# Generation of an oncolytic adenovirus vector combining three cancer targeting strategies and characterization of a new preclinical model for breast cancer virotherapy

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Dedicated to my parents, my sisters, my boyfriend and Jochen († 2010).

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# 1. Introduction

## *1.1 Breast cancer*

Despite early detection methods and advanced conventional treatments, breast cancer is one of the most frequently diagnosed malignancies and the second leading cause of cancer deaths among women worldwide (DeSantis et al., 2011; Jemal et al., 2011; Thomas et al., 2006). The chance of being diagnosed for women is 1 in 8 (http://seer.cancer.gov/csr/1975_2009_pops09/). Every year more than 192,000 women are diagnosed as positive for breast cancer in the United States (Jemal et al., 2010); the incidence rate for breast cancer is still high in the United States as well as Australia/ New Zealand, and Western and Northern Europe (Jemal et al., 2011). Since the last 25 years, breast cancer death rates decreased in several European countries and Northern America due to early detection methods. However, the number of estimated new cases of breast cancer in 2012 is about 226,870 in the United States and the estimated number for breast cancer death is 39,510 (Siegel et al., 2012). According to the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute, responsible for collecting breast cancer data in the United States, 1 in 3 cancers diagnosed in women is breast cancer (DeSantis et al., 2011). Therefore, novel therapies designed with alternative and complementary strategies are sorely needed.

### 1.1.1 Pathophysiology of breast cancer

The breast contains 15-20 lobes which radiating from the nipple surrounded by fibrous connective tissue and fats. Each lobe can be separated into several lobules. The lobules supply milk to the ducts and therefore are also referred to as ductual-lobular. The basic structural units of the breast are the lobules which are surrounded by epithelial cells.

The most common breast cancer originates either in the epithelial cells of the inner lining of the milk ducts (ductal carcinoma) or in the lobules (lobular carcinoma). As with every other cancers, breast cancer developes from different genetic aberrations or aberrant growth factor receptor signaling (Madigan et al., 1995; Taylor et al., 2008; Wosikowski et al., 2000; Ziegler et al., 1993). Furthermore, breast cancer can be inherited, most commonly resulting from a genetic defect in the DNA repair genes BRCA1 and BRCA2 (breast cancer susceptibility gene 1, 2) (Chappuis et al., 1999; Struewing et al., 1997). Besides inherited genetic aberrations and

defects, breast cancer can be initiated through environmental factors (Davis and Sieber, 1997; Madigan et al., 1995; Ziegler et al., 1993).

Development of breast cancer is a multiple process that can be divided into several steps. Precursor lesions that are benign can be divided into non-proliferative and proliferative lesions. For example, non-proliferative lesions are duct ectasia or mild hyperplasia of the normal and simple type (Beckmann et al., 1997). Proliferative lesions can be further divided into proliferative phase without atypia and with atypia. Cells found in regions of proliferative changes without atypia show an extreme cell growth without any abnormal cell structure. Proliferative changes with atypia on the other hand are characterized by regions of cell expansion containing some cells with an abnormal cell structure. Lesions of this type are also called atypical hyperplasia and cells demonstrate a few characteristics of a carcinoma in situ (Beckmann et al., 1997). Carcinoma in situ originated from proliferation of the glandular epithelium demonstrating cellular malignancy and is restricted to a specific area. Carcinoma in situ can be separated in either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). Tumors that aquire the ability to penetrate into the surrounding breast cancer tissue and metastasize to other organs are called invasive breast carcinomas (Lakhani, 1999). The most common type is invasive ductal carcinoma (IDC) (Beckmann et al., 1997).

### 1.1.2 Breast cancer treatments

Several treatment options for breast cancer have been developed during the last decade. These treatment approaches are divided into surgery, chemo-, radiation-, hormonal and targeted therapy. The breast cancer treatment is chosen depending on stage and type of cancer. Most women diagnosed with breast cancer undergo surgery to remove as much cancer tissue as possible.

Breast conservation surgery is the most common surgery, in which depending on the stage of the breast tumor, the breast tumor and a small region of tumor surrounding breast tissue are removed. Segmental or partial mastectomy is another option of surgery during which a larger section of the tumor surrounding breast tissue is removed. Breast cancer patients with more advanced tumor stage need to undergo a radical mastectomy, by removing the entire breast as well as several lymph nodes under the arm. Although, surgery is still the preferred method to remove a tumor successfully, it also bears some disadvantages. The most common

complications observed after breast cancer surgery is the collection of serous fluid under the skin, seroma formation and axillary dead space (Pogson et al., 2003), (Hashemi et al., 2004).

Chemotherapy is another option to treat breast cancer. It involves the use of drugs to kill cancer cells, inhibit cancer cell growth or to avoid cancer recurrence. Some of the commonly used drugs for chemotherapy to treat breast cancer patients are adriamycin (also called doxorubicin), cyclophosphamide, and paclitaxel (Ibrahim et al., 2003); (Hutchins et al., 2005). Usually, chemotherapy can be divided into three different types: 1) Neoadjuvant, given before surgery to shrink the tumor; 2) Adjuvant, given after surgery to prevent recurrence; and 3) Palliativ, given to control cancer in the stage where it is already metastatic. However, treatment with chemotherapeutic reagents induces some mild side effects like fatigue, nausea, hair loss and vomiting and several severe ones such as cardiotoxicity, necrosis of the colon, leukemia and severe infections (Ewertz and Jensen, 2011; Lemieux et al., 2008; Lindley et al., 1999; Sitzia and Huggins, 1998; Yood et al., 2012). The severe side effects can be fatal and cause the death of the breast cancer patient.

Radiation therapy on the other hand exploits high energy X-rays to destroy breast cancer cells or inhibit their proliferation. High energy radiation damages the DNA of cancer cells or forms charged particles like free radicals causing cancer cell killing. There are two types of radiation therapy, external and internal radiation therapy. External radiation therapy is performed with a machine outside of the body whereas internal radiation utilizes radioactive substances injected directly into the tumor or near the cancer occurrence. Radiation therapy is usually given after surgery either alone or in combination with chemotherapeutic reagents to avoid breast cancer recurrence, to kill metastasized breast cancer cells in other organs or residual cancer cells. Radiation therapy is painless but can induce severe side effects like skin irritation, hair loss, and damage to the salvery glands. Those side effects appear because healthy cells can be harmed during this treatment as well (Rutqvist et al., 2003). One of the severe side effects of radiation therapy is the induction of sarcoma. The occurrence rate is very low, but if sarcoma occurrs after the radiation therapy, it is associated with poor clinical outcomes (Sheth et al., 2012).

Besides chemo- and radiation therapy, hormonal therapy is another treatment utilized to treat breast cancer by using drugs to inhibit production of steroid hormones that stimulate cancer cell proliferation. Many breast cancer types express high levels of estrogen and/or progesterone receptors on their cell surface. Breast cancer patients suffering from tumors with high expression of estrogen receptors can undergo hormonal therapy. Drugs usually used for

hormonal therapy include aromatase inhibitors like exemestane (Tomao et al., 2011) and selective estrogen modulators like Tamoxifen (Smith et al., 2000). These drugs block the hormone activity or repress their expression to inhibit breast cancer cell proliferation. Besides killing of cancer cells these drugs show several side effects. For example, treatment with Tamoxifen can cause weight gain, hot flashes, vaginal discharge and severe late side effects such as increasing the risk for blood clots, stroke and invasive endometrial cancer (Perez, 2007). Aromatase inhibitors are most commonly given to postmenopausal women suffering from hormone dependent breast cancer requiring the hormone estrogen to proliferate. Aromatase inhibitors block the aromatase enzyme that converts androgen to estrogen in the body and therefore, prevent cancer cell proliferation (Gibson et al., 2009). Typical aromatase inhibitor side effects are skeletal fractures, hot flashes and development of osteoporosis (Perez, 2007).

Targeted therapy is another therapeutic treatment of breast cancer employing drugs or monoclonal antibodies to recognize and destroy specific cancer cells without damaging healthy cells. For example, monoclonal antibodies are used to recognize specific cancer cell surface markers or factors that are stimulating the tumor growth. Binding of monoclonal antibodies to their target destroys the cancer cell or inhibits cancer cell proliferation/migration. One example of a therapeutic monoclonal antibody is Trastuzumab that acts by blocking the signal cascade of the growth factor protein HER2. HER2 activates a signal cascade that in return induces proliferation of cancer cells (Higgins and Baselga, 2011). Targeted therapy drugs like tyrosine kinase inhibitors are administered to inhibit tumor growth signaling. Lapatinib, a tyrosine kinase inhibitor, prevents the effect of HER2 protein and other proteins inside the tumor cells. PARP inhibitors on the other hand inhibit DNA repair and may trigger cancer cell death. PARP inhibitors are widely studied for triple negative breast cancer (Higgins and Baselga, 2011). Triple negative breast tumors are lacking the expression of estrogen receptors (ER-), progesterone receptors (PR-) and HER2 (HER2-).

Thus, beside several of breast cancer treatment options currently available, there remains a need for novel treatments to reduce mortality rate of breast cancer.

## *1.2 Gene therapy for breast cancer*

Gene therapy is a therapeutic approach that involves the delivery of genetic material to cancer or defective cells and its surrounding tissue. The goal of cancer gene therapy is to cause tumor

cell death or prevent tumor growth. For cells that cause a disease due to a gene defect, gene therapy provides these cells permanently with the missing gene and ideally curing the disease (Cross and Burmester, 2006). Over the last decades, gene therapy became a powerful tool to treat metabolic disorders, cardiovascular diseases and cancer with great success while traditional therapies were limited due to their severe side effects (Kay and Woo, 1994); (Mulavisala et al., 2001; Rakhmilevich et al., 2004; Zhang et al., 1996). Gene therapy can be categorized into five different groups: 1) genetic immunopotentiation, 2) pro-apoptotic gene therapy, 3) anti-angiogenic gene therapy, 4) mutation compensation, and 5) molecular chemotherapy/suicide gene therapy (Cross and Burmester, 2006).

Genetic immunopotentiation increases the immune response against specific tumor antigens. Genes that have been analyzed to use for breast cancer immunotherapy are HER-2/neu protein (Disis et al., 1999; Kawashima et al., 1999), carcinoembryonic antigen (CEA) (Kawashima et al., 1999) and MAGE-1 (Toso et al., 1996).

Pro-apoptotic gene therapy encompasses the combination of a therapeutic drug and specific surface cell death molecules like tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) (Griffith et al., 2000; Lin et al., 2002). This combination increases the apoptotic cell death and at the same time it can improve the therapeutic effect in drug-resistant tumor cells.

Anti-angiogenic therapy, on the other hand, uses the knowledge of the role of angiogenesis in tumor cell growth. Angiogenesis facilitates cancer cell growth by providing them with new blood vessels (Atiqur Rahman and Toi, 2003; Marty and Pivot, 2008). Angiostatin and endostatin were found to have an anti-angiogenic function. It was demonstrated that liposomes complexed to plasmids containing angiostatin and endostatin inhibited breast tumor development in nude mice (Chen et al., 1999; Oga et al., 2003). Breast cancer was induced by the injection of either human breast cancer cells or mice breast cancer cells into the mammary fat pats. Injection of liposome:plasmid complexes was performed intratumorally, intravenous or intramuscular (Chen et al., 1999; Oga et al., 2003).

Mutation compensation is based on the idea to restore defects in a cancer cell that are causing the malignant transformation. For example, one strategy for mutation compensation employs the replacement of a defected tumor-suppressor gene in cancer cells with a working copy such as p53 (Chen and Mixson, 1998) or Rb (Wang et al., 1993). Another strategy is to decrease the tumor cell development by ablating the oncogene function of c-myc (Watson et al., 1991) and c-fos (Arteaga and Holt, 1996) which are used in breast cancer.

Suicide gene therapies are designed to treat cancer cells by delivering a gene that can convert a non-toxic prodrug into a toxic form. Besides killing the targeted cancer cells, suicide gene therapy can also affect surrounding cells of the region. This has been termed a bystander effect, providing the opportunity to destroy cancer cells that might have migrated into the tumor surrounding tissue. Clinical trials were performed with erbB-2 (Pandha et al., 1999) and MetXia-P450 (Braybrooke et al., 2005) as suicide gene therapy for breast cancer.

## *1.3 Adenovirus*

Over the past several years, the field of gene therapy has emerged with a broad variety of applications. Throughout this progress, the use of non-viral and viral vectors as delivery vectors has also widened. One of the most promising candidates for gene therapy is the adenovirus (Ad). Ads were first isolated back in the 1950's and since then their biology has been extensively studied (Hilleman and Werner, 1954; Rowe et al., 1953). Ads have specific features that make them a suitable gene therapy vector (Russell, 2000; Zhang, 1999). First, the generation of high titers required for clinical administration, is no longer a major problem. Furthermore, the Ad genome is really easy to manipulate and large DNA sequences can be inserted without any complications. In addition, Ads are capable to infect a large variety of dividing and non-dividing cells and they are not integrating their viral genome into host chromosomes. Moreover, they show also a low pathogenicity in humans and are causing only mild symptoms during their infection. All these characteristics of Ads for gene therapy as vectors offer a new therapeutic intervention to target breast cancer.

### 1.3.1 Adenoviral structure

At present, there are 53 known human Ad serotypes classified into six species (A-F subgroup) based on their neutralization by specific animal antisera, oncogenic potential and their DNA GC content (Davison et al., 2003; Madisch et al., 2005). Ads are non-enveloped, icosahedral viruses that contain a linear double stranded DNA genome of 36,000 base pairs (Rux and Burnett, 2004). The outer protein layer of the Ad virion is called the capsid that encloses the DNA-protein core complex. The capsid structure is composed of seven polypeptides: the three major proteins hexon (II), penton base (III) and fiber (IV) and the minor proteins IIIa, VI, VIII and IX (Vellinga et al., 2005) as seen in Figure 1. 252 capsomeres are forming the Ad capsid of which 240 are consisting of the hexon protein whereas the remaining 12 capsomeres are

composed of pentons and are found at the 12 vertices. Penton capsomeres are formed by pentameric penton bases and a protruding trimeric fiber (Valentine and Pereira, 1965). Penton bases are important for the capsid stabilization and together with the fiber they initiate and mediate viral attachment and internalization into the host cells. All fiber proteins can be divided into three domains: the N-terminal tail domain, the shaft region of variable length, and the C-terminal knob domain. The trimeric structure of the fiber is formed by sequences of the knob and the shaft domain (Rux and Burnett, 2004). Besides pentons and hexons, the capsid structure is stabilized by the minor proteins IIIa, VI, VIII and IX. Furthermore, the capsid contains also a virus-encoded protease (adenain) that cleaves several precursor proteins like VI, VII, VII, X, and terminal protein (TP) and is essential for the assembly of newly infectious viral particles (Russell, 2009).

The viral genome is enclosed by the capsid and associates with the polypeptides V, VII, X (mu) and TP. This complex builds a condensed core structure. Inverted terminal repeats (ITR) are located at each end of the Ad genome and each 5'-terminus is associated with the TP. Both associations are essential for viral genome replication (Russell, 2000). The Ad genome is separated into regions: the early genes encoding for E1A, E1B, E2A, E2B, E3 and E4; the delayed early genes encoding IX and IVa2 and the late genes encoding L1-L5. Early gene products play an essential role in adenoviral transcription, DNA replication, suppression of host immune response and inhibition of host cell apoptosis whereas the late genes encode for viral structure proteins which are necessary for viral assembly (Russell, 2000). In addition, two genes encode for virus-associated RNAs I and II (VAI and VAII RNAs) which have an important function to inhibit the host protein synthesis during viral replication in infected cells (O'Malley et al., 1986).

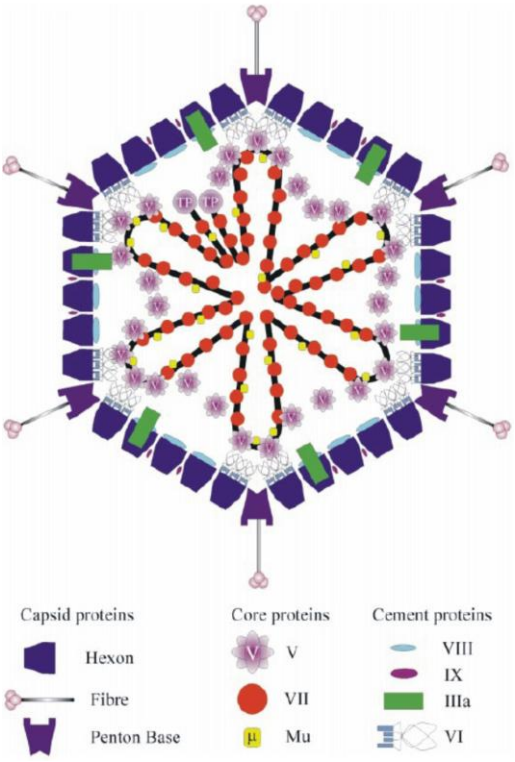


**Figure 1: Adenovirus structure.**

General structure of the Ad: displaying hexons, penton bases, fibers and cement proteins of the Ad capsid. The capsid encompasses the Ad genome and core proteins associated with the Ad genome forming the core of the Ad. Figure was adapted from (Russell, 2000).

### 1.3.2 Adenoviral infection pathway

The Ad infection pathway can be separated into several steps: attachment, internalization, endosome escape, viral replication, assembly and lysis. Ad infection of a host cell is initiated by binding of the fiber knob domain to a cellular attachment receptor on the host cell surface. For most Ad serotypes (except for subgroup B) the primary cellular attachment receptor is the Coxsackie and Adenovirus receptor (CAR) (Bergelson et al., 1997; Roelvink et al., 1998). The expression pattern of CAR on the surface of tissue-specific cells plays a crucial role for the adenoviral tropism. CAR is highly expressed on parenchymal cells in the liver but not exclusively, thus explaining high liver infectivity of Ad vectors upon systemic administration but also the wide Ad vector tropism *in vitro* (Yee et al., 1996).

The first step of Ad infection is the binding of the Ad fiber knob domain to the N-terminal domain of CAR. Besides the binding of the fiber knob to CAR, a second interaction of virion and the cell surface is essential to initiate viral uptake. The interaction between integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the host cell surface and the Arg-Gly-Asp (RGD) motif on the penton base of the

Ad vector is necessary to mediate internalization of the Ad virions (Mathias et al., 1998; Wickham et al., 1993.). Binding of integrins is followed by rapid endocytosis of the virions through clathrin-coated vesicles driven by a cytosolic GTPase (Meier et al., 2002; Roelvink et al., 1998; Roelvink et al., 1999). Once the viral particles are taken up by clathrin-coated vesicles, the endosome acidification process is causing conformational changes of the Ad vector capsid resulting in the release of the virions into the cytoplasm (Wang et al., 1998). The process of viral uptake until viral release into the cytosol is completed within 15 min and therefore rapid (Greber et al., 1993).

Next, the virions are translocated to the nuclear pore complex by a microtubule-dependent trafficking. Subsequently on arrival at the nucleus, Ad vectors interact through hexons with the nuclear pore complex and release their genome into the host cell nucleus to start viral replication. Within one hour more than 90% of Ad vectors are transported to the nucleus and about 18 hours post infection transgene expression can be detected reaching a maximum at 48 hours (Greber et al., 1993). The Ad infection pathway is schematically displayed in Figure 2.

After viral genome replication and translation of the viral mRNA transcripts, Ad vectors need to assemble. Therefore, viral proteins are transported back from the cytoplasm into the nucleus for particle assembly. Final maturation of Ad vectors is achieved by the adenain protease that cleaves the precursor polypeptides of VI, VII, VIII, Mu and TP to obtain infectious virion particles. In the final step of the Ad replication pathway, cells are lysed by the accumulation of ADP (Ad death protein) (Tollefson et al., 1996) and the cleavage of cellular cytokeratins by the L3 protease. Cleaved cytokeratins cause an increased susceptibility of the cell to lyse by disrupting cell integrity (Chen et al., 1993). In addition, in the past several years it has been suggested that autophagy aids the release of progeny virions (Jiang et al., 2008).

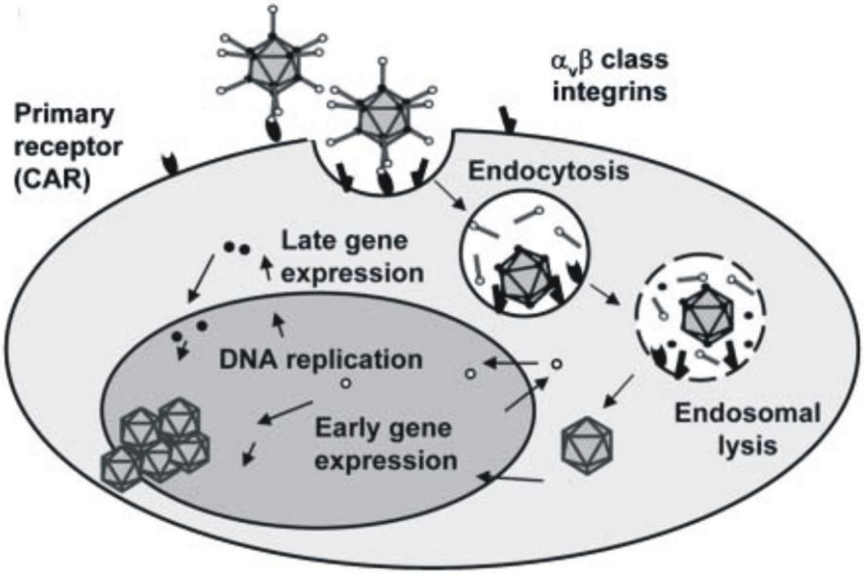


**Figure 2: Ad serotype 5 infection pathway.**

Illustrated are the principal steps of the adenoviral infection. Binding of the Ad fiber knob to the primary cellular attachment receptor is followed by the interaction of penton base with integrins. Binding of integrins initiates viral uptake through clathrin-coated vesicles. In the endosome Ad dissociates in the low pH environment and up on acidification Ads are dismantled and viral particles are released into the cytosol. Viral particles are then transported to the nucleus where they release their viral genome and start viral replication. Figure was adapted from (Kanerva and Hemminki, 2005).

## *1.4 Oncolytic virotherapy of breast cancer*

Because of safety concerns, only replication incompetent viruses were used as vehicle vectors in the beginning of gene therapy. However, clinical studies have shown that using replication incompetent viruses resulted in only a minor proportion of tumor cells infected and did not proffer for therapeutical success (Alvarez and Curiel, 1997). Therefore, employing replication competent viruses to treat malignant tumors was referred to as a virotherapy procedure instead of gene therapy (Alemany et al., 2000; Kirn et al., 2001). Virotherapy exploits tumor specific characteristics for a specific cell targeting, viral infection and replication in tumor cells to kill them while leaving healthy cells unaffected (Alemany et al., 2000; Mathis et al., 2005). The principle of virotherapy is based on the idea that a replication competent virus infects a tumor cell and replicates tumor cell specific leading to tumor cell oncolysis and spread of progeny virions to adjacent tumor cells. Ideally, the tumor specific replication is repeated until all tumor cells are destroyed but not the healthy cells (Alemany et al., 2000; Kirn et al., 2001) (Figure 3).

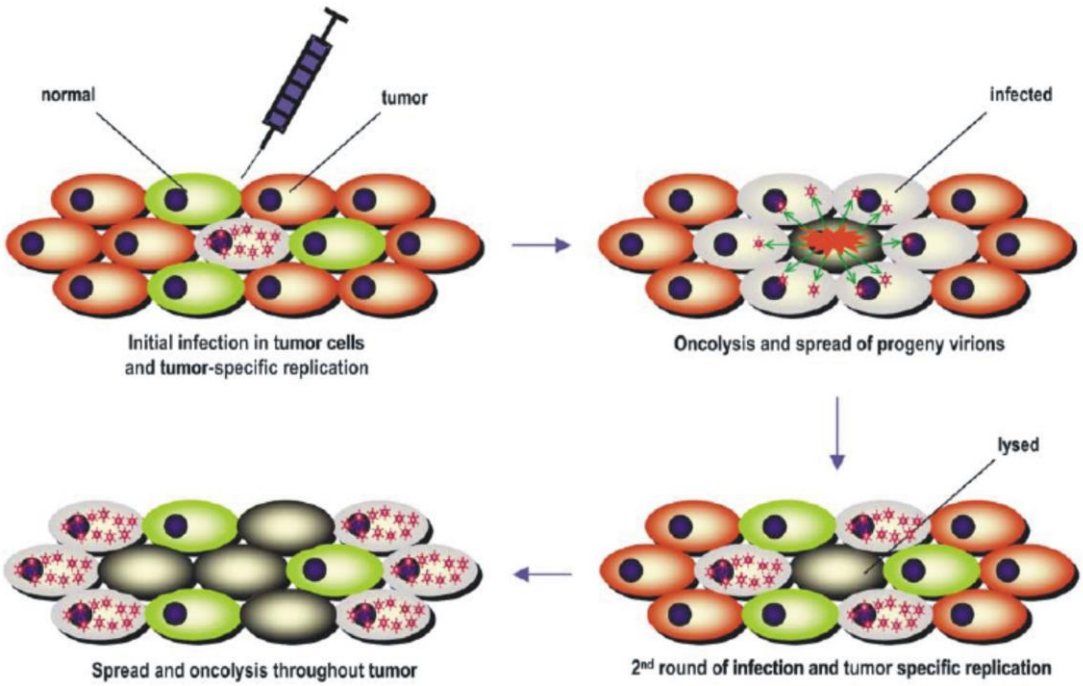


**Figure 3: Oncolytic virotherapy of replicative viruses.**

A replication competent virus infects the target cell, replicates tumor specific and kills the tumor cell by lysis. Progeny virions are spread and can infect neighboring tumor cells. This infection cycle is repeated until all tumor cells are destroyed leaving the healthy cells uninfected. Figure was adapted from (Mathis et al., 2005).

Besides the promising results of virotherapy in the mouse models, the first results in clinical trials revealed a reduced efficiency of Ads to target cancer cells (Douglas et al., 2001; Vasey et al., 2002). It was shown that expression of CAR on the surface of the targeted cell type was a limiting factor. Most tumor types including breast carcinoma displayed a reduced expression of CAR on their cell surface compared to normal cells (Li et al., 1999; Shayakhmetov et al., 2002; Zabner et al., 1997). This was leading to an unspecific targeting of normal cells instead of cancer cells (Glasgow et al., 2006). Ad serotype 2 (Ad2) and Ad serotype 5 (Ad5) are used as vectors for oncolytic virotherapy by the majority of investigators because those two serotypes are the ones which genomes were first sequenced and efficiently studied. Theretofore, modifying the native Ad tropism by altering the Ad fiber structure, the transductional targeting, was developed. Moreover, the Ad targeting can be changed by modifying the Ad replication efficiency on the genetic level. Transcriptional targeting is really important because Ad serotype 5 (Ad5) is able to replicate in a wide variety of cell types. To avoid Ad replication in normal healthy cells, the Ad5 vector can be genetically modified to replicate only tumor specific by incorporating a tumor specific promoter into the Ad genome. In addition to transductional and transcriptional targeting, a third targeting strategy,

translational targeting, was recently established. Ad replication is restricted by controlling the initiation of E1A mRNA translation.

## 1.4.1 Different targeting strategies

### *1.4.1.1 Transductional targeting*

A successful oncolytic virotherapy depends on the efficient binding of the Ad5 vector to the target cancer cells. Low expression of CAR on most cancer types including breast cancer was reported by several investigators to be responsible for reduced infectivity of Ad5 (Glasgow et al., 2006; Li et al., 1999; Shayakhmetov et al., 2002; Zabner et al., 1997). CAR is usually expressed on healthy cells because CAR can induce cell to cell contact, a phenotype that is lost in malignant tumors (Walters et al., 2002). Furthermore, it was shown that Ad vectors were rapidly interacting with factors of the blood system and accumulated in the liver upon intravenous injection (Shayakhmetov et al., 2004). Thus, to provide effective Ad delivery to targeted cells, a large dose of Ad5 was necessary to administer which could induce toxicity.

To circumvent low Ad5 infection efficiency and high accumulation of Ad5 in the liver, the transductional targeting was developed. This targeting strategy alters the native Ad5 tropism to re-target Ad5 vectors to cancer cells. Transductional targeting can be divided into two different categories: 1) genetic targeting and 2) physical targeting (Glasgow et al., 2006).

The concept of adapter-based targeting of Ad vectors is based on the idea that the adapter is forming a molecular bridge between adenoviral vectors and cell receptors. Adapters are composed of bispecific molecules and are used to bypass the native Ad5 CAR tropism. This targeting strategy was explored on the fact, that Ad attachment and entry are two separate steps. Thus, altering the Ad attachment receptor is not changing the entry pathway because entry of Ad vectors is initiated by the binding of cell surface integrins to the Ad penton base (Mathias et al., 1998; Wickham et al., 1993). Bi-specific antibodies, antibody fragments (Fab)-peptide ligand conjugates, Fab antibody conjugates, recombinant fusion proteins (composed of Fabs and peptide ligands), and chemical conjugates between Fabs and cell-specific ligands (Glasgow et al., 2006) are employed as adapters to retarget Ad vectors.

The first successful retargeting of Ad vectors to cancer cells overexpressing folate receptor was established by using a bispecific conjugate that contained an anti-knob neutralizing Fab that was chemically associated to folate (Douglas et al., 1996). Besides this study several other

Fab-ligand conjugates targeted against several cell surface markers such as EGFR (Miller et al., 1998), CD40 (Tillman et al., 1999) and EpCAM (Haisma et al., 1999) have been published to successfully retarget Ad vectors. However, adapter-based delivery systems have a big disadvantage. They are more complex in their kinetics and pharmacodynamics, and their stability has not been investigated in humans so far. That is why a one-component system is preferable to administer into humans for clinical trials (Glasgow et al., 2004).

The second strategy of transductional targeting employs genetical modification of the Ad capsid to circumvent the native CAR-based tropism without destroying molecular functions that are essential for accurate viral replication. Upon basic understanding of Ad attachment, the Ad fiber knob domain became the major site for modifications. Genetical modifications of the Ad capsid can be further divided into three categories: 1) ligand incorporation into the fiber knob, 2) de-knobbing of the fiber and 3) fiber-pseudotyping (Glasgow et al., 2004).

Ligand incorporation into the Ad fiber knob domain was shown to successfully retarget Ad vectors to specific cells. Integrating a polylysine motif (pK7) into the C-Terminus of the fiber knob increased the infection efficiency of tumor cells upon high affinity binding to poly anions like heparansulfate proteoglycans (Wickham et al., 1996). Another integration side for ligands is the HI-loop located in the fiber knob domain. It is possible to incorporate up to 83 amino acids without inhibiting or influencing the adenoviral replication (Belousova et al., 2002). Integration of an Arg-Gly-Asp (RGD)-motif into the HI-loop demonstrated an enhanced binding to integrins and retargeting the modified Ad vector to tumor cells like in ovarian cancer cells (Dmitriev et al., 1998).

De-knobbing of Ad includes the displacement of the entire fiber and substitution with a chimeric knobless fiber. This targeting strategy was developed upon the investigation that Ads without a fiber could be generated (Falgout and Ketner, 1988; Von Seggern et al., 1999). Fiber knob-deleted Ads ablate the native CAR-tropism and can be retargeted to different cell types by incorporating a ligand into the knobless fiber. The maintainance of the fiber trimerization is of main importance for this type of modification because otherwise the modified Ad is unable to assemble correctly and attach to the receptor. Incorporation of a trimeric CD40 ligand resulted in a 100-fold improvement of transduction to human dendritic cells (Belousova et al., 2002). In addition, it is also possible to insert targeting ligands into the capsid proteins like hexon or pIX. A successful insertion of a RGD-motif in the hypervariable region 5 of the hexon protein showed an increased fiber-independent targeting to low-CAR expressing vascular smooth muscle cells (Vigne et al., 1999).

