Arbitrarily Polyomaviruses including human JC, Merkel, and BK polyomavirus, as well as their "famous" simian counterpart, SV40, induce cell transformation in vitro, are tumorigenic in experimental animals, and are highly suspected in the development of some tumors in humans. The most prominent polyomavirus oncoprotein, large transforming antigen (T-antigen), is known to bind several key cellular regulatory proteins including p53, pRb, and IRF-1. The binding dysregulates basic cellular mechanisms responsible for cell proliferation, cell survival and DNA repair. Importantly, JCV T-antigen has been detected in the increasing number of clinical samples of brain tumors including medulloblastoma. In addition to its well-established transforming properties, JCV T-antigen has been recently implicated in energy metabolism, including dysregulation of glycolysis and pentose phosphate pathway (PPP), and inactivation of autophagy. The results of this study demonstrate that medulloblastoma cells expressing JCV T-antigen are characterized by a surprisingly low oxygen consumption rate (OCR - proportional to mitochondrial respiration) and low extracellular acidification rate (ECAR) - proportional to glycolysis when compared to their JCV T-antigen negative medulloblastoma counterparts. These low OCR and ECAR values were detected in spite of higher rate of cell proliferation of T-antigen expressing cells. Instead, these cells demonstrated over 3-fold higher consumption of glutamine, suggesting that glutaminolysis may play a compensatory role in supporting high-energy demands during T-antigen-induced malignant growth. To investigate the molecular details of T-antigen-induced metabolic impacts in medulloblastomas, we are developing a T-antigen inducible system. This is critically important since the observed changes in glucose and glutamine utilization, and mitochondrial respiration, are coming from two different medulloblastoma clones, which are either T-antigen positive or T-antigen negative. Although our data show a strong correlation between the presence of T-antigen and surprisingly low energy metabolism, these differences could also reflect clonal variability. Because of such a possibility, we have decided to construct this JCV T-antigen inducible system, which will allow us to investigate early and late cellular adaptations triggered by the expression of JCV T-antigen. In particular, this inducible expression system permits temporary and tightly regulated expression of JCV large T antigen only in the presence of dioxycyline in the culture medium. The system consists of the inducible promoter pCMV-TetOn3G and pTRE3G-JCVTag-IRES-Cherry-N1, which were consecutively and stably transferred into a T-antigen negative mouse medulloblastoma cell line (Bs-1a). The first stable transfection of pCMV-TetOn3G generates cells expressing a Tet-On 3G transactivator protein under the constitutive CMV early promoter. The second stable transfection introduces the vector co-expressing JCV T-antigen and mCherry fluorescent protein, which are both driven by a tightly regulated TRE3G inducible promoter (pTRE3G). The results in Figures 4-5 demonstrate our initial evaluation of the JCV T-antigen (Tet-on) system in Bs-1a cells.

Figure 1: Characterization of Bs-1a and BsB8 cell lines: A) Western blot analysis of mouse medulloblastoma cell lines showing expression of JCV T-antigen in BsB8, but not Bs-1a cells. The expression of JCV T-antigen protein, which indicates similar loading conditions. B) Comparison of cell cycle distribution between (Bs-1a) and (BsB8) cell lines where the percentage of cells in the cell cycle - proportional to resting cell populations, (2) is the S phase and (3) G2/M phase, which are proportional to proliferating populations. Finally, (4) represents the proportion of dead cells. The data was fixed over night and then plotted at a concentration of 100,000 per 100μg in a 96- well plate. Cells were fixed in 70% ethanol and stained with PI for flow cytometry for analysis. Results show that BsB8 cells have higher proliferation rates compared to 19.6% of cells in G0/G1 compared to 37.2% of Bs-1a cells. Additionally, only 48.5% of BsB8 cells were in the resting (Go/G1) as compared to 57.2% of Bs-1a cells.

