

A Repressive Epigenetic Domino Effect Confers Susceptibility to Breast Epithelial Cell Transformation: Implications for Predicting Breast Cancer Risk

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Abstract

Retinoic acid (RA) is a master epigenetic regulator that plays a pivotal role in both breast morphogenesis and development. Here, we show for the first time that RA, via the RA receptor α (RAR α), epigenetically regulates in a concerted fashion the transcription of two RA-responsive genes, the RA receptor β 2 (RAR β 2) and the cellular retinol-binding protein 1 (CRBP1). Specifically, an impaired RA signal through RAR α in human breast epithelial cells triggers a repressive epigenetic domino effect, involving first RAR β 2 and second CRBP1. The phenotype acquired by breast epithelial cells clearly implies that the resistance to RA-mediated growth inhibition precedes the acquisition of morphological epithelial transformation, thus supporting the occurrence of sequential transcriptional silencing of first RAR β 2 and second CRBP1. The identification of this epigenetic network mechanistically linking RAR β 2 and CRBP1 transcription provides the basis for devising more accurate epigenetic tests for the prediction of breast cancer risk. (Cancer Res 2006; 66(21): 10308-14)

Introduction

Retinoic acid (RA), the bioactive derivative of vitamin A or retinol, is a master epigenetic regulator of gene transcription (1), which plays a pivotal role in postpuberty mammary gland morphogenesis and development (2, 3). Once integrated through the RA receptor α (RAR α), RA signal elicits chromatin modifications that enable transcription of the RA receptor β 2 (RAR β 2; refs. 1, 4). RAR β 2 would then sustain its own transcription (5–7) and the transcription of a few downstream RA-responsive target genes.

By stably impairing the integration of RA signal through RAR α in RA-sensitive breast cancer cells, we found recently that RAR β 2 falls into an aberrant transcriptional inactive status, which is marked by a critical level of repressive chromatin modifications, including, but not limited to, DNA hypermethylation (8). Concomitant with the conversion of RAR β 2 alleles from a permissive transcriptional status into a nonpermissive status, cells are converted to RA resistance (8). These findings led us to originally speculate that the propensity to RAR β 2 epigenetic silencing and RA resistance could be consequent to an altered retinol/RA metabolism, capable of creating an aberrant RA signal through RAR α (8, 9). Indeed, several proteins involved in either retinol or RA metabolism/transport can be found deranged or down-regulated in breast cancer cells

(10–13). One of these proteins is the cellular retinol-binding protein 1 (CRBP1). In this study, we set out to test whether silencing CRBP1 in RA-sensitive cells could mechanistically lead to RAR β 2 silencing and RA resistance. Because CRBP1, in addition of being a retinol transport protein, is also involved in the maintenance of apicobasal-differentiated morphology of human breast epithelial cells (14), we chose to knock down CRBP1 by RNA interference (RNAi) in nontransformed human breast epithelial cells (HME1). However, contrary to our hypothesis, we found that CRBP1 knockdown does not induce RAR β 2 silencing and RA resistance. We found instead that CRBP1 is a downstream RAR-regulated gene, consistent with reports pointing at CRBP1 like one of the few RAR targets (15–19). First, we identified in the human CRBP1 a RA-responsive element (RARE), which is evolutionary conserved (15, 16). Second, we found that both RAR α and RAR β 2 can bind the human CRBP1-RARE region. Finally, when we impaired the integration of RA through either RAR β 2 or RAR α in HME1 cells, we observed the conversion of CRBP1 alleles permissive for transcription into alleles nonpermissive for transcription. A fraction of nonpermissive alleles showed aberrant DNA hypermethylation. Apparently, an impaired integration of RA signal through RAR α leads to a repressive epigenetic “domino effect,” involving first RAR β 2 and second CRBP1. The phenotypic analysis of HME1 clones, showing that the resistance to RA-mediated growth inhibition precedes the acquisition of morphologic phenotypes of epithelial transformation, further supported the occurrence of sequential transcriptional silencing of first RAR β 2 and second CRBP1.

Materials and Methods

Cells and cell cultures. The human telomerase-immortalized non-transformed breast epithelial cell strain HME1 (Clontech, Mountain View, CA) was grown in mammary epithelial growth medium (MEGM) plus bovine pituitary extract (Cambrex, Walkersville, MD) as per manufacturer's instructions. The monkey kidney COS cell line and the human breast cancer MDA-MB-231 cell line (American Type Culture Collection, Manassas, VA) were grown in DMEM plus 5% fetal bovine serum (Invitrogen, Carlsbad, CA).

HME1 cells and derived clones were grown on reconstituted basement membrane in three-dimensional cultures to induce breast epithelial differentiation into acini-like structures essentially as described (20). Briefly, single cells were induced to form acini on chamber slides coated with Matrigel (BD Biosciences, San Jose, CA) in medium plus 2% Matrigel for 10 to 15 days. After fixation with 4% paraformaldehyde, the Golgi apparatus was stained with anti-GM 130 antibody (Ab) (1:400; BD Biosciences) followed by goat anti-mouse Alexa Fluor 546 Ab (1:500; Molecular Probes, Eugene, OR). Integrin was stained with anti-CD49f Ab (1:200; Chemicon, Temecula, CA) followed by anti-rat Alexa Fluor 488 Ab (1:400; Molecular Probes). Nuclei were counterstained with 300 nM 4',6-diamidino-2-phenylindole (Sigma, St. Louis, MO). At least 30 acini per each clone were analyzed by confocal microscopy (SP2 spectral confocal microscope, Leica,

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Microsystem, Wetzlar, DE) to inspect for the presence of a hollow lumen and apicobasal polarization. The phenotype observed in 70% or more of the acini was considered to be the prevalent phenotype.

