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CONTRACTING ORGANIZATION: University of Oregon, Eugene, OR, 97403

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14. ABSTRACT
Medulloblastoma is the most common malignant pediatric brain tumor. Granule neuron precursors (GNPs) in developing cerebellum proliferate exponentially, and the misregulation of which has been linked to medulloblastoma formation. GNPs are unipotent and only give rise to granule neurons. However, using MADM, a mouse genetic mosaic model, we found that medulloblastoma contain glial cells that are trans-differentiated from transformed GNPs. Our preliminary data showed that specific ablation of tumor glia without harming tumor GNPs led to complete tumor remission, suggesting a tumor-supporting role for these trans-differentiated glia. Here we propose to analyze the tumor "social behavior" with two specific aims. First, we will investigate the tumor regressing process at the cellular level *in vivo*, and determine therapeutic parameters of glial ablation for medulloblastoma treatment. Second, we will investigate the molecular basis for glia-tumor crosstalk that sustains the tumor growth. In the past year, we have completed most of the work proposed in aim 1. Our data showed that the glial-ablation treatment not only results in complete remission free of relapses, but also remains quite effective for mice with late-stage tumors. These findings are particularly encouraging since they point to great potentials in targeting glial cells for treating medulloblastoma in human patients.

15. SUBJECT TERMS
Medulloblastoma, granule neuron precursors (GNPs), tumor-derived glial cells, niche support, genetic ablation

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INTRODUCTION:

Medulloblastoma is the most common malignant pediatric brain tumor, resulting from the deviation from normal process of cerebellar development. Immediately after birth, granule neuron precursors (GNPs) on the surface of developing cerebellum proliferate exponentially. The misregulation of GNP proliferation has been linked to medulloblastoma formation. Fate mapping experiments demonstrated that GNPs are unipotent and only give rise to granule neurons. However, using MADM, a mouse genetic mosaic model with lineage tracing capability, we found that medulloblastoma contain glial cells that trans-differentiate from malignantly transformed GNPs. Our preliminary data showed that specific ablation of tumor glia without harming tumor GNPs led to complete tumor remission, suggesting a critical role for these trans-differentiated glia in supporting the growth of tumor GNPs. Here we propose to perform detailed mechanistic analyses for the tumor "social behavior" with two specific aims. First, we will investigate the tumor regressing process at the cellular level *in vivo*, and determine therapeutic parameters of glial ablation for medulloblastoma treatment. Second, we will investigate the molecular basis for glia-tumor crosstalk that sustains the tumor growth.

BODY:

To gain full mechanistic understandings of the "social behavior" in medulloblastoma, two aims of this grant were subdivided into six objectives. 1) Obtain regulatory approval for IACUC protocol to work with mice according to all animal welfare laws and regulations, followed by setting up a mouse breeding colony to supply tumor-bearing mice for proposed experiments [task 1-2]; 2) Perform detailed analysis of cellular events during glial ablation-induce tumor regression [task 3]; 3) Evaluate treatment efficacy to medulloblastoma by ablating glial cells [task 4-6]; 4) Sub-fractionate tumor GNPs and glial cells and establish co-culture assays [Task 7]; 5) Identify candidate genes responsible for glial support to tumor GNPs [task 8-9]; 6) Functional validation of candidate genes using both cell culture and allografting assays [task 10-12]. Among them, we have completed or made significant progression for tasks 1-9 in the first year. We also made very good progresses into task 10 that was originally proposed to be performed in the second year of the project.

For **task 1**, we have worked closely with the IACUC committee at our university, and successfully gone through the regulatory review processes for animal studies to get the approval in the beginning of the funding period prior to starting our animal work.

For **task 2**, we've set up a well-organized mouse colony to generate MADM-based medulloblastoma model with GFAP-TK (referred to as medullo-TK mice hereafter). We've optimized the mouse stocks and established the breeding routines that consistently generate a steady supply of medullo-TK mice for treatment schemes proposed in this grant.

