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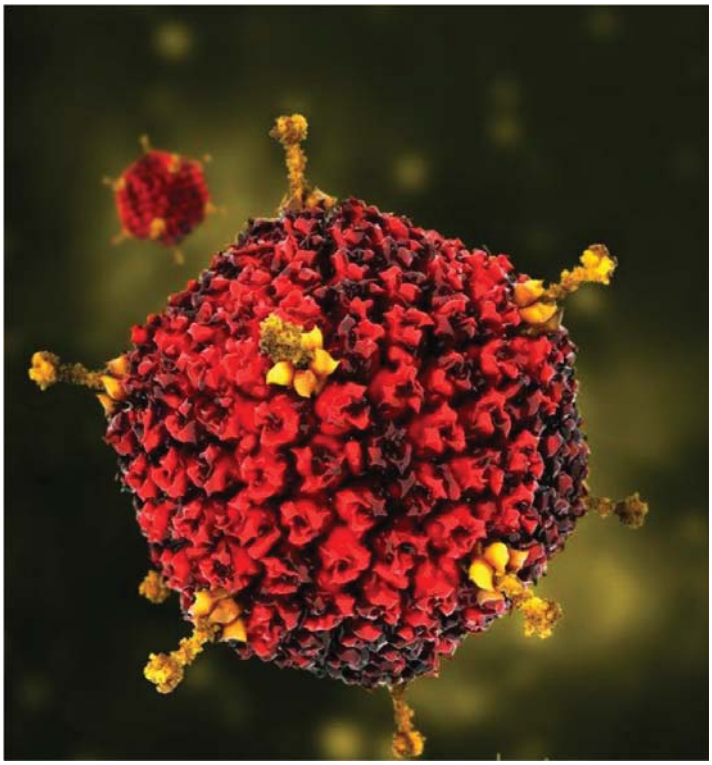
Molecular mechanisms and epigenetic regulation of adenovirus genome structure in persistent infection



Sibel Ciftci

Degree project in molecular biotechnology, 2012
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ABSTRACT

Adenovirus, in particular serotype C, is maintained in lymphocytes of the tonsils and adenoids for long time periods. That is contradictory to its commonly known lytic life cycle. To shed light on the mystery behind the molecular mechanisms of those lytic viruses that establish latent infection, BJAB cells were examined during a 21 days infection time-course. In this thesis, I aim to establish a model system for persistent and/or latent adenovirus infection in BJAB cells and design an experimental set up to study molecular and epigenetic regulation during long-term infection period. BJAB cells infected with mutant (dl309) and wild type adenoviruses were analyzed for potential protein regulation and/or modifications that play significant roles in pathways especially involved in cell cycle progression, such as pRb and p53 pathway proteins. My thesis study also dissects RNAi-microRNA pathway regulation in long-term adenovirus infection and observations indicated distinct regulation than shown in previous studies of lytic adenovirus infection.

The early and late viral gene expression in Ad5 (wt) and Ad5 (dl309) infected cells showed unusual expression patterns during infection course and showed inconsistency with viral genome copy numbers. Therefore, the other major part of my project dissected the potential epigenetic regulation mechanisms in Ad5 (dl309) infected BJAB cells particularly at day 10 dpi and 21 dpi. For this specific aim, chromatin immunoprecipitation assays were performed to study H3 protein deposition as well as H3 acetylation (H3KAc) and H3 lysine 9 trimethylation (H3K9me3) interacting with adenoviral early and late gene promoters. In general, H3 occupancy was slightly decreased at 21 dpi in both adenoviral early and late gene promoters; however, acetylation and methylation status of H3 at 10 dpi and 21 dpi remained identical or insignificantly altered. The findings led me to analyze RNAPII occupancy and its CTD serine 5-phosphorylation status at 10dpi and 21dpi. Interestingly, serine 5 was hypophosphorylated at 21 dpi even though RNAPII occupancy was significantly higher at 21dpi than at 10 dpi. Here, the observed explicit protein and gene expression along with reduced RNAPII efficiency due to hypophosphorylated CTD serine 5 in long-term infection of adenovirus in BJAB cells open up the cloak adenovirus latency secrecy to the scientific community.

Molecular mechanisms and epigenetic regulation of Adenovirus genome structure in persistent infection

Popular science summary

Sibel CIFTCI

Adenoviruses are small sized DNA viruses that can infect a broad range of vertebrates, from fish to humans. The human adenoviruses are mainly classified into subgroups (from A to F) based on their oncogenic capacity in rodents and haemagglutination capacity in humans. The most common adenovirus species C serotypes, namely Ad1, Ad2, Ad5, and Ad6 are known to associated with upper and lower respiratory tract infections such as pneumonia. Adenoviruses are lytic viruses due to their killing capacity of their host cell after replicating their genome. However, the early evidences have shown that species C adenoviruses enter a quiescent stage following the primary infection, which was observed by the intermittent release of the virus in stool. With this atypical life cycle, it has been observed that adenoviruses infect the cells particularly in lymphocytes of the tonsils and adenoids and are maintained as episomes for a long time. Despite this known property of adenoviruses, very little progress has been made on the molecular details of this, due to the lack of reliable cell systems. However, recent studies in this field have provided us with decent model systems to study species C adenovirus persistent/latent infections. In this study, I have established persistent infection by adenovirus serotype C, namely adenovirus 5 in BJAB cells (B lymphocytes). With this system, I dissected molecular mechanisms that may play crucial roles during long-term infection of adenoviruses. Indeed, adenovirus distinctly regulates several cellular pathways and use different strategies in order to reside in the cells persistent and/or latent. Furthermore, to understand the long-term maintenance of quiescent adenovirus genome in persistent infections, the potential molecular mechanisms that might play important roles in epigenetic signaling are also elucidated in this study.

Due to the lack of knowledge on persistent adenovirus infection to the science world, very little has been made on the clinical consequences. Immunocompromised patients have been commonly facing dissemination of the infection and known to cause severe diseases and may end up with death, especially in recipients of organ transplants such as bone marrow, heart, liver, and kidney. Further, new evidence suggest that persistent infection of adenovirus plays a role in obesity and in chronic lung diseases, for example asthma and chronic obstructive lung disease. In therapeutic perspectives, adenoviruses are the most popular viral vectors used in gene therapy applications. Taking all into consideration, it is essential to understand the molecular mechanisms behind adenovirus induced persistent infections to be able to develop new and powerful strategies to struggle with its potential risks and gain knowledge needed for the future construction of better therapeutic vector systems.

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1. INTRODUCTION

1.1 Adenovirus structure and genome organization

1.1.1 Adenovirus structure

Adenoviruses (Ad), members of *Adenoviridae,* are non-enveloped, icosahedral viruses that replicate in the nucleus of the host cell. The adenovirus genome consists of a double stranded linear DNA (dsDNA) molecule. The viral DNA is 26-45 kbp in size and it is encased by a protein structure, called the viral core structure. The core proteins (pVIII, pVII, pV, mu) are associated with viral DNA and play an important role in virus DNA assembly and stabilization. The viral core is in turn surrounded by the capsid structure, which is made up of the adenoviral structural proteins. The capsid proteins are required for interactions with the host cell receptors and they also provide the structural properties of the virion. Thus, the viral core, surrounded by capsid structure, reconstitutes the physical viral particle-the virion (Figure1) [1, 2].

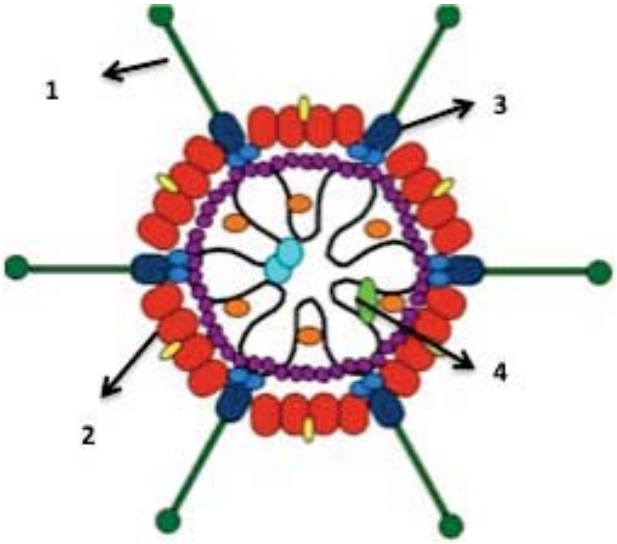


Figure 1. The structure of Adenovirus, kindly provided by Göran Akusjärvi (1: Fiber, 2: Hexon, 3: Penton, 4: Core proteins).

Adenoviral genes are divided into early (E) genes and late (L) genes, based on their expression pattern during the virus life cycle. Thus, the early genes (E1A, E1B, E2A, E2B, E3, E4) are expressed during early and the late genes are predominantly expressed during the late phase of infection. The proteins encoded by the early transcription units encode for proteins, which are mainly involved in the replication of viral DNA a nd in the control of host cellular mechanisms. In contrast viral late transcription unit, called as Major Late Transcription Unit (MLTU) gives rise to the variety of structural proteins. These proteins are essential for the virus assembly and for virus entry (Figure 2).

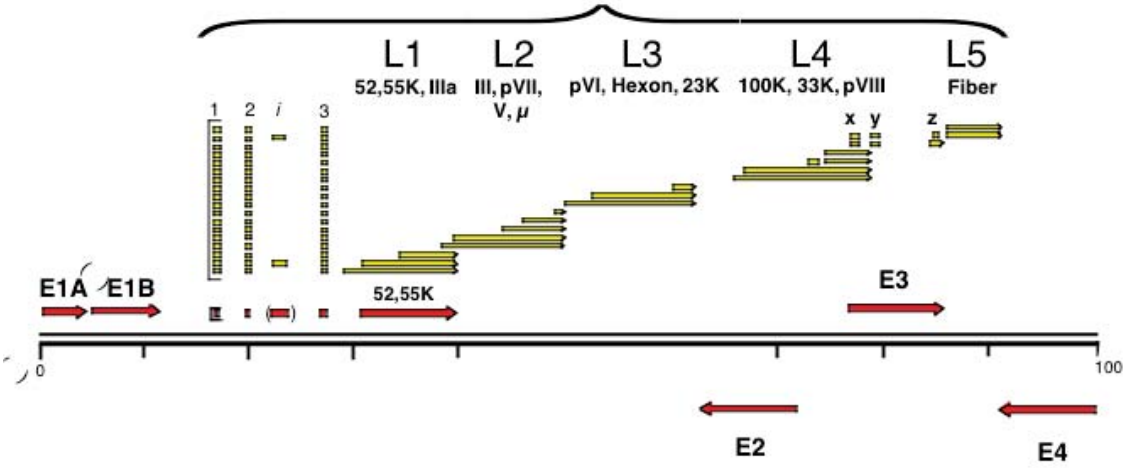


Figure 2. The genome structure of Adenovirus, kindly provided by Göran Akusjärvi

1.1.2 Replication of Adenovirus DNA

The linear adenovirus DNA has the 5′ ends that are covalently attached to a virus-encoded protein, the terminal protein (reviewed in [3]). Virus-encoded proteins; terminal protein, viral DNA polymerase and single-stranded DNA binding protein are required in addition to several cellular proteins and transcription factors for Ad DNA replication. Adenovirus uses its own DNA polymerase, which differs than cellular polymerase in terms of physical and biochemical features. The terminal protein, which locates at the either terminus of the double stranded DNA initiates the replication by a protein priming mechanism, forms the replication fork and moves from one end of the genome to the other. In each replication one of the parental strand is used as template that produces the daughter strand and displaced single strand. The next stage of replication, the displaced single strand serves as the template to synthesize the complementary strand [4].

1.1.3 Transcription of Adenovirus DNA

Adenovirus utilizes the cellular RNA polymerase II (RNAPII) and III (RNAPIII), in order to transcribe its DNA. RNAPII is required for the major part of transcription on viral genome, whereas RNAPIII is for the transcription of virus associated small non-coding RNA, named as VA-RNA. Adenoviruses have the ability of magnifying its small size genome via pre-mRNA splicing process, which produces various numbers of alternatively spliced viral mRNAs. In addition, viral pre-mRNAs undergo various post-transcriptional modifications such as capping, polyadenylation by using host cellular processing machineries [5, 6].

1.2 Adenovirus interaction with host cell

Adenovirus studies have been ongoing for a long time, since it is a precious model for understanding the mechanisms of cell regulation as well as the mechanisms of mRNA transcription and processing. As known from multiple studies, adenoviruses utilize host cells to allow their genome replication. In order to achieve efficient viral DNA replication, adenovirus has to remodel cell cycle progression as well as to subvert the apoptosis; the physical states that limit the virus production. Particularly, adenoviruses tempt investigators owing to the fact that they are easy to work and well characterized as well as posing no risk to researches. Thereby, adenoviruses are attractive models to comprehend and unravel the mechanisms behind cancer since the same cellular pathway is involved in the cell cycle arrest and/or apoptosis being targeted by adenovirus infection and oncogenic processes.

The mammalian cells infected by adenoviruses are introduced to cellular stress, which compel them to cellular arrest and/or apoptosis. However, adenoviruses indispensably depend on the host cell machinery to replicate the viral DNA. Therefore virus encodes proteins that target host cell cycle regulatory proteins and by doing so, they arrange the cellular machinery for their own benefit. Hence, the proteins produced in early infection fundamentally interact with cell pathways to disrupt the cell cycle arrest and/or apoptosis and trigger S-phase transition. Early after adenovirus infection of most cell lines follows the consequent events :(i) pRb (retinoblastoma protein) which is a key mediator protein of the cell cycle is inactivated by the viral E1A protein; (ii) therefore, the pRb bound proteins are activated; (ii) consequently, inappropriate cell cycle progression starts; (iii) pro-apoptotic effects of E1A and p53 are inhibited by the viral proteins encoded by E1B and E4 regions; and (iv) the late genes expression and viral gene replication takes place. A simplified model of the signaling pathway involved in adenovirus infection (A) and tumorigenesis (B) is presented in the following figure.

