Oxidative Stress Suppresses the Expression of 15-Hydroxyprostaglandin Dehydrogenase via Upregulation of DNMT3a/Snail in Human Colon Epithelial Cells

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Abstract

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Overproduction of prostaglandin E_2 (PGE_2) is implicated in pathogenesis of inflammation-associated carcinogenesis. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a key enzyme that catalyzes the conversion of PGE_2 to a biologically inactive 15-keto-PGE_2 metabolite. 15-PGDH has been considered to be a tumor suppressor as its expression is frequently repressed in human malignancies via hypermethylation of its promoter. In our previous study, oxidative stress caused by *Helicobacter pylori* infection is associated with down-regulation of 15-PGDH. This prompted us to investigate whether 15-PGDH expression could also be down-regulated by oxidative stress in human colon epithelial CCD841 cells. When CCD841 cells were treated with H_2O_2, the expression level of 15-PGDH was significantly reduced in both concentration and time dependent manners. H_2O_2 induced methylation of the 15-PGDH promoter in a time dependent manner as determined by methyl specific PCR. H_2O_2-induced down-regulation of 15-

PGDH expression was abrogated by the DNA methyltransferase (DNMT) 3a inhibitor, 5-Aza-2′-deoxycytidine (5-Aza). H_2O_2 generated the reactive oxygen species (ROS), which is attenuated by general antioxidant, N-acetylcysteine (NAC) as determined by DCF-DA staining. In addition, NAC abrogated the down-regulation of 15-PGDH and up-regulation of DNMT 3a expression induced by H_2O_2. Moreover, NAC reduced the methylation level of 15-PGDH promoter induced by H_2O_2. H_2O_2 treatment also elevated expression of Snail and silencing of *snail* restored the 15-PGDH expression suppressed by H_2O_2. Taken together, these finding suggest that ROS down-regulates the expression of 15-PGDH through hypermethylation of CpG island in the 15-PGDH promoter through induction of DNMT3a.

Keyword: 15-Hydroxyprostaglandin dehydrogenase, DNA methyltransferase 3a, Reactive oxygen species, Epigenetic regulation

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Introduction

Inflammation predisposes malignancy and even promotes carcinogenic process. One of hallmarks of chronic inflammation is activation of arachidonic acid pathway, which is catalyzed by cyclooxygenase-2 (COX-2) [1]. Elevated COX-2 expression was found in approximately 50% colonic of adenomas and 85% of adenocarcinomas [2]. Moreover, aberrant overexpression of COX-2 leads to the accumulation of specific prostaglandins, particularly prostaglandin E_2 (PGE_2) [3]. Prostaglandins (PG) play a key role in the generation of the inflammatory response [4]. Their biosynthesis is significantly increased in inflamed tissue and they contribute to the development of acute inflammation [4]. In addition, Overproduction of PGE_2 has been known to be implicated in tumor-associated angiogenesis and metastasis, proliferation of various cancer cells, and resistance to apoptosis as well as inflammatory response [5].

The key enzyme responsible for the biological inactivation of PGE_2 is NAD^+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) [6, 7]. This enzyme oxidizes the 15(S)-hydroxyl group of PGE_2 to generate 15-keto-PGE_2, which exhibits greatly reduced biological activity **(Fig. 1)**. 15-PGDH controls the levels of biologically active PGE_2 and lipoxins in tissues, which may explain an important role of this enzyme in the regulation of inflammation [8, 9]. Several studies demonstrated that 15-PGDH plays as putative a tumor suppressor in various cancers [6, 10, 11]. Overexpression of 15-PGDH inhibits the cell cycle entry, proliferation, invasion and metastasis and tumor growth in various cancer cells [12-16]. In addition, 15-PGDH induced apoptosis and enhanced tumor-induced immune response **(Fig. 2)**. Genetic ablation of 15-PGDH increases intestinal tumor formation in the $APC^{min/+}$ mouse model [17] and overexpression of 15-PGDH inhibits

growth of colon tumor xenografts in immune-deficient mice [10]. 15-PGDH is highly expressed in normal colon mucosa but is ubiquitously lost its expression in human colon cancers [10, 13, 17].

Down-regulation of 15-PGDH in primary tumor has been reported as a result of hypermethylation in its promoter region in gastric cancer [18]. The promoter region of 15-PGDH DNA was 75% methylated in primary prostate tumors and extensively methylated in one cell line [19, 20]. 30% of primary breast tumors cell line was associated with hypermethylation and histone deacetylation of the 15-PGDH promoter [21]. Tumor suppressor genes such as *RUNX3* and *p16* have been known to be hyper methylated in gastric cancer [22, 23], while methylation in the promoter decreases expression of the gene [24]. DNA methylation plays an essential role in maintaining cellular function, and aberrant methylation of DNA (global hypomethylation accompanied by region-specific hypermethylation) is frequently found in tumor cells [25]. DNA methyl transferases (DNMTs) are key enzymes involved in DNA methylation [26]. In mammals, there are three catalytic active DNMTs: DNMT1, which is largely responsible for the maintenance of DNA methylation over replication, and DNMT3a and DNMT3b, which generally perform de novo methylation of either unmethylated DNA or hemimethylated DNA to assist in maintenance [27]. DNA methylation is catalyzed by a group of enzymes called DNMTs that transfer a methyl group from a cofactor molecule *S*-adenosyl-l-methionine (AdoMet or SAM) to the C5 position of the cytosine residues to generate 5-methylcytosine (5 mC) and *S*-adenosyl-l-homocysteine **(Fig. 3)** [28, 29].

Reactive oxygen species (ROS) are constantly generated and eliminated in the biological system, and play important roles in a variety of normal biochemical functions

and abnormal pathological processes [30]. Elevated level of ROS have been detected in almost all cancers, where they activate many signal molecules involved in tumor development and progression [31]. ROS-induced oxidative stress is associated with both aberrant hypermethylation of tumor suppressor gene and global hypermethylation [32]. Therefore, we attempted to determine whether hydrogen peroxide down-regulates expression of 15-PGDH in human colon epithelial CCD841 cells, which is mediated by epigenetic modification of its promoter.

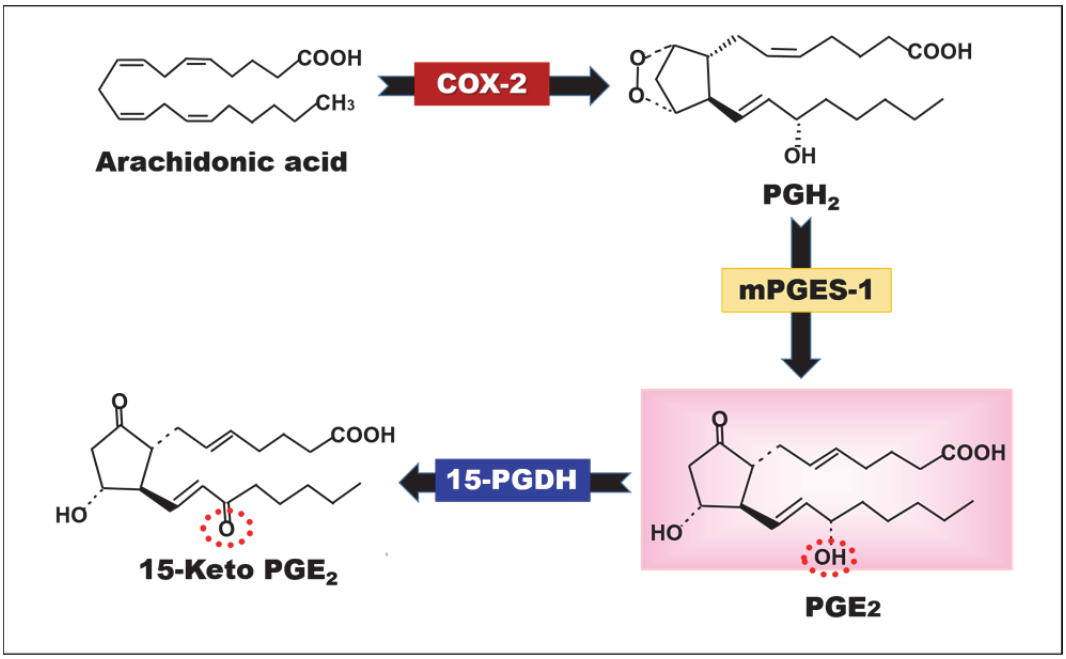

Fig. 1. Prostaglandin biosynthesis pathway and role of PGE_2 in carcinogenesis.

