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Stabilization of SMAR1 mRNA by PGA2 involves a stem–loop structure in the 5’ UTR

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ABSTRACT

Prostaglandins are anticancer agents known to inhibit tumor cell proliferation both in vitro and in vivo by affecting the mRNA stability. Here we report that a MAR-binding protein SMAR1 is a target of Prostaglandin A2 (PGA2) induced growth arrest. We identify a regulatory mechanism leading to stabilization of SMAR1 transcript. Our results show that a minor stem and loop structure present in the 5’ UTR of SMAR1 (φ1-UTR) is critical for nucleoprotein complex formation that leads to SMAR1 stabilization in response to PGA2. This results in an increased SMAR1 transcript and altered protein levels, that in turn causes downregulation of Cyclin D1 gene, essential for G1/S phase transition. We also provide evidence for the presence of a variant 5’ UTR SMAR1 (φ17-UTR) in breast cancer-derived cell lines. This form lacks the minor stem and loop structure required for mRNA stabilization in response to PGA2. As a consequence of this, there is a low level of endogenous tumor suppressor protein SMAR1 in breast cancer-derived cell lines. Our studies provide a mechanistic insight into the regulation of tumor suppressor protein SMAR1 by a cancer therapeutic PGA2, that leads to repression of Cyclin D1 gene.

INTRODUCTION

Most of the cellular transformation processes often involve changes in the gene expression levels rather than changes in the sequence itself. The multiple layers of control include a closely knit transcriptional program and the associated translation of the product. Posttranscriptional regulation (includes mRNA stability and translation) is one such mechanism whereby the organisms control the flow of genetic information into the proteome (1). Within the continuum of posttranscriptional regulation, mRNA stability is considered a major effector of gene regulation (2). For example, the levels of several cell cycle regulatory molecules like Cyclin D1, p21, p27 and cdks are known to oscillate from a high in dividing cells to a low in quiescent cells. Most of these oscillations are due to transcriptional control and regulated protein stability (3). The manifestation of cancer is a consequence of the loss of control of one or more of these regulations that leads to a loss of gene function. Anticancer drugs like flavopiridol and cyclopentenone prostaglandins (PGs) are potent inhibitors of cell cycle in various cell lines (4–6). These compounds cause growth arrest in cultured cells and exhibit antitumor activity in vivo primarily by affecting cell cycle regulatory molecules. Cyclopentenone PGA1 and flavopiridol are shown to affect the transcriptional rate of Cyclin D1 and levels of cdks (7,8). PGA2-mediated growth inhibition is attributed to the downregulation of Cyclin D1 by reduction in the mRNA transcript. Further, microarray-based studies have led to the identification of mRNA species whose transcription and/or steady-state levels are regulated by cyclopentenone PGs (9–11). A number of reports have also documented the role of some RNA-binding proteins such as ELAV family proteins or TIA-1 and TIAR that bind to the 5’ or 3’ UTR of cell cycle regulatory proteins (12,13).

Recently, we have reported that SMAR1 (Scaffold/Matrix attachment region-binding protein 1) is a tumor suppressor MAR-binding protein that downregulates Cyclin D1 expression by recruiting HDAC1-mSin3A co-repressor complex at Cyclin D1 promoter locus (14). SMAR1 is documented to be a tumor suppressor protein that interacts with p53 (15) and retards B16F-10-induced...
melanoma in mouse model (16). It also acts as a transcrip-
tional repressor for MAR-mediated transcription from Eβ loci,
governing V(D)J recombination in T cells. Transgenic mice
overexpressing SMAR1 displays lymphoid organomegaly,
associated with higher infiltration of lymphoid cells (17).
Further, a drastic downregulation of SMAR1 in higher grades of breast cancer and cancer-derived cell lines like MCF-7, HBL-100, ZR 75.3 and ZR 75.1 has also been observed. (14,18).

In the present study, we looked for a possible reason for
downregulation of SMAR1 in breast cancer-derived cell
lines and a therapeutic agent to restore SMAR1 levels. We
find that the stability of SMAR1 mRNA in MCF-7 cells is
low and treatment of this cell line with antitumor agent
PGA2 increases the stability of the transcript. The increase in
SMAR1 mRNA stability is attributed to a 18 bp stem
and loop structure of SMAR1 5’ UTR (φ1-UTR) that
serves as a cognition site for binding of regulatory
proteins, essential for conferring transcript stability.
Interestingly, we identify a variant of SMAR1 5’ UTR
(φ17-UTR) that lacks the stem and loop structure of φ1,
leading to an altered stability. Moreover, we find that the
expression of φ17-UTR, correlated to an elevated Cyclin
D1 level marks the malignant nature of these breast
cancer-derived cell lines. Additionally, the involvement of
SMAR1 in PGA2-mediated growth arrest underscores the
importance of regulation of such tumor suppressor genes
by anticancer agents like PGA2.

MATERIALS AND METHODS

Cell culture and transient transfections

MCF-7 cells were maintained in DMEM supplemented
with 10% fetal calf serum (Invitrogen) in presence of 5%
CO2 at 37°C. Cells were seeded at a density of 1 × 10°/per
35 mm dish and cultured for 24 h before transfection.
MCF-7 cells were treated with PGA2 at various concentra-
tions in fresh complete medium. Cells were harvested
24 h after PGA2 treatment. Actinomycin D (Sigma) was
used at a concentration of 4μg/ml and incubated for
indicated time points.

