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Signaling Pathways for the Maintenance of Glioblastoma In Vivo

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Glioblastoma multiforme (GBM) is one of the most malignant forms of human brain cancer. Despite intensive treatment, the mean survival of glioblastoma patients remains about 1 year. The stem cell antigen, CD133, identifies a tumorigenic population in glioblastoma patients. Tumor xenografts generated in immunocompromised mice by CD133+ cells showed higher resistance to radiation and chemotherapy, suggesting that CD133+ cells could be a more aggressive tumorigenic population. For this study, CD133+ glioblastoma cell lines were generated by CelProgen, Inc. from ten tumor-recurrent patients following radiation and chemotherapy. These cell lines are stable in vitro and express the following markers for at least seven passages when maintained in CelProgen's complete expansion media and matrix: CD133, Telomerase, SSEA3 and 4, Oct4, Nestin, GFAP and Map2. Using this panel of 10 primary glioblastoma cell lines, we have characterized markers for stem cell phenotype and in vivo growth characteristics. Preliminary studies show that that up to 1,000 CD133 positive cells injected subcutaneously generated tumors within 21 days in nude mice. Currently, we are assessing the signaling pathways (JNK, p38, ERK and PI3K/AKT signaling pathways) that are responsible for tumor formation in vivo and maintenance of stem cell characteristics in vitro. Using small molecule inhibitors, we are assessing changes in tumor growth parameters that include the recruitment of host programs that promote angiogenesis, lymph node invasion and metastasis as well as the pathways that contribute to radiation and chemotherapy resistance.

Introduction: It is well documented that patients with GBM have a low median survival of approximately one year and respond poorly to most therapeutic modalities. Standard of care for newly diagnosed GBM includes surgical resection, radiotherapy and Temozolomide administered both during and after radiotherapy. However, most patients develop tumor recurrence or progression after this multimodal treatment. Repeat resection and stereotactic radiosurgery upon recurrence improves outcome only in select patients. GBM tumors are composed of a morphologically heterogeneous mixture of poorly differentiated neoplastic astrocytes and are localized preferentially in the cerebral hemispheres. Studies have revealed that only a small population of cells has the specific ability to reinitiate cancer and may reflect the behavior of brain cancer stem cells (BCSCs), which have been shown to harbor various molecular abnormalities enhancing their ability to both initiate and maintain tumor growth as well as provide resistance to therapy. Infiltrating growth of glioma cells and their resistance to therapy have hampered the development of efficacious treatment. Due to the limited understanding of the biology of the disease, the main therapeutic strategies employed to treat malignant gliomas have remained essentially unchanged. Consequently, an urgent need for more effective, targeted therapies that take into account specific signaling pathways and molecular alterations found in gliomas has emerged. The current research focused on BCSCs as treatment targets by generating an in vivo model system that may guide the discovery of new therapeutic strategies.

Methods: **Donor population and samples**: The donor population from which the Brain Cancer Stem Cells (BCSCs) were extracted were patients having having recurrence of the tumor following resection of the initial tumor combined with radio- and chemotherapy. Tissue was obtained under HIPAA guidelines and after signed informed consent. Ten individual donor samples were selected for this study. The population consisted of 5 males and 5 females ranging in age from 35 to 65 years.

BCSC culture: BCSCs were expanded and proliferated in CelProgen's Complete Expansion Media and Matrix for 7 days and then characterized and selected for CD133 cell population.

Tumorigencicity: Nude mice were subcutaneously injected with CD133 positive or negative BCSCs and evaluated for tumor growth after 21 days. Mice were also evaluated for metastases after 18 days.

Inhibitor Studies: Inhibitors were added to BCSC cultures for up to 5 days at the IC50 values. Inhibitors used were SP600125 (IC50 = 0.11 uM; JNK) and SB431542 (IC50 = 0.25 uM; TGFβ). Cell counts were evaluated by Alamar Blue and CD133 and Nestin by ELISA

Conclusions:

- In vitro inhibition of JNK and TGFβ signaling pathways reduces the expression of the stem cell markers CD133 and Nestin and reduces BCSC viability.
- Subcutaneous injection of only 1,000 CD133+ stem cells generated a tumor within 18 days.
- Extensive infiltration into the brain was also seen at 18 days post subcutaneous injection. This model allows us to study the development of brain tumors without surgical induction and injury.
- We are currently evaluating this in vivo model to determine the characteristics of these cancer cells and the expression of cytokines and other factors (such as TGFβ) that may have important roles in maintaining the stem cell-like pool of cells in GBM and also contribute to angiogenesis, invasion and metastasis.

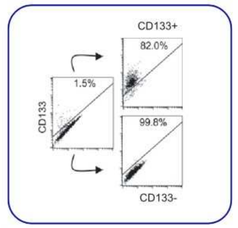

Figure 1: Human Brain Cancer Stem Cell (HBCSC) culture expanded in Celprogen's Expansion Media & Matrix for 7 days. The media and matrix combination enabled to expand CD133 positive cells in the culture system.

