

# Hormonal Regulation of Testicular Steroid and Cholesterol Homeostasis

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The male sex steroid, testosterone (T), is synthesized from cholesterol in the testicular Leydig cell under control of the pituitary gonadotropin LH. Unlike most cells that use cholesterol primarily for membrane synthesis, steroidogenic cells have additional requirements for cholesterol, because it is the essential precursor for all steroid hormones. Little is known about how Leydig cells satisfy their specialized cholesterol requirements for steroid synthesis. We show that in mice with a unique hypomorphic androgen mutation, which disrupts the feedback loop governing T synthesis, that genes involved in cholesterol biosynthesis/uptake and steroid biosynthesis are up-regulated. We

identify LH as the central regulatory molecule that controls both steroidogenesis and Leydig cell cholesterol homeostasis *in vivo*. In addition to the primary defect caused by high levels of LH, absence of T signaling exacerbates the lipid homeostasis defect in Leydig cells by eliminating a short feedback loop. We show that T signaling can affect the synthesis of steroids and modulates the expression of genes involved in *de novo* cholesterol synthesis. Surprisingly, accumulation of active sterol response element-binding protein 2 is not required for up-regulation of genes involved in cholesterol biosynthesis and uptake in Leydig cells. (*Molecular Endocrinology* 22: 623–635, 2008)

STEROID HORMONES PLAY critical regulatory roles in diverse aspects of mammalian physiology including the response to stress, development of secondary sexual characteristics, the control of the menstrual cycle, and spermatogenesis. All steroid hormones are derived from cholesterol in a series of stepwise enzymatic modifications. In mammals, there are two major sites of steroid hormone synthesis: the adrenals, which mainly produce aldosterone and corticosteroids, and the gonads, which synthesize the sex steroids. In females, estradiol and progesterone are the major sex steroids, whereas males synthesize primarily testosterone (T) and its more potent metabolite, dihydrotestosterone (DHT). Steroid signaling is medi-

ated primarily through the nuclear hormone receptor class of ligand-dependent transcription factors (1). T exerts its actions by signaling through the androgen receptor (AR) (2, 3).

The synthesis of steroid hormones is a highly regulated process. The primary site of T production in males is the Leydig cell, the major cell type that populates the interstitial regions of the testis. LH, secreted by the pituitary, binds to the G protein-coupled LH/chorionic gonadotropin receptor (LHCGR) on the surface of Leydig cells (4). Activation of LHCGR stimulates Leydig cell T output by increasing intracellular concentrations of cAMP, thereby increasing the production of proteins necessary for steroidogenesis. T then reduces the synthesis of LH in the pituitary by directly repressing the expression of the LH  $\beta$ -subunit (5). A number of studies also suggest that T can regulate release of the hypothalamic GnRH, the signal that initially stimulates the pituitary to synthesize and release LH (6). This regulatory loop is referred to as the hypothalamic-pituitary-gonadal (HPG) axis.

Transcription of genes encoding proteins involved in steroidogenesis is stimulated by cAMP. The genes encoding steroidogenic enzymes, including *steroidogenic acute regulatory protein* (StAR; *Star*), *cytochrome P450 11a1* (*Cyp11a1*, also known as *cytochrome P450 side chain cleavage*) and *cytochrome P450 17a1* (*Cyp17a1*), are known targets of cAMP-stimulated transcription (7–9). T synthesis in Leydig cells is also regulated by a short feedback loop

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Abbreviations: AG, Aminoglutethimide; ApoE, apolipoprotein E; AR, androgen receptor; DHT, dihydrotestosterone; EL, endothelial lipase; hCG, human chorionic gonadotropin; HDL, high-density lipoprotein; HL, hepatic lipase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR, HMG-CoA reductase; HPG, hypothalamic-pituitary-gonadal;  $3\beta$ HSD,  $3\beta$ -hydroxysteroid dehydrogenase; LDL, low-density lipoprotein; LDLR, LDL receptor; LHCGR, LH/chorionic gonadotropin receptor; LXR, liver X receptor; 25-OHC, 25-hydroxycholesterol; ORO, Oil red O; qRT-PCR, quantitative RT-PCR; SR-B1, scavenger receptor type B1; SREBP, sterol response element-binding protein; StAR, steroidogenic acute regulatory protein; T, testosterone.

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whereby T represses the transcription of genes involved in steroidogenesis. The effect of T on steroidogenic gene expression is well illustrated by studies of *Cyp17a1*, where T represses cAMP-stimulated *Cyp17a1* mRNA in both primary Leydig cell cultures and in MA-10 Leydig tumor cells (10, 11).

The regulation of genes involved in the biosynthesis of cholesterol, the precursor of steroid hormones, is well studied (12). Central to the regulation of intracellular cholesterol levels is the membrane-bound transcription factor, sterol response element-binding protein (SREBP). When concentrations of sterols are low within the cell, SREBP cleavage-activating protein (SCAP) facilitates the cleavage of the membrane-bound SREBP, freeing SREBP to translocate to the nucleus and activate genes encoding *de novo* cholesterol biosynthetic enzymes and receptors mediating cholesterol uptake (13). There are three related SREBP proteins: SREBP1a, which regulates fatty acid and cholesterol biosynthesis; SREBP1c, which regulates fatty acid synthesis; and SREBP2, which primarily regulates genes involved in cholesterol metabolism (14). Several lines of evidence indirectly suggest that the SREBP pathway is active in Leydig cells. First, Leydig cells robustly express *Srebp2* and to a lesser extent *Srebp1c* but not *Srebp1a* (15). Second, activation of steroidogenesis with human chorionic gonadotropin (hCG), an agonist of LHCGR, results in the depletion of intracellular cholesterol reserves in Leydig or Leydig tumor cells (16, 17). Coincident with the depletion of intracellular lipid pools, there is a transient up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) activity, the rate-limiting activity in *de novo* cholesterol biosynthesis, suggesting activation of the SREBP2 pathway.

These circumstantial pieces of evidence implicate the SREBP pathway in the regulation of cholesterol synthesis and uptake in Leydig cells. However, several pieces of evidence argue against a role for SREBP in the regulation of genes involved in cholesterol homeostasis in steroidogenic cells. When the Leydig tumor line MA-10 is treated with 25-hydroxycholesterol (25-OHC), a potent inhibitor of SREBP activation, there is no effect on HMG-CoA synthase (*Hmgcs1*) expression or *de novo* cholesterol biosynthesis (18). This study suggests that steroidogenic tissues may not regulate cholesterol acquisition via the SREBP-mediated sterol-sensing pathway.

To understand better the regulation of steroidogenesis and cholesterol homeostasis in the testis, we used a unique genetic model that disrupts feedback regulation in the HPG axis. Our group has previously generated a novel allele of the *Ar* in the mouse that circumvents the complication of cryptorchidism and sex reversal associated with null mutation of *Ar*. We generated a hypomorphic allele of *Ar*, *Ar<sup>invflox(ex1-neo)/Y</sup>*, by inserting a neomycin resistance

(*neo<sup>R</sup>*) cassette into intron 1 (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>) (19). A cryptic splice acceptor within *neo<sup>R</sup>* results in stochastic splicing into the *neo* open reading frame, leading to reduced *Ar* mRNA. Despite the severe deficiency in AR compared with wild-type, these animals undergo normal testicular descent, and, like the spontaneous null mutant *Ar<sup>tfm</sup>*, they have elevated levels of serum LH. Unlike the *Ar<sup>tfm/Y</sup>* mouse, *Ar<sup>invflox(ex1-neo)/Y</sup>* males produce vast amounts of T in response to the high levels of serum LH. In this study, we use this model to dissect the role of hormonal signaling in the regulation of steroidogenesis and maintenance of cholesterol homeostasis in Leydig cells.

