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# Publizierte Inhalte der Medizinischen Habilitationsschrift

**von**

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# Geschlechtsabhängige Expression renaler und hepatischer Transporter für organische Anionen und Kationen

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2. **Next generation sequencing of sex-specific genes in livers of diabetic ZSF1 rats.** Babelova A, Burckhardt BC, Salinas-Riester G, Pommerenke C, Burckhardt G, Henjakovic M. (*Genomics*; doi: 10.1016/j.ygeno.2015.07.006).

3. **Male-dominant activation of rat renal organic anion transporter 1 (Oat1) and 3 (Oat3) expression by transcription factor BCL6.** Wegner W, Burckhardt BC, Burckhardt G and Henjakovic M. *PLoS One* 7: e35556, 2012.

4. **Transcriptional regulation of human organic anion transporter 1 (OAT1) by B-cell CLL/lymphoma 6 (BCL6).** Wegner W, Burckhardt G, Henjakovic M. *Am J Physiol Renal Physiol*. 307: F1283-F1291, 2014.

# Inhaltsverzeichnis

## Abkürzungsverzeichnis

| | |
|---|---|
| ABC | *ATP binding cassette* |
| Acacb | *Acetyl-CoA carboxylase 2* |
| Acsl4 | *acyl-CoA synthetase long-chain family member 4* |
| AhR | *aryl hydrocarbon receptor* |
| ATF-1 | *activating transcription factor 1* |
| BCL6 | B-*cell CLL/lymphoma 6* |
| cAMP | 3',5'-cyclisches Adenosinmonophosphat |
| CAR | *constitutive androstane receptor* |
| Cd36 | Fettsäuretranslokase |
| cGMP | 3',5'-cyclisches Guanosinmonophosphat |
| CREB-1 | *cAMP response element*-binding protein 1 |
| Cyp | Cytochrome P450 |
| FDA | *U.S. Food and Drug Administration* |
| FXR | *farnesoid X receptor* |
| G6pd | Glucose-6-phosphat-Dehydrogenase |
| Gck | Glucokinase |
| GO | *Gene Ontology* |
| Gstm | *Glutathion S-Transferasen mu* |
| Hnf / HNF | *hepatocyte nuclear factor* (Maus und Ratte / Mensch) |
| Hsd | Hydroxysteroid-Dehydrogenase |
| Lifr | Cytokinrezeptor |
| MDR1 | *Multidrug Resistance Protein 1* |
| Mdr1b | *Multidrug Resistance Protein 1* in der Ratte (Homolog des humanen MDR1) |
| Mrp / MRP | *Multidrug Resistance-associated* Protein (Maus und Ratte / Mensch) |
| NFκB | *nuclear factor kappa B* |
| NGS | *Next generation sequencing* |

| | |
|---|---|
| NSAIDs | *nonsteroidal anti-inflammatory drugs* |
| Oat / OAT | Organische-Anionen-Transporter (Maus und Ratte / Mensch) |
| Oct / OCT | Organische-Kationen-Transporter (Maus und Ratte / Mensch) |
| OK | *Oppossum kidney* |
| PAS | periodic acid-Schiff (Färbung) |
| PCA | *principal component analysis* |
| P-gp | P-Glykoprotein |
| PKA | Proteinkinase A |
| Polr3g | *Polymerase-(RNA)-III-(DNA-Directed)-Polypeptide* G |
| PPARα | *peroxisome proliferator-activated receptor* α |
| PPARδ | *peroxisome proliferator-activated receptor* δ |
| PXR | *pregnane X receptor* (PXR) |
| Sglt | *Sodium-glucose cotransporter* |
| SHHF | *spontaneously hypertensive heart failure* (genetisch veränderter Rattenstamm) |
| SHP | *FXR-induced transcriptional repressor* |
| Slc / SLC | *solute carrier* (Maus und Ratte / Mensch) |
| SNPs | *single nucleotide polymorphisms* |
| Srd5a1 | Steroid 5α-Reduktase 1 |
| Sult2a1 | Gallensäure-Sulfotransferase 1 |
| USF | *upstream transcription factor* |
| ZDF | *Zucker diabetic fatty* (genetisch veränderter Rattenstamm) |
| ZSF1 | *Zucker spontaneously hypertensive fatty* (ZDFxSHHF-Kreuzung, Generation F1) |

# 1 Einleitung

## 1.1 Geschlechtsabhängige Nebenwirkungen von Pharmaka

In einer multizentrischen Studie aus England ergab die Analyse von 513608 Patienten-Daten, dass zahlreiche, häufig verschriebene Medikamente geschlechtsabhängige Nebenwirkungen induzieren [1]. Eine weitere, geschlechtsabhängige Analyse von 2371 Patienten-Daten zeigte häufigere Nebenwirkungen von Antibiotika und anti-inflammatorischen Substanzen bei weiblichen als bei männlichen Patienten in Deutschland und Israel [2].
Bei der Entwicklung und Testung neuer Medikamente wird die Geschlechtsabhängigkeit inzwischen häufiger beachtet als in den vergangenen Jahrzehnten. Die FDA (*U.S. Food and Drug Administration*) empfiehlt mittlerweile ausdrücklich, Frauen in klinische Studien zu integrieren [3]. Dadurch sollen die möglichen geschlechtsabhängigen Nebenwirkungen in der frühen Entwicklungsphase von Arzneimitteln erfasst werden [3].

Die geschlechtsabhängige Pharmakokinetik und Pharmakodynamik einzelner Präparate beruht hauptsächlich auf deren biologischer Verfügbarkeit, der Verteilung, dem Metabolismus und der Ausscheidung [4]. Die genauen Ursachen für die unterschiedliche Verträglichkeit von Arzneimitteln zwischen Frauen und Männern sind bisher unbekannt. Eine der möglichen Ursachen könnte die geschlechtsabhängige Expression renaler und hepatischer Transportproteine sein, die für die Aufnahme der Arzneimittel in die Zellen („Influx-Transporter“) und deren Ausscheidung aus den Zellen („Efflux-Transporter“) verantwortlich sind.

## 1.2 Influx- und Efflux-Transporter

Diverse Transporter, die im Darm, in der Leber, in den Nieren und in der Bluthirnschranke exprimiert sind, wurden 2010 vom internationalen Transporter-Konsortium als klinisch relevant eingestuft [5]. Das Konsortium empfahl diese Transporter bei der Entwicklung und der Testung neuer Arzneimittel zu berücksichtigen [5]. Die klinisch relevanten Transporter gehören zu den Genen der *solute carrier* (SLC)- und der *ATP binding cassette* (ABC)-Familie [5].

Zur SLC-Genfamilie 22 gehören Organische-Anionen-Transporter (OATs) und Organische-Kationen-Transporter (OCTs) [5]. OATs und OCTs sind für die Aufnahme (Influx) von zahlreichen endogenen und exogenen Substanzen aus dem Blut in die proximale Tubuluszellen der Nieren und in die Hepatozyten verantwortlich [6-10].

In der ABC-Genfamilie sind unter anderem *Multidrug Resistance-associated* Proteine (MRPs) und *Multidrug Resistance Protein 1* (MDR1) vertreten [5]. Diese Efflux-Transporter sind für die Ausscheidung verschiedener Arzneimittel und deren Stoffwechselprodukte aus den Zellen heraus verantwortlich [11, 12]. Häufig transportieren MRPs und MDR1 die anionischen bzw. kationischen Substanzen aus den Zellen heraus, die durch OATs und OCTs in die Zellen aufgenommen wurden [13] (Abb. 1 und 2).

### 1.2.1 Influx-Transporter für organische Anionen und Kationen

OAT1 und OAT3 sind in der basolateralen Membran proximaler Tubuluszellen in den Nieren exprimiert und wurden als klinisch relevant eingestuft [6, 7, 10, 14]. Die polyspezifischen Transportproteine OAT1 und OAT3 transportieren endogene und exogene Substanzen (z.B. Arzneimittel) aus dem Blut in die proximalen Tubuluszellen im Austausch gegen intrazelluläres $\alpha$-Ketoglutarat ($\alpha$-$KG^{2-}$) [7, 10, 14]. In verschiedenen Studien wurde gezeigt, dass ACE-Inhibitoren, Angiotensin II Rezeptor-Blocker, Diuretika, Statine, Antibiotika, Zytostatika und nicht-steroidale, anti-inflammatorische Arzneimittel (*nonsteroidal anti-inflammatory drugs*, NSAIDs) mit OAT1 und OAT3 interagieren oder durch diese Transporter in die Zellen aufgenommen werden [7, 15].

OAT2 ist in der sinusoidalen Membran von Hepatozyten und in der basolateralen Membran proximaler Tubuluszellen in den Nieren exprimiert [7, 14]. Bisher wurde gezeigt, dass OAT2 das 3',5'-cyclische Guanosinmonophosphat (cGMP), das Diuretikum Bumetanid und die antiviralen Arzneimittel Acyclovir, Ganciclovir und Penciclovir transportiert und mit verschiedenen Zytostatika, Diuretika und NSAIDs interagiert [16-20]. OAT2 zählt noch nicht zu den klinisch relevanten Transportproteinen [21]. Der Transport-Mechanismus von OAT2 ist weitestgehend unbekannt und wird derzeit auch in unserem Labor untersucht.

OCT1 und OCT2 sind polyspezifische Transportproteine, die bevorzugt positiv geladene Substanzen transportieren und eine überlappende Substrat-Selektivität zeigen [9, 13]. OCT1 ist in der sinusoidalen Membran von Hepatozyten der humanen Leber exprimiert [21]. OCT2 ist beim Menschen hauptsächlich in der basolateralen Membran proximaler Tubuluszellen lokalisiert [5]. OCT2 wurde in die Liste klinisch relevanter Transportproteinen aufgenommen, OCT1 noch nicht [21]. OCT1 und OCT2 interagieren mit zahlreichen Arzneimitteln verschiedener Substanzklassen [9, 22]. Unter anderem vermittelt OCT2 den renalen Transport von Metformin und Cisplatin [9, 23]. Die Rolle von OCT1 bei der Aufnahme, der biologischen Verteilung und Sekretion des Antidiabetikums Metformin wird kontrovers diskutiert [24-26].

### 1.2.2 Efflux-Transporter für organische Anionen und Kationen

Die Efflux-Transporter MRP2 und MRP4 sind in der luminalen Membran proximaler Tubuluszellen in den Nieren exprimiert und vermitteln den ATP-abhängigen Transport von negativ geladenen Substanzen aus den proximalen Tubuluszellen in den Primärharn [11, 27, 28]. Außerdem vermittelt MRP2 den Efflux von Substanzen aus den Epithelzellen im Interstitium und aus den Hepatozyten in die Galle [5, 11, 13]. MRP4 ist in der sinusoidalen Membran von Hepatozyten und in der apikalen Membran von Endothelzellen der Hirnkapillaren lokalisiert und am Efflux zahlreicher Substanzen beteiligt [5, 11, 13]. Die endogenen Substrate von MRP2 und MRP4 sind u.a. Estradiol-17β-D-glucuronid (ein Metabolit von Östrogen) und Leukotrien C4 [11, 29-32]. MRP4 transportiert auch 3',5'-cyclisches Adenosinmonophosphat (cAMP), 3',5'-cyclisches Guanosinmonophosphat (cGMP) und Urat aus den proximalen Tubuluszellen der Nieren in den Primärharn [28, 33]. Der Efflux von Methotrexat aus der Leber und aus den Nieren, wie auch die Chemo-Resistenz verschiedener Krebszellen wird u.a. durch MRP2 und MRP4 vermittelt [11, 34, 35].

MDR1, auch P-Glykoprotein (P-gp) genannt, ist in den apikalen Membranen lokalisiert und vermittelt den ATP-abhängigen Efflux von organischen Kationen in den Epithelzellen im Interstitium, in den Hepatozyten, in den proximalen Tubuluszellen der Nieren und in den Endothelzellen der Gehirn-Kapillaren [5, 13]. Der MDR1-abhängige Transport endogener Substrate ist unklar, weil dieses Transportprotein bisher hauptsächlich bezüglich Chemo-Resistenz untersucht wurde [12]. MDR1 transportiert zahlreiche anti-neoplastische und immunmodulierende Substanzen, wie z.B. Vinblastin, Cyclosporin, Doxorubicin oder Verapamil [12, 13].

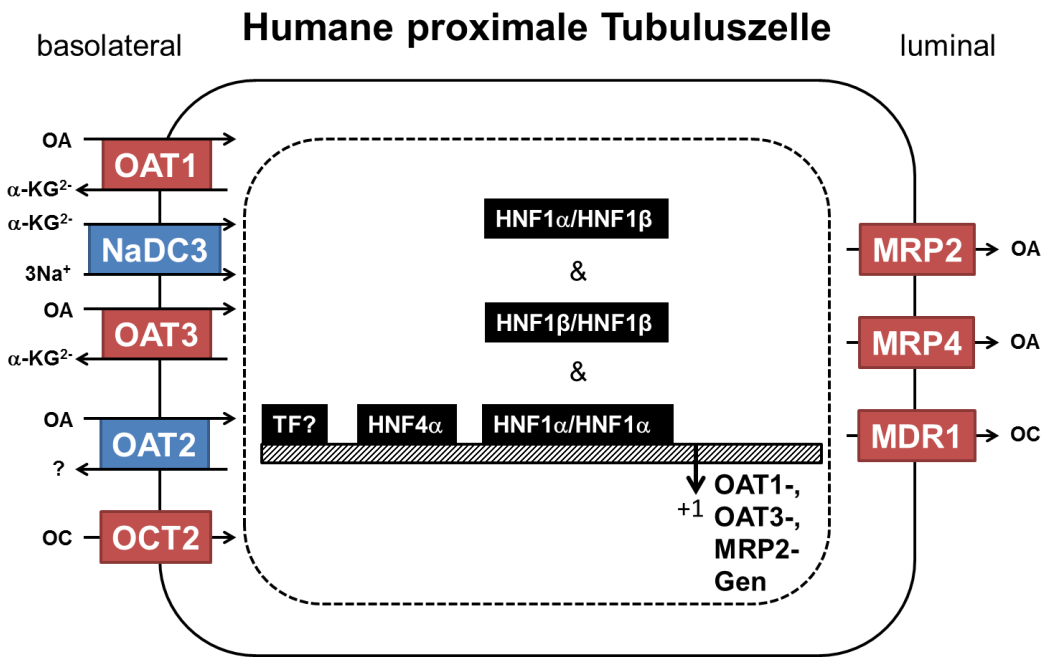


**Abbildung 1: Schematische Darstellung renaler Transporter.** OAT1 und OAT3 transportieren organische Anionen (OA) im Austausch gegen α-Ketoglutarat (α-$KG^{2-}$), welches durch den Natrium-Dicarboxylat-Cotransporter 3 (NaDC3) in die Zellen aufgenommen wird. Der Transport-Mechanismus von OAT2 ist unbekannt. Die OAs werden unter anderem durch MRP2 und MRP4 aus den proximalen Tubuluszellen in den Primärharn abgegeben. OCT2 transportiert organische Kationen (OC) aus dem Blut in die proximale Tubuluszelle der Nieren. MDR1 transportiert OCs, z.B. Zytostatika aus den Zellen in dem Primärharn. Die rot hervorgehobenen Transporter wurden als klinisch relevant eingeordnet, die blau-markierten noch nicht. Die Transkriptionsfaktoren *hepatocyte nuclear factor 1α* (HNF1α) und HNF1β aktivieren die Promotoren entweder als Homo- oder Heterodimere. HNFα kann die Promotoren von OAT1, OAT2 und MRP2 aktivieren. Die Transkription der renalen Transporter wird durch weitere bekannte und unbekannte Transkriptionsfaktoren (TF) beeinflusst. Die rot hervorgehobenen Transporter wurden als klinisch relevant eingeordnet, die blau-markierten noch nicht.

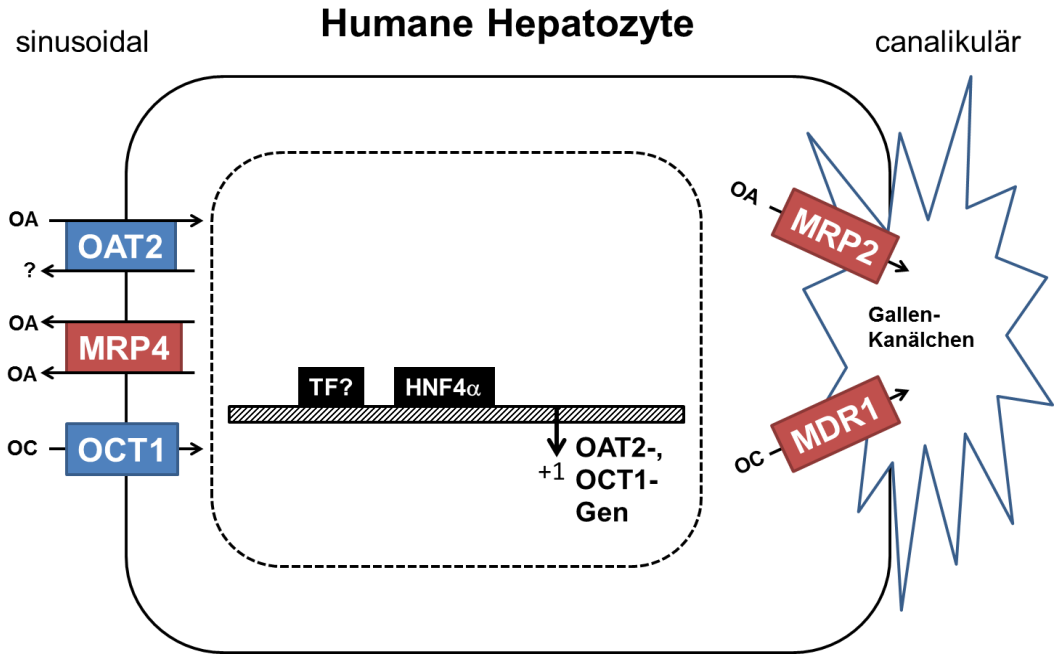


**Abbildung 2: Schematische Darstellung hepatischer Transporter.** OCT1 ist in der sinusoidalen Membran exprimiert und transportiert organische Kationen (OC) aus dem Blut in die Hepatozyten. Der Mechanismus des OAT2-abhängige Transports von organischen Anionen (OA) ist noch unbekannt. MRP2 und MRP4 transportien OAs und MDR1 OCs aus den Hepatozyten raus. Die rot hervorgehobenen Transporter wurden als klinisch relevant eingeordnet, die blau-markierten noch nicht. Der Transkriptionsfaktor *hepatocyte nuclear factor 4α* (HNF4α) aktiviert die Promotoren von OAT2, OCT1 und MRP2 in Hepatozyten. Die Transkription der hepatischen Transporter wird durch weitere bekannte und unbekannte Transkriptionsfaktoren (TF) beeinflusst.

## 1.3 Transkriptionelle Regulation von Transportproteinen

Transkriptionsfaktoren induzieren oder supprimieren die Transkription von Genen im Zellkern. Die Gen-Deletion des Transkriptionsfaktors *hepatocyte nuclear factor 1α* (Hnf1α) induziert niedrigere mRNA-Mengen von Oat1, Oat2 und Oat3 in den Nieren und von Oat2-mRNA in der Leber von Mäusen [36]. In den Nieren von Hnf1α-knockout Mäusen sind die mRNA-Mengen von Mrp2, Mrp4 und Mdr1b dagegen höher als bei den Wildtyp-Tieren [36]. Die hepatische mRNA von Mrp4 ist in den Hnf1α-knockout-Mäusen höher im Vergleich zu den Wildtyp-Mäusen [36].

Es konnte auch *in vitro* nachgewiesen werden, dass Hnf1α und Hnf1β die Promotoren der Mausgene Oat1 und Oat3 aktivieren [37, 38]. Der Einfluss von HNF1α auf die humanen Promotoren von OAT1 und OAT3 ist deutlich höher als von HNF1β [37, 38].

Der Transkriptionsfaktor HNF4α erhöht die Promotor-Aktivität der humanen Gene für OAT1, OAT2 und OCT1 [39-41]. Der Zellkernrezeptor *farnesoid X receptor* (FXR) und der FXR-*induced transcriptional repressor* SHP sind an der HNF4α-abhängigen Regulation von OAT2 beteiligt [40]. Bei der Regulation des Gens für OCT1 interagiert HNF4α mit den evolutionär hochkonservierten Transkriptionsfaktoren *upstream transcription factor 1* (USF1) und USF2 [42]. Die Transkription des humanen MRP2 wird auch durch HNF4α induziert [43]. HNF4α wurde in den proximalen Tubuluszellen der Nieren und in den Hepatozyten identifiziert [44].

Des Weiteren kann die Transkription von OAT3 durch den erhöhten intrazellulären cAMP-Spiegel induziert werden [45]. Die cAMP-aktivierte Proteinkinase A (PKA) phosphoryliert die Transkriptionsfaktoren *cAMP response element-binding protein* (CREB-1) und *activating transcription factor 1* (ATF-1), die an die *cAMP response elements* binden und den OAT3-Promotor aktivieren [45].

Weitere Transkriptionsfaktoren, die die Expression von hepatischen Transportproteinen regulieren können sind *aryl hydrocarbon receptor* (AhR), *constitutive androstane receptor* (CAR), *pregnane X receptor* (PXR), *peroxisome proliferator-activated receptor* α (PPARα) und FXR [46]. AhR, CAR und PXR induzieren die Transkription der humanen Transportproteine MDR1 und MRP2 und inhibieren die Transkription von OAT2 und OCT1. In der Leber von Nagern (Ratte und Maus) beeinflussen die Transkriptionsfaktoren AhR, CAR und PXR die Expression von Mrp2, Mrp4 und Mdr1b, aber nicht von Oat2 und Oct1 [46].

## 1.4 Geschlechtsabhängige Expression von Transportproteinen in Tiermodellen

Oat1 und Oat3 sind in den männlichen Ratten- und Mäusenieren höher exprimiert als in den weiblichen und unterliegen einer Testosteron-abhängigen Regulation [47-50]. Oat2 wurde in den Hepatozyten und in der apikalen Membran proximaler Tubuluszellen in der Ratte identifiziert [51, 52]. In der Leber und in den Nieren ist Oat2 bei weiblichen Ratten höher exprimiert als bei männlichen [48, 51, 52]. Oct2 wird auch in den Rattennieren in der basolateralen Membran proximaler Tubuluszellen exprimiert [13]. Die Oct2-Expression ist in den männlichen Ratten höher und Testosteron-abhängig reguliert [53].

In der Leber weiblicher Ratten wurde eine höhere Proteinexpression von Mrp2 nachgewiesen als bei Männchen [54]. Mrp4 ist in den männlichen Ratten-Nieren höher exprimiert als in den weiblichen [55]. Im Gegensatz dazu wurde in einem Mausmodel ein höherer mRNA-Gehalt von Mrp4 in weiblichen als in männlichen Nieren gezeigt. Mdr1b ist in der männlichen Ratten-Leber zweifach höher exprimiert als in der weiblichen [55]. Diese Mdr1b-Rattendaten entsprechen der humanen Situation, weil eine zweifach höhere MDR1-Expression bei Männern im Vergleich zu Frauen gezeigt wurde [56, 57].

## 1.5 Diabetes Typ 2

### 1.5.1 Geschlechtsabhängigkeit beim Diabetes Typ 2

Diabetes mellitus gehört zu den häufigsten Erkrankungen weltweit [58]. In 90 % aller dokumentierten Diabetes-Fälle wurde bisher Diabetes Typ 2 diagnostiziert [59]. Mehr als 20 % der Patienten entwickeln eine diabetische Nephropathie [60]. In klinischen Studien wurde festgestellt, dass die Nierenfunktion bei zahlreichen Diabetes-Patienten durch die begleitende Hypertension beeinflusst wird [61]. Bei Frauen vor der Menopause wurde ein deutlich niedrigerer Blutdruck beobachtet als bei Männern im gleichen Alter, wobei Östrogen möglicherweise als Vasodilatator agiert [62]. Des Weiteren wurde beschrieben, dass die diabetische Nephropathie in Assoziation mit Diabetes Typ 2 bei Männern häufiger vorkommt als bei Frauen [63].

Der Einfluss der diabetischen Nephropathie auf die Expression von renalen Transporterproteinen ist bisher unbekannt. Die renalen OATs und OCTs sind an der Sekretion häufig verschriebener Arzneimitteln beteiligt [6, 7, 14, 22]. Es wurde gezeigt, dass OAT3 das Arzneimittel Sitagliptin und OCT2 das am häufigsten verabreichte orale Antidiabetikum Metformin transportiert [22, 64]. Basierend auf den Ergebnissen verschiedener *in vitro* Studien ist eine Beteiligung des ABC-Transporters P-gp (MDR1) an der renalen Sekretion von Sitagliptin und Metformin anzunehmen [65].

Die Leber ist der Hauptakteur für die Metabolisierung und die Ausscheidung von Arzneimitteln. Patho-histologische und -physiologische Veränderungen in der humanen Leber sind mit Diabetes Typ 2 assoziiert [66]. Die Patienten mit Diabetes Typ 2 haben eine hohe Prävalenz die nichtalkoholische Fettlebererkrankung, die Leberzirrhose oder das hepatozelluläre Karzinom als Folgeerkrankung zu entwickeln [66-68]. In 427 humanen Leberproben wurden 80 geschlechtsabhängige Gene identifiziert [69]. Dabei waren OAT2 und OCT1 in weiblichen Leberproben höher exprimiert als in den männlichen Proben [69]. Die Antidiabetika Sitagliptin, Repaglinidin und Rosiglitazon interagieren *in vitro* mit dem humanen OCT1 [65, 70].

In Rattenlebern induziert Diabetes bereits im frühen Stadium einen oxidativen Stress in den Zellen, der zu pathologischen Veränderungen der hepatischen Struktur, zu Inflammation und fokaler Nekrose führt [71]. Die geschlechtsabhängige Expression von Genen wurde in den Lebern von gesunden und diabetischen Ratten beschrieben [72, 73].

### 1.5.2 ZSF1-Ratten, ein Model für Diabetes Typ 2 und diabetische Nephropathie

Die *Zucker spontaneously hypertensive fatty* (ZSF1)-Ratten stellen ein Tiermodell für Diabetes Typ 2 und diabetische Nephropathie dar [74]. Die Symptomatik der Erkrankung von ZSF1-Ratten entspricht der humanen Situation [74]. Dieser Tierstamm ist das Produkt der Kreuzung von *Zucker diabetic fatty* (ZDF) und *spontaneously hypertensive heart failure* (SHHF) Ratten der ersten Generation (F1) [74, 75]. Die ZSF1-Ratten sind Träger von zwei Mutationen im Leptin-Gen [74]. In diesem Rattenmodell werden nicht-adipöse ZSF1 Ratten als Kontrolle verwendet. Nicht-adipöse und adipöse ZSF1 Tiere weisen bereits im Alter von acht Wochen einen erhöhten Blutdruck im Vergleich zu anderen Rattenstämmen auf [76, 77]. Der Blutdruck von ZSF1-Männchen erhöht sich mit steigendem Lebensalter [76, 77]. Im Alter von 8 bis 41 Wochen ist die Konzentration von Glukose und von Triglyceriden im Blutplasma von adipösen ZSF1-Ratten höher als in den nicht-adipösen (Kontroll-) Tieren [74]. Nur adipöse ZSF1-Ratten entwickeln mit ansteigendem Lebensalter Diabetes Typ 2 und eine diabetische Nephropathie, die durch erhöhte Kreatinin- und Albumin-Konzentrationen im Urin charakterisiert ist [74, 78]. Die geschlechts- und die Diabetes-abhängige Expression von Transportproteinen und deren transkriptionelle Regulatoren in den Nieren und in der Leber wurde bisher in ZSF1-Ratten nicht untersucht.

# 2 Ergebnisse und Diskussion

## 2.1 Geschlechtsabhängige Expression renaler Transportproteine und Transkriptionsfaktoren in ZSF1-Ratten

(Publikation: **Sex-differences in renal expression of selected transporters and transcription factors in lean and obese zucker spontaneously hypertensive fatty rats.** Babelova A, Burckhardt BC, Wegner W, Burckhardt G, Henjakovic M. *J Diabetes Res*. 2015; Article ID 483238)

Ziel dieser Studie war die Untersuchung der geschlechts- und Diabetes-abhängigen Expression von Oat1-3, Oct1-2, Mrp2, Mrp4, Mdr1b, und deren transkriptionellen Regulation durch den *hepatocyte nuclear factor 1*$\alpha$ (Hnf1$\alpha$), Hnf1β und Hnf4$\alpha$ in den Nieren von ZSF1-Ratten. Zusätzlich wurde die mRNA des Natrium/Glucose-Cotransporter 1 (Sglt1) und 2 (Sglt2) untersucht.

Zur Untersuchung von Transportproteinen und Transkriptionsfaktoren wurden die Nieren von weiblichen und männlichen, adipösen und nicht-adipösen ZSF1-Ratten verwendet. Die ZSF1-Ratten wurden bis zu dem Lebensalter von 16 Wochen in einem Labor von Charles River im Staat New York (USA) unter standardisierten Tierhaltungsbedingungen gehalten. Um mögliche Belastungen der Tiere während des Transports zu vermeiden, wurden im Labor von Charles River die Organe *post mortem* entnommen, in RNA-*later* oder 4 %-igen *para*-Formaldehyd konserviert und gekühlt zu unserem Labor nach Göttingen transportiert. Die RNA wurde aus dem Nierenkortex isoliert und die mRNA von Transportproteinen und Transkriptionsfaktoren durch *Realtime*-PCR untersucht. Für die histologische Untersuchung wurden die Nieren in Paraffin eingebettet, geschnitten und die morphologischen Veränderungen nach der *periodic acid-Schiff* (PAS)-Färbung dokumentiert.

Wir konnten zeigen, dass die Nierenstruktur von weiblichen, nicht-adipösen ZSF1-Ratten den gesunden Nieren anderer Rattenstämme entsprach. In den Nierenschnitten von adipösen ZSF1-Weibchen und -Männchen fanden wir dagegen die histologischen Merkmale der diabetischen Nephropathie. Dabei beobachteten wir eine Glomerulosklerose, eine tubuläre Atrophie und Dilatation, hyaline Protein-Ablagerungen im Tubuluslumen, Druck-induzierte Deformationen der Glomeruli und die Dilatation der Bowman'schen Kapsel. Diese patho-histologischen Merkmale waren in den Nieren männlicher ZSF1-Ratten deutlich stärker ausgeprägt als in den Nieren weiblicher Tiere. Die patho-histologischen Befunde wurden durch den Nachweis der erhöhten Proteinexpression von Fibronektin in den Nieren adipöser Ratten bestätigt. Diese Ergebnisse sind in guter Übereinstimmung mit der aktuellen Literatur [78]. Zusätzlich fanden wir, dass die Basalmembran in den Nieren nicht-adipöser

ZSF1-Männchen verändert und die renale Proteinexpression von Fibronektin im Vergleich zu Kontroll-Weibchen signifikant erhöht ist. Da bekannt ist, dass nicht-adipöse und diabetische ZSF1-Männchen mit steigendem Lebensalter eine Hypertonie entwickeln [79], sind die beobachteten morphologischen Änderungen in den Nieren nicht-adipöser Männchen möglicherweise eine Folge der Hypertonie.

In den Nieren nicht-adipöser ZSF1 Ratten konnten wir im Vergleich zu Männchen bei Weibchen signifikant höhere mRNA-Mengen für die Transporter Sglt1, Sglt2, Oat1, Oat2, Oat3 und Oct1 nachweisen. Zudem zeigten wir erstmalig, dass in weiblichen Rattennieren der Transkriptionsfaktor Hnf1β höher exprimiert wird als in männlichen. Unsere Daten stimmen bezüglich der geschlechtsabhängigen, renalen Sglt1-, Sglt2- und Oat2-Expression mit der Literatur überein [52, 80]. Wir konnten die geschlechtsabhängige Expression von Sglt1, Sglt2 und Oat2 auch in den Nieren adipöser ZSF1-Ratten bestätigen. Im Gegensatz zu unseren Daten ist bekannt, dass die Transportproteine Oat1 und Oat3 in anderen Rattenstämmen in den Nieren männlicher Tiere höher exprimiert sind als in den Weibchen [47-49]. Der Widerspruch unserer Ergebnisse zu den Literaturdaten bezüglich Oat1 und Oat3 bleibt aufzuklären. Des Weiteren zeigen unsere Ergebnisse erstmalig eine höhere Oct1-Expression in den Nieren weiblicher Tiere. In Übereinstimmung mit einer veröffentlichten Studie [81] war die Oct2-mRNA im Nierenkortex männlicher, adipöser ZSF1-Männchen höher als in den weiblichen Ratten. Außerdem ist bekannt, dass der Androgenrezeptor die Expression von Oct2 transkriptionell reguliert [82]. Vergleichbar zur Literatur [55] haben wir auch keine geschlechtsabhängige Expression von Mrp2, Mrp4 und Mdr1b in unserer Studie nachweisen können.

Im Vergleich zu den Kontrolltieren fanden wir in adipösen ZSF1-Weibchen signifikant niedrigere mRNA-Mengen für Sglt1-2, Oat1-3, Oct1-2, Mrp4 und Mdr1b. Im Gegensatz zu unseren Daten wurde in *Zucker obese* (ZO) Ratten gezeigt, dass Diabetes die Expression von Sglt1 und Sglt2 erhöht [80]. Dieser Unterschied ist möglicherweise auf die Verwendung verschiedener Tierstämme zurückzuführen. In Übereinstimmung mit unseren Daten wurde eine erniedrigte Expression von OAT1 und OAT3 in den Nieren von Patienten mit diabetischer Nephropathie nachgewiesen [83]. Wie in unserer Studie festgestellt, wird Oct2 durch Diabetes in Ratten-Nieren reduziert [81]. In einem Streptozotocin-induzierten Ratten-Dabietesmodell wurde die Mrp2- und Mrp4-Expression durch Diabetes Typ 2 erhöht [81]. Es ist anzunehmen, dass die verschiedenen Phasen des Diabetes Typ 2 in den verwendeten Tiermodellen den Grund für diese Diskrepanz darstellen. Im Nierenkortex von adipösen (diabetischen) und nicht-adipösen (nicht-diabetischen) ZSF1-Männchen waren die mRNA-Mengen für Transporterproteine vergleichbar.

Die Transkriptionsfaktoren Hnf1β und Hnf4α waren in den Nieren adipöser ZSF1-Männchen und -Weibchen signifikant niedriger als bei den entsprechenden nicht-adipösen Kontrolltieren. Die Assoziation von Polymorphismen im HNF1β-Gen mit Diabetes mellitus wurde in mehreren Studien nachgewiesen [84-86]. In diabetischen Rattenmodellen wurde bisher keine Änderung von Hnf1β gezeigt. Die Suppression von HNF4α in den Nieren von Patienten mit diabetischer Nephropathie und eine Diabetes-abhängige Reduktion von Hnf4α in Nieren von ZDF-Ratten wurde bereits beschrieben [87].

