

AMERICAN UNIVERSITY OF BEIRUT

CERAMIDE REGULATION BY *E4orf4* GENE DURING
ADENOVIRAL INFECTION

by
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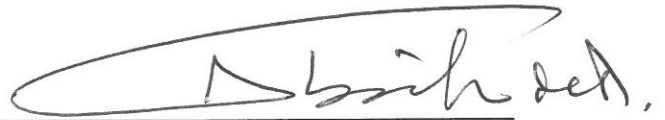
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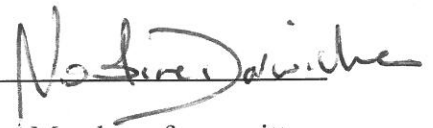
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AN ABSTRACT OF THE THESIS OF

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Title: Ceramide Regulation by *E4orf4* Gene during Adenoviral Infection.

Cancer remains the leading cause of global mortality. Advanced and metastatic cancer often remains incurable by current treatment options underscoring the need for the development of novel approaches. Therapies based on viruses are promising in the field of cancer, including adenovirus type 5 (Ad5). Advantages of adenovirus use include its ability to infect both dividing and arrested cells, and the ease of obtaining high concentrations. *E4orf4*, an early adenoviral gene, is a candidate oncolytic viral gene as it triggers p53- and caspase-independent apoptosis selectively in cancer cells. Yet a deeper understanding of its mechanism of action is necessary to elucidate its function before its clinical application. The potency of *E4orf4*'s lytic activity precludes its stable transfection in cells as they would quickly succumb. For this purpose, we used a T-Rex tetracycline inducible system. The *E4orf4* gene was introduced into a vector pcDNA4/T0, where its expression is repressed by another vector pcDNA6/TR. In theory, this system would allow us to tightly control the expression of *E4orf4* in “stably” transfected A549 cell line and subsequently be able to study its effects on apoptosis and ceramide. Real-time PCR and western blot analysis were used to confirm the expression of *E4orf4* in these cells in response to tetracycline treatment. Analyses of viability by Trypan Blue, cell cycle distribution, expression levels of apoptotic-related proteins and ceramide levels were measured. Surprisingly, we observed that the expression of *E4orf4* in A549 cells did not have any effect on the cell growth, apoptosis, or ceramide levels as compared to the A549-Ø (empty vector) cells. Moreover, *E4orf4* increased the number of cells in the S phase along with an elevation of cyclin A levels in A549-*E4orf4* cells. In an effort to understand these unexpected findings, we examined ceramide levels and the expression of several antiapoptotic and proapoptotic proteins of the BCL2 family in our system. We discovered that the presence of the *E4orf4* gene, even in the presence of the Tet-repressor and the absence of tetracycline treatment, significantly increased the baseline expression of BCL2 and decreased the expression of BAK and BCLX_{s/L}. Moreover, the baseline levels of ceramide were significantly lower than those in A549-WT (wild type) cells. This indicated that the T-Rex system was leaky and that the cells that were eventually cloned following transfection and subsequent selection were those that had the ability to resist apoptosis induced by the minimal expression of *E4orf4*. In order to verify whether *E4orf4* expression was directly driving these changes, we transiently transfected *E4orf4* in A549 cells, and found that it did not alter the expression levels of BAK and BCL2 proteins. Furthermore, transient *E4orf4* expression enhanced the accumulation of ceramide significantly at 6 h post transfection. In conclusion, these results suggest that the T-Rex system is not appropriate for the study of the effects of *E4orf4* on apoptosis and ceramide. Other methodologies will need to be used to determine the exact mechanism of action of this gene and its effect on ceramide regulation.

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ABBREVIATIONS

- Ad5: adenovirus type 5
- ADP: adenovirus death protein
- Alk-SMase: Alkaline Sphingomyelinase
- APL: Acute Promyelocytic Leukemia
- ASMase: Acid Sphingomyelinase
- BSA: bovine serum albumin
- BAD: Bcl-2 Antagonist of Cell death
- BAK: Bcl-2 Antagonist Killer
- BAX: Bcl-2 Associated X
- BCL-xL: B Cell Lymphoma-Long Isoform
- BCL-2: B cell lymphoma 2
- BID: Bcl-2 Interacting Protein
- CAPP: ceramide-activated protein phosphatase
- CAR: coxsackievirus and adenovirus receptor
- DDW: double distilled water
- CDK: Cyclin-Dependent Kinase
- CerS: Ceramide Synthase
- DGK: Diacylglycerol kinase enzyme
- DMEM: Dulbecco's modified Eagle's medium

DNA: deoxy ribonucleic acid

DTT: Dithiothreitol

ER: Endoplasmic Reticulum

E: early

E4orf4: early gene 4 open reading frame 4

ECL: enhanced chemiluminescence

E-coli: Escherichia coli

EGTA: ethylene glycol tetraacetic acid

FBS: fetal bovine serum

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

HCl: hydrochloric acid

HRP conjugated secondary antibodies: Horseradish Peroxidase conjugated secondary antibodies

ITR: inverted terminal repeats

Kbp: kilo base pair

L: late

LiCl: Lithium Chloride

mRNA: messenger RNA

MgCl₂: Magnesium Chloride

MLP: Major late promoter

MOMP: Mitochondrial Outer Membrane Permeabilization

NSMase: Neutral Sphingomyelinase

NaCl: sodium chloride

Na₂HPO₄: Di-sodium hydrogen orthophosphate anhydrous

Orf: open reading frame

PBS: phosphate buffer saline

PP1: protein phosphatase 1

PP2A: protein phosphatase 2A

PVDF: polyvinylidenedifluoride

PARP: Poly (ADP-Ribose) Polymerase

PI: Propidium Iodide

PKC: Protein Kinase C

RGD: arginylglycylaspartic acid

RB: Retinoblastoma

SDS: Sodium dodecyl sulfate

SMS: sphingomyelin synthase

SM: Sphingomyelin

SMase: Sphingomyelinase

S1P: Sphingosine 1 Phosphate

SPT: serine-palmitoyl CoA transferase

SR protein: serine/arginine-rich protein

Tet: tetracycline

TetR: tetracycline repressor

TP: terminal protein

TRAIL: TNF-Related Apoptosis-Inducing Ligand

TRAILR: TNF-Related Apoptosis-Inducing Ligand Receptor

UV: ultraviolet

VP: viral particle

CHAPTER I

LITERATURE REVIEW

A. Cancer

Cancer is a devastating disease causing mortality and morbidity worldwide with an estimated 8.2 million deaths in 2012. This number is predicted to rise by 75% and reach around 25 million over the next two decades. Cancer is a group of diseases characterized by an uncontrolled division of cells [1], and the development of a mass consisting of abnormal cells that have extended outside their normal boundaries (World Cancer Report 2015 by WHO).

Normally, cells of the body follow a systematic pattern of growth, division, and death. However, when this pattern becomes dysregulated, cancer begins to develop. Unlike regular cells, cancer cells continuously grow and divide, but they frequently have a decreased ability to undergo apoptosis [2]. These cells are capable of invading and damaging normal tissues either locally or at distant sites of the body [1], where the latter is referred to as metastasis, a late stage of cancer frequently preceding death. At the molecular level, the development of most cancers occurs as a result of multiple alterations that accumulate progressively over years.

Many internal and external factors contribute to the increase in the global burden of cancer. Internal factors include inherited genetic defects, hormones, aging, and immune conditions; whereas, external factors include tobacco, infectious organisms, obesity, exposure to radiation and chemicals, as well as other behavioral and environmental risks. These factors may act together, or in sequence to promote carcinogenesis [3].

Several treatment options are used to cure cancer including radiation, chemotherapy, surgery, hormone therapy, and immunotherapy [4]. There is no single treatment for cancer. In fact, patients often receive a combination of at least two of these therapies along with palliative care when necessary. Moreover, the selection of these treatments depends on many factors such as the type of cancer, health status of the patient, age, clinical staging and other factors. When the tumor is still localized, treatment is more likely to be successful. However, only a small percent of patients with distant metastasis are responsive to conventional treatments [5] .

Currently used therapies are associated with unbearable side effects that threaten the patients' life instead of saving it [6-7]. Subsequently, the mentioned therapies are often ineffective for advanced and metastatic diseases highlighting the need for development of novel therapies. Malignant tumors gradually gain resistance to traditional treatments [7]. Therefore, there is an urgent need for a more effective therapy that would abolish, or at least control, metastatic diseases and reduce the intrinsic drug resistance. Ideally, these novel cancer therapeutics should specifically target cancer cells, and prevent damaging healthy ones and have minimal side effects [8].

In the past few years, adenoviral (Ad) vectors have drawn the attention of many of those involved in the field of gene therapy for cancer because of their practical advantages and application potential [9-10].

B. Adenovirus and Cancer

1. *Adenoviruses: new hope for cancer gene therapy*

Gene therapy is a new therapeutic approach which involves the transfer of genetic material into a cell in order to treat certain diseases [11]. It can be established through two different methods. The first one is performed by introducing a new gene into the host cell aiding it in defeating the disease. The second way is by knocking out or inactivating the mutated gene which had its proper function affected [12]. Therefore, gene therapy has been considered a treatment for many diseases for the past few decades, employing a variety of vectors of gene delivery.

There are several approaches for cancer gene therapy including the enhancement of immune response against a tumor, repair of cell cycle defects emerging from losses of tumor suppressor genes, disabling the inappropriate activation of certain oncogenes, and induction of cell suicide gene strategies [13].

For a successful application of gene therapy, some elements are a must, including genes, promoters, and regulatory elements, as well as effective vectors [14]. Therefore, gene therapy is applied by the integration of molecular constructs called vectors, which could be either viral or non-viral [15].

The non-viral gene delivery system aids in the delivery of a part of DNA into a cell. This system is established by using chemical methods such as polymers and cationic liposomes or physical methods, such as electroporation, magnetofection and sonoporation. Despite the safety of the non-viral vectors, they provide only transient gene expression [16-17].

Virotherapy, the use of viral vectors, is a new strategy to treat cancer by selectively infecting and killing tumor cells without affecting the normal ones. Oncolytic viruses used in virotherapy can either kill tumor cells by bursting them open or deliver genes that make the cells more susceptible to traditional chemotherapies. They can also be labeled with fluorescent or radioactive tags. Once delivered into the body, they can home in on cancer cells leaving undamaged healthy ones [6, 7]. Viral vectors are more efficient than non-viral ones and, as a result, their use is becoming more widespread [16, 18].

A promising candidate as a viral vector for therapeutic gene transfer is adenovirus type 5 (Ad5), which has been widely used because of its attractive characteristics. The size of its genome is quite large (~ 36kb), which makes it useful for the development of large-capacity vectors with minimal viral sequence [13]. Moreover, it is easily manipulated, purified, and generated [19].

Ad5 is biologically safe compared to other vectors since it is mostly responsible for benign well-characterized infections in humans with no significant side effects following early clinical application or vaccination with live wild-type (wt) Ad [13]. Ad5 has the ability to infect either dividing or quiescent cells without integration into the host cell genome thus presenting low genotoxicity for *in vivo* application. In addition, they are relatively stable in structure and DNA sequence, and elevated viral titers can be produced (up to 10^{13} VP/ml) [20]. Moreover, the vectors can be introduced into different tissues via different routes of administration. They can be injected either intravenously or intratumorally [21]. These are the key features that make adenoviruses such attractive gene transfer vectors for cancer gene therapy.

2. Adenoviruses

Adenoviruses were first isolated in 1953 as pathogenic agents from primary cultures derived from human adenoids, from which the name is derived [22-23]. There are 68 different serotypes in humans divided into 7 species (A-G) that were identified using two traditional methods: hemagglutination and serum neutralization.

Adenoviruses are mainly responsible for mild infections in the upper and/or lower respiratory tract as well as gastrointestinal and ocular infections [24]. These infections, mostly those involving the respiratory tract are common in infants and children aged less than 5 years. Adenoviruses were first used as expression vectors in mammalian cells in 1980's [11] then used in gene therapy in 1990. In 2004, it was reported that *in vivo* administration of Ad expressing ornithine transcarbamylase, crucial for the urea cycle, in mice lacking this enzyme corrected the deficiency [25].

Several generations of Ad vectors have been developed. In the first generation of vectors, the early adenoviral genes E1 and/or E3 were removed. These genes are responsible of the replication of viral DNA and the production of the virus capsid proteins. However, these earlier constructs showed toxic side effects and strong immune responses when applied *in vivo* [19, 26-27].

The second generation vectors had some or all of the early adenoviral genes E2 deleted and hence the capacity to replicate viral DNA was eliminated. The third generation vectors called 'gutless' vectors had the other viral genes or lately all the viral genes deleted and require helper virus for production [26, 28] (Figure 1). Several transgenes can home in this class of viruses with a total of 37 kb [29].

3. *Ad5 structure*

Adenoviruses are large non-enveloped particles possessing a linear double stranded DNA genome. The geometric structure of a virion capsid is icosahedral having a diameter of around 80 kb [30]. Twelve fibers extend outside the capsid corners and their knob portion is responsible for the high affinity adsorption of the virus to target cell receptors [31]. Therefore, the fiber is the first component of the virus to interact with a given host cell contributing significantly to the ability of the virus to bind and infect cells.

In addition to the fibers, the capsid consists of 252 capsomers composed of two major proteins known as hexon and penton base. Hexon proteins occupy the majority of the capsid with a total number of 240 and are located on the facets of the capsid. However, the other type of proteins present at the vertices and from which fiber proteins are projected constitutes the minority of the capsid with a total number of 12. Besides, the minor capsid proteins include IIIa, VI, VIII and IX (Figure 2) [22, 26, 32].

The complexity of the structure lies in its inner core possessing a linear adenoviral DNA that is tightly wrapped around viral proteins V, VII and X [30]. There are six other structural components situated in the virus core, five are associated with the double stranded DNA genome [V, VII, Mu, IVa2 and the terminal protein (TP)], whereas the sixth one is the viral cysteine protease which plays a vital role in the assembly and production of mature infectious virus (Figure 1) [31]. This structural rearrangement is common to all adenoviruses [30].

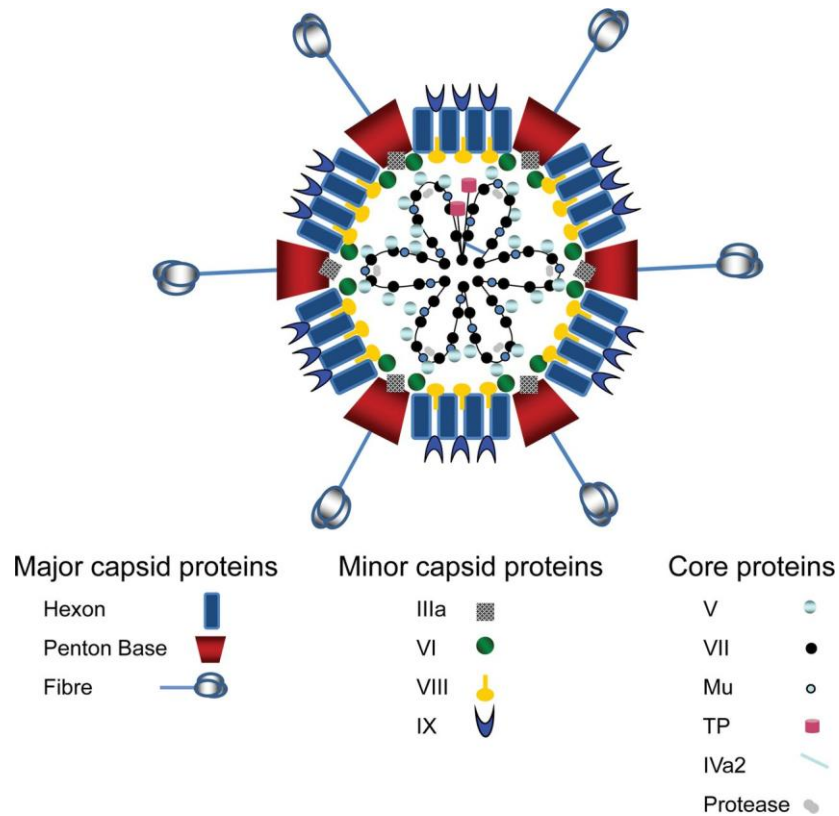


Figure 1: A schematic representation of the Ad capsid [33] .

4. Adenovirus genome

The Ad5 genome is about 36 kb in size and encodes for almost 39 genes, divided into early (E) or late (L) genes depending on their expression either before or after DNA replication (figure 2) [30]. During the early phase proteins are expressed from six different transcription units designated as E1a, E1b, E2a, E2b E3 and E4. The E1 genes involved in the regulation of the other viral regions, whereas E2 encodes proteins necessary for viral DNA replication. E3 gives the adenoviruses the ability to escape from the immune system while E4 plays a role in the cell cycle control [34]. The major late proteins named L1 to L5, result from alternative splicing of a single

transcript MLP (Major late promoter) and encode for the structural proteins while inverted terminal repeats (ITR) are needed in DNA replication where they act as primers [30]. Moreover, the packaging sequence is required for efficient packaging of Ad5 DNA [35] (Figure 2).

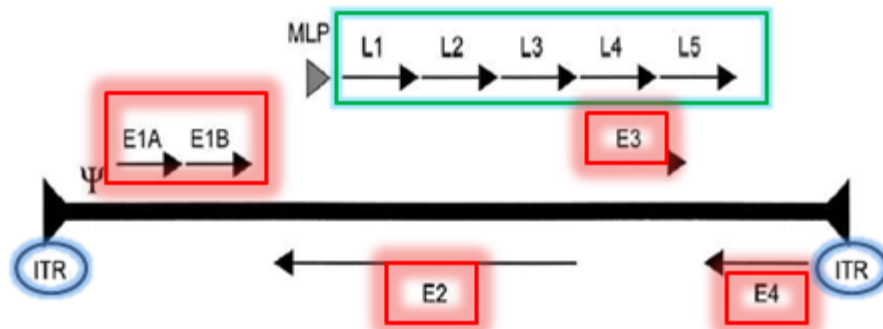


Figure 2: Map of the Ad genome showing the early genes (E1 to E4), late genes (L1 to L5), the packaging sequence (Ψ) and the ITRs. Arrows represent the direction of transcription [36].

