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This current proposal is based on our previous observations that: (1) Interferons (IFNs) are capable of exerting growth inhibition and anti-tumor effects on human cancer cells; and (2) p202 expression alone is sufficient to suppress both cell growth and tumor development of human prostate and breast cancer cells. To further investigate the anti-tumor activity of p202 on prostate cancer and to develop a p202 gene therapy for prostate cancer, we have proposed three specific aims to accomplish our objectives.
Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells;
Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer;
Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclinical gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer animal model.
Success of those aims will constitute a scientific basis for p202-associated anti-tumor effect on prostate cells and will enable us to develop a novel p202 gene therapy strategy against prostate cancer.

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## Table of Contents

## INTRODUCTION:

The interferon family (IFN) is composed of three classes:α, β and γ (1). The IFN family not only plays an integral role in host defense system against certain tumors and foreign antigens such as viruses, bacteria and parasites; but also possesses immunomodulatory and cell growth-inhibitory activities. However, the molecular mechanisms involved in IFN's anti-tumor activity are remained elusive. In a recent study, several IFN-inducible proteins are implicated in the process of tumor suppression (2). Moreover, based on DNA analysis, 19 out of 95 IFN-inducible genes are differentially downregulated during prostate tumor progression (3). The anti-cellular function of IFNs has been attributed to their abilities to induce $G_1$ phase arrest in cell cycle (4-6). P202, an IFN-inducible gene is a primarily nuclear 52kd phosphoprotein, has been shown to have a growth retardation function that was presumably accomplished by its ability to bind several cell-cycle regulatory proteins such as E2Fs, AP1, NFκB and pRb, resulting in the failure of S phase entry (7-9). Using p202 as a therapeutic agent, we have demonstrated that the multiple anti-tumor activities in human cancer xenograft models including breast and pancreatic cancers (10-11). Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype *in vitro* (12). Our ultimate goal is to develop a gene therapy strategy that would specifically deliver p202 to the prostate cancer cells so that the "normal cells" would not be affected by such treatment. To accomplish our goal, three specific aims are proposed (see below). The

success of those aims will constitute a scientific basis for p202-associated anti-tumor effect on prostate cancer cells and will enable us to develop a novel p202 gene therapy strategy against prostate cancer.

**BODY:**

**A. SPECIFIC AIMS: (NO CHANGES)**

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

a. determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells;

b. determine pro-apoptotic activity of p202 in response to therapeutic agents, e.g. TNFα.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

a. determine the effect of p202 on G1/S cell cycle regulators in prostate cancer cells;

b. determine the effect of p202 on G2/M cycle regulators in prostate cancer cells.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

a test the anti-tumor activity of p202 gene for tumors induced by s.c. injection;

c. test the anti-tumor activities of p202 gene for orthotopic prostate model;

d. develop a prostate-specific gene therapy strategy.

**B. STUDIES AND RESULTS:**

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

As reported last year, we have completed the Specific Aim 1 a, the results, which indicated that p202 has anti-tumor and pro-apoptotic activities in prostate cancer cells, are in press in *Molecular Carcinogenesis* (please see the Appendix 1, a copy of galley proof). We have held preliminary discussions with the FDA regarding the feasibility of initiating a phase I clinical trial using the p202 gene. The initial response from the FDA is that a human gene is preferable because of the potential immunogenicity of a murine gene expressed at high levels. For this reason, we have initiated to identify human genes homologous to the mouse p202 and have now cloned by using RT-PCR two genes including the AIM2 (Absent in Melanoma) and NMDA (Myeloid Nuclear Differentiation Antigen). When those two genes were tested their ability to inhibit cell growth using colony formation assay, we have found that AIM2 but not NMDA possesses activity to inhibit breast cancer cells. Since AIM2 is of human origin, and there are obvious advantages to using a human gene in human clinical trials rather than a murine gene such as p202, therefore, we will explore AIM2's anti-tumor activity in prostate cancer cells and in animal models. So far, we have demonstrated that AIM2 has an anti-growth

activity as p202 in both prostate and breast cancer cell lines. (Appendix 2) We will continue to compare antitumor activity of the human AIM2 and mouse p202 gene *in vivo*.

Regarding to the Specific Aim 1b, we have only observed that p202 has minimal response to TNF-α, a therapeutic agent in prostate cancer cells. However, to this end, we recently discovered an interesting compound, Emodin which is a nature product extracted from the roots of numerous plants of the genus Rhamnus and acts as a tyrosine kinase inhibitor, has a profound effect on prostate cancer cells' proliferation and survival. We unexpectedly found that Emodin also inhibits expression of androgen receptor (Appendix 3) and suppresses cell growth and induced apoptosis in prostate cancer cells (Appendix 4). We will continue to study the molecular mechanisms on how Emodin inhibits prostate cancer cell growth and test its antitumor activity in animal models.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

Please see the Appendix 1, we have completed the both subaims proposed in Specific Aim2. Briefly, we have shown that (1) the active form of Rb (hypophosphorylated Rb) is involved in p202-mediated growth arrest, particularly in arresting in $G_1$ of cell cycle in prostate cancer cells; and (2) cyclin B and p55cdc, which are known to be essential for the G2/M transistion and for the normal metaphase-to-anaphase transition in mitotic stage, respectively, are downregulated in p202 expressed prostate cancer cells.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

As reported in the Appendix 1, we have completed three subaims proposed in the Specific Aim 3. However, as mentioned in Specific Aim 1, we have consulted with FDA regarding p202 in future clinical trials, they have indicated that a human gene is preferable to a murine gene. Our data suggest that the human AIM2 may fulfill this requirement. We will investigate the therapeutic efficacy of AIM2 in an orthotopic prostate cancer model.

## KEY RESEARCH ACCOMPLISHMENTS:

- We have completed Specific Aim 1 a, Specific Aim 2 a to b, and Specific Aim 3 a to c.
- By using RT-PCR, we have successfully cloned human gene homologous to p202, AIM2 and NMDA.
- AIM 2 possesses an anti-growth activity as p202 has in both breast and prostate cancer cells.
- Emodin inhibits expression of androgen receptor
- Emodin suppresses cell proliferation and induces cell apoptosis in prostate cancer cells, therefore, it could act as an effective new therapeutic agent in prostate cancer.

