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ORIGINAL ARTICLE

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Hyperacetylation enhances the growth-inhibitory effect of *all-trans* retinoic acid by the restoration of retinoic acid receptor β expression in head and neck squamous carcinoma (HNSCC) cells

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Abstract The chemotherapeutic effects of *all-trans*-retinoic acid (atRA) are mediated by the retinoic acid receptor β (RARβ), but RARβ expression is reduced in a number of head and neck carcinoma (HNSCC) cells which causes resistance to RA treatment in half the patients with HNSCC. The possible mechanism for the reduced RARβ expression has been suggested as the methylation of the CpG islands adjacent to the RA response elements (RARE) in the RARβ promoter and the loss of histone acetylation. The suppressed RARβ expression can be reactivated by a demethylating agent (5-aza-2′-deoxycytidine, 5-AzaC) or a histone deacetylase inhibitor (trichostatin A, TSA). Therefore, we sought to determine if the restoration of RARβ activity, or a combination of these drugs, could restore the sensitivity to RA in RARβ-negative HNSCC cells with an epigenetically methylated RARβ promoter region. SqCC/Y1 cells resistant to atRA showed methylated and unmethylated forms in the RARβ promoter region. RARβ expression of these cells was restored by 5-AzaC or TSA treatment. Also, treatment with TSA and atRA combined synergistically increased the growth-inhibitory effect and highly induced the transcriptional activation of the RARβ promoter compared to atRA treatment in HNSCC cells. Additionally, TSA alone and the combination 5-AzaC and TSA increased lysine-9 (Lys-9) acetylation and Lys-4 methylation of the first exon at the RARβ gene, while decreasing the methylation of Lys-9 in the HNSCC cells.

Keywords Hyperacetylation · Growth-inhibitory effect · Retinoic acid receptor · HNSCC

Abbreviations atRA: *All-trans* retinoic acid · 5-AzaC: 5-Aza-2′-deoxycytidine · ChIP: Chromatin immunoprecipitation · HDAC: Histone deacetylase · HNSCC: Head and neck carcinoma · Lys-9/4: Lysine-9/4 · MSP: Methylation-specific PCR · RARβ: Retinoic acid receptor β · TSA: Trichostatin A

Introduction

Retinoids, a group of vitamin A derivatives, are known to be important in the regulation of normal cellular growth and differentiation. There have been suggestions that they could play a chemopreventative role in the reversible promotion stage of multistep carcinogenesis. Studies in head and neck carcinoma (HNSCC) cells have demonstrated that retinoids can inhibit cell proliferation in vitro and prevent the development of cancer in patients with premalignant oral lesions with previously treated head and neck cancer in vivo [1–4]. The effects of retinoids are known to be mediated by nuclear retinoid receptors [1, 2], retinoic acid receptors (RARs) or retinoid X receptors (RXRs). RA binds and activates the RARs, which is a ligand-inducible transcription factor that activates the transcription of the RA target genes by associating with RA response elements (RARE) located

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in their promoter regions. The complex actions of RAR and RXR isotypes (α, β, γ) mediate the multiple effects of retinoids through the formation of homo- or heterodimers [5]. Of these isotypes, retinoic acid receptor β (RARβ) has been suggested to play an important role in the biological functions of RA in many different types of cancer cells.

RARβ expression is also thought to be associated with cellular sensitivity to retinoid in numerous cancer cells, including HNSCC cells, breast cancer cells, and lung cancer cells [6–9]. However, many HNSCC cells show reduced or absent RARβ expression [10, 11] and exhibit relative resistance to the growth-inhibitory effects of *all-trans* retinoic acid (atRA) [7]. Recent studies have demonstrated that DNA methylation is at least one contributing factor to RARβ inactivity [12–14], which can be alleviated by demethylation using 5-aza-2-deoxycytidine (5-AzaC), restoring RA-induced RARβ transcription. Inhibition of histone deacetylase (HDAC) also forces cancer cells to undergo apoptosis [15] and causes significant suppression of human prostatic tumor growth in nude mice [16]. In studies on trichostatin (TSA), an HDAC inhibitor, and RARβ expression, TSA was found to enhance RARβ expression in epithelial cells [12] and RA-resistant human colon cancer cells [13]. Additionally, methylated DNA is closely linked to the deacetylation of histones through an association with methyl CpG-binding proteins and the recruitment of HDAC [17]. These events cause the loss of the RARβ gene expression, which leads to epigenetically silenced expression in lung and breast cancer cell lines [18, 19]. Recently, other studies have revealed that acetylated lysine-9 (Lys-9), histone H3 and methylated lysine-4 (Lys-4) are increased in euchromatic domains, and correlate with active gene expression, whereas methylated Lys-9 is increased in deacetylated, transcriptionally silent, heterochromatic regions [20, 21]. The above DNA methylation and histone modifications have been hypothesized to determine whether RARβ gene expression is active or inactive.

The aim of this study was to improve the response to atRA in atRA-resistant HNSCC cells and to provide a clinical application of atRA by reducing treatment-related toxicity. Based on these observations, we hypothesized that the demethylating agent 5-AzaC and the HDAC inhibitor TSA would enhance the antineoplastic action of atRA in HNSCC cells. Therefore, we sought to determine whether the RARβ gene could be silenced by methylation-mediated epigenetic mechanisms in RARβ-negative HNSCC cells, and whether combined treatment with 5-AzaC, TSA and atRA could restore RARβ expression in these cells. Moreover, whether treatments using 5-AzaC, TSA and atRA might induce a reduction in the methylated Lys-9 H3 and augmentation of the acetylated Lys-9 and methylated Lys-4, and whether these changes are essential for RARβ restoration in RARβ-negative HNSCC cells were also investigated.

Materials and methods

Cell culture conditions and reagents

The HNSCC cell lines 1483, SqCC/Y1, 11B, 17A, and 17B were obtained from Dr. R. Lotan (University of Texas M.D. Anderson Cancer Center, Houston, Tx.). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimal essential medium (DMEM) and Ham's F12 medium (pH 7.2) (Gibco/BRL, Grand Island, N.Y.), containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The atRA, TSA and 5-AzaC were purchased from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of atRA and TSA, at concentrations of 10 and 1 m *M*, were prepared in 100% DMSO and stored at −80°C and protected from light at all times. The 5-AzaC was dissolved in a solution containing 0.45% NaCl and 10 m *M* sodium phosphate (pH 6.8).

5-AzaC, TSA and atRA treatments

The cells were seeded at 5×10^5 cells per 10-cm plate, with 10% FBS-containing medium. At 70% confluence, they were incubated for 24 h in a 0.5% FBS-containing medium in order to minimize the effect of endogenous RA in the culture medium, and then were treated with 0.1 μ*M* 5-AzaC. After 48 h, the culture medium was changed to a medium containing 50 n *M*TSA. The atRA was added 3 h later directly to the culture medium to a final concentration of 1 μ*M*. After 48 h, the cells were washed twice in phosphate-buffered saline (PBS, pH 7.4) and collected by trypsinization. Control cultures received the same amount of DMSO as the treated cultures. For each experiment, the collected cells were stored at −80°C.

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay

Cell growth rate was determined by a MTT-based colorimetric assay [22]. Cells were grown in 96-well plates (Nunc, Roskilde, Denmark), with 0.5% FBS-containing medium, at an initial density of 2×10^3 cells/well. The cells were treated with 0.05, 0.1, 0.5, 1 and 5 μ*M*5-AzaC for 48 h. The cell growth inhibition rate was directly proportional to the concentration of 5 μ*M* 5-AzaC. For the combined treatment, they were treated as described above. After 48 h, 50 μl MTT solution (Sigma) was added to each well, and the cell viability was measured as recommended by the manufacturer, and the absorbance in individual wells determined at 545 nm using a microplate reader (BioRad, Hercules, Calif.).

