A Novel Method for Construction of New Viral Vectors with Site Specific Modifications

Di Yu

Degree project in applied biotechnology, Master of Science (2 years), 2009
Examensarbete i tillämpad bioteknik 45 hp till masterexamen, 2009
Biology Education Centre and Department of Oncology, Radiology and Clinical Immunology, Uppsala University
Supervisor: Professor Magnus Essand
A Novel Method for Construction of New Viral Vectors with Site Specific Modifications

Di Yu

Abstract

It is getting popular to use recombinant oncolytic adenoviruses for cancer therapy. Adenoviruses can be modified to selectively infect or replicate in cancer cells. However, there is a need to further improve targeting, cancer cell killing capacity and hiding ability of the virus from the host immune system. Due to technical limitations, currently used vector construction systems focus on modification of the adenoviral E1 region and there is a need for systems that allow for genome wide modification. This study presents a new method for circular bacmid rescue of linear wild type viral DNA, which makes it much easier to do viral construction from scratch. In addition, with the help of a counter-selection marker, the new method gives a scarless modification at any desired site regardless of restriction enzyme.
# Index

Introduction ...................................................................................................................................... 1  
Gene Therapy.................................................................................................................................... 1  
Viral Vector....................................................................................................................................... 1  
Adenoviruses...................................................................................................................................... 2  
  Biology of Adenovirus .............................................................................................................. 2  
  Adenoviral vector system ......................................................................................................... 4  
Oncolytic virus used for cancer therapy ........................................................................................... 8  
Recombineering .............................................................................................................................. 9  
Screen, Selection and Counter-selection ....................................................................................... 9  
Aim .................................................................................................................................................. 10  
Results .......................................................................................................................................... 10  
  Bacmid rescue .......................................................................................................................... 10  
  Site specific modification ........................................................................................................ 11  
Discussion ...................................................................................................................................... 13  
Material and Methods .................................................................................................................... 14  
References ................................................................................................................................... 18
Introduction

Gene Therapy

Gene Therapy means introducing a gene into targeted cells or tissues of an individual and that the gene product should cure or lower down the progression of a disease. The first human gene therapy procedure was taken on in 1990 on a child with a severe combined immunodeficiency called adenosine deaminase (ADA). The patient was given a transfusion of T lymphocytes that had been genetically modified to carry a normal copy of ADA gene.\(^1\) Although ADA could be partly controlled by artificial drug, the doctor hoped for a permanent cure. Since then gene therapy has been developing rapidly aiming at treating both acquired and inherited diseases. The ADA treatment is an example of a monogenic inherited disease, where the non-functional gene was replaced or corrected. Gene therapy for acquired diseases such as cancer can involve a gene product or a biological reagent that directly or indirectly kills the malignant cells. Currently more than 1500 clinical trials are ongoing all over the world, most of them in the US and the UK (Available from: http://www.wiley.co.uk/genetherapy/clinical/).

One limiting step for successful gene therapy is gene delivery.\(^2,3\) An efficient vector is needed for therapeutic gene delivery into the target area without any substantial toxicity to other cells. The vector should be easily controlled for targeting when administrated systematically with low immunogenicity to prevent activation of immune response.

Viral Vector

Vectors can be divided into nonviral and viral vectors based on biological activity. Viral vectors are attractive as gene delivery vectors because of their efficient cell entry mechanism and their ability to transduce both dividing and non-dividing cells. According to their molecular features, vectors are also classified into DNA-based and RNA-based vectors. The features of the most commonly used viral vectors are concluded in Table 1. Human adenovirus-based vectors have been widely used for transduction of various cell types in basic research to study gene function, in gene therapy applications and in vaccine development.