5. Adenovirus life cycle

The adenoviruses life cycle can be divided into two phases: early and late phase. The early phase starts as soon as the virus makes its first interaction with the cell and proceeds until the expression of the early gene products necessary for viral replication. These events can take about 6 to 8 hours. The late phase begins with the expression of late viral genes, assembly of progeny virions and ends with cellular release. It is normally more rapid than the early phase, yielding virus in 4 to 6 hours [26, 30]. Typically the whole cycle is completed in 24 to 36 hours [37].

In a natural infection, the interaction with a host tissue probably occurs via an aerosol into either the respiratory or gastrointestinal tracts [33]. First, the fibre protein binds to a primary receptor. There are a number of binding receptors on a variety of cells but the major receptor for most adenoviruses is called Coxsackie Adenovirus Receptor (CAR) (Figure 3). It was named as such because it is essential for the initial binding of coxsackie B virus. CAR is a member of the immunoglobulin super family and is involved in the formation of tight junctions [37-38]. Alternatively, it has been shown that Ad5 can also enter through heparin sulfate proteoglycans either directly by binding to the fibre proteins or indirectly by their interaction with several blood factors.

Second, the RGD peptide consisting of (Arg-Gly-Asp) on the penton base interacts with cellular $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins on the cell surface. This interaction triggers a variety of cellular responses leading to the internalization of the virus through endocytosis into clatherin-coated vesicles. Consequently, this results in the alteration of the cytoskeleton in order to facilitate the virus entrance and packaging into endosomes [39].

Subsequently, once inside the cell, the capsid is dissociated and degraded. The virus translocates to the nuclear pore, where the viral DNA is released into the nucleus of the host cell [24-26] (Figure 3). Afterwards, selective transcription and translation of the early genes take place which results in the maturation of the infectious cycle.

E1A is the first viral transcription unit to be expressed followed by transcription of other early viral genes E1B, E2, E3 and E4. Then the DNA replication is initiated by late genes transcription [40]. This leads to the production of the virus structural components, such as virion proteins and proteins required for the assembly of infectious virions, and its encapsidation [41]. Finally, these new virions migrate and accumulate in the cytoplasm until they provoke host-cell lysis and their liberation in the extracellular matrix. The escape of the adenoviral DNA is facilitated by ADP (Adenovirus death protein), which is required for efficient lysis [26].

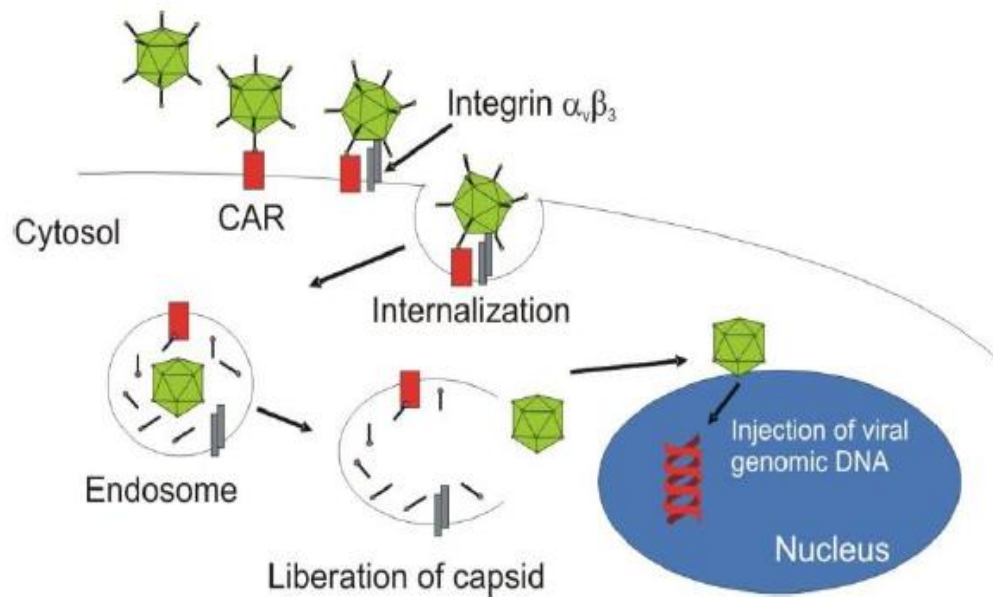


Figure 3: Infectious cycle of Adenovirus 5 (Ad5). (Adopted from Strauss *et al.*, 2013)

C. E4 region

The E4 adenoviral gene is one of the five early adenoviral genes that have drawn the attention of scientists in the field of cancer gene therapy. It consists of seven open reading frames (orfs) encoding at least six proteins noted as E4orf1 to E4orf6/7 (figure 4) [42].

The E4 proteins play several roles in the viral replication, such as control of gene expression at different levels and protection of the virus against host defense mechanisms. Many studies have suggested that E4 is a multifunctional gene required for viral DNA replication, accumulation of late viral mRNAs and proteins, virus particle assembly as well as the inhibition of antiviral defense mechanism. Moreover, it acts to shut off host proteins [43].

E4 promoter is known to be rapidly activated by E1A transcriptional activator protein soon after infection. Transcription persists into the late phase of infection until it is repressed at later times [44].

The *E4orf1* gene product negatively controls the late viral protein synthesis and the levels of viral progeny produced, enhancing the survival of Ad5-infected cells. As for *E4orf2* gene product, there was no specific role or function described.

The *E4orf3* protein regulates Ad5 mRNA splicing, reinforces late viral mRNAs translation, and stimulates cell cycle-independent virus growth. It is also required for viral DNA replication [45]. Moreover, it blocks the activity of interferon induced cellular antiviral defense mechanism [46]. Regarding *E4orf6/7*, it is known to monitor E2F transcription factor.

Concerning our gene of interest *E4orf4*, many studies have demonstrated that it binds to protein phosphatase 2A and induces a non-classical form of p53-independent apoptosis. Its role had been shown to be effective only in cancer cells and not in normal human cells which made it an interesting potential tool in cancer gene therapy [47].

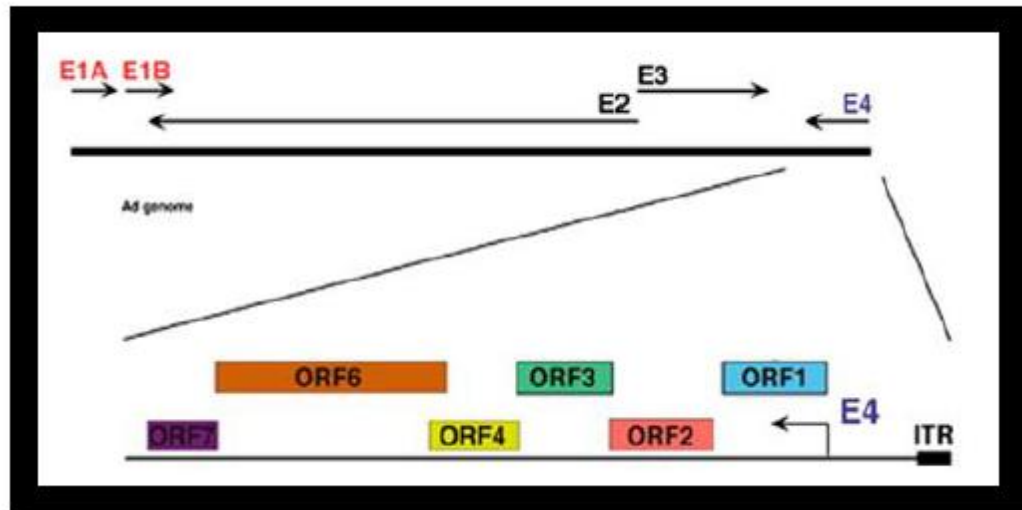


Figure 4: A schematic representation of early transcription units of Ad5 with the different open reading frames of the early region 4 (E4) [48].

1. *E4orf4* protein

E4orf4 protein, a 114-residue product of the early region E4, shares no homology with other viral or cellular proteins [49]. *E4orf4* is highly stable and remains at constant levels late in infection; however, its transcription stops during the late phase. Thus, it controls the progression from early to late phases during viral replication [47].

The *E4orf4* protein is essentially localized in the nucleus of host cells, with small amounts in the cytoplasm [50-51]. The non-phosphorylated *E4orf4* protein is mainly localized in the nucleus whereas phosphorylated *E4orf4* accumulates in the cytoplasm and plasma membrane [51]. *E4orf4* is phosphorylated on its tyrosine residues

by Src kinases family. This phosphorylation leads to caspase-independent membrane blebbing and cell death [52]. This distinctive mode of cell death requires a cytoplasmic subcellular localization [53].

2. *E4orf4* function

To date, all E4orf4 activities during virus infection are linked to its interaction with PP2A [43]. It was described that E4orf4 binds specifically to the B55 subunit of the Ser/Thr protein phosphatases PP2A that are involved in many cellular processes such as cell division, signal transduction, development and gene expression [43]. These enzymes consist of three subunits; the scaffold subunit A, the catalytic subunit C, and the regulatory subunit B. Both subunits A and C interact with a regulatory subunit B.

The binding of E4orf4 to the B α subunit of PP2A is crucial for its ability to induce apoptosis. In fact, a mutated E4orf4 protein that is unable to bind to an active PP2A lacks the cell killing property of transformed cells [54]. Moreover, it has been shown that this interaction alters the activity of PP2A holoenzymes against some substrates by either altering the intracellular localization of PP2A or altering the binding of PP2A with critical substrates. This leads to the hypophosphorylation of these substrates causing growth arrest and cell death [55].

As mentioned earlier, E4orf4 interacts with members of the Src kinases family, leading to its Tyr phosphorylation and to the deregulation of Src signaling, promoting cell death [56]. Thus, both Src and PP2A enzymes collaborate to trigger E4orf4-induced cell death both in mammalian cells and *Saccharomyces cerevisiae* [57].

Furthermore, E4orf4 is considered as a splicing enhancer protein since it contributes to the dephosphorylation of the SR proteins after binding to PP2A. SR are

serine/arginine-rich proteins regulated by phosphorylation and involved in alternative splicing of many apoptotic regulatory genes in response to external death stimuli [58].

For instance, SR proteins are involved in alternative splicing of two key apoptotic genes, *caspase 9* and *Bcl-x*, as well as the adenoviral late L1 gene. Consequently, E4orf4 may stimulate a switch in the late adenovirus splicing pattern [51, 53].

E1A is required to activate all early viral promoters. E4orf4 can autoregulate its own transcription by inhibiting E1A-induced activation of E4 transcription. This is accomplished by the formation of E4orf4/PP2A complex that can dephosphorylate E1A by its phosphatase activity. Once dephosphorylated, E1A activity is repressed resulting in the repression of the transcription of E4 as well as its own transcription [59-60]. This regulation carried out by E4orf4/PP2A complex can have the advantage of limiting the cytotoxic effects during the earlier phases of infection thereby ensuring maximum productivity [60].

3. *E4orf4: a potential tool for cancer therapy*

E4orf4 represents an important candidate in cancer therapy. Its therapeutic potential emerges from its ability to induce selective and efficient cell death in transformed cells but not in normal cells. It has been shown that E4orf4 can exert its killing effect on a variety of cancer cell lines. In fact, around 40 human cancer cell lines were found to be susceptible to killing induced by E4orf4 whereas, a variety of tissue-derived primary human cells were resistant [47].

Interestingly, E4orf4 specificity in killing cancer cells was not only limited to tissue culture cells but also in multicellular organism. A study performed in *Drosophila*

eye disc has shown that E4orf4 can efficiently eradicate cancer clones more than the normal ones [43]. Additionally, it has been reported that when cotransfecting a diverse combination of oncogenes in primary rat embryo fibroblasts, E4orf4 was shown to induce a significant levels of cell death as compared to cells transfected with an empty vector. However, the reason behind this selectivity is still not fully elucidated. Many hypotheses arose regarding this issue [61].

Some suggested that some cancer cells are dependent on specific oncogenic pathways that are targets of E4orf4. When these pathways are inhibited by E4orf4, cancer cells die without affecting the normal ones. Another hypothesis suggested that cell cycle checkpoints are impaired in cancer cells making them easily affected by E4orf4, which is known to disrupt mitotic checkpoints [27].

In addition, some studies demonstrated that E4orf4 expression in transformed cells induces an early appearance of morphological and actin changes, which rapidly cause dramatic membrane blebbing. The changes in actin dynamics occur prior to DNA condensation and upstream in the death pathway [36].

Besides its selectivity, it is noteworthy that E4orf4 induces cell death independently from p53 activation [62]. The majority of human cancers end up with mutations that inactivate p53, and many others are probably defective in downstream components of the p53 pathway. As many current therapies rely on p53-dependent cell killing, these treatments become of limited value in these cases since the loss of p53 function is accompanied with the resistance to these therapies. E4orf4 protein is known to induce cell death regardless of p53 status [55, 63]. In fact, this protein was not found to cause an alteration in p53 protein levels in human embryonic kidney cells HEK 293 cells. In addition, E4orf4 was able to induce cell death in H1299 cells (human non small cell

lung carcinoma cell line), which lack p53 expression [62] . These unique properties of E4orf4 prompted the curiosity of researchers to further understand its mechanism of action.

Moreover, it has been reported that E4orf4-induced cell death is caspase-independent since caspase inhibitors did not prevent E4orf4 cell killing [43] although in some cell lines caspase activation was observed after E4orf4 expression [34]. E4orf4 is potentially involved in a crosstalk between caspase-dependent and caspase-independent cell death inducing signaling pathways that can be related to caspase-dependent apoptosis or mitotic catastrophe-mediated necrosis [27].

Furthermore, the role of E4orf4 in cell cycle disorders has not been fully elucidated; however, many studies have suggested that E4orf4-expressing cells undergo G2/M and possibly G1 arrest [43, 64] .

Minimal *in vivo* trials of E4orf4 have been reported, but the above mentioned studies performed on this gene provide a motivation for scientists to go further and investigate the exact mechanism of action of this gene in a variety of tissue culture cells as well as in animal models [43].

D. Sphingolipids

Sphingolipids, a class of natural lipids, are key components of eukaryotic cellular membranes synthesized in the endoplasmic reticulum (ER) [65]. They have structural properties which have been backed up in recent years with functional properties; this made them bio-effector molecules participating in vital signaling pathways that determine the cell's fate including survival, growth, proliferation, differentiation, and death [66-67].

Moreover, they have a role in inflammatory responses of the immune system. They act as receptors for viruses, bacteria and antibodies [67]. While there are more than 300 species of sphingolipid molecules, the most studied in this family of lipids, are ceramide, sphingosine, sphingosine-1-phosphate, sphingomyelin, and glucosylceramide, and their regulating enzymes.

Ceramide is considered to be the structural backbone of most sphingolipids and has been the most extensively studied. Ceramide acts as a second messenger for a variety of intracellular events that are part of the normal cellular homeostasis and others that are associated with diseases including cancer [66].

1. Ceramide structure

Ceramide is an N-acylsphingosine consisting of an aliphatic amino alcohol known as sphingosine [68-69]. This sphingoid base is an amide linked to a mono-unsaturated or saturated fatty acid chains that vary in length from 14 to 26 carbon atoms [70-71]. Ceramide is the simplest form of sphingolipids; it is a highly hydrophobic molecule having only two hydroxyl groups, which gives it a slight hydrophilic property [66, 72] (Figure 5).

Ceramide is the basic structure of complex sphingolipids that are characterized by additional hydrophilic regions in the R position. For instance, sphingomyelin (SM) contains a phosphorylcholine group, and glycosphingolipids have a carbohydrate group [73].

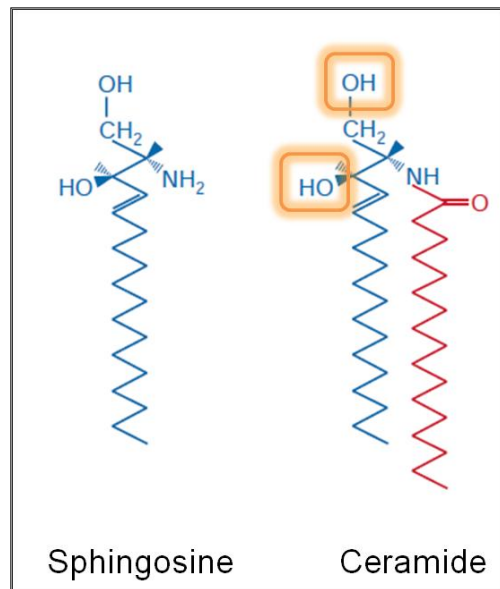


Figure 5: Structure of Ceramide and sphingosine

2. *Ceramide Metabolism*

Ceramide is produced inside the cell through three major metabolic pathways depending on cell type and stimulus [74]. These complex pathways regulate ceramide levels through the involvement of different enzymes. These differ in their sub-cellular location in organelles [71, 75].

