



Suppression of gene expression by tethering KRAB domain to promoter of ER target genes[☆]

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Abstract

Estrogens play an important role in the development and progression of breast cancer. Although estrogen antagonist treatment often results in the arrest or remission of breast cancer growth, most breast cancers recur and become resistant to estrogen ablative therapy. The molecular mechanisms underlying these actions remain largely undefined. It is hypothesized that tumor cells of an advanced stage may develop compensatory pathways to stimulate the expression of estrogen receptor (ER) target genes or downstream events, independent of estrogen action. In this study, we developed a chimeric repressor to turn off ER target genes with the aim of directly investigating the role of ER target genes in tumor progression. The chimeric repressor contains the ER DNA-binding domain that recognizes estrogen response elements (EREs), a Kruppel-associated box (KRAB) repressor domain which silences target genes when tethered to their promoter regions and a truncated progesterone ligand-binding domain which responds only to the exogenous synthetic ligand, RU486. The ability of the chimeric repressor to block ER mediated transcription was assessed in transient transfection assays. ER-induced reporter activity was inhibited by the repressor in a dose-dependent manner, with the maximum effect of more than 80% reduction. The inhibitory activity of the chimeric repressor was tightly under the control of RU486. Effective suppression by the repressor on the natural promoter of ER target gene, complement factor 3 (C3), was also observed. The inhibitory activity was specific to ER, since the repressor has no effect on other nuclear receptor systems tested. Furthermore, the repressor could inhibit the 4-hydroxy-tamoxifen (4OH-T)-induced ER activity. Taken together, our results demonstrate that the inducible repressor we have designed could specifically inhibit ER target gene expression in response to an exogenous synthetic ligand. This repressor will provide a useful tool to study the role of ER target genes in breast cancer progression and it may be potentially useful for gene therapy of breast cancer. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Estrogen receptor target gene; Regulable repressor; Kruppel associated box; Breast cancer

1. Introduction

Estrogens play an important role in the regulation of the development and function of sex organs, including the breast and reproductive tract [1] as well as in the maintenance of bone, liver, fat and cardiovascular functions [2]. In the breast, estrogen promotes epithelial cell proliferation and ductal elongation in normal breast morphogenesis [3]. Dysregulation of estrogenic activities often causes malfunction, and breast cancer development [4]. It is well documented

that cumulative exposure to estrogen is associated with initiation and progression of breast cancer [5, 6]. In addition, there is a strong correlation between reproductive history and the incidence of breast cancer [7]. Endocrine therapies in breast cancer patients could drastically reduce tumor formation and recurrence [8].

Most, if not all, effects of estrogens are mediated by the estrogen receptor (ER). ER belongs to a large family of nuclear receptors that function as ligand-inducible transcription factors. As a feature of nuclear receptors, ER is composed of three major functional domains: a ligand binding domain, a DNA binding domain and a transactivation domain. Upon binding to cognate ligand, ER is activated, dimerizes and binds to specific DNA response elements (EREs) in the regulatory region of target genes, eventually stimulating

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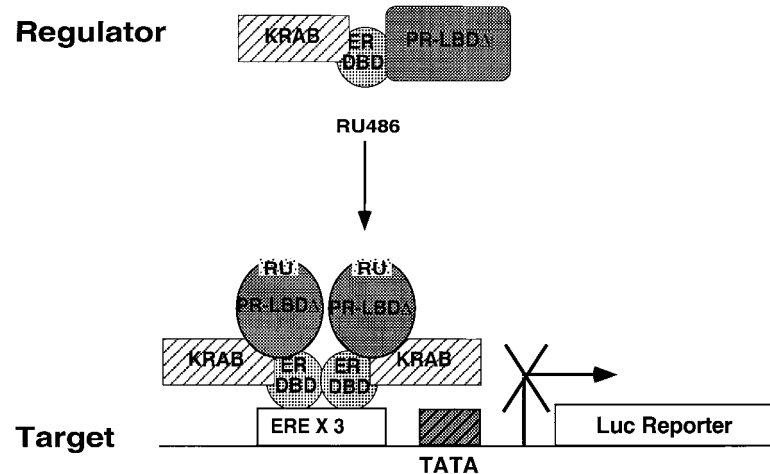
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target gene expression [9, 10]. Among ER target genes are growth factors which act as direct mitogens in a paracrine or autocrine manner to stimulate epithelial proliferation. More than half of breast carcinomas are found to be ER positive and retain some degree of steroid responsiveness [11]. An increased level of ER expression in breast cancer tissue implies that the tumor could have arisen from a subset of cells, which inappropriately express high levels of ER. This could further lead to the stimulation of cell proliferation and accumulation of genetic mutation, resulting in tumor formation and progression into estrogen independent growth.

