

Award Number: **W81XWH-05-1-0270**

TITLE: **Gene therapy for osteolytic breast cancer bone metastasis**

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REPORT DATE: **June 27, 2006 (Report Period: June 01, 2005 – May 31, 2006)**

TYPE OF REPORT: **Annual Report**

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

DISTRIBUTION STATEMENT: **Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) June 28, 2006		2. REPORT TYPE Research Report		3. DATES COVERED (From - To) 01/06/2005 - 05/31/2006	
4. TITLE AND SUBTITLE Gene therapy for osteolytic breast cancer bone metastasis				5a. CONTRACT NUMBER W81XWH-05-1-0270	
				5b. GRANT NUMBER BC044440	
				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) The University of Alabama at Birmingham Department of Pathology 701, 19 th Street S., LHRB 513 Birmingham, AL 35294-0007				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materials Command 504 Scott Street Fort Detrick MD 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. Osteoprotegerin (OPG) is a "decoy" receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. We sought to achieve sustained effects of OPG combining cell therapy and gene therapy approaches. The aims were to determine therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, and to determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis. So far, we produced high-titer recombinant AAV vectors encoding osteoprotegerin, and tested the feasibility of MSC therapy for reducing osteolysis in bone initiated by cancer growth. Also we established a method for bone homing of ex vivo cultured MSC by transient expression of $\alpha 4\beta 1$ integrin. Continuation of the ongoing studies in to next year will provide valuable information on therapeutic effects of this therapy for breast cancer bone metastasis.					
15. SUBJECT TERMS Osteolysis, osteoprotegerin, mesenchymal stem cells, gene therapy, adeno-associated virus					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON Selvarangan Ponnazhagan
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code) 205-934-6731

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INTRODUCTION

Bone is the frequent metastatic site for human breast cancer resulting in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of cancer cells and the bone microenvironment results in the upregulation of factors promoting osteoclastogenesis and osteolytic bone destruction. Thus, osteolysis and tumor cell accumulation can be inhibited by interrupting one or more of the steps involved in the cycle. The major treatment to reduce the burden of bone metastasis in breast cancer patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, while improving the formulations of bisphosphonate compounds, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial.

A better understanding of the molecular events in breast cancer osteolytic bone destruction indicates that the receptor activator of nuclear factor κ B ligand (RANKL), produced by osteoblasts, activated T cells and marrow stromal cells stimulates the recruitment, differentiation, and activation of osteoclasts by binding to RANK. Osteoprotegerin (OPG) is a “decoy” receptor that competes with RANK for RANKL, thus, modulating the effects of RANKL. However, during the metastatic events involving cancer and stromal cell interaction, endogenous OPG levels are markedly reduced. Thus, OPG remains an effective molecule for future therapies for bone metastasis. To achieve sustained effects of OPG, gene therapy is more powerful than pharmacological therapies. Since the process of bone metastasis in breast cancer is a secondary event that occurs in late-stage disease or during recurrence, genetic therapies aimed at controlling this process should be both sustained and localized. Thus, for sustained expression of therapeutic levels of OPG, a vector capable of stable expression of the transgene without vector-associated toxicity and immunity is ideal. The adeno-associated virus vectors (AAV) are more promising to this end. With recombinant AAV vectors, it is possible to obtain significant therapeutic advantage by either systemic or bone-targeted transduction and can be combined with bisphosphonate treatment for synergistic effects.

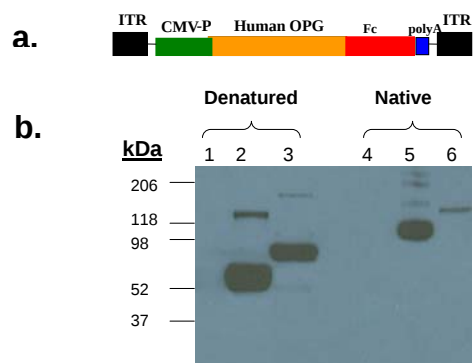
The proposed specific aims of the project are:

- 1) To determine the therapeutic effects of stable OPG expression by rAAV gene therapy in a murine model of breast cancer bone metastasis, *and*
- 2) To determine the synergistic effects of OPG gene therapy with bisphosphonate therapy in a murine model of breast cancer bone metastasis.

