Vitotherapy of prostate cancer and development of monocytes as delivery vehicles of oncolytic adenovirus

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Abstract

This report contains two different projects. The first project considers a virus with replication restricted to prostate cells and it encodes for a protein called neutrophil activating protein (NAP). I have confirmed that prostate cells transduced with the virus contain both NAP mRNA and protein. NAP has been shown to have immune modulating effects on immune cells. I showed by using migration assays that NAP has a chemotactic effect on neutrophils. NAP’s immune modulating effects can possibly enhance the potency of an oncolytic virus that can be used in cancer virotherapy.

The second project considers development of a tumor-targeted, cell-based monocytic carrier system to enhance the delivery of oncolytic adenovirus to tumors after systemic delivery. A pShuttle plasmid, iHREcmv_E1A, was recombined with an adenoviral vector creating the virus Ad5/35[iHREcmv_E1A]. The E1A gene in Ad5/35[iHREcmv_E1A], which function to activate other adenoviral genes, is under transcriptional control of a hypoxia responsive element (HRE). HRE activate transcription of E1A in hypoxia, which is a condition of many tumors. The homing propensity of monocytes against supernatant from neuroendocrine BON tumor cells was evaluated using migration assays. However, the results showed a higher migration capacity of monocytes migrating towards LNCaP cells (Eriksson F. unpublished data) than towards BON cells. In an *in vivo* experiment, I injected Ad5/35[HREcmv-Luc] directly into established subcutaneous BON tumors in mice and the luciferase expression in tumors was compared with mice that got intravenously injected monocytes transduced with the virus. Expression of luciferase was only detected in mice that got intratumoral injection of virus. The idea was to use monocytes as carrier cells for Ad5/35[iHREcmv-E1A] and restrict replication of the virus to the tumor site after systemic delivery.
Introduction

Virotherapy

Viral existence was first recognized in the end of the 19th century. Early case reports reveal that researchers encountered cancer patients with naturally occurring virus infections that had regression in tumor growth. Viruses were used to transmit infections to cancer patients. Most infections were defeated by the immune system and had no impact on tumor growth but in immunosuppressed people, the infection persisted and tumors regressed. However, morbidities were high in these patients and the field was abandoned [1].

Today, genetically engineered viruses are of big interest in cancer therapy. Oncolytic viruses are designed to selectively target and lyse tumor cells. In this way virions are amplified during each infection cycle and new tumor cells are infected [2]. Also, immunogenic viruses developed by genetic manipulation are used in virotherapy. These direct the immune system to an antitumoral immune response.

Adenoviruses in virotherapy

Adenoviruses are one of the most commonly used viruses in virotherapy. They have a relatively low pathogenicity, their genome does not integrate into host genome, they are easily genetically manipulated and relatively stable in the bloodstream which is of importance for systemic administration [3]. Today there are 51 known serotypes which are divided into six subgroups, A to F. One of the most common serotype is adenovirus serotype 5 (Ad5) belonging to subgroup C [4].

Limitations of virotherapy

Virotherapy encounters different obstacles. One obstacle is that viruses are eradicated by the immune system. Adenoviruses have been shown to cause rapid complement activation [5]. As reviewed by Davis et al. [6], one of the most significant problem in virotherapy is neutralization of viruses by circulating antibodies. The outer coat fibers of the virus, for example penton base proteins and hexons, are the antigens that are most susceptible to circulating antibodies. These antigens are detected by B cell receptors that signal to the B cell to propagate and secrete circulating antibodies. Antibodies also cause agglutination of viruses that are then phagocytosed by macrophages and other phagocytes. Another problem is that most oncolytic viruses require local administration in order to be efficient, which can be a problem if the tumor is inaccessible [3]. Also, physical barriers in the form of tumor supporting structures can limit intratumoral spread [7].
**Construction of an oncolytic adenovirus with prostate specific replication**

Tumor specific promoters are often used to regulate expression of different viral genes. E1A is a gene, the functions of which are to control activation of other adenoviral genes [8]. A prostate specific conditionally replicating adenovirus (CRAD) with oncolytic effect in prostate cancer cells has previously been developed [9]. This virus is called Ad5[I/PPT-E1A]. In this virus, E1A expression is under control of the PPT sequence. The PPT sequence is comprised by the prostate specific antigen (PSA) enhancer, the prostate specific membrane antigen (PSMA) enhancer and the T cell receptor γ-chain alternative reading frame protein (TARP) promoter [10]. The PPT sequence is in turn regulated by the mouse H19 insulator [11]. This H19 insulator has been reduced by 1.4 kb to create the Ad5[i/PPT-E1A] [12], which improves the activity of the PPT sequence and allows for insertion of additional therapeutic genes. To reduce background replication of the virus in hepatic cells and thereby preventing liver toxicity, liver specific microRNA (miR122) has been inserted into the virus creating the Ad5[i/PPT-E1A-miR122] [13]. The genetic material of the virus was further reduced by removal of 500 bp in the PPT sequence to allow for insertion of additional therapeutic genes Ad5[i/ppt-E1A-miR122]. The neutrophil activating protein (NAP) gene, linked to E1A via a self cleaving T2A sequence, was inserted which created the final Ad5[i/ppt-NAP-T2A-E1A-miR122] virus [Essand, M and coworkers, unpublished data].

**Neutrophil Activating Protein: an immune modulating agent**

Neutrophil activating protein (NAP) is a virulence factor originating from *Helicobacter pylori*. The protein has a molecular mass of 150 kD and acts via toll like receptor 2 TLR-2 [14]. As reviewed by D’Elios et al. [15], NAP is chemotactic for neutrophils and monocytes, stimulates neutrophil adhesion to endothelial cells and production of oxygen radicals and chemokines. NAP has the ability to stimulate different cell types such as neutrophils, monocytes and dendritic cells to produce IL-12, IL-23 and TNF-α. IL-12 and IL-23 in turn stimulates T helper cells (Th1) cells by affecting T-cell cytokine production and the cytolytic potential of Th clones. The protein has been shown to promote a remarkable decrease of IL-4 secreting cells and an increase of IFN-γ producing T-cells. NAP also stimulates neutrophils to synthesize and release CXCL8 (IL-8), CCL3 (MIP-1α) and CCL4 (MIP-1β). Consequently, NAP promotes an anti tumoral immune response. This makes NAP protein an interesting agent in cancer immunotherapy.
Monocytes as delivery vehicles of oncolytic adenovirus

Immature monocytes circulate in the bloodstream. When monocytes get more mature they start to migrate towards tissues. In tissue they differentiate to macrophages. Macrophages are recruited to tumors by chemokines and are called tumor associated macrophages (TAMs) in tumor tissue [16]. TAMs have a high expression of CD14 receptors and low expression of CD16 receptors (CD14^{high}/CD16^{low}). Another subgroup of macrophages which have low expression of CD14 receptors and high expression of CD16 receptors (CD14^{low}/CD16^{high}) have been shown to have a high expression of Tie2. These monocytes are called Tie2 expressing monocytes (TEMs). Tie2 is an angioprotein required for vascularisation and growth of several tumors. In migration assays, TEMs have been shown to migrate towards angioprotein, which is a ligand released by activated endothelial cells and angiogenic vessels. This suggests a homing mechanism for TEMs to tumors [17].

