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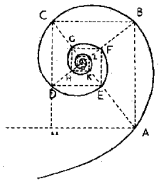

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# MOLECULAR CHARACTERIZATION OF RESISTANCE TO RADIATION IN A SMALL SUBSET OF TUMOR CELLS INDUCED BY JC VIRUS T-AG IN MICE


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## ABSTRACT

*JCV is a small, naked tumor polyomavirus with an icosahedral capsids containing a circular, double-stranded DNA genome. Its genome is divided into early and late genes, separated by a non-coding control region (NCCR) containing the promoter, the origin of replication (ORI) and the enhancer elements. Early genes encode for large T antigen, small t antigen and three different T' proteins called T'135, T'136 and T'165. Late genes encode for the structural proteins VP1, VP2 and VP3, and the non-structural agnoprotein. JCV only replicates in human. In non-permissive cells, they are not able to support viral replication or expression of late genes. In those cells, only the transcription of early genes is observed, such as large T antigen. This leads to genome instability and inactivation of oncosoppressor proteins and eventually tumorigenesis. Cells derived from a murine brain tumor induced by JCV injection have shown different resistance to ionizing radiation. We cultured these BSB8-RR cells and characterized them comparing to the radiation sensitive BSB8 cells. Using MTT assay we showed a resistance to radiation to these cells, compare to the BSB8. We then performed cell-cycle analysis, colony formation assay e soft agar growth assay and we showed that BSB8-RR grow faster and they are more tumorigenic. Since we saw a decrease in the level of large T antigen expression in BSB8-RR, compared to BSB8, we hypothesized an involvement of large T antigen in DNA repair. Using DNA repair assays such as NHEJ and homologous recombination, we suggested that BSB8-RR cells may be resistant to radiation because they have increased homologous recombination activity, compared to the BSB8 cells. We suggested that this increase is due to the lower expression of large T antigen in BSB8-RR, that has been previously reported as inhibitor of homologous recombination. In conclusion, our data showed a more aggressive phenotype of BSB8-RR cells and a resistance to radiation that we suggested is due to the lower level of large T antigen in these cells, compared to BSB8.*

## SOMMARIO

*JCV è poliomavirus di piccole dimensione, nudo, con capside icosaedrico contenente un genoma a DNA circolare a doppio filamento. Il suo genoma si divide in geni precoci e tardivi, separati da una regione di controllo non codificante (NCCR) contentente il promotore, l'origine di replicazione (ORI) e gli elementi enhancer. I geni precoci codificano per la proteina T grande, la proteina t piccola e tre diverse proteine T' T'135, T'136 and T'165. I geni tardive codificano per le proteine strutturali VP1, VP2 e VP3 e la proteina non strutturale agnoproteina. JCV replica solo nell'uomo. Le cellule non permissive non sono in grado di supportare la replicazione virale o l'espressione delle proteine tardive. In queste cellule viene osservata solo la la trascrizione dei geni precoci, come ad esempio la proteina T grande. Questo porta ad una instabilitá genica ed all'inattivazione di oncosoppressori che infine portano a tumorigenesi. Cellule derivate da un tumore murino indotto da iniezione di JCV hanno mostrato resistanza alle radiazioni. Abbiamo coltivato queste cellule denominate BSB8-RR e le abbiamo caratterizzate, confrontandole con le BSB8, cellule sensibili alle radiazioni. Utilizzando il saggio di MTT abbiamo mostrato la resistenza alle radiazioni di queste cellule, rispetto alle BSB8. Abbiamo poi svolto analisi del ciclo cellulare, saggio di formazione di colonie e crescita su soft agar e abbiamo dimostrato che BSB8-RR crescono piu' velocemente e sono piu' tumorigeniche rispetto alle BSB8. Avendo visto una diminuzione del livello di espressione della proteina T grande in BSB8-RR rispetto alle BSB8, abbiamo ipotizzato un ruolo della proteina T grande nel riparo del DNA. Abbiamo utilizzato due assay per studiare il riparo del DNA, il non-homologous end joning (NHEJ) e la ricombinazione omologa (HR) e abbiamo mostrato che le BSB8-RR riparano il DNA con una percentuale piu' alta di ricombinazione omologa rispetto a BSB8 e questo potrebbe essere il motivo per cui sono resistenti alle radiazioni.*

*Concludendo, i nostri dati mostrano un fenotipo più aggressivo delle cellule BSB8-RR e una resistenza alle radiazioni che noi suggeriamo sia dovuta alla minore espressione della proteina T grande in queste cellule, rispetto alle BSB8.*

**TABLE OF CONTENTS**

# 1. INTRODUCTION

## *1.1 Polyomavirus*

Polyomaviruses are small, naked tumor viruses categorized within the *Polyomaviridae* family with icosahedral capsids containing a circular, double-stranded DNA genome. Polyomaviruses name derives from “poly-”, a Greek word meaning many, and “-oma”, that means tumor, because the first isolated member of this family was able to induce various tumors when injected into mice [1, 2]. Polyomaviruses can infect different species, comprising humans, mice, hamsters, birds and monkeys [3].

Studying the phylogenetic associations within polyomaviruses, four genera have been delineated: *Alphapolyomavirus*, *Betapolyomavirus*, *Gammapolyomavirus* and *Deltapolyomavirus*. The studies were based on the amino acid sequence of the large tumor antigen, large T antigen, one of the early viral proteins [4]. Each family member has a restricted host range, in general members of the genera *Alphapolyomavirus*, *Betapolyomavirus* and *Deltapolyomavirus* infect mammals, while members of the genus *Gammapolyomavirus* infect birds [1].

The first polyomavirus was discovered in 1953 [5] and named the murine pneumotropic virus (MptV). In 1959 and 1962 other two similar viruses were discovered and named simian virus 40 (SV40) [6, 7] and baboon polyomaviruses (SA12) [8]. To date, thirteen polyomaviruses have been identified in humans (Table 1). The first two human polyomaviruses have been discovered in 1971, BK virus, isolated from the urine of a patient with renal transplant [9] and JC virus, isolated from the brain of a patient with progressive multifocal leukoencephalopathy [10]. BKV and JCV were named after the patients’ name (initials) involved in the discoveries.

BKV and JCV are widely spread in the human population, about 90% of adults have specific antibodies directed against these two viruses [11].

The human polyomaviruses BK and JC are characterized by a genomic sequence homology of 75%, they are morphologically very similar to murine oncogenic polyomavirus (PyV) and they show a common ancestral to the monkey virus (SV40) [12].

In the last decade, several studies have led to the identification of new members of the *Polyomaviridae* family. In 2007, a study conducted on nasopharyngeal samples from children with infections of the respiratory tract, led to the identification of a new human polyomavirus, called Karolinska Institute Polyomavirus (KIPyV) because of the place where it was discovered. This virus is phylogenetically related to the early genomic region of the other human polyomaviruses, but it has low homology with the late region [13].

Concurrent to the discovery of the KI virus, another group of researchers discovered a fourth human polyomavirus, named Washington University Polyomavirus (WUPyV). They were analyzing genomic sequences found in bronchial washings from patients with severe inflammation of the respiratory tract [14]. Following the sequencing of the entire WU genome, a homology has been found between the structural characteristics of the WUPyV genome and the other human polyomaviruses. Preliminary data showed a similarity in the genomic structure, phylogenetic and tropism between WUPyV and KIPyV, suggesting that these viruses belong to a different branch of the human polyomavirus family [15].

One year after the discoveries of KI and WU viruses, Feng and his collaborators conducted a study on Merkel Cell Carcinoma (MCC), a rare but aggressive skin cancer, to understand the possible role of viral infections in this type of cancer. In the samples analyzed, they discovered the presence of transcripts of the large T antigen of a polyomavirus that was never described. They named this virus as Merkel Cell Polyomavirus (MCPyV) [16]. Analyzing the large T antigen sequence, it has been found the presence of

mutations that are thought to be implicated in the formation of truncated forms of large T antigen. These truncated forms maintain their domain for Rb-binding [17].

Human polyomaviruses 6 and 7 were isolated in 2010 by Schowalter and colleagues in cutaneous tampons [18].

Also in 2010 van der Meijden and his group discovered a human polyomavirus associated with trichodysplasia spinulosa in an immunocompromised patient, named TSPV [19].

The human polyomavirus 9 was isolated in 2011 from a patient with history of kidney transplantation and immunosuppressive therapy [20].

The Malawi Polyomavirus was isolated in 2012 in feces samples of a healthy baby in Malawi and in diarrheal feces of an American child from St. Louis [21].

The human polyomavirus 12 was first discovered in 2013 during a screening of samples from the gastrointestinal tract. It was mostly found in liver samples but also in the colon, in the rectum and in feces [22].

The STLP virus was isolated in 2013, it was also found in fecal samples from a healthy Malawians child [23].

The New Jersey polyomavirus is the last human polyomavirus discovered to date. It was isolated from epithelial cells of a patient who was undergone to pancreatic transplant. A group of researcher led by Lipkin, screened tissue samples from a patient that was presenting retinal blindness and vasculitic myopathy, symptoms derivable from viral causes. They discovered the presence of a fourteenth human polyomavirus [24].

In Table 1 a list of the human polyomaviruses discovered until 2017. The year of first description and the isolation sites are listed, along with the genome size.

| Name | First description | Genome size | Associated clinical diseases / isolation sites | References |
|---|---|---|---|---|
| JC (JVPyV) | 1971 | 5,310 | Progressive multifocal leukoencephalopathy | Padget et al, 1971 |
| BK (BKPyV) | 1971 | 5,153 | Kidney and bladder epithelium, nephritis | Gardner et al, 1971 |
| KI (KIPyV) | 2007 | 5,040 | Respiratory tract | Allander et al, 2007 |
| WU (WUPyV) | 2007 | 5,229 | Respiratory tract | Gaynor et al, 2007 |
| MCPyV | 2008 | 5,387 | Merkel cell carcinoma | Feng et al, 2008 |
| TSPV | 2010 | 5,232 | Trichodysplasia spinulosa-associated skin lesions | van der Meijden et al, 2010 |
| HPyV6 | 2010 | 4,926 | Normal skin and skin tumors; serum and body fluids | Schowalter et al, 2010 |
| HPyV7 | 2010 | 4,952 | Normal skin and skin tumors; serum and body fluids | Schowalter et al, 2010 |
| HPyV9 | 2011 | 5,026 | Kidney and bladder epithelium | Scuda et al, 2011 |
| HPyV10 (Malawi or MWPyV, MXPyV) | 2012 | 4,927 | Healthy stool, acute diarrhea | Siebrasse et al, 2012; Buck et al, 2012; Yu et al, 2012 |
| HPyV12 | 2013 | 5,033 | Liver, gastrointestinal tract | Korup et al, 2013 |
| STLPyV | 2013 | 4,776 | Fecal | Lim et al, 2013 |
| NJPyV | 2104 | 5,108 | Vascular endothelium, muscle tissue | Mishra et al, 2014 |

**Table 1**. Human polyomaviruses information. (Adapted from Cook, 2016 [25])

### *1.2 JC virus*

The John Cunningham virus or JCV is a human polyomavirus belonging to the genus *Betapolyomavirus* [26]. It has a circular genome of around 5 kb, 5130 bp for the Mad-1 strain [27], associated with cellular histone proteins (H2A, H2B, H3 and H4) and packaged into chromatin resembling cellular genomes called minichromosomes [28]. The JCV genome is divided into early and late genes, which are separated by a non-coding control region (NCCR). The NCCR contains the promoter, the origin of replication (ORI) and the enhancer elements [29].

Early and late genes are transcribed in opposite direction: the early region is the first to be transcribed in a counter-clockwise direction, while late genes are transcribed in a clockwise direction (Figure 1) [30]. The early genes encode for large T antigen, small t antigen and three different T' proteins

called $T'_{135}$, $T'_{136}$ and $T'_{165}$, that are T antigen splice variants [31]. The late genes comprise the structural proteins VP1, VP2 and VP3, and the non-structural agnoprotein [32].

JCV infects only humans, in fact it has been suggested that the large T antigen can interact only with the human DNA polymerase, at the level of viral early gene transcription and DNA replication. JCV tissue tropism is specific for human glial cells, kidney epithelial cells, and, with less efficiency, in B lymphocytes, as experimental animals and in vitro analysis suggested [33, 34].

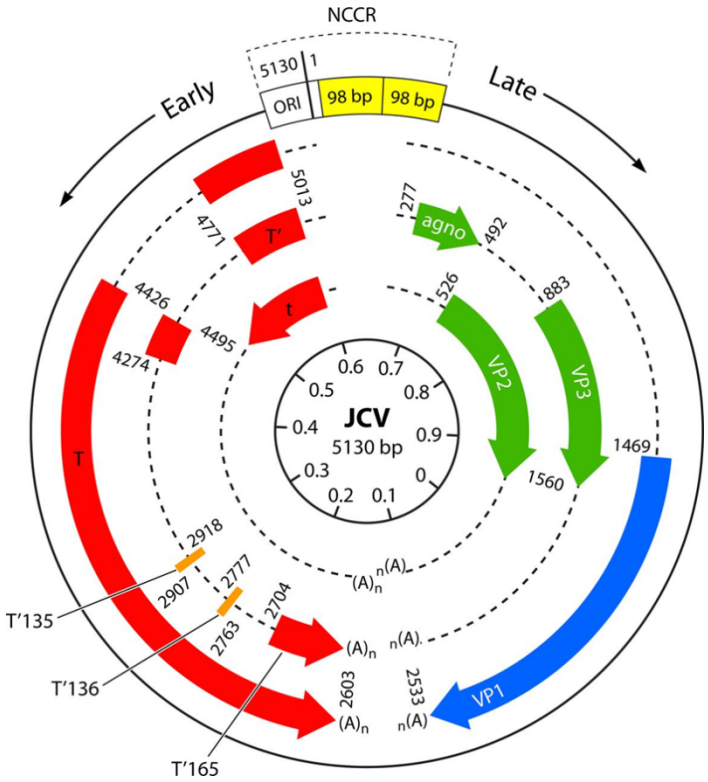


**Figure 1**. Schematic representation of JCV Mad-1 genome organization. The early open reading frame proceeds from the NCCR in the counter-clockwise direction and the late open reading frame in the clockwise orientation. Proteins in the same reading frame are illustrated with the same colour. Start and stop sites are indicated. All transcripts are polyadenylated. Reprinted with permission [30].