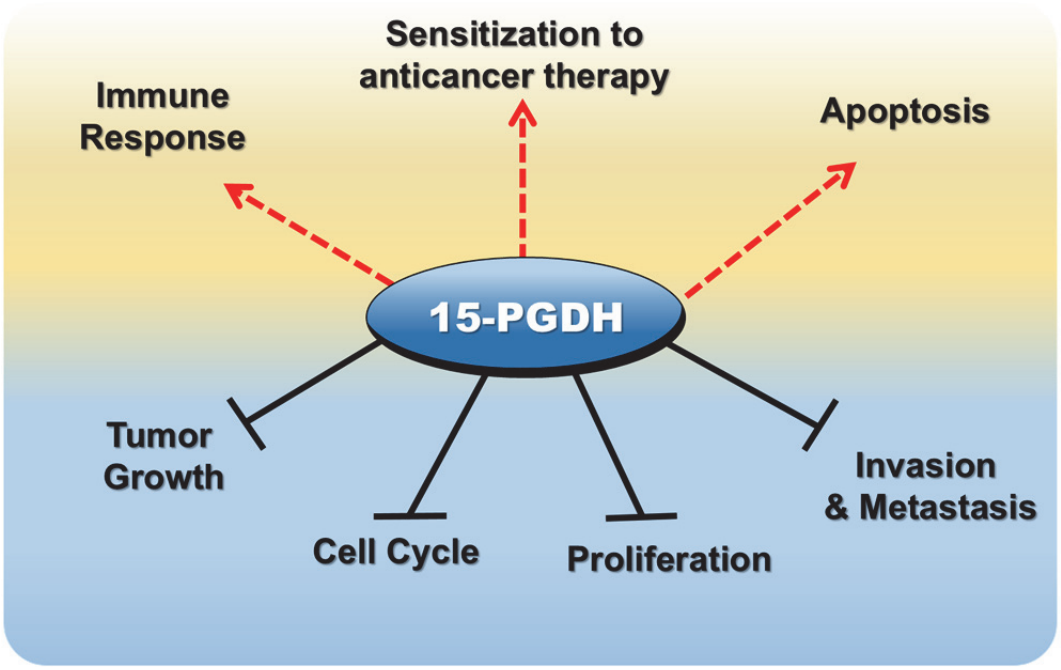

Fig. 2. 15-PGDH plays as a putative tumor suppressor.

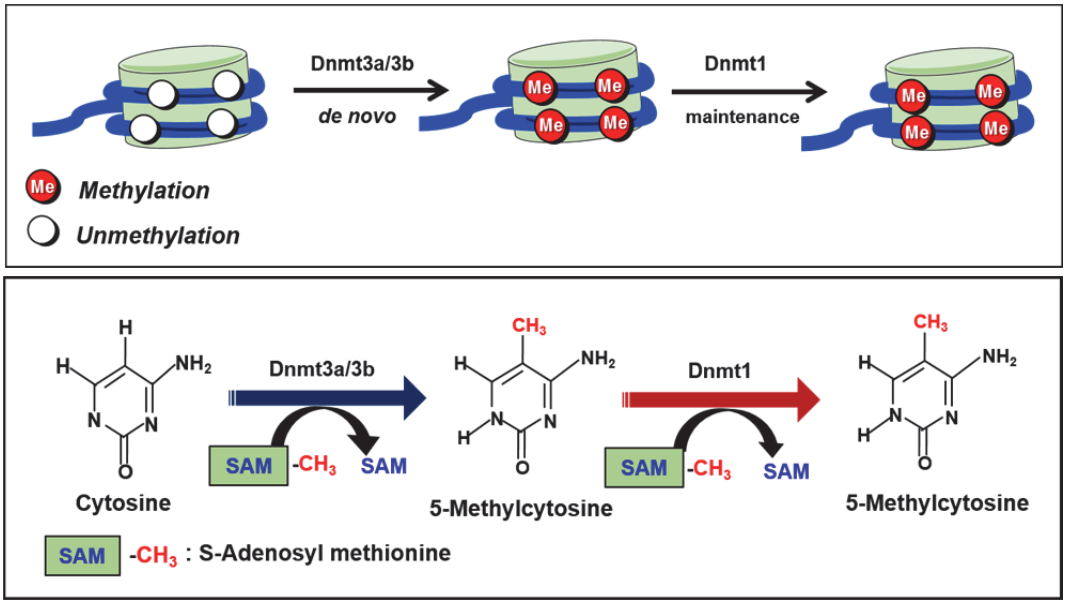

Fig. 3. Methylation of DNA by DNA methyltransferase enzymes (DNMTs) DNMT1, DNMT3a and DNMT3b.

MATERIALS AND METHODS

Materials

Hydrogen peroxide was purchased from Junsei Chemical Co. (Ohmano-Cho, Koshigaya, Japan). Minimum essential medium (MEM), penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (South Logan, Utah, USA). 5-Aza-2'-deoxycytidine and *N*-acetyl-*L*-cystein were obtained from Sigma-Aldrich (St Louis, MO, USA). Primary antibodies against DNMT1 and DNMT3a, GAPDH, Lamin B, α–Tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against 15-PGDH was the product of Cayman Chemical Co. (Ann Arbor, MI, USA).

Cell Culture

CCD 841 cells was obtained from ATCC (Manassas, VA). CCD841 cells were cultured in in MEM containing of 10 % FBS/ 1 % penicillin-streptomycin at 37℃ in a 95 % air and 5 % CO_2 atmosphere. Cells were seeded at density 2 X 10^5 viable cells per 100 mm plate, and the media was changed every other day for 3 days. Cells were harvested by 0.05% trpysin-EDTA (Grand Island, NY, USA), washed in phosphate-buffered saline (PBS), and immediately frozen at 80℃ for subsequent analyses.

Nuclear and Cytoplasmic Protein Extraction

Cytoplasmic proteins were separated in buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.1% NP-40). Nuclear pellets were washed twice with buffer A with inhibitors and then lysed in buffer C (20 mM HEPES, 100 %

Glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). Quantification of protein was performed by Bradford method according to standard protocols.

