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Abstract—DNA methylation patterns are maintained in adult somatic cells. Recent findings, however, suggest that all methylation patterns are not preserved. We demonstrate that stimulatory signals can change the DNA methylation status at a CCAAT/enhancer binding protein (CEBP) binding site and a transcription start site and activate expression of the angiotensinogen gene (*AGT*). A CEBP binding site in the human *AGT* promoter was hypomethylated in tissues with high expression of *AGT*, but not in those with low expression. The transcriptional activity of *AGT* promoter sequences cloned into a reporter plasmid depended on DNA methylation. In cultured human cells, interleukin 6 stimulation caused DNA demethylation around a CEBP binding site and a transcription start site; demethylation was accompanied by increased CEBP-β recruitment and chromatin accessibility of the *AGT* promoter. DNA methylation activity decreased in the nucleus. Excess circulating aldosterone upregulated *AGT* expression and was accompanied by DNA hypomethylation around a CEBP binding site and a transcription start site in human visceral adipose tissue. High salt intake led to upregulation of *Agt* expression, DNA hypomethylation around 2 CEBP binding sites and a transcription start site, and decreased DNA methylation activity in rat visceral adipose tissue. Taken together, CEBP binding initiates chromatin relaxation and transcription, which are followed by DNA demethylation around a CEBP binding site and a transcription start site in the *AGT* promoter. Decreased DNA methylation activity in the nucleus may play a role in DNA demethylation. DNA demethylation switches the phenotype of *AGT* expression from an inactive to an active state. (***Hypertension*. 2014;63:281-288.**) • **Online Data Supplement**

Key Words: angiotensinogen ■ CCAAT-enhancer binding protein-β ■ CEBPB protein, human ■ chromatin ■ DNA methylation ■ transcription initiation site

Angiotensinogen (AGT; serpin peptidase inhibitor, clade A, member 8) is a glycoprotein that is the primary substrate of the renin–angiotensin–aldosterone system (RAAS) and is, therefore, one of the most frequently studied proteins because of its contributions to hypertension. Given that AGT is the primary substrate controlling the rate-limiting step of the production of angiotensin peptides, a rise in AGT levels can lead to a parallel increase in the formation of the physiologically active enzyme angiotensin II and may ultimately result in multiple diseases, such as hypertension, cardiovascular disease, and kidney injury.[1–3]

The *AGT* gene is expressed in many human tissues, as determined by an analysis of cDNA sequences available on the Unigene website. The *AGT* gene is known to be highly expressed in the liver, heart, and brain, whereas weakly expressed in the adrenal gland (www.ncbi.nlm.nih.gov/sites/entrez?db=unigene; Figure S1 in the online-only Data Supplement). The expression of *AGT* is under developmental and hormonal control in a cell type–specific manner.[4] It is generally approved that *AGT* expression is predominantly regulated at the transcriptional level.[5] Multiple *cis*-acting DNA regulatory elements and transcription factors are known to be important in the regulation of *AGT*.[4,6–9] Among those transcription factors, CCAAT/enhancer binding protein (CEBP) has been shown to increase *AGT* promoter activity through binding to the promoter region[6] and is important for the regulation of *AGT* in response to interleukin 6 (IL6).[6,10] The CEBP family facilitates the binding of other transcription factors to shared sites and contributes to efficient chromatin remodeling at some of these sites. This feature of CEBP function indicates that it is a pioneering factor for the assembly of transcription factor complexes at these sites.[11]

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From the Department of Internal Medicine (F.W., Y.C., A.Z., S.K., T.Y., M.Y., Y.T.), Department of Urology (Y.M., M.N.), and Department of Stem Cell Biology (T.A.), Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan; Department of Hygiene, Kanazawa University School of Medicine, Kanazawa, Japan (M.D., K.S.); Department of Respiratory Medicine, Fukui Red Cross Hospital, Fukui, Japan (Y.D.); and Department of Pathology, Tohoku University Hospital, Sendai, Japan (K.O., Y.N., H.S.).

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Correspondence to Masashi Demura, Department of Hygiene, Kanazawa University School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan. E-mail m-demura@med.kanazawa-u.ac.jp

Recent studies implicate a role for adipose AGT in blood pressure regulation in mice.[12–14] Glucocorticoids increase angiotensinogen gene expression,[6,15,16] whereas the effect of mineralocorticoids on angiotensinogen gene expression is unknown in any type of cell. Circulating AGT derived from adipose tissue is increased in obese hypertension.[17–19] High salt intake is associated with obesity,[20,21] suggesting a potential link between high salt intake and increased adipose AGT.

Although many studies have been conducted on the mechanisms of *AGT* gene regulation, little is known about the contribution of DNA methylation to regulation of the *AGT* gene. DNA methylation occurs almost exclusively at cytosines of CpG dinucleotides. The patterns of DNA methylation are faithfully replicated at every cell division once they have been established. DNA methylation is associated with the formation of heterochromatin and gene silencing. MECP2 (methyl-CpG–binding protein 2) is able to directly bind to methylated DNA and repress methylation-based transcription.

In this study, we characterize the epigenetic regulation of the *AGT* gene. The transcriptional activity of the *AGT* gene was found to be dependent on DNA methylation. We also found excess circulating aldosterone and high salt intake as a stimulator of adipose angiotensinogen gene expression in humans and rats, respectively. Stimulatory signals caused DNA to be dynamically demethylated and led to chromatin relaxation in the *AGT* promoter.

Methods

Cell Culture

NCI H295R human adrenocortical cells (ATCC, Manassas, VA) were cultured in DMEM/F12 medium (Sigma Chemical Company, St Louis, MO) supplemented with 2% Ultroser G (Pall Biosepra, NY), 1% ITS+Premix (BD Biosciences, Bedford, MA), and 1% penicillin–streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator.

Human AGT ELISA

To quantify human AGT protein in cell lysate, a human AGT ELISA was performed with a Human angiotensinogen ELISA kit (CUSABIO, Wuhan, China). Cytoplasmic extracts were isolated from H295R cells. Data were normalized to the total protein concentration of the cytoplasmic extracts.

Real-Time Reverse Transcription Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) was performed using the SYBR Green method. *ACTB* (actin, beta) and *Rn18s* (18S ribosomal RNA) were used as the reference genes for humans and rats, respectively. The primer sequences were as follows: human *AGT*: forward 5′-CCCTGGCTTTCAACACCTAC-3′, reverse 5′-GCTGTTGTCCACCCAGAACT-3′; human *ACTB*: forward 5′- TGGC ACCCAGCACAATGAA-3′, reverse 5′- CTAAGTCATAGTCCGCCT AGAAGCA-3′; rat *Agt*: forward 5′-AGCACGGACAGCACCCTATT, reverse 5′-AGAACTCATGGAGCCCAGTCA-3′; rat *Rn18s* RNA: forward 5′-ACGGAAGGGCACCACCAGGA-3′, reverse 5′-CACCAC CACCCACGGAATCG-3′.

Bisulfite Sequencing

Genomic DNA was treated with bisulfite and amplified with primers specific for the human *AGT* and rat *Agt* promoter regions. The human *AGT* promoter regions spanning from −483 to −130 and from −110 to +78 were amplified with the following primer pairs: forward 5′-GTTGAAGGTTATATATTTTATGAG-3′, reverse 5′-AATACCAAAAACGACACTC-3′ (−483 to −130) and forward 5′-GGTTTGGTTAAGTGATGTAA-3′, reverse 5′-AAAAACAAAACCGAAAAA-3′ (−110 to +78). The rat *Agt* promoter regions spanning from −400 to −103 and from −57 to +236 were amplified with the following primer pairs: forward 5′-AATTTGATTAGATGTGTTAGAAG-3′, reverse 5′-TTACATATTTACCCCAATTC-3′ (−400 to −103) and forward 5′-TGAAGTTTATTTGTTATTAGGGTTTTT-3′, reverse 5′-AAACTCAACTCCTACCCAATCA-3′ (−57 to +236). Eight cloned PCR products were chosen for analysis of CpG methylation status, as previously described.[22–24]

Chromatin Accessibility by Real-Time PCR

H295R cells were cultured in 48-well plates to 80% to 95% confluence before they were digested in situ with or without nuclease at 37°C for 1 hour, in accordance with the protocol for the EpiQ Chromatin Analysis Kit (BIORAD, Hercules, CA). The cells were subsequently lysed and genomic DNA was isolated. One hundred nanograms of genomic DNA was used to perform SYBR Green real-time PCR (qPCR) for the *AGT* gene and the reference gene. Each PCR was performed in duplicate. The qPCR data were analyzed with the data analysis tool of the EpiQ Chromatin Analysis Kit to determine the chromatin accessibility within the *AGT* promoter region (Figure 1). The human *AGT* promoter regions were amplified with the following primer pairs: forward 5′- CCCGTTTCTGGGAACCTT-3′, reverse 5′-AGATGCCAGAAGCGACACTC-3′ (R2; CEBP); forward 5′-CAGCTATAAATAGGGCATCGTG-3′, reverse 5′- ACAA GACCGAGAAGGAGCTG-3′ (R1; transcription start site); forward 5′- TTGCCTAAGCAAGACTCTCC-3′, reverse 5′-GGAG ATGTACCCCCAAGAGG-3′ (R3; CpG3); forward 5′-GAGCAG CTGAAGGTCACACA-3′, reverse 5′- GCAGGGGAGAGTCTTG CTTA-3′ (R4; CpG1,2).

