Supporting materials

Materials and Methods

Chemicals

17β-Estradiol (E2), ICI 182,780 (ICI; an ER antagonist), tamoxifen (an ER antagonist), letrozole (an aromatase inhibitor), dehydroepiandrosterone (DHEA), nitroblue tetrazolium salt (NBT), β-NAD, DMSO, Annexin V-FITC and propidium iodide (PI) were purchased from Sigma-Aldrich (Sigma, US). 4,4,4′-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) triphenol (PPT; an ERα selective agonist) and 2,3-bis(4-hydroxyphenyl) propionitrile (DPN; an ERβ selective agonist) were purchased from Tocris Biosciences (Ellisville, US). Soluble mouse Gas6 was purchased from R&D Systems (R&D, US).

Cell lines and flow cytometry assay of PS exposure

The Leydig cell line, mLTC-1 (CRL-2065, ATCC, US), was cultured in RPMI medium (Sigma, US) supplemented with 0.1 mg/ml gentamycin, 4.5% charcoaled-treated heat-inactivated FBS (Bioclear, Wilts, UK) and 9% heat-inactivated horse serum (HS) (Life Technologies, Paisley, Scotland) at 37 °C under 5% CO_2. The treatment design was as follows: cells were treated with either DMSO (Control), 10 nM E2, 10 nM E2+1 μM ICI, 10 nM PPT or 10 nM DPN for 36 h. PS exposure after the treatment with 10 nM E2 for 36 h was assessed by flow cytometry using Annexin V-FITC according to manufacturer's instructions. Briefly, after E2 treatment, 1×10^6 cells/ml were collected by trypsinization, centrifuged at 1000×g for 5 min, washed three times with PBS, resuspended in 1× binding buffer with Annexin V-FITC and protease inhibitors, and incubated for 15 min in the dark. Annexin V-positive cells in the cell suspension were immediately detected by FACSCalibur flow cytometry (BD Bioscience, US). Data were analyzed using Cell Quest software (BD Bioscience, US). Importantly, the cell debris was gated out before analysis on the basis of forward scatter (FSC) and side scatter (SSC).

Culture and treatment of macrophage cell line

The murine macrophage cell line, Raw246.7, was purchased from the Chinese Academy of Sciences Cell Bank (CASCB, under the license of ATCC). Cells were cultured in RPMI containing 10% charcoaled and heat-inactivated FBS (HyClone, US) under 5% CO_2 at 37 °C, and the cells were subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free D-PBS. The treatment design was identical to the mLTC-1 cell treatment *in vitro* as stated above.

Quantitative real time RT-PCR analysis

Total RNA was isolated from the mouse testes, mLTC-1 cells and RAW246.7 macrophages using the TRIzol® reagent (Invitrogen, US). For quantitative real time RT-PCR (qPCR) analysis, 1 μg of total RNA was incubated with 10 U of avian myeloblastosis virus reverse transcriptase (Promega, US), dNTP mix and oligo-dT primers at 37°C for 1 h. qPCR was performed on an ABI 7300 (Applied Biosystems, US) using SYBR Green Supermix (Applied Biosystems, US) according to the manufacturer's protocol. The expression levels of genes were normalized against L19 ribosomal RNA. The primer sets for qPCR are described S-Table 2.

Immunohistochemistry and Western blot analysis

Deparaffinization, rehydration and antigen retrieval were performed as described previously (1). The testis sections (thickness of 5 μm) were treated with 3% H_2O_2 in PBS (pH 7.6) for 10 min and blocked with normal goat serum (1:200 dilution) for 10 min. The sections were then incubated at 37°C for 1-2 h with one of the following goat polyclonal antibodies used at 1:200 and purchased from Santa Cruz Biotechnology: PCNA (sc-7907), 3βHSD (sc-30820). The primary anibodies were subsequently detected by incubation with biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG followed by avidin-biotin-peroxidase (Vector Laboratories, US). Specific binding was visualized using 3',3'-diaminobenzidine tetrahydrochloride. Sections were slightly counterstained with Mayer's hematoxylin.

Western blot analysis was performed as described previously (1). The total

protein of cells or tissues was extracted using RIPA buffer. Aliquots of protein were electrophoresed in 10% SDS-PAGE and transferred to PVDF membrane. Antibodies and dilutions are presented in S-table 4. After washing with TBS-T buffer, the membrane was incubated with HRP-conjugated secondary antibodies (goat anti-mouse IgG, goat anti-rabbit IgG, and rabbit anti-goat IgG; all at 1:5000; Santa Cruz, US). Exposure was obtained using ECL (GE Healthcare, US).