## REPORTABLE OUTCOMES:

"Prostate-Specific Antitumor Activity by Probasin-Directed p202 Expression". Yong Wen, Dipak Giri, Duen-Hwa Yan, Bill Spohn, Ralph G. Zinner, Weiya Xia, Timothy C. Thompson, Robert J. Matusik and Mien-Chie Hung. *Molecular Carcinogenesis*, in press, 2003. (A copy of galley proof is attached as an Appendix 1.)

## CONCLUSIONS:

In the last two years, we have completed most of the Specific Aims (and subaims, please see Appendix 1). We currently examine the anti-growth activity of human AIM2. The

initial results are encouraging (Appendix 2). In addition, we also found that Emodin could potentially be an useful therapeutic agent to treat prostate cancer (Appendix 4). We will continue to study on antitumor activity for both AIM2 and Emodin in prostate cancer animal models.

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**APPENIDX 1: GALLEY PROOF**

**"Prostate-Specific Antitumor Activity by Probasin Promoter-Directed p202 Expression"** ***Molecular Carcinogenesis*****, in press, 2003.**

**Yong Wen, Dipak Giri, Duen-Hwa Yan, Bill Spohn, Ralph G. Zinner, Weiya Xia, Timothy C. Thompson, Robert J. Matusik and Mien-Chie Hung**

MOLECULAR CARCINOGENESIS 9999:1–8 (2003)

# Prostate-Specific Antitumor Activity by Probasin Promoter-Directed p202 Expression

**Yong Wen,[1] Dipak Giri,[1] Duen-Hwa Yan,[1] Bill Spohn,[1] Ralph G. Zinner,[2] Weiya Xia,[1] Timothy C. Thompson,[3] Robert J. Matusik,[4] and Mien-Chie Hung[1]***

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p202, an interferon (IFN) inducible protein, arrests cell cycle at $G_1$ phase leading to cell growth retardation. We previously showed that ectopic expression of p202 in human prostate cancer cells renders growth inhibition and suppression of transformation phenotype in vitro. In this report, we showed that prostate cancer cells with stable expression of p202 were less tumorigenic than the parental cells. The antitumor activity of p202 was further demonstrated by an ex vivo treatment of prostate cancer cells with p202 expression vector that showed significant tumor suppression in mouse xenograft model. Importantly, to achieve a prostate-specific antitumor effect by p202, we employed a prostate-specific probasin ($ARR_2PB$) gene promoter to direct p202 expression ($ARR_2PB$-p202) in an androgen receptor (AR)-positive manner. The $ARR_2PB$-p202/liposome complex was systemically administered into mice bearing orthotopic AR-positive prostate tumors. We showed that parenteral administration of an $ARR_2PB$-p202/liposome preparation led to prostate-specific p202 expression and tumor suppression in orthotopic prostate cancer xenograft model. Furthermore, with DNA array technique, we showed that the expression of p202 was accompanied by downregulation of $G_2$/M phase cell-cycle regulators, cyclin B, and p55cdc. Together, our results suggest that p202 suppresses prostate tumor growth, and that a prostate-specific antitumor effect can be achieved by systemic administration of liposome-mediated delivery of $ARR_2PB$-p202. 

Key words: p202; tumorigenecity; probasin; cyclin B; p55cdc

## INTRODUCTION

The interferon (IFN) family of cytokines plays a crucial role in host defense system against viral, bacterial, and parasitic infections and certain tumors. In addition, they also possess immunomodulatory and cell growth-inhibitory activities. There are three classes of IFN: α, β, and γ [1]. The mechanism involved in tumor suppressor activity of IFNs has not been well established. However, several IFN-inducible proteins were implicated in the process of tumor suppression [2]. Consistent with that notion, a recent report based on DNA array analysis indicates that 19 of 95 differentially down-regulated genes associated with prostate tumor progression are, in fact, IFN-inducible genes [3]. The anticellular function of IFNs has been attributed to their abilities to induce $G_1$ phase arrest in the cell cycle [4–6]. Human prostate cancer cells are also sensitive to the antimitotic action of IFNs [7,8]. Recent studies demonstrate the inhibitory effect of IFN-α on growth [9–11] and colony formation [8] in several human prostate carcinoma cell lines.

Besides the therapeutic effects of IFNs in certain clinical settings, there are also undesirable side effects, viz. fever, chills, anorexia, and anemia associated with high dose IFN, which is often required to obtain a therapeutic response [12,13]. This has impeded IFN as an effective anticancer agent. In an attempt to circumvent this disadvantage and to harvest the benefit of IFN treatment, we explored the possibility of using an IFN-inducible protein, p202 [14], as a potential therapeutic agent. p202 belongs to murine 200 amino-acid protein family. Although the physiological function of p202 is not well defined, the experimental evidence gathered so far suggests its role in cell-cycle control, differentiation, and apoptosis [15,16]. In particular,

Yong Wen, Dipak Giri, and Duen-Hwa Yan contributed equally to this work.

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Abbreviations: IFN, interferon; Rb, retinoblastoma; $ARR_2PB$, modified prostate-specific probasin gene promoter; Luc, luciferase; PEI, polyethylenimine; PBS, phosphate buffer saline; SN, a cationic liposome formulation; AR, androgen receptor.

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ectopic p202 expression in cells results in retardation of growth that is thought to be mediated by E2F/retinoblastoma (Rb) pathway leading to $G_1$ arrest [17,18].

With p202 as a therapeutic agent, we have demonstrated the multiple antitumor activities in human cancer xenograft models including breast and pancreatic cancers [19,20]. Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis, and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype in vitro [21]. However, it has not yet been determined whether p202 expression inhibits the tumorigenicity of prostate cancer cells and whether the p202-based gene therapy is feasible in human prostate cancer xenograft model. In this report, we showed that p202 expression reduced the tumorigenicity of prostate cancer cells. With modified prostate-specific probasin gene promoter ($ARR_2PB$)-p202, a p202 expression vector driven by $ARR_2PB$ promoter [22–24], we showed prostate-specific tumor suppression by $ARR_2PB$-p202. p202 expression was accompanied by downregulation of $G_2$/M phase cell-cycle regulators, cyclin B, and p55cdc [25–27].

## MATERIALS AND METHODS

### Cell Lines and Plasmids

LNCaP, MCF-7, PC-3, and four p202-expressing PC-3 clones, i.e., p202-1, -2, -3, and -4 [21] were cultured in Dulbecco's Minimum Eagle's Medium[Q2]/F12 media supplemented with 10% fetal bovine serum. The p202 expression vector, CMV-p202 [18], is driven by CMV promoter. To construct the $ARR_2PB$-luciferase (Luc) vector, the $ARR_2PB$ promoter element (468 bp), in pBlueScript II SK+ vector [22], was ligated into the KpnI/Sac I site of the PGL3-enhancer vector (Promega, Madison, WI). The $ARR_2PB$-p202 was generated by replacing the *Luc* gene in the $ARR_2PB$-Luc with the p202 coding sequence obtained from CMV-p202 vector [18] by BamHI digest. The correct orientation was confirmed by unique restriction enzyme digestion.

