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RESEARCH ARTICLE

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27-Hydroxycholesterol regulates human *SLC22A12* gene expression through estrogen receptor action

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Abstract

The excretion and reabsorption of uric acid both to and from urine are tightly regulated by uric acid transporters. Metabolic syndrome conditions, such as obesity, hypercholesterolemia, and insulin resistance, are believed to regulate the expression of uric acid transporters and decrease the excretion of uric acid. However, the mechanisms driving cholesterol impacts on uric acid transporters have been unknown. Here, we show that cholesterol metabolite 27-hydroxycholesterol (27HC) upregulates the uric acid reabsorption transporter URAT1 encoded by *SLC22A12* via estrogen receptors

Abbreviations: 27HC, 27-hydroxycholesterol; ABCG2, ATP-binding cassette sub-family G member 2; ER, estrogen receptor; ERE, estrogen response element; GLUT9, glucose transporter 9; iPS, induced pluripotent stem cell; OAT4, organic anion transporter 4; OAT10, organic anion transporter 10; PT, proximal tubule; SERM, selective estrogen receptor modulator; snRNA-seq, single nucleus RNA sequencing; URAT1, urate transporter 1.

Masaya Matsubayashi and Yoshihiko M. Sakaguchi contributed equally to this work.

[Correction added on December 24, 2020, after first Online publication: Copyright has been updated to Online Open with the legal statement.]

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(ER). Transcriptional motif analysis showed that the *SLC22A12* gene promoter has more estrogen response elements (EREs) than other uric acid reabsorption transporters such as *SLC22A11* and *SLC22A13*, and 27HC-activated *SLC22A12* gene promoter via ER through EREs. Furthermore, 27HC increased *SLC22A12* gene expression in human kidney organoids. Our results suggest that in hypercholesterolemic conditions, elevated levels of 27HC derived from cholesterol induce URAT1/*SLC22A12* expression to increase uric acid reabsorption, and thereby, could increase serum uric acid levels.

KEYWORDS
27-hydroxycholesterol, estrogen receptor, transcriptional regulatory element, uric acid

1 | INTRODUCTION

Elevated levels of serum uric acid have been reported as a risk factor for various diseases such as gout, renal diseases, and cardiovascular diseases.[1-5] Increased serum uric acid levels also trigger vascular endothelial dysfunction, which leads to metabolic syndrome and cardiovascular diseases.[6-11] Obesity, insulin resistance, aging, and hormonal imbalance are risk factors for metabolic diseases,[12-15] of which obesity is associated with hypercholesterolemia [16,17] and hyperuricemia.[18,19] Such evidence suggests the existence of a correlation between uric acid levels and cholesterol metabolism. However, the underlying mechanism of this phenomenon is unknown.

There are two causes of hyperuricemia: One is a hepatic overproduction of uric acid, and the other is an under-excretion of uric acid from the kidney and small intestine. Overproduction of uric acid is caused by activated hepatic xanthine oxidoreductase. The excretion of uric acid is regulated by several uric acid transporters, such as urate transporter 1 (URAT1)/*SLC22A12*, ATP-binding cassette sub-family G member 2 (ABCG2)/*ABCG2*, and glucose transporter 9 (GLUT9)/*SLC2A9*.[20-24] Filtered uric acid is reabsorbed into the proximal tubular cells from urine by reabsorption transporters including URAT1 and is secreted into the blood through GLUT9.[24] Uric acid reabsorption transporters play an important role in regulating serum uric acid levels. The expression of Urat1/*Slc22a12* protein/gene is increased in hyperuricemia or obesity in animal models.[25-27] Obesity has also been found to decrease the renal clearance of uric acid in humans.[19] These reports suggest that obesity and metabolic disorder affect the clearance of uric acid by changing URAT1/*SLC22A12* expression. Nevertheless, there is limited information on the mechanism(s) by which URAT1/*SLC22A12* expression is regulated in metabolic disorders conditions.

Previously, we found that the cholesterol metabolite 27-hydroxycholesterol (27HC) acts as an endogenous selective estrogen receptor (ER) modulator, or SERM, and promotes atherosclerotic lesion development in an ER-depending manner.[28] 27HC acts as an agonist or antagonist of ER in a tissue- and cell type-dependent manner.[29,30] In metabolic diseases such as hypercholesterolemia, the concentration of 27HC is increased.[31] Serum 27HC and cholesterol levels are positively correlated with each other, and serum 27HC levels are elevated in humans after 30 years of age.[30-33] Further, studies have observed that circulating 27HC levels are lower in premenopausal females than in males, but these levels are increased after menopause.[30,32] The profile of 27HC resembles that of serum uric acid. Serum uric acid levels are increased with aging and after menopause in females.[34-36] Males also tend to have higher uric acid levels than females.[37,38] These similarities hint at a potential correlation between 27HC and uric acid.

In this study, we investigated how the cholesterol metabolite 27HC impacts uric acid transporter URAT1/*SLC22A12* using evolutionarily conserved analysis, single-nucleus RNA-sequencing (snRNA-seq) analysis, cell culture assays, mouse kidney histology, and human kidney organoid assays.

2 | MATERIALS AND METHODS

2.1 | Alignment of uric acid transporters

Amino acid sequences of human uric acid transporters (URAT1, OAT4, OAT10, ABCG2, NPT1, NPT4, GLUT9, OAT1, and OAT3) were obtained from the NCBI database. Multiple sequence alignment was performed by the MUSCLE program. Based on the alignment, an unrooted phylogenetic tree was generated by the Clustal program using the Neighbor-Joining (NJ) method[39,40] and plotted using DRAWTREE in the PHYLIP software suite.[41] For gene promoter regions of human uric acid transporters, the 1000 bases upstream sequences were identified from the UCSC Genome Browser (http://genome.ucsc.edu/). Multiple alignments of gene promoter sequences and the phylogenic tree were generated by the Clustal program using the NJ method. The alignment of gene promoter sequences was drawn by ES print 3 (http://espript.ibcp.fr/ESPript/ESPript/index.php). We identified sequence similarities depiction parameters as more than 0.75 at a global score.

2.2 | Transcription factor-binding site search and estrogen response element scores

Gene promoter sequences were obtained as described above. We searched potential ER-binding sites in each gene promoter sequence using the TFBIND website (http://tfbind.hgc.jp/). Based on the results corresponding to the consensus sequence, we searched the start and end position of each estrogen response element (ERE) sequence in the promoter sequence. The sequence Logo was made by Web Logo 3 (http://weblogo.threeplusone.com/). ERE score was defined as the calculated similarity score by TFBIND. ERE values were represented as a multiplication of the 10-fold ERE scores.