<table>
<thead>
<tr>
<th>Features</th>
<th>DNA-based Vectors</th>
<th>RNA-based Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. Insert Size (Kb)</td>
<td>Unlimited</td>
<td>7.75</td>
</tr>
<tr>
<td>Concentration (VP/mL)</td>
<td>Unlimited</td>
<td>&gt;10(^10)</td>
</tr>
<tr>
<td>Delivery Route</td>
<td>Ex/In vivo</td>
<td>Ex/In vivo</td>
</tr>
<tr>
<td>Integration</td>
<td>Very poor</td>
<td>Yes</td>
</tr>
<tr>
<td>Expression in vivo</td>
<td>Short</td>
<td>Yes</td>
</tr>
<tr>
<td>Stability</td>
<td>Very good</td>
<td>Good</td>
</tr>
<tr>
<td>Pre-existing host immunity</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>ADV(^1)</td>
</tr>
<tr>
<td></td>
<td>AAV</td>
<td>&gt;10(^{12})</td>
</tr>
<tr>
<td></td>
<td>HHV</td>
<td>&gt;10(^8)</td>
</tr>
<tr>
<td></td>
<td>VACV</td>
<td>&gt;10(^9)</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>7.75</td>
</tr>
<tr>
<td></td>
<td>Lentivirus</td>
<td>7.75</td>
</tr>
<tr>
<td></td>
<td>Alphavirus</td>
<td>5</td>
</tr>
</tbody>
</table>

1 ADV: adenovirus; AAV: adeno-associate virus; HHV: Human herpesvirus; VACV: vaccinia virus; RV: retrovirus.
Adenoviruses

Biology of Adenovirus

Adenoviruses are nonenveloped viruses composed of a protein capsid and a double-strand linear DNA genome (Fig 1). The whole genome of the virus is about 30-38kb in size and contains 30-40 genes. There are over 52 serotypes in humans, which subsequently are divided into 6 subgroups (A-F). Except for serotype 14, which can be lethal to humans, most of the adenoviral infections are nonfatal and cause upper respiratory tract infections and eye infections.4, 5

![Figure 1. A schematic structure of adenovirus](image)
The capsid contains hexon, fiber and penton base proteins. The double stranded DNA is embedding in the capsid with terminal proteins attached at both end of the linear DNA.

During entry of adenovirus into a host cell, there are two sets of interactions between the virus and the host cell. The infection starts with a docking attachment between the adenoviral fiber knob domain and the cell receptor. Two receptors have been studied: CD46 for subgroup B6, 7 and the coxsackie/adenovirus adhesion receptor (CAR) for other human adenovirus serotypes8. There are also reports showing that heparan sulfate glycosaminoglycans (HS-GAGs)9, CD8010, CD8610, sialic acid11-14, MHC-I molecule15, 16 and scavenging receptors (SR)17 are alternative docking routes. The secondary interaction involves the RGD motif on the penton base binding to a αv integrin molecule on the cell surface. This binding activity simulates actin polymerization and is followed by endocytosis of the virus particles via clathrin-coated pits.18
The binding of adenovirus fiber knob to the CAR receptor initiates adenovirus internalization. This is followed by the interaction between the RGD motif on the penton surface with $\alpha_v$ integrins. This co-receptor stimulates virion particle entry into the host cell via receptor-mediated endocytosis. After internalization, the endosomes acidifies, which allows the viral particle to be released into the cytosol and consequently the virion will be translocated into the nucleus to begin gene expression.

Once the adenovirus has successfully entered into the host cell the pH value lowers in the endosome, which leads to disassociation of the fiber and penton base from the capsid. This causes the endosome disruption and is followed by virion escape from the vesicle to the cytoplasm. The virus is translocated to the nuclear pore with the help of cellular microtubules. The adenovirus particle disassembles and the viral DNA is injected in the nucleus. The virus DNA starts to bind to histone molecules and subsequently initiates viral gene expression, viral DNA replication and new virus production (Fig 2).

