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RESEARCH ARTICLE

The *faah* gene is the first direct target of estrogen in the testis: role of histone demethylase LSD1

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Abstract Estrogen (E_2) regulates spermatogenesis, yet its direct target genes have not been identified in the testis. Here, we cloned the proximal 5′ flanking region of the mouse fatty acid amide hydrolase (*faah*) gene upstream of the luciferase reporter gene, and demonstrated its promoter activity and E_2 inducibility in primary mouse Sertoli cells. Specific mutations in the E_2 response elements (ERE) of the *faah* gene showed that two proximal ERE sequences (ERE2/3) are essential for E_2-induced transcription, and chromatin immunoprecipitation experiments showed that E_2 induced estrogen receptor β binding at ERE2/3 sites in the *faah* promoter in vivo. Moreover, the histone demethylase LSD1 was found to be associated with ERE2/3 sites and to play a role in mediating E_2 induction of FAAH expression. E_2 induced epigenetic modifications at the *faah* proximal promoter compatible with transcriptional activation by remarkably decreasing methylation of both DNA at CpG site and histone H3 at lysine 9. Finally, FAAH silencing abolished E_2 protection against apoptosis induced by the FAAH substrate anandamide. Taken together, our results identify FAAH as the first direct target of E_2.

Keywords Endocannabinoids · Estrogen · FAAH · LSD1 · Testis · Spermatogenesis

Abbreviations

AEA	Anandamide
AR	Androgen receptor
BSA	Bovine serum albumin
CB_1	Type-1 cannabinoid receptor
CB_2	Type-2 cannabinoid receptor
ChIP	Chromatin immunoprecipitation
E_2	17β-estradiol
$epiE_2$	17α-estradiol
ERβ	Estrogen receptor β
ERE	Estrogen-response element
FAAH	Fatty acid amide hydrolase
FSH	Follicle-stimulating hormone
HMT	Histone methylase
HDT	Histone demethylase
LSD1	Histone lysine demethylase 1
MSP-PCR	Methylation-specific PCR
Parg	Pargyline
PBS	Phosphate-buffered saline
PFA	Paraformaldheyde
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TMX	Tamoxifen

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Introduction

Spermatogenesis is controlled by gonadotrophins and testosterone and is modulated by a complex network of endocrine and paracrine factors, among which are estrogens [1]. The biological effects of 17β-estradiol (E_2) are mediated by the estrogen receptors α (ERα) and β (ERβ), which belong to the nuclear receptor superfamily and function as ligand-induced transcriptional factors. ERs regulate gene expression in a ligand-responsive manner through a classical nuclear mechanism, by direct interaction with estrogen-response elements (EREs) in the promoter of target genes [2]. DNA-bound ERs undergo conformational changes, which lead to cofactor recruitment and gene transcription by altering chromatin structure. In the testis of adult mice, both ER types have been identified: ERα is localized in Leydig cells, whereas ERβ is expressed in Sertoli cells and most germ cells [3]. Although ERα has also been detected in rat Sertoli cells [4], no clear evidence documented its presence in the same cells of mice. Growing interest in the effects of E_2 in male reproduction is linked to the observation that ERs knockout [5] or disruption of E_2 synthesis [6] impair male fertility. Although some putative direct target genes of E_2 have been found in the testis [7–9], up to now the identification of a testicular cell-specific ER responsive promoter has not been reported.

In this investigation, we identified fatty acid amide hydrolase (FAAH) as a direct target gene of E_2 in mouse Sertoli cells. FAAH hydrolyzes the two most prominent endocannabinoids, *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol. These substances play manifold roles in both the central nervous system and in the periphery [10, 11], reproductive organs included [12], mainly by activating G-protein coupled type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors [13]. In the seminiferous tubules of the testis, both germ cells and somatic Sertoli cells possess a complete biochemical machinery to synthesize, transport, degrade and bind AEA and 2-arachidonoylglycerol [14–16]. Both endocannabinoids affect male reproductive functions: 2-arachidonoylglycerol has a pivotal role in spermatogenesis, by promoting the meiotic progression of germ cells [16]; AEA controls sperm functionality by reducing motility, capacitation, and acrosome reaction [17, 18], and it also reduces the spermatogenic output by inducing apoptosis of Sertoli cells [15]. AEA signaling depends on its endogenous content, that in vivo is strongly controlled by FAAH [19]. Remarkably, recent studies have shown that among the different components of the "endocannabinoid system" (i.e., AEA-binding receptors and metabolic enzymes) in Sertoli cells, only FAAH is a target of follicle-stimulating hormone (FSH) [14, 15]. FSH regulates FAAH expression and activity by triggering both protein kinase A- and aromatase-dependent transduction pathways [15]. Aromatase activation leads to the conversion of androgens to estrogens with the production of E_2, which stimulates FAAH expression at the transcriptional and translational level by unknown molecular mechanisms [15].

In this study, we have characterized the regulation of *faah* gene expression by E_2. We demonstrate that E_2 engages ERβ, which binds to ERE sites in the *faah* proximal promoter, and that the histone demethylase LSD1 is recruited at this site. We also show that E_2 induces demethylation of both DNA at CpG site and histone H3 at lysine 9 (H3K9) in the *faah* proximal promoter, so that E_2-stimulated FAAH expression protects Sertoli cells against apoptosis induced by AEA.

Materials and methods

Chemicals

Chemicals were of the purest analytical grade. 17β-estradiol (E_2), 17α-estradiol (epiE_2), tamoxifen (TMX), Fulvestrant (ICI 182780), pargyline (Parg) hydrochloride and anandamide (*N*-arachidonoylethanolamine, AEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *S*-Adenosyl-*L*-[methyl-^{3}H]methionine (63 Ci/mmol) was from Amersham Biosciences (Buckinghamshire, UK).

Rabbit anti-LSD1 and anti-WT1 antibodies were from Abcam (Cambridge, UK), rabbit anti-ERβ and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit anti-H3K9me3 antibody was from Millipore (Billerica, MA, USA).

Plasmids

The murine *faah* promoter nucleotide spanning from −723 to +10 was amplified by polymerase chain reaction (PCR) using the following primers: forward 5′-GTCCTG AAGTTACCTCACTAG-3′ and reverse 5′-TGCAGGAGA TCATGGTGCTGA-3′. PCR products were cloned into *Xho*I/*Hin*dIII sites of pGL3 basic vector (Promega Corp., Madison, WI, USA). The −560/+10 deletion mutant was obtained by PCR, using specific primers (forward AGACG TAGGTAGATAGCCCAGAGT, and reverse TGCAGG AGATCATGGTGCTGA); the −250/+10 deletion mutant was obtained after digestion with *Sac*I. All the other site-specific mutants were obtained by PCR amplification, using primers mutated in the ERE sequences, as follows: ERE1: 5′-TAGGGTGTGCATTACACTAA-3′; ERE2: 5′-ACCCGACCTATTCACCTCAGCCT-3′; ERE3: 5′-GC CGAGTTAAGGTAAATGGCCGC-3′. Sequence analysis was performed for each construct by Cycle Sequencing (BMR, University of Padova, Italy).

Experimental animals

Random-bread Swiss CD1 mice were reared in our facilities. All animal experimentation described in this investigation was conducted in accordance with accepted standards of human animal care. All experimental protocols were approved by the local committees on animal care and use, and were in keeping with accepted veterinary medical practice.

Cell culture, treatment, and determination of apoptosis

Primary Sertoli cell cultures were prepared as reported [20]. Tissue explants from 7- or 17-day-old Swiss-CD1 mice were cultured at 32 °C in serum-free minimum essential medium (MEM; Invitrogen Ltd, Paisley, UK) for 3 days, and then were treated with hypotonic solution (20 mM Tris–HCl, pH 7.5) to remove remaining germ cells.

Depending on the experimental procedures, primary Sertoli cells were treated with the indicated concentrations of E_2, related compounds, or AEA. In some experiments, pargyline (3 mM) was added to the culture medium 16 h before the treatment with any other substance in order to inhibit LSD1 activity [21].

Apoptotic cell death was quantified after 24-h treatment by ELISA assay (Roche Diagnostics, Mannheim, Germany) based on the evaluation of DNA fragmentation through an immunoassay for histone-associated DNA fragments in the cell cytoplasm. Specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates was performed by measuring the absorbance values at 405 nm.

