

DNA methyl transferase I acts as a negative regulator of allergic skin inflammation

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ABSTRACT

The role of DNA methyl transferase I (DNMT1) in allergic inflammation was investigated. Antigen stimulation decreased expression of DNMT1 in rat basophilic leukemia cells (RBL2H3). The down regulation of DNMT1 induced expression of histone deacetylase 3 (HDAC3). HDAC3 was necessary for allergic skin inflammation, such as triphasic cutaneous reaction and passive cutaneous anaphylaxis. The down regulation of DNMT1 resulted from activation of PKC and rac1 which were necessary for proteasome-dependent ubiquitination of DNMT1 by antigen stimulation. N-acetyl-L-cysteine, an inhibitor of reactive oxygen species production, exerted negative effects on allergic skin inflammation. Antigen stimulation led to increased expression of Tip60, a histone acetyl transferase. Wild type, but not mutant form, Tip60 decreased expression of DNMT1 while increasing expression of HDAC3, suggesting role for acetylation in ubiquitin-dependent proteasomal degradation of DNMT1. In vivo down regulation of DNMT1 increased ear thickness, typical of allergic skin inflammation, induced vascular leakage and promoted angiogenesis in BALB/c mouse. The down regulation of DNMT1 enhanced angiogenic potential of rat aortic endothelial cells (RAEC) accompanied by activation of VEGF-2 and induced interaction between VEGF-2 and syk in RAEC. The enhanced angiogenic potential of RAEC was associated with the induction of VEGF by down regulation of DNMT1 in RBL2H3 cells. The down regulation of DNMT1 induced leukocytes-endothelial cell interaction and expression of various adhesion molecules. Aspirin exerted a negative effect on allergic skin inflammation by indirect regulation on DNMT1 via Tip60. Taken together, these results suggest novel role for DNMT1 in allergic skin inflammation.

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1. Introduction

DNA methyl transferase I (DNMT1) is a maintenance DNA methyl transferase that catalyzes addition of a methyl group to cytosines to adjacent to guanines (Bestor, 1988; Yoder et al., 1997). DNMT1 prefers hemimethylated DNA substrate (Bacolla et al., 1999) although it can catalyze methylation of unmethylated DNA (Jair et al., 2006). DNA methylation and histone modification are major epigenetic modification in various cellular activities (Adcock et al., 2006). DNA methylation is a nonredundant repressor of the Th2 effector program (Makar and Wilson, 2004). DNA methyltransferase 3b recruits HDAC2 to regulate differentiation of PC12 cells (Bai et al., 2005). Histone deacetylation induces DNMT1 expression to silence survivin gene expression (Ma et al.,

2011). Apicidin, an inhibitor of HDAC(s), down regulates DNMT1 (You et al., 2008). Histones acetylation leads to decreased DNMT1 expression (Nandakumar et al., 2011). The above reports suggest interactions among epigenetic factors.

Glucocorticoid resistance in asthma is associated with the reduced HDAC2 activity (Li et al., 2010; Barnes and Adcock, 2009). Corticosteroid function is dependent on HDAC2 (Meja et al., 2008). The reduction of HDAC2 activity has been reported in asthma (Pankaj et al., 2008). Conditional deletion of HDAC1 in T cells enhances Th2 cytokine expression in airway inflammation (Grausenburger et al., 2010). Trichostatin A, an inhibitor of HDAC(s), attenuates airway inflammation in mouse asthma model by decreasing expression of Th2 cytokines (Choi et al., 2005). HDAC inhibitors suppress induction of COX-2, a hall mark protein in allergic inflammation (Yamaguchi et al., 2005). HDAC3 expression is induced by antigen stimulation in RBL2H3 cells via NF-κB (Kim et al., 2010c). Antigen stimulation leads to the decreased expression of HDAC2 while increasing expression of HDAC3 (Kim et al., 2010c).

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Allergic inflammation involves inflammation in relation with angiogenesis (Kim et al., 2011). IL-8 secreted from activated human mast cells contributes to angiogenesis (Kim et al., 2010b). Mast cells interact with endothelial cells to contribute to angiogenesis in multiple myelomas (Nico et al., 2008). S-adenosyl methionine, a methyl group donor, inhibits angiogenesis (Sahin et al., 2011).

In this study, we studied involvement of epigenetic factor(s), such as DNMT1, in allergic skin inflammation. We examined relationship between DNMT1 and HDAC(s). We investigated mechanism of down regulation of DNMT1 in terms of acetylation-driven proteasome-dependent ubiquitination. The role of DNMT1 in angiogenesis in relation with inflammation was investigated by examining effect of down regulation of DNMT1 on endothelial cell signaling, expression of adhesion molecules and angiogenic potential, such as endothelial cell tube formation and aortic ring formation. We showed that down regulation of DNMT1 led to induction of VEGF, which was responsible for enhanced angiogenic potential. In this study, we also investigated mechanism of anti-allergic effect by aspirin in terms of expression regulation of DNMT1. Our study provides novel role of DNMT1 in allergic skin inflammation and the mechanism of expression regulation of epigenetic factors, such as DNMT1 and HDAC2.

2. Materials and methods

2.1. Cell culture

RBL2H3 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified Eagle's medium containing heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, San Diego, CA). Cultures were maintained in 5% CO₂ at 37°C. Rat aortic endothelial cells (RAEC) were isolated and maintained as previously reported (Kim et al., 2011). Bone marrow-derived mouse mast cells were isolated and cultured according to the standard procedures (Kim et al., 2010a).

2.2. Isolation of skin mast cells from mouse

Ears of BALB/c mice were cut into fragments and incubated in RPMI1640 medium supplemented with 25% fetal bovine serum, 1.5 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO), 0.5 mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO), 0.2 mg/ml protease (Sigma-Aldrich, St. Louis, MO) and 0.5 mg/ml DNase I (Sigma-Aldrich, St. Louis, MO) for 60 min at 37°C. Dispersed cells were filtered sequentially through 70 µm and 40 µm cell strainers (Becton Dickinson Labware, Franklin Lakes, New Jersey). The pelleted cells were resuspended in RPMI1640 medium containing 0.1% bovine serum albumin and submitted to a continuous isotonic Percoll gradient (72%) for mast cell isolation. Purified mast cells were resuspended in RPMI-FBS. The cell purity (>96%) and viability (>98%) were evaluated by toluidine blue and trypan blue exclusion staining, respectively.

2.3. Mice

Five week-old female BALB/c mice were purchased from SLC Japan (Shizuoka, Japan) and maintained in SPF condition. All animal experiments were approved by Institutional review Board for animal studies of Kangwon National University.

2.4. Chemicals and reagents

Oligonucleotides used in this study were commercially synthesized by the Bionex Company (Seoul, Korea). DNP-HSA and DNP-specific IgE antibody were purchased from Sigma Chemical Company. Chemicals used in this study were purchased from Sigma Chemical Company. All other antibodies were purchased from Cell Signaling Company (Beverly, MA). Anti mouse and anti rabbit IgG-horse radish peroxidase conjugate antibody was purchased from Pierce Company (Rockford, IL). Lipofectamin and PlusTM reagent for transfection were purchased from Invitrogen (San Diego, CA). Immunohistochemistry staining was performed by using avidin-biotin detection method (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA).

2.5. Preparation of siRNA duplexes

The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, Austin, TX).

2.6. Western blot analysis and immunoprecipitation

For immunoprecipitation, cell lysates were immunoprecipitated with respective antibody (each at 2 µg) on ice for 1 h. Protein G-sepharose was then added and the reaction was performed at 4°C for 2 h on a rotary shaker. Immune complexes were washed three times with lysis buffer, and 2× sample buffer was added to the beads. Boiled samples were then loaded on gels. Western immunoblot analyses were followed according to the standard procedures (Kim et al., 2010a,b,c).

2.7. Rac1 activity assays

Rac1 activity assays were performed according to the well established procedures (Kim et al., 2010a,b,c).

2.8. IgE-dependent Triphasic Cutaneous Reaction (allergic skin inflammation) in the mouse ear

IgE-dependent triphasic cutaneous reaction in the ear of female BALB/c mice was induced as reported previously (Nagai et al., 1999). In brief, mice were passively sensitized by injecting 0.5 µg of mouse anti-DNP monoclonal IgE antibody intravenously. Twenty-four hours later, cutaneous reaction was evoked by painting with 25 µl of 0.15% DNFB acetone-olive oil (3:1) solution onto each surface of both ear lobes. Ear thickness was measured before and after the DNFB challenge by using a digital gauge. To examine effect of HDAC3 on allergic skin inflammation, scrambled siRNA (100 nM) or HDAC3 siRNA (100 nM) was injected i.v. on the day of IgE sensitization and two days after DNFB stimulation. To determine effect of aspirin on allergic skin inflammation, BALB/c mice were given i.p. injection of aspirin (20 mg/kg) before and after DNFB stimulation.

2.9. IgE-dependent passive cutaneous anaphylaxis

BALB/c mice were passively sensitized with an intradermal (i.d.) injection of DNP-specific IgE (0.5 µg/kg) into both ears. The mice were challenged twenty-four hours later with an intravenous (i.v.) injection of DNP-HSA (250 µg/kg) plus 250 µl PBS containing 2% (v/v) Evans blue solution. Thirty minutes after DNP-HSA challenge, the mice were euthanized, and the 2% (v/v) Evans blue dye was extracted from each dissected ear in 700 µl of acetone/water (7:3) at room temperature overnight. The absorbance of Evans blue in the extracts was measured with a spectrophotometer at 620 nm. In order to examine effect of NAC on PCA, BALB/c mice

were passively sensitized with an i.d. injection of DNP-specific IgE (0.5 µg/kg) along with or without NAC (0.1 mM). The mice were challenged twenty-four hours later with an i.v. injection of DNP-HSA (250 µg/kg) plus 250 µl PBS containing 2% (v/v) Evans blue solution.

2.10. Effect of DNMT1 on vascular leakage

Ears of BALB/c mouse were injected with scrambled siRNA (100 nM) or DNMT1 siRNA (100 nM). The next day, the mice were challenged with intravenous injection of 250 µl PBS containing 2% (v/v) Evans blue solution. The mice were killed an hours after injection of Evans blue solution, followed by the removal of an ear for measurement of the amount of dye extravasated. The dye was eluted from the ear in 700 µl of formamide at 63 °C. The absorbance was measured at 620 nm.

2.11. Histological analyses

Ear samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 µm, and then stained with hematoxylin and eosin to examine extent of leukocytes infiltration by in vivo down regulation of DNMT1 or HDAC3. Immunohistochemical staining of ear tissues was then performed using an established avidin-biotin detection method (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA). Briefly, 4- to 6 µm-thick sections of the paraffin-embedded tissue blocks were cut, mounted on positively charged glass slides, and dried in an oven at 56 °C for 30 min. The sections were deparaffinized in xylene and then rehydrated in graded ethanol and water. Endogenous peroxidase was blocked by incubation in 3% (v/v) Hydrogen peroxide for 15 min. Antigen retrieval was accomplished by pretreatment of the sections with citrate buffer at pH 6.0 for 20 min at 56 °C in a microwave oven and then allowing the sections to cool for 30 min at room temperature. Nonspecific endogenous protein binding was blocked using 1% bovine serum albumin (BSA). The sections were then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used for detection of proteins. Anti-DNMT1 (1:100, Abcam, England), anti-HDAC3 (1:100, Abcam, England), anti-HDAC2 (1:100, Abcam, England), and anti-Tip60 (1:50, Abcam, England). After washing, biotinylated secondary antibodies were applied at 1:100 or 1:200 dilutions for 1 h. Color was developed with diaminobenzidine (DAB, Vector Laboratories Inc.). Sections were counterstained with Mayer's Haematoxylin. Sections incubated without primary antibody served as controls. To visualize tissue mast cells, the sections were stained with 0.1% toluidine blue (Sigma) in 0.1 N HCl for 15 min.

2.12. RT-PCR analysis

Total RNA was isolated by Trizol and converted into cDNA using Superscript reverse transcriptase.

2.13. Chromatin immunoprecipitation (ChIP) assay

Assays were performed according to manufacturer's instruction (Upstate). The antibody immunoprecipitates were reverse cross-linked. PCR was done on the phenol-chloroform-extracted DNA with specific primers of HDAC3 promoter-1 [5-AAATAGAGGGGTTGGGGGAG-3(sense)] and [5-CCGAGACTCGAAGTGCACAT-3(antisense)], HDAC3 promoter-2 [5-CAGACGACTGGGAAACCCAG-3(sense)] and [5-TCCGAGGCTTGAGACTTGA-3(antisense)] and HDAC3 promoter-3 [5-GTCTCAAGCCTCGGAACCG-3(sense)] and [5-CCGTAGGAAGTTGCCAC-3(antisense)] sequences were used.

