5-AzaC Treatment Enhances Expression of Transforming Growth Factor-β Receptors through Down-regulation of Sp3*

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We have previously reported that Sp3 acts as a transcriptional repressor of transforming growth factor-β receptors type I (RI) and type II (RII). We now present data suggesting that treatment of MCF-7L breast and GEO colon cancer cells with 5-aza cytidine (5-azaC) leads to down-regulation of Sp3 and the concomitant induction of RI and RII. Western blot and gel shift analyses on 5-azaC-treated MCF-7L and GEO nuclear extracts indicated reduced Sp3 protein levels and decreased binding of Sp3 protein to radiolabeled consensus Sp1 oligonucleotide. Southwestern analysis detected decreased binding of Sp3 to RI and RII promoters in 5-azaC-treated MCF-7L and GEO cells, suggesting a correlation between decreased Sp3 binding and enhanced RI and RII expression in these cells. Reverse transcription-polymerase chain reaction and nuclear run-on data from 5-azaC-treated MCF-7L and GEO cells indicated down-regulation of Sp3 mRNA as a result of decreased transcription of Sp3. We reported earlier that 5-azaC treatment induces RI and RII expression through increased Sp1 protein levels/activities in these cells. These studies demonstrate that the effect of 5-azaC involves a combination of effects on Sp1 and Sp3.

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TGF-β* plays an important role in the regulation of cell proliferation, differentiation, and extracellular matrix remodeling in different cell types (1, 2). TGF-β exerts its effects through high affinity cell surface receptors, which are referred to as types I (RI), II (RII), and III. RI and RII are serine/threonine kinases, and an active receptor complex consists of two molecules each of RI and RII, which are essential for TGF-β signal transduction and inhibition of cell growth (3–6). Recent studies indicate that following TGF-β binding and subsequent activation of RI by RII, RI phosphorylates smad 2 and/or smad 3, which can then associate with smad 4 and translocate to the nucleus, where binding to the target DNA or other DNA-binding proteins occurs. In contrast, smad 7 was found to antagonize the TGF-β signaling pathway by binding to RI, thereby preventing the activation of smad 2 and smad 3 (7).

One of the vital roles of TGF-β is the growth inhibition of normal epithelial cells, as well as some cancer cells. Because RI and RII are important for TGF-β-mediated growth inhibition, a loss of either receptor contributes to TGF-β resistance and subsequent tumor formation and progression (8–10). TGF-β resistance due to a mutation of the RII gene in gastric and colon carcinoma cells (9, 11, 12) or transcriptional repression of RII due to decreased binding of nuclear proteins to the RII promoter in keratinocytes and breast cancer cells was reported (13, 14). TGF-β resistance due to DNA methylation of the RI promoter or RI promoter repression by Sp1 deficiency was reported in a subset of gastric and colon cancer cells (15, 16). RI and RII replacement in cells that lack or show reduced levels of TGF-β receptors led to restoration of TGF-β response and subsequent reversal of malignancy, as seen in breast and colon cancer cells (8, 10).

The promoters for RI and RII have been characterized (17, 18). RI and RII promoters lack distinct TATA boxes, are highly GC-rich, and depend on Sp1 transcription factor for the initiation of transcription. Whereas the RI promoter contains four consensus and several putative Sp1 sites, the RII promoter contains two Sp1 sites. Within the Sp gene family of transcription factors, Sp1, Sp2, and Sp4 are known to be activators of gene transcription, whereas Sp3 is generally considered to be a repressor (19). Sp1 and Sp3 transcription factors recognize the same DNA element and have similar binding affinities. Sp3 has been shown to repress Sp1-mediated trans-activation of several genes (20–22).