### 1.2.1 Non-coding control region (NCCR)

The non-coding control region or NCCR is the most variable portion of the viral genome and it contains the origin of replication (ORI), the TATA box, binding sites for the large T antigen and cellular transcription factors, the bidirectional promoters and the enhancers. It is described as the region between the large T antigen start codon (ATG) and the start of the region encoding the agnoprotein. Unlike the coding regions that are well preserved belong the polyomaviruses, the NCCR region is hypervariable [35].

In a single infected person, it is possible to find the NCCR and multiple variants. JC virus can have an archetype NCCR or rearranged NCCR, depending on the structure. The transmitted form of the JC virus is believed to be the one with the archetype NCCR because it is mostly found in the urine of healthy or diseased individuals and rarely in the brain of patients with progressive multifocal leukoencephalopathy (PML) [36].

The most variable region within the JC virus NCCR is the 98-bp tandem repeat region. JC virus can be divided into two classes: the class I viruses are described as viruses containing the 98-bp tandem repeat within the NCCR, for example the Mad-1 strain. The class II viruses are strains of JC virus that show differences from the NCCR of class I because of the presence of deletions and insertions [37].

The archetype NCCR is divided into 6 regions named Box A (36 bp), B (23 bp), C (55 bp), D (66 bp), E (18 bp) and F (69 bp). Each box contains binding sites for cellular transcription factors involved in viral transcription [38].

The prototype NCCR is a variant isolated from tissues of patients with PML, it is a rearrangement of the archetypal sequence. The first original prototype is the Mad-1 strain, which contains one NCCR consisting of a 98-bp sequence A-C-E, repeated (A-C-E-A-C-E-F), with duplication of the TATA box and binding sites for specific cellular transcription factors. The TATA

boxes are important for the transcription of both early and late viral genes [39]. The absence of the boxes B and D in the Mad-1 strain is involved in the formation of binding sites for cellular transcription factors, including YB-1/Purα and NF-1 [40].

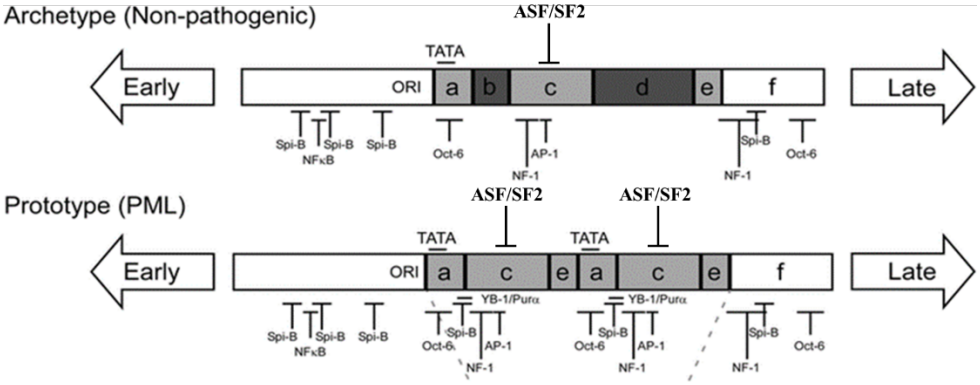


**Figure 2**. JC virus archetype and prototype regulatory region sequences and host transcription factor binding sites. Modified and reprinted with permission [40].

### 1.2.2 Early proteins

The size of the JCV early region is 2.4 kb, it encodes for large T antigen, small t antigen and three different T' proteins. A common pre-messenger RNA (mRNA) precursor is alternatively spliced and transcribed into the early proteins. Large T antigen and its variants have multifunctional tasks, they can interact with both viral and host proteins and DNAs. These proteins are involved in viral transformation, gene regulation and replication and they are required for driving the host cell toward S phase [32, 41].

The large T antigen is the main regulatory protein of polyomaviruses. It is necessary for the initiation of viral DNA replication and it is able to auto-regulate its own promoter and trans-activate the late genes [42].

The large T antigen is a protein of 688 amino acids and it can be structurally divided into different functional domains:

- DnaJ domain that binds cellular Hsc70 and polymerase α;
- LXCXE motif that binds specifically the proteins of pRb family, inactivating their functionality;
- TPPK, threonine-proline-proline-lysine domain;
- NLS domain, necessary for the protein nuclear localization signal;
- Helicase domain;
- p53 binding domain, that binds the p53 cellular suppressor protein.

These domains are listed from the N-terminal to the C-terminal and shown in Figure 3 [43].

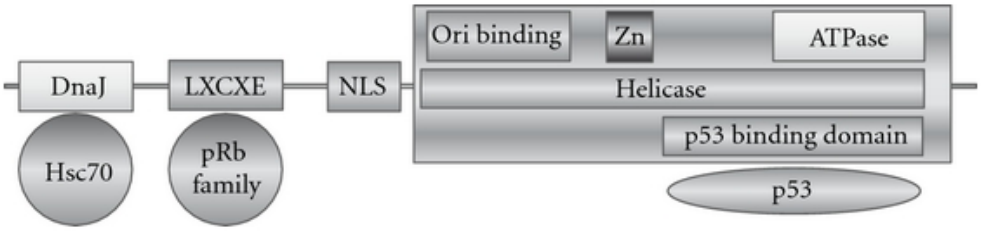


**Figure 3.** Representation of large T antigen structure. Reprinted under the Creative Commons Attribution License [43]

On both the N- and C-terminal, two phosphorylation domains of serine and threonine residues are located: they are essential for large T antigen function in viral DNA replication [44].

The DnaJ domain, at the N-terminal of the large T antigen protein, and the LXCXE motif contribute to viral DNA replication, with a mechanism that is still unknown. The LXCXE motif binds the retinoblastoma protein (pRb) and the pRb-related proteins retinoblastoma-like protein 1 (RLB1) and

retinoblastoma-like protein 2 (RBL2) [45]. The binding of the large T antigen to the pRb disrupts the pRb/E2F complexes, allowing the release of the transcription factor E2F. Its release promotes cell cycle progression, stimulating the resting cells to enter the S-phase of the cell cycle (Figure 4A and 4B) [46].

Following the DnaJ and the LXCXE domains, there is a threonine-proline-proline-lysine (TPPK) domain. The phosphorylation of its threonine residue is important for the replication mediated by the large T antigen [47].

C-terminal to the TPPK motif, there are a nuclear localization sequence (NLS), a DNA-binding domain (DBD) and a helicase domain. The NLS is important for the transport of the newly formed large T antigen proteins from the cytoplasm to the nucleus mediated by specific importins. The DBD and helicase domains are required for viral replication because they recruit cellular factors involved in DNA replication. The DBD can recruit protein such as the DNA polymerase-α catalytic subunit, the replication protein A complex and the DNA primase complex; the helicase domain recruits EP300, CREBBP, p53 and DNA topoisomerase 1 [47]. The helicase domain, in addition to recruit cellular proteins, binds precise regions of the origin of replication (ORI) in the NCCR. This binding permits the initiation of the viral replication [48]. The protein p53 is one of the protein that the helicase domain can bind: this binding results in the block of all the p53 downstream functions, for example the induction of apoptosis and the arrest of cell growth (Figure 4C and 4D) [49].

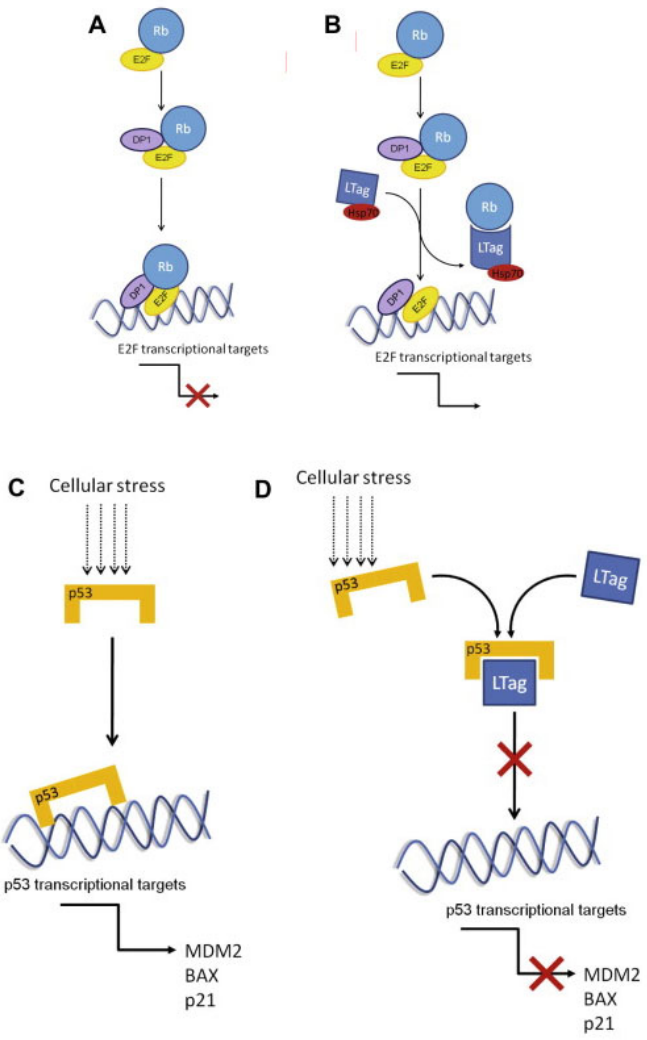


**Figure 4.** Schematic representation of the role of large T antigen on host cell transcription. (A) In absence of mitotic signals, E2F remains attached to Rb and the transcription of E2F targeted genes is inhibited. (B) In the presence of large T antigen, it can bind to Rb, avoiding the formation of the complex E2F/Rb and permitting the entry of the host cell in mitosis. (C) In a normal cell, the p53 pathway is activated and it can promote the transcription of genes involved in DNA repair or apoptosis, such as MDM2, BAX and p21. (D) The presence of large T antigen and its direct binding to p53, prevents the binding between the p53 protein and the host cell DNA. Modified and reprinted with permission [50].

The role of the small t antigen is not yet fully delineated. It has been demonstrated its importance in the JCV life cycle, because it has an active role in the induction of the cells to start the S phase of the cell cycle. In fact, the small t antigen interacts and inactivates the regulatory factors belonging to the Rb family and it sequesters the protein phosphatase 2 (PP2A) [51]. The inhibition of PP2A mediated by the small t antigen prevents the viral late agnoprotein dephosphorylation and this can be an important regulatory mechanism for JCV replication [52].

The T’ proteins (T’135, T’136, and T’165) were discovered in 1995 by Trowbridge and Frisque. At first, it was supposed they were products form the degradation of the large T antigen because they share with it a sequence homology equal to 132 amino acids [31].

Then, it was discovered that the T' proteins are generated by alternative splicing of the mRNA coding for the large T antigen. Indeed, they share the DnaJ domain and LXCXE in the N-terminal portion, but they differ at the C-terminal end because the T’ proteins have a different degree of phosphorylation. This difference affects the binding of the T' proteins with the members of the Rb family, such as pRb, p107 and p130, and with the Hsp70. In particular, this interaction results in the release of the transcriptional factor E2F that promotes the advancement of the cell cycle from G1 to S phase [53, 54].

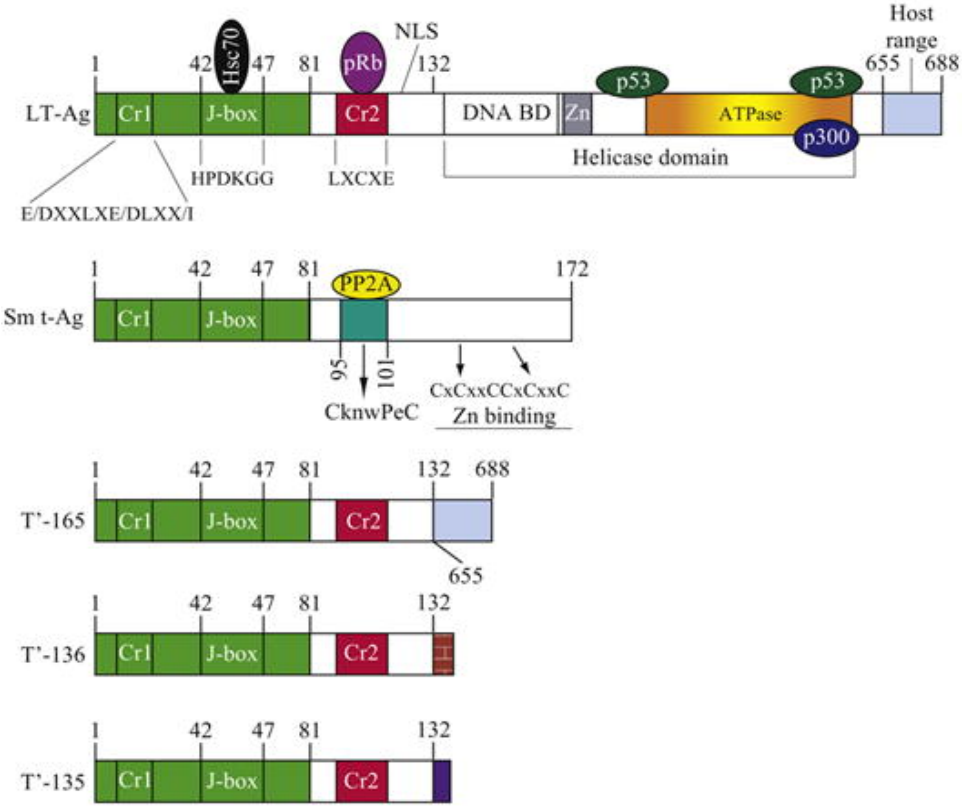


**Figure 5.** Graphical representation of JCV early proteins, large T antigen (LT-Ag), small t antigen (Sm t-Ag) and T′ proteins (T′-135, T′-136 and T′-165) and some functional domains. Reprinted with permission with modification [54]

### 1.2.3 Late Proteins

The size of the JCV late region is 2.3 kb, it encodes for three structural proteins, the major VP1 and the two minor VP2 and VP3, and the agnoprotein. Analogously to the early region, a common late pre-mRNA precursor is alternatively spliced and transcribed into the late proteins. The ORFs of the late proteins overlap: VP1 is a protein composed of 354 amino

acids, its ORF is located at the 3' end of the late protein region. The ORFs for VP2 and VP3 are located between the 3' end of the agnoprotein ORF and the 5' end of the VP1 ORF. VP2 is 344 amino acids and VP3 is arranged from the 225 amino acids at the C-terminal of VP2. Agnoprotein is the smallest protein, it is constituted of 71 amino acids and its ORF begins at nucleotide 277 and terminates at nucleotide 492.

