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14. ABSTRACT
The goal of this project was to use the previously identified peptides with the reported specificity for neovasculature of prostate tumors to genetically modify the natural tropism of human adenovirus type 5 towards designing of tumor-selective gene therapy vectors. The study involved the design of peptide-modified Ad fiber proteins, their expression and characterization in vitro. A panel of recombinant adenovirus vector incorporating the most promising of these protein chimeras has been developed. These new vectors have been tested in vivo using human prostate xenografts established in nude mice as a model of target tumors. Biodistribution of the control and modified adenoviral vectors in these animals has been studied using non-invasive whole body imaging; measurements of the vector-encoded reporter activity in isolated organs and tumors; and also by qPCR-based detection of vector particles. This work showed, however, that none of the designed vectors possessed the tumor specificity expected based onthe reported performance of chosen peptide ligands. Potential reasons for these unexpected findings are discussed.

15. SUBJECT TERMS
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INTRODUCTION:

The goal of this project was to develop novel Ad vectors, which would be targeted to vasculature of prostate tumors via genetic incorporation into their capsid of the previously identified vasculature endothelium-specific peptides ligands. Additionally, tumor selectivity of these vectors was to be further improved by limiting the expression of the therapeutic transgene carried by the vectors, to endothelial cells of tumor vasculature. For this purpose, transcriptional regulation of the transgene expression by the endothelium-specific Flt promoter was proposed. These double-targeted viruses were expected to selectively infect blood vessels within the prostate tumors and destroy the tumors' blood supply by locally expressing a "suicide" transgene.

REPORT BODY

Description of work:

Task 1. Generation of recombinant Ad fiber proteins targeted to prostate vasculature endothelium.

1.1. Modification of the fiber-expressing plasmid vectors. According to the Statement of Work, the project began with the construction of plasmid vectors for subsequent expression of the candidate fiber genes, which are to be designed in the project, in eukaryotic cells. To this end, we first modified the plasmids of the pVS series (5, 13) that contain Ad5 fiber genes with the modified carboxy terminus or the HI loop region. The purpose of these additional modifications was to design a double mutant of the fiber protein (FΔ2) that would not bind the known Ad5 fiber receptors – CAR (8, 20) and heparan sulfate glycosaminoglycans, HSG (18). These mutated constructs would be an optimal platform for the incorporation of tumor-specific targeting peptides, thereby yielding *truly targeted* fibers.

These mutations were introduced by site-directed, PCR-based mutagenesis procedure that replaced the HSG-binding *KKTK* motif in the Ad5 fiber shaft domain with a *GAGA* tetrapepdide (18), and also deleted the *TAYT* sequence within the fiber knob domain (17). Therefore, the plasmid vectors pVSΔ2.Fc.BaeI, pVSΔ2.PB10.BaeI, and pVSΔ2.PB40.BaeI were designed. pVSΔ2.Fc.BaeI was designed to make the fiber genes with targeting ligands positioned at the carboxy terminus of the fiber, whereas pVSΔ2.PB10.BaeI and pVSΔ2.PB40.BaeI were made to design the fiber genes, whose products would incorporate the ligands within the HI loop of the knob domain. Of note, these two plasmids are the derivatives of the pVS.PB10.BaeI and pVS.PB40.BaeI (6), respectively, each containing linker-encoding sequences that flank the site of the ligand insertion. The length of the linkers within the pVS.PB10.BaeI-encoded fibers is 5 amino acid (aa), while the fibers encoded by pVS.PB40.BaeI incorporate 20 aa-long linkers. Each of these vectors contains a unique recognition site for the restriction endonuclease BaeI, which allows for a "seamless" fusion of the ligand with the fiber protein.

1.2. Derivation of expression vectors encoding the ligand-modified fibers. At the next step we selected those peptides (from the previously published), which would allow us to test the central concept of this project by directing the resultant vectors to the vasculature of prostate tumor xenografts. The problem we were facing in making this judgment was that while one of the papers, which we based our strategy on, described only two targeting peptides (1), the other publication listed a family of twenty-five closely related peptide sequences with only one of them being functionally tested (2). Obviously, testing all of these twenty-five peptides in this project would be rather unrealistic. Therefore, the rational selection of a subset of these ligand

candidates had to be done to maximize the chances for the positive outcome of the project. Thus, in addition to using the peptides described by Arap *et al*. (1), *SMSIARL* and *VSFLEYR*, we also decided to include in our study two of the peptides described by Arap *et al*. (2), *GRRAGGS* and *AGGVAGG*. The rationale for selecting *GRRAGGS* was obvious – that was the only peptide fully characterized in the published study. Peptide *AGGVAGG* was also included in our project because its sequence contains two copies of the consensus tripeptide *AGG* motif that is seen in all of the prostate vasculature-specific peptides identified by Arap *et al.* (2). Thus, we reasoned that having this duplication of the core binding motif in one ligand sequence would increase the affinity of the resultant fiber protein and, subsequently, Ad vector for the target receptor.

Additional considerations that were taken into account were warranted by the fact that peptide ligands identified by the phage display technology (1, 2) are constrained by the flanking cysteine (Cys) residues. These Cys residues are introduced into the design of the phage library to stabilize the target-binding configuration of the otherwise flexible peptide ligands via the formation of a disulfide bond. While this constraining approach apparently works for the phage-displayed peptides, its suitability for Ad targeting is questionable. This is because all of the newly synthesized Ad capsid proteins are translocated to the nucleus shortly after translation and, thus, never become available to the disulfide isomerases, the endoplasmic reticulum-localized enzymes that facilitate the formation of the disulfide bonds. Although we chose to use the constrained configurations of each of the four selected peptides (above), we also decided to include in our study the Cys-free versions of those peptides, whereby the Cys residues were either deleted, or replaced with residues of valine (Val) and alanine (Ala). This Val/Ala combination was previously found to most closely mimic the geometry of the Cys-Cys pair (21). Thus, each of the four selected peptide ligands was included in the study in three different configurations, thereby yielding twelve candidate ligands. This rather broad spectrum of the ligand configuration was expected to narrow significantly when the trimerization tests of the designed fibers are completed: based on the previous work by us and others, it could be predicted that a significant proportion of the ligand-carrying fibers would fail to form stable trimers leaving us with a reasonable number of successful leads. The sequences of all peptide ligands used in this work are summarized in Table 1.

The DNA sequences encoding the selected peptides were assembled using oligonucleotide duplexes and cloned into BaeI-cut expression vectors. Upon transformation of *E.coli*, colonies containing recombinant plasmids were identified by PCR using the insert-specific primers. Subsequently, the correct structure of these plasmids was verified by sequencing. In summary, a total of thirty-six plasmid vectors expressing the modified fiber genes have been designed.

1.3. Expression and characterization of modified fiber proteins. The goal of this part of work was to make sure that the ligand-modified fibers can be efficiently expressed in human cells and, most importantly, that the resultant proteins retain the capacity of the wild type Ad5 fiber to form homotrimers. This trimerization ability is a key structural requirement for a functional fiber, because previous studies have shown that monomeric fibers cannot be incorporated into Ad capsids (15).

The newly designed fibers were tested by transfecting 293T cells with the fiber-expressing vectors and then analyzing the resultant products present in the cell lysates using Western blotting. Each of the tested lysates was loaded on the gel in both the fully denatured (boiled for 5 min) or non-denatured form. Comparison of the patterns produced by these samples allowed for identification of those fiber configurations that failed to form trimers under non-denaturing

conditions and thus had to be eliminated from the future work. Samples of the lysed cells expressing the wild type Ad5 fiber were used as a positive control of trimerization. A typical result of such a test is shown in Fig. 1.

Figure 1. Western blot analysis of the transiently expressed fiber proteins. The lysates of the transfected cells expressing the following fiber species were analyzed:

1. wild type Ad5 fiber (native)
2. wild type Ad5 fiber (denatured)
3. double-mutated Ad5 fiber (FΔ2) (native)
4. double-mutated Ad5 fiber (FΔ2) (denatured)
5. FΔ2.PB40-*GRRAGGS* (native)
6. FΔ2.PB40-*GRRAGGS* (denatured)
7. FΔ2.PB40-*CGRRAGGSC* (native)
8. FΔ2.PB40-*CGRRAGGSC* (denatured)
9. FΔ2.PB40-*VGRRAGGSA* (native)
10. FΔ2.PB40-*VGRRAGGSA* (denatured)

M Protein mol. mass standards

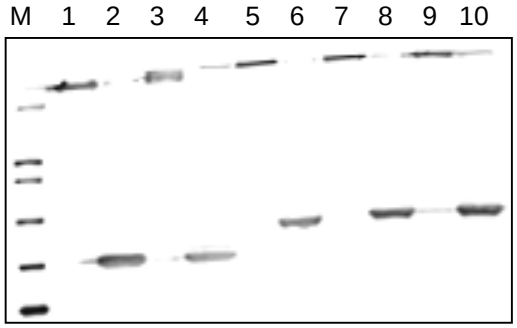


Table 1. Trimerization by various peptide-modified fiber proteins. Scores "good", "fair" and "poor" show the efficacy, with which a given fiber-ligand fusion trimerizes. Three peptides (highlighted with gray) did not score higher than "fair" in any of the tested fusions.

Peptide	Vector (site of ligand insertion)		
	pVSΔ2.PB10.BaeI (extended HI loop)	pVSΔ2.PB40.BaeI (extended HI loop)	pVSΔ2.FcBaeI (carboxy terminus)
SMSIARL	fair	fair	good
CSMSIARLC	good	fair	fair
VSMSIARLA	fair	fair	fair
VSFLEYR	fair	good	good
CVSFLEYRC	poor	fair	fair
VVSFLEYRA	poor	fair	fair
GRRAGGS	good	good	good
CGRRAGGSC	good	good	good
VGRRAGGSA	good	good	good
AGGVAGG	good	poor	good
CAGGVAGGC	good	good	good
VAGGVAGGA	good	good	good

In summary, these experiments showed that, as a general trend, the incorporation of targeting peptides into the fiber had destabilizing effect on the resultant protein with some of the

modified fiber proteins showing more efficient trimerization, than the others. The most important result of this work is, however, that we have identified structurally stable fiber configurations for the majority of the peptide ligands chosen. These protein species are identified by the "good" score in Table 1. The fiber fusions with peptides *VSMSIARLA, CVSFLEYRC, and VVSFLEYRA* scored as "fair" or "poor" and have been excluded from future work.

