AD_____

Award Number: DAMD17-00-1-0312

TITLE: p202 as a Potential Therapeutic Gene for Breast Cancer

PRINCIPAL INVESTIGATOR: Mien-Chie Hung, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

REPORT DATE: April 2004

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Final (1 Apr 2000 - 31 Mar 2004)

4. TITLE AND SUBTITLE

p202 as a Potential Therapeutic Gene for Breast Cancer

5. FUNDING NUMBERS

DAMD17-00-1-0312

6. AUTHOR(S)

Mien-Chie Hung, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

E-Mail: mhung@mdanderson.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The main goal of this proposal is to study the anti-tumor activity of p202 and its application in a breast cancer model system. Three specific aims are 1) To determine the effects of p202 on the sensitivity of breast cancer cells to anticancer agents; 2) To test the anti-tumor activity of p202 in breast cancer cells using preclinical gene therapy strategies in an orthotopic breast cancer animal model; and 3) To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model. We have completed the first two aims in the funding period. However, In an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with FDA and were told that the p202 gene is of mouse origin and it is not favorable for human clinical trials. Hence, we turn our attention to the human 200 family members in a hope to identify a human gene associates with a strong anti-cancer activity as p202 does. To this end, we found that growth arrest of AIM2 is comparable to that of p202. Therefore, in the current status, we intend to further characterize the anti-cancer activity and therapeutic efficacy of AIM2 gene *in vitro* and *in vivo*, and to develop a gene therapy protocol using AIM2 as a therapeutic gene for breast cancer.

14. SUBJECT TERMS

Breast Cancer, gene therapy, tumor suppressor gene, p202

15. NUMBER OF PAGES

26

16. PRICE CODE

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

Table of Contents

Introduction

The Interferon (IFN) family of cytokines plays a crucial role in host defense system against viral, bacterial and parasitic infections and certain tumors (1). In addition, they also possess immunomodulatory and cell growth-inhibitory activities (2-8). Apart from the therapeutic effects of IFNs in certain clinical settings, there were also undesirable side effects (e.g., fever, chills, anorexia, and anemia) associated with a high dose of IFN treatment, which is often required to obtain a significant response (9-11). This has hampered the use of IFNs as effective anti-cancer agents (please see later). In an attempt to circumvent this potential drawback and to maintain the benefit of IFN treatment, we propose in this study to use AIM2, a human IFN-inducible protein, as a potential therapeutic agent. The structurally related human (AIM2, IFI16 and MNDA) and murine (p202, p203, p204 and D3) genes (12) belong to the 200-family, a family consisting of IFN-inducible proteins. Among them, p202 has been well characterized and shown to have a growth inhibitory function (13-15). Furthermore, p202 could inhibit tumorigenicity *in vivo* either by a tumorigenicity assay using p202-expressing PC3 cell lines or in an *ex vivo* tumorigenicity assay using the parental PC3 cells (15). In addition, it prolonged survival for LNCaP orthotopic tumor-bearing mice under a gene therapy setting through intravenous delivery of p202 gene complexed with liposome (15). We have also demonstrated that p202 associates potent anti-cancer activities including suppression of tumorigenicity, metastasis and angiogenesis in orthotopic animal models for breast and pancreatic cancers (16-17). Thus, the p202 gene, a murine member of IFN-inducible 200 family possesses a strong anti-cancer activity in multiple animal models.

In an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with FDA and were told that the p202 gene is of mouse origin and it is not favorable for human clinical trials. Hence, we turn our attention to the human 200 family members in a hope to identify a human gene associates with a strong anti-cancer activity as p202 does. To this end, we have isolated human 200 family genes through RT-PCR including AIM2, IFI16 and MNDA. The initial screening of cell-killing

activity among these three human genes, we found that growth arrest of AIM2 is much potent than the other two; and is comparable to that of the p202 gene. Therefore, in the current status, we intend to further characterize the anti-cancer activity and therapeutic efficacy of AIM2 gene *in vitro* and *in vivo*, and to develop a gene therapy protocol using AIM2 as a therapeutic gene for breast cancer.

Body

A. Specific Aims

Specific Aim 1: To determine the effect of p202 on the sensitivity of breast cancer cells to anti-cancer agents.

Specific Aim 2: To test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model.

Specific Aim 3: To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model.

B. Study and Results

In this final report, we have completed the Aims 1 and 2 (Appendices 1 and 2) in the funding period. As mentioned above, in an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with FDA and were told that the p202 gene is of murine origin and therefore, it is not appropriate for human clinical trials. In the last report, we have shown that AIM2 (Absent In Melanoma), a human homologue of p202 gene, has an anti-growth activity in breast cancer cells. Here, we report that AIM2 exhibits an anti-tumor activity on human breast cancer in an orthotopic mouse model (Appendix 3, Fig.1). Using luciferase report assay, we have demonstrated that, AIM2 expression as expected to be similar to p202, can repress NF-κB-mediated transcription activation in response to TNFα and p65-activated transcription, respectively (Appendix 3, Fig. 2 and 3). Therefore, human AIM2 may suppress tumor growth through a similar mechanism of p202.

To further investigate the growth inhibitory effect of AIM2, we have established stable clones of AIM2 by an inducible tetracycline regulatory system (Tet-off) *in vitro*

(Appendix 3, Fig. 4). In addition, we observed that AIM2 expression under inducible tetracycline regulatory system (Tet-off) decreases the tumor volume on human breast cancer in an orthotopic mouse model (Appendix 3, Fig. 5). Thus, AIM2 could be a potential candidate for future human clinical trials.

Key Research Accomplishments

- AIM2 exhibits a growth inhibitory activity in breast cancer cells.
- AIM2 suppresses tumor growth in an orthotopic animal model.
- AIM2 expression inhibits NF-κB-mediated transcription activation in response to TNFα.
- AIM2 expression represses p65 (NF-κB)-activated transcription.
- Establishment of the stable clones of AIM2 under an inducible tetracycline regulatory system (Tet-off) *in vitro*.
- AIM2 expression under inducible tetracycline regulatory system (Tet-off) inhibits tumor growth *in vivo*.

Reportable Outcomes and Personnel

A. Reportable Outcomes

Publication

1. Wen Y., Yan, D-H., Spohn, B., Deng, J., and Hung, M.-C. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon inducible protein, p202 in breast cancer cells. *Cancer Res.* 60:42-46, 2000.
2. Ding, Y., Wen Y., Spohn. B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., Hung, M.-C. and Yan, D.-H. Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer. *Clin. Cancer Res.* 8:3290-3297, 2002.

Grant Awarded

Under the support of DAMD17-00-1-0312, Dr. Bartholomeusz is awarded a DOD grant in Breast Cancer program, entitled "A novel apoptotic molecule Bok for treatment of

breast cancer", DAMD-17-02-1-0448 (funding period March 1, 2003- February 28, 2005).

B. Personnel

Principal Investigator: Mien-Chie Hung, Ph.D.

Co-Project Investigator: Geoffrey Batholomeusz, Ph.D.

Co-Project Investigator: Jaw-Ching Liu, Ph.D.

Ph.D. Graduate Student: Chi-Ping Day, M.S.

Conclusion and Significance

In this final report, we presented that AIM2 anti-growth activity and its molecular mechanism are similar to that of p202. Furthermore, we have successfully established AIM2 stable clones under an inducible tetracycline regulatory system (Tet-off). Using the newly obtained inducible clones of AIM2, we have demonstrated that AIM2 (Tet-off) inhibits tumor growth on breast cancer in an orthotopic animal model. These results suggest that AIM2 is a potential candidate in a gene therapy setting. We will develop a gene therapy protocol using AIM2 as a therapeutic gene for breast cancer.

References

1. Gutterman, J. U. Cytokine therapeutics: lessons from interferon a. Proc. Natl. Acad. Sci. USA, *91:* 1198–1205, 1994.
2. Lengyel, P. (1993). Tumor-suppressor genes: News about the interferon connection. Proc. Natl. Acad. Sci. USA *90*, 5893-5895.
3. Sen, G. C. (1991). The Hormonal Control of Gene Transcription., P. Cohen, and Foulkes, J. G., ed. (Amsterdam: Elsevier), pp. 349-374.
4. Sen, G. C., and Lengyel, P. (1992). The interferon system-a bird's eye view of its biochemistry. J. Bio. Chem. *267*, 5017-5020.
5. Sica, G., Fabbroni, L., Castagnetta, L., Cacciatore, M., and Pavone-Macaluso, M. (1989). Antiproliferative effects of interferons on human prostate carcinoma cell lines. Urol. Res. *17*, 111-115.
6. Sokoloff, M. H., Tso, C.-L., Kaboo, R., Taneja, S., Pang, S., deKernion, J. B., and Belldegrun, A. S. (1996). In vitro modulation of tumor progression-associated properties of hormone refractory prostate carcinoma cell lines by cytokines. Cancer *77*, 1862-1872.
7. van Moorselaar, R. J., van Stratum, P., Borm G., Debruyne, F. M., and Schalken, J. A. (1991). Differential antiproliferative activities of alpha and gamma-interferon and tumor necrosis factor alone or in combinations against two prostate cancer xenografts transplanted in nude mice. Prostate *18*, 331-344.
8. Hobeika, A. C., Subramaniam, P. S., and Johnson, H. M. (1997). IFN-α induces the expression of the cyclin-dependent kinase inhibitor p21 in human prostate cancer cells. Oncogene *14*, 1165-1170.
9. Ahre A, Bjorkholm, M., Osterberg, A., Brenning, G., Gahrton, G., and Gyllenhammar, H. (1988). High doses of natural alpha interferon in the treatment of multiple myeloma-a pilot study from the myeoloma group of central Sweden. Eur. J. Hematol. *41*, 123-130.
10. Kirkwood, J., Harris, J., Vera, R., Sandler, S., Fischer, D., and Khanderkar, J. (1985). A randomized study of low and high doses of leukocyte alpha-interferon in metastatic renal cell carcinoma: the American Cancer Society collaborative trial. Cancer Res. *45*, 863-871.
11. Mani, S., and Poo, W. J. (1996). Single institution experience with recombinant gamma-interferon in the treatment of patients with metastatic renal cell carcinoma. Am J Clin Oncol *19*, 149-53.
12. Landolfo S, Gariglio M, Gribaudo G, Lembo D. The Ifi 200 genes: an emerging family of IFN-inducible genes. Biochimie. 1998 Aug-Sep;80(8-9):721-8.
13. Xin H, Pramanik R, Choubey D. Retinoblastoma (Rb) protein upregulates expression of the Ifi202 gene encoding an interferon-inducible negative regulator of cell growth. Oncogene. 2003 Jul 31;22(31):4775-85.
14. Yan DH, Wen Y, Spohn B, Choubey D, Gutterman JU, Hung MC. Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202. Oncogene. 18:807-811, 1999.
15. Wen Y, Giri D, Yan DH, Spohn B, Zinner RG, Xia W, Thompson TC, Matusik RJ, Hung MC. Prostate-specific antitumor activity by probasin promoter-directed p202 expression. Mol Carcinog. 37:130-137, 2003.

16. Wen Y., Yan, D-H., Spohn, B., Deng, J., and Hung, M.-C. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon inducible protein, p202 in breast cancer cells. *Cancer Res.* 60:42-46, 2000.
17. Wen, Y., Yan, D.H., Wang, B., Spohn, B., Shao, R., Zou, Y., Ding, Y., Xie, K., and Hung, M.-C. p202, an interferon-inducible protein, mediates multiple anti-tumor activities in human pancreatic cancer xenograft models. *Cancer Res.* 61:7142-7147, 2001.

Appendices

1. Wen Y., Yan, D-H., Spohn, B., Deng, J., and Hung, M.-C. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon inducible protein, p202 in breast cancer cells. *Cancer Res.* 60:42-46, 2000.

2. Ding, Y., Wen Y., Spohn. B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., Hung, M.-C. and Yan, D.-H. Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer. *Clin. Cancer Res.* 8:3290-3297, 2002.

