ΑΛΕΞΑΝΔΡΕΙΟ ΤΕΧΝΟΛΟΓΙΚΟ ΕΚΠΑΙΔΕΥΤΙΚΟ

ΙΔΡΥΜΑ ΘΕΣΣΑΛΟΝΙΚΗΣ

ΣΧΟΛΗ ΕΠΑΓΓΕΛΜΑΤΩΝ ΥΓΕΙΑΣ ΚΑΙ ΠΡΟΝΟΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΩΝ ΕΡΓΑΣΤΗΡΙΩΝ

ΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

Generation of KO cell lines of APOBEC 3A, 3B and 3G using CRISPR/Cas9

πτυχιακή εργασία

της

ΜΑΡΙΝΑΣ ΤΥΠΟΥ

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MAX-PLANCK-GESELLSCHAFT

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List of Abbreviations

APS – Ammonium Persulfate

Ctr – *Chlamydia trachomatis*

Ctr L2 – *Chlamydia trachomatis* serovar L2

DNA – deoxyribonucleic Acid

EB – elementary body

LB – liquid broth

MOI – multiplicity of infection

PCR – polymerase chain reaction

qPCR– quantitative PCR or real-time PCR

RB – reticulate body

RT – room temperature

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel ele*Ctr*ophoresis

SOC – super optimal broth

TEMED – tetramethylethylenediamine

APOBEC– apolipoprotein B mRNA editing enzyme

RNA – Ribonucleic acid

gRNA– guide Ribonucleic acid

CRISPR– Clustered Regularly Interspaced Short Palindromic Repeats

HPV–Human papillomavirus infection

LGV– Lymphogranuloma Venereum

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Preface

Cervical cancer is one of the most common cancers in women with a mortality rate of over 50%. It mainly occurs as two histologically distinct types – adenocarcinoma (ADC) and squamous cell carcinoma (SCC), rarely other entities are seen. SCC accounts for ~90% of cases and has an established causal link to human papilloma virus (HPV) infections. Cervical cancer is the fourth most common cancer globally with a mortality rate of over 50%. Human papilloma virus (HPV) infections are considered as the main cause of cervical cancers. However, only two percent of the HPV infected women develop cervical cancers. Therefore, a number of co-factors are implicated to be essential for carcinogenesis. The gram-negative obligate intracellular bacterial pathogen *Chlamydia trachomatis* (*C. trachomatis) which causes most* frequent sexually transmitted infections is one of the major co-factor in HPV induced cervical carcinogenesis.

Previous studies have indicated that the apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) family members of cytidine deaminases to be upregulated and contribute to the development of mutations found in cervical cancers. Although the HPV oncoproteins E7 is implicated in the enhanced expression of some of these APOBEC family members the molecular mechanisms are not yet clear. Interestingly, transcriptomic analysis of *C. trachomatis* infected mouse, HPV E6E7 positive endocervical primary epithelial cells (END1/E6E7) and HPVe negative primary human ectocervical cells reveled increased expression of APOBEC3A, APOBEC3B and APOBEC3G, irrespective HPV status (*Chumduri, Gurumurthy et al, unpublished*).

This project involves investigation of the mechanism and consequences of upregulation of these APOBEC family members during *C. trachomatis* infection. Towards this the bachelor thesis project encompasses following objectives (a) Generate CRISPR/Cas9 mediated knockout cells lines of Apobec3B, Apobec3A, Apobec3G, (b) Confirm the Knockouts, (c) Investigate the effects of knockouts during *C. trachomatis* infection.

1. Theoretical Part

1.1Cervical Cancer

The cervix is part of a woman's reproductive system. It is in the pelvis and is the lower part of the uterus and is a fibrous tube that brings the uterine body into contact with the vagina.

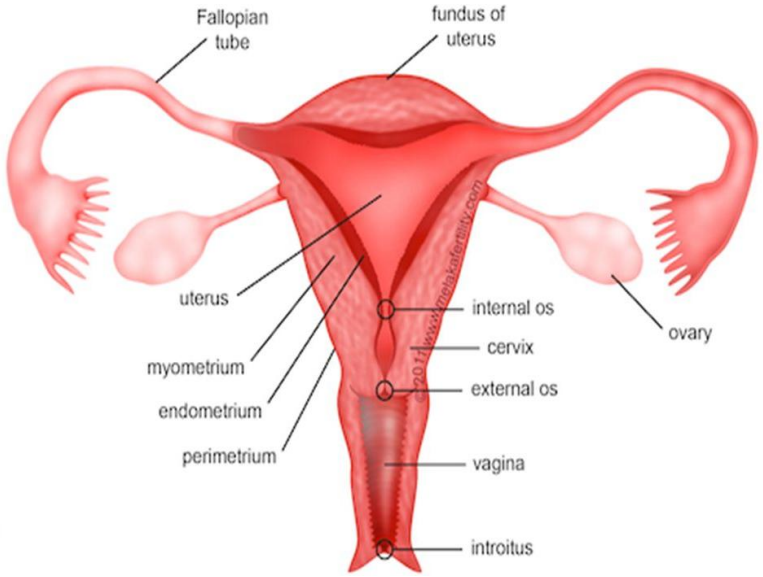

Figure 1. Female reproductive system [4]

Cervical cancer is a malignant tumor in the tissues of the cervix. In general, all types of cancer start in cells, which are the structural elements of the tissues. All organs, including cervix, are made up of tissues. Normal cells are divide and form new cells since our body needs new cells to replace the already old ones. Although, sometimes this process faces some damages. It has been observed that sometimes new cells are born when the body does not need them and the old cells or cells with damages do not die. This has the consequence of the accumulation of cells, which forms the tissue mass known as tumor or growth. There are two kinds of cervix growths [5]

- Benign (not cancer)
- Malignant(cancer)

Cervical cancer begins to develop in the cervical epithelial cells. Over time can spread through the lymph vessels to the lymph nodes as well as to the lungs, bones and liver through the blood vessels.[5]

1.1.1 Statistical data

Cervical Cancer is a major health problem worldwide, according to WHO statistics it is the seventh most common in women in the European Region. In 2008, there were more than 61 000 women who were diagnosed with cervical cancer and about 28 000 deaths. According to these statistics, cervical cancers kills 1 in 25 000 women in the European Region. It is considered the second most common cancer in women between the age of 15 to 44 years.

The pleasing fact is that mortality rates are decreasing over time from 6,35 deaths per 100.000 people (1980) to 4,47 deaths per 100.000 people (2009). There is a big variation of mortality rates among countries, with the higher levels in eastern Europe [6].

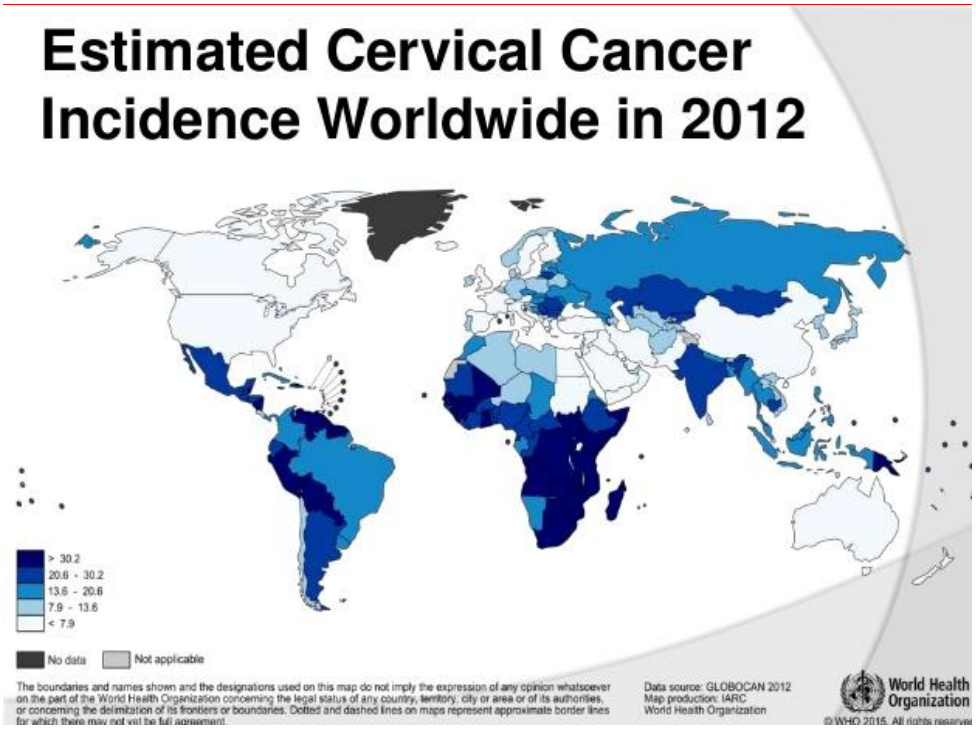

Figure 2. Rate of Cervical Cancer Worldwide, WHO 2012[7]

1.1.2 Symptoms

At an early stage of cancer, there are usually no symptoms. Unlike, at an advanced stage of cancer the most common symptoms are: Vaginal bleeding between periods, bleeding after intercourse, intercourse pain, unusual vaginal discharge, vaginal bleeding after menopause, excessive tiredness, leg pain or swelling and low back pain.[5]

1.1.3 Diagnosis

Examination of the cervix is essential for the detection and diagnosis of cancer. The diagnosis of cancer can be done in the following ways: Lab tests (pap test), Cervical exam (colposcopy), Tissue sample (biopsy) [5].

1.1.4 Treatments

The treatment options depend mainly on the stage of the disease (size of the tumor, depth of filtration, and spread of the disease to adjacent or other organs of the body). Possible treatments for women with cervical cancer can be surgery, radiation therapy, chemotherapy or a combination of these [5].

1.1.5 Stages of cancer

The results of tests like MRI, CT, X-Ray are taken together with the results of the initial tumor biopsy to determine the stage of cervical cancer. Cervical cancer includes the following stages:

Stage 0 - Abnormal cells are found only on the first layer of cells that cover the uterine cervix.

Stage I - The tumor is located only in the tissues of the cervix.

Stage II - The tumor has spread to the cervix, the vagina and the tissues around the cervix.

Stage III - The tumor has spread throughout the pelvic region.

Stage IV - The tumor has spread beyond the pelvic region to nearby organs such as the bladder or the rectum. The tumor can also spread to the lungs, liver or bones, although this is unusual [5].

1.1.6 Risk Factors

Studies have found that infection with the virus of HPV is the major risk factor of almost all cervical cancers. However, there are a number of other risk factors implicated in the development of cervical cancers such as smoking and infections with stecually transmitted infections with *C. trachomatis* [5].

1.2 Human papillomavirus (HPV)

Human papillomavirus (HPV) is a very widespread virus, most people (80%), men and women will come into contact with this virus at some point in their lives without even realizing it. HPV is transmitted by sexual route. Infection is particularly common at the age of 18-22 years. HPV infection does not cause symptoms (except for types 6 and 11 that cause warts). Factors, which increase the likelihood of HPV infection are 1) a large number of sexual partners, 2) smoking and 3) a large number of children. The most effective way of precaution is the vaccination. The treatment is intended to remove - destroy the HPV damage so that they do not lead to cancer. The methods commonly used for treatment are:

- The cryopause (ie destruction of the tissue by freezing), an earlier method that tends to be abandoned because the depth of tissue destruction is not precisely controlled.

- LLETZ / LEEP (tissue removal with diathermy), a very good method for relatively small and symmetrical lesions, with local anesthesia.

- Laser, a very good method for asymmetric and/or major lesions, with local anesthesia

The life cycle of HPV is coupled directly to the biology of their host cell. HPV virions infect the basal layers of the stratified epithelium, through micro wounds and enter cells via interaction with certain receptors such as α-6 integrin for HPV16.

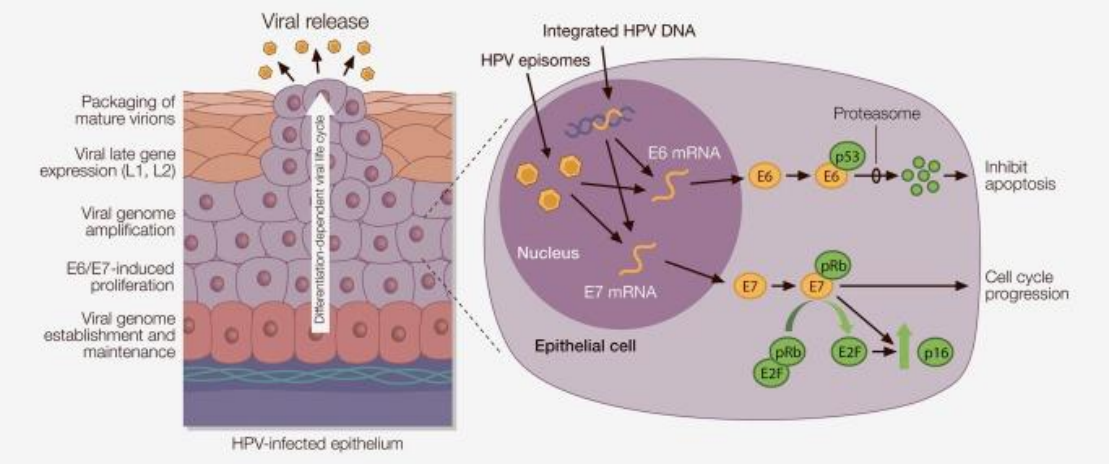

Figure 3. HPV Life cycle [8]

The human papillomavirus (HPV) has a circular double-stranded DNA molecule genome of around 8000 base pairs. So far more than 100 HPV genotypes are described and almost 40 of them infect the genital mucosa. These HPV genotypes are categorized into low or high-risk genotypes, according to the damage they cause in the cervix. Unfortunately, high-risk types of HPV are related with more than 90% of cervical cancers.[9]

1.2.1 Cervical Cancer and HPV

By the year 2000, the epidemiological studies had identified an essential and definite affiliation between HPV infections and cervical cancer. More recently studies, have undoubtedly shown that HPV infection preceeds the development of cervical cancer even for several years. Specifically, HPV DNA was observed in 90–100% of cervical cancer cases. This strongly, shows that most of the cervical cancers are caused by HPV infection [10]. HPV is known as the first, considerable cause of human cancer. In substance, the definition of considerable cause concerns that cervical cancer will not develop in the absence of HPV DNA existence.

HPV TYPE	HSIL		CANCER	
	N° of patients (N° of Studies)	Prevalence % (95% CI)	N° of patients (N° of Studies)	Prevalence % (95% CI)
Global	**2446 (52)**		**5540 (62)**	
Any	1749 (36)	82.5 (77.3–87.1)	3435 (43)	89.0 (84.3–92.9)
Type 6	1415 (29)	4.2 (2.2–6.7)	2274 (32)	1.7 (0.9–2.8)
Type 11	1414 (29)	2.4 (1.3–3.8)	2274 (32)	1.3 (0.5–2.5)
Type 16	2327 (49)	46.5 (41.3–51.7)	5463 (60)	53.2 (49.1–57.2)
Type 18	2194 (45)	8.9 (6.3–11.8)	4962 (56)	13.2 (11.0–15.6)
Type 31	1785 (36)	8.0 (6.0–10.4)	3903 (45)	7.5 (5.5–9.8)
Type 33	1722 (35)	6.5 (4.7–8.5)	3821 (42)	4.3 (3.2–5.5)
Type 35	1228 (24)	3.0 (1.9–4.4)	2332 (31)	2.0 (1.3–2.7)
Type 39	885 (20)	2.4 (1.5–3.5)	1977 (27)	1.8 (1.3–2.4)
Type 45	1077 (24)	3.9 (2.8–5.2)	3389 (37)	4.6 (3.5–5.7)
Type 51	1013 (21)	3.7 (2.1–5.7)	2131 (30)	2.1 (1.1–3.3)
Type 52	1152 (25)	4.9 (2.9–7.4)	2544 (34)	3.2 (2.1–4.4)
Type 56	892 (19)	2.4 (1.5–3.4)	2155 (28)	1.2 (0.8–1.7)
Type 58	1197 (26)	8.7 (6.0–11.9)	2564 (34)	3.0 (2.1–4.1)
Type 59	954 (21)	1.9 (1.2–2.9)	2199 (30)	1.6 (1.1–2.2)
Type 66	926 (20)	1.8 (1.1–2.8)	2095 (28)	1.1 (0.7–1.6)

Figure 4. HPV comprarison to cancer and HSIL(High Grade Squamous Intraepithilial Lesion) [11]

Some types of HPV are referred as "low-risk" types because they very rarely cause lesions that develop into cancer. Types of HPV that are likely to lead to cancer, are referred as "high risk". Both types can cause the growth of abnormal cells, but only "high risk" HPV types lead to cancer. Sexually transmitted HPV types of high risk types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73. These cause neoplasms in the uterine cervix, which are usually flat, almost invisible and appear only with colposcopy. Types of HPV-16 and HPV-18 together cause 70% of cervical cancers. It is very important to note that the vast majority of "high risk" HPV types of infections disappear on their own and do not cause cancer. Furthermore, HPV infection is considered to cause a large number of anal, vulvar, vaginal and penile cancers. Also, studies have shown that there is a great correlation between the existence of HPV and the development of head and neck cancer [12].

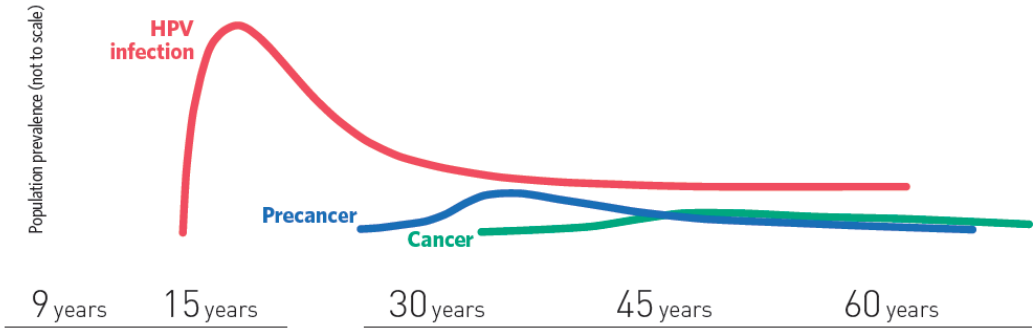

Figure 5. The HPV infection and the appearance of the cancer relatively of the age[6]

Further, it is shown that women, who are positive for HPV16/18/45 they are 4.2 times more prone to develop cervical intraepithelial neoplasia grade 2 than those with other HPV types [13].