Clonogenic assay

Cells were seeded at 200 cells per 60-mm dish in 10% FBS-containing medium, and 24 h after seeding were treated with 5-AzaC, TSA and atRA, as described above. For longer reagent exposure times, the medium containing 5-AzaC, TSA and atRA singly or in different combinations was exchanged every 72 h. As the colonies became visible (2–3 weeks), they were fixed with methanol for 15 min, and washed in PBS. The colonies were then stained with 0.1% crystal violet for 10 min, rinsed with water and counted using a Gel-doc colony counter image analyzer (Bio-Rad, Hercules, Calif.).

Reverse transcriptase-polymerase chain reaction

Total cellular RNA was isolated from cells treated with 5-AzaC, TSA and/or atRA or from untreated cells with TRIREAGENT-RNA isolation reagent (Gibco/BRL, Gaithersburg, Md.), following the manufacturer's instructions. Moloney murine leukemia virus reverse transcriptase (MMLV; Gibco/BRL) and Oligo-d$(T)_{15}$ primer (Roche, Indianapolis, Ind.) were added to aliquots of total RNA (1 μg) to a final volume of 20 μl in order to generate cDNAs. The cDNA product (2 μl) was used for the PCR amplification of RARβ and β-actin. Their primer pairs are listed in Table 1. The PCR reaction mixture (20 μl) consisted of 1× reaction buffer, 2 m $M MgCl_2$, 200 μMdNTP, 0.4 U Amplitaq polymerase and 0.2 μM of each primer. An initial denaturation at 95°C for 5 min was followed by 30 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, and then a final extension step at 72°C for 5 min. The PCR products from each sample were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide and then photographed.

Table 1 Primers for the RARβ gene used in this study

Primer	Sequence	Size (bp)
MSP	For the methylated RARβ gene MF: GGGTTTATCGAAAGTTTATTC MR: TTCCGAATACGTTCCGAAT	231
	For the unmethylated RARβ gene UF: GGTAGGGTTTATTGAAAGTTTATTT UR: AAACCTTCCAAATACATTCCAAAT	241
RT-PCR/ β-actin	For RT-PCR of the RARβ Forward: CATGTTTGACTGTATGGATG Reverse: AGCCCTTACATCCCTCACAG	329
	For β-actin Forward: ACCCAGATCATGTTTGAGACC Reverse: GGAGTTGAAGGTAGTTTCGTG	486
ChIP assay	PCR A: for analysis of the promoter vicinity F-1: TCCTGGGAGTTGGTGATGTCAG R-1: AAACCCTGCTCGGATCGCTC	248
	PCR B: for analysis of the distal region F-2: GCCGAGAACGCGAGCGATCC R-2: GGCCAATCCAGCCGGGGC	146

Northern blots

Total cellular RNA prepared from each sample (30 μg) was subjected to electrophoresis on a 1% agarose gel. The RNA was then transferred overnight to a GeneScreen Plus nylon membrane (NEN Life Science Products, Boston, Mass.) by capillary transfer and immobilized on the membrane by UV crosslinking. The RNA blots were prehybridized and hybridized to an [α-^{32}P]-dCTP-labeled retinoic acid receptor cDNA probe, obtained from Dr. J.M. Kurie (University of Texas, M. D. Anderson Cancer Center, Houston, Tx.) and Dr. R. Evans (Salk Institute, San Diego, Calif.). After an overnight incubation at 65°C, the membranes were washed under high-stringency conditions (0.1× SSC at 65°C) and exposed (below −70°C) to X-ray film for autoradiography overnight. The membrane was also stripped and reprobed with GAPDH cDNA as an RNA loading control.

Western blots

The HNSCC cells were treated as described above. The total cell lysates (30–50 μg) were resolved on 12% SDS-PAGE gel, and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane non-specific binding was blocked using 5% non-fat milk/TBS for 60 min and incubated overnight at 4°C with primary anti-human RARα (Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-human RARβ antibodies (1:500). After washing, a peroxidase-labeled secondary antibody (Amersham Pharmacia Biotech, 1:5000) was added to the membrane and incubated for 1 h at room temperature. After washing again, the bands were visualized using the peroxidase-linked enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Each run was repeated three times.

Methylation-specific PCR (MSP)

The methylation status of the promoter region of the RARβ (−477/+392, GenBank accession numbers S82362 and M96016) in HNSCC cells was analyzed using sodium bisulfite MSP as described previously [23]. The genomic DNA was treated with sodium bisulfite, as described previously [23, 24]. The primer pairs are listed in Table 1. The modified DNA was used as a template for PCR, using primers to differentiate between the methylated and unmethylated DNA for the RARβ promoter region, as previously described [14]. A 50-μl PCR reaction mixture consisted of 1× reaction buffer, 2 m $M MgCl_2$, 200 μMdNTP, 0.2 μM of each primer and 50 ng bisulfite-modified DNA. The reactions were hot-started at 95°C before the addition of 2.5 U of Taq polymerase (Qiagen, Chatsworth, Calif.). Amplification was followed by 30 cycles at 95°C for 30 s, 55°C for 30 s

and 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR products from each sample were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide and then photographed. DLD-1 cells and normal lymphocytes were used as methylated and unmethylated positive controls [25], respectively.

Transient transfections and luciferase reporter assay

The luciferase reporter plasmid was obtained from Dr. H.Y. Lee (University of Texas, M. D. Anderson Cancer Center, Houston, Tx.). Luciferase reporter plasmids containing RAREs (AGTTCA) in direct repeat separated by five nucleotides (DR5) in the context of a TK heterologous promoter (RARE-TK-LUC) and a control plasmid containing the TK promoter but no RARE (TK-LUC) [26, 27] were used. The cells were seeded at a density of 1×10^5 cells per well and cultured in 10% FBS-containing medium. After 24 h, the SqCC/Y1 cells were transfected with 1 μg reporter plasmid by lipofection using the Lipofectamine reagent (Gibco/BRL, Grand Island, N.Y.). The 1483 cells were transfected with RARE-TK-LUC or TK-LUC using a CELL PORATOR electroporation system I (Gibco/BRL) set at 200 V and 1000 μF, as previously described [27]. The following day, the cells were treated with 5-AzaC, TSA and atRA, as described above. After 48 h, their lysates were assayed for luciferase activity using a luciferase assay system (Promega, Madison, Wis.). Luciferase activity was determined with a TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.). The transfection efficiencies among the cells were normalized by comparing the β-galactosidase activity of cells cotransfected with a β-galactosidase expression vector (Promega) as an internal control. β-Galactosidase assays were performed using a luminescent β-gal detection kit II (Clontech, Basingstoke, UK) following the manufacturer's instructions. The results are recorded as the means ± SE of the values obtained from triplicate plates transfected from either the lipofection or electroporation mixture.

Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed as described previously [28]. Briefly, the cells were treated with 5-AzaC, TSA and atRA as described above. Formaldehyde was then added to the cells to crosslink the histones to the DNA. The cells were scraped with 500 μl SDS lysis buffer containing protease inhibitors. The lysates were sonicated to obtain DNA fragments and the sonicated samples were precleared with a salmon sperm DNA/protein A agarose slurry and incubated for 1 h at 4°C with rotation. Following preclearing, 5 μl of anti-Lys-9 acetylated histone H3 antibody, anti-Lys-9 methylated histone H3 antibody or anti-Lys-4-methylated antibody (Upstate Biotechnology, Lake Placid, N.Y.) were incubated overnight at 4°C with rotation. After elution, crosslinks were reversed and the immunoprecipitated DNA was recovered by phenol/chloroform extraction and analyzed using PCR. The primer pairs used for the ChIP analysis of the RARβ promoter (PCR A and PCR B) are listed in Table 1. The PCR reaction mixture (20 μl) consisted of 1× reaction buffer, 2 m $M$$MgCl_2$, 200 μ$M$dNTP, 0.4 U Amplitaq polymerase, 0.2 μM primers and 25 ng immunoprecipitated (bound) DNA, non-immunoprecipitated (input) DNA or no-antibody (control) DNA. An initial denaturation at 95°C for 2 min was followed by 30 cycles of 95°C for 20 s, 62°C for 10 s and 72°C for 30 s, and a final extension step of 5 min at 72°C. To ensure the PCR products for PCR A and PCR B of the RARβ promoter were in a linear amplification range, each reaction was initially set up with different dilutions of input DNA, various amplification cycle numbers, and the final PCR conditions selected. The PCR products from each sample were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide. Quantitative analyses were performed with a Multi-Image light cabinet, and quantified with a Gel-doc image analyzer (Bio-Rad, Hercules, Calif.).