Another category to retarget Ad vectors is fiber-pseudotyping in which the whole fiber or only the fiber knob domain of serotype 5 is exchanged with one of a different human Ad serotype. It was shown that subgroups B and D of human Ads do not use CAR as their primary cellular attachment receptor. CD46, CD80 and CD86 have been identified as cellular attachment receptors for subgroup B Ads like Ad serotype 3, 35 and 7 (Fleischli et al., 2007; Short et al., 2004; Sirena et al., 2004). Binding of Ad3 to these receptors has been brought into question, because several investigators failed to confirm these receptors for Ad3 (Marttila et al., 2005; Segerman et al., 2003). Recently, Wang et al. published that Desmoglein-2 is the cellular attachment receptor of Ad3 (Wang et al., 2011). Desmoglein-2 is a transmembrane glycoprotein which binds calcium and is a member of the cadherin protein family (Steinberg et al., 1987).

The fiber chimera Ad5/3, in which fiber knob of serotype 5 was exchanged with the one of serotype 3, was first established by Krasnykh and colleagues (Krasnykh et al., 1996). Ad5/3 ablated CAR binding in the same manner as Ad3 by itself. Studies including this fiber chimera demonstrated an increased targeting of Ad to the low-CAR ovarian cancer cell lines (Kanerva et al., 2002; Kanerva et al., 2002). In addition, the Ad3 serotype has the same biodistribution, liver toxicity and clearance profile as Ad5 serotype. This suggests that Ad3 has a good safety profile, an important feature for human clinical administration (Kanerva et al., 2002).

#### *1.4.1.2 Transcriptional targeting*

In addition to alter the native Ad tropism with transductional targeting, there is still a need to enhance the cancer cell specific Ad replication due to the wide variety of Ad5 wild type (WT) tissue replication efficiency. Transcriptional targeting involves the genetic modification of the Ad genome to restrict Ad replication to a specific cancer cell type. This method involves the use of a human tumor- or tissue-specific gene promoter (TSP) exchanged with the endogenous E1A gene promoter. Since Ad virions can broadly infect multiple cell types, Ad replication can be restricted by inserting a TSP in place of the E1A promoter.

The E1A protein is needed for initiating Ad transcription and replication and is therefore expressed very early after Ad infection. Incorporation of a TSP upstream of the E1A gene is leading to a promoter-controlled Ad replication in the cells where the TSP is active (Pelka et al., 2008). Ads that are genetically modified to restrict Ad replication are named conditionally replicating adenoviruses (CRAds).

In the last decade, a great number of TSPs were identified for several cancer types. One of the first TSP that was identified and shown to be over expressed in a number of pancreatic, gastric and lung cancers was the carcinoembryonic antigen (CEA) promoter (Tanaka et al., 1996). It was shown that upon the insertion of the CEA promoter liver toxicity after intravascular Ad administration was reduced compared to a control vector (Tanaka et al., 1996).

Moreover, several TSP have been reported to be highly activated in breast cancer cells such as CXCR4 (Zhu et al., 2004), survivin (Zhu et al., 2004) and DF3/MUC1 (Ring et al., 1997). Recent publications suggested a role of the CXCR4/SDF-1α signaling to play a role in progression of numerous cancer types (Moore, 2001; Murakami et al., 2002). In particular, a study performed by Muller *et al.* revealed that CXCR4 was highly expressed in breast cancer cells and metastases while expression in their normal counterparts was low (Muller et al., 2001). Therefore, several groups utilized the CXCR4 promoter and exchanged it with the E1A promoter to successful restrict Ad replication to a specific cancer type such as breast cancer (Haviv et al., 2004; Stoff-Khalili et al., 2005; Zhu et al., 2004).

Besides limiting the Ad replication by controlling the E1A expression through a TSP, another procedure to restrict Ad replication is to delete specific base pairs in the Ad genome. Upon the deletion of specific base pairs, Ad requires tumor-specific cellular factors to restore Ad replication. The first deletion mutant CRAd was ONYX-015 carrying two deletions in the E1B gene, encoding for a 55 kDa protein (Bischoff et al., 1996). Usually the functional protein binds p53, inactivates it and induces S-phase entry upon Ad infection. Mutating two sites in the E1B gene, Ad virions can only replicate in cells that possess an aberrant p53 pathway (Bischoff et al., 1996).

Another deletion mutant CRAd is Ad5 Δ24 that carries a 24 base pair deletion in the constant region 2 (CR2) of the E1A gene (Bischoff et al., 1996). This region is necessary to bind retinoblastoma (Rb) protein to induce S-phase entry. CRAds containing the Δ24bp deletion can only replicate efficiently in cells where this interaction is not necessary because cells are defective in the Rb-p16 pathway (Bischoff et al., 1996).

### *1.4.1.3 Translational targeting*

Besides transductional and transcriptional targeting, there is a clear need for additional and new targeting strategies to optimize the tumor specific Ad replication. Transcriptional targeting bears the disadvantage that most TSPs demonstrate a low background activity in normal and

liver tissues. This background activity could induce problems during clinical studies by increasing the Ad hepatotoxicity. Therefore, there is a clear need for additional and new targeting strategies to optimize the tumor specific Ad replication.

Recently, Stoff-Khalili *et al.* introduced the translational targeting strategy to the virotherapy field (Stoff-Khalili et al., 2008). Translational targeting involves the insertion of a control element upstream of the E1A gene to restrict E1A mRNA translation to a specific cell type. In cancer cells, translational regulation has become a well-recognized mechanism contributing to the neoplastic phenotype. In normal cells eIF4E protein concentration is low, it is bound to the 4E-Binding Protein (4E-BP) and gets inactivated and therefore, translation initiation is regulated. Phosphorylation of 4E-BP results in the release and activation of eIF4E causing the formation of the translation initiation complex and translation initiation itself (De Benedetti and Graff, 2004). Clearly, the eukaryotic initiation factor 4E (eIF4E) is a key player in translational regulation and is critical to the genesis and/or progression of cancer cells based on the fact that it is overexpressed in the majority of solid tumors studied to date (DeFatta et al., 1999; Miyagi et al., 1995; Rosenwald et al., 1999; Stoff-Khalili et al., 2008). In this context, increased expression of eIF4E is leading to elevation of basic fibroblast growth factor (FGF-2) (Kevil et al., 1995) and vascular endothelial growth factor (VEGF) protein synthesis (Kevil et al., 1996). The mRNAs of those two proteins have a long and complex GC-rich structure, named also "weak mRNA". The structure of weak mRNAs reduces the translation efficiency due to time that the translation initiation complex needs to scan the weak mRNA for the start codon. Therefore, weak mRNAs are usually poorly translated in normal cells due to the limited translation initiation whereas strong mRNAs have a short UTR sequence and are highly translated into proteins.

Besides the significance of eIF4E in the early changes of a primary tumor, it has been identified as one of the eight genes which when increased, exhibit a molecular signature of metastatic potential (Ramaswamy et al., 2003). In tumorigenic cells and during cellular transformation the concentration of free eIF4E is increased (DeFatta et al., 1999), causing an enhanced translation of weak mRNAs in cancer cells as seen in Figure 4 (De Benedetti and Graff, 2004). Translational targeting was realized by incorporating of the 5'UTR sequence of rat fibroblast growth factor 2 (FGF2) producing a weak mRNA upstream of the E1A gene. This incorporation is then restricting the E1A mRNA translation to the cells with high concentration of free eIF4E (Stoff-Khalili et al., 2008).

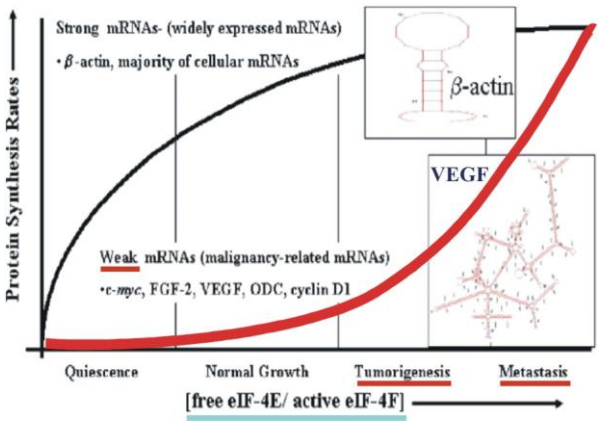


**Figure 4: mRNA translation efficiency in tumor progression.**

Protein synthesis rate of weak mRNAs is increased in a proportional manner with increasing free eIF4E concentration and tumor progression. Modified from De Benedetti and Graff (De Benedetti and Graff, 2004).

### *1.4.1.4 Combination of targeting strategies*

Although, an enhancement of tumor specificity was gained by every single targeting strategy, all targeting strategies have some disadvantages. Hence, combining targeting strategies was reasonable to increase the safety for infection and replication in normal cells. The feasibility of combining either transductional and transcriptional or transcriptional and translational regulation strategies (dual-level targeting CRAd) for the key goal of oncolytic adenovirus replicative specificity has recently been established (Stoff-Khalili et al., 2008; Stoff-Khalili et al., 2007). Both dual-level viruses showed a specific gain in safety of normal cells and controlled viral replication (Stoff-Khalili et al., 2008; Stoff-Khalili et al., 2007), suggesting that the combination of several targeting strategies in a single CRAd is enhancing cancer cell specific replication and safety of normal cells. The US Food and Drug Administration is still anxious about the specific replication of tropism modified Ads due to the possibility of infecting a wide range of normal cells carrying the receptor to which the modified virus can bind (Yamamoto and Curiel, 2010). Consequently, there is still a need to even increase the cancer cell specific replication due to the fact that several TSPs show a background activity in several cell types. Furthermore, translational restriction of E1A would be even more effective, if the virus would be targeted directly to a specific cancer cell type.

### 1.4.2 Animal models for oncolytic virotherapy

After a successful engineering of an Ad vector and gaining cancer cell specificity *in vitro*, a newly created Ad vector needs to be investigated in an adequate *in vivo* animal model. Thus far, the most common animal model used to analyze oncolytic efficiency of newly generated Ad vectors is the human xenograft model in immunodeficient mice (Yamamoto and Curiel, 2010). Disruption of *foxn*1 gene in nude mice is causing deficiency in B-cell development and T-cell activity. Nude mice are usually used as an animal model for oncolytic virotherapy because the immunosuppressed mice allow human cancer cells to from tumors and to treat the developed tumors with different anti-cancer treatments (Davis and Fang, 2005). Certainly, the reliability on data received from this animal model is questionable because the immune system and the associated inflammation are impaired in nude mice. Furthermore, the species specific Ad replication is a disadvantage for the nude mouse model (Ginsberg et al., 1991; Paielli et al., 2000; Rustia and Shubik, 1979). It was shown, that mice are poorly permissive for human Ad replication leading to a limited possibility for investigating human Ad replication in normal organs and hepatotoxicity resulting from virus replication (Duncan et al., 1978; Ginsberg et al., 1991; Hjorth et al., 1988).

Recently, the "Tissue-Slice-Model" was introduced using three-dimensional precision tissue slices from human tissues to evaluate preclinical oncolytic vectors. Tissue slices can be obtained from normal liver and tumor tissue by the Krumdieck tissue slicer (Krumdieck et al., 1980). The "Tissue-Slice-Model" represents an active metabolic system displaying the heterogeneity of the investigated tissue. It was shown, that human CRAds can successfully replicate on those tissue slices and hepatotoxicity of CRAds can be investigated on liver tissue slices (Kirby et al., 2004; Stoff-Khalili et al., 2006, Stoff-Khalili et al., 2007; Stoff-Khalili et al., 2005). This newly invented technique makes it possible to analyze CRAds pre-clinical efficiently and fast (Kirby et al., 2004; Stoff-Khalili et al., 2006). Unfortunately, analyzing the interaction of CRAds with the immune system is impossible with the "Tissue-slice-model". Hence, other pre-clinical animal models are necessary that are permissive to Ad replication and immunocompetent.

It was reported in the past, that Cotton rats' species *Sigmodon hispidus* is permissive for the replication of several human respiratory viruses including Ad5 (Niewiesk and Prince, 2002). Cotton rats were shown to allow Ad5 replication in lungs, nasal passages and cornea and display the same pathology seen in Ad5 infected-humans (Ginsberg et al., 1989; Pacini et al., 1984; Prince et al., 1993; Tsai et al., 1992). Based on these findings, the cotton rat animal

model was employed to investigate *in vivo* spread and toxicity of Ad5 based vectors (Rojas-Martinez et al., 1998). Recently, the cotton rat model was introduced as a semi permissive animal model in the field of oncolytic virotherapy by establishing transplantable cotton rat tumor cell lines (Steel et al., 2007; Toth et al., 2005). Both groups reported that Ad5 replication and spread to neighboring tumor cells is possible in the generated cotton rat cancer cell lines *in vitro* and *in vivo*. Consequently, the cotton rat tumor model was suggested as an animal model to determine replication efficiency, hepatotoxicity and immune response of the Ad5 vectors for oncolytic therapy of tumors. However, a big disadvantage of the use of cotton rats is the difficulty in handling them because they are more aggressive and bite frequently (Niewiesk and Prince, 2002).

Besides the cotton rat model and the tissue slice model, another method to analyze the safety profile of Ads and the immune response, is the exploitation of conditionally replicative canine Ads to investigate in spontaneous dog osteosarcoma treatment (Hay, 2003; Hemminki et al., 2003). This model offers the possibility to gain valuable results about oncolytic activity and toxicity in a natural host and transferable to human application. Nevertheless, employing the canine model is expensive and a connection to veterinarian facilities is essential (Ternovoi et al., 2005), reducing the availability of the canine model for most investigators.

Lately, Thomas et al. introduced the Syrian hamster as a permissive immunocompetent animal model to analyze oncolytic Ad5 vectors (Thomas et al., 2006).

## *1.5 The Syrian hamster animal model*

Previously, the Syrian hamster (*Mesocricetus auratus*) has been introduced to be permissive for human Ad5 replication on the contrary to mainly other species examined (Eggerding and Pierce, 1986; Ginsberg et al., 1991; Silverstein and Strohl, 1986). In cancer cell lines of this animal Ad5 is competent to infect those cells, replicate in them and spread from cell-to-cell (Bortolanza et al., 2007; Thomas et al., 2006; Ying et al., 2009). In addition, it was shown that the adenovirus has important antitumor efficacy after injection in Syrian hamster tumors *in vivo* (Spencer et al., 2009). Significantly, the Ad replicates not only in the tumors, but also in the lungs, liver and other organs (Thomas et al., 2006). Therefore, the "Recombinant DNA Advisory Committee (RAC)" has suggested using the Syrian hamster model to further optimize adenovirotherapy and characterize/identify the basic mechanisms in tumor killing (Lichtenstein et al., 2009).

To date, several established hamster cancer cell lines are available to study oncolytic CRAds but not for all cancer cell types (Bortolanza et al., 2007; Thomas et al., 2006). Recently, Spencer *et al.* developed a new pancreatic carcinoma model to study CRAds in the Syrian hamster (Spencer et al., 2009). In this study transplantable tumors of the Syrian hamster from the National Cancer Institute were employed to establish a permanent pancreatic cancer cell line. It was shown, that the Syrian hamster pancreatic carcinoma (SHPC9) cell line was permissive for Ad5 replication and could induce tumor growth upon subcutaneous injection into the hamsters (Spencer et al., 2009).

Nonetheless, up to now no Syrian hamster breast cancer cell line is available because hamsters are somehow resistant to mammary cancer and only two studies have been reported to induce mammary tumors in hamsters (Della Porta, 1961; Rustia and Shubik, 1979).

### 1.5.1 Induction of breast cancer in female Syrian hamsters

In the past, several different studies of carcinogenesis and cancer chemoprevention have been performed using the Syrian hamster as animal model except for mammary carcinogenesis. The hamster cheek pouch model is one of the studies that was performed in Syrian hamster (Gimenez-Conti, 1993). This model uses the polycyclic aromatic hydrocarbon 7,12-dimethylbenz[a]anthracene (DMBA) applied either as an initiating agent in combination with a tumor promoter such as benzoyl peroxide or as a total carcinogen. Usually, squamous cell carcinomas containing a mutation in the Ha-ras gene are developed after this treatment (Gimenez-Conti, 1993). Furthermore, it was reported that diethyl-stilbestrol (DES) treatment of Syrian hamsters leads to the development of renal carcinomas (Devanesan et al., 2001). Based on this treatment result, Laurent et al. developed a Syrian hamster renal carcinoma cell line (Laurent et al., 1999). In addition to the development of renal carcinomas, Syrian hamsters are capable of developing cholangiocarcinomas, pancreatic cancer and bile duct hyperplasia after exposition to aromatic amines like N-nitrosobis(2-oxopropyl)amine (BOP) (Kaneko et al., 2004; Miyauchi et al., 2000). Pancreatic cancer on the other hand can also be introduced by injecting two to three rounds of ethionine-methionine-BOP into the hamsters and feed them a choline-deficient diet (Mizumoto et al., 1988).

To date, only a few studies reported the development of mammary tumors (Della Porta, 1961; Rustia and Shubik, 1979), but none could establish a breast cancer cell line so far. Hence, it was of great interest to establish a breast cancer cell line that could be used to evaluate the

potency of newly designed oncolytic vectors alone or in combination with chemotherapy or radiation therapy. Therefore, Coburn et al. induced mammary tumor formation in female Syrian hamsters by exposing the hamster to the carcinogen N-methyl-N-nitrosurea (MNU) (Coburn et al., 2011). This study was based on the induction of mammary tumors in rat upon MNU treatment (Steele et al., 1994). Treatment with MNU induced mammary tumors in female Syrian hamster, but only in a limited number (Coburn et al., 2011). Histology of these primary mammary tumors showed abnormal ducts containing secretory products separated by poorly differentiated epithelial cells (Coburn et al., 2011). Mammary tumors and premalignant lesions (ADH) were stained positive for HER-2/neu and pancytokeratin, indicating that the lesions and tumors were of epithelial cell and mammary origin (Coburn et al., 2011).

# Aim of Thesis

Virotherapy employing conditionally replicating adenoviruses (CRAds) represents a promising tool for a wide array of neoplastic diseases and especially to treat breast cancer. Ideally, cancer specific replication of CRAds results in virus mediated oncolysis of infected tumor tissues and release of the progeny virions capable to further propagate in surrounding tumor cells but not in those of normal tissues. Critical for a therapeutic index is selective killing of tumor cells while avoiding killing normal cells. It was recently shown in our group that combination of targeting strategies (dual-level CRAds) increase the tumor on/liver off ratio for breast cancer cells (Stoff-Khalili et al., 2008; Stoff-Khalili et al., 2007).

Based on these findings, the first aim of this study was to generate a new CRAd combining three targeting strategies that are used in oncolytic virotherapy to further increase breast cancer specific replication. For this purpose, it was planned to construct a novel 5/3 fiber chimeric (transductional targeting) virus containing the human tumor-specific promoter CXCR4 as a transcriptional control element and the 5'-untranslated region (5'-UTR) of rat FGF-2 (Fibroblast Growth Factor-2) mRNA as a translational control element upstream of the E1A gene. Background replication efficiency and breast cancer specificity were determined by using cytotoxicitiy assays, quantitative realtime polymerase chain reaction (qRT-PCR) and immunoblot analyses comparing infected breast cancer cells to normal cells.

However, the preclinical evaluation of human Ad vectors has been limited due to the poor replication of human Ads in mice. In this regard, the Syrian hamster has been lately introduced as an immunocompetent and Ad replication permissive animal model. Due to the lack of a breast cancer model in the Syrian hamster to evaluate newly constructed oncolytic Ad vectors, our group recently succeeded in inducing mammary tumors in female Syrian hamsters. Thus, the second aim of this study was to characterize the first Syrian hamster breast cancer cell line for its cancerous and tumorigenic character. Furthermore, this cell line was investigated whether it is a suitable *in vitro* cell line to evaluate oncolytic Ad vectors for their cancer specificity. Therefore, several established CRAds such as Ad5 Δ24 RGD and Ad5/3 WT were used to determine their infection and replication efficiency in this newly established Syrian hamster breast cancer cell line. The final goal is to use the Syrian hamster as a novel breast cancer model for preclinical evaluation that is immunocompetent and permissive for human Ad5 replication.

# 2. Materials

## *2.1 Bacterial Strains*

**BJ 5183** (Stratagene, Agilent Technologies, La Jolla USA)

This bacterial strain is a recombinant competent strain in which the recombination between a vector containing the adenoviral genome and a transfer vector containing the gene of interest takes place only if both vectors share appropriate regions of homology.

Genotyping *end*A1 *sbc*BC *rec*BC *gal*K *met thi*-1 *bio*T *hsd*R **(Strr)** (Hanahan, 1983).

**XL10 Gold** (Stratagene, Agilent Technologies, La Jolla, USA)

The bacterial strain XL10 Gold is used commonly for transformation of large DNA molecules as the adenoviral genome with high efficiency. Genotyping: TetrD(*mcrA*)*183* D(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1* (*lac* Hte [F´ *proAB lacI*qZD*M15* Tn*10*Tetr) Amy Camr].

### 2.1.1 Media for cultivation of bacteria

| **LB-Medium** | | **LB-Agar** | |
|---|---|---|---|
| Trypton | 10 g/l | Trypton | 10 g/l |
| Yeast extract | 5 g/l | Yeast extract | 5 g/l |
| NaCl | 8 g/l | NaCl | 8 g/l |
| adjust pH to 7.5 with NaOH | | Agar | 15 g/l |
| → add $H_2O$ up to 1 liter | | adjust pH to 7.5 with NaOH | |
| | | → add $H_2O$ up to 1 liter | |

## *2.2 Eukaryotic cells*

- **A549** (ATCC, CCL-185$^{TM}$)

  A549 is a human lung carcinoma cell line, isolated from a lung carcinoma of a 58 year old Caucasian man in 1972.

- **Hamster Fibroblasts**

  Fibroblasts were dissected from the lower back a Syrian hamster as a normal control cell line (Kindly provided by Dr. M. Mathis, LSU Health Sciences Center, Shreveport, USA; Present work).

- **HEK-293** (ATCC, CRL-1573™)

  HEK-293 cells were isolated from human embryonic kidneys of a healthy aborted fetus. This cell line expresses the left end of the adenoviral genome of serotype 5 and is excellent for titrating human adenoviruses. The left arm sequence of the Ad5 genome was incorporated through transfection into the human chromosome 19.

- **HMAM4 spindle and round**

  This hamster breast cancer cell line was developed by MNU-treatment in the Syrian Hamster. (Kindly provided by Dr. M. Mathis, LSU Health Sciences Center, Shreveport, USA Present work)

- **HMAM5**

  Hamster breast cancer cell line was developed by MNU-treatment in the Syrian Hamster. (Kindly provided by Dr. M. Mathis, LSU Health Sciences Center, Shreveport, USA Present work)

- **HMEpC – Human mammary epithelial cells** (PromoCell, C-12650)

  HMEpC were isolated from human adult mammary glands and stained positive for cytokeratin.

- **MCF-7** (ATCC, HTB-22™)

  This breast cancer cell line was developed from the mammary glands from an adenocarcinoma of a 69-years old Caucasian woman. Growth of MCF-7 cells is inhibited by tumor necrosis factor alpha (TNF alpha). Furthermore, MCF-7 cells express the estrogen receptor on their surfaces.

- **MDA-MB-231** (ATCC, HTB-26™)

  MDA-MB-231 cells were obtained from the mammary glands from an adenocarcinoma of a 51-years old Caucasian woman. MDA-MB-231 cells express the WNT7B oncogene and are immortalized.

- **MDA-MB-231/N** (Meric et al., 2000)

  MDA-MB-231 cells were stable transfected with a control plasmid pSV-neo.

- **MDA-MB-231/E1A** (Meric et al., 2000)

  MDA-MB-231 cells were stable transfected with pE1A-neo that was carrying the WT E1A gene of Adenovirus serotype 5.

- **NHDF - Normal human dermal fibroblasts** (PromoCell, C-12300 juvenile foreskin)

  To isolate NHDFs the dermis of juvenile foreskin was used from different locations like the breast, the thighs, the face and the abdomen. This primary cell line is not immortalized and can be maximal passaged 16 times.

- **PHF PA2 und PHF 25M**

  Human fibroblasts isolated from human cutaneous skin. (Kindly provided by Dr. B. Akgül, University of Cologne)

- **SK-BR-3** (ATCC, HTB-30™)

  This cell line is a breast cancer cell line, derived from the mammary glands of a 46 year old Caucasian woman. The cell line is immortalized and over expresses the HER2/c-erb-2 gene product (Hudziak RM, et al. Monoclonal antibodies directed to the Her2 receptor. US Patent 5,677,171 dated Oct 14 1997).

- **ZR-75-1** (ATCC, CRL-1500™)

  ZR-75-1 is a breast cancer cell line established from the mammary glands of a ductal carcinoma of a 63-years old Caucasian woman.

### 2.2.1 Cell Culture Media

**Table 1: Cell culture media**

| Cell lines | Cell Culture Medium |
|---|---|
| MDA-MB-231, SK-BR-3, ZR-75-1, MCF-7 | DMEM + 10% FBS + 1% Penicillin/Streptomycin |
| MDA-MB-231/E1A; MDA-MB-231/N | DMEM + 10% FBS + 1% Penicillin/Streptomycin + 250µg/ml G418 |
| HEK-293 | DMEM + 10% FBS + 1% Penicillin/Streptomycin |
| A549 | DMEM + 10% FBS + 1% Penicillin/Streptomycin |
| HMEC | Mammary Epithelial Cell Growth Medium® (PromoCell) |
| NHDF, PHF 25M, PHF PA2 | DMEM + 10% FBS + 1% Penicillin/Streptomycin |
| HMAM5 | DMEM + 2% FBS + 1% Penicillin/Streptomycin |
| HMAM4 spindle and round | DMEM + 10% FBS + 1% Penicillin/Streptomycin |
| Hamster Fibroblasts | RPMI + 10% FBS + 1% Penicillin/Streptomycin |

- **Dulbecco´s modified Eagle´s Medium (DMEM)** (Invitrogen, Karlsruhe, Germany)

  plus GlutamaxTM, 4500 mg/l Glucose without sodiumpyruvate

  FBS 10 % (Invitrogen, Karlsruhe, Germany)

  Penicillin 100 units/ml (Invitrogen, Karlsruhe, Germany)

  Streptomycin 100 µg/ml (Invitrogen, Karlsruhe, Germany)

- **Freezing medium for all cells except HMEC and NHDF**

  DMSO 10% (Invitrogen, Karlsruhe, Germany)

  FBS 90% (Invitrogen, Karlsruhe, Germany)

- **Freezing medium for HMEC and NHDF**

  DMEM 70% (Invitrogen, Karlsruhe, Germany)

  FBS 20% (Invitrogen, Karlsruhe, Germany)

  DMSO 10% (Invitrogen, Karslruhe, Germany)

## *2.3 Nucleic Acids*

### 2.3.1 Synthetic Oligonucleotides

The Oligonucleotides used for polymerase chain reaction (PCR), Sequencing and quantitative PCR (qPCR) were ordered from Operon, Invitrogen, Bioneer, and TIB MOLBIOL.

**Table 2: PCR and sequencing oligonucleotides**

| **Name** | **Method** | **Sequence 5' → 3'** |
|---|---|---|
| Human CXCR4 promoter forward | PCR | CCTCCTTCCTCGCGTCTG |
| 5'UTR rat FGF-2 reverse | PCR | ATGCAACTTTCTCCCTTCCTGC |
| Ad3 fiber knob forward | PCR | TTATGGACAGGTCCAAAACCA |
| Ad3 fiber knob reverse | PCR | TTTTCATTATCTGTTCCCGCA |
| Human CXCR4 promoter forward | Sequencing | TATTGGCCATTGCATACGTT |
| Human CXCR4 promoter reverse | Sequencing | TGGTAACCGCTGGTTCTCCA |
| 5'UTR sequence of FGF-2 forward | Sequencing | TTTGTTTAAACGGCACCCAT |
| 5'UTR sequence of FGF-2 reverse | Sequencing | TGTGCTGGAATTCAGGGCAG |

**Table 3: Oligonucleotides used for qPCR**

| **Name** | **Sequence 5' → 3'** |
|---|---|
| Ad5 E4 gene forward (E4 copy number determination) | GCAAGGCGCTGTATCCAA |
| Ad5 E4 gene reverse (E4 copy number determination) | GGGTCGCCACTTAATCTACCT |
| Ad5 E4 gene forward (viral entry and viral titer) | TCCCGCGTTAGAACCA |
| Ad5 E4 gene reverse (viral entry and viral titer) | GCACTCCGTACAGTAGG |
| Ad5 E1A gene forward | CAGTTGCCGTGAGAGTTGGT |
| Ad5 E1A gene reverse | TGCCCAGGCTCGTTAAGC |
| Ad5 pTP gene forward | GAGGTGCTGCTGATGATG |
| Ad5 pTP gene reverse | CAAGGACATGGTGCTTCT |
| Human HPRT1 forward | TGACACTGGCAAAACAATGCA |
| Human HPRT1 reverse | GGTCCTTTTCACCAGCAAGCT |
| Human GAPDH forward | GAAGGTGAAGGTCGGAGT |
| Human GAPDH reverse | GAAGATGGTGATGGGATTTC |

### 2.3.2 Plasmids used to generate the Ad5/3 CXCR4 UTR

**Table 4: Plasmids used in this study**

| Name of Plasmid | Reference |
|---|---|
| pVK 500 FB5/3 | Kindly provided by Dr. D.T. Curiel, Washington University, St. Louis, USA |
| pShuttle.CXCR4-UTR-E1A | (Stoff-Khalili et al., 2008) |

### 2.3.3 Plasmids used for the standard curve of the qPCR

- **pJET1-hHPRT1** (3,2 kb) human HPRT1 PCR product cloned into pJET1/Blunt to prepare serial dilutions to generate a standard curve in qPCR experiments provided by Dr. B. Akgül.
- **AdEasy-1** (33,5kb) (Agilent Technologies, Santa Clara, CA, USA), standard plasmid for E4 and pTP gene.
- **pShuttle WT pIX RFP** (10,7kb), standard plasmid for E1A gene (kind gift of Prof. David Curiel).
- **pCMV-SPORT6 hGAPDH** (5,7kb)**,** human GAPDH cDNA clone 3869809 cloned into pCMV-SPORT6 to prepare serial dilutions to generate a standard curve in qPCR experiments (Open Biosystems, Lafayette, CO, USA).

