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Advances in Brief

Endogenous Reactivation of the *RARβ2* Tumor Suppressor Gene Epigenetically Silenced in Breast Cancer[1]

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Abstract

Loss of expression of retinoic acid receptor β2 (*RARβ2*), a potent tumor suppressor gene, is commonly observed during breast carcinogenesis. *RARβ2* silencing can be traced to epigenetic chromatin changes affecting the *RARβ* P2 promoter. Here we show that retinoic acid therapy fails to induce *RARβ2* in primary breast tumors, which carry a methylated *RARβ* P2 promoter. DNA methylation leads to repressive chromatin deacetylation at *RARβ* P2. By inducing an appropriate level of histone reacetylation at *RARβ* P2 we could reactivate endogenous *RARβ2* transcription from unmethylated as well as methylated *RARβ* P2 in breast cancer cell lines and xenograft tumors, and obtain significant growth inhibition both *in vitro* and *in vivo*. This study may have translational implications for breast cancer and other cancers carrying an epigenetically silenced *RARβ* P2 promoter.

Introduction

Vitamin A and its active metabolites, including RA,[4] are essential for growth and cell differentiation of epithelial tissue (1). Retinoids exerts their effects mainly via nuclear receptors, the RARs and the RXRs, both of which are members of the nuclear receptor superfamily (1). The human *RARβ* gene is expressed as three isoforms: *β1*, *β2*, and *β4* (2). The biologically active *RARβ2* isoform (1, 2) is under the regulation of the P2 promoter containing a high affinity RA-responsive element RARE (3), which is associated with the transcriptional activation of *RARβ2* by RA in a variety of cells (1).

RARβ2 mRNA expression is greatly reduced in a number of different types of human carcinomas including breast carcinoma (4–7). A growing literature has demonstrated that the anticancer effect of RA is primarily mediated by RARβ2, which is a potent tumor suppressor. Expression of *RARβ2* in *RARβ2*-negative cancer cells restored RA-induced GI and caused decreased tumorigenicity (8). Exogenous expression of *RARβ2* results both in RA-dependent and RA-independent apoptosis, and growth arrest even in breast cancer cell lines with scanty amounts of RARα, the first effector of *RARβ* P2 (4, 5, 9). Inhibition of *RARβ2* expression in *RARβ2*-positive cancer cells abolished RA effects (10). Moreover, *RARβ2* knockouts of F9 teratocarcinoma cells could not undergo growth arrest in the presence of RA, indicating that *RARβ2* is required for the growth inhibitory action of RA (11). Finally, expression of *RARβ2* antisense caused an increased frequency of carcinomas in transgenic mice (12). How RARβ2 exerts its anticancer activity is still largely unknown. Studies in breast cancer cell lines indicate two major RARβ2 antineoplastic mechanisms, namely RA-induced apoptosis and RA-independent antiactivator protein-1 activity (5, 9). Moreover, *RARβ2* may be involved in the enhancement of tumor immunogenicity (13). Thus far, induction of antitumoral effects in concomitance with endogenous *RARβ2* up-regulation in response to retinoids has been successfully achieved only in patients with oral premalignant lesions (14). In contrast, most epithelial tumors, including breast cancer, showed poor or no response to retinoid treatment (15, 16). In a clinical trial of RA in advanced breast carcinoma patients, *RARβ2* was induced only in one-fourth of *RARβ2*-negative breast tumors (16).

The potential causes for progressive decrease in *RARβ2* mRNA expression during breast carcinogenesis (6, 7) and lack of RA response may be both genetic and epigenetic. However, we and others (17–19) have found that lack of *RARβ2* is more often because of DNA methylation affecting the *RARβ* P2 promoter of one or more *RARβ* alleles. This made us hypothesize that silencing of *RARβ2* because of epigenetic changes in the *RARβ* P2 chromatin may hamper *RARβ* P2 inducibility by RA and be a cause of RA resistance (18). Here we show that this is indeed the case. We were able to analyze pathological specimens of primary breast tumors of a clinical trial of RA (16) and found that those tumors, which did not express *RARβ2* at the end of RA therapy, carry a methylated *RARβ* P2. Thus, lack of inducibility of *RARβ2* by RA seems to be because of an aberrant repressive chromatin status at *RARβ* P2.

Apparently, all of the machinery necessary for *RARβ2* reactivation in the presence of RA seems to be intact in breast cancer cells lacking endogenous *RARβ2* expression, because these cells can transcriptionally activate an exogenous *RARβ2* RARE (4). In the presence of RA, a normal *RARβ* P2 is activated first by RARα/RXR heterodimers and cofactors and subsequently by RARβ2/RXR heterodimers (20) via dynamic histone acetylation. We reasoned that provided that at least one genomic copy of *RARβ* is intact, and provided that sufficient cofactors and effectors (for instance RARα/RXR) are available in a cell, endogenous reactivation of *RARβ2* should be feasible by reversing the repressive constraints affecting the P2 promoter. Here we show that by inducing an appropriate level of *RARβ* P2 acetylation we could restore *RARβ2* transcription from both unmethylated and methylated *RARβ* P2 promoters in *RARβ2*-negative carcinoma cells of breast. Endogenous *RARβ2* reactivation resulted in significant GI both *in vitro* and *in vivo*. This study may have translational implications: (*a*) *RARβ* P2 methylation seems to be a "predictor" of RA response in breast cancer; and (*b*) reactivation of *RARβ2* may be a strategy to

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[4] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid responsive element; PB, phenyl butyrate; 5-Aza-CdR, 5-aza-2′ deoxycytidine; TSA, Trichostatin A; GI, growth inhibition; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSP, methylation-specific PCR; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; HAT, histone acetyltransferase; HDACI, histone deacetylase inhibitor.

restore RARβ2 anticancer effects in breast cancer as well as in other epithelial cancers where the *RARβ* P2 promoter is epigenetically silenced.

