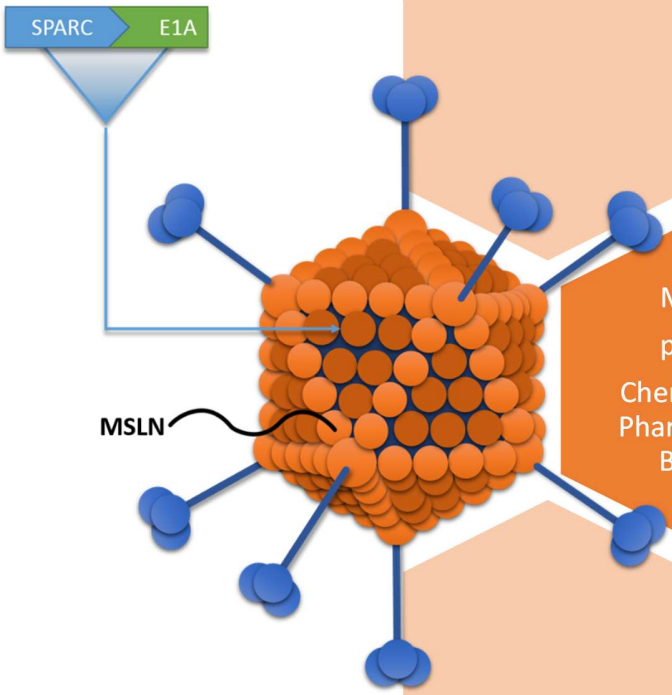
CRAd with capsid incorporated mesothelin for higher anti-tumor immunization



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Summary/Abstract

The adenovirus (Ad) has been widely used as a vaccine because of its unique characteristics as gene transfer vector. The conventional approach for using Ads as vaccine is the insertion of genes encoding the protein of interest into the expression cassette. An alternative approach is the capsid incorporation strategy. This strategy describes the incorporation of antigens into the capsid proteins to achieve higher immunization. To extent the use of the adenovirus, Ads have been modified to work as cancer therapy agents. These conditionally replicative Ad replicate specific in cancer cells, while sparing the normal cells. This project combined the two earlier mentioned strategies to design a vaccine for ovarian cancer. Specifically, the hypothesis testing Ad contains a SPARC promoter and the tumor peptides from mesothelin in capsid protein pIX. The hypothesis of this project is that CRAd with capsid-incorporated mesothelin gives higher anti-tumor immunization then single treatment of CRAd or mesothelin as a transgene. The five Ads for the test of hypothesis are all succesfully produced. It is possible that the mesothelin is not expressed on the capsid surface caused by non-incorporation. Therefore, the expression of mesothelin on the surface should be validated by western blot before immunization can be tested.

Introduction

Adenovirus

The adenovirus (Ad) is a double-strand DNA virus with a naked icosahedral protein capsid. (figure 1) This double-strand DNA is linear oriented and 36 kilobase pairs long. It contains four early regions (E1a/B, E2A/B, E3, and E4) and five late regions (L1-L5) (figure 2). The early regions, like their name indicate, are transcribed early in the viral reproductive cycle. Translation of these early regions results in the production of proteins which are involved in viral transcription and replication of viral DNA. The late regions are expressed later in the viral reproduction cycle and code for compounds of the capsid.

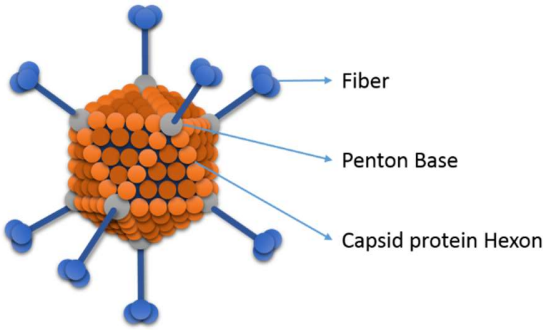


Figure 1: Adenovirus structure

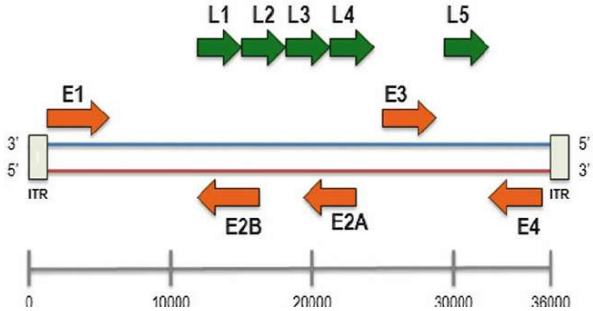


Figure 2: Schematic representation of the genome of the adenovirus. The DNA contains inverted terminal repeats at both ends. Genes are located on both DNA strands. [1]

Replication of the Ad starts with the entry of the Ad into the host cell. This entry is mediated by binding of the fiber knob to the coxsackievirus-adenovirus receptor (CAR).[2] This interaction is followed by a secondary interaction whereby a motif of the penton base interacts with an integrin molecule. [3] (figure 1) These two interactions together generate endocytosis of the virus into the host cell. In the endosome, the capsid components of the Ad will disband by the endosome acidity. The disbanding in combination with the penton toxicity cause elimination of the endosome and the virion is released into the cytoplasm. After the Ad is transported to the nucleus, the virus particles disassemble, and the DNA can enter the nucleus through the nuclear pore.

Adenovirus as Vaccine

The Ad is widely used for gene transfer and as a vaccine. [4] For vaccine applications, many advantages have driven the use. These advantages include the ability to infect a variety of cell types, transduce in replicating and non-proliferating cells, and the Ad genome is easy to manipulate. The essential characteristic is the ability to induce innate and adaptive immune responses. Based on this, many applications have been explored. These applications include malaria, HIV and cancer [5]–[7]. All these application Ads have been tested in clinical trials. Examples of these clinical trials are phase 3 studies with RTS,S/AS01 as a malaria vaccine and HIV-1 adenovirus subtype 35 vector vaccine [8], [9]. These trails have proven the high effectivity and safety of the Ad as a vaccine.

The conventional approach for using Ad as a vaccine is to insert antigen (Ag) genes into the expression cassette of the Ad genome. This insertion is mostly performed by placement of the Ag in the deleted the E1A/B region of the Ad genome. During viral reproduction, the inserted Ag will be produced. [10] The immune system can recognize the expressed Ag, and this results in an adaptive immune response. Importantly, the Ad capsid proteins induce an innate immune response during cell entry. This innate immune response enhances the immunogenicity when the Ad is used as vaccine vectors.

Alternative method for Ad as Vaccine

An alternative approach for inducing Ag immune response is the 'capsid incorporation' strategy. [11] (figure 3) This strategy describes the incorporation of antigen or epitopes into capsid proteins of the Ad. As a result, the Ag is displayed on the capsid proteins. The 'capsid incorporation' strategy result in the processing of the Ag by the exogenous pathway. This process leads to quantitatively and qualitatively distinct active immunization in comparison to the conventional approach. Based on this potential benefit, the capsid incorporation strategy may complement the immunity induced by Ag expressed as a transgene. [12], [13]

Another advantage of the capsid incorporation is the ability to circumvent the preexisting immunity against Ad5. Fifty to ninety percent of the adult population carries neutralizing antibodies (NAbs) against the Ad5. [14] These NAbs are generated against capsid proteins of the Ad5. The presence of these NAbs causes an immune reaction against the Ad5, resulting in Ad5 clearance. The capsid incorporation strategy can circumvent this clearance by disguises the capsid proteins with Ags. Therefore, Ad5 neutralizing epitopes on the capsid are not recognized by the NAbs. [15]–[17]

The capsid proteins of the Ad possess intrinsic flexibility allowing the Ag incorporation. Based on this, several capsid proteins allow the 'capsid incorporation' strategy: hexon (polypeptide II), penton base (polypeptide III), fiber (polypeptide IV), polypeptide VII (pVII) and polypeptide IX (pIX). (figure 4) The successfulness of incorporation depends on the flexibility and display opportunities of the various capsid proteins.

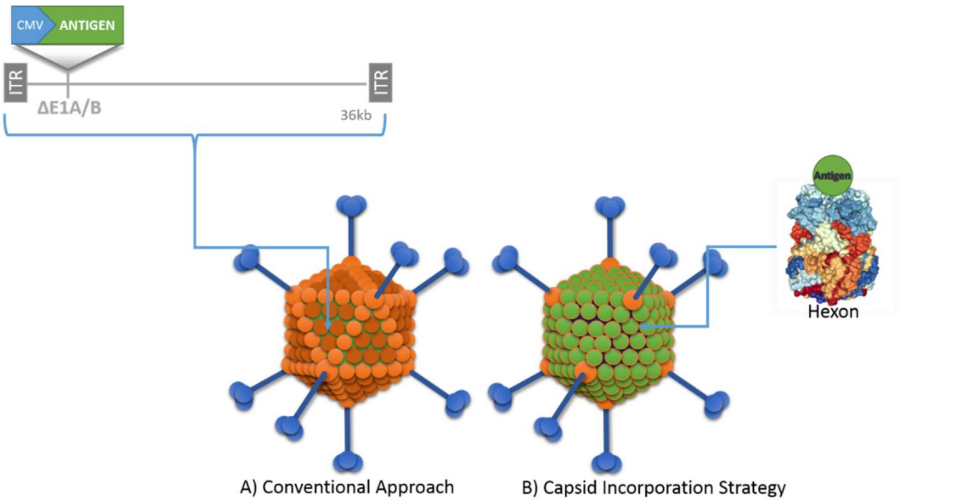


Figure 3: Representation of the conventional versus the capsid incorporated strategy
A) The antigen is inserted into deleted E1A/B and will be expressed during virus replication
B) The Ag is incorporated into the capsid proteins (for example hexon). The capsid proteins are shown in orange, and the Ag is colored green. [18]

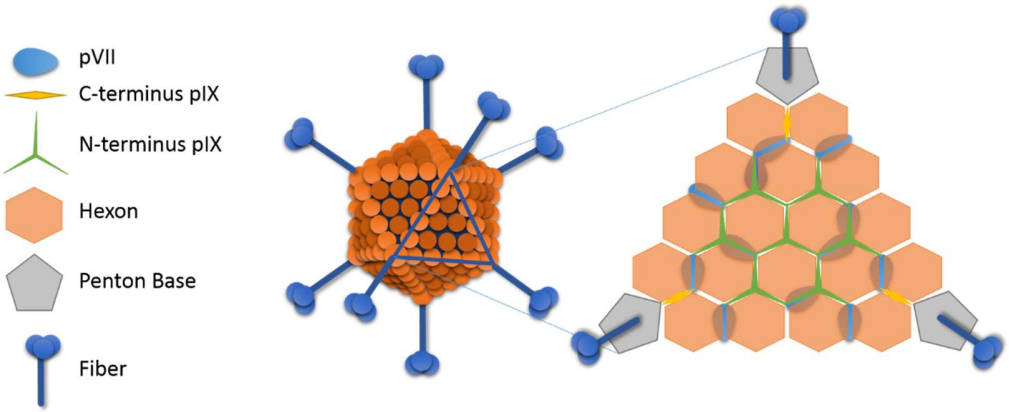


Figure 4: Schematic representation of the capsid proteins that allow Ag incorporation. The hexon, penton base, fiber, and pIX are capsid proteins and pVII is a core protein. [18]

Adenovirus as a Cancer Treatment

There are several clinical approaches for the use of adenovirus vector as a cancer treatment. Firstly, tumor antigen can be configurated into the expression cassette of the Ad genome. Expression of this tumor Ag results in an immune response against its Ag. [19] Secondly, as cancer gene therapy, the Ad is used as a delivery vector for anti-tumor genes into tumor tissue. [20] Lastly, Ad can be designed to specially replicate in tumor cells, while sparing the healthy cells. [21] These conditionally replicative adenoviruses (CRAds) cause cell death specifically in the tumor tissue.

CRAds as Cancer Treatment

As stated before, CRAds can specifically kill cancer cells while sparing healthy cells. The mechanism behind this principle is based on the exploitation of a tumor-specific promoter. The transcription factors which can bind to the tumor-specific promotor are exclusively present in the tumor cells. The transcription and production of new CRAds causes cell death. The new produced CRAds can subsequently infect and lyses surrounding cancer cells. [22] (figure 5) Moreover, CRAds can also activate the immune system to induce anti-tumor immunity. [23], [24] These two mechanisms together make CRAds appropriate cancer treatment vectors.

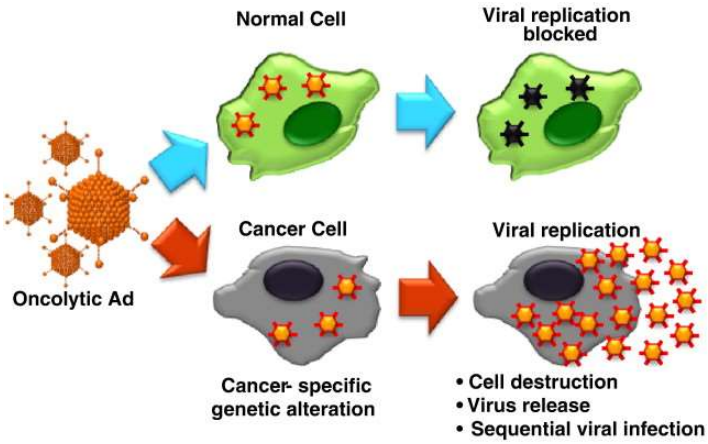


Figure 5: Viral replication of CRAds (oncolytic Ad) in normal and cancer cells [25]

Limits of current model for study of CRAd-based anti-tumor immunization

A mouse model to investigate anti-tumor immunization is not available because the human Ad replicates poorly in murine cells. [26] To overcome this biological block tumor xenograft has been employed. However, it is not possible to study anti-tumor immunity in tumor xenograft model. [27] To investigate the potency of oncolytic viruses, it is required to have an in vitro study accessible. On this basis, an immunocompetent murine cancer model that allows human Ad replication is necessary to investigate CRAd-based anti-tumor immunization.

Study for anti-tumor immunization: ID8 model

Different murine models including the murine syngeneic immunocompetent ID8 ovarian cancer models were tested to find an immunocompetent murine cancer model that allows human Ad replication. Surprisingly, the ID8 murine ovarian cancer cells lines were fully permissive of human adenovirus replication and CRAd-mediated cytolysis.[28] Furthermore, a new improved ID8 model with a knockout of p53 and Brac2 shows better growth, less survival, more immunogenic and better immunobiology. [29] Therefore, the new ID8 model provides a unique opportunity for evaluation of anti-tumor immunity capacity of CRAds.

Hypothesis

The goal of this study is to achieve anti-tumor immunization by the capsid incorporation of a tumor antigen in a CRAd-based vector. To be more specific, the tumor antigen mesothelin is incorporated into capsid protein pIX in a CRAd based on a secreted protein acidic and rich in cysteine (SPARC) promoter. (figure 6) Therefore, the hypothesis is CRAd with capsid-incorporated mesothelin gives higher anti-tumor immunization in the ID8 model then single treatment of CRAd or mesothelin as a transgene. The novel features of this approach are the incorporation of a tumor antigen into pIX and the combination of CRAd with the capsid incorporation of tumor antigen.

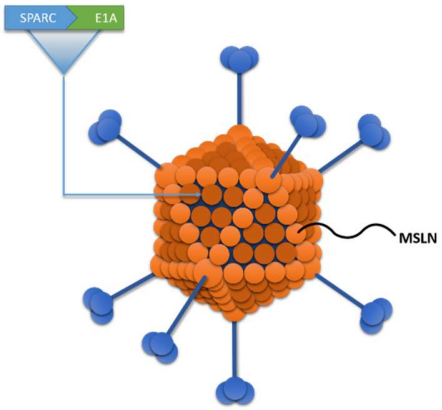


Figure 6: Hypothesis testing CRAd with MSLN incorporated into pIX capsid protein

Design of CRAd for use in ID8 model for test of hypothesis

For this hypothesis, a CRAd based on the tumor-specific promoter SPARC is designed. SPARC is a secreted glycoprotein that is overexpressed in many aggressive human cancers, including ovarian cancer. (figure 7) Furthermore, SPARC also plays an essential role in ovarian cancer growth, apotheosis and metastasis. Importantly, the SPARC promoter has been validated as an effective tumor-specific promoter for ovarian cancer. [30], [31]

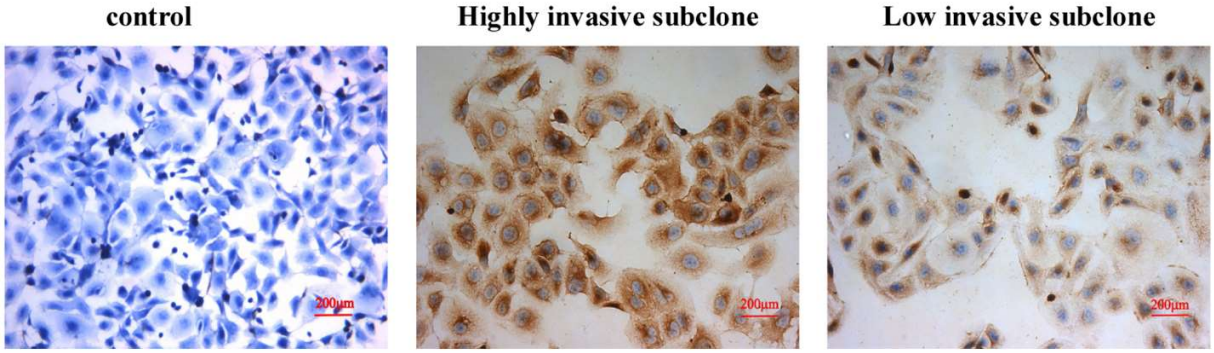


Figure 7: Expression of SPARC in highly invasive and low invasive sub-clone ovarian cancer. Tissue colored by immunohistochemistry. Brown staining indicates SPARC expression.[32]

Mesothelin as tumor antigen for test of hypothesis

The tumor antigen that is used for this test of hypothesis is mesothelin. Mesothelin (MSLN) is produced in the form of a precursor protein. The precursor protein contains two fragments: MSLN (40 kDa) and megakaryocyte-potentiating factor (MPF). The MPF is excreted, and MSLN stays bound to the cell membrane. (figure 8A) Mesothelin (MSLN) is highly expressed in ovarian cancer. (figure 8B) [33], [34] Preclinical studies, as well as results from initial clinical trials, have validated MSLN as an attractive target for cancer therapy with tumor vaccines.

