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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 of 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 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. Additionally, the use of adeno-associated viruses encoding either conventional soluble VEGF receptors or new variants with superior pharmacokinetic properties is described.				
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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 (Becker et al., 2002; Kuo et al., 2002; Kuo et al., 2001; Tseng et al., 2002). 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 (Parks et al., 1996; Zolotukhin et al., 2002). 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 evaluation 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

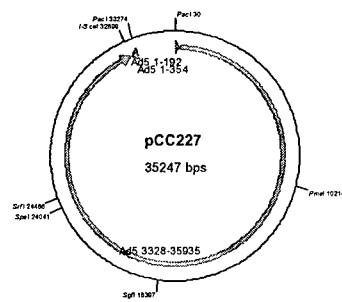
Our progress on the aims of this grant are detailed below. The major work during the past year occurred in Tasks 3 and 4 since Tasks 1 and 2 were largely accomplished over by the last progress report.

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

A plasmid containing the I-SceI-inactivatable helper virus genome has been constructed using homologous recombination in *E. coli* (Chartier, 1996). In the resulting pCC227 (Figure 1), the adenovirus packaging signal (Ψ) has been removed from its natural location at the left end of the genome and cloned after the right ITR. A second ITR has been added to the right end in order to allow viral DNA replication. The viral genome is flanked by two *PacI* restriction sites.

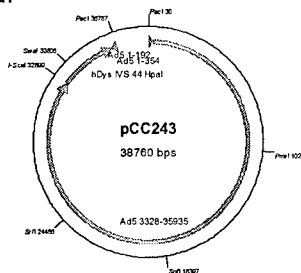
Figure 1:



B. Molecular cloning of recombination-inactivatable helper virus

A plasmid containing a recombination-inactivatable helper virus genome has been constructed. pCC243 (figure 2) presents the same characteristics than pCC227 but for a 3500 bp long DNA fragment (intron 44 of the human dystrophin gene) cloned in between the I-SceI site and the Ψ . This extra DNA fragment is used as a stuffer DNA in order to increase the size of the viral genome up to its packaging size limit. This way, the recombination-generated viral genome with duplicated Ψ (see figure 8 of the grant for putative mechanism) will become too large to be packaged.

Figure 2:



C. Generation of helper viruses

Viruses were rescued after transfection of 293 cells with *PacI*-restricted pCC227 and 243 DNAs. The corresponding viruses were named GHCC227 and 243, respectively, and medium scale batches were purified for each of them. Virus titers are presented in the table bellow.

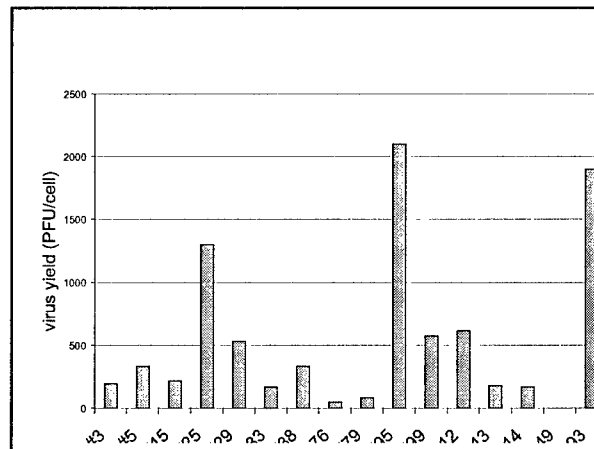
Virus	Titer (PFU/ml)
GHCC227	1.2X10E11
GHCC243	1X10E10

GHCC227 titer was comparable to the titers obtained for the first generation adenovirus vectors routinely produced in the lab. On the contrary, GHCC243 yield was at least 10 fold lower than the titer of an unmodified adenovirus vector. This was expected since GHCC243 was designed to be prone to rearrangements that would make it unable to be packaged. We were therefore encouraged by this first hint that recombination-mediated elimination of the helper virus could be used to get rid of a fraction of the helper viruses.

