Thrombomodulin Is Silenced in Malignant Mesothelioma by a Poly(ADP-ribose) Polymerase-1-mediated Epigenetic Mechanism*

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Malignant mesothelioma (MM) is a relatively rare neoplasm, whose formation is associated with occupational hazard largely due to exposure to asbestos, with rather common metastases to the lungs, liver, bone, and the adrenal gland (1). It has been reported that MM patients frequently suffer from thromboembolic complications (2–5). An increased susceptibility to thrombosis of solid malignant diseases develops as a result of the activating effect of malignant cells on the hemostatic system (6). The development of this activating effect is a consequence of interactions between malignant cells and the various components of the coagulation system, including coagulation factors, platelets, endothelial cells, and the fibrinolytic system, which promotes progression from a prothrombotic state to clinically manifested disorders of the hemostatic system (7).

Among natural anticoagulants, thrombomodulin (TM) plays a critical role due to its anticoagulant activity, and it is also involved in pathological processes, including thrombosis, inflammation, and cancer (8). TM is a glycosylated transmembrane protein with high affinity for thrombin to form a complex that inhibits thrombin activity and accelerates protein C activation (9). Although it is mainly expressed by endothelial cells, it is found in a wide range of other cells (10–12). Heterogeneous expression of TM has been observed in the neoplastic disease, and it is found in a wide range of other cells (10–12). Heterogeneous expression of TM has been observed in cancer, and low or no TM expression is associated with poor prognosis (13–16).

TM is transcriptionally up-regulated by thrombin, the vascular endothelial growth factor, histamine, retinoic acid, cAMP, or heat shock (17, 18). Its transcription is down-regulated by shear stress, hypoxia, and the transforming growth factor-β (19). The ability of the TM gene promoter to bind relevant transcription factors appears to reflect a summation of events that define competence, including nucleosome position, histone modifications, nuclear localization, and the DNA methylation status.

DNA methylation is typically required for active transcription and is frequently developmentally regulated (20). In addition, expression of various genes is modulated via modification of histones. Silencing of the TM gene promoter has been implicated in the down-regulation of TM synthesis, and it was found that methylation of the promoter CpG islands (CGI) is one of the mechanisms causing its low expression (21, 22).

The mechanism by which CGIs are protected from methylation in nonmalignant cells and become susceptible to methylation in tumor cells is unknown. Accumulating evidence indicates a role for poly(ADP-ribose) polymerase-1 (PARP1) in the regulation of methylation patterns. Besides being an enzyme involved in DNA repair, PARP1 also participates in the transcription regulation of various genes. More specifically, the pro-

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§ The abbreviations used are: MM, malignant mesothelioma; 5-aza-dC, 5-aza-deoxycytidine; CGI, CpG island; DNMT, DNA methyltransferase; NM, normal mesothelioma; PARP, poly(ADP-ribose) polymerase-1; qRT-PCR, quantitative RT-PCR; TM, thrombomodulin; TSA, trichostatin; IP, immunoprecipitation.
tein promotes decondensation of the chromatin structure through poly(ADP-ribosyl)lation of histones as well as acting as an enhancer-promoter regulatory complex (23). It has been found that the nuclear balance between unmodified and poly(ADP-ribosyl)ated PARP1, which depends on the dynamics of the PARP and poly(ADP-ribose)glycohydrolase activity, is a key to the maintenance of the genomic methylation pattern (24).

In this study, we analyzed TM expression in biopsies of MM and investigated the involvement of DNA methylation-associated gene silencing in TM expression. To evaluate the role of PARP1 in epigenetic modifications of the TM gene promoter, nonmalignant mesothelial cells (Met-5A) and MM cells (H28) were silenced for PARP1, and DNA methylation and acetylation-associated gene expression were evaluated. We show here, for the first time, that PARP1 regulates TM expression on the epigenic level and that this regulation is involved in MM.