The antiestrogen, tamoxifen, has been developed to block the binding of estrogen to its receptor. Tamoxifen has been used successfully to inhibit ER dependent growth of breast cancer [12]. A major unresolved issue is why most breast cancers that contain ER eventually become resistant to estrogen-ablation therapy [13]. It has been proposed that the mutation of ER to a constitutively active receptor or to a recep-

tor which can be activated by estrogen antagonists, like tamoxifen or other steroidal compounds existing in the blood stream may contribute to the transition from estrogen-dependent to -independent tumor growth [14, 15]. If this hypothesis is correct, attempts to block the interaction between ER and estrogen by antagonists will not be able to suppress ER function. However, half of all advanced breast cancers are estrogen receptor positive but resistant to antiestrogen therapy. Some ER negative tumors behave as if they are ER positive in their expression of ER target genes such as the progesterone receptor. Furthermore, many ER mutations identified in tumor cells are also found in healthy cells of breast cancer patients or healthy individuals. Thus, it remains controversial whether ER mutations have a primary role in the transition from estrogen-dependent to -independent states. An additional explanation is that the activation of ER may be possible through ligand independent pathway. Indeed, growth factors, intracellular protein kinases [16–18], the cell cycle regulator cyclin D1 and

A.



B.

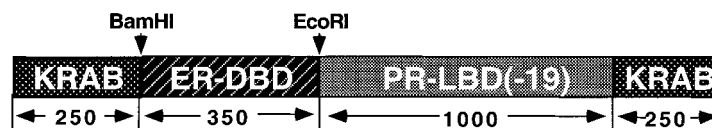


Fig. 1. Inducible repressor system. (A) Model of inducible repressor system. The regulable repressor contains a DNA-binding domain binding to estrogen response element (ERE), a transcription repression domain obtained from Kruppel-associated box (KRAB) and a mutated PR ligand-binding domain which responds to antiprogesterin, RU486. The regulable repressor constructed in this way can compete with wild-type ER for ERE binding and turn off all the ER target genes in breast cancer cells in the presence of RU486. (B) Diagram of regulable repressor KEDPK construct.

cyclin-dependent kinase (cdk2/cyclin A) complex [19–21] have been shown to modulate the activity of unliganded ER in different model systems. It is hypothesized that the unliganded receptor activation occurs via receptor phosphorylation. This phosphorylation leads to changes in receptor conformations whereby the potential to interact with co-activators or other transcription factors may be different from conformations induced by estrogens. This ligand-independent activity may also be enhanced by the addition of the partial agonist tamoxifen.

Though ER could be activated either by its ligand or alternatively by either mutations or ligand-independent pathways, the activated form(s) of ER will ultimately act on the regulatory region of its target genes to exert its biological function. In this study, we constructed a regulable repressor which will bind to an ERE and silence the ER target gene expression in response to exogenous stimuli. A similar strategy has been employed to construct a regulable inducer (GLVP) which activates target gene expression in response to exogenous signal [22,23]. To generate a repressor, we used the Kruppel-associated box (KRAB) which is a highly conserved repression domain in the Kruppel-class zinc finger family of transcription factors [24,25]. When KRAB is linked to a heterologous DNA-binding domain, it can shut off transcription of target genes containing the DNA response element to which the chimeric protein binds [26]. Here we constructed a fusion protein linking KRAB to the estrogen receptor DNA-binding domain to repress all the ER target genes with ERE in their promoters. In order to generate a regulable repressor, we used a truncated ligand binding domain of progesterone receptor which binds specifically to the antiprogestin, RU486 [27]. Upon binding of RU486, the inducible repressor will dimerize and bind to EREs

to suppress the ER target gene expression as depicted in Fig. 1(A). In this study, we demonstrate that the regulable repressor can inhibit the ER dependent gene expression in response to exogenous added ligand, RU486.