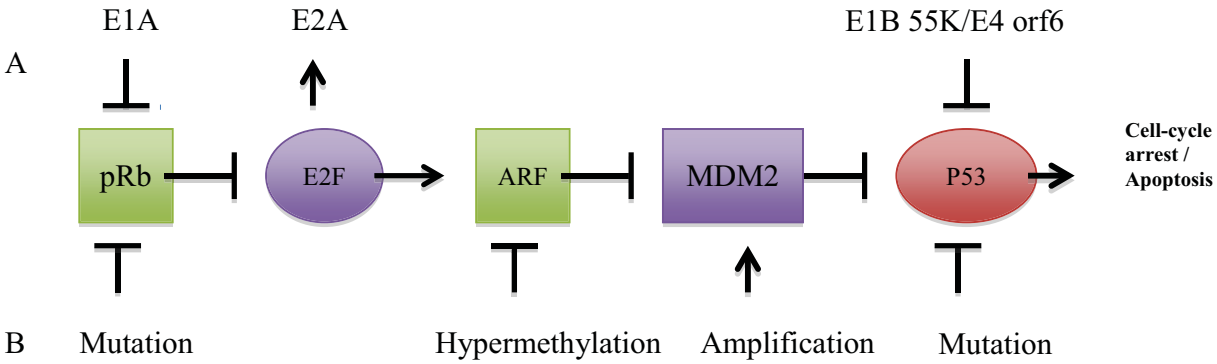


Figure 3. The overview of signaling pathway involved in cell cycle progression.

As illustrated in the model, the overlap in the molecular targeting by adenovirus infection and oncogenesis that influences the cellular regulation such as apoptotic, anti-apoptotic mechanisms and responses to cellular stress led investigators to investigate new pathways which may play significant roles in discovering novel therapeutic targets.

In my project, I have also attempted to investigate these signaling pathways as well as to observe the significant differences in these molecular mechanisms involved in the lymphocytes infected by serotype C adenovirus 5 that shows atypical life cycle of infection.

1.3 Adenovirus life cycle: lytic *versus* latent/persistent

1.3.1 Lytic life cycle

Adenoviruses have been known as lytic viruses due to their conventional lytic replication life cycle divided into early and late phases based on the onset of viral DNA replication. They are dependent on the host cellular machinery to replicate and express their genome. The early phase of the life cycle is initiated by the host cell infection via fiber-host cell receptor interaction and by the adsorption of the viral DNA, which is delivered into cell nucleus. Transcription of the viral genome with the onset of the replication takes place during the early phase of the life cycle in order to express the proteins required for viral genome replication as well as the proteins directly interacting with cellular proteins that restrain the cellular growth. After the onset of viral DNA replication (ca. 12 hours post-infection (hpi)) the virus enters to the late phase of the life cycle when the late genes are mainly expressed to yield immense amounts of the structural proteins essential for virus assembly. Upon virus capsid assembly in the nucleus, the structural proteins in the cytoplasm are transported into nucleus for virus packaging. The life cycle of adenoviruses is completed (app. 30 hpi) by lysing the cell due to the disruption of the cellular cytoskeleton and releases 10^4 -10^5 virus particles per cell.

1.3.2 Persistent/latent life cycle

Adenoviruses are classified into subgroups (A-F) based on their haemagglutination capacity in human, rat and monkey as well as on their oncogenecity to rodents. Thus, subgroup A has large, subgroup B has intermediate and subgroups C to F have weak or unknown oncogenic potential in rodent cells. The most common species C adenoviruses (serotypes Ad1, Ad2, Ad5 and Ad6), which are mostly associated with symptomatic upper respiratory tract and lower respiratory tract infections in human population, also shows a persistent infection characterized by intermitted viral release in stool long after nasopharyngeal shedding is halted. Early studies have focused on isolating viruses from explant of human tonsils and adenoid tissues to culture. Unexpectedly, adenoid tissue and tonsil did not produce infectious virus immediately after explant. Interestingly, infectious virus appeared from these tissues weeks to months. The observations that tonsils and adenoid tissues containing adenovirus do not achieve producing infectious virus, led investigators to assert that these tissues accommodate the virus for long period of time. Present studies have focused on the cell types that harbor persistent or latent species C adenoviruses and suggested that lymphocytes are the major reservoirs of persistent adenovirus infection [7].

Atypical infection life cycle of adenoviruses, namely, persistent and/or latent infection achieved by infecting human lymphocyte cell lines with serotype C adenoviruses provide the virus to maintain in the cells for long time period unlike in lytic infection. In persistent infection, a minute amount of virus may released during infection, which does not cause harm to the cells, whereas in the latent infection, viruses remain in the cells for long time without any release however, lytic infection may emerge late in the infection. In the most recent study, Zhang and colleagues achieved to establish a model system for latent/persistent infection by using four lymphoid cell lines, two B cell lines (BJAB and Ramos) and two T cell lines (Jurkat and KE37 (DMSZ)) and explored the virus-host cell interaction for prolonged periods of time. Importantly, their work has established a valuable cell model system, which would allow to study the persistent adenovirus infection in human B-cells [8].

1.4 Project description

As described earlier, human adenoviruses generally lead to lytic infection, which results in production of infectious virus particles. Research during the last five decades have clearly established the major molecular pathways how adenovirus reprograms host cell molecular pathways to keep the lytic phenotype. It has been also mentioned that recent studies described the existence of persistent/latent infection phenotype caused by the human adenoviruses. However, the molecular mechanisms of adenovirus caused latent/persistent infection are unknown to the scientific community due to mainly the lack of suitable cell model. However, the recent experiments by Zhang et al. (2010) pointed out human B cells as a potential model system to study adenovirus caused latent infection [8].

The aim of my research project is to establish latent infection phenotype in adenovirus-infected cells and to investigate some of the molecular mechanisms that might be related to latent infection. For this purpose, I aimed to establish human adenovirus mediated persistent/latent infection in human B cells (BJAB cell line) and follow the gene expression alterations as well as viral mRNA expression pattern during the infection cycle. Recent developments in adenovirus persistence infection have also reported that in adenovirus infected BJAB cells (B cells) there is a down regulation of the coxsackie adenovirus receptor (CXADR), which might be the reason for extended survival of the persistently infected B cells. Based on these observations, my project also introduces another perspective to dissect the potential CXADR gene regulation. It has been known that most of the epigenetic changes occurs at the histone protein level. Indeed, the histone protein variants as well as their post-translational modifications have significant roles in gene expression regulation. However, the question of how the epigenetic signaling behaves under the different cellular conditions remains to be unraveled. Therefore, it is reasonable to suspect of the involvement of particular epigenetic alterations in persistent adenovirus infection compared to lytic infection life cycle. In that manner, the project also focused on potential chromatin rearrangements occur during the establishment and persistent infection of adenovirus infected BJAB cells.

2. MATERIALS AND METHODS

2.1.0 Cell lines. BJAB (EBV –negative Burkitt´s lymphoma) cell line was obtained from Ingemar Ernberg (Karolinska Institute, Stockholm).

2.1.1 Adenoviruses. Wild type Ad5 and mutant type Ad5dl309 adenoviruses were provided by professor Göran Akusjärvi (Uppsala University). Mutant Ad5dl309 virus expresses all early genes except for E3 RID alpha and beta as well as 14.7K proteins due to lack of genes encoding the proteins.

2.1.2 Growing of BJAB cells. BJAB cells were grown in RPMI-1640 complete media (R5886, "Sigma") supplemented with 2mM L-Glutamate, 10% fetal calf serum and PEST (penicillin-streptomycin) at 37°C, 5% CO_2. They have been grown until they reach confluency ($1x10^6$cells/ml). The cells were counted by using Burker counting chamber and the cell viabilities observed under the microscope.

2.1.3 Freezing cells. $5x10^6$ cells/ml were frozen after reaching to confluence ($1x10^6$cells/ml) in RPMI growth medium containing DMSO ("Sigma") and stored at -80°C.

2.1.4 Infection of BJAB cells with Adenovirus. BJAB cells, which reached the confluency, were counted and $1x\ 10^7$ cells/ml per infection were collected by spinning at 1000 rpm, 5´ at room temperature (RT). Cells were suspended using 25ml serum free (SF) RPMI media, collected by centrifugation and resuspended in 2ml media (SF). Viruses were introduced to the cell suspension at 100 PFU/cell and incubated at 37°C for 3h. Afterwards, infected cells were harvested and washed twice with 14ml of complete media and the final cell density was adjusted to $0.5x10^6$ cells/ml with 20 ml RPMI growth media.

2.1.5 Sample collections from each time points. Samples for protein expression, ChIP assay, viral DNA, total cellular RNA was collected to analyze during the each time point within the 21 da ys time course. Mock samples were collected at the beginning of the infection. After each collection cell density was adjusted to 1.5×10^7 cells/ml for the next time point.
Protein samples- $5x10^6$ cells were harvested for protein expression and spun at 1,000 rpm, 5´ at 4°C. The cells were resuspended in 1ml ice cold PBS. After removing all PBS by spinning at the same speed, the cell pellet was stored at -20°C.

RNA isolation- $1x10^6$ cells were collected for total RNA isolation with TRI reagent ("Sigma-Aldrich", T9424) and the manufacturer instructions were followed.

DNA isolation- $1x10^6$ cells were collected for viral DNA detection. DNA was isolated by QIMamp® DNA Blood Mini Kit (50) ("Qiagen") and the protocol provided with the kit was followed.

Cross-linking cells for ChIP assay– $5x10^7$ cells/ml were harvested and spun at 1,000 rpm, 5' RT. The pellet was suspended in 25 ml 1xPBS (37°C) to density of $1x10^6$ cells. The cells were cross-linked with 37% formaldehyde ("Sigma"-F8775) and incubated on shaker with low speed, exactly 10 m in at room temperature. The cross-linking was terminated by 1.25ml of 2.5M glycine and incubated 5´on the shaker. Cross-linked cells were spun at 1,000 rpm at RT, 5´ and pellet was washed with ice-cold 1xPBS. The pellet after removing all PBS was stored at – 80°C.

2.1.6 PCR quantification of viral genome copy number. 5 µL of DNA samples from each time points including mock were analyzed by quantitative PCR for a Ad5 conserved region, E4orf1, using primer E4.F, 5´-CATCAGGTTGATTCACATCGG-3 and E4.R, 5´-GAAGCGCTGTATGTTGTTCTG-3.

A 10-fold serial dilutions (from 20,000,000,000 molecules/µL to 2,000 molecules/µL) of pCR2.1Ad5E4orf1 plasmid DNA (440 ng/µL) (generously provided by Di Yu, Dept. of Immunology, Genetics and Pathology (IGP), Uppsala University) was used to make a standard curve. The viral DNA samples were undergone four times 10-fold serial dilutions and from 10^{-2} to 10^{-4} were analyzed and performed in triplicate together with pCR2.1Ad5E4orf1 plasmid DNA and average C (t) values were reported. Reagents for qPCR- 10 µL of 2x iQ™ Syber® green super mix from BioRad (the mixture includes SyberGreen dye which binds to double stranded DNA, reaction buffer, dNTPs, Taq DNA polymerase), 1 µL of gene specific primers and 3µL of nuclease free water (Gibco® Life Technologies). The qPCR reactions were performed in 48-well plates in duplicate. A typical three-step real time PCR protocol was followed; Initial denaturation and enzyme activation at 95°C of 3 min. 1 cycle; denaturing at 95°C, 15 sec., annealing at 60°C 1 min., extension 5 sec. 40 cycles; melt curve at 95°C 10 sec. 1 cycle.

2.1.7 cDNA synthesis. Isolated RNAs from uninfected and infected BJAB cells were reverse transcribed to cDNA. RNA samples were subjected to DNase treatment using DNA-free™ Kit (Applied Biosystems) (manufacturer instructions were followed) to eliminate genomic DNA contamination and concentrations were measured by NanoDrop. One µg of total RNA was converted to cDNA by random primers (0.1 µg/µL) using "AffinityScript Multiple Temperature cDNA synthesis Kit" (instruction manual was followed).

2.1.8 RT-qPCR Analysis of mRNA expression in Ad5(wt) and Ad5(dl309) infected BJAB cells.

Converted cDNAs were amplified by qPCR to quantify the mRNA expression levels of human coxsackie adenovirus receptor (CXADR) transcript as well as the viral transcripts for E1A and hexon. The primers used for the CXADR mRNA were the following: forward, 5'- CGACTCTCCCACCTGCTAAG-3, and reverse, 5'-TGCTCTGTGCTGGAATCATC-3 (tp208/209). They were run in triplicates and duplicates and the data were normalized to 18S rRNA.