The three metabolic pathways are: *De novo* synthetic pathway, Sphingomyelin hydrolysis pathway and Salvage pathway.

a. *De novo* synthetic pathway

The *de novo* synthesis of ceramide takes place in the ER through a series of enzymatic reactions. This pathway is first initiated by the rate limiting enzyme known as serine palmitoyl transferase (SPT) which condenses serine and palmitoyl coA yielding 3-ketosphinganine [76-77]. The resulting compound is then reduced by 3-ketosphinganine reductase to sphinganine. This is followed by the acylation of the sphingoid base by ceramide synthase, which consists of 6 isoforms (CerS1-6) leading to the production of a variety of dihydroceramides [78]. Finally, a biologically active ceramide is generated once 4,5-trans double bond is added by the dihydroceramide desaturase [79] (Figure 6).

b. Sphingomyelinase pathway

Ceramide is transported to the golgi apparatus after its *de novo* synthesis in the ER. This transportation is established either by vesicles or by the highly specific ceramide transport protein (CERT) [80]. Afterwards, upon the action of sphingomyelin synthase, ceramide is converted into sphingomyelin (Figure 6); the latter is then reallocated from the golgi to the plasma membrane, where it plays an important role in the formation of lipid rafts along with other sphingolipids [81]. Ceramide can then be regenerated through the action of different sphingomyelinases (SMases). These enzymes have particular phospholipase C activity that hydrolyzes sphingomyelin yielding ceramide and a water-soluble phosphorylcholine [82-83].

Sphingomyelinases exist in several isoforms that can be distinguished depending on their pH optimum. These isoforms can be acidic, neutral or alkaline [70, 72]. Alkaline sphingomyelinases are present in the bile and intestinal mucosa and are not found to be involved in signal transduction pathways. However, acidic and neutral sphingomyelinases are promptly activated by a variety of stress stimuli such as chemotherapeutic agents leading to a rapid increase in ceramide levels in the cell [84-85]. Actually, acidic sphingomyelinases (aSMases) activity is mostly found in humans and it has two isoforms the lysosomal and secretory aSMases [86].

Briefly, both *de novo* and sphingomyelinase pathways produce a biologically active ceramide; however, the time needed to generate this sphingolipid in these pathways is not the same. Actually, ceramide can be formed within few minutes upon SMase activation unlike the De novo pathway that requires several hours [82].

c. Salvage pathway (Recycling)

The “Salvage” pathway is also known as the sphingolipid recycling pathway [87]. It aids in the generation of the majority of endogenous ceramide of which it accounts for around 50% of the sphingolipid pool [70]. In fact, this process involves mainly lysosomal enzymes that utilize complex sphingolipids, glycosphingolipids as well as various ceramide species and target them for degradation. The resulting product is sphingosine [88] (Figure 6) .

Unlike ceramides, this backbone molecule is able to shuttle back into the ER and serves as a substrate for ceramide synthases that are able to acylate it, thus contributing to the production of ceramide [80]. Additionally, several enzymes are known to interfere in this recycling pathway such as ceramidases that hydrolyze ceramide into sphingosine. The latter can be either phosphorylated to sphingosine -1 phosphate (S1P) by the action of sphingosine kinase or reacylated to ceramide [89].

Moreover, ceramide can be converted into more complex sphingolipids such as sulfatides, cerebroside and gangliosides [90]. These molecules are formed by the addition of glycosyl to C1 position, while addition of a phosphorylcholine group to the same position is catalyzed by sphingomyelin synthases (SMS) and results in the generation of sphingomyelin [91-92].

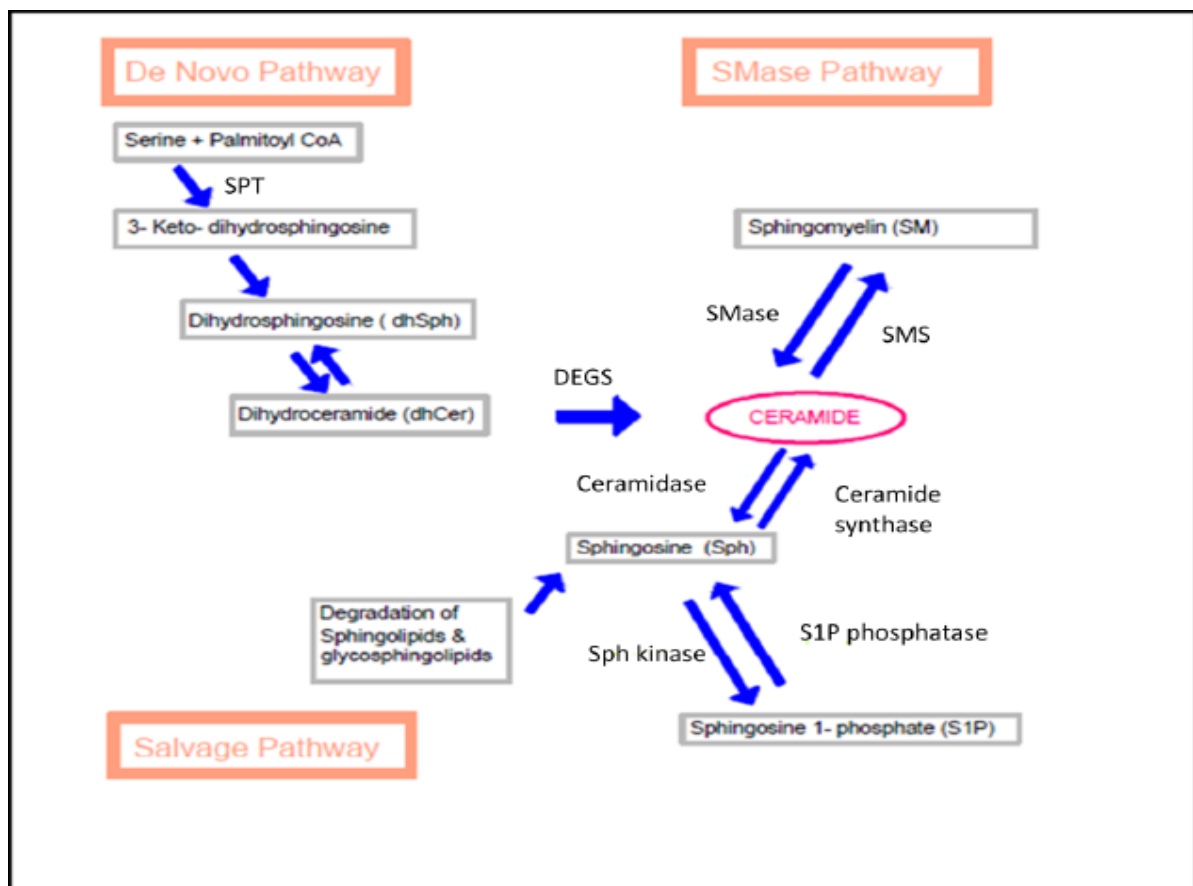


Figure 6: Ceramide synthesis pathways. The scheme shows metabolic pathways for ceramide synthesis composed of the sphingomyelinase pathway, the *de novo* pathway and the salvage pathway (Adopted from Pettus, 2002).

3. Biological implications of Ceramides:

Ceramides are tumor suppressor sphingolipids that act as second messengers for a variety of extracellular stimuli such as heat shock, growth factors, inflammatory mediators (TNF- α and Fas ligand stimulation), ultra violet radiation, γ –irradiation and chemotherapeutic agents [70]. They are signaling molecules that mediate several cellular processes including growth suppression, apoptosis, and differentiation [89, 93-94].

The interference of ceramides in growth suppression mechanism is mediated by c-Fos and AP-1 [95]. The latter molecules lead to the de-phosphorylation of retinoblastoma (Rb) protein, which will consequently suppress growth and induce senescence or cell cycle arrest [95-97]. Moreover, ceramide was found to be involved in the regulation of other cellular processes such as autophagy [98] and phagocytosis [99].

Furthermore, rapid ceramide formation activates diverse apoptotic pathways that can be either p53-dependent [100] or p53-independent [101]. Ceramide interacts with numerous intracellular enzymes including phosphatases, kinases, and proteases. Precisely, the best characterized enzymes shown to be activated by ceramides are Ceramide-activated protein phosphatases (CAPPs) [70, 88]. These include PP1 and PP2A; the former is a serine/threonine protein phosphatase that dephosphorylates serine/arginine-rich proteins such as the splicing protein SRp30a, leading to the generation of pro-apoptotic proteins BCL-xS or caspase 9 [102]. Once activated by ceramide, PP1 is also found to induce growth arrest by dephosphorylating Rb [103].

On the other hand, the second CAPP Protein, PP2A, is involved in the regulation of pro-growth kinases such as PKB (Protein kinase B) and PKC (Protein kinase C) [74]. Furthermore, this phosphatase interferes in the mitochondrial signaling pathways by dephosphorylating anti-apoptotic proteins such as BCL-2 and Bad [97, 104] as well as

activating MEK1 and Ras/Raf1 pro-apoptotic signaling pathways [105] . As a result, the activation of these pathways by Ceramide-induced PP2A activity triggers mitochondrial membrane depolarization, Cytochrome C release, and intrinsic cell death [105].

Regarding its physiological role, ceramide is the major modulator of membrane platforms. Several studies revealed that the presence of ceramide confers rigidity to the lipid bilayer since it aids in the reorganization of the cell membrane and membrane rafts [106]. However, others showed that this sphingolipid molecule can exert many biochemical and morphological manifestations of apoptosis. Ceramide production can regulate many alterations in the cell membrane such as vesiculation, blebbing and externalization of phosphatidylserine, which are the main hallmarks of apoptosis [107].

Additionally, ceramide indirectly contributes to the efflux of Cytochrome C from the mitochondria. This occurs via the formation of ceramide channels on its outer surface that increase the permeability of the mitochondrial outer membrane through the formation of pores [108-109].

4. Ceramide- contribution to apoptosis:

One of the prominent features of cancer cells is their resistance to death receptor ligands that are the key players in mediating the extrinsic form of apoptosis [110-111]. However, ceramide involvement in this process was found to be crucial in increasing the susceptibility of tumor cells for this pathway [79]. CD95, one of the important death receptors of TNF superfamily, was found to accumulate ceramide extracellularly as well as intracellularly in the nucleus upon binding to its specific ligand [112] . Increased levels of ceramide triggered by CD95-ligand interaction results in the activation of SMase and subsequently caspase activation leading to apoptosis [112-113]. Dihydroceramide

synthase is required for the action of CD95 in mediating apoptosis. An inhibitor for this enzyme, Fumonisin B1, limits the activity of CD95 in leukemic cells [114].

The interaction of TRAIL, another important death receptor ligand, with its corresponding receptors TRAILR1 and TRAILR2 [115] was also found to induce apoptosis by accumulating ceramide through SMase activation; however, this process is highly dependent on a specific enzyme known as CerS6 [116]. The latter synthesizes ceramide species with acyl chains containing 14, 16, and 18 carbons in length. It has been shown that the knockdown of Cers6 in colon cancer results in the inhibition of apoptosis triggered by TRAIL [116].

Moreover, reactive oxygen species highly regulate the SMase activity; therefore, the production of ceramide and their clustering in the membrane that are stimulated by TRAIL are inhibited by antioxidants [112].

Besides the engagement of ceramide in the extrinsic pathway of programmed cell death, exogenous administration or endogenous production of this sphingolipid participates more in the intrinsic mitochondrial pathway of apoptosis [79]. Frequently, malignant cells are resistant to apoptosis mediated by the mitochondria. Here lies the potential role of ceramide [79] where it can initiate a cascade of pathways that engage the mitochondria and result in the release of pro-apoptotic proteins that also hinder the energetic function of the mitochondria and hence trigger apoptosis.

It has been shown that ceramide can relocate directly to the mitochondria once it is generated at the plasma membrane from which it forms a platform. This structural composition formed on the outer leaflet of the plasma membrane folds inward and mingles with the mitochondria rendering it non-functional [117-118]. Therefore, ceramide accumulation was found to induce apoptosis in cancer cells by altering the

function of the mitochondria. However, the exact mechanism of ceramide-induced intrinsic apoptosis is still not fully elucidated.

Several experiments done on isolated mitochondria revealed that endogenous ceramide production can be established through the localization of certain enzymes such as CERSs and neutral ceramidases or their association with the mitochondria [119-120]. Additionally, ceramide can also be generated through the salvage pathway or ceramide synthases by ER membranes surrounding the mitochondria [121]. These mitochondria-associated membranes were found to be important in supplying the outer mitochondrial membrane with adequate ceramide levels [121]. Subsequently, ceramide channels are formed on the outer surface and this is associated with the release of pro-apoptotic proteins including cytochrome c which is an apoptotic marker [108, 122].

However, some elaborate studies have demonstrated that this mitochondrial outer membrane permeabilization (MOMP) and the induction of apoptotic signaling pathways are not only triggered by ceramide but rather these events are due to a synergistic role between ceramides and BAX [123-124]. Actually, BAX oligomerization on the outer mitochondrial surface and pore formation requires the presence of ceramide-rich surfaces [125-126]. Moreover, a study performed on histiocytic lymphoma cell line showed that ceramide alters the intracellular pH leading to conformational change in BAX and consequently cell death [127].

On the other hand, some workers demonstrated that ceramide induces the activation and inhibition of certain downstream signaling pathways to mediate MOMP. Ceramide acts by either activating PP2A or inactivating AKT; both pathways result in the activation of a serine-threonine kinase known as glycogen synthase kinase 3 β (GSK-3 β) [128-130]. The latter leads to the cleavage of BID to truncated tBID through the

activation of caspase 2 as well as caspase 8 [131-132]. Afterwards, this membrane-targeted death ligand translocates to the mitochondria and affects its function to antagonize Bcl-2 and promote apoptosis [132]. Furthermore, in prostate cancer cells, ceramide is shown to induce Cytochrome C release as well as caspase 9 activation through the activation of PKC [133]. This influences the normal mitochondrial functions and triggers MOMP and subsequent apoptosis [133].

Another mitochondrial protein that is regulated by ceramide is BCL2. This anti-apoptotic protein [132, 134] is important in regulating the high levels of calcium [131] and cell death receptors [135]. Ceramides triggers mitochondrial apoptosis through downregulation of this protein and activation of PP2A which in turn activates caspase 2 [79]. Overexpression of BCL2 prevents the action of ceramide in inducing MOMP, and low calcium levels counteract the effect of ceramides in activating caspase 2 [132, 136].

To summarize, there is a complex network between ceramide and the mitochondria that requires further investigation due to the engagement of several pathways [79]. The initiation of the apoptotic responses targeted against the mitochondria is mediated through CerSs, as well as acid and neutral SMases [79]. These ceramide-generating enzymes are known as the conductors of intrinsic apoptosis and they react differently in cancer depending on the cell type [137-138].

In conclusion, cancer cells exhibit impaired apoptotic pathways involving both the extrinsic and the intrinsic pathways. In fact, mitochondria hold promise in the field of cancer as a target for cancer therapy [139]. Additionally, ceramide upregulates pro-apoptotic proteins that counteract the effect of BCL2 in inducing cell survival in tumors [140]. This interplay between ceramide and mitochondrial proteins overcomes the resistance of cancer cells to chemotherapy and intrinsic apoptosis [79].

E. E4orf4 and ceramide influence on cell cycle progression:

Numerous adenoviral genes induce changes in different stages of the cell cycle. E4orf4 causes an alteration in the cell cycle depending on the cancer cell type [141]. Previous experiments conducted on HEK293 cells revealed that E4orf4 induced cell cycle arrest at G2/M. This was shown by the accumulation of these cells bearing 4N DNA content that subsequently resulted in the accumulation of apoptotic cells [142-143]. Similarly, other studies showed that E4orf4 also mediated G2/M arrest in *S. cerevisiae* [142, 144].

On the other hand, in H1299 cells, the increased percentage of cells having 4N DNA content was accompanied by increased levels of cyclin E. However, it has been shown that these cells were not in the G2/M phase since they did not contain high levels of mitotic markers including cyclin B1. Therefore, it has been suggested that these cells progressed from mitosis to G1 phase. Moreover, It was shown that this progression is performed in the absence of cytokines [64, 145]. These studies confirm that the influence of E4orf4 on cell cycle progression is cell type specific.

Ceramide has been shown to cause an alteration of different phases of the cell cycle in cancer cells. Previous study reported that exogenous administration of ceramide affects cell cycle progression from G1 to S phase [79, 97]. Normally, this phase is tightly regulated by different cyclin-dependent kinases (CDKs) namely, CDK2, CDK4 and CDK6. These are activated upon interaction with cyclin E and cyclin D resulting in the phosphorylation of RB. Additionally, this phase is controlled by a variety of cell cycle inhibitors such as the CDK inhibitors: p21 and p27 [79].

However, exposure of malignant cells to ceramide alters the normal transition from G1 to S phase. In fact, ceramide reduces the levels of cyclins and CDKs contributing to the activation of this phase. It also decreases RB and upregulates p21 resulting in cell cycle arrest at G1 phase [146-148].

F. Ceramide, Adenovirus and Cancer:

The tumor suppressor protein p53 is the main inducer of apoptosis. However, this gene is mutated in more than 50% of human tumors especially after chemotherapy is initiated leading to great difficulty in achieving successful tumor kill [70]. This strongly motivates scientists to seek cell death mechanisms that are independent of p53 activation. Fortunately, ceramide has been reported as a potent alternative to induce cellular death regardless of p53 activation [74, 149-150]. In fact, many cancerous cells such as colon carcinoma cells were shown to have significant lower levels of ceramide compared to the healthy ones [67].

On the other hand, dysregulation in ceramide metabolism has been found in both solid and non-solid tumors and may lead to chemotherapy resistance [73, 149]. Therefore, manipulation of endogenous ceramide levels through inhibiting enzymes that metabolize it such as ceramidases and glucosyltransferases or the activation of enzymes that trigger ceramide synthesis pathways may increase the effectiveness of some cancer therapies [72, 151].

Besides the relation between ceramide levels and pathogenesis of cancer, adenoviruses are found to interfere in this process. Previous published data from our laboratory revealed that the adenoviral infection resulted in a dose-dependent and a time-dependent accumulation of ceramide over a period of 24 to 96 hours [58]. It was shown that this accumulation is not only important in inducing cell death but also affects SR

protein phosphorylation important for alternative gene splicing and is necessary for the completion of the adenovirus replication cycle since ceramide synthesis inhibitors caused a delay in cell death [58].

