The ATP-binding cassette transporter 1 mediates lipid efflux from Sertoli cells and influences male fertility

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Abstract **The liver X receptor/retinoid X receptor (LXR/RXR)-regulated gene ABCA1 effluxes cellular cholesterol and phospholipid to apolipoprotein A1 (apoA1), which is the rate-limiting step in high-density lipoprotein synthesis. The RXR pathway plays a critical role in testicular lipid trafficking, and RXRβ-deficient male mice are sterile and accumulate lipids in Sertoli cells. Here, we demonstrate that ABCA1 mRNA and protein are abundant in Sertoli cells, whereas germ cells express little ABCA1. LXR/RXR agonists stimulate ABCA1 expression in cultured Sertoli MSC1 and Leydig TM3 cell lines. However, Sertoli TM4 cells lack ABCA1, and TM4 cells or primary Sertoli cells cultured from ABCA1$^{-/-}$ mice both fail to efflux cholesterol to apoA1. Expression of exogenous ABCA1 restores apoA1-dependent cholesterol efflux in Sertoli TM4 cells. In vivo, ABCA1-deficient mice exhibit lipid accumulation in Sertoli cells and depletion of normal lipid droplets from Leydig cells by 2 months of age. By 6 months of age, intratesticular testosterone levels and sperm counts are significantly reduced in ABCA1$^{-/-}$ mice compared with wild-type (WT) controls. Finally, a 21% decrease ($P = 0.01$) in fertility was observed between ABCA1$^{-/-}$ males compared with WT controls across their reproductive lifespans. These results show that ABCA1 plays an important role in lipid transport in Sertoli cells and influences male fertility.**—Selva, D. M., V. Hirsch-Reinshagen, B. Burgess, S. Zhou, J. Chan, S. McIsaac, M. R. Hayden, G. L. Hammond, A. W. Vogl, and C. L. Wellington. **The ATP-binding cassette transporter 1 mediates lipid efflux from Sertoli cells and influences male fertility.** ***J. Lipid. Res.*** **2004.** 45: **1040–1050.**

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High density lipoprotein (HDL) is one of several lipoproteins that transport lipid components in the blood. HDL plays an important atheroprotective role by transporting cholesterol and phospholipids from peripheral tissues to the liver in a process known as reverse cholesterol transport (1–4). High levels of HDL are protective against atherosclerosis even in the presence of elevated low density lipoproteins (LDL) (5–7). HDL-derived cholesterol is also the major source of cholesterol for the synthesis of steroid hormones in steroidogenic tissues such as the testis, adrenal gland, and ovary (8). In mice, fertility can be compromised by inactivation of genes involved in HDL and lipid metabolism, suggesting that appropriate regulation of lipid metabolism is critical for reproductive functions (9–13).

ABCA1 is a member of the ATP-ase cassette superfamily of transporters. The biological function of ABCA1 is to transfer cholesterol and phospholipids from peripheral cells to lipid-free apolipoprotein AI (apoAI), which constitutes the rate-limiting step in HDL biosynthesis (14–19). Homozygous or compound heterozygous mutations in ABCA1 cause Tangier disease (TD), which is characterized by virtually undetectable plasma HDL, tissue deposition of cholesterol esters, and an increased risk of atherosclerosis (15–19). Conversely, overexpression of ABCA1 in mice increases plasma HDL levels and strikingly protects against atherosclerosis (20–22). Recent observations suggest that macrophage ABCA1 plays a major role in protecting from atherosclerosis but has little impact on plasma HDL levels, whereas liver ABCA1 is a major determinant of plasma HDL levels in vivo (21, 23–27). The roles of ABCA1 in other tissues have not yet been addressed.

ABCA1 is as highly expressed in the testis as it is in liver (28, 29), suggesting that ABCA1 may also play a role in

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Abbreviations: apoA1, apolipoprotein A1; HDL-C, HDL cholesterol; LXR, liver X receptor; RXR, retinoid X receptor; TD, Tangier disease; WT, wild-type; SR-BI, scavenger receptor class B type I.

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regulating testicular lipid transport, which is largely separated from the peripheral circulation (30). Tight junctions in testicular capillaries form a barrier for the transfer of plasma lipoproteins to the interstitium and Leydig cells, and a system of plasmalemmal vesicles allows the passage of LDL and HDL to the interstitial compartment (31). Sertoli cells are further separated from interstitial blood capillaries by the basement membrane, which excludes the passage of LDL but allows HDL to enter the seminiferous tubule (32). The molecular pathways that regulate lipid exchange between testis and periphery are currently not well understood but are of interest because of the crucial role of cholesterol in both steroidogenesis and spermatogenesis.

In the testis, reproductive steroid hormones are produced by Leydig and Sertoli cells. Interstitial Leydig cells secrete several androgens including testosterone and dihydrotestosterone, which regulate male sexual development and spermatogenesis (33). Testosterone is taken up by Sertoli cells and converted into estradiol and dihydrotestosterone. Leydig cells can synthesize cholesterol de novo or can take up cholesterol from HDL (34). In contrast, Sertoli cells cannot synthesize cholesterol and are dependent upon androgens transported from Leydig cells or HDL entering from blood vessels (32, 35, 36).

Like macrophages, Sertoli cells are highly phagocytic and require the ability to efflux excess lipids that accumulate after engulfment of membrane-rich structures. One function of Sertoli cells is to endocytose and degrade residual bodies, which are the cytoplasmic portions of elongated spermatids that are shed during extrusion of differentiated sperm into the lumen of the seminiferous tubule (37–39). Additionally, any spermatogenic cells that undergo apoptotic death before they complete maturation into spermatozoa also are phagocytized by Sertoli cells (40–42). Sertoli cells accumulate excess lipids acquired by each of these pathways and require the ability to efflux these lipids. Although the pathways by which Sertoli cells regulate lipid efflux are not yet known, the accumulation of lipid droplets in retinoid X receptor $\beta^{-/-}$ ($RXR\beta^{-/-}$) Sertoli cells demonstrates that RXR-regulated genes are required for the removal of lipids from Sertoli cells.

