

Award Number: DAMD17-03-0223

TITLE: Anti-Angiogenic Gene Therapy for Prostate Cancer

PRINCIPLE INVESTIGATOR: Selvarangan Ponnazhagan, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham AL 35294

REPORT DATE: October 2006

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE 01-10-2006			2. REPORT TYPE Final			3. DATES COVERED 1 Apr 2003 – 30 Sep 2006		
4. TITLE AND SUBTITLE Anti-Angiogenic Gene Therapy for Prostate Cancer						5a. CONTRACT NUMBER		
						5b. GRANT NUMBER DAMD17-03-1-0223		
						5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Selvarangan Ponnazhagan, Ph.D.						5d. PROJECT NUMBER		
						5e. TASK NUMBER		
						5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Alabama at Birmingham Birmingham AL 35294						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. SPONSOR/MONITOR'S ACRONYM(S)		
						11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited								
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.								
14. ABSTRACT In work supported by this funding, we produced high-titer recombinant AAV vectors encoding mouse endostatin and angiostatin, and human osteoprotegerin; established TRAMP mouse breeding colony, and performed in vitro and in vivo studies to determine the effects of anti-angiogenic therapy at two different stages of prostate cancer progression. Additionally, we constructed rAAV encoding human OPG, produced high-titer virus and validated the biological efficacy of the vector encoded protein in inhibiting osteoclastogenesis in vitro. Continuation of the ongoing studies in the next few months will conclude these studies on therapeutic effects of anti-angiogenic gene therapy using adeno-associated virus in prostate cancer growth and metastasis.								
15. SUBJECT TERMS Adeno-associated virus; anti-angiogenesis, angiostatin, endostatin								
16. SECURITY CLASSIFICATION OF:					18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC		
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U					10	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	10
References.....	10
Appendices.....	10

Title of the Grant: Anti-angiogenic Gene Therapy for Prostate Cancer
Award number: DAMD17-03-0223
Principal Investigator: Selvarangan Ponnazhagan, Ph.D.
Final Report: April 01, 2003 – March 31, 2006

INTRODUCTION

One of the major implications of prostate cancer progression is bone metastasis. Primary therapies for neoplastic prostate disease have been prostatectomy followed by chemotherapy and radiation therapy. Although these forms of palliative therapies have been successful in early detected prostate cancers, a problem in majority of the treated cases is the growth of radiation/chemotherapy resistant tumor cells, which become refractory to treatment and exhibit an aggressive growth and metastatic profile. Thus, novel therapies that will control the process of recurrence and metastasis will have a profound clinical implication in the management of prostate cancer patients who undergo primary therapies.

An interesting new target for prostate cancer therapy is tumor angiogenesis, which is vital for tumor growth and metastasis. Since anti-angiogenic therapy targets normal endothelial cells that form neovasculature, long term sustained presence of anti-angiogenic factors is critical for therapeutic significance. Although few drugs and purified proteins have shown preclinical efficacy of this form of therapy, a long-term application of these therapies have been associated with systemic toxicity, limited half life and increasing cost. Thus, stable long-term therapies without these effects would be highly beneficial. Gene therapy approach using recombinant adeno-associated virus vectors (rAAV) encoding anti-angiogenic factors is a very promising form of therapy for prostate cancer recurrence and metastasis. Major advantage of rAAV vectors are 1) long-term transgene expression 2) stable integration, 3) low-immunogenicity or toxicity and 4) non-pathogenicity.

Our recent preclinical evaluation using rAAV encoding angiostatin, endostatin and soluble vascular endothelial growth factor receptor (sFlt-1) indicated long-term protection of mice against the growth of a human angiogenesis-dependent ovarian cancer cells as xenograft. Sustained expression of the anti-angiogenic factors was detected over four months without any systemic toxicity. Based on these data, we proposed in our funded application to evaluate the potential of rAAV-mediated anti-angiogenic gene therapy in a transgenic adenocarcinoma mouse prostate (TRAMP) model, which exhibits most of the pathological features seen in human prostate cancer including a progressive angiogenic phenotype with advancing stages of the disease, bone metastasis and refractiveness of androgen depletion over time. New experiments will include the analysis of bone metastasis of prostate cancer cells following rAAV-mediated anti-angiogenic gene therapy. Further, we will also determine the effects of long-term expression of murine osteoprotegerin as primary and an adjuvant to anti-angiogenic gene therapy for the inhibition of bone metastasis of malignant prostate disease in mouse model.