When HPV infection persists, some cervical cells develop lesions (dysplasia) and these lesions evolve first in precancerous and then invasive cancer. Investigations have shown that there are two important HPV genes (E6, E7) with a crucial role. It is shown that, E6 promotes the degeneration of p53, a tumor suppressor gene, via the interaction with E6AP (an E3 ubiquitin ligase) while the E7 binds the retinoblastoma protein (pRb) and switchon the E2F transcription factor leading to expression of pro proliferative p16. Interestingly, research has shown that these two oncogenes (E6, E7) play an essential role in the development of HPV induced cervical cancer. Increased expression of these genes has been observed, presumably due to the incorporation of viral DNA into the host cell genome in rapidly evolving cancers [9].

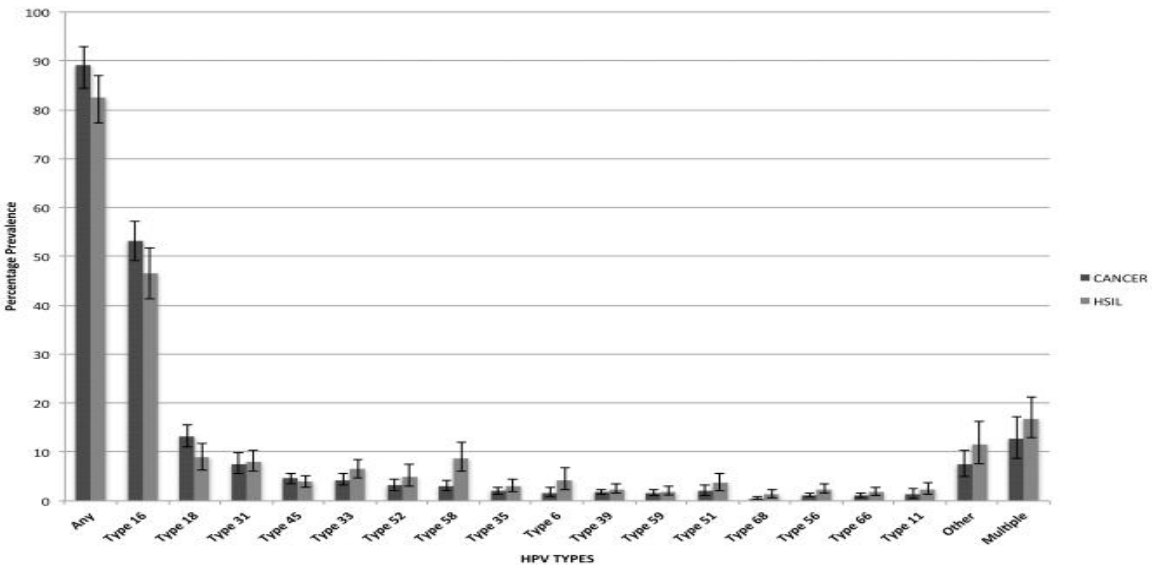

Figure 6.HPV association with cervical cancer and HSIL(High Grade Squamous Intraepithilial Lesion) [11]

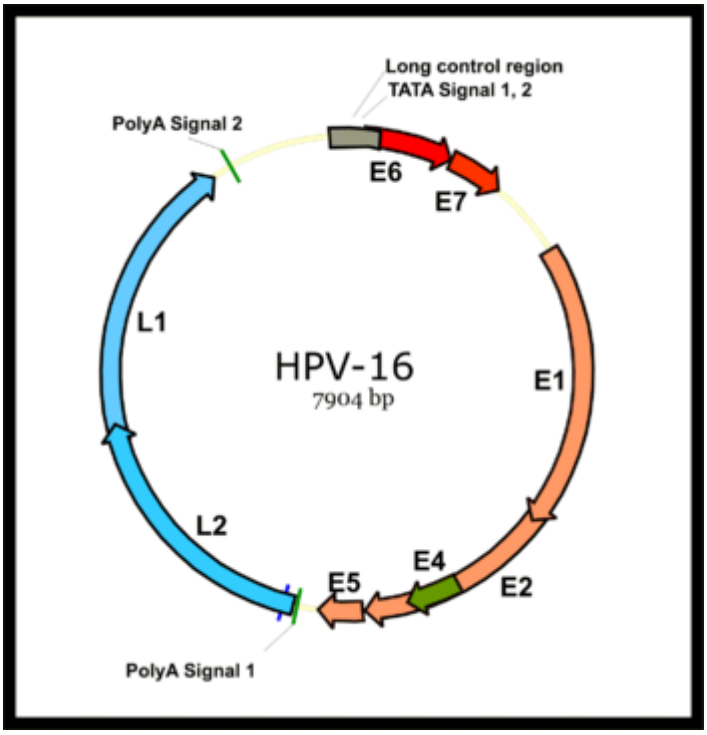

Figure 7. Genome organization of Human papillomavirus type 16[14]

1.3 *Chlamydia spp*

C.trachomatis is a causative agent of wide range of diseases including trachoma a preventable blinding disease and sexually transmitted disease (STD) that can lead to permanent damage to the reproductive system of women as well as ectopic pregnancy.

C.trachomatis is member of the *Chlamydiaceae* family and are small, non-mobile, Gram- negative, obligatory intracellular organisms growing in the host cell cytoplasm. Are sensitive to antibiotic therapy and resemble viruses, requiring live cells to multiply [15] and they consist genome of 1 to 1.2 Mega bases. The genus of *Chlamydia* includes the following species: *Chlamydia (C.) trachomatis, C. muridarum, C. pneumoniae, C. pecorum, C. suis, C. abortus, C. felis, C. caviae,* and *C. psittaci*. However, two genera of Chlamydia are clinical significance for humans the *C. trachomatis* and the *C. pneumoniae* [15]. Nonetheless, studies have shown that there are more than 250 unexplored families of *Chlamydia* [15, 16].

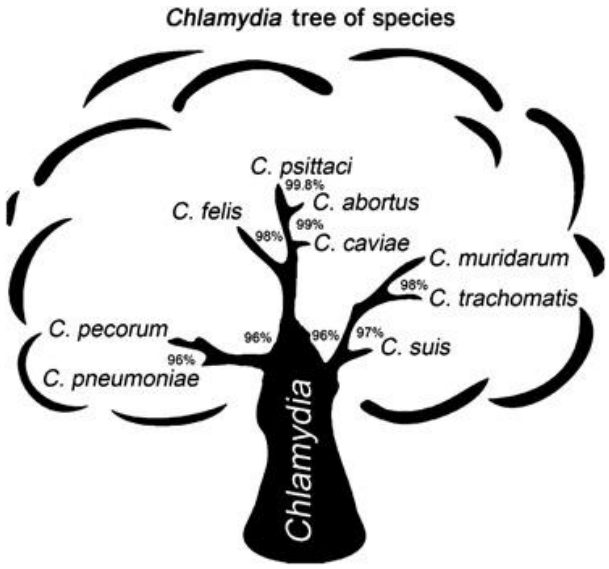

Figure 8. *Chlamydia* family [17]

1.3.1 Life cycle

Although, *Chlamydia* go through an extracellular stage they only replicate in the host cell within and intracellular vacuoles termed as inclusion. Accordingly, *Chlamydia* have biphasic life cycle and is composed of two stages: 1) the elementary bodies (EBs) which are 0.25 to 0.3 μm in diameter and 2) reticulate bodies (RBs) of 0.5 to 0.6 μm in diameter [18, 19]. In the first stage, the EBs infect the eukaryotic cells and are located within a cytoplasmic vacuole, known as inclusion, the EBs are converted to replicable RBs by the host cell entry. RBs then replicate by several cycles binary fission. Towards the end of the life cycle RBs re-differentiate back to EBs, which in turn are released into the extracellular environment through the lysis of the host cell [18].

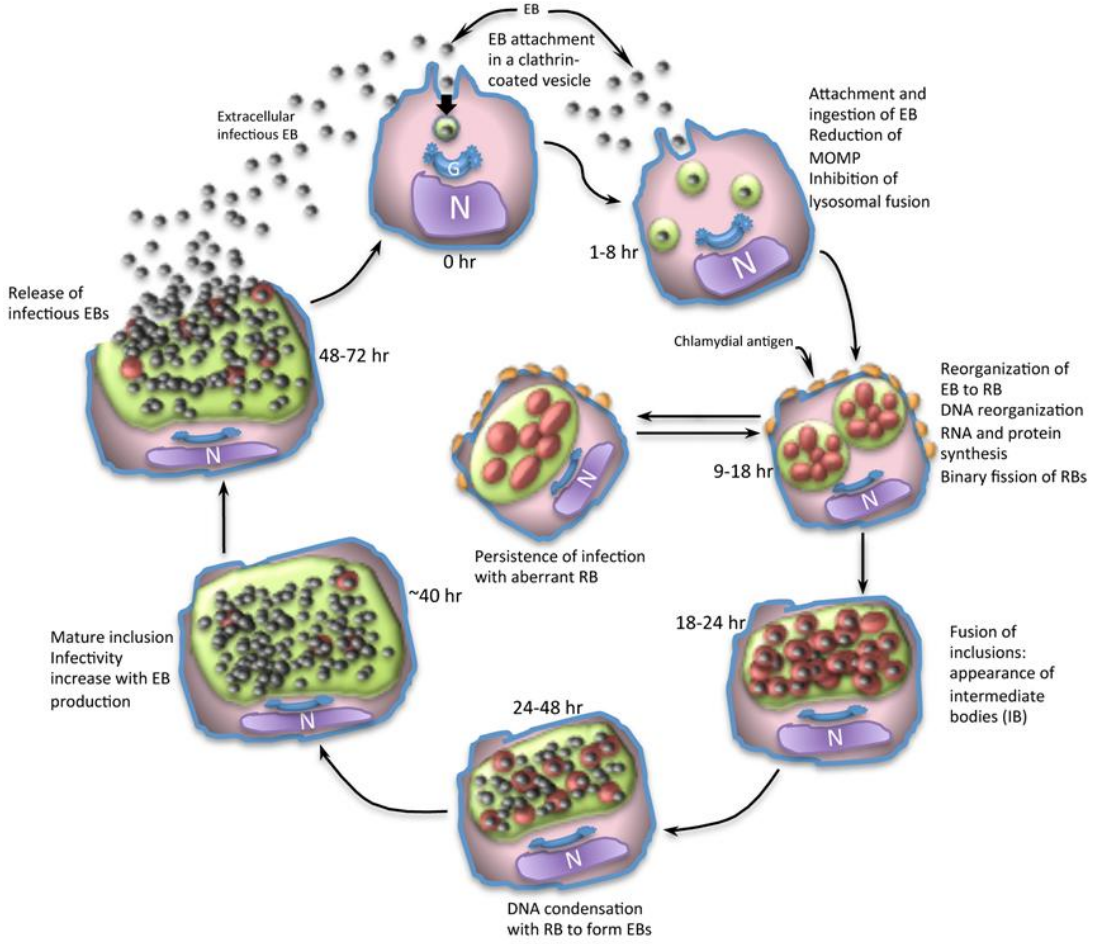

Figure 9. Life cycle of Chlamydia [20]

1.3.2 C. trachomatis

C. trachomatis is a cause of bacterial sexually transmitted disease. According to, WHO it is estimated that every year there is more than 100,000,000 new cases of *C. trachomatis* infections reported worldwide [21].

C. trachomatis can cause blindness, non-gonococcal urethritis and reactive arthritis [22-24]. The manifestations and the prevalence of the disease are worse in women than in men [25]. Only 1/3 of the patients infected with *C. trachomatis* have symptoms such as mucoadhesive cervicitis with yellow exudate. One of the most serious events is the PID (Pelvic inflammatory disease) due to untreated-*Chlamydia* [26] actually 40% of these women will present PID and 1/4 of them will be sterile [27]. Also, studies have also shown that there is an increased risk of *Chlamydia*-infected women becoming infected at some point in their life by HIV(human immunodeficiency virus) [28]. Diagnosis of *C. trachomatis* is based on molecular tests like PCR or ligase chain reaction.

1.3.2.1 Chlamydia trachomatis serotypes

For *C. trachomatis*, 18 different serotypes have been found. Serovars A, B, Ba, and C (clade 3) infect the ophthalmic epithelium and lead to the trachoma a disease-causing blindness while D, Da, E, F, G, H, I, Ia, J, Ja and K (clades 2 and 4) cause urogenital conditions, even infertility [29]. These different serotypes are identified based on the serological reactivity of the epitopes of the main *Chlamydia* membrane protein (MOMP) [30, 31]. These serovars belong to two biological groups (biovars) with apparent clinical infections: 1) lymphatic syndrome, serotypes L1, L2 and L3 cause invasive STDs known as Lymphogranuloma venerum (LGV) (clade 1 and 2) the non-invasive epitheliotrophic trachea biophore, which consists of the categories (clades 2, 3,4) already mentioned [31, 32].

1.3.2.2 Cervical cancer and C. trachomatis

Several studies have shown that there is an essential association between *C. trachomatis* infection and cervical neoplasia. An anti-*C. trachomatis* heat shock protein 60-1 antibodies were predominantly found in cervical cancer samples, signifying that a *C. trachomatis* infection increases the cervical cancer risk [34].

A reasonable system which contributes to *Chlamydia* infection as an agent of increased risk of developing cancer, is the inflammatory response which is related to the infection, therefore leading to increased expression of cytokines, chemokines, reactive oxidative metabolite production and expansion and angiogenic factors, reduced cell-mediated immunity, and the reproduction of free radicals, as a result of DNA damages and non-functional repair of DNA, leading to genetic instability [35, 36]. Moreover, ***C. trachomatis*** infection leads to disintegration of the N-cadherin contingent cell connections and that results in the N-cadherin/β-catenin breakdown complex in human cervical cancer epithelial HeLa cells [37]. Moreover, it was shown that there is a substantial increase of cell proliferation in mice infected with *C. trachomatis*, which might lead to cervical dysplasia [34, 38].

1.3.2.3 C. trachomatis and HPV

In order to conduct a study about the most important risk factors for HPV infection, 12,527 women were tested where 303 of them were positive for HPV, 44% of these women were found to have the same type of HPV DNA, after checking their history for previous infections, it was concluded that the most important risk factor for HPV infection is a history of previous *C. trachomatis* infection [39].

1.4 Apobecs

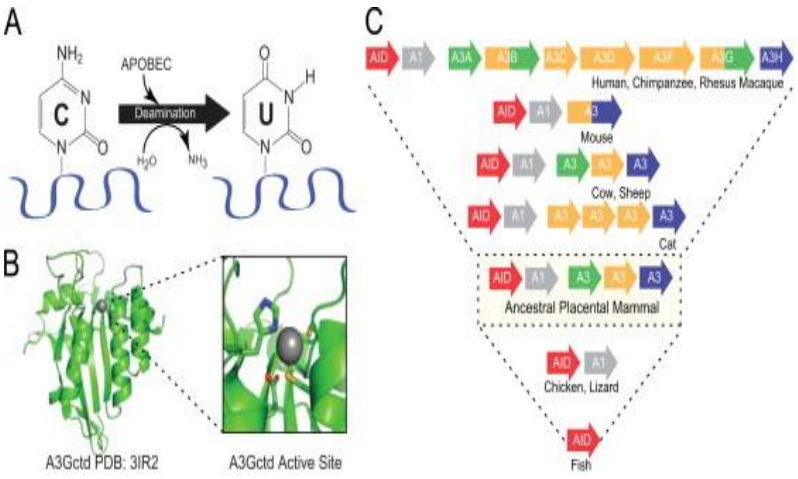

Figure 10. APOBECs structure [40]

APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) is a family of evolutionarily concerved cytidine deaminases. The family of APOBEC proteins is a group of cellular enzymes that catalyze the deamination of cytidine (C) to uracil (U) in single-stranded DNA or RNA [41, 42] and act as antiviral factors in the innate immune system of the host [43]. The APOBEC family in humans comprise at least 11 members [43, 44]. AID and APOBEC1 genes are located on chromosome 12, APOBEC2 gene is located on chromosome 6, seven APOBEC3 proteins (APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, APOBEC3H genes) are located on chromosome 22 and APOBEC4 gene is located on chromosome 1 [41, 43, 45-48]. The APOBEC proteins apart from targeting the viral DNA/RNA target also the host genomic DNA, creating clusters of C-to-T conversions in the genome [49-52].

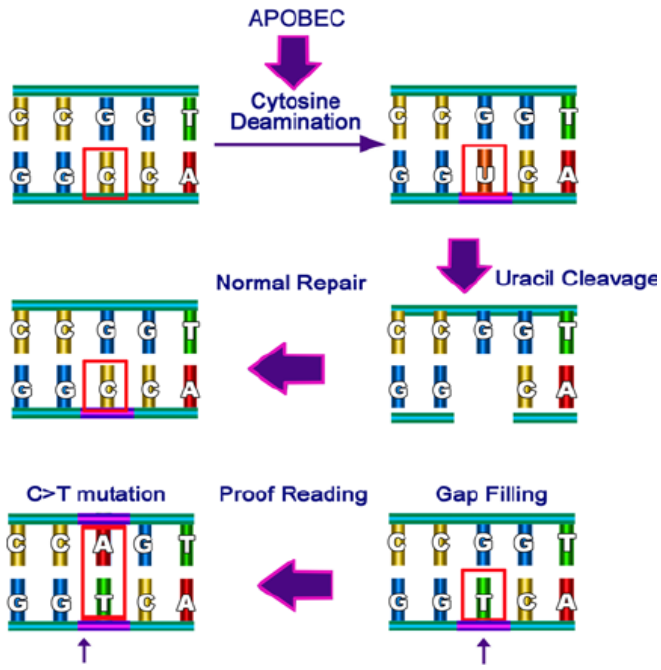

Figure 11.APOBECs function[53]

1.4.1 Cervical Cancer and APOBECs

Recent studies have shown that a subclass of APOBEC cytidine deaminases, may activate mutation clusters in human tumors. Mutation clusters of the whole-genome and exome are adapted to the rigorous criteria notional of an APOBEC mutation pattern. The APOBEC mutation signature in cancer has been characterized as C-to-T

and C-to-G base replacement mutations within 5′-TC dinucleotide pattern and most frequently within 5′-TCA and 5′-TCT trinucleotide contexts. These genes can set incorrectly in cancer cells and cause mutations in human genomic DNA. This mutation can lead to drug resistance and metastasis [54]. Also, research based on carcinogenesis proved that a subclass of APOBEC with a TC mutational specificity has the capability to cause mutations in chromosomal and mitochondrial DNA, thus can play a significant role in carcinogenesis [49, 55, 56]. According to these criteria 954,247 mutations in 2,680 exomes from 14 cancer types, showed that in 68% of all mutations there is a substantial existence of the APOBEC mutation pattern in the bladder, cervical, breast, head and neck, and lung cancers [57].