Based on the high expression of ABCA1 in the testis (28, 29), regulation of ABCA1 expression by the liver X receptor(LXR)/RXR pathway (43–46), and the requirement for cholesterol in reproductive physiology, we sought to investigate the role of ABCA1 in the male reproductive system. In this study, we identify Sertoli cells as a major site of ABCA1 expression in the testis and demonstrate that ABCA1 deficiency results in accumulation of lipids in Sertoli cells in vivo and in vitro. Furthermore, $ABCA1^{-/-}$ male mice have significantly reduced intratesticular testosterone levels as well as reduced sperm counts compared with wild-type (WT) animals at 6 months of age. Finally, although $ABCA1^{-/-}$ mice are not sterile, we observed a significant 21% reduction ($P = 0.01$) in the ability of $ABCA1^{-/-}$ males to sire offspring compared with age-matched littermate controls over their reproductive lifespans. Our results identify ABCA1 as a critical modulator of lipid efflux in Sertoli cells and a contributor to male fertility in vivo.

MATERIALS AND METHODS

Animals

$ABCA1^{-/-}$ mice were generously provided by Omar Francone (Pfizer Global Research and Development), and WT DBA1/J mice were obtained from Jackson Laboratories. Animals were maintained on regular chow (PMI Feeds) for all experiments. All procedures involving animals were performed in accordance with protocols from the Canadian Council of Animal Care and the University of British Columbia Animal Care Committees.

Human tissues

Human tissues were kindly provided from a tissue bank maintained by Dr. M.R. Hayden in accordance with protocols from the University of British Columbia Committee on Research using Human Tissues.

Isolation of primary germ cells

Germ cells were isolated from the testes of WT mice at 10 weeks of age as described (47, 48). Briefly, testes were excised and washed with phosphate buffered saline (PBS) supplemented with penicillin-streptomycin (5 mg/ml), decapsulated, minced for 5 min and incubated in 100 ml of the above PBS solution for 8 min. The medium was removed and the remaining testicular fragments were digested in trypsin (80 mg/ml) in PBS at 33°C for 10 min. The reaction was stopped by adding 25 mg/ml trypsin inhibitor, and the resulting solution was treated with DNase (0.4 mg/ml) at room temperature for 5 min. Isolated tubules were minced and filtered through a 100 μm nylon filter and a 20 μm nylon filter. After centrifugation at 800 rpm for 10 min, the pellet was resuspended in 15 ml DMEM/ F-12 (Canadian Life Technologies, Inc.), supplemented with 10% FBS, and incubated in a tissue culture flask for 5 h. The supernatant, free of Sertoli cells, was recovered and centrifuged. After washing twice with PBS, cell pellets were frozen for the RNA or protein extraction.

Isolation of tubule cells

A mixed population of Sertoli and germ cells (Sertoli-germ cell ratio of approximately 1:13) was isolated from the testes of WT mice at 10 weeks of age. Testes were excised and washed in DMEM/F-12 supplemented with penicillin-streptomycin and amphotericin (5 mg/ml of each). Washed testes were decapsulated and incubated in 0.9 mg/ml collagenase in PBS at 33°C for 10 min with agitation. After centrifugation at 800 rpm for 10 min, the pellets were resuspended in 50 ml of culture medium and were allowed to sediment for 15 min. This was repeated three times, then a second incubation with 0.9 mg/ml collagenase was performed at 33°C for 10 min. After centrifugation at 800 rpm for 10 min, the pellets were washed twice with PBS and frozen for the RNA and protein extraction. For culture of primary Sertoli cells, mixed Sertoli and germ cells isolated from the testes of WT mice and $ABCA1^{-/-}$ mice at 3 months of age were seeded at 900,000 cells per well in a 24-well plate (Falcon) in DMEM culture medium containing 2% FBS and incubated at 33°C at 5% CO_2. After 10 h in culture, the wells were washed once, and the medium was changed to remove germ cells and retain Sertoli cells. Enriched Sertoli cell cultures were incubated for 3–4 days prior to initiating cholesterol efflux assays.

RNA analyses

Total RNA from liver, testes, and isolated testicular cells were extracted from WT mice using TRIzol reagent (Canadian Life Techonologies, Inc.). For RT-PCR analysis, RNA was reverse transcribed at 42°C for 50 min using 3 μg of total RNA and 200 units of Superscript II, together with an oligo(dT) primer and reagents provided by Invitrogen. An aliquot (2 μl) of the RT product was amplified in a 35-μl reaction in the presence of 1 unit of *Taq* polymerase, 0.05 mM $MgCl_2$, 1.25 μM of each dNTP, and 0.2 μM each of an oligonucleotide primer corresponding to a 5′-sequence (5′-cctttctggaagggtttgtgc) and a reverse primer sequence (5′-gatctgccgtaacattctcagg) of the mouse ABCA1 gene. The PCR was performed for 35 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s. A mouse cyclophylin A cDNA was amplified by RT-PCR under the same conditions using 1 μl of the RT and two specific primers (forward 5′-atggtcaaccccaccgtg, and reverse 5′-cagatggggtagggacg) to control for the integrity and relative amounts of mRNA in the samples.

Western blotting

All murine tissues were harvested immediately after sacrifice and snap frozen in liquid N_2 until use. Frozen human tissues were obtained from a preexisting tissue bank, with a postmortem index of less than 12 h. All tissues were homogenized in 20 mmol/l Hepes, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.5% (v/v) Triton X-100, and complete protease inhibitor tablets (Roche Molecular Biochemicals). Homogenates were briefly sonicated and centrifuged at 9,000 rpm for 10 min at 4°C. The supernatant was collected and stored at −70°C. Protein concentrations were determined by Lowry assay. Equal amounts of protein (50 μg/well) were separated on 7.5% SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Corp.) prior to immunodetection with a monoclonal antibody specific for the C terminus of ABCA1 (28), or a monoclonal anti-GAPDH (Chemicon) as a loading control. Immunoreactivity was detected by enhanced chemiluminescence (Amersham). Protein abundance was calculated by densitometry using NIH Image 6.1 software and normalized to GAPDH levels. Densitometry was performed on duplicate or triplicate gels run from at least three independent experiments. Data represent the mean and SD from tissue from at least four age- and sex-matched animals, each analyzed in duplicate.

