Determining glioblastoma proteome changes in response to lateral ventricle neural stem cells

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Background

Glioblastoma

- Glioblastoma (GBM) is the most common and malignant primary tumor in adults.
- GBM tumors located near the lateral ventricle display a more aggressive recurrence pattern, negatively impacting patient survival.
  - Suggests involvement of subventricular zone neurogenic niche in GBM malignancy.

Methionyl-tRNA synthetase (MetRS)

- Mutant MetRS L274G (MetRS*) allows for incorporation of azide-tagged methionine analog azidonorleucine (ANL) into newly formed proteins.

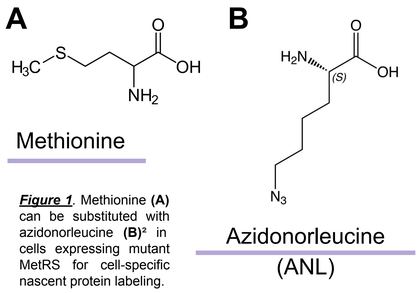


Figure 1. Methionine **(A)** can be substituted with azidonorleucine **(B)**[2] in cells expressing mutant MetRS for cell-specific nascent protein labeling.

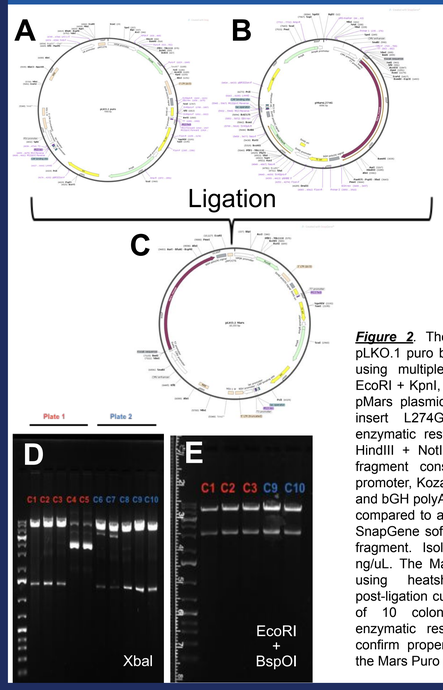
Objectives

- To optimize a tool to determine cell-specific proteomic changes of GBM cells in response to neural stem cell proximity.
- To define the intercellular communication between neural stem cells and GBM cells.

Methods

- Molecular cloning of MetRS* into lentiviral backbone with puromycin selection
- Confirmation of ANL incorporation into multiple MetRS mutant cell lines using western blot and silver stain
  - HEK
  - 1A GBM (patient-derived primary GBM line)
- Lentivirus production from cloned plasmid
  - Quantification and concentration
  - Titration
- Lentivirus validation using puromycin selection
- Co-culture proteomic analysis

Cloning MetRS* into lentiviral plasmid



Sample	Concentration (ng/uL)
LKO.1 puro	6.0
Mars L274G	10.3

Isolated Mars fragment:
- KflI
- EcoRI

Figure 2. The sequential organization of the pLKO.1 puro backbone plasmid (**A**) was verified using multiple enzymatic restriction reactions: EcoRI + KpnI, BshTI + NcoI, NdeI + BamHI. The pMars plasmid **(B)** carrying the mutant MetRS insert L274G was verified using multiple enzymatic restriction reactions: BglII + BamHI, HindIII + NotI, XhoI + SalI. PCR of the Mars fragment consisted of CMV enhancer, CMV promoter, Kozak sequence, 6X His, Mars L274G, and bGH polyA signal. The 35x cycle product was compared to a simulated agarose gel created by SnapGene software to ensure 3676 bp length of fragment. Isolated PCR product yielded 37.8 ng/uL. The Mars puro plasmid **(C)** was formed using heatshock ligation. Two separate post-ligation cultures were plated to select a total of 10 colonies. After MiniPrep, additional enzymatic restriction reactions were used to confirm proper sequencing and organization of the Mars Puro plasmid **(D, E)**.