Chromatin Immunoprecipitation Quantitative PCR

The chromatin immunoprecipitation (ChIP) assays were performed with the LowCell# ChIP Kit (Diagenode Diagnostics, Liège, Belgium) in accordance with the manufacturer's protocol. Anti–CEBP-β (Anti-CEBPB) polyclonal antibody (sc-150; Santa Cruz Biotechnology, Dallas, TX) and IgG (sc-2027; Santa Cruz Biotechnology) were used. Quantification of ChIP DNA was

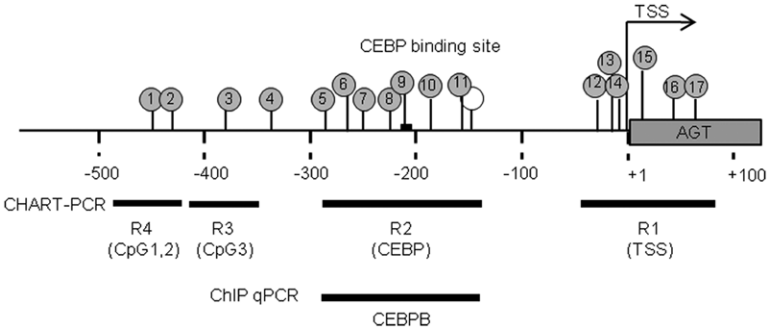

Figure 1. Schema of the experimental design for the human angiotensinogen (*AGT*) promoter. Filled lollipops denote cytosine residues. A CpG dinucleotide, open lollipop was not analyzed. Nucleotide numbers are relative to a TSS. CEBPB indicates CCAAT/enhancer binding protein-β; CHART-PCR, chromatin accessibility by real-time polymerase chain reaction; ChIP qPCR, chromatin immunoprecipitation quantitative PCR; and TSS, transcription start site.

accomplished by qPCR with primers specific for the *AGT* promoter region around the CEBPB transcription factor binding site; the primer sequences are the same as those used for the chromatin accessibility by real-time PCR assay. The results are presented as fold enrichment of IgG. Each sample was amplified in triplicate and all experiments were repeated ≥3×.

DNA Methylation Activity Assay

Using the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigenetic, Farmingdale, NY), DNA methylation activity was quantified as the ability of nuclear extracts to methylate DNA substrate. The methylated DNA was recognized with an anti–5-methylcytosine antibody. Each sample was measured in triplicate.

DNA Demethylation Activity Assay

Using the EpiQuik DNA Demethylase Activity/Inhibition Assay Ultra Kit (Epigenetic), DNA demethylation activity was quantified as the ability of nuclear extracts to demethylate methylated DNA substrate. The methylated DNA was recognized with an anti–5-methylcytosine antibody. Each sample was measured in triplicate.

Statistical Analysis

Statistical significance of the differences between experimental groups was analyzed by using Student *t* test, 1-way ANOVA, or 2-way ANOVA. The data are expressed as mean±SEM. *P* values <0.05 were considered to be statistically significant.

Results

IL6 Treatment Results in DNA Demethylation Around a CEBP Binding Site and a Transcription Start Site

We analyzed the DNA methylation status of 17 of 18 CpG dinucleotides within a 525-bp fragment (–459 to +66) of the human *AGT* promoter, which contains 1 CEBP binding site (Figure 1). We sought to gain insight into dynamic changes in DNA demethylation of the *AGT* promoter. IL6 has been shown to stimulate *AGT* expression.[25,26] IL6 treatment upregulated *AGT* expression in a dose-dependent manner in H295R cells (data not shown). A preliminary comparison of DNA

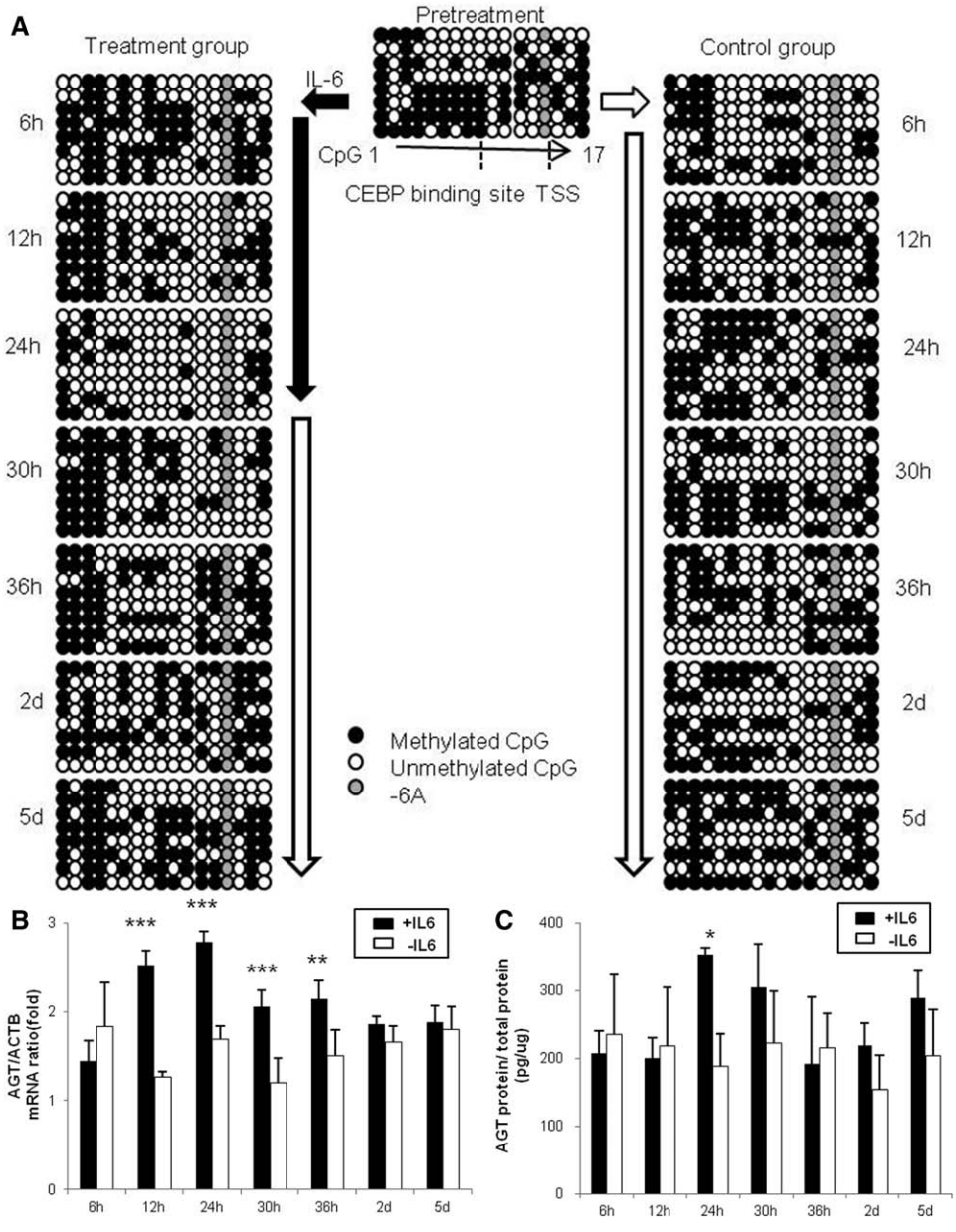

Figure 2. Dynamic changes in DNA methylation in the angiotensinogen (*AGT*) promoter. **A**, Changes in DNA methylation. Gray circles indicate the A allele of the G-6A polymorphism (rs5051). The –6G allele becomes a CpG dinucleotide, a canonical target site for DNA methylation. White circles denote unmethylated cytosine residues; black circles, methylated. **B**, *AGT* mRNA level. Results are given as fold change normalized to ACTB. **C**, AGT protein level. Interleukin 6 (IL6) treatment increased angiotensinogen expression. Data are presented as mean±SEM of 3 independent experiments. **P*<0.05, ***P*<0.005, and ****P*<0.0001 vs –IL6 (2-way ANOVA followed by Bonferroni post tests). CEBP indicates CCAAT/enhancer binding protein; and TSS, transcription start site.

methylation patterns of the *AGT* promoter was made between IL6 stimulation (10 ng/μL) for 24 and 72 hours in H295R cells. DNA demethylation around a CEBP binding site and a transcription start site was observed in both conditions, and the methylation patterns were similar with each other. We concluded that the DNA demethylation required almost 24 hours to reach the highest levels (data not shown) and decided to stimulate H295R cells with IL6 (10 ng/μL) for 24 hours in further experiments. To determine how DNA demethylation and remethylation progress, we treated H295R cells with IL6 (10 ng/μL) for 24 hours and observed the dynamic changes in DNA methylation patterns of the *AGT* promoter for 5 days. Nontreated cells served as a control. In cells treated with IL6, DNA demethylation around the CEBP binding site and the transcription start site was detected within 12 hours after stimulation and reached a maximum level by 24 hours. CpG dinucleotides around the CEBP binding site and the transcription start site in treated cells were remethylated to a level that was similar to that of nontreated cells during the next 24 hours. No obvious changes in CpG methylation were observed in cells of the control group (Figure 2A).

Gray circles indicate the A allele of the G-6A polymorphism (rs5051). The −6G allele becomes a CpG dinucleotide, a canonical target site for DNA methylation. The results strongly indicated that H295R cells had −6AA genotype. Unmethylated or methylated cytosines were observed probably because errors happened in bisulfite sequencing or mutations occurred in H295R cells.

IL6 Treatment Increases AGT Expression

AGT mRNA levels gradually increased during IL6 stimulation and decreased to a level that was similar to that of nontreated cells after stimulation. *AGT* mRNA levels in cells treated with IL6 were significantly higher than those in nontreated cells at 12, 24, 30, and 36 hours after stimulation (Figure 2B). Similar changes were observed in the levels of AGT protein. At 24 hours after stimulation, AGT protein levels in cells treated with IL6 were significantly higher than those in nontreated cells (Figure 2C).

IL6 Treatment Recruits CEBPB to the *AGT* Promoter Region

DNA hypomethylation at a CEBP binding site has been observed in tissues with elevated expression of *AGT*, and the site was demethylated during IL6 treatment. These observations suggest an association between CEBP and changes in DNA methylation. We used a ChIP assay to determine that the binding of CEBPB to the *AGT* promoter in cells treated with IL6 was significantly higher than that in nontreated cells. A steep increase in recruitment of CEBPB to the *AGT* promoter was observed during IL6 treatment. Five days after stimulation, CEBPB binding in cells treated with IL6 had decreased to a level that was similar to levels in nontreated cells (Figure 3).