To overcome the obstacle of neutralization of viruses by the host immune system, the administration of oncolytic virus to tumors can be achieved by the use of carrier cells, such as monocytes/macrophages, which have natural propensities to home to tumor mass [18]. Viruses administrated to mice in carrier cells have showed improved therapeutic efficacy compared to administration of viruses without carrier cells [18, 19].

Replication of viruses in a carrier cell can be activated in different ways. Hypoxia responsive element (HRE) is an element that activates transcription of E1A in hypoxia, which is a condition of many tumors. Previous studies demonstrate that hypoxia can lead to a 100-fold increase in activity of a reporter virus with HRE driven luciferase expression (Essand M., unpublished data).
Aims

This work considers two different projects. In the first project, the aim is to enhance the potency of an oncolytic adenovirus, with prostate specific replication, by insertion of Neutrophil Activating Protein (NAP), an immune modulating agent of Helicobacter pylori.

The aim of the second project is to develop a tumor-targeted, cell-based carrier system to enhance the delivery of oncolytic virus to tumors after systemic administration.
Results

Verification of NAP mRNA and protein in LNCaP cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122]

LNCaP cells were transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122] and Ad5[i/ppt-E1A-miR122]. Cells were harvested, Rnase-treated and cDNA was made. NAP cDNA was amplified by PCR and the product analyzed on a 1% agarose gel. NAP mRNA has a size of 597 bp and showed to be present in cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122] but not in cells transduced with Ad5[i/ppt-E1A-miR122] (Figure 1).

![Figure 1](image)

**Figure 1** PCR analysis showing NAP mRNA in LNCaP cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122] and Ad5[i/ppt-E1A-miR122]. Cells were incubated for 24 hours at MOI 50.

LNCaP cells were transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122], Ad5[i/ppt-E1A-miR122], Ad5 (wt) and a mock virus. Protein was extracted from the cells and analyzed by western blot. E1A protein with the size of 37 kD and 45 kD was detected in cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122], Ad5[i/ppt-E1A-miR122] and Ad5 (wt). No E1A protein was detected in supernatant from cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122] or in cells transduced with mock virus (Figure 2). The NAP gene is linked to E1A, which means that NAP protein was present in the sample with extracted protein from cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122].
Figure 2 Western blot analysis showing detection of E1A protein. LNCaP cells were transduced with Ad5/35[i/ppt-NAP-T2A-E1A-miR122], Ad[i/ppt-E1A-miR122], Ad5 (wt) and a mock virus at MOI 10 and incubated for 72 hours.

**Efficiency of neutrophil isolation**

Neutrophils were isolated from PBMC using dextran sedimentation followed by Ficoll separation. Neutrophils and PBMCs were analyzed by flow cytometry to confirm that the isolation method gave a sufficient purity of neutrophils. Isolated neutrophils stained with CD14 and CD15 antibodies were 85% positive for the granulocytic CD15 marker and expressed low levels of the CD14 monocyte marker. Of PBMCs stained with the same antibodies only 4% were CD15 positive and 20% were positive for CD14 (Figure 3). These results signify that this method can give an 80% enrichment of CD15 positive neutrophils when isolated from PBMCs.
Neutrophil migration against NAP

Neutrophils from two different donors were isolated as described. A migration assay was performed to investigate if NAP has the ability to attract neutrophils. In donor one, 20% more cells migrated towards supernatant containing NAP compared to tumor supernatant without NAP. In the second donor 43% more cells migrated towards tumor supernatant containing NAP compared to tumor supernatant without NAP (Figure 4).
Figure 4 Neutrophil migrations towards tumor supernatant contained 100 µl extracted protein from cells transduced with Ad5/35[<i>i</i>/ppt-NAP-T2A-E1A-miR122] or Ad[<i>i</i>/ppt-E1A-miR122] resuspended in 320 µl fresh medium. Migrated cells were counted after three hours of migration.

**Transduction efficiency of Ad5/35-GFP in CD14<sup>high</sup> monocytes**

CD14<sup>high</sup> monocytes were isolated by magnetic activated cell sorting (MACS). The purity of monocytes was >95% (data not shown). GFP expression was analyzed in monocytes transduced with Ad5/35-GFP to investigate the transduction efficiency of the virus. Untransduced monocytes were used as controls. The background fluorescence in untransduced monocytes, which probably represent autofluorescent dead cells, was 0.68 % (Figure 5a). Monocytes transduced with Ad5/35-GFP showed 59.58% GFP expressing cells (Figure 5b). As a conclusion, Ad5/35-GFP has a transduction efficiency of almost 60 % (Figure 5ab).

![Flow cytometry plots](image)

**Figure 5** GFP expression in monocytes. One million monocytes were transduced with Ad5/35-GFP at MOI 50. 48 hours after transduction, flow cytometry was used to measure GFP expression in both untransduced monocytes and monocytes transduced with Ad5/35-GFP a Untransduced monocytes. b Monocytes transduced with Ad5/35-GFP.
**Isolation efficiency of $CD16^{\text{high}}$ monocytes**

$CD14^{\text{low}}/CD16^{\text{high}}$ monocytes were isolated by MACS. Isolation efficiency of $CD14^{\text{low}}/CD16^{\text{high}}$ monocytes was analyzed by flow cytometry. Three different samples with PBMCs, flow through after negative selection of $CD15^{\text{high}}$ (granulocytes) and $CD56^{\text{high}}$ (NK cells) cells and positive selection of $CD14^{\text{low}}/CD16^{\text{high}}$ cells were compared after MACS isolation. PBMCs consisted of 14% $CD16^{\text{high}}$ monocytes (Figure 6a) and flow through contained 1% $CD16^{\text{high}}$ monocytes (Figure 6b). However, after CD16 cell isolation 72% of the cells were $CD16^{\text{high}}$ monocytes (Figure 6c).

