Histol Histopathol (2010) 25: 1385-1402

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Renal expression of organic anion transporter Oat5 in rats and mice exhibits the female-dominant sex differences

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Summary. The organic anion transporter 5 (Oat5, Slc22a19) was previously localized to the brush-border of proximal tubule (PT) S3 segment in rat and mouse kidneys. Here we report on sex hormone-regulated expression of Oat5 in rat kidneys, after reinvestigating: a) expression of its mRNA by end-point and real time RT-PCR in the tissue, b) abundance of its protein by Western blotting (WB) in isolated membranes, and c) immunolocalization in tissue cryosections. In untreated male (M) and female (F) adult rats, the expression of Oat5 mRNA was predominant in the outer stripe (OS), exhibiting sex differences (M<F), upregulated by castration, and unaffected by ovariectomy. In castrated M, testosterone treatment strongly downregulated, whereas estradiol and progesterone treatment weakly upregulated its expression. By WB, a single protein band of ~72 kDa in variously-treated animals exhibited a density pattern comparable to that of mRNA. By immunostaining, Oat5 protein was localized to the brush-border of S1/S2 in the cortex (CO) (weakly) and in S3 of the OS and medullary rays (strongly) with the F-dominant intensity. In variously-treated rats, the immunostaining pattern matched that of mRNA and WB data. In prepubertal rats, the renal expression of Oat5 mRNA and protein was weak and sex-independent. In adult mice, the sex-dependent pattern of renal Oat5 protein expression was comparable to that in rats. Therefore, the renal expression of Oat5 in rats (and mice) exhibits zonal (CO<OS) and sex differences (M<F), which appear after puberty, largely due to androgen-driven downregulation of its mRNA and protein expression.

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Key words: Gender differences, Kidney, Membrane transporters, Organic anions, Proximal tubule,

Introduction

Organic anion transporters (Oats in animals/OATs in humans; subfamily of Slc22 drug transporters) mediate transport of endogenous and exogenous organic anions (OA), and play a major role in internalization (uptake) and/or extrusion (secretion) of OA in the cells of various mammalian organs, mainly in liver and kidneys. Oats/OATs are thus mediators of body distribution, accumulation in some organs, and final elimination of antibiotics and various other anionic drugs used in therapy of human and animal diseases, but this way they also mediate drug-drug interactions and organ toxicity (Inui et al., 2000; Van Aubel et al., 2000; Sweet et al., 2001; Burckhardt and Burckhardt, 2003; Eraly et al., 2004; Koepsell and Endou, 2004; Lee and Kim, 2004; Miyazaki et al., 2004; Ho and Kim, 2005; Sweet, 2005; Anzai et al., 2006; Li et al., 2006; Sekine et al., 2006; Burckhardt and Rizwan, 2007; Zhou and You, 2007; Sekine et al., 2008).

In the kidney, Oats/OATs are localized to the luminal i.e. brush border (BBM) or basolateral (BLM) membrane of epithelial cells along the nephron, where they mediate transport of various anionic compounds that are either reabsorbed from, or secreted into the tubular fluid. In rats and mice, the rate of renal reabsorption and/or excretion of many OA exhibits sex differences, which can be correlated with the sex-dependent expression of relevant Oats at the level of mRNA and/or protein (Lu et al., 1996; Reyes et al., 1998; Sweet et al., 2001; Buist et al., 2002; Cerrutti et al., 2002; Kato et al., 2002; Kobayashi et al., 2002a,b;

Buist et al., 2003; Burckhardt and Burckhardt, 2003; Morris et al., 2003; Terlouw et al., 2003; Buist and Klaassen, 2004; Koepsell and Endou, 2004; Lee and Kim, 2004; Ljubojevic et al., 2004, 2007; Wright and Dantzler, 2004; Schlattjan et al., 2005; Sekine et al., 2006; Burckhardt and Rizwan, 2007; Sabolic et al., 2007). However, comparable phenomena could not be confirmed for some renal Oats (Oat1 and Oat3) in rabbits (Groves et al., 2006) indicating that the sex-dependent expression of specific renal OA transport/transporters could be species-specific. Organic anion transporter type 5 (Oat5/OAT5) has been studied in human, rat and mouse organs, but the data collected thus far indicate that the human and rodent genes are not orthologs and code for proteins of different characteristics, localization and function(s). In humans, OAT5 (SLC22A10) mRNA was located by Northern blotting exclusively in the liver; the kidneys were negative (Sun et al., 2001; Eraly and Nigam, 2002) whereas in rats and mice the Northern blotting and RT-PCR studies showed an absence of Oat5 (Slc22a19) mRNA in the liver and its presence in the kidney (Youngblood and Sweet, 2004; Anzai et al., 2005; Kwak et al., 2005).

Oat5 from rats (rOat5) and mice (mOat5) has been cloned and characterized. At the level of cDNA and peptide sequences, the mOat5 and rOat5 are identical 88% and 82%, respectively. The mOat5 protein furthermore exhibits: a) limited identity to hOAT4 (42%), hOAT5 (55%), mOat1 (38%), mOat2 (31%) and mOat3 (36%) proteins, b) 10-12 transmembrane domains, c) four potential glycosylation sites, and d) numerous potential phosphorylation sites (Youngblood and Sweet, 2004). By Western blotting of the renal BBM, mOat5 was identified as the ~85 kDa protein band (Kwak et al., 2005), whereas in the rOat5-transfected HEK293 cells, the protein showed up as the ~65 kDa band (Anzai et al., 2005). By RT-PCR in isolated tubules, rOat5 mRNA was localized to the proximal tubule S2 and S3 segments (S2<S3) (Anzai et al., 2005), whereas by immunohistochemistry of the rat and mouse kidney, the transporter was localized to the BBM of proximal tubule straight segment (S3) in the outer stripe (Anzai et al., 2005; Kwak et al., 2005). In an *in vitro* expression system, Oat5 has been defined as a cation-independent, probenecid-sensitive organic anion/dicarboxylate antiporter, which can transport ochratoxin A (OTA), estrone-3-sulfate (ES) and dehydro-epiandrosterone sulfate (DHEA-S), and be inhibited by some sulfate but not glucuronide conjugates. Furthermore, mOat5, but not rOat5, was found to transport salicylate, but none showed a significant affinity for p-aminohippurate (PAH) and various other anionic and cationic drugs (Youngblood and Sweet, 2004; Anzai et al., 2005; Kwak et al., 2005).

Sexual dimorphism in the expression of renal Oat5 has been tested by RT-PCR in rats and mice; in both species, the levels of Oat5 mRNA were found to be similar in male (M) and female (F) animals (Youngblood and Sweet, 2004). However, our preliminary immunochemical studies have indicated the presence of sex differences in the expression of rOat5 protein in rat kidneys. Following these initial observations, we have performed a detailed re-investigation of the expression of renal Oat5 at mRNA and protein level in rats and mice of both sexes. A preliminary communication of this work has been reported (Brzica et al., 2007).

Material and methods

Animals and treatment

Prepubertal (3 weeks old) and adult (12-14 weeks old) M and F rats of Wistar strain were from the breeding colony in the Institute in Zagreb, Croatia. Adult (12-14 weeks old) M and F mice of C57Bl/6 strain were purchased from the breeding colony in the Department of Physiology, School of Medicine (Zagreb, Croatia). Animals were bred and maintained according to the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, 1996). Before and during experiments animals had free access to standard pellet food and tap water. The Institutional Ethic Committee approved the studies.

Prepubertal rats and adult mice were used untreated, whereas the adult rats were used either untreated or sham operated, or gonadectomized. Untreated adult rats were sacrificed at an age of 12-14 weeks. Gonadectomy was performed at an age of 6 weeks; M rats were castrated by scrotal route, whereas F rats were ovariectomized by dorsal (lumbal) approach under proper anesthesia (Narketan, 80 mg/kg b.m./Xylapan, 12 mg/kg b.m., i.p.). The sham-operated animals underwent the same procedure, except the gonads were not removed.

Some gonadectomized or sham-operated rats (4 animals in each group) were left to recover for another 6 weeks before sacrifice. Of castrated males, some animals (4 rats/group) underwent the s.c. treatment with either testosterone enanthate, estradiol dipropionate or progesterone (each: 2.5 mg/kg b.m./day for 14 days; hormones dissolved in sunflower oil). The castrated control and sham-operated rats (4 animals/group) were treated with an equivalent amount of sunflower oil (0.5 mL/kg b.m./day for 14 days, s.c.).

Antibodies and chemicals

Affinity purified, rabbit-raised polyclonal antibody (rOat5-ab) against the synthetic peptide corresponding to C-terminal sequence of the rOat5 protein (amino acids 538-551: REVKKDAVAKVTPF), and the immunizing peptide (rOat5-peptide), were kindly supplied by Transgenic Inc. (Kumamoto, Japan); the use of this antibody in immunochemical studies has been described (Anzai et al., 2005). The use of an affinity-purified polyclonal, chicken-raised anti-V-ATPase 31-kDa subunit antibody (V-ATPase-ab), and of a monoclonal

antibody against α-actin (α-actin-ab; Chemicon Int., Temecula, CA, USA) was described in our previous publications (Sabolic et al., 2002, 2006; Ljubojevic et al., 2007). Secondary antibodies, which included the CY3-labeled (GARCY3) and alkaline phosphatase-labeled (GARAP) goat anti-rabbit IgG, were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA) or Kirkegaard and Perry (Gaithesburg, MD, USA).

Anesthetics (Narketan and Xylapan) were purchased from Chassot AG (Bern, Switzerland). Oil solutions of testosterone enanthate, estradiol dipropionate, and progesterone were from RotexMedica GmbH (Trittau, Germany) and Galenika (Belgrade, Serbia). Protease inhibitors (phenyl-methyl-sulfonyl-fluoride (PMSF), antipain and benzamidine) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were analytical grade and obtained from commercial sources such as Sigma (St. Louis, MO, USA) or Fisher Scientific (New Jersey, NY, USA). The sources of specific reagents and equipment for RNA isolation, end-point and real-time RT-PCR assays, Western blotting, and steroid determination are indicated in the text related to these methods.

Isolation of RNA and synthesis of first strand cDNA

The adult rats were sacrificed by decapitation. The kidneys were removed, decapsulated, cut into ~1 mm thick sagittal slices, and one (middle) slice was immediately submerged into the RNAlater solution (Sigma, St. Louis, MO, USA). The slice was later used either in toto, or the renal cortex and outer stripe tissues were dissected manually and used separately in further isolation steps. Prepubertal rats were sacrificed by cervical dislocation, bled by cutting abdominal aorta, and the kidneys were removed, placed in RNAlater, and used *in toto*. Total cellular RNA from corresponding tissues was extracted using Trizol (Invitrogen) according to manufacturer's conditions. RNA concentration and its purity were estimated by the spectrophotometric measurement of optical density at 260 and 280 nm. The quality and integrity of RNA was estimated by agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light. Isolated RNA was stored at -70°C until use.