The VP1 protein is the main viral capsid protein. For each pentamer of VP1 there is a single molecule of VP2 or VP3, in order to form an individual capsomere that contains a pore at the five-fold axis of symmetry. It is probable that the pore is occupied by the C-terminus of VP2. The VP1 C-terminals extend to anchor together adjacent capsomeres and to form the entire capsid of 72 pentamers [55-57]. The VP1 protein contains the epitopes recognized by the host immune system and it is involved in the identification of the cellular receptors and infection of the target cells [58, 59].

VP2 and VP3 are the minor capsid proteins, they have a role in the transport of the virus to the nucleus and its scavenging [60, 61]. They are also involved in the assembling of the capsid, in fact it has been demonstrated that the binding between VP2 and VP3 proteins, chaperone Hsp70 and large T antigen leads to the formation of deposits of pre-virions within the nucleus of infected cells. Specifically, late proteins VP2 and VP3, associated with Hsp70, can interact with large T antigen in the cytoplasm or in the nucleus. This interaction induces a conformational change in the large T antigen that increases its affinity for the viral origin of replication followed by new DNA synthesis. Newly made DNA is immediately caught by the VP2 and VP3 proteins that can start the virion assembly process. Subsequently, the nucleocapsids are covered by the VP1 protein and they become mature virions [54].

Agnoprotein is active during the last phase of JCV cellular infection. Its function has not been well understood yet. It has been hypothesized that it

could be a viroporine, supporting the release of the virus from the cells [62]. Agnoprotein also interacts with large T antigen in order to reduce the levels of viral replication to promote the translation of the late transcripts and the formation of mature virions. Some authors have shown that agnoprotein can be implicated in tumorigenesis. In fact, in the non-permissive cells infected, agnoprotein acts in cooperation with large T antigen in order to alter the regulation of cellular metabolic by the host cell becoming transformed. In particular, it would contribute to the development of mutations at the level of genomic DNA, blocking the activity of the cellular proteins involved in the mechanisms of DNA repair [63-66].

### 1.2.4 Infectious lifecycle

The infectious lifecycle of JC virus is divided into early and late stages. The early stage starts with the interaction of the virus with the surface of the host cell and lasts until the beginning of viral DNA replication. The late stage comprises all the following events that lead to the assembly of new virions and ends with the release of viral progeny.

The early stage starts with the JC virus attachment to the cell surface receptors, in particular the serotonin receptor $5HT_{2A}$ and alpha (2–6)-linked sialic acid [67]. The attached virus particles enter the cells through clathrin-mediated endocytosis [68, 69]. Following internalization, the virus is being transported to the $Rab5^{+}$ early endosomes, translocated within the endoplasmic reticulum and released into the cytoplasm in order to move towards the nucleus. Entry into the nucleus involves interaction with nuclear pore complexes [30]. The following step is the expression in the nucleus of the viral early genome and then the alternative splicing of the viral transcripts. After splicing events, the early proteins are transported back to the cytoplasm, where they are translated into large T antigen, small t antigen and T' proteins. Large T antigen is transported back to the nucleus where it can

directly recruit the host cell DNA polymerase complex to the viral ORI, in order to initiate bi-directional viral DNA synthesis and trans-activate the viral late promoter. At this point, the production of the three structural capsid proteins, VP1, VP2, and VP3, and the regulatory agnoprotein starts [70, 71]. It has been shown that also the small t antigen has an important role in JCV replication [52]. Once the capsid proteins are translated, they are transported back to the nucleus, where the encapsidation process starts: the proteins are sequentially added to one another and the viral genome can be packaged into the capsids. The last and final step is the release of the virions from infected cells, probably upon the lysis of the infected cells [72].

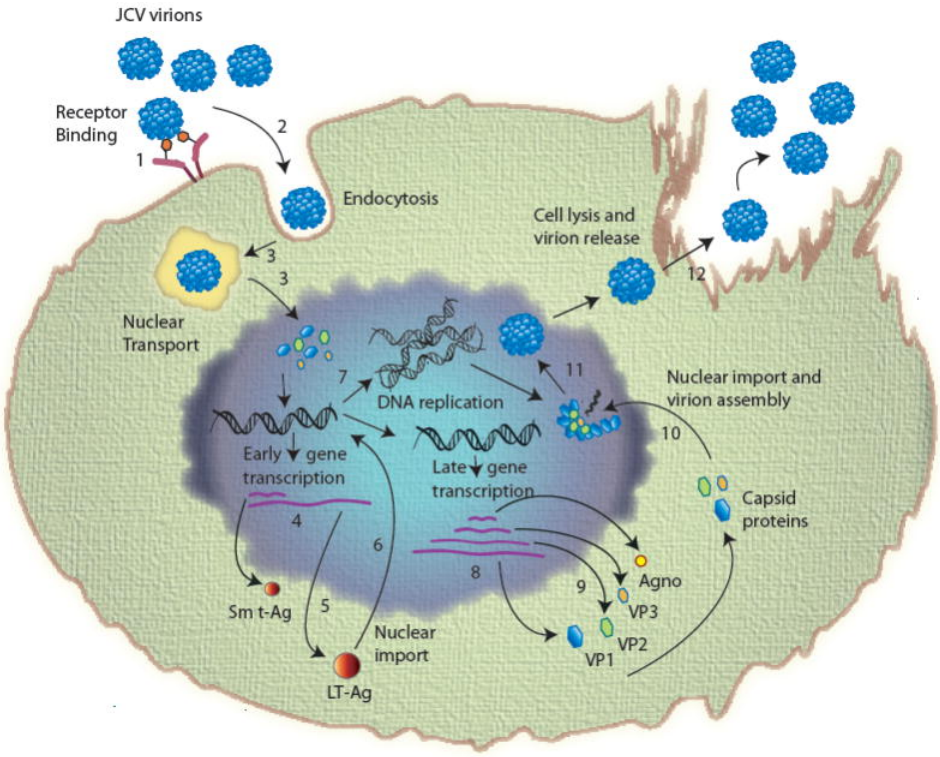


**Figure 6.** Chronological events during JCV life cycle. (1) Binding of virus to the receptors on the cell surface; (2) entry in the cell by clathrin-mediated endocytosis; (3) uncoating of virions, both in endoplasmic reticulum or in the nucleus and nuclear transport; (4) transcription of early genome region; (5) translation of early regulatory proteins; (6) import of Large T antigen (LT-Ag) into nucleus to start viral DNA replication and activation of late genome transcription; (7) replication, (8) transcription and (9) translation of viral late genome; (10) import of capsid proteins in the nucleus; (11) assembly of viral progeny in the nucleus; (12) release of virions from infected cells. Reprinted with permission [72].

### 1.2.5 JCV associated diseases in the central nervous system

Progressive multifocal leukoencephalopathy or PML is a rare but fatal brain demyelinating pathology that results from the JCV lytic infection in oligodendrocytes. These cells produce myelin sheets, which wrap around the axons of the neurons to support the transmission of the neuronal current. The infection causes oligodendrocytes death and, sub-sequentially, lack of myelin. This leads to axonal dysfunction and eventually neurons death. The death of both oligodendrocytes and neurons in the CNS and their loss result in the formation of small plaque. PML lesions are multifocal and they affect the white matter. Areas where these plaques are formed merge and form lesions, as the disease develops. The result is the death of the individual [3]. PML symptoms are: motor dysfunction, visual defects and speech impairment. MRI to visualize multifocal lesions is essential in diagnosis because these symptoms are not specific for PML. MRI is followed by research of JCV DNA and/or proteins in the cerebrospinal fluid and brain biopsies. The first neuropathological description of PML has been reported in 1958 by Astrom and colleagues, where they analyzed brain tissue from patients with chronic lymphatic leukemia and Hodgkin's disease [73]. PML etiology was unknown until the discovery of the presence of viral particles in cerebral lesions typical of PML [74]. These were then isolated in cultures of fetal human brain in the 1971 by Padgett and colleagues. The cerebral tissue used to isolate the virus was taken from the patient John Cunningham, JC, whose initials gave the name to JC virus [10].

Between the 1971 and the early years of the 80s, only around 200 cases had been reported, all connected with lymphoproliferative disorders [75]. PML became more prevalent after the AIDS epidemic, from all HIV-positive patients, around 3% developed PML [76]. The major cause of developing PML in AIDS patients is believed to be linked to the decrease in $CD4^+$ T cells.

These cells are necessary for the maintenance of $CD8^+$ T cells, the most important cells in the immune control of JCV infection [77]. Furthermore, it is possible that the interactions between JCV and HIV may contribute to the development of PML. In particular, HIV protein Tat may stimulate JCV promoter [78, 79].

PML has been diagnosed also in individuals under monoclonal antibody therapy for treating autoimmune diseases or lymphoproliferative disorders. Four monoclonal antibodies have been associated with PML: natalizumab, efalizumab, rituximab and infliximab. These drugs cause the depletion of mature B cells in the periphery, with the consequent recruitment of immature B cells from the bone marrow and the potential spread of the latent virus in the brain [30].

There is currently no effective therapy able to neutralize this pathology.

Natalizumab mechanism to increase the risk of developing PML is not yet known. It has been theorized that the low immune-surveillance of the CNS and the increased presence of B cells and $CD34^+$ progenitor cells together can be advantageous for JCV viral replication [49].

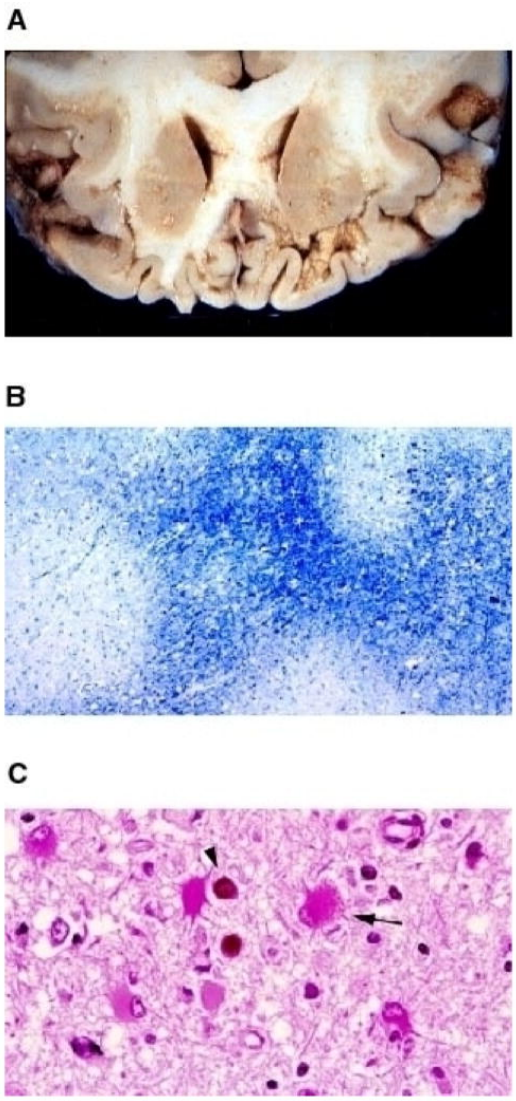


**Figure 7.** (A) Gross examination of JCV- induced lesions occurring at the subcortical white matter. A coronal section of the frontal lobe of the brain from a PML patient is shown. (B) Apparent myelin loss, as result of JCV infection of oligodendrocytes, is made detectable by Luxol blue staining (40×). Demyelinated areas are visibly distinguishable as white plaque areas. (C) Hematoxilin and Eosin staining of the brain sections from a PML patient. Infected oligodendrocytes are indicated with a round dark staining of the eosinophilic inclusion bodies (arrow head). An arrow points to an infected astrocyte (400×). Reprinted with permission from [80].

