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TRANSFORMATION OF B LYMPHOCYTES BY SV40, A SMALL DNA TUMOUR VIRUS

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Introduction

Simian virus 40 (SV40) is a small DNA tumor virus, linked with specific human cancers such as malignant pleural mesothelioma (MPM), brain and bone tumors, leukemia, lymphoma diseases. Its DNA sequences is also detected in healthy blood donors. While the debate on SV40 association, rate of prevalence and detection of its DNA sequences in different human cancers including non-Hodgkin lymphomas (NHL) are at increase, indebt studies on the interaction between lymphocytes and SV40 has not yet been fully described.

The highlighted issue calls for further imperative investigation. Hence, this study investigated the interaction of SV40 virus in healthy blood donors using three different experiments. Experiment I: "Simian virus 40 sequences of healthy individuals from Aviano Cancer center (Italy)." The experiment aimed at detecting SV40 DNA sequences in healthy blood donors. Experiment II: " SV40 infection of human T lymphocyte." It aimed at investigating the rate of susceptibility of purified T lymphocytes to SV40 infection. While the third experiment-"Transformation of human B cells by SV40, a small DNA tumour virus" investigated the interaction of SV40 with purified B lymphocytes to observe the capacity of SV40 to infect, transform, and immortalize B cells. These three studies/experiments indeed, indicated the potential role of SV40 in Lymphomagenesis, its relationship and effects with human lymphocytes. Detailed information of these experiments is depicted in results session, each starting with a short abstract.

1.1: The discovery of the simian virus 40, SV40

SV40 belongs to the Polyomaviridae, a family of small DNA viruses that comprises the human polyomaviruses BK virus (BKV) and JC (JCV). BKV and JCV infect 70-90% of the adult population (1,2,3,4). The family name is derived from the first recognised member of the

Polyomaviridae, the murine polyomavirus (MuPyV), originally discovered in 1953 by Ludwik Gross as a source of salivary gland tumours in mice. MuPyV was initially designated 'polyomavirus' from the Greek 'poly' meaning many and 'oma' meaning tumours, due to its ability to induce a variety of solid tumours in mice (5,6).

SV40 discovery was linked to the development of the anti-polio vaccines in the 1950s and 1960s as poliovirus was grown in primary kidney cells of rhesus and cynomolgus macaques, which were often naturally infected with SV40 (7,8,9). The virus was then inadvertently introduced into the human population through administration of these contaminated anti-poliovirus vaccines. Soon after its isolation, to verify whether SV40 is able (i) to transform animal and human cells and (ii) to induce tumors in animal models this viral agent was intensely studied both in vitro and in vivo. It turned out that SV40 transforms different types of animal and human cells and induce in experimental animals different kind of tumors, depending on the inoculation route. Consequently, SV40 became known as a potent DNA tumour virus. In the past two decades, increasing evidence of the presence of SV40 in human tumours of different histotypes has seen this 'monkey virus' emerge as a potential human pathogen, a topic that is continually under debate (10,11).

1.2: *Polyomavirus Infection in the Natural Host*

There are 22 existing known members of the Polyomaviridae, capable of infecting a range of species including monkeys, rodents and birds. The phylogenetic analysis of the polyomaviruses has revealed three genetically separate groups: (a) avian polyomaviruses, (b) mammalian polyomaviruses linked to MuPyV and (c) mammalian polyomaviruses linked to SV40. The natural host of SV40 is the Rhesus macaque (macaca mulatta), but several related species of monkey are also capable of being infected (12,13,14).

The spreading of SV40 in the monkey is thought to be by viral shedding in the urine, with host infection occurring by the oral, respiratory and subcutaneous routes. In healthy monkeys, SV40 appears to cause a low level persistent infection in the kidney, this demonstrates that SV40 is a nephrotropic virus (14). In immune compromised monkeys with Simian Immunodeficiency Virus (SIV), SV40 is associated with widespread infection, with the virus being detected in the brain, lung, kidney, lymph node, spleen and peripheral blood, suggesting SV40 may also have neurotropic and lymphotropic properties (15,16). Polyomaviruses are also present in the human host. BK virus (BKV) and JC virus (JCV) are exclusive human viruses and were both discovered in 1971 (17,18).

Recently, three new human polyomaviruses have been discovered (Table1, 12); KI virus (KIV), WU virus (WUV) and Merkel cell virus (MCV). The novel polyomaviruses KN and WUV have been established in respiratory fluids in individuals with respiratory infection and MCV is associated with Merkel Cell Carcinoma, a rare but aggressive human cancer of neuroendocrine origin (13,19,20,). Phylogenetically, MCV is the only human polyomavirus that does not belong to the SV40 subgroup (21). This most recently discovered member of the *Polyomaviridae* has been shown to have the highest homology to the mouse polyomavirus subgroup and is most closely related to the lymphotropic polyomavirus (LPV, African green monkey polyomavirus), presumed to be of simian origin. While the polyomaviruses are thought to be highly species-specific and are believed to evolve in close association with their host, the close evolutionary relationship of MCV to LPV and SV40 to BKV and JCV calls this concept into question and may indicate the possibility that host switching can occur (21).

Table 1.1 Members of the *polyomaviridae* family

VIRUS	NATURAL HOST	YEAR OF DISCOVERY
Murine polyomavirus	Mouse	1953
Murine pneumotropic virus	Mouse	1953
SV40	Monkey (rhesus macque)	1960
SA12	Monkey (chacma baboon)	1963
Rabbit polyomavirus	Rabbit	1964
Hamster polyomavirus	Hamster	1968
BK virus	Human	1971
JC virus	Human	1971
Bovine polyomavirus	Cattle	1974
Lymphotropic papovavirus	Monkey (African green monkey)	1979
Avian polyomavirus	Bird	1981
Rat polyomavirus	Rat	1984
Baboon polyomavirus type 2	Baboon	1989
Cynomolgus polyomavirus	Monkey (cynomolgus)	1999
Goose hemorrhagic polyomavirus	Goose	2000
Chimpanzee polyomavirus	Chimpanzee	2005
Crow polyomavirus	Bird (crow)	2006
Finch polyomavirus	Bird (finch)	2006
KT polyomavirus	Human	2007
WU polyomavirus	Human	2007
Squirrel monkey polyomavirus	Monkey (Squirrel monkey)	2008
MC polyomavirus	Human	2008
		(12)

1.3: SV40 infection in humans

SV40 infection in the human host was seen as a rare event and only restricted to people living in contacts with the natural hosts-monkeys such as inhabitants of Indian villages located close to the jungles and workers attending to monkeys in zoos and animal facilities. Its association with the human host dates back to the 1950s and 1960s when SV40 contaminated vaccines occurred due to the ability of the virus to survive the formalin treatment used to inactivate the poliovirus (22). Human populations were exposed to SV40 by contaminated vaccines administered to hundreds of millions of people in United States, Europe Canada, Asia and Africa between the years of 1955-1963 (9, 23). Additionally, it is thought that a major European manufacturer distributed SV40 contaminated vaccines until as recently as 1978 (24).

Soon it was shown that children vaccinated with contaminated oral polio vaccine (Sabin vaccine or OPV) shed infectious SV40 in stools for at least 5 weeks after vaccination (25). Some children who received the same OPV, did not develop neutralizing antibodies even though they may have

received large doses of live SV40 compared with the potential inactivated SV40 in inactivated polio vaccine (Salk vaccine or IPV). Additionally, SV40 human contamination occurred in experimental infection with live respiratory syncytial virus to adult volunteers and a neutralizing antibody response in about two thirds of the volunteers was revealed. Inactivated vaccines against adenoviruses and hepatitis A virus contributed to human exposure to SV40, even though, the amount of SV40 infections was almost certainly lower than that administered with OPV or live respiratory syncytial virus (26, 27, 28).

The discovery of SV40 contaminated vaccine raised concerns and prompted research into the potential risk to human health. Soon after the identification of SV40 as a polio vaccine contaminant, several studies with hamsters started observing its generation of tumours in experimental animals. It was discovered that subcutaneous injection of rhesus monkey kidney cell extracts into newborn hamsters led to the formation of sarcomas at the site of inoculation (29). Afterwards, it was observed that intracranial injection of SV40 into hamsters induce ependymonas, a type of brain cancer (30). The Syrian golden hamster has been developed as an animal model for SV40-induced tumours with primary brain cancers, malignant mesotheliomas (31), bone tumours (32a) and systemic lymphomas (32b), developing in a manner depending on route of SV40 inoculation.

Furthermore, early serologic studies reported the presence of SV40 neutralizing antibodies at different titers, in the population that received IPV. Immune response appeared to correlate with the amount of SV40 present in the vaccine; 30-50% of individuals reached a significant antibody response against formalin-inactivated SV40 after three doses of the vaccine. Antibody titers persisted for a period of up to 3 years post inoculation. Additional serologic studies reported SV40 seropositivity in individuals with no history of immunization with contaminated IPV or other possible routes of SV40 infection. Studies such as Shah et al, detected antibodies to SV40 in adults and children born 1954 and after 1964 respectively, when IPV was free of SV40. This suggests that,

there is the possibility of human infection by SV40 irrespective of initial exposure to contaminated polio vaccine and that SV40 is being transmitted in the population to this day (1, 4, 33, 34).

To date, the route of transmission and prevalence of SV40 infection in human is still largely unknown. Serological studies have yet to provide comprehensive SV40 prevalence data. Nevertheless, recent studies with PCR and serological techniques indicate that SV0 infection occurs both in children and adults since SV40 DNA sequences was detected in normal and neoplatic tissues of people too young (1-30 years) or too old (60-80 years) to have been vaccinated with SV40-contaminated anti-polio vaccine. This finding may explain the lack of difference in cancer incidence between individuals vaccinated with SV40-contaminated and SV40-free anti-polio vaccines. Secondly, SV40 sequences and Tag were detected in blood and sperm specimens from normal individuals, oncological patients and in lymphoblastoid cells, which suggests that human PBMCs may represent a reservoir and vehicle of SV40 spreading in the tissues of the host and among individuals. Thirdly, detection of SV40 sequences in urine and stool samples from both children and adults indicates the possibility of haematic, sexual and or fecal routes of transmission, which are likely to be responsible for SV40 horizontal infection in humans. Furthermore, there has been detection of specific antibodies to SV40 capsid antigens in human sera, though, there was no comparative data on SV40 DNA prevalence in PBMC and antibodies presence to SV40 antigens in sera from the same patients (35).

Significantly, following laboratory experiments using hamsters, SV40 was shown to transform many human cell types in culture (36). In 1964, an interesting albeit unethical study by Jensen et a1., demonstrated that SV40-transformed human cells were able to produce subcutaneous tumours when injected into human volunteers (37). These SV40 transformed human cells grew as subcutaneous nodules for two weeks and then regressed, possibly because of an immune response. These early studies of SV40-mediated tumour induction in animal models indicated that SV40 was a potent cancer-causing virus and fuelled concerns about the exposure of the human population to

the SV40 in contaminated polio vaccines. SV40 has since been detected in various human tissues, both normal and malignant (11, 38, 39, 40).

Presently, the association between SV40 and human cancer remains very controversial. The discovery of the new human polyomavirus, MCV, integrated into merkel cell tumours, and the continuing studies on the cancer association of BKV and JCV, adds weight to the association of this family of viruses with human cancer.

Comparism of SV40, BKV and JCV

BKV and JCV are exclusive human pathogens and were both discovered in 1971 (17, 18). BKV and JCV infection is widespread in humans and usually occurs in childhood. Seropositivity for BKV reaches 90% in children aged five to nine, with JCV seropositivity at 50 to 60% by the age of ten (41). BKV infection usually occurs at an earlier age than JCV (42).

JCV and BKV share 69% sequence homology with SV40. The greatest homology is found in the early region coding for Tag and tags, whereas a lower homology is detected in the regulatory region. SV40 is phylogenetically closely related to JCV and BKV. They also evidence similarity with respect to size (5.2 Kb), genome organization and DNA sequence. The Tags of SV40, BKV and JCV strongly cross-react with the same antisera, while a less strong cross-reactivity is observed in most structural antigenic determinants of the viral proteins, named VP1, 2 and 3. The DNA sequences of SV40 share 70% homology with BKV, and 69% with JCV (41, 42).

BKV was first isolated from the urine of a renal transplant patient with ureteric stenosis (17). Although the virus is ubiquitous, it does not cause disease in the healthy host, but can produce pathological effects in immunocompromised individuals such as renal transplant recipients, in whom it can affect as many as five percent (43, 44). JCV was first isolated from the brain tissue of a patient with progressive multifocal leukoencephalopathy (PML) (18), a demyelinating disease of

the central nervous system caused by a lytic infection of oligodendrocytes (42). Similar to BKV, JCV is also associated with disease in the immunocompromised host, with PML usually only developing in individuals with a severely compromised immune system. PML was rare before the emergence of Human Immunodeficiency Virus (HIV) but this disease now affects about 5% of HIV-infected patients and is considered to be an Acquired Immunodeficiency Syndrome-defining disease (45).

1.4: SV40 Structure and Genome

SV40 is a non enveloped DNA tumour virus . The virion is about 45 nm, an icosahedral particle, with a density of 1.34–1.35 g/cm3. The viral genome is a circular, double-stranded DNA molecule of 5,243 bp in length in the reference strain SV40-776, with slight nucleotide variations in other strains, subdivided into three functional domains (34). On a genomic level SV40 is closely related to BKV and JCV, sharing approximately 70% homology (11). Although these three viruses are closely related, they can be distinguished at the DNA and protein levels and also serologically by neutralisation and haemagglutination inhibition assays (14). The genomic organisation of SV40, BKV and JCV is conserved with the genomes divided into three functional domains: the early region, the late region, and the regulatory region (46, 47, 48). The early genes are transcribed from one strand of the genome and the late genes are transcribed in the opposite direction from the complementary strand (42). The SV40 early viral coding regions (figure 1) comprises; the large T-antigen (Tag), small t-antigen (tag) and the 17kT (tiny T antigen). On the other hand, the late region encodes the structural proteins VP1, VP2 and VP3 and a small regulatory protein called agnoprotein, sometimes referred to as VPx (49). There is also, the pre-micro RNA (miRNA) in every mature virus (48).
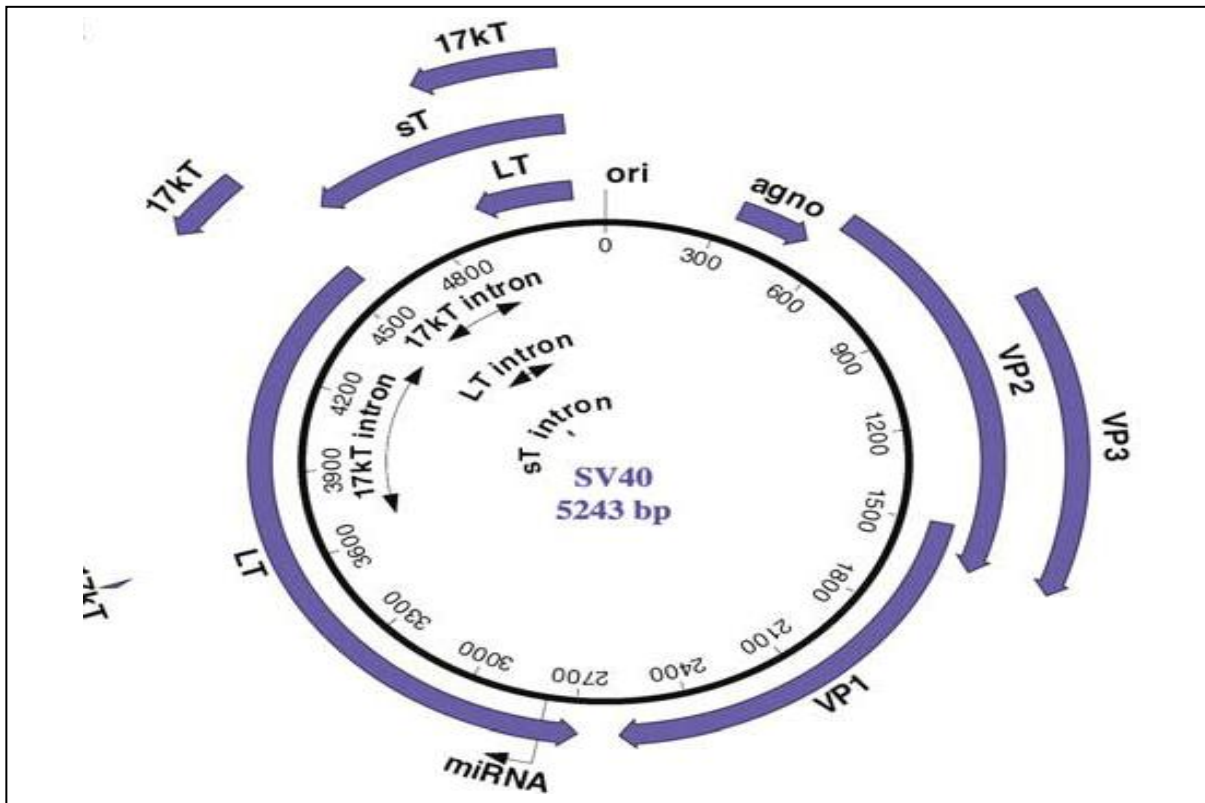
Figure 1.1: simian virus 40 (SV40) genome, showing the Tag, tag and tiny T antigen (17kT) in the early region. In the late region of the viral genome, the capsid proteins VPl, VP2 and VP3 are shown along with the regulatory protein, agnoprotein [agno] and a pre-microRNA (miRNA). The regulatory region (ori) contains sequences for the early and late promoter and the origin of replication (48).

1.4.1: SV40 Late Genome Region

The Capsid Proteins and Functions

The atomic resolution of the SV40 capsid is formed from the VP1(40kD), the major capsid protein forming the pentameric capsomers that make up the surface of the virus particle, the identical minor capsids VP2 (39kD) and VP3 (27kD) (14). The VP1 capsomers along with the VP2/3 complexes are then joined by the C terminal arms of VP1 to form the icosahedral capsid surrounding the minichromosome (Figure 1.3) and the four host-derived core histones [H2A, H2B, H3 and H4] (48, 50).
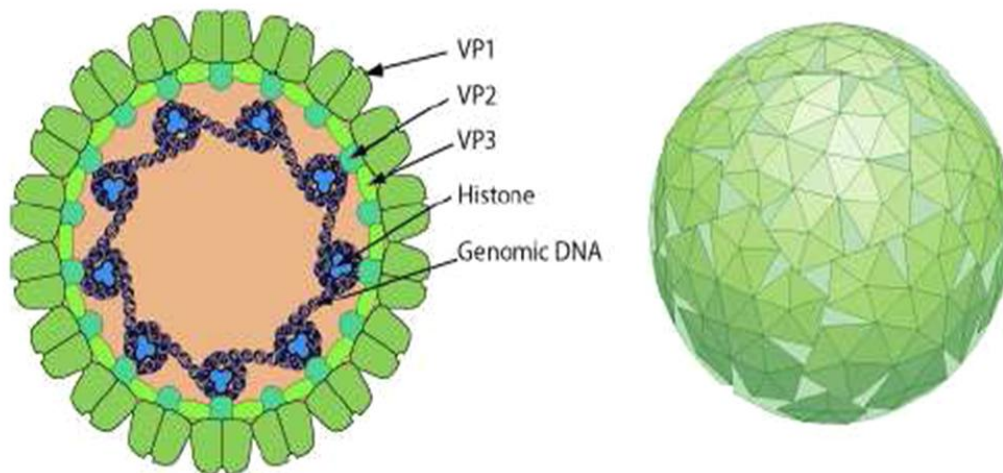
Figure 1.2: *SV40 minichromosomes revealing the structural capsid proteins*

SV40 Entrance into the Cell:

The cell infection starts by the binding of the SV40 virus to a receptor identified as the major histocompatibility complex (MHC) which is present on the plasma membrane of the host cell (51). SV40 binding to the host cell is co-directed by the capsid and VP2 (Figure 1.4). Subsequently, the bound virus traverses the membrane and enters a caveola which are large, 70-100 nm diameter flask-shaped invaginations on the plasma membrane (52), where after ~20mins, it is endocytosed and transported in caveolae-coated vesicles to the caveosome through an actin- and dynamin-dependent process. Each 60-70 nm transported vesicle contains a single SV40 virion (52, 53). SV40 particles tend to bud from the caveosome and traffic to the ER. From studies, the ER has been suggested to be the SV40 disassembling domain since by the aid of the ER-resident molecular chaperones, the genome and VP1 pentamers associated with VP2 and VP3 are liberated (51, 52, 54). Further dissociation of the VP1 pentamers tend to release the bound VP2 and VP3. The VP2 and VP3 are inserted into the ER through oligomarization to form a multimeric complex that aids in transporting the genome across the ER membrane. The VP2 and VP3 complex integrates in the contiguous nuclear and ER membrane to directly transport the genome into the nucleus. Intact SV40 has also been proposed to enter the nucleus through the nuclear pore complex (NPC).

However, the largest macromolecule the NPC has been shown to translocate measures 39 nm in diameter indicating that the NPC would exclude the entrance of the intact 50 nm SV40 capsid (55). Further integration of the VP2 and VP3 complex away from the nuclear boundary transports the genome into the cytoplasm (Figure 1.4), where one of the structural protein, "VPX" utilizes its nuclear localization sequence and DNA-binding domains to traffic the genome into the nucleus (56).

However, uncoating of the viral capsid must occur before the genome can be translocated to the nucleoplasm for replication. Polyomavirus capsid undergoes endocytosis and is transported to the nucleus where the viral DNA is uncoated and transcription of the early region begins. The nuclear envelope poses a significant barrier to viral infection and the trafficking of viral components. The strict size limit for nuclear entry requires large viral capsids to undergo uncoating, unwinding or nucleic acid release prior to nuclear entry (55, 57, 58). Enveloped virions (influenza, retroviruses, calciviruses, flaviviruses and herpesviruses) are also subjected to these constraints, as their lipid envelopes are shed exposing the capsids, which must then release the replication competent genome.
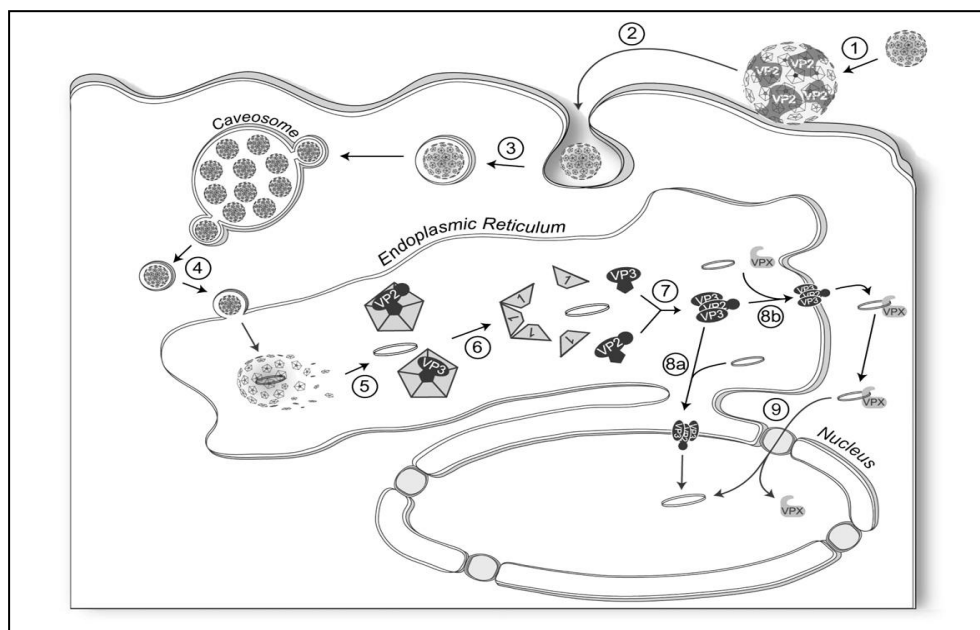
Figure 1.3: *model for SV40 Entry and Penetration of the ER Membrane (56.)*

Thus, capsids are exposed to cellular proteins that may be commanded by viruses to induce capsid uncoating or deformation to support nucleic acid release enabling genome delivery and replication. The nonenveloped dsDNA adenovirus (60-90 nm) employs cellular processes where, molecular chaperones and a virally encoded internal protease (p23) to weaken its capsid, allowing the genome to be released and enter the nucleus (59). The mechanism of uncoating is the most poorly understood stage in the life cycle of SV40 but the size of the SV40 capsid with its genome comprising of dsDNA associated with histones, indicates that the virus requires extensive disassembly before the genome is imported into the nucleus. This suggests that release of the SV40 genome would closely imitate adenovirus, requiring a cellular trigger for disassembly. Since SV40 traffics to the ER during cell entry, the ER likely contains the cellular trigger that initiates viral uncoating (Figure 1.4). The ER provides an oxidizing and calcium rich environment, optimized for the maturation and quality control of secretory proteins. It houses a variety of molecular chaperones, foldases and proteases, which play central roles in these fundamental cellular processes (60).

LT assists in the transition from viral DNA replication to the synthesis of the late transcripts ~30 hr post-infection. Two late viral transcripts are transcribed: a 16S RNA that is transported to the cytoplasm where it translates VP1; and a second 19S transcript, which encodes for VP2, VP3 and the agnoprotein. While VP1 is the central protein that forms the viral capsid, the roles and functions of VP2 and VP3 in the infection process are poorly defined. VP2 is myristylated on its amino terminal glycine residue (39, 60, 61). Deletion of the myristyl group in a mouse polyoma Py mutant strain resulted in a 20 fold decrease in virus infectivity due to an apparent defect at, or prior to virus uncoating (56, 61). In sharp contrast to these findings, deletion of the unique N-terminal portion of VP2, which contains the myristylated residue, has no effect on SV40 viability.

Since viral assembly occurs within the nucleus, newly synthesized viral structural proteins are directed for nuclear import by their intrinsic NLS. During infection, complexes of VP1, VP2 and

VP3 form in the cytosol and are transported into the nucleus for DNA binding and virion assembly. The low calcium concentration and reducing environment of the cytosol likely aids in the assembly of virions by inhibiting the generation of SV40 capsids prior to nuclear import. Once in the nucleus, VP2 and VP3 binding to the genome is proposed to facilitate SV40 assembly by forming a scaffold for VP1 capsomeres to assemble the capsid (62).

Viral lysis and Optimal Spreading

Little is known about the process involved in the dissemination of SV40 and nonenveloped viruses from the cell in general. SV40 infection is thought to result in cell lysis or death through a necrotic pathway due to the cellular trauma that results from the infection (63). However, electron microscopy studies have shown that SV40 virions saturate the apical surface of polarized cells ~48 hr post-infection, ~12-24 hr before cell permeabilization occurs (64).

Observation of SV40 within large smooth membrane vesicles suggest that SV40 utilizes a novel exocytic pathway that involves an unknown sorting signal to reach the cell surface. This would advantageously position the virus for the initial infection of adjacent cells prior to cell permeabilization and support the more efficient dissemination of virus for infection of distant cell populations upon cell permeabilization. This two-step process would enable the initial infections to occur without alerting the immune system, which becomes aware of the virus upon cell permeabilization. The plasma membrane of host cells is unquestionably compromised during infection. However, how this permeabilization is initiated is unclear (56, 60). Several enveloped and non enveloped viruses utilize proteins termed viroporins or virus porins to mediate membrane disruption during viral entry and release.

