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Transgenic Male Mice Expressing Human Hydroxysteroid Dehydrogenase 2 Indicate a Role for the Enzyme Independent of Its Action on Sex Steroids

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Hydroxysteroid (17β) dehydrogenase 2 (HSD17B2) has been shown to inactivate both estrogens and androgens and activate 20α-hydroxyprogesterone to progesterone. In the present study, we generated transgenic (TG) mice ubiquitously expressing human HSD17B2. The TG mice produced showed growth retardation and delayed eye opening at the postnatal age. Disrupted spermatogenesis was evident in the presence of normal serum and intratesticular testosterone, progesterone, and normal circulating LH concentrations. A proper androgen action in the target tissues was confirmed by normal histological appearance of the prostate and epididymis. Furthermore, quantitative RT-PCR analysis indicated only a slight decrease in androgen-dependent gene expression in the prostate. The disrupted spermatogenesis was not associated with increased germ cell apoptosis as analyzed by caspase-3 activation. However, it resulted in infertility in the HSD17B2 TG males after the age of 3 months, and at the age of 6 months the seminiferous tubules showed a Sertoli cell-only phenotype. The data indicate that the growth retardation and disrupted spermatogenesis are not due to a lack of proper estrogen or androgen action. Interestingly, the testicular phenotype and some of the other phenotypic changes described are typically observed in mice with reduced action of retinoic acid signaling. This, together with the rescue of the testis phenotype by a synthetic retinoic acid receptor agonist (4-[(E)-2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid), suggests a role for HSD17B2 in the action of retinoids, in addition to its oxidative HSD17B activity on sex steroids. (*Endocrinology* 148: 3827–3836, 2007)

HYDROXYSTEROID (17β) DEHYDROGENASES (HSD17Bs) catalyze the conversion between 17-keto and 17β-hydroxysteroids using nicotinamide adenine dinucleotide phosphate or oxidation of nicotinamide adenine dinucleotide phosphate as cofactors. Among the sex steroids, the 17β-hydroxy forms (*e.g.* estradiol, testosterone, and 5α-dihydrotestosterone) are the highly active forms, whereas the corresponding 17-keto forms (estrone, androstenedione, and 5α-androstanedione, respectively) are biologically less active. HSD17B enzymes are expressed in many extragonadal tissues and are considered to play important roles in the *in situ* synthesis and metabolism of estrogens and androgens in various peripheral tissues including sex steroid target tissues (1, 2). Local activation and inactivation of sex steroids by HSD17Bs may play a major role in the regulation of the extent of hormone action in the target tissues (3, 4) and thereby have a central role, *e.g.* in the pathogenesis of breast and endometrial cancers (5–7), Alzheimer's disease (8), and endometriosis (9–11).

To date, 12 HSD17B enzymes with different tissue distribution, catalytic preference, substrate specificity, biochemical properties, and subcellular distribution have been identified (12, 13). Most of the HSD17Bs share only limited sequence identity with each other (14), but many of them have overlapping substrate spectra. Among these enzymes, HSD17B2 has been identified and characterized in several mammals including humans (15). The enzyme is expressed in a wide variety of tissues, including steroid target tissues such as breast, uterus, prostate, placenta, and the tissues involved in catabolism and excretion of steroids such as the liver and kidney (16). HSD17B2 is also widely expressed in the surface epithelial cells of the gastrointestinal tract (17, 18). The catalytic properties of HSD17B2 have been studied *in vitro* and in several cultured cell line models. These studies have shown that the enzyme predominantly catalyzes the inactivation of the estrogens and androgens (19, 20). However, the enzyme also activates 20α-dihydroprogesterone to progesterone (20, 21). Despite several studies on the enzyme characteristics *in vitro* and in cultured cell lines, little is known about the physiological role of HSD17B2 *in vivo*. To study its function further and to analyze the role of HSD17B2 in the regulation of local steroid availability in the peripheral target tissues *in vivo*, we generated transgenic (TG) mice ubiquitously expressing human HSD17B2 under the cytomegalovirus-enhanced chicken β-actin promoter. These mice provide a novel tool to study the physiological function of HSD17B2.

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Abbreviations: E_1, Estrone; E_2, [^{3}H]estradiol; HSD17B2, hydroxysteroid (17β) dehydrogenase 2; PFA, paraformaldehyde; q, quantitative; T, testosterone; TG, transgenic; TTNPB, 4-[(E)-2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid; WT, wild type.

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Materials and Methods

Generation of TG mice and animal husbandry

HSD17B2 TG mice (HSD17B2TG) were generated by microinjecting the expression fragment containing the human *Hsd17b2* cDNA (kindly provided by professor Van Luu-The, Laval University, Québec, Canada)

under the chicken β-actin promoter (kindly provided by Professor Junichi Miyazaki, Osaka University, Osaka, Japan). The promoter provides a universal transcriptional control of the transgene (Fig. 1A). Integration of the transgene was verified by PCR screening and Southern blotting using genomic DNA isolated using the phenol-chloroform-isoamylalcohol method (22). Two fragments of the transgene were amplified separately using distinct primer pairs (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org). Of the 15 founder mice born, two mouse lines (HSD17B2TG-141 and HSD17B2TG-011) were generated for a detailed analysis of the postnatal phenotype. All animal experiments were approved by the University of Turku (Turku, Finland). The animals were handled in accordance with the institutional animal care policies of the University of Turku. Because the TG mice were generated and analyzed in the FVB/N inbred strain, in most of the studies, we used wild-type (WT) mice of independent litters as controls. The WT littermates of TG mice were not different from the independent WT mice. However, in the measurement of body growth and eye opening, the non-TG littermates of the same litter were used to standardize the nutritional state. Non-TG littermates were also used in the studies aimed at rescuing the testis phenotype observed in the HSD17B2 TG males.

