

The effects of DNA methyl transferases on antiaging *klotho* gene expression

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Abstract: Several intracellular metabolic pathways in which multiple genes have a role affect the aging process. One important genetic factor associated with aging is *klotho* gene. In some recently published studies, *klotho* gene has also been associated with several types of cancer and identified as a tumor suppressor gene. Therefore, elucidation of the control of *klotho* gene expression has become more important. Results suggesting that the promoter region of human *klotho* gene could be epigenetically controlled by DNA methylation have been reported. In contrast, a study revealing whether a change in the expression level of DNMT enzymes in the cells affects *klotho* gene expression has not been found. Herein, the effects of DNMT3A and DNMT3B enzymes on the expression of *klotho* gene, an important genetic factor relating to the aging process and some human cancer types, were investigated. For this purpose, the expression levels of DNMT3A and DNMT3B in HEK293 cells were artificially changed, and the effect on *klotho* gene promoter activity was investigated using a reporter gene. The results of this research showed that DNMT enzymes have negative regulatory effects on *klotho* gene promoter organized as a chromatin structure, and they have an enhancing effect on promoter activity when it is located on plasmid DNA. These results elucidate the control mechanism of human *klotho* gene expression.

Key words: Aging, DNA methylation, DNMT-3A, DNMT-3B, *klotho*

1. Introduction

The aging process is determined by both environmental and genetic factors. Genes in particular play the biggest role in the aging and lifespan of organisms. *Klotho* gene is an important genetic factor related to aging. This gene, identified during mutagenesis studies in mice, plays a critical role in lifespan and the development of age-related diseases in mammals (Kuro-o et al., 1997). Loss of *klotho* can result in multiple aging-like phenotypes resembling human aging such as arteriosclerosis, osteoporosis, skin atrophy, pulmonary emphysema, and infertility (Kuro-o et al., 1997; Nabeshima, 2002), while the overexpression of *Klotho* extends lifespan by 20%–30% (Kurosu et al., 2005).

In humans, the *klotho* gene localized on chromosome 13 is composed of 5 exons and encodes a type-I single pass transmembrane protein (Matsumura et al., 1998). The *klotho* protein is defined as a multifunctional protein involved in several metabolic pathways including calcium and phosphate homeostasis (Imura et al., 2007; Alexander et al., 2009; Razzaque, 2009), the insulin/IGF-1 signaling cascade (Bartke, 2006), apoptosis (Ikushima et al., 2006), angiotensin-II-induced events in the kidney (Negri, 2005), and oxidative stress (Yamamoto et al., 2005). Recently, *klotho* gene has been associated with the development of several types of cancer (Lu et al., 2008; Usuda et al.,

2011a, 2011b; Wang et al., 2011). In this regard, *klotho* has been defined as a tumor suppressor gene (Xie et al., 2013; Tang et al., 2015). On the other hand, the clinical and histological severity of chronic kidney disease is related to the degree of *klotho* promoter methylation (Chen et al., 2013). It has, therefore, become more important to clarify the control mechanism of *klotho* gene. Consequently, investigations into the regulation of *klotho* gene expression with epigenetic factors have accelerated in recent years (Jin et al., 2015; Luo et al., 2015).

The promoter of the gene and upstream control regions are the most important cis-acting factors regulating gene expression. The upstream region of human *klotho* gene promoter has rich CG islands. The CG islands are candidates for methylation with DNA methyl transferase (DNMT) enzymes. DNA methylation is one of the most important epigenetic factors controlling gene expression in eukaryotic organisms. In mammals, it has a vital role in many events such as cell differentiation and growth control, protection of chromosomal integrity, parental imprinting, inactivation of X chromosome, and regulation of gene expression (Chow and Brown, 2003; Ting et al., 2004). There are some reports considering the role of intracellular DNMT enzymes on *klotho* gene regulation (Lee et al., 2010). However, there are several

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unknown points regarding control of *klotho* gene by DNA methylation. In this study, the effect of DNMT enzymes on *klotho* gene promoter activity was investigated to assemble clues about the epigenetic control of *klotho* gene. For this purpose, the enzyme expression of endogenous DNMT enzymes was decreased with gene-specific siRNA or increased with transient transfection of DNMT-expressing plasmid vectors in human embryonic cells (HEK293). The activity of human *klotho* gene promoter in these cells was detected with previously constructed reporter plasmid vectors encoding luciferase enzyme under the control of human *klotho* gene regulatory elements (Turan and Ata, 2011). The results showed that DNMT enzymes upregulate the activity of *klotho* promoter located on plasmid DNA, but downregulate when it is organized as a chromatin structure in stably transfected cells.

2. Materials and methods

2.1. Cell lines

Human embryonic kidney (HEK293) and mouse fibroblastic Swiss3T3 were used for transient transfection experiments. HEK293 cells were maintained at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM), high glucose (Gibco, USA), supplemented with 10% (v/v) fetal calf serum (Gibco, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Gibco, USA). Swiss3T3 cells were maintained under the same conditions as HEK293 cells with the exception of DMEM (low glucose) (Gibco, USA).

