Transcriptional regulation of urate transportosome member SLC2A9 by nuclear receptor HNF4α

Katharina Prestin,1 Stephanie Wolf,2 Rico Feldmann,2 Janine Hussner,1 Ingrid Geissler,2 Christian Rimbach,2 Heyo K. Kroemer,3 Uwe Zimmermann,4 and Henrette E. Meyer zu Schwabedissen1

1University of Basel, Department of Pharmaceutical Sciences, Biopharmacy, Basel, Switzerland; 2University of Basel, Department of Pharmaceutical Sciences, Biopharmacy, Basel, Switzerland; 3University Medicine, Ernst Moritz Arndt University Greifswald, Center of Drug Absorption and Transport, Institute of Pharmacology, Greifswald, Germany; 4University of Basel, Department of Pharmaceutical Sciences, Biopharmacy, Basel, Switzerland

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Members of the solute carrier group of membrane transport proteins, such as SLC22, SLC17, and SLC2, as well as members of the ATP-binding cassette (ABC) transporter family are part of this network of urate transporters (as recently summarized in Ref. 52).

In the urate transportosome, urate transporter 1 (URAT1; SLC22A12), organic anion transporter (OAT)4 (SLC22A11), and OAT10 (SLC22A13) are assumed to govern urate reabsorption at the apical membrane (3, 8, 11). OAT1 (SLC22A6) and OAT3 (SLC22A8) are expressed at the basolateral membrane and facilitate urate uptake from the circulation into tubular cells (17, 31, 38). Cellular elimination of urate at the apical membrane is mediated by voltage-driven Na+/P+-cotransporter (NPT1) (SLC17A1) and NPT4 (SLC17A3), and the ATP-dependent transporter ABCG2 and ABCC4 (18, 19, 46, 51).

In accordance with the assumed pivotal role of transporters in urate handling, several single-nucleotide polymorphisms (SNPs) located in transporter genes, including ABCG2, SLC17A1, SLC17A3, SLC22A11, and SLC2A12, have been identified in genome-wide association studies as predictive for serum urate levels (35). In addition, SNPs in the PDZ domain containing-protein 1 (PDZK1) gene have been reported to be associated with urate homeostasis. This membrane-associated scaffold protein contains several PDZ domains, which interact with specific C-terminal PDZ-binding motifs of other proteins, thereby coordinating membrane localization, signal transduction, and posttranslational modifications of its targets (21). Functional interaction of PDZK1 has been shown for uptake transporters URAT1 and OAT4 as well as for efflux transporters NPT1 and ABCG2, thereby suggesting that PDZK1 stabilizes the apical urate transporter network (1, 10, 27, 40).

However, the aforementioned genome-wide association studies have shown that genetic variants of solute carrier family 2, member 9 (SLC2A9), are of major relevance for human urate homeostasis, whereby the minor alleles of the identified SNPs were associated with lower serum urate levels, indicating that SLC2A9 is a key factor of the urate transportosome (23, 47).

SLC2A9 belongs to the facilitated glucose transporter protein family class II. In contrast to other members of this transporter superfamily, SLC2A9 not only transports hexoses but also mediates an electrogenic uniport of urate (4, 33, 47, 50). Expression of SLC2A9 has been observed in various embryonic and adult tissues, including the kidney, liver, placenta, and pancreas (33). Importantly, the SLC2A9 gene is a template for several mRNA transcripts coding for at least two

Address for reprint requests and other correspondence: H. E. Meyer zu Schwabedissen, Dept. of Pharmaceutical Sciences, Biopharmacy, Univ. of Basel, Klingelbergstrasse 50, Basel CH-4056, Switzerland (e-mail: h.meyerzuschwabedissen@unibas.ch).
different isoforms with different N-terminal (2). Notably, those transcripts exhibit different cellular localization. Indeed, whereas SLC2A9 isoform 1 is expressed at the basolateral membrane of polarized cells, SLC2A9 isoform 2 has been reported to sort to the apical site (2).

However, even if SLC2A9 is assumed to be a major factor in urate homeostasis and although numerous studies of the past 10 yr have focused on the other functional components of the urate transportosome, little is known about the coordination of the transcellular transport of urate as realized in tubular cells.

