Dartmouth-Hitchcock
NORRIS COTTON
CANCER CENTER

One Medical Center Drive
Lebanon, NH 03756

Phone (603) 653-9000
Fax (603) 653-9003

Cancer.Dartmouth.edu

NORRIS COTTON CANCER CENTER
THE ANDREA CLARK NELSON MEDICAL RESEARCH ENDOWMENT (THE ANDY FUND)
2011-2012 STATUS REPORT

The Andy Fund provides funding essential to Norris Cotton Cancer Center's research focused on cancer of the brain, especially glioblastoma multiforme. The Andy Fund enables investigators to pursue new ideas that may lead to more effective and patient-centered care. Promising scientists can develop preliminary research data that can then be used to pursue grant opportunities from external funding organizations such as the National Institutes of Health. By developing and sharing his/her research through publication and collaborative relationships, the investigator has an impact upon the scientific community at large and contributes to the process of discovery in cancer research.

The Andy Fund is managed by the Cancer Center Administration, and funding recommendations are made by the Cancer Research Committee (CRC) and submitted to the Director of Norris Cotton Cancer Center and the Dean of Dartmouth Medical School for approval. In its deliberations regarding allocation from The Andy Fund for 2011, the CRC recognized the specific purpose of The Andy Fund to support research in malignant brain tumors and chose to support the investigations of Dr. Brenton Paolella working in the laboratory of Dr. Mark Israel.

Dr. Paolella's work has focused on understanding the molecular mechanisms underlying the development of glioblastoma multiforme. Recently the identification of a cell or origin of glioma has been identified unequivocally. While tumors do sometimes mimic characteristics and express markers of specific cell types of the organ in which they arise, the precise cell type in the spectrum of cells from the undifferentiated tissue stem cells, which we now know to be present in organs of adults, to the fully differentiated cells that characterize mature tissues, are known for very few tumor types. The identification of such a target cell is of great importance because it provides an opportunity to understand better the molecular mechanisms underlying the conversion of the precise normal cell in which a particular tumor arises into a malignant tumor cell. The nervous system contains three major unique cell lineages (cell types) that underlie brain function: neurons, astrocytes, and oligodendrocytes. The mature cell in each of these lineages arises from a series of alterations in gene expression that mark the transition not a multi-potent brain stem cell into one of these functional cell types. In each of these developmental pathways there are a series of increasing mature cell types marked by the expression of specific genes that can be used as "markers" of the specific stage in maturation that a particular cell represents. Glioblastoma multiforme has been shown in the past year to arise from an oligodendrocytic precursor cell (OPC) thereby opening the door for mechanistic studies to understand early events in the molecular pathogenesis of this devastating disease.

Inhibitor of DNA binding 2 (Id2) is a helix-loop-helix (HLH) transcription factor expressed by OPCs and known to influence proliferation and cell-fate specification in the central nervous system. Forced expression of Id2 inhibits the maturation of OPCs into oligodendrocytes and promotes proliferation while loss of Id2 results in pre-mature OPC differentiation and cell cycle exit. The tumor suppressor p53 is often inactivated in glioma). Dr. Paolella demonstrated experimentally that Id2 is directly repressed by p53 in NSCs, and loss of p53 results in sustained over-expression of Id2 and Id2-induced proliferation. These findings raise the question of whether sustained Id2 expression associated with p53 inactivation in primitive NSCs contributes to gliomagenesis by influencing the formation of OPCs.

Recent studies utilizing mouse models of glioma have demonstrated that glioma can arise from oligodendrocyte progenitor cells (OPCs) following their malignant transformation. The importance of OPCs in glioma initiation is underscored by the observation that following selective inactivation of the tumor suppressors p53 and NF1 in neural stem cells (NSCs), glioma are initiated specifically by OPCs and not by NSCs themselves or any of their other progeny. The Olig genes (Olig1, Olig2, and Olig3) are critical determinants for the oligodendroglial lineage. The critical role of Olig2 expression in the specification of OPCs is evident in the requirement for Olig2 for the development of NG2-positive cells during development. Olig2 expression is at its highest levels in proliferative OPCs, while still expressed at low levels in more mature oligodendrocytes. OPC proliferation can be stimulated by the platelet-derived growth factor (PDGF). The Cancer Genome Atlas Network has described gain-of-function alterations in PDGF/PDGFRA in glioma suggesting that PDGF/PDGFR signaling contributes to the malignant proliferation tumors arising in OPCs.