### Subcutaneous and Ex Vivo Tumorigenicity Assays

PC-3 vector control (pcDNA3-pool), p202-1, and p202-2 cells ($1 \times 10^6$ each) in 200 μL of phosphate buffer saline (PBS) were injected subcutaneously in 4- to 5-wk-old nude mice (five mice/ten tumors/cell line) on both sides of the abdomen. Tumor sizes were measured with a caliper once a week and tumor volume was calculated with the formula: Vol. = S × S × L/2, where S = the short length of the tumor in cm, and L = the long length of the tumor in cm. For ex vivo experiment: PC3 cells growing in 100 mm dishes were transfected with 10 μg of CMV-p202 DNA complexed with 22.5 μg of polyethylenimine (PEI) for 45 min. PC3 cells were mock transfected with either CMV-p202 alone or PEI alone. After transfection, the cells were washed and incubated for an additional 18 h in complete media. Cells were then trypsinized, washed in PBS, counted, and $1 \times 10^6$ cells were inoculated s.c. in two sites on the flanks of male nude mice. Tumor size was measured weekly and volume calculated.

### Transfection and Luc Assays

Human prostate cancer cell lines LNCaP and PC3, and a human pancreatic cancer cell line (Panc-1) were used for the reporter assay. Cells ($2 \times 10^6$) were plated into 6-well plate the day before transfection. Cells[Q3] with SN2 liposome were transfected with 0.5 μg of $ARR_2$-PB-Luc plasmid, 0.5 μg of CMV-Luc plasmid, and 0.05 μg of pRL-TK. Cells were harvested 36 h after transfection. The Luc activity was determined with the dual Luc protocol (Promega) with a luminometer.

### Immunohistochemistry

The avidin-biotin peroxidase complex technique used in this study was modified from that described previously [28]. Briefly, formalin-fixed tissue sections were deparaffinized and dehydrated in ascending grades of ethanol. The sections were treated with 0.05% trypsin for 15 min, blocked in 0.3% hydrogen peroxidase in methanol for 15 min followed by treatment with 1% (v/v) normal horse serum for 30 min. The slides were incubated overnight at 4°C with anti-p202 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:25 dilution. After liberal washing with PBS, the slides were incubated with biotinylated rabbit antigoat IgG at 1:200 dilution in PBS for 60 min at room temperature. The slides were subsequently incubated for 45 min at room temperature with the avidin-biotin-peroxidase complex diluted 1:100 in PBS. The product of enzymatic reaction was visualized with 0.125% aminoethylcarbazole, which gives a red colored reaction product. For counter staining, Mayer's hematoxylin was used.

### Systemic Gene Therapy in Human Prostate Cancer Xenograft Model

Athymic nude mice (nu/nu) were opened through a single mid-ventral incision under sedation and the prostate gland was exposed. An aliquot of 30 μL of PBS containing $2 \times 10^6$ LNCaP cells were inoculated into the gland with a sterile syringe and 25 G needle. Such an inoculation resulted a small swelling at the site. LNCaP cells under such conditions gave rise to tumors in 100% of animals as observed in a pilot experiment. The abdominal incision was closed with sterile stainless steel clips. A group of four animals was returned to a cage following recovery from the sedation and recruited for the experiment. The

treatment protocol was initiated 7 days after the intraprostatic inoculation of LNCaP cells, a time interval sufficient to give rise to small tumors as observed in the pilot experiment. A dose of 25 μg of $ARR_2PB$-p202 plasmid DNA entrapped in a lipid formulation (SN) [29] at the ratio of 1:1.5 was incubated at room temperature for 30 min. The DNA/liposome complex was intravenously injected into the tail vein. The mice were treated twice a week for a period of 1½ months and then followed by treatment once a week. The Luc control group received an equivalent dose of plasmid DNA ($ARR_2PB$-Luc)/liposome complex. Animals were examined weekly to assess the tumor growth.

### Western Blot Analysis

Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM $Na_2PO_4$ (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM $Na_3VO_4$, 5 mM PMSF, 1% aprotinin, and 10 μg/mL of leupeptin. The antibodies specific for human Rb, cyclin B, p55cdc, and actin (Santa Cruz Biotechnology, Inc.) were used to detect these proteins by Western blot as described previously [19].

## RESULTS AND DISCUSSION

### p202 Suppressed Tumorigenicity of Prostate Cancer Cells

To investigate whether p202 could exhibit growth inhibitory effect on prostate tumor in vivo, two assays were performed. The first assay employed two p202 stable cell lines derived from human prostate cancer cell line, PC-3 [21]. The second was an ex vivo tumorigenicity assay with PC-3 cells transfected with p202. As shown in Figure 1A, 16 wks postimplantation, p202-1 and p202-2 clones generated smaller tumors than that of the control, pcDNA3-pooled cell line. In fact, the p202-2 clone failed to form tumors in mice under identical experimental conditions. The difference in tumorigenesis between p202-1 and p202-2 may be attributed to an inadequate p202 protein expression in the former [21]. To rule out the possible contribution of clonal heterogeneity on the observed effects, we performed an ex vivo tumorigenicity assay in which PC-3 cells were transiently transfected with a p202 expression vector with a PEI vector delivery system. The transfected PC-3 cells were employed to generate subcutaneous xenografts in nude mice. The p202 transfected PC-3 cells interestingly showed no detectable tumor after 10 days (Figure 1B). On the contrary, the DNA control, i.e., CMV-p202 alone, was ineffective in containing tumor growth, indicating that the observed antitumor effect on PC-3 cells is attributable to p202 transfection. The vector controls, i.e., PEI alone, did not significantly affect tumor formation. Together, these results strongly suggested that p202 possesses an antitumor activity against prostate cancer cells. Importantly, it provides a scientific basis for developing a p202-based gene therapy strategy in orthotopic human prostate cancer xenograft model.