1.4.2 Apobecs in HPV

Carcinogenesis induced by HPV exhibits molecular alterations and characterized by gene instability and hypermethylation of tumor suppressor gene promoters in the host genome. APOBEC plays an important role in cervical cancer caused by HPV whose expression is stimulated by viral infection. APOBEC proteins cooperate in the complicated interactions among HPV and the host genome. Through the carcinogenesis connect immunity and viral infection [58]. The expression of APOBEC3A and APOBEC3B tends to increase upon infection with HPV16 in cervical tumors compared to normal tissues, and this increase is believed to be due to HPV16 oncoproteins E6/E7 [59]. There is a strong correlation between cervical cancer and increased expression of APOBEC3B protein. Detailed analysis of the APOBEC mutation patterns in cervical and head and neck cancers have been shown to be caused by the infection of HPV [57].

1.5 Common methods used for Molecular study

1.5.1 PCR

From the point of view of molecular biology, two properties of DNA are of particular interest. The first is the genetic code which is in the form of triplets (triplets of

nucleotides) that define the synthesis of the polypeptide chain. The second characteristic feature of DNA is that it consists of two chains complementary to each other form a twin - helix structure, which is necessary so that the duplicate each daughter cell to get an identical copy. In the laboratory, the usual methodology used for multiplication of a DNA fragment (DNA amplification), is the Polymerase Chain Reaction or PCR. PCR is a biochemical exponential proliferation technique of a DNA sequence of your choice, with the purpose of strengthening it by creating thousands of copies of it. An important advantage of PCR is its astonishing sensitivity. Using the PCR technique the synthesis of a DNA segment that is the target of interest is performed in an in vitro reaction. Essentially PCR functions as an alternative method of cloning because it can reproduce in large quantities and high speed and accuracy of a particular DNA sequence even if that is in a few copies. PCR is based on the detection of the genetic material of interest directly from the sample to be tested. Also, this method is based on the need to use primers for DNA synthesis. The process submits the sample to thermal cycles of three stages, each stage is performed at a different temperature. Each cycle is doubling of DNA, and typically 30-40 cycles that lead to millions of copies of the original DNA segment. A typical PCR procedure involves three steps. The initial stage is the denaturation of double-stranded DNA into two single-stranded chains at a temperature usually of 95 ° C. Followed by annealing, at 56 ° C, i.e. the binding of the primers to the denatured single strand DNA sequences and finally extension at 72 ° C [60, 61]. In more detail the temperature steps:

1) Denaturation of DNA: Originally, the DNA fragments are subjected to a high temperature between 90 ° C to 95 ° C to achieve the denaturation of double-stranded molecules. The maximum temperature of this phase is limited by the activity of DNA polymerase.

2) Annealing of Primers: The initial temperature is reduced to 56 ° C to a value that allows DNA primers to be adapted by hybridization to the complementary DNA sequences of the mixture. Typically, the hybridization temperature is 3-5 degrees lower than the melting temperature of the primers that are used.

3) DNA elongation [61]: DNA extension/elongation is usully carried out at 72^0 C.

1.5.2 Real-time PCR

The measurement of the quantity of the product is carried out throughout the reaction, via monitoring the increase in fluorescence of a fluorescent substance. In this specific case, fluorescence is measured in each PCR cycle, resulting in an amplification plot allows the investigator to monitor the entire process of the reaction. Also, an increase in the fluorescence signal is proportional to the synthesized product is directly related to the amount of the original substrate.

The amplification curve is divided into three phases: exponential, linear and the saturation phase. During the exponential phase, in each cycle of the reaction makes exact doubling of the product, as all the components necessary for the PCR (e.g., dNTPs, primers, polymerase) are in excess (100% efficiency). As the reaction continues, the linear phase occurs during which some of the reagents begin to be depleted, while inhibitors are gradually accumulating. In saturation phase, the response is slowed down, as it decreases its efficiency and finally stops completely, so the fluorescence curve it reaches a plateau. The saturation point differs for eahc sample and depends on their reaction kinetics.

Measurements for quantification relate to its exponential phase reaction. An important parameter for quantification is the Ct value (Cycle threshold). This is the number of cycles of the amplification reaction which are required for the value of the observed fluorescence to approach one specific threshold. The value of this limit is set above corresponding to the non-specific signal (background). Ct is inversely proportional to the original amount of substrate: the lower the Ct value the higher the concentration of the original substrate [62].

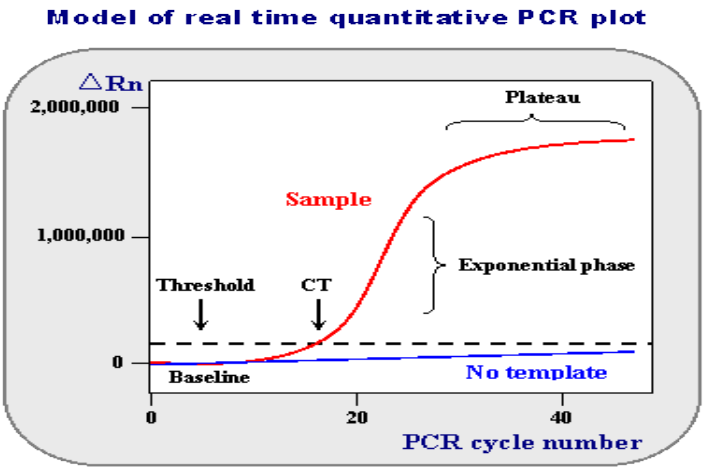

Figure 12. Rt-PCR cycle [1]

1.5.3 Western Blot

SDS-PAGE (Sodium dodecyl sulfate Polyacrylamide gel elec*tr*ophoresis) is a technique that gives the possibility of electrophoretic separation of proteins and nucleic acids based on their mobility. In particular, proteins are denatured due to the presence of SDS detergent. So they lose their physical properties and their three-dimensional structure and are negatively charged. Since a load of proteins no longer plays a role in their separation, they are separated by their size, immigrating from the negative to the positive electrode. The concentration of SDS in the gel is chosen according to the size of the proteins that one intends to separate: proteins with a higher molecular weight need higher SDS concentration to denature [63]. In this work SDS-PAGE was carried out using Bio-Rad Mini-PROTEAN system.

1.5.4 Transduction

The Lentivirus is characterized by their ability to transcribe RNA into DNA the same as the retrovirus. There are numerous steps implicated in the preparation and the transduction of a Lentivirus in a host cell. Initially, the virus uses the glycoproteins which are on the surface for attachment to the external surface of a cell. Particularly,

Lentiviruses adhere to the CD40 ligand glycoproteins on the surface of T-lymphocyte cells. Then the virus genome is injected into the host cell's cytoplasm reverse transcriptase enzyme performs a reverse transcription of the virus RNA genome. The virus DNA then, with the help of the virus enzyme integrase, is integrated into the host cell DNA. Subsequently, the host cell accomplishes transcription and translation to create virus particles and compile virions , which are then released from the host cell [64, 65].

Here we used a two-vector system fot the generation of Lentiviruses expressing the gRNA of interest. For the transfection two vectors are used, the one is the Packaging vector psPAX2, which is the backbone of the virus system. A packaging vector, such as psPAX2 contains a robust CAG promoter for efficient expression of packaging proteins. And the second one is envelope vector pMD2.G (VSVG). An envelope vector, such as pMD2.G.

1.5.5 *E.coli* transformation

In this project we used *E.coli-StbI3* chemically competent cells to amplify the recombinant plasmid LentiCRISPRv2. *E.coli-StbI3* are chemically competent cells that are permissive to take up plasmid DNA. *E.coli- StbI3* chemically competent cells are engineered in order to increase the transformation efficiency. This strain allows a stable replication of high-copy number plasmids [66].

General, competence is the capability of any cell to take up extracellular DNA from its environment, a process known as transformation. The DNA is then transferred through the membrane (for gram-negative bacteria) by means of multi-component protein complexes [67].

1.5.6 CRISPR/Cas9 Technology

CRISPR / Cas systems were for the first time seen in *E. coli* strain K12 as repetitive 29 base sequences, including 32 base-separator sequences. Initially, they were believed to be involved in the development of bacteria, the separation of DNA

molecules after replication and DNA repair [68]. More CRISPR / Cas elements have been discovered in the genomes of about half the bacteria and almost all the archaeobacteria. Their role is to deal with infectious viruses and are characterized by intrusiveness, immune memory and inheritance to affiliates. Their main features are the presence of straightforward iterations, almost identical in size and sequence, the presence of similar sized and variable sequence separators that interpose between iterations, the existence of a leader sequence with a CRISPR transcription regulator role from the absence of open reading frames, the proximity to the locus of the Cas proteins and the presence at the targeting site of a proximal motif in the spacer (PAM) in the sequence Target [68].

1.5.6.1 Classification of CRISPR / Cas *systems*

CRISPR / Cas systems can generally be categorized into three categories, depending on the Cas and other proteins involved in their activity. Cas1 and Cas2 are common to all types of systems and appear to be involved in the insertion of the invading sequences at the CRISPR gene pool.

The Type I CRISPR system comprises a number of different Cas proteins necessary for crRNA maturation and target digestion. Better characterized is the function of Cas3, which has heliCase and endonuclease activity that causes breakdown at target sites, whereas crRNA maturation is usually catalyzed by Cas6 proteins (Cas6, Cas6e or Cas6f), or in IC type systems by Cas5 or Cas7.

The CRISPR type II is the simplest among the CRISPR-Cas systems in which the endonuclease activity required for the cleavage of target DNA is concentrated in a single muldomain protein, Cas9 and co-processed by tracrRNA and crRNA molecules.. The maturation of crRNA is catalyzed by RNase III-specific RNase-specific RNA molecules in the presence of Cas9.

In the CRISPR type III system, crRNA maturation is catalyzed by Cas6, but then the resulting molecule is transferred to a new Cas protein complex, csm in subtype III-A systems, and cmr in type III-B, for further processing of its end. Interest in system III shows that it can be used to target DNA (III-A) and RNA (III-B) [69].

1.5.6.2 Mechanism of operation of the CRISPR/Cas type II system

The first step in acquiring acquired immunity is the insertion of the sequence-separator into the CRISPR gene. The exact mechanism is not known, but Cas7, Cas1 and Cas2 appear to be involved. The insertion does not involve a random segment since it has been found that the viral sequences incorporated are located adjacent to the PAM oligonucleotide sequences in the viral genome. During insertion, the repeating sequence doubles on either side of the divider [70].

The maturation in CRISPR / Cas type II systems presupposes the presence of pre-cRNA, tracRNA, Cas9 and endogenous RNase III. Pre-crRNAs are the single products resulting from the transcription of the CRISPR locus and contain the separators between repeats. TracRNAs are single-stranded RNA molecules encoded by the antisense DNA sequence relative to pre-crRNAs and Cas proteins at a genetic site upstream of the CRISPR locus. Two tracrRNAs, size 171 and 89 nucleotides, in which there is a 25 nucleotide sequence complementary to 24 with a sequence found in the repeats of the CRISPR locus, have been detected. When a hybrid molecule is formed resulting from the binding of a tracrRNA to a repeat sequence it is possible to identify double-stranded RNA structure from RNase III, which catalyzes the hybridization of the hybridoma. For the RNase III catalyzed maturation of crRNA, the presence of Cas9 is required without the mechanistic significance of its presence being known [71].

Cas9 interacts through a lobe structure with the locally duplex sequence formed between crRNA and tracrRNA, forming a three-point system through which targeting is directed. For effective targeting, two elements, the primary targeting sequence, and the PAM sequence are required. The primary targeting sequence is the monoclonal RNA sequence of approximately 13 nucleotides at the 3-end of the total size of 20 nucleotides of the cRNA targeting sequence, while the PAM sequence is a small motif of usually 3 nucleotides in sequence and size depending on the species of organism in which is encountered. The most widely used CRISPR / Cas9 system is from *Streptococcus pyogenes* with 5-NGG-3 PAM sequence, whereby N is any nucleotide. Initial ligation of the system occurs in the double-stranded PAM DNA sequence via

Cas9, and since the main sequence of the crRNA is complementary to the target DNA sequence, Cas9 acts as helicase causing local DNA fusion and DNA-RNA hybrid formation, where it is connected via a second lobe-shaped territory. Cas9 then catalyzes the cleavage of the DNA sequence complementary to crRNA with HNH domain activity and the crRNA homologous displaced DNA sequence through RuvC-like domain activity, causing double-strand break. Data show that in both chains the breakage occurs between the third and fourth nucleotides from the 3-terminus of the complementary crRNA sequence in DNA [2, 72-74].

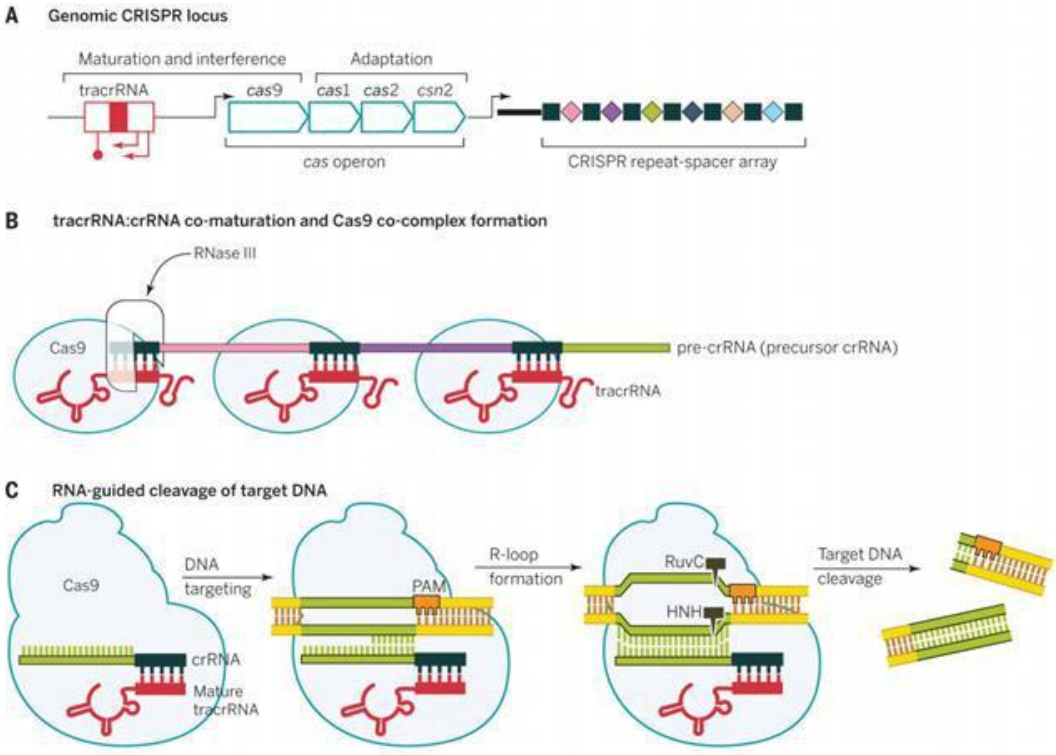

Figure 13. Insertion, maturation and action mechanisms of the CRISPR / Cas9 system [2, 3]**.**

1.5.7 The role of plasmid

Plasmids are double-stranded-circular DNA sequences that are capable of self replicating in a host cell. Plasmid vectors are DNA molecule, which transfers the fabricated foreign genetic material, in our experiment the plasmid vector is carrying gRNA, into another cell, where it can be replicated and expressed. Generally, the vector is a DNA sequence that composes of an insert and a larger sequence that works as the "backbone" of the vector. The function of that vector usually is, to isolate, proliferate or express the insert into the target cell. *E. coli* vectors are usually used for the manipulation of DNA which consists of materials mandatory for their maintenance in *E. coli*. [75]

LentiCRISPRv2 (Plasmid #52961) was the plasmid used to clone the gRNAs in this study. This plasmid is an updated version of the original lentiCRISPR (Addgene plasmid #49535) and can produce 10-fold higher titer virus. The vector can be digested using *EspI3*, and a pair of annealed oligos can be cloned into the single guide RNA scaffold. The oligos are designed based on the target site sequence (20bp) and with the help of the EspI3 enzyme the gRNA take the place of the filler on the 3' end by a 3bp NGG PAM sequence[76]. Total size of the plasmid is 14873 bp of which 10 kb correspond to its primary segment plasmid (backbone). The plasmid is supplemented with two 4200 bp inserts and 600bp. The first insert carries the Cas9 expression gene, the second carries the gene resistance to the antibiotic puromycin. The plasmid also carries multiple cloning site with possibility to restriction digest with various restriction enzymes including *EspI3* restriction sites, a digestion that will allow the cloning of the gRNA sequences in the vector [77].