2.3 | Single-nucleus RNA-sequencing analysis

Single-nucleus transcriptome analysis was performed using a data set of single nucleus RNA-seq from a human adult kidney (GEO: GSE118184).[42] The digital gene expression matrix was processed for quality control, normalization, linear dimensional reduction, and unsupervised clustering of single-cell using Seurat (https://satijalab.org/seurat/) plug-in of R software (https://www.r-project.org/) according to the tutorial in the developer's website (https://satijalab.org/seurat/vignettes.html). Cells were filtered out as low-quality cells based on unique feature counts and mitochondrial counts. After the filtration, we applied the function "Sctransform" normalization to remove confounding sources of variation, mitochondrial mapping percentage, the total number of molecules, and cell cycle heterogeneity[43,44] in order to mitigate confounding effects. Principal component (PC) analysis was conducted and the most significant PCs of the data set were selected for two-dimensional Uniform Manifold Approximation and Projection (UMAP). Unsupervised clustering was performed by functions "FindClusters" and "RunUMAP" at a resolution level of 0.8. Single-cell RNA-seq measures transcripts from both cytoplasm and nucleus, whereas snRNA-seq measures only nuclear transcripts. Nuclei contain only a fraction of total cellular RNA. Although nuclear and cytoplasmic mRNAs are highly correlated,[45] some protein-coding mRNAs are retained in the nucleus.[46] Despite these differences, snRNA-seq data sets predict cell types comparably with high concordance.[47,48]

2.4 | Annotation of the clusters and cell-type identification

The cluster names used for the anatomical structures of the kidney are podocyte, PT: proximal tubule; LH (DL): the loop of Henle (descending loop); LH (AL): the loop of Henle (ascending loop); DCT: distal convoluted tubule; CNT: connecting tubule; PC: principal cell; ICA: intercalated cell type A; ICB: intercalated cell type B; EDC: endothelial cell; and U: undefined. They were identified based on the maker gene expression described in a previous report.[42]

2.5 | Co-expression analysis of three uric acid reabsorption transporters

Co-expression levels of three uric acid reabsorption transporters were shown in scatterplots of these transporters. The scatterplots of two gene expressions after extracting the cells that express either gene were also created using a Seurat function, "FeatureScatter," which is a plug-in of R software (https://www.r-project.org/). The axes represent the expression levels of each gene from count data after log-normalization.

2.6 | Analysis tissue *ESR1* and *ESR2* expression from RNA-seq database

We analyzed *ESR1* and *ESR2* gene expression in various tissues, as well as tested for sex differences in kidney *ESR1* and *ESR2* expression. The expression data referred to GTEx RNA-seq data (https://www.proteinatlas.org/ENSG00000091831-ESR1/tissue and https://www.proteinatlas.org/ENSG00000091831-ESR1/tissue/kidney). We statistically analyzed all male and female kidney sample data in GTEx RNA-seq.

2.7 | *SLC22A12* gene promoter cloning

All plasmid constructs were designed by SnapGene (GSL Biotech, Chicago, Illinois, USA). The human genomic DNA was isolated from U2OS cells using the QuickExtract™ DNA Extraction Solution (Lucigen, Wisconsin, USA). From the gDNA, the *SLC22A12* gene promoter was amplified using KOD One PCR Master Mix (TOYOBO, Osaka, Japan) or PrimeSTAR® Max DNA Polymerase (TAKARA BIO, Shiga, Japan) with primer F: gctcgctagcctcgaaatcagttccaaagagcctctaaagaag, and primer R: ccggattgccaagctagagaggcagctgctcca. After the PCR product was confirmed by the agarose gel electrophoresis, it was purified using QIAquick Gel Extraction Kit (Qiagen) and cloned into a pGL4.17 [luc2/Neo] Vector (Promega, Madison, Wisconsin, USA) using Xho I and Hind III restriction enzyme sites. The ERE sequences of *SLC22A12* gene promoter were amplified to get mutants (TGACC was changed to CAGTA) using PrimeSTAR Mutagenesis Basal Kit (TAKARA BIO, Shiga, Japan) with the following primers: ERE#1 primer F: aagcaggattcagtacaagggctcgcagtgcgt; primer R: cgagcccttgtactgaatcctgcttaaaagcca; ERE#2 primer F:ccagacactgcagtatgagaggccatagctgag; primer R: tggcctctcatactgcagtgtctggcctggaac; ERE#3 primer F: cagctgccagcagtacaagcccacacagagact; and primer R: tgtgggcttgtactgctggcagctgcccagccc. The constructs were amplified, and sequences were confirmed.

2.8 | Cell culture and generation of stable cell line

The human hepatocellular carcinoma cell line HepG2 was purchased from Cellular Engineering Technologies, Inc Cells were maintained in RPMI1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated under a humidified atmosphere containing 5% CO_2 at 37°C. To generate stably expressed cell lines, cells were seeded in six-well plates at 1.0×10^5 cells per well and introduced to linearized luciferase expression plasmid fused with *SLC22A12* gene promoter using Lipofectamine 2000 (Invitrogen, Waltham, Massachusetts, USA). At 24 hours after transfection, the medium was changed to G418 (800 μg/ml)-containing medium. We selected stably expressed cells by removing G418-sensitive cells with changing media every day for two weeks.

2.9 | Quantitation of promoter activity by luciferase assay

The luciferase activity was measured in HepG2 cells that stably express intact and ERE mutant *SLC22A12* gene promoter-regulated luciferase proteins. This was done using One-Glo EX Luciferase Assay System (Promega) and according to the manufacturer's instructions. Cells in ninety-six-well plates were treated with 17β-estradiol, estrone, 27HC, 3β-hydroxy-5-cholestenoic acid, or ICI 182,780 for 24 hours at indicated concentrations. Then, One-Glo EX reagent (80 μl/well) was added to the cells, and luciferase activity was measured with a SpectraMax. All experiments were performed in triplicate.

2.10 | Kidney immunohistochemistry

Kidney samples were collected from 12-month-old *Cyp7b1*$^{+/+}$ and *Cyp7b1*$^{-/-}$ mice on the C57BL/6 background. Collected samples were fixed in 4% formalin for 48 hours at 4°C, processed, paraffin-embedded, and cut into 6 μm-thick sections. After rehydration, the sections were treated in the citrate buffer (pH 6.0) at 95°C for 10 minutes for antigen retrieval, followed by 10% goat serum (Life Technologies) at room temperature for 1 hour. Then, the sections were blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, California, USA) for 15 minutes. Thereafter, an anti-Urat1 antibody (Millipore Sigma, HPA024575, 1:500) was added, and the sections were incubated overnight. After washing the sections, they were blocked again using 3% hydrogen peroxidase (ThermoFisher Scientific) and incubated with biotinylated goat-anti-rabbit antibody (Vector Laboratories, BA-1000, 1:200) for 1 hour. The sections were visualized using an avidin-biotin complex kit (ABC kit, Vector Laboratories) and DAB Quanto chromogen (ThermoFisher Scientific, Waltham, Massachusetts, USA), followed by counterstaining with Harris hematoxylin (ThermoFisher Scientific, Waltham, Massachusetts, USA). Image analysis was performed using Image J/Fiji. Proximal tubules were detected, and the maximum intensity of the brush border membrane in the proximal tubular cells was measured (Figure S5). About 230-240 points per image were measured in a blind manner. Statistical analysis was performed using the average of these intensities per image.