VP4 (Figure 1.5) is a recently described protein and its expression is thought to coincide with viral lysis. VP4 expression is thought to represent a mechanism by which SV40 regulates its life cycle to

enable optimal spreading of the virus (60). The nonenveloped poliovirus VP1 and VP4 proteins also aid in the delivery of viral RNA to the host. The poliovirus 2B protein has been proposed to initiate cell lysis. The 2B and myristylated VP4 proteins are small viral core components and VP1 contains an N-terminal α-helical domain that is also sequestered in the viral core. Upon receptor binding, the capsid undergoes a conformational change enabling VP1 and VP4 to insert into the host cell membrane and facilitate the transport of the viral RNA (65). A single point mutant in VP4 renders the channel ineffective, preventing the release of the viral RNA into the cytosol of the host (65).
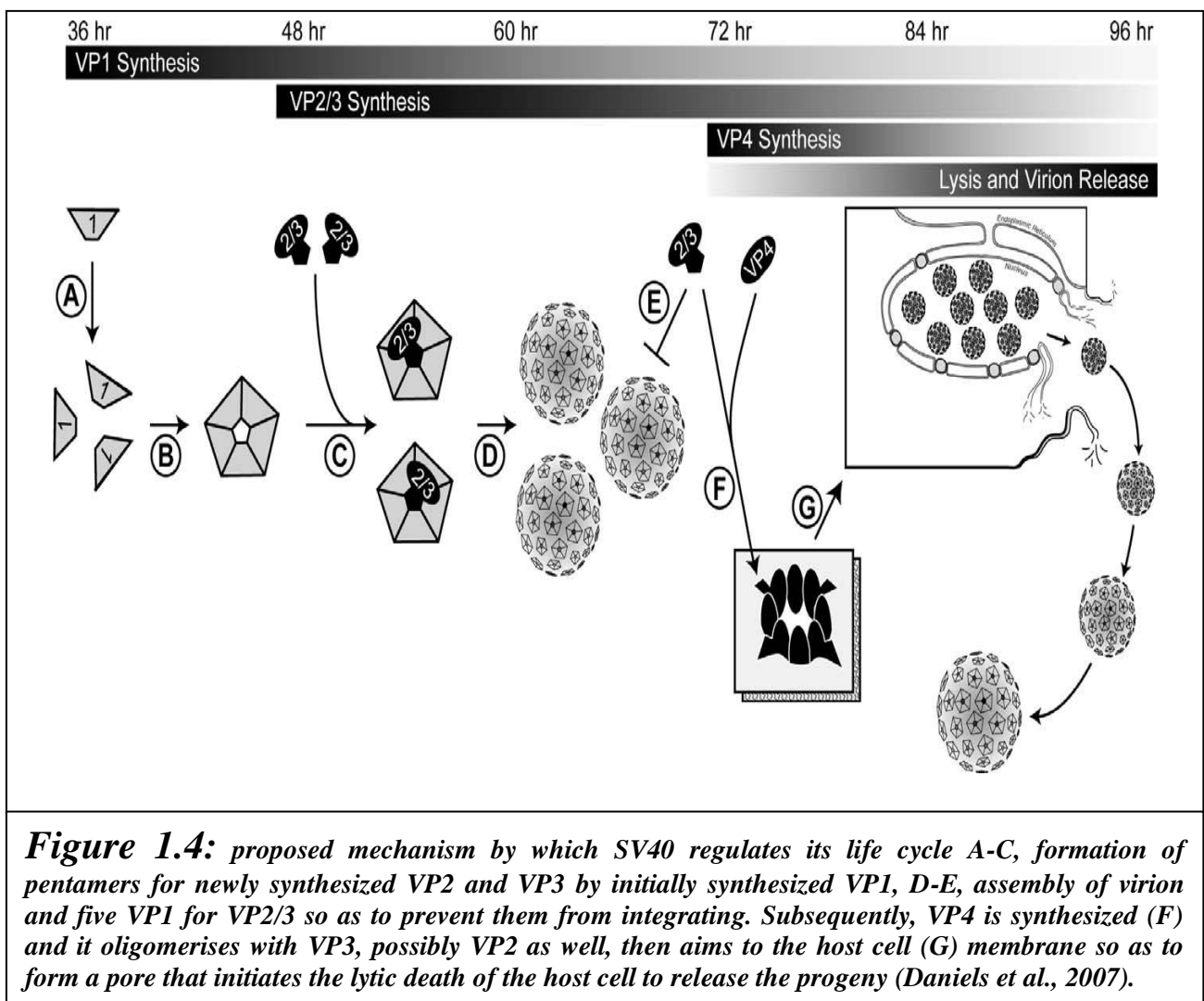
Figure 1.4: *proposed mechanism by which SV40 regulates its life cycle A-C, formation of pentamers for newly synthesized VP2 and VP3 by initially synthesized VP1, D-E, assembly of virion and five VP1 for VP2/3 so as to prevent them from integrating. Subsequently, VP4 is synthesized (F) and it oligomerises with VP3, possibly VP2 as well, then aims to the host cell (G) membrane so as to form a pore that initiates the lytic death of the host cell to release the progeny (Daniels et al., 2007).*

1.4.2:   SV40 Early Genome Region

The Large Tag

With the help of Tag and tag SV40 picks the lock on pivotal-check points in its life cycle control and achieves the transformation of host cells by expressing these two important proteins (Tag and tag) upon infection. This causes the cell to undergo an unrestrained proliferation circle that replicates and produces viral DNA and viral particles respectively.

The large tumour antigen (Tag) of SV40 is a 708 amino acid protein that has many functions. It is the major transforming protein of the virus. T-ag is required for initiation of viral DNA synthesis and stimulates host cells to enter S phase. The oncogenic capacity of SV40 stems largely from the ability of this oncoprotein to bind to and inactivate the cellular tumour suppressor proteins and cell cycle regulatory proteins that include the retinoblastoma proteins such as pRB, p107 and p130/pRB2. The tumour suppressor proteins include p53 and the transcriptional co activators p300 and CBP (23, 66). Tag protein is found predominantly in the nuclei of SV40-infected and/or transformed cells (4).

Tag Transformation Domains

SV40 Tag is a multifunctional oncoprotein that possesses several defined functional domains that play a critical role in cell transformation and tumour induction (14). Figure 1.6 schematically illustrates different functional domains of SV40 large T antigen. The amino terminus of the Tag contains two distinct domains important in cell transformation. The far amino terminus of Tag includes the J domain involved in proper folding of protein complexes. This region shares 82 amino acid residues with small t antigen. The second region of the amino terminus of Tag mediates the binding to pRB and the pRB family members p107 and p130 (67). The J domains aslo coperate with LxCxE motif (residues 103-107) of the Tag to inactivate the functions of pRB family memebers and may also have additional transformation. The transforming ability of SV40 Tag is

sometimes abolished by mutations that disrupt their binding with either pRB or p53 (68). Furthermore, the transforming potentials of SV40 Tag is contributed by its possession of additional features such as, the ATPase and DNA helicase. These features provide specifically binding and unwinding functions to SV40 Tag at its origin of replication, thereby assisting in the viral DNA replication. The DNA binding domain (131-259) is mainly known for its function as a transcriptional transactivator. Tag, in addition to targeting cellular tumour suppressor proteins, also targets nuclear acetylases including CREB-binding protein (CBP), P/CAF and p300 (new family members of transcriptional co-activators). These regulatory proteins function as cofactors and play important roles in transcription and posttranslational modification of cellular tumour suppressor proteins (69, 70, 71).
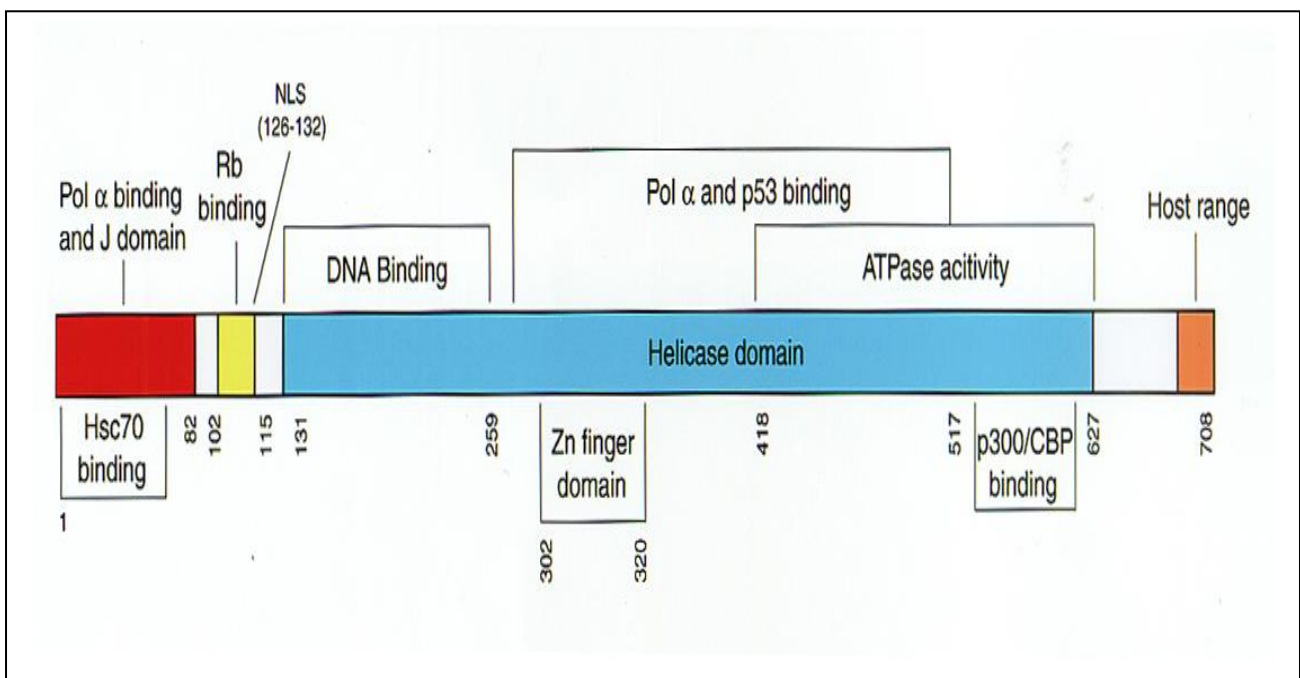
Figure 1.5: *a schematic model representing the functional domains of SV40 Tag. Nearly, minimal regions of T-antigen that retain binding activity to polymerase α-primase (Polα), tumour suppressor proteins Rb and p53, human heat shock protein 70 (hsc70) and coactivators p300 and CBP are illustrated. DNA binding domain, ATPase activity domain, nuclear localization signal (NLS) domain, helicase domain, host range domain, Zn finger domain, and J domain are also depicted.*

Tag interacts with these proteins through multiple regions (Eckner et al, 1996;) and inactivates their important cellular functions. This is also thought to contribute to deregulation of cell cycle progression.

The SV40 Small T antigen (tag)

Small t antigen of SV40, is a 174 amino acid protein (48) produced by alternative splicing of early transcripts. It shows an all α-helix structure with two zinc-binding sites in the unique domain (figure 1.7). Small ag J domain comprises three helices with structure similar to large T antigen crystal structure and NMR structure of poliomavirus DnaJ-like domain (72, 73).
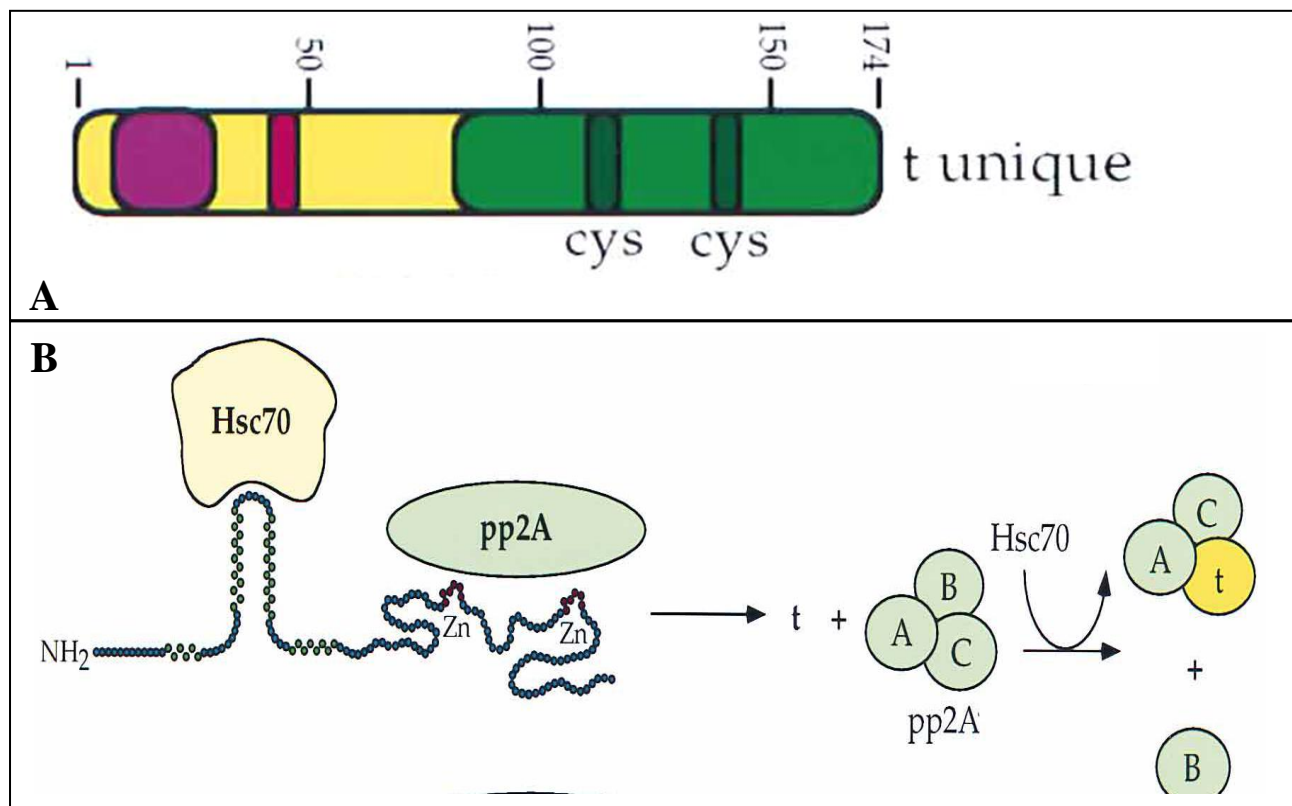
Figure 1.6: *A, The domains of Small tag (St) displaying the 174 amino acid with unique regions and the crystal structures similar to large T antigen and DnaJ-like domain. B, Small t antigen contains a J domain adjacent to a zinc binding domain that directs the association of small t antigen with the trimeric phosphatase pp2A. SV40 small tag interacts with the B subunit-binding region of the PP2A A subunit, resulting in the displacement of the B subunits from the PP2A complex by the small t (72).*

Small tag forms complexes with the regulatory subunit of the protein phosphate 2A (PP2A) family as in (Figure 1.7B) of serine-threonine phospatases (48, 74). This association appears to inhibit the function of PP2A, which in turn leads to more phosphorylated and increased kinase activity of several cellular kinases including MAP kinase and its kinase ERK, Jun N-terminal kinase (JNK) and a key ion transporter, the Na/H anti-porter (75, 76, 77).

Tag assist solemnly on human cell transformation through its inactivation of the pRB and p53 pathways. Studying the tag mutants has provided important clues to the function of tag during cell transformation. Mutations in tag that prevent the interaction with and inhibition of PP2A demonstrate that this interaction is required for tag mediated transformation. On the contrary, a tag mutant that contains only the PP2A inactivation domain (amino acids 88-174) retains the ability to reduce transformation, which suggest that the alterations in PP2A activity is required for the transforming activity of tag (78).

Small tag in cell Proliferation and Transformation

Studies have shown that tag expression results in enhanced proliferation rates which suggests its involvement in the promotion and regulation of cell cycle progression. It supports the modification from G1 to S phase in cell cycle; tag expression or suppression of PP2A B56g enhances the ability of cells to proliferate in low-nutrient conditions (79, 80). Small tag also promotes the up-regulation of AP-1 transcriptional activity by regulating mitogen activated protein kinase (MAPK) through its over expression. Studies have observed the Raf, MEK1/2, ERK1/2 and KSR sites as possible spots MAPK regulation occurs. Additionally, expression of tag also gives rise to expression and elevation of certain proteins such as cyclin D1, B, thymidine kinase and dihydrofolate reductase linked with cell cycle progression (81). Small tag activate other transcriptional factors such as cAMP regulatory element binding protein (CREB), SP-1 and E2F transactivation linked to cell cycle progression. Small t antigen can also regulate transcription by trans activating the Ad E2A

promoter, which is transcribed by RNA polymerase II, and the Ad VA-I promoter, which is transcribed by RNA polymerase III (82).

Furthermore, tag has been observed to stabilize c-Myc that is directly dephosphorylated by PP2A in tag transformation assays by inhibiting the activity of PP2A (Figure 1.8). A stabilized c-Myc mutant is also capable of substituting for tag expression (83, 84). SV40 tag has been also implicated in anti-apoptotic pathways (Figure 1.8) and induction of changes in cytoskeleton. Studies have shown lines of evidence implicating tag in regulation of apoptosis. Transcriptional analysis of human cells expressing tag revealed up-regulation of anti-apoptotic targets of NF-kB (Figure1.8) such as *ALDHI*, *SERPINB2* and surviving. NF-kB is activated by tag regulation through protein kinase and phosphatidylinositol 3-kinase (P13K) signaling (85). It is also involved in the regulation of the P13K/Akt pathway. Genetic substitution experiments have demonstrated that under tag expression either activated PI3K or a combination of the activated PI3K effectors Akt and Rac1 induce human cell transformation (Figure1.8). Equally, blocking PI3K function inhibits tag-mediated transformation (86).

Figure 1.7: *cell transformation mediated by tag downstream expression leading to inhibition of PP2A complexes that activates numerous signaling pathways and regulation of c-Myc, Akt and Anti-apoptotic NF-kB pathways (74).*
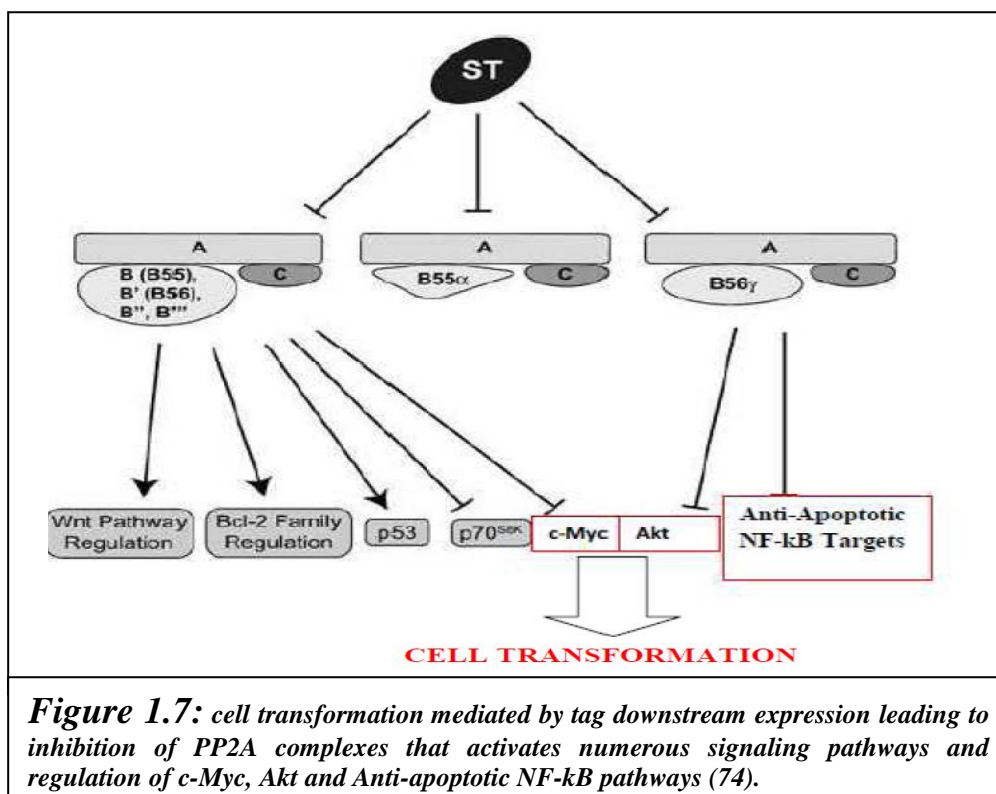
SV40 tag expression also aims at PP2A-dependent processes governing the cytoskeleton. Over expression of tag in epithelial cells induce dramatic F-actin rearrangements with increase Racl-induced membrane ruffling, Cdc42-initiated filopodia and loss of RhoA-dependent stress fibers (87). Studies on transcriptional profiling analyses have shown that a number of genes such as osteopontin, paxillin and gasoline involved in cellular adhesion and motility are up-regulated by the presence of tag. It also causes down regulation of several genes like Plakoglobin, claudin11, ICAM-1 and VCAM-1 involved in cell-cell adhesion and junctional adhesion. This down regulation causes severe defects in formation and barrier properties of tight junction in cells with tag expression (88).

1.4.3: SV40 Regulatory region and Micro RNA

The viral genome of SV40 shows a regulatory region consisting of DNA replication promoter and enhancer elements. A non-coding region, it however functions to orchestrate viral transcription and replication. Its characteristic series of specific nucleotide motifs serve as binding or interaction sites for proteins involved in transcription or DNA replication (91).

The structure of the regulatory region of SV40 viruses can be distinguished in accordance with the number of enhancer elements observed. Hence, two types of regulatory region structure in the SV40 viruses based on the presence of enhancer elements present. Strains having two 72-base pair enhancer elements are referred to as having a 'non-archetypal' or complex regulatory region structure while regulatory regions lacking a duplicated enhancer element are considered 'archetypal' or simple structures (89).

In the case of the reference strain of SV40-776, variants containing one enhancer element are designated 776-1E (-1E virus) while viruses with a duplicated enhancer are called776-2E (-2E virus). These -2E viruses have been shown to replicate faster in tissue culture (89). It has been noted that while natural isolates of SV40 or virus isolated from human tumours generally do not contain enhancer duplicates, laboratory adapted monkey strains of SV40 usually contain two 72-base pair

enhancer elements (90). However, it is thought that a mixture of both archetypal and non archetypal regulatory regions may be present in monkey isolates and that laboratory tissue culture allows the selection of the faster growing variants (91).

Studies have shown that the regulatory region of SV40 may exert a significant influence on tumour incidence. In studies on SV40 tumour induction in Syrian golden hamsters (Mesocricetus auratus), the -lE virus was found to induce tumours in a larger proportion of animals than the -2E virus (92). While the faster speed of viral replication has typically been seen as an advantage for a virus in overcoming the ability of the immune system to control its growth, it has been proposed that slower replicating viruses may evoke a weaker immune response and therefore enhance their likelihood of persistence by circumventing immune surveillance (93). Based on this theory, it is thought that the faster replicating SV40 -2E virus is recognized and cleared more efficiently by the host immune response than the slower replicating -lE virus which would be more likely to evade the immune system and persist (90).

It has also been recently recognized that the viral regulatory region of SV40 may exert effects on vertical transmission in hamsters (94). In a study investigating SV40 transmission in the hamster model, it was found that hamsters inoculated with SV40 strains containing complex regulatory regions (-2E) transmitted virus more frequently than those infected with simple enhancer (-1E) viruses. Further studies are required to determine the relationship between the regulatory region and pathogenesis of SV40 infection, including the possibility that some SV40 strains or variants are more pathogenic than others.

Micro RNA

Since its discovery in the 1990s, relentless study efforts on micro RNA affirmd that SV40 encodes RNA that play important role in the viral life circle (48). Cellular and viral miRNAs are small regulatory RNAs that play critical role in gene regulation. (95).

miRNAs function by binding to complementary sequences in the 3' un-translated regions of messenger RNA (mRNAs), and then mediating mRNA degradation or repressing translation, ultimately leading to reduced protein expression. It has been known for some time that some viral genomes include non-coding RNA such as miRNAs, but their function is only starting to come to light. In fact, recent studies suggest that viral miRNAs may function to evade the host innate immune response, regulate gene expression and possibly contribute to viral-mediated tumourigenesis (96, 97). Micro RNAs are useful to the virus in that they are non-immunogenic, take up a small amount of genomic space and are powerful regulators of gene expression (98).

SV40 encodes a pre-miRNA, designated miR-S1, which is processed into two miRNAs that bind to the early RNAs, thereby directing their cleavage at a late stage of the viral cell cycle (98). The end result of the miRNA action in SV40 is a reduction in the expression of viral T antigens. It is known that some protein products of early SV40 genome, such as Tag, are very immunogenic and generate a strong cytotoxic lymphocyte (CTL) immune response. The function of the SV40 miRNA in down regulating expression of these immunogenic proteins suggests that the miRNA is involved in immune evasion strategies. Studies conducted using a mutant SV40 virus named 776-2E-SM, that lacks the viral miRNA, have shown that target cells infected with the mutant are more sensitive to CTL-mediated death than cells infected with a wild type SV40 (47).

In addition, release of the cytokine interferon-y was also diminished when CTLs encountered cells infected with the wild type SV40. Together these results suggest that SV40 miRNA may play a role in evading the adaptive immune response (47). Viral miRNAs in the herpes viruses Epstein Barr virus (EBV), human cytomegalovirus (HCMV) and Kaposi's sarcoma herpes virus (KSHV), have been shown to function in modulating the virus life cycle and human immune response, thereby underlining the importance of miRNAs in viral infections (99).

1.5:   Life Cycle of SV40 in Host Cells

SV40 cellular infection begins by its binding to the major histocompatibility complex class 1 (MHC class 1) which is present on the plasma membrane of the host cell (Figure 1). The MHC class 1 molecule act as the specific cell surface receptor for SV40 (100, 101). The life cycle of SV40 in human cells is less well understood but the expression of MHC class I by nearly all nucleated cells thus explains the broad tropism of SV40 and its ability to infect and induce transformation in many types of cells and tissues (23). Not all cells expressing MHC class I are, however, susceptible to SV40 infection and differential ability in infecting polarized epithelial cells via the apical or basolateral surfaces have been reported, despite similar MHC expression on both membranes (42). This suggests the possibility of other factors contributing to SV40 tropism such as co-receptors factors for viral entry after binding or additional transcription factors that may need to be present for viral replication to occur (102).

In the natural monkey host, SV40 infection is known to result in a lytic infection. This viral production is controlled by the immune response and the virus thus persists in the kidney where it can be reactivated by immunosuppression (103). Further, after binding to the cell surface, the poliomavirus capsid is transported to the nucleus where uncoating and transcription of the viral DNA into early region of the genome occurs. The alternatively spliced transcription from the early region gives rise to two mRNAs that encode the large Tag and small tag. SV40 large Tag, a nuclear phosphoprotein of 94kD, is an essential factor for viral DNA replication. It binds to the viral origin of replication where it promotes unwinding of the DNA double helix and recruitment of cellular proteins that are required for DNA synthesis, including DNA polymerase-a and replication protein A (39).

SV40 depends on cellular enzymes and cofactors for DNA replication and these proteins are expressed in S phase. Large T antigen modulate cellular signaling path ways to induce cells to enter S phase and is thought to stimulate the cell cycle through its ability to bind to numerous cellular

proteins involved in crucial transduction path ways that control cell cycle progression and apoptosis (104). Small tag does not partake in lytic infection in culture rather assists the Large Tag in transformation of cells by SV40 and increases virus yield in permissive cells. In human cells, through binding to the cellular tumour suppressor proteins of the pRb family, Tag expression leads to the induction of S phase. pRb normally binds the transcription factor E2F to form a transcriptional repressor complex. However, on binding to SV40 Tag, a dissociation of E2F-pRb complexes occurs (106), releasing E2F to activate expression of growth stimulatory genes (Figure 1.9 A).

This pre-emptive signal for S phase induction and cell division and the resulting unbalanced DNA synthesis induces the cell to initiate a p53 response (tumour suppressor gene). p53 normally senses DNA damage and either pauses the cell cycle to repair the DNA or directs the cell to enter the apoptotic pathway, known as programmed cell death. However, Tag overcomes this obstacle by binding to p53 and inhibiting its function (Figure 1.9B). The cell is thus induced to proliferate and the p53-mediated checkpoint is abolished. This Tag-mediated subversion of cell cycle control allows cells with genetic damage to survive and enter S phase, leading to an accumulation of Tag-expressing cells with genomic mutations that may promote tumour growth as an accidental side effect of the viral replication strategy (105, 106, 107).