For **task 3**, we performed histological analysis of cellular events during glial ablation-induced tumor regression. After injecting GCV in medullo-TK mice at P35, we first analyzed tumor brains 6-hour post treatment. For five individual brains that we examined, GFP+ tumor cells were still visible in all of them (Figure 1A shows a representative

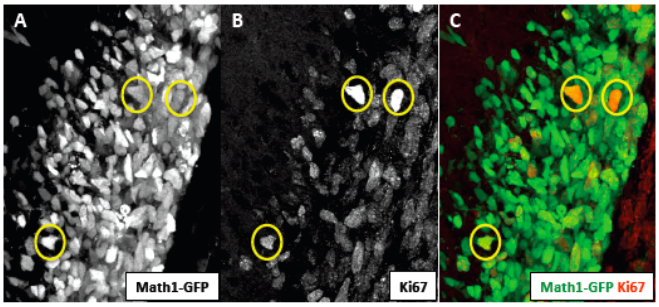

Figure 1. Histological analysis of tumors in medullo-TK mice 6-hour post GCV-injection showed no signs of regression medulloblastoma.

image). When we stained for Ki67, almost all of these cells are still proliferating, evidenced by the positive staining of Ki67 (Figure 1B & C). Therefore, at this early stage, tumor cells have not started reacting to the consequences of glial ablation.

Based on this finding, next we decided to examine a set of brains at 1-day post treatment. Interestingly, at this time point we often observe the progressive death of tumor cells. In one representative brain, there was a massive loss of Math1+ GNPs in the region outlined by the red margin (Figure 2A). However, in other regions there were still significant amount of GFP+ tumor GNPs (Figure 2A). To investigate whether these tumor cells are still proliferative or going through apoptosis, we stained the tissue slice with Ki67 and TUNEL, respectively. While these cells were Ki67 negative indicating the cease of proliferation (data not shown), most tumor GNPs stained positive for TUNEL (Figure 2B). Interestingly, while many cells undergoing apoptosis were green, some of them were neither glial cells (TK negative) nor tumor GNPs (GFP negative, Figure 2C). The identify of these cells were serendipitously revealed while we co-stained Annexin V (another apoptotic marker) with a GFAP antibody raised in the mouse, which can cross-reacting with mouse vasculature during secondary antibody detection (Figure 2D). Further co-staining of CD34 (endothelial cell marker) and Annexin V confirmed the death of vasculature in glia-ablated tumor mass (Figure 2E). Data from this series of experiments showed that while tumor cells still proliferate 6-hour post GCV treatment, by 1-day post treatment, massive cell death has begun. It is interesting that some tumor regions seem to be more susceptible than other areas, and that the death of tumor cells even led to apoptosis of vasculatures within the tumor mass, an effect could be exploited for medulloblastoma therapy if more data support this finding.

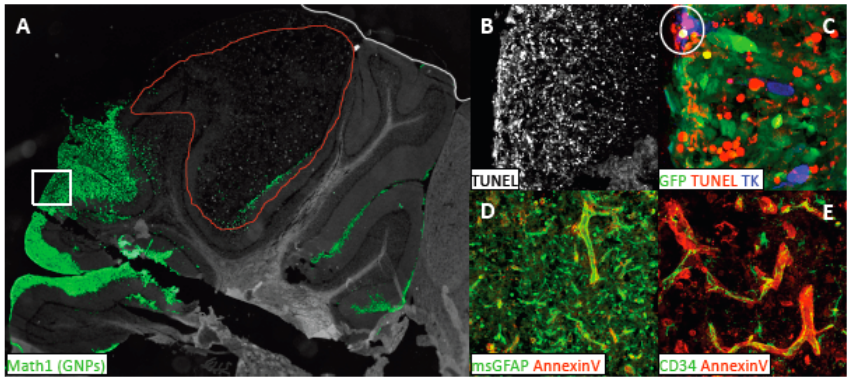

Figure 2. Histological analysis of tumors in medullo-TK mice 1-day post GCV-injection. A) Low magnification image shows that part of the tumor mass regressed while some GFP+ tumor cells persisted in other regions. B) TUNEL staining revealed massive cell death in remaining GFP+ tumor cells. C) Both GFP+ tumor cells and GFP- by-standing cells went through apoptosis. D-E) Endothelial cells in the tumor mass went through apoptosis after glial ablation.