2.11 | Kidney organoid culture

Human kidney organoids were generated from induced pluripotent stem (iPS) cell line CRL1502 according to the procedures reported in our previous study[49] with some modification. Human iPS cells were treated with 8 μM CHIR99021 in a "basal medium," APEL2 (STEMCELL Technologies, Vancouver, Canada) that was supplemented with 1% Protein Free Hybridoma Medium II (PFHM II, GIBCO), for 5 days, and with FGF9 (200 ng/ml) and heparin (1 μg/ml) for an additional 2 days. Then, the cells were

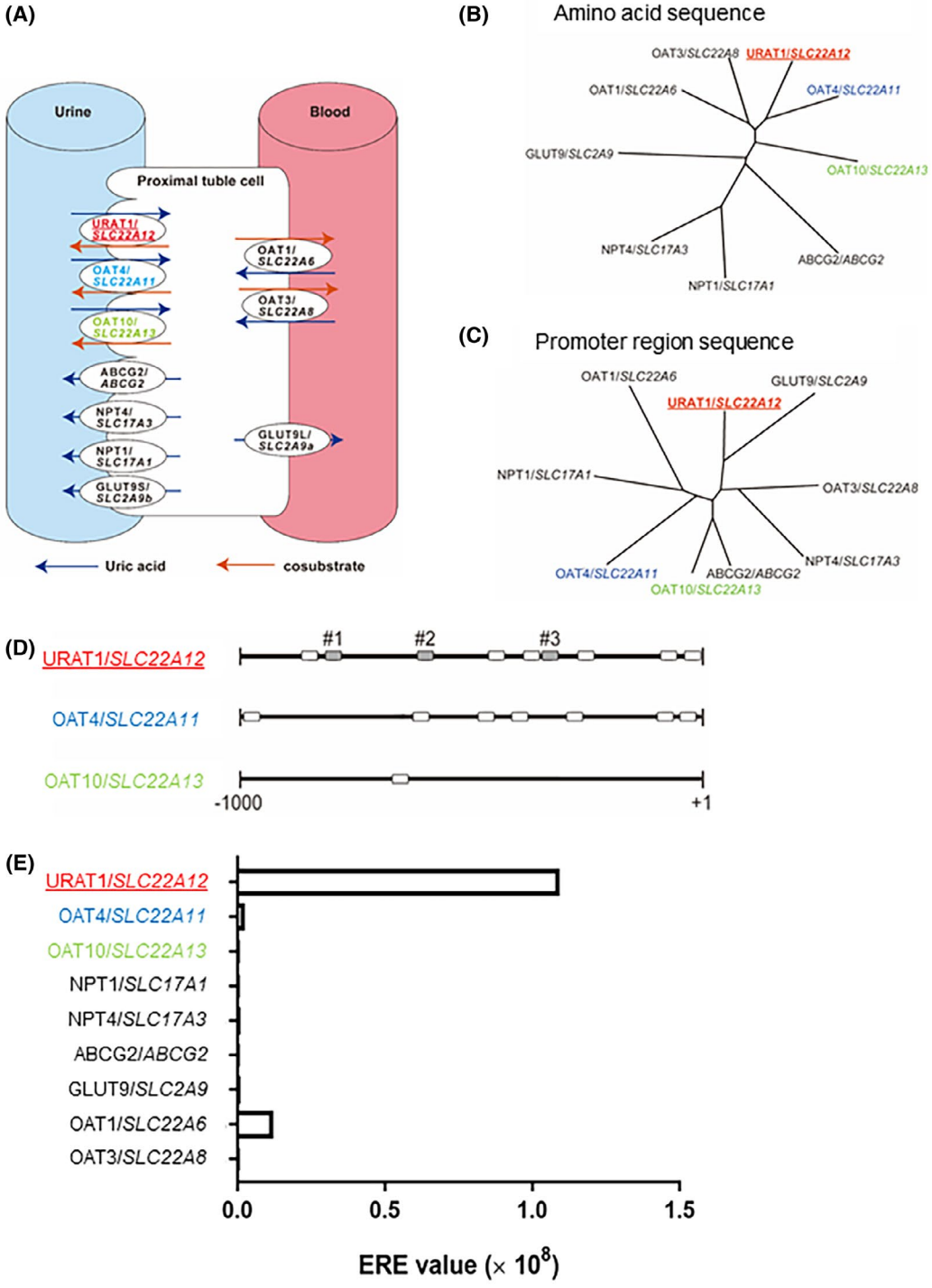

FIGURE 1 Phylogenetic trees of uric acid reabsorption transporters. A, Schema of uric acid transporters in kidney proximal tubular cells. B and C, Phylogenetic trees of the uric acid transporters (B) amino acid sequences and (C) gene promoter sequences. Three reabsorption transporters are indicated in color. D, A diagram of the gene promoter regions. White boxes show potential ERE sequences, and three grey boxes of *SLC22A12*, #1-#3, show the ERE sequences having ERE scores more than 0.8. E, A bar plot showing the ERE values of uric acid transporter gene promoter regions

collected, dissociated into 2.5×10^5 single cells, and spun down at 400 ×*g* for 2 minutes to form a pellet. The pellets were transferred onto a Transwell of 0.4 μm pore membrane (#3450, Corning, USA) to culture in liquid-air interfaces. The cells were cultured with the following factors in the basal media in the following order: 10 μM CHIR99021 for 1 hour, FGF9 (200 ng/ml) and heparin (1 μg/ml) for 5 days, and basal medium only for 13-15 days. Organoids were treated with 10 μM 27HC for the final 2 days before sampling (day 25-27 of differentiation) in order to examine

the effect of 27HC on the *SLC22A12* expression but not on the organoid development.

2.12 | Quantitative RT-PCR

Total RNA was extracted from organoids using Nucleo Spin (MACHEREY NAGEL, Germany) and cDNA was synthesized from 500 ng of total RNA using PrimeScript™ RT Master Mix (TAKARA BIO, Shiga, Japan). Quantitative reverse-transcription PCR (qRT-PCR) was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California, USA) and specific primers for qRT-PCR. The reaction was run in triplicate, and the transcription of each gene was normalized to the mean values of *β-actin* and analyzed using the ΔΔCT method by StepOne Software Ver2.3. The primer sequences for qRT-PCR were designed by Bio-Rad (*SLC22A12*; qHsaCID0011483, *β-actin*; qHsaCED0036269). The data were collected from six independent experiments.

2.13 | Statistics and data analysis

Results of the luciferase assay and qRT-PCR were represented by the ratio against mean control values. All the values obtained represent means ± SEM. Student's *t* test, one-way ANOVA followed by Dunnett's test, or two-way ANOVA followed by Sidak's multiple comparisons test were performed to determine the significance among groups (GraphPad Prism7). A *P* value of less than .05 was considered statistically significant.

3 | RESULTS

3.1 | *SLC22A12* gene promoter is differentially regulated from *SLC22A11* and *SLC22A13* gene promoter through estrogen response elements

As shown in the model of transcellular uric acid transport in the proximal tubular (PT) cells (Figure 1A), several uric acid transporters regulate uric acid flow from/to the PT cell.[50] To investigate the molecular difference among uric acid transporters, we analyzed the amino acid sequences of these uric acid transporters with evolutionarily conserved analysis. The amino acid sequences of uric acid reabsorption transporters, URAT1 encoded by *SLC22A12*, OAT4 encoded by *SLC22A11*, and OAT10 encoded by *SLC22A13* showed a closer evolutionary relationship to each other (Figure 1B and S1A, B), suggesting that these transporters share a similar function.

Although the co-expression profile at single-cell resolutions and the difference in transcriptional regulation of uric acid transporters are important to understand the uric acid flow, these are largely unknown. To evaluate the expression of uric acid reabsorption transporters at single-cell resolutions, we analyzed publicly available snRNA-seq data of human adult kidneys. Each cell type was clearly separated (as shown in Figure S2A and gene markers of cell types shown in Figure S2B), and we focused on the distribution of uric acid transporters in the PT region in the snRNA-seq analysis (Figure S2A-C). The co-expression plots showed that *SLC22A12* was expressed independently of *SLC22A11* or *SLC22A13* genes (Figure S2D-F). In addition, the gene promoter sequence of *SLC22A12* shares a distant evolutionary relationship with *SLC22A11* or *SLC22A13* (Figure 1C and S3A). These analyses suggest that the *SLC22A12* gene is expressed independently of other uric acid reabsorption transporters under specific transcriptional regulation.

