Designer Monotransregulators Provide a Basis for a Transcriptional Therapy for *De Novo* Endocrine-Resistant Breast Cancer

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The main circulating estrogen hormone 17β-estradiol (E2) contributes to the initiation and progression of breast cancer. Estrogen receptors (ERs), as transcription factors, mediate the effects of E2. Ablation of the circulating E2 and/or prevention of ER functions constitute approaches for ER-positive breast cancer treatments. These modalities are, however, ineffective in *de novo* endocrine-resistant breast neoplasms that do not express ERs. The interaction of E2-ERs with specific DNA sequences, estrogen responsive elements (EREs), of genes constitutes one genomic pathway necessary for cellular alterations. We herein tested the prediction that specific regulation of ERE-driven genes by an engineered monomeric and constitutively active transcription factor, monotransregulator, provides a basis for the treatment of ER-negative breast cancer. Using adenovirus infected ER-negative MDA-MB-231 cells derived from a breast adenocarcinoma, we found that the monotransregulator, but not the ERE-binding defective counterpart, repressed cellular proliferation and motility, and induced apoptosis through expression of genes that required ERE interactions. Similarly, the monotransregulator suppressed the growth of ER-negative BT-549 cells derived from a breast-ductal carcinoma. Moreover, the ERE-binding monotransregulator repressed xenograft tumor growth in a nude mice model. Thus, specific regulation of genes bearing EREs could offer a therapeutic approach for *de novo* endocrine-resistant breast cancers.

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INTRODUCTION

The dysregulation of integrated systems that orchestrate the growth and death of breast-ductal epithelial cells leads to breast cancer. 17β-estradiol (E2), the main circulating estrogen hormone, is one agent involved in the initiation and progression of breast cancer (1,2). The effects of E2 are mediated primarily by the estrogen receptors (ERs) α and β (1,2). ERs, as with other transcription factors, are modular proteins in which distinct structural regions display unique functional features of the receptors (1,2). The amino-termini of ERs contain a ligand-independent transactivation function. The central regions are the DNA binding domains followed by flexible hinge domains that contain a nuclear localization signal. The multifunctional carboxyl-termini mediate ligand binding, dimerization and ligand-dependent transactivation functions of the receptors.

Following synthesis, ERs dimerize and mainly translocate to the nucleus independently of E2 (3). The binding of E2 induces major conformational changes in ERs, which expose surfaces that support coregulatory protein interactions critical for transcription activity (1,2). One pathway for E2-ERs to regulate gene expression involves the interaction of E2-ERs with estrogen responsive elements (EREs), which are permutations of a palindromic, GGTCAnnnTGACC, DNA sequence (1,2). This signaling is called the ERE-dependent signaling pathway (1,2). E2-ERs also regulate gene expression through interactions with transcription factors bound to their cognate regulatory elements on DNA, hence the ERE-independent signaling pathway (1,2).

Current treatments for ER-positive breast cancer include inhibitors of enzymes involved in estrogen biosynthesis to reduce/ablate the circulating hormone and on antiestrogenic compounds to alter/prevent ER functions (4–6). These approaches are initially successful in producing remission of established tumors (4,5). However, tumors eventually de-

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velop resistance to such therapies resulting in the acquired endocrine-resistance phenotype (4–6).

Endocrine modalities also are ineffective in the treatment of *de novo* endocrine-resistant breast cancer because of the absence, or loss, of ER expression (4–6). An autonomous regulation of aberrant cell growth signifies *de novo* resistance malignancies (4,5). Several growth factors and their receptors, which are transmembrane tyrosine kinases, are overexpressed and act as autocrine growth stimulators (7). While treatment options for *de novo* endocrine-resistant neoplasms are limited, inhibition of growth factor signaling, including antibody therapy to block ligand binding to receptors and kinase inhibitors to inhibit receptor activity, are being explored as therapeutic approaches (7). In addition, reexpression of ERs by epigenetic approaches or introduction of ERs by gene delivery could provide therapeutic benefits by restoring ligand signaling for ER-negative breast neoplasms (8,9).

Our recent studies aimed at dissecting nuclear estrogen signaling pathways suggest that genomic responses from the ERE-dependent signaling pathway to E2-ER are required to induce phenotypic changes (10,11). These findings raise the possibility that targeted regulation of ERE-driven genes could be a basis for the development of a therapy for ER-negative breast cancers. We herein examined this prediction by using engineered transcription factors specifically designed to regulate the expression of ERE-driven genes independently of ligands, ER status and cell type (12). We previously showed that the intrinsic specificity of the DNA binding domain of ERα to interact with ERE sequences can be exploited to engineer a monomeric ERE-binding module by cojoining two DNA binding domains with the hinge domain (12). Integration of strong transcription activation domains from other transcription factors into the ERE binding module generated monomeric transcription factors, or monotransregulators, with constitutive activity at ERE-driven gene promoters (12). Our present findings from adenovirus infected cells emulating *de novo* endocrine-resistant tumors indicate that a monotransregulator, but not the ERE-binding defective counterpart, altered cellular proliferation, death and motility by mimicking the effects of E2-ERα on gene transcriptions that required ERE interactions. Furthermore, the monotransregulator effectively repressed xenograft tumor growth in nude mice. Emphasizing the critical importance of the ERE-dependent signaling pathway in the induction cellular responses, these findings provide a basis for the development of transcriptional approaches that target EREs of estrogen-responsive genes for the treatment of *de novo* endocrine-resistant breast cancer.