IL6 Treatment Increases Chromatin Accessibility of the *AGT* Promoter

Treatment of H295R cells with IL6 increased recruitment of CEBPB to its binding site in the *AGT* promoter. CEBPB has been characterized as a pioneering factor that binds to chromatin-embedded DNA and recruits remodeling proteins.[27] Chromatin accessibility may be important for dynamic changes in DNA demethylation and remethylation in a locus-specific manner. Chromatin accessibility by real-time PCR revealed that IL6 treatment significantly increased chromatin accessibility around a transcription start site (R1) at 6, 12, and 24 hours after stimulation, with accessibility increasing from 58% to 79% (Figure 4A). Chromatin accessibility around a CEBP binding site (R2) also increased, to ≈80%. At 24 hours after stimulation, the accessibility of treated cells was significantly different from that of cells in the control group (Figure 4B). After stimulation, the accessibility of both regions returned to levels that were similar to those of the control group.

In contrast, chromatin accessibility did not increase around the CpG3 (R3; Figure 4C) or CpG1/2 (R4) sites (Figure 4D). The accessibility of both regions was consistently <40%; this percentage indicates that the chromatin was accessible at a low level or was even highly inaccessible.

IL6 Treatment Reduces DNA Methylation Activity

We used a time-course analysis to determine DNA methylation and demethylation activities in nuclear extracts from H295R cells. IL6 treatment significantly reduced DNA methylation activity at 12, 24, 30, 36, and 48 hours after stimulation (Figure 5A). No obvious differences in DNA demethylation activity were observed between IL6-treated cells and those of the control group (Figure 5B). These results suggest a potential role for decreased DNA methylation activity during the process of DNA demethylation and remethylation.

Discussion

In contrast with readily reversible histone modifications, DNA methylation was generally accepted to be a relatively static epigenetic modification. Recent findings, however, support the emerging concept that DNA methylation patterns are not always stable.[28–30] DNA methylation is a well-known mechanism for gene silencing, but comparatively little is known about where changes in DNA methylation occur and how DNA methylation is rapidly reversed in a locus-specific

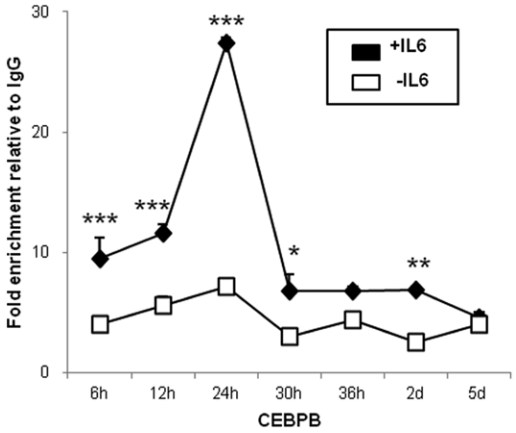

Figure 3. Interleukin 6 (IL6)–induced CCAAT/enhancer binding protein-β (CEBPB) recruitment to the angiotensinogen (*AGT*) promoter. Data are shown as fold enrichment relative to IgG of 3 independent experiments (mean±SEM). **P*<0.05, ***P*<0.005, and ****P*<0.0001 vs –IL6 (2-way ANOVA followed by Bonferroni post tests).

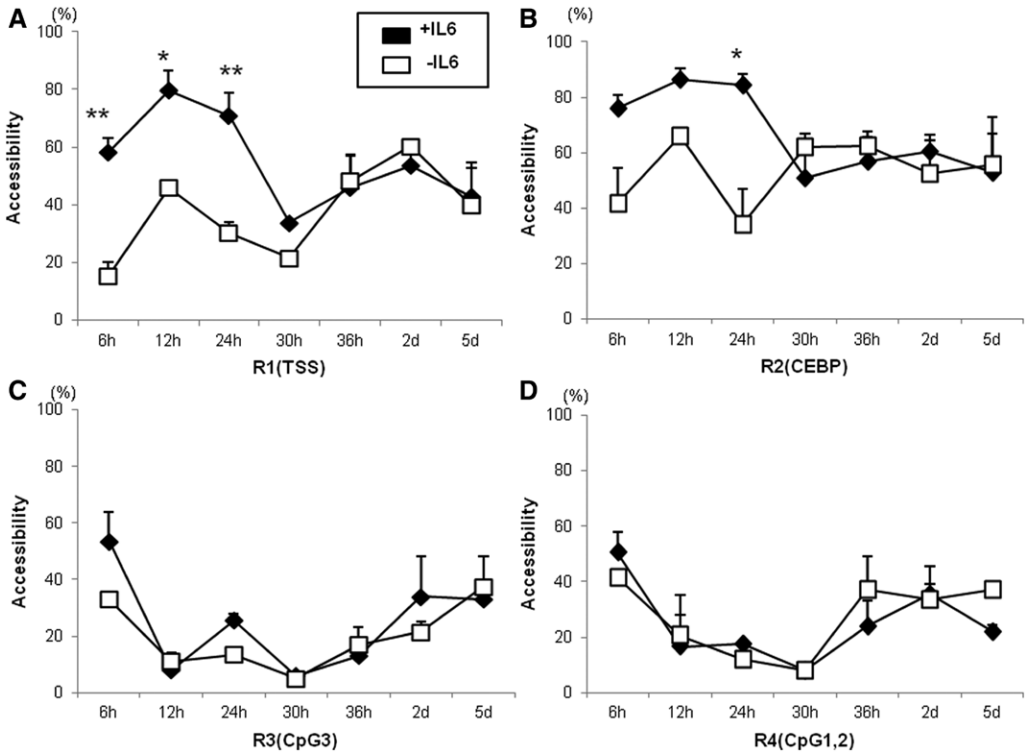

Figure 4. Changes in chromatin accessibility within the angiotensinogen (*AGT*) promoter. **A**, Transcription start site (TSS). **B**, CCAAT/enhancer binding protein (CEBP) binding site. **C**, CpG2 and 3 sites. **D**, CpG1 site. Data are presented as mean±SEM of 3 independent experiments. **P*<0.05, ***P*<0.005 vs – interleukin 6 (IL6; 2-way ANOVA followed by Bonferroni post tests).

manner. Our study revealed that the stimulatory signals of IL6, excess circulating aldosterone, and high salt intake all led to DNA demethylation around a CEBP binding site and a transcription start site, where chromatin structure was relaxed. DNA demethylation was detected within 12 hours after IL6 treatment, whereas the highest level of demethylation was evident by 24 hours.

Irrespective of the type of stimuli, DNA demethylation around a CEBP binding site and a transcription start site in the angiotensinogen gene promoter was accompanied by increased angiotensinogen gene expression in both humans and rats. The CEBP family of proteins has been reported to pioneer chromatin remodeling and maintain chromatin accessibility.[11] Taken together, our findings indicate that CEBP family members initiate chromatin relaxation and transcription, which are followed by DNA demethylation around a CEBP binding site and a transcription start site in the angiotensinogen gene promoter.

The molecular regulatory effects of IL6 on *AGT* expression have been extensively investigated in human liver cells. In addition to CEBP family members, NR3C1, STAT3, and HNF1A that bind to the nucleotide sequence located between −162 and −278 of the *AGT* promoter are involved in IL6-induced activation of *AGT* (Figure S4A).[31] Because the *AGT* promoter was fully unmethylated in human liver HepG2 cells, we were unable to use these cells to analyze changes in DNA methylation (Figure S2). Instead, we used human adrenocortical H295R cells that had a partially methylated *AGT* promoter (Figure 2A). In H295R cells, IL6 treatment upregulated *AGT* expression in a similar manner with HepG2 cells and was accompanied by extensive demethylation from CpG5 (−281/−282) to CpG11 (−152/−153; Figure 2A). These results suggest that transcription factors CEBP, NR3C1, STAT3, and HNF1A may be involved in the upregulation of *AGT* and DNA demethylation of this gene promoter in H295R cells as is the case with human liver cells.

We have shown for the first time that circulating aldosterone in patients with aldosterone-producing adenoma (APA) leads to increased *AGT* expression in visceral adipose tissue (Figure S5C). Aldosterone is ≈1000× less than cortisol in the circulating blood (Table S1). Aldosterone and cortisol, although both corticosteroids, regulate *AGT* differently: aldosterone is a more potent activator of *AGT* expression than is cortisol. RAAS is a system of major importance in the control of fluid and electrolyte homeostasis. Thus, aldosterone seems to exert a physiologically relevant effect on *AGT* expression. This positive feedback effect may be an innate capacity to efficiently retain fluid in land dwelling mammalian species. Excessive salt in contemporary diets depresses the circulating RAAS.[32] DNA hypermethylation of the *AGT* promoter in adipose tissue from NFA (non-functioning adrenocortical adenoma) may reflect depressed levels of the circulating RAAS in contemporary humans.

Two receptor systems, the glucocorticoid receptor system (NR3C1) and the mineralocorticoid receptor system (NR3C2), differentially modulate corticosteroid actions in adipose tissue.[33] Glucocorticoid receptor and mineralocorticoid receptor bind to a common corticosteroid response element. The CEBP binding site (−217 to −225) is also known to interact with corticosteroid receptors.[6] CEBPB and glucocorticoid receptor have been reported to cooperatively and specifically regulate

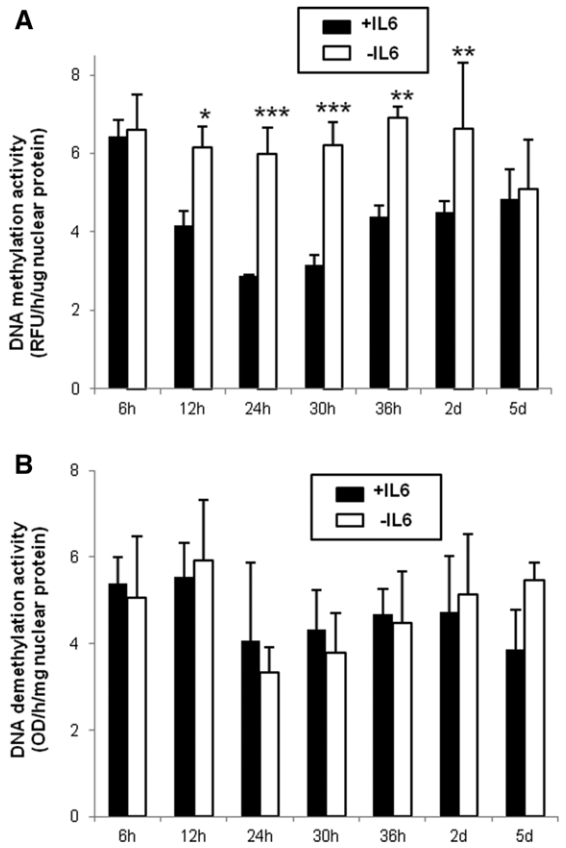

Figure 5. Changes in DNA methylation and demethylation activities. **A**, DNA methylation activity. **B**, DNA demethylation activity. **P*<0.05, ***P*<0.005, and ****P*<0.0001 vs – interleukin 6 (IL6; 2-way ANOVA followed by Bonferroni post tests).

chromatin accessibility and remodeling at co-occupied sites.[11] A CpG dinucleotide at the CEBP binding site (−217 to −225) was hypomethylated in adipose tissue of patients with APA, whereas this site was hypermethylated in the tissue of patients with Cushing syndrome (CS; Figure S5A and S5B). The difference in DNA methylation at the CEBP binding site between patients with APA and those with CS suggests differential actions of corticosteroids in human adipose tissue via interactions between corticosteroid receptors and CEBP.

