Nucleosome Positioning and Gene Regulation of the *SGLT2* Gene in the Renal Proximal Tubular Epithelial Cells[§]

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Received January 16, 2018; accepted June 27, 2018

ABSTRACT

Filtered glucose is mostly reabsorbed by sodium-glucose cotransporter 2 (SGLT2) in the proximal tubules. SGLT2 is predominantly expressed in the human kidney. However, the regulatory mechanisms for *SGLT2* gene expression in the human kidney remain unclear. We in this work elucidated the transcriptional regulatory mechanisms for the *SGLT2* gene by nucleosome occupancy in the *SGLT2* promoter region. Expressions of *SGLT2* mRNA and protein were markedly weaker in human kidney-derived HK-2 cells than the human kidney. The nucleosome occupancy level in the *SGLT2* promoter region was low in the kidney, but high in HK-2 cells. A treatment with a histone deacetylase inhibitor trichostatin A (TSA) decreased nucleosome occupancy in the promoter region and increased *SGLT2*

expression levels in HK-2 cells. The upregulation of *SGLT2* expression by histone acetylation was accompanied by a higher binding frequency of hepatocyte nuclear factor (HNF) 1 α , a transcriptional modulator of *SGLT2* in the human kidney, to the promoter region. The transfection of a HNF1 α expression plasmid into HK-2 cells resulted in the upregulation of *SGLT2* mRNA expression in the presence of TSA, but not in the treatment of dimethylsulfoxide as a control. Nucleosome occupancy in the promoter region was markedly higher in the liver and small intestine than the kidney. Our results indicate that tissue-specific nucleosome occupancy plays an important role in the regulation of *SGLT2* gene expression via HNF1 α binding at the *SGLT2* promoter region.

Introduction

The kidney plays an important role in the reabsorption of glucose from the glomerular filtrate. Approximately 180 g glucose is typically filtered by the kidneys each day and reabsorbed in proximal tubule epithelial cells (PTECs) by sodium-glucose cotransporters (SGLTs) (Wright, 2001; Chen et al., 2010; Gerich, 2010; Pérez López et al., 2010). In the S1 and S2 segments of the proximal tubule, approximately 90% of filtered glucose is reabsorbed through SGLT2 (Gerich, 2010). Previous studies demonstrated that a deficiency in SGLT2 expression decreased blood glucose levels. The knockout of *SglT2* in diabetic mice reduced blood glucose levels and prevented glomerular hyperfiltration (Vallon et al., 2013). Therefore, the inhibition of SGLT2 activity in the kidney is regarded as an effective strategy for glycemic control in diabetic patients. Selective SGLT2 inhibitors have been developed as a new class of oral medication for the treatment of type 2 diabetes mellitus. These drugs reduce glucose reabsorption by SGLT2 in the proximal tubules, thereby increasing urinary

glucose excretion and lowering plasma glucose levels (Jabbour and Goldstein, 2008; Boldys and Okopień, 2009; Misra, 2013).

Previous studies focused on the regulation of *SGLT2* expression by transcription factors. Hepatocyte nuclear factor (HNF) 1 α , which has been characterized as a modulator of *SGLT2* expression, was shown to directly bind to the *SGLT2* 5'-flanking region (5'-FR) and control *SGLT2* gene expression (Pontoglio et al., 2000; Freitas et al., 2008; Zhao et al., 2016). Additionally, HNF4 α and SP1 have been reported to play an important role in *SGLT2* expression (Kothinti et al., 2010; Bonner et al., 2015). Although these transcription factors are expressed in various tissues such as the liver and intestines, *SGLT2* is not. Therefore, another mechanism for the regulation of *SGLT2* expression appears to contribute to tissue-specific gene expression. The nucleosome is a basic unit of chromatin and consists of approximately 147 bp of DNA wrapped around histones. Nucleosome occupancy plays an important role in the epigenetic regulation of gene expression by inhibiting the initiation of transcription at gene promoters (Lorch et al., 1987; Workman, 2006). Previous studies demonstrated that nucleosomes control tissue-specific transcription factor binding to gene promoters, and, thus, contribute to tissue-specific gene expression (Hoffman et al., 2010; Hu et al., 2011; Tsui et al., 2011; Yang et al., 2013; Zhang et al., 2015). However, the relationship between nucleosome occupancy and tissue-specific *SGLT2* expression remains unknown. Therefore, a

This work was supported by Japan Society for the Promotion of Science KAKENHI [Grant JP17K08954].

<https://doi.org/10.1124/mol.118.111807>.

[§] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: 5'-FR, 5'-flanking region; ChIP, chromatin immunoprecipitation; DMSO, dimethylsulfoxide; HNF, hepatocyte nuclear factor; NOME-Seq, nucleosome occupancy and methylome sequencing; NuSA, nucleosome-scanning assay; PCR, polymerase chain reaction; PTEC, proximal tubule epithelial cell; SGLT2, sodium-glucose cotransporter 2; siHNF1 α , HNF1 α siRNA; siRNA, small interfering RNA; TSA, trichostatin A; TSS, transcription start site.

