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A Cell-Autonomous Molecular Cascade Initiated by AMP-Activated Protein Kinase Represses Steroidogenesis

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Steroid hormones regulate essential physiological processes, and inadequate levels are associated with various pathological conditions. In testosterone-producing Leydig cells, steroidogenesis is strongly stimulated by luteinizing hormone (LH) via its receptor leading to increased cyclic AMP (cAMP) production and expression of the steroidogenic acute regulatory (STAR) protein, which is essential for the initiation of steroidogenesis. Steroidogenesis then passively decreases with the degradation of cAMP into AMP by phosphodiesterases. In this study, we show that AMP-activated protein kinase (AMPK) is activated following cAMP-to-AMP breakdown in MA-10 and MLTC-1 Leydig cells. Activated AMPK then actively inhibits cAMP-induced steroidogenesis by repressing the expression of key regulators of steroidogenesis, including *Star* and *Nr4a1*. Similar results were obtained in Y-1 adrenal cells and in the constitutively steroidogenic R2C cells. We have also determined that maximum AMPK activation following stimulation of steroidogenesis in MA-10 Leydig cells occurs when steroid hormone production has reached a plateau. Our data identify AMPK as a molecular rheostat that actively represses steroid hormone biosynthesis to preserve cellular energy homeostasis and prevent excess steroid production.

Adequate sex steroid hormone levels and pulsatility, required for proper development and homeostasis, are tightly regulated by the hypothalamus-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates secretion of the pituitary luteinizing hormone (LH), which leads to androgen production by testicular Leydig cells. Via the negative-feedback loop, steroids act on the hypothalamus/pituitary to inhibit GnRH and LH/follicle-stimulating hormone (FSH) production.

Binding of LH to its G-protein-coupled receptor (LHR) on Leydig cells activates adenylate cyclase, triggering an increase in cyclic AMP (cAMP) levels from the intracellular ATP pool (1). The cAMP pathway accounts for most of the LH-induced steroidogenesis (2) and modulates the expression and activity of the steroidogenic acute regulatory (STAR) protein, a rate-limiting factor in hormone-induced steroidogenesis (3). STAR is essential for the transport of cholesterol, the precursor of all steroid hormones, from the outer to the inner mitochondrial membrane where steroidogenesis is initiated (3, 4). Humans with naturally occurring mutations in the *STAR* gene (5) and *Star*$^{-/-}$ mice (6) show suppressed gonadal and adrenal steroidogenesis and suffer from lipoid congenital adrenal hyperplasia and male pseudohermaphroditism. LH/cAMP-induced *Star* expression, and thus steroidogenesis, involves the protein kinase A (PKA)- and mitogen-activated protein kinase (MAPK)-mediated phosphorylation of several transcription factors, including cAMP response element binding protein (CREB)/CRE modulator (CREM) (7, 8) and GATA4 (9). Furthermore, *de novo* synthesis of the NR4A1 (nuclear receptor subfamily 4, group A, member 1) (NUR77) transcription factor is also required (10). Induction of *Nr4a1* and *Star* in Leydig cells also requires Ca^{2+} release from internal stores (11, 12), leading to activation of the Ca^{2+}/calmodulin-dependent kinase I (CAMKI) (10, 13).

Within Leydig cells, the intensity of the LH response, and thus the steroidogenic output, is attenuated by the conversion of the newly synthesized cAMP into AMP by phosphodiesterase 4, 8A, and 8B (PDE4/8A/8B) (14, 15). In most cells, such an increase in AMP levels activates the AMP-activated protein kinase (AMPK), a ubiquitous serine/threonine kinase (reviewed in reference 16). AMPK is a heterotrimeric complex containing one catalytic subunit (α) and two regulatory subunits (β and γ). The competitive binding of AMP/ADP/ATP on AMPKγ modulates the phosphorylation status of AMPKα at residue Thr172 by the upstream kinases liver kinase B1 (LKB1) (also known as STK11) (17, 18) and Ca^{2+}/calmodulin-dependent protein kinase kinase beta (CAMKKβ) (19).

The role of AMPK in energy balance and metabolism is well-known (16). Mounting evidence support a role for AMPK in male reproduction and steroid hormone production. (i) The AMPK agonist resveratrol impairs human chorionic gonadotropin (hCG)-mediated testosterone production in Leydig cells by targeting STAR expression (20). (ii) Overexpression of the AMPK-related kinase SIK3 (salt-inducible kinase 3) in adrenal steroidogenic Y-1 cells inhibits adrenocorticotropic hormone (ACTH)-induced STAR expression (21). (iii) Activation of AMPK with 5-amino-imidazole-4-carboxyamide-1-β-D-ribofuranoside (AICAR) (22) or metformin (23) decreases FSH-induced progesterone production in granulosa cells by inhibiting the expression of 3-β-hydroxysteroid dehydrogenase (3βHSD) and STAR. (iv) Inactivation of the *Prkaa1* gene (encoding AMPKα1) in mice leads to impaired fertility due to higher circulating testosterone caused by increased levels of steroidogenic proteins and lower LH/FSH levels (24). Together, these studies strongly suggest

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that AMPK negatively regulates steroidogenesis in the adrenal glands and gonads. However, the molecular mechanisms of AMPK action remain poorly understood.

In this study, we show that modulation of AMPK activity either pharmacologically or genetically directly affects hormone-induced STAR and NR4A1 expression and steroidogenesis. Microarrays and quantitative PCRs (qPCRs) revealed that expression of several genes known to be involved in steroidogenesis was affected upon AMPK activation. Our data identify AMPK as a novel gatekeeper of steroidogenesis and a target for altering steroid hormone production.

MATERIALS AND METHODS

Cell culture. Mouse tumor MA-10 Leydig cells (25) were provided by Mario Ascoli (University of Iowa, Iowa City, IA) and maintained in Dulbecco modified Eagle medium with nutrient mixture F-12 (DMEM–F-12) supplemented with penicillin-streptomycin (P-S) and 15% horse serum (HS). The mouse adrenal Y-1 cell line, the mouse Leydig MLTC-1 cell line, and the rat Leydig R2C cell line were obtained from ATCC (Manassas, VA). Y-1 and MTLC-1 cells were maintained in DMEM supplemented with P-S and 10% fetal bovine serum (FBS), while R2C cells were maintained in DMEM–F-12 supplemented with P-S, 5% FBS, and 2.5% HS. All cells were grown at 37°C and 5% CO_2.

Chemicals. The AMPK activators AICAR and metformin were obtained from Tocris Bioscience (Minneapolis, MN) and Cayman Chemical Company (Ann Arbor, MI), respectively. Forskolin (Fsk), 22(*R*)-hydroxycholesterol, and 8-bromo-cAMP (8Br-cAMP) were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada).

Protein purification and Western blots. Nuclear extracts, whole-cell lysates, and Western blots were performed as previously described (10). For each treatment, cells were cultured in serum-free medium in the presence of either dimethyl sulfoxide (DMSO)-ethanol (vehicle), metformin (10 mM), Fsk (10 μM) alone, or Fsk and AICAR (1 mM) for either 1 h (nuclear extracts for NR4A1 detection) or 4 h (whole-cell lysates for STAR detection). Each experiment was performed at least 3 times. The antibodies used for detection of NR4A1 (M-210), STAR (FL-285), and lamin B (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies used for detection of NR5A1 and tubulin were purchased from BD Bioscience Pharmingen (San Diego, CA) and Sigma-Aldrich Canada (Oakville, ON, Canada), respectively. The antibodies for detection of AMPKα, phosphorylated AMPKα (P-AMPKα), acetyl coenzyme A (acetyl-CoA) carboxylase (ACC), phosphorylated ACC (P-ACC) were purchased from Cell Signaling (Beverly, MA), and LKB1 was purchased from Abcam (Cambridge, MA).

Transfections of siRNAs against AMPKα1, LKB1, and PDE8A. For knockdown experiments, 150 nM small interfering RNA (siRNA) oligonucleotides were used per 60-mm plate along with 16 μl of Jetprime/200 μl Jetprime buffer. In some cases, the amounts were scaled down proportionally when smaller wells were used. Knockdown of AMPKα1 was achieved using a 50/50 mix of two complementary pairs of siRNA oligonucleotides (Table 1). For LKB1 knockdown, a 50/50 mix of two complementary pairs of siRNA oligonucleotides was also used (Table 1). For PDE8A knockdown, two different siRNAs were independently used (Table 1).

Plasmids, transfections and reporter assays. The bp −980, −195, −144, −120, −95, and −70 to +16 *Star* reporter constructs and the bp −980 to +16 construct harboring the NBRE/SF1 (steroidogenic factor 1) (NR4A1/5A1) mutation were previously described (10). The bp −747 to +50 *Nr4a1* reporter construct was previously described (13). MA-10 Leydig cells were transfected and lysed, and lysates were analyzed as previously described (10, 13). Prior to lysis, cells were treated with vehicle (DMSO-ethanol), AICAR (1 mM), 8Br-cAMP (0.5 mM), Fsk (10 μM), Fsk plus AICAR (1 mM), or 8Br-cAMP plus AICAR for 4 h. These experiments were performed at least three times, each time in duplicate or triplicate.