Glucocorticoids are well-known activators of angiotensinogen gene expression in humans and rodents.[6,15,16] Three putative corticosteroid response elements (gtccct, −371 to −376; ctctg, −393 to −397; ctctg, −400 to −404) exist in the vicinity of CpG2 and CpG3 (Figure S4A). DNA hypomethylation at CpG2/3 sites and around a transcription start site was observed in visceral adipose tissue of patients with CS. Cortisol-induced recruitment of corticosteroid receptors might cause DNA demethylation at these 2 CpG sites and activate *AGT* transcription in cases of CS. Hypertension is a prominent characteristic of APA and CS. Although elevation in arterial pressure is primarily induced by aldosterone and cortisol in APA and CS, respectively, increased *AGT* expression from visceral adipose tissue may contribute, in part, to the development of hypertension in both conditions.

High salt intake suppresses the circulating RAAS. Salt intake can affect sympathetic nerve activity and lead to changes in the gene expression profile of adipose tissue. *Agt* expression was paradoxically increased in visceral adipose tissue from Wistar-Kyoto rats fed a high-salt diet (Figure S6D). This result suggests that salt-dependent hypertension in humans may be, in part, dependent on increased *AGT* expression in visceral adipose tissue. More importantly, DNA hypomethylation at CEBP binding sites and a transcription start site coincided with increased *Agt* expression in rat visceral adipose tissue (Figure S6A–S6D). This finding indicates that a close association exists between DNA hypomethylation at CEBP binding sites and increased angiotensinogen gene expression in both humans and rats.

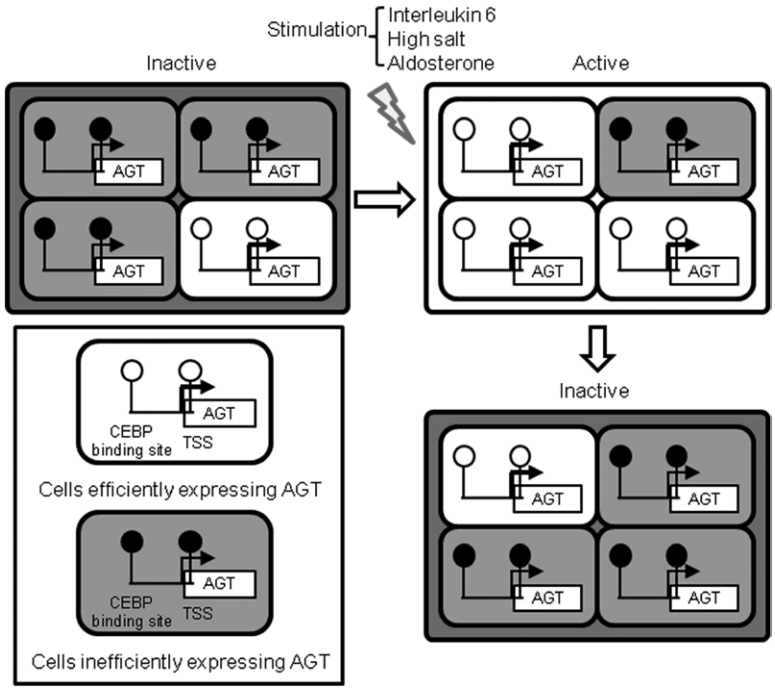

Figure 6. Conversion of the gene expression phenotype. Stimulatory signals switch the gene expression phenotype within a tissue from an inactive to an active state. After stimulation, the gene expression phenotype gradually restores to its former state. AGT indicates angiotensinogen; CEBP, CCAAT/enhancer binding protein; and TSS, transcription start site.

We have shown that DNA methylation at CpG dinucleotides alters gene expression by affecting transcription factor binding activity.[23] However, the effects of CpG methylation on binding activities of transcription factors with the *AGT* promoter were limited (Figure S4B and S4C). CpG methylation increased MECP2 binding activities with the *AGT* promoter (Figure S4D). These observations suggest that MECP2 recruitment to methylated CpG dinucleotides would play a crucial role in transcriptional repression of *AGT* by DNA methylation.

Functional DNA demethylation is accompanied by chromatin accessibility.[34] Subtle change in topology of nucleosomal DNA in promoter regions can play an important role in gene regulation.[35] After IL6 stimulation, we detected an open chromatin structure only around a transcription factor binding site and a transcription start site. Thus, chromatin accessibility plays a crucial role in gene regulation.

DNA methylation is established and maintained by DNA methyltransferases. DNA methyltransferase 3A and 3B are responsible for de novo DNA methylation, whereas DNA methyltransferase 1 maintains DNA methylation patterns through successive rounds of cell division.[36] DNA demethylation can occur through an active or passive pathway. Active DNA demethylation is the postulated process whereby 5-methylcytosine is sequentially modified, excised, and replaced by the DNA repair machinery. By contrast, passive DNA demethylation refers to the loss of the methyl radical from 5-methylcytosine when DNA methyltransferase 1 is inhibited or absent during successive rounds of DNA replication.[37] In our study, DNA demethylation of the *AGT* promoter was associated with decreased DNA methylation activities (Figure 5A; Figure S6E). This association suggests that passive, rather than active, DNA demethylation may be a more likely cause. The balance between DNA methylation and demethylation activities may be a major determinant for DNA methylation status.

Perspectives

Stimulatory signals can orchestrate locally transcription factor recruitment, open chromatin configuration, and DNA demethylation in a locus-specific manner and globally a reduction in DNA methylation activity in the nucleus. Any type of continuous signal could elicit changes in DNA methylation to globally control the balance between DNA methylation and demethylation activities. DNA methylation status can be used to trace the amount of chromatin that is opened by transcription factors at specific sites and functions as a memory to maintain responsiveness of gene expression to additional signals. DNA demethylation converts the gene expression phenotype within a tissue from an inactive to an active state (Figure 6).

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Disclosures

None.

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Novelty and Significance

What Is New?

- We demonstrate dynamic changes of DNA methylation as to where changes in DNA methylation occur and how rapidly DNA demethylation and remethylation progress in the angiotensinogen gene (*AGT*) promoter.
- Excess circulating aldosterone and high salt intake upregulate adipose *AGT* expression in humans and rats, respectively.

What Is Relevant?

- Increased AGT expression from visceral adipose tissue may contribute to the development of hypertension in APA (aldosterone-producing adenoma) and CS.
- Salt-dependent hypertension may depend on increased AGT expression in visceral adipose tissue.

Summary

DNA methylation negatively regulates the *AGT* expression and dynamically changes in response to continuous stimulation in the *AGT* promoter. The methylation patterns function as a memory to maintain responsiveness of gene expression to additional signals. Stimulatory signals convert the gene expression phenotype within a tissue from an inactive to an active state.

Dynamic CCAAT/enhancer binding protein (CEBP)-associated changes of DNA methylation in the angiotensinogen gene

Fen Wang,[1] Masashi Demura,[2] Yuan Cheng,[1] Aoshuang Zhu,[1] Shigehiro Karashima,[1] Takashi Yoneda,[1] Yoshiki Demura,[3] Yuji Maeda,[4] Mikio Namiki,[4] Katsuhiko Ono,[5] Yasuhiro Nakamura,[5] Hironobu Sasano,[5] Tadayuki Akagi,[6] Masakazu Yamagishi,[1] Kiyofumi Saijoh,[2] Yoshiyu Takeda[1]

Short Title: Epigenetic regulation of the angiotensinogen gene

[1] Department of Internal Medicine, Graduate School of Medical Science, Kanazawa University
[2] Department of Hygiene, Kanazawa University School of Medicine
[3] Department of Respiratory Medicine, Fukui Red Cross Hospital
[4] Department of Urology, Graduate School of Medical Science, Kanazawa University
[5] Department of Pathology, Tohoku University Hospital
[6] Department of Stem Cell Biology, Graduate School of Medical Science, Kanazawa University

Supplemental Material

Materials and Methods

Tissue distribution of the *AGT* gene expression

We determined the expression profile of the *AGT* gene by the analysis of expressed sequence tag counts publicly available at the UniGene web site (Supplementary Fig. S1).

AGT promoter constructs

Luciferase reporter plasmids containing various fragments of the 5'-flanking region of the *AGT* promoter were prepared from human genomic DNA using PCR (Figure S3A). After digestion with KpnI and NheI, the reporter constructs were synthesized by PCR amplification of human *AGT* gene using TTTGCACAGGGTAGGCTCTT, TGTGTAACTCGACCCTGCAC and TGGTCTGGCCAAGTGATGTA as the respective forward primers and CCGGCTTACCTTCTGCTGTA as the reverse primer. The amplified fragments contained the nucleotide sequence -511 to +44, -238 to +44 and -112 to +44, respectively. The forward primer had the KpnI restriction site and the reverse primer had the NheI restriction site so that amplified fragments could be subcloned in the pGL4.10 basic vector. Inserts were sequenced to ensure fidelity of the amplified sequences.