EXPERIMENTAL PROCEDURES

Biopsy Sampling—Biopsy specimens were obtained from 22 subjects (aged 69.8 ± 10.1 years; 20 males, 2 females) who underwent thoracoscopy or thoracotomy for suspected MM. The collected tissue was divided into two parts; one was suspended immediately in the RNALater solution (Ambion) and stored at −80 °C until analysis. The other part of the tissue was used for histological examination by the Pathological Anatomy Unit of the Hospital University (Ancona, Italy). According to the diagnosis, the individuals were classified as subjects with MM (the MM group) and as healthy subjects with normal mesothelium (the NM group). The MM group (n = 10) included tissue with clear signs of the pathology, whereas the NM group (n = 5) included nonmalignant tissue. The clinical data were obtained retrospectively and include information on the gender, age, histology, neoadjuvant chemoradiation and therapy administration (before surgery), smoking status, and the pathologic staging. The exclusion criteria were the presence or suspicion of any infectious disease and other malignancies. The patients were not treated with any adjuvant chemotherapy or radiation therapy. All subjects filled in a questionnaire that included their informed consent and provided a blood sample. The study was carried out according to the Helsinki Declaration and approved by the Ethical Committee of the University Hospital of Marche, Italy. The demographic and pathological characteristics of the subjects are summarized in Table 1.

Cell Culture—Nonmalignant immortalized mesothelial cells (Met-5A) and MM cells (H28) were obtained from the American Type Culture Collection. The cell lines were grown in RPMI 1640 medium supplemented with 2 mm l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS and cultured at 37 °C and 5% CO₂ in humidified atmosphere.

Knockdown of PARP1—To establish sub-lines with stably silenced PARP1, 0.2 × 10⁶ exponentially growing Met-5A and H28 cells were transfected with 1 µg of the PARP1 shRNA pRS plasmid, with the PARP1 targeting sequence of TAC CAT CCA GGC TGC TTT GTC AAG AAC AG or 1 µg of empty pRS plasmid (OriGene) using the TransIT-LT1 transfection reagent (Mirus). Selection was carried out with puromycin added to the cells at 1 µg/ml 48 h post-transfection. Puromycin-resistant clones were isolated, expanded, and analyzed for PARP1 protein and mRNA levels as well as PARP activity. Selected clones were maintained in 1 µg/ml puromycin.

Assessment of PARP Activity—PARP activity was assessed using the universal colorimetric PARP kit (Trevigen) based on the incorporation of biotinylated ADP-ribose into histone proteins. Cell and tissue lysates containing 50 µg of protein were loaded into 96-well plates coated with histones and biotinylated poly(ADP-ribose), allowed to incubate for 1 h, treated with streptavidin-HRP, and read at 450 nm in an ELISA plate reader (Sunrise). The PARP activity was expressed as units per mg protein.

DNA Methyltransferase Activity Analysis—The activity of DNA methyltransferases (DNMTs) was evaluated in nuclear extracts of Met-5A and MM H28 cell lines and their PARP1-silenced counterparts (Met-5A and H28 cells) with or without 5-aza-2′-deoxycytidine (5-aza-dC; Sigma) treatment. The cells were seeded in T25 cell culture flasks and treated with 5 µM 5-aza-dC for 96 h. The cells were then harvested, and nuclear extraction was performed (EpiQuik nuclear extraction kit, Epigentek). The DNMT activity was assayed in 5 µM of nuclear extract proteins using the EpiQuik DNA methyltransferase activity kit (Epigentek). The results were expressed as the increase in the OD per hour per mg of protein.

Western Blotting—Cells and tissue were lysed in a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1 mM EDTA (pH 8), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and a mixture of protease inhibitors (2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml pepstatin) and stored at −80 °C until analysis. The protein level was quantified using the Bradford assay (Sigma). 50 µg of protein were separated on a 12.5% SDS-PAGE gel and transferred to nitrocellulose membranes (Protran). After blocking for 1 h with 5% nonfat milk in PBS-Tween, the membranes were incubated with monoclonal anti-PARP1 IgG (C2–10; Trevigen), monoclonal anti-TM IgG (PBS-01; Abcam), and polyclonal anti-β-actin (Bethyl) at 4 °C overnight. After washing with PBS-T, the membranes were incubated with HRP-conjugated secondary antibody for 2 h at room temperature, and the protein bands were visualized using the ECL detection system (Pierce). Band intensities were evaluated by ChemiDoc using Quantity One software (Bio-Rad).