Task 2. Assessment of the receptor-binding capability of the modified fiber proteins.

2.1 The evaluation of the interleukin-11 receptor α (IL-11Rα)-binding. The pVS-derived expression vectors that encoded the fiber proteins modified with the IL-11Rα–specific peptides (*GRRAGGS, CGRRAGGSC, or VGRRAGGSA*) were used to transfect 293T cells to direct the fibers' expression. The expression of the fibers was then confirmed by Western Blot done with the lysates of the transfected cells. These lysates were then used in ELISA with purified IL-11Rα protein (Santa Cruz Biotechnologies, Santa Cruz, CA). In parallel, purified recombinant extracellular fragment of the Ad5 receptor CAR, sCAR, was used as a control for binding of the transiently expressed wild type Ad fiber protein to confirm that our ELISA technique works. Also included in this experiment was the recombinant phage containing the *CGRRAGGSC* peptide (2), which was provided to us by Dr. Renata Pasqualini (University of Texas M.D. Anderson Cancer Center).

This assay showed excellent binding of the wt fiber to sCAR and no binding of the peptide-modified fibers to IL-11Rα. Most surprisingly, however, was the lack of binding to IL-11Rα by the *CGRRAGGSC*-displaying phage vector (Table 1).

Table 2. ELISA with the lysates of 293T cells transiently expressing fiber-peptide proteins. All fiber constructs shown contained the targeting peptide at the carboxy terminus of the double-mutated Ad5 fiber (FΔ2) that is unable to bind to sCAR.

Tested construct	OD_{490}	
	sCAR	IL-11R
none (negative control)	0.0703	0.1222
wt Ad5 fiber (positive control for sCAR binding)	2.2582	0.1214
Fα2, (negative control for sCAR binding)	0.0733	0.1266
Fα2.Fc-*GRRAGGS*	0.0693	0.1191
Fα2.Fc-*CGRRAGGSC*	0.0725	0.1265
Fα2.Fc-*VGRRAGGSA*	0.0725	0.1544
fd-IL11R, phage displaying the peptide	0.2178	0.3310
fd-tet, phage with unmodified pIII (negative control)	0.0603	0.1342
anti-IL11Rα Ab N-20	0.0354	0.0764

In this regard, it should be mentioned that the overall quality of the reagents, which we purchased from Santa Cruz Biotechnologies, was rather poor: contrary to the vendor's claims, none of the several anti-IL-11Rα antibodies (Ab) worked in our hands with the lysate of the IL-11Rα-positive LNCaP cells (24) in Western blot. In addition, these Abs did not detect purified IL-11Rα protein in ELISA, and detected it in Western blot only when the protein was used in the amount of 1μg. Alternative commercial sources of anti-IL-11Rα Ab and IL-11Rα could not be found. Therefore, we hypothesized that the lack of binding to purified IL-11Rα in ELISA, which we had seen with both the cell lysates and the phage, could be due to low quality of this protein target.

To troubleshoot these problems, two additional efforts were undertaken. First, we chose to test our recombinant fiber constructs in the most stringent cellular system, in which two isogenic cell lines that would differ only by the level of the IL-11Rα expression would serve as targets for these fiber proteins. To this end, we made an IL-11Rα–expressing derivative of the IL-11Rα–negative 293 cell line and used it in combination with the parental 293 cells to test our constructs. IL-11Rα cDNA (Open Biosystems, Huntsville, AL) was linked *via* an internal ribosome entry sequence (IRES) to the neomycin aminotransferase gene II (G418 resistance) within the mammalian expression plasmid pIRES-neo3 (Clontech, Mountain View, CA). The resultant plasmid, pIRES.IL11R, was used to transfect 293 cells and the G418-resistant cells were selected. Expression of IL-11Rα in these 293-IL11R cells was demonstrated by quantitative RT-PCR. In addition, we have designed an IL-11Rα-specific recombinant protein probe comprising the Ad5 fiber-T4 fibritin chimera (used here as a scaffold) fused at its carboxy terminus with the human IL-11. This protein was expressed transiently in 293T cells and used in a flow cytometry experiment with 293-IL11Rα cells, which confirmed the presence of the receptor on these cells (data not shown).

Having demonstrated the presence of the target receptor on these 293-IL11Rα cells, we used them for FACS analysis to evaluate the phage containing the *CGRRAGGSC* peptide. Unfortunately, the data obtained in this experiment was no different from the ELISA results presented above: the phage showed no preferential binding to 293-IL11Rα cells (as compared to parental 293 cells) (Table 3).

Table 3. Flow cytometry analysis of phage interaction with the cell-associated target receptor. Binding to IL-11Rα-expressing cells of the GRRAGGS peptide-modified phage (fd-GRRAGGS) was compared to that of the unmodified parental phage, fd-tet. Another phage vector, fuCT/6C6, that contains a single chain Ab to PSMA (prostate specific membrane antigen) and the PSMA-expressing derivative of 293 cells 293/PSMA were used as positive control of binding.

Cells	Phage	Mean fluorescence intensity
293	fd-tet	11.13
293	fd-GRRAGGS	11.15
293	fuCT/6C6	9.47
293/IL11Rα	fd-tet	7.99
293/IL11Rα	fd-GRRAGGS	8.3
293/IL11Rα	fuCT/6C6	12.64
293/PSMA	fd-tet	6.07
293/PSMA	fd-GRRAGGS	11.51
293/PSMA	fuCT/6C6	181.41

The availability of 293-IL11Rα cells also made it possible to test the IL-11Rα binding of the newly made fiber proteins in the context of modified Ad particles. Such additional tests would provide us with a clear answer to whether the incorporation of these modified fibers into an Ad virions makes the viruses IL-11Rα-specific. The viruses containing the peptide-modified fibers were generated by using a fiber trans-complementation method described by Jakubczak *et al.* (12). Briefly, a "tester" Ad vector, Ad5LucF^0, that expresses a firefly luciferase and also lacks the fiber gene in its genome, was amplified in 293/F28 cells that constitutively express wild type Ad5 fiber (13). Next, the pVS-derived plasmids expressing the peptide-modified fibers (above) were used to transfect 293T cells, which were then infected with the Ad5LucF^0. This resulted in simultaneous replication of the fiber-less virus and the expression of the candidate

fibers in each 293T cell that has been both transfected and infected. As a result, the viruses generated in this experiment each contained one of the peptide-modified fiber candidates (confirmed by Western blot of the virions purified on CsCl). These viruses were used for parallel infections of 293 and 293-IL11Rα cells. The result of this experiment corroborated the data, which we previously obtained with the *GRRAGGS*-modified fiber proteins in the ELISA and FACS formats (above): no IL-11Rα-specific gene transfer was observed (Fig. 2).

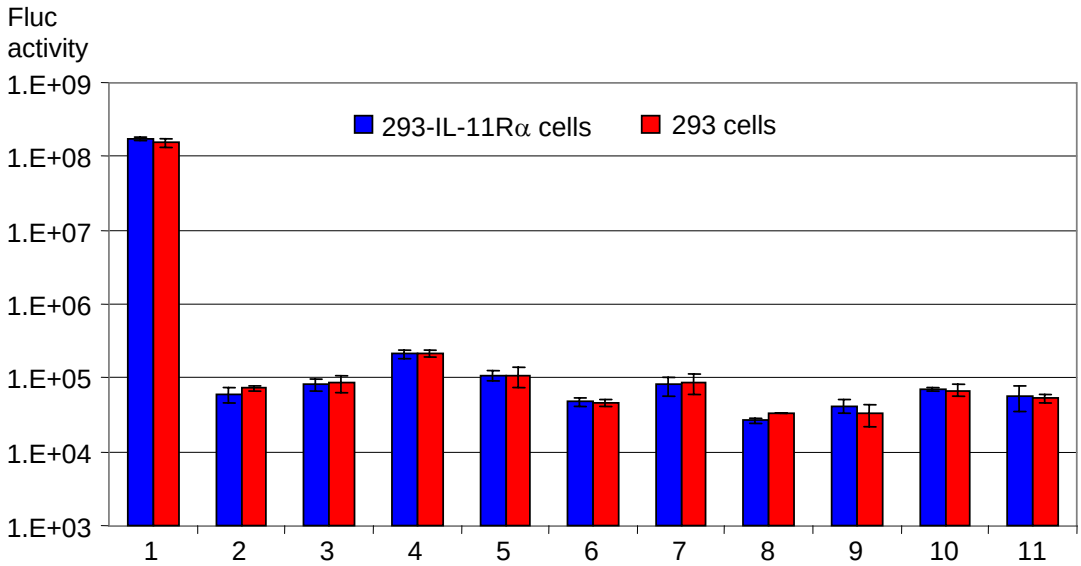


Figure 2. Transduction of 293 and 293/IL-11Rα cells with the fiber-modified Ad vectors. Cells were infected with Ad vectors obtained with the fiber trans-complementation method. Twenty-four hours post infection the cells were collected, lyzed and the activity of the Ad vector-expressed luciferase was measured. Viruses used in this experiment incorporated the fiber proteins with peptides inserted in either the extended HI loop (FΔ2.PB10 or FΔ2.PB40), or the carboxy terminus (FΔ2.Fc). The fiber constructs contained in each of the Ad vector are: 1, wild type Ad5 fiber; 2, mutated FΔ2; 3, FΔ2.PB10-GRRAGGS; 4, FΔ2.PB10-CGRRAGGSC; 5, FΔ2.PB10-VGRRAGGSA; 6, FΔ2.PB40-GRRAGGS; 7, FΔ2.PB40-CGRRAGGSC; 8, FΔ2.PB40-VGRRAGGSA; 9, FΔ2.Fc-GRRAGGS; 10, FΔ2.Fc-CGRRAGGSC; 11, FΔ2.Fc-VGRRAGGSA. Fluc activity is shown in relative light units.

While it is possible that a target-specific peptide identified in a phage biopanning study has lost its binding capability upon genetic fusion with the Ad fiber protein (such failures have been reported previously (23), the lack of any binding to IL-11Rα by the *GRRAGGS*-displaying phage particles made us believe that the identification of IL-11Rα as a target for the *GRRAGGS* peptide (2) was done incorrectly. This conclusion is further supported by the data from Dr. Chun Li (University of Texas M.D. Anderson Cancer Center), who has demonstrated that radiolabelled *GRRAGGS* peptide does not bind to IL-11Rα-expressing cells. However, this potentially incorrect target identification did not compromise our approach of using this particular peptide for Ad tropism modification, because the aforementioned *GRRAGGS*-displaying phage has been shown to bind prostate vasculature (2), thus making the identity of the target cell marker irrelevant for the purpose of our work.