Luciferase assay

SssI methylase (New England Biolabs Japan Inc., Tokyo, Japan) was used for in vitro methylation of *AGT* promoter luciferase constructs in the pGL4.10 vector. In each case, half of the DNA sample was methylated using SssI and the other half was incubated with SssI methylase in the absence of S-adenosylmethionine (mock-methylation). The efficiency of methylation or mock-methylation was determined through digesting modified constructs with restriction endonuclease BsiEi (New England Biolabs Japan Inc., Tokyo, Japan). Adrenocortical H295R cells were transfected using FuGene HD (Roche Laboratories, Basel, Switzerland) with the following plasmids: (1) 100 ng of modified pGL4.10 firefly luciferase reporter plasmid containing methylated or unmethylated AGT promoter and (2) 10 ng of pRL-TK Renilla luciferase control reporter vector that contains a cDNA encoding Renilla luciferase (Promega, Madison, WI, USA) as an internal control for transfection efficiency. The day before transfection, H295R cells were seeded onto a 96-well plate and transfected at 80% confluency. Cells were incubated for 48h following transfection. Firefly and Renilla luciferase assays were performed using 10ul of cell lysates and a dual-luciferase reporter assay system kit (Promega). Results are reported as the average of triplicate assays and are expressed as a ratio to the internal standard Renilla luciferase.

Enzyme-linked immunosorbent assay-based electrophoretic mobility shift assay (NoShift assay)

Nuclear extracts were isolated from H295R cells and HepG2 cells according to the manufacturer's protocol with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Yokohama, Japan). Protein concentration was determined using a BCA protein assay (Thermo Fisher Scientific) with BSA as a standard. The enzyme-linked immunosorbent assay-based electrophoretic mobility shift assay was carried out using

NoShift transcription factor assay kits (TAKARA BIO INC., Otsu, Japan) according to the manufacturer's protocol. 6 μg of nuclear extracts was incubated on ice for 30 min with each of the biotinylated oligonucleotides including J region (HNF4A/NR2F1 binding site), CEBPB/NR3C1 binding site with -217A or G allele and USF1/ESR1 binding site with -20A or C allele in the presence or absence of CpG dinucleotides methylation (10 pmole), together with 500 ng salmon sperm DNA and 0.01U Poly (dI-dC) in 1× NoShift binding buffer. After 1 hour of incubation at 37ºC on streptavidin-coated microassay plates, samples were incubated with anti-USF1 (sc-229; Santa Cruz Biotechnology), anti-ESR1 (sc-544; Santa Cruz Biotechnology), anti-CEBPB (sc-150; Santa Cruz Biotechnology), anti-NR3C1 (sc-8992; Santa Cruz Biotechnology), anti-HNF4A (sc-8987; Santa Cruz Biotechnology), anti-NR2F1 (sc-28611; Santa Cruz Biotechnology) and anti-MECP2 (pAb-052-050; Diagenode s.a., Li□ge, Belgium) antibodies respectively and further incubated at 37ºC for 1 hour. Bound transcription factor was then detected using a specific secondary horseradish peroxidise conjugate antibody. After intensive washing to remove the secondary antibody, TMB substrate and 1N HCL (stop solution) were added before the plates were read at 450nm wavelength. Sequences of the biotinylated double-stranded oligonucleotides are listed as follows: Unmethylated probe with -20A: 5'-AGGGC**A**TCGTGACCCG-biotin-3', Methylated probe with -20A: 5'-AGGGC**A**TC^{m}GTGACCCG-biotin-3', Unmethylated probe with -20C: 5'-AGGGC**C**TCGTGACCCG-biotin-3', Methylated probe with -20C: 5'-AGGGC**C**TC^{m}GTGACCCG-biotin-3', Unmethylated probe with -217G: 5'-TCGACCCTGCACC**G**GCTCACTCTGTTCAGCAGT-biotin-3', Methylated probe with -217G: 5'-TCGACCCTGCACC^{m}**G**GCTCACTCTGTTCAGCAGT-biotin-3', Unmethylated probe with -217A: 5'-TCGACCCTGCACC**A**GCTCACTCTGTTCAGCAGT-biotin-3', Unmethylated probe within J region: 5'-TCTGGGAACCTTGGCCCCGACTC-biotin-3', Methylated probe within J region: 5'-TCTGGGAACCTTGGCCCC^{m}GACTC-biotin-3'.

Procurement of human tissue

Visceral adipose tissue surrounding the adrenal gland was obtained from 27 patients including 10 patients with aldosterone-producing adenoma (APA), 7 patients with Cushing syndrome (CS), and 10 patients with non-functioning adrenocortical adenoma (NFA) (Supplementary Table S1). Human tissue samples were obtained from Kanazawa University Hospital (Kanazawa, Japan), Tohoku University Hospital (Sendai, Japan), and Ishikawa Central Prefectural Hospital (Kanazawa, Japan). All samples were frozen in liquid nitrogen and stored at -80°C. All experimental protocols were approved by the Institutional Review Boards of Kanazawa University, Tohoku University, and Ishikawa Central Prefectural Hospital. The purpose of the study was explained to all patients, and written informed consent was obtained from each study participant.

Animal experiments

Visceral adipose tissue was obtained from Wistar Kyoto (WKY) rats that were fed either low-sodium (0.45%) or high-sodium (7%) chow for 8 weeks (n= 5, each group). All experiments were performed in accordance with the guidelines for the use of experimental animals of the Animal Research Committee of Kanazawa University.

Results

DNA methylation within a CEBP binding site appears to be inversely associated with *AGT* expression in human tissues

AGT expression is high in the liver, heart, and brain, compared to the adrenal gland (Supplementary Fig. S1). Bisulfite sequencing showed that a CpG dinucleotide within the CEBP binding site was hypomethylated in tissues from the liver and heart, whereas hypermethylated in those from the adrenal gland and leukocytes (Supplementary Fig. S2). CpG dinucleotides within the *AGT* promoter region were completely unmethylated in the human liver cell line, HepG2. Thus, methylation status of a CpG dinucleotide within the CEBP binding site appeared to be inversely associated with *AGT* expression.

DNA methylation strongly represses the *AGT* promoter activity

To assess the effect of DNA methylation on the *AGT* transcriptional activity, we synthesized luciferase reporter constructs containing varying lengths of the *AGT* promoter either with or without CpG methylation (Supplementary Fig. S3A). We used constructs with AA at -6 (rs5051), AA at -20 (rs5050) and GG at -217 (rs5049). DNA methylation abolished the *AGT* promoter transcription activities (Supplementary Fig. S3B), indicating the *AGT* promoter was dependent on DNA methylation.

DNA methylation affects DNA-protein interaction in vitro

Several polymorphisms in the 5'-flanking region of the human *AGT* gene including the -20 and -217 polymorphisms affect the *AGT* regulation [1]. Allele-specific transcription factors such as USF1 preferential binding to -20C and ESR1 binding to -20A are associated with increased *AGT* promoter activity[2]. Previous genetic study also indicated that HNF4 may strongly activate the *AGT* transcription through binding J region (HNF4A/NR2F1 binding site) within the *AGT* promoter, but NR2F1, a transcriptional repressor, dramatically represses the human *AGT* transcription [3]. These transcription factors bind to important elements containing CpG dinucleotides which are target sites for DNA methylation (Supplementary Fig. S4A). There is now abundant evidence that DNA methylation affects DNA-protein interactions and may have a function in all processes in which such interactions occur. We performed *in vitro* analysis to determine the functional significance of methylation in the *AGT* promoter region by analysing the binding potential of methylated or unmethylated oligonucleotides (analogous to putative *AGT* regulatory elements) to nuclear proteins from H295R and HepG2. NoShift assay showed DNA methylation impaired USF1 binding at -20 with A or C in both cell lines. However, DNA methylation had little effects on activators ESR1, CEBPB, NR3C1 and HNF4A binding to the corresponding elements (Supplementary Figs. S4B and C). DNA methylation increased NR2F1 binding in HepG2 cells (Supplementary Fig. S4C) and MECP2 binding in both cell lines (Supplementary Fig. S4D).

Excess circulating aldosterone induces DNA demethylation of the *AGT* promoter and increases mRNA levels in human adipose tissue

We simultaneously determined the DNA methylation status and mRNA levels of the *AGT* gene in visceral adipose tissue from patients with APA, CS, and NFA. The *AGT* promoter, including 1 CEBP binding site, was largely unmethylated in patients with APA

(Supplementary Fig. S5A and B); this finding contrasts with those in patients with CS and NFA. *AGT* mRNA levels in adipose tissue were consistently higher in patients with APA than in patients with NFA (Supplementary Fig. S5C). These results demonstrate that excess circulating aldosterone leads to demethylation of the *AGT* promoter, including the CEBP binding site and the transcription start site, and activation of the *AGT* gene in human adipose tissue.

DNA at CpG2/3 and DNA around a transcription start site (CpG12 to 15) were both hypomethylated in adipose tissue of patients with CS, and the levels of methylation were comparable to that in the tissue of patients with APA (Supplementary Fig. S5A and B). Although the difference was not statistically significant, *AGT* mRNA levels in adipose tissue tended to be higher in patients with CS than in patients with NFA (Supplementary Fig. S5C). Although to a lesser extent than with aldosterone, excess circulating cortisol also leads to demethylation and upregulation of *AGT*.

High salt intake induces DNA demethylation of the *Agt* promoter and increases mRNA levels in rat adipose tissue

We analyzed the DNA methylation status of 12 out of 13 CpG dinucleotides within a 571 bp fragment (-364 to +207) of the rat *Agt* promoter, which contains 2 CEBP binding sites (cttgctcca, +2 to +10; ctgggaa, +78 to +84; Supplementary Fig. S6A). We simultaneously determined both DNA methylation status and mRNA levels of the *Agt* gene in visceral adipose tissue from Wistar-Kyoto rats fed either a high- or low-salt diet. CpG dinucleotides around the CEBP binding sites and a transcription start site were hypomethylated in rats fed a high-salt diet (Supplementary Fig. S6B and C). Levels of *Agt* mRNA were consistently higher in rats fed a high-salt diet than in those fed a low-salt diet (Supplementary Fig. S6D). These results demonstrate that high salt intake leads to demethylation of CEBP binding sites and a transcription start site of the *Agt* promoter and activation of the *Agt* gene in rat visceral adipose tissue, as was observed at the *AGT* promoter of human visceral adipose tissue.