Therefore, these findings establish the possibility that adenoviruses stimulate the ceramide pathway in order to induce preferential cellular or viral alternative gene splicing in the late phase of the viral cycle [58].

As a conclusion, the exact link between ceramide, adenovirus and cancer remains ambiguous. Cell-cycle pathways, including the ceramide pathway, are similarly dysregulated in malignant cells as well as in virally infected cells in order to maintain cellular replication machinery with overlapping key players, such as Rb and p53. Accordingly understanding how adenoviral infectious events interfere with ceramide pathways has implications not only on the interaction of virus and host cell but also on discovering potential new targets or approaches for treating cancer.

CHAPTER II

THESIS HYPOTHESIS AND SPECIFIC AIMS

OBJECTIVE OF THE STUDY:

Several genes were suggested to be responsible for ceramide accumulation during adenovirus infection. Two candidates were first identified.

The first candidate was the E1A adenoviral protein. Since E1A is known to induce forced transition into the S phase by binding to Rb, it was demonstrated that when this occurs in cells not prepared for this transition, significant cell stress is induced triggering apoptosis. It was hypothesized by our group that the inhibition of Rb by E1A might trigger a compensatory accumulation of ceramide as a component of the stress response in which ceramide has been shown to play a prominent role. However, preliminary unpublished data from our laboratory showed that infecting MCF7 cells with a mutant adenovirus that expresses E1A12S protein alone, there was no significant increase in ceramide levels up to 96 hours post infection despite adequate expression of E1A (data not shown). Therefore, we concluded that it is unlikely that E1A protein is responsible for inducing ceramide accumulation during adenoviral infection. The second candidate was that E4orf4 protein might be responsible for this accumulation. This protein is able to induce p53-independent cell death; a property shared with ceramide, and thus was suspected to engage the ceramide pathway. In previous studies, two mutant adenoviruses dl1011 and dl1014 were used. The dl1011 virus does not express any of the E4 region proteins whereas dl1014 expresses only the E4orf4 protein but none of the other E4 proteins. Ceramide levels were measured at 24, 48, and 72 hours in MCF7 cells post- infection with the wild type adenovirus (rec700), dl1011, or dl1014. It was shown

that infection with the virus expressing only the E4orf4, d11014, induced the fastest and highest increase in ceramide levels among the three viruses (Figure 7) [58]. These experiments indicated that the E4orf4 could be an important candidate accounting for ceramide accumulation.

Our main aim in this study is to investigate the correlation between E4orf4 expression and ceramide accumulation in inducing cell death. Due to the published potent oncolytic activity of E4orf4, we planned to use an inducible expression system in the human lung adenocarcinoma cell line A549 in order to tightly regulate the expression of E4orf4. We favor the following hypothesis: *E4orf4 is a major inducer of ceramide accumulation and subsequent cell cycle perturbation and apoptosis.*

SPECIFIC AIMS:

1. Establish and characterize an inducible expression system leading to controllable expression of the potentially active E4orf4 protein
2. Determine the effects of E4orf4 expression on cell viability and apoptosis
3. Characterize the effects of E4orf4 expression on the cell cycle
4. Describe the effects of E4orf4 expression on ceramide levels
5. Identify the ceramide metabolic pathways activated by E4orf4

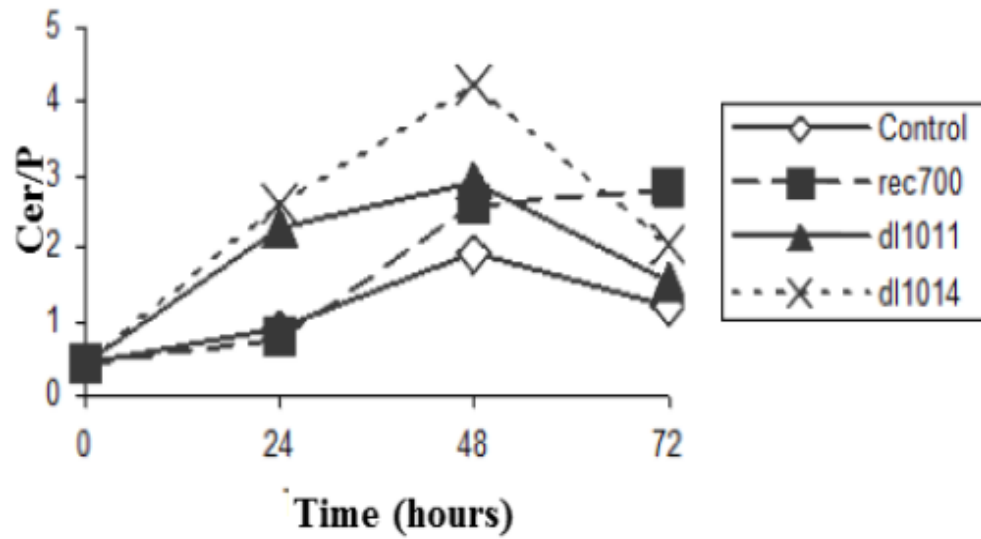


Figure 7: Ceramide accumulation generated from infection of MCF7 cell lines with different adenoviruses: the wild type rec700 and the mutant adenoviruses dl1011 and dl1014. The amount of ceramide is represented by the calculated ratio of ceramide/phosphate.

CHAPTER III

PRELIMINARY RESULTS

Preliminary studies done in our laboratory, showed that the infection of MCF7 (Human breast cancer cells) with the wild type adenovirus rec700 increased ceramide levels in a dose and time dependent manner [58]. The accumulation of ceramide was not accompanied with increase in the cell death (Figure 8A, B). These results were not cell type specific as it was also shown that adenoviral infection of A549 cells also induces ceramide accumulation (data not shown).

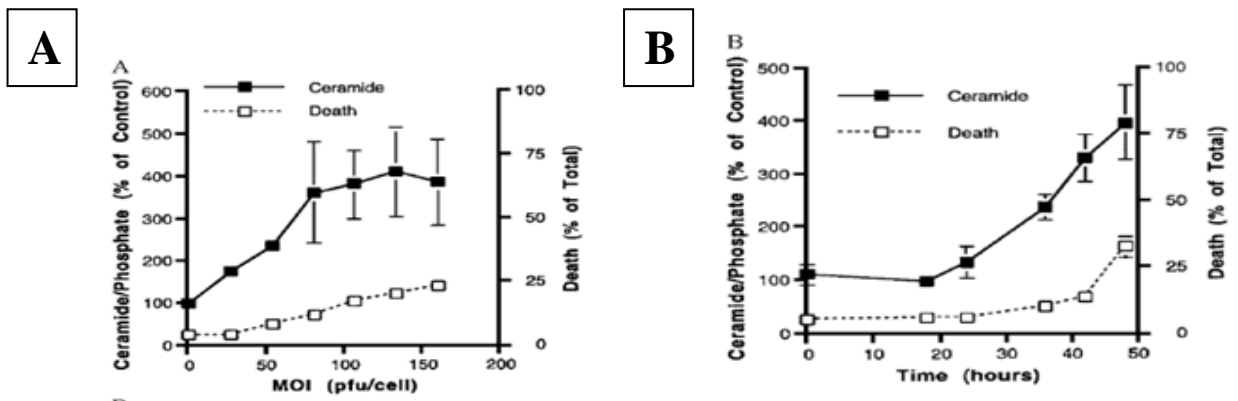


Figure 8: (A) Effect of adenovirus infection on ceramide levels and cell death. MCF7 cells were infected with rec700 adenovirus at the indicated MOIs then collected at 48h post-infection. Ceramide was measured and Cell lysis was determined using the trypan blue exclusion assay [58]. (B) Time-dependent increase in ceramide and cell lysis in MCF7 cells post infection with adenovirus rec700 at an MOI of 160 pfu/cell.

Preliminary studies were done to investigate the role of E4orf4 in A549 cells. Transient transfection of E4orf4 in these cells showed that E4orf4 induced a slight increase in ceramide levels 24 h post-transfection (data not shown). Although the increase in ceramide levels induced by E4orf4 was not significant with respect to the negative controls; however, these results were thought to be promising and the low levels of ceramide were attributed to low efficiency of transfection and potent toxicity of E4orf4 protein to cells that express it. Therefore, it was reasoned that developing an inducible expression system would solve both of these shortcomings of transient transfection as it allows the isolation of a clone of cells capable of expressing E4orf4 in a controlled fashion.

Initially, the Complete Control Inducible Mammalian Expression system from Stratagene was used. This inducible system includes two plasmids pERV3 and pEGSH vectors. The expression of E4orf4 (present in pEGSH) is induced upon the addition of 10 μ M of Ponasterone A 24 h post transfection into the cells.

However, this inducible system was not adequate for conditional expression of E4orf4 since it was very “leaky” leading to continuous expression of E4orf4 resulting in drastic death levels at 48 h post transfection. Therefore, another inducible system T-Rex[™] inducible system (Invitrogen) was chosen specially since it was well recommended by different studies and can be tightly controlled [141].

CHAPTER IV

MATERIALS AND METHODOLOGIES

A. Cell lines and cell culture:

The human lung adenocarcinoma epithelial cell line known as A549 cells were purchased from the American Type Culture Collection ATCC center (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Lonza) free of tetracycline and 1% penicillin (100 units/ml) – streptomycin antibiotics (Gibco). Cells were maintained in a humidified atmosphere containing 5% carbon dioxide at 37°C and passaged twice weekly by trypsinization (Gibco).

B. Expression vectors:

The T-Rex™ System (Invitrogen), a tetracycline-regulated mammalian expression system was used to express our gene of interest, *E4orf4*.

Three types of plasmid were used: pcDNA3-E4orf4, pcDNA™4/T0, pcDNA™6/TR.

➤ pcDNA™4/T0 consists of a complete CMV enhancer-promoter sequence containing two copies of the tetracycline operator TetO2 sequence for high-level regulated expression and a Zeocin™ resistance gene for effective selection of stable mammalian cell lines.

➤ pcDNA™6/TR encodes the Tet repressor (TetR) and expresses the blasticidin resistance gene for rapid selection of mammalian cell lines that stably express this TR plasmid.

The gene *E4orf4* was isolated from a pcDNA3 vector supplied by Dr. Philip Branton (McGill University, Canada). A fusion gene expressing E4orf4 and 2 tags: flag and a green fluorescent protein (GFP) was inserted into the plasmid pcDNA4/T0 .

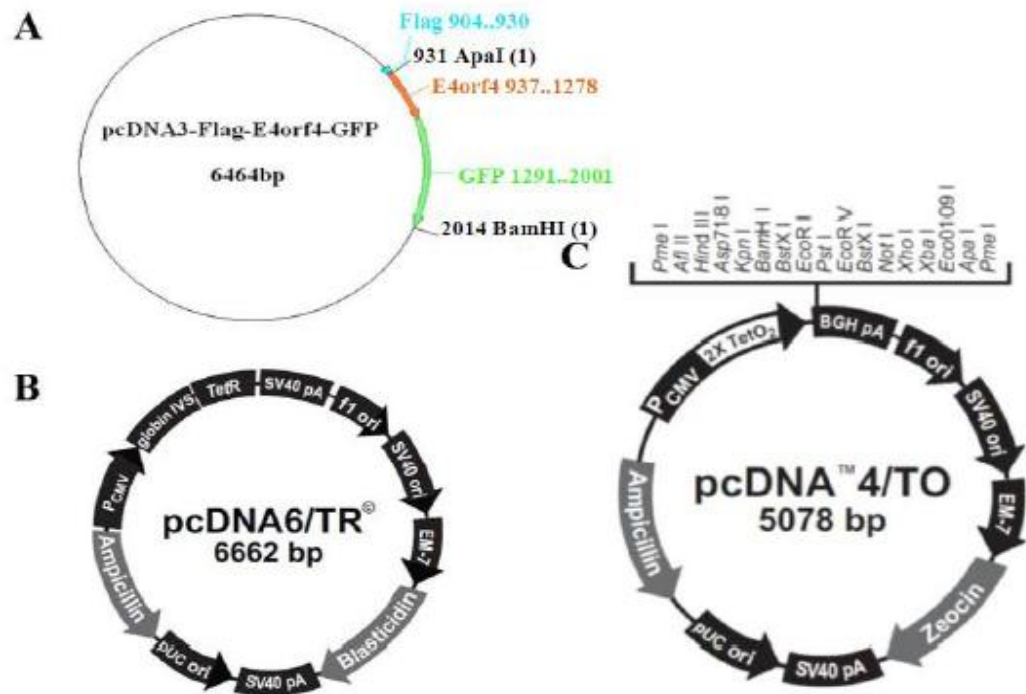


Figure 9: Map of the vector Flag-E4orf4-pcDNA3-GFP (A), the vector pcDNA6 / TR (B) and the vector pcDNA4 / T0 (C) (Invitrogen).

The expression of E4orf4 (present in pcDNA4/T0) is induced upon the addition of tetracycline (Tet) into the cells. The system is based on the fact that pcDNA6/TR expresses the TetR upon the transfection of pcDNA6/TR into the cell. TetR forms a homodimer that binds with extremely high affinity to TetO2 sequence in the promoter of the inducible expression vector, thereby repressing the transcription of the gene of interest. Upon addition, Tet binds with high affinity to TetR homodimers and causes a conformational change in the repressor that renders it unable to bind to the Tet operator.

The TetR-Tet complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest (Figure 10).

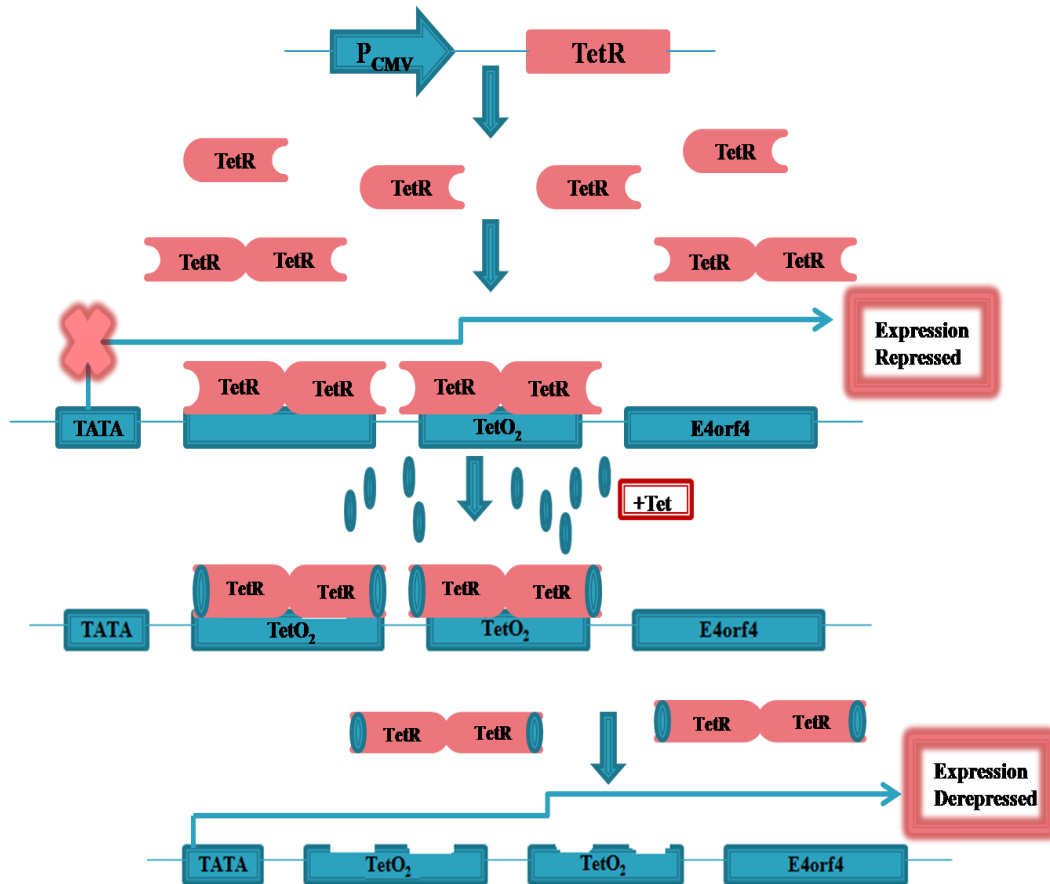


Figure 10: Regulation of transcription in the T-Rex™ system.

C. Transient cell line transfection.

Cells were seeded in a 6 well plate at a density of 500,000 cells/well. 24 hours post seeding, cells were transfected with 4 μ g DNA and 5 μ l lipofectamine 2000 reagent per well. The protocol includes adding DNA and the lipofectamine to 250 μ l opti-MEM (Gibco) reduced serum media each, incubating the mixture for 15-20 minutes at room temperature then adding the lipofectamine 2000 reagent (total volume become 500 μ l) and incubating for 15 minutes at room temperature. Meanwhile, the medium is discarded from the wells, cells were washed by phosphate buffer saline 1X (PBS1X, Gibco) then we added 1.5ml opti-MEM reduced media plus 0.5 ml of the DNA- lipofectamine 2000 mixture to each well. Finally, the medium was replaced by DMEM complete media after 5 hours. Cells were harvested after 20h and 24h post transfection. These experiments were done in duplicates.