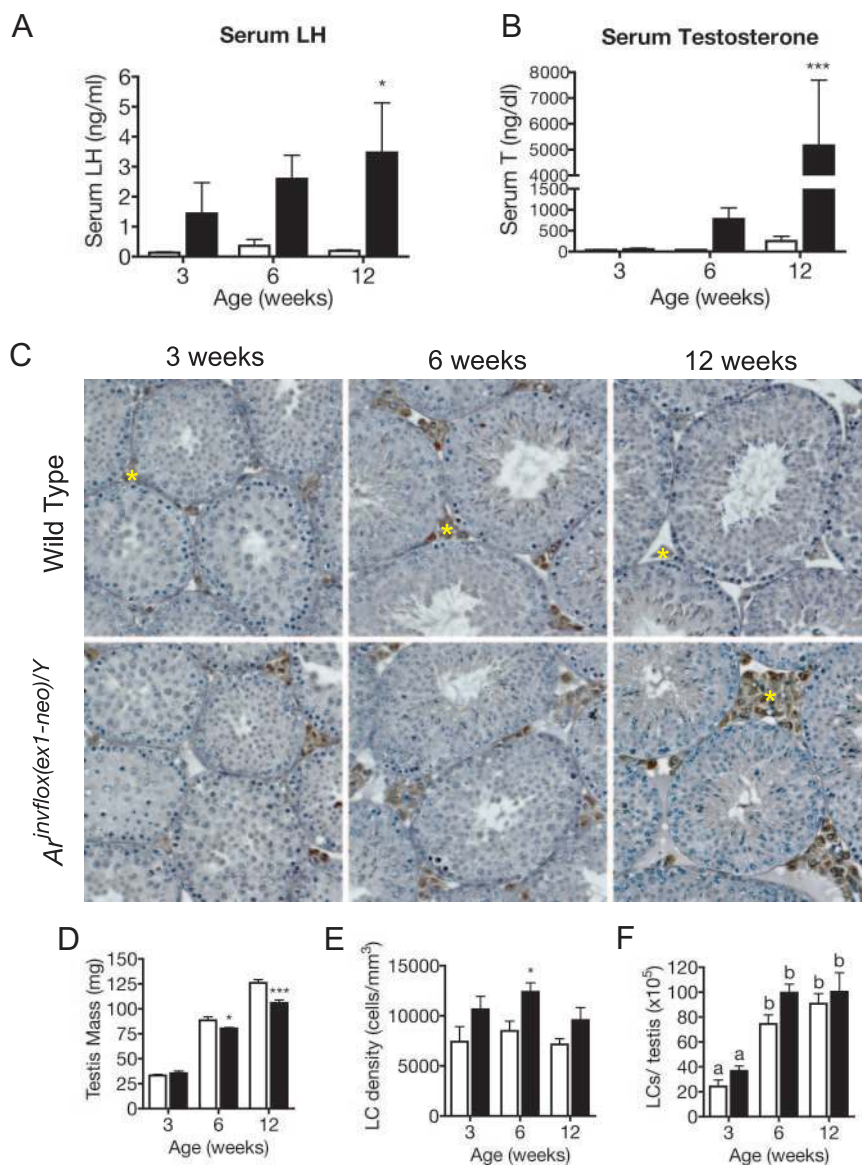
## RESULTS

### Serum Hormone Profiles Reflect Disruption of the HPG Axis

In a previous study, we demonstrated that serum T and LH were dramatically increased in 8-wk-old *Ar<sup>invflox(ex1-neo)/Y</sup>* male mice (19). The high levels of LH, produced in response to the disruption of feedback regulation, resulted in high levels of T produced by *Ar<sup>invflox(ex1-neo)/Y</sup>* Leydig cells. To better understand the cause and consequence of the changes in the context of pubertal maturation, we measured serum LH and T levels from wild-type and *Ar<sup>invflox(ex1-neo)/Y</sup>* mice at 3, 6, and 12 wk of age. In wild-type mice, serum LH levels rise from early to late puberty as expected. However, in *Ar<sup>invflox(ex1-neo)/Y</sup>* mice, LH levels appear elevated as early as 3 wk of age and continue to rise into adulthood, when they show a 21-fold elevation above wild-type LH levels (Fig. 1A). Accompanying the rise in serum LH levels, T levels increase into adulthood for both wild-type and *Ar<sup>invflox(ex1-neo)/Y</sup>* mice. In *Ar<sup>invflox(ex1-neo)/Y</sup>* mice, serum levels of T are approximately 20-fold higher than in adult wild-type mice (Fig. 1B). Thus, the pattern of pubertal increase in serum LH and T is unaffected in *Ar<sup>invflox(ex1-neo)/Y</sup>*, but the magnitude of the changes of these hormones is significantly affected.

### Leydig Cell Numbers Unaltered in *Ar<sup>invflox(ex1-neo)/Y</sup>*

Previous studies suggest that the hormonal milieu of the testis can have a profound effect on Leydig cell numbers (20–22). Because increased Leydig cell number might increase T output in *Ar<sup>invflox(ex1-neo)/Y</sup>* mice, we examined the histology of the testis for gross signs of Leydig cell hyperplasia (Fig. 1C). Using an antibody against the steroidogenic enzyme CYP11A1 to mark Leydig cells, we observed an apparent increase in the number of Leydig cells populating the interstitial regions of the testis in *Ar<sup>invflox(ex1-neo)/Y</sup>* mice. To more



**Fig. 1.** Hormonal Profiles and Leydig Cell Numbers during Pubertal Maturation

Hormonal profiles and Leydig cell numbers are shown for wild-type (white bars) and  $Ar^{invflox(ex1-neo)/Y}$  (black bars) mice during pubertal maturation. A and B, Serum LH (A) and serum T (A) levels. Each bar represents the mean hormone level for five to 10 animals. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  from a two-way ANOVA followed by Bonferroni post test. C, Transverse sections of wild-type and  $Ar^{invflox(ex1-neo)/Y}$  testes from animals 3, 6, and 12 wk of age were stained with an antibody against CYP11A1 to mark Leydig cells (brown) and then counterstained with hematoxylin. Yellow asterisks mark CYP11A1<sup>+</sup> Leydig cells. D, Testis mass is decreased in  $Ar^{invflox(ex1-neo)/Y}$  (black bars) compared with wild-type (white bars). E, Numerical density of Leydig cell nuclei. F, Absolute number of Leydig cells per testis as estimated from numerical density of Leydig cells (LCs) (see *Materials and Methods*). Each bar represents the mean values for six to eight animals. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; letters indicate  $P < 0.05$  for a two-way ANOVA followed by Bonferroni post test.

definitively assess the Leydig cell numbers during and after pubertal maturation, we used a systematic histological method to estimate Leydig cell number (23). Testis mass was significantly reduced in  $Ar^{invflox(ex1-neo)/Y}$  mice by 6 wk of age, probably due to the previously described defect in terminal germ cell differentiation (19). Mass was used

to estimate testis volume (see *Materials and Methods*). As suggested by our initial observations of  $Ar^{invflox(ex1-neo)/Y}$  testis histology, the numerical density of Leydig cells is increased in the mutant testis (Fig. 1E). However, the total number of Leydig cells on a per-testis basis is not significantly affected in  $Ar^{invflox(ex1-neo)/Y}$  (Fig. 1F). The increase in Leydig