As SV40 virus replication proceeds, expression of the late genes begins. The gene products of the late region are the capsid proteins VP1, VP2, VP3, which assemble with the replicated viral DNA to form virions. In infection of the natural monkey host these virions are released upon cell lysis, resulting in death of the host cell (39). The life cycle of SV40 from infection to the production of new virions is thought to take 96 hours in permissive cells (Figure 1.5), resulting in about 300 infectious progeny virions per cell (60, 108).
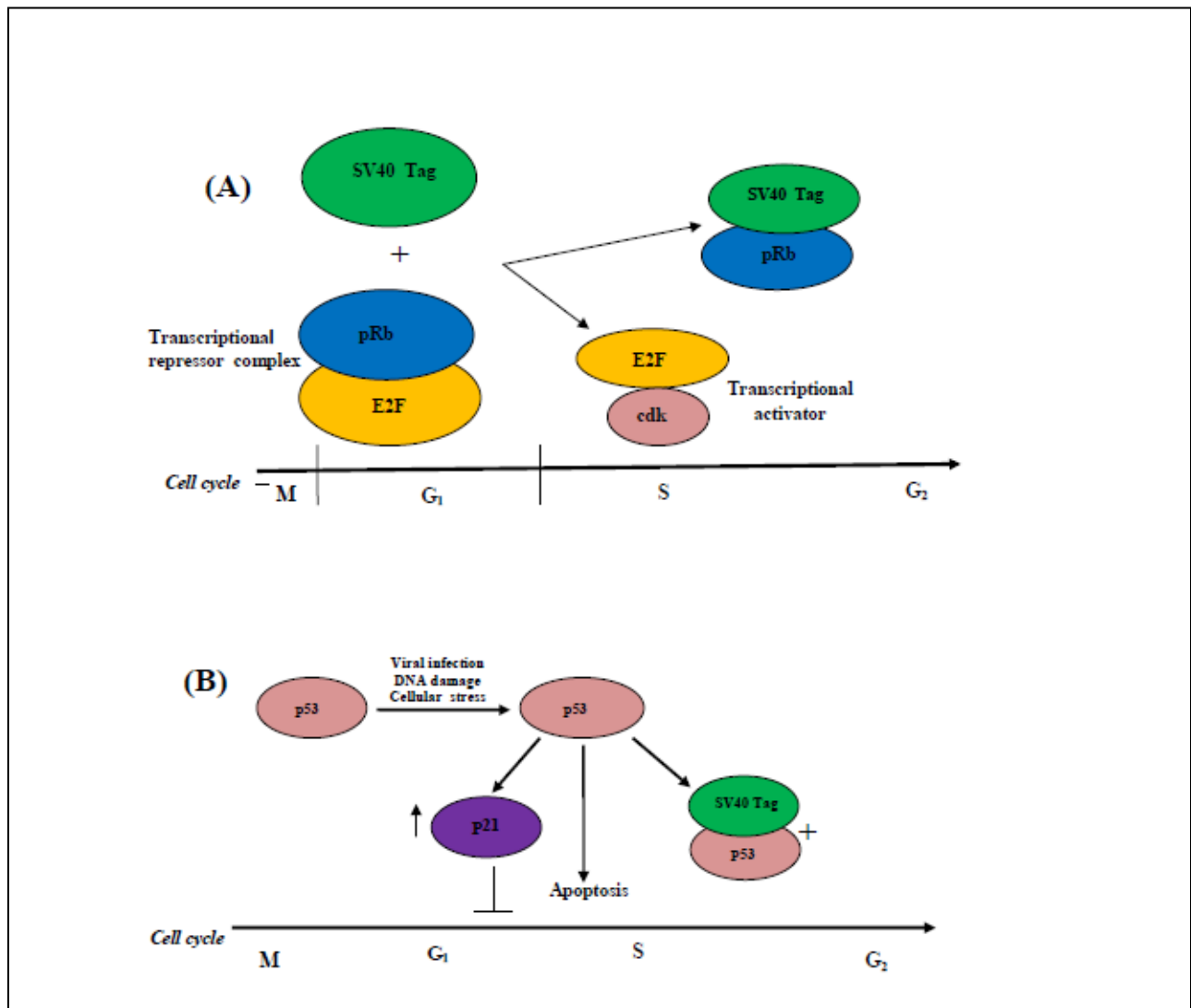
Figure 1.8: *interaction of SV40 Tag with pRb and p53 Schematic representation of the interaction of SV40 Tag with tumour suppressor proteins pRb and p53. (A) pRb normally binds transcription factor E2F, forming a transcriptional repressor complex in early GI phase of the cell cycle. Normally, when pRb is phosphorylated by cyclin-dependent kinases (cdk), E2F is released and activates growth-stirnulatory genes. Tag binding to pRb causes unscheduled dissociation of pRb-E2F complexes, releasing active E2F. (B) The function of p53 is to sense DNA damage and either cause the cell to pause in late GI phase for DNA repair or direct the cell to commit suicide through the apoptotic pathway if repair is not possible. p53 can transcriptionally induce p21, a cyclin-dependent kinase inhibitor, which blocks the activity of GI cyclin-cdk complexes arresting cell cycle progression in late GI phase. Binding of the SV40 Tag results in p53 being sequestered, abolishing its function and allowing cells with genetic damage to survive and enter S phase. As a result, Tag-expressing cells with genomic mutations accumulate that may promote tumourigenic growth (23).*

Although polyomaviruses grow most efficiently in the cells of their natural host species they do not display absolute host specificity (75). Several outcomes are observed in cellular infection by a polyomavirus. In the natural host, cells are permissive to viral infection and are able to support viral replication which results in lytic infection and viral progeny (103). In general, these infections are non-oncogenic for their natural hosts (109). However, SV40 and other turnour viruses may become

more oncogenic when they cross species (2, 4, 110). SV40 DNA can become integrated into the chromosomal DNA of the cell especially upon infection of the non-permissive cells.

Different studies indicate that SV40 can replicate productively in human cells, including spongioblasts, fetal neural cells, new born kidney cells and some tumour cell lines, with poor growth outcome in human fibroblasts. Hence, the human cells are said to semi- permissive to SV40 infection (36, 103, 110). Some human cell types undergo visible cell lyses in response to SV40 infection, whereas other cells fail to exhibit cytopathic changes and produce low virus levels. Studies have shown that while SV40 infection of human fibroblasts leads to a productive outcome with viral replication, the virus establishes a persistent, non-lytic infection in mesothelial cells (3) and becomes transformed at high rate (1000 times higher than that of human fibroblast) by SV40 Tag alone, releasing infected virions due to persistent infection. Similar situation has also been observed in human lymphoblastoid B cell line where SV40 progeny is produced at a low level (38). The behavior of SV40 in mesothelial cell is very uncommon for a DNA tumour virus. Studies have evidenced that DNA tumour viruses either infect permissive cell lines with the production of an infectious viral progeny or infect and transform non-permissive cells without a productive viral cycle. The rate-limiting step to sustained transformation is thought to be the random integration of viral DNA into the cellular genome by non-homologous recombination. If this integration occurs such that the early coding sequences are intact allowing Tag to be expressed, the host cell and its subsequent descendants are transformed (4, 38, 48).
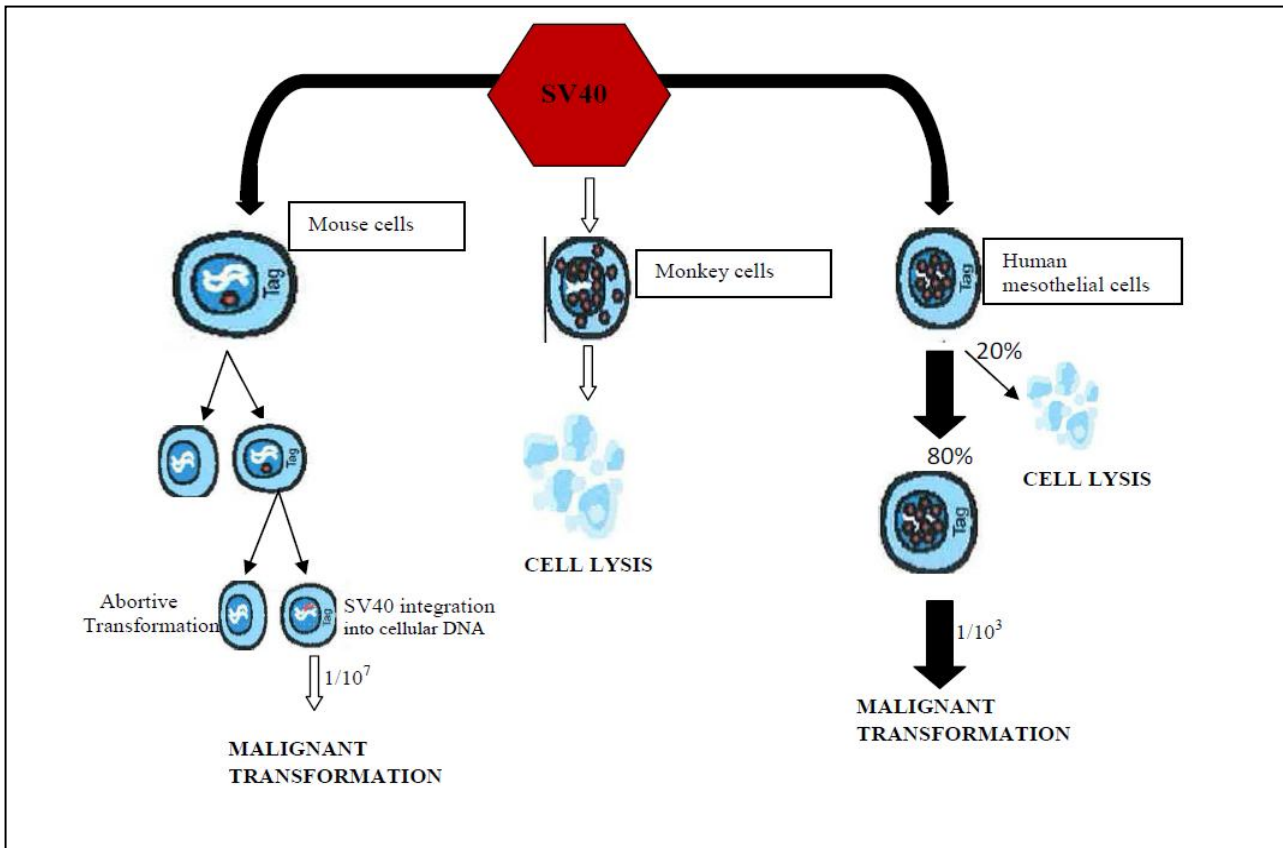
Figure1.9: *possible Outcomes of SV40 infection in different Cells.*
Left—In SV40 infection of non-permissive mouse cells, no production of viral particles and malignant transformation can occur. Middle—Infection of permissive monkey cells with cell lysis that results in the production of progeny or many viral particles. Right—Infection of human mesothelial cells that leads to very limited production of viral particles with limited cell lysis and frequent malignant transformation (110).

Possible Cell Transformation Phases from Primary Cell Growth.

Cell transformation could be described as the changes in types of proteins expressed and growth characteristics that take place in cells infected by some viruses, including tumour formation by retroviruses. Transformation could also be oncological or malignant (111,112). Investigating the process of the cell transformation induced by different agents in vitro can indisputably be a very powerful approach to elucidate this basic mechanism. Currently, this type of experiment has been carried out extensively with animal cell models, while only rarely has been observed with human cell types (113, 114). The difference in susceptibility and transformation between animal and human

cell models has been attributed to DNA repair capacity, response to oxidative stress, epigenetic factors such as DNA methylation (114).
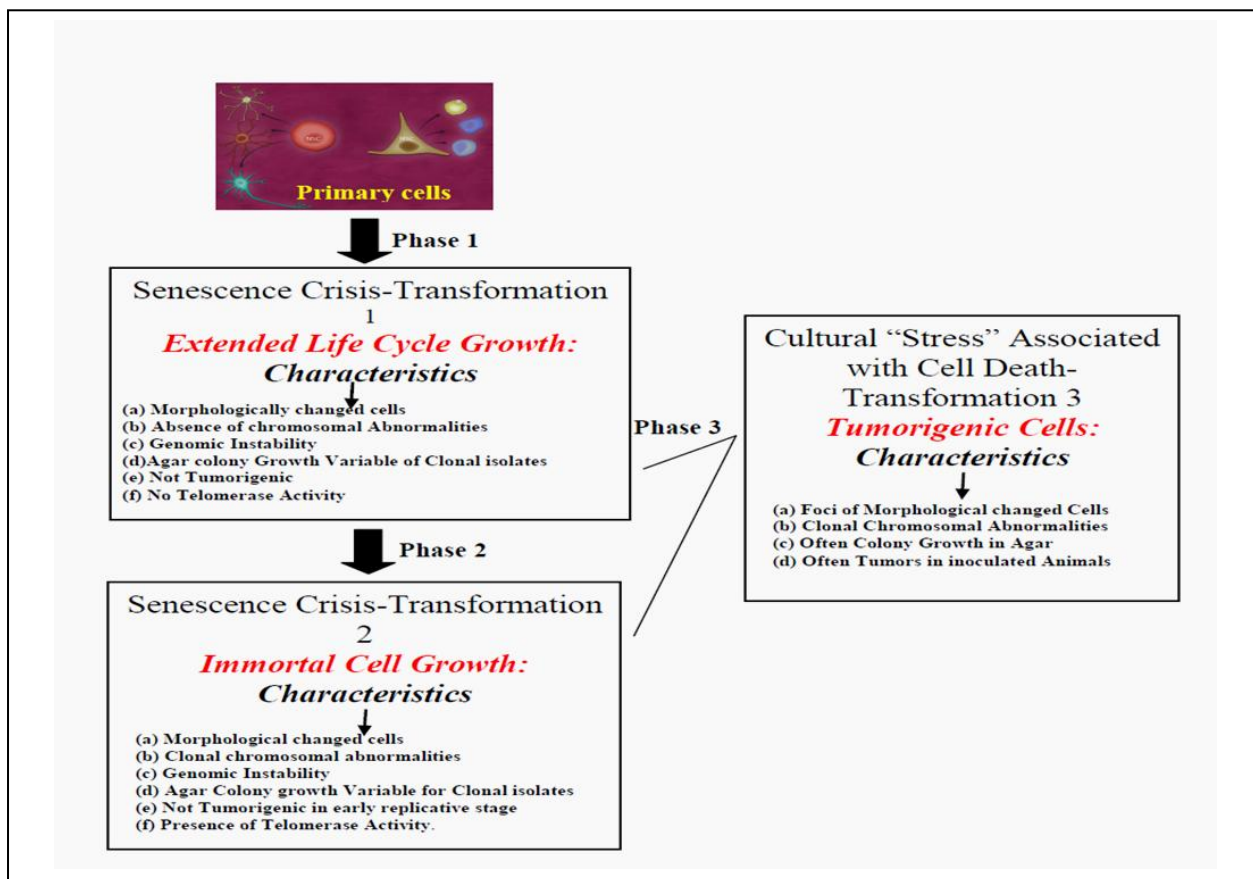
Figure 1.10: *schematic presentation of the three most frequently cited cell transformation Stages/Phases with their characteristics. Phase 1 showing Extended life cells (EL) originated from the primary cells followed by Phase 2- the Immortalized cells (I) and in stressful cultural conditions tumourigenic cells –Phase 3 is developed from I and EL cells.*

In cell transformation, three stages/phases are observed (Figure 1.11): (a) production of cells with an extended life (EL. Cells) from primary diploid cultures that is characterized mainly of diploid primary cells in pre-crisis stage; (b) emergence of immortalized cells (I. Cells) from EL cells after passing through stage two crisis or post crisis stage; (c) development of tumourigenic cells (T. Cells) from EL or I cells in stressful cultural conditions (64, 115,).

Furthermore, transformation and postponement of senescence of cells can be carried out in human cells such as lymphocytes (with short life span in culture) using different experimental approaches such as transforming by tumour viruses (*Herpesvirus saimiri, infection with T-cell leukemia virus-*

type1, transfection with Extein-Barrvirus, EBV), or with viral oncogens such as the large T antigen (Tag) of different Polyomaviruses (116a, 116b). Previously, the transforming capacity of the Tag has been demonstrated in human lymphocytes in vitro (38, 117, 118).

1.6: The Immune Response to SV40

Usually, the viral replication cycle begins with the binding of the virus to the host cell through specific receptors (adsorption). For SV40, the host cell receptor is the MHC class 1 and it determines the tropism and specifity of SV40 virions. The effector in the cellular immune response to virus is the CD8$^+$ cytotoxic T lymphocyte (CTL), which lyses virus-infected cells following recognition of virus encoded epitopes presented on the cell surface by MHC class 1. several factor like the proper generation of the virus epitopes, successful loading and stable binding of virus epitopes to MHC class 1 molecules, proper trafficking of the complex to the cell surface and the presence of T cell in the repertoire capable of recognizing the virus epitopes contribute to successful CD8$^+$ T-cell recognition of virus epitopes (120). The MHC class 1 molecules comprises of a trans-membrane heavy chain that associate non-covalently with β2 microglobulin and a bound peptide representing the T cell recognized epitope. The epitope recognized by CD8$^+$ T cells are typically peptides of 8-10 amino acids in length that can be derived from a variety of virus proteins. This length requirement is imposed by the structure of the peptide binding groove in which the closed ends of the groove accommodate the amino and carboxyl termini of the peptide.

Entry of virus proteins into the MHC class 1 antigen presentation pathway might occur through either direct infection of APCs or by "cross-presentation" in which virus-infected cells or cellular debris are phagocytosed, and the antigens are shuttled into the MHC class 1 processing pathway. Recently, it has been observed that CD4$^+$ T cells prime APCs via signaling through the CD40/ CD40 ligand receptor pair. These activated APCs are then capable of delivering co-stimulatory signals via B7-CD28 or similar engagements for the activation of naïve CD8$^+$ T cells (119).

Studies addressing the requirement for CD4$^+$ T cells help for successful priming of virus-specific CD8$^+$ T-cells responses have suggested that some virus-specific responses are independent of CD4$^+$ T-cell help, perhaps, due to alternate mechanisms for activation of APCs (119, 120).

SV40 Cellular Immune Response During Viral Lytic Cycle

Adaptive and innate immunity are firmly involved in defense against viral infections. However, nature of virus-host interaction determines the participation of the various arms of the immune response. Virus neutralization antibodies play a definitive role in interrupting viral spread while the T lymphocyte-mediated mechanism are involved in the elimination of virus-infected target cells. The CD8$^+$ T lymphocyte (CTL) is responsible for this effect and it lyses virus infected cells by recognizing a viral epitope presented by the MHC class 1 antigens. In a typical immune response to viral infection, natural killer cells that recognize virus-infected cells are recruited followed by a burst of CTL after about 4-7 days. The CTL tends to fall to base line as the virus load declines and then persist as memory T cells which can be recalled upon re-exposure to the viral gene products. The mechanism of CTL effector is very essential for the elimination of virus- infected cells, specifically mainly on those viruses that cause persistent infections (121).

Furthermore, the CD4-mediated T-cells responses are involved in helping B cells produce antibodies and cytokines involved in the proliferation of CD8$^+$ T cells and activation of APC. In human poliomaviruses, only SV40 has an available, reliable model which is the rehesus monkey and as its natural host. For BKV and JCV no animal models in which virus infection leads to its replication in vivo. This particular reason is affecting tremendously the study of T-cell mediated immunity during infection of natural hosts. Recently, a study have reported on the role of CD8$^+$ T cells in interrupting the infectious cycle infection in SV40 in vitro. In this study, the murine CTL clone specific for H2-K^b and H2-D^b were utilized along with restricted SV40 T antigen epitopes as probes for abrogating the SV40 infectious cycle in permissive monkey cells. To utilize the strategy,

a continuous line of monkey kidney cells, TC-7, was transfected with murine H2-K^b and H2-D^b class 1 antigens, thus allowing the presentation of T-antigen epitopes to SV40 T-antigen-specific CTL clones generated by B6 mice. From this study, it was observed that, the interaction of T-antigen-specific CTL clones with SV40 infected TC7/H-2D^b or TC7/H-2K^b cells for 5hrs reduced the SV40 yield by 70-90% as measured by the infectious center assay. This result suggests that: (a) the target of CTL is a non virion protein T antigen, which is synthesized before viral DNA replication; and (b) the CTL mediated events may also take place in the natural host undergoing SV40 infection (122, 123).

1.7: SV40 Association with Human Cancers

It has really been established that SV40 is a potent transforming virus, having observed its induction/generation of tumours in experimental animal models and various types of cells in vitro. More recent evidence from serological and molecular studies revealing its presence in humans raise the possibility that the virus could have transforming capabilities in human cells both in vivo and in vitro.

SV40 in Human Brain Tumours

Detection of SV40 DNA sequences in early studies was in rare cases due to low sensitivity used techniques. However, the studies supported previous findings of SV40 induction of brain tumours in hamsters (124). Recently, improved polymerase chain reaction methods (PCR) as amplification based methods has shown breakthrough in SV40 research, providing a sensitive method of DNA detection in human samples. PCR has been used to detect SV40 sequences in a variety of child hood brain tumours such as ependymoma and choroid plexus tumours, as well as thyroid, pituitary and parotid gland tumours. These human brain tumours correspond to the neoplasms that are induced by SV40 experimental inoculation in rodents or by generation of transgenic mice with the SV40 early region gene directed by its own early promoter-enhancer (39, 125).

There has been numerous studies with continuing reports of SV40 detection in brain tumours (90). Not only has the virus been detected but SV40 Tag has also been found complexed with tumour suppressors p53 and pRb in brain tumours, providing further evidence of a direct functional role for the virus in tumourigenesis (126). In addition to patient studies, an interesting report was published detailing the development of a meningioma in a laboratory scientist who had worked with an SV40-transformed cell line (127). This brain tumour was found to contain SV40 DNA sequences indistinguishable from the laboratory virus, suggesting that laboratory-acquired SV40 infection may have been responsible for turmour development. A meta-analysis of the association of SV40 with brain turmours revealed that samples from patients with brain tumours were almost four times more likely to have evidence of SV40 infection than those from controls (90). Furthermore, SV40 has been detected in multiple histological types of brain tumour, in both children and adults. Indeed, since brain tumours represent 21% of all cancers in children, it has been hypothesised that this high incidence of paediatric brain tumours may suggest acquisition of an infectious agent such as SV40 from the mother (90). However, other studies examining polyomaviruses in brain cancers using primers capable of detecting SV40 sequences have not reported co-infection (128).

SV40 in Malignant Mesothelioma

Malignant mesothelioma is an aggressive tumour of the lung pleura, pericardium or peritoneum, with mean patient survival of only one year from time of diagnosis (129). Mesothelioma has been firmly linked to asbestos exposure. However, since10-20% of occurrence is in people with no known exposure and only 5-10% of those exposed to high levels of asbestos develop this type of cancer, it is thought other factors may be involved in mesothelioma development (3). Asbestos and SV40 have been shown to be co-carcinogens, with SV40 infection lowering the amount of asbestos required for malignant mesothelioma to develop in hamsters. This raises the possibility that SV40 may act as a co-factor in other cancer types (130).

Recent studies have shown that SV40 sequences could be found in up to 60 % of mesotheliomas and that 100 % of the infected cells express SV40 T antigen (Tag). However, the infected cells are not lysed by SV40 due to abnormally high levels of p53 expression in mesothelial cells which binds Tag; and with SV40 there is a possibility of acquisition of genetic mutations and a malignant phenotype (40, 129, 131, 132).

Interestingly, the paracrine mechanism, exerted by the SV40 Tag in murine and canine cells has been also observed in human mesothelial cells. In these mesothelial cells, SV40 Tag activates an autocrine/paracrine loop, involving the hepatocyte growth factor (HGF) and its cellular receptor, which is the product of the oncogenes c-met, as well as the vascular endothelial growth factor (VEGF) and its cellular receptor VEGFR. HGF and VEGF released from SV40-positive human cells, bind their receptors in neighboring and distant SV40-positive and SV40-negative cells, driving them into proliferation and tumourigenesis. In this human cell models only one cell out of 100/1000 cells needs to express the Tag to transform all the cells of the monolayer (133, 134).

Numerous possible roles of SV40 in the pathogenesis of human mesothelioma has been observed: (a) SV40 ability to bind in vivo p53 and RB family proteins in human mesothelioma samples; (b) its activation of Notch-1, a gene promoting cell cycle progression and cell proliferation in primary mesothelial cells; (c) induction of apoptosis in mesothelioma cells transfected with antisense DNA to the SV40 early region genes; (d) presence of SV40 tag-specific cytotoxic T lymphocytes in sera of patients affected by mesothelioma; (e) finally, poorer prognosis of mesotheliomas harboring SV40 early region sequences compared to SV40-negative mesotheliomas. In fact, mesothelial cells are specifically susceptible to infection and transformation by SV40. Some studies give emphasis on the fact that SV40 tumour antigens induce telomerase activity in human mesothelial cells without any effect in fibroblast cells. This suggest that, both SV40 oncoproteins specifically participate in the immortalization of mesothelial cells during mesothelioma development (135,

33

136). The association between SV40 and malignant mesothelioma appears to be the strongest for any SV40-associated cancer (124). Nevertheless, studies such as James et al., have failed to detect SV40 sequences from 43, 30, 17, and 19 mesothelioma cases from Johannesburg-South Africa, Wales-United kingdom, New York and Croatia respectively (137).

SV40 in Non-Malignant Specimens

Previous studies have shown expression of SV40 Tag in certain cancers suggesting the possibility of SV40 etiologic role support in these cancers. Observation of animals with tumours developed antibody against SV40 Tag and neutralization of SV40 with specific antibody before virus inoculation prevented cancer development (32b, 138). Probably, all these findings in experimental models prompted past as well as recent investigators to consider the role of SV40 infections in some human malignances and non-malignances as well.

Studies have reported the localization of SV40 DNA sequences to renal tubular epithelial cell nuclei in renal biopsies of patients with focal segmental glomerulosclerosis using in situ hybridization. In these studies, several strains of SV40 were displayed, these strains included SV40 strain 776 and other strains with mutations in the early and late regions (39, 138). Additionally, other different studies showed that SV40 DNA sequences from the viral regulatory region were detected and identified in the allograft of immune-compromised pediatric renal transplant recipients and in the native kidneys of a young adult lung transplant patient with polyomavirus nephropathy (139). Another study, reported that SV40 and BKV sequences have been co-detected in the kidneys of patients with post-transplantation intestinal nephritis thus suggesting that SV40 may co-operate in the etiopathogenesis of this chronic disease. SV40 sequences was also detected in kidney tissues and urine. Furthermore, SV40 DNA sequences in PBMCs in both oncological patients and normal individuals from various population have also been detected by different study groups (39, 40, 176). These results demonstrate the nephrotropic and lymphotropic properties of SV40 and indicate that the kidney and PBMCs can serve as reservoirs for the virus in humans. Generally, it appears

that patient with acquired and or iatrogenic immunosuppression seems to be at risk for SV40. Although, the frequency, natural history and morbidity of the virus in this increasing patient population are not clear (125).

1.8: Association of SV40 with Lymphomagenesis/Non-Hodgkin's Lymphoma (NHL)

Studies have shown that the types of tumour developed/induced by SV40 in hamsters are in the same range with human tumour with SV40 DNA sequences. Since the past decades, the role of SV40 in lymphomagenesis has been investigated in humans, although, lymphomagenic potential of SV40 has long been established by different studies in experimental animals. Diamandopoulos has shown that, 72% hamsters inoculated with SV40 intravenously developed lymphomas while the control- in-inoculated group no lymphoma result (32b). The histological type of resulting tumours was very consistent with diffuse large cells and the lymphoma was shown to be B-cell origin with cell surface antigen (140).

Past studies observed that lymphomas were one of the most common malignancies induced by SV40 in experimental models (141). Knowledge of these animal model studies prompted the investigation of the possible role of SV40 in human lymphomagenesis. There has been continuous detection of SV40 along with other polyomaviruses BKV, JVC and MCPyV in healthy blood donors and in B cell rich tonsillar tissues of immunocompetent children (40a, 40b, 94, 142,). This suggests the possibility of SV40 having a tropism for the lymphatic system, which is a very important factor to use in trafficking the virus around the body and in pathogens.

Recently, in United States, Lymphoma has been identified as the most common type of blood cancer, the seventh most common cancer in adults and the third most common in children. In the United States and European Union, about 54,000 and 50,000 people are diagnosed annually with

non-Hodgkin lymphoma (NHL) respectively (**http://www.cdc.gov/vaccinesafety/updates/archive/polio...**). There has also been a dramatic increase in NHL incidence in the last 30 years, with an 80% increase in the US noted between 1973 and 1997 (143).

NHL includes a group of more than 20 different lymphoproliferative disorders arising from Lymphocyte cells [B, T, and NK cells] (144). Significant association of SV40 has been established by several studies from the continuous detection of SV40 DNA sequences (Table 1.2). Despite the fact that the exact etiology of NHL is not known, but as EBV is associated to Burkitt's Lymphoma, KSHV associated to primary effusion lymphoma and human T lymphotropic virus type 1 (HTLV-1), adult T cell known to be associated to leukemia/lymphoma—similarly, some infectious agents have been identified to cause NHL by lymphocyte infection. Virus such as hepatitis C has also been implicated as an increasing risk factor of NHL through chronic immune stimulation in a chronic infection (145).

Immunodeficiency is also considered a major risk factor for the development of NHL, and it is a common malignancy in HIV-infected patients as a result of deficient immunosurveillance of oncogenic viruses (146, 176). Additionally, immunosuppressed patients in other poliomavirus related diseases such as BKV and JVC have also shown to be at risk of NHL. This data suggest that SV40 positive NHL does not appear to be dependent of pronounced immunodeficiency in the host (11).

Numerous significant reports have identified SV40 DNA sequences in diffuse large B cells lymphoma and follicular lymphoma, two of the most common histologic types of B cell lymphoma accounting for approximately 50-60% of all cases of NHL (23). Involving SV40 sequences in these particular types of NHL raises the question about the significance of the developmental stage of B cells in the progression to lymphoma and indicates mature B cells to be more susceptible than precursor cells in the transforming potential of SV40.

