AD_____

Award Number: DAMD17-02-1-0143

TITLE: Development of Gutless Adenoviral Vectors Encoding Anti Angiogenic Proteins for Therapy of Prostate Cancer

PRINCIPAL INVESTIGATOR: Calvin J. Kuo, M.D., Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

REPORT DATE: February 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2004	Annual (15 Jan 2003 - 14 Jan 2004)

4. TITLE AND SUBTITLE
Development of Gutless Adenoviral Vectors Encoding Anti Angiogenic Proteins for Therapy of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0143

6. AUTHOR(S)
Calvin J. Kuo, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305-5401

E-Mail: cjkuo@stanford.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The current work describes the development of technologies for long-term viral expression of soluble VEGF receptor anti-angiogenic proteins for therapeutic use in prostate cancer. Two technologies are described, "Gutless" adenoviruses and Adeno-associated viruses (AAV). Gutless adenoviruses have been reported to be capable of > 1 year transgene expression but their large-scale production is severely limited by helper virus and replication-competent adenovirus contamination. Several approaches for generation for pure gutless adenoviruses expressing the anti-angiogenic soluble Flk-1 VEGF receptor, towards achieving therapeutic responses which are more durable than conventional adenoviruses: (1) generation of a gutless adenoviral production method using I-SceI-dependent packaging signal deleting and viral genome recombination to inactivate helper virus, (2) generation of completion cell lines for gutless vectors using episomal or stable expression of helper genomes. Additionally, the use of adeno-associated viruses encoding either conventional soluble VEGF receptors or new variants with superior pharmacokinetic properties is described.

14. SUBJECT TERMS
Prostate cancer, angiogenesis, gene therapy, agenovirys

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Development of Gutless Adenoviral Vectors Encoding Anti-angiogenic Proteins for Therapy of Prostate Cancer

A promising new treatment modality for cancer is represented by anti-angiogenic therapy. In prior work we have developed adenoviral vectors encoding the soluble ligand-binding ectodomains of the Flk-1 and Flt-1 VEGF (Vascular Endothelial Growth Factor) receptors which sequester VEGF and exert systemic anti-angiogenic and anti-tumor effects. Single i/v injections of these adenoviruses produce broad-spectrum suppression (>80%) of tumor growth in a variety of tumor models including human LNCaP prostatic carcinoma orthotopically implanted into SCID mice and TRAMP transgenic mice which develop spontaneous prostatic adenocarcinoma with high penetrance. The duration of expression and tumor suppression from soluble VEGF receptor adenoviruses is limited to 3-6 weeks in immunocompetent hosts due to anti-viral immunity. However, repeat injections in SCID animals is accompanied by repeat expression and therapeutic response.

Two different viral vector systems have been described to produce long-term gene expression: "Gutless" adenoviruses and adeno-associated viruses (AAVs), both of which have been reported to be capable of > 1 year transgene expression. The production of gutless adenoviruses has been hampered by contamination with helper virus and replication-competent adenovirus, which can generate immune responses. We are developing several novel approaches for generation of pure gutless adenoviruses expressing the soluble Flk-1 VEGF receptor, hypothesizing that they will produce more durable therapeutic responses than conventional adenoviruses. As described within, these approaches include (1) generation of a gutless adenoviral production method using I-SceI-dependent packaging signal deletion and viral genome recombination to inactivate helper virus, (2) generation of complementation cell lines for gutless vectors using episomal or stable expression of helper genomes and (3) cloning of the soluble Flk1 VEGF receptor cDNA into gutless vectors and evaulation in murine models of prostate cancer. As an alternative approach, we are also pursuing AAV expression of soluble VEGF receptors, using the soluble Flk1 VEGF receptor as well as a recently-described VEGF Trap with extremely long serum half-life.