TABLE 1 siRNA sequences

Targeted transcript	Pair of siRNA oligonucleotides	siRNA sequence
Ampka1	1st	UCUCUUUCCUGAGGACCCAUCUUAU
		AUAAGAUGGGUCCUCAGGAAAGAGA
	2nd	GACUCUUUCCUGGACGACCACCAUU
		AAUGGUGGUCGUCCAGGAAAGAGUC
Lkb1 (*Stk11*)	1st	GGAGGAGGAGGAGGAAGACUUGUUU
		AAACAAGUCUUCCUCCUCCUCCUCC
	2nd	GCAUCGACUCCACCGAGGUAAUCUA
		UAGAUUACCUCGGUGGAGUCGAUGC
Pde8a	1st	CAUUGACGAGGUUGCUGCCCUCAUU
		AAUGAGGGCAGCAACCUCGUCAAUG
	2nd	CCUUCAUGAUGUCCCACCACGGAUU
		AAUCCGUGGUGGGACAUCAUGAAGG

Progesterone and testosterone quantification. Progesterone and testosterone levels were quantified by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI). Briefly, cells were seeded in 24-well plates at 150,000 cells/well. Two days later (~500,000 cells), the medium was replaced with serum-free medium in the presence of DMSO-ethanol (vehicle), AICAR (1 mM), Fsk (10 μM) alone, Fsk plus AICAR, hCG (20 ng/ml), or hCG plus AICAR for 4 h (unless otherwise specified). The medium (500 μl) was then transferred to 1.5-ml tubes and stored at −80°C until ELISAs were performed. For basal progesterone quantification (without Fsk stimulation), 50 μl of medium was diluted 20 times (MA-10 Leydig cells and Y-1 adrenal cells) or 2,000 times (R2C Leydig cells), 50 μl of which was then used in the ELISA. For Fsk/hCG-stimulated progesterone quantification, 20 μl of the medium was diluted 50 times (MA-10 and Y-1 cells), and 50 μl of the dilution was used in the ELISA. For Fsk/hCG-stimulated testosterone quantification in MLTC-1 cells, 20 μl of the medium was diluted 50,861 times, and 50 μl of the dilution was used in the ELISA.

cAMP quantification. Intracellular cAMP levels were quantified by ELISA as recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI). Briefly, MA-10 Leydig cells were seeded in 24-well plates at 150,000 cells/well. Two days later (~500,000 cells), the medium was replaced with serum-free medium in the presence of DMSO-ethanol (vehicle), Fsk (10 μM), or hCG (20 ng/ml) for the indicated time. The cells were lysed, and cAMP was measured by ELISA.

Coimmunoprecipitation. Following the indicated treatment, MA-10 Leydig cells were lysed, and nuclear extracts were prepared as previously described (10). p300 immunoprecipitation was performed with 500 μg of nuclear proteins (1 μg of protein per 1 μl of buffer) and 3 μg of p300 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) in a coimmunoprecipitation (co-IP) buffer (150 mM NaCl, 1 mM EDTA [pH 8.0], 50 mM Tris-HCl [pH 7.0], 0.15% IGEPAL, and freshly added protease inhibitor cocktail) overnight at 4°C using 40 μl of Dynal Dynabeads (Life Technologies). Following 3 washes with 1 ml of the co-IP buffer, proteins were resolved on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were probed with antibodies against p300 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-CREB (Ser133; Santa Cruz Biotechnology, Santa Cruz, CA). For a negative control, 3 μg of an IgG was used (H-270; Santa Cruz Biotechnology, Santa Cruz, CA).

Chromatin immunoprecipitation assay. Following the indicated treatment, cells were cross-linked with 1% formaldehyde for 10 min at

TABLE 2 Sequences of the primers used in the qRT-PCRs

Transcript	Sequence of the primer	
	Forward	Reverse
Cdk12	ACCTGTGGTCCTGCCTCCTG	AGGGGGCCTCTTCTCTGGTG
Cited4	CCGACCACCTGATGCTCGCC	GGAAAGAGACCGGCGACCCG
Crebl2	GAGCGAAGCCGGCAGAGTGC	GCTTGCTCTGCTCCTCGCCA
cFos	TGCCTGAGGCTTCCACCCCA	TGGCACAGAGCGGGAGGTCT
cJun	GCACCTCCGCGCCAAGAACT	AAGCCCTCCTGCTCGTCGGT
Nr0b1	CGTGCTCTTTAACCCAGACCT	TCCATGCTGACTGCACCAAT
Nr4a1	AGGAGACCAAGACCTGTTGC	TCGATCAGTGATGAGGACCA
Nr4a3	CTCGGCTATGACCCCACGGC	TTGGGTGGCGATGGGAGGCT
Pde8a	GTGCAATTTGGCCCGATGAG	GATGTCATGGAGTTTGTCCTGG
Ppme1	CGTGGCATGGGTGGGCGATT	AGGCCGAGTCTGCGTCTCCA
Rpl19	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC
Scarb1	GCTGCTGTTTGCTGCGCTCG	GGGTCCACGCTCCCGGACTA
Star	GTTCCTCGCTACGTTCAAGC	GAAACACCTTGCCCACATCT

37°C, washed twice with 2 ml of cold phosphate-buffered saline (PBS), scraped, and transferred into microtubes. The pellets were then washed for 10 min at 4°C each time with 1 ml cold buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5]) and 1 ml cold buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5]). The pellets were then resuspended in 200 μl of sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris [pH 8.0], freshly added protease inhibitors) and left on ice for 10 min before sonicating with the Misonix S-4000 instrument with a 3-in. cup-horn for 50 times, 20 s at 80% power (with a 20-s pause between each cycle). The extracts were then centrifuged at 4°C for 10 min at 13,000 rpm, and the supernatants were transferred to a new microtube. Input DNA consisted of a 20-μl aliquot of sonicated extract (10%) added to 180 μl of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.0],167 mM NaCl, and freshly added protease inhibitors) and 8 μl of 5 M NaCl to reverse cross-linking. Following an overnight incubation at 65°C, DNA was purified and loaded on an agarose gel to validate the size of the DNA fragments (around 0.5 kb in length).

To prepare the Dynabead/antibody complex, 40 μl (for each reaction mixture) of the Dynabeads were transferred into a microtube and washed with 500 μl of PBS with 0.5% bovine serum albumin (PBS–0.5% BSA) three times. Following the washes, 500 μl of PBS–0.5% BSA was added to the beads, followed by 5 μg of the antibody. The bead/antibody mixture was incubated overnight at 4°C. The antibodies used in this study are p300 (C-20), CREB (H-74), and phospho-CREB (Ser133) (Santa Cruz Biotechnology), and 5 μg of an IgG was used (H-270; Santa Cruz Biotechnology) as a negative control. The next day, the bead/antibody mixture was washed with 500 μl of PBS–0.5% BSA twice, resuspended in 50 μl PBS–0.5% BSA, and added to 150 μl of sonicated extract plus 1,350 μl of dilution buffer. The reaction mixture was left overnight at 4°C on a rotating wheel. The next day, the beads were washed 4 times with 1 ml chromatin immunoprecipitation (ChIP) wash buffer (100 mM Tris [pH 7.4], 500 mM LiCl, 1% IGEPAL, 1% sodium deoxycholate) for 5 min at 4°C on a rotating wheel, washed with 1 ml TE (10 mM Tris [pH 8], 1 mM EDTA), and eluted twice with 200 μl of freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature with shaking (vortex). The two eluates were pooled (400 μl), and 16 μl of 5 M NaCl was added to reverse the cross-links by incubating the solution overnight at 65°C. The reverse cross-linked solution was then treated with 2 μl of 10-mg/ml proteinase K, the DNA was isolated with a QIAquick PCR purification kit (Qiagen), and a PCR was performed using a 5-μl aliquot of the ChIPed DNA and of the input diluted 10 times. The bp −284 to −3 promoter region of the *Nr4a1* mouse gene was amplified with the following oligonucleotides: forward, 5′-GCA GCG GGC GAG AGG AAA AC-3′; reverse, 5′-TGG CGA GCC CGA CCC ACA TC-3′.

RNA isolation for microarray analysis and quantitative RT-PCR. For each treatment, cells were cultured in serum-free medium in the presence of either DMSO-ethanol (vehicle), AICAR (1 mM), Fsk (10 μM) alone, or Fsk plus AICAR for the specified time. The sequences of the primers used are shown in Table 2. Isolation of RNA, cDNA synthesis, and quantitative reverse transcription-PCR (RT-PCR) were performed as previously described (10). The microarray analyses were carried out with Affymetrix mouse gene 1.0 ST arrays.

DNA microarray analyses. DNA microarray analyses were carried out with the Affymetrix mouse gene 1.0 ST arrays. The array interrogates 28,853 well-annotated genes with 770,317 distinct probes. The design of the Affymetrix mouse gene 1.0 ST array was based on the February 2006 mouse genome sequence assembly (UCSC mm8, NCBI build 36) with comprehensive coverage of RefSeq, Ensembl, and putative complete coding sequence (CDS) GenBank transcripts.

Chips were processed according to the Affymetrix standard protocol. Briefly, total RNA (200 ng per sample) was labeled using the Affymetrix GeneChip whole-transcript (WT) cDNA synthesis and amplification kit protocol and hybridized to the arrays as described by the manufacturer (Affymetrix, Santa Clara, CA). The cDNA hybridization cocktail was incubated overnight at 45°C while rotating in a hybridization oven. After 17 ± 1 h of hybridization, the cocktail was removed, and the arrays were washed and stained in an Affymetrix GeneChip fluidics station 450, according to the Affymetrix-recommended protocol (26). The arrays were scanned using the Affymetrix GeneChip scanner (GCS) 3000 7G and the GeneChip operating software (Affymetrix, Santa Clara, CA) to produce the intensity files. Microarray hybridization was carried out at the Microarray Facility of the Centre Hospitalier Universitaire de Québec (CHUQ) Research Centre of Université Laval (http://www.crchudequebec.ulaval.ca/expressiongenique/index.htm).

Data analysis was performed using Partek software, version 6.6 (Partek Inc., St. Louis, MO). Robust multiarray analysis (RMA) background correction and a logarithmic transformation were applied to data arrays. A false discovery rate (FDR)-corrected *P* value of 0.05 and +1.5- and −1.3-fold changes were set as threshold values. A one-way analysis of variance (ANOVA) was used to assess significant differences between the conditions.