Task 3. Generation and preliminary characterization of Ad vectors incorporating the Flt-1 promoter-controlled dual expression cassette and the fibers modified with targeting peptides.

The overall design of the expression cassettes to be incorporated into Ad vectors was slightly modified compared to the originally proposed. Specifically, instead of inserting two similarly

designed cassettes, each containing one of the two transgenes, into the Ad rescue vectors, we chose to design one cassette to express both transgene products, the HSV TK and firefly luciferase (Fluc). This was done to avoid potential intramolecular recombination events involving the two copies of the promoter and polyadenylation sequences, which otherwise would be present in each expression cassette (4). To obviate the necessity for two expression cassettes, we chose to use a genetic fusion of HSV TK and Fluc, "TL" for short, whose gene was kindly provided to us by Dr. Vladimir Ponomarev (Memorial Sloan-Kettering Cancer Center, New York, NY).

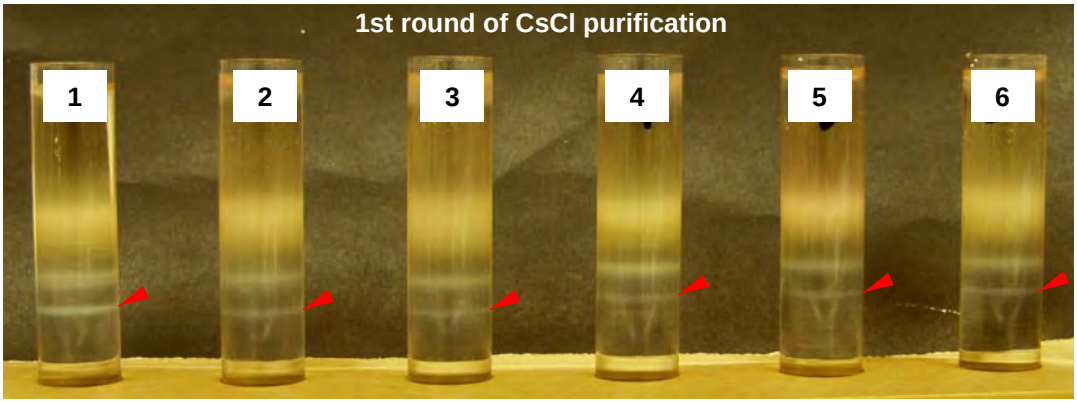
First, the open reading frame of the TL gene was cloned into the pShuttle-CMV vector (Stratagene, La Jolla, CA), resulting in pShuttle.CMV.TL, in which the TL sequence was under transcriptional control of cytomegalovirus (CMV) immediate-early promoter. Next, the promoter-less pShuttle (Stratagene) was used to subloclone an Flt-1 promoter that was provided to us by our Consultant Dr. Paul Reynolds (Royal Adelaide Hospital, North Terrace, Australia), and the TL sequence downstream of it, yielding pShuttle.Flt.TL.

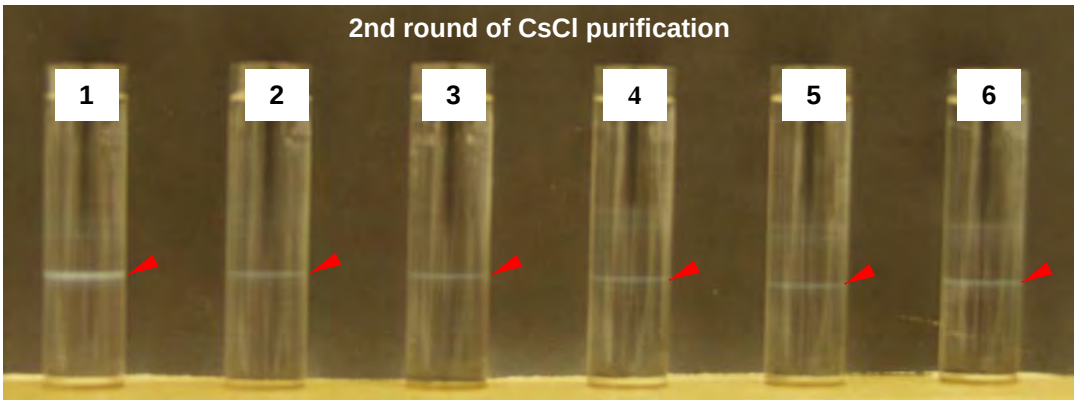
Both expression cassettes were then transferred from these shuttle plasmids into the rescue vector pVK500C (5) to replace the E1 region of Ad5 genome. This was done by homologous DNA recombination in bacteria as previously described (10). The resultant Ad rescue vectors incorporating the CMV promoter- and Flt promoter-containing expression cassettes were designated, pVK514 and pVK515, respectively.

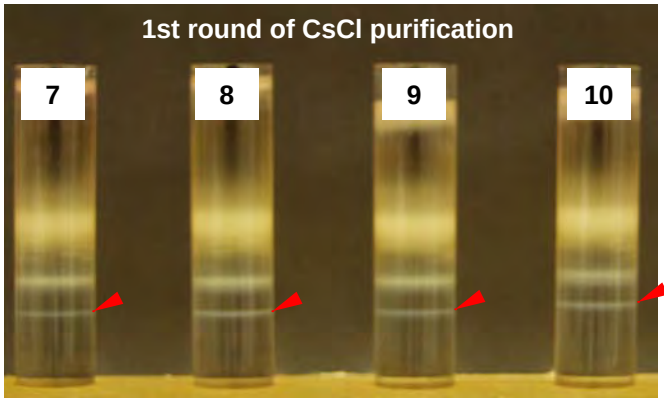
In parallel, we narrowed down the number of the fiber protein candidates, whose genes were considered for subsequent incorporation into Ad genomes. Based on the protein trimerization data generated in Task 1, the priority was given to those fiber-peptide species that showed satisfactory trimerization profiles. Thus, the following nine combinations of peptides and sites of incorporation within the fiber molecule were identified for further evaluation:

- peptides *GRRAGGS, SMSIARL*, *CSMSIARLC*, *VSMSIARLA*, *VSFLEYR*, *CAGGVAGGC*, and *VAGGVAGGA* incorporated within the extended HI loop of the mutated fiber (the scaffold designated FΔ2.PB40)
- peptides *SMSIARL*, *VSFLEYR*, and *AGGVAGG* fused to the carboxy terminus of the mutated fiber (the scaffold designated FΔ2.Fc).

To further narrow our choices we tested the efficacy with which each of these modified fibers incorporates into Ad capsid. For this, we used the above mentioned fiber trans-complementation protocol. In brief, expression plasmids encoding each of the nine fiber candidates was used to complement the fiber deficiency of the tester virus, Ad5LucF^{0} (above). The resultant viruses were purified in two sequential CsCl gradients (Fig. 3), and analyzed by Western blot with anti-fiber Ab (Fig. 4). Based on the results of these assays, the following fiber-peptide constructs were selected for the virus rescue work: FΔ2.PB40-*GRRAGGS*, FΔ2.PB40-*SMSIARL*, FΔ2.Fc-*VSFLEYR*, and FΔ2.PB40-*VAGGVAGGA***.**







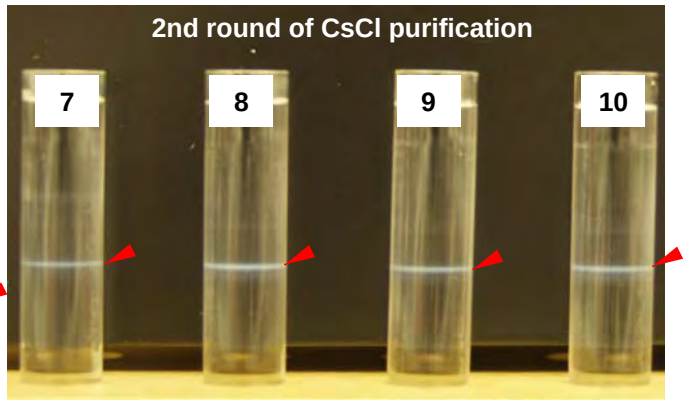


Figure 3. Purification of Ad vectors generated by the fiber trans-complementation method. Bands containing complete Ad virions are shown by red arrowheads. Fiber proteins used in each instance are listed below.

1. wild type Ad5 fiber (control)
2. FΔ2.PB40-*SMSIARL*
3. FΔ2.PB40-*CSMSIARLC*
4. FΔ2.PB40-*VSMSIARLA*
5. FΔ2.PB40-*VSFLEYR*
6. FΔ2.Fc-*SMSIARL*
7. FΔ2.Fc-*VSFLEYR*
8. FΔ2.PB40-*CAGGVAGGC*
9. FΔ2.PB40-*VAGGVAGGA*
10. FΔ2.Fc-*AGGVAGG*

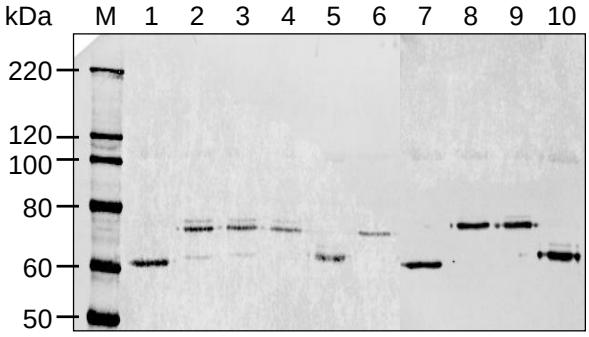


Figure 4. Incorporation of the modified fiber proteins in Ad particles. CsCl-purified Ad vectors produced using the fiber trans-complementation method were fully denatured and analyzed by Western blot with anti-fiber Ab. Fiber proteins tested are listed below. M, protein standards

1. wild type Ad5 fiber
2. FΔ2.PB40-*SMSIARL*
3. FΔ2.PB40-*CSMSIARLC*
4. FΔ2.PB40-*VSMSIARLA*
5. FΔ2.Fc-*SMSIARL*
6. FΔ2.PB40-*VSFLEYR*
7. FΔ2.Fc-*VSFLEYR*
8. FΔ2.PB40-*CAGGVAGGC*
9. FΔ2.PB40-*VAGGVAGGA*
10. FΔ2.Fc-*AGGVAGG*

To make recombinant Ad genomes incorporating the genes encoding these chosen protein constructs they were transferred to the fiber shuttle vector pZ3.1. These genes were then excised from the resultant plasmids together with the flanking Ad DNA sequences, and used for homologous DNA recombination with the linearized forms of either pVK514 and pVK515. The plasmids obtained as a result of these recombinations each contained a complete Ad5 genome with a TL-expressing cassette driven by either the CMV or Flt promoter, and one of the genes encoding for either the wild-type fiber, or a peptide-modified fiber. Therefore, a total of ten recombinant Ad genomes have been constructed.