Semi-quantitative RT–PCR

Seven micrograms of total RNA was subjected to reverse
transcription in 20μl reaction mixture containing 1× random hexanucleotide mix, 1 mM dNTPs, RNase
inhibitor and MuMLV reverse transcriptase (Invitrogen).
PCR reactions were carried out in a 25 μl reaction mixture
using 1 μl cDNA as template. Gene-specific primers used
are listed in Table 1.

Real Time RT–PCR

The quantitative measurements of RNA transcripts was
performed by icycler iQ thermal cycler system (BioRad)
using double-stranded DNA-specific fluorophore SYBR
Green. In a 25 μl PCR reaction, 1 μl of cDNA was
amplified using 1× iQ SYBR Green Supermix (BioRad)
containing 0.4 mM dNTP mix, 1.5 mM MgCl2, 50 pmol of
forward and reverse primer mix, SYBR Green I, 0.5 U
iTag DNA polymerase. Resolution of the product of
interest from nonspecific amplification was achieved by
melt curve analysis. Quantitation was done with three
different sets of cDNA samples. Sigma plot was used for
statistical analysis and plotting graphs.

Cloning of φ1 and φ17 UTR

The φ1 and φ17 UTRs were PCR amplified using specific
primers from φ1 and φ17 templates, described previously
(19). The PCR products were then cloned in and PGEM-T
Easy for φ1 and TOPO-TA vector for φ17. Plasmids
isolated from positive colonies were digested with EcoRI
and cloned in pEGFP vector.

RNase protection assay (RPA)

To determine the half-life of SMAR1, Actinomycin D at a
final concentration of 4μg/ml was added to MCF-7 cells.
For stability assays, PGÅ2 (70 μM) and Actinomycin D
were added simultaneously to the culture media.

Table 1. Different sets of primers and oligos used for assays

<table>
<thead>
<tr>
<th>Figure</th>
<th>Primer/Oligo</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A and 2, 2C, D, 5 B, C</td>
<td>For-AGACAAACACCACGAGAAT</td>
<td>SMAR1 (Predominantly φ1 form)</td>
</tr>
<tr>
<td>1C, 5 B, C</td>
<td>Rev-CGGAGTTCAGGGTGATGATGATGAC</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>1, 2, 5</td>
<td>For-TACCACTGGCATCGTGATGGACT Rev-TTTCTGCATCCTGTCGGCAAT</td>
<td>β-actin</td>
</tr>
<tr>
<td>2 E, G</td>
<td>For-TCCCTCCTCCAAAAATGCCAG Rev-AGAGATGGAAGGGGGAAAGA</td>
<td>Composite UTR</td>
</tr>
<tr>
<td>2 F</td>
<td>For-TACCACTGGCATCGTGATGGACT Rev-TTTCTGCATCCTGTCGGCAAT</td>
<td>φ1</td>
</tr>
<tr>
<td>2 A, B</td>
<td>Rev-CGGAGTTCAGGGTGATGATGATGAC cloned in PGEM-T Easy</td>
<td>φ17</td>
</tr>
<tr>
<td>2 I</td>
<td>Amplicon from For-AGACAAACACCACGAGAAT</td>
<td>SMAR1 (recognizes φ1 form predominantly)</td>
</tr>
<tr>
<td>2 I</td>
<td>Rev-TTCATCATCATCTTCGTCAAGA cloned in PGEM-T Easy</td>
<td>φ1 UTR</td>
</tr>
<tr>
<td>2 I</td>
<td>Amplicon from For-CAGACCCGGACCCACAC Rev-TTCATCATCATCTTCGTCAAGA cloned in PGEM-T Easy</td>
<td>φ17 UTR</td>
</tr>
<tr>
<td>4 D-G</td>
<td>Amplicon from For-CAGACCCGGACCCACAC Rev-TTCATCATCATCTTCGTCAAGA cloned in TOPO-TA</td>
<td>SL1</td>
</tr>
<tr>
<td>4 G</td>
<td>CAGAATTACCCCTCTAAAGGGGAACCACCGTCAGAAGAAC</td>
<td>Mutant 1</td>
</tr>
<tr>
<td>5 F, G</td>
<td>For-TGAAAATGAAAGAAGATGGATGAGTGC</td>
<td>Mutant 2</td>
</tr>
<tr>
<td>5 F, G</td>
<td>Rev-GAACACTTGACAGGGGTTGT</td>
<td>Probe II Cyclin D1 promoter</td>
</tr>
</tbody>
</table>
Cells were harvested at different time points post-treatment, washed in 1× PBS and pelleted at 1000 r.p.m. Total RNA was isolated using Tri-Reagent (Sigma) and used for protection assay. Antisense RNA probes were made by linearizing φI and φ17 SMAR1 templates using NcoI or PsI respectively, and performing in vitro transcription in the presence of SP6 or T7 RNA polymerase. In a 25 μl transcription reaction containing 1 μg of linearized DNA fragment, 1× transcription buffer (Stratagene), 0.4 mM each of ATP, CTP, GTP, 0.25 mM UTP and 50 μCi [α-32P]UTP (BRIT), 40 U of RNasin and 20 U of T7/SP6 RNA polymerase (Stratagene) were used to label RNA. Labeled RNA transcripts were then purified using probe quant G-50 columns (Amersham Pharmacia). Samples were heated at 90°C for 3 min before hybridization. For the hybridization reaction, 10 μg of total RNA was incubated with labeled probe (~10^6 c.p.m.) in hybridization buffer at 37°C for 16 h. The reaction mixtures were then diluted using 300 μl of digestion buffer. Single-stranded RNA was then digested using RNaseT1 (15 μg/ml) and RNaseA (1 μg/ml) for 2 h at 30°C. After phenol/chloroform extraction and ethanol precipitation, samples were analyzed on 6–10% urea gel. Band intensities were quantified using phosphorimager (BioRad).