Statistical analyses. For all single comparisons between two experimental groups, one-tailed paired Student's *t* tests were performed. For all statistical analyses, a *P* of <0.05 was considered significant. When specified, statistical analyses were done using one-way analysis of variance followed by the Bonferroni *post hoc* test to identify significant differences. All statistical analyses were done using the SigmaStat software package (Systat Software, San Jose, CA).

Microarray data accession number. Microarray data have been deposited in the GEO database and can be assessed using the data set accession number GSE50118.

RESULTS

Activation of AMPK inhibits Fsk/hCG-induced steroidogenesis in MA-10 Leydig cells. Treatment of MA-10 Leydig cells with the widely used AMP analogue AICAR (27) led to AMPKα phosphorylation on Thr172 (Fig. 1A). Activation of adenylate cyclase with Fsk, which increases endogenous cAMP (Fig. 1B) and thus ultimately AMP, also led to AMPKα phosphorylation (Fig. 1A). Phospho-AMPKα was active as determined by the phosphorylation of acetyl-CoA carboxylase (ACC) at Ser79 (Fig. 1A), a known AMPKα target (28). To ensure that AMPKα phosphorylation was not due to artificially high cAMP levels (and consequently AMP) triggered by Fsk/hCG in MA-10 Leydig cells, cAMP levels were measured and found to be increased by 5- to 7-fold, with the increase gradually decreasing to ~2-fold 4 h posttreatment (Fig. 1B and C), similar to results for LH-treated rat primary Leydig cells (29, 30). These data indicate that Fsk is as efficient as LH/hCG for endogenous activation of AMPK in Leydig cells. Finally, AICAR had no effect on cell viability up to 24 h posttreatment (Fig. 1D). Altogether, these data validate the MA-10 Leydig cell line as an adequate model to study the regulation of hormone-induced steroidogenesis and the mechanisms of AMPK action.

To determine the impact of AMPKα activation on steroid production by MA-10 and MLTC-1 Leydig cells, androgen levels were measured in the presence of AICAR. Steroid production by MA-10 cells in response to Fsk and hCG (Fig. 2A) was similar to that of LH/hCG-treated primary Leydig cells (30, 31). While activation of AMPKα did not affect basal steroidogenesis, it significantly blunted the production of Fsk- and hCG-induced progesterone (MA-10 cells) and testosterone (MLTC-1 cells) (Fig. 2A and B). The steroidogenic output of MA-10 Leydig cells cotreated with Fsk and the general phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) (to maintain high cAMP and low AMP levels, thus preventing AMPK activation) was dramatically increased compared to treatment with Fsk alone (Fig. 2C). In this context (Fsk plus IBMX, less cAMP-to-AMP conversion), the efficacy of AMPK at repressing steroidogenesis was reduced compared to treatment with Fsk alone (2.4-fold versus 4.5-fold inhibition, respectively).

Several PDEs are present in Leydig cells, including PDE4 and PDE8 (14). Since PDE8 activity is not affected by IBMX, we sought to determine whether PDE8, in addition to IBMX-sensitive PDEs, could also be implicated. Primary Leydig cells from *Pde8a*$^{-/-}$ and *Pde8b*$^{-/-}$ mice were shown to have similar steroidogenic properties, and the regulatory effects of PDE8A and PDE8B on steroidogenesis are independent of each other (14). By using a siRNA knockdown strategy, we first evaluated *Pde8a* expression following transfection of two different siRNAs and showed that its mRNA levels were reduced by more than 50% (Fig. 2D). In contrast to IBMX, reduction of PDE8A did not further increase Fsk-induced steroidogenesis (Fig. 2E). There was a small increase in basal steroidogenesis in PDE8A-depleted MA-10 Leydig cells, but it did not reach statistical significance (Fig. 2E). Taken together, these results indicate that the rate of cAMP-to-AMP conversion by IBMX-sensitive PDEs in hormone-induced conditions modulates AMPK activity and therefore steroidogenesis.

AMPK represses steroidogenesis by targeting the cholesterol transport pathway. The cholesterol analogue 22(*R*)-hydroxycholesterol (OHC) that freely translocates to the inner mitochondrial membrane, thus bypassing the cholesterol transport machinery, was next used to assess whether AMPK inhibits steroidogenesis by preventing cholesterol shuttling into the mitochondria or by af-

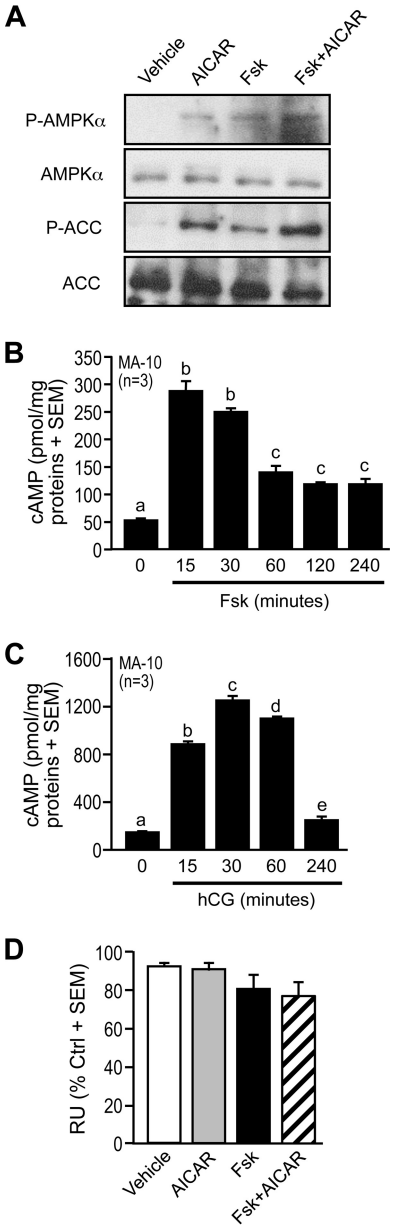

FIG 1 Activation of AMPK by Fsk and time course of cAMP production in MA-10 Leydig cells. (A) P-AMPKα, AMPKα, P-ACC, and ACC levels were determined by Western blotting using whole-cell extracts from MA-10 Leydig cells treated for 2 h with either vehicle (DMSO), AICAR (1 mM), Fsk (10 μM), or Fsk plus AICAR. Representative blots from three independent experiments are shown. (B) Intracellular cAMP levels produced by MA-10 Leydig cells following Fsk treatment (10 μM) at different time points were quantified by ELISA (values are means plus standard errors of the means [SEM] [error bar] for 3 duplicate experiments). A *P* value of <0.05 was considered statistically significant using a one-way ANOVA test. Different letters indicate a statistically significant difference. (C) Same as panel B but with hCG stimulation (20 ng/ml). (D) MA-10 Leydig cells were treated as indicated for 24 h in serum-free medium before performing the cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RU, relative units; Ctrl, control.

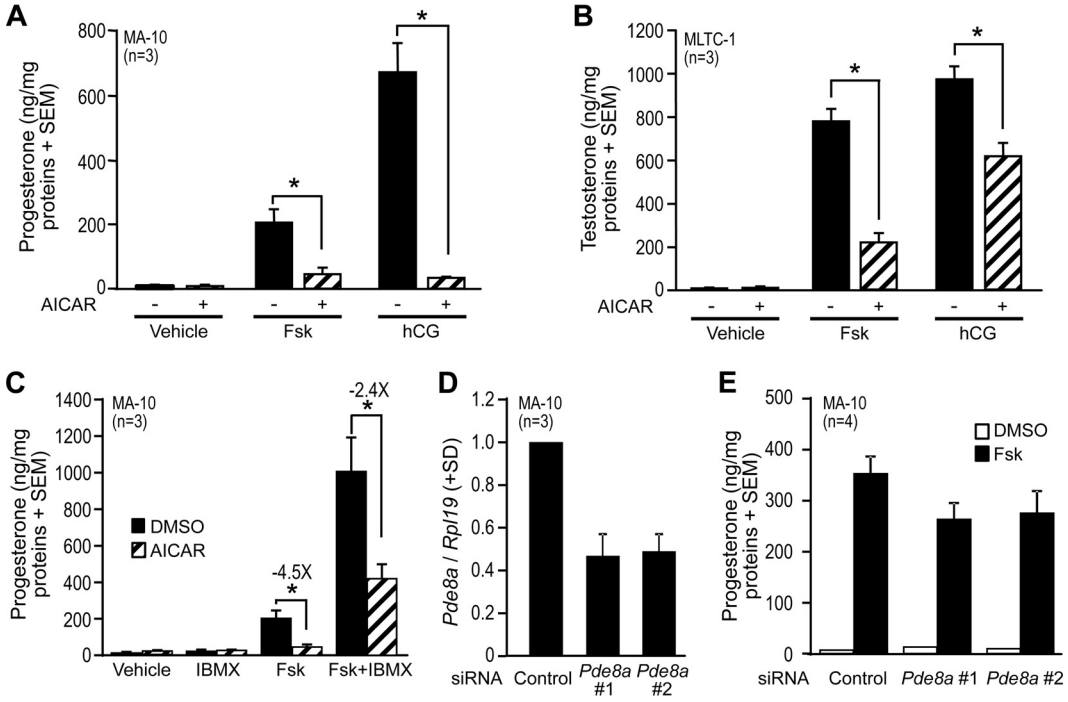

FIG 2 Activation of AMPK blocks steroidogenesis in Leydig cell lines. (A) Progesterone secreted by MA-10 Leydig cells treated for 4 h with vehicle (−), AICAR (+), Fsk, Fsk plus AICAR, hCG, or hCG plus AICAR was quantified by ELISA. (B) Testosterone secreted by MLTC-1 Leydig cells treated for 4 h with vehicle, AICAR, Fsk, Fsk plus AICAR, hCG, or hCG plus AICAR was quantified by ELISA. (C) Progesterone secreted by MA-10 Leydig cells treated for 4 h with vehicle, AICAR, Fsk, Fsk plus AICAR, IBMX, IBMX plus AICAR, Fsk plus IBMX, or Fsk plus IBMX plus AICAR was quantified by ELISA. (D) *Pde8a* mRNA levels were determined by quantitative RT-PCR using total RNA isolated from MA-10 Leydig cells 32 h posttransfection of two distinct siRNAs against *Pde8a*. Values were normalized to *Rpl19* levels, and results are shown as fold induction over siRNA control (values are means plus standard deviations [SD]). (E) MA-10 Leydig cells were transfected for 48 h with two different siRNAs against *Pde8a* and treated for 4 h with vehicle or Fsk, and progesterone production was quantified by ELISA. Values that are statistically significantly different ($P < 0.05$) are indicated by a bar and asterisk.

fecting the expression/activity of steroidogenic enzymes (Fig. 3A). OHC treatment led to a 5- to 7-fold increase in steroidogenesis in MA-10 and MLTC-1 Leydig cells, which was not inhibited by AMPK/AICAR (Fig. 3B and C). Together, these results indicate that AMPK blunts hormone-induced steroidogenesis exclusively at the level of cholesterol transport from the outer to the inner mitochondrial membrane, which is the rate-limiting step in steroidogenesis.