To elucidate the key factors for the unique transcriptional regulation of uric acid transporters, we further analyzed their gene promoter regions. Since the elevated uric acid level is associated with the metabolic condition and sex hormone status,[10,51] we focused on the binding motifs of metabolic or sex hormone-regulated transcription factors on the gene promoter regions. The *SLC22A12* gene promoter contains nine potential sequences of estrogen response elements (EREs) and has the highest ERE value among uric acid transporter genes (Figure 1D, E, and S3B-J). These analyses suggest that *SLC22A12* is most strongly regulated by ER among uric acid transporters.

Since the ERE activates the promoter activity through ER binding, we investigated the expression of ER in the human kidney. ERα/*ESR1* and ERβ/*ESR2* are expressed in the kidney, although the expression levels were ten to a hundred times lower than in tissues with the highest expression, such as the ovary and cervix (Figure S4A). In addition, there was no difference in the *ESR1* and *ESR2* expression between males and females (Figure S4B). These results suggest that the kidney ERs bind to EREs on the *SLC22A12* gene promoter regardless of sex and that the promoter activity is upregulated.

3.2 | Human *SLC22A12* gene promoter activity is upregulated by 27HC

To investigate whether ER regulates *SLC22A12* transcription activity, we created a plasmid construct that includes the *SLC22A12* gene promoter region fused to a luciferase expression vector (Figure 2A) and performed a luciferase assay using this construct. Our study aimed to examine the effect of ligands for ER in the kidney in a physiological condition where the ER expression is low (Figure S4A). This provided

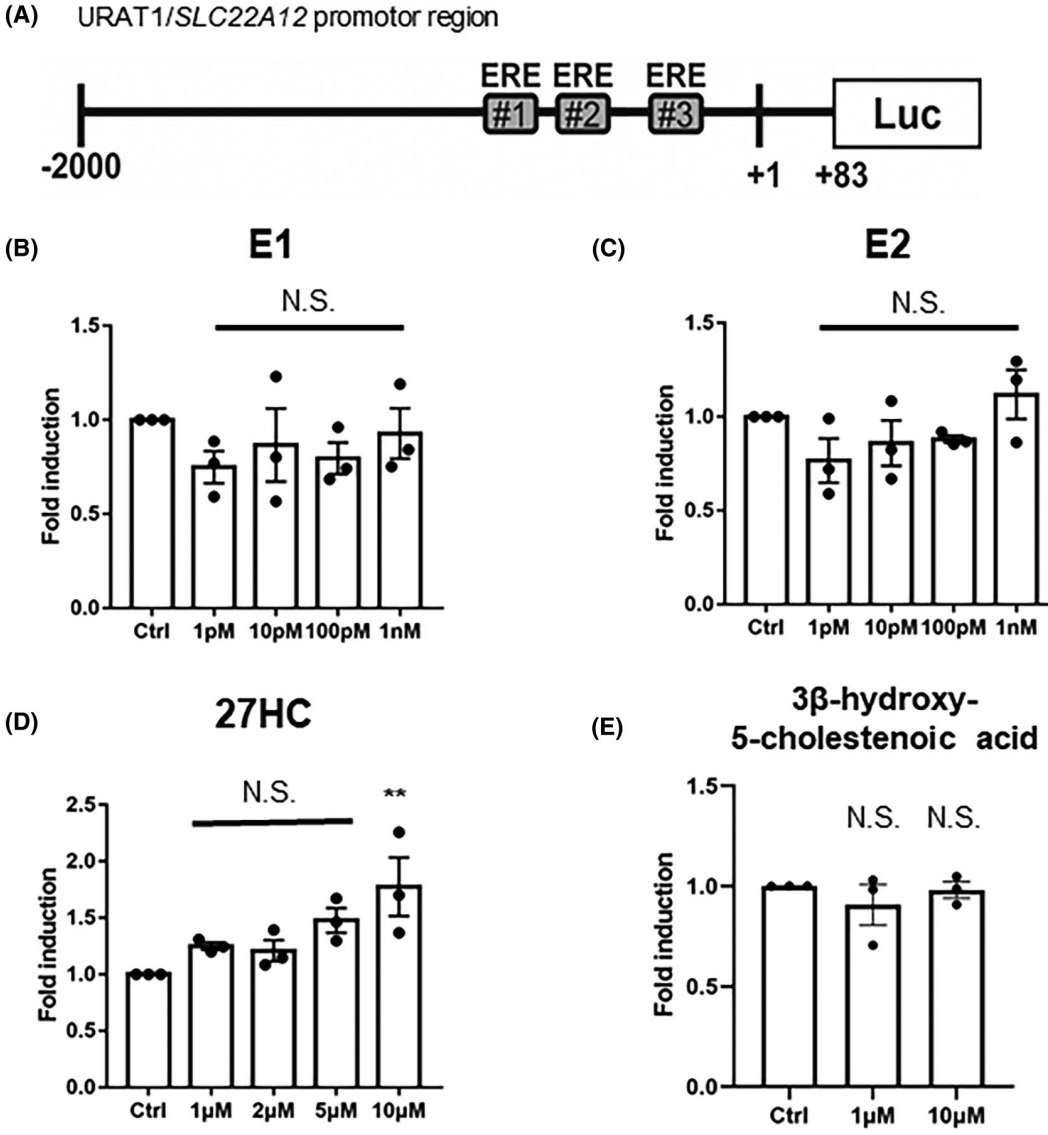

FIGURE 2 *SLC22A12* gene promoter activities. A, Schematic representation of a human URAT1/*SLC22A12* gene promoter-luciferase reporter construct. ERE#1, #2, and #3 represent the same site shown in Figure 1D. B-E, HepG2 cells with stable expression of *SLC22A12* gene promoter-driven luciferase proteins were treated with E1 (B) or E2 (C) at the indicated concentration for 24 hours. D, The effect of 27HC on the *SLC22A12* gene promoter activity in HepG2 cells. E, The effect of 3β-hydroxy-5-cholestenoic acid on the URAT1/*SLC22A12* promoter activity in HepG2 cells. The results were analyzed using one-way ANOVA followed by Dunnett's tests. ** $P < .01$ vs. control (ctrl). NS, nonsignificant. All experiments in triplicate were performed independently three times

the basis for our choice of HepG2 cells that have relatively similar levels of ER expression as in the kidney.

First, we examined whether estrogens, which are major ligands for ER, affected the gene promoter. There are different types of estrogens. 17β-estradiol (E2) is the most potent among the endogenous estrogens[52] and significantly decreases after menopause,[53] whereas estrone (E1) becomes the dominant circulating estrogen in postmenopausal females.[53] E1 and E2 did not change the *SLC22A12* gene promoter at physiological concentrations (E1: $P = .3013$, E2: $P = .0592$; Figure 2B,C). These results suggest that estrogens do not affect *SLC22A12* gene transcription given the physiological conditions in humans.