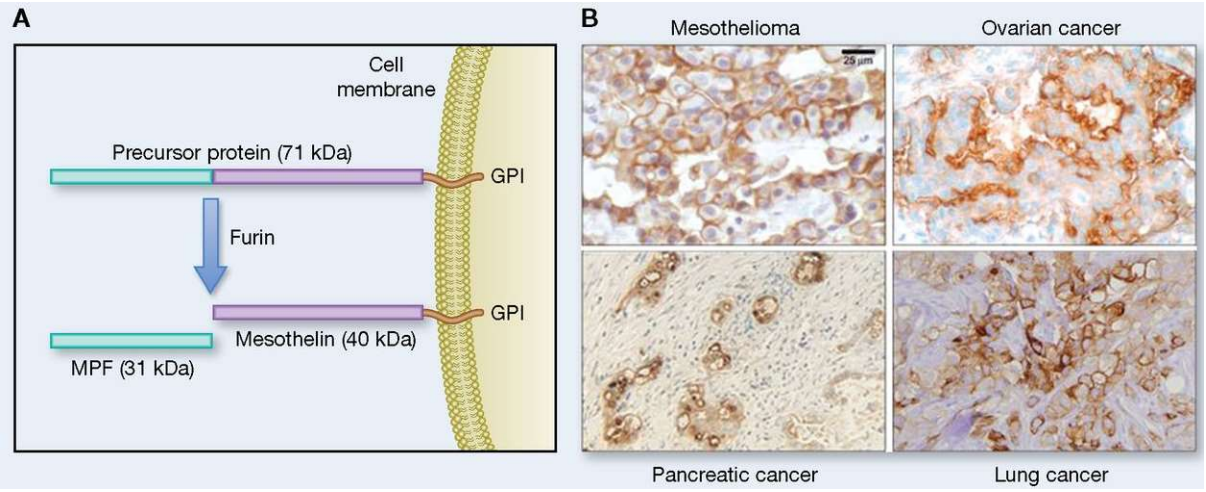


Figure 8A: Precursor protein of MSLN contain MSLN and secreted MPF fragment
Figure 8B: Expression of MSLN in mesothelioma and three cancer types by immunohistochemistry using an anti-mesothelin antibody. The MSLN positive tissue is colored brown by staining.[33]

Capsid incorporation of Mesothelin by pIX fusion for test of hypothesis

pIX is one of the minor capsid protein of the Ad. The pIX protein is located between nine major capsid proteins of the hexon in each facet of the capsid. (figure 9) Therefore, the pIX protein functions as a binding protein and stabilizes the capsid of the Ad. [35] In contrast to the other capsid proteins, pIX tolerates fusion of relatively large and functional proteins. [36], [37] Also, incorporation of proteins into pIX does not affect the function of the pIX protein. The pIX-display technology has also proven to be successful in preclinical vaccination studies for different antigens. [36]–[38]

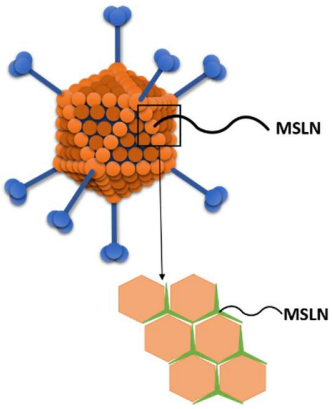


Figure 9: Schematic representation of Mesothelin incorporated in capsid protein pIX

For the test of hypothesis, epitopes of MSLN will be incorporated into pIX. The epitopes are selected based on MSLN-specific T cells derived from WT and MSLN$^{-/-}$ mice. In figure 10 the results of this epitope mapping are shown. The results show five epitopes that give an MSLN-specific T cell response in MSLN$^{-/-}$ mice. These five epitopes will be incorporated into pIX. Although pIX is structurally expendable, the C-terminal addition of excessive length cause non-incorporation. For this reason, an adenovirus with one epitope of MSLN is added to the design for testing the hypothesis. The epitope 406-414 gives an MSLN-specific T cell response in wild type and MSLN$^{-/-}$ mice. Therefore, this epitope will be used for incorporation into pIX. [39] This epitope is 9 amino acids long and contains 27 nucleotides.

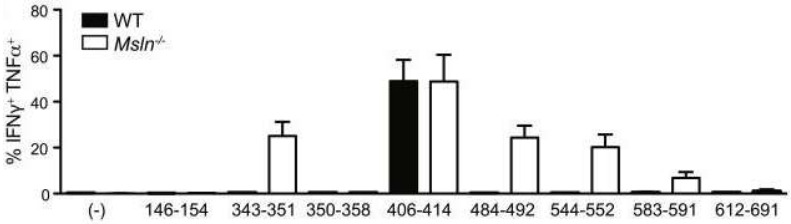


Figure 10: Epitope mapping of MSLN-specific T cells derived from WT and MSLN$^{-/-}$ mice [39]

Design testing hypothesis

The two hypothesis testing Ads are SPARC driven Ad with either one or five epitopes. The three controls Ads are a CRAd Ad with irrelevant peptide incorporated into pIX, a non-replicated Ad with MSLN peptide incorporated into pIX and a conventional Ad that expresses mesothelin. (figure 12) The first control of CRAd with the irrelevant peptide is designed to test the effect of MSLN incorporation. The second control of non-replicative Ad with MSLN incorporated in pIX tests the effect of the SPARC promoter. The last control is designed to test the difference between the capsid incorporation strategy and the conventional approach.

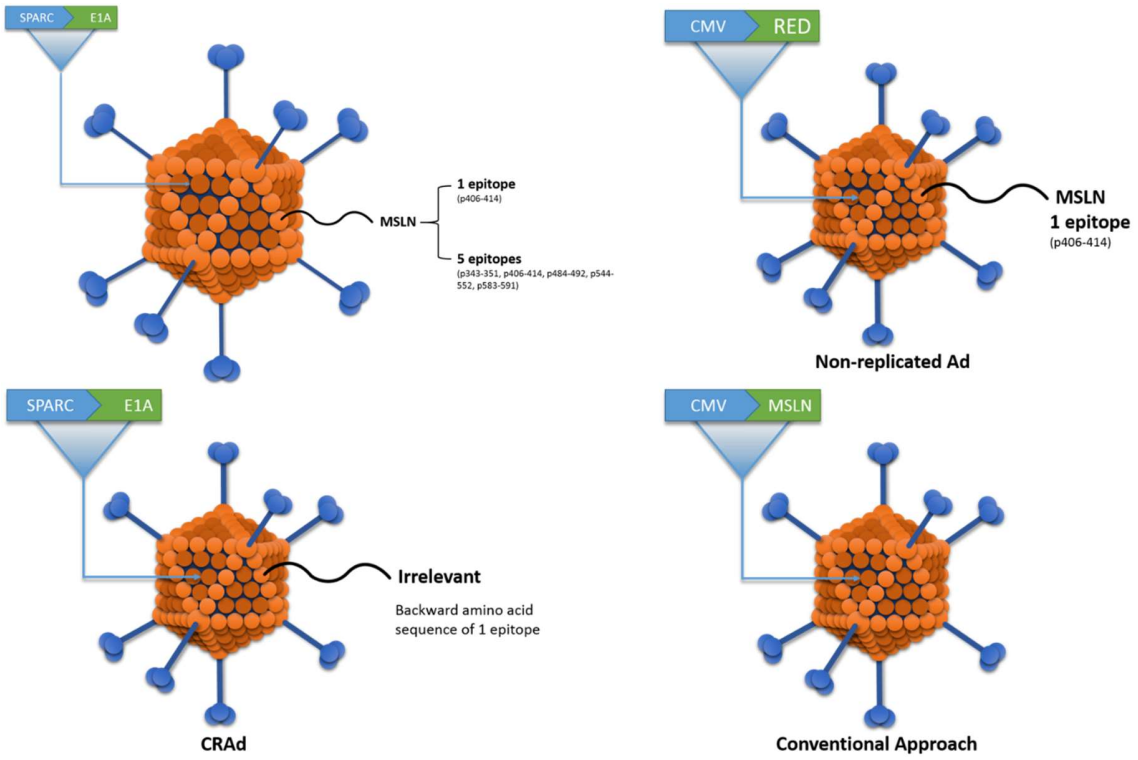


Figure 11: Construction of Adenovirus for test of hypothesis

Methods

Design shuttle vectors

One epitope design

Based on figure 10, the selected one epitope is p406-414 of MSLN. This region of MSLN is translated into an amino acid (AA) sequence. [40] The AA sequence is further translated into a nucleotide sequence by using triplet codes that are familiar in the protein pIX. This will increase the change for successful incorporation of the epitope. With the software Vector NTI®, the most used triplet codes for every AA in pIX are analyzed. (table 1) These results are used to translate the AA code into a nucleotide sequence. An example of this translation selection is AA Q at position 407. In the original MSLN, Q at p407 codes for the triplet CAA. Table one shows that in protein pIX the most used codon for Q is CAG. For this reason, the selected codon in the design is CAG. In addition, a BstBI restriction site is added in the epitope. The restriction site makes it possible to analyze if the epitope is incorporated or not by restriction analysis. The full nucleotide code for epitope p406-414 based on the described analysis is shown in table 2.

Table 1: Triplet code analysis of capsid protein pIX of the Ad

Amino Acid(s)	Codon(s) Used
A (Ala)	GCA(6), GCC(9), GCG(1), GCT(6)
D (Asp)	GAC(2), GAT(5)
E (Glu)	GAA(1), GAG(2)
F (Phe)	TTC(1), TTT(2)
G (Gly)	GGA(2), GGC(1), GGG(2), GGT(1)
I (Ile)	ATT(3)
K (Lys)	AAG(2)
L (Leu)	CTG(5), CTT(3), TTG(8)
M (Met)	ATG(3)
N (Asn)	AAC(2), AAT(3)
P (Pro)	CCA(1), CCC(4), CCG(2), CCT(1)
Q (Gln)	CAA(1), CAG(5)
R (Arg)	CGC(5), CGG(1), CGT(2)
S (Ser)	AGC(6), AGT(1), TCA(3), TCC(6), TCG(1), TCT(5)
T (Thr)	ACA(1), ACC(6), ACG(3), ACT(3)
V (Val)	GTC(2), GTG(5), GTT(3)
W (Trp)	TGG(1)
Y (Tyr)	TAC(1), TAT(1)

Table 2: Nucleotide en AA sequence of epitope p406-414 of MSLN

Epitope p406-414	**AA**	G	Q	K	M	N	A	Q	A	I
	Triplet code	GGA	CAG	AAG	ATG	AAT	GCC	CAG	GCC	ATT

The epitope p406-414 is attached to the capsid protein pIX by a linker. Nadine et al demonstrated in their article that a 45Å-spacer gives better results than a glycine-linker for pIX incorporation. [36] For this reason, a 45Å-spacer is added to the design. In addition, three natural flanking AA are added on both sides of the epitope to promote processing by proteolytic enzymes. (table 3) These natural flanking AA are the three AA on both sides of the original MSLN protein. The natural flanking AAs are the AA at place 403-405 and AA at place 415-417 of MSLN. The AA sequence is translated to a nucleotide sequence with the help of table one. (table 4)

Table 3: Outline of one epitope design with AA sequence

45Å-spacer	3 AA natural flanking	p406-414 AA	3 AA natural flanking	C-terminus pIX
EETRARLSKELQAAQARLGADMEDVCGRLVQYRGEVQA	VSK	GQKMNAQAI	ALV	

Table 4: Natural flanking AA with translated codons for one epitope of MSLN design

Natural flanking p406-414	**AA**	V	S	K	Epitope P406-414	A	L	V
	Codon	GTG	AGC	AAG		GCC	TTG	GTG

One irrelevant epitope design

One of the controls is an Ad with an irrelevant epitope incorporated into pIX. To minimize the variation between the hypothesis testing epitope and the irrelevant epitope, the same AAs are used. The AAs are ordered backward. (figure 5) This order will not cause an immune response against MSLN. Moreover, the same design structure is used as described for the one epitope of MSLN. Thus, the same AA and codons for natural flanking and 45Å-spacer are applied. In addition, a BstBI restriction site is added by a silent mutation.

Table 5: Nucleotide and AA sequence of epitope p406-414 backward of MSLN (irrelevant epitope)

Epitope p406-414 backward	**AA**	I	A	Q	A	N	M	K	Q	G
	Triplet code	ATT	GCC	CAG	GCC	AAT	ATG	AAG	CAG	GGA

Five epitopes design

For the five epitope design, the five epitopes that give an immune response in MSLN$^{-/-}$ mouse are selected: p343-351, p406-414, p484-492, p544-552, p583-591.[39] These epitopes are placed in order of increasing immunity. The same technique is used for selection of natural flanking epitopes as for the one epitope design. In addition, the AAs are translated to triplets as described earlier. In the five epitope design, the BamHI restriction site is added to check the ligation with restriction analyses.

The five epitope design has a length of ~ 200 bp. This length makes it suitable for the DNA HiFi assembly. This method has a higher efficiency than a normal ligation. For the assembly, an overhang of 20 bp on both sites homologous to the shuttle is necessary. For this reason, the overhang is added to the design.

Table 6: Design outline for five epitope design with AA sequence

45Å-spacer	3 AA natural flanking	p583-591	3 AA natural flanking	p544-552	3 AA natural flanking	p484-492 →
	LQG	GIPNGYLVL	DFN VQK	LLGPNIVDL	KTE KAC	SAFQNVSGL
3 AA natural flanking	**p343-351**	**3 AA natural flanking**	**p406-414**	**3 AA natural flanking**	**C-terminus pIX**	
EYF LVN	EIPFTYEQL	SIF VSK	GQKMNAQAI	ALV		

Conventional approach control virus

The conventional approach virus expresses full MSLN under the control of the CMV promoter. Via the company GenScript, a standard shuttle vector with MSLN (NM_0188570) was ordered. Primers were designed for the HiFi assembly. Because of lack of time, the cloning for this construct was put off.

First cloning step

Shuttle vectors preparation

The control with the non-replicative Ad contains a red fluorescence protein in deleted E1A/B. The RedpIX shuttle (figure 12) contains an H7 region after the protein pIX. This H7 can be easy replaced with the gene of interest for pIX capsid incorporation. The CRAd with SPARC promotor is made by the use of the shuttle plasmid pShIE1pIX45H7. In addition, this shuttle also contains an H7 region that can be replaced by the gene of interest. Both shuttle vectors already contain the 45Å-linker.

For removal of the H7 region, 10 µg of both vectors were digested with restriction enzyme NotI. After the digestion, the digestion product was purified by gel electrophoreses. The digested product was run on a gel and the linearized vector was cut out to remove H7 region. The shuttles were then gel extracted with QIAquick gel extraction kit.

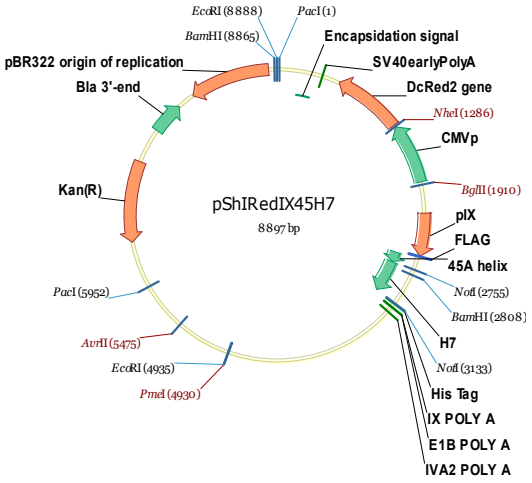


Figure 12: Shuttle vector pShIRedIX45H7

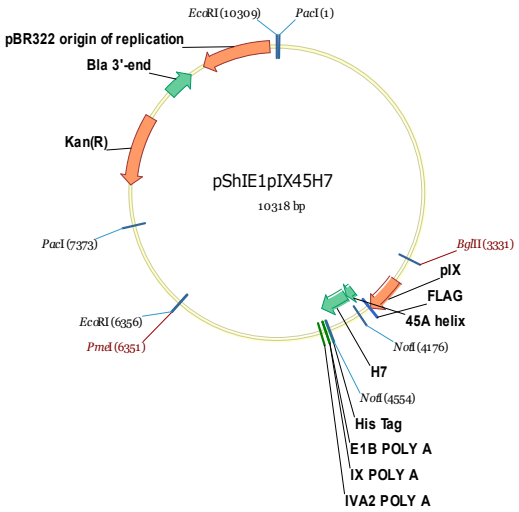


Figure 13: Shuttle vector pShIE1pIX45H7

One and irrelevant epitope design

The sequence of the one epitope was ordered as two oligo's: the 5'-3' sequence and the antisense sequence of this fragment. In addition, two oligo's were ordered for the irrelevant epitope. All the oligo's were diluted in H_2O with 10 mM Tris-Cl (buffer EB) to a final concentration of 100µM. The oligoes are annealed via a special setup PCR program. This program is designed to start with a temperature of 99 °C and decrease by 1 °C with every cycle. Every cycle takes one minute and 90 cycles in total. The final concentration after annealing was 333 ng/µl.

The two oligo's both have overhangs of 3 AAA amino acids on 3' and 5' because the amino acids sequence of NotI is three times A. This is necessary for a ligation into the NotI digested shuttle plasmid. The two oligo's are ligated into the NotI digested pShIRedIX45 and pShIE1pIX45 with T4 ligase. Thereafter, the ligation mixtures are transformed into NEB® 10-beta chemically competent E. coli (High Efficiency). The transformation mixture was spread out on kanamycin agar plates. Eight different colonies of each transformation were grown in 100 ng/mL kanamycin in LB media.