D. Stable cell line transfection.

Cells were seeded in a T25 flask at a density of 1,000,000 cells. 24 hours post seeding, cells were transfected with 8 μ g DNA and 12.5 μ l lipofectamine 2000 reagent. The protocol includes adding DNA and the lipofectamine to 250 μ l opti-MEM (Gibco) reduced serum media each, incubating the mixture for 15-20 minutes at room temperature then adding the lipofectamine 2000 reagent (total volume become 500 μ l) and incubating for 15 minutes at room temperature. Meanwhile, the medium is discarded from the flask. We added 500 μ l opti-MEM reduced media plus 500 μ l of the DNA- lipofectamine 2000 mixture. The medium was then completed with 4ml DMEM complete medium after 5-6 hours. After 24 hours, the medium was replaced with DMEM complete media and cells

were selected with the adequate mammalian antibiotic 48 hours post-transfection, (10µg/ml blasticidin, and 250µg/ml zeocin).

E. Cell cloning:

Cells were seeded at a density of 1 cell/ well in a 96 well plate. In order to establish a single cell cloning, a serial dilution was performed by adding 100µl of cell suspension in each well. Then, cells were incubated at 37°C in a humidified CO₂ incubator. Clones were then checked every 4 to 5 days depending on the growth rate. Wells that contained a single clone were marked and transferred to a larger vessel (24 well plate) and then selected by the suitable antibiotics.

F. Reagents and antibodies:

The reagents and antibiotics that were used in our experiments included reagent A, B and S (Bio-Rad), penicillin 100units/ml (Gibco), streptomycin 100µg/ml (Gibco), protein ladder (Abcam), ampicillin 100mg/ml (Sigma), zeocin 100 mg/ml (Invitrogen), blasticidin 5mg/ml/ (Invitrogen) and Tet 1mg /ml (Invitrogen).

The primary monoclonal antibodies that were used include mouse anti-GFP monoclonal antibody (Invitrogen), mouse anti-GAPDH (glyceraldehydes 3-phosphate dehydrogenase) monoclonal antibody , rabbit anti-BAK, mouse-anti-Bcl2, rabbit anti-Bcl_{X_{S/L}}(Santa Cruz Biotechnology) , rabbit anti-PARP(Cell Signaling), Cytochrome C (Abcam) monoclonal antibodies The Secondary antibodies conjugated to horseradish peroxidase anti-mouse and anti-rabbit (Jackson) were used for western blot analysis.

G. Protein extraction and quantification:

Cells were seeded in a 6-well plate at a density of 5×10^5 cells/well. After scraping (at different time points), cells were centrifuged and resuspended in lysis buffer (0.25 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 2mg bromophenol blue) containing phosphatase and protease inhibitors. Samples were then boiled for 5 minutes at 95°C. The extracted proteins were quantified using Detergent Compatible Bio-Rad Protein Assay (Bio-Rad) where proteins were allowed to interact with reagent A (*alkaline copper tartrate solution*), reagent S then reagent B (*Folin's reagent*) leading to blue color development. The absorbance was read at 750nm on spectrophotometer (Elisa plate reader). The concentration of the proteins was determined with respect to a known protein standard concentration of bovine serum albumin (BSA, Amresco). Finally samples were conserved at -20°C.

H. Western blot:

Protein expression levels were analyzed using 12% or 10% acrylamide gel. The gel was prepared, placed in the apparatus and then filled with TGS 1X running buffer (Tris(hydroxymethyl)- aminomethane 30g, glycine 144g ,and SDS 10g, all supplied from Bio Rad). Samples were prepared using equal amounts of proteins versus loading (Tris-HCl 0.25M (pH6.8), SDS 4%, Glycerol 20% and bromophenol blue 2mg, 5% β -mercaptoethanol) and lysis buffers. Equal amounts of proteins were loaded on the gel and migration was allowed to take place at 70 volts (v) for the stacking gel then 120v for the resolving gel. Following migration, transfer to a polyvinylidene difluoride (PVDF) membrane was done in TGS 1X-20% methanol transfer buffer for 1hour and 30minutes at 100V. The membrane was blocked to prevent non-specific binding by using 5% milk-

TBS 1X (Tris(hydroxymethyl)-aminomethane 12g, 87.8g NaCl, 1L double distilled water (DDW), pH= 8). Following 2 h of blocking, the membrane was incubated with the specific primary antibody diluted in 5% milk- TBS 1X or 5% BSA-TBS 1X overnight at 4°C. The membrane was washed 3 times by TBS 1X-0.1% Tween for 10 minutes on a shaker before we added the HRP-conjugated secondary antibodies diluted in 5% milk-TBS 1X for about 1 hour. Finally the membrane was developed using ECL western blotting reagent (GE health care, UK) as a substrate. A sensitive sheet of photographic film (Agfa) is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. Bands were also imaged by ChemiDoc machine (Bio-Rad) .These experiments were done in duplicates.

I. RNA extraction and RT- PCR

Cells were seeded at a density of 5×10^5 cells/well then harvested post induction with Tet at different time points. Total RNA were extracted from cells using QIAGEN (RNAeasy plus Minikit) as recommended by the manufacturer. The extracted RNA undergoes a reverse transcription using the protocol Revert Aid First Strand c-DNA Synthesis Kit (K1622, Fermentas).

Real-time PCR was performed using 1Q SYBR Green supermix, forward and reverse primers for GAPDH and E4orf4.

The PCR was done using PerkinElmer Gene Amp 9700 PCR Thermal Cycler C1000 (Bio-Rad), by adding the 2 primers of E4orf4:

- The length of E4orf4 is 341 base pair (b.p)

E4.ORF4 - FWD1: 5'-CACTCTCTCAAAGCATCCAGG-3' 176 b.p- 196 b.p

E4.ORF4 – REV1: 5'-CTTATTCTGCGGTGGTGGATG-3' 272 b.p- 252b.p

J. Lipid extraction

Lipids were extracted according to the method of Bligh and Dyer in 1959 [66]. Briefly, cells were seeded at a density of 10^6 cells per well. The cells were then scraped into borosilicate glass tubes, pelleted at 1500 rpm for 10 minutes, washed once with ice cold PBS 1X and repelleted with the same conditions. After that, the cellular pellet was resuspended in 2ml methanol, 1 ml chloroform and left between 2-3 days for fixation at -80°C .

Next, the aqueous and the lipid phases were separated using methanol: chloroform: water ratio of (2.2:1.7) and centrifuged at 3000 rpm for 10 minutes at 4°C . The resulting lipid phase (lower phase) of each sample was then recovered and transferred into a new glass tube where they will be dried using Speed Vac machine. Finally, the invisible pellet was resuspended in 1 ml chloroform and $200\mu\text{l}$ are aliquoted for lipid phosphate determination by the phosphate assay.

K. Phospholipids phosphate assay

Phosphates were measured based on the method of Rouser et al. in 1970 [67]. Lipid aliquots taken after lipid extraction were used to determine the phosphate concentration found initially in the samples. The aliquots were dried to remove the chloroform before adding perchloric acid (70%). The reaction was allowed to proceed for 1 hour at 180°C . After that, the samples were cooled and $830\mu\text{l}$ of water was added per sample. This was followed by adding $170\mu\text{l}$ of ammonium molybdate (2.5%) and $170\mu\text{l}$ of freshly prepared ascorbic acid (10%). The mixture was incubated in a water bath at 50°C then resulting absorbance was read on a spectrophotometer at 820nm. The

concentration of the lipids was determined with respect to a known lipid standards concentration of di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4).

L. Ceramide assay

Ceramide assay was done according to a modified version of diacylglycerol assay [152]. First, 200 μl of lipid samples were dried, and then 20 μl of micelles (made of octyl- β -D-glucoside/dioleoyl phosphatidylglycerol) were added per sample. The tubes were sonicated for 30 minutes in a water bath sonicator. After that, we prepared the reaction mixture made of 50 μl 2x buffer (100mM Imidazole HCl pH=6.6, 100mM LiCl, 25mM MgCl_2 , 2mM EGTA), 0.2 μl of 1M DTT (dithiothreitol), 5 μg of Diacylglycerol kinase enzyme (DGK, prepared by Dr. G. Dbaibo from *E. Coli*) and dilution buffer (10mM imidazole of pH=6.6 and 1mM diethylenetriaminepentaacetic acid pH= 7). Seventy micro liters of the mixture was added per sample, then the reaction was initiated by adding 10 μl of the ATP mixture made of 2.5mM cold ATP (non-radioactive), 1.3 μCi of the [γ - ^{32}P] ATP solution, and water. The reaction was allowed to proceed for 30 minutes after which it was stopped by the addition of 2ml methanol, 1ml chloroform and 0.7ml water. The tubes were allowed to rest for 10 minutes. Then lipids were extracted by adding 1ml chloroform and 1ml water. The lipid phase was transferred to a clean glass tube and dried. Lipids were suspended in a 50 μl of chloroform: methanol mixture of ratio 9:1. Twenty five micro liters of each sample was spotted on a lane of a silica gel thin layer chromatography plate. The plates were placed in a previously prepared elution chamber filled with elution mixture made of chloroform: acetone: methanol glacial acetic acid: water in a ratio of 50:20:15:10:5. Samples migrated for 2 hours then exposed to x-ray film overnight at -70°C . Areas of radioactivity corresponding to the ceramide phosphate band were identified by comparing to a known ceramide standards and scraped

into scintillation vials containing 4 ml of scintillation fluid was added. The radioactivity was read using a liquid scintillation counter. These experiments were done in triplicates.

M. Cell cycle analysis by Flow Cytometry

Cells were seeded at a density of 10^6 cells / well. After trypsinization (at different time points), and centrifugation at 2000 rpm for 5 min at 4°C , cells were fixed by ethanol 70% and stocked at -20°C. On the day of flow, cells were centrifuged under the same conditions, the resulting pellet was resuspended in 800 µl of propidium iodide (PI) (Sigma), an intercalating agent of nucleic acids (5% PI-PBS 1X with RNase 2 µl / ml) for one hour at 4 ° C. Then the cells were analyzed (10,000 events) by flow cytometry (FACS Sorter-Beckman).

CHAPTER V

RESULTS

1. Establishment of inducible expression of *E4orf4* in A549 cell line

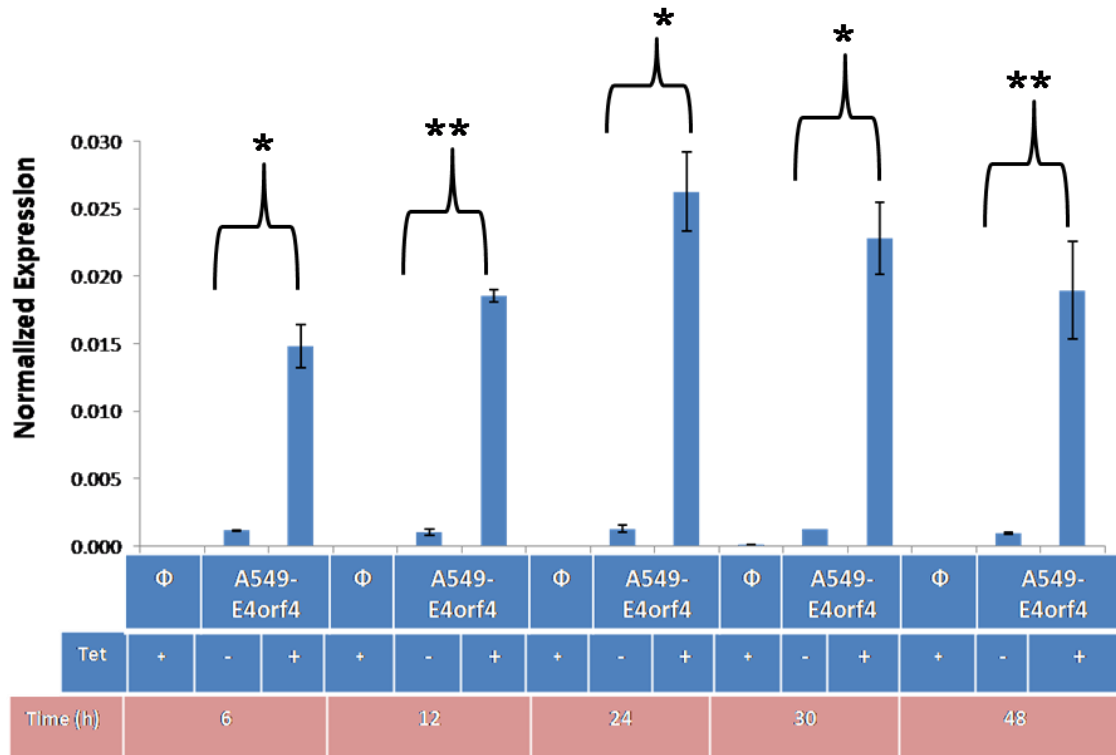
A549 cells were co-transfected with pcDNATM4/T0 and pcDNATM6/TR. Stable transfectants were established by continuous selection with antibiotics, namely zeocin and blasticidin which are selecting agents for cells incorporating pcDNATM4/T0 and cells having the regulatory plasmid pcDNATM6/TR, respectively.

From these stable transfectants, several clones were isolated. A549-E4orf4 was selected showing a significant and reproducible increase in *E4orf4* expression upon tetracycline (Tet) induction (+ Tet) in comparison to the cells transfected with a plasmid containing E4orf4 without induction with Tet (-Tet) as well as cells transfected with an empty plasmid (A549- Φ).

a. Gene expression analysis of E4orf4 by quantitative real time-PCR

The expression of *E4orf4* in A549-E4orf4 cells was analyzed upon induction with Tet (+Tet) (2 μ g/ml) and compared to two negative controls, which are A549-E4orf4 cells without induction with tetracycline (-Tet) and A549- Φ cells. Data were normalized to GAPDH levels.

A



B

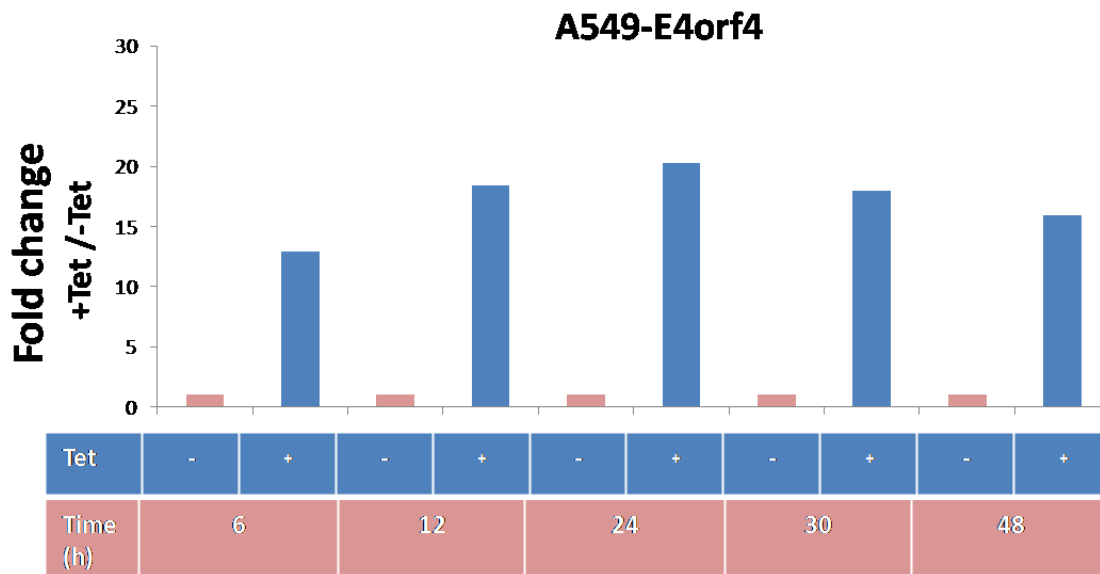


Figure 11: *E4orf4* gene expression in A549-E4orf4. (A) *E4orf4* expression in A549-E4orf4 cells induced with Tet for 6, 12, 24, 30 and 48 hours (h) as compared to non-induced cells or cells transfected with empty plasmid A549- Φ cells. The asterisks *, **, indicate statistically significant differences at $p < 0.05$; $p < 0.01$ respectively. (B) Fold change increase of *E4orf4* expression in A549-E4orf4 cells upon treatment with Tet. Cells were treated with $2\mu\text{g/ml}$ of (Tet) and harvested at the indicated time points. Mean normalized expression of *E4orf4* is relative to GAPDH expression level. Data is representative of 3 independent experiments.

Comparing cells transfected with an empty plasmid (A549- Φ), those non-induced with Tet (-Tet) and those induced with Tet (+Tet) after 6, 12, 24, 30 and 48 h, we observed a significant increase in the expression of *E4orf4* in A549-E4orf4 cells after induction with Tet (Figure 11A). *E4orf4* expression peaks at 24 h with 20 folds increase upon treatment with Tet. However, this expression decreased by 5 folds after 48 h of induction (Figure 11B). The representative bar graphs are shown as the mean \pm SD of duplicates. These results validate the proper functioning of the inducible T-Rex TM System in A549-E4orf4 cells.

b. E4orf4 protein expression

We assessed the expression pattern of E4orf4 in A549-E4orf4 cells for up to 48 h. Because specific E4orf4 antibody is not commercially available, we used the anti-GFP antibody to detect E4orf4 since E4orf4 is tagged to GFP (refer to materials and methods section). Anti-GAPDH was used as a house-keeping gene (Figure 12).