Task 4 and task 5 is to assess the possibility of relapses of glia-ablated tumors after the initial regression observed in the preliminary data of our proposal and in Figure 2 of this report. Specifically, in **task 4**, we stained remnant tumor GNPs with Ki67 and NeuN at 7-day post treatment to see whether they remain proliferative or have fully differentiated (Figure 3). To make sure that the cells that we examined were dividing tumor cells prior to the GCV treatment, we co-injected BrdU with GCV. While we did observe

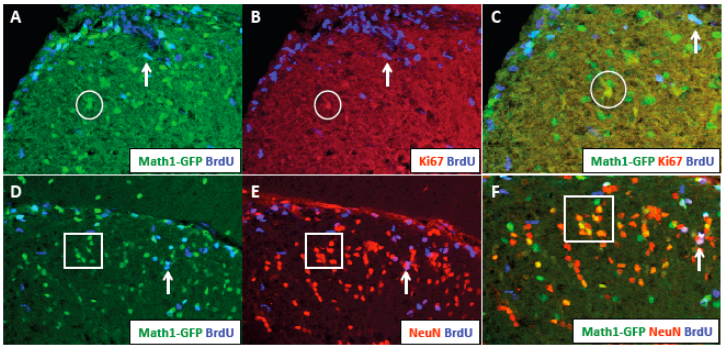

Figure 3. Tumor cells at 7-day post treatment undergo massive neuronal differentiation with few dividing mutant GNPs remaining. (A-C) Few remaining tumor cells are Ki67+ although many of them were dividing at the time of treatment and stained positive for BrdU. (D-F) Many remaining tumor cells are NeuN+, indicating that they are fully differentiated into neuronal fate.

some BrdU positive tumor cells at 7-day post treatment, almost all of them are Ki67 negative, suggesting that they are no longer proliferative (Figure 3, A-C). When we co-stained these cells with NeuN, a mature neuron marker, we found many tumor GNPs have fully differentiated into neurons, further supporting the notion that they lost the relapsing capability (Figure 3, D-F). In **task 5**, we investigated whether GCV-treated, regressed tumors could still relapse by waiting an extended period prior to dissection. We originally planned to complete this task between the 10th and 20th month due to the need for newly generated mice for other tasks. Fortunately, our optimized breeding colony generated mice very efficiently, allowing us to work on this task before the planned dates. In this experiment, we treated medullo-TK mice with GCV at P35, then waited three to four months, at least twice the lifespan of control, untreated mice, to monitor the relapse potentials in these mice. We found that there were no tumors in any mice in the experimental group, suggesting that the treatment leads to complete remission free of any relapses. When we sectioned these brains, we often found cluster of green cells under the meninges, which are most likely remnant cells at original tumor locations (Figure 4A). When we