The DNA is extracted from the E. Coli with QIAprep Plasmid mini-prep and the DNA was precipitated. The extracted DNA is analyzed, to check ligation insertion into the shuttle plasmid, by restriction analysis with BstBI and NFeI for the Red shuttle and BstBI and PmeI for the E1 shuttle. In addition, the colonies that showed the right restriction pattern were analyzed further by sequencing. The sequencing was done with two primers that bind before and after the capsid protein pIX.

The DNA of the colonies that showed the correct sequencing results was up-scaled. This upscaling was performed by adding 250 µl of the glycerol stock to 250 mL LB media with kanamycin. The DNA was extracted with QIAprep Plasmid Midi Kit. After the extraction, the DNA was purified by DNA precipitation. The concentration was measured with a spectrophotometer. The four constructed shuttle plasmids are shown in figure 14 and 15.

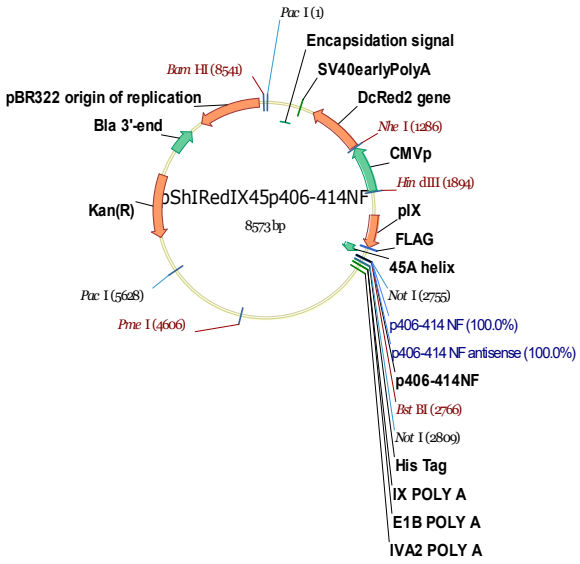


Figure 14A: pShIRedIX45p406-414 (red shuttle with one epitope in pIX)

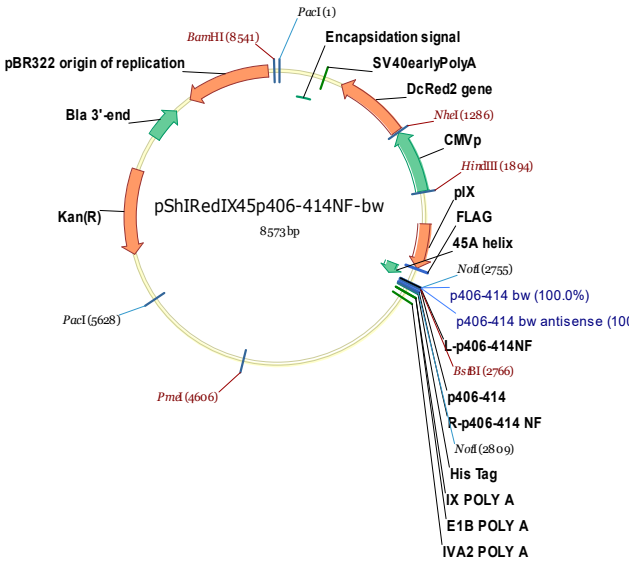


Figure 14B: pShIRedIX45p406-414bw (red shuttle with irrelevant epitope in pIX)

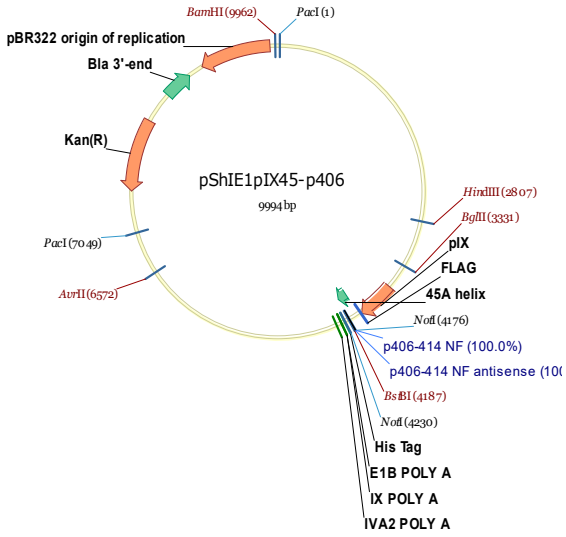


Figure 15A: pShE1pIX45-p406
(E1 shuttle with one epitope in pIX)

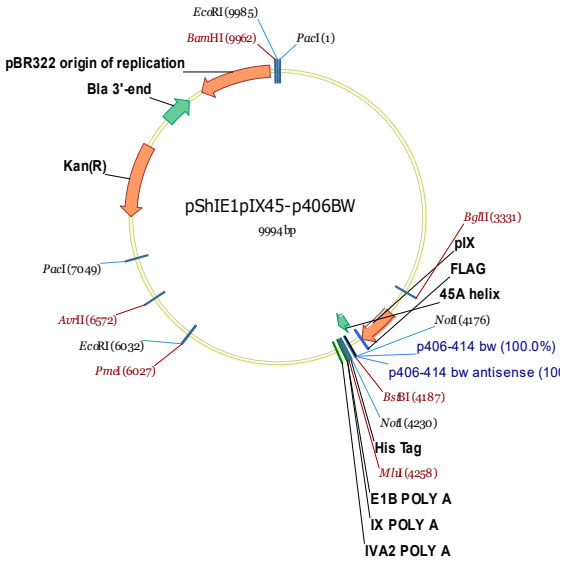


Figure 15B: pShE1pIX45-p406BW
(E1 shuttle with an irrelevant epitope in pIX)

Five epitopes design

The five epitopes insert was ordered as a double-stranded gene block fragment from integrated DNA technologies. As described in the design, the gene block fragment has a 20 bp overhang on both sites that is a complement to the shuttle plasmid. The gene block fragments were dissolved in 50 µl of buffer EB. For the HiFi DNA assembly, 140 ng of the digested pShRedIX45, 160 ng of the digested pShE1pIX45 and 10 ng (0,06 pmol) of the 5 epitope gene block fragment was used. Two reactions were set up, one with the Red shuttle and one with the E1 shuttle. The shuttle, the fragment and the assembly master mix were incubated for 15 min at 50 °C. After the incubation, 2µl of the assembly mixture was transformed into NEB-5-alpha Competent E. coli cells.

After the transformation, the same steps as described with the one epitope design were performed. BamHI and PmeI were used for the restriction analyses. In figure 16, the plasmid maps of the designed shuttle constructs are shown.

After the first cloning step, shuttle vectors: pShRedIX45-p406 and pShRedIX45-5epitopes are ready for the homologous recombination. These shuttles do not need a second cloning step.

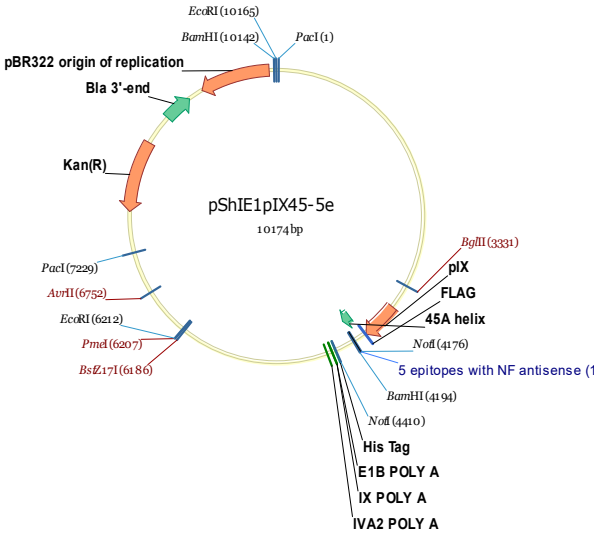


Figure 16A: : Shuttle vector pShIE1pIX45-5e (E1 vector with 5 epitopes in pIX)

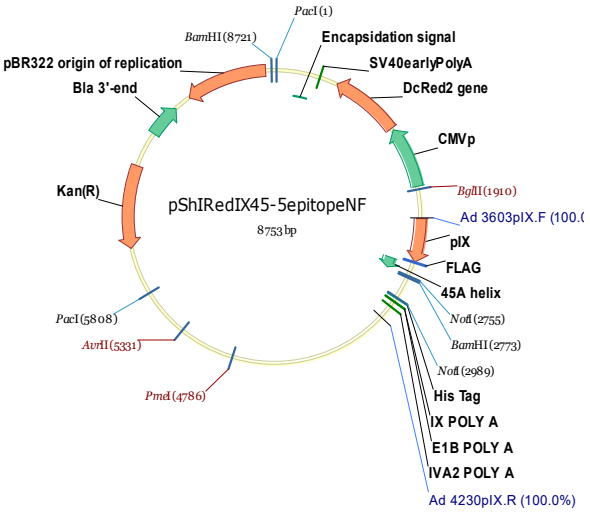


Figure 16B: Shuttle vector pShIRedIX45-5e (Red vector with 5 epitopes in pIX)

Second cloning step

The second cloning step is to replace the CMV promoter with a SPARC promoter. Therefore, the shuttle vector with the SPARC promoter was digested with AvrII and BglII. After the digestion, the digested shuttle was loaded on an electrophorese gel. The 6kb fragment was cut out of the gel and purified with the Qiagen QIAquick gel extraction kit. The same digestion was performed on the shuttle vectors pShE1pIX45-p406, pShE1pIX45-p406BW and pShE1pIX45-5epitopes. However, the 3kb digested fragment was extracted from these vectors.

After the digestion, the ligations as shown in figure 17 are performed. The ligations were accomplished with molecular ratio 1:1 and a T4 ligase. The ligation mixtures were transformed into DH10β chemically competent cells. The cells were spread out on kanamycin Agar plates. Eight colonies were picked form this plates and grown overnight in LB media with kanamycin. The DNA was extracted from these colonies by Qiagen Plasmid Miniprep. After the extraction, the DNA was precipitated.

To check if the ligation mixtures were correct, a restriction analysis was performed with either BamHI, NotI or BstBI in combination with PmeI. The colonies with the right restriction pattern were sequenced. The primers used for this sequence bind to the AvrII and BglII sites. The DNA of the colonies with the correct nucleotide sequence are upscaled with the use of Qiagen Plasmid midi kit.

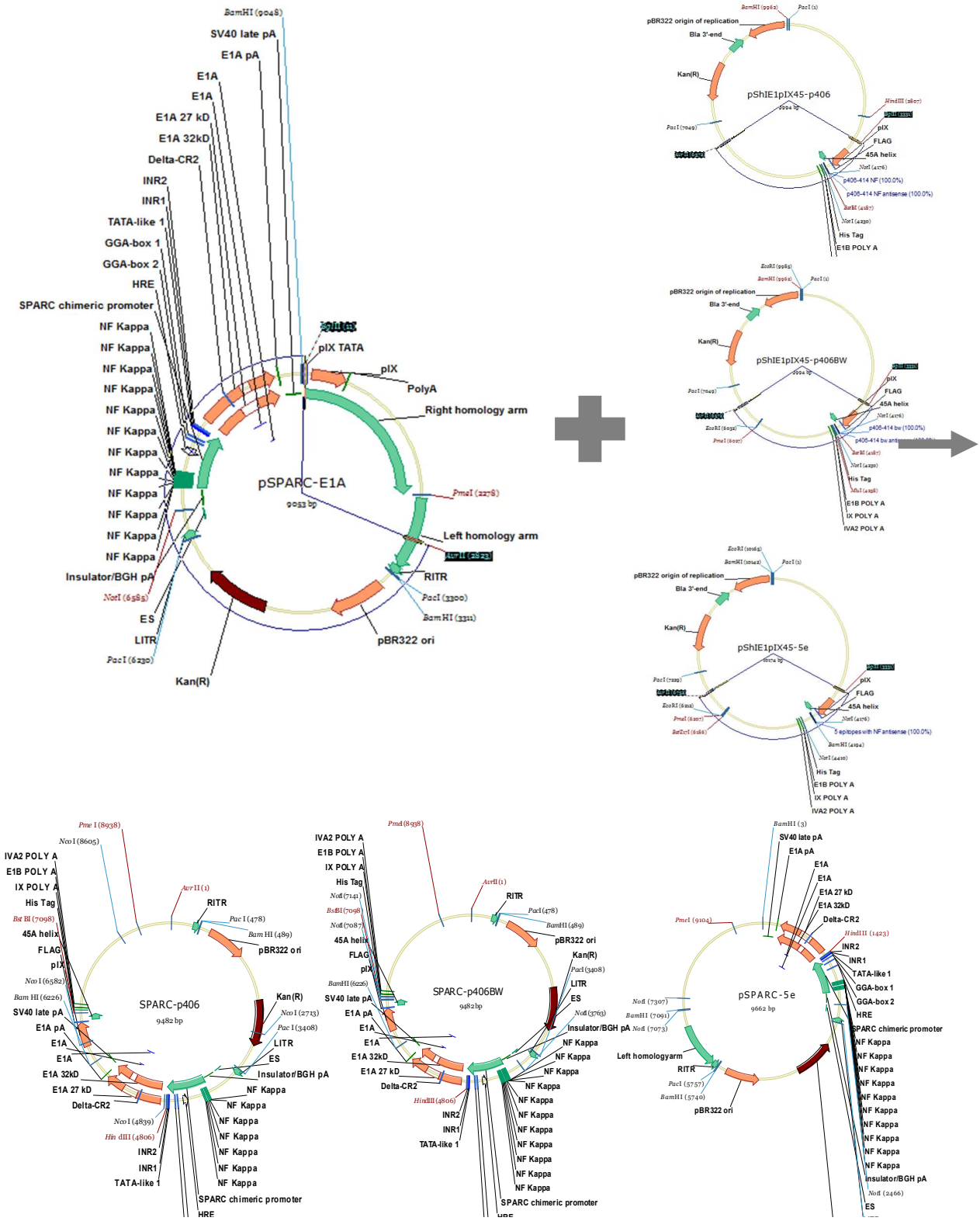


Figure 17: Cloning schema for SPARC cloning

Homologous recombination of production of adenoviral genome

For the generation of the adenovirus genome, Bj-5183-AD-1 electroporation competent cells were used. These cells are recombination proficient bacterial cells carrying the pAdEasy-1 plasmid that encodes the Adenovirus-5 genome. They supply the components necessary to execute a recombination event between the Ad-Easy vector and the shuttle plasmids. Recombination is possible because the shuttle plasmids contain right and left arm homologous regions to the pAd-Easy-1 vector.

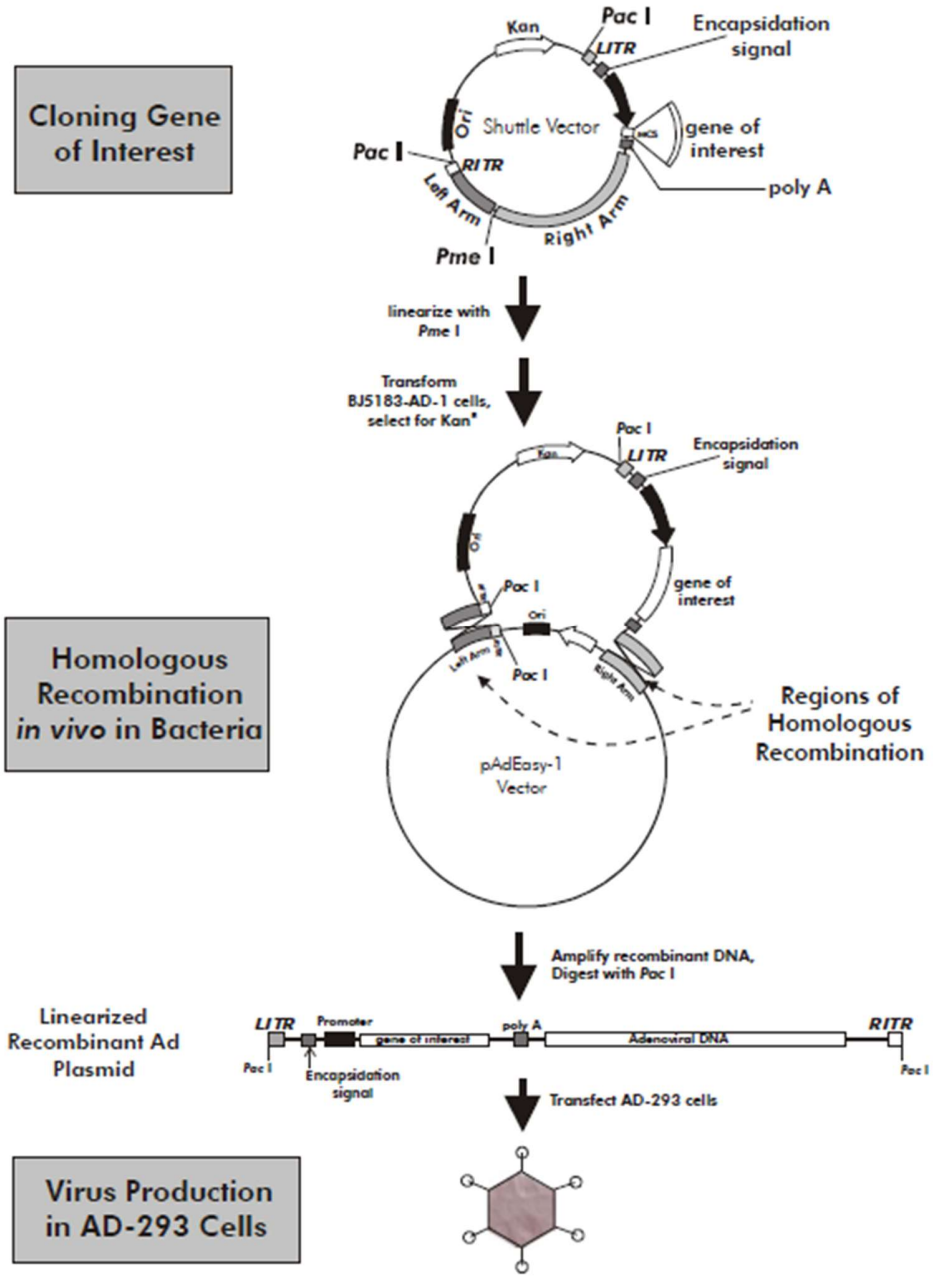


Figure 18: Production of recombinant adenovirus with the Ad-Easy adenoviral system (BJ5183-AD-1) [41]

Preparation shuttle plasmid for homologous recombination

The first step of the homologous recombination is digestion of the shuttle plasmids. (figure 18) 3 µg of all the shuttle plasmids were digested with PmeI. PmeI is a blunt end restriction enzyme. For this reason, the shuttle plasmids were dephosphorylated with Alkaline Phosphatase: Calf Intestinal (CIP). The linearized plasmids were purified by gel extraction with QIAquick gel extraction kit and dissolved in buffer EB.