Therefore, we hypothesized that there should be a mechanism providing synchronized transcriptional regulation of the transporters involved in the network. The aim of the present study was to understand the transcriptional regulation of SLC2A9 and to test whether identified factors might contribute to the coordination of expression and therefore the activity of the urate transportosome.

### MATERIALS AND METHODS

#### Tissue samples

Human kidney tissue samples were obtained from patients undergoing nephrectomy after diagnosis of renal carcinoma. Tissue samples were collected after written informed consent from the patients. This study was approved by the local ethics committee of the Medical Faculty of the University of Greifswald (III UV 12/03). Preparation of nonmalignant transformed and neoplastic samples was followed by snap freezing in liquid nitrogen and storage at −80°C until further use. The snap-frozen samples were pulverized in a mixer mill.

#### Cell culture

HeLa cells (CCL-2, American Type Culture Collection) were maintained in DMEM with GlutaMAX-I and 1 mM sodium pyruvate (Invitrogen, Darmstadt, Germany), and HepG2 cells were cultured in RPMI 1640 with GlutaMAX-I (Invitrogen), each supplemented with 10% FBS (Invitrogen). Cell culture was performed at 37°C with 5% CO2 in a humidified atmosphere.

#### RNA extraction and quantitative real-time RT-PCR

Total mRNA from different human tissues was commercially obtained from BioCat (Heidelberg, Germany) and used for the quantification of tissue-specific gene expression. Total mRNA from human surgical renal tissue and cell lines was isolated using NucleoSpin silica-membrane technology provided by Macherey-Nagel (Dueren, Germany) according to the manufacturer’s instructions. DNase-treated total mRNA (2 μg) was primed with random hexamers and reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed using predeveloped 6-carboxylfluorescein (FAM)-labeled TaqMan probes, as shown in Table 1 (Life Technologies, Darmstadt, Germany), an equivalent of 20 ng cDNA, TaqMan Gene Expression Master Mix (Life Technologies), and the 7900 HT Fast Real-Time PCR System (Life Technologies). Expression was normalized to that of polymerase (RNA) II (DNA directed) polypeptide A using the ΔCt method (where Ct is threshold cycle).

#### For correlation analysis of SLC2A9, the number of transcripts was determined using reference samples each containing defined amounts of plasmid DNA harboring the cDNA sequence of the respective target gene. The copy number of transcripts per microliter of reference sample was determined by calculating the molecular weight of the complementary reference plasmids using an internet-based program (http://www.bioinformatics.org/sms2/reference.html) after spectrophotometric quantification of plasmid DNA content using BioPhotometer plus and μCuvette G1.0 (Vaudaux-Eppendorf, Schönenbuch, Switzerland).

#### Western blot analysis

For protein isolation, pulverized tissue samples were homogenized and solubilized by 15 min of incubation with ice-cold RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.01% sodium deoxycholate, and 0.1% SDS) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany) and by syringing with a 20-gauge needle. Cell debris was removed by 15-min centrifugation at 13,000 g. Protein lysates were stored at −80°C. Protein content was quantified using the Pierce BCA Protein Assay Kit (ThermoScientific, Bonn, Germany) and an Infinite M200 microwell reader (Tecan, Crailsheim, Germany). For Western blot analysis, protein samples were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes using a Mini-PROTEAN tetra transblot electrophoresis and tank blotting system (Bio-Rad, Munich, Germany). Subsequently, membranes were blocked with 5% BSA in 1× Tris-buffered saline-Tween 20 at room temperature for 1 h followed by an overnight incubation with the respective primary antibody at 4°C. I-19 goat polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was used for the detection of β-actin, sc21631 goat polyclonal antibody (Santa Cruz Biotechnology) was used for the detection of SLC2A9, and ab41898 mouse monoclonal antibody (K9218, Abcam, Cambridge, UK) was used for the detection of HNF4α (each diluted 1:1,000). Binding of the specific antibody was visualized using hors eradish peroxidase-labeled secondary antibodies (each diluted 1:2,000), Pierce ECL Plus Western Blotting Substrate (ThermoScientific), and the ChemiDoc XRS imaging system (Bio-Rad).

#### Cloning of wild-type and mutated promoter fragments

The promoter of SLC2A9 was subcloned into pGL3-basic (Promega, Mannheim, Germany) and used for the primers shown in Table 2. After amplification from human genomic DNA, the PCR fragments were purified and restricted using the indicated enzymes (Table 2). After ligation and amplification in Escherichia coli DH5α, the cloned sequence was controlled by Sanger sequencing (MWG Eurofins, Ebersberg, Germany). To introduce sequence variations, the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) was used following the manufacturer’s instructions.

