Crosstalk between FSH and relaxin at the end of the proliferative stage of rat Sertoli cells

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Abstract

Follicle-stimulating hormone (FSH) stimulates the proliferation of immature Sertoli cells through the activation of PI3K/AKT/mTORC1 and MEK/ERK1/2 pathways. Mature Sertoli cells stop proliferating and respond to FSH by stimulating cAMP production. To gain insight into possible mechanisms involved in this switch as well as the impact of paracrine factors that stimulate cell proliferation, we analyzed the effects of FSH and relaxin on intracellular signaling pathways involved with proliferation and differentiation in Sertoli cells from 15-day-old rats, which are close to the transition between the two stages. FSH stimulated ³H-thymidine incorporation and cyclin D1 expression, changes associated with proliferation. In contrast, FSH inhibited AKT and ERK1/2 phosphorylation, activated cAMP production and induced changes in several cell cycle genes that were compatible with differentiation. Relaxin also stimulated ³H-thymidine incorporation but increased phosphorylation of ERK1/2 and AKT. When both hormones were added simultaneously, relaxin attenuated FSH-mediated inhibition of ERK1/2 and AKT phosphorylation and FSH-mediated activation of cAMP production. FSH but not relaxin increased CREB phosphorylation, and relaxin but not FSH shifted NF-κB expression from the cytoplasm to the nucleus. Relaxin did not inhibit the effects of FSH on inhibin α and *Bcl2* expression. We propose that at this time of Sertoli cell development, FSH starts to direct cells to differentiation through activation of cAMP/CREB and inhibition of ERK1/2 and AKT pathways. Relaxin counteracts FSH signaling through the inhibition of cAMP and activation of ERK1/2, AKT and NF-κB, but does not block the differentiation process triggered by FSH.

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Introduction

Sertoli cells are the somatic component of the seminiferous tubules and produce appropriate microenvironment for spermatogenesis (Griswold 1998, Sharpe *et al.* 2003, Petersen & Söder 2006, Sofikitis *et al.* 2008). Sertoli cells are the first somatic cells to differentiate in the testis, and they play a central role in the organization of testis differentiation (Svingen & Koopman 2013). Sertoli cells influence testis cord formation, Müllerian duct regression, and the differentiation and function of germ cells, peritubular myoid cells, fetal Leydig cells and endothelial cells.

Each Sertoli cell supports a limited number of germ cells (Russell *et al.* 1990). Therefore, the total number of Sertoli cells is directly related to the size of the testis, the number of germ cells and sperm production in adult life. In humans, Sertoli cells proliferate during the fetal and neonatal period (12–18 months) and remain quiescent until puberty (10–14 years), when the last period of proliferation occurs (Sharpe *et al.* 2003). In rats, proliferation of Sertoli cells ceases 16–20 days after birth (Orth 1982, Wang *et al.* 1989).

However, adult, mature, Sertoli cells do not seem to be terminally differentiated (Tarulli *et al.* 2012). They can return to the immature state and proliferate after super-expression of ID proteins (inhibitors of differentiation), after being cultured for long periods with 5% serum and after transplantation under the renal capsule (Chaudhary *et al.* 2005, Ahmed *et al.* 2009, Chui *et al.* 2011, Mital *et al.* 2014).

When the mechanisms that promote proliferation and maturation of Sertoli cells fail, the immature state may continue throughout adult life with impairment of spermatogenesis (Sharpe *et al.* 2003). The number of Sertoli cells depends on several factors, including genetic factors, and hormones including the follicle-stimulating hormone (FSH), testosterone, thyroid hormones, luteinizing hormone (LH), 17β-estradiol, growth hormone and several paracrine factors (Buzzard *et al.* 2003, Sharpe *et al.* 2003, Lucas *et al.* 2014a).

FSH is a major regulator of Sertoli cell function. The hormone transmits its signal after coupling with FSH receptor (FSHR), a member of a subfamily of G protein-coupled receptors (GPCRs) family A that also

includes the LH receptor (LHR), the thyroid stimulating hormone receptor (TSHR) and the receptors RXFP1 to 4, for the relaxin family peptides receptors, insulin-like peptide 3 (INSL3), relaxin-3 and INSL5 (Hsu *et al.* 2002, Bathgate *et al.* 2013, Halls *et al.* 2015). The knockout of the FSHR (*Fshr*^{-/-}) does not block fertility (Kumar *et al.* 1997) but reduces gametogenesis and sperm count in mice (Dierich *et al.* 1998). Deletion of the *Fshr* gene in mice reduces the number of Sertoli cells at birth by 22%. The mitogenic effect of FSH continues in the neonatal period and it induces proliferation of cultured Sertoli cells obtained from 5-, 8-, 10-, 12- and 15-day-old rats (Crépieux *et al.* 2001, Khan *et al.* 2002, Cardoso *et al.* 2010, Riera *et al.* 2012) but not from 19-day-old rats (Crépieux *et al.* 2001). The proliferative effect of FSH on Sertoli cells isolated from 5- and 8-day-old rats has been shown to involve the activation of PI3K/AKT/mTORC1 and MEK/ERK1/2 pathways (Crépieux *et al.* 2001, Riera *et al.* 2012). Mature Sertoli cells stop proliferating but they are still responsive to FSH. At this stage, FSH supports cell differentiation and its signaling switches to the activation of cAMP production (Bhattacharya *et al.* 2012).