2.2. Transient transfections

Plasmid DNAs were transiently transfected into HEK293 and Swiss3T3 cells by using 293fectin (Invitrogen, #948289) and 25 kDa branched polyethylenimine (PEI; Sigma-Aldrich, #408727), respectively. The cells were seeded in 12-well or 24-well plates containing complete DMEM and incubated for 20–24 h under standard culture conditions. After incubation, cells were transfected with 1–2 µg of plasmid DNA. In the transfection experiment with PEI, plasmid DNAs were diluted in OPTI-MEM (Gibco, USA) at 20 ng/µL concentration and mixed with an equal volume of PEI solution prepared in OPTI-MEM at 60 ng/µL concentration. DNA transfection with 293fectin was carried out according to the manufacturer's instructions. Forty-eight hours posttransfection, the cells were harvested for Western blot analysis or luciferase enzyme assays or inspected under a fluorescent microscope for gene expression and localization.

2.3. Construction of plasmid vectors

pCAGGS (Niwa et al., 1991) and pCHA plasmids kindly provided by Dr Nagata (Nagata et al., 1998) were used to construct expression vectors. pRL-CMV (Promega, USA) control vector was used for normalization. All

plasmids were amplified in *Escherichia coli* DH5α grown in LB (+ Amp) liquid media and isolated with a Plasmid Midi kit (Qiagen, Germany), according to the manufacturer's instructions. In order to construct pCHA-DNMTs encoding HA-tagged DNMTs, cDNAs of human DNMT3A v.1, DNMT3B v.3, and DNMT3B v.7 were cloned into pCHA plasmid. Human DNMT cDNAs were isolated from HEK293 cells with reverse-transcription PCR. Total RNA was extracted from HEK293 cell by using an RNA extraction kit (Roche, #11828665001, Germany). Then cDNA was prepared with reverse transcription by using oligo-dT primers, according to the manufacturer's instructions. The gene coding regions were amplified from this cDNA with PCR by using specific primers 5'-AAGAATTCAAATGCCCGCCATGCCCTCCAG-3' and 5'-AAGAATTCTTACACACACGCAAATAATC TCCTTCAGCG-3' for DNMT3A v.1 and 5'-AAGAATTCAAATGAAGGG AGACACCAGGC ATC-3' and 5'-AAGAATTTCCTATTC ACATGCAAAGTAGTCCTTCAG for DNMT3B v.3 and DNMT3B v.7. The PCR-amplified DNMT fragments were digested with EcoRI restriction enzyme (New England Biolabs, #R0101S), treated with Klenow enzyme (New England Biolabs, #M0210L), and purified from agarose gel. pCHA plasmid vector was digested with MluI (New England Biolabs, #R0198S), blunted with Klenow, and dephosphorylated with SAP (Fermentas, #EF0511). The linearized vector was ligated with purified DNMT fragments by using a ligation kit (TaKaRa, #6022). The resulting expression vectors were designated pCHA-DNMT3A-v.1, pCHA-DNMT3B-v.3, and pCHA-DNMT3B-v.7. The plasmids encoding the wild-type DNMT enzymes were constructed by subcloning the full-length DNMT3A and DNMT3B fragments into the eukaryotic expression vector, pCAGGS. Full-length DNMT fragments were generated from pCHA-DNMT plasmid vectors by digestion with EcoRI. The resultant DNA fragments were purified with agarose gel and ligated with pCAGGS linearized by EcoRI digestion. The plasmids encoding native DNMT enzymes were designated pCAGGS-DNMT3A-v.1, pCAGGS-DNMT3B-v.3, and pCAGGS-DNMT3B-v.7. The nucleotide sequence of each plasmid was confirmed by DNA sequencing. Reporter plasmid vectors pHKP-Luc, pHKP \square II-Luc, and pHKP \square VII-Luc, encoding luciferase under the control of human *klotho* promoters, were generated as previously reported (Turan and Ata, 2011).

2.4. Western blotting

The expression of DNMT genes cloned in pCHA and pCAGGS plasmid vectors was detected with Western blotting. HEK293 cells were seeded in 12-well plates (1 × 10⁵ cells/well), incubated under standard culture conditions for 20–24 h, and transfected with 2 µg of related expression vectors. After 48 h incubation, the cells were harvested in

200 µL of SDS sample buffer. The proteins were separated by electrophoresis through a 10% polyacrylamide gel in the presence of 0.1% SDS and transferred to a nitrocellulose membrane. First, the membranes were exposed to the specific primary antibodies diluted at a 1:500 ratio in the blocking solution (5% skim milk in Tris-buffered saline): monoclonal mouse anti-HA (Millipore, #05-904) for HA-tagged DNMTs, polyclonal rabbit anti-DNMT3A (Santa Cruz, #H-295/20703) for native DNMT3A, and monoclonal mouse anti-DNMT3B (Santa Cruz, #G-9/376043) for DNMT3B-v.3 and DNMT3B-v.7. Then they were exposed to the biotinylated secondary antibodies (goat anti-mouse IgG; Millipore, #AP124B) or goat anti-rabbit IgG (Thermo Scientific, #31823) diluted at 1:3500 in Tris-buffered saline. The proteins were visualized with streptavidin-conjugated alkaline phosphatase (Millipore, #ES007) and its substrate BCIP/NBT (Millipore, #NMM1581860).