Body

Our progress on the aims of this grant are detailed below:

Task 1: Development of a gutless adenoviral production method using I-SceI-dependent packaging signal deletion and viral genome recombination to inactivate helper virus, Months 1-18

A. Molecular cloning of the I-SceI-inactivatable helper virus: *completed* (see last year's report)

B. Molecular cloning of recombination-inactivatable helper virus: *completed* (see last year's report)

C. Generation of helper viruses: *completed* (see last year's report)

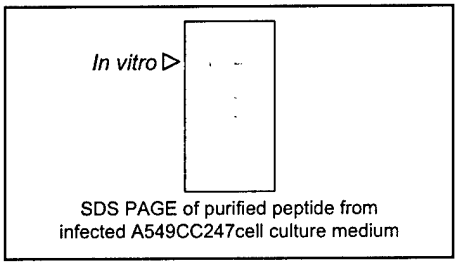
D. Establishment of E1 complementation cell lines with and without I-SceI

Our new E1-complementation cell line, A549CC247, is now routinely used in the lab for the production of Ad5-derived recombinant vectors. During a short trial period, viruses were produced in parallel on 293 cells and the new cell line to make sure the latter reproducibly supported the production of good quality research-grade virus batches using techniques which we have previously described (Becker et al., 2002; Chartier et al., 1996; Imler et al., 1996; Kuo et al., 2001). This line has now been used to produce 7 different viral constructs. A comparison of virus yield using the new A549CC247 versus the conventional 293 line is depicted below and the A549CC247 was superior or equivalent in all cases.

	A549CC247	293
AdKDR/hFc	1.2E+11 PFU/ml	3.4E+10 PFU/ml
AdHA/Flt/His	1.4E+10 PFU/ml	1.7E+10 PFU/ml
AdmB2/GFP	1.8E+10 PFU/ml	3.8E+9 PFU/ml

We confirmed the absence of RCA in the first virus batches produced on A549 cells using the supernatant rescue assay (Dion et al., 1996) that allowed us in the past to detect RCA in about 50% of our 293-produced virus batches.

All the viruses produced on A549CC247 were of course shown to express their transgene both *in vitro* and *in vivo*, as illustrated in the figure 1 for AdKDR/Fc.



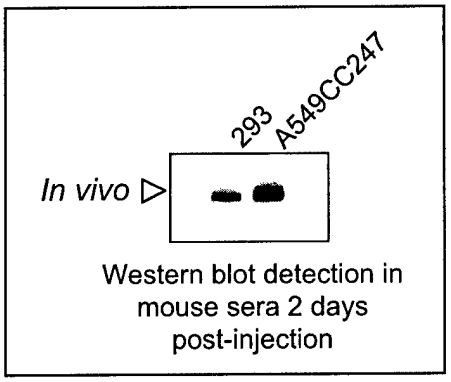


Figure 1: expression of KDR/Fc from A549CC247-produced AdKDR/Fc in tissue culture (left panel) and in mouse serum (right panel).

An I-Sce-I-expressing 293 cell line was established (see last report), but the introduction of an I-SceI expression cassette into A549CC247 was not pursued after we showed that the low level I-SceI-mediated packaging signal excision in the 293-I-SceI cells did not support efficient helper clearance.

However, the A549CC247 cell line which potently supported viral production without contamination with replication-competent adenovirus (see above) was further characterized for the status of its E1-containing DNA copies. Total DNA was extracted from the cells and several other E1-complementing clones and analyzed by Southern blot with an E1-specific DNA probe. The result is presented in Figure 2. The expected 2.3 kb band upon HindIII digestion was detected as well as additional bands that suggested that most of the transfected DNA was integrated in the genome rather than episomally maintained. This confirmation of E1 locus integration indicates that the support of viral replication by this cell line is E1-dependent.

