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Advances in Brief

Loss of *Retinoic Acid Receptor β* Gene Expression Is Linked to Aberrant Histone H3 Acetylation in Lung Cancer Cell Lines[1]

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Abstract

Most lung cancer cell lines do not express retinoic acid receptor (RAR)-β in response to all-*trans* retinoic acid (RA) because of a defect in *RARβ* gene transcription (RA-refractory cells). Here we investigated mechanisms of RA refractoriness in 14 lung cancer cell lines. Eleven cell lines were found to be RA refractory, and in the other three cell lines, *RARβ* levels increased with RA treatment (RA-responsive cells). We observed *RARβ* promoter methylation in 7 of 11 RA-refractory cell lines (64%) and in 0 of the 3 RA-responsive cell lines. Treatment with 5-aza-2′-deoxycytidine restored RA response in two of the seven cell lines with *RARβ* promoter methylation (29%). RA treatment increased acetylation of histones H3 and H4 on chromatin of the *RARβ* promoter in RA-responsive cells. Only histone H4 acetylation increased in RA-refractory cells, including refractory cells with and without evidence of promoter methylation. Thus, loss of histone H3 acetylation consistently correlated with RA refractoriness in lung cancer cell lines. RA refractoriness and aberrant histone acetylation were attributable to *RARβ* promoter methylation in some cell lines but not in others, suggesting that multiple mechanisms contribute to this transcriptional defect in lung cancer cells.

Introduction

All-*trans*-RA[4] is a ligand for the RA nuclear receptor family (RARα, RARβ, and RARγ). RARs function as heterodimers with RXRs and regulate the transcription of genes with RAREs in their promoters, such as *RARβ* (1). Nuclear receptors regulate gene transcription through association with coregulators that maintain local chromatin structure in either a repressive or permissive state. In the absence of ligand, RXR-RAR complexes actively repress transcription by association with receptor corepressors, which recruit histone deacetylases to create a multimeric repressor complex (2). Upon binding to ligand, the receptors dissociate from the corepressors and bind to receptor coactivators, which have intrinsic histone acetyltransferase activity and facilitate the recruitment of RNA polymerase II and the basal transcription machinery (3). In most lung cancer cell lines and tumor biopsy specimens, *RARβ* mRNA is not detectable, and RA treatment does not increase *RARβ* gene transcription (4–7). Recently, methylated CpG islands were identified adjacent to the RARE in the *RARβ* promoter (8). DNA methylation is a common epigenetic mechanism of tumor suppressor gene silencing in cancer (9). The biological consequences of DNA methylation are mediated by a family of methyl-CpG binding domain proteins that interact with a histone deacetylase-containing core complex via recruitment of the Sin3 corepressor (10). Here we investigated mechanisms of RARβ loss in lung cancer cells. We found that loss of histone H3 acetylation on chromatin of the *RARβ* promoter consistently correlated with RA refractoriness in lung cancer cell lines. DNA methylation contributed to aberrant histone acetylation in some cell lines but not in others, suggesting that multiple mechanisms contribute to this transcriptional defect in lung cancer cells.

Materials and Methods

Cell Culture. We tested the following 14 lung cancer cell lines: Calu-1, H226B, H226Br, SK-MES-1, H322, H358, H441, H460, H1299, Calu-6, A549, H596, H661, and H1607 (11). Lung cancer cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% FCS and antibiotics at 37°C in an atmosphere containing 5% CO_2.

Northern Blot Analysis. Lung cancer cells were seeded onto 10-cm plates (10^6 cells) and treated for 12 h with 10^{-6} M RA or medium alone, and total RNA was extracted. Twenty μg of RNA were subjected to electrophoresis on a 1% agarose gel containing 2% formaldehyde. After gels were soaked in 50 mM NaOH/1× SSC for 20 min and then soaked in 10× SSC for 20 min, RNA was transferred onto a Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA) overnight by the capillary transfer method. Membranes were cross-linked and then incubated in the hybridization solution containing [γ-^{32}P]dCTP-labeled probe. The probe was labeled using a Prime-It II Random Primer Kit (Stratagene, La Jolla, CA) and purified by MicroSpin S-300 HR Columns (Amersham Phamacia Biotech Inc., Piscataway, NJ). After overnight incubation, membranes were washed under high-stringency condition and exposed to enhanced chemiluminescence films for autoradiography.