The replication of adenovirus relies on the host’s replication machinery. Adenovirus genes are separated by expression pattern, into two groups: early genes (E1-E4) occurring before, and late genes (L1-L5) occurring after DNA replication. The early gene products are mainly non-structural, regulatory proteins, which are responsible for the activation of other virus genes and induction of host proteins that are necessary for DNA synthesis (E1); viral DNA synthesis (E2); modulation of the host immune response and escape from host immune scavenging (E3) and the regulation of DNA replication, mRNA transport and apoptosis (E4). The late genes (L1-L5) are driven by a major later promoter (MLP). The late genes are mainly coding for structural proteins such as fiber, penton and hexon, which are major proteins of the viral capsid, as well as proteins involved in viral assembly. Once the viral components have successfully been replicated the viral DNA is packaged into its protein shell, the virus capsid. There is an encapsidation sequence at the beginning of the viral genomic DNA, which is necessary for packaging. Finally, virus is released from the cell as a result of virally induced cell lysis.
The first adenoviral gene to be transcribed is E1A, which encodes proteins binding to many cellular proteins such as retinoblastoma (pRb). The binding to pRb leads to release of E2F which can activate DNA replication and synthesis.\textsuperscript{21} To avoid E1A induced premature host cell death, two E1B proteins (19kD, 55kD) are expressed. The 19kD protein acts as anti-apoptotic factor (Bcl2 homolog), while the 55kD protein binds to p53 and inhibits cell apoptosis.\textsuperscript{22} E1A is also needed to induce the other viral genes and hence essential for viral replication. Therefore, adenoviruses lacking the E1A gene are replication defective.

The hexon protein is the most abundant protein of the adenoviral capsid. There are 7 hypervariable regions (HVR1-7) in the hexon containing the majority of sequence variability among adenovirus serotypes. HVRs are coding for amino acids exposed on the outer surface of the adenovirus (Fig 3).\textsuperscript{23} Report shows that these exposed amino acids contain key Ad5-specific neutralizing antibody epitopes.\textsuperscript{24} By replacing the corresponding HVRs from Ad5 to Ad48 (Ad5HVR48), Roberts and colleagues successfully circumvent pre-existing anti-vector immunity.\textsuperscript{24} Recently, Waddington shows that liver transduction of adenovirus serotype 5 is mediated by binding of coagulation factor X (FX) to hexon. However, neither Ad48 nor Ad5HVR48 bind FX and liver toxicity was therefore abolished.

![Figure 3. Ad5 hexon structure (Protein Data Bank 1P30)](image)

Adenoviral vector system

An adenovirus can be modified to a gene delivery vector by replacing the E1 region with a gene expression cassette.\textsuperscript{26} These kinds of viral vectors are produced in a helper cell line such as 911 or 293, where E1 is provided \textit{in trans}. Because of the ability to infect both dividing and non-dividing cells, adenoviral vectors are often used in basic research studies as well as in cancer gene therapy applications. The first generation of replication-defective vectors has E1 and E3 deletion, which allow for a maximum of 7kb of DNA insertion. Such vector can be produced at high titers ($>10^{12}$VP/mL) and are widely used for gene delivery. However, due to the homologous regions
between helper cell lines and viral vector DNA, wild type-like virus (replication-competent adenovirus, RCAds) can occur through recombination during viral production in both 911 and 293 helper cell lines. This gives a limitation on the application of the first generation adenoviral vectors. A later generation of replication-defective adenovirus vector has also been developed, which has further deletions in the on E2 and/or E4 region to lower down the risk by recombination. Moreover, the new generation vectors enlarge the cloning capacity up to 30kb.