3. Recent Data of AIM2: Fig 1-5 and figure legends

[CANCER RESEARCH 60, 42–46, January 1, 2000]

Advances in Brief

Tumor Suppression and Sensitization to Tumor Necrosis Factor α-induced Apoptosis by an Interferon-inducible Protein, p202, in Breast Cancer Cells[1]

Yong Wen,[2] Duen-Hwa Yan,[2] Bill Spohn, Jiong Deng, Shiaw-Yih Lin, and Mien-Chie Hung[3]

Department of Cancer Biology, Section of Molecular Cell Biology [Y. W., D-H. Y., B. S., J. D., S-Y. L., M-C. H.], Department of Surgical Oncology [D-H. Y., M-C. H.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

p202, an IFN-inducible protein, interacts with several important regulatory proteins, leading to growth arrest or differentiation. In this report, we demonstrate that, in addition to inhibiting *in vitro* cell growth, p202 can also suppress the tumorigenicity of breast cancer cells *in vivo*. Furthermore, we found that p202 expression could sensitize breast cancer cells to apoptosis induced by tumor necrosis factor α treatment. One possible mechanism contributing to this sensitization is the inactivation of nuclear factor-κB by its interaction with p202. These results provide a scientific basis for a novel therapeutic strategy that combines p202 and tumor necrosis factor α treatment against breast cancer.

Introduction

IFNs possess a wide variety of biological properties such as antivirus, antiproliferation, immunoregulation, antiangiogenesis, and antineoplasia and have been used in clinical treatment of certain cancers (1). Here, we examined the possibility of using an IFN-inducible protein, p202 (2), as a potential therapeutic substitute for IFNs. p202 is a M_r 52,000 nuclear phosphoprotein known to be a negative transcription modulator that, in most cases, inhibits transcription of its target genes by physically interacting with certain transcription activators (3–8). Like IFN treatment, constitutive expression of p202 causes G_1-S cell cycle arrest in murine fibroblast cells (9, 10). Consistent to that observation, we demonstrated previously that the enforced expression of p202 could significantly retard the *in vitro* growth of prostate cancer cells in both cell culture and soft agar (10). However, it is not known whether p202 expression could exert an antitumor effect on cancer cells. In this report, we demonstrated for the first time that p202 expression was able to inhibit tumorigenicity of human breast cancer cells *ex vivo*. Furthermore, p202 expression can sensitize breast cancer cells to apoptosis induced by TNF[4]-α and that correlates with inactivation of NF-κB by a NF-κB/p202 interaction. These results suggest a potential combined therapy using p202 and TNF-α against breast cancer.

Received 7/27/99; accepted 11/11/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work is partially supported by NIH Grants RO-1 CA 58880 and CA 77858, M. D. Anderson Breast Cancer Research Program, and Faculty Achievement Award (to M-C. H). Y. W. is the recipient of a predoctoral fellowship from the United States Army Breast Cancer Research Training Grant Program (Grant DAMD 17-99-1-9264 to M-C. H.). S-Y. L. is a predoctoral fellow supported by the Department of Defense Army Breast Cancer Research Program, Grant DAMD17-98-1-8242.

[2] These two authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Cancer Biology, Section of Molecular Cell Biology, Box 108, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3630; Fax: (713) 794-0209.

[4] The abbreviations used are: TNF, tumor necrosis factor; NF, nuclear factor; CMV, cytomegalovirus; PEI, polyethylenimine; FACS, fluorescence-activated cell sorter.

Materials and Methods

Cell Culture, Transfection, and Colony-forming Assay. MDA-MB-453 and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection. Cells were maintained in DMEM/F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. Cells were transfected with a p202 expression vector (CMV-p202) or the control vector pcDNA3 (Invitrogen) using lipofectin (Life Technologies, Inc.) and selected in 500 μg/ml G418 (Geneticin; Life Technologies, Inc.). Western blotting using an anti-p202 polyclonal antibody (11) identified p202 stable transfectants.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay and FACS Analysis. These standard assays were done as described previously (12).

[^{3}H]Thymidine Incorporation Assay and Soft-Agar Assay. These standard assays were done as described previously (12).

Tumorigenicity Assay. Female athymic nude mice (*nu/nu*), 4–5 weeks of age, were used in this *ex vivo* experiment. Briefly, MCF-7 cells were transfected with CMV-p202 (10 μg) using PEI. Twenty-four h after transfection, cells (3×10^6) were harvested in 0.2 ml of PBS and injected into the mouse mammary fat pads. 17-β-Estradiol pellets (0.72 mg/pellet, 60-day release; Innovative Research of America, Inc.) were implanted s.c. into the mice 1-day before cell injection. The presence of estrogen is essential for MCF-7 cells to grow in mice. The size of the tumors was measured with a caliper every week, and the tumor volume was calculated using a formula: $V = 1/2 \times S^2 \times L$, where V = volume, S = the short length of the tumor, and L = the long length of the tumor in cm.

Immunoprecipitation and Immunoblotting. MDA-MB-453 (453) and 453-p202 cells were treated with 10 and 20 ng/ml of human TNF-α (R & D Systems, Inc., Minneapolis, MN) for 30 min. Cells with or without TNF-α treatment were extracted in RIPA lysis buffer without SDS on ice. Exacts were sonicated and cleared by centrifugation at 4°C. For immunoprecipitation, equivalent aliquots of cell lysates (1 mg of total protein) were incubated with 1 μg of anti-p65 antibody (Santa Cruz Biotechnology) for 4 h with gentle rotation at 4°C. Protein A-Sepharose beads (50 μl) was added for an additional 1 h. The beads were extensively washed with ice-cold RIPA buffer, and the precipitate was dissolved in a sample buffer for electrophoresis and Western blot.

Results and Discussion

To investigate a potential growth-inhibitory effect of p202 on breast cancer cells, we performed a colony-forming assay by transfecting a p202 expression plasmid driven by CMV promoter (CMV-p202) or a control vector (pcDNA3) containing neomycin-resistance gene into two human breast cancer cell lines, MDA-MB-453 (453) and MCF-7. After 3 weeks of G418 selection, the number of G418-resistant colonies was scored. A dramatic reduction in the number of G418-resistant colonies was seen in cells (MCF-7 and 453) transfected with p202 as compared with that with the control plasmid, pcDNA3 (Fig. 1*a, left panel*). There was at least a 75% reduction in colony number in both p202-transfected cell lines (Fig. 1*a, right panel*). These data suggest that p202 expression may be associated with antiproliferation

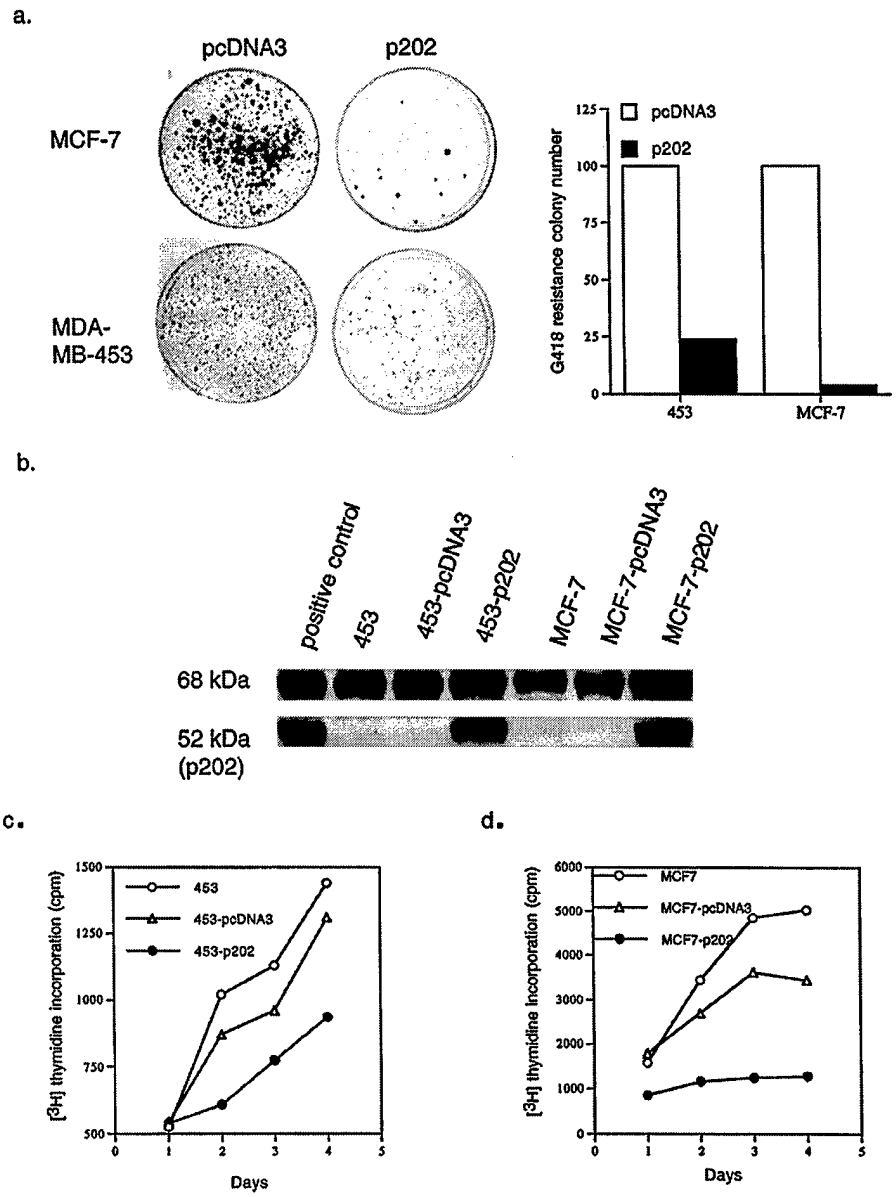

Fig. 1. Expression of p202 inhibits the proliferation of MDA-MB-453 and MCF-7 breast cancer cells. *a*, colony-forming assay. MDA-MB-453 and MCF-7 cells were transfected with either a control vector (pcDNA3) or a p202 expression vector. The colony number obtained from pcDNA3 transfection was set as 100%. *b*, Western blot analysis of the p202 stable transfectants. The M_r 52,000 protein represents p202, and the nonspecific M_r 68,000 protein cross-reacting with the antibody was used as an equal loading control. *c* and *d*, [^{3}H]thymidine incorporation assays. DNA synthesis rate was measured by the amount of [^{3}H]thymidine incorporated into the cells at each time point. The measurement was conducted in quadruplicates, and the variations within each quadruplicate are too small to be of any significance.

and/or proapoptotic activity in these breast cancer cells. To further characterize the biological effects of p202 expression on these cells, we attempted to isolate several lines of p202-expressing stable clones. Using Western blot with a p202-specific antibody (11), we were able to identify one p202-expressing stable clone (of 20) from each cell line, *i.e.*, MDA-MB-453-p202 (453-p202) and MCF-7-p202 (Fig. 1*b*). The low frequency of p202-expressing clones obtained from the G418-resistant colony supports the idea that p202 expression may cause an antiproliferation and/or proapoptotic effect on these cells. To assess these two p202-mediated biological effects, we first measured and compared the mitogenic activity between the p202 stable lines and the control cell lines using [^{3}H]thymidine incorporation assay. The p202-expressing cells (453-p202 and MCF-7-p202) exhibited a reduced DNA synthesis rate as compared with their respective control cell lines, *i.e.*, 453 and 453-pcDNA3; MCF-7 and MCF-7-pcDNA3 (Fig. 1, *c* and *d*). Similarly, the p202-expressing cells also showed a slower growth rate than the control cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Thus, our data strongly suggest that p202 functions as a growth inhibitor in breast cancer cells.

To test whether p202 expression in breast cancer cells may also suppress their *in vitro* transformation phenotype defined by the ability of these cells to grow in soft agar, we then measured the number of colonies formed in soft agar by the p202-expressing cells and the control cells. As shown in Fig. 2, *a* and *b*, both 453-p202 and MCF-7-p202 exhibited >60% reduction (after 3 weeks of incubation) in colony number than those of the parental and pcDNA3 transfectant. The difference in number was not attributable to the slower growth rate of the p202-expressing cells than that of the control cells (Fig. 1, *c* and *d*), because a prolonged (6 weeks) incubation of the same plates did not yield more colonies. Rather, it represents a real loss of anchorage-independent growth, *i.e.*, an *in vitro* transformation phenotype, of these p202-expressing cells.