For the colony formation assay, exponentially growing cells were seeded at 3×10^2 per well in six-well plates and allowed to attach for 48 hours. After treatment with either 0.1 μ M RA or vehicle (ethanol) for 24 hours, the medium was replaced with drug-free medium and cells were allowed to grow for 10 to 14 days, until the appearance of colonies was observed. Colonies were then fixed with methanol, stained with Giemsa, and scored to establish the colony formation index.

For the anchorage-independent colony formation assay, 1×10^4 cells per well resuspended in semisolid medium containing 0.3% agarose were layered on 0.5% agarose in six-well plates and cultured for 4 weeks. Foci were counted in 10 random fields per each triplicate sample under a Nikon (Melville, NY) Eclipse E600 microscope. Each experiment was repeated three times.

Drugs. RA and 5-aza-2'-deoxycytidine (5-Aza; Sigma) were dissolved in 100% ethanol and 0.45% NaCl containing 10 nM sodium phosphate (pH 6.8), respectively, and stored in aliquots at -80°C in the dark.

RNAi. Short hairpin RNA (shRNA) sequences targeting *CRBP1*, *RAR β 2*, and *RAR α* mRNAs were cloned into the pSUPER-retro vector (Oligoengine, Seattle, WA). The sequences targeted by RNAi were as follows: *CRBP1-A*, 5'-GTGCATGACAACAGTGAGC-3'; *CRBP1-B*, 5'-GGTGTGGTCTGCAAGCAAG-3' (Genbank NM_002899, nucleotides 371-389 and 495-513, respectively); *RAR β 2-A*, 5'-GCTGGCTTGTCTGTCATAA-3'; *RAR β 2-B*, 5'-GGGGCA-GAGTTTGTAGGAG-3' (Genbank NM_000965.2, nucleotides 303-321 and 371-389, respectively); and *RAR α -B* (8), 5'-AGCGCACCAGGAAACCTTC-3' (Genbank NM_000964, nucleotides 681-699). The control mock sequence 5'-ACGTACGTACGTAGTGGG-3', which should not recognize any human mRNA, was cloned in the pSUPER-retro vector and used as a control. The silencing effect of each of these sequences was tested by transient cotransfection with the cognate exogenous cDNAs in COS cells. The cDNA sequences included the following: (a) the *RAR α* cDNA cloned into pSG5 plasmid (kindly provided by Dr. Fausto Andreola, NCI, Bethesda, MD); (b) the *CRBP1* cDNA cloned from MDA-MB-468 cell line in frame with the TAG sequence present into the pCMV-TAG vector (Stratagene, La Jolla, CA) after PCR amplification using the primers 5'-TATGGAATTCCTGGCTCCAGTCACTCCCGAA-3' (sense) and 5'-TATCTCGAGCTGATTGGTTGGGA-CAGGTTGTCT-3' (antisense) containing restriction sites for *Eco*RI and *Xho*I, respectively; and (c) the *RAR β 2* cDNA cloned from T47D into pCDNA3.1+ (Invitrogen) after PCR amplification using the primers 5'-TATGGATCCGCAAGGAGATCATGTTT-3' (sense) and 5'-TATAAGCTTT-TATGACGAGTGGTACTG-3' (antisense) containing restriction sites for *Bam*HI and *Hind*III, respectively. Stable transfections in HME1 cells were carried out with Lipofectamine Plus (Invitrogen). Single stable clones were selected in puromycin 1 μ g/mL and four clones per each RNAi sequence were selected for further analysis.

Retroviral infection. Supernatants containing either the *RAR α* dominant-negative LXRAR α 403SN or the empty LX SN (mock) retroviral particles (kindly provided by Dr. Fausto Andreola) were used to infect HME1 cells as described (21). Four independent clones were used for further analysis.

Real-time reverse transcription-PCR. Total RNA obtained with the single-step method using Trizol (Invitrogen) was treated with DNase I (Ambion, Austin, TX). For each sample, 1 μ g total RNA was retrotranscribed with SuperScript First-Strand Synthesis System (Invitrogen) in a 20 μ L reaction. One microliter of the so obtained cDNA was then used for each triplicate in real-time reverse transcription-PCR, which was done on an iCycler (Bio-Rad, Hercules, CA) using the iQ SYBR Green Supermix (Bio-Rad) with primers specific for *CRBP1* [5'-GGTACTGGAAGATGTTGGTC-3' (sense) and 5'-CATCTCTAGGTGCAGCTCAT-3' (antisense)], *RAR β 2* [5'-GACTGTATGGATGTTCTGTCAG-3' (sense) and 5'-ATTGTCCTGGCAGACGA-3' (antisense)], and *RAR α* [5'-TGTGGACTTCGCCAAGCA-3' (sense) and 5'-CGTGTACCGCGTGCAGA-3' (antisense)]. The mRNA levels were normalized to the mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*; 5'-GAAGGTGAAGTCCGGAGTC-3' (sense) and 5'-GAAGATGGTGTGGGATTC-3' (antisense)].