CONCLUSIONS
Our results demonstrate that two different cell types stably expressing viral oncoprotein JCV large T-antigen are characterized by significantly lower mitochondrial respiration and glycolysis when compared to the corresponding cells lacking the expression of this viral protein. We also demonstrated that T-antigen expressing medulloblastoma cells uptake glutamine from the culture medium much more effectively than their T-antigen negative counterparts. To further confirm this important metabolic effect, we are in the process of constructing and testing a JCV T-antigen inducible system (Tel-on), which we are planning to utilize in our future metabolic studies, in which effects of JCV T-antigen on metabolic and signaling parameters could be evaluated independently from the cellular and clonal context.

Figure 2: Effects of JCV T-antigen on oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using Extracellular Flux Analyzer (XF24/Seahorse). Panels A and B: OCR and ECAR values registered in JCV T-antigen negative (R508) and positive (R508T) mouse embryo fibroblasts. 2) Four clones were expanded and screened for inducibility by transfection with luciferase-expressing pTRE3G-Luc and pRL plasmids. 3) The cell line which displayed highest induction was further transfected with pTRE3G-JCVTag-IRES-Cherry-N1 plasmid along with a linear puromycin selection marker. 4) After a second round of drug selection with puromycin and G418, a double resistant cell line was established, which expressed high levels of T-antigen in response to dioxycyline.

Figure 3: Glutamine consumption from the medium high performance liquid chromatography (HPLC) was conducted on deproteinized conditioned media labeled with O-phthalaldehyde (OPA) using a HPLC (1) Dionex Ultimate 3000; Sunnyvale, CA) with a cation exchange column (particle size 3 μM). A) Ammonia and glutamine were analyzed using following reagents: 10% formic acid + ACCLAR 120 (C18). B) Alpha amino acids were analyzed on the basis of standard curves of known amounts.

Figure 4: Establishing Tet-On 3G inducible expression system: 1) Mouse medulloblastoma cells were transfected with pCMV-Tet-On3G plasmid and selected with G418 antibiotic to create a stable cell line expressing Tet-On 3G transactivator protein. 2) Four clones were expanded and screened for inducibility by transfection with luciferase-expressing pTRE3G-Luc and pRL plasmids. 3) The cell line which displayed highest induction was further transfected with pTRE3G-JCVTag-IRES-Cherry-N1 plasmid along with a linear puromycin selection marker. 4) After a second round of drug selection with puromycin and G418, a double resistant cell line was established, which expressed high levels of T-antigen in response to dioxycyline.

Figure 5: Evaluation of Tet-On 3G transactivator using luciferase reporter and pM010. Panel A: Bs-Tet-On clones 6, 7, 8 & 11 were transfected with 1.6μg of pCMV-Tet3g plasmid and selected with G418 antibiotic to. BsB8 mouse medulloblastoma cell lines. Panels C and D: OCR and ECAR values registered in JCV T-antigen negative (R508) and positive (R508T) mouse embryo fibroblasts. Basic metabolic measurements were analyzed using following reagents: 10% formic acid + ACCLAR 120 (C18). Amino acids were analyzed on the basis of standard curves of known amounts.

Figure 6: Western blot analysis documenting JCV T-antigen expression following doxycycline treatment. BsB8 cells were cultured in transient with pCMV-tet-on3G and with the vector containing JCV T antigen cDNA(pN0010) under tet-on operator. At 48 hours following transfection the cells were treated (+) or not treated (-) with doxycycline (100ng/ml) and protein lysates were collected. Loading conditions are indicated by re-probing with α-tubulin antibody.

Figure 7: Luciferase assay documenting JCV T-antigen expression following doxycycline treatment. BsB8 cells were transfected in transient with pCMV-tet-on3G and with the vector containing JCV T antigen cDNA(pN0010) under tet-on operator. At 48 hours following transfection the cells were treated (+) or not treated (-) with doxycycline (100ng/ml) and protein lysates were collected. Loading conditions are indicated by re-probing with α-tubulin antibody.