Culture of testicular cell lines

Immortalized Leydig TM3 and Sertoli TM4 cells were obtained from the American Type Culture Collection, and Sertoli MSC1 cells were a gift from Dr. M.D. Griswold, Department of Biochemistry and Biophysics, Washington State University. Cells were maintained in DMEM with 10% FBS and penicillin-streptomycin, and were stimulated with 10 μm 9-*cis*-retinoic acid and 10 μM 22-R-hydroxycholesterol for 16–18 h as previously described (49). Where indicated, cells were infected with an adenovirus expressing ABCA1 (Ad-ABCA1) (25) in DMEM supplemented with 2% FBS for 60 min, followed by replacement of conditioned media for 24 h. For Oil Red O staining, cells were cultured on glass coverslips in growth media supplemented with 20 μg/ml soluble cholesterol for 24 h prior to staining (21).

Cholesterol efflux

Cells were labeled with 1 μCi/ml of [^{3}H]-cholesterol (New England Nuclear) for 18 h, then washed and equilibrated in DMEM with 0.5% BSA for 1 h. Efflux was initiated by the addition of 10 μg/ml of lipid-free apoA1 (Calbiochem) for 4 h. Media was collected and centrifuged at 12,000 rpm for 5 min. Cells were lysed in 200 μl of 0.1 M NaOH, 0.2% SDS at RT for 20 min, and 200 μl of media, and the total cell lysate was added to scintillation vials containing 400 μl of scintillant and quantified. The percent efflux was calculated as the total counts in the medium divided by the sum of the counts in the medium plus the cell lysate, as previously described (49).

Histology and electron microscopy

For toluidine blue histology and electron microscopy, mice were transcardially perfused with a fixative containing 0.1 M sodium cacodylate 1.5% paraformaldehyde and 1.5% gluteraldehyde (pH 7.3) for 15 min. Following perfusion, the testes were removed and fixed by immersion in this same buffer for up to 24 h. Fixed testes were cut into small pieces, washed 3× (10 min each) in 0.1 M sodium cacodylate (pH 7.3), and then postfixed for 1 h on ice in a solution containing 0.1M sodium cacodylate (pH7.3) and 0.1 M osmium tetroxide. The tissue was washed with distilled water, stained for 1 h with aqueous 1.0% uranyl acetate, and then washed again with distilled water. The tissue was dehydrated through a graded series of ethanol and embedded in Poly/Bed 812. Thick (1 μm) sections were cut, stained with toluidine blue, and photographed on a Zeiss Axioplan 2 microscope using a CCD camera equipped with Metamorph (Universal Imaging Corporation) imaging software. Thin sections were stained with uranyl acetate and lead citrate and then examined and photographed with an AMT Advantage HR digital CCD camera on a Hitachi H7600 transmission electron microscope. For Oil Red O staining, mice were transcardially perfused with PBS containing 4% paraformaldehyde (pH 7.3). Testes were removed, immediately frozen in OCT, and sectioned on a cryostat.

Testosterone measurements

Testes were homogenized in 1 ml of PBS 1× and transferred to a glass tube. After adding 2 ml of diethylether, the samples were vortexed for 1 min and centrifuged 10 min at 2,000 rpm. The lower aqueous phase was frozen in dry ice and the upper organic phase was transferred to a new glass tube, dried under nitrogen, and resuspended in 1 ml of 100% ethanol. Testosterone levels in the ether extracts were measured using a testosterone ELISA kit (Immuno-Biological Laboratories), according to the supplier's instructions.

Isolation of sperm

Immediately after sacrifice, the cauda epididymis was removed, cut into three sections, and placed into 500 μl of PBS at room temperature. Sperm were allowed to swim out for 2 h, then were transferred to a fresh tube for quantitation on a hemacytometer. Blinded triplicate samples from each mouse were quantitated.

Fertility testing

Male ABCA1$^{-/-}$ mice and WT littermate controls were placed with individual WT females and the presence of vaginal plugs was scored 2× daily for 5 days, after which the mice were separated. Males were placed with a second female, and plugged females were individually housed until any resulting litters were weaned. Each male was tested with at least three different females. Fertility was quantitated by scoring the percent of productive matings, which were defined as the proportion of plugged females that produced litters.

Statistical analysis

Data represent the means and standard deviations of at least three independent measurements. Statistical analyses of were performed using Graph-Pad Prism software (version 3.03). For ABCA1 expression, cholesterol efflux, testosterone levels, and sperm counts, data were analyzed using one-way ANOVA with a Neuman-Keuls posttest or with two-tailed Student's *t*-tests as ap-

propriate. For fertility testing, data were analyzed using a repeated-measures, one-way ANOVA with a Bonferroni correction.

RESULTS

ABCA1 expression in the male reproductive system

Previous studies have shown that ABCA1 is highly expressed in testes (28, 29). To define the tubule cell types containing ABCA1 mRNA, WT murine testes were fractionated into a mixed population of Sertoli and germ cells (Sertoli-germ cell ratio is approximately 1:13) and isolated germ cells. Semiquantitative RT-PCR analysis showed that ABCA1 mRNA is as abundant in the Sertoli and germ cell mixture as in whole testicular and liver lysates, and is present only at low levels in isolated germ cells (**Fig. 1A**). These observations suggest that Sertoli cells contain the majority of ABCA1 mRNA in the seminiferous tubule. Western blots were then performed to determine ABCA1 protein levels in male reproductive tissues. Testicular fractionation experiments confirmed that ABCA1 protein is far more abundant in a mixed Sertoli-germ cell preparation compared with isolated germ cells, showing that Sertoli cells contain the majority of ABCA1 (Fig. 1B). In addition, ABCA1 is expressed at low levels in whole lysates prepared from murine epididymis but is absent from seminal vesicle whole lysates (Fig. 1C). In human tissues obtained from two independent individuals, ABCA1 protein levels in whole testicular lysates are comparable to those in whole liver lysates (Fig. 1D).