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

Ad5 E1 sequence encompassing nt 459-3509 was cloned into pREP4 (Invitrogen Corporation, Carlsbad, CA) downstream from the RSV promoter to obtain pCC247. In pCC247, the TK promoter drives the expression of the hygromycin resistance gene. A second plasmid named pCC248 was constructed where the TK promoter was replaced with the E1B promoter. The E1B promoter is inducible by one of the E1A gene products and could therefore help selecting clones that are not only resistant to the drug but also express the E1 transcription unit. The human lung carcinoma A549 cells (Smith et al., 1986) have been shown before to be able to stably express the E1 genes (Imler et al., 1996). We thus stably transfected A549 cells with pCC247 and pCC248 and more than a hundred hygromycin-resistant clones were obtained. The ability of the clones to support E1-deleted adenovirus vectors propagation was then evaluated after infection of the clones with a GFP-expressing adenovirus vector. Approximately 90% of the clones scored positive after the very first screening for AdGFP rescue (see figure 7 of the grant for an example) and at this stage no difference between the two types of plasmid could be noticed. The actual level of E1 complementation of all the GFP positive clones was then scored by an end point dilution titering method. The results obtained have been plotted on the graph below (Figure 3) for the best candidates.

Figure 3: Titering of AdGFP yield on Hygro^R pCC247/48-transfected A549 clones.



One of the clones (#95) allowed recovery of AdGFP to a level comparable or better than 293 cells. This clone had been transfected with pCC247 and has therefore been named A549CC247. A small bank of A549CC247 cells was then made and the cells were further evaluated for the production of a large-scale batch of a humanized version of our 1st generation AdFlk-1/Fc (Kuo et al., 2001) having the human Flk1

ectodomain (KDR) fused to a human IgG1 Fc fragment. At every step of this evaluation, A549CC247 cells performed as well or better than the 293 cells as illustrated in Figure 4.

Figure 4.

	293 cells	A549CC247 cells
Titer (PFU/ml)	3.4X10E10	1.2X10E11
Total PFUs	6.8X10E10	3.6X10E11
PFU/cell	314	720
VP:PFU ratio	108	61

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 5 for AdKDR/Fc, a fully humanized soluble KDR VEGFR ectodomain.

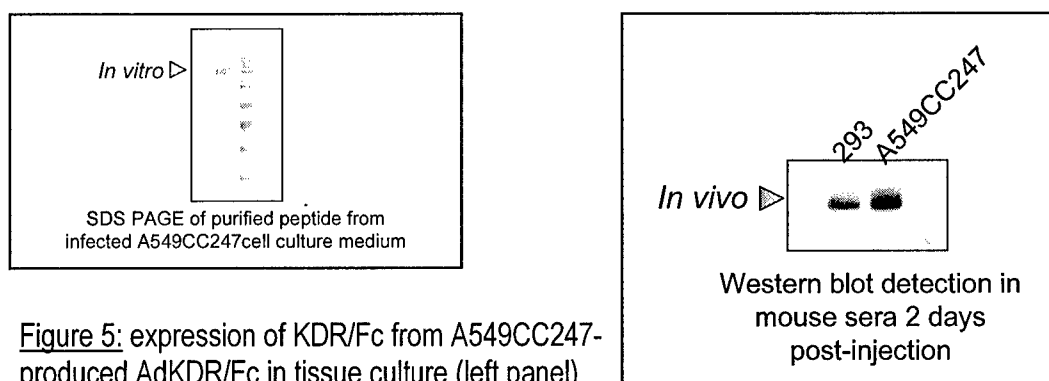
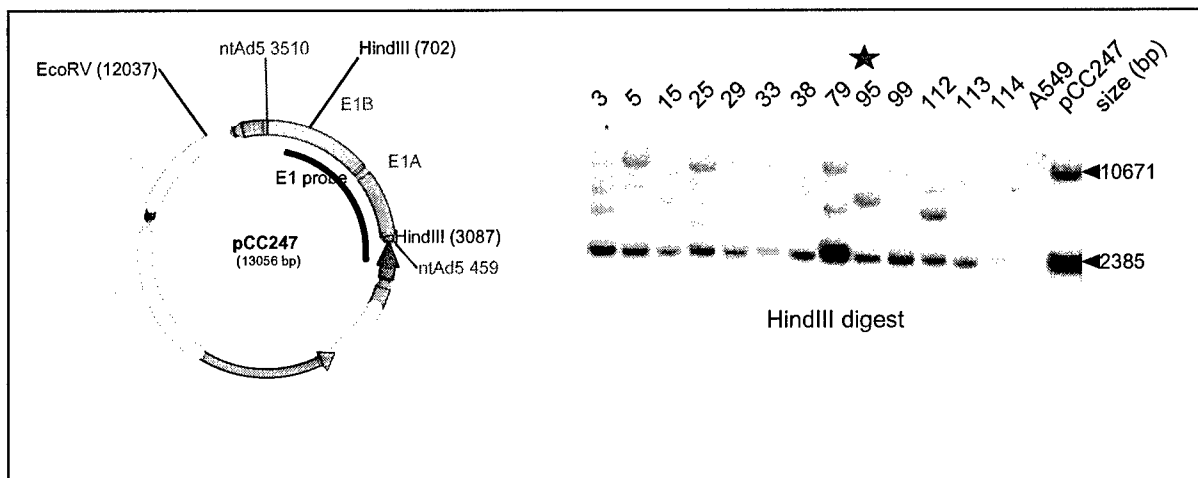