Materials and Methods

Cells and Drug Treatments

Cells. Breast and larynx cancer cell lines were maintained in DMEM with 5% FCS; lung and prostate cancer cell lines were maintained in RPMI 1640 with 5% FCS.

Drug Treatments. Cells seeded at different concentrations and in different vessels according to the objective of the analysis (see details in the different sections) were allowed to attach to the plastic substrate before being treated for periods ranging from 24 h to 6 days with different drug(s) and vehicles. All-*trans*-RA (Sigma, Milan, Italy) dissolved in 95% ethanol was used at final concentrations of 1 and 5 μM; 5-Aza-CdR (Sigma) dissolved in 0.45% NaCl containing 10 mM sodium phosphate (pH 6.8) was used at a final concentration of 0.8 μM; PB (Triple Crown America Inc., Peekasie, PA) dissolved in PBS was used at final concentrations of 2.5 and 5 mM; and TSA (Sigma) dissolved in ethanol was used at final concentrations ranging from 33 to 330 nM.

GI. GI was calculated using the trypan blue method according to standard protocols.

Clonogenicity. Five-hundred to 1000 cells/well were seeded in six-well plates, enabled to attach overnight to the plastic substrate before the addition of the appropriate concentrations of the desired drug(s) or vehicles (controls). The medium were replaced with drug-free medium for the desired time. As the colonies became visible (2–3 weeks), cells were fixed with methanol, stained with Giemsa (1:10 in distilled water), and counted.

Apoptotic Index. Apoptosis was evaluated by the *in situ* cell death and horseradish peroxidase detection kit (Roche, Milan, Italy) according to the manufacturer's recommendations. The apoptotic index was calculated as AC/TC, where AC is the number of apoptotic cells and TC the number of total cells counted under a light microscope.

Breast Tumor Samples. Formalin-fixed, paraffin-embedded sections from breast tumor from patients enrolled in a clinical trial Phase 1B (16) were provided by the Pathology Department, Istituto per lo Studio e la Cura dei Tumori, Genoa (Italy).

DNA and RNA Extraction. Extraction of DNA and RNA from breast cancer cell lines was performed with DNAzol and Trizol, respectively (Invitrogen, Carlsbad, CA). DNA from paraffinated breast cancer samples was extracted from three consecutive sections.

RT-PCR. Real-time RT-PCR was performed on cDNA obtained with Superscript first-strand synthesis kit (Invitrogen) using the ABI PRISM 7700 Sequence Detection System (TaqMan), and the following primers and probes (Applied Biosystems, Foster City, CA) *RARα* sense, 5′-TGTGGAGTTCGCCAAGCA-3′; *RARα* antisense 5′-CGTGTACCGCGTGCAGA-3′; and *RARα* oligoprobe, 5′-FAM-CTCCTCAAGGCTGCCTGCCTGGA-TAMRA-3′; *RARβ* sense 5′-CTTCCTGCATGCTCCAGGA-3′; *RARβ* antisense 5′-CGCTGACCCCATAGTGGTA-3′; *RARβ* oligoprobe 5′-FAM-CTTCCTCCCCCTCGAGTGTACAAACCCT-TAMRA-3′; *GAPDH* sense, 5′-GAAGGTGAAGGTCGGAGTC-3′; *GAPDH* antisense 5′-GAAGATGGTGATGGGATTTC-3′; and *GAPDH* oligoprobe, 5′-FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA-3′.

Quantitation was performed by the comparative threshold cycle C_t method. For semiquantitative RT-PCR, 50 ng of Dnase-treated total RNA was amplified with the Superscript One-Step RT-PCR System (Invitrogen). The β2 and β4 transcripts were identified simultaneously with sense primer 5′-AACGCGAGCGATCCGAGCAG-3′ and antisense primer 5′-ATTTGTCCT GGCAGACGAAGCA-3′; the β1 transcript with the sense 5′-TGACGTCAGCAGTGACTACTG-3′ and antisense: 5′-GTGGT TGAACTGCACATTCAGA-3′ primers; and the actin transcript with the sense 5′-ACCATGGATGATGATATCG-3′ and antisense 5′-ACATGGCTGGGGTGTTGAAG-3′primers.

MSP. Bisulfite modification of genomic DNA and MSP analysis using U3/M3 and U4/M4 *RARβ* P2 primers were as described (18).

ChIP Assay. ChIP analysis was performed with the ChIP kit (Upstate Biotechnology, New York, NY) according to the manufacturer's instructions with minor modifications and anti-acetyl-histone H3, anti acetyl-histone H4, and anti-phospho H3 antibodies (Upstate Biotechnology). Chromatin was immunoprecipitated from 2×10^6 cells treated with different drug (s) or control vehicles. For duplex PCR the primers included: the *RARβ* P2 sense primer 5′-GCCGAGAACGCGAGCGATCC-3′, the *RARβ* P2 antisense primer 5′-GGCCAATCCAGCCGGGGC-3′, the *GAPDH* sense primer 5′-ACAGTCCATGCCATCACTGCC-3′, and *GAPDH* antisense primer 5′-GCCTGCTTCACCACCTTCTTG- 3′.