### 2.3.4 Transfection reagents

Mammalian Transfection Kit (Agilent Technologies, Santa Clara, CA, USA)

### 2.3.5 RT-PCR

FastStart Universal SYBR Green Master Mix (ROX) (Roche, Indianapolis, IN, USA)

### 2.3.6 Loading dyes and DNA Markers

| | |
|---|---|
| Blue/Orange 6x Loading Dye | (Promega, Madison, WI, USA) |
| 1kb DNA Ladder | (Promega, Madison, WI, USA) |
| 1kb DNA plus Ladder | (Life Technologies, Grand Islands, NY, USA) |

## *2.4 DNA Preparation*

| | |
|---|---|
| Plasmid Maxi Kit | (Qiagen, Valencia, CA, USA) |
| PureLink™ HiPure Plasmid Filter Maxiprep Kit | (Invitrogen, Karlsruhe, Germany) |
| QIAmp Blood Tissue Minikit | (Qiagen, Hilden, Germany) |
| QIAquick Gel Extraction Kit | (Qiagen, Valencia, CA, USA) |

## *2.5 RNA Preparation and cDNA Synthesis*

| | |
|---|---|
| RNeasy Mini Kit | (Qiagen, Valencia, CA,USA) |
| RNase-free DNAse Set | (Qiagen, Valencia, CA, USA) |
| iScript$^{TM}$ cDNA Synthesis Kit | (BioRad, Hercules, CA, USA) |

## *2.6 Viruses*

**Table 5: Viruses used in this study**

| Description | Modification | Reference |
|---|---|---|
| Ad5 WT | No changings | Vectorlogics |
| Ad5/3 WT | Fiber chimera, fiber tail and shaft of serotype 5, fiber knob of serotype 3 | Kindly provided by Dr. D.T. Curiel, Washington University, St. Louis, USA |
| Ad5 LacZ | LacZ gene instead of E1A gene | Clontech |
| Ad5/3 luc | Fiber knob of Ad3, Luciferase in the E1A gene | Kindly provided by Dr. D.T. Curiel, Washington University, St. Louis, USA |
| Ad5 CXCR4 | Human CXCR4 promoter upstream of E1A gene | (Stoff-Khalili et al., 2008) |
| Ad5/3 CXCR4 | Human CXCR4 promoter upstream of E1A gene, Ad3 fiber knob | (Stoff-Khalili et al., 2007) |
| Ad5/3 CXCR4 UTR | Human CXCR4 promoter upstream of E1A gene, Ad3 fiber knob, 5'UTR sequence of rat FGF-2 upstream of E1A gene | Present study |
| Ad5 Δ24 | 24bp deletion in the E1A gene | Kindly provided by Dr. D.T. Curiel, Washington University, St. Louis, USA |
| Ad5 Δ24 RGD | 24bp deletion in the E1A gene, RGD motif in the H1-loop of the fiber knob | Kindly provided by Dr. D.T. Curiel, Washington University, St. Louis, USA |

## *2.7 Proteins*

### 2.7.1 Enzymes

Restriction enzymes were purchased from the following companies: New England BioLabs (Ipswich, MA, USA) and Fermentas (St. Leon-Rot, Germany).

| | |
|---|---|
| T4-DNA Ligase | (NEB, Ipswich, MA, USA) |
| Herculase II Fusion Enzyme with dNTPs Combo | (Agilent Technologies, Santa Clara, CA, USA) |
| Platinum® Taq DNA Polymerase | (Invitrogen, Karlsruhe, Germany) |

### 2.7.2 Molecular weight markers

PageRuler$^{TM}$, prestained protein ladder 170-10 kDa (Fermentas, St. Leon-Rot, Germany)

PageRuler$^{TM}$, unstained protein ladder 200-10k Da (Fermentas, St. Leon-Rot, Germany)

### 2.7.3 Antibodies

**Table 6: Specific antibodies used in this study**

| **Antibody** | **Antigen** | **Dilution** | **Reference** |
|---|---|---|---|
| 4EBP-1 | Synthetic N-terminal peptide human 4E-BP1 | 1:1,000<br>5% milkpowder TBS-T | Epitomics |
| eIF4E | Full length of human eIF4E | 1:1,000<br>5% milkpowder TBS-T | Santa Cruz |
| E1A | full length Adenovirus 13S E1A fusion protein | 1:1,000<br>5% milkpowder TBS-T | Santa Cruz |
| β-actin | SDS extract from human myocardium | 1:2,000<br>5% milkpowder PBS-T | Millipore |
| Goat anti-mouse | IgG, F(ab')$_2$ Fragment specific | 1:2,500<br>5% milkpowder PBS-T | Dianova |
| Goat anti-rabbit | IgG, F(ab')$_2$ Fragment specific | 1:10,000<br>5% milkpowder TBS-T | Dianova |
| Anti-Hexon | Adenovirus hexon protein | 1:1,000<br>1% BSA PBS | Clontech Laboratories |

## *2.8 Staining reagents*

Crystal Violet (CV) (Sigma-Aldrich, Steinheim, Germany)

MTT Cell Growth Kit Assay (Millipore, Schwalbach, Germany)

## *2.9 Solutions and Buffers*

**PBS (Phosphate Buffered Saline)**

| | |
|---|---|
| NaCl | 137 mM |
| KCl | 2.7 mM |
| $Na_2HPO_4$ | 4.3 mM |
| $KH_2PO_4$ | 1.4 mM |

**50x TAE buffer (pH 8,0)**

| | |
|---|---|
| Tris-Acetate | 2 M |
| EDTA | 50 mM |

**TE-buffer (pH 8,0)**

| | |
|---|---|
| Tris | 10 mM |
| EDTA | 1 mM |

**Saturated Caesium Chloride**

95-100 g Caesium Chloride

100 ml TE-buffer

**Adenoviral dialysis buffer**

| | |
|---|---|
| Tris pH 7.4 | 10 mM |
| NaCl | 75 mM |
| Sucrose | 5% |
| Polysorbate 80 (Tween 80) | 0.020% |
| $MgCl_2$ | 1 mM |
| EDTA pH 8.0 | 100 µM |
| EtOH | 0.5% |
| L-Histidine | 10 mM |

**10x Running Buffer (1 L)**

| | |
|---|---|
| Tris-base | 30 g |
| Glycine | 144 g |
| SDS | 10 g |

| **Cell Lysis Buffer I** | | **Cell Lysis Buffer II** | |
|---|---|---|---|
| NP-40 | 0.1% | NP-40 | 1% |
| Tris-HCl pH 8.0 | 50 mM | Tris-HCl pH 8.0 | 20 mM |
| KCl | 100 mM | NaCl | 150 mM |
| PMSF | 1 mM | EDTA pH 8.0 | 5 mM |
| DTT | 1 mM | PMSF | 1 mM |
| Protease Inhibitor Mix w/o EDTA | 1x | Protease Inhibitor Mix w/o EDTA | 1x |

| **Stacking-Gel** | | **Separating Gel** | |
|---|---|---|---|
| Tris-HCl pH 6.8 | 0.5 M | Tris-HCl pH 8.8 | 1.5 M |
| SDS | 0.4% | SDS | 0.4% |
| | 29.2% Acrylamide/0.8% Bis-acrylamide | | |
| | TEMED, 10% APS | | |

| **10x Transfer Buffer (1 L)** | | **1x Transfer buffer (1 L)** | |
|---|---|---|---|
| Tris-base | 30 g | 10x Transfer Buffer | 100 ml |
| Glycine | 144 g | MeOH | 20% |
| | | dH2O | adjust to 1 L |

| **4x LÄMMLI-Buffer** | | **Strip-Buffer for Western Blots** | |
|---|---|---|---|
| Tris-HCl pH 6.8 | 200 mM | β-mercaptoethanol | 100 mM |
| SDS | 8% | SDS | 2% |
| Bromphenolblue | 0.4% | Tris-HCl pH 6.7 | 62.5 mM |
| Glycerol | 40% | | |
| β-mercaptoethanol | 10% | | |

## *2.10 Chemicals and reagents*

- Acrylamide (Roth, Karlsruhe, Germany)
- Actinomycin D (Sigma-Aldrich, St. Louis, MO, USA)
- Agarose (Sigma-Aldrich, Steinheim, Germany)
- Ampicillin (Sigma-Aldrich, Steinheim, Germany)
- ß-Mercaptoethanol (AppliChem, Darmstadt, Germany)
- Bradford Reagent (Bio-Rad Laboratories GmbH, Munich, Germany)
- Bromophenol blue (Merck KGaA, Darmstadt, Germany)
- Caesium-Chloride (Sigma-Aldrich, St. Louis, MO, USA)
- Crystal Violet (Sigma-Aldrich, Steinheim, Germany)
- Dimethyl Sulfoxide (DMSO) (AppliChem GmbH, Darmstadt, Germany)
- Di-Sodium hydrogen phosphate (Merck KGaA, Darmstadt, Germany)
- Ethidiumbromide (Roth, Karlsruhe, Germany)
- Fetal Bovine Serum (FBS) (Invitrogen, Karlsruhe, Germany)
- Glycerol (AppliChem GmbH, Darmstadt, Germany)
- Glycine (Roth, Karlsruhe, Germany)
- Isoflurane (Halocarbon products corporation, River Edge, NJ, USA)
- Kanamycin (Gibco, Life Technologies, Grand Island, NY, USA)
- Potassium dihydrogen phosphate (Merck KGaA, Darmstadt, Germany)
- Sodium Dodecyl Sulfate Polyacrylamide (SDS) (Roth, Karlsruhe, Germany)
- Sucrose (Sigma-Aldrich, Steinheim, Germany)
- TEMED (AppliChem GmbH, Darmstadt, Germany)
- Tween 20 (Caesar & Loretz GmbH, Hilden, Germany)
- Tween 80 (Polysorbate 80) (Sigma-Aldrich, Steinheim, Germany)

Plastic ware was purchased from the following companies: Eppendorf (Hamburg, Germany), Falcon BD (Heidelberg, Germany), Greiner (Solingen, Germany), Sarstedt (Nümbrecht, Germany) and TPP (Trasadingen, Switzerland).

## *2.11 Machines used in this study*

- ABI 7900HT (Applied Biosystems, Carlsbad, CA, USA)
- iCycler (BioRad, Hercules, CA, USA)
- LightCycler System (Roche Diagnostics, Mannheim, Germany)
- Microscope (Diavert, Leica Microsystems, Wetzlar, Germany)
- Nano-Drop (PEQLAB, Erlangen, Germany)
- SmartSpec 3000 (BioRad, Hercules, CA, USA)
- SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA)
- Wallac Victor2 1420 Multilabel Counter (Perkin Elmer life Sciences, Rodgau, Germany)

# 3. Methods

## *3.1 Molecular Biology*

Molecular biology methods were performed according to the established protocols (Sambrook, 2001)

### 3.1.1 Generation of the triple-level targeted adenovirus (Ad5/3 CXCR4 UTR)

According to recent established dual-level targeted CRAds Ad5/3 CXCR4 and Ad5 CXCR4-UTR which showed an increase in tumor specific replication (Stoff-Khalili et al., 2008; Stoff-Khalili et al., 2007), in this study a triple-level targeted CRAd (Ad5/3 CXCR4 UTR) was established. The purified Ad5/3 CXCR4 UTR was used to infect breast cancer cells and analyzed for oncolytic activity and viral replication.

#### *3.1.1.1 Generation of Ad5/3 CXCR4 UTR plasmid*

To construct a triple level targeted Ad containing a fiber knob chimera (5/3), the human CXCR4 promoter and the 5'UTR sequence of rat fibroblast growth factor 2 (FGF-2) upstream of the E1A gene, a shuttle vector containing the modified E1A region (pShuttle.CXCR4-UTR-E1A) and the backbone vector pVK500 vector containing the FB5/3 fiber were recombined. The fiber gene of the plasmid pVK500 is composed of the Ad5 serotype tail and shaft sequence and the Ad3 serotype knob sequence. The Ad5/3 CXCR4 UTR plasmid was generated by transforming the bacterial strain BJ5183 with both vectors. Beforehand, the pShuttle.CXCR4-UTR-E1A was *Fse*I linearized and recombined in BJ5183 with *Cla*I digested pVK500 FB5/3 backbone. After recombination a large scale preparation of the DNA was performed by transforming the DNA into XL10 Gold bacteria. Subsequently, the plasmid was verified by sequencing and PCR analyses (3.1.1.2).

To rescue the CRAd virions of Ad5/3 CXCR4 UTR, the pAd.CXCR4-UTR-E1A.FB5/3 plasmid was linearized with *Pac*I and transfected into HEK-293 cells using the calcium phosphate transfection method (3.2.3). The vector was successfully rescued on HEK-293 cells and expanded by several rounds of propagation on sequentially increasing flask size and

numbers. The virus was purified by double CsCl density gradient centrifugation (3.1.1.4) and subsequently dialyzed against Ad dialysis buffer. Final aliquots of virus were analyzed for infectious units (ifu) with the Adeno-X Rapid Titer Kit (Clontech; St-Germain-en-Laye, France) (3.2.4.1). Viruses were finally stored at -80°C.

### *3.1.1.2 Polymerase Chain Reaction (PCR) and sequencing*

To analyze whether both targeting elements, the human CXCR4 Promoter and the 5'UTR-sequence of rat FGF-2 were correctly inserted upstream of the E1A gene, a polymerase chain reaction (PCR) was performed (Saiki et al., 1988). Human CXCR4 promoter forward primer was designed to align only to the human CXCR4 promoter sequence and 5'UTR sequence reverse primer was designed to anneal only to the 5'UTR sequence of rat FGF-2. Due to the design of both primers, only Ads that contain the CXCR4 promoter and the 5'UTR sequence would reveal a PCR product. Furthermore, the presence of the Ad3 fiber knob in the triple level targeted adenovirus was confirmed by performing a PCR. Oligonucleotides are listed in (2.3.1; Table 2) and reactions were performed in Thermocyclers (BioRAD).

Recombinant plasmids and DNA of the triple-level virus after rescue were sequenced according to established the methods (Sanger et al., 1977), to confirm the presence and correct sequence of all inserts.

### *3.1.1.3 Isolation of plasmid DNA from bacterial strains*

Plasmid DNA from transformed bacteria strains was isolated with an alkaline lysis method (Birnboim, 1983) according to previously described protocols.

### *3.1.1.4 Cesium chloride extraction of adenovirus*

To isolate viral particles after assembly of virus in cell culture, infected cells were collected and pelleted by centrifugation for 10 min at 600 x g. The cell pellet was then resuspended in 5 ml of supernatant and three freeze/thawing cycles were performed. During those cycles the cell pellet was thawn in a 37 °C water bath and afterwards, directly frozen again at -80 °C. Those freeze/thawing cycles were lysing the cells, so that the Ads within those cells was released into the supernatant. After the third cycle, a 1/10 volume of 5% sodium deoxycholate

was added and the suspension was incubated for 30 min on ice to further disrupt the cells without destroying the virions. Then, the cellular DNA was sheared by using a probe-type homogenizer. Afterwards, 1.8 ml of saturated CsCl for each 3.1 ml of virus suspension was added. Viral suspension was distributed into tubes and centrifuged for 16-20 h at 4 °C and 99,000 x g. After centrifugation, the virus band was collected in a small volume and dialysed against Ad dialysis buffer overnight (ON) at 4 °C. The next day, dialysed Ad was aliquotted and stored at -80 °C.

### 3.1.2 Quantitative polymerase chain reaction (qPCR)

DNA levels were quantified by qPCR and cDNA levels after reverse transcription of mRNA (qRT-PCR) using the LightCycler System (Roche Diagnostics, Mannheim) and the ABI 7900HT (Applied Biosystems, Carlsbad, CA, USA). To determine viral entry and to titer the viral stock solutions, the LightCycler System was used with the following reaction mix: 1.25 units Platinum Taq Polymerase and the associated buffer (Invitrogen, Karlsruhe), 4 mM $MgCl_2$, 5% DMSO (Applichem, Darmstadt), 1.6 µl of a 1:1,000 dilution of SybrGreen I (Sigma-Aldrich, Steinheim), 500 ng/µl non-acetylated bovine serum albumin (Fermentas, St. Leon-Rot), 0.5 µM forward and backward primer each and 0.2 mM deoxynucleotide triphosphates each (Fermentas, St. Leon-Rot). To analyze E1A and pTP mRNA levels and Ad replication efficiency the ABI 7900HT system was used. To perform this qPCR, the FastStart Universal SYBR Green Master Mix (ROX) of Roche diagnostics was employed. Each reaction mix contained 10 µl SYBR green mix and 0.5 µM of each primer. All samples used in this study were analyzed in duplicates or triplicates together with a plasmid dilution series of plasmids described in (2.3.3). These dilution series were used to generate standard curves.

The cycling protocol conditions were as follows: 10 min at 95 °C (ABI 7900HT) or 60 sec at 95°C (LightCylcer), followed by 40 cycles of 1 sec at 95 °C (20 °C/s), 5 sec at 50 °C (pTP) or 55 °C (20 °C/s), and 15 sec at 72 °C (20 °C/s). At the end of each elongation step the fluorescence was quantified once per cycle. Subsequently, a melting curve protocol was carried out for 15 sec at 95 °C, followed by 20 sec at 70 °C (20 °C/s) and 1 sec at 95 °C (0.2 °C/s). During the heating process at 95 °C, fluorescence was measured continuously. At the end, samples were cooled down to 40 °C for 10 sec. All DNA and cDNA levels were normalized to HPRT1 or GAPDH except for the E4 copy number results, the half life of E1A mRNA and the E1A mRNA levels in the hamster cell lines.

## *3.2 Cell Biology*

### 3.2.1 Cultivation of Cell lines

All human breast cancer cell lines, the Syrian hamster breast cancer cell lines and all human fibroblasts used in this study, were cultured in Dulbeccos Modified Eagle´s Medium containing stable glutamine (DMEM + Glutamax$^{TM}$, 4,500 mg/l glucose, Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). HMEC cells were cultured in Human Mammary Epithelial Growth Medium (PromoCell) supplemented with 0.004 ml/ml Bovine Pituitary Extract, 10 ng/ml Epidermial Growth Factor (recombinant human), 5 µg/ml insulin (recombinant human) and 0.5 µg/ml hydrocortisone. The HMAM5 cell line was additionally cultured in DMEM containing 4,500 mg/l glucose (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and only 2% (v/v) FBS (Invitrogen). Hamster fibroblasts were cultured in RPMI (Invitrogen) supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were cultivated in an atmosphere of 5% $CO_2$ and 90% humidity at 37 °C. FBS containing 10% DMSO were used to stepwise freeze down the cell lines.

### 3.2.2 Adenoviral infection of cell lines

Cell lines were plated one day prior of viral infection. The next day, cells were infected with the indicated viral concentrations. Viruses were diluted in 2% FBS DMEM and added in the specific concentrations on the cell lines, followed by a two hours incubation time at 37 °C in an atmosphere with 5% $CO_2$ and 90% humidity. After the incubation time, the virus containing media was removed and fresh 10% FBS DMEM was added. Cells were then incubated at 37 °C in an atmosphere containing 5% $CO_2$ and 90% humidity until the collection day.

### 3.2.3 Transfection of Ad5/3 CXCR4 UTR vector into HEK-293 cells

For the generation of a functional Ad, the Ad5/3 CXCR4 UTR plasmid was transfected into HEK-293 cells using a calcium phosphate transfection kit from Agilent Technologies (Mammalian Transfection Kit). HEK-293 cells are carrying the left arm of the Ad5 genome incorporated in their genome. Therefore, those cells provide the perfect environment for viral replication and assembly.

Ad5 plasmid DNA was linearized by *Pac*I to reveal the Ad inverted terminal repeats (ITR). Then, the linearized DNA was transfected with a calcium phosphate transfection according to the manufacturer's instructions. Briefly, 0.7 x $10^6$ cells were plated on a T-25 tissue culture flask and grown until 50-70% confluency. 15 µg of *Pac*I digested DNA were diluted with $dH_2O$ until a final concentration of 450 µl. Then, 50 µl of Solution 1 ($CaCl_2$) were added to the DNA sample followed by the addition of 500 µl Solution 2 (BBS), the mixture was gently mixed by tapping the side and then incubated for 10-20 min at RT. Subsequently, cell culture medium was aspirated of the T-25 flask, cells were washed twice with PBS and 5 ml of DMEM without FBS containing 5% of Solution 3 (MBS) was added. Afterwards, the DNA mixture was added drop wise in a circular manner to the flask and cells were incubated for three hours at 37 °C. After incubation, cell culture medium was removed, cells were washed twice with PBS, and fresh medium was added and incubated at 37 °C until 50% of the cells were killed by the newly replicated virus.

## 3.2.4 Titer determination of Adenoviral stocks

### *3.2.4.1 Infectious units per ml*

Infectious units per ml were determined using the Ad titer kit of Clontech (St-Germain-en-Laye, France) according to the manufacturer's instructions. Briefly, 1.25 x $10^5$ HEK-293 cells were plated per well on a 24-well tissue culture plate one day prior to infection. Then, serial dilutions of viral stock solutions were added to the cells and incubated for two hours at 37 °C. After the incubation time, additional 800 µl of 10% FBS DMEM were added and the plate was incubated for 48 h at 37 °C in an atmosphere of 5% $CO_2$ and 90% humidity. At 48 h post infection, medium was removed, cells were dried and fixed with 500 µl ice-cold 100% methanol for 10 min at -20 °C. Subsequently, methanol was removed, cells were washed three times with 1% BSA PBS and then a 1:500 dilution of mouse anti-Hexon antibody was added on each well followed by one hour incubation at 37 °C. After removal of the first antibody, cells were again washed three times and a 1:1,000 dilution of rat anti-mouse antibody (HRP-conjugate) was added for one hour at 37 °C. Before adding the DAB working solution which stains the positively infected cells, the secondary antibody was aspirated, cells were washed and DAB working solution was added for 10 min at RT. Afterwards, positively stained cells were counted per field with the microscope and the infectious units were calculated after the following formula:

$$\frac{\text{(infectious cells/field) x (fields/well)}}{\text{volume of virus (ml) x (dilution factor)}} = \text{ifu/ml}$$

### *3.2.4.2 Copy number per µl*

Copy number of viral stock solutions was verified with qRT-PCR (3.1.2). DNA was isolated form viral stocks by using the QIAamp® DNA Blood Mini Kit from Qiagen (Hilden, Germany). After DNA isolation, the copy number of the viral genome was determined of 1 µl of a 1:100 dilution using the qRT-PCR. A standard curve was generated using the pAdEasy vector containing the E4 gene. E4 copy number/µl of the viral stock solution was calculated according to the standard curve.

## 3.2.5 Defining oncolytic activity

To determine virus-mediated cytotoxicity two assays were performed the crystal violet (CV) (Sigma Aldrich, Munich, Germany) staining and the MTT Cell Growth Kit (Millipore, Schwalbach, Germany).

### *3.2.5.1 Crystal Violet Analysis*

1 x $10^4$ cells were seeded in a 24-well tissue culture plate and infected with the indicated adenoviruses at different titers or mock infected on the next day. Cell killing was visualized by staining the cells with 0.1% crystal violet (CV) in 70% ethanol for 20 min. Residual CV was removed by washing the wells with tab water, after drying, images of the plates were taken with a Canon EOS D30 digital camera (Canon, Krefeld, Germany).

### *3.2.5.2 MTT-Assay*

1 x $10^3$ cells were plated on a 96-well tissue culture plate and infected with Ads at different titers per cell or mock infected on the next day. To visualize viral cytotoxicity, the MTT Cell Growth Kit of Millipore (Schwalbach, Germany) was used according to the manufacturer's instructions. Briefly, six days post infection 10 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to the medium, cells were incubated at 37 °C for

150 min, followed by the addition of 100 µl acidic isopropanol and an incubation time of 20 min shaking on a rotor. The absorbance was measured at 560 nm and 630 nm. The absorbance of 630 nm was substracted from the 560 nm absorption. Results were expressed as percentage of infected cells relative to uninfected cells, setting mock 100%.

### 3.2.6 Quantifying viral cell entry, transcription and replication

Viral uptake was determined by quantifying E4 copy number of total cellular DNA using qPCR (3.1.2). To analyze viral cell entry, 6 x $10^5$ cells were seeded in a 24-well tissue culture plates and infected with the indicated viruses at 1, 10 or 100 ifu/cell or mock infected the next day. At 1 h and 3 h after infection, cells were washed with PBS and trypsinized. Subsequently, cells were collected by centrifugation and washed twice with PBS prior to DNA isolation using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To analyze E1A mRNA expression levels of total cellular mRNA and DNA E4 copy number in the supernatant of infected cells, 1 x $10^5$ cells were plated one day before infection. The next day, cells were infected with the indicated viruses at 10 ifu per cell or mock infected. E1A mRNA expression levels were determined collecting cells 48 h after infection, purifying total RNA with the RNeasy Mini kit (Qiagen, Hilden, Germany), reverse transcribed into cDNA (iScript™ cDNA Synthesis Kit, BioRad, Hercules, CA) and finally, a qRT-PCR (3.1.2) was performed as previously described (Zhu et al., 2004).

E4 copy numbers in the supernatant were determined by collecting 200 µl supernatant of the infected cells daily until 4 days post infection. Viral DNA was isolated using QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) and viral replication was quantified by qPCR (3.1.2).

To monitor Ad5 E1A and Ad5 pTP expression upon infection, 2 x $10^5$ cells were seeded per well of a 6-well tissue culture plate one day before infection. The next day, cells were infected with 10 ifu per cell of the indicated viruses and mRNA was isolated at 6 h, 12 h, 24 h and 48 h post infection using RNeasy Mini kit (Qiagen, Hilden, Germany). Afterwards mRNA was reverse transcribed into cDNA (iScript™ cDNA Synthesis Kit, BioRad, Hercules, CA, USA), followed by qRT-PCR (3.1.2) to analyze the expression levels.

### 3.2.7 Half-life determination of E1A mRNA

Half-life of Ad5 E1A mRNA was determined by treating the cells with 10 µg/ml actinomycin D. Initially, 2 x $10^5$ cells were seeded per well of a 6-well tissue culture plate and the next day cells were infected with the indicated viruses. At 12 h post infection 10 µg/ml of actinomycin D was added to the medium of the infected cells. Cells were collected at several time points, mRNA was isolated which was then reverse transcribed into cDNA and mRNA degradation was determined by using qRT-PCR (3.1.2).

### 3.2.8 Growth characterizations

To verify growth of HMAM4 and HMAM5 cells, 15,000 cells per well were plated on a 6-well tissue culture plate. Cells were counted in triplicates daily for 10 to 12 days with a hemocytometer. As a control cell line, the breast cancer cell line MDA-MB-231 was used. Furthermore, growth of HMAM5 cells cultured in DMEM containing different concentrations of FBS (2%, 5% and 10%) was also determined.

### 3.2.9 Anchorage independence determination

To analyze anchorage independent growth, two layers of agar and media were prepared. A 1 ml 1.2% agar and 1 ml of preheated 10% FBS DMEM was combined receiving the lower layer. 1ml of this solution was then added to each well of a 5 cm tissue culture plate. After the lower layer hardened, the upper layer was prepared. A 0.6% agar solution was preheated in a 45 °C water bath. Afterwards, 1 ml of the 0.6% agar solution was combined with 1 ml of a 2 x $10^4$ cells/ml single cell suspension. Subsequently, 0.5 ml of the upper layer was added on the top of the lower layer. In the last step, 1x DMEM solution was added on the top of the upper layer and the tissue culture plate was incubated at 37 °C for 15 days. Finally, the developed colonies were counted.

## *3.3 Biochemistry*

### 3.3.1 Preparation of protein extracts

For preparation of protein extracts, cells were washed twice with PBS, harvested with trypsin and centrifuged for 5 min at 600 x g at RT. Then, cell pellets were resuspended either in cell lysis buffer I (0.1% NP-40, 50mM Tris-HCl (pH 8.0), 100mM KCl, 1mM DTT, 1mM PMSF) or in cell lysis buffer II (1% NP-40, 20mM Tris-HCl (pH 8.0), 150mM NaCl, 5mM EDTA, 1mM PMSF and 1x Roche Protease Mix). Cells resuspended in lysis buffer I were sonicated for 5 min using the Biorupter and centrifuged for 10 min at 4 °C and 20,800 x g to remove unsolubilized membranes. Protein extracts were then used for a co-immunoprecipitation (3.3.2.3). Cells resuspended in cell lysis buffer II were incubated for 1.5 h on ice, afterwards sonicated for 5 min and finally centrifuged at 1,000 x g and 4 °C for 15 min. The protein concentration for protein extracts was determined using Bradford protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) and Bovine Serum Albumin (BSA) (Sigma Aldrich, Munich, Germany) as a standard. Protein samples were stored at -80 °C until further use.

### 3.3.2 SDS-PAGE and Immunoblotting

Proteins extracted from tissue cultured cells and after co-immunoprecipitation (3.3.2.3) were separated according to their size with SDS-PAGE and transferred to Trans-Blot nitrocellulose (Bio-Rad Laboratories GmbH, Munich, Germany) or polyvinylidene fluoride (BioTrace PVDF) (Pall Corporation, Dreieich, Germany) membranes according to established protocols. After transfer of the proteins to the membrane, membrane was blocked for 1 h with 5% (w/v) milkpowder in 1x TBS-Tween (TBS-T; 0.1% Tween-20). Immunostaining was carried out by diluting the respective antiserum in blocking solution or in 5% (w/v) milkpowder PBS-Tween (PBS-T; 0.1% Tween-20) or in 5% (w/v) BSA (Sigma Aldrich, Munich, Germany) and incubating the membranes overnight (ON) at 4 °C. The next day, membrane was washed twice for 10 min and once for 5 min with either TBS-T or PBS-T. Detection of the primary antibodies was determined by incubating with a secondary antibody that contains a horseradish peroxidase conjugate and is specific for the immunoglobulin G of the first antibodies species, for 1 h at RT. After repeating the washing steps with TBS-T or PBS-T, BM Chemoluminescence Blotting Substrate (POD) reagents (Roche Diagnostics, Mannheim, Germany) were added in a mixture of 1:100 on the membrane for 1 min. Chemoluminescence was detected by light sensitive x-ray films (Super RX, Fuji-Film, Duesseldorf, Germany). The

prestained PageRuler, range 170-10 kDa and the PageRuler unstained protein ladder 200-10 kDa (Fermentas, St. Leon-Rot, Germany) were used as molecular weight standards.

#### *3.3.2.1 Determination of viral protein expression*

To determine the Ad E1A expression level, Western immunoblot analysis was performed. Cells were infected with Ad5 Wild type (WT), Ad5/3 luc, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR at 1 ifu/cell. Cells were harvested 5 days post infection and lysed in cell lysis buffer II (3.3.1). 30 µg of proteins were subjected to a 10% SDS-PAGE and proteins were then transferred to nitrocellulose membranes.

#### *3.3.2.2 eIF4E protein expression level*

eIF4E protein levels in Ad5 WT or Ad5/3 WT infected cells were quantified by Western immunoblot analysis. 5 x $10^5$ cells were plated 1 day before infection on 10 cm tissue culture plate. The next day, cells were infected with 10 ifu/cell of Ad5 WT or Ad5/3 WT. Cells were collected 24 h, 48 h and 72 h post infection. Protein lysates were prepared using cell lysis buffer II (3.3.1) and 30 µg of protein were subjected to a 15% SDS-PAGE. Next, proteins were transferred to a nitrocellulose membrane ON at 30 mA and RT.

#### *3.3.2.3 Co-immunoprecipitation*

To perform a co-immunoprecipitation in which the eIF4E binding partners were precipitated, 3 x $10^6$ MDA-MB-231 cells and 4 x $10^6$ MCF-7 cells were plated 1 day before infection. The next day, cells were infected with 50 ifu per cell of Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 UTR. All cells were collected at the indicated time points. Protein lysates were prepared by adding the appropriate amount of cell lysis buffer I (3.3.1). 700 µg of protein extract was incubated with 5 µl (0.5 µg/ml) agarose beads which are covalently bound to eIF4E antibody (Santa Cruz Biotechnologies, Heidelberg, Germany). Samples were incubated for 1.5 h at 4 °C by rotation. Afterwards, samples were pelleted by centrifugation at 1000 x g for 1 min at 4 °C and supernatant was carefully removed. Then, four washing steps were performed using 1 ml of cell lysis buffer I without Roche protease inhibitor mix. After the final washing step, supernatants were discarded and pellets were resuspended in 20 µl 2 x Lämmli-Buffer. Finally

samples were subjected to a 15% SDS-PAGE and proteins were transferred on a PVDF membrane ON at 30 mA and RT.