After JCV discovery in 1971, JCV was thought to be able to infect only oligodendrocytes and astrocytes in the brain white matter, neurons were not considered susceptible to infection [81]. In 2003, a group of researcher has demonstrated a productive JCV infection of cerebellar granule cell neurons [82] and in 2005, a disease called JCV granule cell neuronopathy (JCV GCN) was described [83]. In 2009, a grey matter disease has been described in a HIV-negative patient presenting lung cancer. This disease has been named JCV encephalopathy (JCVE), and it is characterized by a productive infection in the cortical pyramidal neurons [84]. In 2014, a fatal case of JCV meningitis (JCVM) was described. It was found in an HIV-negative patient with a very high JC viral load in the cerebrospinal fluid and productive infection in leptomeningeal cells [85]. This specific tropism for cerebellar granule cell neurons is caused by a JCV variant with a small deletion in the VP1 protein. This infection results in cerebellar atrophy and associated dysarthria, appendicular, and gait ataxia [86].

JCV granule cell neuronopathy can be manifested both in concomitance or not with PML [82, 83] and it has been described in both HIV-positive and HIV-negative patients [87-89]. The JCV GCN disease is characterized by destruction of cerebellar granule cells. This leads to a subacute or chronic cerebellar dysfunction with several symptoms: ataxia, dysarthria and motor incoordination. MRI shows cerebellar atrophy and changes in the white matter in the cerebellum and brainstem [90]. The definitive diagnosis is based using PCR to detect JCV DNA in CSF, cerebellar biopsy displaying JCV infection of granule cell neurons or immunohistochemistry using an antibody against T antigen in combination with a neuronal marker, such as NeuN or MAP-2. The infected granule cell neurons are expected to have a hyperchromatic aspect with enlarged nucleus [83, 91]. The JCV GCN therapy is identical to the one for PML [92].

In JCV encephalopathy, JCV infection mainly implicates cerebral pyramidal neurons and astrocytes in the cortical grey matter and grey-white junctions. Viral proteins have been found in the nuclei, cytoplasm and axons of neurons, suggesting that JC virus may migrate through axons of infected neurons and spreads in the brain. It is more common to find large T antigen than VP1 in infected neurons, so probably in the cortical pyramidal neurons, the JCV infection is abortive. Wüthrich and colleagues [84] described the first case of JCV encephalopathy in a patient who presented multiple cortical lesions, aphasia, and progressive cognitive decline. RM showed lesions at the level of the grey substance and the viral genome was found through PCR in the liquor. The diagnosis was finally confirmed by the histological examination that showed a JCV correlated lytic infection of cortical pyramidal neurons and astrocytes.

Unlike PML, in JCV meningitis (JCVM) there are no focal lesions of the white matter and the MR shows only a slight dilatation of the cerebral ventricles. Numerous cases have been documented describing JCV as the only pathogen present in the CSF of patients with typical meningeal symptoms. It is not yet clear whether this clinical picture is due to a primary viral infection or to a reactivation of the virus. The diagnosis is confirmed by the research of JCV viral genome in the liquor in the absence of all other neurotrophic viruses. Therapy, even in this case, it is the same as the one for PML [92].

### 1.2.6 JCV and tumors

When the expression of early genes is followed by the replication of viral DNA and expression of late genes, the result is a productive infection. One example is the infection of oligodendrocytes, which are permissive to the lytic infection of JCV and are destroyed during the infectious process, leading to the PML. However, other scenarios are also possible. In the case of non-permissive cell infection, only the expression of early genes is observed. Such cells are not able to support neither viral replication nor the expression of late genes. In this situation, the expression of large T antigen leads to the inactivation of oncosuppressors and to the deregulation of signalling pathways or genome instability, which can contribute to cellular transformation (Figure 8) [33].

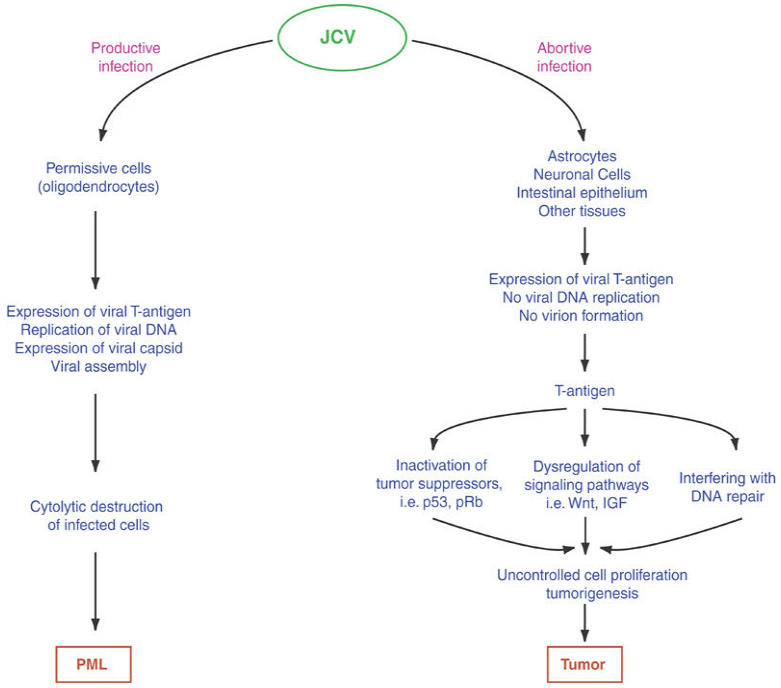


**Figure 8**. Mechanisms of JCV T-antigen-mediated cellular transformation or demyelination. Reprinted with permission [33].

The oncogenic potential of Polyomavirus JC has been extensively demonstrated *in vitro* in non-permissive cells. In these cells, the viral replication cycle is not completed, pushing the cell into transformation. The factors contributing to the permissiveness can be several, such as tissue of origin, point of development, or state of differentiation. This can be observed in the same oligodendrocytes: the mature oligodendrocyte can totally support a lytic infection while an undifferentiated oligodendrocyte can not. The undifferentiated oligodendrocyte might only allow the expression of the large T antigen, leading to the cell transformation and development of an oligodendroglioma [33].

As described above, the large T antigen is the major actor in cell transformation, because its several domains cooperate in binding and inactivating cellular proteins that typically stop the entry into S phase. JCV large T antigen drives the cell cycle from G1 into S phase, promoting cell transformation in non-permissive cells [2]. This transition is the result of the binding between the LXCXE domain on large T antigen and proteins belonging to the Rb tumor suppressor family [45, 93, 94]. The binding between large T antigen, p53 and pRb family leads to the inactivation of cellular tumor suppressors protein and then uncontrolled cell growth. The connection between large T antigen and p53 removes the ability of the cellular protein to enhance transcription of p21/WAF-1, an inhibitor of cyclin kinases. In normal situations, a reduction in the kinase activity of G1/S cyclins:cdks keeps pRb in a hypophosphorylated state. In this state pRb is active and can sequester E2F, an S phase specific transcription factor. The binding between large T antigen and pRb can release E2F from the pRb:E2F complex so it can promote unscheduled transcription of S phase genes (Figure 9) [95].

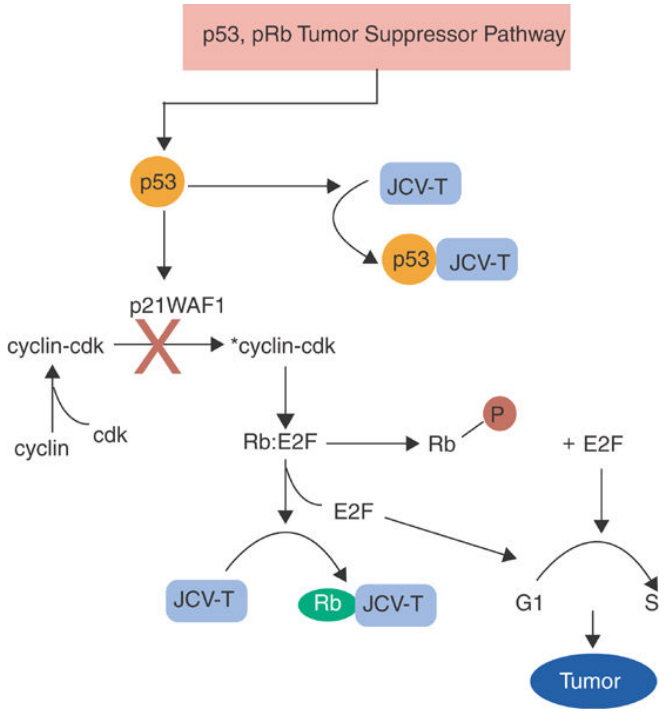


**Figure 9.** Mechanisms of the p53, pRb tumor suppressor pathways. Reprinted with permission [95].

JCV large T antigen can also bind other cellular proteins, such as insulin receptor substrate 1 (IRS-1), β-catenin, the neurofibromatosis type 2 gene product and the antiapoptotic protein survivin [96-100]. IRS-1 is a membrane associated tyrosine kinase, its activation causes cell proliferation and activates antiapoptotic signals. Large T antigen can bind IRS-1, causing its translocation into the nucleus. This translocation is important in one of the mechanisms involved in DNA repair, the homologous recombination (HR). Normally, the Insulin Growth Factor-I Receptor (IGF-1R)/IRS-1 signalling pathway supports HR because of a direct binding between Rad51 and hypo-phosphorylated IRS-1 in the cytoplasm. When IGF-1R is being stimulated, IRS-1 becomes phosphorylated and loses the capacity to complex Rad51. This protein can now translocate into the nucleus, where it is involved in

mechanisms that support HR DNA repair [101, 102]. Large T antigen inhibits HR because it supports the binding between Rad51 and IRS-1 in the nucleus. This leads to an accumulation of DNA mutation and eventually malignant phenotype [96, 101, 103]. Furthermore, IRS-1 is the key component of IGF-1 signalling pathway. The binding with large T antigen leads also to abnormal phosphorylation and activity of MAPK and PI3K, leading to tumor development (Figure 10) [104].

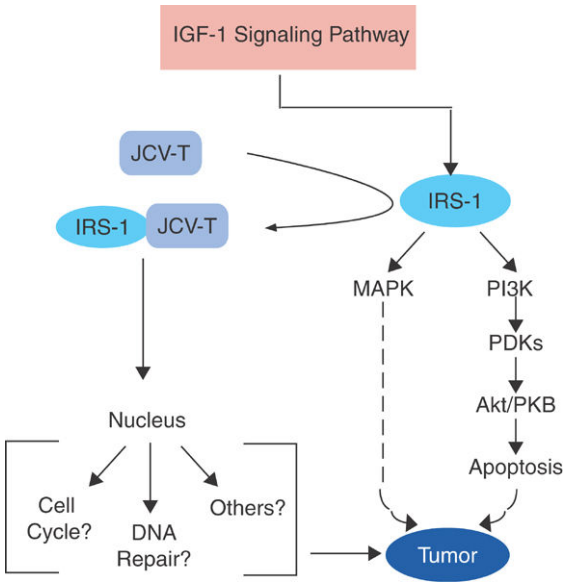


**Figure 10.** Mechanisms of the IGF-1 signalling pathways. Reprinted with permission [95].

Several studies have demonstrated the involvement of Wnt signalling in various cancers associated with JCV. It was theorized that JCV was involved in the deregulation of the Wnt signalling pathway, in particular the large T antigen binding with β-catenin, a protein belonging to the Wnt pathway. Large T antigen induces the stabilization of β-catenin, which increases in protein level and translocates into the nucleus. Here, β-catenin is able to induce the

transcription of C-Myc and cyclin D1, proteins involved in the cell life cycle (Figure 11) [105].

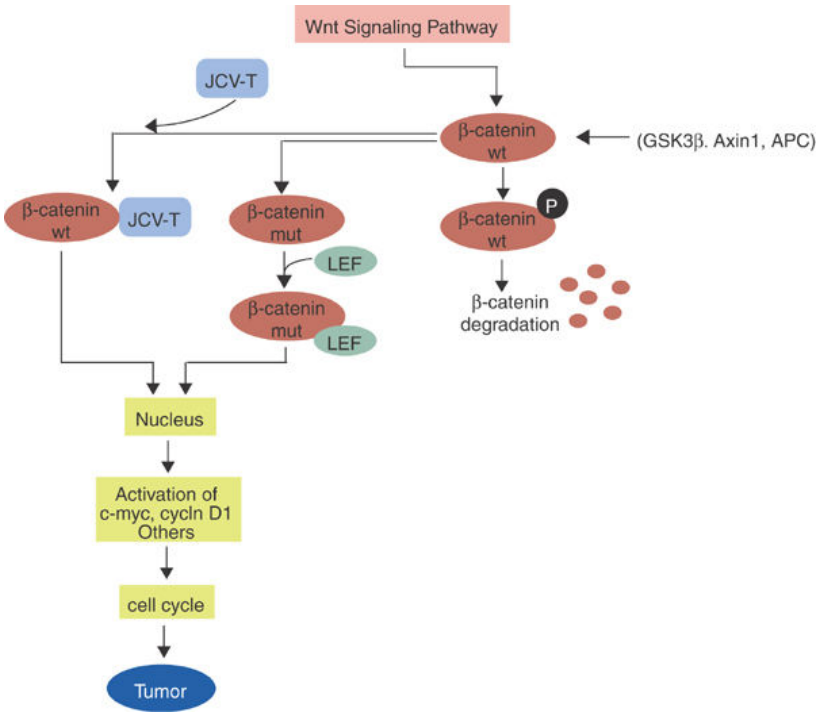


**Figure 11.** Mechanisms of the Wnt signalling pathway. Reprinted with permission [95].

In Figure 12 a schematic summary of the molecular mechanisms of cell transformation mediated by large T antigen.