Figure 5: expression of KDR/Fc from A549CC247-produced AdKDR/Fc in tissue culture (left panel) and in mouse serum (right panel).

A549CC247 cell line which potentially 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 6. 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 6.



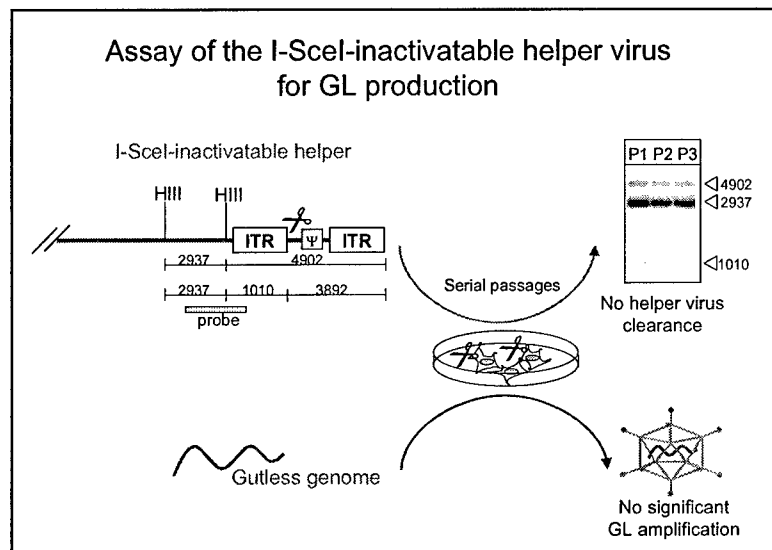
To confirm that the elimination of any sequence of homology between the adenoviral sequences in the cells and the adenoviral sequences in the vectors prevent RCA formation, RCA assays will be performed on material serially passaged on the A549CC247 cells.

In order to generate an I-Sce-I-expressing cell line, 293 cells were transfected with an I-Sce-I-expressing pcDNA3.1+-derived plasmid. One Geneticin^R clone was selected for its ability to allow virus rescue after transfection of a circular plasmid containing an I-Sce-I-flanked AdGFP genome (see original grant for illustration).

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

The I-Sce-I-expressing E1 complementation cells were infected with GHCC227 at a m.o.i. of 1 and serially passaged 3 times. Viral DNA was extracted and analyzed by southern blot for the appearance of a 1 kb fragment specific for the I-Sce-I-generated viral end. The fragment was only very faintly detectable at all 3 passages (Figure 7).