The proposed specific aims of the project are:

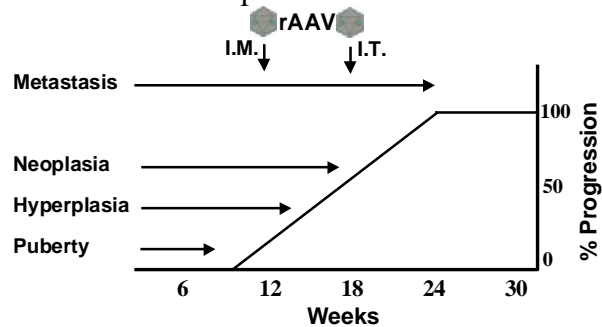
1. To determine long-term therapeutic potential of rAAV-mediated anti-angiogenic gene therapy in bone metastasis of neoplastic prostate disease in the transgenic adenocarcinoma mouse prostate (TRAMP) model *in vivo*.
2. To determine the adjuvant effects of long-term anti-angiogenic gene therapy and osteoprotegerin therapy for androgen-independent recurrence of prostate cancer in the TRAMP model.

BODY

Determination of rAAV-mediated anti-angiogenic gene therapy for early and late stage prostate cancer in mouse model.

During the last year, we have produced high-titer recombinant AAV containing mouse angiostatin and endostatin and initiated *in vivo* studies in male transgenic adenocarcinoma of prostate mouse (TRAMP) model. We have developed a breeding program for obtaining sufficient male TRAMP mice for the studies. The *in vivo* studies have been initiated at two different phases of prostate cancer development in these mice as depicted.

Treatment regimen with rAAV encoding angiostatin and endostatin in TRAMP mice for early and late-stage disease.

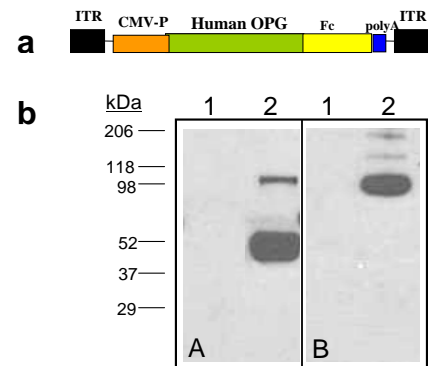


These long-term studies are ongoing and we will have conclusive data in approximately 3 months. However, based on the ongoing trend, it appears that rAAV-mediated anti-angiogenic gene therapy may provide significant therapeutic benefit when administered during early stage disease. Although marginal therapeutic gains are noted in the group treated with rAAV encoding angiostatin and endostatin during 18-weeks of age, the reduction in tumor growth was not dramatic.

Construction of rAAV encoding human OPG and analysis of expression as a soluble factor.

To determine if rAAV-mediated gene therapy can be used to inhibit prostate cancer bone metastasis, a rAAV containing the N-terminal 185 amino acid portion of the human OPG cDNA fused to the human immunoglobulin (Fc) was constructed. The construct was tested for the expression and extracellular secretion of OPG in RAW (a murine macrophage cell line) cell cultures. Results, shown below, indicate the expression of OPG from rAAV transduced cells.

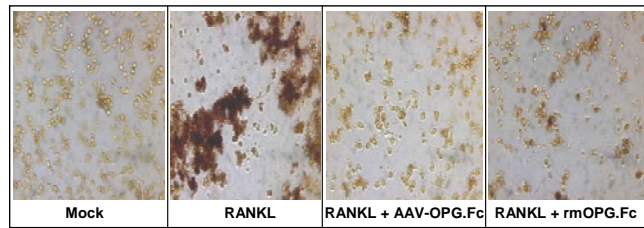
Recombinant AAV encoding the human OPG.Fc (a) and expression of the OPG.Fc from RAW cell supernatant (b). RAW cells were mock-transduced (1) or transduced with rAAV-OPG.Fc (2) construct and the supernatants were analyzed by Western blot using a monoclonal antibody for the human OPG in either denatured gel (A) or non-denatured gel (B).