HSD17B activity measurements in vitro and in vivo

Oxidative HSD17B activity for the transgene construct was verified in cultured cells as previously described (23), with minor modification. Briefly, HEK-293 cells were transfected with the pCAGGS-*hHsd17b2* construct using the lipofectamine method (Invitrogen, Carlsbad, CA) (24). Cells (4×10^5cells/well) were applied into 12-well plates in DMEM/F12–10% fetal calf serum, including penicillin and streptomycin. After overnight culture the medium was aspirated from the wells, and 2 ml of serum free medium containing 200 nM [^{3}H]estradiol (E_2) (2×10^5 cpm/ml; PerkinElmer Life Sciences, Boston, MA) were applied. The cells were then incubated at 37 C for 1, 2, 4, 8, and 24 h in the cell culture conditions. After incubation, the reactions were stopped by freezing the reaction tubes in dry ice-ethanol bath. Steroids were extracted twice with 2 ml of diethyl ether (Merck, Darmstadt, Germany), the extracts were pooled and ether evaporated under nitrogen flow and dissolved in 150 μl of acetonitrile-water (48:52, vol/vol). The amount of E_2 converted to E_1 (estrone) was analyzed by separating E_1 and E_2 using a HPLC system (Waters 2695; Waters, Milford, MA) connected to a flow scintillation analyzer (Packard, Meriden, CT).

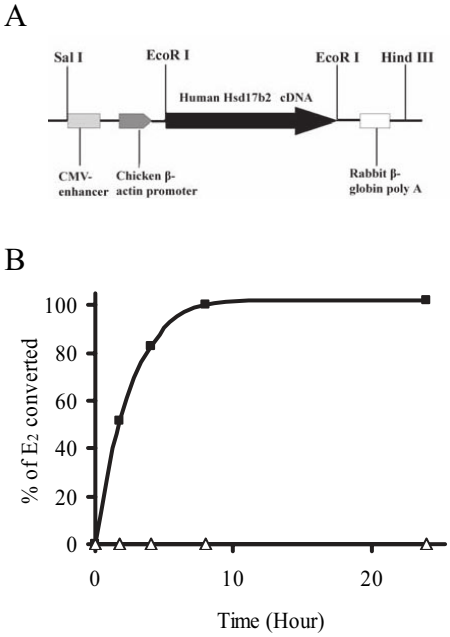

FIG. 1. A, Schematic picture of the HSD17B2 transgene construct (3.6 kb) used in transgenic mice production. B, Oxidative HSD17B activity (E_2 to E_1) in cultured HEK-293 cells transiently transfected with the human pCAGGS-Hsd17b2 construct (*black rectangle*) and with the plasmid only (*white triangle*).

HSD17B activity was measured *in vivo* with 2- to 3-month-old HSD17B2TG-141 male mice. [^{3}H]E_2 in a final concentration of 5.6 μg/kg was used as substrate. The substrate was dissolved in 25% ethanol in water, and an iv injection of 2.5 μl/g body weight was given via the tail vein. The mice were then anesthetized by 300–600 μl Avertin (2,2,2-tribromoethyl alcohol + tert-amyl alcohol; Sigma, St. Louis, MO), blood was collected by cardiac puncture after 18 min, and the mice were euthanized by cervical dislocation. The steroids were extracted from the blood by ether extraction and the amount of E_2 converted to E_1 was analyzed as described above.

Morphological and histological analysis

Growth of the HSD17B2TG-141, -011, and WT mice was analyzed (six mice per group) by weighing the mice twice a month between the ages of 15 d and 6 months. Phenotypic features were mostly analyzed in mouse line 141, with the highest level of transgene expression. In these mice the age of eye opening was analyzed and compared with the WT mice at the age of 12.5–25.5 d. For other phenotype analysis, the males were killed at the age of 4 and 6 months. The mice were anesthetized by ip injection of 300–600 μl 2.5% Avertin, and blood was collected by cardiac puncture. Organs were removed, dry blotted, and weighed. For histological analysis, the tissues including epididymis, prostate (seminal vesicles), pituitary, kidney, liver, intestine, spleen, adrenal, and heart were fixed in 4% paraformaldehyde (PFA) at 4 C overnight. The testes were fixed in Bouin's solution. The fixed tissues were then dehydrated, embedded in paraffin, and sectioned at 5 μm thickness. The sections were stained with Harris hematoxylin and eosin (BDH, Poole, UK). The reproducibility of all the morphological and histological data were verified by similar findings in at least six animals.

RNA analysis

Total RNA was isolated from the heart, kidney, testis, epididymis, adrenal, muscle, uterus, ovary, spleen, seminal vesicle, pituitary, and liver using RNeasy minikit (QIAGEN, Hilden, Germany). Mammary gland and brain RNA were isolated using the RNeasy lipid tissue minikit (QIAGEN). One μg of total RNA was treated with DNase I (Invitrogen). Prostate RNA was extracted by homogenizing the tissue in a 4 M solution of guanidinium thiocyanate including 0.1 M 2-mercaptoethanol and then isolated by applying cesium chloride (CsCl) ultracentrifuge method (25). RT-PCR analysis was used to detect tissue distribution of the transgene. Quantitative (q) RT-PCR analysis was performed to compare the transgene expression in the HSD17B2TG-141 and HSD17B2TG-011 lines for a selected amount of tissues and to analyze androgen-dependent genes in the epididymis and prostate. In addition, qRT-PCR analysis was performed to analyze retinoic acid target genes in the testis. All primers used in RT-PCR and qRT-PCR are described in supplemental Tables 1 and 2, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org. Quantitative RT-PCR were carried out using DNA Engine Opticon system (MJ Research, Waltham, MA) and the QuantiTect SYBR Green RT-PCR kit (QIAGEN) with continuous fluorescence detection. The reverse transcription reaction was 30 min at 50 C, followed by 15 min at 95 C to inactivate reverse transcriptase and activate HotStarTaq DNA polymerase. Thereafter, PCRs were carried out using the conditions described in supplemental Tables 1 and 2. All qRT-PCR data were normalized to the expression of mouse L-19.

Hormone measurements

Blood was at 4 C overnight. Serum samples were collected after centrifugation and stored at −20 C until used for testosterone (T), progesterone, LH, and FSH measurements. Serum FSH and LH were measured by immunofluorometric assays as described previously (26, 27). Serum T was measured from diethyl ether extracts using a RIA (28). Serum progesterone was measured from diethyl ether extracts using a time-resolved fluoroimmunoassay (PerkinElmer Life Sciences, Turku, Finland). Tissue T was determined by homogenizing a piece of testis, prostate, heart, kidney, muscle, and brain in 1 ml PBS buffer. Three hundred microliters of the tissue homogenates were extracted twice in

TABLE 1. Body weights of WT, HSD17B2TG-141, and HSD17B2TG-011 mice

Age	15 d	30 d	2 months	4 months	6 months
WT	11.9 (±1.8)	22.6 (±1.9)	29.8 (±1.9)	33.3 (±6.8)	33.0 (±4.6)
HSD17B2TG-141	7.6 (±1.2)[a]	13.2 (±2.3)[b]	23.3 (±1.5)[a]	27.4 (±1.1)[a]	27.6 (±1.8)[a]
HSD17B2TG-011		14.3 (±2.7)[c]		26.8 (±3.8)[a]	30.9 (±0.8)[a]

Mean ± SD (grams), n = 6–10 animals.
[a] No significant difference between HSD17B2TG-141 and WT or between HSD17B2TG-011 and WT.
[b] HSD17B2TG-141 differed significantly from WT ($P < 0.001$).
[c] HSD17B2TG-011 differed significantly from WT ($P < 0.001$).