**Virus transduction efficiency in $CD16^{\text{high}}$ monocytes**

$CD16^{\text{high}}$ monocytes were transduced with four different viruses Ad5/35, Ad5/3, Ad5 (wt) and a mock virus. All viruses expressed GFP. After transduction, GFP expression was measured by flow cytometry to compare transduction efficiency between the different viruses. Ad5/3, Ad5 (wt) and the mock virus showed almost the same transduction efficiency as untransduced monocytes (Figure 7a-d). However, monocytes transduced with Ad5/35 had around 17% more GFP expressing cells compared to monocytes transduced with Ad5/3, Ad5 (wt) and the mock virus (Figure 7e).
**Production of Ad5/35iHREcmv_E1A**

A pShuttle vector encoding iHREcmv_E1A and an adenoviral plasmid, Ad5/35, were cotransformed by homologous recombination. After preparation of adenoviral DNA for cell transfection the DNA concentration was measured by a spectrophotometer to 87.7 ng/µl. Pac I digestion was used to verify that recombination of adenoviral DNA occurred. The digestion product was analyzed on a 1% agarose gel. Two bands with the size of 4.5 kb and 40 kb were visible which imply that the expected recombination had occurred (Figure 8). After production of the recombinant adenoviral DNA, a fluorescence forming unit (FFU) assay was performed. The number of green cells per grid (0.04 cm²) was counted to 44 cells. To get the total number of green cells in the 8 cm² dish the 44 cells were multiplied with 200. By additional multiplication of the dilution factor $10^6$, the total titer was calculated to $8.8 \times 10^9$ FFU/ml.
Figure 8 PacI was used to digest Ad5/35iHREcmv_E1A to confirm that right recombination had occurred between the pShuttle vector encoding iHREcmv_E1A and the adenoviral plasmid Ad5/35. A 1% agarose gel was used to detect DNA digestion products with the size of 4.5 kb and 40 kb.

Monocyte migration against supernatant from neuroendocrine tumor cells

CD14<sup>high</sup> monocytes were isolated by MACS from three different donors. Migration capacity of CD14<sup>high</sup> monocytes against supernatant from BON cells compared to fresh media was investigated. In donor 1, almost the double amount of monocytes migrated towards supernatant from BON cells compared to the control medium. Donors 2 and 3 showed almost a 3-fold increase in monocytes migrating towards supernatant from BON cells compared to the control medium (Figure 9).

Figure 9 Migration assay with 2x10<sup>5</sup> CD14<sup>high</sup> monocytes migrating against supernatant from BON cells and fresh control medium.
Replication of naked Ad5/35[HREcmv-Luc] virus occurred after intratumoral injection

Nude mice bearing subcutaneous BON tumors were used to compare replication capacity of naked Ad5/35[HREcmv-Luc] and Ad5/35[HREcmv-Luc] inside a monocyctic carrier cell. Naked Ad5/35[HREcmv-Luc] virus was intratumorally injected and monocytes transduced with the virus were injected intravenously. Luciferase expression was detected by the IVIS imaging system in mice intratumorally injected with naked Ad5/35[HREcmv-Luc]. The three mice showed luciferase expression at $1.6 \times 10^6$, $3.3 \times 10^6$ and $5.2 \times 10^6$ photons/second/cm$^2$ (Figure 10a). No luciferase expression was detected in mice intravenously injected with monocytes transduced with Ad5/35[HREcmv-Luc] (Figure 10b).

Figure 10 Luciferase gene expression in tumors three days after virus delivery. Ten minutes after luciferine substrate injection the in vivo luciferase expression was visualized using the IVIS imagine system. a Mice injected intratumoral with 25 µl naked Ad5/35[HREcmv-Luc] ($7.5 \times 10^5$ virus particles). b Mice intravenously injected with $1.5 \times 10^6$ monocytes transduced with Ad5/35[HREcmv-Luc] at MOI 300.
Discussion

Neutrophil activating protein (NAP) present in cells

I have confirmed that NAP mRNA and E1A protein is present in cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122. Because the NAP gene is linked to E1A, NAP is probably present in the cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122 (Figures 1, 2). No E1A protein was found in supernatant from the cells, which indicates that the protein is not actively secreted from the cells (Figure 2). For a therapeutic purpose it is essential that the NAP protein is released from the cells because its function should be to attract immune cells to the tumor site. Using an oncolytic virus, the NAP protein is released upon cell lysis. For the future, an idea is to link the protein with a signal peptide to get the protein secreted from the cells. By doing so, the need for cell lysis can be circumvented.

NAP protein has probably immune modulating effects on neutrophils

Migration of neutrophils against media containing extracted NAP showed an increase in number of migrated cells against extracted NAP compared to protein lysate without NAP. Additional migration assays has to be performed to establish the chemoattractant effects of NAP protein on neutrophils. Also, to provide better proof of NAP’s immune modulating effects, neutrophils should be cultured in extracted NAP from LNCaP cells transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122]. The supernatant from the neutrophils should then be analyzed by CBA. Here, it would be possible to investigate if neutrophils would up or down regulate any cytokines in response to NAP stimulation.

Monocytes have best homing capacity against LNCaP cells

Before the start of my studies, migration assays were performed to investigate the homing capacity of monocytes against LNCaP cells. The amount of monocytes migrated towards supernatant from LNCaP cells were more than six times higher compared to fresh medium (Eriksson F., unpublished data). As we encountered difficulties with the in vitro culture of LNCaP cells, we evaluated the BON tumor cell line as model for monocyte migration. The amount of monocytes migrated towards supernatant from BON cells were the double or almost three times higher compared to fresh medium. As a conclusion, monocytes have a higher migration capacity against supernatant from LNCaP cells then towards supernatant from Bon tumor cells. This makes LNCaP cells more suitable for additional in vivo experiments. The next step to investigate monocytic migration against LNCaP cells in vivo would be to label monocytes with green fluorescence and inject into mice carrying prostate tumors. At certain time points after injection, tumors would be harvested and the amount of monocytes inside the tumor quantified by flow cytometry. Also, untransduced monocytes could be compared to transduced monocytes to ensure that the migration capacity does not get affected by transduction.
**Ad5/35 has the best transduction efficiency of CD14\textsuperscript{high} monocytes**

I have clearly demonstrated that Ad5/35 had the best transduction efficiency on CD14\textsuperscript{low}/CD16\textsuperscript{high} monocytes of the four viruses tested in this study (Figure 7). However, Ad5/35 has an even higher transduction efficiency of CD14\textsuperscript{high} monocytes compared to CD14\textsuperscript{low}/CD16\textsuperscript{high} monocytes (Figures 5b, 7e). This high transduction efficiency is due to the fiber knob of serotype 35. The fiber knob of serotype 35 has a high affinity to the CD46 receptors on monocytes, which facilitate the transduction [20]. It could be that CD14\textsuperscript{high} monocytes have more CD46 receptors on the surface than CD14\textsuperscript{low}/CD16\textsuperscript{high} monocytes, which would explain why the transduction efficiency was higher in CD14\textsuperscript{high} monocytes.

**CD14\textsuperscript{high} monocytes is most suitable as a carrier cell**

Monocytes which were CD14\textsuperscript{low}/CD16\textsuperscript{high} were investigated due to previous results suggesting that this subgroup has a homing mechanism against tumors [17]. Unfortunately, it was hard to isolate appropriate amounts of CD14\textsuperscript{low}/CD16\textsuperscript{high} monocytes. Also, transduction efficiency was much lower compared to CD14\textsuperscript{high} monocytes (Figures 5b, 7e). Due to these problems, CD14\textsuperscript{high} monocytes are better suited for our purpose than CD14\textsuperscript{low}/CD16\textsuperscript{high} monocytes.