Fig. 1. Analysis of transcriptional activity of *SGLT2* 5'-FR and *SGLT2* expression levels in HK-2 cells. (A) Luciferase activity of a series of reporter constructs containing *SGLT2* 5'-FR in HK-2 cells. Results are expressed as fold increases in pGL4.10. (B) *SGLT2* mRNA levels in HK-2 cells and the human kidney were measured by quantitative PCR and normalized to RPL13 mRNA levels. Results are expressed relative to *SGLT2* mRNA levels in the human kidney. (C) Western blot analyses of *SGLT2* and β -actin protein expression in HK-2 cells and the human kidney. *SGLT2* protein levels represent the density ratio for the bands of *SGLT2* and β -actin. Results are expressed relative to *SGLT2* protein levels in the human kidney. Results represent the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$.

clearer understanding of nucleosome occupancy at the *SGLT2* promoter may provide significant insights into transcriptional regulatory mechanisms for the *SGLT2* gene in the human kidney.

In the present study, we focused on the transcriptional regulation of the *SGLT2* gene by nucleosome occupancy in *SGLT2* 5'-FR in the human kidney. We demonstrated that decreasing nucleosome occupancy in the *SGLT2* promoter plays a critical role in the upregulation of *SGLT2* expression. We also revealed that HNF1 α binds to the *SGLT2* promoter in the human kidney, and the activation of *SGLT2* transcription by HNF1 α requires low nucleosome occupancy through histone acetylation in the *SGLT2* promoter.

Materials and Methods

Human Tissue. A human kidney cortex and small intestine obtained from surgical specimens were purchased from Integrated Laboratory Services-Biotech (Chestertown, MD). A human liver sample was obtained from a Caucasian donor at the National Disease Research Interchange (Philadelphia, PA). All assays using human tissue samples were performed by three independent experiments ($n = 3$). The use of human tissues was approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (Fukuoka, Japan).

Cell Culture. HK-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum at 37°C in an incubator with 5% CO₂.

Construction of *SGLT2* Reporter Plasmids. The DNA fragment of *SGLT2* 5'-FR (–3185/+18) was amplified by a polymerase chain reaction (PCR) using primers (described as –3185 and +18 in Supplemental Table 1) and human genomic DNA as a template. The pGL4.10 vector (Promega, Madison, WI) was digested by KpnI and HindIII restriction enzymes and then ligated with the PCR product using the In-Fusion HD Cloning kit (TaKaRa, Shiga, Japan). Deletion constructs [–2320/+18, –1587/+18, –485/+18, –154/+18, –44/+18, and del(–51/–37)] were generated by In-Fusion cloning or the QuikChange II site-directed mutagenesis method (Supplemental Table 1) using the –3185/+18 construct as a template. The sequences of these plasmids were confirmed by direct sequencing using primers (Supplemental Table 2) described previously (Nishimura et al., 2014).

Construction of a HNF1 α Expression Plasmid. The coding region of HNF1 α was amplified by PCR using cDNA synthesized from the human kidney and primers (Supplemental Table 3). The pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) was digested by EcoRI

and EcoRV restriction enzymes and then ligated with the PCR product using the In-Fusion HD Cloning kit. The sequence of the plasmid was confirmed by directed sequencing using primers (Supplemental Table 3).

Luciferase Assay. HK-2 cells were seeded on 24-well culture plates at 0.2×10^5 cells/well and incubated for 24 hours. A series of reporter constructs [equimolar amounts of the empty pGL4.10 plasmid (250 ng), 500 ng HNF1 α expression plasmid or the empty pcDNA3.1(+) plasmid, and 25 ng internal standard pGL4.70 (Promega)] were transfected into HK-2 cells using FuGENE HD Transfection Reagent (Promega), according to the manufacturer's instructions. After 48 hours, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 Luminometer (Promega). Transcriptional activities were expressed as the ratio of *Firefly* luciferase to *Renilla* luciferase activity.

RNA Interference. The transfection mix was prepared in Opti-MEM (Invitrogen) with HNF1 α small interfering RNA (siRNA) (siHNF1 α ; Sigma-Aldrich, St. Louis, MO) or control siRNA (MISSION siRNA Universal Negative Control; SIC-001; Sigma-Aldrich) and Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's directions. The final concentrations of siRNA and lipofectamine added to the cells were 20 nM and 2 μ l/ml, respectively. HK-2 cells were cultured in the presence of the transfection mixture for 24 hours, and cells were treated with dimethylsulfoxide (DMSO) or 1.0 μ M trichostatin A (TSA; Wako, Osaka, Japan) for 24 hours. siHNF1 α sequences were as follows: 5'-CAGUGAGACUGCAGAA-GUAtt-3' (sense sequence) and 5'-TACTTCTGCAGTCTCACTGtt-3' (antisense sequence).