MATERIALS AND METHODS

Engineering of ERE Binding Proteins

Expression vectors were described previously (12,13). $ER\alpha_{EBD}$ contains amino-acid substitutions (Glu203Ala, Gly204Ala and Arg211Glu) in the DNA recognition helix of the DNA binding domain (11). The construction of PV and PV_{EBD} also was described previously (12). Because of an earlier engineering strategy (12), PV_{EBD} contains a CDC with amino acid substitutions replacing Cys202 and Cys205 with His residues. These mutations completely prevent ERE interactions (12,14).

Restriction and DNA modifying enzymes were obtained from New England Bio-Labs (Beverly, MA, USA) or Invitrogen (Carlsbad, CA, USA). 17β-estradiol (E2) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture and Biochemical Assays

MDA-MB-231 cells were cultured as described (10,11). BT-549 cells obtained from ATCC (Manassas, VA, USA) were maintained in RPMI-164 supplemented with 10 μg/mL insulin (Sigma-Aldrich) and with 10% fetal bovine serum (FBS, Invitrogen). Recombinant adenoviruses were generated and tittered as described (10,11).

To assess the functional protein synthesis, we used immunocytochemistry (ICC), Western blot (WB) and electrophoretic mobility shift assay (EMSA) (13).

Endogenous Gene Expression

MDA-MB-231 cells (100,000 cells) were plated in 6-well culture plates in phenol red-free medium containing 10% CD-FBS without E2 for 24 h. Cells were then infected with Ad-ERα or Ad5-$ER\alpha_{EBD}$ and maintained with or without 10^{-9} M E2 for 48 h, while Ad5-PV or Ad5-PV_{EBD} infected cells were maintained for 48 h in the presence of vehicle. The total RNA was subjected to quantitative PCR (qPCR) using custom TaqMan Low Density Arrays (Foster City, CA, USA) (10,11). Relative quantification analysis was performed using the comparative $^{C}_{T}$ method (15).

Cellular Proliferation

MDA-MB-231 (5,000 cells) or BT-549 (10,000 cells) cells were plated in 24-well culture plates in phenol red-free medium containing 10% CD-FBS for 24 h. Cells were infected with or without 10^{-9} M E2 for 6 d. At termination, cells were subjected to counting using a hemocytometer and a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen) (10,11).

Cell Cycle Analysis

Cells (50,000 cells) in 6-well culture plates were infected with or without E2 for 48 h. Cells were subjected to fluorescence-activated cell sorting (FACS) using the EPICS Elite (Coulter Corp, Miami, FL, USA) (10,11).

Annexin V and TUNEL Assays

Cells (100,000 cells) in 6-well culture plates infected with or without E2 for 96 h were subjected to an Annexin V assay (Invitrogen) as described (10,11). Infected cells (25,000 cells) in poly-D-lysine coated 48-well plates with or without E2 for 96 h were also subjected to a TUNEL assay (Promega, Madison, WI, USA) as described (10,11).

Motility Assays

Infected cells (200,000 cells) in 12-well plates were maintained for 48 h with or without E2 to allow cells to reach near confluence. A wound was then generated and the gap closure was imaged at 24-h intervals (10, 11).

For the invasion assay, equal numbers of infected cells (25,000) for 48 h from each treatment group were seeded into BD Matrigel Invasion Chambers (San Diego, CA, USA) for 24 h. Cells on the bottom of the chamber membrane were stained with Diff-Quik (Dade Behring, Newark, DE, USA). Images were captured, and stained cells were counted from images as described (10,11).

Tumor Studies

All animal experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol. MDA-MB-231 cells infected with an adenovirus for 48 h were collected. Live cells (4×10^6 cells/mouse) were resuspended in 100 L base medium and Matrigel (BD Biosciences, Bedford, MA, USA) at a 1:1 ratio to support initial tumor growth. Cell suspensions were inoculated subcutaneously using a 27-gauge needle into the left mammary fat pad of 6-wk-old female athymic NCr-*nu*/*nu* nude mice (NCI, Frederick, MD, USA) housed in microisolator cages and given food and water *ad libitum*. Mice were divided into 5 groups of 10 mice, with the exception of the Ad5-PV-100 group that had 9 mice because of the death of a mouse at an early stage of the study. Groups of mice were inoculated with cells infected with either the parent Ad5 at 900 MOI, Ad5-PV_{EBD} at 900 MOI, Ad5-PV at 100 MOI together with Ad5 at 800 MOI, Ad5-PV at 200 MOI together with Ad5 at 700 MOI or Ad5-PV at 400 MOI together with Ad5 at 500 MOI. The initial tumor growth was monitored by palpation, and tumor size was subsequently measured weekly with calipers. The tumor volume was estimated by using an ellipsoid approximation and the formula of $V = (\pi/6)abc$, wherein a, b and c denote length, width and height, respectively. At termination, tumors were excised, weighed, fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 4 μm thickness were stained with hematoxylin/eosin, and images were captured by light microscopy.