### TABLE 1

**Demographic and pathological characteristics**

| Biopsies | Age (years) | Sex | Smoking (yes/ex/no) | Histotype (EP/B1/SA) | Stage |
|----------|-------------|-----|---------------------|----------------------|-------|
| MM-1     | 68          | M   | Ex                  | EP                   | S-Ib  |
| MM-2     | 63          | M   | No                  | EP                   | S-la  |
| MM-3     | 66          | M   | No                  | EP                   | S-la  |
| MM-4     | 75          | M   | Yes                 | EP                   | S-IV  |
| MM-5     | 81          | F   | Yes                 | EP                   | S-III |
| MM-6     | 70          | M   | Ex                  | EP                   | S-la  |
| MM-7     | 75          | M   | Ex                  | EP                   | S-III |
| MM-8     | 66          | M   | No                  | EP                   | S-III |
| MM-9     | 83          | M   | No                  | SA                   | N.D.  |
| MM-10    | 77          | M   | No                  | EP                   | N.D.  |
| NM-1     | 80          | F   | Yes                 | n.s.                 | n.s.  |
| NM-2     | 58          | M   | Yes                 | n.s.                 | n.s.  |
| NM-3     | 83          | M   | No                  | n.s.                 | n.s.  |
| NM-4     | 60          | M   | No                  | n.s.                 | n.s.  |
| NM-5     | 61          | M   | No                  | n.s.                 | n.s.  |
Epigenetic Regulation of Thrombomodulin Expression

5-Aza-2'-deoxycytidine and Trichostatin Treatment—Met-5A and H28 cells lines and their PARP1-silenced counterparts (Met-5A194 and H281949 cells) were seeded in a six-well plate at a density of 0.1 × 10^6. After an overnight incubation, cells were treated with freshly prepared 5 μM 5-aza-dC for 96 h or 200 ng/ml trichostatin (TSA, Sigma) for 24 h. For the combination treatment, cells were first treated with 5 μM 5-aza-dC for 72 h, then 200 ng/ml of TSA was added, and the cells were incubated for further 24 h.

**Immunohistochemistry**—Formalin-fixed paraffin-embedded tissue of the subjects affected by MM (n = 10) was collected from the Pathological Anatomy Unit of the Hospital University of Ancona, Italy. The normal mesothelial tissue from NM subjects (n = 6) was used as the control. Formalin-fixed paraffin-embedded sections were cut at 5-μm thickness, deparaffinized, and heated in 1× EDTA buffer (pH 8.0) for 15 min. After blocking, the sections were incubated with anti-TM IgG (PBS-01; Abcam) at 4 °C overnight and then with the FITC-conjugated secondary antibody (Pierce). The sections were inspected in a fluorescence microscope (Axiocam MRc5, Zeiss). Hematoxylin and eosin staining was used for histological evaluation.

**Immunocytochemistry**—Met-5A and H28 cells as well as their PARP1-silenced counterparts were placed in 35-mm dishes on glass coverslips. After an overnight incubation, the tissue detected by Western blot analysis and expressed as band density normalized to β-actin. C. representative immunohistochemical analysis of TM expression (right panels) and tissue histology (left panel) of MM and NM tissue.

**Quantitative RT-PCR (qRT-PCR) Analysis**—Total RNA was extracted from biopsies (2–3 mg) and cultured cells (10^6) using the SV total RNA Isolation System (Promega Italia) according to the manufacturer’s instructions. The first-strand cDNA was synthesized using the MMLV Kit (Promega Italia). The qRT-PCR analyses were performed in the final volume of 25 μl using the specific primer sequences: TM, 5'-TAA CGA AGA ACA CAC AGA CTA CGA TT-3' (forward) and 5'-CTA GCC CAC GAG GTG AGA GT-3' (reverse); PARP1, 5'-AAG CCC TAA AGG CTG AGA AC-3' (forward) and 5'-AGG AGG CAC TTG CTG CTT GT-3' (reverse); and GAPDH, 5'-TCC ACT GGC GTC TTC ACC-3' (forward) and 5'-GTC AGA GAT GAT GAC CCT TTT TTT-3' (reverse). The housekeeping GAPDH gene was used as the loading control. The expression of the genes was assessed by the IQ5 qPCR instrument (Bio-Rad) using the RT2 SYBR Green qPCR Master Mix (Invitrogen). The results were expressed as ΔΔCT, and fold changes in relative mRNA expression were calculated using the equation 2^(-ΔΔCT).