Our next goal was to use theses designed Ad genomes to rescue the viruses. Because the viruses to be rescued were expected to contain mutated fibers and, thus, unable to use the natural mechanism of cell infection, the rescue was done using the previously established cell line, 293/F28 (5) that expresses the wild type Ad5 fiber. Transfection of these cells with the designed Ad genomes yielded mosaic virions that incorporated both the wild type and the modified fibers. The presence, of the wild type fiber made these vectors infectious and facilitated further amplification in 293/F28 cells. The last round of amplification was supposed to be done in regular 293 cells (no wt fiber expression) to yield virions equipped with targeted fibers only.

By the time the project reached this point, the data that we have generated in another on-going project, which also involved Ad targeting to tumor, made us think that the idea of mutating the putative HSG-binding site within our fiber constructs was a wrong one. The troubling news was that the double-mutated, ligand-modified fibers that have been designed in that other project and (importantly!) confirmed to bind the target receptor (Her2), proved to be very inefficient in directing Ad virions to this receptor. In contrast, the viruses equipped with the fiber constructs that contained only one mutation (the one in the knob) showed high levels of target cells transduction. This observation made us revisit the entire idea of mutating the fiber shaft as a means to ablate undesirable binding of Ad vectors to HSG. We realized that the site of the mutation previously proposed to ablate this binding (19) is located immediately downstream to the so-called "*hinge*" region within the shaft domain. This hinge is known to give the fiber its flexibility that is a key structural feature of the protein, because it allows a CAR-anchored Ad virion to form the secondary contact with the cell and that in turn triggers Ad internalization. It has been previously shown that the fibers with mutated hinge yield Ad vectors with poor infectivities (22). These considerations led us to the conclusion that the use of Ad vectors with double-mutated fibers in the *in vivo* studies in Task 4 would most likely yield negative results with respect to both the specificity and efficacy of gene transfer. Should this happen at such a late time point in the project, we would not be able to remake the vectors and redo the experiments in animals because there would not be any time and money left. Therefore, we decided to remake these vectors using the fiber genes with only one mutation. To further

support that decision, although in retrospect, we would like to reference the recent studies by Kritz *et al*. (14) and Di Paolo *et al* (11). The former paper reported that the targeted Ad vectors containing the fiber proteins with the mutated shaft domain near the hinge region are ineffective for cell retargeting strategies. The latter study clearly showed that the previously proposed interaction of the KKTK motif within the fiber's shaft with HSGs simply does not take place. Therefore, the concept about the involvement of this motif in undesired uptake of Ad vectors by the liver, which was widely accepted (although never proved directly) at the time this project was conceived, has been proved wrong.

Having made this decision, we went back and redesigned all the fiber genes and the shuttle vectors, remade the rescue plasmids containing the Ad genomes with these modified fiber genes. A total of eight Ad genomes (Table 4) have been remade.

Next, the Ad vectors were rescued and propagated using the fiber-complementing cell line 293/F28 (as described above). Because this work took several months and caused a significant delay in the project, and also consumed extra funds, at this juncture we decided to limit the total number of vectors to be used further in the project to a total of four (shown by gray shading in Table 4). These selected Ad vectors were then re-packaged by an additional round of propagation that was done in 293 cells. Specifically, each of the vector configurations amplified previously in 293/F28 cells was used to infect 2×10^9 293 cells. The re-packaged virions were then purified by equilibrium centrifugation in CsCl gradients yielding the amounts shown in Table 4. To confirm the identities of these Ad vectors, DNA sequencing of the modified fiber genes was done.

Table 4. Summary of Ad vector constructs re-made in Year 3 of the project. Vectors shown by gray shading will be used in the *in vivo* experiments in Task 4. In addition, two control Ad vectors, Ad5CMV-TL.F5 and Ad5Flt-TL.F5, each equipped with the wild type fibers have been produced.

Vector	Promoter that drives the reporter gene	Peptide ligand and its position within the fiber	Total yield in the final preparation (vp)
Ad5CMV-TL.PB40-1.7	CMV	*GRRAGGS* in the HI loop	1.6E+13
Ad5Flt-TL.PB40-1.7	Flt	*GRRAGGS* in the HI loop	1.6E+13
Ad5CMV-TL.PB40-1.12	CMV	*VAGGVAGGA* in the HI loop	1.8E+13
Ad5Flt-TL.PB40-1.12	Flt	*VAGGVAGGA* in the HI loop	1.3E+13
Ad5CMV-TL.PB40-1.1	CMV	*SMSIARL* in the HI loop	
Ad5Flt-TL.PB40-1.1	Flt	*SMSIARL* in the HI loop	
Ad5CMV-TL.Fc3.4	CMV	*VSFLEYR* at the carboxy terminus	
Ad5Flt-TL.Fc3.4	Flt	*VSFLEYR* at the carboxy terminus	

In addition, the efficacy of incorporation of the modified fibers into Ad particles was assessed by Western blot analysis, which showed that the fibers did incorporate into virions very efficiently (Fig. 5). Based on these data, we conclude that the virions of these vectors have adequate structure and thus are suitable for tumor targeting experiments *in vivo*.

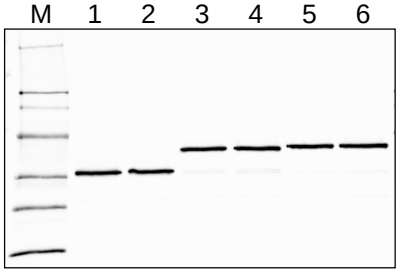


Figure 5. Western blot of purified Ad vectors. Aliquots of CsCl-purified viral preparations were fully denatured and fractionated by SDS-PAGE. After electroblotting, the samples were probed with the anti-Ad fiber antibody. The fibers were detected using the fluorescently-labeled secondary antibody and a near-infrared scanner Odyssey. M, protein mol. weight standards.

1. Ad5CMV-TL.F5
2. Ad5Flt-TL.F5
3. Ad5CMV-TL.PB40-1.7
4. Ad5Flt-TL.PB40-1.7
5. Ad5CMV-TL.PB40-1.12
6. Ad5Flt-TL.PB40-1.12

Task 4. Studies of the* in vivo *distribution and anti-tumor effect of the double-targeted Ad vectors.

4.1. Development of a target cell line for in vivo studies and its preliminary characterization.

A target cell line for subsequent *in vivo* studies has been established as follows. To facilitate the identification of the human tumor xenografts grown in mice and also to allow for non-invasive monitoring of tumor growth, we made a derivative of human prostate carcinoma cell line LNCaP stably expressing a dual-modality reporter gene.

To this end, a recombinant replication-deficient retrovirus vector containing an open reading frame of *Renilla* luciferase (hRLuc) (Promega, Madison, WI) fused with the enhanced green fluorescent protein (EGFP), was assembled within a commercial retroviral backbone, pLEGFP-N1 (BD Biosciences, San Jose, CA). This cloning put the dual reporter gene under transcriptional control of the cytomegalovirus (CMV) promoter. The resultant retro-vector was designated pLhRLuc-eGFP (Fig. 6).

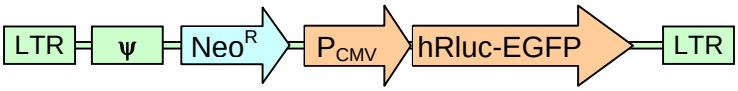


Figure 6. Schematic representation of LhRLuc-EGFP genome. LTR – long terminal repeat, ψ - packaging signal, Neo^R – G418 resistance gene, P_{CMV} – cytomegalovirus promoter, hRluc-EGFP – dual-reporter coding sequence.

This retro-vector was packaged by transfection of 293/GPG cells (16) that yielded pseudo-typed, pan-tropic virions decorated with the protein G of the vesicular stomatitis virus (VSV-G). The rescued vector was then used to transduce LNCaP cells and the stably transduced clones were selected using resistance to G418 as a selection marker (Neo^R).

Since the expression of the G418 resistance gene by this vector is not linked to the expression of the reporter transgene, an additional selection for GFP-producing cells was done using preparative cell sorting by flow cytometry. Two sequential sorting procedures resulted in a brightly fluorescent polyclonal cell line, LNCaP/hRluc-EGFP (Fig. 7).

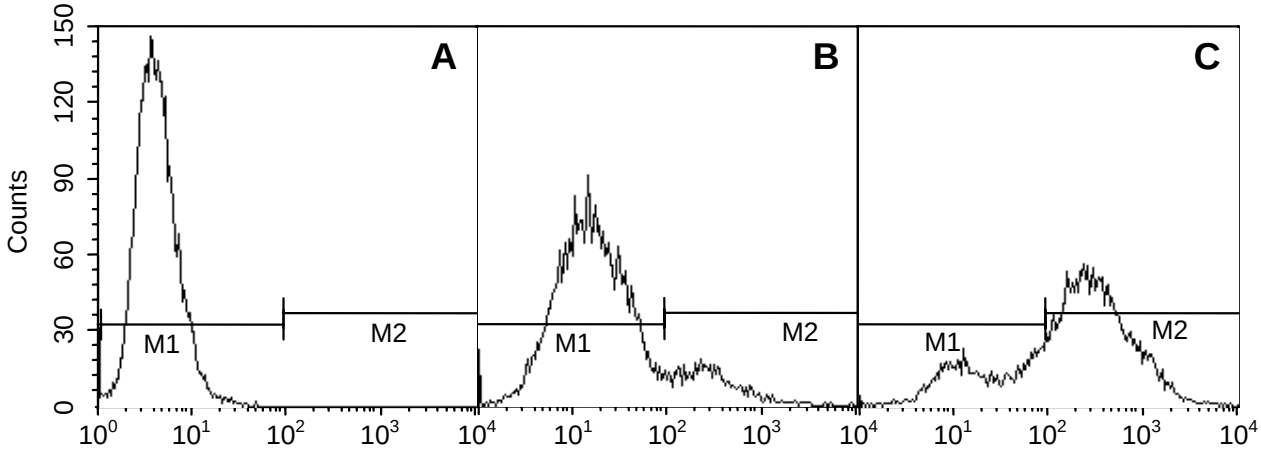


Figure 7. EGFP fluorescence in stably transduced LNCaP cells. Flow cytometry of the parental LNCaP cells (A), LNCaP cells after retroviral transduction (B), and polyclonal line LNCaP/hRluc-EGFP obtained after two rounds of selection by cell sorting (C).