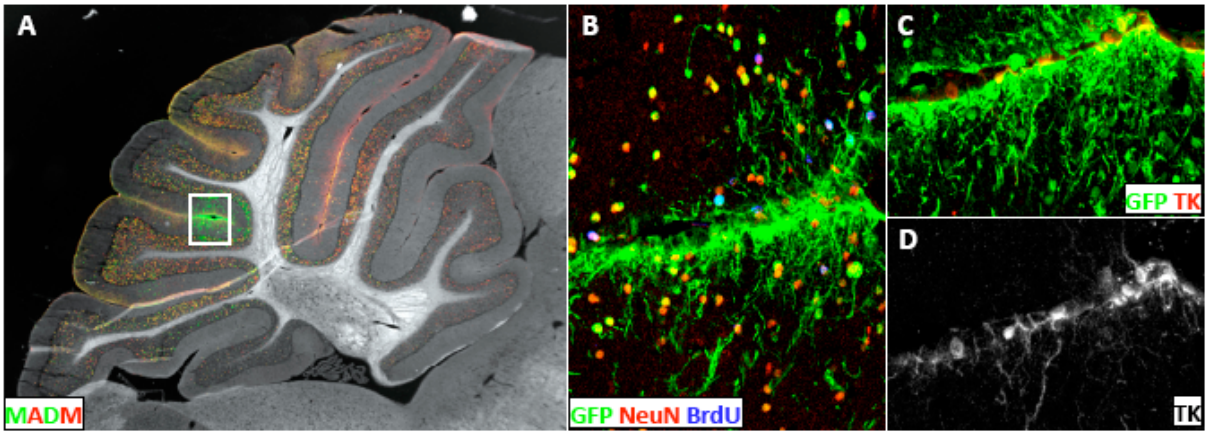

Figure 4. Glia ablation via GCV treatment leads to complete remission of tumors. A) Treated medullo-TK cerebellum showed relatively "normal" tissue organization 3-month following treatment. B) Higher magnification of boxed region A, showing remnant mutant GFP+ cells. Most mutant cells have differentiated into NeuN+ neurons (yellow), some are traced from the treatment period with BrdU (purple) demonstrating that differentiation can occur very early after treatment. C) Remnant tumor glial cells appear not to contribute to further malignancy. D) Nuclei of GFAP-TK+ tumor glia, from panel C, remain at remission stages.

co-stained these cells with NeuN, we found that many of these cells are fully differentiated neurons (Figure 4B). Some of them still retained BrdU that was co-injected during the GCV treatment months ago, suggesting that these cells withdrew from cell cycle completely after the glial ablation (Figure 4B). We also found some remnant tumor cell-derived GFP+ glial cells that evaded the ablation (Figure 4C-D). However, they appear to be harmless since they are neither proliferating nor supporting tumor growth at this time. Overall, the results from these two tasks are very encouraging, suggesting that the treatment paradigm could be highly effective for human patients if it advances into clinics.

The findings from the above two tasks made **task 6** particularly important. Since most of the time human patients come to the clinics when neurological symptoms start to appear, at which time tumor sizes tend to be quite large. In this task, we Injected GCV in medullo-TK mice at different time points to determine the treatment efficacy of glial ablation during different tumor progression stages. We originally planed to perform this task in the second year. But with the efficient management of our mouse colony, we performed these experiments within the first year. In total we examined four experimental groups with at least five mice per group: 1) P25-35 injection of GCV into Medullo-TK mice, when tumors are generally small to medium in size; 2)

P45 injection of GCV into Medullo-TK mice, when tumors are generally very large that often cause mouse death in 1-2 weeks; 3) Medullo-TK mice without any GCV injections; 4) Medullo mice without TK and injected with GCV to control for unlikely tumor-shrinking activity of GCV on its own. We found that while mice from two control groups died at around 2-month of age, GCV-treated mice lived for five months or longer (Figure 5). While all Medullo-TK treated at P25 to P35 lived symptom-free to more than five-month of age, two out of eight Medullo-TK mice treated at P45 died around 2-month of age but the remained six mice lived symptom-free for many months (Figure 5). We postulate that the death of two mice with late treatment could be caused by irreversible neurological damages caused by tumors prior to the treatment, and that large tumors could be treated as long as the brain damage is not permanent. This encouraging data suggest that even treating mice with large tumors could be very effective.

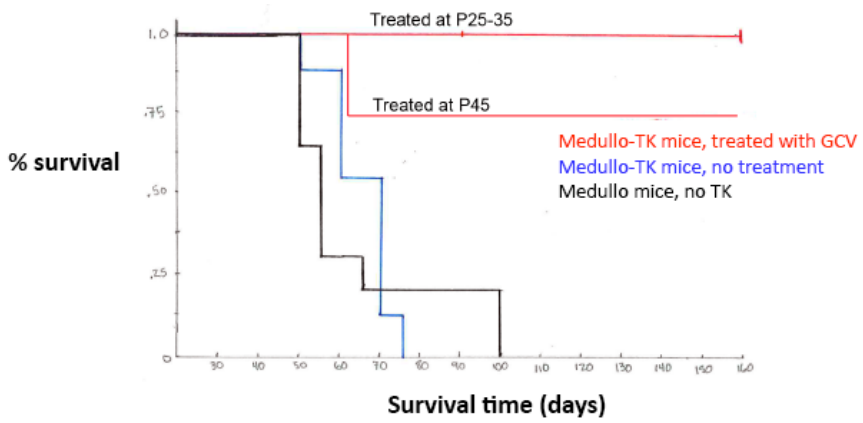

Figure 5. Glial ablation treatment at different tumor progression stages appears to be very effective.

