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The rexinoid bexarotene represses cyclin D1 transcription by inducing the DEC2 transcriptional repressor

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Abstract

Bexarotene is an RXR-selective vitamin A analog that has been shown to prevent ER-negative mammary tumorigenesis in animal models. While investigating the mechanism by which bexarotene prevents ER-negative breast cancer development, we found that the expression of cyclin D1, a critical cell cycle promoter, was repressed by bexarotene in vitro and in vivo. Time course and cycloheximide experiments show that repression of cyclin D1 is a late effect and requires new protein synthesis. Previously we discovered that DEC2 (differentially expressed in chondrocytes-2), a helix–loop–helix transcription repressor, was induced by bexarotene in human mammary epithelial cells. Therefore, we hypothesized that bexarotene represses the transcription of cyclin D1 through induction of DEC2. Luciferase reporter studies demonstrated that either

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Conflict of interest Reid P. Bissonnette is employed in the Department of Retinoid Research, Ligand Pharmaceuticals, Inc., San Diego, CA 92121. W. W. Lamph is a previous employee of Ligand Pharmaceuticals. Powel H. Brown serves as a Scientific Advisory Board member for Susan G. for the Cure, not for profit organization. All other authors have no conflict of interest.

bexarotene treatment or forced expression of DEC2 can repress the transcription of a cyclin D1 promoter reporter by affecting the basal transcriptional activity.

Results from chromatin immunoprecipitation experiments showed that bexarotene treatment causes the recruitment of DEC2 and HDAC1 (histone deacetylase 1) to the cyclin D1 promoter. Co-immunoprecipitation confirms the interaction between DEC2 and HDAC1, suggesting that the recruitment of HDAC1 to the cyclin D1 promoter is through DEC2. Trichostatin A, a HDAC inhibitor, reverses the cyclin D1 repression by bexarotene, suggesting that repression of cyclin D1 involves histone deacetylation. Knock-down of DEC2 by siRNA abolishes the cyclin D1 repression, further supporting our hypothesis. Finally, we demonstrated that overexpression of DEC2 dramatically inhibited cell proliferation and repressed the expression of cyclin D1 in human mammary epithelial cells. These results suggest that bexarotene down-regulates cyclin D1 through induction of DEC2, followed by recruitment of HDAC1 to the cyclin D1 promoter causing transcriptional repression. By elucidating the mechanism by which rexinoids inhibit cell proliferation, it will be possible to develop more effective and less toxic drugs to prevent ER-negative breast cancers.

Keywords

Rexinoid; Bexarotene; Prevention; Breast cancer; Cyclin D1; DEC2

Introduction

Despite aggressive screening to detect breast cancer in early stage, and significant advances in treatment, breast cancer remains a major health threat to women. Results from recent clinical trials of tamoxifen and raloxifene have demonstrated that chemoprevention (the use of pharmacological agents (natural or synthetic) to inhibit or reverse the process of carcinogenesis) is an effective approach to reduce the risk of breast cancer [1]. Both tamoxifen and raloxifene are selective estrogen receptor modulators (SERMs) that target estrogen receptor (ER) signaling. These anti-estrogen drugs prevent the development of ER-positive breast cancer, but they have no effect on the development of ER-negative breast cancer [2]. ER-negative breast cancer accounts for one-third of all the breast cancers and has a particularly poor prognosis. Recently, a number of novel chemopreventive agents targeting estrogen-independent signaling pathways have been shown to prevent ER-negative breast cancer in animal models [3]. Some of the most promising chemopreventive agents are retinoids which have been extensively studied in both preclinical and clinical settings [4–6].

