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Reversion of Transcriptional Repression of Sp1 by 5 aza-2′ Deoxycytidine Restores TGF-β Type II Receptor Expression in the Pancreatic Cancer Cell Line MIA PaCa-2[1]

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ABSTRACT

The pancreatic cancer cell line, MIA PaCa-2 is not responsive to transforming growth factor β (TGF-β) because of a lack of expression of the TGF-β type II receptor (RII). We show that the lack of RII expression is caused by a deficit of the transcription factor Sp1. Nuclear run-off assays and Western immunoblot showed low levels of transcription and protein levels of Sp1, respectively. Treatment of MIA PaCa-2 cells with the DNA methyl transferase inhibitor, 5-aza-2′-deoxycytidine, resulted in an increase in the rate of Sp1 transcription, in Sp1 protein expression, and in the binding of Sp1 to the RII promoter. Ectopic expression of Sp1 cDNA in MIA PaCa-2 cells led to an increase in RII promoter-chloramphenicol acetyltransferase activity and RII expression. Expression of Sp1 cDNA also caused a reduction in both growth and clonogenicity that was associated with restoration of responsiveness to TGF-β. Conversely, cells that express RII (BxPC-3 and MIA PaCa-2 Sp1 transfectants) when treated with mithramycin, an inhibitor of Sp1 binding, showed a reduction in RII mRNA expression. The reduction of RII mRNA was attributed to a decrease in RII promoter-chloramphenicol acetyltransferase activity that was associated with a decrease in Sp1 binding to the RII promoter. These data indicate that transcriptional repression of the *Sp1* gene in MIA PaCa-2 cells plays a role in the transcriptional inactivation of the *RII* gene and thus lack of responsiveness to TGF-β.

INTRODUCTION

TGF-β[3] is a multifunctional polypeptide that governs a number of cellular processes such as cellular proliferation, differentiation, deposition of the extracellular matrix, and immunosuppression (1–4). TGF-βs elicit their effects by binding to three major types of cell surface receptors, *e.g.*, RI, RII and RIII, as demonstrated by affinity labeling (1–4). RI and RII are glycoproteins of M_r 53,000 and 75,000, respectively. These two receptors are serine/threonine kinase receptors that are essential for TGF-β signaling (5–8). RIII is a proteoglycan of M_r 280,000–330,000 and is a membrane protein lacking a cytoplasmic protein kinase domain (9, 10). RIII is reported to enhance the ligand presentation to the TGF-β signaling receptors, although RIII itself was not involved in signal transduction (11, 12).

Lack of sensitivity to TGF-β can provide tumor cells with a selective growth advantage and accelerate malignant progression. Several studies indicate that a variety of carcinoma cells lose their growth inhibitory response to TGF-β because of a loss of TGF-β receptors (13–16). The heteromeric complex of RI and RII is essential for TGF-β signaling, and loss of these receptors can cause a loss of sensitivity to TGF-β. Expression of RII cDNA restored the growth inhibitory effect of TGF-β in a variety of human cancer cell lines that do not express RII (15, 17–19). These studies indicate that *RII* is a tumor suppressor gene (16, 20). Other studies demonstrated that mutations in simple repeats of the *RII* gene that are targeted by microsatellite instability plays a critical role in altering responsiveness to TGF-β in cancer cells, especially in HNPCC (21–23). Additional studies using the dominant negative mutant of *RII* also demonstrated the premise that the *RII* gene is a tumor suppressor (24, 25). Loss of growth-inhibitory response to TGF-β was reported in pancreatic acinar cells from transgenic mice expressing a dominant negative mutant of *RII* (24). Similarly expression of the dominant negative mutant of *RII* in transgenic mice caused an increase in the incidence of carcinoma (25).

Another reason for the lack of TGF-β signaling is mutations of the *DPC4* (*Smad4*) gene. *DPC4*, a candidate tumor suppressor gene, is an important down-stream target of TGF-β signaling (26–28). The *DPC4* gene is reported to be homozygously deleted in ~30% of pancreatic cancers and inactivated in another 20% of these cancers (29). A recent study indicates that loss of TGF-β signaling defects occur in >80% of pancreatic cancers because of genetic inactivation of one of the TGF-β pathway components, including TGF-β receptors and DPC4 (30).

Although mutations in the *RII* gene were found to be responsible for loss of TGF-β signaling in HNPCC, we found the occurrence of such mutations in the *RII* gene to be low (4%) in pancreatic adenocarcinoma (31). Similar observations indicating a low frequency of mutations in *RII* were reported by others for pancreatic cancer (30, 32). We also recently demonstrated that down-regulation of RII mRNA expression was common in pancreatic tumor tissues and human pancreatic cancer cell lines (33). Taken together, these findings suggest that the lack of RII expression in pancreatic cancer cells could be attributable to epigenetic mechanisms. One possible epigenetic mechanism is aberrant DNA methylation of CpG islands of the promoter regions. The promoter of the *RII* gene has been cloned and characterized (34, 35). The RII promoter lacks a TATA box or CAAT box. The promoter of the *RII* gene contains binding sites for Sp1 and AP1 transcription factors (34, 35), a novel *ets*-related transcription factor (36), along with as-yet unidentified positive regulatory elements.

Previously, we showed that the pancreatic carcinoma cell line, MIA PaCa-2, is resistant to TGF-β-induced growth inhibition because of a lack of expression of the *RII* gene (37). Of the three TGF-β receptors, MIA PaCa-2 cells express RI and RIII (33). These cells do not have a homozygous deletion mutation of DPC4 (26) and express DPC4 mRNA (33, 38). Restoration of RII expression in MIA PaCa-2 cells by transfecting the cells with a RII cDNA reduced their tumorigenic

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[3] The abbreviations used are: TGF-β, transforming growth factor β; RI, RII, and RIII, TGF-β receptor types I, II, and III, respectively; RT-PCR, reverse transcription-PCR; HNPCC, hereditary non-polyposis colon cancer; DPC4, deleted in pancreatic cancer; 5-aza-2′-dc, 5-aza-2′deoxycytidine; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LOH, loss of heterozygosity; EMSA, electrophoretic mobility shift assay; PVDF, polyvinylidene difluoride; MAPK, mitogen activated protein kinase; IGF-1R, insulin-like growth factor-1 receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-thiazolyl blue; ets, epithelial specific; p3TP-Lux, TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter construct.

phenotype and rendered these cells sensitive to radiation-induced growth inhibition (39). Hence, the only component of TGF-β signaling that is lacking in MIA PaCa-2 cells is RII expression and, thus, provides the basis for the insensitivity of MIA PaCa-2 cells to TGF-β-induced growth inhibition.

In this study we analyzed the mechanism responsible for the repression of the *RII* gene in MIA PaCa-2 cells. We show that MIA PaCa-2 cells possess a low level of the transcription factor Sp1 because of a *de novo* transcriptional repression. The transcriptional repression of Sp1 was reversed by treating the cells with 5-aza-2′-dc, a methyl transferase inhibitor (40, 41). Treatment of cells with 5-aza-2′-dc caused an increase in the rate of Sp1 transcription and restoration of RII expression. Expression of Sp1 cDNA in MIA PaCa-2 cells also resulted in the induction of RII mRNA expression and restoration of TGF-β sensitivity, demonstrating the direct role of Sp1 to transactivate the *RII* gene. When RII-expressing cells (BxPC-3 and MIA PaCa-2 cells transfected with Sp1 cDNA) were treated with mithramycin, an inhibitor of Sp1 binding (42, 43), it caused a reduction of RII expression and RII promoter-CAT activity, demonstrating the Sp1 requirement for RII transcription. The findings of this study indicate that a deficit of Sp1 protein may be responsible for the transcriptional repression of the *RII* gene in pancreatic cancer cell line MIA PaCa-2.