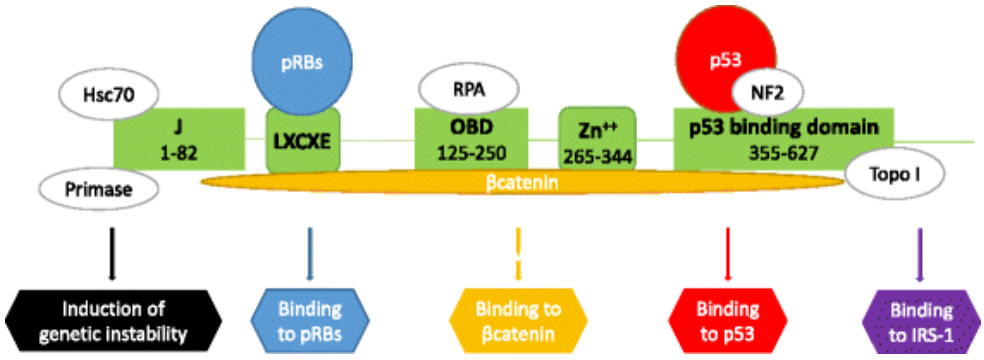


**Figure 12.** Molecular mechanisms of cell transformation mediated by large T antigen. Reprinted with permission [2].

### 1.2.7 JCV-Induced Mouse Medulloblastoma

In order to understand the tissue specificity of JCV and its potential role in inducing diseases, in 1999, Krynska et al [106] generated a transgenic mouse containing the early region of JCV. Using this mouse model, the same group, in 2000 [107], described two distinct sub-populations of cells. These sub-populations were derived from the tissues taken from this animal model, that is very related to the human medulloblastoma.

As shown in Figure 13, the two populations of cells show a very different morphological appearance.

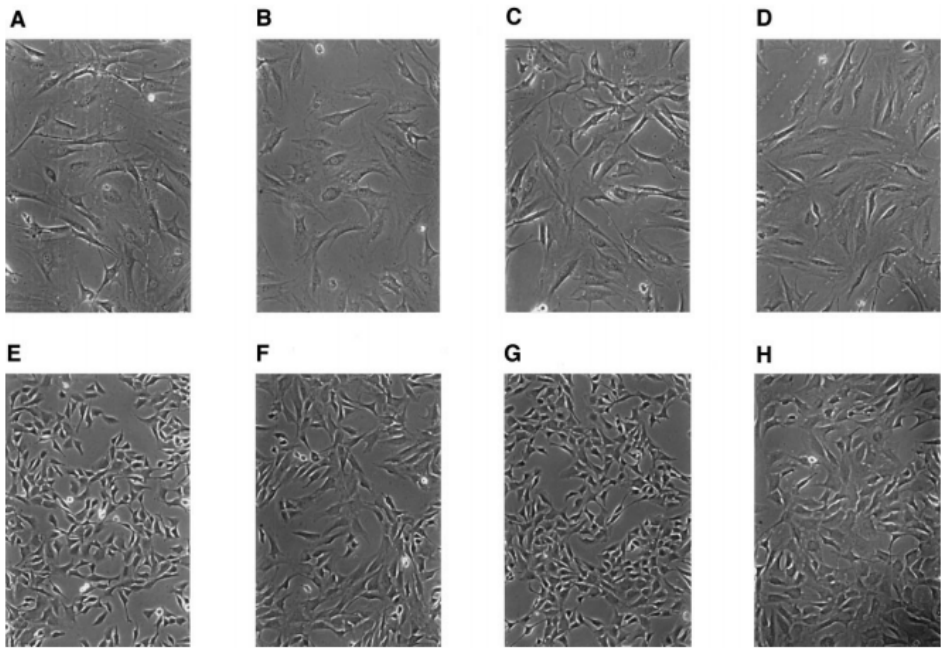


**Figure 13.** Morphological appearance of clonal cell lines derived from a mouse medulloblastoma; From A through D: BS-1a, BS-1b, BS-1c and BS-1f, four clonal cell lines that exhibit a moderate degree of cytoplasm and a process-bearing morphology. From E through H: BS-1B7, BS-1B8, BS-1B13 and BS-B14, four cell lines with scant cytoplasm and shorter processes. Reprinted with permission [107].

In this paper, the authors described a population of cells that was T antigen negative and a population that was T antigen positive. They developed four different cells lines for each population and named BS-1a, BS-1b, BS-1c and BS-1f the T antigen-negative cells, BS-1B7, BS-1B8, BS-1B13 and BS-B14 the T antigen-positive cells. Figure 14 shows the immunohistochemical staining for JCV T antigen, neuronal marker filament, synaptophysin and GFAP in BS-1a and BS-1B7.

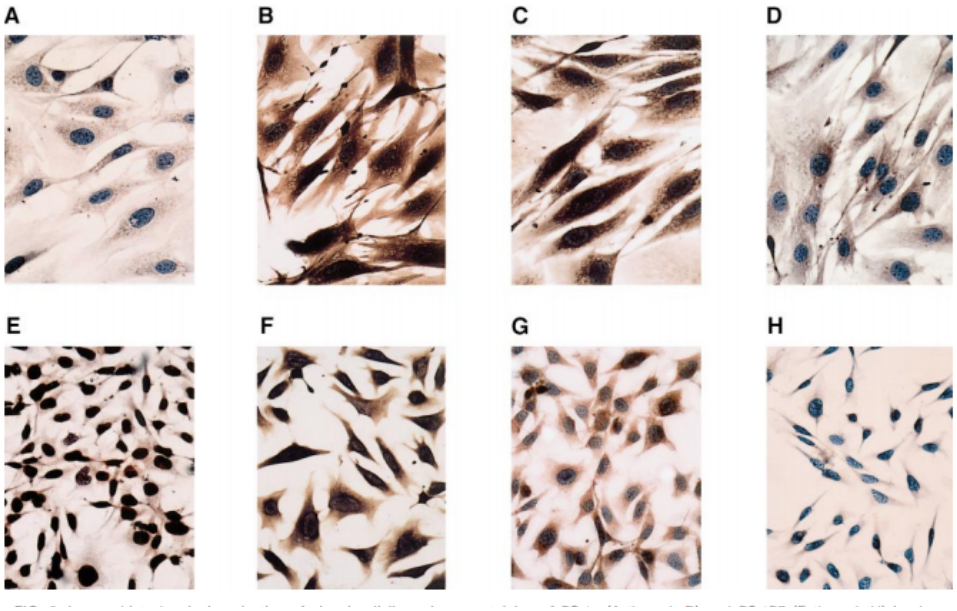


**Figure 14.** Immunohistochemical evaluation of BS-1a (A through D) and BS-1B7 (E through H). Panel A and E reveal the presence of JCV T antigen in nuclei of BS-1B7 cells but the absence in nuclei of BS-1a. Positive staining for neuronal cellular marker (B and F) and synaptophysin (C and G) is observed. Negative immunoreactivity for glial marker GFAP (D and H). Reprinted with permission [107].

After characterization, the authors studied the tumorigenicity of these cells. They observed that all the T antigen-positive cells were able to produce large tumors in nude mice, while only one T antigen-negative cell line was able to produce a small tumor.

## 2. AIMS OF THE STUDY

The human polyomavirus JC (JCV) is a member of the Polyomaviridae family, isolated for the first time in 1971 from the brain of a patient with progressive multifocal leukoencephalopathy [10]. JCV infects only humans, and within human cells it has a tissue-specific tropism. JCV infection in non-permissive cells leads to a transforming phenotype, with the expression of early protein large T antigen [33, 34].

Previously, two population of cells derived from a murine brain tumor induced by JCV injection have been characterized [106, 107]. This tumor is correlated to the human medulloblastoma, a very aggressive and invasive tumor of the cerebellum. It is one of the most common neoplasms in children. Cells characterized within the two populations were either T antigen-negative or T antigen-positive.

Ionizing radiations are one of first treatment in tumor therapies. Since the populations were derived from a tumor, we culture this two populations of cells and we irradiated them to study their viability after irradiation. Surprisingly, after radiation treatment, we noticed that some of the T antigen-positive cells were still viable.

Aims of this thesis are: characterize the radiation resistant cells, named BSB8-RR (Radiation Resistant) and the radiation sensitive cells, named BSB8. After characterization, we aim to understand if JCV T antigen has an effective role in the resistance to radiation. In fact, radiations cause DNA double strand breaks and previously, it has been demonstrated a link between JCV T antigen and mechanisms for repairing DNA [101]. We would like to understand if the expression level of large T antigen changes among BSB8 and BSB8-RR and if this change can affect the resistance to radiation.

## 3. MATERIAL AND METHODS

### 3.1 Cell lines and cultures

The mouse medulloblastoma T-antigen-positive, BSB8, as well as T-antigen-negative, BS1A were previously described [106]. BSB8-Radiation Resistant (RR) were developed irradiating BSB8 cells with 6-Gy energy and culturing resistant cells arose. BS1A, BSB8 and BSB8-RR cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% heat-inactivated FBS and 100 µg/ml penicillin/streptomycin. All the cells were maintained at 37°C in a humidified atmosphere with 7% CO2.

### 3.2 MTT assay for cell viability

BSB8 and BS1A cells were plated in a concentration of 3 x $10^5$ cells/well in 6-well tissue culture plates and irradiated with a different amount of ionizing radiation (3-Gy, 6-Gy or 12-Gy). Forty-eight hours after treatment, cells were incubated with 1 ml of MTT (3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide) at 0.5 mg/ml working solution for 2 hours at 37°C. The converted insoluble purple formazan was solubilized with 1 ml of acidic isopropanol (0.004 M HCl in isopropanol). The absorbance of the converted formazan was measured at a wavelength of 570 nm with a background subtraction at 650 nm. BSB8 and BSB8-RR were plated in 6-well tissue culture plates and irradiated with ionizing radiation (6-Gy). Forty-eight and seventy-two hours after treatment they were processed for MTT assay as described.

### 3.3 Western blotting

Whole cell protein extracts were washed with PBS and lysed with TNN lysis buffer (40 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% NP40, and 1% protease inhibitors cocktail). Protein concentrations were quantified using Bradford reagent. Protein extracts were then heated to 95°C

for 5 min and resolved through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were transferred to nitrocellulose membranes in a transfer buffer containing 25 mM Tris base (pH 7.4), 200 mM glycine, and 20% methanol. After transfer, membranes were blocked for 30 min at room temperature with 10% non-fat dry milk in 1X phosphate-buffered saline containing 0.1% Tween-20 (PBST). After blocking, membranes were incubated with primary antibodies overnight at 4°C. The membranes were subsequently washed three times with PBST and incubated with secondary antibodies at a dilution of 1:5000 for 1 h at room temperature. After secondary antibodies incubation, membranes were visualized with an Odyssey CLx Imaging System (LI-COR).

### *3.4 Cell cycle*

BSB8 and BSB8-RR were harvested at three different time point: 0h, 12h, 24h after FBS deprivation for twenty-four hours. Cells were washed with PBS, resuspended in 1 ml of PBS and added drop-wise to 4 ml of 88% ethanol to fix them, in a final concentration of 70% ethanol. Cells were then washed with PBS, resuspended in 300 µl of PBS and incubated with 10 µg/ml of propidium iodide and 100ug/ml solution of RNase A at 37°C for 30 minutes in the dark. After incubation, cells were cooled at 4°C and data were acquired using a Guava EasyCyte Mini flow cytometer (Guava Technologies).

### *3.5 Colony formation assay*

To assess tumorigenesis, BSB8 and BSB8-RR were transfected with pcDNA3.1 (+) and after twenty-four hours harvested and replated in 100-mm dishes. Cells were kept in DMEM complete medium with G418 for selection until colonies formation. To count the number of colonies, cells were fixed and stained with a solution of with 0.1% crystal violet in 20% (vol/vol) aqueous methanol.

### *3.6 Soft agar growth*

BSB8 and BSB8-RR were transfected with pcDNA3.1 (+) and after twenty-four hours harvested and seeded in 60-mm dishes containing 2 mL of a 0.3% agarose suspension in DMEM complete medium with G418. Plates were then incubated at 37°C for three weeks.

### *3.7 Nonhomologous end joining assay for double strand DNA break repair*

The nonhomologous end joining (NHEJ) assay was performed by a modification of the method of Baumann [108] as previously described [109]. Nuclear extract of BSB8 and BSB8-RR was prepared as follows. Cells were harvested, washed with ice-cold PBS, resuspended in ice-cold hypotonic lysis buffer, denominated Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT and protease inhibitors), and left on ice for fifteen minutes. Then, lysates were centrifuged at 1,800 g for ten minutes and the supernatants were aliquoted as A1-cytoplasmic extract. Cell pellets were resuspended in ice-cold Buffer A and homogenized to 90% cell lysis and centrifuged at max speed for twenty minutes. Supernatants were aliquoted as A2/S100-Cytosolic extract. Cell pellets were resuspended in ice-cold hypertonic nuclear lysis buffer, denominated Buffer C (20 mM HEPES pH 7.9, 0.6 M KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF and protease inhibitors). After forty-five minutes under shaking at 4°C, the nuclear lysates were centrifuged at max speed for thirty minutes. Supernatants were aliquoted as nuclear extract and dialyzed overnight against buffer containing 25 mM Tris HCl (pH 7.5), I mM EDTA, 10% glycerol (vol/vol) and protease inhibitors.

The NHEJ assay was performed as follows. pBlueScript(KS+) was linearized by restriction endonuclease digestion in order to have four nucleotide 5′

overhang at one end, using BamHI, and the other end blunt with EcoRV. The resulting 3 Kb linear DNA was used as DNA substrate for the end-joining reactions. Reactions were set up in 25 mM Tris OAc (pH 7.5), 100 mM KOAc, 10 mM MgOAc, 1 mM DTT, 2 mM ATP and 200 mM dNTPs as reaction buffer. 50 µg of nuclear extracts from BSB8 or BSB8-RR was added to the reaction buffer. The reactions were then incubated for five minutes at 37°C before the addition of 400 ng of DNA substrate and then for one hour at 37°C. DNA products were deproteinized by treatment with proteinase K and analyzed by electrophoresis through a 0.7% agarose gel.