Luciferase assay

The Sertoli cells were transiently transfected in 96-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cells were transfected with 200 ng of DNA, containing a mixture of 196 ng of *faah* promoter constructs and 4 ng of Renilla luciferase reporter gene. After 4 h, cells were washed and cultured in fresh media for 24 h. The cells were then treated for 30 min in media containing or not containing E_2 and ER antagonist. The luciferase activity was quantified using a commercially available kit (dual-luciferase reporter assay system; Promega Corp.) with a Perkin-Elmer Victor2 luminometer.

Gene silencing with small interfering RNAs (siRNAs)

Synthetic ready-to-use small (21 nucleotides) interfering RNA (siRNA), complementary to a region of *lsd1* or *faah*, and non-silencing control siRNAs were custom-synthesized by Qiagen (Tokyo, Japan).

To knock-down *lsd1* gene, Sertoli cells were transfected in 96-well plates with 5 pmol of siRNA and 0.25 µl of Lipofectamine 2000 (Invitrogen Ltd) in 50 µl of Opti-MEM, according to the manufacturer's instructions. After 4 h, cells were washed and cultured in fresh media for 24 h, and then they were transfected with the *faah* promoter construct and Renilla luciferase reporter gene, as described above.

For *faah* gene silencing, Sertoli cells were transfected with 600 pmol of siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), under the same experimental conditions used to knock-down *lsd1*. After 4 h, cells were washed and cultured in fresh media for 24 h. Then, they were cultured for a further 24 h in media containing AEA (1 µM) and β-estradiol (200 nM), and finally qRT-PCR analysis and evaluation of cell death were performed.

Western blotting and immunofluorescence

Western blotting was performed on Sertoli cell extracts, as described previously [16]. Briefly, cell lysates were separated using SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline with 5 % milk and 0.05 % Tween 20, and were probed with primary antibodies at 4 °C overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used for detection with the ECL and ECL-plus systems (GE Healthcare, Little Chalfont, UK). For immunofluorescence experiments, mouse Sertoli cells were fixed in 4 % paraformaldehyde (PFA) and washed with phosphate-buffered saline (PBS). Samples were permeabilized with 0.1 % Triton X-100 for 5 min and incubated for 1 h in 0.5 % bovine serum albumin (BSA). Samples were then washed with PBS and incubated for 2 h at room temperature with antibodies against LSD1 (1:300) and WT1 (Abcam), followed by 1 h of incubation with Alexa Fluor 488 goat anti-rabbit (1:300; Invitrogen) and Alexa Fluor 555 goat anti-mouse (1:300; Invitrogen) [8].

Images were taken from a Leica DMI 6000B deconvolution microscope using an HCX PL Fluotar 63 × 1.25 oil objective. Images were acquired at room temperature using a Leica DFC 350 FX camera and the Leica Las AF application suite v1.8.0 software. Images were exported as RGB 8-bit TIFF files, and Photoshop and Illustrator programs (Adobe, San Jose, CA) were used for composing the panels.

Chromatin immunoprecipitation

Proteins from Sertoli cells cultured for 60 min in the presence or absence of E_2, were cross-linked to DNA by

direct addition to the culture medium of formaldehyde at a 1 % final concentration for 15 min at 37 °C. Cells were lysed to isolate nuclei in a hypotonic buffer containing 5 mM PIPES (pH 8.0), 85 mM KCl, and 0.5 % NP40. Nuclei were then resuspended, lysed in a buffer containing 1 % SDS, 10 mM EDTA, and 50 mM Tris–HCl (pH 8.0), and sonicated to obtain fragmented chromatin samples, which were immunoprecipitated overnight with 2–4 µl of anti-ERβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LSD1 (Abcam, Cambridge, MA, USA) or anti-H3K9me3 (Millipore, Billerica, MA) antibodies, or of rabbit IgGs as a control (Sigma-Aldrich, St. Louis, MO, USA). The protein-DNA complexes were incubated at 65 °C overnight to reverse protein-DNA crosslinks and precipitated DNA was analyzed by quantitative real-time PCR using 1–5 µl aliquots out of 30 µl of DNA. The primers used were the following: for ERE 2/3, forward (5′-TGATGCTGAGACGTTGATAAGG-3′) and reverse (5′-TCGTCTGTTCCGCCTGCCTTAAAA-3′); for distal sequence (−1,630/−1,430), forward (5′-CCATGGCCTG CTTTCTAAGACTCT-3′) and reverse (5′-TCCACACCA GACTTAATCAGCC-3′). PCR amplification was performed using Power SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) in a PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA).