Western blot analysis

CCD814 cells (2×10^5 cells/ml) were plated in a 60 mm dish and treated with H_2O_2 when the cells were grown to approximately 70–80% confluence. After rinse with phosphate-buffered saline (PBS), cell lysates were collected in 1 X cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate , 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin). Lysates were sonicated and centrifuged at 130,000 rpm for 15 minutes at 4°C. The protein concentration was determined by using Bio-Rad Protein Assay (Hercules, USA). Per lane, 15–20 μg of whole-cell lysate was separated on 6% or 12% SDS-acrylamide gels and transferred Immobilon®-P PVDF membrane (EMD Millipore Coperation, Billerica, MA 01821, USA) at 100 mA for 1 h. The membrane was blocked in 5% non-fat dry milk reconstituted in 0.1% Tween 20 in PBS (PBST) for 1 h, followed by incubation with the indicated antibodies in PBST with 3% non-fat dry milk. The membrane was then rinsed three times with PBST buffer for 10min each. The washed membrane was incubated with 1:2000~1:5000 dilution of the horseradish peroxidase- conjugated secondary antibody in PBST for 1 h at room temperature. The membrane was washed again four times in PBST buffer. Immune detection was performed using the ECL Western blot detection system. All of the values subjected to statistical analysis were

normalized to internal control values (α-tubulin or GAPDH) and were determined from at least three independent experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After H_2O_2 treatment, total RNA of human colon epithelial CCD841cells was extracted with TRIzol (Invitrogen, USA) following the manufacturer's instructions. Total RNA (2 μg) was used for cDNA synthesis by using reverse transcriptase of murine leukemia virus (Promega, Madison, WI, USA). The primers used were: GAPDH (F); 5'-AAG GTC GGA AAC GGA TTT-3' GADPH (R); 5'-GCA GTG AGG GTC TCT TCT CT-3', 15-PGDH (F); 5'-GTA AAG CTG CCC TGG ATG AG-3' (R); 5'-AAC AAA GCC TGG ACA AAT GG, DNMT3a (F); 5'-TGA CGA GCC AGA GTA CGA GG-3' (R); 5'-CTG CTT GTT GTA CGT GGC CT-3'.

Transfection

The small interfering RNA (siRNA) of Snail and 15-PGDH and its negative Control siRNA were designed using BLOCK-iT™ RNAi Designer and siRNA oligonucleotides were purchased from Genolution Pharmaceuticals (Seoul, Korea). The strands of Snail and 15-PGDH siRNA were as follows: Snail; 5'-GCG AGC UGC AGG ACU CUA AUU-'3 15-PGDH; 5'- CAA GGU AGC GCU GGU GGA UUG GAA UUU-'3. CCD841 cells (2 x 10^5/6 Well) were transfected with 50 nM of specific or scrambled siRNA oligonucleotides using Lipofectamine RNAiMAX according to manufacturer's instruction (Invitrogen, USA).

Measurement of Intracellular ROS Accumulation

The fluorescent probe DCF-DA was used to monitor the net intracellular accumulation of ROS. After treated with H_2O_2 (300 μM), the CCD841 cells were rinsed with PBS and were loaded with 10 μmol/L DCF-DA. After 30-minute incubation at 37 °C, the intracellular ROS accumulation was determined by fluorescent microscopy (Nikon, Japan) set at 488 nm for excitation and 530 nm for emission.

Immunofluorescent Staining

CCD841 cells (2 x 10^5 cells per well in an 8 chamber plate) were washed in PBS three times and fixed with 4% buffered formalin solution (20 min). After a rinse with PBS, cells were permeabilized with 0.1% triton X- 100 (5 min) and blocked with 10% BSA in PBST (30 min). Anti-DNMT1, anti-DNMT3a, and anti-15-PGDH diluted 1:1000 in 1% bovine serum albumin (BSA) in PBST were applied overnight at 4 °C. This was followed by washing cells in PBS (twice for 5 min each) and incubation for 1 h at room temperature with FITC-goat anti-rabbit IgG secondary antibody (Invitrogen, USA) diluted at 1:1000 in 1% BSA-PBST. After washing (twice for 5 min each), the cells were treated with PI (Invitrogen, USA). The signals were detected using Zeiss Inverted LSM 700 Microscope (Carl Zeiss, Germany).

DNA methylation analysis

Genomic DNA was isolated from CCD841 cells using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). Two microgram of genomic DNA was bisulfite modified using EpiTect Bisulfite kit (Qiagen, Valencia, CA) according to manufacturer's

protocol. Primers used for 15-PGDH Nested-methyl specific PCR (MSP) were nested-forward 5'-GAT ATA TAA TTG ATA TTG ATT-3' and nested-reverse 5' ACT TCA AAA TTC CAA TCC ACC-3'. For MSP, specific oligonucleotides were synthesized to amplify methylated or unmethylated 15-PGDH promoter regions. The primer pairs were as follows (forward and reverse, respectively): Methylation; 5'-GTT TAG GGG GTA GGT GAT ATA GTC-3' and 5'-ACT ACT AAA ACG AAC GAT AAA CGA A-3', Unmethylation; 5'-TTT AGG GGG TAG GTG ATA TAG TTG T-3' and 5'-ACT ACT AAA ACA AAC AAT AAA CAA A-3'. Each PCR product was analyzed using 3% agarose gel.

Statistical analysis

Values were expressed as the mean ± S.D. of at least three independent experiments. Statistical significance was determined by One-way ANOVA and Student's *t* test. The criterion for statistical significance was $*p < 0.05$, $**p < 0.05$ and $***p < 0.005$.

RESULTS

H_2O_2 down-regulates the expression of 15-PGDH

When CCD841 cells were treated with H_2O_2, expression of 15-PGDH was decreased in concentration **(Fig. 4A)** and time-dependent manners **(Fig. 4B)**. In parallel with the reduced protein level of 15-PGDH, the expression of its mRNA transcript was also decreased at H_2O_2 300 μM **(Fig. 4C).** In addition, immunofluorescence staining analysis revealed that H_2O_2 decreased 15-PGDH expression in CCD841 cells **(Fig. 4D).** These findings indicate that ROS negatively regulates 15-PGDH expression.