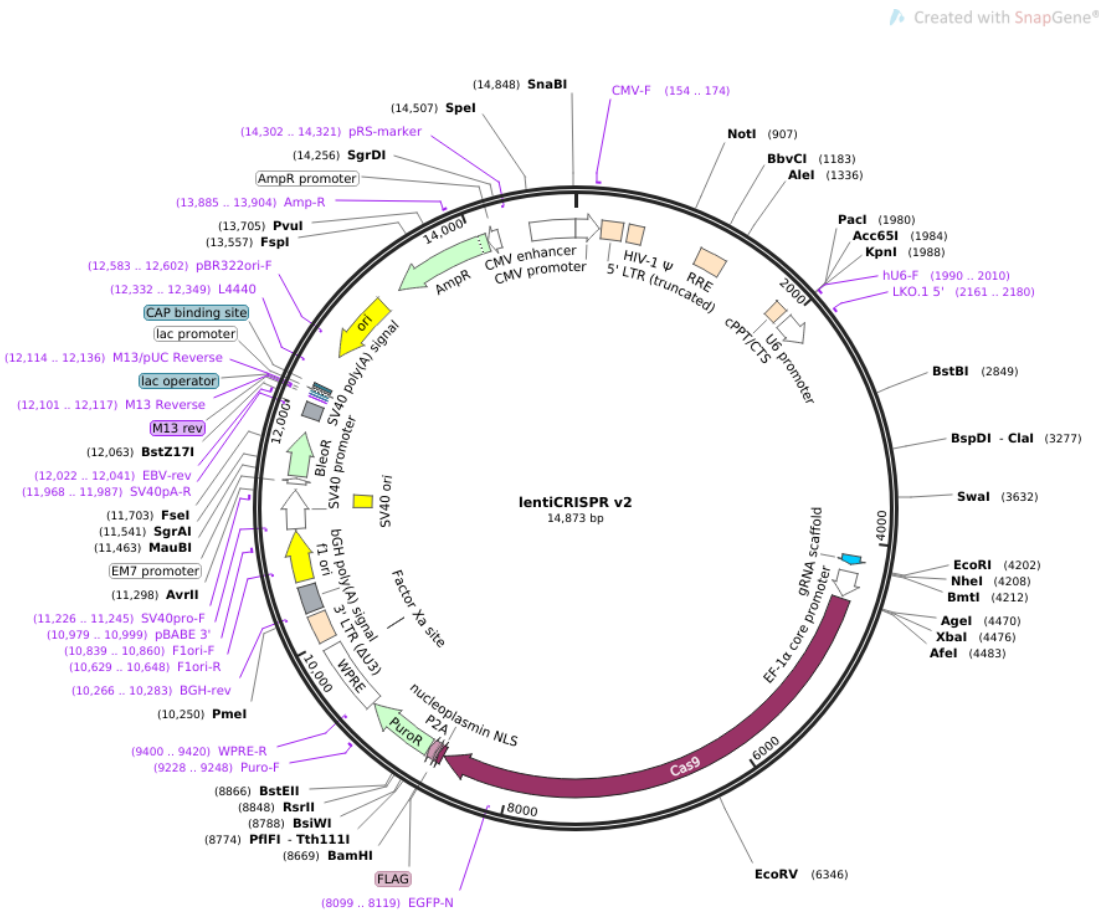

Figure 14. LentiCRISPRv2 plasmid map [77]

2. Experimental Part

2.1 Background&Objectives

Cervical cancer is one of the most common cancers in women with a mortality rate of over 50% (1). It mainly occurs as two histologically distinct types – adenocarcinoma (ADC) and squamous cell carcinoma (SCC), rarely other entities are seen. SCC accounts for ~90% of Cases and has an established causal link to human papilloma virus (HPV) infections (2).

Cervical cancer is the fourth most common cancer globally with a mortality rate of over 50%. Human papilloma virus (HPV) infections are considered as the main cause of cervical cancers [78]. However, only two percent of the HPV infected women develop cervical cancers. Therefore, a number of co-factors are implicated to be essential for carcinogenesis. The gram-negative obligate intracellular bacterial pathogen *Chlamydia trachomatis* **(***C. trachomatis*) which causes most frequent sexually transmitted infections is one of the major co-factor in HPV induced cervical carcinogenesis [39, 79].

Previous studies have indicated that the apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) family members of cytidine deaminases to be upregulated and contribute to the development of mutations found in cervical cancers [59]. Although the HPV oncoproteins E7 is implicated in the enhanced expression of some of these APOBEC family members the molecular mechanisms are not yet clear [80].

Interestingly, transcriptomic analysis of *C. trachomatis* infected mouse, HPV E6E7 positive endocervical primary epithelial cells (END1/E6E7) and HPV negative primary human ectocervical cells reveled increased expression of APOBEC3A, APOBEC3B and APOBEC3G, irrespective HPV status (*Chumduri, Gurumurthy et al, unpublished*).

This project involves investigation of the mechanism and consequences of upregulation of these APOBEC family members during *C. trachomatis* infection.

Towards this, the bachelor thesis project encompasses following objectives:

a. Generate CRISPR/Cas9 mediated knockout cells lines of Apobec3B, Apobec3A, Apobec3G
b. Confirm the Knockouts
c. Investigate the effects of knockouts during *C. trachomatis* infection.

2.2 Materials and Methods

The first step according to CRISPR/Cas9 system was to prepare a plasmid which contains the desirable sequence, Cas9 and at least one antibiotic resistant. Once we had the circular plasmid, we digested and dephosphorylated it , so finally through agarose gel and gel extraction we isolated the desirable linear band of the vector. At the same time we annealed the gRNAs of APOBEC3B.

The second step was to ligate the vector with the gRNAs and transformed into Stbl3 bacteria. After plasmid extraction we send the DNA for sequencing in order to certify that we have the right vector .

Subsequently, we performed a Lentivirus Transduction into END1E6/E7 cells and after puromycin selection we had KO END1E6/E7 cells of APOBEC3B.

The last step was to infect the cells with *C.trachomatis* and certify via WB if the CRISPR/Cas9 system led to succeed KO.

2.2.1 Materials

2.2.1.1 Bacterial strains and Cell lines

Bacterial strains	Derivation
Chlamydia trachomatis LGV L2	Lymphatic isolates (ATCC VR-902B)
E. coli-StbI3	Derived from HB101. For storage of plasmids that have the potential to recombine. Example, the LTRs in lenti- and retro-viral plasmids, endA+, use care in preparing DNA from this strain.

Table 1 Bacterial strains

Cell line	Derivation
End1 E6/E7	Human endocervical epithelium transformed by HPV-16 E6/E7, (ATCC CRL-2615) from American type cell collection.
293T cell	Highly transfectable derivative of 293 cell
THP-1 cell line	Derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia
Hela	Human epithelial cells from cervical adenocarcinoma (ATCC CCL-2) from American type cell collection.

Table 2 cell lines

2.2.1.2 Cell culture medium

Medium	Composition	Manufacturer
Growth medium	1) Complete medium (10% FCS) FCS 50ml - Fetal Calf Serum (Cat.Nr:S0115,Lot.0710x) DMEM 500ml 100mM Na-pyruvate 5ml 200mM L-Glutamine 6ml 2) RPMI (Cat.Nr:21875-034, Lot.1838081)	 Biochrom Gibco Gibco Invitrogen Gibco
Infection medium	Complete medium (5% FCS) 25ml Fetal Calf Serum (Cat.Nr:S0115,Lot.0710x) DMEM 500ml 100mM Na-pyruvate 5ml 200mM L-Glutamine 6ml	 Biochrom Gibco Gibco

		Invitrogen
Cell lines passaging	DPBS (Cat .Nr :8043.2, Lot.various) Trypsin (Cat.Nr: 25300-096, Lot.various)	Gibco Gibco

Table 3 cell culture medium

2.2.1.3 Antibiotics

Antibiotic	Manufacturer
Puromycin (Cat.Nr:A11138-03, Lot.1689314)	Gibco
Ampicillin (Art. Nr: A9518-1006; Lot. 074K0522)	Sigma

Table 4 Antibiotics

2.2.1.4 Enzymes

Enzymes	Manufacturer
FastAP (Cat.Nr:EF0654, Lot.00468647)	Thermo Fisher Scientific
FastDigest *Esp3I (*Cat.Nr:FD0454, Lot.00477845)	Thermo Fisher Scientific
T4 PNK (Cat.Nr:EK0031, Lot.00104797)	NEB
Quick Ligase (Cat.Nr:M2200S)	NEB
RT Enzyme Mix (Cat.Nr:4389988, Lot.1607058)	Applied Biosystems

Table 5 Enzymes

2.2.1.5 Reagents

Reagents	Manufacturer
10X FastDigest Green Buffer (Cat.Nr:B72, Lot.00472159)	Thermo Fisher Scientific
10X T4 Ligation Buffer (Cat.Nr:B0202S)	NEB
2X Quick Ligase Buffer (Cat.Nr:B2200S, Lot.0021509)	NEB
Cryo-SFM (Cat.Nr:C-29910, Lot.407M132)	PromoCell
Dimethyl Sulfoxide-DMSO (Cat.Nr:D2660-100ML, Lot.RNBF5734)	Sigma
LE Agarose (Cat.Nr:840004)	Biozym
Nonfat dried milk powder (Cat.Nr:A0830,1000, Lot.5U010811)	Applichem Panreac
SDS, Sodium laurylsulfate, Sodium dodecyl sulfate, SDS pellets (Cat.Nr:20765.03, Lot.120895)	Serva
TEMED,Tetramethyl-ethylenediamine (Cat.Nr :T9281-25ML, Lot.BCBN1173V)	Sigma
Rotiphorese® Gel 30 (37,5:1) 30 % Acrylamid-, Bisacrylamid-Stammlösung im Verhältnis 37,5:1 (Cat.Nr :3029.1)	Roth
DTT, 1,4-Dithiothreitol 1M (1,84 g DTT +10 ml dH2O)	
Power SYBR Green RT-PCR Mix (Cat.Nr:4388869, Lot.1610059)	Applied Biosystems
Fugene 6 (Cat.Nr:E2692, Lot.000023528)	Promega
OPTI-MEM Reduced Serum Medium (Cat. Nr: 31985062)	Sigma
GeneRuler 50bp (Cat.Nr :5M0371, Lot.00266480)	Thermo Scientific
GeneRuler 1Kb (Cat.Nr :5M0311, Lot.00033708)	Thermo Scientific
PageRuler Plus Prestained (Cat.Nr:26619, Lot.0027822)	Thermo Scientific
ECL Prime Western Blotting Detection Reagent(WB enhanced luminol reagent Cat.Nr: 0RT2755, lot.275-16471, WB oxidizing reagent Cat.Nr: 0RT2655, Lot.265-16471)	Perkin Elmer
Polybrene (1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide)	Sigma

Table 6 Expendable reagents

2.2.1.6 Agarose gel buffer and bacterial media

Buffer	Composition
LB medium	NaCl 5g/L, Tryptone 10g/L, Yeast extract 5g/L
SOC medium	0.5% (w/v) yeast extract , 2%(w/v) tryptone ,10 mM NaCl, 2.5 mM KCl ,20 mM MgSO4
TBE buffer	89.15 mM Tris/HCl, 88.95 mM Boric acid, 2 mM EDTA
LB-agar plates with ampicillin	15g/L Agar, 10g/LTryptone,5 g/L Yeast Extract,5 g/L NaCl

Table 7 Agarose gel buffer and bacteria media

2.2.1.7 Western blot buffer

Buffer	Composition
Resolving Gel	1M Tris-HCL pH8.8 (30.2g Tris in 250ml Dis.H2O)
Staking Gel	1M Tris-HCL pH6.8 (30.2g Tris in 250ml Dis.H2O)
1X Ele*Ctr*ophoresis Buffer (10X Stock)	24mM Tris base 190 mM Glycine 0.1% SDS pH should be approximately 8.3
Wet Blot Transfer Buffer	10X 288g of Glycine+ 60g Tris in 2 L 1x 100ml of 10X Tris/Glycin+200ml Methanol Add 1000 ml H2O
TBS 10x (concentrated TBS)	24.23 g Tris HCl 80.06 g NaCl Mix in 800 ml ultra pure water pH to 7.6 with pure HCl Top up to 1 L
TBST	For 1 L: 100 ml of TBS 10x + 900 ml ultra pure water + 1ml Tween20 (0.05%)

Blocking Buffer	50ml TBST + 2.5 g Milk powder (5%)
3x Laemmmli Buffer	6x LB: 3ml Glycerol; 1,5ml beta-mercaptoethanol; 9ml 10% SDS; 3,75ml staking gel buffer; Pinch of bromophenol blue; 10ml dis. H2O

Table 8 Western Blot buffers

2.2.18 Antibodies

Antigen	Specie	Company	Dilution for WB
Primary Antibodies			
Anti-APOBEC3B	Rabbit	Proteintech (Cat.Nr:14559-1-AP)	1:500
Anti-APOBEC3G	Mouse	Proteintech (Cat.Nr: 60100-1-Ig)	1:500
Anti-APOBEC3A	Goat	Novus Biologicals (Cat.Nr: NB100-93428)	1:500
Anti-PHO1	Rabbit	Abcam (Cat.Nr:ab38641, Lot.GRG7185-5)	1:1000
Anti-ßActin	Mouse	Sigma (Cat.Nr:A5441,clone AC-15, Lot.014114759)	1:10000
Anti-*Chlamydia* Hsp60	Mouse	Enzo Life Sciences (Cat.Nr:ALX-804-072-R100, Lot.a081516	1:5000
Secondary Antibodies			
Anti-Rabbit IgG POD	Donkey	Amersham GE Healthcare (Cat.Nr:NA934-1ML, Lot.9739640)	1:2000
Anti-mouse IgG POD	Sheep	Amersham GE Healthcare (Cat.Nr:NA931-1ML, Lot.9761194)	1:3000
Anti-Goat IgG HRP	Donkey	Thermo Fisher (Cat.Nr: PA1-28664, Lot. SE 2386927)	1:2000

Table 9 Antibodies

2.2.1.9 Primers

The primers were purchased from Sigma-Aldrich

Gene	Primer sequence 5'-3'
APOBEC3B_KO_2: FP	GGAGTCTTAGGGCTTTTGGTTT
APOBEC3B_KO_2: RP	CACCACCCAGAACCTTCTATCT
APOBEC3B_KO_10+11: FP	AGTGTTTCCAGATCACCTGGTT
APOBEC3B_KO_10+11: RP	AGATCCATCCATCTTTCATGCT
APOBEC3B_rt_FP(PMID: 23696735)	TTCGAGGCCAGGTGTATTTCA
APOBEC3B_rt_RP(PMID: 23696735)	CAGAGATGGTCAGGGTGACA
APOBEC3A_rt_FP(PMID: 23696735)	ATGGCATTGGAAGGCATAAG
APOBEC3A_rt_RP(PMID: 23696735)	CAAAGAAGGAACCAGGTCCA
APOBEC3G_rt_FP(PMID: 23696735)	GGTCAGAGGACGGCATGAGA
APOBEC3G_rt_RP(PMID: 23696735)	GCAGGACCCAGGTGTCATTG
AICDA_rt_FP(PMID: 23696735)	AAATGTCCGCTGGGCTAAGG
AICDA_rt_RP(PMID: 23696735)	GGAGGAAGAGCAATTCCACGT
Cas9_FP	CCGAAGAGGTCGTGAAGAAG
Cas9_RP	TCGCTTTCCAGCTTAGGGTA

Table 10 Primers

2.2.1.10 gRNAs

The gRNA was purchased from Sigma-Aldrich

gRNAs	gRNA sequence 5'-3'
Apobec3B_KO_2_1	caccgTGGCGCCGTCGAAGGACCAA
Apobec3B_KO_2_2	aaacTTGGTCCTTCGACGGCGCCAc
Apobec3B_KO_11_1	caccgCATAGTCCATGATCGTCACG
Apobec3B_KO_11_2	aaacCGTGACGATCATGGACTATGc
Apobec3B_KO_10_1	caccgAGGAGCCCGCGTGACGATCA
Apobec3B_KO_10_2	aaacTGATCGTCACGCGGGCTCCTc

Table 11 gRNAs

2.2.1.11 Commercial KITs

Kit	Manufacturer	Use
GeneJET™RNA Purification Kit	Thermo Scientific (Cat.Nr: K0732, Lot.00442650)	RNA purification
Qiagen Plasmid Mini/Midi kit	Qiagen (Cat.Nr:19743, Lot.151042692)	Plasmid isolation
QIAquick Gel Extraction Kit	Qiagen (Cat. Nr:28706, Lot.148039847)	Gel purification
GeneJET™ Genomic DNA Purification Kit	Thermo scientific (Cat.Nr:K0722, Lot.00330156)	DNA purification

Table 12 Commercial KITS

2.2.1.12. Equipment

Name	Type	Manufacturer
Photo-NanoDrop ND-1000	Spektrometer	Thermo Scientific
PTC/225 Peltier Thermal Cycler	Thermocycle	DNA Engine Tetrad
PCR System StepOnePLUS 9 6well RT	RT-PCR SYSTEM	Applied Biosystems

Table 13 Equipment

2.2.1.13 Software

Name	USE	Manufacturer
Photoshop CS6	Image processing	Adobe
Illustrator CS6	Image processing	Adobe
Excel 2016	RT-PCR	Microsoft
PowerPoint 2016	Image processing	Microsoft

Table 14 Software

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2.2.1.14 Material Sterilization

All the material, culture media and solutions suitable to high temperature treatment methods were autoclaved at 121°C and 1 atmosphere, for 20 minutes.

2.2.2. Methods

2.2.2.1 EndoFree Plasmid DNA pereparation

The plasmid colonies were selected from *E.coli* strains from LB agar plates and they were grown in 100ml of LB medium with 100μl of Ampicillin and incubated at 37°C overnight. The plasmid DNA was extracted using Qiagen Midiprep kits according to manufacturer's manuals.

2.2.2.2 DNA Digestion

The DNA digestion was performed using the enzymes from Thermo Fisher Scientific:FastAP (Cat.Nr:EF0654,Lot.00468647), this enzyme has the ability to digest and dephosphorylate at the same time all types of DNA in 10 min at 37°C (waterbath) and FastDigest *Esp3I (*Cat.Nr:FD0454, Lot.00477845) with recognition sequence at the positions 2852 and 4737. In addition, 100mM freshly prepared DTT was used with the purpose to reduce the disulfide bonds of proteins and peptides. Eventually, 10X FastDigest Buffer and ddH2O up to 60μl have completed the reaction. The digestion reaction was performed 30 minutes at 37^{o}C.

2.2.2.3 Agarose Gel Electrophoresis

Electrophoresis in agarose gel is one of the most common techniques to evaluate the size of DNA or RNA fragments. The principle of this technique is based on the migration of nucleic acids fragments from a negative to a positive charge since DNA is negatively charged in neutral pH. The fragments will migrate according to their size, meaning that small fragments will more easily cross the porous matrix produced by agarose in solution. The analysis of the DNA fragments was evaluated by electrophoresis in 0.5% TBE agarose gel with ethidium bromide solution at 80-120 V

for 45-90 minutes. The ladder used for size quantification of the double-stranded fragments were GeneRuler 50bp (Cat.Nr :5M0371, Lot.00266480) and GeneRuler 1Kb (Cat.Nr :5M0311, Lot.00033708) (Thermo Scientific™). The gels were visualized in Gene Genius Bio-Imaging System (SynGene™).