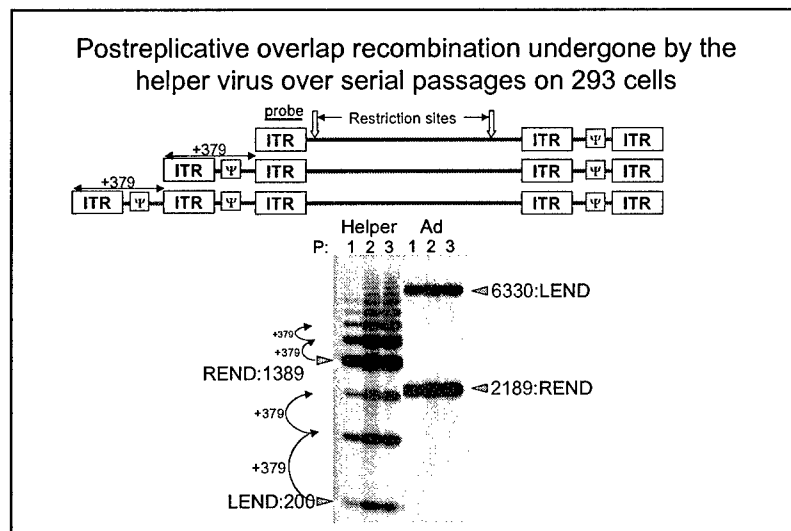
Figure 7.



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

GHCC227 was used to demonstrate the occurrence of recombination between the ITRs upon viral DNA replication. For this, 293 cells were infected with GHCC227 and the viral DNA was analyzed by Southern blot according to the schematic representation of Figure 8. the appearance of a ladder of DNA fragments of regularly increasing sizes above the 2 fragments specific for the virus left and right ends can only be explained by the phenomenon known as 'postreplicative overlap recombination'.

Figure 8

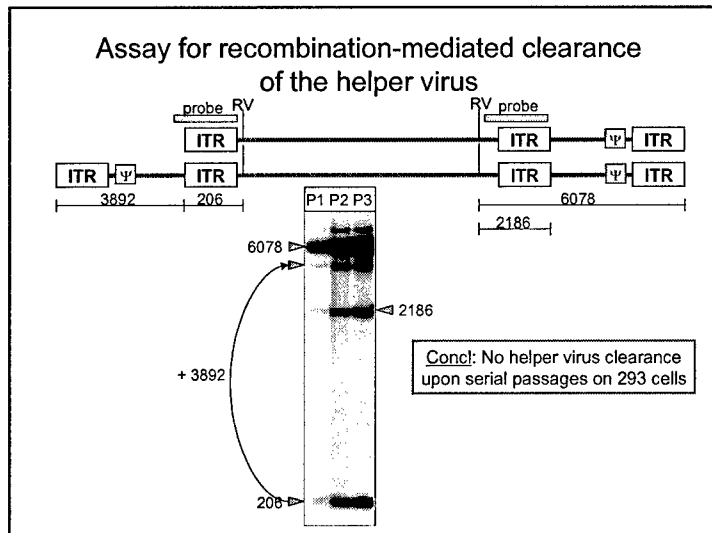


G. Evaluation of helper virus inactivation in (E) and (F)

The fact that the amount of total viral DNA detected in both the experiments described above did not decrease upon passaging Figures 7 and 8) as well as the fact that no diminution of CPE could be observed upon passaging suggest that 1) the I-SceI-mediated cut is very inefficient in our I-SceI-transfected cell line, and 2) the I-SceI-mediated excision of Ψ does not lead to clearance of the helper virus even upon repeated passages. The helper virus clearance was also evaluated using GHCC243 as

the helper virus. As shown in Figure 9, recombination was once again clearly demonstrated but it unfortunately did not induce any detectable virus clearance.