Analysis of Rluc activity in these cells confirmed a very high level of bioluminescence (Fig. 8), thus suggesting that visualization of the LNCaP/Rluc-EGFP-derived tumor xenografts in mice could be done by bioluminescence-based imaging of Rluc activity.

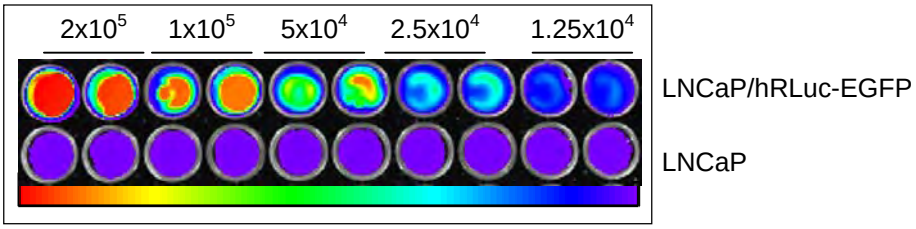


Figure 8. Bioluminescence measured in live LNCaP cells using optical imaging. Cells, either parental LNCaP or retrovirus-transduced and sorted LNCaP/Rluc-EGFP, were seeded in the wells of a 96-well plate in the amounts shown above the wells (in "cell/well"). After attachment, coelenterazine was added (1μg/ml) and the plate was imaged using the IVIS200 imaging system (Xenogen). Pseudo-color scale below the image shows the luminescence intensity from 10^6 photon/sec/cm^2/sr (purple), to $3.8x10^7$ photon/sec/cm^2/sr (red). According to this measurement, the photon output in LNCaP/hRLuc-EGFP cells equals $1.8x10^3$ photon/cell/sec.

In addition, cell sorting was used to isolate individual clones of Rluc-GFP-producing LNCaP cells from this polyclonal line. Three brightly fluorescent clones have survived the selection and have been expanded (Fig. 9). Should the polyclonal LNCaP/hRLuc-EGFP line fail to form xenografts in mice, these clones would be used instead.

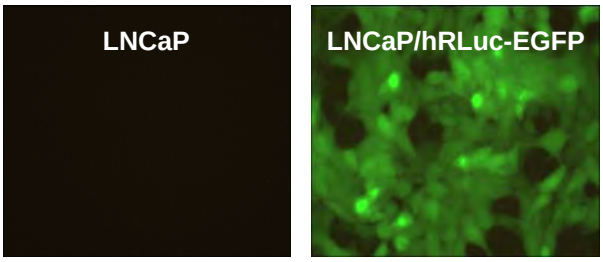


Figure 9. Fluorescent images of the parental LNCaP cells and a monoclonal cell line LNCaP/hRLuc-EGFP.

Next, the ability of polyclonal LNCaP/hRLuc-EGFP line to establish target xenografts in nude mice was tested. This was done to rule out a possibility that the expansion, retroviral transduction and repeated cell sorts might have affected the tumorigenicity of these cells, and would thus make the subsequent *in vivo* studies impossible.

Towards this end, 8-week old male nu/nu mice were injected with either the parental LNCaP, or modified LNCaP/hRLuc-EGFP cells mixed with Matrigel (BD Biosciences). The cells were injected subcutaneously into the flanks of the animals. Each cell line was used at a dose of 10^6 cells per injection site. These experiments showed that while there were substantial animal-to-animal variations in tumor growth, these variations were seen in animals injected with any of the two tested cell lines. More important was the fact that the LNCaP/hRLuc-EGFP formed tumors just as efficiently as did the parental LNCaP cells (Fig. 10). Each of these cell lines yielded xenografts at a rate of 40%.

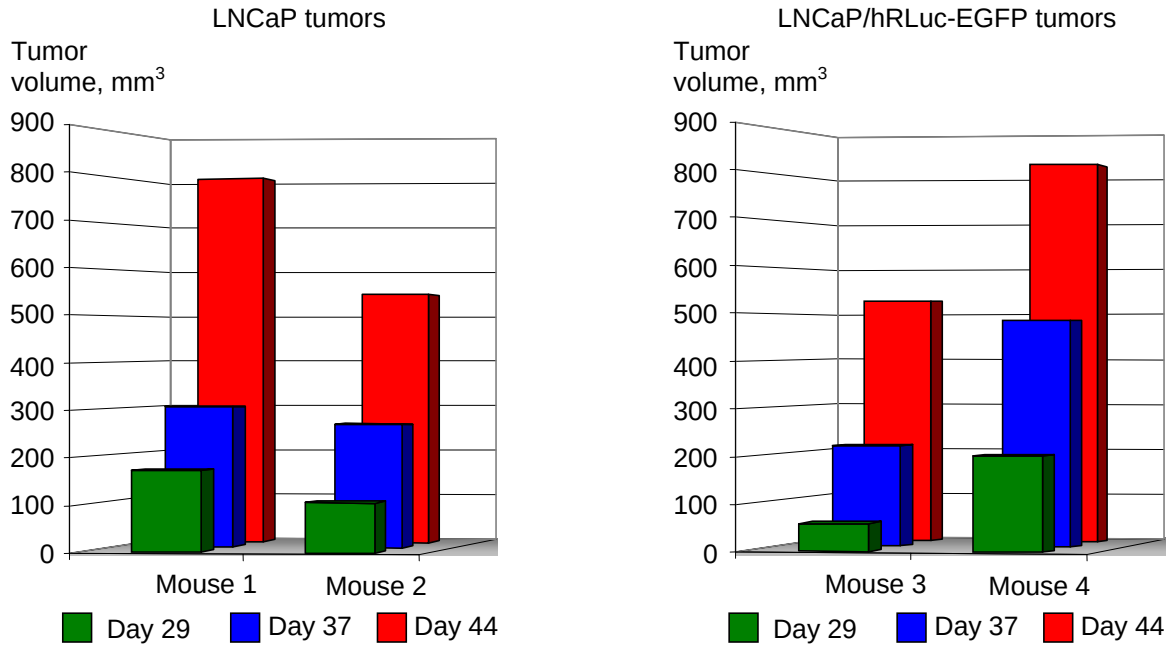


Fig. 10. Growth of LNCaP- and LNCaP/hRLuc-EGFP-derived xenografts in nude mice. Tumor growth in animals injected as described was monitored by measuring them weekly in their longest dimension and at 90º to their longest dimension and calculating their sizes by the formula (length x width^2)/2. Bar graphs show the size of the nodules in mm^3 at days 29, 37, and 44 post-implantation.

4.2. In vivo *gene delivery to prostate tumors by modified Ad vectors.*

The central hypothesis of this project that a stringent control over Ad vector dissemination *in vivo* and targeting of Ad therapy to prostate tumors could be achieved by genetic tailoring of Ad tropism was tested using an experimental design outlined in Fig. 11.

Towards this end, the subcutaneous LNCaP/hRLuc-EGFP xenografts established in nude mice were targeted by the four genetically modified Ad vectors developed in Task 3. To capture the patterns of the vectors' biodistribution shortly after vector administration, a bioluminescent imaging of the Ad-expressed Fluc reporter in live animals was done. This method provided us with the snapshots of the reporter expression in entire animals and identified the loci of the highest vector concentration. While these images provided an important initial guidance, they were not entirely conclusive because the vector-induced Fluc activity in the liver produced very strong luminescent signals, which could potentially mask the signals generated by other organs and the tumors. Such potential overlap could be neither identified, nor resolved because our imaging system did not have a tomographic capability.

To obtain more detailed patterns of the vectors' distribution, the animals were sacrificed, and individual tumors and selected organs were collected, homogenized, lysed, and used as substrates in two separate analyses. First, the Fluc activity in these samples was determined. This assay provided a more accurate quantitative measure of the vector-expressed reporter activity for each individual organ or tumor, which – in contrast to whole body imaging results - was not compromised by the reporter activity in the adjacent tissues.

In addition to *reporter expression* patterns, the biodistribution of *viral particles* was analyzed by detecting Ad genomes in the lysates of harvested tissues.

Therefore, the validity of our conclusions is supported by the results obtained independently using three different assays.