2.2.2.4 DNA Purification from Agarose gel

The extraction and purification of the terget DNA fragment were performed using QIAquick Gel Extraction Kit, Qiagen (Cat. Nr:28706, Lot.148039847), which contains a silica-based membrane technology in the form of a convenient spin column. The DNA fragment of interest was excised from a 0.5% TBE agarose gel with a clean scalpel and placed in a microcentrifuge tube previously weighed. The tube was weighed once again after collecting the band of interest. Binding buffer was added in a proportion of 3:1 (300μL/100mg of agarose gel) to the gel slice and the mixture was incubated on a heat-block at 56°C for 10 minutes with slight shaking. After the gel slice was completely dissolved, isopropanol was added in a proportion of 1:1 (100μL/100mg of agarose gel) the mixture was transferred to a GeneJET purification column and centrifuged for 1 minute. The column was then washed with 750μL of Wash Buffer, centrifuged for 1 minute. The column was transferred to a clean microcentrifuge tube with 50μL of elution buffer. After centrifuge for 1 minute, the purification column was discarded and the purified DNA was stored at -20°C.

2.2.2.5 DNA annealing

To create CRISPR KO in End1 E6/E7 cell lines were used the gRNAs, which already mentioned (1.8.9 gRNAs). In this step, oligos for both strands of gRNA were phosphorylated and annealed. T4 PNK (NEB M0201S) enzyme was used and the T4 Ligation Buffer with ATP. The function of T4 PNK is the repair of RNA, this enzyme has two enzymatic reactions the one is the transformation of the γ phosphate from

ATP to the 5′ OH terminus of RNA and the second one is the hydrolytic removal of a 3′ PO_4 terminus from RNA [81-85].

Phosphorylation/annealing reaction was performed using conditions as follows:

30 minutes at 37°C

5 minutes at 95°C and then reduced to 25°C and ramp down until 5°C with 0.1°C/sec

2.2.2.6 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify DNA fragments. All PCR reactions were performed in a PTC/225 Peltier Thermal Cycler.

In general, an annealing temperature was the average plus 2°C of calculated melting temperatures of primers. Extension of the amplicon was carried out for 30 s for each kb of amplified DNA.

The standard PCR cycling conditions were as follows:

Temp	Time	Cycle
98°C	1 min	1x
98 °C 63 °C 72 °C	10sec 10sec 10sec	35x
72 °C	5 min	1x
4 °C	For ever	1x

2.2.2.7 DNA ligation

Ligation was performed using Quick ligase kit from NEB. The Ligation mix was composed of the insert, backbone, Quick ligase and the buffer according to manufacturer's manual. The ligation reaction was performed 10 minutes at room temperature. Including a negative control, digested plasmid without oligos and a positive control, undigested plasmid. Followed by transformation of chemo-competent bacteria.

2.2.2.8 Preparation of chemically competent cells (StbI3) and transformation of plasmid into StbI3 bacteria

For general cloning purpose, chemical competent *E. coli* Stbl3, which is commonly used strain for lentiviral transfer plasmid system, was used for bacterial transformation. 5µL of the ligation reaction was added to 50µL aliquots of chemically competent cells and incubated on ice for 20-30 minutes. Cells were subjected to heat-shock at 42°C for 45 seconds on a heat-block and immediately placed on ice for 2-3 minutes. 100µL of Soc medium or LB medium was added to each tube and the cells were placed at 37°C with shaking for 1 hour. Finally, the cells were slightly spun down and plated on LB plates containing 100µg/ml ampicillin and incubated overnight at 37°C.

2.2.2.9 Midi-preparation of plasmid DNA **9**

Plasmid DNA from *E. coli* strains StbI3 cells was extracted using Qiagen Plasmid Mini/Midi kit Qiagen (Cat.Nr:19743, Lot.151042692). 100ml of bacterial culture was spun down at 3400rpm for 13 minutes. The supernatant was discarded and the pellet was gently resuspended in 4 mL of resuspension solution and transferred to QIAfilter Cartridge incubate at RT, 10 min. After the cap was removed from QIAfilter column and the column was empty by gravity flow.

The column was washed twice with 10ml wash buffer and eluate with 5mL of elution buffer. The DNA was precipitated by adding 3,5 ml room temperature isopropanol, mix and centrifuge at 10.000 x g for 30 min, 4°C and decant the supernatant. The pellet was washed with 2 ml endotoxin-free 70% ethanol and centrifuge at 10.000 x g for 10 min. After centrifuge air dry pellet for 5-10 min and resolve DNA in 150 μl endotoxin-free H_2O and the purified plasmid DNA was stored at -20°C.

2.2.2.10 Cell culture

End1 E6/E7, 293T, Hela and Hacat cells were incubated in DMEM media (10% FCS) and placed in 37°C incubator with 5% CO2. To split the cells (for 75cm2 flask), remove the old medium and wash with 10ml 1XPBS, afterwards add 2ml trypsin (Trypsin acts by cutting amino acids, specifically lysines or arginies, on their c-termini unless these amino acids are followed by an proline. Most trypsin solutions for cell culture also contain EDTA which acts as a chelator for calcium. By removing calcium from a solution with cells, cadherins which hold cells to each other, are broken and cells separate from each other as well as from the surface of the tissue culture plastic) [86] and place in the incubator for 7-8 minutes, then examine the cells under the microscope to make sure that the cells are detached, add 10ml medium to the cells and resuspend in order to avoid lump of cells. Take 4ml of the cells and transfer into the new 75cm2 flask and add 10ml complete medium, then put in the 37°C incubator. Split the cells every 3-4 days in the ratio 1: 3.

2.2.2.11 Lentiviral Preparation & Transfetion

The first day around $2x10^6$ 293T cells were seeded in the 10cm dish within 13ml DMEM (10% FCS, 2mM L-Glutamine and 1 mM Pyruvate) without Penicillin/Streptomycin.

The second day, Master mix prepared before transfection with 156µl Fugene6 and 1924µl of OPTI-MEM. The master mix was incubated for 5 minutes. The DNA mix includes 2.6 µg DNA (lentiCRSPRv2 with gRNAs), 1.95 µg psPAX2 and 0.65 µg pMD.2G (VSVG) and the volume is made up to 52 µl of OptiMEM. The mixture was incubated for 30 min and added dropwise to the 293T cells. The cells were incubated in the incubator (37^{O}c, 5% CO_2) and after 15h the medium was changed.

$0.3x10^5$ End1 E6/E7 cells were seeded in the 10 cm dish with 2mL DMEM supplemented with 2mM L-Glutamine and 1mM Pyruvate and 10% FCS and were infected with virus supernatant, then were incubated overnight and the medium was changed the second day. After four days infection, mCherry positive cells were selected by puromycin.

2.2.2.12 Long-Term Storage of END1 cells

1×10^6 END1 E6/E7 cells were spun down at 1000rpm for 5 minutes. Supernatant was discarded and the cell pellet was resuspended in 1ml of 90% FCS plus 10% DMSO (Dimethyl sulfoxide, Sigma-Aldrich®) or Cryo-SFM and transferred to a well-labeled cryo-tube (Nunc®). Stocks were stored at -80°C.

2.2.2.13 Genomic DNA purification

The genomic DNA was purified from END1 E6/E7 cells (1×10^6 cells) using the GeneJET™ Genomic DNA Purification Kit, Thermo scientific (Cat.Nr:K0722, Lot.00330156). The cells were harvest by centrifuge at 1000rpm for 5 minutes. The supernatant was removed and the cell pellet was washed in 1ml of cold PBS to remove any media components by centrifuge at 2500rpm for 5 minutes once again. The pellet was resuspended in 200µl of lysis buffer including 50mM EDTA, 1% SDS and 10mg/ml Proteinase K. The resuspended pellet was later incubated at 56°C for 10 minutes and mixed occasionally by vortexing. 20µl RNase A was added and the

mixture was incubated for 10 minutes at room temperature. To the tube was added 400µl of 50% Ethanol. The sample was transferred into the prepared lysate to a GeneJET™ Genomic DNA Purification Column inserted in a collection tube and centrifuged for 1 minutes at 13000rpm.The flow-through solution was discarded and the column was placed into a new 2ml collection tube. The was washed twice with the included wash buffers and after centifuge1 minutes at 13000rpm was added 200 µl of elution buffer and centifuge1 minutes at 13000rpm The final concentration of genomic DNA was measured by NanoDrop®.

2.2.14 C. trachomatis infection

The green fluorescent protein (GFP) expressing *C. trachomatis* serovar L2 was used in this thesis. The *C. trachomatis* serovar L2 isolated from an LGV (ATCC VR-902B) was transformed with the plasmid pGFP:SW2 containing the GFP gene. 97% of *Ctr* L2 ex-pressed the GFP. The GFP-*Ctr* L2 stock used contained $1{,}17x10^9$ infectious units/ml.

Cells were seeded in 6 wells plate, two uninfected and two infected wells per sample ($3x10^5$ cells per well). A day after seeding ($5x10^5$ cells per well), the old medium was removed from each well and the cells were infected by adding 1ml of medium with *C. trchomatis* at MOI 5 and incubated at 35°C with 5% CO^2. After 2 hours, medium in the infected wells was changed with fresh 5% FCS DMEM medium.

2.2.2.15 Microscope and image analysis

Images were analyzed by automated microscope from Olympus Biosystems fluorochromes microscope. The images were captured with 10x and 20x microscope lenses per well, using GFP and BF filters.

2.2.2.16 Western blot

2.2.2.16.1 Preparation of the samples

Between 40-48 hours after the infection removes old medium, wash with cold 1x PBS, add 250µl/well 2X Laemmli buffer, scrape the cells with a cell scraper, collect the lysate into a 2ml Eppendorf tubes. Boil at 95^{o}C for 5-7 minutes.

2.2.2.16.2 SDS-PAGE

Clean the glasses with 70% EtOH and combs, build construction, add water to check for leaking. Prepare resolving gel 12% add 5ml of the gel slowly in the pocket, avoid bubbles, add 1ml 2-Isopropanol to remove bubbles, wait 10 min to solidify. Pour out 2-Isopropanol and prepare 3% stacking gel, add 1ml of the gel and immediately, insert comb, wait for 30 minutes. Fix the gel in the chamber and fill it with electrophoresis buffer. Load the protein samples on the gels and 5 µl of a pre-stained protein ladder was in the first or last lane (or both). After that, run the gel at 70 V for 10-15 min and then run further at 100V.

2.2.2.16.3 Wet Blot transfer

Wet the 2 sponges and the 4 filter papers in transfer buffer, take out the gel from the running support and eliminate the upper gel, activate the membrane for 1minute and 30sec in methanol and incubate later for 5min in transfer buffer. Prepare the transfer stack as follows:

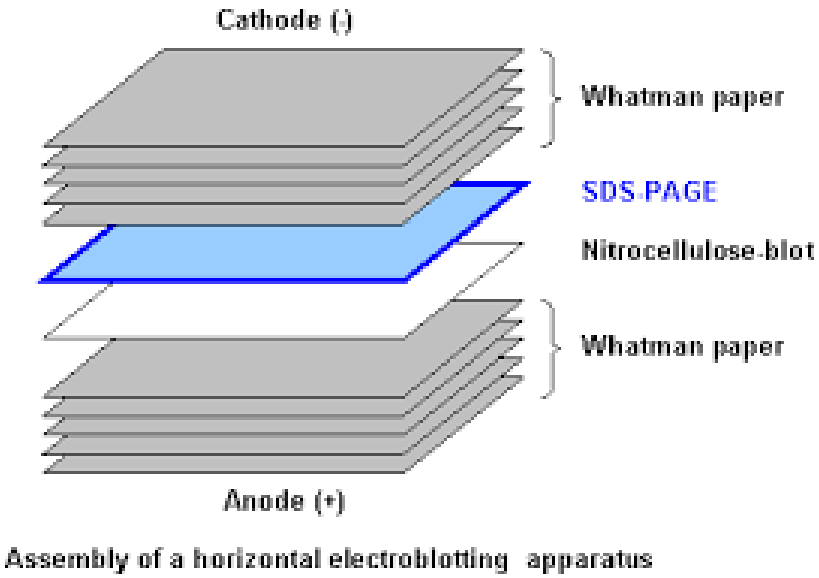

Figure 15.Wet transfer [87]

Close the transfer support and put it in its recipient with a recipient of ice (-80ºC) and transfer buffer until the top, add a magnet to the transfer recipient. The wet transfer occurs at 250mA for 2h (per chamber/1 or 2 gels) in the 4ºC room, spinning OR 15V per gel overnight.

2.2.2.16.4 Developing

Take the membrane out from the TBST, let it dry with a little in a napkin, put the membrane (with proteins up) on the cassette for development, add 1.5mL per membrane of 1:1 of Enhanced Luminol Reagent Plus and Oxidizing Reagent Plus.
Cover the membrane in the transparent plastic wrap and remove excess reagent and acquire an image using darkroom development techniques for chemiluminescence. Exposure times 1min – 2min (and then approximates to the best match) and add on the developing machine.

2.2.2.17 Validation of the gRNA knockout efficiency by qRT-PCR

2.2.2.17.1 Sample preparation

Between 40-48 hours after the infection removes old medium, wash with cold 1x PBS, scrape the cells with a cell scraper, collect the lysate into a 2ml Eppendorf tubes.

2.2.2.17.2 RNA isolation

(supplied with Fermentas GeneJET™ RNA Purification Kit #K0731)
The concentration of the RNA was validated by spectrophotometer.

2.2.2.17.3 qRT-PCR

Component	Volume for One Reaction
RNase-free H2O	4.36 µl
Power SYBR® Green RT-PCT MIX	10 µl
Forward primer (10 mM)	Primer pair 0.5 µl (final concentration 200 nM)
Reverse primer (10 mM)	
RT Enzyme Mix	0.16 µl

REACTION: 15 µl MIX + 10 µl RNA (10 ng/µl)

PCR cycling conditions:

10 min 95°C : inactivation of RTase and activation of Taq

40 cycles:

15 sec 95°C: Denature

30 sec 60°C: annealing

30 sec 72°C : elongation

Melting curve:

15 sec 95°C : Denature

15 sec 60°C: annealing

15 sec 95°C: Denature

2.2.2.18 Single cell cloning

In two 96 well plates, 200 μl of KO11 and KO10 cell suspensions were added additionally to the well A1. From the first well, 100 μl was transferred to the well B1 and these 1:2 dilution was repeated down the entire column, discarding 100 μl from the last well H1. 100 μl of medium was added in column 1. Subsequently, 100 μl from the wells in the first column was transferred to those in the second column. These 1:2 dilution was repeated to the entire plate. Furthermore, 100 μl from each of the wells in the last column was discarded, thus all the wells to end up with 100 μl of the cell suspension. Finally, 100μl of the medium was added to each well, so to have the final volume of all the cells 200 μl (See figure 15).

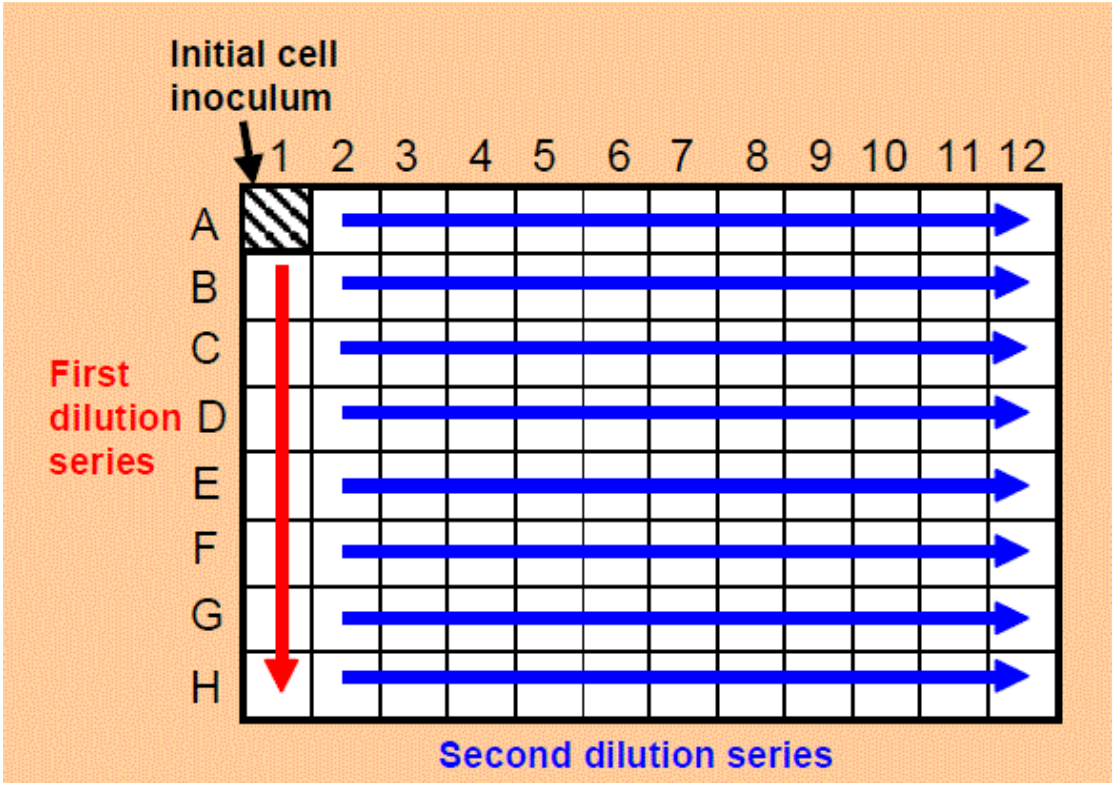

Figure 16.Singel cell cloning template [88]

2.3 Results

2.3.1. Validation of APOBECs regulation by *C. trachomatis* using qPCR

As mentioned earlier, microarray analysis of different cell lines infected with *C. trachomatis* revealed upregulation of APOBEC 3A, 3B and 3G. Therefore, we wanted to validate these reulsts using quantitative- real time PCR (qRT-PCR). For this End1 E6/E7 and human primary ectocervical cells were either uninfected or infected with MOI5 of *C. trachomatis* for 48h and total RNA was extracted. qRT-PCR was performed using target-specific primers indicated in the table below.