Recent studies indicated DNA methylation as a mode of inactivation of several genes, including some that are involved in cell cycle control. DNA methyltransferase inhibitors, 5-aza cytidine (5-azaC) and 5-aza-2’ deoxycytidine are the most commonly used DNA demethylating agents to reverse methylation and reactivate the expression of these genes (23). MCF-7L and GEO cells are resistant to growth inhibition by TGF-β because of the reduced expression of RI and RII (8, 10). We have previously reported that treatment of these cells with 5-azaC induced RI mRNA levels and consequently increased the expression of cell-surface RI and RII (14, 16). Significantly, increased RI and RII expression resulted in the restoration of TGF-β response, as evidenced by the enhanced activity of a TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter in the 5-azaC-treated cells (14, 16). However, Southern analysis following 5-azaC treatment ruled out the demethylation of RI and RII genes as a contributor to RI and RII expression (14, 16). We have shown that MCF-7L and GEO cells were Sp1-deficient and that 5-azaC treatment increased Sp1 protein levels as a result of increased Sp1 protein stability, leading to RI and RII expression (14, 16). Furthermore, we have demonstrated that MCF-7L and GEO cells express high levels of Sp3 protein, which acts as a transcriptional repressor of RII (24). This raises the question of how methyltransferase inhibition affects Sp3 as well as Sp1. We
now report that 5-azaC treatment of MCF-7L breast and GEO colon cancer cells decreases Sp3 protein levels and hence results in decreased binding to RI and RII promoters. This decreased Sp3 binding contributes to enhanced RI and RII expression in these cells. Taken together, the previous and present studies on MCF-7L breast and GEO colon cancer cells indicate that the demethylation-enhanced expression of TGF-β receptors is due to a combination of effects on Sp1 and Sp3. Moreover, whereas modulation of Sp1 was shown to be post-transcriptional, Sp3 repression occurs through decreased transcription of the Sp3 gene.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7L cells were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum (Sigma), amino acids, antibiotics, pyruvate, and vitamins (Life Technologies, Inc.). GEO colon cancer cells were grown in serum-free medium as previously described (10). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂. 5-AzaC was added to growth medium in two 24-h pulses on days 2 and 5. On day 6, 5-azaC-containing medium was replaced with fresh medium, and the cells were grown for another 2 days in the absence of 5-azaC. Cells were used on day 8 for RNA determinations, isolation of nuclear extracts for electrophoretic mobility shift assays (EMSA), and Western blots.

Western Immunoblot Analysis of Sp3—Nuclear extracts (5 μg) were obtained from control and 5-azaC-treated MCF-7L breast and GEO colon carcinoma cells, and Western analysis was performed as described previously (14). Rabbit anti-human Sp3 and c-Jun polyclonal antibodies were purchased from Santa Cruz Biotechnology. EMSA—The consensus Sp1 oligonucleotide was end-labeled using [γ-32P]ATP, and electrophoretic mobility shift assays were performed as described previously (14). Whenever Sp1 and Sp3 antibodies were used, the nuclear extracts were incubated with 2 μg of Sp1 or Sp3 antibody (anti-rabbit, Santa Cruz Biotechnology) for 15 min on ice prior to the addition of 32P-labeled oligonucleotide.

Southern Blotting—Southern analysis was performed as described previously (22). Briefly, nuclear extracts were resolved by 7.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Following transfer, the membrane was blocked overnight with 2.5% (w/v) nonfat dried milk in 25 mM HEPES, pH 8.0, 1 mM dithiothreitol, 10% (v/v) glycerol, 50 mM NaCl, and 1 mM EDTA. The membrane was then incubated with [α-32P]dCTP-labeled RI (~618 bp to the start site) and RII (~274 bp to the start site) probe (antisense primer) and poly(dI-dC) as a competitor for nonspecific binding for 4 h. Later, the membrane was washed with wash buffer (10 μM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol), dried, and autoradiographed.

RT-PCR—Total RNA from control and 5-azaC-treated MCF-7L and GEO cells was reverse-transcribed into cDNA. PCR analysis was then performed to determine the RI, RII, and Sp3 expression levels in control and 5-azaC-treated MCF-7L and GEO cells using the respective cDNAs as templates. Primers for actin were used as controls to determine the RI, RII, and Sp3 expression levels. RT-PCR analysis is a rough estimate of the changes in RNA levels following 5-azaC treatment. A total of 30 cycles of amplification were performed. Primers for RI generate a 685-bp fragment as follows: sense primers, TGC TGG CAT TGG GAT GT; antisense primers, TGG TCC TGG GCT ATT GAA TCA. Primers for RII generate a 1003-bp fragment as follows: sense primers, GCC AAC AAC ATC AAC CAC ACA AAC; antisense primers, TAG TGT TTA GGG AGC CTT CAG. Primers for actin generate a 621-bp fragment as follows: sense primers, ACA CAG TGC TCA TGG ACT AGG; antisense primers, AGG GCC CAG CCT CAG CAT ACT. Primers for Sp3 generate a 450-bp fragment as follows: sense primers, AGG TTC AGG GAG TTG CAA TT; antisense primers, TCT GTG CCT GTG TCT CAG.