Xenograft Mouse Models of Breast Cancer. Female athymic nude mice (Taconic Farms Inc., Germantown, MD) 6 weeks of age were injected with 1.5 mg/kg of body weight depo-estradiol (Florida Infusion Co, Palm Harbor, FL) 2 days before s.c. bilateral inoculation in the flank region with 5×10^6 breast carcinoma cells resuspended in serum-free medium (Invitrogen) and mixed with Matrigel (1:1; BD Biosciences, Bedford, MA) in a final volume of 0.2 ml. Mice for each cell line were randomly placed in groups (5 mice/group). Mice in the control group were treated with i.p. injections of vehicle (DMSO) six times a week. RA (2.5 mg/kg of body weight) and TSA (1 mg/kg of body weight) were administered by i.p. injections six times a week. Treatment was initiated when palpable tumors were established. Tumor volume was measured with a caliper twice a week and calculated according to the formula: A (length) × B (width) × C (height) × 0.5236. Mice were treated for 3–4 weeks, then euthanized. Tumors were harvested for molecular studies.

Statistical Analysis. Data from the trypan blue counts, clonogenicity assays, apoptotic index, and tumor size are presented as means ± SE. Differences between groups were analyzed using the Student's test for independent samples. The level of significance was set at $P < 0.05$.

Results

RA Cannot Induce *RARβ2* Reactivation in Human Primary Breast Tumors Carrying a Methylated *RARβ* P2. Here we provide evidence that primary breast tumors, which do not show *RARβ2* induction after RA-therapy, carry a methylated *RARβ* P2 promoter. By using MSP we analyzed the DNA of 13 breast tumors including 12 invasive ductal carcinoma and 1 lobular adenocarcinoma of patients enrolled in a clinical trial of RA therapy (16). These tumors were characterized previously for estrogen receptor, proliferation index (Ki67 reactivity), and *RARβ2* expression before and after RA therapy (16). Four *RARβ2*-positive tumors carried an unmethylated *RARβ* P2. Of the 9 tumors with very low or negative baseline *RARβ2* transcription, 3 carried an unmethylated P2 and 6 carried a methylated P2 (Fig. 1*A*). On RA treatment, the tumors carrying a methylated P2 did not show *RARβ2* reactivation (Fig. 1*A*). Representative *RARβ* P2 MSPs of an unmethylated tumor (Patient 28) and a methylated tumor (Patient 5) are reported in Fig. 1*B* along with the MSPs of two prototypic breast cancer cell lines, T47D and MCF7, carrying an unmethylated and a methylated *RARβ* P2, respectively. The presence of both unmethylated (U) and methylated (M) products likely reflects a mixture of normal and malignant cells in the tumor sample. These data strongly indicate that a methylated *RARβ* P2 is associated with lack of *RARβ2* inducibility by RA.

Endogenous *RARβ2* Reactivation by RA Is Possible Only When There Is Sufficient Histone Acetylation at *RARβ* P2. By using ChIP and anti-acetyl-H3 and- H4 antibodies we analyzed the *RARβ* P2 acetylation status of three prototypic breast carcinoma cell lines, the *RARβ2*-positive Hs578t line constitutively expressing also *β4* (the other transcript regulated by *RARβ* P2), and the *RARβ2*- negative T47D and MCF7 lines. We found that *RARβ* P2 chromatin was acetylated in the unmethylated, *RARβ2*-positive Hs578t cell line and in the *RARβ2*-negative T47D cell line but not in the *RARβ2*-negative MCF7 cell line. Results of ChIP with the anti-acetyl-H4 antibody is reported in Fig. 1*C*. When we treated the *RARβ2*-negative T47D and MCF7 cell lines with pharmacological doses of RA (1 μM) we observed an increase in acetylation of *RARβ2* transcription in the unmethylated T47D cells but not in the methylated MCF7 cells (Fig. 1*C*). Negative ChIP with anti-acetyl-H3 and -H4 antibodies in MCF7 cells was not because of rearrangements/deletions of the *RARβ* P2