Figure 9:

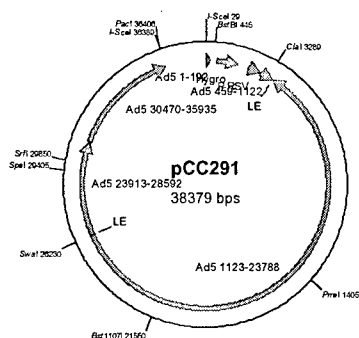


Task 2: Generation of complementation cell lines for gutless vectors, Months 1-18

A. Molecular cloning of conditionally-inactive helper genomes

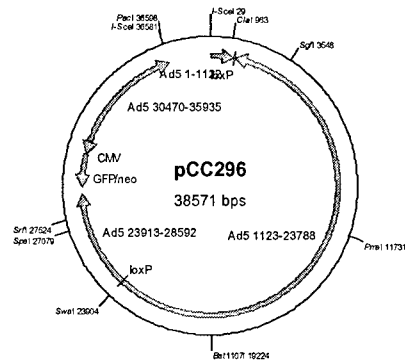
A Ψ -deleted, I-SceI-flanked and E1-E2-flipped adenovirus genome was cloned into a pBR-derived plasmid. In the resulting pCC291 (Figure 10), the E1A promoter whose enhancer overlaps with the packaging signal had to be replaced by a heterologous promoter. We choose the RSV promoter that had been used successfully in the new E1 complementation cell line. The selection marker in this plasmid is the hygromycin resistance gene which has been introduced upstream from E1. Production of GL-FIk1-Fc in the resulting cell line will be evaluated upon induction of I-SceI and cre.

Figure 10:



A packaging-competent version of this construct was also generated, which allows virus rescue and can therefore be used conveniently to test the system. The plasmid is pCC296 (Figure 11). It contains the wild-type E1A regulatory sequence and the selection/reporter gene (a GFP/neo fusion gene) was inserted in place of the E3 region.

Figure 11:



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

A. Cloning of Flk1-Fc cDNA into gutless vectors by homologous recombination

This has been accomplished by inserting our well-characterized Flk1-Fc soluble VEGF receptor cDNA (Kuo et al., 2001) into a gutless adenoviral backbone to generate pCC151 (Figure 10). Similarly to 1st generation adenovirus vectors, pCC151 was generated by homologous recombination in bacteria (Chartier et al., 1996) (Figure 11).

Figure 10:

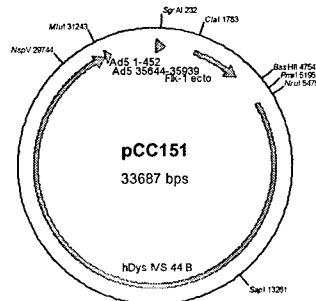
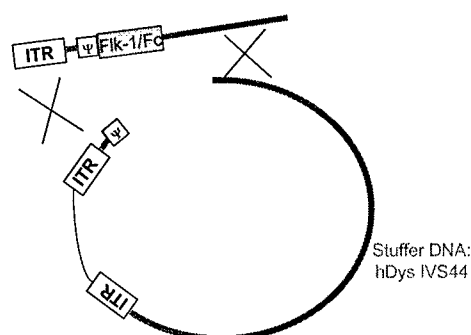


Figure 11:

Construction of a gutless adenovirus vector



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 + pl-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 12.

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.

