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 \( \alpha \) (RAR\( \alpha \)), epigenetically regulates in a concerted fashion the transcription of two RA-responsive genes, the RA receptor \( \beta \) (RAR\( \beta \)) and the cellular retinol-binding protein 1 (CRBP1). Specifically, an impaired RA signal through RAR\( \alpha \)R in human breast epithelial cells triggers a repressive epigenetic domino effect, involving first RAR\( \beta \)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\( \beta \)2 and second CRBP1. The identification of this epigenetic network mechanistically linking RAR\( \beta \)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 \( \alpha \) (RAR\( \alpha \)), RA signal elicits chromatin modifications that enable transcription of the RA receptor \( \beta \) (RAR\( \beta \); refs. 1, 4). RAR\( \beta \)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\( \alpha \)R in RA-sensitive breast cancer cells, we found recently that RAR\( \beta \)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\( \beta \)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\( \beta \)2 epigenetic silencing and RA resistance could be consequent to an altered retinol/RA metabolism, capable of creating an aberrant RA signal through RAR\( \alpha \)Rs (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\( \beta \)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\( \beta \)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\( \alpha \)s and RAR\( \beta \)2 can bind the human CRBP1-RARE region. Finally, when we impaired the integration of RA through either RAR\( \alpha \)2 or RAR\( \beta \)2 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\( \alpha \)s leads to a repressive epigenetic “domino effect,” involving first RAR\( \beta \)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\( \beta \)2 and second CRBP1.

Materials and Methods

Cells and cell cultures. The human telomerase-immortalized nontransformed 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 30 nM 4′,6-diamidino-2-phenyldlde (Sigma, St. Louis, MO). At least 30 acini per each clone were analyzed by confocal microscopy (SP2 spectral confocal microscope, Leica, Germany).

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Microsystem, Wetzlar, DE) to inspect for the presence of a hollow lumen and apico-basal 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 $3 \times 10^4$ per well in six-well plates and allowed to attach for 48 hours. After treatment with either 0.1 μM 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 x 10^4 cells per well was resuspended in semisolid medium containing 0.3% agarose and 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, RARα2, and RARβ mRNAs were cloned into the pSUPER-retro vector (Oligoengine, Seattle, WA). The sequences targeted by RNAi were as follows: CRBP1-A, 5′-GTGCAATGCAACACATGAGC-3′; CRBP1-B, 5′-GGTTGTTGTCGGAGGACAG-3′ (Genbank NM_002899, nucleotides 371-389 and 495-513, respectively); RARα2-A, 5′-CTGTTGCTGTCGTGCAAAT-3′; RARα2-B, 5′-GGGCACAGTTGTTGAGGACG-3′ (Genbank NM_000965.2, nucleotides 303-321 and 371-389, respectively); and RARβ-α (8), 5′-AGGCACACGAGGGAACCTGC-3′; RARβ-β (9), 5′-AGGCACCAGGAAAATCTGC-3′ (Genbank NM_000964, nucleotides 681-699). The control mock sequence 5′-ACGTACGTACGTACGTACGT-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 transfection with the cognate exogenous cDNAs in COS cells. The cDNA sequences included the following (a) the RARβ1 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′-TATTGAGATCCCTGGCTCCAGTGACTCTCAGC-3′ (sense) and 5′-TATCTCGAGCTGGTTGGGCACTGCTGCTGTC-3′ (antisense) containing restriction sites for EcoRI and XhoI, respectively; and (c) the RARβ2 cDNA cloned from T47D (Invitrogen) after PCR amplification using the primers 5′-TATTGAGATCCCTGGCTCCAGTGACTCTCAGC-3′ (sense) and 5′-TATCTCGAGCTGGTTGGGCACTGCTGCTGTC-3′ (antisense) containing restriction sites for BamHI and HindIII, respectively. Stable transfections in HME1 cells were carried out with Lipofectamine Plus (Invitrogen). Single stable clones were selected in puromycin 1 μg/ml and four clones per each RNAi sequence were selected for further analysis.

**Retroviral infection.** Supernatants containing either the RARs dominant-negative LXRαRα403SN 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 μg total RNA was retrotranscribed with SuperScript First-Strand Synthesis System (Invitrogen) in a 20 μl 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′-GGTACGCTAGATTCCTCTCTT-3′ (sense) and 5′-CATCTCTAGTCGACCTCTCT-3′ (antisense)], RARα2 [5′-GACTGCTTTGAAAGTTGTAAC-3′ (sense) and 5′-TTGTTGTTGTCGGAGGACAGC-3′ (antisense)], and RARβ [5′-TGTTGACCGATCAGAAGC-3′ (sense) and 5′-GGTGTAAGGCGGCGAGCA-3′ (antisense)]. The mRNA levels were normalized to the mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase [GAPDH; 5′-GAAGGTGAAAGTCCCTGAC-3′ (sense) and 5′-GAAGATGGTTAGGGATTC-3′ (antisense)].