#### Reporter gene assays

For reporter gene assays, cells were seeded at a density of 1 × 10^5 in serum-free media in 24-wells 24 h before transfection. Cells were transfected with 975 ng of the indicated variants of reporter gene vector pGL3-basic, 25 ng of pRL-TK (Promega) for normalization of transfection efficiency, and the indicated amounts of the eukaryotic expression vector pEF6/V5-HNF4α, hepatocyte nuclear factor (HNF4α); NR2A1, nuclear receptor subfamily 2, group A, member 1; POLR2A, polymerase (RNA) II (DNA directed) polypeptide A; OAT, organic anion transporter; URAT1, urate transporter 1; NPT, Na+-Pi cotransporter; ABC, ABC2, ATP-binding cassette; BCRP, breast cancer resistance protein; MRP4, multidrug resistance protein 4; PDZK1, PDZ domain containing-protein 1; NR0B2, nuclear receptor subfamily 0, group B, member 2; SHP1, small heterodimer partner 1.

### Table 1. Gene symbol and assay identifiers of TaqMan assays (Life Technologies, Darmstadt, Germany) used for quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>TaqMan Assay</th>
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<tbody>
<tr>
<td>SLC2A9 (isoform 1)</td>
<td>Hs0119173_m1</td>
</tr>
<tr>
<td>SLC2A9 (isoform 2)</td>
<td>Hs0120684_m1</td>
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<tr>
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<td>SLC22A6 (OAT1)</td>
<td>Hs00191220_m1</td>
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<td>SLC22A8 (OAT3)</td>
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<td>PDZK1</td>
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<tr>
<td>NR0B2 (SHPI)</td>
<td>Hs00222677_m1</td>
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Table 2. Primers (Eurofins MWG Operon, Ebersberg, Germany) used for PCR amplification and enzymes used for cloning of SLC2A9 promoter fragments into pGL3-basic

<table>
<thead>
<tr>
<th>Promoter Fragment</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>pGL3-iso2 (-29,737 to -28,887 bp)</td>
<td>5'-CGCGGAGCTCGAGAGAGAGTGAAACACAG-3' (SacI)</td>
<td>5'-CGCGGCTACGTTGCTAGAACTCTTTGAGGCTAG-3' (NotI)</td>
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<tr>
<td>pGL3-iso2 (-15,113 to -14,140 bp)</td>
<td>5'-CGCGGAGCTCACTGAGAGAGTGAAACACAG-3' (SacI)</td>
<td>5'-CGCGGCTACGTTGCTAGAACTCTTTGAGGCTAG-3' (NotI)</td>
</tr>
<tr>
<td>pGL3-iso1 (-976 to -55 bp)</td>
<td>5'-CGCGGCTACATGGTGAATGACAGGACAGAAGAG-3' (KpnI)</td>
<td>5'-CGCGGCTACATGGTGAATGACAGGACAGAAGAG-3' (KpnI)</td>
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<tr>
<td>pGL3-iso1 (-617 to -55 bp)</td>
<td>5'-CGCGGCTACCTGCACTTTGCAGAGTAAAGAGAG-3' (KpnI)</td>
<td>5'-CGCGGCTACCTGCACTTTGCAGAGTAAAGAGAG-3' (KpnI)</td>
</tr>
<tr>
<td>pGL3-iso1 (-231 to -55 bp)</td>
<td>5'-CGCGGCTACATGGTGAATGACAGGACAGAAGAG-3' (KpnI)</td>
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<tr>
<td>pGL3-iso1 (-447 to -212 bp)</td>
<td>5'-CGCGGCTACATGGTGAATGACAGGACAGAAGAG-3' (KpnI)</td>
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</tr>
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Fig. 1. Positive correlation of SLC2A9 isoform 1 mRNA expression and expression of other urate transporters of the urate transportosome and hepatocyte nuclear factor (HNF)4α in the human kidney. mRNA expression levels were assessed in renal tissue samples using quantitative real-time RT-PCR and reference samples for the quantification of numbers of transcripts of the corresponding transporters. Spearman regression analysis was conducted to test the correlation of SLC2A9 isoform 1 and SLC2A12 [urate transporter (URAT1); A], SLC2A11 [organic anion transporter (OAT)4; B], SLC2A13 (OAT10; C), and HNF4α (D). Expression was normalized to that of polymerase (RNA) II (DNA directed) polypeptide A (n = 29). R, Spearman coefficient. Dotted lines show the confidence intervals (CI).
Chromatin immunoprecipitation assay. For chromatin immunoprecipitation (ChIP), 300 ng snap-frozen, pulverized tissue or 2 × 10^6 cells and the EZ-ChIP Kit were used (Merck, Zug, Switzerland). In detail, chromatin was fixed with 1% formaldehyde for 15 min before the in vivo cross-linking was quenched with glycine. Cells were washed with PBS twice and lysed with 1.6 ml SDS lysis buffer. Lysates were sheared on ice with 35 repeats of 30-s pulses set to 0.9 and 90% amplitude followed by 45-s rests using the ultrasonic processor (UP200S) and a 1-mm tip (Hielscher, Teltow, Germany). Cross-linked protein/DNA complexes were immunoprecipitated using anti-RNA polymerase II antibody (no. 05-623B, Merck) as a positive control, anti-HNF4a antibody (K9218, Abcam), and ChIP-blocked protein G-conjugated agarose beads. Treatment with mouse IgG (no. 12-371B, Merck) served as a negative control. Protein-DNA cross-linking was reversed, and DNA was purified using silica membrane spin columns. Purified DNA (0.5 μl) was applied by PCR using 0.1 μl Platinum Taq DNA polymerase, 1× PCR buffer, 1.5 mM MgCl2, 400 μM dNTP mix, 0.3 μM sense primer (5′-CATGATCACAATTGCCA-CACCTCC-3′), and 0.3 μM antisense primer (5′-GACCCCA-GACTCTGCAAG-3′) in a total reaction volume of 25 μl. The temperature profile of the ChIP-PCR was as follows: preincubation at 95°C (2 min) followed by 40 cycles of 95°C (30 s), 60°C (30 s), and 72°C (30 s), and final extension at 72°C (2 min). The resultant PCR products were separated by 2.5% agarose electrophoresis.