APOBEC3B RT-PCR Primers (PMID: 23696735)	
APOBEC3B_rt_FP	TTCGAGGCCAGGTGTATTTCA
APOBEC3B_rt_RP	CAGAGATGGTCAGGGTGACA
APOBEC3A RT-PCR Primers (PMID: 23696735)	
APOBEC3A_rt_FP	ATGGCATTGGAAGGCATAAG
APOBEC3A_rt_RP	CAAAGAAGGAACCAGGTCCA
APOBEC3G RT-PCR Primers (PMID: 23696735)	
APOBEC3G_rt_FP	GGTCAGAGGACGGCATGAGA
APOBEC3G_rt_RP	GCAGGACCCAGGTGTCATTG
AICDA RT-PCR Primers (PMID: 23696735)	
AICDA_rt_FP	AAATGTCCGCTGGGCTAAGG
AICDA_rt_RP	GGAGGAAGAGCAATTCCACGT

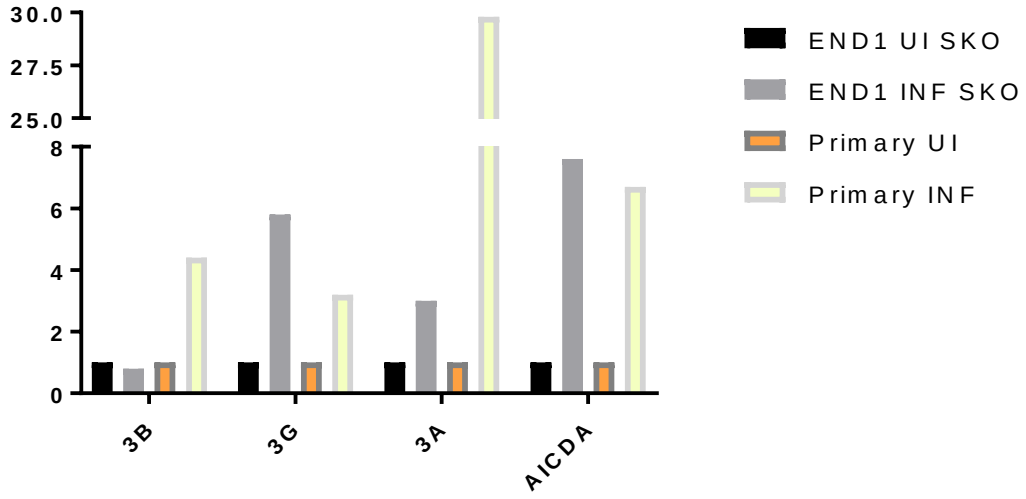

Figure 17.qPCR of uninfected and infected APOBEC3B, APOBEC3A, APOBEC3G and AICDA in END1/E6E7 and human primary ectocervical cells .

Our results indicate significant up regulation in both infected End1/E6E7 and primary cells at APOBEC3G,3A and AICDA in both cell systems. However, APOBEC3B was mainly found to be upregulated in primary cells (The RNA from End1/E6E7 and primary cells was provided by Stefanie Koster, MPIIB). These results thus confirm the observations from the micro array experiments.

2.3.2 CRISPR Knock-out of APOBEC3B

2.3.2.1 DNA preparation for CRISPR

As mentioned earlier in background and objectives section, expression of APOBEC3B, APOBEC3A and APOBEC3G genes was increased after the infection with *C. trachomatis* in different cell systems. Thus, in order to study the role of these proteins during *C. trachomatis* infection our first goal is to generate knock-out cell line using the CRISPR/Cas9 technology. For this we started with the generation of knock-out cell lines of the APOBEC3B in End1 E6/E7 cells.

Therefore, the first step of this part of the project was to prepare sufficient quantities of the LentiCRISPRv2 plasmid that which would be used for the cloning of the

gRNA. The plasmid possesses the antibiotic resistance genes for ampicillin and puromycin that can be used for the selection of transformats in E.coli and host cells respectively, an origin of replication in *E. coli*, a promoter and terminator. *E. coli* were transformed with LentiCRISPRv2 and the transformants were selected by ampicillin resistance, the plate with the transformed colonies is show in Figure 18. From this one colony was picked using a sterile toothpick and inoculated in to sterile LB medium and used for the preparation of the plasmid midi preparation.

Figure 18. Ccolonies of LentiCRISPRv2 transformed E.Coli

Once, the LentiCRISPRv2 plasmid was isolated in the midi-prep the next step was to digest it with the Esp3I restriction enzyme with recognition sequence at the positions 2852 and 4737 in the plasmid. This restriction digestion leads to the digestion of the plasmid at two positions, releasing two bands with a size of 2Kb and 12,873bp. The digested plasmid was subjected to agarose gel electrophoresis and the 12,873bp was isolated by gel purification.

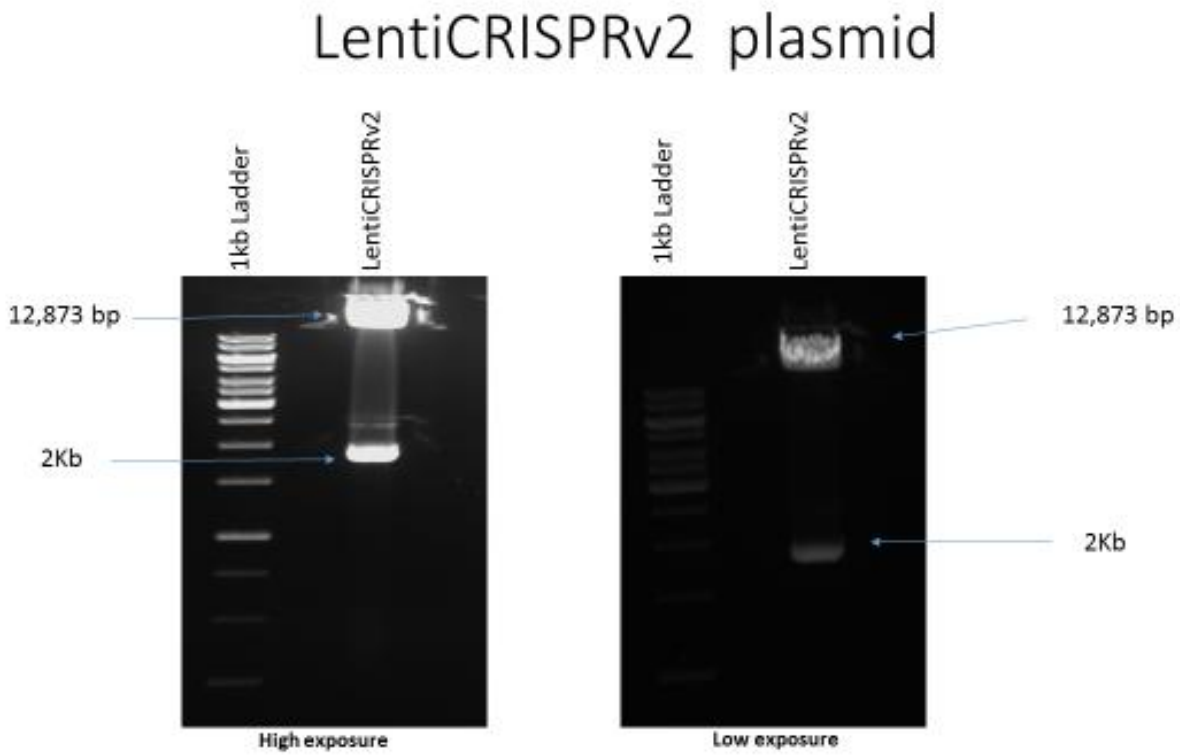

Figure 19.LentiCRISPRv2 plasmid in agarose gel after digestion and dephosphorylation. The plasmid was digested in two positions generating two bands. One larger 12.873 bp and one smaller 2Kb

The second step of the cloning was to anneal APOCEC3B_KO2, APOCEC3B_KO11 and APOCEC3B_KO10 gRNAs oligos to generate the gRNA for ligation in the linearized LentiCRISPRv2 vector.

Apobec3B Knock-out gRNA: (Eg. 2,11,10-Chopchop ranking)	
Apobec3B_KO_2_1	caccg**TGGCGCCGTCGAAGGACCAA**
Apobec3B_KO_2_2	aaac**TTGGTCCTTCGACGGCGCCA**c
Apobec3B_KO_11_1	caccg**CATAGTCCATGATCGTCACG**
Apobec3B _KO_11_2	aaac**CGTGACGATCATGGACTATG**c
Apobec3B_KO_10_1	caccg**AGGAGCCCGCGTGACGATCA**
Apobec3B_KO_10_2	aaac**TGATCGTCACGCGGGCTCCT**c

After, generating the gRNA's by annealing I next performed the ligation of the linearized LentiCRISPRv2 vector with the gRNA to generate the circularized plasmid with the desirable sequences (gRNA sequences).

2.3.2.2 Transformation ligation product in to E. coli StbI3

As mention earlier in the methods section - preparation of chemically competent cells (StbI3) and transformation plasmid into StbI3 bacteria, lentiviral plasmids contain Long-Terminal Repeats (LTRs) and thus must be transformed into recombination-deficient bacteria. Therefore, the ligation product was transformed in *E. coli* StbI3 and transformanats were selected by ampicillin resistance. We obtained numerous transformed colonies. From these we picked two to three colonies and plasmid DNA DNA was purified by midiprep.

Once, the DNA was prepared, I sent it for sequencing using hU6-F (5'-GAGGGCCTATTTCCCATGATT-3') to confirm if the gRNA was cloned correctly.

Results of sequencing

Ergebnisse Sequenzierung:

Sequenzvergleich zu pEZ252 (CEA.02, wurde für STZ kloniert, sgRNA: **caccgAGAGACCATGGAGTCTCCCT**

Vektor LentiCRISPR V2 ; sgRNAs sind in Position 7638-7658

sgRNAs:

Apobec3B_KO¬_2_1 caccgTGGCGCCGTCGAAGGACCAA

Apobec3B_KO¬_11_1 caccgCATAGTCCATGATCGTCACG

Apobec3B_KO¬_10_1 caccgAGGAGCCCGCGTGACGATCA

pEZ252 7600 tcttggctttatatatcttgtggaaaggacgaaacaccg-agagaccatg

KO-2_1-1.hU6-F 176 tcttggctttatatatcttgtggaaaggacgaaacaccg-tggcgccgtc

KO-2_2-1.hU6-F 172 tcttggctttatatatcttgtggaaaggacgaaacaccg-tggcgccgtc

KO-10_1-1.hU6-F 170 tcttggctttatatatcttgtggaaaggacgaaacaccg-aggagcccgc

KO-10_2-1.hU6-F 166 tcttggctttatatatcttgtggaaaggacgaaacaccg-aggagcccgc

KO-11_1-1.hU6-F 173 tcttggctttatatatcttgtggaaaggacgaaacaccgcatagtccat-

KO-11_2-1.hU6-F 172 tcttggctttatatatcttgtggaaaggacgaaacaccgcatagtccat-

pEZ252 7649 gagtctccctgttttagagctagaaatagcaagttaaaataaggctagtc

KO-2_1-1.hU6-F 225 gaaggaccaagttttagagctagaaatagcaagttaaaataaggctagtc

KO-2_2-1.hU6-F 221 gaaggaccaagttttagagctagaaatagcaagttaaaataaggctagtc

KO-10_1-1.hU6-F 219 gtgacgatcagttttagagctagaaatagcaagttaaaataaggctagtc

KO-10_2-1.hU6-F 215 gtgacgatcagttttagagctagaaatagcaagttaaaataaggctagtc

KO-11_1-1.hU6-F 222 gatcgtcacggttttagagctagaaatagcaagttaaaataaggctagtc

KO-11_2-1.hU6-F 221 gatcgtcacggttttagagctagaaatagcaagttaaaataaggctagtc

As shown in the figure above all the colonies sequenced were found to contain the desired sequences.

2.3.2.3 Production of lentivirus and transduction.

In order to generate the knockout clonal cell lines of APOBECs, using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology. The LentiCRISPRv2 vector consisting of one of gRNA for the APOBEC was used for the production of lentiviral particles. The produced lentiviruses were used for transduction of End1 E6/E7 cells. The transduced cells were selected with puromycin, the selection of the cells containing the vector was complete after 3-4 days of transduction.

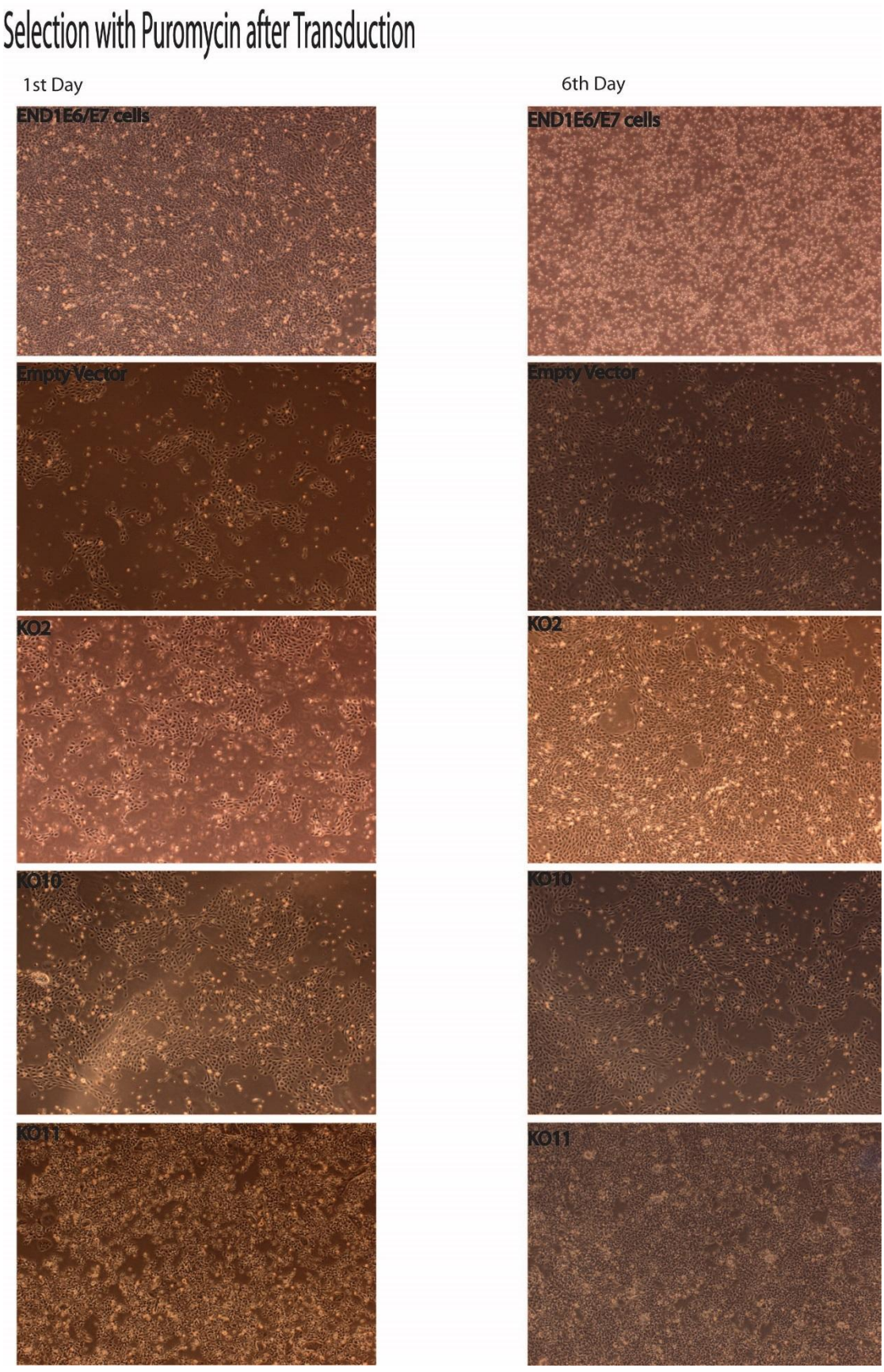

Figure 20. END1E6/E7 cells after transduction

Interestingly, the End1 E6/E7 cells transduced with the APOBEC3B_KO11 gRNA showed significantly different morphological phenotype compared to the control cells. Thus, we assumed that in case of this gRNA ww might have a KO of the target gene in a majority of the cells.

2.3.3 Infection of the APOBEC knockout pools with *C. trachomatis*

In order to, verify the whether the knock-out was successful or not, we investigate the expression of APOBEC genes after infection cell cells either uninfected or infected with C. trachomatis. For this, pools of the APOBEC knockout End1 E6/E7 cells were either uninfected or infected with MOI 5 of GFP expressing *C. trachomatis L2* in a 6 well plate. As shown in Figure 21-22, around 80% of the cells were found to be infected revealed by the presence of a large *C. trachomatis* inclusions shown in green.