Nuclear Run-on Assay—Isolation of nuclei and nuclear run-on assays were performed as described previously (16). Briefly, control and 5-azaC-treated MCF-7L and GEO cells were lysed, and the nuclei were pelleted by brief centrifugation. The pellet was resuspended in 200 μl of reaction buffer (150 mM potassium acetate; 10 mM MgCl₂; 0.1 mM dithiothreitol; 50 mM HEPES (pH 8.0); 10% glycerol, 0.5 mM each of ATP, CTP, and GTP; 13 μM UTP; and 100 μM of [γ-32P]UTP (3000 Ci/mmol), PerkinElmer Life Sciences) and incubated for 30 min at 30 °C. The [32P]RNA was isolated by cesium gradient centrifugation, and an equal amount of radioactivity in 5 μl of hybridization buffer was added to each filter and incubated for 2 days at 45 °C. Filters were prepared using the Schleicher and Schuell slot blot system. Each slot was loaded with 10 μg of linearized, alkali-denatured plasmid and then washed with 1 M ammonium acetate. The Sp3 plasmid contained a 3.14-kilobase linearized NotI cDNA fragment. The actin plasmid with a 1.6-kilobase linearized BamHI-HindIII cDNA fragment and vector without Sp3 insert were used as controls. The plasmid DNA was immobilized on nitrocellulose filters by baking at 80 °C for 1.5 h in a vacuum oven. After hybridization, filters were washed twice in 2× SSPE (sodium chloride, sodium phosphate, and EDTA) solution at 45 °C for a total of 60 min and then transferred to 2× SSPE solution containing RNase A (10 μg/ml), incubated for 20 min at 37 °C, air-dried, and exposed to Kodak XAR-5 film with intensifying screens.

Stable Transfections—The CMV-Sp3 cDNA vector or the CMV control vector without Sp3 cDNA was stably transfected into MCF-7L cells using the Fugene 6 chemical method (Roche Molecular Biochemicals). The control vector-transfected cells are referred to as MCF-7L Neo, and Sp3 transfectedants are referred to as MCF-7L Sp3 cells.

RESULTS

Effect of 5-AzaC on RI and RII Expression—RT-PCR analysis using RI and RII primers was performed on total RNA from control and 5-azaC-treated MCF-7L and GEO cells to determine whether treatment with the DNA methyltransferase inhibitor 5-aza cytidine leads to RI and RII expression. RI and RII transcripts were induced in the 5-azaC-treated MCF-7L and GEO cells (Fig. 1). Actin, which was used as a control, was not affected, thus confirming the selectivity of 5-azaC effects on RI and RII expression.

5-AzaC Effects on Sp3 Protein—We have previously reported that Sp3 acts as a transcriptional repressor of RI and RII in MCF-7L and GEO cells (24). To determine whether 5-azaC-mediated Sp3 down-regulation is leading to RI and RII expression in MCF-7L and GEO cells, Western immunoblot analysis using Sp3 antibody was performed on the control and 5-azaC-treated MCF-7L and GEO cells. Sp3 antibody recognizes a doublet at 115 kDa and two 68-kDa species. The Sp3 protein doublet at 115 kDa may be the result of differential post-translational modification, as seen in the case of Sp1 (14). The 68–70-kDa species are the result of differential internal translation initiation (25). Only the 115-kDa species has been reported to be biologically active. 5-AzaC treatment reduced the expression of all the Sp3 isoforms, whereas there was no difference in the c-Jun levels, indicating selectivity of Sp3 modulation (Fig. 2).