RESULTS

Profiling of the urate transportosome in human kidney samples. Expression of the genes assumed to be part of the urate transportosome, namely, SLC22A6 (OAT1), SLC22A8 (OAT2), SLC22A11 (OAT4), SLC22A13 (URAT1), SLC17A1 (NPT1), SLC17A3 (NPT3), ABCG2 (breast cancer resistance protein), and ABC4 (multidrug resistance protein 4) were assessed in 29 human kidney samples using quantitative real-time RT-PCR. Reference samples each containing a defined amount of plasmid DNA harboring the cDNA sequence of the respective transporter were used to define the copy number of the corresponding transcripts. All transporters were detected in the tested tissue samples, including SLC2A9 isoforms 1 and 2.

However, in the following reported experiments, we focused on the analysis of SLC2A9 isoform 1. Importantly, as shown in Fig. 1, A–C, Spearman regression analysis revealed a statistically significant positive correlation of SLC2A9 isoform 1 mRNA expression with apical urate uptake transporters involved in renal urate reabsorption, such as SLC22A12 (URAT1, Spearman coefficient (R) = 0.7276 [confidence interval (CI): 0.4836–0.8666], P < 0.0001), SLC22A11 (OAT4, R = 0.6808 [CI: 0.4093–0.8415], P < 0.0001), and SLC22A13 (OAT10, R = 0.6394 [CI: 0.3464–0.8188], P = 0.002). Similar results were obtained for the apical urate efflux transporters SLC17A1 [NPT1, R = 0.5473 (CI: 0.2152–0.7659), P = 0.0021] and SLC17A3 [NPT4, R = 0.7803 (CI: 0.5791–0.8941), P < 0.0001] and the basolateral urate uptake transporter SLC22A6 [OAT1, R = 0.7365 (CI: 0.4982–0.8713), P < 0.0001; data not shown].