## *3.4* In vivo *experiments*

### 3.4.1 Establishment of an *in vivo* syngeneic tumor model

The protocol number for this experimental setup was P-07-050. To verify tumor take efficacy in syngeneic female Syrian hamsters, cultured HMAM4B and HMAM5 cells were subcutaneously injected into each hind flank of the animals. Animals were shaved at the site of injection, anesthesized with isoflurane and then 1 x $10^7$ cells were injected subcutaneously. Tumor development and growth was monitored daily using a digital caliper. Finally, the tumor volume was quantified by measuring the smallest ($d$) and the largest ($D$) diameter with the caliper. The absolute tumor volume was then determined using the following formula: $V = D \times d^2 \times 0.52$

# 4. Results

## *4.1 Triple-level targeted oncolytic Adenovirus, combining transductional, transcriptional and translational targeting*

To date, virotherapy is a promising tool to treat cancer. However, optimization of tumor specific targeting and replication of virions used as anticancer agents is still needed. Therefore, several targeting strategies have been established in the past years, but unfortunately every single targeting strategy exhibits some disadvantages. The disadvantage of transductional targeting is the infection of normal cells expressing the cellular attachment receptor of the tropism modified Ad vector on their surface. Tropism modified vectors do not distinguish between cancer and normal cells; they bind to cells expressing their cellular attachment receptor. Once, they entered the cell the vectors start to replicate and induce cytotoxicity through cell lysis. Their replication is not cancer cell restricted. Tumor specific promoters (TSP) are used to restrict Ad replication to cancer cells. Nevertheless, they demonstrate also a small background activity in normal cells so that replication in some normal cells is possible. Theretofore, combining targeting strategies was a reasonable approach to increase the cancer specificity profile in normal cells. Recently, it was shown by Stoff-Khalili et al. that the combination of either transductional and transcriptional targeting or transcriptional and translational targeting enhanced the cancer specificity profile and at the same time maintained the replication efficiency in cancer cells (Alemany et al., 2000; Stoff-Khalili et al., 2008; Stoff-Khalili et al., 2007). Assembling and replication efficiency was not impaired in both virions upon inserting two control elements into the viral genome. These results indicated that the combination of several modifications in the Ad genome is feasible without affecting the Ad replication. Based on these findings, the aim of this study was to construct a conditionally replicating adenovirus (CRAd) that is combining all three targeting strategies. The combination of transductional, transcriptional and translational targeting was hypothesized to further increase specificity of CRAd replication for breast cancer cells. Construction of a CRAd containing three levels of control is a novel approach to gain breast cancer specificity in the field of virotherapy. The systemic administration of triple-level targeted CRAds could be enabled because of the reduction of unspecific replication in normal cells. In this study, a triple-level targeted CRAd was constructed carrying the Ad3 fiber knob domain (transductional targeting), the human CXCR4 promoter (transcriptional targeting) and the 5'UTR sequence of

rat FGF-2 (translational targeting) upstream of the Ad5 E1A gene. This newly constructed CRAd was named Ad5/3 CXCR4 UTR (Figure 5).

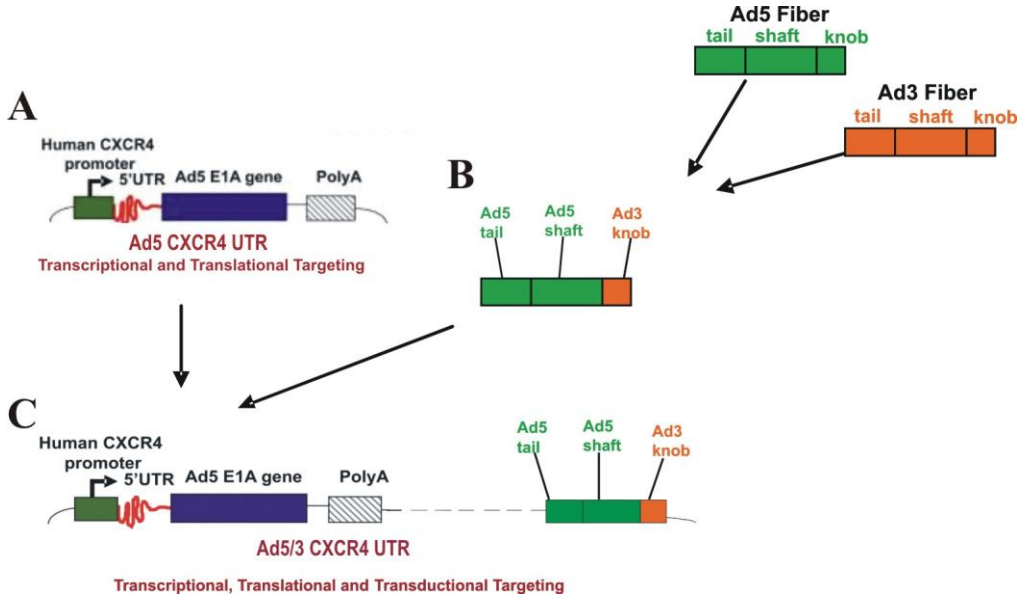


**Figure 5: Strategy to develop translational and transcriptional targeting as an addition to transductional targeting.**

**A)** Shuttle plasmid harboring a CXCR4-promoter-controlled E1A gene with the FGF2 5'UTR-sequence, pShuttle.CXCR4-UTR-E1A. **B)** A backbone vector containing a fiber knob domain of serotype 3 instead of serotype 5 whereas the shaft and the tail remain from the serotype 5. **C)** Both vectors were used in an *E.coli* recombination system to attain recombinant genomes with the pShuttle.CXCR4-UTR-E1A vector to produce the pAd.CXCR4-UTR-E1A.Fb5/3 vector.

### 4.1.1 Incorporation of Ad3 fiber knob, human CXCR4 promoter and rat 5'UTR-sequence of FGF-2 mRNA into Ad5 genome

The backbone plasmid pVK 500 FB5/3 and the shuttle plasmid pShuttle.CXCR4-UTR-E1A were recombined using the *E.coli* strain BJ5183. The virus was rescued on HEK-293 cells after transfection of the viral plasmid DNA and further propagation was carried out on A549 cells. After the successful rescue of the newly constructed triple-level CRAd Ad5/3 CXCR4 UTR, several PCRs were performed, to analyze whether all control elements were incorporated within the genome of Ad5/3 CXCR4 UTR. PCR primers were designed to specifically align to the Ad3 fiber knob region and another set was designed to align upstream in the CXCR4 promoter region and downstream in the 5'UTR region of the FGF-2. DNA of the viral stock solutions was isolated and analyzed via PCR. PCR products for Ad3 fiber knob were detected in samples containing the DNA of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR at the

expected size of 350 bp except for Ad5 WT, shown in Figure 6A. Clearly, this result demonstrated that all viruses carrying the Ad5/3 fiber chimera incorporated the Ad3 fiber knob in their genome. Furthermore, a PCR product of the human CXCR4 promoter and the 5'UTR sequence of FGF-2 was only observed in the DNA sample of Ad5/3 CXCR4 UTR (Figure 6B), indicating that incorporation of the CXCR4 promoter and the 5'UTR sequence upstream of the Ad5 E1A region was successful. Moreover, sequencing analyses of the modified regions in the Ad5/3 CXCR4 UTR genome confirmed the incorporation of the control elements and revealed that no mutations were present within those regions.

Taken together, PCR and sequencing results demonstrated that the Ad5 fiber knob was successfully exchanged with the Ad3 fiber knob and that the human CXCR4 promoter and the 5'UTR sequence of FGF-2 were incorporated into the Ad5/3 CXCR4 UTR genome.

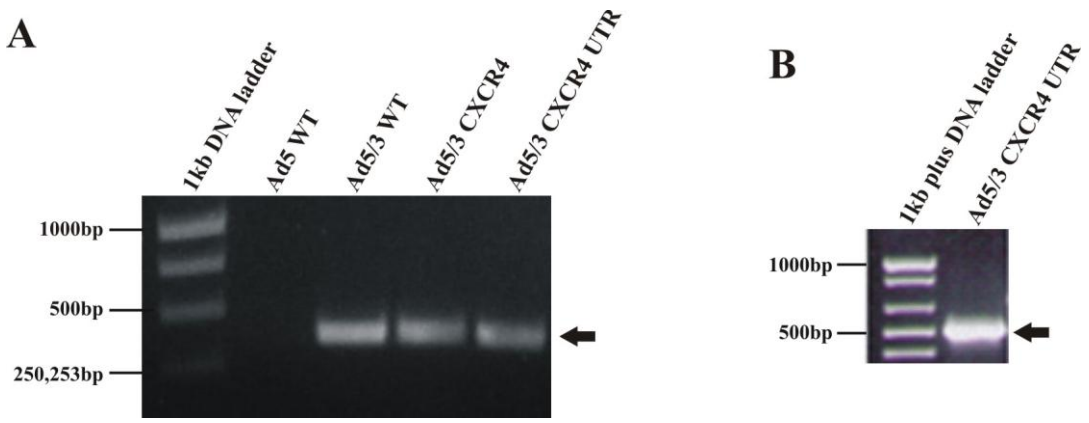


**Figure 6: Incorporation of all three control elements in Ad5/3 CXCR4 UTR after recombination.**

DNA was purified from viral stock solutions of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR and examined for the presence of **A)** Ad3 fiber knob and **B)** CXCR4 promoter and 5' UTR sequence of FGF-2 in PCR. The correct band for the PCR product is indicated with an arrow at **A)** 350 bp and **B)** 513 bp.

### *4.1.2* Ad5/3 CXCR4 UTR gains differential oncolytic activity in breast cancer cells compared to normal cells *in vitro*

The concept of oncolytic virotherapy is based on cancer cell specific infection, replication and spread of released progeny virions to neighboring cancer cells. Modification of Ad vectors is an easy approach to target and restrict Ad replication to breast cancer cells. Thus, after modification of Ad vectors, the analysis of the replication efficiency and the oncolytic activity on breast cancer cells needs to be evaluated to confirm the hypothesized enhancement of specificity.

To investigate oncolytic activity of Ad vectors cytotoxicity assays were performed: Crystal Violet (CV) staining (Figure 7) and the MTT assay (Figure 8). Therefore, cytolytic activity of Ad5/3 WT, Ad5 CXCR4, Ad5/3 CXCR4 and the newly constructed Ad5/3 CXCR4 UTR was determined in different breast cancer cell lines (MDA-MB-231, MCF-7 and ZR-75-1) and compared to the one observed in normal human cell lines (human fibroblasts and HMEC). In the breast cancer cell lines MCF-7 and ZR-75-1, the observed oncolytic activity of the Ad5/3 CXCR4 UTR virus was different compared to that observed in breast cancer cell line MDA-MB-231 (Figure 7C). For oncolytic potency, Ad5/3 CXCR4 UTR displayed a lesser cytotoxic activity compared to Ad5/3 CXCR4 and Ad5/3 WT in MCF-7 and ZR-75-1 breast cancer cells; whereas in MDA-231-MB cells Ad5/3 CXCR4 UTR showed an oncolytic activity that was nearly comparable to Ad5/3 CXCR4 (Figure 7C). In MCF-7 cells, cell killing by Ad5/3 CXCR4 UTR was detectable at a 0.1 MOI whereas in ZR-75-1 cells, cytotoxicity was only detectable at 10 MOI. Furthermore, cell killing by Ad5/3 CXCR4 UTR was only one order of magnitude potency less effective in MCF-7 cells compared to Ad5/3 CXCR4 and Ad5/3 WT. In ZR-75-1 cells on the other hand, the oncolytic activity of Ad5/3 CXCR4 UTR was decreased at around three potencies compared to the one of Ad5/3 CXCR4 and Ad5/3 WT (Figure 7C). Moreover, in MDA-MB-231 a nearly comparable oncolytic activity of Ad5/3 CXCR4 UTR and Ad5/3 CXCR4 was observed at 1 MOI as seen in Figure 7C. However, the oncolytic activity of Ad5/3 CXCR4 UTR was enhanced in MCF-7 compared to Ad5 WT, but was reduced in MDA-MB-231 and ZR-75-1 cells.

In contrast to this result, in human fibroblasts (NHDF), the cytotoxicity of Ad5/3 CXCR4 UTR was attenuated compared to the cytotoxicity of Ad5/3 CXCR4 and Ad5/3 WT, about two to three potencies (Figure 7B). In addition, Ad5/3 CXCR4 UTR displayed a lower oncolytic activity compared to Ad5 WT in NHDF infected cells. In HMEC infected cells then cell killing by Ad5/3 CXCR4 UTR was detected at a concentration of 10 MOI. The cytolytic activity of Ad5/3 CXCR4 UTR was decreased about one order of magnitude potency compared to Ad5/3 CXCR4 and at around two potencies compared to Ad5 WT and Ad5/3 WT in HMECs. Ad5 WT and Ad5/3 WT displayed cytotoxicity even at the lowest concentration of 0.01 MOI in HMEC cells and 0.01 MOI for Ad5/3 WT and 0.1 MOI for Ad5 WT in NHDF cells (Figure 7B). In this regard, Ad5/3 CXCR4 UTR showed a reduced cytotoxic potential compared to Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 in human fibroblasts and HMEC cells.

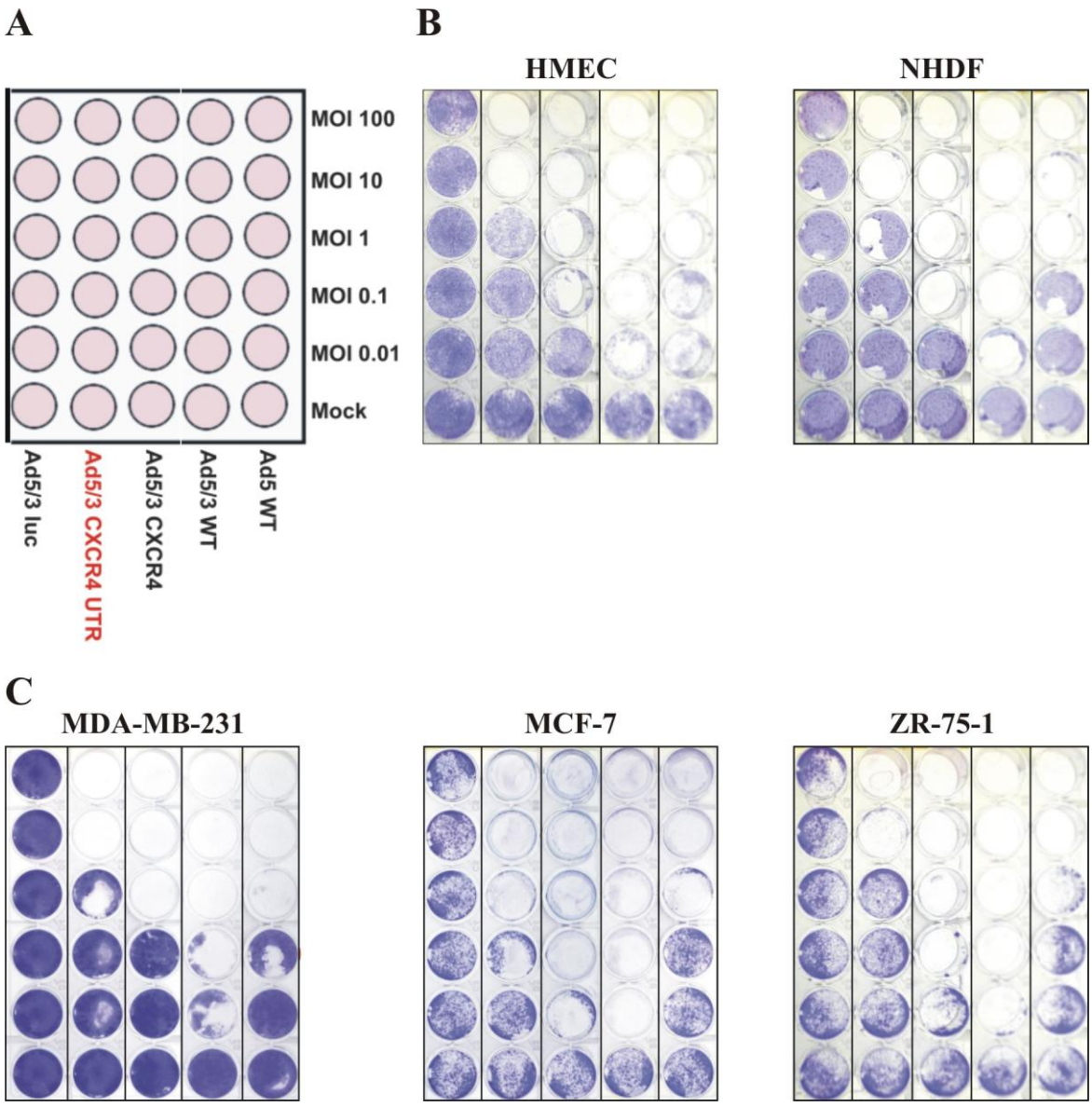


**Figure 7: Reduced oncolytic activity of Ad5/3 CXCR4 UTR in breast cancer and normal cells.**

**A)** Scheme of CV-plate. **B)** Normal human cell lines human fibroblasts (NHDF) and HMEC and **C**) Human breast cancer cell lines MDA-231-MB, MCF-7 and ZR-751 were infected with Ad5/3 luc (non-replicating control), Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR at indicated virus concentrations. Oncolytic activity was monitored by staining viable cells with 0.1% CV. Representative data of three independent CV stainings.

To confirm the results of the CV staining, a different technique was employed to determine cytotoxicity, the Millipore MTT assay. In this assay, MTT is added to the culture medium. Viable cells metabolize MTT in their mitochondria to formazan visible as violet crystals. The crystals are then dissolved in acidic isopropanol and the absorbance of the formazan is measured. Therefore, the lack of color is an indicator for cell cytotoxicity.

At the highest concentration of 100 MOI infection with Ad5/3 CXCR4 UTR displayed an oncolytic activity in MDA-MB-231, MCF-7 and ZR-75-1 cells, which was the same to the one

seen for Ad5/3 WT and Ad5/3 CXCR4 (Figure 8A). However, at a concentration of 10 MOI of Ad5/3 CXCR4 UTR cell killing was only about 20 to 35% in the breast cancer cell lines whereas Ad5/3 WT and Ad5/3 CXCR4 displayed around 80% cell killing (Figure 8A). Also, more than 50% of cell killing was obtained using Ad5/3 WT at a concentration of 1 MOI in MCF-7 and ZR-75-1 cells. Cell killing by Ad5/3 CXCR4 UTR mimicked the one of Ad5 WT in all three breast cancer cell lines and demonstrated an even stronger oncolytic activity at the highest concentration of 100 MOI (Figure 8A). In addition, in MCF-7 and ZR-75-1 cells the difference in cytotoxicity between Ad5/3 WT and Ad5/3 CXCR4 was greater than compared to the one seen in MDA-MB-231 cells.

In human fibroblasts NHDF the cytolytic activity did not reflect the one detected with the CV staining. The highest cytotoxicity was measured in Ad5/3 CXCR4 UTR infected NHDFs at 100 MOI as seen in Figure 8B. Ad5 WT and Ad5/3 WT showed only 20% cell killing at 100 MOI and Ad5/3 CXCR4 did not demonstrate any cytolytic activity. In the lower concentrations of all viruses cytotoxicity was not detectable in NHDFs whereas in the CV staining the oncolytic activity was clearly present upon infection with these viruses (Figure 7B). Therefore, the results of the MTT-Assay and the CV staining were contradictory for NHDFs. As a result, another human fibroblast cell line, PHF Pa2, was included. In human fibroblast PHF Pa2 and HMEC on the other hand, infection with Ad5/3 CXCR4 UTR showed a gain of security compared to Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 (Figure 8B). Ad5/3 CXCR4 UTR demonstrated only 30% cell killing at a concentration of 1 MOI in HMECs whereas the cell killing by Ad5 WT and Ad5/3 CXCR4 was about 60% and the one for Ad5/3 WT was even around 90%. In PHF Pa2 cells, cytolytic activity of Ad5/3 CXCR4 UTR was only 20% at the highest concentrations of 10 and 100 MOI (Figure 8B). In contrast, Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 displayed 80% cell killing at 100 MOI and 60% cell killing at 10 MOI.

Taken together, Ad5/3 CXCR4 UTR demonstrated the same oncolytic activity in breast cancer cells at 100 MOI as the other Ad vectors whereas the results for the lower concentrations were different in the MTT assay compared to the CV staining. This result could be explained by the different incubation times of the assays. In addition, oncolytic activity of Ad5/3 CXCR4 UTR was greater than Ad5 WT in MDA-MB-231 cells and the same in MCF-7 and ZR-75-1 cells. It was shown that Ad5/3 CXCR4 UTR gained cancer specificity and at the same time lost oncolytic potency for breast cancer cells.

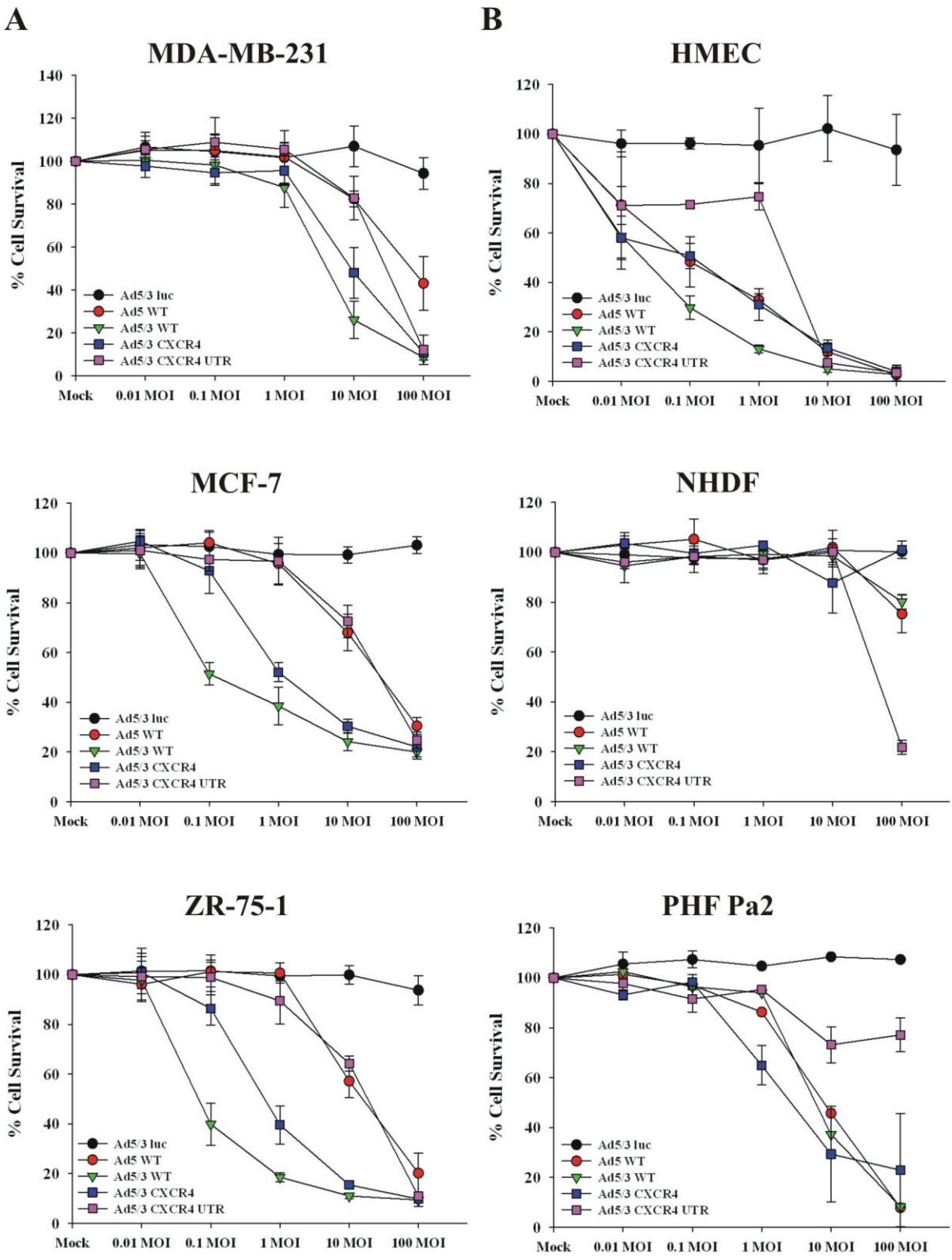


**Figure 8: Comparable oncolytic potency of Ad5/3 CXCR4 UTR to Ad5 WT in breast cancer cells.**

**A)** Breast cancer cell lines, MDA-MB-231, MCF-7, ZR-75-1 and **B)** Normal cell lines, human mammary epithelial cells (HMEC) and human fibroblasts (NHDF and PHF Pa2) were infected with Ad5/3 luc (non replicating control), Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR at the indicated virus concentrations. Cell survival was determined by using MTT. Percentage of survived cells was calculated setting non-infected cells (mock) as 100%. Average ± standard deviation of three independent MTT-assays is displayed. Experiments were performed in quadruplicates.

### 4.1.3 E1A protein expression is reduced in Ad5/3 CXCR4 UTR infected cells

To determine how the integration of the 5'UTR sequence into the Ad5 genome altered the Ad5 E1A protein expression, E1A protein concentrations were analyzed in infected MDA-MB-231 cells by immunoblot analyses. Cells were infected with 1 MOI of the indicated viruses, harvested 5 days post infection and E1A protein was detected using a specific anti-Ad5 E1A antibody. It was expected that the gain of security would result from reduced E1A translation due to the insertion of the 5'UTR sequence in normal cells and would be less in breast cancer cell lines. Indeed, in normal cell lines, human fibroblasts (NHDF) and HMECs, the E1A protein expression of Ad5/3 CXCR4 UTR was nearly absent compared to Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4, as seen in Figure 9B. In contrast, Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 displayed nearly the same high expression of E1A protein in NHDFs and in HMECs. In breast cancer cell lines the Ad5 E1A protein of Ad5/3 CXCR4 UTR was detectable after infection (Figure 9 A). However, compared to the very high E1A expression in Ad5/3 WT and Ad5/3 CXCR4 infected breast cancer cells, the E1A protein concentration of Ad5/3 CXCR4 UTR was significantly reduced. Ad5 WT expression of E1A protein was only slightly reduced compared to Ad5/3 WT and Ad5/3 CXCR4 infected breast cancer cells, but significantly increased compared to Ad5/3 CXCR4 UTR (Figure 9A). The highest expression of Ad5/3 CXCR4 UTR E1A was detectable in infected MDA-MB-231 cells. These results reflected the CV staining and MTT-Assays in which the oncolytic activity of Ad5/3 CXCR4 UTR was higher in MDA-MB-231 cells at 1 MOI compared to HMEC and NHDF, in which the oncolytic activity was only detectable at 10 MOI.

To summarize, Ad5 E1A protein expression was reduced in Ad5/3 CXCR4 UTR infected breast cancer cells more than expected.

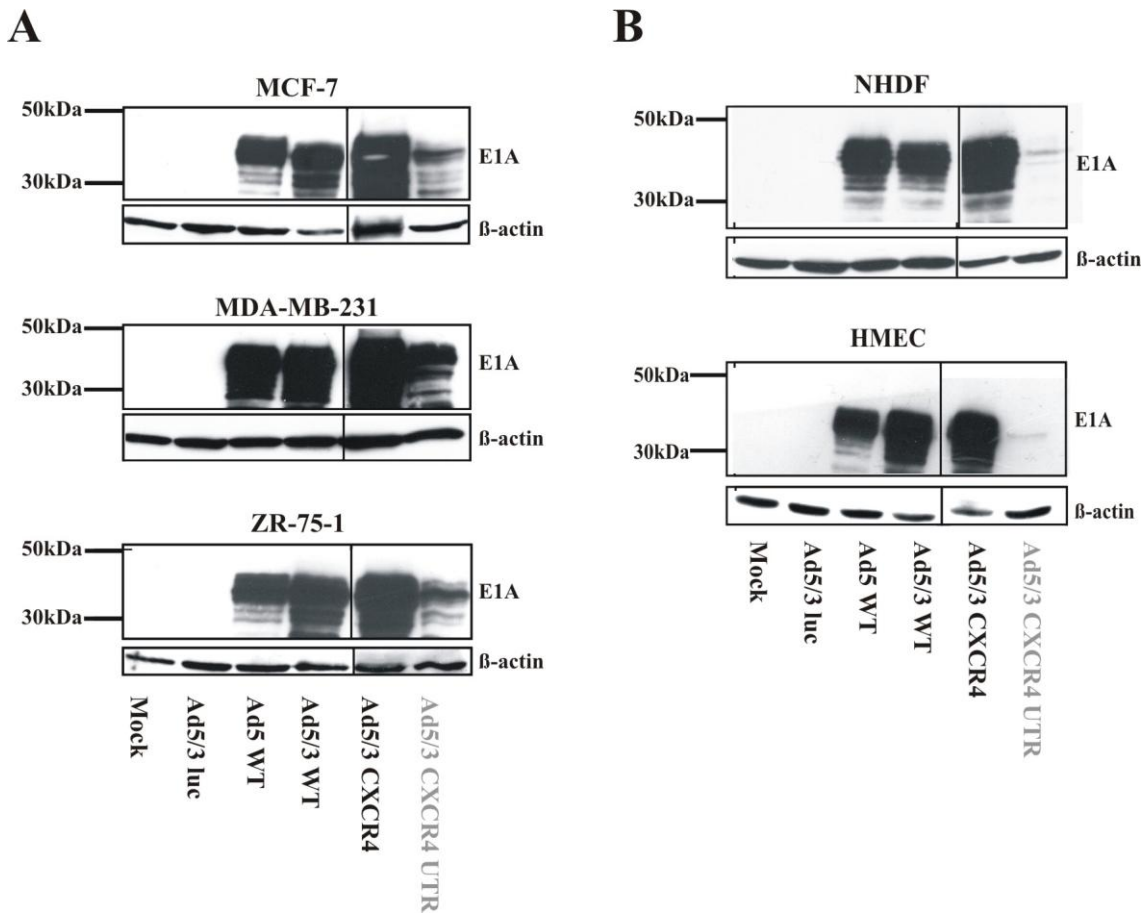


**Figure 9: Decreased Ad5 E1A protein levels in Ad5/3 CXCR4 UTR infected cells.**

**A)** Breast cancer cell lines MDA-MB-231, MCF-7 and ZR-75-1 and **B)** Normal cell lines human fibroblasts and HMEC. Cells were infected with Ad5/3 luc (non-replicating Ad), Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR at a concentration of 1 ifu per cell. Protein samples were prepared after 5 days of infection, proteins were then separated on a 10% SDS-PAGE and protein levels of Ad5 E1A were finally determined performing immunoblot analyses with a specific anti Ad5 E1A antibody. Detection of β-actin served as loading control. These results are representative data of three independent immunoblot experiments.