**DNA Isolation and Bisulfite Modification**—Genomic DNA was isolated from five noncancerous and 10 cancerous biopsy specimens and from cultured cell lines treated with 5-aza-dC and TSA alone or in combination, using the Methylamp isolation and modification coupled DNA Kit (Epigentek) according to the manufacturer’s protocol. The extraction of genomic DNA from cultured cells was performed using the DNeasy Blood and Tissue Kit (Qiagen). The isolated DNA was stored at −20 °C before use. The genomic DNA (3 μg) was digested by EcoRI (Promega Italia) and purified using the Wizard DNA Clean-up system (Promega Italia). 500 ng of the digested and purified DNA were subjected to bisulfite modification using the Imprint DNA Modification Kit (Sigma) according to the manufacturer’s instructions. 100 ng of modified DNA isolated from biopsies and cell lines were used in PCR analysis for bisulfit sequencing. To study the methylation of TM genes, a CpG island in the promoter, 5'-UTR, and a first exon region containing 44 CpG dinucleotides, respectively, was analyzed and amplified by PCR using AmpliTaq Gold (Applied Biosystems). For the TM gene, the primer sequences were as follows: 5'-GAT TAA GAG ATG AAA GAG GGT TGT A-3' (forward) and 5'-AAA CCA AAA CCC CAA ACA TAT TA-3' (reverse), and the annealing temperature was fixed at 58 °C. In the PCR reactions, the forward primer was added after the first eight cycles followed by 35 more cycles. The PCR product was purified using the Silence Bead DNA Gel Extraction Kit (Fermentas), cloned into p-GEM-T-easy (Promega Italia), and transferred into JM109 competent cells according to the manufacturer’s instructions. Clones containing the plasmid with the insert were selected using isopropyl 1-thio-β-d-galactopyranoside/X-Gal plates and verified by PCR using the M13 primers. The plasmid DNA was subsequently extracted using the Wizard Plus SV Miniprep DNA purification System (Promega Italia) according to the manufacturer’s instructions; 10 clones for each biopsy and cell line were sequenced. The results were expressed as methylation percentage of the 44 CpG dinucleotides.

**Co-immunoprecipitation**—Imunoprecipitation (IP) was performed using the Cross-link immunoprecipitation kit (Pierce). Briefly, Met-5A and H28 cell lines (3 × 10^6) were lysed in the IP buffer. Aliquots normalized for total protein (1 mg) were incubated overnight with anti-DNMT1 (Epigentek) previously cross-linked to protein A/G plus agarose. The resin was washed with the IP buffer, and the bound protein was eluted. Western
blots were performed according to standard procedures. Antibodies against PARP1, DNMT1, and normal mouse IgG (Epigentek) were used to detect the individual proteins.

ChIP—The ChIP assay was performed using the EpiQuik chromatin immunoprecipitation kit (Epigentek). Met-5A and H28 cells as well as their PARP1-silenced counterparts (0.5 × 10^6) were cross-linked with 1% formaldehyde in the RPMI medium. The reaction was stopped with 1.25 M glycine. The cells were harvested and incubated in the lysis buffer containing protease inhibitors, followed by DNA sonication. After centrifugation (14,000 × g for 10 min), an aliquot of the supernatant was incubated (2 h) with anti-DNMT1 IgG previously cross-linked to the 96-well strips. An aliquot of each supernatant was used as the input control. Positive and negative controls were processed using anti-RNA polymerase II IgG and anti-normal IgG, respectively. The immunocomplexes and input controls were incubated with proteinase K at 65 °C, and the samples were transferred to the column and washed with 70 and 90% ethanol, and the purified DNA was eluted. To verify the binding of DNMT1 to the TM promoter, PCR was carried out according to standard procedures. TM primers were designed for the promoter region (352 to −287). The sequences were as follows: 5′-AGG GCA GGG TTT ACT CAT CC-3′ (forward) and 5′-TAC TCG GAT TGC TGG GTT CT-3′ (reverse). The annealing temperature was fixed at 55 °C. GAPDH primers were used as a positive control. The band intensities were evaluated by ChemiDoc using Quantity One software.

Statistical Analysis—Results were expressed as mean values ± S.D. Comparisons between groups were performed using the Mann-Whitney U test for unpaired samples and the Kruskal-Wallis analysis for multiple comparisons. A probability value of p < 0.05 was considered significant. All statistical analyses were performed with the SPSS statistical software (version 15).

RESULTS

TM Expression in Malignant Mesothelioma—The expression of TM mRNA was ~12-fold lower in MM tissue when compared with NM tissue with high statistical significance (p < 0.001) (Fig. 1A). Although all MM surgical specimens lacked TM immunoreactivity, strong cell membrane TM staining was documented for NM tissue (Fig. 1B). Low TM protein expression in MM tissue was further corroborated by Western blot analysis (Fig. 1C).
Methylation Status of TM Promoter Region—Bisulfite sequencing was performed in MM and NM biopsies to define the status of DNA methylation. A CpG-rich section of the TM gene spanning the region from the promoter at $-298$ to the first exon at $+149$ was analyzed for its methylation status. Hypermethylated TM was found in the pathological tissue (80%), whereas 20% of methylation was observed in NM tissue (Fig. 2), suggestive of epigenetic regulation of TM expression of normal and malignant mesothelial tissue.