MATERIALS AND METHODS

Cell Lines. The pancreatic cancer cell lines, MIA PaCa-2 and BxPC-3, were purchased from American Type Culture Collection (Rockville, MD). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum. A limiting dilution clone of MIA PaCa-2 (clone 6) was established in our laboratory and used in this study. The pancreatic cancer cell line BxPC-3 was used as a control cell line in this study. Among a battery of pancreatic cancer cell lines that we have tested, this cell line expresses appreciable levels of the *RII* gene and is comparable with most nontumor tissues of pancreas and a normal epithelial cell line, Hs 578N (33). To assess the effect of inhibition of methylation, cells were treated with 5-aza-2′-dc. The day of seeding the cells was counted as day 0. Cells were treated with 5-aza-2′-dc at a concentration of 2 μg/ml (equivalent to 8.77 μM) for 24 h on days 2 and 5. Cells were used for RNA extraction, nuclear run-off assays, transfection, or Western immunoblots on day 7. To assess the effect of mithramycin, an inhibitor of Sp1 binding, cells were treated with the drug at the indicated concentrations. Cells were harvested 24 h after the treatment to assess RII promoter-CAT activity and RII mRNA expression by RT-PCR.

mRNA Expression of RII. RNA was extracted using TRI ZOL reagent (Life Technologies, Inc., Gaithersburg, MD). One μg of total RNA was reverse transcribed into cDNA using a commercially available kit (Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer's instructions. Specific primers were used to determine the mRNA expression of the *RII* and *β-actin* genes, as described previously (33). Expression of *β-actin* gene levels were used as an internal control to normalize the expression levels. To determine the effect of 5-aza-2′-dc or mithramycin, cells were treated as detailed above in the cell culture section. RNA was extracted from untreated or 5-aza-2′-dc-treated MIA PaCa-2 cells and reverse transcribed into cDNA. Similarly RII mRNA expression was analyzed from MIA PaCa-2 (Neo) or MIA PaCa-2 (Sp1) cells. Wherever appropriate, RNA extracted from BxPC-3 cells was used as a control for RII mRNA expression. The PCR products were run in 2% agarose gels, stained with ethidium bromide, and photographed.

DNA Sequencing of *RII* cDNA. RT-PCR was performed as described above to amplify the complete cDNA of *RII* as two overlapping fragments using two sets of primers. The primers were designed for the *RII* gene based on the published sequence (GenBank accession no. M85079). The first set of primers are: sense primer RII.297U, 5′-CGC TGG GGG CTC GGT CTA TG-3′; and antisense primer-RII.1139D, 5′-ACT GCC ACT GTC TCA AAC TGC TCT-3′. The second set of primers are: sense primer-RII.1029U, 5′-GCC AAC AAC ATC AAC CAC AAC ACA-3′; and antisense primer-RII.2061D, 5′-GGG GCA GCC TCT TTG GAC AT-3′. The PCR resulted in 866-bp and 1052-bp fragments, respectively. The yield of PCR product in MIA PaCa-2 cells was always undetectable in agarose gels stained with ethidium bromide. Hence, these PCR products were purified by using QiaQuick columns (Qiagen, Valencia, CA), and subjected to a second round PCR using the same set of primers. This procedure yielded sufficient quantities of PCR products to perform DNA sequencing. The purified PCR products were sequenced in both directions by using an automated Applied Biosystems 373A DNA sequencer and dye terminator cycle sequencing with DNA Taq polymerase fluorescent sequencing.

Nuclear Run-off Transcription Assays. Nuclear run-off transcription assays were performed as described previously (44), with some modifications to determine the rate of transcription of *RII* and *Sp1* genes in MIA PaCa-2 cells. BxPC-3 cells were used as a control to determine the rate of transcription of RII. Briefly, blots were prepared by using plasmid DNA immobilized on positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany) using a Schleicher and Schuell slot blot system (Schleicher & Schuell, Keene, NH). The amount of RII or Sp1 cDNA loaded were 2.0 and 4.0 μg/slot. An equal amount of empty vector was also loaded to check the background hybridization. As a loading control, GAPDH cDNA was used. The amount of GAPDH cDNA loaded was 0.5 and 1.0 μg/slot. Slots were loaded with the appropriate quantities of alkali-denatured plasmids, as indicated, and washed with 2 M ammonium acetate (pH 7.0). After drying the blots at room temperature, the plasmid DNA was UV-cross-linked in a Stratalinker UV-cross-linker (Stratagene, La Jolla, CA). Nuclei were prepared from exponentially growing cells. After trypsinization, cells were suspended in NP40 lysis buffer [10 mM TrisCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP40] and homogenized in a dounce homogenizer. Nuclei were pelleted after a brief spin at 2500 rpm for 1 min at 4°C. The isolated nuclei were stored in storage buffer [0.02 M Tris (pH 8.0), 20% glycerol, 0.14 M KCl, 0.01 M $MgCl_2$, and 14 mM β-ME] at −80°C until used for transcription reactions. An equal number of nuclei were suspended in 100 ml of transcription reaction buffer [0.02 M Tris (pH 8.0); 10% glycerol; 0.14 M KCl; 0.01 M $MgCl_2$; 14 mM β-ME; 1 mM each of ATP, CTP, GTP; and 100 μCi of (α-^{32}P)-UTP [(3000 Ci/mmol); Amersham Pharmacia Biotech Inc., Piscataway, NJ], and the reaction was incubated at 30°C for 30 min. The ^{32}P-labeled nascent RNA was extracted using TRI ZOL reagent (Life Technologies, Inc.) and precipitated in the presence of glycogen as a carrier. Equal amounts of radiolabeled RNA were hybridized to blots in ULTRAhyb hybridization buffer (Ambion, Inc., Austin, TX). Hybridization was performed at 45°C for 48 h. Blots were sequentially washed twice in 2× SSC for 10 min and in 0.2× SSC- 0.1% SDS at 65°C for 1 h. Routine autoradiographic techniques were performed to develop the blots.

Protein-DNA Binding Studies. EMSA, competitive analyses, and antibody supershifts were carried out using specific wild-type and mutant oligonucleotides representing the two Sp1-binding regions, as described previously (45). These two oligonucleotides were referred to as Sp1 (−37/−8) and Sp1 (−155/−125), based on the nucleotide positions relative to transcriptional initiation site (35). To determine the effect of mithramycin on Sp1 binding to the RII promoter, oligonucleotide probes were preincubated for 1 h at 4°C in the presence of mithramycin (0.1 μM) before adding to the nuclear extracts, as described previously (43).