**RNA electrophoretic mobility shift assays (EMSAs)**

PBK-CMV φI and φ17 were linearized with XhoI and in vitro transcribed with T3 polymerase to yield the respective 5' UTR probes. The wild-type and mutant UTR minor stem and loop sequences were synthesized as forward and reverse complementary oligonucleotides (Genomechanix), containing T3 polymerase site. They were then annealed with respective pairs and in vitro transcribed with T3 polymerase to yield the probes. The sequences are:

**SL1-Fwd:** CGAAATTAACCCTCAACTAAAGGGACC CACGGTCGACAGAACC

**Mutant 1-Fwd:** CGAAATTAACCCTCACTAAAGGG ACCACACGCTCGACAGGACC

**Mutant 2-Fwd:** CGAAATTAACCCTCACTAAAGG GAACCACATAATCGACAGGACC and their reverse complementary. EMSAs were performed in a 10 μl binding reaction containing 10 μg of MCF-7 cell extract, either untreated or treated with PGA2 and 12 fmol of transcript, 10 mM Tris (pH 7.4), 15 mM KCl, 5 mM MgCl2 and 10% glycerol. For competition assays, cold competitors were mixed with the hot probe 10 min prior to incubation with lysate. All reactions were carried out on ice for 20 min and then run on 8% native gel, resolved, dried and processed for autoradiography.

**Immunoblotting and antibodies**

Cells were scraped in 1× PBS, collected at various time points and lysed using DIGNAM buffer. Equal amount of proteins were taken for immunoblotting. Following SDS-PAGE, the resolved proteins were transferred to PVDF membrane (Amersham). Blocking was carried out with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membranes were probed with primary and respective secondary antibody. Proteins were detected using ECL plus chemiluminescence substrate (Amersham). Antibodies used are Cyclin D1 and Actin (Santa Cruz). SMAR1 polyclonal antibody was raised in house as described earlier (14). Mouse secondary HRP and rabbit secondary HRP were purchased from BioRad.

**Immunostaining**

MCF-7 cells were seeded on cover slips and after 24 h, the cells were treated with indicated amount of PGA2. Posttreatment, cells were washed with ice-cold 1× PBS and the cells were fixed with 2% PFA for 15 min at room temperature. After subsequent quenching and blocking in PBS containing 10% FCS, SMAR1 antibody was added onto the cover slips and incubated for an hour. After three washes with cold PBS, cells were incubated with secondary rabbit FITC in dark for 45 min. Cells were then washed three times with ice-cold PBS and mounted using Antifade (DABCO) from Sigma. The mounted slides were then visualized using confocal laser microscope (LSM-510, Zeiss, Thornwood, NY).

**Luciferase reporter assay**

For luciferase assays, 1 μg of Cyclin D1 luciferase promoter construct (CD1) was cotransfected with φI or φ17 SMAR1 with an internal GFP control in MCF-7 cells. Luciferase assays were performed 24 h posttransfection or PGA2 treatment. For SMAR1 knockdown experiments, 100 nM siRNA was cotransfected with Cyclin D1 promoter construct. Luciferase activity was measured using Luclite substrate (Perkin Elmer, USA) and assay performed using Top-Count luminometer (Packard Life sciences, USA). Equal amounts of protein were used for luciferase assays and relative light units plotted for luciferase activity.

**Chromatin Immunoprecipitation (ChIP) analysis**

ChIP assay kit (Upstate Biotechnology) was performed following manufacturer’s instructions. 1×10^6 cells were plated per 30 mm dish and treated with PGA2 or vehicle. After treatment, DNA–protein complexes were fixed with 1% formaldehyde at 37°C for 10 min at various time points. ChIP assays were carried out using anti-SMAR1, anti-HDAC1, anti-H3K9, anti-H4K10, RNA pol II, H3 pSer10 and H3K9 methyl antibodies (Cell Signaling). Input DNA, rabbit IgG (r-IgG), and mouse IgG (m-IgG) pulled DNA served as controls for all the experiments. Immunoprecipitated DNA was then subjected to 28 cycles of PCR using primers for probe II described earlier (14).

