

AD _____

Award Number: DAMD17-03-1-0290

TITLE: Novel Therapeutic Approach for Breast Cancer

PRINCIPAL INVESTIGATOR: Devanand Sarkar, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10027

REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

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.

20041215 026

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

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 Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

| | | | | |
|--|---|--|--|--|
| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE June 2004 | 3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 2003 - 31 May 2004) | |
| 4. TITLE AND SUBTITLE Novel Therapeutic Approach for Breast Cancer | | | 5. FUNDING NUMBERS DAMD17-03-1-0290 | |
| 6. AUTHOR(S) Devanand Sarkar, Ph.D. | | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Columbia University New York, New York 10027 E-Mail: Ds2039@columbia.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) Despite advances in the detection and treatment of breast cancer, the final impact on survival rates has not altered significantly. Based on these considerations, novel therapeutic approaches, such as gene-based therapy, offer promise for successfully treating this prevalent female disease. I am constructing replication competent bipartite adenoviruses (RCBA) in which the replication is controlled by the promoter of the PEG-3 gene (PEG-Prom) that shows cancer-selective activity and which simultaneously express either the cancer-selective apoptosis-inducing gene melanoma differentiation associated gene-7 (mda-7)/IL-24 or an immunomodulatory gene such as interferon- γ or IL-2. I have demonstrated that PEA-3 and AP-1, transcription factors involved in regulating oncogenesis and tumor progression, are involved in the regulation of PEG-prom activity in breast cancer cells. I have also demonstrated that RCBA in which replication is controlled by PEG-Prom and which expresses mda-7/IL-24 (Ad.PEG-E1A.CMV-mda-7) kills breast cancer cells without harming normal breast epithelial cells. However, RCBA in which replication is controlled by CMV promoter (Ad.CMV-E1A.CMV-mda-7) kills both breast cancer and normal cells indicating the breast cancer-selective killing property of Ad.PEG-E1A.CMV-mda-7. This exciting initial observation indicates that Ad.PEG-E1A.CMV-mda-7 might be a valuable tool for actual therapeutic application in patients with breast cancer. | | | | |
| 14. SUBJECT TERMS Targeted cancer gene therapy, cancer specific gene expression cassettes, replication competent bipartite adenoviruses | | | 15. NUMBER OF PAGES 12 | |
| | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited | |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

| | |
|-----------------------------------|----|
| Cover..... | 1 |
| SF 298..... | 2 |
| Introduction..... | 4 |
| Body..... | 5 |
| Key Research Accomplishments..... | 8 |
| Reportable Outcomes..... | 9 |
| Conclusions..... | 11 |
| References..... | 12 |
| Appendices..... | |

Introduction

Despite advances in the detection and treatment of breast cancer, the final impact on survival rates has not altered significantly. Currently, one in nine American women can be expected to develop breast cancer, of which one third will die of this disease (1). A high priority exists for developing approaches to improve the long-term disease-free status and overall survival of patients. The objective of the proposal is to develop effective adenovirus-based gene therapy approach for the treatment of breast cancer. This approach contains three basic elements. (1) It utilizes PEG-Prom, the promoter region of the rodent Progression elevated gene-3 (PEG-3), which has selectively high activity in cancer cells, including breast cancer cells, while having very low to no activity in normal cells, including primary breast epithelial cells (2, 3 and unpublished data). (2) The PEG-Prom is used to drive the expression of E1A and E1B genes of adenovirus, which are necessary for its replication. This strategy allows conditional replication of adenovirus only in cancer cells but not in normal cells. (3) These conditionally replicating adenoviruses express pro-apoptotic or immuno-modulatory genes such melanoma differentiation associated gene-7 (*mda-7*)/IL-24 or interferon- γ or IL-2 thus allowing profound anti-tumor apoptotic and immune response. Importantly, multiple studies have shown that *mda-7*/IL-24 has cancer-selective apoptosis-inducing properties without having any adverse effect on normal cells (4-8). Thus the combination of PEG-Prom and *mda-7*/IL-24 will provide cancer-specific expression of transgene and cancer-specific killing. The specific aims of the three-year proposal are (1) To molecularly characterize the PEG-Prom to understand how it functions and to more effectively use it to target human breast cancers. (2) To construct replication competent bipartite adenovirus (RCBA) vectors in which the PEG-Prom controls expression of E1A and a cytomegalovirus (CMV) promoter controls expression of *mda-7*/IL-24 or an immunomodulatory gene (IFN- γ or IL-2). (3) To evaluate the ability of RCBA's to inhibit *in vivo* growth of established human breast cancer xenografts in athymic nude mice. Specific aim 1 would be accomplished in Year 1, specific aim 2 in Year 2 and specific aim 3 in Year 3. However, because of the complex technical methods of making RCBA's, I have started to perform experiments that encompass both Specific aims 1 and 2 and have partially accomplished both these aims. I am continuing my experiments so that both the aims are finished by Year 2.