DNA methylation activity in nuclear extracts was significantly lower in rats fed a high-salt diet than in those fed a low-salt diet (Supplementary Fig. S6E). No significant differences in DNA demethylation activity were observed between rats fed either a high- or low-salt diet (Supplementary Fig. S6F). A high salt intake-induced decrease in DNA methylation activity may play a crucial role in DNA demethylation of the *Agt* promoter.

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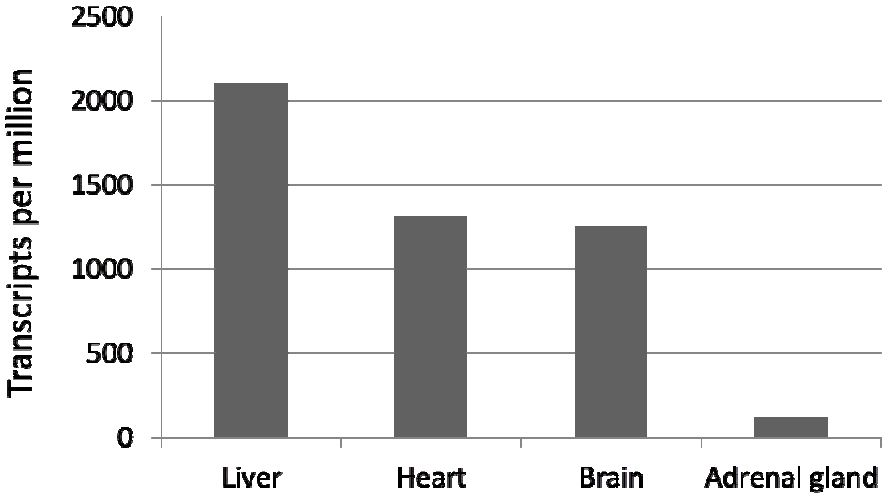
Supplementary Table S1. Characteristics of patients with adrenocortical adenoma

Variables	APA	CS	NFA
Age, yr	53.4±10.2	52.0±19.1	55.0±9.43
Systolic blood pressure, mmHg	134.3±10.9	147.0±20.7	129.1±25.1
Diastolic blood pressure, mmHg	79.3±16.8	91.6±13.9	75.4±15.2
Plasma aldosterone, pmol/L	474.5±231.0	127.2±57.9	234.5±133.7
(ng/dL)	(17.1±8.3)	(4.6±2.1)	(8.5±4.8)
Serum cortisol, nmol/L	342.1±99.6	561.7±256.9	386.9±243.1
(μg/dL)	(12.4±3.6)	(20.4±9.3)	(14.0±8.8)

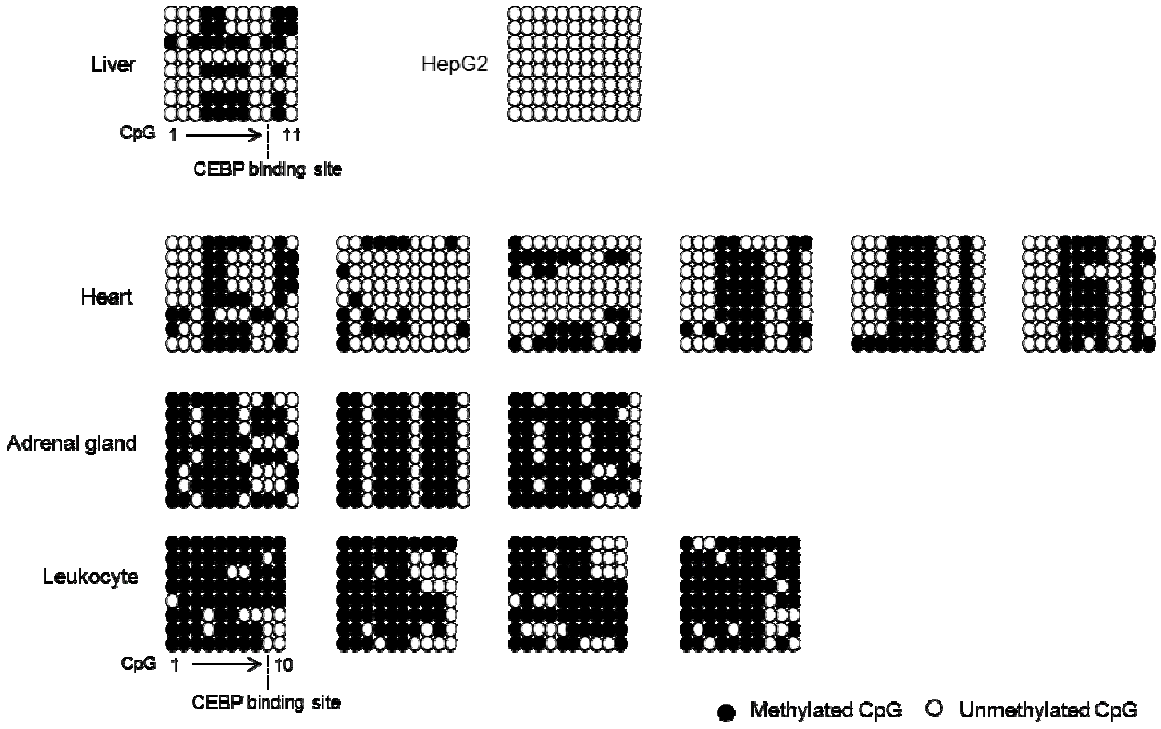
Data are shown as mean±S.D. APA indicates aldosterone-producing adenoma; CS, Cushing syndrome; NFA, non-functioning adrenocortical adenoma.

Supplementary Figure S1



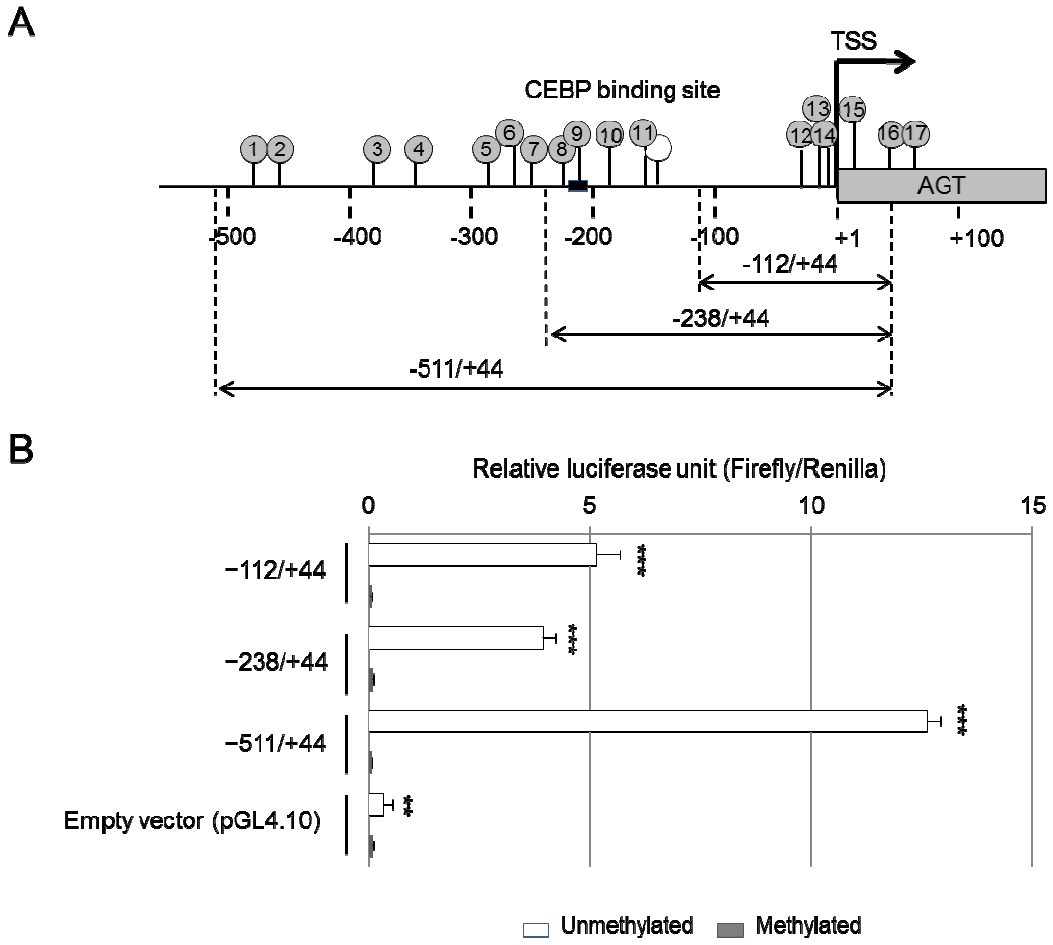
Expressed sequence tags profile of the *AGT* gene. Those data were obtained from the UniGene web site (www.ncbi.nlm.nih.gov/sites/entrez?db=unigene).