### 4.1.4 Replication efficiency is decreased in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells

Protein expression of E1A was relatively low in Ad5/3 CXCR4 UTR infected breast cancer cells. To confirm impairment in Ad replication, the replication efficiency of Ad5/3 CXCR4 UTR infected cells was examined. Replication efficiency of Ads can be detected by analyzing viral genome copies in the supernatant of infected cells in a time course. Based on the number of viral DNA copies detected in the supernatant, it is possible to determine how efficiently progeny virions are released from the infected cells. This method is usually used to analyze if modifications within the viral genome are interfering with the assembly and release of progeny virions.

In the following experiment, the human breast cancer cell line MDA-MB-231 was infected with 10 MOI of the indicated viruses and supernatant was collected daily for 4 days after infection. Viral DNA copy numbers in the supernatant were determined using qRT-PCR. As shown in Figure 10, replication efficiency of Ad5/3 CXCR4 UTR was significantly reduced compared to the one of Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4. At day 2 the copy numbers started to increase for Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 (Figure 10). The release of progeny virions in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells started to increase at day 3. At 4 days after the infection, the concentration of virions present in the supernatant of Ad5/3 CXCR4 UTR infected MDA-MB-231 cells was drastically increased in the supernatant compared to day three.

In summary, the release of progeny virions demonstrated a lag-phase in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells indicating that the replication cycle of Ad5/3 CXCR4 UTR was inhibited during early infection. This experiment confirmed the previous results of reduced E1A gene transcription and decreased oncolytic activity of Ad5/3 CXCR4 UTR in breast cancer cell lines.

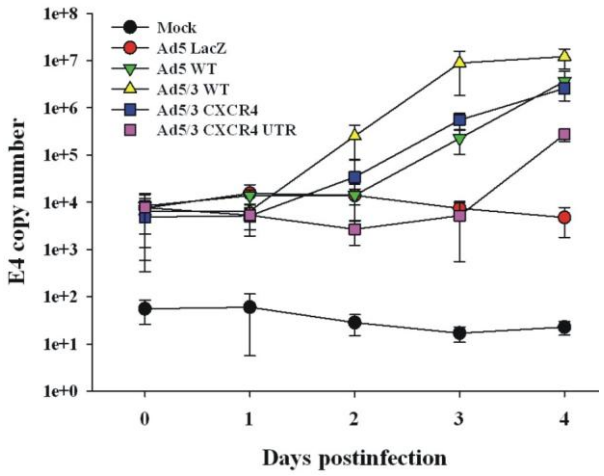


**Figure 10: Ad5/3 CXCR4 UTR showed a lag phase in replication efficacy.**

MDA-231-MB cells were infected with Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4, Ad5/3 CXCR4 UTR and a non-replicating virus (Ad5 LacZ) at a viral concentration of 10 MOI. Growth medium was collected at days 0, 1, 2, 3 and 4 post infection. DNA was isolated and E4 copy number was quantified by qRT-PCR. Experiment was repeated twice and triplicates were measured with the qRT-PCR of each sample. Average values ± standard deviation is displayed in the figure.

### 4.1.5 Different titration method does not reveal inaccurate relative Ad titers

Determining Ad titers of the viral stock solutions is an important step in the field of virotherapy. Titrating of Ad stocks can be performed using different techniques divided into two categories: quantification of either infectious or physical particle titer. One of the widely used methods is the determination of infectious units using the embryonic kidney cell line HEK-293. However, titration for infectious units of Ad using HEK-293 cells produces a disadvantage for Ad vectors that are modified in the fiber knob region. This is because binding of fiber-modified Ads to a different cellular receptor that have a lower expression on HEK-293 cells compared to CAR could result in an inaccurate ifu concentration of the assayed Ad vector. The HEK-293 cells were initially immortalized by transfecting these cells with the Ad5 genome. The left arm of the Ad5 genome was incorporated into the chromosome 19 of HEK-293 cells during this process; therefore this cell line provides several Ad proteins in *trans*. The expression of these proteins can lead to a second disadvantage to determine the real infectious unit of a modified oncolytic Ad vector. A different technique to titer a viral stock solution is to analyze the viral DNA copy/particle number by performing qPCR (Heim et al., 2003). Determining Ad titers using qPCR has several advantages: it is a reproducible and sensitive method, and it is cell line and receptor independent. However, a major disadvantage of this method is that it does not distinguish between vectors with incomplete Ad assembly and non-encapsidated DNA. Therefore, the results of the qPCR may not present an infectious titer.

To examine whether titration for infectious units using HEK-293 cells resulted in an inaccurate titer of Ad5/3 CXCR4 UTR, viral copy number of viral stocks was determined using qRT-PCR. Viral DNA was isolated from viral stocks and then viral E4 gene copy number was determined by performing qPCR. After quantification of viral copy number, MDA-MB-231 cells were infected with the indicated MOIs of a non-replicating Ad5 (Ad5 LacZ), with replicating Ads (Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR), or were mock infected. In this analysis, CV staining was performed to monitor oncolytic activity. The cells were infected with a higher viral copy number per cell than infectious units because quantification of DNA copy numbers also included the non-encapsidated DNA and partially replicated DNA. Therefore, MDA-MB-231 cells were infected with higher MOIs to reach the same result as observed when ifu titers were used to infect the cells.

As presented in Figure 11, Ad5/3 CXCR4 UTR exhibited a diminished oncolytic activity in MDA-MB-231 cells compared to Ad5/3 WT and Ad5/3 CXCR4 infecting the cells with viral

copy number whereas on the other hand it showed the same oncolytic activity as Ad5 WT. Both Ad5 WT and Ad5/3 CXCR4 UTR demonstrated cytotoxicity above 20 MOI. On the other hand, infection of MDA-MB-231 cells with viral copy numbers of Ad5/3 WT and Ad5/3 CXCR4 displayed an equal oncolytic activity for both viruses at 0.2 MOI (Figure 11).

Taken together, the two different titration methods resulted in a similar oncolytic activity of Ad5/3 CXCR4 UTR in the breast cancer cell line MDA-MB-231. These results indicated that titration for infectious units did not result in an inaccurate relative titer of the viral stocks.

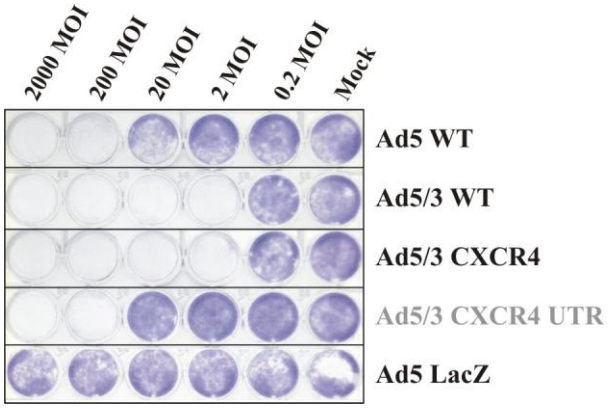


**Figure 11: Reduced oncolytic activity of Ad5/3 CXCR4 UTR in MDA-MB-231 upon viral copy number titer infection.**

MDA-MB-231 cells were infected with Ad5 LacZ (non-replicating Ad), Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR at the indicated titers of viral copy number per cell. Oncolytic activity was monitored 2 weeks after infection, staining viable cells with 0.1% CV. Shown is a representative result of three independent experiments.

### 4.1.6 Viral cell entry of Ad5/3 CXCR4 UTR is comparable to the one of Ad5/3 WT and Ad5/3 CXCR4

Titration of viral stock solutions with a different method could not reveal the reason for the reduced replication efficiency and E1A protein expression in Ad5/3 CXCR4 UTR infected breast cancer cells. To exclude that an impaired viral cell entry caused the delayed viral replication and E1A protein expression, viral cell entry of Ad5/3 CXCR4 UTR was investigated. Consequently, MDA-MB-231 breast cancer cells were infected with the indicated viral concentrations of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR or mock infected. At 1 h and 3 h after addition of the viruses, cells were collected and DNA was isolated. E4 copy number was then determined in 100 ng of total cellular DNA using qPCR.

As shown in Figure 12, viral cell entry efficiency of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR was comparable after 1 h and 3 h of viral addition for all viral concentrations. In addition, the concentration of E4 copy number in total cellular DNA was increased 3 h after viral addition compared to the concentrations seen after 1 h (Figure 12A and B). The lowest viral entry was detectable in the lowest viral concentration and increased with increasing viral concentration. Viral cell entry of Ad5 WT on the other hand, was reduced compared to the other three viruses as displayed in Figure 12A and B. Ad5 WT demonstrated nearly the same level of viral cell entry efficiency at a concentration of 10 ifu per cell which was seen for the other viruses at a concentration of 1 ifu per cell. In addition, viral cell entry at 100 ifu per cell of Ad5 WT was comparable to the one seen at 10 ifu per cell for Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR (Figure 12A and B). This result was expected due to the lower expression of CAR on the surface of MDA-MB-231 cells compared to the high expression of the Ad3 fiber knob receptor.

Taken together, viral cell entry of Ad5/3 CXCR4 UTR was not altered compared with Ad5/3 WT and Ad5/3 CXCR4 in MDA-MB-231 breast cancer cells and could be excluded as potential explanation for delayed Ad5/3 CXCR4 UTR replication.

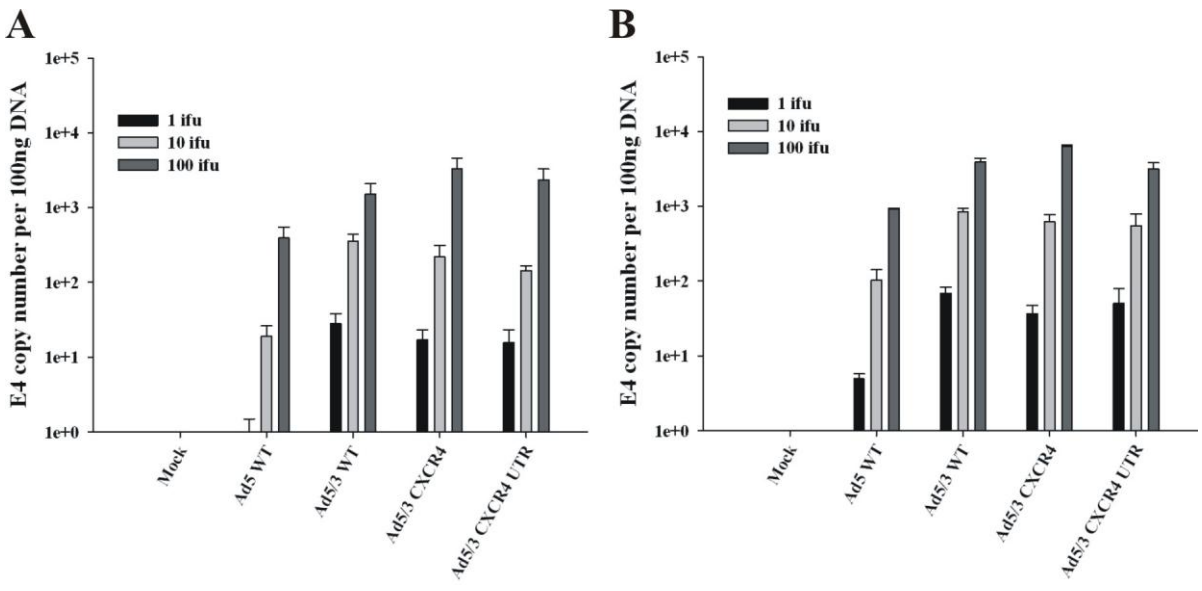


**Figure 12: No impairment of Ad5/3 CXCR4 UTR cell entry in MDA-MB-231 cells.**

Shown are: Ad5 E4 copy numbers of total cellular DNA after **A)** 1 h and **B)** 3 h of viral addition to MDA-MB-231 breast cancer cells. MDA-MB-231 cells were infected with 1 ifu, 10 ifu or 100 ifu per cell of Ad5 WT, Ad5/3 WT, Ad5/3 CXR4 and Ad5/3 CXCR4 UTR and incubated with virus for 1 or 3 h. Cells were harvested and total DNA was isolated. Ad5 E4 copy number was subsequently determined in 100 ng of total cellular DNA employing qPCR. qPCR samples were measured in duplicates and average values ± standard deviation of three independent experiments are displayed.

### 4.1.7 eIF4E protein expression is not dysregulated by Ad3 fiber knob binding

The constructed Ad5/3 CXCR4 UTR revealed a reduced expression of Ad5 E1A gene, as well as replication efficiency and oncolytic activity compared to the dual-level targeted oncolytic Ads (Ad5/3 CXCR4 and Ad5 CXCR4 UTR). Nonetheless, the Ad5/3 CXCR4 UTR demonstrated cancer specificity by maintaining breast cancer oncolytic activity over normal cells. The only difference between the triple-level targeted Ad5/3 CXCR4 UTR and the dual-level targeted oncolytic Ads is the addition of the Ad 3 fiber knob to Ad 5 CXCR4 UTR and addition of the 5'UTR sequence to Ad5/3 CXCR4. Therefore, it was hypothesized that the binding of Ad3 fiber knob to its cellular attachment receptor could activate an intracellular signaling cascade. This cascade could dysregulate eIF4E protein expression, leading to a reduced translation efficiency of E1A in Ad5/3 CXCR4 UTR infected cells. Overexpression of eIF4E in infected cells is essential for efficient translation an E1A mRNA that has incorporated a complex GC rich 5'UTR sequence. Inhibition or repression of eIF4E expression upon Ad3 fiber knob binding could explain the reduced replication efficiency and expression of E1A as seen in Ad5/3 CXCR4 UTR infected breast cancer cells compared to Ad5/3 CXCR4 infected breast cancer cells. Thus, to investigate the underlying mechanism of the reduced translation of Ad5/3 CXCR4 UTR E1A, the eIF4E protein level was analyzed in Ad5 WT and Ad5/3 WT infected breast cancer cells. MDA-MB-231 and MCF-7 cells were infected with 10 ifu of the viruses per cell and cell lysates were collected daily up to 72 h after infection. Immunoblot analyses were performed to detect the eIF4E protein expression. As shown in Figure 13, the eIF4E protein expression was not affected upon the binding of Ad3 fiber knob to its cellular attachment receptor in both breast cancer cell lines. Infection of Ad5 WT or Ad5/3 WT did not alter the eIF4E protein expression at 24 h, 48 h or 72 h after infection. Furthermore, eIF4E protein concentrations were comparable in Ad5 WT infected MDA-MB-231 (Figure 13A) and MCF-7 (Figure 13B) cells compared to Ad5/3 WT infected cells.

Hence, binding of Ad3 fiber knob to breast cancer cells did not induce a signaling cascade that in return interfered with the eIF4E protein expression. This result led to the suggestion that the free concentration of eIF4E might be deregulated due to an impaired phosphorylation of 4E-BP.

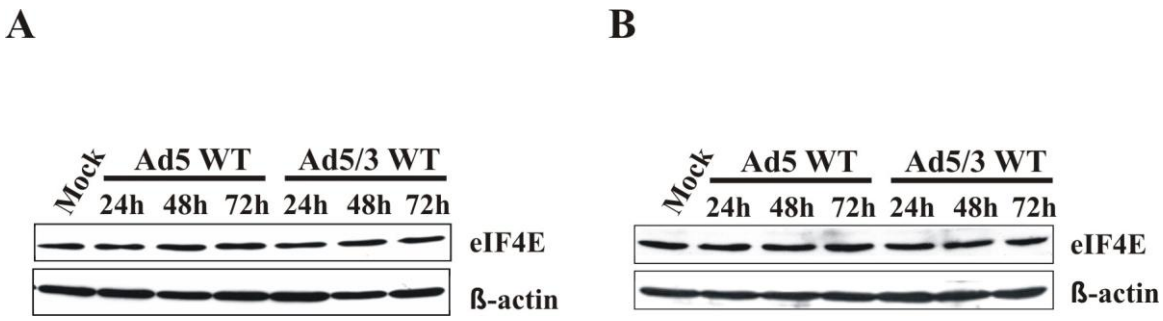


**Figure 13: No influence on the eIF4E protein expression upon Ad5 WT or Ad5/3 WT infection in breast cancer cells.**

Immunoblot analyses of **A)** MDA-MB-231 and **B)** MCF-7 cells infected with 10 ifu per cell of Ad5 WT or Ad5/3 WT are displayed. Cells were collected at the indicated time points after infection, protein lysates were prepared and 30 µg of protein was subjected to a 10% SDS-PAGE. eIF4E protein expression was monitored using a specific eIF4E antibody. B-actin served as a loading control. Shown is a representative data of three independent experiments.

### 4.1.8 Phosphorylation of 4E-BP1 is delayed in Ad5/3 CXCR4 UTR infected breast cancer cells

The phosphoprotein eIF4E is present at a limiting quantity within a normal cell and crucial for translation initiation (Duncan et al., 1987). Usually eIF4E activity is regulated by the 4E-BP by binding eIF4E and therefore inactivating it (Fadden et al., 1997; Pause et al., 1994). Phosphorylation of 4E-BP on several sites is diminishing the interaction with eIF4E leading to the release of eIF4E and resulting in enhanced translation initiation (Fadden et al., 1997; Pause et al., 1994). Upon release from 4E-BP, eIF4G can bind to eIF4E and form eIF4F essential for translation initiation. The ras-ERK and PI3 kinase/Akt pathway are both involved in the phosphorylation of 4E-BP at multiple sites (Gingras et al., 2001). It was shown in the past, that upon Ad5 infection phosphorylation of 4E-BP1 and 4E-BP2 was increased resulting in the liberation of eIF4E and formation of the translation initiation complex (Gingras and Sonenberg, 1997). Furthermore, the authors could display that phosphorylation of 4E-BP was impaired upon deletion or mutation of the Ad5 E1A gene suggesting a role of Ad5 E1A in the 4E-BP phosphorylation upon Ad5 infection (Gingras and Sonenberg, 1997). In addition, it was published that Ad5 binding and infection activates the PI3K pathway and therefore, inducing phosphorylation of 4E-BP (Li et al., 1998).

Based on those publications it was hypothesized that binding of Ad3 fiber knob to its cellular attachment receptor is interfering with the phosphorylation of the 4E-BP and therefore reducing the translation of E1A in Ad5/3 CXCR4 UTR infected cells. A reduced concentration

of free eIF4E could diminish the translation of the Ad5/3 CXCR4 UTR E1A gene containing the long GC-rich UTR-sequence of rat FGF-2. Immunoblot analyses detecting eIF4E protein expression after infection revealed that binding of Ad3 fiber knob did not influence the eIF4E protein expression. Thus, it was speculated that if Ad3 fiber knob binding would inhibit phosphorylation of 4E-BP, the concentration of free eIF4E would be reduced and in return the E1A gene expression of Ad5/3 CXCR UTR.

Consequently, the phosphorylation level of 4E-BP1 was analyzed in Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 UTR infected MCF-7 and MDA-MB-231 breast cancer cells. Cells were infected with 50 ifu per cell and harvested at the indicated time points post infection. Subsequently, a co-immunoprecipitation for eIF4E was performed to determine the level of unphosphorylated 4E-BP still bound to eIF4E. The different phosphorylation forms of 4E-BP1 can be displayed by using a specific anti 4E-BP antibody. Hyperphosphorylated forms (γ and δ) demonstrate a slow mobility on SDS-PAGEs compared to the unphosphorylated (α) and hypophosphorylated (β) forms (Beretta et al., 1996; Fadden et al., 1997; Gingras et al., 1996; Pause et al., 1994).

As shown in Figure 14A, only two bands of 4E-BP1 were detected, indicating that the co-immunoprecipitation worked and that only unphosphorylated 4E-BP forms were precipitated together with the eIF4E. Unphosphorylated 4E-BP1 concentration started to decrease after 6 h of Ad5 WT addition to MDA-MB-231 cells. In contrast to this result, infection with Ad5/3 WT induced phosphorylation of 4E-BP1 already at 3 h after viral addition. Unphosphorylated 4E-BP1 was nearly absent after 24 h of Ad5/3 WT addition on MDA-MB-231 cells. Infection of MDA-MB-231 cells with Ad5/3 CXCR4 UTR displayed the same time dependent phosphorylation as Ad5/3 WT but demonstrating a lag phase of 3 h. At 24 h after addition of virions, unphosphorylated 4E-BP1 was still detectable in Ad5/3 CXCR4 UTR infected cells whereas it was completely absent in Ad5/3 WT infected cells (Figure 14A). In MCF-7 cells on the other hand, infection with Ad5 WT did not demonstrate a phosphorylation of 4E-BP due to the detection of the same concentration 24 h after viral addition compared to the one seen at time point zero (Figure 14B). However, infection with Ad5/3 WT and Ad5/3 CXCR4 UTR showed a decline in the unphosphorylated forms of 4E-BP1 after 6 h of viral addition (Figure 14B).

To summarize, phosphorylation of 4E-BP1 upon infection with Ad5/3 CXCR4 UTR was delayed compared to infection with Ad5/3 WT but displayed an improved phosphorylation of 4E-BP compared to Ad5 WT infected breast cancer cells. Therefore, delayed phosphorylation

of 4E-BP1 in Ad5/3 CXCR4 UTR could be explained by the suggestion that Ad5 E1A is involved in the initiation of 4E-BP phosphorylation upon Ad infection (Gingras and Sonenberg, 1997).

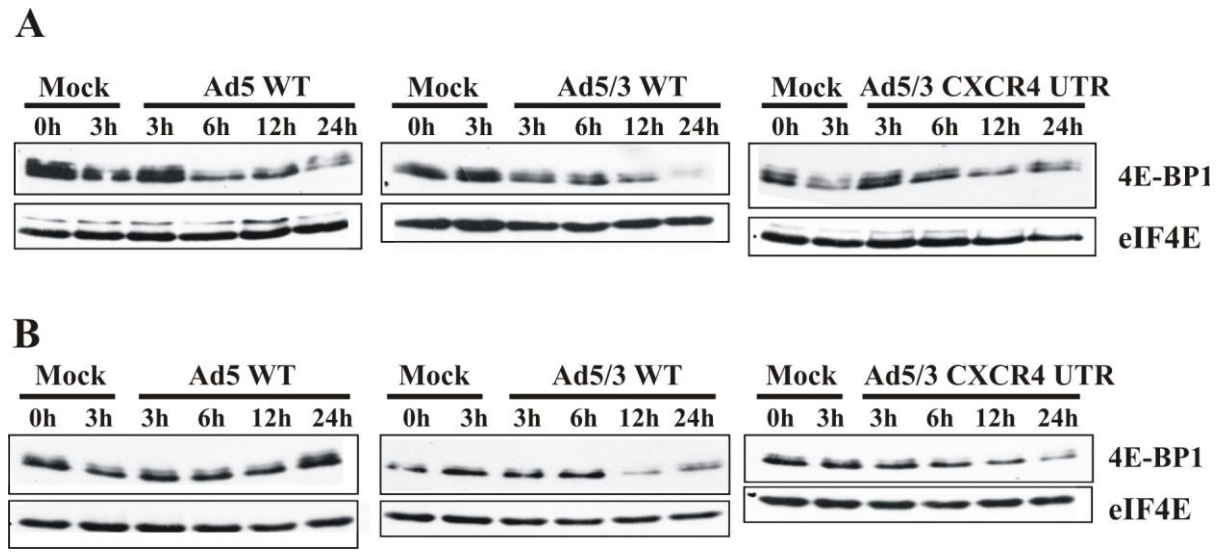


**Figure 14: 4E-BP phosphorylation was time dependent in Ad5/3 CXCR4 UTR infected breast cancer cells.**

**A)** MDA-MB-231 and **B)** MCF-7 cells were infected with 50 ifu per cell of Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 UTR. Cells were collected at indicated time points after the addition of Ads on the breast cancer cell lines. Protein lysates were prepared and 800 µg of protein was used to perform a co-immunoprecipitation against eIF4E. Samples were then subjected to a 15% SDS-PAGE and immunoblot analyses were performed to detect non-phosphorylated 4E-BP1 with a specific antibody against 4E-BP1. eIF4E served as a loading control. Each figure represents a representative of two individual performed experiments.

### 4.1.9 Delayed expression of E1A and pTP of Ad5/3 CXCR4 UTR in MDA-MB-231 cells

The eIF4E protein expression and phosphorylation of 4E-BP were not altered upon the Ad3 fiber knob binding as hypothesized and therefore not likely responsible for the decreased E1A expression. Detection of a reduced E1A protein concentration and delayed release of progeny virions of Ad5/3 CXCR4 UTR in breast cancer cell lines indicated an impaired E1A gene transcription. To determine whether E1A gene transcription was decreased, E1A mRNA expression levels were quantified by qRT-PCR in infected MDA-MB-231 cells. Cells were infected with 10 MOI of the indicated viruses and harvested 48 h post infection. Subsequently, RNA was isolated and concentration of E1A mRNA was determined by performing qRT-PCR. As seen in Figure 15, the E1A mRNA expression of Ad5/3 CXCR4 UTR was significantly

decreased in MDA-MB-231 cells compared to Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4. Ad5/3 WT and Ad5/3 CXCR4 displayed a higher E1A gene transcription than Ad5 WT, indicating a higher transcription efficiency of Ad5/3 WT and Ad5/3 CXCR4. However, E1A gene transcription of Ad5/3 CXCR4 UTR was increased compared to the non-replicating Ad5 LacZ. This result showed that Ad5 E1A transcription is reduced compared to Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4, but still present within the breast cancer cell.

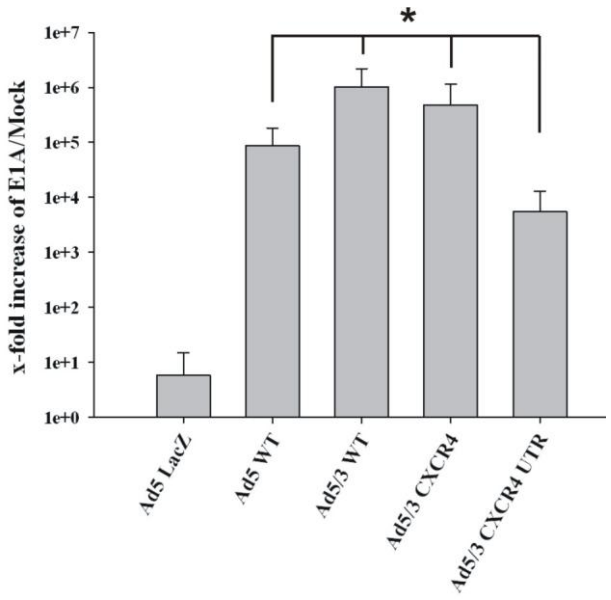


**Figure 15: Significant reduction of Ad5/3 CXCR4 UTR E1A mRNA levels in MDA-MB-231 cells.**

MDA-MB-231 breast cancer cells were infected with Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4, Ad5/3 CXCR4 UTR and non-replicating Ad5 LacZ at a virus concentration of 10 ifu per cell. Cells were collected 48 h post infection, RNA was isolated and reverse transcribed into cDNA. E1A levels were then analyzed via qRT-PCR. The experiment was repeated twice and triplicates of cDNA samples were evaluated by qRT-PCR. *, $p < 0.05$ Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 versus Ad5/3 CXCR4 UTR.

To further investigate the decreased transcription of E1A gene, the Ad5 E1A and pTP mRNA levels were investigated at different time points after infection. A time course could reveal whether Ad5 E1A gene expression is present at the beginning of Ad5/3 CXCR4 UTR and is then inhibited during the process of Ad replication or if Ad5 E1A gene expression is inhibited right from the beginning. Ad5 E1A is the earliest gene that is expressed during Ad5 infection cycle. Ad5 E1A protein is necessary to induce transcription of the early genes that are located downstream of the E1A gene (Berk et al., 1979) and essential to stimulate the cell gene expression and proliferation as modulator. Thus, with an impaired or reduced Ad5 E1A transcription, the whole Ad5 replication pathway is also affected. The transcription of the Ad5 E2 gene is also initiated by Ad5 E1A. Ad5 E2 transcription is necessary to start Ad DNA

replication because Ad5 E2B encodes for the proteins Ad Pol and Ad pTP. Both proteins form a stable heterodimer that is critical for initiation of viral genome replication (Liu et al., 2003).

In this experiment, the transcription levels of Ad5 E1A and pTP genes were analyzed to investigate Ad5 genome replication. MDA-MB-231 cells were infected with 10 ifu per cell of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR or mock infected. Cells were collected at the indicated time points after viral infection and RNA was isolated. The mRNA levels of Ad5 E1A and pTP were then analyzed performing qRT-PCR and results were normalized to human GAPDH levels. As seen in Figure 16 at time point 0 (2 h after addition of virus) Ad5 E1A mRNA levels were already slightly elevated in MDA-MB-231 cells infected with Ad5/3 WT compared to Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR. At 6 h post infection Ad5 E1A expression started to increase in Ad5/3 WT and Ad5/3 CXCR4 infected MDA-MB-231 cells while it decreased in Ad5/3 CXCR4 UTR infected cells (Figure 16). Following infection, Ad5 E1A mRNA levels steadily increased in Ad5/3 WT and Ad5/3 CXCR4 infected MDA-MB-231 cells at a similar rate, showing nearly the same E1A mRNA concentration after 48 h of infection. Ad5/3 CXCR4 UTR on the other hand showed a 24 h lag phase in E1A expression that started to increase 24 and 48 h after the infection with Ad5/3 CXCR4 UTR. A comparable time dependent mRNA transcription rate between the viruses as seen for Ad5 E1A was observed for Ad5 pTP (Figure 16). At 12 h post infection the expression of Ad5 pTP increased in Ad5/3 WT and Ad5/3 CXCR4 infected MDA-MB-231 cells at an equal rate whereas pTP expression was delayed in Ad5/3 CXCR4 UTR infected cells. The pTP mRNA concentration increased around 36 h post infection in MDA-MB-231 cells infected with Ad5/3 CXCR4 UTR.

In summary, these results demonstrated that Ad5/3 CXCR4 UTR E1A and pTP mRNA transcription was delayed in MDA-MB-231 cells compared to Ad5/3 WT and Ad5/3 CXCR4. This result showed that the delayed expression of E1A reduces the replication efficiency of Ad5/3 CXCR4 UTR in breast cancer cells.

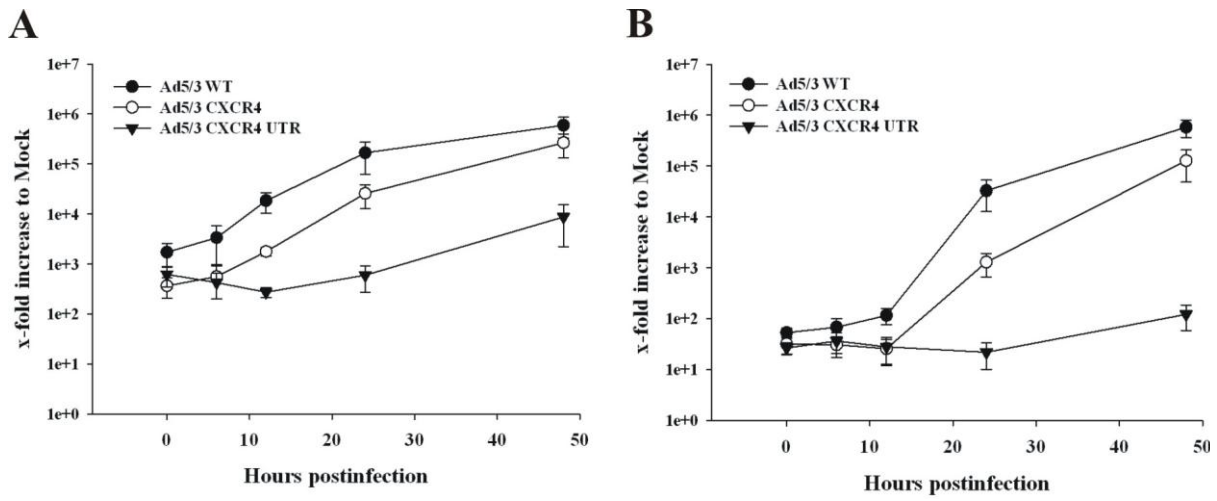


**Figure 16: Ad5/3 CXCR4 UTR displayed a lag phase in E1A and pTP gene transcription.**

**A)** E1A mRNA and **B)** pTP mRNA level were analyzed by qRT-PCR of infected MDA-MB-231 cells. Cells were infected with 10 ifu per cell of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR and 0 h, 6 h, 12 h, 24 h and 48 h after infection cells were harvested, RNA was isolated and 0.6 µg of mRNA were then transcribed into cDNA, which was analyzed in triplicates by qRT-PCR. Figure shows the average value ± standard deviation of three independent experiments.