We have shown that relaxin and its receptor RXFP1 are co-expressed in the rat testis, and relaxin seems to play a paracrine or autocrine role in this tissue (Filonzi *et al.* 2007, Cardoso *et al.* 2010, Nascimento *et al.* 2012, Pimenta *et al.* 2015). Relaxin and RXFP1 were present in Sertoli cells from 15-day-old rats, and relaxin-stimulated Sertoli cell proliferation by activation of the MEK/ERK1/2 and PI3K/AKT pathways (Cardoso *et al.* 2010, Nascimento *et al.* 2012). The proliferative effect of relaxin on Sertoli cells from 15-day-old animals was not additive or synergistic with FSH (Cardoso *et al.* 2010).

Sertoli cells from 15-day-old rats continue to proliferate although they are close to ceasing proliferation and starting differentiation. To gain insight into possible mechanisms involved in this switch as well as the impact of paracrine factors that stimulate cell proliferation, we analyzed the effects of FSH and relaxin on intracellular signaling pathways involved with proliferation and differentiation in Sertoli cells from 15-day-old rats, which are close to the transition between the two stages.

Materials and methods

Hormones

Human FSH was obtained from Dr A F Parlow, from the National Hormone and Peptide Program (Torrance, CA, USA). Recombinant human relaxin H2 (RLN) was from Phoenix Pharmaceuticals Company (Burlingame, CA, USA).

Animals

Male Wistar rats, 15-day-old, born and housed in the Animal Facility at the Instituto Nacional de Farmacologia e Biologia

Molecular, Universidade Federal de São Paulo, were used. Rats were kept at 23°C on a 12 h light:12 h darkness schedule, with food and water freely available. The experimental procedures were approved by the Research Ethics Committee from UNIFESP (1311/11).

Primary culture of Sertoli cells

The testes were removed and decapsulated and Sertoli cells were prepared as described previously (Skinner & Fritz 1985, Lucas *et al.* 2008). The cells were plated at a density of approximately 4×10^6 cells/mL in phenol-red free Ham's F12/Dulbecco's modified Eagle medium (F12/DMEM 1:1, Gibco, Invitrogen) containing 0.02 g/L gentamicin (Sigma Chemical Co.), pH 7.2–7.4 and supplemented with 10 µg/mL insulin, 10 µg/mL transferrin, 10 ng/mL sodium selenite and 10 ng/mL epidermal growth factor (EGF, Sigma). The cells were grown in a humidified atmosphere of 5% CO₂:95% air at 35°C. After 48 h cultures were treated with 20 mM Tris-HCL, pH 7.4, to lyse residual germ cells (Galdieri *et al.* 1981), and allowed to grow for another 24 h. Culture medium was replaced by one without supplements 20 h before the experiments. At this stage, the cells were 90–95% confluent and viability determined by trypan blue exclusion was higher than 90%. The presence of other cell types was evaluated by several criteria, and was negligible (Lucas *et al.* 2008).

Determination of cyclic AMP production

Cells (4×10^6) were plated in 6-well dishes and cultured in the absence of supplements for 16 h before the assay. Endogenous phosphodiesterases were inhibited with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mM; Calbiochem) for 30 min. Cells were incubated with phosphate buffered saline (PBS; control), FSH (100 ng/mL) for 30 min or relaxin (RLN, 50 ng/mL; 8 nM) for 5, 10, 15 or 30 min. To evaluate whether relaxin modulates the response to FSH, cells were first treated with FSH for 30 min and then with relaxin for different periods, or treated simultaneously with FSH and relaxin for 30 min. The reaction was stopped on ice and the nutrient medium was aspirated. After extraction with 1.0 mL absolute ethanol, the ethanol phase was transferred to Eppendorf tubes and dried in speed vacuum. The concentration of intracellular cAMP was determined by enzyme immunoassay in 96-well microplates, with the Biotrak RPM 225 kit (GE Healthcare) according to the instructions of the manufacturer. Duplicate 100 µL aliquots of the samples and standard curve (12.6–3200 fmol cAMP/mL) were resuspended in lysis buffer 1B (0.25% w/v dodecyltrimethylammonium bromide in 0.05 M acetate buffer pH 5.8, with 0.002% w/v bovine serum albumin and 0.01% w/v sodium azide). Reaction was developed with 150 µL peroxidase substrate for 1 h, at room temperature. Reaction was interrupted with 100 µL 1 M H₂SO₄, and the optical density was read at 450 nm (VersaMax Elisa Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The results were analyzed with GraphPad Prism 5.01 software (GraphPad Software).