Positive correlation of mRNA levels of SLC2A9 isoform 1 and HNF4α in the human kidney. Based on the observed correlation of SLC2A9 isoform 1 expression with other members of the urate transportosome, we hypothesized that there should be a factor coordinately regulating a gene network summarizing the urate transportosome. It seems noteworthy that the nuclear receptor HNF4α has been previously reported to be involved in the modulation of expression of some of the above-mentioned urate transporters, namely, SLC17A1 (NPT1), SLC17A3 (NPT4), SLC22A6 (OAT1), and SLC22A8 (OAT3) (9, 30, 31, 48). To provide evidence that this nuclear receptor could play a role in the regulation of the renal urate transporter gene network, expression of HNF4α was tested in human kidney samples, revealing expression levels similar to hepatic tissue (data not shown).

In addition, Spearman regression analysis showed a statistically significant positive correlation of SLC2A9 isoform 1 and HNF4α mRNA expression [R = 0.6103 (CI: 0.0337–0.8024), P = 0.0004] in kidney tissue samples, suggesting that there might be an involvement of HNF4α in the modulation of SLC2A9 expression (Fig. 1D).

Transfection with the HNF4α expression vector induces endogenous SLC2A9 isoform 1 mRNA expression levels in HeLa cells. Transactivation experiments were conducted to test whether HNF4α modulates SLC2A9 expression in a cellular system. In detail, 48 h after transfection of HeLa cells with a eukaryotic expression vector encoding for the nuclear receptor HNF4α, detection of endogenous SLC2A9 isoform 1 mRNA expression revealed enhanced endogenous expression of this transporter (mean mRNA expression relative to pEF6/V5-His control ± SD: 67.5 ± 4.5; Fig. 2). However, SLC2A9 isoform
transcriptional regulation of SLC2A9 by HNF4α

1 expression was further enhanced by cotransfection of RXRα (mean mRNA expression relative to pEF6/V5-His control ± SD: 164.9 ± 9.9). Enhanced expression in the presence of HNF4α, which was further increased in the presence of RXRα, was also observed for the well-known HNF4α target gene nuclear receptor subfamily 0, group B, member 2 (NR0B2), which is also called small heterodimer partner 1 (mean mRNA expression relative to pEF6/V5-His control ± SD: 24.0 ± 0.7 for HNF4α and 40.0 ± 0.6 for HNF4α + RXRα; Fig. 2).

Identification of two putative gene-regulating modules of SLC2A9. Initially, to identify regulative promoters of SLC2A9 isoforms 1 and 2, respectively, fragments of the intronic regions upstream of exons 0a, 1a, 2a, and 1b were subcloned into pGL3-basic (Fig. 3A). The subcloned SLC2A9 promoter fragments were used in cell-based dual luciferase reporter gene assays and showed no transcriptional activity upstream of the first transcribed exons (0a and 1a) of isoform 2 (Fig. 3B). However, there was an increased basal activity of the reporter gene in HeLa cells transfected with SLC2A9 promoter fragments −740 to +161 bp, representing a fragment 5'-upstream of exon 2a (isoform 2), and −976 to −55 bp, representing a fragment 5'-upstream of exon 1b (isoform 1). Basal transcriptional promoter activity of SLC2A9 isoform 1 was further restricted to a fragment encompassing −231 to −55 bp using 5'-deletion constructs of the identified promoter fragment in cell-based luciferase assays (Fig. 3C).

In silico analysis of the core promoter of SLC2A9 isoform 1 and in vitro mutation of putative transcription factor-binding sites. As shown in Fig. 4A, several potential transcription factor-binding motifs in the SLC2A9 isoform 1 promoter fragment (−231 to −55 bp) were identified by in silico analysis using internet-based programs such as TFSEARCH and NUBISCAN (14, 34). In addition to CCAAT/enhancer binding protein (C/EBP), octamer transcription factor 1 (Oct1), c-Myb, E26 transformation specific (Ets), PU.1, and NF-kB binding motifs, an inverted/a direct repeat of one hexanucleotide (IR1, DR0), a putative HNF4α response element, was identified in position −84 to −73 bp of the promoter fragment. Sequence alignment showed high similarity of this motif with HNF4α cis elements in the promoters of known target genes of this nuclear receptor, such as human α1-antitrypsin or coagulation factor X (Fig. 4B) (12, 16).

