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**14. ABSTRACT**

Gliomas comprise ~60% of cases among pediatric brain tumors. To date, there are few resources available to study and manipulate pediatric brain tumor cells, to evaluate whether pediatric tumor will have fundamental different responses to the new therapeutic regimes. Since glioma stem cell lines have been successfully isolated from adults, in this proposal we aim to isolate GSC population from pediatric patients. Through a collaboration with Dr. Xiao-Nan Li at Texas Children's Hospital, in the past year, Dr. Paddison's group has successfully derived and cultured eight patient-derived pediatric glioma stem cell lines and has begun molecular and phenotypic characterization of these lines. This characterization has included analysis of gene expression and patient-specific gene mutations, and also proof-of-concept shRNA screens. As a direct result of these efforts, we have begun to observe similarities and differences among pediatric glioma isolates among genes and networks required for proliferation and survival – the key objective of this grant.

**15. SUBJECT TERMS**
Glioblastoma multiforme , glioma stem cell, brain tumor-initiating cell, pediatric brain tumor, RNAi, functional genetics

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Title: Pediatric Glioblastoma therapies based on patient-derived stem cell resources
Contract Number: W81XWH-11-1-0756

PI: Patrick J. Paddison, PhD, Fred Hutchinson Cancer Research Center
Progress Report September 2012 (YEAR 1)

## INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive and common form of brain cancer in adults, and is among the deadliest cancers with a median survival period of 12-14 months. GBM tumors appear to be hierarchically organized suggestive of a cancer stem cell origin. Consistent with this notion, tumor-initiating, glioma stem cells (GCS) have recently been isolated that retain the development potential and specific genetic alterations found in the patient's tumor. When used to generate tumors in the cortex of mice, these cells give rise to patient-specific molecular signatures and histological features.
Gliomas comprise ~60% of cases among pediatric brain tumors. To date, there are few resources available to study and manipulate pediatric brain tumor cells, to evaluate whether pediatric tumors will have fundamental different responses to the new therapeutic regimes. Since glioma stem cell lines have been successfully isolated from adults, in this proposal we aim to isolate GSC population from pediatric patients. Through collaboration with Dr. Xiao-Nan Li at Texas Children's Hospital, Dr. Paddison's group has access to ten orthotopic mouse lines harboring tumors derived from ten different pediatric glioma cases. In these orthotopic xenograft models, patient tumor samples are injected directly into the cortex of recipient NOD-SCID mice, where upon the patient tumor regrows with similar molecular and pathological characteristics observed in the tumor of origin. In this grant, we are deriving and characterizing pediatric GSC lines and assessing whether they diverge from adult GSCs with respect to genes and networks required for proliferation and survival.

## BODY

In this proposal, Dr. Paddison takes a step towards defining new therapeutic strategies for pediatric glioma by applying adult GSC isolation and culture techniques to derive pediatric glioma stem cells and then assessing whether pediatric isolates diverge from adult GBM patients with respect to genes and networks required for proliferation and survival. There are three specific aims:

1. To isolate pediatric glioma stem cell populations in defined monolayer growth culture conditions.
2. To perform molecular and phenotypic characterization of pediatric glioma stem cell isolates.
3. To determine whether adult and pediatric GSCs share common proliferation and survival networks.

**Study Design:** Pediatric GSC lines will be isolated from ten pediatric patient tumors. All ten lines will be characterized for stem cell properties, including expression of progenitor makers, capacity for multilineage differentiation, and tumor formation. Finally, lines will be examined for requirement of 6 genes essential to adult GSCs and, more broadly, for pathways required for proliferation and/or survival using a shRNA shot-gun screening approach.

**Progress on each Aim is reported according to Aim below.**

**Progress on Aim 1 (year 1)**

**In aim 1,** we proposed to isolate pediatric glioma stem cell lines from existing orthotopic mouse lines harboring tumors derived from different pediatric glioma cases. Adult GSC lines retain patient-specific molecular signatures and karyotype when isolated and cultured in monolayers on laminin coated plastic in the serum-free culture media supplemented with EGF and FGF-2 [5,6]. While multiple adult GSC lines have been successfully isolated [5,6], there are currently no pediatric GSC lines available for

comparison studies. The isolation of pediatric lines will allow identification of pediatric-specific glioma networks and vulnerabilities, which may be absent or altered in adult tumors.