2. Materials and methods

2.1. Plasmid construction

The KRAB domain and E2F1 DNA binding domain were amplified from pBXG1/Kid-1N (kindly provided by Dr. J. V. Bonventre, Massachusetts General Hospital, Charlestown, MA) and pCMV-E2F1 and ligated together by PCR. The resultant fragment was cut by *Xba*I and *Eco*RI and inserted into MCS sites of pBS-KSII(+) to construct pKS-KE. The truncated PR-LBD(–19)-KRAB fusion fragment was amplified from pCEP4-GL914KRAB [23] by PCR. The PCR product was cut by *Xba*I and *Eco*RI, terminal ends filled by klenow, then ligated into the *Eco*RV site of pKS-KE. The resultant plasmid pKS-KEPK contains the functional KRAB domain at both N- and C-termini of the chimeric construct. E2F1 DNA binding domain was excised with *Bam*HI and *Eco*RI and replaced by a PCR amplified ER DNA-binding domain to generate regulable repressor of ER target genes pKS-KEDPK. The plasmid pKS-KEDPK was then subcloned into the pCMX and pCEP4 (Invitrogen, Carlsbad, CA) expression vectors. Plasmid DNAs were sequenced and checked by *in vitro* transcription/translations. The primers used for PCR amplification (5' primer; 3' primer) are listed as follows:

KRAB: AAGCTTCTAGACTGCAGCTCGAGGCCACCATGGCTCCTGAGCAAAG;

CCGCTTCACGGGATCCTCTCCTTGCTG.

E2F1-DBD: GAGGATCCCGTGAAGCGGAGGCTGGAC;

CCGGAATTCGGAGATCTGAAAGTTCTC.

ER-DBD: CGCGGATCCTATGGAATCTGCCAAGGAG;

CCGGAATTCAGACCCCACTTCACCCCTG.

PR-LBD and KRAB fusion:

CGCGGATCCTTTAAAAAGTTCAATAAAGTCAGAG;

CCGGAATTCTCATCCTTGCTGCAACAGGGAG.

2.2. *In vitro* transcription/translation of the regulable repressor KEDPK

1 μ g of the KEDPK expression vector was translated *in vitro* in a TNT Coupled Reticulocyte Lysate Systems (Promega) together with an empty expression vector and ER expression vector at 30°C for 30 min. The translated products were then separated in 10% SDS-PAGE gel, fixed, dried and developed following autoradiography.

2.3. Gene transfer and luciferase assay

HeLa cells were routinely maintained in Dulbecco's Modified Medium (DMEM; GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). Cells were seeded 24 h before transfection in 6-well tissue culture plates (2×10^5 cells/well) in phenol red-free DMEM containing 10% charcoal/dextran treated FCS. DNA was transiently transfected into cells with lipofectin reagent (GIBCO, Gaithersburg, MD). Cells were transfected for 6 h and then washed with phosphate buffer to remove reagents. Cells were then incubated for an additional 24 h in phenol red-free medium containing 10% charcoal/dextran treated FCS in the absence or presence of hormones. Cell extracts were prepared by adding 300 μ l lysis buffer (Promega, Madison, WI) and assayed for luciferase activities (Monolight 2010 Luminometer, Analytical Luminescence Laboratory, MI). All determinations were performed in quadruplicate at least twice.

The reporter plasmid (ERE)₃TATA-Luc contains 3 copies of the vitellogenin ERE fused upstream of E1A TATA box linked to luciferase (Luc). The C3-Luc reporter, which contains 1.8 kb of the human C3 gene promoter (–1807 to 58) [28], was kindly provided by Jon Rosen (Ligand Pharmaceuticals).