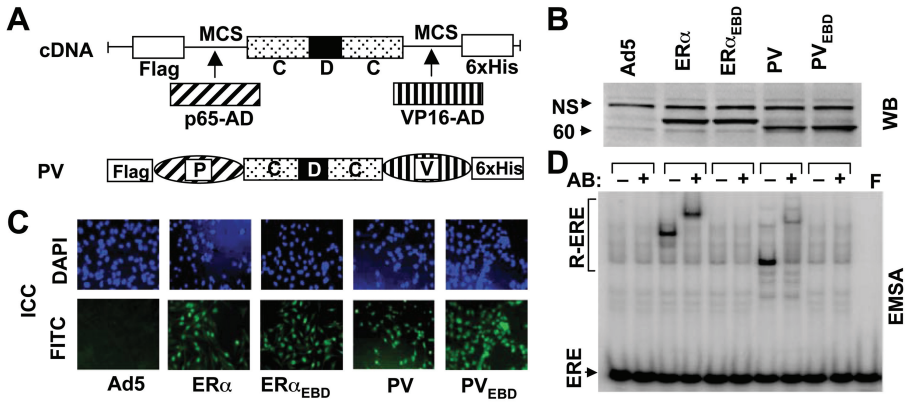

Figure 1. Engineering of monotransregulators. (A) The activation domains (ADs) of p65 and VP16 proteins were genetically joined through multiple cloning sites (MCS) to the 5′ and 3′ ends, respectively, of the CDC-cDNA to generate the monotransregulator PV. The construct also contains sequences encoding the Flag and 6xHis epitopes at the amino- and carboxyl-termini, respectively. (B) MDA-MB-231 cells were infected with the parent recombinant adenovirus (Ad5) at MOI 900, a recombinant adenovirus bearing cDNA for ERα at 50, $ER\alpha_{EBD}$ at 150, PV at 200 and PV_{EBD} at 900 MOI. For all infections, the total MOI was adjusted to 900 by supplementing with the parent Ad5. Extracts (10 μg) of infected MDA-MB-231 cells at 48 h were subjected to Western blotting (WB) using a horseradish peroxidase-conjugated monoclonal Flag antibody. Molecular mass in kDa is indicated. NS denotes nonspecific protein detection. (C) Intracellular localization of receptor proteins was examined by immunocytochemistry (ICC). A fluorescein isothiocyanate-conjugated Flag antibody (FITC) localizes constructs to the nucleus stained with 4′,6-diamidino-2-phenylindole (DAPI) at 48 h after infection. (D) Cell extracts (10 μg) at 48 h after infection also were subjected to electrophoretic mobility shift assay (EMSA) with (+) or without (–) a Flag antibody (Ab). ERE specifies unbound and R-ERE denotes receptor-bound radiolabeled ERE. F in panel B indicates the radiolabeled ERE only. In all experiments a representative result from three independent experiments is shown.

Statistical Analysis

Results were presented as the mean ± standard error of the mean (SEM). Significance was determined using a two-tailed unpaired t test with a confidence interval of 95%.

All supplementary materials are available online at www.molmed.org.

RESULTS

Synthesis of Monotransregulators

Our previous studies have demonstrated that genomic responses from the ERE-dependent pathway induced by E2-ERs are required to elicit cellular alterations (10,11). We therefore anticipated that the regulation of ERE-bearing genes by monotransregulators would emulate the effects of E2-ERα on cellular phenotypes. To examine this prediction, we used the montransregulator p65-CDC-VP16, or PV (12). PV bears single copies of the activation domains of the p65 subunit of nuclear factor κB (16) and the herpes simplex virion protein-16 (17) genetically fused to the amino- and carboxyl-termini of the ERE binding module CDC (Figure 1A). CDC is composed of two DNA binding (C) domains cojoined with the hinge (D) domain to simulate the interaction of each ERα monomer in the ERα dimer with one ERE half-site (18,19) (see Figure 1A). We also used the ERE binding defective PV (PV_{EBD}), which contains amino-acid replacements in the ERE binding module to prevent ERE interactions (12).

We utilized ER-negative MDA-MB-231 cells derived from human breast adenocarcinoma as a model (8,10,11). The recombinant adenovirus bearing ERα-cDNA (Ad5-ERα) was used at 50 multiplicity of infection (MOI), with which infected cells synthesize ERα at a level that requires E2 to function (11). The concentration of Ad5-PV was based on preliminary studies (Supplemental Figure 1A–E). Ad5-PV at 200 MOI synthesized an amount of functional PV that altered the growth and cell cycle phases similar to those observed with E2-ERα (see Supplemental Figure 1A–E). The adenovirus expressing ERE binding defective ERα ($ERα_{EBD}$) or PV (PV_{EBD}) cDNA was used at 150 or 900 MOI, respectively. At these MOIs, infected cells produced mutants at levels comparable to those of parent proteins that reached maximal amounts at 48 h after infection (see Supplemental Figure 1). For all experiments, the total MOI was adjusted to 900 by supplementing with the parent adenovirus (Ad5).

Western blot (WB) analysis using an antibody specific to the Flag epitope at the amino-terminus of each construct indicated that cells synthesize monotransregulators with a molecular mass of ~63 kDa (Figure 1B). As ERs, PV and PV_{EBD} locate to the nucleus as assessed by immunocytochemistry (ICC) (Figure 1C). An electrophoretic mobility shift assay (EMSA) further verified that PV, like ERα, but not PV_{EBD} or $ERα_{EBD}$, interacts with ERE (Figure 1D).