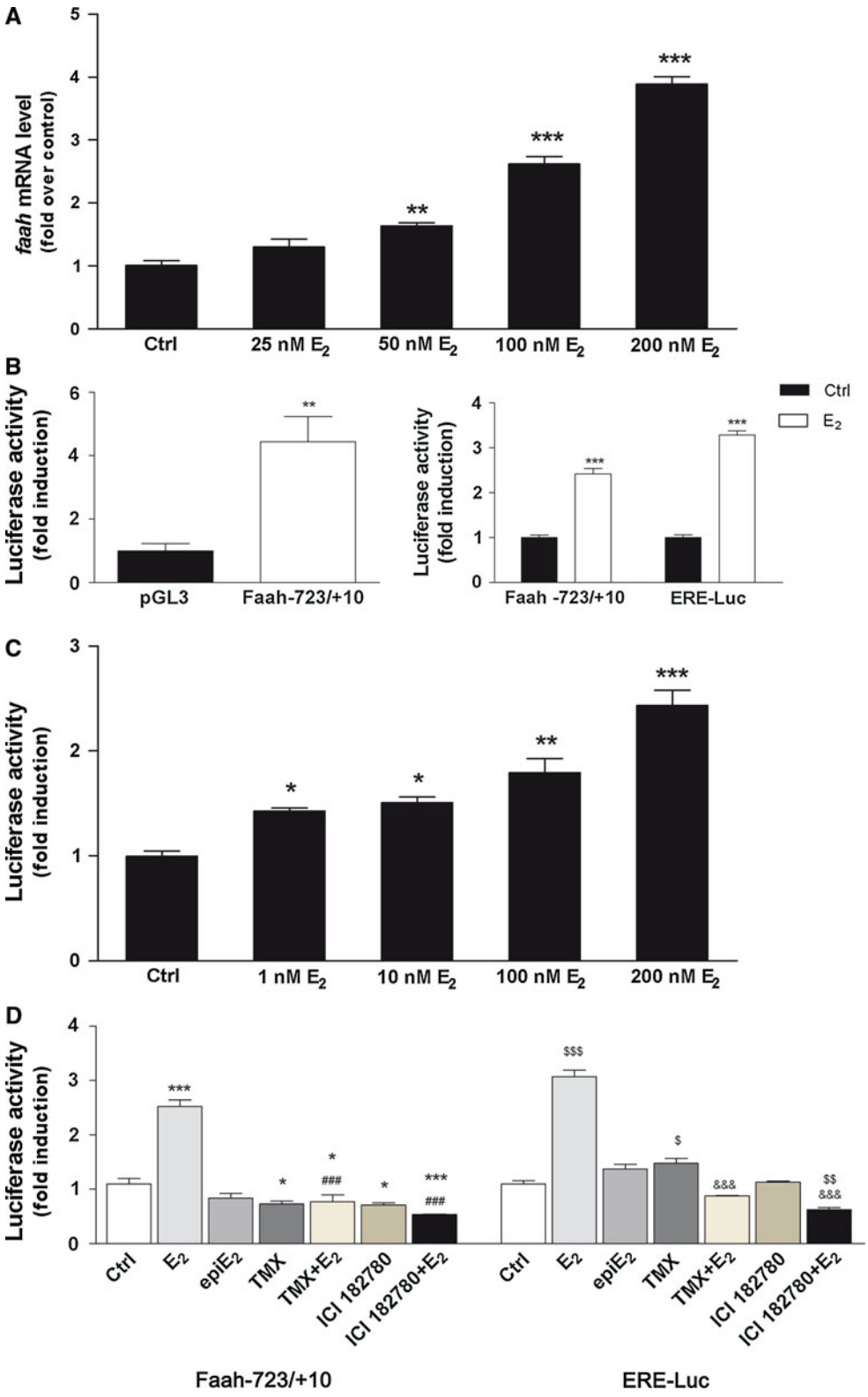
Quantitative real-time PCR

RNA was extracted from Sertoli cells using the RNeasy extraction kit (Qiagen, Crawley, UK). Quantitative real-time (qRT) PCR reactions were performed using the SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen Ltd). Briefly, 1 µg of total RNA was used to produce cDNA with 10 U/µl SuperScript III reverse transcriptase, in the presence of 2 U/µl RNaseOUT, 1.25 µM oligo$(dT)_{20}$, 1.25 ng/µl random hexamers, 5 mM $MgCl_2$, 0.5 mM dNTP mix, and diethyl pyrocarbonate-treated water. The reaction was performed using the following RT-PCR program: 25 °C for 10 min, 42 °C for 50 min, 85 °C for 5 min, then after addition of 0.1 U/µl of *Escherichia coli* RNase H, the product was incubated at 37 °C for 20 min. The target transcripts were amplified by means of an ABI PRISM 7700 sequence detector system (Applied Biosystems, Carlsbad, CA, USA), using the following primers (Invitrogen): mouse *faah* F (5′-AGATTGAGATG TATCGCCAG-3′), and R (5′-CTTCAGAATGTTGTCCC AC-3′); mouse LSD1 F (5′-TGGGCCCGGGGCTCCTA TTC-3′), and R (5′-GGGATTGGCTGTGGGGCACC-3′); mouse β-actin F (5′-TGTTACCAACTGGGACGA-3′), and R (5′-GTCTCAAACATGATCTGGGTC-3′). β-Actin was used as housekeeping gene for quantity normalization. One microliter of the first strand of cDNA product was used (in triplicate) for amplification in 25 µl of reaction solution containing 12.5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 10 pmol of each primer. The following PCR program was used: 95 °C for 10 min; 40 amplification cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s.

Fig. 1 Estrogen stimulation of *faah* expression. **a** *faah* mRNA expression was significantly stimulated by E_2. Real-time PCR analysis of *faah* expression in Sertoli cells from 17-day-old mice after treatment with different concentrations of E_2 for 4 h as indicated. ***Denotes $p < 0.001$ versus Ctrl; **denotes $p < 0.01$ versus Ctrl. **b** *Left* histogram of luciferase activity in Sertoli cells transiently transfected with pGL3-FAAH or with the promoterless pGL3 plasmid. Luciferase activity is expressed as fold increase over the control vector. *Right* histogram of luciferase activity in Sertoli cells transiently transfected with pGL3-$FAAH_{-723/+10}$ or with reporter construct containing three copies of consensus ERE (ERE-Luc), in the presence or not of 200 nM E_2 for 30 min. Luciferase activity is expressed as fold increase over the untreated control cells. ***Denotes $p < 0.001$ versus Ctrl; **denotes $p < 0.01$ versus pGL3. **c** Luciferase activity in Sertoli cells transiently transfected with pGL3-$FAAH_{-723/+10}$ after treatment with different concentrations of E_2 for 30 min. *denotes $p < 0.05$ versus Ctrl; **denotes $p < 0.01$ versus Ctrl; ***denotes $p < 0.001$ versus Ctrl. **d** Effects of $epiE_2$, TMX and ICI 182780 (all used at 200 nM) on E_2-dependent transcriptional activation in Sertoli cells. These cells were transfected with $Faah_{-723/+10}$ or with ERE-Luc and, after 18 h, they were treated with $epiE_2$, TMX or ICI 182780 in the presence or absence of 200 nM E_2. For $FAAH_{-723/+10}$: ***denotes $p < 0.001$ versus Ctrl; *denotes $p < 0.05$ versus Ctrl; $^{\#\#\#}$denotes $p < 0.001$ versus E_2; for ERE-Luc: $^{\$\$\$}$denotes $p < 0.001$ versus Ctrl; $^{\$\$}$denotes $p < 0.01$ versus ctrl; $^{\$}$denotes $p < 0.05$ versus Ctrl; $^{\&\&\&}$denotes $p < 0.001$ versus E_2

Bisulfite DNA modification and methylation-specific PCR

Genomic DNA was isolated from Sertoli cells using the DNeasy kit (Qiagen, Crawley, UK). After DNA extraction, 2 µg of DNA was treated with bisulfite, using the MethylDetector Bisulfite modification Kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's protocol. PCR analysis was performed as previously described [22]. Two microliters of bisulfite-modified DNA was amplified by using PCR master mix (Promega Corporation, Madison, WI, USA) containing 25 U/ml of TaqDNA polymerase, 400 µM dNTPs, 1.5 mM $MgCl_2$, and 0.4 µM of each primer. The amplification program was as follows: 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The primers used for FAAH promoter amplification (M: methylation specific, U: specific for unmethylated sequence) were the following: *faah*P MF (5′-TAGAGTTAGGAAAAATGAT GTTGAGAC-3′), *faah*P MR (5′-TTTTAAAACAAACGA AACAAACGA-3′), *faah*P UF (5′-TTAGAGTTAGGAAA AATGATGTTGAGAT-3′), *faah*P UR (5′-TTTTAAAAC AAACAAAACAAACAAA-3′), and β-actin F (5′-TGTT


A
faah mRNA level (fold over control)
Ctrl
25 nM E2
50 nM E2
100 nM E2
200 nM E2
B
Luciferase activity (fold induction)
pGL3
Faah-723/+10
Faah -723/+10
ERE-Luc
Ctrl
E2
C
Luciferase activity (fold induction)
Ctrl
1 nM E2
10 nM E2
100 nM E2
200 nM E2
D
Luciferase activity (fold induction)
Ctrl
E2
epiE2
TMX
TMX+E2
ICI 182780
ICI 182780+E2
Faah-723/+10
ERE-Luc

ACCAACTGGGACGA-3′), β-actin R (5′-GTCTCAAAC ATGATCTGGGTC-3′). PCR products were loaded on a 1.8 % agarose gel containing ethidium bromide, and were visualized under UV illumination.

Statistical analysis

The data reported in this study are the mean ± SEM of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by the non-parametric Student's *t* test and ANOVA test followed, when necessary, by post hoc Newman-Keuls test (GraphPad Software for Science, San Diego, CA, USA).

Results

The proximal 5′ flanking region of *faah* gene is activated by E_2

To determine whether the expression of FAAH mRNA was regulated by E_2 in mouse Sertoli cells, we treated primary cell cultures with different doses of E_2 for 4 h. mRNA expression, evaluated by qRT-PCR (Fig. 1a), increased significantly at 50 nM E_2 and reached a maximum fourfold increase at 200 nM E_2.

In order to study the transcriptional regulation of FAAH, we cloned the 5′ flanking proximal region of the *faah* gene, −723 bp to +10 nt with respect to the translational start site (atg), upstream of the luciferase reporter gene in pGL3 basic vector, and we transiently transfected Sertoli cells. Our construct showed a basal activity three- to fourfold higher than the promoterless vector (Fig. 1b, left panel), indicating that elements responsible for the basal expression of FAAH were present in this region. Then, we observed that treatment with 200 nM E_2 stimulated luciferase activity by ∼3-fold (Fig. 1b, right panel), thus suggesting that ERE sequences in the proximal promoter could mediate E_2 effects. As positive control, we transfected the cells with pERE-luc plasmid containing three ERE consensus sequences upstream of the luciferase gene. In Fig. 1c we show that E_2 was able to significantly increase *faah* proximal promoter activity in a dose-dependent manner. Lower concentrations of E_2 (1–10 nM) had already a significant effect on luciferase activity, but the maximum increase was observed at the highest (200 nM) E_2 concentration (Fig. 1c). Thus, the latter dose was used in further experiments. Incidentally, it should be noted that concentrations in the high-nM range have been largely used to investigate gene transcriptional regulation by E_2 [23–27] or metabolic effect of this hormone [28]. Moreover, it has been shown that intratesticular concentrations are much higher than serum levels of E_2 [29]. The specificity of E_2-dependent FAAH transcription was assessed by using 17-α-estradiol (epiE_2), which is an epimer of E_2 unable to bind ER, or specific ER antagonists such as tamoxifen (TMX) and Fulvestrant (ICI 182780) [28]. Sertoli cells were transfected with plasmid FAAH −723/+10 and were then treated with these substances. Figure 1d shows that epiE_2 had no effect on promoter activity, and so did TMX and ICI 182780 alone; instead, both ER antagonists abolished the E_2-induced luciferase activity, suggesting that E_2 regulation of the *faah* gene was mainly mediated through a canonical estrogen receptor signaling.