Transduction of rAAV-OPG.Fc inhibits osteoclast differentiation *in vitro*. The biological activity of rAAV produced OPG was determined in osteoclast forming assay using RAW cells. Briefly, 10^5 RAW cells were plated in 24-well tissue culture plates and grown in medium containing 10% FBS, 20 ng/ml M-CSF, and 50 ng/ml RANKL in the presence or absence of conditioned medium from 293 cells transduced with rAAV-OPG.Fc. The growth medium plus

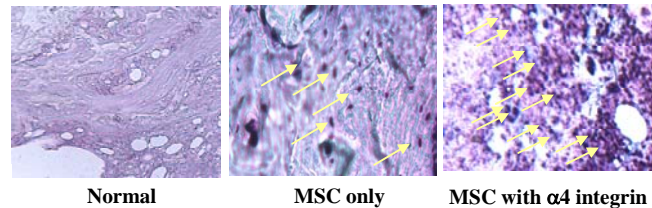
additives were changed every alternate day. After five days of culture, the cells were fixed and stained for tartrate-resistant alkaline phosphatase (TRAP), a marker for multinucleated osteoclasts. Results, shown below, demonstrate that rAAV produced OPG is biologically active and effectively inhibits osteoclastogenesis.

Figure 2. TRAP staining of RAW cells following rAAV-OPG.Fc transduction.



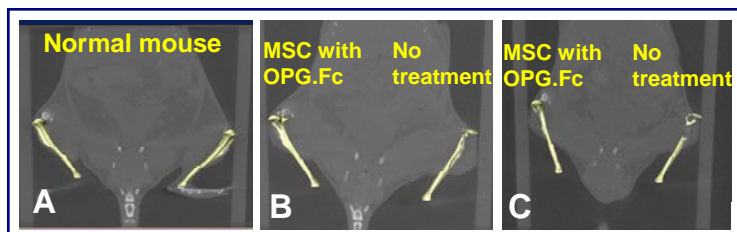
We established that mesenchymal stem cells (MSC), to be used in the proposal as cell therapy and gene therapy vehicle, can be efficiently transduced by AAV (1). In order to increase their efficacy in bone-specific homing by ex vivo method, we adapted a strategy to transiently express $\alpha 4\beta 1$ integrin on MSC cell surface. By this modification, we were able to successfully increase bone specific homing and retention of MSC upon in vivo transfer. This is shown in Figure 3.

Figure 3: *In situ* hybridization analysis of bone marrow stromal cells for the identification of donor MSC repopulation. Two weeks after sham-transplantation (Normal), or transplantation with mock-transfected (MSC only) or transfected with plasmid encoding $\alpha 4$ integrin (MSC with $\alpha 4$ integrin) to mouse stem cells from male mice, animals were sacrificed for analysis. Femur bones were decalcified and sectioned to 5 μ m thickness. The sections were deparaffinized and rehydrated through alcohol gradation series. Following denaturation at 85o C in hybridization buffer, the sections were probed using DIG-labeled Y-chromosome specific DNA probe. The slides were gently counterstained with eosin.



To test the efficiency of OPG-expressing MSC in protecting osteolytic lesions due to cancer bone metastasis, encountered initially in prostate cancer patients prior to the appearance of osteoblastic lesions, we transfected OPG expression vector in mouse MSC and transplanted them to tibial bones of nude mice harboring osteolytic prostate cancer cell line, PC-3. These cells were stably transfected with luciferase gene, hence, allowed non-invasive imaging of the cancer cell growth. Micro-CT analysis of the bone following the therapy indicated remarkable retention of bone architecture after the OPG-expressing MSC were therapeutically implanted. Representative data is shown in Figure 4.

Figure 4. Radiographic images of mice tibia following treatment with MSC producing OPG.Fc. Approximately 10^5 osteolytic bone metastatic cancer cell line PC-3 were implanted in mouse tibia (B & C). Seven days after tumor cell implantation, MSC producing OPG.Fc was injected in one side and the other side left untreated. Picture shown in panel A is from a normal mouse without any tumor or MSC.



Following this, we constructed recombinant AAV plasmid with OPG-Fc to produce virus. However, we encountered technical difficulties initially in the purification of the virus due to the heparin binding property of the transgenic protein. We have recently overcome this and have produced adequate amount of the vector for testing *in vivo*. We hope to finish this in the next few months. The DoD has kindly allowed a 6-month no cost-extension of the project and that would be very helpful to accomplish the task.