AMPK represses STAR expression. STAR is a key protein in hormone-induced cholesterol transport and steroidogenesis (3, 4) and thus a possible target of AMPK. As expected (8, 10), Fsk/cAMP caused an increase in STAR protein levels, which was abolished by AMPK activation (Fig. 4A), as previously suggested (22). Furthermore, Fsk-induced *Star* mRNA levels (Fig. 4B) and promoter activity (Fig. 4C) were also inhibited upon AMPK activation. The widely used antidiabetic drug metformin is known to activate AMPK in various

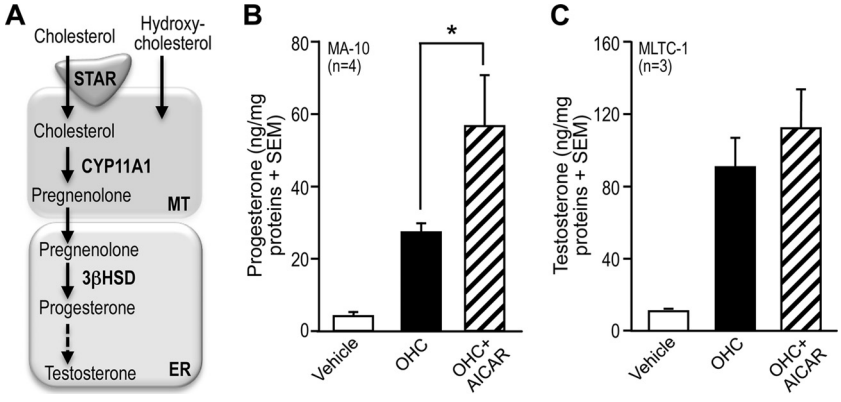

FIG 3 AMPK blocks steroidogenesis by targeting the cholesterol transport machinery. (A) Diagram representing the mode of action of 22(*R*)-hydroxycholesterol (OHC), which diffuses through the mitochondrial inner membrane bypassing the cholesterol transport machinery. MT, mitochondria; ER, endoplasmic reticulum. (B) Progesterone secreted by MA-10 Leydig cells treated for 4 h with vehicle, OHC, or OHC plus AICAR was quantified by ELISA. (C) Testosterone secreted by MLTC-1 Leydig cells treated for 4 h with vehicle, OHC, or OHC plus AICAR was quantified by ELISA.

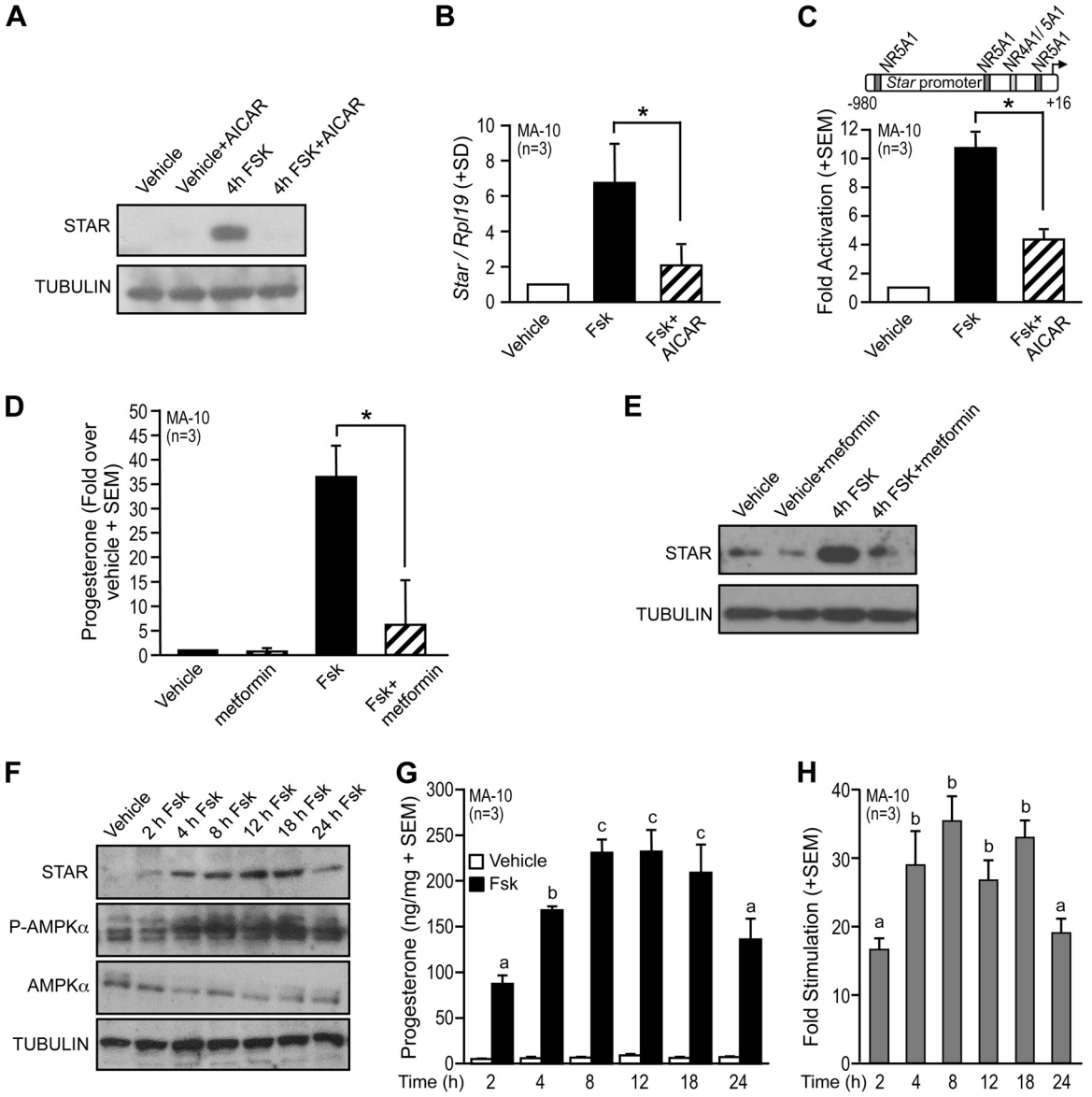

FIG 4 AMPK blocks Fsk-induced steroidogenesis by targeting *Star* expression in MA-10 Leydig cells. (A) STAR and tubulin protein levels were determined by Western blotting using whole-cell extracts from MA-10 Leydig cells treated for 4 h with vehicle, AICAR, Fsk, or Fsk plus AICAR. (B) *Star* mRNA levels were determined by quantitative RT-PCR using total RNA isolated from MA-10 Leydig cells treated for 2.5 h with vehicle, Fsk, or Fsk plus AICAR. Values were normalized to *Rpl19* levels, and results are shown as fold induction over the value for vehicle (mean plus SD). (C) Transient transfections of the murine *Star* promoter (bp −980 to +16) reporter were performed in MA-10 Leydig cells treated for 4 h with vehicle (DMSO), 8Br-cAMP (0.5 mM), or 8Br-cAMP plus AICAR. Previously characterized NR5A1 (bp −980, −135, −100, and −42) and NR4A1/5A1 (bp −100) elements are indicated. Results are shown as fold activation over the value for vehicle (mean plus SEM). (D) Progesterone secreted by MA-10 Leydig cells treated for 4 h with vehicle, metformin (10 mM), Fsk (10 μM), or Fsk plus metformin was quantified by ELISA. (E) MA-10 Leydig cells were treated for 4 h with vehicle, metformin, Fsk, or Fsk plus metformin. and STAR and tubulin protein levels were determined by Western blotting. (F) STAR, P-AMPKα, AMPKα, and tubulin protein levels were determined by Western blotting using whole-cell extracts from MA-10 Leydig cells treated for 2 to 24 h with vehicle or Fsk. (G) Progesterone secreted by MA-10 Leydig cells treated for 2 to 24 h with vehicle or Fsk was quantified by ELISA. (H) The Fsk-dependent induction of progesterone production is shown as a ratio of the progesterone levels in the presence of Fsk over vehicle, shown in panel G.

cell types, including granulosa steroidogenic cells (23). Since metformin is used in animal studies and to treat humans, and as such may represent a more physiological compound than AICAR, we performed experiments using metformin in MA-10 Leydig cells. In the presence of metformin, Fsk-induced progesterone levels (Fig. 4D) and STAR expression (Fig. 4E) were dramatically reduced, as observed with AICAR (Fig. 2A and 4A). These data indicate that AMPK targets *Star* gene expression.