**In vivo studies**

To test the hypothesis of hypoxia induced gene expression, I injected naked Ad5/35[HREcmv-Luc] reporter virus intratumorally into neuroendocrine BON tumors growing in nude mice. Luciferase expression in tumors was compared to mice that got \textit{in vitro} injection of monocytes transduced with Ad5/35[HREcmv-Luc]. Luciferase expression was detected in mice intratumorally injected with naked virus. This confirms that HRE got activated by hypoxia and that expression of luciferase occurred inside the tumor. Unfortunately, no expression of luciferase was detected in tumors in mice \textit{in vitro} injected with transduced monocytes, meaning that the monocytes were not able to migrate to this type of tumor. \textit{In vitro} migration assays with monocytes have showed higher migration capacity against LNCaP cells than BON cells (Eriksson F., unpublished data), which could be a reason why this \textit{in vivo} study was not successful. Additional \textit{in vivo} studies will be performed using LNCaP tumors and anti tumor effects will be evaluated using the oncolytic Ad5/35[iHREcmv_E1A] virus. The E1A gene in Ad5/35[iHREcmv_E1A] is under transcriptional control of HRE, which means that this oncolytic virus would start to replicate in hypoxia.
Material and methods

Cell culture conditions
Flasks with CellBIND surface (Corning, Lowell, MA, USA) were used for culturing prostate tumor cells (LNCaP cells). LNCaP cells were growing in media containing 450 ml RPMI-1640 with 2mM L-glutamine, 50 ml 10% Fetal Bovine Serum, 5 ml 10mM (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) HEPES, 500 µl 1 mM sodium pyruvate and 2.5 ml 10000U/ml penicillin streptomycin (PEST) Neuroendocrine BON tumor cells and 911 cells do not need flasks with Corning CellBIND Surface. The media for culturing BON cells contained 112.5 ml DMEM, 112.5 ml F12K, 25 ml 10% Fetal Bovine Serum, 2.5 ml mM sodium pyruvate and 2.5 ml 10000U/ml PEST. Media for culturing 911 cells contained 450 ml DMEM with 50 ml 10% Fetal Bovine Serum, 5 ml 1 mM sodium pyruvate and 5 ml 10000U/ml PEST. All reagents for media preparation were purchased from Invitrogen, Carlsbad, CA, USA. Renewal of medium was performed two to three times a week. The cells were split or subcultured at the confluence of 80% with a ratio of 1:3 and incubated at 37°C with 5% CO₂ content.

Transduction of LNCaP cells
One million prostate tumor cells were transferred to 25 cm flasks with Corning CellBIND Surface. After 24 hours when cells were attached to the bottom of the wells they were transduced with the desired multiplicity of infection (MOI). MOI means the ratio of infectious viruses to cells. Medium was removed from the cells and 1 ml medium containing the virus was added to each flask. Cells were transduced for 2 hours at 37°C with 5% CO₂ content. After 2 hours 4 ml medium were added to each flask and incubated for the desired time at 37°C with 5% CO₂ content.

Isolation of total RNA
Cells and media were collected in a tube and centrifuged for 5 minutes at 1500 rpm. Supernatant was removed and the pellet washed one time in 5 ml 1 x PBS. An RNeasy Kit (QIAGEN, Valencia, CA, USA) was used to isolate total RNA. The pellets were resuspended in 350 µl Buffer RLT and homogenized by passing the lysate 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. One volume 70% ethanol was added to each sample and mixed well by pipetting. From the samples 700 µl was transferred to RNeasy spin columns placed in collection tubes. The tubes were centrifuged for 15 seconds at 8000 x g. Flow through was discarded. To each column 700 µl buffer RW1 were added followed by centrifugation 15 seconds at 8000 x g. Flow through were discarded. To each column 500 µl buffer RPE were added and the tubes centrifuged for 15 seconds at 8000 x g. Flow through were discarded. An additional 500 µl buffer RPE was added and the tubes centrifuged for 2 minutes at 8000 x g. RNeasy spin columns were placed in a new collection tube and 50 µl RNase-free water were added followed by centrifugation 1 minute at 8000 x g to elute the RNA.
**DNase digestion**

RNase-Free DNase set (QIAGEN) was used to digest contaminating DNA. Distilled water (dH₂O) was added to 40 µl RNA solution to a final volume of 87.5 µl. The RNA solution was mixed with 10 µl buffer RDD and 2.5 µl DNase I stock solution followed by incubation for 10 minutes at room temperature. RNase Mini Kit was used to clean up RNA. First, 350 µl buffer RLT was mixed with the RNA solution. Then, 250 µl 99.5% ethanol was mixed with the RNA sample. From the samples 700 µl was transferred to RNeasy spin columns placed in collection tubes. The tubes were centrifuged for 15 seconds at 8000 x g. Flow through were discarded. To each column 500 µl buffer RPE were added and the tubes centrifuged for 15 seconds at 8000 x g. Flow through were discarded. An additional 500 µl buffer RPE was added and the tubes centrifuged for 2 minutes at 8000 x g. The RNeasy spin columns were placed in a new collection tube and 50 µl RNase-free water were added followed by centrifugation 1 minute at 8000 x g to elute the RNA.

**cDNA synthesis**

cDNA synthesis was carried out using the Superscript II kit from Invitrogen. Each sample contained total RNA in the range of 1 ng to 5 µg. To each sample 1 µl 500 µg/ml oligo dt primer, 2 µl 10 mM dNTP, 2 µl total RNA and 7 µl dH₂O was added and the solution incubated for 5 minutes at 42°C. Tubes were put on ice to cool down and centrifuged a few seconds to spin down all liquid. To each tube 4 µl 5 x first strand buffer, 2 µl 0.1 M DTT and 1 µl 40 units/µl RNase Out was added and incubated 2 minutes at 42°C. Finally 1 µl 200 units/µl super script II RT was added followed by incubation for 50 minutes at 42°C. Temperature was raised to 70°C for 5 minutes to inactivate reverse transcriptase.

**PCR**

A master mix was made containing 0.5 µl Taq polymerase, 5 µl buffer, 37 µl water, 1 µl 25 µM forward primer, 1 µl 25 µM reverse primer, 2 µl 5 mM dNTP and 1.5 µl 50 mM MgCl₂ for each sample. A final volume of 48 µl master mix and 2 µl template was used for PCR. Primers were designed for amplification of NAP cDNA. Forward primers were designated 5´-TAAAACATTGCAAGCGGATG-3´ and reverse primers 5´-CGGCCATTTCTTCGGTAAT-3´. The PCR program contained 35 cycles with 30 seconds at 94°C, 30 seconds at 64°C and 1 minute at 72°C.