Total RNA Isolation and Capture of Nascent RNAs. HK-2 cells were seeded on a six-well plate at 1.5×10^5 cells/well and incubated for 24 hours, and cells were then transfected with 500 ng HNF1 α expression plasmid or pcDNA3.1(+) plasmid using the FuGENE HD Transfection Reagent (Promega). After 48 hours, cells were treated with DMSO or 1.0 μ M TSA, together with 0.2 mM 5-ethynyl uridine for 24 hours. Total RNA was isolated from the human kidney and HK-2 cells using ISOSPIN Cell & Tissue RNA (Nippon Gene, Tokyo, Japan). Ethynyl uridine-labeled nascent RNA was biotinylated and captured using a Click-iT Nascent RNA Capture Kit (Invitrogen), according to manufacturer's instructions.

cDNA Synthesis and Quantitative PCR. Total RNA (500 ng) or nascent RNA (200 ng) was transcribed using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (TaKaRa) under the following conditions: 95°C for 2 minutes, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. *SGLT2* and HNF1 α mRNA levels were normalized to 60S ribosomal protein L13 (RPL13) mRNA levels. The primers used in this study are listed in Supplemental Table 4.

Fig. 2. Analysis of epigenetic regulation in *SGLT2* 5'-FR in HK-2 cells and the human kidney. (A and B) NOME-Seq data of *SGLT2* 5'-FR in HK-2 cells and the human kidney. The arrow indicates the *SGLT2* TSS. (A) Vertical lines indicate CpG sites. White circles represent unmethylated CpG sites, and black circles represent methylated CpG sites. (B) Vertical lines indicate GpC sites. White circles represent unmethylated GpC sites, and black circles represent methylated GpC sites. Deep gray bars represent nucleosome occupancy, which is the region of consecutive unmethylated GpC sites over 147 bp. (C) NuSA data of 5'-FR in HK-2 cells (black circles) and the human kidney (white circles). Relative nucleosome occupancy is expressed relative to the level of nucleosome occupancy in each sample without the micrococcal nuclease treatment and indicated by the midpoints of each amplicon. Results represent the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ for HK-2 cells versus the human kidney.

Nucleosome Occupancy and Methylome Sequencing. Nucleosome occupancy and methylome sequencing (NOME-Seq) were performed according to the manufacturer's instructions (Active Motif, Carlsbad, CA), with slight modifications. Approximately 50 mg frozen human tissues and 7.5×10^5 of HK-2 cells were cross-linked by 1% formaldehyde at room temperature for 15 and 5 minutes, respectively, and cross-linking was quenched by adding 1.25 M glycine. Cells were homogenized in lysis buffer with 40 strokes of a Potter–Elvehjem homogenizer (Wheaton Industries, Millville, NJ). Homogenized kidney tissues were filtered through an 80- μ m Nylon Woven Net Filter (Merck Millipore, Darmstadt, Germany) to remove tissue debris. Chromatin was sheared to fragments larger than 1 kb. Sheared

chromatin was treated with 0 or 20 U GpC methyltransferase at 37°C for 4 hours. Reactions were stopped by the addition of stop solution and incubated at 95°C for 15 minutes. Bisulfite conversion was then performed on DNA. Bisulfite-converted DNA was used for PCR amplification with primers for *SGLT2* 5'-FR (Supplemental Table 5). PCR products were cloned into the pGEM-T easy vector (Promega). Twenty colonies in GpC methyltransferase-treated DNA and 10 colonies in nontreated DNA were subjected to colony PCR, followed by sequencing with primers (Supplemental Table 5) for the assessment of nucleosome occupancy and CpG methylation, respectively. GpCpG sites were removed from the analysis of nucleosome occupancy as they are both a GpC and CpG context.

Fig. 3. Influence of the TSA treatment on *SGLT2* expression and histone acetylation in *SGLT2* 5'-FR in HK-2 cells. HK-2 cells were treated with DMSO or TSA for 24 hours. (A) *SGLT2* mRNA levels were measured by quantitative PCR and normalized to RPL13 mRNA levels. Results are expressed relative to *SGLT2* mRNA levels in DMSO-treated cells (control). (B) Scheme of the ChIP assay in 5'-FR. The upper arrow indicates the *SGLT2* TSS. Lower arrows indicate two sets of PCR primers targeting 5'-FR, designated as distal and proximal. (C) ChIP analyses targeting 5'-FR in DMSO- or TSA-treated cells using antibodies against normal IgG (white bars) and acetylated histone H3 (black bars). (D) Semiquantitative analyses of acetylated histone H3 enrichment in two regions in ChIP analyses in (C). Results are expressed as the percentage of the immunoprecipitate over total input DNA. Results represent the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$.