In a time course experiment, we found that STAR protein levels were increased by 2 h, peaked, and stabilized between 8 and 18 h before decreasing (Fig. 4F), a profile that correlates with the level of progesterone accumulated in the culture medium over time (Fig. 4G). The ratio of stimulated over basal progesterone levels (i.e., Fsk-mediated fold enhancement of steroidogenesis or rate of steroidogenesis) revealed that the stimulation of progesterone production has reached a plateau by 4 h and does not further

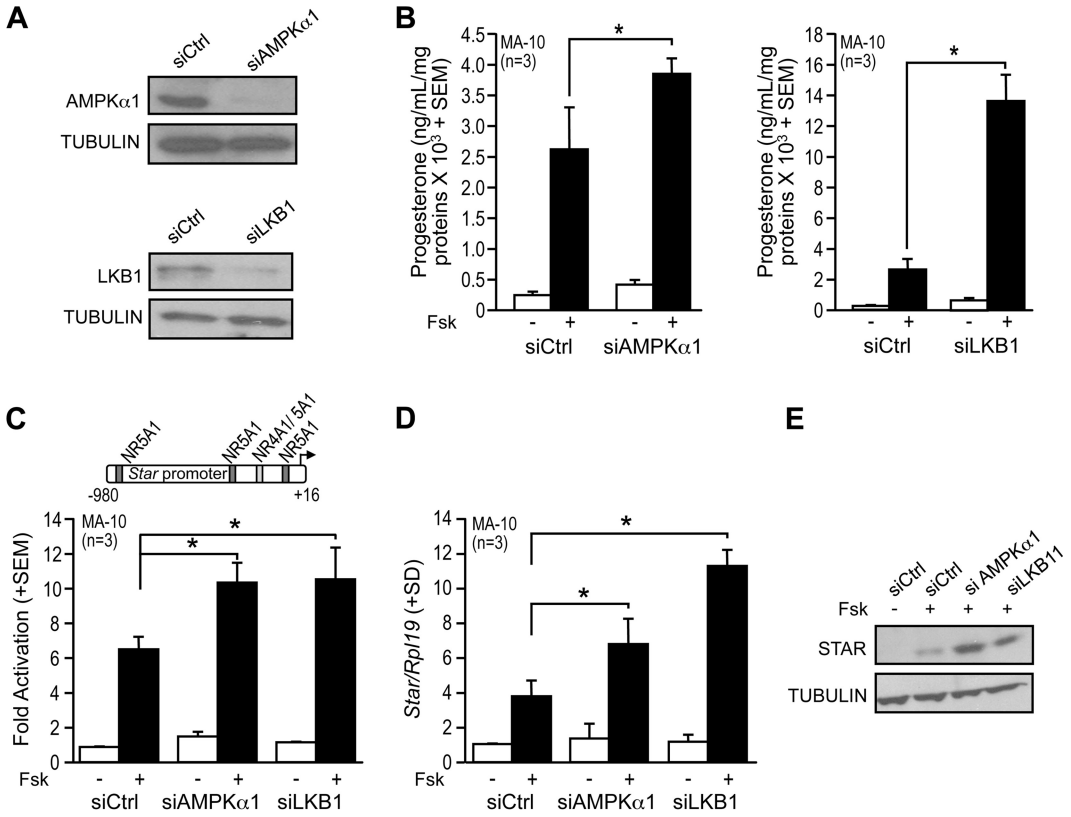

FIG 5 siRNA-mediated knockdown of AMPKα1 or LKB1 increases steroidogenesis and STAR expression in MA-10 Leydig cells. (A) AMPKα, LKB1, and tubulin protein levels were determined by Western blotting using whole-cell extracts from MA-10 Leydig cells 2 days posttransfection of the siRNAs against AMPKα1 or LKB1. (B) Progesterone secreted by MA-10 Leydig cells treated for 4 h with vehicle, Fsk, or Fsk plus AICAR was quantified by ELISA 2 days posttransfection of the siRNAs against AMPKα1 or LKB1. (C) Transient transfections of the murine *Star* promoter (bp −980 to +16) reporter were performed in MA-10 Leydig cells treated for 4 h with either vehicle (DMSO) or Fsk (10 μM) 24 h following transfection of siRNA against AMPKα1 or LKB1. Results are shown as fold activation over vehicle (mean plus SEM). (D) *Star* mRNA levels were determined by quantitative RT-PCR using RNA isolated from MA-10 Leydig cells treated for 2.5 h with vehicle or Fsk (10 μM) 2 days after transfection of the siRNAs against AMPKα1 or LKB1. Values were normalized to *Rpl19* levels, and results are shown as fold activation over vehicle (mean plus SD). (E) STAR and tubulin protein levels were determined by Western blotting using whole-cell extracts from MA-10 Leydig cells treated with Fsk (10 μM) or not treated with Fsk 2 days posttransfection of the siRNAs against AMPKα1 or LKB1.

increase (Fig. 4H). Conversely, phospho-AMPKα levels were increased by 4 h following Fsk stimulation and remained higher than basal levels for up to 24 h after Fsk stimulation (Fig. 4F). The 2-h delay between STAR increase (at 2 h) and AMPK activation/phosphorylation (at 4 h) is consistent with a permissive state allowing the initial increase in STAR expression and steroid synthesis followed by activation of AMPK to limit the increase in STAR expression and steroidogenesis. Thus, maximal AMPK activation levels correspond to the maximal rate of progesterone synthesis, which then gradually decreases due to the inhibition of Fsk-induced STAR expression by activated AMPK.

To further confirm the involvement of AMPK in the regulation of steroidogenesis, siRNAs were used to knock down AMPKα1, the main catalytic subunit expressed in Leydig cells (24), as well as its upstream kinase LKB1 (Fig. 5A). In MA-10 Leydig cells depleted of AMPKα1 or LKB1, the Fsk-induced progesterone levels were significantly increased compared to cells treated with nontargeting siRNAs (Fig. 5B). Furthermore, *Star* promoter activity (Fig. 5C), mRNA levels (Fig. 5D), and STAR protein (Fig. 5E) levels were concomitantly increased in MA-10 Leydig cells depleted of AMPKα1 or LKB1. These results indicate that AMPK activation prevents overproduction of steroids following hormonal stimulation by directly inhibiting *Star* expression.

Activated AMPK modulates expression of factors important for Leydig cell function. A microarray approach was used to elucidate the transcriptional network targeted by AMPK leading to *Star* repression and decreased steroidogenesis. Using a false discovery rate (FDR)-corrected *P* value of 0.05 and a −1.3- to +1.5-fold change, 390 genes, including several known to regulate cell proliferation and metabolic/rhythmic processes, were differentially regulated following AMPK activation (Fig. 6A). Of these genes, 10 were validated by qPCR (Fig. 6B). As expected (Fig. 4B), Fsk-induced *Star* expression was strongly inhibited by AICAR/AMPK (Fig. 6B). In addition to *Star*, Fsk-induced expression of the scavenger receptor b1 (*Scarb1*), known to be important for cholesterol import in Leydig cells (32, 33), was also impaired by AMPK activation. In addition to genes encoding proteins implicated in cholesterol transport (*Star* and *Scarb1*), AMPK activation impaired the expression of several transcriptional activators (*Nr4a1*, *Nr4a3*, *Crebl2*, *Cited4*, and *cJun*), some known to positively regulate steroidogenesis (8, 34). On the other hand, expression of *cFos*, a repressor of steroidogenesis (35, 36), was increased (Fig. 6B). Expression of *Ppme1* (protein phosphatase methylesterase 1) and *Cdk12* (cyclin-dependent kinase 12) was not affected, confirming the specificity of AICAR/AMPK action (Fig. 6B).

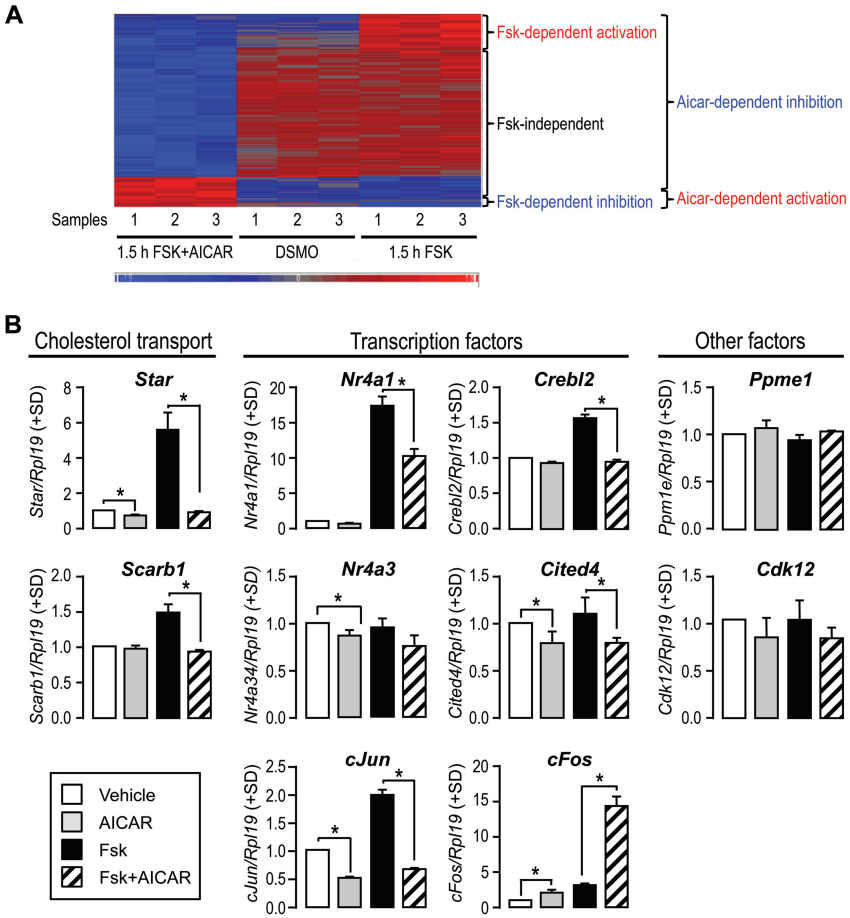

FIG 6 Activation of AMPK impairs the expression of several prosteroidogenic genes in MA-10 Leydig cells. (A) Heatmap showing the impact of AMPK activation on the transcriptome of MA-10 Leydig cells. The expression of 390 genes significantly affected by AICAR treatment in the presence of Fsk is shown (false discovery rate [FDR] of 0.05 and fold induction of ≥1.5 and less than or equal to −1.3). (B) Validation of genes affected by AICAR treatment was performed by quantitative RT-PCR using RNA isolated from MA-10 Leydig cells treated for 1.5 h with vehicle, AICAR, Fsk, or Fsk plus AICAR. Values were normalized to *Rpl19* levels, and results are shown as fold activation over vehicle (mean plus SD) ($n = 3$). Values that are statistically significantly different are indicated by a bar and asterisks as follows: ∗, $P < 0.05$.