2.14. Endothelial cell tube formation assay

Growth factor-reduced matrigel was added to 24-well plates (200 µl matrigel per well) and polymerized for 30 min at 37 °C. Rat aortic endothelial cells were first incubated in M199 containing 1% FBS for 1 h, followed by the addition of conditioned medium of each cell transiently transfected with control siRNA (10 nM) or DNMT1 siRNA (10 nM), before antigen stimulation. After 6 to 8 h of incubation at 37 °C, the endothelial cells were photographed using an inverted microscope (magnification, ×100; Olympus).

2.15. Aortic ring formation assays

Rat aortic ring assay was performed according to the standard procedures (Kim et al., 2011).

2.16. Whole mount staining

BALB/c mice were given i.v. injection of scrambled siRNA (100 nM) or DNMT1 siRNA (100 nM) twice in a total of five days. Mouse ears were fixed in 4% (v/v) paraformaldehyde and blocked with TNB buffer (NEN Life Science Products) containing 0.3% Triton X-100. Rabbit anti-CD31 (1:100, PECAM-1) was diluted in TNB buffer, and ear samples were incubated with primary antibody overnight at 4 °C. The samples were then washed three times and subsequently incubated with the secondary antibody, anti-rabbit IgG Alex 488 (1:100, Molecular probes). In order to examine effect of allergic skin inflammation condition on angiogenesis, DNP-specific IgE (0.5 µg) was injected into ears of BALB/c mice. The next day, the mice were challenged with intravenous injection of a 250 µg of DNP-HSA in 250 µl PBS. The mice were injected with DNP-HSA twice in a total of six days before whole mount staining employing anti-PECAM-1 antibody. Signals were visualized and digital images were obtained by using a Zeiss Apotome microscope and a Zeiss LSM 510 confocal microscope equipped with argon and helium-neon lasers (Zeiss). Area densities (percentage of tissue area) of blood vessels were measured by PECAM-1 immunopositive blood vessels at a magnification of ×200 in five regions, each 0.21 mm² area, per mount or section.

2.17. β-hexosaminidase activity assays

β-hexosaminidase activity assay was performed according to the standard procedures (Kim et al., 2010c).

2.18. Statistical analysis

Data were analyzed and graphed using GraphPad Prism statistics program (GraphPad Prism Software). Results are presented as mean ± SE. Statistical analysis was performed using *t* tests with differences between means considered significant when *P* < 0.05.

2.19. Histamine release assay

Histamine level was measured according to manufacturer's instruction (SPI-Bio). To measure cellular histamine level, culture supernatants were used.

2.20. Cellular adhesion assay

The IgE-sensitized RBL2H3 cells or BMMCs were stimulated with or without DNP-HSA (100 ng/ml) for 1 h. The RBL2H3 cells or BMMCs (each at 10⁴/ml) were added and incubated with RAEC for 15 min, non-adhering cells were removed by gentle washing with

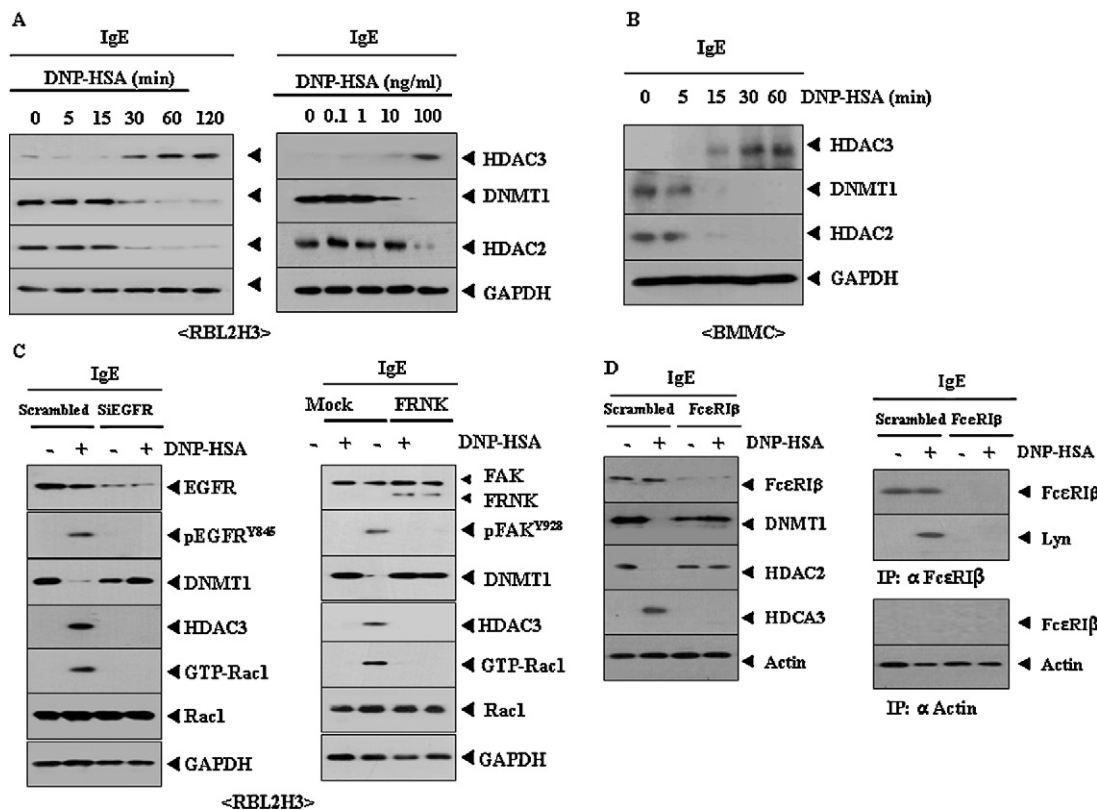


Fig. 1. DNMT1 is down regulated by antigen stimulation. (A) The DNP-specific IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA (100 ng/ml) for various time intervals (left panel) or stimulated with various concentrations of DNP-HSA for 30 min. Western blot is a representative figure of three independent experiments. (B) The IgE-sensitized bone marrow-derived mouse mast cells (BMMCs) were treated with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates were subjected to Western blot analysis. Western blot is a representative figure of three independent experiments. (C) RBL2H3 cells were transiently transfected with scrambled siRNA (10 nM) or EGFR siRNA (10 nM). The next day, cells were sensitized with DNP-specific IgE for 24 h, followed by stimulation with DNP-HSA for 1 h (left panel). RBL2H3 cells were transiently transfected with control vector (1 μ g) or FRNK (1 μ g), an endogenous inhibitor of FAK. The next day, cells were sensitized with DNP-specific IgE for 24 h, followed by stimulation with DNP-HSA for 1 h (right panel). Cell lysates prepared were subjected to Western blot analysis. Western blot is a representative figure of three independent experiments. (D) RBL2H3 cells were transiently transfected with Fc ϵ RI β siRNA (10 nM) or scrambled siRNA (10 nM). The next day, cells were sensitized with DNP-specific IgE for 24 h, followed by stimulation with DNP-HSA for 1 h. Cell lysates were prepared and subjected to Western blot analysis (left panel). Cell lysate were also immunoprecipitated with the indicated antibody (2 μ g/ml), followed by Western blot analysis (right panel). Western blot is a representative figure of three independent experiments.

PBS, and the number of adherent cells was determined in triplicate per $\times 40$ magnification field.

3. Results

3.1. Antigen stimulation in RBL2H3 cells leads to the down regulation of DNA methyltransferase I (DNMT1)

We reported the induction of HDAC3 in antigen-stimulated RBL2H3 cells (Kim et al., 2010c). We were interested to examine the expression of other epigenetic factor(s) during allergic inflammation. Antigen (DNP-HSA) stimulation decreased the expression of DNMT1 while increasing that of HDAC3 in a time and dose-dependent manner in RBL2H3 cells (Fig. 1A). The decreased expression of DNMT1 preceded induction of HDAC3 (Fig. 1A). The decreased expression of DNMT1 was also observed in antigen-stimulated bone marrow-derived mouse mast cells (BMMC) (Fig. 1B). We previously reported role for EGFR and FAK in allergic inflammation (Kim et al., 2011). The decreased expression of DNMT1 and increased expression of HDAC3 by antigen stimulation was dependent on EGFR and FAK (Fig. 1C). EGFR and FAK were also necessary for activation of rac1 by antigen stimulation (Fig. 1C). The decreased expression of DNMT1 was also dependent on Fc ϵ RI β (Fig. 1D). Taken together, these results indicate that DNMT1 is involved in allergic inflammation.

3.2. The down regulation of DNMT1 leads to the induction of HDAC3, and HDAC3 is necessary for allergic skin inflammation

Because the down regulation of DNMT1 preceded induction of HDAC3, we examined possibility of DNMT1 as a negative regulator of HDAC3. 5-aza-2-deoxycytidine, an inhibitor of DNMT1, decreased expression of DNMT1 and increased the expression of HDAC3 (Fig. 2A). The down regulation of DNMT1 by RNA interference induced expression of HDAC3 while decreasing expression of HDAC2 in RBL2H3 cells (Fig. 2B). ChIP assay showed displacement of DNMT1 from HDAC3 promoter sequences by antigen stimulation, and also showed the direct binding of DNMT1 in the absence of antigen stimulation (Fig. 2C). We examined the role of HDAC3 in allergic skin inflammation. For this, BALB/c mouse model of allergic skin inflammation was employed. In vivo i.v. injection of scrambled siRNA or HDAC3 siRNA was performed before and after DNFB stimulation. The in vivo down regulation of HDAC3 exerted a negative effect on allergic skin inflammation (Fig. 2D) and H&E staining also showed decreased ear thickness by down regulation of HDAC3 (Fig. 2E). Western blot analysis of BALB/c mouse ear tissue lysates showed decreased expression of hall marks of allergic inflammation, such as COX-2, MMP-2, Snail, pERK and PGES, regardless of routes of injection of HDAC3 siRNA (Fig. 2F). Ear skin mast cells isolated from BALB/c mouse at each time point after DNFB stimulation showed down regulation of DNMT1 and induction of tryptase, a marker of mast cell activation, and HDAC3 (Fig. 2G). We examined