Sequence analysis of the proximal 5′ flanking region of the *faah* gene showed the presence of three potential estrogen receptor-binding sites that were named ERE1 (−614), ERE2 (−381), and ERE3 (−301) (Fig. 2a). Although the proximal promoter did not contain palindromic ERE consensus sequences (GGTCA nnn TGACC), all three EREs contain a well-conserved half-ERE 5′-TG ACC-3′ or 5′-GGTCA-3′ core motif. A series of mutants of the proximal promoter were generated to investigate the role of these ERE sites in transcriptional activation by E_2 (Fig. 2b). Deletion of the promoter until −560 nt from the translational start site, which determined loss of the ERE1 site, caused a significant decrease (∼50 %) of promoter stimulation by E_2 (Fig. 2c). By contrast, deletion of the promoter until nucleotide −250, that eliminates all ERE sequences, determined a complete loss of E_2 inducibility, still maintaining promoter basal activity (Fig. 2c). Moreover, the introduction of site-specific mutations showed that mutation in ERE1 site (TGACCC mutated to T**T**AC**A**C) still maintained E_2 transcriptional activation, but reduced by ∼50 %, while mutations in ERE2 (ATCTGTTCTGACC mutated to A**C**CTGTTC**ACCT**C) or ERE3 sequences (GGTCAAGGAAACC mutated to **A**GT**T**AAGG**CTTA**C) completely abolished gene activation by E_2 (Fig. 2c). These observations indicate that ERE2 and ERE3 are responsible for *faah* gene regulation by E_2 in Sertoli cells, while ERE1 is required to enhance the transcriptional response.

E_2 induces ERβ binding to ERE2/3 sites in the proximal promoter and activation of histone demethylase LSD1

To assess whether ERβ associated in vivo with the ERE sites in the *faah* proximal promoter, genomic DNA from Sertoli cells obtained from 17-day-old mice and treated or not with E_2 was subjected to chromatin immunoprecipitation (ChIP) assays, using anti-ERβ antibodies followed by qRT-PCR. E_2 treatment determined a ∼2.5-fold increase of ERE2/3 DNA (region a) immunoprecipitated with anti-ERβ antibodies (Fig. 3a), indicating that ERβ was associated with the ERE2/3 sequences in a ligand-dependent manner. Instead, the receptor was not associated with a 5′

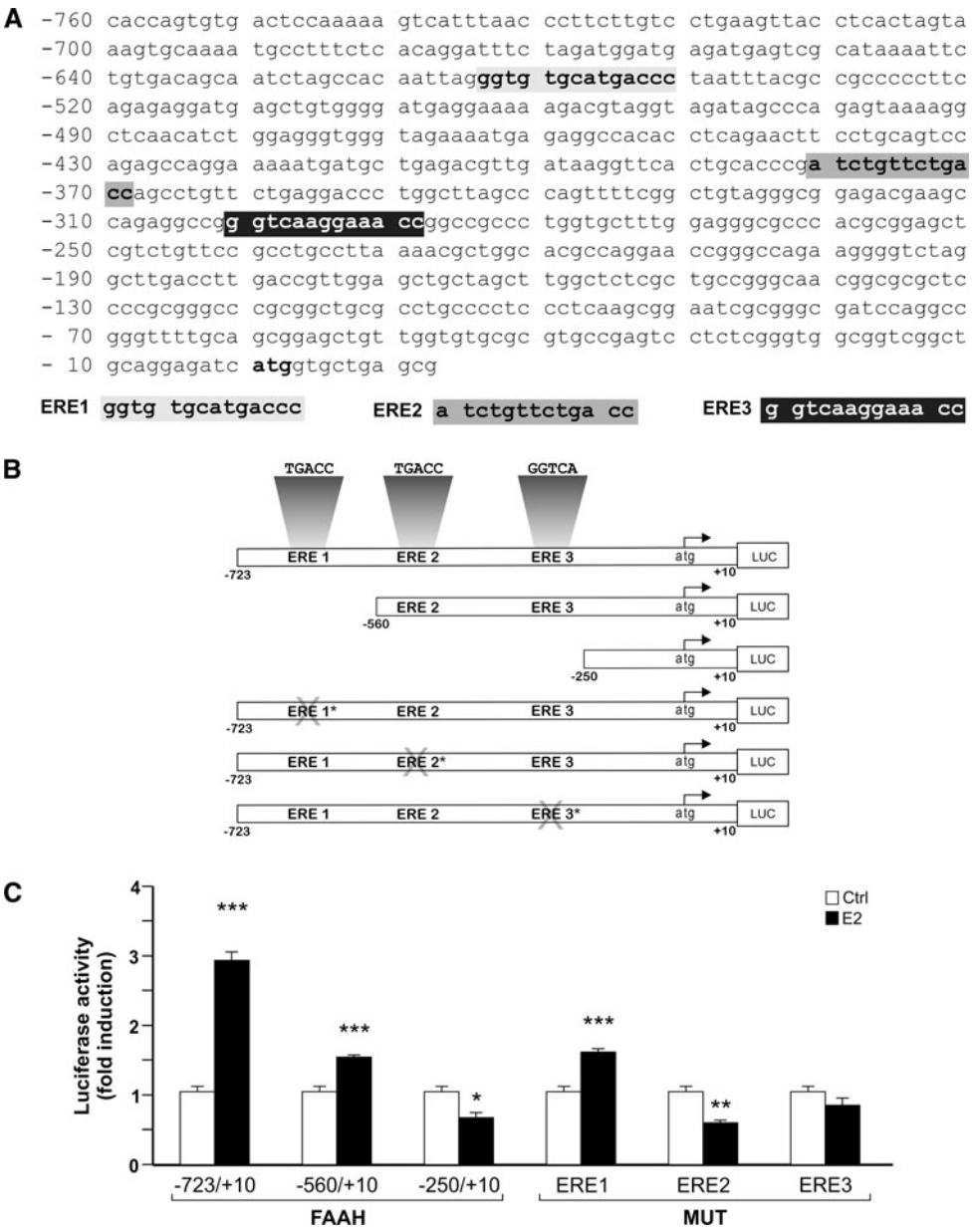


Fig. 2 ERE-binding sites in the *faah* proximal promoter are required for activation by E_2. **a** Nucleotide sequence of the proximal 5′ flanking region of the mouse *faah* gene from −723 to +13 nt from the translational start site. *faah* sequence from −723 to +10 was PCR amplified using primers reported in "Materials and methods" and Ref. [45]. Putative ERE sites are underlined and indicated as ERE1–3. The translational start codon (atg) is in *bold*. **b** Schematic representation of *faah* promoter 5′ sequential deletion constructs and site-specific mutants at ERE1, ERE2, or ERE3 sites. Half ERE sites are indicated. **c** Sertoli cells were transfected with 0.2 μg of each *faah* promoter construct, and were treated or not with 200 nM E_2 for 30 min. After incubation, dual-luciferase reporter assays were performed. ***Denotes $p < 0.001$ versus Ctrl; **denotes $p < 0.01$ versus Ctrl; *denotes $p < 0.05$ versus Ctrl

upstream promoter region between −1,630 and −1,430 from the translational start site (region b), demonstrating the specificity of the ERβ binding to the proximal region of the *faah* gene (Fig. 3a).

Nuclear hormone receptors are known to activate transcription by recruiting coactivators that can eventually modify chromatin structure and allow the assembly of the RNA PolII transcriptional machinery at the promoter site. More recently, the lysine-specific demethylase 1 (LSD1) has been demonstrated to control nuclear hormone receptor-dependent gene expression, by activating ER target genes [21, 30], as well as androgen-receptor target genes like *PSA* [31]. On this background, we investigated the role of LSD1 in estrogen-regulated expression of the *faah* gene in Sertoli cells. By means of qRT-PCR and immunoblotting analyses, we found that Sertoli cells express LSD1 (Fig. 4a, b), and that this enzyme is mainly localized in the nuclear compartment as shown by immunofluorescence (Fig. 4c). To demonstrate the recruitment of LSD1 to the ERE2/3 sites of *faah* promoter, we performed ChIP analysis using anti-LSD1 antibodies to immunoprecipitate Sertoli cell chromatin. We found that LSD1 was specifically associated with chromatin at ERE2/3 sites and that E_2 did not modify its binding (Fig. 3a).