First strand cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Fermentas Int., Ontario, Canada) following the manufacturer's instructions. Total cellular RNA (3 μg) was denatured at 70°C for 5 min in the reaction mixture containing 0.5 μg oligo dT(18) and reverse transcribed in total volume of 20 μL reaction mixture containing 1x reverse transcription buffer, 20 units of ribonuclease inhibitor, 1 mM of dNTP mix, and 40 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase. The reaction mixture was incubated at 37°C for 60 min followed by incubation at 72°C for 10 min. cDNAs were diluted 5x in DNase/RNase free water (Gibco-BRL, Grand Island, NY, USA) and stored at -20°C until use.

End-point RT-PCR

The end-point RT-PCR studies were performed with the cDNA samples obtained from 2-4 independent RNA preparations from each experimental group of rats in order to expose relative levels of rOat5 mRNA expression in the renal tissues which, however, could not be quantitatively analysed. PCR was performed in total volume of 20 μL using: 1 μL of 5x diluted first strand cDNA, 0.4 μM specific primers and ready to use PCR Master Mix (Fermentas Int., Ontario, Canada) following instructions by the manufacturer. To avoid amplification of genomic DNA, intron over-spanning primers were used. Custom primers for rOat5 and ß-actin were purchased from Invitrogen (Online). The sequences of specific primers used for RT-PCR reactions and predicted RT-PCR product sizes are defined in Table 1. Reaction conditions used for PCR were: initial denaturation for 3 min at 94°C, denaturation for 30 sec at 95°C, annealing for 30 sec at 57°C and elongation for 45 sec at 72°C. The non-template control (NTC) reactions, where the cDNA was substituted with DNase/RNase free water, were included in each PCR reaction in order to check possible contamination; the PCR products were not detected in NTC reactions, indicating an absence of relevant contamination in our tests (data not shown). RT-PCR products were resolved by electrophoresis in 1% agarose gel stained with ethidium bromide, and visualized under ultraviolet light. The housekeeping gene ß-actin was used as a control for variations in the input of RNA. To obtain comparable results, preliminary experiments were done to determine the optimal number of the PCR cycles within the exponential phase of the PCR reaction; 27 and 23 cycles were optimal for rOat5 in the cortex and outer stripe,

Table 1. Primer sequences used for end-point PCR.

Gene	Forward (f) / Reverse (r) Primers (5'-3')	Accession No. Gene Bank	Location	PCR Product size (bp)
rOat5	f: GGAGGCAGCAGAGACAAAAC r: TTGCTCCTCCTAATGATGCC	XM_342011.2	1104-1123 1431-1450	347
ß-actin	f: GTCGTACCACTGGCATTGTG r: AGGAAGGAAGGCTGGAAGAG	NM_031144.2	518-537 862-881	364

respectively, whereas 30 cycles were optimal for ß-actin in both tissue zones.

Real-time RT-PCR

In order to quantify and statistically analyse the rOat5 mRNA expression in the renal tissues, the real-time RT-PCR was performed with the cDNA samples obtained from 3 independent RNA preparations in each experimental group of rats. Real-time RT-PCR was performed in a 50 μL volume using 3.3 μL (100 ng) of the first-strand cDNA template, 2.5 μL of 20x TaqMan Gene Expression Assays mix, 25 μL of the 2x TaqMan Universal PCR Master Mix (all from Applied Biosystems, Foster City, CA, USA) and 19.2 μL of nuclease-free water. Primers and probes were designed by Applied Biosystems and supplied as TaqMan Gene Expression Assays Mix containing a 20x mix of unlabeled PCR forward and reverse primers, as well as TaqMan MGB probe. Assay IDs were Rn00696699_m1 and Rn00667869_m1 for rOat5 and ß-actin, respectively (http://www.appliedbiosystems.com). Amplification and detection were performed using the 7500 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions included an initial 2 min at 50°C, then 10 min at 95°C, followed by 40 two-step cycles of denaturation (15 sec at 95°C) and annealing/extension (1 min at 60°C). The NTC reactions, where the cDNA was substituted with nuclease-free water, were included in each run to screen for possible contamination. To standardize the input of cDNA amount, an endogenous control, i.e., the housekeeping gene ß-actin was run, quantified, and the results were normalized to these values. Each sample was performed in duplicate. Quantification of rOat5 mRNA amount (mean fold changes ± S.E.M of duplicate measurements) was accomplished by comparative Ct-method using the Relative Quantification Study Software (Applied Biosystems), and the experimental data are shown relative to the mean value (= 1 arbitrary unit) in the corresponding control samples.

Tissue fixation and immunofluorescence staining

Fixation of the rat organs with 4% p-formaldehyde *in vivo*, as well as cutting 4-μm thick tissue cryosections were described in detail in our recent publications (Sabolic et al., 2006; Ljubojevic et al., 2007). To obtain optimal immunostaining of rOat5 in tissue cryosections, in preliminary experiments we tried different antigen retrieval techniques in order to maximize the antibody binding, and found out that the conditions, recently described for Oat2 in the rat and mouse kidney (Ljubojevic et al., 2007), were also optimal for rOat5. In brief, the rehydrated cryosections were heated in a microwave oven for 20 min in 10 mM citrate buffer (pH 6), rinsed in PBS, incubated for 20 min with 1% bovine serum albumin to block the nonspecific antibody binding, incubated with the rOat5-ab (diluted 1:100 in PBS) at 4°C overnight, rinsed, incubated with GARCY3 (1.6 μg/mL) for 60 min at room temperature, rinsed, covered with a fluorescence fading retardant Vectashield (Vector Laboratories Inc., Burlingame, CA, USA), and inspected for immunofluorescence.

To test the staining specificity, rOat5-ab was blocked with the rOat5-peptide (final concentration of the peptide was 0.5 mg/mL) for 4 hours at room temperature prior to use in an immunofluorescence assay as described above.

The stained sections were examined and photographed with an Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany) using a Spot RT Slider camera and software (Diagnostic Instruments, Sterling Heights, MI, USA). The photos were imported into Adobe Photoshop 6.0 for conversion of the CY3-related red fluorescence into black and white mode, and for further processing and labeling.

Preparation of tissue homogenates and membranes

Adult rats were sacrificed by decapitation. The kidneys were removed, decapsulated, and either used *in toto* or sagitally sliced, and the renal cortex and outer stripe tissues were dissected manually and used as separate tissue pools. Mice and prepubertal rats were sacrificed by cervical dislocation, bled by cutting abdominal aorta, and the kidneys were removed and used *in toto*. The respective tissues were homogenized (10% homogenate) in a chilled homogenization buffer (300 mM mannitol, 5 mM EGTA, 12 mM Tris-HCl (pH 7.4), 1 mM PMSF, 0.1 mM benzamidine, and 0.1 μg/mL antipain) with a Powergen-125 homogenizer (Fisher Scientific, New Jersey, NJ, USA) at the maximal setting (1 min homogenization - 2 min pause - 1 min homogenization). Total cell membranes (TCM) were isolated from the tissue homogenates by differential centrifugation using the refrigerated high speed centrifuge (Sorvall RC-5C; rotor SS34) and ultracentrifuge (Sorvall OTD-Combi, rotor T-875) (both from Sorvall Instruments, Newton, CT, USA). The cell debris was removed by centrifugation at 6,000 x g for 15 min; the pellet was discarded, and the supernatant was centrifuged at 150,000 x g for 1 hour. The resulting pellet (TCM) was resuspended in a chilled buffer (150 mM mannitol, 2.5 mM EGTA, 6 mM Tris-HCl, pH 7.4).

BBM from the cortical and outer stripe tissue (rats) or from the total kidney (mice, some rats) homogenates were isolated by the Mg^{2+}/EGTA precipitation method (Biber et al., 1981). The protein was determined by the Bradford assay (Bradford, 1976), and the membranes were stored at -70°C until further use for Western blotting.

SDS-PAGE and Western blotting

The membrane samples were thawed at 37°C, mixed with loading buffer containing (final concentrations) 1% SDS, 12% glycerol, 30 mM Tris-HCl, pH 6.8, without

(non-reducing conditions) or with 5% ß-mercaptoethanol (ß-ME; reducing conditions). Samples were incubated at 95°C for 5 min or 65°C for 15 min, or 37°C for 30 min. Proteins (the amount of membrane protein (μg/lane) is indicated in the respective figure legends) were separated through 10% SDS-PAGE mini gels, and then electrophoretically wet-transferred to an Immobilon membrane (Millipore, Bedford, MA, USA), using the equipment from Bio-Rad Laboratories (Hercules, CA, USA). Following transfer, the Immobilon membrane was (in sequence) briefly stained with Coomassie Brilliant Blue to check the efficiency of transfer, destained, blocked in blotto-buffer (5% non-fat dry milk, 0.15 M NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.4) for 1 hour, incubated overnight in the same buffer containing either rOat5-ab (1:1000, in blotto-buffer) or V-ATPase-ab (1:20), or α-actin-ab (1:1000), washed with blotto-buffer, incubated for 60 min at room temperature with GARAP (0.1 μg/mL in blotto-buffer), washed in blotto-buffer and PBS, and the protein bands were visualized by the alkaline phosphatase activity-mediated reaction in a buffer that contained 20 mM Tris-HCl, pH 9.0, 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 1.65 mg/mL) and nitro blue tetrazolium (NBT; 3.3 mg/mL).

To show the specific labeling, the rOat5-ab was preincubated with the corresponding immunizing peptide (final concentration of the peptide was 0.5 mg/mL) for 4 hours at room temperature before use in the Western blot analysis as described above.

The relative molecular mass (Mr) of the labeled Oat5 protein band was estimated by using Protein Ladders (Fermentas Int., Ontario, Canada). The labeled protein bands were evaluated by densitometry using the Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc.). An area of the strongest protein band in the blot of control samples was marked, and an equal area was applied to all other bands in the same blot. The area density of each band was expressed in arbitrary units, relative to the strongest band density (= 1) in the corresponding control samples.

Presentation of the data

The end-point RT-PCR data were obtained with cDNA samples from independent RNA isolations from two adult (untreated, gonadectomized and hormone-treated gonadectomized rats) or four prepubertal animals in each experimental group. The real time RT-PCR measurements were performed with cDNA samples from independent RNA isolations from three animals in each experimental group. The immunocytochemical and Western blotting data represent findings in four animals (rats and mice) in each experimental group. The numeric data were expressed as means ± SEM and statistically evaluated by using either Student's *t*-test or ANOVA with Duncan and/or Mann-Whitney tests at the 5% level of significance.