**Cell cycle analysis**

MCF-7 cells were plated at a density of 1×10^5 on a 35 mm dish. After 6 h of serum starvation, synchronized cells were transfected with SMAR1 siRNA, φI or φ17 SMAR1 plasmid constructs using Lipofectamine 2000 (Invitrogen). Twelve hours posttransfection, cells were treated with 30 and 70 μM PGA2 in case of siRNA transfection and cells were harvested 36 h posttransfection. After trypsinization, cells were collected and washed.
in PBS and pelleted at 1000 r.p.m. The resulting pellet was then re-suspended in 0.2 ml 1× PBS containing 2% fetal bovine serum and fixed in 0.8 ml 70% ethanol. Cells were kept in the fixative for 2 h and then centrifuged for 5 min at 1000 r.p.m. Cells were then treated with RNase A (75 U/ml) and re-suspended in 0.5 ml PBS containing 50 mg/ml propidium iodide (PI) and 0.1% Triton X-100. This was incubated at room temperature for 30 min and subjected to flow cytometry. PI-stained cells were analyzed for cell cycle profiles by FACS Vantage (Becton Dickinson) using Cell Quest.

RESULTS

PGA2 induces SMAR1 expression

The negative regulation of Cyclin D1 by SMAR1 and PGA2 are well documented, though the mechanisms reported are different (8,14). This prompted us to check the interdependence of these pathways and look for a possible regulation of SMAR1 by PGA2. To evaluate this study, we employed MCF-7 cell line derived from mammary epithelia that is characterized by low invasive potential and induced Cyclin D1 level correlated to reduced SMAR1 expression (14). MCF-7 cells were treated either with vehicle (ethanol) or PGA2 (0–100 μM).

Total RNA was isolated 24 h posttreatment and the cDNA obtained was subjected to RT–PCR and Real time RT–PCR analysis. SMAR1 transcript levels increased up to 45 μM PGA2 treatment, after which a steady-state level of the transcript was observed (Figure 1A). Quantitation by Real time RT–PCR showed that SMAR1 transcript increased 1.8- to 2-fold at 30–100 μM PGA2 (Figure 1B). The transcript profile was normalized using β-actin. PGA2-induced SMAR1 protein levels were checked using western blot analysis using 30 and 70 μM PGA2. At these concentrations, the protein expression increased by 3.8- and 4.5-fold respectively, while the protein levels were very low in untreated and vehicle-treated cells (Figure 1C and D). The steady increase in the protein amounts were then visualized using confocal microscopy. MCF-7 cells were treated with PGA2 (15, 30 and 70 μM) and the protein levels were monitored using SMAR1 antibody. A steady increase in the amount of SMAR1 was observed from 15 to 70 μM PGA2 concentrations (Figure 1E).

PGA2 stabilizes SMAR1 mRNA

It is well known that most of the reported prostaglandin-mediated effects occur through alteration in mRNA half-life. Results from the previous section show that PGA2
treatment increased SMAR1 transcript by 2-fold but the protein level by 4-fold, indicating a possible involvement of mRNA stability. To address this issue, half-life of endogenous SMAR1 transcript was verified by blocking cellular transcription using Actinomycin D (4 μg/ml) and cells were collected at indicated time points (Figure 2A). Total RNA was isolated and subjected to RPA as described in ‘Materials and Methods’ section. As shown in Figure 2A upper panel, SMAR1 transcript level decreased after 4 h in Actinomycin D-treated samples. Interestingly, upon addition of Actinomycin D and PGA2, we observe that the levels of SMAR1 transcript remained steady till 24 h (Figure 2B, upper panel). This indicates that PGA2 stabilizes SMAR1 mRNA and maintains the steady-state levels of the transcript. The mRNA available at the given time points were normalized to the actin transcript and quantification represented as bar graph (Figure 2A and B).

**SMAR1 has two 5’ UTR variants in MCF-7 cells**

The stability and translation of most of the transcripts are associated with the untranslated regions, both at 5’ and 3’ UTRs. Primers were designed to amplify the sequence from the start of exon1 and a part of exon 2 that was expected to yield an intact SMAR1 5’ UTR of 142 bp (18). The amplimer of 5’ UTR from the cDNA of MCF-7 cells untreated and treated with PGA2 were checked on a 10% polyacrylamide gel. Interestingly, untreated cells showed a single amplimer (Figure 2C, lane 1) while in case of PGA2 treatment (30 and 70 μM), there was an additional amplimer (Figure 2C, lanes 2 and 3). The fast migrating amplimer (lower band) was common to both untreated and PGA2 treated cells, while there was an induction of the slower migrating amplimer (upper band) specifically in PGA2-treated cells. Thus, the upper and the lower bands represent two variants of 5’ UTR. Earlier, we have reported the identification of three clones of SMAR1 from mouse thymocyte cDNA library (19). Out of these, two clones shared the same open reading frame and same translational reading frame of downstream sequence, but differed only by 18 bases in 5’ UTR. The two clones were denoted as φ1 (containing full-length 5’ UTR) and φ17 (containing variant 5’ UTR lacking 18 bases). When checked using φ1 and φ17 template controls (Figure 2D, lanes 2 and 4), we find that the size of upper band obtained upon PGA2 treatment corresponded to the φ1 form (the upper band in Figure 2C, lanes 2 and 3; Figure 2D, lane 5) and the lower band in untreated and PGA2-treated cells corresponds to φ17 form (the lower band in Figure 2C, lanes 1–3, Figure 2D, lane3). This was further verified by cloning the corresponding PCR products in PGEMT-Easy vector and DNA sequencing. RT–PCR analysis of cDNA from MCF-7 cells (untreated or treated with 70 μM PGA2, lanes 1 and 2, respectively) with primers specific for φ1-UTR and φ17-UTR forms was performed. The results revealed that upon PGA2 treatment there was no apparent change in the φ17 form but there was an increase in the φ1 form of SMAR1 (Supplementary Figure S1A). The map of SMAR1 gene and the primers used are depicted in Supplementary Figure S1B.