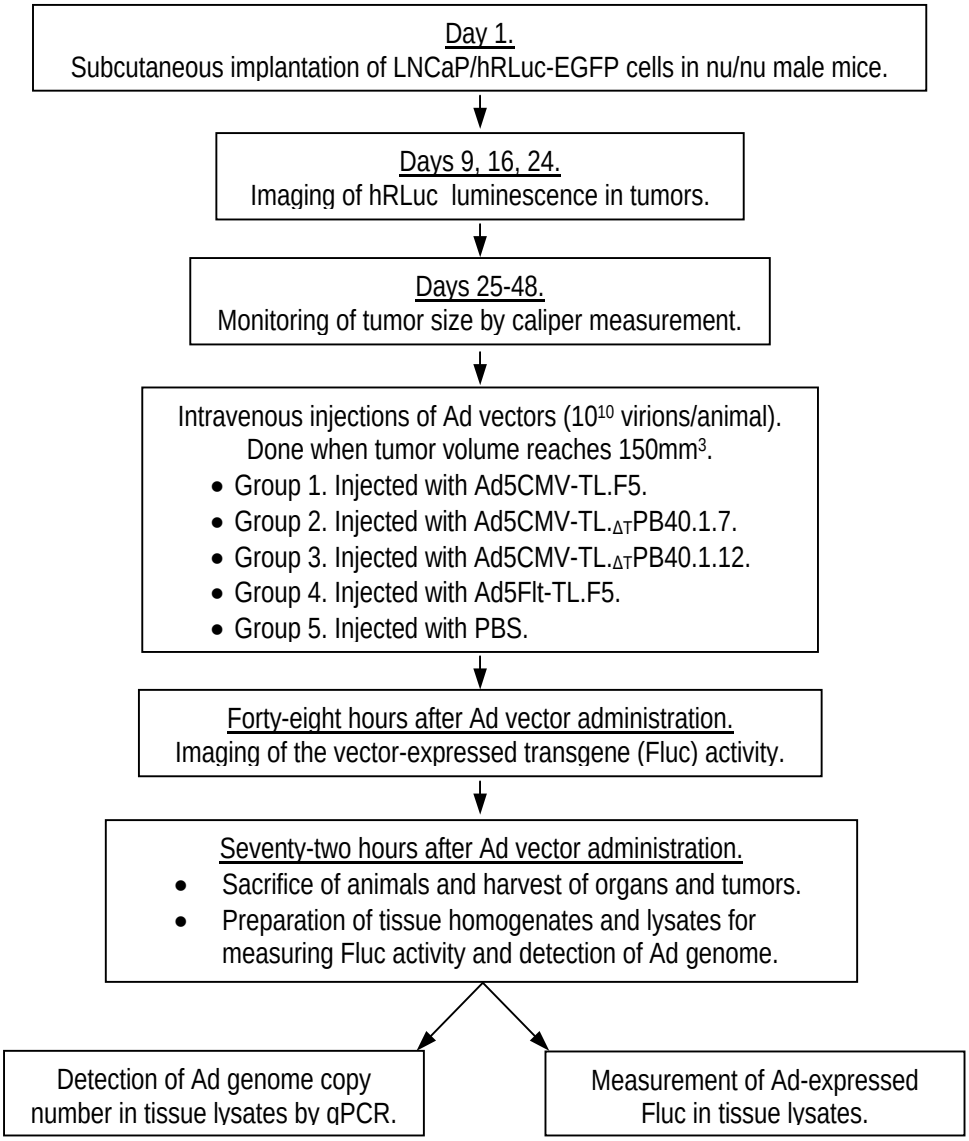


Figure 11. Overview of the *in vivo* experiments. On Day 1, prostate tumors were established in mice by injecting them with LNCaP/hRLuc-EGFP cells. At early stages of tumor development (Days 9-24), their growth was monitored using bioluminescent imaging of the tumor-expressing Rluc activity. On Day 25, the measurements of the most developed tumors with caliper began. From this time point and until Day 48, those animals that developed tumors were used for Ad vector injections. Forty-eight hours post-injection, the biodistribution of vector-directed transgene expression (Fluc) was done by injecting the mice with the Fluc substrate, D-luciferin and imaging them as before. Seventy-two hours post vector administration, the animals were sacrificed and their organs and tumors collected. These tissue samples were used for the Fluc activity measurements and qPCR detection of Ad DNA.

4.2.1. Optical imaging of tumor growth and Ad vector biodistribution.

The target prostate tumor xenografts were established in 8-week old nude male mice by injecting the animals subcutaneously with LNCaP/hRLuc-EGFP cells (2×10^6 cells per injection site) mixed with Matrigel. On Days 9-24, optical imaging was done to identify those mice, in which the tumor implantation was successful. For this, the mice were injected via tail vein with the substrate for the tumor-produced Rluc, coelenterazine (20 μg per injection), and imaged in an IVIS200 imaging system. Examples of such images and the quantitative representation of the intensities of the acquired signals are shown in Fig. 12.

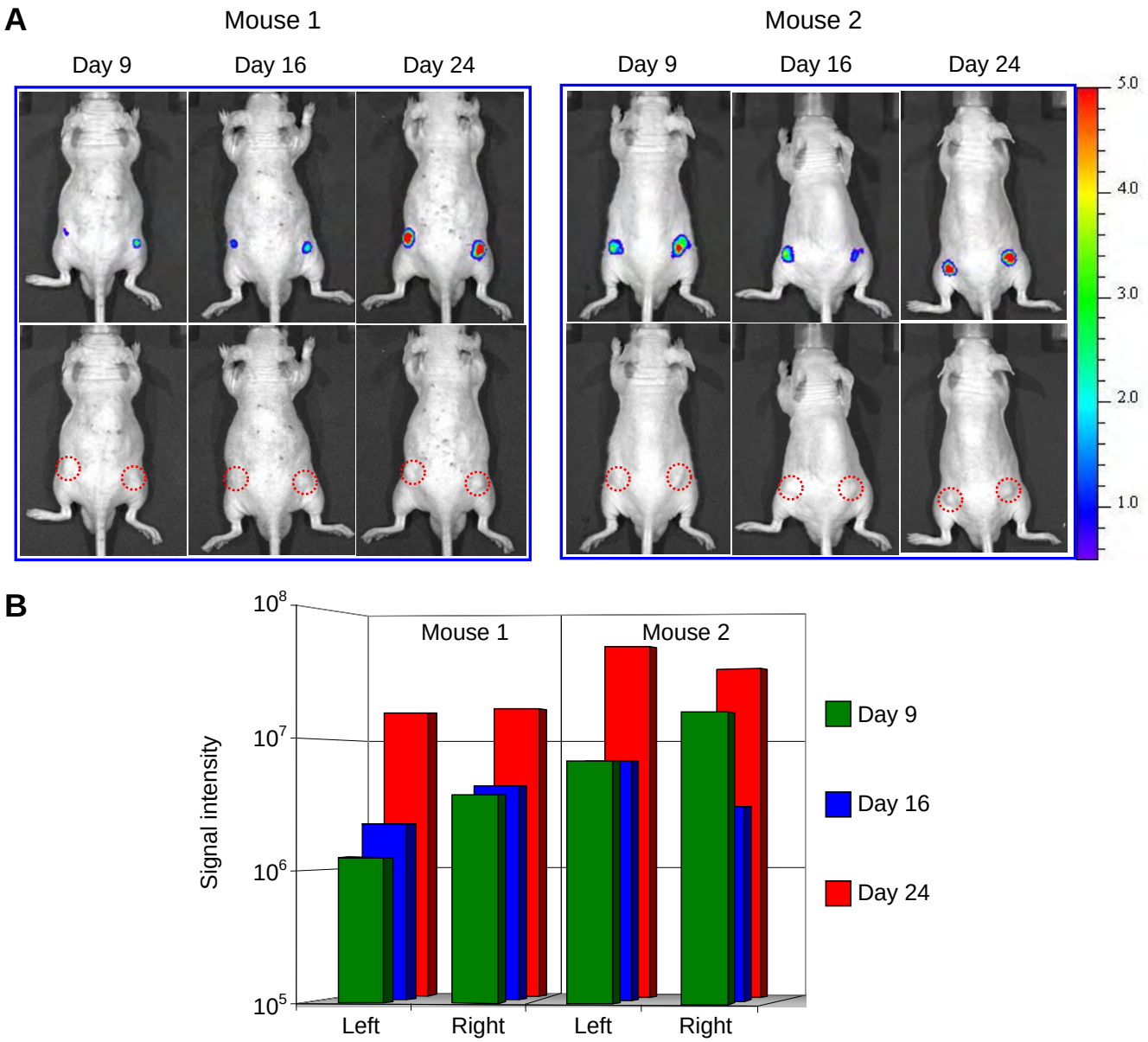


Figure 12. Monitoring of xenograft growth in nude mice using optical imaging of tumor-expressed *Renilla* luciferase. A. Photographic images either alone (lower rows), or with the superimposed bioluminescent signals (upper rows) of two representative animals taken on days 9, 16, and 24 are shown. Red contours in the lower images show the sites of xenografts' growth. The pseudo-color scale on the right shows the range of bioluminescent signal intensities in 10^6 of photon/sec/cm^2/radian^2. B. Graph representation of the intensities of bioluminescent signals (in photon/sec/region of interest) registered in tumors on either the left or the right flank of the animals shown in A.

On Day 25, the measurements of the most developed tumors with caliper began. At the same time, the animals showing no tumor development were excluded from the experiment.

Injections with viral vectors were done when a given mouse developed at least one tumor of 150 mm^3 in size; at which point the aliquots of Ad vectors (100μl in PBS) were injected into the animal's tail vein. Forty-eight hours post-injection, the biodistribution of vector-directed transgene expression (Fluc) was done by injecting the mice (tail vein) with D-luciferin (3 mg/injection) and imaging them in an IVIS200 imager.

This whole body imaging revealed robust bioluminescence in the livers of all animals injected with Ad vectors that contained the reporter under control of CMV promoter. However, no Fluc expression was seen anywhere else including the tumors (Fig. 13). Animals injected with the vector whose reporter gene was controlled by an Flt promoter showed no significant signals anywhere in the body.

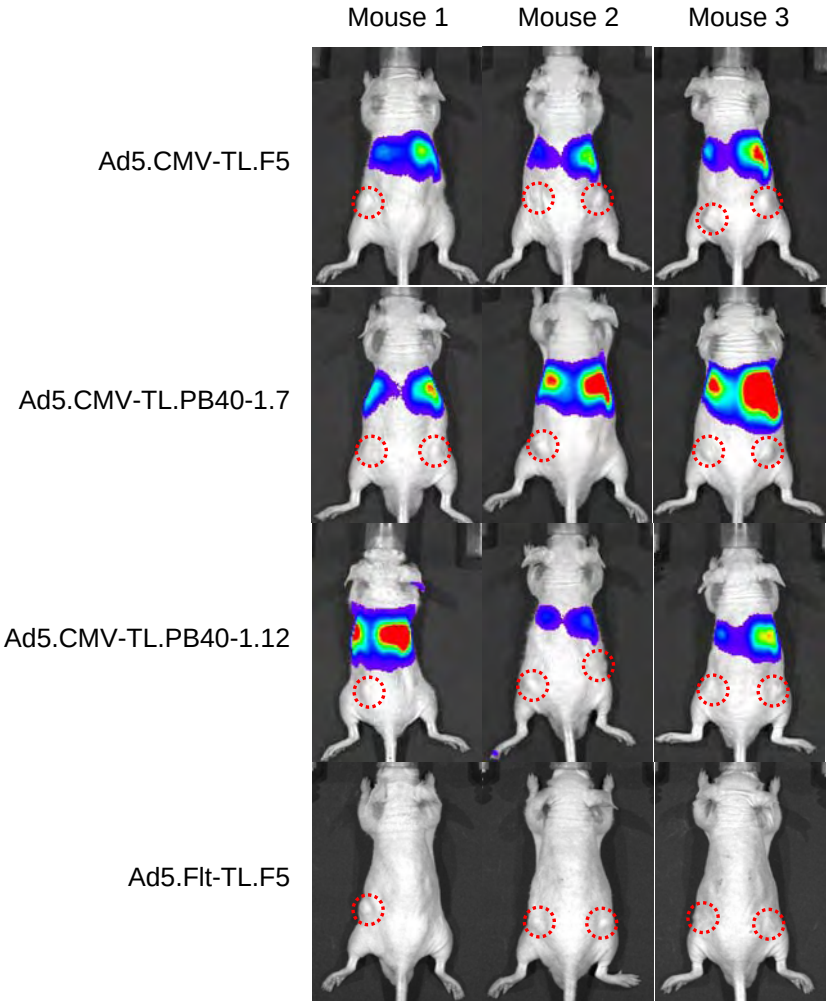


Figure 13. Whole body imaging of tumor-bearing animals injected with Fluc-expressing Ad vectors. Each of the four cohorts of mice injected with one of the tested viral vectors is represented by a group of three animals arbitrary labeled as mouse 1, 2, and 3. Viral vectors used in this study (listed to the left of the image panels) were: Ad5.CMV-TL.F5 (unmodified wt fiber, reporter expression under control of CMV promoter), Ad5.CMV-TL.PB40-1.7 and Ad5.CMV-TL.PB40-1.12 (fibers containing the ΔTAYT deletion and one of the two targeting peptides – 1.7 or 1.12, reporter expression under control of CMV promoter), Ad5.Flt-TL.F5 (unmodified wt fiber, reporter expression under control of Flt promoter). Color shading shows the areas of bioluminescence, while the red contours show the sites of xenografts' growth.