Table 1.2: detection of SV40 DNA sequences in human lymphoploriferative disorders/NHL

Study	Sample	Technique Used	Country	SV40 DNA in Lymphoproliferative disorder/NHL No Positive/no.tested (%)
Martini et al., 1998b	Human lymphoproliferative disorders obtained from human-immunodeficiency-virus (HIV)-seronegative and HIV-infected patient	PCR, Filter hybridization and immunochemistry	Italy	11/79 (13.9)
Shivapurkah et al., 2002	68 Non Hodgkin Lymphoma and 31 Hodgkin Lymphoma patients	PCR and Southern blot	USA	NHL patients 29/68 (43) H L patients 3/31 (9)
MacKenzie et al., 2003	152 NHL samples, from patients in the U.K. with lymphadenopathy and lymphoid leukemia	PCR assay	UK	0/152 (0)
Nakatsuka et al., 2003	122 cases with HNL	Immunohistochemistry, PCR, Southern blot and in situ Hybridization	Japan	14/122(11)
Engels et al., 2004	724 incident NHL case patents and 622 control subjects from a population based U.S. case control study	Enzyme immunoassays	USA	52/724 (7.2)
Butel et al., 2003	154 NHL patients, 186 non-malignant lymphoid samples and NHL from HIV-I-positive patients	PCR, Southern blot, Hybridization analyses	USA	64/154 (42) 0/186 (0) 33% of NHL from HIV-l-positive patients
Dana et al., 2005	119 B cell NHL main samples	Elisa, logistic regression	USA	24/119 (16.8) 33/184 (15.2)
Meneses et al., 2005	125 Lymphoma and control specimens from HIV negative patients	PCR, Southern blot and immunohistochemistry	Costa Rica	30/125 (24) 0% from Control Samples
Po-Min Chen et al., 2006	19 frozen lymph nodes from NHL patients	PCR, Southern blot, hybridization analysis	Taiwan	13/91 (14.3)
Amara et al., 2007	108 diffuse large B-cell type lymphoma (DLBCL) samples	PCR assays	Tunisia	63/108 (56)
Toracchio et al., 2009	171 archival paraffin-embedded lymphoma specimens	PCR analysis and immunohistochemistry	USA	Part A=10/44 (23) Part B= 4/127 (3)
Zekri et al., 2007	Lymphoma samples and Control samples	PCR	Egypt	83/158 (54) 4/34 (12) Control

(147).

Different studies have provided evidence for the detection of sequences of SV40 DNA with human lymphomas and other lymphoproliferative disorders (Table 1.2). However, many studies still could not detect SV40 sequences in their samples (148, 149). The inability of these studies to detect SV40

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DNA in their samples could be attributed to laboratory technical difficulties in the detection of SV40; and to geographic variation relating to the distribution of SV40 contaminated polio vaccines and/or characteristics of a particular population along with existence of variation in the ethnic susceptibility to SV40.

In fact, the role of some viral agent such as EBV have been strongly implicated in certain lymphoproliferative disorders. The main question now is: can SV40, just like in the case of mesothelioma where it has been identified as co-carcinogen with asbestos (130), be also identified as a causative agent?, or can it have a role to play in lymphomagenesis/ NHL?. Other questions raised by studies investigating the role of SV40 in lymphoproliferative disorders include: "is the association of SV40 with NHL causal or incidence?; does SV40 have a hand in the proliferation of tumourigenic events in NHL?; do tumours offer a microenvironment that favours viral replication in humans with latent SV40 infection?" (103). Honestly, the answer to the above questions are yet to be strongly established experimentally.

Nonetheless, there have been previous and present studies investigating the presence of SV40 in human lymphoproliferative disorders though only few studies such as Dolcetti et al., and Shaikh et al., have focused on the SV40 interaction with human B lymphocytes (38, 118). Despite the limitations of Shaikh et al in their study, they tried to detect SV40 DNA sequences using samples collected at only one early time point when little effects of SV40 would be expected. The results of the above two studies indicated the potential role of SV40 in Lymphomagenesis and call for further investigation to examine the relationship and effects of SV40 with human lymphocytes.

2. Objective of the experimental thesis

Simian Virus 40 (SV40), a small DNA tumour virus of approximately 5.2 kb, has been fully characterized as a transforming and tumourigenic viral agent. It transforms different types of animal and human cells, and when inoculated by different routes in experimental animals, it induces bone and brain tumours, mesothelioma and lymphoproliferative disorders. SV40 footprints in humans have been found to be associated at high prevalence with specific human tumours of same as are induced by SV40 in rodents. It has also been detected, although at lower prevalence, in blood samples from healthy donors. These SV40 properties are mainly related to the activities of its two oncoproteins, the large Tumour antigen (Tag) and the small tumour antigen (tag), which display multiple functions. In cell transformation and tumourigenesis, Tag target key cellular products, such as the tumour suppressor p53 and pRB family proteins, inactivating their functions.

However, different studies have provided no evidence of detecting SV40 sequences in their experiments/samples and its ability to infect blood cells, probably due to laboratory technical difficulties in detection of SV40, geographic variation relating to the distribution of SV40 contaminated polio vaccines and/or characteristics of a particular population along with existence of variation in the ethnic susceptibility to SV40. As consequence of these conflicting results, considerable debate has developed in science community. The problems related to SV40 infection in the human population and its contribution to human cancer have been summarized in an evaluation by the "Immunization Safety Review Committee" established by the Institute of Medicine of the American National Academies. Detailed investigations aimed at verifying whether SV40 may infect human lymphocyte cells from healthy individuals are of paramount importance in revealing the capability of SV40 to spread in humans and in studying its contribution in the onset/progression of lymphomagenesis.

Moreover, the relevance of viral infections to the onset/progression of human hematologic malignancies and other blood diseases is still a matter of active investigation. In as much as contrasts of opinion on the role/association of SV40 in Lymphoproliferative disorders has continued for an age, indebt studies on the interaction between lymphocytes and SV40 has not yet been fully described. My research aimed to analyze the effect of SV40 infection on human B and T lymphocytes so as to gain insights into the pathogenesis of SV40 in human disease. The project was therefore designed to experimentally verify (a) SV40 DNA sequences in 60 healthy blood donors from Aviano Cancer Center, (b) characterize infection of strain 776 SV40 in T and B cells and transfection with pSV3neo plasmid (a plasmid with early region of SV40) in B cells obtained from healthy blood donors so as to understand the possibility of susceptibility of B and T cells, their transformation/ immortalization and production of viral progeny.

In my method, continuous analysis of extracted DNA samples from healthy blood donors, SV40-infected/transfected B and T lymphocytes then allowed: (i) examination of the effect of SV40 infection on the cell proliferation and viability of B/T-lymphocytes using trypan blue analysis/Alamar blue assays on cultured cells; (ii) examination of the presence and expression of viral sequences with quantitative/qualitative PCR, RT-PCR and FACs analysis; (iii) examination of the morphological and immunological characteristics with immunoflorescence and scanning electron microscope; and (iv) production of viral progeny using cytopathic effects assay and plaque assay.

3. Materials and methods

3.1: PBMCs and Isolation B/T cells from buffy coats

Buffy coats were obtained from the blood bank of St. Anna's University-Hospital of Ferrara. PBMCs (n=1x10^9) were isolated by density gradient centrifugation over Ficoll-Histopaque 1077 (Sigma-Aldrich, Milan, Italy). Then, PBMCs were cultured overnight with RPMI 1640 (Euro Clone, Milan, Italy) supplemented with 10% Fetal bovine Serum (Euro Cline Milan) and 2% Penicillin/Streptomycin (P/S) at 37°C and 5% CO_2 in a humidified atmosphere. Human B and T cells were isolated from PBMCs by deplation method using immunomagnetic DYNAbeads (Invitrogen Milan, Italy) according to the instruction of the manufacturer. Human B and T cells were maintained in culture in RPMI 1640 medium (InVitrogen, Milan, Italy) supplemented with 10% FBS (Euroclone, Milan, Italy), 2 mM L-glutamine, 2% (P/S) and 2% phytohemagglutinin (PHA) (Gibco, Invitrogen, Milan, Italy) at 37°C and 5% CO_2 in a humidified atmosphere, in 6 well plates at 2 x10^6 /ml cells. Trypan blue assay was employed to assess the proportion of viable cells by an optical microscope. Cell count (n= 2x10^6/ml) was used for percentage survival calculation. Percentage survival= number of viable cells at test time point/number of viable cells at time zero x 100.

3.2: Flow cytometric analysis, infection and transfection of human B and T cells

Flow cytometer of BD FACS CALIBUR was used to analyse the expression of human B and T cells markers isolated by immunomagnetic dynabeads. B cells were investigated with the monoclonal antibodies, CD19, anti-CD45 and anti-CD3, then labelled fluorescein-isothiocyanate (FITC)-conjugated (Becton Dickinson, Milan, Italy).

SV40 infection was carried out under Biosafety Level 2 safety precautions. Human B lymphocytes (n=2x10^6/ml) were infected with the SV40 strain 776 in RPMI supplemented with 1% FBS and 1%

of P/S. Viral inoculum, diluted in medium with 1% FBS was employed at m.o.i. of 0.1 p.f.u./cell. Incubation was carried out for 2hrs at 37°C, 5% CO_2, with occasional rocking, in 6 well plates. The viral inoculum was then removed, and after rinsing with 1% saline, cells were maintained in culture in complete medium at 37°C, 5% CO_2 in a humidified incubator. Different assays were carried out at different time post infection (p.i.) to verify the SV40 infection of B cells. The culture medium was changed twice a week.

pSV3neo DNA (figure 3.1), 500 ng, was transfected into B cells (n=$2x10^6$/ml) in six well plates using Lipofectamine^{TM} LXT and PLUS^{TM} Reagents (Invitrogen, Milan Italy) according to manufacturers instruction. Transfected B cells were maintained in culture in the same manner as described above for the SV40-infected cells.

Figure 3.1: structure of the pSV3-neo plasmid with intact SV40 early region. The pSV3neo plasmid is composed of pBR322 origin of DNA replication (pBR322 ori) a β-lactamase gene (AMP^R); a neo gene (1.4 kb fragment); and the SV40 DNA sequence. The SV40 origin of DNA replication (SV40 ori) and SV40 early promoter are present on a small fragment immediately 5' to the neo segment (150).
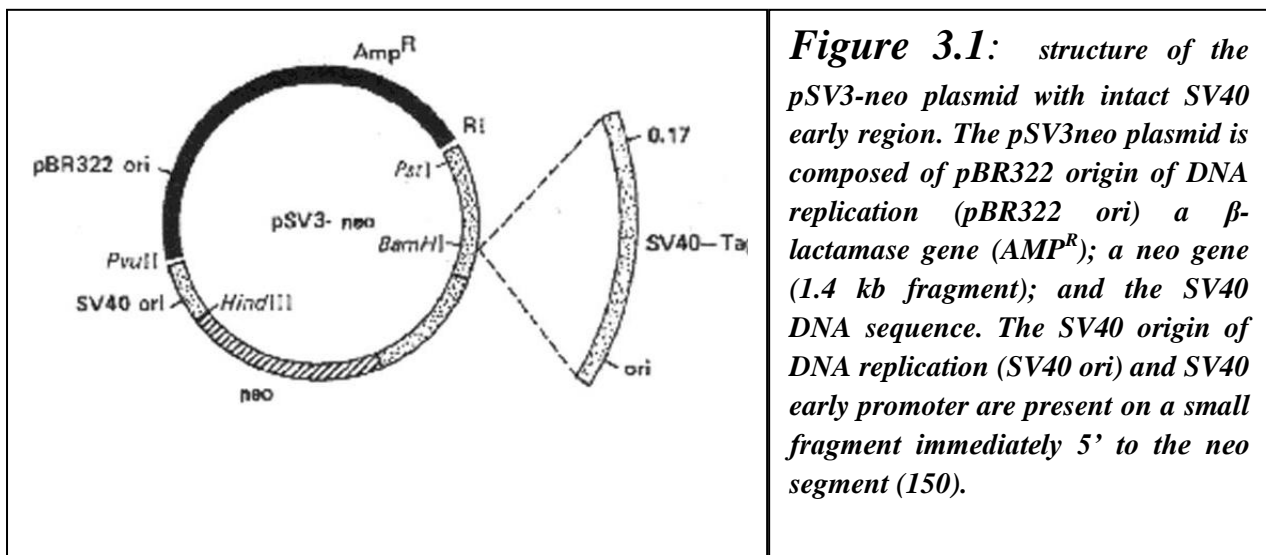
3.3: Cell lines

CV-1 (Figure 3.2A) cell line was initiated in March of 1960 by F. C Jensen and his colleagues with a tissue section excised from kidney of a normal adult male African green monkey (Cercopithecus aethiops). CV-1 cell lines exbits the morphology of fibroblast and has the capacity to grow adherently to glass or plastic surfaces. CV-1 cells are known to be subject to several viruses such as SV40, poliovirus 1, herpes simplex, california encephalitis and Estern and Western Equine

encephalitis. Being permissive to SV40 infection, were used as positive controls to verify the C.P.E. CV-1 cells, were grown in Dulbecco's modified Eagle's medium–F12 (DMEM) (Euroclone, Celbio, Milan, Italy), supplemented with 10% FBS and 2% P/S.

WI38- VA13 (Figure 3.2B) is an SV40 transformed cell line derived from embryonic lung human strain WI-38 subline 2RA fibroblast cells (151, 152). WI-38 VA13 is used as a Tag positive control and were maintained in culture with Minimum Essential Medium (EMEM) (Euroclone, Celbio, Milano Italy) supplemented with 10% FBS and 2% P/S.

Figure 3.2: *positive control Cell lines. (A), Normal African Green Monkey kidney Fibroblast cells (CV-1), (B) Embryonic lung Human Strain WI-38 Subline 2RA Fibroblast cells (WI38-VA13).*
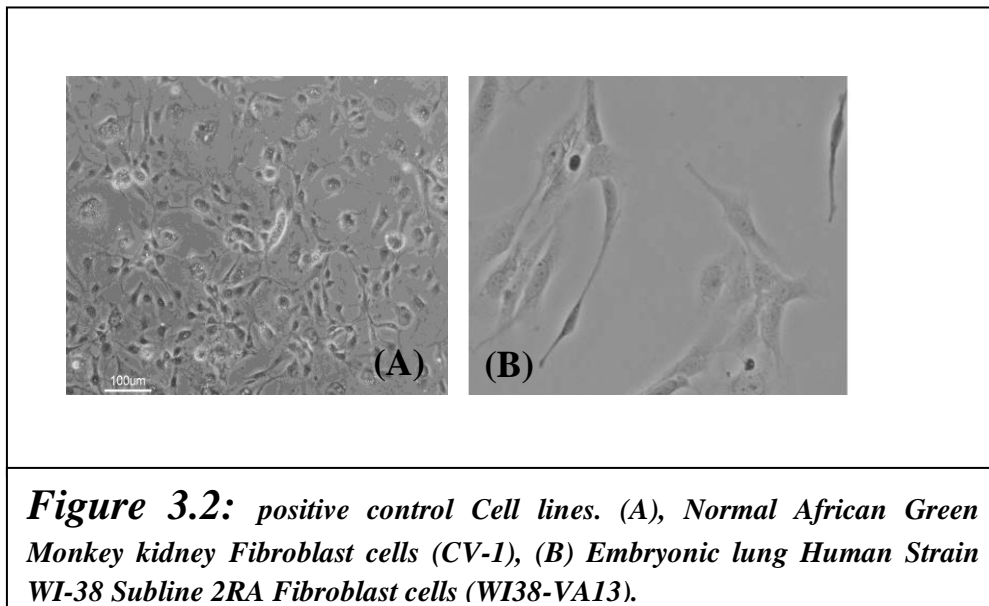
3.4: Alamar Blue assay for cell viability and proliferation

Normal, SV40-infected and pSV3neo transfected B-cells were collected from culture flasks, counted with Hemacytometer, brought first to a concentration of $2x\,10^6$ cells/ml and subsequently seeded in duplicate wells of a 24-well plates with 1 ml of RPMI medium containing 1% FBS and 5% Alamar blue (AB), plates were maintained at standard culture conditions of 5% CO_2 in air at 37°C. Optical density of 100 μl of the medium in 96 well plates was measured at 570 nm and 620 nm with a standard spectrophotometer at 5 hours in different days of p.i. As a negative control, AB was added to medium without cells (153, 154) . The percentage reduction of Alamar blue was

calculated using the formula: = [ALW - (AHW x R0)] x 100,   Where: ALW = absorbance at lower wavelength minus the media blank, AHW = absorbance at higher wavelength minus the media blank and R0 = Correction factor [Absorbance of AB in media − Absorbance of media only at lower wavelength/Absorbance of AB in media − Absorbance of media only at higher wavelength] (155).

3.5: *Population doubling time (PDT)*

To determine the population doubling time (PDT) of transformed cells, B transfected and infected cell numbers from 80-d.p.i. were determined in triplicate using haemocytometer. PDT was calculated at 48 and 72 hours intervals using the formular: PDT= t log 2/ log Nt- log No, where t is time period, Nt is number of cells at time t and No is initial number of cells (156, 157).

3.6.1: DNA Purification (*FOR EXPERIMENT I*)

The subjects under investigation were 60 healthy blood donors. All the 60 subjects were from the same area with residual environmental asbestos pollution (Aviano). The persistent environmental pollution of asbestos is being suspected as the cause of a higher mortality from MM among the inhabitants of the area regardless of occupational history. Blood from 60 subjects were collected and stored at −80°c till the time of use for laboratory analysis. 500μl of the blood was digested with 1% SDS and 500μg/ml protinase K (Sigma, Milano Italy). The DNA was purified using a mixture of phenol-chloroform-Isoamyl alcohol in the ratio of 25:24:1 and dialyzed overnight with TEN buffer (10mM Tris, pH7.5, 1mM EDTA, 1M Nacl). It was further purified overnight with TE buffer in the absence of Nacl

3.6.2: *DNA and RNA extraction* **(FOR EXPERIMENT II and III)**

DNA was extracted using a commercial kit (Qiagen kit, Invitrogen, Milano Italy) as directed by the supplier, using $1 \times 10^{6/}$ml cells density of lymphocyte cells. DNA was also extracted from cell sample pellets (B and T infected and normal lymphocyte cells, WI-38 VA13 cultured cells and pSV3neo transfected B cells). Pellet was digested with protein kinase buffer, RNase (10μg/ul) and SDS of 10%, DNA was purified using a mixture of phenol-chloroform-isoamyl alcohol in the ratio of 25:24:1 and dialyzed overnight with TEN buffer (10mM Tris, pH7.5, 1mM EDTA, 1M Nacl). It was further purified overnight with TE buffer in the absence of NaCl (40a).

Total RNA was extracted from 5×10^{6} WI38-VA13 cells, infected, transfected and normal samples of B cells, using TRIzol reagent (Gibco/BRL, Milan, Italy) according to the manufacturer's instructions.

3.7.1: *Polymerase Chain Reaction [PCR]* **(FOR EXPERIMENT I)**

SV40 DNA from 776 strain was used as the positive control in PCR amplification.500ng DNA samples from 60 healthy blood donor were initially verified for PCR analysis using a control reaction designed to amplify β-globin gene sequences to check for PCR Compatibility. The parameter cycle for β -globin gene amplification were 5 min at 94°C., 30 s at 56°C., 30 s at 72°C. for 35 cycles (40a). All samples were further investigated for amplification of SV40 sequences. To avoid PCR contaminations, all reagents for both DNA extraction and PCR analysis were exclusively used for the experiments of this study. All samples were analyzed for SV40 Tag sequences of SVINTfor-SVINTrev and SV.for2-SV.rev specific for SV40 early region sequ and later sets of primers served for nested PCR (nPCR). The SV.for2-SV.rev has the cycle parameter of 5mins at 94°c, 1min at 94°c, 1min at 55°c, 1min at 72°c for 45cycles and SVINTfor-SVINTrev with cycle parameter of 15mins at 94°c, 1min at 94°c, 45s at94°c, 45s at 60°c, 1min at 72°c for

40cycles. Ten micro liters of each PCR reaction were loaded on 2% agarose gel and eletrophoresed in 0.5$_x$ TAE buffer [40 mM Tris- acetate EDTA (pH 8)], stained with ethidium bromide, and photographed. The molecular weight marker was the gene ruler 100bp. DNA from samples that were positive for SV40 by PCR were analyzed and sequenced three times in both directions using the primers according to the manufacturer's protocol.

3.7.2: PCR techniques (FOR EXPERIMENT II and III)

For PCR analysis, 200ng of DNA extracted from WI-38 VA13, infected, transfected and normal B cells. DNA extracted from both WI38- VA13 cells and whole SV40 DNA wild-type strain 776 were used as positive controls while normal B cells were used as negative control (40a). PCR amplification of the DNA samples was determined using SV.for2-SV.rev (single step PCR) and Sv.for1/Sv.rev1(nested PCR). Polymerase chain reaction products (10 ul sample) were migrated in a 1% electrophoresis agarose gels in the presence of ethidium bromide, using a TAE 0.5X buffer containing 40 mM Tris-acetate, 1mM EDTA, pH 8 and visualized under U.V. light using the Gel Doc 2000 System (Bio-Rad, Milano, Italy). Molecular weight markers were the Gene ruler 100 bp (Fermentas, Milan, Italy).

3.8: RT-PCR analysis

For reverse transcriptase (RT), 0.3μg of total RNA of the samples were respectively resuspended in 10 μl of a reaction mixture made from 10x reaction buffer with MgCl$_2$, 1 U DNase I-RNase-free (Fermentas, Milan, Italy) in water diethylpyrocarbonate (DEPC) and incubated at room temperature (RT) for 15 min. Dnase I was inactivated by adding 1 μl of 25 mM EDTA and incubating at 65°C. for 10 min. DNA-free total RNA was transcribed. cDNA was obtained using SuperScript II (Invitrogen, Milano, Italy) following the manufacturer's instructions. cDNA was then amplified by

single PCR with primers SV.for2-SV.rev and analyzed by gel electrophoresis. A house keeping gene, NAPDH (159) was also used to control the efficiency and reliability of the cDNA samples.

3.9: *Ultrastructural studies (Experiment II-III)*

For ultrastructural studies, Cultured infected, transfected, and normal B cells were fixed overnight at 4°C in 2·5% gluteraldehyde and post fixed for 2 hrs at room temperature in 1% osmium tetroxide and dehydrated with increasing concentration of ethanol before embedding in Araldite II resin. Transversally cut sections were then analyzed using transmission and scanning electron microscopy (TEM and SEM, Cambridge UK, model Stereoscan S-360) as described before (158, 160, 161, 162).

3.10: *Indirect immunofluorescence*

WI-38 VA13 expressing SV40 Tag was used as positive control in this experiment (163). WI38 - VA13 cells were grown in 12 mm coverslip glass. T and B cells were infected or transfected as described above, then stained for immunofluorescence at different times p.i. Samples were sedimented by cytospin and analyzed by indirect immunofluorescence. Briefly, cells were fixed in methanol acetone (1:1) for 10 min at RT, washed in PBS, and reacted with the mouse monoclonal antibody against Tag, C-terminus epitopes, with Pab101 (Santa Cruz Biotecnology, Santa Cruz, CA, USA) (epitope between residues 512 and 708) for 1h at Room Temperature (RT) and then with anti-mouse IgG-FITC-conjugated (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h at Room Temperature. To verify the presence of SV40 capsid proteins (VPs), cells were reacted with goat anti-SV40 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h at RT, and then with secondary antibodies of donkey anti-goat IgG-FITC-conjugated (Santa Cruz Biotecnology, Santa Cruz, CA, USA) for 1h at RT (B. Burmeiser et al., 2004). Cells were counter-stained with 0.5 µg/ml of 4',6-diamino-2-phenylindole (DAPI) (Sigma, Milano, Italy) and observed

with a Nikon TE2000E microscope (Nikon S.p.A., Florence, Italy), while digital images were captured using the ACT-1 and ACT-2 software for the DXM1200F digital camera (Nikon S.p.A., Florence, Italy).

3.11: Cytopathic effect of infected T cells, B transfected and infected with titer determination

Permissive CV-1 cells were used to verify the presence of SV40 virions at day 10, 40 in T cells and 10, 40 and 60 days p. i. in infected B cells. Sonicated pellet and medium of SV40-infected T, B cells were employed as inoculums in CV-1 to investigate the typical SV40 cytopathic effects (C.P.E.). SV40 infected T, B-cells and medium were subjected to three cycles of freezing and thawing and then sonicated. Subsequently, lysated and medium were inoculated into monolayers of CV-1 cells (60-70% confluent) (138) and incubated for 2 hrs at 37°C, 5% CO_2 with occasional rocking in DMEM supplemented with 1% FBS and antibiotics. After incubation, CV-1 cells were cultured in 24 well plates in DMEM supplemented with 1% FBS for 15 days for observation of C.P.E appearance. CV-1 cells infected with 10 to 10^6 pfu/ml and uninfected CV-1 represented the positive and negative control respectively. SV40 776 strain was used as the viral working stock as described previously (158).

3.12: Plaque assay

Virus plaque assay was carried out as previously done in (164, 165, 166). Briefly, SV40 776 strain virus stock solution (10^7) was used to infect $2x10^6$/ml purified B and T lymphocytes at 0.1 pfu/cell. Absorption was carried out for 2hrs at 37°C, 5% CO_2 in a humidified incubator in 2 ml DMEM medium supplemented with 1% FBS. Cells were washed with 1% PBS. 200ul dilution of 10^6 of infected cells in DMEM medium supplemented with 1% FBS were inoculated into monolayer CV-1 cells in a 6 well plates and incubated for 6hrs at 37°C. Subsequently, cell medium was aspirated from the plates and plated with DMEM medium solution supplemented with 1% FBS and 1.0 %

agarose gel (Sigma, Milan, Italy) prepared by autoclaving at 121°C. for 20 minutes. After incubation for 9 days at 37°C with addition of 500ul medium every 3 days, the agarose layer was stained with 0.1% crystal violet. Plaques were counted and expressed as number of PFU/10^6 cells. SV40 infected CV-1 at 10^1 to 10^6 viral titre and uninfected CV-1 represented positive and negative controls, respectively (164, 165, 166).

3.13: Statistical Significance

The results of Trypan blue and percentage expression of B cells from different donors were tested for significance using the student's t-test (two-tailed). Differences were considered significant at p < 0.05 percentage and the values of the parameters analyzed are expressed as mean ± standard deviation (SD). The 95% confidence intervals of samples (C.I) was also reported.

4. Results

Experiment I

Simian Virus 40(SV40) Sequences in Blood Specimens Of Healthy Individuals From Aviano.

SV40 is a DNA virus found to be cocarcinogenic to asbestos in the pathogenesis of Mesothelioma. Human infected mesotheliomal cells are also found to be susceptible to SV40 with 100% of the infected cells expressing the SV40 T antigen (Tag). This Study investigated the presence of SV40 sequences in 60 healthy Subjects from Aviano, an Italian town with asbestos pollution history and high prevalence of mesothelioma. PCR result on the purified DNA samples from the subjects using SV40 as the positive control showed that 17% (7.4–27CI) were SV40 positive. It showed that 17% of 60 subjects can be distinguished as possible potentially asbestos infected individuals and that SV40 with asbestos are really cofactors in the pathogenesis of mesothelioma. It also indicated that SV40 virus is being circulated in the human population. This study could serve as an epidemiological study that can bring SV40 virus closer to clinical practice.

4.1: SV40 DNA sequences in 60 Subjects of Aviano

DNA from blood sample of 60 subjects suitable for PCR analysis were analyzed by single PCR to determine the presence of SV40 sequences. SV40 Tag NH_2-terminal coding sequences were investigated with primer sets Sv.for2-Sv.rev with 575bp. The single PCR analyses indicated that SV40 Tag sequences were not present in any of the 60 DNA samples. Further analysis of the DNA samples with primers set SVINTfor-SVINTrev and SV.for-SV.rev by nPCR for SV40 Tag intron sequences (Table 4. 2). The result showed that 10 (17%) out of 60 samples were positive for SV40

intron sequences. A control using 15 samples negative for SV40 DNA samples analysed for nPCR with SV.for-SV.rev and SVINTfor-SVINTrev were also found negative. A subject is considered to be positive if it was detected two or more times in the nPCR with SVINTfor-SVINTrev set of primers

SUBJECTS	SINGLE PCR			N° PCR in BB2-BB3			N° PCR in SVINTfor-SVINTrev			FINAL RESULT
3	-	+	-	-	-	+	+	-	+	+
5	-	+	-	-	-	-	+	+	+	+
15	-	-	+	+	+	+	+	-	+	+
16	-	+	-	+	+	+	+	+	-	+
17	-	-	+	+	-	+	+	+	+	+
18	-	+	-	+	-	+	-	+	+	+
19	-	+	-	+	-	+	+	-	+	+
32	-	-	-	-	-	-	+	+	+	+
51	-	-	+	-	-	-	-	+	+	+
53	+	-	-	+	+	-	+	+	+	+

Table 4.1: *subject numbers detected SV40 DNA sequences from the 60 examined subjects. The experiment was carried out trice in each set of primers and a subject was considered to be positive using the second amplification (SVINTfor-SVINTrev) with occurrence of at least two times. BB2-BB3 (SV.for1-SV.rev 1, N° PCR (number of PCR).*

Table 4.2: Oligonucleotides Used as Primers in PCR and RT-PCR Analysis.