MSP Assay. The MSP assay was performed as described by Virmani *et al.* (8), which was a modification of previously described methods (12), in which a 146-bp, CpG-rich genomic region (position 950-1076) adjacent to the two RAREs (positions 792–797 and 803–808) was amplified. Briefly, genomic DNA was digested with 200 μg/ml proteinase K (Life Technologies, Inc.) and purified by phenol extraction. Then, 1 μg of DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C, 30 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite (pH 5.0; both from Sigma Chemical Co., St. Louis, MO) were added, and the solution was incubated at 50°C for 16 h. The treated DNA was then purified with a Wizard DNA Purification System (Promega Corp., Madison, WI). The bisulfite-modified DNA was amplified with primers specific for either methylated or unmethylated *RARβ* promoter P2 as described elsewhere (8). The PCR products were analyzed on 2% agarose gels.

RT-PCR Analysis. Lung cancer cells found to have methylation of the *RARβ* promoter were seeded at a density of 10^6 cells/10-cm plate and incubated for 6 days in medium with 1 μM RA, 10 μM 5-Aza-CdR, or both (Sigma Chemical Co.), with medium changes on days 1, 3, and 5. Under these

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[4] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; MSP, methylation-specific PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; 5-Aza-CdR, 5-aza-2′-deoxycytidine; RTK, receptor tyrosine kinase; RT-PCR, reverse transcription-PCR.

Table 1 *MSP assay for RARβ gene*

Cell line	RARβ mRNA	RARβ methylation
Squamous cell carcinoma		
Calu-1	−	Neg
H226B	−	Neg
H226Br	−	Pos
SK-MES-1	−	Pos
Adenocarcinoma		
H322	−	Neg
H358	−	Pos
H441	−	Pos
Large cell carcinoma		
H460	−	Pos
H661	+	Neg
H1299	+	Neg
Anaplastic carcinoma		
Calu-6	+	Neg
Bronchioloalveolar cell carcinoma		
A549	−	Neg
Adenosquamous carcinoma		
H596	−	Pos
Small cell carcinoma		
H1607	−	Pos

MSP analysis was performed on lung cancer cell lines with different histopathological features. Cell lines were characterized as sensitive (+) or resistant (−) to the effects of RA on *RARβ* mRNA levels. MSP was performed using methylated- and unmethylated-specific primers, to identify cell lines with (Pos) and without (Neg) *RARβ* promoter methylation. The experiment was performed twice and produced the same results each time.

conditions, at the completion of treatment the cells had undergone at least 4 population doublings and were subconfluent (70–80%), permitting incorporation of 5-Aza-CdR throughout treatment. The cells were harvested at the end of the 6th day for extraction of total cellular RNA with the High Pure RNA Isolation kit, as described above. Reverse transcription was performed with *RARβ2*- and *GAPDH*-specific primers. Five μl of reverse transcription reaction mixture was used as a template for PCR with nested primers for *RARβ*. *GAPDH* primers were used to monitor the integrity of isolated RNA. The primers used for RT-PCR were as follows: 5′-TTATTGCACGAGTGGTGACTGACTGA-3′ (antisense *RARβ*); 5′-CAGACATTCAGTGCAAGGGAGATC-3′ (sense *RARβ*) and 5′-AATTTGGTCTGCGATGGTCCAGCC-3′ (antisense *RARβ*) for nested PCR; and 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (sense *GAPDH*) and 5′-AGCCTTCTCCA TGGTGGTGGTGAAGAC-3′ (antisense *GAPDH*). The amplified DNAs were separated on a 2% agarose gel and analyzed by Southern blotting with human *RARβ* cDNA used as a probe.

