THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 18, pp. 14456–14466, April 27, 2012

Deregulated Hepatic Metabolism Exacerbates Impaired Testosterone Production in Mrp4-deficient Mice*[S]

Received for publication, November 4, 2011, and in revised form, February 8, 2012 Published, JBC Papers in Press, February 28, 2012, DOI 10.1074/jbc.M111.319681

Jessica A. Morgan[‡§], Satish B. Cheepala[‡], Yao Wang[‡], Geoff Neale[¶], Masashi Adachi[‡], Deepa Nachagari[‡], Mark Leggas[‡], Wenchen Zhao[‖], Kelli Boyd[**], Raman Venkataramanan[‖], and John D. Schuetz[‡1]

From the [‡]Department of Pharmaceutical Sciences, [¶]Hartwell Center for Bioinformatics and Biotechnology, and [**]Department of Pathology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, the [‖]School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and the [§]Integrated Program in Biomedical Sciences, The University of Tennessee Health Science Center, Memphis, Tennessee 38163

Background: The role of the ABC transporter, Mrp4, in regulating Leydig cell testosterone synthesis is unknown.
Results: In a murine genetic model the absence of Mrp4 impairs testosterone production by disrupting cAMP signaling.
Conclusion: Mrp4 absence disrupts Leydig cell cAMP homeostasis by impairing luteinizing hormone-activated testosterone production.
Significance: These findings reveal a mechanism for unexplained side effects of altered androgen production by therapeutics disrupting Mrp4 function.

The physiological role of multidrug resistance protein 4 (Mrp4, Abcc4) in the testes is unknown. We found that Mrp4 is expressed primarily in mouse and human Leydig cells; however, there is no current evidence that Mrp4 regulates testosterone production. We investigated its role in Leydig cells, where testosterone production is regulated by cAMP, an intracellular messenger formed when the luteinizing hormone (LH) receptor is activated. Because Mrp4 regulates cAMP, we compared testosterone levels in *Mrp4*$^{-/-}$ and *Mrp4*$^{+/+}$ mice. Young *Mrp4*$^{-/-}$ mice had significantly impaired gametogenesis, reduced testicular testosterone, and disruption of Leydig cell cAMP homeostasis. Both young and adult mice had impaired testosterone production. In *Mrp4*$^{-/-}$ primary Leydig cells treated with LH, intracellular cAMP production was impaired and cAMP-response element-binding protein (CREB) phosphorylation was strongly attenuated. Notably, expression of CREB target genes that regulate testosterone biosynthesis was reduced in *Mrp4*$^{-/-}$ Leydig cells *in vivo*. Therefore, Mrp4 is required for normal Leydig cell testosterone production. However, adult *Mrp4*$^{-/-}$ mice are fertile, with a normal circulating testosterone concentration. The difference is that in 3-week-old *Mrp4*$^{-/-}$ mice, disruption of gonadal testosterone production up-regulates hepatic Cyp2b10, a known testosterone-metabolizing enzyme. Therefore, defective testicular testosterone production de-regulates hepatic Cyp-mediated testosterone metabolism to disrupt gametogenesis. These findings have important implications for understanding the side effects of therapeutics that disrupt Mrp4 function and are reported to alter androgen production.

The main function of the testes is gametogenesis and testosterone synthesis (1) in Leydig cells. Testosterone is primarily responsible for secondary sex characteristics and spermatogenesis (2), and its production is regulated by pituitary luteinizing hormone (LH)[2] (3). The main intracellular messenger of LH, cAMP, activates cholesterol and steroidogenic pathways (4). However, regulation of systemic testosterone levels is complex and likely to involve unknown, LH-independent factors.

The multidrug resistance-associated protein Mrp4 (Abcc4) is the fourth member of the ABCC subfamily of ABC transporters (5). Mrp4 was functionally defined as a nucleotide monophosphate efflux transporter (6) and later found to export cyclic nucleotides in an energy-dependent fashion (7, 8) because of latter role of Mrp4 and the effect of Mrp4 substrates (*e.g.* thiopurines (7) and methotrexate (9)) and inhibitors (nonsteroidal anti-inflammatory drugs (10)) on androgen response (11) and fertility (12, 13), we hypothesized that Mrp4 regulates testosterone production.

Here we show that Mrp4 is expressed primarily in the testicular Leydig cells in humans and mice. Using *Mrp4* knock-out mice, we demonstrate that Mrp4 deficiency leads to early impairment of gametogenesis strongly linked to reduced intratesticular testosterone and specific up-regulation of a testosterone-metabolizing cytochrome P450 (Cyp) enzyme. Our findings demonstrate that Mrp4 plays a crucial role in testosterone production and suggest a feedback process whereby factors that impair testosterone biosynthesis induce changes in hepatic enzyme biosynthesis that enhance testosterone degradation.

EXPERIMENTAL PROCEDURES

Animals—Mrp4 mice used in this study were generated in our laboratory as previously described on a mixed C57BL6/129-SVJ and maintained on this background by intercrossing littermates (14). *Mrp4*$^{-/-}$ mice were compared with age-matched

* This work was supported by National Institutes of Health Grants 2R01GM60904, P30CA21745, and CA21865 and by the American Lebanese Associated Charities (ALSAC).
[S] This article contains supplemental Figs. S1–S7 and Tables S1–S4.
[1] To whom correspondence should be addressed: MS 313, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678. Tel.: 901-595-2174; Fax: 901-595-3125; E-mail: John.Schuetz@stjude.org.

[2] The abbreviations used are: LH, luteinizing hormone; CREB, cAMP-response element-binding protein; KO, knock-out; LHR, LH receptor.

littermates (WT mice were littermate controls) housed and fed under identical conditions. This study was approved by the St. Jude Animal Care and Use Committee.

Leydig Cell Purification—Primary Leydig cells were isolated as previously described (15, 16). Testes from *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ mice were decapsulated, and cells were detached from tissues by collagenase treatment. After filtration through gauze, macrophages were removed by incubation at 37 °C for 30 min. Nonadherent cells were then centrifuged through a Percoll density gradient at 3000 × *g* for 20 min to harvest Leydig cells (38–52% density component). Leydig cell viability (typically >90%) was determined by trypan blue dye exclusion. The purity of the fraction was assessed by immunoblotting for HSD3b1, a Leydig cell-specific marker (17).

Testosterone, LH, and Androstenedione Assays—Testes and liver were harvested from 3-week-old and adult mice, homogenized in 1× PBS with a glass Dounce homogenizer, and centrifuged at 5900 × *g* for 5 min. The supernatant was combined with an equal volume of diethyl ether, and the organic phase was dried under a stream of N_2 gas at room temperature and reconstituted in 1× PBS. The recovery of testosterone by this procedure was estimated by spiking samples with radiolabeled testotsterone. The recovery of radiolabel was ~50%. Testosterone (ng/mg) was measured by ^{125}I-labeled radioimmunoassay (RIA) (MP Biomedicals) according to the manufacturer's instructions. Serum testosterone (ng/ml) was directly measured by the same RIA.

Serum LH and androstenedione concentrations (ng/ml) were assayed by the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia School of Medicine (Charlottesville, VA). LH was measured by immunoradiometric sandwich assay and androstenedione by RIA.

Ki67 Staining—At the St. Jude Veterinary Pathology Core, formalin-fixed, paraffin-embedded slides of testes harvested at each time point from three 3-week-old and three adult *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ mice were prepared by standard methods and stained with antibody to Ki67 (cell proliferation marker) and hematoxylin using the LabVision720 autostainer (ThermoShandon). Total Leydig cells were enumerated in five random fields per slide at ×40 magnification. Cell proliferation was reported as the mean number of Ki67-positive cells per field.

Microarray Expression Analyses—RNA was purified from Leydig cells and testes of 3-week-old and adult *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ mice with TRIzol reagent (Invitrogen). Whole testis analysis used three mice per genotype and Leydig cell analyses used nine 3-week-old and five adult mice per genotype. RNA from each age and genotype group was combined before analysis. After RNA quality was confirmed (Agilent Bioanalyzer 2100), RNA was processed using the Affymetrix one-cycle target labeling protocol and hybridized overnight to the Affymetrix Mouse 430 2.0 array. Array signals were summarized using the MAS 5 algorithm and $\log_2$-transformed prior to analysis. Microarray analysis of liver gene expression used three mice per genotype and age group; samples were quality assessed as described above, then processed using the Affymetrix 3′IVT express protocol. Labeled targets were hybridized on the HT_MG-430_PM plate array and processed using the GeneTitan system. Array signals were summarized using the RMA algorithm with Affymetrix Expression Console software.