Among the different kinds of adenoviral vector systems, the most widely used one is called AdEasy and was developed by Tongchuan He in the laboratory of Bert Vogelstein. The AdEasy is a replication-defective vector system, where the construction is based on homologous recombination. The system has a backbone plasmid called pAdEasy, which contains most of the wild type adenovirus serotype 5 genome, and a pShuttle vector which is designed for cloning genes of interest or gene expression cassettes into the E1 region (Fig 4). The full length adenoviral DNA is generated by recombination between the pShuttle vector and the pAdEasy backbone vector in the homologous recombination prone E.coli strain BJ5183.
Figure 4 Generation of recombinant adenovirus using the AdEasy system | The gene of interest is cloned by an ordinary cut-ligation procedure into the pShuttle transfer vector. This is followed by a Pmel restriction enzyme linearization of the shuttle vector and co-transformation together with the pAdEasy I backbone plasmid into E.coli BJ5183, where recombination occurred and the viral genomic construction is achieved. After PacI digestion of the circular recombinant plasmid, the linear double strand DNA is transfected into mammalian packaging cells (911 or 293), which contain E1 in trans, to produce the viral vector.

In Parallel, Mizuguchi and colleagues developed an in vitro ligation-based adenoviral vector system which simplifies the cloning procedure during virus construction. The main principle is that this system utilizes the unique homing enzymes I-CeuI and PI-SceI to allow insertion of the gene of interest by in vitro ligation (Fig 5).
Figure 5. Construction of recombinant adenovirus vectors based on an in vitro ligation method. The gene of interest is first cloned into the multiple cloning site of the pHM3 shuttle plasmid. Then the gene cassette is moved into the adenovirus backbone vector using I-CeuI and PI-SceI enzyme digestion. The ligation is performed in vitro and the recombinant adenoviral vector is amplified in a normal E. coli strain DH5α. Finally, the virus is also packaged in a helper cell line such as 911 or 293.

Another system called the RAPAd expression system was developed by Anderson and colleagues. This system has a shuttle plasmid for insertion of the gene of interest and a backbone plasmid which lack the left ITR, encapsidation signal sequence and E1 region. The linear whole adenoviral genome is rescued by a recombination occurring in a helper packaging cell line such as 293. With this system, the work labor during viral production is dramatically reduced. The general idea of how this system works is illustrated in figure 6.
Figure 6. An illustration showing the main principle of the RAPAd expression system. First, the gene of interest is cloned into the multiple cloning site (MCS) of shuttle vector. Then, the shuttle vector and backbone vector are linearized by PacI. This is followed by a co-transfection into the packaging cell line 293. The viral DNA is rescued by recombination in the packaging cell line.

**Oncolytic virus used for cancer therapy**

For virus-based cancer gene therapy, the main idea is to use oncolytic viruses to kill the cancer cells by the lytic capacity during the virus infection stage. Conditionally replicating adenoviruses (CRAds) are often used. CRAds contain at least the native E1, E2, E4 and late genes. They have an
intact ability to infect cells, replicate therein, lyse the cells and release the new viral particles to get a secondary infection of neighboring cells. By combining three types of modification: targeting, arming and shielding, oncolytic viruses can obtain high specificity to cancer cells without losing lytic capacity. Targeting improves safety, arming through the expression of therapeutic genes improves efficacy and coating with low immunogenic molecules provides shielding from the host immune response with increased circulation time and as a consequence improved targeting.

**Recombineering**

Recombination-mediated genetic engineering, termed as recombineering, is a method to construct or modify DNA vectors based on homologous recombination in vivo (usually in an E. coli strain) using recombinase from λ phage. It is a powerful method for fast and efficient construction and modification of vectors. Episomes in the bacteria can be modified using linear PCR products (double stranded) or synthetic oligonucleotides (single stranded) as substrate with as short as 35-40 base pair homologous region at both ends.