LSD1 is an amine oxidase, and thus its activity can be inhibited by monoamine oxidase inhibitors like pargyline (Parg) [21, 31]. We found that treatment with Parg, as well as knock-down of *lsd1* gene in Sertoli cells transfected with

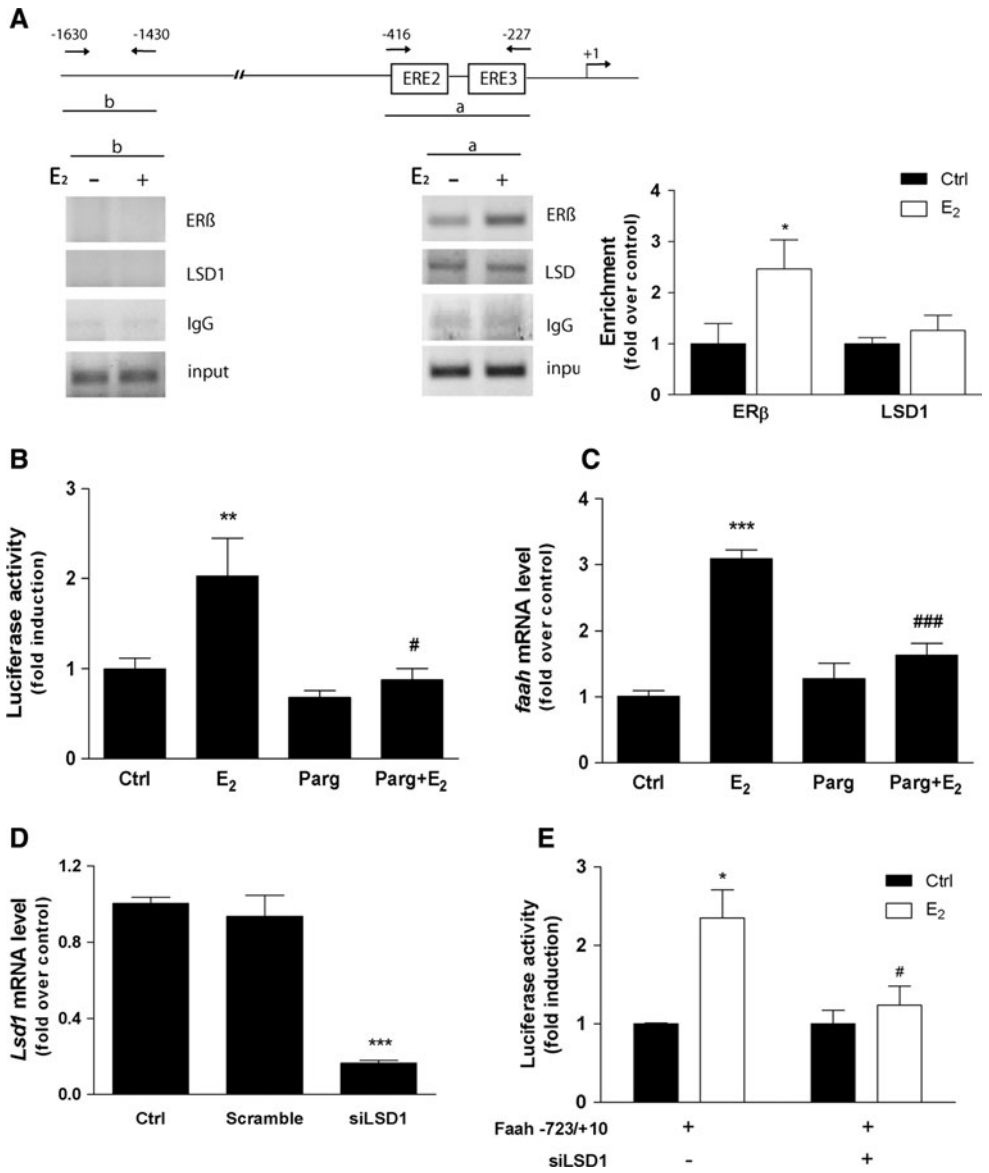


Fig. 3 E_2 induces ERβ binding and LSD1 activation at ERE sites in the *faah* proximal promoter. **a** Diagram of mouse *faah* 5′-flanking region with the location of primers used for ChIP-qPCR in the indicated regions *a* and *b*. Chromatin immunoprecipitation (ChIP) assays were performed with anti-ERβ and anti-LSD1 antibodies in Sertoli cells treated or not with 200 nM E_2 for 1 h, and analyzed by quantitative real-time PCR. A representative agarose gel of PCR products of ERE2/3 region of *faah* promoter is shown. Input chromatin, indicating sample before immunoprecipitation, and no primary antibody (IgG) were included as positive and negative controls, respectively. *Bar graph* represents fold enrichment of ERβ and LSD1 bound to ERE2/3 in E_2-treated cells relative to that in the control cells. Results were normalized for input and non-immune rabbit IgG, and *denotes $p < 0.05$. **b** Pargyline reduces E_2 stimulation of *faah* promoter in Sertoli cells, as analyzed by luciferase assay. Sertoli cells were transfected with $FAAH_{-723/+10}$, and were treated with 3 mM pargyline for 16 h, and then for 30 min with 200 nM E_2. After incubation, dual-luciferase reporter assays were performed. **Denotes $p < 0.01$ versus Ctrl; $^{\#}$denotes $p < 0.05$ versus E_2. **c** Pargyline reduces E_2-induced expression of endogenous *faah* gene in Sertoli cells, analyzed by qRT-PCR. ***Denotes $p < 0.001$ versus Ctrl; $^{\#\#\#}$denotes $p < 0.001$ versus E_2. **d** Sertoli cells were transfected with *lsd1* siRNA. Knock-down of endogenous LSD1 was ascertained by qRT-PCR. ***Denotes $p < 0.001$ versus Ctrl. **e** LSD1 silencing reduced E_2 stimulation of *faah* promoter in Sertoli cells, analyzed by luciferase assay. *Denotes $p < 0.05$ versus Ctrl; $^{\#}$denotes $p < 0.05$ versus E_2

the FAAH −723/+10 plasmid, blocked the E_2-induced *faah* promoter activity (Fig. 3b, e). Moreover, Parg abolished E_2 stimulation of endogenous FAAH expression (Fig. 3c). Incidentally, transfection of Sertoli cells with the *lsd1* siRNA effectively knocked-down the expression of the target gene (Fig. 3d).

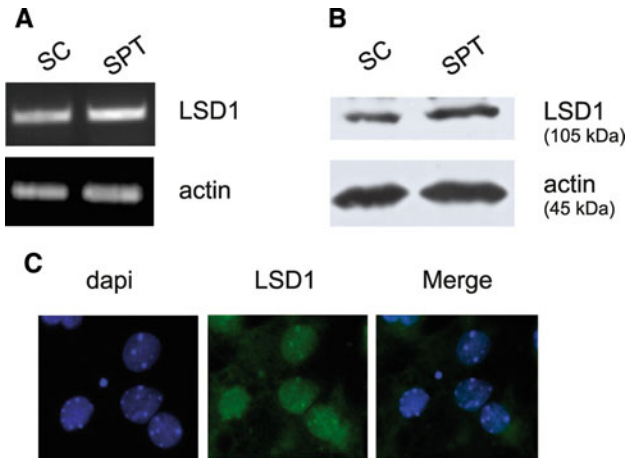


Fig. 4 LSD1 expression in Sertoli cells. **a** PCR and Western blotting (**b**) show the expression of LSD1 in Sertoli cells. Spermatids were used as positive controls. **c** Immunofluorescence shows nuclear localization of LSD1 in Sertoli cells

Altogether, these results indicate the engagement of LSD1 in regulating E_2-induced expression of *faah* gene.