Supplementary Figure S2



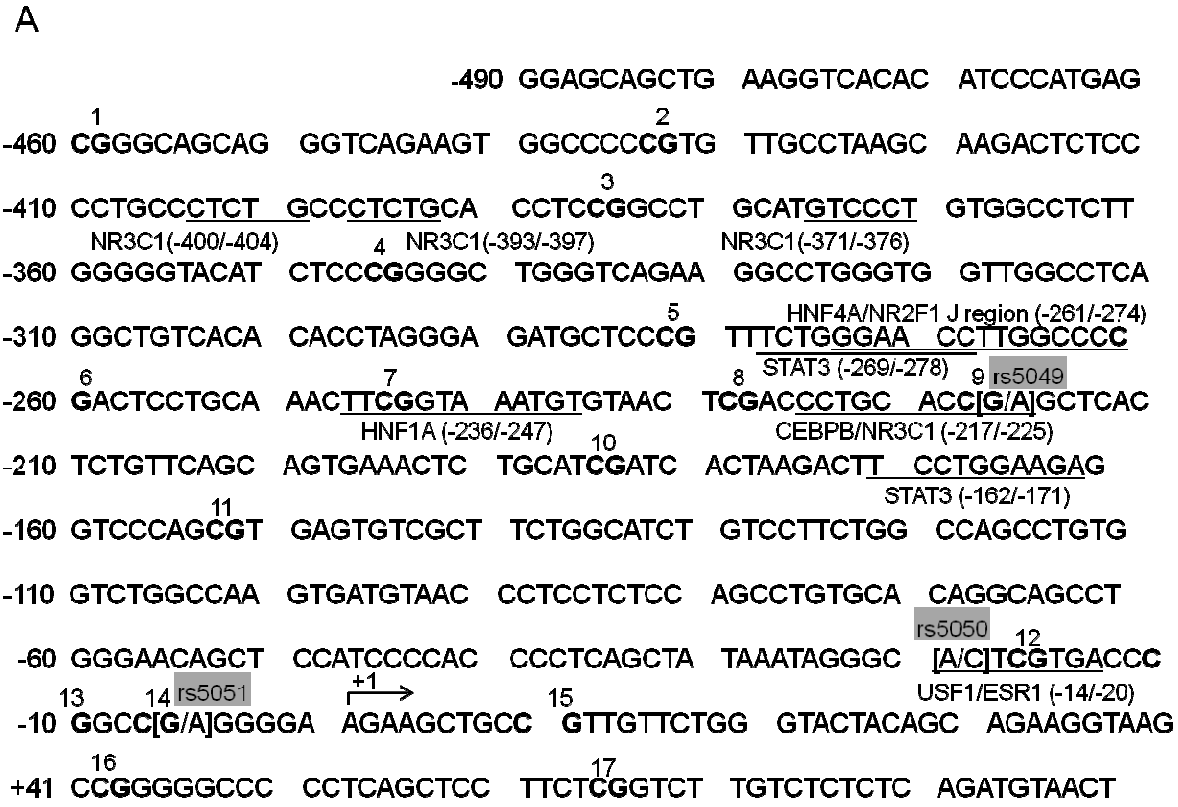
DNA methylation status in human cells and tissues. Horizontally-aligned circles represent one clone with either CpG 1-11 or CpG1-10 (Fig. 1). White and black circles indicate unmethylated and methylated cytosines of CpG sites, respectively. The CEBP binding site is indicated by dotted line.

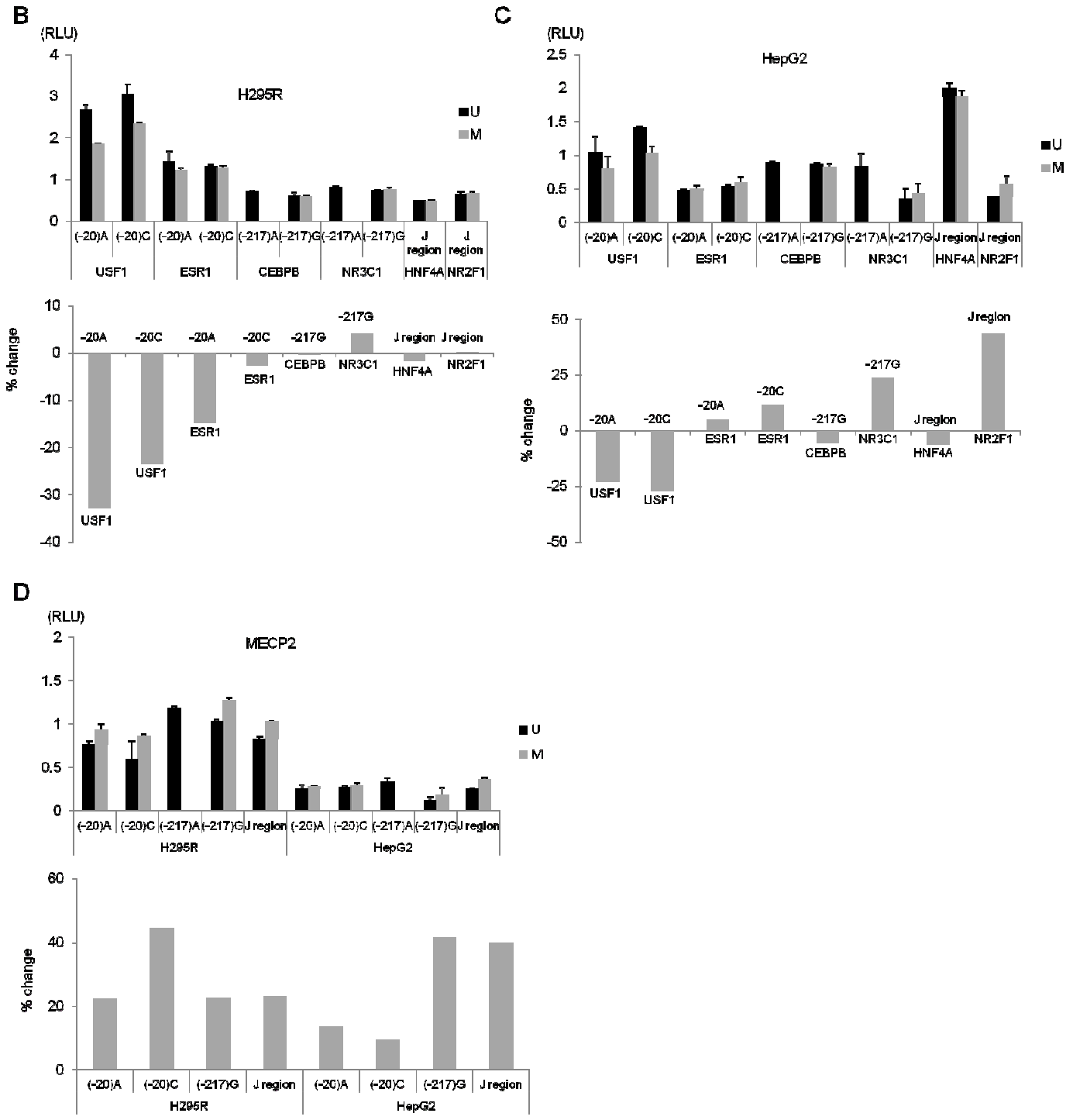
Supplementary Figure S3



Repression of the *AGT* promoter activity by DNA methylation. A, Luciferase constructs containing various *AGT* promoter regions. **B,** Effect of DNA methylation on *AGT* promoter activity. Various luciferase constructs containing in vitro methylated or mock methylated *AGT* promoter regions were transfected into H295R cells. Results are given as luciferase activity normalized to cotransfected Renilla luciferase activity and are reported as the mean± SEM of triplicate replicates. Nucleotide numbering was based on the transcription start site of the AGT gene. $^{**}p < 0.005$, $^{***}p < 0.0001$ compared to methylated constructs (2-tailed unpaired t test).