ChIP Analysis. ChIP analysis was performed as described elsewhere (13). Briefly, lung cancer cells were treated for 12 h with 1 μM RA or medium alone. The cells were then cross-linked (histones to DNA) by adding formaldehyde directly to the culture medium (1% final concentration) for 10 min at 37°C. Cells were harvested and lysed with lysis buffer [25 mM Tris (pH 8.1),10 mM EDTA, and 1% SDS] containing protease inhibitors. Immunoprecipitation was performed on extracts from equal numbers of cells with anti-acetyl-histone H3 or anti-acetyl-histone H4 antibodies (Upstate Biotechnology Inc., Lake Placid, NY) overnight at 4°C, and immune complexes were isolated with salmon sperm DNA/protein A-agarose slurry (Upstate Biotechnology Inc.) as described previously (13). Protein-bound immunoprecipitated DNA was washed with LiCl wash buffer and Tris-EDTA, and immune complexes were eluted by adding elution buffer (1% SDS and 0.1 N $NaHCO_3$). The cross-links were reversed by treatment with 20 mM NaCl-1% SDS, and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR was performed to amplify the *RARβ* promoter with primers (antisense, 5′-AAACCCTGCTCGGATCGCTC-3′; sense, 5′-TCCTGGGAGTTGGTGATGTCAG-3′) that amplify *RARβ* promoter sequences from positions 732 to 979. PCR conditions were as follows: 94°C for 5 min to denature, 58°C for 1 min to anneal, and 72°C for 1 min to elongate, for 30 cycles. An aliquot of the whole cell protein-DNA complex (2% of immunoprecipitated volume) was subjected to PCR analysis as a control for input. The PCR products were analyzed on 2% agarose gels and subjected to Southern blotting with a 1.6-kb *Pst*I-*Bam*HI human *RARβ* genomic fragment including 1.47 kb of promoter sequences 5′ of the TATA box.

Western Blot Analysis. Exponentially growing (60% confluent) lung cancer cell lines were lysed in lysis buffer as described elsewhere (14), and 50 μg of sample were used for Western blot analysis. Polyclonal antibodies against human TIF2, SRC1, RAC3, SMRT, and NcoR were generated and used in Western blotting as described previously (15). Dr. Sharon Roth (The University of Texas M. D. Anderson Cancer Center) generously provided polyclonal antibody against human GCN5, and we purchased antibody to CBP (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Sigma Chemical Co.).

Results

RARβ Promoter Methylation. We performed MSP analysis to examine the methylation status of the *RARβ* gene promoter in a panel of 14 lung cancer cell lines. In two independent experiments, we found *RARβ* promoter methylation in 7 of the 14 lung cancer cell lines tested, which included cell lines of multiple histologies (Table 1). We also examined *RARβ* expression in response to RA treatment. Most cell lines