**The responsiveness of 5’ UTR to PGA2 is essential for SMAR1 mRNA stability**

Next we checked if the responsiveness of SMAR1 UTR to PGA2 contributed to the stability of SMAR1 transcript. The antisense UTR transcript specific to each form was hybridized with the total RNA from MCF-7 cells untreated or treated with PGA2, as described in ‘Materials and Methods’ section. Protected bands revealed that the φ17 form was predominant in untreated cells, unlike the φ1 form (almost 3–4 times lower than φ17, verified by densitometry). An increase in protection of φ1 transcript was observed upon PGA2 treatment while φ17 form remained almost unchanged (Supplementary Figure S1C). To validate the transcript profiles of φ1-UTR and φ17-UTR containing SMAR1, cDNA obtained was subjected to Real time RT–PCR analysis using primers specific to the UTRs and melt curve analysis performed. The transcript profile obtained after Actinomycin D treatment showed that the half-life of φ1 was 4–6 h while that of φ17 varied between 14–16 h (Figure 2E). Though the transcript level of φ1 remained stable after PGA2 and Actinomycin D treatment, φ17 transcript levels started to decline from 12 h, as verified by melt curve analysis (Figure 2F). Quantification of transcripts revealed that φ1-SMAR1 level increased 2-fold at 6 h time point of PGA2 treatment and remained steady till 24 h, while there was a negligible change in φ17-SMAR1 level (Figure 2G). These results point out at the early response of φ1-UTR to PGA2 treatment that stabilizes the transcript till 24 h. To further assess the response of the two UTRs to PGA2, we performed reporter assays, where the UTRs from both forms were cloned upstream of gfp gene in pEGFP vector. In case of φ1-UTR transfection, we observed a 2–3-fold increase in GFP expression upon PGA2 treatment compared to the untreated cells (Figure 2H, lanes 3–5). φ17-UTR transfection however showed only a minor change in the basal expression of GFP, showing that φ1 and not φ17 is responsive to PGA2 (Figure 2H, lanes 7–9). Thus, we demonstrate that in MCF-7 cells, φ1 form of SMAR1 and not φ17 form responds to PGA2.