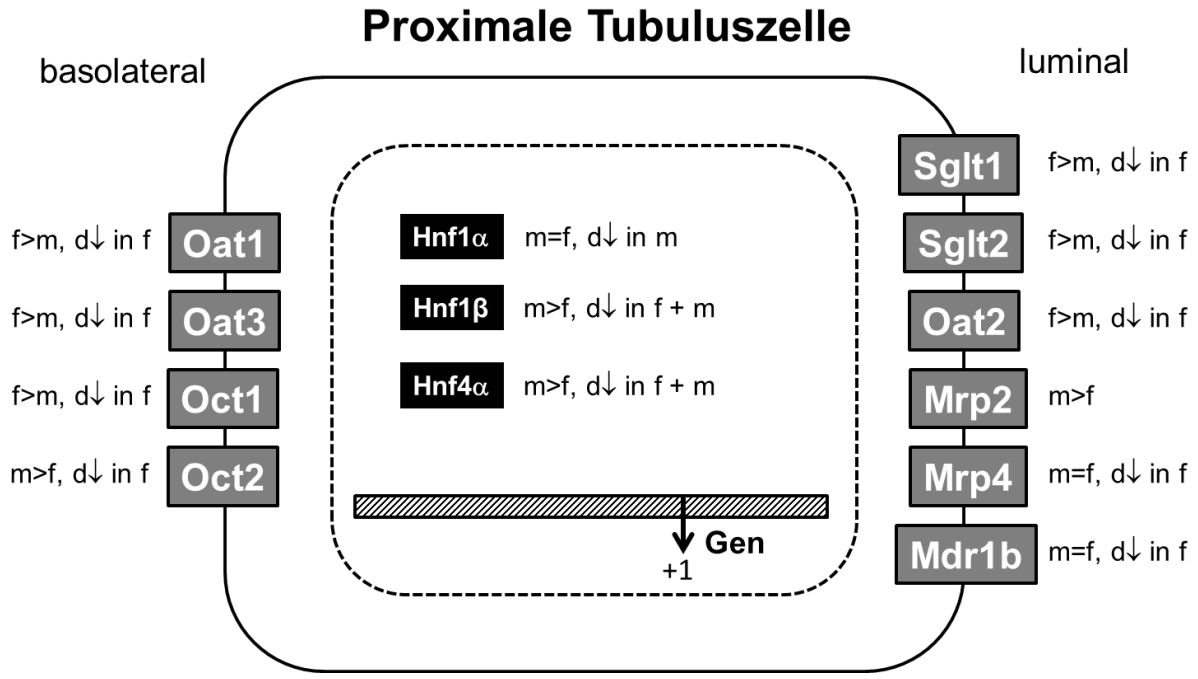


**Abbildung 3: Schematische Darstellung renaler Transporter und Transkriptionsfaktoren in ZSF1-Ratten.** Dargestellt sind Organische-Anionen-Transporter (Oat1, Oat2 und Oat3), Organische-Kationen-Transporter (Oct1 und Oct2), *Sodium-glucose cotranporter* (Sglt1 und Sglt2), *Multidrug Resistance-associated* Proteine (Mrp2 und Mrp4), *Multidrug Resistance Protein 1b* (Mdr1b) und *hepatocyte nuclear factors* (Hnf1α, Hnf1β und Hnf4α) in den proximalen Tubuluszellen von ZSF1-Ratten. Dabei wurde die geschlechtsabhängige (weiblich versus männlich; f: *female*; m: *male*) und die Diabetes (d: *diabetes*)-abhängige Expression gekennzeichnet, die wir identifiziert haben.

In unserer Studie waren die geschlechts- und die Diabetes-abhängigen Unterschiede für Oat2-mRNA am stärksten ausgeprägt. Aus diesem Grund haben wir den OAT2-vermittelten Transport von verschiedenen endogenen Metaboliten, Antidiabetika und Blutdruck-senkenden Mitteln untersucht. Hierzu haben wir HEK293-Zellen, die mit dem humanen OAT2 stabil transfiziert sind, eingesetzt. Der OAT2-vermittelte Transport des radioaktiv-markierten Modellsubstrats [$^3$H]cGMP [17] wurde in An- und Abwesenheit dieser Substanzen gemessen.

Die Konzentration der Stoffwechselprodukte Adipat und Suberat ist im Urin von diabetischen Patienten erhöht [88]. Die OAT2-vermittelte [$^3$H]cGMP-Aufnahme wurde durch Adipat und

Suberat nicht beeinflusst. Im Urin von Patienten mit diabetischer Nephropathie wurde eine deutlich niedrigere Konzentration der Stoffwechselprodukte Glykolat, Citrat, 3-Hydroxy-iso-Butyrat, *cis*-Aconitat und Homovanillat nachgewiesen [83]. Da nur Homovanillat den OAT2-induzierten [$^3$H]cGMP-Transport um etwa 20 % reduziert, kann ausgeschlossen werden, dass OAT2 für den Transport dieser Metabolite verantwortlich ist.

In weiteren Versuchen wurde die Interaktion von OAT2 mit den Antidiabetika (Sitagliptin und Miglitol), mit den ACE-Hemmern (Captopril und Enapril) untersucht. Im Vergleich zu dem OAT2-Inhibitor Indomethacin [89] ist der Einfluss von Sitagliptin, Miglitol, Captopril und Enapril auf die OAT2-Funktion sehr gering.

Zur Reduktion des Blutdrucks werden Patienten mit chronischen Nierenerkrankungen Schleifendiuretika, wie z.B. Furosemid oder Bumetanid verschrieben [90]. Es wurde gezeigt, dass die Halbwertszeit von Furosemid im Blut von Patienten mit einer Niereninsuffizienz deutlich erhöht ist [91]. Wir konnten erstmalig zeigen, dass Furosemid den OAT2-vermittelten [$^3$H]cGMP-Transport mit hoher Affinität hemmt ($IC_{50}$: 10,9 µM). In einer Studie von Hasannejad *et al.* (2004) wurde der OAT2-Transport von $PGF_{2\alpha}$ durch Furosemid mit einer $IC_{50}$ von 603 µM inhibiert [92]. Der Grund für diese Diskrepanz zu unseren Daten ist höchstwahrscheinlich die Verwendung von verschiedenen Zellmodellen und Modellsubstraten. Die OAT2-vermittelte Aufnahme von [$^3$H]cGMP wurde in unseren Zellen mit moderater Affinität durch Bumetanid inhibiert ($IC_{50}$: 130 µM), was gut mit veröffentlichten Daten übereinstimmt [92].

Diese Studie zeigte eine geschlechtsabhängige Ausprägung der Nierenschäden in adipösen ZSF1-Ratten. Außerdem war die geschlechtsabhängige Expression von einzelnen Transportproteinen und deren transkriptioneller Regulatoren durch Diabetes Typ 2 verändert. Die höchsten Unterscheide zwischen den ZSF1-Gruppen wurden für Oat2 identifiziert. In einem *in vitro* Model wurde die Funktion des humanen OAT2 untersucht. OAT2 interagierte mit Sitagliptin und Enapril ausschließlich in sehr hohen, therapeutisch nicht relevanten Konzentrationen, aber hochaffin mit dem häufig verschiebenden Schleifendiuretikum Furosemid. Die Daten dieser Studie zeigen, dass bei der Testung von Arzneimitteln in präklinischen Studien beide Geschlechter des Tiermodells untersucht und die Expression renaler Transportproteine berücksichtigt werden sollte.

## 2.2 Geschlechtsabhängige hepatische Expression in ZSF1-Ratten

(Publikation: **Next generation sequencing of sex-specific genes in livers of diabetic ZSF1 rats.** Babelova A, Burckhardt BC, Salinas-Riester G, Pommerenke C, Burckhardt G, Henjakovic M. (*Genomics*; doi: 10.1016/j.ygeno.2015.07.006)

Das Ziel dieser Studie war es, die geschlechtsabhängigen Gene in der Leber adipöser ZSF1-Ratten zu identifizieren. Dabei sollten auch die *solute carrier* 22a (Slc22a)-Gene für hepatische Transportproteine mit eingeschlossen werden.

Die ZSF1-Ratten wurden bis zu einem Lebensalter von 16 Wochen im Labor von Charles River im Staat New York (USA) unter standardisierten Tierhaltungsbedingungen gehalten. Um mögliche Belastungen der Tiere während des Transports zu vermeiden, wurden im Labor von Charles River die Organe *post mortem* entnommen, in RNA-*later* oder 4 %-igen *para*-Formaldehyd konserviert und gekühlt zu unserem Labor nach Göttingen transportiert. Die RNA wurde aus den konservierten Leberproben von jeweils sechs adipösen ZSF1-Weibchen und -Männchen isoliert. Die geschlechtsabhängigen Unterschiede wurden mit Hilfe der RNA-Sequenzierung (*Next generation sequencing*, NGS) untersucht.

Durch die *principal component analysis* (PCA) der RNA-Sequenzierungsdaten konnten wir eine statistisch signifikante Trennung der weiblichen und der männlichen Tiergruppe zeigen. Dabei waren 469 von 15.027 untersuchten Genen geschlechtsabhängig exprimiert. Die geschlechtsspezifische Genexpression in der Leber stimmt gut mit den Literaturreferenzen überein [69, 72, 73, 93]. Nachdem wir die Gene ausgeschlossen haben, die sehr niedrig exprimiert sind (base mean < 1000), waren 40 hepatische Gene höher in Weibchen und 63 höher in Männchen exprimiert.

In einer Studie wurde gezeigt, dass zahlreiche geschlechtsspezifische Gene in der Leber auf autosomalen Chromosomen lokalisiert sind [93]. Wir fanden die höchste Anzahl geschlechtsabhängiger Gene auf den Chromosomen 1, 2 und 5. Wie in der Veröffentlichung von Zhang et al [93] sind die meisten, in unserer Studie identifizierten, weiblich-spezifischen Gene auf Chromosom 1 lokalisiert. Die höchste Anzahl männlich-spezifischer Gene sind auf Chromosom 5 zu finden.

Mit Hilfe der *Gene Ontology* (GO)-Analyse zeigten wir, dass die höchste Anzahl geschlechtsabhängiger Gene mit den GO-Signalwegen „*response to chemical stimulus*", „*lipid metabolic process*" und „*response to organic substances*" assoziiert ist. Dieses Ergebnis bestätigt die Analysen aus zwei Studien, die in der humanen bzw. Ratten-Leber die Assoziation geschlechtsspezifischer Gene zu diesen Signalwegen identifiziert haben [93, 94]. In geringerer Anzahl waren die geschlechtsabhängigen Gene den Kategorien „*response*

*to external stimulus*", „*steroid metabolic process*", „*retinol metabolism*" oder „*response to organic cyclic compound*" zugeordnet.

In der Leber von ZSF1-Männchen höher exprimiert waren Gene, die für die Cytochrome P450 (Cyp2c11, Cyp4a2), die Glutathion S-Transferasen *mu* (Gstm1, Gstm2, Gstm7), die Hydroxysteroid-Dehydrogenasen (Hsd11b1, Hsd17b2) und den Transporter Oat3 (Slc22a8) codieren. Die höhere Expression von Genen, die im Fettsäure-Signalweg (Acsl4, Acacb, Cd36) oder in der Glykolyse (Gck, G6pd) involviert sind, wurde in der Leber von ZSF1-Weibchen nachgewiesen. Zusätzlich herausragend in der weiblichen Leber war die RNA-Menge des Cytokinrezeptors Lifr, der Steroid 5α-Reduktase Srd5a1 und der Gallensäure-Sulfotransferase Sult2a1.

Zur Verifizierung der RNA-Sequenzierungsdaten von adipösen ZSF1-Ratten wurden acht geschlechtsspezifische Gene in der Realtime-PCR untersucht. Der geschlechtsabhängige Unterschied der männlich-spezifischen Gene für Gstm2, Hsd11b1, Cyp4a2, Cyp2c11 und der weiblich-spezifischen Gene Lifr, Srd5a1, Gck, Gpam wurde in der Realtime-PCR-Analyse bestätigt. Es wurde gezeigt, dass diese Gene auch in der Leber von nicht-adipösen ZSF1-Ratten geschlechtsabhängig exprimiert sind.

Das männlich-dominante Gen Cyp4a2 und die weiblich-spezifischen Gene Srd5a1 und Gpam waren in der Leber adipöser ZSF1-Männchen höher exprimiert als in den nicht-adipösen Männchen. In adipösen ZSF1-Weibchen war die Menge der Hsd11b1-mRNA höher und die mRNA von Gstm2, Lifr und Srd5a1 niedriger im Vergleich zu den gesunden, weiblichen ZSF1-Ratten.

Gstm2 ist für die Detoxifizierung elektrophiler, endogener und exogener Substanzen in der Leber verantwortlich [95] und wird in der Leber von ZDF-Ratten Diabetes-abhängig supprimiert [12]. Es bleibt unklar, warum die Gstm2-Expression in adipösen ZSF1-Weibchen, nicht aber in den adipösen Männchen reduziert war.

In der Leber ist Hsd11b1 für die enzymatische Aktivierung von Glukokortikoiden verantwortlich [96]. In einem anderen Ratten-Stamm wurde ebenfalls eine männlich-spezifische Expression des hepatischen Hsd11b1 nachgewiesen [73]. Eine geschlechtsspezifische Hsd11b1-Expression existiert nicht in der humanen Leber [69]. Diese Diskrepanz zwischen den humanen und Ratten-Daten zeigt, dass die Hsd11b1-Expression die humane Situation nicht widerspiegelt.

Die männlich-spezifische Expression der Fettsäure-ω-Hydroxylase Cyp4a2 in der Rattenleber [97] ist bekannt und wurde in unserer Arbeit bestätigt. Ähnlich wie in einem Ratten-Modell des Streptozotocin-induzierten Diabetes [98] wurde in der Leber von ZSF1-Männchen Cyp4a2-mRNA Diabetes-abhängig hochreguliert. Dieses Cytochrom hydroxyliert

Testosteron und ist an der hepatischen Metabolisierung zahlreicher Arzneimittel beteiligt [99]. Die geschlechtsabhängige Cyp2c11 Expression in der Leber stimmt mit den Literaturdaten überein [100]. Die hepatische Cyp2c11-Expression wird in den Tiermodellen für Diabetes Typ 1, aber nicht in Ratten mit Diabetes Typ2 induziert [101-103]. Unsere Cyp2c11-Daten bestätigen die gute Vergleichbarkeit von ZSF1-Ratten mit den anderen Tiermodellen für Diabetes Typ 2.

Die Differenzen der Lifr-mRNA zwischen den ZSF1-Gruppen stimmen mit den Veröffentlichungen überein, die zeigen, dass die Östrogen-abhängige Lifr-Expression in der Leber durch Diabetes Typ 2 reduziert wird [104, 105].

Die weiblich-exprimierte Steroid-5$\alpha$-Reduktase Srd5a1 spielt bei der hepatischen Metabolisierung von Testosteron und Kortikosteron eine wichtige Rolle und wird in der Leber diabetischer ZDF-Ratten nicht verändert [103]. Der Grund für die Reduktion des Srd5a1-mRNA-Gehalts durch Adipositas in weiblichen ZSF1-Ratten bleibt unklar.

Die Glucokinase (Gck) wird in der weiblichen Rattenleber höher exprimiert als bei Männchen und wurde in den diabetischen ZDF-Ratten nicht Diabetes-abhängig verändert [106, 107], was unsere Ergebnisse bestätigen.

Das weiblich-spezifische Gpam [73] ist ein Schlüsselenzym der hepatischen Lipogenese. Wir haben erstmalig gezeigt, dass die hepatische Gpam-mRNA in ZSF1-Männchen Diabetes-abhängig erhöht wird. Dieser Aspekt stellt ein interessantes Forschungsziel für die Zukunft dar, da sie Hinweise auf das Entstehen der Fettleibigkeit bei Männern erbringen könnte.

Die NGS-Daten für alle sequenzierten Transportproteine der Slc22a-Genfamilie wurden separat analysiert. In der Leber adipöser ZSF1-Ratten wurde die RNA für die Transporter Oct1, Octn2, Oat2 und Oat3 nachgewiesen. Die RNA-Mengen von Oct2, Oat1, Urat1 und Oat10 waren vernachlässigbar niedrig. Nur Oat3 war in der Leber adipöser ZSF1-Männchen höher exprimiert als bei den Weibchen.

Die Oat3-, Oat2 und Oct1-mRNA wurde in der Leber von adipösen und Kontrolltieren mit Hilfe der Realtime-PCR untersucht. Ein vielfach höherer Oat3-mRNA-Gehalt wurde auch in der Leber nicht-adipöser ZSF1-Männchen nachgewiesen. Damit wurden die Literaturdaten bestätigt, die eine geschlechtsabhängige Oat3-Expression in der Rattenleber zeigten [48]. In den nicht-adipösen Ratten war die Oat2- und Oct1-mRNA-Menge höher in der weiblichen als in der männlichen Leber, welches mit den humanen Daten bezüglich weiblich-spezifischer Expression von OAT2 und OCT1 übereinstimmt [69]. Bezüglich Oat2- und Oct1-Expression wurden keine Differenzen zwischen den adipösen und nicht-adipösen Männchen nachgewiesen. Im Gegensatz dazu konnten wir erstmalig zeigen, dass die Oat3-mRNA in adipösen ZSF1-Weibchen signifikant höher ist als in den gesunden Weibchen. Da OAT3 in

der humanen Leber nicht exprimiert ist, sollte die geschlechtsabhängige Oat3-Expression in prä-klinischen Ratten-Studien beachtet werden.

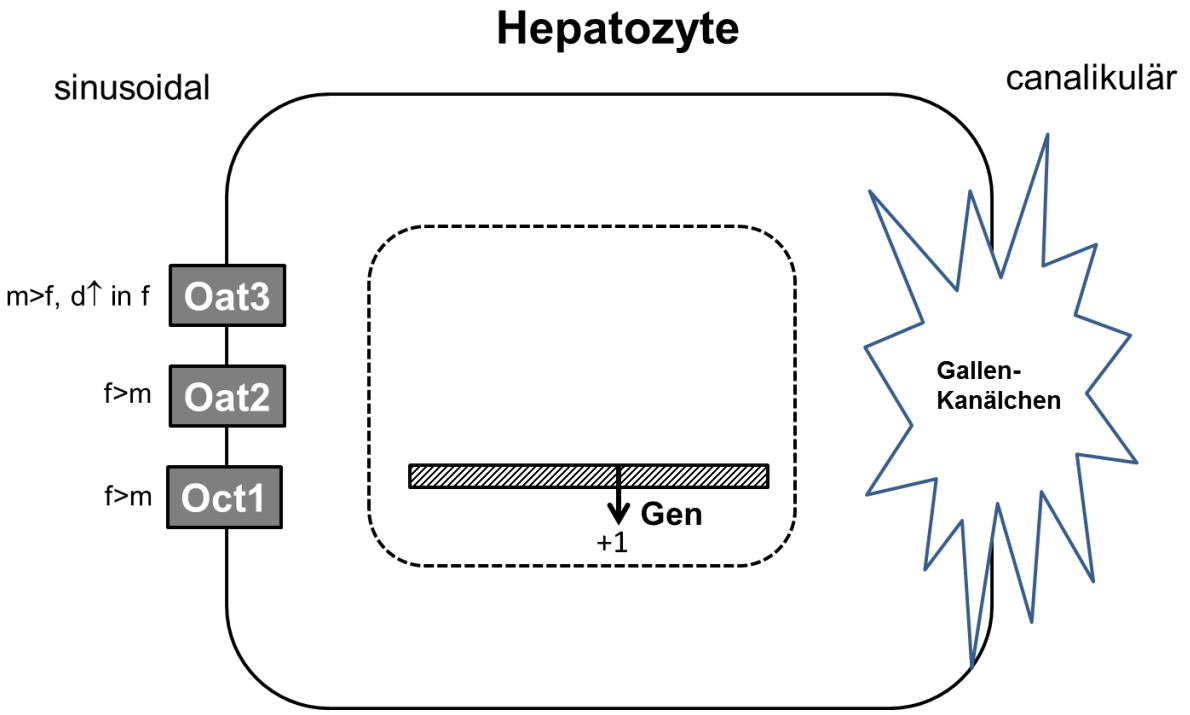


**Abbildung 4: Schematische Darstellung der geschlechtsabhängigen hepatischen Tranporter-Expression in ZSF1-Ratten.** Die Abkürzungen stehen für: Organische-Anionen-Transporter (Oat2), Oat3 und des Organische-Kationen-Transporter 1 (Oct1) in den Hepatozyten von ZSF1-Ratten. Dabei wurde die geschlechtsabhängige (weiblich versus männlich; f: *female*; m: *male*) und die Diabetes (d: *diabetes*)-abhängige Expression gekennzeichnet, die wir identifiziert haben.

In dieser Studie wurden zahlreiche geschlechtsspezifische Gene in der Leber von ZSF1-Ratten identifiziert. Unsere Daten stehen in sehr guter Übereinstimmung mit den Studien in anderen Tiermodellen für Diabetes Typ 2 und reflektieren in vielen Fällen die humane Situation. Die geschlechtsabhängige Gen-Expression hepatischer Transportproteine und Enzyme, die für die Metabolisierung exogener Substanzen verantwortlich sind und deren Differenz zur humanen Gen-Expression sollte in der Entwicklung und Testung von neuen Arzneimitteln berücksichtigt werden.

## 2.3 Transkriptionelle Regulation der Gene für Oat1 und Oat3 durch den Transkriptionsfaktor B-cell CLL/lymphoma 6 (BCL6)

(Publikation: **Male-dominant activation of rat renal organic anion transporter 1 (Oat1) and 3 (Oat3) expression by transcription factor BCL6.** Wegner W, Burckhardt BC, Burckhardt G and Henjakovic M. *PLoS One* 7: e35556, 2012)

In der basolateralen Membran proximaler Tubuluszellen wurde die Expression der Transporterproteine OAT1 und OAT3 in den humanen Nieren und die Expression der homologen Proteine Oat1 und Oat3 in den Rattennieren nachgewiesen [108-111]. In den Nieren von männlichen Ratten wurde eine höhere und Testosteron-abhängige mRNA- und Protein-Konzentration von Oat1 und Oat3 gezeigt [47-49]. Die Transkription von Oct2, welcher auch in der basolateralen Membran proximaler Tubuluszellen exprimiert wird, wurde durch einen Androgenrezeptor und Testosteron aktiviert [82]. Basierend auf diesen Literaturdaten ist zur Beginn dieser Studie angenommen worden, dass die Promotoren von Oat1 und Oat3 durch die Anwesenheit des Androgenrezeptor-Testosteron-Komplexes induziert werden.

Zur Untersuchung dieser Hypothese haben wir Oat1- und Oat3-Promotorfragmente in einem *Luciferase*-Vektor kloniert und deren Aktivität in An- und Abwesenheit des Androgenrezeptor-Testosteron-Komplexes untersucht. Dazu wurden die Nieren-Modellzellen (*Oppossum kidney*, OK) mit den Promotorfragmenten und mit einem Androgenrezeptor-Vektor oder Kontrollvektor transfiziert. Anschließend wurden die OK-Zellen in An- oder Abwesenheit von Testosteron inkubiert. Überraschenderweise wurde die Oat1- und Oat3-Promotoraktivität durch den Androgenrezeptor-Testosteron-Komplex nicht verändert. Wir konnten zeigen, dass der Androgenrezeptor in OK-Zellen transfiziert wurde und als Protein exprimiert war. Als Positivkontrolle wurde der Probasin-Promotor der Ratte [112] verwendet, der in unserem System erfolgreich aktiviert wurde. Aus diesem Grund konnten wir die direkte Interaktion der Oat1- und Oat3-Promotoren mit dem Androgenrezeptor-Testosteron ausschließen.

Im Folgenden haben wir die geschlechtsabhängige Expression in den Rattennieren durch eine *Microarray*-Analyse untersucht. Das Ziel dieser Analyse war es, geschlechtsabhängige Gene zu identifizieren, die an der transkriptionellen Regulation von Oat1 und Oat3 beteiligt sind.

Im ersten Schritt wurde die RNA aus dem Nieren-Kortex von weiblichen und männlichen Ratten isoliert und die Oat1- und Oat3-mRNA in der Realtime-PCR untersucht. Übereinstimmend mit veröffentlichen Daten [47-49] haben wir im Nieren-Kortex von männlichen Ratten höhere Oat1- und Oat3-mRNA-Mengen nachgewiesen und damit die

geschlechtsabhängige Expression bestätigt. Anschließend wurde in diesen RNA-Proben die geschlechtsabhängige Gen-Expression mit Hilfe einer *Microarray*-Analyse untersucht.

Nach der statistischen Datenanalyse, dem Vergleich mit der Trankriptom-Datenbank „*Rat Proximal Tubule Transcriptome Database*“, dem Ausschluss von doppelt-detektierten Genen und den Genen, die niedrig exprimiert sind, wurden 56 geschlechtsabhängige Kandidatengene identifiziert. Von diesen Kandidatengenen waren 13 weiblich- und 43 männlich-dominant exprimiert.

Zur Verifizierung von *Microarray*-Daten wurden sieben renale Gene mittels Realtime-PCR analysiert. Die *Microarray*-Daten für die Gene des Androgenrezeptors, der Transkriptionsfaktoren *hepatocyte nuclear factor 1*$\alpha$ (Hnf1$\alpha$), Hnf1β, Hnf4$\alpha$, Polymerase-(RNA)-III-(DNA-Directed)-Polypeptide G (Polr3g), Hydroxysteroid-17β-Dehydrogenase 1 (Hsd17b1) und B-cell CLL/lymphoma 6 (BCL6) wurden in der Realtime-PCR-Analyse bestätigt. Der mRNA-Gehalt des Androgenrezeptors und der Mitglieder der Hnf-Genfamilie waren in weiblichen und männlichen Rattennieren vergleichbar. Die mRNA-Mengen von Polr3g, Hsd17b1 und BCL6 waren signifikant höher in den Nieren männlicher Ratten im Vergleich zu den weiblichen. Für die Gene Polr3g, Hsd17b1 und BCL6 haben wir in dieser Studie erstmalig eine geschlechtsabhängige Expression in den Rattennieren nachgewiesen.

Ein vielversprechender Kandidat, der möglicherweise in die geschlechtsabhängige Regulation von Oat1 und Oat3 eingreift, stellt der Transkriptionsfaktor BCL6 dar. BCL6 wurde in den proximalen Tubuluszellen männlicher Rattennieren im Vergleich zu den weiblichen Nieren höher exprimiert. Der Transkriptionsfaktor BCL6 wurde in B-Lymphozyten identifiziert und charakterisiert [113]. Die BCL6-Expression wurde auch in anderen Zelltypen, wie z.B. T-Lymphozyten, Hepatozyten und Adipozyten nachgewiesen [114-116]. In der Leber wurde eine höhere BCL6-Expression in männlichen als in weiblichen Ratten beschrieben [115]. In den bisher publizierten Experimenten führte die BCL6-Bindung an die DNA oder die Interaktion mit anderen Proteinen zu einer Hemmung von anderen Transkriptionsfaktoren (Repressoren) und damit indirekt zu einer Aktivierung von Zielgenen [113, 116, 117].

Zur Untersuchung der BCL6-abhängigen Promotoraktivität wurden die Oat1- und Oat3-Promotorfragmente mit einem BCL6-Plasmid oder dem Kontrollvektor pcDNA3 in OK-Zellen ko-transfiziert. In den BCL6-transfizierten OK-Zellen wurde durch die Immunfluoreszenz-Analyse das BCL6-Protein in 23,3 % der transfizierten OK-Zellen nachgewiesen. Wir konnten zeigen, dass BCL6 die Transkription von Oat1 und Oat3 unabhängig von der Promotorfragment-Länge und Anzahl der berechneten BCL6-Bindungsstellen (*in silico* Analyse durch Genomatix-*Software*) aktiviert.

In dieser Studie wurde gezeigt, dass der Androgenrezeptor mit den Promotoren von Oat1 und Oat3 nicht interagiert. Im Rahmen einer *Microarray*-Analyse haben wir den

Transkriptionsfaktor BCL6 als einen potentiellen Modulator der Oat1- und Oat3-Transkription identifiziert. In weiterführenden Studien sollte der Effekt von BCL6 auf die humanen OAT1- und OAT3-Promotoren untersucht werden.

## 2.4 BCL6-abhängige Regulation der humanen OAT1-Transkription

(Publikation: **Transcriptional regulation of human organic anion transporter 1 (OAT1) by B-cell CLL/lymphoma 6 (BCL6).** Wegner W, Burckhardt G, Henjakovic M. *Am J Physiol Renal Physiol*. 307: F1283-F1291, 2014)

Die transkriptionelle Regulation des humanen, renalen Transporterproteins OAT1 ist noch nicht vollständig aufgeklärt. In verschieden Promotoranalysen wurde gezeigt, dass die Transkriptionsfaktoren HNF1$\alpha$, HNF1β und HNF4$\alpha$ in den proximalen Tubuluszellen der Nieren exprimiert sind und die OAT1-Transkription aktivieren [38, 39]. In einer früheren Studie [118] konnten wir zeigen, dass der Transkriptionsfaktor BCL6 die Oat1- und Oat3-Transktiption aktiviert. Unsere Ergebnisse wurden durch eine Forschungsgruppe in Japan bestätigt [119]. Der Einfluss von BCL6 auf die renale Expression des humanen OAT1-Transportproteins wurde bisher nicht untersucht.

In dieser Studie sollte der Effekt von BCL6 auf den humanen OAT1-Promotor untersucht werden. Durch die *in silico* Analyse wurden sechs potentielle BCL6-Bindungsstellen vor der Transkriptionsstart-Sequenz des OAT1-Gens identifiziert. Entsprechend wurden verschiedene OAT1-Promotorfragmente kloniert, die sich in der Länge und der Anzahl potentieller BCL6-Bindungsstellen unterschieden. Nach der Transfektion der Promotorfragmente mit einem BCL6-Plasmid oder dem Kontrollvektor pcDNA3 wurde eine BCL6-abhängige Aktivierung des OAT1-Promotors nachgewiesen.

Die BCL6-abhängige Aktivierung des OAT1(-1982/+88)-Promotorfragments mit drei potentiellen BCL6-Bindungsstellen war deutlich höher als die der kürzeren Fragmente. Aus diesem Grund wurden die potentiellen Bindungsstellen einzeln und kombiniert im OAT1(-1982/+88)-Fragment mutiert. Überraschenderweise wurde die BCL6-abhängige Aktivität des Promotorfragments durch diese Mutationen nicht verändert, obwohl eine BCL6-Bindung an den Promotor nicht mehr möglich war. Dieses Ergebnis führte uns zu der Annahme, dass BCL6 die OAT1-Transkription indirekt aktiviert.

In den meisten bekannten Fällen bindet BCL6 direkt an die Bindungssequenz im Promotor und agiert als Repressor [113]. In einigen Fällen ist für die BCL6-abhängige Genregulation eine Protein-Protein-Interaktion mit anderen Transkriptionsfaktoren nötig, wie z.B. mit einer Untereinheit des *nuclear factor kappaB* (NF$\kappa$B) in B-Zellen oder mit dem *peroxisome proliferator-activated receptor delta* (PPAR$\delta$) in Kardiomyozyten [120, 121].

In einer Studie wurde die Protein-Protein-Interaktion des Transkriptionsfaktors BCL6 und HNF1α nachgewiesen [122]. Aus diesem Grund haben wir die potentielle Mitwirkung von BCL6 und HNF1α bei der transkriptionellen Regulation von OAT1 getestet. Wir konnten zeigen, dass in unseren Modellzellen (*Oppossum kidney*, OK) BCL6 und HNF1α im Zellkern ko-exprimiert sind. Die OAT1-Promotorfragmente wurden in Anwesenheit von BCL6 oder HNF1α aktiviert. In Anwesenheit beider Transkriptionsfaktoren konnten wir einen kooperativen und additiven Effekt auf die Aktivität des OAT1-Promotors nachweisen. Ein additiver Effekt von BCL6 und Transkriptionsfaktor Maf auf einige T-Zell-assoziierte Gene wurde bereits früher beschrieben [123]. Zudem wurde der OAT1-Promotor nach der Mutation der HNF1α-Bindungsstelle (Position im OAT1-Gen: -57/41 bp; [38]) durch BCL6 nicht aktiviert.

Die von uns verwendeten OK-Zellen exprimieren endogen das HNF1α-Protein. Aus diesem Grund war es möglich, die Bindungsaktivität des endogenen HNF1α-Proteins an den OAT1-Promotor in An- und Abwesenheit von BCL6 zu testen. Die Bindungsaktivität wurde mit Hilfe des *electrophoretic mobility shift assay* (EMSA) untersucht. Hierzu haben wir OAT1-Promotorfragmente verwendet die eine intakte oder eine mutierte HNF1α-Bindungsstelle enthalten und die Zellkernextrakte von BCL6- und pcDNA3-transfizierten OK-Zellen. In mehreren EMSA-Analysen konnten wir beweisen, dass in Anwesenheit von BCL6 die Bindungsaktivität des HNF1α-Proteins an die Bindungsstelle im OAT1-Promotor erhöht wird. Im Westernblot wurde nachweisen, dass BCL6 die endogene Proteinexpression von HNF1α mindestens verdoppelt.

In dieser Studie konnten wir darlegen, dass BCL6 den OAT1-Promotor indirekt aktiviert. Außerdem erhöht BCL6 die Bindungsaktivität und die endogene Expression von HNF1α in den Zellen. Es bleibt unklar, ob BCL6 für die Aktivierung der OAT1-Transkription eine Protein-Protein-Interaktion mit dem Transkriptionsfaktor HNF1α benötigt.

In folgenden Studien sollte daher die Protein-Protein-Interaktion von HNF1α und BCL6 untersucht werden, weil HNF1α die Transporter in den Nieren [124] und in der Leber [125] reguliert. Geplant ist die Untersuchung des BCL6-Effektes auf die Transkription von OAT2, OAT4, OAT5, OAT7 und URAT1 im Rahmen eines bewilligten DFG-Projektes.

## 3 Zusammenfassung

Die Transportproteine der *solute carrier* (SLC)- und *ATP binding cassette* (ABC)-Gen-Familie sind für die Aufnahme und die Sekretion von Substanzen, wie z.B. von Arzneimitteln verantwortlich. Zu den klinisch-relevanten, renalen SLCs gehören die Organische-Anionen-Transporter 1 (OAT1), OAT3 und Organische-Kationen-Transporter 2 (OCT2), die Substanzen aus dem Blut in die proximalen Tubuluszellen der Nieren aufnehmen. Die ABC-Transporter *Multidrug Resistance-associated Protein 2* (MRP2), MRP4 und *Multidrug Resistance Protein 1* (MDR1) sind in den Nieren und der Leber exprimiert und transportieren Substanzen aus den Zellen.