TM Expression and Its Epigenetic Modulation in Cultured Cells—The gene and protein expression levels of TM were analyzed in the nonmalignant Met-5A and the MM H28 cells by qRT-PCR and immunocytochemistry (Fig. 3). Expression of TM was markedly lower in the MM cells when compared with nonmalignant cells (>200-fold difference, Fig. 3A). To investigate the role of the methylation and/or acetylation, the cells were treated with 5-aza-dC and TSA, respectively. The DNMT inhibitor 5-aza-dC, and partially the deacetylation inhibitor TSA, increased the TM expression in the MM cells. On the other hand, treatment with the individual epigenetic inhibitors separately did not affect the TM expression in Met-5A cells, whereas its level decreased upon combined treatment of the cells with the two agents (Fig. 3B). The methylation involvement was confirmed by assessing the DNMT activity. As shown in Fig. 3C, the activity of DNMT was significantly higher in the MM cells relative to Met-5A cells, and the 5-aza-dC treatment markedly inhibited the DNMT activity in both cell lines. The level of the TM protein expression in Met-5A and H28 cells and the effect of the epigenetic modulators 5-aza-dC and TSA was confirmed by immunocytochemical analysis (Fig. 3D).

TM Expression Is Regulated by PARP1—To examine the role of PARP1 in the methylation- and/or acetylation-mediated silencing of TM, the nonmalignant mesothelial cells Met-5A and the MM cells H28 were stably silenced for PARP1 protein expression to generate the Met-5A1947 and H281949 sub-lines. Silencing of PARP1 in Met-5A cells (Fig. 4A) resulted in a lower PARP activity (Fig. 4B). We then tested these cells for the level of expression of TM mRNA. Fig. 4C documents that silencing of the PARP1 protein expression caused a massive down-
regulation in the TM transcript. The lower level of TM mRNA in the Met-5A1947 cells was restored to the level in Met-5A cells transfected with the empty vector (Met-5ApRS cells) when they were treated with the epigenetic modulators, in particular 5-aza-dC (Fig. 4D). The PARP1-silenced Met-5A1947 cells also exhibited enhanced DNMT activity, which was completely inhibited by 5-aza-dC treatment (Fig. 4E). The level of the TM protein expression is shown in Fig. 4F. Finally, we found a much higher level of methylation within the −298 to +149 region of the TM gene in the Met-5A1947 cells compared with their counterparts transfected with the empty vector (Fig. 5).

In the next set of experiments, we investigated the effect of PARP1 silencing on the regulation of TM expression in the MM H28 cells. The H281949 cells with down-regulated PARP1 gene (Fig. 6A) and with low PARP1 activity (Fig. 6B) exerted considerable up-regulation of the TM mRNA and protein with no additional effect of the epigenetic modulators 5-aza-dC and TSA (Fig. 6, C, D, and F). Silencing of PARP1 in H28 cells significantly reduced the DNMT activity.

FIGURE 4. The effect of knocking down PARP1 on TM expression in NM cells. A, relative PARP1 gene expression was detected by qRT-PCR and Western blot analysis (inset) of Met-5A cells transfected with empty pRS plasmid (Met-5ApRS cells) and Met-5A cells transfected with PARP1 shRNA pRS plasmid (Met-5A1947 cells). B, PARP activity in Met-5ApRS and Met-5A1947 cells. C, TM mRNA fold change in Met-5A1947 with respect to Met-5ApRS cells. D, TM mRNA fold change in Met-5A1947 cells treated with epigenetic modulators with respect to nontreated cells. E, DNMT activity in Met-5ApRS and Met-5A1947 cells incubated with or without 5-aza-dC (right panel). F, TM protein levels were evaluated in Met-5ApRS and Met-5A1947 cells incubated with or without 5-aza-dC (5 μM, 96 h) or TSA (200 ng/ml, 24 h). The results are expressed as mean values ± S.D. of three independent experiments performed in duplicate or as representative immunocytochemical images (magnitude, 60×). The asterisk represents significant differences between Met-5A1947 and Met-5ApRS cells, the degree symbol between 5-aza-dc-treated versus untreated cells, with p < 0.05.
The MM cells with silenced PARP1 also showed massive loss of CpG methylation in the TM locus when compared with their counterparts transfected with the empty plasmid (Fig. 7).