Here we report that each of the PDX-pediatric brain tumor models listed in Table 1 successfully used for isolate tumor sphere cultures in GSC expansion media (N2B27 neural basal media (Stemcell Technologies) supplemented with EGF and FGF-2 (20ng/mL each)[6]). Since ability to generate tumor spheres during in vitro culture correlates with tumor initiating cell activity [7,8], this suggests that we have successfully isolated and cultured tumor initiating cells from each tumor. Importantly, we have been successful at passaging, expanding and freezing tumor sphere cultures, achieving a key goal and milestone for aim 1. We are now in the process of converting the tumor sphere cultures into monolayer cultures, which will enable high throughput screening and additional experimental manipulation (Table 1).

**Progress on Aim 2 (years 2-3)**

**In aim 2,** we proposed to perform molecular and phenotypic characterization of pediatric isolates from Aim 1 to determine whether they: (1) harbor glioma stem cell characteristics; (2) fall into one or more adult GBM subclasses; and (3) are capable of initiating tumors. We have made significant progress for this aim.

We have begun to assess pediatric isolates in terms of their ability to form tube-like endothelial structures in vitro, which may be important for tumor-microenvironment interactions and represent a novel tumor-specific lineage [9,10]. So far the 1406 but not the 1502 line shows ability to differentiate and form tube-like structures in endothelial tube formation assays. We've also started access degree of expression of endothelial cell markers such as CD105, which may correlate with tumor aggressiveness in adult GBMs.

We are now attempting to classify pediatric GSC isolates according to the scheme proposed for adult GBMs [11,12] based on an 840 gene list predictive of Proneural, Neural, Classical and Mesenchymal subtypes. In order to classify GSC isolates by tumor subtypes according epigenetic signatures produced by The Cancer Genome Atlas (i.e., classical, mesenchymal, neural, and proneural) [11,13], we developed the following procedure during the last year. We first performed RNA-seq on GSC cultures (n=3) using an Illumina HiSeq 2000 according to the manufacturer's instructions (FHCRC Genomics Shared Resource). RNA-Seq reads are then aligned to the GRCh37/hg19 assembly using Tophat [14] and counted for gene associations against the UCSC genes database with HTSeq, a python package for analysis of high-throughput sequencing data [15]. The R language of statistical computing is then used for further analysis [16]. All data is combined and normalized using a trimmed mean of M-values (TMM) method from the R package, edgeR [17-19]. Normalized counts are then log transformed, and the means across all the cell lines were used to calculate relative gene expression levels. The GSC line data is then clustered using a Manhattan distance complete-linkage method to establish leaflets. Previously 173 glioma cell lines were subtyped using the expression of 840 signature genes [11]. Our samples are clustered using 790 of these genes. The associations of our cell lines to those in publication are determined by minimum Manhattan distance to expression centroids produced by ClaNC. If a gene is expressed consistently in a particular subtype by absolute distance, then that is counted as a 1 and the number of associated genes in each category is summed. As a validation, the four subtypes are clearly distinguished when the method is applied to the 173 glioma lines described previously [11]. We are currently applying this analysis to each of our adult and pediatric GBM isolates.

**Progress on Aim 3 (year 3)**

**In aim 3,** we proposed to examine RNAi hits that have been validated as essential for adult GSC proliferation or survival in pediatric GSC isolates. In addition, we further proposed to perform preliminary shRNA "shot gun" screens tol be performed on two pediatric GSC lines to obtain functional genetic "finger prints" to compare to adult GSCs screen results.