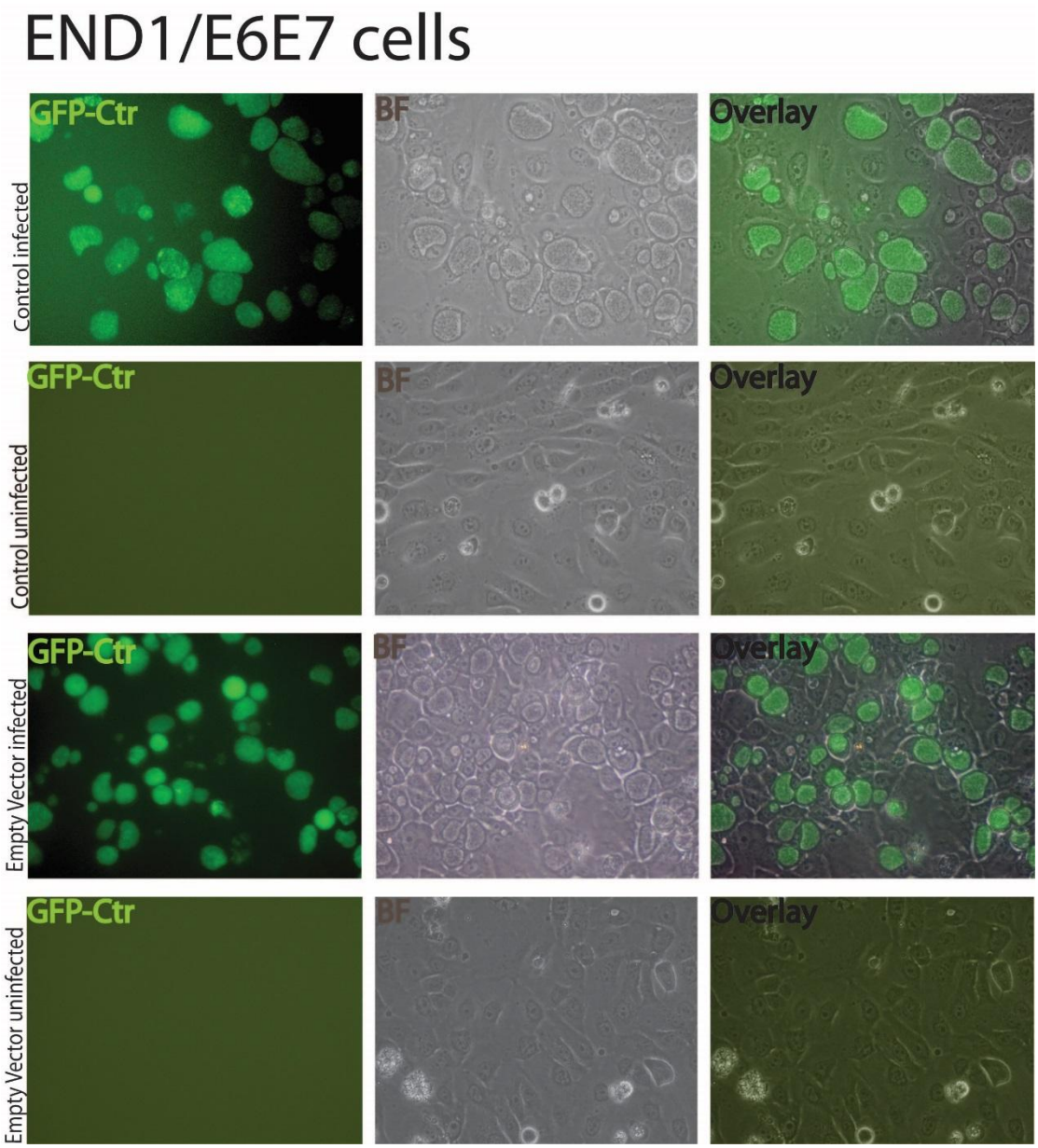

Figure 21. Infected and uninfected END1E6/E7 cells and Empty Vector

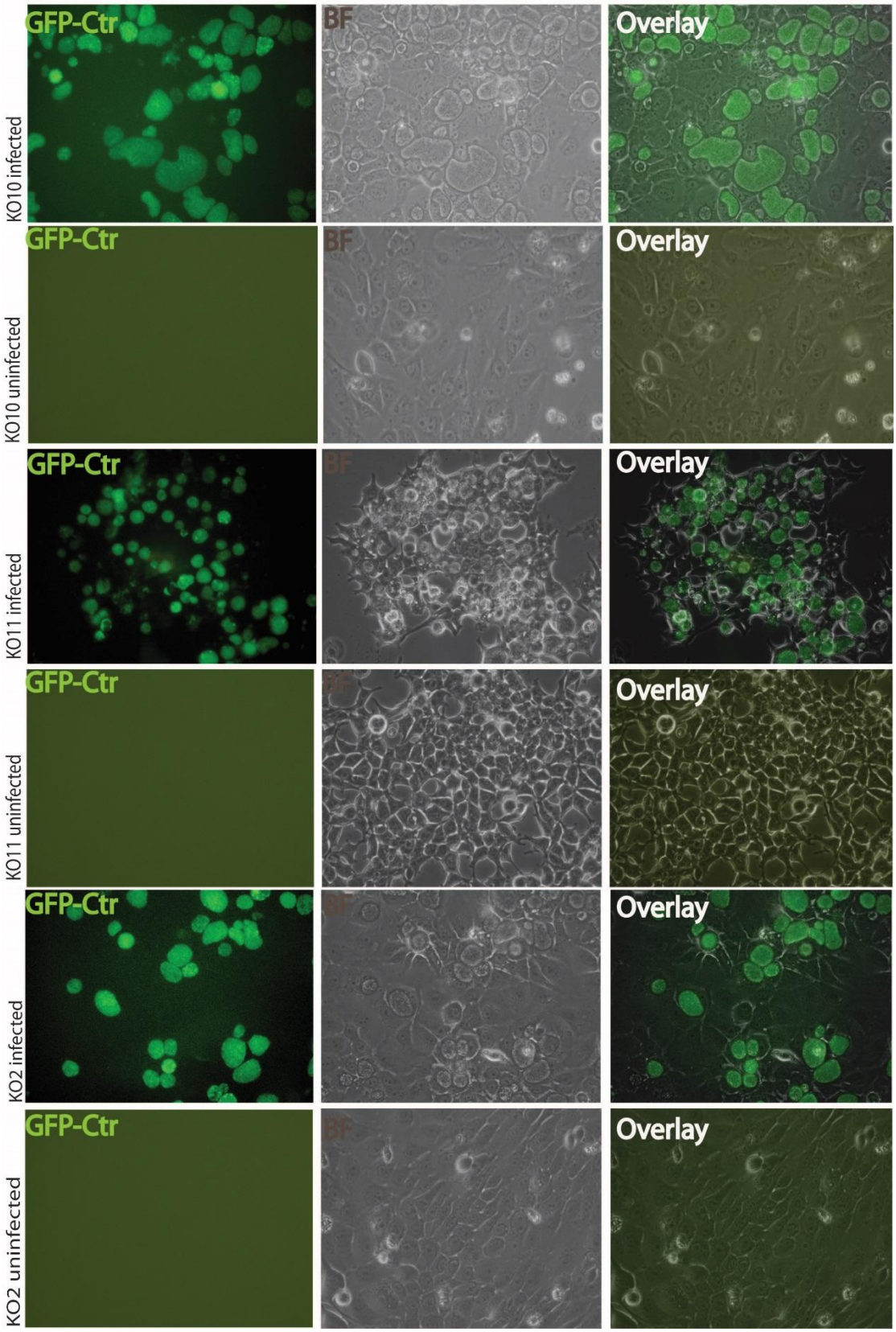

Figure 22. Infected and uninfected KO10, KO11, KO2

2.3.4 Evaluation of knockout by Western blotting

Whole cell lysates were prepared from uninfected and *C. trachomatis* infected control as well as the APOBEC KO pools using Lamili buffer. The cell lysates were subjected to Western Blot using specific antibody targeting APOBEC3B and beta.actin was used as a loading control. Our results show bands for APOBEC3B in the control as well as the KO pools after *C. trachomatis* infection. to all infected KO.

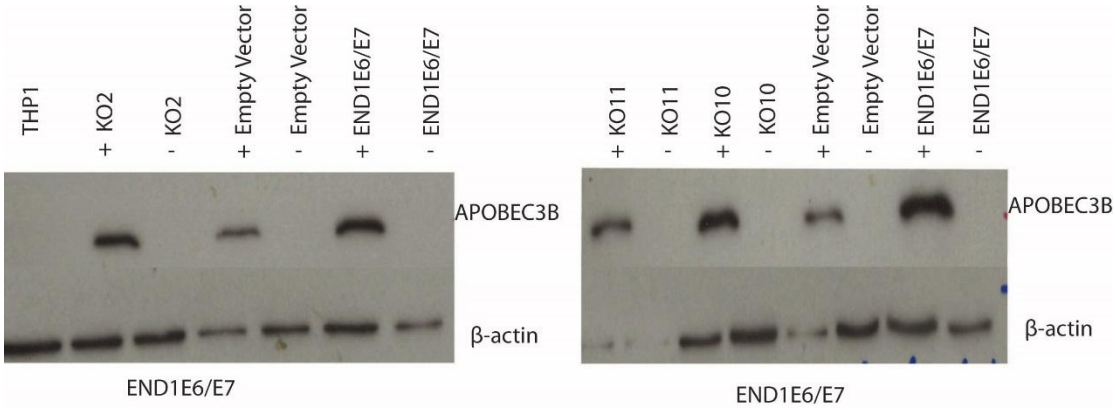

Figure 23. Western Blot. End1E6/E7 cells uninfected or Ctr infected for 40h were subjected to western blotting analysis . B-Actin antibodies were used as loading control.

2.3.5 Single cell clones expansion

Since, we could not see a significant amount of loss of the APOBEC3B protein from the KO cell pool compared to the control cells. We decided to generate single cell clones and identify the clones that shown complete KO for the target gene. For this purpose I performed a single cell cloning by serial dilution using $1x10^6$ cells of END1E6/E7 KO11 and KO10 cells. The dilution steps were carried out as mentioned in the methods (single cell cloning). 10-15 days after the dilution and plating I was expected to see single cell clones as shown in the example shown in Figure 25.

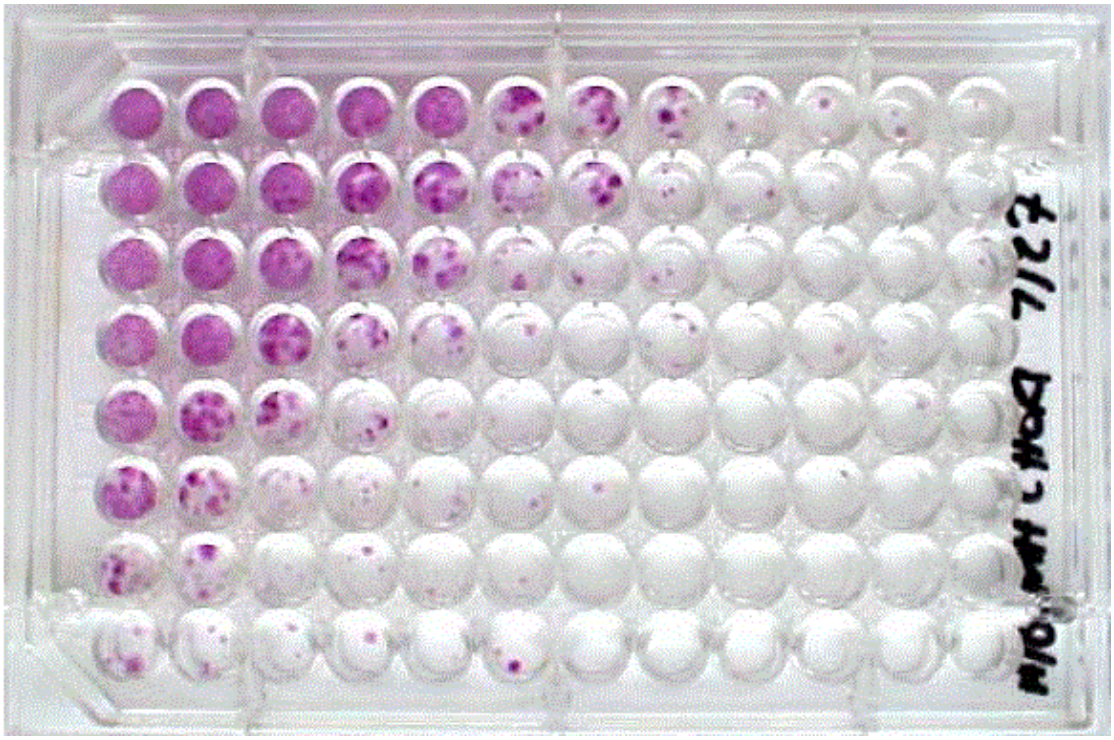

Figure 24.Example of a plate containing dilution of CHO-K1 cells [88]

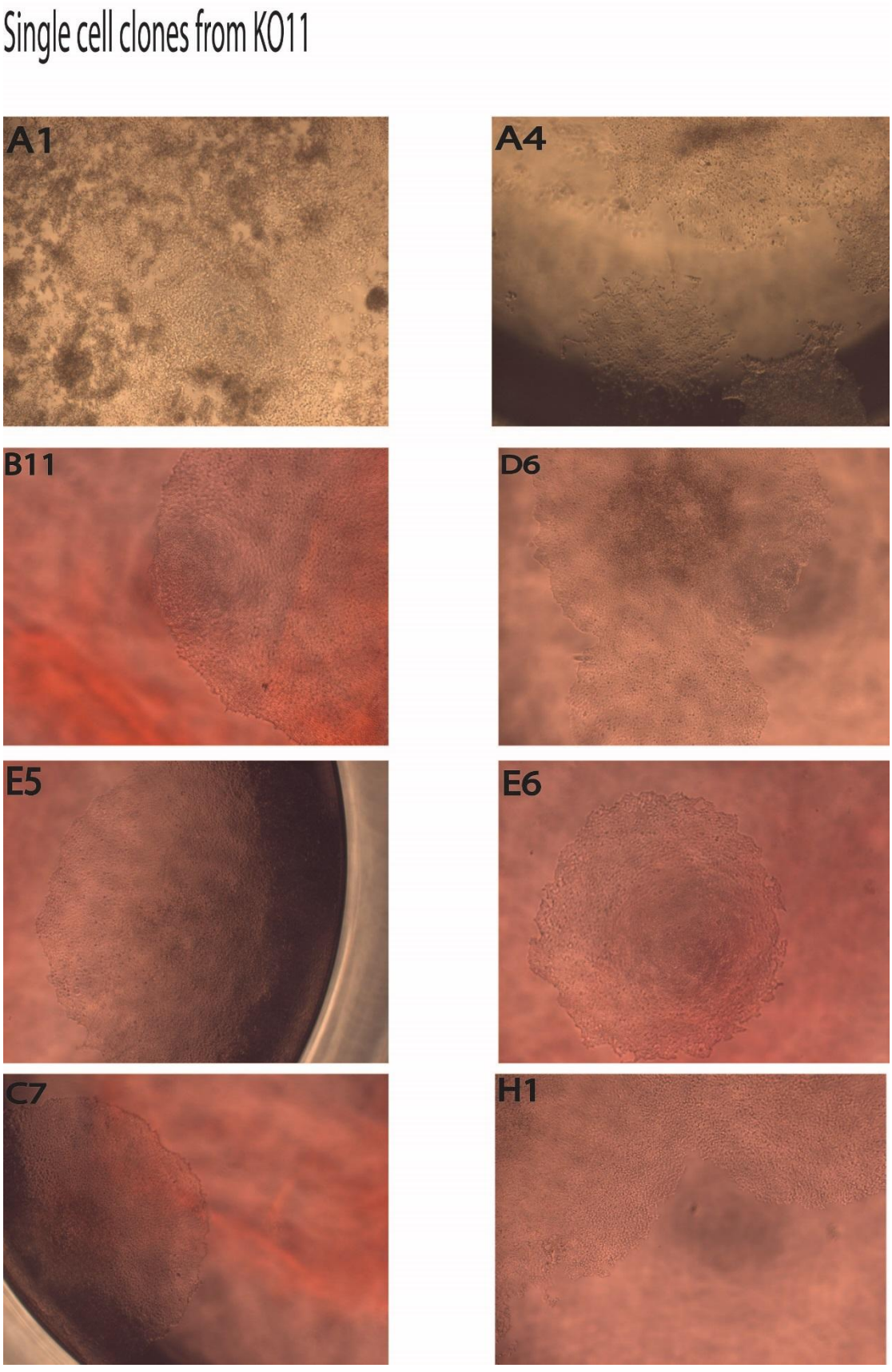

Figure 25.Single cell clones of KO11 in 96 well plate 15 days after the dilution in A1, A4, B11, D6, E5, E6, C7 and H1 wells

Once the single cell colonies started to grow and became confluent in the 96 well, I transferred the single cell clones to 24 well plate. Further, they were transferred from 24 to 12 well plate then to 6 well plate and finally to T75 flasks. The clones thus selected were used for investigating the knockout status of the APOBEC3B using western blotting in uninfected and *C. trachomatis* infected cells.

2.3.6 Infection of single cell clones of KO10 and KO11 with *C. trachomatis*

Here, we wanted to verify the knockout status of the APOBEC3B in each of the single cells clones generated using the gRNA 10 (KO10) and gRNA 11 (KO11). Thus each of the single cell clones were either uninfected or infected with MOI 5 of *C. trachomatis* in a 6 well plate. After 40 hours of the infection, the cells were harvested with 2X Laemmli buffer for WB.

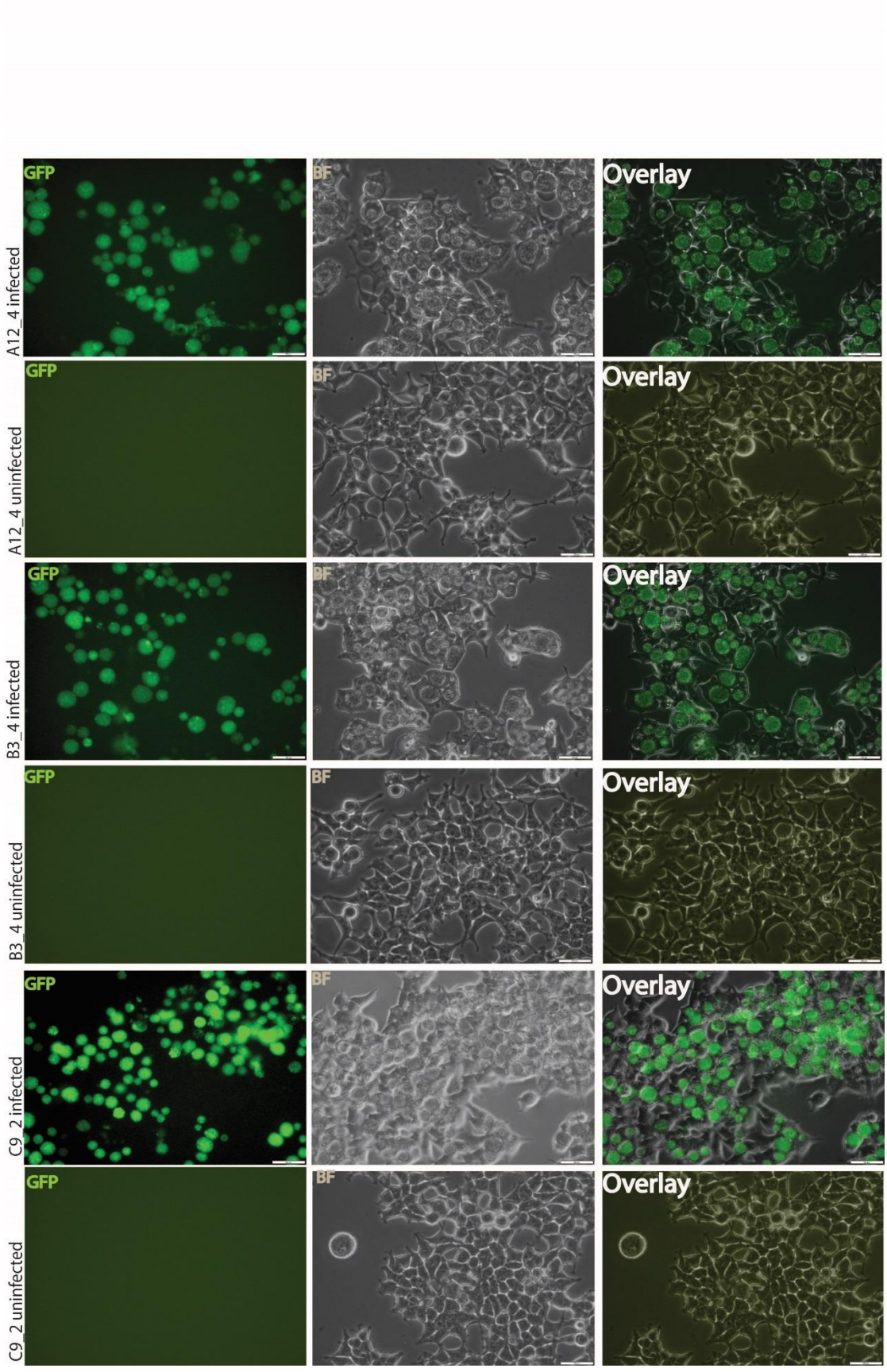

Figure 26. Single cell clones infected with GFP expressing C. trachomatis**.** Representative of 22 single cell KO11 clones are shown.