Retinoids are vitamin A analogs that play important roles in regulating cellular proliferation, differentiation, and apoptosis [7]. They exert their activity through binding to the nuclear retinoid receptors RARs and/or RXRs. The ligand-bound receptors form dimeric complexes which interact with DNA at specific retinoid responsive elements and regulate the transcription of their target genes. Based on their nuclear receptor specificity, retinoids can be categorized as RAR-selective, RXR-selective (i.e., rexinoids), or as pan-agonists, which bind both RAR and RXR. Previous study had demonstrated that pan-agonist 9cRA was effective in preventing ER-negative mammary tumorigenesis in a mouse model, but had significant toxicity [5]. The RAR-selective retinoid TTNPB displayed modest chemopreventive activity with high toxicity [4], whereas the RXR-selective retinoid, bexarotene (LGD1069), suppresses mammary tumorigenesis with no apparent side effects [4]. Therefore, chemopreventive efficacy of retinoids can be separated from toxicity using receptor-selective retinoids. We previously reported that rexinoid bexarotene significantly delayed ER-negative mammary tumor formation in multiple mouse models [4, 6]. Thus,

rexinoids represent more effective and tolerable agents than retinoids for the prevention of ER-negative breast cancer.

Although significant progress has been made toward understanding the RAR/RXR-mediated signaling pathway, the mechanism by which bexarotene suppresses tumorigenesis is still poorly understood. Recently, we reported that bexarotene prevents breast cancer primarily through inhibition of proliferation [8]. We also reported that bexarotene blocked the cell cycle progression from G0/G1 to S phase, and decreased the protein levels of cyclin D1 in both HMEC cells and MMTV-erbB2 mice [9]. Therefore, suppression of cyclin D1 may play a critical role in mediating bexarotene-induced growth suppression. Consistent with this, Yu et al. and Landis et al. [10, 11] demonstrated that MMTV-erbB2 mice lacking cyclin D1 activity failed to develop lactating mammary glands and were protected from erbB2-induced tumors. In this report, we investigated the mechanism by which bexarotene suppresses the expression of cyclin D1. We found that bexarotene repressed the transcription of cyclin D1 through an indirect mechanism by inducing DEC2, a helix-loop-helix transcription repressor. DEC2 then bound to the cyclin D1 promoter and recruited histone deacetylase 1 (HDAC1), resulting in the repression of cyclin D1 transcription. These findings suggest that DEC2 is a critical mediator in the retinoid/rexinoid signaling pathway. These results help us understand how rexinoids down regulate their target genes and inhibit mammary epithelial proliferation.

Materials and methods

Cell lines and chemicals

Normal human mammary epithelial cells (HMEC) were obtained from Clonetics (San Diego, CA). HMECs were maintained in Mammary Epithelial Basal Medium (MEBM) supplemented with the Mammary Epithelial Growth Media (MEGM) kit (Cambrex Corporation, East Rutherford, NJ). Medium was changed every 2 days. MCF-7 cells were cultured in DMEM containing 5% FBS. Bexarotene was obtained from Ligand Pharmaceuticals, Inc. (San Diego, CA). Bexarotene experiments were conducted at 1 μ M. Cycloheximide was obtained from Sigma.

DNA constructs

Cyclin D1 deletion promoter constructs (−973, −742, −543, −254) were obtained from Dr. R Müller [12]. The pA3 luciferase construct was obtained from Dr. Richard and G. Pestell [13]. Several deletion and mutant promoter fragments (−254 and fragments shorter than −254) were generated by PCR and subcloned into pA3 luciferase vector with restriction enzymes *KpnI* and *HindIII*. These mutants had a specific sequence deleted or mutated. 59-CRE mutant promoter has a mutation in the CRE element from −48/−41 (TAACGTCA to CGAACCTT). The pBabe-DEC2 retrovirus construct was created by two steps. First, the DEC2 coding sequence (lacking the N-terminal fragment) was cut from Flag-DEC2 construct [14] by *EcoRI* and *SaI*, and ligated into pBabe-puro3 vector digested with the same enzymes. Second, the N-terminal fragment of DEC2 was amplified using the following primers: 5′-TCT AGA GGA TCC GCC ACC ATG GAC TAC AAA GAC GAT GAC GAC -3′ and 5′-ATC TAT CGA TGA ATT CGC GG -3′. Both the N-terminal fragment of DEC2 and the construct prepared from first step were digested by *BamHI* and *EcoRI* followed by ligation. All the DNA constructs were confirmed by DNA sequencing.