Construction of GAS6 luciferase reporter plasmid and luciferase assays

Gas6-LUC fusion genes were constructed in the pGL3-Basic vector (Promega, US) according to a previous report (2). Briefly, the Gas6 promoter region was amplified from mouse genomic DNA with the following primers: Fprimer, 5'-AGCTAGGGTACCTTCTCCAGAGAGGAGTATCC-3'; and Rprimer, 5'-CTAGCTAGATCTAGAGCGAAGGGGCCATGGCG-3'. The PCR product was inserted into pGL3-Basic KpnI/BglII sites to generate Gas6 promoter-LUC.

mLTC-1 cells were plated in RPMI containing 10% FCS in six-well plates and were cultured for 24 h before transfection. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, US) with the pCMV-β β-gal vector (Invitrogen, US) as an internal control for transfection efficiency. Cell extracts were prepared 36 h after transfection and were assayed for LUC and β-gal activities.

3D live cell imaging

Leydig cells isolated from AROM+ and WT testes were cultured in RPMI complete medium at 37 °C with 5% CO_2. Peritoneal macrophages were grown in RPMI complete medium at 37°C under 5% CO_2. Leydig cells labeled with CFSE (Sigma, US) were added to macrophages at a 4:1 ratio. Video microscopy was conducted at 37°C with a DeltaVision core microscope (API, US), and images were captured every 2 min for 10 h.

Leydig cell number estimation

A known fraction of the tissue was sampled systematically at random from each testis in a stepwise sampling procedure (3) : a) Each testis was cut into 4-mm-thick slabs; b) Every 2^{nd}- 3^{rd} slab was sampled systematically randomly and cut into 4-mm-thick bars providing 6-10 bars; c) every 2^{nd}- 3^{rd} bar was sampled and cut into cubes; d) every 4^{th} to 6^{th} of those cubes were sampled. The sampled tissue was embedded in 2-hydroxy-methacrylate (Technovit 7100®) and stained with hematoxylin and eosin where the Leydig cells and Sertoli cells can be recognized. The blocks of methacrylate, each containing 8-10 cubes of testicular tissue, were cut into 40 µm-thick sections. Approximately 10 sections were sampled from each testis and the optical fractionator principle was used to estimate the total number of Sertoli and Leydig cells in a known fraction of the tissue. To avoid bias from cutting artifacts, a disector height of 15 µm was chosen. By this sampling procedure, the coefficient of error (CE=SEM/mean) at each sampling level was estimated and kept below 10%. The volume was chosen to count approximately 150 cells of each cell type in each testis. The total number of Sertoli and Leydig cells was estimated by multiplication of the counted number of cell ($\sum Q$) by the inverse of the sampled fration. For example, in one testis every 2^{nd} slab, every 3^{rd} bar, every 6^{th} cube, and every 12^{th} methacrylate section were sampled, and the Leydig cells were counted in 1/398 of tissue section. Thus, The estimated number of Leydig cells was equal to 2x3x6x12x398x the counted number of Leydig cells.

Murine Leydig cell isolation

Mice were anesthetized and sacrificed by cardiac puncture. Testes were dissected and then washed twice with Dulbecco's PBS (D-PBS), and the tunica-free testes were transferred to a Petri dish with fresh medium. Mouse Leydig cells were isolated according to a modified method (4). The tunica-free testes were dissociated and transferred immediately into 10 ml Enzymatic Solution 1 (enriched DMEM:F12, 200 µg/ml deoxyribonuclease I, 0.5 mg/ml collagenase IA, C-0130, Sigma, US). The tubules were shaken for 15 min (75 cycles/min) at 34 °C, and then then they were

layered over 40 ml 5% Percoll (Sigma-Aldrich, USA)/95% 1× Hank's balanced salt solution (HBSS; Invitrogen, USA) in a 50-ml conical tube and allowed to settle for 20 min. Leydig cells were isolated from the top 35 ml Percoll. This top 35 ml Percoll was aliquoted into two 50- ml conical tubes (17.5 ml each), and 32.5 ml 1×D-PBS was added to each tube. The tubes were centrifuged for 10 min at 500 × g. The pellet was resuspended in 55% Percoll/45% 1×Hank's balanced salt solution (HBSS; vol/vol) and centrifuged at 20,000 × g for 1.5 h at 4°C. The top 2 ml Percoll containing cellular debris was discarded, and 5- ml fractions were collected from the top of the tube. Each fraction was diluted with 35 ml 1×HBSS and centrifuged at 500× g for 10 min at 4°C. The supernatant was discarded, and cells were resuspended in the residual 1×HBSS (approximately 400 µl). The cells were centrifuged at 500× g for 10 min at 4°C, and rall residual 1×HBSS was removed. The purified Leydig cells were used for subsequent experiments.

To determine the purity of Leydig cell preparations, 5 µL cells were mixed with 50 µL 4% PFA and smeared on a glass slide. The air-dried smears were rinsed with 1×D-PBS, blocked with 10% donkey serum (Abcam, USA) in 1×D-PBS for 1h in a humidified chamber, and rinsed with 1×D-PBS for 5 min. Cells were incubated with primary antibodies diluted with 3% serum in 1×D-PBS at 4°C overnight (1:100 dilution of anti-3βHSD; Santa Cruz, USA). After rinsed with 1×D-PBS for 5 min, and cells were incubated with secondary antibodies diluted with 3% serum in 1×D-PBS for 1 h in a humidified chamber (1:400 dilution of Alexa Fluor 594 donkey anti-goat IgG; Invitrogen). After 1×D-PBS wash, the slide was stained with DAPI and visualized on a Nikon Eclipse TE2000-U microscope. The Leydig cell preparations were determined to be 85% to 90% in purity.