The bacterial strains used for recombineering should be able to express the bacteriophage recombination system, which involves the genes gam, exo and bet. gam prevents degradation of linear DNA introduced into the host bacteria. exo has 5'-3' exonuclease activity that creates single-stranded overhangs on introduced linear DNA. bet protects these overhangs and help the following recombinant process. Donald Court and colleagues developed an E.coli strain, SW102, containing the λ recombination system in its genome. These genes are transcribed by a λPL promoter and tightly controlled by a temperature-sensitive cI857 repressor. When the bacteria are cultured at low temperature (30-32°C), recombination proteins are repressed. However, after about 15 minutes incubation at 42°C, the promoter is activated and a sufficient amount of recombinase are produced for the recombination process. The linear DNA substrate, with sufficient homologous ends at both 5’ and 3’ to the target vector already present in the bacteria, can be easily introduced into the bacteria by electroporation. Recombinants are obtained by selection against antibiotic markers.

**Screen, Selection and Counter-selection**

As the name implies there are two ways for selection, positive and negative. A positive selection is based on introducing a gene into E.coli which makes the bacteria resistant to antibiotics, while a negative selection is based on introducing a gene which makes the bacteria sensitive to some kind of substrate. In the present study, we use the bla gene for ampicillin resistance as a positive selection, and the sacB gene, which makes the E.coli sensitive to sucrose, is used as a counter-selection marker. lacZ is a gene involved in the lac operon for lactose catabolism, which naturally is controlled by the lacI repressor naturally. Here, we use IPTG to induce the expression of the lacZ gene and the gene product β-galactosidase could digest the substrate X-Gal and make the colony appear blue.
Aim

The aim of the investigation presented in the thesis is to characterize and develop a rapid method for viral vector construction from wild type linear DNA with the possibility to modify sequence anywhere in the genome, that is more potent and flexible than the ones currently used for gene therapy.

Results

Bacmid rescue

The natural adenoviral DNA is linear and hard to manipulate. If the DNA is inserted into a selectable plasmid/bacmid, it will become much easier to modify and to scale up by replication E.coli. To construct the bacmid harboring the full-length wild type adenovirus serotype 5 (Ad5) genomic DNA, the E.coli strain SW102 was used for mini-λ-mediated recombineering. A bacmid backbone was PCR amplified from pAL1143. Linear genomic adenovirus DNA was together with the PCR product co-electroporated into heat-shock induced SW102. The obtained bacmid called pBACAd5s(wt) was rescued by antibiotic selection and verified by restriction enzyme digestion (Fig 7). The bacmid contains an expression cassette with a RSV promoter driving the homing enzyme I-SceI in its backbone and two I-SceI sites at both ends of its sequence. This enables the bacmid to self-split after transfection into the mammalian cell to release the linear viral DNA.
Figure 7 A schematic illustration of the circular bacmid rescue | By co-transforming the PCR amplified bacmid backbone together with the viral DNA into heat-shock induced SW102, the circular bacmid is rescued by recombineering. Positive colony is selected out by Cm resistance.

Site specific modification

pBACAd5s(wt, HVR5_FWKT) was generated by selection, counter-selection modification. First, a selection cassette consisting of the ampicillin resistance gene, the lacZ gene and the sacB gene (als) was amplified by PCR to introduce the homologous region to the targeted site, in our case
the hypervariable region 5 (HVR5) of the adenoviral hexon protein. By recombineering, the als cassette was successfully inserted and verified by restriction enzyme digestion. Thereafter, a small fragment containing four amino acids from the somatostatin molecule (FWKT) was PCR amplified again to introduce the homologous region to the right insertion site. The idea being to retarget adenovirus infection to tumor cells that are over-expressing somatostatin receptors and at the same time block the binding of blood factors to the adenovirus capsid. By a second recombineering, the als cassette was replaced by the FWKT motif and positive recombinants was selected against sucrose resistance according to the counter-selection marker (Fig 8). The positive recombinant was designated pBACAd5s(wt, HVR5_FWKT).