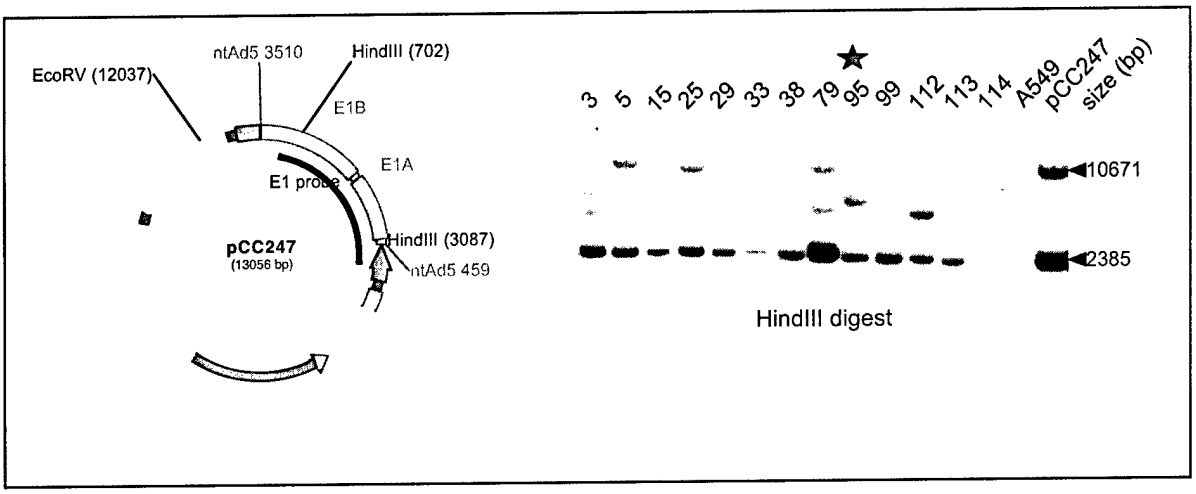


Figure 2: Southern blot analysis of A549-CC247 clones with an E1-specific DNA probe.

E. Evaluation of packaging signal excision in the I-SceI-expressing E1 complementation cell line

I-SceI mediated packaging signal excision was shown last year in our 293-I-SceI cells (see last report).

F. Evaluation of helper genome recombination in the E1 cell line without I-SceI

Efficient 'postreplicative overlap recombination' was shown to take place between the ends of our specially designed GHCC243 helper virus (see last report).

G. Evaluation of helper virus inactivation in (E) and (F)

Neither I-SceI expression nor recombination supported the clearance of the helper viruses to a detectable level, leading us to conclude this first part of the work and to concentrate this year efforts towards the establishment of a cell line expressing all adenovirus functions for GL production. As one more idea to improve this process we are investigating the improvement of I-SceI expression by using lentivirus or retrovirus to generate stable I-SceI cell lines as opposed to conventional tranfection.

Conclusion of task 1: The tools towards the development of novel helper-dependent systems for the production of gutless viruses were generated. The I-SceI- and recombination-inactivatable helper viruses were shown to work very efficiently although neither mechanism has yet attained an inactivation level high enough to allow quantitative clearance upon gutless production. This could be improved by higher-expressing I-SceI cell lines as described above. The new E1 complementation cell line that was established in order to prevent any risk of recombination with the helper viruses has turned out to be extremely valuable tool for the laboratory and some effort was dedicated to the characterization and banking of this new cell line which appears to be superior to conventional 293 cells.

Task 2: Generation of complementation cell lines for gutless vectors, Months 1-18

A. Molecular cloning of conditionally inactive helper genomes: *completed (see last year's report).*

B. Evaluation of the I-SceI- and cre-dependent activation of helper genomes in transient transfection

A number of AdCC296 rescue experiments were performed in order to verify that the silent genome that was to be introduced in the complementation cell line could be reverted to a functional virus by I-SceI and Cre recombinase. Virus rescue was tested at both the DNA and virion levels. 293 cells were transiently co-transfected with pCC296 + pI-SceI + pMC-Cre. DNA and virus were harvested 72 hours later. PCC295, the non-floxed control version of pCC296 was included in all the experiments. DNA was analyzed by Southern blot using an E1-specific DNA probe after treatment with DpnI to eliminate the input plasmid DNA. The analysis strategy and result are presented in Figure 3.

The 3973 bp fragment specific for the replicating reconstituted E1 region of AdCC296 was detected only after co-transfection with all 3 plasmids. This showed that I-SceI-mediated excision of the viral genome along with Cre-mediated flip of the E1 and E2 sequences could efficiently rescue adenovirus DNA replication.

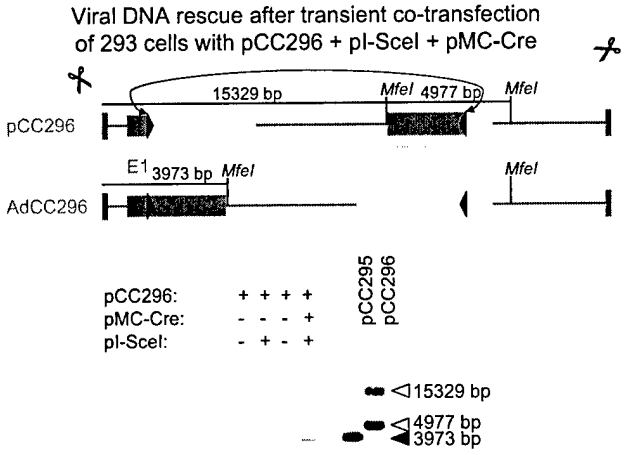


Figure 3: Diagram and result of the Southern blot analysis of viral DNA after transient co-transfection of 293 cells with pCC296 + pI-SceI + pMC-Cre. The scissors represent the cutting by I-SceI. The long double-head arrow represents the Cre-mediated flip of the E1 and E2 sequences.