SV40 DNA Regions	Oligonucleotides	Reference Position	Annealing. Cycle (bp)	Temp °C Number	Size
TAG NH₂. Terminal Region.	SV. for2: 5'-TTTGGAGGCTTCTGGGATGCAACT-3'	nt 4945-4921			
	SV. rev : 5'- GCATGACTCAAAAAACTTAGCAATTCTG-3'	nt 4372-4399	55	45	575
	SVINTfor: 5'- AAGTAAGGTTCCTTCACAAAG-3'	nt 4904-4924	60	40	235
	SVINTrev: 5'- AACTGAGGTATTTGCTTCTTC-3'	nt 4690-4710			
	SV.for1: 5'-TAGATTCCAACCTATGGAACTGA-3'	nt 4552-4574	56	35	172
	SV.rev1:5'-GAAAGTCCTTGGGGTCTTCTA-3'	nt 4403-4424			
	Control Gene in RT-PCR				
NAPDH	5'TGGTATCGTGGAAGGACTCATGAC-'3	95-332	55	45	238
	5'ATGCCAGTGAGCTTCCGGTTAGC-3'				

Experiment II

*S*imian virus 40 Infection of Human T Lymphocytes.

Transfection with SV40 Tag may be a valuable method for obtaing long term human T cell lines for studies of both aging and immunology though it does not fully reveal the ability of SV40 virions to be able to infect T lymphocytes. The present study investigated human T lymphocytes isolated by immunomagnetic separation from peripheral blood mononuclear cells (PBMCs) of healthy blood donors, infected with simian virus 40 (SV40) and then monitored for evidence of a productive infection. Trypan blue analysis up to 80 days post infection revealed viability and plorification capacity of the infected cells. SV40 sequences were detected in T infected cells up to 80 days post infection (p.i.) with PCR tecnique. Immunofluorescence images showed the presence of SV40 Tag and Viral proteins in T lymphocytes from 3 days p.i to 40 days p.i. Hence, our data indicated that human T lymphocytes can be infected by this small DNA tumor virus.

Human T cells purified by immunomagnetic separation from the PBMCs of blood donors had a population purity of > 98%, as determined by flow cytometry using CD3 and CD45 mAb, as membrane markers. This remarkably showed that, the used T cells were normal human T cells of healthy donors. Then, the T lymphocytes were infected by SV40 and monitored in culture for 80 days post infection.

4.2: To investigate the Viability of the SV40 Infected T lymphocyte cells

The viability and proliferation rate between the normal and infected T cells was quite distinguishable.   The trypan blue exclusion analysis employed showed increase in the number of T SV40 infected cells from the initial starting number of 80×10^6 total of every infected cells (Figure

4.1). In the normal cells, drastic cell death was observed and the cells lasted for about 5 days in culture (Figure 4.1), and the T infected cells continued to be viable in culture but exhibiting slow decrease in number of SV40 viral cells as observed in (118).

Figure 4.1. *Figure 1. Normal (■) and SV40-infected (▲) T lymphocytes. In our culture conditions, the majority of normal T cells (with PHA ●, or without PHA ■) remained viable for up to 5 and 3days, respectively, while SV40-infected T lymphocytes were viable for up to 80 p.i. The error bars represent averages of results of T cell cultures obtained from 6 donors.*
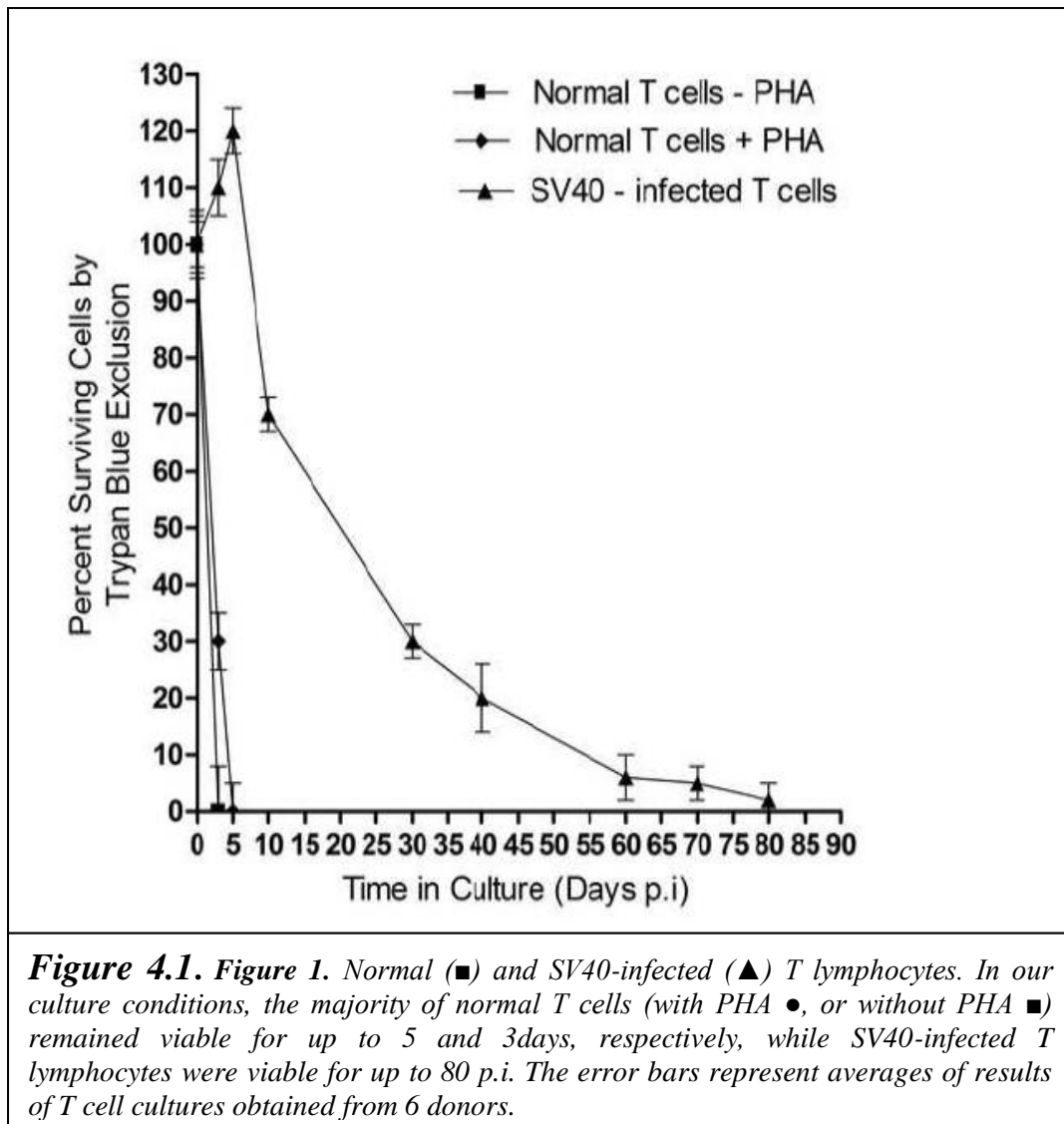
4.3: SV40 sequence analysis in infected T cells by single and nested PCR

In further assess of the SV40 specificity, the highly conserved SV40 Tag domains in PCR analysis is similar to the SV40-776 wild type repeatedly detected in human tumors and healthy human tissues by many investigators. The PCR analysis of the SV40 DNA from infected cells using B-globing control gene primers indicated that DNA of the infected cells were of SV40 776 viral

particle, (Figure not shown). This indicated that no deletions or rearrangements occurred during the long-term cultures (40, 64).

 DNA sequences was present in T cells culture infected by SV40 (Figure 4.2). SV40 Tag sequences is found with single PCR using SV.for2-SV.rev set of primers (Pancaldi et al., 2009) up to 30 p.i both in supernatant and pellet (Figure 4. 2a). Then, in further analysis up to 80 days post infection (p.i.) in pellet with nested PCR (Figure 4.2b) using set of primers specific for SV40 early region sequences (Table 4.2), SV40 DNA sequences was detected. Tag was also detected by RT-PCR using primers specific for SV40 early regions. The WI38-VA13 used as positive control with SV40 776 also tested positive while T normal cells used as negative control was negative as expected.

Figure 4.2: *Single and Nested PCR Analysis of SV40 DNA Sequences up to 80 days Post Infection using set of Primers SV.for2-SV.rev (A) and SV.for1-SV.rev1(B), (C) RT-PCR of infected T cells up to 40d.p.i. DNA and cDNA (WI38-VA13 used as positive control), M(molecular weight maker-100bp), C(Normal T cells), R(PCR Control).*
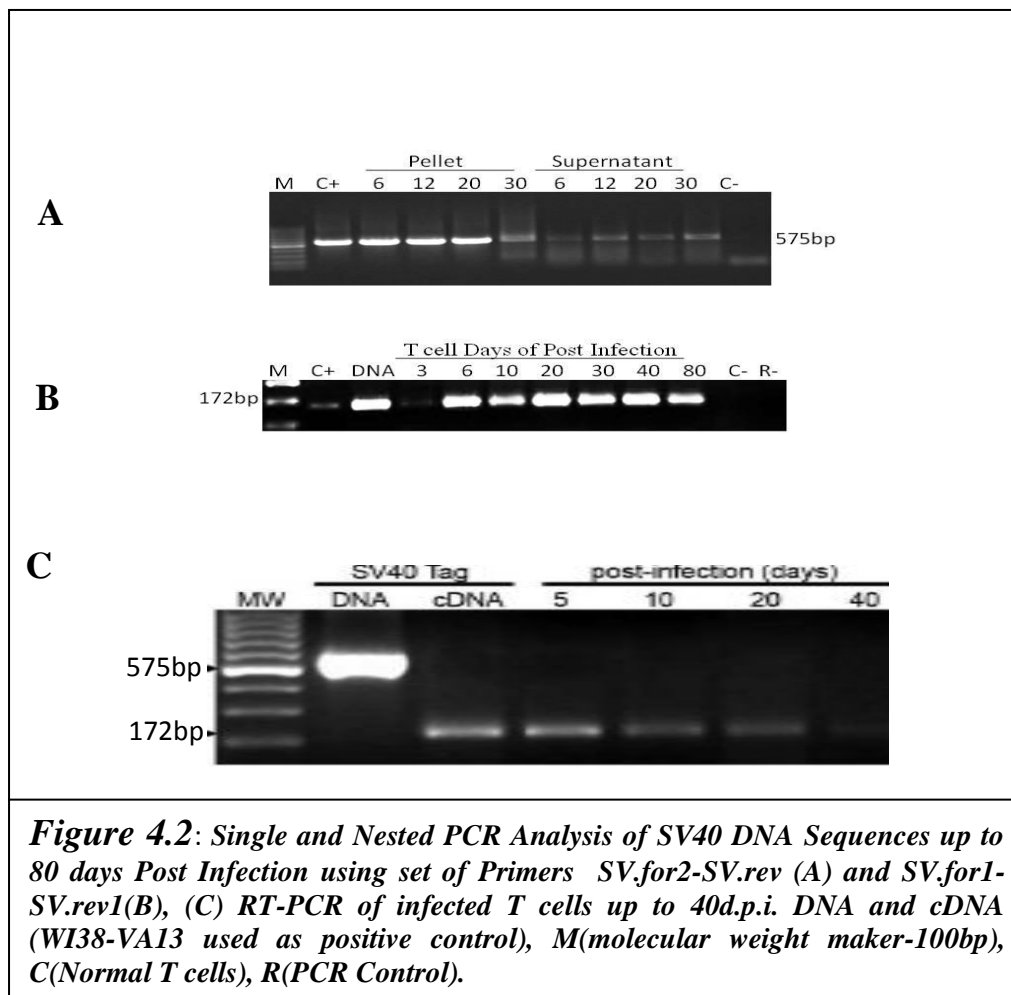
4.4: SV40 Tag oncoprotein and capsid VPs expression in T lymphocytes

For the presence of SV40 Tag and VPs expression the indirect immunoflourescence was employed (Figure 4.3). SV40 Tag and VPs was observed in T SV40 infected cells up to 50-d.p.i. Immunofluorescence images revealed Tag protein in human T cell infected by SV40 virions up to 50-d.p.i. (Figure 4.3a). In human T cell SV40-infected, VP1 were detected by immunofluorescence, up to 50 days (Figure 4.3b). T infected cells showed vacuole cytoplasmic expression in both Tag and VPs. This showed that early and late viral genes were expressed in T SV40 infected cells. After 30-50 days infection with SV40, T cells decreased in number dramatically. After this period, the viral proteins were not checked again using immunoflourescence staining.

Figure 4.3:
Immunoflourescence staining of T cell (a) Tag, A,C,D, (T cell infected with SV40), B,D,F, (WI38-VA13 used as positive control). C,D (4', 6-diamino-2-phenylindole (0.5 mg/ml)[DAPI] used to visualize the nucleus), E,F(Merged Image). (b) VP1, A,C,E(T cell infected with SV40) D,E,F (WI38-VA13),C,D (DAPI), E,F (Merged).
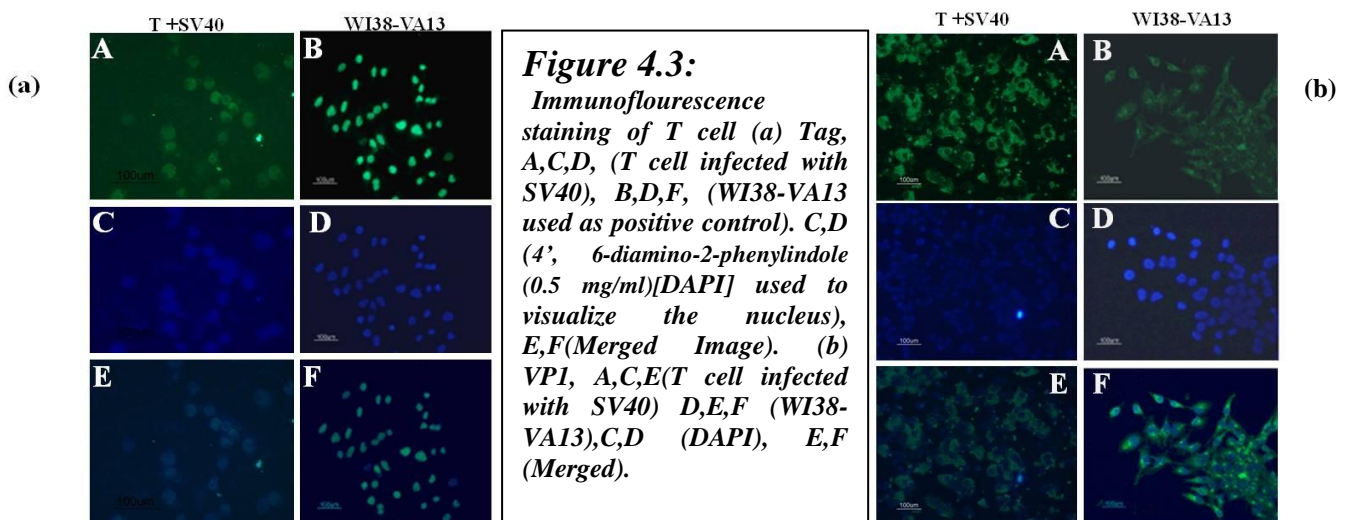
4.5: SV40 Effective Production Of Progeny by infected T cells

Cytopathic effect was employed to observe the rate of multiplicity and progeny release in T SV40 infected cells of 10 and 40 d.p.i with CV-1 monolayer cells. Aliquots of infected cells (10^5) and its medium (5ml), sonicated and not sonicated, were used as inoculums to CV-1 permissive cells. Indeed the SV40 infected T cells showed cytopathic effect with CV-1 at different viral titers (Table 4.3). The cytopathic effect was characterized with cytoplasmic vacuoles surrounding the nuclear membrane which is a typical SV40 cytopathic effect.

Type of cell cultures	Post infection day	Type of sample	Titer
Human T Lymphocyte SV40 infected	10	cells	10^3 pfu/ml
Human T Lymphocyte SV40 infected	40	cells	10^3 pfu/ml
Human T Lymphocyte SV40 infected	10	medium	10^2 pfu/ml
Human T Lymphocyte SV40 infected	40	medium	10^2 pfu/ml

Table 4.3: *Viral titers showed by SV40 776 strain released by infected T human lymphocytes and CV-1 cells (45-54-2$_{CV-1}$).*

4.6: Structure distortion in SV40 infected T cells

To observe ulteration in the structure of the T infected cells, a transverse electron microscope (TEM) was employed and the images were noted as mentioned in materials and method. Indeed, visible changes in transverse structure was detected in transfected and infected cells (Figure 4.4). Changes was observed from 10d.p.i. Different changes were observed in the nucleus and cytoplasm which were very similar to that of SV40 infected cells (Figure 4.4B). Characteristics observed was also related to that seen in Leukemia cells (Figure 4.4B-E).

Figure 4.4: *TEM results of T cells (A) Normal human T cell x6,300, (B) typical T cell SV40 infected after 10p.d.i, Cell with hairy projection similar to hairy cells of Leukemia, x13.000 (C) T cell of 10d.p.i with large size, voluminous indented nucleus and clear cytoplasm, x5.000.(D)Filamentous projection of the cytoplasm. typical structures found in hairy cells, x40,000. (E) Cytoplasm with many organelles, multivasicular bodies and ribosomal- lamella,*

Evidently, after an initial phase of the semi-permissive T cells, as evidenced by Tag, Vps expression, and production of virions, there followed a second phase of T cells fully permissive to the virus. After 50- d.p.i, in fact, there seems to appear a distinct phase of permissive cells to the virus, the lytic cycle and cell death. The PHA stimulated T cell replication and the establishment of a semi-permissive stage for the development of cell culture propagation for T cell. The study also indicated that SV40 can establish a persistent infection in human T cells lasting after the "crisis" stage.

Experiment III

Transformation of human B cells by SV40, a small DNA tumor virus

Simian virus 40 (SV40) is a small DNA virus linked with specific human cancers such as malignant pleural mesothelioma, brain and bone tumors, leukemia, lymphoma diseases. It could also be detected in healthy blood donors. While debate and rate of prevalence/detection of DNA sequences in human cancers and non-Hodgkin lymphomas (NHL) is at increase, indebt studies on the interaction between lymphocytes and SV40 has not yet been fully described. Hence, our effort to bring forward this study with purified human B lymphocytes isolated from the peripheral blood mononuclear cells of healthy blood donors, experimentally infected and transfected, monitored for evidence of infection, transformation/immortalization and release of progeny using continuous analysis with different parameters.

SV40-positive B lymphocytes extended their lifespan up to 100-d.p.i. Expression of SV40 Tag was detected in infected and transfected B cells up to 90-d.p.i with immunoflourescence while the viral capsid protein VP1 was detected up to 50-d.p.i. in infected cells. SV40 viral progeny was continuously produced from day 10 to 60 d.p.i in wild SV40 strain infected cells. SV40 DNA sequences were detected in both infected and transfected cells up to 100-d.p.i. These data indicate that human B lymphocytes can be efficiently infected with SV40. Ultra structural morphology were detected in both infected and transfected cells. Normal B human cells lasted about 6 days in culture. More than 50% of these B lymphocytes were transformed using the B cell ultra-structural analysis and characteristics of the cells in culture.

This study detailed the SV40 infected and pSv3neo transfected B lymphocyte realized from Peripheral blood mononuclear cells (PBMCs) of healthy blood donors and observed in culture for three months . The cells observation commenced from 3 days of post infection to 100 days.

4.7: FACs analysis, infection and transfection

To determine the population number of purified human B lymphocytes, isolated from PBMCs of blood donors using immunomagnetic dynabeads, a FACs analysis was employed. B cells showed different expression percentage with maximum population of 95.1% purity from the 6 different donors (Figure 4.4), as determined by flow cytometry using the monoclonal antibodies, CD19 with anti-CD45 and anti-CD3 (Table 4.4).

Subsequently, the purified normal B lymphocytes were infected with SV40 or transfected with pSV3neo as described in materials and methods, then observed in culture for 100-d.p.i.

% expression of B Cell population in different Donors

90,1   95,1   92,1   95   94   94,2

■ B cell
■ T cell

4,5   3,4   4   3   3,1   3,2

1st   2nd   3rd   4th   5th   6th

Donors

Figure 4.5: *FACs results of % population of normal B cells isolated from PBMCs of 6 Healthy blood donors buffy coat. Depicted also is little % population of T cells (red). Differences in the % of B cell expression of the different donors was not statistically significant. P < 0.05 .*

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Antibody	Type	Protein/ Cell Target	Manufacturer
CD19	Monoclonal marker	B normal Cells	Sigma (Milano, Italy)
CD3	Anti-membrane marker	T normal Cells	”
CD45	”	Hematopoetic cells	”
PAB101	Mouse monoclonal, primary	Tag (B transfected and infected cells)	Santa Cruz Biotechnology, CA, USA.
Goat SV40 anti-serum	”	VP1 (B transfected and infected cells)	”
IgG-FITC	Anti-mouse, secondary	Tag (”)	”
Donkey anti-goat IgG-FITC	Anti-goat, secondary	VP1 (”)	”

Table 4.4: *list of antibodies used in FACs and Immunoflourescence Analysis*

4.8: Cell viability and proliferation capacity

To monitor the viability and proliferation capacity of infected and transfected B cells, trypan blue and Alamar blue assays were carried out, respectively. Cells were followed in culture at different d.p.i. with trypan blue. Indeed, SV40-infected B cells and pSV3neo transfected cells showed increase in number at day 5 of p.i. from the initial number of cells; after this period, there was gradual decrease and death of cells until 80-d.p.i (Figure 4.5), with pSV3neo transfected cells retaining more cells at 80-d.p.i in culture than B cells infected with SV40. Suprisely, continous culturing of the cells gave rise to cell increse between 80 – 90 d.p.i. with drastic but constant decline between 95- 100-d.p.i. In all, there was no statistical significance in viability of B cells infected and transfected, while the infected and transfected cells showed prounounced statistical

significance with p= 0.02 and 95% confidence interval of – 71.94-173.4 over normal B cells. The normal B cells lasted between 1 to 6 days in culture (Fig.4.5).

Similarly, Alamar blue assay was also performed at 3 and 30-d.p.i for the cells in culture so as to monitor the proliferation and viability as well. Alamar blue asssay showed the percentage of reduction in cell samples to be the same with trypan blue analysis (Figure 4.6), with reduction in the ability of the cells to reduce alamar blue at advanced stage of post infection in culture. The graph linear of the cells depicted a rise at 3-d.p.i. in the transfected and infected cells but down stream arrow linear at 30-d.p.i. in culture (Figure 4.6AB, CD). In B normal cells, a fall in the linear lines was observed at 3-d.p.i. and were totally off in culture as at 30-d.p.i. (Figure 4.6E).

Figure 4.6:*Trypan blue analysis depicting % survival of normal, transfected and infected B cells in culture from 5-100 p.i. Normal B cell (triangular line) lasted between 1-6 days in culture. B+ SV40 (B SV40 infected cells), B+ pSV3neo (B pSV3neo transfected cells).*
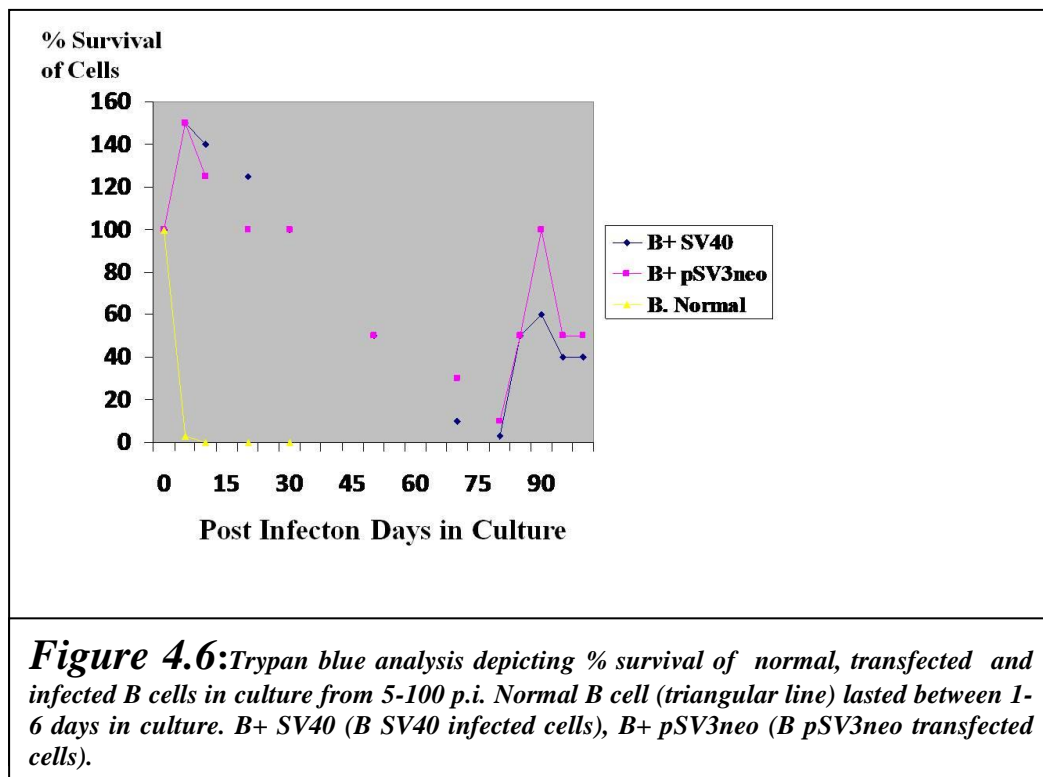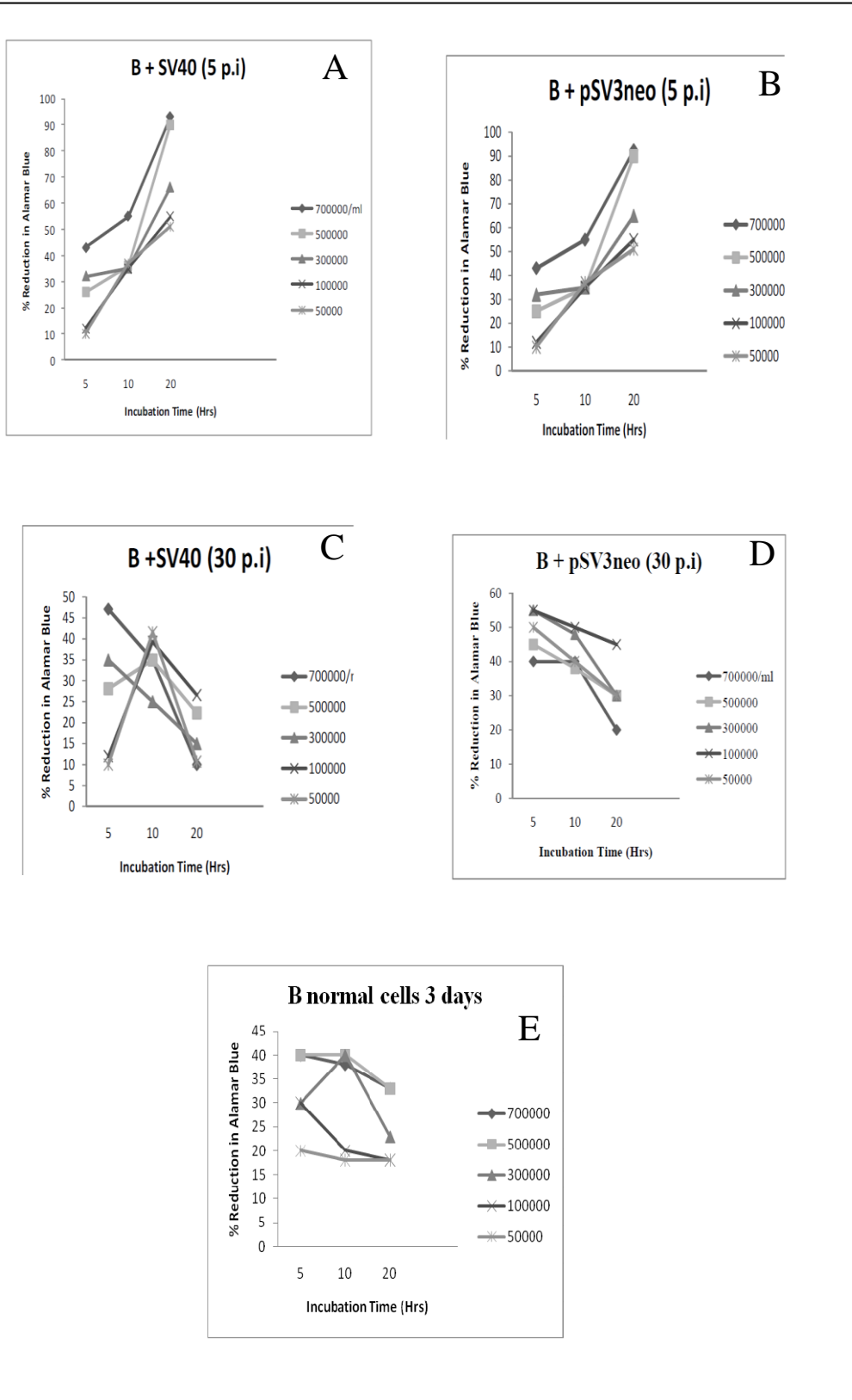
Figure 4.7: *graphs showing percentage of alamar blue reduction of B cells with different incubation periods and at different initial cell densities (A), B cell SV40 infected 5 p.i, (B), B cell pSV3neo transfected 5 p.i, (C), B cell SV40 infected 30 p.i, (D), B cell pSV3neo transfected 30 p.i, (E), B normal cells 3 days in culture.*

4.9: *Growth Behaviour of Infected and Transfected Cells*

To have more insight on the proliferation and viability of the infected and transfected cells, the growth behavior investigation was carried out by finding the population doubling time as described in materials and methods. Subsequently, infected and transfected cells from 80-d.p.i. doubling time was calculated at 48 and 72 hours intervals. Figure 4.4 represents the linear of the mean results from the two interval times experiment. The B cell SV40 infected cells (Figure 4.7A) showed a slight difference in both doubling time and growth capacity from B cell pSV3neo transfected cells (Figure 4.7B).