Nucleosome Scanning Assay. A nucleosome-scanning assay (NuSA) was performed using the EpiScope Nucleosome Preparation Kit (TaKaRa), according to the manufacturer's protocol, with slight modifications. Approximately 20 mg frozen human kidney or 2.0×10^6 of HK-2 cells was washed with ice-cold phosphate saline buffer and incubated in lysis buffer for 10 minutes. Tissue samples were homogenized and filtered, as described above. The nuclei released were treated with 0 or 4 U micrococcal nuclease for 30 minutes at 37°C and purified using NucleoSpin Gel and PCR Clean-up (TaKaRa). Nucleosomal DNA enrichment was evaluated by quantitative PCR with overlapping primer pairs (Supplemental Table 6), producing a high-resolution, quantitative map of the nucleosome location and occupancy. Relative nucleosome occupancy was presented by the ratio to the nucleosomal DNA enrichment of LINE-1 as a reference gene using the Pfaffl method (Pfaffl, 2001).

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation (ChIP) assays were performed using the EpiQuik ChIP or Tissue ChIP kit (Epigentek, Farmingdale, NY), according to manufacturer's instructions. Briefly, approximately 50 mg human kidney or 1.0×10^6 of HK-2 cells were cross-linked, homogenized, and filtered, as described above. Chromatin was sheared to fragments ranging from 300 to 600 bp. The sonicated lysate (5 μ l) was used to quantify the total amount of DNA (input). Immunoprecipitation was performed for 90 minutes with 2 μ g antibodies: anti-acetylated histone H3 (Merck), anti-HNF1 α (sc-393925; Santa Cruz Biotechnology, Santa Cruz, CA), or normal mouse IgG as the negative control. Immunoprecipitated DNA and input DNA were analyzed by semi-quantitative PCR or quantitative PCR using primers for *SGLT2* 5'-FR (Supplemental Table 7). Semiquantitative PCR was performed using Gene RED PCR Mix Plus (Nippon Gene). PCR products were resolved by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensity of bands was quantified with the Lumino Image Analyzer LAS-3000 and Multi Gauge (Fujifilm, Tokyo, Japan).

Western Blot Analysis. The human kidney was pulverized and dissolved in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS). HK-2 cells were harvested and homogenized with radioimmunoprecipitation assay buffer 24 hours after the TSA treatment. Deglycosylation was performed by incubation with peptide-N-glycosidase F (New England Biolabs, Ipswich, MA). Lysate samples were separated on 9% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes by Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA). Membranes were blocked in phosphate-buffered saline with 0.1% Tween 20 and 5% nonfat dry milk at room temperature for 20 minutes and incubated with the primary anti- β -actin (ab6276, diluted at 1:5000; Abcam, Cambridge, UK), anti-SGLT2 (sc-393350, diluted at 1:200; Santa Cruz Biotechnology), and anti-HNF1 α (sc-6547, diluted at 1:1000; Santa Cruz Biotechnology) antibodies at 4°C overnight. After washing, the membrane was incubated at room temperature for 1 hour with the ECL Mouse IgG, horseradish peroxidase-linked whole Ab (NA931, diluted at 1:10,000; GE Healthcare, Little Chalfont, UK), or donkey anti-goat IgG-horseradish peroxidase (sc-2020, diluted at 1:10,000; Santa Cruz Biotechnology). After washing the membrane, immune complexes were visualized using a chemiluminescence reagent (Amersham ECL Select; GE Healthcare) and captured with the Lumino Image Analyzer LAS-3000.

Statistical Analysis. Results from three independent experiments are expressed as the mean $\pm$ S.D. Statistical analyses were performed with the statistical software program R. The means of two groups were compared with an unpaired Student's *t* test (two-tailed). Comparisons of the means of multiple groups were performed with the Tukey-Kramer test. Two-way analysis of variance was used to examine the effects of two factors on an experimental response. All tests were considered significant at $P < 0.05$.

Results

Analysis of SGLT2 mRNA Levels and Nucleosome Occupancy in HK-2 Cells and Human Kidney Tissue. *SGLT2* is primarily expressed in the cortex of the kidney and located in the S1 and S2 segments of the proximal tubule. The HK-2 cell line, an immortalized PTEC line from a normal adult human kidney, is available as a human PTEC model (Ryan et al., 1994; Wilmer et al., 2010). However, a luciferase assay with HK-2 cells showed no significant changes in the luciferase activity of *SGLT2* 5'-FR reporter constructs (Fig. 1A). Quantitative PCR with cDNA synthesized from total RNA and Western blot analyses demonstrated that *SGLT2* mRNA and protein levels were markedly lower in HK-2 cells than in the human kidney (Fig. 1, B and C). These results suggest different regulatory mechanisms for *SGLT2* gene