To confirm actual production of virus particles upon activation of pCC296 by I-SceI and Cre, the medium harvested 72 hours post-co-transfection was used to infect A549 cells. Because a GFP expression

cassette was cloned in the E3 region of pCC296, the infection of A549 cells with the potentially rescued AdCC296 would result in the production of fluorescence by these cells. The A549 cells were monitored for GFP fluorescence 48 hours post-infection. On this basis, fluorescence as a surrogate for rescued viral particles was observed only after infection with the product of the co-transfection with pCC296 + I-SceI + Cre, but not if products from transfections missing any one of these three plasmids were present. This likely represents a >200 fold-induction with the combination pCC296 + I-SceI + Cre versus other combinations (Figure 4). This showed that complete adenoviral functions could be successfully rescued by inducing the excision of the viral genome and the flip of the E1 and E2 sequences of pCC296, validating the use of pCC291 as a non-packageable helper virus for the production of gutless vectors.

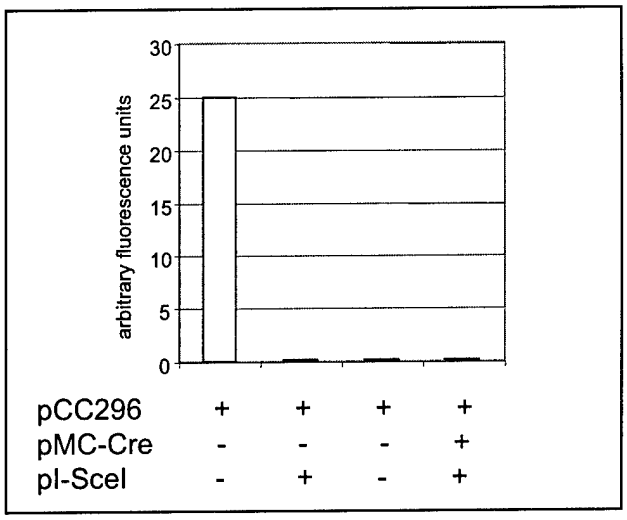


Figure 4. Cre- and I-SceI-dependent rescue of virus production by I-SceI-mediated excision of the viral genome and Cre-mediated flip of the E1 and E2 sequences of pCC296.

C. Evaluation of transient rescue of gutless virus production by helper genomes

PCC179 was constructed that contains the genome of a LacZ-expressing gutless vector. The rescue of GLCC179 was attempted several times by transient co-transfection of 293 cells with pCC179 + pCC296 + pI-SceI + pMC-Cre. We have not yet been successful at the transduction of LacZ activity to A549 cells infected with the product of the co-transfection, although efforts are ongoing. If unsuccessful, this may be secondary to either AdCC296 not providing the GL vector with the viral functions in *trans* or the efficiency of rescue of the helper functions not being high enough to *trans*-complement for the GL vector in this transient assay. As an alternative approach, the establishment of stable cell lines containing pCC291 was pursued in order to address this issue (see below).

D. Establishment of cell lines inducibly expressing I-SceI and cre

A gutless vector that would express both I-SceI and Cre in addition to the transgene has been designed. The replacement plasmid has been assembled and named pCC298 (Figure 5). It contains a pyTK-Cre cassette and a CMV-I-SceI cassette in addition to the Gas5-LacZ cassette. The corresponding GL should be useful for the rapid screening of complementation cell line candidates.

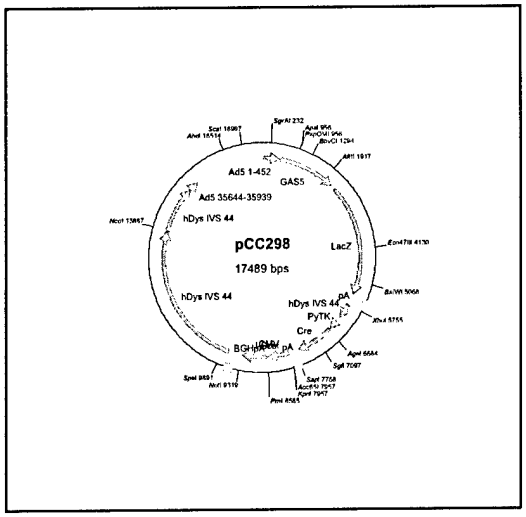


Figure 5: Replacement plasmid for the construction of a gutless vector expressing LacZ, I-SceI and Cre recombinase.

