma	1	1
F	45	Astrocytoma	1	2
F	34	Astrocytoma	1	1
F	30	Astrocytoma	1	1
F	49	Astrocytoma	1	1
F	35	Astrocytoma	2	1
F	63	Astrocytoma	2	1
M	32	Astrocytoma	2	1
M	27	Astrocytoma	2	2
M	44	Astrocytoma	2	0
F	26	Astrocytoma	2	1
M	24	Astrocytoma	2	2
M	43	Astrocytoma	2	4
M	51	Astrocytoma	2	0
F	18	Astrocytoma	2	1
M	39	Astrocytoma	2	3
M	48	Oligodendroma	2	0
M	43	Glioblastoma	2/3	1
M	66	Astrocytoma	3	4
M	42	Astrocytoma	3	2
F	39	Glioblastoma	3	2
M	37	Glioblastoma	3/4	4
F	49	Glioblastoma Multiforme	4	2
M	36	Glioblastoma	4	2
F	58	Glioblastoma	4	4
F	43	Glioblastoma Multiforme	4	3
F	63	Glioblastoma	4	2
M	65	Glioblastoma	4	2
F	61	Glioblastoma (with Necrosis)	4	3
M	42	Glioblastoma	4	1
M	9	Glioblastoma	4	1
F	44	Glioblastoma	4	2
M	59	Glioblastoma (with Necrosis)	4	1
M	25	Glioblastoma	4	1
F	8	Adjacent Normal Tissue	N/A	1
M	77	Adjacent Normal Tissue	N/A	0
M	63	Adjacent Normal Tissue	N/A	0
M	30	Adjacent Normal Tissue	N/A	0
F	49	Adjacent Normal Tissue	N/A	0

Table of tissue microarray analysis results. Scoring was 1 = 0-25% cells staining positive, 2=26-50% cells staining positive, 3=51-75% cells staining positive, 4=76-100% cells staining positive.

Supplemental Figure 4-S1
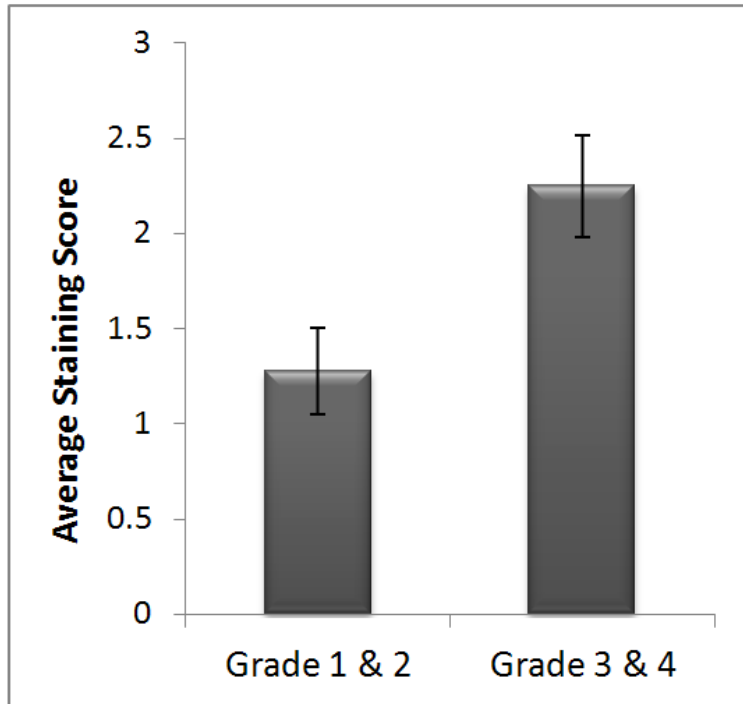
Average of scoring for low grade (1 & 2) versus high grade (3 & 4) tumors. p-value = 0.01 by Student's t-test. N= 18 for low grade (Male, Age 43 Grade 2/3 was excluded), N=16 for high grade. Error bars are Standard Error of the Mean.