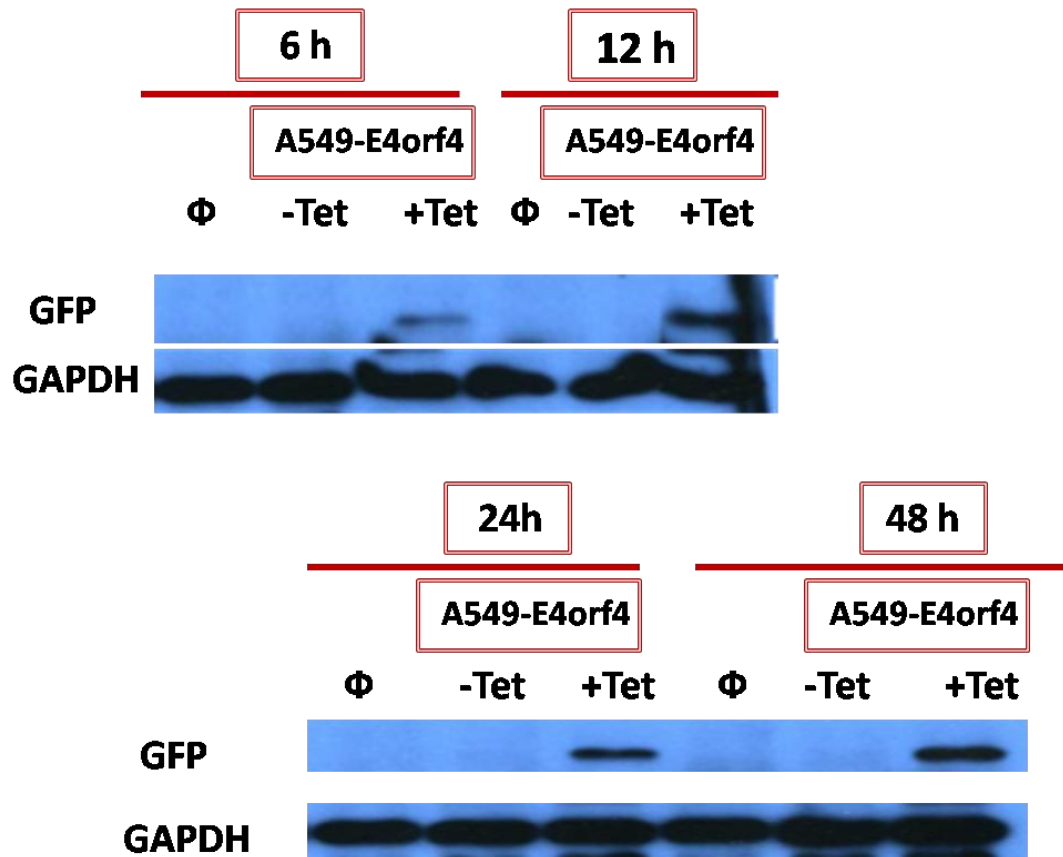
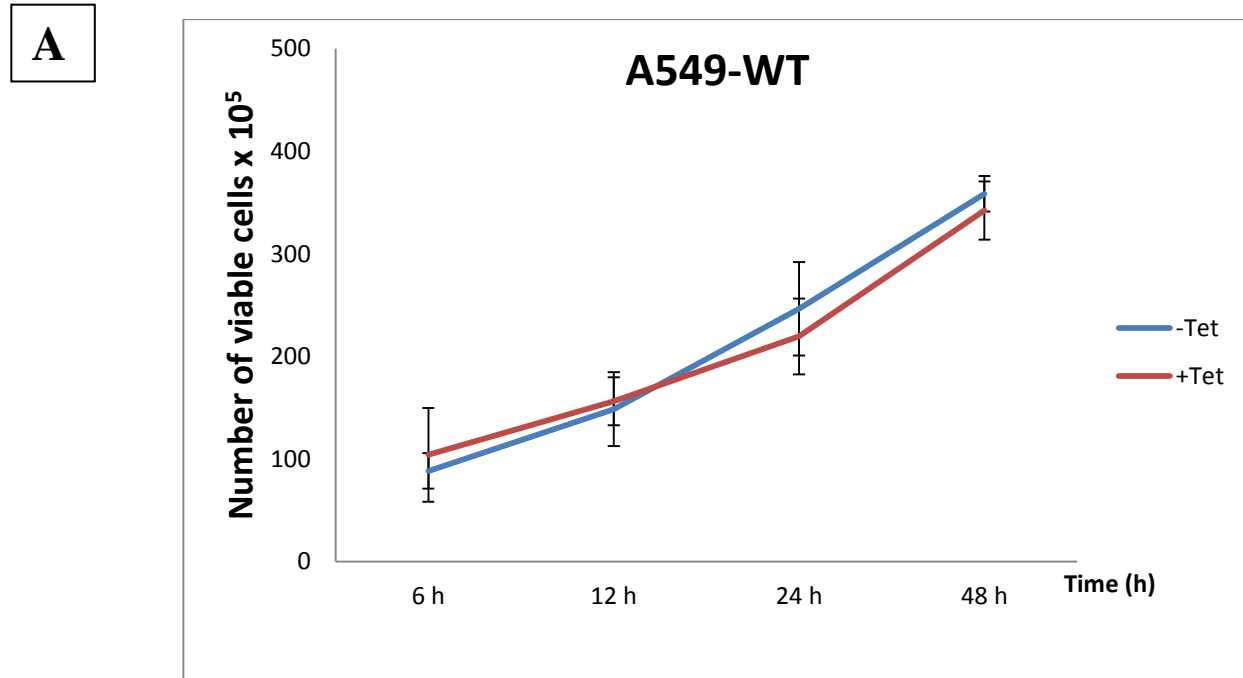


Figure 12: GFP expression in A549-E4orf4 cells and A549- Φ cells. Expression levels of E4orf4 protein by Western blotting using anti-GFP antibody, on A549- Φ and A549 – E4orf4 with or without induction by Tet at different time points. Results were normalized to GAPDH levels.

These results showed that there is a time-dependent increase in E4orf4 expression (Figure 12). In concordance with genomic expression of *E4orf4*, this expression decays after 48 h post induction. This decay may be probably due to the loss of (Tet) effect overtime. Therefore, we re-induced cells at 24 h and then these cells were harvested at 30 h where E4orf4 expression increased again at the protein level as well as at the genomic level (data not shown).

2. Tetracycline is not toxic to A549 cells:

Preliminary data revealed that 2 $\mu\text{g/ml}$ of Tet is the optimal concentration required to induce *E4orf4* and is not toxic to A549 cells. In order to determine the effect of our gene of interest and to verify that the resulting effect is not due to the drug itself or the effect of transfection with empty vector, we performed Trypan Blue assay on A549-WT cells in the presence and in the absence of Tet in order to determine the effect of treatment on cell viability for up to 48 h. As shown in Figure 13A, the number of cells without treatment is almost the same as treated cells. Similarly, tetracycline was not found to affect cell viability in A549- Φ (Figure 13B). Based on these results we were able to study the effect of E4orf4 alone on cell viability using the T-RexTM inducible system.



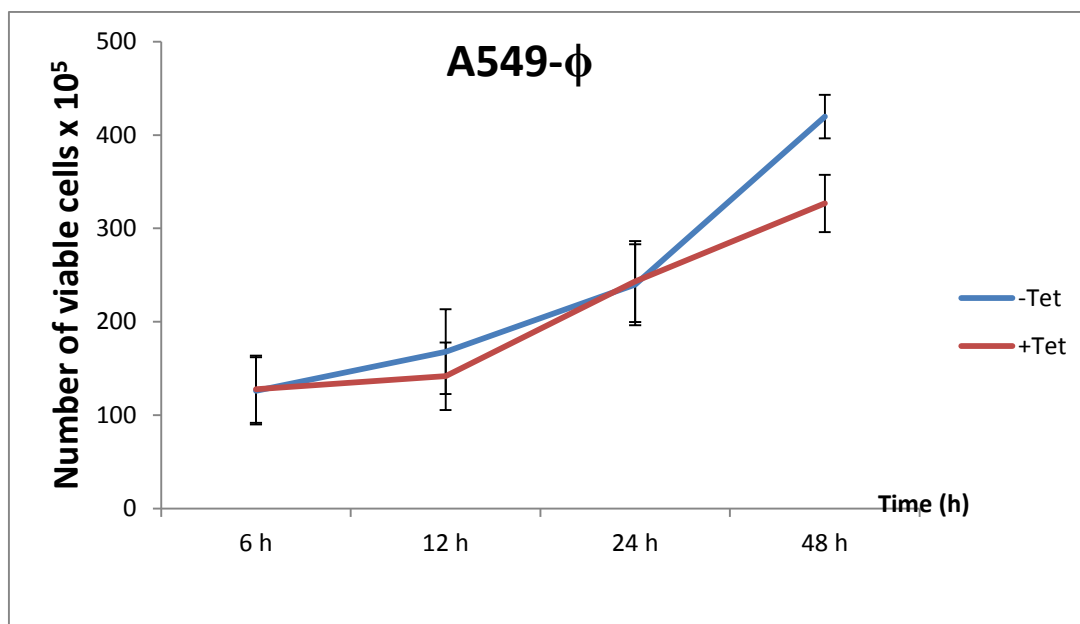
B

Figure 13: Cell viability of A549 cells by Trypan Blue assay. (A) Number of A549-WT cells for up to 48 h in the presence of Tet (+Tet) and in the absence of Tet (-Tet). (B) Cell viability in A549- Φ cells before and after induction with Tet at the above mentioned time points. These figures illustrate the number of viable cells $\times 10^5$. This experiment is performed twice in duplicates.

3. E4orf4 expression does not affect the viability of A549 cells

In order to determine the effect of E4orf4 on cell viability, we performed Trypan Blue assay at different time points pre (-Tet) and post (+Tet) induction with Tet. As shown in Figure 13C there was no significant difference in cell number upon treatment with Tet. Instead, there was a slight increase in cell number after 48 h of induction. Moreover, a negligible number of dead cells was observed within the 48 h of Tet induction (data not shown). These results revealed that E4orf4 expression does not affect cell viability in our model.

We then investigated whether apoptosis occurred in response to E4orf4 expression by assaying several markers of apoptosis. We checked for caspase 3 and caspase 9 activation in our model. These are executioner proteases with an important role in apoptosis that are activated by their proteolytic cleavage. Results showed that there was neither caspase 3 nor caspase 9 cleavage at the above mentioned time point (data not shown). Furthermore, we checked for PARP cleavage (poly ADP ribose polymerase), which is also an indicator of apoptosis and results showed that there was no PARP cleavage following E4orf4 expression for up to 48 h (Figure 13 D). Therefore, we ruled out the possibility that E4orf4 can induce the classical form of apoptosis in A549 cells.

C

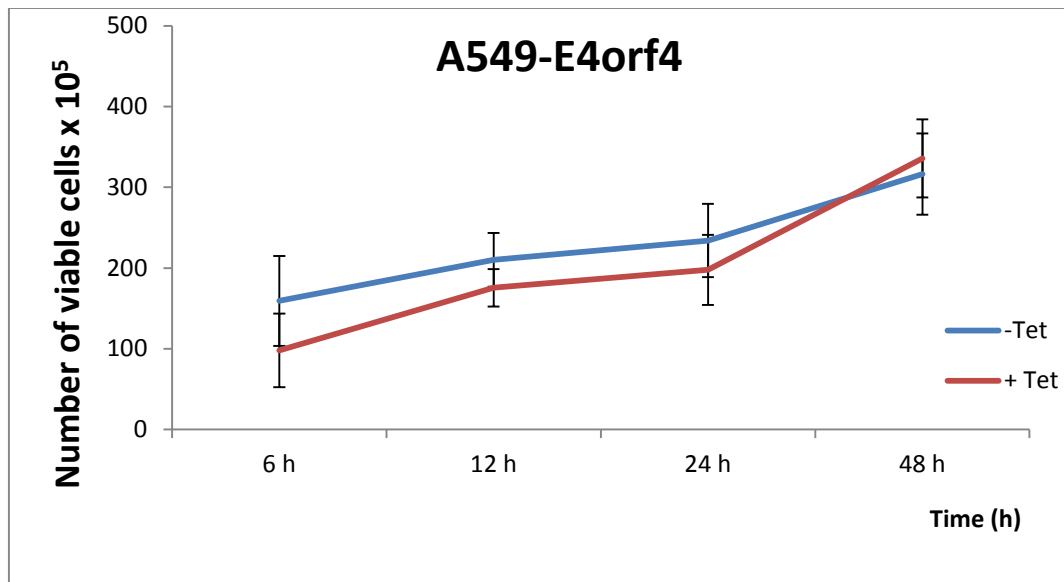


Figure 13C: Cell viability by Trypan Blue assay in A549-E4orf4 cells in the presence or absence of Tet. Cells were harvested at 6, 12, 24 and 48 h and Trypan Blue assay was performed before and after treatment with Tet. This figure shows the number of viable cells x10⁵. This experiment is representative of two independent experiments done in duplicates.

D

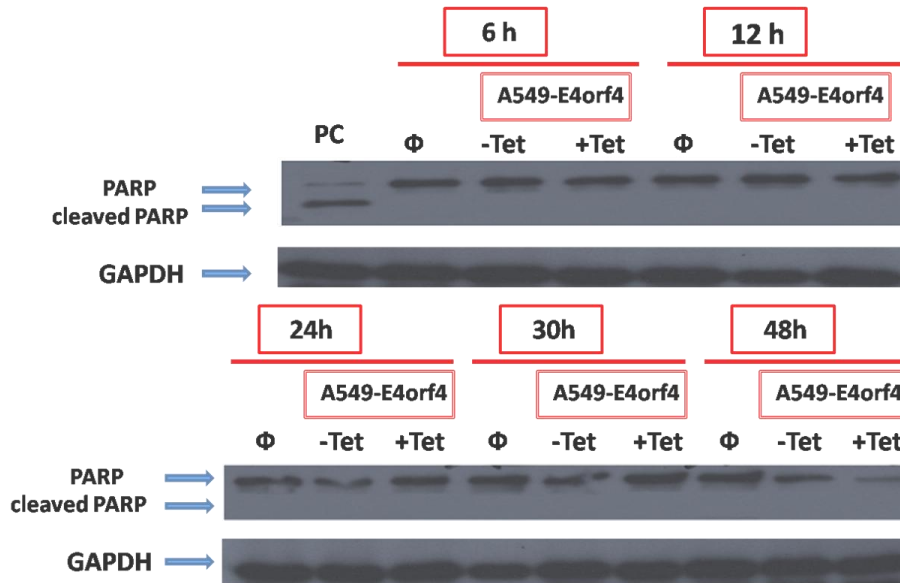


Figure 13 D: No PARP cleavage in A549-E4orf4 cells and in A549- Φ cells for up to 48 h as compared to a positive control (PC). Cells were collected at the indicated time points before (-Tet) and after (+Tet) treatment with Tet. Whole cell lysate of 50 μ g of proteins were loaded and 10% SDS-PAGE gel was performed. PC (γ - irradiated molt 4 cells harvested after 24 h). The two diagrams show uncleaved PARP (upper panel) of 116 kDa size and cleaved PARP (lower panel) of 89 kDa size. Data shown is a representative of two independent experiments.

4. INDUCED E4ORF4 DOES NOT INCREASE CERAMIDE LEVELS IN A549 CELLS:

In order to investigate the effect of E4orf4 protein on ceramide levels, A549-E4orf4 cells were seeded then harvested at 6, 12 and 24 h post-induction with Tet. We chose to investigate the levels of ceramide at these time points specifically since we have demonstrated that E4orf4 have the best expression at 24 h post-induction. Lipids were extracted and ceramide levels were measured by (DGK) assay as described in materials and methods. In order to compare the amounts of ceramide found in the samples at different time points, the measured ceramide amounts that were normalized to the amount of total lipid phosphate in each sample.

Our results showed that Tet had no effect on ceramide levels in A549- Φ cells as well as on A549-E4orf4 cells (Figure 14 A) indicating that inducing E4orf4 expression in the latter did not induce ceramide accumulation. However, as seen in Figure 14B, we observed that the baseline levels of ceramide at all time points were significantly lower in A549-E4orf4 cells irrespective of Tet induction when compared to those observed in A549- Φ cells. Several publications revealed that *E4orf4* induces cell cycle arrest in many cell lines. Therefore, we decided to investigate its effect on the cell cycle progression in our model.

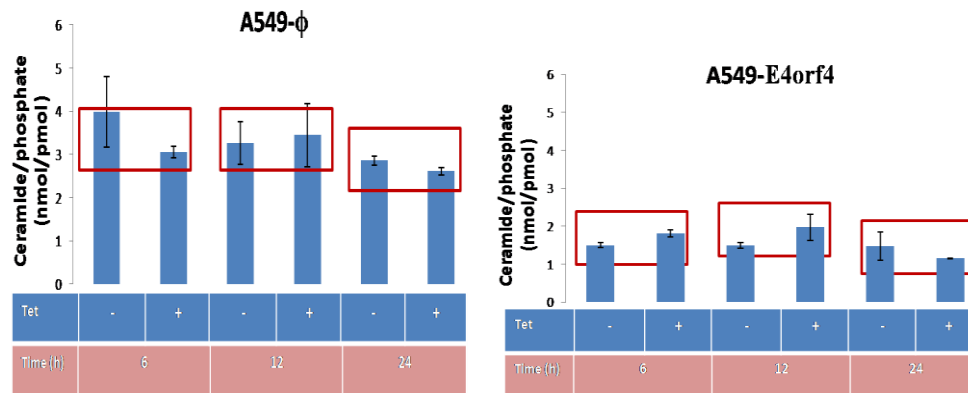
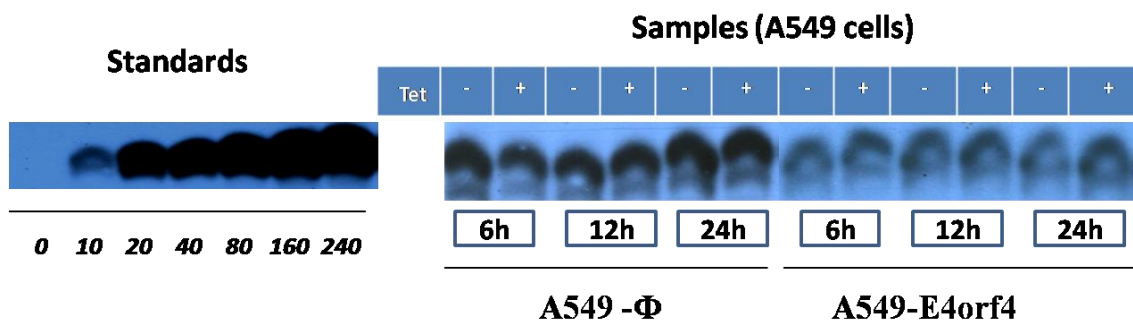
A**B**

Figure 14: Ceramide levels in A549-E4orf4 cells and A549- Φ cells before and after treatment with Tet. Cells seeded at a density of 10^6 /well were collected after 6, 12 and 24 h pre and post induction with Tet. Then, ceramide levels were measured by DGK assay. Ceramide values were normalized to lipid phosphates (A) Histograms showing ceramide levels in A549- Φ cells and A549-E4orf4 cells before and after induction with Tet at the indicated time points. (B) Representative X-ray film showing ceramide levels before and after induction with Tet in A549- Φ cells as well as A549-E4orf4 cells. Data shown are representative of three independent experiments, each done in duplicates.