E. Establishment of cell lines containing inactivated helper genomes

PCC291 and 296 (packaging-proficient version of pCC291) both contain a neomycin resistance gene expression cassette for the positive selection of stably transfected clones. Analysis of geneticin-resistant 291 and 296 single clones (>100) and a small number of 291 and 296 pools of clones (6 of each) are undergoing analysis by Southern blot using an E1-specific DNA probe.

F. Production of gutless adenovirus upon induction of I-SceI and cre and rescue of helper functions: *in progress.*

Task 3: Evaluation of gutless adenoviruses expressing soluble Flk1 VEGF receptor cDNA in murine models of prostate cancer, Months 19-36

A. Cloning of Flk-1/Fc cDNA into gutless vectors by homologous recombination: *completed (see last year's report).*

B. Comparison of pharmacokinetics of gutless and conventional Flk1-Fc adenoviruses

It would be expected that conventional GL preparations, with helper virus contamination, would exhibit poor long-term gene expression. This was confirmed as a batch of GLCC151 (GLFlk-1/Fc) was produced using the conventional helper virus system (Parks et al, 1996). Transgene production and duration of expression were assayed with this conventional GL Flk-1/Fc compared to the corresponding first generation Ad vector after tail vein injection to C57/Bl6 mice. The results were reported on the graph below.

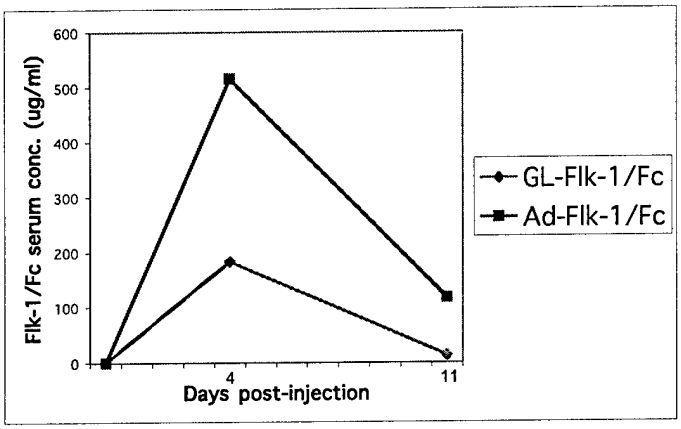


Figure 6. Comparison of a conventionally-produced, helper-contaminated GL versus 1st generation adenovirus. This transient expression would not be expected with a helper-free gutless virus as will be produced by this project.

GLCC151 drove lower level of expression of Flk-1/Fc. This could be due to a slight over-estimation of the GL vector titer. As expected, this initial experiment indicated that the conventionally produced GL vector did not support a longer lasting expression of the transgene. Indeed, Flk-1/Fc levels returned to low/undetectable 12 days post-injection for both the first generation Ad vector and the GL vector. The batch of GLCC151 was carefully characterized and found to be contaminated with both helper virus and RCA that could be responsible for triggering an immune response against the GL-infected cells and the rapid clearance of these cells. This expected result clearly underlines the need for a helper-free GL production system, as is one of the major aims of the current grant.

C. Evaluation of gutless Flk1-Fc adenoviruses in orthotopic LNCaP mice: *in progress*

D. Evaluation of gutless Flk1-Fc adenoviruses in TRAMP transgenic mice: *in progress.*

Task 4: Evaluation of pseudotyped AAV vectors encoding Flk1-Fc as alternatives to gutless adenoviruses (months 18-36).

As described, the conventional production of gutless adenoviruses can be complicated by helper virus contamination and poor scalability. In the current proposal, we are pursuing several approaches to circumvent these obstacles using novel strategies for helper virus inactivation as well as generation of helper-independent systems (i.e. complementation cell lines). As a parallel approach we have been exploring the use of adeno-associated viruses (AAV) as an alternative viral system which could also confer durable and high-level expression.