Homologous recombination transformation

The purified linearized plasmids were transformed into BJ5183-AD-1 cells by electroporation. Between 120-150 ng of the plasmids, 20 µl of the BJ cells and 3µl water was used. The electroporation method is time sensitive. It is important that the DNA and cells are mixed just before electroporation. In addition, SOC media should be added to the electrophoresed mixture as soon as possible. After the electroporation, the bacteria mixture was incubated for 1 hour at 37 °C and spread out on kanamycin agar plates.

The Ad-Easy DNA is ampicillin resistance and cannot grow on the kanamycin agar plates. (figure 18) For this reason, the colonies that grow on the kanamycin agar plates were either shuttle plasmids or recombinant DNA. Recombinant DNA has three times higher molecular weight and grows slower because of this. Consequently, ten small colonies were picked on the agar plates. These colonies were grown overnight in 2 mL LB media with kanamycin. The DNA was extracted from the overnight media with the help of Qiagen mini-prep with DNA precipitation.

Analysis recombination colonies

The mini-prep DNA was loaded on a 0,8% agarose gel to check the molecular weight of the colonies. Only the high molecular weight colonies were analyzed further by polymerase chain reaction (PCR). For Ad5-SPARC-5e, Ad5-Red-5e and Ad5-E1-5e, primers Ad3603pIX.F and Ad4230pIX.R were used. For Ad5-SPARC-p406, Ad5-Red-p406, primers Ad3603.pIX.F and the oligo p406 antisense. For Ad5-SPARC-p406BW, primers Ad3606.pIX.F and oligo p406BW antisense were used. (table 7) The inserted protein in the one epitope design is only 50 bp. Therefore, it is not possible to see a difference in PCR product of pIX between the Ad-Easy shuttle and the plasmid shuttle. For this reason, the oligo's are used as primers. These oligo's can only bind to the recombination plasmid. The PCRs were performed with Q5 polymerase master mix. The temperature of annealing was calculated with NEB Tm calculator. In addition, the annealing time was adjusted for the size of produced PCR product.

Table 7: Primers used for the analysis of recombinant DNA

Plasmid	Primer sequence
Ad5-SPARC-5e **(SPARC promoter with 5 epitopes of MSLN)**	*Ad3603.pIX.F :* GCCGCCATGAGCACCAAC *Ad4230.pIX.R :* ATGAAGCTCTGCAGTGGTGCTACCT
Ad5-Red-5e **(Red fluorescence with 5 epitopes of MSLN)**	
Ad5-E1-5e **(E1 (replicative) with 5 epitopes of MSLN)**	
Ad5-SPARC-p406 **(SPARC promoter with one epitope of MSLN)**	*Ad3603.pIX.F:* GCCGCCATGAGCACCAAC *Oligo p406 antisense:* GGCCGCCACCAAGGCAATGGCCTGGGCATT CATCTTCTGTCCCTTCGAAACAGC
Ad5-Red-p406 **(Red fluorescence with one epitope of MSLN)**	

Ad5-SPARC-p406BW **(SPARC promoter with one irrelevant epitope)**	*Ad3603.pIX.F:* GCCGCCATGAGCACCAAC *Oligo p406BW antisense:* GGCCGCCACCAAGGCTCCCTGCTTCATATT GGCCTGGGCAATCTTCGAAACAGC

DNA upscaling for transfection

DH10β Transformation

The BJ5183-AD-1 are sufficient for recombination but do not produce enough DNA for the transfection. Therefore, the confirmed DNA was transformed into DH10β cells to upscale the DNA. For this transfection, 1µl of DNA, 5µl of water and 10µl of cells was used. The DH10β are like the BJ cells, electroporation competent cells. Therefore, this procedure is also time-sensitive. After the electroporation and incubation at 37 °C, the mixture was diluted 100 times with SOC media. 100 µl was spread out on kanamycin agar plates. Four colonies were picked for the plates and grown overnight in LB media with kanamycin. The DNA was extracted for the overnight grow colonies with Qiagen Plasmid mini kit.

Analysis of DH10β colonies

The mini-prep DNA was analyzed by loading on a 0,8% agar gel, HindIII digestion. In addition, the DNA was digested with PacI to test the sites of recombination. If recombination took place between the left and right arm, PacI digestion results in a ~30kb and 3,0kb fragment. If recombination took place at the origins of replication and right arm, digestion with PacI results in ~30kb fragment and 4,5kb fragment. The adenovirus genome maps with the correct recombination are shown in figure 19.

In addition, the same PCRs as in figure 7 were performed. The DNA with the correct PCR product and restriction patterns were up-scaled by addition of 250µl in 250 mL LB media with 1% kanamycin (100 mg/mL). This mixture was grown overnight in a shaking incubator at 37 °C. The DNA was extracted by Qiagen Plasmid Midi-Kit. The DNA was precipitated after the extraction. The concentration of DNA was measured with a spectrophotometer.

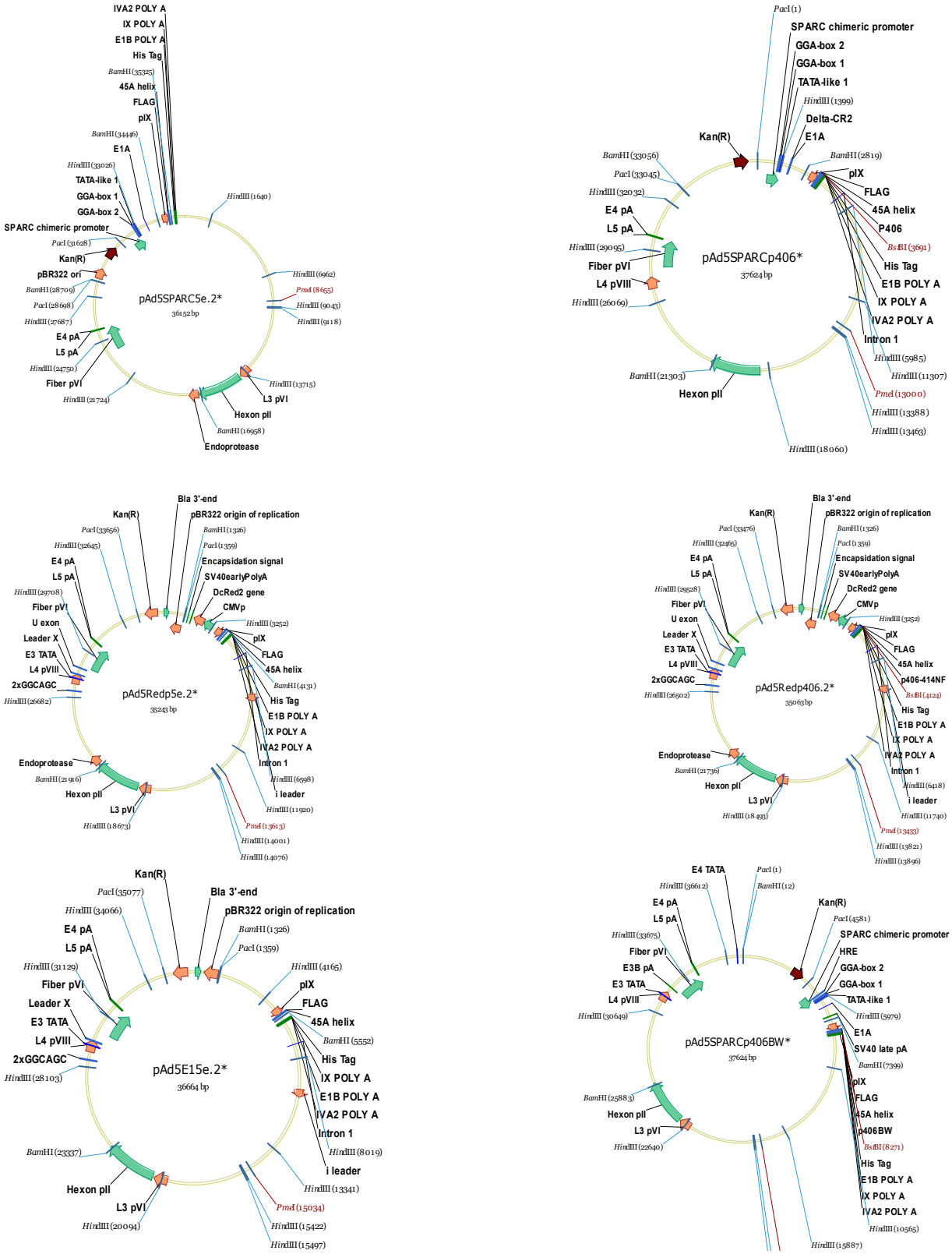


Figure 19: Simplified plasmid maps of the various adenovirus, which are produced during the project

Transfection into HEK-293 cells

DNA preparation for transfection

For transfection, the DNA should be digested with PacI to linearize the DNA. 10 µg of the midi-prep DNA was digested with 2 µl of PacI and incubated overnight in a shaking incubator at 37°C. The digestion was checked by gel electrophorese. The digested DNA was precipitated to remove toxins and toxicity DNA.

Cells preparation for transfection

For transfection, HEK293 cells are used because they are able to propagate adenovirus. For the preparation of the cells, HEK293 cells were thawed in a water bath. The thawed cells were mixed with 5 mL of 10% FBS 1% ampicillin/streptomycin DMEM-F12 media. The cells were spun down at 1200 rpm for 5 min. The media was removed and new media was added. The cells were grown in a T75 flask. The media was refreshed the next day. Wait at least a week before using cells for transfection after thawing. The cells were split during that week to prevent overgrowth.

Transfections are performed in 6-wells plates. Preparation of the cells for seeding in 6-wells plates was done by washed the cells with phosphate buffered saline (PBS) and trypsinized them with trypsin. The cells were spun down at 1200 rpm for 5min and resuspended in new 10mL 10% media. The cells were counted with a bright-line hemacytometer. A tissue culture 6 wells plate was seeded with $1{,}7 \times 10^6$ cells per well.

Transfection

For transfection, the amount of media should be 1 mL instead of 2 mL. This enhances the migration of the DNA with transfection agent attractene complexed to the cells. In addition, the concentration of FBS in the media is lower during transfection because it can interfere with the DNA complexes.
For formation of the complexes, 425 µl 0% media, the PacI digested DNA (50 µl) and 25 µl of attractene was added to an Eppendorf tube. It is important that this order is followed. The mixture was incubated at room temperature for 15 min. The control mixture contains 95 µl 0% media with 5 µl attractene.
In the meantime, the 10% media with amp/strep is removed from the 6-wells plate. The wells were washed with 2mL of 0% media in each well and 1 mL of 2% media without amp/strep was added. After 15 minutes incubation, 100 µl was added dropwise to the 5 wells. The remaining well was the control. After 5h, the media was removed and 2 mL 10% media with amp/strep was added.

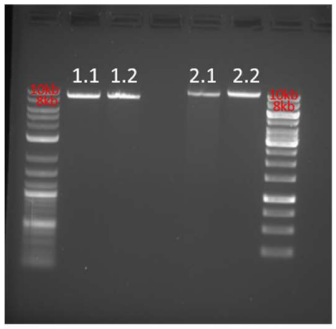
Harvest adenovirus

After 24 hours, the red fluorescence expressing adenoviruses were checked on the microscope. The cells should show red fluorescence because this means that the adenovirus DNA is inside the cell. The 6-well was checked every day to check the progress of the development of plaques. When the plaques were clearly visible (after 10-14 days), the cells were harvest into a 15 mL tube and stored at -80 °C. The cell mixture was thawed and frozen for 3-4 times to lyse the cells and releasement of the virus into the supernatant. After 3-4 times thawing and freezing, the cells were spun down at 4000 rpm for 20 min at 4 °C. The supernatant was collected. This supernatant can be used to infect new cells and upscale the virus titer.

Results

First cloning step

Shuttle vectors preparation



1.1 pShIRedIX45H7 NotI digested tube 1
1.2 pShIRedIX45H7 NotI digested tube 2

8519: pShIRedIX45H7: NotI(3133) - NotI(2755)
378: pShIRedIX45H7: NotI(2755) - NotI(3133)

2.1 pShIE1AIX45H7 NotI digested tube 1
2.2 pShIE1AIX45H7 NotI digested tube 2

9940: pShIE1pIX45H7: NotI(4554) - NotI(4176)
378: pShIE1pIX45H7: NotI(4176) - NotI(4554)

Figure 20: pShIRedIX45H7 and pShIE1AIX45H7 digested with NotI and gel extracted the shuttles without H7 part

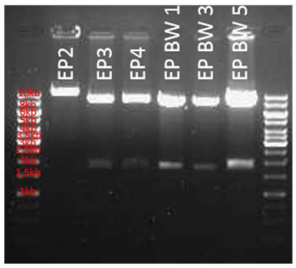
The shuttle plasmids pShIRedIX45H7 and pShIE1AIX45H7 were successfully gel extracted (figure 20). The H7 region is deleted in the shuttles by the Not I digestion and gel extraction.

One epitope and irrelevant design E1 vector

The ligation mixture was transformed into chemically competent E. Coli cells. A few colonies grown on the kanamycin Agar plats with shuttle plasmids pShE1pIX45-p406 and pShE1pIX45-p406BW. These colonies were analysis by restriction analysis. Colonies 2 and 3 of pShIE1IX45-p406 and colonies 1, 3 and 5 of pShIE1IX45-p406BW showed the correct restriction pattern. (figure 21)

The sequence results showed that epitope p406-414 in pShE1pIX45-p406 colony 3 and 4 was ligated in the wrong direction, the opposite direction. Colony 1 of pShE1pIX45-p406BW showed the correct sequence results. The epitope is ligated at nucleotides 4176 to 4229 (figure 22). Colony 3 of pShE1pIX45-p406BW did not have the right nucleotides on the ligation place and colony 5 was ligated in the wrong direction.

Unfortunately, the E. Coli cells were in total used for the analysis and there was no glycerol stock made. Because of this, colony 1 of pShE1pIX45-p406BW was transformed into DH5α E. Coli. Four colonies were analyzed with the same restriction analyses. All four colonies had the right restriction pattern. (figure 23) In addition, one of the colonies was sequenced and this result was the same as in figure 22.



EP2 pShIE1pIX45-p406 colony 2
EP3 pShIE1pIX45-p406 colony 3
EP4 pShIE1pIX45-p406 colony 4

8154: pShIE1pIX45-p406: PmeI(6027) - BstBI(4187)
1840: pShIE1pIX45-p406: BstBI(4187) - PmeI(6027)

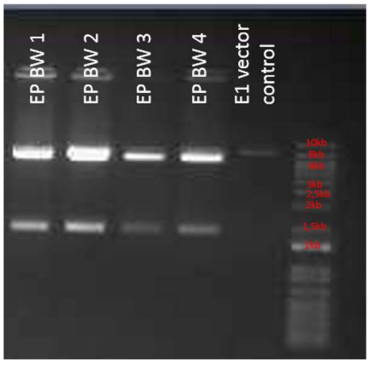
EP BW 1 pShIE1pIX45-p406BW colony 1
EP BW 3 pShIE1pIX45-p406BW colony 3
EP BW 5 pShIE1pIX45-p406BW colony 5

8154: pShIE1pIX45-p406BW: PmeI(6027) - BstBI(4187)
1840: pShIE1pIX45-p406BW: BstBI(4187) - PmeI(6027)

Figure 21: Restriction Analysis with BstBI and PmeI of different colonies of pShIE1pIX45-p406 and pShIE1pIX45-p406BW

```
(4119) 4119      4130      4140      4150      4160      4170      4180      4190      4200      4210      4220      4230
E1 p406BW 1 F (184) GGCTGATATGGAAGATGTCTGTGGGCGACTGGTCCAGTACCGAGGAGAGGTGCAGGCGGCCGCTGTTTCGAAGATTGCCCAGGCCAATATGAAGCAGGGAGCCTTGGTGGCGGCCG
pShIE1pIX45-p406BW(4119) GGCTGATATGGAAGATGTCTGTGGGCGACTGGTCCAGTACCGAGGAGAGGTGCAGGCGGCCGCTGTTTCGAAGATTGCCCAGGCCAATATGAAGCAGGGAGCCTTGGTGGCGGCCG
Consensus(4119) GGCTGATATGGAAGATGTCTGTGGGCGACTGGTCCAGTACCGAGGAGAGGTGCAGGCGGCCGCTGTTTCGAAGATTGCCCAGGCCAATATGAAGCAGGGAGCCTTGGTGGCGGCCG
```

Figure 22: Sequencing results of pShIE1pIX45-p406BW colony 1. The first row is the sequence result and the second row is the vector designed in Vector NTI. The epitope is ligated into nucleotides 4176-4229.

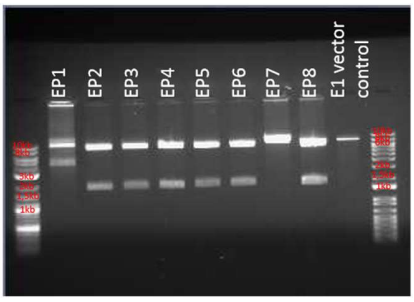


EP BW 1 pShIE1pIX45-p406BW colony 1
EP BW 2 pShIE1pIX45-p406BW colony 2
EP BW 3 pShIE1pIX45-p406BW colony 3
EP BW 4 pShIE1pIX45-p406BW colony 3

8154: pShIE1pIX45-p406BW: PmeI(6027) - BstBI(4187)
1840: pShIE1pIX45-p406BW: BstBI(4187) - PmeI(6027)

Figure 23: Restriction Analysis with BstBI and PmeI of pShIE1pIX45-p406BW colony 1 transformed into DH5α

A new transfection with the ligation mixture pShE1pIX45-p406 was performed. Eight colonies were analyzed with restriction analysis. Colonies 2-6 and 8 showed the correct restriction pattern. (figure 24) These colonies are also sequenced. Colonies 5,6 and 8 showed the right nucleotide order. One example of this nucleotide order is shown in figure 25. The other three colonies were ligated in the wrong direction.