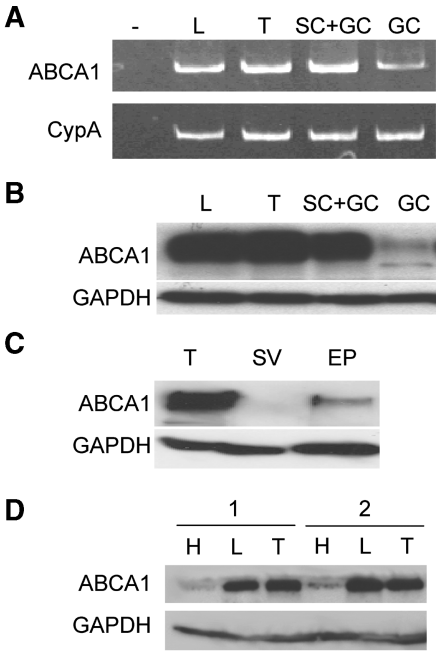

Fig. 1. Expression of ABCA1 in the male reproductive system. A: RT-PCR analysis of murine ABCA1 expression in wild-type (WT) liver (L), whole testes (T), mixed Sertoli/germ cells (SC+GC), and isolated germ cells (GC), showing the relative expression of murine ABCA1 compared with cyclophin A, which was used as an internal control. B: Western blot showing ABCA1 protein levels in lysates of WT murine liver (L), whole testes (T), mixed Sertoli/germ cells (SC+GC), and isolated germ cells (GC). C: Western blot showing ABCA1 protein levels in lysates of WT murine testes (T), seminal vesicle (SV), and epididymis (EP). D: Western blot showing ABCA1 protein levels in human heart (H), liver (L), and testis (T). All Western blots were probed with an ABCA1-specific antibody as well as a GAPDH antibody to control for protein loading.

ABCA1 is required for lipid efflux from Sertoli cells in vitro

ABCA1 expression and activity was next evaluated in murine testicular cell lines. Endogenous ABCA1 was present in Sertoli MSC1 and Leydig TM3 cells and could be induced by treatment with LXR/RXR agonists in both cell types (**Fig. 2A**), demonstrating that regulation of ABCA1 in testicular cells is similar to that in other periph-

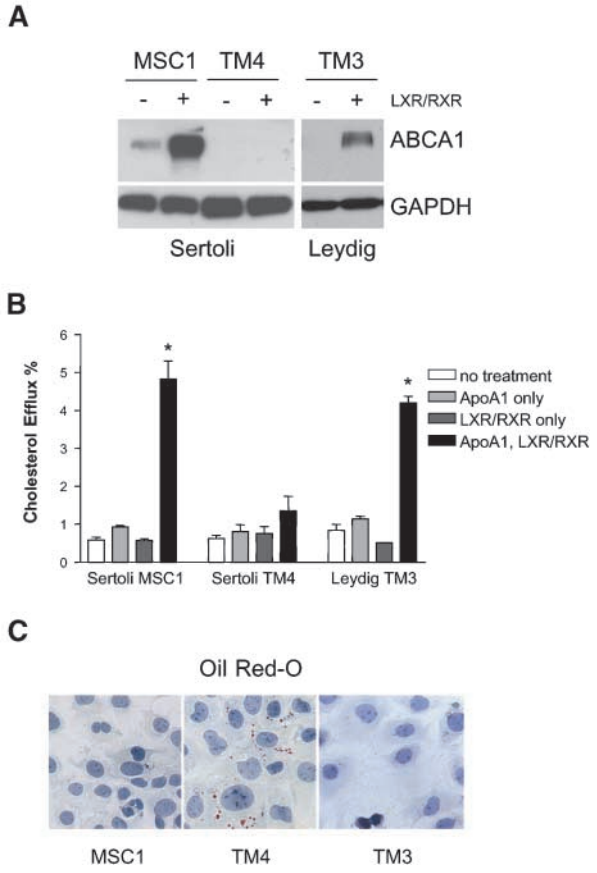

Fig. 2. ABCA1 expression and activity in testicular cell lines. A: ABCA1 expression was tested in the murine Sertoli cell lines, MSC1 and TM4, and the murine Leydig cell line, TM3, by Western blot. Total cell lysates were prepared before (−) and after (+) stimulation with liver X receptor/retinoid X receptor (LXR/RXR) agonists and probed with ABCA1 and GAPDH antibodies. MSC1 and TM3 cells induce ABCA1 expression after treatment with LXR/RXR agonists, but TM4 cells do not. B: Cholesterol efflux in each testicular cell line, with and without treatment with LXR/RXR agonists, and in the presence or absence of 10 μg/ml of apolipoprotein AI (apoAI) as a cholesterol acceptor. Data represent the means and SDs of at least two independent experiments, each performed in triplicate. * $P < 0.001$. C: Oil Red O staining of each cell line, showing accumulation of intracellular lipid droplets only in TM4 cells that lack ABCA1 expression and efflux activity.

eral cells. However, ABCA1 was undetectable in Sertoli TM4 cells even after stimulation with LXR/RXR agonists (Fig. 2A). Consistent with ABCA1 expression levels, both MSC1 and TM3 cells displayed robust cholesterol efflux to lipid-free apoA1, demonstrating that ABCA1 expression mediates cholesterol efflux in cultured Sertoli and Leydig cells (Fig. 2B). In contrast, cholesterol efflux was impaired in Sertoli TM4 cells that lacked ABCA1 (Fig. 2B). Furthermore, TM4 cells accumulated Oil Red O lipid droplets when cultured in the presence of exogenous cholesterol, whereas lipids did not accumulate in efflux-competent MSC1 and TM3 cells (Fig. 2B). These in vitro results suggest that ABCA1 is required for lipid efflux from testicular cells.