**Purification of PCR production**

![Diagram of PCR production process](image)

*Figure 8. A schematic illustration of the selection, counter-selection method for adenovirus modification* | First, a 4.2kb selection, counter-selection cassette containing ampr, lacZ and sacB (als) is PCR amplified and...
homologous sequence of the desired region is induced from the primer sequence. The als cassette is inserted by recombineering into the site where the modification would take place, in our case in the hypervariable region 5 (HVR5) of the adenoviral hexon protein. The next step is the replacement of the als cassette by the desired sequence using 6% sucrose LB selection.

**Discussion**

We have developed a straight-forward and efficient method for constructing viral vectors from natural viral genomic linear DNA. Theoretically, this method is fit for inserting all kind of linear DNA into a selectable plasmid or bacmid backbone. Moreover, by using a selection, counter-selection cassette, any site on any kind of single copy bacmid can be modified.

The most widely used adenovirus for gene therapy applications is serotype 5. The pAdEasy system, which is the most used system in basic research and application research utilize Ad5 and employs the homologous recombination machinery in E. coli BJ5183, which need nearly 1kb of homologous region for the recombination. A recombinant adenovirus is generated by co-transformation of an adenoviral backbone vector and a shuttle vector carrying the gene of interest in the E1 region. In parallel, Mizuguchi and colleagues developed an in vitro ligation-based method to generate E1-deleted adenovirus by using two homing enzyme I-CeuI and PI-SceI, which recognition sites are seldom present in most of the genes. This method reduces the plasmid construction time and improves the throughput of the Ad-constructions. The RAPAd expression system has the adenoviral backbone lacking the left ITR, encapsidation sequence and E1 gene. After co-transfection the backbone plasmid recombines with the shuttle vector, in the packaging cell line. This rapid system gives as high fidelity as the AdEasy system during viral production and reduces the work labor during recombinant selection compared to the AdEasy system or the ligation-based system.

However, because all the systems described above are based on the serotype 5 and only modifies E1, shortcomings still remain. First, almost all adults and 23% of the infants have antibodies against adenovirus serotype 5 and more than half of them (57%, n=53) have neutralizing antibodies. Because of pre-existing antibodies, the virus will be cleared by host immune response, when gene-modified adenovirus is given systematically. To overcome this shortcomings, scientists now try to develop adenoviruses based on serotype 35 and serotype 11, but both of the new vector constructions are based on a serial of PCR and subclonings. It costs labor work and is time consuming. The new method presented herein simplifies the process to insert the viral DNA into a bacmid. By a single step of PCR amplification of the bacmid backbone to introduce the homologies, the circular bacmid can be rescued after recombineering in SW102. Moreover, the method is available for any kind of viral DNA, which allows changing the serotype of adenovirus delivery vector.

Second, Ad5 based vectors have a CAR-dependent tropism, which lowers the transduction efficiency of CAR negative or CAR low-expressing cells, which means that it narrows down the application. For example, endothelium, smooth and adult skeletal muscle, brain tissue, differentiated airway epithelial tissue, primary tumors and hematopoietic cells are low in CAR expression. Chimeric recombinant adenovirus 5 with altered the tropism such as Ad5/f35, which have an Ad5 viral capsid and a fiber from Ad35 has been developed. However, to construct...
such a chimeric virus by traditional subcloning (restriction enzyme/ligation) is hard because of the large size of the viral DNA. Moreover, all the adenovirus vector systems so far have been made for insertion of a foreign gene into the E1 region. It is almost impossible to modify other sites of the viral genome. As a matter of fact for cancer therapy application, it is desirable to make the virus with improved targeting, and better shielding of host immune response. This can be done through modification of the hexon to abolish coagulation factor X binding and reduce liver toxicity\textsuperscript{52, 53} or by modification of the capsid protein pIX for viral tracking\textsuperscript{54} or by displaying peptides on the fiber knob for altered tropism\textsuperscript{55} or improved transduction efficiency.\textsuperscript{56} All these mentioned modification can hardly be done in the currently used vector systems.