2.5. Immunofluorescence assay

Swiss3T3 cells were grown on a glass coverslip in a 12-well plate (2.5×10^4 cells/well) incubated at standard culture conditions for 20–24 h. After 20–24 h incubation the cells grown in each well were transfected with 1 µg of HA-tagged DNMT or native-DNMT-expressing plasmids by using PEI. At 48 h posttransfection, cells were fixed with 3% paraformaldehyde solution, permeabilized with 0.1% NP-40, washed twice with PBS, and then treated with 1% skim milk. The cells were incubated with monoclonal mouse anti-HA, polyclonal rabbit anti-DNMT3A, or monoclonal mouse anti-DNMT3B at 1:500 dilutions in 1% skim milk for 60 min and washed twice with 0.1% NP-40 and once with PBS. Then cells were stained with FITC-conjugated goat anti-mouse IgG (Chemicon, #AB308F) or anti-rabbit IgG (Millipore, #AP307F) at 1:500 dilutions in 1% skim milk. The coverslip was washed with 0.1% NP-40 and mounted in 0.1% p-phenylenediamine and 80% glycerol. Expressions and localizations of DNMTs in the cells were visualized under a fluorescence microscope (Olympus BX50).

2.6. Establishing of the stably transfected HEK293 cells with reporter luciferase vectors

HEK293 cells were stably transfected with reporter luciferase gene cloned under the control of human *klotho* gene promoter. The cells were seeded in a 6-well plate (2×10^5 cells/well) and grown for 20–24 h. Reporter plasmid vectors were linearized with NdeI (NEB, #R0111S) (pHKP-Luc and pHKPDII-Luc) or BglI (Fermentas, #ER0071) (pHKP-DVII-Luc) restriction enzymes. Each of the HEK293 cell cultures grown in 6-well plates were co-transfected with 3 µg of one of the reporter vectors and 0.3 µg of a hygromycin-resistant plasmid. Twenty-four hours posttransfection, the cells were transferred into 6-cm petri dishes and incubated in DMEM containing 75

µg/mL hygromycin B. The culture medium was replaced with fresh medium containing hygromycin B at 3–4-day intervals. The incubation continued until hygromycin-resistant clones were formed. The resistant clones were isolated with glass rings and grown in a 24-well plate. The luciferase activity of stably transfected clones was detected with commercial luciferase reagent.

2.7. Knock down of DNMT3A in HEK293 cells with DNMT3A-siRNA

HEK-293 or stably transfected HEK293 cells were transfected with siRNA specific for DNMT3A (Santa Cruz, #sc-37757) for downregulation of endogenous DNMT3A gene expression. The cells were grown in a 6-well plate (2.5×10^5 cells/well) and incubated for 20 h. After the incubation period, the cells were transfected with 100 pmol DNMT3A-siRNA (Santa Cruz, #sc-37757) or negative control siRNA (Santa Cruz, #sc-37007) by using Lipofectamine RNAiMAX (Invitrogen, #13778-075) transfection reagent, according to the manufacturer's instructions. Forty-eight hours post transfection, the cells were transferred to 6-cm petri dishes containing fresh DMEM medium and incubated for an additional 48 h. Then a quarter of the cells were used for total RNA isolation and semiquantitative RT-PCR. The remaining cells were transfected with DNMT expression vectors or directly used for quantification of reporter luciferase activity in the stably transfected cells.

2.8. Analysis of DNMT3A transcript in siRNA-transfected cells

The DNMT3A transcripts were detected with semiquantitative PCR. Total RNA was isolated from HEK293 cells transfected with siRNA, as mentioned above (2.3). Reverse transcription was carried out with oligo-dT primer. DNMT3A cDNA was amplified with PCR by using specific primers of DNMT3A: 5'-AGCTGCCTACGCACCACCTC-3' and 5'-GCCGCAGCAGCCCGTAGGTA-3'. PCR products were analyzed with 7.5% native polyacrylamide gel. PCR samples were mixed with 6X DNA loading buffered at a 1:5 ratio and loaded to gel. Electrophoresis was carried out in TBE buffer at 80 V for 60 min. DNA bands were visualized by staining the gel in deionized distilled water containing EtBr at 0.2 µg/mL concentration.

2.9. Firefly and Renilla luciferase assays

The luciferase activities were determined using commercially available reagents. Briefly, transiently or stably transfected HEK293 cells grown in 24-well plates were harvested, washed twice with PBS, and lysed in a Renilla lysis buffer (Promega, #E291A). Luminescence was detected with a single tube luminometer (20/20n single tube luminometer; Promega), according to the manufacturer's instructions. The Firefly luciferase activity was normalized relative to the Renilla luciferase activity.

2.10. Statistical analysis

The sample groups were compared using the nonparametric Mann–Whitney test. Statistical analyses were performed using SPSS for Windows, version 17.0 software. $P < 0.05$ defined statistical significance.