**φ1-UTR stem and loop structure is critical for PGA2-induced complex formation**

The natural tendency of RNA is to form highly stable secondary and tertiary structures and the alteration in these structures represent a well-known regulatory mechanism for many cellular processes (20). To determine the differences in the secondary structure that could implicate a functional significance, we analyzed the φ1 and φ17 UTR sequences as shown in Figure 3A. Prediction of the secondary structures of the two UTRs was done using M-fold secondary structure prediction software (21). After energy minimizations, we observed that φ17-UTR lacks a small stem and loop structure, pertaining to those missing 18 bases (Figure 3B).
Figure 2. PGA2 stabilizes SMAR1 mRNA. (A) Equal number of MCF-7 cells were seeded in 35mm dishes and 24h later treated with 4μg/ml Actinomycin D. Cells were collected after indicated time points and total RNA isolated using Tri reagent. In case of PGA2 treatment, 70μM PGA2 was added to cells 12h prior to Actinomycin D treatment. Stabilization of SMAR1 transcript by PGA2 was verified using RPA. Antisense probe from Nco1 digested SMAR1 fragment from PGEM-T Easy was used and protection assays were performed as described in ‘Materials and Methods’ section. Upper panel shows that the half-life of SMAR1 in MCF-7 cells is about 4h. (B) Upper panel shows protection of SMAR1 mRNA over the indicated time intervals upon Actinomycin D and PGA2 treatment. The lower panels show actin mRNA levels used as control. The bar graphs both in A and B represents the quantification of transcripts normalized to actin. (C) RT–PCR analysis of cDNA from untreated and PGA2-treated MCF-7 cells using composite UTR primers reveals the amplification of a lower size fragment in cells (lane1) while in treated sample, the upper band becomes prominent with increasing PGA2 concentration (lanes 2 and 3). (D) Amplification of specific UTRs of cDNA from control and PGA2-treated cells reveals that φ17 is present in untreated cells (lane 3) and φ1 in PGA2-treated samples (lane 5) as checked by amplification with template control (lanes 2 and 4). The pUC mix marker (Fermentas) in lane 1 was used to identify the molecular size of the fragments. (E) Quantitation of transcript derived from φ1 and φ17 after Actinomycin D treatment reveals that the half-life of φ1 is between 4 and 6h and that of φ17 between 14 and 16h. The transcripts were normalized to β-actin control. (F) Real-time–PCR analysis of φ1 and φ17 transcript upon PGA2 and Actinomycin D treatment reveals that φ1 form remains constant while the φ17 form does not respond to PGA2 treatment and there is a decline in the transcript.
One microgram of each of the 17-UTR upon PGA2 treatment (Figure 4D). This reveals the importance of SMAR1 mRNA, the product of which results in Cyclin D1 repression. To confirm this, we performed luciferase reporter assays where Cyclin D1 regulation by 1 and PGA2 were compared. Cotransfection of 0.5 and 1 μg of 1 along with Cyclin D1 promoter construct (CD1-luc) revealed that 1 downregulated Cyclin D1 by 1.5- and 2.5-fold, comparable to PGA2-treated samples. Moreover, PGA2 treatment after knockdown of SMAR1 using the specific siRNA showed inefficient downregulation of Cyclin D1 (Figure 5B). This reveals the importance of SMAR1 produced from 1 transcript in PGA2-mediated downregulation of Cyclin D1.
repression of Cyclin D1. As discussed earlier, the recruitment of SMAR1 and the associated corepressor complex to Cyclin D1 promoter is well documented, and hence we verified if SMAR1 occupied Cyclin D1 promoter in response to PGA2 (Figure 5C). We observed the interaction of HDAC1 and SMAR1 upon PGA2 treatment (Supplementary Figure S2A and B). Chromatin immunoprecipitation experiments using MCF-7 cells either untreated or treated with 70 μM PGA2 were performed. Amplification of probe II region in SMAR1 and HDAC1 immunoprecipitated from PGA2 treated cells (30 and 70 μM) showed the recruitment of SMAR1 corepressor complex on Cyclin D1 promoter (Figure 5D, lanes 2 and 3, 9 and 10,). siRNA-treated, SMAR1-pulled chromatin was used to verify the specificity of the recruitment (Figure 5D, lanes 6 and 7, 12 and 13). These results are consistent with the direct recruitment of HDAC1 and SMAR1 on Cyclin D1 promoter that is responsible for the observed repressive effects. Upon over expression, SMAR1 is shown to deacetylate the histones at H3K9 and H4K8 loci that is accompanied by dephosphorylation of H3-phospho-ser 10 at Cyclin D1 promoter.
Figure 5. Responsiveness of SMAR1 5' UTR is essential for exerting its transcriptional modulatory function. (A) RT–PCR analysis of SMAR1, Cyclin D1, and β-actin transcript levels in PGA2-treated (lanes 2 and 3 at 30 and 70 μM PGA2) or vehicle-treated MCF-7 cells (lane 1). Actin was used as loading control (lower panel). (B) Luciferase activity from Cyclin D1 promoter was studied after transfection with the indicated plasmids, siRNA or PGA2 treatment and relative light units were plotted in the bar graph. Experiments were performed as three independent sets and error bars denote the mean deviation. From below, Bar 1 denotes the luciferase activity of CD1 promoter. Bars 2 and 3 denote the repression of Cyclin D1 by the intact UTR containing SMAR1 while bars 4 and 5 indicate down regulation of Cyclin D1 by the 30 and 70 μM PGA2. Use of SMAR1 specific siRNA and PGA2 treatment shows inefficient downregulation of Cyclin D1 luciferase activity. (C) Schematic representation of Cyclin D1 promoter and SMAR1-binding site (SBS) in probe II region. (D) Chromatin immunoprecipitation was performed as described in ‘Material and Methods’ section. Recruitment of SMAR1 (lanes 2 and 3) and HDAC1 (lanes 9 and 10) on probe II region of Cyclin D1 promoter was studied upon PGA2 treatment in MCF-7 cells using antirabbit SMAR1 and antimouse HDAC1 antibody. Endogenous SMAR1 was depleted using SMAR1 siRNA and studied for recruitment of SMAR1 (lane 7) and HDAC1 (lane 13) in MCF-7 cells treated with PGA2 (70 μM). Scrambled siRNA (scr) was used as negative control that did not alter SMAR1 or HDAC1 recruitment (lanes 6 and 12, respectively). Chromatin immunoprecipitation using mouse IgG, rabbit IgG or no antibody are used as negative control (lanes 15–17). Reverse cross-linked sheared genomic DNA is used as positive control (input) for amplification (lanes 1, 4, 7 and 10). Probe III region was used as control to verify the specificity of SMAR1 binding to the promoter region.
locus. Therefore, we checked if these modifications persist upon PGA2 treatment or if any additional histone modifications are involved in repression of Cyclin D1. We observed a decrease in acetylation at H3K9 (Figure 5E, lanes 5 and 6), H4K8 (Figure 5E, lanes 8 and 9) and phosphorylation of H3p-ser 10 (Figure 5E, lanes 11 and 12) in PGA2-treated cells compared to mock-treated cells. In addition to this, H3K9 monomethylation status that is well documented to have a role in transcriptional activation was investigated. As shown in Figure 5E, lanes 14 and 15, PGA2 treatment decreased the methylation of probe II region compared to mock treatment. To establish the repression of transcription brought about by SMAR1, the recruitment of RNAP II on Cyclin D1 promoter locus was studied. Decreased amplification of probe II region in RNAP II-pulled samples compared to mock treated suggests decreased transcription from Cyclin D1 promoter locus (Figure 5E, lanes 2 and 3). Nonspecific probe III was used as negative control (lanes 17 and 18). Mouse and rabbit IgG served as negative controls (Figure 5D, lanes 15 and 17).