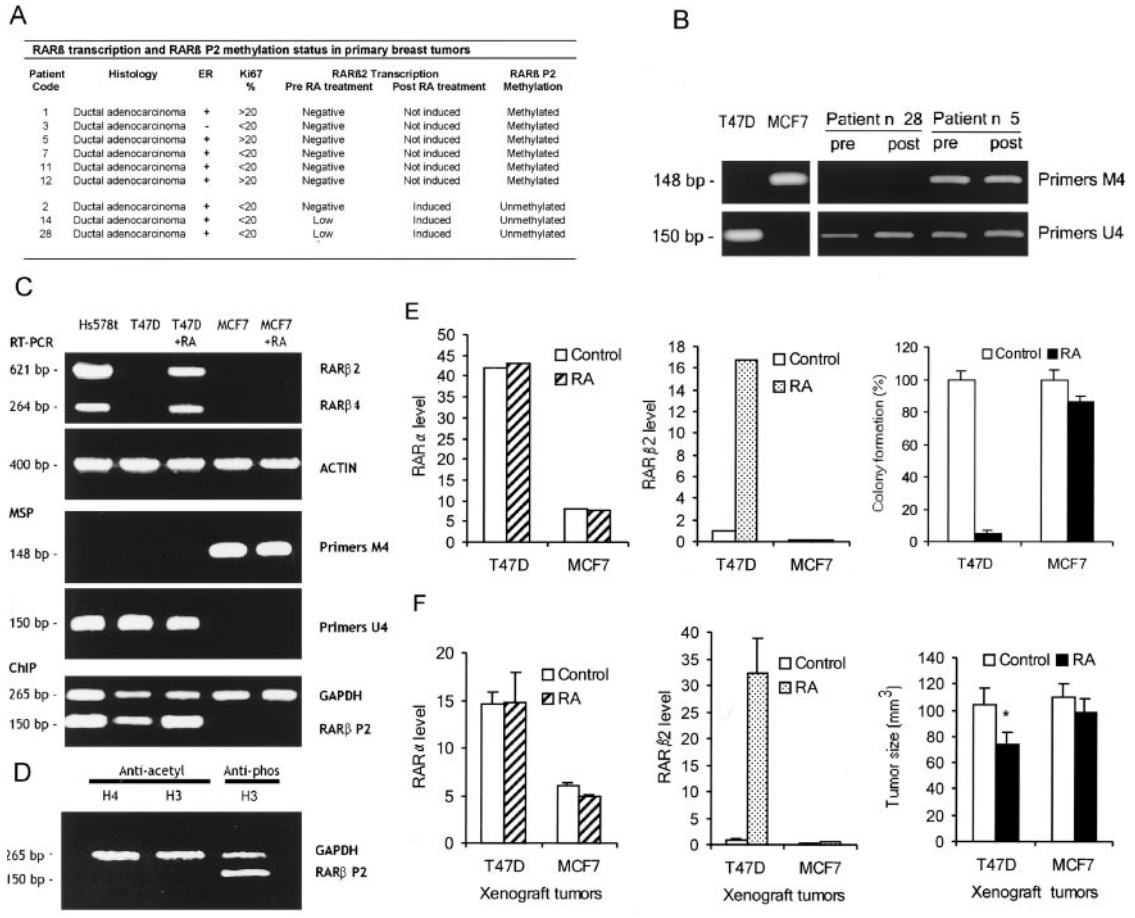

Fig. 1. *RARβ2* inducibility by RA treatment and *RARβ* P2 methylation/acetylation status in breast cells, and primary and xenograft breast tumors. *A*, RA treatment fails to reactivate *RARβ2* in primary breast tumors with a methylated *RARβ* P2 promoter; *B*, MSP analysis of a RA-inducible tumor where *RARβ2* was observed after 3 weeks of RA therapy shows the presence of an unmethylated promoter (Patient 28). In contrast, a tumor where RA therapy failed to reactivate *RARβ2* carries a methylated P2 (Patient 5); *C*, *RARβ2* transcription (*RT-PCR*) and *RARβ* P2 acetylation (*ChIP analysis*) of *RARβ2*-positive (*Hs578t*) and -negative (*T47D, MCF7*) cell lines. RA treatment enhanced *RARβ* P2 reacetylation and induced *RARβ2* in unmethylated T47D cells but not in methylated MCF7 cells; *D*, control ChIP with antiphosphorylated H3 shows the integrity of MCF7 *RARβ* P2; *E* and *F*, *RARβ2* (but not *RARα*) reactivation by RA treatment in T47D cells and xenograft tumors but not in MCF7 cells and xenograft tumors. Endogenous *RARβ2* reactivation is associated with significant loss of clonogenicity and tumor GI; *bars*, ±SD.

region because ChIP with the antiphospho-H3 antibody gave a positive signal (Fig. 1*D*).

Thus, *RARβ2* transcription seems possible only when there is an adequate level of histone acetylation of *RARβ* P2. Treatment with pharmacological concentrations of RA alone can increase acetylation in a hypoacetylated *RARβ* P2 (T47D), but not in a deacetylated *RARβ* P2.

Endogenous *RARβ2* Reactivation from an Unmethylated *RARβ* P2 Is Associated with Significant GI both *in Vitro* and *in Vivo*. Reacetylation at *RARβ* P2 and endogenous *RARβ2* reactivation were found associated with biological effects *in vitro* and *in vivo* (Fig. 1, *E* and *F*). *RARβ2* but not *RARα* expression (evaluated by real-time RT-PCR) after RA treatment in both T47D cells and xenograft tumors (Fig. 1, *E* and *F*) correlated with complete loss of clonogenicity (Fig. 1*E*) and significant GI in xenograft tumors (∗, $P < 0.05$; Fig. 1*F*). Identical RA treatment did not induce *RARβ2* in MCF7 cells and xenograft tumors where the observed GI can be interpreted as because of *RARβ2*-independent effects.

Reacetylation of H3 and H4 Histones at *RARβ* P2 Restores *RARβ2* Transcription from a Methylated *RARβ* P2. Next, we tried to reactivate *RARβ2* from a methylated *RARβ* P2 by modulating the promoter acetylation status in two cell lines carrying a methylated *RARβ* P2, MCF7 and MDA-MB-231 (18). We induced chromatin reacetylation at *RARβ* P2 by using two reacetylating agents, PB, a short fatty acid, and TSA, a hydroxamic acid-based hybrid polar compound (21), as well as a DNA-demethylating agent, 5-Aza-CDR. Promoter reacetylation and transcriptional activation induced by 5-Aza-CDR treatment (0.8 μM for 96 h; Fig. 2*B*) occurred in concomitance to *RARβ* P2 demethylation (Fig. 2*B*). In contrast, promoter reacetylation (Fig. 2*A*) and transcriptional activation induced either with PB (2.5 mM for 72 h) or TSA (33–330 nM for 24–48 h) in combination with RA (1 μM; Fig. 2*B*) occurred from a *RARβ* P2 methylated promoter. In Fig. 2*B* (right and middle panels) we show the results of an experiment of RAR*β2* reactivation using 330 nM TSA and 1 μM RA. Thus, *RARβ* P2 reacetylation is necessary and sufficient to restore the promoter susceptibility to RA action even in the presence of persisting methylation. Interestingly, *RARβ2* reactivation was possible also in breast cancer cells (MDA-MB-231) with very low endogenous *RARα*.