Results

Expression of rOat5 mRNA in the rat kidney; sex differences and effect of gonadectomy

Previous Northern blotting and end-point RT-PCR studies in M and F rats and mice demonstrated similar expression of Oat5 mRNA in the whole kidney tissue (Youngblood and Sweet, 2004). To re-investigate these findings, in our preliminary experiments (data not shown) we optimized the end-point RT-PCR expression signal for rOat5 mRNA at 27 and 23 cycles in the rat kidney cortex and outer stripe, respectively, and for ß-actin mRNA at 30 cycles in both tissues. A lower optimal number of cycles for end-point RT-PCR in the outer stripe indicated zonal differences (cortex < outer stripe) in the expression of rOat5 mRNA. With the same method we then compared the expression of rOat5

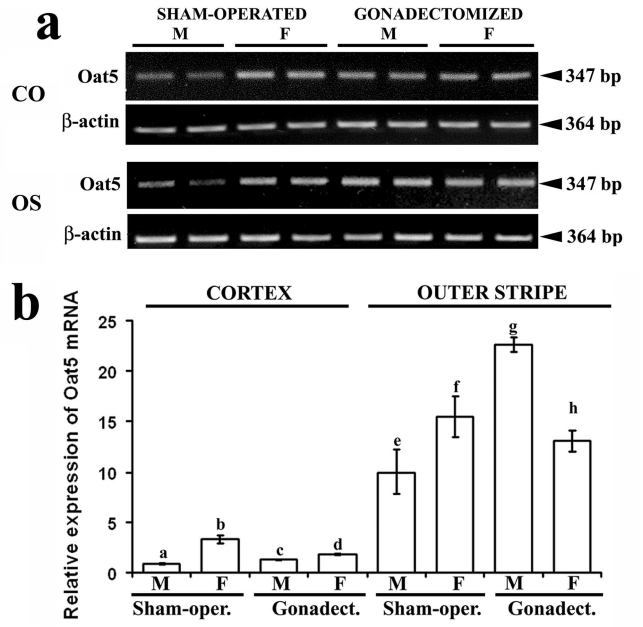

Fig. 1. End-point RT-PCR analysis of rOat5 and ß-actin mRNAs **(a)**, and real-time RT-PCR analysis of rOat5 mRNA **(b)** in the cortex (CO) and outer stripe (OS) of adult M and F rats; sex differences and effect of gonadectomy. **a.** The expression of rOat5 mRNA in both tissue zones was higher in F and upregulated by castration, whereas ovariectomy weakly downregulated in the CO but showed no significant effect in the OS. The expression of ß-actin mRNA was similar in both tissue zones of both genders, and was not affected by gonadectomy. Shown are the data for two independent RNA preparations from the animals of both sexes in each experimental group. **b.** The real-time RT-PCR data of rOat5 mRNA expression in the CO and OS tissue proved the presence of sex differences (F>M, ~3.5-fold in the CO and ~60% in the OS), upregulation by castration in the CO (~50%) and OS (~2.2-fold), and downregulation by ovariectomy in the CO (~50%) and less in the OS (~15%). The data (mean ± SEM) were obtained with the cDNA samples from independent RNA preparations from 3 animals of each experimental group. Statistics (ANOVA): a:b, a:c, a:e, b:d, b:f, e:f, and e:g: P<0.05; f:h, N.S.

mRNA and ß-actin mRNA in the kidney cortex and outer stripe of sham operated and gonadectomized adult M and F animals, and proved its unchangeability by sham operation (data not shown). Therefater, sex differences in, and the effect of gonadectomy on the rOat5 mRNA expression were tested in the renal cortex and outer stripe in more detail using the same (end-point) and real-time RT-PCR methods. A representative experiment with end-point RT-PCR (Fig. 1a) indicated that the expression of rOat5 mRNA in both tissue zones was: a) higher in F, b) upregulated by castration, and c) weakly downregulated (cortex) or unaffected (outer stripe) by ovariectomy, whereas the expression of ß-actin mRNA was unaffected by sex and gonadectomy in both tissue zones. Comparable and quantitative data were obtained with real-time RT-PCR in the cortex and outer stripe (Fig. 1b), showing: a) the Oat5 mRNA expression in the cortex is much lower than in the outer stripe; in sham-operated M, the expression in the outer stripe was ~11-fold higher than in the cortex, whereas in sham-operated F, the expression in the outer stripe was ~5-fold higher than in the cortex, b) sex differences exist in Oat5 mRNA expression (M<F) in both cortex and outer stripe; the difference was much higher in the cortex (~3.5-fold higher in F) than in the outer stripe (~60% higher in F), and c) in the cortex, castration significantly upregulated (~50%) and ovariectomy downregulated (~50%) the expression of rOat5 mRNA, whereas in the outer stripe, the changes followed the same pattern (~2.2-fold upregulation and ~15% downregulation, respectively). Further studies were designed to characterize the role of sex hormones in causing sex differences in renal rOat5 at the level of protein and mRNA.

Characterization of rOat5-ab by Western blotting and immunocytochemistry in rats

The immunochemical analysis of rOat5 in the rat kidney was performed with the rOat5-ab after proper characterization (Fig. 2). Western blotting of TCM isolated from the F kidney outer stripe in reducing (+ßME) and non-reducing (-ßME) conditions at different heating temperatures gave very different results. After heating at 95°C for 5 min in reducing conditions, only a weak band at ~85 kDa was visible, whereas in other conditions, the antibody labeled one major, peptide-blockable protein band of ~72 kDa, which was best exposed after heating at 37°C for 30 min in non-reducing conditions (Fig. 2a). In some conditions, a few other protein bands of much weaker density and lower relative molecular mass (Mr) were also observed. The major protein band was not compact, but rather consisted of a few bands concentrated around 72 kDa. After applying the optimal retrieval technique in tissue cryosections (details described in Material and Methods), the rOat5-ab strongly stained the brush-border of proximal tubule S3 segments in the outer stripe (Fig. 2b, -P); this staining was abolished by the immunizing peptide (Fig. 2b, +P).

In an additional testing of the antibody specificity, we performed immunochemical studies with isolated TCM from, and with cryosections of the rat liver tissue. Previous studies indicated an absence of Oat5 mRNA in this organ (Youngblood and Sweet, 2004). The negative results with both methods (data not shown) confirmed an absence of a significant rOat5 protein content in the rat liver. The optimal conditions, defined in the preceding experiments in rat kidneys, were used in all further immunochemical studies.

Zonal and sex differences in the renal expression of rOat5 protein in rats

Zonal and sex differences in the expression of renal rOat5 protein were studied by Western blotting in isolated membranes from the cortex, outer stripe, or whole kidney, and by immunocytochemistry in tissue cryosections (Fig. 3). As shown in Fig. 3a and b, in TCM from the renal cortex and outer stripe the rOat5-related 72 kDa protein band was stronger in F than in M. Densitometric evaluation of these bands revealed ~5-fold and ~60% higher abundance of the protein in the cortical and outer stripe membranes, respectively, in F compared with M, which is a good match with the data of mRNA expression shown in Fig. 1. Sex differences in the abundance of rOat5 protein were also visible in BBM isolated from the whole kidney homogenates; BBM from the F kidneys contained twice as much rOat5 protein as the membranes from the M kidneys, whereas the abundance of α-actin protein was the same in membranes from both genders (Fig. 3c,d). Localization in BBM, zonal expression, and sex differences in the rOat5 protein were further confirmed by immunostaining of tissue cryosections (Fig. 3e); in M, the staining was apical but weak in some cortical convoluted proximal tubules (S1/S2 segments; Cortex, arrows) and stronger in S3 segments localized in the medullary rays (not shown) and outer stripe (arrows). In F, the apical domain of most cortical convoluted proximal tubules was more or less stained (Cortex, arrows), while the S3 segments in medullary rays (not shown) and outer stripe (arrows) were strongly stained. Other parts of the nephron in rats of both sexes were not stained (data not shown). These data thus indicate that in the rat kidney, rOat5 is localized to the brush-border along the entire proximal tubule, being strongest in the S3 segment, thus causing zonal differences in its expression (cortex < outer stripe), and exhibits strong F-dominant sex differences in its abundance in both tissue zones.

Effect of gonadectomy on the renal expression of rOat5 protein in rats

M and F rats were either sham operated or gonadectomized, the abundance of rOat5 protein was determined by Western blotting in isolated TCM from the kidney cortex and outer stripe, and the intensity of immunostaining in the cortical and outer stripe tubules

was estimated in tissue cryosections (Fig. 4). As shown in Fig. 4a (Western blots) and 4b (densitometric evaluation of the protein bands), in comparison with the respective data in sham-operated animals, castrated M exhibited a significantly higher abundance of the rOat5 protein in isolated TCM from both the cortex and outer stripe, whereas ovariectomy in F weakly (vs. sham operated, N.S.) downregulated this expression only in