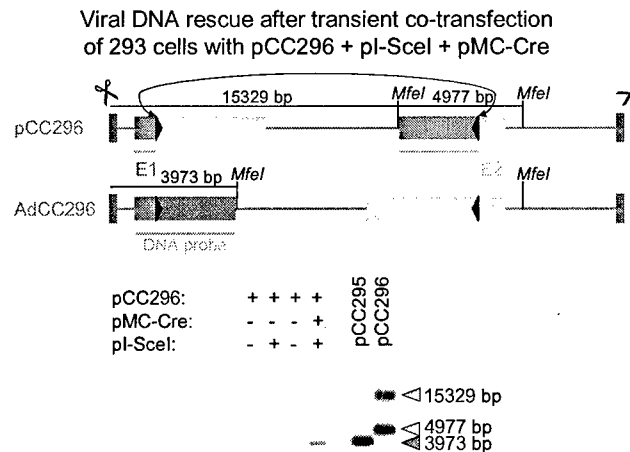


Figure 12: Diagram and result of the Southern blot analysis of viral DNA after transient co-transfection of 293 cells with pCC296 + pl-SceI + pMC-Cre. The scissors represent the cutting by I-SceI. The long double-head arrow represents the Cre-mediated flip of the E1 and E2 sequences.

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 13). 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.

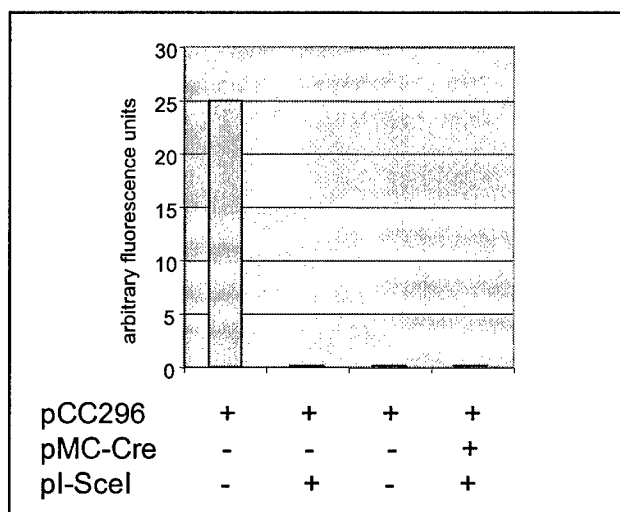


Figure 13. Cre- and I-SceI-dependent rescue of virus production by I-SceI-mediated excision of the viral genome and Cre-mediated flip of the E1 and E2 sequences of pCC296.

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 + pl-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.

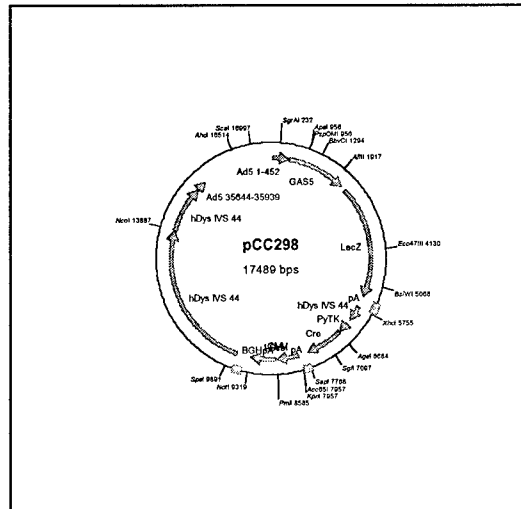


Figure 14: Replacement plasmid for the construction of a gutless vector expressing LacZ, I-SceI and Cre recombinase.

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*.

Overall, we have completed the construction of conditionally inactive helper genomes and demonstrated I-SceI-mediated excision of the viral genome along with Cre-mediated flip of the E1 and E2 sequences followed by efficient rescue of adenovirus DNA replication. Additionally, we demonstrated in the prior progress report 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. We continued to experience difficulty with stable transfection of PCC179 containing the genome of a LacZ-expressing gutless vector, possibly 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.

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

We had previously inserted Flk-1/Fc cDNA, encoding a soluble ectodomain of the Flk1 VEGF receptor into a gutless adenoviral vector to produce GLCC151 (GLFlk-1/Fc). Production of this virus using a conventional virus system. Not surprisingly, this conventionally produced GLFlk1-Fc virus did not support a longer lasting expression of the transgene. To circumvent the likely deleterious contamination of the GLFlk1-Fc with helper virus, we are currently attempting two approaches: (1) a helper-free GL

production system as in Task 2, and (2) an alternative approach of evaluation of AAV and alternative VEGF receptors with improved pharmacokinetics as in Task 4. These alternative approaches will allow the eventual assessment of long-lasting viral vectors in animal models of prostate cancer.