**Cell cycle arrest function of PGA2 mediated by SMAR1 depends on the 5' UTR**

Previous studies by Bhuyan et al. (22) showed that PGA2 exerts growth inhibitory activity in many human cell lines. Consistent with this, we found that PGA2 treatment caused G1 phase arrest by DNA content analysis using flow cytometry. We then checked if this growth inhibitory effect of PGA2 is mediated by SMAR1. Hundred-nanomolar SMAR1 siRNA was transfected in MCF-7 cells and 16h posttransfection, PGA2 was added to the culture media. Cell cycle analysis was done 24h post-PGA2 treatment, after checking the knockdown of SMAR1 (data not shown). There was a marked shift of the cells from G1 to S and G2 phase, indicating that PGA2 was no longer able to arrest cells in G1 phase in the absence of SMAR1 (Figure 6A). The results so far suggest the involvement of PGA2 in stabilizing p1 SMAR1 and it is also clear that SMAR1 is required for PGA2-mediated growth arrest. This prompted us to investigate the difference in growth arrest function of SMAR1, in context of the two UTRs it can host. For this, MCF-7 cells were transiently transfected with equal amounts of p1 or p17 SMAR1 and analyzed for cell cycle progression. DNA content analysis revealed a 1.2–2.5-fold higher number of cells in G1/S phase in case of p1-SMAR1 transfection in comparison to p17- SMAR1 (Figure 6B). This hints that the growth inhibitory effect of PGA2 is mediated at least partially by p1-SMAR1. To check if the PGA2-mediated induction of SMAR1 specifically leads to Cyclin D1 downregulation, western blot analysis to check the status of other cyclins was performed. The results were then verified using siRNA treatment of SMAR1. The specificity of SMAR1 upregulation upon PGA2 treatment was also verified using siRNA treatment (Supplementary Figure S3A). The status of other cyclins (Cyclin A, B and associated cyclin kinases cdks 4 and 6) was unaltered (Supplementary Figure S3B). This result was corroborated using cDNA microarray results (Supplementary Figure S4).

**Breast cancer-derived cell lines from mammary epithelia primarily express p17 form**

To find out if the mechanism of downregulation of SMAR1 by altering the mRNA stability is common to breast cancer-derived cell lines, we screened for the presence of the two forms of SMAR1 UTR in ZR75-1, SKBR-3, T47D and MDA-MB-231. Different cell lines were cultured, RNA isolated and limited cycle RT–PCR analysis was performed. RT–PCR analysis showed that ZR-75-1 failed to show the product that corresponds to p1-UTR form but expresses p17-UTR only (Figure 6C, lane 2 upper and middle panel). This was similar to MCF-7 cells where a very low amplification of p1-UTR transcript was observed (Figure 6C, lane 6, upper and middle panel). Other cell lines like MDA-MB-231, SKBR3 and T-47D expressed the p1-UTR form predominantly and p17-UTR form to a lower extent (Figure 6C, lanes 1, 4 and 5, upper and middle panel). HEK-293 cells, where p1 form is expressed predominantly and p17-UTR is undetectable was used as control (Figure 6C, lane 3, upper and middle panels). The transcript profile we have obtained is in accordance with our previous result, where we have observed that ZR-75-1 and MCF-7 express very low amount of endogenous SMAR1 protein, whereas other cell lines showed a relatively high SMAR1 expression (14). Since the UTR profile of ZR-75-1 and MCF-7 cells was similar, we checked the response of this cell line to PGA2 treatment and compared to MCF-7 cell line. Upon 30μM PGA2 treatment, we found a significant induction of the p1-UTR form compared to the untreated cells (Figure 6D, lanes 1 and 3, upper panel). This was strikingly similar to MCF-7 cells where PGA2 treatment leads to induction of p1-UTR form (Figure 6D, lanes 2 and 4, upper panel). Cyclin D1 profiles in these cell lines untreated or treated with PGA2 showed that the repression of Cyclin D1 occurred in case of PGA2-treated samples that correlate with the emergence of p1-UTR form.

**DISCUSSION**

The primary effect of hormonal therapeutics like PGA2 has been documented through alteration of half-life of mRNA (23,24). The significant role of mRNA stability in gene expression makes it an essential component in pathways whereby tissues and organs respond to stress. Several anticancer agents like PGA2 rely on their ability to alter the mRNA stability of target cell cycle regulatory molecules, to govern the cell cycle fate. The essence of mRNA stability is the balance between regulation by destabilization determinants or translational inhibitory structures that are rendered favorable for transcription upon appropriate stimuli (25).