EP1 pShIE1pIX45-p406 colony 1
EP2 pShIE1pIX45-p406 colony 2
EP3 pShIE1pIX45-p406 colony 3
EP4 pShIE1pIX45-p406 colony 4
EP5 pShIE1pIX45-p406 colony 5
EP6 pShIE1pIX45-p406 colony 6
EP7 pShIE1pIX45-p406 colony 7
EP8 pShIE1pIX45-p406 colony 8

8154: pShIE1pIX45-p406: PmeI(6027) - BstBI(4187)
1840: pShIE1pIX45-p406: BstBI(4187) - PmeI(6027)

Figure 24: Restriction Analysis with BstBI and PmeI of different colonies of pShIE1pIX45-p406 after the second transformation

```
(4093) 4093      4100      4110      4120      4130      4140      4150      4160      4170      4180      4190      4200      4210      4220      4230
Assembly E1 p406 8 (936) GAACTGCAGGCCGCTCAGGCACGACTGGGGGCTGATATGGAAGATGTCTGTGGGCGACTGGTCCAGTACCGAGGAGAGGTGCAGGCGGCCGCTGTTTCGAAGGGACAGAAGATGAATGCCCAGGCCATTGCCTTGGTGGCG
pShIE1pIX45-p406 (4090) GAACTGCAGGCCGCTCAGGCACGACTGGGGGCTGATATGGAAGATGTCTGTGGGCGACTGGTCCAGTACCGAGGAGAGGTGCAGGCGGCCGCTGTTTCGAAGGGACAGAAGATGAATGCCCAGGCCATTGCCTTGGTGGCG
Consensus (4093) GAACTGCAGGCCGCTCAGGCACGACTGGGGGCTGATATGGAAGATGTCTGTGGGCGACTGGTCCAGTACCGAGGAGAGGTGCAGGCGGCCGCTGTTTCGAAGGGACAGAAGATGAATGCCCAGGCCATTGCCTTGGTGGCG
```

Figure 25: Sequencing results of pShIE1pIX45-p406 colony 8. The first row is the sequence result and the second row is the vector designed in Vector NTI. The epitope is ligated into nucleotides 4176-4229.

One epitope and irrelevant design Red vector

pShIRedIX45-p406 transformed in E.Coli did not grow on the kanamycin agar plate. For the first ligation, the molecular ratio between vector and insert was 1:600. A new ligation with a molecular ratio of 1:2 and 1:50 was performed in DH10β chemically competent cells and XL-1 Blue chemically competent cells. All these ligations did not grow on kanamycin agar plates. All the controls grow on the kanamycin agar plates. The ligation is not working. Consequently, a new approach has been developed. The ligation into the E1 vector worked. The ligated E1 vector with the one epitope design is used to clone the p406-414 epitope into the Red

vector. Specifically, the pShE1pIX45-p406 and pShIRedIX45H7 were digested with AvrII and BglII. The epitope part was extracted from pShE1pIX45-p406 and the vector part from pShIRedIX45H7. (figure 26) The epitope part and the vector part was ligated and transformed. The restriction analyzes and one example of the sequence results are shown in figure 27 and 28. All the colonies showed the correct restriction pattern. Colony 7 was sequenced and contain the right nucleotides.

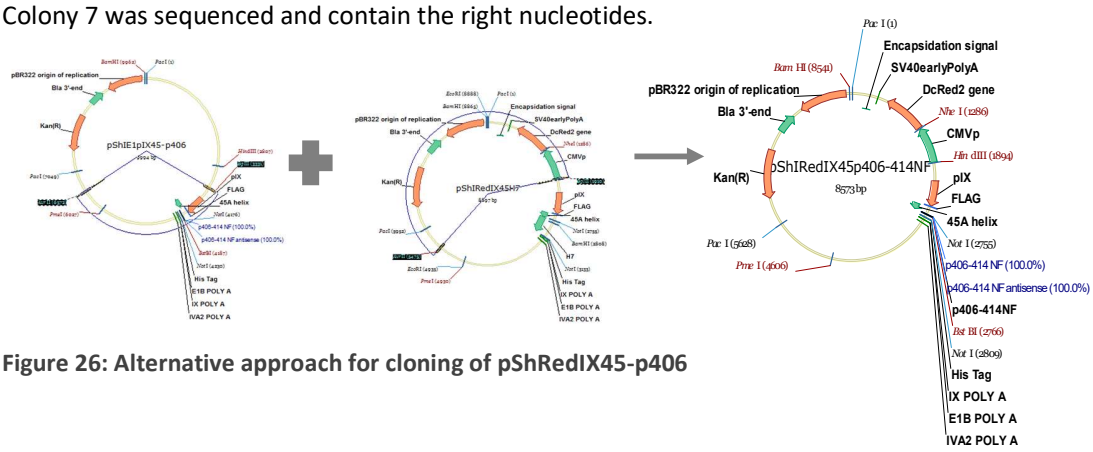
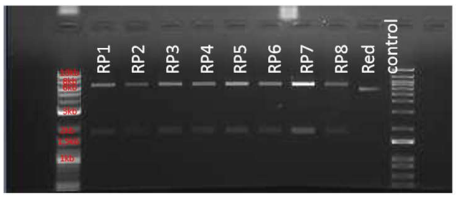


Figure 26: Alternative approach for cloning of pShRedIX45-p406



RP1 pShRedIX45-p406 colony 1
RP2 pShRedIX45-p406 colony 2
RP3 pShRedIX45-p406 colony 3
RP4 pShRedIX45-p406 colony 4
RP5 pShRedIX45-p406 colony 5
RP6 pShRedIX45-p406 colony 6
RP7 pShRedIX45-p406 colony 7
RP8 pShRedIX45-p406 colony 8

6733: pShIRedIX45p406-414NF: PmeI(4606) - BstBI(2766)
1840: pShIRedIX45p406-414NF: BstBI(2766) - PmeI(4606)

Figure 27: Restriction Analysis with BstBI and PmeI of different colonies of pShRedIX45-p406 with the new cloning approach

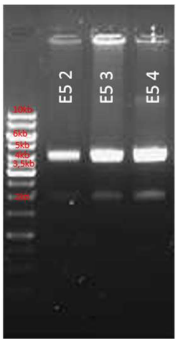
```
                    (5065) 5065  5070      5080      5090      5100      5110      5120      5130      5140      5150      5160      5170      5180
Copy of AWL204_PREMIX (c) (905) GCGTAGAGACAACATTACAGCCCCCATAGGAGGTATAACAAAATTAATAGGAGAGAAAAACACATAAACACCTGAAAAACCCTCCTGCCTAGGCAAAATAGCACCCTCCCGCTCCAGAAC
pShIRedIX45p406-414NF(5063) GCGTAGAGACAACATTACAGCCCCCATAGGAGGTATAACAAAATTAATAGGAGAGAAAAACACATAAACACCTGAAAAACCCTCCTGCCTAGGCAAAATAGCACCCTCCCGCTCCAGAAC
Consensus(5065) GCGTAGAGACAACATTACAGCCCCCATAGGAGGTATAACAAAATTAATAGGAGAGAAAAACACATAAACACCTGAAAAACCCTCCTGCCTAGGCAAAATAGCACCCTCCCGCTCCAGAAC

                    (1854) 1854  1860      1870      1880      1890      1900      1910      1920      1930      1940
Copy of AWL203_PREMIX (c) (942) ACCCCGTAATTGATTACTATTAATAACTAATGCAGGCATGCAAGCTTGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGC
pShIRedIX45p406-414NF(1852) ACCCCGTAATTGATTACTATTAATAACTAATGCAGGCATGCAAGCTTGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGC
Consensus(1854) ACCCCGTAATTGATTACTATTAATAACTAATGCAGGCATGCAAGCTTGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGC
```

Figure 28: Sequencing results of pShRedIX45-p406 colony 7. The upper diagram shows restriction site AvrII at 5151bp. The lower diagram shows restriction site BglII at 1910bp. In both diagrams, the first row is the sequence result and the second row is the vector designed in Vector NTI.

Five epitope design in E1 and Red vector

For cloning of the 5 epitope design, NEBuilder HiFi DNA Assembly Cloning Kit was used. The restriction analysis of the assembly is shown in figure 29 and 30. All colonies demonstrate the correct restriction pattern. In addition, the colonies were checked with sequencing. (figure 31 and 32) These results were confirmed with the designed plasmids.

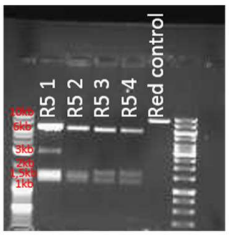
As described under one and irrelevant design E1 vector, there was no glycerol stock made of the transformation bacteria. For this reason, a new transformation into DH5α was necessary. The results for this transformation are shown in figure 33. Clones E1 5e colony 3 and 4 are correct and all clones of Red 5e are correct. The sequence results are the same as shown in figure 31 and 32.



E5 2 pShE1pIX45-5epitopes colony 2
E5 3 pShE1pIX45-5e colony 3
E5 4 pShE1pIX45-5e colony 4

4226: pShIE1pIX45-5e: BamHI(10142) - BamHI(4194)
3935: pShIE1pIX45-5e: PmeI(6207) - BamHI(10142)
2013: pShIE1pIX45-5e: BamHI(4194) - PmeI(6207)

Figure 29: Restriction Analysis with BamHI and PmeI of different colonies of pShE1pIX45-5epitopes with HiFi assembly method



R5 1 pShRedIX45-5epitopes colony 1
R5 2 pShRedIX45-5e colony 2
R5 3 pShRedIX45-5e colony 3
R5 4 pShRedIX45-5e colony 4

5948: pShIRedIX45-5epitopeNF: BamHI(2773) - BamHI(8721)
1487: pShIRedIX45-5epitopeNF: NheI(1286) - BamHI(2773)
1318: pShIRedIX45-5epitopeNF: BamHI(8721) - NheI(1286)

Figure 30: Restriction Analysis with BamHI and NheI of different colonies of pShRedIX45-5epitopes with HiFi assembly method

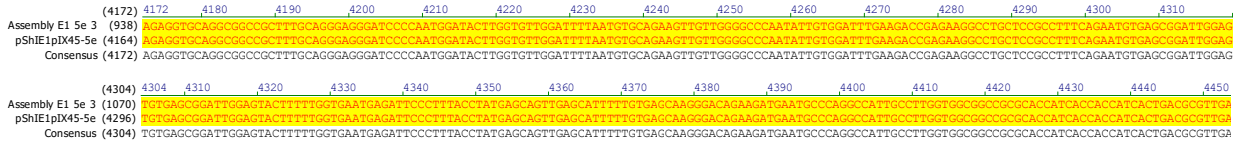


Figure 31: Sequencing results of pShE1pIX45-5epitopes colony 3. The epitope is first row is the sequence result and the second row is the vector designed in Vector NTI. The epitope is between 4176-4409 bp.

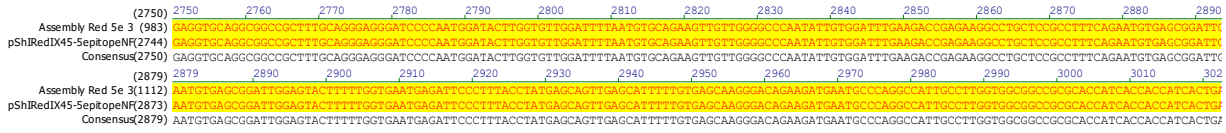


Figure 32: Sequencing results of pShRedIX45-5epitopes colony 3. The epitope is first row is the sequence result and the second row is the vector designed in Vector NTI. The epitope is between 2755-2988 bp.

E5 1 pShE1pIX45-5epitopes colony 1
E5 2 pShE1pIX45-5e colony 2
E5 3 pShE1pIX45-5e colony 3
E5 4 pShE1pIX45-5e colony 4

4226: pShIE1pIX45-5e: BamHI(10142) - BamHI(4194)
3935: pShIE1pIX45-5e: PmeI(6207) - BamHI(10142)
2013: pShIE1pIX45-5e: BamHI(4194) - PmeI(6207)

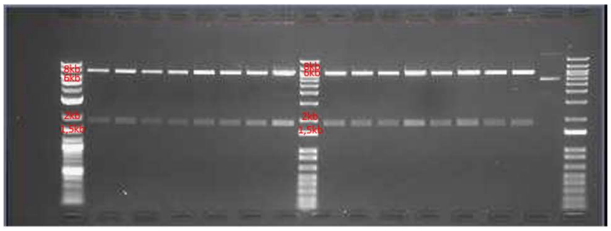
R5 1 pShRedIX45-5epitopes colony 1
R5 2 pShRedIX45-5e colony 3
R5 3 pShRedIX45-5e colony 3
R5 4 pShRedIX45-5e colony 4

3935: pShIRedIX45-5epitopeNF: PmeI(4786) - BamHI(8721)
2805: pShIRedIX45-5epitopeNF: BamHI(8721) - BamHI(2773)
2013: pShIRedIX45-5epitopeNF: BamHI(2773) - PmeI(4786)

Figure 33: Restriction Analysis with BamHI and PmeI of different colonies of pShRedIX45-5epitopes and pShE1pIX45-5epitopes after the second transformation into DH5-α

Second cloning step

The second step of cloning is the addition of the SPARC promoter. The restriction analyses of this cloning step are shown in figure 34 and 35. All colonies are correct according to the restriction analysis. One clone of each construct was sequenced. A summary of the sequence results is shown figure 36-38. The restriction sites contained the predicted nucleotide, which indicates that the ligation worked.



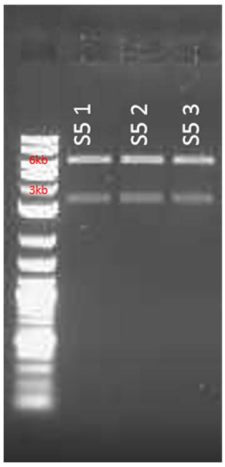
SP 1-8 pSPARC-p406 colony 1-8

7642: SPARC-p406: PmeI(8938) - BstBI(7098)
1840: SPARC-p406: BstBI(7098) - PmeI(8938)

SPB 1-8 pSPARC-p406 BW 1-8

7642: SPARC-p406BW: PmeI(8938) - BstBI(7098)
1840: SPARC-p406BW: BstBI(7098) - PmeI(8938)

Figure 34: Restriction Analysis with BamHI and PmeI of different colonies of pSPARC-p406 and pSPARC-p406BW



S5 1-3 pSPARC-5epitopes colony 1-3

6104: SPARC-5eNF: NotI(3914) - NotI(356)
3324: SPARC-5eNF: NotI(356) - NotI(3680)
234: SPARC-5eNF: NotI(3680) - NotI(3914)

Figure 35: Restriction Analysis with NotI of different colonies of pSPARC-5epitopes

```
                (6166) 6166      6180      6190      6200      6210      6220      6230      6240      6250      6260      6270
AWL196_PREMIX  (117) GAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCC
SPARC-p406(6166) GAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCC
Consensus(6166) GAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCC
```

Figure 36: Sequencing results of pSPARC-p406 colony 5. The epitope is first row is the sequence result and the second row is the vector designed in Vector NTI. Restriction site BglII is located at 6242 bp.

```
                (6191) 6191      6200      6210      6220      6230      6240      6250      6260      6270      6280      6290
AWL199_PREMIX  (137) AGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCC
SPARC-p406BW(6191) AGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCC
Consensus(6191) AGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCC
```

Figure 37: Sequencing results of pSPARC-p406BW colony 6. The epitope is first row is the sequence result and the second row is the vector designed in Vector NTI. Restriction site BglII is located at 6242 bp.

```
                (6203) 6203      6210      6220      6230      6240      6250      6260      6270      6280      6290      6300
Copy of AWL194_PREMIX (c) (938) TACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCGAGTGTGGC
SPARC-5eNF(6202) TACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCGAGTGTGGC
Consensus(6203) TACAAATGTGGTAAAATCGATAAGGATCCGTCGACTCGAAGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCGAGTGTGGC
```

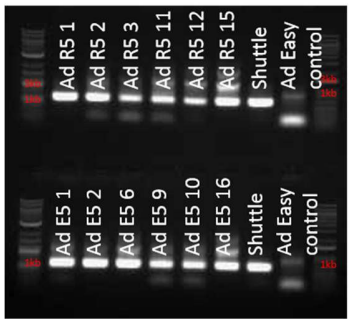
Figure 38: Sequencing results of pSPARC-5epitopes colony 4. The epitope is first row is the sequence result and the second row is the vector designed in Vector NTI. Restriction site AvrII is located at 6256 bp.

Homologous recombination of production of adenoviral genome

Homologous recombination with shuttle vectors pShIRedIX45-5e and pShE1pIX45-5e

The original design for the different viruses did not include the Ads: non-replicative and replicative Ad with 5 epitopes in pIX. Both Ads were ready after the first cloning step. To gain some practice in the new technique, the Ads are used to practice the new techniques during the second cloning step.

The shuttle plasmids were successfully digested with PmeI. The linearized plasmids were transformed into BJ5183-AD-1 with electroporation and 300 µl of the SOC media with bacteria was spread out on kanamycin agar plates. The small colonies were picked and grown overnight. The molecular weight of the colonies was analyzed. The colonies with high molecular weight were further analyzed with PCR. (figure 39) If the homologous recombination occurred correctly, the expectation is that the PCR product is the same as the Shuttle plasmid. Because this means that, the epitopes are present in the Ad colonies. All the clones showed the expected PCR product.