Chromatin immunoprecipitation assay

ChIP assays were performed as described previously [15]. HMEC cells were synchronized with MEBM without growth supplements but contained 0.5 μ g/ml of Anti-EGFR antibody (NeoMarkers, MS-269-PABX) for 48 h before bexarotene treatment. Antibodies used were:

DEC2 (from Dr. Bingfang Yan); HDAC1 (Cell signaling, 2062); Polymerase II (Santa Cruz, SC-899); cJun/AP-1 (Oncogene, PC06); p300 (Santa Cruz, SC-585); normal rabbit IgG (Santa Cruz, SC-2027). Cyclin D1 promoter primers were: 5'-CTCCCGCTCCCATTTCTCTG-3', 5'-TCGGCTCTCGCTTCTGCTG-3'. Negative control primers were MMP1 non-specific primers as previously described [16].

siRNA transfection

siRNAs for DEC2 were synthesized from Sigma with the following sequences: 5'-GCCUAACCAUUAGUACUUATT-3' and 5'-UAAGUACUAAUGGUUAGGCTT-3'. The negative non-specific control siRNA was obtained from Dharmacon. siRNA transfection was conducted in 24-well plates by using a final concentration of 10 nM siRNA and 0.5 µl of DharmaFECT (Dharmacon) for each transfection. HMEC cells were synchronized with MEBM without growth supplements but containing 0.5 µg/ml of Anti-EGFR antibody (NeoMarkers, MS-269-PABX) for 48 h before transfection.

Co-immunoprecipitation

MCF-7 cells were transfected with Flag-DEC1, Flag-DEC2, or Flag-CMV2 empty vectors using Fugene 6 (Roche) according to the manufacture's protocol. 24 h after transfection, total cell lysates were prepared with cell lysis buffer (50 mM Tris HCl pH7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, Protease inhibitor cocktail). The lysates containing equal amounts of total protein (200 µg) were incubated with 30 µl of agarose beads conjugated with either Flag antibody (Sigma #3165) or normal mouse IgG (Sigma A0919) overnight. The beads were washed with TBS (10 mM Tris pH 8.0, and 150 mM NaCl) three times. The proteins were eluted with western blotting loading buffer without 2-mercaptoethanol at 95°C for 5 min followed by western blotting.

Retrovirus infection

DEC2 cDNA was cloned into retroviral vector pBabe-puro3 (from Dr. Aubrey Thompson, Mayo Clinic). HMEC cells were infected with retrovirus pBabe or pBabe-DEC2 produced using Phoenix A packaging cells, according to Dr. Garry Nolan's protocol (Stanford University, Stanford, CA). DEC2 expression cells were screened for resistance in the presence of 10 µg/ml puromycin.

Proliferation assay

HMEC or MCF-7 cells were seeded in 96-well plates at the density of 1000 cells/well. The proliferation assay was conducted using MTS assays (Promega, Inc) as previously described [3].

Luciferase reporter assay

For bexarotene treatment experiments, HMEC cells were plated in 100-mm dishes at 50% confluence and synchronized in MEBM medium containing 0.5 µg/ml of Anti-EGFR antibody for 48 h. Cells were then transfected with cyclin D1 promoter reporters (6 µg/dish) with Fugene 6 (18 µl/dish). 6 h after transfection, cells were split and treated with either DMSO or 1 µM of bexarotene and harvested by passive lysis buffer (Promega) at different time points. For DEC2 expression experiments, HMEC cells were plated in 24-well plates and synchronized in MEBM containing 0.5 µg/ml of Anti-EGFR antibody for 48 h. Cells were transfected with flag-DEC2 (150 ng/well), cyclin D1 promoter construct (150 ng/well), and pRL-TK renilla luciferase construct (20 ng/well) using Fugene 6 (1 µl/well). Cells were harvested at 24 h after transfection. Cyclin D1 promoter activity was measured using the Dual-luciferase Reporter assay (Promega, Madison, WI, USA) as described previously [17].