TSA and RA Needs To Be Administered Simultaneously to Obtain *RARβ2* Reactivation from a Methylated *RARβ* P2. TSA is known to induce transient chromatin acetylation of ~2% of genes in a human cell (21, 22). We compared the occurrence of *RARβ P2* reactivation in MCF7 cells either treated for 24 h with TSA (330 nM) followed by 24 h with 1 μM RA or treated for 24 h with TSA (330 nM) in combination with 1 μM RA. We observed that both histone H3 and histone H4 acetylation faded on removal of TSA (Fig. 3*A*) likely because of the ability of DNA-methylated sites to reattract HDAC

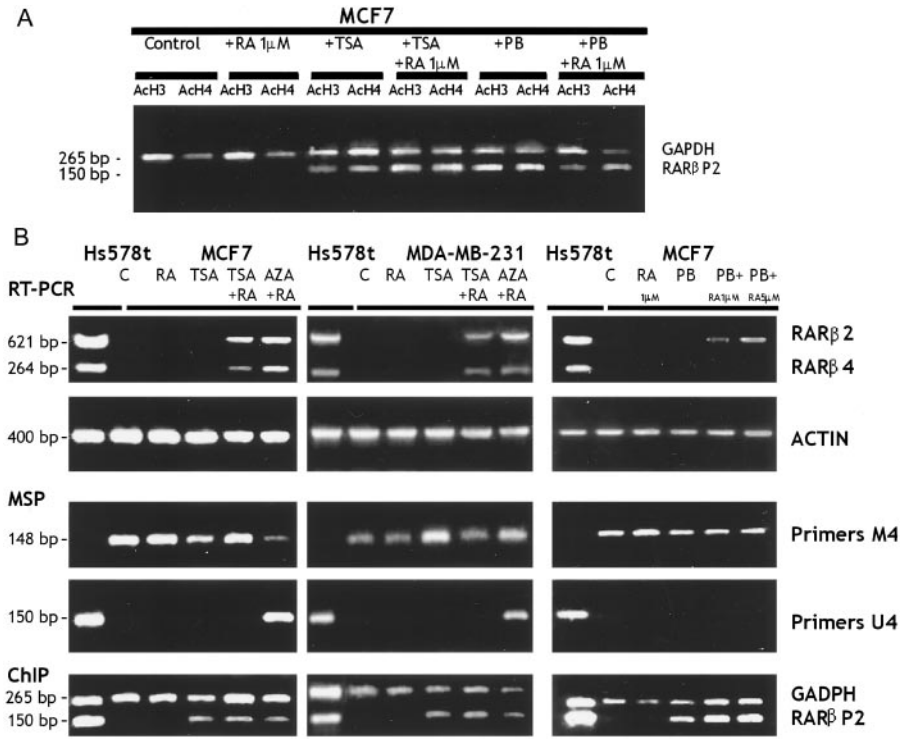

Fig. 2. Reacetylation of methylated *RARβ* P2 is sufficient and necessary for *RARβ2* reactivation by RA. *A*, reacetylation of a methylated *RARβ* P2 (*MCF7 cells*) is induced at both H3 and H4 histones with two HDACIs, PB and TSA. *B*, promoter reacetylation (*ChIP*) and *RARβ2* reactivation (evaluated by *RT-PCR*) occurs, in concomitance with *RARβ* P2 demethylation (evaluated by *MSP*) with 5-Aza-CdR treatment and without demethylation (*MSP*) with combined TSA/RA treatment in both MCF7 (*left*) and MDA-MB-231 (*middle*) cells; PB needs to be used at a much higher concentration than TSA to induce *RARβ2* reactivation in MCF7 (*right*).

complexes. The best strategy for *RARβ2* reactivation was to use both RA and TSA simultaneously (Fig. 3*A*). Apparently, *RARβ* P2 chromatin needs to be maintained "sufficiently relaxed" to enable RA-induced *RARβ* P2 transactivation from a methylated promoter.

Combined TSA and RA Specifically Target Transcription from *RARβ* P2 but not the Adjacent *RARβ* P1 Promoter. One of the major criticisms of the potential harmful effects of chromatin remodeling drugs (demethylating and reacetylating agents) concerns their nonspecific modulation/reactivation of many gene promoters in a cell, particularly the developmentally inactivated promoters. For this reason, we liked to compare the effects of TSA ± RA and 5-Aza-CDR ± RA on the reactivation of *RARβ* P1, the promoter adjacent to *RARβ* P2, which is a developmentally inactivated promoter (2). P1, differently from P2, does not contain a RARE. Treatment with 5-Aza-CdR (0.8 μM) ± RA (1 μM) for 96 h but not TSA (330 nM) ± RA (1 μM) for 48 h induced transcription from *RARβ* P1 in MDA-MB-231 cells (Fig. 3*B*). The NCI H69 *β1* served as a positive control for *β1* transcript expression/size. Apparently TSA cannot restore the activity of P1, whereas 5-Aza-CDR can reactivate both promoters. Thus, by extrapolating from the effects on P1 and P2, it is possible that a TSA-based treatment is less likely to randomly reactivate developmentally inactivated promoters (like P1) than recently inactivated promoters (like P2).

***In Vitro* and *in Vivo* Biological Effects Associated with *RARβ2* Reactivation from a Methylated *RARβ* P2.** Different concentrations of TSA (33–330 nM) combined with RA (1 μM) for 48 h result in *RARβ2* reactivation and significant GI in MCF7 cells (Fig. 3*C*). RA treatment alone was ineffective, whereas treatments with different concentrations of TSA alone (33–330 nM) result, *per se*, in consistent GI. Nevertheless, RA (1 μM) significantly ($P < 0.05$) potentiated the TSA growth inhibitory action (Fig. 3*C*). A combined RA and TSA treatment significantly affected also the proapoptotic action of RA or TSA alone (Fig. 3*D*). Thus, nM concentrations of TSA can modulate the response to pharmacological levels of RA in cells with a methylated *RARβ* P2 inducing profound antiproliferative and apoptotic effects.