EMSA—EMSA were performed using control and 5-azaC-treated nuclear extracts with [32P]-labeled consensus Sp1 oligonucleotide to determine the DNA binding activities of Sp3. One high mobility complex and one low mobility complex were detected in the control MCF-7L and GEO nuclear extracts (Fig. 3a, lanes 1 and 2). Preincubation of the control nuclear extracts with Sp3 antibody prior to the addition of [32P]-labeled oligonucleotide completely depleted the high mobility complex and a major portion of the low mobility complex, indicating that those complexes contain Sp3 protein (Fig. 3a, lanes 3 and 8). 5-AzaC-treated MCF-7L and GEO nuclear extracts either with or without Sp3 antibody preincubation show loss of binding of the high mobility complex but not the low mobility complex (Fig. 3a, lanes 4, 5, 9, and 10). Sp1 and Sp3 proteins recognize the same
GC element and have similar DNA binding affinities (19). We have previously reported that 5-azaC-treated MCF-7L and GEO nuclear extracts show enhanced Sp1 binding to radiolabeled Sp1 oligonucleotide (14, 16). To determine whether the low mobility complex in the 5-azaC-treated MCF-7L and GEO nuclear extracts contains Sp1, we have preincubated the 5-azaC-treated MCF-7L and GEO nuclear extracts with Sp1 antibody prior to the addition of 32P-labeled Sp1 oligonucleotide. The data indicate depletion of low mobility complex in the 5-azaC-treated MCF-7L and GEO nuclear extracts (Fig. 3a, lanes 6 and 11), thus confirming the presence of Sp1 in the complex. Consequently, 5-azaC-treated MCF-7L and GEO nuclear extracts show decreased Sp3 binding but enhanced Sp1 binding to the radiolabeled Sp1 oligonucleotide. Preincubation of the control MCF-7L and GEO nuclear extracts with Sp1 antibody prior to the addition of 32P-labeled Sp1 oligonucleotide did not deplete the high mobility complex and depleted only a minor portion of the low mobility complex (Fig. 3b, lanes 3 and 5). These complexes were depleted when preincubated with Sp3 antibody (Fig. 3a, lanes 3 and 8), which indicates the specificity of the Sp1 and Sp3 antibodies used.

**Effect of 5-AzaC on Sp3 Binding to RI and RII Promoters**—We have previously reported that Sp3 binding to RI and RII promoters is a contributor to RI and RII repression in MCF-7L breast and GEO colon cancer cells (24). Southwestern analysis using radiolabeled RI and RII promoters was carried out to determine whether the decreased Sp3 binding to RI and RII promoters was contributing to RI and RII induction in the 5-azaC-treated MCF-7L and GEO cells. The data indicate a significant loss of Sp3 binding to RI promoter (Fig. 4) and RII promoter (Fig. 5) in the 5-azaC-treated MCF-7L and GEO cells. We observed the binding of 95–100-kDa protein species to RI and RII promoters in the control and 5-azaC-treated cells. However, these protein species were not influenced by 5-azaC treatment.

**Effect of 5-AzaC on Sp3 Expression**—We carried out RT-PCR analysis using Sp3 primers on the total RNA from control and 5-azaC-treated MCF-7L and GEO cells to determine whether the 5-azaC-mediated decrease in Sp3 protein levels (Fig. 2) was due to decreased Sp3 mRNA expression. The Sp3 message was down-regulated in the 5-azaC-treated MCF-7L and GEO cells (Fig. 6). Actin expression levels were not affected, thus confirming the selectivity of 5-azaC effects on Sp3 expression. Transcriptional analyses of Sp3 using nuclear run-on assays were performed to determine whether 5-azaC was affecting Sp3 transcription, resulting in down-regulation of Sp3 mRNA. The data show repression of Sp3 transcription in the 5-azaC-treated MCF-7L and GEO cells (Fig. 7). Transcription of actin, which was used as a control, was not affected, thus confirming the selectivity of 5-azaC effects on Sp3 transcription.

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**FIG. 2. Western immunoblot analysis of Sp3.** Nuclear extracts (5 μg) from control and 5-azaC-treated MCF-7L and GEO cells were resolved by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit anti-human Sp3 and c-Jun antibodies. The Sp3 antibody recognizes a doublet at 115 kDa and 68–70-kDa species. The 68–70-kDa species result from differential internal translational initiation.