Viral transcripts, E1A and hexon, were also quantified by qPCR in triplicate and normalized to 18S rRNA. The primers used for E1A (13S) quantification were as

following: forward, 5'-CTTGGGTCCGGTTTCTATGC-3, and reverse, 5'-CCCGTATTCCTCCGGTGATA-3 (tp200/201). The hexon transcripts were amplified with the following primers: forward, 5'-CACATCCAGGTGCCTCAGAA-3, and reverse, 5'-AGGTGGCGTAAAGGCAAATG-3 (tp202/tp203). The average of triplicates was normalized to 18S.
18S and GAPDH mRNA transcripts were amplified in triplicates with target transcripts to normalize the data. The primers (10μM) used for 18S were as following: forward, 5'-CCCCTCGATGCTCTTAGCTG-3, and reverse, 5'-TCGTCTTCGAACCTCCGACT-3 (s (tp206/tp207). GAPDH mRNA primers (10μM) were following: forward, 5′-ACTCCTCCACCTTTGACGC-3, and reverse, 5′-GTTGCTGTAGCCAAATTCGTT-3. 5 μL of diluted cDNA samples (4ng/μl) from each time points including mock were tested for mRNA expression levels for specific genes with the mentioned primers above in either triplicate or duplicate and the normalized data was reported. The same SyberGreen master mix ("BioRad") as well as PCR programme (three-step PCR programme) was applied as performed in absolute quantification PCR for viral genome quantification.

2.1.9 Flow cytometry. Infected BJAB cells were tested for hexon expression by FACS. The monoclonal mouse antibody (catalog number MAB8052) to adenovirus hexon protein was purchased from Chemicon. The secondary antibody was a goat F(ab′)2 anti-mouse immunoglobulin conjugated with phycoerythrin (PE), purchased from Southern Biotech. Intracellular staining was performed based on Zhang et al., 2010. $1x10^6$ cells were taken and permeabilized with 2% formaldehyde with PBS and incubated with diluted anti-hexon primary antibody in 9 μl of 2%FCS with PBS, room temperature for 30 min. Cells were washed twice with FACs buffer (2%FCS with PBS). Secondary antibody conjugated with PE was added to the cells, incubated at room temperature for 30 min, followed by two times washing and analysis by flow cytometry with generous helps from Di Yu and Xui Hui.

2.1.10 Western blot analysis. $5x10^6$ cells were collected from infected and uninfected cells and lysed using RIPA buffer, containing 25mM Tris-HCl at pH 7.4, 150mM NaCl, 1% NP40, 1% Sodium Deoxycholate, 0.1%SDS, and supplied with complete EDTA-free tablet (Roche). Cell lysates were incubated on ice for five minutes and sonicated with Bioruptor as following settings; three times 30 sec. ON/OFF at high output and 4°C. The protein concentrations were measured with Bradford assay ("BioRad") by following manufacturer instructions. Equal amounts of lysate was mixed with 2xSDS loading dye ("Fermentas") and boiled at 95°C, 5 min. and separated by SDS-PAGE gel (AnyKD gel "BioRad" and 8%-10% home-made gels "recipe calculator Chang Bioscience") at 150V. Separated proteins were transferred to nitrocellulose membrane (Whatman®Protran®, Germany) and immunoblotted to viral and cellular proteins from wild type and mutant adenovirus infected BJAB cells as well as uninfected cells (mock). Proteins were visualized with LI-COR® Odyssey® Infrared Imaging System (Biosciences) based on the manufacturer`s instructions. Protein sizes were compared to Page Ruler™ Prestained Protein Ladder ("Thermo Scientific").

2.1.11 Antibodies. The polyclonal primary rabbit antibody to adenovirus type 5 (ab6982) was purchased from Abcam. The anti-adenovirus 2 E1A mouse monoclonal antibody (M73) was from Calbiochem® Millipore. The polyclonal goat antibody to beta-actin (sc-1616, Santa Cruz) used for loading control. P21 (F-5): sc-6246, PKR (K-17): sc-707, eIF2α (C-20): sc-7629, p53 (FL-393): sc-6243, p53 (FL-393): sc-6243, p21 (F5): sc-6246, pRB (IF8): sc-102, E2F-1 (C-20): sc-193, E2F-6 (E-20): sc-8366, MDM2 (H-221): sc-7918 were purchased from Santa Cruz Biotechnology. The secondary antibodies, which are conjugated to fluorophore, were used this experiment as following; donkey anti-rabbit, goat anti-rat, donkey anti-mouse, donkey anti-goat were purchased by Odyssey ® LI-COR.

2.1.12 Chromatin immunoprecipitation (ChIP) assay. The total amounts of $5x10^7$ cells/ml were collected from each time point of wild type and mutant infected BJAB cells as well as uninfected BJAB cells, cross-linked with 37% formaldehyde treatment and stored as pellets at -80°C until reaching the end time point of sample collection. Further ChIP analysis was performed as following; the frozen pellets were thawed and lysed in Buffer D (with complete protease inhibitor "Roche"), including 1%SDS, 10mM EDTA and 50mM Tris-HCL (pH8.0), to get the solution of $3x10^7$ cells/ml. 300µl of Lysates from each samples were transferred to low DNA binding tubes (Eppendorf Tubes®) for sonication ("Bioruptor") with the following program; 30´´ON/OFF, high energy settings, 12 cycle at 4°C. 100µl sonicated lysates were used for each ChIP reaction. Sonicated lysates, after diluted in ChIP dilution buffer, were pre-cleared with Pierce Protein A/G magnetic beads ("cat.88802") (pre-incubated with tRNA) to avoid the unspecific bindings, incubated at 4°C for 1hour. Magnetic beads were removed by standing on magnetic stand for 1 min. and lysates were transferred into low DNA binding tubes and 1% of the lysate (INPUT) from each sample were removed and stored at -20°C for future analysis. ChIP lysates were introduced to antibodies which were H3 (total) (Ab1791-Abcam), H3Ac (06-599-Millipore), H3K9me3 (Ab8898-Abcam) (2-4µg/$3x10^6$ cells, depending on the affinity of the antibody), incubated overnight at 4°C and next day Pierce Protein A/G magnetic beads were added, incubated 3 hour s at 4°C. Treated samples with magnetic beads and antibodies were washed to eliminate the unspecific bindings as following; Low salt wash buffer, high salt wash buffer, LiCl wash buffer, TE buffer (twice), respectively and finally samples were eluted with elution buffer (1%SDS, 100mM $NaHCO_3$, 10mM DTT), called IP (immunoprecipitates). Immunoprecipitates as well as input materials were reversed as following; IP eluates were treated with RNaseA, incubated at 37°C for 30 minutes and a master mix including 0.5M EDTA, 1MTris HCl (pH 6.5), Proteinase K (20 µg/µl), 5M NaCl were introduced to eluates, incubated overnight at 65°C. The same procedure was applied to input samples after elution buffer were added. Finally, DNA samples were purified by extraction with phenol: chloroform in order to be analyzed by conventional PCR and quantitative PCR.

2.1.13 Sonication test. The shear size of DNA was evaluated by performing a sonication test as following; same procedures were applied for sonication test sample as done in reversion and extraction of input DNA and run on 1% agarose gel. The expected shear size of DNA should be around 100 to 1000bp.

2.1.14 Quantification-PCR analysis of ChIP samples. Quantification PCR analyses were performed on E 1A promoter and major late promoter (MLP) of mutant type adenovirus 5 (Ad5dl309) infected BJAB cells. Histone proteins, RNA polymerase II, III and serine5 phosphorylation of C terminal domain RNA polymerase were analyzed. ChIP samples, the DNA of mutant type infected BJAB cells cross-linked to RNA polymerase II-III and serine5 phosphorylation, were prepared by following the same protocol as done for histone proteins. The antibodies used for RNA polymerases and serine5 phosphorylation were RNAP II (N-20) (sc-899, SantaCruz) and S5P (3E8, D. Eick).
Primers used for ChIP-qPCR reactions were following; E1A promoter forward primer, 5-GGGTCAAAGTTGGCGTTTTA-3 and reverse primer, 5-CAAAATGGCTAGGAGGTGGA-3 (tp200/tp201); Major Late Promoter forward primer 5-AGGTGATTGGTTTGTAGGTGTAGG-3 and reverse primer, 5-CTCCTCGTTTTTGGAAACTGAC-3 (tp202/tp203). PCR reactions were performed in triplicate as following the regular qPCR protocol in this project and the data was reported as average of %input.

3. RESULTS AND DISCUSSION

3.1 Experimental set-up for long-term infection. This study mainly consists of two experimental set-ups with the same experiment design. The first set-up of the study was performed with Ad5 (wt) infected BJAB cells and the second experiment was performed with both Ad5 (wt) and Ad5 (dl309) infected BJAB cells. Infected cells were collected every third day to analyze protein expressions/modifications, viral genome copy numbers, mRNA expression levels and chromatin modifications during 21-day post infection period, as shown on figure 4.

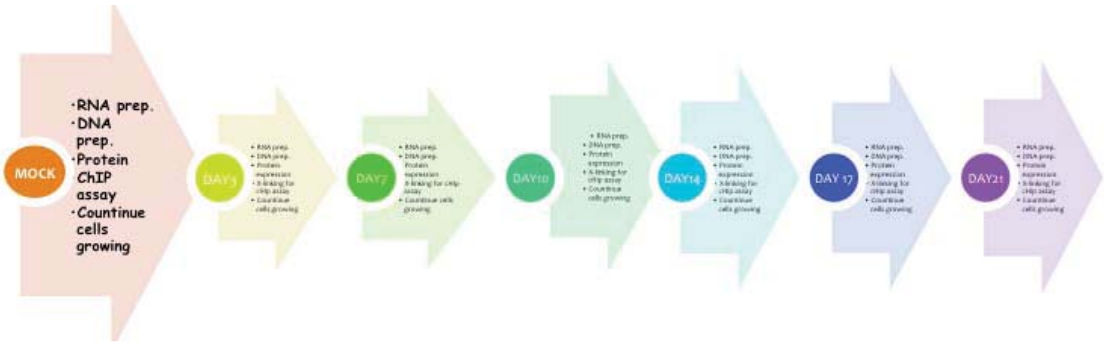
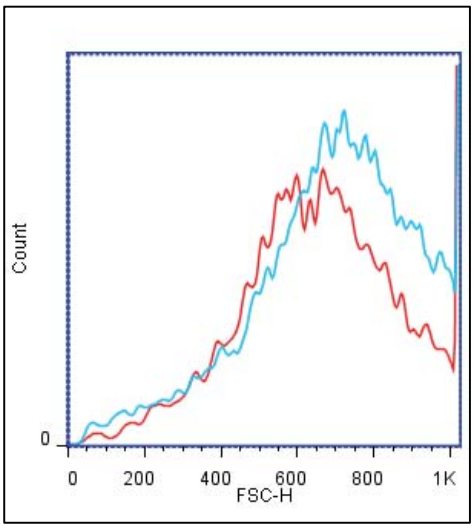


Figure 4. Experimental set-up to study persistent/latent adenovirus infection (The figure summarizes the experiments performed during 21 days post infection. Every 3 days post infection, samples were collected; RNA, DNA, protein and $5x10^7$ cells were cross-linked for ChIP assay. After each collection cell density was adjusted to 1.5 x 10^7 cells/ml for the next time point).

3.2 BJAB cells are permissive for adenovirus infection. Previous studies have shown that human tonsil and adenoid tissues can maintain adenovirus DNA for long time. Specific B cell line, named as BJAB cell line, was used for the present study to monitor Ad5 virus replication during the long period of infection. The BJAB cells were infected by both Ad5 (wt) and Ad5 (dl309) viruses at 100 PFU/cell and grown in suspension culture for 21 days. It was observed that cells remained viable and continued to divide at almost the same rate as the uninfected cells (Mock) based on the Burker chamber cell counting and visual inspection. The infection status was confirmed by the FACS analysis (figure 5A-B) as well as western blot analysis (figure 5C) performed for the hexon protein expression.

A



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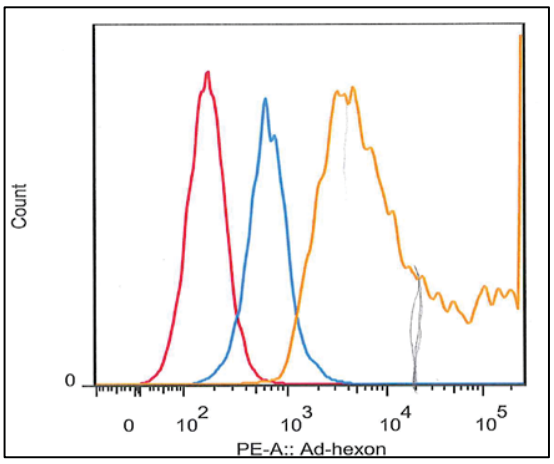

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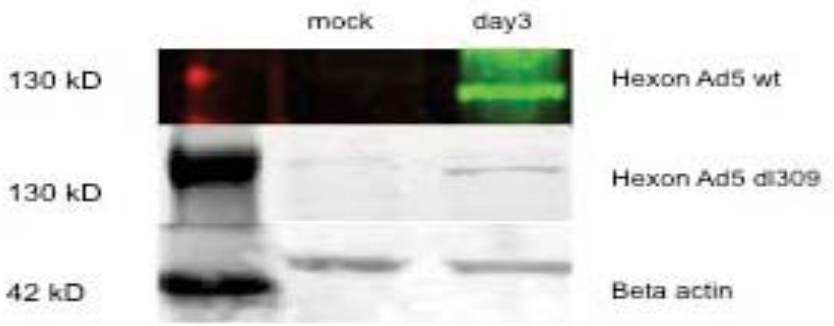


Figure 5. Viral late gene (hexon protein) expression in adenovirus infected BJAB cells, analyzed by flow cytometry (A-B) and western blot (C). (A) The hexon protein expression was monitored after 11-days post infection with Ad5 (wt) (Red) and Ad5 (dl309) (Blue) viruses. The hexon protein expression was detected after 6-days post infection with Ad5 (wt) virus. The intracellular staining of the hexon protein was performed by using anti-hexon antibody followed by detection with secondary antibody conjugated with phycoerythrin (PE). (B) Stained (Blue) and unstained (Red) mock samples were monitored as control. The density of the expressed hexon protein (Yellow) versus the number of cells was reported. (C) The upper blot shows hexon protein expression in Ad5wt infected BJAB cells and the lower blot indicates hexon expression in Ad5dl309 infected BJAB cells at 3-day post infection. The mock sample (uninfected BJAB cells) is negative control for hexon expression. The first lane of the blots indicates 130kD (hexon) and 42kD (beta actin) molecular size marker.