Western Immunoblots. Western immunoblots were prepared to analyze the protein levels of Sp1, MAPK, IGF-1R, RII, and human β-actin. MIA PaCa-2 and BxPC-3 cells were harvested at indicated time points after treatment with 5-aza-2′-dc, and total cellular proteins were extracted by using Laemmli buffer. Fifty μg of total cellular proteins from cells were electrophoresed on a 7.5% SDS-polyacrylamide gel and then transferred to Hybond-P, PVDF membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The blots were probed with specific antibodies for the above-mentioned proteins (Sp1, RII, and IGF-1Rβ; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; MAPK; New England Biolabs, Beverly, MA; and human β-actin; Sigma Chemical Co., St. Louis, MO). Bound antibodies were detected by an enhanced chemiluminescence detection system (NEN Life Science Products, Boston, MA).

Determination of Sp1 Protein Stability. To determine the stability of Sp1 protein, MIA PaCa-2 cells, either untreated or treated with 5-aza-2′-dc, were treated with cycloheximide (10 μg/ml) for 0, 3, 6, 9, and 12 h. Cells were harvested at indicated time points, and protein extractions were analyzed for Sp1 as described in the Western immunoblot section.

CAT Assays. RII promoter-CAT constructs were as described previously (35). Two different RII promoter-CAT constructs (−274/+50 and −47/+50;

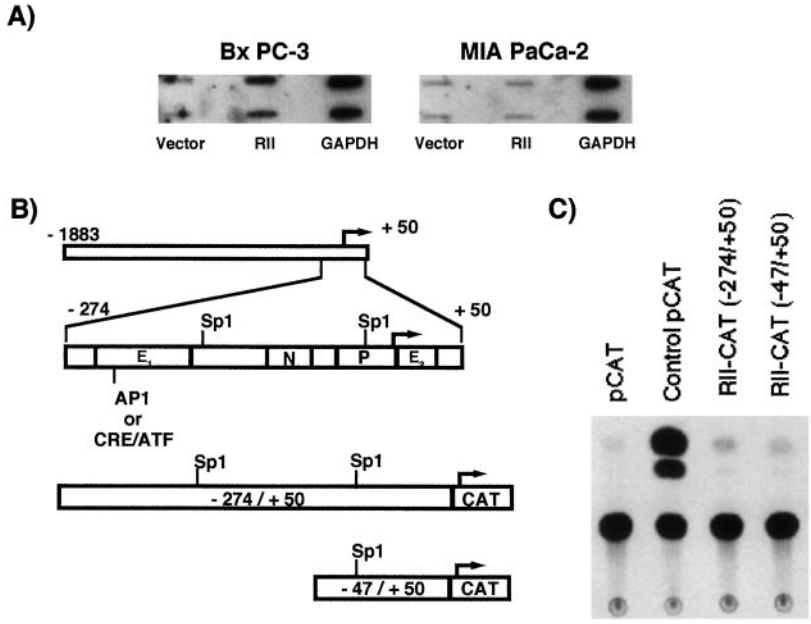

Fig. 1. The transcription rate of the *RII* gene and RII promoter-CAT activity are low in MIA PaCa-2 cells. *A*, nuclear run-off assays were performed as described in "Materials and Methods" to determine the rate of RII transcription in MIA PaCa-2 cells. As a comparison, the rate of RII transcription was analyzed in BxPC-3 cells that express RII mRNA. The amount of RII cDNA or of an empty vector loaded per slot was 4.0 and 2.0 μg in *top* and *bottom Lanes*, respectively. As a loading control, GAPDH levels were also measured. The amount of GAPDH cDNA loaded was 1.0 and 0.5 μg in the *top* and *bottom Lanes*, respectively. *B*, schematic representation of the multiple regulatory elements within the RII promoter and RII promoter-CAT constructs (not to scale). Relative positions of the four identified regulatory elements are shown. *E1* and *E2* are the first and second positive regulatory elements, respectively. *P*, the core promoter. *N*, a negative regulatory element. *Arrows* indicate a +1 transcriptional start site (35). *C*, results from a representative CAT assay. MIA PaCa-2 cells were transiently transfected with a promoterless vector (pCAT), a positive-control CAT vector with SV40 promoter (Control pCAT) to determine general transcriptional activity; RII promoter-CAT vector that contains the core promoter region with two Sp1 binding sites corresponding to the nucleotides −274/+50, and RII promoter-CAT vector that contains one Sp1 binding site corresponding to the nucleotides −47/+50. Cells were harvested after 48 h of transfection, and CAT assays were performed as detailed in "Materials and Methods."

Fig. 1*B*) were used to assess basal RII promoter-CAT activity in MIA PaCa-2 cells. The RII promoter-CAT construct (−47/+50) contains one Sp1 binding site and the other construct, −274/+50, contains the core promoter region with two Sp1 binding sites and other positive/negative regulatory elements. As a control, MIA PaCa-2 cells were transfected with a positive control vector (pCAT-control; Promega, Madison, WI) to monitor the general transcriptional activity. Transfections were performed by using commercially available FuGENE6 transfection reagent (Boehringer Mannheim). To assess the overexpression of Sp1 on RII promoter activity, cells were cotransfected with Sp1 cDNA and RII promoter-CAT construct (−274/+50). Cells were harvested after 48 h of transfection and normalized by the β-gal activity, and standard CAT-assays were performed using TLC plates. However, for cells stably transfected with Sp1 cDNA, normalization was based on the total protein level as measured by using the Bradford-Lowrey reaction (Bio-Rad, Hercules, CA). This procedure was adopted because, in pilot experiments, cotransfection with pSV-β-galactosidase control vector (Promega) showed higher β-galactosidase activity in cells overexpressing Sp1, probably because of the presence of several Sp1 binding sites in the promoter of pSV-β-galactosidase vector. Hence, for the cells transfected with Sp1, total protein levels were measured for normalization.

Stable Transfections. MIA PaCa-2 cells were transfected with a vector that expresses Sp1 cDNA (46) or with an empty vector without the Sp1 cDNA insert by using commercially available FuGENE6 transfection reagent (Boehringer Mannheim). Cells were cultured further and selected in medium containing 620 μg/ml G418. Individual G418-resistant colonies were isolated by ring cloning during drug selection, established as individual clones, and used for additional analysis. MIA PaCa-2 cells stably transfected with an empty vector and a vector with Sp1 cDNA were denoted as MIA PaCa-2 (Neo) and MIA PaCa-2 (Sp1) clone cells, respectively.

Luciferase Assay to Determine TGF-β Responsiveness. Responsiveness of MIA PaCa-2 cells to exogenously added TGF-β was determined as described previously (6). Briefly, cells were transiently transfected with a TGF-β responsive reporter construct, p3TP-Lux. Cells were grown in 6-cm culture dishes for 24 h after transfection and treated with 10 ng/ml of human recombinant TGF-β_1 (R&D Systems, Minneapolis, MN) for 24 h. Cells were harvested in 300 μl of reporter lysis buffer (Promega) after 48 h of transfection. Luciferase activity was measured with 20 μl of lysate using a luciferase assay kit (Promega), as per the manufacturer's suggestion, in a luminometer (TROPIX, Inc., Bedford, MA) and expressed as relative units after being normalized to protein levels.