### 4.1.10 E1A mRNA half life is not altered in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells

After investigating that Ad5 E1A transcription was repressed early in Ad5/3 CXCR4 UTR infection, it was hypothesized that E1A mRNA might be degraded early during the Ad infection cycle. This degradation could be activated upon Ad3 fiber knob binding. After a period of time induced degradation of E1A mRNA would diminish and E1A expression could be initiated. To examine this hypothesis, the E1A mRNA half life in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells was analyzed and compared to E1A mRNA half lives in Ad5/3 WT and Ad5/3 CXCR4 infected MDA-MB-231 cells.

Therefore, MDA-MB-231 cells were infected with 100 ifu per cell of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR for 12 h. Subsequently, cells were treated with 10 µg/ml actinomycin D. Actinomycin D is a chemical reagent that intercalates into DNA and blocks RNA synthesis by hindering the RNA polymerase enzyme to elongate RNA chains (Goldberg et al., 1962). E1A mRNA half life was finally quantified performing qRT-PCR. Actinomycin D was dissolved in DMSO. Thus, to exclude the possibility that DMSO itself induced the degradation of Ad5 E1A mRNA or Ad5 pTP mRNA, DMSO alone was added to another set of cells infected with Ad5/3 WT in the same concentration as actinomycin D. These cells served as negative control.

As displayed in Figure 17, the E1A mRNA concentration started to decrease after 2 h of incubation with actinomycin D for all three viruses. No difference was observed in the degradation rate of E1A mRNA of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR (Figure 17). Upon actinomycin D treatment the E1A mRNA was degraded for all three viruses at a comparable rate whereas Ad5/3 WT infected cells not treated with actinomycin D demonstrated a time dependent increase of E1A mRNA. This result demonstrated that DMSO alone does not induce Ad5 E1A mRNA degradation and that the effect seen in the treated cells was mediated by actinomycin D.

Taken together, E1A mRNA of Ad5/3 CXCR4 UTR revealed the same half life in MDA-MB-231 cells compared to Ad5/3 WT and Ad5/3 CXCR4. Thus, degradation of Ad E1A mRNA that is activated by Ad3 fiber knob binding was not induced and cannot explain the repression of E1A transcription in Ad5/3 CXCR4 UTR infected breast cancer cells.

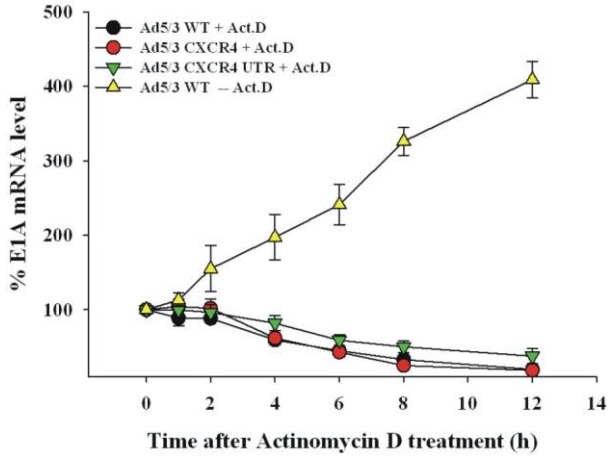


**Figure 17: The E1A mRNA half life of Ad5/3 CXCR4 UTR was not impaired in MDA-MB-231 cells.**

MDA-MB-231 cells were infected with 100 ifu per cell of Ad5/3 WT, Ad5/3 CXCR4 or Ad5/3 CXCR4 UTR. 12 h post infection cells were treated with 10 µg/ml actinomycin D and collected at the indicated time points. For each time point MDA-MB-231 cells infected with Ad5/3 WT and not treated with actinmycin D were collected as a negative control. RNA was isolated, transcribed into cDNA and half life was measured with qRT-PCR. Samples were measured in triplicates. Average values ± standard deviation of three independent experiments are shown. Values were normalized to background levels of E1A in samples containing no reverse transcriptase. Results are expressed as percentage of the results after addition of actinomycin D relative to time point 0 h with the E1A mRNA concentration at 0 h set as 100%.

### 4.1.11 Efficient cytotoxicity of Ad5/3 CXCR4 UTR in HEK-293 cells

As previously mentioned, HEK-293 cells contain the left arm of the Ad5 genome incorporated into its chromosome 19. Recombinant Ads containing restricted expression or deletion of E1A should display a comparable oncolytic activity in this cell line as wild-type Ad. It was shown, that transcription of Ad5/3 CXCR UTR was repressed early during infection and after a 24 h lag phase transcription was initiated. This result suggested that the repressed E1A gene expression is the limiting factor in the Ad5/3 CXCR4 UTR infection pathway. Thus, it was proposed that Ad5/3 CXCR4 UTR should demonstrate comparable oncolytic potency to Ad5/3 WT or Ad5/3 CXCR4 in HEK-293 cells because these cells express the E1A gene and several other genes of the Ad5 genome necessary to support Ad replication.

Therefore, HEK-293 cells were infected with indicated MOIs of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR and cytolytic activity of viruses was monitored using CV staining. As seen in Figure 18, Ad5/3 CXCR4 UTR and Ad5/3 WT demonstrated the same cytolytic potency in HEK-293 cells, displaying cell killing at the lowest concentration of 0.01 MOI. In addition, both mediated killing in HEK-293 cells more efficiently than Ad5 WT and Ad5/3 CXCR4, suggesting that the additional expression of specific Ad5 proteins such as the E1A protein, are necessary to increase the Ad5/3 CXCR4 UTR oncolytic activity for breast cancer cells. Transcription of E1A is a crucial step in the Ad replication pathway since E1A initiates the transcription of the other early genes which are essential for adenoviral genome replication.

Taken together, Ad5/3 CXCR4 UTR showed an increased cytolytic activity in HEK-293 cells compared to breast cancer cells. This result suggested that expression of Ad5 WT E1A gene induced the transcription efficiency of Ad5/3 CXCR4 UTR, causing increased cytolytic activity.

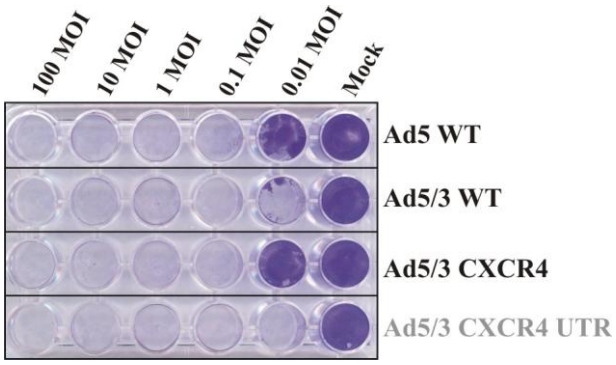


**Figure 18: Increased oncolytic activity of Ad5/3 CXCR4 UTR in HEK-293 cells.**

HEK-293 cells were infected with the indicated titers of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR. One week after infection, cells were stained with 0.1% CV to monitor oncolytic activity. This figure is a representative of three independent experiments.

To confirm the hypothesis whether the stable expression of Ad5 WT E1A in HEK-293 cells induced the enhanced cytolytic activity of Ad5/3 CXCR4 UTR, it was investigated if the stable expression in MDA-MB-231 cells would result in the same outcome. Meric et al. established the MDA-MB-231/E1A cell line (Meric et al., 2000), expressing Ad5 WT E1A and the MDA-MB-231/N cell line transfected with an empty vector and served as control cell line. Hence, MDA-MB-231/E1A and MDA-MB-231/N cells were infected with indicated MOIs of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR. Oncolytic activity was monitored by staining viable cells with CV.

Immunoblot analyses of protein lysates from MDA-MB-231/E1A and MDA-MB-231/N revealed Ad5 WT E1A expression in MDA-MB-231/E1A while this expression was absent in MDA-MB-231/N cells transfected with the empty vector (Figure 19A). Unexpectedly, the presence of the Ad5 WT E1A protein in MDA-MB-231 cells did not enhance the oncolytic potency of Ad5/3 CXCR4 UTR compared to the one of Ad5/3 WT and Ad5/3 CXCR4. In fact, transfected MDA-MB-231 cells were somehow less sensitive to Ad5/3 CXCR4 UTR and Ad5 WT infection (Figure 19B and C) compared to non-transfected MDA-MB-231 cells (Figure 7). Furthermore, Ad5/3 CXCR4 UTR displayed a higher oncolytic activity in MDA-MB-231/E1A cells compared to MDA-MB-231/N cells. Oncolytic activity of Ad5 WT was only detectable at the highest concentration of 100 MOI in MDA-MB-231/N cells. However, no difference in the oncolytic activity was observed between Ad5/3 WT and Ad5/3 CXCR4 in MDA-MB-231/E1A or MDA-MB-231/N infected cells. In both cell lines cell killing of Ad5/3 WT was detectable at a concentration of 0.01 MOI and for Ad5/3 CXCR4 at 0.1 MOI (Figure 19B and C).

To summarize, Ad5 WT E1A protein expression in MDA-MB-231 cells was not sufficient to restore the oncolytic activity of Ad5/3 CXCR4 UTR compared to the level seen in HEK-293 cells.

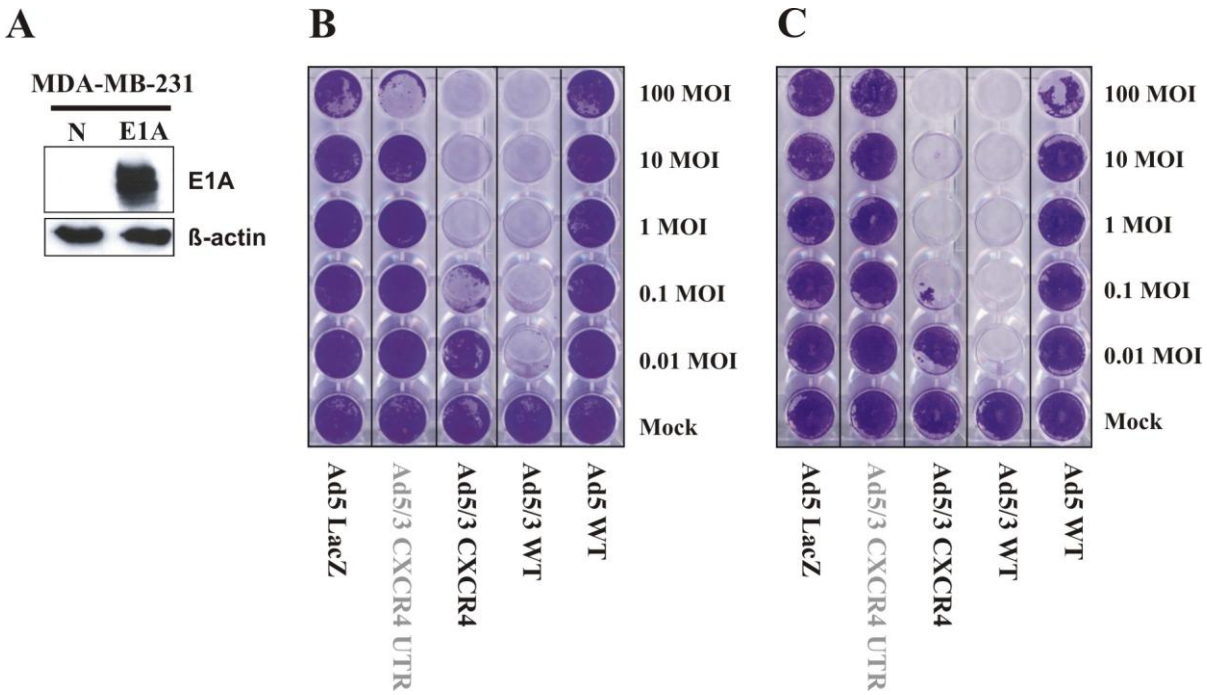


**Figure 19: Stable expression of E1A did not enhance oncolytic potency of Ad5/3 CXCR4 UTR.**

**A)** Western Blot analysis of MDA-MB-231 stable transfected with either Ad5 E1A gene (MDA-MB-231/E1A) or an empty vector (MDA-MB-231/N). Cells were harvested, protein lysates were prepared and then 30µg of protein was subjected to a 10% SDS-PAGE. Detection of Ad5 E1A protein was performed with an antibody specific for Ad5 E1A. B-actin served as a loading control. Crystal violet analyses of infected **B)** MDA-MB-231/E1A and **C)** MDA-MB-231/N cells. Cells were infected with the indicated titers of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4, Ad5/3 CXCR4 UTR and the non-replicating Ad5 LacZ. Oncolytic activity was determined staining viable cells with 0.1% CV. These figures are representatives of three independent experiments.

One disadvantage of titering fiber knob modified Ad vectors on cell lines is the expression pattern of the compatible cellular attachment receptor. A disadvantage to titer Ad vectors containing a transcriptional or translational control element on HEK-293 cells is the incorporated left arm of the Ad5 genome that supports Ad replication and abolishes the restriction incorporated into the genome. It was proposed that titration for infectious units of Ad5/3 CXCR4 UTR on a different cell line that is not stably expressing Ad proteins, would result in a different ifu concentration of the viral stocks. Therefore, viral stocks of Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR were quantified using A549 cells to analyze whether ifu titers would be different compared to the ones titered using HEK-293 cells.

Ifu titers quantified on A549 cells were nearly the same for Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4 compared to the ones determined on HEK-293 cells. The only exception was the ifu concentration for Ad5/3 CXCR4 UTR. Ifu concentration of Ad5/3 CXCR4 UTR was significantly reduced after titration on A549 cells, about three potencies (not shown). Next, MDA-MB-231, MDA-MB-231/N and MDA-MB-231/E1A breast cancer cells were infected with the indicated viral concentrations and oncolytic activity was investigated by staining viable cells with CV solution. An enhanced oncolytic potency of Ad5/3 CXCR4 UTR was obtained in all three cell lines (Figure 20) compared to the oncolytic potency observed with ifus titered on HEK-293 cells (Figure 7 and Figure 19). Oncolytic activity of Ad5/3 CXCR4 UTR was detectable until the concentration of 0.01 MOI. Furthermore, Ad5/3 CXCR4 UTR killed breast cancer cells with nearly the same efficiency as Ad5/3 WT and Ad5/3 CXCR4 and with a higher efficiency than Ad5 WT (Figure 20). In contrast, Ad5 WT did not display any increase in oncolytic activity in MDA-MB-231/E1A and MDA-MB-231/N cells but showed a high oncolytic activity on control MDA-MB-231 cells. This result indiciated that transfection of this cell line might have altered the receptor expression of CAR on its surface.

In summary, the ifu quantification using A549 cells that do not support Ad5 replication due to stable Ad5 gene expression resulted, in an improved oncolytic activity of Ad5/3 CXCR4 UTR in MDA-MB-231 cells. Thus, this result suggested that HEK-293 cells provide a specific Ad5 protein impaired in Ad5/3 CXCR4 UTR that is essential to obtain efficient Ad replication in cells.

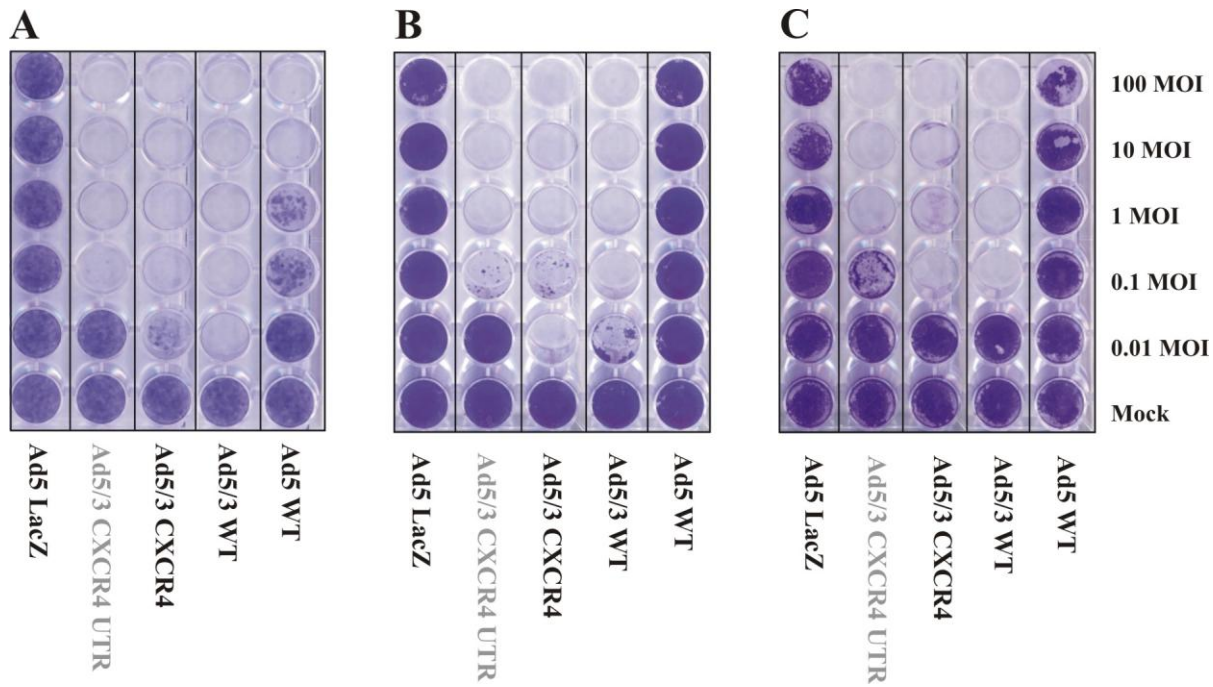


**Figure 20: Titration on A549 cells increased oncolytic activity of Ad5/3 CXCR4 UTR.**

**A)** MDA-MB-231, **B)** MDA-MB-231/E1A and **C)** MDA-MB-231/N cells were infected with Ad5 WT, Ad5/3 WT, Ad5/3 CXCR4, Ad5/3 CXCR4 UTR and Ad5 LacZ (non-replicating Ad) at indicated titers. The ifu concentrations of viral stock solutions were quantified on A549 cells. Viable cells were stained with 0.1% CV to visualize oncolytic potency. Figures are representative data of three independent experiments.

## *4.2 Characterization of the Syrian hamster breast cancer cell line*

The second aim of this study was to establish a novel breast cancer animal model that is permissive for Ad replication and also immunocompetent. Recently, it was reported that the Syrian hamster is permissive for Ad5 replication and immunocompetent (Thomas et al., 2006). Unfortunately, until to date there was no Syrian hamster breast cancer model available to analyze CRAds specifically designed to treat breast cancer. Therefore, mammary tumors were induced in Syrian hamsters upon MNU treatment in our research group (Coburn et al., 2011). Tumor sections stained positive for several breast cancer markers such as HER-2/neu and revealed epithelial origin indicating that developed tumors were originated from mammary epithelial cells. One of the primary tumors was used to establish a Syrian hamster breast cancer cell line in cell culture. Two different morphologic phenotypes of cells were observed after initiating the tumor cells into cell culture, a uniformly round and a more elongated shaped one. Those two cell types were cultivated separately as HMAM4A (round morphology) and HMAM4B (elongated morphology) (Coburn et al., 2011).

### 4.2.1 Syrian hamster breast cancer cells induce tumor formation in syngeneic Syrian hamsters

To analyze whether the established Syrian hamster breast cancer cell line HMAM4B was capable to induce tumor formation, 1 x $10^7$ HMAM4B cells were injected subcutaneously into both hind flanks of syngeneic untreated female Syrian hamsters and tumor formation was then monitored.

After injection of HMAM4B cells *in vivo*, a significant tumor growth rate was monitored with a tumor take rate about 50% (Figure 21A). Furthermore, a lag phase of tumor detection between the time point of HMAM4B injection and tumor development was detected. This lag phase was variable between the different animals and injection sides, and ranged from 15 to 36 days (Figure 21A). Tumors developed after injection of HMAM4B cells were histopathologically analyzed by a pathologist. One of the developed tumors was further passaged into cell culture and the cell line HMAM5 was established. HMAM5 cells were also subcutaneously injected into the hind flanks of syngeneic untreated female Syrian hamsters and tumor formation was monitored. The tumor take rate (around 70%) after injection of the HMAM5 cell line was greater compared to the one after HMAM4B injection (Figure 21A and B). Mean body weight growth characteristics of hamsters (Figure 21C) and tumor volumes *in vivo* (Figure 21B) revealed that after a lag phase of 21 days, tumor growth was similar in all animals until they were sacrificed at the upper limit allowed in the protocol (20% of total body weight).

Immunohistochemical analyses for the molecular markers p63 and cytokeratin were performed on tumors that were induced upon HMAM4B and HMAM5 subcutaneous injection to determine the molecular subtype of the hamster mammary tumors. Breast cancer can be divided into four distinct molecular classifications: luminal A (ER+, PR+, HER2-), luminal B (ER+, PR+, HER2+), basal (ER-, PR-, HER2-) and basal-like HER-2/neu positive (ER-, PR-, HER+). P63 and cytokeratin are molecular markers for basal categorized breast cancers. It was shown that most luminal breast cancer tumors are stained negative for these two markers and basal breast cancer tumors are stained positive (Matos et al., 2005; Ribeiro-Silva et al., 2005). Tumors derived after subcutaneous injection of HMAM4B or HMAM5 cells were negative for both basal molecular markers p63 and cytokeratin 5/6, indicating that those tumors can be categorized as luminal breast cancer tumors (Coburn et al., 2011).

Taken together HMAM4B and HMAM5 Syrian hamster breast cancer cells were able to induce tumor formation in syngeneic untreated female Syrian hamsters upon subcutaneous injection

into the hind flanks. In addition, it was distinguished that mammary tumors developed from MNU-treatment were originated from mammary epithelial cells and can be categorized as luminal breast cancer.

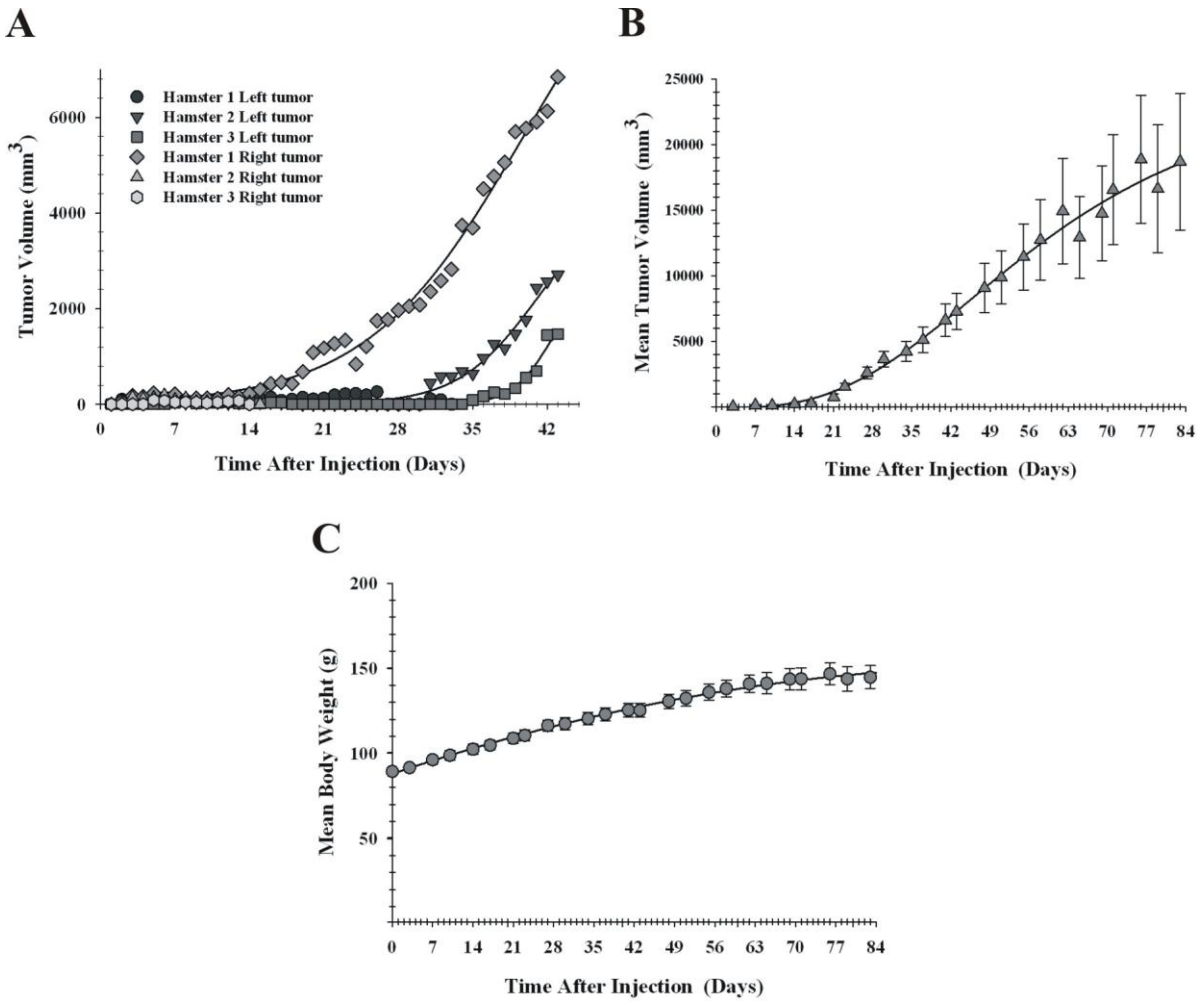


**Figure 21: HMAM4B and HMAM5 cells induced tumor formation in syngeneic female Syrian hamsters.**

Shown are: **A)** Tumor formation after the injection of HMAM4B cells, **B)** mean tumor growth (n =7) at each site after s.c injection of HMAM5 cells and **C)** mean body weight (n = 10) of syngeneic untreated female Syrian hamsters injected with HMAM5 cells. 1 x $10^7$ HMAM4B or HMAM5 cells were s.c. injected into the hind flanks of syngeneic untreated female Syrian hamsters. Tumor growth was monitored from the day of injection with a digital caliper. For injection of HMAM4B cells a total of three animals were injected whereas the animal number for the HMAM5 injection was ten. Two hamsters in which HMAM5 cells were injected were euthanized at day 65 (E1) and at day 79 (E2) due to excessive tumor burden. Each data point in B and C represents the average value ± standard error at each time point.

### 4.2.2 HMAM5 cells displayed anchorage independent growth and 24 h doubling time during exponential growth phase

HMAM4B cells were able to be passaged more than 15 times and HMAM5 cells were even passaged more than 40 times, suggesting that both cell lines were immortal carcinoma cell lines and not passage-limited like primary cells. To determine whether both cell lines have cancerous characteristics several experiments were performed. First, the anchorage independence assay was used to investigate whether the newly established Syrian hamster breast cancer cell lines could grow in soft agar like other breast cancer cell lines. Anchorage independent growth is one of the best properties to study *in vitro* correlation with tumorigenecity (Colburn et al., 1978; Freedman and Shin, 1974; Shin et al., 1975). To proliferate, normal cells require growth factors and cell adhesion to an underground. Cancer cells on the other hand gained the capacity to proliferate without adhesion to an underground. This independent growth is a key marker for tumorigenicity.

Therefore, HMAM4A, HMAM4B, HMAM5 and MDA-MB-231 were plated on soft agar and incubated for three weeks. Anchorage independent growth was quantified by counting colony formation with a cell number greater than approximately 50 cells. HMAM4A, HMAM4B and HMAM5 each displayed an anchorage independent growth on soft agar indicated by the formation of colonies (Figure 22). The number of colonies formed by HMAM5, HMAM4A and HMAM4B was similar to the one detected of the human breast cancer cell line MDA-MB-231, indicating a cancerous character for the established Syrian hamster breast cancer cell lines.

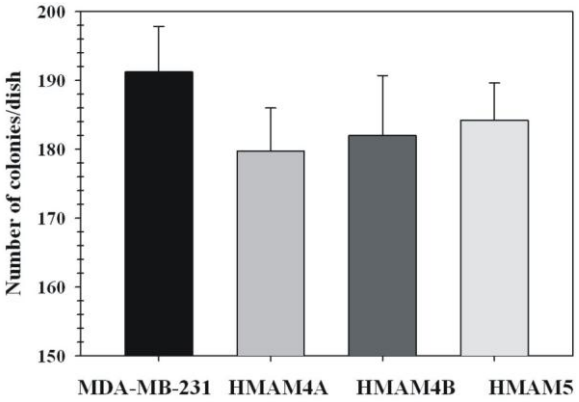


**Figure 22: Syrian hamster breast cancer cell lines showed anchorage independent growth.**

0.5 x $10^4$ cells of human MDA-MB-231, HMAM4A, HMAM4B and HMAM5 cell lines were plated on 0.5% soft agar in complete DMEM containing 10% FBS and incubated at 37°C. Positive colonies greater than 50 cells were counted after three weeks in culture. Each data point represents the average ± standard error of three independent experiments.

Furthermore, cell doubling time of HMAM5 and HMAM4B cells during the exponential phase was quantified by performing a growth curve for both cell lines. 1 x $10^4$ cells were plated and cell number of triplicates was determined daily until 10 to 12 days after plating the cells. Figure 23A presents the growth curve of HMAM5, HMAM4B and MDA-MB-231 cells. Both Syrian hamster breast cancer cell lines displayed a quick growth with a doubling time of approximately 24 h during the exponential phase, which was comparable to the observed growth curve of human MDA-MB-231 cells.

In addition, growth of HMAM5 cells was analyzed in medium containing different percentages of FBS (2%, 5% and 10%). This was examined, to determine whether the growth rate of HMAM5 cells could slowed down by reducing the FBS concentration without any morphological changes of the cells. 1.5 x $10^4$ HMAM5 cells were plated and cell numbers were determined daily in triplicates with a hemocytometer. HMAM5 cells cultured in medium containing 5% or 10% FBS demonstrated a similar growth rate whereas cells cultured in 2% FBS containing medium displayed a slower doubling time (Figure 23B). Furthermore, no morphological changes were detectable between the cells cultured in 10%, 5% or 2% FBS containing media.