Next, we attempted *RARβ2* reactivation in MCF7 xenograft tumors. Preliminarily, we observed that TSA was not toxic in female nude mice when administered six times a week for 4 weeks at concentrations ranging from 0.5–5 mg/kg of body weight (data not shown). These data confirmed that TSA is a drug with lack of toxicity *in vivo* (23). Then, we treated groups of five 6–8 week-old female nude mice bearing MCF7 xenograft tumors with i.p. injections of the lowest concentrations of TSA (0.5 and 1 mg/kg body weight) and RA (2.5 mg/kg body weight) alone or in combination six times/week for 4 weeks. Tumor growth and general animal conditions (body weight/behavior) were measured and monitored for the entire duration of treatment. At the end of week 4, animals were sacrificed. Tumors of mice receiving 1 mg/kg of TSA in combination with RA (2.5 mg/kg of body weight) showed consistent *RARβ2* reactivation evaluated by RT-PCR (Fig. 3*F*). TSA treatment, which alone also induced GI, significantly modulated the response of RA (Fig. 3*E*).

***RARβ2* Reactivation Can Be Induced by Combined TSA and RA Treatment in a Variety of Epithelial Carcinoma Cells.** We analyzed the correlation between methylation and acetylation status at *RARβ* P2 in additional breast cancer cell lines as well as carcinoma cell lines of other tissues (prostate and larynx). Partial/complete P2 methylation (evaluated by MSP analysis before and after 5-Aza-CDR treatment) was always associated with a *RARβ* P2 deacetylated status (evaluated by ChIP with anti-acetyl-H3 and -H4 antibodies). The presence of an epigenetically modified *RARβ* P2 always correlated with transcriptional silencing (Fig. 4*A*). TSA (33–330 nM) and RA (1 μM) treatments always resulted in reactivation of endogenous *RARβ2* from an epigenetically silenced *RARβ* P2 (Fig. 4*A*).

Discussion

There is mounting evidence in epithelial cancer cell lines and animal models (5, 8–13) of the potent anticancer effects of the tumor suppressor *RARβ2*. It has also been demonstrated that effective restoration of endogenous *RARβ2* can be a powerful strategy to treat premalignant oral lesions (14). Here we report that endogenous

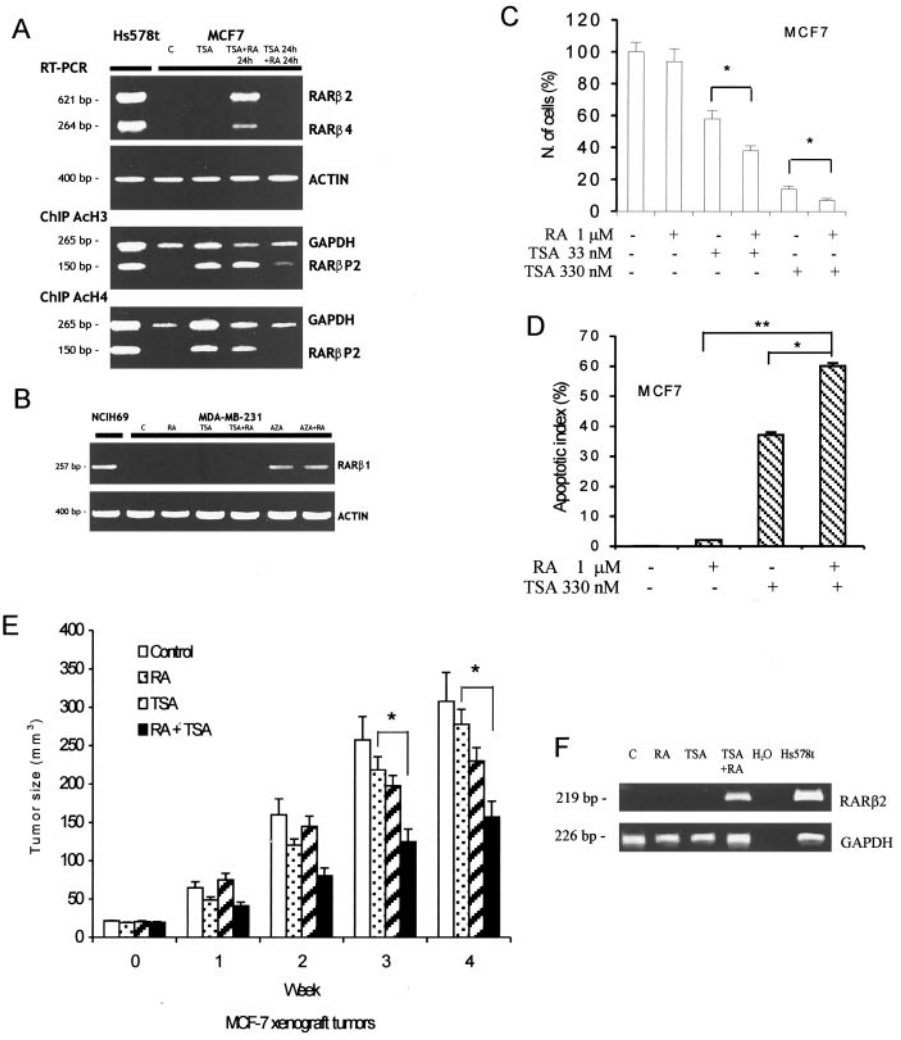

Fig. 3. Effects of combined RA and TSA treatment on *RARβ2* reactivation from a methylated *RARβ* P2 *in vitro* and *in vivo*. *A*, TSA and RA must be administered simultaneously to reactivate *RARβ2* from a methylated *RARβ* P2; *B*, TSA, differently from 5-Aza-CDR, does not reactivate the developmentally inactivated *RARβ* P1 promoter, 5′to *RARβ* P2; *C* and *D*, combined TSA (33–330 nM) and RA (1 μM) treatments can reactivate *RARβ2* and significantly affect both GI and apoptotic index of MCF7 cells; *E*, significant tumor GI was observed, in concomitance with endogenous *RARβ2* reactivation as evaluated by RT-PCR (*F*), in MCF7 xenograft tumors after 4 weeks of combined TSA (1 mg/kg body weight) and RA (2.5 mg/kg body weight) treatment; *bars*, ±SD.