One of the most critical biological properties determining the potential application of a tumor suppressor gene in cancer therapy is its ability to reduce tumorigenicity *in vivo*. To test a possible antitu-

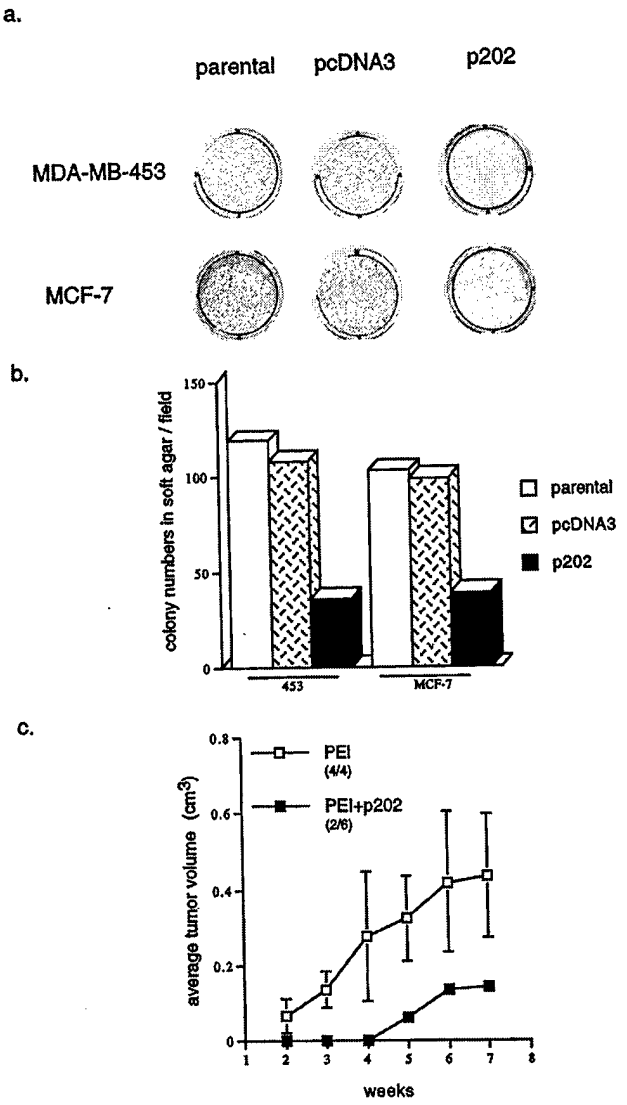

Fig. 2. p202 inhibits the transformation phenotype of breast cancer cells. *a*, colony formation in soft agar. MDA-MB-453, 453-pcDNA3, 453-p202, MCF-7, MCF-7-pcDNA3, and MCF-7-p202 cell lines were subjected to anchorage-independent growth in soft agar. *b*, number of colonies formed in soft agar as shown in *a*. The number represents the average of five random microscopic fields from each cell line. *c*, p202 *ex vivo* experiment. MCF-7 cells were transfected with p202 expression vector using PEI. After 24 h, cells were harvested, and the p202 (*PEI+p202*) or mock (*PEI*) transfected cells (3×10^6 cells/injection) were injected into the mammary fat pad of female nude mice. 17-β-Estradiol pellets were implanted s.c. into the mice 1-day before inoculation. Tumor formation was monitored every week. *Bars*, SE.

mor activity of p202, we performed an *ex vivo* tumorigenicity assay in an orthotopic breast cancer model. Briefly, CMV-p202/PEI or PEI alone (PEI is a polymer vector used for transfection) was transfected into MCF-7 cells before injection into the mammary fat pads of the estrogen-supplemented nude mice. The p202 transfection caused a drastic reduction of tumorigenesis of MCF-7 cells as compared with that of the mock transfection (PEI alone; Fig. 2*c*). Our data demonstrated, for the first time, that the p202 expression is associated with an antitumor activity in animals.

In an attempt to identify therapeutic agents that may cooperate with p202 to synergize the antitumor effect on breast cancer cells, we used FACS analysis (Fig. 3) to determine a potential synergism in inducing apoptosis. We found that the p202-expressing cells were more susceptible to TNF-α-induced apoptosis than the control cells, *i.e.*, after treatment with TNF-α (0, 10, 20 ng/ml) for 48 h, more 453-p202 cells were undergoing apoptosis (sub-G_1 population) than the parental 453 cells and 453-pcDNA3 control cells in a dose-dependent manner (Fig. 3*a*). Likewise, MCF-7-p202 cells were also found to be more sensitive to TNF-α-induced apoptosis than the parental MCF-7 cells in a dose-dependent manner (Fig. 3*b*). These results suggested that p202 expression could sensitize cells to TNF-α-induced apoptosis.

One possible mechanism of the p202-mediated sensitization to TNF-α-induced apoptosis is that p202 could antagonize the antiapoptotic function of NF-κB (13–15). To test that hypothesis, we tested whether p202 expression could affect the NF-κB-mediated transcription activation in response to TNF-α treatment. We cotransfected CMV-p202 and a NF-κB-activatable promoter-reporter construct (κB-luc), *i.e.*, an IκB promoter-driven luciferase gene, into 453 cells in the presence of TNF-α (Fig. 4*a*). As expected, κB-luc was readily activated in the presence of TNF-α. However, this TNF-α-induced transcription activation was repressed by p202 in a dose-dependent manner. To test whether p202 acted on the NF-κB molecule to elicit such transcription repression, we cotransfected CMV-p202 with a Rel-A (a p65 subunit of NF-κB) cDNA expression vector and κB-luc. As shown in Fig. 4*b*, whereas p202 expression alone has no effect on κB-luc, it could greatly repress NF-κB (Rel-A)-activated IκB pro-

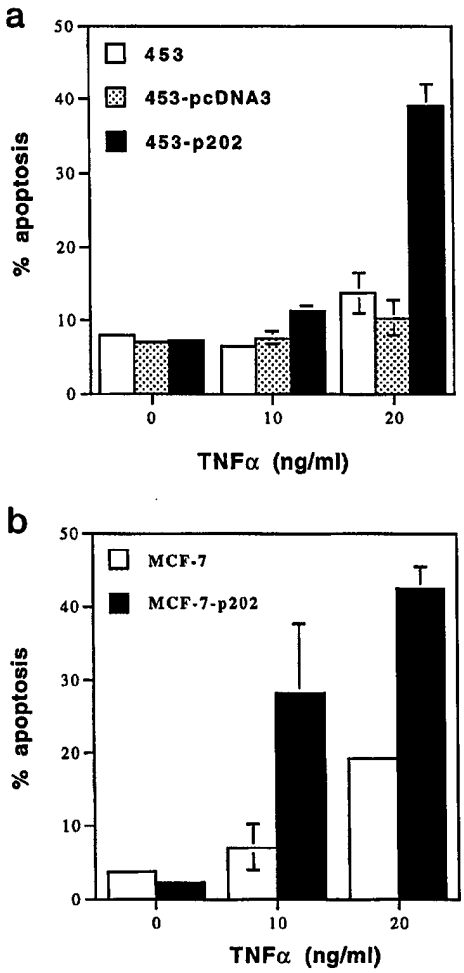

Fig. 3. p202 sensitizes breast cancer cells to apoptosis induced by TNF-α in a dose-dependent manner. *a*, 453, 453-pcDNA3, and 453-p202 cell lines were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. *Bars*, SE. *b*, MCF-7 and MCF-7-p202 were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. Cells were fixed and stained with propidium iodide. Apoptosis was quantitated by FACScan cytometer. *Bars*, SE.

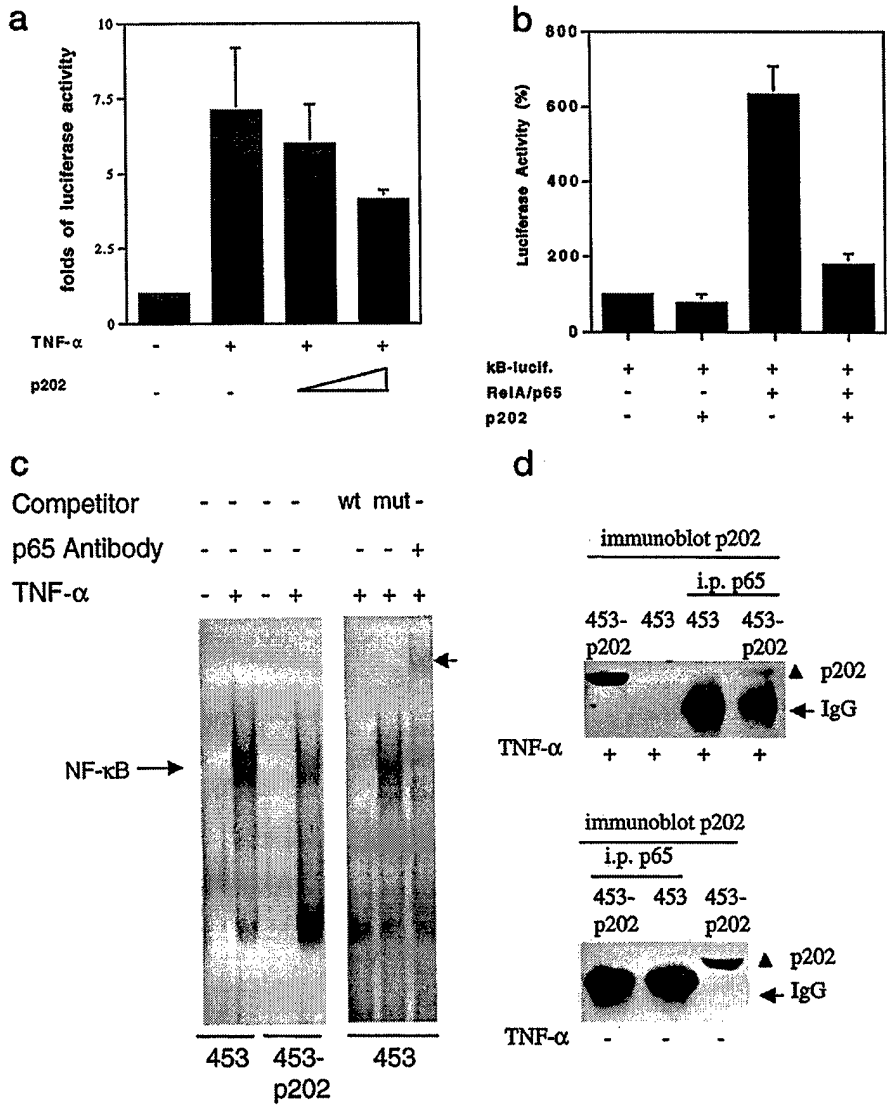

Fig. 4. The interaction and inactivation of NF-κB by p202 is responsible for the p202-mediated sensitization to TNF-α-induced apoptosis. *a*, p202 expression represses NF-κB-mediated transcription activation in response to TNF-α. IκB-Luciferase reporter gene (0.2 μg) and CMV-p202 (0, 0.8, or 2 μg) were cotransfected into MDA-MB-453 cells. Thirty-six h after transfection, cells were either left untreated or stimulated with TNF-α (20 ng/ml) for 6 h. The fold difference in IκB-Luciferase expression was calculated with respect to IκB-Luciferase expression in the absence of TNF-α and p202. *b*, p202 expression represses Rel-A (p65)-activated transcription. MDA-MB-453 cells were cotransfected with κB-luc and ± NF-κB (p65) expression vector. The inhibitory activity of p202 on the induction of IκB promoter activity by p65 was assessed by cotransfection with p202 expression vector. Luciferase activity was measured 48 h after transfection. The data represent an average of two independent experiments after normalization; *bars*, SE. *c*, gel-shift assay. 453 and 453-p202 nuclear extracts, used in this assay, were isolated from TNF-α-treated cells (20 ng/ml of for 30 min). *Left panel*, the activated NF-κB (p65/p50) induced by TNF-α is indicated by an *arrow*. *Right panel*, competition assay was performed in the presence of a 70-fold excess of wild-type or mutant oligonucleotides containing NF-κB binding site. A polyclonal Rel-A antibody supershifted the NF-κB complex to a slower-migrating position, as indicated by an *arrow*. *d, top panel*, p202 is physically associated with p65. 453 and 453-p202 cells were treated with or without TNF-α (20 ng/ml for 30 min). Cell lysates (1 mg) were used in the subsequent immunoprecipitation with anti-p65 antibody. Immunoprecipitated complexes were analyzed by SDS-PAGE, followed by immunoblotting with p202 antibody. *Bottom panel*, immunoblots of p202 protein using untreated 453 and 453-p202 cells serve as negative and positive controls, respectively. ▲, p202 band. IgG band is also indicated.

moter activity. These results suggest that the transcriptional repression of TNF-α-mediated gene expression by p202 may be attributable to the inactivation of NF-κB by p202.