### *3.8 Homologous recombination for double strand DNA break repair*

The homologous recombination (HR) events were measured by a modification of a method previously described [110]. For the transient transfection, BSB8 and BSB8-RR cells were electroporated with different amount of an expression plasmid containing DR-GFP recombination substrate denominated pDRGFP, pCBASceI expression plasmid encoding I-SceI endonuclease and pDsRedI-Mito (Clontech, Paolo Alto, CA). The expression of I-SceI causes a double strand break (DSB) in the specific restriction site included in the DR-GFP cassette and HR events can restore the functional GFP expression. The co-transfection with pDsRedI-Mito, a plasmid that encodes for a red fluorescent protein with a mitochondrial localization, was used to calculate the efficiency of the transfection. Seventy-two hours after electroporation, cells were harvested and GFP and RFP expression was detected and quantified using Guava EasyCyte Mini flow cytometer (Guava Technologies). Stable cells for DRGFP were created as follow: BSB8 and BSB8-RR were electroporated with pDRGFP. Forty-eight hours after transfection cells were harvested and replated in 100-mm dishes with DMEM and puromycin for selection. BSB8-DRGFP and BSB8-RR-DRGFP were then electroporated with different amount of pCBASceI and

pDsRedI-Mito. After five days, cells were harvested and GFP and RFP expression was detected and quantified. The HR efficiency was established as the percentage of cells showing both green nuclear fluorescence and red mitochondrial fluorescence versus cells showing only red fluorescence.

# 4. RESULTS

## *4.1 Impact of large T antigen on resistance to radiation in JCV induced tumors*

### 4.1.1 Radiation induces apoptosis in BSB8 cells

First, we examined whether ionizing radiation has an impact on BSB8 and BS1A cells viability. Cells were irradiated with three different radiation doses: 3-Gy, 6-Gy and 12-Gy and MTT assay was performed seventy-two hours after treatment. As shown in Figure 15 panel A, radiations had no impact on BS1A cells, but a reduction in BSB8 viability was observed. The reduction was visible even at the lowest dose of radiation used. Since MTT assay does not discriminate between apoptosis and necrosis, we performed a western blot to check the expression levels of proteins involved in apoptosis and autophagy, such as caspase-3 and cleaved caspase-3, Bag3 and LC3. BS1A and BSB8 cells were irradiated with two different radiation doses: 6-Gy and 12-Gy. Seventy-two hours after treatment, whole cell protein content was extracted and analyzed. As we can see in Figure 15 panel B, there is an increase in the expression level of Cleaved Caspase-3 and a slight decrease of Bag3 level in BSB8 cells, compared to BS1A. This means that radiations stimulate apoptosis in BSB8 but not in BS1A, in a dose dependent manner. Autophagy was stimulated in both BSB8 and BS1A cells, as demonstrated by the conversion from LC3-I to LC3-II. Looking at the level of large T antigen in BSB8 after radiation, we observed that treatment reduces the expression level of this protein, suggesting that it may have a role in the sensibility to ionizing radiation.

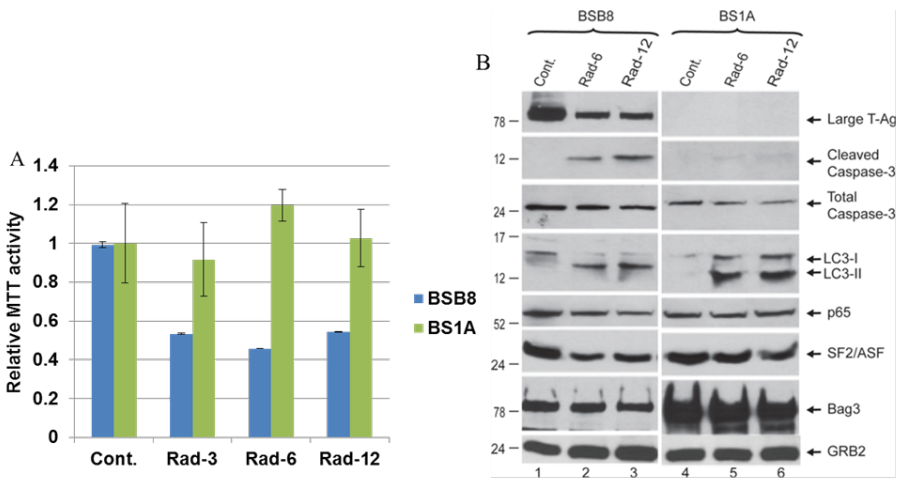


**Figure 15.** Effect of radiation on apoptosis and autophagy. Panel A: MTT assay of BS1A and BSB8 cells after treatment with ionizing radiation. Panel B: Expression of proteins involved in apoptosis and autophagy in BS1A and BSB8 cells after radiation treatment.

### 4.1.2 A small population of BSB8 cells survives after radiation

While performing our experiments, we observed that 10% of BSB8 cells were still viable seventy-two hours post-radiation. We then decided to culture these cells because we wanted to understand why they were still viable and we denominated them as BSB8-Radiation Resistant (BSB8-RR). In panel A of Figure 16 we showed the outline we used to culture these RR cells. We first irradiated BSB8 cells for MTT assay. We noticed some cells were still viable and we cultured them for some time and we confirmed the resistance to the radiation of these cells performing another viability assay after radiations. The validation of the resistance to radiation by BSB8-RR is shown in panel B and C of Figure 16: BSB8 and BSB8-RR were irradiated with a radiation dose of 6-Gy and a MTT assay was performed forty-eight and seventy-two hours after treatment. At forty-eight hours (panel B), there is no difference in cells viability between control and irradiated cell in both BSB8

cells and BSB8-RR cells. At seventy-two hours, irradiated BSB8 cells show a strong decrease in their viability, compare to the BSB8 cells not irradiated (panel C). BSB8-RR viability is not affected by radiation: in fact, even after seventy-two hours, viability of BSB8-RR irradiated is comparable to BSB8-RR not irradiated (control) viability.

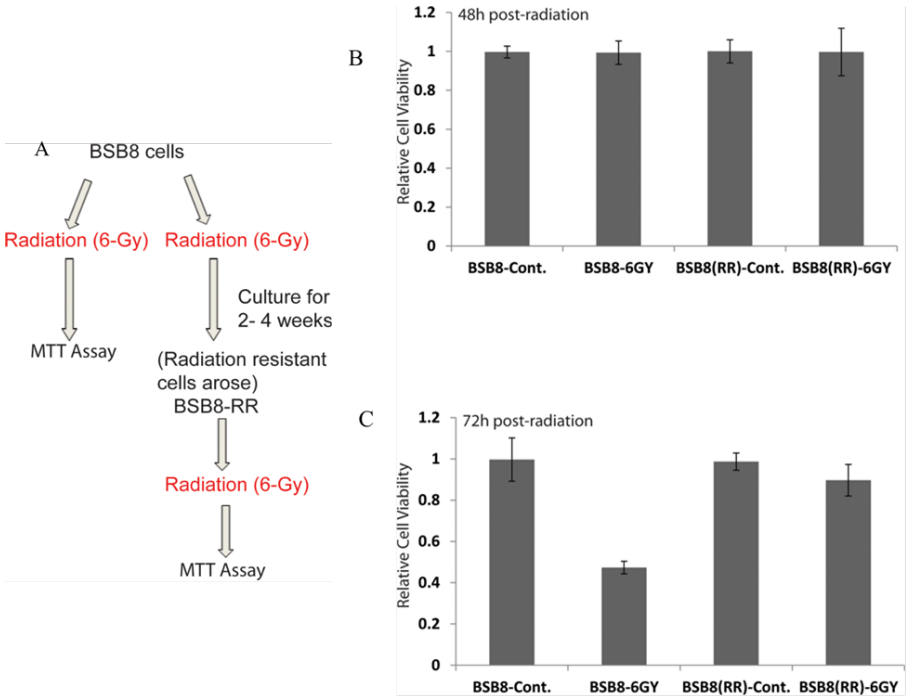


**Figure 16**. BSB8-RR cells show resistance to second radiation. Panel A: Outline of BSB8 and BSB8-RR viability assay after treatment with radiation. Panel B: MTT assay on BSB8 and BSB8-RR control or irradiated with dose of 6-Gy at forty-eight hours after radiation. Panel C: MTT assay on BSB8 and BSB8-RR control or irradiated with dose of 6-Gy at seventy-two hours after radiation.

### 4.1.3 BSB8-RR cells express reduced levels of JCV early proteins

Given this ability to resist radiation, we then investigated the role of JCV early proteins, large T antigen and small t antigen, in BSB8-RR cells. First, we extracted whole cell protein from BS1A, BSB8 and BSB8-RR and we determined the expression level of JCV early proteins. We also investigated the basal expression level of a protein linked with large T antigen, p53, and Bag3. As shown in Figure 17 panel A, the expression level of both large T antigen and small t antigen proteins is lower in BSB8-RR cells compared to BSB8 cells. As expected, BS1A cells were negative for large T antigen. Furthermore, differences in the level of p53 and Bag3 were observed. In particular, BSB8 and BSB8-RR cells expressed higher level of p53 compared to BS1A cells, probably because of the presence of large T antigen. In fact, BSB8 cells level of p53 is higher compare to BSB8-RR cells. On the other hand, BS1A cells expressed higher level of Bag3 compare to BSB8 and BSB8-RR cells. Next, we performed a time point assay on BSB8 and BSB8-RR cells to monitor the level of protein expression after radiation treatment. We irradiate the cells with 6-Gy dose of radiation and we did a whole cell extraction forty-eight and seventy-two hours post treatment. Changing in JCV early proteins levels was evaluated through western blot. As shown in Figure 17 panel B, forty-eight hours after radiation, the level of large T antigen, as well as the level of small t antigen, in BSB8 irradiated cells was lower compared to the BSB8 control, while the level of large T antigen and small t antigen in BSB8-RR irradiated cells was similar to the BSB8-RR control. Interestingly, seventy-two hours after radiation, the level of large T antigen in BSB8-RR irradiated was higher than the BSB8-RR control, while the level in BSB8 irradiated was still lower than BSB8 control.

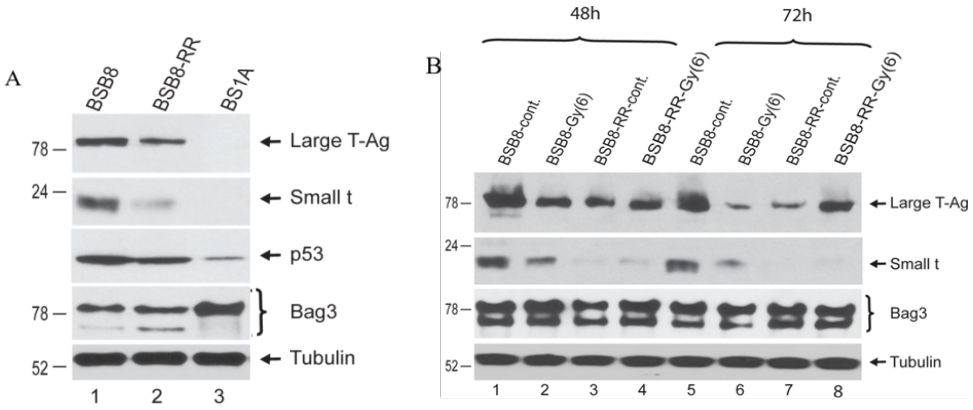


**Figure 17.** Large T antigen expression is suppressed in BSB8-RR cells. Panel A: Basal expression level of JCV early proteins in BS1A, BSB8 and BSB8-RR cells. Panel B: expression level of JCV early proteins in BSB8 and BSB8-RR cells after radiation treatment.

### 4.1.4 BSB8-RR cells show increased cell-cycle and grow faster than BSB8 cells

Since BSB8 are cells derived from medulloblastoma, we wanted to evaluate the growth characteristic and tumorigenesis capacity of BSB8 and BSB8-RR cells. In order to investigate their cell cycle, we starved the cells for twenty-four hours, culturing them in DMEM without FBS. We replaced FBS and we harvested cells at three time points, 0h-12h-24h, after replacing FBS. Cells were then fixed, incubated with propidium iodide and cell cycle analysis was performed using flow cytometry. Cell cycle analysis of BSB8 and BSB8-RR. is shown in panel A of Figure 18: at time point 0h and 12h BSB8 and BSB8-RR cells are at the same cell cycle phase. Interestingly, at time point 24h, we observed that about 70% of BSB8-RR cells were in G1/G0 phase, and 15% were in G2/M phase. Regarding BSB8 cells, only about 50% were in G1/G0 phase at time point 24h, while 30% of BSB8 cells were G2/M phase. BSB8 cells maintained the same cell cycle among the three time points. Following

cell cycle analysis, we investigate the tumorigenesis capacity of these cells. We performed a colony formation assay and soft agar growth assay and, as seen in panels B and C of Figure 18, BSB8-RR cells are able to make more colonies in both assays, compared to BSB8 cells. The colony intensity of BSB8-RR cells is 4 times higher than BSB8 (panel B) and the number of colonies formed by BSB8-RR cells is two third than BSB8 (panel C). These results suggest that BSB8-RR cells proliferate faster than BSB8 cells and they have a more aggressive phenotype.