Supplemental Figure 4-S2:
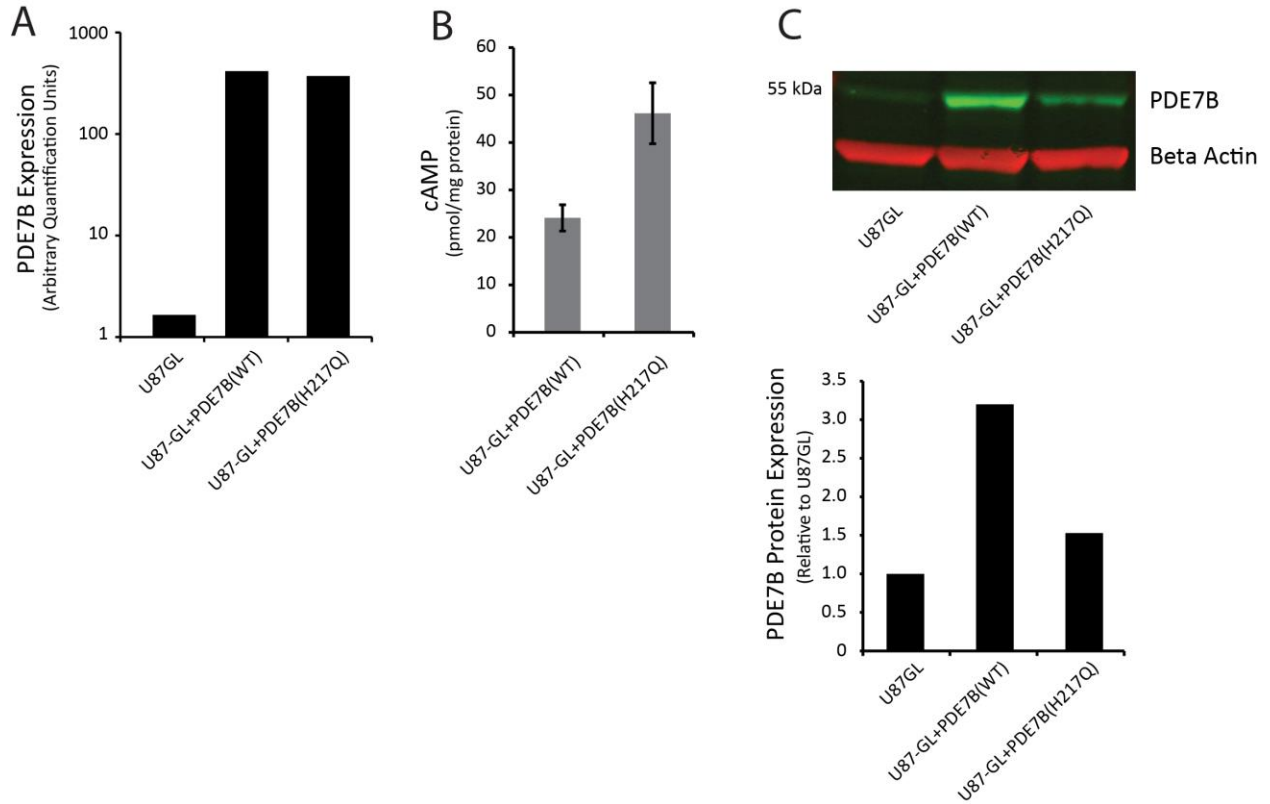
Validation of PDE7B overexpression cell lines. (A) qRT-PCR for PDE7B showed a 415 fold (Wild type) and 372 fold (H217Q) overexpression in the two cell lines. (B) cAMP measurements in the two cell lines grown in vitro.   N=5 (WT), N=6 (H217Q). p-value is 0.017 by Student's t-test. (C) A Western for PDE7B showed a ~3 fold overexpression of PDE7B protein in the wild type line and a 1.5 fold overexpression in the catalytically inactive H217Q line.

Supplemental Figure 4-S3:
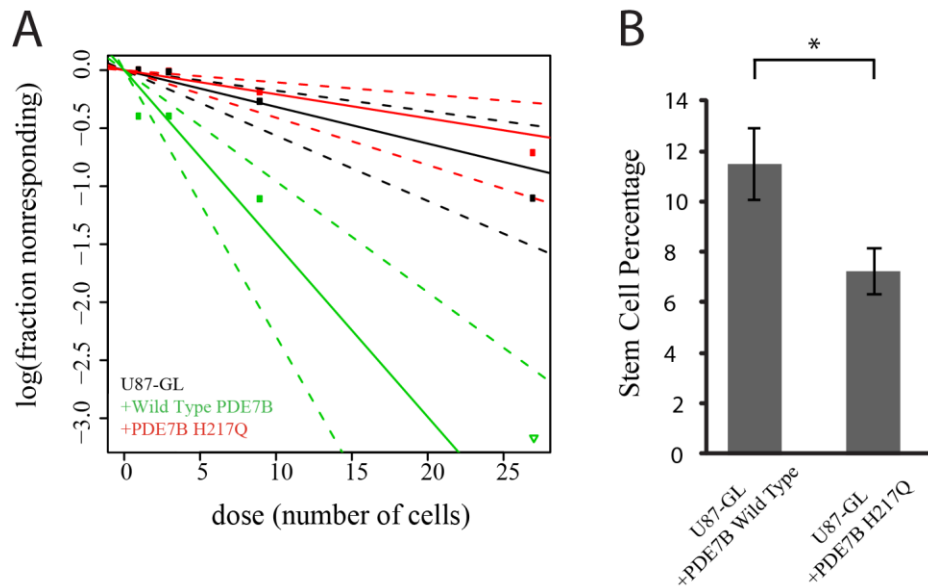
PDE7B overexpression increases glioma stem-like percentage *in vitro*. (A) Representative curve from one biological replicate of an Extreme Limiting Dilution Analysis (ELDA) to determine the percentage of cancer stem cells in a U87 line with overexpression of wild type PDE7B compared to overexpression of a catalytically inactive H217Q mutation. (B) Quantification of the stem like cells in shows that overexpression of wild type PDE7B results in significantly more stem-like cells than the catalytically inactive H217Q line. N=5, *p-value = 0.03