To determine the effects of anti-angiogenic gene therapy in multi-stage prostate cancer in a spontaneously occurring preclinical animal model, we evaluated the potential of recombinant adeno-associated virus (rAAV)-mediated stable expression of angiostatin and endostatin during early and late stages of prostate cancer progression in TRAMP mice. Cohorts of 8 week-old and 18 week-old male TRAMP mice received either no virus or 1.2×10^{11} genomic particles of rAAV encoding green fluorescent protein (GFP) or mouse endostatin plus angiostatin by intramuscular (i.m.) injection. The effects of therapy were determined by periodic immunohistochemical analyses of the prostate for apoptotic index, endothelial cell growth and tumor proliferation. The levels of endostatin and angiostatin in systemic circulation were measured by ELISA and survival index was recorded as the endpoint.

Results of these studies indicated significant tumor-free survival following rAAV endostatin + angiostatin therapy only when the vector was given at earlier time (5 weeks and 10 weeks) compared to treatment at 18 weeks. Both the untreated and rAAV-GFP treated mice died by 35 weeks of age, and that treated with rAAV endostatin + angiostatin at 5 or 10 weeks remained alive up to 70 weeks. Immunohistochemical analysis of prostate from two mice sacrificed from the rAAV endostatin + angiostatin treatment group during 35 weeks, longest time of survival of the untreated or rAAV-GFP treated mice, indicated significantly low endothelial cell proliferation, and more tumor cell apoptosis. The mean weight of prostate in untreated group at the time of death was 9.0 ± 2.1 gm whereas mice from rAAV-endostatin + angiostatin treatment had prostate with a mean weight of 4.8 ± 0.6 gm even at 60 weeks. Organ metastasis was significantly less in rAAV endostatin + angiostatin treated mice compared to untreated, or rAAV-GFP treated mice when mice in control group died. However, the therapeutic effects were not significant if rAAV administration was given by i.m. injection during 18 weeks of age when the disease is in advanced stage with enlarged prostate and distant metastasis. These studies indicate that stable gene therapy by rAAV-endostatin + angiostatin may be more effective as an adjuvant therapy with significant effects for minimally invasive tumors rather than advanced-stage disease. Figures below indicate some of the findings.

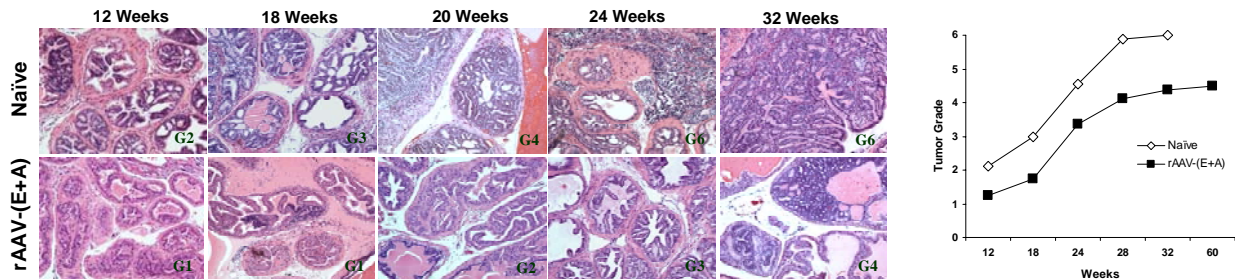


Figure 5. Histologic analysis of prostate tissue from TRAMP mice with or without rAAV-(E+A) treatment. Cohorts of TRAMP mice were sacrificed at indicated weeks following no treatment or treatment with rAAV-EA and prostate tissues sectioned and stained for histopathologic analysis. The slides were evaluated by a pathologist and graded as follows: Grade 1 (G1): Normal prostate; G2: low grade of prostatic intraepithelial neoplasia; G3: high grade of prostatic intraepithelial neoplasia; G4: well differentiated tumor; G5: moderately differentiated tumor; and G6: poorly differentiated tumor. The graph on right side indicates composite data of tumor grade at different weeks in naïve and rAAV-(E+A) treated mice.