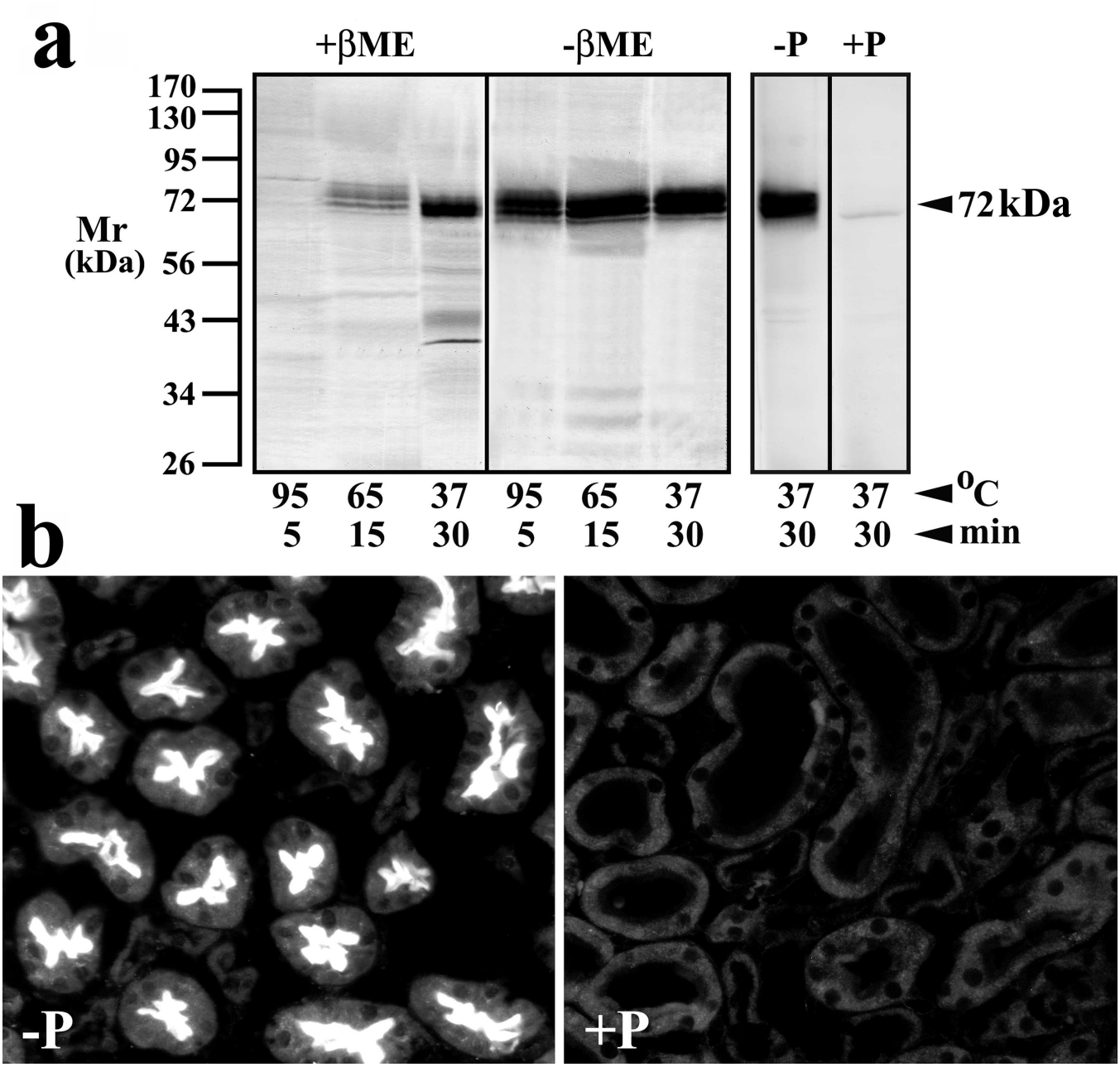

Fig. 2. Testing of rOat5-ab specificity by immunoblotting **(a)** and immunocytochemistry **(b)** in the samples from rat kidney. **a.** TCM, isolated from the F kidney outer stripe, were prepared in reducing (+ßME)) or nonreducing (-ßME) conditions at the indicated heating temperatures for 5 min (95°C), 15 min (65°C) or 30 min (37°C), and blotted with the rOat5-ab (-P) or with the antibody that had been preincubated with the immunizing peptide (+P). In reducing conditions, the intensity of several labeled bands was weak and increased with decreasing temperature, whereas in nonreducing conditions, only a single band of ~72 kDa was strongly labeled. In optimal nonreducing conditions (-ßME, 30 min at 37°C), the 72 kDa protein band (-P) was absent after the peptide block (+P). The lanes contained 60 μg protein. **b.** By immunocytochemistry, the rOat5-ab strongly stained the brush border in proximal tubule S3 segments in the outer stripe (-P), and this staining was blocked by the immunizing peptide (+P).

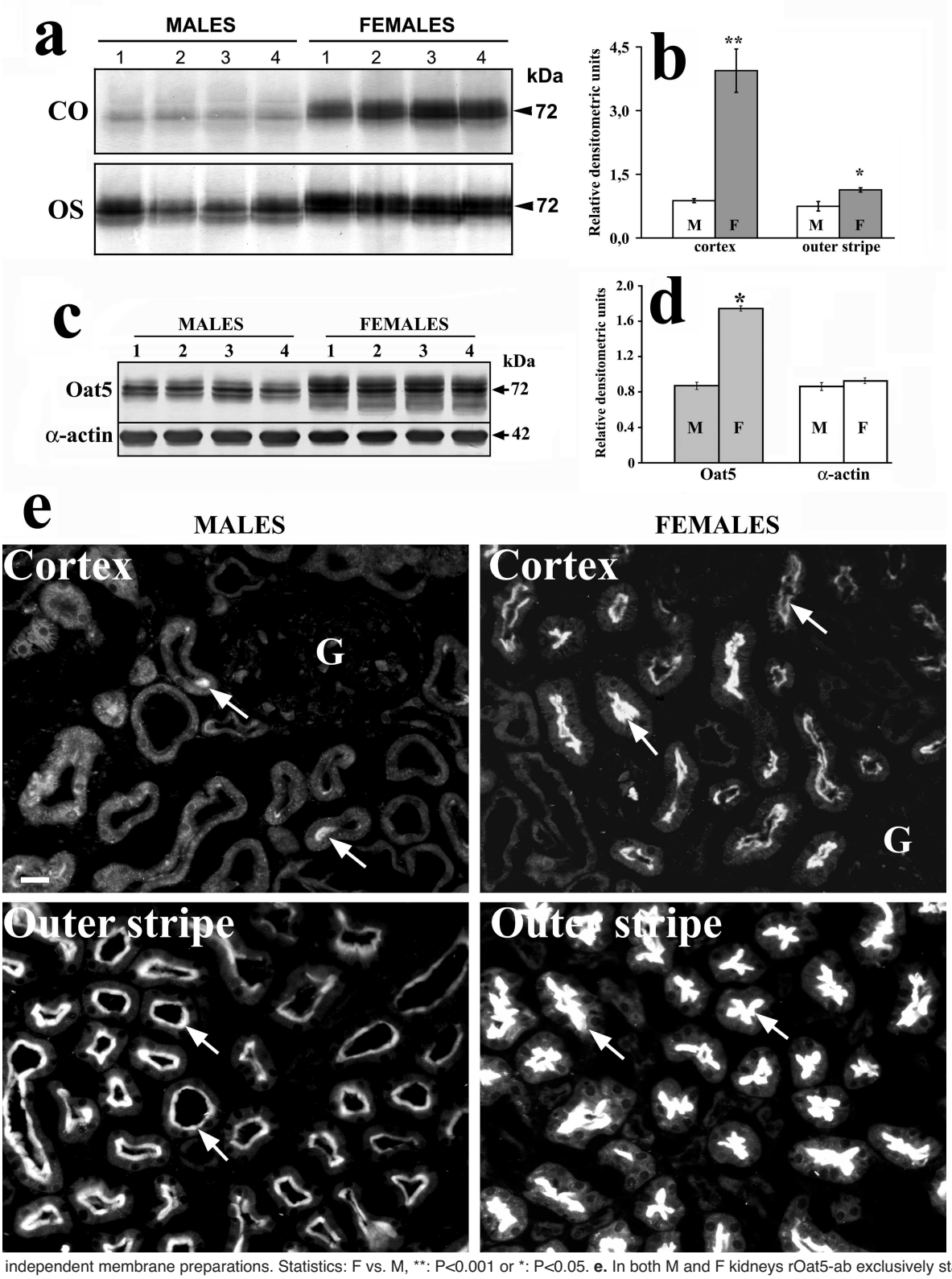

Fig. 3. Zonal and sex differences in the expression of rOat5 protein in the rat kidney; demonstration by Western blotting in isolated membranes (**a-d**) and by immuno-cytochemistry in tissue cryosections **(e)**. **a.** TCM, isolated from the cortex (CO) and outer stripe (OS) of male (M) and female (F) kidneys, were blotted with the rOat5-ab. In both tissue zones, the labeled 72 kDa protein band was stronger in F, indicating the presence of sex differences (M<F). In both sexes, the band was stronger in the OS, reflecting the presence of zonal differences (CO<OS). **b.** Densitometric data of the bands shown in **(a)**. **c.** BBM, isolated from the total M and F kidneys, were blotted with the rOat5-ab and α-actin-ab; the 72 kDa protein band (rOat5) was stronger in F, whereas the 42 kDa α-actin protein band had similar density in the membranes from both sexes. **d.** Densitometric data (means ± SEM; N = 4 in each bar) of the bands shown in **(c)**. The lanes contained 60 (TCM) or 40 (BBM) μg protein of the independent membrane preparations. Statistics: F vs. M, **: P<0.001 or *: P<0.05. **e.** In both M and F kidneys rOat5-ab exclusively stained the brush-border of proximal tubules (arrows); glomeruli (G), distal segments of the nephron, and various non-nephron structures (not labeled), were not stained. In M kidneys, the brush-border of cortical convoluted tubules was stained weakly (Cortex; arrows), whereas the brush-border of S3 segments in the outer stripe (arrows) and medullary rays (not shown) was stained stronger. In F kidneys, the pattern of brush-border staining in proximal tubules exhibited similar zonal differences (cortical convoluted tubules < S3 segments in the OS and medullary rays), but the staining intensity was stronger than in M, proving the presence of sex differences (M < F) in both zones. Bar: 20 μm.