The new method presented herein could easily make scarless modification in any desired site of the genome using an appropriate counter-selection marker such as sacB. It reduces the construction time to only 2 weeks. This hopefully could improve the usage of viral vector in cancer gene therapy.

## Material and Methods

### Material

E. coli strain SW102 and bacmid pAL1143 were kind gifts of Richard Stanton (Cardiff University, UK). Wild type adenovirus serotype 5 genomic DNA was prepared using High Pure Viral Nucleic Acid Kit from Roche (Cat. 11858874001). pUC_FWKT was an artificial synthesized plasmid purchased from Genescript. All restriction enzyme were purchased from New England Biolabs. E.Z.N.A.® MicroElute® Gel Extraction Kit was from Omega, Inc. Lysogeny broth (LB) medium was produced from Rudbeck Lab technique support. IPTG, X-Gal, and all antibiotics were from Sigma-Aldrich. All selection LB media and selection LB agar plates are produced by adding an appropriate antibiotic into LB or LB agar. Concentration of ampicillin (Amp) is 100μg/mL. Chloramphenicol (Cm) is at a concentration of 15μg/mL.

### PCR

All PCR was performed using Phusion DNA polymerase (Finnzymes, Cat. F-548S). The product is then digested by DpnI for 3 hours and purified by gel extraction. The purified DNA was quantified by Nanodrop.

### Induction of recombination system by heat-shock

A single colony of SW102 containing desired bacmid was inoculated into 3 ml LB(Cm) and cultured overnight at 32°C, 225rpm. 0.5ml of the overnight culture was then transferred into 10ml LB(Cm) and cultured until the OD\textsubscript{600} value reached 0.4. This is followed by a 15 minutes incubation at 42°C with shaking and then the culture was cooled down by shaking the tube in ice-water slurry for 15-20 minutes.

### Competent Cell Preparation

The bacteria were collected by 5 minutes centrifugation at 2000xg, 0°C and washed twice using ice-cold H\textsubscript{2}O. The pellet was then resuspended in 400uL ice-cold H\textsubscript{2}O by gently shaking.
Transformation by electroporation
A 100μL aliquot of the induced competent SW102 was mixed with 100ng purified PCR product and transferred into pre-cooled 0.2cm cuvette. Electroporation was performed at 2.50kV, 200ohm, 25μF (Gene Pulser II, Bio-Rad). The bacteria were recovered in 1ml LB for 1 hour at 32°C with shaking, unless specified differently. All bacteria were plate onto LB agar plate with appropriated antibiotic/s and incubated for 30 hours at 32°C.

Bacmid minipreparation
Since the bacmid used here is remaining in a single copy, the minipreparation is modified as follows using the Sigma miniprep kit. Single colonies were inoculated into 5ml LB(antibiotic) and grow overnight at 32°C. 4.5 mL bacteria were pelleted by centrifugation. The cell pellet was resuspended in 200μl buffer P1, lysed by adding 200μl buffer P2. Cell debris were aggregated by adding 200μl P3 buffer and followed by 10 minutes centrifugation at 15000×g. The clear supernatant containing DNA was transferred into a new tube. And the DNA was precipitated by adding 600μl isopropanol with a centrifugation at 15000xg for 10 minutes. DNA pellet was washed using 70% ethanol and redissolved in 30μl 10mM Tris pH8.5.

Bacmid rescue from viral genomic DNA
pAL1143 contains pBeloBAC11 derived bacmid backbone and a RSV promoter driven I-SceI expression cassette. A 8 Kb fragment containing bacmid backbone and the I-SceI expression cassette was amplified using a pair of primers as follow: pBACs_F(GCG CCA CGT CAC AAA CTC CA); pBACs_R(CAC GTC ACA AAC TCC ACC CC ). The PCR cycling conditions were 98°C for 30s followed by 35 cycles of 98°C for 8s, 67°C for 15s, 72°C for 4min, and a final extension step of 72°C for 10 min in a PTC-200 thermal cycler (MJ Research, USA). The PCR product was treated by DpnI and gel purified. Wild type adenovirus 5 genomic DNA was isolated using the Roche kit and quantified. The linear viral DNA and the PCR product bacmid backbone was mixed in a mole ratio at 1:100. Then the mixture was co-electroporate into heat-shock induced SW102. Cells were placed on LB agar plate containing Cm for overnight culture. The day after, 8 colonies were checked by miniprep and restriction enzyme digestion. SW102 with positive bacmid was maintained in -80°C with 30% glycerol supplied. The new bacmid was designed pBACAd5s(wt).