(A)

Time	No of Cells/ml
0	500000
48	1500000
72	3000000

Growth Rate = 0.0255
Doubling Time = 26.0

(B)

Time	No of Cells/ml
0	800000
48	1000000
72	4000000

Doubling Time = 27.0
Growth Rate = 0.0247

Figure 4.8: population doubling time of cells. (A), B cells SV40 infected (B+ SV40), (B), B cells pSV3neo transfected (B+pSV3neo).
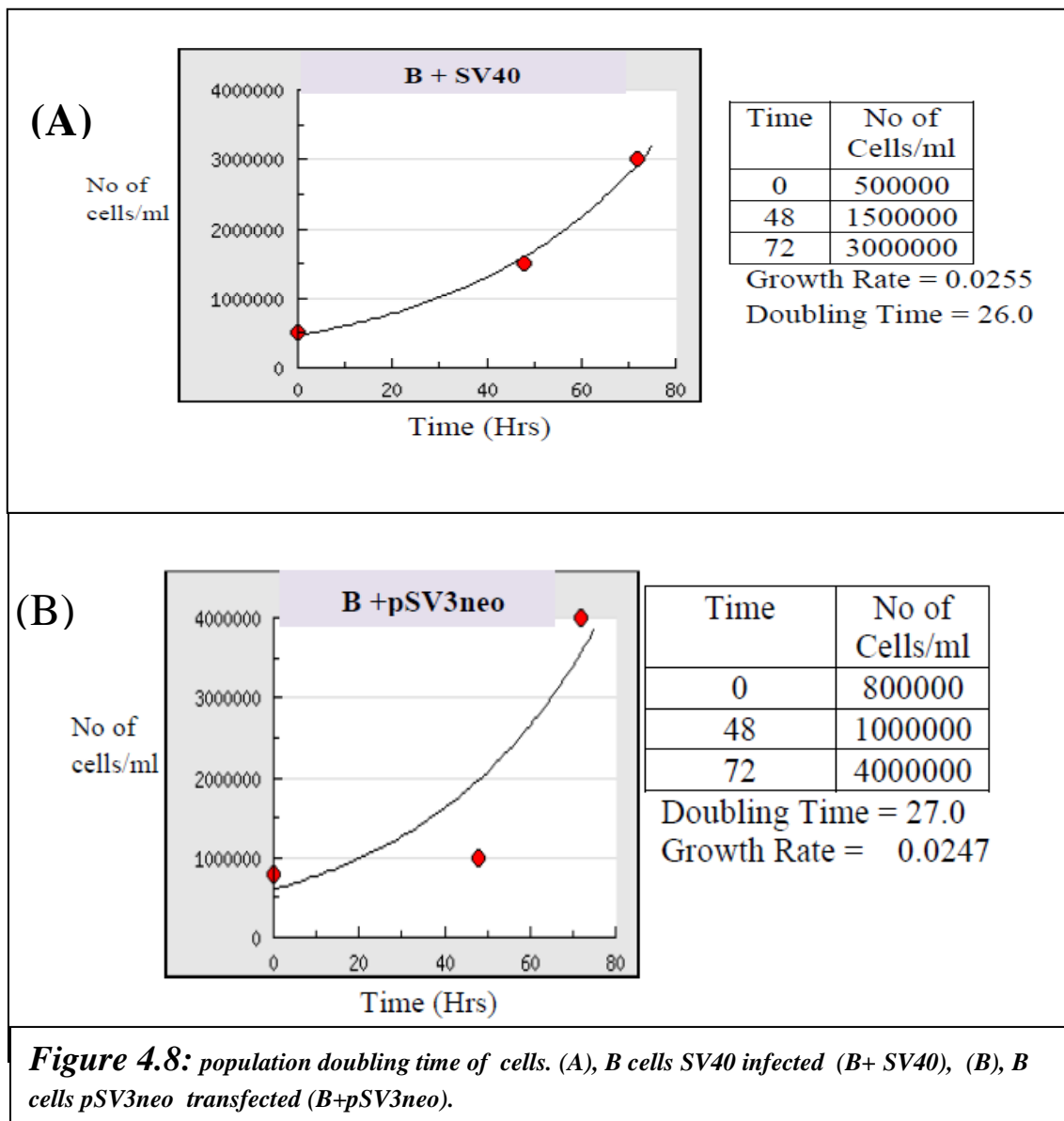
4.10: *Detection of SV40 DNA sequences in infected and transfected B cells*

SV40-infected and pSV3neo transfected B-cells were investigated for SV40 DNA sequences by single PCR using primers specific for SV40 sequences (Table 4.2) for the period of 3-100-d.p.i. PCR detected Tag NH2-terminal coding sequences with product size of 575 base pair (bp) in both SV40 infected and pSV3neo transfected B-cells up to 100-d.p.i. (Figure 4.8 A,B). The result generally showed that SV40 sequences were detected in infected and transfected B-cells at every inspected time, and consistent during cell culture even when cells entered into crisis (Figure 4.8C). This indicated stable integration of SV40 Tag into B cells. As anticipated, DNA samples from normal human B cells were SV40-negative.

Figure 4.9: *PCR and RT-PCR analysis for the infected and transfected B cells (A), PCR of B cells SV40 infected of 3-100 p.i, (B), PCR ofB cells pSV3neo transfected of 3-100 p.i, (C), RT-PCR of B cell SV40 infected, (D), B cell pSV3neo transfected, B+ pSV3neo (tranfected b cells), B+SV40(infected B cells), C+ (SV40 776- positive control), DNA (WI38-VA13), cDNA(WI38-VA13 cDNA), C- (B cells untransfected- negative control, R (PCR control), bp (base pair), MW (Molecular weight marker-100bp).*

4.11: Expression of SV40 Tag and VP1 in Human transfected and infected B cells

Having verified the SV40 sequences with PCR in the transfected and infected B cells, expression of SV40 Tag and VP1 was assessed in the cells using RT-PCR and immunoflourescence parameters respectively. A single RT-PCR was performed on total cytoplasmic RNA from SV40 infected and pSV3neo transfected B cell samples using a primers specific for the Tag sequences SV.for2-SV.rev located upstream and downstream of the Tag intron. The cDNA samples for B cell infected with SV40 produced a fragment of 229bp and the viral DNA of 575 as well (Figure 4.8C). The pSV3neo transfected B cell was absent (Figure 4.8D) probably due to small concentration of cDNA. Only the DNA fragment was detected with nested PCR amplification reaction with primers Sv.for1/Sv.rev1 yielding an amplification of 172bp (Figure not shown). Tag mRNA was detected up to 20-d.p.i. in infected cells.

Immunoflourescence experiments established the presence of SV40 Tag proteins in both SV40 infected and pSV3neo transfected B cells and in SV40 transformed cells of WI38-VA13 human fibroblast (Figure 4.9). The Tag was detected in both the infected and transfected cells from 8 to 90-d.p.i. (Figure 4.9 a-o). This indicated stable integration of SV40 Tag into B cells. As anticipated, Tag was also detected in WI38-VA13 used as the positive control (Figure 4.9,g ,h, i.).

Correspondingly, VP1 protein was also expressed in SV40 infected B cells but absent in pSV3neo transfected cells (Figure 4.10). The VP1 expression was detected up to 30-d.p.i. in the infected cells and exhibited mainly dense VP1 staining at the cytosol (4.10,a,d,g), which is very common with SV40 infected cells. The B cells showed large B cell morphology with diffuse cytoplasmic staining.
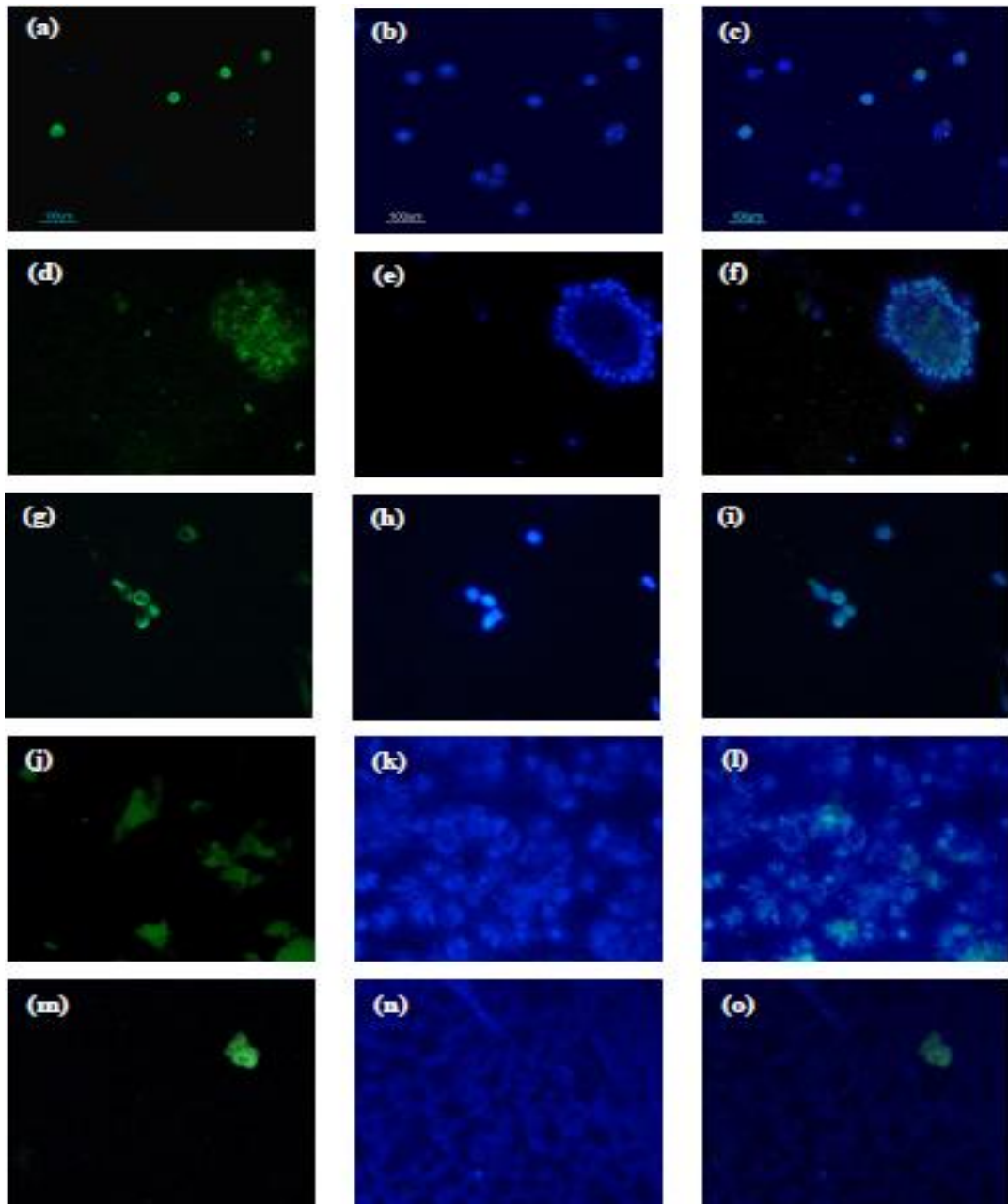
Figure 4.10: *Immunofluorescence detection of SV40 Tag in infected, transfected B cells and WI38-VA13. Fixed cells were incubated with PAB101 to localize the Tag, an adequate secondary antibody conjugated with FITC was used to reveal the signals (a,d,g,j,m). Nuclei were visualized with 4', 6-diamino-2-phenylindole (0.5 mg/ml) DAPI (b,e,h,k,n). Depicted also is merge Images of Tag proteins in B+SV40, B+PSV3neo and WI38-VA13 cell lines (c,f,i,l,o). B+ SV40 (B cell SV40 infected of 8p.i(a,b,c) and 90p.i (j,k,l). B+pSV3neo (B cell pSV3neo transfected cells of 8p.i (d,e,f) and 90p.i*

MAGNIFICATION 20x
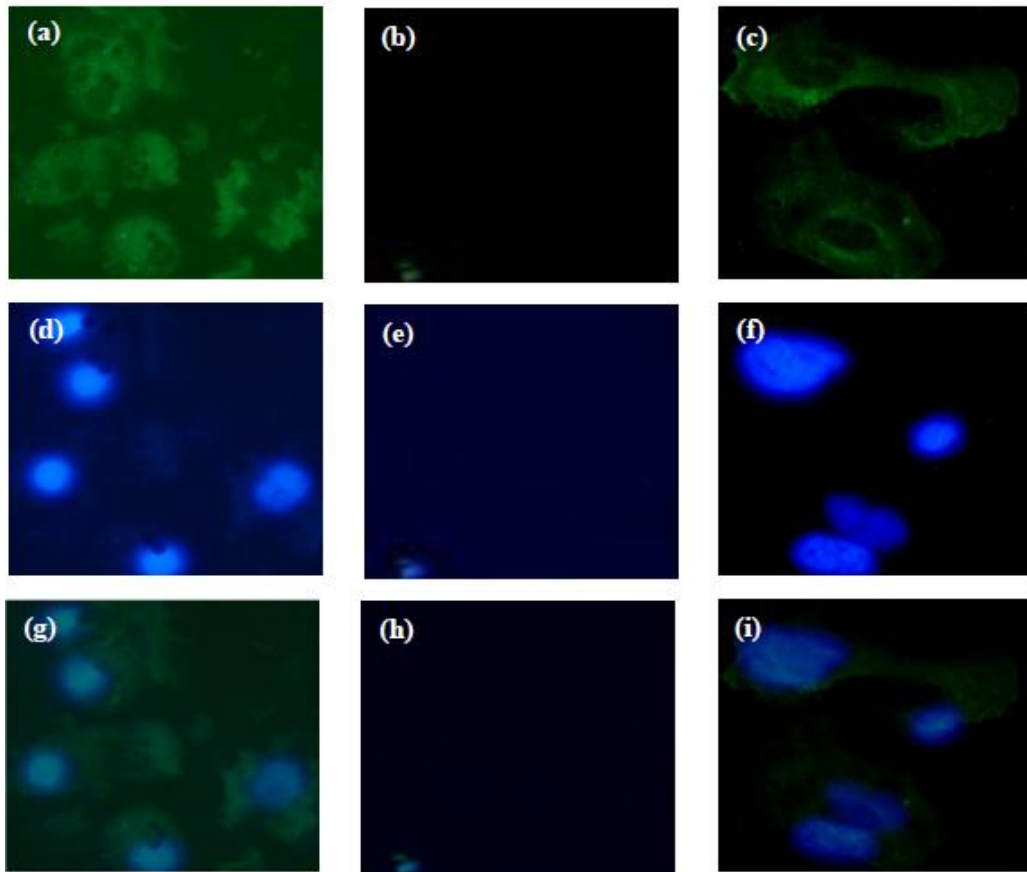
Figure 4.11: *Detection of VP1 in infected and transfected human B cells (m,n,o). WI38-VA13 (positive control- g, h,i,). B, Cellular localization of VP1 proteins I of B+SV40, B+PSV3neo and SV40-immortalized fibroblasts WI38-VA13(Control). Fixed cells were incubated with goat anti-SV40 serum to localize the VP1 with adequate secondary antibody conjugated with FITC to reveal signals. FITC (a,b,c). DAPI (d,e,f) Merge (g,h,i).*

MAGNIFICATION 40x

4.12: Structure distortion in transfected and infected B cells

To observe the possibility of a change in the structure of the B cells infected and transfected, a transverse electron microscope (TEM) was employed and the images were noted as mentioned in materials and method. Indeed, visible changes in transverse structure was detected in transfected and infected cells (Figure 4.11). The pSV3neo transfected B cells of 50-d.p.i. showed large but

many vacuole with some particles, indent condensed and clear nuclei. The electron dense reaction product is seen to be confined to chromatin portion of the nucleus which is often in striking contrast to the B normal cells (Figure 4.11A). In B cells infected with wild 776 SV40 strain of 3 and 6-d.p.i. showed more larger vacuoles with indented and empty or clear nucleoli as observed in plasmid tranfected cells (Figure 4.11 B,D). Visible also, is hair projection of the cytoplasm (D). The hairy projection feature is more pronounced in the pSV3neo transfected B cells of 10-d.p.i. (4.11,C). Some B transfected and infected cells of 50-d.p.i. depicted clear cytoplasm with indented and perforated nucleoli respectively (Figure 4.11, E, F). Ruptured cell wall with more lax chromatin, no cytoplasm and reduced indented nucleoli was also observed. The advanced stage of nuclear membrane restitution without any evidence of cytoplasmic division suggests the formation of a binucleated cells (F, x 10,00). Figure 4.11G, shows normal B cells or uninfected, untransfected cells (x 10,00).

Generally, B cells transfected and infected depicted features similar in other SV40 infected lymphocytes and in lyphomagenesis disorders such as Leukemia, Hodgkin deseases, and HTLV-1 infected cells (167, 168).

Figure 4.12: transmission electron microscope for ultrastructural observation in B infected and transfected cells. A, C, E, B are pSV3neo transfected cells (X 15.0, 9.00, and 15.0 respectively), B, D, F, B are cells infected with wild SV40 776 (X 5.00,8.00 and 10.0), G, B normal cells (X 7.00).
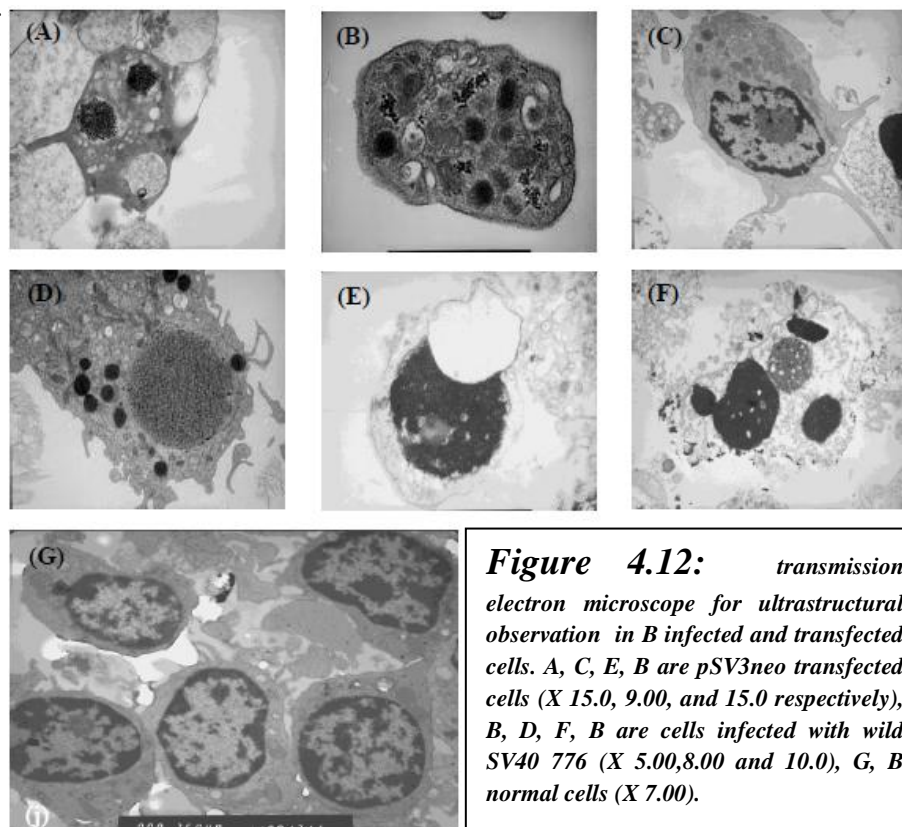
4.13: *Effective production of progeny in infected and transfected B cells with CV-1 permissive monolayer cells*

Cytopathic effects (C.P.E.) assay was employed to observe the rate of multiplicity of B infected and transfected human cells. Based on this, aliquots of the cells (10^5) and their medium of 10, 40 and 60 d.p.i. were inoculated to CV-1 monolayer cells and monitored for 15 days. In this experiment, the C.P.E. observed in CV-1 cells infected at different multiplicity of infection (MOI) from 10^1-10^6 served as positive control (Figure 4.12 A [10^6], B[10^1]). As anticipated, the SV40 infected B cells showed C.P.E. with CV-1 which can be differentiated with cytoplasmic vacuoles surrounding the nuclear membrane, which is the main characteristics of SV40 infected monolayer cells (4.12,G-L). C.P.E. appeared as plaques on the 10th day in plates of 10-d.p.i. in B cell SV40 infected cells (4.12,G, J) and later spread across the cell monolayer over longer periods in culture. Apparently, C.P.E. was observed in pSV3neo transfected B cells at 10-d.p.i. but no C.P.E. in both cell and medium of 40-d.p.i. (Figure 4.12 C, E, F). The C.P.E. was also visible in infected cells of 40 and 60-d.p.i. cell and medium (H, I, K, L). No C.P.E. was observed in CV-1 uninfected cells used as negative control (D). Additionally, different titers were observed in the cells that showed C.P.E. with CV-1 using end-point dilution method (Table 4.5).

4.14: *Plaque assay viral titer*

Additionally, the study tried to observe viral titer using plaque assay as did in cytopathic effect analysis. For this purpose, infected and transfected B cells were treated as mentioned in materials and methods section and incubated with DMEM medium containing 1.0% of agarose gel for 6 days. Plaque assay was visualized with 0.1% crystal violet (Figure 4.13). Indeed plaque was observed in B cell SV40 infected of 10-d.p.i. with titer of 4×10^4 in cell and 1×10^3 in medium (Figure 4.13C, E), while apparent plaque was observed in B cells SV40 infected of 60-d.p.i. samples (Figure 4.13 D,F). CV-1 cells infected at moi 10^6pfu/ml was used as positive control. As anticipated, no plaque was observed in uninfected CV-1 cells used as negative control (Figure 4.13B).

Cell culture Medium Type	Days of Post Infection	Titer
Pellet (B+SV40)	10	10^3 pfu/ml
Medium (B+SV40)	10	10^2 pfu/ml
Pellet (B+PSV3neo)	10	10^1 pfu/ml
Medium (B+PSV3neo)	10	-
Pellet (B+SV40)	40	10^2 pfu/ml
Medium(B+SV40)	40	10^2 pfu/ml
Pellet (B+PSV3neo)	40	-
Medium (B+PSV3neo	40	-
Pellet (B+SV40)	60	10^1 pfu/ml
Medium (B+SV40)	60	-

Table 4.5: *viral titers determined in CV-1 permissive cell monolayers (10^6 cells/monolayer) using 50% Estimated Endpoint dilution technique. B+SV40(B cells SV40 infected), B+pSV3neo(B cells pSV3neo transfected).*
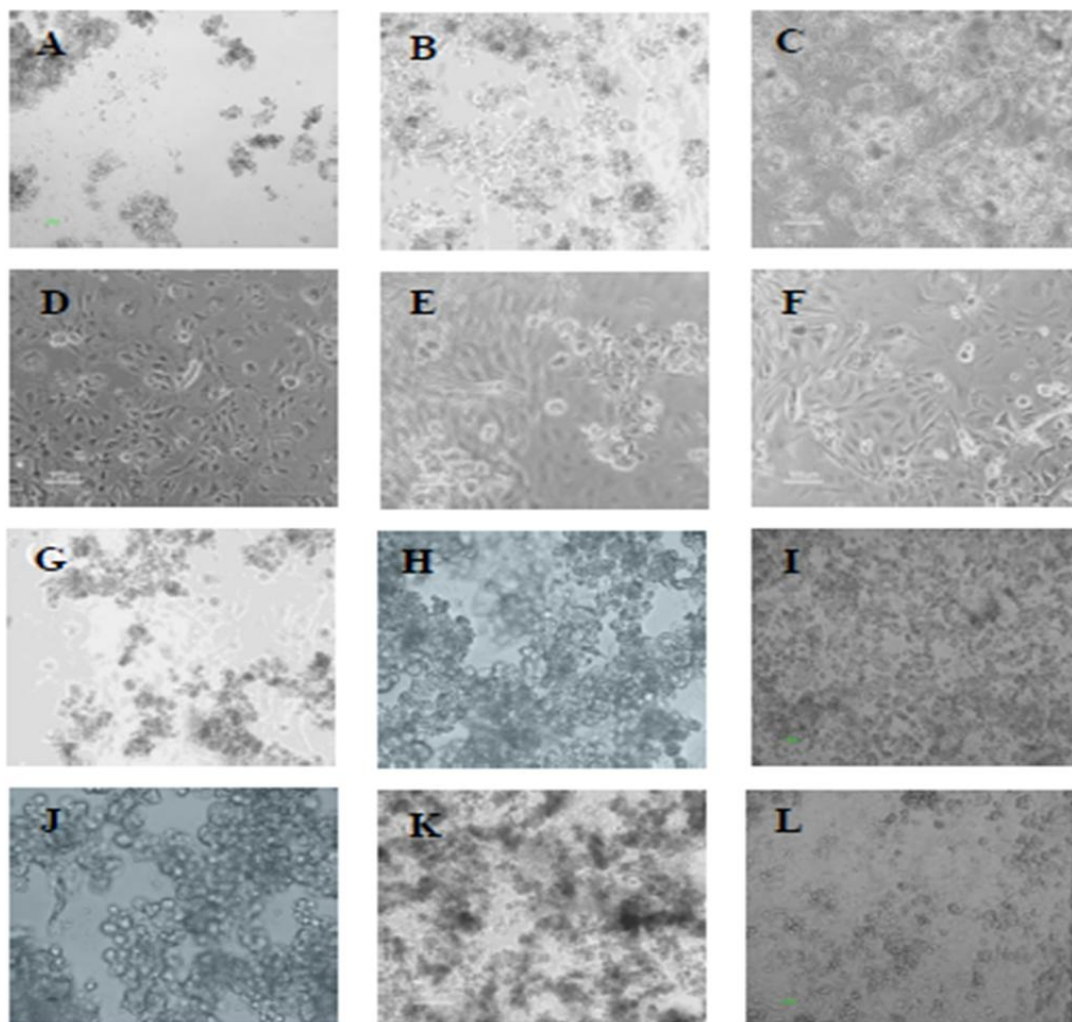
Figure 4.13: *C.P.E observed in B infected and transfected cells with CV-1 monolayers.) AB, CV-1 positive control, D, CV-1 uninfected monolayer-negative control, C,E, F, B cell pSV3neo transfected of 10 and 40 p.i respectively. G,H,I, B cell SV40 strain 776 infected cells of 10, 40 and 60p.i respectively. J,K,L, medium of B infected cells of 10, 40, and 60 p.i.*
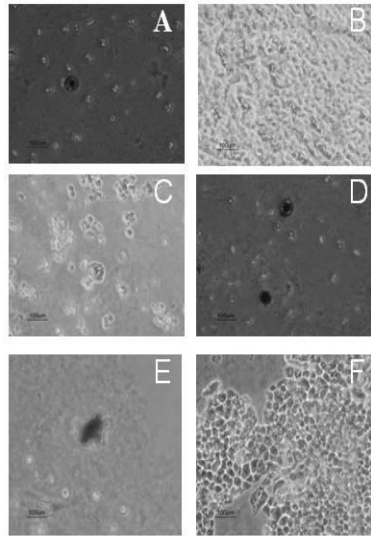
Figure 4.14: *Plague assay, A (CV-1 infected with SV40- control positive), B (CV-1 uninfected cell-negative control), CD (B cell SV40 infected of 10 p.i) E,F (B cell SV40 transfected of 60 p.i) . Magnification 20x.*

5 Discussion

Cellular transformation and tumorigenic activities of oncogenes have been intensively investigated (113, 114). Evidence of an association between SV40 infection and human cancers has been accumulated during the past 40 years with its major controversy dividing the research community in pro/counter groups. Numerous questions have been imposed by some investigators concerning the association of SV40 with human diseases, thereby jeopardizing further serious investigation concerning SV40 and certain human cancers. However, many investigations have been carried out on the mechanisms of transformation of SV40 Tag in animal and human cells; on cell differentiation and on the ability of SV40 Tag to bind and inactivate p53.