3. Results

3.1. Generation of an inducible repressor

Based on the modular nature of transcription factors we generated chimeric repressors in attempt to specifically turn off the ER target genes in the presence of exogenous ligand. Regulable repressors contain a KRAB domain either at both N- and C-termini (KEDPK) or only at the C-terminus (EDPK), an ER DNA-binding domain and a truncated progesterone receptor ligand-binding domain (–19) as shown in Fig. 1(B). The KRAB domain we used is a conserved region of 75 amino acids present in the N-terminus of Kid-1, a member of the Kruppel class of transcription factors isolated from rat kidney [29]. Fusion of this

KRAB domain to DNA binding domains of the LacI/Z, Gal4 or TetR domains has been shown to be able to suppress the expression of respective reporters which contain corresponding binding site in their promoters [24–26]. Here, the KRAB domain was linked to an ER fragment (aa 175 to 282) comprising minimal ER DNA binding domain [30] to make sure that this construct can specifically bind to ERE with affinity compatible to the wild-type ER. The truncated PR ligand binding domain has been found to activate rather than repress reporter activity in the presence of antagonist RU486 [27]. Fusion of this mutated ligand binding domain to heterologous protein has been shown to render it under the control of exogenous ligand RU486 [22, 23]. The specific feature of these regulable repressors is that they should only inhibit the target genes containing an ERE in the presence of RU486.

To ensure that correct proteins are made from the regulable repressor, we performed *in vitro* transcription/translation in parallel with the parental cloning vector and ER expression vector (66 kDa) as controls. As shown in Fig. 2, no protein was translated from the empty vector. The expected size of translated proteins was produced by EDPK and KEDPK expression vectors. These data suggested that the recombinant

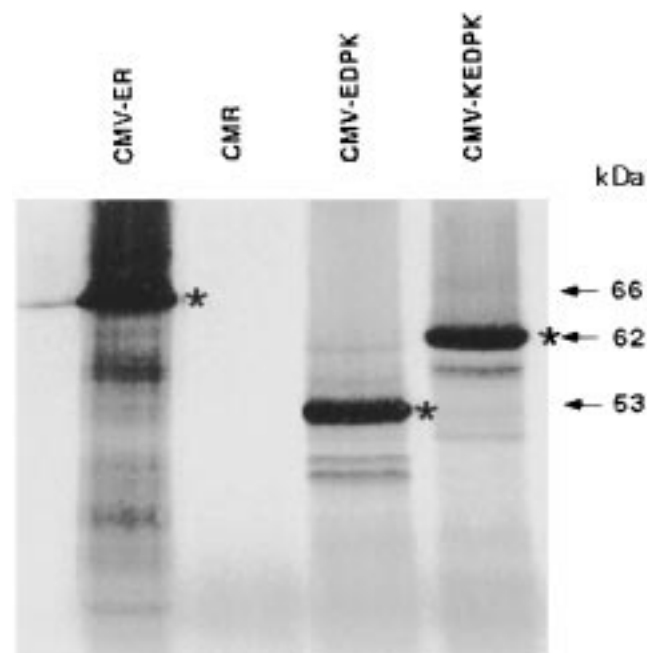


Fig. 2. *In vitro* transcription/translation of the regulable repressor, KEDPK. A TNT coupled reticulocyte lysate system was used to express the KEDPK regulator. An empty expression vector and ER expression vector were included as controls. The translated products were separated in 10% SDS-PAGE gel. * denotes the expected size of translated proteins.

DNA constructs were able to express the full-length chimeric proteins.

3.2. The inducible repressor inhibits ER dependent transcription in the presence of progesterone antagonist, RU486

To test the functional properties of the repressor, HeLa cells were co-transfected with repressor KEDPK and (ERE)₃TATA-Luc reporter plasmids together with a human ER expression vector. Luciferase activity in response to a saturating dose of E₂ (10⁻⁹ M) and RU486 (10⁻⁸ M) was measured in the absence or presence of repressor plasmids. As shown in Fig. 3(A), there is a seven-fold increase in ER dependent transcription in the presence of E₂. ER dependent transcription was not affected by addition of RU486. The

chimeric repressor KEDPK has no significant effect on the reporter gene expression in the absence of exogenous ligand, RU486. However, KEDPK could effectively inhibit more than 80% of ER-mediated transcription in the presence of RU486 (10 nM). Thus, KEDPK could compete effectively for the ERE binding site with wild-type ER to suppress the transcription. EDPK could also inhibit ER induced transcription but was less potent than that of KEDPK (data not shown).