Faah gene expression is not regulated by E_2 in immature Sertoli cells

Sertoli cells undergo extensive changes during the postnatal period, and these modifications are critical for the establishment of spermatogenesis and the development of the adult pattern of testicular function. Indeed, it has been demonstrated that FAAH expression and activity in Sertoli cells decrease in an age-dependent manner [14]. To ascertain whether regulation of FAAH expression by E_2 was modified during postnatal development, we analyzed by qRT-PCR FAAH mRNA expression in Sertoli cells obtained from 7-day-old mice. We found that FAAH mRNA expression was not stimulated by E_2 in immature cells, either after 4 h or after 24 h of treatment (Fig. 5a). Transfection experiments with FAAH −723/+10 plasmid in immature Sertoli cells confirmed that proximal promoter had basal transcriptional activity, but it was not significantly stimulated by E_2 (Fig. 5b). One possible reason for the loss of regulation of *faah* gene by E_2 in immature cells could be the absence of either ERβ or LSD1, which mediate this activity of E_2 in the mature (17-day-old) cells. ERβ was found to be expressed to a similar protein level at both ages, as analyzed by Western blotting (Fig. 5c), whereas LSD1 was not expressed in immature Sertoli cells, as shown by qRT-PCR (Fig. 5d), immunoblotting (Fig. 5c) and immunofluorescence (Fig. 5e). These results point to the lack of LSD1 as one of the possible causes of the loss of *faah* promoter inducibility by E_2 in immature (7-day-old) versus mature (17-day-old) Sertoli cells.

E_2 induces epigenetic modifications of the chromatin at 5′ flanking region of the *faah* gene

DNA methylation and histone methylation are important epigenetic modifications that are strictly related to gene transcription. Actively transcribed genes are typically unmethylated at CpG islands in the promoter region, and are marked by H3K9 hypomethylation. Thus, we investigated the effect of E_2 on these epigenetic modifications in the *faah* proximal promoter. DNA methylation was examined by using an MSP-PCR assay, which is sensitive and specific for methylation of any CpG site located within CpG islands [22] (Fig. 6a). Control Sertoli cells showed a marked methylation of *faah* promoter (Fig. 6b), consistent with a low expression of this gene, while E_2 treatment dramatically decreased the methylation levels of the proximal region, in line with the observed increase of expression levels.

Histone methylation of the *faah* proximal promoter was investigated in vivo by ChIP assays using antibodies against H3K9me3. After 60 min of 200 nM E_2 stimulation, we observed a decrease in H3K9me3 levels in ERE2/3 sites (region a) of the *faah* promoter with respect to untreated cells (Fig. 6c). In order to demonstrate that lower E_2 doses (10 nM) could be sufficient to induce the same molecular effects observed at 200 nM E_2, we investigated the chromatin modifications in the *faah* proximal promoter by analyzing H3K9 methylation by ChIP assays. We found that 10 nM E_2 decreased the H3K9me3 level in the ERE2/3 sites (region a) of the *faah* promoter in Sertoli cells in a similar manner compared to 200 nM E_2 (Supplementary Fig. 1 and Fig. 6c). This observation further supports the in vivo physiological relevance of the effects of E_2 on *faah* promoter in mouse Sertoli cells. Altogether, our results indicate that E_2 triggers epigenetic modifications in the *faah* promoter that favor a more transcriptionally competent configuration of chromatin.

FAAH silencing abolishes E_2-mediated protection of Sertoli cells against AEA-induced apoptosis

Previous studies have clearly demonstrated the effect of AEA in inducing apoptosis of Sertoli cells, and the crucial role of FAAH in promoting survival of these cells by degrading AEA itself [15]. To provide evidence for the biological relevance of FAAH regulation by E_2, we analyzed apoptosis induced by AEA in Sertoli cells, after knocking down the *faah* gene through siRNA. Figure 7a shows that transfection of Sertoli cells with the FAAH siRNA effectively knocked down the expression of the *faah* gene, and erased its induction by E_2. Furthermore, as reported in Fig. 7b the addition of AEA to Sertoli cells for 24 h significantly increased the extent of apoptosis,

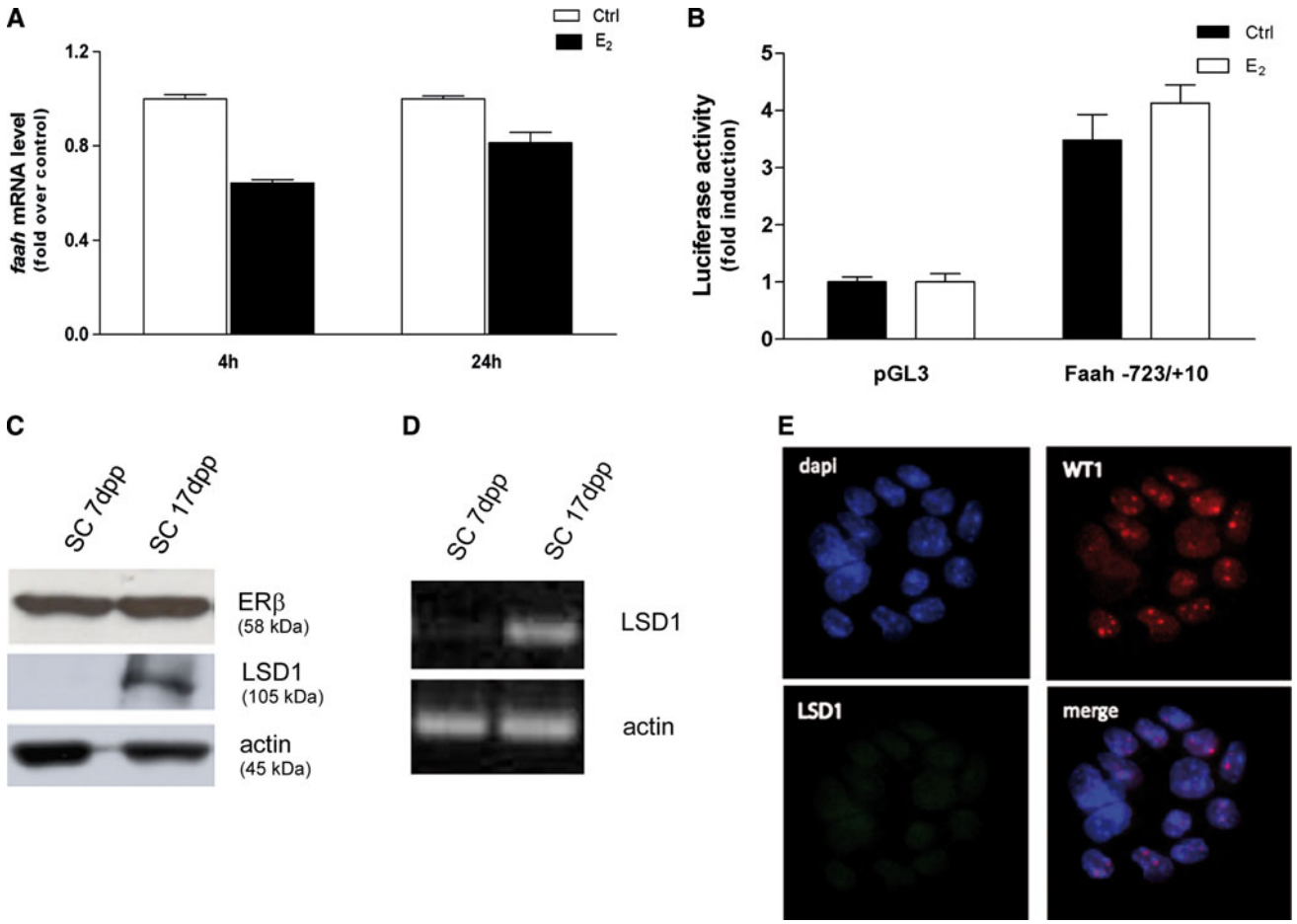


Fig. 5 *FAAH* is not regulated by E_2 in immature Sertoli cells. **a** *Faah* mRNA expression was not stimulated by 200 nM E_2 in Sertoli cells from 7-day-old mice. Quantitative RT-PCR analysis of *faah* expression in Sertoli cells from 7-day-old mice after treatment with 200 nM E_2 for 4 or 24 h as indicated. **b** Sertoli cells were transiently transfected with $Faah_{-723/+10}$ or with the promoterless pGL3 plasmid, and were stimulated with E_2. Luciferase activity is expressed as fold increase over the untreated cells. **c**, **d** LSD1 expression in immature Sertoli cells from 7-day-old mice was analyzed by RT-PCR (**c**) and by Western blotting (**d**), and was compared to mature (17-day-old) Sertoli cells. **e** The absence of LSD1 was verified by immunofluorescence analysis of Sertoli cells from testes of 7-day-old mice, using anti-LSD1. Anti-WT1 antibodies were used as Sertoli cell-specific marker

evaluated as DNA fragmentation [15] and, as expected, the latter process was further increased by FAAH silencing. On the other hand, a strong increase in DNA fragmentation of FAAH-silenced cells was observed upon treatment with a combination of AEA and E_2 (Fig. 7b).