MTT and Soft Agar Assays. The growth rate of MIA PaCa-2 (Neo) and two different clones of MIA PaCa-2 (Sp1) cells was determined by MTT assays. Cells (1×10^3) were plated in 96-well plates and MTT assays were performed at days 1, 2, 4, and 5. On the day of assay, the growth media was replaced with serum-free medium containing 0.5 mg/ml MTT (Sigma Chemical Co.) and incubated at 37°C for 2 h. At the end of the incubation period, cells were solubilized in 200 μl of DMSO, and colorimetric determination was performed at 570 nm absorbance with a plate reader. The data were represented as the mean values from four independent experiments. The tumorigenic potential of cells was determined in soft agar assays as described previously (33). Briefly, MIA PaCa-2 (Neo) or MIA PaCa-2 (Sp1) cells were suspended in 0.4% NOBLE agar in DMEM medium containing 10% fetal bovine serum and plated on top of 1 ml of 1% agar in the same medium in six-well tissue culture plates. Wherever necessary, 10 ng/ml of TGF-β_1 (R&D Systems) was added to the medium. The cells were then incubated for 14 days at 37°C in a CO_2 humidified incubator. Colonies were visualized by staining with 0.5 mg/ml MTT and photographed.

RESULTS

Lack of RII mRNA Expression in MIA PaCa-2 Cells Is Not Due to Mutations in the Coding Region of *RII* Gene. We previously showed that MIA PaCa-2 cells lack RII expression (33, 37). MIA PaCa-2 cells, however, do express the other two TGF-β receptors, RI and RIII, as well as DPC4 (Smad4), an important down-stream target of the TGF-β signaling pathway. Previous reports indicate that mutations in the coding region of the *RII* gene cause a down-regulation of RII expression in colon cancer cell lines (16, 21). Hence, we assessed the possibility of the presence of mutations in the coding region of the *RII* gene of MIA PaCa-2 cells. The entire coding region of the *RII* gene was sequenced by amplifying the complete cDNA as two overlapping fragments by RT-PCR. DNA sequence analysis showed no evidence of mutations in the coding region of the *RII* cDNA (data not shown).

Transcriptional Rate of RII Is Low in MIA PaCa-2 Cells. Because there were no mutations found in the coding region of the *RII* gene, we determined whether a dysfunction of transcriptional activation might account for the lower level of RII expression in MIA PaCa-2 cells. Nuclear run-off assays were used to assess whether there was any impairment of the endogenous RII promoter activity. Nuclear run-off assays showed that the rate of transcription of *RII* gene is low in MIA PaCa-2 cells compared with the control cell line BxPC-3 (Fig. 1*A*). Bx PC-3 cells that express higher levels of RII mRNA showed a higher rate of RII transcription (Fig. 1*A*).

One possibility for the lower rate of RII transcription in MIA

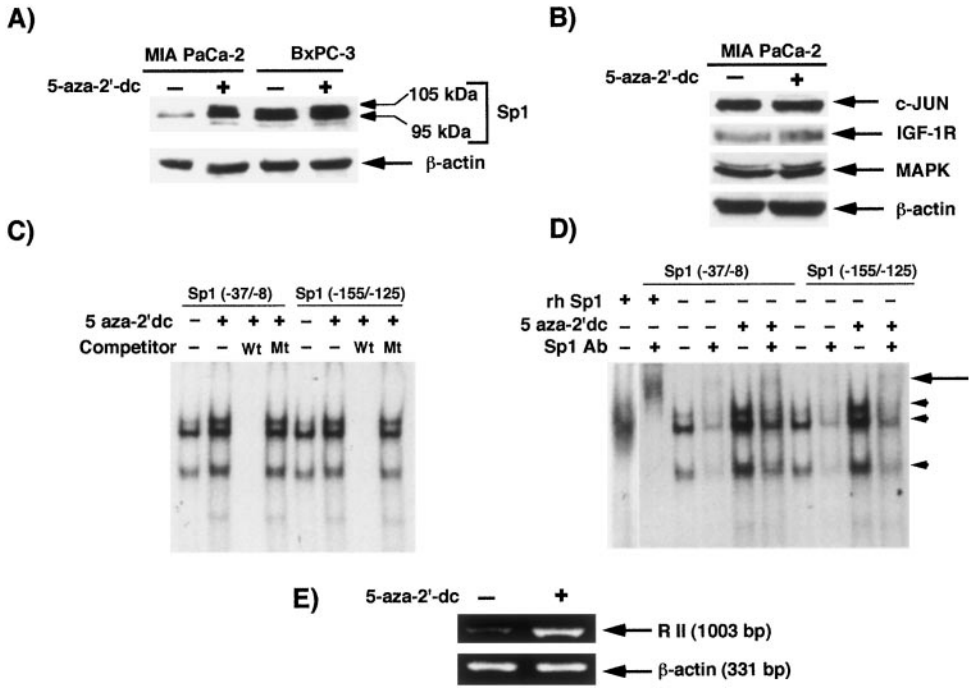

Fig. 2. Treatment of MIA PaCa-2 cells with 5-aza-2′-dc causes an increase in Sp1 protein levels that is associated with an increase in RII mRNA expression attributable to an increase in the binding of Sp1 to RII promoter. *A*, Western immunoblot showing the Sp1 protein levels in untreated or 5 aza-2′-dc (2 μg/ml) -treated MIA PaCa-2 cells. Total cellular proteins were extracted and resolved on a 10% SDS-PAGE, transferred to PVDF membrane, and probed with Sp1 antibody as described in "Materials and Methods." As a control, protein levels of Sp1 were analyzed in the other pancreatic cancer cell line, BxPC-3, that expresses normal levels of the *RII* gene. *B*, Western immunoblot showing no change in the levels of c-JUN, IGF-IR, and MAPK protein levels in MIA PaCa-2 cells either untreated or treated with 5-aza-2′-dc (2 μg/ml). Total cellular proteins were extracted, and Western blots were prepared as mentioned above and probed with specific antibodies. *C*, EMSA was performed using two oligonucleotide probes that contain the Sp1 consensus sequence in the RII promoter, as described in "Materials and Methods." Radiolabeled oligonucleotide probes were incubated with nuclear extracts from MIA PaCa-2 cells either untreated or treated with 5-aza-2′-dc (2 μg/ml). When competed with mutant oligonucleotides that contain mutated Sp1 consensus sequence, no decrease in the bound-protein complexes was observed. *D*, to determine that the proteins bound to the oligonucleotide probes contain Sp1 protein, supershift analysis was performed using a Sp1 antibody. Commercially available human recombinant Sp1 protein was used as a control. EMSA shows an increased binding of nuclear proteins from cells treated with 5-aza-2′-dc when compared with untreated cells. A depletion of specific bands (*arrowheads*) and supershifted band (*arrow*) was observed, indicating that the bound nuclear complexes contain Sp1 protein. *E*, MIA PaCa-2 cells were treated with 5 aza-2′-dc (2 μg/ml) as described in "Materials and Methods." Total RNA was extracted and RT-PCR was performed to analyze mRNA expression of RII. Constitutively expressing *β-actin* gene RT-PCR was performed as an internal control.