4.2.2. Additional vector localization studies.

Since the whole body imaging failed to detect any tumor-localized expression of the vector-encoded reporter, additional studies were done in an effort to detect either the transgene expression, or the physical presence of the vector particles in tissues of the Ad-injected mice.

Towards this end, seventy-two hours post vector administration, the animals (described in 4.2.1) were sacrificed and their organs and tumors were collected. These tissue samples were homogenized in PBS using TissueLyser homogenizer (Qiagen, Valencia, CA). Aliquots of these samples were then lysed and used as substrates in two separate analyses.

First, the Fluc activity in these samples was determined using Luciferase Assay kit (Promega) and tube luminometer (Berthold, Oakridge, TN). The data generated in this assay largely corroborated the results of the whole body imaging: expression of the Fluc reporter was almost entirely localized to the liver of the animals with only trace amounts of activity seen in the heart, kidney, and spleen (Fig. 14). Consistent with both the previous reports and our whole body imaging data, the virus, whose reporter was controlled by Flt promoter, consistently showed lower levels of expression in all organs.

These findings led us to a conclusion that neither the ΔTAYT mutation, nor the incorporation of targeting peptides 1.7 and 1.12 into the fiber have significantly changed the biodistribution of the modified vectors among various organs, which remained essentially the same as seen for unmodified control vector AdCMV-TL.F5.

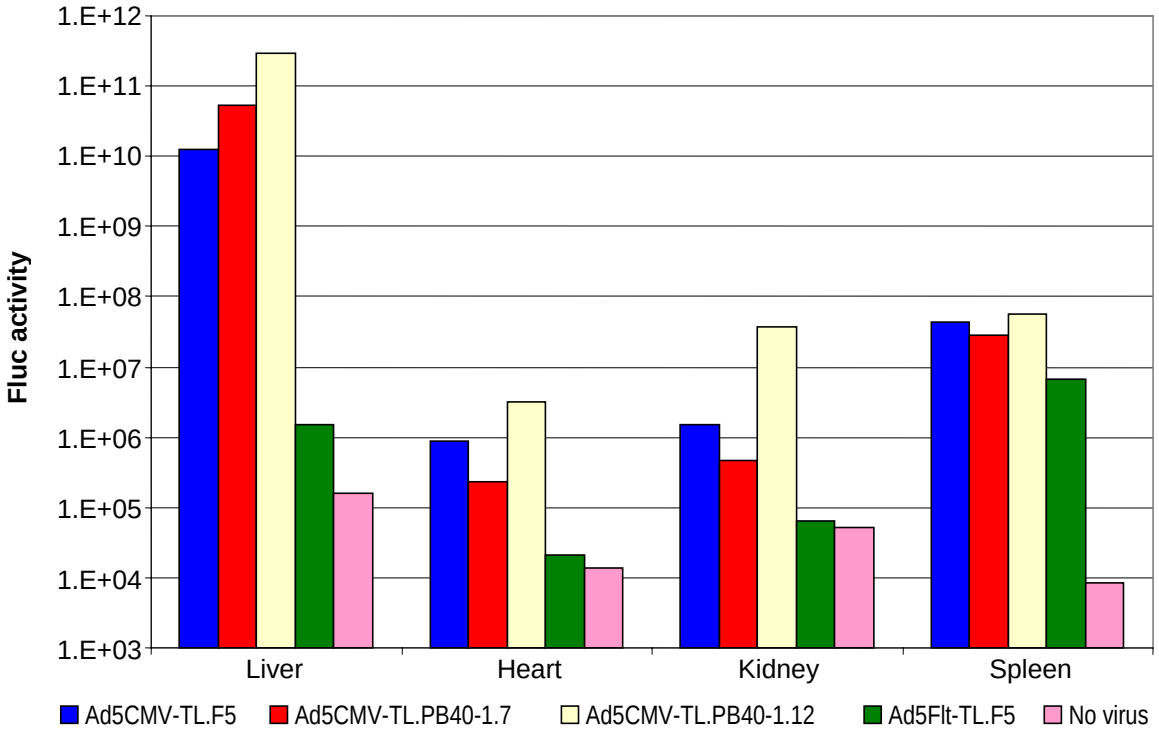


Figure 14. Fluc activity in isolated organs of the Ad vector-injected mice. The levels of the vector-expresses Fluc in each of the four tested organs is shown in relative light unit per entire organ. “No virus” data points are the readings of Fluc in organs collected from mice injected with PBS.

The higher sensitivity of this assay (compared to the whole body imaging) provided us with the measurements of Fluc activity in tumors, which were compared to bioluminescence registered in the livers (Fig 15). This comparison further confirmed that the capsid modification of Ad5CMV-TL.PB40-1.7 and Ad5CMV-TL.PB40-1.7 has not made these vectors tumor-specific: both the absolute levels of reporter expression directed by these viruses in tumors, and the ratio of these activity to that detected in the livers was essentially the same as seen for the control vector, Ad5CMV-TL.F5.

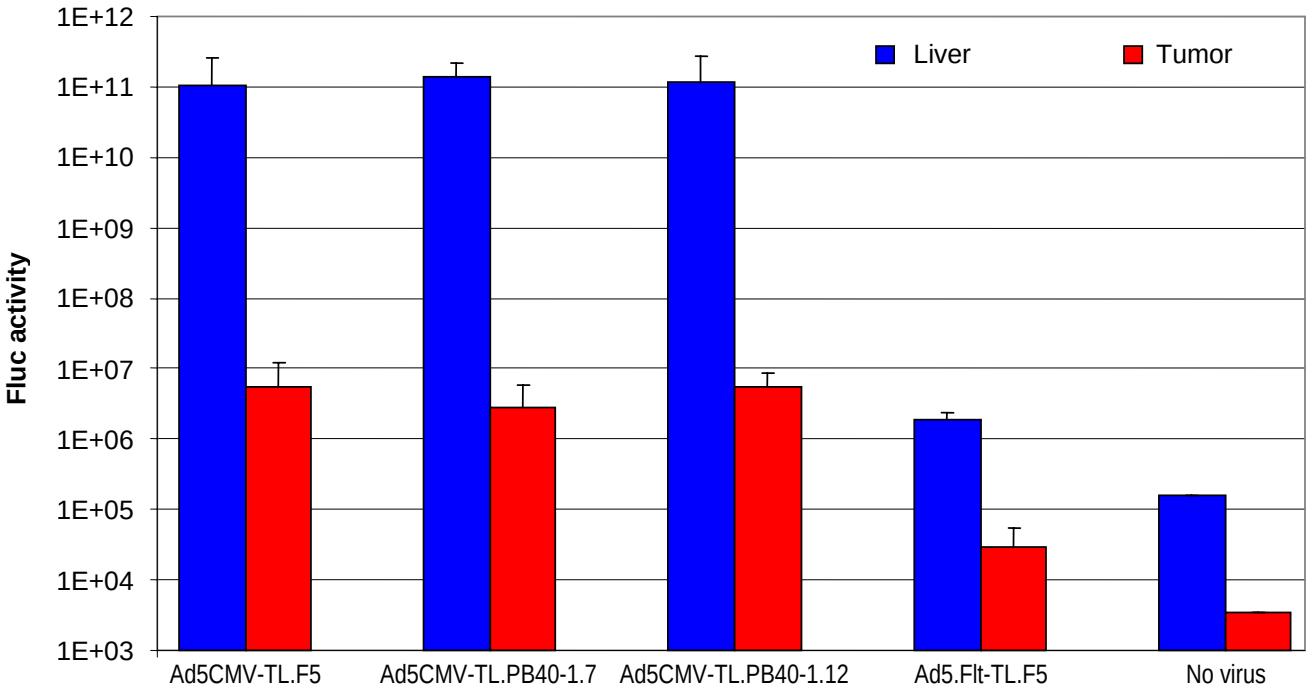


Figure 15. Comparison of Fluc activity levels in tumors and livers of Ad-treated mice. Reporter activity measured in tissue lysates is shown in relative light units per liver or tumor. "No virus" data points are the readings of Fluc in organs collected from mice injected with PBS. Error bars show standard deviations of data points within a cohort of mice injected with a given Ad vector.

Next, tissue homogenates were used as templates for quantitative PCR (qPCR) to assess the spread of Ad particles by detecting viral genomes. For this, aliquots of the tissue homogenates (above) were lyzed using SDS and proteinase K. The residual proteinase K activity was heat-inactivated, and the samples were diluted 1:100 in TE buffer. (This simple sample preparation technique has been chosen over the more sophisticated purification of total DNA from the tissue samples because, due to its simplicity, the sample-to-sample variations in DNA yields were minimal, thus allowing for accurate determination of the Ad copy number not only in the analyzed aliquots of lysates, but, most importantly, the accurate extrapolation of these data to entire organ or tumor.) Aliquots of these diluted lysates were then used as templates for qPCR with the primers (forward - TGGCTTCGGGTTCTATGTAAACTC, reverse - TTCTGCGGTGGTGGATGTTA) and probe (TTCATGCGCCGCTGCCCTG) specific for the E4 region of Ad5 genome, which had been designed and synthesized using the Applied Biosystems' proprietary Major Groove Binding™ technology.