Fluorescent cytometry analysis (FACS) indicated a clear overlap between hexon expression of Ad5 (wt) and Ad5 (dl309) infected BJAB cells after 11 days of infection (figure 5A). The overlap area was gated based on the negative control (mock) (data is not shown), which refers only the cells expressing hexon protein. However, the peak at hexon expression of Ad5 (wt) infected cells is observed before than of the mutant infected cells. This variation in cell numbers can be explained due to the technical lost of cells during the sample preparation. The FACS analysis from the experiment set-up 1 performed on the Ad5 (wt) infected cells indicates a clear shift of hexon expression in infected samples comparing to control (Mock) at 6-day post infection (dpi) (figure 5B) confirming that Ad5 (wt) rapidly established productive infection of BJAB cells as measured by late protein, hexon, expression. Further analysis by western blot also confirmed the Ad5 (wt) and Ad5 (dl309) infected BJAB cells were successfully infected and expressed viral late protein (hexon) at 3 dpi . The phenotype of delayed hexon protein expression of Ad5 (dl309) infected BJAB cells compared to Ad5 (wt) infected cells were also observed with western blot analysis. Interestingly, the hexon protein expression of Ad5 (dl309) virus infected cells is considerably less than the hexon protein expression of Ad5 (wt) infected cells. FACS and the western blot analysis findings are suggesting that BJAB cells were successfully infected by Ad5 (dl309) and Ad5 (wt) viruses and also confirmed that the infected cells were still hosting viruses at 6-11 days post infection.

3.3 Ad5 (dl309) infected, but not Ad5 (wt) infected lymphocytes can be maintained in cell culture for extended time-period. BJAB cells infected with Ad5 (wt) and Ad5 (dl309) viruses were maintained in suspension culture and were monitored for the viral late protein expressions and viral DNA presence periodically during 21 da ys post infection (figure 4).

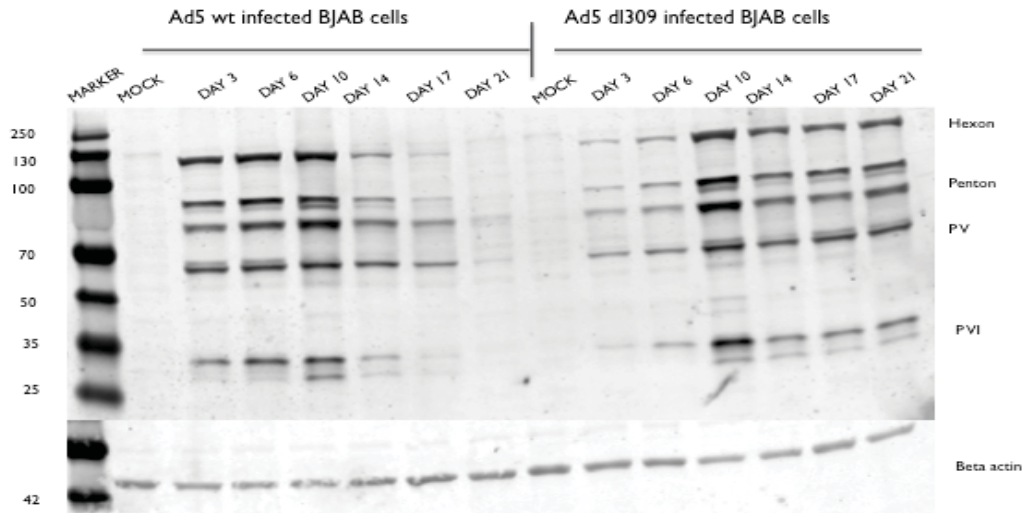


Figure 6. Viral capsid protein expression in Ad5 (wt) and Ad5 (dl309) infected BJAB cells. The late viral

protein expression was detected with antibody against viral capsid proteins on western blot. Antibody against beta-actin was used to monitor equal protein loading on the SDS-PAGE.

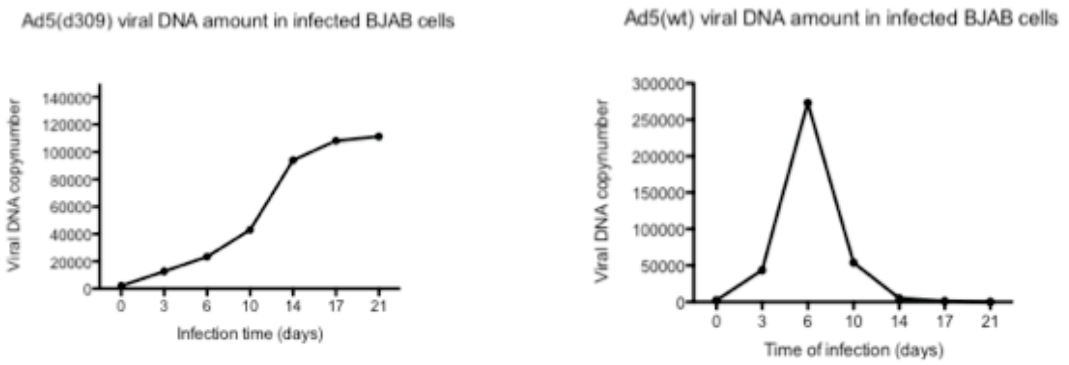


Figure 7. Absolute quantification Ad5 (wt) and Ad5 (dl309) genome copy number in infected BJAB cells as a function of time (days post infection).

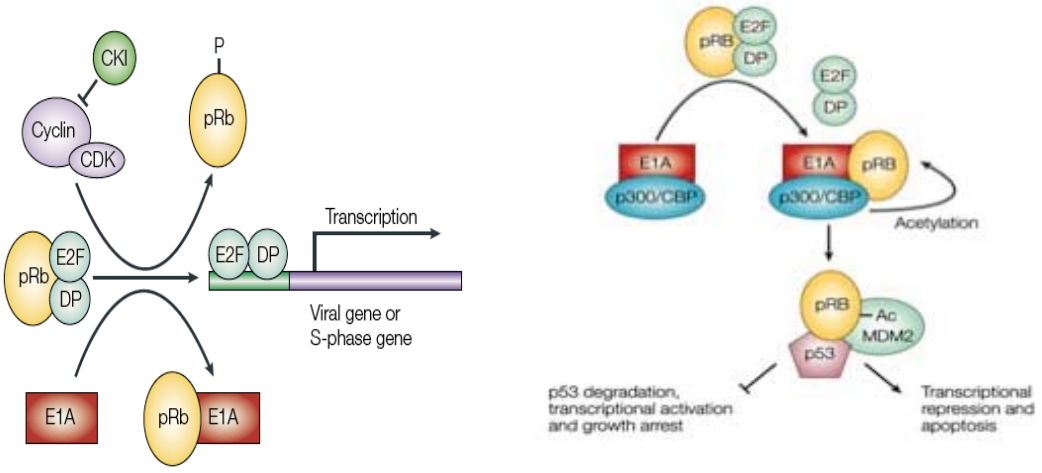
In Ad5 (dl309) infected BJAB cells, viral DNA levels increased up to 100,000 copies within two weeks and by the end of the 21 day post infection, the viral genome levels reached at 120,000 copies (figure 7). In contrast, the viral DNA levels in Ad5 (wt) infected BJAB cells dropped abruptly within two weeks of initial infection after peaking at 6 dpi and DNA levels fell below the detection limit by the end of infection (figure 7). The earlier study by Zhang et al, (2010) also reported that in Ad5 dl309 infected BJAB cells, viral DNA levels reached up to 100,000 copies per cell within 10 days of initial infection and after peaking around day 23 dpi, a gradual loss of genomes was observed over the following 3 months [8]. However, the interesting phenomenon with the Ad5 (wt) infected BJAB cells lost the viral genome by the 21 day of initial infection was never published before. Additionally, a striking expression patterns of the viral late proteins expression in the Ad5 (wt) and Ad (dl309) infected BJAB cells were observed in this study (figure 6). Consistent with the viral DNA levels, the Ad5 (dl309) infected BJAB cells maintained the late proteins expression during observed time of infection whereas Ad5 (wt) infected BJAB cells lost the capsid proteins expression by the end of the 21 dpi (figure 6). It is also noteworthy to mention that BJAB cells infection with the Ad5 (dl309) virus occurred with 6 days delay however the Ad5 (wt) infection was established rapidly. These findings led me to conclude that Ad5 (dl309) and Ad5 (wt) infected BJAB cells provided an extended period of infection time compared to other commonly used cell lines such as, HeLa, 911, HEK293, which release the virus particles within 72hpi, due to the lytic feature of adenovirus. Together with these results, the astonishing differences in viral genome levels and late proteins expression patterns (figure 7) indicate that the persistent infection can be established in the Ad5 (dl309) infected BJAB cells while Ad5 (wt) infected BJAB cells showed rather delayed lytic infection pattern.

3.4 Distinctive protein regulation in Ad5 (wt) and Ad5 (dl309) infected BJAB cells.

Viruses must remodel their host cell molecular machinery at many levels during the infection for mainly two reasons. At first, the virus needs to create an optimal environment for its replication to assure efficient production of progeny virus. Secondly, the virus must overcome the host cell antiviral defense mechanisms to increase the possibility to release its progeny. Therefore, there has to be different mechanisms that viruses use to usurp the cellular molecular machinery for efficient viral DNA replication, RNA transcription, mRNA processing and transport, protein synthesize and posttranslational modification of proteins. While virus reprograms host cellular machinery, the host cell also responds to virus infection via cellular antiviral defense mechanisms to inhibit virus amplification. Thereby, the virus and host cell interactions seem to be particularly complicated at the level of protein synthesis. In this study, the viral and host cellular protein modifications and regulation were studied to lighten the question of what strategies adenoviruses use to be able to remain for long period of time in lymphocytes or what sort of protein regulation could take place in persistent infection. Previous studies performed for the purpose of comprehending protein modifications (sumoylation, phosphorylation, methylation, ubiquitination) and protein stability in short time infection (lytic infection) by adenoviruses were essentially taken into consideration in my study to investigate further and observe distinctive mechanisms in both Ad5 (wt) and Ad5 (dl309) infected BJAB cells which may contribute to long term infection. Therefore, the present study focused on the regulation/modification of viral and cellular proteins, which play significant roles in cell cycle regulation, apoptosis regulation and transcriptional activation/repression during 21-day time-course in Ad5 (wt) and Ad5 (dl309) infected BJAB cells.

3.4.1 E1A, the early viral protein has noticeably different regulation in Ad5 (wt) and Ad5 (dl309) infected BJAB cells

The early region 1A of adenoviruses is immediately transcribed with the onset of infection to activate the viral transcription. The E1A protein interacts with a large number of cellular proteins that control gene expression, cell growth, transcriptional co-activators (p300/CBP family and E2F) and repressors (CtBP) as well as cell cycle regulatory proteins (p53 and pRb) to immortalize the host cell, which is essential for productive viral propagation (figure 8).



A B

Figure 8. (A) The expression of E1A protein counteracts the normal cell control by binding to pRB and releasing E2F transcription activator. (B) E1A protein also allows cell apoptosis via the acetylated pRB-MDM2 and tumor repressor p53 complex (Figure adapted from [9]).

Due to the significance of E1A protein during adenovirus lytic infection, its regulation/modification was dissected in this study, which might have contributed to the long-term infection in Ad5 (wt) and Ad5 (dl309) infected BJAB cells (figure 9). As suspected, E1A protein is distinctly regulated in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21dpi. The E1A protein was abundantly expressed within 6 days of post infection in BJAB cells. Similarly the viral genome copy number was peaking afterwards. The expression of the E1A protein as well as viral genome copy number was commenced to be down regulated at 10 dpi and disappeared at 17 dpi. Interestingly, the E1A protein expression in Ad5 (dl309) infected BJAB cells was incompatible with the viral gene expression levels. The E1A protein was extensively expressed during 6-10 days post- infection. This phenomenon was also followed by the viral gene expression. However, afterwards, it was expected that the E1A protein would be up regulated with a crescendo until the end of infection day due to the increasing viral gene copy number. Even though viral genome copy number was increasing exponentially, the E1A protein was down regulated at 14 dpi and interestingly appeared with its abundant expression 3 days later (17dpi) and again returned to the same levels as at 14 dpi by the end of infection (see also figure7). This explicit expression pattern of the E1A protein in Ad5 (dl309) infected BJAB cells, which was not observed before, might be one of the reason for the establishment of persistent infection. Therefore the findings suggested that E1A protein regulation might play a crucial role in adenovirus long-term infection.