AMPK suppresses *Star* expression by targeting the nuclear receptor NR4A1. To identify the regulatory elements in the *Star* promoter involved in the AICAR/AMPK-mediated repression, MA-10 Leydig cells were transiently transfected with a series of deletion constructs of the mouse *Star* promoter, stimulated with cAMP (to increase promoter activity without activating AMPK), and then tested for AMPK/AICAR responsiveness. As shown in Fig. 7A, AMPK/AICAR repressed the cAMP-induced activity of the bp −980, −195, −144, and −120 to +16 *Star* reporters but not of the bp −95 and −70 to +16 *Star* reporters. These data indicate that the AMPK-sensitive element(s) is located between bp −120 and −95 of the *Star* promoter. This region contains a binding site at bp −100 (ATCCTTGA) for the nuclear receptors NR5A1 (SF1) (37) and NR4A1 (NUR77) (10). This NR4A1/NR5A1 element is required for maximal cAMP-dependent activation of the *Star* promoter (10). When a mutation known to prevent binding of NR4A1 and NR5A1 (ATCCTTGA to ATAATTGA [mutant nucleotides are underlined]) was introduced into this element in the context of the bp −980 to +16 *Star* reporter, the AICAR/AMPK-dependent inhibition was significantly reduced compared to the wild-type reporter (Fig. 7B). This suggests that the nuclear receptor NR4A1 and/or NR5A1 could be targeted by AMPK, leading to repression of *Star* expression.

We therefore tested the impact of AMPK activation on NR4A1 and NR5A1 protein levels. As shown in Fig. 8A, we found that the Fsk-induced NR4A1 protein levels were reduced upon activation of AMPK with AICAR, while NR5A1 protein levels were not affected. However, we cannot formally exclude the possibility that posttranslational modifications affect SF1 activity following AMPK activation. Furthermore, Fsk-induced *Nr4a1* mRNA levels (Fig. 8B) as well as the cAMP-induced *Nr4a1* promoter activity (Fig. 8C) were also reduced by AICAR/AMPK. Finally, knocking down AMPKα1 or its upstream kinase LKB1 potentiated the Fsk-dependent induction of *Nr4a1* promoter activity (Fig. 8D), mRNA levels (Fig. 8E), and NR4A1 protein levels (Fig. 8F), while NR5A1 protein levels remained unchanged (Fig. 8F).

AMPK is known to reduce p300 recruitment to its target promoter (38, 39). Since p300 is required for *Nr4a1* transcription in other cells (40, 41), ChIP assays were performed to test whether activated AMPK impairs p300 recruitment on the *Nr4a1* promoter. Treatment of MA-10 Leydig cells with Fsk led to an increase in p300 recruitment, which was prevented by activation of

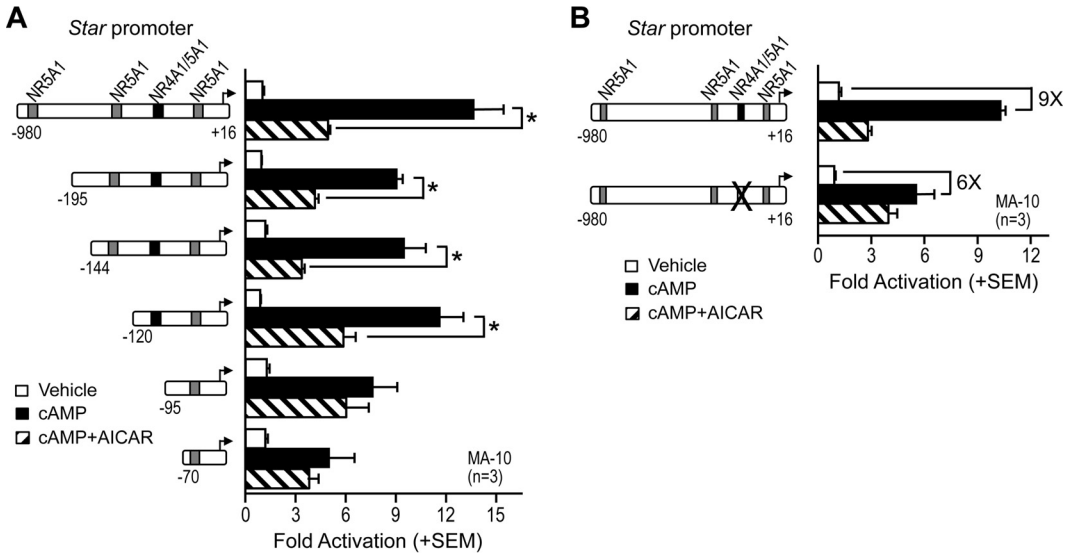

FIG 7 AMPK impairs *Star* expression by targeting the NR4A1/NR5A1 promoter element located at bp −100. (A) To locate the AICAR responsive element within the murine *Star* promoter, MA-10 Leydig cells were transiently transfected with a series of 5′ deletion constructs (bp −980, −195, −144, −120, −95 and −70 to +16) of the mouse *Star* promoter and treated with vehicle, 8Br-cAMP (0.5 mM), or 8Br-cAMP plus AICAR (1 mM) for 4 h. Results are shown as fold activation over vehicle (mean plus SEM). An asterisk indicates a statistically significant difference ($P < 0.05$). (B) MA-10 Leydig cells were transiently transfected with the bp −980 to +16 mouse *Star* promoter, either wild type or containing a mutation in the NR4A1/NR5A1 element at bp −100 (the mutation is indicated by a large X). Cells were then treated with vehicle, 8Br-cAMP (0.5 mM), or 8Br-cAMP plus AICAR (1 mM) for 4 h. Results are shown as activity relative to the activity of the wild-type reporter treated with vehicle, which was arbitrarily set at 1. The fold stimulation by cAMP is indicated.

AMPK with AICAR (Fig. 8G). Furthermore, the Fsk-induced recruitment of phospho-S133 CREB to the *Nr4a1* promoter, which activates this promoter (42–45), was reduced following AMPK activation, while total CREB was not affected (Fig. 8G). Activation of AMPK was also found to disrupt the Fsk-induced p300/phospho-S133 CREB interaction (Fig. 8H), which is in agreement with the ChIP data. Together these results strongly suggest that the repressive effect of AMPK on *Star* expression and steroidogenesis is mediated, at least in part, by modulating the expression of the nuclear receptor *Nr4a1*, an important activator of hormone-induced *Star* transcription and steroidogenesis (10, 46).

Activation of AMPK suppresses steroidogenesis in adrenal cells. Since adequate hormonal stimulation of adrenal steroidogenesis also requires STAR and NR4A1 (47, 48), we evaluated whether AMPK also repressed steroidogenesis in Y-1 adrenal cells. These cells express AMPK that can be activated by AICAR (Fig. 9A), causing a reduction in Fsk-induced progesterone synthesis (Fig. 9B). This reduction in progesterone synthesis can be explained by a significant reduction in the Fsk/cAMP-induced STAR (Fig. 9C to E) and NR4A1 (Fig. 9F to H) gene expression and protein levels. These results indicate that activation of the AMPK pathway also modulates adrenal steroidogenesis via *Star* and *Nr4a1* expression.

Activation of AMPK reduces steroid production in uncontrolled steroidogenic cells. Cells in several hormone-dependent pathologies (prostate, breast, ovarian cancers as well as polycystic ovarian syndrome [PCOS]) aberrantly produce steroid hormones that act as a mitogen for these cells (49; reviewed in references 50 and 51). We used the R2C Leydig cell line, which constitutively produces high levels of steroid hormones without any stimulation (52, 53) to test the possibility that activation of AMPK reduces steroid hormone production. When R2C cells were treated with AICAR, progesterone production was decreased by 50% at 8 h and 24 h posttreatment (Fig. 10A), indicating that activated AMPK significantly reduces steroid production in constitutively steroidogenic cells. In agreement with data obtained in MA-10, MLTC-1, and Y-1 cells, we found that *Star* and *Nr4a1* mRNA levels were decreased following AMPK activation (Fig. 10B and C). In addition, expression of *Nr0b1* (*Dax1*), a repressor of steroidogenesis and *Star* transcription (54), was significantly increased in AICAR-treated R2C cells (Fig. 10D).

DISCUSSION

Steroid hormone synthesis is a meticulously regulated process, since insufficient or excess production is detrimental. While the stimulation of steroid synthesis in response to tropic hormones has been well studied, the detailed inhibitory mechanism(s) preventing excess steroidogenesis remained largely unknown. Here we show that stimulation of Leydig and adrenal cell steroidogenesis activates the energy-sensing kinase AMPK which then actively represses steroidogenesis by inhibiting STAR and NR4A1 expression. Furthermore, AMPK activation in constitutively steroidogenic Leydig cells led to a significant reduction in steroid hormone production.