Mouse macrophage isolation

Mice were anesthetized and sacrificed by cardiac puncture. The peritoneal cells were collected with 5 ml of ice-cold D-PBS. The cells were cultured in RPMI

containing 10% FBS at 37 °C under 5% CO_2. After 2 h, non-adherent cells were removed by washing with PBS, and a portion of adherent macrophages were first identified by F4/80 (Biolegend, US) immunostaining for purity. The remaining macrophages were used for subsequent experiments.

Gene expression microarray analysis on an Agilent platform

Total RNA was extracted from whole testes using Trizol reagent (Invitrogen) and purified with mirVana miRNA Isolation kit (Ambion, Austin, TX, USA). RNA purity and concentration were determined from OD260/280 readings using spectrophotometer (NanoDrop ND-1000) and RNA integrity was determined by 1% formaldehyde denaturing gel electrophoresis. Half of the RNA was used for the Agilent platform, and the second half of the RNA was used later for qPCR validation studies. cDNA labeled with a fluorescent dye (Cy5 and Cy3-dCTP) was produced by Eberwine's linear RNA amplification method and subsequent enzymatic reaction. The labeled RNA was purified and hybridized to microarrays according to the Agilent manufacturer's instructions. Images were scanned with the Agilent microarray scanner (Agilent), gridded and analyzed using Agilent feature extraction software version 10.10. The scanned images were ananlyzed with Feature Extraction Software 10.10 (Agilent) using default parametersto obtain background subtracted and spatially detrended processed signal intensities. Features flagged in Feature Extracton as Feature Non-uniform outliers were excluded. A total of 6 samples divided into two groups (AROM+ and WT; n=3/group) were used in the statistical test. At first, we created a list of the 200 most upregulated or downregulated candidate genes on the basis of fold changes compared to WT, and we selected genes over the threshold of 2.0-fold. The top 200 candidate genes were classified by the IPA bioinformatics tool (Ingenuity Systems) using the criteria for genes involved in the control of cell growth, cell cycle, cell death, tumorigenesis, and the E2/ER pathway. Over 30 genes were analyzed in the validation study, and 3 housekeeping genes were used as internal controls. The results were represented as normalized relative quantification (NRQ), and 8 major genes of interest were further evaluated.

Measurements of [Ca^{2+}] by F-4500

The membrane-permeable Ca^{2+} indicator, Fura-2/AM (Beyotime, China), was used to monitor the intracellular calcium [Ca^{2+}] by measuring the emitted fluorescence. Briefly, mLTC-1 cells at 10^6 cells/ml (untreated or treated with E2) were loaded with 4 μm Fura-2/AM for 50 min at 37°C, and cells were resuspended at the starting concentration in HANK'S buffer after two washes. Fluorescence was measured using a double emission microfluorescence system (F-4500) by exciting alternately at 340 and 380 nm and detecting emission at 510 nm. The [Ca^{2+}]i was calculated using the following equation: [Ca^{2+}]i=Kd ［(R-Rmin)/(Rmax-R)］(Fmin/Fmax); where Kd is the dissociation constant of the Ca^{2+}•fura-2 complex (224 nm); R represents the fluorescence at F340/F380; Rmax represents the maximum fluorescence of F340/F380; Rmin represents the minimum fluorescence of F340/F380; Fmax represents the maximum fluorescence (obtained by treating cells with 2.5% Triton X-100); and Fmin represents the minimum fluorescence (obtained for ionophore-treated cells in the presence of 1 mM EDTA). Each experiment was repeated three times.

Measurement of serum E2 and Gas6 levels

Serum E2 concentration was measured by a commercial radioimmunoassay kit (Immunotech; Beckman Coulter, Marseille, France) as described previously (5).

A human Growth Arrest-Specific 6 (Gas6) ELISA kit (DGAS60, R&D, US) was used to measured the human serum Gas6 concentration according to the manufacturers' procedure. Briefly, the calibration curves were made according to the four-parameter logistic equation. The absorbance was immediately read at 450 nm with a reference wavelength at 570 nm, and values shown in the dot represent the mean of two replicates. The ELISA plate reader was obtained from TECAN Inc. (Durham, NC, US).

Detection of mitochondrial changes

For detection of mitochondrial membrane potential (MMP) changes, a mitochondrial staining kit was purchased (cat No. CS0390, Sigma, US). Briefly, mLTC-1 cells were suspended (0.6-0.8×10^6 cells/ml) in RPMI medium with 1 ml of staining solution and incubated for 20 min at 37 °C under 5% CO_2. The cells were then collected and washed with 5 ml of ice-cold 1×JC-1 staining buffer, and the fluorescence intensity was determined by a fluorescence spectrophotometer (F-4500, Hitachi, Japan). The red fluorescence of aggregated JC-1 represents intact mitochondria, and the green fluorescence of monomeric JC-1 represents disrupted mitochondria. The ratio of red to green fluorescence intensity reflects the level of changes in MMP. Each experiment was repeated three independent times.