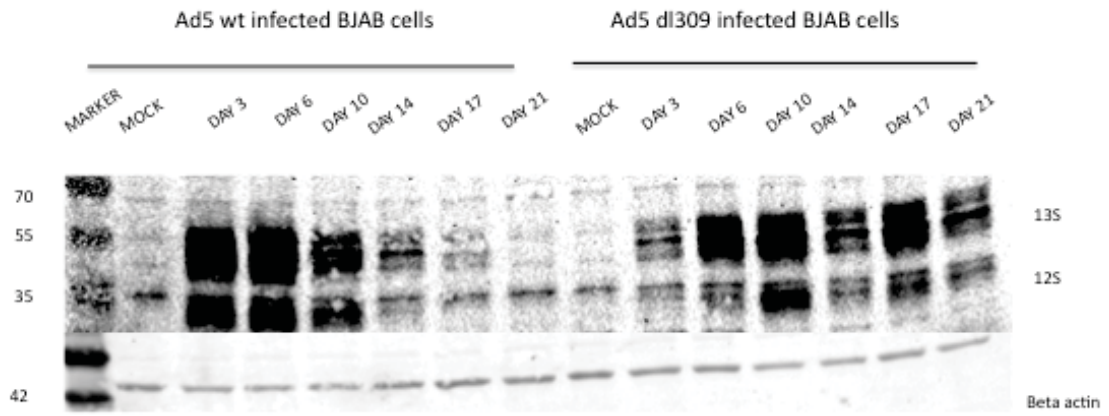


Figure 9. The E1A protein with two splice forms (13S and 12S) was detected by western blot analysis in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21-day infection time-course. Antibody against beta-actin was used as loading control.

3.5 The human cell growth control mechanism in long-term infection

Viruses depend on the host cell mechanisms subvert the host cell scrutiny to replicate the viral genome and propagate. On the other hand, host cells also regulate the cell cycle under the cellular stress such as viral infection and either undergoes growth arrest and/or apoptosis. Therefore, viruses firstly attack to the cellular growth control mechanisms in order to immortalize their host. In adenovirus, the viral E1A and E1B early genes products yield significant acts in the cellular pathways mainly involved in cell cycle progression control, apoptosis and in specific tumor suppressor proteins p53 and pRB. The previous results of this study announced that the viral E1A protein was distinctly regulated in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during long-term infection. Due to the peculiar regulation of E1A and the importance of tumor suppressor genes which are highly targeted by the Ad5 early genes, the p53 and pRB pathways and their regulation and/or modifications in Ad5 (wt) and Ad5(dl309) infected BJAB cells during 21 dpi were analyzed.

3.5.1 The p53 pathway and its regulation in Ad5 (wt) and Ad5 (dl309) infected BJAB cells.

The p53 protein is known as a critical player in synchronizing the cellular response to cellular stresses induced by DNA damage, chromosomal distortions, chemicals, oncogene activation, hypoxia and virus infections. Studies have shown that controlled signaling from and to p53 protein is needed for the maintenance of the cell integrity. The known mechanism by which p53 can, accomplish its transcription activation and repression is by up regulating and down regulating the expression of genes involved in cell cycle control and apoptosis. In order to overcome with this scrutiny, adenoviruses encode proteins that hamper with the p53 pathway. However, p53 firstly and directly interacts with the early viral protein 55K (E1B55K), which blocks the ability of p53 to activate transcription on cell cycle arrest responsible gene such as p21/CDKN1A (fig.11).

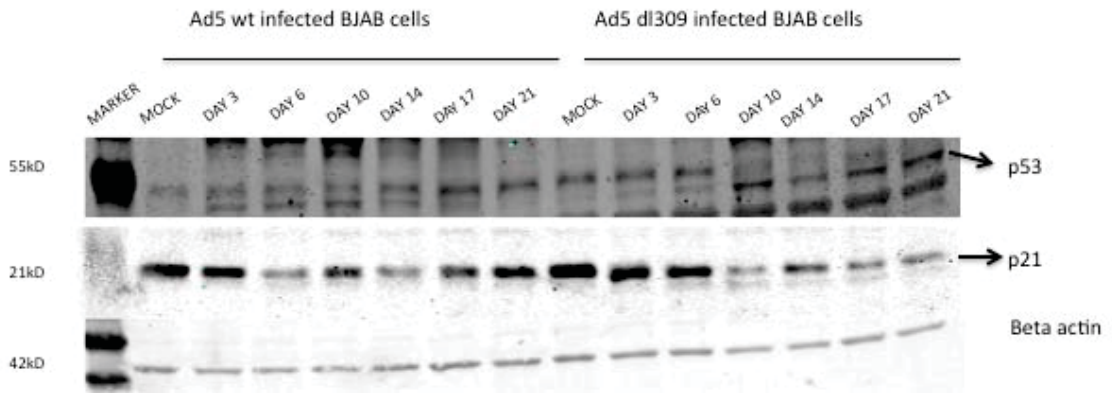


Figure 10. Western blot analysis of p53 and p21 cellular proteins in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21-days infection. Beat-actin was used to control equal loading of the protein samples.

The western blot results have been showed that p53 pa thway is differently regulated in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21 dpi (figure 10). Furthermore, posttranslational modifications of the p53 protein in long-term infection were also observed in this study. As clearly seen, the p53 protein underwent an unknown posttranslational modification (lower band) during the infection and it was regulated in a different fashion compared in Ad5 (wt) and Ad5 (dl309) infected BJAB cells. In Ad5 (wt) infected cells, a novel form of the p53 was rapidly introduced by the Ad5 (wt) infection while conserving its typical form (upper, slower migrating band). These two forms of p53 were maintained during 14 dpi. Later on, as clearly seen, p53 was returned to its slower migrating form as in the mock cells (upper band) at 17 and 21 dpi. However, in Ad5 (dl309) infected BJAB cells, the new form of p53 appeared at 6 dpi and stayed constant till th e end of infection time-course (figure 10). The following periods of infection, the faster migrating p53 form was maintained while the slower migrating form was lost, which was vice versa compared to Ad5 (wt) infected BJAB cells. These findings suggested that in long-term infection of adenovirus, p53 undergoes post-translational processing, which might play a critical role in the infection cycle. It has been known that in lytic infection of adenovirus, p53 undergoes a variety of modifications including phosphorylation, sumoylation that might regulate the p53 protein functions. However, there is no e vidence on p53 pr otein regulation during long-term infection of adenovirus. Here, I have showed that specific regulation of p53 takes place during the long-term Ad5 infection in BJAB cells.

The levels of the p21 protein, which is best-known p53 target gene, were also analyzed in my study (figure 10). As known in general, the p21 protein inhibits the enzymes Cdk2 and Cdc2, which are required for cell cycle progression into the S phase and M phase, respectively. Therefore, p53 regulates the expression of p21 to induce cell cycle arrest. My observations showed that p21 protein is particularly regulated by the modified p53 i ntroduced by the adenovirus infection in BJAB cells. Indeed, less

expression of the p21 protein was only observed in the existence of the specific, faster migrating form of p53. Additionally, Ad5 (dl309) infected BJAB cells showed slight modifications of p21 in between 10 and 21dpi, which might be unique to the establishment of persistent infection.

My results led me to conclude that the p53 protein undergoes specific processing, which might be important for establishment of persistent infection in Ad5 infected BJAB cells.

3.5.2 The pRB pathway and its regulation in Ad5 wt and Ad5 dl309 infected BJAB cells.

As mentioned earlier, adenovirus expresses its early genes, namely E1A and E1B to interfere with host cellular proteins, which play crucial roles in cell cycle progression. In previous section, the specific regulation of the p53 pathway in long-term infection by Ad5 has been shown. However, there is another pathway called Rb pathway, which is also critical in cell cycle regulation. The simplified view of Rb pathway indicates that the Rb proteins are interacting with cellular transcription factors, E2Fs, which negatively regulate the cell cycle progression during the G_0/G_1 and G_1/S phase transitions. However, in normal cells, this repressive obstacle is accomplished by the phosphorylation of Rb proteins through CDKs that releases transcriptionally active E2F proteins. Consequently, free active E2F proteins can activate transcription of a b road range of genes that are involved in cell cycle progression and DNA replication. In adenovirus-infected cells, the adenoviral early protein E1A undertakes the role of CDKs and similarly it binds to Rb proteins to liberate transcriptionally active E2Fs (Figure 11).

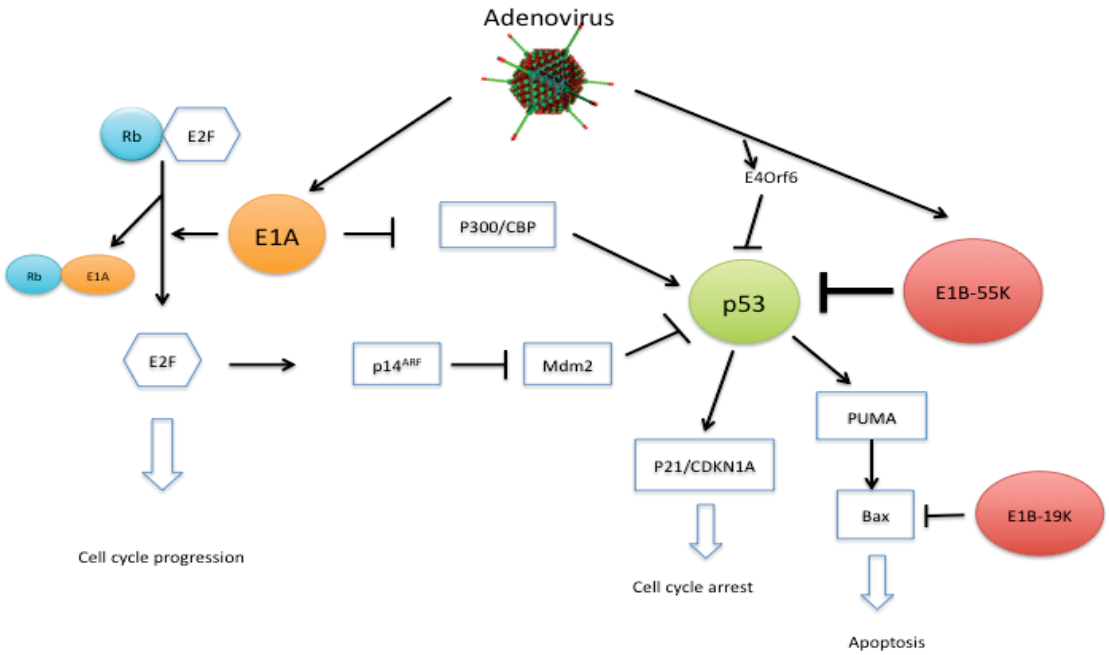


Figure 11. A simplified model of p53 and pRb pathways. In lytic infection of adenovirus, pRB and p53 are targeted by the E1A and E1B proteins, respectively.

In this study, the regulation mechanisms of Rb pathway in the long-term infection of Ad5 were also investigated. The pRB protein along with the cell transcription factors E2F-1 and E2F-6 were particularly studied by the virtue of their critical roles in the pathway. The results in figure 12 showed that by the infection occurred, in Ad5 (wt) and Ad (dl309) infected BJAB cells, pRB protein was up regulated and highly expressed during the over period of infection. However, it was unexpected to observe similar expression patterns of pRb protein in both Ad5 (wt) and Ad5 (dl309) infected BJAB cells due to its regulatory adenoviral protein E1A unique expression patterns observed during my time-course study. It has been known that E2F-1 is one of the strongest transcription factors and it directly binds to the pRb protein and in lytic infection of Ad5 studies have shown that RNAs encoding E2F-1 increased significantly in concentration from 24h post infection (Miller et. Al). However, in my study, I could not observe striking changes of E2F-1 amounts at the protein level during the 10 dpi, yet a posttranslational modification of E2F-1 appeared (seen as two bands) during this period of infection in Ad5 (wt) infected BJAB cells, whereas this particular modification in Ad5 (dl309) infected BJAB cells can be seen only at 10dpi. Later on, only one form of E2F-1 is expressed, slightly down regulated in Ad5 dl309 infected BJAB cells whereas fairly well expressed in Ad5 (wt) infected BJAB cells. On the other hand, E2F-6 that is known to cause a delay in the transition of G1/S phase of the cell cycle in case of its overexpression showed different regulation pattern in Ad5 (wt) and Ad5 (dl309) infected BJAB cells. It was observed that after BJAB cells infected by Ad5 (wt), the expression of E2F-6 was down regulated and maintained in low amounts during 21-infection time. Whereas, E2F-6 did not really change its expression and was constantly expressed during 21 dpi in Ad5 (dl309) infected BJAB cells.

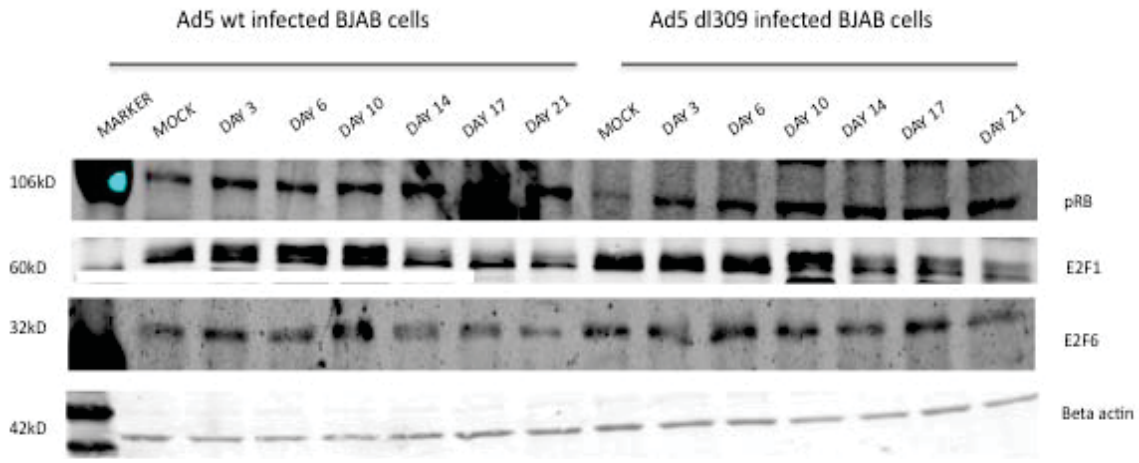


Figure 12. The protein expression analysis of pRb and its potential targets E2F-1 and E2F-6 in the Rb pathway, in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21 dpi. Beta actin was used as a loading control. The expected sizes of the proteins were detected and shown.