Ad R5 1-3, 11-12, 15 Ad5RedIX45-5epitopes colonies 1-3, 11-12, 15

Ad E5 1-2, 6, 8-10, 16 Ad5E1pIX45-5epitopes colonies 1-2, 6, 8-10, 16

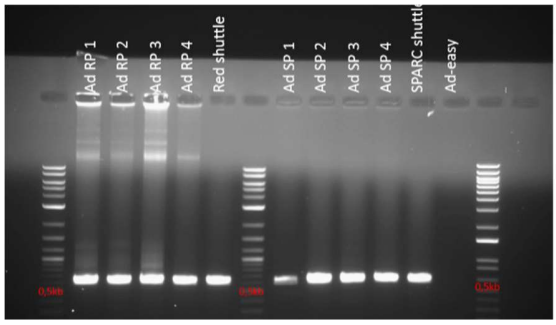
Primers: Ad3904pIX.F, Ad4060pIX.R

PCR product in shuttle: 846 bp
PCR product in Ad easy control: 156 bp

Figure 39: PCR analysis to test homologous recombination with primers Ad3904pIX.F (AGTTGACGGCTCTTTTGGCACA) and Ad4060pIX.R (CACTTGCTTGATCCAAATCC)

Homologous recombination with shuttle vectors pSPARCpIX45-p406 and pShRedIX45-p406

In the first try for homologous recombination, no colonies grew on the agar plate. The remaining SOC media with cells were spun down, concentrated and spread out. This also did not show growth on the kanamycin agar plate. A new transformation was performed with an increased amount of cells (30µl vs 20µl) and increased amount of water (4µl vs 3µl). After this transformation, all the cells were spun down and concentrated before spreading it on Agar plates. The next day, a few small colonies formed. Only 4 colonies on the pSPARCpIX45-p406 plate and on the pShRedIX45-p406 plate. All colonies were high molecular weight colonies. The colonies were analyzed by PCR. (figure 40) All clones demonstrate the expected PCR product. This PCR was also optimized to find the best annealing temperature. The oligo primer is longer than the forward primer. This makes it hard to find an optimal temperature. The optimal temperature for this reaction was 71,9°C.



Ad RP Ad5RedIX45-p406 BJ clones
Ad SP Ad5SPARCpIX45-p406 BJ clones

Primers: Ad3603pIX.F and Oligo p406 as.R

PCR product in shuttles: 576 bp
PCR product in Ad easy control: -

Figure 40: PCR analysis to test homologous recombination with primers Ad3603pIX.F (GCCGCCATGAGCACCAAC) and Oligo p406 as.R (GGCCGCCACCAAGGCAATGGCCTGGGCATTCATCTTCTGTCCCTTCGAAACAGC)

Homologous recombination with shuttle vectors pSPARCpIX45-5epitopes and pSPARCpIX45-p406BW

The same problem during the first transformation occurred with the pSPARCpIX45-5epitopes and pSPARCpIX45-p406BW vectors. During the second transformation with an increased amount of BJ5183-AD-1 cells and DNA, four colonies of pSPARCpIX45-5epitopes and no colonies grow of pSPARCpIX45-p406BW. All four colonies did not contain the five epitopes of MSLN (figure 41). Another BJ-5183-AD-1 transformation with the same condition was set up. This transformation resulted in another four colonies of pSPARCpIX45-5epitopes and five small colonies of pSPARCpIX45-p406BW. The colonies formed after 24 hours, this longer than the expected 12-18 hours. Clone 7 of pSPARCpIX45-5epitopes contained the five epitopes of MSLN (figure 41). All five clones of pSPARCpIX45-p406BW contained the irrelevant epitope (figure 42).
The optimal temperature for primer combination Ad3603pIX.F and Ad4230pIX.R was 70 °C. For Ad4230pIX.R and oligop406BW was this temperature 72 °C.

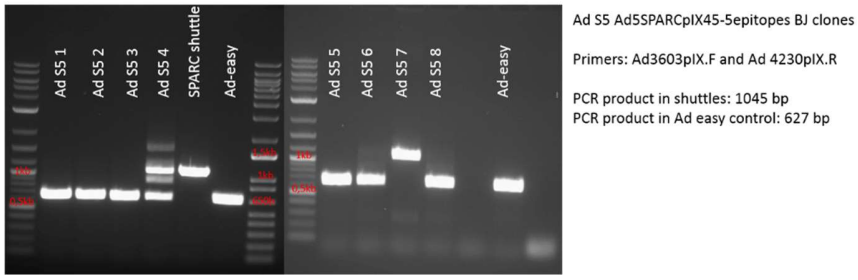


Figure 41: PCR analysis to test homologous recombination with primers Ad3603pIX.F (GCCGCCATGAGCACCAAC) and Ad4230pIX.R (ATGAAGCTCTGCAGTGGTGCTACCT)

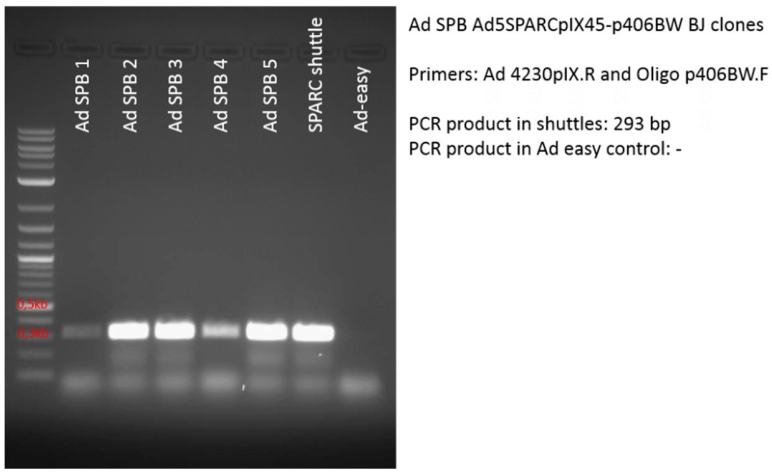
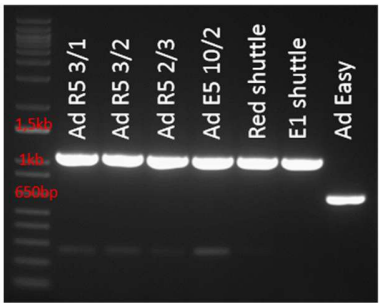


Figure 42: PCR analysis to test homologous recombination with primers Oligop406BW.F (GGCCGCTGTTTCGAAGATTGCCCAGGCCAATATGAAGCAGGGAGCCTTGGTGGC) and Ad4230pIX.R (ATGAAGCTCTGCAGTGGTGCTACCT)

DNA upscaling for transfection

DH10β upscaling with shuttle vectors Ad5IRedIX45-5e and Ad5E1pIX45-5e

To produce more DNA, two BJ colonies were transformed into DH10β. After this transfection, three colonies of each transformation were analyzed. The PCR results in figure 43 show that all clones contain the 5 epitopes. As a final check, a HindIII and PacI digestion are also performed. (figure 44 and 45) The PacI digestion was performed to check the configuration of the Ad because there are two possible recombination possibilities. The two possibilities give either a 3kb or a 4,5kb product when PacI digested. All gels show that the clones have the 3kb configuration and show the predicted restriction pattern.



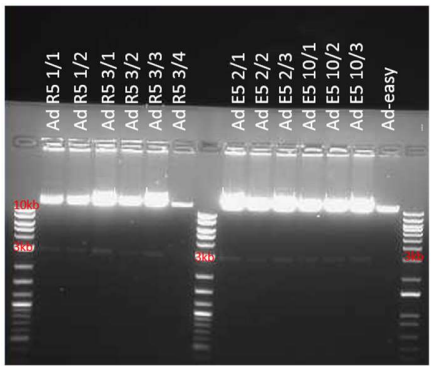
Ad R5 Ad5RedIX45-5epitopes colonies

Ad E5 Ad5E1pIX45-5epitopes colonies

Primers: Ad3603pIX.F and Ad4230pIX.R

PCR product in shuttles: 1045 bp
PCR product in Ad easy control: 627 bp

Figure 43: PCR analysis to test retransformation into DH10β with primers Ad3603pIX.F and Ad4230pIX.R

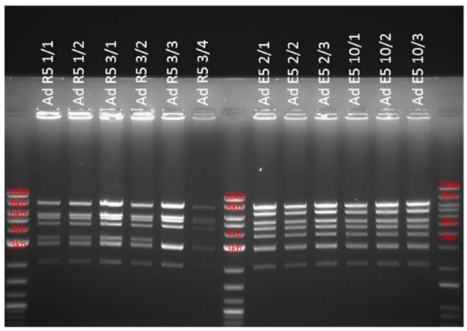


Ad R5 Ad5RedIX45-5epitopes
Ad E5 Ad5E1pIX45-5epitopes

32297: pAd5Redp5e.2*: PacI(1359) - PacI(33656)
2946: pAd5Redp5e.2*: PacI(33656) - PacI(1359)

33718: pAd5E15e.2*: PacI(1359) - PacI(35077)
2946: pAd5E15e.2*: PacI(35077) - PacI(1359)

Figure 44: Restriction Analysis with PacI different DH10β clones of Ad5RedIX45-5epitopes and Ad5E1pIX45-5epitopes



Ad R5 Ad5RedIX45-5epitopes
Ad E5 Ad5E1pIX45-5epitopes

8009: pAd5Redp5e.2*: HindIII(18673) - HindIII(26682)
5850: pAd5Redp5e.2*: HindIII(32645) - HindIII(3252)
5322: pAd5Redp5e.2*: HindIII(6598) - HindIII(11920)
4597: pAd5Redp5e.2*: HindIII(14076) - HindIII(18673)
3346: pAd5Redp5e.2*: HindIII(3252) - HindIII(6598)
3026: pAd5Redp5e.2*: HindIII(26682) - HindIII(29708)
2937: pAd5Redp5e.2*: HindIII(29708) - HindIII(32645)
2081: pAd5Redp5e.2*: HindIII(11920) - HindIII(14001)
75: pAd5Redp5e.2*: HindIII(14001) - HindIII(14076)

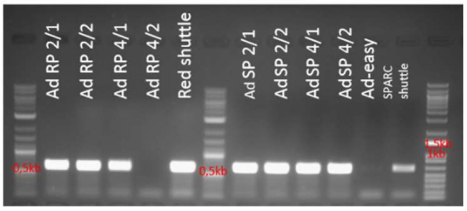
8009: pAd5E15e.2*: HindIII(20094) - HindIII(28103)
6763: pAd5E15e.2*: HindIII(34066) - HindIII(4165)
5322: pAd5E15e.2*: HindIII(8019) - HindIII(13341)
4597: pAd5E15e.2*: HindIII(15497) - HindIII(20094)
3854: pAd5E15e.2*: HindIII(4165) - HindIII(8019)
3026: pAd5E15e.2*: HindIII(28103) - HindIII(31129)
2937: pAd5E15e.2*: HindIII(31129) - HindIII(34066)
2081: pAd5E15e.2*: HindIII(13341) - HindIII(15422)
75: pAd5E15e.2*: HindIII(15422) - HindIII(15497)

Figure 45: Restriction Analysis with HindIII different DH10β clones of Ad5RedIX45-5epitopes and Ad5E1pIX45-5epitopes

DH10β upscaling with shuttle vectors Ad5SPARCpIX45-p406, Ad5RedIX45-p406, Ad5SPARCpIX45-5epitopes and Ad5SPARCpIX45-p406BW

Two different colonies of each construct and one colony of Ad5SPARCpIX45-5epitopes are transformed into DH10β. The PCR analysis demonstrates that all clones are correct, expect Ad5RedIX45-p406 4/2 (figure 46 and 47). A PacI and HindIII digestion are performed on the correct colonies as a final test. The PacI digestion showed that Ad5SPARCpIX45-5epitopes and Ad5RedIX45-p406 have the 3kb configuration and Ad5SPARCpIX45-p406 and Ad5SPARCpIX45-p406BW have the 4,5kb configuration. (figure 48) Clone Ad5SPARCpIX45-p406 4/1 is not fully digested with HindIII. All the other clones contain the right restriction pattern. (figure 49)

To make absolutely sure that the epitopes are incorporated into the pIX DNA, the DNA vectors were sent for sequencing. The sequence results confirmed the incorporation of the epitopes in the pIX capsid protein. The same primers (Ad3904 and Ad4060) were used as during the first sequencing round. The results were exactly the same as in figure 22 (Ad5SPARCpIX45-p406BW), figure 25 (Ad5SPARCpIX45-p406), figure 28 (Ad5RedIX45-p406) and figure 31 (Ad5SPARCpIX45-5epitopes).

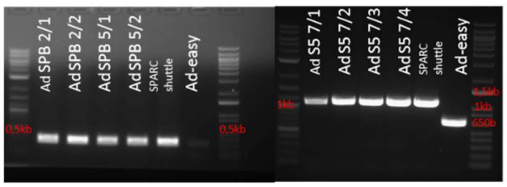


Ad RP Ad5RedIX45-p406 DH10β clones
Ad SP Ad5SPARCpIX45-p406 DH10β clones

Primers: Ad3605pIX.F and Oligo p406 as.R

PCR product in shuttles: 576 bp
PCR product in Ad easy control: -

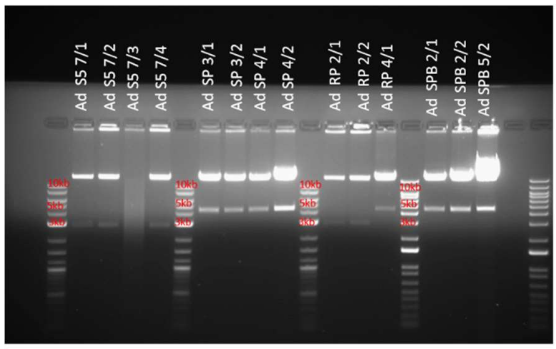
Figure 46: PCR analysis to test retransformation into DH10β with primers Ad3603pIX.F and Oligop406as.R



Ad SPB Ad5SPARCpIX45-p406BW DH10β clones
Primers: Ad 4230pIX.R and Oligo p406BW.F
PCR product in shuttle: 293 bp
PCR product in Ad easy control: -

Ad S5 Ad5SPARCpIC45-5epitopes DH10β clones
Primers: Ad3603pIX.F and Ad4230pIX.R
PCR product in shuttle: 1045 bp
PCR product in Ad easy control: 627 bp

Figure 47: PCR analysis to test retransformation into DH10β with primers Oligop406BW.F and Ad4230pIX.R or Ad3603pIX.F and Ad4230pIX.R



Ad S5 Ad5SPARCpIX45-5epitopes
Ad SP Ad5SPARCpIX45-p406
Ad RP Ad5RedIX45-p406
Ad RPB Ad5SPARCpIX45-p406BW

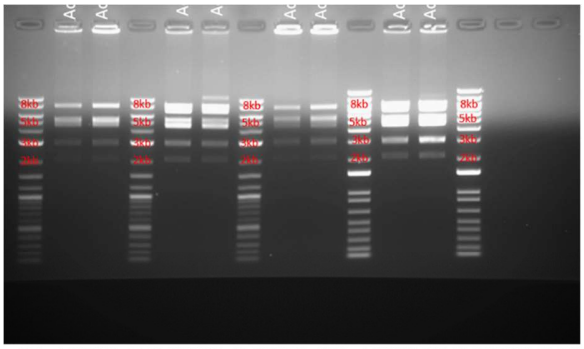
33222: pAd5SPARC5e.2*: PacI(31628) - PacI(28698)
2930: pAd5SPARC5e.2*: PacI(28698) - PacI(31628)

33044: pAd5SPARCp406*: PacI(1) - PacI(33045)
4580: pAd5SPARCp406*: PacI(33045) - PacI(1)

32117: pAd5Redp406.2*: PacI(1359) - PacI(33476)
2946: pAd5Redp406.2*: PacI(33476) - PacI(1359)

33044: pAd5SPARCp406BW*: PacI(4581) - PacI(1)
4580: pAd5SPARCp406BW*: PacI(1) - PacI(4581)

Figure 48: Restriction Analysis with PacI different DH10β clones of Ad5SPARCpIX45-5epitopes, Ad5SPARCpIX45-p406, Ad5RedIX45-p406 and Ad5SPARCpIX45-p406BW



8009: pAd5SPARC5e.2*: HindIII(13715) - HindIII(21724)
5339: pAd5SPARC5e.2*: HindIII(27687) - HindIII(33026)
5322: pAd5SPARC5e.2*: HindIII(1640) - HindIII(6962)
4766: pAd5SPARC5e.2*: HindIII(33026) - HindIII(1640)
4597: pAd5SPARC5e.2*: HindIII(9118) - HindIII(13715)
3026: pAd5SPARC5e.2*: HindIII(21724) - HindIII(24750)
2937: pAd5SPARC5e.2*: HindIII(24750) - HindIII(27687)
2081: pAd5SPARC5e.2*: HindIII(6962) - HindIII(9043)
75: pAd5SPARC5e.2*: HindIII(9043) - HindIII(9118)

8009: pAd5SPARCp406*: HindIII(18060) - HindIII(26069)
6991: pAd5SPARCp406*: HindIII(32032) - HindIII(1399)
5322: pAd5SPARCp406*: HindIII(5985) - HindIII(11307)
4597: pAd5SPARCp406*: HindIII(13463) - HindIII(18060)
4586: pAd5SPARCp406*: HindIII(1399) - HindIII(5985)
3026: pAd5SPARCp406*: HindIII(26069) - HindIII(29095)
2937: pAd5SPARCp406*: HindIII(29095) - HindIII(32032)
2081: pAd5SPARCp406*: HindIII(11307) - HindIII(13388)
75: pAd5SPARCp406*: HindIII(13388) - HindIII(13463)

8009: pAd5Redp406.2*: HindIII(18493) - HindIII(26502)
5850: pAd5Redp406.2*: HindIII(32465) - HindIII(3252)
5322: pAd5Redp406.2*: HindIII(6418) - HindIII(11740)
4597: pAd5Redp406.2*: HindIII(13896) - HindIII(18493)
3166: pAd5Redp406.2*: HindIII(3252) - HindIII(6418)
3026: pAd5Redp406.2*: HindIII(26502) - HindIII(29528)
2937: pAd5Redp406.2*: HindIII(29528) - HindIII(32465)
2081: pAd5Redp406.2*: HindIII(11740) - HindIII(13821)
75: pAd5Redp406.2*: HindIII(13821) - HindIII(13896)

8009: pAd5SPARCp406BW*: HindIII(22640) - HindIII(30649)
6991: pAd5SPARCp406BW*: HindIII(36612) - HindIII(5979)
5322: pAd5SPARCp406BW*: HindIII(10565) - HindIII(15887)
4597: pAd5SPARCp406BW*: HindIII(18043) - HindIII(22640)
4586: pAd5SPARCp406BW*: HindIII(5979) - HindIII(10565)
3026: pAd5SPARCp406BW*: HindIII(30649) - HindIII(33675)
2937: pAd5SPARCp406BW*: HindIII(33675) - HindIII(36612)
2081: pAd5SPARCp406BW*: HindIII(15887) - HindIII(17968)
75: pAd5SPARCp406BW*: HindIII(17968) - HindIII(18043)

Figure 49: Restriction Analysis with HindIII different DH10β clones of Ad5SPARCpIX45-5epitopes, Ad5SPARCpIX45-p406, Ad5RedIX45-p406 and Ad5SPARCpIX45-p406BW

Transfection into HEK-293 cells

DNA preparation for transfection

All the constructs were linearized with PacI before the transfections. These linearizations were checked with gel electrophoresis and were all successful.