The specificity of repressor activity was assessed by co-transfection with reporter plasmid containing glucocorticoid response element (GREtk-Luc) which is palindromic DNA sequences similar to the ERE. Since RU486 has been reported to have glucocorticoid receptor (GR) antagonistic property, a constitutively active form of GR (GR*) with ligand binding domain trun-

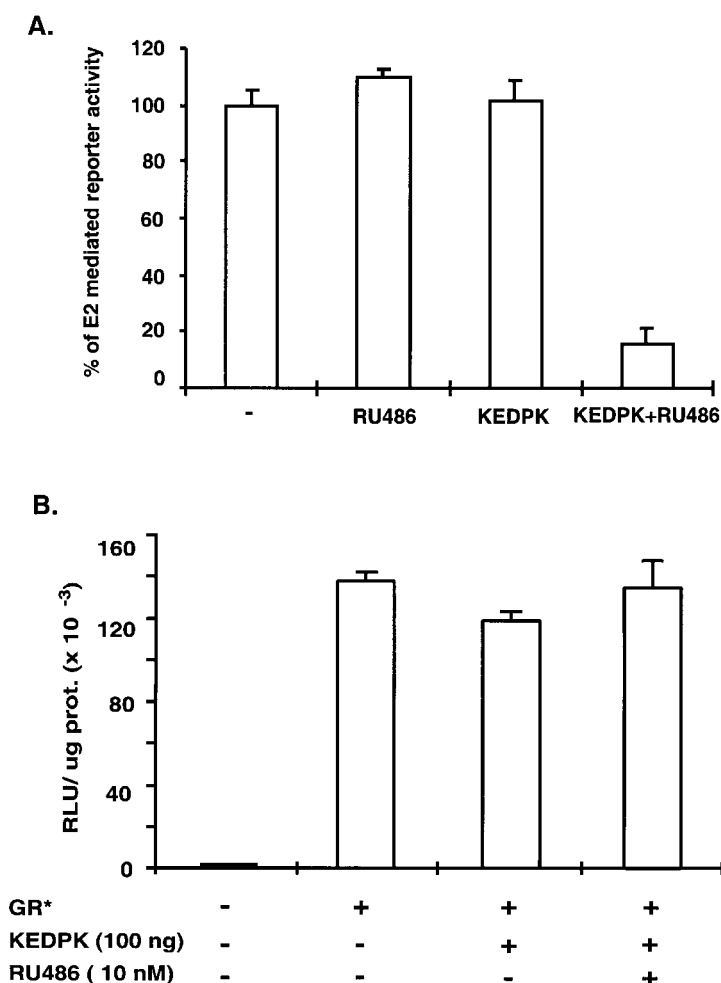


Fig. 3. Specific inhibition of ER-dependent activation by the regulable repressor, KEDPK. Panel A: an (ERE)₃TATA-Luc reporter construct (100 ng) and a human ER expression plasmid (50 ng) was transfected along with or without regulable repressor, KEDPK (200 ng), into HeLa cells using lipofectin. After 6 h of transfection, cells were washed and incubated in the presence of E₂ (1 nM) for an additional 24 h with or without RU486 (10 nM), as indicated. The magnitude of ER activation by E₂ alone was set at 100%. Panel B: HeLa cells were transfected with 50 ng of reporter GREtk-Luc, 50 ng of GR* (LBD truncation) with or without 100 ng regulable repressor, KEDPK. Luciferase activity was assayed 24 h after treatment with or without 10 nM RU486. Luciferase activity was normalized for protein quantitation. A single experiment representative of at least two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

cation was used to exclude the effect of RU486 on GR activity [31]. The truncated GR (GR*) stimulated GR reporter gene expression over 50-fold. The repressor has no effect on the GR* induced transcription either in the absence or in the presence of RU486 [Fig. 3(B)]. Thus, the repressor specifically inhibited ER mediated transcription in transient transfection assays.