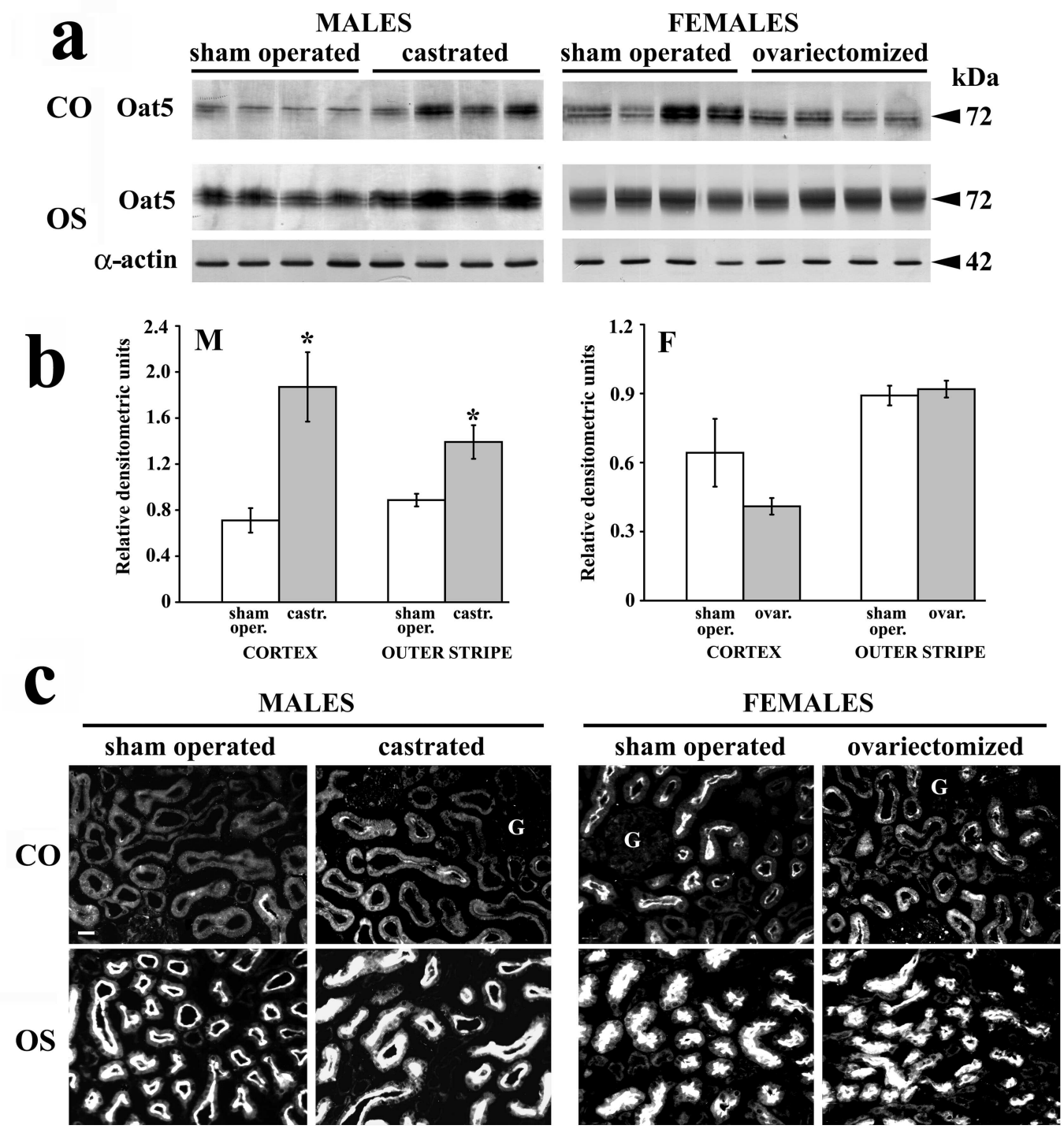

Fig. 4. Effect of gonadectomy in M and F rats on the expression of rOat5 protein in the kidney; Western blot **(a, b)** and immunocytochemical **(c)** analysis. **a.** TCM, isolated from the cortex (CO) and outer stripe (OS) of sham operated and castrated M or sham-operated and ovariectomized F, were blotted with rOat5-ab. α-Actin was used as an indicator of protein loading. **b.** Densitometric data (means ± SEM; N=4 in each bar) of the 72 kDa protein bands shown in **a**. In M, castration significantly upregulated the density of rOat5 protein band in both tissue zones (vs. sham operated, *P<0.05), whereas in F, ovariectomy caused a limited (25%), but nonsignificant downregulation of the band density in the CO, and no effect in the OS. The α-actin protein band (42 kDa) was not affected by gonadectomy, and indicated similar protein loading in the lanes. **c.** In comparison with the brush-border immunostaining in sham-operated animals, in castrated M the intensity of staining was stronger in both CO and OS, whereas in ovariectomized F, the staining was not visibly changed in both CO and OS. Bar: 20 μm.

the cortex. The 42 kDa α-actin protein band in the cortex (not shown) and outer stripe (Fig. 4a) was not affected by gonadectomy. By immunocytochemistry (Fig. 4c), in the cortex of sham-operated males only some tubules were weakly apically positive, whereas in castrated M, most cortical tubules were more or less weakly positive. In the outer stripe of castrated rats, the apical domain of S3 segments was stained much stronger than in sham-operated animals. In F, however, the staining intensity was not visibly affected by ovariectomy in both tissue zones. The data from this experiment indicate that androgens may be major determinators (inhibitors) of the renal Oat5 expression in rats.

Renal expression of rOat5 mRNA and protein in sex hormone-treated castrated rats

To further define the effects of sex hormones on renal rOat5, castrates were treated with either oil (controls) or various sex hormones (testosterone, estradiol, progesterone) for two weeks, and the expression of rOat5 mRNA by end-point and real-time RT-PCR (Fig. 5) and its protein in the renal cortical and outer stripe tissues and isolated membranes by immunochemical methods (Fig. 6) was determined.

The representative data of end-point (Fig. 5a) and real-time RT-PCR data (Fig. 5b, shown only for measurements in the outer stripe) showed that the expression of rOat5 mRNA in both tissue zones was strongly upregulated by castration (in agreement with the data in Fig. 1), whereas in castrated animals it was downregulated by testosterone treatment, and additionally upregulated by estrogen (weakly) and progesterone treatment, while the expression of ß-actin mRNA was not clearly affected by hormonal treatment. A similar pattern of the data was obtained by Western blotting of isolated TCM (Fig. 6a,b) and by immunostaining of tissue cryosections (Fig. 6c). As shown in Fig. 6a and b, the abundance of rOat5 protein in TCM from both tissue zones was upregulated by castration (in agreement with the data in Fig. 4a,b), whereas the enhanced protein expression in castrates was completely reversed by testosterone treatment. However, the treatment with estradiol and progesterone resulted in no effect in the cortex and in only a limited stimulatory effect (20-30%) in the outer stripe (Fig. 6a,b). The abundances of α-actin protein and 31 kDa V-ATPase subunit were not affected by hormonal treatment (Fig. 6a). The immunoblotting pattern of rOat5 protein was reproduced by the staining intensity in proximal tubules (Fig. 6c). Overall, these experiments showed that in the rat kidney, the expression of rOat5 is strongly inhibited by androgens and weakly stimulated by F sex hormones.

Expression of rOat5 mRNA and protein in the kidney of prepubertal rats

To test sex differences in the renal rOat5 in prepubertal rats, its expression at mRNA and protein levels was compared in TCM and RNA isolated from whole kidneys and in tissue cryosections from prepubertal and adult animals (Fig. 7).

As shown in Fig. 7a and b, the density of the rOat5 protein band in TCM from the adult M and prepubertal M and F was similar and much lower than in the adult F. A comparable pattern of data was observed for the staining intensity of proximal tubule S3 segments in the outer stripe (Fig. 7c), and for the rOat5 mRNA expression determined by end-point (Fig. 7d) and real-time RT-PCR (Fig. 7e). Therefore, the expression of renal rOat5 in prepubertal rats was low, similar to that in adult M, and exhibited no sex differences.

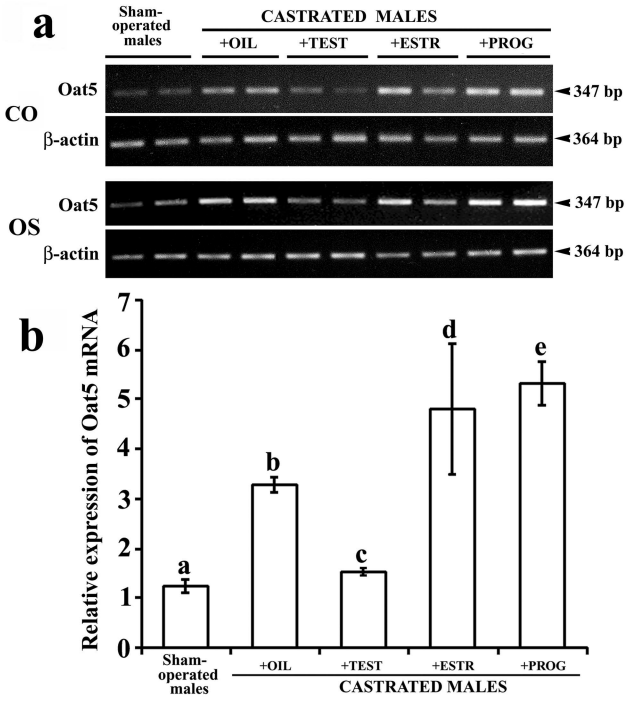

Fig. 5. Effect of the sex hormone treatment in castrated rats on the expression of rOat5 and ß-actin mRNA in the kidney cortex (CO) and outer stripe (OS); end-point RT-PCR **(a)** and real-time RT-PCR **(b)** analysis. **a.** End-point RT-PCR data: in both tissue zones, the expression of rOat5 mRNA was upregulated by castration (c.f. sham-operated, oil-treated M with the oil-treated castrates). In comparison with the expression in oil-treated castrated rats, testosterone strongly downregulated, whereas estradiol and progesterone upregulated the mRNA expression. The expression of ß-actin mRNA was not hormone dependent, and indicated similar loading of cDNA. **b.** The real-time RT-PCR data show the expression of rOat5 mRNA in the OS, and were performed with the cDNA samples from independent RNA preparations from 3 animals in each experimental group. The rOat5 mRNA expression (mean ± SEM) in oil/hormone-treated castrated rats was shown relative to that in sham-operated M; the expression was upregulated by castration, reversed by testosterone treatment, and additionally upregulated (40-50%) by estrogen and progesterone treatment. Statistics (ANOVA): a: b, b: c, and b: e, $P<0.05$; a: c, b: d and d: e, N.S.