Fig. 4. Influence of the TSA treatment on nucleosome occupancy in *SGLT2* 5'-FR in HK-2 cells. (A) NOME-Seq data of *SGLT2* 5'-FR in DMSO- or TSA-treated cells. Vertical lines indicate GpC sites. White circles represent unmethylated GpC sites, and black circles represent methylated GpC sites. Deep gray bars represent nucleosome occupancy, which is the region of consecutive unmethylated GpC sites over 147 bp. (B) NuSA data of 5'-FR in DMSO (black circles)- or TSA (white circles)-treated cells. Relative nucleosome occupancy is expressed relative to the level of nucleosome occupancy in each sample without the micrococcal nuclease treatment and indicated by the midpoints of each amplicon. Results represent the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ for DMSO group versus TSA group.

expression between HK-2 cells and the human kidney. Therefore, we performed NOME-Seq to identify endogenous DNA methylation and the distribution of nucleosome occupancy in 5'-FR in HK-2 cells and the human kidney. NOME-Seq results of CpG methylation profiles showed that 5'-FR was highly methylated in HK-2 and the human kidney (Fig. 2A). In NOME-Seq, GpC methyltransferase may artificially methylate GpC sites that are not protected by nucleosomes or DNA-binding proteins. GpC methylation patterns represent the sites of a genome occupied by nucleosomes. To confirm whether GpC sites in GpC methyltransferase-treated samples were successfully methylated, we assessed GpC sites in the *GAPDH* promoter. These GpC sites were highly methylated in extracted DNA samples (Supplemental Fig. 1). The 5'-FR was occupied by more nucleosomes in HK-2 cells than in the human kidney (Fig. 2B; Supplemental Fig. 2A). We then performed NuSA to confirm nucleosome occupancy in *SGLT2* 5'-FR. Relative nucleosome occupancy was markedly higher in HK-2 cells than in the human kidney (Fig. 2C). These results indicated that *SGLT2* 5'-FR in HK-2 cells is highly occupied by nucleosomes.

Influence of the TSA Treatment on *SGLT2* Expression and Nucleosome Occupancy in *SGLT2* 5'-FR in HK-2 Cells. Histone acetylation is a well-known mechanism

for epigenetic regulation that upregulates gene expression. Histone deacetylase inhibitors stimulate gene expression by enhancing histone acetylation. In the present study, HK-2 cells were treated with TSA, the most common histone deacetylase inhibitor, to evaluate the effects of histone acetylation on *SGLT2* expression and nucleosome occupancy in *SGLT2* 5'-FR. Quantitative PCR with cDNA synthesized from total RNAs showed that the TSA treatment significantly upregulated *SGLT2* mRNA expression (Fig. 3A). The ChIP assay showed that the TSA treatment induced the acetylation of histone H3 in the region between -145 and +135 bp upstream of the *SGLT2* transcription start site (TSS), whereas histone H3 acetylation between -547 and -212 bp upstream of the TSS was not significantly induced (Fig. 3, B-D). We then performed NOME-Seq and NuSA to assess changes in nucleosome occupancy in 5'-FR. The nucleosome occupancy level within approximately 300 bp upstream of the TSS was decreased by the TSA treatment, whereas nucleosome occupancy was not changed in the region between -500 and -300 bp upstream of the TSS (Fig. 4; Supplemental Fig. 2B). NOME-Seq results also showed that the TSA treatment had no effect on endogenous DNA methylation in 5'-FR (Supplemental Fig. 3). These results indicate that

Fig. 5. Analysis of HNF1α expression and HNF1α-binding frequency after the TSA treatment in HK-2 cells. (A) ChIP analyses with quantitative PCR targeting the HNF1α binding site in *SGLT2* 5'-FR in DMSO- or TSA-treated cells and the human kidney using antibodies against normal IgG (white bars) and HNF1α (black bars). The relative binding frequency of HNF1α was measured by quantitative PCR and normalized to that of the input. (B) HK-2 cells were reverse transfected with negative control or siHNF1α for 24 hours and then treated with DMSO or TSA for 24 hours. Western blot analyses representing HNF1α and β-actin protein expression in HK-2 cells. (C) Protein levels represent the density of the ratio for the bands of HNF1α and β-actin in (B). Results were normalized to HNF1α protein levels in DMSO-treated cells (control). (D) *SGLT2* mRNA levels were measured by quantitative PCR and normalized to RPL13 mRNA levels. Results are expressed relative to *SGLT2* mRNA levels in DMSO-treated cells. Results represent the mean ± S.D. of three independent experiments. **P* < 0.05.

histone acetylation by the TSA treatment upregulates *SGLT2* expression and decreases nucleosome occupancy levels within approximately 300 bp upstream of the TSS.