To determine if restoration of ABCA1 expression rescued lipid efflux from ABCA1-deficient Sertoli TM4 cells, exogenous ABCA1 was delivered to TM4 cells using an adenovirus expressing human ABCA1 (Ad-ABCA1). Ad-ABCA1 has previously been shown to result in increased ABCA1 expression and efflux activity in vitro and in vivo, whereas no increase in ABCA1 expression or activity is observed with a control adenovirus expressing alkaline phosphatase (25). As a positive control, MSC1 cells were also treated with Ad-ABCA1. A dose-dependent increase in ABCA1 expression was observed in both cell types (**Fig. 3A**). Although Ad-ABCA1 resulted in an increase in ABCA1 expression in MSC1 cells, we observed a slight but nonsignificant increase in cholesterol efflux activity under the conditions used in these experiments, suggesting that 10 μg/ml apoA1 may be limiting for cholesterol efflux. However, Ad-ABCA1 treatment of TM4 cells resulted in a significant increase ($P < 0.01$) in apoA1-dependent cholesterol efflux at a multiplicity of infection of 1,000 compared with uninfected cells, using 10 μg/ml apoA1 as an exogenous cholesterol acceptor (Fig. 3B). Although Ad-ABCA1 is very effective at transducing exogenous ABCA1 expression as shown by Western blot, we noted that the increase in efflux activity was moderate compared with the amount of efflux activity observed in cells treated with LXR/RXR agonists (compare MSC1 efflux activity in Figs. 2 and 3). Although the reasons for this phenomenon are not fully understood, it is possible that LXR/RXR stimulation could induce the expression of other gene products that act in concert with ABCA1 to facilitate cholesterol efflux. LXR/RXR stimulation could also result in changes in ABCA1 subcellular localization or posttranslational modification that enhance its functional efflux activity. In contrast, Ad-ABCA1 is expressed from the heterologous cytomegalovirus promoter, and we have previously noted that it is comparably less effective than endogenously-regulated ABCA1 in mediating efflux activity despite equivalent protein levels (25). Nevertheless, sufficient cholesterol efflux activity was restored in Ad-ABCA1-treated TM4 cells to reduce the accumulation of Oil Red O-positive lipid droplets by approximately 70% (data not shown). These experiments show that apoA1-dependent cholesterol efflux could be rescued in a Sertoli cell line by selective expression of exogenous ABCA1.

The ability of exogenous ABCA1 to rescue cholesterol efflux from TM4 cells supports a direct role for ABCA1 in mediating cholesterol efflux from Sertoli cells. To confirm that selective deficiency of ABCA1 is sufficient to block apoA1-dependent cholesterol efflux from Sertoli cells, efflux assays were performed on primary Sertoli cells cultured from ABCA1$^{-/-}$ and WT littermate controls (N = 2 for each genotype). Whereas WT cells exhibited a 1.4-fold increase in apoA1-dependent cholesterol efflux ($P < 0.05$), ABCA1$^{-/-}$ primary Sertoli cells exhibited no significant efflux activity in the presence of apoA1 ($P > 0.05$) (**Fig. 4**, N = 2 mice with quadruplicate measurements for

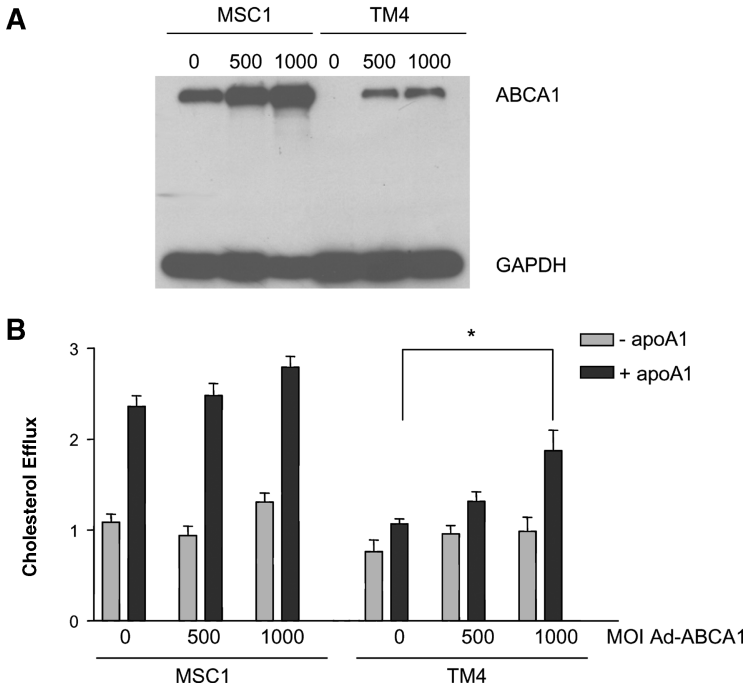

Fig. 3. Exogenous ABCA1 restores cholesterol efflux from ABCA1$^{-/-}$ Sertoli cells. A: Western blot of MSC1 and TM4 cells cultured in the absence of Ad-ABCA1 (−) or treated with Ad-ABCA1 at a multiplicity of infection (MOI) of 500 or 1,000. Blots were probed with ABCA1 and GAPDH antibodies. B: Cholesterol efflux in MSC1 and TM4 cells, with and without Ad-ABCA1 infection, using 10 μg/ml of apoAI. * $P < 0.01$. Data represent the means and SDs of a representative experiment performed in triplicate.