**FIG. 3. EMSA.** EMSA was performed using 32P-labeled consensus Sp1 oligonucleotide and nuclear extracts from control and 5-azaC-treated MCF-7L and GEO cells. Whenever Sp1 and Sp3 antibodies were used, the nuclear extracts were preincubated with 2 μg of either Sp1 or Sp3 antibody prior to the addition of 32P-labeled oligonucleotide.

**FIG. 4. Detection of Sp3 binding to RI promoter.** Southwestern analysis was performed by resolving control and 5-azaC-treated MCF-7L and GEO nuclear extracts using 7.5% SDS-PAGE and probing the nitrocellulose membrane following protein transfer with radiolabeled RI promoter probe.
5-AzaC Induces TGF-β Receptors

**DISCUSSION**

TGF-β receptors RI and RII are key players in the TGF-β-mediated growth suppression of normal epithelial cells, as well as some cancer cells. Loss of expression of either RI or RII has been associated with TGF-β resistance, leading to tumor formation and progression (8, 9, 10, 12, 26, 27). GEO colon and MCF-7-L breast cancer cells show reduced levels of RI and RII (14, 16). Ectopic TGF-β receptor expression in these cells reduced tumorigenicity in athymic nude mice, thus suggesting the role of TGF-β receptors as tumor suppressors (8, 10). We have previously reported the induction of RI and RII expression in GEO and MCF-7-L cells through increased Sp1 protein levels/activities by the DNA methyltransferase inhibitor 5-azaC.

**Effect of 5-AzaC on RI and RII Expression in MCF-7L Neo and MCF-7L Sp3 Cells—5-AzaC treatment of MCF-7L cells led to TGF-β receptor induction through a combination of increased Sp1 protein levels/activities (14) and decreased Sp3 protein levels/activities (Fig. 2 and 3a). To further confirm that 5-azaC-mediated Sp3 down-regulation is required for the TGF-β receptor induction in MCF-7L cells, we stably expressed Sp3 cDNA under the control of a CMV promoter in MCF-7L cells and analyzed the 5-azaC effects on TGF-β receptor induction in MCF-7L Neo and MCF-7L Sp3 cells. If 5-azaC-mediated Sp3 down-regulation was contributing to TGF-β receptor induction in MCF-7L cells, we would expect to see the TGF-β receptor induction in MCF-7L Neo and MCF-7L Sp3 cells. If 5-azaC-mediated Sp3 down-regulation was contributing to TGF-β receptor induction in MCF-7L cells, we would expect to see the TGF-β receptor induction in MCF-7L Neo and MCF-7L Sp3 cells. Consequently, 5-azaC-treated MCF-7L Neo nuclear extracts showed decreased Sp3 binding but enhanced Sp1 binding leading to TGF-β receptor expression. In contrast, ectopic Sp3 expressing MCF-7L Sp3 cells did not show loss of Sp3 binding or TGF-β receptor expression following 5-azaC treatment, indicating continued presence of Sp3 as a cause for the blockade of TGF-β receptor induction in MCF-7L Sp3 cells.

**Effect of 5-AzaC on Sp3 transcription—**Sp3 transcriptional analyses were performed in control and 5-azaC-treated MCF-7L and GEO cells using nuclear run-on assays as described under “Experimental Procedures.”

**Effect of 5-AzaC on Sp3 mRNAs levels—**Total RNA from control and 5-azaC-treated MCF-7L and GEO cells was reverse-transcribed into cDNA, and PCR analysis was performed using primers for Sp3 and actin as described under “Experimental Procedures.”