My results led me to speculate that due to the similar expression and modification patterns of E2F-1 and different regulation of E2F-6 in the Ad5 (wt) and Ad5 (dl309) infected BJAB cells, E1A preferentially targets E2F-6 rather than E2F-1. Further, the same expression pattern of pRB protein observed in both Ad5 wt and dl309 infected BJAB cells may suggest that E1A regulates p53 pa thway rather than Rb pathway. However, other proteins in the target pathway should be also taken into consideration in the future studies for further investigations.

3.6 The adenoviral control of the RNAi-microRNA pathway in long-term infection

MicroRNAs are class of non–coding small RNAs (21-22 nucleotides long) that play significant roles in posttranscriptional gene expression regulation. The evidences have implicated that miRNAs are widely involved in cellular processes including cell differentiation, proliferation and apoptosis. In mammals, the initial long miRNA transcripts (pri-miRNA) undergoes two major processing mechanism held by RNase III endonucleases, Drosha and Dicer. The first process takes place in the nucleus where Drosha is involved and creates precursor microRNAs (pre-miRNA). The resulting processed miroRNAs are recognized by exportin5 and transports to the cytoplasm where the second process occurs. In the cytoplasm, Dicer creates a s mall duplex of miRNA namely miRNA/miRNA* by cleaving pre-miRNA. Eventually, the guide miRNA strand is recognized by the RNA-induced silencing complex (RISC), which contains Argonoaute (Ago) protein as core component whereas the other strand (miRNA*) is probably degraded. Consequently, miRNA guides the RISC complex to the target mRNA to down regulate the expression of target mRNAs by either inhibiting the mRNA translation or by direct cleaving of mRNA (degradation). The direct cleavage of mRNAs is specifically achieved by Ago2 due to its unique endonucleolytic activity. There are studies have shown that adenovirus specific non coding RNAs so-called viral associated I RNA (VAI RNA), resembles to microRNA precursors, can inhibit the microRNA pathway in cells at mainly three levels; (i) it inhibits the transport of pre-microRNA by blocking Exportin5 (ii) it c an act as a competitive substrate for Dicer and replace itself with the guide strand of microRNA to create mivaRNAI duplex and (iii) the generated duplexes will saturate the RISC complex in the late infection. Adenoviruses also use their non-coding RNAs to inhibit cellular defense mechanism. During virus infection, protein kinase R (PKR) is highly expressed as a r esult of increased levels of type I interferon. PKR is activated with the interaction of dsRNA which is followed by homodimerization and autophosphorylation of the PKR. The activated PKR leads to block the translation machinery in infected cells. Wael Kamel has announced that Ago2 levels were rapidly reduced whereas the levels of Dicer remained stable in the lytic infection of adenovirus. Therefore, due to the paramount importance of Ago2 and Dicer proteins in the microRNA pathway and the critical role of PKR in the infected host cell translational mechanism were analyzed to discern the regulation of these proteins in the long-term infection of adenovirus.

The western blot results in figure 13 showed that PKR protein expression was up regulated by the infection occurred and maintained fairly stable during 21dpi of Ad5 (wt) infected BJAB cells whereas it was started to be down regulated from 10 dpi in Ad5

(dl309) infected BJAB cells. On the other hand, Dicer protein was slightly up regulated after infection and at magic 10 dpi it was abundantly expressed for both Ad5 (wt) and Ad5 (dl309) infected BJAB cells. Afterwards, the same expression levels appeared and maintained to the end of infection. It was noted earlier that in Ad5 (wt) lytic infection the Dicer protein was down regulated at the late infection; however in this study it was shown that Dicer protein is specifically regulated and maintains its low level expression during the long-term adenovirus infection. On the contrary to rapid reduction of Ago2 protein in early lytic infection of adenovirus (W.Kamel, personal communication), my results showed that the Ago2 protein was expressed during the long term adenovirus infection and fairly more in Ad5 (wt) infected BJAB cells compared to Ad5 (dl309) infected BJAB cells.

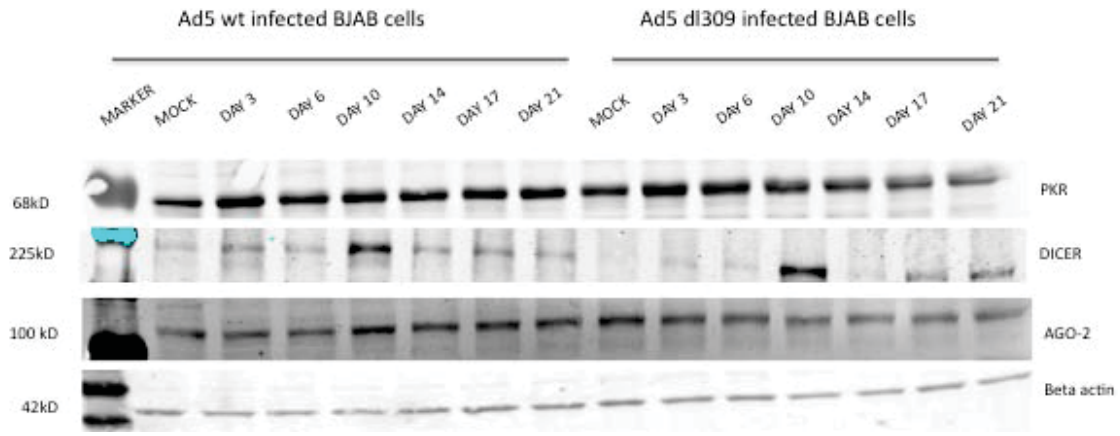


Figure 13. The protein expression of Ago2, PKR and Dicer in Ad5 wt and Ad5 dl309 infected BJAB cells during 21dpi. Beta actin was used as loading control.

The protein expressions and regulation of the crucial proteins in microRNA pathway were analyzed here and the western blot results showed that there are significant differences in the expression of these particular proteins in Ad5 infected BJAB cells during long term of infection. However, further analyses such as the expression levels of mivaRNAs and its regulation during the long-term infection of adenovirus should be performed.

3.7 Coxsackie Adenovirus Receptor (CXADR)

The life cycle of virus initiates with cell entry where it propagates its genome and terminates with leaving its host cell so that it can infect other cells. Viruses achieve the cell entry through receptor recognition. Previous studies have shown that many viruses attack to receptors that are specialized as cell-cell adhesion proteins (Walters R., 2002). Adenoviruses infect epithelia generally via the receptor called Coxsackie adenovirus

receptor (CXADR). CXADR belongs to immunoglobulin receptor superfamily which is consists of two extracellular Ig-like domains, namely, D1 and D2. Due to the homodimerization of D1 domains located on adjacent cells, CXADR mediates the cell adhesion and specialized as intracellular tight junctions. By the adenovirus infection of the cell, the fiber protein, which is adenoviral capsid protein, binds to CXADR by disrupting the CXADR: CXADR homodimer form due to its high affinity (figure 14).

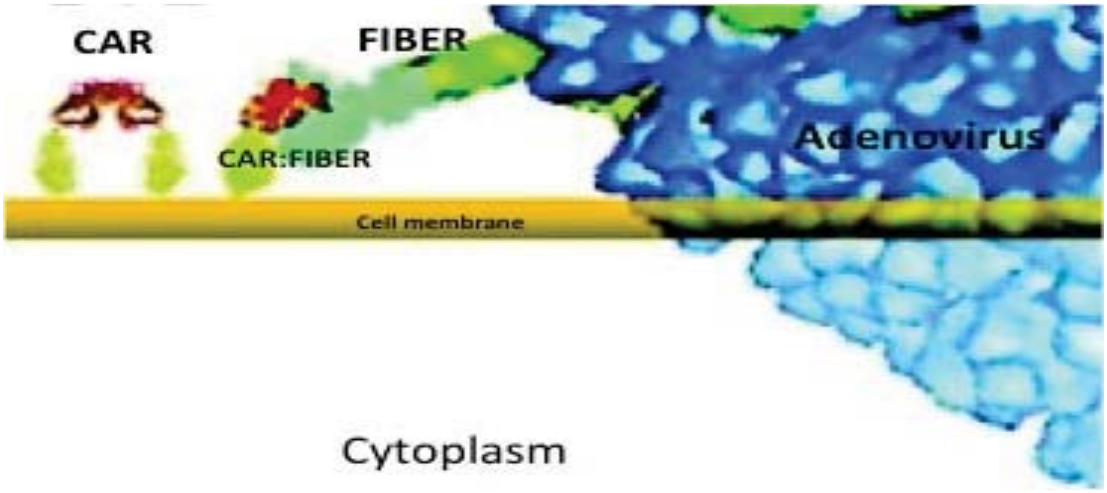


Figure 14. Interaction of fiber and CXADR receptor occurs at the onset of adenovirus infection.

Zhang and his colleagues described that CXADR is down regulated in adenovirus-infected B and T lymphocytes. In their flow cytometry analysis, they observed a rapid loss of surface CXADR in Ad5 (dl309) infected BJAB cells within 18 to 24hpi and remained low over 100 dpi . They claimed that the initial loss of surface CXADR could be due to binding and internalization of CXADR: fiber complex. Indeed, their observations showed the loss of CXADR correlates with the expression of fiber protein which can bind to CXADR and either segregate within the cell or mask its recognition by the flow cytometry. On the other hand, they expand the CXADR study to longer period of infection to examine the CXADR expression and regulation at late infection. Interestingly, very little amount of CXADR expression was observed in infected lymphocytes up to 300 dpi even though viral late genes were undetectable. To investigate more, they performed RT-PCR and western blot to analyze CXADR gene expression and protein expression, respectively. RT-PCR results showed that the levels of CXADR transcripts do not considerably change but at the 14 dpi CXADR transcripts decreased and could not be detected by 32 dp i. Additionally, the reduced levels of cellular CXADR protein were confirmed at late times in persistent infection. They concluded that CXADR mRNA and protein levels were down regulated at late times of persistent infection in Ad5 (dl309) infected BJAB cells [8]. In my study, the CXADR protein expression and gene expression were analyzed during 21 dpi in Ad5 (wt) and Ad5 (dl309) infected BJAB cells.

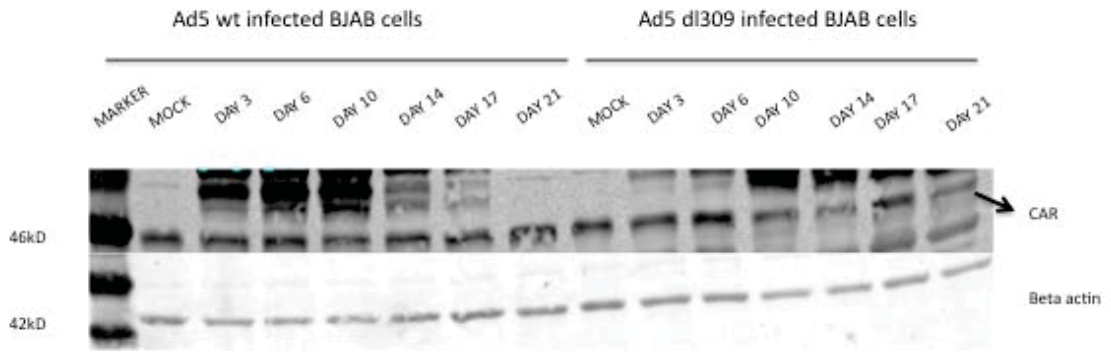


Figure 15. The western blot analysis of CXADR protein in Ad5 wt and Ad5 dl309 infected BJAB cells during 21 dpi . The expected size of CXADR (46kD) was detected. Beta actin and mock was used as loading control and positive control, respectively.

The western blot results in figure 15 showed that CXADR protein expression levels were maintained nearly same during 21 dpi in Ad5 wt infected BJAB cells even though the viral late protein expressions changed during that time period of infection (see also figure 6). However, CXADR protein expression in Ad5 (dl309) infected BJAB cells during 21dpi was subjected to particular regulation such that CXADR was considerably up regulated at 6pi and down regulated at 10dpi which was maintained to the end of infection time. At 17 dpi, a surprising effect was observed where CXADR was distinctly up regulated comparing to the former and latter CXADR protein expressions. This particular regulation had been also observed for E1A in Ad5 (dl309) infected BJAB cells (see also figure 9). To understand further whether this unique effect is on transcriptional level or not, I have performed qRT-PCR in Ad5 dl309 infected BJAB cells. The CXADR mRNA expression levels were normalized to 18S rRNA and average of technical triplicates is shown.

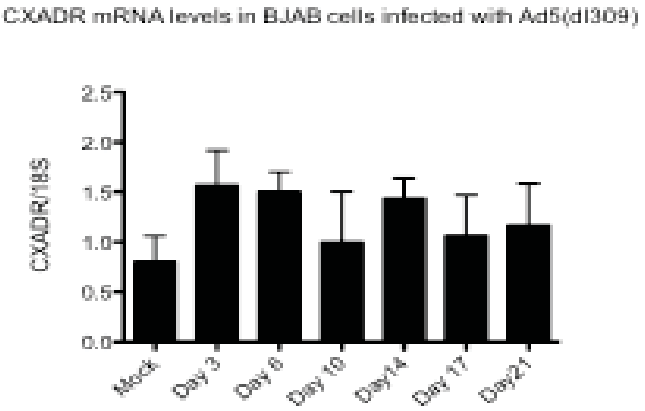


Figure 16. Gene Expression of Coxsackievirus and adenovirus receptor (CXADR) in Ad5 dl309 infected BJAB cells during 21dpi. Average of a technical triplicate was reported. The data was normalized to 18S rRNA.