Microarray analysis was performed by using the Spotfire DecisionSite version 9.1 (TIBCO). For genes interrogated by multiple probe sets, a single expression value was calculated using the maximum signal reported across redundant probe sets. Genes associated with steroid metabolism were identified by annotations curated from Entrez Gene, Gene Ontology, and GeneGO Metacore terms (18). For analysis of cAMP target genes, the PKA, CREB, and cAMP pathways were curated for known direct and indirect target genes. Transcriptional targets of the androgen receptor were identified using curated terms from GeneGo and Metacore pathways. Cytochrome P450 genes were identified using the Affymetrix Gene annotations.

Liver Microsome Isolation—Approximately 50–100-mg liver samples from 3-week-old and adult mice were homogenized in homogenization buffer (100 mM Tris base, 100 mM KCl, 1 mM EDTA, and 20 μM butylated hydroxy-toluene BHT) using a Potter pestle attached to an electric drill motor, and centrifuged at 18,500 × *g* for 15 min at 4 °C. The supernatant was centrifuged at 106,000 × *g* for 1 h at 4 °C. The pelleted microsomal fraction was resuspended in microsomal storage buffer (100 mM potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 20 μM BHT, 20% glycerol). Microsome protein concentration was determined by the Bradford assay method (19).

Immunoblotting and Immunohistochemistry—Testes from 3-week-old and adult animals were ultrasonically disrupted in M-Per (Thermo Scientific) plus protease inhibitor mixture (Roche Diagnostics). Protein concentration was determined by the Bradford assay method. Protein extracts were size fractionated by SDS-PAGE as described previously (14). Leydig cells isolated from *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ testes and purified as described above were assayed for expression of StAR (Santa Cruz), Pbr (Everest Biotech), Cyp11a1 (Chemicon International), 3-β-HSD1 (Abcam), and Dhcr7 (Santa Cruz). Liver microsomes were assessed for expression of Cyp2b10, Cyp3a11, and Cyp1a2 by using antibodies (a gift from Dr. Erin Schuetz). Relative protein quantities were determined by densitometry using ImageJ software. Immunohistochemical detection of Mrp4 was performed as previously described (14) using the Mrp4 monoclonal antibody M4-I_{10} (a gift from Dr. George Scheffer).

cAMP Measurement—Freshly isolated Leydig cells were immediately lysed in 0.1 M HCl and assayed for cAMP content (pmol per plated cell number) by using the acetylated version of a cAMP enzyme immunoassay (Enzo Life Sciences) according to the manufacturer's instructions. To assess cAMP concentration under stimulated conditions, the Leydig cells were treated with 10 mIU of LH plus isobutylmethylxanthine (a phosphodiesterase inhibitor), and cAMP was assayed as described above.

Real-time PCR—Real-time PCR was performed in 20 ng of diluted cDNA with SYBR Green (Invitrogen) and 300 nM gene-specific primers, using the ABI 7900HT sequence detection system (ABI). Supplemental Table S4 lists primer sequences, designed using Primer Express (ABI). After 10 min of denaturation, 40 cycles of two-step PCR were performed (15 s at 95 °C and 60 s at 60 °C) followed by a dissociation step of 15 s at 95 °C,

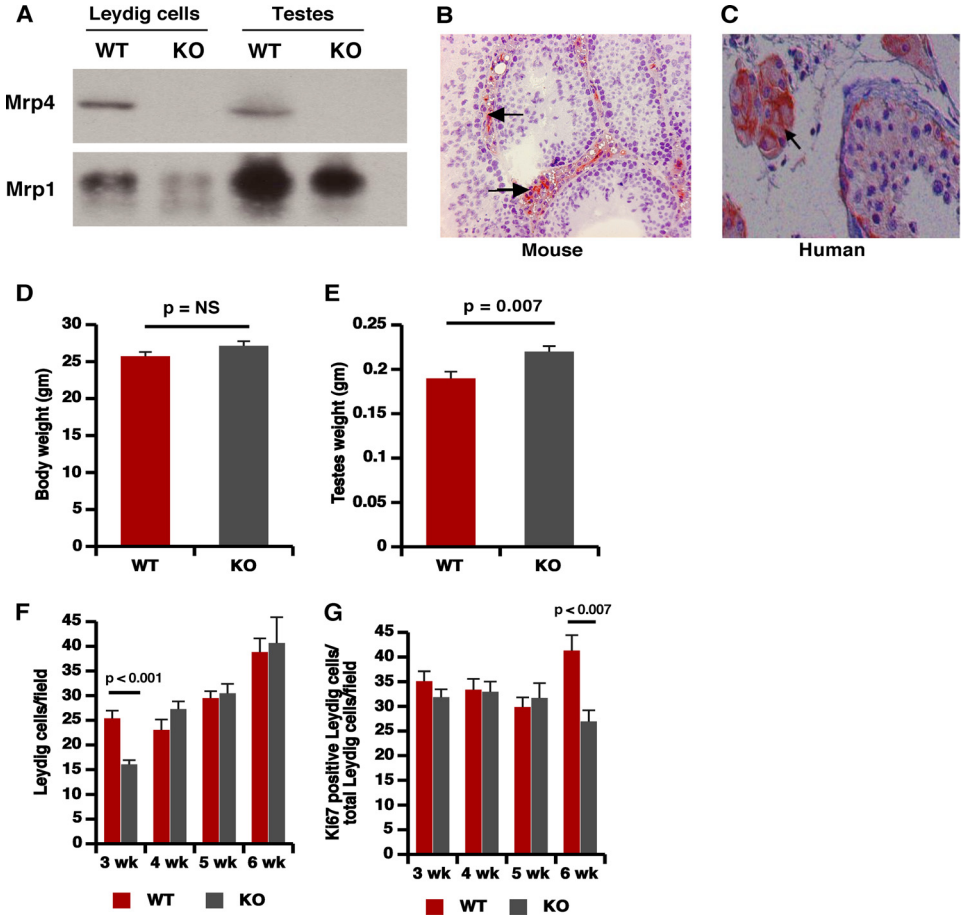

FIGURE 1. **Mrp4 is expressed in Leydig cells.** Mrp4 and Mrp1 protein was assessed in adult *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ mice by immunoblot (*A*) and immunohistochemistry (*B* and *C*) (see supplemental data). Mrp4 (*red* stain, see *arrows*) is visible in mouse (*B*) and human (*C*) Leydig cells (magnification ×400). *D*, mean body weight and *E*, testicular weight of adult *Mrp4*$^{+/+}$ (n = 37) and *Mrp4*$^{-/-}$ (n = 37) mice. *F*, mean testicular Leydig cell counts (n = 3 mice per age and genotype). *G*, mean testicular Leydig cell proliferation (Ki67+ cells per total Leydig cell number) (n = 3 mice per age and genotype). All *error bars* = S.E.

15 s at 60 °C, and 15 s at 95 °C. The fold-difference in expression, normalized to housekeeping genes, was calculated by the $\Delta\Delta C_t$ method.

Testosterone Metabolism—Mouse liver microsomes were suspended in 10 mM phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$, and 2.4 mM NADPH. Testosterone was added (200 μM final concentration) followed by a 1-h incubation at 37 °C. The reaction was terminated by the addition of and equal volume of cold methanol and precipitated proteins were separated by centrifugation at 13,000 × g for 5 min at RT. One hundred microliters of sample were injected in the HPLC system for metabolite analysis. HPLC was performed using Waters 600-717-2487 systems (Waters) and chromatographic separation was achieved using a reverse phase Hibar RT 250-4 pre-packed column (Merck). The mobile phase was 50% methanol, 50% water (v/v) with a run time of 50 min. Data acquisition and analysis was performed using Empower 2 software. The standard curve was linear from 0.02 to 1 μg/ml for 6α-hydroxytestosterone and 16α-hydroxytestosterone and from 0.05 to 1 μg/ml for 16β-hydroxytestosterone and 7α-hydroxytestosterone. The CV for the assay was less than 10%. Concentrations of the all the metabolites were calculated from the standard curve and the observed concentrations in the microsomal incubates were within the concentration ranges for the standard curve. The rate of formation of each metabolite is expressed as picomole/mg/min. The following standards used: 6α-hydroxytestosterone, 6β-hydroxytestosterone, 16α-hydroxytestosterone, 16β-hydroxytestosterone, 7α-hydroxytestosterone, and testosterone obtained from Steraloids Inc.

Statistics—Data are expressed as mean ± S.E. of at least three animals per age and genotype for each experiment. The statistical significance of the difference between mean values was determined by Student's t test.