**Effect of 5-AzaC on Ectopic Sp3 Binding Affinities—**EMSAs were performed using MCF-7L Neo and MCF-7L Sp3 nuclear extracts with 32P-labeled consensus Sp1 oligonucleotide to determine the DNA binding activities of Sp3. One high mobility complex and one low mobility complex were detected in the MCF-7L Neo and MCF-7L Sp3 cells (Fig. 10a, lane 1, and b, lane 1). Preincubation of the MCF-7L Neo nuclear extracts with Sp3 antibody prior to the addition of 32P-labeled oligonucleotide completely depleted the high mobility complex and a major portion of the low mobility complex, indicating that both complexes contain Sp3 protein (Fig. 10a, lane 2). 5-AzaC-treated MCF-7L Neo nuclear extracts either with or without Sp3 antibody preincubation showed loss of binding of the high mobility complex but not the low mobility complex, indicating that only the high mobility complex contains Sp3 protein (Fig. 10a, lanes 3 and 4). Preincubation of the 5-azaC-treated MCF-7L Neo nuclear extracts with Sp1 antibody showed depletion of the low mobility complex, indicating that the complex contains Sp1 (Fig. 10a, lane 5). In contrast, preincubation of 5-azaC-treated MCF-7L Sp3 nuclear extracts with Sp3 antibody showed only minor loss of binding of the low mobility complex, indicating that this complex contains low amounts of Sp1 protein (Fig. 10b, lane 3). However, preincubation of the 5-azaC-treated MCF-7L Sp3 nuclear extracts with Sp3 antibody led to the depletion of the high mobility complex and a major portion of the low mobility complex, indicating that both complexes contain Sp3 protein (Fig. 10b, lane 4). Consequently, 5-azaC-treated MCF-7L Neo nuclear extracts showed decreased Sp3 binding but enhanced Sp1 binding leading to TGF-β receptor expression. In contrast, ectopic Sp3 expressing MCF-7L Sp3 cells did not show loss of Sp3 binding or TGF-β receptor expression following 5-azaC treatment, indicating continued presence of Sp3 as a cause for the blockade of TGF-β receptor induction in MCF-7L Sp3 cells.

**FIG. 5. Detection of Sp3 binding to RI promoter.** Southwestern analysis was performed by resolving control and 5-azaC-treated MCF-7L and GEO nuclear extracts using 7.5% SDS-PAGE and probing the nitrocellulose membrane following protein transfer with radiolabeled RI promoter probe.

**FIG. 6. 5-AzaC decreases Sp3 mRNA levels.** Total RNA from control and 5-azaC-treated MCF-7L and GEO cells was reverse-transcribed into cDNA, and PCR analysis was performed using primers for Sp3 and actin as described under “Experimental Procedures.”

**FIG. 7. Effect of 5-azaC on Sp3 transcription.** Sp3 transcriptional analyses were performed in control and 5-azaC-treated MCF-7L and GEO cells using nuclear run-on assays as described under “Experimental Procedures.”

**FIG. 8. 5-AzaC effect on RI and RII expression in MCF-7L neo and MCF-7L Sp3 cells.** Total RNA from control and 5-azaC-treated MCF-7L Neo and MCF-7L Sp3 cells was reverse-transcribed into cDNA, and PCR analysis was performed using primers for RI, RII, and actin as described under “Experimental Procedures.”
cytidine (14, 16). Furthermore, we have shown that another member of the Sp gene family, Sp3, acts as a transcriptional repressor of RI and RII in these cells (24). We now report that in addition to increased Sp1, 5-azaC treatment down-regulates Sp3 expression, thus contributing to RI and RII induction in MCF-7L and GEO cells. These studies demonstrate that the effect of 5-azaC involves a combination of effects on Sp1 and Sp3.

The RI and RII promoters lack distinct TATA boxes. However, they contain multiple GC boxes and depend upon Sp1 for the initiation of transcription (17, 18). MCF-7L and GEO cells are resistant to growth inhibition by TGF-β because of the reduced expression of RI and RII (8, 10). We have previously reported that treatment of these cells with 5-azaC induced TGF-β receptor mRNA levels and consequently increased the expression of cell-surface RI and RII (14, 16). Significantly, increased RI and RII expression resulted in the restoration of TGF-β response as evidenced by the enhanced activity of a TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter in the 5-azaC-treated cells (14, 16). However, Southern analysis following 5-azaC treatment ruled out the demethylation of RI and RII genes as a contributor to RI and RII expression (14, 16). We have shown that MCF-7L and GEO cells were Sp1-deficient and that 5-azaC treatment increased Sp1 protein levels as a result of increased Sp1 protein stability, leading to RI and RII expression (14, 16). However, 5-azaC treatment also decreased Sp3 protein levels in MCF-7L and GEO cells (Fig. 2). The Sp3 protein doublet at 115 kDa may be the result of differential posttranslational modification as seen in the case of Sp1 (14). The 115-kDa Sp3 protein is the biologically active form, and the inactive 68–70-kDa species arises as a result of differential internal translational initiation (25). However, as opposed to direct promoter repression, Sp3-derived 68–70-kDa species can bind GC elements and thus act as inhibitors of Sp1-mediated gene activation (25).