**PARP1-DNMT1 Complex and DNMT1/TM Promoter Localization**—Coimmunoprecipitation was performed to evaluate whether DNMT1 is associated with PARP1. Met-5A and H28 cell extracts were immunoprecipitated with anti-DNMT1 IgG, and the presence of PARP1 was evaluated. PARP1 was found to be associated with DNMT1 in Met-5A cells (Fig. 8A), whereas no association was found in H28 cells. Next, the co-localization of DNMT1 on the TM promoter and the effect of PARP1 silencing on DNMT1/TM promoter interaction were investigated in Met-5A and H28 cells as well as their PARP1-silenced counterparts using ChIP assay. Cross-linked chromatin was immunoprecipitated with anti-DNMT1 IgG, and the region of DNA within the TM promoter was evaluated by PCR. Fig. 8B shows that DNMT1 did not localize in the TM promoter in Met-5A cells, whereas PARP1 silencing markedly increased the DNMT1-TM promoter complex formation. Conversely, DNMT1 specifically localized within the region of the TM promoter in H28 cells, and PARP1 silencing reduced the DNMT1/TM promoter interaction.

**DISCUSSION**

Tumor cells lacking TM feature low anti-coagulant activity, facilitating adhesion to the endothelium of target tissue and, as a consequence, metastatic spread and thrombus formation (6, 25). Thrombosis, a well-recognized complication of cancer, is an early manifestation of malignancy and is associated with a high rate of morbidity and mortality (26, 27). Clinical observations have documented that malignant mesothelioma patients are highly susceptible to thromboembolic events, including arterial thrombosis (3). MM patients may show increased production of procoagulant factors associated with a reduced secretion of molecules with anticoagulant activity.

To this effect, this study documents that TM expression is silenced in surgical MM specimens (cf. Fig. 1). To evaluate possible involvement of epigenetic modification in this process, we first analyzed the methylation status of CGIs in the TM pro-
moter region and its proximity in the MM and NM tissues, more specifically within the locus spanning the promoter region (from position -298) and the first exon (position +149). We show here that CGIs are methylated in the “TM-silent” MM tissue and demethylated in the TM-expressing NM tissue (cf. Fig. 2). The involvement of epigenetic modifications in the TM silencing was then confirmed by treating immortalized mesothelial cells and MM cells with epigenetic agents. Of the epigenetic modulators tested, only the demethylating agent 5-aza-dC substantially restored TM expression in MM cells, while not affecting mesothelial cells (cf. Fig. 3, A and B). Conversely to what we observed in MM cells, the TM promoter of the nonmalignant mesothelial cells was found to be in a demethylated state (cf. Figs. 5–7). Thus, inhibition of DNMT1 by the epigenetic agent 5-aza-dC did not induce any further demethylation with, as anticipated, no additional effect on TM expression.

It is well established that epigenetic events are controlled by a specific sub-group of proteins, which include DNMTs, his...
Acetyltransferases, histone lysine methyltransferases, or histone deacetylases. These enzymes modulate the methylation or acetylation patterns of the promoter regions of a variety of genes, thereby controlling their expression (29–33). Recently, components of chromatin, including histones, have been documented as targets for poly(ADP-ribosyl)lation (34), a post-transcriptional modification of specific proteins mediated by PARPs. Accumulating evidence links poly(ADP-ribosyl)lation with DNA methylation (24, 35–37). This then epigenetically regulates expression of different genes. Consistent with this notion, we found that PARP1 associated with DNMT1 (cf. Fig. 8A) in the nonmalignant mesothelial cells Met-5A. Silencing of PARP1 thus liberates DNMT1 to methylate DNA. In fact, DNMT1 was found to localize in the TM promoter (cf. Fig. 8B), which induces its methylation, resulting in the inhibition of the TM gene and protein expression associated with enhanced DNMT activity (cf. Figs. 4 and 5). This mechanism of the regulation of TM expression was further confirmed by treatment of the cells with the epigenetic modulatory agent 5-aza-dC, an inhibitor of DNMT.