Quantitative reverse-transcriptase PCR (QRT-PCR)

QRT-PCR assays of cyclin D1 and DEC2 genes were conducted as previously described [18].

Western blotting analysis

Western blotting was conducted as previously described [18]. DEC2 antibody was provided by Dr. Bingfang Yan. HDAC antibody was obtained from Cell signaling (2062). Flag antibody was obtained from Sigma.

Results

The expression level of cyclin D1 is correlated with cell proliferation

To investigate the mechanisms by which rexinoids prevents ER-negative breast cancer development, we previously identified several rexinoid-modulated genes [8, 18]. Cyclin D1, a critical cell cycle regulator, was repressed by bexarotene both in vitro and in vivo [18]. We hypothesized that cyclin D1 plays an important role in bexarotene-induced growth suppression. To test this, we examined the growth suppressive effect of bexarotene and cyclin D1 expression levels in HMEC and MCF-7 cells. As seen in Fig. 1a, b, bexarotene halts the proliferation of HMEC cells while down regulating the expression of cyclin D1. In MCF-7 cells, bexarotene has little effect on the proliferation or expression of cyclin D1 (Fig. 1c, d). The growth suppression is closely related with cyclin D1 expression levels, suggesting that cyclin D1 is a key mediator for rexinoid-induced growth suppression.

Bexarotene down-regulates cyclin D1 expression through transcriptional repression

We next performed a cyclin D1 promoter luciferase reporter assay with HMEC cells. As shown in Fig. 2a, bexarotene treatment repressed the cyclin D1 promoter reporter by 30–50%. The smallest promoter reporter 254 shows significant repression. Therefore, we hypothesize that the response element of cyclin D1 repression is located downstream of –254 within the promoter region. To further identify which specific response element plays a role in cyclin D1 repression, we prepared a series of deletion and mutation promoter constructs as shown in Fig. 2b. Most of the deletion or mutation reporters demonstrate similar repression. The deletion of CRE site within the –254 promoter region resulted in an additional 30–40% decrease of promoter activity. The shortest deletion construct, +24, loses the basal transcriptional activity. These data suggest that bexarotene represses the transcription of cyclin D1 depending on the presence of elements within the proximal region of the cyclin D1 promoter.

Down-regulation of cyclin D1 requires new protein synthesis

In our luciferase reporter experiments, we observed that the repression of cyclin D1 transcription was more significant at late time points (24 and 48 h) than at early time points (<24 h) following bexarotene treatment (data not shown). Consistent with this, the RNA level of cyclin D1 is only reduced at 48 h of treatment, but not at the earlier time points (Fig. 2c). Therefore, the repression of cyclin D1 transcription is a late effect, possibly by an indirect mechanism. Pretreatment with cycloheximide, a protein synthesis inhibitor, totally abolished the cyclin D1 repression by bexarotene (Fig. 2c), demonstrating that the repression of cyclin D1 by bexarotene requires new protein synthesis.

Bexarotene induces DEC2, a transcription repressor

Since bexarotene-induced repression of cyclin D1 transcription requires new protein synthesis, we investigated whether bexarotene induced proteins that might affect cyclin D1 transcription. In our previous studies, we found that DEC2, a helix–loop–helix transcription