PaCa-2 cells is that there is a defect in the endogenous RII promoter. This defect could be attributable to a mutation within the promoter region or to the silencing of gene expression by aberrant methylation or be caused by a lack of appropriate transcriptional activation. Previous studies showed the presence of two Sp1 binding sites in the RII promoter and absence of TATA or CAAT boxes (Fig. 1*B*; 34, 35). To ascertain the possibility of any defects of the RII promoter, we initially performed CAT assays using two wild-type RII promoter-CAT constructs. The first construct (−274/+50) contains two Sp1 binding sites along with positive regulatory elements, and the second construct (−47/+50) has one Sp1 binding site. When MIA PaCa-2 cells were transfected with either of these two different wild-type RII promoter-CAT constructs, a negligible level of RII promoter-CAT activity was observed in these cells irrespective of the constructs used (Fig. 1*C*), suggesting a lack of appropriate transcriptional activation. To rule out the possibility of any artifacts in transfections, MIA PaCa-2 cells were transfected with a positive-control CAT vector that contains SV40 promoter to monitor the general transcriptional activity and transfection. The positive control showed a significant CAT activity indicating that MIA PaCa-2 cells failed to show RII promoter-CAT activity, irrespective of the constructs used.

Sp1 Protein Levels Are Low in MIA PaCa-2 Cells and Can Be Restored by Treatment with 5-aza-2′-dc. Because the CAT assays with wild-type RII promoter-CAT constructs showed very low levels of CAT activity, we further assessed the possibility of the absence of the transcriptional activator Sp1 in MIA PaCa-2 cells. Western immunoblot analysis of MIA PaCa-2 cells showed very low levels of Sp1 protein expression (Fig. 2*A*). The control pancreatic cancer cell line BxPC-3 that expresses higher levels of RII mRNA (33) also showed higher levels of Sp1 protein (Fig. 2*A*). Thus, a deficiency in Sp1 expression might account for the lack of RII expression in MIA PaCa-2 cells.

After ascertaining that the RII repression possibly was attributable to low levels of Sp1 protein in MIA PaCa-2 cells, we assessed the basis for the low level of Sp1. We determined whether treatment of MIA PaCa-2 cells with 5-aza-2′-dc, a methyl transferase inhibitor (40, 41), would increase the level of Sp1 protein, inasmuch as such a treatment caused an increase in the stability of Sp1 protein in MCF-7 L cells (45). Treatment of MIA PaCa-2 cells with 5-aza-2′dc resulted in a dramatic increase of Sp1 protein levels (Fig. 2*A*). We next determined whether treatment of MIA PaCa-2 cells with 5-aza-2′-dc caused a general increase of the protein levels. Western immunoblot analysis showed that the levels of MAPK, IGF-1R, and another general transcription factor, AP-1 (c-jun), were not dramatically altered by this treatment (Fig. 2*B*). This suggests that 5-aza-2′-dc did not result in a global increase in the levels of various proteins.

5-aza-2′-dc Treatment Caused Increased Binding of Sp1 Protein to the RII Promoter in MIA PaCa-2 Cells. EMSAs were performed to analyze the nuclear protein complexes that bind to the Sp1 consensus elements in the promoter of the *RII* gene. The two oligonucleotides (Sp1 −37/−8 and Sp1 −155/−125) used in this study were described previously (45). Nuclear extracts from untreated and 5-aza-2′-dc-treated MIA PaCa-2 cells were subjected to gel-shift analysis. Nuclear proteins from 5-aza-2′-dc-treated MIA PaCa-2 cells contain greater amounts of DNA-protein complexes than the nuclear proteins from untreated MIA PaCa-2 cells (Fig. 2*C*). The increase in

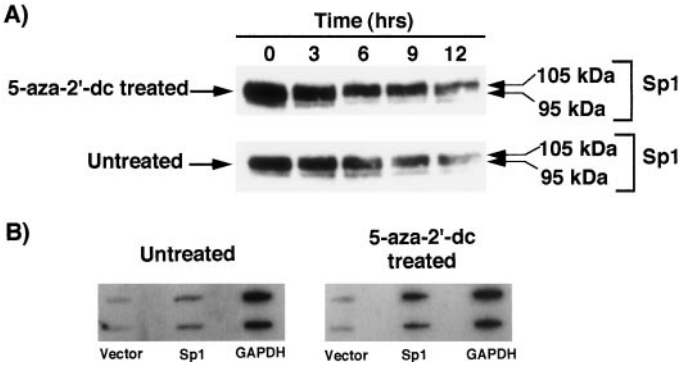

Fig. 3. 5-aza-2′-dc treatment does not change the stability of Sp1 protein in MIA PaCa-2 cells but increases the rate of Sp1 transcription. *A*, a representative Western blot showing the levels of Sp1 protein in MIA PaCa-2 cells either untreated or treated with 5-aza 2′-dc (2 μg/ml) were treated with cycloheximide (10 μg) for the indicated time periods. Total cellular proteins were extracted and resolved on a 10% SDS-PAGE, transferred to PVDF membrane, and probed with Sp1 ,antibody as described in "Materials and Methods." Excessive proteins were loaded from untreated cells to visualize Sp1 protein. *B*, a representative nuclear run-off assays showing the increased rate of Sp1 transcription in MIA PaCa-2 cells treated with 5-aza 2′-dc (2 μg/ml). The amount of Sp1 cDNA or an empty vector loaded per slot was 4.0 and 2.0 μg in *top* and *bottom Lanes*, respectively. As a loading control, GAPDH levels were also measured. The amount of GAPDH cDNA loaded was 1.0 and 0.5 μg in *top* and *bottom Lanes*, respectively.

the binding of DNA- protein complexes was observed for both the oligonucleotides representing the two Sp1 consensus elements of RII promoter. The protein-DNA complexes represent the specific binding of proteins to Sp1 consensus elements, because addition of excess unlabeled wild-type oligonucleotides showed a decrease in the binding complexes that was not seen with a mutant oligonucleotide (Fig. 2*C*). To assess whether these DNA-protein complexes contain Sp1 protein, supershift assays were carried out by incubating the oligonucleotide DNA-protein complexes with an antibody to Sp1. The addition of Sp1 antibody caused a shift for human recombinant Sp1 protein (Fig. 2*D*). However, for nuclear protein complexes from MIA PaCa-2 cells, the shifted complex was low (Fig. 2*D, arrow*), but a large depletion of bound protein complexes to the oligonucleotides was observed (Fig. 2*D, arrowheads*). This depletion of bound complexes with Sp1 antibody suggests that the bound complexes contain Sp1 protein.

Because 5-aza-2′-dc treatment caused an increase in Sp1 binding as determined by EMSA, we next tested whether these cells also show an increase in RII mRNA expression. RT-PCR analysis revealed that 5-aza-2′-dc treatment caused an increase in RII mRNA expression in MIA PaCa-2 cells (Fig. 2*E*).

5-aza-2′-dc Treatment Increases the Rate of Transcription of *Sp1* Gene, but not the Stability of Sp1 Protein in MIA PaCa-2 Cells. To determine the mechanism by which 5-aza-2′-dc increases the expression of Sp1, MIA PaCa-2 cells were treated with cycloheximide (10 μg/ml), and proteins were extracted at 0, 3, 6, 9 and 12 h after treatment. Western immunoblots were performed to detect Sp1 protein levels. Treatment with 5-aza-2′-dc did not lead to any change in the half-life of Sp1 protein when compared with untreated cells (Fig. 3*A*). However, treatment of MIA PaCa-2 cells with 5-aza-2′-dc had led to an increase in the rate of transcription of the *Sp1* gene as determined by nuclear run-off assays (Fig. 3*B*).