**Protein extraction**

LNCaP cells were transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122], Ad5[i/ppt-E1A-miR122], Ad5 (wt) and a mock virus at MOI 10 and incubated for 72 hours. To collect cells that had detached from the bottom of the well, the medium was centrifuged for 5 minutes at 1500 rpm, supernatant discarded and pellet resuspended in 400 µl RIPA buffer (5 ml 1M tris-cl, 30 ml 5 M NaCl, 5 ml 20% TRITON-X, 5 ml 10 % sodium deoxycholate, 0.5 ml 20% SDS and 50 ml ddH2O). The solution with pellet resuspended in RIPA buffer was added back to the wells with attached cells followed by incubation for 30 minutes at 4°C on a rocker. A cell scraper was used to scrape off cells and the samples were collected in 1.5 ml microcentrifuge tubes followed by centrifugation for 10
minutes at 13000 rpm, 4°C. Protein was now present in the supernatant, ready to be analyzed by western blot. For cytokine solution in neutrophil migration assay, LNCaP cells were transduced with Ad5[i/ppt-NAP-T2A-E1A-miR122] or Ad5[i/ppt-E1A-miR122] at MOI 10. Three days later, cells were lysed by freezing, thawing and vortex instead of using RIPA buffer and extracted protein was resuspended in 1 ml 1xPBS.

**Coomassie plus protein assay**

The coomassie plus protein assay is based on a BSA concentration standard curve. A mastermix was prepared which contained 1 x PBS and coomassie plus protein assay reagent (Fisher Scientific, Göteborg, Sweden) in a ratio of 1:1. To eight cuvettes 1 ml master mix were added. Different volumes of BSA with a concentration of 200 µg/ml were added to one cuvette each and the concentrations were determined by using a spectrophotometer (Table 1).

Table 1 Different volumes of BSA were added to 1 ml mastermix. A spectrophotometer was used to measure OD600 at different concentrations of BSA.

<table>
<thead>
<tr>
<th>BSA (µl)</th>
<th>BSA (µg) in 1 ml</th>
<th>OD600</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,25</td>
<td>0,25</td>
<td>0,003</td>
</tr>
<tr>
<td>2,5</td>
<td>0,5</td>
<td>0,019</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0,037</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>0,076</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>0,154</td>
</tr>
<tr>
<td>40</td>
<td>8</td>
<td>0,258</td>
</tr>
<tr>
<td>80</td>
<td>16</td>
<td>0,397</td>
</tr>
</tbody>
</table>

BSA concentration was plotted against OD600 and a trend line was calculated using Microsoft excel (Figure 11). OD600 was measured in the protein samples and OD600 were used to calculate protein concentrations by using the trend line equation (Table 2).

![Figure 11](image_url)

**Figure 11** BSA concentration was plotted against OD600 and a trend line was calculated using Microsoft excel.
Table 2: To determine protein concentrations in the samples a volume of 1 µl was added to one cuvette each, mixed and placed in the spectrophotometer. OD<sub>600</sub> of the samples were in the range of the BSA concentration curve. The trend line equation (y = 0.0262x + 0.0243) was used to calculate protein concentration in the four samples by inserting OD<sub>600</sub> for each sample in the equation. DNA concentrations of the samples were multiplied with the dilution factor to get the final protein concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>vol. (µl)</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>value (µg/ml)</th>
<th>dilution factor</th>
<th>protein (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP supernatant</td>
<td>1</td>
<td>0.304</td>
<td>11.26</td>
<td>1000</td>
<td>11.26</td>
</tr>
<tr>
<td>Ad5/35[i/ppt-NAP-T2A-E1A-miR122]</td>
<td>1</td>
<td>0.179</td>
<td>6.36</td>
<td>1000</td>
<td>6.36</td>
</tr>
<tr>
<td>Ad[i/ppt-E1A-miR122]</td>
<td>1</td>
<td>0.13</td>
<td>4.44</td>
<td>1000</td>
<td>4.44</td>
</tr>
<tr>
<td>Mock virus</td>
<td>1</td>
<td>0.185</td>
<td>6.56</td>
<td>1000</td>
<td>6.59</td>
</tr>
<tr>
<td>Ad5 (wt)</td>
<td>1</td>
<td>0.192</td>
<td>6.87</td>
<td>1000</td>
<td>6.87</td>
</tr>
</tbody>
</table>

**SDS-PAGE and Western blot detection**

A volume of 200 µl 4 x SDS sample buffer containing 200 mM Tris-HCl, 8% SDS, 40% glycerol and 0.1% bromophenol blue was mixed with 10 µl 100% β-mercaptoethanol. 4 x SDS sample buffer containing β-mercaptoethanol were added to each sample (Table 3). All samples were boiled for 5 minutes at 95°C and cooled down to room temperature thereafter loaded on a SDS-PAGE gel. 1 x running buffer containing 900 ml deionized H<sub>2</sub>O and 100 ml 10x Tris-glycine running buffer (184g TG-SDS powder diluted in 1 l distilled H<sub>2</sub>O) was used. The voltage was set to 70 V and raised to 120 V when samples reached the resolving part of the gel.

Table 3: Samples containing 50 µg protein were prepared. Protein concentrations were used to calculate sample loading volumes. 4 x SDS sample buffer containing β-mercaptoethanol were added to each sample with a volume of one third of the sample loading volume.

<table>
<thead>
<tr>
<th>sample</th>
<th>protein (µg/µl)</th>
<th>loading (µg)</th>
<th>Sample loading vol.</th>
<th>4xSDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP supernatant</td>
<td>11.26</td>
<td>50</td>
<td>4.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Ad5/35[i/ppt-NAP-T2A-E1A-miR122]</td>
<td>6.36</td>
<td>50</td>
<td>7.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Ad[i/ppt-E1A-miR122]</td>
<td>4.44</td>
<td>50</td>
<td>11.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Mock virus</td>
<td>6.59</td>
<td>50</td>
<td>7.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Ad5 (wt)</td>
<td>6.87</td>
<td>50</td>
<td>7.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

An iBLOT was used to transfer protein on to the membrane. A copper anode was placed in the bottom of the iBLOT. Bottom buffer matrix and a stack integrated with 0.2 µm nitrocellulose membrane was placed on top of the copper anode. The SDS-PAGE gel was placed on top of the nitrocellulose membrane. Above the gel top buffer matrix and a copper cathode was placed. After transfer the SNAP i.d Protein Detection System (Millipore, Billerica, MA) was used for detection. The blot membrane was pre-wet with dH<sub>2</sub>O and placed in the centre of the blot holder. A roller was used to gently remove air.
bubbles. The spacer was placed on top of the blot membrane and the roll blot was used to remove air bubbles. The blot holder was closed and placed in the system chamber followed by closing and latch of the system lid. Blocking solution containing 30 ml 1xTBS with 0.1% Tween and 1% β-mercaptoethanol was added to the well and vacuum turned on. When the well was emptied vacuum was turned off and 3 ml of primary antibody (3 ml 1xTBS with 0.1% Tween, 1% β-mercaptoethanol, 0.02% sodium azide and 1 μl primary antibody, mouse-anti-human Ad-E1A (1:600 dilution) (NeoMarkers, Fermont, CA) was added to the well followed by incubation for 10 minutes in dark. Vacuum was turned on and the blot was washed with 30 ml 1xTBS with 0.1% Tween and 1% β-mercaptoethanol. When the blot holder was empty vacuum was turned off. Secondary antibody (3 ml 1xTBS with 0.1% Tween, 1% β-mercaptoethanol and 1 μl infrared dye labelled secondary antibody rabbit-anti-mouse-680 (LI-CORE Bioscience, Lincoln, Nebraska USA)) was applied to the well followed by incubation for 10 minutes in dark. Vacuum was turned on and the blot was washed with 30 ml 1xTBS with 0.1% Tween and 1% β-mercaptoethanol. The blot membrane was taken out from the SNAP i.d Protein Detection System and scanned in an Odyssey Infrared Imagine System (LI-CORE Bioscience).