Although the past few years have witnessed a large number of publications on the presence of SV40 sequences in human tumors, surprisingly little information exists on the proportion of SV40 infection in the blood of healthy individuals (40a, 23). The published frequency of SV40 DNA in normal tissues or in people without cancer is quite variable. In Italy, different studies have found different frequencies of 13%, 23%, and 29% though some study groups failed to find any positive control samples among their subjects. In (experiment 1) of this work, a total of 17% positivity for SV40 was observed from buffy-coats of 60 healthy individuals from Aviano using the nested PCR approach. Notwithstanding the low frequency of SV40 sequences in this study, the data confirms and extends previous results on the presence of SV40 sequences in DNA from different cohorts of healthy blood donors in Italy.

Additionally, the study is in support of the evidence that SV40 is circulating among healthy individuals and this calls for further research in larger cohorts, and from different global regions, so as to resolve the issue of how and when SV40 infection occurs among humans and to investigate the routes of infection.

While recent reports suggest the presence of SV40 DNA sequences in different lymphoproliferative disorders, little is known about the main interaction of SV40 with lymphocyte cells. Hence, this study further investigated the interaction between SV40 and B/T lymphocytes to verify if SV40 infects, transforms, and immortalizes B cells.

Isolated and purified B and T cells were found to be positive for CD19 with anti-CD45 and anti-CD3 (Table 4.1). Although little variations in % expression of cells and almost no statistically significant variation was observed in percentage population of the 6 donors (Figure 4.4) using FACs analysis, this experiment shows that the collected buffy coat of the healthy blood donors and the subsequent purified B and T cells were normal, implying that with this percentage expression rate, problems opposed by variability in different donors is ruled out in the experiment. In effect, the use of primary human lymphocyte B and T cells could also offer more mechanistic insight than already existing immortalized B cell lines.

Proliferation and viability capacity of the infected and transfected B/T lymphocytes were analyzed from 5-d.p.i. using trypan blue and alamar blue assays. Both pSV3neo and wild strain 776 SV40 induced proliferation with more than 100% increase in cell survival when compared to uninfected B and T lymphocytes (Figures 4.1 and Figure 4.5). The cell survival graph indicated that the proliferation and viability capacity of normal B cells to survive in culture lies between 1 to 5 days. The data showed that, the pattern of infection in B and T cells is in line with the semi-permissibility usually depicted by SV40 in human cells (3, 4). It proofs to be efficient strategy to infect lymphocytes notwithstanding the drastic fast reduction/death of the infected cells in culture. Though, the cells experienced cell death, the little number of cells that passed crisis stage continued

to multiply up to 80-d.p.i. in T cells and after 80-d.p.i. in B cells with the same pattern of initial cell increase and decline experienced in early stage of proliferation. While increase in cell proliferation was observed in B and T lymphocytes infected with SV40 than in B lymphocyte transfected with pSV3neo, a slight retention of cells in culture was observed in the later than the former.

Increased proliferation reflects the alteration in cell cycle control and extension of life span of cell when the cells are transformed (115). Probably, B and T lymphocytes infected with wild SV40 strain were affected more by culture stress than pSV3neo transfected cells. With this, the infected lymphocytes tend to fall into the third characteristics of transformed cells that "experience more cell death due to cultural stress and tends to be more tumorigenic if inoculated to animal models" (115). This effect of the virus may play a role in cancer development and may support the hypothesis that additional factors may be required to act in synergy with SV40 infection for the induction of a cancerous process in lymphocytes. In other cancer types, the role of an SV40 co-factor is strongly indicated. It has been suggested that SV40 is a co-carcinogen with asbestos in the development of malignant mesothelioma as demonstrated in vitro with human mesothelial cells (3). Another example is the risk of developing HBV—including liver cancer, greatly increased by other co-factors such as the hepatotoxin aflatoxin (169).

Nacchtigal et al.,(1990) found pSV3neo to be a stable transfection plasmid with lipofectamine. Similarly, our results showed that B lymphocytes can be stably transfected with the pSV3neo, an SV40 Tag coding plasmid.

Similarly, the alamar blue assay results/data (Figure 4.6) also depicted cell viability with increased cell viability at early stage of post infection (3-d.p.i.) before drastic fall in graph row, signifying cell death experienced at 30-d.p.i. Unlike infected and transfected cells, decrease in reduction of alamar blue was observed in normal B cells from 3-d.p.i. (Figure 4.6, E). Although percentage survival in

B cells infected with wild SV40 were higher than B cells transfected with pSV3neo, yet pSV3neo transfected cells depicted slight higher percentage of alamar blue reduction at 30-d.p.i. than B cells infected with wild-type 776 SV40 strain. Nevertheless, both results showed reduction in viability at 30-d.p.i. which suggest a certain degree of inhibitory response in these cells.

Alamar blue (AB) is among the vital dyes which are non-toxic to cells and offer the advantages of keeping the cells in culture to observe changes over time. AB has been utilized in various tests such as, measuring the proliferation of human lymphocytes, primary rat hepatocytes, various human cell lines, in measuring cell-mediated cytotoxicity of human T lymphocytes, as well as cell viability in BeWo and JEG-3 choriocarcinoma cells (154).

Furthermore, to have more insight in the transfected and infected B cells growth behavior, their population doubling time was observed. This experiment was carried out at least three times, for accuracy confirmation. B cells SV40 infected was found to grow at approximately one doubling every 26 hours with growth rate of 0.0255 based on quantification of cell number using microscope counting. The transfected B cells make one doubling every 27 hours with growth rate of 0.0247. Additionally, the cells exhibited a short resting phase in culture that normally leads to cell doubling/increase. This characteristics is described by Kim et al as a resting phase (G0) shown by cells that have temporally stopped dividing (156). This suggests that the drastic reduction in number experienced by B cells infected and transfected at 80-d.p.i. in culture was not actually cell death rather resting (G0) phase that gave rise to cell increase after 80-d.p.i.

Another key feature in analyzing the ability of SV40 to infect human lymphocytes is the replication of viral DNA. SV40 DNA sequences was measured during the infection time course from 3-80 days post infection for T cell and 3-100 days for B cells by single PCR using primers specific to SV40. In the infected and transfected cells throughout their period in culture, SV40 sequences were

detected and this suggested stable integration of the SV40 Tag in the infected and transfected B and T cell samples.

Previous and recent studies have implicated SV40 in different human cancers such as mesothelioma, brain tumors and NHL diseases (39, 40a, 90, Table 1.2), and have regularly detected its DNA sequences in healthy blood donors. Malignant pleural mesothelioma (MM) is a fatal human tumor and its onset is related to asbestos fiber inhalation, which is a tumorigenic natural mineral and an immunosuppressor agent (171, 172). According to investigating research studies, prediction of the number of patients who will die of MM in Western Europe each year, will almost double over the next 20 years with a total of about 250,000 death until 2029 before experience in decline (173). SV40 has long been identified as a co-carcinogenic factor with asbestos in the proliferation of mesothelioma. Additionally, NHL disease incidence have dramatically increased in the last 30 years with an 80% increase in the US noted between 1973 and 1997 (143). The reasons for the increase in incidence are not fully understood. However, many different viruses with oncogenic potentials have been found to be associated with non-Hodgkin lymphoma, including SV40 (174, Table 1.2).

The continuous detection of SV40 DNA sequence in B and T cells infected and transfected throughout the time course with PCR technique in this experiment is a strong support to previous studies that detected SV40 sequences in their experiment. The time course of this particular experiment was repeated a number of times to confirm results and elimination of possible contamination. Although, mounting evidence supporting SV40 DNA prevalence in human cancers is building up, many groups failed to detect SV40 in their experiment in different human cancers like brain tumors, mesothelioma and lyphoproliferative disorders (128, 148). This was attributed to some PCR results with contamination of common laboratory plasmids containing SV40 sequences. The positivity of SV40 DNA sequences in this present experiment confirms that

not all investigators that reported SV40 positive results contaminated their results. Nevertheless, some SV40 negative reports can be attributed to geographical and population factors, while other negative reports possibly resulted from technical limitations in the laboratory. A particular research team which has consistently challenged the presence of SV40 in human tumours and has reported negative findings, has recognized sensitivity problems that raise questions about these negative reports (175).

Expression of Tag and VP1 seems to be one of the most important analysis to confirm SV40 infection and transformation. The in vitro detection of SV40 Tag in SV40 infected and transfected B and T cells is a key finding in the study of SV40 involvement in lymphomagenesis. The Tag indicates expression of viral early genes. In fact, Tag detection in transfected and infected B cells up to 90-d.p.i. suggested the real integration of SV40 infection into the genome of B cells even at crisis stage. pSV3neo transfected cell expressed almost nuclear expression of Tag in the immunoflourescence experiment with its absent in the RT-PCR experiment. B and T cells SV40 infected expressed both Tag and VP1 in immunoflourescence analysis and Tag up to 20 and 40-d.p.i. in RT-PCR respectively. Two reasons can be responsible for the absence of SV40 Tag in the RT-PCR experiment: (a) the transcribed RNA is probably too small to be detected with used parameter in transfected cells since low viral load has been known to be associated in most SV40 infected lymphocytes as observed in (38). (b) SV40 may be exhibiting the "heat and run" nature of infection in pSV3neo transfected B cells but only functions by initiating the infection. Subsequent progression of infection sees Tag as not necessary since it has fulfilled its role in initiating the infection/transformation process. This mechanism has been proposed in relation to SV40-associated lymphoma development based on the finding of higher SV40 prevalence in reactive lymphadenopthies than in overt lymphoma, supporting a role for the virus in the early phases of lymphomagenesis (176).

B and T cells SV40 infected showed both nuclear and cytosol expression of VP1 at 8-d.p.i. which was absent in pSV3neo due to its consisting of only early region genes of SV40. Additionally, this difference in B cells transfected and infected response could be due to genetic differences between the two B cell lines. The plasmid virus, with its increased expression of Tag, suggests that Tag is playing a role in the observed expression changes and may hint a potential function of Tag to participate in immune evasion.

Studies have observed that SV40 Tag is required for viral replication and cellular transformation, and is only expressed after entry into a host cell (109). Therefore, analyzing Tag expression in infected and transfected B lymphocytes in this experiment is a critical component in testing the hypothesis that SV40 plays a role in human lymphomagenesis.

The adoption of two detection methods in this experiment, for evidence of SV40 as applied in recent studies: a PCR-based method and immunohistochemical staining for Tag. The agreement of these two independent detection methods almost guarantees against laboratory contamination being responsible for the presence of SV40 (11).

 Studies have detected that several DNA viruses encode unique oncoproteins that target pRb and p53 popularly known as "tumor suppressor gene", emphasizing the central power these two proteins exert over cell cycle control. SV40 Tag is the only viral oncoprotein with the ability to interact with both p53 and pRb family members, underlining its potency and power as a multifunctional transforming protein. Complex-formation of p53 and pRb with Tag has been observed in SV40-positive brain tumours and mesotheliomas. Similarly, the co-incident detection of SV40 Tag and p53 alterations in SV40 infected lymphocytes and lymphoma diseases as well would be informative (177). Furthermore, immunoflourescenc technique was used to detect the p53 expression in SV40 infected B cells in this study. Using PAB240 antibody as primary with FITC conjugated secondary antibody specific for human p53. p53 expression was observed at the cytoplasm and nucleus of the

normal B cells as observed by (178)[Figure not shown], but absent in the infected, tranfected B cells. The full thick staining of the Dapi staining of p53 in the normal B cells was also absent in the W138-VA13 (positive control). This data suggested the possibility of p53 being disactivated in the SV40 infected and transfected B cells and its transformation caused by the presence of SV40 Tag (49). The short coming of this experiment was its failure to use a cell line known to express p53 as a second positive control. This therefore, is strongly recommended for future work as this will clarify and firmly confirm result. It is of note that the tumour suppressor proteins p53 and pRb are commonly lost, over expressed or mutated in human tumours. The SV40 transforming mechanism in binding of Tag to p53 and pRb proteins is similar to the functional role of SV40 in lymphomagenesis because the functional inactivation of genes that monitor genome integrity is a trait of lymphomas (23). This has particularly been observed in patients with diffused large B cell lymphoma and follicular lymphomas, which are the two types of NHL in which SV40 most frequently found, often have detectable p53 alterations.

While the precise mechanism of SV40 transformation and immortalization has not yet been fully clarified, its Tag is known to allow cells to bypass the G1 cell cycle block, most likely by interacting with p53 and pRb tumor suppressor proteins. The complex formation between Tag and these cellular proteins leads to the inactivation of these tumor suppressor gene products, resulting in unregulated DNA replication and cell division. It is very likely that SV40 T antigen–mediated inactivation of pRb and p53 pathways extends the B cell lifespan which gave rise to its transformation and perhaps short immortalization. However, the life cycle of B cells infected by SV40 has been proposed to last up to 4-6 months in culture (38).

In lymphomagenesis disorders, chromosomal translocations and structural aberrations of cells are the most frequently recognized oncogenic events (176). Indeed, ultrastructural verification of B and T infected and transfected cells with TEM depicted different abnormal formations in the DNA, cytoplasm, vacuoles and cell wall right from 3-d.p.i. in infected B cells (Figure 4.11). Generally, B

cells transfected and infected depicted features distinguished in other SV40 infected lymphocytes and in lymphomagenesis disorders such as Leukemia, Hodgkin diseases, and HTLV-1 infected cells (161, 167, 168). This result strongly confirmed transformation of the B and T cells by wild SV40 strain and pSV3neo plasmid.

Additionally, from the immunoflourescence results, an interesting observation was noted in terms of morphological aspect. At day 8-50 d.p.i., 20% of the SV40-infected B and T cells displayed large B cell morphology with dense SV40 VP1 staining in the cytosol compartment (Figure 4.b and Figure 4.10). This morphological structure of large B and T cells reminds that of Large Cell Lymphoma (LCL), which is very cantankerous and rapidly rising cancer of either B-or T-cells. LCL is known to be one of the most common types of lymphoma, accounting for 31% of all non-Hodgkin's disease (179).

Vertical and horizontal transmissions have been postulated as additional possible sources of infection along with proliferation of archetypal and non archetypal SV40 transmission (39). It has been postulated that SV40 is transmitted through environmental contact. Indeed, it has been shown that this virus is present in the urine, stool, tonsil and blood specimens of children and adults, suggesting different ways of transmission, such as urine, oro-foecal, respiratory and haemapatic routes are responsible for SV40 infection. The observation of this present study is in support of the results obtained by past studies on subjects administered with SV40-contaminated vaccines by different routes. In fact, SV40 was detected in these subjects after a number of weeks or days in their stools and tonsils depending on oral or nasal spray administration of contaminated vaccines (39, 104).

Indeed, SV40-infected human B and T cells produced SV40 viral progeny, from 10 to 60-d.p.i., with different titers. SV40 induced lyses in almost 80% of B and T cells in culture. Our experimental data indicate that infected human B and T cells are virus progeny producers, but then

they lose the ability to shed the virus later than 60-d.p.i. It is therefore possible that before the "crisis" stage, SV40 induces an "acute" infection in human B and T cells, which is somehow controlled by those cells which take over during the crisis, resulting in a sort of persistent infection. Interestingly, this result indicate that B and T cells can be infected by SV40, while the lifespan of B and T cells, which resisted the lyses, is greatly extended. In reality, normal human B cells from healthy blood donors have been distinguished to proliferate in culture roughly eight days. SV40 DNA persisted in infected and transfected B and T cells, as indicated by the presence of Tag sequences.

The etiology of the many different types of lymphoma is multi-factorial in nature, and viruses have been shown to be involved in some cases. Given the strong oncogenicity of SV40, if it is present in human lymphocytes then it could possibly function as a co-factor in lymphomagenesis. The indication of a co-carcinogenic role for SV40 is further strengthened given that this theory has been proven for other transforming viruses. For instance, EBV has long been linked with Burkitt's lymphoma but presence of the virus is only one factor involved in disease development (180).

CONCLUSION

Although limited in size, the study of 60 subjects from Aviano, gives an epidemiological support of presence/prevalence of SV40 in the PBMC of healthy donors from Aviano. The findings of this study is similar to those of SV40 prevalence detected previously by earlier studies carried out in blood specimen of healthy subjects, not minding the difference in frequencies, which may be due to the employment of different protocols. Furthermore, the study suggests that oncogenic SV40 virus is being disseminated in the human population. Further research in larger cohorts, and from different global regions, is needed to resolve the issue of how and when SV40 infection enters and spreads among the human species. This will also help to reduce the predicted incidence rate of 250,000 cases of mesothelioma over the next 40 years. Obviously, the use of SV40 as a diagnostic marker in mesotheliomal cancers and asbestos is promising and this encourages epidemiological molecular study to help in bringing SV40 closer to clinical practice.

Relating SV40 with human B and T cells denotes an innovative that should be put into consideration when assessing the biological activity of this viral agent in the human host. The characteristics of SV40 in B and T cells indicate the possibility of natural SV40 infection in humans and calls for further investigation especially in hematological diseases and other malignancies that involves oncogenic viruses such as HTLV-1, EBV and HIV. More studies in human T and B cells with SV40 infections are necessary to test the specificity of the relationship of SV40 with lymphoproliferative disorders.

Indeed, human B and T cells are semi permissible to SV40 infection and can last in culture between 4-6 months as speculated before in (38). In this study, the SV40 infected lymphocyte cells have depicted the usual characteristics of transformed cells as proposed in (115), though how tumorigenic the infected and transfected cells are, is yet to be revealed. Inasmuch as a full transformation of infected/transfected B cells have been achieved in this study, my data therefore

suggests that SV40 may play a role in lymphomagenesis and in spreading the virus to other organs in the body. Additional investigations are needed to establish whether SV40 act together with co-factors, including micro-environmental, genetic and epigenetic factors in the B cell transformation.

ABBREVIATIONS

AB	Alamar Blue
APCs	Antigen presenting cells
AHW	Absorbance of higher wavelength
ALW	Absorbance of lower wavelength
BB2-BB3	SV.for1-SV.rev 1 primers
B+ SV40	SV40 infected B cells
B +pSV3neo	pSV3neo transfected B cells
BKV	BK virus
CTL	Cytotoxic T lymphocyte
CBP	CREB-binding proteins
cDNA	Complementary Deoxyribonucleic acid
CREB	cAMP regulatory element binding proteins
C.P.E	Cytopathic effect
DEPC	diethylpyrocarbonate
DMEM	Dulbeccon modified minimal essential medium
d.p.i	Days of post infection
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
DLBCL	Diffuse large B-cell type lymphoma
EBV	Epstein- Barr virus
EL cells	Extended life cells
EMEM	Eagles minimal essential medium
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACs	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
HCMV	Human Cytomegalovirus
HGF	Hepatocyte growth factor
HTLV-1	Human T Lymphotropic virus type-1
HIV	Human Immunodeficiency virus
I cells	Immortalized cells
IPV	Inactivated polio vaccines
JCV	JC Virus
JNK	Jun N-terminal Kinase
KIV	KI virus

KSHV	Kaposi's Sarcoma Herpes virus
LPV	Lymphotropic polyomavirus
MAPK	Mitogen Activated Protein Kinase
MCV	Merkel Cell Virus
MHC	Major Histocompatibility Complex
miRNA	Micro ribonucleic acid
MOI	Multiplicity of Infection
MPM	Malignant Pleural Mesothelioma
MuPyV	Murine Polyomavirus
MW	Molecular weight marker
NHL	Non-Hodgkin's lymphoma
NK cells	Natural killer cells
NLS	Nuclear Localization Signal
NPC	Nuclear pore complex
nPCR	Nested Polymerase chain reaction
OPV	Oral Polio vaccine
P13K	Phosphatidylinositol 3-kinase
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDT	Population doubling time
PFU	Plague forming units
PHA	Phytohemagglutinin
PML	Progressive multifocal leukoencephalopathy
PP2A	Protein phosphate 2A
p53	tumor protein 53
pRb	retinoblastoma protein
RNA	Ribonucleic acid
RO	Correction factor
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
RT-PCR	Reverse-transcriptase polymerase chain reaction
SEM	Scanning electron microscope
SIV	Simian immunodeficiency virus
SV40	Simian virus 40
Tag /LT	Large T antigen
tag /ST	Small T antigen
TEM	Transmission Electron Microscope
T +SV40	SV40 infected T cells
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VP1	Viral protein 1
WUV	WU virus

REFERENCES

1. Padgett BL, Walker DL (1973). **Prevalence of antibodies in human sera against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy**. J Infect Dis 127:467–470.

2. Kim B, Song J, Jeon Y, Jeog B, Yun S, Cho H, Carp R, Kim Y (2009). **Spontaneous immortalization of oligodendroglial cells derived from an SV40 T antigen-positive human glioblastoma multiforme.** Cancer Letters 283:212-221.

3. Bocchetta M, Di Resta I, Powers A et al. (2000). **Human mesothelial cells are unusually susceptible to simian virus 40-mediated transformation and asbestos cocarcinogenicity**. Proc Natl Acad Sci USA 97: 10214-10219.

4. Carbone M, Rizzo P, Pass HI. (1997). **Simian virus 40, poliovaccines and human tumors: a review of recent developments.** Oncogene 15: 1877-1888.

5. Gross L. (1953). **A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice**. Proc Soc *Exp* Biol Med 83: 414- 421.

6. Stewart SE, Eddy BE, Borgese N. (1958). **Neoplasms in mice inoculated with a tumor agent carried in tissue culture.** JNatl Cancer Inst 1958; 20: 1223- 1243.

7. Gazder AF, Butel JS, Carbone M. (2002). **SV40 and human tumours: myth, association or causalty?** Nature Rev Cancer 2:957-964.

8. Garcea RL, Imperiale MJ. (2003). **Simian virus 40 infection of humans**. J Virol 77: 5039-5045.

9. Stratton K, Almario DA, McCormick MC. (2002). **Immunization safety review: SV40 contamination of polio vaccine and cancer.** Washington DC: The National Academies Press, 2002.

10. Taguchi F, Kajioka J, Miyamura T (1982). **Prevalence rate and age of acquisition of antibodies against JC virus and BK virus in human sera**. Microbiol Immunol 26:1057-1064.

11. Vilchez RA, Brayton CF, Wong C et al (2004). **Differential ability of two simian virus 40 strains to induce malignancies in weanling hamsters.** Virology 2004; 330: 168-177.

12. Dalianis T, Ramqvist T, Andreasson K, Kean JM, Garcea RL. (2009). **KI, WU and Merkel cell polyomaviruses: a new era for human polyomavirus research.** Semin Cancer Biol 19: 270-275.

13. Feng H, Shuda M, Chang Y, Moore PS. (2008). **Clonal integration of a polyomavirus in human Merkel cell carcinoma.** Science 319: 1096-1100.

14. Butel JS, Lednicky JA. (1999). **Cell and molecular biology of simian virus 40: implications for human infections and disease.** J Natl Cancer Inst. 91: 119-134.

15. Newman JS, Baskin GB, Frisque RJ. (1998). **Identification of SV40 in brain, kidney and urine of healthy and SIV-infected rhesus monkeys.** J Neurovirol 4: 394-406.

16. Lednicky JA, Arrington AS, Stewart AR et al., (1998). **Natural isolates of simian virus 40 from immunocompromised monkeys display extensive genetic heterogeneity: new implications for polyomavirus disease.** J Virol. 72: 3980-3990.

17. Gardner SD, Field AM, Coleman DV, Hulme B. (1971). **New human papovavirus (B.K.) isolated from urine after renal transplantation.** Lancet 1 : 1253- 1257.

18. Padgett BL, Walker DL, ZuRhein GM, Eckroade RJ, Dessel BH. (1971). **Cultivation of papovalike virus from human brain with progressive multifocal leucoencephalopathy.** Lancet 1: 1257- 1260.

19. Allander T, Andreasson K, Gupta S et al., (2007). **Identification of a third human polyomavirus.** J Virol. 81: 4130- 4136.

20. Gaynor AM, Nissen MD, Whiley DM et al., (2007). **Identification of a novel polyomavirus from patients with acute respiratory tract infections.** PLoS Pathog. 3: e64.

21. Viscidi RP, Shah KV (2008). **Cancer. A skin cancer virus?** Science. 319 :1049-1050.

22. Shah KV. (2007). **SV40 and human cancer: a review of recent data**. Int J Cancer 120:21 5-223.

23. Vilchez RA, Butel JS (2003). **SV40 in human brain cancers and non-Hodgkin's lymphoma.** Oncogene 22:5164-5172.

24. Cutrone R, Lednicky J, Dunn G et al. (2005). **Some oral poliovirus vaccines were contaminated with infectious SV40 after 1961**. Cancer Res 65: 10273-10279.

25. Melnick JL, Stinebaugh S. (1962). **Excretion of vacuolating SV-40 virus (papova virus group) after ingestion as a contaminant of oral poliovaccine**. Proc Soc Exp Biol Med. 109: 965-968.

26. Rollinson DE, Page WF, Crawford H, Gridley G, Wacholder S, Martini J, Miller R, Engels EA. (2004). **Case-control study of cancer among US Army veterans exposed to Simian virus 40-contaminated adenovirus vaccine**. Am J Epidemol. 160:317-324.

27. Richmond JE, Parry JV, Gardner SD. (1984). **Characterisation of a polyomavirus in two foetal rhesus monkey kidney cell lines used for the growth of hepatitis A virus**. Arch Virol. 80: 131- 146.

28. Morris JA, Johnson KM, Aulisio CG, Chanock RM, Knight V. (1961). **Clinical and serologic responses in volunteers given vacuolating virus (SV-40) by respiratory route**. Proc Soc Exp Biol Med. 08: 56-59.

29. Eddy BE, Borman GS, Berkeley WH, Young RD. (1961). **Tumors induced in hamsters by injection of rhesus monkey kidney cell extracts.** Proc Soc Exp Biol Med.107: 191 -197.

30. Kirschstein RL, Gerber P. (1962). **Ependymomas produced after intracerebral inoculation of SV40 into new-born hamsters.** Nature 195: 299-300.

31. Cicala C, Pompetti F, Nguyen P, Dixon K, Levine AS, Carbone M. (1993). **SV40 small t deletion mutants preferentially transform mononuclear phagocytes and B lymphocytes in vivo.** Virology 190: 475- 479.

32a. Diamandopoulos GT. (1973). **Induction of lyrnphocytic leukemia, lymphosarcoma, reticulum cell sarcoma, and osteogenic sarcoma in the Syrian golden hamster by oncogenic DNA simian virus 40.** JNatl Cancer Inst. 50: 1347- 1465.

32b. Diamandopoulos GT. (1972). **Leukemia, lymphoma, and osteosarcoma induced in the Syrian golden hamster by simian virus 40.** Science 176: 173-175.

33. Gardner SD. (1973). **Prevalence in England of antibody to human polyomavirus (BK).** Br Med J 1: 77-78.

34. Butel JS, Jafer S, Wong C, Arrington AS, Opakun AR, Finogold MJ, Adam E. (1999). **Evidence of SV40 infection in hospitalized children.** Hum Pathol. 30: 1496-1502.

35. Engels EA, Viscidi RP, Galloway DA, Carter JJ, Cerhan JR, Davis S, Cozon W, Severson RK, de Sanjoso S, Colt JS, Hartge P. (2004). **Case control study of simian virus 40 and non-Hodgkin lymphoma in the United States.** J Natl Cancer Inst. 96: 1368-1374.

36. Schein HM, Enders, JF. (1962). **Transformation induced by simian virus 40 in human renal cell cultures. I. Morphology and growth characteristics**. Proc Natl Acad Sci USA 48:1164-1172.

37. Jensen F, Koprowski H, Pagano JS, Ponten J, Ravdin RG. (1964**). Autologous and homologous implantation of human cells transformed in vitro by SV40.** J Natl Cancer Inst 32:917-932.

38. Dolcetti R, Martini F, Quaia M, Gloghini A, Vignocchi B, Cariati R, Martinelli M, Carbone A, Boiocchi M, Tognon M (2003) **Simian virus 40 sequences in human lymphoblastoid B-cell lines**. J Virol 77:1595-1597.

39. Martini F, Corallini A, Balatti V, Sabbioni S, Pancaldi C, Tognon M (2007) **Simian virus 40 in humans.** Infect Agent Cancer 2:13.

40a. Pancaldi C, Balatti V, Guaschino R, Vaniglia F, Corallini A, Martini F, Mutti L, Tognon M (2009) **Simian virus 40 sequences in blood specimens from healthy individuals of Casale Monferrato, an industrial town with a history of asbestos pollution.** J Infect 58:53-60.