This hypothesis was further supported by a subsequent observation that p202 expression was associated with a reduced level of the active NF-κB (p65/p50) molecule as measured by a gel-shift assay (Fig. 4*c, left panel*). As expected, the level of active NF-κB was found to be significantly increased in both the p202-expressing (435-p202) and the parental (453) cells treated with TNF-α (20 ng/ml). However, the level of activated NF-κB was greatly reduced in 453-p202. Using either a wild-type or mutant NF-κB DNA binding sequence as a competitor, we showed that the DNA/protein complex was indeed NF-κB specific in that only wild-type, but not mutant, sequence could compete with the NF-κB/DNA complex. Moreover, the fact that this complex could be supershifted in the presence of an anti-p65 antibody (Fig. 4*c, right panel*) further confirms the identity of this DNA/protein complex being NF-κB-specific. Thus, these data support the idea that p202 expression may impede the formation of active p65/p50 heterodimer. That, in turn, represses transcriptional activation induced by NF-κB.

It is possible that p202 may interact with p65, forming a p202/p65 complex, which may significantly reduce the concentration of free p65 in p202-expressing cells. To test that possibility, we performed a coimmunoprecipitation assay. As shown in Fig. 4*d, upper panel*, with TNF-α treatment, p202 could be coimmunoprecipitated with p65 by an anti-p65 antibody in 453-p202 nuclear extract but not 453 extract. As a control, no detectable p202 was observed in either cell line without TNF-α treatment (Fig. 4*d, lower panel*). These data strongly indicate that p202 and p65 are physically associated in the same complex upon TNF-α stimulation. The p65 protein level is comparable between 453 and 453-p202 cells with TNF-α treatment (data not shown), indicating that p202 may not regulate p65 expression.

The above observation presents a possible scenario that TNF-α-induced NF-κB activation could be antagonized by p202 via a p202/p65 interaction. That, in turn, causes subsequent transcriptional repression of genes, the activation of which requires active NF-κB. Although it has been reported previously that p202 could bind both p50 and p65 *in vitro* and p50 *in vivo* (6), our data are the first demonstration of an *in vivo* association between p202 and p65 upon TNF-α stimulation. Taken together, our results provide a possible mechanism that accounts for the p202-mediated sensitization to TNF-α-induced apoptosis in breast cancer cells.

Inflammatory cytokines, *e.g.*, TNF family members, can transduce apoptotic signals in certain tumor cells and have been tested in a number of clinical trials (16). Despite the promising data in animal models, unsatisfactory results have been observed in many clinical trials (17). It might be attributable to the resistance of many cancer cells to TNF-α-induced apoptosis, presumably, by the activation of NF-κB and the subsequent induction of survival factors that counteract apoptosis. In this report, we demonstrated that p202 expression not only exerted strong growth retardation and tumor suppression activities in breast cancer cells but also is able to sensitize these cells to TNF-α-induced apoptosis, and that sensitization is associated with inactivation of NF-κB via a p202/p65 interaction. Thus, our data implicate a potential therapeutic application of a combined treatment of TNF-α and p202 gene therapy for cancer patients.

References

1. Gutterman, J. U. Cytokine therapeutics: lessons from interferon α. Proc. Natl. Acad. Sci. USA, *91:* 1198–1205, 1994.
2. Choubey, D., Snoddy, J., Chaturvedi, V., Toniato, E., Opdenakker, G., Thakur, A., Samanta, H., Engel, D. A., and Lengyel, P. Interferons as gene activators–indications for repeated gene duplication during the evolution of a cluster of interferon-activatable genes on murine chromosome 1. J. Biol. Chem., *264:* 17182–17189, 1989.
3. Choubey, D., Li, S-J., Datta, B., Gutterman, J. U., and Lengyel, P. Inhibition of E2F-mediated transcription by p202. EMBO J., *15:* 5668–5678, 1996.
4. Choubey, D., and Gutterman, J. U. Inhibition of E2F-4/DP-1-stimulated transcription by p202. Oncogene, *15:* 291–301, 1997.
5. Datta, B., Li, B., Choubey, D., Nallur, G., and Lengyel, P. p202, an interferon-inducible modulator of transcription, inhibits transcriptional activation by the p53 tumor suppressor protein, and a segment from the p53-binding protein 1 that binds to p202 overcomes this inhibition. J. Biol. Chem., *271:* 27544–27555, 1996.
6. Min, W., Ghosh, S., and Lengyel, P. The interferon-inducible p202 protein as a modulator of transcription: inhibition of NFκB, c-Fos, and c-Jun activities. Mol. Cell. Biol., *16:* 359–368, 1996.
7. Datta, B., Min, W., Burma, S., and Lengyel, P. Increase in p202 expression during skeletal muscle differentiation: inhibition of MyoD protein expression and activity by p202. Mol. Cell. Biol., *18:* 1074–1083, 1998.
8. Choubey, D., and Lengyel, P. Binding of an interferon-inducible protein (p202) to the retinoblastoma protein. J. Biol. Chem., *270:* 6134–6140, 1995.
9. Lembo, D., Angeretti, A., Benefazio, S., Hertel, L., Gariglio, M., Novelli, F., and Landolfo, S. Constitutive expression of the interferon-inducible protein p202 in NIH3T3 cells affects cell cycle progression. J. Biol. Regul. Homeostatic Agents, *9:* 42–46, 1995.
10. Yan, D-H., Wen, Y., Spohn, B., Choubey, D., Gutterman, J. U., and Hung, M-C. Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202. Oncogene, *18:* 807–811, 1999.
11. Choubey, D., and Lengyel, P. Interferon action: cytoplasmic and nuclear localization of the interferon-inducible 52-kD protein that is encoded by the *Ifi202* gene from the gene 200 cluster. J. Interferon Res., *13:* 43–52, 1993.
12. Shao, R., Karunagaran, D., Zhou, B. P., Li, K., Lo, S-S., Deng, J., Chiao, P., and Hung, M-C. Inhibition of nuclear factor-κB activity is involved in E1A-mediated sensitization of radiation-induced apoptosis. J. Biol. Chem., *272:* 32739–32742, 1997.
13. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB [see comments]. Science (Washington DC), *274:* 784–787, 1996.
14. Beg, A. A., and Baltimore, D. An essential role for NF-κB in preventing TNF-α-induced cell death [see comments]. Science (Washington DC), *274:* 782–784, 1996.
15. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB [see comments]. Science (Washington DC), *274:* 787–789, 1996.
16. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A., Jr., and Shepard, H. M. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. Science (Washington DC), *230:* 943–945, 1985.
17. Frei, E. D., and Spriggs, D. Tumor necrosis factor: still a promising agent. J. Clin. Oncol., *7:* 291–294, 1989.

Proapoptotic and Antitumor Activities of Adenovirus-mediated p202 Gene Transfer[1]

Yi Ding, Yong Wen, Bill Spohn, Li Wang, Weiya Xia, Ka Yin Kwong, Ruping Shao, Zheng Li, Gabriel N. Hortobagyi, Mien-Chie Hung, and Duen-Hwa Yan[2]

Departments of Molecular and Cellular Oncology [Y. D., Y. W., B. S., L. W., W. X., K. Y. K., R. S., Z. L., M-C. H., D-H. Y.], Breast Medical Oncology [G. N. H.], and Surgical Oncology [M-C. H., D-H. Y.]. The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Purpose and Experimental Design: **p202, a mouse IFN-inducible protein, is a member of the 200-amino acid repeat family. Enforced p202 expression in stable cancer cell lines resulted in growth inhibition *in vitro* and tumor suppression *in vivo*. However, to study the immediate effect of p202 and test the potential efficacy of p202 treatment, an efficient gene delivery system for p202 is required. For these purposes, an adenoviral vector expressing the p202 gene (Ad-p202) was generated. We examined the effects of Ad-p202 infection on human breast cancer cells. Furthermore, we tested the efficacy of Ad-p202 treatment on breast and pancreatic cancer xenograft models.**

Results: **We found that Ad-p202 infection induces growth inhibition and sensitizes the otherwise resistant cells to tumor necrosis factor α-induced apoptosis. In addition, we demonstrated for the first time that Ad-p202 infection induces apoptosis and that activation of caspases is required for the full apoptotic effect. More importantly, we showed the efficacy of Ad-p202 treatment on breast cancer xenograft models, and this antitumor effect correlated well with enhanced apoptosis in Ad-p202-treated tumors.**

Conclusions: **We conclude that Ad-p202 is a potent growth-inhibitory, proapoptotic, and tumor-suppressing agent. Ad-p202 may be further developed into an efficient therapeutic agent for human cancer gene therapy.**

INTRODUCTION

IFN is known to exert antiproliferative and antiviral actions. It has both direct and indirect (immunological) antitumor activity in several human malignancies, including leukemia and lymphomas as well as solid tumors. Aside from the therapeutic effects of IFN in certain clinical settings, there are also undesirable side effects (*e.g.*, fever, chills, anorexia, and anemia) associated with the high-dose IFN treatment that is often required to obtain a significant response (1, 2). This has hampered IFN as an effective anticancer agent. In an attempt to circumvent this potential drawback and maintain the benefit of IFN-mediated antitumor activity, we have begun to explore the possibility of using an IFN-inducible protein, p202, as a potential therapeutic agent (3–5). p202 is a mouse IFN-inducible, chromatin-associated protein. It belongs to the 200-amino acid repeat family (6, 7). The unique feature of p202 is illustrated by its ability to interact with several important transcriptional regulators that include E2Fs, Rb, pocket proteins p130 and p107, Fos/Jun, c-Myc, NF-κB,[3] and p53BP-1 (reviewed in Ref. 8), resulting in transcriptional repression of genes that are up-regulated by these transcriptional regulators. The exact role of p202 in the IFN-mediated signal pathway is not well defined. However, consistent with the multiple antitumor activities of IFN (9), enforced expression of p202 in stable murine fibroblasts and human cancer cell lines leads to retardation of cell growth and suppression of transformation phenotype (3, 5, 10, 11). Furthermore, breast cancer cells stably transfected with p202 are sensitized to TNF-α-induced apoptosis (5), and that effect is associated with inactivation of the TNF-α-induced NF-κB via p202-NF-κB interaction. We postulated that p202 sensitizes cancer cells to TNF-α-induced apoptosis by inactivating NF-κB, which, in turn, turns off NF-κB-activated antiapoptotic gene expression, leading to enhanced TNF-α-induced cell killing (5).

To generate a p202-based therapeutic agent for efficacy study in animal models and a tool to study the biological function of p202, we constructed Ad-p202. In this study, we

Received 1/22/02; revised 6/26/02; accepted 6/27/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the University Cancer Foundation at the University of Texas M. D. Anderson Cancer Center, Department of Defense Grant DAMD17-99-1-9270, and Texas Advanced Technology Program under Grant 003657-0082-1999 (to D-H. Y.); NIH Grant CA77858 and Department of Defense Grants DAMD17-00-1-0312 and DAMD17-01-1-0071 (to M-C. H.); the Breast Cancer Research Foundation/Estee Lauder Foundation (G. N. H. and M-C. H.); and Cancer Center Core Grant 16672. Y. W. is a recipient of a predoctoral fellowship from the Department of Defense Breast Cancer Research Training Grant DMAD17-99-1-9264.

[2] To whom requests for reprints should be addressed, at Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3677; Fax: (713) 794-0209; E-mail: dyan@mdanderson.org.