Analysis of HNF1α Expression and HNF1α-Binding Frequency After the TSA Treatment in HK-2 Cells. Transcription factors capable of binding to *SGLT2* 5'-FR were screened by the Database Center for Life Science Galaxy (Institute in Research Organization of Information and Systems, Chiba, Japan). A binding site of HNF1α, which is a transcriptional modulator of *SGLT2* in the human kidney, was found between -51 and -37 bp (-51/-37) upstream of the *SGLT2* TSS. Chromatin immunoprecipitated (ChIP) assays with quantitative PCR demonstrated that HNF1α binding was detected in the region between -144 and +26 bp upstream, including the predicted HNF1α binding site (-51/-37), in the human kidney (Fig. 5A). TSA-treated HK-2 cells showed a significantly higher binding frequency than DMSO-treated cells (Fig. 5A). We next performed RNA interference with siHNF1α to evaluate a role for HNF1α in regulating *SGLT2* gene expression in HK-2 cells. Quantitative PCR and Western blot analyses showed that the TSA treatment caused the upregulated HNF1α mRNA (Supplemental Fig. 4) and protein expression (Fig. 5, B and C). The transfection of siHNF1α in DMSO- or TSA-treated HK-2 cells significantly decreased HNF1α mRNA (Supplemental Fig. 4) and protein expression (Fig. 5, B and C). The knockdown of HNF1α decreased *SGLT2* mRNA expression in TSA-treated HK-2 cells, whereas no significant changes were observed in DMSO-treated HK-2 cells (Fig. 5D). These results suggest that the TSA treatment upregulates *SGLT2* gene expression by increased HNF1α binding in *SGLT2* 5'-FR in HK-2 cells.

Deletion Analysis of *SGLT2* 5'-FR in HNF1α-Expressing HK-2 Cells. We performed luciferase assays using HNF1α-expressing HK-2 cells to clarify whether the predicted HNF1α binding site contributes to the transcription of the *SGLT2* gene (Fig. 6). The transfection of the HNF1α-negative plasmid (pcDNA3.1) showed that luciferase activity levels were not significantly different in the four reporter constructs containing *SGLT2* 5'-FR from the control reporter plasmid (pGL4.10). The transfection of the HNF1α expression plasmid significantly enhanced the luciferase activities of the -154/+18 construct and -485/+18 construct, whereas no significant differences were observed in luciferase activities between -154/+18 and -485/+18. These constructs contained the predicted HNF1α binding site (-51/-37). We then measured the luciferase activity of the HNF1α binding site-deleted construct [del(-51/-37)]. Cells transfected with del(-51/-37) demonstrated that the deletion of the binding site resulted in loss of HNF1α-mediated luciferase activity. These results suggest that the predicted HNF1α binding site (-51/-37) is essential for HNF1α-mediated transcription of the *SGLT2* gene.

Quantitative Analysis of Effects of the TSA Treatment on *SGLT2* mRNA Levels in HNF1α-Expressing HK-2 Cells. Quantitative PCR with cDNA synthesized from total RNA and nascent RNA was performed to evaluate the effects of the TSA treatment on *SGLT2* mRNA expression in HNF1α-expressing HK-2 cells (Fig. 7). In the present study, the nascent RNA was defined as RNA transcribed after the DMSO or TSA treatment. Total and nascent *SGLT2* mRNA expression levels were significantly higher in HNF1α-expressing cells treated with TSA than in pcDNA3.1-transfected cells. Nascent

Fig. 6. Deletion analysis of *SGLT2* 5'-FR in HNF1 α -expressing HK-2 cells. HK-2 cells were transfected with a series of reporter constructs or a control reporter plasmid (pGL4.10), together with the HNF1 α -negative plasmid (pcDNA3.1, white bars) or HNF1 α expression plasmid (black bars). The position of the deleted region is indicated with Del(-51/-37). Relative luciferase activity is expressed as a fold increase in pGL4.10 and represents the mean $\pm$ S.D. of three independent experiments. * P < 0.05.

SGLT2 mRNA levels were 1.8-fold higher than total *SGLT2* mRNA in HNF1 α -expressing cells treated with TSA. In contrast, no significant changes in *SGLT2* mRNA expression levels were observed in HNF1 α -expressing cells treated with DMSO. These results indicate that histone acetylation induced by the TSA treatment plays an important role in the HNF1 α -mediated upregulation of the *SGLT2* gene.

Analysis of Nucleosome Occupancy in Human Kidney, Liver, and Small Intestine Tissues. HNF1 α is expressed in various tissues, such as the liver and intestines, whereas *SGLT2* is not. We attempted to identify nucleosome occupancy in 5'-FR containing the HNF1 α binding site in the human liver and small intestine. The region within approximately 300 bp upstream of the *SGLT2* TSS showed markedly higher nucleosome occupancy in the liver and small intestine than in the kidney (Fig. 8). These results suggest that tissue-specific nucleosome occupancy in 5'-FR plays an important role in the regulation of *SGLT2* expression via HNF1 α binding.