To identify candidate glioma-lethal targets, we performed parallel RNAi screens for proliferation genes in three human GSC lines and the CB660 NSC line [6,20], using a previously published pooled "shotgun" shRNA screen strategy targeting 713 kinases and also a genome-wide library targeting ~19,000 genes [1,2]. To facilitate analysis of these hits, in collaboration with Dr. Jun Zhu's group at Sage

Bionetworks (FHCRC), we next asked whether candidate screen targets had general relevance to GBM by analyzing them in GBM-specific molecular networks created from multiple genomic data of >300 GBM tumor samples. From this work we have focused on two biological stories. In one, we observed that in adult GBM and genetically transformed cells, but not untransformed cells, BUB1B, a key mitotic checkpoint gene, protects cells from lethal kinetochore-microtubule instability not apparent in untransformed cells (resubmitted to Cancer Discovery). In the other, we found that adult GBM cells have added sensitivity to inhibition of PHF5A, a highly conserved PHD-finger domain protein that facilitates interactions between the U2 snRNP complex and ATP-dependent helicases (under review at Cell). We have examined inhibition of both of these genes and other adult GBM-lethal screen hits in pediatric GBM-1052 isolates (Table 1). The results were interesting.

We found that 1052 cells were resistant to BUB1B knockdown similar to untransformed cells. For GBM and genetically transformed cells sensitive to BUB1B knockdown, mechanistic studies revealed that these cells have altered sister KT dynamics during metaphase, which likely favor KT-MT instability. We investigated this possibility that 1052 cells lack this KT conformational change during mitosis. This was indeed the case. As shown in Figure 1, the interkinetochore distances (IKDs), or the maximum distance achieved between sister KTs when stable end-on MT attachment has occurred [4], in 1502 cells fell into the range of cells insensitive to BUB1B inhibition. This result emphasizes the notion that pediatric brain tumors may differ substantially from those observed in adults. We are currently analyzing other pediatric isolates for short and long IKDs and sensitivity to BUB1B knockdown. If successful, these studies may identify IKDs as a biomarker for pediatric GBM that predicts sensitivity to perturbation of KT-MT interactions.

At the same time, we performed a proof-of-concept shRNA screen in 1502 cells. This screen assayed ~20 genes that scored as being differentially required for adult GBM cell expansion, as compared to human NSCs. For this assay, a human mini-pooled lentiviral shRNA library of ~100 shRNAs was used to infect 1502 cells and one NSC cell (CB660) at a representation of ~1000 fold and a multiplicity of infection (MOI) of ~1 in parallel. Cells were then selected by puromycin to remove uninfected population and divided into 3 replicates. Cells were kept for three weeks, encompassing approximately 10 cell doublings. Afterwards, DNA was harvested and viral inserts containing unique target sequence were PCR amplified. The PCR product went through a column purification procedure to remove primers and genomic DNA and then subjected to Illumina high-throughput sequencing. In this analysis, shRNAs lost in the GSC population represent candidate gene targets that may be required by tumor outgrowth. The shRNA screen and Bar-code array analysis will be performed as previously described [1-3]. This analysis revealed that multiple candidate genes differentially required in 1502 cells (Figure 2). In particular, we find PHF5A as a common target between adult GBM isolates and 1502. This is an exciting result, since we have shown that PHF5A knockdown in adult GSCs inhibits RNA splicing of an unusual class of exons with distinctive 3' splice sites, leading to defects in constitutive and alternative splicing in thousands of essential genes, including many required for cell cycle progression (under peer review at Cell). This suggests that at least this pediatric GBM isolate has the same novel requirement for PHF5A to maintain proper exon recognition. This could suggest that classes of compounds affecting 3' splice recognition may be effective against pediatric brain tumors.