It was found earlier that normal cells with active PARP1 and, consequently, increased level of poly(ADP-ribosyl)lation, are characterized by widespread DNA hypomethylation (37). Conversely, inhibition of the PARP activity introduces an abnormal hypermethylated pattern in the genomic DNA (35) as well as in the CGI regions (36). This suggests that in the absence of polymers of ADP-ribose, certain DNA regions are no longer protected from methylation. The reason is that PARP1 in its poly(ADP-ribosyl)ated form makes DNMT1 catalytically inactive and thus inefficient in DNA methylation (38). In this model, the poly(ADP-ribose) groups present on PARP1 interact monovalently with DNMT1, suppressing its enzymatic activity.

A completely different behavior was observed in MM cells. Coimmunoprecipitation analysis revealed that DNMT1 and PARP1 are not associated in H28 cells (cf. Fig. 8A), allowing DNMT1 to methylate DNA; DNMT1 localizes within the TM

![Figure 7. TM promoter methylation status of H28pRS and H281949 cells.](image-url)
promoter in these cells. The methylated status of H28 cells was reversed by silencing PARP1, resulting in enhanced TM expression. The detailed mechanisms by which CGIs are protected from methylation by poly(ADP-ribosyl)ated PARP1 (39). It is possible that under the condition of poly(ADP-ribose) levels too low to inhibit the DNMT1 activity, new methyl groups were added onto the DNMT1 promoter, which resulted in the down-regulation of the gene. However, this hypothesis was not confirmed by our results. We did not find a reduction in DNMT1 gene expression in PARP1-silenced H28 cells with respect to the wild-type counterparts (data not shown). Therefore, an indirect role of PARP1 could be involved.

In conclusion, a dual role of PARP1 has been established in TM gene regulation in the context of mesothelioma. Under physiological conditions, PARP1 is involved in the maintenance of a demethylated status leading to TM expression. In tumors, PARP1 induces TM promoter methylation, thus resulting in TM silencing. We believe that this report shows, for the first time, that PARP1 regulates TM expression on the epigenetic level and that this regulation is involved in MM. These findings suggest a possible benefit of using PARP inhibitors to improve the outcome of MM patients that appears rather grim at this stage (40 – 42).

REFERENCES
1. Hillerdal, G. (1983) Br. J. Dis. Chest 77, 321–343
2. Chahinian, A. P., Pajak, T. F., Holland, J. F., Norton, L., Ambinder, R. M., and Mandel, E. M. (1982) Ann. Intern. Med. 96, 746–755
3. Nguyen, D., Lee, S., Libby, E., and Verschraegen, C. (2008) Ann. Thorac. Surg. 85, 1032–1038
4. Ames, P. R., and Aye, W. W. (2008) Thromb J. 6, 1–3
5. Ferrero, E., Ferri, M., Viazzo, A., Gaggiano, A., Berardi, G., Piazza, S., Cumbo, P., and Nesi, F. (2010) Ann. Vasc. Surg. 24, 257.e9–12
6. Noble, S., and Pasi, I. (2010) Br. J. Cancer 102, S2–9
7. Sierro, E., Wojtukiewicz, M., Zawadzki, R., Zimnoch, L., and Kisiel, W. (2010) Thromb. Res. 125, 71–75
8. Weiler, H., and Isermann, B. H. (2003) J. Thromb. Haemost. 1, 1515–1524
9. Esmon, C. T. (1989) J. Biol. Chem. 264, 4743–4746
10. Maruyama, I., Bell, C. E., and Majerus, P. W. (1985) J. Cell Biol. 101, 363–371
11. Obama, H., Obama, K., Takemoto, M., Soejima, Y., Shirahama, T., Oh, Y., Yoshida, H., Qzawa, M., Muramatsu, T., and Maruyama, I. (1999) Anticancer Res. 19, 1143–1147
12. Lager, D. J., Callaghan, E. J., Worth, S. F., Raife, T. J., and Lentz, S. R. (1995) Am. J. Pathol. 146, 933–943
13. Matsushita, Y., Yoshiie, K., Imamura, Y., Ogawa, H., Imamura, T., Takao, S., Yonezawa, S., Aikou, T., Maruyama, I., and Sato, E. (1998) Cancer Lett. 127, 195–201
14. Ogawa, H., Yonezawa, S., Maruyama, I., Matsushita, Y., Tenzuka, Y., Toyoyama, H., Yanagi, M., Matsumoto, H., Nishijima, H., Shimotakahara, T., Aikou, T., and Sato, E. (2000) Cancer Lett. 149, 95–103
15. Suehiro, T., Shimada, M., Matsumata, T., Taketomi, A., Yamamoto, K., and Sugimachi, K. (1995) Hepatol. Cell 21, 1285–1290 s
16. Liu, P. L., Tsai, J. R., Chiu, C. C., Hwang, J. J., Chou, S. W., Wu, S. J., Chen, Y. L., Chen, W. C., Chen, Y. H., and Chong, I. W. (2010) Mol. Carcinog. 49, 874–881
17. Calnek, D. S., and Grinnell, B. W. (1998) Exp. Cell Res. 238, 294–298
18. Niforas, P., Chou, M. D., and Bird, P. (1996) Gene 176, 139–147
19. Sperry, J. L., Deming, C. B., Bian, C., Walinsky, P. L., Kass, A. D., Kolodgie, F. D., Virmani, R., Kim, A. Y., and Rade, J. J. (2003) Circ. Res. 92, 41–47
20. Wang, Y., Yu, Q., Cho, A. H., Rondeau, G., Welsh, J., Adamson, E., Mercola, D., and McClelland, M. (2005) Neoplasia 7, 748–760
21. Furuta, J., Kaneda, A., Umebayashi, Y., Otsuka, F., Sugimura, T., and Ushijima, T. (2005) Melanoma Res. 15, 15–20
22. Kaneda, A., Kaminishi, M., Yanagihara, K., Sugimura, T., and Ushijima, T. (2002) Cancer Res. 62, 6645–6650
23. Krishnakumar, R., and Kraus, W. L. (2010) Mol. Cell 39, 8–24
24. Caiafa, P., Guastafurro, T., and Zampieri, M. (2009) FASEB J. 23, 672–678
25. Conway, E. M., Van de Wouwer, M., Pollefeyt, S., Jurk, K., Van Aken, H., De Vriese, A., Weitz, J. I., Weiler, H., Hellings, P. W., Schaeffer, P., Herbert, J. M., Collen, D., and Theilmeier, G. (2002) J. Exp. Med. 196, 565–577
26. Lee, A. Y., and Levine, M. N. (2003) J. Clin. Invest. 112, 1535–1542
27. Prandoni, P., Piccioli, A., and Girolami, A. (1999) Haematologica. 84, 437–445
28. Rusch, V. W. (1995) Chest 108, 1122–1128
29. Kouzarides, T. (2007) Cell 128, 693–705
30. Zhang, Y., and Reinberg, D. (2001) Genes Dev. 15, 2343–2360
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31. Sterner, D. E., and Berger, S. L. (2000) *Microbiol. Mol. Biol. Rev.* **64**, 435–459
32. Nowak, S. J., and Corces, V. G. (2004) *Trends Genet.* **20**, 214–220
33. Shilatifard, A. (2006) *Annu. Rev. Biochem.* **75**, 243–269
34. Faraoe-Mennella, M. R. (2005) *Biochem. Cell Biol.* **83**, 396–404
35. de Capoa, A., Febbo, F. R., Giovannelli, F., Niveleau, A., Zardo, G., Marenzi, S., and Caiafa, P. (1999) *FASEB J.* **13**, 89–93
36. Zardo, G., and Caiafa, P. (1998) *J. Biol. Chem.* **273**, 16517–16520
37. Guastafierro, T., Cecchinelli, B., Zampieri, M., Reale, A., Riggio, G., Sthandier, O., Zupi, G., Calabrese, L., and Caiafa, P. (2008) *J. Biol. Chem.* **283**, 21873–21880
38. Reale, A., Matteis, G. D., Galeazzi, G., Zampieri, M., and Caiafa, P. (2005) *Oncogene* **24**, 13–19
39. Zampieri, M., Passananti, C., Calabrese, R., Perilli, M., Corbi, N., De Cave, F., Guastafierro, T., Bacalini, M. G., Reale, A., Amicosante, G., Calabrese, L., Zlatanova, J., and Caiafa, P. (2009) *PLoS One* **4**, e4717
40. Robinson, B. W., Musk, A. W., and Lake, R. A. (2005) *Lancet* **366**, 397–408
41. Fennell, D. A., Gaudino, G., O’Byrne, K. J., Mutti, L., and van Meerbeeck, J. (2008) *Nat. Clin. Pract. Oncol.* **5**, 136–147
42. Tomasetti, M., Amati, M., Santarelli, L., Alleva, R., and Neuzil, J. (2009) *Curr. Mol. Pharmacol.* **2**, 190–206