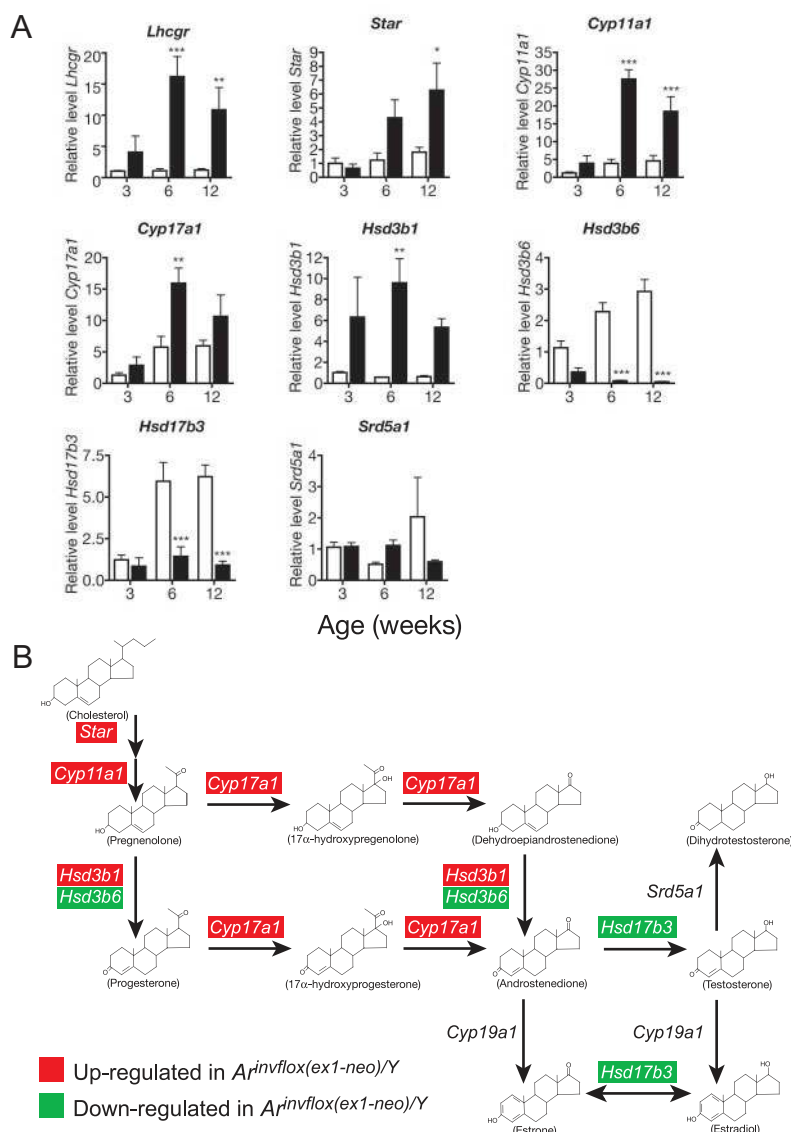
cell numerical density was taken into account when analyzing the expression of Leydig cell-specific transcripts (see *Materials and Methods*).

### Genes Involved in Steroidogenesis Are Misregulated in *Ar* Hypomorphs

Because Leydig cells become increasingly responsive to LH during pubertal maturation, we measured *Lhcgr* expression in wild-type and *Ar<sup>invflox(ex1-neo)/Y</sup>* males at an early-puberty time point (3 wk), a late-puberty time point (6 wk), and adulthood (12 wk) by

quantitative RT-PCR (qRT-PCR). We observed that *Ar<sup>invflox(ex1-neo)/Y</sup>* Leydig cells express increasing amounts of *Lhcgr* during pubertal development, reaching 11-fold up-regulation over wild-type by adulthood (Fig. 2A). Elevated levels of *Lhcgr* mRNA are compatible with an increase in LH-stimulated increase in T production.

As suggested by the high levels of T, many transcripts encoding steroidogenic enzymes were highly up-regulated (Fig. 2A). Notably, the transcript encoding the protein that catalyzes the rate-limiting step of steroidogenesis, *Star*, was up-regulated nearly 5-fold



**Fig. 2.** Misregulation of Transcripts Encoding Proteins Involved in T Biosynthesis

A, Expression of genes involved in steroidogenesis in wild-type (white bars) and *Ar<sup>invflox(ex1-neo)/Y</sup>* (black bars) testes. Levels of mRNA were normalized between samples based on the levels of *Actb* in the case of Northern blots and *Rps2* for transcripts analyzed by real-time qRT-PCR. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  from a two-way ANOVA followed by Bonferroni post test. B, The pathway of T biosynthesis from cholesterol. Red highlights indicate that the transcript was present at significantly higher levels in *Ar<sup>invflox(ex1-neo)/Y</sup>* testes at one or more time points during development, whereas green indicates the transcript was present at lower than wild-type levels at one or more time points.



over wild-type levels. However, not all transcripts encoding steroidogenic proteins were up-regulated in  $Ar^{invflox(ex1-neo)/Y}$ . Interestingly,  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase-6 (*Hsd3b6*) and hydroxysteroid (17 $\beta$ ) dehydrogenase-3 (*Hsd17b3*) were both significantly down-regulated despite the high levels of LH and *Lhcgr*. Collectively, the expression of these transcripts shows that the regulation of steroidogenic gene expression is similar in wild-type and  $Ar^{invflox(ex1-neo)/Y}$  at 3 wk of age but diverges during puberty into adulthood. By this time, every key transcript in the T biosynthetic pathway is affected (summarized in Fig. 2B).

### Up-Regulation of Genes Involved in Cholesterol Uptake and de Novo Biosynthesis

Cholesterol is the essential precursor for all steroid hormones, suggesting that steroidogenic cells have specialized requirements for maintaining proper cholesterol homeostasis. To determine how Leydig cells meet their cholesterol requirements for steroid synthesis, we measured the expression levels of genes involved in cholesterol synthesis and receptor-mediated uptake in wild-type testis and compared them with the levels in the  $Ar^{invflox(ex1-neo)/Y}$  testis, which synthesizes approximately 20-fold higher levels of T (Fig. 1B). All genes involved in *de novo* cholesterol synthesis [*Hmgcs1*, sterol C4 methyl-oxidase-like (*Sc4mol*), sterol C5 desaturase (*Sc5d*), and 7-dehydrocholesterol reductase (*Dhcr7*)] that we analyzed showed a statistically significant increase in expression in  $Ar^{invflox(ex1-neo)/Y}$  testis compared with wild-type testis by 6 wk of age (Fig. 3A). The two receptors involved in uptake of extracellular cholesterol, the low-density lipoprotein (LDL) receptor (LDLR; *Ldlr*) and scavenger receptor type B1 (SR-B1; *Sr-b1*), both showed a significant elevation by 6 wk of age in  $Ar^{invflox(ex1-neo)/Y}$  testis. However, *Ldlr* expression decreased into adulthood, whereas *Sr-b1* mRNA remained elevated compared with wild-type. These data suggest that Leydig cells meet their cholesterol needs through both *de novo* synthesis and uptake of extracellular cholesterol (summarized in Fig. 3B).

### Disruption of Leydig-Cell Lipid Homeostasis in *Ar* Hypomorphs

Up-regulation of pathways involved in cholesterol synthesis and uptake in the testes of  $Ar^{invflox(ex1-neo)/Y}$  mice is consistent with an enhanced requirement for cholesterol for steroidogenesis. To determine whether the effects of *Ar* hypomorphism on cholesterol requirements were systemic or restricted to the gonad, we profiled the composition of plasma lipids. Plasma cholesterol and triglyceride levels were similar between genotypes, as were plasma lipid profiles (Fig. 4, A and B). In addition, testicular cholesterol concentrations were similar between the genotypes. These data suggest that alterations in cholesterol requirements in