Die Expression dieser Transporter wird in den Nieren und der Leber gesunder Ratten geschlechtsspezifisch durch einen zunächst unbekannten molekularen Mechanismus reguliert. Wir konnten die geschlechtsabhängige Expression renaler Transporter in ZSF1-Ratten (einem Model für Diabetes Typ 2) nachweisen. Die größten Änderungen wurden für Oat2 gefunden. Das humane OAT2-Protein interagierte in einem *in vitro* Model hochaffin mit dem Schleifendiuretikum Furosemid. In der Leber von ZSF1-Ratten war die mRNA-Menge für Oat3 und für zahlreiche Enzyme, die an der hepatischen Metabolisierung beteiligt sind, geschlechtsabhängig exprimiert.

Wir konnten zeigen, dass der Androgenrezeptor-Testosteron-Komplex die Transkription der männlich-dominant exprimierten Oat1- und Oat3- Transporter der Ratte nicht beeinflusst. Der Transkriptionsfaktor B-cell CLL/lymphoma 6 (BCL6) wurde als potentieller Aktivator von Oat1 und Oat3 identifiziert. BCL6 aktivierte auch den humanen OAT1-Promotor. Die BCL6-abhängige transkriptionelle Regulation war vom *hepatocyte nuclear factor 1*$\alpha$ (HNF1$\alpha$) abhängig.

Da HNF1$\alpha$ die Promotoren verschiedener SLC- und ABC-Transporter beeinflusst, muss die Interaktion von HNF1$\alpha$ und BCL6 bezüglich deren geschlechtsabhängigen Regulation weiter untersucht werden. Die geschlechtsabhängige Transporter-Expression sollte bei der Entwicklung und der Testung neuer Arzneimittel berücksichtigt werden.

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*Research Article*

# Sex-Differences in Renal Expression of Selected Transporters and Transcription Factors in Lean and Obese Zucker Spontaneously Hypertensive Fatty Rats

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The aim of this study was to identify sex-dependent expression of renal transporter mRNA in lean and obese Zucker spontaneously hypertensive fatty (ZSF1) rats and to investigate the interaction of the most altered transporter, organic anion transporter 2 (Oat2), with diabetes-relevant metabolites and drugs. Higher incidence of glomerulosclerosis, tubulointerstitial fibrosis, and protein casts in Bowman's space and tubular lumen was detected by PAS staining in obese male compared to female ZSF1 rats. Real-time PCR on RNA isolated from kidney cortex revealed that Sglt1-2, Oat1-3, and Oct1 were higher expressed in kidneys of lean females. Oct2 and Mrp2 were higher expressed in obese males. Renal mRNA levels of transporters were reduced with diabetic nephropathy in females and the expression of transcription factors Hnf1$\beta$ and Hnf4$\alpha$ in both sexes. The highest difference between lean and obese ZSF1 rats was found for Oat2. Therefore, we have tested the interaction of human OAT2 with various substances using tritium-labeled cGMP. Human OAT2 showed no interaction with diabetes-related metabolites, diabetic drugs, and ACE-inhibitors. However, OAT2-dependent uptake of cGMP was inhibited by furosemide. The strongly decreased expression of Oat2 and other transporters in female diabetic ZSF1 rats could possibly impair renal drug excretion, for example, of furosemide.

## 1. Introduction

Diabetes mellitus is one of the most common diseases, with 346 million affected individuals worldwide in 2012 and represents the seventh leading cause of death in the United States [1]. Type 2 diabetes accounts for about 90% of all diagnosed cases [2]. More than twenty percent of patients with type 2 diabetes develop diabetic nephropathy [3]. Moreover, clinical studies reported a high prevalence of hypertension for patients in both early and late stages of this disease, which potentiates further progression of kidney damage [4]. Vice versa, the decline in kidney function contributes to elevated blood pressure in patients with type 2 diabetes [4]. Premenopausal women typically have lower blood pressure than age-matched men, possibly mediated by estradiol which appears to act as a vasodilator [5]. This is in line with a higher incidence of diabetic nephropathy associated with type 2 diabetes observed in men compared to age-matched women [6].

An unresolved issue is the association of diabetic nephropathy with expression of transport proteins responsible for renal secretion of drugs. Members of the solute carrier 22 (Slc 22) gene family, organic anion transporters (human OAT; rat and mouse Oat), and organic cation transporters (human OCT; rat and mouse Oct) are expressed in the kidneys and take up endogenous and exogenous compounds, including frequently prescribed drugs, from the blood into proximal

Table 1: Blood pressure (BP), blood glucose, plasma glyceride, and urine albumin/creatinine ration in young and old male ZSF1 rats.

| | Lean ZSF1 | Obese ZSF1 | Reference |
|---|---|---|---|
| Systol. BP (mmHg)<br>8 weeks of age | | 132 ± 19 | [21] |
| Systol. BP (mmHg)<br>20 weeks of age | 156 ± 14 | 176 ± 23 | [21] |
| Diastol. BP (mmHg)<br>8 weeks of age | | 88 ± 8 | [21] |
| Diastol. BP (mmHg)<br>20 weeks of age | 101 ± 9 | 103 ± 11 | [21] |
| Blood glucose (mg/dL)<br>8 weeks of age | 114 ± 2.8 | 147 ± 3.9 | [19] |
| Blood glucose (mg/dL)<br>29 weeks of age | 115 ± 11 | 424 ± 37 | [24] |
| Plasma triglyceride (mg/dL)<br>8 weeks of age | 49 ± 3 | 483 ± 46 | [19] |
| Plasma triglyceride (mg/dL)<br>29 weeks of age | 194 ± 23 | 5200 ± 702 | [24] |
| Urine albumin/creatinine ratio<br>8 weeks of age | 0.03 ± 0.001 | 0.23 ± 0.04 | [19] |
| Urine albumin/creatinine ratio<br>16 weeks of age | 0.048 ± 0.007 | 1.203 ± 0.118 | [23] |

tubular cells [7–10]. Among antidiabetic drugs, OCT2 is involved in proximal tubular secretion of metformin, and OAT3 transports sitagliptin [9, 11]. For rat kidneys, androgen-dependent expression of Oat1, Oat3, and Oct2 and higher expression of Oat2 in females was reported, suggesting sex-dependent renal drug handling at least in this species [12, 13].

ATP-dependent efflux transporters, multidrug resistance-associated protein 2 (Mrp2), Mrp4, and P-glycoprotein (Mdr1b) are localized in the apical membrane of renal proximal tubules and are responsible for the secretion of organic anions and cations from the proximal tubular cells into the urine [14]. Human gene promoters of OAT1, OAT2, and MRP2 are activated by the transcription factor hepatocyte nuclear factor 4$\alpha$ (HNF4$\alpha$) [15–17]. Interestingly, single nucleotide polymorphisms (SNPs) associated with type 2 diabetes were found in the gene encoding HNF4$\alpha$ [18].

The aim of this study was to identify, at the level of mRNA, potential sex- and diabetes-dependent changes of Oats, Octs, ATP-dependent efflux transporters, and the transcriptional regulators, Hnf1$\alpha$, Hnf1$\beta$, and Hnf4$\alpha$. Additionally, the levels of sodium-dependent glucose cotransporter 1 (Sglt1) and Sglt2 were investigated. We used obese Zucker spontaneously hypertensive fatty rats (ZSF1) as an established animal model for type 2 diabetes and diabetic nephropathy. Lean ZSF1 rats served as a model-specific control.

ZSF1 rats were previously developed by crossing rat strains with different mutations in the leptin receptor gene, Zucker diabetic fatty (ZDF) rats, and spontaneously hypertensive heart failure (SHHF) rats [19, 20]. Lean and obese ZSF1 rats had similar mean arterial blood pressure at seven weeks of age and elevated blood pressure (BP) at 20 weeks of age (Table 1) [21, 22]. The concentration of blood glucose and plasma triglycerides were higher in obese than in lean ZSF1 rats at eight weeks of age (Table 1) [19]. However, only obese ZSF1 rats developed type 2 diabetes with diabetic nephropathy, characterized by elevated urine albumin/creatinine ratios (Table 1) [19, 23]. Our hypothesis was that, in addition to sex-dependences, the renal expression of Oats and Octs may be altered in diabetic nephropathy that might influence the renal secretion of metabolites and exogenous substances.

## 2. Material and Methods

*2.1. Animals and Kidney Preservation.* Kidneys from obese male and female ZSF1 (ZSF1-*Lepr*$^{fa}$*Lepr*$^{cp}$/Crl) rats, and their lean controls, were obtained from Charles River (Stone Ridge, NY). Animals were kept in the animal facility of Charles River Laboratories under conventional housing conditions (22°C, 55% humidity, and 12 h day/night cycle) with free access to water and rat chow. Kidneys of adult (16-week-old) ZSF1 rats were removed postmortem in accordance to federal law, conserved in paraformaldehyde (4%) or RNA*later*, and shipped to our laboratory.

*2.2. Morphological Study.* Rat kidneys fixed in paraformaldehyde (4%) were processed for embedding in paraffin. Serial sections (3 $\mu$m) of kidneys were stained with periodic acid-Schiff (PAS).

*2.3. RNA Isolation.* After removal of the kidney capsule, cortical slices were prepared, from which total RNA was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. Quality and quantity of the extracted RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Boeblingen, Germany) and NanoDrop ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, Wilmington,

NC), following the manufacturer's protocol. RNAs with RNA integrity number (RIN) > 8 were used for further experiments.

*2.4. cDNA Synthesis and TaqMan Real-Time PCR.* Superscript II Reverse Transcriptase (Life Technologies, Darmstadt, Germany) and Oligo dT-Primers (Eurofin MWG Operon, Ebersberg, Germany) were used for reverse transcription of RNA. Genes of interest were analyzed using TaqMan Master Mix and TaqMan Gene Expression Assays (Life Technologies): Sodium-dependent glucose cotransporter 1 (Sglt1), Rn00564718_m1; Sglt2, Rn00574917_m1; Oat1, Rn00568143_m1; Oat2, Rn00585513_m1; Oat3, Rn00580082_m1; Oct1, Rn00562250_m1; Oct2, Rn00580893_m1; Mrp2, Rn00563231_m1; Mrp4, Rn01465702_m1; P-glycoprotein (Mdr1b), Rn00561753_m1; Hnf1$\alpha$, Rn00562020_m1; Hnf1$\beta$, Rn00447453_m1; Hnf4$\alpha$, Rn00573309_m1. The mRNA levels of hypoxanthine phosphoribosyltransferase 1 (Hprt1, Rn01527840_m1), $\beta$-actin (Rn00667869_m1), and cyclophilin A (Rn00690933_m1) were tested as housekeeping control genes for sample normalization. For all tested genes, PCR conditions were as follows: 2 min at 50°C followed by 10 min at 95°C and 40 amplification cycles (95°C for 15 s and 60°C for 60 s), using Mx3000P real-time PCR cycler (Agilent Technologies). Signals detected between 35–40 amplification cycles were defined as low gene expression. The amplification efficiencies of used assays were 100% (+/− 10%), in accordance with manufacturer's information. The real-time PCR data were analyzed as ΔCt = housekeeping gene (Hprt1)-gene of interest (Figure 2), using the $2^{-\Delta\Delta Ct}$ method (see Supplementary Figures in Supplementary Material available online at http://dx.doi.org/10.1155/2015/483238) [25].

*2.5. Transport Studies.* The uptake of cGMP in HEK293 cells stably transfected with human OAT2 (kindly provided by PortaCellTec Biosciences GmbH, Göttingen, Germany) was investigated in the absence and presence of metabolites known to be accumulated in diabetic patients and therapeutics for treatment of diabetes and hypertension. First, OAT2- and vector-transfected HEK293 cells were seeded at a density of $2 \cdot 10^5$ cells/well in a 24-well cell culture plate and incubated for ~72 h in Dulbecco's modified Eagle medium-high glucose (DMEM HG, D5796, Sigma Aldrich) culture medium supplemented with 10% fetal bovine serum (number 10270, Life Technologies), 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin (PAA Laboratories GmbH, Austria). The cells in each well were washed with PBS and mammalian Ringer solution containing 130 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $Mg_2SO_4$, 1 mM $NaH_2PO_4$, 20 mM HEPES, and 20 mM D-glucose, pH 7.4. The uptake of cGMP was tested after incubation of the cells for 5 min with 100 nM [$^3$H]cGMP (PerkinElmer, Hamburg, Germany) and 9.9 $\mu$M unlabeled cGMP (BioLog, Bremen, Germany), with and without potential inhibitors at 37°C. Substances investigated for their inhibitory potential were adipic acid (Sigma Aldrich), suberic acid (Sigma Aldrich), glycolic acid (Sigma Aldrich), citric acid (Merck), 3-hydroxyisobutyrate (Fluka), *cis*-aconitic acid (Sigma Aldrich), homovanillic acid (Sigma Aldrich), indomethacin (Sigma Aldrich), sitagliptin (Santa Cruz Biotechnologies), miglitol (Santa Cruz Biotechnologies), captopril (Sigma Aldrich), enalapril (Sigma Aldrich), furosemide (Sigma Aldrich), and bumetanide (Sigma Aldrich). After incubation with radio-labeled cGMP and potential inhibitors, cells were washed three times with PBS at 4°C, and cell lysis was induced by incubation for 2 h with 500 $\mu$L of 1 M NaOH. Thereafter, cell lysates were transferred to scintillations vials, 2.5 mL Lumasafe scintillation solution was added to each vial, and radioactivity was counted by a liquid scintillation counter (Tri-Carb 1500; PerkinElmer). Total protein concentrations were determined by the Bradford protein assay, and the cGMP uptake was calculated per milligram of total protein.

*2.6. Statistical Analysis.* Real-time PCR data and data of transport experiments are presented as mean ± SEM. Statistical analysis of real-time PCR data was performed with two-way analysis of variance (ANOVA). Following two-way ANOVA, Bonferroni test was used for multiple comparison of males versus females and of lean versus obese rats (GraphPad Prism 4, version 4.03; GraphPad Software, La Jolla, CA). Data of transport experiments were statistically analyzed with two-tailed unpaired $t$-test (GraphPad Prism 4). Differences were considered significant at the level of $P < 0.05$.

## 3. Results

*3.1. Structural Changes in the Kidneys of Lean and Obese ZSF1 Rats.* Light microscopy of periodic acid-Schiff stained sections revealed differences between lean females and lean males in the structure of glomeruli and tubuli of ZSF1 rats (Figure 1, le-female, le-male). Whereas the structure of glomeruli was similar between the two sexes, the tubular basement membrane of lean males appeared to be thicker than that of lean females (arrows). Sex-differences became more obvious in obese rats where renal damage was more prominent in obese males than in obese female ZSF1 rats. In kidneys of obese male and female ZSF1 rats, glomerulosclerosis, extensive mesangial matrix accumulation, and mesangial hypercellularity were detected (Figure 1, §§§). In obese males, in addition, a dilatation of Bowman's capsule and tuft-to-capsule adhesion was present (§§). Tubular injury was indicated by the thickening of tubular basement membrane, tubular dilatation with epithelial cell flattening, tubular lesions, and lumen containing protein as well as by atrophic tubuli (#) with hyaline casts (Figure 1, ##).

Excessive depositions of fibronectin confirmed the development of interstitial fibrosis in kidneys of obese ZSF1 rats. Protein expression of fibronectin was higher in kidneys of lean males than of lean females (data not shown).

*3.2. Differences between Lean and Obese ZSF1 Rats and Sex-Differences in Renal Cortical mRNA Expression of Transporters and Transcription Factors.* The housekeeping genes $\beta$-actin and cyclophilin A were differently expressed between lean and obese animals and between the sexes (data not shown). In contrast, the expression of Hprt1 did not differ between

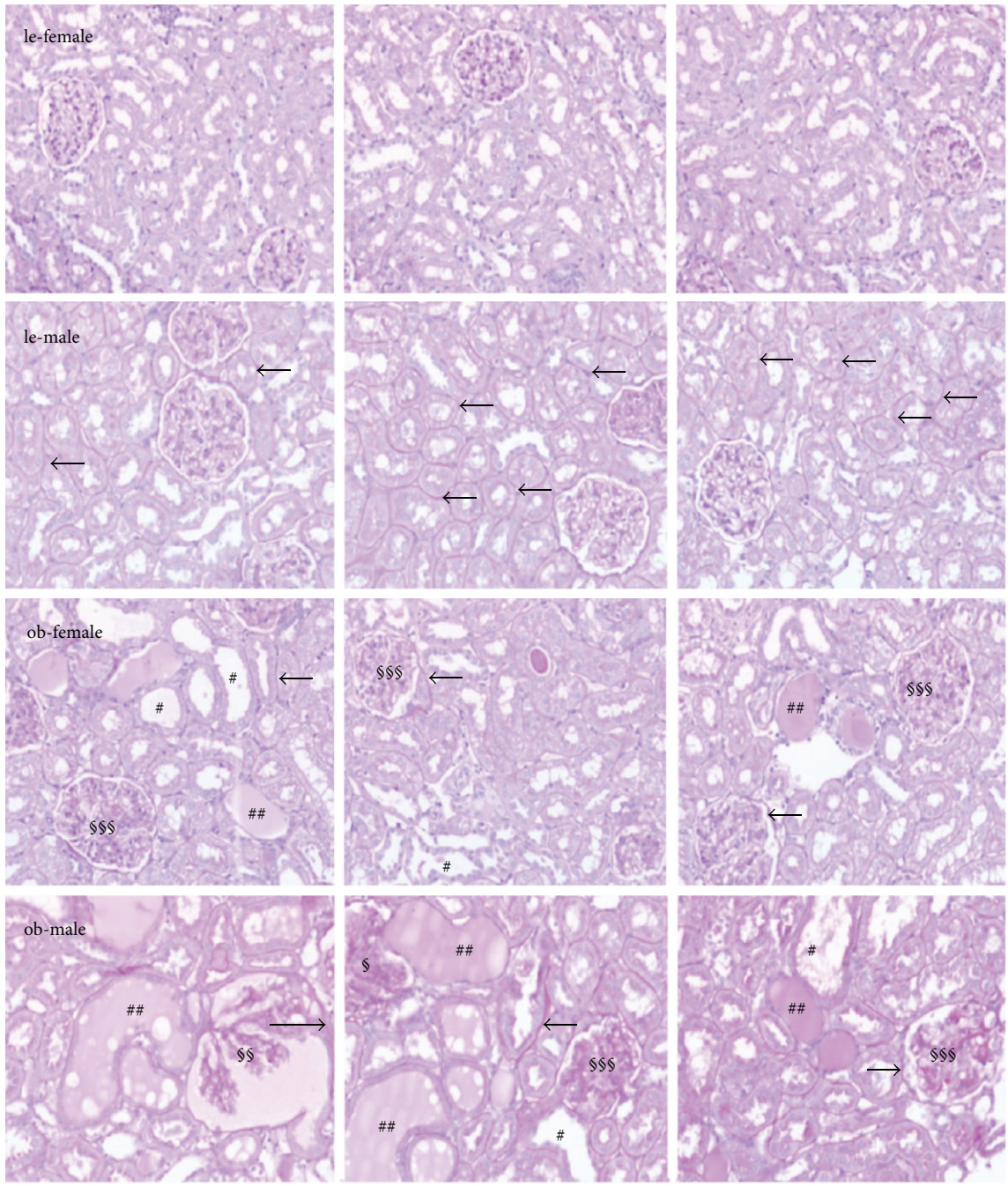


Figure 1: PAS staining in renal cortical slices. Renal sections of lean (le) and obese (ob) ZSF1 rats were stained with PAS and structural changes were analyzed. Examples for histological changes in renal tissue are marked as follows: #, tubular atrophy and dilatation; ##, hyaline protein casts in tubular lumen; §, pressure-induced deformation of glomerulus; §§, dilatation of Bowman's capsule and protein cast in Bowman's space; §§§, glomerulosclerosis. The arrows mark the thickening of tubular and glomerular basement membranes. Magnification: 200x. Representative images from three different rats under each condition are shown.

experimental groups and was, therefore, used as a reference gene in our study.

Sex dependences as well as differences in mRNA expression between lean and obese rats are presented as ΔCt values in Figure 2 and are summarized as $2^{-\Delta\Delta Ct}$ values in Supplementary Figures 1 and 2, respectively. In Figure 2, white bars correspond to lean and black bars to obese ZSF1 rats, respectively. Negative bars indicate that more PCR cycles were needed to reach the threshold for the gene of interest than for the reference gene Hprt1, that is, the gene of interest shows a lower expression than Hrpt1. Conversely, positive bars in Figure 2 indicate a lower number of PCR cycles for the gene of interest than for Hprt1, that is, a higher gene expression as compared to Hprt1. In general, the more

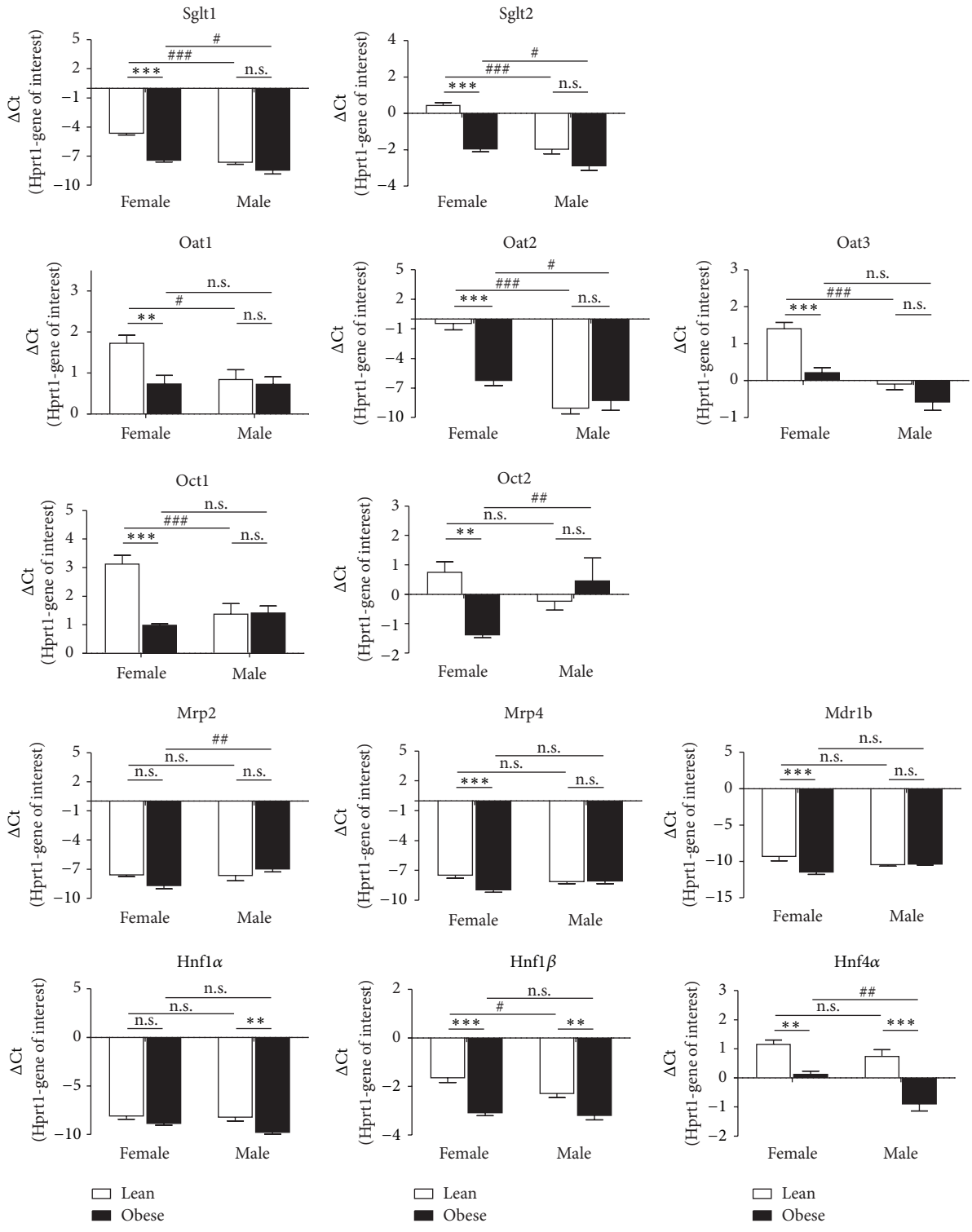


Figure 2: Diabetes- and sex-dependent renal gene expression in lean (le) and obese (ob) ZSF1 rats. Gene expressions were analyzed using TaqMan real-time PCR and presented as mean ± SEM. $n = 6–8$. n.s., not significant; $^{**}P < 0.01$; and $^{***}P < 0.001$, for the comparison of ΔCt values between lean and obese ZSF1 rats. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; and $^{\#\#\#}P < 0.001$, for comparison of ΔCt values between females and males.

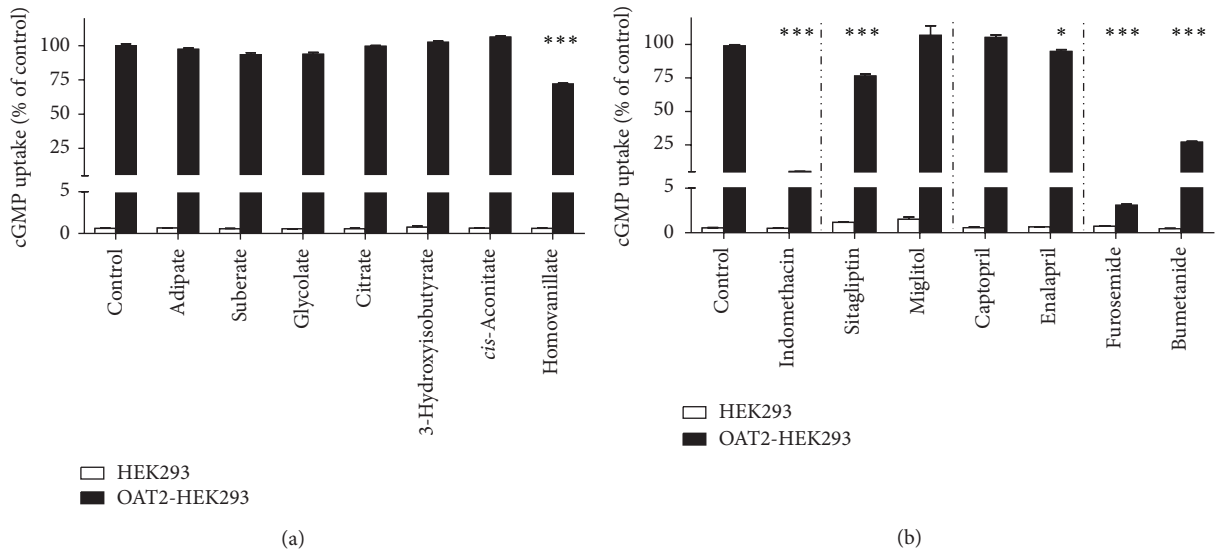


Figure 3: Influence of dicarboxylates, metabolites, and drugs on OAT2-mediated cGMP uptake in HEK293 cells. The uptake of cGMP was determined after 5 min incubation with 10 $\mu$M cGMP (0.1 $\mu$M [$^3$H]cGMP + 9.9 $\mu$M unlabeled cGMP) alone (controls) or in the presence of 500 $\mu$M potential inhibitors at 37°C. (a) Dicarboxylates and metabolites; (b) drugs. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$; $^{***}P < 0.001$, compared to control.

positive (or the less negative) the ΔCt values are, the higher the expression of the gene of interest is.

In lean ZSF1 rats, mRNAs coding Sglt1 and Sglt2 were higher expressed in females, because more PCR cycles were needed to reach the threshold in males (Figure 2). In terms of $2^{-\Delta\Delta Ct}$ values, sex differences amounted to 8.35 ± 1.02-fold for Sglt1, and to 5.51 ± 0.68-fold for Sglt2 (Supplementary Figure 1A). Female expression of Sglt1 and Sglt2 mRNA was retained in obese animals but was less pronounced (Figure 2). Interestingly, Sglt1 and Sglt2 expression was higher in female lean ZSF1 rats than in female obese rats. In males, no significant differences between lean and obese ZSF1 rats were observed for Sglt1 and Sglt2 (Figure 2).

Similarly, levels of Oat1, Oat2, and Oat3 were higher in kidneys of lean females than lean males (Figure 2). These sex-differences vanished for Oat1 and Oat3 in obese rats (Figure 2). In contrast, Oat2 mRNA was higher in obese female than in obese males, with a 6.70 ± 1.58-fold difference (Supplementary Figure 1B). In female obese ZSF1 rats, a decreased expression of Oat1, Oat2, and Oat3 was observed in comparison with lean females (Figure 2). Interestingly, expression of Oat2 showed the strongest difference between lean and obese females. For Oat1, Oat2, and Oat3 mRNA levels no significant differences were detected between male lean and obese ZSF1 rats (Figure 2).

In lean female ZSF1 rats, the mRNA expression level of Oct1 was significantly higher than in their male counterparts and this sex dependence vanished in the kidneys of obese animals (Figure 2). Renal mRNA expressions of Oct1 and Oct2 were significantly reduced in female obese ZSF1 rats as compared to lean females. No significant changes in Oct1 and Oct2 levels were detected between lean and obese male rats. In contrast, significantly higher Oct2 expression was detected in obese males as compared to obese females (Figure 2).

Lean female rats showed a higher Mrp4 and Mdr1b expression than obese females; however, no change in Mrp2 mRNA. In male ZSF1 rats, expression of Mrp2, Mrp4, and Mdr1b remained unchanged in obese rats compared to leans (Figure 2). Renal expression of Mrp2, Mrp4, and Mdr1b showed no significant sex dependence in lean ZSF1 rats. In obese animals, Mrp2 mRNA was higher in males than in females (Figure 2).

The expression of the transcription factor Hnf1$\alpha$ was low in all tested animal groups with a detection limit beyond 35 amplification cycles. Hnf1$\beta$ and Hnf4$\alpha$ mRNA expressions were significantly reduced in both female and male obese ZSF1 rats as compared to lean controls (Figure 2). The expression of Hnf1$\beta$ was slightly higher in lean females as compared to lean males but was similar in kidneys of obese females and obese males. The transcription factor Hnf4$\alpha$ was higher expressed in females as in males only in obese ZSF1 rats (Figure 2).

*3.3. Inhibition of OAT2 Transport Function.* Given the large changes in Oat2 expression, the inhibitory potential of metabolites (adipate, suberate, glycolate, citrate, 3-hydroxyisobutyrate, *cis*-aconitate, and homovanillate) associated with diabetic kidney disease [26, 27], drugs for treatment of diabetes (sitagliptin, miglitol), and hypertension (captopril, enalapril, furosemide, and bumetanide) on human OAT2 expressed in HEK293 cells was investigated.

The OAT2-dependent accumulation of radioactive labeled cGMP, a known substrate for OAT2 [28], was not affected by dicarboxylates adipate and suberate (Figure 3(a)). In addition, cGMP uptake was not inhibited by the metabolites glycolate, citrate, 3-hydroxyisobutyrate, and *cis*-aconitate. In contrast, cGMP uptake was significantly decreased in

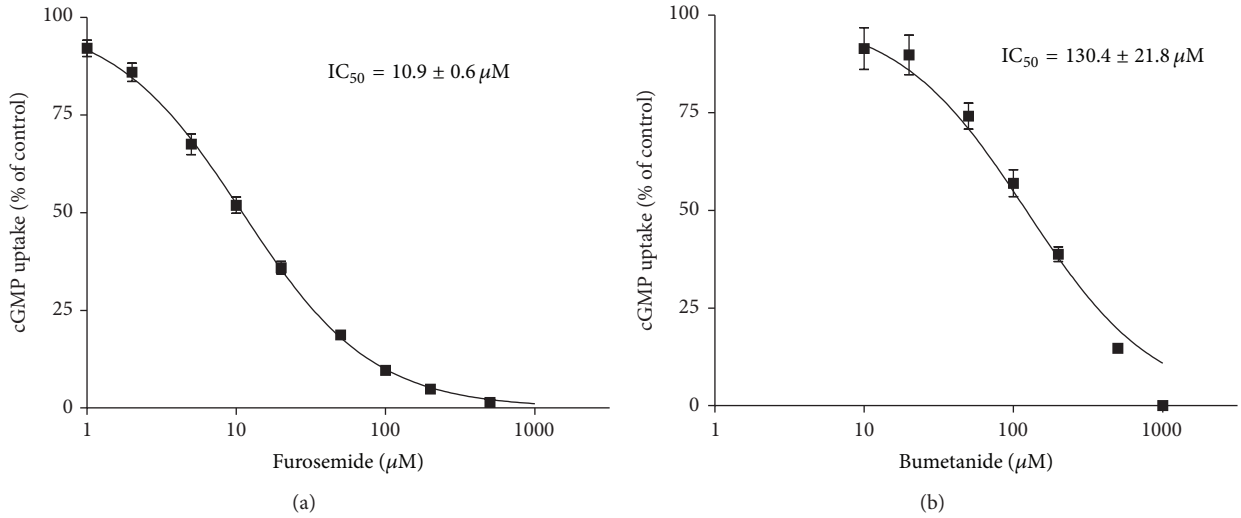


Figure 4: $IC_{50}$ determination for the inhibition of OAT2-mediated cGMP uptake by furosemide and bumetanide. In HEK293 cells stably transfected with OAT2 or empty vector, intracellular cGMP accumulation was determined after coincubation with 10 $\mu$M cGMP (0.1 $\mu$M [$^3$H]cGMP + 9.9 $\mu$M unlabeled cGMP) and 1–1000 $\mu$M furosemide (a) or 10–1000 $\mu$M bumetanide (b), respectively, for 5 min at 37°C. The furosemide and bumetanide concentrations causing half-maximal inhibitory effect ($IC_{50}$) on cGMP accumulation in OAT2 expressing cells were calculated. Data are presented as mean ± SEM. $n_{\text{furosemide}}$ = 2–4; $n_{\text{bumetanide}}$ = 2.

the presence of the dopamine metabolite homovanillate (Figure 3(a)).