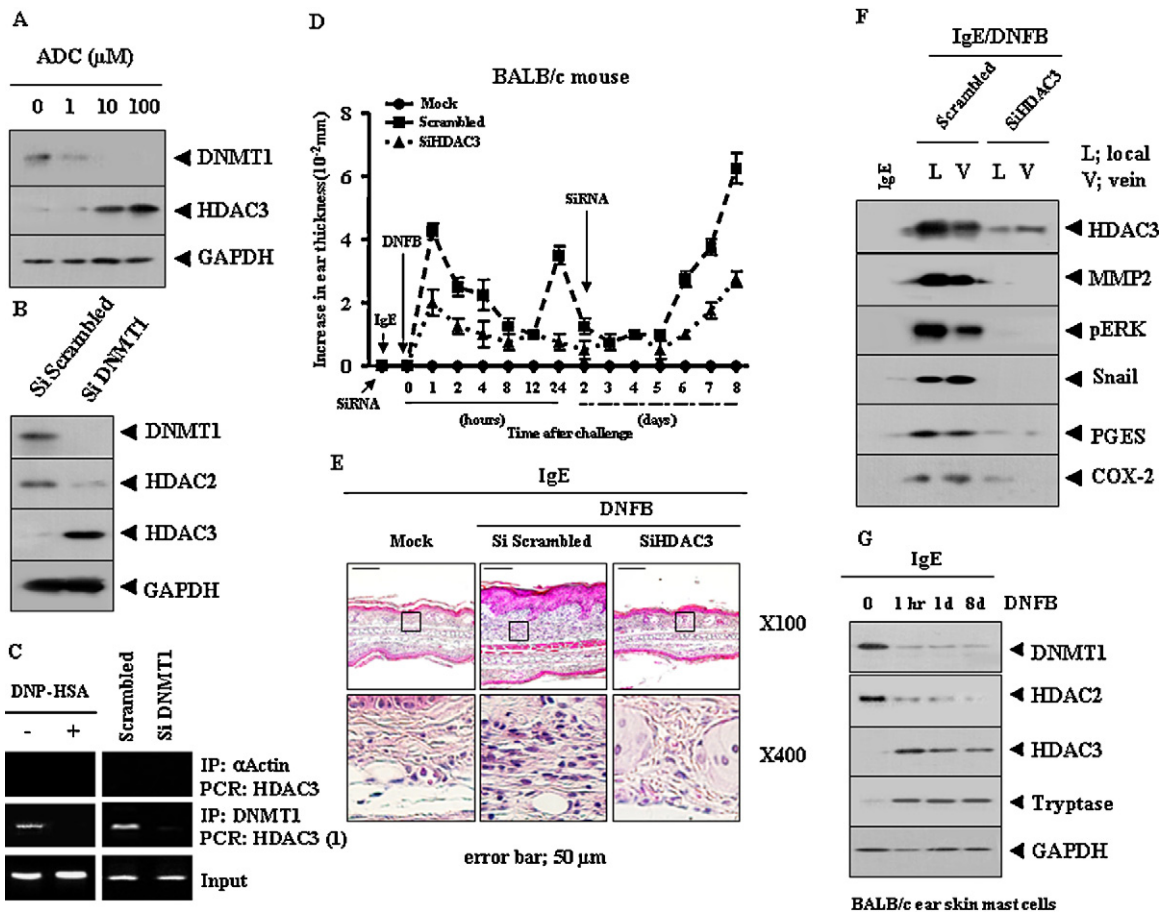


Fig. 2. HDAC3, negatively regulated by DNMT1, is necessary for allergic inflammation. (A) RBL2H3 cells were treated with various concentrations of 5-aza-2-deoxycytidine (ADC) for 24 h. Western blot analysis was performed. (B) RBL2H3 cells were transiently transfected with scrambled siRNA (10 nM) or DNMT1 siRNA (10 nM). At 48 h after transfection, Western blot was performed. (C) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA (100 ng/ml) for 1 h (left panel). RBL2H3 cells were transiently transfected with scrambled siRNA or DNMT1 siRNA. The next day, cells were sensitized with DNP-specific IgE for 24 h, followed by stimulation with DNP-HSA for 1 h (right panel). ChIP assays were performed by using the indicated antibody. Number in parenthesis denotes primer 1 site of HDAC3. (D) BALB/c mice were injected i.v. with DNP-specific IgE antibody (10 μ g) along with siRNA (each at 100 nM). The next day, cutaneous reaction was evoked by painting with 25 μ l of 0.15% DNFB acetone-olive oil (3:1) solution onto each surface of both ear lobes. Ear thickness was measured for eight days. Each value represents average values obtained from five BALB/c mice of experimental group. Means $\pm$ SEM of three independent experiments are shown. (E) Eight days after stimulation with DNFB, ear of each mouse was subjected to H&E staining employing paraffin section. (F) is the same as (D) except that BALB/c mouse was also given i.d. (intradermal) injection of scrambled (100 nM) or HDAC3 siRNA (100 nM) before and after DNFB stimulation. Eight days after stimulation with DNFB, tissue lysates from ear of each BALB/c mouse were subjected to Western blot analysis. Western blot is a representative figure of three independent experiments. (G) BALB/c mice were given intravenous (i.v.) injection of DNP-specific IgE antibody (10 μ g/kg). The next day, both ears of mice were painted with 2,4-dinitrofluorobenzene (DNFB) or DMSO. At each tie point after DNFB stimulation, ear skin mast cells were isolated and cell lysates were prepared. Cell lysates prepared were subjected to Western blot analysis. Western blot is a representative figure of three independent experiments.

mechanism of induction of HDAC3 by down regulation of DNMT1. The down regulation of DNMT1 led to induction of SP1 and p65, a subunit of NF- κ B in RBL2H3 cells (Supplemental Fig. 1A). Antigen stimulation also led to induction of SP1 and p65 (Supplemental Fig. 1B). HDAC3 promoter contains binding site transcription factors, such as AP1, NF- κ B, Snail and GATA-1 (Supplemental Fig. 1C). ChIP assays showed the binding of DNMT1 to SP1/Snail/GATA-1 site of HDAC3 promoter sequences in the absence of antigen stimulation (Supplemental Fig. 1C). Antigen stimulation induced binding of SP1 to Snail/SP1 site of HDAC3 promoter sequences and also induced binding of p65 to AP1/NF- κ B site of HDAC3 promoter sequences (Supplemental Fig. 1C). Taken together, these results indicate that DNMT1 may act as a negative regulator of HDAC3, and HDAC3 is necessary for allergic skin inflammation.

3.3. *Rac1* is necessary for the induction of HDAC3, down regulation of DNMT1

Reactive oxygen species are necessary for allergic inflammation (Kim et al., 2010c). We examined role of reactive oxygen

species in expression regulation of DNMT1. Activation of *rac1* preceded induction of HDAC3 (Fig. 3A). The decreased expression of DNMT1 and HDAC2 occurred at the same time as the activation of *rac1* by antigen stimulation (Fig. 3A). The down regulation of DNMT1 was correlated with DNMT1 modifications, such as phosphorylation, acetylation and tyrosine nitrosification (Fig. 3B). MG132, an inhibitor of proteasomal degradation, prevented antigen from decreasing expression of DNMT1 and HDAC2 in RBL2H3 cells (Fig. 3C). The dominant negative *rac1* construct (*rac1N17*) partially prevented antigen from decreasing expression of DNMT1, HDAC2 and prevented antigen from increasing expression of HDAC3 (Fig. 3D), suggesting that down regulation of DNMT1 may also occur independently of *rac1*. *Rac1N17* prevented antigen from inducing ubiquitination and tyrosine nitrosification of DNMT1 (Fig. 3E). Antigen stimulation decreased expression of DNMT1 and HDAC2 and disrupted interaction between DNMT1 and HDAC2 (Fig. 3F). The reduction of HDAC2 results from post translational modification, such as tyrosine nitrosification (Adenuga et al., 2009). Antigen stimulation induced ubiquitination of HDAC2 (Fig. 3G, left panel). *Rac1N17* also prevented antigen from inducing

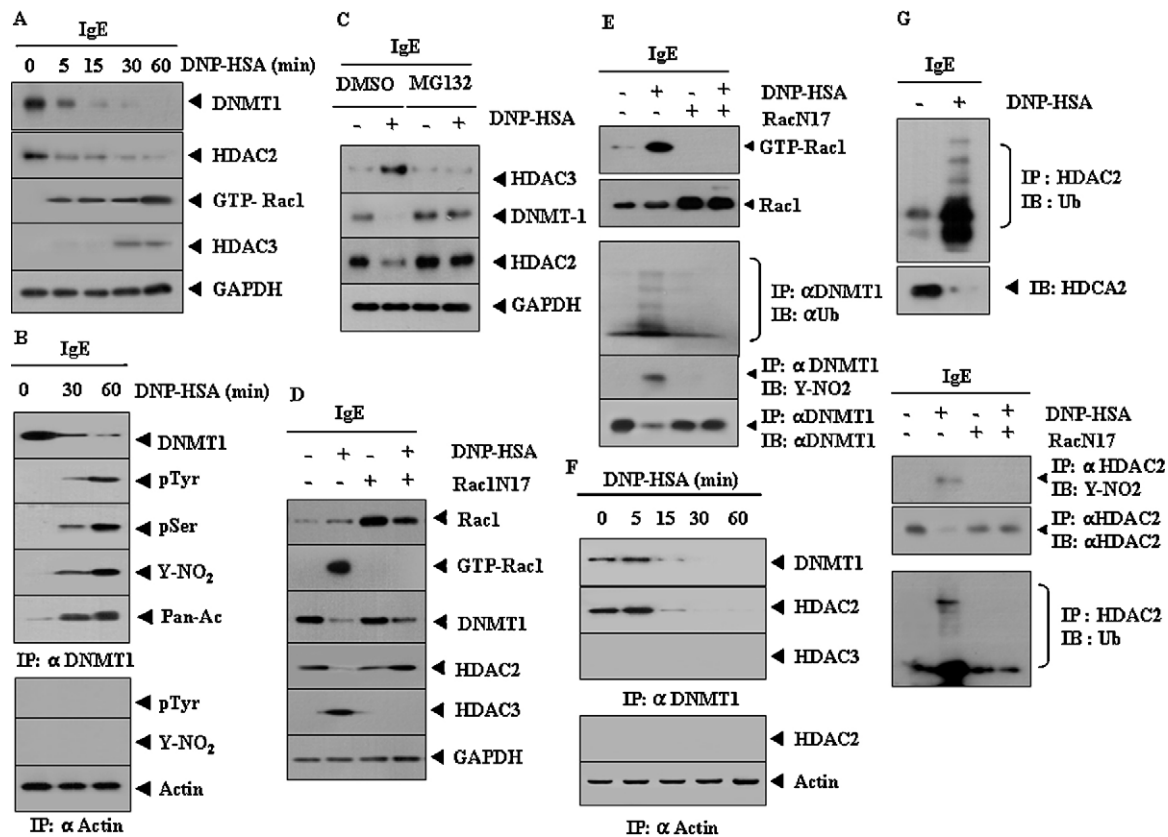


Fig. 3. Rac1 is responsible for proteasomal degradation involving ubiquitination of DNMT1. (A) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for various time intervals. Cell lysates were subjected to Western blot analysis. (B) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for various time intervals. Cell lysates were immunoprecipitated with anti-DNMT1 antibody (2 μ g/ml), followed by Western blot analysis. (C) The IgE-sensitized RBL2H3 cells were pretreated with or without MG132 (1 μ M), an inhibitor of proteasomal degradation, for 1 h, prior to stimulation with DNP-HSA for 1 h. Cell lysates were subjected to Western blot analysis. (D) Control vector (1 μ g) or dominant negative rac1 construct (rac1N17) (1 μ g) was transfected into RBL2H3 cells prior to sensitization with DNP-specific IgE. Cell lysates prepared after stimulation with DNP-HSA for 1 h were subjected to Western blot analysis. (E) is the same as (D) except that cell lysates prepared were immunoprecipitated with anti-DNMT1 antibody (2 μ g/ml), followed by Western blot analysis employing the indicated antibodies. Rac1 activity assay was also performed as described. (F) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for 1 h. Cell lysates were prepared and immunoprecipitated with anti-DNMT1 antibody (2 μ g/ml) or anti-actin antibody (2 μ g/ml), followed by Western blot analysis. (G) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for 1 h. Cell lysates were prepared and immunoprecipitated with anti-DNMT1 antibody (2 μ g/ml), followed by Western blot analysis (left panel). Control vector (1 μ g) or rac1N17 (1 μ g) was transfected into RBL2H3 cells prior to sensitization with DNP-specific IgE. Twenty four hours after, cells were then stimulated with DNP-HSA for 1 h. Cell lysates prepared were immunoprecipitated with anti-HDAC2 antibody (2 μ g/ml), followed by Western blot analysis (right panel).

ubiquitination and tyrosine nitrosification of HDAC2 (Fig. 3G, right panel). These results suggest that Rac1 is at least partially responsible for expression regulation of DNMT1 by inducing tyrosine nitrosification and proteasome-dependent ubiquitination. Next, we examined whether reactive oxygen species would be necessary for in vivo allergic skin inflammation. For this, we employed BALB/c mouse model of allergic skin inflammation. N-acetyl-L-cysteine (NAC), an inhibitor of reactive oxygen species production, exerted a negative effect on increased ear thickness resulting from allergic skin inflammation (Supplemental Fig. 2A). BALB/c mice showed triphasic cutaneous reaction, typical of allergic skin inflammation, in response to DNFB stimulation (Supplemental Fig. 2A). Western blot analysis showed that NAC prevented induction of HDAC3 and tryptase, a marker of mast cell activation, and restored expression of HDAC2 and DNMT1 in ear skin mast cells isolated from DNFB-challenged BALB/c mouse (Supplemental Fig. 2B). NAC prevented increase in rac1 activity in DNFB-challenged BALB/c mouse (Supplemental Fig. 2B). NAC also restored interaction between HDAC2 and DNMT1 in DNFB-challenged BALB/c mouse (Supplemental Fig. 2B). We also employed BALB/c mouse model of passive cutaneous anaphylaxis (PCA) to examine effect of NAC on allergic skin inflammation. NAC exerted a negative effect on increased vascular permeability accompanied by PCA in BALB/c mouse (Supplemental Fig. 3A). NAC exerted negative regulations on expression of HDAC3

and tryptase (Supplemental Fig. 3B). NAC restored expression of DNMT1 and HDAC2 and also restored interaction between DNMT1 and HDAC2 (Supplemental Fig. 3B). NAC exerted a negative effect on rac1 activity in DNP-HSA-challenged BALB/c mouse (Supplemental Fig. 3B).

PKC signaling is necessary for activation of rac1 and is involved in allergic inflammation (Kim et al., 2010c). Inactivation of PKC α or PKC δ prevented antigen from inducing tyrosine nitrosification and phosphorylation of DNMT1 and HDAC2, and also prevented antigen from decreasing expression of DNMT1 and HDAC2 in RBL2H3 cells (Supplemental Fig. 4). These results suggest that expression regulation of DNMT1 involves modification by PKC and rac1 and reactive oxygen species are necessary for allergic skin inflammation.