3. Results

3.1. Construction of expression vectors

Human *DNMT3A* gene is located on the short arm of chromosome 2 at 23.22 position and consists of 22 introns and 23 exons. The *DNMT3B* gene, having the same number of exons, is located on the long arm of chromosome 20 at 11.22 position (Figure 1). This work started with cloning of *DNMT* cDNAs into mammalian expression vectors. Gene-specific cDNAs were amplified by PCR by using HEK293 cell cDNA. A single variant of *DNMT3A* (*DNMT3A-v.1*) and two variants of *DNMT3B* (*DNMT3B-v.3* and *DNMT3B-v.7*) were obtained. *DNMT* cDNAs were cloned in both pCAGGS and pCHA plasmid vectors for expression of native DNA methyl transferase (DNMT) enzymes, and HA-tagged DNMT enzymes for distinguishing endogenous enzymes, respectively. The expression of *DNMT*s cloned on plasmid vectors in HEK293 cells was analyzed by Western blotting. The localization of these proteins in transiently transfected cells was demonstrated with immunofluorescence assay.

The results showed that *DNMT3A* and *DNMT3B* enzymes encoded by expression vectors were efficiently synthesized in transiently transfected HEK293 cells (Figure 2A) and localized in the nucleus of Swiss3T3 cells. The localization of *DNMT* enzymes was not affected by the presence of HA tag (Figure 2B).

3.2. The effect of overexpression of DNMTs on a reporter gene under the control of human *klotho* promoter

The quantities of *DNMT3A* and *DNMT3B* enzymes in HEK293 cells were increased by transient transfection. The effects of overexpression of *DNMT* enzymes were analyzed by quantitation of luciferase expression from a reporter plasmid carrying the luciferase gene under the control of human *klotho* gene promoter. In the experiments, three different reporter vectors (pHK-luc, pHKP[VII]-luc, and pHKP[VIII]-luc) with different sized upstream regions of human *klotho* gene promoter (Turan and Ata, 2011) were used. The relative luciferase activities, as a ratio of Firefly luciferase to Renilla luciferase in lysates prepared from cells co-transfected with *DNMT* expression vectors and reporter vectors, are given in Figure 3.

Unexpectedly, the increase in *DNMT* gene expression in the cells did not lead to a reduction in reporter luciferase activity, as shown in Figure 3A. On the contrary, a statistically significant increase ($P < 0.05$) of luciferase activity was observed in the cells overexpressing *DNMT*

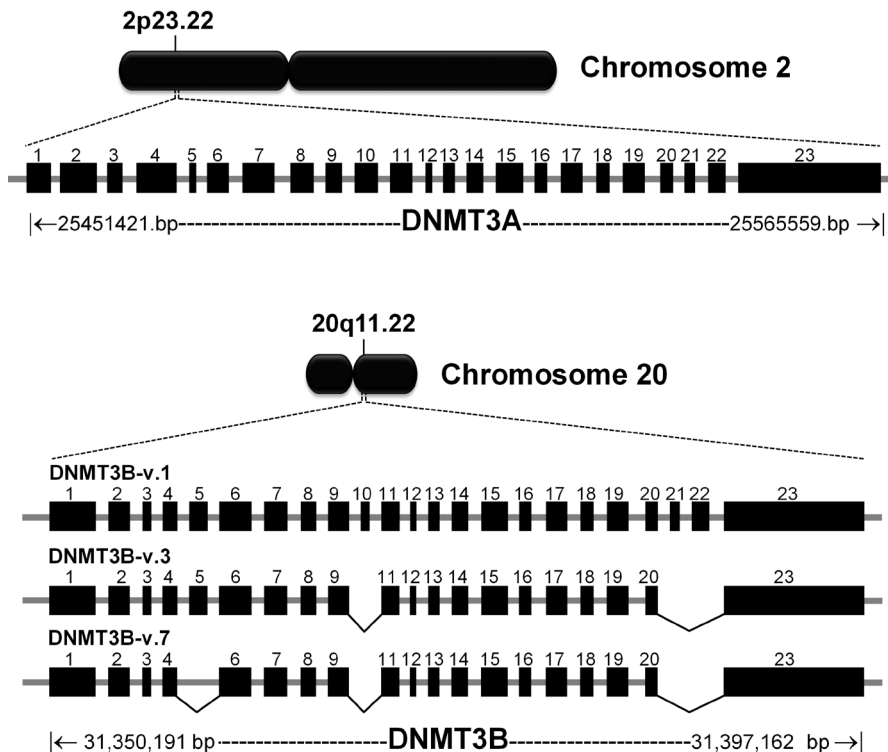


Figure 1. Genomic localization and structure of *DNMT3A* (locusID-NCBI, 1788) and *DNMT3B* genes (locusID-NCBI, 1789).