Statistical analysis

The results are presented as the means ± SE for at least three separate experiments performed in triplicate. The significance of differences between values was determined by classical one-way ANOVA tests.

Results

Methylation status of RARβ promoter in HNSCC cell lines

To identify the methylation status of the RARβ promoter region in various HNSCC cell lines, we amplified the unmethylated and methylated PCR products of the RARβ promoter region in these cell lines using MSP methods (Fig. 1a). Specific primer pairs were constructed to detect the methylation status of the RARβ promoter region (Table 1), as described previously [29]. SqCC/Y1 and 17A cells showed both methylated and unmethylated PCR products, suggesting partial methylation in the RARβ promoter region. Neither 1483 cells nor 11B cells showed any methylated form, but 17B cells showed a fully methylated form. These findings are in accordance with those of another recent study [30]. From these cell lines we selected the partially methylated SqCC/Y1, the unmethylated 1483 cells, and the fully methylated 17B cells to compare atRA sensitivity with methylation status of the RARβ promoter. Basal expression of RARβ mRNA in SqCC/Y1 and 17B cells was not shown (Fig. 1c, d). RARβ partial methylation was found, with concomitant loss of mRNA expression

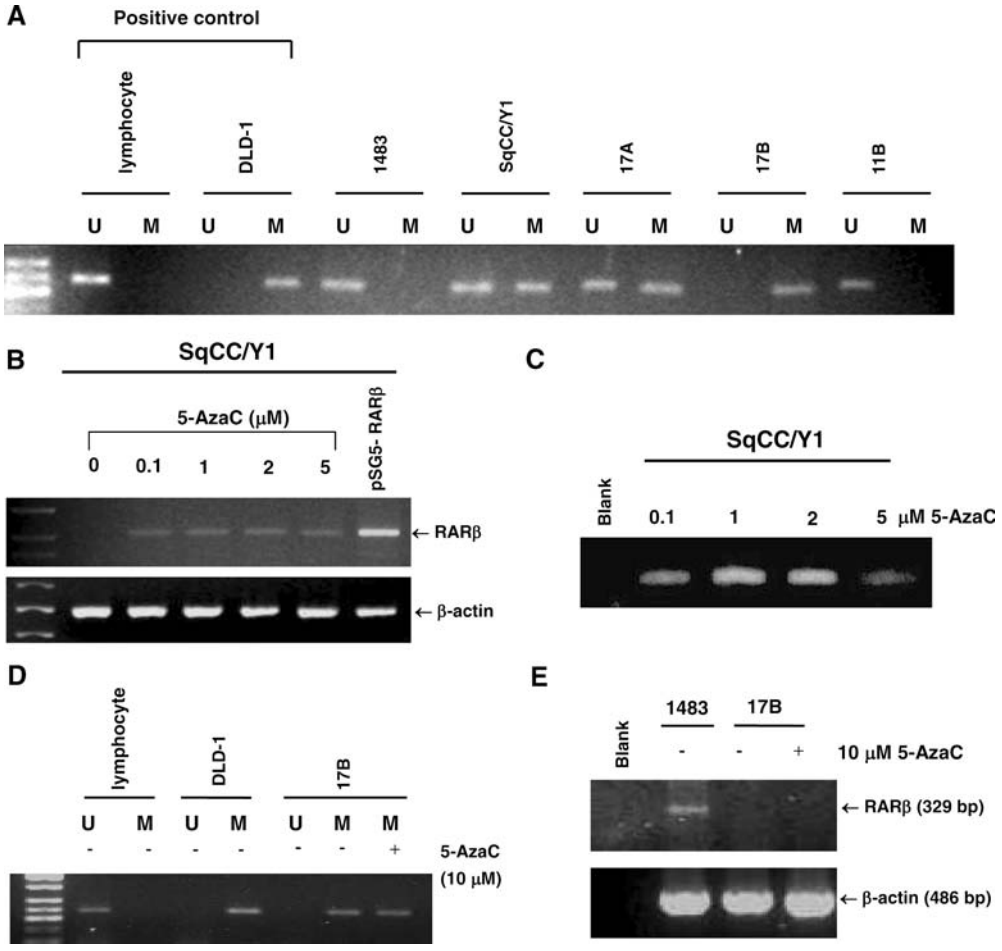

Fig. 1 DNA methylation status of the HNSCC cell lines and RARβ expression in SqCC/Y1 and 17B cells treated with 5-AzaC. **a** MSP was used to assess the methylation status of the RARβ promoter region in HNSCC cell lines. Reactions specific for unmethylated DNA (*U*) and methylated DNA (*M*) are indicated. The positive controls were normal lymphocytes (for unmethylated form) or DLD-1 cells (for methylated form). **b** The methylation status of the RARβ promoter in 17B cells treated with 10 μ*M* 5-AzaC. **c**, **d**RARβ mRNA expression in SqCC/Y1 cells treated with 5-AzaC at 0, 0.1, 1, 2 and 5 μ*M* and in 17B cells treated with 5-AzaC at 10 μ*M* assessed by RT-PCR. pSG5-RARβ (RARβ expression vector) was transiently transfected into SqCC/Y1 cells compared to endogenous expression. β-Actin was used as an internal control. **e** Methylated form of SqCC/Y1 cells treated with 5-AzaC at 0, 0.1, 1, 2 and 5 μ*M*. The results show clear signs of promoter in the cells for RARβ and the partial demethylation of methylated DNA following treatment with 5 μ*M* 5-AzaC, but the relative intensity of the unmethylated DNA did not change (data not shown)

of the RARβ in SqCC/Y1 cells. After treatment with various concentrations of 5-AzaC, RARβ mRNA expression was restored in SqCC/Y1 cells, even at low concentrations. However, a dose response effect of 5-AzaC was not observed between treatment level and induced expression level in SqCC/Y1 cells (Fig. 1d). In addition, 5-AzaC treatment had no effect on the demethylation of the RARβ promoter region in SqCC/Y1 cells. At the highest concentration of 5 μ*M*, 5-AzaC slightly reduced the methylated band but was not able to induce the fully unmethylated form in the partially methylated RARβ promoter of SqCC/Y1 cells (Fig. 1e). In 17B cells, which have loss of the 3p arm and full methylation of the residual allele [31], treatment with 5-AzaC at the highest concentration of 10 μ*M* failed to restore RARβ mRNA expression or induce the demethylation at the RARβ promoter (Fig. 1b, c).