Body

During the past year I have obtained significant data with regards to specific aims 1 and 2 which are enumerated below.

I. PEA-3 and AP-1 are important for regulation of PEG-Prom activity in breast

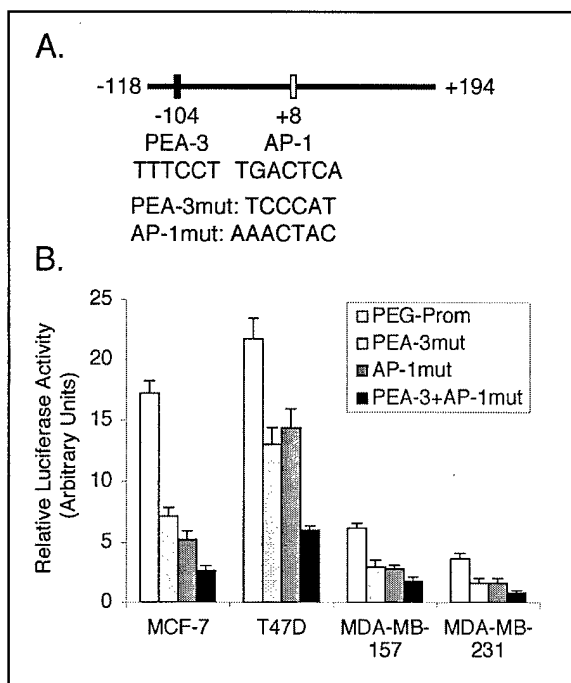


Fig. 1. PEA-3 and AP-1 are involved in the regulation of PEG-Prom activity. **A.** Schematic representation of PEG-Prom showing the position of PEA-3 and AP-1 sites. The numbers represent the relative positions of the nucleotide when the transcription start site is regarded as +1. **B.** The indicated cell lines were transfected with the different promoter-luciferase constructs together with a β -galactosidase expression construct. Luciferase assay was performed 48 h post-transfection and luciferase expression was normalized by β -galactosidase expression. The data represent mean \pm S.D. of two independent experiments

cancer cells. In rodent system, it was observed that PEA-3 and AP-1 transcription factors play important roles in controlling the activity of PEG-Prom (2, 3). I, therefore, checked the involvement of these two factors in

regulation of PEG-Prom activity in breast cancer cells. This is especially important since PEA-3 has been shown to be overexpressed in breast carcinoma cells (9, 10). Mutant constructs were made in which either PEA-3 or AP-1 or both sites were mutated and the luciferase activity of these constructs were analyzed in a number of breast cancer cells. In all the cell lines, mutation of either PEA-3 or AP-1 site decreased the promoter activity by \sim 50%, while mutation of both sites reduced it to \sim 25% (**Fig. 1**). The basal promoter activity was much higher in MCF-7 and T47D cells than in MDA-MB-157 and MDA-MB-231 cells. I am currently examining if there is any correlation between the promoter activity and the expression levels of PEA-3 and AP-1 in each cell line.

II. Construction of Ad.PEG-E1A.CMV-mda-7 and Ad.CMV-E1A.CMV-mda-7. I

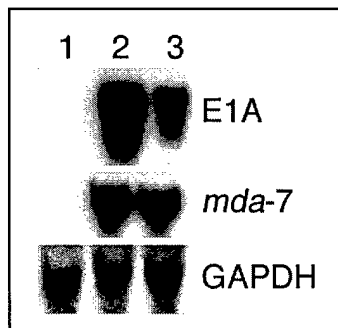


Fig. 2. RCBA's generate E1A and mda-7 mRNAs. T47D breast cancer cells were infected with Ad.vec (lane 1), Ad.CMV-E1A.CMV-mda-7 (lane 2) and Ad.PEG-E1A.CMV-mda-7 (lane 3) at an M.O.I. of 100 pfu/cell. The expressions of E1A, mda-7 and GAPDH were analyzed by Northern blot analysis 24 h post-infection

constructed Ad.PEG-E1A.CMV-mda-7 in which PEG-Prom drives the expression of adenoviral E1A gene and CMV promoter controls the expression mda-7 gene in the E3 region of the adenovirus. As a control I constructed Ad.CMV-E1A.CMV-mda-7 in which