Western blot. Western blot analysis of *RAR β 2*, FLAG-CRBP1, and GAPDH protein expression was done with standard protocols using an anti-

RAR β 2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-FLAG M2 monoclonal antibody (Sigma) recognizing the TAG encoded by the CMV-TAG vector, and an anti-GAPDH antibody (Santa Cruz Biotechnology). Appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) were used for detection.

Quantitative chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was done using reagents purchased from Upstate (Lake

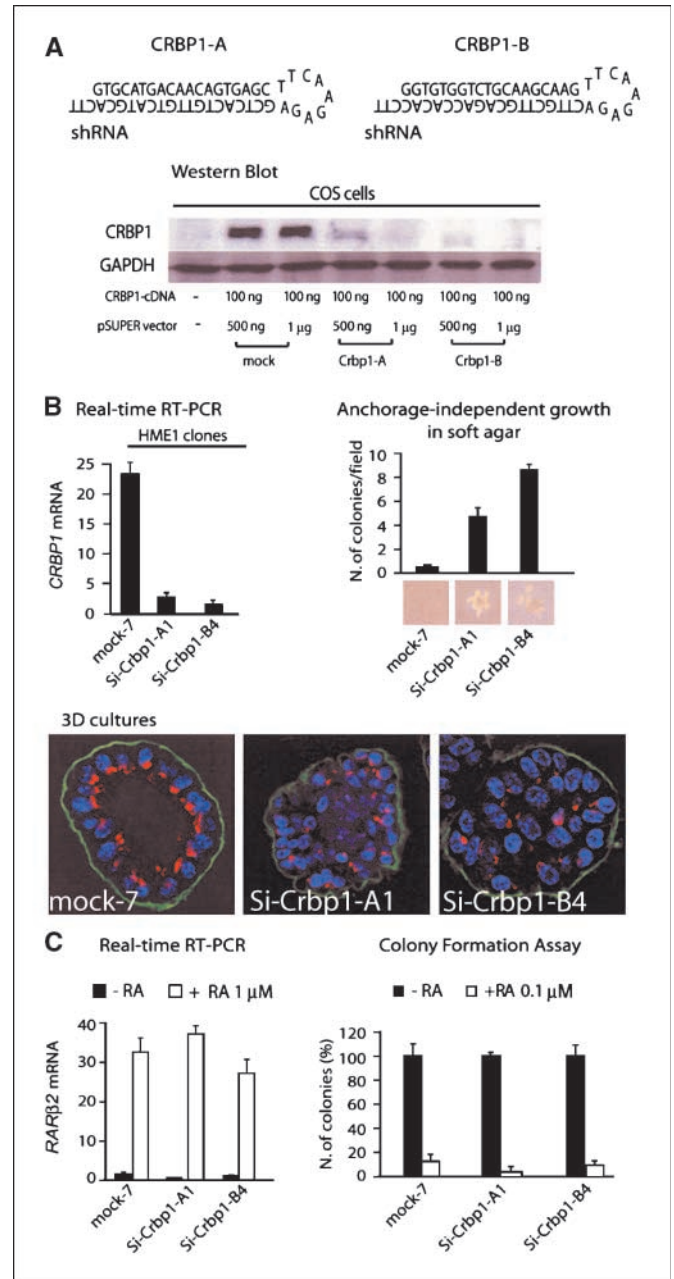


Figure 1. *CRBP1* silencing does not induce *RAR β 2* silencing and RA resistance. **A**, two sequences, *CRBP1-A* and *CRBP1-B* (top), cloned in the pSUPER-retro vector and able to efficiently abrogate the expression of exogenous *CRBP1* in COS cells transfected with human *CRBP1* cDNA (bottom), were stably transfected into HME1 cells. **B**, two HME1 clones, Si-Crbp1-A1 carrying *CRBP1-A* and Si-Crbp1-B4 carrying *CRBP1-B*, showing significant ($P < 0.001$) *CRBP1* transcriptional down-regulation compared with the control clone, mock-7, carrying a scrambled sequence (top, left), displayed both anchorage-independent growth (top, right) and an impaired epithelial polarization in three-dimensional culture [blue, nuclei; red, Golgi apparatus; green, integrin (bottom)]. **C**, Si-Crbp1-A1 and Si-Crbp1-B4 with unaffected *RAR β 2* transcriptional activity (left) are RA sensitive (right).