**Western blot.** Western blot analysis of RARβ2 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 Ossipee, NH). Figure 1. CRBP1 silencing does not induce RARα2 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 mock, -2, 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 RARα2 transcriptional activity (left) are RA sensitive (right).
Placid, NY) according to the manufacturer’s protocol. Chromatin was immunoprecipitated with antibodies against acetyl histone H4 (Upstate), RARα (Santa Cruz Biotechnology and Active Motif (Carlsbad, CA)), and RARβ (Santa Cruz Biotechnology). The immunoprecipitated DNA was amplified by real-time PCR with specific primers encompassing the CRBP1-RARE [5'-AGCTTTTG ATAAAGGTGAGGGTTGAGGATA-3' (sense) and 5'-CCCTGGCTCCCTCACCAT-3' (antisense)], a control region 8.7 kb upstream of the CRBP1-RARE [5'-GAACCTTTGAGGTGGAGGATA-3' (sense) and 5'-CCCTGGCTCCCTCACCAT-3' (antisense)], and the GAPDH promoter region [5'-GGGGCTGGCCAAGCAG-3' (sense) and 5'-AAAGAGAGTCCCCTGCAG-3' (antisense)] and a region encompassing a CpG stretch, which we defined as the RARβ2 methylation epicenter (RME; ref. 8). To sequence single alleles, the PCR products were cloned into the pCR4-TOPO plasmid vector (Invitrogen).

**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'-AGCTTTTG ATAAAGGTGAGGGTTGAGGATA-3' (sense-1), 5'-GGGGCTGGCCAAGCAG-3' (sense-2), and 5'-AAAGAGAGTCCCCTGCAG-3' (antisense)] and a region encompassing a CpG stretch, which we defined as the RARβ2 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 sCRBP1 transcript sequence from the Ensemble database (ENST0000232219) 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 RP11-533K23 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 RARβ2 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 RARβ2 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 prototypic control clone, mock-7, carrying a scrambled sequence, we did not induce RARβ2 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 RAR\(^b\) (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 CRBP1 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 \(\mu\)M, 72 hours) activated both RAR\(^b\) and CRBP1 transcription in HME1 cells (Fig. 2B, top) in association with a significant increase of histone H4 acetylation at both the RAR\(^b\)-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 RAR\(^b\) and CRBP1 in MDA-MB-231 cells were transcriptionally silent (Fig. 2C, top), hypomethylated at histone H4 (Fig. 2C, middle), and showed DNA hypermethylation (Fig. 2C, bottom). The transcription of both genes was concomitantly reactivated by RA (1 \(\mu\)M) after partial reversion of the DNA hypermethylated status by 5-Aza (0.8 \(\mu\)M, 72 hours; Fig. 2C, top). Altogether, these data implied that the transcription of both RAR\(^b\) and CRBP1 is epigenetically regulated in a concerted fashion in response to RA. This supposition was reinforced by ChIP analysis with anti-RAR\(^b\)-specific antibodies, which detected RAR\(^b\) binding at the CRBP1-RARE but not at an adjacent 5' control region (Fig. 2D, left). In addition, the level of RAR\(^b\) binding increased in response to RA, concomitant with a decrease of RAR\(\alpha\) binding at the CRBP1-RARE (Fig. 2D, right). These data prompted us to test whether CRBP1 transcription is under direct RAR regulation.

RAR\(^b\) knockdown in HME1 cells leads to CRBP1 epigenetic silencing. When we down-regulated RAR\(^b\) transcription in HME1 by RNAi, we observed down-regulation of CRBP1 transcription, which remained unresponsive to RA. We used two sequences, RAR\(^b\)-2-A and RAR\(^b\)-2-B (Fig. 3A, top), which efficiently silenced RAR\(^b\) expression from an exogenous RAR\(^b\) cDNA transfected in COS cells (Fig. 3A, bottom), to stably transfect HME1 cells. Four independent clones, two per each sequence, showing significant RAR\(^b\) transcriptional down-regulation and RA resistance (Fig. 3B, top, left and right, respectively) yet unaffected levels of RAR\(\alpha\) transcription (Fig. 3B, bottom) relative to a representative control clone, mock-8, were selected for further analysis. The clones tested included Si-\(\beta\)-A8, carrying the RAR\(^b\)-2-A sequence, and Si-\(\beta\)-B4, carrying the RAR\(^b\)-2-B sequence (Fig. 3B, left). In both Si-\(\beta\)-A8 and Si-\(\beta\)-B4 CRBP1 alleles were clearly nonpermissive for transcriptional down-regulation.