Construction of Ad vector with somatostatin receptor (SSTR) binding peptide FWKT in HVR5 region
A 4.2kb cassette (HVR5_als) containing the ampicillin resistant gene, the lacZ gene and the sacB gene was amplified from pAL1143 using primers as follows: HVR5_als_F (AAT GGA AAG CTA GAA AGT CAA GTG GAA ATG CAA TTT TTC CCT GTG ACG GAA GAT CAC TTC G); HVR5_als_R (CCA CTT TAG GAG TCA AGT TAT CAC CAT TGC CTG CGG CTG CCT GAG GTT CTT ATG GCT CTT G ). The PCR cycling conditions were 98°C for 30s followed by 35 cycles of 98°C for 8s, 61°C for 15s, 72°C for 1min 20s, and a final extension step of 72°C for 10 min in a PTC-200 thermal cycler (MJ Research, USA). The HVR5_als amplicons harbored a 50bp homology at both ends to the target sequence after amplification, which were induced by the primers. The HVR5_als was then transformed into the heat-shock induced SW102 which already contain pBACAd5s(wt). Positive colonies were selected against ampicillin resistance. With the help of LacZ gene, the positive colonies were also screened out as blue one and designed pBACAd5s(wt, HVR5_als). A 200bp fragment
(HVR5_FWKT) containing FWKT motif and with 80bp homologies at both ends to HVR5 region was also amplified using primers: HVR5_FWKT_F (AAG CAA CAA AAT GGA AAG C) and HVR5_FWKT_R (ATG AGT GTC TGG GGT TTC TAT). After heat-shock induction of SW102 containing pBACAdSs(wt, HVR5_als), the HVR5_FWKT fragment was electroporated in. Bacteria were selected against sucrose sensitivity by plating all the cells on 6% sucrose LB agar plate with Cm. White colonies were checked by restriction enzyme digestion.
Acknowledgements

I would like to thank all the persons who helped me finish this project and made my life enjoyable.

Magnus Essand, My supervisor and friend. Thank you a lot for giving me the opportunity; for all the help during this project. You touched me a lot not only your enthusiasm to science, but also your kindness and brilliant personality. You always have time to talk with me about the project, teach me how to drink wine. As a student, I still have a lot to learn from you, rock and roll for instance.

Berith Nilsson, Thank you very much for helping me with all the protocols and for being a kind person to be with.

Justyna Leja, PhD student and my secondary supervisor. Thank you so much for the help, not only concerning the experiment design, the critical thinking, but also how to use English to make a joke...

Angelika Danielsson, The first one to bring me into the project since day 1, and opened the door for me to get into the GIG corridor.

Nadim Majdalani, scientist working at the National Cancer Institute (NCI) in US. You are always available online to answer my questions. And teach me how to set up the counter-selection protocol. I will never forget that you reply my email with all the specifics and details with patience and prudence.

Linda Gustavsson, Ole Forsberg, Arian Sadeghi and all the other persons at our group of clinical immunology. It’s great that you all are very nice to a foreigner.

Chuan Jin, your love, your support for me, your patience and your understanding with me. Without you I am incomplete. Thank you!

最后，衷心感谢我的父亲，母亲。感谢你们几年来对我的养育之恩，一直以来对我的理解，支持以及关爱。

Di Yu
References