Novel AAV technologies have demonstrated the potential to overcome prior obstacles to the widespread use of AAV, namely, low expression levels and large-scale production. Most significantly, capsid proteins from other AAV strains (i.e. AAV1, AAV5) have been used to pseudotype the conventional AAV2 virus strain, resulting in increased infectivity to muscle (AAV1) and liver (AAV5). The use of transient transfection to provide helper functions (as opposed to simultaneous adenoviral infection), as well as the replacement of CsCl gradients with iodixanol gradient/anion exchange chromatography protocols have both dramatically improved production yields of AAV (Zolotukhin et al., 2002).

A novel soluble VEGF receptor, "VEGF Trap" has recently been described by Regeneron which has dramatically prolonged serum half-life after systemic administration relative to conventional soluble

VEGF receptors. This may be particularly advantageous when combined with a persistent virus strategy (i.e. AAV or gutless adenovirus). We have produced a cDNA representing the VEGF Trap has been assembled containing Ig domain 2 of Flt1 and Ig domain 3 of KDR along with an immunoglobulin Fc fragment. This VEGF Trap cDNA has been cloned into the E1 locus of conventional adenovirus to establish functional activity of the transgene in VEGF corneal micropocket angiogenesis assays. Additionally, together with our long-time collaborator Richard Mulligan at Harvard, the VEGF Trap cDNA has been inserted into an ITR-flanked AAV vector.

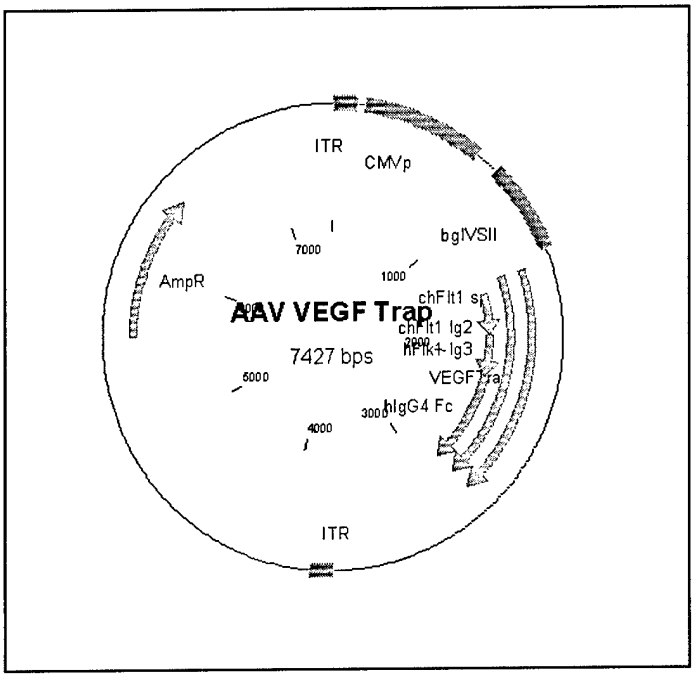


Figure 7.

The AAV VEGF Trap plasmid is currently being evaluated for protein secretion in transient transfection. If this looks positive then the secreted product will be evaluated for activity in vitro in inhibition of endothelial proliferation. A similar strategy will be pursued for AAV Flk1-Fc. Subsequently, pseudotyped virus will be produced with AAV1 or AAV5 capsid proteins (Zolotukhin et al., 2002) and the pharmacokinetics, anti-tumor and anti-angiogenic efficacy of AAV1 and AAV5 pseudotyped AAVs expressing soluble Flk1-Fc or VEGF Trap will be evaluated using ELISA, prostate xenograft models, and VEGF corneal micropocket assays. It will be interesting to compare the duration of expression of AAVs and Ads expressing Flk1-Fc versus VEGF Trap given the prolonged circulating half-life of VEGF Trap protein. Additionally, if we are able to validate the VEGF Trap cDNA this could be inserted into gutless vectors as well.

Key research accomplishments:

1. Construction and analysis of an novel E1 complementation cell line for adenovirus production (A549CC247) bearing only minimal E1 regions and demonstration that this line supports adenoviral production to levels exceeding that of 293 cells.
2. Construction and analysis of a matched vector set lacking homology with the E1 locus integrated into A549CC247.
3. Demonstration of absence of replication-competent adenovirus in preps made using (1) and (2) described above.

4. Construction of recombination-inactivatable helper viruses bearing duplicated packaging signals
5. Construction of I-SceI-inactivatable helper virus genomes
6. Construction of I-SceI stably-transfected 293 cell lines
7. Evaluation of recombination and I-SceI-mediated helper virus clearance
8. Construction of a packaging-competent, Ψ-deleted, I-SceI-flanked and E1-E2-flipped adenovirus genome
9. Construction of a replacement plasmid for the construction of a gutless vector expressing LacZ, I-SceI and Cre recombinase.
10. Construction of a cDNA encoding VEGF Trap and insertion into adenovirus and an AAV shuttle vector.