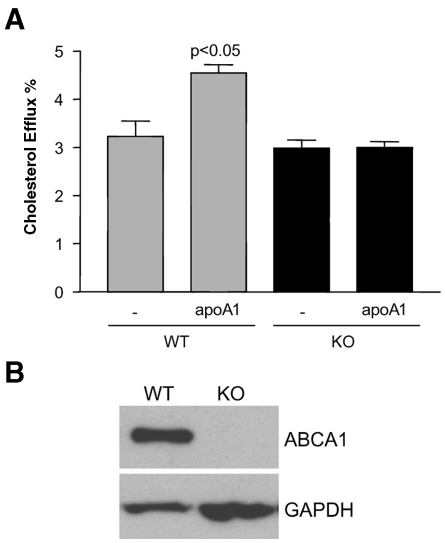

Fig. 4. ABCA1 is required for apoAI-dependent cholesterol efflux from primary Sertoli cells. A: Cholesterol efflux in primary Sertoli cells cultured from WT and ABCA1$^{-/-}$ (KO) mice. Cells were treated with LXR/RXR agonists for 24 h and assayed for cholesterol efflux using 10 μg/ml of apoAI as a cholesterol acceptor where indicated. Data represent the means and SDs of quadruplicate measurements from two independent mice of each genotype. B: Western blot of primary Sertoli cells after LXR/RXR stimulation, demonstrating ABCA1 protein expression in WT but not in ABCA1$^{-/-}$ cells.

each). This experiment was performed in the presence of LXR/RXR agonists, which demonstrates that no other LXR/RXR-inducible gene can compensate for ABCA1 and restore apoA1-dependent cholesterol efflux in primary ABCA1$^{-/-}$ Sertoli cells. ABCA1 protein was confirmed in WT but not ABCA1$^{-/-}$ primary Sertoli cells by Western blot (Fig. 4B).

ABCA1$^{-/-}$ mice display abnormal testicular lipid distribution

Semi-thin sections stained with toluidine blue revealed that ABCA1$^{-/-}$ testes were spermatogenic, and that tubules at all stages of spermatogenesis were present (**Fig. 5**). However, when compared with WT testes, there was a dramatic accumulation of lipid droplets near the base of the seminiferous epithelium in ABCA1$^{-/-}$ tubules at 7 months of age (Fig. 5D). Furthermore, extensive lipid deposition (asterisk) and tubule vacuolization (arrow) are evident in ABCA1$^{-/-}$ mice by 11–12 months of age (Fig. 5B, C). These phenotypes are not observed in WT animals that do not display lipid accumulations at any age examined up to 24 months (Fig. 5A, B, C). Oil Red O staining confirmed abnormal testicular distribution in ABCA1$^{-/-}$ compared with WT testes (data not shown). These observations suggest that the inability to efflux lipids from ABCA1$^{-/-}$ Sertoli cells can lead to progressive tubule vacuolization, particularly in older animals.

Ultrastructural analysis confirmed the presence of abnormal lipid distribution in ABCA1$^{-/-}$ testes. Lipid droplets were present in the cytoplasm of ABCA1$^{-/-}$ Sertoli cells at 2 months of age (**Fig. 6A**, B). Furthermore, ABCA1$^{-/-}$ Leydig cells were depleted of lipid droplets, which are normally present in WT mice and act as a cholesterol reservoir for steroid synthesis (Fig. 6C, D). Because ABCA1$^{-/-}$ mice have virtually no circulating HDL, these observations indicate that HDL serves as a primary

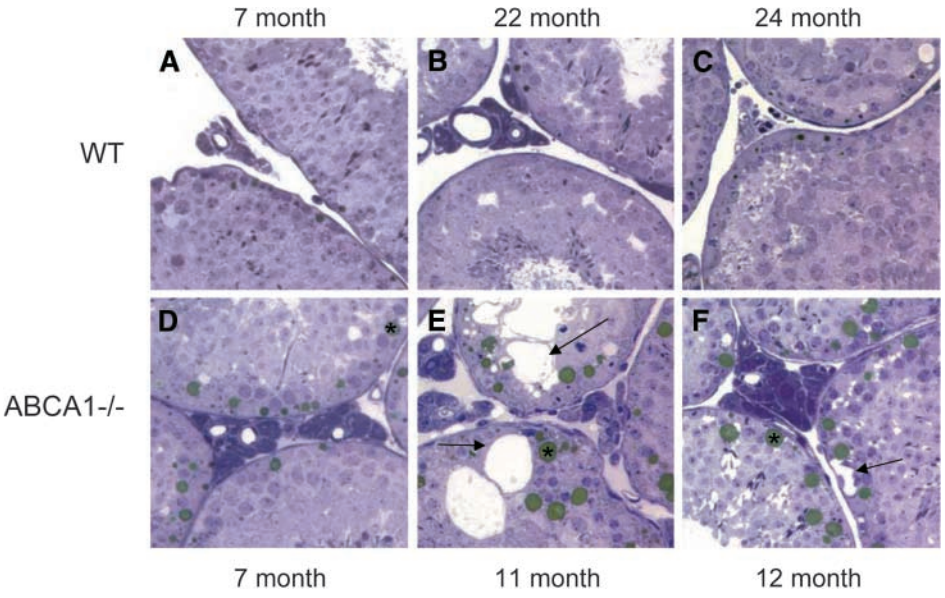

Fig. 5. Age-dependent accumulation of lipids in ABCA1$^{-/-}$ tubules. Testes from ABCA1$^{-/-}$ and WT mice at the indicated ages were permeated with osmium tetroxide, sectioned, and stained with toluidine blue. Panels A, B, and C are WT testes, and panels D, E, and F are ABCA1$^{-/-}$ testes, photographed at 400×. Lipid droplets (brown, asterisk) are present in Sertoli cells near the tubule basement membrane in the ABCA1$^{-/-}$ animals at 7 months of age, and extensive accumulation of these lipids is evident in 11- and 12-month-old animals. Additionally, variable amounts of testicular vacuolization and degeneration are visible in 11- and 12-month-old ABCA1$^{-/-}$ mice (arrows). The architecture of WT testes is normal up to 24 months of age.