Expression of Sp1 cDNA in MIA PaCa-2 Cells Causes an Increase in Sp1 Protein, RII Promoter-CAT Activity, and RII Expression. To confirm further that Sp1 deficiency causes the repression of *RII* gene expression, we transfected MIA PaCa-2 cells with a vector containing the *Sp1* cDNA under the control of a cytomegalovirus promoter (46). Transient transfection of MIA PaCa-2 cells with Sp1 cDNA showed increased levels of Sp1 protein as analyzed by Western immunoblots (Fig. 4*A*). MIA PaCa-2 cells cotransfected with the Sp1 cDNA and RII promoter-CAT construct showed an increase in RII promoter-CAT activity, indicating the transcriptional up-regulation of RII in cells that express the Sp1 cDNA (Fig. 4*B*).

Because transient expression of Sp1 cDNA led to an increase in

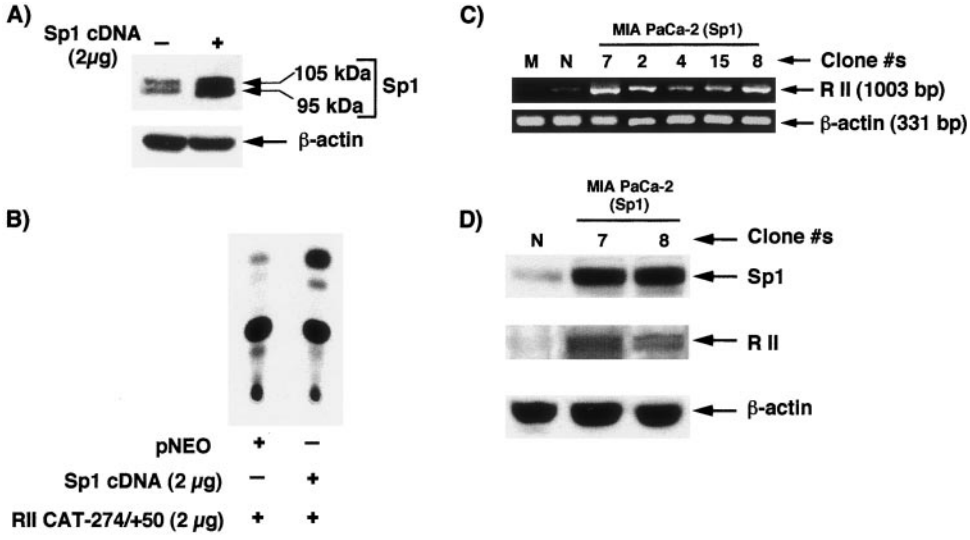

Fig. 4. Expression of Sp1 cDNA in MIA PaCa-2 cells causes an increase in Sp1 protein levels, RII promoter-CAT activity, and RII expression. *A*, Western immunoblot showing the levels of Sp1 protein in MIA PaCa-2 cells transiently transfected with Sp1 cDNA. Cells were harvested after 48 h of transfection. Total cellular proteins were extracted, resolved on a 10% SDS-PAGE, transferred to PVDF membrane, and probed with Sp1 antibody, as described in "Materials and Methods." *First Lane*, the Sp1 protein levels in MIA PaCa-2 cells transfected with an empty vector; *second Lane*, the Sp1 protein levels in MIA PaCa-2 cells transiently transfected with 2 μg of Sp1 cDNA. *B*, a representative CAT assay showing an increase of RII promoter-CAT activity in MIA PaCa-2 cells transiently transfected with Sp1 cDNA (2 μg). MIA PaCa-2 cells were transiently cotransfected with a vector that expresses Sp1 cDNA or an empty vector (*pNEO*) and 2 μg of RII promoter-CAT construct, −274/+50, that contains the core promoter region of the *RII* gene with two Sp1 binding sites. Cells were harvested after 48 h of transfection, and CAT assays were performed as detailed in "Materials and Methods." *C*, stable transfection of MIA PaCa-2 cells with Sp1 cDNA causes an increase in RII expression. RT-PCR was performed with appropriate primers from total RNA extracted to determine the mRNA expression of the *RII* gene in parental MIA PaCa-2 (*M*) or MIA PaCa-2 (Neo) cells (*N*) and different clones of MIA PaCa-2 (*Sp1*) cells. β-actin mRNA levels were measured as an internal control. *D*, Western immunoblot showing the Sp1 and RII protein levels in MIA PaCa-2 (Neo) cells (*N*) and MIA PaCa-2 (*Sp1*) clone 7 and 8 cells. Total cellular proteins were extracted and resolved on a 10% SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies as described in "Materials and Methods."

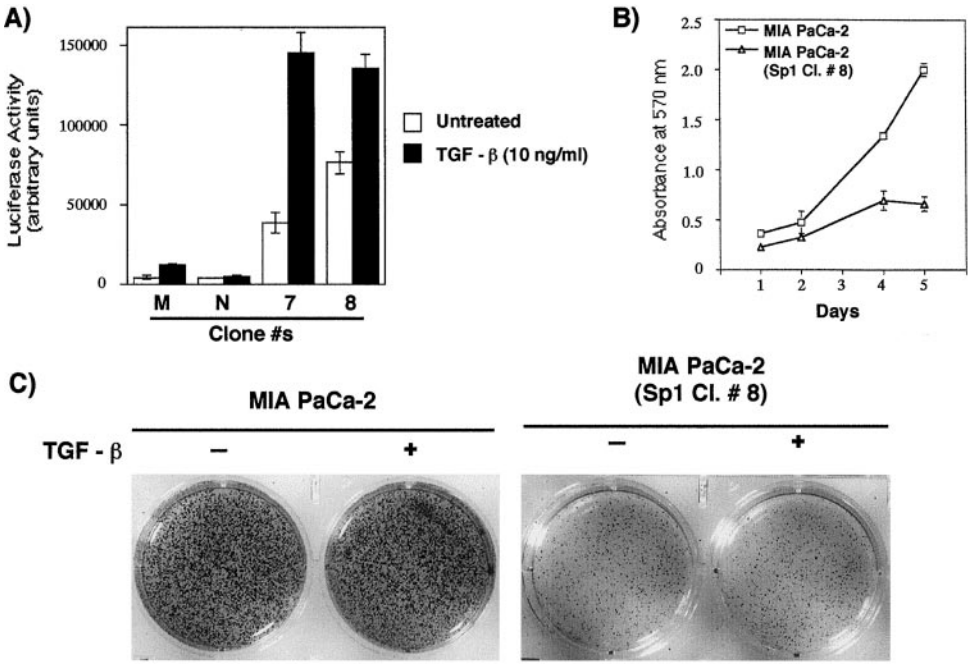

Fig. 5. Expression of Sp1 cDNA in MIA PaCa-2 (*Sp1*) cells restores responsiveness to TGF-β and causes a reduction in growth and clonogenicity. *A*, a representative luciferase activity assay indicating the restoration of responsiveness to TGF-β. MIA PaCa-2 (*Sp1*) clone 7 and clone 8 cells (*7* and *8*) or MIA PaCa-2 (Neo) cells (*N*) or parental MIA PaCa-2 cells (*M*) were transiently transfected with a TGF-β responsive promoter construct, p3TP-lux. After 24 h of transfection, cells were untreated or treated with TGF-β_1 (10 ng/ml) for 24 h. Luciferase activity was determined after 48 h of transfection, as detailed in "Materials and Methods." *B*, MTT assays were performed as described in "Materials and Methods" to determine the rate of growth of MIA PaCa-2 (Neo) and MIA PaCa-2 (Sp1) cells. The rate of growth was measured up to five days after plating of the cells. MIA PaCa-2 (Sp1) cells show a reduced growth as compared with MIA PaCa-2 (Neo) cells. *C*, clonogenic assays were performed to assess the anchorage-independent growth characteristics of MIA PaCa-2 (Sp1) cells. MIA PaCa-2 (Neo) and MIA PaCa-2 (Sp1) cells were grown in soft agar in the presence or absence of TGF-β_1 (10 ng/ml) for 14 days.

