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TITLE: Social behavior in Medulloblastoma: Functional Analysis of Tumor-Supporting Glial Cells

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Medulloblastoma is the most common malignant pediatric brain tumor. Granule neuron precursors (GNPs) in the 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.
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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 establish a co-culture system to investigate the molecular basis for glia-tumor crosstalk that sustains the tumor growth.

BODY:

Because our lab moved from University of Oregon to University of Virginia in early 2013, in revised task 1 we submitted IACUC protocol to UVa and ACURO documents to USAMRMC Office of Research Protections and got the approval in mid-October, 2013. Upon the approval, we successfully established the mouse colony to start producing MADM mice to generate medulloblastoma (revised task 2) for experiments in other tasks.

In revised task 3-6, we originally planned to acquire a mouse strain containing DN-SNARE transgene to block the secretion of growth factors from niche glial cells, and to test the effects on tumor growth. However, experts in the field advised us to avoid that approach, because the effectiveness of that transgene is questionable, thus unlikely to yield interpretable data. Therefore, we focused our energies on molecular analysis and co-culture assays to investigate the molecular mechanisms of niche support from glial cells. The strategy paid off greatly (see below).

One of the biggest hurdles in this project is to obtain pure population of niche cells from a massive amount of tumor cells. Therefore, we devoted significant manpower on the revised task 7. We went through many rounds of trials and errors to sub-fraction tumor cells from niche cells to establish the co-culture system. Because niche glial cells are low in abundance in the tumor mass, we decided to first co-culture tumor cells (red in Figure 1A and B) with normal glial cells (green in Figure 1B). We found that tumor cells cultured alone went through massive cell death and the surviving ones form self-aggregates (Figure 1A). On the contrary, in the presence of glial cells, tumor cells proliferated actively and formed intimate interactions with glial cells (Figure 1B). Quantitative analysis clearly demonstrated that tumor cells co-cultured with glial cells had increased proliferation, decreased cell death and differentiation compared to tumor cells cultured alone (Figure 1C), indicating the importance of glial cells for tumor progression. While it is exciting, we’d like to point out that the glial cells used in the co-culture assay were from normal brain regions rather than medulloblastoma. To make the final conclusion, we will need to perform the co-culture experiment with purified niche glial cells from the tumor mass. Our initial attempt of niche glia purification has been fruitful in terms of purity (see below) but the quantity is still too low for the co-culture assay. Therefore, in the next period of this grant, we will devote major efforts to purify enough niche glial cells by pooling multiple tumor samples so that we can repeat the co-culture experiment with bona fide tumor-derived glial cells.
For revised task 8, we set out to identify the candidate factors secreted from niche glial cells. Using qRT-PCR, we screened through a panel of candidate growth factors, including EGF, PDGFa, PDGFb, NT-3, IGF1, HGF, KitL and FGF. We reasoned that any suitable candidate should fulfill three criteria: first, it should express at a significantly higher level in tumor mass than in normal cerebellum; second, it should show consistent elevation in multiple tumor samples; and third, the candidate factor should not show elevated expression in tumor GNPs since that would make it an autocrine rather than niche factor. Based on our qRT-PCR results from 5 tumor samples, we found that IGF1, HGF, and PDGFa fit the first two criteria (Figure 2). To verify the third criterion, we then compared the expression levels of these three factors in tumor mass versus in tumor GNPs purified with percoll gradient method. We found that IGF1 has significantly high level expression in tumor mass, but low level expression in tumor GNPs.
(comparable to the level in normal cerebellum). To a lesser extent, HGF also had similar expression pattern, however with higher variation. PDGFa is a typical example of a non-“niche” factor as its expression is equally elevated in both tumor mass and tumor GNPs (Figure 3). Therefore, we chose IGF1 for further analysis since it passed all screening criteria for a consistent niche factor in medulloblastoma. In situ analysis showed that the pattern of IGF1 expression is sporadic, suggesting its secreting source likely to be niche rather than the bulk of tumor cells (Figure 4). We have been optimizing the 2-color in situ protocol, and should be able to determine whether the cell type that produces IGF1 is niche glia by the end of this grant period.

For revised task 9, we have collected some RNA samples from dissociated tumor and niche glial cells. Because of the low abundance of niche glial cells (~1% in the tumor mass), we employed highly selective immunopanning procedure with anti-Itgb5 to isolate tumor glia (1) (Figure 5, left panel). To verify the enrichment of glial cells samples before submitting for RNAseq, we performed qRT-PCR analysis with known astrocyte gene Aldh1L1 and tumor GNPs with Math1. The tumor astrocyte (TuAstrocyte) fraction was enriched for Aldh1L1 and depleted for Math1 expression compared to the tumor GNP fraction, suggesting that they are pure enough for further analysis. Currently we have them in queue so should obtain the data for analysis in the coming grant period.

For revised task 10, we have constructed a few lentiviral vectors that contain shRNA against different regions of IGF1R (Figure 6). To avoid the off-target effect, we designed multiple shRNA probes targeting different regions spanning the coding sequence (Figure 6A). We designed an RNAi reporter system similar to that

Figure 2. qRT-PCR screening of growth factors in multiple medulloblastoma samples from our mouse model. Tumor mass was harvested and total RNA was extracted. First strand cDNA was synthesized with iScript transcriptase and used as template for aRT-PCR with SyBR green mixture. Expression of growth factors was examined in five individual Medulloblastoma and level was normalized to that of normal cerebellum. tu: Medulloblastoma.