**Isolation of PBMCs by Ficoll separation**

 Buffy coats were used for isolation of PBMCs. From one buffy coat 12.5 ml blood was mixed with 17.5 ml 1xPBS to a final volume of 30 ml, which was carefully added to a tube containing 15 ml Ficoll (GE Healthcare, Uppsala, Sweden). It is important that the blood does not get mixed with the Ficoll. The tube was centrifuged at 1500 rpm for 30 minutes. After centrifugation, the top layer with plasma was discarded and PBMCs collected. PBMCs were washed twice in 50 ml 1xPBS and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet containing PBMCs were resuspended in 20 ml 1xPBS.

**Isolation of neutrophils by dextran and Ficoll separation**

Equal volumes of 3 % dextran solution (distilled H₂O (dH₂O) with 0.9 % NaCl and 3 % dextran (Sigma-Aldrich, Stockholm, Sweden)) and whole blood from a buffy coat were mixed in 50 ml colonial tubes followed by incubation for 20 minutes at room temperature. The straw-colored, leukocyte rich, erythrocyte poor upper layer was transferred to a 50 ml colonial tube. Leukocytes were pelleted by centrifugation for 10 minutes at 500g at 4°C. The pellet was resuspended in 10 ml 0.9 % saline solution. A 50 ml colonial tube was prepared with 10 ml Hypaque-Ficoll. The cell solution was carefully added on top of the Ficoll and the tube centrifuged for 40 minutes at 400g at room temperature. Two bands should appear after centrifugation. The lighter band contains mononuclear cells, whereas the denser band has both granulocytes and erythrocytes. Supernatant above the denser band was removed and the rest resuspended in 10 ml d H₂O for 28 seconds. Tonicity was restored by adding 10 ml 1.8 % saline. The solution was centrifuged for 5 minutes at 500g at 4°C. Supernatant was discarded and the pellet resuspended in 20 ml 1 x PBS.
Migration assay

A 96 well plate was placed in a transwell migration chamber (Neuroprobe, Gaithersburg, MD, USA). Positive pipetting was used to add 420 µl attractant solution to wells on to the 96 well plate. A slight positive meniscus was formed which prevents air bubbles from being trapped when the filter makes contact. The filter was hold by the edges and placed with the filter down on the bottom plate. The top plate was closed. To the top wells 2x10^5 cells in 100 µl were added. The chamber was incubated for 4 hours at 37°C with 5% CO₂ content. By shaking the chamber up side down the fluid in the top wells were removed and 100 µl 2 mM EDTA in PBS was added to each well followed by incubation for 30 minutes at 4°C. Fluid in the top wells were removed by shaking and the chamber was opened. The 96 well plate was centrifuged for 5 minutes at 2000 rpm to remove migrated cells from the bottom side of the filter into the wells of the plate. The filter was removed and 200 µl attractant solution from each well was discarded. Cell pellets were resuspended in the small volume of attractant solution that was left and collected in microcentrifuge tubes. The tubes were centrifuged and all supernatant discarded followed by resuspension of the cell pellet in 100 µl 1 x PBS. Cells were counted using a Bürker chamber.

Isolation of CD14<sup>high</sup> monocytes by magnetic activated cell sorting (MACS)

PBMCs were washed one time with 10 ml 1xPBS, centrifuged for 5 minutes at 1500 rpm and the supernatant was discarded. A second wash was performed exactly as the first one but with MACS buffer (1xPBS with 0,5% bovine serum albumin (BSA) and 2mM EDTA) instead of 1xPBS. The pellet was resuspended in 80 µl MACS buffer per 10⁷ total cells and 20 µl MACS CD14 Micro beads (Miltenyi Biothech, Bergisch Gladbach, Germany) was added per 10⁷ total cells, followed by incubation for 15 minutes at 6-12°C. Cells were washed one time with 10 ml MACS buffer, centrifuged for 10 minutes at 300 g and supernatant was removed. The pellet was resuspended in 500 µl MACS buffer per 10⁸ total cells. A selection LS column (Miltenyi) was placed in the magnetic field of a MACS separator and the column was washed one time with 3 ml MACS buffer. The PBMC sample was added to the column followed by three washes with 3 ml MACS buffer. All effluent liquid was collected in a 15 ml colonial tube. To collect CD14<sup>high</sup> cells the column was placed on a new tube, 5 ml MACS buffer was added and the cells were flushed through with a plunger supplied with the column.

Isolation of CD14<sup>low</sup>/CD16<sup>high</sup> monocytes by magnetic activated cell sorting (MACS)

PBMCs were isolated by density gradient centrifugation and the number of cells in the sample was counted. One hundred million cells were transferred to a new tube and washed once with 10 ml 1xPBS, centrifuged for 5 minutes at 1500 rpm and the supernatant was discarded. A second wash was performed exactly as the first one but with MACS buffer instead of 1xPBS. The pellet was resuspended in 300 µl MACS buffer per 10⁸ total cells. An additional 100 µl FcR blocking reagent and 100 µl non-monocyte depletion cocktail which labels CD15<sup>+</sup> granulocytes and CD56<sup>+</sup> NK cells was added per
10^8 total cells, followed by incubation for 15 minutes at 4-8°C. Cells were washed one time in 10 ml 1x PBS and centrifuged for 10 minutes at 300xg and the supernatant removed by pipetting. The pellet was resuspended in 500 µl MACS buffer and transferred to a LD column placed in the magnetic field of MACS separator. The column was washed one time with 3 ml MACS buffer. PBMCs were added followed by three washing steps with 3 ml MACS buffer. Total effluent was collected and centrifuged for 10 minutes at 300xg and the supernatant removed by pipetting. The pellet was resuspended in 400 µl MACS buffer and 100 µl CD16 microbeads was added, followed by incubation for 15 minutes at 4-8°C. Cells were washed one time in 10 ml 1x PBS and centrifuged for 10 minutes at 300xg and the supernatant removed by pipetting. The pellet was resuspended in 500 µl MACS buffer and transferred to a new LS column. The effluent was collected. To collect CD14<sub>low</sub>/CD16<sub>high</sub> cells the column was placed on a new tube, 5 ml MACS buffer was added and the cells were flushed through with a plunger supplied with the column.