Since sustained high levels of Id2, a known regulator of OPC proliferation, is observed in p53 inactivated NSC, Dr. Paolella along with Dr. Matt Havrda in the Israel Lab sought to determine the functional consequences of Id2 deregulation in NSCs that may contribute to gliomagenesis. They found that deregulated Id2 expression in differentiating NSCs resulted in the accumulation of proliferative OPCs. Using a novel mouse model, they extended these studies to show that OPC enriched cultures cells gave rise to high-grade glioma in a PDGF-rich microenvironment. Mechanistically, they found that OPC accumulation was associated with increased Olig2 expression resulting from inhibition of Hey1, which they identified as a novel direct transcriptional repressor of Olig2. These findings identify an oncogenic function for Id2 and a novel mechanism for OPC maturation arrest in the development of glioblastoma multiforme, and suggest that Id2 and the pathway it mediates in fostering the formation of OPCs may be a novel target(s) for the development of new therapeutic interventions.

Sincerely,

Mark A. Israel, M.D.
Director, Norris Cotton Cancer Center
Professor of Pediatrics and Genetics
Geisel School of Medicine at Dartmouth

The Andy Fund: Progress Report for 2011-2012

Dr. Brenton Paoella, Ph.D. and Dr. Mark A. Israel, M.D.
Departments of Pediatrics and Genetics
Norris Cotton Cancer Center
Geisel School of Medicine

Project Title:

Poor oxygenation of glioblastoma tumor tissues promotes enhanced malignancy through a molecular pathway in which tumor stem cell replication regulated by HIF-1α mediated STAT3 activation.

Id proteins promote proliferation and influence cell-fate determination in the central nervous system. In particular, Id2 is important for oligodendroglial differentiation promoting the expansion of OPCs. Also, Id2 has been implicated as a marker of OPCs. Cultured NSCs provide an opportunity to critically assess oligodendrocytic differentiation *in vitro*, and we examined the effect of Id2 loss on the expression of oligodendrocyte markers during NSC differentiation.

Id2 expression promotes NSC differentiation along the oligodendrocytic lineage while blocking maturation of oligodendrocyte precursor cells

NSC from WT and *Id2*$^{-/-}$ mice were differentiated *in vitro* and examined for markers of the major neural lineages including the astrocytic marker, GFAP, and the neuronal marker, Tuj1. These observations are consistent with previous *in vivo* findings from our lab indicating that the loss of Id2 increased the differentiation of NSCs along the astrocytic lineage and was required for normal numbers of cells expressing the neuronal marker Tuj1. To examine oligodendrocyte maturation in these cultures, we evaluated the expression of both a marker of the oligodendroglial lineage that is expressed early and throughout subsequent stages of differentiation, Olig2, and a "late" marker expressed on post-mitotic oligodendrocytes prior to myelination, O4. After 8 days of culture under differentiation conditions (see Methods), cultures of *Id2*$^{-/-}$ NSCs had dramatically fewer cells expressing either Olig2 or O4 than did cultures of differentiated WT NSCs (Figure 1A and 1B). The finding of decreased numbers of Olig2 and O4 expressing cells in differentiating *Id2*$^{-/-}$ NSC was observed at multiple other time points between 3 and 15 days of differentiation (data not shown). Our observation of decreased Oligo2 expression, as measured by immunoblotting (Figure 1C), in cultures of differentiating *Id2*$^{-/-}$ NSC, was consistent with these findings.

To determine if Id2 expression was sufficient to restore Olig2 expression to differentiated *Id2*$^{-/-}$ NSCs, we cultured WT, *Id2*$^{-/-}$ and *Id2*$^{-/-}$ NSCs in which Id2 expression had been restored (*Id2*$^{-/-}$ pBMN-Id2) in differentiation media. As before (Figure 1A), we found that WT NSC cultures contained numerous Olig2-positive cells, while *Id2*$^{-/-}$ NSC cultures contained significantly fewer Olig2-positive cells (Figure 1F). However, stable expression of *Id2* in *Id2*$^{-/-}$ NSCs following transfection of pBMN-Id 2 resulted in cells expressing Olig2 in cultures incubated under differentiation conditions (Figure 1F and 1G), although Olig2 is not expressed in NSC prior to *in vitro* differentiation (data not shown).