repressor, was induced by bexarotene both in vitro and in vivo [8, 18]. In Fig. 3a, b, we measured the protein levels of DEC2 in HMEC and MCF-7 cells treated with DMSO and bexarotene. As expected, bexarotene induced DEC2 in HMEC cells, but not in MCF-7 cells. This result is consistent with our previous results that demonstrate that bexarotene down-regulates cyclin D1 and inhibits proliferation in HMEC cells but not in MCF-7 cells (Fig. 1). To investigate whether bexarotene induces DEC2 expression, we performed a time course study and found that DEC2 RNA expression was induced by bexarotene as early as 6 h of treatment (Fig. 3c). Cycloheximide treatment did not abolish the induction, indicating that induction of DEC2 by bexarotene does not require new protein synthesis (Fig. 3c). All of these data suggest that induction of DEC2 occurs prior to the repression of cyclin D1. In addition, published data [19] showed that forced expression of DEC2 can repress the transcription of cyclin D1 in C2C12 cells, suggesting that DEC2 may also be responsible for bexarotene-induced cyclin D1 repression.

DEC2 represses the transcription of cyclin D1 in HMEC cells

To test our hypothesis that DEC2 plays a role in cyclin D1 repression, we performed cyclin D1 reporter assays to determine whether DEC2 can repress cyclin D1 promoter activity in HMEC cells. As shown in Fig. 4a, b, overexpression of DEC2 repressed the activity of all the cyclin D1 reporter constructs except the shortest construct (+24). The repressive activity of DEC2 correlates well with that seen after bexarotene treatment (Fig. 2a, b). It is interesting to note that promoter constructs 742 and 543 show similar repression. Construct 543 lacks the E-Box response element, which is a typical DEC protein binding site, suggesting that this specific E-Box element is not required for cyclin D1 repression. These data support our hypothesis that bexarotene can repress the transcription of cyclin D1 through DEC2 induction.

DEC2 binds to cyclin D1 promoter

As a helix-loop-helix transcription factor, DEC proteins bind directly to the promoter region of target genes [20]. Since the E-box sequence was not responsible for the cyclin D1 repression, we investigated whether DEC2 can bind to the cyclin D1 promoter in HMEC cells. We therefore, performed a ChIP assay after immunoprecipitating DEC2 and other transcription factors involved in regulating basal transcriptional activity (Fig. 5a). As expected, DEC2 binding to the promoter of cyclin D1 (at the proximal promoter from -114 to +90) was induced by bexarotene treatment. c-Jun, a known transcription factor that binds to the cyclin D1 promoter, was also included as a positive control. c-Jun recruitment to this promoter was not changed by bexarotene treatment. However, we did observe decreased binding of polymerase II to the cyclin D1 promoter after bexarotene treatment. This result further supports our conclusion that cyclin D1 down-regulation occurs at the transcription level. We also observed increased binding of HDAC1 to the cyclin D1 promoter after bexarotene treatment. Figure 5a shows western blot staining results demonstrating DEC2 induction and cyclin D1 repression in the ChIP samples. The recruitment of HDAC1 to the promoter of cyclin D1 suggests that bexarotene represses the transcription of cyclin D1 through a HDAC-dependent mechanism.

Bexarotene represses the transcription of cyclin D1 through a HDAC-dependent mechanism

A recent study showed that DEC proteins can regulate the transcription through both HDAC-dependent and HDAC-independent manners [21, 22]. To test the HDAC-dependency of cyclin D1 repression, HMEC cells were treated with bexarotene in the presence or absence of TSA, a histone deacetylase inhibitor. In the absence of TSA, bexarotene down-regulated cyclin D1 by approximately 40%. In contrast, in the presence of TSA, the repression of cyclin D1 was totally abrogated (Fig. 5b). However, the underlying mechanism by which

cyclin D1 expression is repressed is unknown. These results suggest that the repression of cyclin D1 transcription is through histone deacetylation. To determine whether the recruitment of HDAC1 to the cyclin D1 promoter is through binding to DEC2, we performed a co-immunoprecipitation experiment. Breast cancer cells were transiently transfected with Flag conjugated DEC expression constructs, and Dec2 was immunoprecipitated with anti-Flag antibodies. Western blot analysis was then performed using anti-HDAC1 and anti-Flag antibodies (Fig. 5c). As shown in Fig. 5c, both DEC1 and DEC2 can interact with HDAC1.