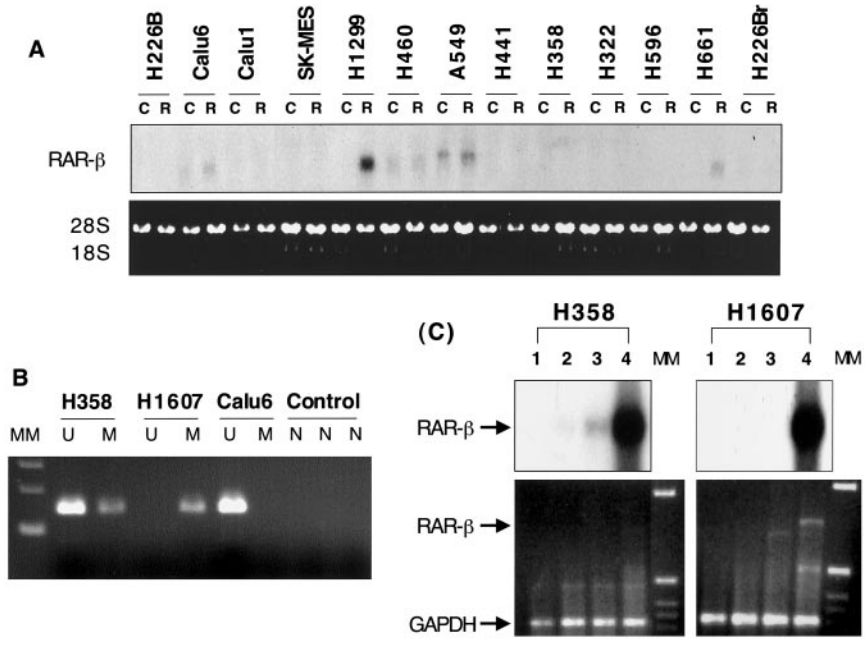

Fig. 1. *A*, *RARβ* expression was examined by Northern blot analysis (20 μg total RNA/lane) of the indicated lung cancer cells treated with media alone (*C*) or 1 μM RA (*R*). A photograph of the ethidium bromide-stained gel illustrates relative amounts of RNA loaded/lane, with the indicated positions of 28S and 18S RNase bands. *B*, *RARβ* promoter methylation in the RA-refractory cell lines H358 and H1607. The RA-responsive cell line Calu-6 was included as a negative control. Genomic DNA was subjected to MSP analysis, using unmethylated-specific (*U*) and methylated-specific (*M*) primers to the *RARβ* gene as described in "Materials and Methods." Control PCR reactions were performed using no template DNA (*N*) with unmethylated-specific, methylated-specific, and no primers, respectively. *MM*, molecular weight marker. *C*, RT-PCR analysis of *RARβ* and *GAPDH* expression was performed on RNA extracted from H358 and H1607 cells treated for 6 days with media alone (*Lane 1*), RA alone (*Lane 2*), 5-Aza-CdR alone (*Lane 3*), and RA plus 5-Aza-CdR (*Lane 4*). *RARβ*- and *GAPDH*-specific PCR products were electrophoresed on an agarose gel, stained with ethidium bromide (illustrated in the *bottom panels*), transferred to a nylon membrane, and hybridized to a *RARβ* cDNA (illustrated in the *top panels*). Molecular weight markers (*MM*) are indicated.

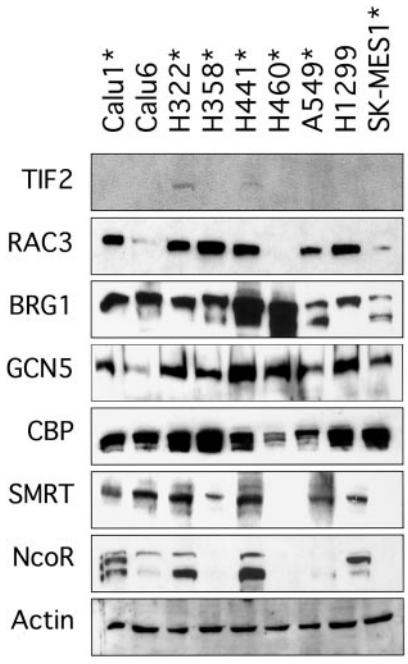

Fig. 2. Western analysis of coactivators and corepressors. Fifty μg of cell lysates were prepared from the indicated lung cancer cell lines, loaded onto a 7.5% SDS-PAGE gel, and immunoblotted to antibodies against the indicated proteins, including actin as a control for relative amounts of sample loaded/lane. RA-refractory cells are indicated by *asterisks*.

expressed no detectable *RARβ* in the basal state. RA treatment increased *RARβ* expression in Calu-6, H661, and H1299 cells (RA-responsive cells), or 23% of the lung cancer cell lines examined (Fig. 1*A*). In the other 11 cell lines examined, *RARβ* levels did not increase with RA treatment (RA-refractory cells; Fig. 1, *A* and *C*). In most RA-refractory cells, *RARβ* mRNA was not detectable by Northern blotting, but in A549 and H460 cells, *RARβ* was expressed basally and did not change with RA treatment. Of the RA-refractory cells, 64% (7 of 11 cell lines) had *RARβ* promoter methylation. Thus, we observed RA refractoriness in cell lines with and without *RARβ* promoter methylation.

We examined the contribution of *RARβ* promoter methylation to RA refractoriness. We treated the seven RA-refractory cell lines for 6 days with 5-Aza-CdR, RA, or both. Under these conditions, at the completion of treatment the cells had undergone at least 4 population doublings and were subconfluent (70–80%), permitting incorporation of 5-Aza-CdR throughout treatment. MSP analysis demonstrated that 5-Aza-CdR treatment decreased *RARβ* promoter methylation by at least 50% but was still detectable (data not shown). Treatment with 5-Aza-CdR restored RA responsiveness in two of the seven cell lines (29%) with *RARβ* promoter methylation (Fig. 1, *B* and *C*). Thus, 5-Aza-CdR treatment was sufficient to restore RA responsiveness in some cells with *RARβ* promoter methylation, but not in others.