CMV promoter controls the expression of both E1A and mda-7 genes. Two additional constructs were created as controls, Ad.CMV-E1A and Ad.PEG-E1A in which the expression of E1A was controlled by CMV promoter and PEG-Prom, respectively,

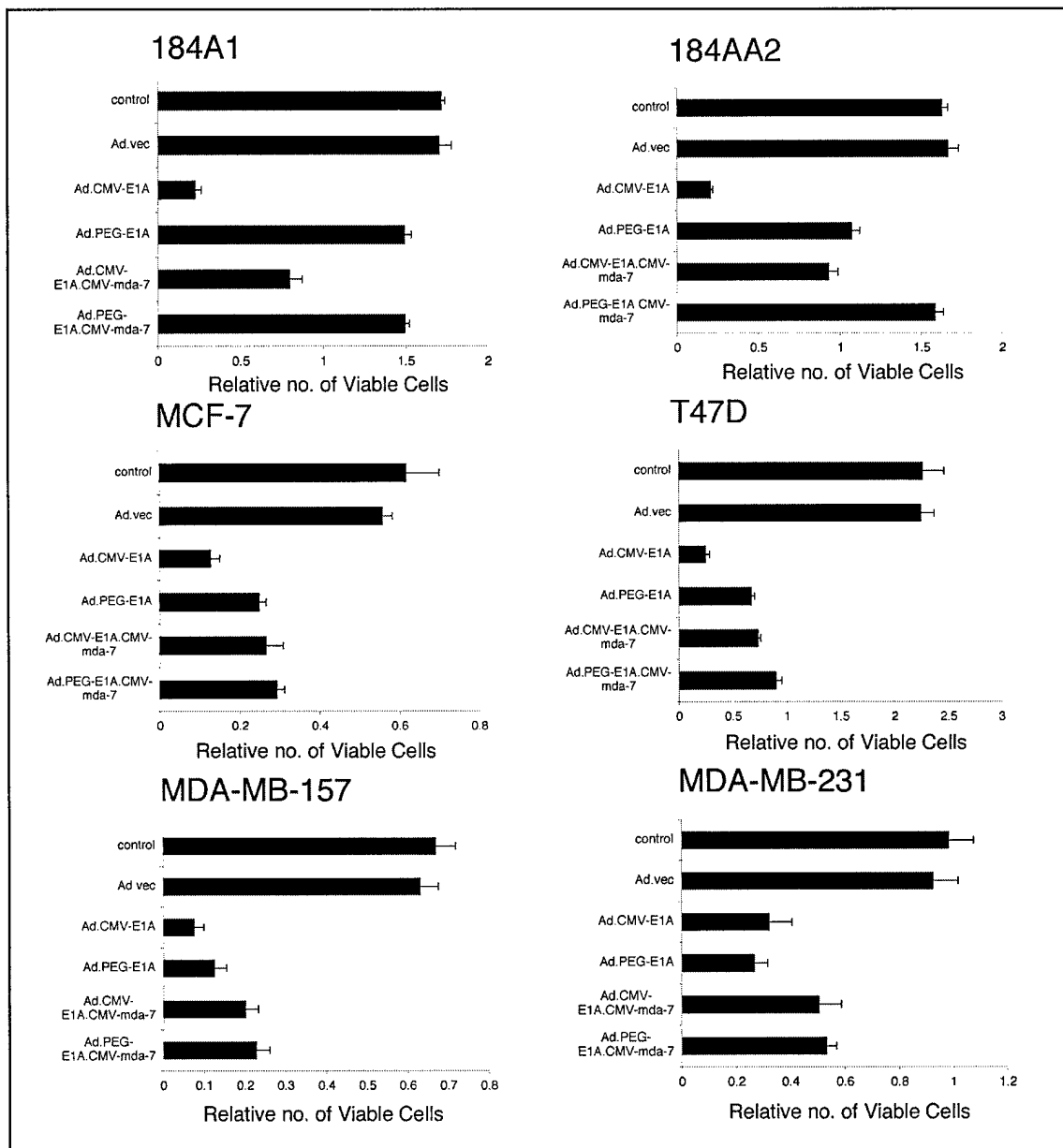


Fig. 3. Breast cancer specific killing of PEG-Prom-driven adenoviruses. Different cell lines were infected with the indicated adenoviruses at an M.O.I. of 100 pfu/cell and cell viability was analyzed by standard MTT assay 4 days post-infection. The data represent mean±S.D. of two independent experiments.