3.4. Acetylation leads to down regulation of DNMT1

Acetylation drives proteasomal degradation involving ubiquitination (Du et al., 2010). DNMT1 showed acetylation in response to antigen stimulation in RBL2H3 cells (Fig. 3B). We examined the effect of acetylation on expression of DNMT1. Antigen stimulation led to increased expression of Tip60, a histone acetyl transferase in RBL2H3 cells (Fig. 4A). The down regulation of Tip60 decreased expression of HDAC3 while increasing expression of DNMT1 and HDAC2 (Fig. 4B). Over expression of Tip60, but not mutant form

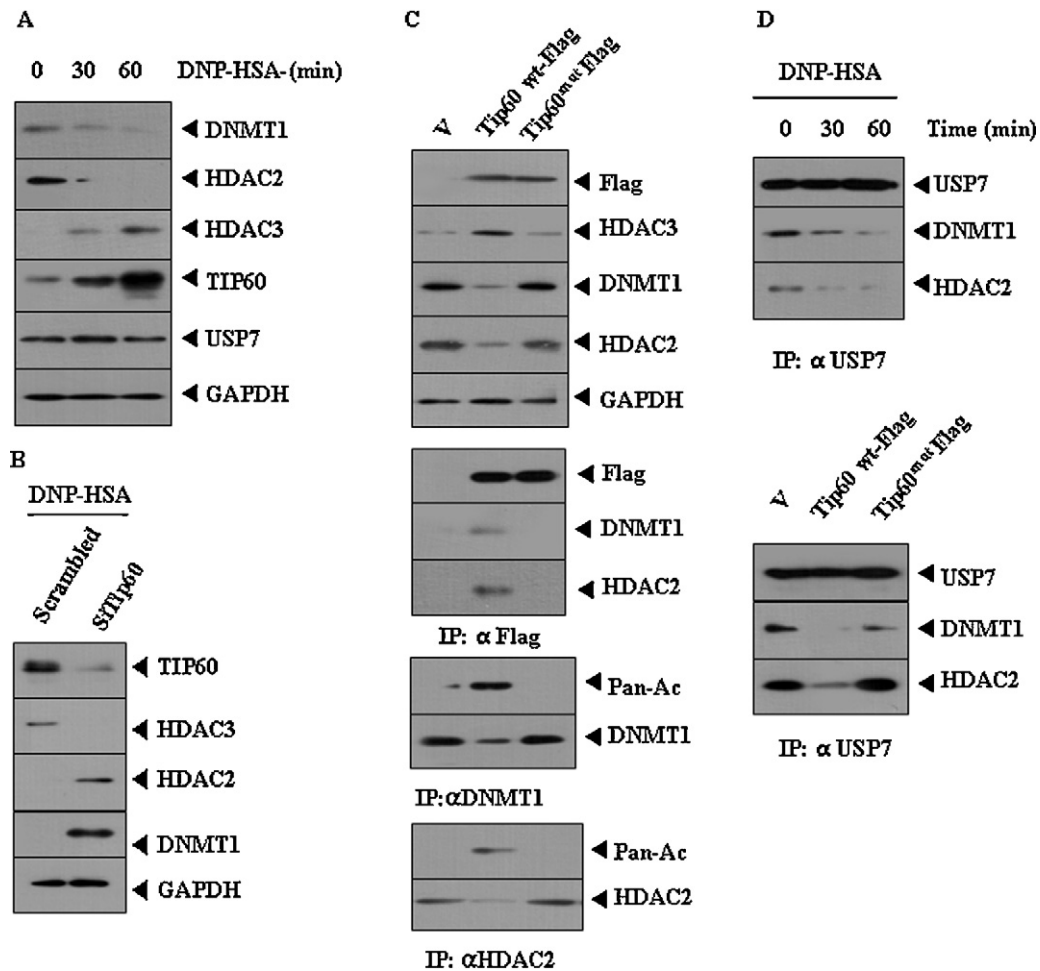


Fig. 4. Acetylation of DNMT1 by Tip60 is responsible for its down regulation of DNMT1. (A) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for various time intervals. Western blot analysis was performed. (B) RBL2H3 cells were transiently transfected with scrambled siRNA or Tip60 siRNA, prior to sensitization with DNP-specific IgE. After sensitization, cells were then stimulated with DNP-HSA for 1 h, followed by Western blot analysis. (C) RBL2H3 cells were transiently transfected with control vector (1 μ g), Tip60 wt-Flag (1 μ g) or Tip60^{mut}Flag (1 μ g). At 48 h after transfection, Western blot analysis was performed (upper panel). The same cell lysates were immunoprecipitated with anti-Flag (2 μ g/ml), anti-DNMT1 antibody (2 μ g/ml) or anti-HDAC2 antibody (2 μ g/ml), followed by Western blot analysis (lower panel). (D) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for various time intervals. Cell lysates were immunoprecipitated with anti-USP7 antibody (2 μ g/ml), followed by Western blot analysis (upper panel). RBL2H3 cells were transiently transfected with the indicated construct (each at 1 μ g). At 48 h after transfection, cell lysates were immunoprecipitated with anti-USP7 antibody (2 μ g/ml), followed by Western blot analysis (lower panel).

of Tip60 (Q377E, G380E), decreased expression of DNMT1 and HDAC2 while increasing expression of HDAC3 (Fig. 4C), suggesting that acetylation of DNMT1 leads to down regulation of DNMT1 and thereby increases expression of HDAC3. Immunoprecipitation showed interaction between Tip60 and DNMT1 and also showed interaction Tip60 and HDAC2 (Fig. 4C), suggesting that Tip60 may directly induce acetylation of DNMT1 and HDAC2. Immunoprecipitation also showed acetylation of DNMT1 and HDAC2 by wild type Tip60 (Fig. 4C). Antigen stimulation disrupted interaction between USP7, a deubiquitinating enzyme, and DNMT1 and also disrupted interaction between USP7 and HDAC2 (Fig. 4D, upper panel), suggesting that USP7 may stabilize DNMT1 and HDAC2 in the absence of antigen stimulation. Over expression of wild type Tip60, but not mutant Tip60, prevented interaction between USP7 and DNMT1 and interaction between USP7 and HDAC2 (Fig. 4D, lower panel). This suggests that Tip60 induces destabilization of DNMT1 and HDAC2 to result in degradation of DNMT1 and HDAC2. These results indicate that Tip60 is responsible for degradation of DNMT1 and HDAC2 via acetylation-driven proteasome-dependent ubiquitination. It would be also interesting to examine effect of other histone acetyl transferases on expression of DNMT1.

3.5. *In vivo* allergic inflammation involves decreased expression of DNMT1

Because antigen stimulation led to the decreased expression of DNMT1, we examined whether DNMT1 would be involved in *in vivo* allergic inflammation. DNFB stimulation after i.v. injection of DNP-specific IgE led to typical triphasic pattern of ear swelling (Fig. 5A). H&E staining of paraffin section showed increased ear thickness and leukocytes infiltration by DNFB stimulation (Fig. 5B). Western blot analysis of ear tissue of BALB/c mouse showed decreased expression of DNMT1 and also showed increased expression of HDAC3 and Tip60 (Fig. 5C, upper panel). Immunoprecipitation showed increased acetylation of DNMT1 and showed decreased interaction between DNMT1 and USP7 by DNFB stimulation (Fig. 5C, lower panel). Immunoprecipitation also showed increased interaction between DNMT1 and Tip60 (Fig. 5C, lower panel). Immunohistochemistry staining of paraffin section showed that DNFB stimulation decreased expression of DNMT1 and HDAC2 while increasing expression of Tip60 and HDAC3 (Fig. 5D). These results indicate that the down regulation of DNMT1 is correlated with the development of *in vivo* allergic skin inflammation.

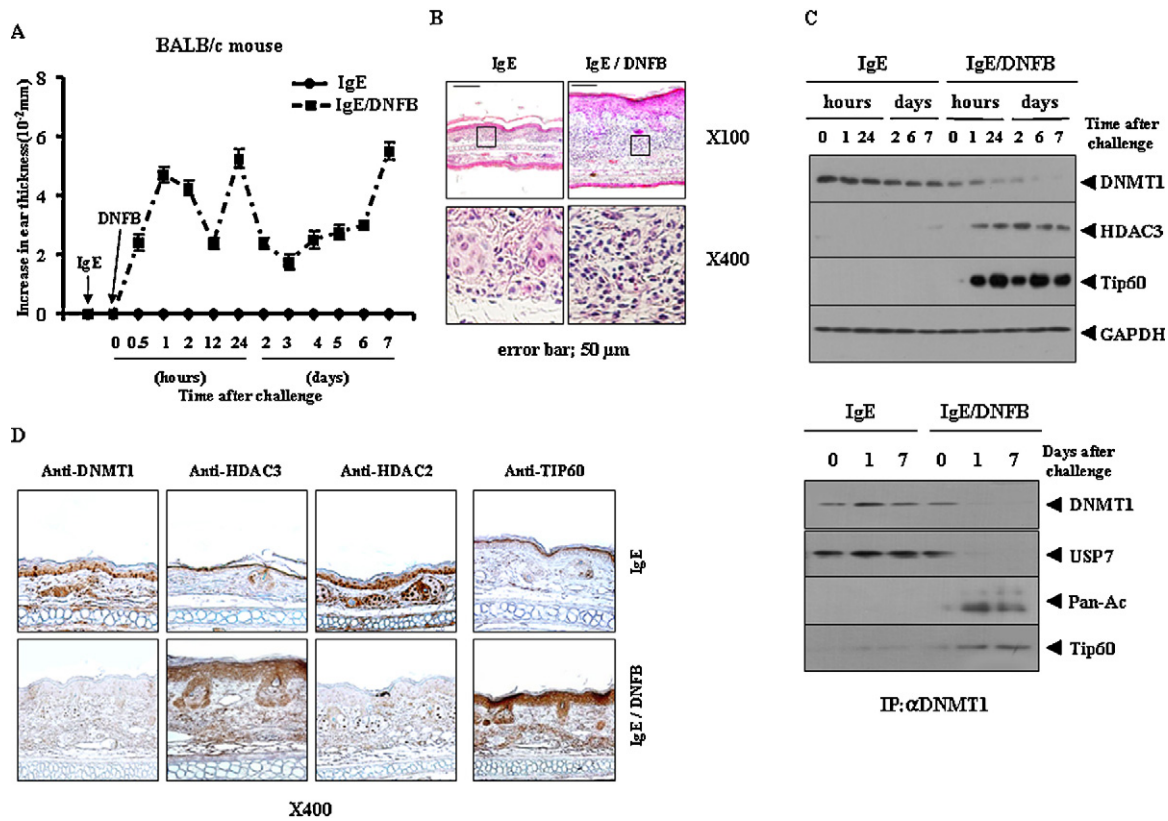


Fig. 5. In vivo allergic inflammation involves decreased expression of DNMT1. (A) BALB/c BALB/c mice were injected i.v. with DNP-specific IgE antibody (10 μ g). The next day, ears of mice were painted with 2, 4-dinitrofluorobenzene (DNFB). Ear thickness was measured for seven days as described. Each value represents average values obtained from five BALB/c mice of experimental group. Means $\pm$ SEM of three independent experiments are shown. (B) Paraffin sections (4–6 μ m thickness) of the ear prepared at seven days after DNFB-challenge were also subjected to H&E staining. Representative images from four animals for each experimental group are shown. DNFB challenge increases ear thickness and infiltration of leukocytes. (C) At each time point after DNFB stimulation, tissue lysates from ear of BALB/c mouse were subjected to Western blot analysis (upper panel). The same tissue lysates were immunoprecipitated with anti-DNMT1 antibody (2 μ g/ml), followed by Western blot analysis (lower panel). (D) The IgE-sensitized BALB/c mice were challenged with 0.15% DNFB in olive oil/acetone as described. The sensitized BALB/c mice challenged with olive oil/acetone without DNFB were used as a control. Paraffin sections (4–6 μ m thickness) of the ears of BALB/c mice prepared at seven days after DNFB-challenge were stained with the indicated antibodies. Representative images from four animals for each experimental group are shown (magnification, 400 $\times$; Olympus).

3.6. DNMT1 acts as negative regulator of allergic inflammation

Because the down regulation of DNMT1 was correlated with the development of allergic skin inflammation, we examined the possibility of DNMT1 as a negative regulator of allergic inflammation. In vivo i.v. injection of DNMT1 siRNA increased ear thickness in BALB/c mice (Fig. 6A). H&E staining showed that in vivo down regulation of DNMT1 increased ear thickness and leukocytes infiltration (Fig. 6B). Immunohistochemistry staining of paraffin sections of BALB/c mouse ear tissues showed decreased expression of DNMT1 and HDAC2 while expression of HDAC3 was increased by down regulation of DNMT1 (Fig. 6C). Western blot analysis of BALB/c mouse ear tissue lysates showed that the in vivo down regulation of DNMT1 induced expression of HDAC3, and also increased expression of adhesion molecules, such as integrin α 5, ICAM-1 and VCAM-1 (Fig. 6D), suggesting that down regulation of DNMT1 may affect vascular permeability and cellular interaction in relation with allergic inflammation. We next examined tissues that reflect DNMT1 activity. For this, we employed BALB/c mouse model of PCA (passive cutaneous anaphylaxis). Antigen (DNP-HSA) stimulation enhanced β -hexosaminidase activity (Supplemental Fig. 5A) and histamine release activity (Supplemental Fig. 5B) in ear, lung and spleen, but not in liver tissue. Injection of DNMT1 siRNA (i.v.) enhanced β -hexosaminidase activity (Supplemental Fig. 5A) and histamine release activity (Supplemental Fig. 5B) in ear, lung and spleen, but not in liver tissue. We found increased expression of HDAC3 and decreased expression of DNMT1 and

HDAC2 in ear, lung and spleen, but not in liver tissue, by antigen stimulation (Supplemental Fig. 5C) or by in vivo DNMT1 siRNA injection (Supplemental Fig. 5D). The down regulation of DNMT1 enhanced β -hexosaminidase activity and histamine release activity in RBL2H3 cells (Supplemental Fig. 5E). These results suggest that the down regulation of DNMT1 may activate mast cells in ear, lung and spleen. Activation of mast cells in these tissues may enhance vascular permeability and therefore cellular interaction. Taken together, these results indicate involvement of DNMT1 in allergic skin inflammation and further suggest role for DNMT1 in allergic inflammation-induced cellular interaction and angiogenesis.