.

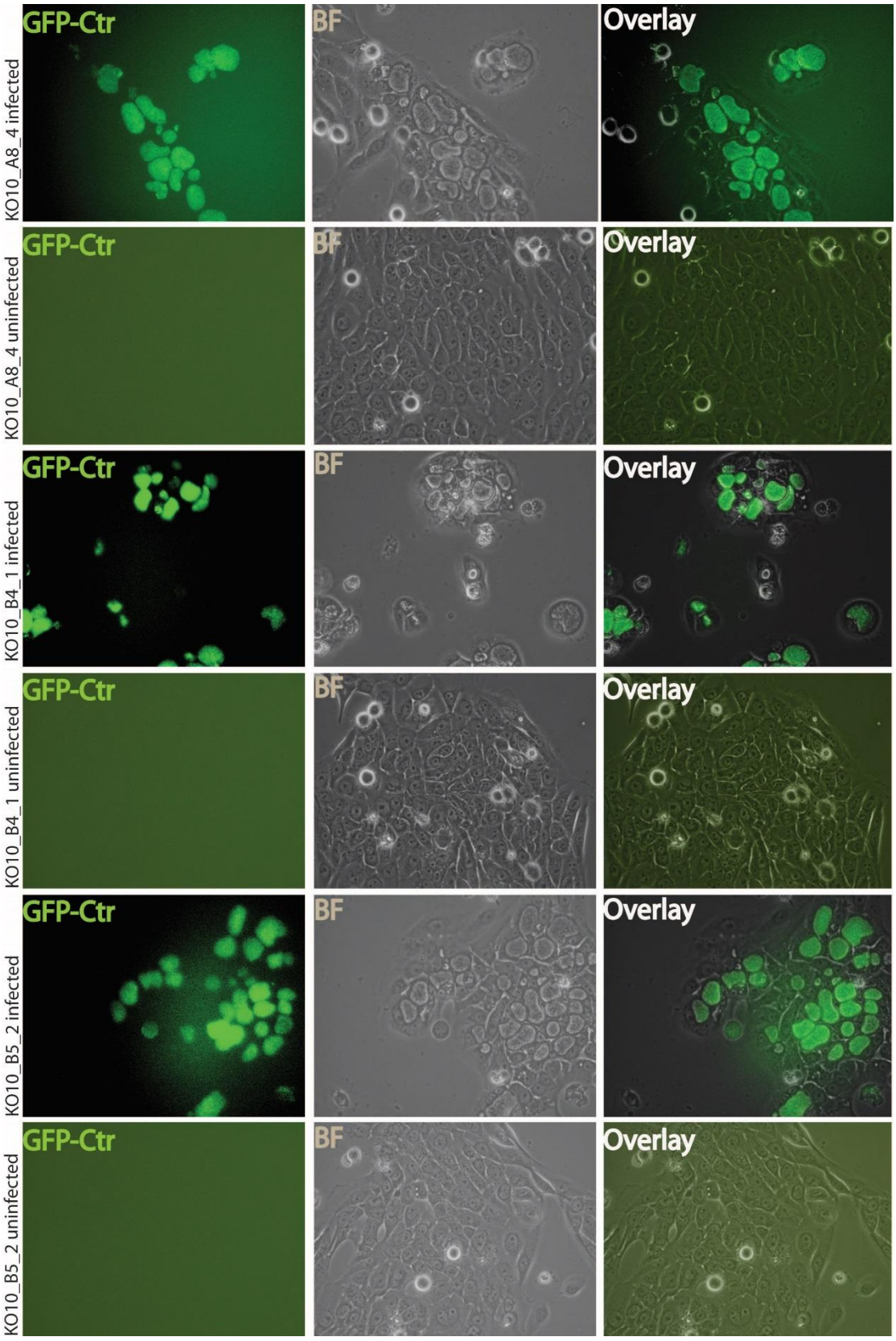

Figure 27. Single cell clones infected with GFP expressing C. trachomatis.Representative of 22 single cell KO10 clones are shown**.**

2.3.7 Evaluation of knockout after infection of single cell clones by WB

The lysate from the single cell of KO11 (H1/4, B10/1, F4/3, D6/3, B3/4, H3/2) either uninfected or infected by *C. trachomatis* were harvested and subjected to WB using the APOBEC3B antibody

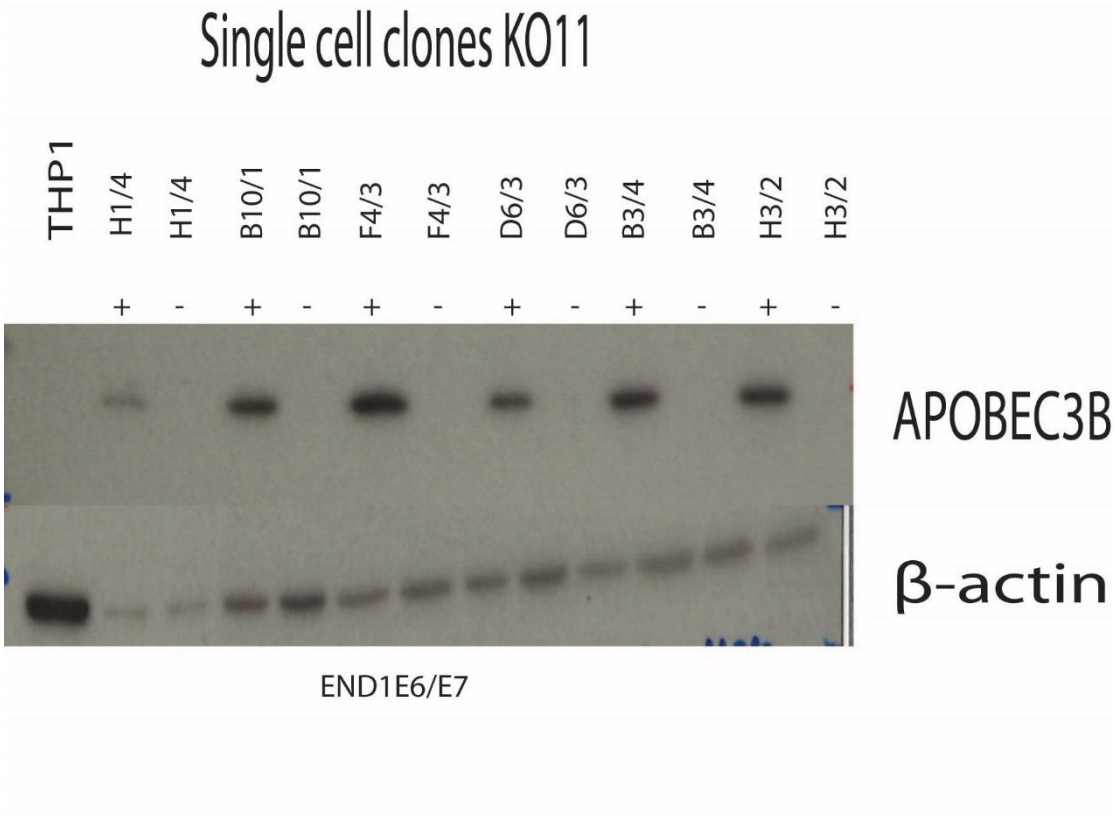

Figure 28. Western Blot for single cell clones of KO11 . Single cells clones uninfected or *C. trachomatis* infected for 40h were subjected to western blotting analysis. B-Actin antibodies were used as a loading control.

2.3.8 Deep sequencing for the evaluation of indels

The genomic DNA from the pool and single cell clones was isolated and PCR was performed using a set of primers flanking the gRNA site in the genome. The amplicon was then sent for sequencing for the analysis of Indels to determine the efficacy of

CRISPR/Cas9. APOBEC3B KO2 pool, there is about 65% indel frequency. All other samples do not show any relevant indel proportions.

2.4 Discussion

To study the effect of APOBEC3 genes unregulated during the *C. trachomatis* infection we decided to generate CRISPR/Cas9 mediated knockout cell lines. For this purpose END1E6/E7 cells were used, these cells are appropriate to study the knockouts effects of APOBEC3 during *C. trachomatis* infection as they are isolated from human endo cervix and especially and were transformed with HPV-16 E6/E7 oncogenes [89, 90].

The first part of this thesis consisted of cloning gRNAs in to suitable lentivirus vectors and generation of the lentivirus for transduction of the target cells. We chose the LentiCRISRv2, it consisted ampicillin resistance gene for selection in *E. coli* as well as bleomycin and puromycin resistence genes for selection in the host cells . Also, the vector consists of a multiple cloning site for rapid and efficient cloning of Taq-amplified PCR products. The ampicillin resistance gene for selection in *E. coli* for high-copy replication and maintenance of the plasmid [77].

The second part of the thesis was the lentiviral transduction into the host cells, generation of single cell clones and verification of the knockout status. There are different ways to introduce DNA (the gRNA or gRNA containing plasmid DNA and Cas9 expression construct) into eukaryotic cells like as 1) lipofection where you mix gRNA and Cas9 with lipid vesicles (liposomes) to form liposome-gRNA/Cas9 complexes. Then you add the complexes directly to your cell cultures which are taken up by the cells. 2) Electroporation is a procedure that uses an electrical pulse (300~400 mV for less than a millisecond) to create temporary pores in the cell membrane so that DNA or RNA can enter and 3) Lentiviral based transduction as already described in methods-Lentiviral preparation and transduction. We chose the lentiviral transduction because the delivery efficiency is high even in the hard to transfect cell lines [91-95].

In the third part END1E6/E7 KO2, KO10, KO11 cells were infected with GFP expressing *C. trachomatis.* From all the different serotypes of *Chlamydia trachomatis*, I already described in the introduction- *Chlamydia trachomatis* serotypes, the most suitable for our experiment was considered the serotype L2. The reason was, that only

the Serovars L1, L2, and L3 cause invasive STDs and specifically, lymphogranuloma venereum. The decision to use this serotype for the infection was based solely on ease of use. Interestingly, the fluorescence microscope images showed significant diffee´rences in the morphology of the host cells for KO11 pools and also *C. trachomatis* inclusions seemed to be considerably smaller.

Nevertheless, the phenotype by itself is not a sufficient to conclude that the knockout was successful. Therefore, we performed a Western Blot analysis using a specific antibody for APOBEC3B. The APOCE3B proteins was found to be still expressed both in the KO2, KO10 and KO11 pools. These observations raised the question, why we can see such a different phenotype for host cells and also *C. trachomatis* growth in KO11 cells although we do not have knockout. This aspect needs to be further investigated.However, a possible explanation may be that we have a CRISPR off-target mutation.

In conclusion, we succefulls cloned gRNA constructs in the lentivirus vectors which also consist the Cas9 expression cassette. Further, we were successful in generating the lentiviruses for three independent gRNAs targeting APOBEC3B and transduction of END1/E6E7 cells. Further, both the pools and single cells were selected using puromycin selection strategy. However, subsequent analysis by Western Blotting reveled that we do not have knockouts with single cells clones among the once analysed. Therefore, we should repeat the experiment maybe with different more efficient gRNAs. Also, we will use primary cells in additionally to END1/E6E7, since our qRT-PCR results shows clear increase of the expression of APOBEC3G, APOBEC3A and AICDA in infected primary cells comparative to END1/E6E7 cells. Last but not least, considering to deep sequencing results our next more efficiency step is to make single cell clones for KO2, because the overall we could observe 65% on-target mutation rate from the pool of the KO2.

Περίληψη

Ο καρκίνος του τραχήλου της μήτρας είναι ο τέταρτος συνηθέστερος καρκίνος στις γυναίκες και αποτελεί ένα από τα σημαντικότερα προβλήματα υγείας παγκοσμίως. Είναι σήμερα γνωστό ότι σχετίζεται με πολλές βακτηριακές λοιμώξεις με την μεγαλύτερη συσχέτιση με την HPV λοίμωξη. Συγκεκριμένα, οι υψηλού κινδύνου τύποι του ιού έχουν βρεθεί σε πάνω από 70% καρκίνων του τραχήλου της μήτρας. Ωστόσο, ο HPV δεν είναι η μόνη παθολογική κατάσταση που έχει συσχετιστεί με τον καρκίνο του τραχήλου της μήτρας. Πολλές σημαντικές επιστημονικές έρευνες βασίζονται στη σχέση μεταξύ του *C. trachomatis,* μιας κοινής σεξουαλικά μεταδιδόμενης νόσου και του καρκίνου του τραχήλου της μήτρας. Μεγάλο ενδιαφέρον παρουσιάζουν οι μελέτες που περιγράφουν τα *C. trachomatis* ως ένα από τους κύριους συν-παράγοντες στην καρκινόγονη επαγόμενη από HPV. Έτσι, δημιουργείτε μια αλληλεπίδραση μεταξύ του καρκίνου του τραχήλου της μήτρας, του HPV και του *C.trachomatis*. Προηγούμενες μελέτες έχουν δείξει ότι οι απολιποπρωτεΐνες φαίνεται να αυξάνουν και να συμβάλλουν στην ανάπτυξη των μεταλλάξεων που βρέθηκαν στους καρκίνους του τραχήλου. Αν και οι HPV ογκοπρωτεΐνες E7 εμπλέκονται στην ενισχυμένη έκφραση κάποιων μελών της οικογένειας APOBEC, οι μοριακοί μηχανισμοί δεν είναι ακόμη σαφείς.

Ενδιαφέρον παρουσιάζει, η ανάλυση των HPV E6E7 θετικών ενδοτραχηλιακών πρωτογενών επιθηλιακών κυττάρων (END1 / E6E7) ποντικιού που έχουν μολυνθεί από *C. trachomatis* , και των εξωτραχηλιακών αρνητικών σε HPV πρωτογενών ανθρώπινων κυττάρων, όπου παρατηρείται αυξημένη έκφραση APOBEC3A, APOBEC3B και APOBEC3G, ανεξαρτήτως κατάστασης HPV (*Chumduri, Gurumurthy et al, unpublished*).

Η έρευνα αυτή περιλαμβάνει τη διερεύνηση του μηχανισμού και των συνεπειών της αυξημένης ρύθμισης των παραπάνω μελών της οικογένειας APOBEC κατά τη διάρκεια της λοίμωξης από C. trachomatis. Για το σκοπό αυτό, η πτυχιακή αυτή στοχεύει (α) Στη δημιουργία ,μέσω CRISPR / Cas9 τεχνολογίας, Knockout κυτταρικών γραμμών Apobec3B, Apobec3A, Apobec3G, (β)Στην επιβεβαίωση των Knockouts, (γ) Στη διερεύνηση των επιδράσεων των knockouts κατά τη διάρκεια λοίμωξης από C. trachomatis.Με επιτυχία δημιουργήσαμε gRNA κλώνους σε

πλασμίδιο που περιέχει την Cas9 αλληλουχία. Επίσης πετύχαμε την δημιουργία END1/E6E7 κυτταρικών γραμμών με την αλληλουχία στόχο των gRNAs για το APOBEC3B. Ωστόσο, περαιτέρω ανάλυση έδειξε ότι δεν έχουμε knockouts.

Abstract

Cervical Cancer is the fourth most common cancer and a major health problem worldwide. Cervical cancer has been associated with various pathological conditions with the major association with HPV infection. Actually, "high risk" types of HPV cause more than 70% of cervical cancers. However, HPV is not the only pathological condition who have been associated with cervical cancer. Considerable scientific research is based on the relationship between *C. trachomatis* , a common sexually transmitted disease, and cervical cancer. Interestingly, the studies have shown that *C. trachomatis* is one of the major co-factor in HPV induced cervical carcinogenesis. Thus, there is an interaction between Cervical cancer, HPV and *C. trachomatis* infection. A big concern in several studies was the behaviour of APOBECs gene, which is lead to mutations, 68% of them are in the bladder, cervical, breast, head and neck, and lung cancers.

Previous Experiments based on transcriptomic analysis of *C. trachomatis* infected mouse END1E6/E7 and HPV negative primary human ectocervical cells, performed at Department of Molecular Biology from Max Planck Institute for infection biology were based on the role of Chlamydia infection in the development of cervical cancers. These experiments showed that the expression of APOBEC3A, APOBEC3B and APOBEC3G genes after Chlamydia trachomatis infection was upregulated at both RNA and DNA level (*Chumduri, Gurumurthy et al, unpublished*).

The aim of this research project is a)Generate CRISPR/Cas9 mediated knockout cells lines of Apobec3B, Apobec3A, Apobec3G b)Confirm the Knockouts and c)Investigate the effects of knockouts during *C. trachomatis* infection. We successful cloned gRNA constructs in the lentivirus vectors which also consist the Cas9 expression cassette. We were also succed in generating the lentiviruses for three independent gRNAs targeting APOBEC3B and transduction of END1/E6E7 cells. However, subsequent analysis by Western Blotting revealed that we do not have knockouts.

3. References

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