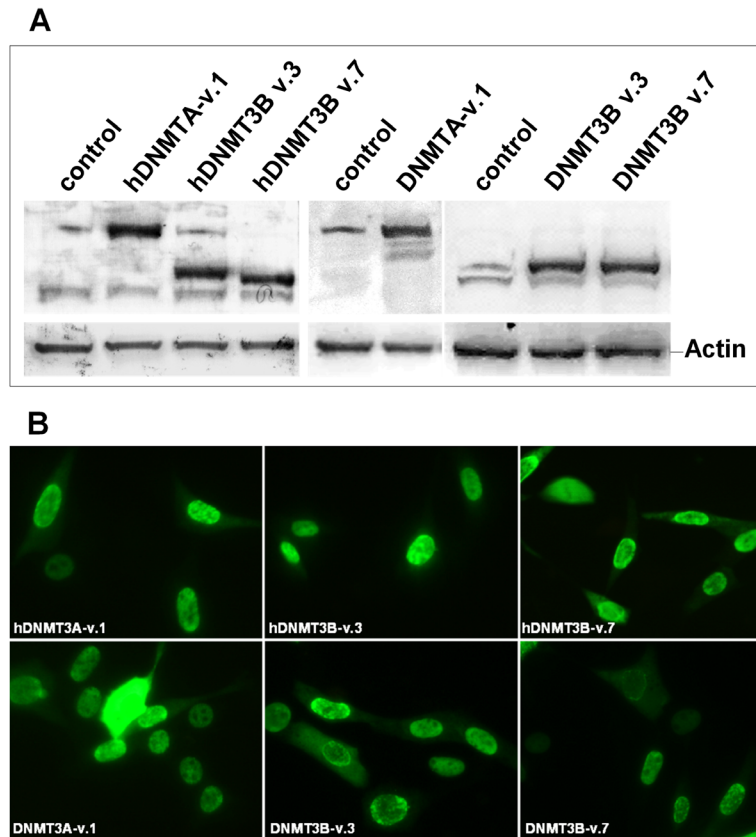


Figure 2. A. Western blot analysis of DNMT expression in HEK293 cells. The native DNMT3A and DNMT3B enzymes were visualized with rabbit polyclonal anti-DNMT3A and mouse monoclonal anti-DNMT3B antibodies, respectively. The DNMT enzymes tagged with the HA epitope were visualized with monoclonal anti-HA antibodies. The protein bands were visualized using SA-AP and the chromogenic substrate with BCIP-NBT. B. Expression and localization of DNMT enzymes in Swis3T3 cells. HA-tagged DNMT enzymes were visualized with monoclonal anti-HA antibody and FITC-conjugated monoclonal anti-mouse IgG antibodies. For visualization of native DNMT enzymes, rabbit polyclonal anti-DNMT3A, mouse monoclonal anti-DNMT3B, and FITC-conjugated secondary antibodies were used.

enzymes. The overexpression of DNMT3A resulted in a much greater increase in reporter activity. Similar reporter activity profiles were observed in experiments carried out with plasmid vectors encoding HA-tagged DNMT enzymes (data not shown). The dose-dependent effect of DNMT3A on human *klotho* promoter is shown in Figure 3B. A correlation was observed between the increase in DNMT3A enzyme and reporter luciferase activity.

3.3. The effect of DNMTs on human *klotho* promoter in stable transfected HEK-293 cells

The DNA molecules of eukaryotic cells are packaged as chromatin in the nucleus. In contrast, plasmid DNA in transiently transfected cells will not be organized as a chromatin structure. HEK293 cells stably transfected with reporter vectors were established because DNA

molecules behaved differently, either relaxed or organized as chromatin via protein factors. The stably transfected cell clones were selected in the presence of hygromycin antibiotic. The cell clones were controlled for transgene by using genomic PCR, and the luciferase activities of lysates prepared from these cells were measured (data not shown). DNMT enzymes were overexpressed in stably transfected HEK293 cells with transfection of expression vectors encoding HA-tagged DNMT (hDNMT) enzymes. The Firefly luciferase activities in lysates of cells of overexpressing DNMT enzymes were normalized with Renilla luciferase activity (Figure 4). The results showed that the increase of hDNMT3A enzyme in the stably transfected cells with pHKP-luc reporter vector had no significant effect on human *klotho* promoter ($P = 0.74$).