Reportable outcomes:

1. Construction of E1 complementation cell line for adenovirus prodcution bearing only minimal E1 regions and demonstration that this line supports adenoviral production to levels exceeding that of 293 cells.
2. Attenuation of helper virus encapsidation by post-replicative overlap recombination.

Conclusions:

We have taken several approaches towards achieving durable VEGF receptor expression via gene therapy, with the ultimate goal of long-term anti-angiogenic therapy of cancer. In the first approach, we have developed methodology to produce helper-free gutless adenoviruses. A well-characterized E1 complementation cell line has been derived which generates superior yields of several different adenoviruses to levels equal to or exceeding that of conventional 293 cells. When used with a matched vector, this line lacks overlap homology with the plasmid E1 locus and, resulting in undetectable contamination with replication-competent adenovirus. As described last year we have also demonstrated that can we attenuate helper virus encapsidation after a single passage using helper viruses engineered to undergo post-replicative overlap recombination. Our experience with the recombination-mediated mechanism shows that a very high percentage of viral genome needs to be impaired in order for it to translate into viral clearance. We have also constructed a gutless vector that expresses both I-SceI and Cre in addition to the transgene in order to simplify the number of complementation functions necessary for gutless production in addition to helper viruses or complementation cell lines. The corresponding gutless virus will greatly facilitate the rapid screening of complementation cell line candidates. Finally, as a second approach we are exploring the utility of pseudotyped AAV vectors to provide long-term viral expression of soluble VEGF receptors. This approach is still in its early phases but will be used in conjunction with a novel soluble VEGF receptor with superior pharmacokinetics (VEGF Trap), which should improve expression duration of AAV, gutless adeno or conventional adenoviruses.

So What? Anti-angiogenic therapy is a potentially new therapeutic modality for prostate cancer, for which novel therapies have been lacking. The chronic treatment of cancer with anti-angiogenic agents raises both the necessity for long-term delivery systems and issues of economic cost. To address these issues, we are using virus technology, including gutted adenoviruses and adeno-associated virus, to achieve long-term and economic delivery of soluble VEGF receptors, which antagonize the dominant growth factor controlling the growth of tumor vasculature.

Additionally, such long-term delivery strategies could facilitate the scientific examination of tumor resistance mechanisms to VEGF antagonism.

References:

Becker, C.M., F.A. Farnebo, I. Iordanescu, D.J. Behonick, M.C. Shih, P. Dunning, R. Christofferson, R.C. Mulligan, G.A. Taylor, C.J. Kuo, and B.R. Zetter. 2002. Gene therapy of prostate cancer with the soluble vascular endothelial growth factor receptor Flk1. *Cancer Biol Ther.* 1:548-53.

Chartier, C., E. Degryse, M. Gantzer, A. Dieterle, A. Pavirani, and M. Mehtali. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. *J Virol.* 70:4805-10.

Dion, L.D., J. Fang, and R.I. Garver, Jr. 1996. Supernatant rescue assay vs. polymerase chain reaction for detection of wild type adenovirus-contaminating recombinant adenovirus stocks. *J Virol Methods.* 56:99-107.

Imler, J.L., C. Chartier, D. Dreyer, A. Dieterle, M. Sainte-Marie, T. Faure, A. Pavirani, and M. Mehtali. 1996. Novel complementation cell lines derived from human lung carcinoma A549 cells support the growth of E1-deleted adenovirus vectors. *Gene Ther.* 3:75-84.

Kuo, C.J., F. Farnebo, E.Y. Yu, R. Christofferson, R.A. Swearingen, R. Carter, H.A. von Recum, J. Yuan, J. Kamihara, E. Flynn, R. D'Amato, J. Folkman, and R.C. Mulligan. 2001. Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. *Proc Natl Acad Sci U S A.* 98:4605-10.

Zolotukhin, S., M. Potter, I. Zolotukhin, Y. Sakai, S. Loiler, T.J. Fraites, Jr., V.A. Chiodo, T. Phillipsberg, N. Muzyczka, W.W. Hauswirth, T.R. Flotte, B.J. Byrne, and R.O. Snyder. 2002. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods.* 28:158-67.