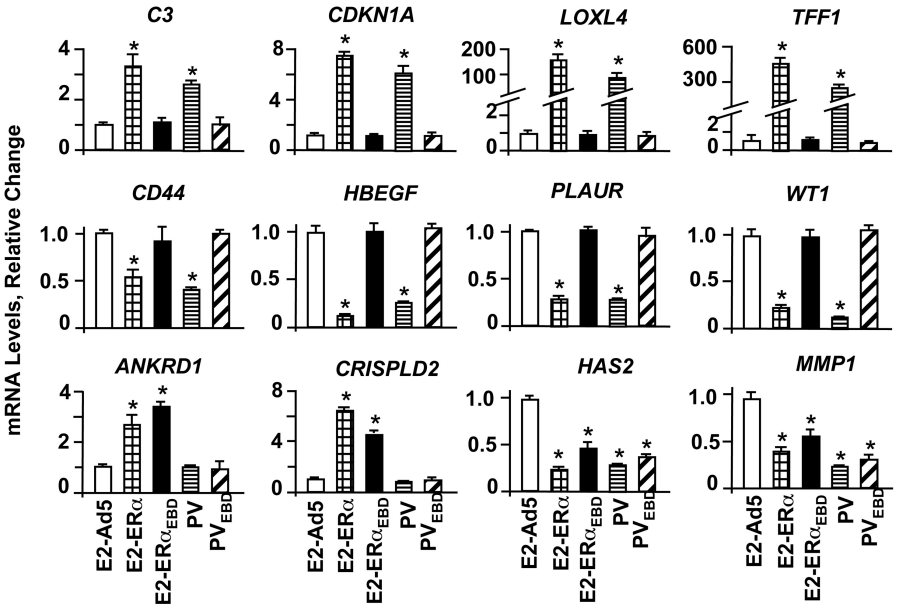

Figure 2. Regulation of endogenous gene expression by monotransregulators. MDA-MB-231 cells infected with the adenovirus bearing none (Ad5), ERα or $ERα_{EBD}$ cDNA were treated without (data not shown) or with 10^{-9} M E2 for 48 h, while cells infected with the adenovirus bearing Ad5-PV or Ad5-PV_{EBD} were treated with vehicle. Total RNA was subjected to qPCR for the analysis of estrogen responsive gene expressions. Results, which are the mean ± SEM of three independent determinations, depict relative change in mRNA levels compared with those observed in cells infected with Ad5 in the absence of E2, which is set to 1. Asterisk (*) indicates significant change.

Effects of Monotransregulators on Gene Expression

E2-ERs activate or repress the expression of responsive genes through the ERE-dependent and ERE-independent signaling pathways. To assess the effects of monotransregulators on gene expression, total RNA from MDA-MB-231 cells at 48 h after infection was subjected to quantitative PCR. E2-ERα induced the expression of the *C3* (*complement component 3*) *CDKN1A* (*cyclin dependent kinase inhibitor, p21*), *LOXL4* (*lysyl oxidase-like 4*) and *TFF1* (*trefoil factor 1*; *pS2*) genes, as we previously reported (11). On the other hand, E2-ERα repressed the transcription of the *CD44* (*CD44 antigen*), *HBEGF* (*heparin-binding EGF-like growth factor*), *PLAUR* (*plasminogen activator, urokinase receptor*) and *WT1* (*Wilms tumor 1*) genes (Figure 2). We found that PV mirrored the effects of E2-ERα on these gene expressions, while E2-$ERα_{EBD}$ or PV_{EBD} did not affect the transcription of these genes. Thus, the regulation of this subset of genes by PV, like E2-ERα, requires ERE interactions.

We also observed that the expression of *ANKRD1* (*ankyrin repeat domain 1 [cardiac muscle]*), *CRISPLD2* (*cystein-rich secretory protein LCCL domain containing 2*), *HAS2* (*hyaluronan synthase 2*) and *MMP1* (*matrix metallopeptidase 1 [interstitial collagenase]*) genes is independent of ER-ERE interactions as E2-$ERα_{EBD}$ and E2-ERα modulated the transcription of these genes, as we showed previously (11). In contrast, PV or PV_{EBD} had no effect on the *ANKRD1* or *CRISPLD2* gene expression. This suggests that the expression of these genes is ERα-specific. All constructs, however, repressed the transcription of the *HAS2* or *MMP1* gene, implying the generality of action.

Mediation of Cellular Growth

Our previous studies indicated that the ERE-dependent genomic responses to E2-ERα are necessary for the induction of phenotypic alterations (10,11). Since PV mirrored the effects of E2-ERα on gene expressions that require ERE interactions, we asked whether PV alters cellular growth similar to E2-ERα. Analysis of histograms generated by cell sorting of MDA-MB-231 cells at 48 h after infection revealed that ERα only in the presence of 10^{-9} M E2, but not $ERα_{EBD}$, altered cell cycle kinetics compared with the parent Ad5 (Figure 3A, Supplemental Figure 2A). PV also increased the number of cells accumulated in G1 phase and