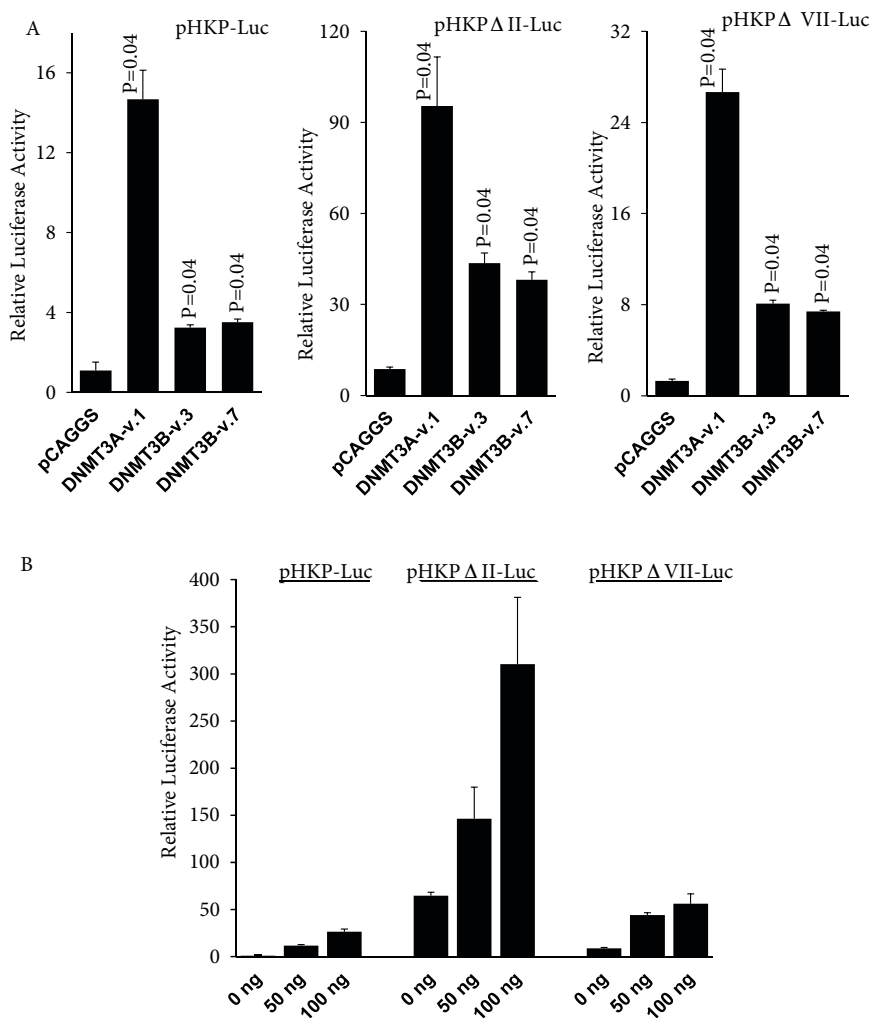


Figure 3. A. The effects of overexpressed DNMT enzymes on luciferase reporter gene expression in HEK293 cells. The cells were grown in 24-well plates containing complete medium for 20–24 h and co-transfected with 100 ng of DNMT expression vector, 100 ng of reporter luciferase vector, and 50 ng of control pRL plasmid vector (for each well), as shown in figures. B. Dose-dependent effect of DNMT3A on reporter gene expression. HEK293 cells grown in 24-well plate were co-transfected with 100 ng of reporter luciferase vector, 50 ng of control pRL plasmid, and different amounts of DNMT3A expression vectors mentioned in figures. Total amount of plasmid DNA for each well was adjusted to 300 ng with pCAGGS vector. Error bars represent standard deviations.

In contrast, hDNMT3B variants lowered more than 50% of reporter luciferase activity in the same cells ($P < 0.05$). The increase in hDNMT3B variants in the two other cell lines showed similar effects on reporter gene expression, but lower than in pHKP-luc cells. However, the effects of hDNMT3B variants were statistically significant compared with the control (vector) ($P < 0.05$).

3.4. The effect of knock down of endogenous DNMT3A on *klotho* gene promoter activity in stably transfected HEK293

In order to reduce the amount of endogenous DNMT3 enzyme, HEK-293 cells and the cells stably transfected with reporter plasmids, were transfected with the siRNA of DNMT3A. The reporter luciferase gene expression

levels in DNMT3 knocked-down cells were determined. First, the amounts of endogenous DNMT3 transcripts in cells transfected with siRNA were determined by semiquantitative RT-PCR. The results given in Figure 5A showed significant reduction of DNMT3A transcripts in cells transfected with specific siRNA. Then reporter luciferase activity in these cells was measured (Figure 5B). The background activity in the control HEK-293 cell lysates was equalized to 1, and the luciferase activities of other samples were compared to that. The results showed a statistically significant increase in reporter luciferase activity in DNMT3A-siRNA-transfected HEK-293 cells having stable reporter genes ($P < 0.03$).

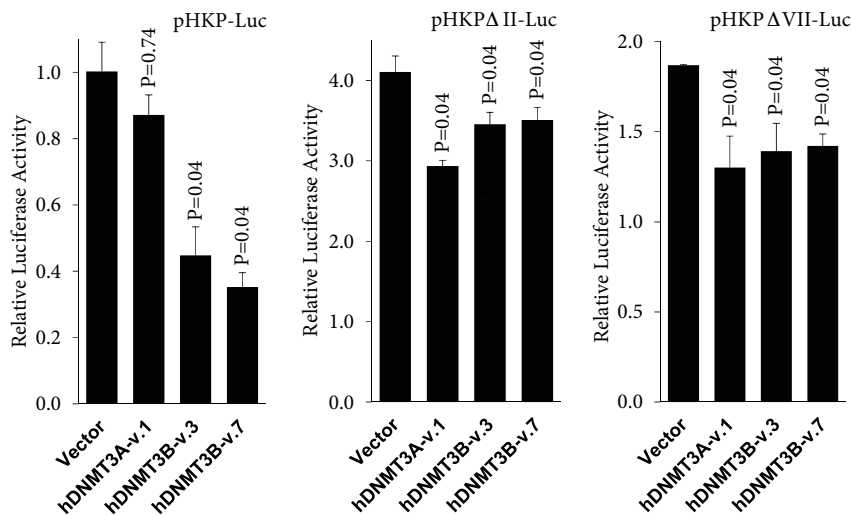


Figure 4. The effects of DNMT enzymes on luciferase reporter gene expression in stably transfected HEK293 cells. The cells were grown in a 24-well plate and co-transfected with 100 ng of DNMT expression vectors, 50 ng of control pRL plasmid, and 100 ng of pCHA. After 48 h incubation, the cells were harvested and luciferase activities in lysates were detected. Error bars represent standard deviations.