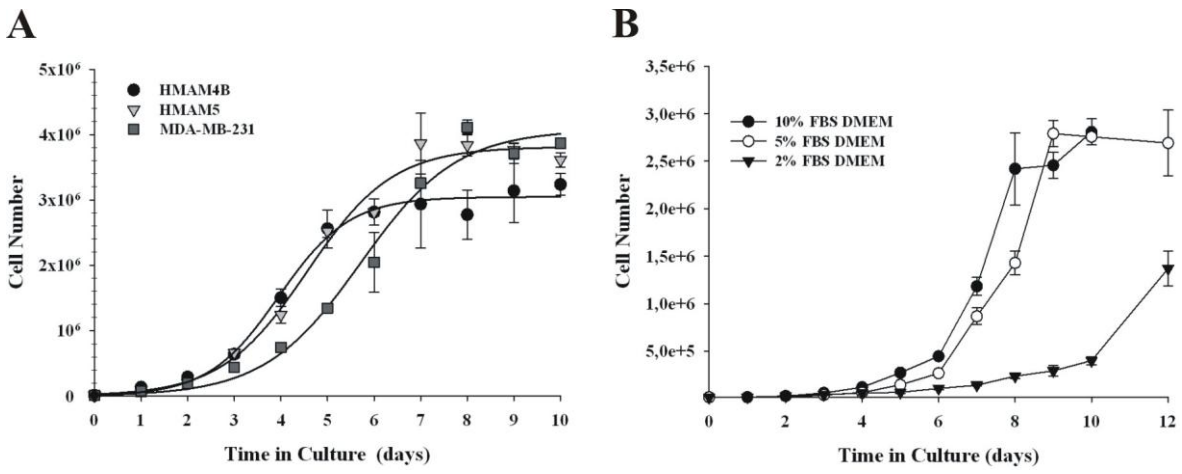


**Figure 23: HMAM5 and HMAM4B cells displayed a 24h doubling time during exponential growth.**

Shown are: **A)** Representative growth curve of HMAM4B, HMAM5 and MDA-MB-231 cells and **B)** representative growth curve of HMAM5 in DMEM containing either 2%, 5% or 10% FBS. 1 x $10^4$ cells were plated to monitor growth rates of the newly established HMAM5 and HMAM4B cell lines compared to MDA-MB-231 cells. To analyze the growth rate of HMAM5 cells cultured in DMEM containing different concentrations of FBS 1.5 x $10^4$ cells were plated. Cell number of triplicates was determined daily. Each data point represents the average ± standard error of triplicates.

Besides the determination of anchorage independent growth and the doubling time of the newly established Syrian hamster breast cancer cell lines, it was of interest to quantify the chromosome number of the cell line. Aberrations in the chromosome numbers are good indicators for cancerous characteristic in a cell line. For that reason, cytogenetic analyses were performed on the HMAM5 cell line by Applied Genomics Laboratories, Inc. (Melbourne, FL, USA) using a standard giemsa banding method (Seabright, 1971). Syrian hamsters contain usually 44 (2n) chromosomes (Romanenko et al., 2007). Metaphases of HMAM5 cells were analyzed performing a cytogenetic analysis. A total of 100 metaphases was examined and the average chromosome number of these metaphases was 74 (Coburn et al., 2011). This result was also observed for HMAM4B cells upon cytogenetic analysis. Furthermore, 5 metaphases of HMAM5 cells were further karyotyped and multiple copies of chromosomes A3, C11, C14, C15, E20 and F21 were detected as well as a number of unknown chromosomes (UC) (Coburn et al., 2011). Several copy numbers of chromosomes indicated that the established HMAM4B and HMAM5 Syrian hamster breast cancer cell lines have cancerous character.

Immunohistochemical staining demonstrated already that the mammary tumors in the Syrian hamster originated from epithelial cells and could be categorized as luminal breast cancer. To further determine if the Syrian hamster breast cancer cell line derived from a luminal A or luminal B tumor, the estrogen dependence of HMAM5 cells was determined in our laboratory. Several commercially available anti-ER antibodies were examined whether they cross react with the Syrian hamster breast cancer cell line; unfortunately none of the antibodies did. Consequently, the estrogen dependence was determined using the anti-estrogen agent tamoxifen. Tamoxifen is a non-steroidal estrogen antagonist that inhibits the growth of estrogen-responsive cells (Smith et al., 2000). Human breast cancer cell line MCF-7 was used as a positive control for an ER positive breast cancer and human breast cancer cell line MDA-MB-231 as ER negative breast cancer cells to determine the responsiveness to tamoxifen. Treatment with tamoxifen decreased the proliferation equally in MCF-7 and HMAM5 cells in a dose-dependent manner (Coburn et al., 2011). MDA-MB-231 cells on the other hand, were rather insensitive to tamoxifen treatment, and proliferation was only slightly inhibited at high concentrations of tamoxifen. These results suggested that the Syrian hamster breast cancer cell line HMAM5 was derived from a luminal B subtype mammary carcinoma.

Taken together, HMAM5 and HMAM4B cells showed anchorage independent growth and a similar growth curve compared to human MDA-MB-231 cells. Furthermore, it was shown that cell growth was reduced by culturing HMAM5 cells in medium containing 2% FBS. Cytogeneic analyses and anchorage independent growth indicated tumorigenic characteristics

for the Syrian hamster breast cancer cell lines. In addition, it was shown, that the HMAM5 cell line derived from a mammary tumor categorized as luminal B.

## *4.3 Replication efficiency of oncolytic Ads on HMAM5 cells*

The HMAM5 breast cancer cell line including the Syrian hamster *in vivo* model was proposed to be a useful animal model to determine toxicity and cancer specificity profile of newly generated oncolytic vectors for virotherapy. To investigate this proposition several *in vitro* experiments were performed, analyzing the replication efficacy of established CRAds on the Syrian hamster breast cancer cell line. The main goal of this study was to verify the compatibility of the model to investigate newly constructed Ad vectors specifically designed to target breast cancer. Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD and Ad5/3 CXCR4 viruses were used to infect HMAM5 cells and analyze the oncolytic efficiency in HMAM5 cells compared to hamster fibroblasts isolated from a female Syrian hamster.

In this regard, the Ad5-Δ24-RGD is an oncolytic Ad of great interest because it is the first fiber modified conditionally replicative competent human Ad used in a clinical trial (Page et al., 2007). A 24bp deletion in the constant region 2 (CR2) of the E1A gene is present in the Ad vectors Ad5 Δ24 and Ad5 Δ24 RGD. Those Ads are unable to bind retinoblastoma (Rb), a tumor suppressor/cell cycle regulator protein. Interaction of Ad5 E1A with Rb allows Ad to induce S-phase in the infected cell. Therefore, viruses containing this deletion are reduced in their ability to overcome the $G_1$-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, e.g., tumor cells defective in the *Rb-p16* pathway, or in rapidly cycling normal cells featuring phosphorylation of Rb (Fueyo et al., 2000; Heise et al., 2000). This pathway may be inactive in all human tumors (Sherr, 1996).

In Ad5 Δ24 RGD the 24bp deletion is combined with a genetic modification in the HI loop to overcome the CAR-dependent infection. The HI-loop is exposed on the fiber knob and it is possible to incorporate up to 83 amino acids into the HI-loop without deleterious effects on viral replication. Incorporation of the arginine-glycine-aspartate (RGD) sequence motif to the HI-loop targeted the Ad in particular towards $\alpha_v\beta$ cell surface integrins expressed at high levels on most cancer cells (Mathias et al., 1998; Wickham et al., 1993). In the past, studies have shown successful results in cancer treatment using the RGD-modified Ads (Dmitriev et al., 1998; Kasono et al., 1999). Bauerschmitz et al. showed an increased cellular infectivity and an enhanced progress in the therapeutic effects of a preclinical ovarian cancer model with the use

of Ad5-Δ24-RGD (Bauerschmitz et al., 2002). Studies about its efficacy have been performed in order to translate this approach into an early-phase clinical trials for patients with ovarian cancer.

### 4.3.1 Ad5 Δ24 RGD displays a significant E1A mRNA and protein expression in HMAM5 cells

To determine viral oncolytic potency it is of great importance to analyze the E1A mRNA and protein expression in infected cells. E1A is a key player during the Ad replication cycle because it is essential to initiate the transcription of the other early genes and the viral genome replication. Thus, if expression of E1A is at a low level, the replication efficiency in return will be also reduced. Therefore, Ad5 E1A mRNA levels of several established Ad vectors were analyzed in HMAM5 cells and hamster fibroblasts. Both cell types were infected with 10 ifu per cell of Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD, Ad5/3 CXCR4 and a non-replicating Ad (Ad5/3 luc or Ad5 LacZ). 48 h post infection, cells were harvested, RNA was isolated and E1A mRNA levels were determined by qRT-PCR.

Ad5 Δ24 RGD displayed higher E1A mRNA levels in HMAM5 cells compared to hamster fibroblasts as presented in Figure 24A and 24B. Furthermore, high levels of Ad5 WT E1A mRNA were detectable in HMAM5 cells after 48 h of infection (Figure 24B). Ad5/3 WT and Ad5 CXCR4 E1A mRNA levels were comparable to Ad5 WT E1A mRNA levels in hamster fibroblasts (Figure 24A), but significantly reduced in HMAM5 cells (Figure 24B). E1A levels of Ad5 Δ24 and Ad5/3 CXCR4 on the other hand were increased in HMAM5 cells and comparable to the ones of Ad5 WT, Ad5/3 WT and Ad5 CXCR4 in the hamster fibroblasts. The most prominent E1A mRNA levels were detectable in Ad5 Δ24 RGD infected HMAM5 cells after 48 h.

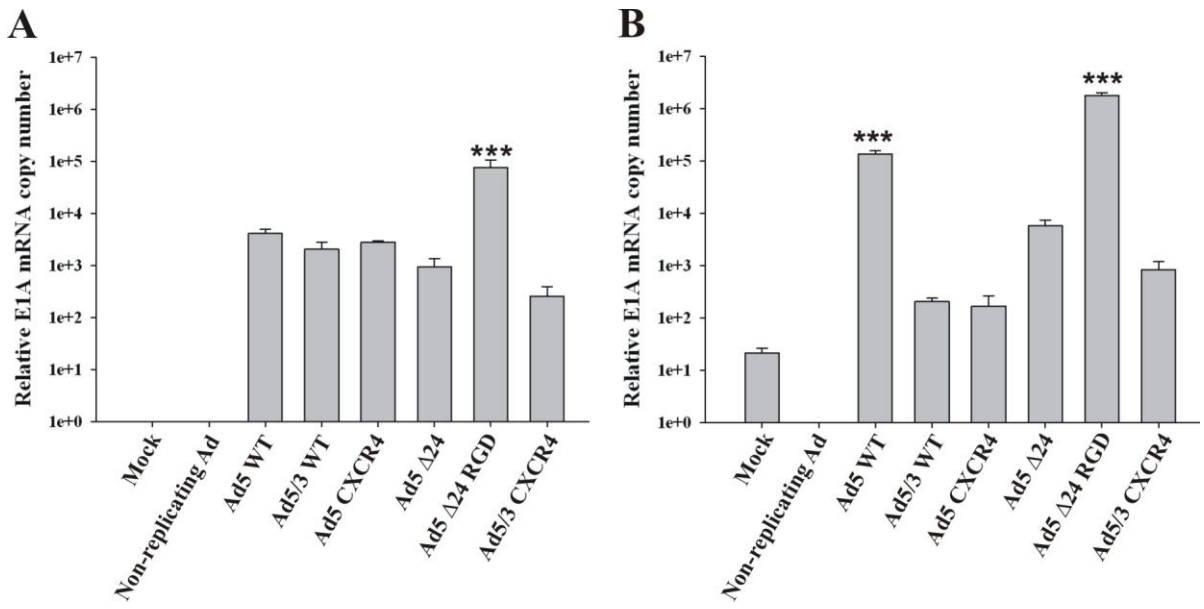


**Figure 24: E1A mRNA expression of Ad5 Δ24 RGD was significantly increased in HMAM5 cells.**

**A)** Hamster fibroblasts and **B)** HMAM5 cells were infected with 10 ifu per cell of Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad Δ24, Ad5 Δ24 RGD, Ad5/3 CXCR4 and a non-replicating Ad5 or mock infected. 48 h post infection mRNA was isolated, transcribed into cDNA and E1A mRNA levels were quantified in triplicates by qRT-PCR. Average values ± standard deviation of two independent experiments are displayed. ***, $p < 0.0005$.

After the determination of successful E1A transcription for some Ad vectors in HMAM5 cells, the E1A protein expression was investigated by infecting hamster fibroblasts and HMAM5 cells. Cells were infected with the same viruses and with the same concentration as for the E1A mRNA quantification. At 48 h after infection, cells were harvested and protein levels were detected performing immunoblot analyses with a specific antibody for Ad5 E1A. As seen in Figure 25A and 25B, Ad5 Δ24 RGD displayed a significant higher protein expression in hamster fibroblasts and in HMAM5 cells compared to the other Ad vectors. However, in hamster fibroblasts E1A protein expression of Ad5 WT and Ad5 Δ24 was detectable as a faint signal whereas in HMAM5 cells, E1A protein expression was only detectable in Ad5 Δ24 RGD infected cells (Figure 25A and B). E1A protein expression in Ad5/3 WT, Ad5 CXCR4 and Ad5/3 CXCR4 infected hamster fibroblasts and HMAM5 cells, was not detectable, even after long exposure times.

Taken together, immunoblot analyses results were in accordance with the results observed for the E1A mRNA expression, demonstrating an increased transcription and translation of the E1A gene in Ad5 Δ24 RGD infected cells. Besides, the significant high expression of Ad5 Δ24 RGD E1A in hamster fibroblasts, the discrepancy of E1A expression to the other Ad vectors was higher in HMAM5 cells. These results were suggesting that Ad5 Δ24 RGD was

able to infect specifically HMAM5 cells and initiate E1A expression and viral genome replication compared to other Ad vectors.

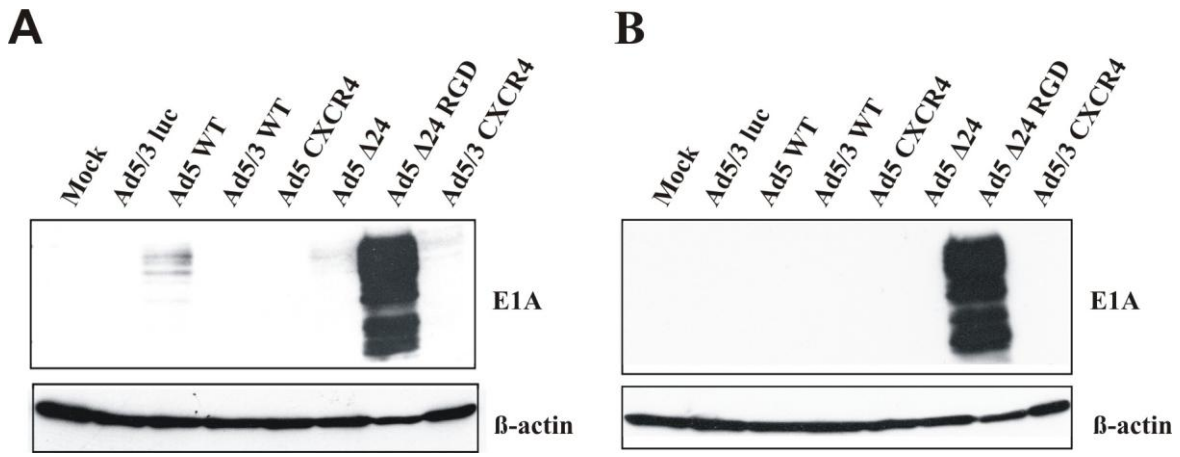


**Figure 25: E1A protein expression was dominant in Ad5 Δ24 RGD infected HMAM5 cells.**

Ad5 E1A protein expression was determined in **A)** Hamster fibroblasts and **B)** HMAM5 cells infected with 10 ifu per cell of Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD, Ad5/3 CXCR4 and Ad5/3 luc (non-replicating virus) or mock infected. 48 h post infection cells were harvested and 30 µg of protein were subjected on a 10% SDS-PAGE. Afterwards proteins were transferred onto a nitrocellulose membrane and immunodetection was carried out by using anti-Ad5 E1A and anti-β-actin (loading control). Shown are representative results of three independent experiments.

### 4.3.2 Viral replication of Ad5 Δ24 RGD in HMAM5 is significantly increased compared to Ad5 WT

After successful E1A expression and viral genome replication, the analysis of viral release is essential to determine the replication efficiency of constructed Ad vectors for virotherapy. Therefore, the E4 copy number in the supernatant of infected hamster fibroblasts and HMAM5 cells was quantified using qPCR.

In this experiment, 1 x $10^5$ cells were plated and infected the next day with 10 MOI of Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD, Ad5/3 CXCR4 and a non-replicating Ad or mock infected. Supernatant of infected cells was collected daily, DNA was isolated and viral release was analyzed by determining the Ad5 E4 copy number by qPCR. Viral replication efficacy of Ad5 CXCR4, Ad5 Δ24, Ad5/3 CXCR4 and Ad5 LacZ were not detectable in hamster fibroblasts as presented in Figure 26A. However, a slight increase in Ad5 E4 copy number was detectable for Ad5 WT, Ad5/3 WT and Ad5 Δ24 RGD at day 4 after infection. In HMAM5 cells on the other hand, great replication efficiency was observed for

Ad5 Δ24 RGD starting at day 1, until it reached a plateau phase at day 3 and day 4 after infection (Figure 26B). Both Ad5 WT and Ad5 Δ24 displayed similar release of progeny virions but were significantly reduced compared to Ad5 Δ24 RGD at day 4. As seen in Figure 26A and B, viral release of Ad5/3 WT and Ad5/3 CXCR4 was not detectable in HMAM5 cells and in hamster fibroblasts. Ad5 CXCR4 on the other hand showed a slight increase of E4 copy number at day 4 in HMAM5 cells, but in hamster fibroblasts the release of progeny virions was not detectable.

To summarize, Ad5 Δ24 RGD displayed specific viral replication in the Syrian hamster breast cancer cell line HMAM5 compared to hamster fibroblasts. In addition, Ad5 WT and Ad5 Δ24 demonstrated also a specificity of viral replication in HMAM5 cells but to a lower extent than Ad5 Δ24 RGD. All other viral vectors demonstrated no release of progeny virions in HMAM5 cells and hamster fibroblasts, indicating that those viruses can not replicate in the Syrian hamster cells.

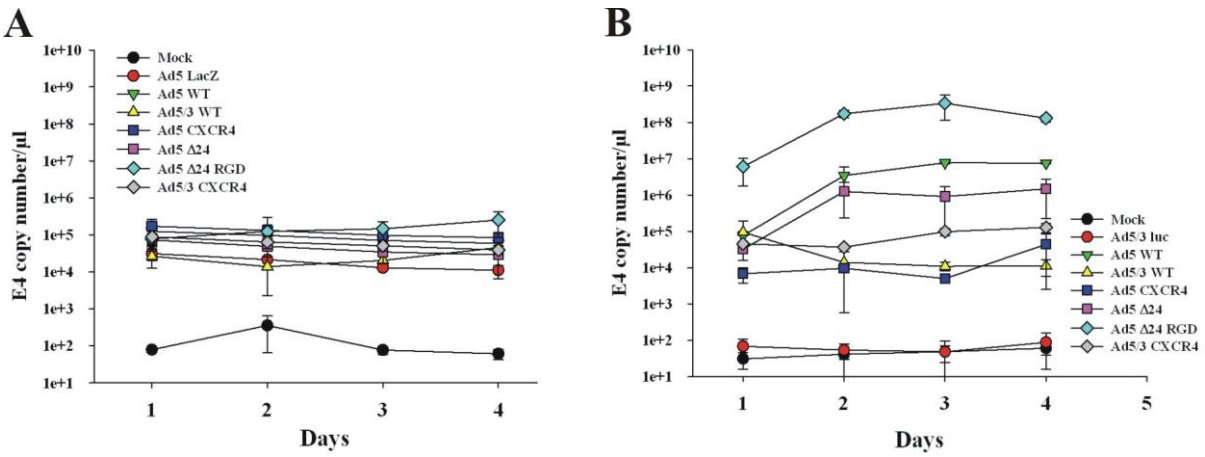


**Figure 26: Increased replication efficacy of Ad5 Δ24 RGD in HMAM5 cells.**

Shown are: E4 copy numbers detected in the supernatant of **A)** Hamster fibroblast and **B)** HMAM5 cells infected with Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD, Ad5/3 CXCR4 and a non-replicating Ad. 1 x $10^5$ cells were plated and on the next day infected with 10 ifu per cell of the indicated viruses. 200 µl of supernatant was collected daily, DNA was isolated and E4 copy number was determined in triplicates using qPCR. Average values ± standard deviation of three independent experiments are shown.

### 4.3.3 Enhanced oncolytic activity of Ad5 Δ24 RGD in HMAM5 cells

It was already shown that Ad5 Δ24 RGD displayed a significantly increased E1A mRNA and protein expression plus viral replication in HMAM5 cells and therefore, oncolytic activity was determined using the CV method. Briefly, 5 x $10^3$ cells were plated and infected the next day

with the indicated titers of Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD, Ad5/3 CXCR4 and the non-replicating Ad5/3 luc. Oncolytic activity was then monitored by staining viable cells with CV solution. Figure 27 presents the results of the CV staining in hamster fibroblasts and HMAM5 cells. Cytolytic activity of Ad5 WT and Ad5 Δ24 RGD was observed at 1,000 MOI in hamster fibroblasts and at 100 MOI for Ad5 Δ24 RGD whereas no cell killing was detectable in hamster fibroblasts infected with Ad5/3 WT, Ad5 Δ24, and Ad5/3 luc (Figure 27A). In contrast, in HMAM5 cells Ad5 Δ24 RGD demonstrated oncolytic activity at 10 MOI, revealing a gain of specificity for HMAM5 breast cancer cells compared to hamster fibroblasts (Figure 27B). Furthermore, cell killing was also observed in Ad5 Δ24 infected HMAM5 cells at a viral concentration of 1,000 MOI, suggesting also a gain in specificity for HMAM5 breast cancer cells compared to hamster fibroblasts. Ad5 WT displayed the same cytolytic activity at 1,000 MOI in HMAM5 cells as observed in hamster fibroblasts (Figure 27A and B).

To summarize, Ad5 Δ24 and Ad5 Δ24 RGD showed an enhanced oncolytic activity in HMAM5 cells compared to hamster fibroblasts with Ad5 Δ24 RGD displaying the greater infectivity rate than Ad5 Δ24. The same oncolytic activity of Ad5 WT was observed in hamster fibroblasts and in HMAM5 cells whereas Ad5/3 WT and Ad5/3luc did not display any oncolytic potency for both cell lines.

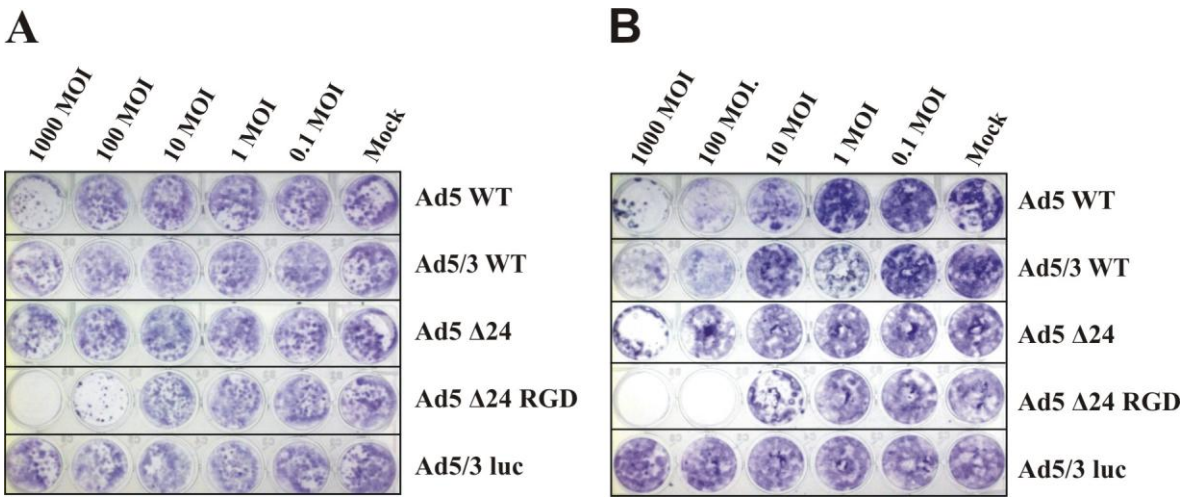


**Figure 27: Oncolytic activity of Ad5 Δ24 RGD was increased in HMAM5 cells.**

Crystal violet analysis was performed on **A)** Hamster fibroblasts and **B)** HMAM5 cells infected with Ad5/3 luc (non-replicating Ad5), Ad5 WT, Ad5/3 WT, Ad5 CXCR4, Ad5 Δ24, Ad5 Δ24 RGD and Ad5/3 CXCR4. Cells were infected with indicated MOIs and stained 2 weeks later with 0.1% CV. Figures are representative data from three independent experiments.

# 5. Discussion

## *5.1 Construction of an oncolytic adenovirus containing transductional, transcriptional and translational targeting*

Virotherapy is an innovative approach to treat breast cancer which is still the second leading cause of cancer death in women (Jemal et al., 2011). The Ad-based virotherapy represents a therapeutic method to achieve efficient tumor cell oncolysis by cancer specific replication and release of the progeny virions that are capable of further propagating in surrounding tumor cells but not in those of normal tissues (Alemany et al., 2000; Biederer et al., 2002; Mathis et al., 2005; Stoff-Khalili et al., 2006; Vile et al., 2000). Nevertheless, the overall efficacy of Ad-based virotherapy needs to be improved with different approaches such as transductional, transcriptional or translational targeting, due to sub-optimal vector delivery and unspecific replication. Each targeting strategy by itself bears some disadvantages such as background replication efficiency in normal cells. Clearly, the specificity of Ads as anti-tumor agents in cancer therapy is desperately necessary, so that they can accomplish their safety profile and achieve their full benefit in terms of clinical efficacy. In the present study, a novel oncolytic Ad harboring transductional, transcriptional and translational targeting was successfully constructed to gain tumor specificity in virotherapy and reduce the Ad background replication efficiency in normal cells. The Ad5 fiber knob was replaced with the serotype 3 knob to achieve transductional targeting. Furthermore, the Ad5 E1A promoter was exchanged with the human CXCR4 promoter (transcriptional targeting) and finally, we introduced the translational control of Ad5 E1A by inserting the 5'-UTR sequence of FGF-2

After successful rescue of the triple-level targeted Ad construct, designated Ad5/3 CXCR4 UTR, PCR and sequencing analyses demonstrated the correct insertion of all three control elements into the genome of the Ad5/3 CXCR4 UTR virus. An essential step in developing of a novel oncolytic Ad is to determine the functionality for killing the specific cancer type *in vitro*. In normal cells a gain of cancer specificity was detectable after the infection of Ad5/3 CXCR4 UTR. Unfortunately, Ad5/3 CXCR4 UTR displayed a reduced oncolytic activity between one to two orders of magnitude depending on the breast cancer cell line treated compared to other Ad vectors including Ad5/3 WT and Ad5/3 CXCR4. However, compared to Ad5 WT, the Ad5/3 CXCR4 UTR demonstrated an equal oncolytic activity using breast cancer cells and a lower cytolytic activity using normal cells. Moreover, the E1A protein expression was expected to be reduced in normal cells compared to breast cancer cells

due to the incorporation of the 5'UTR sequence. Indeed, the E1A protein concentration was shown to be nearly absent in normal cells while it was clearly detectable in infected breast cancer cell lines. Nevertheless, the E1A protein expression of Ad5/3 CXCR4 UTR and therefore also E1A translation was reduced in all breast cancer cell lines included in this experimental setup compared to the other Ad vectors. Furthermore, it was possible to show that release of progeny virions was significantly reduced in MDA-MB-231 cells infected with Ad5/3 CXCR4 UTR compared to Ad5 WT, Ad5/3 WT and Ad5/3 CXCR4. Those experiments confirmed the lower efficacy of Ad5/3 CXCR4 UTR to kill breast cancer cells compared to the other Ad vectors. The disadvantages of a different receptor expression pattern and the expression of several Ad genes in HEK-293 cells can lead to an inaccurate titer of fiber or replication modified Ads. Re-titration of viral stocks for viral DNA copy number was cell line independent, but did not result in a change in the oncolytic activity of Ad5/3 CXCR4 UTR in MDA-MB-231 cells observed compared to Ad5/3 WT and Ad5/3 CXCR4. Overall, Ad5/3 CXCR4 UTR still demonstrated a lower oncolytic activity than Ad5/3 CXCR4. This result revealed that the titration method was not the limiting factor that induced reduced oncolytic activity of Ad5/3 CXCR4 UTR compared to other Ads. Furthermore, it was demonstrated that viral cell entry efficiency of Ad5/3 WT, Ad5/3 CXCR4 and Ad5/3 CXCR4 UTR in MDA-MB-231 cells was comparable using different concentrations at either 1 h or 3 h post infection. As expected, viral cell entry of Ad vectors containing the Ad5/3 fiber chimera was increased compared to Ad5 WT, due to the higher expression of the Ad3 cellular attachment receptor on breast cancer cells than the Ad5 receptor CAR. This finding clearly showed that the viral cell entry of Ad5/3 CXCR4 UTR was not impaired and even greater compared to Ad5 WT, leading to the speculation that an unknown cellular factor might be responsible for the delayed replication efficiency of Ad5/3 CXCR4 UTR in breast cancer cells.

Incorporation of all three targeting strategies involved the new combination of Ad3 fiber knob with the 5'UTR sequence of FGF-2. Desmoglein-2 was recently characterized as the primary cellular attachment receptor for Ad serotype 3 (Wang et al., 2011). However, nothing is known so far regarding the interaction of Ad3 fiber knob and the Desmoglein-2 receptor to mediate activation of intracellular signaling cascades. These cascades could alter eIF4E protein expression or the phosphorylation level of 4E-BP, resulting in a reduced translation of E1A mRNA and therefore, reduced replication efficiency of Ad5/3 CXCR4 UTR. However, the investigation of eIF4E protein expression levels after Ad5/3 WT and Ad5 WT infection in two different breast cancer cell lines, MDA-MB-231 and MCF-7, did not reveal any effect.

Even after a longer incubation time, the expression pattern of Ad5 WT and Ad5/3 WT infected breast cancer cells was not altered, indicating that Ad3 fiber knob binding is not dysregulating the protein expression of eIF4E. The phosphorylation of 4E-BP in MDA-MB-231 cells was shown to be initiated upon Ad5/3 WT or Ad5/3 CXCR4 UTR infection and occurred at a rate even faster compared to Ad5 WT. On the other hand, phosphorylation of 4E-BP in MCF-7 cells was absent after Ad5 WT infection and clearly detectable after Ad5/3 WT and Ad5/3 CXCR4 UTR infection. One reason for this result could be a lower expression of CAR on the surface of MCF-7 cells compared to MDA-MB-231 cells. Another possibility is exemplified by the finding that in several breast cancer cell lines, the PI3K pathway is altered and enhanced phosphorylation of 4E-BP already exists (Hernandez-Aya, 2011). Evidence for this possibility is indicated in the results in which a lower concentration of non-phosphorylated 4E-BP was detected at time point zero compared to MDA-MB-231 cells. These findings showed that Ad3 fiber knob binding does not alter 4E-BP phosphorylation in a negative manner, and therefore, Ad5/3 CXCR4 UTR translation efficiency is not likely to be impacted.