2 ml diethyl ether and evaporated. After reconstitution into PBS buffer, the amount of T per milligram of tissue was measured (28).

Immunohistochemistry

For immunohistochemistry all testes were fixed in the 4% PFA overnight at 4 C. Five-micrometer-thick paraffin sections were cut from 4% PFA-fixed testis. After deparaffinization and rehydration in xylene and ethanol, the sections were placed in 10 mM sodium citrate buffer (pH 6.0), followed by heating in a microwave oven for antigen retrieval. The endogenous peroxidase was blocked by incubating the sections in 1% H_2O_2 for 20 min. Sections were then incubated for 4 h with the antibody for androgen receptor (1:350 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at +4 C in PBS containing 3% BSA. The bound primary antibody was detected by using biotinylated goat antirabbit IgG, followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Color was developed with diaminobenzidine tetrahydrochloride as a substrate (Zymed Laboratories, San Francisco, CA), and the sections were slightly counterstained with Mayer's hematoxylin, dehydrated, and mounted. To clarify whether the germ cell degeneration was associated with apoptosis, a rabbit polyclonal antibody for cleaved caspase-3 (Asp175) was used (1:100 dilution; Cell Signaling Technology, Beverly, MA) overnight. The staining procedure was identical with that described above.

4-[(E)-2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB) treatment

HSD17B2TG and WT males of the same litters were treated with vehicle solution or a retinoic acid receptor agonist (TTNPB; Sigma), eight mice per group. The TTNPB was stored at a concentration of 3 mg/ml in dimethylsulfoxide (Sigma) and kept in the dark at −20 C. For the treatment, the TTNPB was diluted with oil to a final concentration of 1.5 μg/ml. The mice were treated with 1.5 μg TTNPB per kilogram body weight twice a week from the age of 20 d until the age of 4 months. Every second TTNPB injection was given sc, and the other was ip. Before every injection, the body weights of all mice were measured. At the age of 4 months, the mice were anesthetized with 300–600 μl 2.5% Avertin, ip, after which the blood was collected by cardiac puncture and organs were removed after cervical dislocation. The testes were fixed overnight at 4 C in Bouin's solution. The fixed testes were dehydrated, embedded in paraffin, and sectioned at 5 μm thickness. The sections were stained with Harris hematoxylin and eosin. All the seminiferous tubules in three independent sections from one testis of all mice were evaluated under the microscope with Leica IM500 software (Leica Microsystems Digital Imaging, Cambridge, UK). The histological appearances of the seminiferous tubules were graded in five categories, depending on the severity of the damage observed in the spermatogenesis: very severe, severe, moderate, mild, and normal.

TABLE 2. Tissue weights in WT, HSD17B2TG-141, and HSD17B2TG-011 mice

Animal line	Epididymis	Testis
4 months of age		
WT	38 (±2.8)	97 (±5.2)
HSD17B2TG-141	20 (±2.3)[a]	41 (±2.3)[a]
HSD17B2TG-011	32 (±3.9)	83 (±5.4)
6 months of age		
WT	38 (±2.0)	97 (±4.5)
HSD17B2TG-141	19 (±0.9)[a]	29 (±3.6)[a]
HSD17B2TG-011	34 (±1.9)	93 (±3.5)

Mean ± SD (milligrams), n = 6–10 animals.
[a] HSD17B2TG-141 differed significantly from WT ($P < 0.001$).

Statistical analysis

Statistical analyses were performed using the SigmaStat program (Systat Software Inc., Point Richmond, CA). The results of qRT-PCR, eye opening, HSD17B activity *in vivo*, and the rescue of testis phenotype were analyzed by *t* test or Mann-Whitney rank sum test. Hormone data, body and organ weights, and qRT-PCR data of RA-target genes were analyzed by one-way, repeated-measures ANOVA and then followed by the Holm-Sidak test (multiple comparison options for a one-way ANOVA). Significance was set as $P < 0.05$ and the values were presented as mean ± SD.

Results

Generation of mice expressing human HSD17B2

HEK-293 cells were transiently transfected with the *pCAGGS-hHsd17b2* construct (Fig. 1A) and the conversion of E_2 to E_1 was followed up to 24 h. With a substrate concentration 0.4 nmol E_2 per 10^5 cells, the 17HSD17B2-expressing cells converted most of the E_2 to E_1 within 4 h, whereas an opposite reaction was not detected (Fig. 1B). This indicated that the chicken β-actin promoter-driven human HSD17B2 catalyzed oxidative HSD17B activity in cultured cells, similar to that previously reported.

Fifteen HSD17B2 founder mice were obtained (seven females and eight males). Progenies from two independent founders were selected for further studies by their growth retardation phenotype and the genomic Southern blot analysis showing the integration of several copies of transgene (HSD17B2TG-141 mice with a higher copy number of transgene and HSD17B2TG-011 mice with a lower copy number of transgene). Other mouse lines did not present any obvious phenotype. At the age of 4 months, qRT-PCR analyses were carried out in RNA extracted from heart, kidney, liver, and testis, and the results indicated a different level of transgene expression in HSD17B2TG-141 and HSD17B2TG-011 mice (Fig. 2A). Furthermore, RT-PCR analysis in HSD17B2TG-141 and HSD17B2TG-011 indicated ubiquitous transgene expression in various mouse tissues (Fig. 2B).

To test the activity of HSD17B2-transgene in the HSD17B2TG-141 mice, the conversion of E_2 to E_1 was followed *in vivo* by injecting [^{3}H]E_2 into the tail vein. Analyzing the concentration of [^{3}H]E_1 and [^{3}H]E_2 in blood circulation, a significant increase in oxidative HSD17B activity was detected in the TG mice, compared with WT ($P < 0.05$, Fig. 3). The results confirmed that the transgene possesses oxidative HSD17B activity *in vivo* in the mouse.