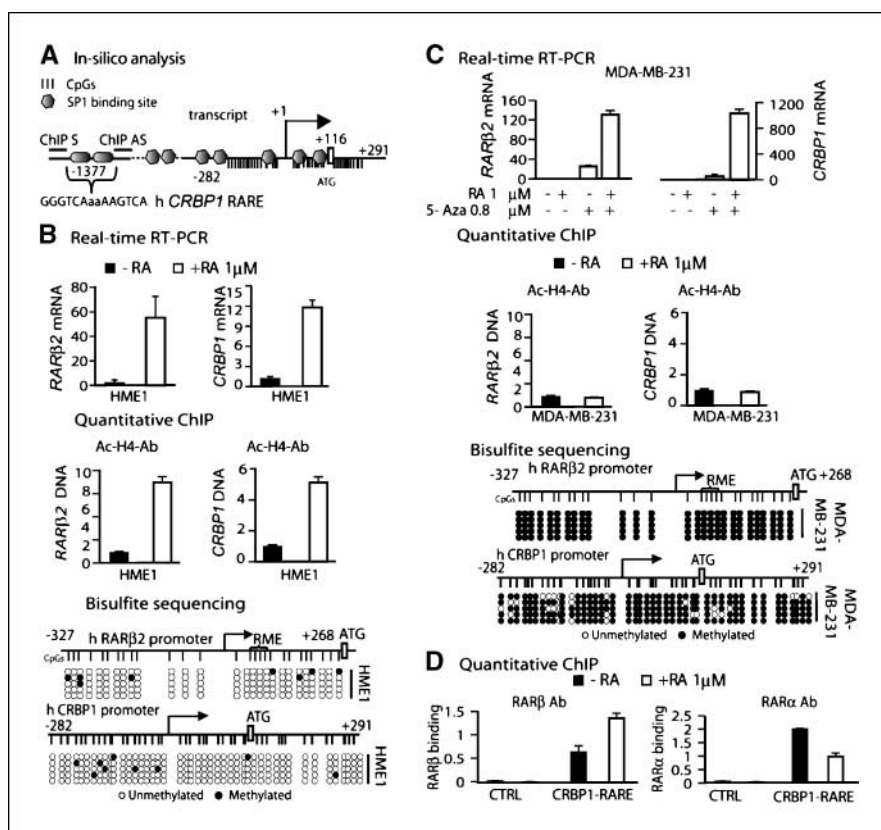


Figure 2. CRBP1 is epigenetically regulated in response to RA. *A*, *in silico* analysis of the human CRBP1 showing a RARE 1,377 bp upstream of the transcription start site. *B*, RA significantly induces both transcription ($P < 0.001$; top) and histone H4 acetylation ($P < 0.05$; middle) of both RAR β 2 and CRBP1 in HME1 cells where both genes are not hypermethylated (bottom). *C*, both RAR β 2 and CRBP1 remain transcriptionally silent in response to RA in MDA-MB-231 cells (top) consistent with lack of histone H4 acetylation (middle) and the presence of DNA methylation (bottom). RAR β 2 RA-induced and CRBP1 RA-induced transcription can be concomitantly reactivated after treatment with 5-Aza (top). *D*, ChIP analysis with either anti-RAR α or anti-RAR β antibodies showing that both RAR α and RAR β 2 bind to the CRBP1-RARE in HME1 cells significantly ($P < 0.05$) more than to an adjacent [control (CTRL)] region 8.7 kb 5' to the RARE. RAR β 2 binding and RAR α binding are increased and decreased, respectively, in response to RA.

Placid, NY) according to the manufacturer's protocol. Chromatin was immunoprecipitated with antibodies against acetyl histone H4 (Upstate), RAR β [Santa Cruz Biotechnology and Active Motif (Carlsbad, CA)], and RAR α (Santa Cruz Biotechnology). The immunoprecipitated DNA was amplified by real-time PCR with specific primers encompassing the CRBP1-RARE [5'-AGCTGCACCTGTGAGAACACAT-3' (sense) and 5'-CCACCAAGTAGATGACATAATCA-3' (antisense)], a control region 8.7 kb upstream of the CRBP1-RARE [5'-GGACCTTGGTGGTGGAGGATA-3' (sense) and 5'-CCCTGCAGGTCTCCACTAT-3' (antisense)], and the GAPDH promoter region [5'-GGTGCCTGCCAGTTGAACCA-3' (sense) and 5'-AAAGAA-GATGCGGCTGACTGTGCA-3' (antisense)]. The relative enrichment of immunoprecipitated DNA was calculated by normalizing the PCR signals of the samples to both the input and the no antibody controls. In ChIP experiments with anti-acetyl histone H4 antibody, we used as an internal control the amplification of the GAPDH promoter. In control ChIPs with anti-RAR β and anti-RAR α antibodies, we amplified a region 8.7 kb upstream of the CRBP1-RARE as a negative control.

DNA methylation analysis. Genomic DNA was extracted with DNaZol (Invitrogen) and modified with sodium bisulfite as described previously (22). We amplified by seminested PCR a 449-bp region of the CRBP1 CpG island encompassing 45 CpG sites using specific primer sets [5'-AGGTTT-AGATAAAGGTTTGTAAAGT-3' (sense-1), 5'-GTGGTTGTGAGTGTGA-GAAG-3' (sense-2), and 5'-CACCAAACCACAACCTACCAAA-3' (antisense)] and a region encompassing a CpG stretch, which we defined as the RAR β 2 methylation epicenter (RME; ref. 8). To sequence single alleles, the PCR products were cloned into the pCR4-TOPO plasmid vector (Invitrogen).