Sp1 protein levels and RII promoter-CAT activity, we stably transfected MIA PaCa-2 cells with Sp1 cDNA. MIA PaCa-2 cells stably transfected with an empty vector and a vector with Sp1 cDNA were denoted as MIA PaCa-2 (Neo) and MIA PaCa-2 (Sp1) clone cells, respectively. Transfected cells were cloned and screened for RII mRNA expression by RT-PCR analysis. Most of the MIA PaCa-2 (Sp1) clones showed an increase in RII mRNA expression compared with MIA PaCa-2 (Neo) cells (Fig. 4*C*). Among the numerous MIA PaCa-2 (Sp1) clones established, two individual clones (clone 7 and 8) were used for additional analysis to rule out the possibility of any clonal variation. These two clones showed an increase in Sp1 protein levels and a concurrent increase of RII protein levels when compared with MIA PaCa-2 (Neo) cells (Fig. 4*D*). These two clones also showed an increase in RII promoter-CAT activity similar to that observed in MIA PaCa-2 cells transiently transfected with Sp1 cDNA (data not shown) and were assessed for growth, clonogenicity, and responsiveness to TGF-β as determined by the p3TP-Lux-reporter assay.

Ectopic Expression of Sp1 cDNA in MIA PaCa-2 Cells Restores Responsiveness to TGF-β and Causes a Decrease in Growth and Clonogenicity. The responsiveness to TGF-β was analyzed by the p3TP-Lux-reporter assay. Expression of Sp1 increased 3TP-lux activity in MIA PaCa-2 (Sp1) cells (Fig. 5*A*). The level of responsiveness was increased further by the addition of exogenous TGF-β (Fig. 5*A*). However, parental MIA PaCa-2 cells or MIA PaCa-2 (Neo) cells did not show considerable p3TP-Lux activity even after the addition of exogenous TGF-β (Fig. 5*A*). The increased basal levels of p3TP-Lux-reporter activity in MIA PaCa-2 (Sp1) cells suggests restoration of autocrine TGF-β activity after an increase in the expression of the RII receptor. Similar restoration of autocrine TGF-β signaling attributable to an increase of RII was also observed in other cell systems (47, 48).

To evaluate the biological significance of Sp1 expression in restoring TGF-β responsiveness, growth assays were performed. The rate of growth was compared for MIA PaCa-2 (Neo) and MIA PaCa-2 (Sp1) cells by MTT assays. Expression of Sp1 cDNA caused a reduction in the rate of growth as compared with MIA PaCa-2 (Neo) cells (Fig. 5*B*). The clonogenic potential of MIA PaCa-2 (Sp1) cells was assessed by soft agar assays. MIA PaCa-2 (Sp1) cells showed a dramatic reduction in clonogenicity as compared with MIA PaCa-2 (Neo) cells (Fig. 5*C*). Exogenous TGF-β did not add to a further-pronounced reduction of growth in soft agar. This suggests that restoration of autocrine TGF-β signaling by Sp1 expression was sufficient for the reduction of growth in soft agar. Because the observations on MTT and soft agar assays were similar for both MIA PaCa-2 (Sp1) clones 7 and 8, the data are shown only for MIA PaCa-2 (Sp1) clone 8 in Fig. 5.

Mithramycin Decreases Binding of Sp1 to the RII Promoter and Inhibits RII Promoter-CAT Activity and RII mRNA Expression. To ascertain the dependency of RII promoter on Sp1, additional experiments were performed by treating cells with mithramycin, an inhibitor of Sp1 binding (42, 43). Initially, EMSA assays were performed to determine the effect of mithramycin treatment on the binding of Sp1 protein to the RII promoter that contains the Sp1 consensus element. Nuclear extracts from MIA PaCa-2 (Neo) cells and MIA PaCa-2 (Sp1) cells were used for the study. The nuclear extracts from MIA PaCa-2 (Sp1) cells showed more binding of protein-DNA complexes than those from the nuclear extracts from MIA PaCa-2 (Neo) cells. The increase in the bound complexes were found for both the oligonucleotides (Sp1 −37/−8 and Sp1−155/−125). When oligonucleotides were preincubated with mithramycin, a considerable reduction of the bound protein-DNA complexes were observed (Fig. 6*A*). This suggests that mithramycin reduced the binding of Sp1 to the oligonucleotide probes that contain the Sp1 consensus elements of the RII promoter. MIA PaCa-2 (Sp1) cells also showed an inhibition in the RII promoter-CAT activity upon treatment with mithramycin (Fig. 6*B*). When Bx PC-3 or MIA PaCa-2 (Sp1) cells that show RII expression were treated with mithramycin, a reduction in the RII mRNA expression was observed (Fig. 6*C*).

DISCUSSION

In the present study we used the human pancreatic cancer cell line, MIA PaCa-2 to investigate the mechanism responsible for a lack of RII expression. Previously, we showed that MIA PaCa-2 cells are highly tumorigenic and resistant to TGF-β-induced growth inhibition, presumably because of a lack of *RII* gene expression (37). Although

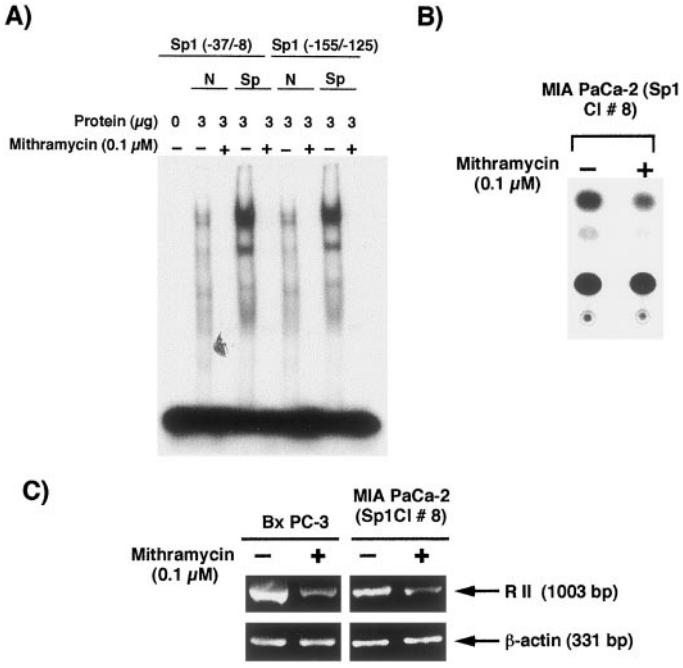

Fig. 6. Mithramycin inhibits the binding of Sp1 to RII promoter, RII promoter-CAT activity, and RII mRNA expression. *A*, EMSA showing inhibition of Sp1 binding to RII promoter. The oligonucleotides were pretreated with mithramycin, and nuclear extracts from MIA PaCa-2 cells were transfected with an empty vector (*N*) and MIA PaCa-2 cells transfected with Sp1 cDNA (*Sp*) were incubated as detailed in "Materials and Methods." Pretreatment of oligonucleotides with mithramycin dramatically reduces the binding of Sp1 to RII promoter. *B*, CAT assay showing a reduction of RII-promoter-CAT activity in MIA PaCa-2 (Sp1) cells treated with mithramycin. *C*, RT-PCR analysis showing reduced RII mRNA expression in MIA PaCa-2 (Sp1) and in BxPC-3 cells treated with mithramycin.