[3] The abbreviations used are: NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor α; i.t., intratumor; CMV, cytomegalovirus; GFP, green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOI, multiplicity of infection; PARP, poly-(ADP-ribose) polymerase; pfu, plaque-forming unit(s); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PI, postinfection; fmk, fluoromethyl ketone; Z-VAD, N-benzyloxycarbonyl-Val-Ala-Asp; Z-DEVD, N-benzyloxycarbonyl-Asp-Glu-Val-Asp.

show that Ad-p202 infection of breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. Interestingly, we found that Ad-p202 infection alone induces apoptosis in breast cancer cells, and the activation of caspases is critical for this process. More importantly, we demonstrated the efficacy of Ad-p202 treatment in human breast cancer xenograft models through either i.t. or i.v. injection. This antitumor activity correlated well with p202 expression and apoptosis in Ad-p202-treated tumors. Together, our results suggest that Ad-p202 is a potent growth-inhibitory, proapoptotic, antitumor agent that could be further developed to become an effective therapeutic agent for cancer gene therapy treatment.

MATERIALS AND METHODS

Generation of Ad-p202. Ad-p202 was constructed according to the protocol described previously (12). p202 cDNA (11) was subcloned into an adenovirus vector (pAdTrack-CMV) that carries a CMV promoter-driven GFP. A separate CMV promoter directs p202 cDNA. A control virus, an adenoviral vector expressing luciferase gene and GFP (Ad-Luc), was likewise generated. The expression of GFP gene enabled us to monitor the infection efficiency by direct observation using a fluorescence microscope.

***In Vitro* Growth Assays.** MDA-MB-468 human breast cancer cells were maintained in DMEM/Ham's F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. MTT is a pale yellow substrate that can be cleaved by living cells (but not dead cells) to yield a dark blue formazan product. The extent of MTT cleavage determined colorimetrically (at 570 nm) can be used to measure cell proliferation. Briefly, 2×10^3 cells were plated in 96-well culture plates in 0.1 ml of culture medium. Ad-p202 or Ad-Luc was added at a MOI of 200 on the next day. At the different times indicated, 20 μl of MTT (5 mg/ml stock solution) were added to each well. Cells were cultured for an additional 2 h, and then 100 μl of lysis buffer [20% SDS in 50% *N,N*-dimethylformamide (pH 4.7)] were added to each well, followed by 5 h of incubation, and then absorbance was measured at 570 nm. [^{3}H]Thymidine incorporation assay was performed as described previously (13).

Apoptosis Assays. For flow cytometry analysis, cells were collected at the indicated times PI, washed once with PBS, and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a nylon mesh and then adjusted to a final concentration of 0.1% (w/v) RNase and 50 μg/ml propidium iodide. Apoptotic cells were quantified by FACScan cytometer. The DNA fragmentation assay was carried out as described previously (13).

Western Blot Analysis. MDA-MB-468 cells treated with or without TNF-α (R&D Systems, Inc., Minneapolis, MN) were infected with Ad-p202 or Ad-Luc at a MOI of 200. Seventy-two h PI, cells were lysed with radioimmunoprecipitation assay lysis buffer. The protein extracts were subjected to SDS-PAGE followed by Western blotting according to the procedure described previously (5). Goat anti-p202 polyclonal antibody and anti-PARP antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Laboratories (Lexington, KY), respectively. Caspase inhibitors Z-VAD and Z-DEVD-fmk were purchased from Enzyme Systems Products (Livermore, CA).

Gel-Shift Assay. The NF-κB gel-shift assay was performed as described previously (13).

Ad-p202 Gene Therapy in Human Cancer Xenograft Models. For the orthotopic breast cancer xenograft model, MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads (2 tumors/mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: group 1, Ad-Luc; and group 2, Ad-p202. For i.t. injection, 1×10^9 pfu viruses/treatment was administered. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for 7 weeks and once a week thereafter. For tail vein injection, 5×10^8 pfu viruses/treatment were administered. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for 5 weeks and once a week thereafter.

Immunohistochemical Analysis of p202 Expression and Apoptosis. Mice were sacrificed 24 h after the last treatment. Tumors obtained from Ad-p202- or Ad-Luc-treated mice bearing either breast or pancreatic tumors were then excised and fixed with formalin and embedded in paraffin. Immunohistochemical analysis of p202 protein expression was performed according to the protocol described previously (14). Tumor sections were incubated with goat polyclonal antibody specific for p202 (Santa Cruz Biotechnology) followed by incubation with biotinylated rabbit antigoat IgG and subsequent incubation with avidin-biotin peroxidase before visualization. TUNEL assay was performed to detect the ends of degraded DNA fragments induced by apoptosis according to the protocol described previously (15).

RESULTS

Ad-p202 Mediates p202 Expression in Breast Cancer Cells. To test the efficiency and monitor the expression of p202 protein by Ad-p202 infection, we infected MDA-MB-468 breast cancer cells with either Ad-p202 or Ad-Luc followed by fluorescence microscopy and Western blot analysis, respectively. As shown in Fig. 1*A*, Ad-p202 and Ad-Luc infection at a MOI of 200, at 24-h PI, exhibited >90% infection efficiency as indicated by the GFP-positive cells shown in a representative field (Fig. 1*A*, *right panels*). The same cells are shown in phase-contrast images (Fig. 1*A*, *left panels*). The mock-infected cells (*Control*) showed no GFP expression. In addition to MDA-MB-468, we found that Ad-p202 could infect a panel of other human breast cancer cell lines (*e.g.*, MDA-MB-453, MDA-MB-435, MDA-MB-231, and MCF-7), albeit with various infection efficiency rates (data not shown). We chose the MDA-MB-468 cell line for subsequent studies because it is tumorigenic in the mouse xenograft model and has relatively high infection efficiency by Ad-p202. The expression of p202 protein in Ad-p202-infected cells was further analyzed by Western blot using p202-specific antibody. Fig. 1*B* shows that whereas the mock- and Ad-Luc-infected cells have no p202 expression, Ad-p202 infection efficiently directed p202 expression in MDA-MB-468 cells in a dose-dependent manner. These results clearly demonstrate that Ad-p202 infection adequately directs p202 expression in MDA-MB-468 cells.

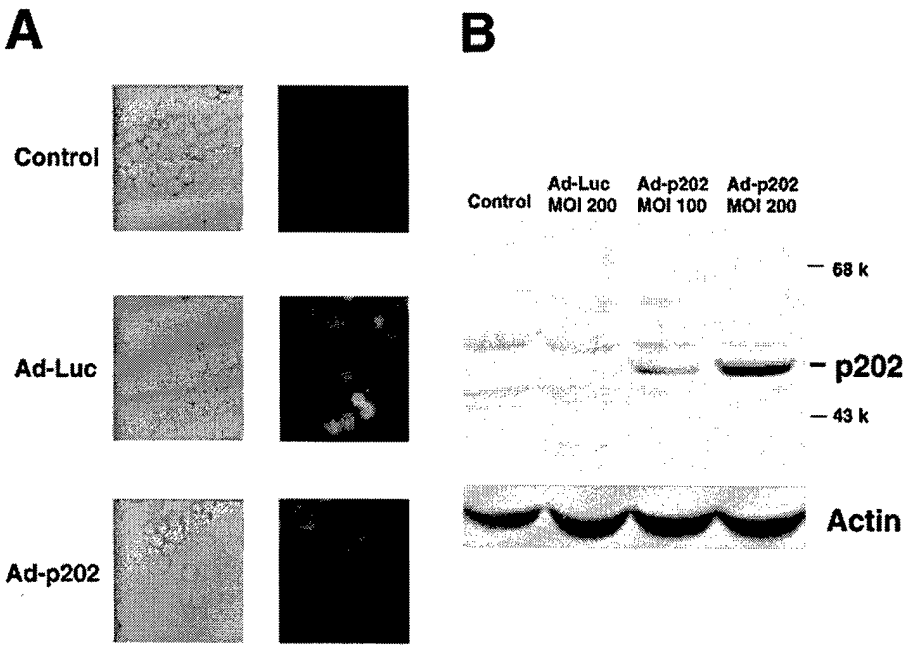

Fig. 1 Ad-p202 construction, p202 expression, and infection efficiency. *A,* Ad-p202 was generated according to the protocol described previously (12). The pAdTrack-CMV vector contains two independent CMV promoter-driven transcription units, one for GFP and one for p202 cDNA. MDA-MB-468 human breast cancer cells were infected by Ad-Luc or Ad-p202 at a MOI of 200. Twenty-four h PI, >90% of cells were found to be GFP positive as visualized by fluorescence microscopy (*right panels*), indicating that the infection efficiency is >90%. *Left panels,* phase-contrast microscopy. *Control,* mock-infected cells. *B,* p202 protein is expressed in Ad-p202 infected cells. MDA-MB-468 cells infected with Ad-Luc or Ad-p202 for 72 h were analyzed for p202 protein expression by Western blot. *Control,* mock-infected cells. Actin protein was used as an equal loading control.

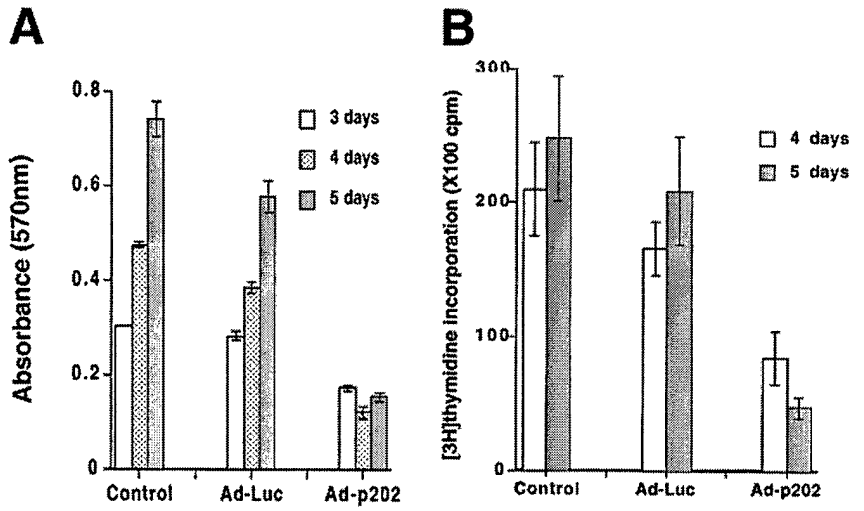

Fig. 2 Ad-p202 infection inhibits cell proliferation. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at a MOI of 200. Cell growth was monitored at the indicated PI time (3–5 days) by (*A*) MTT assay [means ± SD (n = 3)] or (*B*) [^{3}H]thymidine incorporation assay; data are presented as the means of quadruplicates.

Ad-p202 Infection Reduces Breast Cancer Cell Growth. To assess the effect of Ad-p202 infection on cell growth, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc followed by *in vitro* growth assays such as the MTT assay and [^{3}H]thymidine incorporation assay at different time points, *i.e.*, 3–5 days PI. As shown in Fig. 2, whereas the mock infection (*Control*) and Ad-Luc infection have no growth-inhibitory effect on MDA-MB-468 cells, Ad-202 infection significantly hampered cell growth (Fig. 2*A*) and DNA synthesis rates (Fig. 2*B*). This observation strongly indicates that Ad-p202 infection inhibits cell growth in breast cancer cells and is congruent with our previous findings using stable cancer cell lines (3, 5).