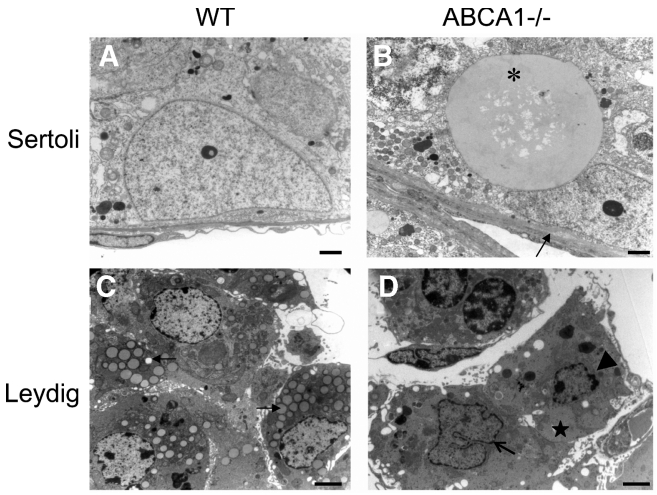

Fig. 6. Ultrastructure of WT versus ABCA1$^{-/-}$ testes. Testes from 2-month-old animals were immersion fixed in 1.5% paraformaldehyde, 1.5% gluteraldehyde, postfixed in 1% osmium tetraoxide, Epon embedded, and sectioned for electron microscopy. Panels A and C are WT, and panels B and D are ABCA1$^{-/-}$ testes, photographed at 60,000× with the bar representing 2 μm. A, B: Sertoli cells, showing accumulation of a large lipid droplet resulting in nuclear indentation in the ABCA1$^{-/-}$ mouse (*), as well as a thickened tubule wall in the ABCA1$^{-/-}$ testis (arrow). C, D: Leydig cells. Compared with WT Leydig cells with normal levels of lipid droplets (arrows), the Leydig cells of the ABCA1$^{-/-}$ mouse are depleted of lipid, have clumping of the smooth endoplasmic reticulum (star), and display extensive invaginations of the nuclear membrane (open arrow) or increased chromatin condensation and margination (arrowheads).

source of cholesterol in WT Leydig cells. Furthermore, the lack of HDL in the absence of ABCA1 suggests that steroidogenesis in ABCA1$^{-/-}$ mice is supported by de novo synthesis of cholesterol.

ABCA1 deficiency reduces testicular function and compromises fertility

Testosterone assays were performed to determine if testicular function was impaired in ABCA1$^{-/-}$ males. At 5 months of age, intratesticular testosterone levels and sperm counts were significantly reduced in ABCA1$^{-/-}$ males compared with WT controls ($P < 0.05$, N = 8–10) (**Fig. 7**). To determine if reduced sperm counts in ABCA1$^{-/-}$ mice were severe enough to compromise fertility, ABCA1$^{-/-}$ male mice were mated to at least three different WT females and the frequency of productive matings (litters born to plugged females) were scored. Fertility was tested using mice aged 4–5 months, 7–8 months, and 11–13 months. At each age tested, the frequency of productive matings was reduced in ABCA1$^{-/-}$ mice compared with age-matched WT littermate controls (**Table 1**). Relative to the WT mice, fertility in ABCA1$^{-/-}$ males is decreased by 21% ($P = 0.01$) across their reproductive lifespans. These observations demonstrate that the reduced testosterone levels and sperm counts observed in aged ABCA1$^{-/-}$ mice are sufficient to significantly compromise fertility.

DISCUSSION

In this study, we provide in vivo and in vitro evidence that ABCA1 plays a role in testicular lipid metabolism. In the seminiferous tubule, ABCA1 is enriched in Sertoli cells, where it mediates lipid efflux. As early as 2 months of age, testes from ABCA1$^{-/-}$ males display abnormally large lipid droplets within Sertoli cells, suggesting that ABCA1 participates in the removal of excess lipids acquired by Sertoli cells during normal phagocytosis of residual bodies or apoptotic spermatids. Additionally, cultured Sertoli cells that lack ABCA1 fail to efflux cholesterol to apoA1 and accumulate Oil Red O-positive lipid droplets, and these phenotypes can be reversed by addition of exogenous ABCA1. These observations suggest that ABCA1 is a key component of a lipid removal pathway in Sertoli cells.

Lipid homeostasis in Sertoli cells is regulated by a balance between HDL-mediated cholesterol uptake and ef-

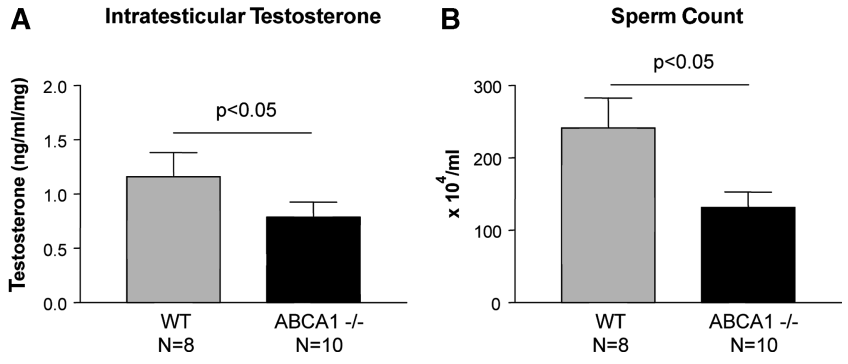

Fig. 7. Reduced testosterone levels and sperm count in ABCA1$^{-/-}$ mice. A: ABCA1$^{-/-}$ males and WT controls were sacrificed at 6 months of age (N = 8 for WT, and N = 10 for ABCA1$^{-/-}$). Intratesticular testosterone is significantly reduced in 6-month-old ABCA1$^{-/-}$ mice relative to WT control mice. B: Using the same animals used for testosterone measurements, spermatozoa from the cauda epididymis were obtained and quantitated using duplicate samples from each animal. Significantly fewer sperm were released from ABCA1$^{-/-}$ epididymis compared with WT controls.

TABLE 1. Fertility in ABCA1$^{-/-}$ mice

Age	Genotype		Plug	Plug/No Pregnancy	Plug/Pregnancy	Productive Matings
						%
4–5 months	WT	(N = 4)	10	1	9	90
	ABCA1$^{-/-}$	(N = 3)	6	2	4	67
7–9 months	WT	(N = 6)	12	4	8	67
	ABCA1$^{-/-}$	(N = 6)	11	5	6	54
11–13 months	WT	(N = 6)	10	4	6	60
	ABCA1$^{-/-}$	(N = 3)	6	3	3	50

WT, wild-type.

flux. Although apoA1 is not synthesized within the testis, HDL penetrates the testicular lamina propria and serves as the primary source of cholesterol for Sertoli cells (50). Uptake of cholesterol by cultured Sertoli cells is facilitated by the presence of apoA1 and apoE, and Sertoli cells express both scavenger receptor class B type I (SR-BI) and LDL receptor-related protein receptors (32, 51–53). Although cholesterol uptake in cultured Sertoli cells is primarily mediated through an apoE-dependent compared with an apoA1-dependent pathway, cholesterol efflux is approximately twice as efficient with apoA1 compared with apoE (32).