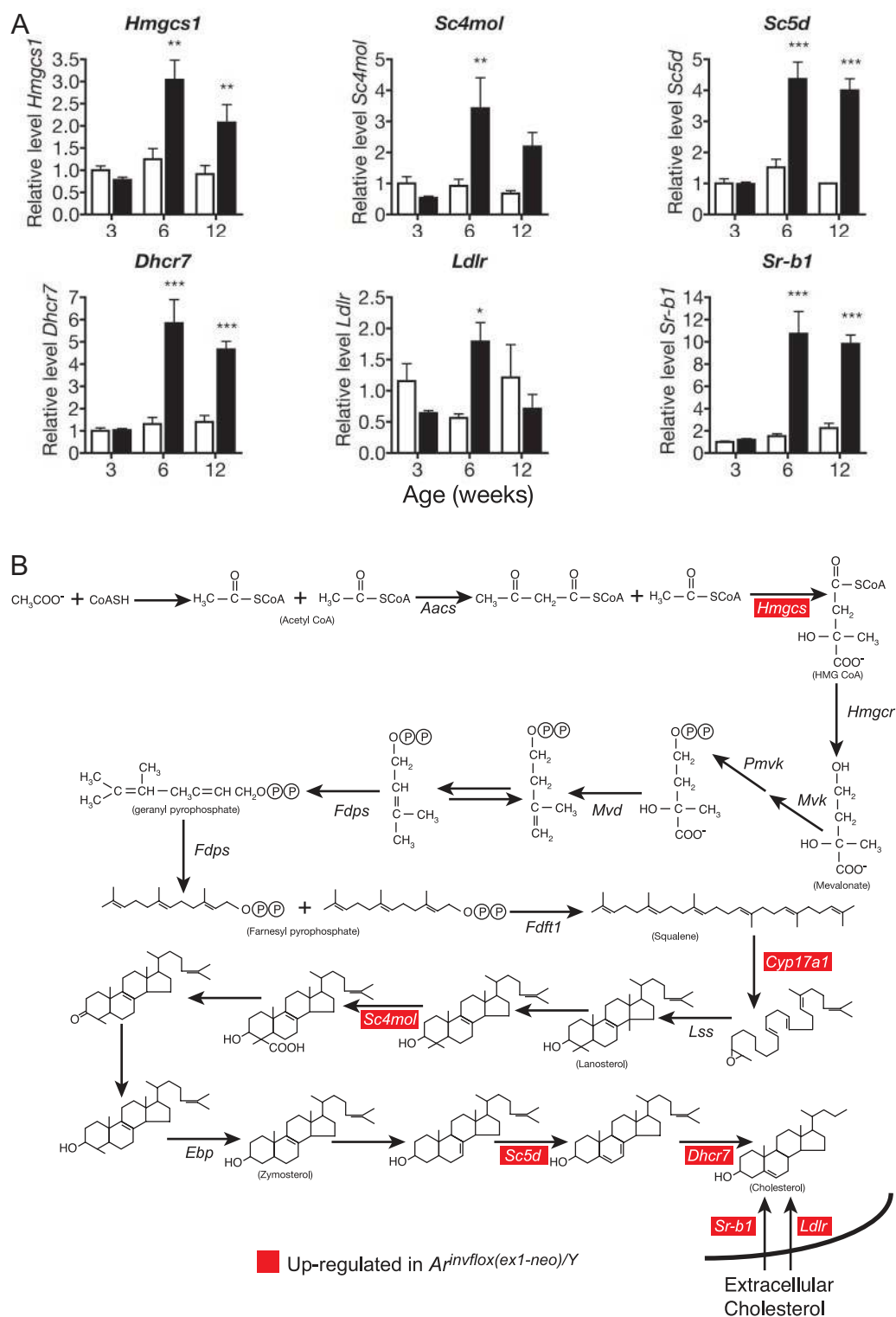
the  $Ar^{invflox(ex1-neo)/Y}$  testis is not due to global alterations in lipid homeostasis.

Enhanced testicular requirements for cholesterol may also be reflected in an increase in enzymes required for the uptake of high-density lipoprotein (HDL) cholesterol via the selective uptake pathway. Hepatic lipase (HL) and endothelial lipase (EL) both facilitate the flux of cholesterol from the HDL particle. Despite the enhanced cholesterol requirements of the testes of *Ar* hypomorphs, there was no significant difference in either testicular HL activity (Fig. 4C) or EL protein levels (Fig. 4D, quantified in 4E). Testicular levels of apolipoproteins that mediate uptake of extracellular sources of cholesterol were also unaltered between genotypes (Fig. 4D, quantified in 4E). However, as suggested by its mRNA levels, we observed dramatic up-regulation of SR-B1. LDLR protein was also highly up-regulated despite the absence of an increase in mRNA levels. As suggested by our profiling of genes involved in steroidogenesis, there was an increase in the levels of StAR, the key protein that facilitates the use of cholesterol for steroid synthesis. These observations show that Leydig cell intrinsic components involved in uptake and use of cholesterol are clearly up-regulated.

Because of the low abundance of the steroidogenic Leydig cells within the testis (roughly 3% of cells/testis), alterations in Leydig cell lipid content would not necessarily be observed by measuring total testicular lipid content. Therefore, we used Oil red O (ORO) staining on frozen sections of testis from wild-type and  $Ar^{invflox(ex1-neo)/Y}$  mice to assess the relative distribution and abundance of cholesterol. Wild-type animals demonstrated weak, but detectable, ORO staining in Leydig cells, identified definitively in serial sections stained with hematoxylin and eosin (Fig. 4F). Strikingly, there was a dramatic increase in the lipid content of Leydig cells in  $Ar^{invflox(ex1-neo)/Y}$  mice. Analysis of blindly scored ORO-stained sections showed a statistically significant increase in the lipid content of Leydig cells of *Ar* hypomorphs (Fig. 4G). The high lipid content in  $Ar^{invflox(ex1-neo)/Y}$  Leydig cells suggests that despite the additional requirements for cholesterol for steroidogenesis, the increase in *de novo* synthesis and uptake results in abnormal accumulation of lipids.

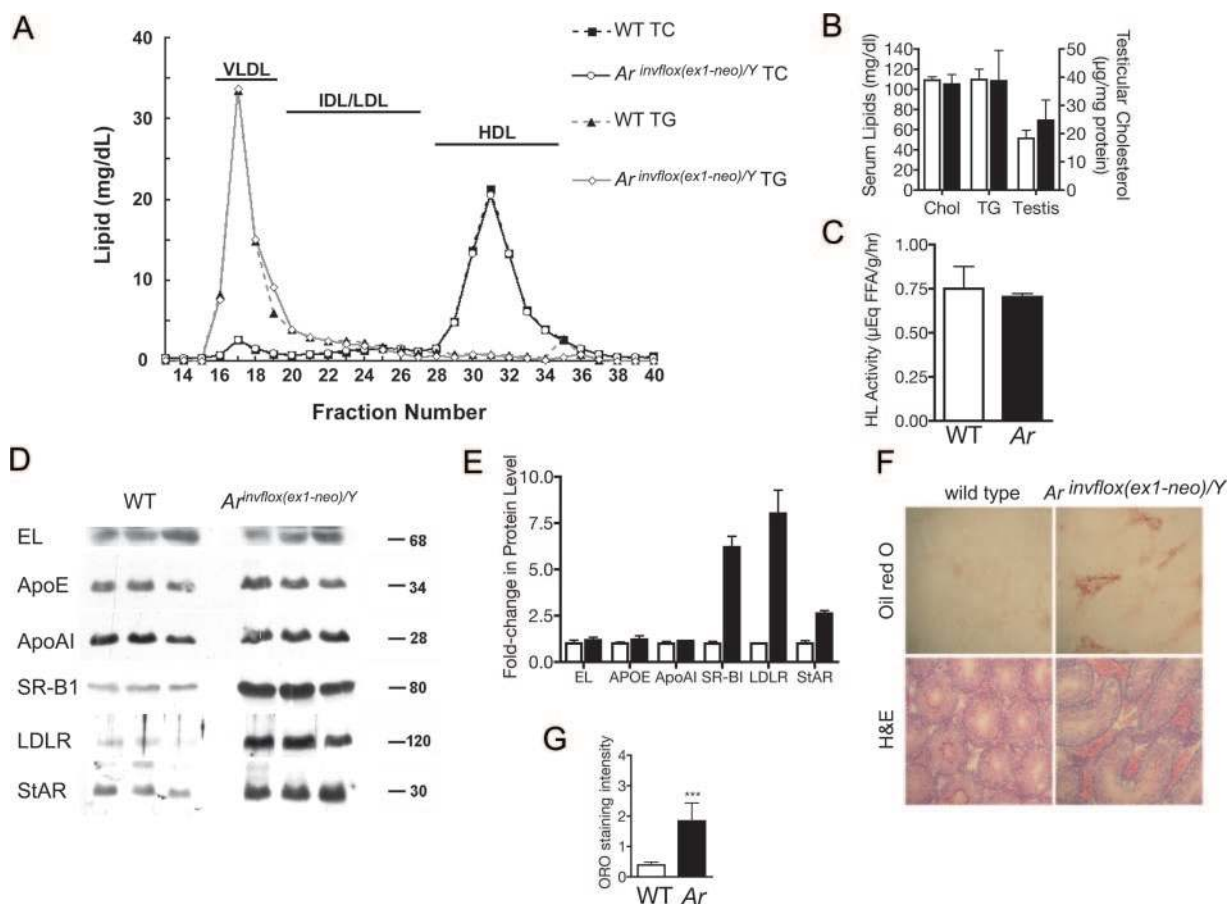
### Regulation of Steroid and Cholesterol Biosynthesis and Uptake by cAMP and DHT