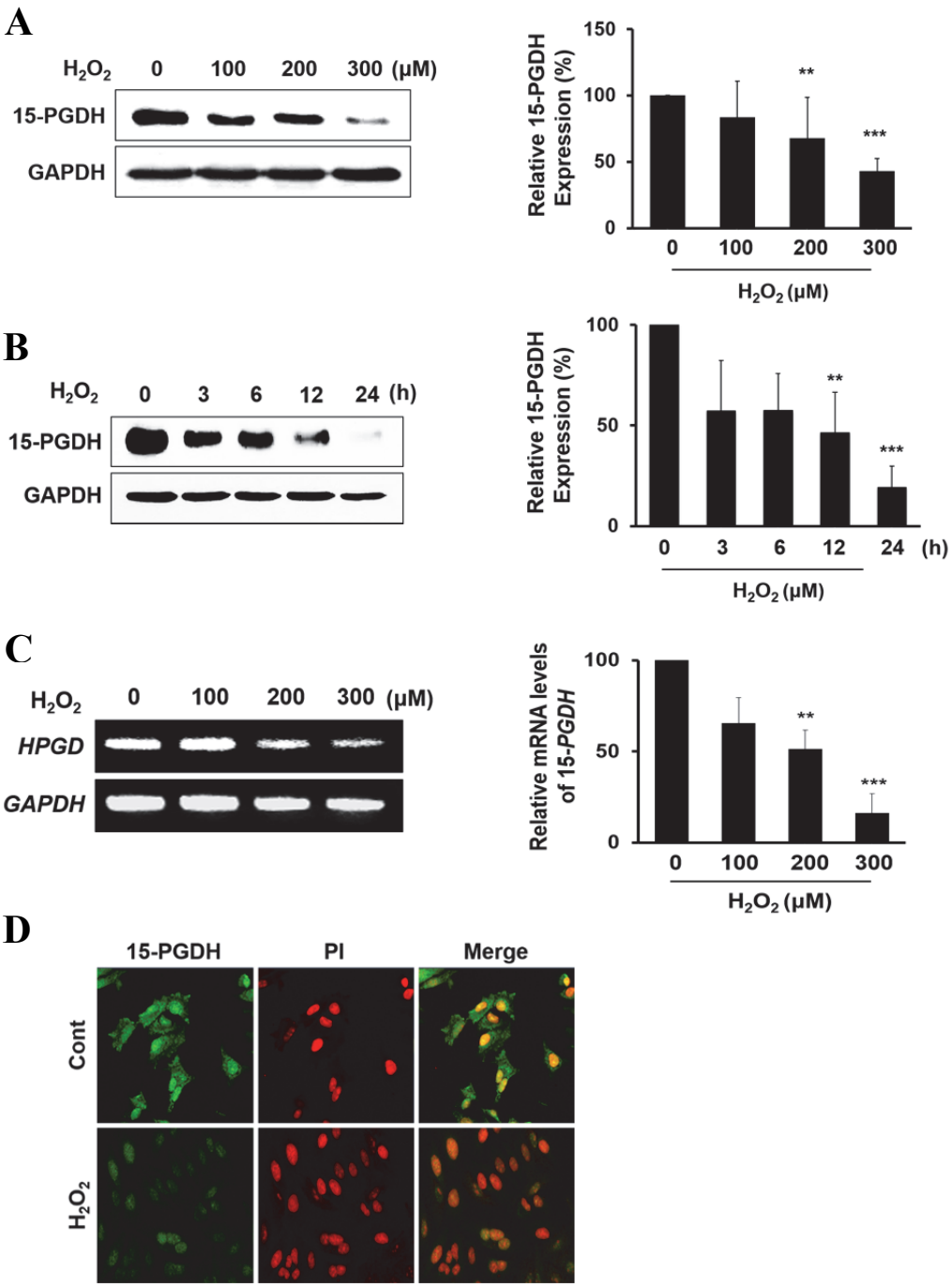

Fig. 4. H_2O_2 suppresses the expression of 15-PGDH in human colon epithelial CCD841 cells. (A) CCD841 cells were treated with H_2O_2 (100, 200, and 300 μM) for 24

h and total protein isolated from cell lysates was subjected to Western blot analysis for the measurement of 15-PGDH expression. GAPDH was used as an equal loading control for normalization. ***p <0.001 **(B)** CCD841 cells were treated with H_2O_2 (300 μM) for the indicated time. Protein levels were determined by Western blot analysis. GAPDH was used as loading control. ** p <0.05 **(C)** The mRNA level of 15-PGDH was determined by *RT-PCR.* The mRNA level of GAPDH was used as an internal control**. (D)** Expression of 15-PGDH was observed by immunofluorescence staining.

H_2O_2 induces hypermethylation of 15-PGDH through up-regulation of DNMT3a

To determine whether down-regulation of 15-PGDH is associated with hypermethylation of its promoter region, the level of 15-PGDH promoter methylation was verified by methylation-specific PCR (MSP) in the CCD841 cells treated with H_2O_2 for 0, 3, 6, 9, or 12 h. H_2O_2 increased the methylation level of 15-PGDH in a time dependent manner. **(Fig. 5A)**. Four active DNA methyltransferases, DNMT1, DNMT2, DNMT3a, and DNMT3b, have been reported in mammals [33]. DNMT3a is a de novo DNA methyltransferase that modifies unmethylated DNA. In contrast, DNMT1 shows high preference for hemimethylated DNA [34]. ROS may induce site-specific hypermethylation via either the up-regulation of expression of DNMTs or the formation of a new DNMT containing complex [32]. We observed that H_2O_2 increased the expression of DNMT1/3a in a concentration-dependent manner in the total protein **(Fig. 5 B)**. There was an increase in DNMT3a, but not in the level of DNMT1, in the nucleus extract of 6 h treatment **(Fig. 5C)**. In addition, immunofluorescence staining analysis revealed that H_2O_2 increased DNMT3a expression in CCD841 cells **(Fig. 5D)**. These finding suggest that ROS induces expression of DNMT3a in CCD841 cells.

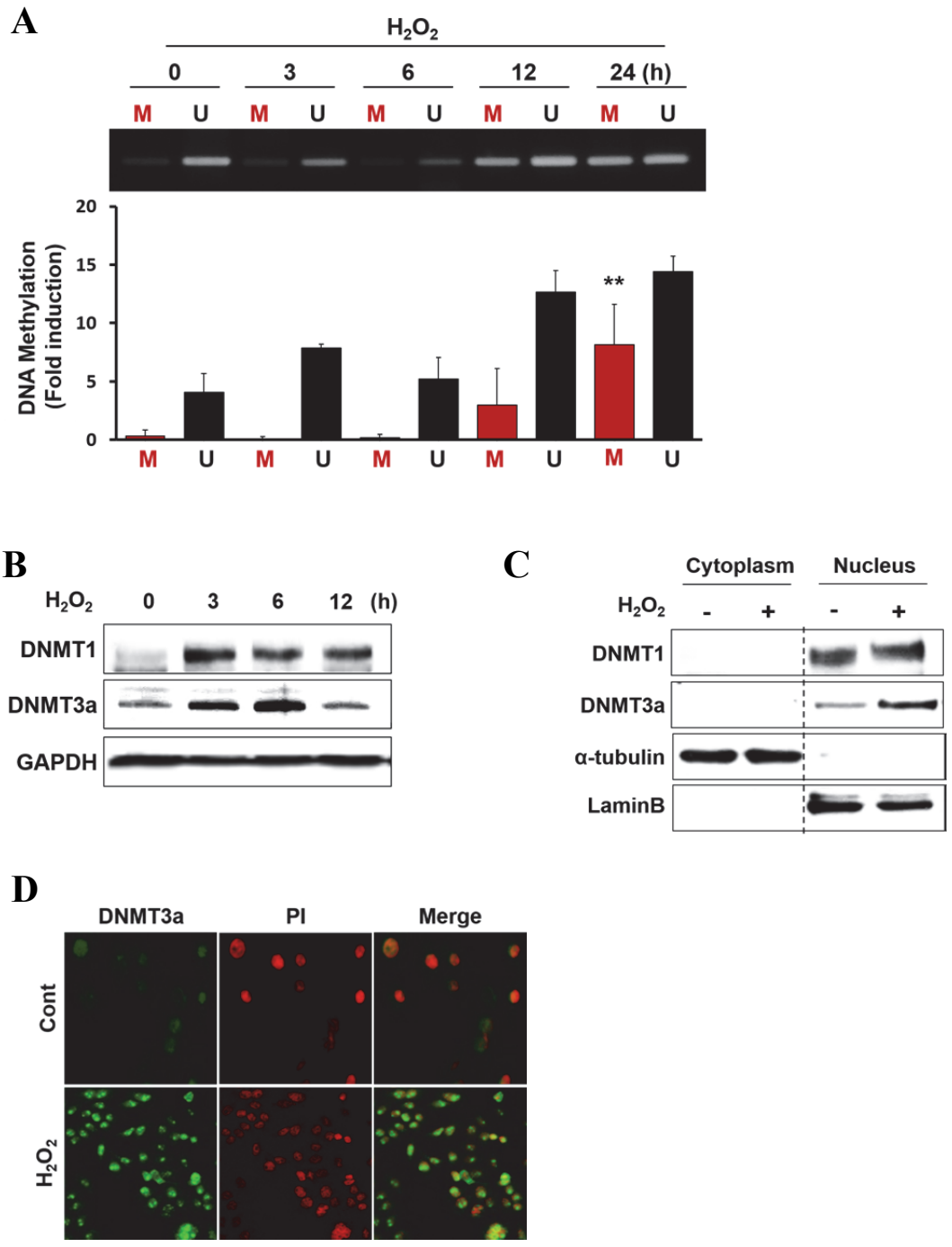

Fig. 5. H_2O_2 induces hypermethylation of 15-PGDH promoter via up-regulation of DNMT3a. (A) Effect of H_2O_2 on the level of methylation of CpG islands present in the