Because ABCA1 is required for efflux of lipid to apoA1, these observations suggest that ABCA1 may directly mediate the majority of lipid efflux from Sertoli cells. This is supported by observations that both apoA1$^{-/-}$ and ABCA1$^{-/-}$ mice have greatly reduced plasma HDL and apoA1 levels due the inability to synthesize HDL, which leads to hypercatabolism of apoA1 in the absence of ABCA1 (9, 54–56). Notably, Sertoli cells from apoA1$^{-/-}$ mice do not accumulate lipids, suggesting that lack of circulating HDL is not sufficient by itself to result in Sertoli cell lipid accumulation (57). In contrast, accumulation of tubule lipids in ABCA1$^{-/-}$ mice supports a direct role for ABCA1 in regulating Sertoli cell lipid efflux. However, because our study used a model of ABCA1 deficiency in which ABCA1, plasma HDL, and apoA1 levels are all reduced, our analysis does not rule out the possibility that lipid accumulation in ABCA1-deficient Sertoli cells may also be caused by insufficient circulating apoA1 to act as a lipid acceptor.

Absence of ABCA1 also results in depletion of lipid from Leydig cells, a phenotype also observed in apoA1$^{-/-}$ mice (57). HDL is the primary source of cholesterol for steroidogenic tissues in rodents, and apoA1 plays a critical role in SRBI-mediated selective uptake of HDL cholesterol (HDL-C) that normally accumulates in lipid droplets in the adrenal gland, testis, and ovary (34, 58). Because lipid droplets are depleted in Leydig cells in both ABCA1$^{-/-}$ as well as apoA1$^{-/-}$ mice, we hypothesize that the lack of circulating HDL is the primary cause of lipid depletion in ABCA1-deficient Leydig cells, rather than a specific function of ABCA1 in Leydig cells per se. Nevertheless, Leydig cell function is partially compromised in the absence of ABCA1. ABCA1$^{-/-}$ males produce less testosterone and generate fewer spermatozoa compared with WT controls.

Collectively, the impairment of Leydig and Sertoli cell functions in the absence of ABCA1 compromises male fertility, but does not render ABCA1$^{-/-}$ mice completely sterile. Interestingly, elimination of RXRβ in mice results in lipid accumulation in Sertoli cells, decreased spermatogenesis, abnormal spermatid morphology, and male infertility (13). We show that ABCA1 expression is induced by LXR/RXR agonists in both Sertoli and Leydig cells, suggesting that ABCA1 is one gene that fails to be induced in RXRβ-deficient mice. In addition, we show that some, but not all, of the phenotypes of RXRβ-deficient males are reproduced in ABCA1$^{-/-}$ males. Specifically, selective elimination of ABCA1 results in Sertoli cell lipid accumulation, decreased spermatogenesis, and reduced fertility, but does not affect sperm morphology. Our findings suggest

TABLE 2. Children of TD fathers

TD Patient	Mutation	Father's Age	Number of Children	Youngest Child's Age	Father-Child Age Difference	Reference
		years		*years*	*years*	
TD1 (III:01)	C1477R, splice	43	1	6	36	(17)
TD1 (II:5)	G1764del-635X	63	2	21	42	(16)
TD2 (II:4)	3′ del-1834X	52	2	<14	>38	(16)
TD3 (II.4)	N935S	66	3	28	38	(16)
TD5 (III:4)	A877V, W530S	52	1	31	21	(59)
II-2	1,284X	62	4	21	41	(60)
P	R1680W	48	3	5	43	(61)

TD, Tangier disease.

that ABCA1 may be one RXRβ-regulated gene that is essential for mediating lipid efflux from Sertoli cells, but that RXRβ-responsive genes other than ABCA1 may play primary roles in the regulation of normal sperm morphology in mice.

A review of the literature shows that men with TD are fertile (16, 17, 59–61). Examination of the published pedigrees shows that between one and five children have been born to TD fathers (**Table 2**). It is not known if men with TD also develop testicular degeneration and reduced sperm counts similar to those observed in ABCA1$^{-/-}$ mice. However, because clinical infertility can be observed in men with as little as a 10% reduction in spermatozoa (62), it is possible that TD men may experience a higher incidence of age-related reproductive difficulties compared with normolipidemic subjects.

Abnormalities in human lipid and lipoprotein metabolism can affect fertility, although the mechanisms by which they do are largely undefined. For example, women with polycystic ovary syndrome have increased cardiovascular disease risk factors compared with controls, including increased total cholesterol (TC), LDL-C, and triglyceride (TG) levels, as well as lower HDL-C levels (63, 64). Additionally, men with hypotestosteronemia are reported to have depressed HDL-C levels compared with controls (65, 66), and high levels of TC and TG are associated with poor semen quality and azoospermia (67). In one report, a dyslipidemia incidence of 65% was found in a set of 106 men with reproductive concerns (68). Despite these observations, lipids are not routinely tested in patients referred to fertility clinics, even though a decline in male reproductive fitness over the past 20 years, particularly for older men, may be related to the increased prevalence of dyslipidemia observed in Western populations over this time period. Specifically, it may be important to know if particular types of dyslipidemias occur frequently in patients with reproductive concerns, and if these correlate to specific genes with established roles in lipid metabolism.

Our results suggest that ABCA1 has a significant role in mediating lipid efflux from Sertoli cells in vivo and in vitro. A deeper understanding of the roles of ABCA1 and additional RXR-regulated genes involved in lipid transport in reproductive tissues may offer new insights for the development of potential therapeutic approaches for reproductive disorders based on modulation of lipid metabolism.

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