RARβ2 expression can be reactivated in breast cancer cells and xenograft tumors, and correlates with GI *in vivo* and *in vitro*. We show that *RARβ2* reactivation can be tailored to a specific breast cancer by using either pharmacological concentrations of RA alone or in combination with chromatin remodeling drugs based on the knowledge of the epigenetic status of the *RARβ* P2 promoter, which contains the RARE.

We observed that failure of *RARβ2*- negative breast tumors to respond to RA therapy does correlate with the methylation status of the *RARβ* P2 promoter (Fig. 1, A *and B*). Specifically, breast tumors, which failed to re-express *RARβ 2* after RA therapy, carried a methylated *RARβ* P2 promoter, whereas breast tumors carrying an unmethylated *RARβ* P2 re-expressed *RARβ2* after 3 weeks of RA treatment (18). These data paralleled what we observed in xenograft tumors of T47D and MCF7 cells, carrying an unmethylated and methylated *RARβ* P2, respectively (Fig. 1*F*). These data clearly indicated that methylation at *RARβ* P2 is a major hurdle for successful RA therapy.

It is known that DNA methylation can induce repressive chromatin remodeling by causing massive histone deacetylation at the methylated sites (24–27). By using prototypic *RARβ2*-negative breast cancer cell lines carrying either an unmethylated *RARβ* P2 (T47D) or a methylated *RARβ* P2 (MCF7 and MDA-MB-231) we observed that RA treatment alone (1 μg/ml) induced *RARβ2* reactivation, concomitant with an increase of promoter histone acetylation, only in cells carrying an unmethylated *RARβ* P2 (Fig. 1*C*). In contrast, we did not obtain *RARβ2* reactivation by the same RA treatment in cells carrying a methylated/deacetylated *RARβ* P2. These results corroborated our hypothesis (18) that differential RA resistance in cancer cells may be because of differential levels of repression at *RARβ* P2. Repression consequent to differential levels of HDAC accumulation at the promoter is perhaps due to an altered RA metabolism and/or decreased levels of RARα, or other cofactors, essential for *RARβ* P2 activity. It is possible that an inactive, hypoacetylated promoter (in our case *RARβ* P2) may be capable to attract additional epigenetic changes like DNA methylation leading to additional deacetylation, ultimately resulting into gene silencing (24). Both defects of RA metabolism and low levels of RARα have indeed been detected in breast carcinoma cells (28–31). In particular, MCF7 line carries at least two defects, which can lead to low intracellular concentrations of RA, namely altered expression of lecithin:retinol acyl transferase and aldehyde dehydrogenase 6, whereas MDA-MB-231 line presents a very low level of endogenous *RARα*.

To reverse deacetylation of *RARβ* P2 and test whether we could obtain endogenous *RARβ2* reactivation in MCF7 and MDA-MB-231 cells with a methylated/deacetylated promoter we used different chromatin remodeling drugs including 5-Aza-CDR, PB, and TSA. All of the three drugs were capable of inducing reacetylation at P2 (Fig. 2, *A* and *B*). Reacetylation was obtained in concomitance with demethylation with 5-Aza-CDR and in the presence of methylation with either TSA or PB (Fig. 2, *A* and *B*). TSA, expected to reactivate ~2%

A

Epigenetic status of *RARβ* P2 and *RARβ* transcription in epithelial cancer cell lines

Cell line	Tissue	*RARβ* P2 Methylation	*RARβ* P2 Acetylation	*RARβ* transcription -RA	+RA	+TSA	+TSA +RA
Hs578t	Breast	Unmethylated	Acetylated	Positive	nd	nd	nd
HCC 2185	Breast	Unmethylated	Acetylated	Positive	nd	nd	nd
T47D	Breast	Unmethylated	Hypo Acetylat	Negative	Induced	nd	nd
DU 145	Prostate	Partially Meth.	Deacetylated	Negative	Not Induced	Not Induced	Induced
PC 3	Prostate	Partially Meth.	Deacetylated	Negative	Not Induced	Not Induced	Induced
HCC 712	Breast	Methylated	Deacetylated	Negative	Not Induced	Not Induced	Induced
MCF7	Breast	Methylated	Deacetylated	Negative	Not Induced	Not Induced	Induced
LNCaP	Prostate	Methylated	Deacetylated	Negative	Not Induced	Not Induced	Induced
Hep2	Larynx	Methylated	Deacetylated	Negative	Not Induced	Not Induced	Induced