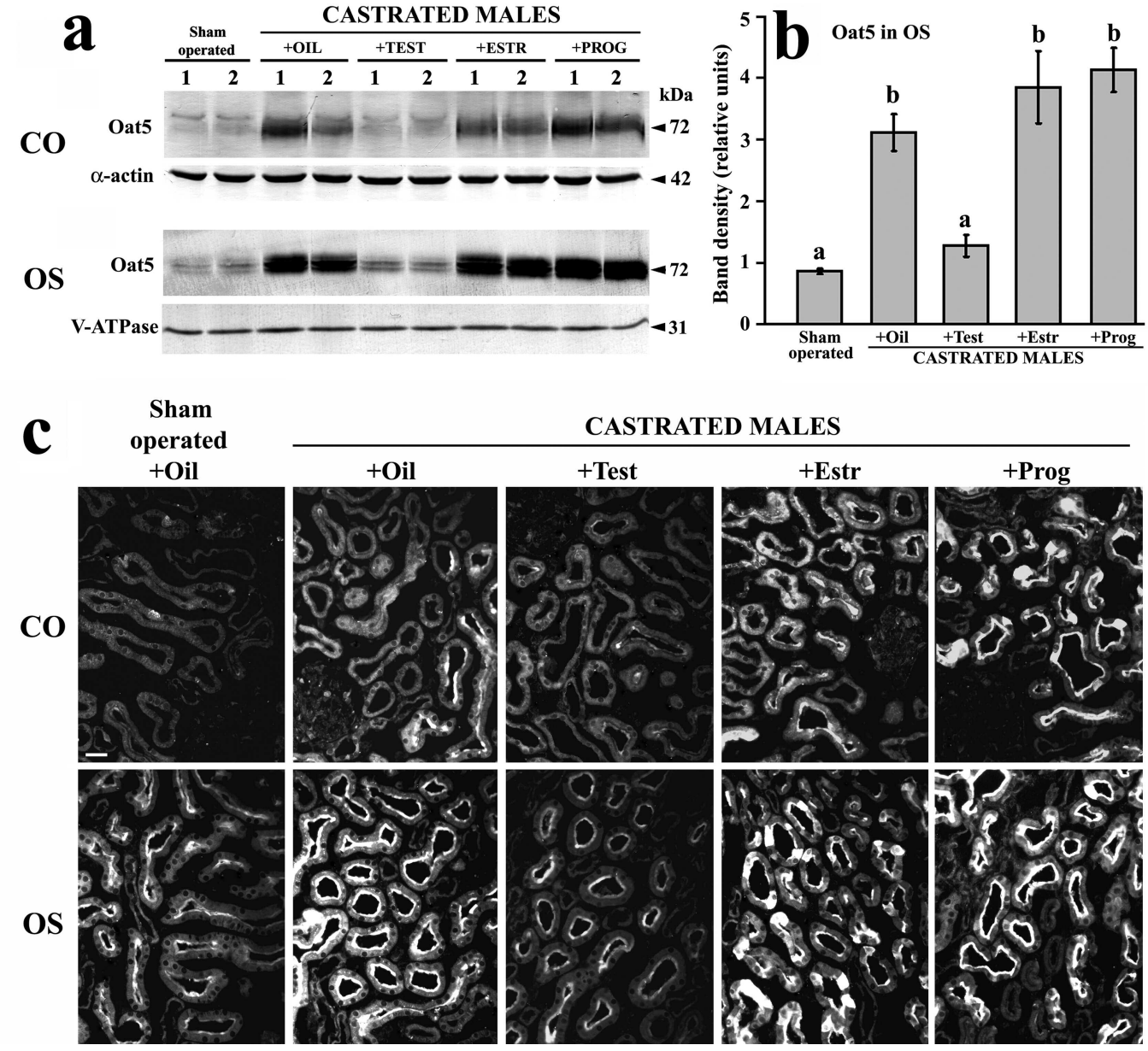

Fig. 6. Effect of the sex hormone treatment in castrated rats on the expression of rOat5, α-actin, and V-ATPase proteins in the kidney tissue zones; Western blot **(a, b)** and immunocytochemical **(c)** analysis. **a.** TCM, isolated from the cortex (CO) and outer stripe (OS) of sham-operated (oil-treated) and oil- or hormone-treated castrated M, were blotted with the rOat5-ab. The labeling with α-actin-ab and V-ATPase-ab was used as a control of protein loading. In both CO and OS, in comparison with the rOat5 protein band (72 kDa) in sham-operated (oil-treated) M, the density of this band in castrated animals was: a) strongly upregulated in oil-treated rats, b) reversed by testosterone treatment, and c) weakly (25-30%; vs. oil-treated catrates, N.S.) upregulated by estradiol and progesterone treatment. The abundances of α-actin (CO) and V-ATPase (OS) proteins were not affected by hormonal treatment, and indicated similar protein loading in the lanes. Each lane contained 60 μg proteins of the independent membrane preparations. **b.** Densitometric data (means ± SEM; N=4 in each bar) of the 72 kDa (rOat5) protein band in the OS. Statistics (ANOVA): a: b, $P<0.05$; a: a and b: b, N.S. **c.** The immunostaining of rOat5 in the CO and OS proximal tubules. In comparison with the brush-border staining in sham-operated (oil-treated) M, the staining in castrated (+oil-treated) M was stronger. The testosterone treatment decreased, whereas the estradiol and progesterone treatment slightly increased the staining in castrated M. In oil-, estradiol-, and progesterone-treated castrated M, many proximal tubule cells also exhibited a strong intracellular granular staining (c.f. panel of estradiol-treated animals, OS). Bar: 20 μm.

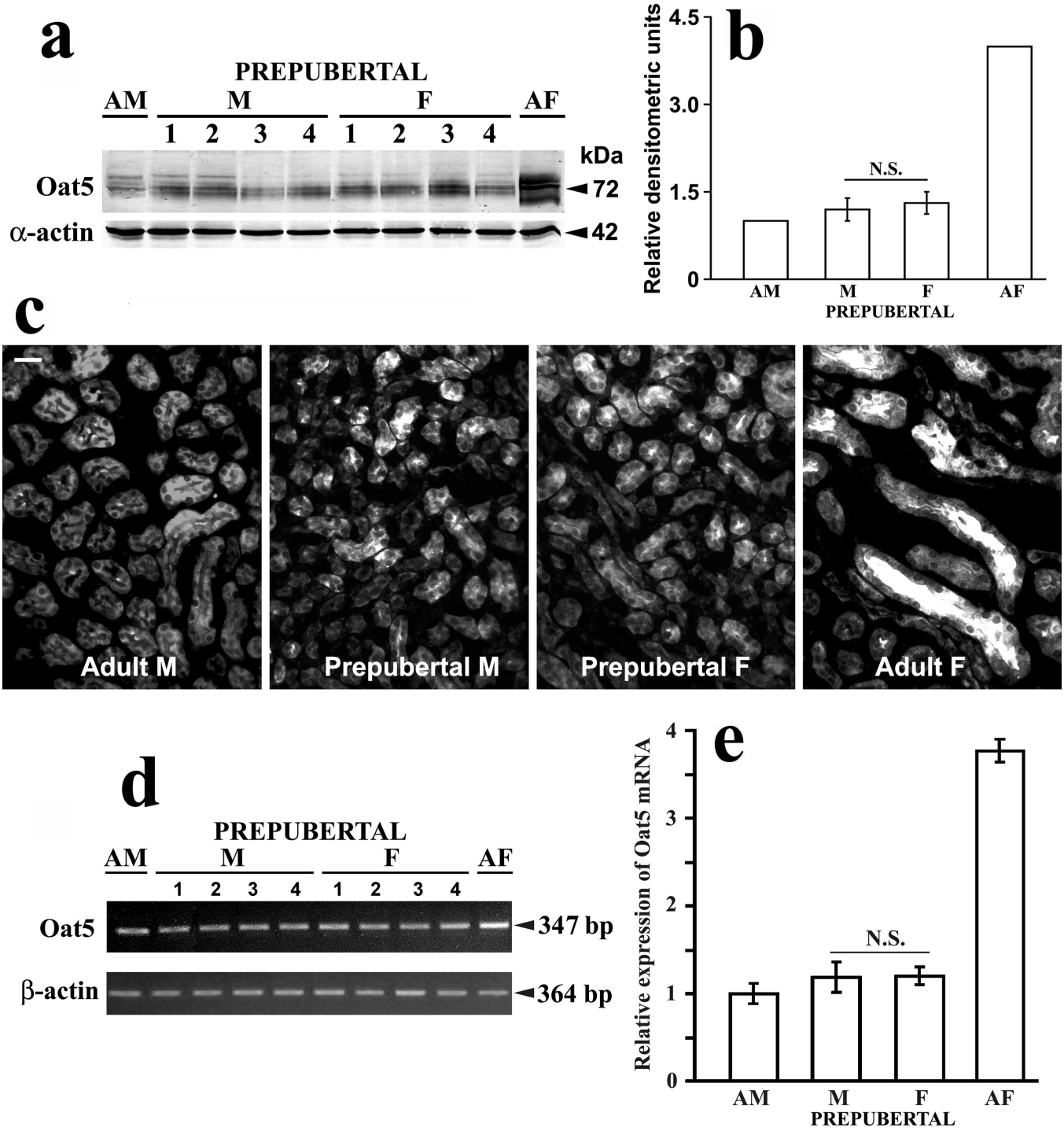

Fig. 7. Expression of rOat5 in the kidney of prepubertal male (M) and female (F) rats: Western blotting (**a, b)**, immunocytochemical (**c**), end-point RT-PCR (**d**), and real-time RT-PCR **(e)** analysis. **a.** Western blot: TCM were isolated from the whole kidneys of four prepubertal M and F rats and, for comparative reasons, from (one of each) adult M (AM) and F (AF) rats, and blotted with the rOat5-ab and α-actin-ab. The density of 72 kDa Oat5 protein band in prepubertal M and F rats was comparable to that in the adult M but much weaker than that in the adult F, and exhibited no sex difference. The α-actin protein band was similar in all membrane preparations, indicating proper loading of the lanes. Each lane contained 60 μg proteins of the independent membrane preparations. **b.** Densitometric data of the 72 kDa protein band shown in **a**. Adult M (AM) and F (AF) are represented by a single datum, whereas the bars for prepubertal M and F indicate the band density (mean ± SEM) in 4 independent membrane preparations. N.S., not significant. **c.** The staining intensity of rOat5 in brush-border of the OS tubules (S3) in prepubertal M and F rats was similar, sharper that in the adult M, but much weaker than in the adult F. Bar: 20 μm. **d.** End-point RT-PCR was performed with RNA isolated from the whole kidneys; the rOat5 mRNA band in prepubertal rats was: a) similar in M and F, b) similar to that in the adult M, and c) weaker than that in the adult F. **e.** The real-time RT-PCR data (mean ± SEM of a duplicate measurement in individual cDNA preparations) were related to the mRNA expression in the adult M (1±0.2 relative unit, N=2). The expression in prepubertal M (1.2±0.2; N=4) and F (1.2±0.1; N=4; vs. prepubertal M, N.S.) was similar to that in the adult M, but much lower than in the adult F (3.8±0.1 relative units, N=2).

Immunochemical characterization of rOat5-ab in the mouse kidney

By checking the proper database (http://www.ncbi.nlm.nih.gov/entrez, BLASTP), we have found out that the immunizing synthetic peptide corresponding to C-terminal sequence of the rOat5 protein (XP_342012; amino acids 538-551: REVKKDAVAKVTPF) is 71% (10 out of 14 amino acids) similar to the corresponding peptide sequence in the mouse Oat5 (NP_659034; amino acids 538-551: KEAKKDVVAKVTPL; the underlined amino acids are different from those in the rOat5 peptide, but have similar chemical properties). We have therefore tested the rOat5-ab for its efficiency to recognize the same protein (mOat5) in the mouse kidney.