Disrupting the regulation of the HPG axis by reducing *Ar* message has an impact on both AR and LH signaling. Therefore, changes in gene expression observed in  $Ar^{invflox(ex1-neo)/Y}$  mice could be the result of reduced AR signaling, increased LH signaling, or both. To address these possibilities, we used the easily manipulated MA-10 mouse Leydig tumor line (24). MA-10 cells express most genes involved in steroidogenesis but lack *Hsd17b3* expression, preventing the synthesis of T. However, in response to LH or its second



**Fig. 3.** Expression of Genes Involved in Cholesterol Synthesis and Uptake

Expression of genes involved in cholesterol synthesis and uptake in wild-type (*white bars*) and *Ar<sup>invlox(ex1-neo)</sup>/Y* (*black bars*) testis. A, Each *bar* represents the relative mean expression level for the given transcript from six individual animals. Levels of mRNA were normalized between samples based on the levels of *Actb* in the case of Northern blots and *Rps2* for transcripts analyzed by real-time qRT-PCR. All mRNA levels are normalized to the density of Leydig cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  from a two-way ANOVA followed by Bonferroni post test. B, The pathway of synthesis of cholesterol from acetate.



**Fig. 4.** Global and Testicular Lipid Phenotype of Adult *Ar*<sup>invflox(ex1-neo)/Y</sup> Mice

A, Fast-performance liquid chromatography profiles of plasma lipoprotein cholesterol and triglycerides from wild-type and *Ar*<sup>invflox(ex1-neo)/Y</sup> mice. Plasma (100  $\mu$ l) from three mice from each genotype was fractionated by Superose 6 chromatography. Fractions were assayed for cholesterol and triglyceride concentrations using standard colorimetric assays. Horizontal bars indicate lipoprotein distributions. B, Total serum cholesterol and triglycerides were not different between genotypes ( $n = 3$ ). Testicular cholesterol was slightly elevated in *Ar* hypomorphs compared with wild-type, the difference was not significantly different. C, Comparison of hepatic lipase activity between genotypes. D, Western blot analysis for EL, ApoE, ApoA1, SR-B1, LDLR, and StAR comparing levels between wild-type and *Ar*<sup>invflox(ex1-neo)/Y</sup> total testis extracts. E, Laser-densitometry quantification of protein levels in Western blots shown in D. F, ORO staining in the Leydig cells of *Ar*<sup>invflox(ex1-neo)/Y</sup> mice. G, Blindly scored randomly selected sections from *Ar*<sup>invflox(ex1-neo)/Y</sup> and wild-type testes. Sections were scored as follows: 0, no staining in Leydig cells; 1, weak/diffuse staining; 2, intermediate staining in some Leydig cells; 3, strong staining in some Leydig cells; 4, strong staining in all Leydig cells. Error bars in all panels reflect SEM. \*\*\*, Significant difference at  $P < 0.001$ , Student's  $t$  test.

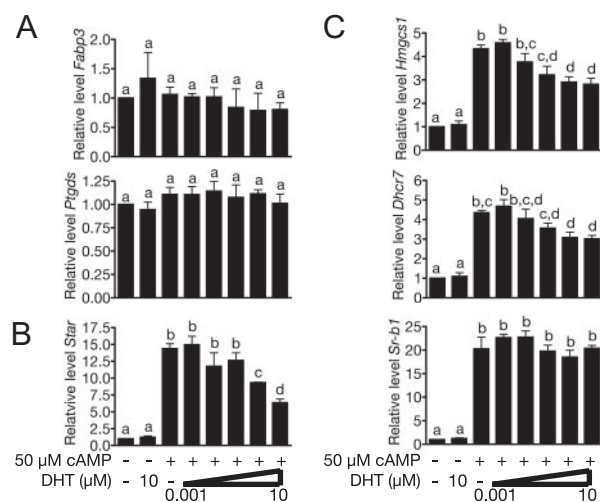
messenger cAMP, MA-10 cells synthesize large amounts of P and its metabolites.

To define which changes in gene expression are due to perturbation of the testicular hormonal milieu, we treated the cells with DHT, 8-Br-cAMP, or both. Expression of markers of Leydig cell maturation is unaffected by treatment with 8-Br-cAMP or DHT (*Ptgds* and *Fabp3*, Fig. 5A). This is consistent with their designation as markers of Leydig cell development and not merely targets of AR or LH signaling. In contrast, genes involved in steroid and cholesterol synthesis and uptake were up-regulated in response to treatment with 8-Br-cAMP (Fig. 5, B and C). Interestingly, none of these genes were up-regulated in response to DHT, but in the case of *Star*, *Dhcr7*, and *Hmgcs1*, DHT treatment attenuated

cAMP-stimulated gene expression. These data suggest that T signaling modulates cAMP-stimulated cholesterol and steroid synthesis by regulating the expression of target genes.

#### cAMP-Stimulated Expression of Genes Involved in Cholesterol Synthesis and Uptake Is Independent of the Nuclear Recruitment of SREBP2

Cholesterol synthesis and uptake is regulated in most mammalian cells by the SREBP2 pathway. To address this possible mechanism of gene regulation, we treated MA-10 cells with cAMP in the presence or absence of 25-OHC, a potent inhibitor of SREBP pathway activation. The addition of 10  $\mu$ M 25-OHC was



**Fig. 5.** Regulation of Steroid Synthesis from *de Novo* Synthesized Cholesterol by cAMP and DHT in the MA-10 Leydig Tumor Line

A, Genes previously identified as markers of Leydig cell maturation; B, genes required for steroid biosynthesis; C, genes required for cholesterol synthesis and uptake. Letters indicate  $P < 0.05$  for a one-way ANOVA followed by Newman-Keuls post test.

unable to suppress 8-Br-cAMP-stimulated expression of *Hmgcs1*, *Dhcr7*, or *Sr-b1*, all known targets of SREBP2 (Fig. 6A). Despite the addition of 25-OHC at a concentration sufficient to block processing of SREBP2 (Fig. 6B), we actually observed a modest increase in the levels of cAMP-stimulated *Hmgcs1* and *Sr-b1* mRNA. These results suggest that SREBP2 processing is not required for the increased expression of genes involved in cholesterol synthesis and uptake.

Because depletion of intracellular cholesterol pools for steroid synthesis could trigger activation of SREBP2, we used a second, independent method to address the possible role of SREBP2 activation of gene expression in response to cAMP stimulation. We tested whether inhibiting steroid synthesis with aminoglutethimide (AG) in cAMP-stimulated MA-10 cells would prevent the up-regulation of cholesterol biosynthetic genes. As observed under treatment of MA-10 cells with 25-OHC, AG did not repress the expression of *Hmgcs1*, *Dhcr7*, or *Sr-b1* (Fig. 6C). Again, this suggests that depletion of intracellular sterol stores is not required for cAMP-stimulated expression of genes involved in cholesterol uptake and synthesis. Finally, we assessed the levels of processed SREBP2 in untreated MA-10 cells as well as cells treated with DHT or 8-Br-cAMP alone or in combination. MA-10 cells untreated or treated with cAMP, DHT, or both show a constant amount of processed SREBP2 (Fig. 6D). These data indicate that cAMP-stimulated expression of cholesterol biosynthesis and uptake gene expression is unlikely due to the accumulation of active SREBP2.