leaving the E3 region empty. Both Ad.CMV-E1A.CMV-mda-7 and Ad.PEG-E1A.CMV-mda-7 successfully generated E1A and mda-7 mRNAs (Fig. 2) indicating the authenticity of these constructs.

III. Breast cancer specific killing of PEG-prom-driven adenoviruses. I infected 184A1 and 184AA2, two immortal but non-tumorigenic breast epithelial cells, and MCF-

7, T47D, MDA-MB-157 and MDA-MB-231 breast cancer cells with Ad.vec (control empty adenovirus), Ad.CMV-E1A, Ad.PEG-E1A, Ad.CMV-E1A.CMV-mda-7 and Ad.PEG-E1A.CMV-mda-7 and analyzed cell viability by standard MTT assay. Ad.PEG-E1A and Ad.PEG-E1A.CMV-mda-7 showed little to no adverse effect on the viability of 184A1 and 184AA2 cells since PEG-Prom does not function in normal cells and since mda-7 induces apoptosis only in cancer cells but not in normal cells (**Fig. 3**). However, Ad.CMV-E1A and Ad.CMV-E1A.CMV-mda-7 could inhibit the growth of these cells indicating the specificity of action of PEG-Prom in cancer cells. All the adenoviruses could significantly inhibit the growth of all the breast cancer cells. These findings indicate that Ad.PEG-E1A.CMV-mda-7 might be developed as an effective tool for eradication of breast cancer. I am currently making other RCBA's which I will characterize in the year to come.

Key Research Accomplishments:

1. Identification of PEA-3 and AP-1 transcription factors as key elements regulating the activity of PEG-Prom in breast cancer cells.
2. Construction of RCBAAs, Ad.PEG-E1A.CMV-mda-7 and Ad.CMV-E1A.CMV-mda-7.
3. Demonstration of breast cancer-specific killing of Ad.PEG-E1A.CMV-mda-7 without having adverse effect on normal breast epithelial cells.

Reportable Outcomes:**Publications:**

1. Sarkar D, Kang D-C, Goldstein MI, Fisher PB. Approaches for gene discovery and defining novel protein interactions and networks. *Curr Genom.* 2004, 5:231-244.
2. Emdad L, Sarkar D, Su Z-Z, Boukerche H, Bar-Eli M, Fisher PB. Progression Elevated Gene-3 (PEG-3) induces pleiotropic effects on tumor progression: modulation of genomic stability and invasion. *J Cell Physiol.* 2004, in press.
3. Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Ann Rev Immunol.* 2004, 22:929-979.
4. Sarkar D, Leszczyniecka M, Kang D-C, Lebedeva IV, Valerie K, Dhar S, Pandita TK, Fisher, PB. Downregulation of Myc as a potential target for growth arrest induced by human polynucleotide phosphorylase (*hPNPase^{old-35}*) in human melanoma cells. *J Biol Chem.* 2003, 278:24542-24551.
5. Lebedeva IV, Sarkar D, Su Z-Z, Kitada S, Dent P, Stein CA, Reed JC, Fisher PB. Bcl-2 and Bcl-xL differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, *mda-7/IL-24*. *Oncogene.* 2003, 22:8758-8773.
6. Lebedeva IV, Su Z-Z, Sarkar D, Kitada S, Dent P, Waxman S, Reed JC, Fisher PB. Melanoma differentiation associated gene-7, *mda-7/interleukin-24*, induces apoptosis in prostate cancer cells by promoting mitochondrial dysfunction and inducing reactive oxygen species. *Cancer Res.* 2003, 63:8138-8144.
7. Leszczyniecka M, Su Z-Z, Kang D-C, Sarkar D, Fisher, PB. Expression regulation and genomic organization of human polynucleotide phosphorylase, *hPNPase^{old-35}*, a type I interferon inducible early response gene. *Gene.* 2003, 316: 143-156.
8. Su Z-Z, Leszczyniecka M, Kang D-C, Sarkar D, Chao W, Volsky DJ, Fisher PB. Insights into glutamate transporter regulation in human astrocytes: cloning and characterization of the promoter for excitatory amino acid transporter 2 (EAAT2). *Proc Natl Acad Sci USA.* 2003, 100:1955-1960.
9. Su Z-Z, Lebedeva IV, Sarkar D, Gopalkrishnan RV, Sigmon C, Yacoub A, Valerie K, Dent P, Fisher PB. Melanoma differentiation associated gene-7, *mda-7/IL-24*, selectively induces growth suppression, apoptosis and radiosensitization in malignant gliomas in p53-independent manner. *Oncogene.* 2003, 22: 1164-1180.
10. Lebedeva IV, Su Z-Z, Sarkar D, Fisher PB. Restoring apoptosis as a strategy for cancer gene therapy: focus on *p53* and *mda-7*. *Sem Cancer Biol.* 2003, 13: 169-178.
11. Sauane M, Gopalkrishnan RV, Sarkar D, Su Z-Z, Lebedeva IV, Dent P, Pestka S, Fisher PB. MDA-7/IL-24: novel cancer growth suppressing and apoptosis inducing cytokine. *Cytokine Growth Factor Rev.* 2003, 14: 35-51.
12. Sauane M, Gopalkrishnan RV, Lebedeva IV, Mei MX, Sarkar D, Su Z-z, Kang D-c, Dent P, Pestka S, Fisher PB. Mda-7/IL-24 induces apoptosis of diverse cancer