As shown in Fig. 8a, the immunoblot of BBM isolated from the whole F mouse kidney showed that the rOat5-ab labeled a single, immunizing peptide-blockable ~72 kDa protein band. In the outer stripe of F kidney, the antibody strongly stained the brush-border and (weaker) some intracellular organelles in the proximal tubule S3 segments (Fig. 8b); this staining was also blocked by the immunizing peptide (Fig. 8c). In similarity with the findings in rat liver, the antibody did not label a protein band in TCM isolated from the mouse liver, and exhibited no staining in tissue cryosections of this organ (data not shown). We thus conclude that the rOat5-ab can be used to label/stain the respective transporter also in the mouse kidney.

Zonal and sex differences in the renal expression of Oat5 protein in mice

The Western blot of BBM isolated from the whole mouse kidneys showed the presence of F-dominant gender differences in the abundance of mOat5 protein (Fig. 9a); the ~72 kDa protein band was ~4-fold stronger in F than in M kidneys (Fig. 9b). By immunocytochemistry (Fig. 9c): a) only the proximal tubules (all segments) in the cortex and outer stripe were stained; other parts of the nephron were negative, b) proximal tubules in both zones exhibited staining at the apical (brush-border) domain and also a variable intracellular granular staining, which was most prominent in the tubules of F outer stripe (c.f., inset), c) in M mice, the staining of proximal tubules in the cortex was weaker than in the outer stripe, d) in F mice, the apical and intracellular staining in the proximal tubules was much weaker in the cortex than in the outer stripe, and e) the staining in both zones was stronger in F than in M. These data thus showed that zonal (cortex < outer stripe) and sex (M < F) differences in the renal expression of Oat5 exist also in mice.

Discussion

Using two independent methods to study the expression of mRNA (end-point and real-time RT-PCR) and two immunochemical methods to study the expression of proteins (Western blotting and immuno-

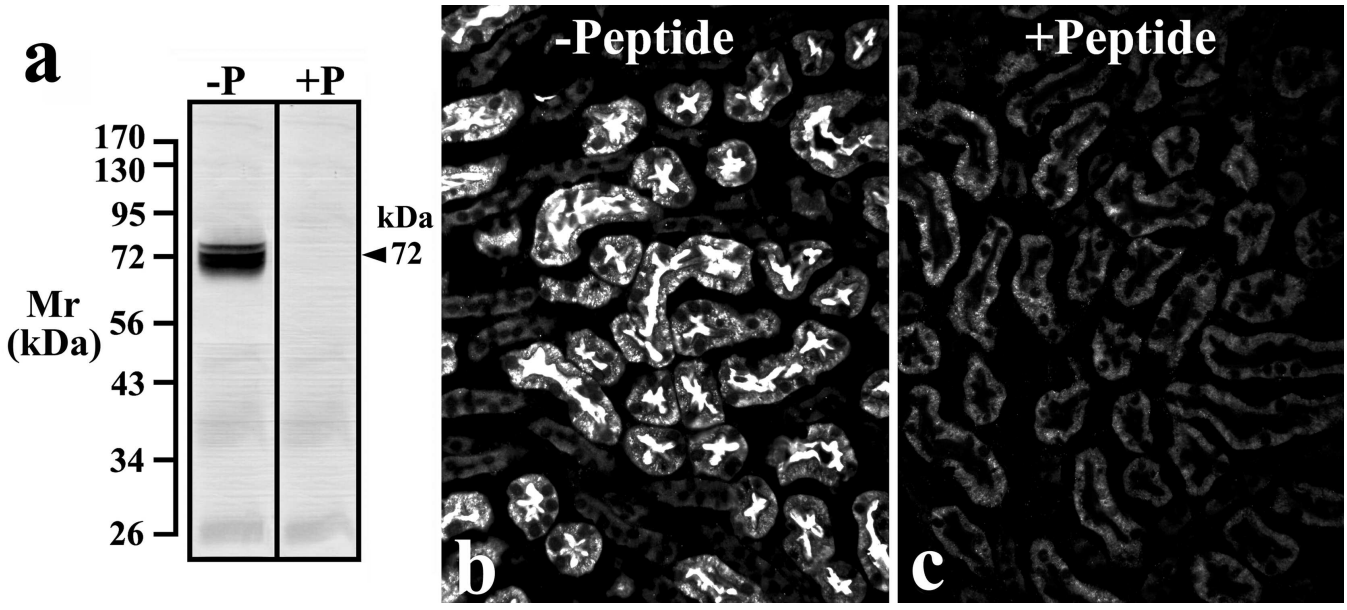

Fig. 8. Testing of rOat5-ab specificity by immunoblotting **(a)** and immunocytochemistry **(b, c)** in the samples from mouse kidney. **a.** BBM (40 μg protein/lane), isolated from the total F kidney homogenate, were prepared (-ßME, 30 min, 37°C) and blotted with the rOat5-ab (-P) or with the antibody that had been preincubated with the immunizing peptide (+P); only one band of ~72 kDa was strongly labeled that was blocked by the peptide. By immunocytochemistry, the rOat5-ab stained brush-border (brightly) and nonidentified intracellular organelles (weaker) in the proximal tubule S3 segments in the OS **(b)**. This staining was abolished by the immunizing peptide **(c)**.

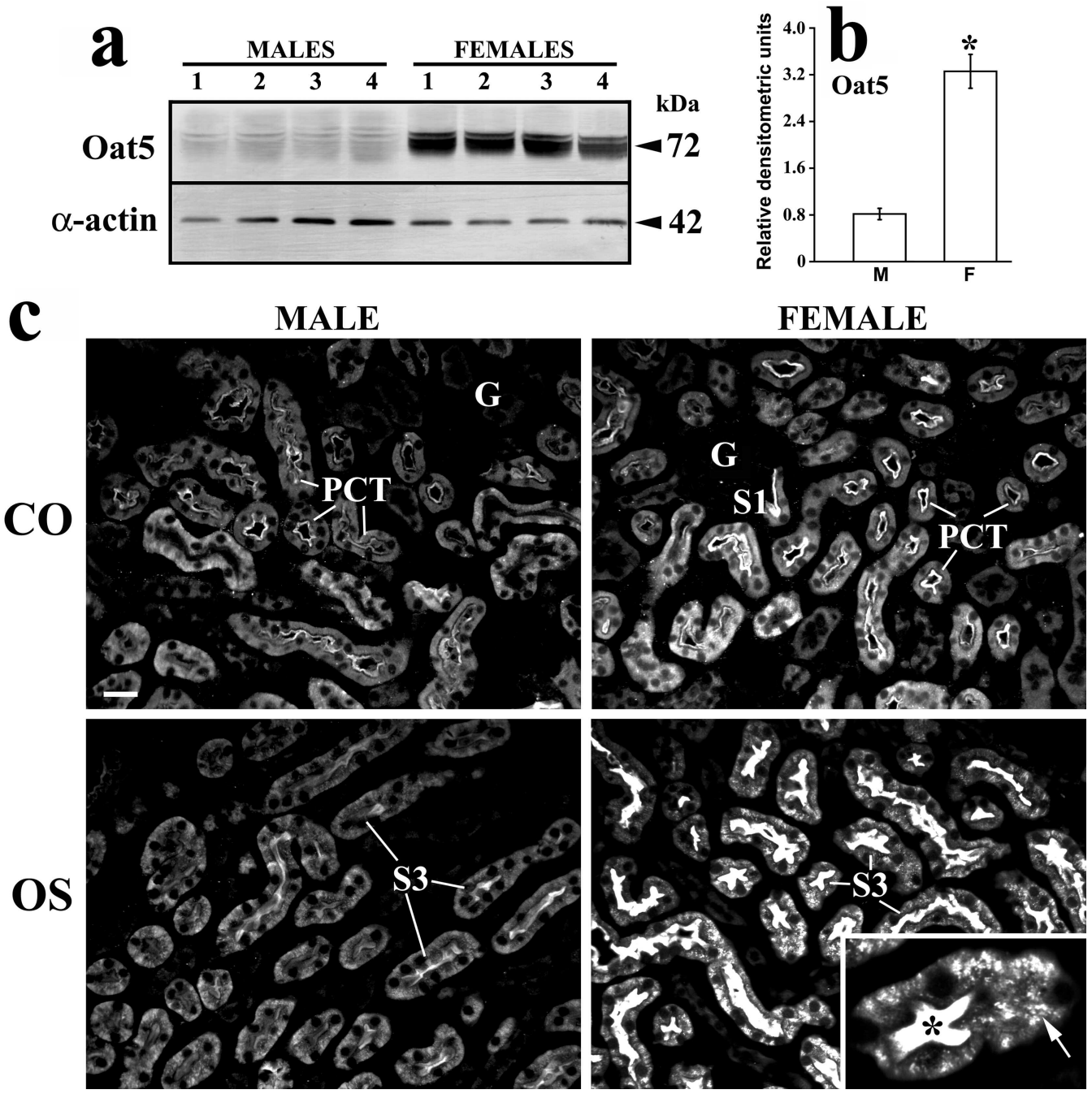

Fig. 9. The expression of Oat5 protein in the mouse kidney as revealed by Western blotting **(a, b)** and immunocytochemical **(c)** analysis. **a.** Sex differences in the expression of Oat5 protein in BBM isolated from the whole mouse kidney; the Oat5-related 72 kDa protein band in M was much weaker than in F, whereas the 42 kDa α-actin protein band showed no major differences, indicating similar loading of the lanes. Each lane contained 60 μg protein of the independent membrane preparations. **b.** Densitometric evaluation of the 72 kDa protein band (mean ± SEM; N=4 in each experimental group) shown in **a**. *Vs. M, P<0.01. **c.** Immunolocalization of Oat5 protein in the kidney cortex (CO) and outer stripe (OS) of M and F mice; zonal and sex differences. In both sexes, only the brush-border and intracellular organelles in proximal tubules were stained. The staining in proximal convoluted tubules in the CO (PCT) was weaker than in the OS (S3), showing zonal differences. In both zones, the staining was stronger in F. Inset: In the S3 in F, brightly stained was brush-border (asterisk) and intracellular organelles (granular staining indicated by an arrow). G, glomerulus; S1, the initial proximal tubule segment; S3, the straight proximal tubule segment; Bar: 20 μm.

cytochemistry), in this work we re-investigated in detail the expression of rOat5 in M and F rat kidneys. In addition, the immunochemical methods were used to characterize the expression of Oat5 protein in M and F mouse kidneys.