In silico analysis of human CRBP1. To analyze the DNA region 5' to hCRBP1, the hCRBP1 transcript sequence from the Ensemble database (ENST00000232219) and the National Center for Biotechnology Information (NCBI) database (NM_002899.2) were compared with both the sequence encoding human DNA for CRBP1 and exons 1 and 2 (and joined coding sequence) from the NCBI database (X07437), and the complete sequence of the human 3 BAC RP11-553K23 clone (Roswell Park Cancer Institute

Human Bacterial Artificial Chromosome Library, Buffalo, NY) deposited at the NCBI database (AC046134).

Statistical analysis. Statistical significance was calculated based on three independent experiments using the Student's *t* test.

Results

CRBP1 knockdown in HME1 cells does not lead to RAR β 2 silencing and RA resistance. CRBP1 is a protein critically involved in the transport and mobilization of retinol, the precursor of RA (23, 24). Previously, we speculated that lack/derangement of proteins involved in retinol/RA metabolism could alter the intracellular concentration of RA, thus leading to RAR β 2 epigenetic silencing (8, 9). The findings presented here discount that this might be the case for CRBP1. When we simulated the occurrence of CRBP1 epigenetic down-regulation in HME1 cells by knocking down CRBP1 transcription by RNAi with two sequences, CRBP1-A and CRBP1-B (Fig. 1A, top), selected to efficiently silence an exogenously expressed human CRBP1 protein in COS cells (Fig. 1A, bottom), we did not induce RAR β 2 silencing, and consistently, we did not observe the conversion to RA resistance (Fig. 1C). Four independent HME1 clones, two per each sequence, showing significant CRBP1 transcriptional down-regulation relative to a prototypic control clone, mock-7, carrying a scrambled sequence, were analyzed. Here, we show that for two representative clones, Si-Crbp1-A1 and Si-Crbp1-B4, which were stably transfected with the sequences CRBP1-A and CRBP1-B, respectively (Fig. 1B, top, left), the induction of CRBP1 knockdown confers the two phenotypes expected for CRBP1 down-regulation (14, 25), including anchorage-independent growth in soft agar (Fig. 1B, top, right) and impaired apicobasal polarization when grown on basement

membrane in three-dimensional cultures. Both clones formed mainly acini with a filled lumen (Fig. 1B, bottom); however, they maintained a transcriptionally active *RARβ2* (Fig. 1C, left) and remained sensitive to the growth-inhibitory action of RA (Fig. 1C, right).

Evidence that *hCRBP1* is a direct RAR target, epigenetically regulated in response to RA. Previous reports implied that *CRBP1* might be a direct RAR target gene (15–19). We located by *in silico* analysis (see Materials and Methods) a human *CRBP1*-RARE 1,377 bp upstream of the *hCRBP1* transcription start site (Fig. 2A), which is highly homologous to both mouse and rat *CRBP1*-RARE sequences (15, 16). Second, we found that RA (1 μM, 72 hours) activated both *RARβ2* and *CRBP1* transcription in HME1 cells (Fig. 2B, top) in association with a significant increase of histone H4 acetylation at both the *RARβ2*-RARE and the *CRBP1*-RARE chromatin regions (Fig. 2B, middle), which is consistent with the scanty DNA methylation in just a minority of HME1 alleles (Fig. 2B, bottom). Conversely, both *RARβ2* and *CRBP1* in MDA-MB-231 cells were transcriptionally silent (Fig. 2C, top), hypoacetylated at histone H4 (Fig. 2C, middle), and showed DNA hypermethylation (Fig. 2C, bottom). The transcription of both genes was concomitantly reactivated by RA (1 μM) after partial reversion of the DNA hypermethylated status by 5-Aza (0.8 μM, 72 hours; Fig. 2C, top). Altogether, these data implied that the transcription

of both *RARβ2* and *CRBP1* is epigenetically regulated in a concerted fashion in response to RA. This supposition was reinforced by ChIP analysis with anti-*RARβ2*-specific antibodies, which detected *RARβ2* binding at the *CRBP1*-RARE but not at an adjacent 5' control region (Fig. 2D, left). In addition, the level of *RARβ2* binding increased in response to RA, concomitant with a decrease of *RARα* binding at the *CRBP1*-RARE (Fig. 2D, right). These data prompted us to test whether *CRBP1* transcription is under direct RAR regulation.

***RARβ2* knockdown in HME1 cells leads to *CRBP1* epigenetic silencing.** When we down-regulated *RARβ2* transcription in HME1 by RNAi, we observed down-regulation of *CRBP1* transcription, which remained unresponsive to RA. We used two sequences, *RARβ2*-A and *RARβ2*-B (Fig. 3A, top), which efficiently silenced *RARβ2* expression from an exogenous *RARβ2* cDNA transfected in COS cells (Fig. 3A, bottom), to stably transfect HME1 cells. Four independent clones, two per each sequence, showing significant *RARβ2* transcriptional down-regulation and RA resistance (Fig. 3B, top, left and right, respectively) yet unaffected levels of *RARα* transcription (Fig. 3B, bottom) relative to a representative control clone, mock-8, were selected for further analysis. The clones tested included Si-β-A8, carrying the *RARβ2*-A sequence, and Si-β-B4, carrying the *RARβ2*-B sequence (Fig. 3B, left). In both Si-β-A8 and Si-β-B4 *CRBP1* alleles were clearly nonpermissive for transcription