CXADR mRNA levels in Ad5 (dl309) infected BJAB cells during 21dpi interestingly were not correlated with its protein expression pattern. As seen in figure 16, CXADR gene expression was considerably increased by the infection and later on fluctuations of CXADR mRNA levels were observed. However, the particular regulation of protein expression observed at 17 dpi appeared at 14 dpi for CXADR mRNA levels. Additionally, the mRNA levels were never decreased below the mock CXADR mRNA levels.

3.8 Adenoviral late gene (hexon) and early gene (E1A) expression in Ad5 wt and Ad5 dl309 infected BJAB cells during 21 dpi.

Due to observed distinct protein regulation of early and late genes together with the viral genome copy number difference in Ad5 (wt) and Ad5 (dl309) infected BJAB cells, further analyses were performed by RT-qPCR to evaluate the differences at transcriptional level. Therefore, total RNA extracts from each time point of 21 days infection including mock were analyzed by relative quantification RT-PCR. The mRNA levels of the late gene hexon was well correlated with its protein expression profile as well as the viral genome copy number in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21 dpi.

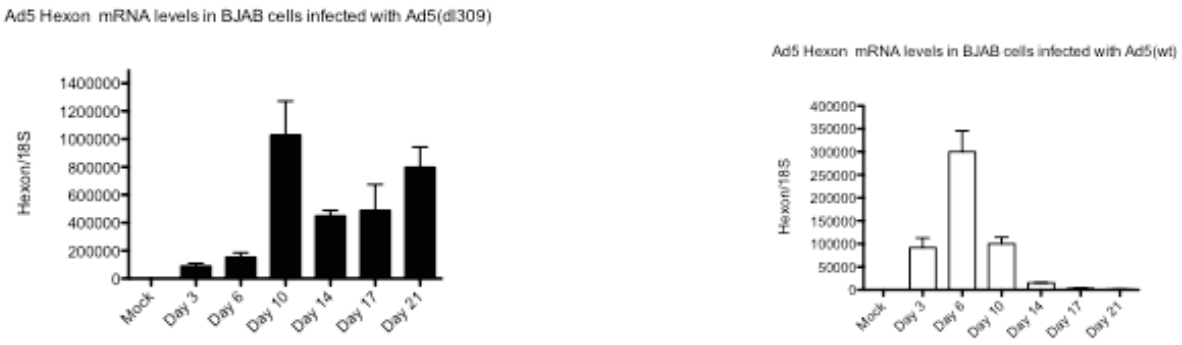


Figure 17. Expression of Ad5 Hexon mRNA in BJAB cells infected with Ad5 wt and dl309. Average of a technical triplicate was reported. The data was normalized to 18S rRNA.

As seen in figure 17, the hexon gene mRNA expression in Ad5 (wt) and Ad5 (dl309) in BJAB cells was considerably distinct from protein expression. Particularly, the hexon gene expression was slightly down regulated and disappeared after peaking at 6 dpi in Ad5 (wt) infected BJAB cells (see also figure 6). In Ad5 (dl309) infected BJAB cells hexon gene expression was down regulated after peaking at 10dpi and maintained at substantial levels until the end time point of infection. This effect was also observed at protein level; however, it was not consistent with viral genome copy number since increasing viral genome copies were observed after 10 dpi (see also figure7). This particular effect suggests that transcription was somehow silenced or down regulated which could be due to epigenetic regulation. To test further the hypothesis chromatin immunoprecipitation assay was performed on major late promoter, which is in charge of the adenoviral late genes expression (e.g. hexon).

Figure 18. Expression of Ad5 E1A (13S) mRNA in BJAB infected cells with Ad5 wt and dl309. Average of a technical triplicate was performed and relatively normalized to 18S rRNA.

The adenoviral early gene E1A expression was also examined owing to its particular protein expression regulation (figure 18). The E1A gene expression followed the similar pattern as the hexon in both Ad5 (wt) and Ad5 (dl309) infected BJAB cells (see also figure 17). In other words, the adenoviral E1A gene expression levels were decreased and lost after peaking at 6 dpi, which was also concordant with E1A protein expression levels and the viral genome copy number in BJAB cells infected with Ad5 (wt) virus (see also figure 7 and 9). However, in Ad5 (dl309) infected BJAB cells, the E1A gene expression levels were not mutually related to the viral genome copy number and protein expression regulation in terms of two differences; (i) increasing copy number after 10 dpi but less E1A gene expression was observed and (ii) a rapid up- and down-regulation of E1A protein expression at 17 dpi did not reflect to gene expression levels. Therefore, the increasing viral genome copy numbers after 10 dpi but decreasing mRNA levels led me to ascertain that epigenetic regulation might have occurred. The abundant protein expression particularly at 17 dpi even though in the presence of less mRNA levels suggested transcription of E1A gene might be enhanced. To investigate further, E1A promoter, which regulates E1A gene expression, was also consulted to ChIP assay.

3.9 EPIGENETIC REGULATION OF ADENOVIRUS GENOME STRUCTURE IN PERSISTENT INFECTION

The term of epigenetics refers heritable changes in gene expression caused by mechanisms other than changes in nucleotide sequence. It has been known that even though cells of multicellular organisms are genetically identical, they are different in genome structure and function due to the different gene expression. These stable alterations seemed to be inherited in short term without involving mutations in the nucleotide sequence. Researches over the past years have focused on the potential mechanisms that involve in epigenetic regulation. Consequently, it has been seen that

epigenetic regulation confine gene expressions by modifying genome regions to either silence or activate genes. This is accomplished by direct chemical alterations of DNA regions and by modifications of proteins that interact with gene locus. Environmental changes seem to provoke these mechanisms to moderate gene expressions as a response. It has been well known that eukaryotic gene expression is considerably complex process, which requires tight regulation at different levels (Reviewed from [10]). An effective gene expression can rely on gene transcription, RNA processing and positioning in the cell and on the output information of the mRNA by the translation machinery. Therefore, the transcription is the first and pivotal step leading to the gene expression in this cascade where the information stored in DNA is encoded to different forms of RNAs. In my study, I have dissected the potential epigenetic mechanisms that might have played significant roles in particular alterations distinctly observed in Ad5 (wt) and Ad5 (dl309) infected BJAB cells during 21dpi. There are strong evidences showing that in infected cells, the chromatin structure of adenovirus genome DNA plays significant roles in its genome functions [2]. However, how the viral chromatin structure is regulated during the long-term adenovirus infection remains enigmatic.

In this study, I performed chromatin immunoprecipitation assay (ChIP) in adenovirus infected BJAB cells to enlighten the regulatory mechanism of how the chromatin structure is formed during the long-term infection of adenovirus. In addition, I was interested in understanding how are the E1A and Hexon gene expression regulated in Ad5 (dl309) infected BJAB cells.

3.9.1 Chromatin immunoprecipitation assay (ChIP)

Direct and indirect protein-DNA interactions are known to play critical roles in regulation of chromosomal functions such as DNA replication, gene expression, and chromosome segregation. Therefore, understanding protein and DNA interactions can illuminate the mechanisms that execute chromosomal functions, structure and organization. Chromatin immunoprecipitation is an elegant technique to study the interactions between modified or unmodified proteins and specific genomic regions within the natural ambiance of the cell. In order to designate proteins located in the chromatin and specific genomic regions to which they interact, the chromatin is first divided into workable pieces and proteins of interest are fished out. Eventually, isolated DNA from precipitated chromatin can be analyzed by either conventional PCR or quantitative PCR.

3.9.2 Overview of the method

Proteins are cross-linked to DNA with formaldehyde followed by chromatin fragmentation by sonication. The proteins of interest cross linked to DNA are immunoprecipitated with specific antibodies attached to magnetic beads. The precipitated cross-links are reversed. Eventually, the isolated DNA is analyzed by absolute quantification PCR or conventional PCR.

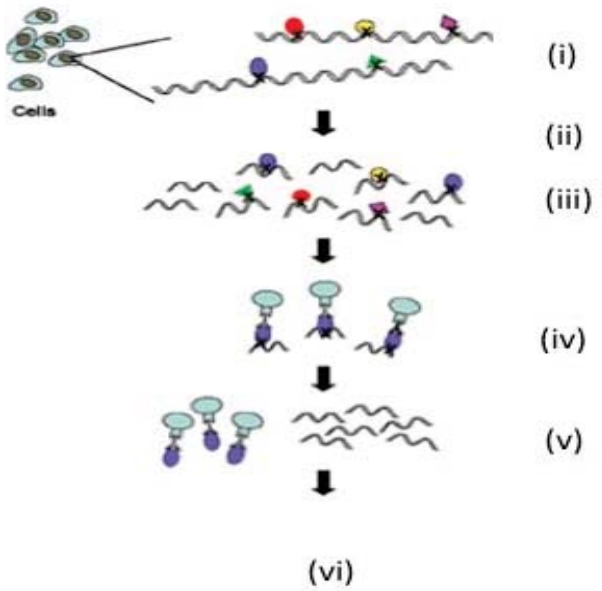


Figure 19. The chromatin immunoprecipitation assay is done in the following order; (i) cross linking, (ii) cell lysis, (iii) sonication, (iv) immunoprecipitation, (v) reversion of cross-links, and (vi) analysis with PCR.

In my study, I collected samples ($5x10^7$ cells/ml) from each time point during the 21 days infection course and cross-linked cells with 1% formaldehyde for 10 minutes. Afterwards, cells were lysed with lysis buffer (RIPA buffer). An efficient cell lysis affects the yield of genomic DNA and it is crucial to reliably compare results among a group of samples. The released cross-linked genomic DNA by cell lysis was sheared by sonication into small sizes (150-350 bp). Sonication is a critical step in ChIP assay to achieve a good resolution between DNA sequence that is bound to protein of interest and nearby DNA sequence that does not bind to that protein. Therefore, the shear size should be tested by electrophoresing the sonicated DNA on 1% agarose gel. After confirming the sonication efficiency, the sheared chromatin, which governs the specific DNA region bound to protein of interest, was subjected to immunoprecipitation with high affinity antibodies. The concentration and specificity of antibodies are critical to obtain accurate amounts of protein-DNA complexes of interest. In order to prevent unspecific bindings, samples should be pre-cleared with magnetic beads before adding antibodies. The complex was co-precipitated by adding antibodies specific proteins and captured with protein A/G coupled magnetic beads. The precipitated protein-DNA complex was eluted with elution buffer from antibodies. The outcome elutes consist of only DNA-protein

complex of interest. To be able to analyze these specific genomic regions, the cross-link should be reversed. Therefore, the elutes were subjected to enzyme (proteinase K) and heat (65^0C, O/N) to digest the cross-linked proteins. Finally the eluates were analyzed by quantitative PCR (qPCR).

3.9.3 Analysis of precipitated material by QPCR and data normalization

In this study three control samples specific for ChIP experiment were included: the input sample, the bead control sample and the mock sample. The input sample is one of the most commonly used control and data normalization method in ChIP assay. It indicates the presence and amount of chromatin in the ChIP reaction. Therefore, 1 % of input sample was taken from chromatin before pre-clearing steps. Bead control consists of the chromatin sample with no antibody is used to obtain information about the amount of background signal relative to the level of the signal of ChIP sample which can be generated during the ChIP assay. Therefore, bead samples were taken from the step where chromatin samples were incubated with beads without addition of antibodies and used as negative control. The normalized data can be announced as fold enrichment, which refers the ChIP signal as a fold increase in signal relative to the background signal. In this study, the data is shown as "% of input", which reflects the chromatin recovery percentages by the antibodies. The bead control was used to observe background signals. Another control included in this ChIP assay was the mock sample. The mock sample includes the chromatin from uninfected cells therefore it is a good negative control which should not yield any PCR product with primers specific to viral genome.

I have performed ChIP qPCR on E1A and MLP of Ad5 (dl309) infected BJAB cell samples taken from 10 dpi and 21 dpi, where the viral mRNA accumulation and viral DNA amount seemed to undergo a regulation (Figures 7, 17 and 18).

3.9.4 Histone deposition at adenoviral E1A and MLP promoters at 10 dpi and 21 dpi in Ad5 (dl309) infected BJAB cells

As mentioned earlier, transcription is regulated at different levels; histone modification and chromatin remodeling is one of the most important epigenetic mechanism that plays significant roles in gene expression. In the cell nucleus, the genomic DNA is organized into chromatin that is made of nucleosomes. The nucleosomes are basic units of chromatin, consisting of 146 bp of DNA wrapped around four histone core proteins (H2A, H2B, H3 and H4). Furthermore, the core histones exhibit the histone tails, which protrude from the nucleosomes. The deposition of histones and the modulation of the chromatin structure are critical for the regulation of gene expression, since the packaging of DNA prevents the accessibility of transcription factors to DNA. Furthermore, the chemical modifications of chromatin such as DNA methylation and posttranscriptional modifications such as phosphorylation, acetylation, methylation and ubiquitylation of histone tails can also alter the chromatin structure and act on gene expression. However, the regulation of chromatin structure are not only restricted to the cellular genome, some viruses also have chromatin like structures with their own genome. The adenovirus genome is incorporated with core proteins that are

known to pack its DNA. The recent report by Komatsu and Nagata showed that cellular histone proteins were also involved in the viral genome in the early infection of adenovirus [2]. Taken all together, here, I have dissected the molecular mechanisms involved in the chromatin regulation in Ad5 (dl309) infected BJAB cells. Particularly, H3 protein deposition as well as H3 acetylation (at residues H3K9 and H3K14) and H3 lysine 9 trimethylation (H3K9me3) were studied. In general, acetylations of histone H3 tail are known to play critical roles in the initiation and enhancement of transcription whereas the methylation of histone 3 on lysine 9 signals gene repression.