Notably, after mutation of this particular binding site, an intense loss of basal luciferase activity of promoter fragments −231 to −55 bp as well as −976 to −55 bp was observed in HepG2 cells containing high endogenous mRNA expression of HNF4α (Fig. 5A). However, no such effect was observed when we tested the impact of the mutated HNF4α-binding site on the

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**Fig. 3.** Identification of gene-regulating modules of SLC2A9. **A:** genomic structure of the SLC2A9 gene with exons (boxes), introns (lines), alternative translational start sites (arrows), and alternative exons specific for isoform 1 (exon 1b) and isoform 2 (exons 0a, 1a, and 2a). Fragments of the intronic regions 5'-upstream of exons 0, 1a, 2a, and 1b were subcloned into luciferase vector pGL3-basic. **B:** subsequent dual luciferase reporter gene assays with these promoter fragments in HeLa cells revealed two gene-regulating modules in the SLC2A9 gene, with one being 5'-upstream of the first exon (exon 1b) of SLC2A9 isoform 1. *P < 0.05 by paired t-test (two-tailed). **C:** using deletion constructs, basal activity of the module of isoform 1 was restricted to a region from −231 to −55 bp relative to the translational start site of isoform 1. Data in **B** and **C** are means ± SD from three independent experiments performed as triplicates. ***P < 0.0005 by ANOVA with Dunnett’s multiple-comparison test.
basal promoter activity in HeLa cells harboring low endogenous HNF4α (Fig. 5B).

Furthermore, the binding sites of C/EBP, Oct1, c-Myb, NF-κB, and Ets were mutated to determine their involvement in basal promoter activity. Mutation of C/EBP, Oct1, and c-Myb motifs did not alter luciferase activity of both promoter fragments in HeLa cells (data not shown). However, there was a loss of promoter activity of both fragments by 64 ± 8% and 58 ± 9% when the NF-κB motif in position -107 to 96 bp was mutated and subsequently used in a dual reporter gene assay. Importantly, mutation of the Ets-binding motif in position -119 to -114 bp resulted in a significant decrease of reporter gene activity of promoter fragments -976 to -55 bp and -231 to -55 bp by 94 ± 2% and 91 ± 1%, respectively (data not shown).

Transfection with the HNF4α expression vector enhances activity of the putative core promoter of SLC2A9 isoform 1, and transactivation is reduced by mutation of the putative HNF4α-binding site. In dual reporter gene assays, subcloned SLC2A9 isoform 1 promoter fragments cotransfected with

Fig. 4. In silico analysis of promoter fragment -231 to -55 bp of SLC2A9 isoform 1. A: internet-based programs (Softberry NSITE and TFSearch) revealed putative transcription factor-binding sites for CCAAT/enhancer binding protein (C/EBP), octamer transcription factor 1 (Oct1), c-Myb, E26 transformation specific (Ets), PU.1, NF-κB, and liver factor A1 (LF-A1 synonym HNF4α). NUBIScan analysis identified a hexanucleotide [direct repeat (DR) and inverted repeat (IR)] as being a putative HNF4α-binding site. B: sequence alignment of the HNF4α-binding motif in the SLC2A9 gene with HNF4α motifs of known HNF4α target genes. The consensus sequence was determined using ORegAnno (http://www.pazar.info/cgi-bin/proj_res.cgi) (28). FX, factor X; CYP7A1, cytochrome P-450 7A1.

Fig. 5. Mutational analysis of the putative HNF4α-binding site. In dual reporter gene assays, luciferase activity of promoter fragments harboring the mutated (Mut) HNF4α-binding motif [deletion of 6 nucleotides (-6nt) or 12 nucleotides (-12nt)] was determined in HepG2 (A) and HeLa (B) cells 48 h after transfection. Data are means ± SD relative to the activity of wild-type (WT) promoter fragments from three independent experiments. *P < 0.05 and **P < 0.005 by ANOVA with Dunnett’s multiple-comparison test.
eukaryotic HNF4α expression vector showed significantly higher luciferase activity than promoter fragments cotransfected with pEF6/V5-His control vector (mean luciferase activity relative to pEF6/V5-His control ± SD: 8.2 ± 1.8; Fig. 6A). Similar results were obtained using eukaryotic RXRα expression vector, which had increased activity relative to pEF6/V5-His control (3.4 ± 1.2). Simultaneous cotransfection with both HNF4α and RXRα resulted in an even higher enhancement of promoter activity (mean luciferase activity relative to pEF6/V5-His control ± SD: 607.0 ± 162.4). Importantly, reduced transactivation by HNF4α was observed when we performed mutational analysis of the putative HNF4α-binding site in the core promoter of SLC2A9 isoform 1 (Fig. 6B). In detail, dual luciferase reporter gene assays of subcloned SLC2A9 isoform 1 promoter fragments −231 to −55 bp and −976 to −55 bp with the mutated sequence of the HNF4α-binding site showed significant differences in the induction of luciferase activity by 50% compared with the wild type when cotransfected with the eukaryotic HNF4α expression vector.