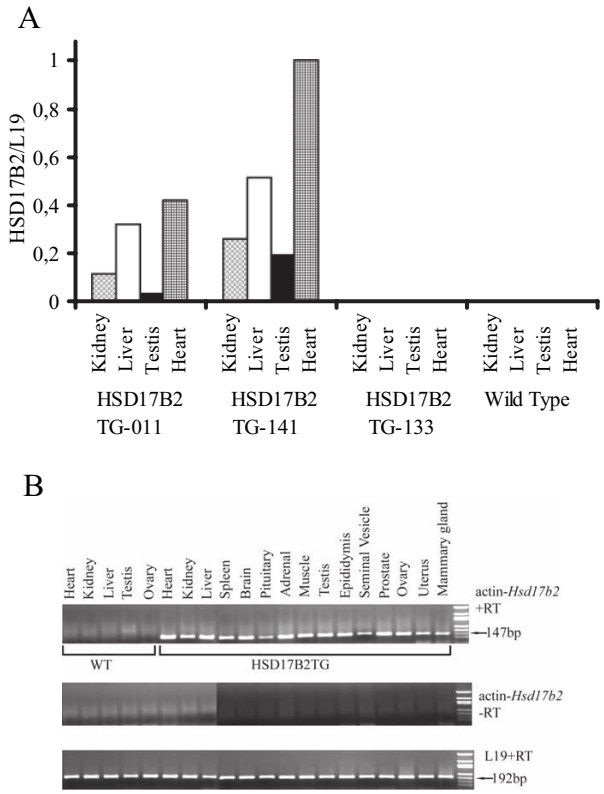

FIG. 2. A, Transgene expression in HSD17B2TG-141, HSD17B2TG-011, HSD17B2TG-133 (no expression), and WT males, as measured by qRT-PCR. The total RNAs for each tissue were pooled from six male mice. B, Distribution of transgene expression measured by RT-PCR in various tissues obtained from HSD17B2TG-141 mice.

Body and reproductive organ weights

Evaluation of the males in HSD17B2TG-141 and HSD17B2TG-011 mouse lines revealed a reduced body weight, compared with WT littermates. The difference in body weight became significant at the age of 1 month. At this age the weight of HSD17B2TG-141 and HSD17B2TG-011 males was 40–50% reduced as compared with WT males ($P < 0.001$, Table 1). However, the life span of HSD17B2TG males was normal. The body weight in the HSD17B2TG-141 mouse line, with the stronger transgenic expression, remained smaller, albeit not significantly, throughout the 6 months follow-up, whereas the mice of the HSD17B2TG-011 line had a normal body weight at the age of 6 months (Table 1). A significant ($P < 0.001$) reduction in organ weight was detected only for the testes and epididymides in the HSD17B2TG-141 males at the age of 4 and 6 months (Fig. 4 and Table 2). HSD17B2TG-141 mice also displayed delayed eye opening (Table 3, $P < 0.001$). At the age of 13.5 d, 75% (82 of 109) of the WT mice had both eyes opened, whereas only 6% (four of 64) of the TG mice did. In some TG mice, one of the eyes showed a squinting phenotype several days after the eye opening (data not shown). All the WT mice had both eyes opened at the age of 15.5 d, whereas for the TG mice, this was true at the age of 25.5 d.

Mating and fertility

Continuous mating was carried out to analyze the fertility of the HSD17B2TG males up to 6 months of age. The fertility

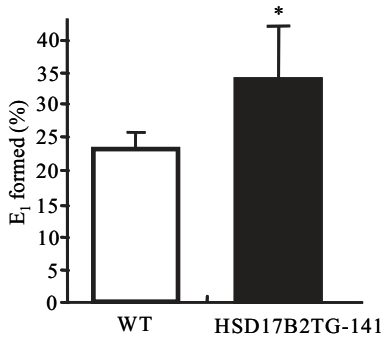

FIG. 3. HSD17B activity (E_2 to E_1) measured in the WT (n = 5) and HSD17B2TG-141 males (n = 6) *in vivo*. *, $P < 0.05$.

of the HSD17B2TG-011 males was proven to be normal, whereas 50% of the HSD17B2TG-141 males were subfertile and 50% infertile at the age of 2 months, and at about 3 months of age, all the TG males became infertile. No difference was found in serum LH, FSH, and progesterone concentrations or in intratesticular T and serum T concentrations among WT, HSD18B2TG-141, and HSD17B2TG-011 mice analyzed at 4 and 6 months of age (data not shown). For example, at the age of 4 months, the serum T values (n = 5) were 2497.5 ± 2578.0 pg/ml for WT, 1442.5 ± 1422.4 pg/ml for HSD17B2TG-141, and 2243.8 ± 1257.1 pg/ml for HSD17B2TG-011 mice. No significant changes were observed in intratissular T concentrations between HSD17B2TG-141 and WT males measured in the prostate, kidney, heart, muscle, and brain at 4 months of age (data not shown). Although the HSD17B2TG-141 males were infertile, the prostate and seminal vesicle size (Fig. 4) and their histology were normal, indicating proper androgen action. An androgen action within normal range was also confirmed by analyzing the mRNA expression of the androgen-dependent genes in the prostate. The expression of four of five genes analyzed by qRT-PCR was not significantly different be-

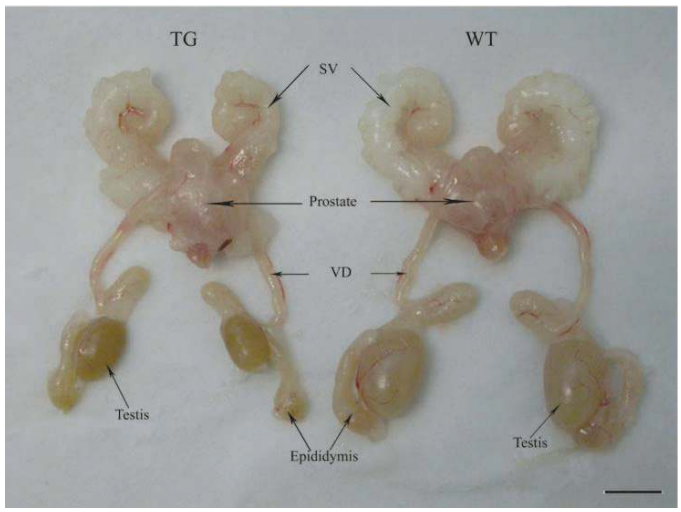

FIG. 4. Macroscopic anatomy of the reproductive tract in the HSD17B2TG-141 and wild-type males at the age of 6 months. HSD17B2TG-141 males presented with highly decreased testis and epididymis size, whereas the seminal vesicles and prostate size and structure were similar in TG and WT mice. SV, Seminal vesicle; VD, vas deferens. *Bar*, 1 cm.