While it is important to understand the effectiveness of treatment in the mouse model, it is as important to understand the molecular mechanisms for tumor-derived glial cells to support tumor GNP growth. To achieve this goal, we first need to complete **task 7** to sub-fractionate glial cells and tumor GNPs. We have made great progresses according to the original plan to complete this task by the middle of the second year. By adapting and optimizing a Ficoll-gradient based centrifugation method, we have successfully isolated tumor GNPs to high purity, free of glial cell contamination (Figure 6A-B). On the other hand, although efficient isolation and culturing of wildtype glial cells have been achieved, we are still in the process of protocol adjustment for isolate glial cells from the tumor mass due to more complex cellular composition and probably distinct extracellular matrix proteins in tumors. A recent discussion with a postdoc in our collaborator Ben Barres' lab pointed to a few subtle but critical procedural issues, such as the extreme gentleness during cell dissociation and steps to optimize antibody-based affinity capture technique, etc.

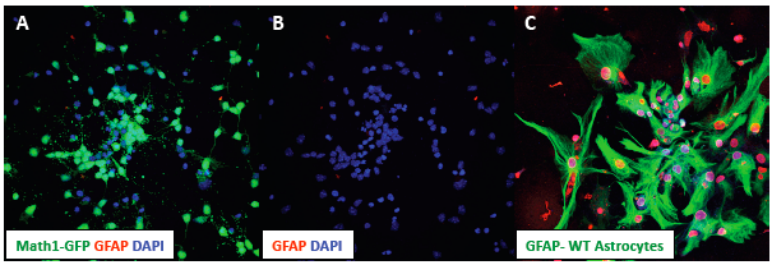

Figure 6. Sub-fractionate glial cells and GNPs from mixed tumor mass. (A-B) Tumor GNPs can be sub-fractionated to high purity, free to GFAP+ glial cell contamination. (C) Successful culturing of WT glial cells.

The understanding of molecular mechanisms also requires us to identify candidate genes. In **task 8** we proposed to use *in situ* analysis of candidate trophic factors in tumor glial cells. For this task, we recruited a new graduate student Kate Karfilis to first refine the technique to be able to assign the expressed genes to a particular cell type. Because her research interest is

tumor angiogenesis, she used Angiopoietin 1 (Ang-1) as her candidate probe. In the past year, she first conquered one major hurdle caused by the disintegration of tumor tissues during the high-temperature incubation process. Then she designed multiple probes to examine the expression pattern of Ang-1 in developing and tumor-bearing cerebellum. She found that Ang-1 is highly expressed in Purkinje neurons and Bergmann glial cells based on their cell body location and possibly protoplasmic astrocytes in the inner granule layer (Figure 7, top panels). She also found high-level of Ang-1 expression in tumors (Figure 7, bottom panels), although the exact cell type(s) that express the factor is still unclear. She is currently developing multi-color *in situ* based on the protocol published by Peter Mombaerts' group [1] so that she could soon co-stain cell identity marker (such as GFAP or Aldh1L1 for glial cells, or Math1 for GNPs) with growth factors. We expect to make this important technical breakthrough for our team early in the second project year since it is the best way to examine cell-type specific expression of secreted factors without the need of isolating individual cell types to high purity.

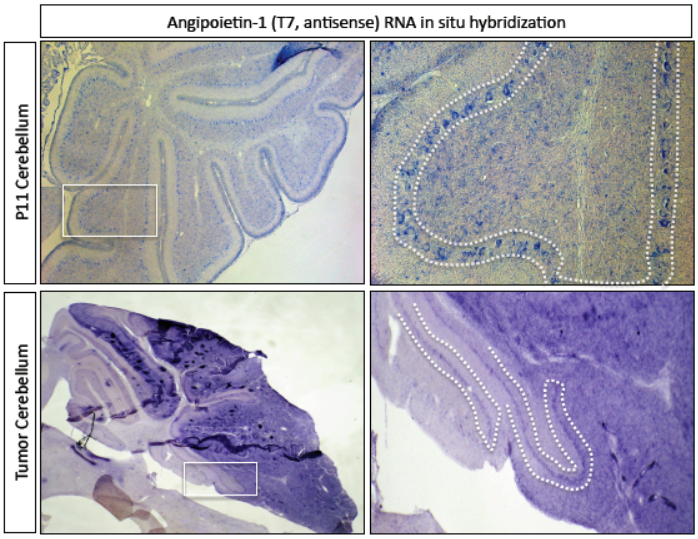

Figure 7. In situ hybridization of Ang-1 in normal developing cerebellum (top panels) and medulloblastoma (bottom panels).

