The Effects of JCV T-antigen on Tumor Cell Metabolism

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ABSTRACT Primate 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 IRS-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 doxycycline in the culture medium. The system consists of two vectors, pCMV-TetOn3G and pTRE3G-JCVTag-IRES-Cherry-N1, which were consecutively and stably transfected 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.

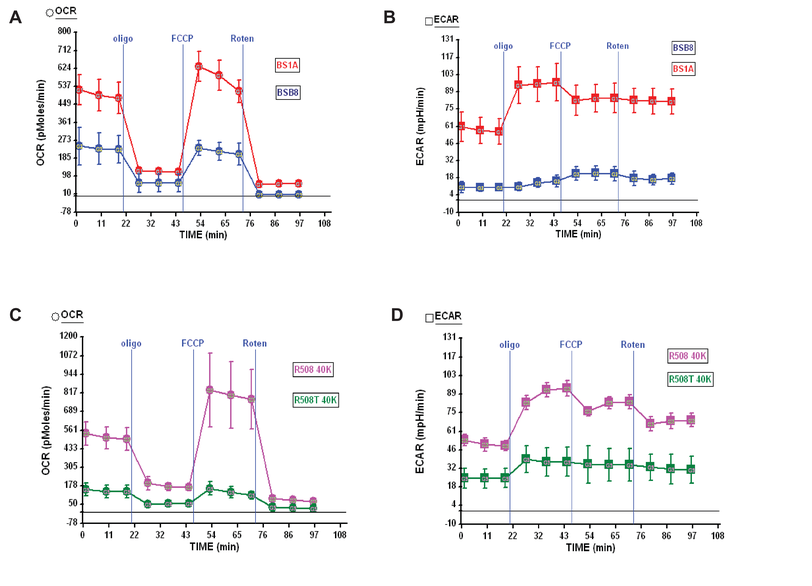


Figure 2: Effects of JCV T-antigen on oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using Extracellular Flux Analyzer (XF24;Seahorse). Panels A and B: OCR and ECAR values registered in JCV T-antigen negative (Bs-1a) and positive (BsB8) mouse medulloblastoma cell lines. **Panels C and D:** OCR and ECAR values registered in JCV T-antigen negative (R508) and positive (R508T) mouse embryo fibroblasts. Basic metabolic responses were analyzed using following metabolic toxins: Oligomycin (Oligo); FCCP (uncoupling factor); and rotenone (Roten). Before measurement, the cells were plated at $5x10^4$ cells/well and cultured in the presence of 10% FBS for 24 hrs. An immediate OCR (**Panels A and C**) and ECAR (**Panels B and D**) values were registered in serum-free assay medium following sequential injection of oligomycin, FCCP and rotenone. Data represent average values SD from three measurements (n=3).