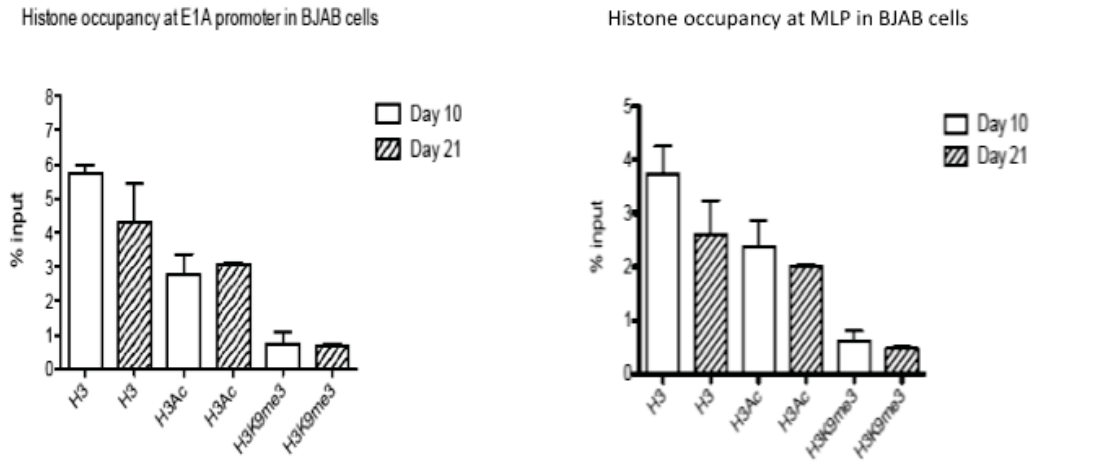


Figure 20. Histone occupancy at E1A and major late promoter in Ad5 (dl309) infected BJAB cells, particularly at 10dpi and 21dpi.The data is shown as % of input. The average of technical triplicates is shown. The Mock was not included due to lack of adenovirus genome in this sample.

In Ad5 (dl309) infected BJAB cells at 10 dpi and 21 dpi , unexpectedly, no significant changes in H3 occupancy could be observed (figure 20). However, the total histone H3 occupancy at 21 dpi was slightly less than 10 dpi for both E1A and major late promoters. In general, the acetylated H3 (H3Ac) was detected more than methylated histone 3 (H3K9me3) for both promoters, which is reasonable since E1A and hexon genes were intensely expressed at that times (see also viral genome copy numbers and gene expressions on figures). The decrement of histone 3 occupancy between 10 dpi and 21 dpi was also reflected to gene expression level at which E1A and hexon genes were down regulated. However, the findings were not sufficient to explain the reason why E1A and hexon gene expressions were down regulated in the presence of high viral genome copy number at 21 dpi even though the histone occupancy negligibly changed between 10 dpi and 21 dpi. These results led me to question another mechanism, which might have particularly involved at that time points. To test further, I performed ChIP assay on RNA polymerase II (RNAPII) and particularly serine5 phosphorylation at carboxy-terminal domain of RNAPII at day 10 dpi and 21 dpi in Ad5 (dl309) infected BJAB cells. As known to date, RNAPII transcribes protein-encoding genes to produce mRNA. The RNAPII largest subunit has a particular feature by presenting a heptapeptide sequence

(YSPTSPS) repeat (52 times repeated in humans) at the carboxy-terminal domain (CTD) of the subunit. The CTD undergoes intensive serine phosphorylation and dephosphorylation during transcription, particularly at serine 2 (Ser2P) and 5 (Ser5P). The transcription is initiated with hypo-phosphorylated RNAPII at the promoter and hyperphosphorylation at serine 5 o f CTD is required for promoter clearance and transition from initiation to elongation step of transcription [11]. It has been known that alterations in the phosphorylation status of specific CTD residues affect the recruitment of transcription factors together with RNAP II processivity and activity. In infected cells, adenovirus utilizes the cellular RNAPII to transcribe its genome. Therefore, the performance of RNAPII could have contributed to the particular gene expression pattern observed in infected BJAB cells after 10dpi and 21dpi.

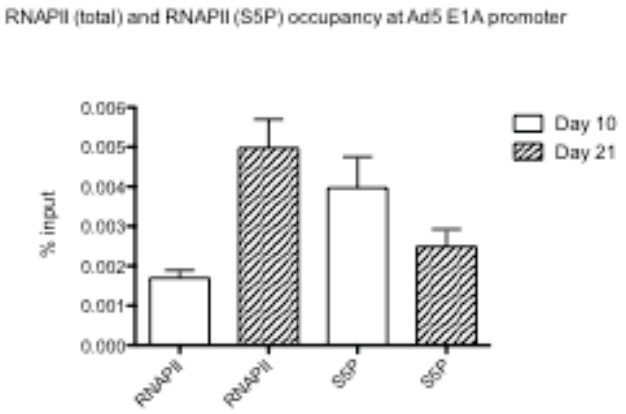


Figure 21. RNAPII occupancy at E1A promoter and CTD phosphorylation on serine 5 in Ad5 (dl309) ınfected BJAB cells. ChIP was performed with anti-RNAPII and S5P antibodies The data is expressed as 1% input and the average of technical triplicate was performed.

The ChIP results showed that the RNAPII occupancy at E1A promoter was considerably increased from 10 dpi to 21 dpi in Ad5 (dl309) infected BJAB cells. This observation was consistent with the viral genome copy number, increasing from 10 dpi to 21 dpi. However, CTD Ser5 phosphorylation showed an astonishing pattern at 10 dpi and 21 dpi. As seen in figure 21, phosphorylation on serine 5 was significantly reduced at 21 dpi. Due to the facts mentioned about the serine5 phosphorylation, which plays critical roles in the regulation of gene expression, the finding can enlighten the question why the reduction in gene expression occurred at 21 dpi in the presence of high viral genome copy number. Apparently, RNAPII activity was demolished by the dephosphorylation on serine 5 of CTD and consequently the gene expression of E1A was down regulated at 21 dpi. To ensure whether this modification was unique to adenoviral gene and adenovirus infection, a c ellular gene (GAPDH) was tested. The GAPDH, which is a housekeeping gene, should be constitutively expressed even in adenovirus-infected cells. Therefore, ChIP assay was performed at GAPDH promoter to analyze the RNAPII occupancy and specifically Ser5 phosphorylation in the mock sample as well as Ad5 (dl309) infected BJAB cells at 10 dpi and 21 dpi. Indeed, the particular phosphorylation regulation of Ser5 observed at 21 dpi was unique to viral genome and adenovirus infection. As seen in

figure 22, Ser5 phosphorylation of RNAPII at GADPDH promoter at 21 dpi was more than that at 10 dpi. This observation was just the opposite of what have been observed in Ad5 (dl309) infected BJAB cells at 10 dpi and 21 dpi. Additionally, by the infection, the Ser5 phosphorylation was inhibited; however the RNAPII occupancy at the GAPDH promoter did not change as seen at the same levels in the mock and in the infected cells, suggesting that Ser5 phosphorylation is preferentially and particularly regulated in the adenovirus infected cells.

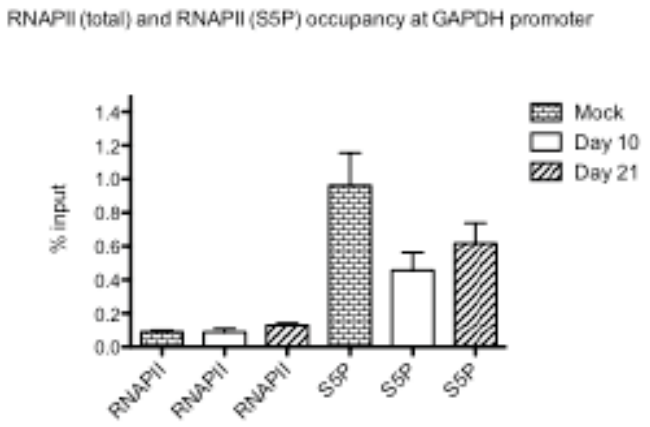


Figure 22: ChIP with anti-RNAPII and Ser5P antibodies on GAPDH promoter. The data is shown as % of input from the average of technical triplicates.

Previous studies have indicated that the phosphorylation status of serine residues of RNAPII carboxy-terminal domain is specifically regulated by virus infection. In a supporting manner, my results showed that Ad5 (dl309) infection in BJAB cells led to a striking loss of Ser5 phosphorylation on the RNAPII CTD. I speculate that this helps to create *de novo* hypophosphorylated form of RNAPII that can affect the activity of transcription and consequently reduce the efficiency of adenovirus gene expression. Therefore, I concluded from my ChIP assay experiments that remodeling chromatin structure and/or histone modifications do not play significant roles in the regulation of adenovirus gene expression rather it causes the diminution of RNAPII CTD Ser5 phosphorylation, which is probably required for the establishment of persistent infection. This interesting observation should be further confirmed by performing the same ChIP assay on the lytic infection of adenovirus to observe whether this effect is unique to adenovirus persistent infection.

4. CONCLUSIONS and FUTURE PERSPECTIVES

The scopes of this thesis were to establish a long-term adenovirus infection model system in BJAB cells (B cells) and to elucidate both molecular mechanisms and epigenetic regulation underlying the adenovirus persistent infection that is not familiar to science community. The project was treated in several perspectives to unravel the mechanism behind the adenovirus persistency in lymphocytes. For this purpose, the project was supplemented with variety of methods to analyze the samples collected from both wild type and mutant type adenovirus infected BJAB cells during 21 days of post infection along with uninfected BJAB cells. The following conclusions for the project can be drawn:

Firstly, I achieved to set up a model system which provides an extended period of infection time compared to other cell lines commonly used in adenovirus studies and show lytic infection (short-term infection) such as HeLa, 911, HEK293 cell lines. I also provided two significant features of adenovirus during the observed time period of infection in Ad5 wt and Ad5 (dl309) infected BJAB cells as shown by viral genome copy numbers. Due to the fact that viral DNA levels in Ad5 (wt) infected BJAB cells fell below the detection limit by the end of infection whereas it was increased and maintained high levels of copy numbers at 21dpi in Ad5 (dl309) infected BJAB cells, I concluded that persistent infection can be established in the Ad5 (dl309) infected BJAB cells while Ad5 (wt) infected BJAB cells behaved as lytic infection in long term. This achievement should be further analyzed by prolonging the infection period to see the changes in viral copy number after 21 d pi where it peaked. After establishing long-term infection of adenovirus in BJAB cells, I have studied the viral and host cellular protein regulation/modifications as well as their interactions to investigate their potential crucial roles in long-term infection. The findings concluded that p53 pathway is distinctively, yet significantly regulated in long-term infection of adenovirus comparing to pRb pathway. Additionally, conspicuous *de novo* posttranslational modifications of proteins were discovered in this study, which were also regulated differently in Ad5 (wt) and Ad5 (dl309) infected BJAB cells. In future studies, the de novo modifications should be specifically determined in order to define their essential roles in adenovirus latency. In order to obtain better understanding of the function of p53 and pRb proteins in long-term adenovirus infection, their expression profiles as well as potential modification status should be assessed in lytic infected cells. In addition to cellular proteins that play significant roles in cell cycle progression, Ago2, Dicer and PKR proteins assigned in RNAi-microRNA pathway and in host cell translation machinery were also dissected in my project. My results suggested that microRNA pathway is crucially regulated in long-term adenovirus infection and also differently regulated in Ad5 (wt) and (dl309) infected BJAB cells. To analyze further its significance in adenovirus latency, the studied proteins should be also analyzed in lytic infected cells.

The coxsackie adenovirus receptor (CXADR) specifically and commonly targeted by serotype C adenovirus to initiate infection was also studied in order to observe its regulation on protein level as well as on transcription level during long term infection of

adenovirus. On protein level, CXADR was not significantly regulated in Ad5 (dl309) infected cells by comparison to Ad5 (wt) infected cells. However, this feature could not be observed on mRNA level. To test the inconsistency between the gene expression and the protein expression further, chromatin immunoprecipitation assay should be performed in future studies.

The second part of my project focus on the epigenetic regulation in order to understand how adenovirus regulates its genome and reprograms the host chromatin during persistent/latent infection. Additionally, down regulated gene expression pattern of E1A and hexon after 10dpi while the viral genome copy number significantly increased after 10 dpi until the end of infection period in Ad5 (dl309) BJAB cells was also dissected by chromatin immunoprecipitation assay. ChIP results have shown that histone proteins are intact with adenoviral genome and they have undergone posttranslational modifications such as methylation and acetylation. Interestingly, the histone depositions and modifications were not significant between 10dpi and 21dpi in which the most striking and major alterations of gene expression occurred. The alternative explanation was revealed by another ChIP assay performed on s erine 5 phosphorylation of CTD of RNAPII. The hypophosphorylated serine 5 at 21 dpi most likely reduced the polymerase activity and consequently adenoviral E1A gene could not be successfully transcribed. This striking and unique phenomenon observed on phosphorylated serine 5 may elucidate the mechanism of adenovirus persistency in lymphocytes. However, the phenomena should be further confirmed with prolonged infection period and also evaluated in lytic infected cells to ensure that it is specific to long-term adenovirus infection. Additionally, serine 2 a nd 7 phosphorylation that also play crucial roles in RNAPII activity and processivity should be tested for further understanding of serine phosphorylation status in adenovirus persistent infection.

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