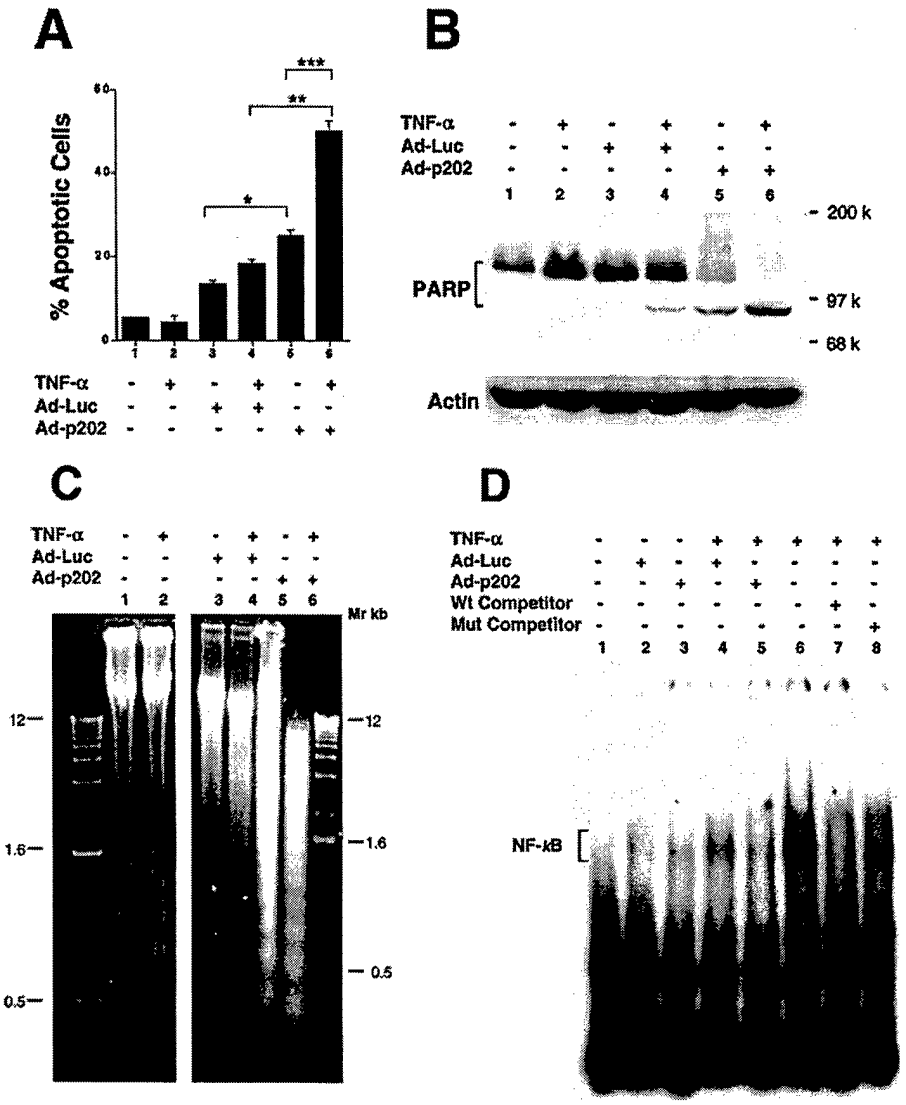

Fig. 3 Ad-p202 infection induces apoptosis and sensitizes cells to apoptosis induced by TNF-α. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at a MOI of 200. *A*, 24 h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 48 h (72 h PI). Apoptosis was then monitored by flow cytometry analysis (done in triplets; *bars*, SD). *t* test: *, $P < 0.001$; **, $P < 0.0002$; and ***, $P < 0.0005$. *B*, PARP cleavage assay. Twenty-four h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h PI. The PARP protein (M_r 116,000) was cleaved into M_r 85,000 product in the event of apoptosis. *C*, DNA fragmentation assay. Twenty-four h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h PI. *D*, Ad-p202 infection inhibits TNF-α-induced NF-κB DNA binding activity. MDA-MB-468 cells were infected with Ad-p202 or Ad-Luc in the presence or absence of TNF-α (50 ng/ml) 24 h PI for 30 min. The nuclear extracts were then isolated and incubated with a radioactive-labeled oligonucleotide containing NF-κB binding site (13). The excess cold wild-type or mutant NF-κB binding site was added to the incubation to demonstrate the specific NF-κB DNA binding activity. The NF-κB-DNA complex is indicated.

Ad-p202 Infection Induces Apoptosis in Breast Cancer Cells. Without stress signals, the p202 stable cancer cell lines do not exhibit apoptotic phenotype (3, 5). It is possible that p202 stable cell lines isolated after a vigorous selection process may possess a physiologically tolerant level of p202. The fact that only a small number of p202 stable cell lines were obtained by colony-forming assay (3, 5) raises the possibility that p202 expression alone may induce apoptosis. To test that possibility, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc followed by flow cytometry analysis at 72-h PI to detect apoptosis by measuring the cell population in the sub-G_1 phase of cell cycle. As shown in Fig. 3*A*, although Ad-Luc infection induced modest apoptosis (compare *Lane 1* with *Lane 3*), Ad-p202 infection (*Lane 5*) caused significantly more apoptosis (>20%) than Ad-Luc infection (*Lane 3*; $P < 0.001$, *t* test). That observation was further confirmed by two other apoptosis assays: (*a*) the PARP cleavage assay, in which full-length PARP (M_r 116,000) is cleaved by caspases into a fragment of approximately M_r 85,000 (Fig. 3*B*); and (*b*) a DNA fragmentation assay that is based on the activated endonucleases during apoptosis (Fig. 3*C*). Ad-p202 infection resulted in a marked increase of the PARP cleavage product (M_r 85,000; Fig. 3*B*, *Lane 5* and Fig. 4*A*, *Lane 3*) and an enhancement of DNA fragmentation (Fig. 3*C*, *Lane 5*). In contrast, Ad-Luc infection yielded a minimum amount of M_r 85,000 PARP cleavage product (Fig. 3*B*, *Lane 3* and Fig. 4*A*, *Lane 2*) as well as a near basal level of DNA fragmentation (Fig. 3*C*, compare *Lanes 1* and *3*). Together, our results strongly indicate that Ad-p202 alone induces apoptosis in MDA-MB-468 cells. Given that the MDA-MB-468 cell line harbors mutant p53 (16), Ad-p202-mediated apoptosis thus appears to be independent of p53 status.

p202-mediated Apoptosis Is Caspase-dependent. Because caspases are activated during apoptosis and have a variety

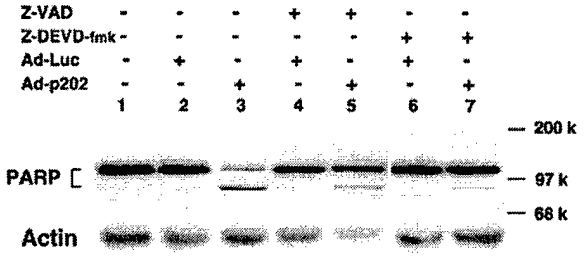

Fig. 4 The activation of caspases is critical for Ad-p202-mediated apoptosis. Z-VAD (100 μM) and Z-DEVD-fmk (80 μM) inhibit Ad-p202-mediated apoptosis in MDA-MB-468 cells. Western blot analysis of PARP cleavage and actin expression was performed 48 h PI. The intensity of full-length PARP and PARP cleavage product bands was measured using NIH Image 1.62 software.

of substrates including PARP (17), the cleavage of PARP in Ad-p202-infected cells suggests that the activation of caspase may be involved in Ad-p202-induced apoptosis. To test that hypothesis, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc in the presence or absence of a pan-caspase inhibitor, Z-VAD. At 48 h PI, the intensity of full-length PARP and PARP cleavage product bands on Western blot was measured using NIH Image 1.62 software. The percentage of M_r 85,000 product was calculated by setting the total intensity of both M_r 116,000 and M_r 85,000 bands in each lane at 100%. As shown in Fig. 4, the addition of Z-VAD attenuates Ad-p202-induced apoptosis, as indicated by the reduced (but not completely eliminated) level of PARP M_r 85,000 cleavage product from 57.4% (*Lane 3*) to 13.3% (*Lane 5*), whereas Z-VAD has no effect on PARP cleavage in Ad-Luc-infected cells (*Lane 4*). This result supports the idea that the activation of caspases, at least in part, is required for Ad-p202 to induce full apoptotic effect. Because PARP is also a substrate for caspase-3, which is considered to be a crucial enzyme commonly activated during apoptosis (17), we examined whether the activation of caspase-3 plays a role in Ad-p202-induced apoptosis. To that end, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc in the presence of a caspase-3-specific inhibitor, Z-DEVD-fmk (18). As shown in Fig. 4, the level of PARP cleavage product in Ad-p202-infected MDA-MB-468 cells is significantly reduced to 8.9% (*Lane 7*) with Z-DEVD-fmk treatment as compared with that without Z-DEVD-fmk (57.4%; *Lane 3*). As a control, no detectable PARP cleavage was observed in Ad-Luc-infected cells treated with Z-DEVD-fmk (*Lane 6*). Thus, this result suggests that the activation of caspase-3 is critical for Ad-p202-mediated apoptosis. To further confirm this observation, we infected a caspase-3-null breast cancer cell line, MCF-7 (19), with Ad-p202 or Ad-Luc, followed by flow cytometry analysis. We observed that although p202 was readily expressed as determined by Western blot, the Ad-p202-infected MCF-7 cells yielded the similar level of apoptosis as that of the controls, *i.e.*, mock and Ad-Luc infection (data not shown). Therefore, our data suggest that the activation of caspases is critical for Ad-p202 to exert full apoptotic effect.

Ad-p202 Infection Sensitizes Breast Cancer Cells to TNF-α-induced Apoptosis. We tested whether Ad-p202 infection could also sensitize breast cancer cells to TNF-α-induced apoptosis (5). Although MDA-MB-468 cells appear to be resistant to TNF-α (50 ng/ml; added at 24 h PI for 48 h)-induced apoptosis (Fig. 3*A*, *Lanes 1* and *2*; Fig. 3*B*, *Lanes 1* and *2*; and Fig. 3*C*, *Lanes 1* and *2*), the combination of TNF-α and Ad-p202 induced massive cell killing [compare Fig. 3*A*, *Lanes 5* and *6* ($P < 0.0005$); Fig. 3*B*, *Lanes 5* and *6*; and Fig. 3*C*, *Lanes 5* and *6*]. These results suggest that Ad-p202 infection sensitizes MDA-MB-468 cells to TNF-α-induced apoptosis. In contrast, the apoptosis resulting from the combined treatment of TNF-α and Ad-Luc (Fig. 3*A*, *Lane 4*) was significantly less than that from combined treatment with TNF-α and Ad-p202 (Fig. 3*A*, *Lane 6*; $P < 0.0002$). This observation was confirmed by PARP cleavage and DNA fragmentation assays (Fig. 3*B*, *Lanes 4* and *6*; Fig. 3*C*, *Lanes 4* and *6*). These data indicate that the sensitization to TNF-α-induced apoptosis is specific to p202 expression. Because p202-mediated sensitization to TNF-α-induced apoptosis correlated with the inactivation of NF-κB, specifically, via the loss of NF-κB DNA binding activity (5), we next tested whether Ad-p202 infection affects TNF-α-induced NF-κB DNA binding activity. At 24 h PI, TNF-α was added for 30 min, and the nuclear extract was then isolated and subsequently incubated with a radioactive-labeled oligonucleotide containing the NF-κB binding sites. A gel-shift assay was then performed to detect the NF-κB DNA binding activity. As shown in Fig. 3*D*, we observed a complete abolishment of TNF-α-induced NF-κB DNA binding activity in Ad-p202-infected MDA-MB-468 cells (Fig. 3*D*, compare *Lanes 1, 3, 5,* and *6*). As controls, TNF-α-induced NF-κB DNA binding activity (*Lane 6*) can be readily competed by cold wild-type NF-κB DNA binding site (*Lane 7*) and, to a lesser extent, by cold mutant probe (*Lane 8*). Ad-Luc infection also reduces the TNF-α-induced NF-κB DNA binding activity somewhat, but to a lesser extent than Ad-p202 (*Lanes 4* and *5*). Together, our data suggest that Ad-p202 infection could sensitize otherwise resistant MDA-MB-468 cells to apoptosis induced by TNF-α, which correlates with a loss of TNF-α-induced NF-κB DNA binding activity.