Discussion

Many studies have underlined the importance of estrogens in male fertility [5, 32–34], as demonstrated by the expression of ERs and aromatase in the testis and by the fact that mice lacking either ERα [5] or aromatase [6] show altered spermatogenesis and infertility. However, evidence for a direct action of E_2 on the seminiferous epithelium is still missing, and remarkably genes that are regulated by E_2 through classical ERE sequences remain unknown in the testis. Yet, the latter organ contains all cofactors necessary to directly activate genes in response to E_2, as demonstrated by using luciferase reporter gene assays under the control of three tandem ERE elements in testis of transgenic mice [35].

Here we demonstrate that the *faah* gene is the first direct target of E_2 in Sertoli cells. We show that E_2 induced a ~4-fold increase of FAAH mRNA in Sertoli cells and that this stimulation occurred at the transcriptional level. However, we cannot exclude that estrogen might also modulate mRNA stability. In the same context, in our study we found that supraphysiological (200 nM) and physiological (10 nM) E_2 concentrations have the same effects on the molecular mechanisms involved in the stimulation of *faah* promoter activity; yet, caution should be taken because at higher concentrations, E_2 might also have off-target effects. The proximal 5′ flanking region of the mouse

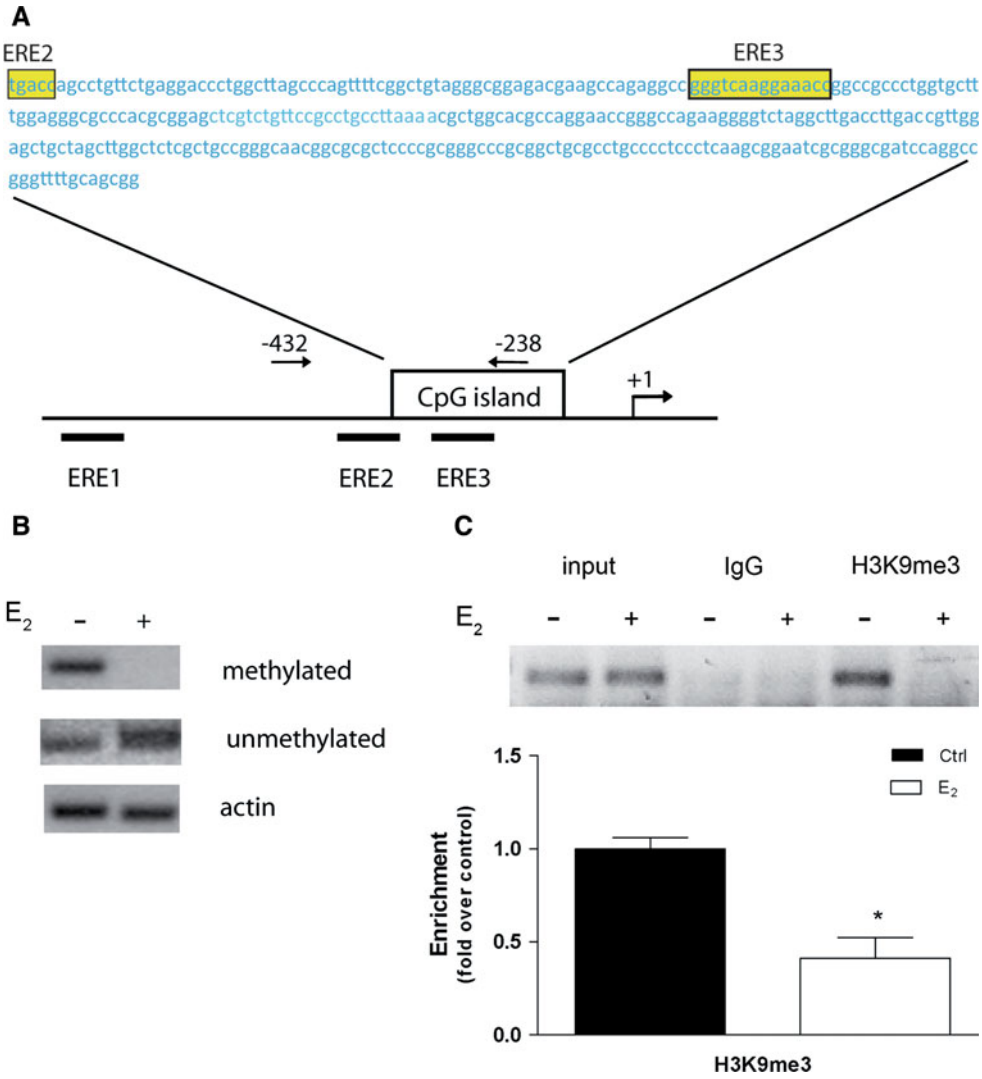


Fig. 6 DNA and histone demethylation is induced by E_2 at 5′ flanking region of the *faah* gene. **a** Schematic representation of the CpG island identified in the FAAH promoter. ERE sites and localization of primers for PCR amplification are indicated. **b** Sertoli cells were treated or not with 200 nM E_2 for 30 min, and then DNA was extracted and analyzed by PCR, using specific primers as indicated in "Materials and methods". **c** ChIP assay was performed with anti-H3K9me3 antibody in Sertoli cells treated or not with 200 nM E_2 for 1 h, and analyzed by qRT-PCR. A representative agarose gel of PCR products of ERE2/3 sites (region **a**) of *faah* promoter is shown. Input chromatin, indicating sample before immunoprecipitation, and no primary antibody (IgG) were included as positive and negative controls, respectively. *Bar graph* represents fold enrichment of H3K9me3 in E_2-treated cells relative to controls. Results were normalized for input and non-immune rabbit IgG

faah gene contained the elements necessary for basal and inducible transcription in Sertoli cells. Through the use of selective estrogen receptor modulators like TMX or ICI 182780, we demonstrated the specificity of estrogen regulation. In fact, both drugs exhibited a complete antagonist effect on E_2-mediated stimulation of the FAAH promoter and they did not have any direct effect when given alone. This is in agreement with a previous study reporting that TMX exhibited a pure antagonism in an ERβ expressing system [36]. Consistently with these data epiE_2, which is ineffective at ERs [28, 29], had no effect on FAAH expression. In the proximal promoter, at least three potential ERE sequences can be identified. Two ERE sites, named ERE2 and ERE3, are at a distance of 60 bp from each other, and proximal to the transcriptional start site; the third, ERE1, is located upstream of them, at position −615 from the translational start site. These are all imperfect ERE sites because they do not contain canonical palindromic ERE sequences, yet they show half-ERE consensus sequences. Although it has been reported that half-ERE sites confer weak E_2 responsiveness, genes are known for which E_2-induced transcription is mediated by two or more ERE half-sites [37]. The importance of these sites in mediating the E_2 effect on *faah* promoter activity has been investigated by mutant analysis, demonstrating that ERE2 and ERE3 are necessary for promoter stimulation by E_2; indeed, deletion of one of these sites or mutations in their sequence completely abolished the E_2 effect. Instead, ERE1 is involved in enhancing the E_2 response of the proximal promoter, since its deletion or mutation caused a reduction of the promoter inducibility.