Inhibition of DEC2 expression abolishes the repression of cyclin D1 by bexarotene

To further determine whether DEC2 is required for cyclin D1 down-regulation by bexarotene, we inhibited DEC2 expression using siRNA (Fig. 6a). Transfection of DEC2 siRNA significantly repressed the expression of DEC2 in HMEC cells. In the control cells which were transfected with a non-specific siRNA, bexarotene induced DEC2 and repressed cyclin D1. However, in HMECs which were transfected with DEC2 siRNA, the repression of cyclin D1 was abolished as demonstrated by both RNA and protein expression.

We also determined whether knocking down DEC2 can reverse the proliferation inhibition by bexarotene (Fig. 6b). We first measured DEC2 RNA levels 2 and 4 days after siRNA transfection in the cells and observed roughly 80% DEC2 knockdown at day 2; however at day 4, DEC2 RNA expression returned to close to control level (data not shown) (Fig. 6b). In the control cells, bexarotene decreased cell proliferation at day 2. However, in DEC2 knock-down cells, bexarotene's growth inhibitory effect was reversed (Fig. 6b). These results suggest that DEC2 mediates bexarotene's growth inhibitory effect via down-regulating cyclin D1 expression.

Forced expression of DEC2 inhibits proliferation in HMEC cells

We next investigated whether DEC2 overexpression also causes suppression of HMEC growth. For these experiments, we overexpressed the DEC2 gene in HMEC cells using retrovirus infection of a DEC2 expressing virus. Figure 6c demonstrates DEC2 protein expression in HMEC cells infected by p-Babe empty vector and p-Babe-DEC2 construct. As expected, p-Babe-DEC2 dramatically induced the expression of DEC2 and repressed the expression of cyclin D1. Cell proliferation experiments (Fig. 6c) showed that overexpression of DEC2 significantly inhibited proliferation in HMEC cells.

Discussion

We previously reported that bexarotene significantly delayed ER-negative breast cancer development in MMTV-erbB2 mice [6]. However, the mechanisms by which retinoids prevent cancer development are not fully understood. In these studies, we investigated the molecular mechanisms by which bexarotene repressed the expression of cyclin D1, a key cell cycle regulator. In this study, we demonstrated that bexarotene represses the transcription of cyclin D1 by inducing the expression of a helix-loop-helix transcription repressor, DEC2. DEC2 then binds to the promoter of cyclin D1 and recruits HDAC1, which results in histone deacetylation and transcriptional repression, ultimately causing growth suppression (as shown in Fig. 7).

Our previous studies suggest that bexarotene prevents mammary tumorigenesis primarily through proliferation inhibition, resulting from G1 cell cycle blockage [8, 9]. The chronic treatment of bexarotene causes significant down-regulation of cyclin D1 in the mammary gland. The role of cyclin D1 in cancer development has been well documented and reviewed [23]. Our current data suggest that retinoids prevent mammary tumorigenesis in part by

suppressing cyclin D. Recently, Dmitrovsky and colleagues reported that bexarotene treatment reduces the expression of biomarkers including cyclins D1 and D3 in non-small cell lung cancer [24]. We also observed a reduction of cyclin D1 expression in postmenopausal women who are at high risk of developing breast cancer and took bexarotene in a breast cancer prevention trial [25].

Given the fact that RXR is such a promiscuous receptor that dimerizes with a variety of nuclear receptors, it is likely that RXR-specific ligands regulate cell growth through multiple signaling pathways. Repression of cyclin D1 clearly is one mechanism by which bexarotene suppresses growth. However, bexarotene also affects other independent growth-regulatory pathways including Cox-2, AP1, IGF-BP6, and RAR β . Induction of IGF-BP6 and RAR β can cause growth suppression. Bexarotene down-regulates the transcriptions of Cox-2 and AP1 in part through sequestration of transcriptional coactivators [15]. Each of these genes can affect growth independently, and together they effectively cause a G1 cell cycle block. Thus, bexarotene can be considered as a “multi-functional” chemopreventive agent that prevents cancer by affecting many independent growth regulatory pathways. Given the complex nature of carcinogenesis which involves aberrant regulation of multiple signaling pathways, multi-functional chemopreventive agents such as rexinoids represent promising agent to prevent the development of ER-negative breast cancer.