OAT2 transport function was abolished by indomethacin, a verified inhibitor of OAT2-mediated cGMP uptake [29] (Figure 3(b)). The antidiabetic drug sitagliptin inhibited OAT2-mediated cGMP accumulation by approx. 25% (Figure 3(b)). Miglitol showed no effect on OAT2-dependent cGMP uptake (Figure 3(b)). No or a small significant inhibition of OAT2-dependent cGMP accumulation was observed in presence of the ACE-inhibitors captopril and enalapril (Figure 3(b)). The uptake of cGMP in OAT2-expressing HEK293 cells was strongly reduced by the diuretics furosemide and bumetanide (Figure 3), showing $IC_{50}$ values of 10.9 ± 0.6 $\mu$M (Figure 4(a)) and 130.4 ± 21.8 $\mu$M (Figure 4(b)), respectively.

## 4. Discussion

Lean and obese ZSF1 rats are hypertensive, but only obese animals develop type 2 diabetes and diabetic nephropathy, exhibiting symptoms comparable to humans [19]. In this study, we show sex-dependent morphological changes in the renal cortex and in the expression of selected proximal tubular transport proteins and transcription factors in lean and obese ZSF1 rats. In addition, we investigated the impact of several metabolites found in the urine of diabetic patients and of drugs used in the treatment of diabetes and diabetes related diseases on the human organic anion transporter 2 (OAT2).

Our histological results are in line with evidence that the symptoms of diabetic renal disease, for example, renal injury, glomerulosclerosis, interstitial fibrosis, and elevated urine albumin/creatinine ratios, were more pronounced in adult male compared to female diabetic ZSF1 rats [23]. Hypertension, which was reported to be also stronger in male compared to female ZSF1 and spontaneously hypertensive heart failure (SHHF) rats was associated with higher rates of progression of glomerulosclerosis and increased fibronectin expression [30]. Thickening of tubular basement membrane observed in our study was evident not only in kidneys of obese but also in kidneys of lean male ZSF1 rats.

It has already been shown in the kidneys of Wistar rats that Sglt1 and Sglt2 proteins are higher expressed in females than in males [31]. Accordingly, in our study, lean female rats showed considerably higher mRNA levels of Sglt1 and Sglt2 than lean male rats. Recent data suggested that expression of both glucose transporters was increased in obese male Zucker rats at the age of 21 weeks [32]. We found that diabetic nephropathy in obese animals resulted in a decline in the expression of both glucose transporters. The explanation for this discrepancy is possibly the different stage of diabetic nephropathy in the Zucker obese (ZO) rat model, described in published study and in the ZSF1 rat model, used in our experiments. Our histological data confirm the published ZSF1 studies, which showed renal damage, as characteristic for diabetic nephropathy, in obese ZSF1 rats already before 21 weeks of age [19, 23].

In patients with diabetic nephropathy, a significantly downregulated renal OAT1 and OAT3 gene expression and impaired secretion of organic anions were observed [27]. In agreement with these human data, significant downregulation of Oat1 and Oat3 was detected in obese female ZSF1 rats compared to lean females. In contrast to published data [13], higher Oat1 and Oat3 mRNA levels were detected in kidneys of female compared with male ZSF1 rats. The unexpectedly low expression of Oat1 and Oat3 in lean males could be due to structural changes as visualized by the thickening of tubular basement membrane in these animals.

Our results showed for the first time higher expression of Oct1 in lean female compared with male ZSF1 rats. This finding may have some implications on drug evaluation using rats as opposed to humans, because OCT1 is not expressed in renal tubular cells of human kidneys [14]. OCT2/Oct2, well-known for transport of the antidiabetic drug, metformin, was identified at high levels in human and rat kidneys [33]. In this study, obese males, but not lean male ZSF1 rats, showed higher Oct2 expression compared with female counterparts. In accordance with published data [34], Oct2 was decreased by diabetic nephropathy in obese females, but not in obese male ZSF1 rats.

Using another model of diabetes, Nowicki and colleagues showed increased levels of the efflux transporters, Mrp2 and Mrp4, in Western blots from whole kidneys of male rats with streptozotocin-induced type 2 diabetes [34]. We found in renal cortex no changes for Mrp2 and decreased Mrp4 mRNA levels in obese ZSF1 females compared to lean controls. Similarly, Mdr1b expression was decreased by diabetic nephropathy in our female obese rats. No changes were observed between obese and lean male ZSF1 rats. Additionally, no sex-differences were found for Mrp2, Mrp4, and Mdr1b expressions in lean animals, which is in line with already published data [35]. However, we observed higher Mrp2 expression in obese males compared with female ZSF1 rats. The reason for this sex-dependent Mrp2 expression in ZSF1 rat strain remains to be clarified.

The expression of Sglt1, Sglt2, Oct1, Oat1, and Oat3 was shown to be transcriptionally regulated by Hnf1$\alpha$ homodimers and Hnf1$\alpha$/$\beta$ heterodimers [36–39]. Low content of Hnf1$\alpha$ mRNA was detected in renal cortex of ZSF1 rats. Thus, it appears unlikely that Hnf1$\alpha$ plays a dominant role in the transcriptional regulation of transporter mRNAs. The mRNA level of Hnf1$\beta$ was significantly decreased in obese ZSF1 rats compared with their lean counterparts. Furthermore, the promoters of rat Oat1, Oat3, and Oct1 can be activated by Hnf4$\alpha$ [40]. The expression of Hnf4$\alpha$ was significantly higher in obese females than in obese male ZSF1 rats and was decreased in both sexes in comparison to lean controls. HNF4$\alpha$ has been found to be suppressed in kidneys of patients with diabetic nephropathy as well as diabetic Zucker diabetic fatty (ZDF) rats, a model for diabetic nephropathy different from that used in this study [41]. Our data showed a decline in Hnf4$\alpha$ expression in male and female obese diabetic ZSF1 rats which is in accordance with published results [41]. The observed sex-dependent Hnf4$\alpha$ expression in obese ZSF1 rats was possibly induced by a higher degree of diabetic nephropathy in male animals as compared to females. Interestingly, despite of reduced Hnf4$\alpha$ expression in obese male rats, there was no change in expression of renal transporters potentially regulated by Hnf4$\alpha$ compared to the lean animals. Given the fact that lean males exhibited a lower transporter expression compared to lean females, we assume that obesity or diabetes induced further reduction in Hnf4$\alpha$ was not sufficient to decrease transporter expression even more.

In accordance with our results, higher renal Oat2 expression was found in females than in males [13, 42]. Diabetic nephropathy significantly decreased Oat2 mRNA in renal cortex of obese female ZSF1 rats. Decreased Oat2 mRNA in female animals is probably the consequence of a higher degree of renal damage in kidneys of obese ZSF1 rats. In males, Oat2 mRNA expression was already very low in lean animals and did not decrease further by diabetes. This result is contradictory to previously published data, which showed increased levels of Oat2 protein in whole kidneys of male diabetic Sprague-Dawley rats [34]. It remains to be clarified why Oat2 expression is different in diabetic kidneys of different rat strains.

In human kidneys, OAT2 is located to the basolateral membrane of proximal tubule cells [14] and is, hence, involved in the uptake of metabolites and drugs from the blood into proximal tubule cells. The renal excretion of adipate and suberate is known to be increased in ketotic episodes of diabetes [26], but neither adipate nor suberate affected OAT2, suggesting no significant role of OAT2 in excretion of these anions in diabetes. The concentrations of glycolate, citrate, 3-hydroxyisoburyrate, *cis*-aconitate, and homovanillate were decreased in the urine of patients with diabetic kidney disease [27]. Our experiments excluded an interaction of these metabolites with OAT2, because only homovanillate was able to inhibit cGMP uptake, but this inhibition was very weak.

The weak inhibition of OAT2-mediated cGMP uptake by high concentrations of sitagliptin and enalapril and the absence of any effect of miglitol and captopril on OAT2 function indicate that OAT2 most likely does not contribute to renal excretion of these antidiabetic and antihypertensive drugs. On the other hand, the loop diuretics furosemide and bumetanide inhibited significantly cGMP uptake by OAT2, in our study. The plasma half-life of furosemide was prolonged in patients with renal insufficiency [43]. For example, patients with mild acute kidney injury (AKI) showed a better response to furosemide than patients with severe AKI [44]. In addition, the effect of furosemide on the fractional excretion of potassium in the urine was higher in healthy volunteers compared to patients with stage III chronic kidney disease [45]. Nevertheless, loop diuretics like furosemide are appropriate in chronic kidney disease for reduction of blood pressure [46].

Our data suggest for the first time the involvement of human OAT2 in the secretion of this loop diuretic because of its high affinity ($IC_{50}$ 10.9 $\mu$M). In mouse $S_2$ cells transfected with human OAT2, an $IC_{50}$ of 603 $\mu$M was published for furosemide [47]. The reason for the disagreement between our results and published data for the interaction of furosemide with human OAT2 is unclear but most likely due to the usage of different expression systems, and different radio-labeled substrates (cGMP versus $PGF_{2\alpha}$). Bumetanide inhibited OAT2 with moderate affinity ($IC_{50}$ 130 $\mu$M), in good agreement with previous data ($IC_{50}$ 77.5 $\mu$M) [47]. These two $IC_{50}$ values are much higher than the free plasma concentration of bumetanide [47], indicating that OAT2 does not appreciably contribute to the renal excretion of bumetanide.

## 5. Conclusion

Kidneys obtained from male and female obese ZSF1 rats revealed tissue damage and significant changes in mRNA content of transporters involved in glucose absorption and drug excretion, for example, Sglt1/2, Oat1/2/3, and Oct1/2.

These changes are most probably due to diabetes type 2 in obese ZSF1 rats. Discrete signs of damage were found in lean males, resulting from other, nondiabetic pathophysiological changes, most probably the hypertension. A number of proximal tubular transporters showed higher mRNA expression in females compared with male ZSF1 rats. Diabetes in obese animals decreased transporter expression more in female than in male rats. The highest difference was found for Oat2. Additional experiments showed for the first time the possible involvement of human OAT2 in secretion of furosemide, an often prescribed diuretic for patients suffering from diabetes, hypertension, and related kidney diseases. The altered expression of renal transporters may have an impact on sugar and drug excretion in diabetic nephropathy.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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# Next generation sequencing of sex-specific genes in the livers of obese ZSF1 rats

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ABSTRACT

Type 2 diabetes induces pathophysiological changes in the liver. The aim of this study was to identify differently expressed genes in the livers of male and female ZSF1 rats (ZDFxSHHF-hybrid, generation F1), a model for type 2 diabetes.
Gene expression was investigated using next-generation sequencing (NGS). Selected candidate genes were verified by real-time PCR in the livers of obese and lean rats.
103 sex-different genes, associated to pathways "response to chemical stimulus", "lipid metabolism", and "response to organic substance", were identified. Male-specific genes were involved in hepatic metabolism, detoxification, and secretion, e.g. cytochrome P450 2c11 (Cyp2c11), Cyp4a2, glutathione S-transferases mu 2 (Gstm2), and Slc22a8 (organic anion transporter 3, Oat3). Most female-specific genes were associated to lipid metabolism (e.g. glycerol-3-phosphate acyltransferase 1, Gpam) or glycolysis (e.g. glucokinase, Gck).
Our data suggest the necessity to pay attention to sex- and diabetes-dependent changes in pre-clinical testing of hepatic metabolized and secreted drugs.



## 1. Introduction

Type 2 diabetes is associated with histopathological and pathophysiological changes in the human liver [1]. A high prevalence to develop nonalcoholic fatty liver disease, liver cirrhosis, and hepatocellular carcinoma was identified in patients with type 2 diabetes [1–3]. In rat liver, oxidative stress, pathological changes of hepatic architecture, inflammation and focal necrosis were shown to be induced in early stages of diabetes [4].

The liver is the major organ responsible for drug metabolism and drug clearance. Women have a greater risk of suffering adverse drug reactions (ADRs) than men [5]. In 427 human liver samples, 80 sex-dependently expressed genes were identified and genes encoding hepatic drug transporter, organic anion transporter 2 (OAT2; gene name: SLC22A7) and organic cation transporter 1 (OCT1; SLC22A1), were found to be female-specifically expressed [6]. In patients with polymorphisms in SLC22A1 lower effects of metformin, an antidiabetic drug and OCT1 substrate, were observed [7]. Other antidiabetic drugs, such as sitagliptine, repaglinide, and rosiglitazone, inhibited OCT1-mediated transport of metformin in vitro [8,9]. In our previous study, only a weak inhibition of OAT2-mediated cGMP uptake by sitagliptin was observed, and OAT2 function was not affected by miglitol [10]. In contrast, sitagliptin transport was shown to be OAT3-mediated [11]. Sex-differences were found for the expression of genes involved in several hepatic drug detoxification and metabolism pathways, in human liver as well as in several rat models for diabetes [6,12,13]. Furthermore, it was postulated that the increased bile acid pool in diabetic rats stimulated the transcription of anion transporters in the sinusoidal membrane of hepatocytes [14].

The aim of this study was to identify transcriptional differences between male and female rat livers, with the focus on hepatic drug metabolizing enzymes and drug transporters from the Slc22a gene family. For this purpose, liver samples of male and female obese ZSF1 rats (ZDFxSHHF-hybrid, generation F1) were investigated using next-generation sequencing (NGS). ZSF1 rats are first generation hybrid between rat strains with different mutations in the leptin receptor gene, Zucker diabetic fatty (ZDF) rats and spontaneously hypertensive heart failure (SHHF) rats [15]. The initial designation of this rat model was ZDFxSHHF-hydrid rats [16]. ZSF1 rats are an established animal model for type 2 diabetes, hyperlipidemia, nephropathy, and metabolic syndrome, exhibiting symptoms comparable to humans [15,17]. Male obese (diabetic) ZSF1 rats were reported to have significantly higher body weight, liver weight, plasma cholesterol, and plasma triglycerides at 8 and 41 weeks of age compared to their lean (non-diabetic) controls

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[15,17–19]. At 8 weeks of age, lean and obese male ZSF1 rats have similar blood glucose level [18]. Elevated blood glucose levels were observed between 12 and 41 weeks of age in obese, but not in lean male ZSF1 rats [17,18]. In general, liver weight, blood glucose, plasma cholesterol, and plasma triglycerides were lower in obese female compared to obese male ZSF1 rats, but higher compared to lean males at 41 weeks of age [17]. Sex-dependent changes of hepatic gene expression were not investigated in lean and obese ZSF1 rats so far.

## 2. Material and methods

### 2.1. Animals and liver preservation

Obese male and female ZSF1 (ZSF1-*Lepr*$^{fa}$*Lepr*$^{cp}$/Crl) rats, and an equivalent number of lean controls for each sex were kept in the animal facility of Charles River Laboratories in New York State under conventional housing conditions (22 °C, 55% humidity, and 12 h day/night cycle) with free access to water and conventional rat chow till 16 weeks of age. The livers of ZSF1 rats were removed post mortem in accordance to US federal law, conserved in RNAlater® (Life Technologies, Germany), and shipped to our laboratory. For the experimental design ≥6 rat individuals for each group to be compared were tested, which is known for achieving robust RNA-Seq results [20]. Technical batch effects were minimized by preparing samples, sequencing libraries and sequencing run at the same time, respectively.

### 2.2. RNA isolation, cDNA library preparation, and next-generation sequencing (NGS)

For NGS experiments total RNA was isolated from the livers of 6 male and 6 female obese ZSF1 rats using TRIzol® reagent (Life Technologies) according to the manufacturer's recommendations and was digested with RNase-Free DNase I in order to remove DNA contamination (Sigma Aldrich, Germany). Quantity and quality of extracted RNA were analyzed using microfluidic electrophoresis (Bioanalyzer; Agilent Technologies USA) and NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, USA), following the manufacturer's protocol. 800 ng of each total RNA samples with a RNA integrity number (RIN) >8 was used as starting material. TrueSeq RNA Sample Preparation Kit (Illumina, Netherlands) was used to prepare samples for NGS analysis. Accurate quantitation of cDNA libraries was performed by using the QuantiFluorTM dsDNA System (Promega, Germany). The size range of cDNA libraries was determined applying the DNA 1000 chip on the Bioanalyzer 2100 from Agilent (280 bp). cDNA libraries were amplified and sequenced by using the cBot and HiSeq 2000 from Illumina (SR, 1 × 50 bp, 10–45 million reads per sample). Sequence images were transformed with Illumina software BaseCaller, which were demultiplexed with CASAVA (version 1.8.2). Quality check was done via FastQC (Babraham Bioinformatics, United Kingdom).

### 2.3. Gene expression analysis

The sequenced reads were mapped to the rat UCSC reference genome (rn4) [21] using bowtie2 (2.0.2) [22]. Conversion of SAM to BAM and corresponding sorting was done via SAMtools [23]. Counting the reads to each gene was done via HTSeq (0.5.3p3) (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). Normalization of read counts to the library size, estimation of dispersions and testing for differentially expressed (DE) genes based on a statistical test assuming negative binomial data distribution were computed in the R/Bioconductor environment (version 2.15.2) loading DESeq (1.10.1) [24] and biomaRt (2.14.0) [25] packages. DESeq normalized data were shown to produce the smallest coefficient of variation in terms of diverse library sizes and compositions compared to other common analysis methods [26]. Significant sex-specific genes were determined as log2 fold change (log2FC) >1 or <−1, base mean <1000, and false discovery rate (FDR)-corrected p value <0.05 with multiple testing correction according to Benjamini and Hochberg [27]. The data were generated conforming to *MIAME* standards and have been submitted to the Gene Expression Omnibus (GEO) database (GSE57598).

### 2.4. cDNA synthesis and TaqMan® real-time PCR

For real-time PCR experiments RNA was used from the livers of 6 lean and obese males, and 8 lean and obese female ZSF1 rats. Reverse transcription of RNA was performed using Superscript™ II Reverse Transcriptase (Life Technologies) and Oligo dT-Primers (Eurofin MWG Operon, Germany). TaqMan® Master Mix and TaqMan® Gene Expression Assays (Life Technologies) were used for the analysis of genes of interest: glutathione S-transferase mu 2 (Gstm2), Rn00598597_m1; 11β-hydroxysteroid dehydrogenase type 1 (Hsd11b1), Rn00567167_m1; cytochrome P450 4a2 (Cyp4a2), Rn01417066_m1; Cyp2c11, Rn01502203_m1; leukemia inhibitory factor receptor (Lifr), Rn00579104_m1; 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (Srd5a1), Rn00567064_m1; glucokinase (Gck), Rn00561265_m1; glycerol-3-phosphate acyltransferase 1 (Gpam), Rn00568620_m1; Oct1, Rn00562250_m1; Oat2, Rn00585513_m1; Oat3, Rn00580082_m1. Hypoxanthine phosphoribosyltransferase 1 (Hprt1, Rn01527840_m1) and β-actin (Rn00667869_m1) were analyzed as housekeeping genes for sample normalization. Real-time PCR conditions were: 2 min at 50 °C followed by 10 min at 95 °C and 40 amplification cycles (95 °C for 15 s and 60 °C for 60 s) using Mx3000P™ real-time PCR cycler (Agilent Technologies). The amplification efficiencies of the TaqMan® Gene Expression Assays were 100 ± 10%, in accordance with manufacturer's information. The data were analyzed as ΔCt = housekeeping gene (Hprt1)-gene of interest, according to the $2^{-\Delta\Delta Ct}$ method [28].

### 2.5. Statistical analysis of real-time PCR data

Real-time PCR data are presented as mean ± SEM. For multiple comparisons, males vs. females, and lean vs. obese ZSF1 rats, the statistical analysis was performed with two-way analysis of variance (ANOVA) and followed by Bonferroni test (GraphPad Prism 4, version 4.03; GraphPad Software, USA). Differences were considered as significant at the level of $p < 0.05$.

## 3. Results

Sex-dependent expression of all investigated genes in the livers of obese ZSF1 rats was summarized in the volcano plot, showing statistically significant female- and male-specific genes marked in red (Fig. 1A). The principal component analysis (PCA) plot shows individual variations of liver samples revealing no differences between male and female livers (Fig. 1B). Interestingly, the gene expression of one male liver (L4) was found to be different in comparison to other males (L1, L2, L3, L5, and L6) (Fig. 1B).

Within male and female liver samples from obese ZSF1 rats, 469 out of 15,027 genes sequenced were sex-dependently expressed. The exclusion of genes with low expression (base mean < 1000) resulted in 103 sex-differently expressed hepatic genes in obese male and female ZSF1 rats (Table 1).

The largest portion of sex-dependent genes was localized on chromosome 1, followed by genes localized on chromosome 2 and 5 (Fig. 2). Most female-specific genes were localized on chromosome 1 (42.5% of 40 female-specific genes) and the highest number of male-specific genes were localized on chromosome 5 (19% of 63 male-specific genes) (Fig. 2).

In addition, candidate genes were analyzed using Gene Ontology (GO) [29]. The highest number of sex-dependent genes was associated to the GO terms “response to chemical stimulus”, “lipid metabolic

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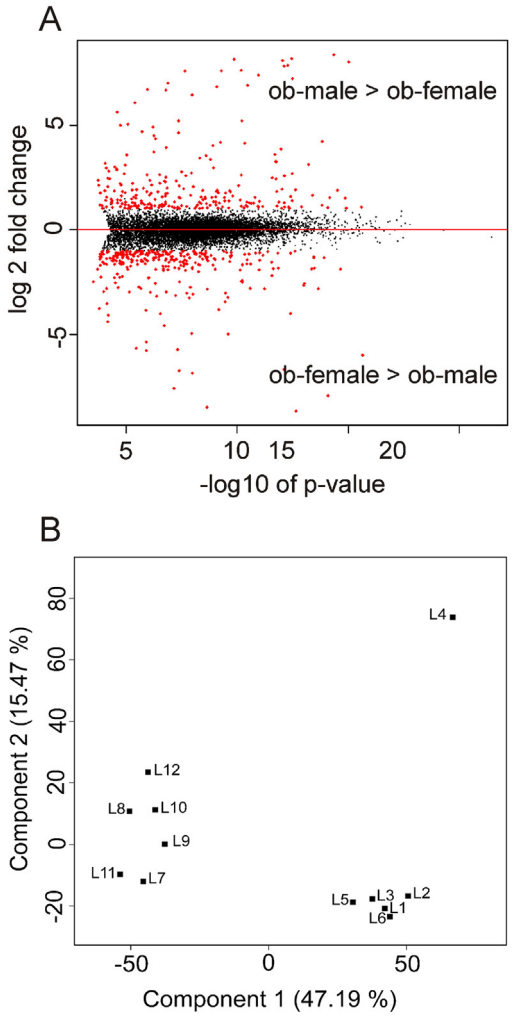


**Fig. 1.** Statistical overview of NGS data using volcano plot and principal component analysis (PCA). A) 15,027 sequenced probes in the NGS analysis is shown in a volcano plot. The negative log10 of the adjusted p-value (−log10 of p-value) is plotted on the x-axis, and the log2 fold-change is plotted on the y-axis. Each gene is represented as a dot. Red points represent possible sex-specifically expressed genes in the livers of 6 male and 6 female obese (ob) ZSF1 rats, and have a log2 fold-change ≤−1 or ≥1, and a p-value <0.05. The black points are characterized by log2 fold-change between −1 and 1 and p-value >0.05, and represent genes similarly expressed in male and female livers. Male-specifically expressed genes have a positive log2 fold-change (red points at the top of the plot) and female-specifically genes have a negative log2 fold-change (red points at the bottom of the plot). B) The potential individual variations of the liver samples were represented in the PCA plot. The component 1 on the x-axis represents 47.19% (optimal range 30–60%) and component 2 on the y-axis represents 15.47% (optimal range <40%) of total variation in the sequencing data. L1–L6: liver samples of obese male ZSF1 rats; L7–L12 liver samples of female obese ZSF1 rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

process", and "response to organic substances" (Table 2). On the other hand, lower number of hepatic sex-dependent genes belonged to the GO terms "response to external stimulus", "steroid metabolic process", "retinol metabolism" or "response to organic cyclic compound". Amongst others, male-specific genes associated to these GO terms were members of cytochrome P450 (Cyp2c11, Cyp4a2), glutathione S-transferases mu (Gstm1, Gstm2, Gstm7), hydroxysteroid dehydrogenases (Hsd11b1, Hsd17b2), and the gene encoding transport protein Oat3 (Slc22a8). Female-specific genes with highest fold-change belonging to enriched GO terms were enzymes involved in fatty acid pathways (Acsl4, Acacb, Cd36) or glycolysis (Gck, G6pd). In addition, cytokine receptor Lifr, steroid 5α-reductase Srd5a1 or bile salt sulfotransferase Sult2a1 were female-specific expressed (Table 2).

Eight candidate genes were verified by real-time PCR, male-specific Gstm2, Hsd11b1, Cyp4a2, and Cyp2c11 and female-specific Lifr, Srd5a1, Gck, and Gpam. Expression of β-actin was significantly altered between lean and obese animals (data not shown). Hprt1 did not differ between animal groups and was therefore used as reference gene (data not shown). Real-time PCR data were calculated as $2^{-\Delta\Delta Ct}$, converted to log2FC, and compared to NGS data in Table 3. In accordance with NGS results, similar log2FC was identified using real-time PCR (Table 3).

Additionally to RNA samples from obese ZSF1 rats, these candidate genes were investigated in the livers of lean controls and presented as $2^{-\Delta\Delta Ct}$ in Fig. 3A–C. The sex-specific expression of candidate genes was also identified in the livers of lean ZSF1 rats (Fig. 3A). Gstm2 expression was equal in lean and obese males, and was decreased in obese compared to lean females (Fig. 3B–C). In contrast, the mRNA level of Hsd11b1 was higher in obese females than in lean females with no significant difference between the livers from lean and obese males. Cyp4a2 expression was higher in obese than in lean males, but without a significant difference between lean and obese females. In the livers of male and female ZSF1 rats, no significant differences between lean and obese were observed for Cyp2c11. Lifr was reduced in obese females as compared to lean females, with no differences between lean and obese males. Srd5a1 mRNA was slightly lower in obese compared to lean females, but was enhanced 2.81 ± 0.74 fold in the livers of obese compared to lean male ZSF1 rats. No significant changes of hepatic Gck mRNA were detected between lean and obese animals. Gpam mRNA remained unchanged in obese compared to lean female, but was significantly higher in obese than in lean male ZSF1 rats, with 8.73 ± 1.05 fold difference (Fig. 3B–C).

The gene encoding Oat3 (Slc22a8) was identified to be higher expressed in the livers of obese male ZSF1 rats compared to obese females (Fig. 4A). Therefore, NGS data for Slc22a genes, a transporter family involved in transport of endogenous and exogenous substances are summarized in Fig. 4A. The mRNA levels of Slc22a2 (Oct2), Slc22a6 (Oat1), Slc22a12 (Urat1), Slc22a13 (Oat10), Slc22a15, and Slc22a25 were low in the livers of males and females, with no significant sex differences. On the other hand, hepatic Slc22a1 (Oct1), Slc22a5 (Octn2), Slc22a7 (Oat2), Slc22a17 (Boct1), Slc22a18, and Slc22a23 (Boct2) mRNA levels were higher in the livers of ZSF1 rats, without showing significant sex differences.

The mRNA levels of three Slc22a transporters with established protein function in the liver [30] were verified by real-time PCR and presented in Fig. 4B–E. In agreement to NGS results, no significant sex-differences were detected for mRNA levels of Slc22a1 (Oct1) and Slc22a7 (Oat2) and male-specific expression was identified for Slc22a8 (Oat3) in the livers of obese ZSF1 rats (Fig. 4B). In lean controls, Slc22a1 (Oct1) and Slc22a7 (Oat2) mRNA levels were higher in females than in males and Slc22a8 (Oat3) was male-specific expressed (Fig. 4C). No significant differences of Slc22a1 (Oct1) and Slc22a7 (Oat2) were detected either in the livers of male or female lean and obese ZSF1 rats (Fig. 4D–E). Additionally, female Slc22a8 (Oat3) expression was increased in obese rats as compared to lean animals (Fig. 4E).

## 4. Discussion

Using NGS analysis, we show sex-dependent differences between male and female obese ZSF1 rats in the hepatic expression of enzymes and transporters involved in cellular response to organic anions and cations, which are possibly involved in functional changes in the diabetic liver. Furthermore, the difference in genes between obese and lean ZSF1 rats for each sex, was investigated choosing sex-specific genes from NGS analysis.

In our study, 103 sex-specific gene expressions were identified in the livers of obese ZSF1 rats. These findings are in line with the high number of hepatic genes, which were reported to be sex-dependently expressed in the human and in the rodent (rat and mouse) liver [6,12,13,31]. In addition to sex-dependent genes localized

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**Table 1**
103 sex-dependent expressed genes in livers of obese ZSF1 rats.

| Gene | Description | Base mean | | log2FC | FDR-p value |
|---|---|---|---|---|---|
| | | Female | Male | | |
| Akr1b7 | Aldose reductase-related protein 1 | 12,800 | 30 | −8,67 | 6,54E-266 |
| A1bg | Alpha-1B-glycoprotein | 34,686 | 140 | −7,94 | 0,00E + 00 |
| Lifr | Leukemia inhibitory factor receptor | 8734 | 85 | −6,67 | 1,81E-65 |
| Aldh1b1 | Aldehyde dehydrogenase X, mitochondrial | 1508 | 46 | −4,99 | 3,69E-25 |
| Sult2a2 | Alcohol sulfotransferase A | 10,213 | 635 | −4,01 | 1,14E-72 |
| Trim24 | Transcription intermediary factor 1-alpha | 1084 | 76 | −3,82 | 3,81E-124 |
| Ifi47 | Interferon gamma inducible protein 47 | 1293 | 118 | −3,44 | 1,66E-09 |
| Pnpla5 | Patatin-like phospholipase domain-containing protein 5 | 1279 | 156 | −3,03 | 1,13E-23 |
| Ces2e | Carboxylesterase 5 | 4209 | 594 | −2,82 | 6,08E-40 |
| Srd5a1 | 3-oxo-5-alpha-steroid 4-dehydrogenase 1 | 22,591 | 3208 | −2,82 | 2,33E-43 |
| Gck | Glucokinase | 2692 | 385 | −2,80 | 8,22E-21 |
| Gpam | Glycerol-3-phosphate acyltransferase 1, mitochondrial | 14,170 | 2231 | −2,67 | 7,09E-18 |
| Oat | Ornithine aminotransferase, mitochondrial | 14,255 | 2387 | −2,58 | 1,66E-59 |
| Pla2g16 | Group XVI phospholipase A2 | 1976 | 356 | −2,47 | 2,93E-18 |
| Rtn4 | Reticulon-4 | 2025 | 378 | −2,42 | 2,08E-68 |
| Acacb | Acetyl-CoA carboxylase 2 | 1787 | 467 | −1,93 | 4,72E-15 |
| G6pd | Glucose-6-phosphate 1-dehydrogenase | 3220 | 851 | −1,92 | 6,82E-12 |
| Sult2a1 | Bile salt sulfotransferase | 40,590 | 11,005 | −1,88 | 2,67E-14 |
| Sult2al1 | Alcohol sulfotransferase A | 16,452 | 4492 | −1,87 | 3,32E-33 |
| Elovl6 | Elongation of very long chain fatty acids protein 6 | 4965 | 1367 | −1,86 | 1,09E-07 |
| Serpina6 | Corticosteroid-binding globulin | 17,757 | 4957 | −1,84 | 4,34E-27 |
| Scd | Acyl-CoA desaturase 2 | 5768 | 1614 | −1,84 | 1,04E-07 |
| Cd36 | Platelet glycoprotein 4 | 1841 | 577 | −1,67 | 7,80E-08 |
| UST4r | Integral membrane transport protein UST4r | 2365 | 761 | −1,64 | 4,07E-31 |
| Fdps | Farnesyl pyrophosphate synthase | 4490 | 1529 | −1,55 | 4,03E-17 |
| Tm7sf2 | Delta(14)-sterol reductase | 1206 | 420 | −1,52 | 1,96E-12 |
| Sall1 | Sal-like protein 1 | 2052 | 758 | −1,44 | 1,54E-19 |
| Cyp2a1 | Cytochrome P450 2A1 | 16,068 | 6335 | −1,34 | 1,29E-13 |
| Dgat2 | Diacylglycerol O-acyltransferase 2 | 22,072 | 8743 | −1,34 | 1,91E-27 |
| Fdx1 | Adrenodoxin, mitochondrial | 1190 | 482 | −1,30 | 2,43E-22 |
| Aifm2 | Apoptosis-inducing factor, mitochondrion-associated 2 | 1165 | 490 | −1,25 | 1,66E-22 |
| Tspan7 | Tetraspanin-7 | 1211 | 516 | −1,23 | 3,86E-13 |
| Acss2 | Acetyl-coenzyme A synthetase, cytoplasmic | 3204 | 1372 | −1,22 | 9,33E-16 |
| Alcam | CD166 antigen | 1802 | 798 | −1,17 | 2,65E-17 |
| Secisbp2l | SECIS binding protein 2-like | 2056 | 918 | −1,16 | 7,73E-10 |
| Lipa | Lysosomal acid lipase/cholesteryl ester hydrolase | 7452 | 3423 | −1,12 | 4,09E-26 |
| Abhd2 | Abhydrolase domain-containing protein 2 | 4967 | 2287 | −1,12 | 3,71E-15 |
| Ces2a | Carboxylesterase 2A | 2824 | 1315 | −1,10 | 2,08E-16 |
| RGD1309079 | Similar to Ab2-095 (RGD1309079), mRNA | 1163 | 546 | −1,09 | 6,95E-14 |
| Acsl4 | Long-chain-fatty-acid--CoA ligase 4 | 8367 | 4112 | −1,02 | 6,87E-21 |
| Tmem176a | Transmembrane protein 176A | 3420 | 6994 | 1,03 | 3,52E-15 |
| Rup2 | Urinary protein 1 | 20,136 | 41,390 | 1,04 | 1,87E-06 |
| Akr7a3 | Aflatoxin B1 aldehyde reductase member 3 | 512 | 1063 | 1,05 | 9,05E-10 |
| Wfdc2 | WAP four-disulfide core domain protein 2 | 824 | 1715 | 1,06 | 1,58E-22 |
| Akr1d1 | 3-oxo-5-beta-steroid 4-dehydrogenase | 11,196 | 23,464 | 1,07 | 2,81E-18 |
| Got1 | Aspartate aminotransferase, cytoplasmic | 1284 | 2697 | 1,07 | 1,82E-20 |
| Serpina3n | Serine protease inhibitor A3N | 31,620 | 67,262 | 1,09 | 1,33E-11 |
| Gls2 | Glutaminase liver isoform, mitochondrial | 3262 | 6945 | 1,09 | 6,14E-22 |
| Gstm1 | Glutathione S-transferase Mu 1 | 4862 | 10,404 | 1,10 | 5,09E-13 |
| Pald | Paladin | 891 | 1927 | 1,11 | 1,92E-23 |
| RGD1311122 | Uncharacterized protein C4orf34 homolog | 1436 | 3108 | 1,11 | 1,52E-23 |
| Hsd17b2 | Estradiol 17-beta-dehydrogenase 2 | 1234 | 2698 | 1,13 | 2,56E-08 |
| Gstm7 | Glutathione S-transferase Yb-3 | 731 | 1600 | 1,13 | 3,10E-11 |
| Gldc | Glycine dehydrogenase | 2217 | 4891 | 1,14 | 1,92E-19 |
| Nucb2 | Nucleobindin-2 | 837 | 1872 | 1,16 | 2,80E-11 |
| Cyp3a23/3a1 | Cytochrome P450 3A1 | 8881 | 20,035 | 1,17 | 7,67E-06 |
| Fahd1 | Fumarylacetoacetate hydrolase domain-containing protein 1 | 935 | 2243 | 1,26 | 1,82E-19 |
| Apon | Apolipoprotein N | 1007 | 2437 | 1,27 | 1,84E-26 |
| Amacr | Alpha-methylacyl-CoA racemase | 2585 | 6328 | 1,29 | 9,17E-32 |
| Oplah | 5-oxoprolinase | 796 | 1999 | 1,33 | 1,05E-27 |
| Hacl1 | 2-hydroxyacyl-CoA lyase 1 | 2147 | 5395 | 1,33 | 1,59E-21 |
| Slc9a3r1 | Na(+)/H(+) exchange regulatory cofactor NHE-RF1 | 442 | 1151 | 1,38 | 9,37E-10 |
| Cyp2d3 | Cytochrome P450 2D3 | 3131 | 8236 | 1,39 | 3,36E-37 |
| Csrp1 | Cysteine and glycine-rich protein 1 | 613 | 1648 | 1,43 | 5,45E-29 |
| Orm1 | Alpha-1-acid glycoprotein | 15,424 | 44,206 | 1,52 | 7,06E-35 |
| Phyh | Phytanoyl-CoA dioxygenase, peroxisomal | 9380 | 27,333 | 1,54 | 2,02E-29 |
| Mettl7a | Methyltransferase-like protein 7A | 351 | 1029 | 1,55 | 1,72E-22 |
| Aqp9 | Aquaporin-9 | 911 | 2671 | 1,55 | 2,31E-20 |
| Cyp2a2 | Cytochrome P450 2A2 | 2702 | 8172 | 1,60 | 8,70E-16 |
| Ddah1 | N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 | 438 | 1348 | 1,62 | 8,27E-30 |
| LOC299282 | Serine protease inhibitor 2.1 | 5192 | 16,302 | 1,65 | 3,62E-05 |
| Akr1c12l1 | Aldo-keto reductase family 1, member C12-like 1 | 326 | 1109 | 1,76 | 4,65E-24 |
| Nnmt | Nicotinamide N-methyltransferase | 367 | 1295 | 1,82 | 4,62E-30 |
| Car3 | Carbonic anhydrase 3 | 1543 | 5490 | 1,83 | 5,95E-06 |