RESULTS AND DISCUSSION

Mrp4 Expression in Leydig Cells—Immunoblot analysis of testicular homogenates revealed Mrp4 expression (Fig. 1*A*). Similar analysis of purified Leydig cells detected abundant amounts of Mrp4 but not Mrp1 (highly expressed in the seminiferous tubules) (Fig. 1*A*). Immunohistochemical analysis with an Mrp4-specific monoclonal antibody confirmed that Mrp4 is localized to the Leydig cells in mouse (Fig. 1*B*) and human (Fig. 1*C*) testes. Adult body (Fig. 1*D*) and testicular (Fig. 1*E*) weight were slightly greater in KO mice than in WT littermates; however, the absolute number (Fig. 1*F*) and proportion of proliferating (Fig. 1*G*) Leydig cells were similar except in 3-week-old KO mice (30% fewer Leydig cells). Therefore, Mrp4 is highly

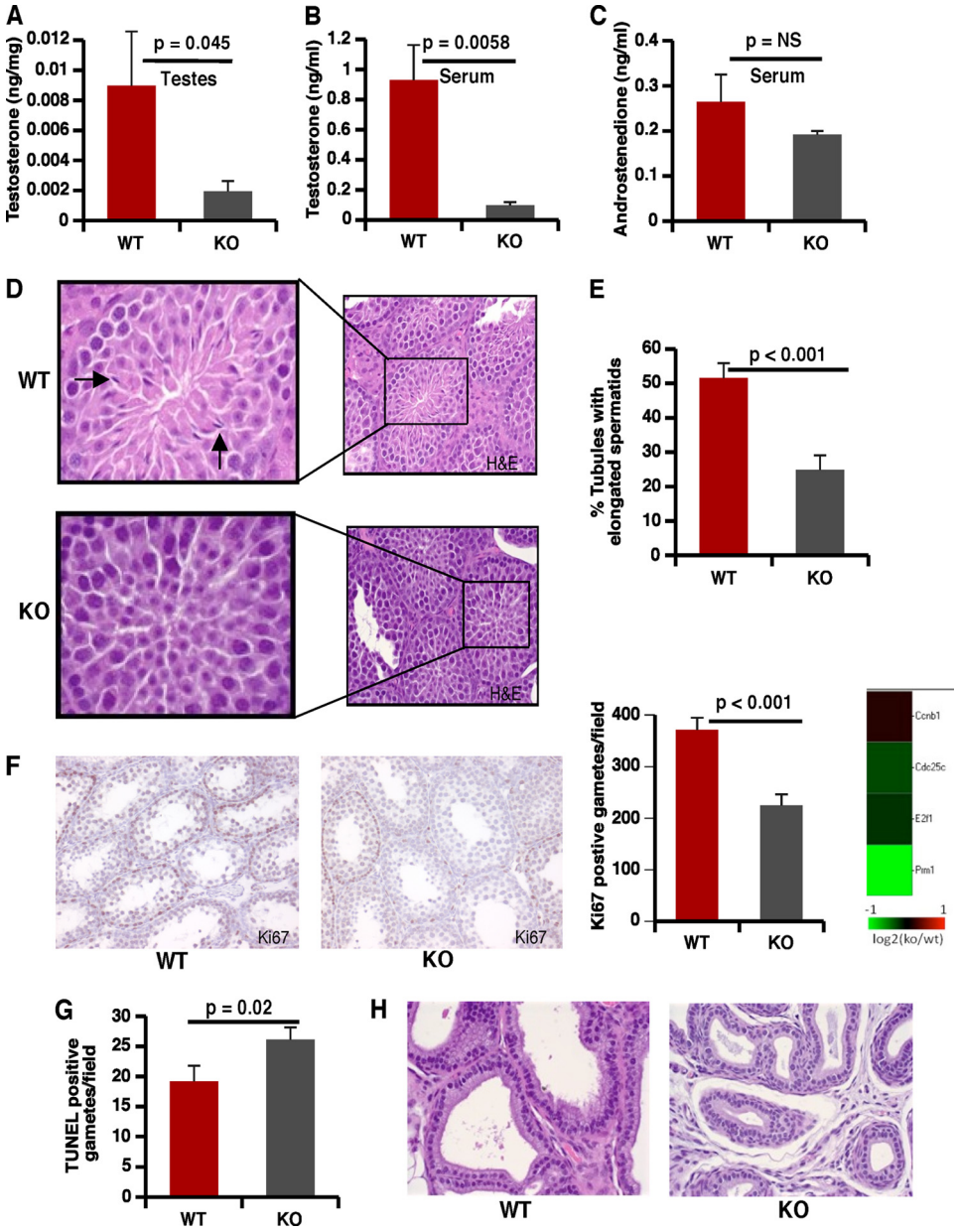

FIGURE 2. **Three-week-old *Mrp4*$^{-/-}$ mice are deficient in testicular and serum testosterone.** Testosterone concentration in testes (*A*) (*Mrp4*$^{+/+}$, $n = 9$; *Mrp4*$^{-/-}$, $n = 4$) and serum (*B*) (*Mrp4*$^{+/+}$ $n = 7$; *Mrp4*$^{-/-}$ $n = 10$) by RIA. *C*, serum androstenedione concentration by RIA ($n = 2$/genotype). *D*, testicular morphology (H&E stain, magnification ×400). *Arrows* indicate elongated spermatids in *Mrp4* WT mice but immature spermatogonia in *Mrp4* KO mice. *Enlarged insets* show these cells in the seminiferous tubule lumen. *E*, percentage of seminiferous tubules per high-power field (HPF) containing elongated spermatids. *F*, quantification of Ki67+ cells seen in *D* (mean number/HPF) (×40). Heat map represents genes crucial to gamete mitosis. *G*, mean number of apoptotic germ cells/HPF (×40) in TUNEL assay. *H*, prostate morphology (H&E, magnification ×400). All data are means; *error bars* are S.E.

expressed in Leydig cells but its absence does not generally affect Leydig cell number or proliferation.

Absence of Mrp4 Reduces Testicular Testosterone—The mean testicular testosterone concentration in prepubertal (3-week-old) KO mice was only 20% of that in WT mice (Fig. 2*A*), much lower than predicted by Leydig cell numbers, and the serum testosterone concentration was less than 10% of WT (Fig. 2*B*). The serum testosterone concentration corresponded to the *Mrp4* gene copy number (*Mrp4*$^{+/+}$ *versus* *Mrp4*$^{+/-}$ *versus* *Mrp4*$^{-/-}$), such that *Mrp4*-heterozygous mice had approximately half that of WT littermates (supplemental Fig. S1). Because Mrp4 does not transport androgens (20), reduced Mrp4 protein was unlikely to directly cause this difference. Serum androstenedione concentration was not significantly reduced in the KO mice (Fig. 2*C*), suggesting its unimpaired peripheral formation, and the serum progesterone concentration was similar in WT and KO mice (WT 2.8 ng/ml ± 0.5 *versus* KO 2.9 ng/ml ± 0.5 S.E.). However, light microscopy revealed disrupted gametogenesis in 3-week-old KO testes, which contained predominantly rounded, immature spermatogonia (Fig. 2*D*).

To assess how Mrp4 absence affects gamete maturation, we analyzed the proportion of seminiferous tubules containing elongated spermatids (gametes). At age 21 days, WT mice had a significantly higher proportion of seminiferous tubules containing gametes (50 *versus* 24%) (Fig. 2*E*), consistent with the

availability of testosterone (21). Because germ cell maturation is controlled by proliferation and apoptosis (22), we tested whether these were altered in KO animals. Immunohistochemical analysis using the proliferation marker Ki67 indicated significant (~33%) reduction of germ cell proliferation in the seminiferous tubules in the absence of Mrp4 (Fig. 2*F*), suggesting that germ cell mitosis is impaired in KO testes. To test this possibility, we conducted an expression analysis of testicular RNA from KO and WT mice. Expression of genes important in germ cell mitosis (*ccnb1*, *cdc25c*, and *E2f*) was slightly reduced, whereas that of *pRM*1 (important in gamete chromatin assembly) was markedly reduced (23) (Fig. 2*F*, *right*). These findings are likely to explain the reduced Ki67 immunoreactivity of KO germ cells and are consistent with the role of testosterone in spermatogenesis (21). Because of the marked reduction of proliferating germ cells, we tested whether the rate of apoptosis had changed. TUNEL staining showed a >30% increase in the number of apoptotic germ cells per tubule in KO animals (Fig. 2*G*).