Supplementary Figure S4

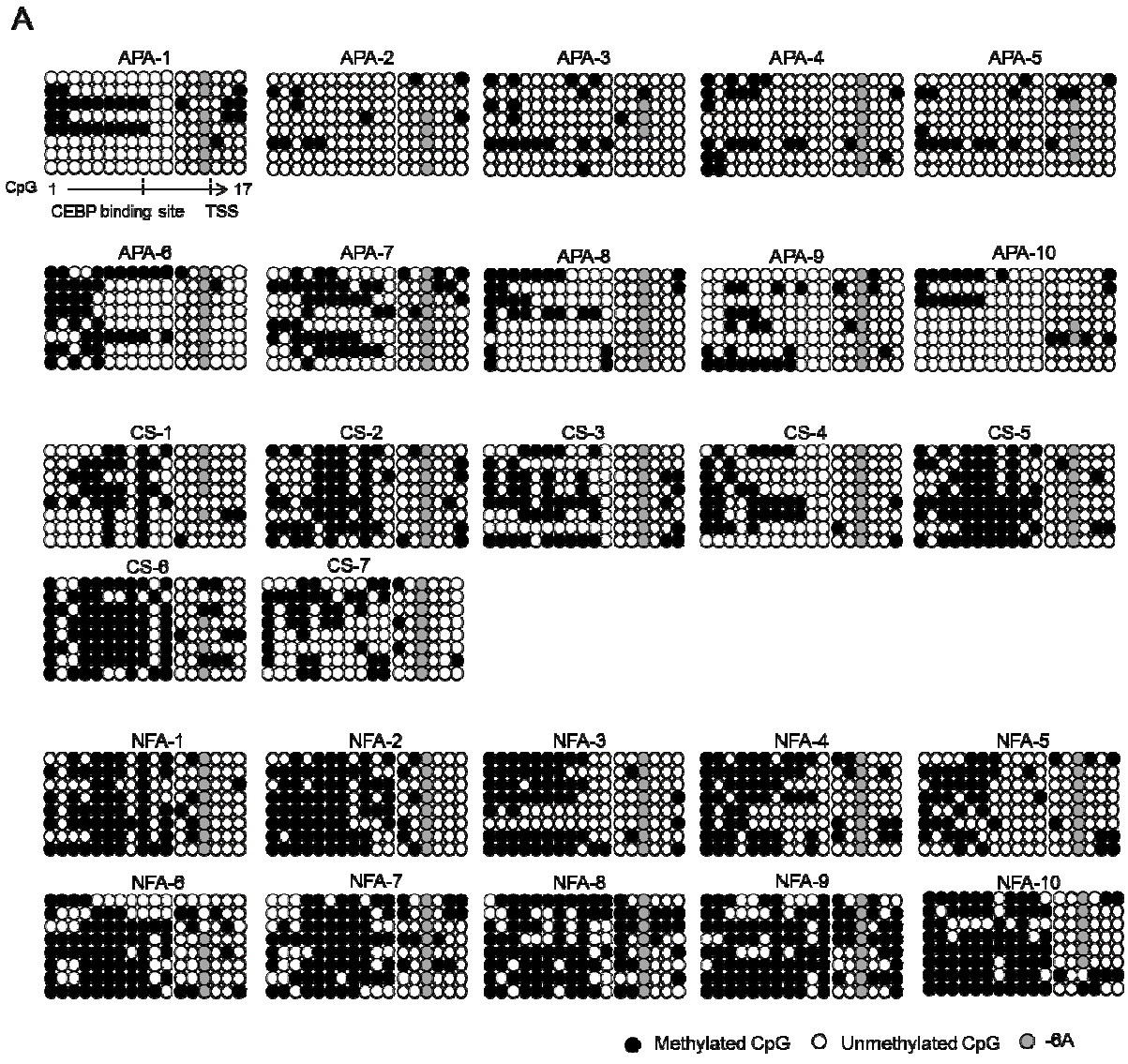


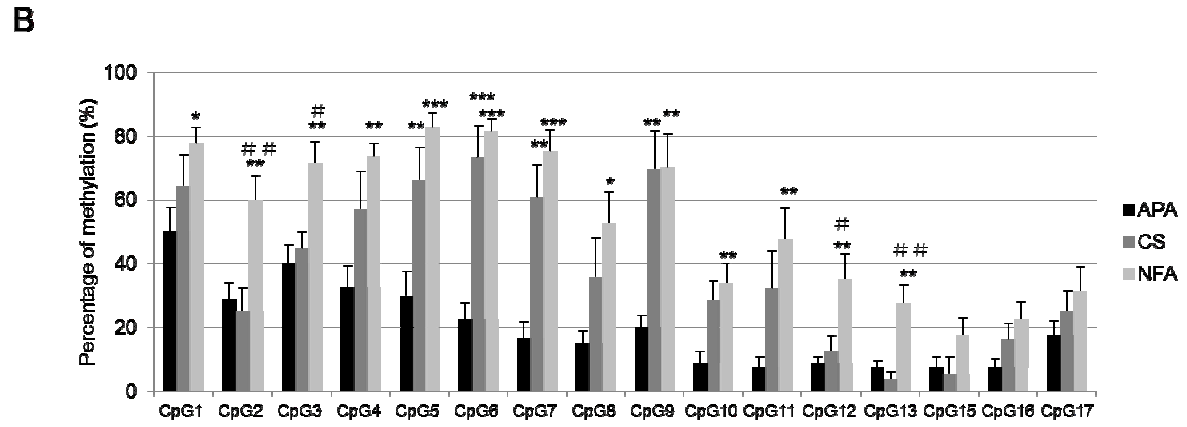


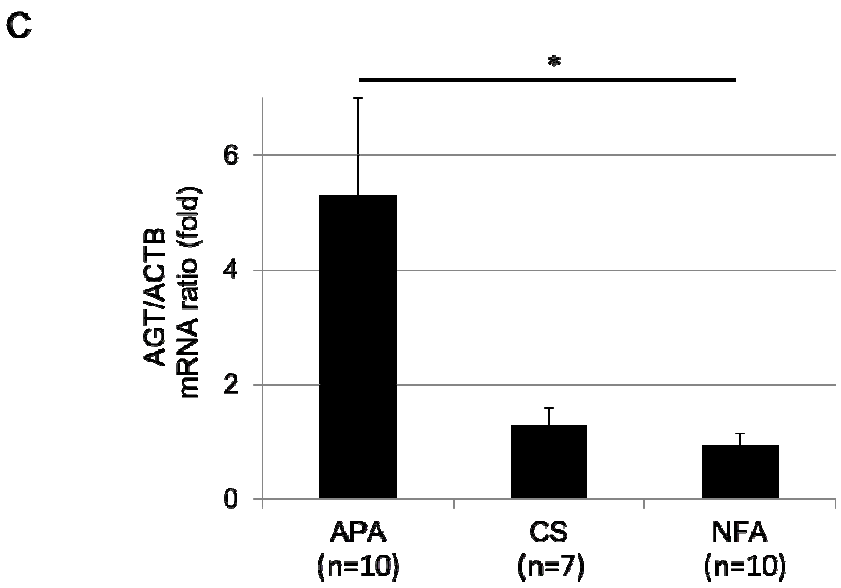
Binding of transcription factors with *AGT* promoter region *in vitro*. A, Nucleotide sequence corresponding to the *AGT* promoter. An arrow indicates the transcription start site (TSS). Nucleotide numbers are relative to the TSS. Transcription factors binding sites are underlined. CpG dinucleotides (in bold) examined in bisulfite sequencing are numbered from the 5' to 3' ends. B, Transcription factors binding activities in H295R cells. **C,** Transcription factors binding activities in HepG2 cells. **D,** MECP2 binding activities. NoShift assay was

performed using antibodies against USF1, ESR1, CEBPB (C/EBPβ), NR3C1 (GR), HNF4A, NR2F1 and MECP2 in both H295R and HepG2 cells. Percent change in transcription factors binding activities with methylated probes compared to those with unmethylated probes is shown.

Supplementary Figure S5

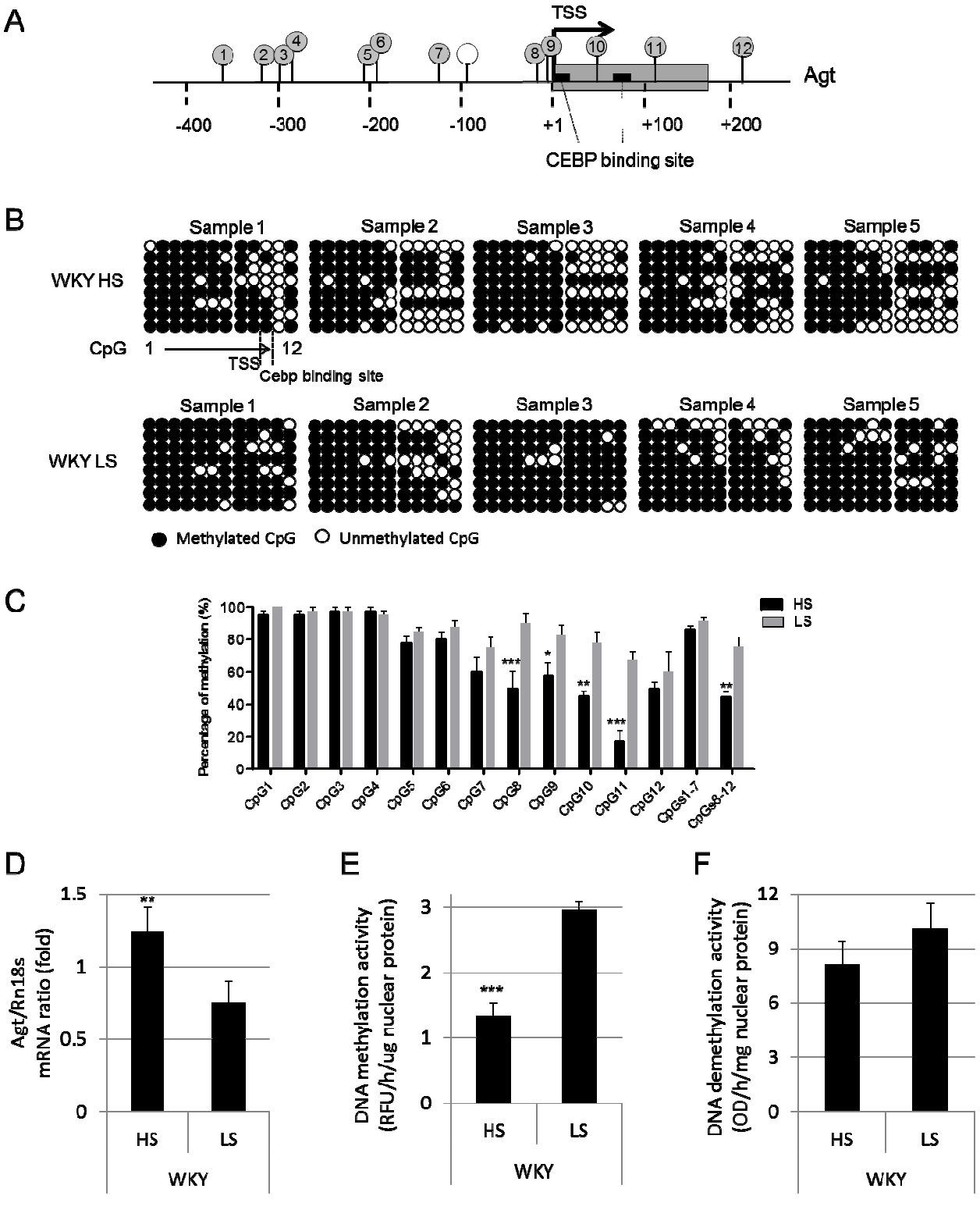






Excess circulating aldosterone-induced DNA demethylation with increased *AGT* expression in human visceral adipose tissue. A, DNA methylation status of the *AGT* promoter. Gray circles indicate the -6A allele of the G/A polymorphism (rs.5051). White circles denote unmethylated cytosine residues; black circles, methylated. APA, aldosterone-producing adenoma; CS, Cushing syndrome; NFA, non-functioning adrenocortical adenoma. **B,** DNA methylation ratios. Data are presented as mean±SEM (n=10, APA; n=7, CS; n=10, NFA). **P* < 0.05, ***P* < 0.005 and ****P* < 0.0001 compared to APA. #*P* < 0.05, ##*P* < 0.005 compared to CS (1-way ANOVA followed by Bonferroni multiple comparison test). **C,** *AGT* mRNA level. Data are presented as mean±SEM. **P* < 0.05 (1-way ANOVA followed by Bonferroni multiple comparison test).

Supplementary Figure S6



High salt intake-induced DNA demethylation with increased *Agt* expression in rat visceral adipose tissue. A, CpG dinucleotides within the rat Agt promoter. Filled lollipops denote cytosine residues. A CpG dinucleotide, white lollipop was not analysed. TSS, transcription start site. Nucleotide numbers are relative to a TSS. **B,** DNA methylation status of the *Agt* promoter. **C,** DNA methylation ratios. Data are presented as mean±SEM. **P* <0.05, ***P* < 0.005 and ****P* < 0.0001 vs LS (2-way ANOVA followed by Bonferroni posttests). **D,** *Agt* mRNA level. Results are given as fold change normalized to Rn18s. **E,** DNA methylation activity. **F,** DNA demethylation activity. WKY, Wistar-Kyoto rats; HS, high salt diet; LS, low salt diet. Data are presented as mean±SEM. ***P* < 0.005, ****P* < 0.0001 (2-tailed unpaired t test).