3.7. Down regulation of DNMT1 induces vascular leakage and angiogenesis

The down regulation of DNMT1 led to increased expression of various pro-inflammatory cytokines, such as TNF- α , IL-13 and IL-18 (data not shown). Expression of IL-13, a Th2 cytokine, is subjected to epigenetic regulation (Lim et al., 2011). TNF- α induces expression of adhesion molecules and vascular permeability (Lee et al., 2012). Because the down regulation of DNMT1 induced expression of various adhesion molecules (Fig. 6D), we hypothesized that the down regulation of DNMT1 would induce vascular leakage. To examine this possibility, DNMT1 siRNA (100 nM) or scrambled siRNA (100 nM) was injected into ears of BALB/c mouse. The next day, Evans blue solution was injected into tail vein of BALB/c mouse. The

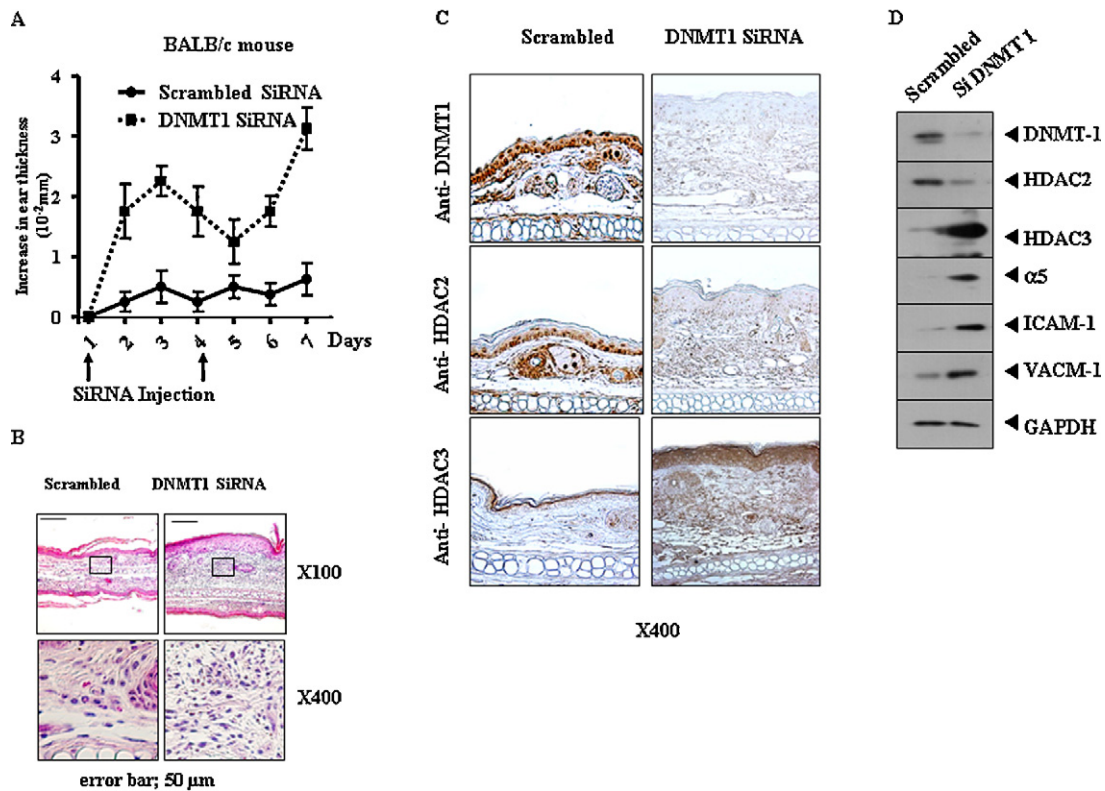


Fig. 6. The down regulation of DNMT1 increases ear thickness and expression of cellular adhesion molecules. (A) BALB/c mice were given i.v. injection of scrambled siRNA (100 nM) or DNMT1 siRNA (100 nM) mouse twice a week. Ear thickness was measured by using digital gauge. Each value represents average value obtained from five BALB/c mice. Means $\pm$ SEM of three independent experiments are shown. (B) Paraffin sections prepared from BALB/c mice that were given i.v. injection of siRNA were subjected to H&E staining. The down regulation of DNMT1 increases ear thickness and leukocytes infiltration. (C) Seven days after injection of siRNA, ear tissue of BALB/c mice were subjected to immunohistochemistry staining employing the indicated antibodies. Paraffin sections were prepared for immunohistochemistry staining (magnification, 400 $\times$; Olympus). (D) Seven days after injection of siRNA, tissue lysates prepared from ears of BALB/c mice were subjected to Western blot analysis.

down regulation of DNMT1 enhanced vascular leakage (Fig. 7A). Next, we examined whether allergic inflammation involved angiogenesis. Whole mount staining of BALB/c mouse ear tissue showed increased expression of PECAM-1, an angiogenic marker protein, by DNP-HSA stimulation (Fig. 7B). The whole mount staining showed that the down regulation of DNMT1 promoted angiogenesis in the ears of BALB/c mouse, as evidenced by increased expression of PECAM-1 (Fig. 7C, left panel). Western blot of BALB/c mouse ear tissue lysates showed induction of angiogenic marker proteins, such as PECAM-1 and ICAM-1, by down regulation of DNMT1 (Fig. 7C, right panel). Taken together, these results suggest that the down regulation of DNMT1 may promote angiogenesis.

3.8. Down regulation of DNMT1 induces changes in endothelial cell signaling

Because the down regulation of DNMT1 led to enhanced vascular leakage (Fig. 7A) and angiogenesis in BLAB/c mouse (Fig. 7B), we examined the effect of DNMT1 on endothelial cell signaling. Conditioned medium of RBL2H3 cells with the down regulation of DNMT1 induced endothelial cell (RAEC) tube formation (Fig. 8A) and rat aortic ring formation (Fig. 8B). AVASTIN, a VEGF-neutralizing antibody, prevented effects of down regulation of DNMT1 on endothelial cell tube formation (Fig. 8A) and aortic ring formation (Fig. 8B). We next examined whether the down regulation of DNMT1 would affect expression of angiogenic proteins, such as VEGF. The down regulation of DNMT1 in RBL2H3 cells induced expression of VEGF (Fig. 8C), suggesting that induction of VEGF resulting from down regulation of DNMT1 in activated basophils may activate endothelial cells. Conditioned medium of RBL2H3 cells with the down

regulation of DNMT1 increased expression of PECAM-1, ICAM-1 and VCAM-1, enhanced interaction between ICAM-1 and LFA-1 (integrin $\alpha_1\beta_2$), interaction between VCAM-1 and integrin α_4 , induced phosphorylation of VEGFR 2 and interaction between VEGFR2 and syk (Fig. 8D, left panel) in RAEC. This suggests that the down regulation of DNMT1 in RBL2H3 cells may induce signaling changes in RAEC. Conditioned medium of RBL2H3 cells with the down regulation of DNMT1 increased expression of VEGF in RAEC as evidenced by Western blot and RT-PCR (Fig. 8D, right panel). This indicates that the down regulation of DNMT1 leads to activation of VEGFR signaling by inducing expression of VEGF in RAEC. The down regulation of DNMT1 did not affect expression of VEGFR1 or VEGFR2 (Fig. 8D, right panel). We performed cellular adhesion assays to examine potential interaction between leukocytes and endothelial cells. Antigen stimulation of RBL2H3 cells or BMMCs led to enhanced interaction between RBL2H3 cells and RAEC also interaction between BMMC and RAEC (Supplemental Fig. 6). Taken together, these results suggest that the down regulation of DNMT1 leads to the enhanced cellular interaction and angiogenesis by regulating expression of inflammatory cytokines. It would be interesting to identify these inflammatory molecules.

3.9. Aspirin regulates expression of DNMT1 to exerts a negative effect on allergic inflammation

Aspirin inhibits angiogenesis by suppressing VEGF expression in human colon cancer (Ouyang et al., 2008). Aspirin inhibits expression of MCP-1 expression in TNF-alpha stimulated human umbilical vein endothelial cells (Yang et al., 2004). Aspirin inhibits

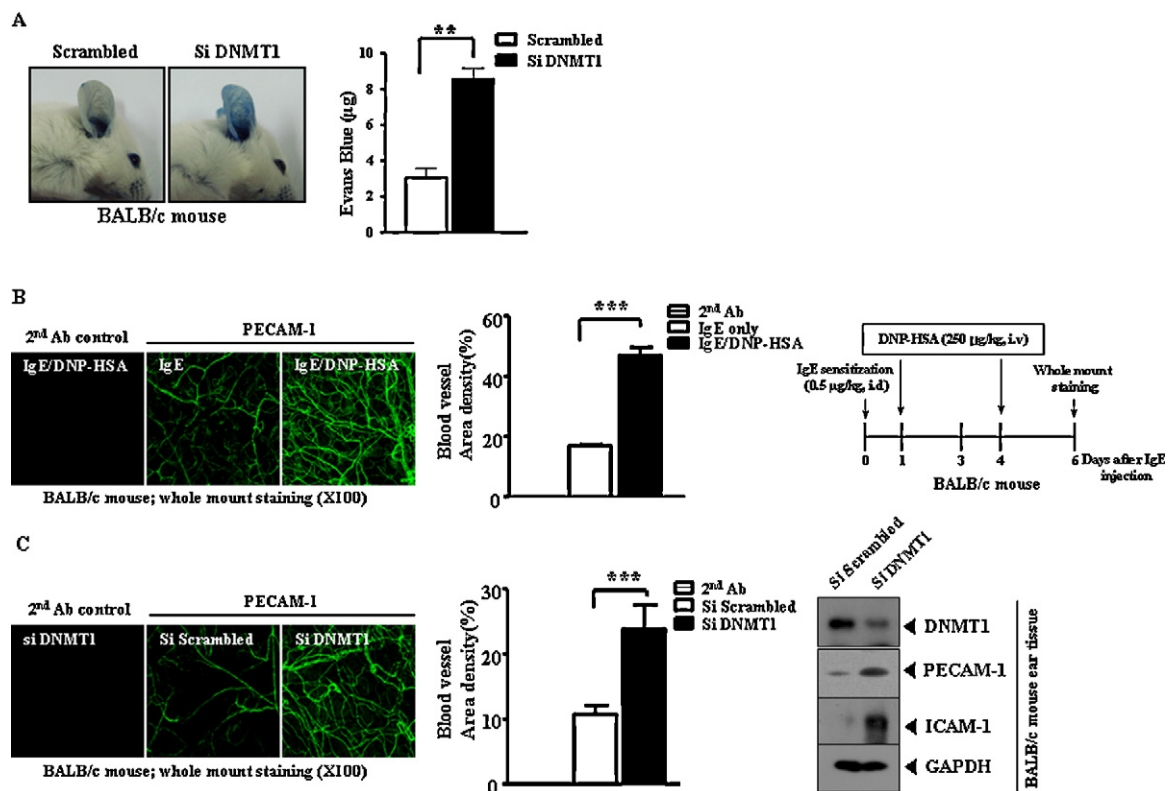


Fig. 7. The down regulation of DNMT1 induces vascular leakage and promotes angiogenesis in BALB/c mouse. (A) BALB/c mice were given i.v. injection of scrambled siRNA (100 nM) or DNMT1 siRNA (100 nM). The next day, 2% (v/v) Evans Blue solution was injected via tail vein to visualize extent of vascular leakage. The absorbance was measured at 620 nm. Representative images from five animals for each experimental group are shown. Each value represents average value obtained from five BALB/c mice. Means $\pm$ SEM of three independent experiments are shown. $^{**}p < 0.005$. (B) BALB/c mice were given i.d. injection of DNP-specific IgE (0.5 μ g). The next day, mice were given i.v. injection of DNP-HSA (250 μ g) or PBS. BALB/c mice were given i.v. injection of DNP-HSA twice in a total of six days. Representative images from four animals for each experimental group are shown. Whole mount staining using anti-PECAM-1 was performed (magnification, 100 $\times$; Olympus). Three independent experiments were performed. $^{***}p < 0.0005$. (C) Scrambled siRNA (100 nM) or DNMT1 siRNA (100 nM) was injected into vein of each BALB/c mouse twice in a total of five days. Five days after injection of siRNA, ears of BALB/c mice were excised and subjected to whole mount staining (left panel). Representative images from five animals for each experimental group are shown (magnification, 100 $\times$; Olympus). Three independent experiments were performed. $^{***}p < 0.05$. Lysates from ear of BALB/c mouse were subjected to Western blot analysis (right panel). Western blot is a representative figure from three independent experiments.