Discussion

In the present study, we focused on the transcriptional regulatory mechanisms of the *SGLT2* gene in the human

kidney. *SGLT2* is known to be expressed in PTECs of the human kidney. Some PTEC lines, such as LLC-PK1, KPT2, and MDCK cells, have been used to analyze expression and regulation of the *SGLT2* gene in the kidney (Da Costa-Pessoa et al., 2014; Jiang et al., 2014; Zapata-Morales et al., 2014). The human kidney-derived HK-2 cell line, which is available as a human PTEC model (Ryan et al., 1994; Wilmer et al., 2010), has been used to evaluate the regulatory mechanism for the *SGLT2* gene in PTECs of the human kidney (Panchapakesan et al., 2013). The principal portion of the kidney was divided into two distinct regions: renal cortex and renal medulla. The expression level was extremely higher in renal cortex than the renal medulla (You et al., 1995; Chen et al., 2010). The proximal tubule, which contains PTECs, is a portion of the renal cortex. Therefore, we performed in vitro analyses using the HK-2 cells derived from PTECs and the renal cortex to reveal the transcriptional profiles of the *SGLT2* gene in the human kidney.

The regulatory mechanisms for *SGLT2* gene expression in the human kidney remain unclear. Our results showed that HK-2 cells exhibited weak transcriptional activity for the *SGLT2* gene (Fig. 1A). We demonstrated that the levels of *SGLT2* mRNA and protein expression were extremely lower in HK-2 cells than the human kidney (Fig. 1, B and C). These results suggest that *SGLT2* expression is not activated in HK-2 cells, which is derived from the PTEC of the human kidney. DNA methylation, which is a well-known epigenetic mechanism for transcriptional silencing (Jones and Baylin, 2007), plays an important role in the regulation of tissue-specific gene expression by inhibiting the binding of transcription factors to DNA (Song et al., 2005; Moore et al., 2013; Medvedeva et al., 2014). Previous studies have reported that kidney-specific expression of various genes is associated with DNA methylation of the promoter region around the TSS (Aoki et al., 2008; Kikuchi et al., 2010; Azuma et al., 2012). Our results of the assessment of CpG methylation using NOME-Seq demonstrated that all CpG sites in *SGLT2* 5'-FR were highly methylated in HK-2 cells and the human kidney (Fig. 2A), suggesting that endogenous DNA methylation is not involved in the regulation of *SGLT2* expression. In contrast, we found that 5'-FR in HK-2 cells was occupied by more nucleosomes than in the human kidney (Fig. 2B). The highly occupied 5'-FR in HK-2 cells was confirmed by NuSA (Fig. 2C). Nucleosome occupancy may regulate transcription by inhibiting the access of transcription factors to promoters (Lorch et al., 1987; Kornberg and Lorch, 1999). These results suggest that the weak *SGLT2* transcriptional activity of HK-2 cells is due to high nucleosome occupancy in *SGLT2* 5'-FR.

Fig. 7. Quantitative analysis of effects of the TSA treatment on *SGLT2* mRNA levels in HNF1 α -expressing HK-2 cells. HK-2 cells were transfected with the HNF1 α -negative plasmid (pcDNA3.1, white circles) or HNF1 α expression plasmid (black circles) and treated with DMSO or TSA for 24 hours. Total and nascent *SGLT2* mRNA levels were measured by quantitative PCR and normalized to RPL13 mRNA levels. Results are expressed relative to *SGLT2* mRNA levels in empty pcDNA3.1-transfected cells treated with DMSO. Results represent the mean $\pm$ S.D. of three independent experiments. * P < 0.05 for HNF1 α group versus pcDNA3.1 group.

Fig. 8. Analysis of nucleosome occupancy in human kidney, liver, and small intestine tissues. NOME-Seq data of *SGLT2* 5'-FR in the human kidney (white squares), liver (white circles), and small intestine (black circles). Graph represents the proportion of nucleosome-occupied GpC sites in 5'-FR in the three tissues. The arrow indicates the *SGLT2* TSS. Vertical lines indicate GpC sites.

Our results showed that the TSA treatment upregulated *SGLT2* mRNA expression, induced the acetylation of histone H3, and decreased nucleosome occupancy in 5'-FR within approximately 300 bp upstream of the *SGLT2* TSS (Figs. 3 and 4). TSA strongly increases the acetylation of the N-terminal tails of histone H3, which is a well-established acetylation target in nucleosome dynamics and gene activation (Choi and Howe, 2009; Hansen et al., 2010). Previous studies reported higher levels of histone H3 acetylation and lower nucleosome occupancy at active genes in promoter regions (Strenkert et al., 2011). Histone acetylation alters the nucleosomal conformation, which may increase the accessibility of transcriptional factors (Norton et al., 1989; Lee et al., 1993). Most transcribed genes have low nucleosome occupancy at promoters (Bell et al., 2011). These findings suggest that decreased nucleosome occupancy in *SGLT2* 5'-FR altered by the TSA-induced acetylation of histone H3 is involved in the upregulation of *SGLT2* expression via the binding of transcription factors to 5'-FR.