TABLE 3. Age of eye opening in WT and HSD17B2TG-141 mice

Age	12.5 d	13.5 d	14.5 d	15.5 d	25.5 d
WT mice (n = 109)					
Eyes closed (%)	94 (86)	22 (20)	2 (2)	0 (0)	
One eye opened (%)	0 (0)	5 (5)	0 (0)	0 (0)	
Eyes opened (%)	15 (14)	82 (75)	107 (98)	109 (100)	
TG mice (n = 64)[a]					
Eyes closed (%)	64 (100)	52 (81)	10 (15)	4 (6)	0 (0)
One eye opened (%)	0 (0)	8 (13)	8 (13)	4 (6)	0 (0)
Eyes opened (%)	0 (0)	4 (6)	46 (72)	56 (88)	64 (100)

[a] The eye opening in both HSD17B2TG-141 male and female mice differed significantly from WT ($P < 0.001$).

tween the TG and WT mice (Fig. 5A), whereas *Srd5a2* showed slightly reduced expression in TG, compared with WT, mice. However, despite the normal histological appearance, *lcn8*, *lcn5*, and *Rnase 9* mRNA expression in the epididymis was significantly different between TG and WT mice (Fig. 5B). Especially the *lcn8* and *lcn5* mRNAs were markedly down-regulated.

Testis histology

Reduced testicular weight was caused by germ cell degeneration, and this was also the cause of the infertility. At the age of 6 months, seminiferous tubules were severely atrophic and contained mostly only Sertoli cells (Fig. 6, E and F). Spermatogenesis was more extensively disrupted in older males, compared with those at the age of 4 months, indicating a progressive degenerative process. This was in line with the observed progression of the subfertility to infertility in 50% of the TG males. Immunohistological staining for an apoptotic marker (cleaved caspase-3) indicated that there was no significant difference in the apoptosis rate in the germ cells between HSD17B2TG-141 and WT males at the age of 2 months (Fig. 7). However, this did not exclude the possibility of an increased apoptotic rate at other time points. The TG testis expressed androgen receptor in the Sertoli, Leydig, and myoid cells (Fig. 7). In wild-type mice, androgen receptor staining is strongest at stage VII-VIII, whereas in HSD17B2TG staining is more even in all stages, suggesting that stage specificity typically observed in mice may depend on the presence of germ cells.

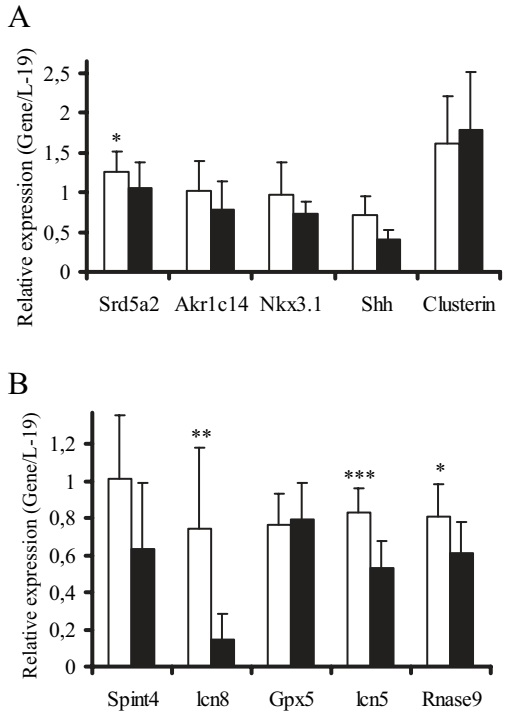

FIG. 5. A, Expression of androgen-dependent, prostate-specific genes in WT (*white bars*) and HSD17B2TG-141 male mice (*black bars*). The data indicated similar expression for *Akr1c14*, *Nkx3.1*, *Shh*, and *Clusterin* in the prostate, whereas expression of *Sdr5a2* was significantly reduced. B, Expression of androgen-dependent, epididymis-specific genes in the WT (*white bars*) and TG mice (*black bars*). The data indicated that the expression of *lcn8*, *lcn5*, and *Rnase 9* in the epididymis were significantly reduced in HSD17B2TG-141 mice, compared with WT mice. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Histological analysis at the spermatogenetic cycles identified that full spermatogenesis was first established, but already at the age of 4 months, the signs of germ cell degeneration were evident (Fig. 6, C and D). The cellular contents of the seminiferous epithelium varied in different cross-sections of seminiferous tubules showing missing spermatogonia, spermatocytes, and spermatids. The changes were not specific for stages of the seminiferous epithelial cycle. Between the age of 4 and 6 months, the degeneration proceeded quickly and Sertoli cell-only histology ensued (Fig. 6H). In many seminiferous tubules, early phases of spermatogenesis (spermatogonia and spermatocytes) were missing, whereas spermatids were still present, suggesting a block in proliferation (Fig. 6G).

Rescue of testis phenotype

The TTNPB is a highly effective synthetic retinoid acid analog, having a high affinity for all retinoic acid receptor isoforms (α, β, γ) but not for the retinoid X receptors. As a synthetic drug, it is not considered to metabolize by HSD17B2. To investigate whether HSD17B2 was involved in the metabolism of retinoids and test whether the testis phenotype was due to the lack of retinoid action, we treated HSD17B2TG males with TTNPB for 3.5 months. Although the TTNPB treatment did not result in normal spermatogenesis completely, the seminiferous tubule damage was significantly reduced. The amount of tubules with very severe and severe damage were reduced from 39.6 and 34.1% in placebo-treated mice to 14.6 and 19.6% in TTNPB-treated mice, respectively. Accordingly, the amount of only moderately or mildly damaged tubules increased, and the amount of normal tubules increased from 1.3 to 6.5% with the TTNPB treatment (Table 4 and Fig. 8). Furthermore, the expression of the *Rbp1*, *Rara*, and *Stra8* (three retinoic acid target genes) in the testes of HSD17B2TG males increased significantly