Antitumor Activity of Ad-p202 in Cancer Xenograft Models. To test the efficacy of Ad-p202 treatment in an orthotopic breast cancer xenograft model, we implanted MDA-MB-468 cells (2×10^6 cells) into mammary fat pads of female nude mice. Treatment began when tumor size reached ~0.5 cm in diameter (about 2 weeks after implantation). We then treated tumor-bearing mice (7 tumors/treatment group) with either Ad-p202 or control virus Ad-Luc (1×10^9 pfu/treatment) via i.t. injection. Treatments were administered twice per week for 7 weeks and once a week thereafter. Tumor size was measured by using the following formula: tumor size = $1/2 \times L \times S^2$, where L and S are the longest and shortest diameters measured, respectively. The tumor size distribution with Ad-p202 or Ad-Luc treatment at two time points (day 25 and day 67) is presented. Whereas there was little difference at the early stage of treatment (Fig. 5*A*, day 25; $P = 0.13$), the Ad-p202-treated tumors grew significantly slower than those treated with Ad-Luc on day 67 ($P = 0.04$). This result supports the idea of a p202-based gene therapy in breast cancer treatment. Because breast cancer is a metastatic disease, it is critical to develop a systemic delivery system for p202 gene transfer. Although the antitumor effect by i.t. treatment is encouraging, no report has shown a therapeutic effect by systemic administration of p202 in a cancer xenograft model. We then performed systemic gene therapy

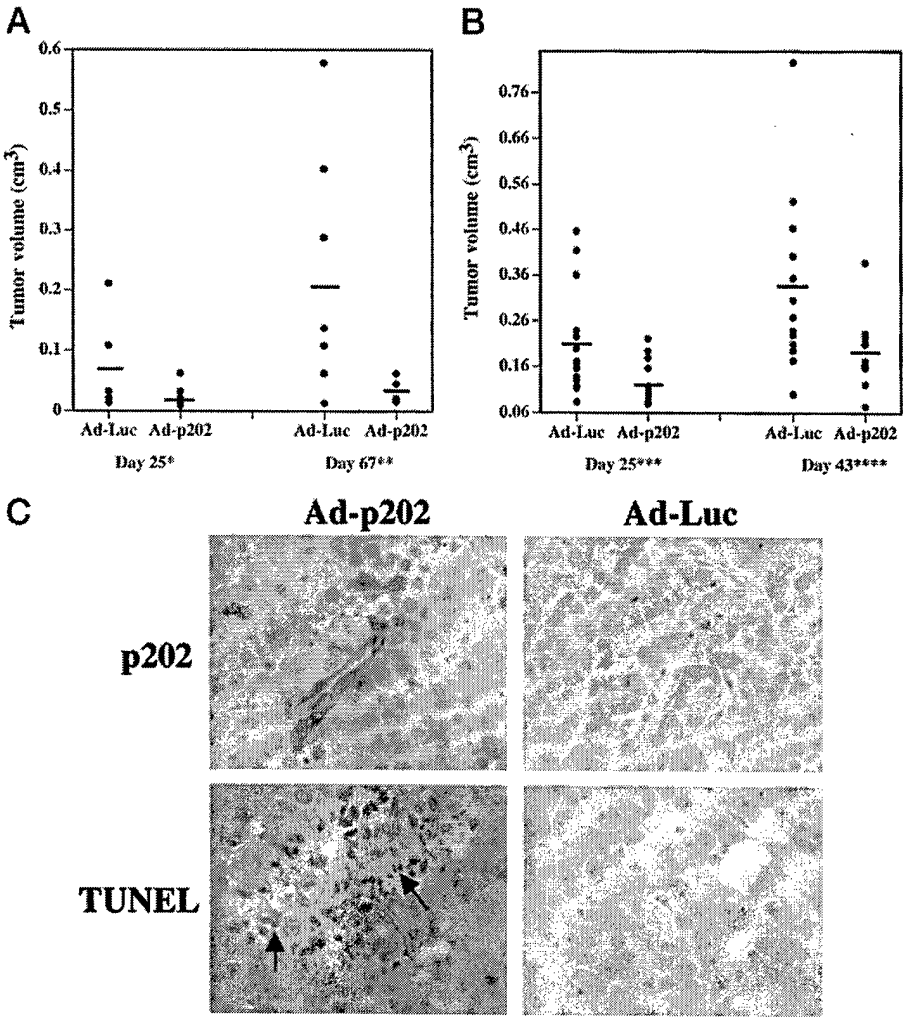

Fig. 5 Antitumor effect by systemic delivery of Ad-p202 in an orthotopic breast cancer xenograft model. *A,* Ad-p202-mediated antitumor effect on breast cancer xenografts by i.t. treatment. MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads of each female nude mouse. Tumor-bearing mice were divided into two treatment groups, Ad-Luc (total, 7 tumors) and Ad-p202 (total, 7 tumors), at 1×10^9 pfu/treatment via i.t. injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for 7 weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, *i.e.*, day 25 and day 67. *t* test: *, $P = 0.13$; and **, $P = 0.04$. *B,* Ad-p202-mediated antitumor effect on breast cancer xenografts by systemic treatment. MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads (2 tumors/mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups, Ad-Luc (total, 14 tumors) and Ad-p202 (total, 14 tumors), at 5×10^8 pfu via tail vein injection. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for 5 weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, *i.e.*, day 25 and day 43. *t* test: ***, $P = 0.0097$; and ****, $P = 0.014$. *C,* apoptosis correlates with p202 expression in Ad-p202-treated breast tumors. Mice were sacrificed 24 h after the last systemic treatment as described above. Tumors were then excised and fixed for the subsequent immunohistochemical analysis. p202 expression was analyzed by using an antibody specific for p202 on tumor samples obtained from Ad-p202- or Ad-Luc-treated mice (14). The TUNEL assay was also performed to detect apoptotic cells in these tumors (15). The *arrows* indicate the representatives of apoptotic cells.

experiments by treating tumor-bearing mice with Ad-p202 or Ad-Luc (5×10^8 pfu/treatment, 14 tumors/group) through tail vein injection. Treatments were administered twice per week for 5 weeks and once a week thereafter. As shown in Fig. 5*B*, Ad-p202-treated mice had a significantly reduced tumor growth rate as compared with the Ad-Luc-treated mice on day 25 ($P = 0.0097$) and day 43 ($P = 0.014$). The above-mentioned observation strongly suggests the feasibility of using a systemic p202-based gene therapy treatment for breast cancer. Because Ad-p202 induces apoptosis *in vitro* (Fig. 3), it is likely that the observed antitumor activity may correlate with enhanced apoptosis in Ad-p202-treated tumors. To test this possibility, we examined the presence of apoptosis in breast tumors treated systemically with Ad-p202. Immunostaining for p202 protein and apoptotic cells were performed 24 h after the last Ad-p202 and Ad-Luc treatment. As shown in Fig. 5*C*, p202 expression was readily detected by immu-

nohistochemical staining in Ad-p202-treated tumors but not in tumors treated with Ad-Luc. Interestingly, strong p202 expression was found in endothelial cells of a tumor blood vessel. It may be due to systemic delivery of Ad-p202. As predicted, apoptosis, as determined by TUNEL assay, is prevalent in Ad-p202-treated tumors but not in Ad-Luc-treated tumors (Fig. 5*C*). The *arrows* indicate the representatives of apoptotic cells. This observation is consistent with our *in vitro* data showing that p202 expression induces apoptosis (Fig. 3). We have also performed a similar Ad-p202 preclinical gene therapy treatment in a human pancreatic cancer xenograft model (4). Consistent with the data presented here, Ad-p202 treatment (by i.t. injection) inhibited tumor growth and induced apoptosis in tumors (data not shown). Taken together, the above-mentioned observations strongly indicate that p202 is a potent tumor-suppressing agent, and its apoptosis-inducing activity contributes to the multiple p202-mediated antitumor activities.

DISCUSSION

In this report we showed that, consistent with our previous findings using p202 stable breast cancer cell lines (5), Ad-p202 infection in MDA-MB-468 breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. Importantly, we demonstrated for the first time that Ad-p202 infection alone induces apoptosis *in vitro*. The correlation between p202 expression and enhanced apoptosis observed in Ad-p202-treated tumors also supports the *in vitro* observation. However, it is possible that the apoptosis could be the result of an artifact caused by coexpression of p202 and adenoviral proteins. We ruled out that possibility because Ad-Luc infection of a p202 stable cell line, 453-p202 (5), did not result in enhanced apoptosis as compared with that of the vector control cells infected by Ad-Luc (data not shown). This result thus strongly suggests that the Ad-p202-induced apoptosis is not likely due to cooperation between p202 and certain adenoviral proteins during infection. Rather, it indicates that a certain cellular apoptotic pathway was activated by p202 expression. Indeed, as shown in Fig. 4, Ad-p202-induced apoptosis requires caspase-3 activation to achieve a full apoptotic effect.

Here, we demonstrated the feasibility of using Ad-p202 in preclinical gene therapy settings. In particular, Ad-p202 treatment by i.t. or i.v. injection resulted in significant tumor suppression in an orthotopic breast cancer xenograft model. Our data are consistent with that reported previously using p202 delivery systems other than adenoviral vector, *i.e.*, polymer and liposome (4, 5). The efficacy of systemic Ad-p202 treatment is encouraging because it shows that Ad-p202 had overcome immunological (nude mice possess immune response, albeit much reduced), physiological, and structural barriers inside and outside the blood vessels to reach tumor cells and unloads the p202 therapeutic gene (20). This result is the first demonstration of efficacy by systemic treatment of p202. It is possible that the systemic Ad-p202 treatment may affect normal tissues the same way it affects tumor tissues. One way to minimize the potential cytotoxicity of the p202 effect on normal tissues is to develop a tumor-specific p202 expression system using a breast cancer-specific promoter to direct p202 expression. This effort is currently in progress. Although toxicity, if any, associated with Ad-p202 treatment remains to be determined, our results nevertheless raise the possibility of using p202-based gene therapy in systemic cancer treatment. In Ad-p202-treated tumors, we also found a reduced level of an angiogenic marker, vascular endothelial growth factor (data not shown). This observation is consistent with the ability of p202 to inhibit angiogenesis (4).

In addition to prostate (data not shown) and breast cancer xenograft models (this study and Ref. 5), the fact that Ad-p202 treatment resulted in an antitumor effect on a pancreatic cancer xenograft model (data not shown; Ref. 4) suggests a general application of p202-based gene therapy in cancer treatment. In addition, because p202 sensitizes cells to TNF-α-induced apoptosis (this study and Ref. 5), our data further support the possible use of Ad-p202/TNF-α combined therapy to achieve better efficacy, especially for cancer cells that are resistant to TNF-α therapy. Experiments are under way to test this possibility in animal models. Taken together, the data we present here strongly suggest that Ad-p202 is a potent therapeutic agent suitable for further development in cancer gene therapy.

REFERENCES

1. Ahre, A., Bjorkholm, M., Osterberg, A., Brenning, G., Gahrton, G., and Gyllenhammar, H. High doses of natural α interferon in the treatment of multiple myeloma: a pilot study from the myeloma group of central Sweden. Eur. J. Hematol., *41:* 123–130, 1988.

2. Kirkwood, J., Harris, J., Vera, R., Sandler, S., Fischer, D., and Khanderkar, J. A randomized study of low and high doses of leukocyte α-interferon in metastatic renal cell carcinoma: the American Cancer Society collaborative trial. Cancer Res., *45:* 863–871, 1985.

3. Yan, D-H., Wen, Y., Spohn, B., Choubey, D., Gutterman, J. U., and Hung, M-C. Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202. Oncogene, *18:* 807–811, 1999.

4. Wen, Y., Yan, D-H., Wang, B., Spohn, B., Ding, Y., Shao, R., Zhou, Y., Xie, K., and Hung, M-C. p202, an interferon-inducible protein, mediates multiple anti-tumor activities in human pancreatic cancer xenograft models. Cancer Res., *61:* 7142–7147, 2001.

5. Wen, Y., Yan, D. H., Spohn, B., Deng, J., Lin, S. Y., and Hung, M. C. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon-inducible protein, p202, in breast cancer cells. Cancer Res., *60:* 42–46, 2000.

6. Landolfo, S., Gariglio, M., Gribaudo, G., and Lembo, D. The Ifi 200 genes: an emerging family of IFN-inducible genes. Biochimie (Paris), *80:* 721–728, 1998.

7. Johnstone, R. W., and Trapani, J. A. Transcription and growth regulatory functions of the HIN-200 family of proteins. Mol. Cell. Biol., *19:* 5833–5838, 1999.

8. Choubey, D. p202: an interferon-inducible negative regulator of cell growth. J. Biol. Regul. Homeost. Agents, *14:* 187–192, 2000.