The induction of DEC2 by bexarotene suggests a novel mechanism by which rexinoids can repress the expression of its target genes. DEC2 is a member of DEC protein subfamily which consists of DEC1 and DEC2. DEC1 was originally identified as a retinoid-induced gene [26, 27], while DEC2 was first identified through EST analysis [28]. DEC1 and DEC2 are reciprocally regulated, and possess an inverse protein expression pattern as observed in multiple tissues [20]. We found that bexarotene induces DEC2, but not DEC1 [8]. Both proteins are known transcription repressors that inhibit cell proliferation in several cell lines [29–31]. Our results are supported by previous study [32], in which forced expression of DEC2 inhibited cell proliferation and down-regulated cyclin D1 expression in C2C12 myoblasts.

DEC proteins can directly bind to the promoter region of their target genes, and repress the transcription through HDAC-dependent and/or independent mechanisms [22]. The repression of cyclin D1 by bexarotene is likely to be through a HDAC-dependent mechanism, as TSA can totally abolish the transcriptional repression. Consistent with this theory, our ChIP results show that HDAC1 binding to the cyclin D1 promoter is increased by bexarotene treatment. The direct interaction of DEC2 and HDAC1 proteins also indicates that the recruitment of HDAC1 to the cyclin D1 promoter is through DEC2. The decreased polymerase binding to the promoter can also be explained by the histone deacetylation, which in turn interferes with the formation of the basal transcriptional machinery.

It is interesting to note that the E-Box response element, a typical DEC protein binding site, does not appear to play a role in the cyclin D1 repression (Fig. 2a). Recently, we found that DEC proteins can also bind to the SP1 binding site and regulate transcription [33]. Sequence analysis revealed that there are multiple putative SP1 binding sites in the proximal region of cyclin D1 promoter, including the region of –30 to +24, which is required for the basal transcription activity of cyclin D1. Hence, taken together, this suggests that DEC2 likely binds to the cyclin D1 promoter at the Sp1-binding sites.

Current studies have demonstrated that the RXR ligand, bexarotene, suppresses breast cell growth though inducing DEC2 expression, which in return suppresses the expression of cell cycle regulator, cyclin D1. Promoter region of the DEC2 gene has a typical RAR response element, and the early induction of DEC2 after bexarotene treatment also supports this

mechanism. In addition, other indirect mechanisms likely contribute to the growth suppression induced by bexarotene. We previously reported that bexarotene inhibited AP-1-dependent transcription and indirectly suppressed the transcription of the *cox-2* gene through sequestration of CBP/p300 toward the CRE response element [15]. In our ongoing studies, we are exploring the mechanisms by which rexinoids regulate cell growth and prevent the development of breast cancer.

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Abbreviations

HMEC	Human mammary epithelial cells
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
RT-PCR	Reverse transcriptase PCR
ER	Estrogen receptor
SERM	Selective estrogen receptor modulator
DEC2	Differentially expressed in chondrocyte 2
HDAC1	Histone deacetylase 1

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Fig. 1.

Cyclin D1 expression levels correlate with cell proliferation rate. Both HMEC and MCF-7 cells were plated in a 96-well plate at the density of 1000 cells/well. Proliferation assay (MTS assay, Promega, Inc.) was conducted as described in "Materials and methods". Western blotting samples were prepared from cells treated with vehicle (DMSO, 0.01%) or bexarotene (1 μ M) for 48 h. Protein bands specific for cyclin D1 were densitometrically measured and a ratio to control actin density was obtained. Relative density averages were from three separate experiments, shown one representative blot. Error bars represent standard deviation

Fig. 2.