In addition to the candidate gene approach above, in **task 9** we proposed to analyze the whole transcriptome for a global view of gene misregulation in both tumor GNPs and glial cells. We have performed pilot experiment with purified tumor GNPs in comparison to control WT GNPs. Rather than using microarray analysis, we decided to use RNA sequencing with the NexGen sequencer available to us at our local genomic facility. Initial data showed great dynamic range. We will soon analyze data in great depth and with statistical significance after we collect more samples for analysis. The purification of glial cells in task 7 in the coming year would be very important to provide materials for the molecular profiling of these cells.

While identifying gene expression changes is an important first step to understand molecular events in the tumor development, it is even more important to master techniques for molecular perturbation so we could investigate cause-and-consequence problems. With that in mind, we advanced our work along the line of **task 10**, which was originally planned for the work in year 2. We have successfully constructed lentiviral vectors and optimized viral production and infection procedures. After the successful purification of tumor GNPs, we tested the infection on them and got a ~40% of efficiency (Figure 8). Although this

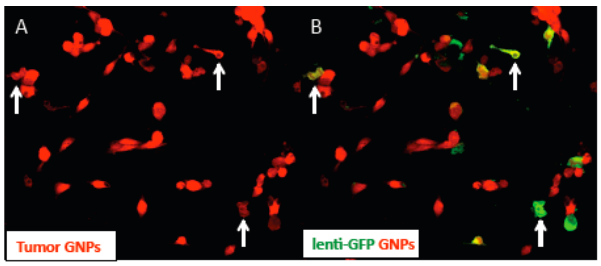

Figure 8. Infection of tumor cells with GFP-expressing lentiviral vector. A) Purified tumor GNPs from red MADM tumor. B) Expression of GFP in infected tumor GNPs.

efficiency is sufficient for many experiments, we will further optimize the procedure in the coming year to increase the infection efficiency for more challenging experiments.

KEY RESEARCH ACCOMPLISHMENTS:

Since this is the first year of a three-year grant, the accomplishments are mixed between scientific findings and technical breakthroughs.

- We observed a fast collapse of tumor mass by analyzing cellular events along the timeline after the GCV treatment for glial ablation.
- We demonstrated that glial ablation induced tumor remission is complete and free of relapses.
- We demonstrated that glial ablation even in late stage tumors remain effective.
- We now can isolate tumor GNPs to high purity, and made significant progresses in optimizing the procedure to purify tumor glial cells.
- We have started performing RNA seq with purified tumor GNPs.
- We have optimized the in situ protocol and observed the upregulation of Ang-1 in the tumor mass.
- We have established a lentiviral-based system that can efficiently infect tumor GNPs.

REPORTABLE OUTCOMES:

- Selected Speaker at Glia in Health and Disease, Cold Spring Harbor Laboratories – July 2012. Title: “Medulloblastoma Builds Its Own Glial Niche.”
- Selected Speaker at Models and Mechanisms of Cancer, Salk Institute – August 2011. Title: “Mosaic Analysis with Double Markers (MADM) in Mice Reveals Community Building Behaviors in Medulloblastoma.”

CONCLUSION:

In the past year, we have completed most of the work proposed in aim 1 and gained a firm grasp of the treatment effect of glial ablation in our mouse model for medulloblastoma. Our data showed that the glial-ablation treatment not only results in complete remission free of relapses, but also remains quite effective for mice with late-stage tumors. These findings are particularly encouraging since they point to great potentials in targeting glial cells for treating medulloblastoma in human patients. However, we did notice that tumor cells went into massive cell death within 1-day of GCV treatment, raising the possibility that the GFAP-TK could have leaky expression in tumor GNPs that results in direct killing. The fact that GFAP promoter often is active in neural stem cells heightens our concerns even further. Considering the importance of this potential caveat, we plan to construct an Aldh1L1-tTA mouse (or a similar transgenic line that express tTA specifically in astroglial cells). This transgenic line can be used in combination with a TetO-DTA line that is currently available in our lab to ablate glial cells specifically. If we could reach similar or identical findings to the work done with GFAP-TK, we would be much more confident to pursue translational direction to apply our findings to human patients.