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Chapter 5: Conclusions and Future Directions

The amount of research done at the interface between cancer biology and computational biology is only going to increase in the foreseeable future. Certainly cancer research shows no signs of slowing down. Since the war on cancer began in the 1970s, we've made good progress in treating certain types of cancer while other types have had no significant advances (1). So there is certainly plenty of work left to do. At the same time, computers are getting faster and faster, with Moore's law (2), the theory that computer speed (i.e. the number of transitors per CPU) doubles approximately every 2 years having been true since it was published in 1965, and showing no signs of slowing down. This combined with the fact that next-gen sequencing data is becoming so popular that its rate of generation is outpacing Moore's law, therefore making the bottle neck in analyzing the data the computational power available (3) (4).

The amazing power that next-generation sequencing will bring to cancer biology will revolutionize cancer to an extent which would have been hard to imagine even just a few years ago. In the not-too-distant future there will be thousands of matched cancer-normal genomes sequenced for all of the most common cancer types. This will give unprecedented power to researchers. Knowledge of the spectrum of mutations and transcriptional profiles present will allow refinement of the molecular subtypes of cancers. Many of which were once thought to be the same disease, or at best distinguished only by pathology, and now known to be distinct types. This process has already been done for some cancers such as glioblastoma (5) and medulloblastoma (6). In the meantime, pharmaceutical and biotech companies will develop a new generation of drugs and therapies for specific driver mutations. Patients, upon initial diagnosis, will have a platform of sequencing done including whole genome, exome, and

transcriptional analysis to better determine which cocktail of drugs will best treat those

mutations.

Next-gen sequencing will also have implications for basic cancer biology research. Studying the

epigenetics of cancer biology, including changes to methylation, histone modification,

transcription etc, is a huge field. The manipulation of cancer cells and determination of the

changes to these biological processes is a fundamental part of cancer research and as sequencing

becomes cheaper, it will become common to switch out qRT-PCR, methylation specifc PCR, and

other low throughput assays in favor of RNA-Seq, Chip-Seq, RBBS, and other high-throughput

techniques to get as much information as possible.

Our assay sought to apply computational methods to the fundamental question of, "What are the

reciprocal interactions between glioblastoma cancer cells and endothelial cells?". Since we were

primarily interested in quantifying changes in transcriptional levels, RNA-Seq was unnecessary,

and microarrays were an ideal alternative. As mentioned earlier, not only is the glioblastoma

tumor microenvironment a complex mixture of many different cell types, but each other tumor

type has a unique combination of cells present. So our assay serves as a prototype system for

looking at other combinations of cell types in coculture, both in glioblastoma and other cancers.

However, there are a number of minor improvements which could be made to improve the

system. Future experiments could increase the number of cell types from 2 to 3 or 4. Each

progressive cell type addition would complicate the analysis and diminish the power to detect

changes, so the limit to the number of cell types would need to be determined empirically, but

each progressive addition of a cell type would make the system more and more suitable as a model for their *in vivo* counterparts. Another improvement would be switching to RNA-Seq instead of using microarrays. This would allow detection of not only the quantification of each transcript in general, but also changes in alternative splicing, allelic expression, and expression of rare gene-fusion products. The deconvolution used on the microarrays could just as easily be applied to RNA-Seq data, though each library might need to be sequenced to a higher depth than a traditional RNA-Seq library to allow for as many genes as possible to show expression above background and therefore be used in determination of the computationally mixed dataset. Finally, another option that was considered, but ultimately passed up in favor of using computational deconvolution, would be using a coculture of mouse and human cell types. RNA-Seq would be required to analyze this dataset, since there are no hybrid human-mouse microarrays, but RNA-Seq analysis can determine the difference between a read from a human transcript versus a mouse transcript when there are SNPs which differ between the two species (7,8). Again, larger than normal datasets would be needed since reads that contained no human-mouse SNPs would be ambiguous and therefore wouldn't really provide significantly to the analysis, but this technique would have the advantage of not needing further experimentation to determine which cell type any gene expression changes had occurred in. The analysis would automatically differentiate between human derived changes and mouse derived changes.