In rat kidneys, the data obtained with all four methods showed a congruent pattern of rOat5 mRNA and protein expression, indicating that rOat5 in this organ is principally regulated by sex hormones at the level of transcription. Thus, at the level of mRNA and/or protein the rat renal rOat5 exhibited: 1) a temperature- and reducing conditions-sensitive protein band of ~72 kDa, with a complex appearance, 2) in adult rats, distinct localization of the protein in BBM of the cortical and outer stripe proximal tubule segments (S1 ~ S2 < S3), 3) strong zonal (cortex < outer stripe) and sex differences (M < F) in the expression, 4) in adult rats, strong upregulation of the expression by castration, and no significant effect of ovariectomy, 5) in castrated adult rats, strong downregulation of the expression by testosterone treatment and weak but variable upregulation by estrogen and progesterone treatment, and 6) in prepubertal rats, low and similar expression to that in the adult M, and sex-independent. The immunochemical experiments in the mouse kidneys showed that the mOat5 protein: 1) exhibits also a complex band of ~72 kDa, 2) is localized to the BBM along the entire proximal tubule, with low expression in M (S1 = S2 = S3) and higher expression in F (S1 = S2 < S3), and also in intracellular organelles, which are most abundant in the S3 cells, and 3) exhibits zonal differences in F (cortex < outer stripe) and sex differences in both tissue zones (M < F).

Unlike previous findings, which on the Western blot indicated Mr of ~65 kDa (Anzai et al., 2005) and ~85 kDa (Kwak et al., 2005) protein bands for rOat5 and mOat5, respectively, in our optimal conditions the renal protein in both species appeared as the ~72 kDa band. The intensity of the band was weaker at harsher denaturing conditions, obviously due to higher sensitivity of the antibody-binding epitope to reducing conditions at higher temperatures. Accordingly, in reducing conditions similar to those used by Kwak et al. (2005) (heating at 95°C/5 min, +,ME), we also observed only a faint protein band of ~85 kDa. In other conditions, the complex ~72 kDa band probably reflected different glycosylation states of the parent protein. The rodent Oat5 protein consists of 551 amino acid residues (Youngblood and Sweet, 2004) which in nonglycosylated state should give a band of Mr ~61 kDa, and which may have been detected in a previous *in vitro* study in the transfected cells (Anzai et al., 2005). However, *in vivo*, four asparagine residues (Asn-39, Asn-56, Asn-62, and Asn102) within the protein may be glycosylated to a different extent and give molecules with slightly different electrophoretic mobility. Similar observations were recently obtained for the renal rOat1 rOat2, rOat3, and mOat2 proteins (Ljubojevic et al., 2004, 2007).

Previous immunohistochemical studies localized the Oat5 protein to the BBM of proximal tubule S3 segments in the outer stripe of the rat and mouse kidney (Anzai et al., 2005; Kwak et al., 2005), whereas the end-point RT-PCR study in isolated tubules from the rat kidney localized the Oat5 mRNA predominantly in the proximal tubule S2 and S3 segments (S2 < S3), but a small expression was also detected in the medullary ascending limb and cortical collecting duct (Anzai et al., 2005). The sex of the animals used in those studies was not indicated, whereas possible sex differences in the expression of renal Oat5 mRNA in rats and mice were previously tested, but not detected (Youngblood and Sweet, 2004). In our immunochemical studies, however, we demonstrated the Oat5 protein largely in the BBM of proximal tubules, but with different, sex-dependent abundances in various tubule segments in adult rats and mice. In adult rats, the transporter was localized to the proximal convoluted tubules (S1/S2 segments) in the cortex, and to the proximal straight tubules (S3 segments) in the outer stripe and medullary rays, with the sequence S1 ~ S2 < S3 that caused zonal differences (cortex < outer stripe). In all these segments, F had a significantly higher protein expression than M, thus resulting in sex differences (M < F). In adult mice, a slightly different distribution and expression was observed; the mOat5 protein was also localized in the BBM of the entire proximal tubule, but exhibited the expression that was in M very low and similar in all segments (S1 = S2 = S3), whereas the expression in F was much higher than in M, similar in the S1 and S2, and highest in the S3. Except in the BBM, mOat5 in the F S3 was also detected in numerous intracellular organelles. The origins of these organelles were not further studied; they may represent a recycling pool of mOat5 that regulates the abundance of transporters in the apical membrane by endo- and exocytosis.

Our data in gonadectomized rats and in castrated rats treated with various sex steroids showed that androgen hormones clearly inhibit the expression of renal rOat5, while estrogens and progesterone may have only a weak stimulatory effect. These opposite hormonal effects may thus be responsible for the F-dominant sex differences in the expression of rOat5 in adult animals. The low and similar expression of rOat5 in prepubertal rats of both sexes indicates that sex differences appear after puberty. However: a) the low expression in prepubertal animals was similar to that in the adult M, but much lower than that in the adult F, and b) ovariectomy had none, whereas the treatment of castrates with F sex hormones had only a limited upregulating effects. Overall these data indicate that after puberty, the role of androgens is to keep the renal rOat5 expression low in M, whereas in F some other factors, possibly growth hormone or some other hormones, or a combination of different hormones, may additionally stimulate the expression of this transporter. With such a pattern of sex hormone-dependency, rOat5 closely resembles rOat2 in the rat kidney, which in the BBM of S3 also exhibits sex

differences (M < F) due to strong androgen inhibition and weak estrogen and progesterone stimulation (Ljubojevic et al., 2007). A finding of similar, F-dominant sex differences in the expression of renal Oat5 in mice indicate that the sex hormone-driven regulation of the transporter in mice is basically similar to that in rats, as also found for Oat2 in the mouse kidney (Ljubojevic et al., 2007).

All the rOat5-related data on zonal and sex differences in adult and prepubertal rats, as well as the data in variously treated rats, were confirmed at the level of both mRNA and protein expression, and were largely congruent, indicating that sex steroids play a principal role in controlling the renal rOat5 gene transcription, predominantly in the S3 segment. Although these effects of sex hormones are impossible to correlate with the expression of relevant sex hormone receptors in the rat (and mouse) proximal tubules (Sabolic et al., 2007), our recent search of the relevant database showed that the promotor region of the renal Oat5 in rats has 3 androgen-responsive elements (AREs), 2 estrogen-responsive elements (EREs) and 2 progesterone-responsive elements (PREs), whereas in mice, it has 4 AREs and 3 PREs, but no ERE (Sabolic et al., 2007). As shown by others, the sex hormone REs in the target gene promotor can exist in one or more copies, and represent binding sites for the respective sex hormone-receptor complexes that mediate their genomic actions via mobilization of various nuclear cofactors (coactivators and/or corepressors) and modification of local chromatin architecture. These local modifications result in inhibition (repression/downregulation) or activation (de-repression/upregulation) of the target gene transcription (Hall et al., 2001; Lee and Chang, 2003; Björnström and Sjöberg, 2005; Geserick et al., 2005). In rats, a strong androgen-driven inhibition and weak estrogen- and progesterone-driven stimulation of the rOat5 mRNA and protein expression indicate that at least one copy of each RE may be active; in rats the AREs act as inhibitory, whereas the EREs and PREs as stimulatory regulators. In mice (without EREs), however, the corresponding regulation may be mediated by the AREs (inhibitory) and PREs (stimulatory).

The functional role of Oat5 in the rodent kidneys *in vivo* is at the present unclear. When expressed in *Xenopus* oocytes, this succinate/OA exchanger exhibited no significant affinity for PAH, urate, and various other anionic and cationic drugs, but it showed strong affinity for OTA (2 μM), ES (2.2 μM) and DHEAS (3.8 μM) (Youngblood and Sweet, 2004; Anzai et al., 2005; Kwak et al., 2005). Having a high affinity for OTA, Oat5 may contribute to intracellular accumulation of this mycotoxin, which in rats was found to be highest in the proximal tubule S3 segment (Dahlmann et al., 1998), and which may be the initial site of OTA nephrotoxicity (Sweet, 2005; Zlender et al., 2009). The *in vivo* role of sex-dependent expression of Oat5 in the rodent proximal tubules is even less clear. Much higher renal expression of Oat5 in F rodents may have physiological relevance in homeostasis of ES and DHEAS. In the blood, ES and DHEAS may represent a pool (reservoir) of inactive steroids, which in the tissues can be enzymatically transformed into active hormones, such as estrone, estradiol, and DHEA (Eberling and Koivisto, 1994; Ranadive et al., 1998). Since both sulfated steroids are reabsorbed in the mammalian kidney (Kellie and Smith, 1957; Wright et al., 1978), proximal tubules in the F rodents may internalize the filtered ES and DHES (in exchange for intracellular succinate and α-ketoglutarate) with a higher rate due to a higher expression of Oat5 in the luminal membrane. The internalized compounds may exit the tubules via Oat3 localized in the proximal tubule BLM; Oat3 also exhibits a high affinity for ES and DHEAS (Sekine et al., 2006), and, based on the recent data in Oat3 KO mice, it plays a major role in the transport of sulfated OA through the BLM (Sweet et al., 2002). However, other studies have documented a number of other transporters that reside in the mammalian nephron and may contribute to overall renal handling of ES and DHEAS (Li et al., 2006, and references there in; Yokoyama et al. 2008). Therefore, the exact role and importance of the sex-dependent expression of Oat5 for reabsorption of ES, DHEAS, and other OA in the rodent proximal tubules should be studied in the Oat5 KO animals. The existence of such animals has not been reported thus far.

In humans, an average blood concentration of ES was also higher in F, and was dependent on the phase in menstrual cycle; in the follicular phase, the levels in F and M were similar, whereas in the luteal phase, the level in F was 100% higher than in M (Ranadive et al., 1998). Moreover, M was found to excrete more steroid metabolites, including DHEA, in the urine than F (Shamim et al., 2000). However, since hOAT5 is absent from the human kidney, a similar function in transporting OTA, ES, DHEAS, and various other OA, may be served by hOAT4, which is also localized to the proximal tubule BBM (Ekaratanawong et al., 2004; Sekine et al., 2006; Burckhardt and Rizwan, 2007), but possible sex differences in its expression in the human kidneys are unknown.

Acknowledgements. We thank Mrs. Eva Heršak for excellent technical assistance, the company Transgenic Inc. (Kumamoto, Japan) for providing the rOat5-ab and the immunizing peptide, Dr. Sylvie Breton from Massachusetts General Hospital/Harvard Medical School in Boston, MA (U.S.A.) for donating the V-ATPase-ab, and Dr. Douglas H. Sweet from Department of Pharmaceutics, Virginia Commonwealth University, U.S.A., for critical reading of the manuscript. This work was supported by grant No. 0022-0222148-2146 from the Ministry of Science, Education and Sports, Republic of Croatia (I.S.).

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Accepted April 29, 2010