5-AzaC and TSA restore RARβ gene expression despite partial methylation at the RARβ promoter in SqCC/Y1 cells

To investigate the loss of RARβ expression by epigenetic suppression, we analyzed the level of RARβ mRNA in RARβ-negative SqCC/Y1 cells treated with 5-AzaC, TSA and atRA using RT-PCR and Northern blot analysis. The RNA extract of 1483 cells was used as a positive control for endogenous RARβ mRNA, in which these cells expressed basal or atRA-induced RARβ mRNA levels (Fig. 2a) [6]. RARβ was detected in untreated 1483 cells but not in untreated SqCC/Y1 cells. Using atRA alone, no RARβ restoration was detected in SqCC/Y1 cells. However, 5-AzaC and TSA alone, or combined 5-AzaC and TSA and/or atRA induced the expression of the RARβ gene. We sought to determine if combined treatment with 5-AzaC and/or

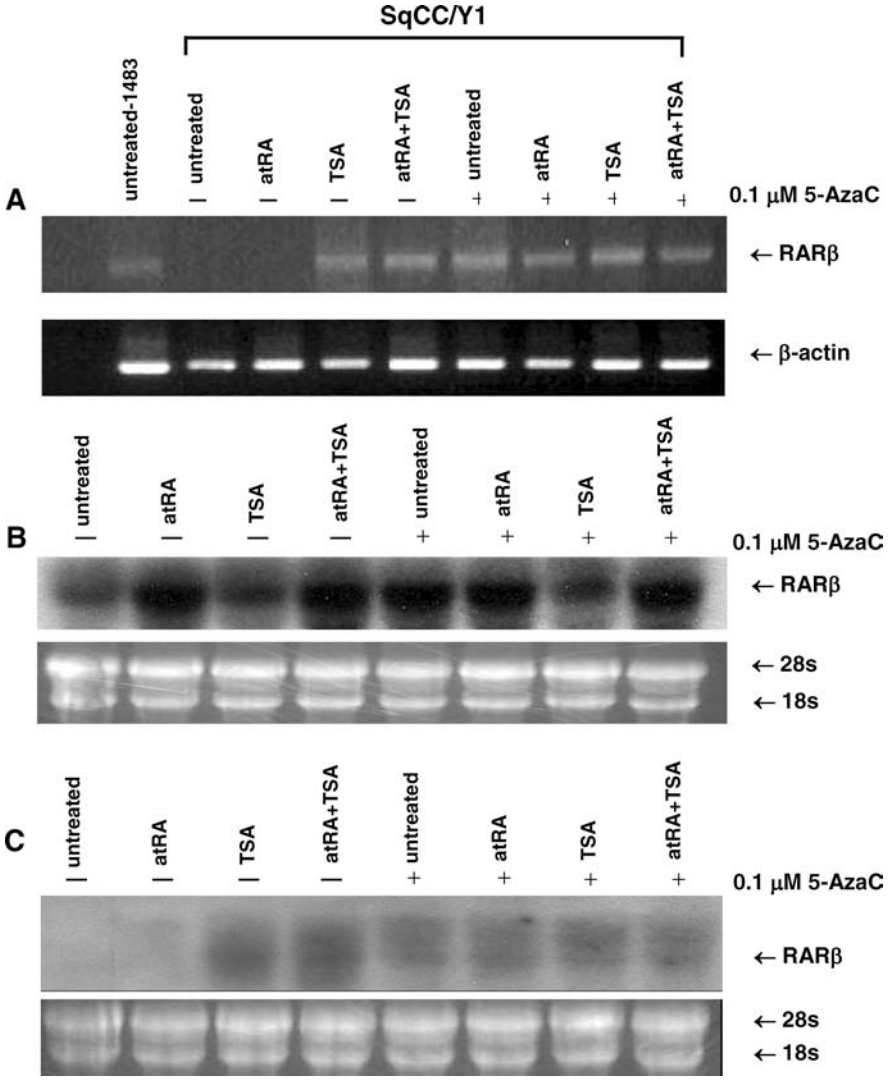

Fig. 2 Expression of RARβ in HNSCC cells treated with atRA, 5-AzaC and TSA singly or combined. **a** RT-PCR analysis of RARβ expression in SqCC/Y1 cells; β-actin was used as an internal control. Untreated 1483 cells are shown as the RARβ-positive control. **b**, **c** Northern blot analysis of RARβ expression in 1483 cells (**b**) and SqCC/Y1 cells (**c**). The 28s and 18s rRNA was used as an internal loading control. **d**, **e**Western blot analysis of RARβ and RARα expression in 1483 cells (**d**) and SqCC/Y1 cells (**e**); β-actin was used as an internal loading control

TSA and atRA could enhance the restoration of RARβ expression compared to the drugs alone in both cell types (Fig. 2b, c). Treatment with atRA or 5-AzaC alone highly increased RARβ expression in 1483 cells but not in SqCC/Y1 cells. The combination of atRA and TSA greatly increased RARβ expression in SqCC/Y1 cells but only slightly increased atRA-induced RARβ expression in 1483 cells. Next, we performed a Western blot analysis on both HNSCC cell lines to detect expression of RARβ protein following treatment with 5-AzaC and TSA. Compared to untreated 1483 cells (RARβ-positive), the expression of RARβ protein was greatly induced in 1483 cells treated with atRA (Fig. 2d). Moreover, the level of RARβ protein induced by the combination of atRA and TSA and/or 5-AzaC was higher than the level induced by atRA alone. In contrast, when SqCC/Y1 cells were treated with atRA alone, no RARβ expression was induced (Fig. 2e), whereas TSA alone slightly induced RARβ expression. Also, combined treatment with atRA and TSA significantly increased RARβ expression in these cells. These results indicate that the combination of atRA and TSA highly increased the level of RARβ protein expression in RARβ-negative SqCC/Y1 cells.

Sensitivity to atRA, 5-AzaC and/or TSA is related to RARβ expression

In the next step the appropriate concentrations of 5-AzaC to get the lowest drug toxicity possible were determined by treating the cells with 5-AzaC only. Therefore, HNSCC and 1483 cells were treated with various concentrations of 5-AzaC (Fig. 3a). The concentration of 5-AzaC was directly proportional to the growth inhibition in both cell types; 50% growth inhibition was achieved with 1 μ*M* 5-AzaC. However, higher concentrations increased the drug toxicity too much. 5-AzaC at 0.1 μ*M* restored RARβ expression in SqCC/Y1 cells (Fig. 1d), although this concentration resulted in about 30% growth inhibition. To further study the combined treatment with atRA and/or TSA, we used 0.1 μ*M* AzaC to treat both cell types.