REFERENCES:

1. Protocols for two- and three-color fluorescent RNA in situ hybridization of the main and accessory olfactory epithelia in mouse. Ishii T, Omura M, Mombaerts P. J Neurocytol. 2004 Dec;33(6):657-69

APPENDICES: meeting abstracts of our selected talks (5 pages)

Microglial voltage-gated proton channel, Hv1, contributes to brain damage in ischemic stroke
Long-Jun Wu, Gongxiong Wu, Edward P. Feener, David E. Clapham.
Presenter affiliation: Harvard Medical School, Boston, Massachusetts. 131

TGF-β signaling regulates complement C1q expression and developmental synaptic refinement
Allison M. Rosen, Arnaud Frouin, Alexander Stephan, Ben A. Barres, Beth Stevens.
Presenter affiliation: Children's Hospital Boston, Boston, Massachusetts; Harvard Medical School, Boston, Massachusetts. 132

Bone marrow transplant arrests disease in an Mecp2-null mouse model of Rett syndrome—Microglia as potential key players in Rett
NC Derecki, JC Cronk, J Kipnis.
Presenter affiliation: University of Virginia, Charlottesville, Virginia. 133

SUNDAY, July 22

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

MONDAY, July 23—9:00 AM

SESSION 10 ROLE OF GLIA IN CNS INJURY AND DISEASE

Chairperson: **R. Armstrong,** Uniformed Services University of the Health Sciences, Bethesda, Maryland

Phenotypic conversions of "protoplasmic" to "reactive" astrocytes in Alexander disease
Alexander Sosunov, Eileen Guilfoyle, Xiaoping Wu, Guy McKhann, James E. Goldman.
Presenter affiliation: Columbia University, New York, New York. 134

MEDULLOBLASTOMA BUILDS ITS OWN GLIAL NICHE

Brit Ventura, Kate Karfilis, Kelsey Wahl, Hui Zong

University of Oregon, Institute of Molecular Biology, Eugene, OR, 97403

Understanding the interactions between tumor cells and their microenvironment are paramount for the design of novel treatments. However, conventional research methods, including mouse cancer models, lack the in vivo single-cell resolution to tease apart tumor versus niche cell types for mechanistic insights. To circumvent this problem, our lab makes use of a novel mouse genetic system termed MADM (Mosaic Analysis with Double Markers) to model cancers. Starting with a mouse heterozygous for a tumor suppressor gene (TSG), MADM can generate sporadic mutant cells that are null for candidate TSG(s) with unequivocal GFP labeling, enabling us to trace the lineage of mutant cells and distinguish them from other niche cells.

In this study, we used MADM to model medulloblastoma, the most prevalent type of pediatric brain tumors. It is known that such tumors, especially the desmoplastic subtype relying on Shh pathway hyperactivation, originate from granule neuron precursors (GNPs). It is puzzling that although GNPs are unipotent toward the granule neuron lineage, the tumor mass contains many glia. Moreover, medulloblastomas are often highly vascularized even though GNPs are known not to produce angiogenic factors. Using GNP-specific Math1-Cre, the MADM model generated GFP+ p53-null GNPs in a heterozygous patched mutation background, resulting in fully penetrant cerebellar tumors. Although the majority of GFP+ cells in the tumor mass resemble GNPs, a closer look revealed green cells with large cell bodies, reminiscent of astroglial morphologies. We further confirmed their glial identity by staining with multiple astrocyte markers. Importantly, these cells are GFP+ suggesting that they are derived from mutant GNPs. It's surprising since normal GNPs are known not to give rise to any glial cells. To investigate the molecular mechanisms that enable mutant GNPs to generate glia within the tumor, we performed transcriptome analysis of tumor GNPs in comparison to wildtype GNPs. This revealed increased expression of transcription factors that are normally restricted to a small time window during embryonic GNP lineage development. It suggests that tumorigenic transformation may lead to a reversion of the fate of some tumor GNPs to an earlier developmental stage with broader potentials. In addition to investigating the mechanisms of trans-differentiation, we are also analyzing the functional roles of tumor glia, in particular their roles in inducing angiogenesis to support tumor GNPs.