First transfections

Constructs Ad5Red-5epitopes and Ad5E1-5epitopes were transfected on 1 May. After 24 hours, Ad5Red-5epitopes was checked on the microscope for red fluorescence. Red fluorescence spots were visible in the cells. This means that the transfection worked. After 12 days, Ad5Red-5epitopes was contaminated with fungus in one of the wells. (figure 50) The fungus was removed and the other wells were harvested. There were no plaques visible yet. Ad5E1-5epitopes was harvest after 14 days when there was no plaque visible yet. The construct was harvested because the change for infection with the fungus was high. Both constructs (Ad5Red-5epitopes and Ad5E1-5epitopes) were transferred to a T25 flask with HEK-293. However, there was no plaques or cells death visible after 5 days. Both transfections did not result in virus rescue.

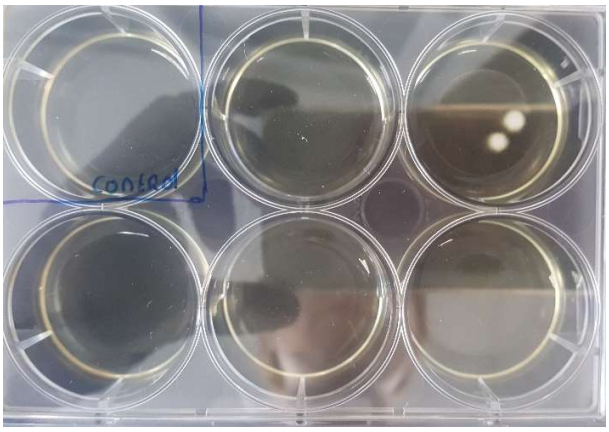


Figure 50: Transfection plate of Ad5Red-5epitopes after 12 days. One well is contaminated with fungus.

Second transfections

During the second round of transfections, constructs Ad5SPARC-5epitopes, Ad5SPARC-p406, Ad5SPARC-p406BW and Ad5Red-p406 were transfected. In addition, the earlier failed constructs were transfected again. After 24 hours, the constructs with red fluorescence were checked under the microscope. The two red fluorescence construct both showed red spots in the cells. This means the transfection worked and the viral DNA is inside the cells. The cells were harvest when plaques were clearly visible. This was after 10-14 days. (table 8) An example of plaques is shown in figure 51 and a red fluorescence picture in figure 52.

Construct Ad5E1-5epitopes was infected with the same virus as Ad5Red-5epitopes. The E1 virus is not necessary for the test of the hypothesis. This virus was a practice virus. Because of this and the lack of time, the construct was not transfected for the third time.

Table 8: Schedule of transfection, harvest, and reinfection of the constructs

	Transfection	**Harvest 6-wells**	**T25**	**Harvest T25**
Ad5SPARC-p406 clone 3/1	24/05/18	05/06/18	08/06/2018	15/06/18
Ad5SPARC-p406 clone 4/1	24/05/18	05/06/18	08/06/2018	No plaques
Ad5SPARC-5epitopes	21/05/18	01/06/18	08/06/2018	15/06/18
Ad5SPARC-p406BW	21/05/18	04/06/18	08/06/2018	18/06/18
Ad5Red-p406	25/05/18	08/06/18	08/06/2018	18/06/2018
Ad5Red-5epitopes	18/05/18	01/06/18	08/06/2018	18/06/2018
Ad5E1-5epitopes	06/06/18	Infection		
	Upscaling		**Harvest upscaling**	
Ad5SPARC-p406 clone 3/1	T75: 18/06/18		25-06-18	
Ad5SPARC-5epitopes	T25: 19/06/18		28-06-18	
Ad5SPARC-p406BW	T25: 19/06/18		25-06-18	
Ad5Red-p406	T25: 19/06/18		28-06-18	
Ad5Red-5epitopes	T25: 19/06/18		25-06-18	

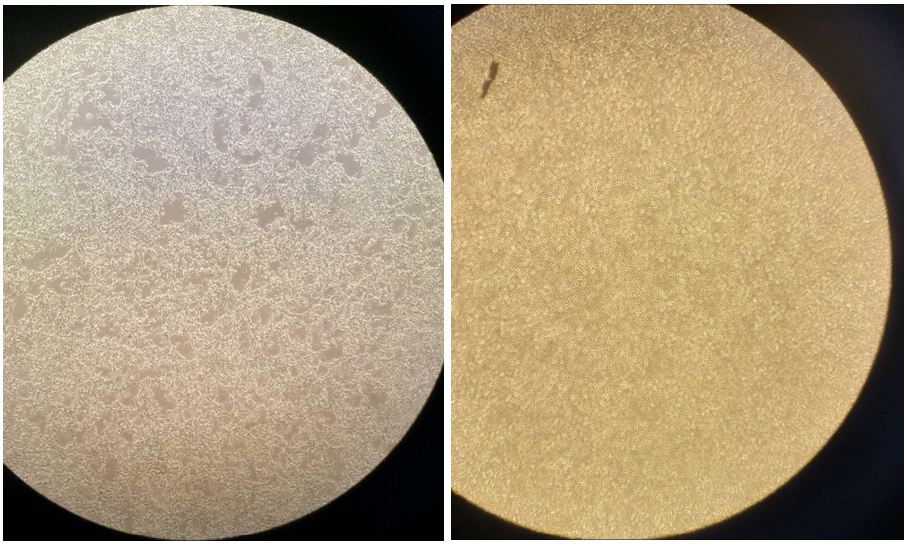


Figure 51: Left picture is plaque formation of Ad5SPARC-p406 3/1 12 days post transfection. The right picture is the control well. (10x magnification)

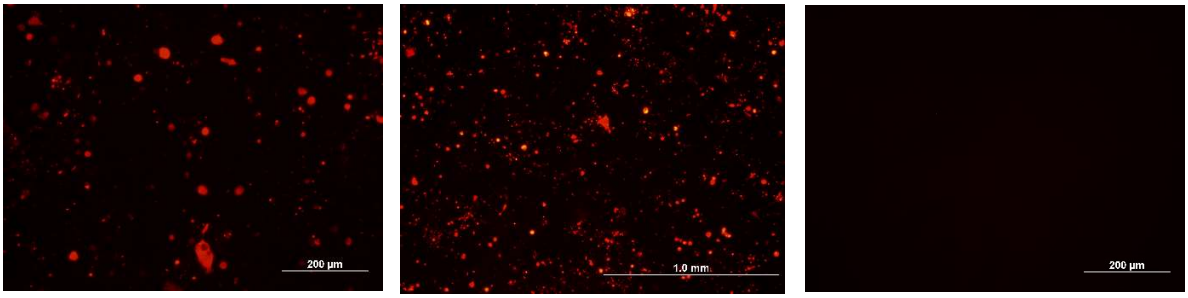


Figure 52: Left picture shows Ad5Red5epitopes 14 days post transfection at zoom 10x. Middle picture is the same construct at 4x magnification. Right picture is the control.

Re-infection into new T25 and upscaling

The harvested virus from the well with plaques was re-infected in a T25 flask with HEK-293 cells. (table 8) The virus was harvested from the T25 flask in 7 to 10 days based on their plaques and cell death. (figure 53, 54 and 55) The cells died slower than expected. For this reason, the same size flask was used for another re-infection. Only Ad5SPARCp406 3/1 was re-infected in T75 because this virus showed more cell death.

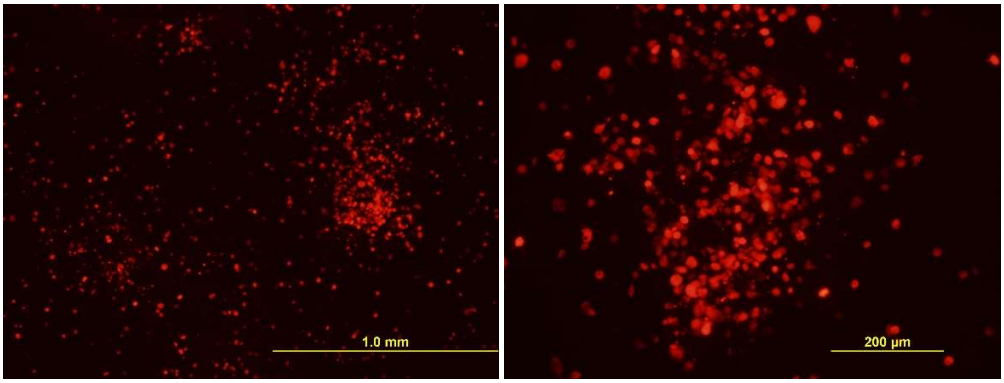


Figure 53: Ad5Red-5epitopes 10 days post infection in T25

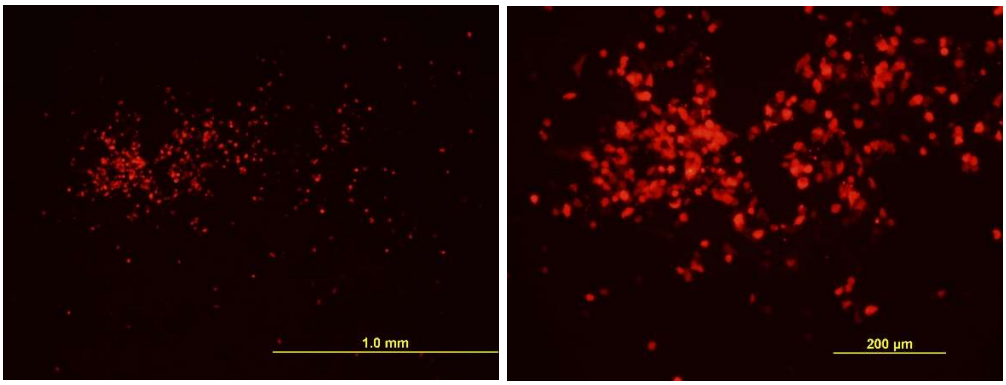


Figure 54: Ad5Red-p406 10 days post infection in T25

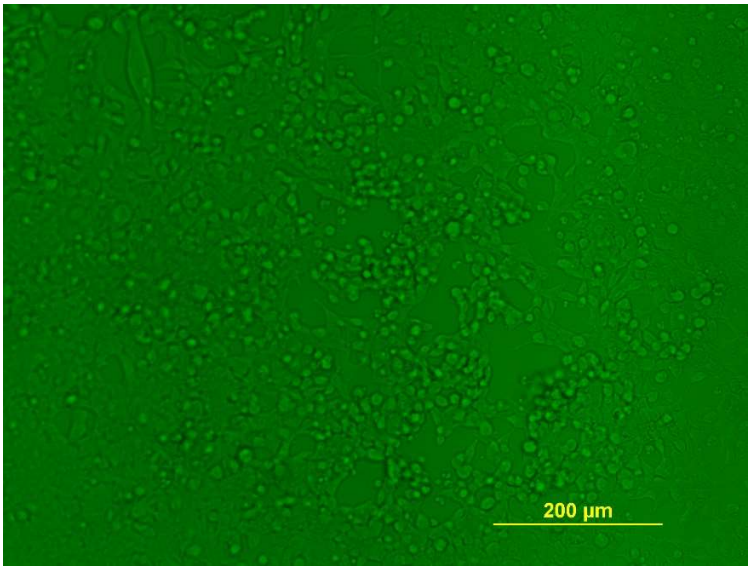


Figure 55: Ad5SPARCp406BW 10 days post infection, green light filter

Discussion

The aim of this project was testing the anti-tumor immunization of a CRAd based on the SPARC promoter with MSLN peptides incorporated into minor capsid protein pIX. The SPARC promoter as well as MSLN has been proven effective ovarian cancer targets. [32], [33] In addition, pIX incorporation has been proven to be able to induce an innate and adaptive immune response.

First cloning step

During the first cloning step, the ligation of one (p406-p414) epitope and the irrelevant epitope into the digested pShRedIX45 did not work. However, the control plasmid formed colonies on the kanamycin agar plate. The control plate demonstrates that the cells are viable. The DNA fragment of 54 bp was successfully inserted into the other vector, pShE1pIX45. This shows that the insert was not toxic to the cells and that the right antibiotic was used. The most logical reason for the failed transfection is inefficient ligation. [42]

Inefficient ligation may be caused by unfavorable molecular ratios of vector to insert, contaminants in the ligation mixture, not long enough overhangs or low quality of DNA. Firstly, in the first ligation a molecular ratio vector/insert of 1:600 was used. This high excess of the insert was chosen because the insert size is uncommonly small. [43] In addition, multiple insertions are not common with small inserts. The same ratio was used in the E1 vector and this worked. Standard ligations are performed with molecular ratios between 1:1 to 1:10. [44] For this reason, ratios 1:2 and 1:150 were also used. However, these ratios did not result in a successful ligation either. Secondly, the ligation mixture may be contaminated. This is unlikely since the reaction has been performed for three times with the same reagents that worked in other ligations. Thirdly, the overhang was 3 AA. The same overhang was used in the E1 vector ligation and worked here. Fourthly, the quality of the ShRedIX45 vector may be low. In the HiFi assembly with the five epitope insert, the Red vector assembly resulted in an observable lower amount of colonies. The HiFi assembly is a more effective ligation method than a normal T4 ligation. The combination of the low quality of the Red vector and the size of the inserted fragment together can be the possibility for the failed ligation. The quality of the Red vector can be affected by UV-light. During the removal of the H7 region, the Red vector was exposed to UV light. Moreover, the optimal molecular ratio may also be explored.

The first ligation with pShE1pIX45 and annealed oligo p406 showed backward configuration. On both sides of the oligo, the sticky overhang is AAA. Because this overhang is the same on both side, the oligo can be inserted in two possible directions. There is a 50% change at every configuration.

Second cloning step

During the second cloning step, the used molecular ratio between vector and insert was 1:1. This ratio was chosen because of the large size of the insert (~3-3,5kb) in comparison to the vector (~6kb). This ligation and transformation worked at the first try.

Homologous recombination

All the homologous recombination with the Ad-easy system formed fewer colonies than expected. Shuttle plasmids pShIX45-5e and pShE1pIX45-5e formed colonies after the second try. For the other four constructs, plenty of different conditions were used. During the different conditions, the constructs formed one, two colonies or no colonies. By performing plenty of recombination and the formation of a few colonies every time, there were enough colonies in the end. All the different conditions used are:

- different amounts of cells (20/30/40 µl)
- more water to reduce the salt concentration
- higher concentration of DNA

- concentrated incubation mixture by spinning down the cells
- different tubes and boxes of BJ-5183-Ad-1 cells
- two different electroporation machines
- two different kanamycin concentration in the agar plates

In addition, the BJ-5183-Ad-1 are spread out on Agar plates with no antibiotics to check the condition of the cells. These plates formed plenty of colonies.

Fortunately, every construct had at least one colony where the homologous recombination was successful. Nevertheless, the number of colonies was astonishingly low. Possible explanations for this observation are:

- Transformation conditions are not optimal

The protocol for BJ5183-Ad-1 was closely followed. Also, a senior researcher who has experience with the Ad-easy homologous recombination performed one of the transformations and had the same results.

- The incorrect antibiotic is used or antibiotic concentration is too high

The Ad-easy genome is ampicillin resistance and the shuttle plasmid is kanamycin resistance. When recombination occurs the created adenoviral genome is kanamycin resistance. The used antibiotic was kanamycin with a concentration of 100 $\mu g.ml^{-1}$ and 20 $\mu g.ml^{-1}$. The standard concentration is 50 $\mu g.ml^{-1}$. The low concentration of 20 $\mu g.ml^{-1}$ gave the same amount of colonies as the 100 $\mu g.ml^{-1}$ concentration.

- DNA preparations are not optimal

Lou et al recommend using alkaline lysis miniprep procedure instead of gel-purifying PmeI-digested shuttle vectors. [45] In this project, the PmeI-digested shuttle vector was purified by gel extraction. According to Lou et al's article, this may be an explanation for the observed amount of colonies.

- Competence of BJAd-Easier cells is not sufficient or optimal

The BJ5183-Ad-1 cells came with a control plasmid. This control plasmid was used for analysis during previous projects. For this reason, it was not possible to do a control homologous recombination. The competence of the BJ-5183-Ad-1 may be the cause for the observed number of colonies.

In conclusion, the DNA preparations or the competence of the BJAd-easy cells can both be the cause of the low efficiency of the homologous recombination. [45]

DNA upscaling for transfection

The adenoviral genomes are transformed into DH10β cells. In this transformation, all colonies should contain identical DNA. Multiple colonies were analysed to make sure nothing went wrong during the transformation. All the colonies showed the correct restriction pattern. In addition, the restriction analyses were also performed to identify the homologous regions. Recombination on the left arm can occur on two places: left arm homologous region of origin of replication. If recombination took place between the left arms, PacI digestion results in a 3kb. If recombination occurs at the origins of replication, PacI digestion gives a 4,5kb fragment.[41] (figure 56) This means that Ad5Red-5e, Ad5E1-5e, Ad5SPARC-5e, and Ad5PARC-p406BW were constructed by homologous recombination between the left arm and Ad5SPARC-p406, Ad5Red-p406 in the origins of replication.