In vivo binding of HNF4α to the SLC2A9 isoform 1 promoter region (−231 to −55 bp). To test whether HNF4α binds to the SLC2A9 isoform 1 promoter, a ChIP assay was performed using DNA isolated from human kidney samples, which revealed an in vivo interaction of this nuclear receptor with the herein identified promoter region of SLC2A9 isoform 1, as shown in Fig. 7D.

Dedifferentiation of the urate transportosome in human renal cell carcinoma. Since we were able to show that HNF4α is a transcriptional modulator of SLC2A9 isoform 1 expression and since HNF4α expression has been previously described as being deregulated in renal cell carcinoma, we asked whether human renal cell carcinogenesis is also associated with altered expression of the herein reported target gene SLC2A9 and other components of the urate transportosome.

Real-time quantitative RT-PCR and Western blot analysis comparing paired nontransformed and malignant transformed renal tissue samples showed lower expression of both SLC2A9 isoform 1 and HNF4α in the carcinoma samples (Fig. 8). Those differences in mRNA expression were also observed for PDZK1 and apical and basolateral urate uptake transporters OAT1 (SLC22A6), OAT3 (SLC22A8), OAT4 (SLC22A11), OAT10 (SLC22A13), and URAT1 (SLC22A12) as well as apical efflux transporter NPT1 (SLC17A1), with lower mRNA levels in renal tumor samples (Fig. 8, A–G). There was no significant difference in expression in tumor samples and paired controls for NPT4 (SLC17A3; Fig. 8H). In contrast, ABCG2 expression showed slightly lower expression in nonmalignant transformed tissue than in tumor samples (Fig. 8I).

**DISCUSSION**

Taken together, we studied the transcriptional regulation of SLC2A9 isoform 1, which is part of the urate transportosome characterized by a functional network of urate uptake and efflux transporters (8). Our investigations not only support the notion that the transcriptional activity of SLC2A9 is regulated by two promoters but also show that the nuclear receptor HNF4α is one of the factors transcriptionally modulating the expression of isoform 1 of this urate transporter.

The assumption of a HNF4α-dependent regulation of SLC2A9 was initially supported by expression studies revealing a positive correlation of SLC2A9 isoform 1 and HNF4α mRNA levels in the human kidney. Further evidence was provided by the observations that transient HNF4α overexpression induced endogenous SLC2A9 isoform 1 mRNA levels in cells and enhanced reporter gene activity of vector constructs harboring the promoter region of SLC2A9 isoform 1. The involvement of HNF4α in the regulation of SLC2A9 isoform 1 was further supported by the in silico identification of a potential HNF4α-binding motif in the core promoter of the transporter gene. Importantly, site-directed mutagenesis of this binding motif
reduced HNF4α-dependent transactivation of the promoter. The high similarity of the herein studied HNF4α response element with sequences previously identified in the 5′-untranslated region of known HNF4α target genes, such as α-antitrypsin, or coagulation factor X indicates that the identified element is a representative HNF4α-binding motif (6, 12, 16, 25, 43).

HNF4α has not only been shown to be expressed in the human kidney but also has been reported to be significantly downregulated in human kidney tumors, thereby playing a role in carcinogenesis and tumor progression (26, 39, 41). Similar results have been observed in hepatocellular carcinoma (24).

The herein reported findings of an in vivo binding of HNF4α to the SLC2A9 isoform 1 promoter region together with the findings on the downregulation of SLC2A9 and HNF4α in human renal cell carcinoma samples correlate well with the function of HNF4α as a modulator of SLC2A9 expression in vivo.

Notably, several components of the urate transportosome, including NPT1 (SLC17A1), NPT4 (SLC17A3), OAT1 (SLC22A6), and OAT3 (SLC22A8), have been previously reported to be target genes of the nuclear receptor HNF4α, thereby providing the basis for the hypothesis that HNF4α is one major determinant of the urate transportosome gene network (9, 30, 31, 48).