**Transduction of monocytes**

Monocytes were enriched by MACS. One million monocytes in 100 µl DC medium (100 ml RPMI-1640, 1 ml human serum, 1 ml 10000U/ml PEST, 0.5 ml 2mM L-glutamine, 250 µl Fungizone, 40 µl β-mercaptoethanol and 1 ml 10mM HEPES) were transferred to microcentrifuge tubes and 5x10<sup>7</sup> viruses (MOI 50) in 200 µl were added. The monocytes were incubated for 2 hours at 37°C with 5% CO<sub>2</sub> content. After transduction the monocytes were transferred to a 12 well plate and DC medium added to a final volume of 2 ml followed by incubation for 48 hours at 37°C with 5% CO<sub>2</sub> content.

**Fluorescence Activated Cell Sorting (FACS)**

One million cells were diluted in 100 µl 1xPBS. For FACS analysis of neutrophils and PBMCs one sample from each were unstained and to one sample from each 2 µl of anti-CD14-FITC and-CD15-APC antibodies (BD Biosciences, Stockholm, Sweden) were added. For analysis of isolated CD16<sub>high</sub> monocytes 5 µl anti-CD14-FITC and-CD16-PE (BD) antibodies were added to PBMCs, cells from flow through and isolated CD16<sub>high</sub> monocytes. Samples were incubated for 20 minutes at 4°C in dark. After incubation cells were washed once by centrifugation at 1500 rpm for 5 minutes followed by addition of 250 µl 1xPBS. Samples were acquired and analyzed using a FACSCalibur and the CellQuest Pro software (BD).

**Preparation of DNA for cell transfection**

Electroporation was used to retransform the recombinant plasmid Ad5/35iHREcmv_E1A in DH10B bacteria. A 2 mm cuvette, pipette tip and electroporator slide was pre-chilled to -20°C. BioRad electroporator was set to 2.5 kV, 25 µF and low range resistance 200 ohms. The electroporated solution contained 1 µl of plasmid DNA from the last made minipreparation and 50 µl DH10B bacteria. After electroporation, the solution was transferred to a 15 ml tube and 300 µl LB medium (1 l distillate H<sub>2</sub>O, 10 g tryptone, 5 g yeast extract and 10 g NaCl) was added followed by incubation at 37°C, 225 rpm for 1 hour. Two LB agar plates with kanamycin were used to plate 10 µl and 100 µl of the
culture. The plates were incubated at 37°C for 18 hours. Four colonies were picked from the plate with 10 µl culture and each colony inoculated in 3 ml LB and 3 µl kanamycin at 37°C, 225 rpm for 18 hours. A DNA mini preparation was performed with 2.8 ml of the cultures, the rest 200 µl was saved for later use.

For minipreparation GenElute Plasmid Miniprep Kit (Sigma-Aldrich) was used. The culture was centrifuged at 12000 g for 1 minute and the supernatant discarded. Bacterial pellet was resuspended in 200 µl resuspension solution. Resuspended cells were lysed by addition of 200 µl lysis solution and mixed by gentle inversion 6-8 times followed by addition of 350 µl Neutralization/Binding solution, mixed by inversion 4-6 times and centrifuged at 12000g for 10 minutes. A GenElute Miniprep Binding column was inserted into a microfuge tube and 500 µl Column Preparation Solution was added and spun down at 12000 g for 1 minute. The cleared lysate was added and centrifuged at 12000 g for 1 minute. Flow-through was discarded. A wash step where 750 µl Wash Solution was added followed by centrifugation at 12000 g for 2 minutes removed salts and other contaminants. The column was transferred to a new collection tube and 100 µl reagent water added followed by centrifugation at 12000 g for 1 minute. DNA was now present in the eluate.

PacI (New England Biolabs, Ipswich, MA, USA) digestion was performed and restriction was analyzed using a 1% agarose gel. Each of the four tubes contained 2 µl plasmid, 3 µl 10 x buffer 1, 0.3 µl 100 x BSA, 1µl PacI and 23.7 µl dH2. The sample was incubated at 37 °C for 1 hour followed by analysis on a 1% agarose gel.

A new miniprep was done with six tubes, each containing 4 ml of bacterial culture. This time 75 µl molecular biology reagent water was added in the eluting step. After centrifugation all samples were mixed together to one sample and PacI digestion was used to control that the expected recombinations had occurred between the pShuttle vector encoding iHREcmv_E1A and the adenoviral plasmid Ad5/35. The tube for PacI digestion contained 4 µl plasmid, 3 µl 10 x buffer 1, 0.3 µl 100 x BSA, 1µl PacI and 21.7 µl water. PacI digestion was confirmed by analysis on a 1% agarose gel. DNA concentration was determined by a spectrophotometer and the DNA was stored at -20°C.

Recombinant adenovirus production (Ad5/35iHREcmv_E1A)

Recombinant AdEasy DNA was digested by Pac I at 37°C for 16 hours. The concentration of the DNA was earlier measured by a spectrophotometer to 87.7 ng/µl. An appropriate amount of DNA to use in recombinant adenovirus production is 6 µg. To calculate the volume of DNA that should be used, 6000 ng was divided by 87.7 ng/µl. For PacI digestion 34 µl DNA, 5 µl 10 x buffer 1, 0.5 µl 100 x BSA, 0.5 µl Pac I and 10 µl water were used. The sample was incubated at 37°C for 16 hours followed by heat inactivation at 65°C for 20 minutes. A 1 % agarose gel was used to confirm the complete digestion of DNA.

911 cells were plated in a 60 mm Petri dish in D-MEM with 10% FBS and incubated at 37°C in a 5% CO2 incubator. Cells in the Petri dish were transfected with the Ad5/35 HRE-cmv-E1A plasmid when the cell confluence was 80 % after one day. Two solutions were prepared for the transfection. The first solution contained 400 µl OPTI-MEM medium (Invitrogen) and 100 µl of the plasmid. The second solution contained 500 µl
OPTI-MEM medium and 10 µl Lipofectamine (Invitrogen) that was dropwise added. Both solutions were incubated for 5 minutes at room temperature. The first solution was dropwise added to the second solution followed by incubation for 30 minutes at room temperature. Finally 4 ml DMEM with 10 % FBS without antibiotics was added. D-MEM medium in the 60 mm Petri dish was removed and 5 ml DNA/liposome solution was carefully added to the inside wall of the plate. The plate was incubated at 37°C for 16 hours in a 5% CO₂ incubator. After 16 hours the medium was changed to a fresh D-MEM medium and the cells were cultured for ten days.

The adenoviral particles were harvested before the first amplification. Cells in the 60 mm diameter Petri dish and medium were collected using a cell scraper and transferred to a 15 ml conical tube, followed by centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and pellet resuspended in 1 ml of 0.1 M Tris-HCL, pH 8.0. Viral stocks were prepared by freezing-thawing-vortexing procedures repeated four times. Finally the cells were centrifuged at 12000 g for 8 minutes. Supernatant containing viral particles were used for the first amplification of the virus. A part of the supernatant, 200 ml was used for transduction and 800 µl was stored in 4 % sucrose at -80°C. In the first amplification 5 ml medium was added to 200 µl virus supernatant. The solution carefully added to the inside wall of a 10 cm diameter Petri dish with the confluence of 80 % 911 cells that was prepared the day before, followed by incubation at 37°C in a 5% CO₂ incubator for five days.