There is a correlation between serum uric acid levels and metabolic conditions.[12-15] Cholesterol metabolite 27HC acts as an endogenous ER ligand and regulates transcriptional activities through EREs.[33,54] Thus, to investigate whether metabolic disorders affect *SLC22A12* transcriptional activity, we examined the effect of 27HC on this gene promoter construct. Normal circulating levels of 27HC are typically measured in a range of 0.15-0.73 μM,[33,54] and the concentration of 27HC reaches millimolar levels in metabolic diseases such as hypercholesterolemia.[31] Gene promoter activity of SLC22A12 was increased by 27HC in a dose-dependent manner and induced significantly at 10 μM ($P = .007$; Figure 2D). Our result suggests that 27HC causes the elevated expression of

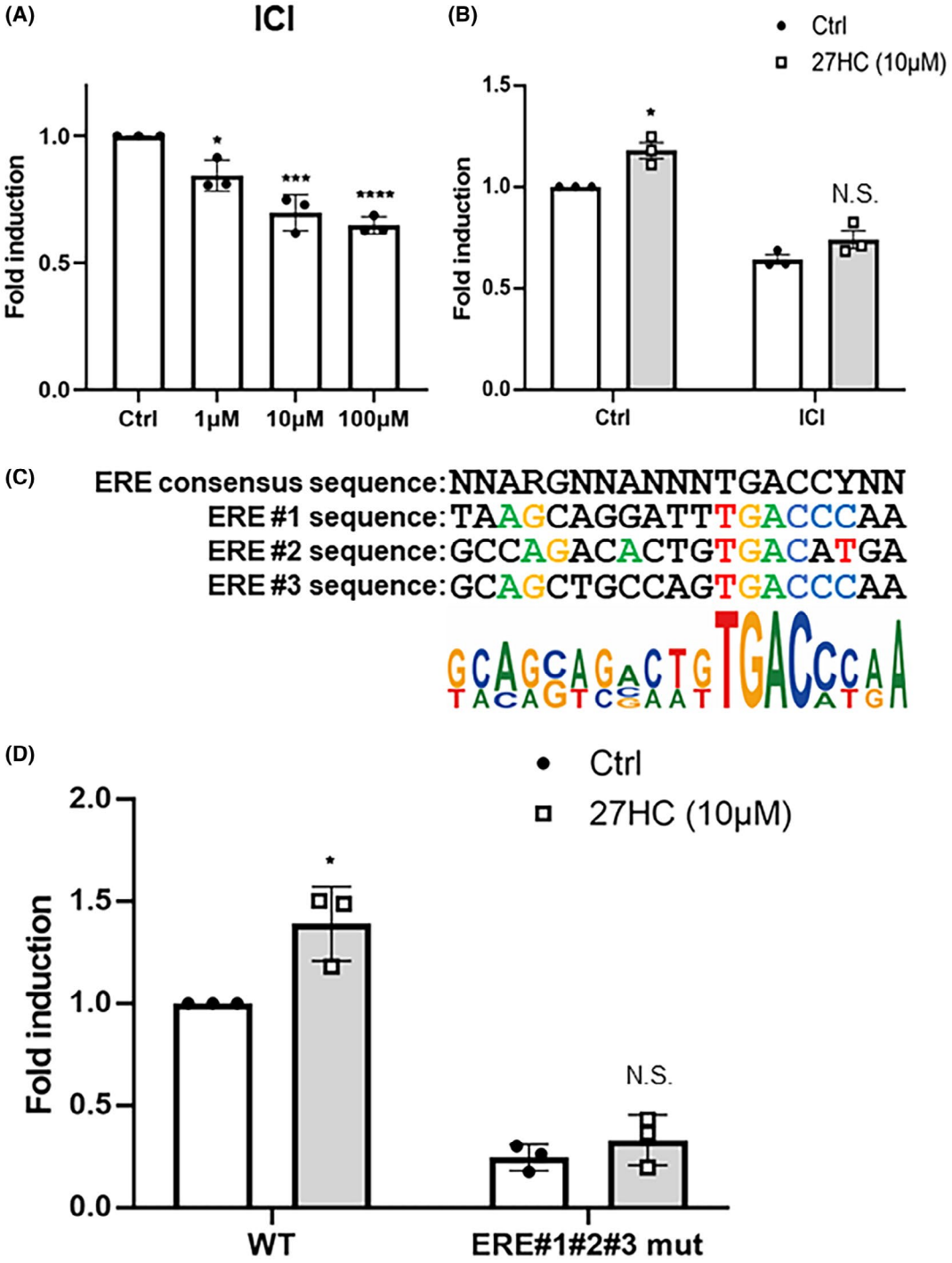

FIGURE 3 Involvement of ER and EREs in the *SLC22A12* gene promoter activity. A, HepG2 cells with stable expression of *SLC22A12* gene promoter-regulated luciferase proteins were treated with ICI 182,780. The results were analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests. * $P < .05$, *** $P < .001$, **** $P < .0001$ vs. ctrl. B, HepG2 cells were treated with 27HC in the absence or presence of 100 μM ICI 182,780 for 24 hours. The results were analyzed using two-way ANOVA followed by Sidak's multiple comparisons tests. * $P < .05$ vs. vehicle control for 27HC. Open column; vehicle control, gray column; 27HC-treated. C, Consensus ERE sequence, potential ERE sequences on the SLC22A12 gene promoter, and sequence logo of the three EREs. The largest letter (TGAC) shows the conserved sequence among the sequences. D, The effect of 27HC on ERE mutant gene promoter activity. The two HepG2 cell lines that stably express intact or ERE mutant gene promoter-driven luciferase proteins were treated with 10 μM 27HC for 24 hours. The results were analyzed using two-way ANOVA followed by Sidak's multiple comparisons tests. * $P < .05$ compared with nontreatment of 27HC. Open column; vehicle control, gray column; 27HC-treated. All experiments in triplicate were performed independently three times

the *SLC22A12* gene at the concentration in metabolic disease patients.

To examine whether the effect of 27HC was caused by 27HC itself or its metabolites, we further tested 3β-hydroxy-5-cholestenoic acid, a metabolite of 27HC. The treatment of 3β-hydroxy-5-cholestenoic acid did not increase *SLC22A12* gene promoter activity even in a high dose ($P = .5729$; Figure 2E). This result suggests that 27HC, rather than its metabolite, upregulates *SLC22A12* gene transcription.

3.3 | 27HC regulates human *SLC22A12* transcription via ER through EREs

To determine the impact of 27HC on *SLC22A12* gene promoter through ER, we treated cells with an ER antagonist, ICI 182,780.[55] ICI 182,780 alone decreased the *SLC22A12* gene promoter activity in a dose-dependent manner ($P < .0001$; Figure 3A), suggesting that this ER antagonist inhibits gene promoter basal activity through endogenous ER. Next, we treated cells with ICI 182,780 in the presence of 27HC. Since 27HC had a higher affinity for ERα than ICI 182,780,[54,56] we treated the higher concentration of ICI 182,780 (100 μM) rather than the concentration of 27HC (10 μM). The effect of 27HC was suppressed by a co-treatment with ICI 182,780 (Ctrl vs. 27HC, $P = .0217$, ICI vs. 27HC + ICI, $P = .3042$; Figure 3B). These results indicate that 27HC increases *SLC22A12* gene transcription via ER activity.

The *SLC22A12* promoter region has nine potential ERE sequences (Figure S3B), and we postulated that 27HC acted on the *SLC22A12* gene promoter through EREs. We created a mutant plasmid construct, in which three sequences with the highest ERE scores on the *SLC22A12* gene promoter region were mutated (Figure 3C). These ERE mutations significantly decreased the baseline of gene promoter activity ($P < .0001$; Figure 3D), indicating that ERE sequences play a major role in the *SLC22A12* transcription. ERE mutation also diminished the effect of 27HC on the *SLC22A12* gene promoter activity (ctrl vs. 27HC in intact ERE, $P = .0183$, ctrl vs. 27HC in ERE mutation, $P = .9495$, 27HC x ERE mutation interaction $P = .0492$; Figure 3D). These results suggest that 27HC regulates the *SLC22A12* gene promoter through ERE.