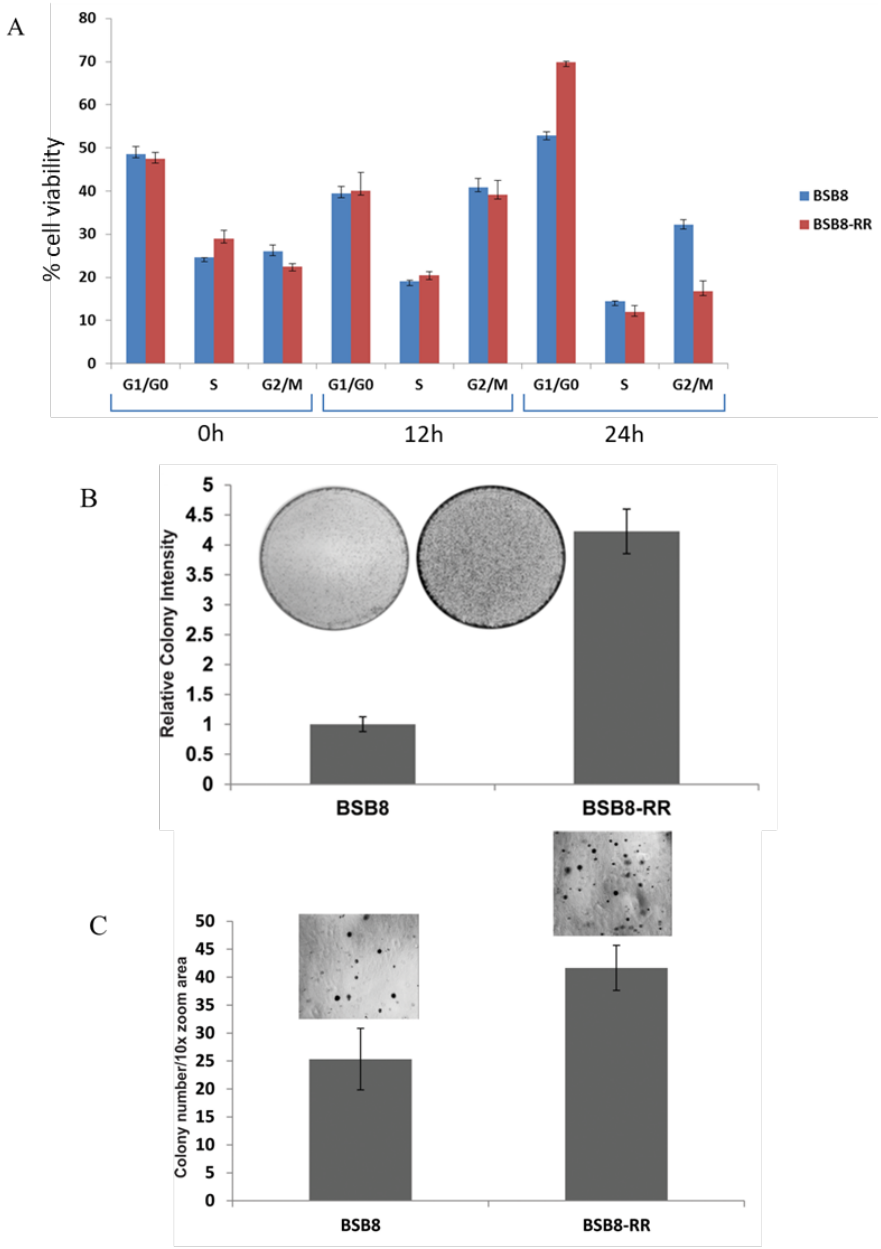


**Figure 18**. Growth characteristic of BSB8 and BSB8-RR. Panel A: Cell cycle analysis of BSB8 and BSB8-RR cells at three time point after FBS deprivation. Panel B: Colony formation assay after transfection with pcDNA3.1 and selection with G418. Panel C: Soft agar growth after transfection with pcDNA3.1 and selection with G418.

### 4.1.5 BSB8-RR cells show reduced NHEJ but increased HR activity than BSB8 cells

Since ionizing radiation causes DNA breaks and JCV large T antigen is involved in some DNA repair pathways, we wanted to investigate if the different expression level of T antigen in BSB8 cells and BSB8-RR has a role in DNA repair. We performed two types of DNA repair assay to evaluate the efficiency in repairing DNA: non-homologous end joining (NHEJ) and homologous recombination (HR). For the NHEJ assay, we prepared a nuclear extract from BSB8 and BSB8-RR cells. Figure 19 shows that nuclear extracts from BSB8-RR cells, lane 4, have lower NHEJ activity than the nuclear extracts from BSB8 cells, lane 3. In fact, in both lane 3 and 4 we can see the monomeric linear plasmid DNA at 3000 bp but in lane 3 we can see more multimeric DNA concatamers.

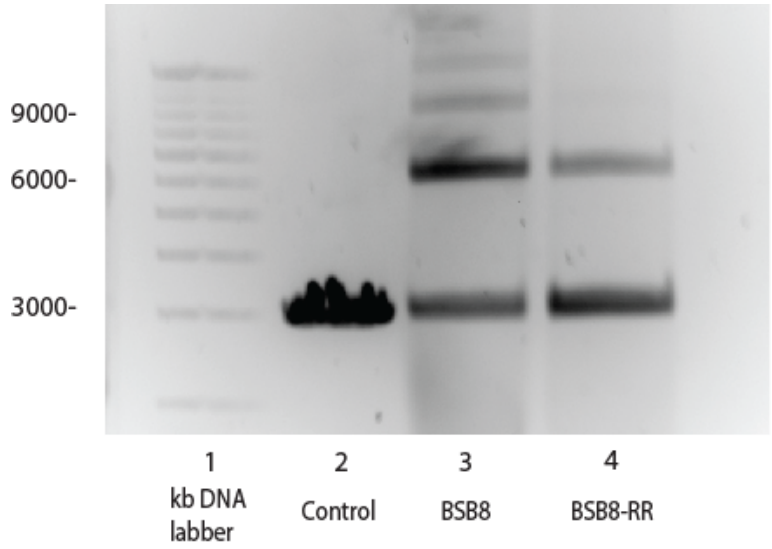


**Figure 19.** Non homologous end-joining (NHEJ) activity in nuclear extracts from BSB8 and BSB8-RR. Lane 2 is negative control without any nuclear extract. Lane 3 is with nuclear extracts of BSB8 cells. Lane 4 is nuclear extracts of BSB8-RR cells.

For the HR assay, we first performed a transient assay, transfecting cells with both pDRGFP and pCBASceI plasmids and pDsRedI-Mito for transfection efficiency. We transfected the cells with two different amount of pDRGFP plasmid: 3 μg and 6 μg. Seventy-two hours after transfection, we harvested the cells and flow cytometry was used to calculate the percentage of green and red positive cells. We harvested the cells at one time point because, since the transfection is transient, if we wait more days, we will lose the plasmid expression. The efficiency in repairing DNA using homologous recombination was established as the percentage of cells positive for both green and red fluorescence normalized with cells positive only for red fluorescence. In panel A of Figure 20, representative scatter plots of untransfected cells, transfected BSB8 and BSB8-RR with pCBASceI and pDsRedI-Mito but not pDRGFP (these are the cells only red positive) and BSB8 and BSB8-RR transfected with all three plasmids (these are cells both positive for green and red fluorescence). In panel B of Figure 20, quantification of GFP positive cells, normalized to the transfection efficiency. Quantification shows a lower ability of BSB8 cells, compared to BSB8-RR cells, to repair DNA using homologous recombination.

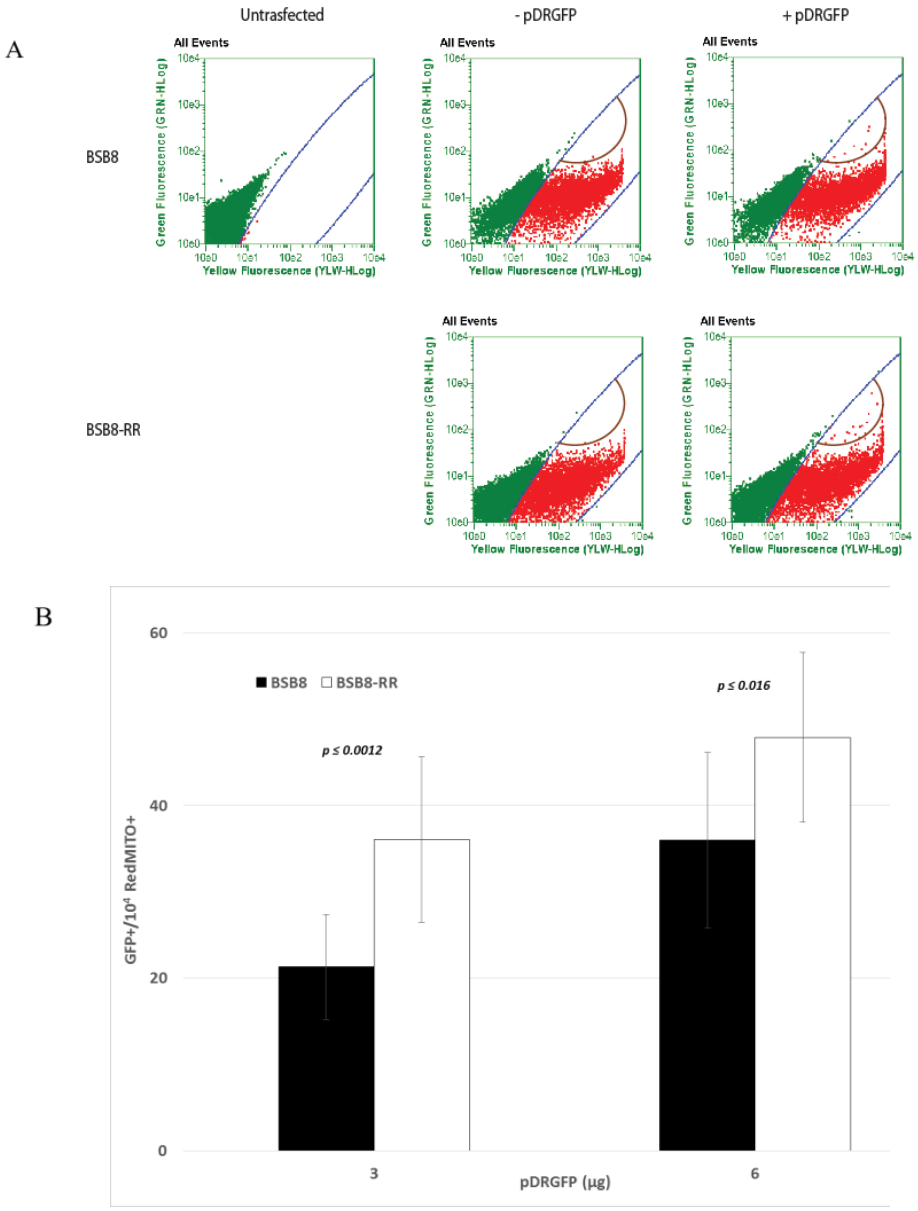


**Figure 20.** Panel A: Representative scatter plots of untransfected cells, transfected BSB8 and BSB8-RR with pCBAScel and pDsRedl-Mito but not pDRGFP and BSB8 and BSB8-RR transfected with pCBAScel + pDsRedl-Mito + pDRGFP. Panel B: Quantification of green positive cells.

Following transient transfection, we noticed the low percentage of green cells so we thought it would be better create stable cells for DRGFP. Once we grew stable cells for DRGFP, we transfected both BSB8-DRGFP and BSB8-RR-DRGFP with pDsRedI-Mito only or with pCBASceI. We harvested the cells at five days post transfection and we performed flow cytometry analysis as done for transient transfection. In Figure 21, panel A, representative scatter plots of BSB8 and BSB8-RR DRGFP stable cells transfected with or without pCBASceI. In Figure 21, panel B, quantification of HR events shows that also in DRGFP stable cells, BSB8-RR cells have a better ability to repair DNA through homologous recombination.

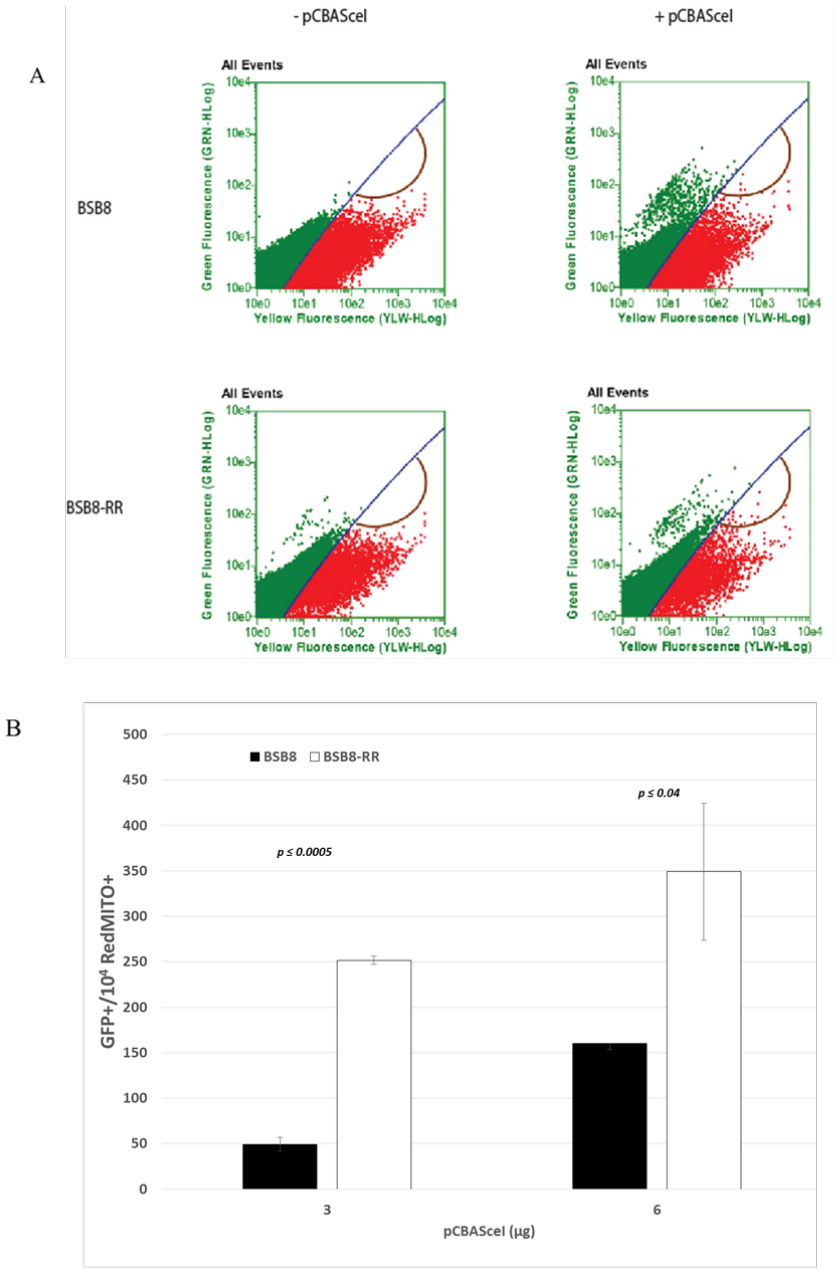


**Figure 21.** Panel A: Representative scatter plots of transfected BSB8 and BSB8-RR with pDsRedI-Mito and BSB8 and BSB8-RR transfected with pCBAScel + pDsRedI-Mito. Panel B: Quantification of green positive cells.