Light microscopy of the prostate gland (dependent on exogenous testosterone for maturation (24) revealed disorganized tubular structure and enlarged interstitial space in 3-week-old KO mice (Fig. 2*H*). The decrease in germ cell proliferation and in expression of genes required for spermatocyte mitosis in KO mice suggests that low testosterone levels impair mitosis of germ cells, which are also reduced by enhanced apoptosis. Notably, expression of genes required for spermatocyte meiosis (*e.g. Stra8*) (25, 26) was not altered in *Mrp4*$^{-/-}$ testes (supplemental Table S3). Genes that are up-regulated or down-regulated in association with testicular development (27, 28) were expressed similarly in KO and WT mice (supplemental Fig. S2), suggesting that nontestosterone-dependent testicular development is not generally impaired. Apoptosis of germ cells and morphologic changes in prostate tissue are expected consequences of the low systemic testosterone concentration (29).

Molecular Basis of Impaired Testosterone Production in Mrp4$^{-/-}$ *Leydig Cells*—The expression of Mrp4 in Leydig cells, the strong relationship between the quantity of Mrp4 and intratesticular and serum testosterone concentration (supplemental Figs. S1 and S3), and the regulation of cyclic nucleotides by Mrp4 (6) led us to hypothesize that expression of steroid and/or cholesterol biosynthetic genes is altered in KO Leydig cells (Fig. 3*A*). Because testosterone biosynthesis is regulated by cAMP after LH receptor (LHR) activation (30), we examined the expression of steroid biosynthetic genes in adult and 3-week-old WT and KO mice by microarray analysis of RNA isolated from purified Leydig cells and hierarchical cluster analysis of "steroid" genes curated from EntrezGene, Gene Ontology, and Metacore (18) (supplemental Fig. S4). The genes (supplemental Table S1) were selected on the basis of $p \leq 0.05$ and a 1.5-fold or greater difference in expression in KO *versus* WT Leydig cells. The heat map (supplemental Fig. S4) showed striking similarities in the expression of affected steroid genes in 3-week-old and adult KO Leydig cells. We hypothesized that because testosterone biosynthesis in Leydig cells is regulated by CREB phosphorylation after LH-mediated cAMP formation (30), Leydig cell expression of other genes whose promoters contain CREB-binding sites (StAR, Cyp17, Cyp11a, and 3-β-HSD) (31, 32) might be altered. Real-time PCR analysis of Leydig cell RNA indicated substantial reduction in expression of StAR (>60%) and 3-β-HSD in *Mrp4*$^{-/-}$ Leydig cells but minimal change in Pbr, a cholesterol importer on the outer mitochondrial membrane (32, 33) (Fig. 3*B*). Because StAR, a cholesterol chaperone and rate-limiting enzyme in cholesterol import, is crucial in early steroidogenesis (34), we evaluated StAR protein expression in Leydig cells. Of the mitochondrial proteins evaluated (VDAC, StAR, and Pbr), only StAR was reduced (>55%) (Fig. 3*C*). This finding and reduction of 3-β-HSD1 (>50%) are consistent with the reduced testicular testosterone concentration in *Mrp4*$^{-/-}$ mice (35). Notably, this effect appears to be specific to Leydig cells, as StAR expression was not reduced in the adrenal gland (supplemental Fig. S5).

Given the critical role of cAMP in regulating testosterone biosynthesis (4), we assessed the concentration of cAMP and testosterone in Leydig cells. Cells from adult KO mice showed strong reduction (>60%) of testosterone and basal cAMP (Fig. 3, *D* and *E*). Moreover, in isolated Leydig cells stimulated with LH at physiological concentration (10 mIU/ml), cAMP concentration was elevated by a factor >5 in WT cells but increased only modestly (by a factor <2) in KO cells (Fig. 3*E*). We reasoned that impaired cAMP formation might affect CREB activation by LH in KO Leydig cells. Freshly isolated Leydig cells from WT and KO mice were incubated with 10 mIU/ml of LH for various intervals. Phosphorylation of CREB was rapid (within 5 min) in WT cells but slow in KO cells (Fig. 3*F*), consistent with the reduced capacity to increase intracellular cAMP. Notably, CREB protein and mRNA levels were similar in WT and KO Leydig cells. These findings suggest that the absence of Mrp4 impairs LH-mediated cAMP formation in Leydig cells, disrupting CREB-mediated transcription. We curated target genes activated by PKA, CREB, or cAMP to determine whether gene expression downstream of CREB was altered in Leydig cells *in vivo*. After elimination of redundant probe sets, 38 genes (supplemental Table S2) whose expression differed 1.5-fold in either direction were identified. Expression of these 38 genes was generally strongly reduced in 3-week-old and adult *Mrp4*$^{-/-}$ cells, consistent with impaired cAMP formation (Fig. 3*G*).

Because the LHR regulates Leydig cell testosterone synthesis and is itself regulated by cAMP levels (36, 37), we evaluated LHR mRNA by real-time PCR and immunoblot analysis. LHR mRNA and protein were strongly reduced in the absence of Mrp4 (Fig. 3*H*), whereas LH concentration was similar in WT and KO mice (Fig. 3*I*), suggesting that the absence of Mrp4 alters LHR expression in Leydig cells. The reduced Leydig cell number at 3 weeks (Fig. 1*F*) was also consistent with the reduced LHR level, as the postnatal Leydig cell number initially depends on LHR signaling (38). Because LHR signaling is essential for steroidogenesis and cholesterol homeostasis (39), we tested whether expression of two genes important in *de novo* cholesterol biosynthesis, *Hmgcs1* and *Dhcr7* (40), were altered by loss of Mrp4. Real-time PCR of RNA from WT and KO Leydig cells showed >70% reduction of *Dhcr7* expression in KO cells (Fig. 3*J*). This apparent defect in LH-regulated cholesterol synthesis appears specific to Leydig cells, as KO mice did not have reduced serum cholesterol (Fig. 3*K*).