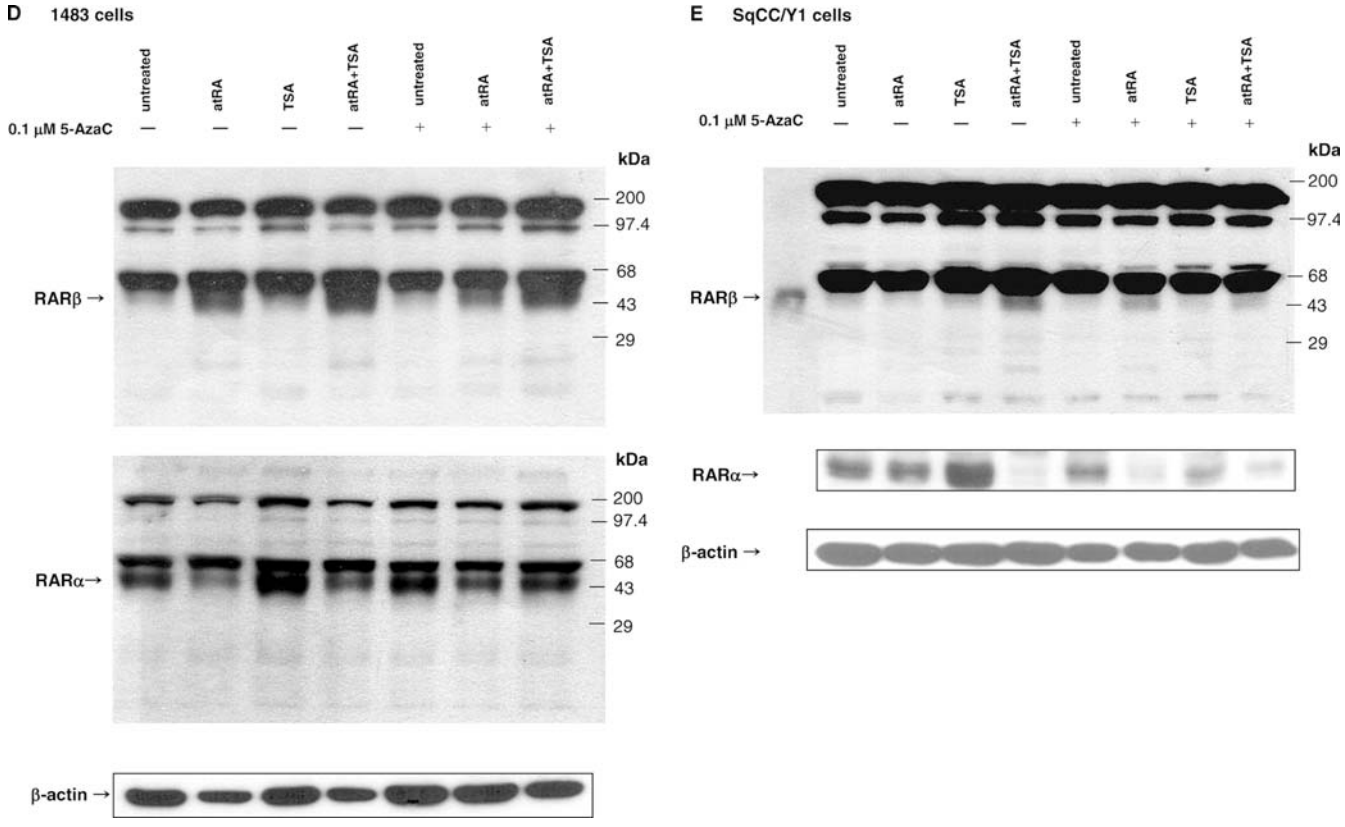

Fig. 2 (Contd.)

Growth-inhibitory effects of atRA, 5-AzaC and TSA treatment were also observed in relation to methylated or unmethylated states of the RARβ promoter regions in RARβ-negative SqCC/Y1 and 17B cells, and in RARβ-positive 1483 cells. The MTT assay showed that atRA alone did not influence the rate of growth inhibition of SqCC/Y1 and 17B cells, whereas it increased growth inhibition by 35% in 1483 cells (Fig. 3b). TSA and atRA in combination acted synergistically in inhibiting the growth of SqCC/Y1 cells. The dose-effect relationship of this synergism was analyzed using a combination index-isobologram (CI 0.73, data not shown).

Unlike SqCC/Y1 cells, fully methylated 17B cells were more resistant to atRA, and so the growth-inhibitory effects of single or combined treatment with these agents were not significantly different in these cells. Even treatment with 5-AzaC at a high concentration could not induce RARβ mRNA expression in 17B cells as shown Fig. 1e. Therefore, 17B cells were excluded from future studies. Although combining 5-AzaC and atRA did not affect the growth inhibition in SqCC/Y1 cells, their effects were more than additive in 1483 cells. Treatment with 5-AzaC and TSA in combination increased the growth inhibition in SqCC/Y1 cells compared to treatment with 5-AzaC alone. In 1483 cells, treatment with 5-AzaC and TSA in combination, or with 5-AzaC, TSA and atRA in combination slightly increased growth inhibition compared to treatment with each drug alone. The effect of treatment with 5-AzaC, TSA and atRA in combination on growth inhibition was not superior to the effect of treatment with 5-AzaC and TSA in combination in SqCC/Y1 cells. Similar results were obtained with the clonogenic assay (Fig. 3c). The clonogenic assay, performed in agar, showed about a 30% reduction in the colony-forming ability of SqCC/Y1 cells treated with the combination of atRA and TSA. 5-AzaC alone markedly reduced the colony-forming ability of both SqCC/Y1 and 1483 cells by up to 50%. Likewise, in 1483 cells, treatment with each drug singly decreased the colony-forming ability of the cells; this was most striking with TSA alone, which caused a reduction of approximately 70%.

Effects of TSA and 5-AzaC on atRA-induced transcriptional activity of the RARβ gene

Next, we sought to determine if the transcriptional activation of the RARβ gene promoter was affected by treatment with atRA, 5-AzaC and TSA, and if this transcriptional activation was caused by the regulation of RARE ligand binding in atRA-sensitive 1483 and atRA-resistant SqCC/Y1 cells. The transcriptional activation of the RARβ promoter was detected as the enzymatic luciferase activity of the cell extract (Fig. 4). Although treatment with atRA alone markedly induced the RARE-TK-LUC (ligand-dependent) transcriptional activity in atRA-sensitive 1483 and atRA-resistant SqCC/Y1 cells, it did not affect the TK-LUC (ligand-independent) activity. TSA alone slightly but not