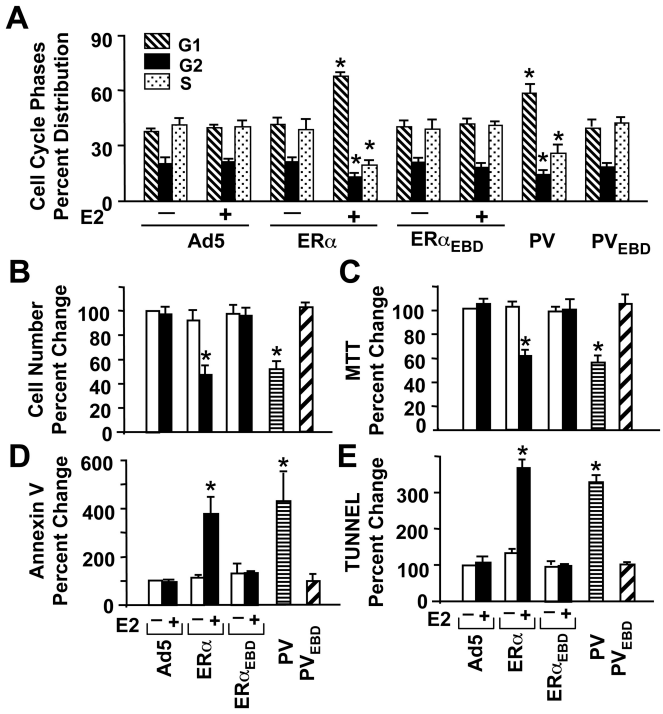

Figure 3. Effects of monotransregulators on cellular growth. (A) MDA-MB-231 cells infected with recombinant adenoviruses in the presence (+) or absence (–) of 10^{-9} M E2 for 48 h were subjected to fluorescence-activated cell sorting for the cell cycle analysis. Results depicted as the percentage of cells in G1, G2 and S phases are the mean ± SEM of three independent experiments. (B, C) Infected cells maintained in the presence (+) or absence (–) of 10^{-9} M E2 (E2) for 6 d were subjected to (B) cell counting or (C) MTT assay. The mean ± SEM, which depicts three independent experiments performed in duplicate, indicates percentage (%) change in cell number compared with those observed in cells infected with Ad5 in the absence of E2, which is set to 100. (D, E) MDA-MB-231 cells also were subjected to (D) Annexin V and (E) TUNEL assays at 96 h after infection for the effects of proteins on cellular death. Results, which are the mean ± SEM of three independent experiments, are summarized as percentage (%) change in the number of cells bound to Annexin V or as the number of cells that incorporated fluorescein isothiocyanate-conjugated dUTP into the fragmented DNA (TUNEL) obtained in comparison with the cells infected with Ad5 in the absence of E2, which is set to 100. Asterisk (*) indicates significant change.

decreased cell numbers in S and G2 phases, whereas PV_{EBD} had no effect on the distribution of cycle phases compared with Ad5.

These results suggest that alterations in cell cycles require ERE interactions, which was also reflected in the cellular proliferation. ERs without E2 did not affect cell growth assessed by cell counting (Figure 3B) or MTT assay (Figure 3C), shown at d 6 after infection. However, in response to 10^{-9} M E2, ERα, but not $ER\alpha_{EBD}$, effectively suppressed proliferation. Similarly, PV, but not PV_{EBD}, repressed cellular growth in comparison with Ad5.

Regulation of Cell Death

The effect of PV on proliferation was the inverse of its effect on apoptosis. PV, like E2-ERα, augmented apoptosis, as revealed by an increase in cell number stained with Annexin V. As a marker for the midstages of apoptosis (20), Annexin V assesses the integrity of the cellular membrane, with significant changes occurring at 96 h after infection (Figure 3D and Supplemental Figure 2B). A TUNEL assay, which catalytically incorporates fluorescein-conjugated nucleotides into the fragmented DNA as one late-stage characteristic of apoptosis (20), further corroborated the results. PV and E2-ERα, but not ERE binding defective counterparts, induced DNA fragmentation at 96 h after infection (see Figure 3D, Supplemental Figure 2C) compared with Ad5.

Effects of Monotransregulators on Cell Type

To ensure that the effect of PV on cellular growth is not cell type specific, we also infected ER-negative BT-549 cells derived from a breast-ductal carcinoma. While mutants were synthesized at levels comparable to those of the parent proteins (Figure 4A), ERα and PV, but not $ER\alpha_{EBD}$ or PV_{EBD}, interacted with ERE (Figure 4B). As with MDA-MB-231 cells, ERα in the presence of 10^{-9} M E2 and PV repressed the growth of BT-549 cells (shown at d 6 after infection). On the other hand, $ER\alpha_{EBD}$ with or without E2 or PV_{EBD} had no effect on cell growth (Figure 4C). Repression of cellular proliferation by E2-ERα or PV involves apoptosis since both increased the cell population stained with Annexin V compared with Ad5 (Figure 4D).

Modulation of Cellular Motility

Breast cancer cells that synthesize ERs endogenously are less motile and invasive than ER-negative cells (8). Introduction of ERs into ER-negative cells hinders cellular motogenesis in an ERE-dependent manner as the ERE binding defective ERs induce no effect (8,10,11). We therefore anticipated that PV, like E2-ERα, would inhibit the motility and invasiveness of MDA-MB-231 or BT-549 cells. Infected cells with or without 10^{-9} M E2 were grown for 48 h to near confluence. A wound was then created and the rate of wound closure was assessed as a function of time. The unliganded ERα delayed the wound closure of MDA-MB-231 cells, which was further delayed with E2, shown at 96 h, in comparison with Ad5 (Figure 5A, Supplemental Figure 2D). Similarly, PV hindered the wound heal-