- cell lines through JAK/STAT-independent pathways. *J Cell Physiol.* 2003, 196: 334-345.
13. Yacoub A, Mitchell C, Lister A, Lebedeva IV, Sarkar D, Su Z-Z, Sigmon C, McKinstry R, Ramakrishnan V, Qiao L, Broaddus WC, Gopalkrishnan R, Grant S, Fisher PB, Dent P. Melanoma differentiation-associated 7 (interleukin 24) inhibits growth and enhances radiosensitivity of glioma cells *in vitro* and *in vivo*. *Clin Cancer Res.* 2003, 9:3272-3281.
 14. Yacoub A, Mitchell C, Brannon J, Rosenberg E, Qiao L, McKinstry R, Linehan WM, Su Z-Z, Sarkar D, Lebedeva IV, Valerie K, Gopalkrishnan R, Grant S, Fisher PB, Dent P. MDA-7 (Interleukin-24) inhibits the proliferation of renal carcinoma cells and interacts with free radicals to promote cell death and loss of reproductive capacity. *Mol Cancer Ther.* 2003, 2: 623-632.
 15. Yacoub A, Mitchell C, Lebedeva IV, Sarkar D, Su ZZ, McKinstry R, Gopalkrishnan RV, Grant S, Fisher PB, Dent P. mda-7 (IL-24) inhibits growth and enhances radiosensitivity of glioma cells *in vitro* via JNK signaling. *Cancer Biol Ther.* 2003, 2: 347-353.

Presentations at Scientific Meetings

1. Sarkar D, Su Z-Z, Lebedeva IV, Fisher PB. Exploiting defects in molecular circuitry as a novel approach for the therapy of pancreatic cancer. LustGarten Foundation, June, 2004.
2. Sarkar D, Leszczyniecka M, Kang D-C, Valerie K, Pandita TK, Fisher, PB. Induction of senescence-like growth arrest in melanocytes and malignant melanoma cells by human polynucleotide phosphorylase (*hPNPase^{old-35}*). 95th Annual Meeting of American Association for Cancer Research, Proceedings, #2413, Orlando, March, 2004.
3. Lebedeva IV, Su Z-Z, Sarkar D, Gopalkrishnan RV, Waxman S, Yacoub A, Dent P, Fisher PB. Strategy for promoting apoptosis selectively in pancreatic cancer cells. 95th Annual Meeting of American Association for Cancer Research, Proceedings, #2945, Orlando, March, 2004.
4. Su Z-Z, Lebedeva IV, Sarkar D, Kitada S, Dent P, Reed JC, Fisher PB. Radiation reverses resistance to mda-7/IL-24 in prostate cancer cells over-expressing the anti-apoptotic proteins Bcl-2 or Bcl-xL. 95th Annual Meeting of American Association for Cancer Research, Proceedings, #5574, Orlando, March, 2004.
5. Sarkar D, Leszczyniecka M, Kang D-C, Su Z-Z, Valerie K, Fisher, PB. Cloning and characterization of *hPNPase^{old-35}*, a human polynucleotide phosphorylase, in the context of terminal differentiation and senescence by an overlapping pathway screening strategy. 94th Annual Meeting of American Association for Cancer Research, Proceedings, #5183, Toronto, April, 2003.
6. Dent P, Yacoub A, Grant S, Gopalkrishnan R, Lebedeva I, Sarkar D, Su Z-Z, Lister A, Broaddus WC, Fisher, PB. MDA-7/IL-24 inhibits the growth and enhances the radiosensitivity of glioma cells, *in vitro* and *in vivo*. 94th Annual Meeting of American Association for Cancer Research, Proceedings, #5507, Toronto, April, 2003.