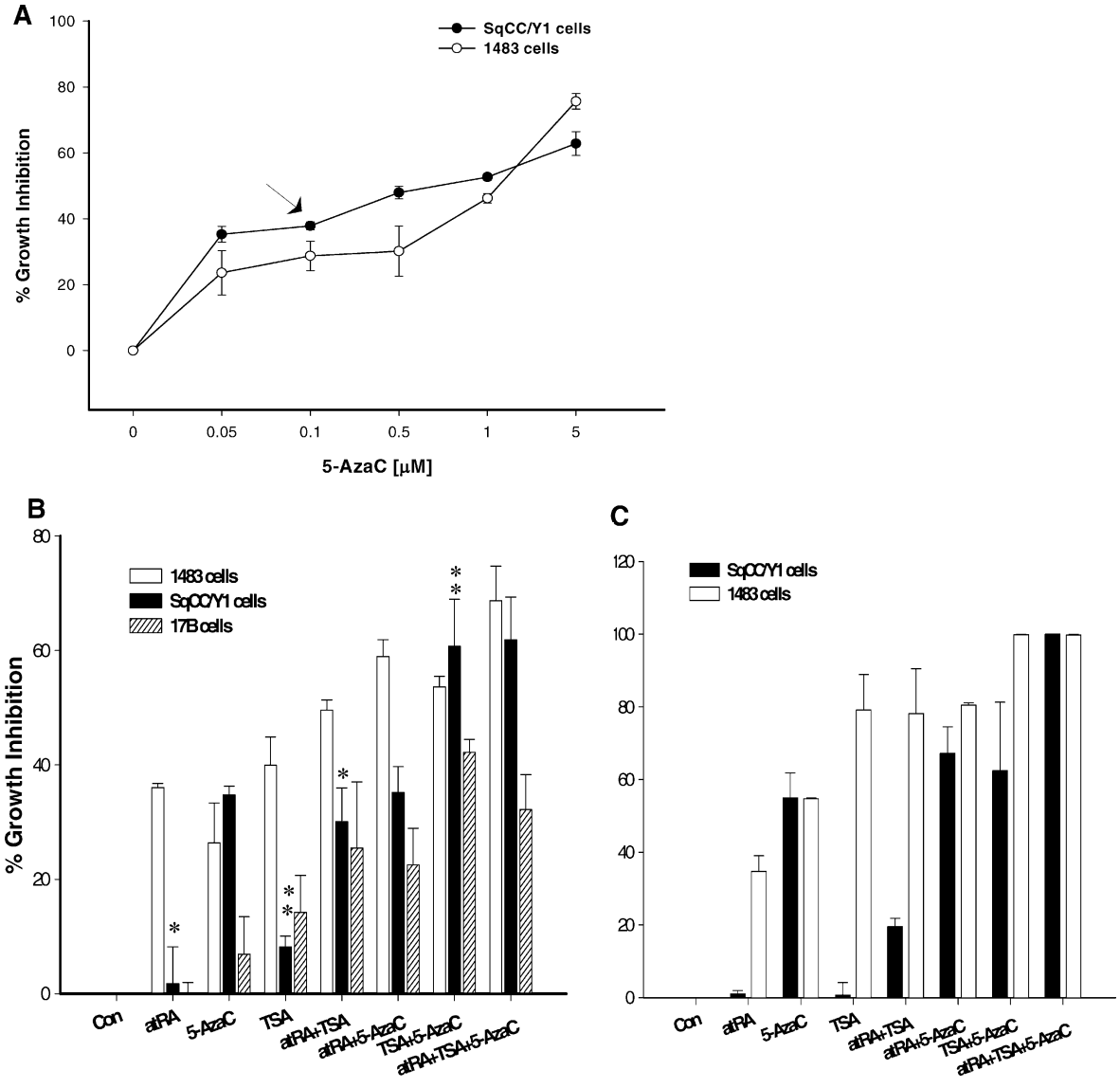

Fig. 3 Growth-inhibitory effects of treatment with various concentrations of 5-AzaC and of combined treatment with 5-AzaC and/or TSA in HNSCC cells. **a**, **b** MTT assays. The cells were treated with 0.05, 0.1, 0.5, 1 and 5 μ*M* 5-AzaC for 48 h. The *arrow* indicates the 5-AzaC concentration chosen for reversal of RARβ expression with simultaneously low cellular toxicity. $*P < 0.05$, vs atRA alone; $**P < 0.001$, vs TSA alone. **c** Clonogenic assay; a cut-point of 100 μm for colony diameter was fixed. The data presented are the reduction in colony-forming ability in relation to untreated control cultures, and are the means of three independent experiments (*error bars* standard deviation)

significantly induced the RARE-TK-LUC activity in both cell types compared to the baseline activity in the untreated controls. In contrast, treatment with 5-AzaC alone, or in combination with atRA, did not affect the RARE-TK-LUC activity in either cell type. These results indicate that exogenous RARβ activity is increased by atRA treatment by activation of transcription in a RARE ligand-dependent mechanism. Combined treatment with atRA and TSA induced a higher RARE-TK-LUC transcriptional activation in both cell types than atRA alone. Also, the combination of 5-AzaC, TSA and atRA was less effective than the combination of atRA and TSA in both cell types.

TSA induces high levels of histone H3 Lys-9 acetylation in the first exon of the RARβ gene

To examine the differential chromatin changes in the RARβ promoter regions caused by 5-AzaC, TSA or atRA, the CpG island region of the RARβ gene was divided into the region in the vicinity of the promoter (PCR A; 248 bp) and the distal region (PCR B; 146 bp), which contained the RARE and the first exon region, respectively. The schemes for the PCR A and PCR B of the 5′-untranslated RARβ region are depicted in Fig. 5a. In SqCC/Y1 cells atRA alone slightly increased the level of acetylated Lys-9 histone H3 in the vicinity of the promoter (PCR A containing RARE) (Fig. 5b). TSA alone greatly increased the acetylation of the first exon (threefold) (PCR B), but did not induce acetylation in

the vicinity of the promoter. 5-AzaC alone and atRA and 5-AzaC combined produced the same acetylation status as atRA alone. Treatments with TSA and atRA combined and 5-AzaC and TSA produced showed similar results in the first exon as those produced by TSA treatment. In unmethylated 1483 cells, treatment with any drug alone did not produce a significant change in the acetylation of Lys-9 histone H3 in any region, but treatment with 5-AzaC + TSA + atRA combined greatly increased the levels of acetylation by approximately threefold in the distal region containing the first exon only (Fig. 5c). Thus, in 1483 cells TSA had a minimal effect on the acetylation of the Lys-9 histone H3 at the locus where no DNA methylation was observed, but had dramatic effects on acetylation in partially methylated SqCC/Y1 cells.

TSA alone or combined with TSA and atRA reduces histone H3 Lys-9 methylation in RARβ gene promoter regions

Methylation of the Lys-9 histone H3 in the two regions was analyzed using the anti-methylated Lys-9 antibody (Fig. 5d,e). In SqCC/Y1 cells, a high level of methylated Lys-9 in the untreated control was seen in both regions (Fig. 5d). Treatment with 5-AzaC, or TSA and 5-AzaC combined or atRA, TSA and 5-AzaC combined decreased the methylation of Lys-9 histone H3, especially in the distal region containing the first exon. Treatment with 5-AzaC and TSA combined or with 5-AzaC alone produced minimal reductions in Lys-9 methylation in the vicinity of the promoter containing only RARE. However, TSA treatment failed to reduce the high levels of methylation of Lys-9 histone H3 in the distal region containing the first exon of unmethylated 1483 cells (Fig. 5e).

Treatment with TSA and 5-AzaC combined significantly induces high levels of histone H3 Lys-4 methylation in the vicinity of the promoter and the distal region of the RARβ gene

To examine the effect of 5-AzaC, TSA, and atRA on other histone modifications, we analyzed the methylation of the Lys-4 histone H3 using an anti-methylated Lys-4 antibody. When SqCC/Y1 cells were treated with TSA and 5-AzaC combined and atRA, TSA and 5-AzaC combined, methylation of the Lys-4 histone H3 was highly increased in both regions (Fig. 5f). In unmethylated 1483 cells, treatment with TSA alone had no effect on the methylation of the Lys-4 histone H3 in either region, whereas 5-AzaC alone greatly increased the levels of methylation by approximately fourfold in the vicinity of the promoter containing the RARE (Fig. 5g). The combination 5-AzaC, TSA and atRA had dramatic effects on the methylation in both regions. Thus, in 1483 cells TSA had no effect on the methylation of the Lys-4 histone H3 at loci where no DNA methylation was observed.

Discussion

Suppression of RARβ is associated with an increase in the methylation of CpG islands in the RARβ promoter, and this increase may cause RARβ gene silencing [32]. Since resistance to atRA treatment may be caused by loss of RARβ expression, we looked at the basal constitutive expression of RARβ and the methylation status of the RARβ promoter region in atRA-resistant SqCC/

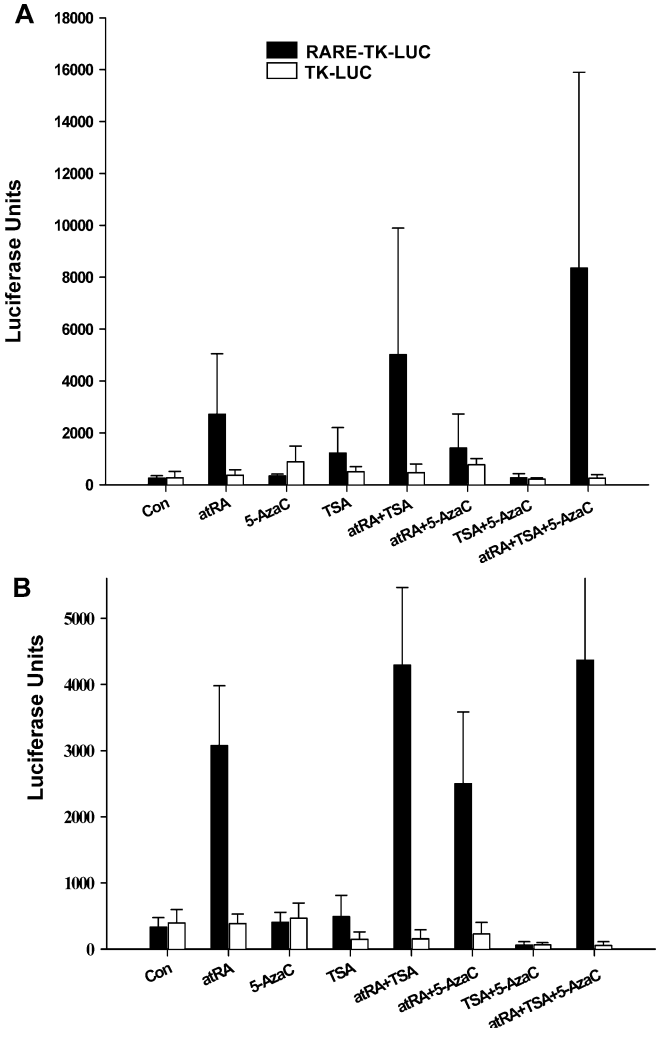

Fig. 4 Effects of single or combined treatments with 5-AzaC and/or TSA and atRA on transcriptional activation of reporter gene in 1483 cells (**a**) and SqCC/Y1 cells (**b**). RARE-TK-LUC and TK-LUC together with the β-galactosidase expression vector were transiently cotransfected into both cell types. Transfection efficiency was normalized for β-galactosidase activity. Experiments were repeated three times to ensure reproducibility, and the data presented are the means of these experiments (*error bars* standard deviation)