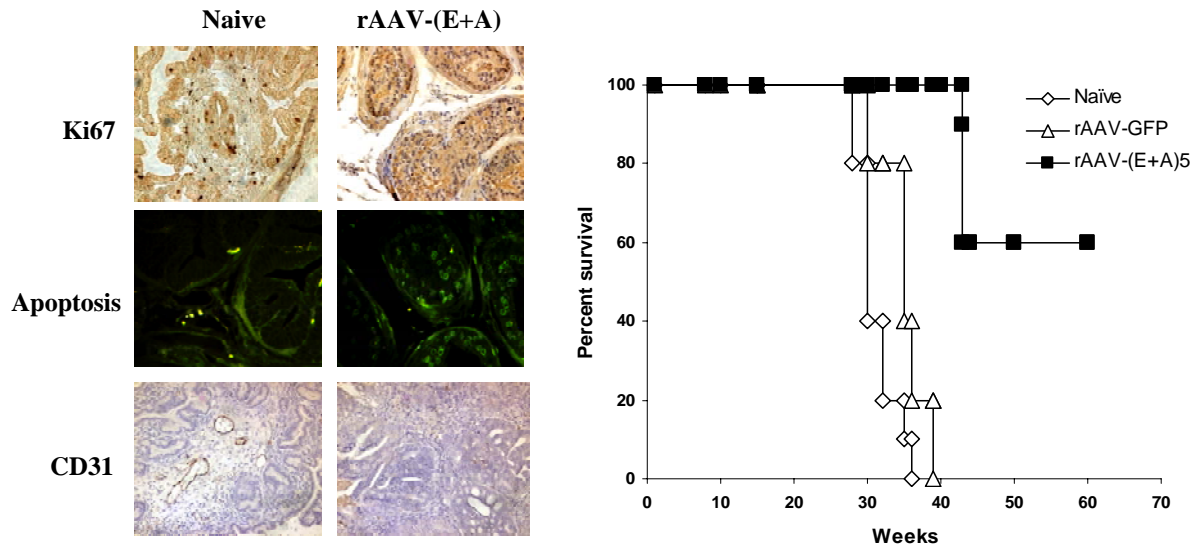


Figure 6. Immunohistochemistry for proliferation, apoptosis and microvessel density, and survival index in naive and rAAV-(E+A) treated TRAMP mice. Prostate tissues were obtained from naive and rAAV-(EA) treated mice. In the pictures presented, samples from mice were of comparable histological grade. Immunohistochemical staining of paraffin-embedded sections was performed with anti-Ki67 monoclonal antibody for proliferation, anti-PARP p85 fragment polyclonal antibody for apoptosis and CD31/PECAM-1 antibody for endothelial cells. The slides were minimally counterstained with hematoxylin. **Survival index of TRAMP mice following rAAV treatment is shown on the right side.** Cohorts of mice in each group were monitored for survival following rAAV treatment at early time point 5 and 10 weeks of age.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed rAAV encoding human OPG, produced high-titer virus and validated the biological efficacy of the vector encoded protein in inhibiting osteoclastogenesis in vitro.
- Developed strategy to increase bone-specific homing of MSC.
- Established that MSC expressing OPG greatly reduce osteolytic effects of cancer growth in bone.
- rAAV encoding secretable forms of mouse endostatin and angiostatin or GFP was injected intramuscularly in 5-week old, 10-week old or 18-week old TRAMP male mice.
- Cohorts of mice from each group were sacrificed at defined stages of tumor progression in untreated TRAMP mouse.
- Weight of the genitourinary system following treatment with rAAV-(E+A) was significantly lower than untreated mice.
- rAAV6 mediated expression of endostatin and angiostatin resulted in increased apoptosis, decreased proliferation and CD31 expression in the prostate tumor, compared to naive mice.
- rAAV6 mediated expression of endostatin and angiostatin delay the appearance of prostate cancer metastasis but did not eliminate tumor onset.
- Interestingly, slow progression of the disease in rAAV-(E+A) treated mice was noticed until well-differentiated tumor grade (grade 4) and remained in that state for several weeks, beyond the time when all the control mice died.
- These studies indicate that stable gene therapy by rAAV-endostatin + angiostatin may be more effective as an adjuvant therapy with significant effects for minimally invasive tumors rather than advanced-stage disease.

REPORTABLE OUTCOMES

(Papers published or communicated that are supported by this award)

Isayeva, T., and Ponnazhagan, S. Anti-angiogenic gene therapy for cancer. *Int. J. Oncol.* 2004, 25: 335-343.

Mahendra, G., Mahasreshti, P., Curiel, D.T., Stockardt, R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., and Ponnazhagan, S. Anti-angiogenic gene therapy through adeno-associated virus 2-mediated stable expression of soluble Flt-1 receptor *Cancer Gene Ther.* 2005, 12: 26-34.

Isayeva, T., Ren, C., and Ponnazhagan, S. Recombinant adeno-associated virus 2-mediated anti-angiogenic prevention in a mouse model of intraperitoneal ovarian cancer. *Clin. Cancer Res.* 2005 11:1342-1347.