### $ARR_2PB$ Promoter Directed Prostate–Specific p202 Expression and Tumor Suppression

To achieve prostate specific p202–mediated antitumor activity, we first tested whether an androgen receptor (AR)-responsive promoter could direct a Luc reporter gene expression in prostate cells. Because $ARR_2PB$ promoter contains two copies of androgen response regions located upstream from a minimum PB promoter, it is highly responsive to androgen-dependent transcriptional activation [22]. We generated $ARR_2PB$-Luc and transfected it into two prostate cancer cell lines with (LNCaP) or without (PC-3) endogenous AR expression. We used a

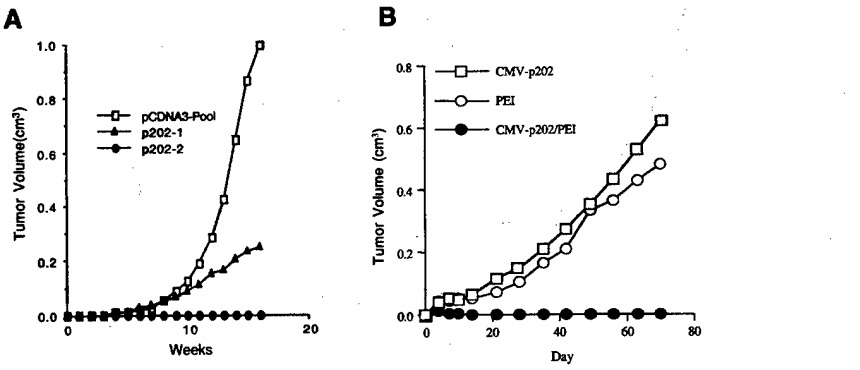


Figure 1. The antitumor function of p202 in PC-3 cells. (A) Reduced tumorigenicity of p202-expressing PC-3 cells. Nude mice (n = 5 per cell line) were injected subcutaneously with $1 \times 10^6$ cells in each of the two sides on the abdomen. Tumor size was measured each week and the tumor volume was calculated. The average tumor size at the indicated time points is presented. (B) p202 reduces the tumorigenicity of PC-3 cells ex vivo. PC-cells were transfected with CMV-p202/polyethylenimine (PEI) complex, PEI alone, or CMV-p202 alone. Eighteen hours after transfection, $1 \times 10^6$ cells were s.c. injected in both sides on the abdomen of a nude mouse.

pancreatic cancer cell line, Panc-1, as a nonprostatic control cell. As shown in Figure 2A, the relative Luc activity of $ARR_2PB$-Luc/CMV-Luc (a Luc gene expression vector driven by a constitutively active CMV promoter) was the highest in the AR-positive LNCaP cells, but not in AR-negative PC-3 and Panc-1 cells. This result suggested that $ARR_2PB$ promoter activity was indeed AR-dependent [22], and thus confirmed the utility of $ARR_2PB$ promoter to direct AR-specific gene expression in prostate cancer cells [23,24].

To test the AR-specific p202-mediated antitumor activity in orthotopic prostate cancer xenograft model, we generated a p202 expression vector driven by $ARR_2PB$ promoter ($ARR_2PB$-p202). Likewise, $ARR_2PB$-Luc served as a negative control. The orthotopic prostate cancer xenograft model was established according to the procedure described previously [30,31]. After initiation of treatment 7 days after orthotopic tumor cell implantation in the prostate, survival time was prolonged in mice treated by $ARR_2PB$-p202. All mice treated with $ARR_2PB$-Luc were sacrificed on the 108th day post-treatment because they carried massive tumors and had reached the institutional permissible limit for tumor burden (Figure 2B). In contrast, 100% of $ARR_2PB$-p202-treated mice were alive and healthy. Sixty percent (three mice) of the $ARR_2PB$-p202-treated mice survived on the 150th day post-treatment. To assess the antitumor activity, in an interim sacrifice protocol, three mice each from $ARR_2PB$-p202 and $ARR_2PB$-Luc treatment groups were euthanized and prostate glands dissected at day 77 of treatment. $ARR_2PB$-p202-treated tumors were remarkably reduced in size than those treated by the control vector, $ARR_2PB$-Luc (Figure 2C). This observation explains the prolonged survival seen in mice treated by $ARR_2PB$-p202. The use of $ARR_2PB$ promoter to direct expression of p202 predicts the specificity of effect. Therefore, we examined the p202 expression on tumors and organs isolated from $ARR_2PB$-p202-treated mice by immunohistochemical staining. The p202 protein was detected in the cytoplasm as a red colored reaction product from the enzymatic reaction with aminoethylcarbazole as the chromogen. Note abundant intracytoplasmic expression of p202 in the tumor from the mouse treated with $ARR_2PB$-p202 (Figure 3, left panel). The mouse treated with $ARR_2PB$-Luc had undetectable p202 (Figure 3, right panel). Given that p202 is primarily a nuclear protein [32], the exact reason for the predominant cytoplasmic staining of p202 is not clear. However, it is probably due to the robust expression of p202 that causes accumulation of p202 in the cytoplasm. Alternatively, because the induced p202 localizes in the cytoplasm for 30–36 h after IFN treatment before translocated into the nucleus [32], it is likely that p202 could still remain in the cytoplasm 20-h post-$ARR_2PB$-p202 treatment. We also examined the expression of p202 in multiple organs such as lung, liver, kidney, and heart to ascertain the nonprostatic expression, if any. There was no extraprostatic expression of p202 except the reticuloendothelial cells of lung and liver from both $ARR_2PB$-p202 and $ARR_2PB$-Luc-treated mice (data not shown and Figure 3). The p202-positive mouse reticuloendothelial cells is probably the endogenous level of p202 expression because all 200 amino-acid protein family members are expressed in hematopoietic cells [33]. Together, the results strongly suggested that systemic delivery of $ARR_2PB$-directed expression vector by SN liposome could result in prostate and AR-specific antitumor activity in prostate cancer.

$ARR_2PB$ promoter-mediated therapeutic gene expression is primarily useful for targeting AR-positive prostate cancer, which consists of a significant portion of prostate cancer patient population. Although AR-negative prostate cancer is insensitive to androgen, in many of these cases, AR is still active [34]. It is conceivable that $ARR_2PB$ promoter could be activated in these androgen-independent prostate tumors. In addition, $ARR_2PB$ promoter is also responsive to glucocorticoids which have been routinely used to improve the quality of life in prostate cancer patients that failed androgen deprivation therapy [22,35]. Thus, $ARR_2PB$-p202 could be potentially used to achieve a prostate-specific therapeutic effect on androgen-independent prostate cancer patients who are treated with glucocorticoids.