These qPCR assays produced the results in agreement with the levels of Fluc activities in the same tested samples. In essence, no preferential accumulation of the fiber-modified vectors within the tumor samples was seen (Fig. 16): the copy numbers of modified Ad vector

genomes in the tumors were equal or lower than the copy numbers for the control Ad vector genomes (Fig. 16). Overall biodistribution patterns of the fiber-modified Ad vectors were virtually indistinguishable from that of the control Ad containing unmodified fibers (Fig. 16B). Expectedly, virtually the same distribution pattern was documented for Ad5Flt-TL.F5, thus suggesting that the distinct reporter expression profile seen for this vector (Fig. 14 and 15) was due to silencing of Flt promoter in no-target cells.

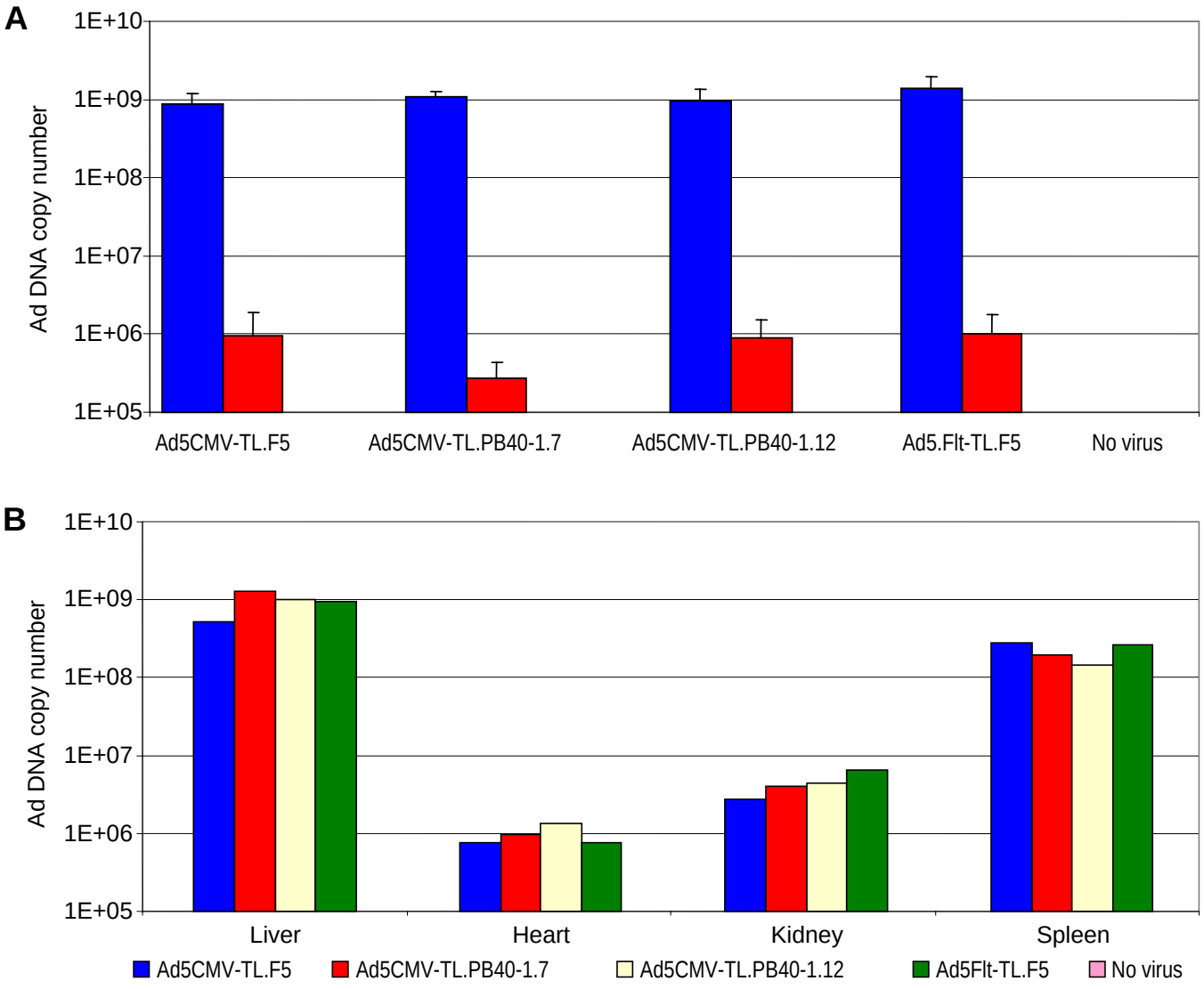


Figure 16. Biodistribution of Ad particles in tumor-bearing animals. A. Comparison of the amounts of Ad virions detected in the livers and tumors. B. Ad vector spread among tested organs. Ad virions were detected in the tissue lysates using qPCR as described in the text. The bars show the copy number of a given Ad vector per entire organ or tumor.

KEY RESEARCH ACCOMPLISHMENTS:

- A panel of thirty-six plasmid vectors encoding the fiber-ligand fusion proteins has been generated.
- Thirty-six candidate fiber-ligand proteins have been transiently expressed in cultured human cells and their ability to self-trimerize has been tested by Western blotting. Lead fiber-ligand constructs have been identified.
- The previously reported (2) specificity of the lead peptide ligand, *GRRAGGS*, for IL-11Rα has been tested. Towards this end, a test cell line stably expressing IL-11Rα and a recombinant protein "probe" displaying IL-11 have been generated. Using these new reagents, we have been able to prove that *GRRAGGS* does not bind IL-11Rα.
- The most promising fiber-peptide combinations identified in Task 1 of the project have been tested in a fiber trans-complementation assay that has identified those fiber candidates that had the highest probability of targeting in the context of a complete Ad particle.
- Recombinant genomes of six Ad vectors containing both the reporter gene cassettes and the genes encoding the most promising modified fiber proteins have been constructed. Two similarly structured Ad genomes have been made to generate the control, non-targeted Ad vectors.
- A panel of eight recombinant Ad vectors expressing a dual-function transgene has been generated. The viruses have been amplified to the scale necessary for subsequent *in vivo* studies. The identities of these vectors have been confirmed by Western blotting and genome sequencing.
- A recombinant retrovirus vector expressing a dual-modality reporter gene (*Renilla* luciferase and green fluorescent protein) has been designed and produced in a pan-tropic configuration.
- Dual reporter-expressing poly- and monoclonal derivatives of the target cell line, LNCaP, have been produced to facilitate the subsequent *in vivo* evaluation of the designed Ad vectors. The expression of the dual reporter in these lines, and their tumorigenicity has been confirmed and found adequate for the *in vivo* studies.
- Target xenografts have been established in nude mice and used to test a panel of the designed Ad vectors.
- Biodistribution of Ad vectors in these animals has been studied using three methods: whole body bioluminescent imaging, *in vitro* analysis of Fluc expression in isolated organs, qPCR detection of Ad particles.
- The results of these assays have unambiguously demonstrated that none of the developed Ad vectors has the expected capacity of tumor-specific gene delivery.

REPORTABLE OUTCOMES:

A manuscript describing our findings is presently in preparation.

CONCLUSIONS:

Despite the fact that our efforts in designing recombinant fibers containing tumor targeting peptides and development, rescue and manufacturing of Ad vectors incorporating these proteins have largely succeeded, none of the developed vectors has been able to deliver its gene payload to target tumors. Therefore, the central concept of this project has not been proved true. The potential reasons for this unfortunate outcome are reviewed below.

This is not the first time when peptides identified by phage biopanning failed to function as targeting ligands upon genetic incorporation into Ad fiber protein (23). The generally accepted interpretation of such failures is that when transferred from the context of one carrier protein (phage's pIII or pVIII) into another protein molecule (Ad fiber), these poorly structured peptides loose the configuration necessary for successful binding to the desired target. "Constraining" of these peptides by flanking them with cysteine residues that is frequently used in the design of phage display libraries cannot, unfortunately, be used in the context of Ad fiber. This is because of the fact that the Ad fibers translocate to the cell's nucleous immediately after their biosynthesis and, as a result, never enter the endoplasmic reticulum where disulfide isomerases form disulfide bonds in proteins. Because of these reasons, the peptides, once believed to be the ligands of choice for Ad tropism modification, are no longer viewed as promising ligands for Ad. Since the submission of this proposal (April, 2003) there has been a conceptual shift in the field of Ad targeting from using peptide ligands towards the artificial tailor-designed proteins often referred as "antibody mimics" (9). These new types of protein molecules are fully compatible with Ad protein biosynthesis and intracellular trafficking. Our laboratory has been very successful in developing this new avenue and has shown that the ligands representing one such new class of molecules, the so-called affibodies, can be used to design fully-functional Ad vectors targeted to a major molecular marker of human tumors, the Her2 (7). We could not, however, employ this new strategy in the context of this proposal, because due to the novelty of the affibody technology it has not yet produced any ligands that would be suitable for targeting tumor vasculature. We continue to be very interested in testing the central hypothesis of this project and will certainly do so as soon as we have the necessary targeting molecules in hand. Of note, we are currently collaborating with Dr. Andreas Pluekthun (University of Zurich, Switzerland) on developing designed ankyrin repeat protein ligands (DARPins) specific for Ad fiber knob domain, which will be used as the virus-binding components of bi-specific targeting protein adapters. For instance, such bi-specific proteins could be designed to contain single-chain VEGF (3), which would make them suitable for targeting Ad vectors to neovasculature of tumors.

Lastly, we cannot rule out a possibility that the peptide ligands that we used in this study are the artifacts of the original *in vivo* phage biopanning studies. While both ligand selection studies in which these molecules were identified have been done in the laboratories that are highly regarded in the field, there are two considerations that make us doubt their targeting potential. First, except for one additional paper (24) there has been virtually no spin-off to those studies, which is somewhat alarming considering the magnitude of the originally reported findings and the potential usefulness of the identified peptides for both the basic and the applied research. Second, it was greatly disappointing to find out that, contrary to reported

binding of the *GRRAGGS* peptide to IL-11Rα, no such binding has been seen in our own experiments, which we tend to trust fully.

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