15-PGDH promoter. CCD841 cells were treated with H_2O_2 (300 μM) for indicated time, followed by isolated the genomic DNA. DNA was subjected to MSP analysis with specific primers of 15-PGDH gene. The PCR-products labeled with ‘‘M’’ were generated by methylation-specific primers, and those labeled with ‘‘U’’ by primers specific for unmethylated DNA. **(B)** CCD841 cells were treated with H_2O_2 (300 μM) for indicated time and expression of DNMT1 and DNMT3a was determined by Western blot analysis. **(C)** Nuclear and cytosol extract were prepared from CCD841 cells treated with H_2O_2 for 6 h. DNMT1 and DNMT3a levels in cytosol and nuclear extracts were determined by Western blot analysis. Lamin B and α-tubulin serve as nuclear and cytoplasmic marker, respectively. **(D)** CCD841 cells were treated with H_2O_2 (300 μM) for 4 h and fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. Cells were immunoblotted with anti-DNMT3a. The localization of DNMT3a was observed by confocal microscopy.

Inhibitor of DNA methylation, 5-aza-2′-deoxycytidine (5-Aza), abrogates the down-regulation of 15-PGDH expression by suppressing the expression of DNMT3a

To further investigate the down-regulation of 15-PGDH by H_2O_2 is associated with high level of DNMT3a, the cells were treated with H_2O_2 in the presence or absence of demethylating agent 5-Aza. Western blot analysis revealed that the down-regulation of 15-PGDH expression induced by H_2O_2 were abrogated by 5-Aza. 5-Aza attenuated the expression of DNMT3a in the total protein extract **(Fig. 6A)** and nucleus one as determined by Western blot analysis **(Fig. 6B)**. Immunofluorescence analysis also revealed the increased accumulation of DNMT3a in nucleus and the abolished expression of DNMT3a by 5-Aza **(Fig. 6C)**. Furthermore, MSP revealed that treatement 5-Aza suppressed up-regulation of 15-PGDH methylation induced by H_2O_2 in CCD 841 cells **(Fig. 6D)**. These findings suggested that oxidative stress induced methylation of the 15-PGDH promoter through up-regulation of DNMT3a.

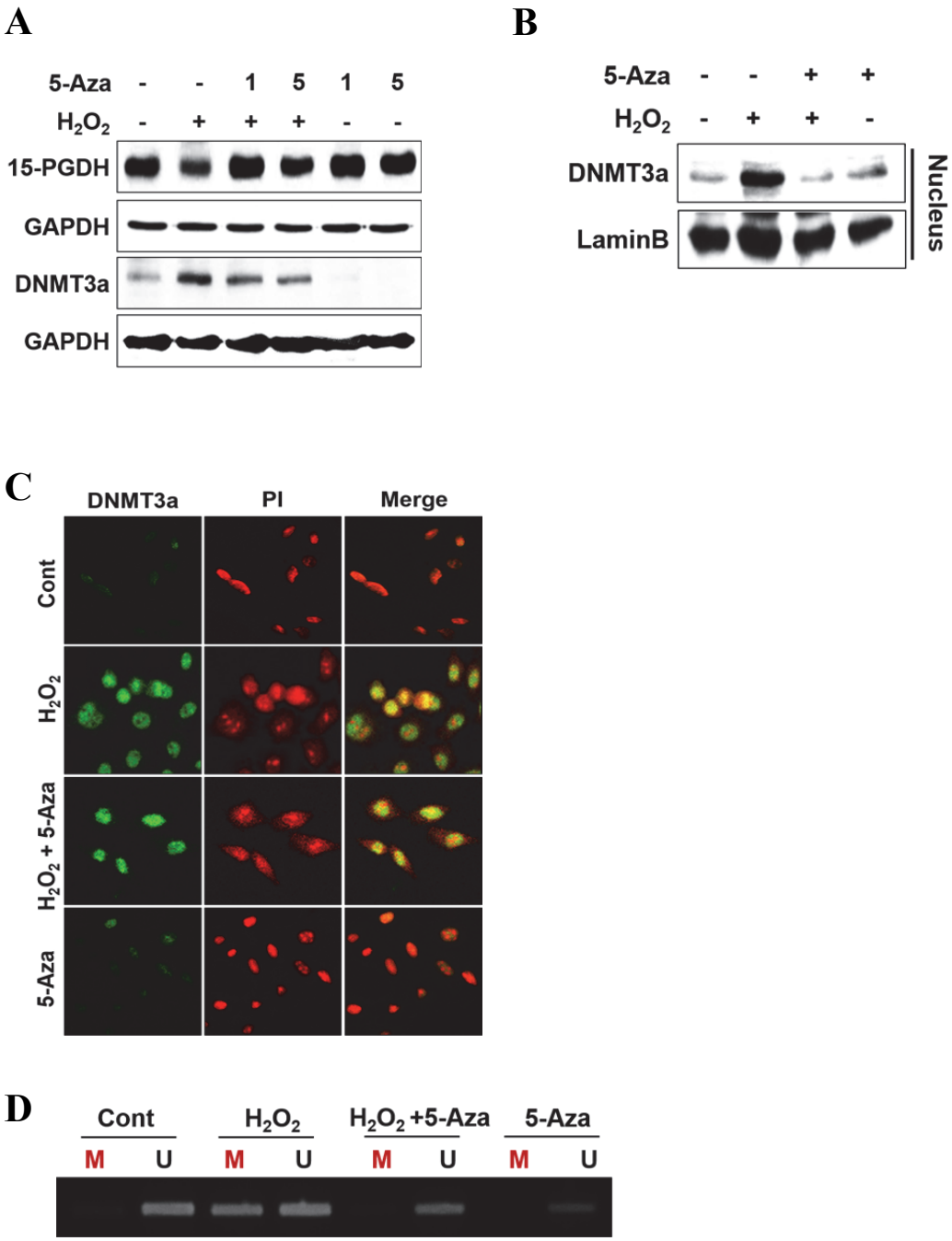

Fig. 6. Effects of the DNA methyltransferase inhibitor, 5-Aza, on expression of 15-PGDH and DNMT3a in CCD841 cells. (A) CCD841 cells were treated with 5-Aza (1 and 5 μM) in the presence or absence of H_2O_2 (300 μM) for 24 h (15-PGDH) and 6 h

(DNMT3a) exposure. The expression of DNMT3a, and 15-PGDH was determined by Western blot analysis. **(B)** Nuclear and cytosolic extracts were prepared from CCD841 cells treated with H_2O_2 (300 μM) in the absence or presence of 5-aza (5 μM). Expression of DNMT3a was measured by Western blot analysis. Lamin B was used as an equal loading control for normalization. **(C)** CCD814 cells were treated with 5-Aza (5 μM) in the absence or presence of H_2O_2 (300 μM) for 4 h and then expression of DNMT3a was detected by immunofluorescence staining. **(D)** CCD841 cells were treated with H_2O_2 (300 μM) in the presence or absence of 5-Aza (5 μM) for 6h followed by isolation of genomic DNA and MSP were conducted against 15-PGDH