All the possible alternatives that were proposed to explain the reduced E1A translation were disproven experimentally. E1A is the earliest gene that is transcribed and initiates the transcription of all other early genes within the Ad genome. Therefore, impaired E1A transcription affects the Ad replication efficiency. It was possible to clearly show that E1A transcription was significantly reduced in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells compared to the other Ad vectors. A time dependent analysis of E1A and pTP (essential in a complex with Ad DNA Polymerase for Ad genome replication) gene expression displayed a delayed expression in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells between 24 h to 48 h. These findings tempted to speculate that an unknown cellular factor might restrict the transcription of Ad5/3 CXCR4 UTR early in infection. Moreover, the degradation of E1A mRNA could be induced due to the incorporation of the 5'UTR-sequence into the Ad5 genome upon Ad3 fiber knob binding resulting in low levels of E1A mRNA early during infection. The Ad5/3 CXCR4 UTR E1A mRNA stability was not altered by comparing the half-lives of Ad5/3 WT and Ad5/3 CXCR4 E1A with the one of Ad5/3 CXCR4 UTR This result showed that a change in the E1A mRNA stability in the Ad infection cycle is not responsible for the decreased oncolytic activity of Ad5/3 CXCR4 UTR compared to other Ads in breast cancer cells.

As mentioned earlier, HEK-293 cells express several Ad5 proteins due to incorporation of the left arm of the Ad5 genome. Hence, Ad vectors that contain a control element upstream of the E1A open reading frame to restrict E1A expression to tumor cells, should demonstrate the

same cytolytic activity in HEK-293 cells. It was shown that Ad5/3 CXCR4 UTR displayed the same cytolytic activity as Ad5/3 WT in infected HEK-293 cells and even a greater cyolytic activity than Ad5/3 CXCR4. Surprisingly, stable expression of the E1A gene could not induce an enhanced oncolytic activity because Ad5/3 CXCR4 UTR still displayed a reduced oncolytic activity in MDA-MB-231 cells stably expressing E1A (MDA-MB-231/E1A) compared to Ad5/3 WT and Ad5/3 CXCR4. Moreover, atypical cell killing was detected in MDA-MB-231/E1A cells and MDA-MB-231 cells transfected with the empty vector (MDA-MB-231/N) after Ad5 WT infection. This result led to the speculation that the transfection of MDA-MB-231 cells reduced the expression of CAR on the cell surface resulting in a decreased Ad5 WT entry and cell killing. Furthermore, the morphology of the transfected MDA-MB-231 cell lines was changed which underlines the assumption that transfection might have altered also receptor expression on the surface. In addition, Ad5/3 CXCR4 UTR displayed also a lower efficacy in cell killing in the transfected cell line compared to the untransfected cell line. This finding indicated that the transfection of the cell line was changing a factor that further reduced the oncolytic activity of Ad5/3 CXCR4 UTR. Nevertheless, it was possible to demonstrate that E1A by itself is not sufficient to restore the oncolytic activity using Ad5/3 CXCR4 UTR.

Moreover, re-titration of viral stocks on A549 cells, which are not providing Ad5 proteins, revealed a three orders of magnitude lower infectious titers Ad5/3 CXCR4 UTR compared to the infectious titer using HEK-293 cells. MDA-MB-231 cells infected with the viruses displayed a greater oncolytic activity of Ad5/3 CXCR4 UTR compared to that seen when titers were determined using HEK-293 cells. These results support the speculation that HEK-293 cells provide an unknown factor that can diminish the delayed expression of Ad5/3 CXCR4 UTR observed using breast cancer cells. Most likely, this unknown factor is the combination of several Ad proteins that are stably expressed in this cell line. Besides, the E1A gene that was not able to enhance oncolytic activity by itself, the E1B and the E2 genes are also expressed in HEK-293 cells. The E1B gene encodes two proteins: the E1B 19K and the E1B 55K protein. The functions of these proteins involve the inhibition of apoptosis and the further modification of the intracellular environment to enable viral protein production (Chinnadurai, 1998; White, 1998). The E2 gene encodes three proteins: the Ad DNA polymerase (Ad pol), the pTP, and the DNA binding protein (DBP). DBP encompasses several functions including early and late gene expression and viral DNA replication (Hay et al., 1995; Van der Vliet, 1995). In addition, the heterodimer complex of Ad pol and pTP is critical for viral DNA replication (Hay et al., 1995; Van der Vliet, 1995). Since these Ad proteins embody

essential functions during the Ad replication cycle, it can be speculated that they provided an enhanced oncolytic activity of Ad5/3 CXCR4 UTR in HEK-293 cells, thereby overcoming any restricting factors observed in the other cell lines tested.

Findings within this study were leading to the assumption, that virus trafficking in infected cells is altered due to Ad3 fiber knob modification. This trafficking alteration could result in impairment of E1A early transcription. Viral entry and trafficking studies have demonstrated that Ads can use clathrin-dependent endocytosis, macropinocytosis, phagocytosis, caveolin-dependent endocytosis or a combination of all these mechanisms to enter the cell; this use is dependent on the cell type and the binding receptor (Henaff et al., 2011; Meier et al., 2002). The primary internalization of Ad5 is mediated through clathrin-coated vesicles by integrin activation *in vitro* (Mathias et al., 1998; Wickham et al., 1993). In contrast, group B Ads (including Ad3 and Ad35) can employ macropinocytosis for internalization into cells (Amstutz et al., 2008; Kalin et al., 2010). Moreover, accumulation of receptors on the cell surface that use clathrin can disrupt the cell membrane and induce macropinocytosis (Doherty and McMahon, 2009). Trafficking studies of Ads containing fiber chimera illustrated that upon exchange of the fiber, the trafficking of Ad5 was changed to the serotype from which the fiber was derived (Miyazawa et al., 2001; Miyazawa et al., 1999). Furthermore, it has been shown that the pH susceptibility is different for Ad serotypes and therefore the time of viral escape from the endosome. Subgroup C Ads escape from early endosomes (Gastaldelli et al., 2008), whereas Ad37 (a member of subgroup D) is transferred to the lysosome before escape (Leopold and Crystal, 2007); and Ad3 (subgroup B member) has a slower escape from the endosome than Ad2 (a subgroup C member) (Amstutz et al., 2008). In addition, Amstutz et al. demonstrated that clathrin plays only a minor role in Ad3 endocytosis.

The trafficking of Ad5/3 fiber chimeras has not yet been investigated. It is possible that the internalization and the trafficking pathway of an Ad5/3 fiber chimera mimic that of Ad serotype 3 rather than Ad serotype 5. Until recently, the primary cellular attachment receptor of Ad serotype 3 has been disputed. Initially, CD46 and CD80 and CD86 have been identified as receptors for Ad serotype 3 (Fleischli et al., 2007; Short et al., 2004; Sirena et al., 2004), but their function was debated (Marttila et al., 2005; Segerman et al., 2003). However, Wang et al. recently published that Desmoglein-2 is the primary cellular attachment receptor (Wang et al., 2011) and so far it is only known that multimerization of the Ad3 fiber knob is necessary to bind efficiently to the receptor and to induce opening of epithelial junctions (Wang et al., 2011). Hence, little is known to date about Ad3 internalization upon binding to Desmoglein-2 and the subsequent trafficking of Ad3 in endosomes. Furthermore, it is not clear whether Ad3

employs also macropinocytosis upon Desmoglein-2 binding as it was shown for CD46 binding. Another potential reason for reduced oncolytic activity of Ad5/3 CXCR4 UTR is that binding of Ad3 fiber knob activates an unknown factor, which inhibits E1A expression by binding in the 5'UTR sequence of rat FGF-2 and works as a repressor. It is speculated that this inhibition is diminished with time after the infection, and then E1A transcription can be initiated.

Alternatively, a mutation of a gene within the Ad5/3 CXCR4 UTR genome essential to initiate the early transcription of E1A could be a different explanation. Amstutz et al. investigated the endocytotic uptake of Ad3 in epithelial and hematopoietic cells. These investigators showed that Ad3 uptake into cells upon CD46 binding occurs through macropinocytosis and that this uptake is controlled by the activation of the C-terminal binding protein 1 (CtBP1) which is a transcriptional co-repressor (Amstutz et al., 2008). CtBP activation and translocation during Ad infection overlaps with transcriptional suppression of cellular genes (Barnes et al., 2003). Some of the targets of CtBP include the histone acetyl transferases, the E1A associated protein p300, and Ad5 E1A itself (Boyd et al., 1993; Chinnadurai, 2002; Senyuk et al., 2005). Studies of CtBP binding to E1A were performed using plasmids that either contained the sequence for 12S E1A or the whole E1A gene (Zhang et al., 2000). It was shown that CtBP binds to the sequence motif Pro-Leu-Asp-Leu-Ser (PLDLS) located at the C-terminus of E1A. Binding of CtBP represses the E1A transcription which can be reversed by acetylation of the Lys-239 (Zhang et al., 2000). Mutation of this amino acid to Arg increased the binding of CtBP compared to WT and caused a repression of E1A transcription (Zhang et al., 2000). These findings led to the hypothesis that a mutation in the CtBP binding region of E1A could enhance the repression effect of CtBP and cause a reduced E1A transcription of Ad5/3 CXCR4 UTR early during the Ad infection cycle. Sequencing of the whole Ad5/3 CXCR4 UTR genome is in progress and should answer this possibility.

In conclusion, the generation of a triple-level targeted oncolytic Ad was achieved and a gain in cancer specificity was detected in normal cells. However, at the same time the triple-level Ad displayed a lower oncolytic activity on breast cancer cells than previously generated dual-level or single-level targeted oncolytic Ad vectors. Furthermore, the examinations of several hypotheses could show that a repressed E1A transcription in Ad5/3 CXCR4 UTR infected cells was the limiting factor. The presence of several Ad5 proteins could omit this restriction and induce a great cytolytic activity of Ad5/3 CXCR4 UTR in HEK-293 cells. Unfortunately, it was not possible to identify the factor(s) responsible for inhibiting E1A transcription in Ad5/3 CXCR4 UTR infected cells. Therefore, further experimental investigations are needed to address this question. Furthermore, trafficking studies and analysis of the CtBP binding site

could reveal whether those two aspects are responsible for the reduced oncolytic activity of Ad5/3 CXCR4 UTR in breast cancer cells. Sequencing analyses of the whole Ad5/3 CXCR4 UTR genome should give new understandings whether a mutation in a specific Ad gene is triggering the delayed expression of Ad5/3 CXCR4 UTR or not. Identifying the factor that represses the transcription of the Ad5/3 CXCR4 UTR E1A gene could give new insights into the transcriptional regulation of oncolytic Ad vectors. Revealing this factor could lead to an optimization of the Ad5/3 CXCR4 UTR vector. To finally conclude, the combination of the Ad5/3 fiber chimera and the 5'UTR sequence in this study demonstrated a reduced efficacy for the Ad vector. This combination is not a suitable approch for a triple-level targeted Ad due to the decreased oncolysis in breast cancer cells. Future intentions to construct a triple-level targeted Ad should either use a different transductional targeting or a different translational targeting. Incorporation of specific motifs into the HI-loop or incorporation of a specific cell ligand could be an alternative to the serotype fiber chimera. An alternative for cancer specific control of *E1A* expression is the addition of a 3'-UTR element that controls the cancer-selective mRNA stability as described by Ahmed *et al. (Ahmed et al., 2003).* Furthermore, another possibility to improve the triple-level construction is the insertion of a specific miRNA target sequence which's activity is reduced in breast cancer cells and up regulated in normal cells. Ultimately, an optimized triple-level Ad vector would be a promising tool to specifically target and kill breast cancer cells. A future goal in combining different targeting strategies is to identify specificity to kill also breast cancer stem cells. Breast cancer stem cells or cancer stem cells in general are thought to induce cancer development. It will be important to understand the oncolytic activity of triple-level Ad vectors for this cell population.

## *5.2 Establishment of a Syrian hamster breast cancer cell line*

In the field of cancer biology, well-characterized cancer cell lines are an important tool to study cancer biology and investigate new treatment options *in vitro*. For *in vivo* examinations in the development of breast cancer and in preclinical studies of a new anticancer therapeutic agent, human xenograft cells are injected into immuno-compromised animals. In spite of this, differences in gene expression, gene activity and function between species can result in disparities in toxicity and efficacy outcome (Mirzaee et al., 2010; Quintieri et al., 2008). Therefore, novel breast cancer models are clearly needed. In the past several years, Syrian hamsters have been employed for a number of diverse cancer chemoprevention and

carcinogenesis models, but none included the mammary carcinogenesis. Thus, our group used MNU to promote the development of spontaneous mammary tumors in female Syrian hamsters (Coburn et al., 2011). It was possible to demonstrate that the developed primary tumors were of mammary and epithelial origin.

In this study, the first immortalized mammary carcinoma cell line from female Syrian hamsters was established. Two cell lines, HMAM4B and HMAM5, were passaged more than 30 times and re-cultured *in vitro*. In addition, both cell lines showed a similar growth rate compared to the human breast cancer cell line MDA-MB-231 and displayed anchorage independent growth by colony formation in soft agar. The rapid growth during the exponential phase and the anchorage independent growth are two markers characteristic of cancer cells. Multiple copy numbers of chromosomes were determined in both Syrian hamster breast cancer cell lines with an average chromosome number of 74 in both whereas the normal chromosome number for Syrian hamster is 44 (2n) (Romanenko et al., 2007). Those *in vitro* data indicated that the Syrian hamster cell lines HMAM4B and HMAM5 were immortalized breast cancer cell lines. In addition, upon reinjection of the cell line HMAM4B into recipient hamsters, tumor formation was induced with a take rate of about 50%. Cells isolated from developed tumors, were able to grow in cell culture (HMAM5) and were also transplantable into recipient hamsters with a tumor formation take rate of 70%. These *in vivo* experiments showed that both breast cancer cell lines are able to induce tumor development upon subcutaneous injection into syngeneic Syrian hamsters, which is an indicator for tumorigenicity. Furthermore, negative staining for the molecular markers p63 and cytokeratin 5 demonstrated that the tumors induced by the subcutaneous injection of HMAM4B and HMAM5 cells were of luminal origin. Moreover, it was shown that the cell lines were derived from a luminal B subtype mammary tumor due to the susceptibility to tamoxifen.

Usually, evaluation for efficacy of newly constructed oncolytic Ad vectors is performed in immunodeficient mice using human xenograft tumor models. Unfortunately, biodistribution and toxicity of oncolytic vectors in this animal model does not accurately reflect that observed in humans because Ad replication is species specific. Recently, it has been demonstrated that Syrian hamsters are permissive for human Ad5 replication and that several Syrian hamster cancer cell lines except for breast cancer were established to study oncolytic Ad vectors (Bortolanza et al., 2007; Spencer et al., 2009; Thomas et al., 2006). In this study, two new Syrian hamster breast cancer cell lines, HMAM4B and HMAM5, were successfully established. Importantly, these cell lines can be used as a syngeneic model in evaluating the efficacy of newly designed oncolytic Ad vectors.

Here, in this study it was demonstrated that Ad5 Δ24 RGD, an oncolytic Ad previously tested in clinical trials for ovarian cancer, showed increased killing of HMAM5 cells compared to Ad5 WT. The Ad5 Δ24 vector also showed an increase in specificity to lyse HMAM5 cells, since Syrian hamster fibroblasts were lysed with a lower efficacy. In addition, E1A mRNA transcription and Ad replication efficiency of Ad5 Δ24 RGD were significantly increased in HMAM5 cells compared to the other viruses tested. Surprisingly, Ad5/3 WT, Ad5 CXCR4 and Ad5/3 CXCR4 did not display replication efficiency or oncolytic activity in the HMAM5 cells or the hamster fibroblasts. This finding was likely due to the possibility that Syrian hamster breast cancer cells and normal cells do not express Desmoglein-2, the Ad3 receptor. Furthermore, Syrian hamster breast cancer cells may not express the CXCR4 gene. Because no commercial antibodies are available that cross-react with Syrian hamster CXCR4 or Desmoglein-2, the expression levels of these proteins in HMAM5 cells could not be measured. Also, the genome of the Syrian hamster is not fully sequenced and consequently, the cDNA sequences for Syrian hamster CXCR4 or Desmoglein-2 are not available. Thus, mRNA levels of these genes could not be determined. Stable transfection of the HMAM5 cell line with a plasmid expressing the human Desmoglein-2 sequence could be used to circumvent the lack of receptor problem.

Taken together, these results showed that a novel immortalized Syrian hamster breast cancer cell line was established. This cell line maintained the ability to initiate tumor formation upon subcutaneous injection into syngeneic female Syrian hamsters. To conclude, this immortalized cell line could be very useful to the research community because spontaneous primary mammary carcinomas in Syrian hamsters are rare. Furthermore, this cell line is a promising test system to preclinically evaluate some oncolytic Ad vectors for their efficacy, which do not contain a serotype chimeric fiber and no human specific incorporation in their genome. This Syrian hamster breast cancer cell line could be further improved for fiber knob chimeras by genetic transfections of the corresponding receptors. A future goal would be that the Syrian hamster breast cancer model could serve as a novel animal system besides mice and rats to study chemopreventive agents in an intact immune system. Nevertheless, before the introduction of the Syrian hamster breast cancer model in the field of virotherapy as a suitable animal model to preclinically evaluate some oncolytic Ad vectors, *in vivo* experiments need to be performed to confirm this assumption and the previous results of the *in vitro* studies. A pilot study including six female Syrian hamsters was performed to determine the dose of viral injection. The results of this pilot study revealed promising effects on tumor reduction after the injection of Ad5 Δ24 RGD. This pilot study needs to be expanded to include a larger

number of animals per treatment arm as well as control treatment arms. The final future goal is to employ the Syrian hamster breast cancer model to evaluate the biodistribution and toxicity of oncolytic Ads in a preclinical animal model.

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# 7. List of figures and tables

# 8. Abstract

Breast cancer is still one of the most frequently diagnosed cancer types in women. Although a variety of treatment options and early detection methods are now available, breast cancer remains one of the leading causes of cancer death among women. Therefore, new therapy approaches are sorely needed. Virotherapy employing oncolytic adenoviruses (Ads) represents a promising tool for treating a wide array of neoplastic diseases including breast cancer. Critical for an effective therapeutic index is selective killing of breast cancer cells while avoiding killing normal cells. Besides the generation of cancer specific oncolytic vectors, new animal models which are immunocompetent and permissive for adenovirus replication are desperately needed to evaluate oncolytic Ad vector functions in a preclinical model. Herein, a novel oncolytic Ad was constructed to target breast cancer by incorporating three cancer control elements into the Ad5 genome: 1) transductional, 2) transcriptional and 3) mRNA translational targeting. A modified triple-level targeted oncolytic Ad (Ad5/3 CXCR4 UTR) incorporating a serotype 5/3 chimeric fiber was generated. This construct used the human CXCR4 gene promoter and the 5'-untranslated region (UTR) from rat Fibroblast Growth Factor-2 mRNA to regulate Ad E1A gene transcription and translation. It was hypothesized that this novel vector would improve the therapeutic index by selective targeting of human breast cancer cells. While it was achieved to construct a replication competent Ad5/3 CXCR4 UTR, this vector displayed a lower oncolytic activity in breast cancer cells compared to Ad5/3 WT and Ad5/3 CXCR4. Since multiple vector modifications could negatively influence virus replication, we examined their effects. We could detect no effects of Ad5/3 knob modification on eIF4E protein expression or on phosphorylation of 4E-BP, which are necessary for the translational targeting element. Furthermore, stable expression of E1A in MDA-MB-231 cells could not provide an increased oncolytic activity of Ad5/3 CXCR4 UTR to the level observed in HEK-293 cells. However, we could show that E1A transcription was delayed in Ad5/3 CXCR4 UTR infected MDA-MB-231 cells compared to the other viruses infected MDA-MB-231 cells. Thus, this may lead to the assumption that HEK-293 cells provide an unknown factor that is ameliorating the combination effect of transductional, transcriptional and mRNA translational targeting that limits oncolytic activity of Ad5/3 CXCR4 UTR.

In the next step, our group succeeded in establishing two Syrian hamster breast cancer cell lines (HMAM4B and HMAM5) from N-methyl-N-nitrosurea (MNU)-induced mammary tumors of female Syrian hamsters. It could be demonstrated that these two cell lines maintained characteristic cancerous and tumorigenic features. Furthermore, these cell lines induced tumor

formation in syngeneic female Syrian hamsters upon subcutaneous injection. Functional *in vitro* assays of oncolytic Ads on HMAM5 cells, demonstrated a specificity of oncolytic Ad replication and oncolysis in the HMAM5 cell line compared to normal Syrian hamster fibroblasts. These results showed that the HMAM5 cell line can be used to evaluate the breast cancer efficacy of oncolytic Ad vectors in an immunocompetent preclinical model.

# 9. Zusammenfassung

Das Mammakarzinom ist immer noch der häufigste maligne Tumor der Frau weltweit. Obwohl es mittlerweile eine große Anzahl von Therapiemöglichkeiten und Methoden zur frühen Erkennung gibt, bleibt das Mammakarzinom eine der Haupttodesursachen unter Krebspatientinnen. Deswegen sind neue Therapieansätze dringend notwendig. Die Virotherapie stellt eine viel versprechende neue Therapieform für ein breites Spektrum von Krebserkrankungen inklusive Brustkrebs dar. Eine Art der Virotherapie setzt die so genannten onkolytischen Adenoviren (Ad) ein. Kritisch für einen effektiven therapeutischen Index ist das selektive Abtöten von Tumorzellen durch Ads bei gleichzeitiger Schonung von gesundem Gewebe. Neben der Generierung neuer onkolytischer Vektoren für tumorspezifische Zellzerstörung, ist die Entwicklung eines immunkompetenten und für Ad Replikation permissiven Tiermodells von großer Notwendigkeit, um onkolytische Vektoren präklinisch zu testen. In dieser Arbeit wurde erstmals ein onkolytisches Ad generiert, das spezifisch Mammakarzinomzellen zerstören soll, indem die drei folgenden Targeting Strategien in das adenovirale Genom inkorporiert wurden: 1) Transduktionales, 2) Transkriptionales und 3) Translationales Targeting. Das neu generierte onkolytische triple-level Ad (Ad5/3 CXCR4 UTR) beinhaltet einen serotypischen 5/3 chimären Fiber als transduktionales Targeting, den humanen CXCR4 Genpromoter als transkriptionales Targeting und die 5'UTR Sequenz des Fibroblast Growth Factor-2 (FGF-2) der Ratte als translationales Targeting upstream des viralen E1A Genes. Dieser virale Vektor sollte durch ein gesteigertes selektives Targeting von Mammakarzinomzellen den therapeutischen Index erhöhen. Bedauerlicherweise wurde im Vergleich mit Ad5/3 WT und Ad5/3 CXCR4 eine geringere onkolytische Aktivität des replikationskompetenten Ad5/3 CXCR4 UTR in Brustkrebszellen festgestellt. Einen Einfluss der Bindung des Ad3 Fiber Knobs auf die eIF4E Protein Expression und die 4E-BP Phosphorylierung, welche wichtig für die Translationinitiation sind, konnte nicht festgestellt werden. Allerdings konnte gezeigt werden, dass die verringerte onkolytische Aktivität des Ad5/3 CXCR4 UTR auf eine verzögerte E1A Transkription zurückzuführen war. Die Annahme, dass ein Abbau der E1A mRNA durch die Ad3 Fiber Knob Bindung stattfindet, wurde in dieser Arbeit durch mRNA Stabilitätsanalysen widerlegt. Des Weiteren konnte gezeigt werden, dass es trotz einer stabilen E1A Expression in MDA-MB-231 Zellen zu keiner erhöhten onkolytischen Aktivität des Ad5/3 CXCR4 UTR kommt, welche in HEK-293 Zellen erkennbar war. Aus diesem Grund ist anzunehmen, dass HEK-293 Zellen einen unbekannten

Faktor exprimieren, der zu einer erhöhten onkolytischen Aktivität des Ad5/3 CXCR4 UTR führt.

Im nächsten Schritt, konnte unsere Gruppe erfolgreich zwei Zelllinien (HMAM4B and HMAM5) aus N-methyl-N-nitrosurea (MNU)-induzierten Mammakarzinomen des weiblichen Goldhamsters isolieren. Die Eigenschaften von Krebszellen konnten in diesen Zelllinen erhalten werden und es konnte gezeigt werden, dass die HMAM4B und die HMAM5 Zelllinie immortalisiert sind. Außerdem konnten nach subkutaner Injektion dieser Zellen Mammakarzinome in genetisch identischen weiblichen Goldhamstern induziert werden. Funktionalitätsuntersuchungen *in vitro* von verschiedenen etablierten onkolytischen Ads in der HMAM5 Zelllinie zeigten, dass eine spezifische Vermehrung des Ad5 Δ24 RGD und Onkolyse in den HMAM5 Zellen stattfindet im Gegensatz zu normalen Goldhamster Fibroblasten. Die hier neu etablierte Goldhamster Mammakarzinomzelllinie HMAM5 kann somit zur Evaluierung von humanen onkolytischen Ad für das Mammakarzinom eingesetzt werden. Zukünftige *in vivo* Studien könnten zeigen, dass das neu generierte Mammakarzinom Modell des Goldhamsters als immunokompetenes Tiermodell eingesetzt werden kann, um onkolytische Ad Vektoren präklinisch zu testen.

# 10. Abbrevations

| | |
|---|---|
| Ad | adenovirus |
| ADP | adenovirus death protein |
| Ad2 | adenovirus serotype 2 |
| Ad3 | adenovirus serotype 3 |
| Ad5 | adenovirus serotype 5 |
| Ad35 | adenovirus serotype 35 |
| Ad5/3 | adenovirus Type 5, containing a fiber chimera, carrying the fiber knob of Ad3 |
| Ad5 CXCR4 | adenovirus serotype 5, human CXCR4 promoter instead of E1A promoter |
| Ad5/3 CXCR4 | adenovirus serotype 5, 5/3 fiber chimera, human CXCR4 promoter |
| Ad5 Δ24 | adenovirus serotype 5, 24bp deletion in E1A gene |
| Ad5 Δ24 RGD | adenovirus serotype 5, 24bp deletion in E1A gene, RGD-motif in H1-loop |
| bp | base pair |
| BRCA1, 2 | breast cancer susceptibility gene 1, 2 |
| BSA | bovine serum albumin |
| CAR | coxsackie and adenovirus receptor |
| CEA | carcinoembryonic antigen |
| CRAd | conditionally replicating adenovirus |
| CR2 | constant region 2 of E1A |
| CtBP1 | C-terminal binding protein 1 |
| CV | crystal violet |
| DMEM | dulbeccos modified eagles medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| ER | estrogen receptor |
| eIF4E | eukaryotic initiation factor 4E |
| Fab | antibody fragment |
| FBS | fetal bovine serum |
| FGF-2 | fibroblast growth factor-2 |
| g | gravitational acceleration |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| h | hour |
| HER2 | human epidermal growth factor receptor 2, erb-B2 |
| HPRT1 | hypoxanthine phosphoribosyltransferase 1 |
| HRP | horseradish peroxidase |
| Ifu | infectious units |
| kDa | kilo Dalton |
| kbp | kilo basepairs |
| l | liter |
| µg | microgram |
| µl | microliter |
| µM | micro Molar |
| mA | milli Amper |
| mg | milligramm |
| ml | milliliter |
| mM | milli Molar |
| min | minute |
| MNU | N-methyl-N-nitrosourea |
| MOI | multiple units of infection |
| mRNA | messenger ribonucleic acid |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| ng | nanogramm |

| | |
|---|---|
| nm | nanometer |
| ON | over night |
| PAGE | polyacrylamide gel electrophoresis |
| PARP | poly (ADP-ribose) polymerase |
| PBS | phosphate-buffered saline |
| PBS-T | phosphate-buffered saline containing tween |
| PCR | polymerase chain reaction |
| PR | progesterone receptor |
| qPCR | quantitative polymerase chain reaction |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| pTP | preterminal protein |
| Rb | retinoblastoma |
| RGD | Arg-Gly-Asp motif |
| RNA | ribonucleic acid |
| RT | room temperature |
| SDS | sodium dodecyl sulphate |
| sec | second |
| TBS | tris-buffered saline |
| TBS-T | tris-buffered saline containing tween |
| TP | terminal protein |
| TSP | tumor specific promoter |
| U | units |
| UTR | untranslated region |
| VEGEF | vascular endothelial growth factor |
| WT | wild type |
| 4E-BP | eIF4E Binding Protein |

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# 12. Eidesstattliche Erklärung

Ich versichere, dass ich die mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten, und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

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Teile der vorliegenden Dissertation sind unter folgenden Titeln veröffentlicht worden:

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..... .....

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# 13. Lebenslauf

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|---|---|
| **Promotionsarbeit**:<br>(seit 03/2009) | AG Stoff-Khalili, Institut für Virologie, Universität zu Köln<br>„Oncolytic Virotherapy for Breast Cancer" |
| **10/2003 bis 01/09**<br>Diplomarbeit:<br>Titel: | Diplom Biologie an der Universität zu Köln, Note: sehr gut (1)<br>AG Prof. Thomas Langer, Universität zu Köln<br>„Characterization of PRELI-like proteins in mouse embryonic fibroblasts" |
| **2003 Schulabschluss** | Städt. Johann-Gottfried-Herder-Gymnasium, Köln<br>Abitur: 2,2 (Leistungskurse: Mathematik, Biologie) |

**Auslandserfahrungen**

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|---|---|
| **Auslandssemester SS 2006** | Study Abroad, Science, University of Sydney |
| **Collaboration**:<br>(30.03.2009 - 23.09.2009,<br>05.09.2011 – 31.03.2012) | LSU Health Sciences Center, Department of Cellular Biology and Anatomy, Group of Prof. Dr. M. Mathis, Gene Therapy, Shreveport, LA, USA |

**Meetings**

| | |
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| **21-25.11.2009** | ESGCT Congress in Hannover, Germany<br>Poster: **Triple level targeting of an oncolytic adenovirus for effective killing of breast cancer cells,** Sabrina Brueggemann, Rosa Franco Guo, J. Michael Mathis, Mariam Stoff-Khalili |
| **22-25.10.2010** | ESGCT Congress in Milan, Italy<br>Poster: **Generation of an adenoviral chimeric fiber 5/3 incorporating a transcriptional and translational control element,** Sabrina Brueggemann, Rosa Franco Guo, Ute Sandaradura de Silva, Baki Akguel, H. Pfister, M.A: Stoff-Khalili, J.M. Mathis |

**Praktika**

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|---|---|
| 23.10.2000 – 3.11.2000 | Behindertenheim in Cork (Irland), Tätigkeit: Betreuer |
| 16.03.2005 – 24.03.2005 | Gemeinschaftspraxis für Laboratoriumsmedizin<br>Dres. med. Wisplinghoff und Kollegen, Tätigkeit: Laborantin |

**EDV-Kenntnisse** Microsoft Word, Excel und Powerpoint, CorelDraw, Vector NTI, Sigma Plot, BD Quantity One

**Sprachkentnisse:** Englisch (sehr gut), Französisch (Schulkenntnisse), Spanisch (Schulkenntnisse)

**Nebentätigkeiten** seit Jul. 2000 , Reisswolf GmbH, Köln, Büroaushilfe
seit Juni 2003 - 2007, TOYS'R'US Köln, Kassiererin/ Aushilfe

**Freizeitinteressen** Basketball (Damenmannschaft, TV Dellbrück seit 2005)
Tanzen (Standardtänze, Hip Hop seit 2003)

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Datum Sabrina Brüggemann