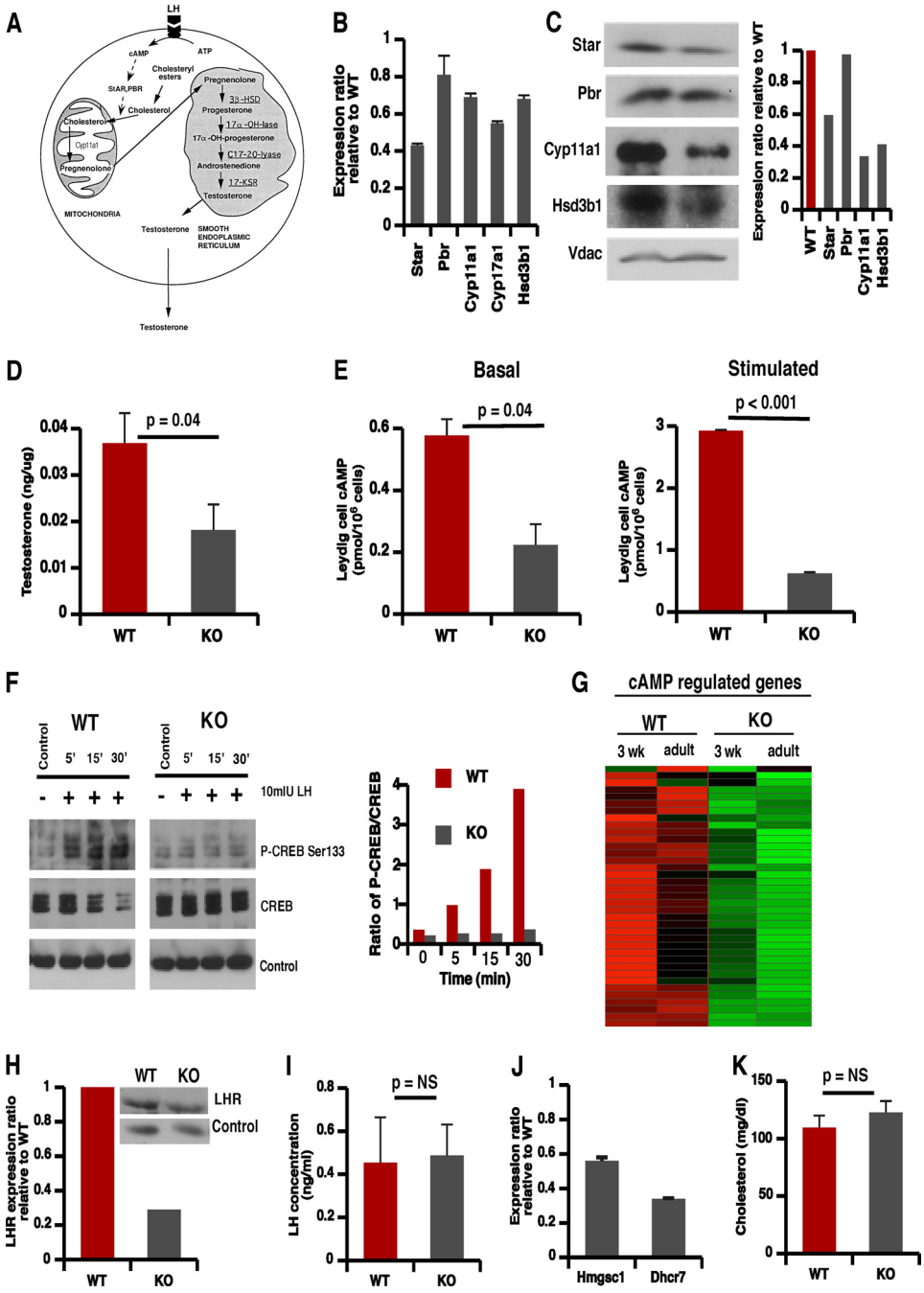

FIGURE 3. **Testicular testosterone biosynthesis is impaired in *Mrp4*$^{-/-}$ mice.** *A*, schematic of testosterone biosynthesis in Leydig cells. *B*, real-time PCR analysis of testosterone biosynthesis gene expression (normalized to GAPDH) in *Mrp4*$^{-/-}$ Leydig cells. *C*, immunoblot analysis of testosterone biosynthesis gene expression (Vdac loading control). At *right*, expression ratios plotted from absolute band intensity. *D*, mean ± S.E. testosterone concentration in adult Leydig cells ($n = 3$ mice/genotype) by RIA. *E*, mean ± S.E. basal and stimulated cAMP concentration in Leydig cells by EIA. *F*, immunoblot analysis of CREB and P-CREB (Ser-133) at the indicated time points in Leydig cells treated as in *panel F*. The loading control was an unknown protein cross-reactive with the antibody that remained constant among samples. *Graph at right* shows ratios of P-CREB to CREB. *G*, selection of cAMP-regulated genes for query. Heat map represents expression of these genes in *Mrp4* KO and WT Leydig cells. *H*, immunoblot shows LHR protein expression in Leydig cells. A nonspecific band was used as loading control. *Graph below* expresses ratios of band intensity. *I*, mean ± S.E. serum LH concentration by RIA ($n = 3$/genotype). *J*, cholesterol gene expression in KO Leydig cells by real-time PCR analysis, presented as the mean ± S.D. ratio of expression in WT cells. *K*, mean serum cholesterol concentration by enzymatic assay (*Mrp4*$^{+/+}$, $n = 10$; *Mrp4*$^{-/-}$, $n = 8$).

Gametes Differentiate Normally in Adult Mrp4$^{-/-}$ *Mice*—As expected from the microarray data, adult KO mice had a 6-fold reduction of testicular testosterone concentration (Fig. 4*A*). However, paradoxically, their serum testosterone concentration was similar to that of WT animals (Fig. 4*B*) and did not reflect elevated serum androstenedione (Fig. 4*C*). Although the KO animals were fertile, the average litter size (Fig. 4*D*) was slightly smaller (five in KO mice *versus* six in WT mice), suggesting an inherent minor reproductive defect. Nonetheless, light microscopy revealed the same proportion of mature sperm cells in KO and WT seminiferous tubules (Fig. 4*E*). Furthermore, germ cell proliferation was comparable in WT and

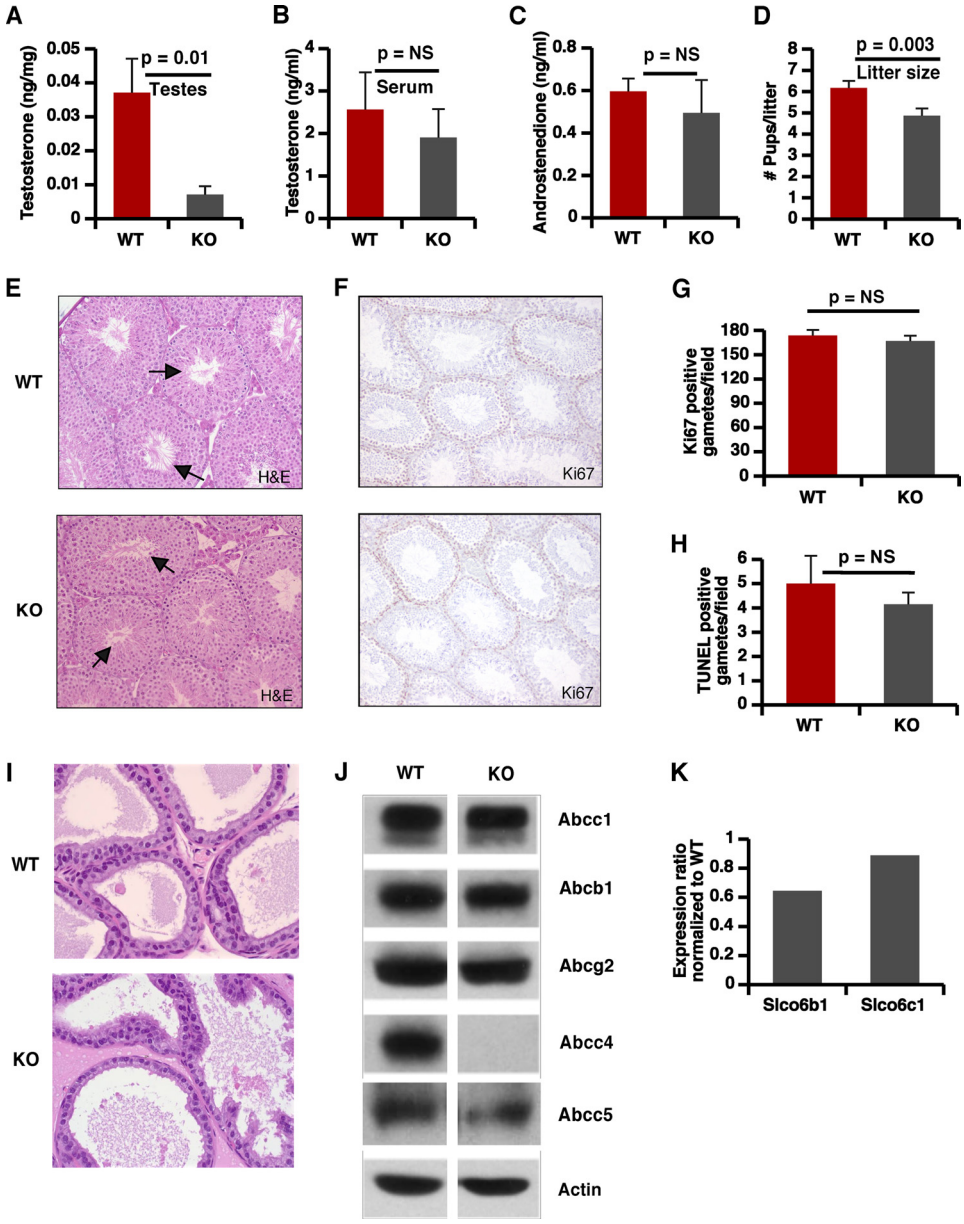

FIGURE 4. **Serum testosterone concentration is normal in adult *Mrp4*$^{-/-}$ mice and related deficiencies are absent.** Mean ± S.E. testosterone concentration in testes (*A*) (*Mrp4*$^{+/+}$, $n = 7$; *Mrp4*$^{-/-}$, $n = 4$) and serum (*B*) (*Mrp4*$^{+/+}$, $n = 10$; *Mrp4*$^{-/-}$, $n = 15$) by RIA. *C*, mean ± S.E. serum androstenedione concentration by RIA ($n = 2$ per *Mrp4* genotype). *D*, mean ± S.E. number of pups per litter (*Mrp4*$^{+/+}$, $n = 51$ litters; *Mrp4*$^{-/-}$, $n = 53$ litters). *E*, adult testicular morphology (H&E, ×200). *Arrows* indicate mature sperm tails in the seminiferous tubule lumen. *F*, immunohistochemical analysis of cell proliferation with Ki67 antibody. *G*, quantification of Ki67-positive cells in *panel F* (mean ± S.E. number of Ki67-positive cells/field) (×400). *H*, quantification of apoptotic cells (mean ± S.E.) (TUNEL-positive cells/field) (×400). *I*, prostate gland morphology (H&E, ×400). *J*, immunoblot analysis of ABC transporters in adult testes, with β-actin as loading control. *K*, mRNA expression of Slco6b1 and Slco6c1 (normalized to Rsp16 expression) in Leydig cells by real-time PCR.

KO testes (Fig. 4, *F* and *G*) and apoptosis was minimal (Fig. 4*H*), indicating adequate intratesticular testosterone. Notably, the testosterone concentration was more than 3-fold greater in adult than in 3-week-old KO testes and was similar to that in 3-week-old WT testes (Figs. 2*A* and 4*A*); this finding is likely to explain the fertility of adult KO mice. Adult WT and KO prostate glands were morphologically identical (Fig. 4*I*), indicating sufficient circulating testosterone for prostate epithelial cell maturation.