mutations in simple repeats of the *RII* gene cause loss of TGF-β signaling in HNPCC (21–23), our study (31) and reports by others (30, 32) show a low incidence of such mutations in the *RII* gene of pancreatic adenocarcinoma. However, we found that the lack of RII expression was common in pancreatic cancer (33). MIA PaCa-2 cells express the other two TGF-β receptors (RI and RIII) and DPC4 (Smad4), an important down stream component of TGF-β signaling (33). We sequenced the complete coding region of the *RII* gene of MIA PaCa-2 cells and did not find any mutations. Nuclear runoff assays showed a low level of RII transcription in MIA PaCa-2 cells. This raises the possibility that mutations in the promoter region of the *RII* gene, or other mechanisms regulating the transcription of the *RII* gene, may be responsible for the lack of expression of the *RII* gene in this cell line. Wild-type RII promoter-CAT reporter studies revealed only a low level of RII promoter activity in MIA PaCa-2 cells. These results suggest that RII transcription is suppressed in MIA PaCa-2 cells.

Transcriptional repression of a gene may be attributable to several mechanisms, including mutations within the promoter region, absence of transcriptional regulation, or aberrant DNA methylation of CpG islands. Recent studies indicate that the pathways of inactivating genes that led to the development of carcinogenesis could be attributable to the silencing of regulatory genes, especially tumor suppressor genes (49–52). The RII promoter lacks TATA and CAAT boxes, but does contain binding sites for Sp1, AP1, and (34, 35), *ets*-related transcription factor (36), as well as other unidentified positive regulatory elements (35).

The importance of Sp1 for basal transcriptional activity was suggested for promoters that lack TATA or CAAT boxes (53, 54). We found by Western blot analysis that MIA PaCa-2 cells express a lower level of Sp1 protein than BxPC-3 cells that express RII. Treatment of MIA PaCa-2 cells with 5-aza-2′-dc caused an increase in the expression of Sp1 protein. However, treatment with 5-aza-2′-dc did not result in a generalized increase of other protein levels such as MAPK, a constitutively expressed protein, IGF-1R, a growth factor signaling protein, and another general transcription factor, AP-1 (c-Jun). A recent study (45) indicated that RII expression was increased in MCF-7 L cells treated with 5-aza-2′-dc because of an increase in Sp1 protein stability (45). In contrast to the previous study on MCF-7 L cells, treatment of MIA PaCa-2 cells with 5-aza-2′-dc did not result in a prolonged stability of Sp1 protein, but resulted in an increase in the rate of Sp1 transcription. This indicates that a deficit of Sp1 protein occurs because of separate mechanisms in different cancer cell types. However, at this point it is not clear how 5-aza-2′-dc treatment causes a transcriptional up-regulation of the Sp1 message in MIA PaCa-2 cells. It is possible that the low levels of Sp1 may be attributable to the methylation of the Sp1 promoter or of the promoter of a transactivator that regulates the expression of Sp1. In this study, however, we have not determined these possibilities, because a cloned Sp1 promoter is not available.

Furthermore, we demonstrated that RII expression is specifically mediated by Sp1 by transfecting MIA PaCa-2 cells with a vector expressing the Sp1 cDNA. Transient transfection of MIA PaCa-2 cells with the Sp1 cDNA led to an increase in Sp1 protein levels and RII promoter-CAT activity. MIA PaCa-2 (Sp1) cells stably transfected with the Sp1 cDNA showed an increase in RII mRNA and protein expression. The biological significance of the expression of Sp1 in MIA PaCa-2 (Sp1) cells was also assessed by growth characteristics. MTT assays showed a reduction in the rate of growth of MIA PaCa-2 (Sp1) cells as compared with MIA PaCa-2 (Neo) cells. The clonogenicity of MIA PaCa-2 (Sp1) cells was analyzed by soft agar assays. One of the characteristic features of MIA PaCa-2 cells is the colony formation in soft agar assays (33, 37). Also, these cells demonstrate an anchorage-independent growth in the presence of TGF-β. Expression of Sp1 cDNA caused a dramatic reduction of clonogenicity in MIA PaCa-2 (Sp1) cells even in the absence of exogenously supplied TGF-β, indicating a reduction of their tumorigenic phenotype. The increase in responsiveness to TGF-β was also observed in p3TP-lux assays. The reduced rate of both growth and clonogenicity that is associated with an increase in the basal levels of 3TP-lux reporter activity in MIA PaCa-2 (Sp1) cells are probably the result of restoration of autocrine TGF-β activity after the restoration of the RII receptor (47, 48).

Conversely, we reasoned that inhibition of Sp1 causes a decrease in RII expression. Mithramycin has been shown to prevent the binding of Sp1 to its consensus sequences in promoters and to inhibit Sp1-dependent gene expression in several cell types (42, 43, 55, 56). EMSAs showed that treatment of mithramycin caused a reduction of Sp1 binding to its consensus sequence of the RII promoter. When MIA PaCa-2 (Sp1) cells that show RII mRNA expression were treated with mithramycin, a reduction in RII promoter-CAT activity and RII mRNA expression was observed. These studies further support the notion that transcriptional activation by Sp1 is critical for *RII* gene expression. We suggest that the *RII* gene may represent a unique subpopulation of genes that require threshold levels of Sp1 for transcriptional activation. It is probable that other transcription factors or cofactors, or activators that interact with Sp1, may efficiently drive the transcription of many genes that depend on Sp1 in cells such as MIA PaCa-2, which have low levels of Sp1. The essential role of Sp1 for RII transcription in other pancreatic cancer cell lines is being investigated currently in this laboratory.

In summary, this study indicates that RII expression in MIA PaCa-2 cells is attributable to suboptimal levels of the transcriptional factor Sp1 that is essential for RII transcription. The increase of expression

of Sp1 by treatment of MIA PaCa-2 cells with a methyl transferase inhibitor (5-aza-2′-dc) or by ectopically overexpressing Sp1 increased the transcription of the *RII* gene. Conversely, RII expression was reduced in pancreatic cancer cells that express RII after treatment with mithramycin, a drug that blocks the binding of Sp1 to its consensus binding sequence. Thus, this study suggests that a threshold level of Sp1 is required for the expression of the *RII* gene. Clearly, in the case of MIA PaCa-2, 5-aza-2′-dc treatment increased Sp1 expression by up-regulating the transcription rate of the *Sp1* gene. Reversion of transcriptional repression of Sp1 causes an increase in RII expression and responsiveness to TGF-β in MIA PaCa-2 cells. Modulation of Sp1 expression may prove a useful target in restoration of RII expression and TGF-β signaling in cells that otherwise possess an intact TGF-β signaling pathway.

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