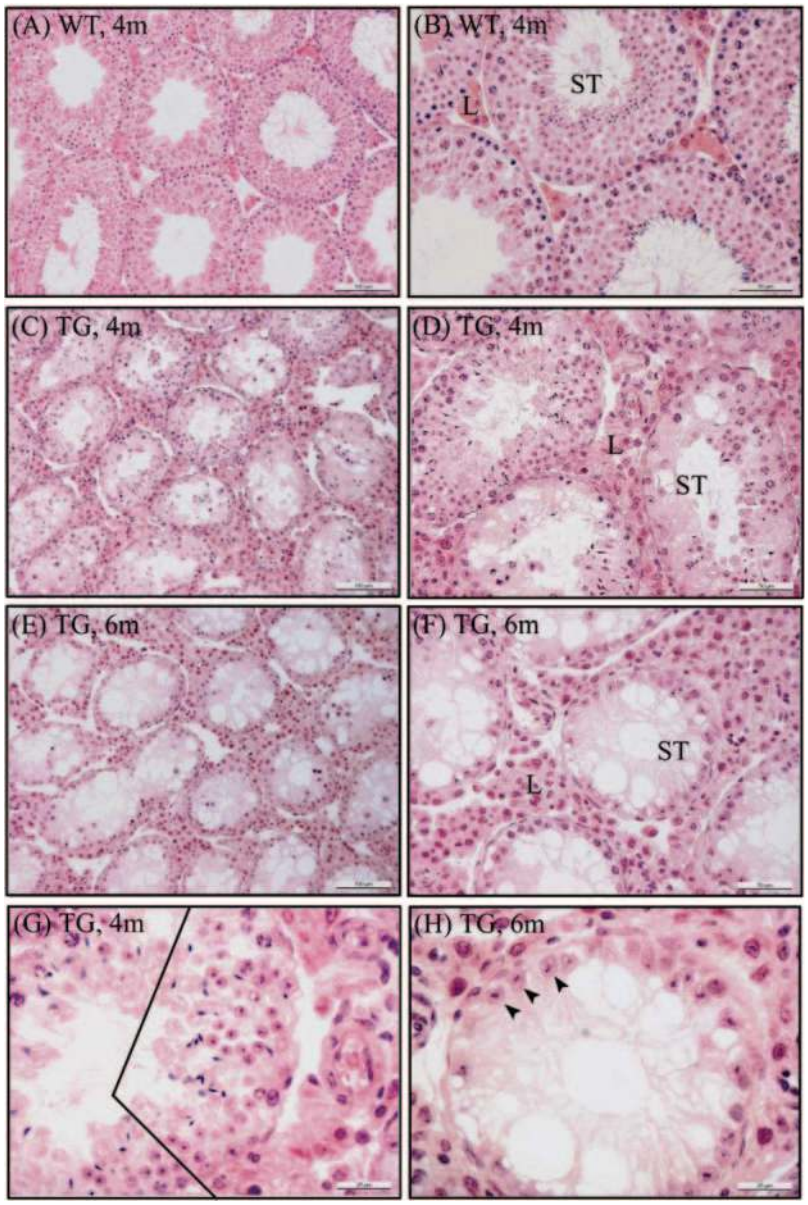

FIG. 6. Histology of the testes of HSD17B2 TG-141 and WT mice. A and B, Four-month-old WT mice. C, D, and G, Four-month-old TG mice. E, F, and H, Six-month-old TG mice. Degeneration of the seminiferous epithelium is detected in the TG mice (C–H). G, The area between two lines indicates that there are no spermatogonia or spermatocytes, but there are still round and elongated spermatids. H, *Arrowheads* indicate that only Sertoli cells survive in the seminiferous tubules in HSD17B2TG mice at the age of 6 months. ST, Seminiferous tubules; L, Leydig cells. *Bar*, 100 μm (A, C, and E); 50 μm (B, D, and F); 25 μm (G and H).

after the TTNPB treatment but not in the placebo-treated mice (Fig. 9). As a control experiment, treating the nontransgenic littermates with TTNPB and placebo did not affect the spermatogenesis (data not shown).

Discussion

HSD17B enzymes have been suggested to play pivotal roles in the regulation and maintenance of the activities of sex steroids (29, 30). The broad tissue distribution (16, 31) together with the broad substrate specificity (19) and predominant oxidative activity of HSD17B2 indicate that the enzyme would play an essential role in the inactivation of highly active 17β-hydroxysteroids in the peripheral tissues, thereby protecting the tissues from excessive sex hormone influence.

In the present study, TG mice with chicken-β actin promoter-driven human *Hsd17b2* were generated to further understand the function of the enzyme in mammalian physiology. The most obvious findings in the HSD17B2TG males were postnatal growth retardation and disrupted spermatogenesis. Interestingly, the phenotype observed did not mimic the phenotypes of mice deficient in estrogen or androgen action. Previous data have shown that the body weight was unchanged in *Esr1*- (estrogen receptor-α) (32, 33) and *Esr2* (estrogen receptor-β)-deficient mice (34, 35), whereas the *Ar* (androgen receptor)-deficient male mice have female-like appearance and reduced body weight (36).

The lack of signs of inappropriate sex steroid action in HSD17B2TG males, and the lack of correlation between HSD17B2 expression and the hormonal status in female rats (37), prompted us to seek for other pathways putatively affected by the HSD17B2 expression. *Hsd17b2* is a close homolog for a number of retinoic acid metabolizing enzymes (38). Thus, it is interesting to note that the growth retardation in HSD17B2TG males resembles the phenotype of *Rara*- (reti-