Receptor Coregulator Expression. Previous studies have shown that RA refractoriness in lung cancer cells is not due to loss of specific RAR or RXR family members (4, 6, 7). However, other components of the retinoid receptor complex, including coactivators and corepressors, have not been investigated in lung cancer cells. We tested the hypothesis that RA refractoriness is caused by coactivator loss or corepressor overexpression. We performed Western blot analysis of the coactivators TIF2, RAC3, BRG1, GCN5, and CBP and the corepressors SMRT and NcoR, all of which are known to associate with RXR, RAR, or both (16–19). In the case of BRG1, SMRT, CBP, and NcoR, we observed multiple immunoreactive bands (Fig. 2), perhaps because the mRNA products of these genes are alternatively spliced, and the protein products are phosphorylated (20–22). The cell lines examined in this study expressed multiple coactivators. We detected CBP, BRG1, and GCN5 in all cell lines. The other coactivators (RAC3 and TIF2) were detected in fewer cell lines, including both RA-responsive and RA-refractory cells. Among the corepressors, we detected SMRT and NcoR in all cell lines except for the RA-refractory cell lines H460 and SK-MES-1. Thus, our findings did not support the hypothesis that RA refractoriness is secondary to loss of a specific coactivator or overexpression of a corepressor.

Histone Acetylation. After binding to ligands, retinoid nuclear receptors recruit coactivators, which induce histone acetylation, chromatin remodeling, and assembly of the general transcription machinery. We hypothesized that in RA-refractory cells, RA treatment would not activate these events on the *RARβ* promoter. ChIP analysis of the RA-responsive cell lines Calu-6 and H661 revealed that RA treatment increased acetylation of histones H3 and H4 at the *RARβ* promoter (Fig. 3). In contrast, RA treatment did not increase acetylation of histone H3 in any of the RA-refractory cell lines examined, whereas histone H4 acetylation increased in all of the RA-refractory cells except SK-MES-1. The *RARβ* promoter was methylated in all of these RA-refractory cell lines except H322. Thus, we observed aberrant

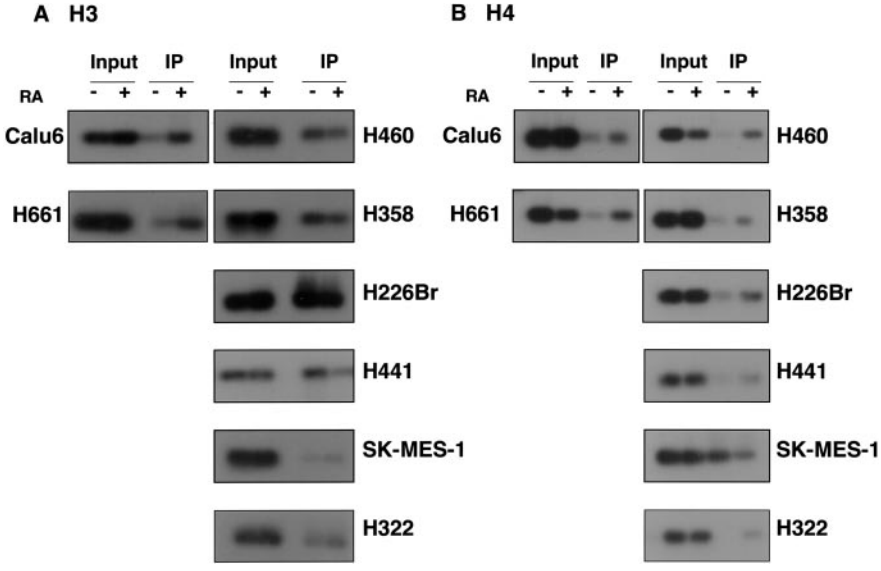

Fig. 3. ChIP analysis was performed to examine whether RA treatment increased acetylation of histones H3 and H4 on the *RARβ* promoter in non-small cell lung cancer cells that are RA responsive (Calu-6 and H661) or RA refractory (H460, H358, H226Br, H441, SK-MES-1, and H322). Lung cancer cell lines were treated for 12 h with RA (+) or media alone (−). Protein-DNA complexes were cross-linked in the cells by treatment with formaldehyde. Cells were lysed, and lysates were subjected to immunoprecipitation (*IP*) with anti-acetyl-histone H3 (*A*) or anti-acetyl-histone H4 (*B*) antibodies followed by PCR to amplify *RARβ* promoter sequences. PCR products were subjected to Southern blotting using a *RARβ* genomic fragment containing the promoter region as a probe. Inputs represent the relative amounts of protein-DNA complex subjected to immunoprecipitation; PCR analysis of the *RARβ* gene promoter was performed on an aliquot of whole cell extract (2% of sample volume subjected to immunoprecipitation).

acetylation of *RARβ* promoter-associated histones in RA-refractory cell lines with and without *RARβ* promoter methylation.