In summary, our MADM-based medulloblastoma model seems to have revealed a community building behavior of tumor cells, through which some of them convert their fate to non-proliferative glial cells, which in turn provide cues for the establishment and maintenance of the tumor microenvironment.

Saturday, August 13 - 9:00 am

SESSION VI: TUMOR MICROENVIRONMENT
Chair: MARTINE ROUSSEL

46 Martine Roussel
St. Jude Children's Research Hospital
Role of Myc Genes in Pediatric Medulloblastoma

47 P. Britten Ventura, Kate Karfilis, Peter Kim, Kelsey Wahl, and Hui Zong
University of Oregon
Mosaic Analysis with Double Markers (MADM) in Mice Reveals Community Building Behaviors in Medulloblastoma

48 Sourav Ghosh, Yael Kusne, Anthony S. Perry, Maurice Jabbour, Edward Mandell, Michael E. Berens, Joseph C. Loftus, and Elisabeth J. Rushing
The University of Arizona, Tucson
aPKC-dependent EGFR and NF-kappaB Signaling Cooperate to Promote Glioblastoma Invasion

49 Vicki A. Sciorra, Michael A. Sanchez, Emily Ho, and Andrew E. Wurmser
University of California, Berkeley
Glioma Cells Participate in the Formation Tumor Capillaries by Competitively Inducing Endothelial Cell Death

Mosaic Analysis with Double Markers (MADM) in Mice Reveals Community Building Behaviors in Medulloblastoma

P. Britten Ventura, Kate Karfilis, Peter Kim, Kelsey Wahl, and Hui Zong
Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

Medulloblastoma is the most common pediatric brain cancer manifesting in the developing cerebellum. The etiology of desmoplastic medulloblastoma implicates that perturbation in the Sonic Hedgehog signaling pathway can lead unipotent granule neuron precursors (GNPs) to overproliferate and tumor formation. To investigate the course of tumorigenesis originating in GNPs, we have employed a mouse genetic mosaic model termed Mosaic Analysis using Double Markers (MADM). MADM-induced loss of heterozygosity of the tumor suppressor, *p53*, mediated by the GNP-specific *Math1*-Cre, in a *Ptc+/-* background leads to consistent and fully penetrant medulloblastoma formation. The permanent labeling of both mutant (GFP+) and wildtype (RFP+) GNPs allows us to visualize the behaviors of mutant GNPs compared to wildtype GNPs originating from the same cell. To our surprise, when we looked at the cellular level in GFP+ tumors, we noticed that although the majority of the tumor mass is made up of dividing mutant GNPs, there was also a significant population of larger GFP+ cells with glial morphology. Marker staining for glial antigens confirmed that these large cells are indeed glial cells. This observation suggests that transformed unipotent GNPs can switch fates in the tumor to produce glia in addition to GNPs. Given this interesting fate switching phenomenon we sought to determine if these glia were important for tumor progression with a genetic ablation experiment to selectively kill the tumor glia using a *Gfap* promoter driven thymidine kinase (TK) transgene. Conversion of the substrate ganciclovir (GCV) into cytotoxic agents by TK in both early and late stage tumors resulted in complete tumor remission, demonstrating the essential role of these glial cells for tumor maintenance. Preliminary results indicate that using this method of ablation, we can cause not only tumor regression but complete tumor remission. This evidence suggests that these fate-switched glia play an important role in the tumor community and its successful growth and maintenance. Currently we are focusing on determining the molecular mechanisms by which tumor glia are generated via a fate-switch process, and by which they facilitate and sustain tumor growth. Dissecting the mode of support and tumor regression afforded by tumor-generated glia should provide targets for more specific therapeutic strategies, circumventing the devastating side effects of current surgical, radio- and chemotherapies.