General antioxidant N-acetylcysteine (NAC) restored the expression 15-PGDH by suppressing the expression of DNMT3a

NAC is potent antioxidant that inhibit oxidative stress by directly scavenging ROS [35]. To investigate whether down-regulation of 15-PGDH is associated with ROS, the cells were treated with H_2O_2 and NAC alone or in combination. H_2O_2 produced the ROS in CCD841 cells as determined by DCF-DA staining. NAC treatments resulted in marked reduction in ROS production induced by H_2O_2 **(Fig. 7A)**. In addition, NAC restored down-regulation of 15-PGDH induced by H_2O_2 as determined by Western Blot analysis. Moreover, up-regulation of DNMT3a induced by H_2O_2 was blocked by NAC in the total protein extract **(Fig. 7B)** and in the nucleus **(Fig. 7C)** as determined by Western blot analysis. Immunofluorescence analysis also revealed that the increased accumulation of DNMT3a in nucleus induced by H_2O_2 was abolished by NAC **(Fig. 7D)**. Furthermore, MSP also revealed that NAC decreased the level of methylated 15-PGDH similar to that attained with 5-Aza **(Fig. 7E)**. These findings suggest that H_2O_2 upregulated the methylation of CpG islands present in the 15-PGDH promoter region by regulating the expression of DNMT3a.

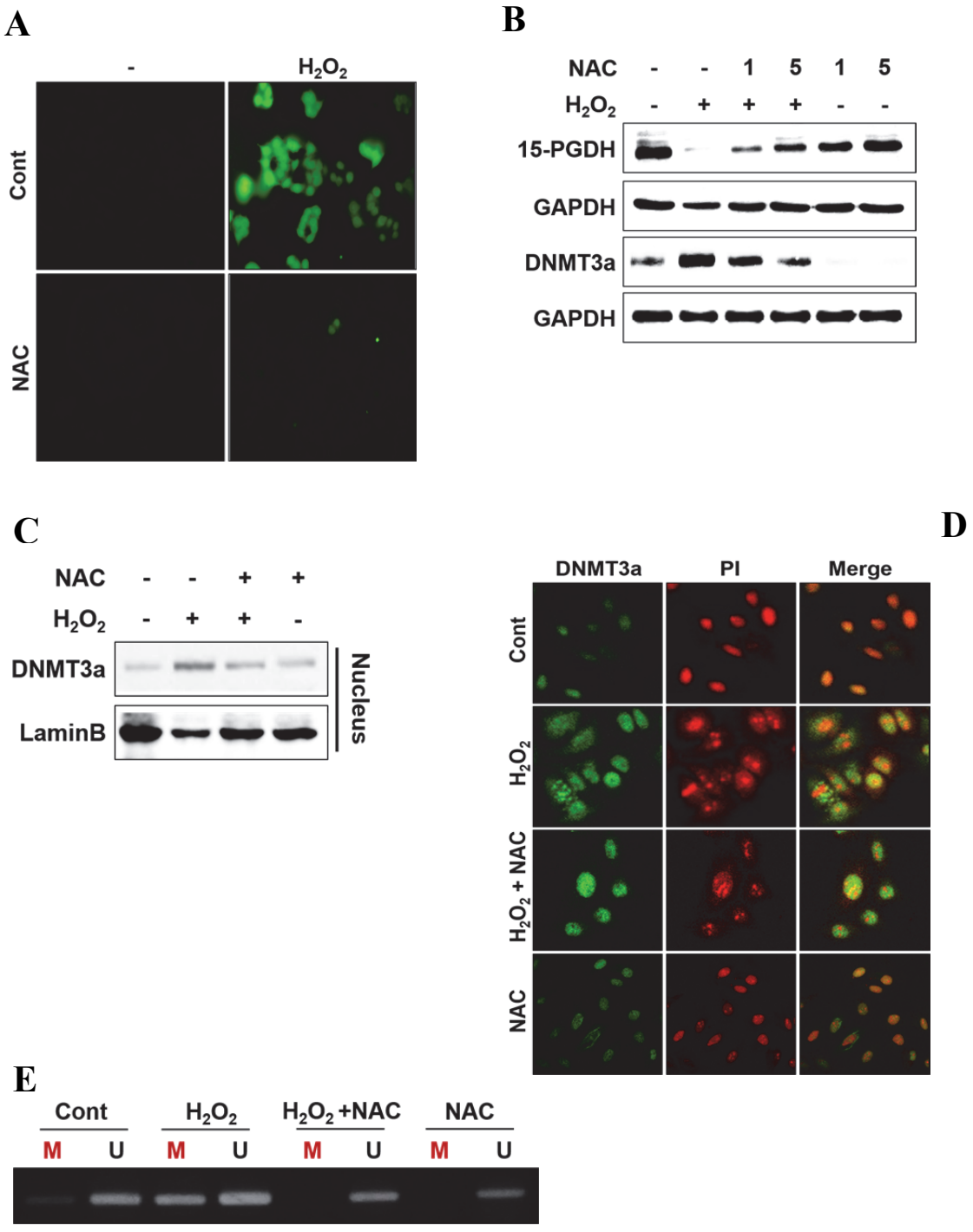

Fig. 7. Effects of general antioxidant NAC on expression of 15-PGDH, DNMT3a and generation of reactive oxygen species in CCD841 cells. (A) CCD814 cells were treated

with NAC (5 mM) for 4 h in the presence of H_2O_2 (300 μM), and the intracellular ROS level was measured by DCF-DA staining. **(B)** CCD841 cells were treated NAC (1 and 5 mM) for 6 h and the12 h in the presence of H_2O_2 (300 μM) for 24h (15-PGDH) and 6h (DNMT3a) exposure. The expression of DNMT3a, and 15-PGDH was measured by Western blot analysis. **(C)** Nuclear and cytosolic extracts were prepared from CCD841 cells treated with H_2O_2 (300 μM) in the absence or presence of NAC (5 mM). Expression of DNMT3a was measured by Western blot analysis. Lamin B was used as an equal loading control for normalization. **(D)** CCD841 cells were treated with NAC (5 mM) in the absence or presence of H_2O_2 (300 μM) for 4 h and then incubated with anti-DNMT3a for immunofluorescence staining. **(E)** CCD841 cells were treated with H_2O_2 (300 μM) in the presence or absence of NAC (5 mM) for 6 h followed by isolation of genomic DNA and MSP were conducted against 15-PGDH.

Snail plays an important role in down-regulation of 15-PGDH expression

Snail is a zinc-finger transcriptional repressor controlling EMT during embryogenesis and tumor progression [36]. Snail induced DNA methylation of the E-cadherin promoter by recruiting histone deacetylase 1 and DNMT1 in hepatocellular carcinoma [37]. Yeon-Mi et al revealed that *H pylori* suppress 15-PGDH protein levels by activating Snail [38]. Therefore, we examined whether H_2O_2 induced expression of Snail in CCD 841 cells. We observed that H_2O_2 induced expression of Snail at 12 and 24 h treatment **(Fig. 8A)**. To determine whether H_2O_2-mediated down-regulation of 15-PGDH was associated with Snail, the CCD 841 cells were transfected with si-Control si-Snail. Snail siRNA partially restored down-regulation of 15-PGDH expression induced by H_2O_2 **(Fig. 8B)**. In contrast, silence of 15-PGDH by its si-RNA induced the expression of Snail in the CCD841 cells, which was enhanced by H_2O_2 treatment **(Fig. 8C)**. Taken together, these findings suggested that up-regulation of Snail by H_2O_2 contribute to down-regulation of 15-PGDH. In addition, there is a reciprocal regulation between 15-PGDH and Snail.