AMPK actively inhibits steroid hormone synthesis. So far, a few studies have reported a potential role for AMPK in repressing steroidogenesis (20, 22–24). However, the molecular mechanisms of AMPK action remained poorly characterized. In the present work, we propose that the increase in the AMP/ATP ratio triggered by the interplay of adenylate cyclase and IBMX-sensitive phosphodiesterase (most likely PDE4 [14]) is responsible for AMPK activation by its upstream kinases like LKB1. Once activated, AMPK then acts as a molecular brake to prevent excessive ATP consumption, which otherwise leads to cell death (55), as well as to prevent excessive steroid hormone production. The fact that maximal AMPKα phosphorylation was reached at ~4 h following Fsk stimulation, which coincides with a decrease in cAMP

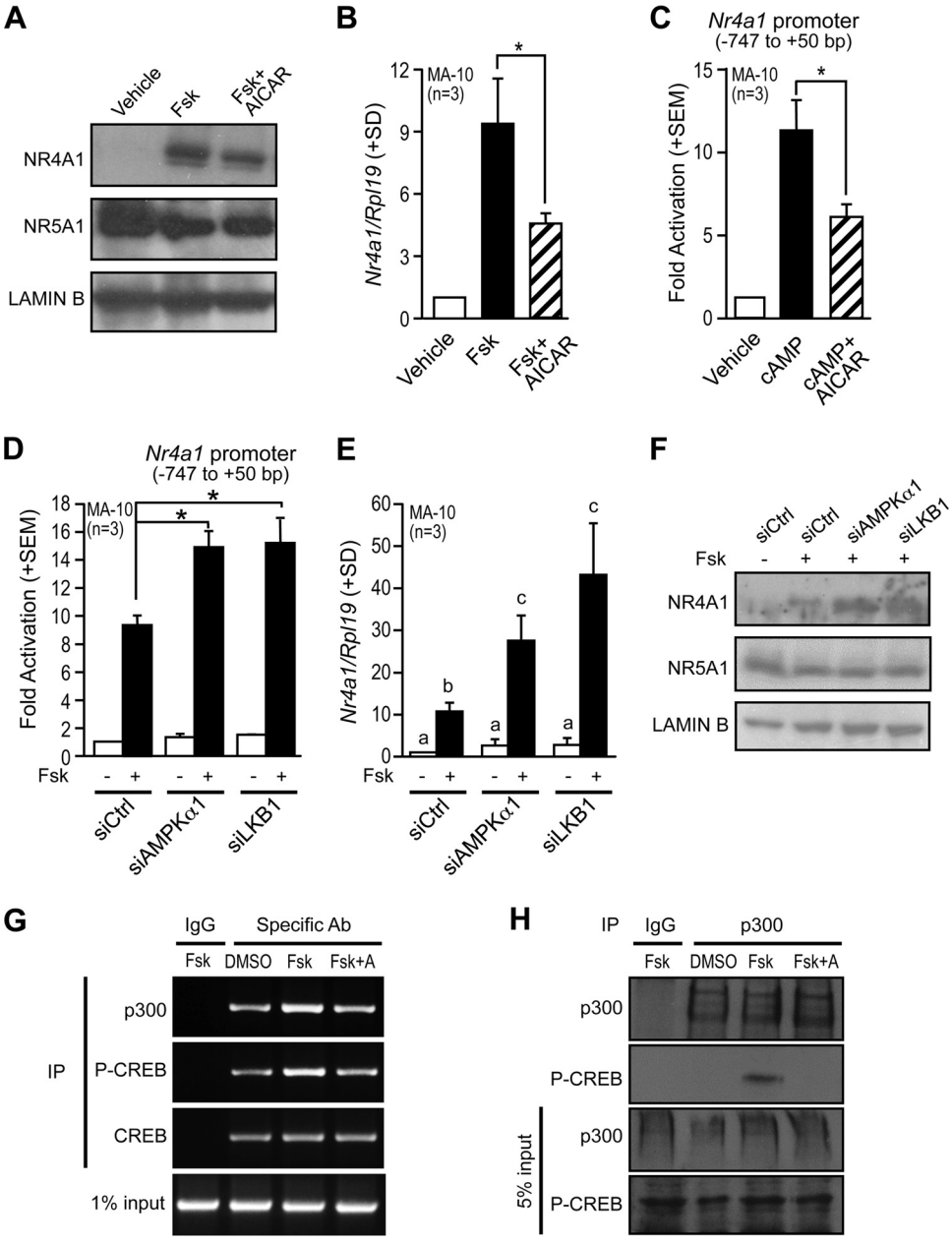

FIG 8 AMPK impairs STAR expression by targeting NR4A1 in MA-10 Leydig cells. (A) NR4A1, NR5A1, and lamin B protein levels were determined by Western blotting using nuclear extracts from MA-10 Leydig cells treated for 2 h with vehicle, Fsk, or Fsk plus AICAR. (B) *Nr4a1* mRNA levels were determined by quantitative RT-PCR using total RNA isolated from MA-10 Leydig cells treated for 1 h with vehicle, Fsk, or Fsk plus AICAR. Values were normalized to *Rpl19* levels, and results are shown as fold induction over vehicle (mean plus SD). (C) Transient transfections of the murine *Nr4a1* promoter (bp −747 to +50) were performed in MA-10 Leydig cells treated for 4 h with either vehicle, 8Br-cAMP (0.5 mM), or 8Br-cAMP plus AICAR. Results are shown as fold activation over vehicle (mean plus SEM). (D) Transient transfections of the murine *Nr4a1* promoter (bp −747 to +50) reporter were performed in MA-10 Leydig cells treated for 4 h with either vehicle (DMSO) or Fsk (10 μM) 24 h following transfection of siRNA against AMPKα1 or LKB1. Results are shown as fold activation over vehicle (mean plus SEM). (E) *Nr4a1* mRNA levels were determined by quantitative RT-PCR using RNA isolated from MA-10 Leydig cells treated for 1 h with vehicle or Fsk 2 days posttransfection of siRNAs against AMPKα1 or LKB1. Values were normalized to *Rpl19* levels, and results are shown as fold induction over vehicle (mean plus SD) ($n = 3$ duplicates). (F) NR4A1/NR5A1 and lamin B protein levels were determined by Western blotting using nuclear extracts from MA-10 Leydig cells 2 days posttransfection of siRNAs against AMPKα1 or LKB1. (G) MA-10 Leydig cells were treated for 30 min with DMSO, Fsk, or Fsk plus AICAR (Fsk+A) in a serum-free medium, and a chromatin immunoprecipitation (IP) experiment was performed to assess recruitment of p300, P-CREB, and total CREB to the bp −284 to −3 region of the *Nr4a1* promoter. An IgG was used as a negative control, and 1% of the sonicated extract was used as input. Ab, antibody. (H) Coimmunoprecipitation experiments were performed on nuclear extracts of MA-10 Leydig cells treated for 30 min with DMSO, Fsk, or Fsk plus AICAR (Fsk+A). Part (5%) of the nuclear extracts was used as input control.