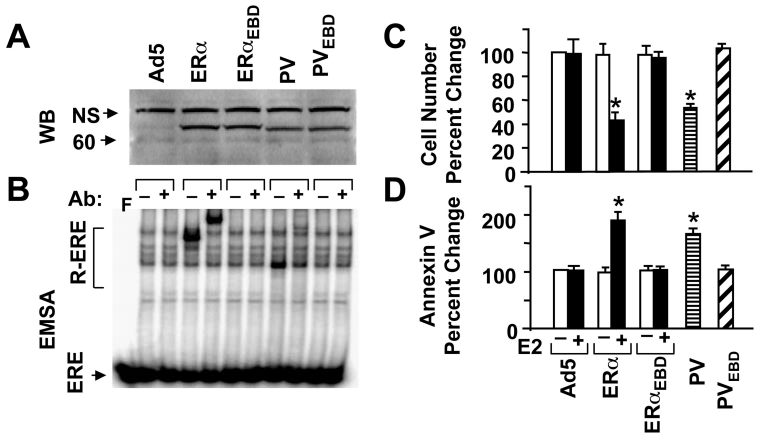

Figure 4. PV mimics the effects of E2-ERα on responses from BT-549 cells. (A & B) Synthesis of functional receptor proteins. BT-549 cells were infected with an adenovirus bearing none (Ad5) or a cDNA for ERα, $ERα_{EBD}$, PV, $ERα_{EBD}$ at 80, 20, 20, 60 and 80 MOIs, respectively. The total MOI was adjusted to 80 MOI by supplementing with Ad5. (A) Extracts of infected BT-549 (25 μg) cells at 48 h after infection were subjected to WB using the horseradish peroxidase-conjugated monoclonal Flag antibody. Molecular mass in kDa is indicated. (B) Cell extracts were also subjected to EMSA in the presence (+) or absence (–) of a Flag antibody. ERE indicates the unbound and R-ERE depicts the receptor-bound radiolabeled ERE. F denotes the radiolabeled ERE only. (C, D) Infected cells in the presence (E2) or absence (–) of 10^{-9} M E2 for 6 d were subjected to (C) cell counting for cellular proliferation or (D) Annexin V assay at 96 h after infection for cellular death. The mean ± SEM, which represents three independent experiments, indicate percentage (%) change in cell number compared with those observed in cells infected with Ad5 in the absence of E2, which is set to 100. Asterisk (*) indicates significant change.

ing, whereas PV_{EBD} or $ERα_{EBD}$ had no effect. PV and E2-ERα also repressed the motility of BT-549 cells as compared with Ad5 at 48 h after infection (see Figure 5A).

We also examined the effects of receptor proteins on cellular invasiveness using an invasion assay that assesses the capacity of cells to migrate through a reconstituted basement membrane. Equal numbers of infected cells were seeded for 48 h on the top of membranes of invasion chambers (Figure 5B, Supplemental Figure 2E). After 24 h, cells on the bottom of the membrane were stained and imaged. PV, like E2-ERα, suppressed the invasiveness of both cell lines, which required ERE interactions, as $ERα_{EBD}$ or PV_{EBD} did not affect migration of infected cells compared with Ad5.

Effects of Monotransregulators on Tumor Formation

Our collective results *in situ* indicated that PV effectively represses the growth of ER-negative cells. We therefore anticipated that the introduction of PV to MDA-MB-231 cells would impair tumor formation *in vivo*. Since the extent by which PV affects cellular growth correlated with the amount of protein synthesized as a result of the Ad5-PV concentration used (see Supplemental Figure 1), we also wanted to address whether graded doses of Ad5-PV would influence the number and growth of tumors. To accomplish this, we infected cells with Ad5 or Ad5-PV_{EBD} at 900 MOI. We also infected cells with Ad5-PV at 100, 200 or 400 MOI supplemented with Ad5 to bring the total MOI to 900. At 48 h after infection, 4×10^6 cells/mouse were implanted subcutaneously into the left mammary pads of athymic NCr-*nu*/*nu* mice (10 mice/group). The implantation site was monitored weekly for 10 wks. Solid tumors in mice implanted with cells infected with Ad5 or PV_{EBD} were palpable by wk 3, and the experiments were terminated at wk 10. Tumor volumes (Figure 6A) and weights (Figure 6B) were determined at this time, and tumors were removed for sectioning and histological preparations using hematoxylin/eosin staining (Supplemental Figure 3). The incidences of primary tumors (indicated in parentheses, Figure 6A) in the Ad5 or Ad5-PV_{EBD} group were 80% and 90%, respectively. On the other hand, mice implanted with cells infected with Ad-PV at various MOIs resulted in slower onset and progression of tumor growth and showed a substantially reduced tumor incidence. The Ad5-PV-100 group had a 44% tumor incidence, versus 10% for the Ad5-PV at 200 MOI. No tumor growth was detected at any time in the Ad5-PV-400 group, nor were *post mortem* tumors observed at the implantation site. These results indicate that PV also represses the development of tumors in xenograft models through a signaling pathway that requires ERE interactions.

DISCUSSION

Current endocrine approaches for ER-positive breast cancer that target E2 synthesis and/or ER functions are successful in the management of the disease in an adjuvant setting or at advanced stages. However, these modalities are ineffective in the treatment of ER-negative endocrine-resistant cancer that accounts for about one-third of all breast neoplasms. An insufficient understanding of the underlying mechanisms responsible for the disease limits treatment options.