![Figure 3.](Image)

**Figure 3.** RAR\(^b\) knockdown in HME1 cells leads to CRBP1 epigenetic silencing. A. the RAR\(^b\)-2-A and RAR\(^b\)-2-B sequences (top) efficiently silenced exogenous RAR\(^b\) expression in COS cells cotransfected with human RAR\(^b\) cDNA (bottom). B. two HME1 clones, Si-\(\beta\)-A8 carrying RAR\(^b\)-2-A and Si-\(\beta\)-B4 carrying RAR\(^b\)-2-B, showing specific and significant down-regulation of RAR\(^b\) (\(P < 0.01\); top, left) but not of RAR\(\alpha\) (bottom) acquired RA resistance (top, right) compared with the control clone mock-8, carrying a scrambled sequence. C. both Si-\(\beta\)-A8 and Si-\(\beta\)-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-\(\beta\)-A8 clone (bottom). E. Si-\(\beta\)-A8 and Si-\(\beta\)-B4 clones showing anchorage-independent growth (left) and impaired epithelial cell differentiation in three-dimensional cultures (right).
CRBP1

H4 hypoacetylation in response to RA (shown here are the alleles of the Si-

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vector (Fig. 4 carrying a scrambled sequence, and LX C3, carrying an empty
transcription relative to the respective control clones, mock-1,
RAR played stable repression of endogenous

Figure 4. Functional inhibition of RARα induces concomitant CRBP1 and RARβ2 epigenetic silencing. A, HME1 stable clones, such as the prototypical Si-α-1, carrying the RARα-B RNAi sequence (top, left), or the prototypical DN α4, carrying the dominant-negative construct RARα403 (top, right), showing significant (P < 0.01) down-regulation of both RARβ2 (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 RARβ2-RARE and CRBP1-RARE. C, left, distribution of CpG methylated sites in 10 RARβ2 (top) and 10 CRBP1 (bottom) nonpermissive hypermethylated alleles, respectively, out of the nonpermissive alleles of S1-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 α4 clones.

(38x383)10

b10

CRBP1

H4 hypoacetylation in response to RA (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, RARβ2 transcriptional repression leads to both CRBP1 epigenetic repression and associated morphologic phenotypes. These data led us to test whether an impaired RA signal at RARα can trigger a repressive epigenetic “domino effect” involving both RARβ2 and its target CRBP1.

An impaired RA signal at RARα leads to concomitant RARβ2 and CRBP1 epigenetic repression. Interference with either RARα transcription by RARα RNAi or RARα function with a dominant-negative RARα mutant (RARα403; ref. 21) in HME1 cells confirmed that indeed this was the case. Both the RARα knockdown clone Si-α-1, carrying the RARα-B silencing sequence (Fig. 4A, top, left; ref. 8), and the DN α4 clone, carrying the dominant-negative RARα403 mutant (Fig. 4A, top, right), displayed stable repression of endogenous RARβ2 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 RARα, but not in the mock controls.

The phenotypes acquired by both Si-α-1 and DN α4 clones fully recapitulated the phenotypes observed after RARβ2 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 RARα403 mutant (26, 27).

We conclude that an impaired RA signal through RARα can cause a coordinated, epigenetic repression of RARβ2 and its direct target CRBP1.

Discussion

In this study, we show that an interference with RA signal at RARα in RA-sensitive, untransformed, human mammary epithelial cells converts in a coordinated succession two RA-responsive genes, RARβ2 and its direct target CRBP1, into a status nonpermissive for transcription. Interestingly, a fraction of nonpermissive RARβ2 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 RARα in RA-sensitive, transformed breast cancer cells leads to RARβ2 epigenetic silencing (8). Apparently, an interference with RA signal through RARα 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 CRBP1 would trigger the silencing of genes that are normally epigenetically regulated by RA. Furthermore, 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 CRBP1 would trigger the silencing of genes that are normally epigenetically regulated by RA.

Our study also provides useful information for improving a breast cancer prediction test, thus far based on RARβ2 hypermethylation analysis alone (34). In light of what we showed here, RARβ2 hypermethylation cannot distinguish between cells heterozygous for RARβ2 nonpermissive alleles, thus still sensitive to RA, from cells homozygous for RARβ2 nonpermissive alleles, thus RA resistant. Only the latter would have tumor phenotypic features. Combining RARβ2 and CRBP1 epigenetic analysis might provide a better test for predicting breast cancer susceptibility.

Acknowledgments

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References

4. Dilworth FH, Chambon P. Nuclear receptors coordinate transcriptionally inactive autosomal gene regions. Apparent, only a fraction of nonpermissive RARβ2 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 it” in the status of nonpermissive alleles.

Interestingly, here we show that RARα, via RARβ2, controls the transcription of CRBP1, a gene involved both in retinol transport and breast epithelial cell morphogenesis. We found recently that RARα, via RARβ2, 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-RARα 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.


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