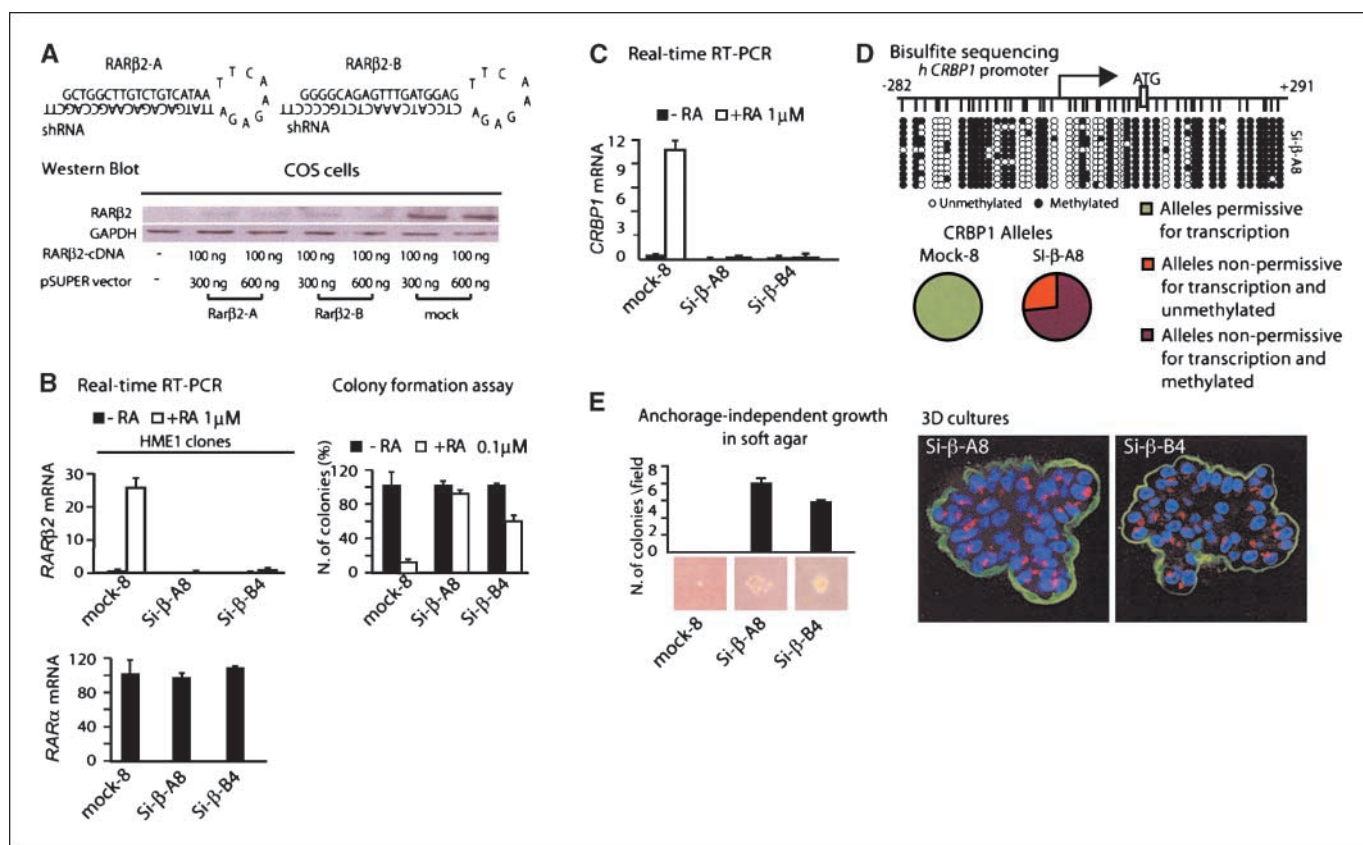


Figure 3. *RARβ2* knockdown in HME1 cells leads to *CRBP1* epigenetic silencing. *A*, the *RARβ2*-A and *RARβ2*-B sequences (top) efficiently silenced exogenous *RARβ2* expression in COS cells cotransfected with human *RARβ2* cDNA (bottom). *B*, two HME1 clones, Si-β-A8 carrying *RARβ2*-A and Si-β-B4 carrying *RARβ2*-B, showing specific and significant down-regulation of *RARβ2* ($P < 0.01$; top, left) but not of *RARα* (bottom) acquired RA resistance (top, right) compared with the control clone mock-8, carrying a scrambled sequence. *C*, both Si-β-A8 and Si-β-B4 show significant ($P < 0.001$) *CRBP1* transcriptional down-regulation. *D*, distribution of CpG methylated sites (top) in *de novo* methylated, nonpermissive alleles of the Si-β-A8 clone (bottom). *E*, Si-β-A8 and Si-β-B4 clones showing anchorage-independent growth (left) and impaired epithelial cell differentiation in three-dimensional cultures (right).