40b. Pancaldi C, Corazzari V, Maniero S, Mazzoni E, Comar M, Martini F, Tognon M (2011) **Merkel cell polyomavirus DNA sequences in the buffy coats of healthy blood donors**. Blood 117:7099-7101

41. Jiang M, Abend JR, Johnson SF, Irnperiale MJ (2009). **The role of polyomaviruses in human disease.** Virology 384: 266-273.

42. Imperiale MJ (2001). **The human polyoma viruses: an overview. In Human Polyomaviruses: Molecular and Clinical Perspective**. Khalili K, Stoner GL. Wiley-Liss, New York. 53-77.

43. Randhawa PS, Demetris AJ (2000). **Nephropathy due to polyomavirus type BK**. N Engl J Med 342: 1361-1363.

44. Hirsch HH, Knowles W, Dickenmann M et al (2002). **Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients**. N Engl J Med 347:488-496

45. Berger JR (2003). **Progressive multifocal leukoencephalopathy in acquired immunodeficiency syndrome: explaining the high incidence and disproportionate frequency of the illness relative to other immunosuppressive conditions**. J Neurovirol 9: 38-41.

46. Fiers W, R. Contreras, G. Haegemann, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert (1978). **Complete nucleotide sequence of SV40 DNA.** Nature 273:113–120.

47. Sullivan CS, Grundhoff AT, Tevethia S, Pipas JM, Ganem D (2005). **SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells**. Nature 435: 682-686.

48. Ahuja D, Saenz-Robles MT, Pipas JM (2005) **SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation.** Oncogene 24:7729-7745

49. Poulin DL, DeCaprio JA (2006). **Is there a role for SV40 in human cancer?** J Clin Oncol 24: 4356-4365.

50. Liddington RC, Yan Y, Moulai J, Sahli R, Benjamin TL, Harrison SC (1991). **Structure of simian virus 40 at 3.8- A resolution.** Nature 354:278-284.

51. Norkin LC (1999). **Simian virus 40 infection via MHC class I molecules and caveolae**. Immunol Rev 168: 13-22.

52. Pelkmans L, Kartenbeck J, Helenius A (2001). **Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER**. Nat. Cell Biol. 3: 473–483.

53. Pelkmans L and Helenius A (2002). **Endocytosis via caveolae.Traffic** 3:311–320.

54. Richards, A. A., E. Stang, R. Pepperkok, and R. G. Parton (2002). **Inhibitors of COP-mediated transport and cholera toxin action inhibit simian virus 40 infection**. Mol. Biol. Cell 13:1750–1764.

55. Pante N, Kann M. (2002). **Nuclear pore complex is able to transport macromolecules with diameters of ~39 nm**. Mol. Biol. Cell 13, 425-434.

56. Daniel R, Rusan NM, Wilbuer AK, Norkin LC, Wadsworth P, Hebert DN (2006). **Simian virus 40 late proteins possess lytic properties that render them capable of permeabilizing cellular memebrane.** J Virol 80:6575-6587.

57. Dean FB, Bullock P, Murakami Y, Wobbe CR, Weissbach L, Hurwitz J (1987). **Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of eplication**. Proc Natl Acad Sci USA 1987, 84:16-20.

58. Smith AE and Helenius A (2004). **How viruses enter animal cells**. Science 304:237–242.

59. Niewiarowska J, D'Halluin JC, Belin MT (1992). **Adenovirus capsid proteins interact with hsp70 proteins after penetration in human or rodent cells.** Exp.Cell Res. 201:408-416.

60. Daniels R, Sadowicz D, Hebert DN (2007). **A very late viral protein triggers the lytic release of SV40**. PLoS Pathog 3: e98.

61 Carswell SJ, Resnick, Alwine JC. (1986). **Construction and characterization of CV-1P cell lines which constitutively express the simian virus 40 agnoprotein: alteration of plaquing phenotype of viral agnogene mutants.** J. Virol. 60:415–422.

62. Griffiths DJ, Nicholson AG, Weiss RA (1998). **Detection of SV40 sequences in human mesothelioma.** Dev Biol Stand. 94:127-136.

63. Gordon-Shaag AY, Yosef M, Abd El-Latif, Oppenheim A (2003). **The abundant nuclear enzyme PARP participates in the life cycle of simian virus 40 and is stimulated by minor capsid protein VP3.** J. Virol. 77:4273–4282.

64. Morelli C, Barbisan F, Iaccheri L, Tognon M (2004) **SV40-immortalized human fibroblasts as a source of SV40 infectious virions.** Mol Med 10:112-116.

65. Danthi S, Enyeart JA, Enyeart JJ (2003). **Modulation of native TREK-1 and Kv1.4 channels by polyunsaturated fatty acids and lysophospholipids**. J Membr Biol 195:147–164.

66. Hamid Ali S, DeCaprio JA (2001). **Cellular transformation by SV40 large T antigen: interaction with host proteins.** Cancer Biology 11:15-22.

67. Fanning E, Knippers R. (1992). **Structured and function of simian virus 40 large tumor-antigen.** Annual Rev. Biochem 61: 55-85.

68. Eckner R, Ludlow JW, Lill NL, Oldread E, Arany Z, Modjtahedi N, DeCaprio JA, Livingston DM, Morgan JA. (1996) **Association of p300 and CBP with simian virus 40 large T antigen**. Mol Cell Biol. 16:3454-4364.

69. DeCaprio JA, Ludlow JW, Figgo J, Show JY, Huang CM, Lee WH. Marsilo E, Paucha E, Livingston DM. (1988) **SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene**. Cell 54:275-283.

70. Ewen ME, Ludlow JW, Marsilio E, DeCaprio JA, Milikan RC, Chang SH, Paucha E, Livingston DM. (1989) **An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both p110Rb and a second cellular protein, p120**. Cell 58:257-267.

71. Campbell KS, Mullano KP, Aksoy LA. StuBdal H, Zalvido J, Pipas JM, Silver PA, Robert TM, Schaffhausen BS, DeCaprio JA. (1997) **DnaJ/hsp40 chaperon domain of SV40 large T antigen promotes efficient viral DNA replication.** Genes Dev. 11:1098-1110.

72. Srinivasan A, McClellan AJ, Vartikar J, Marks I, Cantalupo P, Li Y, Whyte P, Rundell K, Brodsky JL, Pipas JM. (1997) **The amino-terminal transforming region of SV40 large and small T antigens functions as a J-domain**. Mol Cell Biol 17:4761-4773.

73. Serger YR; Garcia-Cao M, Piccinin S, Cunsolo CL, Doglioni C, Blasco MA, Hannon GJ, Maostro R (2002), **Transformation of normal human cells in the absence of telomerase activation.** Cancer cell 2:401-413.

74. Arroyo JD, Hahn WC (2005). **Involvement of PP2A in viral and cellular transformation**. Oncogene. 24:7746–7755.

75. Krurnbholz A, Bininda-Emonds OR, Wutzler P, Zell R (2009). **Phylogenetics, evolution, and medical importance of polyomaviruses.** Infect Genet Evol 9: 784-799.

76. Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM (1990). **Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A.** Cell.60 (1):167–176.

77. Yang SI, Lickteig RL, Estes R, Rundell K, Walter G, Mumby MC (1991). **Control of protein phosphatase 2A by simian virus 40 small-t antigen.** Mol Cell Biol. 4 :1988–1995.

78. Porras A, Bennett J, Howo A, Tokos K, Bouck N, Hanglein B, Sathyamamgalam S, Thimmapaya B, Rundell K. (1996) **A novel simian virus 40 early-region domain mediates transactivation of the cyclin a promoter by small t-antigen and is required for transformation in small t-antigen dependent assay.** J Virol. 70:6902-6908.

79. Hahn WC, Dessain SK, Brooks MW, King JE, Elenbaas B,Sabatini DM, DeCaprio JA, Weinberg RA. (2002). **Enumeration of the simian virus 40 early region elements necessary for human cell transformation.** Mol.Cell. Biol., 22, 2111–2123.

80. Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC (2004). **Identification of specific PP2A complexes involved in human cell transformation.** Cancer Cell 5: 127-136.

81. Howe AK, Gaillard S, Bennett JS, Rundell K (1998**). Cell cycle progression in monkey cells expressing simian virus 40 small t antigen from adenovirus vectors**. J Virol 72: 9637-9644.

82. Loeken, M. R., I. Bikel, D. M. Livingston, and J. Brady (1988). **Trans-activation of RNA polymerase II and III promoters by SV40 small t antigen.** Cell 55:1171-1177.

83. Oren M, Levine AJ (1981). **Immunoselection of simian virus 40 large T antigen messenger rnas from transformed cells.** Virology. 113 (2):790–793.

84. Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR, Sears R (2004). **A signalling**

pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol. 6(4):308-18.

85. Ballou LM, Jiang YP, Du G, Frohman MA, Lin RZ (2003). **Ca(2+)- and phospholipase D-dependent and –independent pathways activate mTOR signaling.** FEBS Letters 550:51-56.

86. Zhao JJ, Gjoerup OV, Subramanian RR, ChengY, Chen W, Roberts TM, Hahn WC (2003). **Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase**. Cancer Cell 3, 483-495.

87. Suzuki K, Chikamatsu Y, Takahashi K (2005). **Requirement of protein phosphatase 2A for recruitment of IQGAP1 to Rac-bound beta1 integrin.** J Cell Physiol. 203(3):487-92.

88. Graessmann A, Graessmann M, Tjian R, Topp WC (1980). **Simian virus 40 small-t protein is required for loss of actin cable networks in rat cells.** J virol 1980. 33: 1182-1191.

89. Lednicky JA, Wong C, Butel JS (1995). **Artificial modification of the viral regulatory region improves tissue culture growth of SV40 strain 776**. Virus Res 35: 143-153.

90. Vilchez RA, Brayton CF, Wong C et al (2004). **Differential ability of two simian virus 40 strains to induce malignancies in weanling hamsters.** Virology 330:168-177.

91. Lednicky JA, Butel JS (2001). **Simian virus 40 regulatory region structural diversity and the association of viral archetypal regulatory regions with human brain tumors**. Semin Cancer Biol 11: 39-47.

92. Sroller V, Vilchez RA, Stewart AR, Wong C, Butel JS (2008). **Influence of the viral regulatory region on tumor induction by simian virus 40 in hamsters.** J Virol 82: 871-879.

93. Bocharov G, Ludewig B, Bertoletti A et al (2004). **Underwhelming the immune response: effect of slow virus growth on CD8+-T-lymphocyte responses.** J Virol 78: 2247-2254.

94. Patel NC, Halvorson SJ, Sroller V et al (2009). **Viral regulatory region effects on vertical transmission of polyomavirus SV40 in hamsters.** Virology 386: 94- 101.

95. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008). **Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?** Nut Rev Genet 9: 102-114.

96. Cullen BR (2006). **Viruses and microRNAs.** Nut Genet 38: S25-30.

97. Sarnow P, Jopling CL, Norman KL, Schiitz S, Wehner KA (2006). **MicroRNAs: expression, avoidance and subversion by vertebrate viruses.** Nat Rev Microbiol 4: 651-659.

98. Sullivan CS (2008). **New roles for large and small viral RNAs in evading host defences**. Nat Rev Genet 9: 503-507.

99. Swaminathan S (2008). **Noncoding RNAs produced by oncogenic human herpesviruses**. J Cell Physiol 216: 321-326.

100. Breau WC, Atwood WJ, Norkin LC (1992). **Class I major histocompatibility proteins are an essential component of the simian virus 40 receptor.** J Virol 66: 2037-2045

101. Norkin LC (1999). **Simian virus 40 infection via MHC class I molecules and caveolae**. Immunol Rev 168: 13-22

102. Atwood WJ (2001). **Cellular Receptors for the Polyomaviruses. In Human Polyomaviruses: Molecular and Clinical Perspective**. Khalili K, Stoner GL. Wiley-Liss, New York. p. 179-196.

103. White MK, Khalili K (2004). **Polyomaviruses and human cancer: molecular mechanisms underlying patterns of tumorigenesis.** Virology 324:1-16.

104. Barbanti-Brodano G, Sabbioni S, Martini F, Negrini M, Corallini A, Tognon M (2004) **Simian virus 40 infection in humans and association with human diseases: results and hypotheses.** Virology 318:1-9.

105. Craford LV, Pim DC, Lamb P.(1984). **The cellular protein p53 in human tumours**. Mol. Biol. Med. 2: 261.

106. Bartek J, Vojteˇsˇek B, Picksley SM, Lane DP. (1992). **Abnormal expression of wild type p53 in normal cells of a cancer family patient.** Lancet 340, 259-263.

107. Lilyestrom W, Klein MG, Zhang R, Joachimiak A, Chen XS. (2006) **Crystal structure of SV40 large T-antigen bound to p53: Interplay between a viral oncoprotein and a cellular tumor suppressor.** Genes and Dev.20: 2373-2382.

108. Saenz-Robles MT, Sullivan CS, Pipas JM (2001) **Transforming functions of Simian Virus 40**. Oncogene 20:7899-7907

109. zur Hausen H (2008). **Novel human polyomaviruses--re-emergence of a well known virus family as possible human carcinogens.** Iat J Cancer 123: 247-250.

110. Carbone M, Pass HI, Miele L, Bocchetta M (2003). **New developments about the association of SV40 with human mesothelioma.** Oncogene 22: 5173-5180.

111. Sergen, J. (2002). **Concise Dictionary of Modern Medicine.** New York: McGraw-Hill.

112. James L Bennington. (2006). **Saunders dictionary and encyclopedia of laboratory medicine and technology.** MEMPHIS, TN USA.

113 Holiday R (1996). **Neoplastic transformation: the contrasting stability of human and mouse cells.** cancer survey 28:103-115.

114. Akagi T, Sasai K, Hanafusa H (2003). **Refractive nature of normal human diploid fibroblasts with respect to oncogen-mediated transformation.** PNAS 100:13567-13572.

115. Walen KH (2002). **The origin of transformed cells: Studies of spontaneous and induced cell transformation in cell cultures from marsupials, a snail, and human aminocytes**. Cancer Genetics and Cytogenetics 133:45-54.

116a. Ryan QC, Goonewardene IM, Murasko DM (1992) **Extension of lifespan of human T lymphocytes by transfection with SV40 large T antigen.** Exp Cell Res 199:387-391

116b. Ryan QC, Goonewardene IM, Murasko DM (1993) **Maintenance of normal T lymphocyte function after transfection with SV40 large T**. Cell Immunol 149:65-81

117. Kanki, T (1994). **Immortalization of human primary B lymphocytes by simian virus 40 early region DNA.** <u>Hybridoma</u> 13(4):327-330.

118. Shaikh S, Skoczylas C, Longnecker R, Rundell K (2004). **Inability of simian virus 40 to establish productive infection of lymphoblastic cell lines.** J Virol 78: 491 7-20.

119. Reudl C, Kopf M, Bachmann MF. (1999). **CD8^{+} T cells mediate CD40-independent maturation of dendritic cells in vivo.** J Exp Med 189: 1875-1884.

120. Andreason SO, Christensen JE, Marker O, Thomsen AR (2000). **Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8^{+} effector T cell responses**. J Immunol 164: 3689-3697.

121. Borrow P, Olstone MBA (1997). **Lymphocytic choriomeningitis virus. In viral pathogenesis**. Nathanson N, Rafi A Eds. Lippincott-Raven: Philadelpha, pp593-627.

122. Drummond JE, Shah KV, Donnenber AD (1985). **Cell-mediated immune responses to BK virus in normal individuals.** J Med Virol 17:237-247.

123. Bates MP, Jennings SR, Tanaka Y, Tevethia MJ, Tevethia SS (1988). **Recognition of Simian virus 40 T antigen synthesized during viral lytic cycle in monkey kidney cells expressing mouse H-2b- and H-2Db-transfectd gene by SV40-specific cytotoxic T lymphocytes leads to the abrogation of virus lytic cycle.** Virology 162:197-205.

124. Garcea RL, Imperiale MJ (2003). **Simian virus 40 infection of humans.** J Virol 77: 5039-45.

125. Sweet BH and Hilleman MR (1960). **The vacuolating virus, S.V. 40.** Proc Soc Exp Biol Med 105: 420-427.

126. Zhen HN, Z h g X, Bu XY et al (1999). **Expression of the simian virus 40 large tumor antigen (Tag) and formation of Tag-p53 and Tag-pRb complexes in human brain tumors.** Cancer 86: 2124-2132.

127. Arrington AS, Moore MS, Butel JS (2004). **SV40-positive brain tumor in scientist with risk of laboratory exposure to the virus.** Oncogene 23: 223 1-5.

128. Carter JJ, Madeleine MM, Wipf GC, Garcea RL, Pipkin PA, Minor PD, Galloway DA (2003). **Lack of serologic evidence for prevalent simian virus 40 infection in humans**. J Natl Cancer Inst 95:1522-1530.

129. Rizzo P, Bocchetta M, Powers A et al (2001). **SV40 and the pathogenesis of mesothelioma.** Semin Cancer Biol 11: 63-71.

130. Kroczynska B, Cutrone R, Bocchetta M et al (2006). **Crocidolite asbestos and SV40 are cocarcinogens in human mesothelial cells and in causing mesothelioma in hamsters**. Proc Natl Acad Sci USA 103: 14128-14133.

131. Cristaudo A, Foddis R, Vivaldi A, Buselli R, Gattini V, Guglielmi G, Cosentino F, Ottenga F, Ciancia E, Libener R, Filiberti R, Neri M, Betta PG, Tognon M, Mutti L, Puntoni R (2005), **SV40 enhances the risk of malignant mesothelioma among people exposed to asbestos: A molecular epidemiologic case-control study.** Cancer Res 65(8): 3049-3052.

132. Alaribe FN, Maniero S, Pancaldi C (2010). **Malignant Pleural Mesothelioma Prognostic Marker: A Review of Osteopontin and Soluble Mesothelin Peptides**. Trop J Pharm Res 9 (6): 605-613.

133. Catalano A, Romano M, Martinotti S, Procopio A (2002). **Enhanced expression of vascular endothelial growth factor (VEGF) plays critical role in the tumor progression potential induced by simian virus 40 large T antigen**. Oncogene 21:2896-2900.

134. Cacciotti P, Strizzi L, Vianale G, Iaccheri L Libener R, Porta C, Tognon M, Gaudino G, Mutti L (2002). **The presence of simian virus 40 sequences in mesothelioma and mesothelial cells is associated with high levels of vascular endothelial growth factor.** Am J Respir Cell Mol Biol 26: 189-193.

135. Waheed I, Guo ZS, Chen GA, Weiser TS, Nguyen DM, Schrump DS (1999). **Antisense to SV40 early gene region induces growth arrest and apoptosis in T-antigen-positive human pleural mesothelioma cells.** Cancer res 59:6068-6073.

136. Bright RK, Kimchi ET, Shearer MH, Kennedy RC, Pass HI (2002). **SV40 Tag-specific cytotoxic T lymphocytes generated from the peripheral blood of malignant pleural mesothelioma patients.** Cancer Immunol Immunother 50: 682-690.

137. Manfredi J. James, Jianli Dong, Wen-jun Liu, Lois Resnick-Silverman, Rui Qiao, Philippe Chahinian, Marko Saric, Allen R. Gibbs, James I. Phillips and J. Murray. (2005). **Evidence against a Role for SV40 in Human Mesothelioma.** Cancer Res., 65: 7.

138. Li RM, Branton MH, Tanawattanacharoen S, Falk RA, Jennette JC, Kopp JB (2002) **Molecular identification of SV40 infection in human subjects and possible association with kidney disease.** J Am Soc Nephrol 13:2320-2330.

139. Milstone A, Vilchez RA, Geiger X, Fogo AB, Butel JS, Dummer S (2004). **Polyomavirus simian virus 40 infection associated with nephropathy in a lung-transplant recipient**. Transplantation 77:1019-1024.

140. Coe JE, Green I (1975). **B-cell origin of hamster lymphoid tumors induced by simian virus 40.** J Natl Cancer Inst 1975; 54: 269-270.

141. Cicala C, Pompetti F, Carbone M (1993)**. SV40 induces mesotheliomas in hamsters**. Am J Path 142: 1524-1533.

142. David H, Mendoza S, Konishi T, Miller CW (2001). **Simian virus 40 is present in human lymphomas and normal blood.** Cancer Lett 162: 57-64.

143. Staudt LM, Wilson WH (2002). **Focus on lymphomas**. Cancer Cell 2002; 2: 363-366.

144. Grulich AE, Vajdic CM (2005). **The epidemiology of non-Hodgkin lymphoma**. Pathology 37: 409-419.

145. Engels EA (2007). **Infectious agents as causes of non-Hodgkin lymphoma**. Cancer Epidemiol Biomarhzrs Prev 16: 401-404.

146. Butel, J.S (2009). **SV40, human infections, and cancer: emerging concepts and causality considerations. In**: Khalili, K. and Jeang, K.T. (ed.), **Viral Oncology**: Basic Science and Clinical Applications, Wiley-Blackwell.

147. Alaribe FN, Manfrini M, Mazzoni E (2011). **Simian Virus 40 contamination Vaccine: Correlation with present prevalence of lymphomas.** Advs. in Bioresearch 2 (1): 159-165.

148. Sui LF, Williamson J, Lowenthal RM, Parker AJ (2005). **Absence of simian virus 40 (SV40) DNA in lymphoma samples from Tasmania, Australia**. Pathology 37: 157-159.

149. Schuler F, Dolken SC, Hirt C et al (2006). **No evidence for simian virus 40 DNA sequences in malignant non-Hodgkin lymphomas.** Int J Cancer 118: 498-504.

150. Southern PJ, Berg P (1982). **Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter**. J Mol AppI Genetics 1:327-341.

151. Girardi AJ, Weinstein D, Moorhead PS (1966). **SV40-transformation of human diploid cells. A parallel study of viral and karyologic parameters.** Ann. Med. Exp.Biol. Fenn. 44: 242-254.

152. Tao L, Dong Z, Zannis-Hadjopoulos M (2001). **Immortalization of human WI38 cells is associated with differential activation of the c-myc origins**. J Cell Biochem. 82(3):522-534.

153. Nakayama, G.R. et al. (1997) **Assessment of the Alamar Blue assay for cellular growth and viability in vitro.** J Immunol Meth 204:205–208.

154. AI-Nasiry S, Geusens N, Hanssens M, Luyten C, Pijnenborg R (2007). **The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells**. Human Reproduction 22:1304-1309.

155. Barbero A. *et al*. (2005). **Experimental and Mathematical Study of the Influence of Growth Factors on the Growth Kinetics of Adult Human Articular Chondrocytes**. *J. Cell. Physiol.* **204**: 830-838.

156. Kim J, Aberg C, Salvati A, Dawson K (2011). **Role of cell cycle on the cellular uptake and dilution of nanoparticles in a cell population.** Nature Nanotechnology vol. 7

157. Meng X, Ichim TE, Zhong J, Rogers A, Yin Z, Jackson J, Wang H, Ge W, Bogin V, Chan KW, Thebaud B, Riordan NH (2007). **Endometrial regenerative cells: A novel stem cell population.** J. Trans. Med. 5:57

158. Morelli C, Barbisan F, Iaccheri L, Tognon M (2004) **Simian virus 40 persistent infection in long-term immortalized human fibroblast cell lines.** J Neurovirol 10:250-254.

159. Bertrand FE, Eckfeldt EC, Lysholm AS, LeBien TW (2000). **Notch-1 and Notch -2 exhibited unique patterns of expression in human B-lineage cells.** Leukemia 14:2095-2102.

160. Secchiero P, Bertolaso L, Casareto L, Gibellini D, Vitale M, Bemis K, Aleotti A, Capitani S, Franchini G, Gallo RC, Zauli G (1998) **Human herpesvirus 7 infection induces profound cell cycle perturbations coupled to disregulation of cdc2 and cyclin B and polyploidization of CD4(+) T cells**. Blood 92:1685-1696.

161. Geiger R, Andritschke D, Friebe S, Herzog F, Luisoni S, Heger T, Helenius A (2011). **BAP31 and BiP are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol**. Nature Cell Biology 13:1305–1314.

163. Jakobovits EB, Abulafia R, Aloni Y (1982). **Temperature-sensitive B mutant of SV40 Disassemble intracellular encapsidation particles at elevated temperature**. Virology 121:95-106.

163. Martinelli M, Martini F, Rinaldi E, Caramanico L, Magri E, Grandi E, Carinci F, Pastore A, Tognon M (2002) **Simian virus 40 sequences and expression of the viral large T antigen oncoprotein in human pleomorphic adenomas of parotid glands**. Am J Pathol 161:1127-1133.

164. Kasahara T, Shiosri-Nakano K,Sugiura A (1977) **Detection of mitogen-activated T and non-T lymphocytes by virus plaque assay. Virus plaque assay on the cells fractionated by unit gravity sedimentation.** Immunology 32: 875.

165. Swetly P, Brodano GB, Knowles B, Koprowski H (1969) **Response of simian virus 40-transformed cell lines and cell hybrids to superinfection with simian virus 40 and its deoxyribonucleic acid.** J Virol 4:348-355.

166. Herzog P, Drosten C, Muller MA (2008). **Plaque assay for human coronavirus NL63 using human colon carcinoma cells.** Virology J. 5:138.

167. Archibald RB, Frenster JH (1973) **In: The cell nucleus,** Busch H (ed) 1:565-580 (1994) Academic Press, New York.

168. Pais-Correia A, Sachse M, Guadagnini S, Robbiati V, Lasserre R, Gessain A et al., (2010) **Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses.** Nature Medcine 16:83-89.

169. Javier RT, Butel JS (2008). **The history of tumor virology**. Cancer Res 68: 7693-7706.

170. Nachtigal M, Legrand A, Nagpal Ml, Nachtigal SA, Span PG (1990). **Transformation of rabbit vascular smooth muscles cells by transfection with early region of SV40 DNA**. American Journal of Pathology 136:297-306.

171. Robinson BW, Musk AW, Lake RA (2005). **Malignant mesothelioma**. Lancet 366:397-408.

172. Peto J, Decarli A, La Vacchia C, Levi F, negri E (1999). **The European mesothelioma epidemic.** Br J cancer 79:666-672.

173. Carbone M, Pass HI, Rizzo P, Marinetti M, Di Muzio, Mew DJ, Levine AS, Procopio A (1994). **Simian virus 40-like DNA sequences in human pleural mesothelioma.** Oncogen 9:1781-1790.

174. Mclaren BR, Haenel T, Stevenson S, Mukherjee S, Robinson BW, Lake RA (2000). **Simian virus (SV) 40 like sequences in cell lines and tumour biopsies from Australian malignant mesothelioma.** Aust N Z J Med 30:450-456.

175. MacLachlan DS (2002). **SV40 in human tumors: new documents shed light on the apparent controversy.** Anticancer Res 22: 3495-3499.

176. Martini F, Dolcetti R, Gloghini A, Iaccheri L, Carbone A, Boiocchi M, Tognon M (1998) **Simian-virus-40 footprints in human lymphoproliferative disorders of HIV- and HIV+ patients**. Int J Cancer 78:669-674.

177. Malkin D (2002). **Simian virus 40 and non-Hodgkin lymphoma**. Lancet 359: 812-813.

178. Katsumoto T, Higaki K, Ohno K, Onodera K (1995). **Cell-cycle dependent biosynthesis and localization of p53 protein in untransformed human cells. Biology of the Cell 84:167–173.**

179. Raetz E, Perkins S, Davenport V, Cairo MS (2003) **B large-cell lymphoma in children and adolescents.** Cancer Treat Rev 29:91-98.

180. Thorley-Lawson DA, Allday MJ (2008). **The curious case of the tumour virus: 50 years of Burkitt's lymphoma.** Nat Rev Microbiol. 6(12):913-924.

Publications

(1). Alaribe FN, Maniero S, Pancaldi C (2010)**. Malignant Pleural Mesothelioma Prognostic Marker: A Review of Osteopontin and Soluble Mesothelin Peptides**. Trop J Pharm Res 9 (6): 605-613.

(2). Alaribe FN, Manfrini M, Mazzoni E (2011). **Simian Virus 40 contamination Vaccine: Correlation with present prevalence of lymphomas.** Advs. in Bioresearch 2 (1): 159-165.

(3). Mazzoni E, Rigolin GM, Alaribe FN, Pancaldi C, Maniero S, Comar M, Martini F, Tognon M. (2012). **Simian Virus 40 efficiently infects human T lymphocytes and extended their lifespan**. Article in Press (Journal of hematology

Manuscripts in Preparation:

1. Alaribe FN, Mazzoni E, Rizzotto L, Maniero S, Manfrini M, Martini F, Tognon M.
Interaction of Simian Virus 40 (SV40) with normal human B Lymphocytes

2. Alaribe FN and Bononi I
Recent software tools and alogorithms relevant for next generation sequencing application.