3.4 | 27HC increases Urat1 expression in the mouse kidneys

To evaluate the role of 27HC on Urat1 encoded by *Slc22a12* expression in the mouse kidneys, we applied immunohistochemistry using kidney samples from *Cyp7b1*$^{+/+}$ and *Cyp7b1*$^{-/-}$ mice. In this mouse model, the deficiency of the 27HC-catabolizing enzyme Cyp7b1 results in elevated serum and tissue 27HC levels without affecting cholesterol and bile acid metabolism.[57] Urat1 protein expression was observed in the apical membrane of the PT region (Figure S5A). The expression levels were observed to be increased in male *Cyp7b1*$^{-/-}$ mice kidneys compared with male *Cyp7b1*$^{+/+}$ mice (Figure 4A, B and S5B), suggesting that elevated 27HC levels induce Urat1 expression in mice.

Given the impact of 27HC on Urat1 expression via ER, there might be sex differences on the effect of 27HC. When we compared the results between sexes, *Cyp7b1*$^{-/-}$ male mice had significantly elevated expression of Urat1 ($P = .0057$, *t* test; Figure 4A,B and S5B), whereas females did not show a significant change of Urat1 expression levels between *Cyp7b1*$^{+/+}$ and *Cyp7b1*$^{-/-}$ genotypes ($P = .0737$, t test; Figure 4A,B and S5B). These results suggest sex differences might potentially influence Urat1 expression.

3.5 | Extracellular 27HC increases *SLC22A12* gene expression in human kidney organoids

From the analysis of promoter activity (Figure 2D) and immunohistochemical analysis in mice kidneys (Figure 4), we postulated that 27HC impacts URAT1/*SLC22A12* in human kidneys. The ERE value of the human *SLC22A12* gene promoter was higher than that of mice (Figure 5A and S6), suggesting that the effect of 27HC on the *SLC22A12* gene promoter activity in humans is greater than that in mice. To estimate the potential for the effect of 27HC on URAT1/*SLC22A12* in human kidneys, we performed kidney organoid culture from human iPS cells and examined the effect of 27HC on the *SLC22A12* expression (Figure 5B). 27HC significantly increased *SLC22A12* mRNA expression ($P = .043$) (Figure 5C). These results indicate that 27HC increases *SLC22A12* expression in human kidneys.

4 | DISCUSSION

In this study, we demonstrated that 27HC, an endogenous SERM, regulates the expression of URAT1/*SLC22A12*. In the basal condition, URAT1/*SLC22A12*, OAT4/*SLC22A11*, and OAT10/*SLC22A13* are involved in the reabsorption of uric acid into blood (Figure 6A). In hypercholesterolemic conditions such as cardiovascular and metabolic diseases, elevated levels of 27HC induce URAT1/*SLC22A12* expression, which can increase uric acid reabsorption and circulating uric acid levels (Figure 6B). Our findings provide new information on the relationship between hyperuricemia and metabolic syndromes.

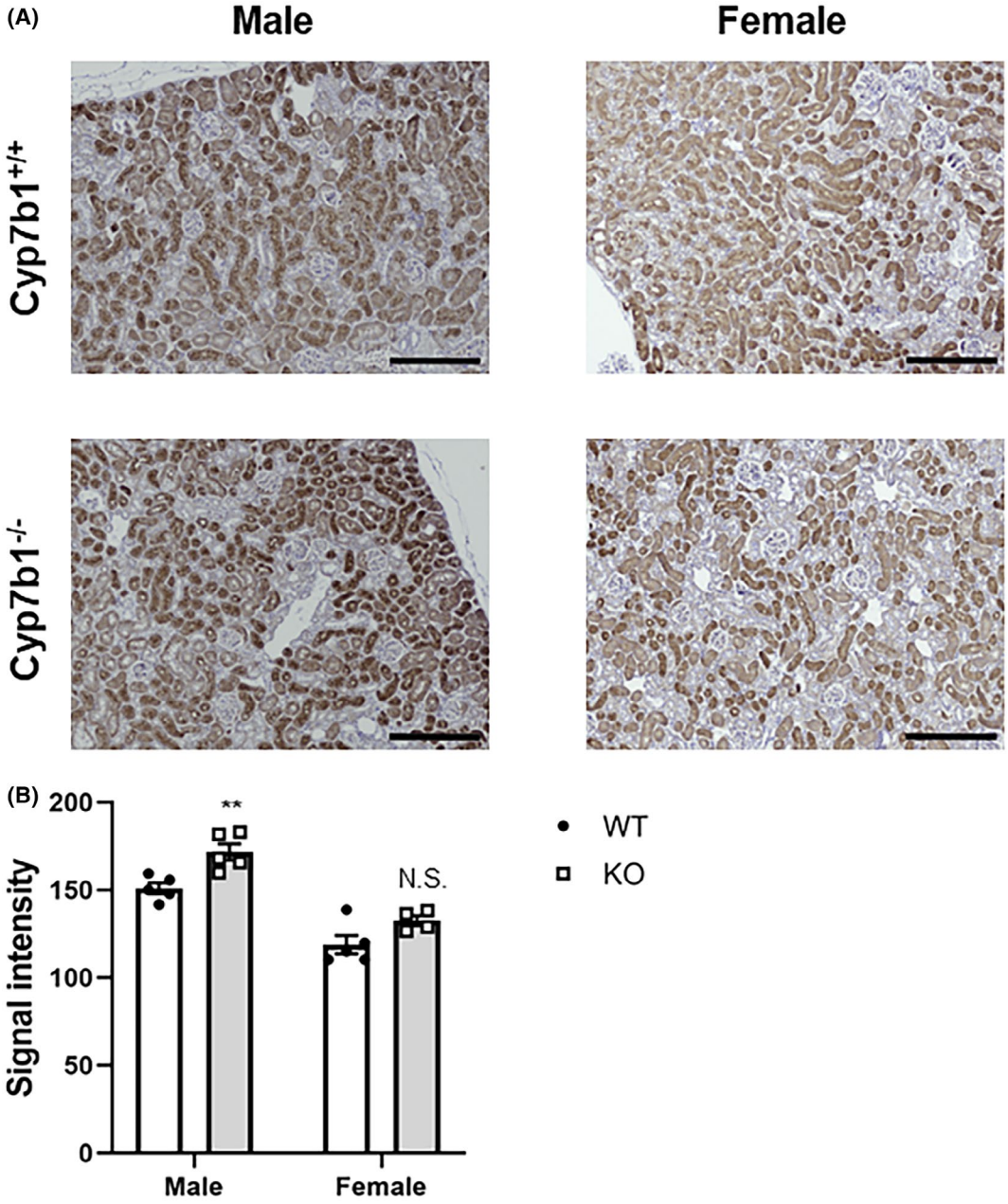

FIGURE 4 Immunohistochemical analysis of kidneys from *Cyp7b1*$^{+/+}$ and *Cyp7b1*$^{-/-}$ mice. A, Immunohistochemical analysis using an anti-Urat1 antibody. Upper panel: *Cyp7b1*$^{+/+}$ (WT), lower panel: *Cyp7b1*$^{-/-}$ (KO). Scale bar showed 200 μm. B, Image analysis on the signal intensities of Urat1 immunostaining in male and female PT cells. Open column; vehicle control, gray column; 27HC-treated. The results were analyzed using a two-tailed student's *t* test. $^{**}P < .01$ vs. WT. Open column; WT mice, gray column; KO mice

4.1 | Elevated 27HC in metabolic disorders increases *SLC22A12* gene promoter activity