Immunflourecence staining

Frozen testes sections were fixed with acetone at 4°C for 15 min and subsequently permeabilized with 0.3% Triton X-100 in PBS for 15 min. After blocking with 10% normal donkey serum in PBS at 37°C for 1 h, the sections were incubated with the double primary antibodies at 4°C overnight. The cells were washed three times with PBS and then incubated with the appropriated double secondary antibodies for 30 min at 37°C. The sections were washed three times with PBS and counterstained for nuclei using 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruze, US) following the manufacturer's protocol. The antibodies were listed in the supporting material (S-Table 3)

Immunocytochemistry

mLTC-1cells were fixed with 4% paraformaldehyde and quenched with 50Mm ammonium chloride. Following permeabilization with 0.1% (v/v) Triton X-100 and blocking 10% rabbit serum, cells were incubated with primary antibody for 1 h followed by FITC-conjugated secondary antibody (Zhongshan Co., Beijing, China) for 40 to 60 min at room temperature. The secondary antibody was used at a dilution

of 1: 50. DAPI staining was used for detection of nuclei.

mLTC-1 cells on coverslips were washed with PBS for 3 times with PBS and fixed with 4% paraformaldehyde for 30 min. The slides were dried in RT for 5 min and washed 3 times in PBS. After permeabilization with 0.1% (v/v) Triton X-100, cells were incubated with 3% H_2O_2 and blocked with 10% rabbit serum. Cells were incubated with the anti-Gas6 primary antibody (Santa Cruz, US) at 4°C overnight, followed by biotinylated rabbit anti-goat IgG (Zhongshan Co., Beijing, China) and avidin-biotin-peroxidase (Vector Laboratories, US) for 30min at 37°C. Specific binding was visualized using 3',3'-diaminobenzidine tetrahydrochloride. Sections were slightly counterstained with Mayer's hematoxylin (Zhongshan Co., Beijing, China).

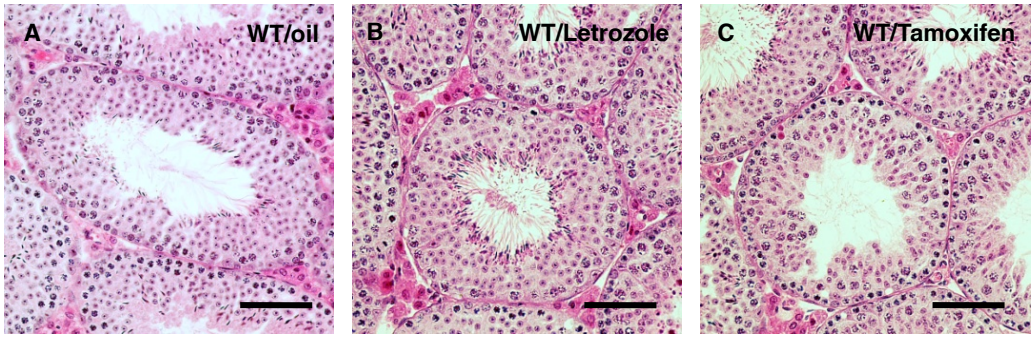
Macrophge and Leydig cell co-culture and staining

Isolated murine macrophages were co-cultured with mLTC-1 cells with a ratio of 1:4. The cocultured cells were treated with DMSO (Cont), E2, E2+ICI, PPT or DPN for 34h. The cells were fixed in 4% PFA for 20 min, washed in D-PBS twice, and then were stained with Hematoxylin for 10 min and Eosin for 2 min.

S-Figure legends

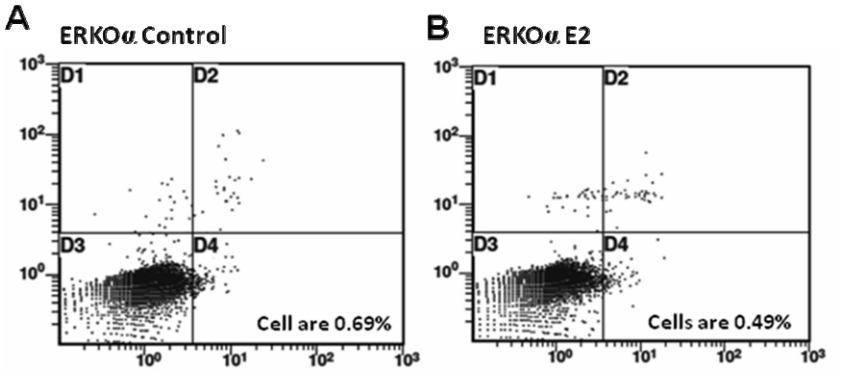
S-Fig. 1 Tamoxifen and Letrozole have no affect on WT testes phenotype. No pathological abnormalities were observed.

Testicular histopathology from WT mice treated with other (A) oil (control), (B) Tamoxifen (0.4 mg/kg/d) and (C) Letrozole (2.0 mg/kg/d). Bar, 50 μm.