Because some ABC transporters (*e.g.* Abcg2) interact with steroids structurally similar to testosterone (41), we hypothesized that increased expression of an ABC transporter(s) or a testis-specific testosterone transporter might account for the rise in serum testosterone in mature KO mice. Immunoblot analysis showed no up-regulation in protein expression of the efflux transporters Mrp1, Mrp5, Pgp, or Abcg2 in the testes (Fig. 4*J*), whereas Mrp2 was undetectable. Real-time PCR showed no Leydig cell up-regulation of Slco6b1 and Slco6c1, recently suggested to be androgen efflux transporters (42). These findings suggest that the rise in systemic testosterone in adult KO mice despite impaired synthesis does not reflect enhanced expression of testosterone efflux transporters.

Lower Serum Testosterone in Young Mrp4$^{-/-}$ *Mice Results from Increased Testosterone Metabolism*—Serum testosterone concentration in adult KO mice was relatively high without

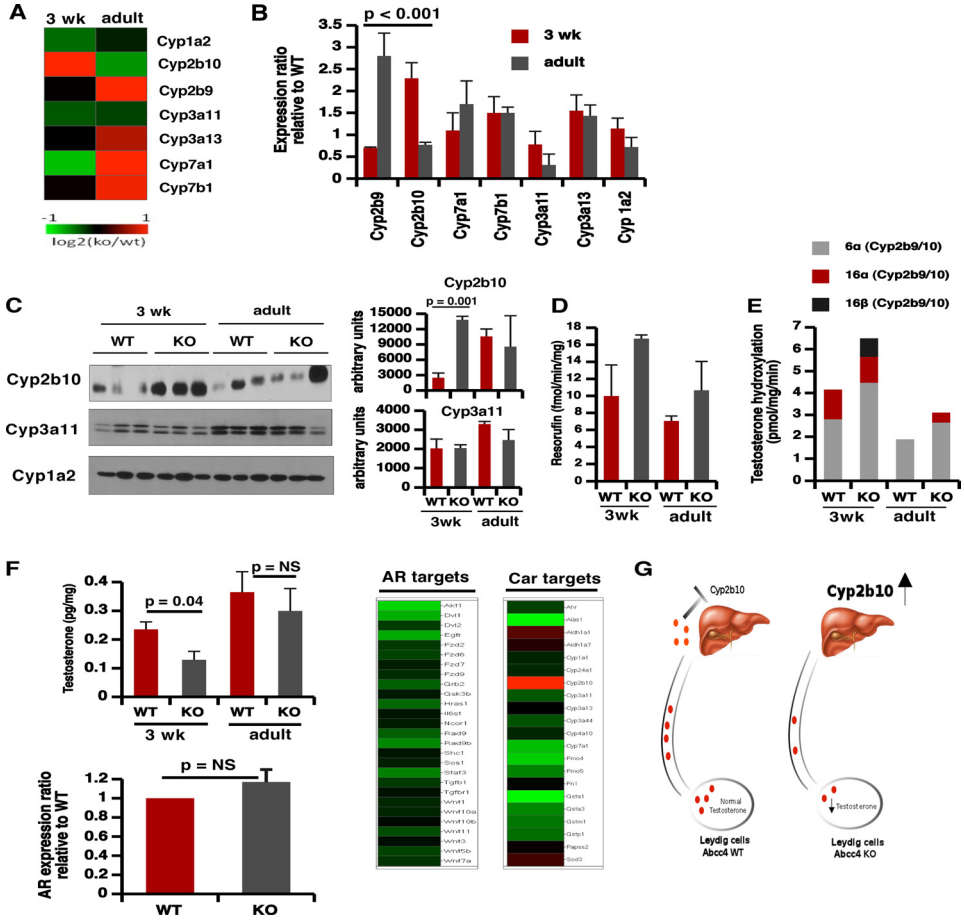

FIGURE 5. **Serum testosterone compensation in adult Mrp4$^{-/-}$ mice results from decreased testosterone metabolism.** *A*, expression heat map of testosterone-metabolizing Cyp450 genes in liver ($n = 3$ per age and genotype). *B*, hepatic expression of testosterone-metabolizing enzymes in juvenile and adult Mrp4$^{-/-}$ mice ($n = 3$ per age group and genotype). Real-time PCR data normalized to Gapdh expression are presented as the ratio of expression in Mrp4$^{+/+}$ counterparts. *C*, representative immunoblot analysis of Cyp2b10 and Cyp3a11 proteins in liver microsomes ($n = 3$ mice per age and genotype). Cyp1a2 was the loading control. Mean ratios of band intensity from two separate experiments are shown ($n =$ total 6 animals). *D*, Cyp2b enzyme activity in Mrp4$^{+/+}$ and Mrp4$^{-/-}$ liver microsomes determined by pentoxyresorufin *O*-dealkylase (PROD) assay. *E*, hydroxylated testosterone metabolites were measured from mouse liver microsomes ($n = 3$ mice per age and genotype) using HPLC analysis. Cyp450s in *parentheses* represent the major Cyp responsible for the corresponding metabolite. *F*, *top*, total testosterone concentration in 3-week-old and adult Mrp4$^{+/+}$ and Mrp4$^{-/-}$ liver measured by RIA. *Bottom*, hepatic androgen receptor mRNA expression (by real-time PCR, normalized to GAPDH expression) in 3-week-old Mrp4$^{-/-}$ relative to Mrp4$^{+/+}$ mice. *Right*, heat maps represent expression of androgen receptor targets (*left*) and Car/Pxr targets (*right*) in 3-week-old WT and KO mice. *G*, model schematic representing the relationship between testicular testosterone concentration and Cyp2b10 expression and activity in Mrp4$^{+/+}$ and Mrp4$^{-/-}$ mice.

up-regulation of testosterone or ABC efflux transporter synthesis. Because the liver is a major site of testosterone metabolism and many stereospecific testosterone hydroxylation reactions are mediated by hepatic Cyp enzymes (43), we reasoned that a Cyp might be up-regulated in 3-week-old but not adult KO liver.

We analyzed hepatic RNA expression by microarray in 3-week-old and adult KO and WT mice and hierarchically clustered the mammalian Cyps (109 probe sets) (supplemental Fig. S6). Among the testosterone-metabolizing Cyps (Cyp1a2, Cyp3a11, Cyp3a13, Cyp7a1, Cyp7b1, and Cyp2b9), only Cyp2b10 showed greater expression in 3-week-old KO *versus* 3-week-old WT liver, and it returned to WT levels in adult KO mice (Fig. 5*A*). Real-time PCR confirmed Cyp2b10 as the only testosterone-metabolizing Cyp strongly increased at 3 weeks (~2.5-fold, $p < 0.001$) (Fig. 5*B*). Notably, Cyp2b9 was not elevated in the 3-week-old KO liver arguing against a general activation of the Cyp2b locus. Cyp3a subfamily members also participate in testosterone metabolism (44), but Cyp3a11 mRNA was not increased at 3 weeks; Cyp3a13 was slightly increased in liver from both 3-week-old and adult mice (Fig. 5*B*). Similar to the mRNA analysis, immunoblot analysis indicated that Cyp2b10 was strongly up-regulated in 3-week-old KO liver microsomes; densitometry indicated a mean 4-fold greater expression in KO than WT 3-week-old liver (Fig. 5*C*), whereas the mean adult levels were similar. Cyp3A11 expression rose as expected in adult mice (43, 45) but did not differ between the WT and KO mice at 3 weeks or in adulthood. Cyp1a2 levels were constant regardless of age or *Mrp4* status (Fig. 5*C*). We then evaluated Cyp2b catalytic activity in KO and WT liver microsomes by examining 7-pentoxyresorufin *O*-dealkylation, a major activity of Cyp2b10 (46) (Fig. 5*D*) and found a greater than 160% increase in activity in 3-week-old KO mice. To determine whether the testosterone metabolism was altered in microsomes we evaluated formation of hydroxylated metabolites of testosterone (6α-hydroxytestosterone, 16α-hydroxytestosterone, 16β-hydroxytestosterone, and 7α-hydroxytestosterone) (Fig. 5*E*). Testosterone hydroxylation (6α-, 16α-, and

16β-) increased by 62.5% in the 3-week-old KO mice, a value in close agreement with the increased activity measured by resorufin formation. Notably, the formation of 7α-hydroxytestosterone was comparable for 3-week-old WT and KO mice (supplemental Fig. S7). These findings indicate that young KO mice have an increased capacity for Cyp2b-mediated testosterone hydroxylation.