Studies have shown that prevention of ER binding to ERE sequences of responsive genes by exogenously introduced ERE decoys (21) or electrophilic agents that disrupt zinc-fingers of ERα (22–24) represses E2-mediated growth of ER-positive breast cancer cells. Complementing this, we recently reported that genomic responses from the ERE-dependent pathway mediated by E2-ER are required to suppress the growth of ER-negative cells as well (10,11). Collectively, these findings indicate the importance of ER-ERE interactions in the proliferation of

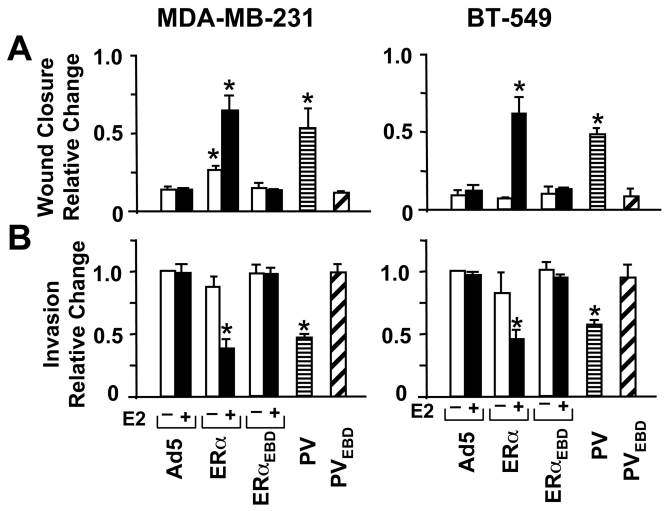

Figure 5. Effects of receptor proteins on cellular motility. (A) Wound-healing assay. Infected MDA-MB-231 or BT-549 cells were incubated in the presence (+) or absence (–) of 10^{-9} M E2 as indicated for 48 h to allow cells to reach near confluence. A wound was then created and images were captured at 0 h and at 24 h intervals. Results, representing the mean ± SEM of three independent experiments performed in duplicate, summarize the wound closure at 96 h for MDA-MB-231 cells and 48 h for BT-549 cells relative to 0 h, which is set to 1. (B) Invasion assay. Cells were infected with recombinant adenoviruses with (+) or without (–) 10^{-9} M E2 for 48 h as depicted. Cells were collected and counted. The same number of cells from each group was then seeded on the upper section of the invasion chamber membrane with the corresponding treatment. After 24 h incubation, cells on the bottom of chamber membrane as an indication of invasiveness were stained and counted. Results, which represent the mean ± SEM of three independent experiments performed in duplicate, are relative changes compared with cells infected with Ad5 in the absence of E2, which is set to 1. Asterisk (*) indicates significant change.

cells synthesizing ERs. We therefore anticipated that targeted regulation of ERE-driven genes independent of ligand-ER signaling and cellular context could provide a basis for developing a therapeutic approach for endocrine-resistant breast cancers. Consistent with this prediction, we demonstrate here that monotransregulator PV suppressed *in situ* and *in vivo* growth of cells derived from ER-negative breast neoplasms by regulating gene expression that rely on ERE interactions.

Studies using global gene expression profiling have revealed that E2-ER regulates the expression of genes involved in various cellular functions, including signal transduction, cellular proliferation, apoptosis and motility (10,11,25). ER, as with other transcription factors, regulates gene transcription through events that involve covalent modifications of chromatin within which responsive gene promoter resides (26). The binding of E2-ER to an ERE sequence of a responsive gene generates a platform for an ordered and combinatorial recruitment of coregulator complexes, including protein and chromatin modifiers (26). The integrated effects of these complexes induce local chromatin alterations that restructure the promoter for the recruitment of basal transcription machinery to activate gene expression (26). E2-ER, as with other transcription factors, also represses the expression of genes. Although the mechanisms are unclear, recruitment of corepressor complexes or squelching of coactivators by the ERE-bound E2-ER is suggested to induce chromatin modifications that counteract the effects of activating signals, thereby repressing the gene expression (27,28). Our results demonstrate that PV mirrored the ability of E2-ERα to regulate the transcription of responsive genes that rely on ERE-interactions. While PV and E2-ERα enhance the expression of the *C3*, *CDKN1A*, *LOXL4* and *TFF1* genes, both suppress the *CD44*, *HBEGF*, *PLAUR* or *WT1* gene expression. These findings suggest that PV and ERα utilize common mechanisms to activate as well as repress the expression of ERE-driven genes. Studies showed that the activation domains of p65 or VP16 induce transcription by orchestrating the recruitment of coactivator proteins, the majority of which also partner with E2-ER (29,30). Since PV and E2-ERα activate or repress the expression of the same genes by interacting with ERE sequences, it appears that the nature of the promoter of the estrogen responsive gene within which an ERE is located determines the direction of gene expression.

PV also regulated the expression of a set of estrogen responsive genes that do not require ERE interactions. In the ERE-independent signaling pathway, E2-ER interacts with transcription factors bound to their cognate regulatory elements on DNA to modulate gene expression in a ligand-, promoter- and cell-context–dependent manner (31,32). Although mechanisms are unclear, direct interactions between surfaces in the amino- and/or carboxyl-termini of ERα and the DNA-bound transcription factor or indirect interactions with coregulators recruited with either transcription factor could be responsible for gene regulation (31,32). We observed that PV or PV_{EBD} had no effect on the transcription of the *ANKRD1* or *CRISPLD2* gene, whereas both ERα and $ERα_{EBD}$ induced the expression of these genes in response to E2. This suggests that the regulation of these genes is dependent upon ER-specific structural features. On the other hand, PV and PV_{EBD}, as ERα and $ERα_{EBD}$, modulated the expression of the *HAS2* and *MMP1* genes. This implies a generality of action, which could involve structural/mechanistic features shared by other transcription factors or hormone receptors. Indeed, *HAS2* gene expression also is regulated by retinoic acid signaling (33,34). Similarly, glucocorticoid, progesterone, retinoic acid and thyroid hormone