Bexarotene represses the transcription of cyclin D1. HMEC cells were synchronized with MEBM containing 0.5 $\mu\text{g/ml}$ of EGFR antibody for 48 h before transfection of cyclin D1 promoter constructs. 6 h after transfection, cells were split and treated with either vehicle (DMSO, 0.01%) or bexarotene (1 μM) for 48 h. **a** Bexarotene repressed the activity of all four cyclin D1 promoter reporters. **b** Promoter activity in the downstream of -254. **c** QRT-PCR of cyclin D1 in HMEC cells treated with DMSO or bexarotene in the presence or absence of cycloheximide (100 $\mu\text{g/ml}$). The expression levels of cyclin D1 were normalized by cyclophilin

Fig. 3.

Bexarotene induces DEC2. **a, b** Western blot of DEC2 in HMEC and MCF-7 cells treated with vehicle (DMSO, 0.01%) or bexarotene (1 μ M) for 24 h. Protein bands specific for DEC2 were densitometrically measured and a ratio to control actin density was obtained. Relative density averages were from three separate experiments, shown one representative blot. Error bars represent standard deviation. **c** QRT-PCR of DEC2 in HMEC cells treated with DMSO or bexarotene in the presence or absence of cycloheximide (100 μ g/ml). The expression levels of DEC2 were normalized by cyclophilin

Fig. 4.

Forced expression of DEC2 represses the transcription of cyclin D1. **a** DEC2 repressed the activity of all four cyclin D1 promoter reporters. HMEC cells were co-transfected cyclin D1 promoter, Flag-DEC2 construct, and pRL-TK dual luciferase/renilla vector. Cells were harvested 24 h after transfection. Firefly luciferase activities were normalized by the activities of renilla. **b** Cyclin D1 promoter activity downstream of -254. The repressive activities of bexarotene and DEC2 show similar patterns

Fig. 5.

Transcriptional repression of cyclin D1 is HDAC-dependent. **a** Chip analysis of cyclin D1 in the proximal promoter region in HMEC cells. PCR results of representative bands conducted at 38 cycles except for polymerase II, which was conducted at 32 cycles. Negative control primers target the MMP1 coding region [16]. Western blotting of cyclin D1 and DEC2 in the sample cells treated with vehicle (DMSO, 0.01%) and bexarotene. **b** TSA treatment abolishes the cyclin D1 repression. Synchronized HMEC cells were treated with DMSO or bexarotene for 48 h in the presence or absence of TSA (1 μ M). Expression levels of cyclin

D1 were determined by QRT-PCR and normalized by cyclophilin. **c** DEC proteins interact with HDAC1 as demonstrated with immuno-precipitation and followed by western blotting

Fig. 6.

DEC2 is required for the cyclin D1 repression and growth suppression. HEMC cells were transfected with either DEC2 siRNAs or non-specific siRNAs. Cells were then treated by vehicle (DMSO, 0.01%) or bexarotene (1 μ M) for expression and proliferation studies. **a** Knockdown of DEC2 abolished cyclin D1 repression. HMEC cells were synchronized before siRNA transfection and harvested 48 h after bexarotene treatment. RNA and protein levels of cyclin D1 and DEC2 were measured by QRT-PCR and western blot. **b** Growth suppressive effect of bexarotene was reduced after DEC2 knockdown. HMEC cells were split 24 h after siRNA transfection and bexarotene treatment started 24 h after split. The efficiency of siRNA knockdown was demonstrated by the RNA levels of DEC2 at day 2

after treatment. Proliferation rates were measured by MTS assay. **c** Overexpression of DEC2 was conducted by retrovirus infection. Expression of DEC2 in cells infected with pBabe or pBabe-DEC2. Protein levels were measured by western blot. Proliferation rate of cells infected with pBabe or pBabe-DEC2 was measured by MTS assay

Fig. 7.
Schematic diagram of the mechanisms by which bexarotene represses cell proliferation