Ren, C., Kumar, S., Shaw, D.R. and Ponnazhagan, S. Genomic stability of self-complementary adeno-associated virus 2 during early stages of transduction in mouse muscle in vivo. *Human Gene Therapy.* 2005, 16: 1-11.

Isayeva, T., Ren, C., and Ponnazhagan, S. Intraperitoneal transduction of adeno-associated virus 2 expressing angiostatin and endostatin synergistically augments paclitaxel therapy and tumor-free survival in a mouse model of epithelial ovarian cancer. *Gene Therapy* 2006 (in press).

Isayeva, T., Chanda, D., Eltoun, I., and Ponnazhagan, S. Effects of sustained anti-angiogenic therapy in multi-stage prostate cancer in TRAMP mice. 2006 (in communication).

(Results presented in conferences)

Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D., Stockard, C.E., and Grizzle, W.E. Adeno-associated virus 2-mediated gene therapy: long-term efficacy of a combination vector over individual therapy. 94th Annual Meeting of the American Society for Cancer Research, Washington D.C., 2003.

Zinn, K.R, Chaudhuri, T.R., Stargel, A., Kumar, S., and Ponnazhagan, S. Gamma camera imaging of Tc-99m-labeled AAV vector followed by bioluminescence imaging of luciferase expression. 6th Annual Meeting of the American Society for Gene Therapy, Washington D.C. 2003.

Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D., Stockard, C.E., Grizzle, W.E., and Meleth, S. Adeno-associated virus 2-mediated gene therapy: long-term efficacy of a combination vector over individual therapy. 6th Annual Meeting of the American Society for Gene Therapy, Washington D.C. 2003.

Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D.R., Stockard, C.R., Grizzle, W.E., and Meleth, S. Adeno-associated virus 2-mediated anti-angiogenic cancer gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. 95th Annual Meeting of the American Society for Cancer Research, Orlando, FL, 2004.

Ren, C., Kumar, S. and Ponnazhagan, S. Genomic stability of self-complementary recombinant

adeno-associated virus 2 in mouse muscle. 7th Annual meeting of the American Society of Gene Therapy, Minneapolis, MN, June 2004.

Isayeva, T., Ren, C., and Ponnazhagan, S. Recombinant adeno-associated virus 2 -mediated anti-angiogenic gene therapy in a mouse model of intraperitoneal ovarian cancer. 7th Annual meeting of the American Society of Gene Therapy, Minneapolis, MN, June 2004.

Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D.R., Stockard, C.R., Grizzle, W.E., and Meleth, S. Adeno-associated virus 2-mediated anti-angiogenic cancer gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. 7th Annual meeting of the American Society of Gene Therapy, Minneapolis, MN, June 2004.

Isayeva, T., Ren, C., and Ponnazhagan, S. Intraperitoneal transduction of adeno-associated virus 2 expressing angiostatin and endostatin synergistically augments paclitaxel therapy and tumor-free survival in a mouse model of epithelial ovarian cancer, 96th Annual Meeting of the American Association for Cancer Research, April 2005 Anaheim, CA.

Isayeva, T., Ren, C., and Ponnazhagan, S. Intraperitoneal transduction of adeno-associated virus 2 expressing angiostatin and endostatin synergistically augments paclitaxel therapy and tumor-free survival in a mouse model of epithelial ovarian cancer, 8th Annual Meeting of the American Society for Gene Therapy, St. Louis, MO, June 2005.

Isayeva, T., Chanda, D., and Ponnazhagan, S. Effects of sustained anti-angiogenic gene therapy in multi-stage prostate cancer in TRAMP mice. 97th Annual Meeting of the American Association for Cancer Research, April 2006, Baltimore, MD.

CONCLUSIONS

In work supported by this funding, we produced high-titer recombinant AAV vectors encoding mouse endostatin and angiostatin, and human osteoprotegerin, established TRAMP mouse breeding colony, and performed in vitro and in vivo studies to determine the effects of anti-angiogenic therapy at two different stages of prostate cancer progression. Additionally, we constructed rAAV encoding human OPG, produced high-titer virus and validated the biological efficacy of the vector encoded protein in inhibiting osteoclastogenesis in vitro. Continuation of the ongoing studies in the next few months will conclude these studies on therapeutic effects of anti-angiogenic gene therapy using adeno-associated virus in prostate cancer growth and metastasis.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.

Diptiman Chanda, Ph.D.

Gene Siegal, MD., Ph.D.

REFERENCES N/A

APPENDICES N/A