### p202 Upregulated the Hypophosphorylated Rb and Downregulated Cyclin B and p55cdc

To investigate the underlying mechanisms of the p202-mediated growth inhibition and tumor suppression in prostate cancer cells, we set out to determine if (1) Rb phosphorylation was involved in p202-mediated growth arrest because IFN treatment increases the level of hypophosphorylated (active) form of Rb [36–38]; and (2) other regulatory genes responsible for the p202-mediated growth retardation and tumor suppression that can be identified by DNA array technology. To examine the effect on Rb phosphorylation by p202, we employed Western blot with a Rb-specific antibody to analyze the phosphorylation status of Rb in both parental and p202-expressing prostate cancer cells. Figure 4A shows that the p202-expressing cells, i.e., p202-1, -2, and -3, exhibit an elevated level of hypophosphorylated form (faster migrating band) of Rb as compared to the control, i.e., pcDNA3-pooled, in which the hyperphosphorylated form (slower migrating band) of Rb is most prevalent. Thus, one possible mechanism by which p202 induces cell growth arrest in PC-3 is by enhancing the level of hypophosphorylated Rb. Presumably, the active Rb would then inhibit E2F transactivation function by forming an Rb/E2F complex. Thus, the E2F-mediated transcription of S-phase genes might

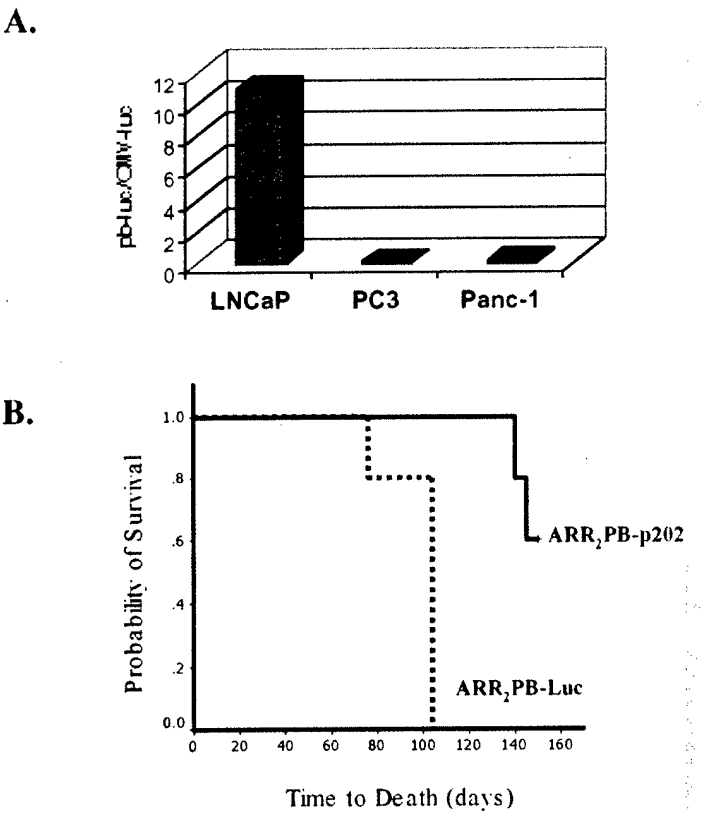


Figure 2. (A) Modified prostate–specific probasin gene promoter ($ARR_2PB$) activity is androgen receptor (AR)-dependent. $ARR_2PB$-luciferase (pb-Luc) (0.5 μg) and CMV-Luc (0.5 μg) were transfected into two prostate cancer cell lines with, for example, LNCaP, or without, for example, PC3, endogenous AR expression. A pancreatic cancer cell line, Panc-1, served as a nonprostatic cell control. The ratios of Luc activity resulted from $ARR_2PB$-Luc and CMV-Luc transfections were measured. pRL-TK (50 ng) was co-transfected and served as an internal control for transfection efficiency with dual Luc assay (Promega, Madison, WI). The data shown here is the average of two independent experiments. (B) Prolonged survival by $ARR_2PB$-p202 treatment. LNCaP orthotopic tumor-bearing mice (n = 5 per treatment group) were intravenously treated with $ARR_2PB$-p202 or $ARR_2PB$-Luc/SN liposome complexes. Survival rates were measured by Kaplan-Meier analysis. (C) Antitumor activity by systemic $ARR_2PB$-p202 treatment. Tumor suppression by $ARR_2PB$-p202 treatment. Representative LNCaP tumors (n = 3 per treatment group) are shown from mice treated with $ARR_2PB$-p202 or $ARR_2PB$-Luc/lipid formulation (SN) liposome complexes on day 77 post-treatment.