We determined whether elevated Cyp2b10 expression in KO liver reflects general activation of constitutive androstane receptor (CAR) target genes. First, we determined that expression of the androgen receptor was similar in WT and KO livers (Fig. 5*F*, *left*). Consistent with the lower liver testosterone concentration (Fig. 5*F*, *top*), the 3-week-old KO liver showed reduced expression of genes regulated by the androgen receptor (AR) (Fig. 5*F*, *center*). *In vivo* studies have shown that testosterone administration strongly suppresses Cyp2b10 (46, 47), possibly by suppressing CAR activity (47). To test if general de-repression of CAR target genes was secondary to reduced hepatic testosterone in KO mice, we analyzed the 3-week-old liver microarray data for expression of manually curated constitutive androgen receptor target genes (Fig. 5*E*, *right*); most of these genes were similarly expressed in WT and KO animals, with the exception of the strong Cyp2b10 up-regulation in KO liver. Therefore, general CAR de-repression was unlikely to explain increased hepatic Cyp2b10 expression in the 3-week-old KO mice. In total, these results support a model whereby systemic testosterone is dramatically reduced in young prepubertal *Mrp4* KO mice by a combination of reduced testicular testosterone production and increased hepatic testosterone metabolism (Fig. 5*G*). However, the adult *Mrp4* KO mice, despite a sustained reduction in testosterone production recover almost normal levels of testosterone due to reduced testosterone metabolism.

Our use of a murine genetic model revealed a new and essential role for Mrp4 in regulating Leydig cell testosterone synthesis downstream of LHR activation. Testosterone synthesis is disrupted in the absence of Mrp4 because of the attenuation of cAMP formation after LHR activation, which appears secondary to impaired CREB activation. Because Mrp4 exports cyclic nucleotides (7), this finding was unexpected. A plausible explanation is that the constitutive absence of Mrp4 produces specific Leydig cell adaptations (*e.g.* elevated phosphodiesterase levels) that down-regulate the LH pathway of cAMP formation (48). Our findings suggest that deficiency or impaired function of Leydig cell Mrp4 reduces testosterone production. Indeed, animal studies show that the Mrp4 substrate methotrexate reduces testosterone concentration by inhibiting steroidogenesis (11). Another Mrp4 substrate, ganciclovir (49), reduces sperm count and damages the testes of rodents (50). Furthermore, humans receiving aspirin (an Mrp4 inhibitor and substrate (10, 51)) show impaired LHR-activated steroidogenesis (52). Likewise, aspirin decreased the number of spermatids in rats, producing a morphology consistent with impaired spermatocyte maturation (53). These findings strongly support our findings and suggest that factors that disrupt Mrp4 function deregulate testosterone production. Because several human *Mrp4* alleles are functionally defective (54–56), this conclusion might be genetically evaluated in humans by assessing the relationship of serum testosterone to these variant alleles.

Our finding that disruption of Leydig cell testosterone biosynthesis affects hepatic metabolism provides a new understanding of testosterone homeostasis *in vivo*. Previous studies in rodents indicated that expression of hepatic drug-metabolizing enzymes can be modified by pituitary growth hormone secretion (57). The role of growth hormone in regulation of human drug-metabolizing enzymes (*e.g.* CYP3A4) has been recapitulated in primary human hepatocyte cultures (58). Our findings extend this view by showing that deficient gonadal production of testosterone activates hepatic Cyp expression in mice. Currently, it is unknown whether acute or sustained disruption of human testicular testosterone production alters the activity of drug-metabolizing enzymes. If so, these findings have important therapeutic implications.

REFERENCES

1. Shalet, S. M. (2009) Normal testicular function and spermatogenesis. *Pediatr. Blood Cancer* **53,** 285–288
2. Walker, W. H. (2009) Molecular mechanisms of testosterone action in spermatogenesis. *Steroids* **74,** 602–607
3. Ruwanpura, S. M., McLachlan, R. I., and Meachem, S. J. (2010) Hormonal regulation of male germ cell development. *J. Endocrinol.* **205,** 117–131
4. Payne, A. H., and Youngblood, G. L. (1995) Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol. Reprod.* **52,** 217–225
5. Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F., and Borst, P. (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (*MRP1*), in human cancer cell lines. *Cancer Res.* **57,** 3537–3547
6. Zelcer, N., Reid, G., Wielinga, P., Kuil, A., van der Heijden, I., Schuetz, J. D., and Borst, P. (2003) Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). *Biochem. J.* **371,** 361–367
7. Chen, Z. S., Lee, K., and Kruh, G. D. (2001) Transport of cyclic nucleotides and estradiol 17-β-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J. Biol. Chem.* **276,** 33747–33754
8. Li, C., Krishnamurthy, P. C., Penmatsa, H., Marrs, K. L., Wang, X. Q., Zaccolo, M., Jalink, K., Li, M., Nelson, D. J., Schuetz, J. D., and Naren, A. P. (2007) Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia. *Cell* **131,** 940–951
9. Chen, Z. S., Lee, K., Walther, S., Raftogianis, R. B., Kuwano, M., Zeng, H., and Kruh, G. D. (2002) Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4). MRP4 is a component of the methotrexate efflux system. *Cancer Res.* **62,** 3144–3150
10. El-Sheikh, A. A., van den Heuvel, J. J., Koenderink, J. B., and Russel, F. G. (2007) Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport. *J. Pharmacol. Exp. Ther.* **320,** 229–235
11. Badri, S. N., Vanithakumari, G., and Malini, T. (2000) Studies on methotrexate effects on testicular steroidogenesis in rats. *Endocr. Res.* **26,** 247–262
12. Polifka, J. E., and Friedman, J. M. (2002) Teratogen update. Azathioprine and 6-mercaptopurine. *Teratology* **65,** 240–261
13. Bendre, S. V., Shaddock, J. G., Patton, R. E., Dobrovolsky, V. N., Albertini, R. J., and Heflich, R. H. (2005) Lymphocyte Hprt mutant frequency and sperm toxicity in C57BL/6 mice treated chronically with azathioprine. *Mutat. Res.* **578,** 1–14
14. Leggas, M., Adachi, M., Scheffer, G. L., Sun, D., Wielinga, P., Du, G., Mercer, K. E., Zhuang, Y., Panetta, J. C., Johnston, B., Scheper, R. J., Stewart, C. F., and Schuetz, J. D. (2004) Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol. Cell. Biol.* **24,** 7612–7621
15. Schumacher, M., Schäfer, G., Holstein, A. F., and Hilz, H. (1978) Rapid