The absence of a requirement for active SREBP2 accumulation for cAMP-stimulated cholesterol biosynthetic gene expression was surprising. To determine whether these observations made using the MA-10 Leydig tumor model are consistent with *in vivo* regulation of cholesterol biosynthetic genes, we collected protein extracts from a Leydig cell-enriched population isolated from wild-type and *Ar<sup>invflox(ex1-neo)/Y</sup>* mice. The *Ar<sup>invflox(ex1-neo)/Y</sup>* mice have elevated levels of LH (Fig. 1A) and increased levels of cAMP in Leydig-enriched cell populations when compared with wild-type-derived Leydig cell populations (data not shown). In the Leydig-enriched protein extracts, we observed no appreciable amount of processed SREBP2 in either wild-type or *Ar<sup>invflox(ex1-neo)/Y</sup>* (Fig. 6E), yet *Ar<sup>invflox(ex1-neo)/Y</sup>* Leydig cells still retain elevated expression of HMGCR, a well-defined target of SREBP2 (Fig. 6F). These data reaffirm the conclusion that SREBP2 activation and nuclear accumulation is not required for cAMP-mediated induction of genes involved in cholesterol biosynthesis and uptake.

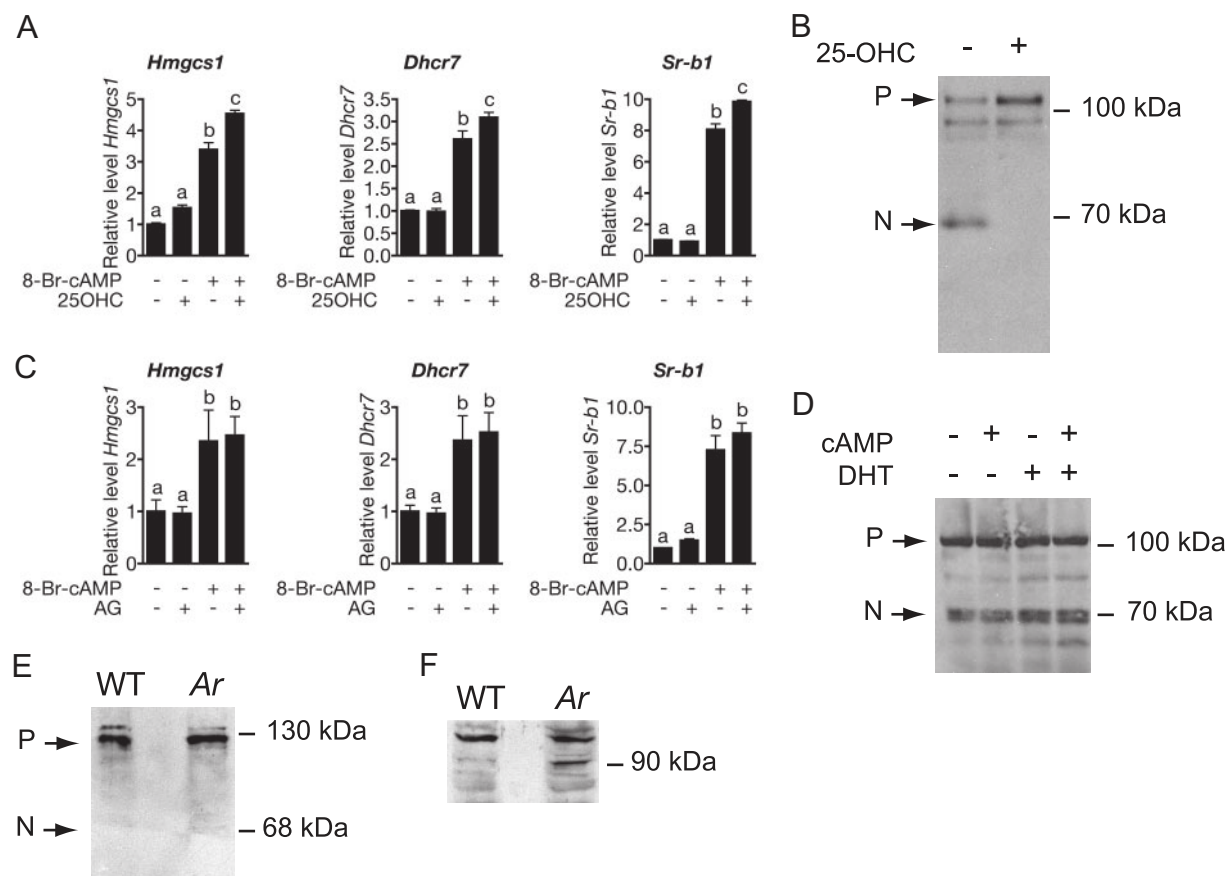
## DISCUSSION

Regulation of testicular steroid and cholesterol homeostasis is critical to the fertility of mammals. In this investigation, we showed that disruption of T-mediated feedback regulation within the HPG axis dramatically disrupts the normal steroid and cholesterol homeostatic mechanisms within the testis. We showed that 1) global reduction of AR signaling results in increased serum LH and T, 2) elevated LH levels increases the expression of genes involved in steroid and cholesterol synthesis and receptor-mediated cholesterol uptake, 3) T modulates the expression of these genes, 4) T's effect on gene expression is associated with suppression of steroids synthesized from *de novo* sources of cholesterol, and 5) the regulation of genes involved in cholesterol biosynthesis and uptake is not mediated by the canonical SREBP2-mediated sterol-sensing mechanism in Leydig cells.

## AR and Regulation of Pubertal Acquisition of Steroidogenic Potential

It has been long recognized that mutations in *Ar* have effects on the development and physiology of the Leydig cell, including steroidogenesis (reviewed in Ref. 25). Interestingly, we found decreased levels of two steroidogenic enzymes, *Hsd17b3* and *Hsd3b6*. Previous studies have shown that the expression of *Hsd3b6* is associated with the pubertal maturation of adult-type Leydig cells (26). Characterization of Leydig cell gene expression in *Ar<sup>tfm/Y</sup>* has shown that AR is required for the onset of *Hsd3b6* expression during puberty (27). The absence of *Hsd3b6* expression and *Ins3* and *Ptdgs*, transcripts associated with mature





**Fig. 6.** cAMP-Stimulated Expression of Genes Involved in Cholesterol Synthesis and Uptake Is Not Mediated by Accumulation of Processed SREBP2

A, Relative mRNA levels in MA-10 cells treated with combinations of 50  $\mu$ M 8-Br-cAMP and 10  $\mu$ M 25-OHC. B, These concentrations of 25-OHC are capable of blocking cholesterol-starved HEK-293 cells from activating SREBP2. The 125-kDa precursor form (P) of SREBP2 and 70-kDa nuclear form (N) are visible in whole-cell extracts of lipid-starved HEK-293 cells. These data are representative of duplicate experiments. C, Relative mRNA levels in MA-10 cells treated with combinations of 50  $\mu$ M 8-Br-cAMP and 5  $\mu$ g/ml AG. Each bar represents the mean expression level for three independent experiments. Letters indicate  $P < 0.05$  for a one-way ANOVA followed by Newman-Keuls post test. Levels of mRNA were normalized between samples based on the levels of *Actb* transcript. D, Treatment of MA-10 cells with cAMP or DHT, alone or in combination, has no effect on levels of the 68-kDa nuclear form of SREBP2. These data are representative of experiments performed in triplicate. E, SREBP2 is not processed in Leydig cell-enriched protein extracts from wild-type or *Ar*<sup>invflox(ex1-neo)/Y</sup> testes. F, HMGCR levels are elevated in Leydig cell-enriched protein extracts from wild-type or *Ar*<sup>invflox(ex1-neo)/Y</sup> testes. Cells used to generate protein extracts in E and F contained more than 60% Leydig cells as determined by  $\beta$ HSD cytochemistry.

adult Leydig cells, led O'Shaughnessy et al. (27) to conclude that AR is required for normal adult Leydig cell differentiation. We have observed a similar phenotype in *Ar*<sup>invflox(ex1-neo)/Y</sup> mice, despite the incomplete inactivation of *Ar* (Fig. 2 and unpublished data). Strikingly, unlike *Ar*<sup>invflox(ex1-neo)/Y</sup> mice, *Ar*<sup>tfm/Y</sup> mutants produce low levels of serum T in response to elevated levels of LH (28). Our results show that LH-stimulated T production is separable from the acquisition of markers of Leydig cell maturation.