BODY

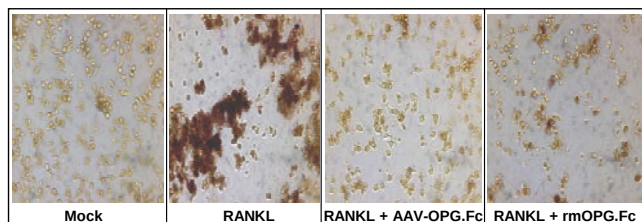
Production of adeno-associated viral vectors encoding human OPG and analysis of expression as a soluble factor. We constructed recombinant adeno-associated virus vectors (rAAV) encoding osteoprotegerin (OPG), either as a fusion protein to the human immunoglobulin (Fc) or without Fc. The constructs were tested initially for the expression and extracellular secretion of OPG in RAW (a murine macrophage cell line) cell cultures. Results, shown in Figure 1, indicate the expression of OPG from rAAV transduced cells.

Figure 1. Recombinant AAV encoding the human OPG.Fc (a) and expression of the OPG.Fc from RAW cell supernatant (b). RAW cells were mock-transduced or transduced with rAAV-OPG.Fc construct and the supernatants were analyzed by Western blot using a monoclonal antibody for human OPG in either denatured gel or non-denatured gel. Lane assignments: Lane 1&4: Mock; 2: s-hOPG-Fc; 3: hOPG-Fc; 4: Mock 293; 5: s-hOPG-Fc; 6: hOPG-Fc



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 in Figure 2, demonstrate that rAAV produced OPG is biologically active and effectively inhibits osteoclastogenesis.

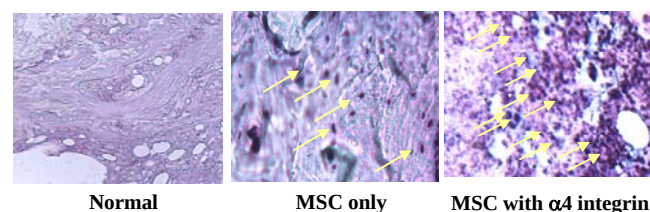
Figure 2. TRAP assay of RAW cells following rAAV-OPG.Fc transduction.



Recently, 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.

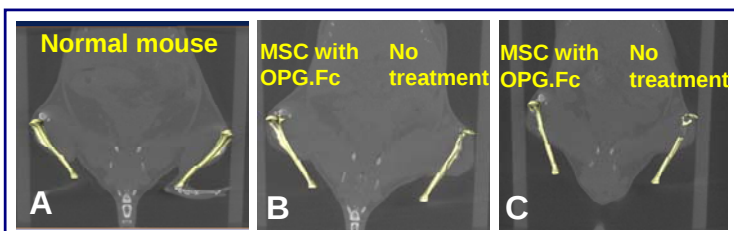
Figure 3: *In situ* hybridization analysis of bone marrow stromal cells for the identification of donor MSC repopulation.

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 commonly in breast cancer patients, we transfected OPG expression vector in mouse MSC and transplanted them to tibial bones of nude mice harboring osteolytic 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.



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.

REPORTABLE OUTCOMES

(Papers published or communicated)

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 Ther. 2006 (in revision).

(Results presented in conferences)

Isayeva, T., Ren, C., and Ponnazhagan, S. Adeno-associated virus mediated anti-angiogenic gene therapy. Department of Defense – Breast Cancer Research Program, 4th Era of Hope Meeting, Philadelphia, PA, 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.

32.

CONCLUSIONS

So far, we produced high-titer recombinant AAV vectors encoding osteoprotegerin, and tested the feasibility of MSC therapy for reducing osteolysis in bone initiated by cancer growth. Also we established a method for bone homing of ex vivo cultured MSC by transient expression of $\alpha 4\beta 1$ integrin. Continuation of the ongoing studies in to next year will provide valuable information on therapeutic effects of this therapy for breast cancer bone metastasis.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.

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1. Kumar, S., Mahendra, G., Nagy, T.R., and **Ponnazhagan, S***. Osteogenic differentiation of recombinant adeno-associated virus 2-transduced murine mesenchymal stem cells and establishment of an immunocompetent murine model for ex vivo osteoporosis gene therapy. Hum. Gene Ther. 2004, 15: 1196-1207.

APPENDICES

N/A