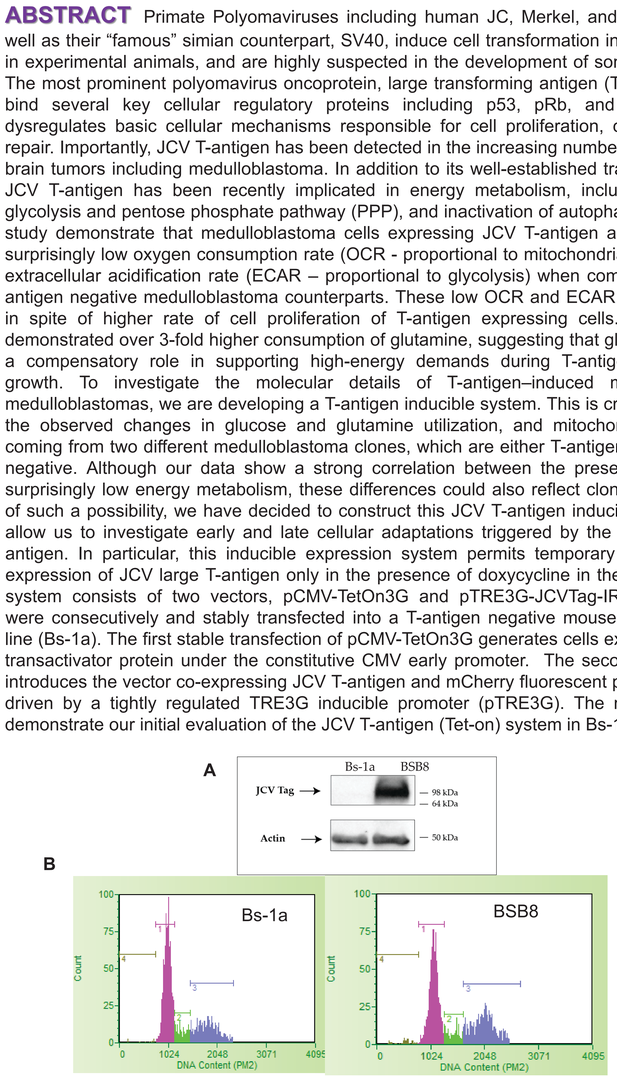


Figure 1: Characterization of Bs-1a and BsB8 cell lines: A) Western blot analysis of mouse medulloblastoma cell lines showing expression of JCV T-antigen in BsB8, but not Bs-1a cells. The expression of housekeeping protein, actin, indicates similar loading conditions**. B)** Comparison of cell cycle distribution between Bs-1a (T-) and BsB8 (T+) cell lines where (1) indicates cell population in Go/G1 phase of 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 cells were fixed over night and then plated at a concentration of 100,00 cells per 100µl in a 96-well plate. Cells were stained with 200µl of propidium iodide and inserted into Guava flow cytometer for analysis. Results show that BsB8 cells have higher proliferation rates with 38.6% of cells in G2/M, compared to 31.2% 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.

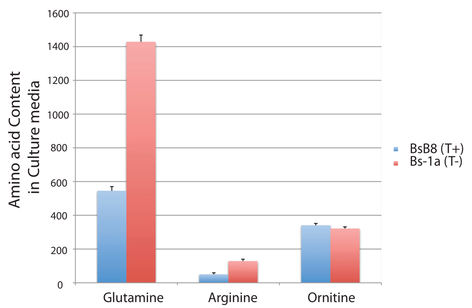


Figure 3: Glutamine consumption from the medium. High performance liquid chromatography (HPLC) was conducted on deproteinized conditioned media labeled with O-phtaldialdehyde (OPA) using a HPLC (Dionex Ultimate 3000; Sunnyvale, CA) with a photodiode array UV detector. Chromatographic separation was achieved using a 150 by 4.6-mm (inner diameter) C18 reverse-phase column (particle size, 3 µm; ACCLAIM 120 C-18). Amino acids were eluted with 100 mM sodium acetate buffer, pH 5.0, with a linear gradient consisting of methanol and acetonitrile. Detection was monitored with UV at an absorbance of 338 nm. The concentration of indicated amino acids were calculated on the basis of standard curves of known amounts.

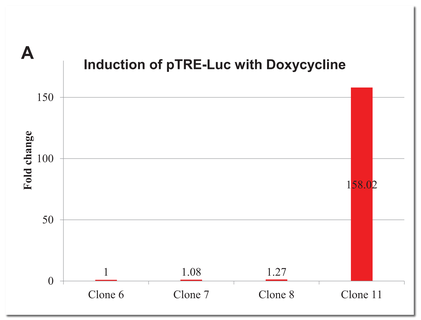
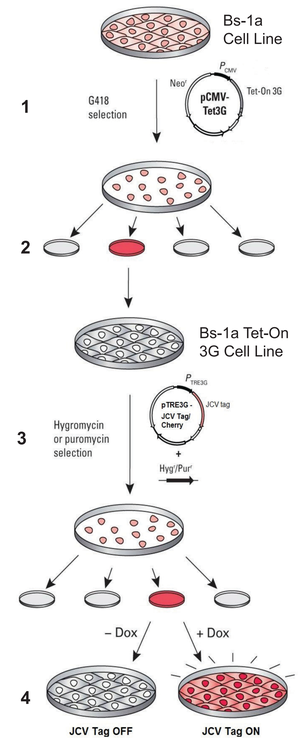
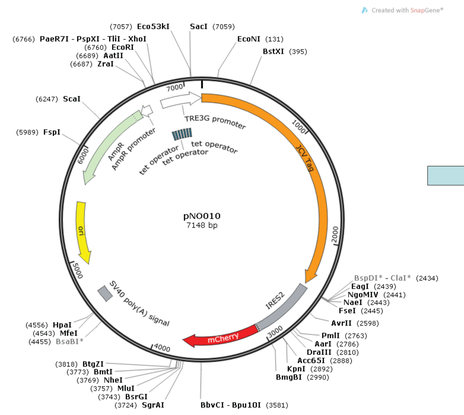


Figure 5: Evaluation of Tet-On 3G transactivator using luciferase reporter and pN0010. Panel A: Bs-1a(T-) clones 6,7,8 & 11 were transfected with 1.6µg of pTRE3G-Luc and 0.4µg of pRL plasmids using Amaxa Nucleofection. Tet-On 3G transactivator protein induction was quantified by inducing luciferase expression with 100ng/ml of doxycycline. After 24 hours, luciferase bioluminescence was measured using luminotometry (Synergy 2). Induction of clone 11 was over 150-fold higher than clones 6,7,& 8, and therefore selected for further experiments. **Panel B:** Inducible expression of JCV T-antigen. Western blot analysis documenting JCV T-antigen expression following doxycycline treatment. Bs-1a 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 the same blot with anti-α-tubulin antibody.

Figure 4: Establishing Tet-On 3G inducible expression system: 1) Mouse medulloblastoma Bs-1a cells were transfected with pCMV-Tet3g 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 doxycycline.





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 have 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 (Tet-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.