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**Table 1** (*continued*)

| Gene | Description | Base mean | | log2FC | FDR-p value |
|---|---|---|---|---|---|
| | | Female | Male | | |
| Pck1 | Phosphoenolpyruvate carboxykinase, cytosolic [GTP] | 9939 | 36,911 | 1,89 | 2,85E-14 |
| Fgl1 | Fibrinogen-like protein 1 | 2652 | 10,010 | 1,92 | 1,12E-06 |
| Gpt | Alanine aminotransferase 1 | 1722 | 6616 | 1,94 | 1,39E-57 |
| Olr59 | Olfactory receptor 51E2 | 402 | 1744 | 2,11 | 1,80E-29 |
| Gstm2 | Glutathione S-transferase Mu 2 | 1075 | 5812 | 2,43 | 1,29E-32 |
| Rdh2 | Retinol dehydrogenase 2 | 923 | 5196 | 2,49 | 2,04E-110 |
| Akr1c13 | aldo-keto reductase family 1, member C13 | 1279 | 7668 | 2,58 | 1,00E-35 |
| Lrtm2 | Leucine-rich repeat and transmembrane domain-containing protein 2 | 327 | 2012 | 2,62 | 3,02E-31 |
| Hsd11b1 | Corticosteroid 11-beta-dehydrogenase isozyme 1 | 1530 | 9903 | 2,69 | 7,19E-31 |
| Spink1 | Serine protease inhibitor Kazal-type 3 | 657 | 6288 | 3,26 | 3,06E-27 |
| Cyp3a18 | Cytochrome P450 3A18 | 637 | 6144 | 3,27 | 3,37E-69 |
| Hao2 | Hydroxyacid oxidase 2 | 505 | 6140 | 3,60 | 5,36E-53 |
| Ust5r | Integral membrane transport protein UST5r | 139 | 2464 | 4,14 | 1,51E-257 |
| Acsm3 | Acyl-coenzyme A synthetase ACSM3, mitochondrial | 63 | 1157 | 4,19 | 1,34E-105 |
| Cyp4a2 | Cytochrome P450 4A2 | 1474 | 27,624 | 4,23 | 4,62E-116 |
| Sds | L-Serine dehydratase/L-threonine deaminase | 61 | 2314 | 5,22 | 2,05E-29 |
| Acnat2 | Acyl-coenzyme A amino acid N-acyltransferase 2 | 8 | 1082 | 6,85 | 2,59E-101 |
| Slc22a8 | Solute carrier family 22 member 8 | 22 | 2825 | 6,91 | 1,10E-67 |
| LOC298111 | Alpha-2u globulin PGCL5 | 75 | 11,422 | 7,23 | 0,00E + 00 |
| Dhrs7 | Dehydrogenase/reductase (SDR family) member 7 | 22 | 3964 | 7,41 | 1,33E-132 |
| Sult1c3 | Sulfotransferase 1C1 | 5 | 1247 | 7,61 | 0,00E + 00 |
| Mup5 | Major urinary protein 5 | 11 | 2320 | 7,63 | 4,62E-30 |
| Obp3 | Alpha-2u globulin PGCL4 (Obp3), transcript variant 1 | 38 | 8835 | 7,83 | 1,15E-126 |
| LOC259244 | Alpha-2u globulin PGCL3 | 41 | 9905 | 7,87 | 4,90E-41 |
| LOC298109 | Alpha-2u globulin PGCL2 | 252 | 66,296 | 8,03 | 8,34E-96 |
| Mup4 | Major urinary protein 4 | 30 | 8434 | 8,10 | 1,22E-34 |
| LOC259245 | Alpha-2u globulin PGCL5 | 6 | 1865 | 8,16 | 6,87E-36 |
| Hsd3b5 | 3 Beta-hydroxysteroid dehydrogenase type 5 | 37 | 11,161 | 8,19 | 3,22E-83 |
| Cyp2c11 | Cytochrome P450 2C11 | 124 | 41,618 | 8,38 | 2,10E-90 |

Female-specific genes: negative log2 fold change (log2 FC) and male-specific genes: positive log2FC; FDR-p value: false discovery rate-corrected p-value.

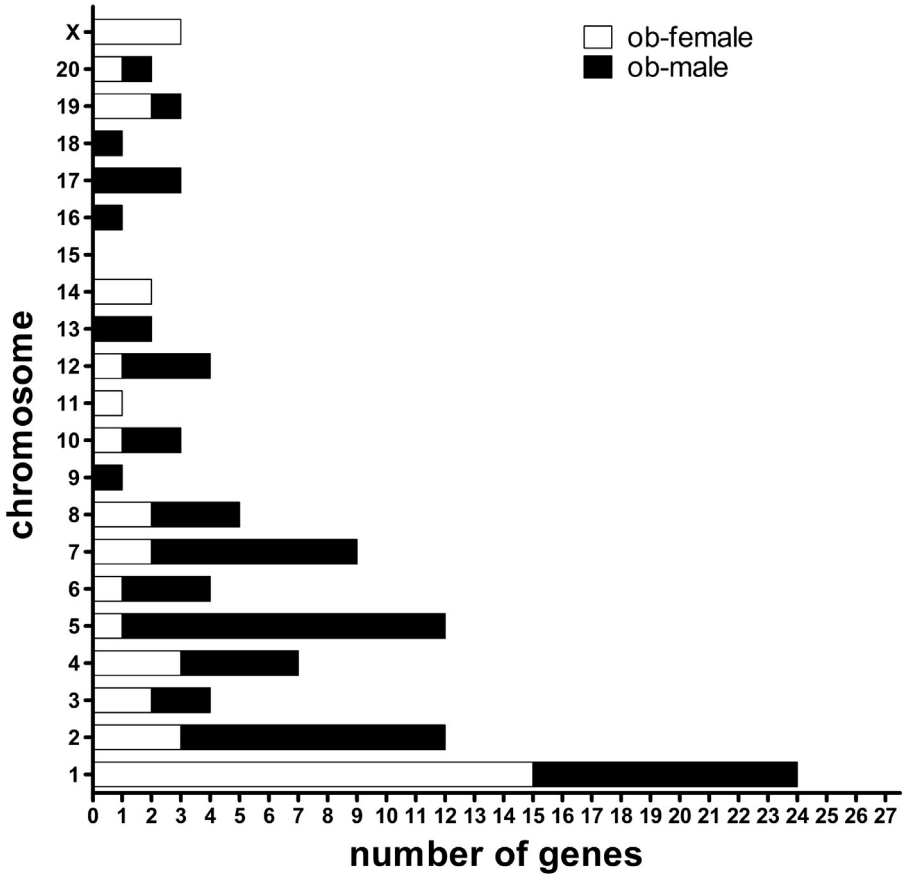


**Fig. 2.** Genomic distribution of sex-specific candidate genes. The graph depicts the number of male- (black) and female (white)-specific genes localized on autosomal rat chromosomes 1–20 and sex-chromosome X. The localization of 103 sex-specific candidate genes, identified in the livers of 6 obese (ob) male and 6 ob-female ZSF1 rats, was analyzed.

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**Table 2**
Genes that play roles in GO pathways whose expression in liver of obese ZSF1 rats was significantly altered by sex.

| GO term | Process | FDR | Male-specific | Female-specific |
|---|---|---|---|---|
| GO:0042221 | Response to chemical stimulus | 6.59E-09 | Akr7a3, Aqp9, Car3, **Cyp2c11**, Cyp3a23/3a1, **Cyp4a2**, Fgl1, Got1, Gstm1, **Gstm2**, Gstm7, **Hsd11b1**, Hsd17b2, Nnmt, Olr59, Orm1, Pck1, Sds, Serpina3n, **Slc22a8** | Acacb, Acsl4, Alcam, Cd36, Cyp2a1, Dgat2, Fdps, Fdx1, G6pd, **Gck**, **Gpam**, **Lifr**, **Srd5a1**, Sult2a1 |
| GO:0006629 | Lipid metabolic process | 7.80E-08 | Acnat2, Acsm3, Akr1d1, Amacr, **Cyp2c11**, Cyp2d3, **Cyp4a2**, Hacl1, Hao2, **Hsd11b1**, Hsd17b2, Hsd3b5, Pck1, Phyh | Acacb, Acsl4, Acss2, Cd36, Ces2a, Cyp2a1, Ddat2, Elovl6, Fdps, Fdx1, G6pd, **Gpam**, Lipa, Pla2g16, Pnpla5, Scd, Serpina6, **Srd5a1**, Sult2a1 |
| GO:0010033 | Response to organic substance | 2.19E-07 | Aqp9, Car3, Cyp3a23/3a1, **Cyp4a2**, Fgl1, Got1, Gstm1, **Gstm2**, Gstm7, **Hsd11b1**, Hsd17b2, Nnmt, Orm1, Pck1, Sds, Serpina3n, **Slc22a8** | Acacb, Acsl4, Cd36, Dgat2, Fdps, Fdx1, G6pd, **Gck**, **Gpam**, **Lifr**, **Srd5a1**, Sult2a1 |
| GO:0009605 | Response to external stimulus | 2.17E-06 | Cyp3a23/3a1, **Hsd11b1**, Orm1, Sds, Serpina3n | Acsl4, Alcam, Cd36, G6pd, **Gck**, **Gpam**, Rtn4, **Srd5a1**, Sult2a1 |
| GO:0008202 | Steroid metabolic process | 3.06E-06 | Akr1d1, Amacr, **Cyp2c11**, Cyp2d3, **Hsd11b1**, Hsd17b2, Hsd3b5 | Cyp2a1, Fdps, Fdx1, G6pd, Lipa, Serpina6, **Srd5a1**, Sult2a1 |
| KEGG:00830 | Retinol metabolism | 3.08E-06 | **Cyp2c11**, Cyp3a18, Cyp3a23/3a1, **Cyp4a2** | Cyp2a1 |
| GO:0014070 | Response to organic cyclic compound | 5.22E-06 | Aqp9, Cyp3a23/3a1, Fgl1, Got1, **Hsd11b1**, Orm1, Sds, Serpina3n, **Slc22a8** | Acacb, Cd36, Fdps, Fdx1, G6pd, **Srd5a1**, Sult2a1, **Gpam** |
| GO:1901700 | Response to oxygen-containing compound | 7.32E-06 | Aqp9, Car3, Got1, Gstm1, **Hsd11b1**, Hsd17b2, Orm1, Pck, Sds, Serpina3n, **Slc22a8** | Cd36, Dgat2, Fdps, Fdx1, G6pd, **Gck**, **Gpam**, **Srd5a1**, Sult2a1 |
| GO:0070887 | Cellular response to chemical stimulus | 7.59E-06 | Aqp9, **Cyp2c11**, Got1, Gstm1, **Gstm2**, Gstm7, **Hsd11b1**, Hsd17b2, Orm1, Serpina3n | Cd36, Dgat2, Fdps, Fdx1, G6pd, **Gck**, **Gpam**, **Lifr**, **Srd5a1**, Sult2a1 |
| KEGG:00140 | Steroid hormone biosynthesis | 1.97E-05 | Akr1d1, Cyp3a18, Cyp3a23/3a1, **Hsd11b1**, Hsd17b2, Hsd3b5 | **Srd5a1** |
| KEGG:00982 | Drug metabolism — cytochrome P450 | 3.33E-05 | **Cyp2c11**, Cyp2d3, Cyp3a18, Cyp3a23/3a1, Gstm1, **Gstm2**, Gstm7 | Cyp2a1 |
| KEGG:00980 | Metabolism of xenobiotics by cytochrome P450 | 9.43E-05 | **Cyp2c11**, Cyp3a18, Cyp3a23/3a1, Gstm1, **Gstm2**, Gstm7 | |
| GO:0048731 | System development | 1.07E-04 | Csrp1, Cyp2d3, **Cyp4a2**, Ddah1, **Hsd11b1**, Hsd17b2, Nnmt, Orm1, Tmem176a | Acsl4, Alcam, Cd36, Dgat2, Fdps, Fdx1, G6pd, **Lifr**, Lipa, Rtn4, Sall1, Scd, **Srd5a1** |
| GO:0031667 | Response to nutrient levels | 1.08E-04 | Cyp3a23/3a1, **Hsd11b1**, Orm1, Sds, Serpina3n | Acsl4, Cd36, G6pd, **Gck**, **Gpam**, **Srd5a1**, Sult2a1 |
| GO:0042493 | Response to drug | 1.29E-04 | **Cyp2c11**, Cyp3a23/3a1, **Cyp4a2**, Gstm1, Nnmt, Orm1 | Acacb, Cd36, Cyp2a1, Fdps, **Srd5a1** |
| GO:0009991 | Response to extracellular stimulus | 1.29E-04 | Cyp3a23/3a1, **Hsd11b1**, Orm1, Sds, Serpina3n | Acsl4, Cd36, G6pd, **Gck**, **Gpam**, **Srd5a1**, Sult2a1 |
| GO:0044707 | Single-multicellular organism process | 1.29E-04 | Akr1d1, Csrp1, Cyp2d3, **Cyp4a2**, Ddah1, Gstm1, **Gstm2**, **Hsd11b1**, Hsd17b2, Nnmt, Olr59, Orm1, Slc9a3r1, Tmem176a | Acsl4, Alcam, Cd36, Dgat2, Fdps, Fdx1, G6pd, **Gpam**, **Lifr**, Lipa, Rtn4, Sall1, Scd, Slc9a3r1, **Srd5a1** |
| GO:0033993 | Response to lipid | 1.56E-04 | Cyp3a23/3a1, Got1, **Hsd11b1**, Hsd17b2, Orm1, Serpina3n | Cd36, Dgat2, Fdps, **Srd5a1**, Sult2a1 |
| GO:0032787 | Monocarboxylic acid metabolic process | 1.81E-04 | Acnat2, Acsm3, Akr1d1, Amacr, **Cyp2c11**, **Cyp4a2**, Hacl1, Hao2, Pck1, Phyh, Sds | Acacb, Acsl4, Acss2, Cd36, Elovl6, **Gpam**, Lipa, Scd |
| GO:0050896 | Response to stimulus | 2.71E-04 | Akr7a3, Aqp9, Car3, **Cyp2c11**, Cyp3a23/3a1, **Cyp4a2**, Fgl1, Got1, Gstm1, **Gstm2**, Gstm7, **Hsd11b1**, Hsd17b2, Nnmt, Olr59, Orm1, Pck1, Sds, Serpina3n, **Slc22a8**, Slc9a3r1 | Abhd2, Acacb, Acsl4, Alcam, Cd36, Cyp2a1, Dgat2, Fdps, Fdx1, G6pd, Gck, **Gpam**, Ifi47, **Lifr**, Lipa, Rtn4, Sall1, Scd, **Srd5a1**, Sult2a1 |

Candidate genes selected for the validation by real-time PCR have been highlighted in bold.

on sex-chromosomes, numerous hepatic sex-biased genes were identified on autosomal chromosomes in the human liver [31]. In our study, a higher number of female-specific genes were localized on chromosome 1 in the livers of obese ZSF1 rats. Similarly, significant enrichment of female-specific hepatic genes was previously found on chromosome 1 in the human liver [31]. The highest enrichment of sex-dependent hepatic genes in obese ZSF1 rats was identified to be involved in xenobiotic metabolism, i.e. associated to GO pathways "response to chemical stimulus", "response to organic substance", "response to external stimulus" and to be involved in lipid metabolism. These results are in accordance with recently described pathways with most sex-specific genes in rat and human liver [31,32].

Out of the identified sex-specific candidate genes, eight genes were selected for the verification using real-time PCR and comparison

**Table 3**
Comparison of NGS analysis and real-time PCR data for selected candidate genes in livers of obese male and female ZSF1 rats.

| Gene name | Entrez gene ID | NGS analysis (log2FC) | Real-time PCR (log2FC) |
|---|---|---|---|
| Glutathione S-transferase Mu 2 (Gstm2) | 24424 | 2.43 | 2.31*** |
| Corticosteroid 11-beta-dehydrogenase isozyme 1 (Hsd11b1) | 25116 | 2.69 | 3.17*** |
| Cytochrome P450 4A2 (Cyp4a2) | 24306 | 4.23 | 3.26*** |
| Cytochrome P450 2C11 (Cyp2c11) | 29277 | 8.38 | 15.81*** |
| Leukemia inhibitory factor receptor (Lifr) | 81680 | −6.67 | −6.06*** |
| 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (Srd5a1) | 24950 | −2.82 | −2.97*** |
| Glucokinase (Gck) | 24385 | −2.80 | −2.76*** |
| Glycerol-3-phosphate acyltransferase 1 (Gpam) | 29653 | −2.67 | −1.35* |

log2FC: log2 fold change.
* $p < 0.05$.
*** $p < 0.001$.

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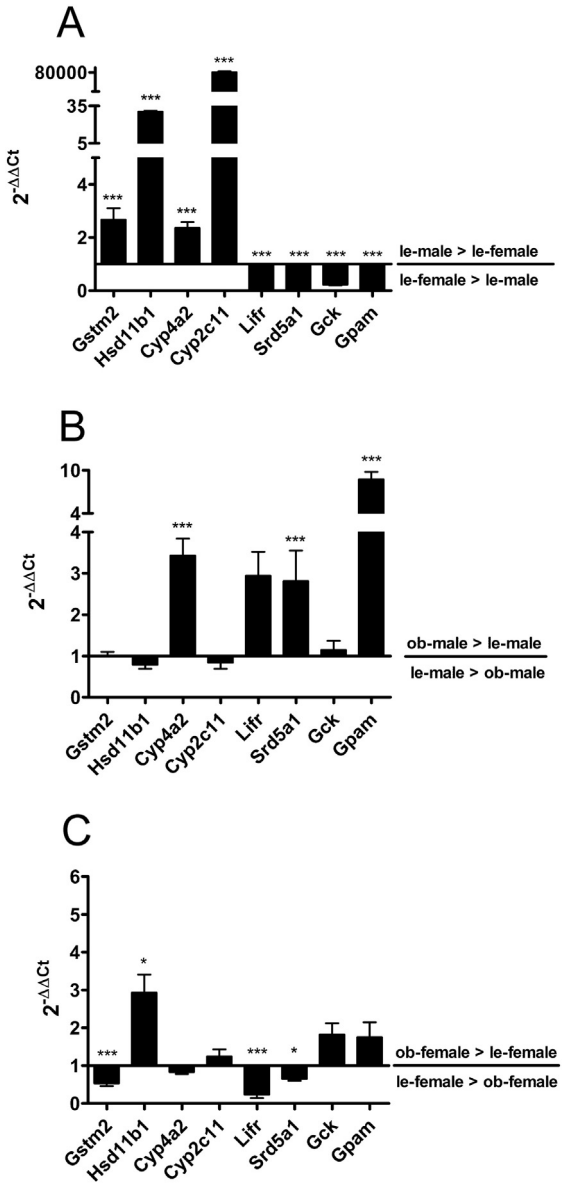


**Fig. 3.** Selected candidate genes in the livers of lean and obese ZSF1 rats. The expression of selected sex-specific candidate genes was verified by TaqMan® real-time PCR in RNA isolated from the livers of lean (le) and obese (ob) ZSF1 rats. Sex-differently expression of candidate genes was investigated in lean ZSF1 rats (A). Differences of candidate genes between lean and obese animals were analyzed in male (B) and female (C) ZSF1 rats. The results of all genes were presented as mean ± SEM. $n_{le\text{-}male} = 6$, $n_{ob\text{-}male} = 6$,$n_{le\text{-}female} = 8$, and $n_{ob\text{-}female} = 8$. *, $p < 0.05$; ***, $p < 0.001$.

between obese and lean ZSF1 rats. The lean and obese male ZSF1 rats are hypertensive [33]. Therefore, the differences in their gene expression observed here are most probably due to diabetes type 2 in obese ZSF1 rats.

Gstm2, a gluthathione S-transferase of class mu isozymes was male-specifically expressed in the livers of lean and obese ZSF1 rats. Glutathione S-transferases are responsible for the detoxification of electrophilic endogenous and exogenous substances, e.g. therapeutic drugs in the liver [34,35]. In accordance with published data [12], hepatic Gstm2 mRNA level was reduced in obese female rats compared to control females. The male-specific hepatic expression of Gstm2 suggests possible additionally female-specific mechanism for the handling of toxic substances.

Hsd11b1 is a microsomal enzyme, mostly expressed in the liver, and responsible for the activation of glucocorticoids, i.e. reduction of cortisone to cortisol [36]. The higher gene expression of Hsd11b1, found in our study in the livers of lean and obese male ZSF1 rats, was previously published in another rat strain, but not in the human liver [6,13]. In agreement with the human situation, Hsd11b1 was enhanced in the liver of obese female ZSF1 rats. In contrast, no significant increase of Hsd11b1 expression was observed in the livers of obese male ZSF1 animals compared with lean males. These discrepancies between rat and human data regarding Hsd11b1 expression suggest that results obtained in established rat models of diabetes cannot be transferable to the human situation.

Male-specific expression of cytochrome P450 4a2 (Cyp4a2), which acts as fatty acid ω-hydroxylase, has been identified in the rat liver [37]. Similar to this published data, we found significantly higher Cyp4a2 mRNA levels in male than in female livers of ZSF1 rats. The mRNA level of Cyp4a2 was shown to be increased by streptozotocin (STZ)-induced diabetes in the livers of male Sprague-Dawley rats [38]. An increase in Cyp4a2 expression in the livers of male diabetic ZSF1 rats compared to their lean controls was detected, which is in accordance with the literature [38].

Cytochrome P450 2c11 (Cyp2c11) has been reported to hydroxylate testosterone and to be involved in the metabolism of several drugs in the liver [39]. We found male-specific gene expression for Cyp2c11 in obese ZSF1 rats and in their lean controls, which is in accordance with literature [40]. In both animal models of type 1 diabetes, STZ- and alloxan-induced diabetes, Cyp2c11 expression was impaired in the liver [41]. In our type 2 diabetes rat model, no significant differences of hepatic Cyp2c11 expression was found between male obese (diabetic) and lean (non-diabetic) ZSF1 rats. The difference between published and our results seems to be based on the type of diabetes model, because similarly no significant diabetes-induced changes of hepatic Cyp2c11 gene expression was identified in ZDF rats or Goto-Kakizaki rats, two other established type 2 diabetes rat models [42,43].

Unfortunately, diabetes-related differences between female obese and lean controls or between male and female obese ZSF1 rats are so far rarely reported in the literature or investigated. Dominguez and his colleagues showed that obese females are not diabetic at 16 weeks of age [17]. The reason for the differences between obese and lean female ZSF1 rats remains to be clarified.

The female-specifically expressed gene Lifr in ZSF1 liver is an integral component of the glycoprotein-LIFR signaling complex, acts as signal receptor for cytokine leukemia inhibitory factor and other cytokines, and is an estrogen-responsive gene [44]. Our result shows that hepatic Lifr mRNA level was significantly lower in the livers of obese compared to lean females. Similarly, decreased mRNA levels of Lifr were identified in the liver of the diabetic Nagoya–Shibata–Yasuda mouse, another model for type 2 diabetes [45].

In accordance with our results, higher hepatic expression of steroid 5α-reductase Srd5a1 was found in female than in male rats [13]. This female-specific enzyme in the liver is responsible for the metabolically activation of testosterone and able to inactivate corticosterone, a mineralocorticoid that exerts an anti-natriuretic action in rats [46,47]. However, our results showed also a weak decrease of hepatic Srd5a1 in obese compared with lean female ZSF1 rats. No significant diabetes-induced change of Srd5a1 was detected in ZDF rats [43]. The reason for the discrepancy between our results and published data remains to be clarified.

Glucokinase (Gck) is a key enzyme of glycolysis, responsible for the first enzymatic step of glycolysis, the phosphorylation of glucose in hepatic cells [48]. Gck mRNA does not seem to be the target of diabetes in the liver, because Gck levels were not changed in adult, diabetic ZDF rats (14, 20, and 26 week of age) compared to their non-diabetic controls [48]. Our data in the livers of 16 weeks old ZSF1 rats confirm this finding. In addition, our results are in line with the observation that hepatic Gck mRNA has higher expression in adult female compared to male rats [49].

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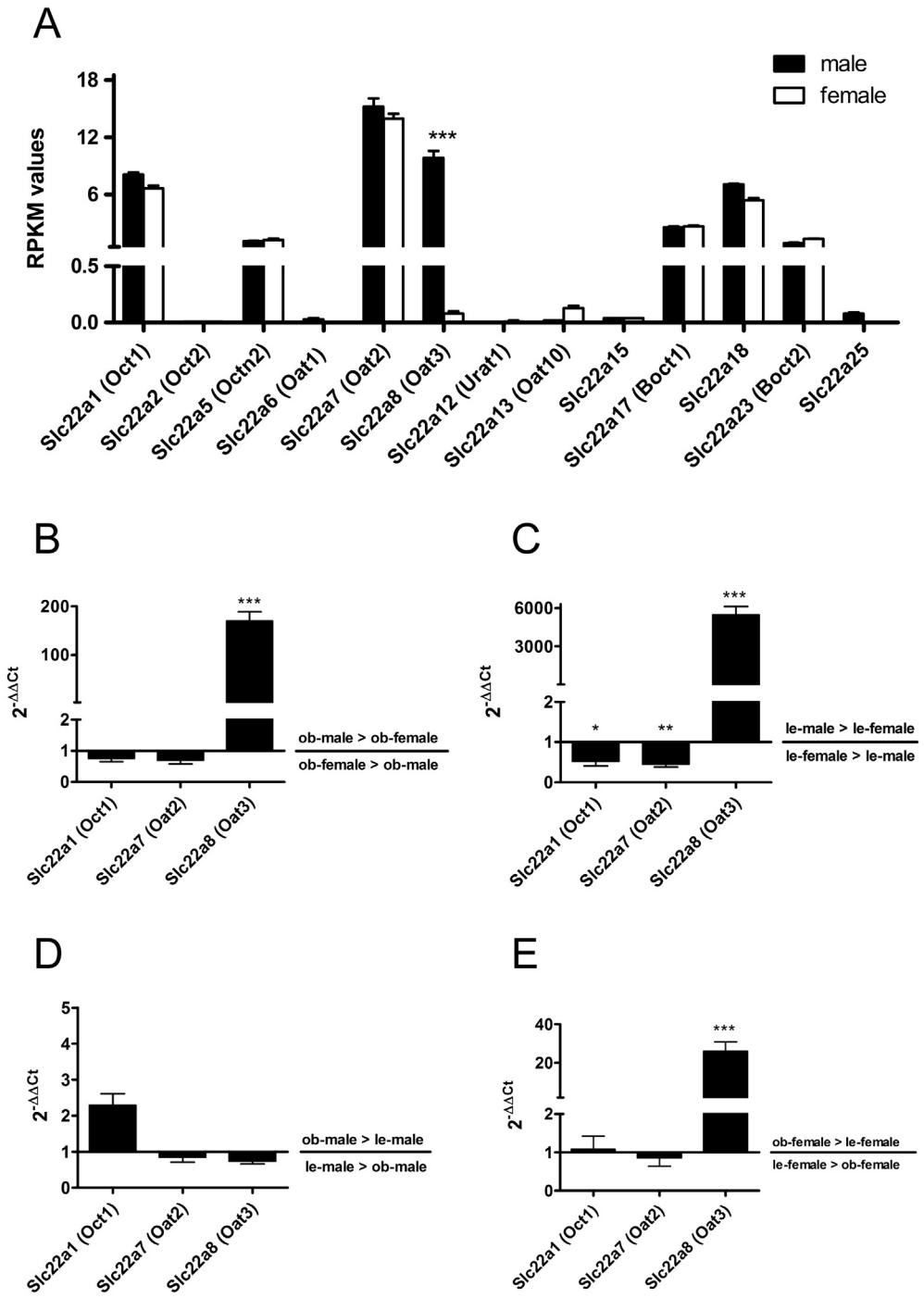


**Fig. 4.** Expression of members of Slc22a gene family in the liver of ZSF1 rats. A) The RPKM (reads per kilobase per million mapped reads) values of Slc22a genes detected in NGS analysis were compared between male and female livers of obese (ob) ZSF1 rats. $n_{male} = 6$, $n_{female} = 8$. ***, $p < 0.001$. B–E) The expression of Slc22a1, Slc22a7, and Slc22a8 was verified by TaqMan® real-time in livers of obese and lean (le) ZSF1 rats. Sex-differently expression of candidate genes was investigated in obese (B) and lean ZSF1 rats (C). Differences of candidate genes between lean and obese animals were analyzed in male (D) and female (E) ZSF1 rats. The results were presented as mean ± SEM. $n_{le\text{-}male} = 6$, $n_{ob\text{-}male} = 6$, $n_{le\text{-}female} = 8$, and $n_{ob\text{-}female} = 8$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The mRNA level of the key enzyme of hepatic lipogenesis, Gpam, was female-specifically expressed in lean and obese ZSF1 rats in accordance with published results [13]. Hepatic Gpam mRNA content was not different between lean and obese female ZSF1 rats, but was clearly enhanced in the livers of obese male ZSF1 rats compared to their lean control animals. In contrast, mitochondrial Gpam enzyme activity was described to be negatively regulated by diabetes [50,51]. However, further investigations of diabetes-mediated effects on hepatic Gpam expression are necessary.

All detected members of Slc22a gene family in the livers of obese ZSF1 rats were separately analyzed. The transport proteins, Oats and Octs encoded by Slc22a genes are known or were postulated to be involved in the uptake of drugs from the blood, based on their in vitro transporter-drug interaction [30]. The role of Oct1, Oat2, and Oat3 in hepatic drug handling was previously investigated and discussed [30,52,53]. Only hepatic Oat3 mRNA was significantly sex-specifically expressed in obese ZSF1 rats. The result of NGS analysis for mRNAs of Oct1, Oat2 and Oat3 was confirmed using real-time PCR analysis.

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In accordance with published data [54], male-biased Oat3 mRNA was also found in the livers of non-diabetic (lean) ZSF1 controls. In a study of age- and sex-dependent effects of hepatic mRNA levels, Oat3 was classified as an protein of phase 3 xenobiotic metabolism and identified to be male-specific in livers of adult rats till 52 weeks of age [32]. The previously published interactions of anti-diabetic drugs and drugs from several other drug groups, and the confirmed sex-specific Oat3 expression in rat liver suggest that the role of Oat3 should be considered in drug development and pre-clinical testing. The consideration of sex-specific Oat3 is important, because human OAT3 interacts with numerous hepatically secreted or metabolized drugs and the sex-difference of human OAT3 remains to be clarified.

## 5. Conclusion

In this study, the sex-dependence of gene expression in obese ZSF1 rats, a rat model for type 2 diabetes which reflects the human disease, was investigated for the first time. In the livers of obese ZSF1 rats, sex-differences were identified for mRNA content of enzymes and transporters involved in GO pathways “response to chemical stimulus”, “lipid metabolism”, and “response to organic substance”. Sex- and diabetes-dependent changes of candidate genes, e.g. Gstm, Hsd11b1, Cyp4a2, in livers of ZSF1 rats are in good agreement with data from published studies performed in other rodent type 2 diabetes models and are clearly distinct from type 1 diabetes models. The known sex-dependent mRNA level of drug transporter Oat3 healthy rats was for the first time identified in livers of diabetic animals. Our results suggest the necessity to use male and female rats in pre-clinical drug development and testing.

## Conflict of interests

The authors have declared that no competing interests exist.

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# Male-Dominant Activation of Rat Renal Organic Anion Transporter 1 (Oat1) and 3 (Oat3) Expression by Transcription Factor BCL6

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## Abstract

***Background:*** Organic anion transporters 1 (Oat1) and 3 (Oat3) mediate the transport of organic anions, including frequently prescribed drugs, across cell membranes in kidney proximal tubule cells. In rats, these transporters are known to be male-dominant and testosterone-dependently expressed. The molecular mechanisms that are involved in the sex-dependent expression are unknown. Our aim was to identify genes that show a sex-dependent expression and could be involved in male-dominant regulation of Oat1 and Oat3.