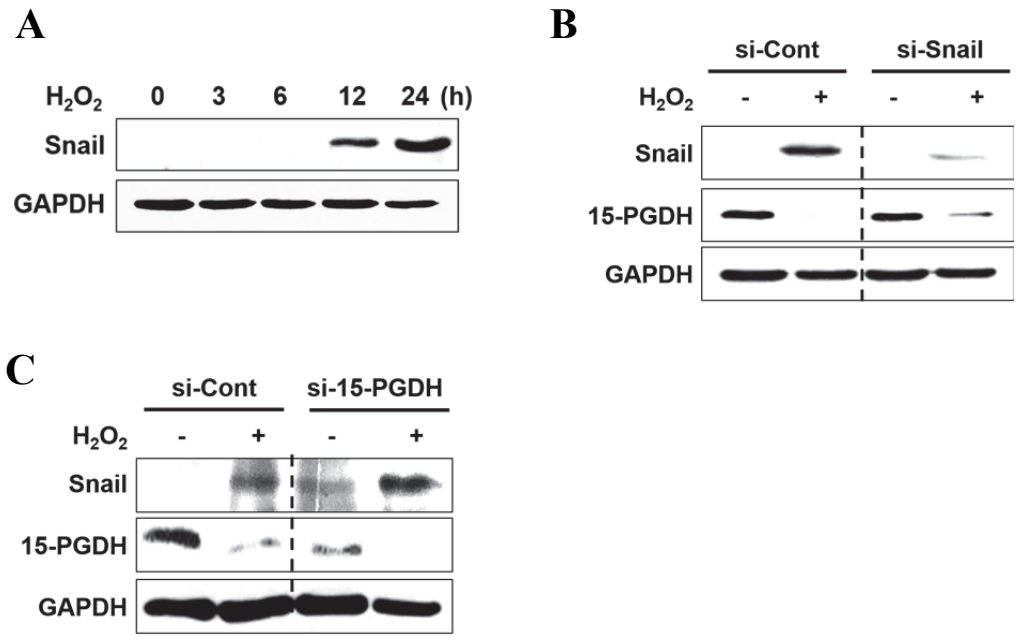

Fig. 8. Snail plays an important role in down-regulation of 15-PGDH. (A) CCD841 cells were treated with H_2O_2 (300 μM) for the indicated time. Indicated protein levels were determined by Western blot analysis. GAPDH was used as loading control. **(B, C)** CCD814 cells were transfected with scrambled siRNA or snail or 15-PGDH siRNA and treated with H_2O_2 (300 μM) for 24 h. The expression of 15-PGDH and Snail was detected by Western blot analysis. GAPDH was used as an equal loading control for normalization.

DISCUSSION

The NAD+-dependent 15-PGDH is the enzyme catalyzing the oxidation of the 15(S)-hydroxyl group of PGE_2 to its oxidized form 15-keto-PGE_2 and conferring PGE_2 biological inactive [39]. Several studies identified a tumor suppressor activity of 15-PGDH in colon, lung, breast and bladder cancers [10, 12, 13, 21, 40]. The expression of 15-PGDH is ubiquitously down-regulated in various cancers [13, 41]. But, regulation of 15-PGDH expression at the translational and post-transcriptional levels remains poorly understood.

Approximately 70% of genes possess promoter-associated CpG islands that mostly remain unmethylated in normal cells unlike the remainder of the genome, which tends to be heavily methylated [42]. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is now firmly established as an important mechanism for gene inactivation [43]. For example, the *p16/CDKN2* tumor suppressor gene may be inactivated by methylation of its 5′ CpG island in transitional cell carcinomas of the bladder [44]. *RUNX3* is a tumor suppressor that is silenced in cancer following hypermethylation of its promoter [45]. *RUNX3* methylation is frequently found in human cancers, including gastric cancer, and is mostly cancer specific, with the exception of the stomach [46]. Yang et al found that methylation of tumor suppressor gene promoters was a frequent event in hepatocellular carcinoma (HCC), as 82% cases of HCC had at least one tumor suppressor gene promoter methylated [47]. The most frequently methylated genes were *SOCS-1*, *GSTP*, *APC*, *E-cadherin*, and *p15*. In comparison to non-tumor liver tissues, methylation of these genes was specifically associated with HCC [47]. One of the well-known mechanism of down-regulation of 15-PGDH is the transcriptional repression

by promoter CpG island hypermethylation [21]. We observed that H_2O_2 induces methylation of 15-PGDH promoter in CCD841 cells.

The *de novo* methyltransferases DNMT3a and DNMT3b are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites, whereas the maintenance methyltransferase DNMT1 copies pre-existing methylation patterns onto the new DNA strand during DNA replication [48]. The levels of DNMTs, especially those of DNMT3a, DNMT3b, and DNMT3l, are often increased in various cancer tissues and cell lines, which may partially account for the hypermethylation of promoter CpG-rich regions of tumor suppressor genes in a variety of malignancies [49]. Promoter methylation, catalyzed by DNMTs, plays an established role in silencing key genes in multiple DNA damage repair pathways; inactivation of these pathways may predispose to a large array of tumors [50]. In addition, DNMT1, DNMT3a and DNMT3b are cooperatively involved in determining the extent of HCCs, and that DNMT protein overexpression in HCCs may be a predictive factor for poor survival [49, 51]. We observed that H_2O_2 induced the expression of DNMT3a.

Inflammation-related cancers are known for their multiple occurrences, and aberrant DNA methylation is known to be present even in noncancerous tissues [52]. DNA methylation is important in the regulation of inflammatory genes [53]. Recent studies have demonstrated that inflammatory cytokines suppress the 15-PGDH expression in inflammatory bowel disease (IBD) [54]. For example, 15-PGDH protein and mRNA are markedly reduced in the inflamed mucosa of patients with IBD [54]. Hemokinin-1, a tachykinin produced by immune cells and upregulated in IBD, was shown to stimulate *COX2* gene expression and repress 15-PGDH protein expression in colonic mucosal

explants [55]. Inflammation can produce ROS, which may lead to methylation of CpG islands in gene of methylation located on gene promoters [56]. We observed that ROS down-regulates the expression of 15-PGDH at the transcriptional level in CCD841 cells. General antioxidant NAC restored the down-regulation of 15-PGDH induced by H_2O_2. 15-PGDH down-regulation mediated by ROS is associated with methylation of the 15-PGDH promoter through up-regulation of DNMT3a.

Snail genes also have additional cellular functions that sometimes occur independently of the induction of EMT [57]. Snail plays a central role in CpG methylation of the E-cadherin promoter via a specific recognition site to which HDAC1 and DNMT1 are recruited [37]. Pioglitazone suppressed expression of both Egr-1 and Snail and also inhibited the binding of Snail to the 15-PGDH promoter [58]. Overexpression of Snail blocked pioglitazone-mediated induction of 15-PGDH and restored the PGE_2 levels in the medium of primary human visceral preadipocytes [58]. We observed that H_2O_2 induced the expression of Snail, knock down of 15-PGDH induces the expression of Snail and *vice versa.*

In conclusion, ROS directly affect the methylation status of the 15-PGDH promoter through the expression of DNMT3a and Snail **(Fig. 9)**. This may contribute to promotion of colon carcinogenesis.