One colony of each construct was up-scaled for the transfection. The transfection efficiency is low. For this reason, it is better to infect HEK-293 cells with two different recombination clones of each construct. During this project, this was not possible because there were no glycerol stocks made of multiple clones. Only for Ad5SPARC-p406 two glycerol stocks were made. For this reason, both clones were infected in HEK-293 cells

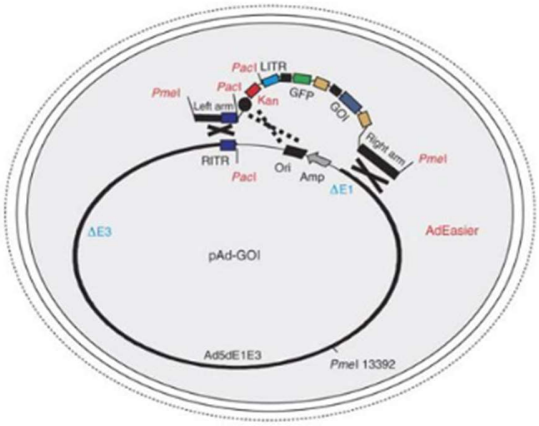


Figure 56: Homologous recombination in BJ5183 with Ad-easy genome inside [45]

Transfection into HEK-293 cells

During the first transfection with Ad5Red-5e, the 6-well plate got infected with fungus. A possible reason for infection with the fungus is the acidity of the media. As shown in figure 50, the media turned from pink to yellow. The DMEM-F12 media contains phenol red as pH indicator. When the media turn yellow, the acidity increased. This enhanced fungus growth. In addition, the media contains ampicillin and streptomycin, but no anti-fungi substances. The acidity probably changed because the cells were too confluent during the transfection. The crowed cells use the nutrients and their metabolites are acidic. For example, the metabolite of glucose is CO2, which forms carbonic acid with H2O. [46]

The remaining wells without the fungus infection were collected and re-infected in new cells. The virus did not cause the new cells to form plaques or cause cell death. A possible reason for this that the virus was not present. The virus was likely not formed because the harvest was too soon. Specifically, the harvest was 12 days post infection (normally 14 days) and no plaques were present during the harvest. In addition, Ad5E1-5e also did not have plaques in between the cells during the harvest, which mean that the virus is also not produced here. Another reason for no plaques formation after the re-infection in T25 can be that it took longer than the 5 days. The T25 flask was thrown away after 5 days. It may be that the concentration of virus was still too low after 5 days to see the plaques formation.

All constructs, except Ad5SPARC-p406 clone 4/1, resulted in successful virus harvest. There are several reasons that the Ad5SPARC-p406 clones 4/1 could not be harvested. The virus formed plaques during the transfection into a 6-well plate. When the virus was harvest from the 6-well plate, the cells were spun down and re-suspended in media. It is possible that the virus was mainly in the supernatant and not inside the cell. Another possible reason is that the plaques looked like plaques but were not plaques. The cells stayed for 14 days in the wells-plate and the media was not changed in those 14 days. The cells could be dying. This means that the plaques were dying cells because of the poor conditions. The efficiency of the transfection is also low because of different factors that can affect the transfection. For example, the number of passages of the cells, the point of infection, the point of harvest and the multiplicity of infection. [47], [48] Moreover, the virus could also be present in the T25 flask, but in a too low titer to causes plaques formation.

Another observation is the low progression of virus growth after re-infection. If there were plaques visible before the harvest, then the virus is present. After 7 to 10 days, the virus causes cell death. The expectation was that the virus will cause cell death after 3-4 days because the virus is already present and can infect cells immediately. The observed incubation time necessary for plaque formation is not conform to the expectation. There are a few reasons that the cytotoxicity caused by the virus took longer: the virus titer is too low, virus loss during the procedure, virus destabilization or slow virus replication caused by the promoter.

A second re-infection in the same size flask was performed to upscale the virus. The expectation for cells death was again 3-4 days. However, it took 9 days for the virus to cause cytotoxicity. During both re-infections, similar time intervals were necessary to cause cytotoxicity by the virus. This indicated that the virus procedure and the virus titer is not the cause of this observation, because it occurred in both re-infections. The third explanation can be virus destabilization. Capsid protein pIX stabilize the adenovirus capsid. Incorporation of foreign proteins can destabilize the capsid. Destabilization of the capsid results in difficulties with the harvest, like slower replication of the virus. However, several other studies with different peptide incorporated into pIX results that incorporation up to 12-30 kDa does not affect the capsid stability. [38], [49] For example, Meulenbroek et al investigate whether the Ad pIX can be used to present large protein on the Ad capsid. They generated an Ad with green fluorescent protein (GFP) incorporated into pIX. They concluded that the incorporation did not affect propagation or infectivity of the virus. Based on this and the articles mentioned above, it is not likely that the capsid is destabilized by MSLN-peptides incorporation into pIX.

The last possible reason is that the SPARC promotor reduced the infection rate of the virus. The SPARC promotor replicates slower than a non-modified vector. This observation is not documented in other SPARC related research paper. However, there is not an article that mentions the replication rate. The articles are focussed on cell death caused by the CRAd. [50], [51]This mean that the SPARC promoter may be the reason for slow replication.

Conclusion

Unfortunately, there was not enough time to fully upscale the Ads and test them in an immunization assay. For this reason, the hypothesis, CRAd with capsid-incorporated mesothelin gives higher anti-tumor immunization in the ID8 model then single treatment of CRAd or mesothelin as a transgene, could not be tested. However, Ad5SPARC-p406, Ad5SPARC-p406BW, Ad5SPARC-5e, Ad5Red-p406 and Ad5Red-5e are successfully harvest after the transfection. The SPARC promoter may be interfering with the replication speed of the Ad which causes lower cell death in the HEK-293 cells. The Ads titers should be up-scaled and the Ads should be purified. To determine if the epitopes are expressed on the adenovirus capsid, a western blot analysis on the His-tag incorporated in pIX should be performed.

In conclusion, epitope display based on pIX for immunization, MSLN as a tumor antigen and the SPARC promoter specificity for ovarian cancer cells are all validated. Taken all this together, the expectation is that the immunization assays support the hypothesis: tumor antigen mesothelin incorporated into capsid protein pIX enhances anti-tumor immunization based on CRAd. However, there is a possibility that the epitopes are not incorporated into capsid protein pIX.

Reference

[1] B. I. Milavetz and L. Balakrishnan, “Viral epigenetics.,” *Methods Mol. Biol.*, vol. 1238, pp. 569–96, 2015.

[2] Y. Zhang and J. M. Bergelson, “Adenovirus receptors.,” *J. Virol.*, vol. 79, no. 19, pp. 12125–31, Oct. 2005.

[3] C. Lyle and F. McCormick, “Integrin alphavbeta5 is a primary receptor for adenovirus in CAR-negative cells.,” *Virol. J.*, vol. 7, p. 148, Jul. 2010.

[4] N. Tatsis and H. C. J. Ertl, “Adenoviruses as vaccine vectors,” *Mol. Ther.*, vol. 10, no. 4, pp. 616–629, Oct. 2004.

[5] N. J. Schuldt and A. Amalfitano, “Malaria vaccines: Focus on adenovirus based vectors,” *Vaccine*, vol. 30, no. 35, pp. 5191–5198, 2012.

[6] D. H. Barouch, “Novel adenovirus vector-based vaccines for HIV-1,” *Curr. Opin. HIV AIDS*, vol. 5, no. 5, pp. 386–390, Sep. 2010.

[7] I. K. Choi and C. O. Yun, “Recent developments in oncolytic adenovirus-based immunotherapeutic agents for use against metastatic cancers,” *Cancer Gene Ther.*, vol. 20, no. 2, pp. 70–76, 2013.

[8] S. Biologicals, “A Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Infants,” *N. Engl. J. Med.*, vol. 367, no. 24, pp. 2284–2295, 2012.

[9] J. Kopycinski *et al.*, “Broad HIV Epitope Specificity and Viral Inhibition Induced by Multigenic HIV-1 Adenovirus Subtype 35 Vector Vaccine in Healthy Uninfected Adults,” *PLoS One*, vol. 9, no. 3, p. e90378, Mar. 2014.

[10] R. G. Crystal, “Adenovirus: the first effective in vivo gene delivery vector.,” *Hum. Gene Ther.*, vol. 25, no. 1, pp. 3–11, Jan. 2014.

[11] S. I. Michael, J. S. Hong, D. T. Curiel, and J. A. Engler, “Addition of a short peptide ligand to the adenovirus fiber protein.,” *Gene Ther.*, vol. 2, no. 9, pp. 660–8, Nov. 1995.

[12] Q. L. Matthews, “Capsid-Incorporation of Antigens into Adenovirus Capsid Proteins for a Vaccine Approach,” *Mol. Pharm.*, vol. 8, no. 1, pp. 3–11, Feb. 2011.

[13] Q. L. Matthews *et al.*, “Optimization of Capsid-Incorporated Antigens For A Novel Adenovirus Vaccine Approach,” *Virol. J.*, vol. 5, no. 1, p. 98, Aug. 2008.

[14] S. M. Sumida *et al.*, “Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein.,” *J. Immunol.*, vol. 174, no. 11, pp. 7179–85, Jun. 2005.

[15] Y. S. Ahi, D. S. Bangari, and S. K. Mittal, “Adenoviral vector immunity: its implications and circumvention strategies.,” *Curr. Gene Ther.*, vol. 11, no. 4, pp. 307–20, Aug. 2011.

[16] E. E. Thacker, L. Timares, and Q. L. Matthews, “Strategies to overcome host immunity to adenovirus vectors in vaccine development,” *Expert Rev. Vaccines*, vol. 8, no. 6, pp. 761–777, Jun. 2009.

[17] A. K. Zaiss, H. B. Machado, and H. R. Herschman, “The influence of innate and pre-existing immunity on adenovirus therapy,” *J. Cell. Biochem.*, vol. 108, no. 4, pp. 778–790, Nov. 2009.

[18] C. A.J. van Winkel, A. Moreno, and D. T. Curiel, “Capsid-incorporation strategy to display antigens for an alternatieve adenoviral vector vaccine approach,” *Mol. Pharm. (in submission)*.

[19] C. Larocca and J. Schlom, “Viral vector-based therapeutic cancer vaccines.,” *Cancer J.*, vol. 17, no. 5, pp. 359–71, 2011.

[20] D. T. Rein, M. Breidenbach, and D. T. Curiel, “Current developments in adenovirus-based cancer gene therapy.,” *Future Oncol.*, vol. 2, no. 1, pp. 137–43, Feb. 2006.

[21] R. Alemany, “Oncolytic Adenoviruses in Cancer Treatment.,” *Biomedicines*, vol. 2, no. 1, pp. 36–49, Feb. 2014.

[22] Y. Jounaidi, J. C. Doloff, and D. J. Waxman, “Conditionally replicating adenoviruses for cancer treatment.,” *Curr. Cancer Drug Targets*, vol. 7, no. 3, pp. 285–301, May 2007.

[23] R. Fan *et al.*, “Enhanced antitumoral efficacy and immune response following conditionally replicative adenovirus containing constitutive HSF1 delivery to rodent tumors.,” *J. Transl. Med.*, vol. 10, p. 101, May 2012.

[24] J. De Munck, A. Binks, I. A. McNeish, and J. L. Aerts, “Oncolytic virus-induced cell death and immunity: a match made in heaven?,” *J. Leukoc. Biol.*, vol. 102, no. 3, p. jlb.5RU0117-040R, 2017.

[25] P. P. Claudio, C. Howard, and R. Nande, “Ultrasound-mediated oncolytic virus delivery and uptake for increased therapeutic efficacy: state of art,” *Oncolytic Virotherapy*, vol. 4, p. 193, Nov. 2015.

[26] G. E. Blair, S. C. Dixon1, S. A. Griffiths, and M. E. Blair Zajdel, “Restricted replication of human adenovirus type 5 in mouse cell lines,” *Virus Res.*, vol. 14, no. 4, pp. 339–346, Dec. 1989.

[27] L. Zhang, F. Hedjran, C. Larson, G. L. Perez, and T. Reid, “A novel immunocompetent murine model for replicating oncolytic adenoviral therapy.,” *Cancer Gene Ther.*, vol. 22, no. 1, pp. 17–22, Jan. 2015.

[28] H. Jiang *et al.*, “Delta-24-RGD oncolytic adenovirus elicits anti-glioma immunity in an immunocompetent mouse model.,” *PLoS One*, vol. 9, no. 5, p. e97407, 2014.

[29] J. Walton *et al.*, “CRISPR/Cas9-mediated Trp53 and Brca2 knockout to generate improved murine models of ovarian high-grade serous carcinoma,” *Cancer Res.*, vol. 76, no. 20, pp. 6118–6129, 2016.

[30] M. V. Lopez *et al.*, “A tumor-stroma targeted oncolytic adenovirus replicated in human ovary cancer samples and inhibited growth of disseminated solid tumors in mice,” *Mol. Ther.*, vol. 20, no. 12, pp. 2222–2233, 2012.

[31] A. L. Alfano *et al.*, “Oncolytic Adenovirus-Loaded Menstrual Blood Stem Cells Overcome the Blockade of Viral Activity Exerted by Ovarian Cancer Ascites,” *Mol. Ther. - Oncolytics*, vol. 6, no. September, pp. 31–44, 2017.

[32] J. Chen *et al.*, “SPARC Is a Key Regulator of Proliferation, Apoptosis and Invasion in Human Ovarian Cancer,” *PLoS One*, vol. 7, no. 8, p. e42413, Aug. 2012.

[33] I. Pastan and R. Hassan, “Discovery of mesothelin and exploiting it as a target for immunotherapy.,” *Cancer Res.*, vol. 74, no. 11, pp. 2907–12, Jun. 2014.

[34] N. Scholler *et al.*, “Soluble member(s) of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma,” *Proc. Natl. Acad. Sci.*, vol. 96, no. 20, pp. 11531–11536, 1999.

[35] R. J. Parks, “Adenovirus protein IX: a new look at an old protein.,” *Mol. Ther.*, vol. 11, no. 1, pp. 19–25, Jan. 2005.

[36] N. C. Salisch *et al.*, “Antigen capsid-display on human adenovirus 35 via pIX fusion is a potent vaccine platform.,” *PLoS One*, vol. 12, no. 3, p. e0174728, 2017.

[37] I. P. Dmitriev, E. A. Kashentseva, and D. T. Curiel, “Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX.,” *J. Virol.*, vol. 76, no. 14, pp. 6893–9, Jul. 2002.

[38] Q. L. Matthews *et al.*, “Epitope Capsid-Incorporation: New Effective Approach for Vaccine Development for Chagas Disease.,” *Pathog. Immun.*, vol. 1, no. 2, pp. 214–233, 2016.

[39] I. M. Stromnes *et al.*, “T Cells Engineered against a Native Antigen Can Surmount Immunologic and Physical Barriers to Treat Pancreatic Ductal Adenocarcinoma,” *Cancer Cell*, vol. 28, no. 5, pp. 638–652, Nov. 2015.

[40] E. Zervos, S. Agle, A. G. Freistaedter, G. J. B. Jones, and R. L. Roper, “Murine mesothelin: characterization, expression, and inhibition of tumor growth in a murine model of pancreatic cancer,” *J. Exp. Clin. Cancer Res.*, vol. 35, no. 1, p. 39, 2016.

[41] W. W. Web, “AdEasy XL Adenoviral Vector System Instruction Manual For Research Use Only. Not for use in diagnostic procedures. AdEasy XL Adenoviral Vector System.”

[42] New England BioLabs, “Troubleshooting Transformation Reactions | NEB.” [Online]. Available: https://www.neb.com/tools-and-resources/troubleshooting-guides/troubleshooting-transformation-reactions. [Accessed: 18-Jun-2018].

[43] BROAD Institute, “Protocol : Cloning of oligos for sgRNA (CRISPR) or shRNA constructs,” *Protoc. Cloning oligos sgRNA or shRNA Constr.*, no. August, pp. 1–8, 2015.

[44] M.-C. Hung and P. C. Wensink, “Different restriction enzyme-generated sticky DNA ends can be joined in vitro,” *Nucleic Acids Res.*, vol. 12, no. 4, 1984.

[45] J. Luo *et al.*, “A protocol for rapid generation of recombinant adenoviruses using the AdEasy system,” *Nat. Protoc.*, vol. 2, no. 5, pp. 1236–1247, May 2007.

[46] M. Arora, “Cell Culture Media: A Review,” *Mater. Methods*, vol. 3, Mar. 2013.

[47] M. Park, M. Lee, S. Kim, E.-C. Jo, and G. Lee, “Influence of culture passages on growth kinetics and adenovirus vector production for gene therapy in monolayer and suspension cultures of HEK 293 cells,” *Appl. Microbiol. Biotechnol.*, vol. 65, no. 5, pp. 553–8, Oct. 2004.

[48] Reece-Ford Matthew, Hitchcock Anthony G., and Lipinski Kai S., “Aspects of process development for virus vector production to imporve quality and quantity,” *Pharmacuetical Technol. Eur.*, vol. Jan, 2018.

[49] R. A. Meulenbroek, K. L. Sargent, J. Lunde, B. J. Jasmin, and R. J. Parks, “Use of adenovirus protein IX (pIX) to display large polypeptides on the virion - Generation of fluorescent virus through the incorporation of pIX-GFP,” *Mol. Ther.*, vol. 9, no. 4, pp. 617–624, 2004.

[50] M. V. Lopez *et al.*, “Expression of a suicidal gene under control of the human secreted protein acidic and rich in cysteine (SPARC) promoter in tumor or stromal cells led to the inhibition of tumor cell growth,” *Mol. Cancer Ther.*, vol. 5, no. 10, pp. 2503–2511, Oct. 2006.

[51] M. V. Lopez *et al.*, “Tumor Associated Stromal Cells Play a Critical Role on the Outcome of the Oncolytic Efficacy of Conditionally Replicative Adenoviruses,” *PLoS One*, vol. 4, no. 4, p. e5119, Apr. 2009.