Since Id2 typically functions to inhibit the transcription of other proteins and had previously been shown to inhibit oligodendroglial differentiation, the finding that *loss* of Id2 resulted in decreased oligodendrocyte differentiation (Figure 1A) and decreased Olig2 expression (Figure 1A) was unexpected, and suggested that the role of Id2 in the differentiation of oligodendrocytes from multipotent NSCs may be more complex that previously proposed. To examine further the effect of dysregulated Id2 expression on oligodendrocyte differentiation we used recombinant *Id2* retroviral vectors to prepare multiclonal NSC cultures that constitutively expressed Id2. As expected, *in vitro* differentiation of WT NSCs resulted in numerous Olig2^{+} and O4^{+} cells indicating easily detectable oligodendroglial differentiation of cells within these cultures (Figure 1D and 1E). In contrast, NSCs constitutively expressing Id2 gave rise to significantly increased numbers of Olig2^{+} cells compared to WT NSCs, but no O4^{+} ells (Figure 1D and 1E) suggesting inhibition of differentiation in this lineage between the OPC marked by Olig2 expression and the more mature oligodendrocyte marked by O4. This finding is consistent with data indicating that Id2 can inhibit oligodendrocytic differentiation, but suggests that Id2 can also increase the number of precursor cells, OPCs, expressing Olig2.

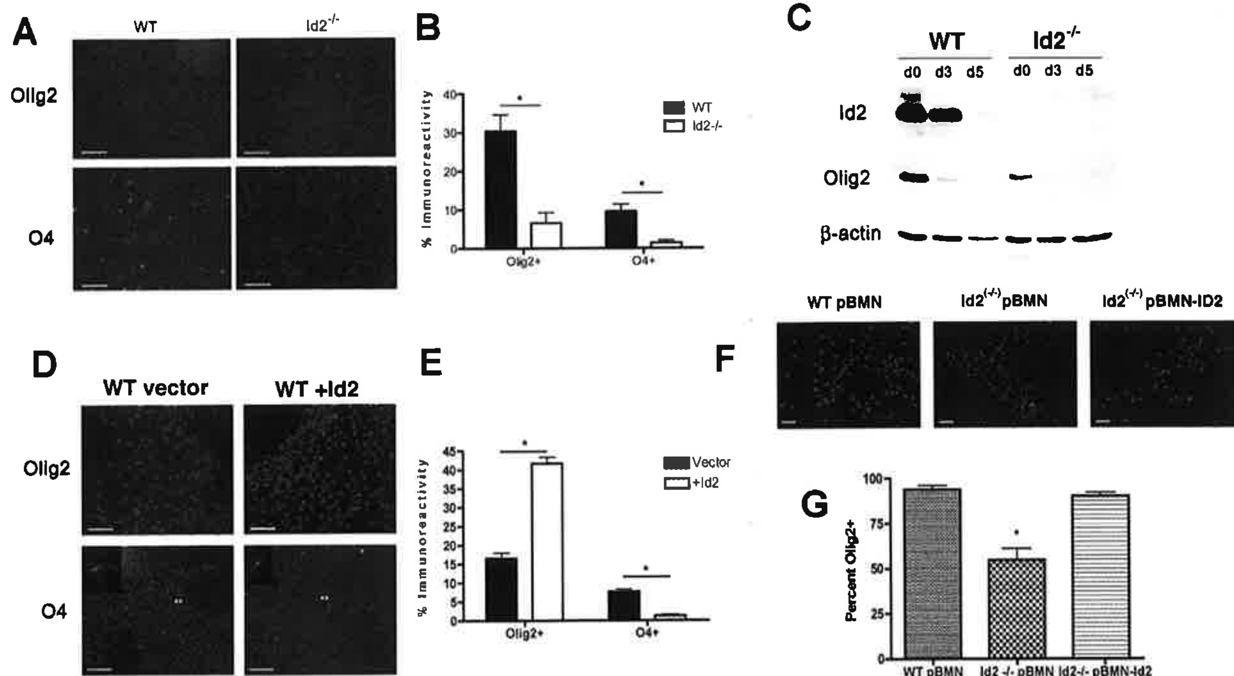

Figure 1. Id2 promotes Olig2 expression during NSC differentiation. (A) Olig2 and O4 immunofluorescence in WT and Id2-/- NSCs 8 days after serum-induced differentiation. Representative images from 3 independent experiments. Olig2 images scale bars represent 25 μm, O4 images scale bars represent 50 μm. (B) Quantification of immunocytochemistry in (A), $p<0.01$. Data represent mean +/- SD from 3 independent experiments. (C) Id2 and Olig2 western blot from WT and Id2-/- NSCs during serum-induced NSC differentiation time course. (D) Immunofluorescent staining of Olig2 and O4 following 8 days of serum-induced differentiation of NSCs expressing exogenous Id2 or the vector alone. Olig2 images scale bars represent 25 μm, O4 images scale bars represent 50 μm. Asterisks indicate the location of cells shown at higher magnification in the inset. (E) Quantification of immunocytochemistry in (D), $*p<0.05$. Data represent mean +/- SD from 3 independent experiments. (F) Immunofluorecent Olig2 staining in WT, Id2-/- and Id2-/- pBMN-Id2 rescued NSCs in OPC differentiation conditions. Scale bars represent 25 μm. (G) Quantification of Olig2 immunostain in (I). Data represent mean +/- SD of a representative experiment performed in duplicate and quantified from 8 random microscopic fields, $*p<0.05$.