## KEY RESEARCH ACCOMPLISHMENTS

- **Isolation and propagation of tumor initiating cells from eight pediatric brain tumor patients.**
- **Development of methodology for classification of GBM tumors from RNA-seq data**
- **Preliminary mutation scan for two GBM tumor isolates**
- **Preliminary examination of GBM-lethal candidates from adult GBM isolate screens in a pediatric GBM isolate**

## REPORTABLE OUTCOMES

- manuscripts, abstracts, presentations:
  Ding et al., **A cancer-specific requirement for BUB1B/BubR1 in human brain tumor isolates and genetically transformed cells?** Cancer Discovery
- licenses applied for and/or issued: none during this reporting period
- degrees obtained that are supported by this award: none during this reporting period
- development of cell lines, tissue or serum repositories:
  **Isolation and propagation of tumor initiating cells from eight pediatric brain tumor patients**
- infomatics such as databases and animal models, etc.:
  **Development of methodology for classification of GBM tumors from RNA-seq data**
- funding applied for based on work supported by this award: none during this reporting period
- employment or research opportunities applied for and/or received based on experience/training supported by this award: none during this reporting period

## CONCLUSION

In summary, we have made substantial progress towards developing in vitro cell models for pediatric brain tumors. We have isolated, successfully cultured, and created a repository for tumor initiating cells from eight pediatric brain tumor patients. Importantly, this repository will be the foundation for all future work for this grant, and represents achievement of a critical milestone for Aim 1 for year 1. In addition, we have also made advanced progress on Aims 2 and 3 through developing methods for typing GBM tumors using TCGA criteria and also performing preliminary RNAi screens on one pediatric GSC isolate. These preliminary studies suggest that, one, there will likely be both commonalities and significant differences in genes required for GSC expansion between adult and pediatric isolates, and, more importantly, that our overall functional genetic approach (Aim 3, year 3) will likely be successful in pin-pointing pediatric brain tumor molecular vulnerabilities.

If successful, these studies will lead to tangible progress for pediatric glioma by creating in vitro resources, where few exist, and specifically addressing the question of whether aggressive pediatric brain tumors have similar vulnerabilities to adults. Moreover, the distribution of these resources to other research centers will lead to better models and improved therapies for pediatric glioma. Lastly, the integration of functional genetic data from comprehensive RNAi screens in defined populations of glioma-initiating cells will act as a valuable comparison data set for genomic data sets arising for pediatric glioma (e.g., TCGA-style profiling).

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## SUPPORTING DATA

Table 1: Pediatric Brain Tumor Models Used for Aims 1-3

| Pediatric Model | Patient | Sex | Neurosphere | Monolayer | RNA-seq | Mutation | shRNA |
|---|---|---|---|---|---|---|---|
| IC-1128GBM-VI | 8 y 7 mo | Male | Y | N | | | |
| IC-1406GBM-V | 5 y | Female | Y | Y | Y | Y | |
| IC-1502GBM-I | 4 y 8 mo | Female | Y | Y | Y | Y | Y |
| IC-1621GBM-IV | 6 y | Male | Y | nd | | | |
| IC-2305GBM | 9 y | Male | Y | Y* | | | |
| IC-3704GBM-III | 12 y | Male | Y | Y | | | |
| IC-3752GBM-rIV | 5 y | Female | Y | nd | | | |
| IC-A46GBM-r V | 16 y | Female | Y | nd | | | |

Y=Yes; N=No; nd=not determined

*doubling time >60hrs

**Figure 1: Measurement of inter-kinetochore distance (IKD) in BUB1B resistant and sensitive cells.** To identify new candidate therapeutic targets for Glioblastoma multiforme (GBM), we combined functional genetics and GBM network modeling to identify kinases required for the growth of patient-derived GBM stem cells, but which are dispensable to proliferating human neural stem cells (NSCs). This approach yielded BUB1B/BUBR1, a critical mitotic spindle checkpoint player, as the top scoring GBM-lethal kinase. Mechanistic studies revealed that BUB1B's GLEBs domain activity is required to suppress lethal kinetochore-microtubule (KT-MT) attachment defects in GBM isolates and genetically transformed cells with altered sister KT dynamics, which likely favor KT-MT instability. We found that 1502 pediatric GSC cells were resistant to BUB1B knockdown. To determine whether KT dynamics were altered in resistant and sensitive cells, we measured inter-kinetochore distance (IKD): the maximum distance achieved between sister KTs when stable end-on MT attachment has occurred [4]. Unlike most adult GBM cells, and also Ras-transformed cells, 1502 cells did not show the characteristic conformation change in KTs, i.e., short IKDs, which correlates with BUB1B sensitivity. This suggests that for pediatric brain tumor targeting BUB1B activity and/or KT-MT function would not be a good therapeutic strategy. More generally, it suggests that pediatric brain tumors may differ from adults in their genetic vulnerabilities.