Previous reports suggest that the expression levels of mouse Urat1/*Slc22a12* protein/gene are related to metabolic disorders, such as in insulin resistance and obesity mouse models.[25,27] However, it was unclear how metabolic conditions impact human URAT1/*SLC22A12* expression and function. 27HC is the most abundant oxysterol in human circulation,[30] and its circulating levels are elevated with hypercholesterolemia and metabolic or cardiovascular dysfunction such as atherosclerosis.[30] As the *SLC22A12* gene promoter contains EREs, we postulated that estrogens and SERM would affect *SLC22A12* expression. In our study, although estrogens and 27HC bind to ER,[54] estrogens (E1 and E2) did not alter *SLC22A12* gene promoter activity at physiological concentrations (Figure 2B,C), while 27HC induced *SLC22A12* gene expression in a dose-dependent manner (Figure 2D). We also observed the involvement of ER in the *SLC22A12* gene expression using ER antagonist ICI 182,780 and ERE mutants (Figure 3). Previously, we demonstrated that E2 and 27HC cause

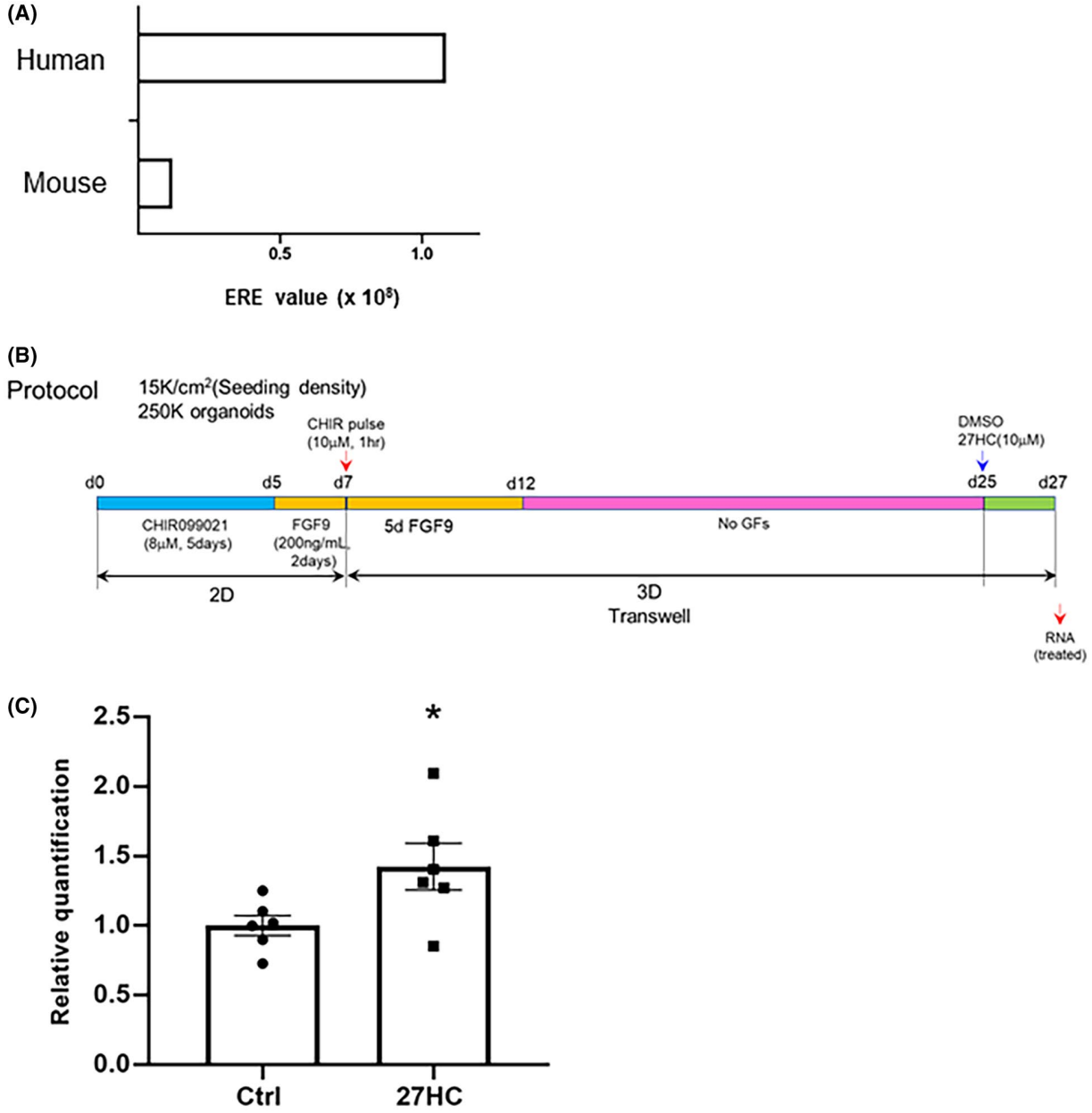

FIGURE 5 *SLC22A12* expression in human kidney organoids. A, A bar plot showing ERE values of human *SLC22A12* and mouse *Slc22a12* gene promoter regions. B, The experimental scheme of human kidney organoid culture with 27HC treatments. C, The effect of 27HC on *SLC22A12* mRNA expression in the human kidney organoids. The experiment was performed in six independent experiments. *$P < .05$ vs. ctrl by two-tailed student's *t* test

unique conformational changes of ERα upon their bindings to ER.[54] Therefore, 27HC may upregulate *SLC22A12* gene promoter activity via ER, while estrogens do not, due to the difference in ERα conformational changes. Our current findings suggest that elevated 27HC levels in metabolic disorders induce *SLC22A12* transcription via ER through EREs.

As discussed in the Introduction, serum 27HC levels correlate well with serum cholesterol levels, and high cholesterol diet-feeding or metabolic dysfunction increases serum 27HC levels.[31] In addition, patients with spastic paraplegia type 5 (SPG5), who have mutations in the gene coding for CYP7B1, have elevated serum 27HC levels with 2-5 μM.[58-60] Also, the deficiency of Cyp7b1 increased 27HC levels in mice.[28,57] As elevated 27HC in *Cyp7b1*$^{-/-}$ mice increased Urat1 expression levels (Figure 4), it is plausible that these *CYP7B1* mutations also cause increased URAT1 expression in humans. The activity of 27HC on the *SLC22A12* gene promoter was mild at 5 μM ($P = .089$, Figure 2D). Thus, *CYP7B1* gene mutation alone may not have strong effects on *SLC22A12*; however, with high cholesterol diets and/or metabolic dysfunction, the serum 27HC levels can easily reach the levels that increase URAT expression enough to modulate serum uric acid levels. Further investigation into the relationship between human