TNF α expression in murine tissue macrophages (Shackelford et al., 1997). Aspirin exerts anti-inflammatory effects through inhibition of Ikappa B-alpha expression (Ricciotti et al., 2010). We found the down regulation of DNMT1 led to induction of TNF α and MCP-1 (data not shown). We hypothesized that aspirin would regulate expression of DNMT1 to exert anti-inflammatory effects. Aspirin prevented antigen from increasing expression of HDAC3 and prevented antigen from decreasing expression of DNMT1 (Fig. 9A). Aspirin prevented antigen from increasing expression of hall marks of allergic inflammation, such as monocyte chemoattractant protein 1 (MCP1) and COX-2 (Fig. 9A). The down regulation of HDAC3 led to decreased expression of COX-2 in antigen-stimulated RBL2H3 cells (data not shown), suggesting that aspirin may target DNMT1 and HDAC3 to regulate expression of COX-2. Aspirin exerted a negative effect on increased β -hexosaminidase activity in antigen-stimulated RBL2H3 cells (Fig. 9B) and exerted a negative effect on allergic skin inflammation by DNFB stimulation (Fig. 9C). Aspirin also exerted a negative effect on leukocytes infiltration by DNFB stimulation (Fig. 9D). Immunohistochemistry staining of paraffin sections of BALB/c mice ears showed that aspirin prevented DNFB from regulating expression of Tip60, HDAC3 and DNMT1 (Fig. 9E). We examined whether effect of aspirin on expression of DNMT1 was specific. For this, we examined effect of washout of aspirin on expression of DNMT1. Aspirin restored expression of DNMT1 and

decreased expression of HDAC3 and Tip60 in antigen-stimulated RBL2H3 cells (Supplemental Fig. 7A). Aspirin did not regulate expression of DNMT1 in the absence of antigen stimulation (Supplemental Fig. 7A), suggesting that aspirin may exert indirect regulation on expression of DNMT1. At each time point (1, 2, 4, 8, 12 and 24 h) after washing out of aspirin, DNP-HSA was added to IgE-sensitized RBL2H3 cells. From four hours after washing out of aspirin, DNP-HSA decreased expression of DNMT1 and increased expression of HDAC3 and Tip60 (Supplemental Fig. 7A), suggesting that a negative effect of aspirin on allergic inflammation may be related with its specific regulation on DNMT1, HDAC3 and Tip60. Over expression of mutant Tip60, but not wild type Tip60, prevented antigen from decreasing expression of DNMT1, suggesting that Tip60 may regulate expression of DNMT1 (Supplemental Fig. 7B). Aspirin exerted a negative effect on expression of exogenous Tip60 thereby to restore expression of DNMT1 in antigen-stimulated RBL2H3 cells (Supplemental Fig. 7B). This suggests that aspirin affects Tip60 to regulate expression of DNMT1. It would be necessary to examine possibility of proteasome-dependent degradation of Tip60 by aspirin. It would be also interesting to examine whether aspirin could influence transcriptional regulators to regulate expression of Tip60. Taken together, these results show that aspirin exerts a negative effect on allergic inflammation by regulating expression of DNMT1 via Tip60.

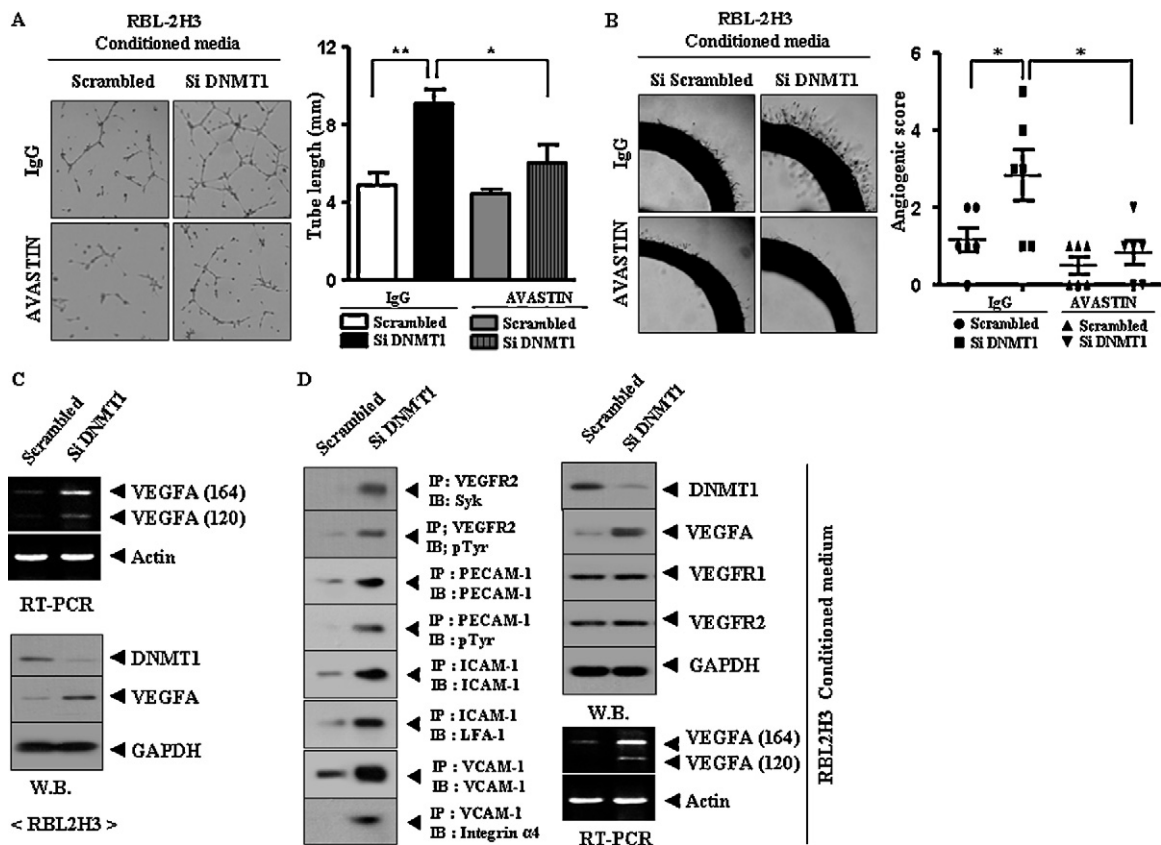


Fig. 8. The down regulation of DNMT1 induces changes in endothelial cell signaling (A) RBL2H3 cells were transiently transfected with scrambled siRNA or DNMT1 siRNA. At 48 h after transfection, conditioned medium was prepared and IgG (4 μ g/ml) or AVASTIN (4 μ g/ml) was added to conditioned medium and incubation continued for 1 h. Conditioned medium was then added to rat aortic endothelial cells (RAEC). After 6 to 8 h of incubation at 37 °C, the endothelial cells were photographed using an inverted microscope for observation of tube formation (magnification, $\times 100$; Olympus). Means $\pm$ SEM of three independent experiments are shown. ** $p < 0.005$; * $p < 0.05$. Three independent experiments were performed. (B) Ninety six -well plates were coated with 30 μ l of matrigel per well and polymerized in an incubator. Aortas isolated from 6-wk-old male Sprague-Dawley rats were cleared of periaortic fat and connective tissues in cold PBS and cut into rings of 1 to 1.5 mm in circumference. The aortic rings were randomized into wells and sealed with a 30 μ l overlay of matrigel. Conditioned medium of antigen-stimulated RBL2H3 cells transiently transfected with control siRNA (10 nM) or DNMT1 siRNA (10 nM) was added. After 6 days, the extent of micro vessel sprouting was determined by using an inverted microscope (magnification, $\times 100$; Olympus). * $p < 0.05$. Three independent experiments were performed. The assay was scored from 0 (least positive) to 5 (most positive) in a double-blind manner. Each data point was assayed six times. (C) RBL2H3 cells were transiently transfected with the indicated siRNA. At 48 h after transfection, total RNAs prepared were subjected to RT-PCR (upper panel) or cell lysates prepared were subjected to Western blot analysis (lower panel). (D) Conditioned medium (1 ml) from RBL2H3 cells transiently transfected with the indicated siRNA was added to RAEC. Four hours later, cell lysates were prepared from RAEC and immunoprecipitated with the indicated antibody (2 μ g/ml each), followed by Western blot analysis (left panel). The same cell lysates were subjected to Western blot analysis (right panel) or total RNAs were prepared from RAEC and subjected to RT-PCR (right panel).

4. Discussion

Although DNMT1 regulates expression of Th2 cytokines (Makar and Wilson, 2004), the role of DNMT1 in allergic inflammation has not been studied. Because allergic inflammation involves increased expression of Th2 cytokines, we wanted to investigate role of DNMT1 in allergic inflammation and mechanisms in relation with DNMT1. 5-aza-2-deoxycytidine, an inhibitor of DNMT1, decreases expression of DNMT1 while increasing expression of HDAC3 (Fig. 2A). 5-aza-2-deoxycytidine (ADC) degrades DNMT1 via proteasomal degradation (Patel et al., 2010; Ghoshal et al., 2005), suggesting that the down regulation of DNMT1 maybe associated with proteasomal degradation of DNMT1. ChIP assay shows the binding of DNMT1 to HDAC3 promoter sequences in the absence of antigen stimulation (Fig. 2C), suggesting that DNMT1 indeed acts as a negative regulator of HDAC3. ADC increases expression of some genes without changes in methylation (Ohm et al., 2007; Mohammad and Baylin, 2010). The displacement of DNMT1 from HDAC3 promoter sequences (Fig. 2C) may result from degradation of DNMT1. DNMT1 acts as a transcriptional repressor without its catalytic activity (Rountree et al., 2000). DNMT1 represses transcription from E2F-responsive promoters (Robertson et al., 2000).

Because ADC causes DNMT1 degradation, it is reasonable that transcriptional repressive activity of DNMT1 may not be associated with its catalytic activity.

DNMT1 phosphorylation by Akt and/or PKC leads to disruption of DNMT1-PCNA-UHRF1 interaction to promote tumorigenesis (Hervouet et al., 2010). PKC α and PKC δ are responsible for phosphorylation of DNMT1 (Supplemental Fig. 4). Phosphorylation status of DNMT1 determines its stability (Estève et al., 2011). Antigen stimulation leads to various post translational modification of DNMT1, such as phosphorylation, acetylation and tyrosine nitrosylation (Fig. 3B). The inhibition of proteasomal degradation by MG132 prevents antigen from decreasing expression of DNMT1 while MG132 prevents antigen from increasing expression of HDAC3 (Fig. 3C). This suggests that expression regulation of DNMT1 and HDAC3 by antigen stimulation involves ubiquitination-dependent proteasomal degradation.