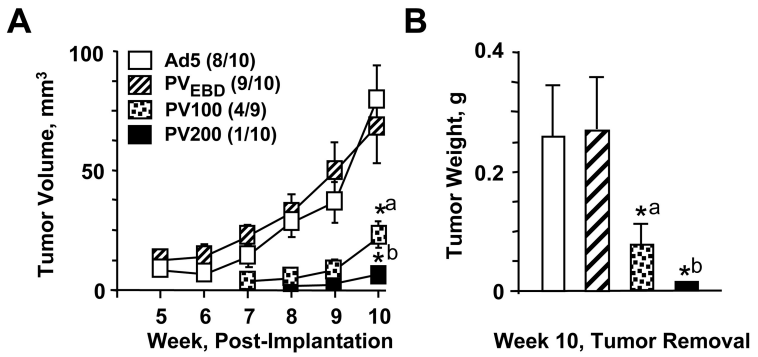

Figure 6. Effects of monotransregulators on tumor growth in xenografts. MDA-MB-231 cells were infected with Ad5 or Ad5-PV$_{EBD}$ at 900 MOI for 48 h. A group of cells was also infected with Ad5-PV at 100 or 200 MOI supplemented with 800 or 700 MOI of Ad5, respectively. Infected cell suspensions (4 × 10^6 cells/mouse) were subcutaneously inoculated into the left mammary fat pads of female athymic NCr-*nu/nu* mice (ten mice/group, with the exception of the Ad5-PV-100 group that contained nine mice). (A) The tumor size was measured with calipers weekly and the tumor volume was calculated. Tumor incidence for each group is shown in parenthesis. Points denote the mean ± SEM of tumor volume of each group. *[a] denotes significant change ($P < 0.041$) compared with Ad5. *[b] indicates significant change ($P < 0.0161$) compared with Ad5. (B) At wk 10, mice were killed. Tumors were removed and weighed. Bars are the mean ± SEM of tumor weight from each group. *[a] denotes significant change ($P < 0.008$) compared with Ad5. *[b] indicates significant change ($P < 0.001$) compared with Ad5.

signaling pathways modulate *MMP1* gene expression (35–38).

Despite the ability of PV$_{EBD}$ to modulate the expression of a subset of estrogen responsive genes, the ERE binding defective monotransregulator had no effect on the proliferation, apoptosis or motility of model cells. Cell proliferation, for example, is dependent upon transcriptional as well as non-transcriptional events to activate the cascade of cyclin-dependent kinases critical for progression through cell cycle phases (39). The activities of cyclin-dependent kinases are modulated by kinase inhibitors that include p21 protein encoded by the *CDKN1A* gene. p21 suppresses proliferation by promoting cell cycle arrest in the G1/S phase transition (39). Increased expression of *CDKN1A* mediated by PV and E2-ERα but not the ERE-binding defective counterparts may be one key event that contributes to the arrest in cell cycle progression, and consequently, the repression of cellular proliferation. Hyaluronan (HA), a major glycosaminoglycan present in the extra cellular matrix, is critical for structural integrity of cells (40). HA is synthesized by the hyaluronan synthase family of transmembrane glycosyltransferases that include hyaluronan synthase 2, a product of the *HAS2* gene (40). The interaction of HA with its surface receptor CD44 encoded by the *CD44* gene initiates a signaling cascade that is critical for cellular proliferation, adhesion and migration (40). PV as E2-ERα regulated the expression of both the *CD44* and *HAS2* genes. On the other hand, the transcription of only the *HAS2* gene was modulated by PV$_{EBD}$ and E2-ERα$_{EBD}$, both of which had no effect on phenotypic features of cell models. These results further highlight the canonical importance of the ERE-driven gene network in the induction of cellular responses. Thus, the ability of the DNA binding domain of ERα to decode ERE sequences allowed PV to induce cellular responses by mimicking E2-ERα to modulate the transcription of genes whose regulations require ERE interactions independent of ligands. The monotransregulator approach therefore provides proof of principle for the development of a transcription therapy for *de novo* endocrine-resistant breast neoplasms.

Counteraction of the beneficial effects of endocrine approaches by the tumor cells that synthesize ERs leads to acquired endocrine-resistant phenotypes, in which antiestrogenic compounds are no longer capable of inhibiting cellular growth (41,42). Although mechanisms are unclear, changes in the relative levels of ER subtypes, ER-isoforms or coregulatory proteins, alterations in the pharmacokinetics of antiestrogenic compounds or aberrations in signaling pathways that cross-talk with E2-ER could contribute to the development of *acquired* endocrine resistance (4–6). Since ER plays a pivotal role in both the initial response and subsequent resistance to antiestrogenic compounds, additional therapeutic benefits could be achieved by preventing ER synthesis or function. Repression of ER synthesis by inhibitory RNA technologies (43) or interference with endogenous ER functions by future generations of antiestrogenic compounds (44) or ER-specific electrophilic agents (22–24) could constitute strategies for the treatment of acquired endocrine-resistant breast cancers. Since these approaches target ER, however, circumvention of ER-dependent events by growth signaling pathways could lead to tumor progression. Monotransregulators with repressor functions that effectively suppress the expression of ERE-driven genes, independent of ligand, receptor status or other signaling pathways could provide an approach for the treatment of *acquired* endocrine-resistant neoplasms as well. The monotransregulator design could also be extended to steroid/thyroid hormone receptors that show modular structure. Targeted regulation of endogenous genes by specific responsive element binders with activator or repressor functions may provide novel approaches to biology and medicine.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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