Conclusions:

I have demonstrated that RCBA in which the replication is regulated by cancer-selective PEG-Prom and that expresses cancer-selective apoptosis inducing cytokine mda-7/IL-24 (Ad.PEG-E1A.CMV-mda-7) specifically kills breast cancer cells without harming normal breast epithelial cells. However, RCBA in which replication is controlled by CMV promoter (Ad.CMV-E1A.CMV-mda-7) kills both breast cancer and normal cells. These preliminary findings indicate that Ad.PEG-E1A.CMV-mda-7 might be developed as a valuable reagent for gene therapy of breast cancer. The efficacy of the RCBA will be tested in eradicating breast cancer xenografts in nude mice that will pave the way for translating this RCBA to actual therapeutical application in breast cancer patients.

References:

1. Hortobagyi, G.N. (1998) Treatment of breast cancer. *N Engl J Med*, **339**, 974-984.
2. Su, Z., Shi, Y., Friedman, R., Qiao, L., McKinstry, R., Hinman, D., Dent, P. and Fisher, P.B. (2001) PEA3 sites within the progression elevated gene-3 (PEG-3) promoter and mitogen-activated protein kinase contribute to differential PEG-3 expression in Ha-ras and v-raf oncogene transformed rat embryo cells. *Nucleic Acids Res*, **29**, 1661-1671.
3. Su, Z., Shi, Y. and Fisher, P.B. (2000) Cooperation between AP1 and PEA3 sites within the progression elevated gene-3 (PEG-3) promoter regulate basal and differential expression of PEG-3 during progression of the oncogenic phenotype in transformed rat embryo cells. *Oncogene*, **19**, 3411-3421.
4. Su, Z.Z., Madireddi, M.T., Lin, J.J., Young, C.S., Kitada, S., Reed, J.C., Goldstein, N.I. and Fisher, P.B. (1998) The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. *Proc Natl Acad Sci U S A*, **95**, 14400-14405.
5. Lebedeva, I.V., Su, Z.-Z., Chang, Y., Kitada, S., Reed, J.C. and Fisher, P.B. (2002) The cancer growth suppressing gene mda-7 induces apoptosis selectively in human melanoma cells. *Oncogene*, **21**, 708-718.
6. Madireddi, M.T., Su, Z.Z., Young, C.S., Goldstein, N.I. and Fisher, P.B. (2000) Mda-7, a novel melanoma differentiation associated gene with promise for cancer gene therapy. *Adv Exp Med Biol*, **465**, 239-261.
7. Lebedeva, I. V., Sarkar, D., Su, Z.Z., Kitada, S., Dent, P., Stein, C.A., Reed, J.C. and Fisher, P.B. (2003) Bcl-2 and Bcl-xL differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, mda-7/IL-24. *Oncogene*, **22**, 8758-8773.
8. Sarkar, D., Su, Z.Z., Lebedeva, I.V., Sauane, M., Gopalkrishnan, R.V., Valerie, K., Dent, P. and Fisher, P.B. (2002) mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc Natl Acad Sci U S A*, **99**, 10054-10059.
9. Trimble, M.S., Xin, J.-H., Guy, C.T., Muller, W.J. and Hassell, J.A. (1993) PEA3 is overexpressed in mouse metastatic mammary adenocarcinomas. *Oncogene*, **8**, 3037-3042.
10. Benz, C.C., O'Hagan, R.C., Richter, B., Scott, G.K., Chang, C.-H., Xiong, X., Chew, K., Ljung, B.-M., Edgerton, S., Thor, A. and Hassell, J.A. (1997) HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. *Oncogene*, **15**, 1513-1525.