# 5. DISCUSSION

## *5.1 Impact of large T antigen on resistance to radiation in JCV induced tumors*

JCV is a small, naked tumor polyomavirus with an icosahedral capsid containing a circular, double-stranded DNA genome. Its genome is divided into early and late genes, separated by a non-coding control region (NCCR) containing the promoter, the origin of replication (ORI) and the enhancer elements. Early genes encode for large T antigen, small t antigen and three different T' proteins called T'135, T'136 and T'165. Late genes encode for the structural proteins VP1, VP2 and VP3, and the non-structural agnoprotein [49]. JCV shows specie-specificity to humans, probably due to the large T antigen can interact only with the human DNA polymerase. JCV has also tissue tropism for specific human cells, such as glial cells and kidney epithelial cells [34].

One example of human cells infection is the lytic infection of oligodendrocytes, that are destroyed during the process, leading to the PML, the progressive multifocal leukoencephalopathy, a rare but fatal demyelinating pathology in the brain [35]. If the expression of early genes is followed by the replication of viral DNA and expression of late genes, we have a productive infection. If the infection occurs in non-permissive cells, they are not able to support viral replication or the expression of late genes. In those cells, only the expression of early genes, such as large T antigen, is observed. The expression of this viral protein leads to genome instability and inactivation of oncosuppressor proteins that eventually can lead to tumorigenesis [2].

In 1999, Krynska et al, induced brain tumors in transgenic mice injecting the JCV early region in mouse embryos [106]. This brain tumor was very similar to human medulloblastoma in location, histological appearance and

expression of markers for differentiation. Medulloblastoma is a malignant, very aggressive tumor of the cerebellum and it is one of the most common cancer in children. In 2000, Krynska et al, derived two distinct populations of cells from the JCV T antigen transgenic mice previously described [107]. These two populations showed a very different morphological features in cytoplasm and processes but, most important, they have different expression of JCV early proteins. One population is T antigen-negative (BS1A cells), the other one is T antigen-positive (BSB8 cells).

Radiotherapy is part of cancer treatment in many patients with some form of cancer. In fact, radiotherapy is probably the most important treatment of localized tumors, after surgery. Since BS1A and BSB8 are tumor derived cell lines, we first examined whether ionizing radiation has an impact on their viability. We irradiated those cells with different doses of radiation and we performed an MTT viability assay. From our results, ionizing radiations had no impact on BS1A cells, but we had a reduction in BSB8 viability. The reduction is visible even at the lowest dose of radiations. BS1A are resistant to radiation because they are immortal but not transformed cells. In fact, after they populate, they have contact inhibition. They do not replicate and radiation is effective only on replicating cells. To complete the understanding of how radiations impact viability in BS1A and BSB8, we looked at the expression of proteins involved in apoptosis and autophagy. Our results show a stimulation of apoptosis by radiation in BSB8 but not in BS1A, in a dose dependent manner. The activation of Caspase-3 and its pathway in the cells is a signal of apoptosis [111]. Autophagy is stimulated in both BSB8 and BS1A cells, as we can see from the conversion of LC3-I to LC3-II. These results show a reduced viability in BSB8 after ionizing radiation because of apoptosis pathways have been activated. Surprisingly, the level of large T antigen in BSB8 after radiation is reduced, suggesting that it may have a role in the sensibility to ionizing radiation.

While performing the viability assay, we noticed that a very small sub-population of BSB8 cells were still viable after radiations. We decided to try to culture these cells to see if they were able to repopulate. These cells were growing very fast and we decided to denominate them as BSB8-Radiation Resistant (BSB8-RR). In order to characterize this sub-population, we first confirmed that they were able to resist to a second radiation. We plated BSB8 and the new BSB8-RR and we irradiated them with ionizing radiations. As expected, BSB8-RR viability did not change after radiations, while BSB8 viability dropped because of cell death. We did confirm the resistance to radiation of BSB8-RR cells.

After confirmed that BSB8-RR cells were resistant to radiations, we wanted to investigate if JCV early proteins may have a role in this resistance. First, we examined the basal expression level of the antigens in BS1A, BSB8 and BSB8-RR. As expected, no antigens level has been detected in BS1A. Interestingly, in BSB8-RR cells the levels of both large T antigen and small t antigen are lower compared to their levels in BSB8 cells. Furthermore, we saw differences in the level of p53, with a higher level of p53 in BSB8 and BSB8-RR compare to BS1A. This result was expected because p53 is being known to interact with large T antigen[112]. The binding between large T antigen and p53 results in uncontrolled cell spread. T antigen removes the ability of p53 to enhance transcription of p21/WAF-1, an inhibitor of cyclin kinases. In normal condition, a reduction in the kinase activity of G1/S cyclins:cdks keeps pRb in a hypophosphorylated state so it can sequester E2F. If E2F is no longer bound to pRb, it can promote unscheduled transcription of S phase genes [95]. To gain insight into the role of large T antigen and its expression level in response to irradiating radiation, we evaluated its expression level in a time-point treatment. We irradiated cells and we harvested them forty-eight and seventy-two hours after radiation. The fact that the level of large T antigen changes in response to radiation during

the time-point analysis suggested an implication of this protein in the resistance to radiation. The level in BSB8 cells decreases dramatically, as these cells are dying. The level in BSB8-RR changes between treatment, maybe because of a reactivation in their tumorigenic phenotype.

The second step in the characterization of BSB8-RR cells was to analyze their cell-cycle after a starvation period and their tumorigenic capacity. BSB8 and BSB8-RR are cells derived from medulloblastoma, known to be a very aggressive tumor. We were expecting a very aggressive phenotype but we wanted to see if the resistance to radiation had impacted this capacity. Large T antigen is the major player in cell transformation, its domains can bind and inactivate cellular proteins, that typically stop the entry into S phase, to force the cell cycle from G1 into S phase [2]. Since large T antigen levels are higher in BSB8 cells then BSB8-RR cells, we predicted more BSB8 cells in G2/M phases compare to BSB8-RR that we were expecting in G1/G0 phases. Our results on cell cycle after FBS starvation show that both cell lines share the same cell cycle phase in the first twelve hours, where they are synchronized after starvation. After twenty-four hours, there are more BSB8 cells in G2/M phase, confirming the ability of large T antigen to drive cells in S and then G2 phase. In contrast to this analysis, we observed a faster growth in cell culture flask of BSB8-RR than BSB8, suggesting a more tumorigenic phenotype. To validate this observation, we performed two assays to evaluate this tumorigenesis. The first assay was a colony formation assay, where we transfected cells with a plasmid carrying a gene for resistance to a specific antibiotic. Using this antibiotic in the culture medium we were able to select cells that were transfected and we let them form colonies. After three weeks we fixed, stained and counted the colonies. A higher number of colonies formed means a more tumorigenic phenotype. The second assay performed was similar to the colony formation assay but the culture media was supplemented with agar. This assay is a method for characterizing the

ability of transformed cells to grow independently of a solid surface, an ability called anchorage-independent growth [113]. This assay is considered to be one of the most stringent tests for malignant transformation in cells. Both cells are able to form colonies in soft agar, meaning that these cells show anchorage-independent growth ability. Normal cells are dependent on the contact between cells and extracellular matrix in order to grow and divide. Conversely, transformed cells are able to grow and divide independently of the environment where they are. If we count the number of colonies formed by BSB8 and BSB8-RR, it is very clear that BSB8-RR showed a more tumorigenic phenotype compared to BSB8.

The last step in BSB8-RR characterization was to understand why these cells are resistant to radiation. As already said, radiations can cause DNA double strand breaks (DSBs), and cells that are not able to repair DNA damage usually follow a cell death fate. We then hypothesized that BSB8-RR may be resistant to radiation because they are able to repair better and/or faster the DNA DSBs caused by ionizing radiations. Two of the major pathways to repair DNA DSBs are: non-homologous end joining (NHEJ) and homologous recombination (HR). HR seems to be preferred in proliferating cells, while NHEJ seems to be preferred in quiescent cells [114]. NHEJ is "non-homologous" because a homologous template is not necessary, the break is repaired with a direct ligation between the two ends. Homologous recombination needs a homologous sequence to perform the repair. Previous studies from our laboratory have shown an attenuation of homologous recombination in cells presenting large T antigen [101]. Indeed, another protein that large T antigen can bind is the insulin receptor substrate 1 (IRS-1). This protein is involved in the mechanism of DNA repair, in fact the IGF-1R/IRS-1 signaling pathway supports homologous recombination because of a direct binding between Rad51 and hypo-phosphorylated IRS-1. When IGF-1R is stimulated, IRS-1 becomes phosphorylated and, in this

phosphorylated status it cannot interact with Rad51. Rad51 can translocate into the nucleus and stimulate homologous recombination [101, 102]. Large T antigen has an inhibitory role in HR because it sustains IRS-1:Rad51 complex in the nucleus at the DNA damage site, preventing HR. This leads to an impairment in homologous recombination but NHEJ is still functional [101]. To assess if the lower expression of large T antigen in BSB8-RR could lead to a major functionality in HR and so in repair DNA DSBs we performed DNA repair assays, NHEJ and HR. Our results show a functional NHEJ repair assay in both cells, but with a lower NHEJ activity in BSB8-RR compare to BSB8. This result was predictable, because BSB8 are more in a quiescent status and large T antigen has no impact on NHEJ functionality. On the other hand, HR is more efficient in BSB8-RR cells, in both transient transfection and stable cells line. We decided to create a stable cell line for DRGFP because HR assay needs time. In this assay we evaluated cell cycle related DNA repair assay, cells need time to grow and divide in order to perform HR. Transient transfection shows the same results as stable cell lines but the count of GFP positive cells per RED positive cells was very low in transient transfection. The presence of higher level of large T antigen in BSB8 cells may lead to an impairment in the repair of DSBs caused by ionizing radiations. Thus, when we irradiate BSB8 cells with ionizing radiations, their viability is compromised, they start dying because they can not repair the breaks in the DNA. Unlike BSB8, the lower level of large T antigen in BSB8-RR permits a more efficient, and error-free, repair of DSBs caused by ionizing radiations, allowing BSB8-RR cells to survive and proliferate.

## 6. CONCLUSION

In this thesis we characterized a new type of BSB8 cells, denominated BSB8-RR. These cells show a resistance to ionizing radiation, compared to the wild-type BSB8. We first demonstrated the effective resistance to a second radiation of BSB8-RR. We then investigated the level of large T antigen expression in these two cell lines and we characterized their growth characteristic and their tumorigenesis, anchorage-dependent or independent. After showing a more tumorigenic phenotype of BSB8-RR compared to the BSB8 cells, we investigated the ability of these cells in repairing DNA DSBs. BSB8 cells have an increase in NHEJ pathway while BSB8-RR perform a better HR repair. We hypothesized a direct effect of large T antigen in DNA repair, because it has been demonstrated that this protein inhibits HR. We confirmed that the difference in large T antigen level in BSB8 and BSB8-RR has consequences on homologous recombination efficacy. As a consequence of the lower level of large T antigen in BSB8-RR cells and better DNA repair, this protein may not even be necessary for their growth. Our future directions are to knock-down large T antigen in both BSB8 and BSB8-RR cells using shRNA for large T antigen and CRISPR/Cas9 system, previously developed in our department [115]. Our idea is that BSB8-RR viability is now independent from large T antigen and, even without this protein, BSB8 are able to grow and divide. We will knock-down large T antigen and we will perform colony formation assay. Furthermore, we want to study the effect of splicing factors protein on T antigen expression. We created different plasmid, for transient and stable expression, of all the twelve splicing factors protein known so far. We would like to investigate if over-expression of these splicing factors protein can influence large T antigen expression, as already described in our lab for SRSF1 [116-118].

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## 8. SCIENTIFIC PRODUCTS

Sariyer, R., De-Simone, F. I., **Donadoni, M.**, Hoek, J. B., Chang, S. L. Sariyer, I. K. Alcohol-Mediated Missplicing of Mcl-1 Pre-mRNA is Involved in Neurotoxicity. Alcohol Clin Exp Res. 2017 Oct;41(10):1715-1724.

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