B

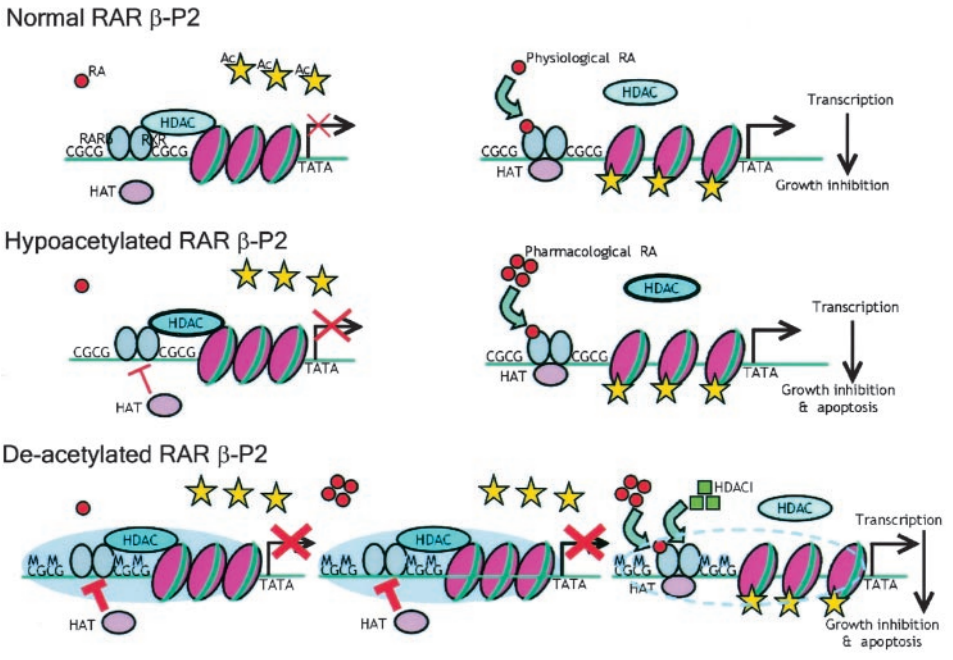

Fig. 4. Reactivation of *RARβ2* in different epithelial cancer cells where *RARβ2* is epigenetically silenced. *A*, reacetylation of *RARβ* P2 and *RARβ2* reactivation was induced by TSA plus RA in epithelial carcinoma cell lines from different tissues showing partial or complete *RARβ* P2 methylation; *B*, a model by which progressive deacetylation at *RARβ* P2 likely occurs during epithelial carcinogenesis. Both mild and severe deacetylation at *RARβ* P2 in *RARβ2*-negative epithelial cancer cells can be reversed pharmacologically by RA alone (*middle panel*) or a combination of HDACIs and RA (*bottom panel*), respectively.

of inactive genes in a tumor cell (21, 22, 32) is, in our opinion, the most desirable of the three drugs to modulate *RARβ2* reactivation and RA response from a methylated *RARβ* P2. To be effective TSA needs to be administered in concomitance with RA, probably to maintain the chromatin status sufficiently transparent to enable RAR/RXR access (Fig. 3*A*). Apparently, TSA can modulate reacetylation of *RARβ* P2 and RA response at far lower concentration (33 nM) than PB (2.5 mM). TSA alone or in combination with RA differently from 5-Aza-CDR is ineffective at reactivating P1, the developmentally inactivated promoter adjacent to P2 in the *RARβ* gene (Fig. 3*B*). This finding suggests that TSA may spare to reactivate developmentally inactivated promoters, and, therefore, is likely to produce fewer harmful effects than 5-Aza-CDR when used *in vivo*.

According to a recent report and our experience TSA is nontoxic and nonteratogenic in mice (23), and for this reason may have potential clinical value. We were successful in obtaining *RARβ2* reactivation in xenograft tumors of MCF7 cells containing a methylated *RARβ* P2 by treating tumor-bearing mice with combined TSA (1 mg/kg body weight) and RA (2.5 mg/kg body weight) for 4 weeks. *In vivo RARβ2* reactivation by RA+TSA (Fig. 3*F*) was associated with consistent tumor GI (Fig. 3*E*). Even if the combined TSA and RA treatment seems to be optimal in achieving *RARβ2* reactivation both *in vitro* and *in vivo*, in some cell lines and xenograft tumors, occasionally, we observed *RARβ2* reactivation using TSA alone. This might be because of re-expression of *RARβ2* from a minimal basal promoter, independent of the RA-responsive element as already reported (33).

We also tested whether endogenous reactivation was possible in other *RARβ2*-negative epithelial cancers cell lines. *RARβ2* inducibility was observed in additional breast cancer cell lines (HCC 2185 and HCC 712) as well as three prostate cell lines (PC-3, DU 145, and LNCaP) and one larynx carcinoma cell line (Hep2; Fig. 4*A*). In all of the lines tested thus far, we observed that endogenous reactivation of *RARβ2* by TSA (33–330 nM) and RA (1 μg/ml) correlated with significant *in vitro* GI and apoptosis.

Our overall data suggest a general model where *RARβ* P2, normally regulated by a dynamic HDAC/HAT balance in the presence of physiological levels of RA, (Fig. 4*B, top panel*) undergoes increased HDAC accumulation during epithelial cell tumorigenesis (Fig. 4*B*). Both mild hypoacetylation at *RARβ* P2 (like the one observed in T47D cells) and severe deacetylation at *RARβ* P2 (like the one detected in all of the other epithelial cell lines) can be reversed but require different pharmacological treatments. RA treatment alone (Fig. 4*B, middle panel*) can reactivate transcription from a mildly hypoacetylated *RARβ* P2, whereas treatment with an HDACI, like TSA, is required to make the promoter susceptible to RA action (Fig. 4*B, bottom panel*).

Other novel HDACIs (21, 32) need to be tested to see whether we can additionally improve the efficiency of reacetylation of methylated *RARβ* P2 and, consequently, the susceptibility to RA response. However, we anticipate that also other HDACIs will affect the acetylation of multiple promoters and proteins like TSA does. Thus, there is a need to engineer different, extremely specific, chromatin remodeling reagents to obtain specific promoter targeting, leaving unaffected the chromatin of all other genes.

At the present time our study provides useful information for potential translational applications for breast cancer and other epithelial cancers. A methylated *RARβ* P2 can be used as a "predictor marker" of RA responsiveness. *RARβ* P2 methylation can be detected at an early stage of breast carcinogenesis, and on minimum quantities of breast ductal lavage cells (34), making it possible to identify breast cancer patients with tumors that may benefit from endogenous *RARβ2* reactivation therapy.

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