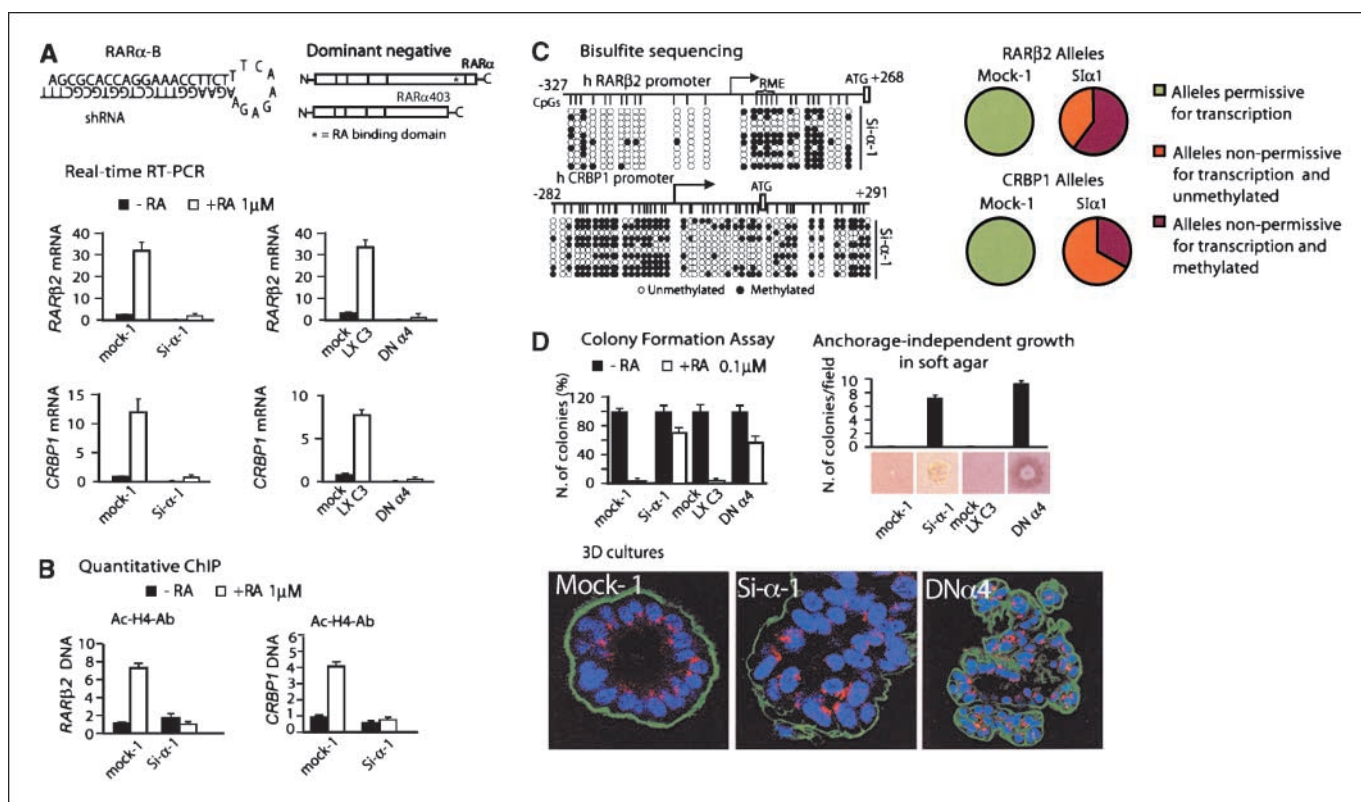


Figure 4. Functional inhibition of $RAR\alpha$ induces concomitant $CRBP1$ and $RAR\beta2$ epigenetic silencing. **A**, HME1 stable clones, such as the prototypical Si- α -1, carrying the $RAR\alpha$ -B RNAi sequence (top, left), or the prototypical DN α 4, carrying the dominant-negative construct $RAR\alpha403$ (top, right), showing significant ($P < 0.01$) down-regulation of both $RAR\beta2$ (middle) and $CRBP1$ (bottom) transcription compared with the cognate control clones. **B**, Si- α -1 clone showing significant histone H4 hypoacetylation in response to RA ($P < 0.05$) at both $RAR\beta2$ -RARE and $CRBP1$ -RARE. **C**, left, distribution of CpG methylated sites in 10 $RAR\beta2$ (top) and 10 $CRBP1$ (bottom) nonpermissive hypermethylated alleles, respectively, out of the nonpermissive alleles of Si- α -1 shown in the pie graphs (right). **D**, evidence of RA resistance (top, left), anchorage-independent growth (top, right), and impaired epithelial polarization in three-dimensional cultures (bottom) in both Si- α -1 and DN α 4 clones.

(Fig. 3C). A fraction of the alleles nonpermissive for transcription (shown here are the alleles of the Si- β -A8 clone) acquired *de novo* DNA methylation over the $CRBP1$ CpG island (Fig. 3D, top and bottom).

Both Si- β -A8 and Si- β -B4 failed to form polarized, hollow lumen acini when grown on reconstituted basement membrane (three-dimensional cultures; Fig. 3E, right), and both displayed anchorage-independent growth (Fig. 3E, left). Apparently, $RAR\beta2$ transcriptional repression leads to both $CRBP1$ epigenetic repression and associated morphologic phenotypes. These data led us to test whether an impaired RA signal at $RAR\alpha$ can trigger a repressive epigenetic “domino effect” involving both $RAR\beta2$ and its target $CRBP1$.