In the second amplification, adenoviral particles were harvested exactly as before the first amplification. After centrifugation at 12000 g for 8 minutes, 200 µl supernatant was mixed together with 40 ml DMEM medium. The solution was divided in two and carefully added to the inside wall of two 15 cm diameter Petri dishes with the confluence of 80% 911 cells that was prepared the day before. The two plates were incubated at 37°C in a 5% CO₂ incubator. Leftover from the supernatant was stored at -20°C.

In the second amplification, adenoviral particles were harvested exactly as before the first amplification. After centrifugation at 12000 g for 8 minutes, 200 µl supernatant was mixed together with 40 ml DMEM medium. The solution was divided in two and carefully added to the inside wall of two 15 cm diameter Petri dishes with the confluence of 80% 911 cells that was prepared the day before. The two plates were incubated at 37°C in a 5% CO₂ incubator. Leftover from the supernatant was stored at -20°C.

After two days the cells were harvested as described earlier and centrifuged at 12000 g for 8 minutes. Five 25 x 25 cm plates were prepared four days before the third amplification. Two 75 cm² flasks with the confluence of 80% cells were trypsinized and medium added to a volume of 10 ml. Cells from two 75 cm² flasks were added to one 25x25 cm plate together with 50 ml medium. In the third amplification 200 µl viral supernatant was added to 50 ml DMEM medium and 10 ml of this solution was distributed to the five 25 x 25 cm plates. Before the virus was added half of the DMEM medium was exchanged to fresh medium. The five plates were incubated at 37°C in a 5% CO₂ incubator.

Two days later the cells were harvested by collecting cells and medium in eight 50 ml tubes. Tubes were centrifuged for 5 minutes at 1000 rpm. Supernatant was carefully removed and the pellet resuspended in 650 µl 0.1 M Tris-HCL, pH 8.0. The cell suspensions were then transferred to eight 1.5 ml microcentrifuge tubes and the four cycles of freezing/thawing/vortex was performed followed by centrifugation at 12000 g for 8 minutes. All the supernatants were pooled in one 15 ml tube.
**Purification of Ad5/35iHREcmv_E1A**

Purification of the virus was performed using the discontinuous CsCl gradient. Three CsCl solutions were prepared by adding different amounts of CsCl to 50 ml 10 mM Tris, pH 7.9. The first solution contained 27.42 g CsCl (density 1.41), the second 22.71 g (density 1.34) and the last solution 18.47 g (density 1.27). CsCl with two different densities was added to an ultra clear centrifuge tube. First 6 ml CsCl with a density of 1.41 was added and the volume level marked with a pen on the tube followed by carefully addition of 10 ml CsCl with density 1.27. The freezed/thawed/vortexed virus was diluted with 10 mM Tris to a final volume of 10 ml and gently loaded on the top of the discontinuous gradient. A balance tube with an identical CsCl gradient was prepared and the swinging bucket rotor was pre-cooled. The ultracentrifuge was running for 2 hours at 25000 rpm at 4°C. After centrifugation a virus band was visible between the two different CsCl solutions, at the top of CsCl with density 1.41 and below CsCl with density 1.27. The tube was punctured with a needle and 2 ml of the virus band was collected in a 5 ml syringe.

A slide-A-Lyser cassette (Thermo Scientific) with a buoy was pre-wet in a 1 litre glass beaker filled with 800 ml dialysis buffer (10 mMTris-HCl (pH 7.9), 2 mM MgCl₂, 4 % sucrose). Virus was applied into a corner entrance of the cassette. The cassette was again placed in the beaker, which contained a magnetic stirrer at low speed. After two hours, the buffer was changed followed by dialysis overnight at 4°C.

**Ad5/35iHREcmv_E1A titer determination**

Three 60 mm Petri dishes were coated with 1 ml 25µg/ml collagen and incubated at room temperature for 30 minutes. The collagen solution was removed and the plates dried for 5 minutes. 911 cells were plated in the three dishes and incubated overnight at 37°C in a 5% CO₂ incubator.

Next day, a 10 fold dilution series was prepared by mixing 5 µl virus together with 495 µl DMEM medium creating a 10² dilution. From this dilution 110 µl was transferred to 1000 µl medium resulting in the dilution 10³. By repeating this four times dilutions was made down to 10⁷. Media was removed from 911 cells and 1 ml of dilution 10⁷, 10⁶ and 10⁵ were added to one plate each and incubated for 2 hours under standard culture conditions. In each plate the virus containing media was exchanged by 2 ml of fresh media and incubated for 48 hours under standard culture conditions.

Medium was removed and cells carefully washed with 1 ml 1xPBS. The cells were air dried for 20 minutes. To fix the cells, 1 ml of 4 % paraformaldehyde (PFA) (Apoteksbolaget, Sweden) was added and the dishes incubated for 2 minutes in the hood. PFA was removed and the cells were carefully washed with 1 ml 1xPBS. To each dish 650 µl anti-Ad hexon antibody (Millipore, Billerica, MA) made in mouse with a dilution of 500 in 1xPBS. Dishes were incubated for 1 hour at room temperature. Antibody solution was removed and cells washed two times with 1 ml 1xPBS. Rabbit anti-mouse-FITC was diluted 200 times in 1xPBS and 650 µl added to each dish. Aluminium foil was used to cover the dishes during 1 hour incubation at room temperature. After incubation, cells were washed one time in 1 ml 1xPBS and 1 ml 1xPBS was added to each dish and leaved during counting of green cells under a fluorescence microscope.
**In vivo experiment**

BON cells were subcutaneously injected into six NMRI nude mice. Three mice got $3 \times 10^6$ cells and the rest got $1 \times 10^7$ cells. After 10 days the mice were treated. Mice injected with $3 \times 10^6$ BON cells got 25 µl containing $7.5 \times 10^5$ Ad5/35HREcmv_Luc virus particles directly injected into the tumor and mice injected with $1 \times 10^7$ BON cells got $1.5 \times 10^6$ monocytes transduced with Ad5/35HREcmv_Luc injected intravenously. Before injection, the monocytes were transduced during two hours with MOI 300. Three days after treatment with naked virus and with monocytes transduced with virus, 150 µl luciferin substrate was intraperitoneally injected. Ten minutes after injection of luciferine substrate the mice were subjected to the IVIS imaging system (Caliper, Mainz, Germany). All experiments using mice were approved by the Swedish National Board for laboratory animals.
Acknowledgement

I would like to give the greatest thanks to my supervisor Fredrik Eriksson for excellent supervision and firm optimism during the project. I am also thankful for the guidance and enjoyable company from the people working in the lab, especially Di Yu who has helped me a lot. Likewise, thanks to Magnus Essand in whose research group I have been working.
References