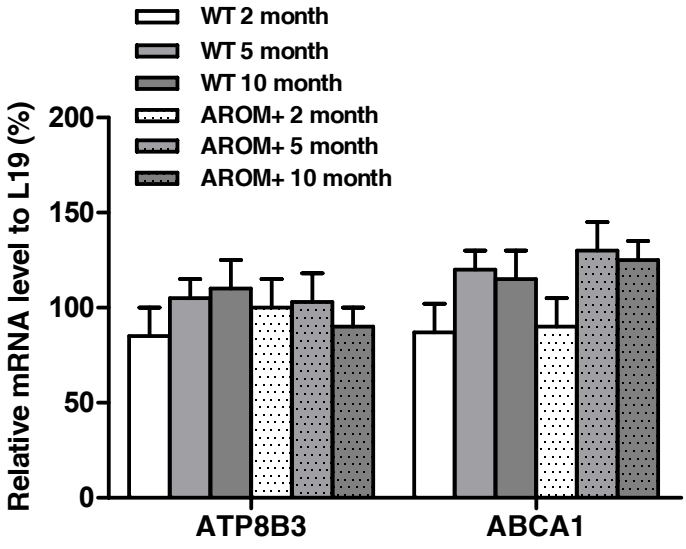
S-Fig. 2 E2/ERα has no effects on PS-extrusion in ERαKO Leydig cells

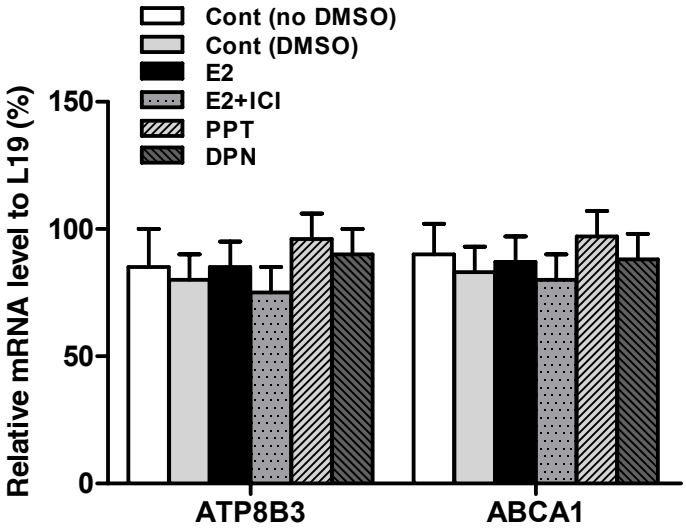
(**A-B**) PS-exposure on Leydig cell surface was measured by Annexin V-FITC staining (x-axis), and apoptosis by PI staining (y-axis), without (A) or with (B) E2 treatment



S-Fig. 3 Expression of *ATP8B3*, *ABCA1* in AROM+ mice testes and E2-treated mLTC-1 cells

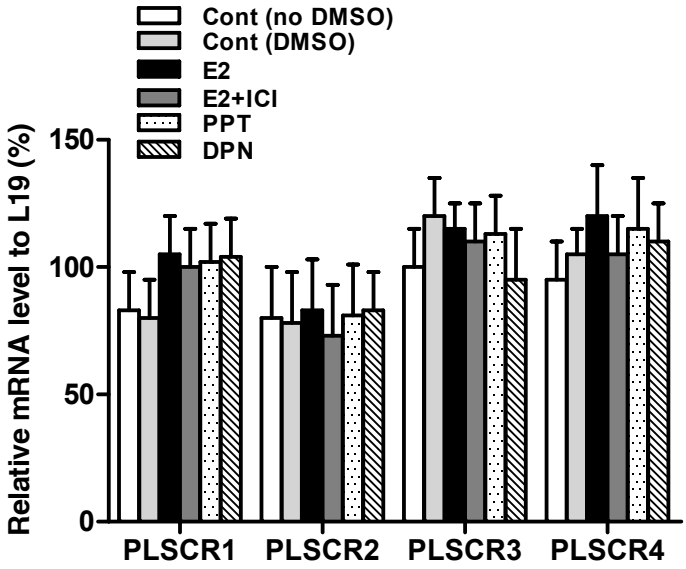
(A-B) Testicular mRNA levels of *ATP8B3 and ABCA1* from AROM+ mice at 2 mo, 4 mo, and 9 mo-old, normalized to L19. (C-D) mRNA levels of *ATP8B3 and ABCA1* from mLTC-1 cells nontreated (no DMSO) and treated with DMSO (Cont), or with E2, E2+ICI, PPT, or DPN for 34 h, normalized to L19.