***Methodology/Principal Findings:*** Promoter activities of Oat1 and Oat3 were analyzed using luciferase assays. Expression profiling was done using a SurePrint G3 rat GE 8×60K microarray. RNA was isolated from renal cortical slices of four adult rats per sex. To filter the achieved microarray data for genes expressed in proximal tubule cells, transcription database alignment was carried out. We demonstrate that predicted androgen response elements in the promoters of Oat1 and Oat3 are not functional when the promoters were expressed in OK cells. Using microarray analyses we analyzed 17,406 different genes. Out of these genes, 56 exhibit a sex-dependent expression in rat proximal tubule cells. As genes potentially involved in the regulation of Oat1 and Oat3 expression, we identified, amongst others, the male-dominant hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1), B-cell CLL/lymphoma 6 (BCL6), and polymerase (RNA) III (DNA directed) polypeptide G (Polr3g). Moreover, our results revealed that the transcription factor BCL6 activates promoter constructs of Oat1 and Oat3.

***Conclusion:*** The results indicate that the male-dominant expression of both transporters, Oat1 and Oat3, is possibly not directly regulated by the classical androgen receptor mediated transcriptional pathway but appears to be regulated by the transcription factor BCL6.

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## Introduction

There are several known differences in the mode of drug action between men and women, however the effect of sex-dependent dosages is just beginning to be explored [1]. The reasons for different drug actions are not entirely clear. Sex differences are found in the absorption, distribution, metabolism and elimination of drugs [1–3]. The liver and kidneys are responsible for drug elimination. Hepatocytes in the liver and proximal tubule cells in the kidneys express several transport proteins such as members of the ATP-binding cassette (ABC) transporters, e.g. multidrug resistance proteins (MRPs), or members of the solute carrier (SLC) family 22A, e.g. organic anion transporters (OATs) and organic cation transporters (OCTs), that are involved in the excretion of endogenous and exogenous substrates [4–6]. Sex differences in the transport of substrates have been shown and could contribute to interindividual variations in drug efficacy [7]. In 2010, "The International Transporter Consortium" published a recommendation as to which of the transport proteins clinically important in drug absorption and elimination have to be analyzed in drug development [8]. Amongst others, the human organic anion transporter 1 (OAT1) and 3 (OAT3) were mentioned as clinically relevant transporters in the kidneys [8]. Immunohistochemical experiments revealed that OAT1 and OAT3 are expressed at the basolateral membrane of proximal tubule kidney cells in humans [9,10] and rats [11,12]. OAT1 and OAT3 are responsible for the uptake of their substrates from the blood into the cells and interact with several drugs, e.g. analgesics, antibiotics, and antivirals [5,13–15]. For example, the often consumed analgesic ibuprofen is transported by human OAT1 as well as by OAT3 [16] and causes more adverse drug reactions (ADRs) in women than in men [17]. For a multitude of different drugs, e.g. analgesics, ACE-inhibitors, and antihistamines, women have a 1.6-fold higher risk of getting an ADR compared to men [18]. This phenomenon could be partially due to sex-dependent differences in the expression of transporters like OAT1 and OAT3. A lower expression of OAT1 or OAT3 may decrease drug excretion. Rats that are often used in preclinical trials exhibit sex-dependent

differences in Oat1 and Oat3, with a higher expression in males compared to females [19]. In rats, expression of Oat1 and Oat3 is increased by testosterone and decreased by estradiol [19]. The molecular mechanisms of sex-dependent expression of Oat1 and Oat3 are still unclear.

The purpose of this study was to identify genes that demonstrate a sex-dependent expression in rat proximal tubule cells and could be related to male-dominant expression of Oat1 and Oat3. We identified as a promising candidate gene BCL6 that shows a male-dominant expression, and might be involved in the regulation of Oat1 and Oat3.

## Materials and Methods

### Cloning of the 5′-regulatory regions (promoter) of Oat1- and Oat3-genes into pGL3-Enhancer

The transcriptional start site of Oat1 and Oat3 was identified by the alignment of genomic and mRNA sequences from each gene (GenBank accession numbers: Oat1 genomic, NW_047563.2; mRNA, NM_017224.2; Oat3 genomic, NW_047563.2; mRNA, NM_031332.1). Based on rat genomic sequence, promoter fragments of varying lengths were amplified by polymerase chain reaction (PCR) using rat genomic DNA (BioCat, Germany) as templates. Primers shown in Table 1 were designed according to sequences in the 5′-regulatory region. Primer numbering indicated their position relative to the transcriptional start site (designated as +1). Amplified PCR products were digested with their corresponding restriction enzymes (New England BioLabs GmbH, Germany) and ligated into the pGL3-Enhancer vector (Promega, Germany) (Table 1). The full length promoter construct of Oat1 is referred to as −2252/+113, the next shorter one as −1666/+113, and the shortest one as −1226/+113. The promoter constructs for Oat3 are designated as −2567/+12 for the full length one, −752/+12 for the next shorter one, and −444/+12 for the shortest promoter construct.

### *In silico* analyses of putative transcription factor binding sites

For determination of predicted transcription factor binding sites, based on genomic sequence, 3 kilobase (kb) upstream of the transcription start site of Oat1 (NW_047563.2) and Oat3 (NW_047563.2) were investigated. The analyses were performed using MatInspector (http://www.genomatix.de/).

### Cell culture, transfection, and luciferase assay

Opossum kidney (OK) cells were purchased from LGC Standards (Germany; European distributor for "ATCC cultures and bioproducts"; ATCC® number: CRL-1840™). OK cells were cultured in Quantum 286 medium (complete medium for epithelial cells with L-glutamine, PAA Laboratories GmbH, Austria) with 100 units/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories GmbH, Austria) at 37°C in a 5% $CO_2$-95% air humidified incubator. Cells ($0.75\times10^5$ cells/well) were seeded into 24-well culture plates in their appropriate culture medium without antibiotics, incubated up to 24 h and then transiently transfected using Lipofectamine™ 2000 (Invitrogen, Germany) according to the manufacturer's recommendations. OK cells were transfected at a confluence of approximately 70% with 0.5 µg promoter construct (Oat1, Oat3) or pGL3-Enhancer (Promega, Germany), 0.5 µg rPb-Luc, the minimal promoter of rat probasin cloned in pGL3-Basic (kind gift from Silke Kaulfuss, Institute of Human Genetics, University of Göttingen, and described in [20]) or

**Table 1.** Cloning of different promoter constructs of Oat1 and Oat3.

| Position | Sequence | Enzyme | Backbone vector |
|---|---|---|---|
| **Oat1 (−1226/+113)** | | | |
| −1226 to −1207 | 5′-*gg***GCTAGC**AGTGAGATGACAGGCAAAGG-3′ | NheI/XhoI | pGL3-Enhancer |
| +94 to +113 | 5′-*gg***CTCGAG**ATGGTGACCTGGATCAACTG-3′ | | |
| **Oat1 (−1666/+113)** | | | |
| −1666 to −1647 | 5′-*gg***GCTAGC**CAGAGTGAGTTCCAGGACAG-3′ | NheI/NdeI | Oat1 (−1226/+113) |
| −1131 to −1111 | 5′-*gggggg*GCATGGAGGTCTGAGGATAAC-3′ | | |
| **Oat1 (−2252/+113)** | | | |
| −2252 to −2232 | 5′-*gg***GCTAGC**CTGCCCAAGATGATTTTGAAC-3′ | NheI/BbvCI | Oat1 (−1666/+113) |
| −1545 to −1526 | 5′-*gcgcg*ATTGGAGCTGAGGACTGAAC-3′ | | |
| **Oat3 (−444/+12)** | | | |
| −444 to −425 | 5′-*gg***GAGCTC**CCCTAACATTCTCCACGAAC-3′ | SacI/XhoI | pGL3-Enhancer |
| −7 to +12 | 5′-*gg***CTCGAG**AGGACAGCTCAGCTCTAAC-3′ | | |
| **Oat3 (−752/+12)** | | | |
| −752 to −732 | 5′-*gg***GAGCTC**GGAGAACAGACAACCACATAC-3′ | SacI/XhoI | pGL3-Enhancer |
| −7 to +12 | 5′-*gg***CTCGAG**AGGACAGCTCAGCTCTAAC-3′ | | |
| **Oat3 (−2567/+12)** | | | |
| −1823 to −1802 | 5′-*ggg***GAGCTC**GGCCAACTAGGAACTAGAAAAG-3′ | SacI/AvrII | Oat3 (−752/+12) |
| −685 to −665 | 5′-*gcggcggg*CAAGCCAACACAAACTGAATC-3′ | | |
| −2631 to −2613 | 5′-TGAGATGAGGACAACGGAC-3′ | SacI/PstI | Oat3 (−1823/+12) |
| −1702 to −1681 | 5′-TTAAATTATACTCCAAGGCCGC-3′ | | |

Oat: organic anion transporter; bold nucleotides: artificial restriction sites (not included in numbering); small italic nucleotides: adjustment of primer melting temperature (not included in numbering).

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pGL3-Basic (Promega, Germany), 0.5 μg of the B-cell CLL/lymphoma 6 (BCL6) expression vector pcDNA3-BCL6 (kind gift from Giovanna Roncador, Monoclonal Antibodies Unit Centro Nacional de Investigaciones Oncológicas, Spain, and described in [21]) or 0.5 μg empty vector pcDNA3, 0.1 μg of the rat androgen receptor (rAR) expression vector pSG5-rAR (kind gift from Olli A. Jänne, Institute of Biomedicine, University of Helsinki, Finland, and described in [22]) or 0.1 μg empty vector pSG5 and 25 ng pRL-TK (*Renilla reniformis* vector, Promega, Germany) as indicated in the figures. Five hours after transfection of OK cells, medium was changed to complete medium with antibiotics in case of BCL6-transfection. For rAR-transfection medium was supplemented with 100 nM testosterone (Fluka, Germany) dissolved in dimethyl sulfoxide (DMSO) (AppliChem, Germany), or control 0.0003% DMSO, in the absence of antibiotics. Incubations were stopped after 43 h and firefly and *Renilla* luciferase activity were determined using the Dual-Luciferase® Reporter Assay System (Promega, Germany). The luminescences were measured using Mithras LB940 luminometer (Berthold, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are presented as the fold increase over pGL3-Basic or pGL3-Enhancer activity.

## Immunofluorescence analysis of BCL6- and rAR-expression

OK cells were seeded ($0.75\times10^5$ cells/well) on poly-D-lysine (Sigma Aldrich, Germany) coated cover slips into 24-well culture plates. Cells were transiently transfected with either 0.1 μg pSG5-rAR or control pSG5 for rAR-expression or with 0.5 μg pcDNA3-BCL6 or control pcDNA3 for BCL6-expression as described above. 43 h after transfection cells were fixed in 3.7% paraformaldehyde (Merck, Germany) in phosphate buffered saline (PBS) (Applichem, Germany) for 8 min at room temperature (RT), and subsequently permeabilized for 5 min at RT in a buffer consisting of 50 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.4; 0.5 mM NaCl, 0.3% Triton X-100 (Carl Roth, Germany). For staining, antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA) (Carl Roth, Germany). Cells were first incubated for 2 h at RT with 0.5 μg/ml rabbit anti-rat AR polyclonal antibody (Santa Cruz, USA) or 0.5 μg/ml mouse anti-human BCL6 monoclonal antibody (Santa Cruz, USA), blocked with 1% BSA for 15 min at RT, incubated for 1 h at RT with 8 μg/ml Alexa Fluor® 488 labeled goat anti-rabbit IgG (H+L) (Invitrogen, Germany) or 8 μg/ml Alexa Fluor® 488 labeled goat anti-mouse IgG (H+L) (Invitrogen, Germany), and finally incubated for 5 min at RT with 300 nM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen, Germany). Cells were washed with PBS and investigated by fluorescence microscopy. For evaluation of transfection efficiency, cells were investigated under a 20× magnification and five pictures across one cover slip were made. Using Image J (version 1.44, National Institutes of Health, USA), DAPI (blue) stained cells were counted and set as 100%. BCL6- and rAR-positive cells, stained in green, were further counted and their ratio to DAPI stained cells was calculated. The mean of all five pictures was estimated and is referred as transfection efficiency in %.

## Ethic statement

N/A

## Animals

RCCHan™:WIST rats were obtained from Harlan Laboratories (Venray, Netherlands). Animals were kept in the animal facility of the University Medical Center Göttingen under conventional housing conditions (22°C, 55% humidity, and 12 h day/night cycle) given free access to water and rat chow. Acclimatization period was at least 14 days. Four male and four female rats were anesthetized with $CO_2$ and euthanized by cervical dislocation. The kidneys were extracted post mortem in accordance with the guidelines of the "German Animal Welfare Act" (German: Tierschutzgesetz; §4 Abs. 3). We did not need formal approval, because the study did not involve any treatments or harmful procedures. The experiments were notified by animal welfare office of University Medical Center Göttingen, Germany.

## Tissue preparation and RNA isolation

The kidneys were extracted directly post mortem, washed in cold PBS and cooled on ice. After removal of the kidneys capsules, cortical slices were prepared and stored overnight in RNA*later*® (Applied Biosystems, Germany) at 4°C. Total RNA was isolated from kidney cortical slices using RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations.

Quality and quantity of extracted RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, USA) following the manufacturer's protocol. RNAs with a RNA integrity number (RIN) >9 were used for further experiments.

## Microarray procedure and data analyses

For microarray experiments, total RNA from four male and four female rat cortical kidney slices, each slice as a separate sample, were analyzed. Microarray preparation was performed as described previously by Opitz *et al.* [23] with the following exceptions: 200 ng of total RNA as starting material, the "Low Input Quick Amp Labeling Kit, one-color" (Agilent Technologies, USA), the SurePrint G3 Rat GE 8×60K Kit (Agilent Technologies, USA), and Agilent DNA microarray scanner (G2505C) at 3 micron resolution were used.

Data analyses were also performed as described previously [23] with the exception that Agilent's Feature (FE) software version 10.7.3.1 was used. All microarray data were generated conforming to the Minimum Information About a Microarray Experiment (MIAME) guidelines and have been submitted to the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/ (accession number: GSE34565). For evaluation of sex-different expression profiles four adjustments were made: 1) $p$-value$<0.05$, 2) log2 fold-change (FC)$\geq1$ (higher expressed in male rats) and log2 fold-change (FC)$\leq-1$ (higher expressed in female rats), 3) false discovery rate (FDR) <5%, and 4) duplicated genes were removed and the ones with the lower log2 fold-change were kept. To identify genes that are expressed in the rat proximal tubules candidate genes were aligned with the "Rat Proximal Tubule Transcriptome Database" (http://dir.nhlbi.nih.gov/papers/lkem/pttr/). Volcano plot for sex-dependent gene expression was performed by using plot function in "R" (http://cran.r-project.org/).

## TaqMan® real-time polymerase chain reaction

For gene expression analysis, RNA of the four male and four female rat cortical kidney slices were reverse transcribed using oligo dT-primer (Eurofins MWG Operon, Germany) and Superscript™ II Reverse Transcriptase (Invitrogen, Germany). The detection of genes of interest was realized by TaqMan® Gene Expression Assays (Applied Biosystems, USA). As endogenous control, mRNA expression of housekeeping gene beta-actin (β-actin) or hypoxanthine phosphoribosyltransferase 1 (Hprt1) was

monitored. Following TaqMan® Gene Expression Assays were used: β-actin, Rn00667869_m1; Hprt1, Rn01527840_m1; hepatocyte nuclear factor 1α (Hnf1α), Rn00562020_m1; hepatocyte nuclear factor 1β (Hnf1β), Rn00447453_m1; hepatocyte nuclear factor 4α (Hnf4α), Rn00573309_m1; androgen receptor (rAR), Rn00560747_m1; Oat1, Rn00568143_m1; Oat3, Rn00580082_m1; polymerase (RNA) III (DNA directed) polypeptide G (Polr3g), Rn01494192_m1; hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1), Rn00563388_g1; B-cell CLL/lymphoma 6 (BCL6), Rn01404339_m1. TaqMan® real-time PCR analysis was performed in a Mx3000P$^{TM}$ real-time PCR cycler (Agilent Technologies, USA) with the following parameters: 2 min heating step at 50°C, 10 min hot start at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amplification efficiency of all used TaqMan® Gene Expression Assays was 100% (+/−10%), indicating that during PCR amplification the starting material was amplified by a factor of two during every cycle. Relative expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method [24], where ΔΔCt is the difference between the selected ΔCt value of a sample and the ΔCt of a control sample. Evaluation of $2^{-\Delta\Delta Ct}$ for the samples indicates the fold change in expression relative to the control.

### Data analyses not related to microarray experiments

Data in the figures are presented as mean ± S.E.M. of either three or four different experiments. Statistical analysis was performed using the two-sided unpaired *t*-test (GraphPad Prism 4, version 4.03). Differences were designated as indicated in the figures.

## Results

### Effect of testosterone on Oat1- and Oat3-promoter constructs in OK cells

*In silico* analysis of 3 kb upstream of the transcription start site of Oat1 and Oat3 promoters were investigated using MatInspector. In both promoters two putative androgen response elements (AREs) were postulated (Table 2), however none of which showed a perfectly conserved binding site (consensus sequence: GGTACAnnnTGTTCT) [25,26]. The role of testosterone in the regulation of Oat1 and Oat3 promoter activity were investigated using luciferase assays as described in material and methods (Figure 1). Cellular localization of transfected rAR revealed an expression with an exclusive nuclear localization (Figure 1A). Transfection efficiency of rAR was 39.1%. The functional activity of rAR was confirmed by rat probasin promoter (rPb-Luc), used as positive control. rPb-Luc was significantly activated by 100 nM testosterone (Figure 1B). In the absence of rAR, rat probasin promoter was not enhanced by testosterone, demonstrating that OK cells do not express endogenous rAR (Figure 1B). The activation of rPb-Luc is regulated via two functional ARE (Table 2) [27]. No significant induction by 100 nM testosterone of Oat1- and as well as Oat3-promoter activities were observed (Figure 1C). The comparison of Oat1- and Oat3-promoter activity revealed a stronger basal promoter activity of Oat3 as compared to Oat1 (Figure 1C).

### Sex-dependent expression of Oat1 and Oat3 in Han Wistar rats

Based on TaqMan® real-time PCR we investigated the sex-dependent expression of Oat1 and Oat3 in cortical kidney slices from four male and four female rats. Since sex differences were investigated, two reference genes β-actin and Hprt were used. The mRNA levels of both reference genes were not altered under the experimental conditions (Figure 2A). TaqMan® real-time PCR revealed sex-dependent expression of Oat1 and Oat3 (Figure 2B). Thereby the sex-dependent difference of Oat1 between male and female was higher as compared to Oat3 (Figure 2B).

### Sex-dependent gene expression profiling

The gene expression profiles of cortical kidney slices from the four male and four female rats used previously, were analyzed using the SurePrint G3 Rat GE 8×60K Microarray Kit from Agilent Technologies. The volcano plot gives a first overview of gene expression between males and females (Figure 3). Out of 22,863 investigated probes, 572 exhibit significant [−log10(adjusted *p*-value)<0.05] sex-dependent expression (log2 fold-change≤−1 or ≥1) (Figure 3). 293 probes were higher expressed in females (log2 fold-change≤−1), and 281 probes were higher expressed in males (log2 fold-change≥1) (Figure 3). After exclusion of probes with a FDR >5% and probes without Gene ID, 160 genes were higher expressed in females and 175 genes higher in males (Table S1). The alignment with the "Rat Proximal Tubule Transcriptome Database" and the removal of duplicated genes revealed 56 genes with significant sex-dependent expression in proximal tubule cells (Table 3). Out of these 56 genes, 13 genes showed a higher expression in females whereas 43 genes showed a higher expression in males. Genes were classified into different groups according to their function included: twenty-one enzymes (18×M>F, 3×F>M), nine membrane proteins and receptors (7×M>F, 2×F>M), two transcription factors (1×M>F, 1×F>M), six transport proteins (4×M>F, 2×F>M), six signal transduction (4×M>F, 2×F>M) and twelve other genes (9×M>F, 3×F>M) (Table 3). TaqMan® real-time PCR was used to verify selected microarray results. Seven genes were chosen as a result of their possible involvement in the transcriptional regulation of the sex-dependently expressed Oat1 and Oat3: Hnf1α, Hnf1β, Hnf4α, rAR (GSE34565), Hsd17b1, BCL6, and Polr3g (bold letters in Table 3). All three validated hepatocyte nuclear factors (Hnf1α, Hnf1β, and Hnf4α) showed no sex-dependent expression (Figure 4), confirming the microarray results (GSE34565). Validation of rAR showed a significant male-dominant expression (Figure 4). This result did not coincide with that indicated by microarray where rAR showed no sex-dependent expression (GSE34565). The levels of Polr3g, Hsd17b1, and BCL6 were clearly higher in males as compared to females (Figure 4), confirming the microarray results (Table 3).

### *In silico* analyses of potential BCL6 binding sites in the Oat1- and Oat3-promoter region

In the analyzed Oat1-promoter there are five potential BCL6 binding sites postulated none of which was a perfectly conserved binding site (consensus sequence: TTCCT(A/C)GAA) [28] (Table 2). *In silico* analysis of the Oat3-promoter revealed six potential BCL6 binding sites, of which none did correlate completely with the consensus sequence (Table 2). The promoter construct Oat1 (−1266/+113) included two predicted BCL6 binding sites, the Oat1 (−1666/+113) construct included four predicted BCL6 binding sites and the Oat1 (−2252/+113) included four predicted BCL6 binding sites (Table 2).

The shortest promoter construct Oat3 (−444/+12) has two predicted BCL6 binding sites (Table 2). In the Oat3 promoter construct (−752/+12), three BCL6 responsive elements are located. The longest Oat3 promoter construct (−2567/+12) included six BCL6 binding sites (Table 2).

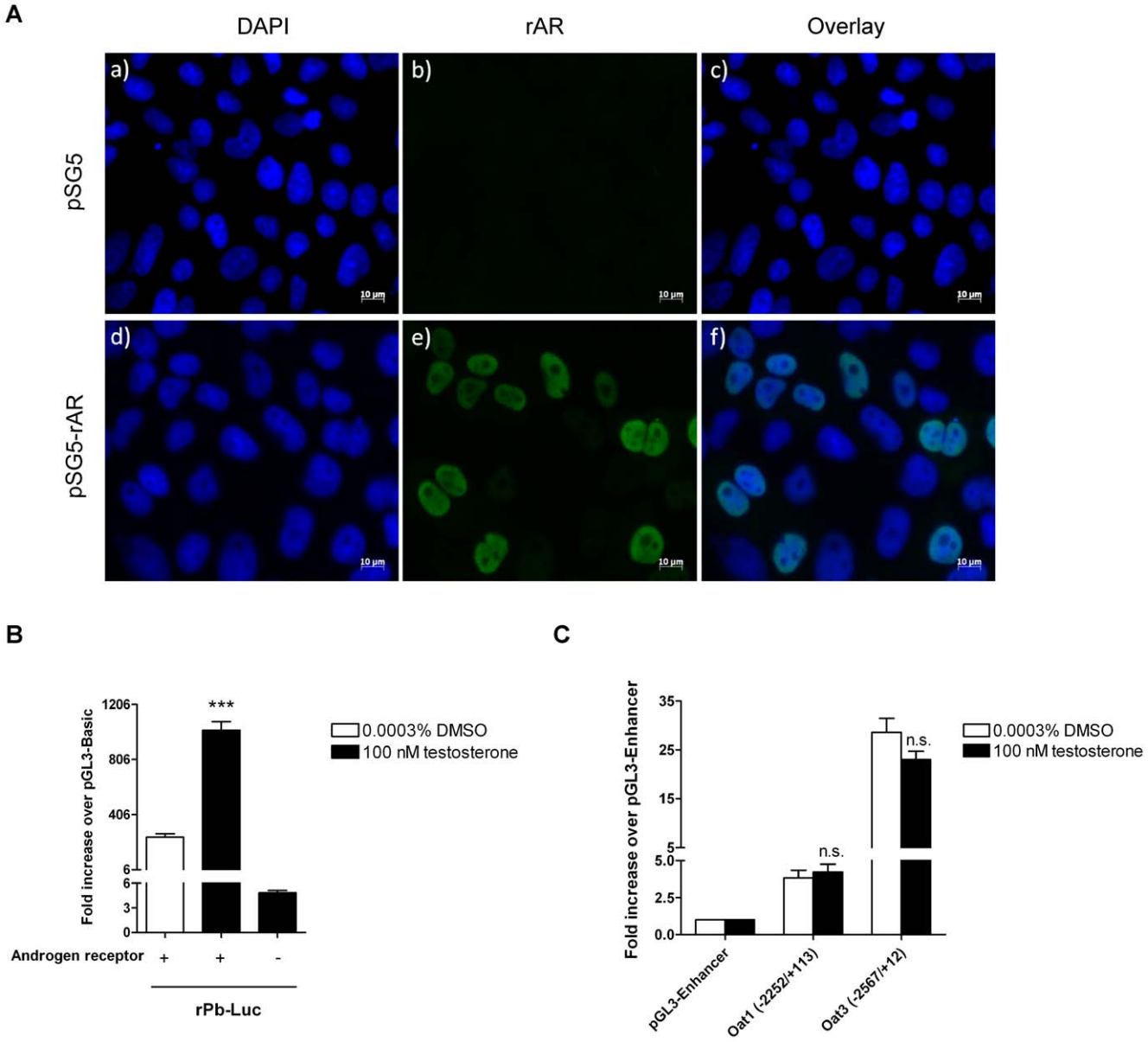


**Figure 1. Effect of testosterone on probasin, Oat1- and Oat3-promoter activity.** OK cells were either transfected with pSG5 or pSG5-rAR, and rAR expression and cellular localization were analyzed using immunofluorescence staining (green color rAR, blue color DAPI staining; excitation wavelength 488 nm and 365 nm) (1A). Promoter constructs of rat probasin, Oat1 and Oat3, and the expression vector for rAR were transiently transfected into OK cells (1B and 1C). Cells were cultured 43 h with either 100 nM testosterone (black bars) or with 0.0003% DMSO as control (white bars) (1B and 1C). Luciferase activity was measured and firefly luciferase was normalized to *Renilla* luciferase. Data are reported as the fold increase presented as mean ± S.E.M.; n = 4; n.s.: not significant; ***: $p<0.001$, significantly different from control (0.0003% DMSO).
doi:10.1371/journal.pone.0035556.g001

## Activation of Oat1- and Oat3-promoter by BCL6

As a positive control for transfection and expression in OK cells, BCL6 expression was investigated using immunofluorescence (Figure 5A). Characterization of BCL6 cellular localization revealed an exclusive nuclear localization (Figure 5A). Transfection efficiency of BCL6 was 23,3%. To test for a possible influence of BCL6 on the promoters of Oat1 and Oat3, OK cells were transfected with different promoter constructs, pcDNA3-BCL6 or empty pcDNA3 vector, and their luciferase activity were measured (Figure 5B and 5C). All three Oat1-promoter constructs showed a significant activation by BCL6 compared to the control (Figure 5B). The promoter constructs Oat3 (−444/+12) and (−752/+12) were activated by BCL6 (Figure 5C). The promoter activity of the longest Oat3 construct (−2567/+12) was not significantly altered but showed a positive trend for an activation by BCL6 (Figure 5C).

## Discussion

The aim of this study was to identify sex-dependently expressed genes in the rat proximal tubule cells that may be involved in the transcriptional regulation of Oat1 and Oat3. Since 1955 it is known that the uptake of the typical Oat1 substrate, *p*-aminohippurate (PAH), by rat renal cortical slices is higher in

**Table 2.** *In silico* analyses of Oat1 and Oat3 promoter.

| Promoter (3 kb) | Binding site | Sequence | Position |
|---|---|---|---|
| **Oat1** | ARE | 5′-gcaggactcagtgtttttgt-3′ | −2006 to −1988 |
| | ARE | 3′-gtagccctggctgtcctgg-5′ | −1655 to −1637 |
| | BCL6 | 5′-tctttcctggtaagaga-3′ | −2352 to −2336 |
| | BCL6 | 5′-gagttccaggacagcca-3′ | −1660 to −1644 |
| | BCL6 | 5′-taatttttagaaataaa-3′ | −1295 to −1279 |
| | BCL6 | 5′-ctgttcctacaagttat-3′ | −1143 to −1127 |
| | BCL6 | 5′-tatttcccagagcccag-3 | −492 to −476 |
| **Oat3** | ARE | 5′-ggctgcctacctgttctgt-3′ | −2863 to −2845 |
| | ARE | 5′-gttgggctctgtgtactct-3′ | −1923 to −1905 |
| | BCL6 | 3′-accttccgtgaaaaaca-5′ | −2055 to −2039 |
| | BCL6 | 5′-gggtccctggaaatagt-3′ | −1366 to −1350 |
| | BCL6 | 5′-gacttcatagaaaactc-3′ | −1174 to −1158 |
| | BCL6 | 5′-atactcatagaaataaa-3′ | −614 to −598 |
| | BCL6 | 3′-aggttcgtggagaatgt-5′ | −439 to −423 |
| | BCL6 | 5′-cccttcccagattctct-3′ | −279 to −263 |
| **Probasin minimal promoter (−426 to +28 bp)** | ARE a) | 5′-tgatagcatcttgttctta-3 | −243 to −225 |
| | ARE a) | 5′-taggttcttggagtacttt-3′ | −138 to −120 |
| **consensus sequence:** | ARE | ggtacannntgttct [25,26] | |
| **consensus sequence:** | BCL6 | ttcct(a/c)gaa [28] | |

Oat: organic anion transporter; ARE: androgen response element; BCL6: B-cell CLL/lymphoma 6;
n: any nucleotide, a) functional ARE published in [27].
doi:10.1371/journal.pone.0035556.t002

males compared to females [29]. Testosterone stimulated the uptake [30] possibly by having a positive influence on the expression of functional transporting proteins [31]. PAH is absorbed from the blood into proximal tubule cells by Oat1 and Oat3 in rat kidneys [32–36] and, in rat renal proximal tubule cells, a higher expression of Oat1 and Oat3 in males compared to females has been reported [19,37]. Moreover, it has been shown that Oat1 and Oat3 expression is increased by testosterone [19]. Two different AREs were predicted in the promoter of Oat1 and Oat3 in our *in silico* analysis. Surprisingly, testosterone showed no stimulating effect on the promoter activity of Oat1 and Oat3, although the minimal promoter of rat probasin (rPb-Luc) was activated under the same conditions. These results suggest that the predicted AREs in the promoter of Oat1 and Oat3 are not functional, and that these transport proteins are not directly activated by the classical androgen receptor mediated transcriptional pathway. Given the known sex-dependent expression of Oat1 and Oat3 in rat proximal tubule cells [19,37], we concluded that hitherto unknown factors are involved in the transcriptional male-dominant expression of Oat1 and Oat3.

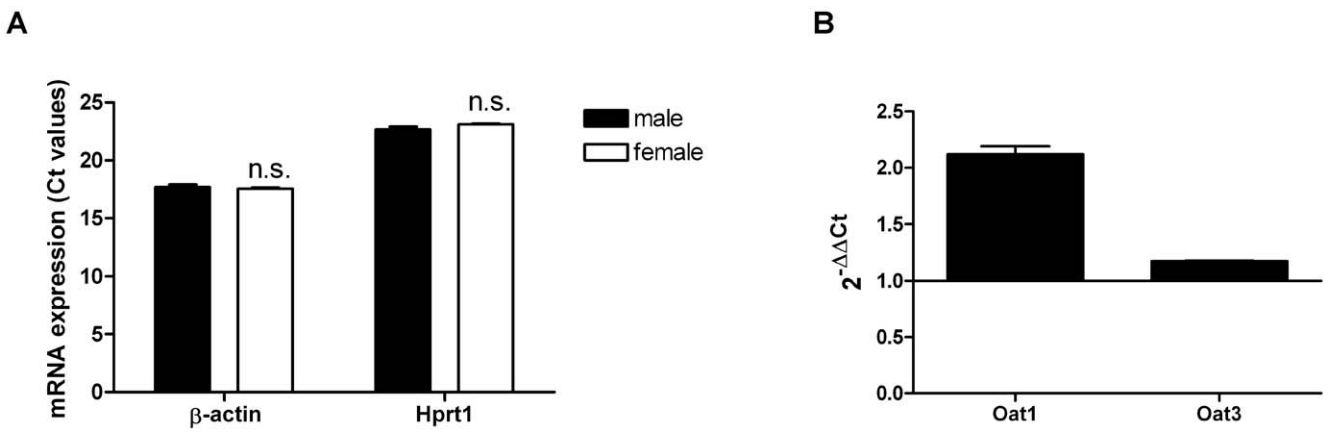


**Figure 2. Sex-dependent expression of Oat1 and Oat3 in rat cortical kidney slices.** Levels of β-actin, Hprt1, Oat1 and Oat3 were analyzed by TaqMan® real-time PCR in total RNA isolated from four male and four female cortical kidney slices. The mRNA expression of reference genes β-actin and Hprt1 were investigated by comparing their Ct values between male and female (2A). Levels of Oat1 and Oat3 were determined using $2^{-\Delta\Delta Ct}$ method, at which β-actin was the reference gene (2B). ΔΔCt values were calculated as ΔCt male - ΔCt female. $n_{male} = 4$; $n_{female} = 4$.
doi:10.1371/journal.pone.0035556.g002

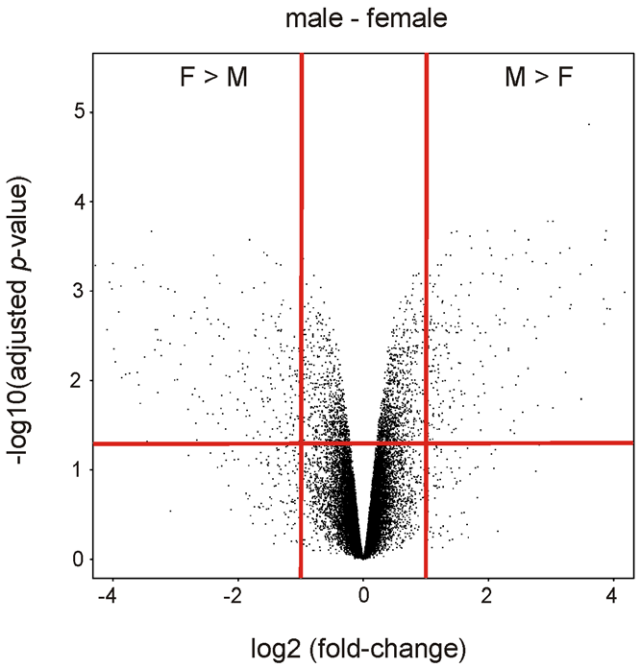


**Figure 3. Volcano plot of microarray analysis.** In this microarray a total of 22,863 probes were analyzed. On the y-axis the negative log10 of the adjusted *p*-value and on the x-axis the log2 of the fold-change is plotted. Each probe is represented as a dot. Low *p*-values (highly significant) are localized at the top of the plot. Probes that are expressed higher in females have a negative log2 fold-change appearing at the left side and probes that are expressed higher in males have a positive log2 fold-change appearing at the right side. The horizontal red line denotes the threshold for $p = 0.05$. The vertical red lines denote the two-fold thresholds.
doi:10.1371/journal.pone.0035556.g003

Using microarray analysis, we examined the expression of 17,406 different genes and found that only 56 genes were significantly sex-dependently expressed. This relatively small number of sex-dependently expressed genes is due to the fact that we only analyzed genes that are localized within the proximal tubule cells. The promising Oat1 and Oat3 regulators, Hnf1α, Hnf1β, and Hnf4α are expressed in the proximal tubule cells (http://dir.nhlbi.nih.gov/papers/lkem/pttr/), but revealed no sex-dependent expression in our microarray analysis and real-time PCR. For human OAT1-promoter the activation by HNF4α was demonstrated [38]. The promoters of mouse Oat1, human OAT1 and OAT3 are activated by HNF1α and HNF1β [39,40].