9. Ferrantini, M., and Belardelli, F. Gene therapy of cancer with interferon: lessons from tumor models and perspectives for clinical applications. Semin. Cancer Biol., *10:* 145–157, 2000.

10. Lembo, D., Angeretti, A., Benefazio, S., Hertel, L., Gariglio, M., Novelli, F., and Landolfo, S. Constitutive expression of the interferon-inducible protein p202 in NIH3T3 cells affects cell cycle progression. J. Biol. Regul. Homeost. Agents, *9:* 42–46, 1995.

11. Choubey, D., Li, S-J., Datta, B., Gutterman, J. U., and Lengyel, P. Inhibition of E2F-mediated transcription by p202. EMBO J., *15:* 5668–5678, 1996.

12. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. USA, *95:* 2509–2514, 1998.

13. Shao, R., Karunagaran, D., Zhou, B. P., Li, K., Lo, S-S., Deng, J., Chiao, P., Hung, M-C. Inhibition of nuclear factor-κB activity is in-

volved in E1A-mediated sensitization of radiation-induced apoptosis. J. Biol. Chem., *272:* 32739–32742, 1997.

14. Xia, W., Lau, Y. K., Zhang, H. Z., Xiao, F. Y., Johnston, D. A., Liu, A. R., Li, L., Katz, R. L., and Hung, M. C. Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. Clin. Cancer Res., *5:* 4164–4174, 1999.

15. Shao, R., Hu, M. C., Zhou, B. P., Lin, S. Y., Chiao, P. J., von Lindern, R. H., Spohn, B., and Hung, M. C. E1A sensitizes cells to tumor necrosis factor-induced apoptosis through inhibition of IκB kinases and nuclear factor κB activities. J. Biol. Chem., *274:* 21495–21498, 1999.

16. Hunt, K. K., Deng, J., Liu, T. J., Wilson-Heiner, M., Swisher, S. G., Clayman, G., and Hung, M. C. Adenovirus-mediated overexpression of the transcription factor E2F-1 induces apoptosis in human breast and ovarian carcinoma cell lines and does not require p53. Cancer Res., *57:* 4722–4726, 1997.

17. Thornberry, N. A., and Lazebnik, Y. Caspases: enemies within. Science (Wash. DC), *281:* 1312–1316, 1998.

18. Pastorino, J. G., Chen, S. T., Tafani, M., Snyder, J. W., and Farber, J. L. The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J. Biol. Chem., *273:* 7770–7775, 1998.

19. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem., *273:* 9357–9360, 1998.

20. Nishikawa, M., and Huang, L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum. Gene Ther., *12:* 861–870, 2001.

Appendix 3: 5 Figures

Fig.1 AIM2 exhibits an anti-tumor effect on human breast cancer cell in an orthotopic mouse model. Six week-old female nude mice (ten mice/group) were inoculated with $2x10^6$ MDA-MB-435 breast cancer cells under mammary fat pad. Intra-tumoral gene therapy with AIM2/Liposome (20μg/8μl, every other day) started after tumors being established. Tumor sizes were measured with a caliper twice a week. The tumor volume was calculated using the formula: Volume SxS*x*L/2 where *S* is the short length of the tumor in cm and *L* is the long length of the tumor in mm ($p<0.01$).

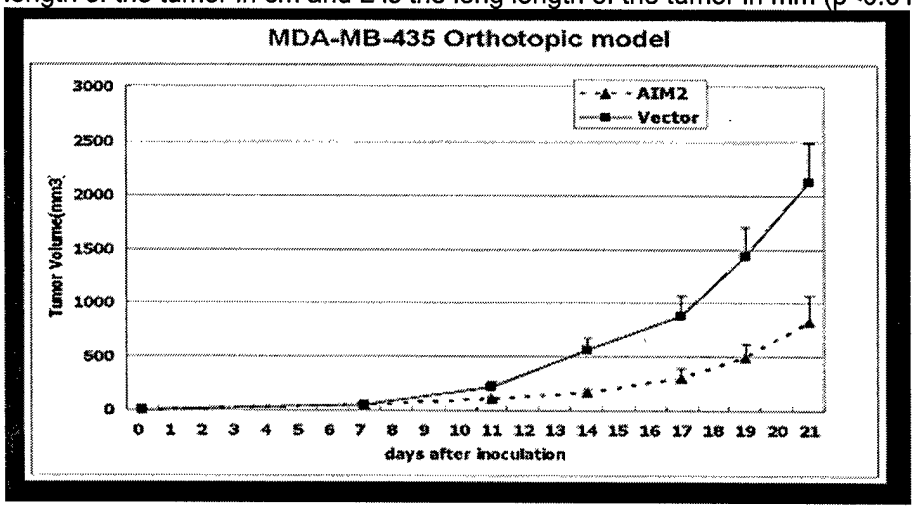

Fig. 2 AIM2 expression represses NF-κB-mediated transcription activation in response to TNF-α. κB-Luciferase reporter gene (0.2 μg) and CMV-Flag-AIM2 (0, 0.5, or 1.5 μg) were cotransfected into MDA-MB-453 and MDA-MB-435 cells. Thirty-six hours after transfection, cells were either left untreated or stimulated with TNFα (20ng/ml) for 6 hours. The relative luciferase activity in IκB-Luciferase expression was calculated by setting κB-Luciferase expression in the absence of TNFα and AIM2 as 100%. It is worthwhile to mention that the NF-κB activity in the presence of AIM2 is even lower than the basal activity without TNFα stimulation. It suggests that AIM2 strongly inhibits NF-κB activity including both basal level and TNFα-induced NF-κB activity.

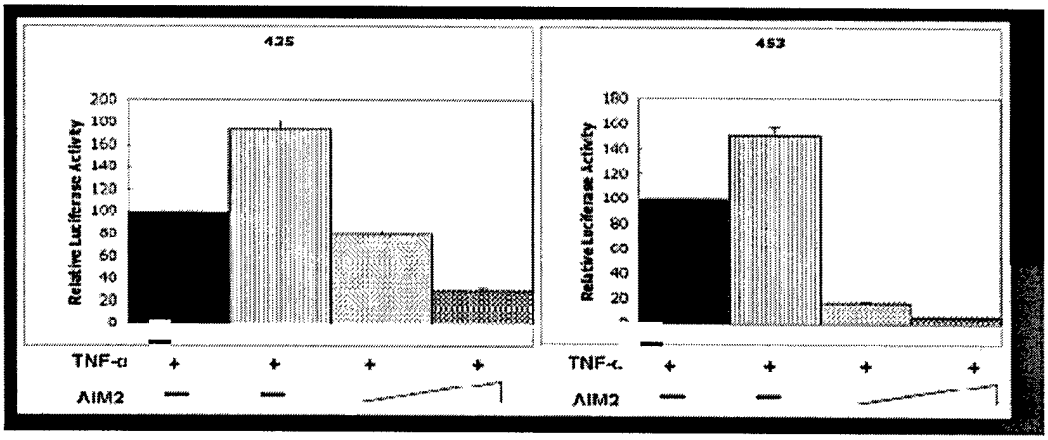

Fig. 3. AIM2 expression represses p65-activated transcription. MDA-MB-453 and MDA-MB-435 cells were cotransfected with κB-luc and NF-κB (p65) expression vectors. The inhibitory activity of AIM2 on the induction of IkB promoter activity by p65 was assessed by cotransfection with AIM2 expression vector. Luciferase activity was measured 48 hrs after transfection. The data represent an average of two independent experiments after normalization.

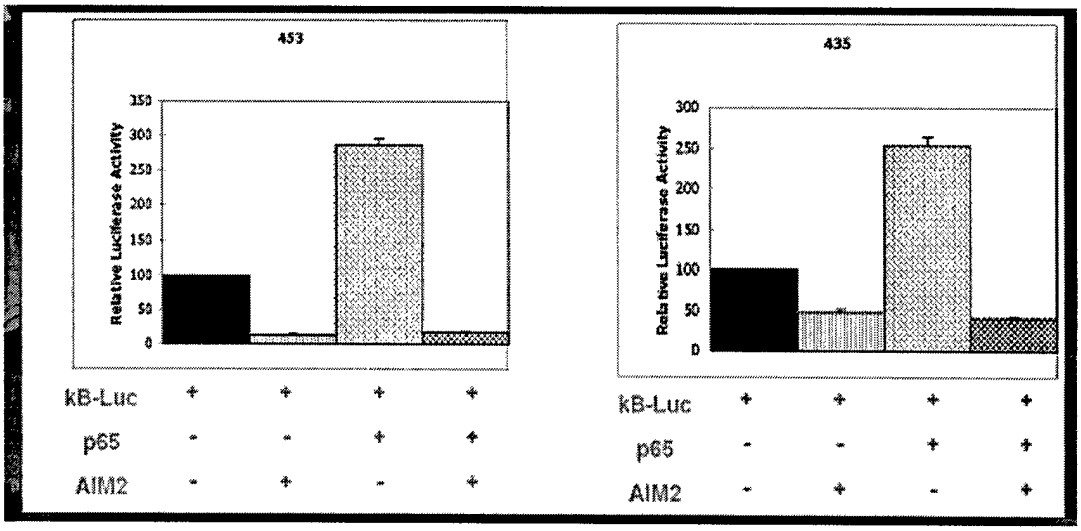

Fig. 4. The AIM2 suppressed breast cancer cells growth under inducible condition. Two stable clones, #13 and #25, of MCF-7 under tetracycline regulation system (tet-off) were selected. AIM2 expression levels were detected in panel (A). Cells were kept in medium containing with doxycycline (1mg/ml). After seven days remove of doxycycline in medium, cells were lysed and Western block with anti-Flag antibody to detect AIM2 expression level. (B) The growth rate was measured by cell number versus time of growth. $2x10^4$ cells were plated under either with or without doxycycline, cells were collected and accounted every other days. Cell numbers were plot in panel B.

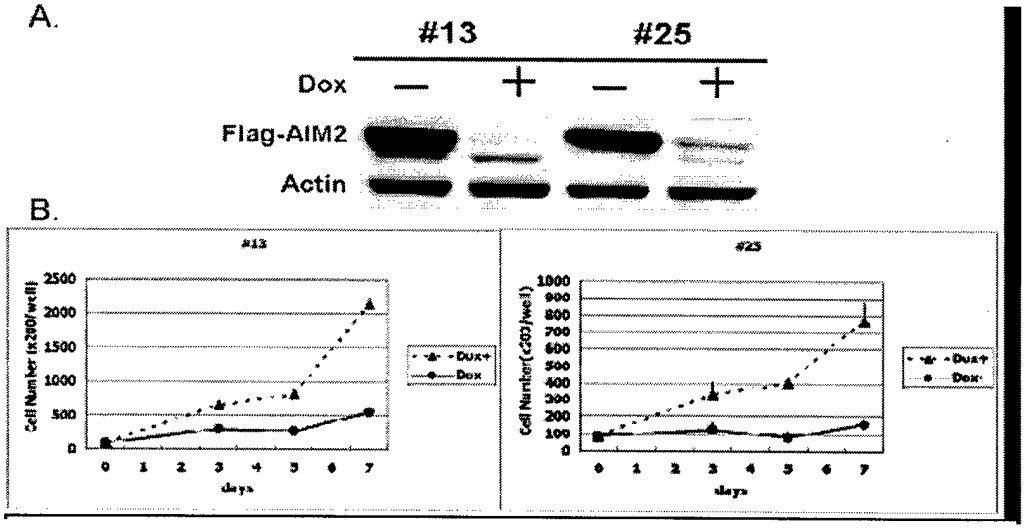

Fig. 5. Tet-off AIM2 exhibits an anti-tumor effect on human breast cancer cell in an orthotopic mouse model. Six week-old female nude mice (ten mice/group) were inoculated with $2x10^6$ cells of MCF-7 stable clones which gene expression under tetracycline regulation system (MCF-Luc: stably express luciferase after removal of textracycline/Doxicycline from media; MCF-AIM2: stable clone of AIM2 tet-off system). The mice were fed with sucrose water with/without Doxicycline (2mg/ml). Tumor sizes were measured with a caliper twice a week. The tumor volume was calculated using the formula: Volume SxSx*L*/2 where *S* is the short length of the tumor in cm and *L* is the long length of the tumor in mm (*bar, SE*).