Our analysis discovered a number of differentially expressed pathways, including those which we expected like proliferation and angiogenesis, and those which were relatively unexpected such as PDE7B, which we chose to focus on for further experiments. PDE7B is a phosphodiesterase which specifically cleaves cyclic adenosine monophosphate (cAMP) into a

non-active, non-cyclic form. cAMP is a ubiquitous intracellular signaling molecule, found all the way down to bacteria and can affect a large number of cellular processes including cell proliferation and stemness (9). cAMP has been well established as an important mediator of brain tumor biology (10) (11,12). Protein Kinase A (PKA) is the main downstream signaling molecular through which cAMP can affect gene expression, and EPAC1 is another important signaling molecule directly influenced by levels of cAMP. One of the main ways in which cAMP is regulated is through very specific subcellular concentration gradients. cAMP is synthesized at the plasma membrane by g-coupled protein receptors which activate adenyl cyclases, which convert AMP into cAMP (13). This cAMP then diffuses throughout the cytoplasm. Its degradation, on the other hand, is mediated by phosphodiesterases, such as PDE7B, which have very specific subcellular localizations. These PDEs can cause local levels of cAMP to drop dramatically and can overcome the rate of cAMP diffusion to create strong gradients (14).

So while the specific mechanism of PDE7B is yet to be uncovered, it is easy to create a model where surface contact by a glioblastoma cell with an endothelial cell causes the glioblastoma cell to change a number of pathways, including the upregulation of PDE7B. This leads to even lower levels of cAMP in some critical subcellular compartment, leading to less PKA activation and changes in some downstream signaling cascade resulting in increased growth and tumorigenesis.

There are a number of future experiments to help elucidate the full function of PDE7B. Given a large amount of time I would start on the outside of the glioblastoma cell and work in. Meaning,

I would first try to figure out what is on the endothelial cell that mediates PDE7B upregulation. There are some obvious candidate molecules I would focus on, such as integrins associated with tumor:endothelial interactions like $\alpha_V\beta_3$ (15). Integrin $\alpha_V\beta_3$ binds to vitronectin (16) which has been shown to be important in glioma cell migration (17). Another possible integrin is $\alpha_4\beta_1$ which has been shown to interact with vascular cell adhesion molecular 1 (VCAM1), an important cell adhesion molecular on endothelial cells (18). By knocking these factors out individually it should be possible to see which one, if any, is required for the upregulation of PDE7B in glioma cells. Depending on ligand is important on the surface of the glioma cell, it might be possible to make an educated guess about the downstream pathway, but perhaps more importantly would be skipping ahead to looking at the specific subcellular localization of PDE7B. This could easily be done with immunofluorescent microscopy using labeled antibodies to PDE7B. Once determined, the importance of that subcellular localization could be ascertained by first knocking down, or out, PDE7B, and then introducing various PDE7B constructs with mislocalization domains which would cause PDE7B to be localized to the plasma membrane, golgi apparatus, or a number of other locations. Similar experiments have already been done with PDE4 to determine the important of its subcellular localization (19) (20). The various constructs could then be assayed for *in vitro* growth, *in vivo* growth, tumorigenesis, cancer stem cells frequency, angiogenic potential, etc to determine the effect of different subcellular localizations on PDE7B function. Another interesting set of experiments might be to look at any synergistic effects between PDE7B and another phosphodiesterase called PDE4A1. It has been previously shown that in the background of the tumor predisposition syndrome neurofibromatosis type 1 (NF1), that overexpression of PDE4A1 is sufficient to drive tumorigenesis (12). By overexpressing and knocking down both PDE7B and PDE4A1 in various

combinations it would be possible to tell if they work in concert to lower levels of cAMP and
drive tumorigenesis.

While there seem to be no current inhibitors of PDE7B that are only specific for it, there are a
number of inhibitors that have strong inhibition of PDE7B with only minor inhibition of other
PDEs (21). We believe that PDE7B is a prime candidate for future studies, in both basic cancer
biology and translational medicine. Hopefully inhibition of PDE7B can slow tumorigenesis and
provide yet another weapon in the fight against glioblastoma.

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