The current study stems from the fact that SMAR1 transcript is found to be drastically downregulated in higher grades of breast cancer and breast cancer-derived cell lines (14,18). Our aim was to decipher the reason for
downregulation of SMAR1 in breast cancer-derived cell lines, specifically in context to MCF-7 cell line. Considering that both SMAR1 and PGA2 are documented to halt cell cycle at G1/S phase, it was an interesting possibility that PGA2 regulates SMAR1, thereby bringing about the repression of a common target gene, i.e. Cyclin D1. MCF-7 cell line is characterized by low levels of SMAR1 transcript that is attributed to a half-life of 4 h, which in turn leads to a poor endogenous protein level. PGA2 treatment, however, renders the transcript stable till 24 h and elevates SMAR1 level in the cell line. Since majority of the cases involving mRNA stability rely on the ability of the UTR present on the 5’ or 3’ end of the transcript to respond to varying stimuli, the aim was to decipher the role of SMAR1 UTR in conferring stability to the transcript. Amplification of the 5’ UTR end of SMAR1 lead to the identification of a variant form of SMAR1-UTR in MCF-7 cells. This has been previously identified as ϕ17-SMAR1 and possesses the same ORF and reads out SMAR1 protein similar to the ϕ1 clone. SMAR1 transcript profile also reveals that this form is predominant in MCF-7 cells, contributing to almost 65% of the observed total transcript (data not shown). The half-life of the transcript with respect to the two UTR forms is also different, with ϕ1 having a half-life of 4–6 h while that of ϕ17 is 14–16 h. Since the secondary structure of RNA is thought to play a significant role in imparting stability and hence dictating the protein translation, we analyzed the secondary structure of both the UTRs. The ϕ1 UTR of SMAR1 is a thermodynamically stable structure and differs from its variant form (ϕ17) in hosting a minor stem–loop structure, the one formed by the missing 18 bases. Recent studies highlight the importance of binding of regulatory proteins to different structures posed by the RNA (26). Further investigations indicated that this sequence is critical for SMAR1
stabilization brought about by nucleoprotein complex formation on the stem–loop structure. The competition studies using different mutants showed that the secondary structure of the loop was important. It is thus possible that upon PGA2 treatment certain proteins that recognize this fold, bind to the stem and loop structure and regulate mRNA stability, resulting in increased SMAR1. This could be an alternate selection mechanism in tumor cells, whereby a variant UTR containing a transcript, encoding a tumor suppressor protein is retained for yet other unknown functions. Interestingly, we also find that the growth-inhibitory response of cells to PGA2 is in part due to the stabilization of SMAR1 mRNA by 5′ UTR, as the knock down of SMAR1 leads to a compensation in the growth-arrest function of PGA2. Considering that we have identified a 5′ UTR variant that is most likely a splice variant of the full-length UTR, it is tempting to hypothesize that RNA-binding factors involved in splicing regulate SMAR1 mRNA stability. This possibility cannot be ruled out as the splicing site between first and second exon junction of SMAR1 that spans 23 kb, hosts the 18 base stem–loop structure critical for SMAR1 stabilization. It is plausible that the recruitment of ribonucleoproteins like TIA-1 that aid in 5′ UTR splicing (27) is regulated by another stabilizing factor. This is essential for splicing of the 60 bases from the 3′ end of first exon to 5′

Figure 7. Diagrammatic representation of SMAR1 regulation by PGA2. (A) The genomic organization of SMAR1 shows that the untranslated exon 1 and a part of exon 2 (23 kb apart) forms the intact 5′ UTR of SMAR1. The junction of these exons is marked by the stem and loop structure of 5′ UTR. The intact 5′ UTR sequence of SMAR1. (B) In MCF-7 cells, φ17, a variant 5′ UTR of SMAR1 is present in higher amount compared to the φ1 form. (E) Factors induced by PGA2 bind to the stem and loop structure of φ1, stabilize the mRNA and altered SMAR1 protein level. SMAR1 protein then recruits HDAC1 corepressor complex to Cyclin D1 promoter, represses transcription, and hence causes G1/S phase arrest.
end of the second exon, to form the composite stem–loop structure (Figure 7A). In breast cancer-derived cells, low level of endogenous SMAR1 and subsequently high level of Cyclin D1 leads to cell proliferation, malignant transformation, and hence neoplasia. Under conditions that favor proliferation or lead to malignant transformation, cancer cells might have evolved such strategies to downregulate tumor suppressor proteins like SMAR1, as witnessed by low levels of the protein in breast cancer-derived cell lines. Treatment with anticancer therapeutics that lead to cell cycle arrest, reverse this effect and proteins like SMAR1 that downregulate proliferative genes are upregulated. It is also possible that SMAR1 transcript stabilized by PGA2 has a definitive link with the estrogen responsiveness of cell line, at earlier stages of transformation. In case of advanced cancer cases, however, this posttranscriptional control mechanism might be defective. Therefore, in spite of having d1-SMAR1 and expressing SMAR1 protein, the existence of alternative pathways that nullify the effect of SMAR1 cannot be ruled out. Thus, our studies provide a mechanism where a therapeutic agent PGA2 used to treat malignancies, alters the mRNA stability of tumor suppressor protein SMAR1, that downregulates the proliferative gene Cyclin D1.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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