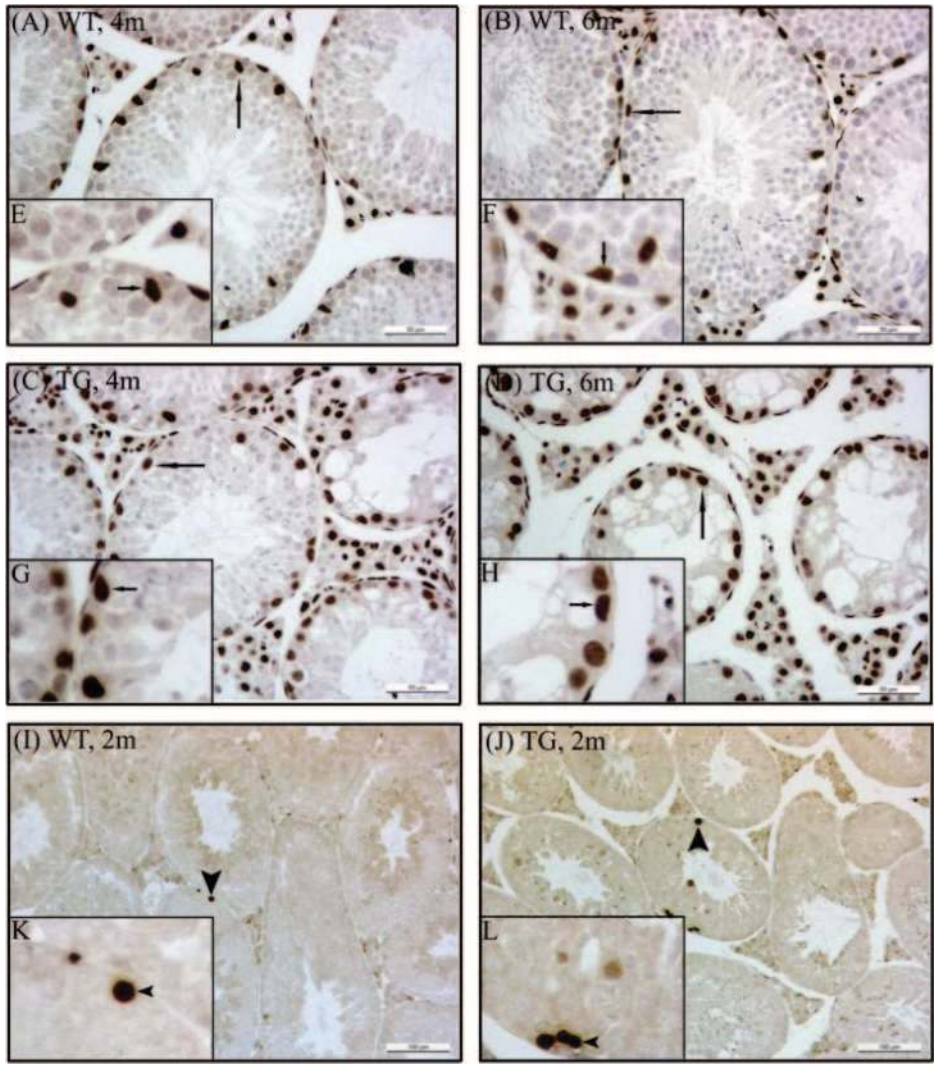

FIG. 7. Immunohistochemical analysis of androgen receptor (A–H) and cleaved caspase-3 (I–L) in the testis. A and E, WT testes at 4 months of age. B and F, WT testes at 6 months of age. C and G, HSD17B2TG-141 testis at 4 months of age. D and H, HSD17B2TG-141 at 6 months of age. All testes from both age groups show positive staining for androgen receptor. *Arrows* indicate densely stained nuclei. There is no significant difference in apoptosis, as determined by cleaved caspase-3 staining, between WT (I) and HSD17B2TG-141 mice (J) at 2 months of age. *Arrowheads* indicate apoptotic cells. *Bar*, 50 μm (A–D); 100 μm (I and J).

noic acid receptor-α) and *Rarg* (retinoic acid receptor-γ)-deficient mice (39, 40). The *Rara* and *Rarg*-deficient mice are not embryonic lethal, and no obvious malformations or lesions were macroscopically or histologically detected. However, they exhibited a slower growth rate and postnatal lethality; only 4% of *Rara* null and less than 40% *Rarg* null mice survived to the age of 2 months. Those mice that survived until the age of 2 months, however, were similar in size, compared with their WT littermates.

In addition to the growth retardation, the HSD17B2TG-141 males had severe defects in reproductive function. Whereas no significant changes were found in the reproductive endocrine parameters, the testis and epididymis were smaller in size as a result of disrupted spermatogenesis. Our data suggest that the germ cell degradation at different developing stages was not associated with germ cell apoptosis dependent on cleaved caspase-3. At the age of 6 months, the seminiferous epithelium of the testis was atrophic, with the presence of mostly Sertoli cells only. Interestingly, similar changes have been observed in the testis of *Rara*-deficient

TABLE 4. Rescue of seminiferous tubule damage with TTNPB in the HSD17B2TG male mice

Type of tubular damage	Control group (%)		TTNPB group (%)	
	Average[a]	Max/min	Average[a]	Max/min
Very severe	39.6 (±12.9)	58.1/25.5	14.6 (±10.7)[b]	29.5/0
Severe	34.1 (±3.7)	42.0/30.7	19.6 (±7.1)[c]	23.7/5.3
Moderate	16.7 (±5.9)	22.6/6.3	30.7 (±2.9)[c]	33.8/25.1
Mild	8.2 (±4.0)	12.3/1.5	28.6 (±14.0)[b]	55.4/13.9
Normal	1.3 (±1.3)	5.5/0	6.5 (±4.3)[d]	14.1/0

[a] Mean ± SD (percent), the ratio of degraded seminiferous tubules, and total seminiferous tubules (n = 8 animals).
[b] TTNPB group differed significantly from control group, $P < 0.001$.
[c] TTNPB group differed significantly from control group, $P < 0.01$.
[d] TTNPB group differed significantly from control group, $P < 0.05$.