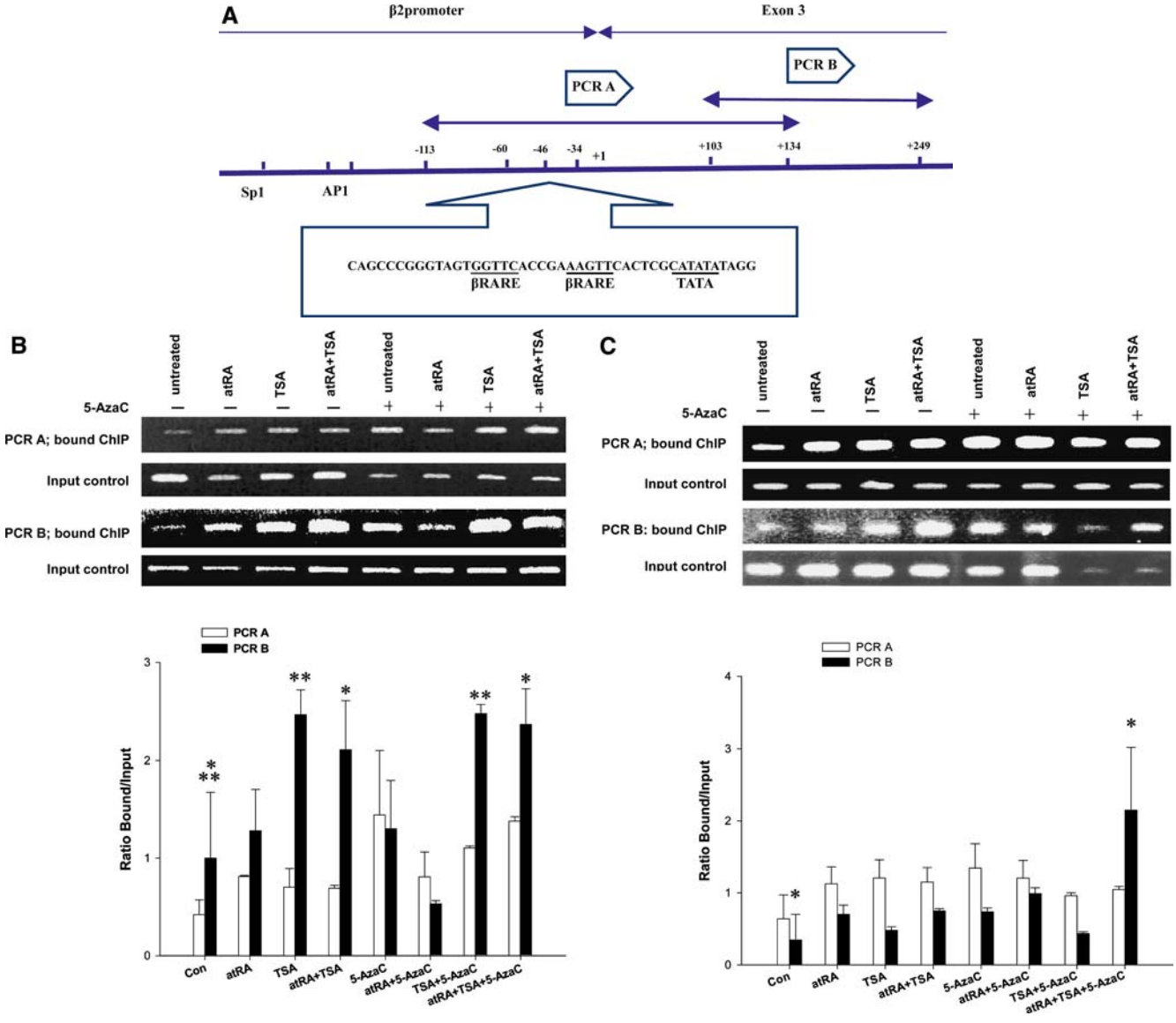

Fig. 5 Effects of a single or combined treatment with 5-AzaC and/or TSA, and atRA on acetylation or methylation of histone H3 at RARβ gene in both cell types. **a** A map of the 5′-untranslated region of RARβ gene. **b–c** ChIP analysis with anti-acetylated histone H3 Lys-9 antibody. **b** SqCC/Y1 cells; *, significantly higher than the value for untreated control ($P < 0.05$); **, significantly higher than the value for untreated control ($P < 0.01$). **c** 1483 cells; *, significantly higher than the value for untreated control ($P < 0.05$). **d–e** ChiP analysis with anti-methylated histone H3 Lys-9 antibody. **d** SqCC/Y1 cells; *, significantly higher than the value for untreated control ($P < 0.05$); **, significantly higher than the value for untreated control ($P < 0.01$). **e** 1483 cells. **f–g** ChIP analysis with anti-methylated histone H3 Lys-4 antibody. **f** SqCC/Y1 cells; *, significantly higher than the value for untreated control ($P < 0.05$). **g** 1483 cells; *, significantly higher than the value for untreated control ($P < 0.05$)

Y1 and atRA-sensitive 1483 cells. We found that TSA combined with atRA highly induced the expression of the RARβ gene in RARβ-negative SqCC/Y1 cells. However, full demethylation was not achieved by treating the partially methylated SqCC/Y1 cells with 5-AzaC. Also, TSA and 5-AzaC did not induce either RARβ expression or growth inhibition in the fully methylated 17B cells. This finding, that TSA cannot induce restoration of expression of highly methylated genes through hyperacetylation, has already been reported. For example, Cameron et al. have shown that TSA fails to restore the expression of methylated MLH1 and TIMP3 in the colorectal carcinoma cell line RKO, whereas it elevates the expression of unmethylated CDKN2B [33]. However, our findings may suggest that the RARβ gene of the other unmethylated allele may work for TSA-induced RARβ expression. In the case of partial methylation, histone acetylation may be important in the restoration of RARβ expression in a methylation-independent manner. Thus, our findings show that TSA enhances atRA-induced RARβ expression, and that the entire demethylation of the RARβ promoter is not an absolute requirement for restoring RARβ.