The biological effects of E_2 are mediated by ERα and ERβ [36], and it has been previously demonstrated that mouse Sertoli cells express only ERβ [3]. By using a ChIP assay with Sertoli cells, we examined the ligand-dependent recruitment of ERβ to the *faah* promoter *in vivo*, and we found that E_2 increased ERβ binding to ERE2/3 promoter sites. This observation is the first to highlight a functional

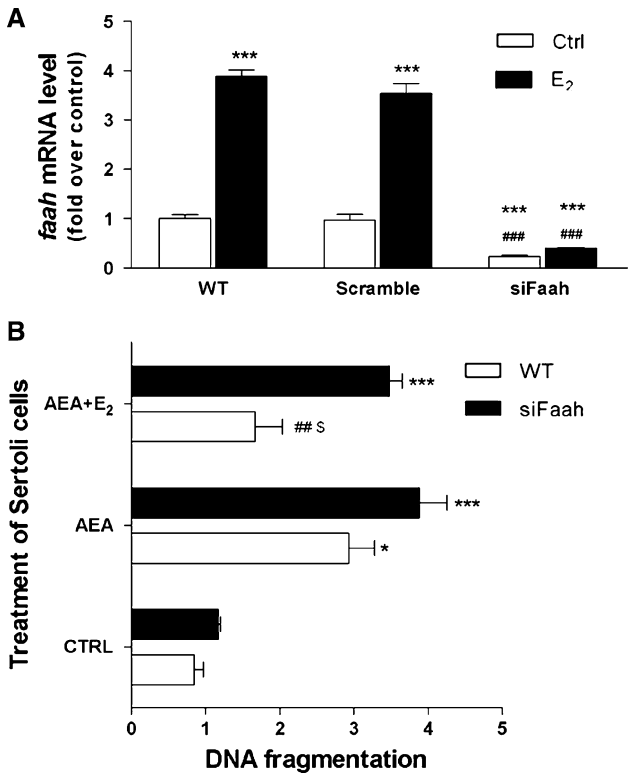


Fig. 7 Faah knockdown increases AEA-induced apoptosis in Sertoli cells. **a** Sertoli cells were transfected with siRNA *faah* and stimulated with 200 nM E_2 for 24 h. Knockdown of endogenous FAAH was ascertained by qRT-PCR. siFaah abolished E_2 induction of *faah* gene expression. ***Denotes $p < 0.001$ versus ctrl; $^{###}$denotes $p < 0.001$ versus E_2. **b** Effect of FAAH silencing on DNA fragmentation induced by 1 μM AEA in Sertoli cells. *Denotes $p < 0.05$ versus Ctrl wt; ***denotes $p < 0.001$ versus Ctrl wt; $^{\$}$denotes $p < 0.05$ versus AEA wt; $^{\#\#}$denotes $p < 0.01$ versus AEA siFaah

role of ERβ in somatic cells of the testis as ligand-activated nuclear transcription factors.

One mechanism by which nuclear hormone receptors can activate transcription is the recruitment of coactivators that can modify chromatin structure and allow the assembly of the RNA PolII transcriptional machinery to the promoter. Recent studies highlight the importance of these chromatin modifications in the transcriptional regulation mediated by nuclear hormone receptors, and suggest that histone modifiers could be nuclear receptor coregulators [38]. LSD1 was identified as the first histone lysine demethylase that acts on either lysine 4 residues (H3K4) or lysine 9 (H3K9) on histone 3 [30, 31] in a context-dependent manner, and acts as a transcriptional inhibitor or activator, respectively. LSD1 contains a SWIRM domain, which functions as a putative protein–protein interaction motif, and an amine oxidase domain that harbors the demethylase activity. Recent evidence demonstrates that LSD1 participates in androgen receptor (AR) and estrogen receptor-dependent gene expression. LSD1 is implicated in H3K9 demethylation associated with *PSA* gene activation in an AR-dependent manner [30], and with activation of most ERα gene targets in MCF7 cells [31]. In our study, we show by qRT-PCR and immunoblotting that LSD1 is expressed in Sertoli cells and is recruited in vivo at the ERE2/3 sites of the *faah* gene both in the presence and in the absence of the ligand. Remarkably, depletion of LSD1 in Sertoli cells by RNAi experiments or inhibition of its activity by pargyline completely abolished stimulation by E_2 of FAAH promoter activity. Furthermore, pargyline abolished the E_2-induced expression of endogenous FAAH, thus indicating that LSD1 activity is important in E_2 transcriptional activation of the *faah* gene. It has been suggested that LSD1 interacts with different partners, and could act by silencing or activating gene transcription. We speculate that in Sertoli cells LSD1 interacts with ligand-bound ERβ at ERE sites of target genes, and that this interaction is required for potentially conferring to promoter a transcriptional competence.

In our investigation, we also document that *faah* proximal promoter is not regulated by E_2 in immature proliferating Sertoli cells. We hypothesized that a low level of expression of ERβ in immature cells or the absence of LSD1 could account for this lack of effect. Interestingly, we found that immature cells express ERβ at the same level as mature cells, but they do not express LSD1. Thus, we suggest that the absence of the latter histone demethylase could contribute to the loss of E_2 inducibility of FAAH promoter at this age. In mammary tumor cell lines it has been demonstrated that ERα binding to target regulatory sequences is impaired by DNA hypermethylation of CpGs, and that the first step needed to stimulate E_2-regulated gene expression is to eliminate epigenetic barriers, thus allowing ERα access to regulatory sequences [39]. DNA cytosine methyltransferase 1 (DNMT1) is the most abundant and catalytically active DNA methyltransferase, and is able to induce covalent addition of a methyl group to the 5′ position of cytosine, predominantly within CpG dinucleotides, which are generally localized in promoter regions [40]. More recently, the interaction between DNMT1 and ERβ has been demonstrated in the testis, suggesting that E_2 signaling could control DNA methylation [8]. On this background, we analyzed the DNA methylation in the *faah* promoter and we observed a dramatic demethylation of the CpG island located in the proximal region after E_2 treatment.

In addition, it has been reported that demethylation of histone 3 at lysine 9 is an important mark associated with transcriptional activation [30]. Here, we document that E_2-stimulated transcription of *faah* gene is accompanied by a decline in H3K9me3 at the ERE2/3 sites, suggesting indeed a role for these modifications in the transcriptional activation of *faah* promoter by E_2. In light of this observation, it is

possible to speculate that E_2-induced expression of the *faah* gene might require, in addition to LSD1, the recruitment of other histone methylases (HMTs) and demethylases (HDMs) in order to promote gene transcription.

Recent data suggest that specific gene regulation requires the assembly and coordinate action of HMTs and HDMs with distinct substrate specificities. Wissmann and colleagues [41] have demonstrated that JMJD2C, which demethylases H3K9me3, and LSD1, which specifically demethylases H3K9 me2/me1, interact and cooperatively stimulate AR-dependent gene transcription. Recently it has been also reported, in ERα regulated transcription, a coordinated interaction among ERα and the H3K9me3 demethylase JMJD2B, and the H3K4 methyltransferase MLL2 complex has been recently reported [42]. Previous studies have underlined the importance of FAAH in male fertility [18, 43], where this hydrolase controls the endogenous levels of AEA, and hence its signaling. For instance, high levels of AEA adversely affect sperm motility, capacitation and acrosome reaction [17, 18], and reduce spermatogenic output by inducing Sertoli cell apoptosis [15]. In line with this, genetic loss of FAAH in mouse leads to elevated levels of AEA in the male reproductive tissues, thus impairing sperm fertilizing capacity [43]. In the testis, Sertoli cells play a crucial role in supporting and regulating germ cell development, and their number is strictly correlated with sperm production [44]. It is also well documented that germ cell apoptosis is physiologically required for the maintenance of an optimal ratio of germ cells to Sertoli cells in the seminiferous epithelium. In this context, it should be recalled that after a first wave of prepuberal cell division, Sertoli cells stop dividing and their number remains constant through the lifespan. Keeping this in mind, we can speculate that the control of Sertoli cell apoptosis could be important to maintain their population size, and consequently, to sustain a normal spermatogenic output. Therefore our findings, showing that E_2 can protect Sertoli cells against AEA-induced apoptosis via activation of *faah* gene expression, suggest that E_2 could be a pro-survival hormone for Sertoli cells.

In conclusion, in this investigation we have identified FAAH as the first direct target gene of E_2 in Sertoli cells, showing that enhancement of *faah* promoter activity engages ERβ and histone demethylase LSD1 and requires chromatin configuration competent for transcription. These findings support an unprecedented role for E_2 as pro-survival hormone for Sertoli cells, with a clear impact on spermatogenesis. They also call for further studies on the cross-talks between steroid hormones and endocannabinoids that might orchestrate signaling pathways well beyond reproductive organs.

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