Levels of *Hsd17b3* message are similarly down-regulated in *Ar*<sup>tfm/Y</sup> and *Ar*<sup>invflox(ex1-neo)/Y</sup> testes (27), suggesting that high levels of this mRNA are not critical for the synthesis of T. Despite the absence of *Hsd3b6* message, there is no significant difference in the levels of  $\beta$ -hydroxysteroid dehydrogenase

( $\beta$ HSD) activity between wild-type and *Ar*<sup>tfm/Y</sup> testis homogenates (29). Although there is a significant reduction of *Hsd3b6* mRNA in *Ar*<sup>invflox(ex1-neo)/Y</sup> mice, there is a reciprocal up-regulation of another *Hsd3b* isoform, *Hsd3b1*. It is unclear whether up-regulation of *Hsd3b1* is critical to the high levels of T synthesized in *Ar*<sup>invflox(ex1-neo)/Y</sup> as compared with *Ar*<sup>tfm/Y</sup>. A null mutation of *Ar* has no effect on the level of *Hsd3b1* mRNA but results in a severe reduction in *Hsd3b6* levels (27). Enzymatically, the key difference between *Ar*<sup>invflox(ex1-neo)/Y</sup> and *Ar*<sup>tfm/Y</sup> is probably the levels of CYP17A1. Levels of CYP17A1 activity in testis slices from *Ar*<sup>tfm/Y</sup> mice are reduced greater than 40-fold compared with wild-type, which is accompanied by a significant reduction in *Cyp17a1* mRNA (28, 29). In contrast, *Cyp17a1* mRNA levels are elevated in the

testes of  $Ar^{invflox(ex1-neo)/Y}$  mice. Another important factor that promotes steroidogenic activity and T production in  $Ar^{invflox(ex1-neo)/Y}$  mice is the normal positioning of the testis. Cryptorchidism alone has a strong effect on steroidogenic activities and may contribute to the differences observed between  $Ar^{tfm/Y}$  and  $Ar^{invflox(ex1-neo)/Y}$  mutants (28).

### Regulation of Cholesterol Synthesis and Uptake in Leydig Cells

Negative feedback regulation of steroidogenic gene expression is well documented for a number of genes (11, 30–32). However, AR-mediated feedback on cholesterol biosynthetic gene expression has not previously been demonstrated. Our results suggest that T modulates the expression of a subset of genes that are required for *de novo* cholesterol synthesis, adding an additional layer of regulation on T synthesis that had not previously been appreciated.

Earlier *in vitro* studies have demonstrated stimulation of genes involved in cholesterol synthesis and uptake in Leydig cells by treatment with hCG or cAMP. In both cultured rat Leydig cells and MA-10 Leydig tumor cells, a large dose of hCG causes a sharp increase, followed by a rapid decline in HMG-CoA reductase activity (16, 17). This decline in activity is associated with the depletion of LHCGR at the cell surface, the so-called desensitization effect. Soccio and colleagues (33) suggest that the up-regulation of *Hmgcr* is due to the depletion of intracellular sterols by steroidogenesis, thus triggering SREBP-mediated up-regulation of *de novo* cholesterol biosynthesis. Our data offer a different mechanistic explanation for increase in cholesterol biosynthetic gene expression. Treatment of MA-10 cells with 25-OHC to inhibit SREBP activation, or AG to inhibit steroidogenesis, had no effect on cAMP-stimulated expression of *Hmgcs1* or *Dhcr7* expression. Perhaps more convincingly, cAMP had no effect on the accumulation of processed SREBP2, indicating that increased nuclear SREBP2 is not the mechanism by which cAMP stimulates increased expression of genes involved in *de novo* cholesterol biosynthesis. The accumulation of neutral lipids in the Leydig cells of  $Ar^{invflox(ex1-neo)/Y}$  mice also argues against the SREBP-mediated sterol-sensing pathway activation of the *de novo* cholesterol synthesis and uptake pathways. Instead, our data strongly support direct activation of these pathways via LH signaling.

Although increased nuclear SREBP2 is not sufficient to explain the up-regulation of cholesterol biosynthetic gene expression, it remains a possibility that SREBP2 is necessary for the expression of these genes. An additional cAMP-dependent cofactor(s) could be required for the maximal cAMP-stimulated expression of SREBP target genes. One candidate for this role is the cAMP response element-binding protein (CREB), which has been shown to be an effective cofactor for SREBP-dependent activation of the *Hmgcs1* promoter

(34). Alternatively, expression of cholesterol biosynthetic genes could be independent of the SREBP pathway. Cells overexpressing the steroidogenic cell transcription factor SF1 express *Hmgcs1*, even in the face of treatment with 25-OHC (18). This suggests that in steroidogenic tissues, expression of cholesterol biosynthetic genes may be driven in an SF1-dependent manner, independent of SREBP activation.

Uptake of extracellular sources of cholesterol is mediated by LDLR and SR-B1, both of which are transcriptional targets of SREBP2. Rats treated for 4 d with hCG show a massive up-regulation of SR-B1 expression accompanied by a more modest up-regulation of LDLR in Leydig cells (35). These results are consistent with the very high levels of *Sr-b1* and modest change in *Ldlr* mRNA in  $Ar^{invflox(ex1-neo)/Y}$  testes. Despite the modest increase in *Ldlr* message in the current study, there was a large increase in testicular LDLR protein. This may reflect translational control of the *Ldlr* mRNA. As we observed for *de novo* synthesis genes in MA-10 cells, *Sr-b1* expression was responsive to cAMP stimulation, and its expression was unaffected by 25-OHC or AG, again suggesting that the up-regulation of these genes does not require additional accumulation of active SREBP2.

Recently, a number of studies have demonstrated the importance of another group of nuclear hormone receptors, the liver X receptors (LXRs), in the maintenance of cholesterol homeostasis in the adrenals and the testis. The null mutation of both *Lxrα* and *Lxrβ* leads to profound accumulation of cholesterol in the adrenals and extremely high levels of serum corticosteroids (36). The overt phenotypic similarity between the *Lxrα/β*<sup>−/−</sup> adrenals and Leydig cell phenotype of  $Ar^{invflox(ex1-neo)/Y}$  share some similar molecular details as well: increased expression of *Star* and *Cyp11a1*, which probably lead to increased steroidogenesis. However, there are no changes in the expression of genes involved in either *de novo* cholesterol synthesis or uptake, but rather the cholesterol accumulation in these mutants is due to dysfunctional cholesterol efflux in the *Lxrα/β*<sup>−/−</sup> adrenals. Like the adrenals, the testes of *Lxrα/β*<sup>−/−</sup> mice accumulate large amounts of cholesterol (37), as evidenced by increased cholesterol in total testis extracts. We did not observe an increase in cholesterol in total testis extracts but instead observed an increase in neutral lipids in Leydig cells by ORO staining. Surprisingly, the high levels of cholesterol observed in the *Lxrα/β*<sup>−/−</sup> testis is not localized in Leydig cells but rather in the Sertoli cells, suggesting that LXRs regulate different aspects of cholesterol homeostasis than the LH-T-AR loop described in this study.

Taken together, our data suggest that LH directly stimulates the transcription of genes involved in steroidogenesis and genes required for cholesterol synthesis and uptake. Also, our data indicate that a subset of cholesterol biosynthetic genes can be down-regulated by T, providing an additional point of feedback regulation in Leydig cells. We have shown that targets

of LH signaling are affected in an HPG axis disruption model *in vivo*. We also have shown that elevated expression levels of cholesterol biosynthesis and uptake genes are associated with high LH levels *in vivo* and cAMP stimulus *in vitro*. By disrupting these points of regulation in the Leydig cell, we have significantly perturbed steroid and cholesterol homeostasis in the testis as observed by high levels of serum T and accumulation of lipids in Leydig cells. Lastly, the up-regulation of genes involved in cholesterol homeostasis and steroidogenesis appears to happen in the absence of activation of SREBP2, a major regulator of cholesterol biosynthesis in nongonadal tissues.