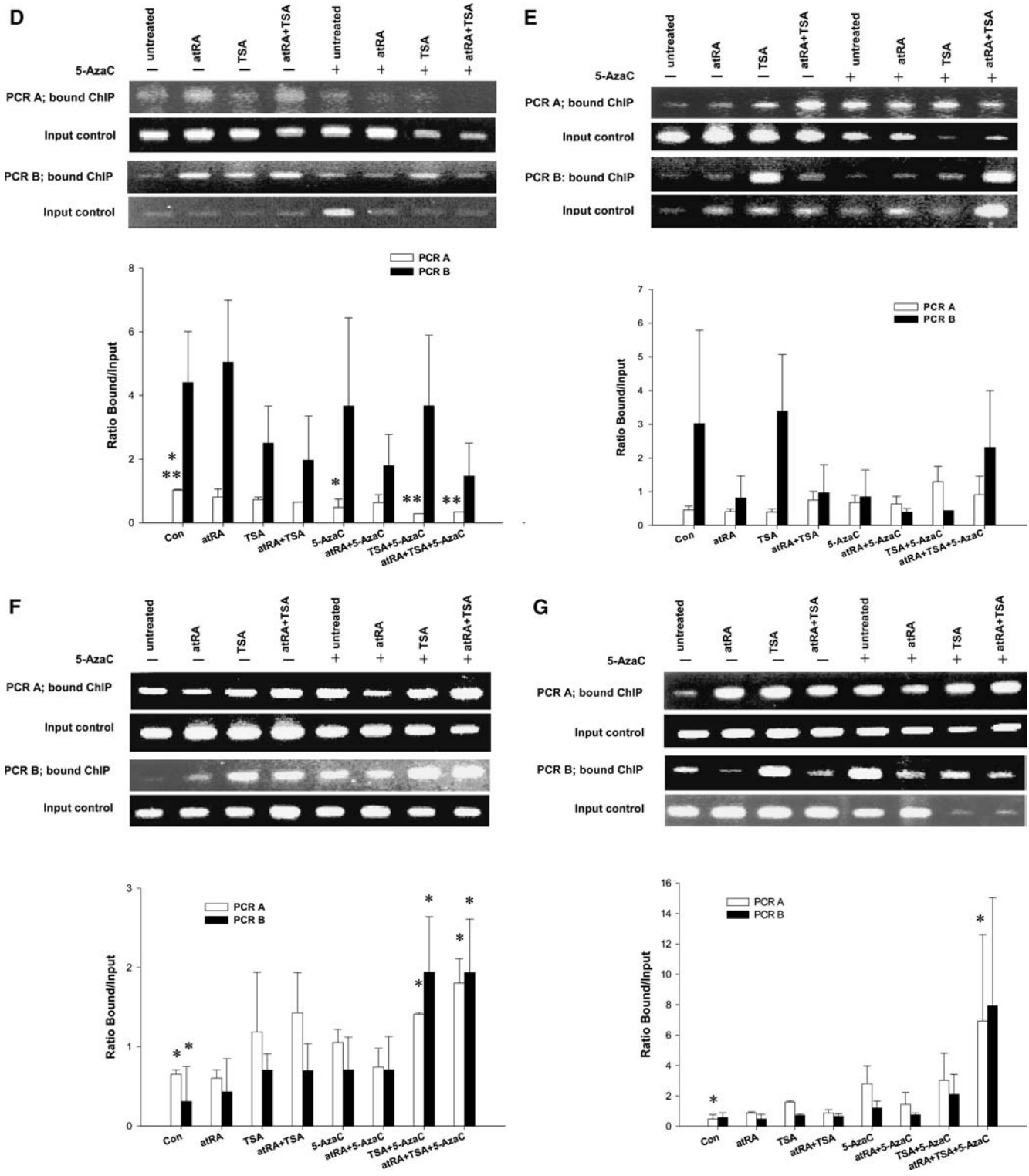

Fig. 5 (Contd.)

Interestingly, in this study, the level of RARα protein expression in SqCC/Y1 cells was increased by treating the cells with atRA or TSA alone. Also, a RARE-TK-LUC reporter system showed that atRA induced RARE-TK-LUC transcriptional activity in RARβ-negative SqCC/Y1 cells. In other study, Arapshian et al. have shown that RA induces exogenous RARβ promoter transactivation in RARβ-negative MCF-7 cells. Also, the βRARE site has been known to be conserved in the murine β2 and human RARα promoters [34]. Therefore, we conclude that atRA may induce the expression of RARα in RARβ-negative SqCC/Y1 cells and that the binding of RARα and RARE can enhance the transcriptional activity of RARE-TK-LUC gene.

Also, it seems that TSA can enhance this transcriptional activity through the induction of both RARα and RARβ gene expression. Another mechanism through which TSA could restore the RARβ gene in RARβ-negative cell lines is by hyperacetylation of histone. TSA increases the endonuclease sensitivity within the RARβ promoter, suggesting that TSA treatment may alter the local chromatin environment, enhancing the assembly of RAR/RXR heterodimers. Sirchia et al. have proved that TSA affects the chromatin state of the RARβ promoter, resulting in the restoration of RA-induced RARβ transcription in RA-resistant breast cancer cells [19].

In this study, we sought to determine if demethylation or reacetylation of the methylated RARβ promoter region could restore RARβ expression and consequently improve the response to RA in RA-resistant HNSCC cells. Treatment with atRA alone did not inhibit the growth of SqCC/Y1 and 17B cells. In addition, 17B cells were less sensitive than SqCC/Y1 cells to single or combined treatment with atRA, TSA, and 5-AzaC. However, in SqCC/Y1 cells TSA and atRA in combination acted synergistically in increasing growth inhibition. In previous studies, combinations of inhibitors of histone deacetylases (such as sodium butyrate, phenyl acetate, TSA and CBHA) and retinoids (atRA, or 13-*cis*-RA) have been shown to act synergistically in vitro in APL cells containing PML-RAR [35] as well as PLZF-RAR [36], AML cells [37], and prostate carcinoma and neuroblastoma cells [38, 39]. However, 5-AzaC, TSA, atRA, or any combinations of these agents could not restore RARβ mRNA expression in fully methylated 17B cells. Thus, we suggest that a demethylating or hyperacetylating agent can restore suppressed RARβ expression in only cancer cells with a partially methylated RARβ promoter.

In this study, RARβ expression was restored by 5-AzaC treatment even though the 5-AzaC (at 0.1–5 μ*M*) did not completely induce the demethylation of the RARβ promoter region in partially methylated SqCC/Y1 cells. This finding has also been reported by others in gastric cancer cell lines [31]. As shown by ChIP, 5-AzaC was able to restore RARβ expression by inducing the acetylation of Lys-9 and the methylation of Lys-4. Although 5-AzaC failed to achieve complete demethylation, it restored the expression of RARβ through other mechanisms.

Recent evidence shows the functional significance of histone acetylation and/or methylation to a transcriptional control, with the target of acetylation or methylation being specific Lys-9 residues in the H3 tails [40, 41]. Also, it has been reported that the downstream/exon regions of a gene become aberrantly methylated more easily than the promoter regions in normal and cancer cells [42]. In our experiments, TSA greatly increased the resultant Lys-9 H3 acetylation in the first exon, but did not induce acetylation in the vicinity of the promoter in either cell type, suggesting that the chromatin alterations caused by TSA are region-specific. In addition, Lys-9 methylation has been demonstrated to be inversely correlated with Lys-9 acetylation in partially methylated SqCC/Y1 cells. 5-AzaC or combined TSA and 5-AzaC reduced methylation of Lys-9 H3 in the first exon. However, TSA induced minimal reduction of Lys-9 methylation in the vicinity the promoter containing RARE. This region shows methylation of CpG sites in SqCC/Y1 cells, and TSA may not induce hyperacetylation in the methylated CpG region. Therefore, we suggest that TSA can restore RARβ expression through hyperacetylation of the RARβ promoter of the unmethylated allele or the first exon (containing no methylation). Also, we showed that combined treatment with TSA and 5-AzaC markedly increased the methylation of the Lys-4 histone H3 at all loci. This combination may act synergistically in modifying histones to affect RARβ expression in SqCC/Y1 cells, showing that the interaction of TSA and 5-AzaC may increase the methylation of Lys-4 histone H3 in both regions.

From a clinical point of view, TSA and 5-AzaC treatment may restore RA sensitivity in RA-resistant RARβ-negative HNSCCs with partially methylated RARβ promoter regions. However, although it has been reported that treatment with TSA or 5-AzaC singly at high concentrations is clinically needed to restore the RARβ gene, using high concentrations may cause severe toxicity. Thus, in this study, we showed that the RARβ gene and retinoid sensitivity can be restored without severe toxicity by combining low doses of TSA and 5-AzaC due to the synergistic growth inhibitory effect of these agents.

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