S-Fig. 4 Expression of *PLSCRs* in treated ERαKO Leydig cells

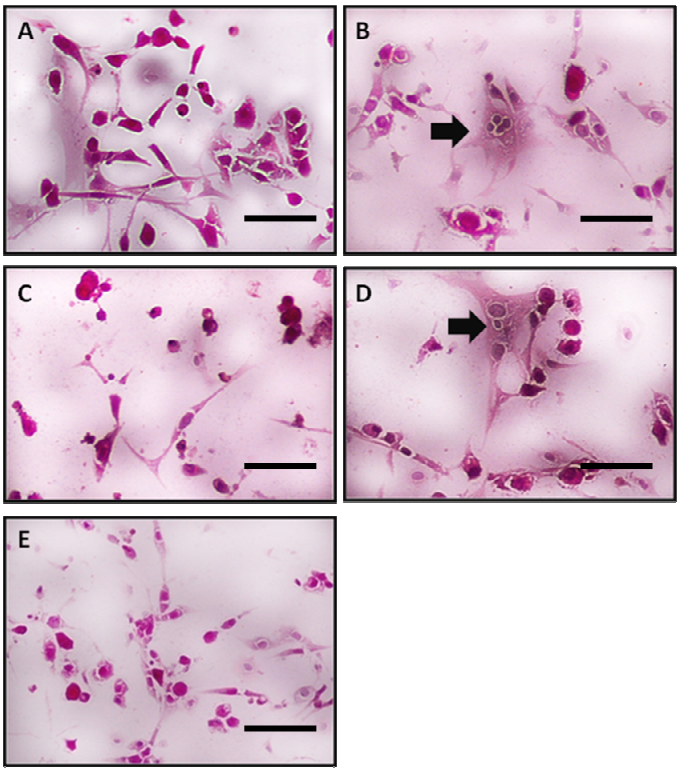
(**A**) No changes in mRNA levels of PLSCR1 (**B**), PLSCR2 (**C**), PLSCR3 (**D**) and PLSCR4 (**E**) in ERαKO Leydig cells nontreated (no DMSO) and treated with DMSO (Cont), or with E2, E2+ICI, PPT, or DPN treatment and normalized to L19.



S-Fig. 5 Phagocytosis of Leydig cells by macrophages is induced by E2/ERα

signaling *in vitro*

Co-culture macrophages and viable Leydig cells from WT testes either without E2 (**A**) or treated with E2 (**B**), E2+ICI (**C**), PPT (**D**), or DPN (**E**). Several cells were observed inside the digitating macrophage (arrow) upon E2 or PPT preincubation. Bar, 50 µm.



Video legends

Unlabeled, irregular-shaped macrophages were mixed with Leydig cells labeled with the green dye, CFSE, and images were collected every 2 min for 8 h. The resulting video is displayed at 15 frames/s. Viable Leydig cells were identifiable by the rapid movement of filopodial protrusions on their surface.

S-Video 1. Wild-type (WT) macrophages and living Leydig cells isolated from WT mice testes were co-cultured without E2 preincubation

S-Video 2. Wild-type (WT) macrophages and living Leydig cells isolated from AROM+ mice testes were co-cultured with E2 preincubation

S-Video 3. Wild-type (WT) macrophages and living Leydig cells isolated from WT mice testes were co-cultured with E2 preincubation.

S-Video 4. Wild-type (WT) macrophages and living Leydig cells isolated from WT mice testes were co-cultured with Gas6 antibody preincubation

S-Video 5. Wild-type (WT) macrophages and living Leydig cells isolated from AROM+ mice testes were co-cultured with Annexin V preincubation

S-Video 6. Wild-type (WT) macrophages and living Leydig cells isolated from WT mice testes were co-cultured with Axl antibody preincubation.

S-Table 1 List of up-regulated and down-regulated genes from Microarray analysis of AROM+ testes

	Gene symbol	Description	Fold Change
NM_053110.2	Gpnmb	glycoprotein (transmembrane) nmb (Gpnmb)	27.0
NM_010705.1	Lgals3	lectin, galactose binding, soluble 3 (Lgals3)	16.2
NM_017372.2	Lyzs	lysozyme (Lyzs)	7.5
NM_007409.2	Adh1	alcohol dehydrogenase 1 (class I) (Adh1)	5.7
NM_019521.1	Gas6	growth arrest specific 6 (Gas6)	5.6
NM_008605.1	Mmp12	matrix metalloproteinase 12 (Mmp12)	4.8
XM_358915.1	LOC385699	similar to Ab2-162 (LOC385699)	4.8
NM_008597.2	Mglap	matrix gamma-carboxyglutamate (gla) protein (Mglap)	4.8
NM_019564.1	Prss11	protease, serine, 11 (Igf binding) (Prss11)	4.7
NM_009853	CD69	CD69 antigen (Cd69)	4.7
NM_007798.1	Ctsb	cathepsin B (Ctsb)	4.3
NM_019394	Mia1		4.2
NM_010686.2	Laptm5	lysosomal-associated protein transmembrane 5 (Laptm5)	4.2
NM_144551.3	Trib2		4.1
XM_356873.1	LOC383099	similar to Ab2-162 (LOC383099)	4.1
NM_144551.3	Trib2		4.0
NM_009778.1	C3	complement component 3 (C3)	3.2
NM_010240.1	Ftl1	Gene Bank	3.2
XM_135820.3	Pfc	properdin factor, complement (Pfc)	3.2
XM_149178.1	Ptpns1	protein tyrosine phosphatase,	3.2