Figure 3. qRT-PCR quantification of growth factor expression in tumor mass versus tumor GNPs. RNAs from tumor mass and its corresponding tumor GNPs were extracted and transcribed into first-strand cDNA with iScript transcriptase. Real time PCR was performed with SyBr green mixture containing primers specific for IGF1, HGF and PDGFa respectively. Expression was normalized to the level in normal cerebellum. 9 tumors were analyzed. Statistical analysis was performed on expression in tumor mass vs tumor GNP groups. IGF1 (p=0.00001) and HGF1 (p=0.008) were of significant high expression in tumor mass compared to tumor GNP while PDGFa was not.

Figure 4. Sparse in situ pattern of IGF1 in the tumor mass.
described by Fellmann et al. (2) to quickly evaluate the efficacy of shRNAs to any target of interest. In this reporter system a 50bp-DNA fragment from the target gene (shRNA recognition site) was fused at the 3’ end of a cDNA encoding the red fluorescent protein mCherry (Figure 6B). A shRNA that effectively target that fragment will suppress the expression of mCherry, which serves as a surrogate marker to indicate the potency of the shRNA. Using this system, we found that shRNA-3329 is moderately effective while shRNA-3437 is fully effective in suppressing their targets based on the reduction of mCherry expression (figure 6C, top and middle panels, respectively). To further validate the potency of these shRNA in knocking down the endogenous IGF1R, we transiently transfected these shRNA-encoding vectors into tumor cells and performed the Western blot experiments. Concordant with the results by using the surrogate reporter system, data in Figure 6D clearly demonstrate the endogenous IGF1R were significantly knocked down by both shRNAs.

Figure 5. Purified niche glial cells from medulloblastoma.

Figure 6. Construction of lentiviral vectors to knockdown IGF1R. A. Diagram to show the two shRNAs against different regions in the mouse IGF1R coding sequence. An shRNA against luciferase was used as a non-specific control. B. Diagram to show the working mechanism of the surrogate reporter system to evaluate the potency of the shRNAs. C. Efficacy of the two shRNAs specific to mouse IGF1R gene to knockdown the cognate targets in HEK293T cells by using the reporter system. D. Same set of shRNA effectively knockdown the endogenous IGF1R protein in tumor cells, shown in western blots.
To perform functional assays in revised task 11, currently we are optimizing the transduction efficiency of lentiviral vectors into tumor cells. In addition to shRNA method, there are many other methods to block IGF signaling (IGF1-blocking antibody, IGFBP3 to titrate IGF1 level, and IGF1R kinase inhibitor). We plan to test their anti-tumor activities as well to firm up our findings with shRNA vectors.

KEY RESEARCH ACCOMPLISHMENTS:

- We firmly established the tumor-supporting role of glial cells with the co-culture assay.
- We had initial success in enriching tumor-derived niche glial cells, which are critical for global gene expression analysis of these cells and the ultimate co-culture experiment.
- Through a qRT-PCR based screening and logic deductions, we identified IGF1 as the candidate niche factor.
- We have constructed lentiviral vectors that can effectively knockdown IGF1R.

REPORTABLE OUTCOMES:

- **Invited speaker.** In vivo analysis of genetic contribution to glial development and functions at cellular resolution using MADM mouse model. XI European Meeting on Glial Cells in Health and Disease, Berlin, Germany, July 3-6, 2013
- **Invited speaker.** Use MADM, a mouse genetic mosaic model, to study how tumor cells attack. Mini-symposium on Cancer Biology, University of Chile, Santiago, Chile, January 10, 2014
- **Invited speaker.** UC Santa Cruz, Department of Molecular, Cell, and Developmental Biology, Santa Cruz, CA, April 14, 2014

CONCLUSION:

Understanding the interaction between tumor and niche cells not only is of great interest for basic biology, but also carries great potential in developing novel and effective therapeutic strategies. In this grant, based on the observation of tumor-to-niche reprogramming behavior, we set out to study the contribution of niche cells to tumor progression. The most important questions for these niche cells boil down to: a) whether they support tumor cells; b) if yes, what they use to support tumor cells; and c) can we stop tumor progression by blocking the supporting factor. In the past year, we had breakthrough in all three areas. Our co-culture system clearly demonstrated the supportive role of glial cells to tumor cells; our rationale-based low throughput screening pinpointed IGF1 as the candidate niche factor; and we have constructed shRNA vectors that can effectively knockdown IGF1R for the functional experiment. We also made a good decision to hold off the use of "secretion-blocking" genetic tool to perform mouse-modeling work. First of all, the tool has intrinsic problems so may not function as it is claimed, based on field experts’ advice. Even if it works, the analysis will be lengthy, and still can’t inform us the actual niche factor for follow-up studies anyway. Now with the identification of IGF1 as the candidate niche factor, we plan to focus our efforts on this lead in the final year of this grant. We would greatly appreciate the approval from the contract officer for this modification.

REFERENCES: