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Role of poly(ADP-ribose) glycohydrolase silencing in DNA hypomethylation induced by benzo(a)pyrene

Haiyan Huang [a,1], Gonghua Hu [a,b,1], Jianfeng Cai [a,1], Bo Xia [a], Jianjun Liu [a], Xuan Li [a], Wei Gao [a], Jianqing Zhang [a], Yinpin Liu [a], Zhixiong Zhuang [a,*]

[a] Key Laboratory of Modern Toxicology of Shenzhen, Shenzhen Center for Disease Control and Prevention, Guangdong, China
[b] Department of Preventive Medicine, Gannan Medical College, Jiangxi, China

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ABSTRACT

Benzo(a)pyrene (BaP) is a known carcinogen cytotoxic which can trigger extensive cellular responses. Many evidences suggest that inhibitors of poly(ADP-ribose) glycohydrolase (PARG) are potent anticancer drug candidates. However, the role of PARG in BaP carcinogenesis is less understood. Here we used PARG-deficient human bronchial epithelial cell line (shPARG cell) as an in vitro model, and investigated the role of PARG silencing in DNA methylation pattern changed by BaP. Our study shows, BaP treatment decreased global DNA methylation levels in 16HBE cells in a dose-dependent manner, but no dramatic changes were observed in shPARG cells. Further investigation revealed PARG silencing protected DNA methyltransferases (DNMTs) activity from change by BaP exposure. Interestingly, Dnmt1 is PARylated in PARG-null cells after BaP exposure. The results show a role for PARG silencing in DNA hypomethylation induced by BaP that may provide new clue for cancer therapy.

1. Introduction

Epigenetic alterations in multiple genes are believed to play a crucial role in carcinogenesis. Epigenetic inactivation of genes by promoter hypermethylation has been recognized as an important mechanism by which tumor suppressor genes are shut down during development of tumors [1]. DNA methylation is the most characterized epigenetic mechanism that appears to play an important role in the epigenetic modulation of gene expression. There are three types of enzymatically active DNA methyltransferases (DNMTs), designated Dnmt1, Dnmt3a and Dnmt3b, which have different capacities for maintenance and de novo methylation [2,3]. Although DNA methylation change is a key contributor to human oncogenesis [4], the mechanisms that introduce widespread hypomethylation in cancer cells are still unknown.

Poly(ADP-ribosyl)ation is a post-translational modification of proteins mediated by poly(ADP-ribose) polymerases (PARPs), which plays important roles in many physiological and pathophysiological processes, including inter- and intracellular signaling, transcription, DNA repair pathways, cell cycle regulation, and mitosis, as well as necrosis and apoptosis [5–8]. ADP-ribosylation reaction is reversible owing to the endo- and exo-glycosidic activity of poly(ADP-ribose)glycohydrolase (PARG) [9,10]. The interplay between PARPs and PARG is responsible for the transient and dynamic nature of the ADP-ribose polymers (PARs) [11].

Recent data reinforce the hypothesis that a physiological level of poly(ADP-ribosyl)ation is crucial in maintaining the genomic methylation pattern. There are recent evidences for an interplay between poly(ADP-ribosyl)ation and DNA methylation [12,13]. One example is the binding of the Dnmt1 to PARP1-bound polymers through the PAR-binding motif [14]. In addition, Dnmt1 is part of a protein family able to bind, in a noncovalent way, long and branched ADP-ribose polymers and that ADP-ribose polymers, either free or PARP1 bound, are able to inhibit DNA methyltransferase activity.

Benzo(a)pyrene (BaP), one of the most widely studied PAHs, is a known carcinogen cytotoxic and/or genotoxic to lung, stomach, and skin tissue in the body [15,16]. BaP exposure can trigger extensive cellular responses [17]. Our previous data showed that poly(ADP-ribosyl)ation played an important role in the cytotoxicity and/or genotoxicity induced by BaP, and DNA methylation pattern was regulated by PARP1. This finding is of great interest, considering that an anomalous DNA methylation induces by BaP associated with poly(ADP-ribosyl)ation.

* Corresponding author. Address: Key Laboratory of Modern Toxicology of Shenzhen, Shenzhen Center for Disease Control and Prevention, No. 8 Longyuan Road, Nanshan District, Shenzhen 518055, China.
E-mail address: zxzhuang2013@126.com (Z. Zhuang).
[1] Haiyan Huang, Gonghua Hu and Jianfeng Cai have contributed equally.

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Thus, the aim of the current study was to investigate how PARG controls and/or protects the DNA methylation pattern after BaP treatment. For this purpose, we used a PARG-deficient human bronchial epithelial cell line (shPARG) as an in vitro model to demonstrate the participation of the PAR-degrading activity of PARG in this process. We have showed that BaP treatment decreased global DNA methylation levels in 16HBE cells in a dose-dependent manner, but no dramatic changes were observed in shPARG cells. The data reported here provide a clue to understanding the molecular mechanism(s) connecting poly(ADP-ribosyl)ation with DNA methylation, giving a possible explanation as to how DNA methylation modulated by poly(ADP-ribosyl)ation as the posttranslational modification. Thus, maintaining of genomic methylation pattern by PARG silencing may have important implications for cancer therapy.

2. Materials and methods

2.1. Cell lines and cell culture

Human bronchial epithelial (16HBE) cells were obtained from the School of Public Health at Sun Yat-sen University, and shPARG cells were previously established by our work [18]. Cells were cultured in minimum essential Eagle's medium (MEM) containing 10% fetal bovine serum (FBS). Penicillin (100U/ml) and streptomycin (100 μg/ml) were added to the culture media. The cultured cells grown to 80% confluence were treated with BaP (BaP was dissolved in DMSO, and the final concentration of DMSO in the culture was 0.1%). Cells were washed twice with PBS before cell lysis. All media and supplements used for cell cultures were obtained from Gibco®.

2.2. 5-mC immunofluorescent detection

Cells grown on glass coverslips were treated with different concentrations of BaP for 24 and 72 h, and then washed with PBS and fixed 15 min with 4% formaldehyde in PBS at room temperature. Briefly, cells were permeabilized with 0.1% Tween (v:v) in PBS, washed with PBS for 3 times. The cells were then incubated overnight at 4 °C with PBS, 0.1% Tween (v:v), containing 1 mg/ml BSA and Anti-5-methylcytosine mouse mAb (1:1000, Calbiochem, Gibbstown, NJ). Followed by sequential incubation of cells with secondary antibodies (sc-2005, 1:2000, Santa Cruz, USA) for 90 min at room temperature. After washing 3 times with PBS, 0.1% Tween (v:v), DNA was counterstained with Dapi. Pictures were taken with Confocal Laser Scanning Microscopy (Leica Tcs sp5, Leica Microsystems).

2.3. Flow cytometric analysis of DNA methylation

Cells were treated with various concentrations of BaP for 72 h, then harvested and washed with PBS for 3 times. Cells from each treatment group were fixed and stained according to Giraldo et al. [19]. Relative levels of DNA methylation of each group were detected at 530/30 nm in the FL-1 channel (green fluorescence). Quantification of flow cytometric analysis of DNA methylation was performed following the procedures described by Giraldo et al.

2.4. Quantitative mRNA analysis of DNMTs using real-time PCR

Total RNA was extracted from the cells of different treatment groups using Trizol Reagents Kit (Invitrogen) and complementary DNA was synthesized through the reverse transcription reaction (iScript complementary DNA Synthesis Kit, Bio-Rad Laboratories). Quantitative real-time PCR analysis was performed using ABI 7900HT sequence detector system (Applied Biosystems) with Power SYBR Green PCR Mix according to the manufacturer's protocol. The relative abundance of mRNAs was calculated by the comparative cycle of threshold (CT) method with GAPDH mRNA as the internal control. The primers are as follows: GAPDH forward AGAAGGCTGGGGCTCATTTG, reverse AGGGGCCATCCACAGTCTTC; DNMT1 forward ACGACCCTGACCTCAAATAT, reverse CCATTAAC ACCACCTTCAAGA; DNMT3a forward CACAGAAGCATATCCAGGAG, reverse CACATTCTCAAAGAGCCAGA; DNMT3b AGTATCAG GATGGGAAGGAG, reverse CGATAGGAGACGAGCTTATTG; MBD2 forward ACTATAAGTGCCCTCTGTGT, reverse TCAGAGTCTCCTT CATGTACTT. All data were collected in triplicate and normalized to GAPDH levels. All data were collected in triplicate and normalized to GAPDH levels. Gene-specific relative mRNA levels were computed by the standard equation $2\wedge-(\Delta CT\ sample-\Delta CT\ control)$.

2.5. Cell lysates and Western blotting

Analysis of protein levels was performed using Western blotting. Cells from different treatment groups were washed once in PBS and collected by scraping into 200 μl ice-cold lysis buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitors. The extracts were sonicated, and supernatants were collected after centrifugation. Proteins (20 μg) were electrophoresed and then transferred onto nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris buffered saline (TBS) for 90 min, incubated overnight at 4 °C with primary antibodies: a mouse monoclonal IgG1 anti-Dnmt1 (H-12, sc-271729, Santa Cruz Biotechnology), mouse monoclonal IgG1 anti-Dnmt3a (64B1446, sc-52921, Santa Cruz Biotechnology), mouse monoclonal IgG1 anti-Dnmt3b (52A1018, sc-52922, Santa Cruz Biotechnology), mouse monoclonal IgG1 anti-GAPDH (6C5, sc-32233, Santa Cruz Biotechnology), rabbit polyclonal IgG anti-MBD2 (H-70, sc-10752, Santa Cruz Biotechnology). Then followed by secondary antibodies at 1:4000 dilution: goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (sc-203l, Santa Cruz Biotechnology). After washing in TBST, blots were incubated in Supersignal® West Dura Extended Duration Substrate (Thermo scientific) and detected with ECL™ western blotting detection system (ImageQuant™ RT, GE Healthcare).

2.6. Co-IP (immunoprecipitation)

The shPARG cells were seeded onto 750 cm^2 cell culture bottle and grown up to 80–90% confluency. Nuclei were collected from trypsinized and PBS-washed cells by centrifugation (at 16,000*g* for 10 s at 4 °C) following incubation (30 min) in isolation buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 50 mM NaF and 0.5 mM DTT (dithiothreitol)]. Nuclear fraction was lysed in RIPA buffer [50 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P40 and 1 mM EDTA). Cell lysates (1.5 mg) were pre-cleared with protein A (for IP anti-Dnmt1 and anti-PAR) agarose beads (Upstate Laboratories) on a rotative shaker at 4 °C for 2.5 h. Pre-cleared lysates were incubated with specific antibodies [rabbit polyclonal anti-Dnmt1 (H-300, sc-20701, Santa Cruz Biotechnology) and mouse monoclonal anti-pADPr antibody (ab14459, Abcam, USA)] and with normal rabbit or mouse IgG (Santa Cruz Biotechnology) on a rotative shaker at 4 °C. The agarose beads, previously saturated with BSA (1 μg/μl) overnight, were added to the lysate/Ab solutions and incubated for 2 h on a rotative shaker at 4 °C. Subsequently, beads were washed in IP buffer and boiled in SDS/PAGE sample buffer. The eluted proteins were analyzed by SDS/PAGE (8% gel) and Western blotting.

2.7. Double immunofluorescence detection

Cells grown on glass coverslips washed with PBS and fixed 15 min with 4% formaldehyde at room temperature. Incubated cells in the mixture of two primary antibodies: a mouse monoclonal anti-pADPr antibody (ab14459, Abcam, USA) and a rabbit polyclonal anti-DNMT1 (1:1000, Santa Cruz Biotechnology) in 1% BSA in PBST in a humidified chamber for 1 h at room temperature. After washing, cells were incubated with the mixture of two secondary antibodies which are raised in different species for 90 min at room temperature. After washing 3 times with PBS, 0.1% Tween (v:v), DNA was counterstained with Dapi. Pictures were taken with Confocal Laser Scanning Microscopy (Leica Tcs sp5, Leica Microsystems).

2.8. Statistical analysis

All experiments were performed at least 3 times on different days. Statistical ANOVA was applied, followed by the Turkey multiple comparison test to determine the statistical difference between treatment groups. The results of the real-time PCR are expressed as the means ± standard deviation. The data were considered significant if $P < 0.05$.

3. Results

3.1. Effect of PARG silencing on global DNA methylation level in the 16HBE cells and shPARG cells

First to evaluate the influence of PARG in global DNA methylation, we assayed PAR-mediated global DNA methylation of a 16HBE cell line constitutively expressing shRNA directed against the catalytic domain of all PARG isoforms (shPARG). The 16HBE cell line was used as a control. Two cells were treated with various concentrations (0, 10, 20, 40 μmol/L) of BaP, and then harvested for 5-mC immunofluorescent detection, which immunocytostaining was performed to detect 5-mC-positive cells. We found BaP treatment resulted in reduction of 5-mC-positive cells in 16HBE cells compared with shPARG cells (Fig. 1). The global DNA methylation levels in 16HBE cells were significantly reduced after BaP treatment for 72 than 24 h. Note that no dramatic changes in global DNA methylation were observed in shPARG cells between BaP treatment for 24 and 72 h.

To make further understand the effect of poly(ADP-ribosyl)ation, we detected global DNA methylation by flow cytometric analysis. We found that BaP treatment resulted in reduction of the level of DNA methylation in 16HBE cells in a concentration-depen-

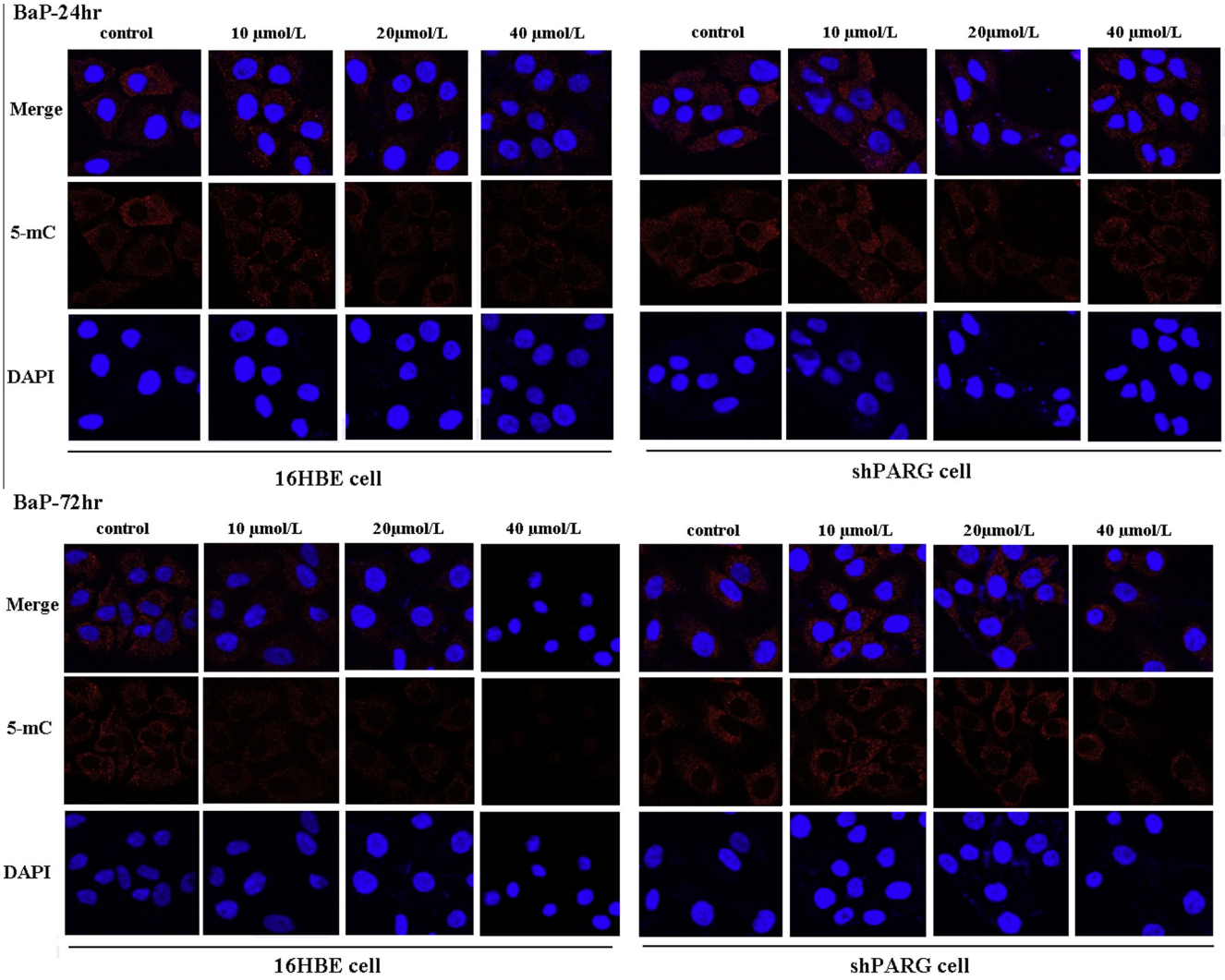

Fig. 1. Effect of PARG on global DNA methylation in 16HBE and shPARG cells treated with various concentration of BaP for 24 and 72 h. Immunocytostaining of 5-mC-postive cells using 5-mC-specific antibody after BaP treatment for 24 and 72 h.

dent manner compared with the control. However, BaP did not change the level of DNA methylation in shPARG cells (Fig. 2). These results suggest that PARG has a broad effect on DNA methylation pattern in cells.

3.2. Effect of PARG silencing on mRNA and protein expression of DNMTs

DNMTs are responsible for maintaining methylation status in the genome, to verify whether PARG effects on the expression of DNMTs, we examined the effect of PARG on mRNA and protein expression of DNMTs. For this purpose, the 16HBE and shPARG cells were treated with various concentrations of BaP for 72 h. At the termination of the experiments, cells were harvested and mRNA and protein expression of DNMT1, DNMT3a, DNMT3b and MBD2 were detected. The mRNA expression of the DNMTs were detected by real-time PCR (Fig. 3A), we found that treatment of 16HBE cells with BaP decreased the mRNA level of DNMT1, whereas increased the level of DNMT3b and MBD2, but did not change the mRNA level of DNMT3a. In contrast, treatment of shPARG cells with BaP decreased the mRNA level of DNMT3a but did not alter the level of DNMT1, but the mRNA levels of DNMT3b and MBD2 were significantly increased ($P < 0.05$ to $P < 0.01$). The increase in mRNA expression of DNMT3b and MBD2 in 16HBE cells were greater than shPARG cells. Similar to mRNA expression, protein reactivation levels were also determined using western blot analysis (Fig. 3B). There was also an increase in protein expression levels of DNMT3b and MBD2 after the treatment of cells with BaP for 72 h, and the increase of the protein expression was greater in 16HBE cells than shPARG cells. BaP treatment decreased the protein expression levels of DNMT1 in the 16HBE cell, whereas BaP treatment decreased the protein expression levels of DNMT3a in shPARG cells, in a concentration-dependent manner.

3.3. PAR interaction with DNMT1

The capability of DNMTs to be modified by PARylation was addressed by IP assays performed using specific Abs against PARs (Fig. 4A). The results indicate that DNMT1 interact with polymers or, at least, that they are complexed with other PARylated proteins. To test the interactions of DNMT1 and PARs, DNMT1/PAR double immunofluorescence assays were performed on the shPARG cells. The results showed in Fig. 4B, indicate that DNMT1 does indeed interact with PARs and exhibits strong signal with low background in the cell nuclei.

4. Discussion

Poly(ADP-ribose) glycohydrolase is the primary enzyme that catalyzes the hydrolysis of poly(ADP-ribose) (PAR). By regulating the hydrolytic arm of poly(ADP-ribosyl)ation, PARG participates in a number of biological processes, including the repair of DNA damage, chromatin dynamics, transcriptional regulation, and cell death [20]. Increasing evidences suggest that inhibitors of PARG are potent anticancer drug candidates [21]. Moreover, Cancer is a manifestation of both genetic and epigenetic modification. DNA methylation and posttranslational modification are important epigenetic events in regulation of gene expression and maintenance of cellular function and that may contribute to cancer development [22,23].

Poly(ADP-ribosyl)ation promotes epigenetic changes in several ways also through a direct control of DNA methylation patterns [24–26]. This evidence prompted to investigate how PARG controls DNA methylation patterns. In the present paper, we investigated DNA methylation regulation by PARG and elucidated the underlying mechanisms using the shPARG cell line which was conducted previously as an in vitro model system. Also, we used BaP, as a well-known carcinogen. Previous study we have demonstrate that preventing the degradation of PAR polymers via PARG depletion protect mammalian cells from damaged induced by BaP [18].

In the beginning of experiments, we compared global DNA methylation pattern in 16HBE cells with shPARG cells after BaP treatment. Our study reveals that BaP significantly decreased the levels of global DNA methylation in 16HBE cells, and the effects were concentration dependent. However, we observed that global DNA methylation remained unchanged in shPARG cells. We also examined the DNMTs activity because DNMTs play a crucial role in DNA methylation. The results showed that BaP decreased DNMTs activity of 16HBE cells. However, we detected no change in shPARG cells. These results indicate that PAR polymers have

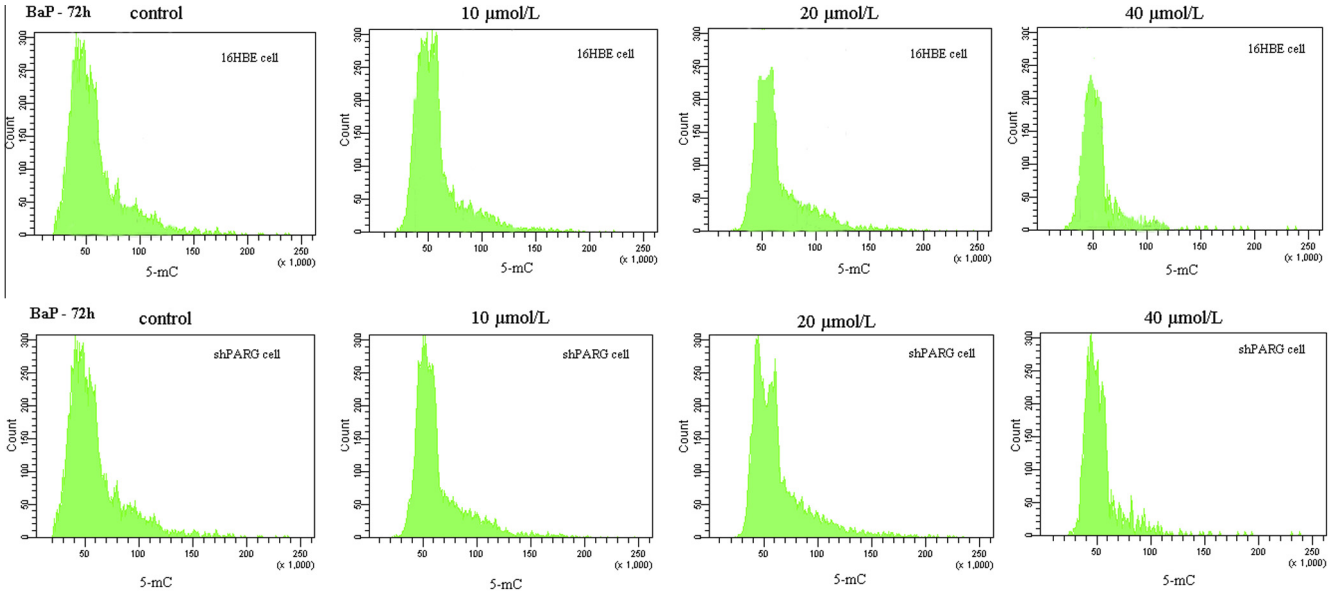

Fig. 2. Flow cytometric analysis of 5-mC in 16HBE and shPARG cells after treated cells with various concentration of BaP for 72 h.

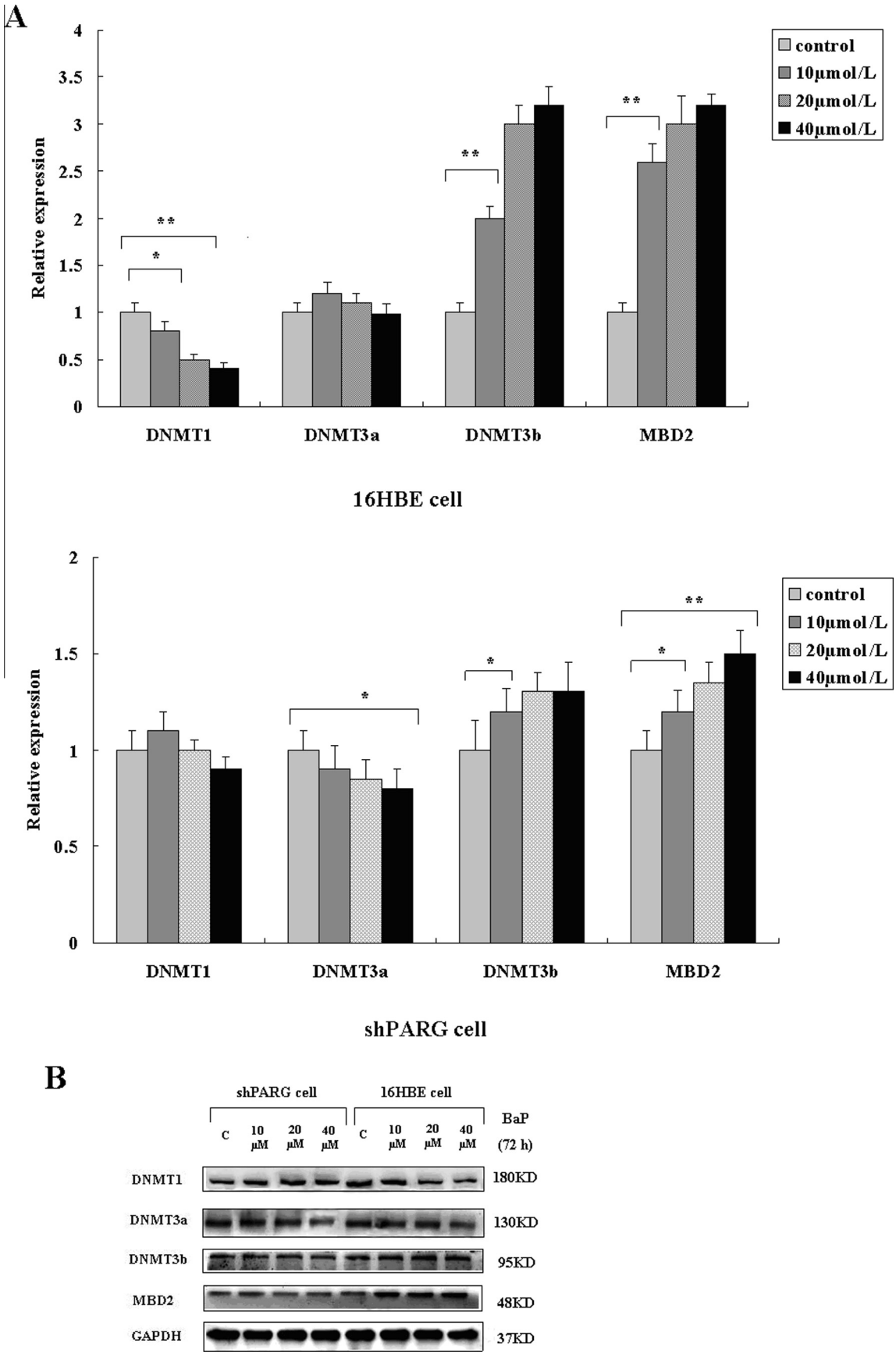

Fig. 3. Effect of PARG silencing on mRNA and protein expression of DNMTs in 16HBE and shPARG cells after treatment with various concentrations BaP for 72 h. (A) Quantitative real-time PCR analysis of mRNA levels of DNMT1, DNMT3a, DNMT3b and MBD2. The results are presented as the expression of the individual mRNA with normalization to GAPDH and as mean values ± standard deviations, $n = 3$. Significant difference versus non-BaP-treated controls; $^{*}P < 0.05$, $^{**}P < 0.01$. (B) Protein expression levels of DNMT1, DNMT3a, DNMT3b and MBD2 in cell lysates were determined using western blot analysis. Representative blots are shown from three experiments.

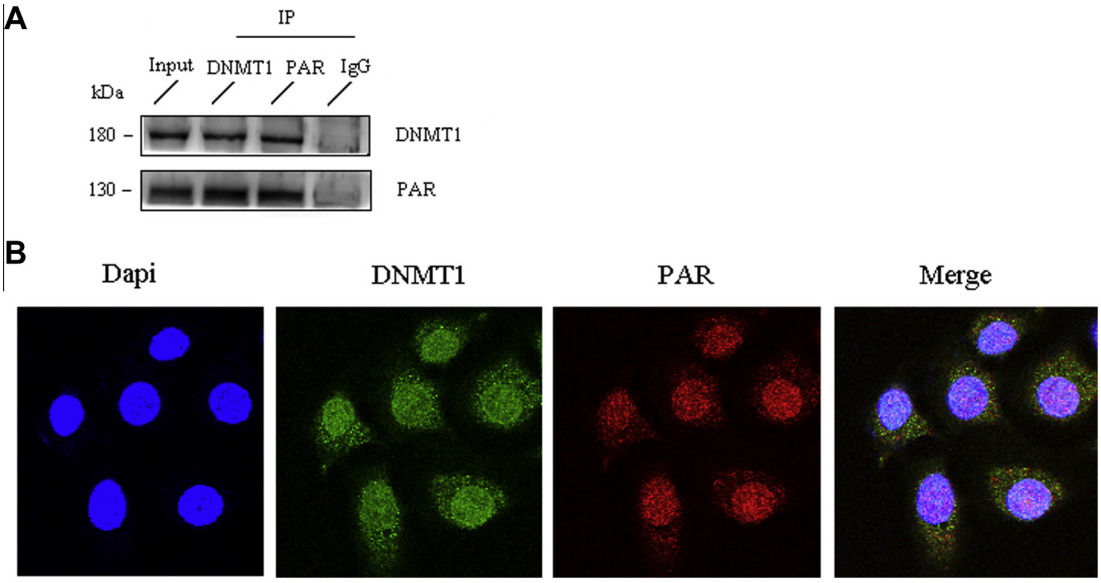

Fig. 4. Analysis of DNMT1 interaction with PARs. (A) Reciprocal co-IP of PARs and DNMT1 in nuclear lysates. Non-specific mouse IgGs were used as negative control. (B) Double immunofluorescence assay of PARs and DNMT1 in the shPARG cells, Scale bar: 20 μm.

has a broad effect on DNA methylation and DNMTs activity. DNA methylation plays an important role in carcinogenesis, which includes genome-wide hypomethylation and hypermethylation of CpG islands in DNA [27,28]. The likelihood that poly(ADP-ribosyl)ation is involved in maintenance of DNA methylation level and prevents cancer development induced by BaP.

The mammalian DNMTs family is divided into maintenance and de novo methyltransferases, including DNMT1, DNMT3a and DNMT3b. MBD2, the founding member of the DNA-binding domain proteins (MBDs) family, bind to methylated DNA and suppress transcription from a methylated target gene, promoting interactions with other proteins [29]. There are increasing evidences indicating that all three DNMTs may possess both de novo and maintenance functions in vivo [30,31]. To uncover the initial mechanism of global genomic hypomethylation, we investigated the mRNA and protein expressions of DNMT1, DNMT3a, DNMT3b and MBD2 in shPARG cells and 16HBE cells after BaP treatment.

DNMT1 is responsible for maintaining the DNA methylation pattern during DNA replication. It has been previously proposed that DNMT1 overexpression might be triggering cellular transformation by methylation-dependent and independent mechanisms [32]. Our data demonstrate that BaP significantly decreases DNMT1 in 16HBE cells after the treatment for 72 h, but it did not change the level of DNMT1 in shPARG cells. Based on this study and previous reports above, it is possible that PARG defection help to maintain global DNA methylation through the DNMT1 expression balance. The interaction between PARP1 and DNMT1 was reported previously [14], and in the present paper we showed DNMT1 interacted with polymers and DNMT1/PARs were co-localized in shPARG cells nuclei. Our results support that DNMT1 was modified by PARylation, which could involve in regulating global DNA hypomethylation induced by BaP.

Since DNMT3b s able to mediate de novo DNA methylation and has been shown to be over-expressed in numerous types of cancer [33–37], this methyltransferase has been postulated to be an important factor in cancer, and it has been shown that DNMT3b polymorphisms are in fact significantly associated with the risk of developing lung cancer [38,39]. Accumulation evidences have revealed an association of over-expressed DNMT3b levels and the development of breast cancer [40] and other malignancies [33–37]. MBD2, a protein reported previously to be implicated in DNA demethylation, which was overexpressed in human cancer [41]. In this study, our data demonstrate that the levels of DNMT3b and MBD2 increasing are a significant difference among shPARG cells and 16HBE cells after BaP treatment for 72 h, and the increase of DNMT3b and MBD2 in 16HBE cells were greater than shPARG cells. These observations suggest that PARG may regulate the expression of DNMT3b and MBD2 to prevent tumor formation.

In conclusion, our results showed that poly(ADP-ribose) glycohydrolase correlates with DNMTs and involves in regulating global DNA hypomethylation induced by BaP, which would help to elucidate the underling molecular mechanism of the role of PARG during DNA methylation, and may have important implications for cancer therapy.

Conflict of interest statement

No conflict of interest by any of the authors is present due to this work.

Acknowledgments

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