Reactive oxygen species play critical role in allergic inflammation and PKC α and PKC δ are responsible for activation of Rac1 (Kim et al., 2010c). Because antigen leads to tyrosine nitrosylation of DNMT1 (Fig. 3B), we hypothesized that rac1 might be responsible for proteasomal degradation of DNMT1. Rac1 is responsible for ubiquitination of DNMT1 (Fig. 3E). Tyrosine nitrosylation of

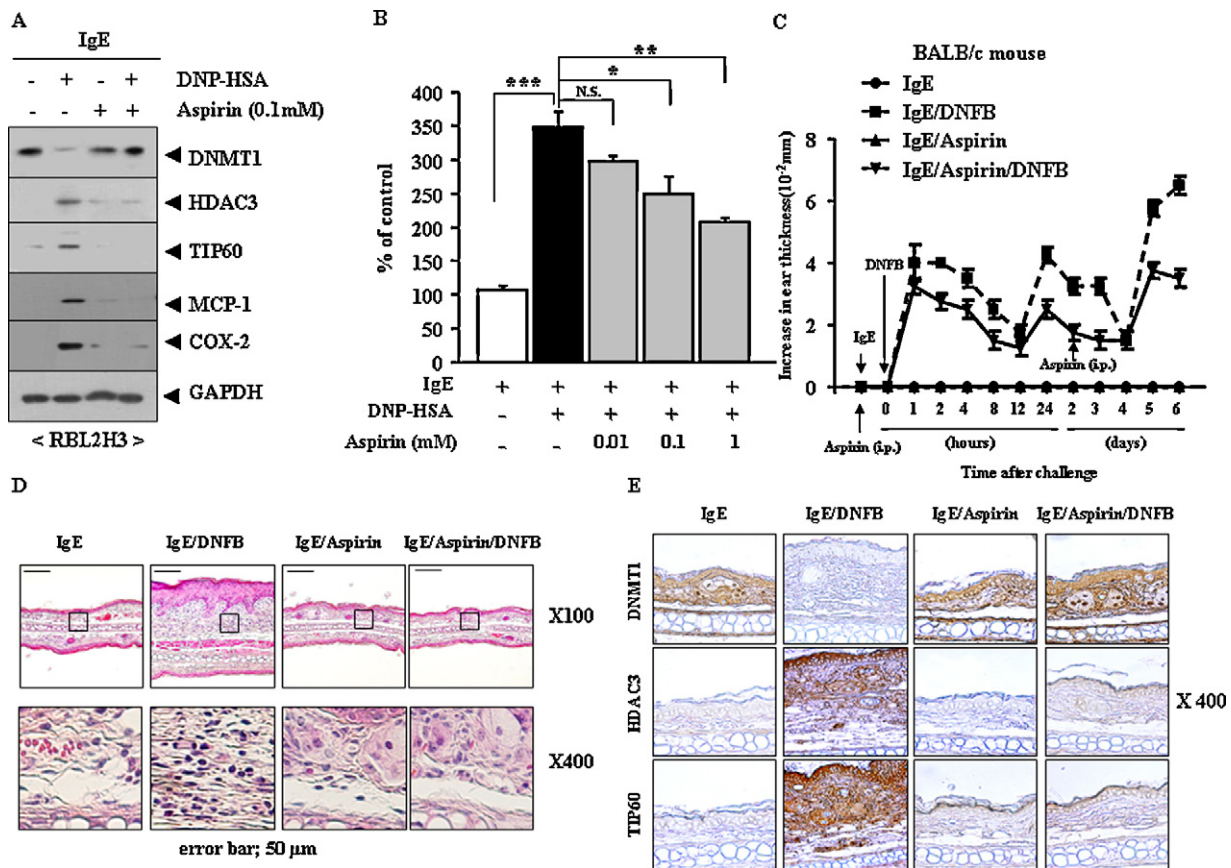


Fig. 9. Aspirin regulates expression of DNMT1 and exerts a negative effect on allergic inflammation. (A) The IgE-sensitized RBL2H3 cells were pretreated with or without aspirin (0.1 mM) for 30 min, followed by antigen stimulation for 1 h. Cell lysates were subjected to Western blot analysis. (B) The IgE-sensitized RBL2H3 cells were pretreated with various concentrations of aspirin for 30 min, followed by antigen stimulation for 1 h. Cells were then subjected to β -hexosaminidase activity assays. Each value represents average of three independent experiments. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. (C) BALB/c mice were injected i.v. with DNP-specific IgE antibody (10 μ g) while BALB/c mice were given i.p. injection with aspirin (20 mg/kg). The next day, cutaneous reaction was evoked by painting with 25 μ l of 0.15% DNFB acetone-olive oil (3:1) solution onto each surface of both ear lobes. BALB/c mice were given i.p. injection of aspirin twice in a total of seven days. Ear thickness was measured for eight days as described. Each value represents average of five mice in each group. Means $\pm$ SEM of three independent experiments are shown. (D) Six days after DNFB stimulation, paraffin sections of BALB/c mice were prepared and subjected to H&E staining (magnification, 400 $\times$; Olympus). (E) Paraffin sections (4–6 μ m thickness) of the ears of BALB/c mice prepared at six days after DNFB stimulation were stained with antibodies against DNMT1, HDAC3, HDAC2 or Tip60. Representative images from four animals for each experimental group are shown (magnification, 400 $\times$; Olympus).

DNMT1 (Fig. 3E), dependent on rac1, may lead to proteasomal degradation. It would be interesting to examine effect of rac1 on binding of DNMT1 to HDAC3 promoter sequences.

The inhibition of HDAC promotes proteasomal degradation of DNMT1 (Zhou et al., 2008). DNMT1 shows interaction with HDAC2 in the absence of antigen stimulation in RBL2H3 cells (Fig. 3F). DNMT1 binds to HDAC2 to form a complex at replication foci (Rountree et al., 2000). DNMT1 associates with histone deacetylase activity (Fuks et al., 2000). Therefore it is reasonable that HDAC2-DNMT1 complex may exert a negative regulation on HDAC3 expression. DNMT3B interacts with HDAC2 (Geiman et al., 2004). In this study, we found that the down regulation of HDAC3 led to the induction of HDAC2 (personal observation), suggesting that HDAC3 and HDAC2 may cross regulate each other. This also suggests that HDAC3 and DNMT1 may cross regulate each other. It would be interesting to examine binding of HDAC3 to HDAC2 promoter sequences.

Tip60 is responsible for DNA damage-dependent acetylation and ubiquitination of H2AX (Ikura et al., 2007). DNMT1 stability is regulated by proteins controlling acetylation-driven ubiquitination (Du et al., 2010). Antigen stimulation induces expression of Tip60 (Fig. 4A), and wild type Tip60, but not mutant Tip60, induces acetylation of DNMT1 (Fig. 4C). This suggests that Tip60 is responsible for acetylation-driven ubiquitination-dependent proteasomal

degradation of DNMT1. USP7, a deubiquitinating enzyme, promotes DNA-binding activity of p53 (Sarkari et al., 2010) and stabilize p53 (Qin et al., 2011). The above reports led us to hypothesize that USP may stabilize DNMT1. Antigen stimulation in RBL2H3 cells disrupts interaction between USP7 and DNMT1 (Fig. 4D, upper panel). Tip60 disrupt interaction between USP7 and DNMT1 (Fig. 4D, lower panel), suggesting that Tip60 may be responsible for destabilization of DNMT1.

In vivo allergic inflammation involves decreased expression of DNMT1 in various tissues, such as ear, lung and spleen, but not in liver tissue (Supplemental Fig. 5C). In vivo allergic inflammation involves interaction between Fc ϵ RI β and Lyn in ear, lung and spleen, but not in liver tissue (personal observation). In vivo down regulation of DNMT1 enhances β -hexosaminase activity (Supplemental Fig. 5A) and histamine release in these tissues (Supplemental Fig. 5B). It is reasonable that the down regulation of DNMT1 may activate mast cells in these tissues. In vivo down regulation of DNMT1 induces interaction between Fc ϵ RI β and Lyn in ear, lung and spleen (personal observation), suggesting that the down regulation of DNMT1 may be necessary for activation of mast cells in these tissues. This suggests that the down regulation of DNMT1 is necessary for initiation and maintenance of allergic inflammation.

Angiogenesis is necessary for allergic inflammation (Detoraki et al., 2009; Choi et al., 2009). Integrin α 5 interacts with EGFR and

is necessary for allergic inflammation in relation with angiogenesis (Kim et al., 2011). Mast cell tryptase and chymase are angiogenic (Ribatti et al., 2011). VEGF secretion during hypoxia requires mast cells (García-Román et al., 2010). Mast cells play essential role for angiogenesis in cervical carcinogenesis (Utrera-Barillas et al., 2010). The inhibition of HDACs reduces monocytes adhesion to endothelium through suppression of VCAM-1 expression (Inoue et al., 2006). Based on the above reports, we hypothesized that allergic inflammation involving expression changes in DNMT1 and HDAC(s), as seen in this study, would be associated with cellular interaction and angiogenesis. In vivo down regulation of DNMT1 leads to increased expression of integrin $\alpha 5$, ICAM-1 and VCAM-1 (Fig. 6D), indicating that the down regulation of DNMT1 may induce cellular interaction. Allergic inflammation involving vascular leakage results from cellular interaction (Oschatz et al., 2011). In vivo down regulation of DNMT1 induces vascular leakage in BALB/c mouse (Fig. 7A), suggesting that allergic inflammation involves activation of vascular endothelium and cellular interaction. DNA methyl transferase inhibition induces differentiation of embryonic stem cells into endothelial cells (Banerjee and Bacanamwo, 2010). We hypothesized that DNMT1 might act as a negative regulator of angiogenesis in relation with allergic inflammation. In vivo down regulation of DNMT1 enhances blood vessel formation in BALB/c mouse (Fig. 7C). It would be necessary to examine effect of down regulation of DNMT1 on expression of various angiogenic proteins.

Angiogenesis involves interaction between leukocytes and endothelial cells (Kim et al., 2011). Conditioned medium from RBL2H3 cells with down regulation of DNMT1 enhances tube formation in RAEC (Fig. 8A) and induces rat aortic ring formation (Fig. 8B). DNMT1 is involved in the regulation of VEGF gene expression (Achour et al., 2008). In fact, the down regulation of DNMT1 induces expression of VEGF in RBL2H3 cells (Fig. 8C). Conditioned medium from RBL2H3 cells with down regulation of DNMT1 induces activation of VEGFR-2 and also interaction between VEGFR-2 and syk (Fig. 8D). Syk is necessary for activation of VEGFR-2 signaling in HUVEC (Kazerounian et al., 2011). Antigen stimulation enhances adhesion between activated basophils and endothelial cells and also enhances adhesion between activated mast cells and endothelial cells (Supplemental Fig. 6). These results indicate that DNMT1 exerts regulation on angiogenesis by inducing expression of VEGF. It would be necessary to examine effect of VEGF on adhesion between activated mast cells and endothelial cells. The down regulation of DNMT1 induces expression of pro-inflammatory cytokines, such as TNF- α , IL-13 and IL-18 (personal observation). It is reasonable that angiogenesis induced by down regulation of DNMT1 is mediated in part by increased inflammation. Mechanism of induction of these pro-inflammatory cytokines by down regulation of DNMT1 remains unknown. It would be also necessary to examine effect of these pro-inflammatory cytokines on cellular interaction and angiogenesis by blocking these cytokines. Because HDAC3 is induced by DNMT1, it would be interesting to examine role of HDAC3 in angiogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2012.06.010>.

References

- Achour, M., Jacq, X., Rondé, P., Alhosin, M., Charlot, C., Chataigneau, T., Jeanblanc, M., Macaluso, M., Giordano, A., Hughes, A.D., Schini-Kerth, V.B., Bronner, C., 2008. The interaction of the SRA domain of ICBP90 with a novel domain of DNMT1 is involved in the regulation of VEGF gene expression. *Oncogene* 27, 2187–2197.
- Adcock, I.M., Ford, P., Barnes, P.J., Ito, K., 2006. Epigenetics and airways disease. *Respiratory Research* 7, 21.
- Adenuga, D., Yao, H., March, T.H., Seagrave, J., Rahman, I., 2009. Histone deacetylase 2 is phosphorylated, ubiquitinated, and degraded by cigarette smoke. *American Journal of Respiratory Cell and Molecular Biology* 40, 464–473.
- Bacolla, A., Pradhan, S., Roberts, R.J., Wells, R.D., 1999. Recombinant human DNA (cytosine-5) methyltransferase. II. Steady-state kinetics reveal allosteric activation by methylated dna. *Journal of Biological Chemistry* 274, 33011–33019.
- Bai, S., Ghoshal, K., Datta, J., Majumder, S., Yoon, S.O., Jacob, S.T., 2005. DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2. *Molecular and Cellular Biology* 25, 751–766.
- Banerjee, S., Bacanamwo, M., 2010. DNA methyltransferase inhibition induces mouse embryonic stem cell differentiation into endothelial cells. *Experimental Cell Research* 316, 172–180.
- Barnes, P.J., Adcock, I.M., 2009. Glucocorticoid resistance in inflammatory diseases. *Lancet* 342, 1905–1917.
- Bestor, T.H., 1988. Cloning of a mammalian DNA methyltransferase. *Gene* 74, 9–12.
- Choi, G.S., Park, H.J., Hur, G.Y., Choi, S.J., Shin, S.Y., Ye, Y.M., Park, H.S., 2009. Vascular endothelial growth factor in allergen-induced nasal inflammation. *Clinical and Experimental Allergy* 39, 655–661.
- Choi, J.H., Oh, S.W., Kang, M.S., Kwon, H.J., Oh, G.T., Kim, D.Y., 2005. Trichostatin A attenuates airway inflammation in mouse asthma model. *Clinical and Experimental Allergy* 35, 89–96.
- Detoraki, A., Staiano, R.I., Granata, F., Giannattasio, G., Prevete, N., de Paulis, A., Ribatti, D., Genovese, A., Triggiani, M., Marone, G., 2009. Vascular endothelial growth factors synthesized by human lung mast cells exert angiogenic effects. *Journal of Allergy and Clinical Immunology* 123, 1142–1149.
- Du, Z., Song, J., Wang, Y., Zhao, Y., Guda, K., Yang, S., Kao, H.Y., Xu, Y., Willis, J., Markowitz, S.D., Sedwick, D., Ewing, R.M., Wang, Z., 2010. DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci Signaling* 3, ra80.
- Estève, P.O., Chang, Y., Samaranayake, M., Upadhyay, A.K., Horton, J.R., Feehery, G.R., Cheng, X., Pradhan, S., 2011. A methylation and phosphorylation switch between an adjacent lysine and serine determines human DNMT1 stability. *Nature Structural and Molecular Biology* 18, 42–48.
- Fuks, F., Burgers, W.A., Brehm, A., Hughes-Davies, L., Kouzarides, T., 2000. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature Genetics* 24, 88–91.
- García-Román, J., Ibarra-Sánchez, A., Lamas, M., González Espinosa, C., 2010. VEGF secretion during hypoxia depends on free radicals-induced Fyn kinase activity in mast cells. *Biochemical and Biophysical Research Communications* 401, 262–267.
- Geiman, T.M., Sankpal, U.T., Robertson, A.K., Zhao, Y., Zhao, Y., Robertson, K.D., 2004. DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. *Biochemical and Biophysical Research Communications* 318, 544–555.
- Ghoshal, K., Datta, J., Majumder, S., Bai, S., Kutay, H., Motiwala, T., Jacob, S.T., 2005. 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Molecular and Cellular Biology* 25, 4727–4741.
- Grausenburger, R., Bilic, I., Boucheron, N., Zupkovic, G., El-Housseiny, L., Tschis-marov, R., Zhang, Y., Rembold, M., Gaisberger, M., Hartl, A., Epstein, M.M., Matthias, P., Seiser, C., Ellmeier, W., 2010. Conditional deletion of histone deacetylase 1 in T cells leads to enhanced airway inflammation and increased Th2 cytokine production. *Journal of Immunology* 185, 3489–3497.
- Hervouet, E., Lallier, L., Debien, E., Cheray, M., Geairon, A., Rogniaux, H., Lous-souart, D., Martin, S.A., Vallette, F.M., Cartron, P.F., 2010. Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells. *PLoS One* 5, e11333.
- Ikura, T., Tashiro, S., Kakino, A., Shima, H., Jacob, N., Amunugama, R., Yoder, K., Izumi, S., Kuraoka, I., Tanaka, K., Kimura, H., Ikura, M., Nishikubo, S., Ito, T., Muto, A., Miyagawa, K., Takeda, S., Finkel, R., Igarashi, K., Kamiya, K., 2007. DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. *Molecular and Cellular Biology* 27, 7028–7040.
- Inoue, K., Kobayashi, M., Yano, K., Miura, M., Izumi, A., Mataka, C., Doi, T., Hamakubo, T., Reid, P.C., Hume, D.A., Yoshida, M., Aird, W.C., Kodama, T., Minami, T., 2006. Histone deacetylase inhibitor reduces monocyte adhesion to endothelium through the suppression of vascular cell adhesion molecule-1 expression. *Arteriosclerosis, Thrombosis and Vascular Biology* 26, 2652–2659.