In accordance are the findings of our expression experiments that showed a positive correlation of SLC2A9 isoform 1 expression with those of other members of the urate transportosome in human nonmalignant transformed kidney samples, indicating that members of a physiological network might also be transcriptionally connected. The findings that HNF4α expression was significantly reduced in renal cell carcinoma and that mRNA expression of SLC2A9 and expression of other urate transporters followed the same trend support the notion that correlated targets might be regulated in the same way following the same trend of regulation, although a low level of HNF4α expression might not be the only reason of downregulation of urate transporters.

However, it seems noteworthy that the role of HNF4α is not limited to the regulation of urate transporter expression, as it has been shown to be a regulator of other proximal tubule functions and, importantly, embryonic tubule maturation in general (9).

Taken together, our findings support the idea that nuclear receptor family member HNF4α is not only a modulator of SLC2A9 isoform 1 expression in the kidney but might also be a central regulator of the gene network summarizing the urate transportosome, thereby supporting the above-mentioned hypothesis that expression and activity of genes involved in renal urate handling could be coordinated by a shared regulator or regulatory pathway.

Interestingly, nuclear receptors as transcriptional regulators of gene networks have been widely studied. For example, nuclear receptor family members farnesoid X receptor (NR1H4) and peroxisome proliferator-activated receptor-α (NR1C1) are modulators of physiological processes such as bile acid synthesis or lipid homeostasis, respectively (7). Moreover, in the context of
drug/toxin elimination, the ligand-activated xenosensor pregnane X receptor (PXR; NR1I2) has been a major focus. In general, after binding of activating ligands, which include a variety of drugs in clinical use, PXR translocates into the nucleus, where it enhances expression and therefore activity of a target gene network. This network summarizes uptake transporters, metabolizing enzymes, and efflux transporters (44). It is therefore assumed that PXR is a key factor adjusting hepatic metabolic activity to drug exposure, thus being a driving force of drug-drug interactions. One example of clinical relevance is the observed loss of therapeutic efficacy of the immunosuppressant cyclosporine A in the presence of the antidepressant St. John’s wort, resulting in graft rejection of transplanted patients (36). Indeed, PXR activation by hyperforin, a constituent of St. John’s wort, induces cytochrome P-450 (CYP)3A4 and P-glycoprotein (ABCB1) expression, thereby increasing hepatic metabolism and elimination of their substrate cyclosporine A (29, 32).

Similar to PXR, the nuclear receptor family member HNF4α dimerizes and binds to specific response elements in the 5’-untranslated region of target genes, as reported by Sladek and colleagues (41). Interestingly, it has been previously reported that HNF4α is important for the above-mentioned PXR-mediated transactivation of CYP3A4 (45). In addition, HNF4α has
been shown to be a major modulator of hepatic gene expression and to directly regulate several drug transporters and metabolizing enzymes, indicating its regulatory role in drug disposition gene expression (20).

Taken together, the nuclear receptor HNF4α may not only coordinate homeostasis of the physiological substrate urate but also the renal clearance of pharmacological compounds and therefore pharmacokinetics by modulating expression of membrane proteins involved in physiological and pharmacological transport. However, it is still unclear whether activity of HNF4α is modulated by ligand-mediated activation or by transcriptional induction. On the one hand, there is evidence that fatty acyl-CoA thioesters and linoleic acid act as potent activators of HNF4α (5, 15, 49). On the other hand, it is known that HNF4α itself is a target of the homeobox transcription factor HNF1α (13). Since HNF1α itself is a target of HNF4α and has been previously reported to modulate the transcription of several components of the urate transportosome, it seems beyond the scope of this report to decide on the role of HNF1α in this regulatory pathway (22, 37, 39, 42, 48).

In conclusion, we demonstrate that the nuclear receptor HNF4α is one of the factors transcriptionally modulating SLC2A9, thereby providing further evidence that this transporter is part of the urate transportosome. Additionally, we show that the urate transporter network is disturbed in human renal cell carcinoma.

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Present address of S. Wolf: Dept. of Gastroenterology, Institute of Hepatology and Infectious Disease, University Hospital, Heinrich Heine Univ. of Düsseldorf, Düsseldorf, Germany.

Present address of C. Rimmich: Reference and Transplantation Center for Cardiac Stem Cell Therapy, Univ. of Rostock, Rostock, Germany.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

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