Confirmation of ANL incorporation

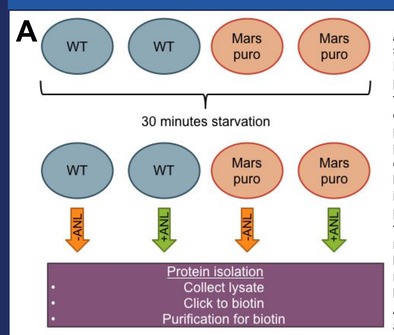
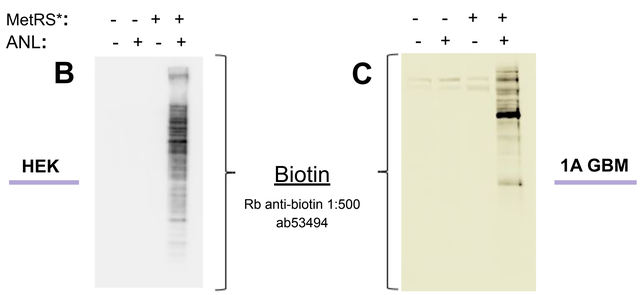


Figure 3. Western blots displayed successful ANL incorporation by mutated MetRS L274G cells. Cells are cultured then placed in methionine-free media to starve for 30 minutes. Post-starvation, cells were divided into two groups where one was placed into ANL-free media and the other placed into media with 4 mM ANL concentration. After 24 hour incubation, cell lysate was collected to begin protein isolation. A biotinylation Click reaction was performed followed by protein purification for biotin **(A)**. This was performed using a mutant MetRS and wild type (WT) HEK cell line (Human embryonic kidney) as well as mutant MetRS and WT primary 1A GBM cell line. Protein analysis showed successful ANL incorporation in both mutant HEK **(B)** and mutant 1A GBM **(C)** cell lines based on Western blot results.



Lentivirus production

Titration

LPS/uL	1.46 x 10^8
MOI Fetal cells	50
MOI GBM cells	100
LPS for 1x10^6 Fetal cells	5.00 x 10^7
LPS for 1x10^6 GBM cells	1.00 x 10^8
uL for Fetal cells	0.34
uL for GBM cells	0.68

Quantification

Average	0.9885
pg/mL in well (1:1^6 dilution)	117.17
pg/mL virus	1.17 x 10^8
Adj. conc.. virus pg/mL	1.17 x 10^7
LPS/mL	1.46 x 10^{11}
Approx. IFU/mL	1.46 x 10^9

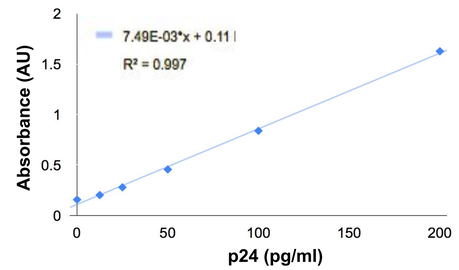


Figure 4. The Mars puro plasmid was used to create a lentivirus (LV) for stable integration of puromycin resistance and MetRS* L274G insert into primary cell genome. The psPAX2 plasmid was used for LV packaging and pMD2.G was used for LV enveloping. After the second harvest of virus, the LV was concentrated then titrated to find optimized quantity needed to transduce 1A GBM cell line and hfNSC cell line (human fetal neural stem cell), F50.