3.3. Dose-dependent inhibition of ER-mediated transcription by the inducible repressor

To further characterize the potency of the repressor on ER-dependent transcription, different amounts of KEDPK plasmid were co-transfected with the ER expression vector. Results shown in Fig. 4 indicate that inhibitory effect of the repressor on ER transcription was dose-dependent. A 50% reduction of ER-mediated transcription was observed when equal amounts of ER and repressor KEDPK plasmids were cotransfected. Maximum inhibition of the ER mediated transcription was observed when repressor was in 4-fold excess of ER. Further increase in the amount of repressor has an inhibitory effect on the reporter even in the absence of RU486 (data not shown), implying that the excess repressor is binding to the ERE site of the reporter gene in the absence of exogenous added ligand. Next, we assessed the effective dose of RU486 in inducing the repressor activity. As shown in Fig. 5, the repressor exhibited a RU486 dose-dependent regulation of suppressive activity. The maximum inhibition of ER mediated transcription appeared at concentration of 10 nM, which is below the concentration of RU486

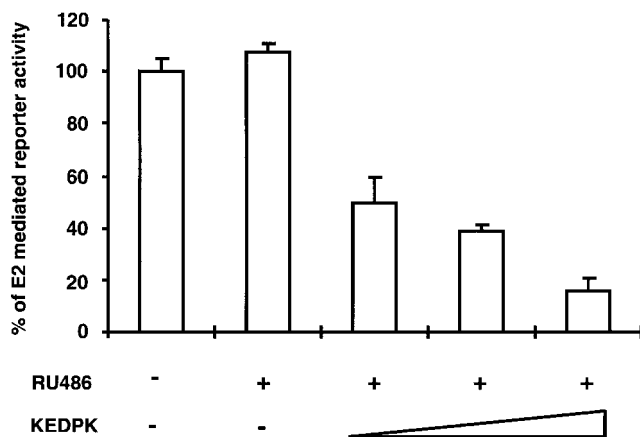


Fig. 4. Inhibition of ER activity by KEDPK in a dose-dependent manner. Cells were transfected with (ERE)₃TATA-Luc reporter (100 ng), human ER expression plasmid (50 ng) and an increasing amount of KEDPK repressor construct (50, 100, 200 ng). Cells were treated with 10 nM 17 β -estradiol and 100 nM RU486 for 24 h as indicated. The magnitude of ER activation by E₂ alone was set at 100%. A single experiment representative of three independent experiments is detailed above. The data shown indicates the mean \pm SEM of quadruplicate estimations.

required to antagonize any progesterone and glucocorticoid activity. Similar results were observed by co-transfection of repressor and reporter plasmids into the breast cancer cell line MCF-7 (data not shown). These results suggested that RU486 could be used as ligand to regulate KEDPK repressor activity with minimal effects on other steroid hormones.

3.4. Inducible repressor that antagonizes ER mediated transcription is independent of cellular and promoter context

ER contains two transactivation domains, AF1 and AF2, which operate in a cell and promoter-specific manner to mediated ER action. Tamoxifen, the most widely used agent in endocrine therapy of breast cancer, acts as a partial agonist of ER in a cell type specific manner. The partial agonist activity of tamoxifen has been proposed to relate to its ability to activate the AF1 of ER [32]. To examine whether the repressor we developed is capable of inhibiting tamoxifen-activated transcription, the repressor plasmid and a (ERE)₃TATA-Luc reporter were transfected together with an ER expression vector into HepG2 (human hepatocellular carcinoma) cells where AF1 activity of ER was proven to be dominant [33]. As shown in Fig. 6, 4-hydroxy-tamoxifen (4OH-T) treatment of HepG2 cells resulted in an 11-fold induction of ER mediated transcription, which is about 10% of the response elicited by E₂. The repressor KEDPK inhibited the 4HOT-induced ER activity in the presence of RU486 as efficiently as it inhibited E₂ induced activity.

In addition, the efficacy of repressor function was examined on a natural estrogen responsive promoter. In this case we chose the estrogen responsive complement factor 3 (C3) promoter which contains putative ERE [34]. The E₂ was able to stimulate luciferase reporter expression from the natural C3 promoter (Fig. 7). Transcription was almost completely blocked in cells transfected with repressor after treatment of RU486 (10 nM). Taken together, these results indicated that repressor KEDPK could block the ER activity independent of cellular and promoter context.