5. Increase in S Phase and cyclin A in A549-E4orf4 cells

For the cell cycle analysis, both adherent and floating cells were combined and prepared for analysis by flow cytometry. As shown in Figure 15A we observed a significant increase in the percentage of cells in the S phase in A549-E4orf4 cells (around 13%) after 6, 12 and 24 h as compared to A549- Φ cells. On the other hand, there was no significant difference in the percentage of cells in the S phase between untreated and treated -A549-E4orf4 cells at the indicated time points. This indicated that the increase might be either due to the defect in DNA replication or a delay in the exit from the S phase.

Since cyclins and CDKs are important regulators of the cell cycle, and we found an increase in the S phase in A549-E4orf4 cells with respect to A549- Φ cells, we checked for the levels of cyclin A, which is an important promoter for the progression into the S phase.

As shown in Figure 15 B, cyclin A expression in A549-E4orf4 cells increased after 6, 12 and 24 h as compared to A549- Φ cells. The presence of high levels of cyclin A in E4orf4-expressing cells correlates with the increase of S phase in these cells. Additionally, this increase in the S phase and cyclin A followed the same trend in A549-E4orf4 cells before and after induction with Tet.

Figure 15A shows a slight increase in the S phase after 6 h and 12 h in A549-E4orf4 cells treated with Tet as compared to untreated A549-E4orf4 cells. Similarly, cyclin A expression levels were higher in A549-E4orf4 cells after induction with Tet (+Tet) at these indicated time points as compared to untreated cells (-Tet) (Figure 15 B).

Moreover, after 24 h both the percentage of cells in the S phase and the cyclin A expression in A549-E4orf4 cells were almost the same in Tet-treated cells as compared to the untreated cells. Along with the ceramide data above, these findings suggested that the A549-E4orf4 cells were inherently different from the A549- Φ cells.

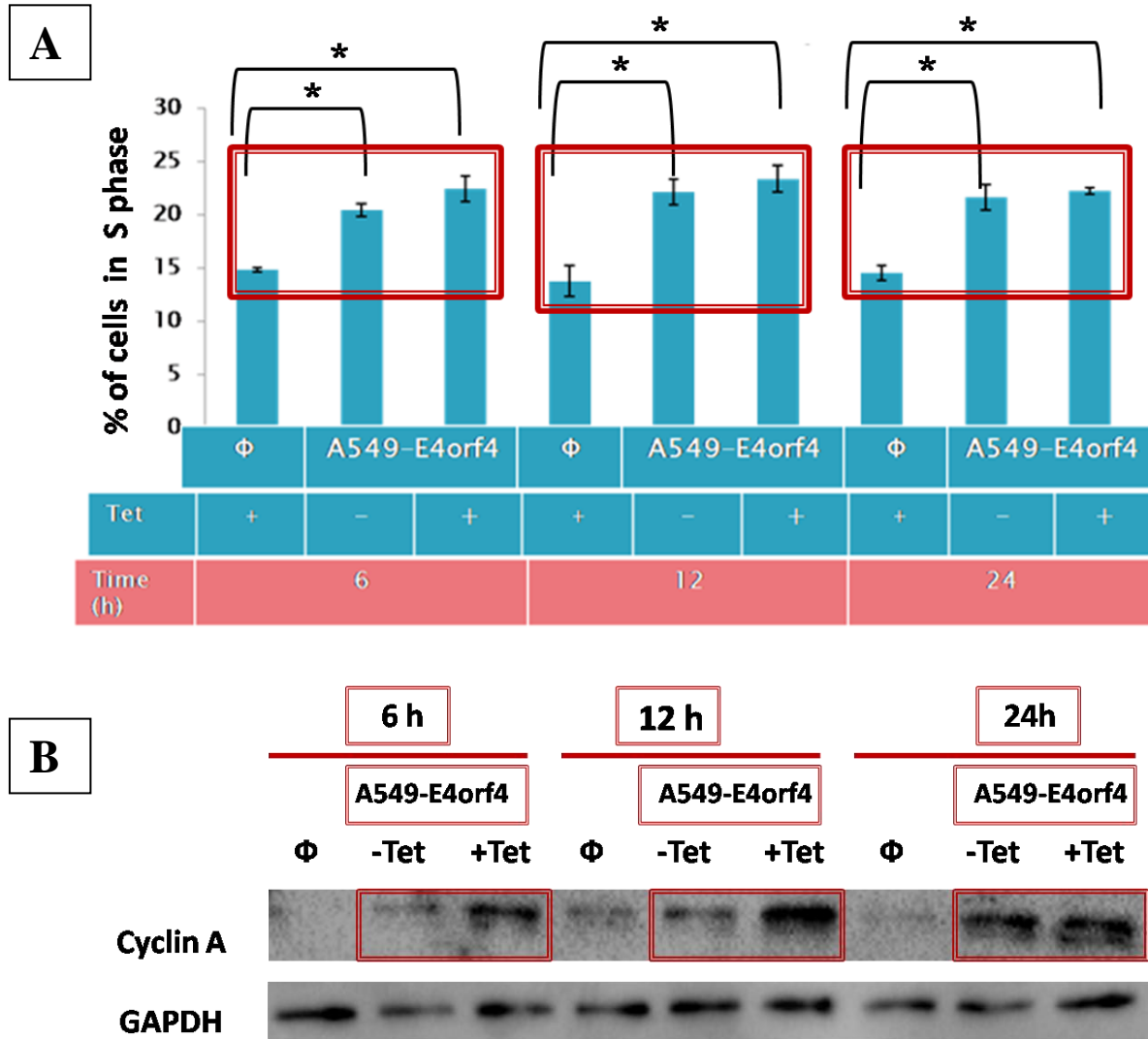


Figure 15: Cell cycle analysis and cyclin A levels in A549-E4orf4 cells and A549- Φ cells before and after induction with Tet. (A) The percentage of cells in the S phase of the cell cycle. Cells were harvested at 6, 12 and 24 h, fixed in 70% ethanol, and stained with propidium iodide (PI) for FACS analysis of DNA content. The asterisk * indicate statistically significant differences at $p < 0.01$. Data are representative of three independent experiments performed in duplicates. (B) Expression of cyclin A in A549-E4orf4 cells and A549- Φ cells. After treatment with Tet, A549-E4orf4 cells as well as A549- Φ cells significantly increased cyclin A levels at 6, 12 and 24 h. Whole cell lysate of 50 μ g of A549-E4orf4 cells as well as A549- Φ cells were determined by Western blot analysis. GAPDH was used as a loading control. Data shown is a representative of two independent experiments.

6. Baseline E4orf4 expression induces BCL2 upregulation and BAK downregulation

The surprising lack of apoptosis and ceramide accumulation in response to E4orf4 induction in our system were puzzling. The lower levels of baseline (i.e. without Tet induction) ceramide and higher levels of baseline cyclin A in the A549-E4orf4 cells compared to the A549- Φ cells suggested to us that the cloned A549-E4orf4 were selected due to inherent properties that allowed them to survive even the small amounts of E4orf4 expression that are inevitable in the “leaky” inducible expression systems. Therefore, we decided to characterize these cells further, specifically, check for pro-apoptotic and anti-apoptotic protein levels in order to observe what is happening at the mitochondrial level in relation to ceramide levels.

Our results revealed a significant increase in BCL2 expression in A549-E4orf4 cells irrespective of treatment with Tet as compared to negative controls (A549- Φ cells) at the indicated time points. This expression peaks after 24 and 48 h in these cells while no expression of BCL2 was detected in A549- Φ cells (Figure 16A). The upregulation of BCL2 expression was accompanied by an inhibition of BAK expression in A549-E4orf4 cells as compared to A549-WT cells as well as A549- Φ cells that significantly express BAK and do not express BCL2 (Figures 16A and 16B).

Moreover, the proapoptotic gene BCLX_{s1} was downregulated in A549-E4orf4 cells as compared to A549-WT cells as well as A549- Φ cells at the studied time points (Figure 17A). The overall expression of Cytochrome C in a whole cell lysate was found to be downregulated in A549-E4orf4 cells as compared to its controls (Figure 17B).

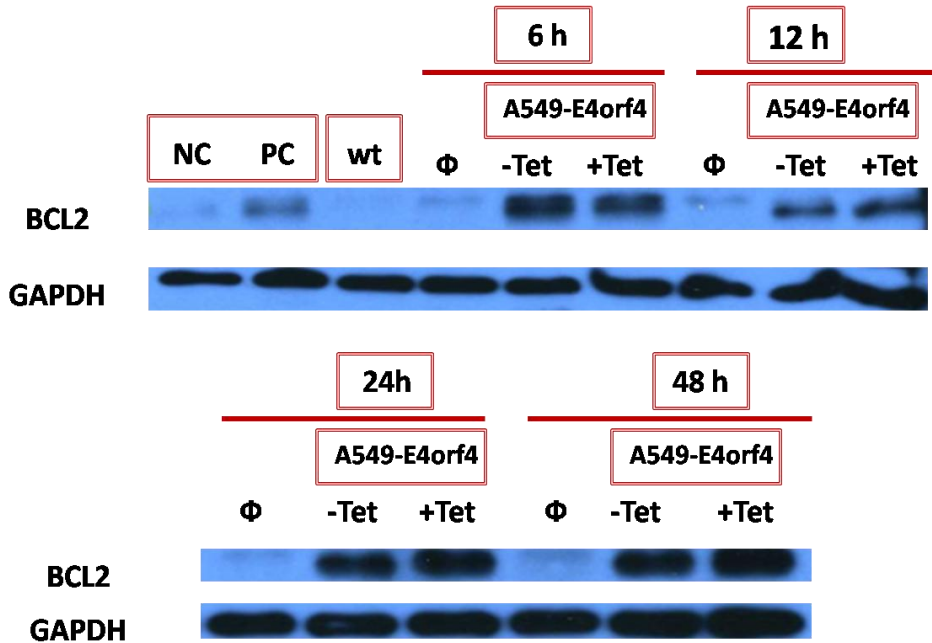
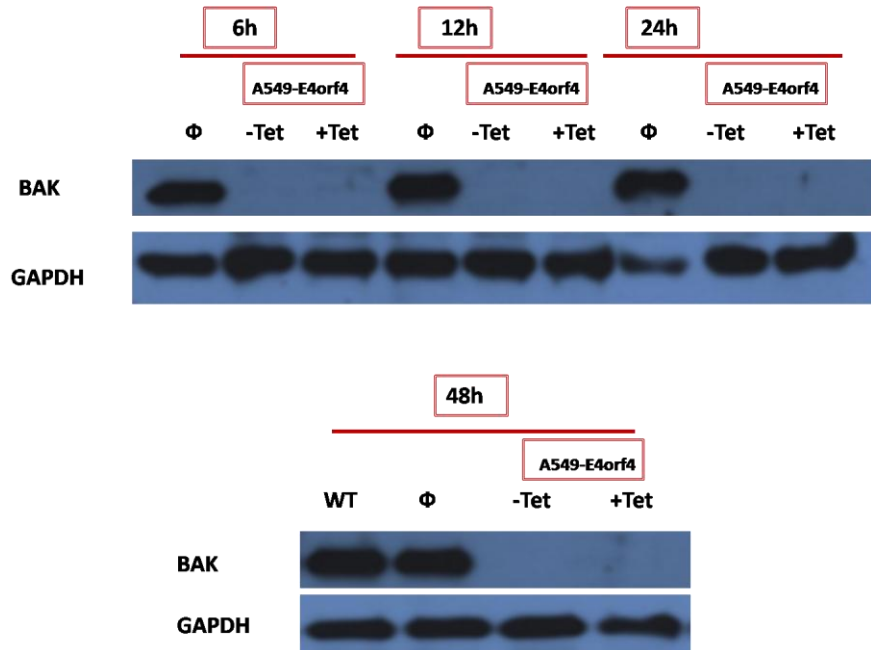
A**B**

Figure 16: BCL2 and BAK expression levels in A549-E4orf4 cells and A549- Φ cells. Cells induced with Tet were harvested before and after induction, 50 μg of whole cell lysate were loaded on 12% SDS-PAGE gel. (A) BCL2 expression in A549-E4orf4 cells compared to A549-WT cells and A549- Φ cells. PC and NC represent γ -irradiated molt 4 cells that are positive and negative to anti-BCL2, respectively. (B) BAK expression in A549-WT cells, A549- Φ cells and A549-E4orf4 cells. GAPDH was used as a loading control. Data are representative of two independent experiments.

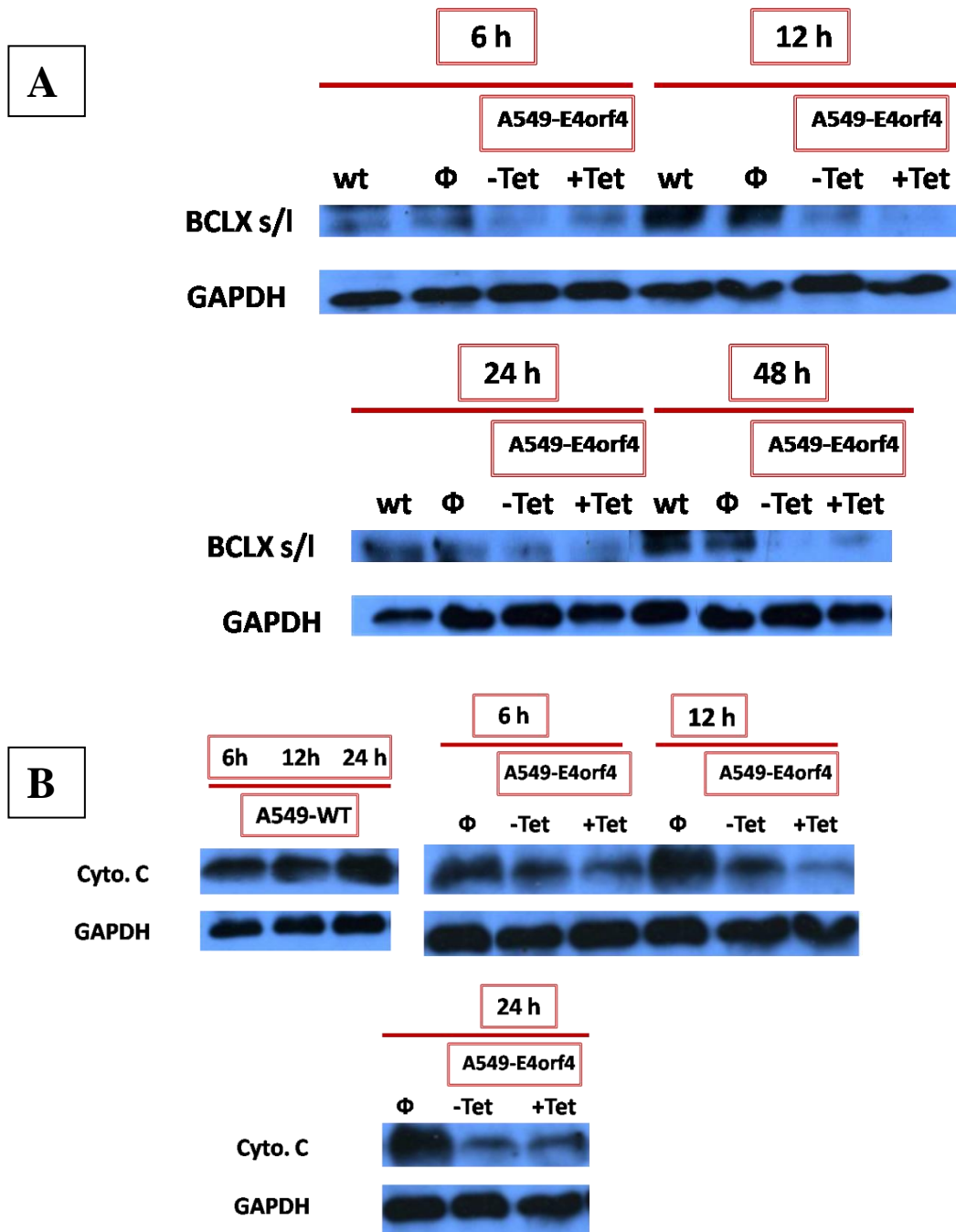


Figure 17: E4orf4 triggers downregulation of BCLX *s/l* and Cytochrome C levels in A549-E4orf4 cells. Cells induced with Tet were harvested before and after induction, 50µg of whole cell lysate were loaded on 12% SDS-PAGE gel. (A) Expression levels of BCLX *s/l* in A549-E4orf4 cells before and after induction with Tet as compared to A549-Φ cells and A549-WT cells at the indicated time points. (B) Cytochrome C levels in A549-E4orf4 cells before and after induction with Tet as compared to A549-Φ and A549-WT cells after 6, 12 and 24 h. GAPDH was used as a loading control. Data are representative of two independent experiments.