Discussion

Several findings presented here provide evidence that RARβ expression depends on DNA methylation in some lung cancer cell lines but not in others. First, 36% of RA-refractory cells had no evidence of *RARβ* promoter methylation. Second, 5-Aza-CdR treatment restored RA responsiveness in only 29% of RA-refractory cells with *RARβ* promoter methylation. An alternative interpretation of this finding is that RA refractoriness in the presence of 5-Aza-CdR is related to persistent *RARβ* promoter methylation in these cells. Third, RA treatment induced aberrant histone acetylation at the *RARβ* promoter in RA-refractory cells with and without *RARβ* promoter methylation. Previous studies support a role for other mechanisms in RA refractoriness. Nur77, an orphan receptor that is overexpressed in lung cancer, heterodimerizes with RXR and competes with RXR-RAR complexes for binding to RAREs, blocking RA-induced expression of target genes (23). Furthermore, RTK pathways, which are activated in lung cancer, can inhibit RXR-RAR transactivation through at least two mechanisms. RTK pathways stimulate the AP-1 transcription factor, which competes with RXR-RAR complexes for binding to the coactivator CBP, blocking coactivator recruitment to retinoid receptors (24). Furthermore, RTK pathways activate stress kinases, which phosphorylate RXR and inhibit ligand-induced RXR-RAR activation (14). Thus, several mechanisms could contribute to RA refractoriness in lung cancer.

In breast and ovarian cancer cells, the *AIB-1* coactivator gene is amplified and overexpressed, and high *AIB-1* levels contribute to hormonal dependence in these tumor types (25). We examined the expression of coactivators and corepressors as a potential cause of RA refractoriness in lung cancer cells. We found no evidence that RA-refractory cells have reduced expression of a specific coactivator or overexpress a specific corepressor. These findings are consistent with previous reports that coactivators have partially overlapping functions (26); according to this hypothesis, reduced levels of a specific coactivator will not lead to loss of nuclear receptor function in cells that express multiple coactivator family members.

Cells use histones to compress DNA into chromatin, and the core histone proteins (two each of histones H2A, H2B, H3, and H4), along with the DNA that wraps around them, define the nucleosome. RA treatment leads to acetylation of histones H3 and H4, and histone acetylation is required for the transcription of retinoid receptor target genes (13). In support of this hypothesis, we found that RA-induced histone H3 acetylation on the *RARβ* promoter was disrupted in all RA-refractory lung cancer cells examined. Interestingly, among the cell lines with *RARβ* promoter methylation, we observed aberrant H3 acetylation in 100% and aberrant H4 acetylation in only 17%, suggesting that DNA methylation selectively targets histone H3. Supporting this hypothesis, H3 is the histone specifically involved in methylation-induced silencing of proviral DNA and the imprinted genes *Snrpn* and *U2af1-rs1* (27, 28). Histone H3 has a long NH_2-terminal tail extending well outside the nucleosome that is subject to acetylation, phosphorylation, and methylation. Whether this characteristic or gene locus-specific factors predispose H3 to selective targeting by histone deacetylases is not known at this time.

In acute promyelocytic leukemia cells, which do not express RARβ, the *RARβ* promoter is methylated by the promyelocytic leukemia protein-RAR fusion protein, which recruits DNA methyltransferases to the *RARβ* promoter (29). In contrast to its effect in acute promyelocytic leukemia cells, RA treatment in lung cancer cells did not restore RARβ expression (Fig. 2) or reverse *RARβ* promoter methylation (data not shown), suggesting that the *RARβ* promoter is methylated through distinct mechanisms in these two types of cancer cells.

Interestingly, we found histone H3 hypoacetylation in one cell line (H322) without evidence of *RARβ* promoter methylation, indicating that mechanisms other than DNA methylation contribute to aberrant histone H3 acetylation in lung cancer cells. Recent data have shown that histone H3 hypoacetylation is linked mechanistically to methylation of histone H3 at lysine 9 (30). This covalent modification leads to the organization of chromatin regions into higher-order structures (heterochromatin) that are transcriptionally silent (30). Thus, the aberrant histone acetylation observed in certain lung cancer cells may be linked to other histone modifications that regulate chromatin structure and gene expression.

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