## MATERIALS AND METHODS

### Animals

All animals were housed in a specific pathogen-free environment and cared for under Institutional Animal Care and Use Committee guidelines. Generation of the hypomorphic *Ar* allele *A<sup>invflox(ex1-neo)</sup>* has been previously described (19). Male mice carrying the hypomorphic allele and their wild-type littermates were killed by CO<sub>2</sub> asphyxiation at the ages of 3, 6, and 12 wk. Animals were genotyped using PCR as previously described (19).

### Phenotypic Analysis

Aortic blood was collected from all animals using a heparin-coated 23-gauge needle. Serum T and LH levels were measured by RIA at the University of Virginia Ligand Core facility. RNA and protein from one testis were prepared as previously described (19). The second whole testis was immersion fixed overnight in Bouin's fixative for histological analysis.

Leydig cell number was determined using the method described by Mori and Christiansen (23). In brief, Bouin's fixed testes were cut into four sections and embedded in paraffin blocks. Five-micrometer sections were collected in a systematic method from each block to represent different regions of the testis. Leydig cells were unambiguously identified by staining with a rabbit antirat cytochrome P450 side chain cleavage antibody (1:250 of Ab1244; Chemicon, Temecula, CA) and then counterstained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, MO). Eight fields were randomly selected from four sections from each testis, and Leydig cell nuclei were counted to estimate nuclear density. Because the density of the testis is approximately 1 mg/ml, testis mass was used as an estimate of testis volume.

### Analysis of Gene Expression

For Northern analysis, 15  $\mu$ g total RNA was run on a 1.5% agarose formaldehyde gel. The RNA was then transferred overnight in 20 $\times$  SSC to Genescreen Plus hybridization membrane (PerkinElmer, Norwalk, CT) and UV cross-linked. Blots were exposed on a Phosphor Storage Screen (GE Healthcare, Piscataway, NJ) and imaged using Storm Scanner 580 (GE Healthcare). Band intensities were measured using ImageQuant (GE Healthcare).

qRT-PCR was performed using the Brilliant SYBR-Green kit (Stratagene, La Jolla, CA). All cDNAs were synthesized from 1  $\mu$ g of total RNA using random hexamers and Superscript III (Invitrogen, Carlsbad, CA) in a 20- $\mu$ l reaction volume. Resulting cDNA was diluted 1:10 in water and subjected to

qRT-PCR using a Stratagene MX-3000P thermocycler. Primers for most transcripts are listed in Ref. 38 with the exception of ribosomal protein S2 (*Rps2*) primer, which is listed in Ref. 39. Novel primers were developed for *Ldlr* (5'-GAG GAG CAG CCA CAT GGT AT-3' and 5'-GCT CGT CCT CTG TGG TCT TC-3'). Relative expression levels were calculated as described elsewhere using levels of *Rps2* to normalize RNA input (39). Normalized gene expression values were then multiplied by the relative density of Leydig cells in the testis to account for differences in Leydig cell number due to age and genotype.

### Lipid and Lipoprotein Characterization

For lipid and lipoprotein characterization, blood was collected from three 12-wk-old mice of each genotype. Individual plasma cholesterol and triglyceride concentrations were determined. In addition, pooled plasma lipoproteins were fractionated using fast protein liquid chromatography as described previously (40). Cholesterol and triglyceride levels in whole plasma and in eluted fractions were determined with standard enzymatic assays for cholesterol (Abbot Spectrum, Abbott Park, IL) and triglyceride (GPO-PAP kit; Boehringer Mannheim, Indianapolis, IN). Lipoprotein cholesterol and triglyceride concentrations were obtained by adding the concentrations of fractions 16–19 (very-low-density lipoprotein), 20–22 (intermediate-density lipoprotein), 23–27 (LDL), and 28–34 (HDL).

Testes were harvested and tissue cholesterol was extracted according to Folch *et al.* (41). Cholesterol concentrations were determined in quadruplicate using standard colorimetric assays, and protein was measured in duplicate. Cholesterol recovery was determined as previously described with minor modifications (42). One half-testis from three individual mice of each genotype was homogenized in 1 ml chloroform/methanol (2:1, vol/vol) containing trace amounts of [<sup>14</sup>C]cholesteryl-oleate (as an internal standard for recovery) and was processed as previously described for adrenal cholesterol measurements (43).

### ORO Staining

To assess Leydig cell cholesterol content, consecutive frozen sections (10  $\mu$ m) from testis were stained with either ORO to detect neutral lipids or with hematoxylin and eosin to visualize tissue morphology. Testes from seven wild-type and six *A<sup>invflox(ex1-neo)</sup>/Y* mice were examined. For each testis, three to five sections were stained with ORO and three to five sections were stained with hematoxylin and eosin.

### Cell Culture

MA-10 cells were obtained as a gift from Jose Teixeira (Massachusetts General Hospital, Boston, MA) with the permission of Mario Ascoli (24) and cultured as previously described (32). Cells were pretreated with varying concentrations of DHT (Sigma) for 1 h before exposure to 50  $\mu$ M 8-Br-cAMP (Sigma). Cells were then cultured for 16 h before harvesting total RNA using 1 ml Trizol (Invitrogen) in accordance with the manufacturer's protocol.

Human embryonic kidney (HEK-293) cells were cultured in DMEM supplemented with L-glutamine, penicillin, streptomycin, and 10% horse serum. For visualizing SREBP2 by Western blot, cells were cultured for 9 h in medium containing 10% lipoprotein-deficient horse serum and 50  $\mu$ M mevastatin (Sigma) in the presence or absence of 10  $\mu$ M 25-OHC (Sigma). Lipoprotein-deficient serum was prepared as previously described (17).

## Immunoblotting

Aliquots of testicular protein extract were analyzed by Western blotting as described (43). The concentration of testis protein extracts was measured using the Lowry method. Equal loading between lanes was verified by Ponceau S staining of the Western blot membrane. After transfer, each blot was incubated with rabbit polyclonal antimouse EL antibody (1:500, C18PEP4 no. 3, a gift from Thomas Quertermous, Stanford University), polyclonal rabbit anti-SR-B1 antiserum (1:1000; Novus Biologicals, Littleton, CO), rabbit polyclonal antihuman LDLR antibody (1:10,000, a gift from Sandra Erickson and Janet Boyles, University of California, San Francisco, San Francisco, CA), or rabbit polyclonal antiserum against StAR (1:1000, a gift from Dr. Douglas Stocco, Texas Tech University, Lubbock, TX). Plasma was collected from four to five mice of each genotype. The membranes were incubated with a goat antimouse apolipoprotein E (ApoE) antibody that also reacts with mouse ApoA1 (a gift from Dr. Karl H. Weisgraber, Gladstone Institute of Cardiovascular Disease). Western blots were visualized with enhanced chemiluminescence substrate (GE Healthcare).

Protein from MA-10 and HEK-293 cells was collected by scraping cells from 10-cm plates in 500  $\mu$ l protein lysis buffer [10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1% SDS]. The lysate was then passed through a 23-gauge needle 11 times and vortexed for 20 min at room temperature. Protein from the cell lysates was quantitated using the BCA assay (Pierce, Rockford, IL). Primary antibodies used were mouse antihuman SREBP2 1C6 (1:25; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit antimouse SREBP2 (7.5  $\mu$ g/ml; a gift of Dr. Jay Horton).

## Leydig Cell Purification

Purification of mouse Leydig cells was carried out according to the method in Ref. 44 with some modification. Eight testes per genotype were removed, and testicular cells were dispersed by treating the decapsulated testis with collagenase (0.25 mg/ml; Sigma) in M199 medium (Invitrogen) at room temperature for 20 min with gentle shaking. After incubation, the dispersed tissues were diluted with M199, and the solution was filtered. Interstitial cells were precipitated by centrifugation of the filtrate and washed once with M199 and twice with PBS. Enrichment for Leydig cells was estimated by 3 $\beta$ HSD cytochemistry.

## Statistical Analysis

All statistical analyses were performed using Prism (version 4.0c; GraphPad Software, San Diego, CA). For comparison between wild-type and mutant animals at each time point, two-way ANOVA was conducted and significance of differences assessed by Bonferroni post test. For comparisons of gene expression between MA-10 cells treated under various regimes, one-way ANOVA followed by Newman-Keuls post test was used.

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