An impaired RA signal at $RAR\alpha$ leads to concomitant $RAR\beta2$ and $CRBP1$ epigenetic repression. Interference with either $RAR\alpha$ transcription by $RAR\alpha$ RNAi or $RAR\alpha$ function with a dominant-negative $RAR\alpha$ mutant ($RAR\alpha403$; ref. 21) in HME1 cells confirmed that indeed this was the case. Both the $RAR\alpha$ knockdown clone Si- α -1, carrying the $RAR\alpha$ -B silencing sequence (Fig. 4A, top, left; ref. 8), and the DN α 4 clone, carrying the dominant-negative $RAR\alpha403$ mutant (Fig. 4A, top, right), displayed stable repression of endogenous $RAR\beta2$ and $CRBP1$ transcription relative to the respective control clones, mock-1, carrying a scrambled sequence, and LX C3, carrying an empty vector (Fig. 4A, middle and bottom). The chromatin of both genes underwent histone H4 hypoacetylation. We conclude that this is

the case because RA did not raise significantly histone H4 acetylation (Fig. 4B, left and right). In addition, we observed *de novo* DNA methylation in the RME (8) and $CRBP1$ CpG island (Fig. 4C, top and bottom) in clones impaired for $RAR\alpha$, but not in the mock controls.

The phenotypes acquired by both Si- α -1 and DN α 4 clones fully recapitulated the phenotypes observed after $RAR\beta2$ and $CRBP1$ silencing, including resistance to RA, anchorage-independent growth, and aberrant epithelial polarization when cells were grown as acini in three-dimensional cultures (Fig. 4D). Interestingly, these phenotypes were described previously in a human mammary epithelial cell strain transfected with the $RAR\alpha403$ mutant (26, 27).

We conclude that an impaired RA signal through $RAR\alpha$ can cause a coordinated, epigenetic repression of $RAR\beta2$ and its direct target $CRBP1$.

Discussion

In this study, we show that an interference with RA signal at $RAR\alpha$ in RA-sensitive, untransformed, human mammary epithelial cells converts in a coordinated succession two RA-responsive genes, $RAR\beta2$ and its direct target $CRBP1$, into a status nonpermissive for transcription. Interestingly, a fraction of nonpermissive $RAR\beta2$ and $CRBP1$ alleles acquires also chromatin repressive modifications, including *de novo* DNA methylation. This is the first

evidence of a repressive epigenetic domino effect involving two RA-responsive genes consequent to an impaired RA signal. As a consequence of this epigenetic domino effect, human breast epithelial cells acquire in a succession RA resistance and distinct phenotypes of breast epithelial transformation, such as loss of epithelial polarization, inability to form a hollow lumen in three-dimensional basement membrane cultures, and anchorage-independent growth.

Recently, we showed that an impaired RA signal through RAR α in RA-sensitive, transformed breast cancer cells leads to *RAR β 2* epigenetic silencing (8). Apparently, an interference with RA signal through RAR α would trigger the silencing of genes that are normally epigenetically regulated by RA. Further, transcriptional silencing is clearly marked by aberrant repressive chromatin changes not only in transformed but also in untransformed cells. We do not know yet by which mechanism the transcriptionally inactive status created by lack of integration of RA signal through RAR α can invoke repressive modifying enzymes capable of imposing both histone and DNA repressive changes at RAR-regulated genes. We are tempted to speculate that, as it happens during the heterochromatinization of the transcriptionally inactive chromosome X (28, 29), an RNA-mediated mechanism may play a role in the heterochromatinization of transcriptionally inactive autosomal gene regions. Apparently, only a fraction of nonpermissive *RAR β 2* or *CRBP1* alleles develops aberrant *de novo* DNA methylation, which would indicate that it is the nonpermissive status that recalls DNA methylation and not the other way around. As for chromosome X, DNA methylation would "lock in" the silent status of nonpermissive alleles.

Interestingly, here we show that RAR α , via *RAR β 2*, controls the transcription of *CRBP1*, a gene involved both in retinol transport and breast epithelial cell morphogenesis. We found recently that

RAR α , via *RAR β 2*, controls the transcription of another RA-responsive gene, *CYP26A1*, an enzyme implicated in RA catabolism and neural morphogenesis (ref. 30 and references within). Thus, RA-RAR α signal seems to control, through a concerted epigenetic mechanism, at least two RA-responsive genes involved in retinol/RA metabolism as well as morphogenesis. The gradient of retinoids is critical for symmetry and morphogenesis during organismal development (31–33). This could also be true during postnatal development of the mammary gland. The results shown here lend support to the hypothesis that retinoid metabolism is intertwined with retinoid-dependent morphogenesis. Specifically, RA itself would epigenetically regulate through its own metabolism critical morphogenetic programs.

Our study also provides useful information for improving a breast cancer prediction test, thus far based on *RAR β 2* hypermethylation analysis alone (34). In light of what we showed here, *RAR β 2* hypermethylation cannot distinguish between cells heterozygous for *RAR β 2* nonpermissive alleles, thus still sensitive to RA, from cells homozygous for *RAR β 2* nonpermissive alleles, thus RA resistant. Only the latter would have tumor phenotypic features. Combining *RAR β 2* and *CRBP1* epigenetic analysis might provide a better test for predicting breast cancer susceptibility.

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