Puromycin selection of transduced cells

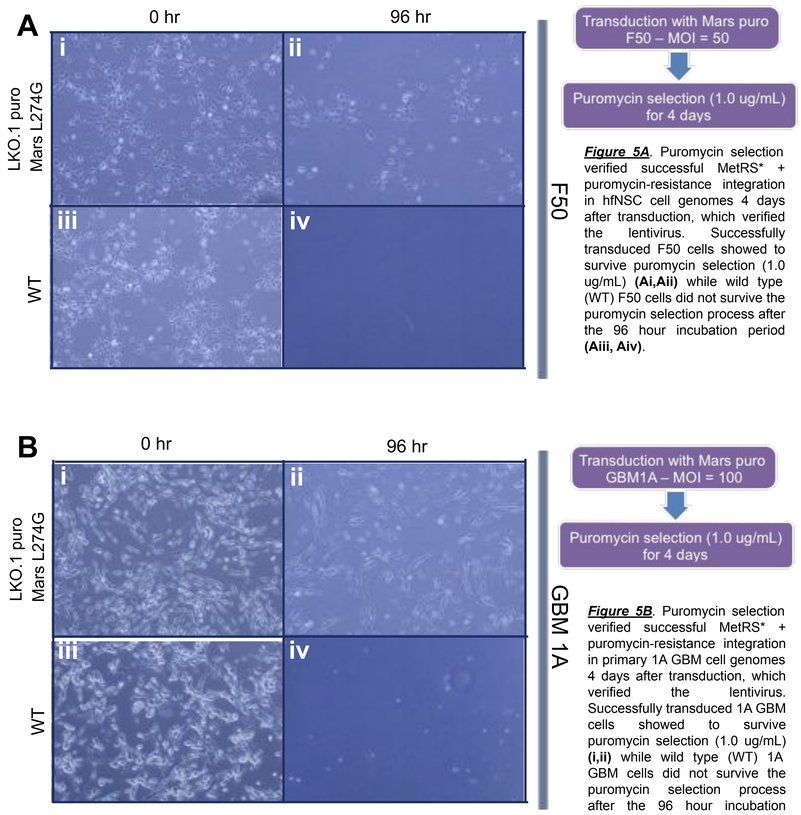


Figure 5A. Puromycin selection verified successful MetRS* + puromycin-resistance integration in hfNSC cell genomes 4 days after transduction, which verified the lentivirus. Successfully transduced F50 cells showed to survive puromycin selection (1.0 ug/mL) **(Ai,Aii)** while wild type (WT) F50 cells did not survive the puromycin selection process after the 96 hour incubation period **(Aiii, Aiv)**.

Figure 5B. Puromycin selection verified successful MetRS* + puromycin-resistance integration in primary 1A GBM cell genomes 4 days after transduction, which verified the lentivirus. Successfully transduced 1A GBM cells showed to survive puromycin selection (1.0 ug/mL) **(i,ii)** while wild type (WT) 1A GBM cells did not survive the puromycin selection process after the 96 hour incubation period **(Biii, Biv)**.

Proteomic changes in GBM cells co-cultured with neural stem cells

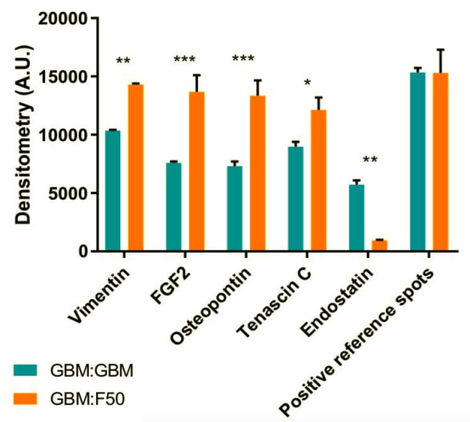


Figure 6. Co-Culture Oncology XL Proteome Profiler array results suggest an upregulation in malignancy promoting proteins. Co-cultures of F50 and 1A GBM were utilized to simulate the environment of glioblastoma neighboring neural stem cells.

Vimentin (upregulation): Component of intermediate filament cytoskeleton. Contributes towards processes including migration, metastasis, and cholesterol signaling. Usually overexpressed in cancers (cell invasion, metastatic tumor spread) and best known in cancers as a marker of cellular epithelial mesenchymal transition (EMT).

FGF2 (upregulation): Commonly expressed in malignant tumors. This protein can promote cell motility, proliferation, increase tumor angiogenesis and inhibit apoptosis.

Osteopontin (upregulation): Typically mediates normal physiological function including cell adhesion, migration, and tissue repair. Specifically in glioblastoma, this protein can act as a chemokine by recruiting macrophages to the GBM tumor site. Also mediates crosstalk between GBM cells.

Tenascin C (upregulation): Extracellular matrix glycoprotein. An upregulation of this protein has been seen as a biomarker for a high grade glioma, Diffuse intrinsic pontine glioma(DIPG).[1]

Endostatin (downregulation): Specifically inhibits endothelial proliferation while significantly inhibits angiogenesis and tumor growth.

Conclusions

- MetRS* metabolic labeling can be successfully cloned into a lentivirus and utilized as a tool for cell-specific proteomics with the use of ANL .
- GBM cells within close proximity of neural stem cells show an increase of proteins representative of malignant cancer spread.

References

1. Qi, J., Esfahani, D.R., Huang, T. *et al.* Tenascin-C expression contributes to pediatric brainstem glioma tumor phenotype and represents a novel biomarker of disease. *acta neuropathol commun* 7, 75 (2019). https://doi.org/10.1186/s40478-019-0727-1
2. Link, A. James; Vink, Mandy K. S.; Tirrell, David A. Journal of the American Chemical Society (2004), 126(34), 10598-10602. DOI:10.1021/ja047629c;
3. Sinnaeve J, Mobley BC, Ihrie RA. Space invaders: brain tumor exploitation of the stem cell niche. Am J Pathol. 2018;188(1):29–38. doi: 10.1016/j.ajpath.2017.08.029.