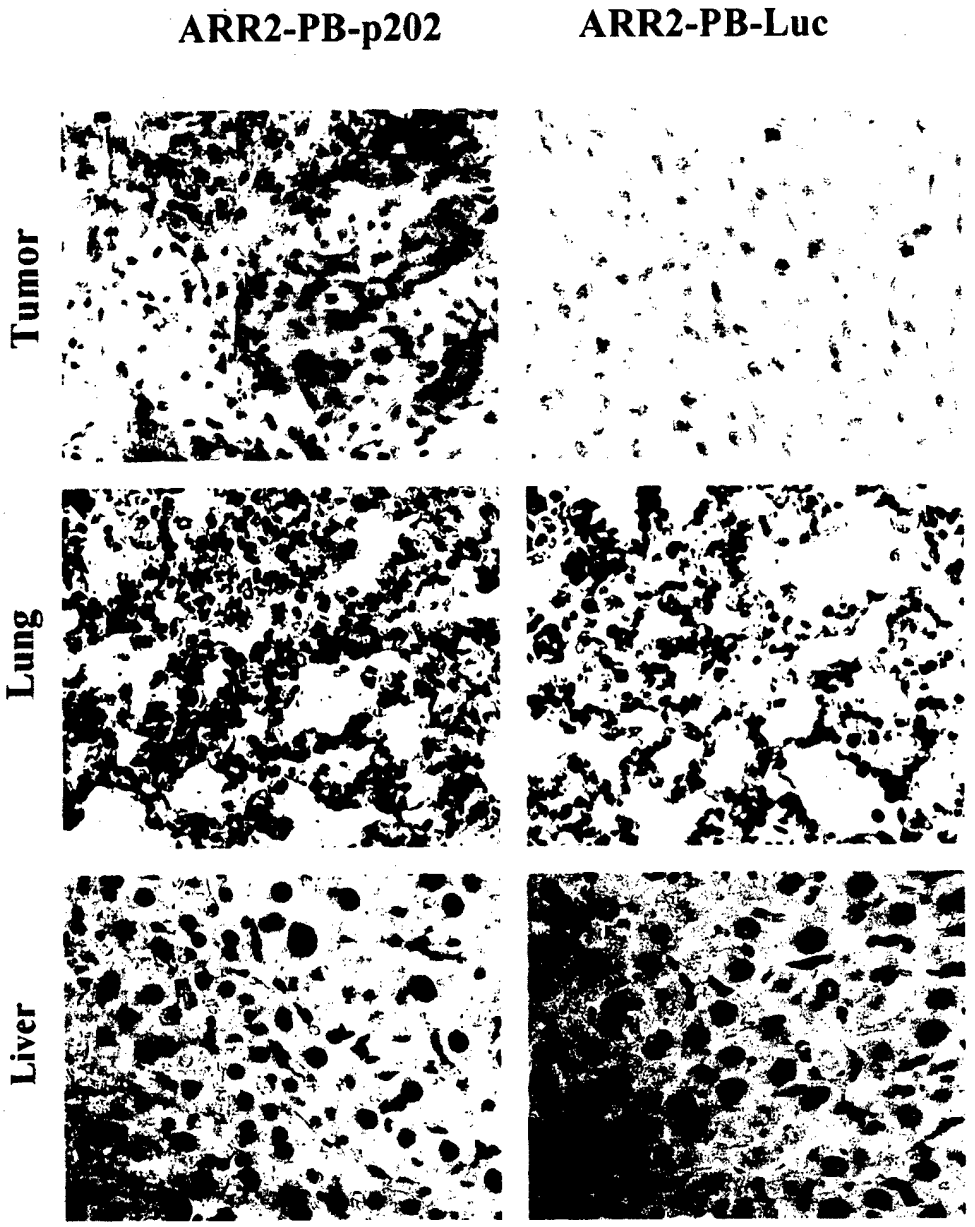


Figure 3. $ARR_2PB$ promoter directs prostate–specific p202 expression. Formalin-fixed tumors, lung, and liver from mice 20-h post-treatment with $ARR_2PB$-p202 or $ARR_2PB$-Luc were sectioned and stained for p202 employing polyclonal anti–p202 antibodies as described in "Materials and methods."

be inhibited causing $G_1$-phase arrest. Because p202 is a transcription modulator, it is possible that p202 could regulate certain gene expression which might be important in p202–mediated growth arrest and tumor suppression in prostate cancer cells. To identify other critical genes involved in p202–mediated antigrowth and antitumor activities, we employed DNA array technology. With RNA products obtained from PC-3 (parental control) and p202-2 (a representative p202-expressing prostate

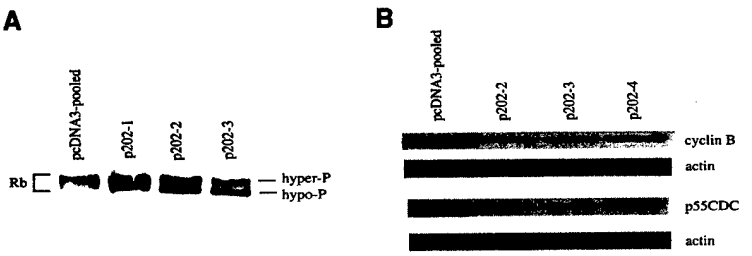


Figure 4. p202 enhances hypophosphorylated retinoblastoma (Rb) and reduces cyclin B and p55CDC expression. Cell lysates obtained from pcDNA3-pool and p202-expressing PC-3 cell lines (p202-1, -2, -3, and -4) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently analyzed by Western blot with antibodies against Rb, cyclin B, p55cdc, and actin. The actin bands served as loading control.

cancer cell) to hybridize with DNA array filters (Clontech[Q4]) containing 588 known genes that are involved in various cellular regulatory pathways including those of cell-cycle control, we were able to identify several candidate genes whose expressions were found significantly influenced by p202 expression. Two such genes have been confirmed by Western blot, i.e., cyclin B and p55cdc (Figure 4B), which showed a reduced level of expression in p202-expressing cells, as compared to the control, pcDNA3-pooled. The sample loading was similar as indicated by the actin control. In light of the well documented p202–mediated $G_1$ arrest, the reduction of cyclin B and p55cdc in p202-expressing cells is rather surprising because cyclin B is involved in $G_2$/M phase transition [25] and p55cdc is required for normal metaphase-to-anaphase transition involved in late mitotic events [26,27]. It is likely that the downregulation of these two genes by p202 may contribute to the p202–mediated cell-cycle arrest. This is the first time that p202 is implicated in involvement in $G_2$/M phase cell-cycle control. It is possible that the p202–associated cyclin B and p55cdc downregulation may contribute, in part, to the p202–mediated growth arrest.

In this report, we showed that p202 expression suppressed the tumorigenicity of prostate cancer cells. A subsequent ex vivo experiment with either CMV-p202/PEI complex also inhibited prostate cancer cell growth in xenograft model. The utility of p202 as a potential therapeutic gene for prostate cancer treatment was demonstrated by the observation that prostate-specific antitumor activity can be achieved by systemically treating the prostate tumor-bearing mice with a p202 expression vector driven by a composite probasin promoter, $ARR_2PB$. Thus, in addition to local and systemic treatment of breast and pancreatic tumors, respectively, by using a p202 expression vector driven by a constitutively active promoter such as CMV promoter [19,20], our results suggested the feasibility of using a tissue-specific promoter to achieve p202–mediated antitumor activity in those cancer types as well. Experiments are underway to test that possibility. Given that p202 is involved in $G_0/G_1$ transition by targeting E2F/Rb pathway [16], it is interesting to note that $G_2$/M cell-cycle regulators such as cyclin B and p55cdc are downregulated by p202. Further analysis on the p202 effect on $G_2$/M transition by downregulation of cyclin B and p55cdc will shed light into how p202 inhibits cell proliferation.