isolation of mouse Leydig cells by centrifugation in Percoll density gradients with complete retention of morphological and biochemical integrity. *FEBS Lett.* **91,** 333–338
16. Asada, S., Koga, M., Nagao, K., Tabata, M., and Takeda, K. (2001) *J. Health Sci.* **47,** 60–64
17. Bain, P. A., Yoo, M., Clarke, T., Hammond, S. H., and Payne, A. H. (1991) Multiple forms of mouse 3β-hydroxysteroid dehydrogenase/δ5-δ4-isomerase and differential expression in gonads, adrenal glands, liver, and kidneys of both sexes. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 8870–8874
18. Nikolsky, Y., Kirillov, E., Zuev, R., Rakhmatulin, E., and Nikolskaya, T. (2009) Functional analysis of OMICs data and small molecule compounds in an integrated "knowledge-based" platform. *Methods Mol. Biol.* **563,** 177–196
19. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254
20. Ho, L. L., Kench, J. G., Handelsman, D. J., Scheffer, G. L., Stricker, P. D., Grygiel, J. G., Sutherland, R. L., Henshall, S. M., Allen, J. D., and Horvath, L. G. (2008) Androgen regulation of multidrug resistance-associated protein 4 (MRP4/ABCC4) in prostate cancer. *Prostate* **68,** 1421–1429
21. McLachlan, R. I., Wreford, N. G., Tsonis, C., De Kretser, D. M., and Robertson, D. M. (1994) Testosterone effects on spermatogenesis in the gonadotropin-releasing hormone-immunized rat. *Biol. Reprod.* **50,** 271–280
22. Wolgemuth, D. J., Laurion, E., and Lele, K. M. (2002) Regulation of the mitotic and meiotic cell cycles in the male germ line. *Recent Prog. Horm. Res.* **57,** 75–101
23. Wykes, S. M., and Krawetz, S. A. (2003) The structural organization of sperm chromatin. *J. Biol. Chem.* **278,** 29471–29477
24. Marker, P. C., Donjacour, A. A., Dahiya, R., and Cunha, G. R. (2003) Hormonal, cellular, and molecular control of prostatic development. *Dev. Biol.* **253,** 165–174
25. Baltus, A. E., Menke, D. B., Hu, Y. C., Goodheart, M. L., Carpenter, A. E., de Rooij, D. G., and Page, D. C. (2006) In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat. Genet.* **38,** 1430–1434
26. Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M. J., Rossant, J., Hamada, H., and Koopman, P. (2006) Retinoid signaling determines germ cell fate in mice. *Science* **312,** 596–600
27. O'Shaughnessy, P. J., Willerton, L., and Baker, P. J. (2002) Changes in Leydig cell gene expression during development in the mouse. *Biol. Reprod.* **66,** 966–975
28. Ge, R. S., Dong, Q., Sottas, C. M., Chen, H., Zirkin, B. R., and Hardy, M. P. (2005) Gene expression in rat leydig cells during development from the progenitor to adult stage. A cluster analysis. *Biol. Reprod.* **72,** 1405–1415
29. Kim, J. M., Ghosh, S. R., Weil, A. C., and Zirkin, B. R. (2001) Caspase-3 and caspase-activated deoxyribonuclease are associated with testicular germ cell apoptosis resulting from reduced intratesticular testosterone. *Endocrinology* **142,** 3809–3816
30. Dufau, M. L. (1988) Endocrine regulation and communicating functions of the Leydig cell. *Annu. Rev. Physiol.* **50,** 483–508
31. Zhang, X., Odom, D. T., Koo, S. H., Conkright, M. D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J. R., Emerson, B., Hogenesch, J. B., Unterman, T., Young, R. A., and Montminy, M. (2005) Genome-wide analysis of cAMP-response element-binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 4459–4464
32. Krueger, K. E., and Papadopoulos, V. (1990) Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. *J. Biol. Chem.* **265,** 15015–15022
33. Papadopoulos, V. (1993) Peripheral-type benzodiazepine/diazepam binding inhibitor receptor. Biological role in steroidogenic cell function. *Endocr. Rev.* **14,** 222–240
34. Stocco, D. M., and Clark, B. J. (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocr. Rev.* **17,** 221–244
35. O'Shaughnessy, P. J., Johnston, H., Willerton, L., and Baker, P. J. (2002) Failure of normal adult Leydig cell development in androgen receptor-deficient mice. *J. Cell Sci.* **115,** 3491–3496
36. Segaloff, D. L., Wang, H. Y., and Richards, J. S. (1990) Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization. *Mol. Endocrinol.* **4,** 1856–1865
37. Kishi, H., Minegishi, T., Tano, M., Abe, Y., Ibuki, Y., and Miyamoto, K. (1997) Down-regulation of LH/hCG receptor in rat cultured granulosa cells. *FEBS Lett.* **402,** 198–202
38. Mendis-Handagama, S. M., Watkins, P. A., Gelber, S. J., and Scallen, T. J. (1998) The effect of chronic luteinizing hormone treatment on adult rat Leydig cells. *Tissue Cell* **30,** 64–73
39. Eacker, S. M., Agrawal, N., Qian, K., Dichek, H. L., Gong, E. Y., Lee, K., and Braun, R. E. (2008) Hormonal regulation of testicular steroid and cholesterol homeostasis. *Mol. Endocrinol.* **22,** 623–635
40. Soccio, R. E., and Breslow, J. L. (2004) Intracellular cholesterol transport. *Arterioscler. Thromb. Vasc. Biol.* **24,** 1150–1160
41. Janvilisri, T., Venter, H., Shahi, S., Reuter, G., Balakrishnan, L., and van Veen, H. W. (2003) Sterol transport by the human breast cancer resistance protein (ABCG2) expressed in *Lactococcus lactis*. *J. Biol. Chem.* **278,** 20645–20651
42. Suzuki, T., Onogawa, T., Asano, N., Mizutamari, H., Mikkaichi, T., Tanemoto, M., Abe, M., Satoh, F., Unno, M., Nunoki, K., Suzuki, M., Hishinuma, T., Goto, J., Shimosegawa, T., Matsuno, S., Ito, S., and Abe, T. (2003) Identification and characterization of novel rat and human gonad-specific organic anion transporters. *Mol. Endocrinol.* **17,** 1203–1215
43. Zhang, Y. Y., and Yang, L. (2009) Interactions between human cytochrome P450 enzymes and steroids. Physiological and pharmacological implications. *Exp. Opin. Drug Metab. Toxicol.* **5,** 621–629
44. Choi, M. H., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (2005) Characterization of testosterone 11β-hydroxylation catalyzed by human liver microsomal cytochromes P450. *Drug Metab. Dispos.* **33,** 714–718
45. Sakuma, T., Takai, M., Endo, Y., Kuroiwa, M., Ohara, A., Jarukamjorn, K., Honma, R., and Nemoto, N. (2000) A novel female-specific member of the CYP3A gene subfamily in the mouse liver. *Arch. Biochem. Biophys.* **377,** 153–162
46. Honkakoski, P., Kojo, A., and Lang, M. A. (1992) Regulation of the mouse liver cytochrome P450 2B subfamily by sex hormones and phenobarbital. *Biochem. J.* **285,** 979–983
47. Kawamoto, T., Kakizaki, S., Yoshinari, K., and Negishi, M. (2000) Estrogen activation of the nuclear orphan receptor CAR (constitutive active receptor) in induction of the mouse *Cyp2b10* gene. *Mol. Endocrinol.* **14,** 1897–1905
48. Vasta, V., Shimizu-Albergine, M., and Beavo, J. A. (2006) Modulation of Leydig cell function by cyclic nucleotide phosphodiesterase 8A. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 19925–19930
49. Adachi, M., Sampath, J., Lan, L. B., Sun, D., Hargrove, P., Flatley, R., Tatum, A., Edwards, M. Z., Wezeman, M., Matherly, L., Drake, R., and Schuetz, J. (2002) Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. *J. Biol. Chem.* **277,** 38998–39004
50. Faqi, A. S., Klug, A., Merker, H. J., and Chahoud, I. (1997) Ganciclovir induces reproductive hazards in male rats after short-term exposure. *Hum. Exp. Toxicol.* **16,** 505–511
51. Nozaki, Y., Kusuhara, H., Kondo, T., Iwaki, M., Shiroyanagi, Y., Nakayama, H., Horita, S., Nakazawa, H., Okano, T., and Sugiyama, Y. (2007) Species difference in the inhibitory effect of nonsteroidal anti-inflammatory drugs on the uptake of methotrexate by human kidney slices. *J. Pharmacol. Exp. Ther.* **322,** 1162–1170
52. Conte, D., Romanelli, F., Fillo, S., Guidetti, L., Isidori, A., Franceschi, F., Latini, M., and di Luigi, L. (1999) Aspirin inhibits androgen response to chorionic gonadotropin in humans. *Am. J. Physiol.* **277,** E1032–1037
53. Didolkar, A. K., Patel, P. B., and Roychowdhury, D. (1980) Effect of aspirin on spermatogenesis in mature and immature rats. *Int. J. Androl.* **3,** 585–593
54. Krishnamurthy, P., Schwab, M., Takenaka, K., Nachagari, D., Morgan, J., Leslie, M., Du, W., Boyd, K., Cheok, M., Nakauchi, H., Marzolini, C., Kim, R. B., Poonkuzhali, B., Schuetz, E., Evans, W., Relling, M., and Schuetz, J. D. (2008) Transporter-mediated protection against thiopurine-induced hematopoietic toxicity. *Cancer Res.* **68,** 4983–4989

55. Janke, D., Mehralivand, S., Strand, D., Gödtel-Armbrust, U., Habermeier, A., Gradhand, U., Fischer, C., Toliat, M. R., Fritz, P., Zanger, U. M., Schwab, M., Fromm, M. F., Nürnberg, P., Wojnowski, L., Closs, E. I., and Lang, T. (2008) 6-Mercaptopurine and 9-(2-phosphonyl-methoxyethyl) adenine (PMEA) transport altered by two missense mutations in the drug transporter gene ABCC4. *Hum. Mutat.* **29,** 659–669
56. Abla, N., Chinn, L. W., Nakamura, T., Liu, L., Huang, C. C., Johns, S. J., Kawamoto, M., Stryke, D., Taylor, T. R., Ferrin, T. E., Giacomini, K. M., and Kroetz, D. L. (2008) The human multidrug resistance protein 4 (MRP4, ABCC4). Functional analysis of a highly polymorphic gene. *J. Pharmacol. Exp. Ther.* **325,** 859–868
57. Waxman, D. J., and Holloway, M. G. (2009) Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol. Pharmacol.* **76,** 215–228
58. Dhir, R. N., Dworakowski, W., Thangavel, C., and Shapiro, B. H. (2006) Sexually dimorphic regulation of hepatic isoforms of human cytochrome p450 by growth hormone. *J. Pharmacol. Exp. Ther.* **316,** 87–94