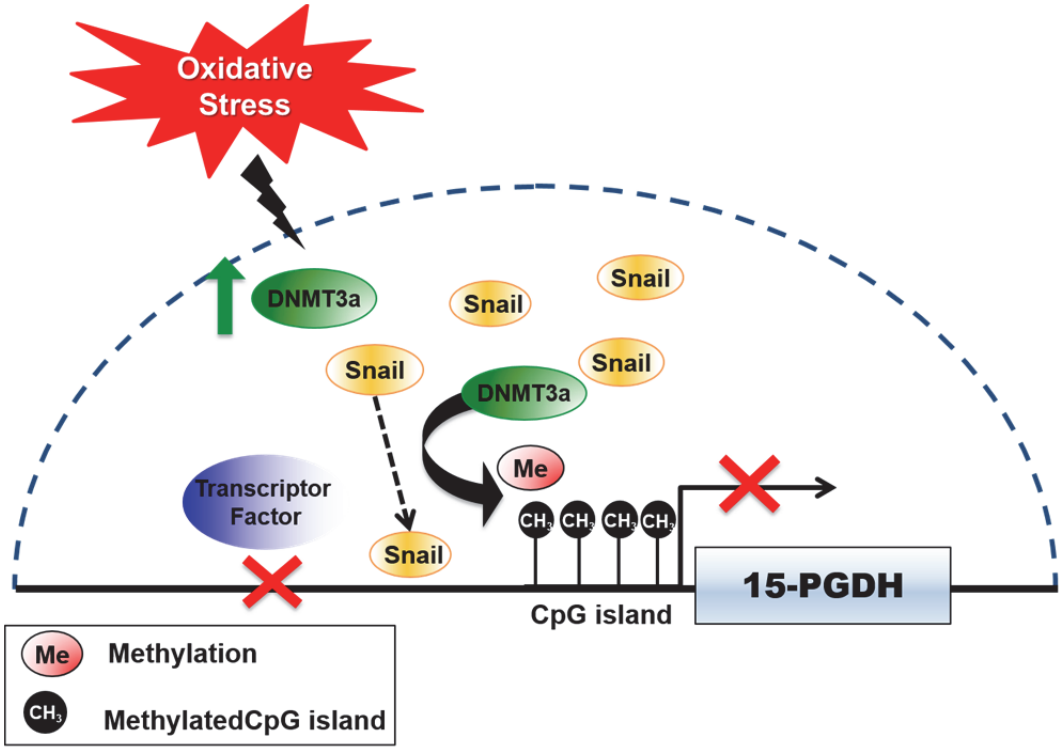

Fig. 9. A proposed mechanism underlying ROS-mediated epigenetic regulation of 15-PGDH.

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국문초록

염증 반응 시 유도되는 Cycolooxygease-2 (COX-2)의 산물인 prostaglandin E_2 (PGE_2)는 암세포의 증식, 혈관 신생 및 침윤과정을 통해 암화과정을 촉진한다. 따라서 PGE_2 의 과잉 생성을 막는 것은 염증 반응 및 염증 유래 암화과정을 조절하는 데 중요하다. 15-hydroxyprostaglandin dehydrogenase (15-PGDH)는 PGE_2 를 15-keto-PGE_2 로 전환 시키는 효소로서, PGE_2 를 비활성화한다. 15-PGDH 는 위암, 폐암, 유방암 등 다양한 암종에서 발현이 낮으며, 암세포에서 과발현 시 암화 과정을 저해하는 종양 억제유전자 (Tumor Suppressor)로서 알려졌다. 활성 산소종 (ROS; Reactive Oxygen Species)은 정상세포에서보다 암세포에서 많이 생성되며 암세포증식에 연루되는 신호전달 단백질을 활성화함으로써 종양 발달을 촉진한다. 또한 ROS 는 종양 억제유전자의 promoter 부분에 존재하는 CpG island 를 과메틸화 시킴으로써 유전자 발현을 저해하여 궁극적으로 암화과정을 촉진하는 것으로 알려졌다. 다양한 암세포와 암 조직에서 15-PGDH 의 발현 감소는 15-PGDH 유전자 promoter 부위의 과메틸화 즉 epigenetic modification 에 의해 매개되는 것으로 알려졌다. 그러나 아직까지 15-PGDH 의 발현 감소에 대한 분자적 작용기전에 대해서는 잘 알려져 있지 않다. 본 연구에서는 대표적인 활성 산소종인 과산화수소(Hydrogen

peroxide, H_2O_2)에 의해 15-PGDH 의 발현이 조절되는지를 epigenetic 측면에서 규명하고자 하였다.

인간 정상대장상피세포인 CCD841 에 과산화수소를 처리 시 농도·시간 의존적으로 15-PGDH 의 발현이 감소됨을 확인하였다. DNA methylation 에 관여하는 DNA methyltransferases (DNMTs)는 종양 억제 유전자 프로모터 CpG island 과메틸화를 조절한다. 과산화수소는 DNA methylation 을 유지하는 주요한 효소인 DNMT3a 의 발현을 유도하였으나 DNMT1 의 발현에는 변화가 없었다. 과산화수소는 15-PGDH 유전자 promoter 부위의 CpG island methylation 정도를 시간 의존적으로 증가하였다. DNA methylation 억제제인 5-Aza-2′-deoxycytidine (5-Aza)는 과산화수소에 의해 증가된 DNMT3a 의 발현을 저해한 동시에 감소된 15-PGDH 의 발현을 회복함을 Western blot analysis 를 통해 확인하였다. 또한 과산화 수소에 의해 증가한 15-PGDH promoter 의 methylation 이 5-Aza 에 의해 감소함을 methyl Specific PCR (MSP)을 이용해 확인하였다. 기 도출된 결과를 바탕으로 과산화수소에 의해 증가된 DNMT3a 의 발현이 15-PGDH 프로모터의 부위의 과메틸화를 유도함으로써 15-PGDH 의 발현 저해를 유도함을 알 수 있었다. 과산화수소에 의한 15-PGDH 의 발현 감소가 산화적 스트레스와 관련이 있는지 알아보기 위해 ROS scavenger 인 N-acetylcysteine (NAC)을 처리하여 확인한 결과, 과산화수소에 의해 증가된

DNMT3a 의 발현은 NAC 에 의해 감소된 반면 감소된 15-PGDH 의 발현은 회복되었다. 또한 NAC 은 과산화 수소에 의해 증가된 15-PGDH promoter 의 methylation 정도를 감소하였다. 상피-간엽전환 (Epithelial-mesenchymal transition, EMT)와 관련된 유전자인 Snail 은 15-PGDH 발현과 역 상관관계가 있는 것으로 알려졌다. 정상대장상피세포에서 과산화수소에 의해 Snail 의 발현이 증가하였다. 15-PGDH 의 발현 감소가 Snail 과 관련되어있는지 알아보기 위해 siRNA 로 Snail 발현을 저해 시 15-PGDH 의 발현이 회복되고, 15-PDGH 의 발현을 siRNA 로 저해 시 Snail 이 발현되었다. 결론적으로 ROS 는 DNMT3a 와 Snail 의 발현 유도를 통해 15-PGDH promoter 의 과메틸화를 촉진함으로써 15-PGDH 의 발현을 저해하는 것으로 사료된다.

주요어 (Keyword): 15-Hydroxyprostaglandin Dehydrogenase, DNA methyltrasferase 3a, Reactive Oxygen Species, Epigenetic regulation