## ACKNOWLEDGMENTS

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and gamma-interferon and tumor necrosis factor alone or in combinations against two prostate cancer xenografts transplanted in nude mice. Prostate 1991;18:331–344.
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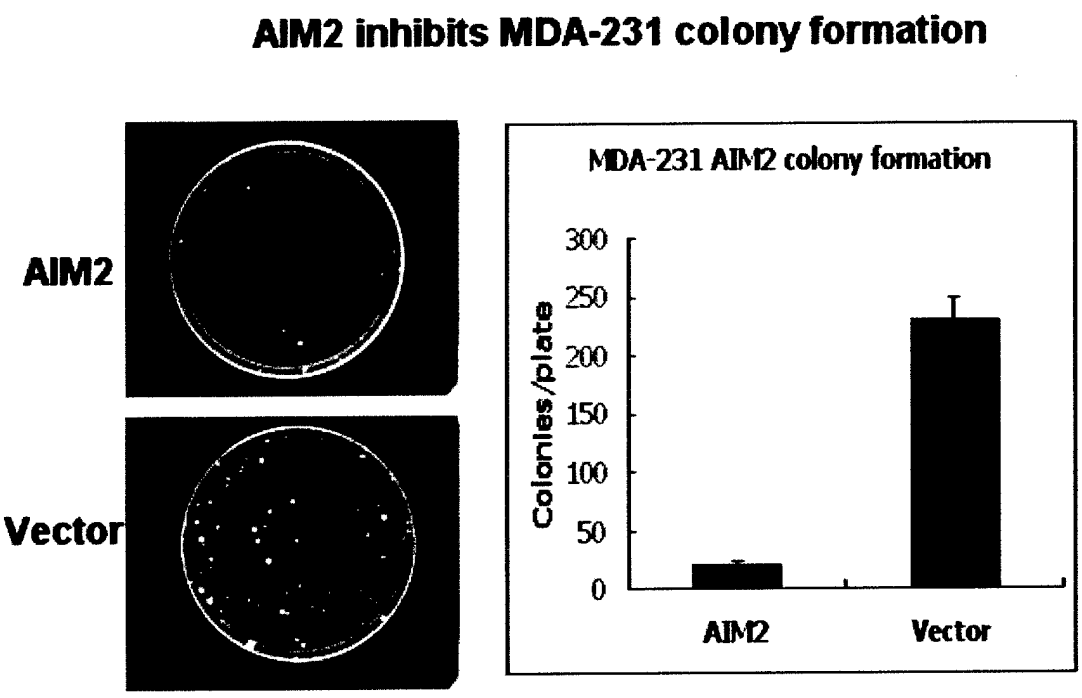
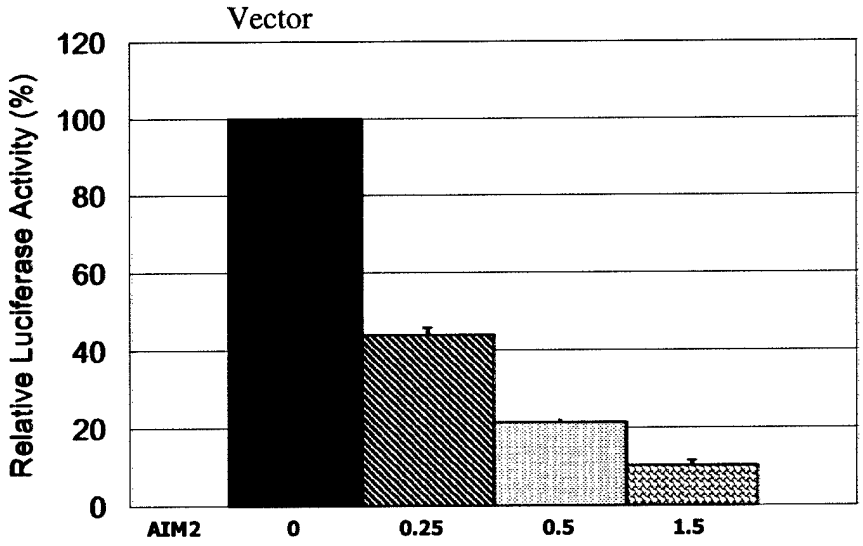
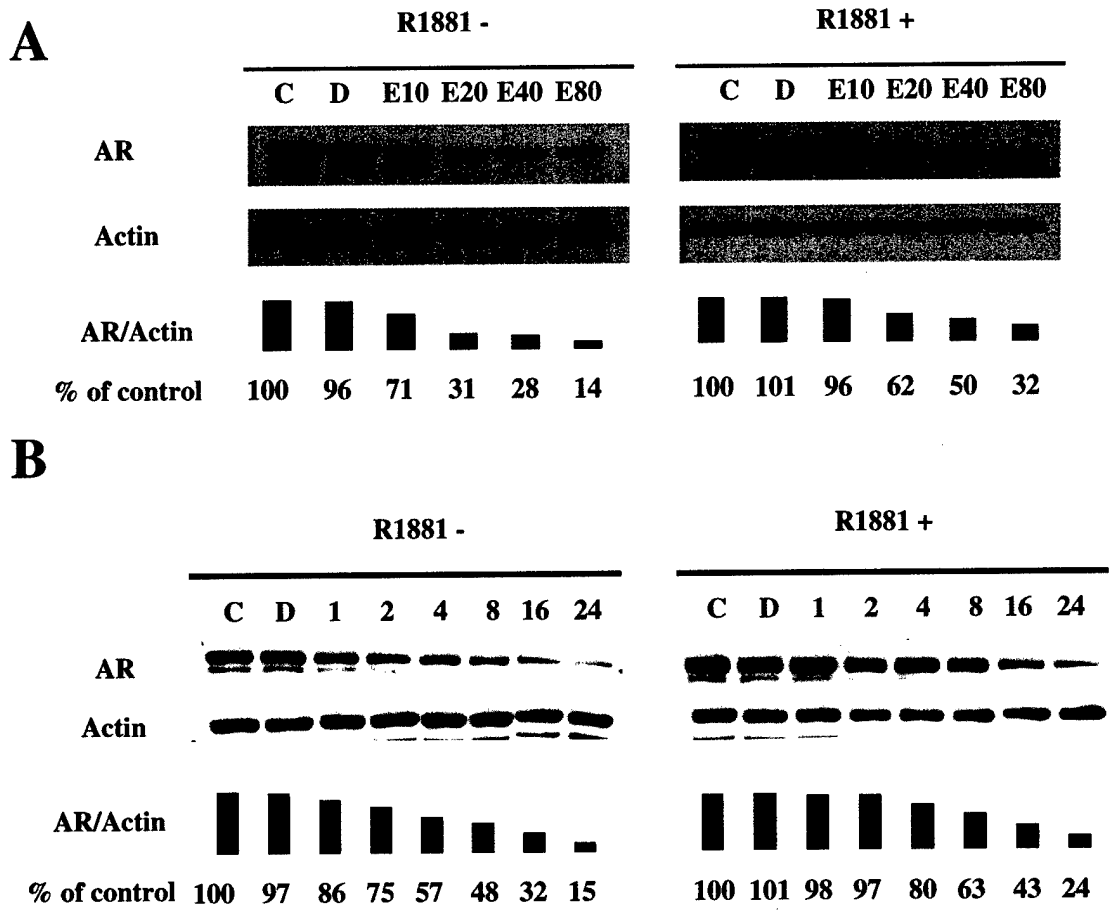
**Appendix 2: AIM2, human gene of p202 familu has anti-growth activity in breast and prostate cancer cells.**