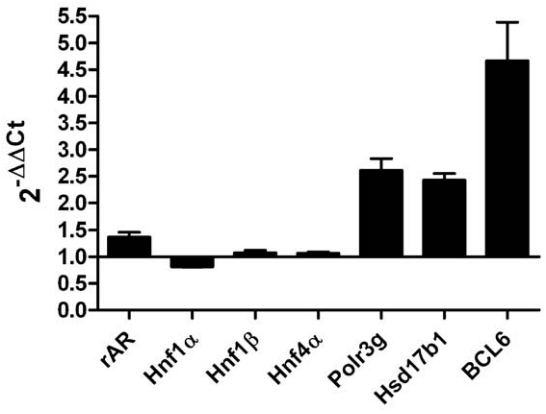


**Figure 4. Verification of microarray results using TaqMan® real-time PCR.** Gene expressions were verified by TaqMan® real-time PCR in total RNA isolated from four male and four female cortical kidney slices. Levels of all genes were determined using $2^{-\Delta\Delta Ct}$ method, at which β-actin was the reference gene. ΔΔCt values were calculated as ΔCt male - ΔCt female. $n_{male} = 4$; $n_{female} = 4$.
doi:10.1371/journal.pone.0035556.g004

The possible Oat1 and Oat3 regulator, rat androgen receptor (rAR) that is expressed in the proximal tubule cells (http://dir.nhlbi.nih.gov/papers/lkem/pttr/) showed no sex-dependent expression in the microarray, but demonstrated a significant male-dominant expression by real-time PCR. The decreased sensitivity of microarrays in comparison to real-time PCR may explain the different results. It is known that both real-time PCR and microarray analysis have inherent pitfalls that could influence the data obtained from each method, resulting in disagreement [41]. The false discovery rate (FDR) of rAR was about 24%, indicating a false positive microarray result that was verified by real-time PCR revealing a significant male-dominant expression. Despite the higher expression of rAR in the male kidneys, rAR is probably not a direct regulator of Oat1 and Oat3 promoters, as we could demonstrate in our study.

Out of the 56 sex-dependently expressed genes three genes that showed a male-dominant sex-dependent expression in the microarray, Polr3g, Hsd17b1, and BCL6 were selected for verification. The real-time PCR analysis clearly confirmed the microarray results for these three genes.

The first validated gene was Polr3g that is a subunit of the dissociable RNA polymerase (pol) III subcomplex [42]. Pol III transcribes especially short non-coding RNAs, for example tRNA, 5S RNA, 7SK RNA, U6 small nuclear RNA and H1 RNA that are involved in processing of pre-rRNA, mRNA and tRNA [42,43]. This subcomplex is responsible for the initiation of Pol III transcription [42]. This so far unknown male-dominant Polr3g expression could enhance the initiation of pol III transcription, leading possibly to a rather indirect effect on the expression of Oat1 and Oat3.

Hsd17b1 was the second validated gene and represents a promising candidate gene for sex-dependent Oat1 and Oat3 regulation. Members of the 17β-hydroxysteroid dehydrogenase (HSDs) family play an important role in the estrogen and androgen steroid biosynthesis [44–46]. Human HSD17b1 selectively converts estrone to estradiol [44]. The rat Hsd17b1 mediates the reduction of androstenedione to testosterone as efficiently as the analogous reduction from estrone to estradiol [47]. The male-dominant renal Hsd17b1 expression could be responsible for an increased testosterone concentration in proximal tubule cells. The increase of Oat1 and Oat3 protein expression by testosterone was published [19], but this increase is potentially not mediated by the classical androgen receptor mediated transcriptional pathway as demonstrated in our contribution. Possibly, the elevated testosterone concentration could activate a, till now unknown, transcription factor, which then activates Oat1 and Oat3 expression.

The third real-time PCR validated gene was the transcription factor BCL6, that was identified and characterized in B-cells [48]. BCL6 is necessary during embryonic development, plays a role in germinal center formation, and is essential in the immune response [28,48]. BCL6 has also been reported to be a proto-oncogen, whereby the intact coding sequence of BCL6 is often translocated, obtaining a new changed 5′ non-coding promoter region [28]. In many cases BCL6 acts as a transcriptional repressor due to its ability to recruit the known transcriptional repressors nuclear receptor corepressor (N-CoR), silencing mediator of retinoid and thyroid receptor (SMRT), and BCL6 interacting corepressor

**Table 3.** Sex-dependently expressed genes in rat proximal tubule cells.

| Symbol | Description | log2 FC (m- f) | P. Value (m-f) | FDR (m-f) |
|---|---|---|---|---|
| | **ENZYMES** | | | |
| Aldh1a1 | aldehyde dehydrogenase 1 family, member A1 | 1,01 | 3,85E-06 | 0,09% |
| Baat | bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) choloyltransferase)chcchcholoyltransferasecholoyltransferasecholoyltransferase) | −1,24 | 1,22E-03 | 2,40% |
| Cth | cystathionase (cystathionine gamma-lyase) | −1,01 | 1,24E-04 | 0,61% |
| Cyp2d4v1 | cytochrome P450, family 2, subfamily d, polypeptide 4 | 1,11 | 4,39E-06 | 0,10% |
| Cyp4a2 | cytochrome P450, family 4, subfamily a, polypeptide 2 | 3,03 | 9,25E-04 | 2,04% |
| Ddx19a | DEAD (Asp-Glu-Ala-Asp) box polypeptide 19a | 1,05 | 6,57E-05 | 0,43% |
| Dnm3 | dynamin 3 | 2,64 | 2,16E-07 | 0,03% |
| Eepd1 | endonuclease/exonuclease/phosphatase family domain containing 1 | 1,02 | 2,67E-04 | 0,98% |
| Es22 | esterase 22 | 2,02 | 3,16E-07 | 0,03% |
| F3 | coagulation factor III (thromboplastin, tissue factor) | 1,10 | 2,80E-05 | 0,27% |
| Hao1 | hydroxyacid oxidase (glycolate oxidase) 1 | 1,88 | 1,24E-04 | 0,61% |
| Hmgcs2 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial) | 3,30 | 9,19E-06 | 0,16% |
| **Hsd17b1** | **hydroxysteroid (17-beta) dehydrogenase 1** | **1,52** | **1,93E-05** | **0,23%** |
| Oat | ornithine aminotransferase (gyrate atrophy) | −1,36 | 3,03E-05 | 0,28% |
| Pecr | peroxisomal trans-2-enoyl-CoA reductase | 1,15 | 9,75E-05 | 0,53% |
| Proc | protein C | 1,01 | 2,22E-07 | 0,03% |
| Ptgds | prostaglandin D2 synthase (brain) | 2,19 | 2,46E-06 | 0,08% |
| Rasa2 | RAS p21 protein activator 2 | 1,04 | 5,92E-05 | 0,41% |
| Rasl12 | RAS-like, family 12 | 2,18 | 2,12E-05 | 0,24% |
| Rdh2 | retinol dehydrogenase 2 | 1,56 | 4,49E-04 | 1,31% |
| Usp9x | ubiquitin specific peptidase 9, X-linked | 2,35 | 2,53E-03 | 3,60% |
| | **MEMBRANE PROTEINS/RECEPTORS** | | | |
| Asgr1 | asialoglycoprotein receptor 1 | 1,49 | 3,35E-05 | 0,29% |
| B4galt5 | UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5 | 1,00 | 1,23E-05 | 0,18% |
| Cd1d1 | CD1d1 molecule | −1,14 | 7,03E-04 | 1,74% |
| Kifc1 | kinesin family member C1 | 1,37 | 3,41E-05 | 0,29% |
| Prlr | prolactin receptor | 1,14 | 1,82E-04 | 0,78% |
| Sectm1b | secreted and transmembrane 1B | 1,21 | 5,43E-05 | 0,40% |
| Stra6 | stimulated by retinoic acid gene 6 | −1,16 | 9,07E-06 | 0,16% |
| Tmem144 | transmembrane protein 144 | 1,02 | 3,89E-05 | 0,32% |
| Trim59 | tripartite motif-containing 59 | 1,02 | 5,78E-06 | 0,12% |
| | **TRANSCRIPTION FACTORS** | | | |
| Arid3b | AT rich interactive domain 3B (Bright like) | −1,48 | 1,65E-04 | 0,73% |
| **Bcl6** | **B-cell CLL/lymphoma 6** | **2,18** | **1,61E-05** | **0,21%** |
| | **TRANSPORT PROTEINS** | | | |
| Abca7 | ATP-binding cassette, sub-family A (ABC1), member 7 | −1,35 | 1,40E-05 | 0,19% |
| Abcb1b | ATP-binding cassette, sub-family B (MDR/TAP), member 1B | −2,66 | 3,96E-03 | 4,65% |
| Ostalpha | organic solute transporter alpha | 1,13 | 3,06E-05 | 0,28% |
| Slc10a1 | solute carrier family 10 (sodium/bile acid cotransporter family), member 1 | 1,12 | 3,98E-04 | 1,21% |
| Slc30a2 | solute carrier family 30 (zinc transporter), member 2 | 1,26 | 7,12E-06 | 0,13% |
| Ust5r | integral membrane transport protein UST5r | 1,07 | 1,85E-04 | 0,79% |
| | **SIGNAL TRANSDUCTION** | | | |
| Cklf | chemokine-like factor | −1,03 | 6,79E-05 | 0,44% |
| Cks2 | CDC28 protein kinase regulatory subunit 2 | 1,06 | 3,81E-03 | 4,55% |
| Dock5 | dedicator of cytokinesis 5 | −1,58 | 1,75E-03 | 2,93% |
| Fgf13 | fibroblast growth factor 13 | 1,12 | 3,32E-06 | 0,09% |
| Gas2 | growth arrest-specific 2 | 1,40 | 5,73E-07 | 0,04% |

**Table 3.** Cont.

| Symbol | Description | log2 FC (m- f) | P. Value (m-f) | FDR (m-f) |
|---|---|---|---|---|
| Nrep | neuronal regeneration related protein | 1,16 | 2,97E-04 | 1,02% |
| | **OTHERS** | | | |
| Cldn1 | claudin 1 | −1,54 | 5,66E-07 | 0,04% |
| Ddit4l | DNA-damage-inducible transcript 4-like | −1,76 | 3,62E-03 | 4,40% |
| Espn | espin | 1,21 | 1,31E-03 | 2,51% |
| Klhl14 | kelch-like 14 (Drosophila) | −1,31 | 2,68E-05 | 0,27% |
| Mlph | melanophilin | 1,91 | 1,53E-06 | 0,06% |
| Obfc2a | oligonucleotide/oligosaccharide-binding fold containing 2A | 1,17 | 2,45E-05 | 0,26% |
| Picalm | phosphatidylinositol binding clathrin assembly protein | 1,14 | 2,96E-06 | 0,08% |
| **Polr3g** | **polymerase (RNA) III (DNA directed) polypeptide G (32 kD)** | **1,34** | **3,14E-05** | **0,28%** |
| Rpp25 | ribonuclease P 25 subunit (human) | 1,77 | 4,64E-05 | 0,35% |
| Rufy3 | RUN and FYVE domain containing 3 | 1,06 | 2,06E-03 | 3,20% |
| Spc25 | SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae) | 1,02 | 1,73E-03 | 2,92% |
| Tox | thymocyte selection-associated high mobility group box | 1,07 | 1,81E-05 | 0,22% |

FC: log2 fold-change (FC)≤−1 or ≥1; FDR: false discovery rate <5%; P. value: p<0.05; (m-f): male-female. Genes are grouped, and displayed within these groups in an alphabetical order. Genes shown with a negative log2 FC are higher expressed in females while genes with a positive log2 FC are higher expressed in males. The expression of all genes was aligned with (http://dir.nhlbi.nih.gov/papers/lkem/pttr/). Bold marked genes were selected for verification by TaqMan® real-time PCR.
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(BCoR) [28,48]. These three transcriptional repressors recruit histone deacetylases 1 and 2 [48]. BCL6 inhibits the expression of different genes like p21 and cyclin D2 [28,48]. Moreover it could repress the activity of NFκB by a direct protein-protein interaction between BCL6 and the subunits of NFκB [49]. BCL6 could also act as an activator leading to alteration in the growth cycle of the cell [50]. In our study, a considerably higher BCL6 expression in males compared to females was found. A comparable male-dominant expression of BCL6 has been demonstrated in the rat liver, whereas a possible role of BCL6 in the regulation of Oats has not been investigated [51]. Although all three validated genes are promising candidate genes for sex-dependent regulation of Oat1 and Oat3, we decided to focus on the possible involvement of BCL6 as a result of predicted BCL6 binding sites in the Oat1 and Oat3 promoter region.

BCL6 is known to bind to a specific DNA sequence with a core sequence of TTCCT(A/C)GAA [28]. Luciferase activity assays revealed a significant activation of Oat1 as well as Oat3 promoter by BCL6. All three Oat1 as well as Oat3 promoter constructs contain predicted BCL6 binding sites. Regarding the amplitude of fold induction, all Oat1 constructs were activated to a comparable amount, indicating that the first and/or second BCL6 binding site is may be the critical site for activation of Oat1 promoter constructs by BCL6. Oat3 promoter constructs were also activated to a comparable extent by BCL6 and possibly directly regulated via first and/or second BCL6 binding site. Our results implicate that male-dominant BCL6 is a promising transcription factor in the promoter activation of sex-dependently expressed Oat1 and Oat3. Interestingly, BCL6 is able to repress the activity of NFκB at the post-transcriptional level due to protein-protein interaction [49]. In renal ischemia the expression of rat renal Oat1 and Oat3 was decreased [52], and that of NFκB was increased [53]. Therefore it is possible that male-dominant BCL6 expression suppresses the activity of NFκB, leading indirectly to the higher expression of Oat1 and Oat3 in males. NFκB itself is expressed in the proximal tubule cells (http://dir.nhlbi.nih.gov/papers/lkem/pttr/) but exhibited no sex-dependent expression in our microarray analysis (GSE34565).

This study presents a first description of sex-dependent gene expression in the kidneys and especially in the proximal tubule cells (GSE34565). The sex differences were identified in several gene groups, e.g. enzymes, membrane proteins and receptors, transcription factors, transport proteins, genes involved in the signal transduction pathways, and others. Some candidate genes like Polr3g, Hsd17b1 and BCL6 might be directly or indirectly involved in sex-dependent expression of Oat1 and Oat3. Our results showed that BCL6 activated the promoters of Oat1 and Oat3 and constituted a promising candidate gene for their sex-dependent regulation. To further elucidate the involvement of BCL6 in the sex-dependent expression of Oat1 and Oat3, additional experiments are required.

## Supporting Information

**Table S1 Sex-dependently expressed genes in rat cortical kidney slices.** FC: log2 fold-change (FC)≤−1 or ≥1, FDR: false discovery rate, (m-f): male-female. Genes are displayed in an alphabetical order, starting with negative log2 FC representing genes higher expressed in females, followed by genes with a positive log2 FC representing higher expression in males. Probes with a FDR >5% and probes with no Gene ID are excluded in this list.
(XLS)

## Author Contributions

Conceived and designed the experiments: WW BCB GB MH. Performed the experiments: WW MH. Analyzed the data: WW MH. Contributed reagents/materials/analysis tools: BCB GB MH. Wrote the paper: WW BCB GB MH.

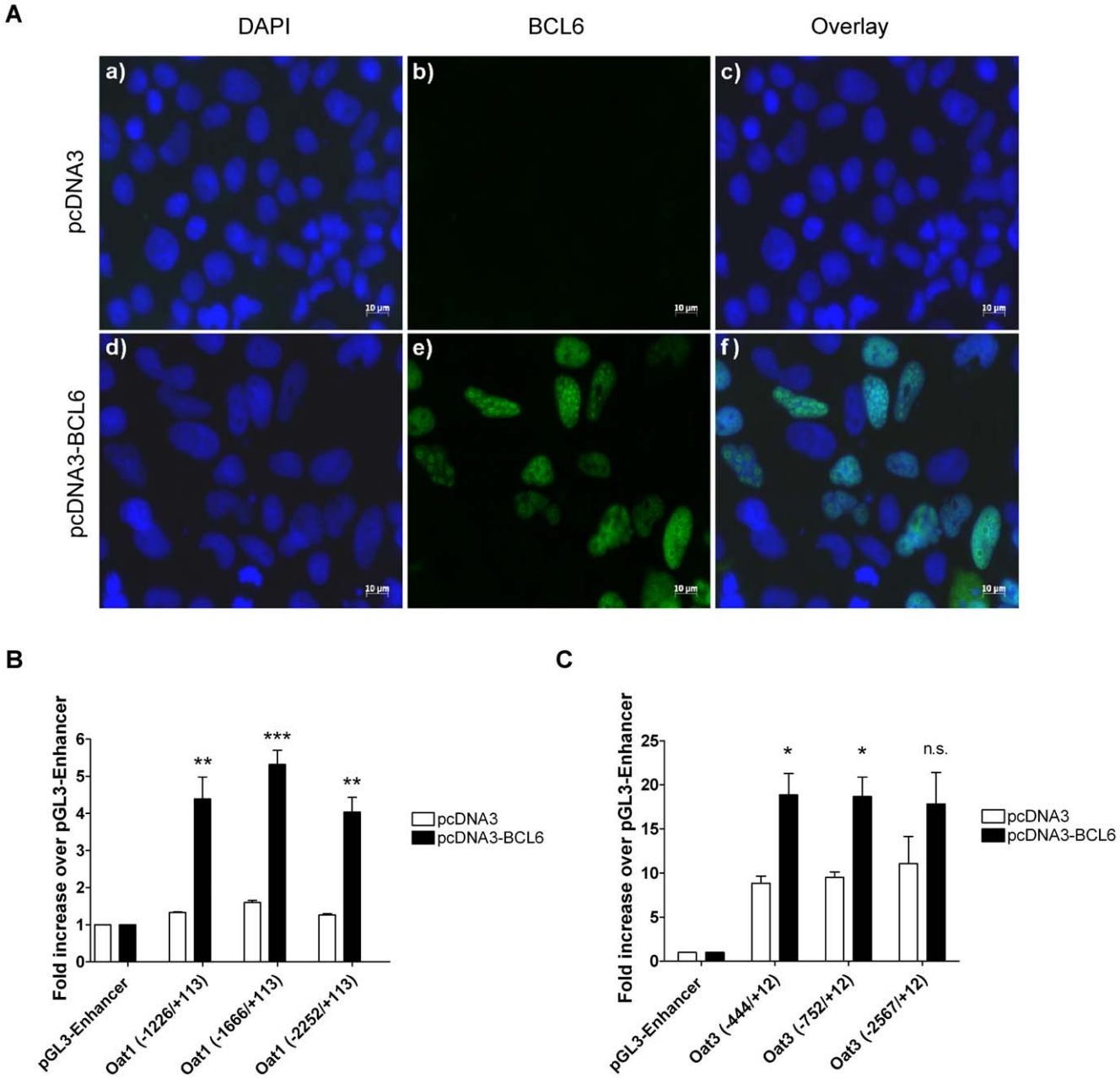


**Figure 5. Effects of BCL6 on Oat1- and Oat3-promoter activity.** OK cells were either transfected with pcDNA3 or pcDNA3-BCL6, and BCL6 expression and cellular localization were analyzed using immunofluorescence staining (green color BCL6, blue color DAPI staining; excitation wavelength 488 nm and 365 nm) (5A). Promoter constructs of Oat1 and Oat3, and the expression vector for BCL6 were transiently transfected into OK cells (5B and 5C). Luciferase activity was measured and firefly luciferase was normalized to *Renilla* luciferase. Data are reported as the fold increase over pGL3-Enhancer and presented as mean ± S.E.M.; n = 3; n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, significantly different from control (pcDNA3).

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# Transcriptional regulation of human organic anion transporter 1 by B-cell CLL/lymphoma 6

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**Wegner W, Burckhardt G, Henjakovic M.** Transcriptional regulation of human organic anion transporter 1 by B-cell CLL/lymphoma 6. *Am J Physiol Renal Physiol* 307: F1283–F1291, 2014. First published September 18, 2014; doi:10.1152/ajprenal.00426.2014.—The human organic anion transporter 1 (OAT1) is crucial for the excretion of organic anions in renal proximal tubular cells and has been classified as a clinically relevant transporter in the kidneys. Our previous study indicated that renal male-predominant expression of rat Oat1 and Oat3 appears to be regulated by transcription factor B-cell CLL/lymphoma 6 (BCL6). The aim of this study was to characterize the effect of BCL6 on human OAT1 promoter and on the transcription of OAT1 mediated by hepatocyte nuclear factor-1α (HNF-1α). Luciferase assays were carried out in opossum kidney (OK) cells transiently transfected with promoter constructs of OAT1, expression vectors for BCL6 and HNF-1α, and the empty control vectors. BCL6 and HNF-1α binding on OAT1 promoter was analyzed using electrophoretic mobility shift assay (EMSA). Protein expression of HNF-1α was investigated by Western blot analysis. Site-directed mutagenesis was used to introduce mutations into BCL6 and HNF-1α binding sites within the OAT1 promoter. BCL6 enhanced the promoter activity of OAT1 independently of predicted BCL6 binding sites but was dependent on HNF-1α response element and HNF-1α protein. Coexpression of BCL6 and HNF-1α induced an additive effect on OAT1 promoter activation compared with BCL6 or HNF-1α alone. BCL6 does not bind directly or indirectly to OAT1 promoter but increases the protein expression of HNF-1α and thereby indirectly enhances OAT1 gene transcription. BCL6 constitutes a promising candidate gene for the regulation of human OAT1 transcription and other renal and/or hepatic drug transporters that have been already shown to be activated by HNF-1α.

renal drug transporter; transcriptional regulation; organic anion transporter 1; B-cell CLL/lymphoma 6; hepatocyte nuclear factor-1

THE SECRETION OF NEGATIVELY CHARGED endogenous metabolites and exogenous substances, e.g., clinically important drugs, is dependent on specific transport proteins in the liver and the kidneys. Organic anion transporter 1 (OAT1), a member of the solute carrier 22A gene family, is localized in the basolateral membrane of proximal tubule cells in human kidneys and is responsible for the transport of numerous, structurally unrelated, organic anions from the blood into proximal tubule cells (6, 13, 20, 32). In several independent in vitro studies, interactions of OAT1 with different drug classes, e.g., nonsteroidal anti-inflammatory drugs, antiviral drugs, diuretics, and the antineoplastic drug methotrexate, have been shown (6, 20, 32). Moreover, OAT1-deficient mice exhibited significantly decreased secretion of different negatively charged metabolites, reduced excretion of the model OAT1 substrate para-aminohippuric acid, and decreased renal mercury toxicity (8, 30, 31). However, little is known so far about in vivo OAT1 drug interactions in patients. Among others, the renal OAT1 transporter was classified as clinically important in drug secretion and was recommended to be analyzed during drug development (10).

The promoter activity of human OAT1 has been shown to be regulated by hepatocyte nuclear factor (HNF)-1α homodimer, HNF-1α/HNF-1β heterodimer, DNA methylation, and HNF-4α (23, 27). Furthermore, HNF-1α-deficient mice showed a reduced renal Oat1 expression compared with wild type (19). A transcriptional regulatory effect for HNF-1α has been reported in liver, kidney, intestine, and pancreas, where the transcription factor itself is expressed endogenously (2).

Male-dominant and testosterone-dependent Oat1 expression has been observed in rat kidneys (5, 18). In our previous study, the male-dominant expressed transcription factor B-cell CLL/lymphoma 6 (BCL6) was identified as a possible sex-dependent regulator of Oat1, and enhancer of Oat1 promoter activity in rat kidneys (33). BCL6 is known to be expressed and active as transcription factor in germinal center B-cells, mature cardiac myocytes, testis, pulmonary epithelium, and hepatocytes (3, 14, 21, 29, 35).

The aim of this study was to investigate the effect of BCL6 on human OAT1 promoter. For this purpose we used opossum kidney (OK) cells, a cell model that exhibits characteristic cell morphology and cell polarity of proximal tubular cells (17). In OK cells, the uptake of the typical organic anion model substrate para-aminohippuric acid and the organic cation model substrate tetraethylammonium was described (24, 28), suggesting functional expression of OATs and organic cation transporters. In our previous studies, three other known kidney cell models (human embryonic kidney-293, NRK-52E, and HK-2) were tested in terms of OAT1 mRNA or protein but showed no endogenous expression. Promoter fragments of OAT1, all differing in their length and number of predicted BCL6 binding sites, were generated and cotransfected with BCL6 expression vector into OK cells. Promoter activity was elucidated by using luciferase assays. In addition, the activity of predicted BCL6 binding sites was tested in mutated OAT1 promoter constructs using luciferase assay and electrophoretic mobility shift assay (EMSA). In proteomic analyses of large B-cell lymphocytes, the protein-protein interaction between BCL6 and HNF-1α has already been identified (22). Accordingly, BCL6-dependent OAT1 promoter activities were tested in the presence and absence of HNF-1α. Additionally, the effects of BCL6 on HNF-1α binding to specific DNA motif and the changes in HNF-1α protein expression were analyzed.

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## MATERIALS AND METHODS

*Cloning of different human OAT1 5′-promoter sequences into pGL3-Enhancer vector.* The transcription start site of human OAT1, designated as +1, was mapped according to Ogasawara et al. (23). A series of different promoter fragments, all varying in their lengths of 5′ regulatory sequences, was generated by using polymerase chain reaction (PCR) with gene specific primers listed in Table 1 and human genomic DNA (Promega). Obtained PCR products were further digested with their corresponding restriction enzymes (New England Biolabs) and ligated into the luciferase reporter vector pGL3-Enhancer (Promega) (Table 1). For the OAT1, the following six promoter constructs were generated: OAT1 (−198 to +88), OAT1 (−342 to +88), OAT1 (−746 to +88), OAT1 (−1,419 to +88), OAT1 (−1,982 to +88), and OAT1 (−3,049 to +88).

*In silico analysis.* Computational analysis of putative transcription factor-binding sites was done using MatInspector software (http://www.genomatix.de/). Based on the genomic sequence (OAT1: NT_167190.1), three kb 5′ upstream of human OAT1 transcriptional start sites (+1) were investigated.

*Cell culture, transfection, and luciferase assay.* OK cells were obtained from LGC Standards (European distributor for "ATCC cultures and bioproducts"; ATCC no.: CRL-1840). OK cells were grown in culture medium Quantum 286 for epithelial cells supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories) at 37°C and a 5% $CO_2$ humidified atmosphere. For transient transfection, cells were seeded at a density of $1 \times 10^5$ cells/well in a 24-well culture plate and incubated for ~20 h in the above culture medium, but without antibiotics. The next day, cells were transiently transfected at a confluency of ~70%, with each well containing 25 ng of the Renilla reniformis vector pRL-TK (Promega), a 0.5-μg promoter construct or pGL3-Enhancer vector (Promega), 0.5 μg of the BCL6 expression vector pcDNA3-BCL6 [kind gift from Giovanna Roncador, Monoclonal Antibodies Unit Centro Nacional de Investigaciones Oncológicas, and described in Garcia et al., 2006 (9)], 0.5 μg of empty control vector pcDNA3, or 0.2 μg of the HNF1α expression vector pCMV-HNF-1α (SC300093, OriGene Technologies; distributor: AMS Biotechnology Europe), as indicated in the figures, by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's recommendation. Five hours posttransfection, the medium was changed to the complete culture medium Quantum 286, including antibiotics. After an incubation period of 43 h, cells were harvested and lysed, and the activities of firefly and Renilla luciferase were investigated using Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions. Luciferase activity was measured on a Mitras LB940 luminometer (Berthold), and firefly activity was normalized to Renilla activity. Data are presented as the fold increase of promoter construct over pGL3-Enhancer.

RAJI cells were obtained from DSMZ (ACC 319; German Collection of Microorganisms and Cell Cultures) and cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (PAA Laboratories).

*Immunofluorescence analysis of BCL6 and HNF-1α coexpression.* Coexpression and transfection efficiency of BCL6 and HNF-1α were investigated in OK cells, cultivated on polylysine-coated coverslips in 24-well plates, and transiently cotransfected with 0.5 μg of pcDNA3-BCL6 and 0.2 μg of pCMV-HNF-1α. Transfected OK cells were fixed in 3.7% paraformaldehyde (Merck) for 8 min and permeabilized in a buffer consisting of 50 mM $Na_2HPO_4$-$NaH_2PO_4$, pH 7.4, 0.5 mM NaCl, and 0.3% Triton X-100 (Carl Roth) at room temperature (RT) for 5 min. Subsequently, OK cells were incubated with 1 μg/ml mouse anti-human BCL6 monoclonal antibody (sc-365618; Santa Cruz Biotechnology) and 2 μg/ml polyclonal rabbit anti-human HNF-1α (sc-10791; Santa Cruz Biotechnology) for 2 h at RT, blocked with 1% BSA for 15 min at RT, and incubated for 1 h at RT with 8 μg/ml Alexa Fluor 546 donkey anti-mouse IgG (H + L; Invitrogen) and 8 μg/ml Alexa Fluor 488 goat anti-rabbit IgG (H + L; Invitrogen). Stained OK cells were mounted and their nuclei counterstained at the same time with Roti-Mount FluorCare DAPI (an aqueous mounting medium which contains nuclear stain 4′,6-diamidino-2-phenylindole dihydrochloride; Carl Roth). Transfection efficiencies of BCL6 and HNF-1α were analyzed using ImageJ (version 1.44; National Institutes of Health).

*Preparation of nuclear extracts and EMSA.* Nuclear extracts were prepared from OK cells and transiently transfected with 0.5 μg of pcDNA3-BCL6, 0.5 μg of pcDNA3, or 0.2 μg of pCMV-HNF-1α and from nontreated/nontransfected RAJI cells. Preparation was done by using a nuclear extraction kit (Panomics/Affimatrix) according to the manufacturer's instructions. For EMSA, sequence-specific 3′-biotin-labeled oligonucleotides with the native or mutated putative binding sites of BCL6 or HNF-1α were purchased

Table 1. *Generation of various OAT1 promoter constructs*

| Position | Sequence | Enzyme | Backbone Vector |
|---|---|---|---|
| OAT1 (−198 to +88) | | | |
| −198 to −177 | 5′-*gc***GCTAGC**GGTCCAATAGATCCCACTCTGG-3′ | *Nhe*I/*Xho*I | pGL3-Enhancer |
| +88 to +64 | 5′-*gg***CTCGAG**TCCCTTGCAGCTTCTCCTCACTTTG-3′ | | |
| OAT1 (−342 to +88) | | | |
| −342 to −319 | 5′-*gg***GCTAGC**TTTGACTGGGCACCCTGTAATTTC-3′ | *Nhe*I/*Xho*I | pGL3-Enhancer |
| +88 to +64 | 5′-*gg***CTCGAG**TCCCTTGCAGCTTCTCCTCACTTTG-3′ | | |
| OAT1 (−746 to +88) | | | |
| −746 to −723 | 5′-*gg***GCTAGC**GGGGAGGGGAGAAGAGAAGAAAAC-3′ | *Nhe*I/*Xho*I | pGL3-Enhancer |
| +88 to +64 | 5′-*gg***CTCGAG**TCCCTTGCAGCTTCTCCTCACTTTG-3′ | | |
| OAT1 (−1,419 to +88) | | | |
| −1,419 to −1,445 | 5′-*gg***GCTAGC**AAAATAACACTATGGCTGGGTGCG-3′ | *Nhe*I/*Xho*I | pGL3-Enhancer |
| +88 to +64 | 5′-*gg***CTCGAG**TCCCTTGCAGCTTCTCCTCACTTTG-3′ | | |
| OAT1 (−1,982 to +88) | | | |
| −1,982 to −1,959 | 5′-*gg***GCTAGC**ACCGCCAGTCAAAAATCACCGTTC-3′ | *Nhe*I/*Pst*I | OAT1 (−1,419/+88) |
| −1,177 to −1,152 | 5′-*gg*CCAGCCTGGAGTGTAGTGGCACAATC-3′ | | |
| OAT1 (−3,049 to +88) | | | |
| −3,049 to −3,026 | 5′-*gg***GCTAGC**CATCCCTTCAACAGCAAGACGGAG-3′ | *Nhe*I/*Nde*I | OAT1 (−1,982/+88) |
| −1,867 to −1,842 | 5′-*gggggg***AGCTCATATGCCCTGTAATTCAGTGG-3′ | | |

OAT, organic anion transporter. Nucleotides are in boldface (artificial restriction sites; not included in numbering). Lowercase italic letters represent small nucleotides (adjustment of primer melting temperature; not included in numbering). Indicated positions are relative to transcription start site (+1) of GeneBank accession nos. OAT1: NT_167190.1.

Table 2. *Primer used for human OAT1 site-directed mutagenesis*

| Cloning of OAT1 (−1,982/+88) | Primer |
|---|---|
| BCL6-Mut1 | 5′-CAGAGCCCAGTCTCC**GGA**CT**CACC**GGAAGACAAATAG GTC-3′ |
| | 5′-GACCTATTTGTCTTCC**GGTGAG**T**CC**GGAGACTGGGCT CTG-3′ |
| BCL6-Mut2 | 5′-CAAGAGGCCACCACACT**GAA**C**CTC**GCCCAGTCTCCTTC-3′ |
| | 5′-GAAGGAGACTGGGC**GAGGTTC**AGTGTGGTGGCCTCTTG-3′ |
| BCL6-Mut3 | 5′-GGAGCCAAAACTCTGT**C**GGC**GTCC**TTCACGTGATTGTC TTAG-3′ |
| | 5′-CTAAGACAATCACGTGAA**GGA**CGCC**G**ACAGAGTTTTGGC TCC-3′ |
| Mut HNF-1α | 5′-GCGTGCTAGCTTGGAGG**ACCGGC**CCTTCTGATACCAA GTC-3′ |
| | 5′-GACTTGGTATCAGAAGG**GCCGGT**CCTCCAAGCTAGCA CGC-3′ |

BCL6, B-cell CLL/lymphoma 6; Mut, mutated; HNF-1α, hepatocyte nuclear factor-1α. Boldfaced nucleotides indicate mutated nucleotides of respective BCL6 or HNF-1α binding sites.

from Thermo Scientific: B-BCL6 probe (positive control) (21), 5′-GAAAATTCCTAGAAAGCATA-3′, B-OAT1_−66/−34, 5′-TCCTTGGAGGGTTAATCCTTCTGATACCAAGTC-3′, and mut_B-OAT1_−66/−34, 5′-TCCTTGGAGG*ACCGGC*CCTTCTGATACCAAGTC-3′.