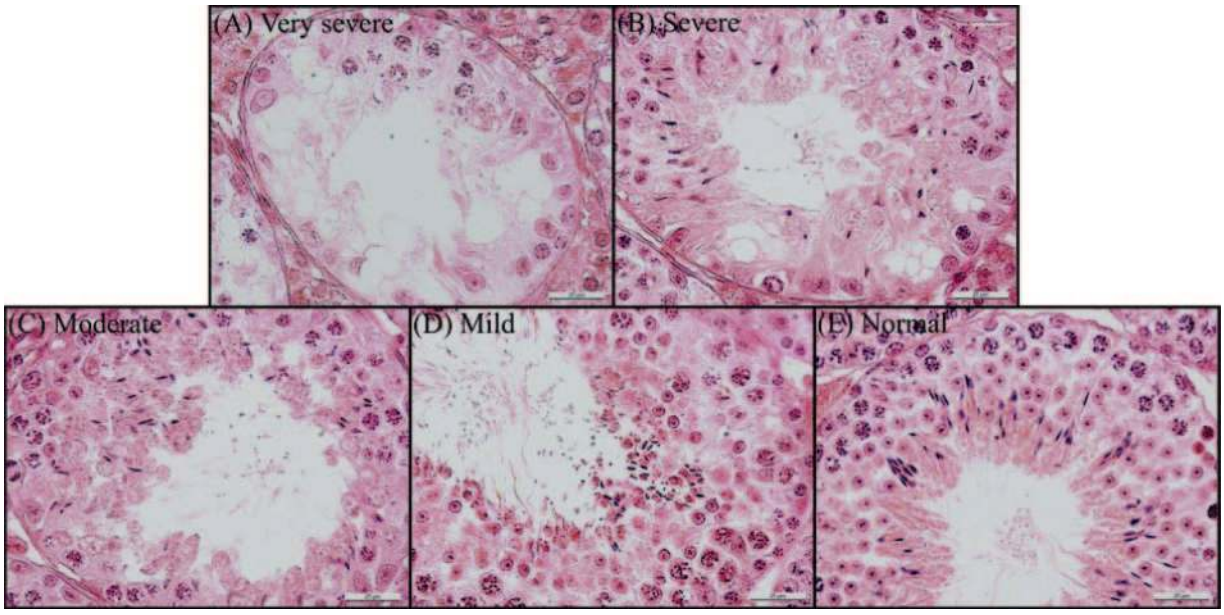

FIG. 8. Testis histology in HSD17B2TG-141 mice with various levels of the seminiferous tubule damage. A, Very severe damage, indicated by only a few germ cells left. B, Severe damage, indicated by marked loss of germ cells. C, Moderate damage, indicated by apparent loss of germ cells. D, Mild damage, indicated by loss of a few germ cells. E, Normal seminiferous tubule.

male mice (40) and vitamin A-deficient male mice (41–43). Most frequently, the early stages of spermatogenetic cell differentiation (spermatogonia and primary spermatocytes) were affected in the *Rara*-deficient male mice, similar to that observed in HSD17B2TG males. Although infertility is also associated to the *Esr1* and *Ar* knockout male mice, the reproductive phenotypes in these mice are different from that of the HSD17B2 TG males. In the *Ar* null males (36), the external genitalia are ambiguous or show feminized appearance, including small and cryptorchid testis. *Gnrh1* (hpg, GnRH), *Ar*, and *LH receptor* null males (LuRKO mice; with highly reduced testosterone production) show that the stages before meiotic division are not androgen dependent (36, 44–46). In the *Esr1* null males, the testis phenotype with dilated seminiferous tubules and the spermatogenetic failure is due to fluid buildup and an impaired efferent ductule and rete testis function (47–49), whereas in *Esr2*-deficient mice, the testicular phenotype and fertility are normal (50). These types of defects were not observed in the HSD17B2TG male mice.

The epididymis of HSD17B2TG was histologically normal (data not shown), but mRNA expression for two members of lipocalin superfamily, *lcn8* (mouse epididymal protein) and *lcn5* (murine epididymal retinoic acid binding protein) (51, 52), were markedly down-regulated. The biological significance of lipocalins in the epididymis is uncertain, but *lcn8* and *lcn5* have been suggested to be involved in retinoid trafficking in the specific region of epididymis (53, 54). In line with this, there is evidence that retinoids also play an important role in regulating gene expression in the epididymis (55). However, the putative regulation of *lcn5* and *lcn8* by retinoids remains to be clarified because several epididymal genes are under complex regulation by androgens, other testicular factors, and the presence of germ cells in the epididymis.

The HSD17B2TG-141 mice displayed delayed eye opening, and in some TG mice the eyes had a squinting phenotype several days after the normal age of the eye opening. A similar phenotype has been reported in vitamin A-deficient mice (42, 56), whereas it is not reported in *Esr1* (57), *Esr2* (50), or *Ar*-deficient mice (36). It is known that the eye is the most sensitive organ to retinol deprivation, and it is often the only site of malformations in less severely affected vitamin A-deficient fetuses (58, 59). Moreover, the analysis of the various retinoid receptor mutant mice (59, 60) has implicated the role of retinoid signaling at most steps of the prenatal eye morphogenesis and eye development.

Vitamin A is essential for male reproduction. Vitamin A deficiency induces early cessation of spermatogenesis, whereas the only remaining germ cells are the undifferentiated spermatogonia. Administrating a high dose of retinoic acid to vitamin A-deficient animals is sufficient to restore and

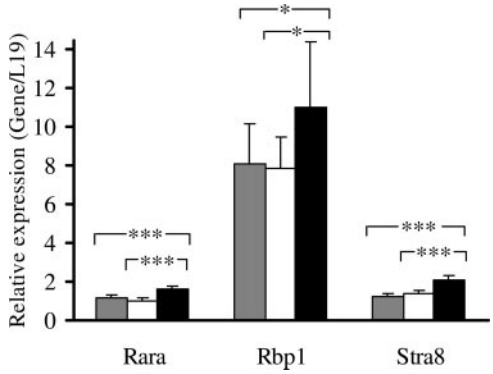

FIG. 9. Quantitative RT-PCR analysis of *Rbp1*, *Rara*, and *Stra8* expressions in the testis of TTNPB-treated (*black bars*), placebo-treated (*white bars*), and nontreated (*gray bars*) adult HSD17B2TG mice. ***, $P < 0.001$;*, $P < 0.05$.

synchronize spermatogenesis in the seminiferous tubules (41–43). To test whether HSD17B2 enzyme participates in the metabolism of retinoids, a rescuing experiment was performed with TTNPB. Interestingly, TTNPB efficiently reduced the damage of the seminiferous tubules observed in the HSD17B2TG males. This, together with the phenotypes, strongly indicates that HSD17B2 enzyme has a novel role in the action of retinoids.

In conclusion, TG mice ubiquitously expressing human HSD17B2 presented with growth retardation with the strongest effect at the prepubertal period. Furthermore, delayed eye opening and severely disrupted spermatogenesis were detected in mice with the highest transgene expression. The data suggest that reduced action of androgens or estrogens is not the cause of the phenotypes observed, and in addition of being involved in the inactivating sex steroids the enzyme has other major metabolic functions. Several of the phenotypes observed are highly similar to those found in mice with deficient retinoid action, and furthermore, the disrupted spermatogenesis was partially restored with an retinoic acid receptor agonist. The data thus suggest a novel direct or indirect role for HSD17B2 in the metabolism of retinoids.

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