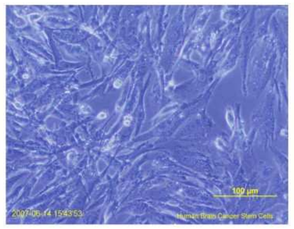

Figure 2: HBCSC culture expanded and proliferated in Celprogen's HBCSC Complete expansion Media and ECM, passage 0, donor #1.

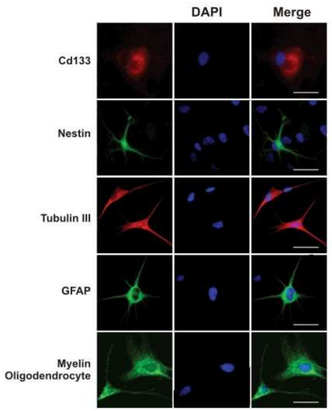

Figure 3: HBCSC immunocytochemistry profile: CD133 positve cells, Nestin, Tubulin III, GFAP and MBP. These cells were characterized for the above mentioned markers up to 7 passages.

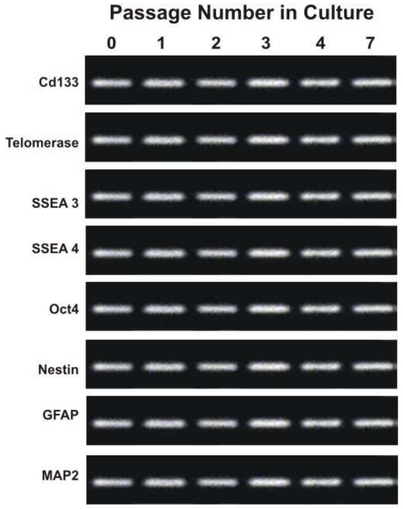

Figure 4: . HBCSC gene expression profile of up to seven passages when maintained in Celprogen's Un-Differentiation Media and ECM.

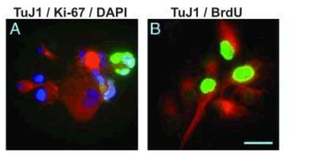

Figure 5: HBCSC immunocytochemistry profile: for neuronal markers These cells were characterized for the above mentioned markers up to 7 passages.

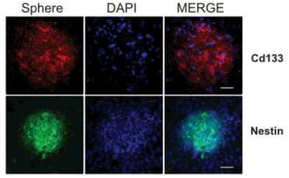

Figure 6: HBCSC immunological profile of neuronal spheres CD133 & Nestin positive.

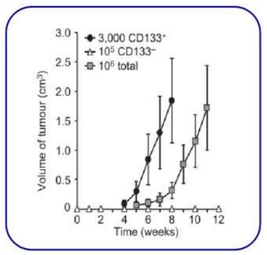

Figure 9: Volume of Subcutaneous tumor generation (cm3) per weeks in Nude mice injected with CD133 positive cells.

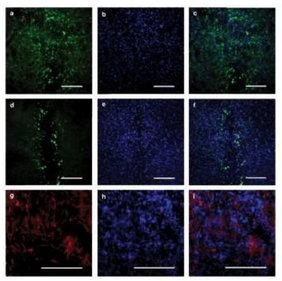

Figure 10: HBCSC immunological profile of tumor formation in nude mice. Green: Nestin+ Cells, Red: CD133+ Cells, Blue: DAPI + Cells.

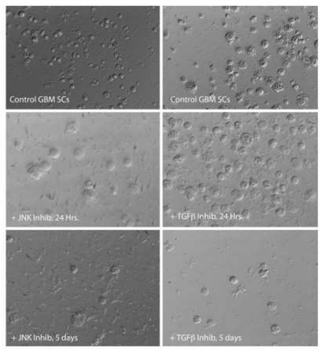

Figure 7: BCSCs treated with JNK (SP600125) or TGFβ (SB431542) inhibitors at their IC50 values for 24 hours and 5 days.

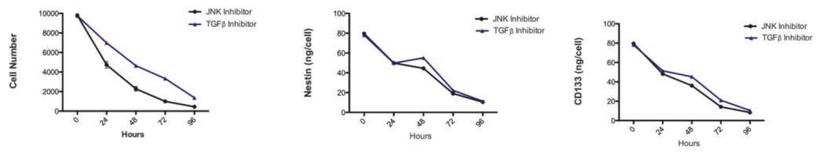

Figure 8: Reduction in levels of CD133 and Nestin following JNK or TGFβ inhibition (IC50 values). Values are normalized to Alamar Blue cell numbers and are expressed as ng/cell.

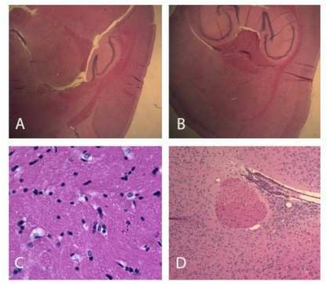

Figure 11: Hematoxylin & Eosin stained brain sections from a mouse 18 days after subcutaneous injection of 1,000 CD133+ BCSCs. BCSCs rapidly infiltrate the brain parenchyma. 2x images **(A/B)** show widespread BCSC infiltration and tumor formation. 40x image **(C)** from the center of the tumor shown in **(D)**.