EMSA procedure was done using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's recommendation. In brief, 4 μg of nuclear extract was incubated with 20 fmol of biotin-labeled probe, 50 ng/μl poly-dIC (in 10 mM Tris and 1 mM EDTA, pH 7.5), 2.5% glycerol, 5 mM $MgCl_2$, 0.05% NP-40, and 2 μl of 10× binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DTT, pH 7.5) in a total volume of 20 μl for 20 min at room temperature. For supershift assay, 1 μg/ml of HNF-1α antibody (sc-10791X; Santa Cruz Biotechnology) was additionally added to the reaction mixtures. After incubation, probes were transferred onto a 6% native polyacrylamide gel, electrophoresed at 100 V until the bromophenol blue dye had migrated aprroximately two-thirds to three-quarters down the length of the gel, and further blotted and cross-linked on a positively charged nylon membrane (Roche). Finally, biotin-labeled DNA protein complexes were detected by chemiluminescence (LightShift Chemiluminescent EMSA Kit; Thermo Scientific).

*Site-directed mutagenesis of OAT1 promoter constructs.* Mutation of the postulated BCL6 and HNF-1α binding sequences within the human OAT1(−1,982 to +88) promoter was done using QuickChange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's recommendation. Insertions of various point mutations were performed by using internal mutated primers (Table 2). Promoter constructs harboring more than one mutation were generated by consecutive rounds of mutagenesis.

Table 3. *In silico analyses of human OAT1 promoter in terms of BCL6 binding sites*

| Promoter (3 kb) | Sequence | Position, bp |
|---|---|---|
| OAT1 | 3′-tatttccacgaaccccc-5′ | −2,577 to −2,561 |
| | 5′-gggttcgtggaaatatt-3′ | −2,575 to −2,559 |
| | 3′-gaattgcttgaacctgg-5′ | −2,395 to −2,379 |
| | 3′-gaattcctccaacagag-5′ | −1,494 to −1,478 |
| | 5′-cacttcccagagcccag-3′ | −241 to −225 |
| | 5′-tccttcctaaaaggaag-3′ | −222 to −206 |
| BCL6 consensus sequence | ttcct(a/c)gaa | Ref. 7 |

*Western blot analysis.* For analysis of HNF-1α in OK cells transfected with pcDNA3, pcDNA3-BCL6, and pCMV-HNF1α, 40 μg of protein of each nuclear extract probe was used. Nuclear extracts were denaturated in loading buffer (5 mM Tris·HCl, 10% glycerol, 1% SDS, and 1% β-mercaptoethanol, pH 6.8) for 10 min at 95°C. Denaturated probes were electrophoresed in 10% SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene difluoride membrane (Roche). The membrane was incubated for 1 h in blocking buffer (5% nonfat dry milk in 0.05% Tween 20-PBS solution) at room temperature and followed by an overnight incubation with 1 μg/ml polyclonal rabbit anti-human HNF-1α antibody at 4°C (sc-10791; Santa Cruz Biotechnology). For labeling of specific bands, the membrane was incubated for 2 h with 0.04 μg/ml goat anti-rabbit horseradish peroxidase IgG (sc-2004; Santa Cruz Biotechnology) at RT. The expression patterns of housekeeping proteins β-actin and histone H3 were investigated using 0.2 μg/ml monoclonal mouse anti-β-actin antibody (DLN-07274; Dianova) or 0.4 μg/ml polyclonal rabbit anti-histone H3 antibody (sc-10809; Santa Cruz Biotechnology) and their corresponding secondary antibodies 0.1 μg/ml polyclonal goat anti-mouse horseradish peroxidase IgG (catalog no. 401215; Calbiochem) or 0.1 μg/ml polyclonal goat anti-rabbit horseradish peroxidase IgG (sc-2004; Santa Cruz Biotechnology). Marked protein bands were detected by enhanced chemiluminescence, using Proxima 2700 (Biostep).

*Statistical analysis.* All data are represented as means ± SE. A minimum of three independent experiments were performed to establish each single data point. Statistical analyses were conducted using the two-sided unpaired *t*-test (GraphPad Prism4, version 4.03). Significance was set at $P < 0.05$.

## RESULTS

*Activation of OAT1 promoter by BCL6.* The OAT1 promoter included six predicted BCL6 binding sites (Table 3). Several 5′-truncated promoter fragments of human OAT1, differing in their length and number of predicted BCL6 binding sites, were

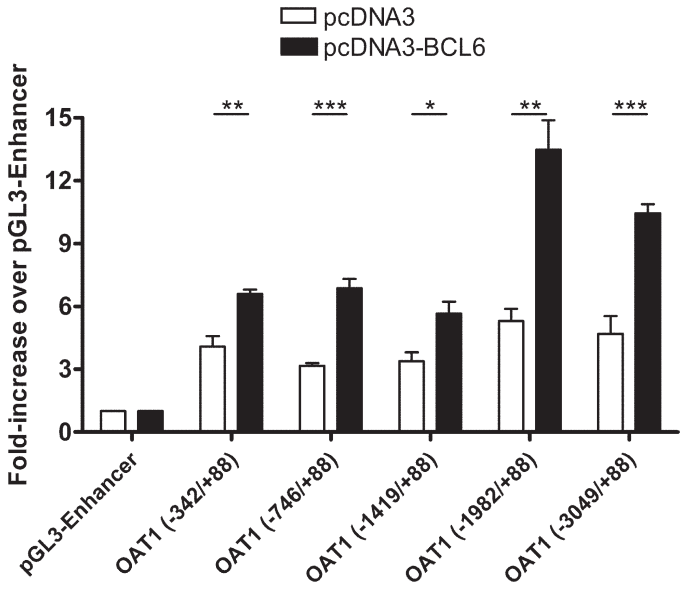



Fig. 1. Activation of organic anion transporter (OAT)1 promoter by B-cell CLL/lymphoma 6 (BCL6). Promoter constructs of human OAT1 and pcDNA3-BCL6 (black bars) or pcDNA3 (open bars) were transfected into opposum kidney (OK) cells. Firefly luciferase was normalized to Renilla luciferase activity. Data are reported as the fold increase over pGL3-Enhancer and presented as means ± SE; $n = 4$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, significantly different from control (pcDNA3).

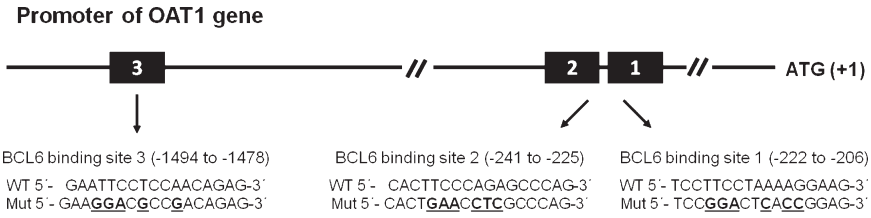
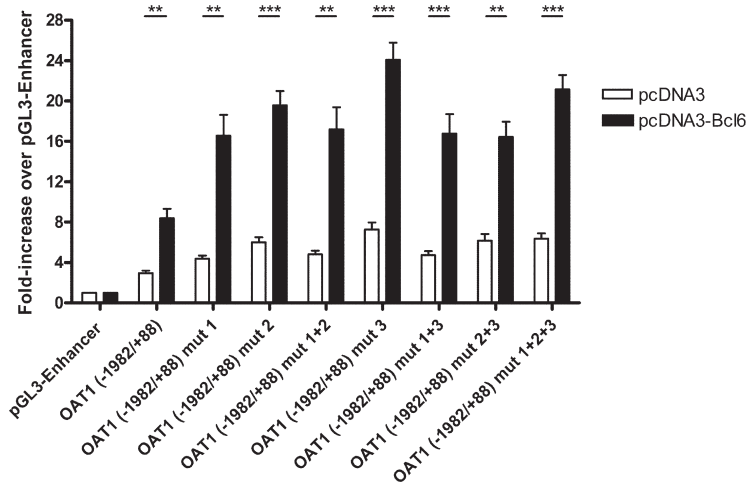



Fig. 2. Mutation of BCL6 binding sites in human OAT1 promoter. Three different BCL6 binding sites were mutated in OAT1 promoter construct OAT1 (−1,982/+88). Mutated and wild-type promoter constructs were cotransfected with pcDNA3-BCL6 (black bars) or pcDNA3 (open bars) in OK cells. Firefly luciferase was normalized to Renilla luciferase activity. Data are reported as the fold increase over pGL3-Enhancer and presented as means ± SE; $n = 4$. $^{**}P < 0.01$ and $^{***}P < 0.001$, significantly different from control (pcDNA3).




cloned into the firefly luciferase vector pGL3-Enhancer. Promoter constructs OAT1 (−342 to +88), OAT1 (−746 to +88), and OAT1 (−1,419 to +88) included two predicted BCL6 binding sites located between −241 to −206 bp upstream of the transcription start (Table 3).

OAT1 (−1,982 to +88) contained three and OAT1 (−3,049 to +88) five BCL6 binding sites. The activities of OAT1 promoter constructs were investigated in OK cells cotransfected with BCL6 expression vector or empty control vector. All tested OAT1 promoter constructs were activated significantly by BCL6 (Fig. 1). OAT1 (−3,049 to +88) promoter construct showed no additional enhancement of BCL6-dependent activity, suggesting that BCL6 binds within 1,982 bp upstream of the transcriptional start site (Fig. 1).

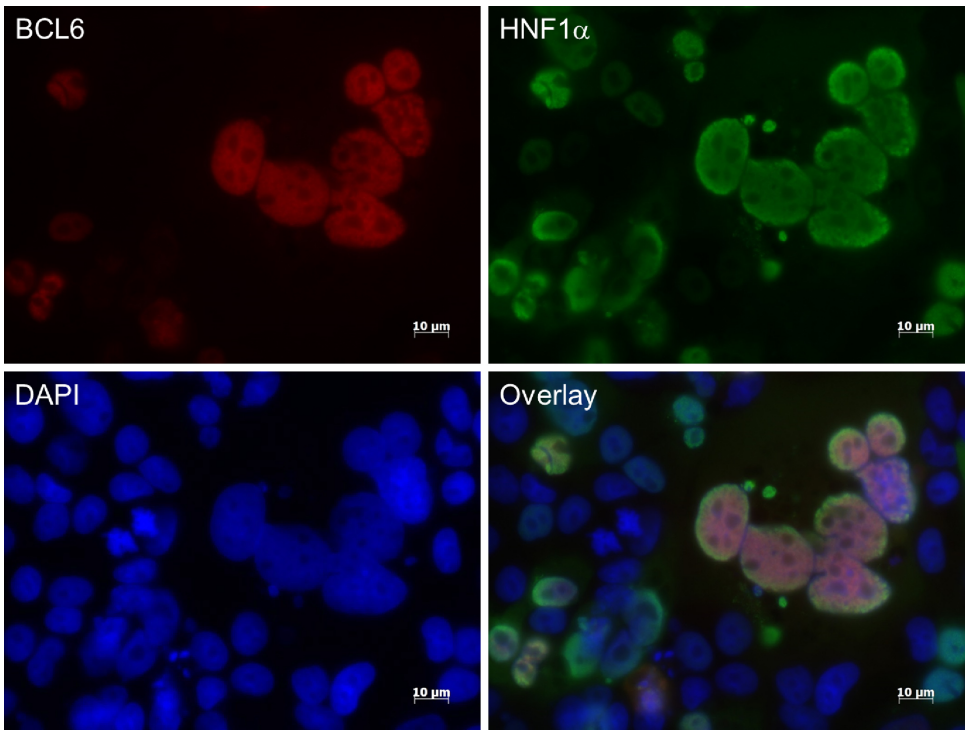



Fig. 3. Coexpression of BCL6 and hepatocyte nuclear factor-1α (HNF-1α) in OK cells. The cells were transiently transfected with a combination of expression vectors for BCL6 and HNF-1α. BCL6 and HNF-1α expression and cellular localization were analyzed using immunofluorescence staining (red, BCL6; green, HNF-4α; blue, DAPI; excitation wavelengths: 546, 488, and 365 nm, respectively). Data are representative for ≥3 independent experiments.

The predicted BCL6 binding sites in the OAT1 (−1,982 to +88) promoter construct were modified by introducing five to six point mutations, resulting in the complete deficiency within in silico analyses and leading to seven mutated promoter constructs (Fig. 2). Wild-type or mutated OAT1 (−1,982 to +88) promoter fragments were cotransfected with either the BCL6 expression vector or control vector (pcDNA3) into OK cells, and luciferase activities were measured. All mutated OAT1 (−1,982 to +88) promoter constructs were activated by BCL6 overexpression compared with pcDNA3 control vector (Fig. 2). No significant differences in BCL6-dependent activation between wild-type and mutated OAT1 promoter constructs were found (Fig. 2).

*Role of HNF-1α in BCL6-dependent activation.* The protein-protein interaction of BCL6 with HNF-1α, a known OAT1 gene activator, was identified previously in a tandem mass spectrometry analysis (22). The involvement of HNF-1α in BCL6-dependent OAT1 promoter activation was investigated by cotransfection of OAT1 promoter constructs with empty vector pcDNA3, BCL6 expression vector, a combination of expression vectors for BCL6 and HNF-1α, and HNF-1α expression vector alone. The coexpression and localization of BCL6 and HNF-1α proteins was characterized in OK cells transiently transfected with expression vectors for BCL6 and HNF-1α using immunofluorescence. Most transfected OK cells coexpressed BCL6 and HNF-1α proteins exclusively in cell nuclei (DAPI stained), with a transfection efficiency of ~30% (Fig. 3).

OAT1 (−198 to +88) and OAT1 (−342 to +88) were significantly activated by BCL6 compared with pcDNA3-transfected cells (Fig. 4). No significant enhancement of BCL6-dependent activation was observed between promoter construct without BCL6 binding sites OAT1 (−198 to +88) and OAT1 (−342 to +88), which include two predicted BCL6-binding sites (Fig. 4). The activities of both promoter constructs were strongly induced by HNF-1α cotransfection (Fig. 4). The combination of BCL6 and HNF-1α resulted in significant additional activity of both OAT1 promoter constructs compared with cells transfected with expression vectors for either BCL6 or HNF-1α (Fig. 4).

The cooperative effect of BCL6 and HNF-1α suggested that BCL6-induced OAT1 promoter activation depended on HNF-1α activity. To test this idea, the known HNF-1α binding site (−56 to −44) (27) was modified in OAT1 (−198 to +88), and the response of the mutated and wild-type promoter construct to BCL6 was compared (Fig. 5). In contrast to wild-type OAT1 (−198 to +88), the promoter construct, which included six exchanged nucleotides in the HNF-1α response element, was not activated by BCL6 cotransfection (Fig. 5).

*HNF-1α binding to OAT1 promoter in BCL6-transfected cells.* The binding of BCL6 and HNF-1α proteins at the HNF-1α binding motif in the OAT1 promoter was investigated by using EMSA. Biotin (B)-labeled oligonucleotides containing the wild-type or mutated HNF-1α binding site (B-OAT1 −66 to −34 and Mut_B-OAT1 −66 to −34) were generated. Nuclear extracts of OK cells transiently transfected with pcDNA3, pcDNA3-BCL6, and pCMV-HNF-1α and nuclear extracts of RAJI cells, which are known to contain high levels of endogenous BCL6 protein (16), were prepared.

The OAT1 promoter wild-type biotin-labeled probe B-OAT1 −66 to −34 containing the known HNF-1α binding site

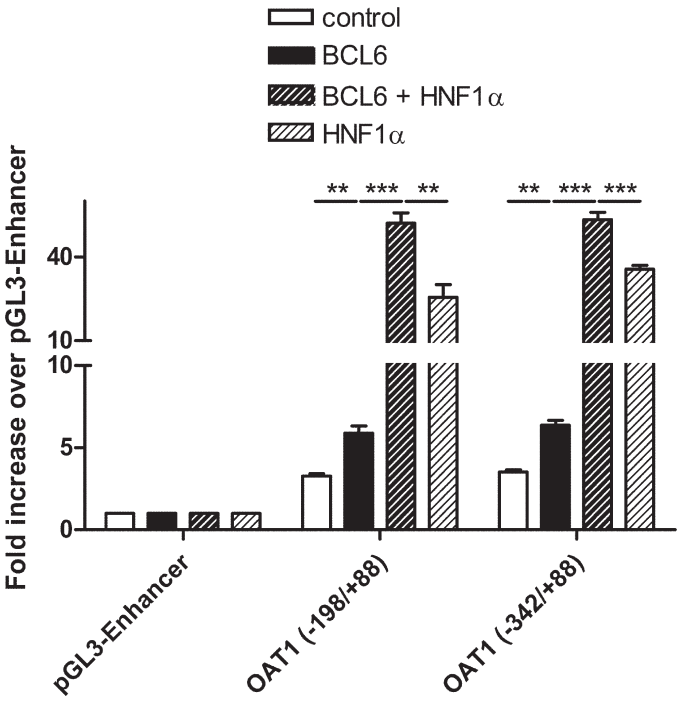



Fig. 4. Cooperative effects of HNF-1α and BCL6 on OAT1 promoter with and without BCL6 binding sites. Promoter constructs OAT1 (−198/+88; without BCL6 binding site) and OAT1 (−342/+88; with BCL6 binding site) were cotransfected with empty vector (open bars), expression vector for BCL6 (black bars), a combination of BCL6 and HNF-1α expression vectors (black and white hatched bars), or expression vector for HNF-1α (black and gray hatched bars) into OK cells. Firefly luciferase was normalized to Renilla luciferase activity. Data are reported as the fold increase over pGL3-Enhancer and presented as means ± SE; $n = 4$. $^{**}P < 0.01$ and $^{***}P < 0.001$, significantly different from control (pcDNA3).

(−56 to −44) (27) was incubated with nuclear extract of RAJI cells in either the presence or absence of a specific BCL6 antibody. Furthermore, this was analyzed by EMSA to investigate the binding of BCL6 protein at the designated HNF-1α motif (Fig. 6). In addition, a second probe with a known BCL6 binding site (7) was generated as positive control for the validation of the testing system. BCL6 protein formed a DNA complex, with the probe used as positive control (Fig. 6). Furthermore, this complex was supershifted on the addition of the BCL6 antibody (Fig. 6). No supershifted bands were detected when the nuclear extract of RAJI cells was incubated with wild-type probe (B-OAT1 −66 to −34), suggesting that BCL6 did not bind directly or indirectly at the OAT1 promoter (Fig. 6). Furthermore, no interactions between BCL6 and OAT1 promoter (−198 to +1) were observed in supershift assays (investigated using different probes with an approximate length of 30 bp, corresponding up to 198 bp upstream of OAT1 transcription start site; data not shown).

Several EMSAs were performed to investigate the binding of HNF-1α protein to HNF-1α motif in BCL6 overexpressing OK cells. Nuclear extract of OK cells transiently transfected with pcDNA3, pcDNA3-BCL6, and pCMV-HNF-1α were incubated with wild-type OAT1 probe (B-OAT1 −66 to −34) or a probe that contains a modified HNF-1α binding site (Mut_B-OAT1 −66 to −34) with or without HNF-1α antibody (Fig. 7). No specific shifted bands were observed when the probe with mutated HNF-1α binding site was incubated with

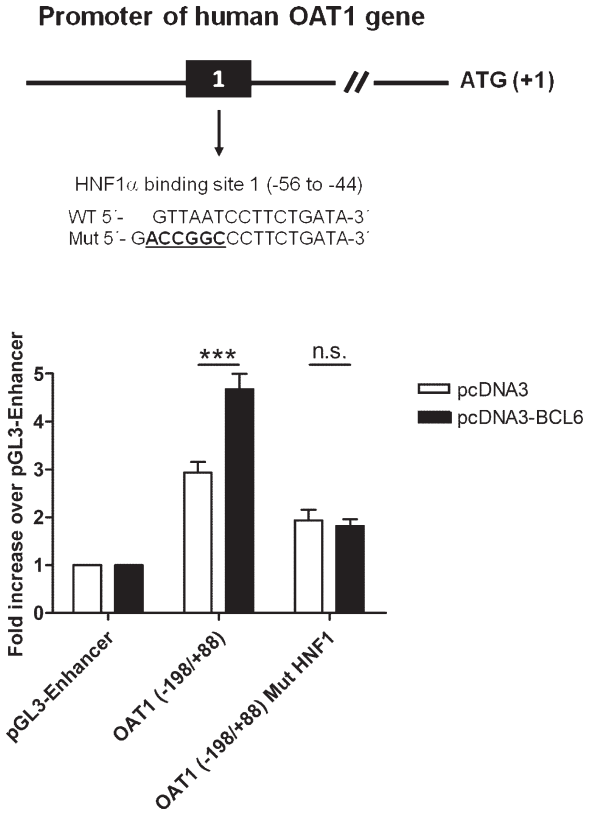



Fig. 5. Effect of HNF-1α binding site mutation on BCL6-dependent OAT1 promoter activation. HNF-1α binding site (−56 to −44) was modified in the OAT1 (−198/+88) promoter construct. Wild-type (WT) and mutated (mut) OAT1 (−198/+88) were cotransfected with pcDNA3 (open bars) or pcDNA3-BCL6 (black bars) into OK cells. Firefly luciferase was normalized to Renilla luciferase activity. Data are reported as the fold increase over pGL3-Enhancer and presented as means ± SE; $n = 4$. ***$P < 0.001$, significantly different from control (pcDNA3). NS, not significant.

nuclear extract of pCMV-HNF-1α-transfected OK cells (Fig. 7, *preparation no. 1*, *lanes 1* and *2*). The formation of specific bands was shown after incubation of wild-type OAT1 probe (B-OAT1 −66 to −34) with nuclear extract of HNF-1α overexpressing OK cells, whereas the addition of an HNF-1α antibody induced the supershift of this band (Fig. 7, *preparation no. 1*, *lanes 3* and *4*). In addition, the formation of specific shift and supershift of HNF-1α-DNA complex and HNF-1α-DNA-antibody complex was shown in lanes containing nuclear extract of pcDNA3- and pcDNA3-BCL6-transfected cells in independent EMSAs with three different nuclear extract preparations (Fig. 7, *preparation no. 1*, *lanes 5–8*; *preparation no. 2*, *lanes 3–6*; and *preparation no. 3*, *lanes 3–6*). The supershifted bands formed by nuclear extract of BCL6-transfected cells with OAT1 probe (B-OAT1 −66 to −34) and HNF-1α antibody showed higher intensity compared with bands formed using nuclear extracts of pcDNA3-transfected cells (Fig. 7, *preparation no. 1*, *lanes 5–8*; *preparation no. 2*, *lanes 3–6*; and *preparation no. 3*, *lanes 3–6*), suggesting an elevated content of endogenous HNF-1α protein in BCL6-overexpressing OK cells.

*Protein expression of HNF-1α in BCL6-transfected OK cells.* Western blot analyses were performed to confirm the difference in endogenous HNF-1α protein expression between BCL6-transfected and control OK cells observed in supershift assays. Nuclear extracts of OK cells transiently transfected with pcDNA3, expression vector for BCL6, and expression vector for HNF-1α were used for Western blots (Fig. 8). In nuclear extracts of BCL6-transfected OK cells, significantly higher HNF-1α protein expression was detected compared with pcDNA3-transfected cells (Fig. 8).

## DISCUSSION

In humans and rodents, the inhibition or deficiency of renal OAT1/Oat1 protein decreases the secretion of organic anions, e.g., endogenous metabolites and drugs (8, 20, 31, 32). Transcriptional regulation of the renal polyspecific transporter OAT1 has not been investigated intensively so far. OAT1 promoter was shown to be regulated by members of the hepatocyte nuclear factor family and DNA methylation (12). Additional transcriptional OAT1 activators are so far unknown. BCL6-dependent promoter activities of rat Oat1 and Oat3 identified in our previous study were verified by a group in Japan (11, 33).

The aim of this work was to characterize the influence of BCL6 on human OAT1 promoter and on the activity of HNF-1α, a known OAT1 regulator. In accord with rat Oat1, human OAT1 promoter constructs were significantly activated by transcription factor BCL6. In most cases, BCL6 directly binds to its consensus sequence and acts as an transcriptional repressor (3). In some cases, BCL6-induced gene regulation is dependent on protein-protein interaction with other transcription factors, e.g., the subunits of nuclear factor-κB in B-cells and peroxisome proliferator-activated receptor-δ in cardiomyocytes (1, 26). Most transcription factors are able to act as activators and/or repressors (summarized in Ref. 34). For example, the induction of BCL6 expression in human CD4 T

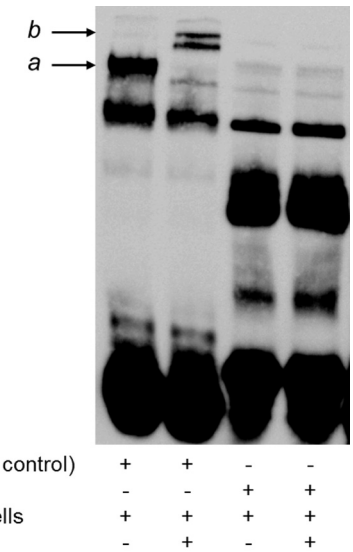



Fig. 6. BCL6 binding at HNF-1α motif in OAT1 promoter. Biotin (B)-labeled oligonucleotide corresponding to human OAT1 promoter and containing a WT HNF-1α binding site (−56 to −44) was created. BCL6 probe, including BCL6 binding motif, was generated and used as positive control for formation of the BCL6-DNA complex. Nuclear extracts of RAJI cells were incubated with biotin-labeled probes in presence or absence of BCL6 antibody. Protein-nucleic acid (*a*: shift) and protein-nucleic acid-BCL6 antibody (*b*: supershift) complexes were detected in electrophoresis mobility shift assay (EMSA). Representative EMSA showing the results of ≥3 independent experiments.

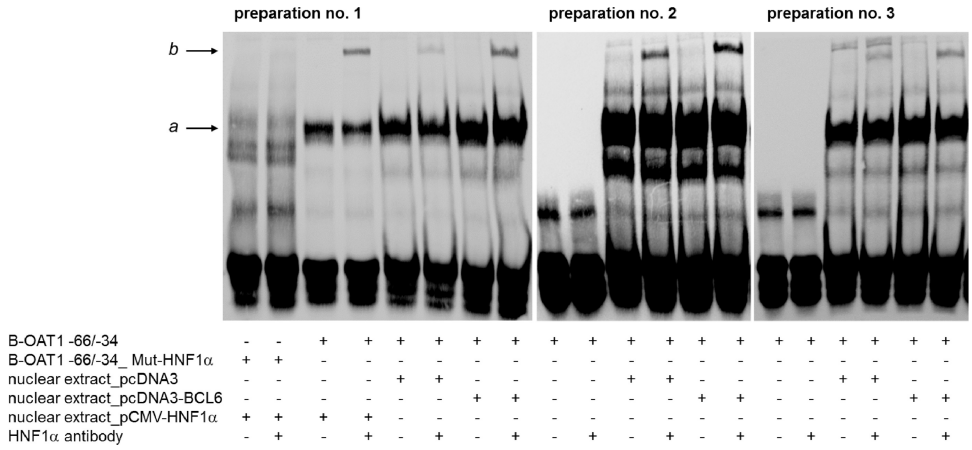



Fig. 7. Effect of BCL6 on HNF-1α binding to OAT1 promoter. B-labeled oligonucleotides corresponding to human OAT1 promoter and containing WT or mut HNF-1α binding site (−56 to −44) were created. Nuclear extracts of OK cells transfected with pcDNA3, pcDNA3-BCL6, or pCMV-HNF-1α were incubated with WT and mut probes in the presence or absence of an HNF-1α antibody. Protein-nucleic acid (*a*: shift) and protein-nucleic acid-HNF-1α antibody (*b*: supershift) complexes were detected in EMSA. Data represent EMSAs with 3 independent nuclear extract preparations.

cells enhances the expression of proteins important for human T cell differentiation and function (15), suggesting an activating role of BCL6 in gene regulation.

Surprisingly, in our study, the BCL6-dependent OAT1 promoter activation was not affected by mutation of predicted BCL6 response elements within the investigated OAT1 promoter. Additionally, the OAT1 (−198 to +88) promoter fragment, which did not include any predicted BCL6 binding sites, was significantly activated by BCL6. Furthermore, in our in silico analysis of the OAT1 promoter, three different HNF-1α binding sites were predicted within 60 bp upstream of the transcription start, but HNF-1α binds only between −57 and −41 bp in these promoter region (27). Therefore, and based on analysis of BCL6-interacting proteins by tandem mass spectrometry, where HNF-1 was identified as a direct interacting partner of BCL6 (22), the effect of BCL6 on HNF-1α-induced OAT1 promoter activity was investigated in OK cells.

In humans and rodents, HNF-1α has been shown to regulate hepatic acute-phase proteins and proteins involved in metabolism of bile acid, cholesterol, lipoprotein, glucose, and fatty acid (2). A large number of hepatic and renal transport proteins is reduced in HNF-1α-deficient mice (19). Promoter of human transporters OAT1, OAT3, OAT4, and urate transporter 1, which is localized in proximal tubule of the kidneys, are also direct targets of HNF-1α (12).

In the presence of BCL6, HNF-1α-mediated OAT1 promoter activation was significantly enhanced independent of predicted BCL6 binding sites, suggesting an interaction between HNF-1α and BCL6. The cooperative effect of BCL6 and HNF-1α was confirmed by the absence of BCL6-dependent activation of OAT1 promoter fragment, which included a modified HNF-1α binding motif. In contrast to our expectations, neither direct nor indirect binding of BCL6 to HNF-1α DNA sequence was exhibited in supershift assays. Nonbinding of BCL6 suggested that BCL6 might influence the HNF-1α activity and/or protein expression and in this manner the promoter activity of OAT1. HNF-1α binding to its specific DNA sequence in OAT1 promoter was enhanced in BCL6-transfected cells compared with control cells. This result correlated well with data from Western blot analysis, which showed a significant increase in endogenous HNF-1α protein expression in BCL6-expressing OK cells. In accord with our results, the coexpression of BCL6 and transcription factor Maf exhibited additive effects on expression for some T cell-associated genes (15). The characteristics of BCL6 and Maf interaction in this cooperative gene activation was not described (15). The molecular mechanism for BCL6-dependent induction of HNF-1α also remains unclear.

Depending on its DNA binding site, transcription factor Y (NFY) has been shown to act as an activator or as a repressor (4, 25). Similar to NFY, but beside its known repressor function, BCL6 can possibly act as an activator for HNF-1α transcription. The activating effect of BCL6 on genes mediated by directly binding to promoter sequences has not been de-

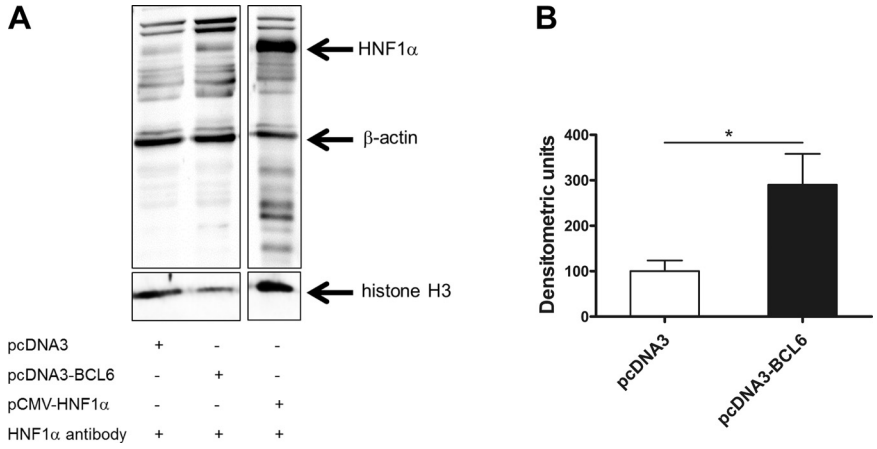



Fig. 8. Protein expression of HNF-1α in BCL6-transfected OK cells. Nuclear extracts of OK cells transfected with pcDNA3, pcDNA3-BCL6, or pCMV-HNF-1α were prepared, and protein expression of HNF-1α was investigated by immunoblotting. β-Actin and histone H3 were used as loading controls. *A*: representative immunoblot showing the results of ≥3 independent experiments. *B*: the intensities of each HNF-1α and histone H3 band were calculated, and the relative values were normalized by averaged intensities of pcDNA3 nuclear extract. Data are presented as means ± SD; $n = 5$. *$P < 0.05$, significantly different from control (pcDNA3 nuclear extract).

scribed so far. However, it is more likely that BCL6 binding to its consensus sequence resulted in a repression of gene transcription. The interaction of BCL6 with an unknown HNF-1α repressor, which might lead to an increase in HNF-1α expression, is also possible.

In summary, human OAT1 promoter constructs were activated by BCL6 independent of any predicted BCL6 response elements. We demonstrated that BCL6 and HNF-1α synergistically induce OAT1 gene transcription. In addition, the BCL6-activating influence on human OAT1 promoter is dependent on the presence of an intact HNF-1α DNA binding sequence and HNF-1α protein without a direct interaction of BCL6 with the OAT1 promoter or HNF-1α complex.

Our findings show for the first time that BCL6 is able to enhance the expression and thereby the activity of HNF-1α, a transcription factor that seems to be important for the regulation of numerous genes, e.g., drug transporter of SLC gene family or proteins involved in several metabolisms expressed in kidney or liver.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## DISCLOSURES

The authors declare that no competing interests exist. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## AUTHOR CONTRIBUTIONS

W.W. and M.H. performed experiments; W.W. and M.H. analyzed data; W.W., G.B., and M.H. drafted manuscript; W.W., G.B., and M.H. edited and revised manuscript; G.B. and M.H. conception and design of research; G.B. and M.H. interpreted results of experiments; G.B. and M.H. approved final version of manuscript; M.H. prepared figures.

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