4. Discussion

Nuclear hormone receptors are inducible transcription factors that regulate gene expression in response to their cognate ligands. They usually contain a transactivation or repression domains, a DNA binding domain and a ligand binding domain. Upon ligand binding, nuclear hormone receptors selectively bind through their DNA binding domains to respective response elements in the promoters and enhancers of target genes and directly interact with transcription

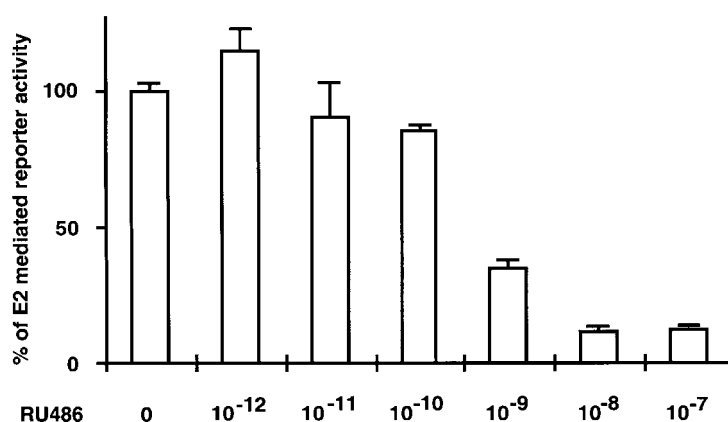


Fig. 5. RU486 dose-dependent curve on KEDPK inhibition of ER activity. HeLa cells were transiently transfected with 50 ng of the (ERE)₃TATA-Luc reporter, 50 ng of the human ER expression plasmid and 200 ng of the repressor construct, KEDPK. Cultures were treated with 17 β -estradiol (10 nM) and different concentrations of RU486 for 24 h as indicated. The magnitude of ER activation by E₂ alone was set at 100%. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean \pm SEM of quadruplicate estimations.

initiation complexes, coactivators or corepressors to activate or repress transcription of their target genes [35]. By taking advantage of the modular nature of nuclear hormone receptors, several regulable gene expression systems have been developed to express exogenous gene products [23, 36, 37]. The basic strategy of these regulable systems relies on two components: a chimeric transactivator genetically engineered from bacterial repressor proteins or eukaryotic receptors to function as a gene switch in response to exogenous ligands and a corresponding heterogeneous promoter, whose activity is under the control of the chimeric transactivator. By linking the gene of interest to a response element of the chimeric transactivators, the ex-

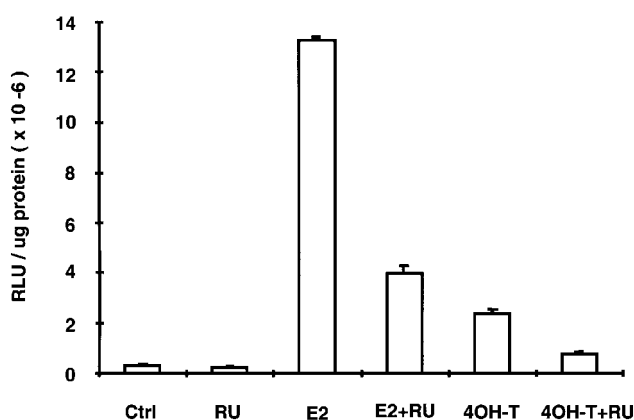


Fig. 6. The effect of KEDPK on 4-hydroxy-tamoxifen (4OH-T) stimulated ER activity. The repressor plasmid (200 ng) and (ERE)₃TATA-Luc reporter (100 ng) were transfected together with an ER expression vector (100 ng) into HepG2 (human hepatocellular carcinoma) cells. Luciferase activity was normalized for quantity of protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean \pm SEM of quadruplicate estimations.

pression of particular gene can be regulated using exogenous ligand. These regulable systems have been used in studying the function of specific protein *in vivo* and in producing therapeutic proteins for the treatment of human disease through gene therapy.