- Jair, K.W., Bachman, K.E., Suzuki, H., Ting, A.H., Rhee, I., Yen, R.W., Baylin, S.B., Schuebel, K.E., 2006. De novo CpG island methylation in human cancer cells. *Cancer Research* 66, 682–692.
- Kazerounian, S., Duquette, M., Reyes, M.A., Lawler, J.T., Song, K., Perruzzi, C., Primo, L., Khosravi-Far, R., Bussolino, F., Rabinovitz, I., Lawler, J., 2011. Priming of the vascular endothelial growth factor signaling pathway by thrombospondin-1, CD36, and spleen tyrosine kinase. *Blood* 117, 4658–4666.
- Kim, D.Y., Jeoung, D., Ro, J.Y., 2010a. Signaling pathways in the activation of mast cells cocultured with astrocytes and colocalization of both cells in experimental allergic encephalomyelitis. *Journal of Immunology* 185, 273–283.
- Kim, G.Y., Lee, J.W., Ryu, H.C., Wei, J.D., Seong, C.M., Kim, J.H., 2010b. Proinflammatory cytokine IL-1 β stimulates IL-8 synthesis in mast cells via a leukotriene B $_4$ receptor 2-linked pathway, contributing to angiogenesis. *Journal of Immunology* 184, 3946–3954.
- Kim, Y., Eom, S., Kim, K., Lee, Y.S., Choe, J., Hahn, J.H., Lee, H., Kim, Y.M., Ha, K.S., Ro, J.Y., Jeoung, D., 2010c. Transglutaminase II interacts with rac1, regulates production of reactive oxygen species, expression of snail, secretion of Th2 cytokines and mediates *in vitro* and *in vivo* allergic inflammation. *Molecular Immunology* 47, 1010–1022.
- Kim, Y., Kim, K., Park, D., Eom, S., Park, H., Lee, H., Lee, Y.S., Choe, J., Hahn, J.H., Kim, Y.M., Jeoung, D., 2011. Integrin α (5) interacts with EGFR, is necessary for Fc ϵ RI signaling and is necessary for allergic inflammation in relation with angiogenesis. *Molecular Immunology* 48, 1035–1045.
- Lee, J.E., Lee, A.S., Kim, D.H., Jung, Y.J., Lee, S., Park, B.H., Lee, S.H., Park, S.K., Kim, W., Kang, K.P., 2012. Janex-1, a JAK3 inhibitor, ameliorates tumor necrosis factor α -induced expression of cell adhesion molecules and improves myocardial vascular permeability in endotoxemic mice. *International Journal of Molecular Medicine* 29, 864–870.
- Li, L.B., Leung, D.Y., Martin, R.J., Goleva, E., 2010. Inhibition of histone deacetylase 2 expression by elevated glucocorticoid receptor beta in steroid-resistant asthma. *American Journal of Respiratory and Critical Care Medicine* 182, 877–883.
- Lim, E.J., Lu, T.X., Blanchard, C., Rothenberg, M.E., 2011. Epigenetic Regulation of the IL-13-induced Human Eotaxin-3 Gene by CREB-binding Protein-mediated Histone 3 Acetylation. *Journal of Biological Chemistry* 286, 13193–13204.
- Ma, A.N., Lu, J., Zhou, X.J., Wang, Y.X., 2011. Histone deacetylation directs DNA methylation in survivin gene silencing. *Biochemical and Biophysical Research Communications* 404, 268–272.
- Makar, K.W., Wilson, C.B., 2004. DNA methylation is a nonredundant repressor of the Th2 effector program. *Journal of Immunology* 173, 4402–4406.
- Meja, K.K., Rajendrasozhan, S., Adenuga, D., Biswas, S.K., Sundar, I.K., Spooner, G., Marwick, J.A., Chakravarty, P., Fletcher, D., Whittaker, P., Meqson, I.L., Kirkham, P.A., Rahman, I., 2008. Curcumin restores corticosteroid function in monocytes exposed to oxidants by maintaining HDAC2. *American Journal of Respiratory Cell and Molecular Biology* 39, 312–323.
- Mohammad, H.P., Baylin, S.B., 2010. Linking cell signaling and the epigenetic machinery. *Nature Biotechnology* 28, 1033–1038.
- Nagai, H., Ueda, Y., Tanaka, H., Hirano, Y., Nakamura, N., Inagaki, N., Takatsu, K., Kawada, K., 1999. Effect of overproduction of interleukin 5 on dinitrofluorobenzene-induced allergic cutaneous response in mice. *Journal of Pharmacology and Experimental Therapeutics* 288, 43–50.
- Nandakumar, V., Vaid, M., Katiyar, S.K., 2011. (–)-Epigallocatechin-3-gallate reactivates silenced tumor suppressor genes, Cip1/p21 and p16INK4a, by reducing DNA methylation and increasing histones acetylation in human skin cancer cells. *Carcinogenesis* 32, 537–544.
- Nico, B., Mangieri, D., Crivellato, E., Vacca, A., Ribatti, D., 2008. Mast cells contribute to vasculogenic mimicry in multiple myeloma. *Stem Cells Development* 17, 19–22.
- Ohm, J.E., McGarvey, K.M., Yu, X., Cheng, L., Schuebel, K.E., Cope, L., Mohammad, H.P., Chen, W., Daniel, V.C., Yu, W., Berman, D.M., Jenuwein, T., Pruitt, K., Sharkis, S.J., Watkins, D.N., Herman, J.G., Baylin, S.B., 2007. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nature Genetics* 39, 237–242.
- Oschatz, C., Maas, C., Lecher, B., Jansen, T., Björkqvist, J., Tradler, T., Sedlmeier, R., Burfeind, P., Cichon, S., Hammerschmidt, S., Müller-Esterl, W., Wülfle, W.A., Nilsson, G., Renné, T., 2011. Mast cells increase vascular permeability by heparin-initiated bradykinin formation *in vivo*. *Immunity* 34, 258–268.
- Ouyang, N., Williams, J.L., Rigas, B., 2008. NO-donating aspirin inhibits angiogenesis by suppressing VEGF expression in HT-29 human colon cancer mouse xenografts. *Carcinogenesis* 29, 1794–1798.
- Pankaj, B., Tehreem, A., Ian, M.A., 2008. The role of histone deacetylases in asthma and allergic diseases. *Journal of Allergy and Clinical Immunology* 121, 580–584.
- Patel, K., Dickson, J., Din, S., Macleod, K., Jodrell, D., Ramsahoye, B., 2010. Targeting of 5-aza-2'-deoxycytidine residues by chromatin-associated DNMT1 induces proteasomal degradation of the free enzyme. *Nucleic Acids Research* 38, 4313–4324.
- Qin, W., Leonhardt, H., Spada, F., 2011. Usp7 and Uhrf1 control ubiquitination and stability of the maintenance DNA methyltransferase Dnmt1. *Journal of Cellular Biochemistry* 112, 439–444.
- Ribatti, D., Ranieri, G., Nico, B., Benagiano, V., Crivellato, E., 2011. Trypsin and chymase are angiogenic *in vivo* in the chorioallantoic membrane assay. *International Journal of Developmental Biology* 55, 99–102.
- Ricciotti, E., Dovizio, M., Di Francesco, L., Anzellotti, P., Salvatore, T., Di Francesco, A., Sciuili, M.G., Pistrutto, G., Monopoli, A., Patrignani, P., 2010. NCX 4040, a nitric oxide-donating aspirin, exerts anti-inflammatory effects through inhibition of I kappa B-alpha degradation in human monocytes. *Journal of Immunology* 184, 2140–2147.
- Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., Wolffe, A.P., 2000. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nature Genetics* 25, 338–342.
- Rountree, M.R., Bachman, K.E., Baylin, S.B., 2000. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nature Genetics* 25, 269–277.
- Sahin, M., Sahin, E., Gümüslü, S., Erdoğan, A., Gültekin, M., 2011. Inhibition of angiogenesis by S-adenosylmethionine. *Biochemical and Biophysical Research Communications* 408, 145–148.
- Sarkari, F., Sheng, Y., Frappier, L., 2010. USP7/HAUSP promotes the sequence-specific DNA binding activity of p53. *PLoS One* 5, e13040.
- Shackelford, R.E., Alford, P.B., Xue, Y., Thai, S.F., Adams, D.O., Pizzo, S., 1997. Aspirin inhibits tumor necrosis factor- α gene expression in murine tissue macrophages. *Molecular Pharmacology* 52, 421–429.
- Utrera-Barillas, D., Castro-Manrreza, M., Castellanos, E., Gutiérrez-Rodríguez, M., Arciniega-Ruiz de Esparza, O., García-Cebada, J., Velázquez, J.R., Flores-Reséndiz, D., Hernández-Hernández, D., Benítez-Bribiesca, L., 2010. The role of macrophages and mast cells in lymphangiogenesis and angiogenesis in cervical carcinogenesis. *Experimental and Molecular Pathology* 89, 190–196.
- Yamaguchi, K., Lantowski, A., Dannenberg, A.J., Subbaramaiah, K., 2005. Histone deacetylase inhibitors suppress the induction of c-Jun and its target genes including COX-2. *Journal of Biological Chemistry* 280, 32569–32577.
- Yang, Y.Y., Hu, C.J., Chang, S.M., Tai, T.Y., Leu, S.J., 2004. Aspirin inhibits monocyte chemoattractant protein-1 and interleukin-8 expression in TNF- α stimulated human umbilical vein endothelial cells. *Atherosclerosis* 174, 207–213.
- Yoder, J.A., Soman, N.S., Verdine, G.L., Bestor, T.H., 1997. DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *Journal of Molecular Biology* 270, 385–395.
- You, J.S., Kang, J.K., Lee, E.K., Lee, J.C., Lee, S.H., Jeon, Y.J., Koh, D.H., Ahn, S.H., Seo, D.W., Lee, H.Y., Cho, E.J., Han, J.W., 2008. Histone deacetylase inhibitor apicidin downregulates DNA methyltransferase 1 expression and induces repressive histone modifications via recruitment of corepressor complex to promoter region in human cervix cancer cells. *Oncogene* 27, 1376–1386.
- Zhou, Q., Agoston, A.T., Atadja, P., Nelson, W.G., Davidson, N.E., 2008. Inhibition of histone deacetylases promotes ubiquitin-dependent proteasomal degradation of DNA methyltransferase 1 in human breast cancer cells. *Molecular Cancer Research* 6, 873–883.