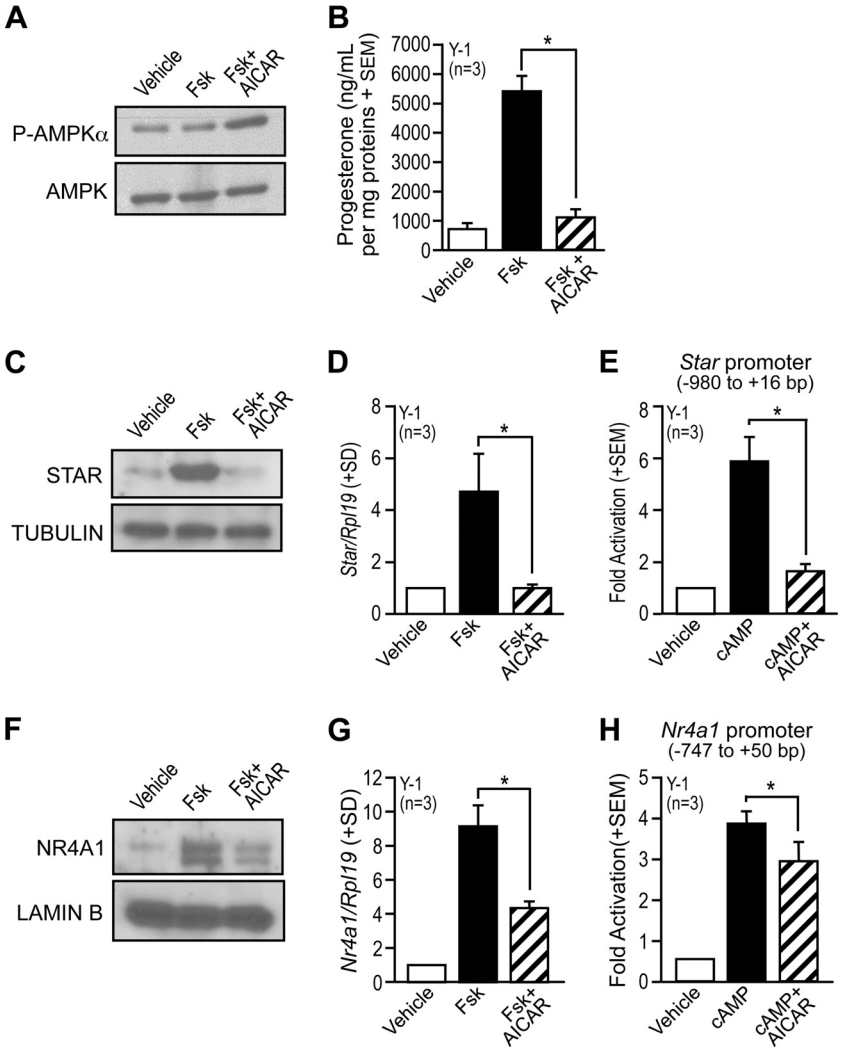

FIG 9 AMPK impairs steroidogenesis by blunting STAR and NR4A1 expression in the Y-1 adrenal cell line. (A) P-AMPKα and AMPKα levels were determined by Western blotting using whole-cell extracts from Y-1 adrenal cells treated for 1 h with vehicle (DMSO), Fsk, or Fsk plus AICAR. (B) Progesterone secreted by Y-1 adrenal cells treated for 4 h with vehicle, Fsk, or Fsk plus AICAR was quantified by ELISA. (C) STAR and tubulin protein levels were determined by Western blotting using whole-cell extracts from Y-1 adrenal cells treated for 4 h with vehicle, Fsk, or Fsk plus AICAR. (D) *Star* mRNA levels were determined by quantitative RT-PCR using total RNA isolated from Y-1 adrenal cells treated for 2.5 h with vehicle, Fsk, or Fsk plus AICAR. Values were normalized to *Rpl19* levels, and results are shown as fold induction over vehicle (mean plus SD). (E) Transient transfections of the murine *Star* promoter (bp −980 to +16) were performed in Y-1 adrenal cells treated for 4 h with vehicle, 8Br-cAMP (0.5 mM), or 8Br-cAMP plus AICAR. Results are shown as fold activation over vehicle (mean plus SEM). (F) NR4A1 and lamin B protein levels were determined by Western blotting using nuclear extracts from Y-1 adrenal cells treated for 2 h with vehicle, Fsk, or Fsk plus AICAR. (G) *Nr4a1* mRNA levels were determined by quantitative RT-PCR using total RNA isolated from Y-1 adrenal cells treated for 1 h with vehicle, Fsk, or Fsk plus AICAR. Values were normalized to *Rpl19* levels, and results are shown as fold induction over vehicle (mean plus SD). (H) Transient transfections of the murine *Nr4a1* promoter (bp −747 to +50) were performed in Y-1 adrenal cells treated for 4 h with vehicle, 8Br-cAMP (0.5 mM), or 8Br-cAMP plus AICAR. Results are shown as fold activation over vehicle (mean plus SEM).

levels to almost basal levels, further supports our model. In addition, maximal AMPK activation coincided with the peak and plateau in steroidogenesis and STAR expression. Together, these data strongly suggest that the delay in AMPK activation (~4 h) following stimulation of steroidogenesis is necessary for increased STAR expression and steroidogenesis (occurs within 2 h). In support of this, when AMPK was activated by AICAR concomitantly with Fsk stimulation, phospho-AMPK was detected as early as 1 to 2 h after treatment, while Fsk-induced STAR expression and steroidogenesis were abolished by 4 h posttreatment. This is significantly shorter than the decrease observed 24 h after Fsk stimulation in the absence of AICAR.

Mechanisms of AMPK action. We found that AMPK inhibits steroidogenesis by repressing genes encoding proteins involved in cholesterol transport, SCARB1 and STAR. For *Star* repression, several mechanisms that are not mutually exclusive are involved. First, AMPK decreases the expression of c-Jun and NR4A1, two activators of steroidogenesis (10, 46, 56–59). Although our data confirmed NR4A1 as a key target of AMPK, our analysis of the *Star* promoter revealed that other elements/factors are involved in mediating AMPK responsiveness, since mutation of the NR4A1 binding site largely decreased, but did not completely abrogate, AMPK repressive effects. One of these factors could be CREB, an activator of *Star* transcription (8) that is targeted by AMPK on the

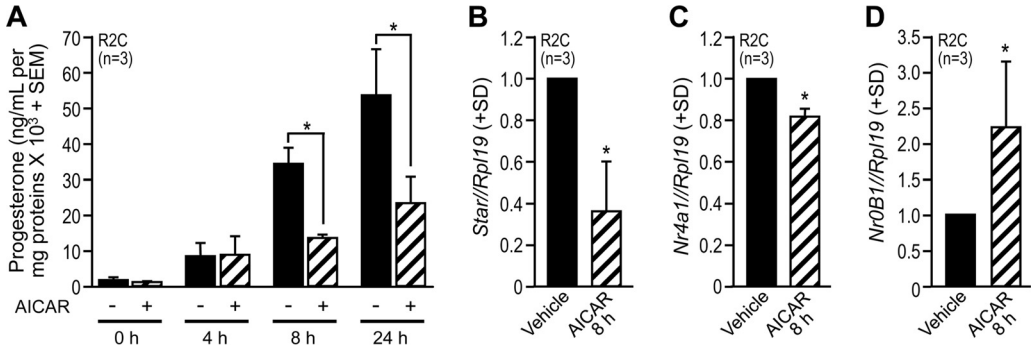

FIG 10 AMPK activation decreases steroid production in the constitutively steroidogenic R2C Leydig cells. (A) Progesterone secreted by the constitutively steroidogenic R2C Leydig cells treated for 0, 4, 8, or 24 h with vehicle (−) or AICAR (+) was quantified by ELISA. (B to D) *Star* (B), *Nr4a1* (C), and *Nr0b1* (D) mRNA levels were determined by quantitative RT-PCR using total RNA isolated from R2C Leydig cells treated for 8 h with vehicle or AICAR. Values were normalized to *Rpl19* levels, and results are shown as fold induction over vehicle (mean plus SD).

Nr4a1 promoter (this work) and by the AMPK-related kinase SIK1 on the *Cyp11a1* promoter in adrenal steroidogenic cells (60).

While AMPK activation did not affect SF1 protein levels, its involvement in AMPK-mediated silencing of *Star* expression cannot be excluded. SF1 is known to be phosphorylated at S203 by MAPK kinase, which leads to an increase in its transactivation potential (61). Thus, activated AMPK could decrease SF1 activity on the *Star* promoter by interfering with its phosphorylation status.

The implication of NR4A1 is further supported by a recent study showing that in hepatocytes NR4A1 prevents AMPKα activation by sequestering its upstream kinase LKB1 in the nucleus (62). Our current results are in agreement with this since we found that the Fsk-dependent activation of AMPKα is maximal at ~4 to 8 h poststimulation which correlates with a decrease in nuclear NR4A1 levels (10), a decrease that would allow LKB1 translocation to the cytoplasm where it can phosphorylate AMPKα, leading to inhibition of steroidogenesis. This would suggest that NR4A1 stimulates steroid synthesis by reducing AMPKα activation in addition to directly activating steroidogenic gene expression.

The second mechanism by which AMPK represses *Star* expression and steroidogenesis is by increasing the expression of repressors of steroidogenesis such as c-Fos and NR0B1. c-Fos is known to inhibit steroidogenesis by repressing *Star* expression (36) and NR5A1 (SF1) transactivation properties (63). c-Fos was also found to be underexpressed in patients with polycystic ovarian syndrome (PCOS), a condition where patients produce excess androgens (64). NR0B1 is an atypical nuclear receptor known to repress steroidogenesis and *Star* transcription (54). In hormone-responsive cells, its expression is decreased in response to stimulation, thus allowing for an increase in *Star* expression and steroid production (54). R2C cells are unique in that they produce large amounts of steroids without any stimulation, and their cAMP levels are similar to those of unstimulated MA-10 Leydig cells (52). The levels of NR0B1 mRNA and protein are low in R2C cells, which explains, at least in part, the high steroidogenic output of these cells (54). In R2C cells, we found that activation of AMPK significantly increases *Nr0b1* expression, leading to repression of steroidogenesis. Our data further strengthen the role of NR0B1 as a repressor of steroid hormone biosynthesis and define both AMPK and NR0B1 as promising targets for the development of novel therapeutic approaches for the treatment of hormone-dependent pathologies.

While the first two mechanisms to explain how activated AMPK represses steroidogenesis focused on *Star* gene expression, a third possible mechanism could involve modulation of STAR protein activity. Phosphorylation of STAR at residue S194 has been shown to be required for its maximal steroidogenic activity (65, 66). Thus, it is possible that AMPK activation impairs steroidogenesis by reducing STAR protein activity via modulation of its phosphorylation status.

Implications for human health. In addition to their role in male fertility, androgens are also essential for male sexual health and libido (67). It is not uncommon for males treated with antidiabetic drugs (metformin or pioglitazone) to exhibit decreased libido and fertility, as these drugs suppress testosterone levels in adults (68, 69). In contrast, the use of metformin has been shown to improve the risk of ovarian cancer and ameliorate pregnancy rates in women with polycystic ovarian syndrome (70, 71), a condition characterized by chronic ovulatory dysfunction and hyperandrogenism (49). Interestingly, androgen levels in women suffering from PCOS or breast cancer treated with metformin are decreased (72, 73), suggesting that metformin improves the health of these patients by repressing steroidogenesis via AMPK activation. Consistent with this, we found that metformin also represses Fsk-induced steroid production and STAR induction in our Leydig and adrenal cell line model. Furthermore, our results with the constitutively steroidogenic R2C Leydig cells indicate that pharmacological AMPK activation could also be an effective way to inhibit steroidogenesis and thus slow hormone-dependent tumor growth, which is consistent with the tumor suppressor role of activated AMPK (74).

In conclusion, our identification of a novel active repression mechanism involving AMPK within all steroidogenic cells complements the other mechanisms known to passively reduce steroid hormone synthesis such as the systemic negative-feedback loop that inhibits LH secretion, the attenuation of LH signaling by LHR endocytosis, and the reduction in *Lhr* expression (75, 76). Our data strongly support the role of AMPK as a rheostat that modulates steroidogenesis when the energy balance is altered and provide novel insights into the mechanisms by which steroidogenesis is decreased in patients treated with antidiabetic drugs that activate AMPK.

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We declare that we have no conflicts of interest.

H.S.A. and J.J.T. designed the experiments, H.S.A. performed all experiments with the MA-10 Leydig cells, MLTC-1 Leydig cells, and Y-1 adrenal cells. F.B. performed the experiments with the R2C Leydig cells and the experiments in MA-10 Leydig cells involving metformin and *Pde8a* siRNA. H.S.A. and J.J.T. analyzed the microarray data and wrote the manuscript.

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