B. Transient expression of E4orf4

1. E4orf4 doesn't alter BAK and BCL2 expression

In order to compare the transient effect of E4orf4 in A549 cells with the stable ones, we transiently transfected cells with E4orf4 (A549-E4orf4) or empty vector (A549- Φ). Then, the cells were collected at 6, 12, 24 and 48 h post-transfection. As shown in Figure 16A, the expression of GFP increases gradually with time in A549-E4orf4 cells and reaches its maximum at 48 h post-transfection as compared to A549-WT cells as well as A549- Φ cells. GAPDH was used to confirm the equal loading of the protein samples.

Unlike the case when we inducibly expressed E4orf4 in A549 cells, BAK expression was highly expressed in A549-WT cells and A549- Φ cells as well as in A549-E4orf4 cells during the 48 h of transfection (Figure 16A). Moreover, BCL2 expression was almost absent in transient transfectants as compared to a positive control (PC) (Figure 16B). Therefore, transient expression of E4orf4 in A549 cells does not alter the normal expression of BAK and BCL2.

Cell cycle analysis revealed no significant difference in the different stages of cell cycle in A549-E4orf4 cells as compared to A549- Φ cells. Also, the significant increase in S phase that was present in the inducible expression system having E4orf4 was absent in the transient ones (data not shown).

In concordance with the data using an inducible system, Trypan Blue assay revealed no significant difference in cell viability in A549-E4orf4 cells as compared to A549- Φ cells. The number of A549-WT cells was slightly higher than the cells transfected with either an empty vector (A549- Φ) or Flag-E4orf4-GFP-pcDNA3-vector (A549-E4orf4) . This may be due to the effect of transfection, since there was no difference in the survival of cells between A549- Φ cells and A549-E4orf4 cells (data not shown).

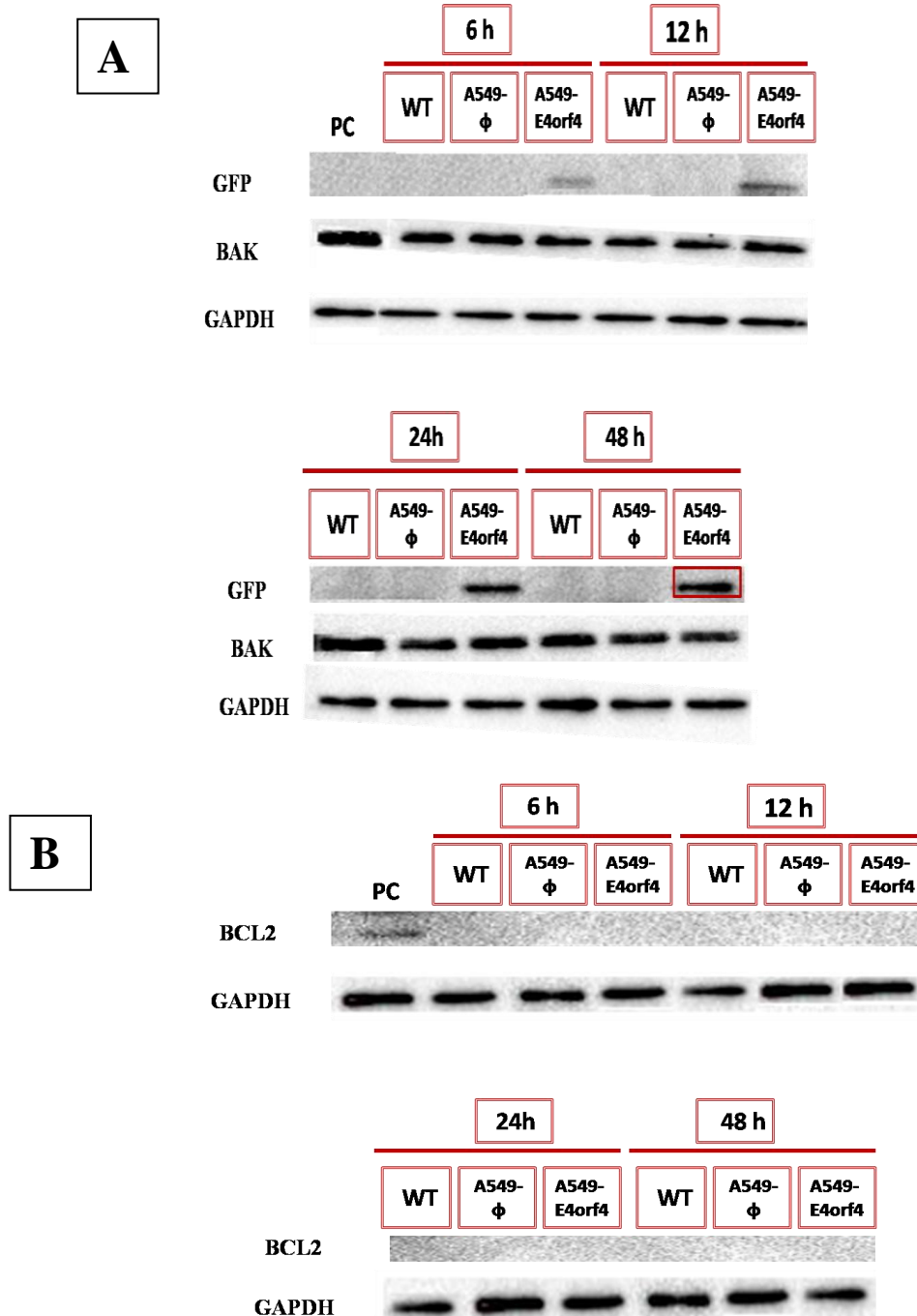


Figure 18: GFP, BAK and BCL2 expression levels in transiently transfected cells. Cells were harvested at 6, 12, 24 and 48 h post transfection, 50 μ g of whole cell lysate was loaded on 12% SDS-PAGE gel. (A) GFP and BAK expression levels in A549-E4orf4 cells, A549-WT cells and A549- Φ cells. (B) Expression levels of BCL2 in A549-WT cells, A549- Φ cells and A549-E4orf4 cells. PC represents A549 cells that are positive to anti-BAK and anti-BCL2, respectively. GAPDH was used as a loading control. Data are representative of two independent experiments.

2. E4orf4 induces ceramide accumulation in transiently transfected A549 cells

In order to investigate the effect of E4orf4 protein on ceramide levels in transiently transfected A549 cells (A549-E4orf4), cells were seeded then harvested at 6, 12 and 24 h post-transfection. We chose to examine the levels of ceramide at these time points specifically in order to compare the effect of E4orf4 in transient transfectants with the results obtained when we used the inducible expression system. Lipids were extracted and ceramide levels were measured by (DGK) assay. The measured ceramide amounts were normalized to the amount of total phosphate in each sample.

Our results showed that ceramide levels increased by around 3 folds after 6 h of post transfection in A549-E4orf4 cells as compared to the control A549- Φ cells. This accumulation of ceramide decreased after 12 h post transfection in pcDNA-E4orf4 cells and but went up again after 24 h post-transfection (Figure 19).

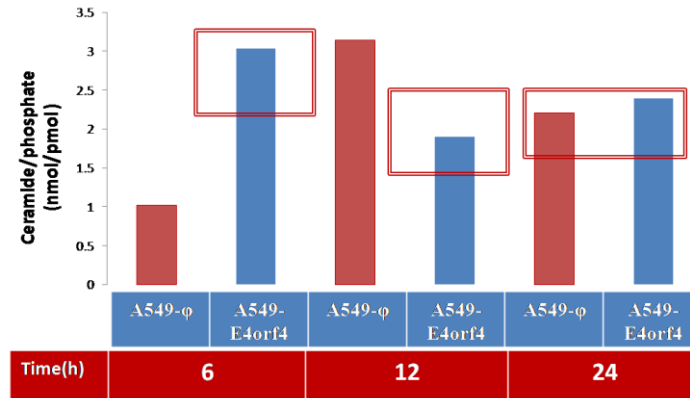
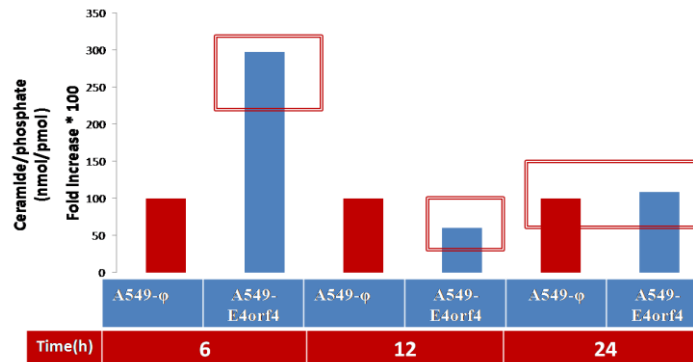
A**B**

Figure 19: Ceramide levels in transiently transfected A549-E4orf4 cells and A549- Φ cells. Cells seeded at a density of 10^6 /well were collected after 6, 12 and 24 h post-transfection. Then, ceramide levels were measured by DGK assay. Ceramide values were normalized to lipid phosphates. (A) Histogram showing ceramide levels in A549- Φ cells and A549-E4orf4 cells after 6, 12 and 24 h post-transfection. (B) Histogram showing fold change of ceramide level in A549- Φ cells and A549-E4orf4 cells at the indicated time points. Data shown are representative of one experiment.

CHAPTER VII

DISCUSSION AND FUTURE PERSPECTIVES

Cancer develops when there is an imbalance between cell proliferation and cell death. In fact, defects in the genes that control cell death as well as those that regulate cell growth trigger the formation of malignant tumors [63]. Many cancer therapies induce p53-dependent cell death and prove ineffective in human tumors with defective, mutated or silent p53. Thus, a lot of research is being conducted to find therapies that act independently of p53 [54].

The adenoviral early gene *E4orf4* can overcome the above obstacles since it was described to kill a wide range of cancer cells independently of p53 [54]. Studies on *E4orf4*-induced cell death revealed several unique and interesting features of cell killing. The contribution of *E4orf4* to cell death differs in a cell line-dependent manner. *E4orf4* initiates the activation of the caspase-independent signaling in CHO cell line that can be linked to different cell death pathways, such as caspase-dependent apoptosis in H1299 and 293T cell lines or mitotic catastrophe-mediated necrosis in H1299 cells [51, 63, 153-154]. Furthermore, *E4orf4*, in collaboration with its major partners such as PP2A and Src kinases, induces several alterations in the nucleus and the cytoplasm, which result in nuclear condensation and cell death in CHO cells [63].

In our study, we have attempted to characterize additional signaling pathways that could potentially play a role in mediating the apoptotic effects of E4orf4, such as the ceramide pathway. Due to the previously described potent pro-apoptotic effects of E4orf4, we reasoned that controlled expression of E4orf4 would be desirable in order to allow experimental evaluation of its effects. Efforts in our laboratory to develop an inducible E4orf4 expression system extended over several years without success.

Transfection with vectors containing *E4orf4* always led to death of transfected cells with no cells surviving selection with specific antibiotics. When cells did survive, E4orf4 was always lacking after (and before) induction. We reasoned that the systems used were too leaky such that transfected cells were dying from the effects of E4orf4 even without induction because minimal E4orf4 expression was sufficient to kill transfected cells. When we finally were successful in developing an inducible expression system as described in the current work, this was reason for jubilation. However, as shown in the current work, our hypothesis that induction of E4orf4 expression would induce ceramide accumulation and apoptosis in this system did not pan out. Induced expression of E4orf4 in A549 cells did not have any effect on the cell growth as compared to the WT cell line as well as to those transfected with the empty vector. This result is not coherent with previously published studies done on E4orf4 that showed that E4orf4 induces cell death in HEK239 and H1299 cells over an extended period [64]. Two explanations are possible for our results. First, none of the previous studies were done on A549 cells, which are pulmonary epithelial cells that are natural host cells for adenovirus. Thus, A549 cells could possibly be resistant to E4orf4 effects and might be responding differently than other cell lines. Second, we used the tetracycline inducible system to express E4orf4 and not adenoviral vectors such as in other published studies [155]. Thus,

the mode of delivery of E4orf4 may have influenced our results. Moreover, some studies used adenoviral mutants lacking the complete E4 region as a negative control and compared it to another adenoviral mutant having only E4orf4 and not all the other open reading frames.

Furthermore, the expression of E4orf4 in our model increased the number of A549-E4orf4 cells in the S phase with no changes in the sub G0 or G2/M phases. This increase in the S phase was accompanied with an elevation of cyclin A, which is an important regulator of the S phase in the cell cycle. Importantly, the increase in S phase cells and in cyclin A was seen even in uninduced A549-E4orf4 cells but became more evident with induction with Tet. Therefore, these results may indicate that E4orf4 causes perturbations of the cell cycle as shown previously. For instance, E4orf4 induces cell cycle arrest at G2/M phase in HEK-293 cell line [142-143] and in yeast *S. cerevisiae* [142, 144] as well as G1 arrest of both tetraploid and diploid cells due to a failure to initiate DNA synthesis in H1299 cells [64].

In addition, we have shown that induced expression of E4orf4 did not accumulate ceramide in our system. In fact, we observed that the uninduced presence of E4orf4 decreases baseline ceramide levels compared to the A549-Ø cells by 2-3 folds. Similarly, when we developed a similar system in MCF7 cells, uninduced E4orf4 was shown to decrease ceramide levels in stably transfected MCF7 cells as compared to MCF7 cells transfected with an empty vector (data not shown).

The unexpected findings in our system with no measurable cell death, apoptosis, or increase in ceramide levels following induction of E4orf4 expression led us to investigate the reasons behind these surprising results. Clues to a possible explanation came from our observations that cells stably transfected with the E4orf4 vector concomitantly with the Tet-Repressor vector behaved differently from cells transfected with the empty vector concomitantly with the Tet-Repressor vector. We found that there was a significantly higher fraction of cells in the S phase, higher cyclin A levels, and significantly lower baseline ceramide levels in cells stably transfected with E4orf4, even without Tet induction, compared to cells transfected with empty vector. Coupled with our previous difficulties in developing the E4orf4 inducible expression system, we reasoned that our eventual success in establishing this system came at the cost of selecting for a subpopulation of A549 cells that were resistant to cell death induced even by the minute amounts of E4orf4 that were expressed, i.e. leaked, in our system in the absence of Tet induction. Therefore, we aimed to decipher the mechanism behind this survival. We examined the regulation of apoptosis-related proteins. Our study demonstrated for the first time that when stably expressing E4orf4 in A549 cells, even without Tet induction, there was a marked increase in the expression of the anti-apoptotic BCL2 protein, and a drastic decrease in the expression of the pro-apoptotic proteins such as BAK and BCLX_{s/L}. In our model, caspases 9 and 3 as well as PARP were not cleaved; additionally, the overall expression of cytochrome c was found to be downregulated in A549 cells. Thus, our cells that were finally successfully transfected with E4orf4 turned out to be a very specific population with very low apoptotic potential based on their high expression of BCL2, low expression of BAK, BCLX_{s/L}, and cytochrome C, and significantly lower levels of ceramide. This is consistent with findings showing that BCL2/BCL-X_L

protected cells against E4orf4 cytotoxicity when transfected in rodent fibroblasts such as CHO LR73 and HyA4 cells [63]. This is also consistent with other studies showing that defects in ceramide generation, e.g. cells expressing the Tax transgene, are resistant to chemotherapy-induced apoptosis.

To further confirm these results, we examined the effect of E4orf4 stable transfection without Tet induction in another clone from the same cell line that was also selected for its significant expression of E4orf4 upon Tet induction. Similarly, E4orf4 triggered downregulation of BAK and upregulation of BCL2 in cells transfected with E4orf4. Additionally, we tested the effect of E4orf4 in one clone of MCF7 since we already characterized MCF7 cell lines depending on E4orf4 expression levels. Likewise, MCF7 cells transfected with E4orf4 displayed a significant increase in BCL2 levels as compared to cells transfected with an empty vector. However, the only difference between the two different cell lines was that the expression of BCL2 was also high in MCF7-WT cells but not in A549-WT cells (data not shown).

Therefore, our results showed that the presence of E4orf4 in A549 cells using an inducible system modulates the balance between antiapoptotic and proapoptotic proteins of the BCL2 family as well as ceramide leading to cell survival. This may be a cell type-specific response or more likely due to the selection process implemented during the development of the inducible expression system where only cells capable of withstanding the toxic effects of low-level E4orf4 “leaky” expression can survive the selection process. Moreover, it may depend on the physiological state of the cells and their genetic or proteomic content.

In conclusion, the E4orf4 inducible expression system is not a valid system to study the apoptotic effects of E4orf4 or its effects on ceramide as the adaptive mechanisms acquired by the cell in order to withstand the long-term expression of even low levels of E4orf4 that leak through preclude the study of these relationships. However, this system could still be useful in the study of non-apoptotic functions of E4orf4.

Further experiments should be done to examine the role of other members of the BCL2 proteins such as BAD and BAX. In parallel, we will examine the effect of E4orf4 on ceramide levels in our stable transfectants by inhibiting BCL2 by using ABT-737, a Bcl-2 Homology 3 (BH3)-mimetic drug [156].

We will also follow another approach where we will be selecting a clone from our stable transfectants that express E4orf4 at lower levels than the clone studied in the current work because we expect the lower expression of E4orf4 not to induce the adaptive mechanisms of increased BCL2 and decreased BAK and ceramide.

Additionally, reliance on transient transfection experiments may be necessary in order to follow through with the remaining specific aims of this study, such as determining the mechanisms of ceramide accumulation.

Our work illustrates the potency of E4orf4 activity and motivates us to develop further approaches to circumvent the difficulties encountered during the current project. Achieving a thorough understanding of how E4orf4 promotes cell death will hopefully allow us to regulate it in a beneficial manner in the treatment of cancer.

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