4. Discussion

DNA methyl transferase enzymes have great importance for the epigenetic regulation of gene expression in humans and other mammalian organisms. Several reports indicate that methylation of the promoter region downregulates *klotho* gene expression. It is suggested that this situation has an important role in the occurrence of diseases such as breast and gastric cancers (Wang et al., 2011; Rubinek et al., 2012). Hypermethylation in the promoter regions of genes leads to repression of the related gene's expression, whereas methylation in the transcribed region has a variable effect on gene expression (Singal et al., 2002). In human cells, three different enzymes, DNMT1, DNMT3A, and DNMT3B, which are responsible for DNA methylation, have been characterized (Okano et al., 1998; Robertson, 2002). Among these enzymes, DNMT1 is predominantly responsible for copying and maintaining DNA methylation patterns after DNA replication (Spada et al., 2007). However, DNMT3A and DNMT3B are involved in the establishment of new DNA methylation (de novo methylation) patterns (Okano, et al. 1998). In this work, the effects of DNMT3A and DNMT3B, which are responsible for de novo methylation in *klotho* gene promoter activity, were evaluated.

The upstream of *klotho* gene, including the promoter region, is very rich in terms of CpG repeats, which are the target sequences for DNMTs. It is suggested that *klotho* gene expression is strictly regulated by DNA methylation, as an epigenetic factor (Wolf et al., 2008; Lee et al., 2010; Wang et al., 2011). In contrast, there are limited data showing the direct effect of DNA methylation on control of *klotho* gene

expression. Here, human *klotho* gene promoter activity in the overexpression of DNMT enzymes or knock-down cells for these genes was detected with reporter luciferase gene under the control of *klotho* promoter. Reporter plasmid vectors carrying the luciferase gene under the control of human *klotho* promoter were co-transfected with DNMTs expressing vectors. Reporter plasmids would not carry a human-specific DNA methylation pattern because of amplification in *E. coli*. Therefore, it is thought that plasmid DNA will be a target for the DNMT enzymes responsible for de novo methylation in mammalian cells. As shown in Figure 3A, contrary to expectations, the increase of DNMT enzymes in transfected cells upregulated human *klotho* promoter activity. These results were confirmed with the dose-response effect of DNMT3A, as shown in Figure 3B. In contrast, the research on epigenetic regulation of *klotho* gene and some antitumor genes showed that hypermethylation in the promoter region reduced gene expression (Jones and Baylin, 2002; Wang et al., 2011; Rubinek et al., 2012). The overexpression of DNMT3A resulted in a much greater increase in reporter activity compared with DNMT3B enzymes. In pHKP-Luc vector, the reporter luciferase is under the control of about ~2.1 kbp upstream of human *klotho* gene, whereas pHKPDII-Luc and pHKPDVII-Luc contain about 400 bp upstream of the promoter region of *klotho* gene. The difference between pHKPDII-Luc and pHKPDVII-Luc is the presence of a short enhancer sequence in the former plasmid (Turan and Ata, 2011). The results shown in Figure 3 suggested that the DNMT3A and 3B enzymes used in this work affect a common *klotho* promoter region in the reporter vector.