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

In this year, we have begun to explore the use of adeno-associated viruses (AAV) as an alternative viral system to obtain long-term expression of soluble VEGF receptors. Additionally, to further boost duration of expression, we have decided to combine AAV technology with a novel soluble VEGF receptor, "VEGF Trap" described by Regeneron, which has dramatically prolonged serum half-life after systemic administration relative to conventional soluble VEGF receptors.

A. Validation of VEGF Trap insert.

The VEGF Trap insert was cloned into the E1 region of adenovirus type 5 for functional validation in vivo. We developed a capture ELISA to document plasma expression of VEGF Trap following Ad VEGF Trap injection i.v. Accordingly, plasma levels of ~15 mg/ml were observed after single i.v. injection into immunocompetent C57Bl/6J mice, tapering thereafter. The dose response of VEGF inhibition by VEGF Trap was examined by progressive titration of Ad VEGF Trap (10^6 - 10^9 pfu) in a VEGF-dependent murine corneal micropocket model. This data indicated that Ad VEGF Trap doses of as little as 10^7 pfu, corresponding to circulating levels of ~80 ng/ml, could produce nearly quantitative (93%) VEGF inhibition in the corneal assay, consistent with appropriate functionality. We also administered Ad VEGF Trap i.m. to mimic intramuscular AAV administration (see below). This demonstrated that we could obtain quantitative VEGF corneal inhibition and persistent expression lasting > 60 days, with peak levels of ~ 600 µg/ml. (Figures 15, 16).

Virus	Dose (pfu)	Day 3 plasma levels (mg/ml)	% inhibition in cornea assay
VEGF Trap	10^9	14.4 ± 1.4	100 ± 0
	3×10^8	5.6 ± 1.2	99.1 ± 1.4
	1×10^8	1.9 ± 0.3	97.1 ± 1.4
	3×10^7	0.3 ± 0.1	93.2 ± 2.6
	1×10^7	0.08 ± 0.003	93.3 ± 3.9
	3×10^6	0.003 ± 0.0003	48.1 ± 8.2
	1×10^5	0.002 ± 0.0009	19.4 ± 8.9

Figure 15. Correlation of Ad VEGF Trap dose (i.v.) with day 3 plasma VEGF Trap levels and % inhibition in VEGF-dependent corneal micropocket assay.

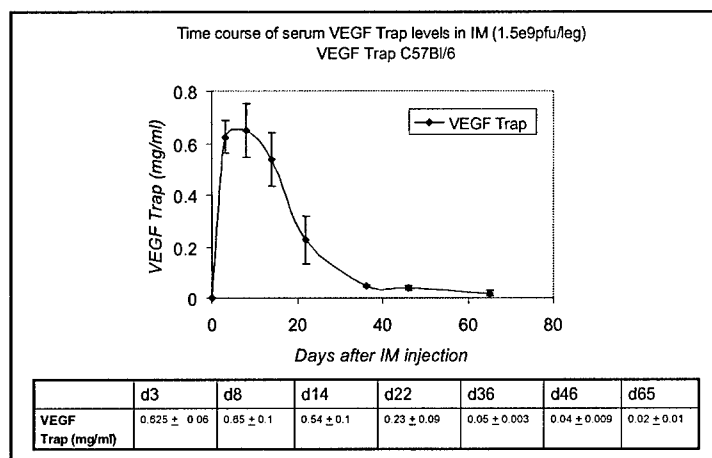


Figure 16. Plasma VEGF Trap levels following intramuscular administration of Ad VEGF Trap.