NSCs expressing dysregulated Id2 are tumorigenic in a PDGF-rich microenvironment

OPCs are a cell of origin for glioma (REF). Based on the observation that Id2 is expressed in human glioma (REF) and our finding that Id2 expression enhanced the production of OPCs (Figure 1) and sustained PDGF responsiveness, we sought to assess whether Id2-mediated alterations in oligodendrocytic differentiation combined with enhanced PDGF stimulation could promote glioma *in vivo.* To expose NSCs over-expressing Id2 to a PDGF-rich microenvironment we utilized a mouse model of glioma previously reported by our laboratory in which the human PDGF-beta (hPDGFB) gene is over-expressed in the CNS under control of the glial fibrillary acidic protein (GFAP) promoter (C57BL/6-Tg(GFAP/hPDGFB)) (Hitoshi et al. 2008). Using syngeneic primary WT NSCs, we prepared NSCs infected with two retroviruses resulting in stable NSC lines constitutively expressing *Id2* along with GFP for *in vivo*

tracing (Id2-GFP). We inoculated these NSCs, and WT NSC expressing only GFP (WT-GFP), into the ventricular zone of neonatal WT or C57BL/6-Tg (GFAP/hPDGFB) (Figure 2A). When Id2-GFP or WT-GFP NSCs cells were injected into WT recipient animals, GFP positive cells could be readily detected in needle tracts, the rostral migratory stream, and the olfactory bulb of asymptomatic mice confirming that injected NSCs survived intraventricular injection, engrafted within the SVZ, and participated in adult neurogenesis. Only NSCs expressing the Id2 transgene (Id2-GFP) inoculated into mice over-expressing PDGF-B in the CNS (C57BL/6-Tg (GFAP/hPDGFB)) led to the development of brain tumors. We found that of 28 animals injected with Id2-GFP NSCs 14 animals that were sufficiently disabled to sacrifice (Figure 2). Each of these animals had an easily detected invasive, malignant brain tumor that expressed GFP (Figure 2B, 2C, 2D). Each of these tumors occurred ipsa-lateral to the injection site. The observation of high-grade glioma in hPDGFB over-expressing animals inoculated with Id2 over-expressing NSCs was in stark contrast to WT littermates that received identical engraftments but never developed CNS tumors (Figure 2C). hPDGFB over-expressing animals that received inoculations of WT-GFP cultures also never developed tumors (Figure 2D). These data indicate that elevated *Id2* expression can enhance tumorigenesis or neural precursor cells in a PDGF-rich microenvironment.