		non-receptor type substrate 1 (Ptpns1)	
XM_129176	Mpeg1	macrophage expressed gene 1 (Mpeg1)	2.8
NM_009465.2	Axl	Mus musculus AXL receptor tyrosine kinase (Axl)	2.0
NM_013564.3	Insl3*	insulin-like 3	-13.2
NM_008491.1	Lcn2	lipocalin 2	-12.1
NM_010642.1	Klk21	kallikrein 21	-8.5
NM_020268.1	Klk27	kallikrein 27	-7.5
NM_008963.1	Ptgds	prostaglandin D2 synthase (brain)	-7.1
NM_007469.2	Apoc1	apolipoprotein C-I	-6.1
NM_010643.1	Klk24	kallikrein 24	-4.6
NM_023455.2	Cml4	camello-like 4	-3.6
NM_053200.1	Ces3	carboxyesterase 3	-3.4
NM_007469.2	Apoc1	apolipoprotein C-I	-3.3
NM_010356.2	Gsta3	glutathione S-trasferase, alpha 3	-3.2
NM_007494.2	Ass1	argininosuccinate synthetase 1	-3.2
NM_010742.1	Ly6d	lymphocyte antigen 6 complex, locus D	-3.1
NM_010915.1	Ngfa	nerve growth factor alpha	-2.9
AK016465	Vcam1	vascular cell adhesion molecule 1	-2.8
NM_016668.2	Bhmt	betaine-homocysteine methyltransferase	-2.6
NM_010174.1	Fabp3	fatty acid binding protein 3	-2.5
NM_008291.1	Hsd17b3	hydroxysteroid (17-beta) dehydrogenase 3	-2.5
XM-130987.3	Rarres1		-2.5
NM_153782.1	BC029169	cDNA sequence BC029169	-2.5
NM_007809.2	Cyp17a1*	cytochrome P450, family 17, subfamily a, polypeptide 1	-2.3
NM_025743.1	4933400A11Rik	RIKEN cDNA 4933400A11 gene	-2.2
NM_008272	Hoxc9	homeo box C9	-2.1
NM_009127.2	Scd1	stearoyl-Coenzyme A desaturase	-2.1
NM_028069.1	Mucdhl	mucin and cadherin like	-2.1
XM_130140.2	4933411C14Rik	RIKEN cDNA 4933411C14 gene	-2.1
AK006263	Siat10	sialyltransferase10 (alpha-2,3-sialyltransferase VI)	-2.0
NM_031192	Ren1*	renin 1, structural	-2.0

S-Table 2 The sequences of oligonucleotide primers and amplified product of real-time PCR

Gene	Sequence of primer (5'- 3')	Amplified Product (bp)
3β-hsd	F: 5'- AGCTCTGGACAAAGTATTCCGA -3' R: 5'- GCCTCCAATAGGTTCTGGGT -3'	234

L19	F: 5'-CCAATGCCAACTCCCGTCA -3'	246
	R: 5'-TCTTCTTGGATTCCCGGTATCT -3'	
Cyp17a1	F: 5'- GTCGCCTTTGCGGATAGTAGT-3'	120
	R: 5'- TGAGTTGGCTTCCTGACATATCA-3'	
Cyp11a1	F: 5'- AGGTCCTTCAATGAGATCCCTT-3'	137
	R: 5'- TCCCTGTAAATGGGGCCATAC-3'	
Star	F: 5'- CGGGTGGATGGGTCAAGTTC -3'	188
	R: 5'- GCACTTCGTCCCCGTTCTC -3'	
PCNA	F: 5'- TTGCACGTATATGCCGAGACC -3'	183
	R: 5'- GGTGAACAGGCTCATTCATCTCT -3'	
Axl	F: 5'- GGAACCCAGGGAATATCACAGG-3'	118
	R: 5'- AGTTCTAGGATCTGTCCATCTCG -3'	
Tyro3	F: 5'- AGATGACCGTGTCTCAGGGG-3'	177
	R: 5'- ACCGTTCCACTGACTTTAGGC-3'	
Mer	F: 5'- TGCGTTTAATCACACCATTGGA -3'	142
	R: 5'- TGCCCCGAGCAATTCCTTTC -3'	
IL-6	F: 5'- CTGCAAGAGACTTCCATCCAG -3'	131
	R: 5'- AGTGGTATAGACAGGTCTGTTGG -3'	
17β-HSD3	F: 5'- TTAGTCGGACACTGGAAAAGC -3'	224
	R: 5'- ATTCTGGCTCTCACCGGAAGT -3'	
TNFα	F: 5'- CCTGTAGCCCACGTCGTAG-3'	148
	R: 5'- GGGAGTAGACAAGGTACAACCC -3'	
CD69	F: 5'- AGGCTTGTACGAGAAGTTGGA -3'	210
	R: 5'- AGTTCACCAGAATATCGCTTCAG -3'	
CCL2	F: 5'- TAAAAACCTGGATCGGAACCAAA -3'	120
	R: 5'- GCATTAGCTTCAGATTTACGGGT-3'	
Mpeg1	F: 5'- AGACATGGGACGGGTGATG -3'	193
	R: 5'- AAGGGCGAGTTCTGTGTTGAT -3'	
ERα	F: 5'-CCTCCCGCCTTCTACAGGT-3'	128
	R: 5'-CACACGGCACAGTAGCGAG-3'	
Lgals3	F: 5'- AGACAGCTTTTCGCTTAACGA -3'	210
	R: 5'- GGGTAGGCACTAGGAGGAGC -3'	
Gas6	F: 5'- CCGCGCCTACCAAGTCTTC -3'	110
	R: 5'-CGGGGTCGTTCTCGAACAC-3'	
ABCa1	F: 5'-GCTTGTTGGCCTCAGTTAAGG-3'	135
(Floppase)	R: 5'-GTAGCTCAGGCGTACAGAGAT-3'	
ATP8b3	F: 5'-TCGGGGAGAACCTTGAGGATA-3'	197
(Flippase)	R: 5'- TCGATGGAACTGCTCGTACAG-3'	
PLSCR1	F: 5'-GGTATCCCCCTCCGTATCCAC-3'	155
	R: 5'-GCCACCACCTGCATAACCT-3'	
PLSCR2	F: 5'-ACTGCTGTACTCGAAATTGCTG-3'	226
	R: 5'-TCGTTCTGAAGAGTGAGCTTTG-3'	
PLSCR3	F: 5'-TCGTTCTGAAGAGTGAGCTTTG-3	102
	R: 5'-CGTTTCCACTCGTTCAGCCT-3'	