Previous studies demonstrated that the TSA-mediated upregulation of gene expression is associated with histone H3 acetylation and the recruitment of HNF1 α to the promoter (Yang et al., 2010). Our results using TSA-treated HK-2 cells showed an enhanced HNF1 α -binding frequency in *SGLT2* 5'-FR (Fig. 5A). The knockdown of HNF1 α decreased HNF1 α protein levels in DMSO- or TSA-treated HK-2 cells (Fig. 5, B and C), but *SGLT2* mRNA expression level was significantly decreased in TSA-treated cells, not in DMSO-treated cells (Fig. 5D). Furthermore, the HNF1 α overexpression, which was confirmed by Western blot analysis (Supplemental Fig. 5), significantly enhanced the transcriptional activity of 5'-FR (Fig. 6). These results suggest that the upregulation of *SGLT2* mRNA expression after the TSA treatment is associated with the enhanced HNF1 α -binding frequency in 5'-FR. In contrast, the loss of HNF1 α -mediated transcriptional activity was caused by the deletion of the predicted HNF1 α binding site (Fig. 6). Previous studies reported that a promoter region of the rodent *Sglt2* gene is located in 5'-FR around the HNF1 α binding site (Pontoglio et al., 2000; Freitas et al., 2008). In addition, reduction in the HNF1 α -binding frequency at the binding site suppresses *SGLT2* mRNA expression (Zhao et al., 2016). Collectively, our results suggest that 5'-FR around the HNF1 α binding site is a promoter region of the *SGLT2* gene.

The upregulation of *SGLT2* mRNA expression by HNF1 α was observed in TSA-treated HK-2 cells (Fig. 7). The TSA treatment also caused a decrease in nucleosome occupancy in 5'-FR containing the HNF1 α binding site (Fig. 4). Histone acetylation decreases nucleosome occupancy and enhances the accessibility of transcription factors to promoter regions

(Li et al., 2005; Zampetaki et al., 2007; Haberland et al., 2009). These results indicate that nucleosome occupancy in 5'-FR plays a critical role in interfering with the binding of HNF1 α , thereby regulating *SGLT2* expression.

Previous studies reported that *SGLT2* is predominantly expressed in the human kidney (Chen et al., 2010; Wright et al., 2011), whereas HNF1 α is strongly expressed in the human kidney, liver, and small intestine (Cereghini, 1996; Harries et al., 2009; D'Angelo et al., 2010). Our results revealed markedly higher nucleosome occupancy in the human liver and small intestine than in the kidney (Fig. 8). In contrast, the results of the assessment of CpG methylation demonstrated that all CpG sites in *SGLT2* 5'-FR were highly methylated in the human kidney, liver, and small intestine (Fig. 2A; Supplemental Fig. 3B). These results suggest that the different nucleosome occupancy levels in these tissues may affect tissue-specific HNF1 α -binding frequency and, thus, lead to tissue-specific *SGLT2* expression. This hypothesis is supported by previous findings demonstrating that tissue-specific gene expression is associated with tissue-specific nucleosome occupancy in the upstream region of the TSS (Kikuchi et al., 2010; Chen et al., 2017).

The inhibition of *SGLT2* has been reported as a new therapeutic strategy for diabetes (Chao and Henry, 2010; Terami et al., 2014). Previous studies demonstrated that *SGLT2* expression levels are increased in patients with type 2 diabetes (Rahmoune et al., 2005; Wang et al., 2017). In contrast, the recent study reported that *SGLT2* expression was significantly higher in healthy controls than participants with type 2 diabetes (Solini et al., 2017). Decreased *SGLT2* expression levels were observed in patients with maturity onset diabetes of the young type 3 (Pontoglio et al., 2000), caused by mutations in the *HNF1 α* gene (Menzel et al., 1998). However, the mechanisms responsible for the regulation of *SGLT2* expression in these diabetic patients remain unclear. Our results demonstrated that *SGLT2* expression levels are associated with the HNF1 α -binding frequency in 5'-FR. An increased HNF1 α -binding frequency in the *Sglt2* promoter region is involved in the diabetes-induced overexpression of *Sglt2* in the rat kidney (Freitas et al., 2008). In addition, a high sodium intake suppresses *SGLT2* expression by reducing the HNF1 α -binding frequency in 5'-FR (Zhao et al., 2016). Therefore, a clearer understanding of the HNF1 α -binding frequency in 5'-FR in the human kidney will provide new insights into how *SGLT2* expression is regulated in diabetic patients.

In conclusion, our results clearly demonstrate that the transcriptional regulatory mechanisms for the *SGLT2* gene are controlled by nucleosome occupancy and HNF1 α binding

in the *SGLT2* promoter. This is the first study on epigenetic mechanisms to regulate *SGLT2* expression in human tissues. Further studies are required to investigate in more detail the histone modifications causing alterations in nucleosome occupancy in the *SGLT2* gene related to the regulation of *SGLT2* expression.

Acknowledgments

We thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University, for technical support.

Authorship Contributions

Participated in research design: Takesue, Hirota, Ieiri.

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