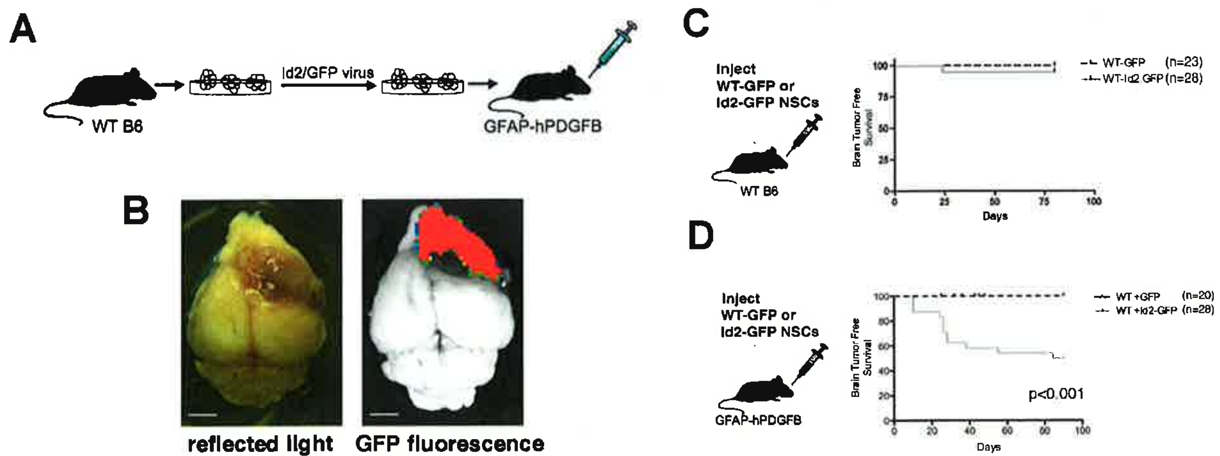

Figure 2: Id2 is gliomagenic in a PDGF rich microenvironment. (A) Schematic of orthopic NSC engraftment into GFAP-hPDGFB animals. (B) Representative image of Id2-driven tumor visualized using reflected light and GFP fluorescence. Scale bars represent 2.5 mm. (C) Kaplan-Meier survival curve of WT-GFP or Id2-GFP NSCs engrafted into WT mice. (D) Kaplan-Meier survival curve of WT-GFP or Id2-GFP NSCs engrafted into GFAP-hPDGFB mice, $p<0.001$.

OPCs are tumorigenic in a PDGF-rich microenvironment

To more closely examine the importance of OPCs in the initiation of glioma occurring in this PDGF-dependent model system, we directed NSC differentiation along the oligodendroglial lineage using culture conditions well characterized to enhance such differentiation and injected these cultures orthotopically into C57BL/6 Tg(GFAP/hPDGFB) mice as described above (Figure 3). We validated the enrichment of OPCs in these cultures of Id2-GFP NSCs by identification of Ng2/Ki67 double-positive cells (data not shown) and a greatly enhanced fraction of cells expressing PDGFRa, a marker of OPCs. Engraftment of these Id2-GFP OPC-enriched cells into Tg(GFAP/hPDGFB) mice resulted in significantly increased tumorigenicity, a shorter latency period, and decreased survival time compared to differentiated WT NSCs and or Id2-GFP NSCs (Figure 3B). These data also provide strong support for the work of others suggesting that OPCs are a cell of origin for glioma.

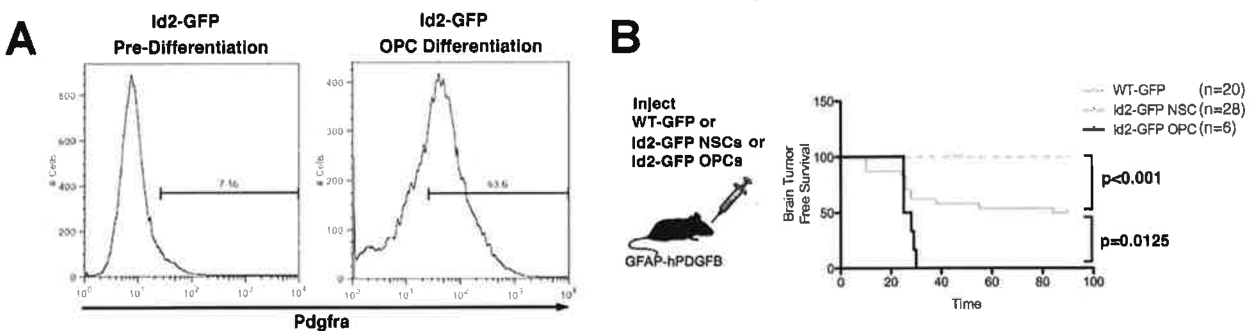

Figure 3: Enrichment for OPCs increases Id2/PDGF-dependent tumor penetrance. **(A)** Live cell PDGFRa-labeling detected by flow cytometry in NSCs differentiated for 3 days under OPC differentiation conditions. Major peak represents PDGFRa-positive cells. **(B)** Kaplan-Meier survival plot from Id2-GFP NSCs after OPC directed differentiation compared to undifferentiated WT–GFP and Id2-GFP NSCs as initially presented in Figure 2.

Sustained expression of the OPC marker *Id2* occurs as a consequence of *p53* inactivation in adult-tissue derived subventricular zone neural stem cells (NSCs). Dr. Paoella has shown that sustained Id2 expression alone is sufficient to enhance the formation of OPCs from differentiating NSCs. When exposed to a platelet derived growth factor (PDGF)-rich microenvironment in vivo, NSCs constitutively expressing Id2 could initiate gliomas. Glioma latency and penetrance could be decreased by enrichment of Id2 over-expressing cells for OPCs and tumors contained numerous OPCs that were resistant to differentiation. These data identify a novel mechanism of OPC specification and identify a role for Id2-mediated expansion of OPCs in the development of glioma. Such results identify a pathway of importance for the development of targeted therapies for glioma and will likely be relevant to other tumors in which p53 inactivation contributes to malignancy.