In this paper, we converted the transactivator to a repressor as a means to repress the endogenous gene expression in a regulable fashion. A number of transcription repressors have been characterized that negatively regulate specific genes transcribed by RNA polymerase II. Analyses of the mechanisms of transcription repression have revealed distinct classes of repression including steric hindrance of transcriptional activators from DNA binding, direct interaction with basal transcription machinery or squelching a specific activator proteins. Like transcriptional activators, many transcriptional repressors are modular factors. One of the best characterized repressor domains, KRAB, can be separated from DNA binding domain. It has been shown that when fused to a heterogeneous DNA binding domain, KRAB suppresses the activating function of various defined transcription activators [26].

In the present study, we used the KRAB domain to create a chimeric transcription repressor directly targeted to the endogenous promoter of a gene of interest. The endogenous target genes we chose are ER target genes. It is well known that ER can regulate its target gene expression by binding to the specific DNA sequences known as estrogen response elements (EREs). The constructed repressor is supposed to effectively inhibit the expression of ER target genes by competing for ERE binding sites with ER and directly interacting with basal transcription factors to interfere with RNA polymerase II-mediated transcription. ER target genes, such as pS2 [38] and C3 [34] have been

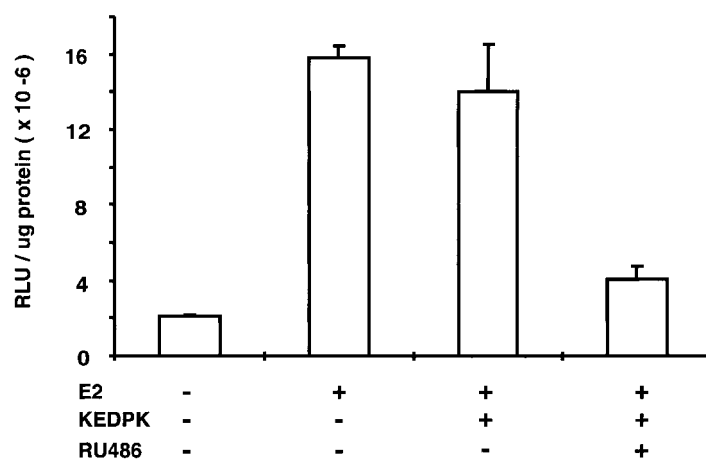


Fig. 7. The effect of KEDPK on ER mediated natural promoter activity of complement factor 3 (C3). HeLa cells were transfected with C3-Luc promoter (100 ng), ER expression plasmid (100 ng) and KEDPK repressor construct (200 ng). Cells were treated with 10 nM 17 β -estradiol and 100 nM RU486 for 24 h as indicated. Luciferase activity was normalized for quantity of protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean \pm SEM of quadruplicate estimations.

identified and found to contain conserved palindromic EREs in their promoter regions, which provide reliable markers to examine the efficiency of regulable repressor of ER target genes. Since the roles of ER and its target genes in the development of hormone-dependent or -independent breast cancers have not been determined, the designed repressor can bypass ER to block the expression of ER target genes. In this way, we should be able to examine directly the role of ER target genes in breast cancer progression.

The repressor we constructed was shown to effectively inhibit ER mediated transcription in transient transfection assay. More than 80% inhibition of ER activity could be achieved by increasing the amount of repressor plasmid. The inhibitory activity of the repressor was shown to be tightly controlled by exogenous ligand, RU486, with the maximum effect at 10 nM. This result is similar to the concentration observed in the transactivation system (GLVP) developed in this lab [22]. No effect of repressor on ERE promoter was observed in the absence of ligand, suggesting that ligand binding domain could control repressor dimerization and binding to the specific DNA sequence. Since the repressor specifically inhibits ER mediated transcription, it is unlikely that the suppressive effect is due to the sequestration of specific activator protein. Most likely, the inducible repressor dimerizes and competes for DNA binding sites with ER in the presence of RU486. Furthermore, by tethering KRAB to the promoter, it can then carry out its repressive function through interacting with basal transcription machinery and silencing the promoter activity.

Taken together, the chimeric repressor, we designed, is effective and specific in inhibiting ER target gene expression in response to exogenous ligand. Therefore, this repressor will provide a useful tool for studying

the role of ER target genes in breast cancer progression and be potentially useful for gene therapy of breast cancer.

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