Gpnmb	F: 5'-AGAAATGGAGCTTTGTCTACGTC-3' R: 5'- CTTCGAGATGGGAATGTATGCC-3'	170
PLSCR4	F: 5'-AAGAGCTGGAAGTGCAATGTC-3' R: 5'-ACCCCTCACTCTCATCATACTC-3'	125

F: Forward; R: Reverse; 3βHSD : hydroxy-delta-5-steroid dehydrogenase; L19: ribosomal protein L19; Cyp17a1: Cytochrome P450 17A1; Cyp11a1: Cytochrome P450 11A1; Star: steroidogenic acute regulatory protein; PCNA: proliferating cell nuclear antigen; Axl: AXL receptor tyrosine kinase; Tyro3: TYRO3 protein tyrosine kinase 3; Mer c-mer proto-oncogene tyrosine kinase; IL-6: interleukin 6; 17β-HSD3:hydroxysteroid (17-beta) dehydrogenase 3; TNFα: tumor necrosis factor; CD69: CD69 antigen; CCL-2: chemokine (C-C motif) ligand 2; Mpeg1: macrophage expressed gene 1; ERα: estrogen receptor 1 (alpha); Lgals3: lectin, galactose binding, soluble 3; Gas6: growth arrest specific 6; ABCa1: ATP-binding cassette, sub-family A, member 1; ATP8b3: ATPase, class I, type 8B, member 3; PLSCR1: phospholipid scramblase 1; PLSCR2: phospholipid scramblase 2; PLSCR3: phospholipid scramblase 3; PLSCR4: phospholipid scramblase 4; Gpnmb: glycoprotein (transmembrane) nmb

S-Table 3 List of the antibodies and dilutions for Immunohistochemistry and Immunflourecence staining

Antibody	Dilution	Cat.nr.	Source	Company
Gas6	1:400	sc-1936	goat polyclonal IgG	Santa Cruz
Mer	1:200	sc-67281	goat polyclonal IgG	Santa Cruz
TNFα	1:200	sc-1350	goat polyclonal IgG	Santa Cruz
Tyro3	1:200	sc-1094	goat polyclonal IgG	Santa Cruz
F4/80	1:100	bs-7058R	Rabbit polyclonal IgG	Bioss Biotechnology
PCNA	1:1000	sc-7907	Rabbit polyclonal IgG	Santa Cruz
3βHSD	1：500	sc-30820	goat polyclonal IgG	Santa Cruz
Axl	1:200	sc-1096	goat polyclonal IgG	Santa Cruz
Cyp19	1:400	sc-14245	goat polyclonal IgG	Santa Cruz

S-Table 4 List of the antibodies and dilutions for western blot

Antibody	Dilution	Cat.nr.	Source	Company
L19	1:500	sc-100830	mouse monoclonal IgG	Santa Cruz
β-actin	1:1000	sc-47778	mouse monoclonal IgG	Santa Cruz
Mer	1:500	sc-67281	goat polyclonal IgG	Santa Cruz
Tyro3	1:500	sc-1094	goat polyclonal IgG	Santa Cruz

PCNA	1:1000	sc-7907	Rabbit polyclonal IgG	Santa Cruz
3βHSD	1:500	sc-30820	goat polyclonal IgG	Santa Cruz
Axl	1:500	sc-1096	goat polyclonal IgG	Santa Cruz
Cyp19	1:400	sc-14245	goat polyclonal IgG	Santa Cruz

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