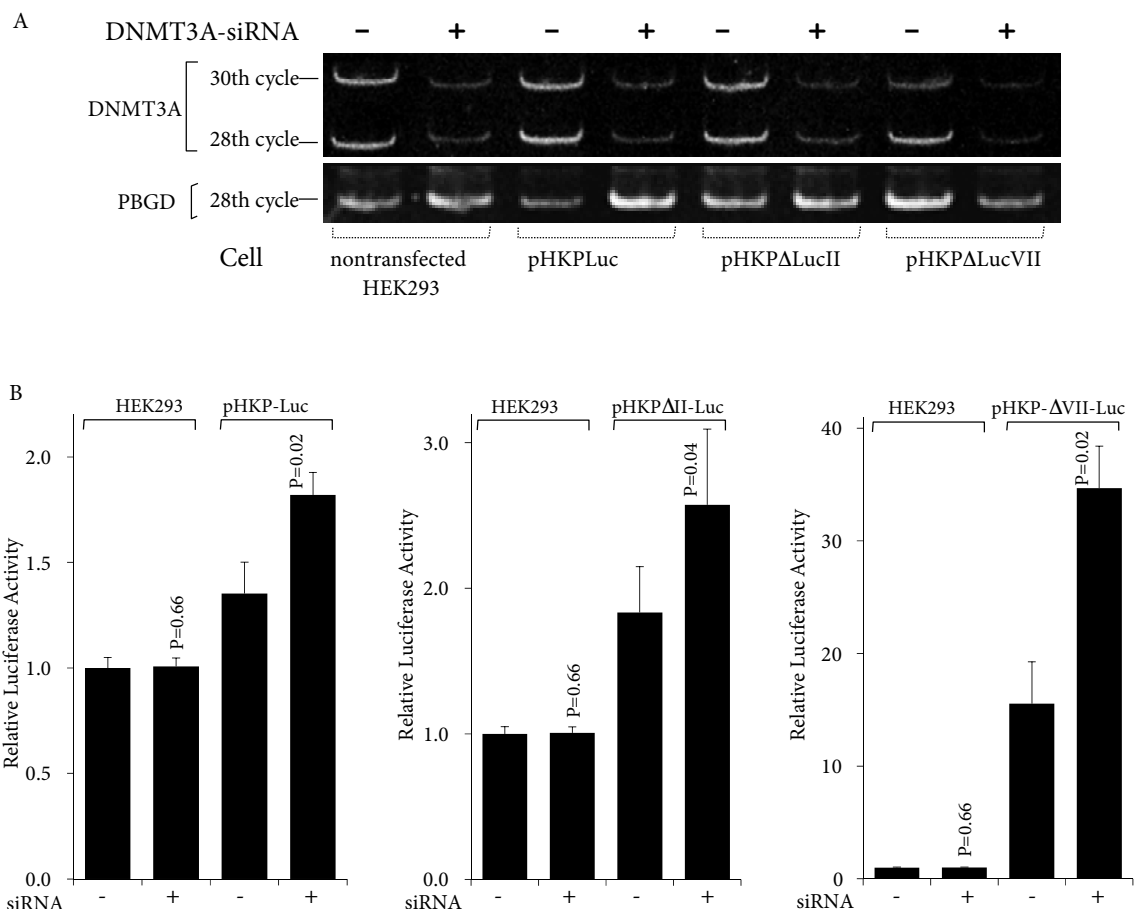


Figure 5. A. Quantitation of DNMT3A transcript in stably transfected HEK-293 that knocked down with DNMT3A-siRNA. DNMT3A transcripts amplified with RT-PCR were analyzed with 7.5% native polyacrylamide gel. B. Reporter luciferase activity in the HEK293 and stably transfected HEK293 cells after 48 h of transfection with DNMT3A-specific siRNA.

It is suggested that DNMT enzymes downregulate gene expression by at least two different mechanisms. First, they change the chromatin structure by stimulating the binding of methyl-CpG binding protein (MeCP) on CpG sequences (Nan et al., 1996). Two types of MeCP (MeCP1 and MECP2) proteins specifically binding to methylated CpG sites have been characterized (Bhakat and Mitra, 2003; Wang et al., 2003). These proteins facilitate the binding of histone deacetylase complex and suppress gene expression. When considered in this context, a reporter gene located on a plasmid vector can give a different response to DNA methylation because it is not organized into chromatin. However, Bhakat and Mitra (1993) reported that methylation of O⁶-methylguanine DNA methyl transferase (MGMT) gene promoter located on the plasmid decreased the reporter gene expression at 10–12-fold, in contrast with our results (Figure 3). Therefore, indirect mechanisms for up- or downregulation of the promoter region carried on the plasmid by DNA

methylation should be considered. The second mechanism considered effective in suppressing gene expression with DNMT enzymes facilitates binding of transcription inhibitors or blocking of transcriptional factors that contribute to recruitment of RNA polymerase II and other transcriptional factors (Hiroi et al., 2013; Wang et al., 2014). Therefore, regulation of gene expression by DNA methylation involves quite complex mechanisms. As a speculative approach, the upregulation of *klotho* gene promoter located on plasmid vector by overexpression of DNMT3A and DNMT3B enzymes may occur as a result of the blockage of transcriptional inhibitors.

The effects of DNMT enzymes on human *klotho* gene expression were investigated in HEK293 cells stably transfected with reporter plasmid vectors. The results showed that the overexpression of hDNMT3B variants decreased more than 50% of reporter luciferase activity in the stably transfected cells with pHKP-luc reporter vector, while these variants showed negligible effects on

klotho promoter activity in the other stably transfected cells (Figure 4). No significant inhibition of reporter luciferase activity was observed in pHKP-luc cells expressing hDNMT3A. hDNMT3A expression in other stably transfected cells showed a statistically important decrease in *klotho* promoter activity. Different levels of a downregulating effect of DNMT enzymes may depend on the expression levels or different action mechanisms of these enzymes in the cells. As an alternative, the endogenous DNMT3A level in stably transfected cells decreased with gene-specific siRNA transfection (Figure 5A) and the measured reporter luciferase activities. A statistically significant increase in reporter luciferase activity in the cells was observed (Figure 5B). These results suggest that the expression of human *klotho* gene organized as chromatin is downregulated with DNA methylation.

In conclusion, we investigated the effects of DNMT enzymes responsible for de novo methylation in human

klotho gene expression. The results showed that DNMT enzymes responsible for de novo methylation in human cells upregulated the human *klotho* gene promoter located on plasmid vectors when it was not organized as a chromatin structure. In contrast, *klotho* gene promoter organized as a chromatin structure was downregulated by DNMT enzymes. These results suggest that the DNA methylation as an epigenetic factor has great importance for human *klotho* gene expression, and that chromatin structure is necessary for DNA methylation with DNMT3A and DNMT3B enzymes.

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