EMSA analyses using Sp3 antibody on the 5-azaC-treated MCF-7L and GEO nuclear extracts indicated the depletion of a high mobility complex but not the low mobility complex (Fig. 3a, lanes 5 and 10). However, this low mobility complex was depleted when preincubated with Sp1 antibody (Fig. 3a, lanes 6 and 11). Consequently, 5-azaC treatment of MCF-7L and GEO cells leads to enhanced Sp1 activity but decreased Sp3 activity. We have previously reported that Sp3 binds to RI and RII promoters and acts as a transcriptional repressor in MCF-7L and GEO cells (24). The decreased Sp3 activity in the 5-azaC-treated MCF-7L and GEO cells was also reflected in the Southwestern analysis, in which decreased Sp3 binding to native RI and RII promoters was observed (Figs. 4 and 5). This resulted in enhanced RI and RII expression (Fig. 1). We observed the binding of 95–100-kDa protein species to RI and RII promoters in the control and 5-azaC-treated cells. However, these protein species were not influenced by 5-azaC treatment, thus suggesting the selectivity of 5-azaC effects on Sp3. Sp3 was also reported to repress the Sp1-mediated transcription of several genes (19–22). However, Sp3 was able to trans-activate c-fos and c-myc promoters (28). Consequently, availability of specific co-activators, co-repressors, or other transcription factors may determine whether Sp3 activates or inhibits transcription of a specific gene. It was previously reported that Sp3 could repress the activity of multiple Sp1 sites contained in the dihydrofolate reductase promoter but not the single Sp1 site contained in the thymidine kinase promoter (21). Thus, Sp3 effects may also depend on the context and/or the number of Sp1 binding sites.

5-azaC treatment of MCF-7L and GEO cells increased Sp1 protein levels as a result of increased Sp1 protein stability. However, it did not affect Sp1 transcription because the Sp1 mRNA levels remained the same in control and 5-azaC-treated MCF-7L and GEO cells (14, 16). In contrast, the Sp3 mRNA levels were down-regulated in the 5-azaC-treated MCF-7L and GEO cells (Fig. 6). The actin mRNA levels were not affected, thus confirming the selectivity of 5-azaC effects on Sp3. The decreased Sp3 mRNA levels in the 5-azaC-treated cells was a result of decreased Sp3 transcription in the 5-azaC-treated MCF-7L and GEO cells (Fig. 7). The inhibition of DNA methylation following 5-azaC treatment is generally associated with enhanced expression of target genes. However, it is interesting to note that we have observed a down-regulation of Sp3 expression in the 5-azaC-treated MCF-7L and GEO cells. We would expect to see an increase in the Sp3 expression levels if the 5-azaC was directly affecting Sp3 promoter or enhancing the activity of a transcription factor required for Sp3 transcription. In contrast, if 5-azaC activates the transcription of a repressor molecule of Sp3 promoter, we would expect to see the down-regulation of Sp3 mRNA levels. The Sp3 promoter, like the Sp1 promoter, has not been cloned and hence is not available to characterize the 5-azaC affects on Sp3 promoter. Overall, the previous and present 5-azaC studies on MCF-7L breast and GEO colon cancer cells suggest that 5-azaC treatment increases Sp1 protein levels (14, 16) but decreases Sp3 protein levels (Fig. 2), leading to RI and RII transcription in these cells. The constitutive Sp3 expression under the control of a CMV promoter (CMV-Sp3 cDNA) blocked the 5-azaC-mediated RI and RII induction in MCF-7L cells (Fig. 8). This indicated that...
5-azaC could affect the endogenous Sp3 expression but not ectopic Sp3 expression, which was under the control of a CMV promoter (Fig. 9). Moreover, the data further confirm that 5-azaC-mediated Sp3 down-regulation is required for RI and RII induction in MCF-7L cells. Consequently, transcriptional control of TGF-β receptor expression is dependent upon the Sp1/Sp3 protein levels/activities and cancer cells can gain a growth advantage by favoring receptor repression through a combination of reduced Sp1 and elevated Sp3 expression.

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