B. Evaluation of AAV VEGF Trap

In collaboration with Richard Mulligan's lab at Harvard, the VEGF Trap cDNA has been inserted into an ITR-flanked AAV vector. Research grade batches pf AAV VEGF Trap virus have been produced. Single i.m. injections of AAV VEGF Trap produce persistent plasma VEGF Trap expression > 6 months post injection of ~100 ng/ml or greater. Because of the data in section A. above, these circulating levels should be sufficient to produce long-term VEGF inhibition. The next steps will be to implant corneal VEGF pellets in these animals at various times post AAV administration to document long-term VEGF neutralization. Subsequently, the ability of AAV VEGF Trap to inhibit human prostate tumor growth will be evaluated using LNCaP and PC3 xenograft models.

C. Unanticipated polycythemia following Ad VEGF Trap administration.

Interestingly, adult mice receiving Ad VEGF Trap by either i.v. or i.m. routes developed unanticipated polycythemia, with hematocrit of >70% from a baseline of ~45-50%. This was both dose- and time-dependent and appears to be linked to elevated plasma erythropoietin levels. An intriguing aspect of this finding is that polycythemia is only associated with high grade VEGF blockade in vivo (~95% or greater) (Figure 17). Similar polycythemia appears with adenoviruses encoding soluble ectodomains of Flt1/VEGFR1 and Flk1/VEGFR2. We are currently examining the mechanistic basis for this phenomenon, but these data appear to implicate endogenous VEGF as a negative regulator of Epo production, with VEGF blockade de-repressing Epo expression followed by polycythemia.

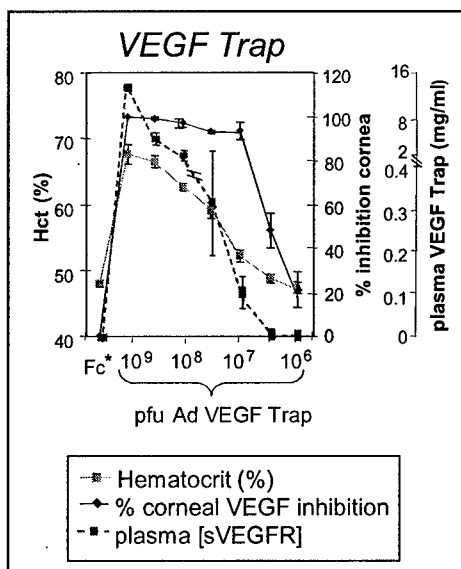


Figure 17. Induction of polycythemia by Ad VEGF Trap correlates with stringent levels of in vivo VEGF blockade. Simultaneous determination of hematocrit (d 14) and VEGF-dependent corneal angiogenesis (d 6) was evaluated in adult C57Bl/6 mice following i.v. injection of Ad VEGF Trap doses as indicated in the figure.

Key research accomplishments:

1. Construction and analysis of a 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 AAV
11. ELISA assays for monitoring VEGF Trap expression in vivo.
12. Dose dependency of VEGF inhibition by VEGF Trap in corneal micropocket assays.
13. Demonstration of polycythemia following stringent VEGF inhibition in vivo.

Reportable outcomes:

1. Construction of E1 complementation cell line for adenovirus production 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.
3. Dose dependency of VEGF inhibition by VEGF Trap
4. Polycythemia following VEGF inhibition in vivo.

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 in

previous years we have also demonstrated that we can 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.

In a second approach, in collaboration with Richard Mulligan's lab at Harvard, we have explored soluble VEGF receptors with improved pharmacokinetic properties in the context of pseudotyped AAV vectors to provide long-term soluble VEGF receptor expression. Substantial progress in this parallel approach has been made in the past year. The VEGF Trap insert has been initially explored in adenovirus to document threshold plasma levels necessary for effective VEGF inhibition *in vivo*. Preliminary experiments with AAV VEGF Trap indicate that long term plasma expression of sufficient magnitude for tumor VEGF blockade will be achievable. An unanticipated result has been the documentation of increased Epo production following high-grade VEGF blockade with VEGF Trap. The mechanistic basis for this is currently under investigation but the current data implicate endogenous VEGF as a negative regulator of Epo production and erythropoiesis *in vivo*.

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.

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