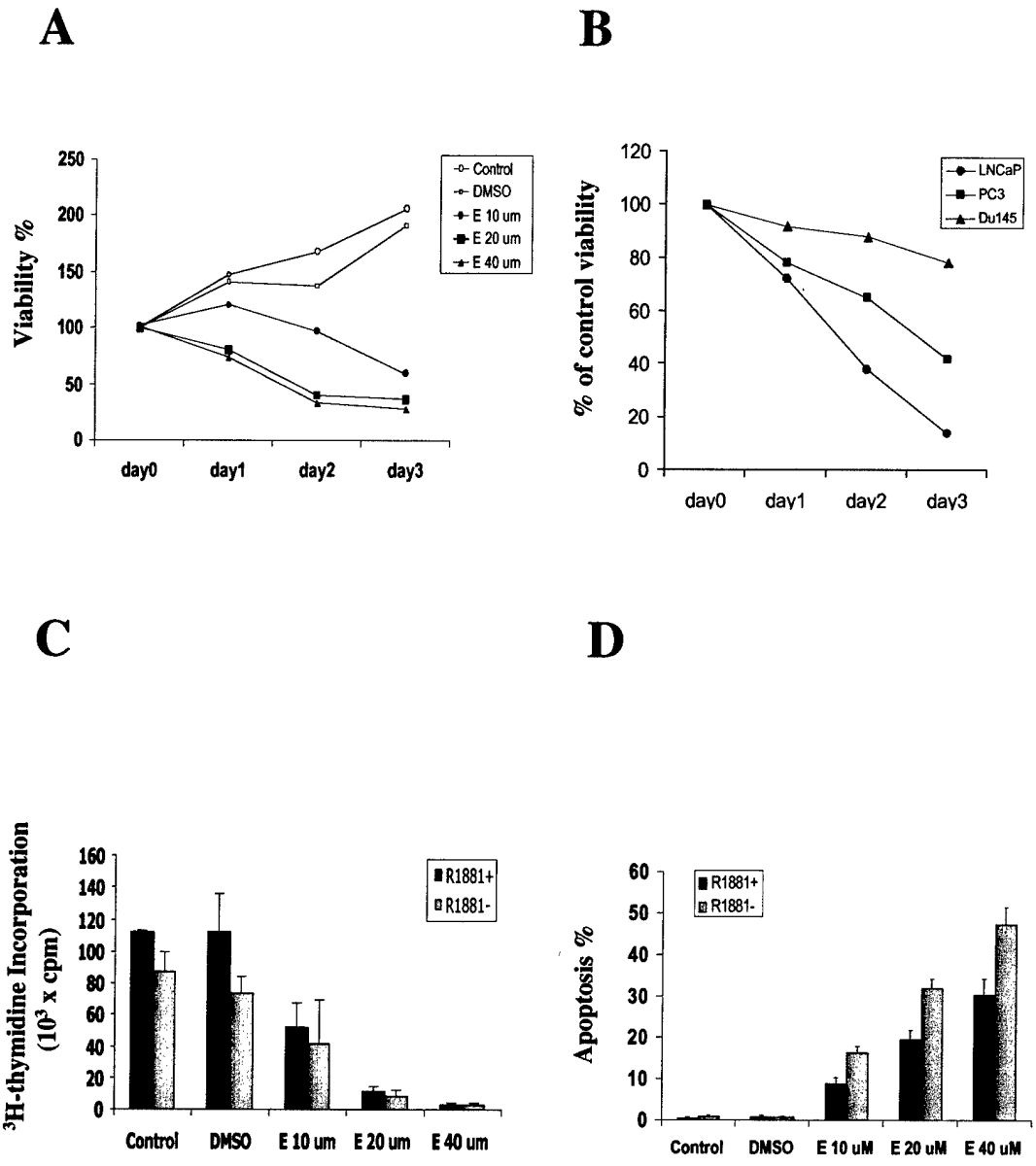

**Survival suppression of prostate cancer cells by AIM2 transfection in a dose-dependant manner.** CMV-Luciferase(2ug) and Flag-AIM2(0,0.25,0.5 or 1.5ug) were cotransfected into PC-3 cells. Thirty-six hours after transfection, luciferase activity was measured. The relative activities were calculated by setting the luciferase activity obtained from vector at 100%. The data represent an average of three independent experiments; *bars,* SD.

**Appendix 3 Emodin inhibits expression of androgen receptor**




**Effect of emodin on Androgen Receptor (AR) expression.** **a**, a prostate cancer cell line, LNCaP cells were treated with solvent (DMSO, labeled D) and various concentrations of emodin 10, 20, 40, or 80 μM, labeled E10-E80 for 18 h with or without R1881, a synthetic androgen. The AR protein level was analyzed by Western blot and quantitated by Bio-Rad PDQUEST Image software and plotted as the percentage of the control (without emodin) after normalization with actin. **b**, LNCaP cells were treated with 40 μM emodin for various lengths of time. The AR protein level was measured by immunoblotting

**Appendix 4: Emodin as a potential therapeutic agent for prostate cancer**




**Effect of emodin on cell proliferation and survival.** **a**, LNCaP cells were treated with various concentrations of emodin in the presence of synthetic androgen, 0.1 nM R1881 for 24, 48, and 72 h. The cell densities were determined by MTT assay. **b**, LNCaP, PC3, and DU145 cells were treated with or without 40 M emodin for 24, 48, and 72 h. The cell densities were determined by MTT assay. **c**, LNCaP cells were treated with various concentrations of emodin in the presence or absence of 0.1 nM R1881. The [$^3$H] thymidine incorporation rates were performed to measure DNA synthesis. **d**, LNCaP cells were treated with various concentrations of emodin in the presence or absence of 0.1 nM R1881 for 48 h. The cells collected were fixed with ethanol, stained with propidium iodide, and analyzed by flow cytometry software to determine the percentage of apoptotic cells.