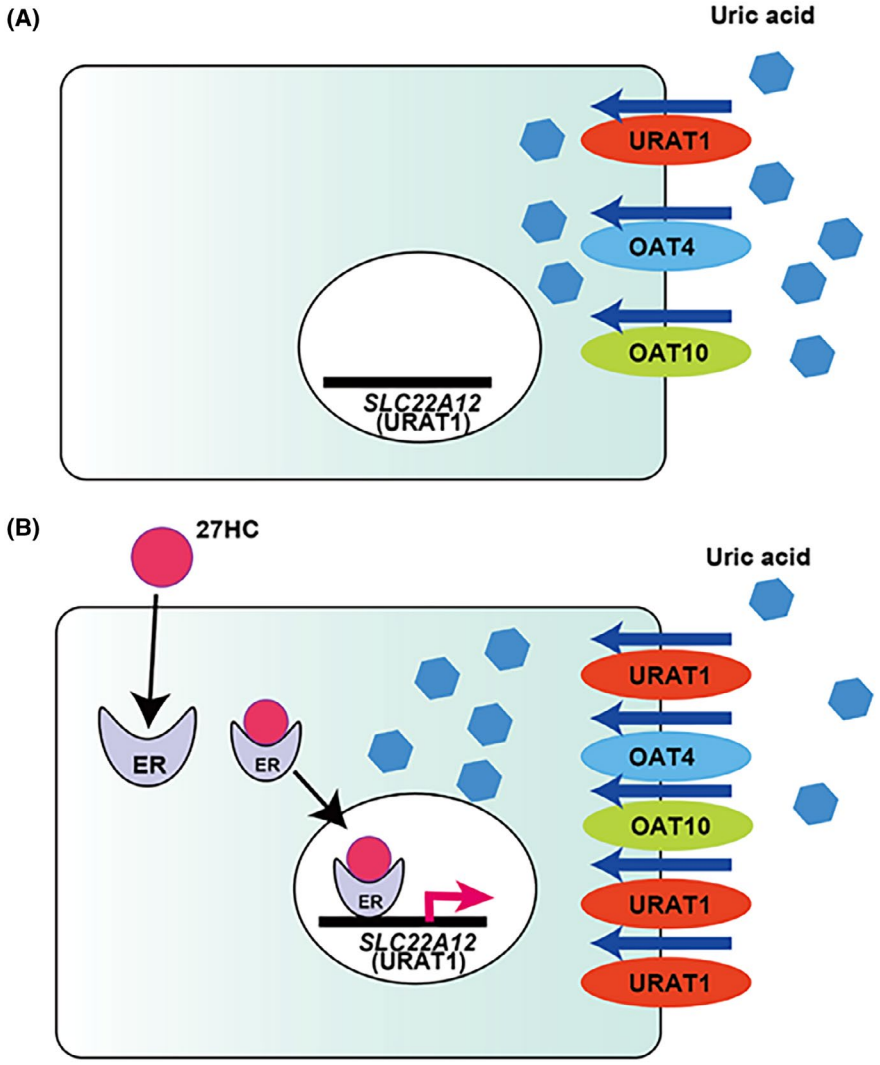

FIGURE 6 Graphical abstract. 27HC binds to ER and induces URAT1/*SLC22A12* expression. This results in an increase of URAT1 in the apical membrane, and more uric acid is transported into the cells, leading to increased serum uric acid levels. The red arrow demonstrates the upregulation of *SLC22A12* transcription. Blue arrows show the movement of uric acid. (A) basal and (B) metabolically dysfunctional condition

CYP7B1 gene mutation and URAT1/*SLC22A12* expression is required.

4.2 | Sex differences on the effect of 27HC on URAT1 expression

Given the impact of 27HC on *SLC22A12* gene promoter via ER, it is possible that sex differences on the effect of 27HC on *SLC22A12* activity are relevant. Our study showed that in *Cyp7b1*$^{-/-}$ mouse kidneys, there was a significant increase in Urat1 expression level in male mice, but not in female mice (Figure 4). This suggests the presence of sex differences in the effect of 27HC on Urat1 expression.

Although the mechanism underlying this sex difference is unclear, we deduce that the balance between estrogens and 27HC is important. For instance, 27HC and estradiol co-treatment halted the suppression of tyrosine hydroxylase expression by 27HC.[61] Estradiol did not change *Slc22a12* mRNA, but it suppressed Urat1 protein expression levels in mice.[62] In fact, Urat1 protein expression in wild-type male mice was greater than that in females (Figure 4 and previous report[63]). Given these facts, the effect of 27HC on Urat1 protein expression may be counteracted by the inhibitory effect of estradiol in female mice.

Despite clear sex differences in the effect of 27HC on mouse Urat1 expression, sex differences on the effect of 27HC on human URAT1 expression remain unclear. Existing studies have found that females, especially before menopause, are less likely to develop uric acid associated morbidities, such as cardiovascular and renal disorders, compared with males in human.[64,65] There is also evidence that E2 suppresses inflammatory responses, while 27HC increases them in the vasculature.[28] Hence, it is possible that the 27HC-induced upregulation of URAT1/*SLC22A12* expression in humans is indirectly antagonized with estrogens, and that it influenced the sex differences in the effect of 27HC. Further studies are required to reveal the relationship between 27HC and estrogens on URAT1/*SLC22A12* expression in humans.

4.3 | Relationship between serum 27HC levels and uric acid levels

As elevated URAT1 expression by 27HC potentially induces the reabsorption of uric acid, serum uric acid levels might be increased. However, in our study, we failed to detect differences in *Cyp7b1*$^{+/+}$ and *Cyp7b1*$^{-/-}$ mice (data not shown). Uric acid is metabolized to allantoin by uricase in rodents, but the activity of uricase had been lost in humans.[66] The basal serum uric acid levels in rodents are much lower than that in humans.[67] Also, the ERE score of the mouse *Slc22a12* gene promoter was much less than that of the corresponding human gene promoter (Figure 5A). In addition, human URAT1 has a fivefold higher affinity for uric acid than mouse Urat1, presumably a result of the evolutionary process for greater uric acid retention and more precise control of serum uric acid levels in humans than mice.[67] Thus, we speculated that it would be difficult to detect changes in serum uric acid levels by 27HC in mice.

Control of serum uric acid levels consists of the balance production and excretion of uric acid. As shown in Figure 1A, the excretion of uric acid is strictly regulated by several uric acid transporters, reabsorption, and secretion transporters in the kidney. Although our findings showed that 27HC increased the activity of the *SLC22A12* gene promoter, the effect of 27HC on other uric acid transporters remains elusive. The ERE value of the *SLC22A12* gene promoter is much higher than that of other uric acid transporter gene promoters (Figure S3), suggesting that URAT1/*SLC22A12* is the only transporter regulated by 27HC among uric acid transporters. Hence, the reabsorption of uric acid by URAT1 could be more dominant than secretion by secretion transporters, such as NPT1/4 and ABCG2, and excretion of uric acid to urine may be reduced.

The relationship between serum 27HC levels and uric acid levels in humans remains unknown. Given that rare variants of the human *SLC22A12* gene affect serum uric acid levels,[68] URAT1 might be important in serum uric acid control. It is quite likely that changes in wild-type URAT1/*SLC22A12* expression levels will impact serum uric acid levels. Further study is warranted to investigate the relationship between 27HC levels and serum uric acid levels.

5 | CONCLUSION

Based on our findings, it is plausible that elevated 27HC levels induce elevated URAT1 expression and potentially increase serum uric acid levels. People with high URAT1 expression levels are susceptible to diseases caused by hyperuricemia and hypercholesterolemia, which can be detrimental to their health. Our findings help to elucidate the correlation between hyperuricemia and metabolic syndromes and provide a potential therapeutic approach toward hyperuricemia-related diseases by modifying 27HC levels.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

MM, YMS, MU, and EM designed research. MM, YMS, YS, HN, AA, LB, RN, and ME performed research. MM, YMS, YS, and S. Kikuchi analyzed data. A.A, LB, MM, MN, and MU performed immunohistochemical analysis. MM, YMS, MU, and EM wrote the paper. S. Kikuchi, MN, S. Kobashigawa, TKM, G.K, MH, ST, K.T, KS, MT, SN, MU, and TN helped to analyze and interpret the data, and critically revised the manuscript. MU and EM conceptualized the study, developed study design, supervised the authors throughout the study, and provided expertise in manuscript preparation. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

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