*Figure panels:*

(A) Cartoon showing IKD measurement. (B) Measurement of IKDs in BTICs, NSCs, MEF, MEF-Ras, RPE and Hela cells using immunofluorescent staining of kinetochores. Constitutive associated centromere network (CCAN/CREST) proteins (red) and outer kinetochore protein, Hec1, (green) were visualized to identify kinetochore pairs. IKDs were measured between Hec1 centroids using Applied Precision Softworx software package. (C-D) Quantification of IKDs from (B). *denotes p<.001 by student's t-test.

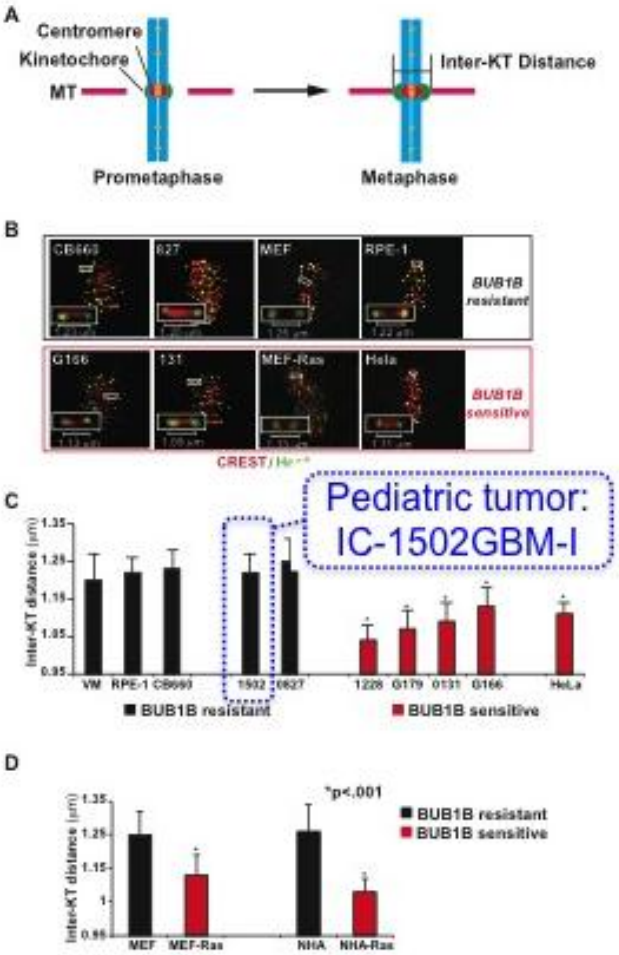

**Figure 2: Preliminary validation screen of adult GBM-lethal genes in Pediatric GBM isolates.** Pediatric GSC 1502 cells and human neural stem cells were infected with lentiviral shRNA pool targeting ~20 adult GBM lethal gene candidates at a representation of ~1000 fold and a multiplicity of infection (MOI) of ~1 in parallel. Cells were then selected by puromycin to remove uninfected population and divided into 3 replicates. Cells were grown for three weeks. Afterwards, DNA was harvested and viral inserts containing unique target sequence were PCR amplified. The PCR product went through a column purification procedure to remove primers and genomic DNA and then subjected to Illumina high-throughput sequencing. In this analysis, shRNAs lost in the GSC population represent candidate gene targets that may be required by tumor outgrowth. The shRNA screen was performed as previously described [1-3].

The graph, below, shows the relative representation of various shRNAs at the end of the expansion period relative to human NSCs. These shRNAs also scored as specifically lethal to three adult GSC lines. Thus, these may represent common therapeutic targets between pediatric and adult brain cancers. If true, downstream efforts, e.g., drug development, would prioritize such targets, which would provide the best therapeutic windows and target both adult and pediatric cancers.