Western blotting for detection of ERK1/2 and AKT phosphorylation

On culture day 4, Sertoli cells were incubated in the absence (control) and presence of 100 ng/mL FSH and 50 ng/mL relaxin (RLN), for the indicated times at 35°C. To evaluate the participation of cAMP, PKA or Epacs (exchange proteins directly activated by cAMP ortholog) on ERK1/2 and AKT phosphorylation, before exposure to FSH or RLN cells were pretreated or not with the phosphodiesterase inhibitor IBMX (0.5 mM, 30 min), the PKA inhibitor [2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89, 2 μ M, 2 h; Sigma Chemical Co.) or the inhibitor of Epac1/2, 5-*tert*-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]-3-oxo-propionitrile (ESI-09; 2 μ M, 30 min, Sigma Chemical Co.). This concentration of ESI-09 was chosen based on previous studies, which demonstrated that this compound specifically blocks intracellular Epac-mediated Rap1 activation and AKT phosphorylation with an apparent IC₅₀ of 1.4 μ M, and Epac1 GEF activity with an apparent IC₅₀ of 3.2 μ M (Almahariq *et al.* 2013).

Medium was removed, the cells were washed with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF, 10 mM Na₄P₂O₇), as described previously (Lucas *et al.* 2008, Nascimben *et al.* 2012). Protein concentration was determined with a BioRad protein assay, using BSA as standard (Bio Rad Laboratories).

Total cellular proteins (30 μ g/lane for AKT, and 60 μ g/lane for ERK1/2) were incubated with sample buffer containing β -mercaptoethanol and subjected to 10% (w/v) SDS/PAGE. Proteins were electrotransferred onto PVDF membranes overnight, 20V at 4°C. Membranes were blocked in TBS containing 0.1% Tween 20 (TBS-T) and 5% (w/v) nonfat dry milk, pH 7.6, for 2 h at room temperature. Membranes were washed in TBS-T and incubated overnight at 4°C with primary antibody. To mark total and phosphorylated MAP kinase, we used rabbit polyclonal antibodies against rat p44/p42 MAP kinase (#9102, Cell Signaling Technology) and phospho-p44/p42 MAP kinase (Thr202/Tyr204, #9101, Cell Signaling Technology), diluted 1:2000 and 1:1000, respectively, in blocking solution. Total and phosphorylated AKT were labeled with rabbit monoclonal antibody against a fragment in the C-terminal sequence of mouse AKT (pan-AKT, #4691; Cell Signaling Technology) and polyclonal antibody against phospho-Ser473 AKT (p-AKT, Ser473, #9198; Cell Signaling Technology), diluted in blocking solution, 1:2000 and 1:1000, respectively.

Membranes were next incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (GE Healthcare), and proteins were visualized with enhanced chemiluminescence reagent (ECL, GE Healthcare). Apparent molecular weights were compared with molecular weight standards (New England Biolabs, Boston, MA, USA), and band intensities were quantified by densitometry analysis of linear-range immunoblots by using Epson Expression 1680 scanner

(Epson America Inc., Long Beach, CA, USA) and Quick Scan 2000 WIN software (Helena Laboratories, Beaumont, TX, USA). Results were normalized by total ERK1/2 or AKT expression, and plotted (mean $\pm$ S.E.M.) in relation to control (=1).

Western blotting for detection of phospho-CREB (Ser133), NF- κ B/p65 and I κ B- α

On culture day 4, Sertoli cells plated in 100 mm dishes were incubated in the absence (control) and presence of 100 ng/mL FSH and/or 50 ng/mL relaxin, for the indicated times at 35°C. Nuclear fractions were obtained as described previously (Arai *et al.* 2006, Royer *et al.* 2012). Briefly, the cells were washed with ice-cold PBS and lysed in ice-cold lysis buffer A (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% v/v Nonidet P40, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF, 10 μ g/mL leupeptin and 10 μ g/mL aprotinin). After 20 min incubation on ice, the lysate was slowly added to lysis buffer A containing 1.0 M sucrose. After centrifugation at 13,000 g for 30 min at 4°C, the supernatant was centrifuged again (13,000 g, 30 min, 4°C) to obtain the cytoplasmic fraction, and the pellet, containing the nuclear fraction, was washed and resuspended in 100 μ L lysis buffer B (10 mM HEPES, 200 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, pH 7.9, 5% v/v glycerol, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF, 10 μ g/mL leupeptin and 10 μ g/mL aprotinin). The nuclear fraction was the supernatant obtained after centrifugation at 13,000 g, for 30 min at 4°C.

Cytoplasm or nuclear extracts (20 μ g/lane) were incubated with sample buffer containing DTT and subjected to 10% (w/v) SDS/PAGE. Proteins were electrotransferred onto PVDF membranes as described before, and membranes were blocked in TBS-T containing 10% w/v nonfat dry milk, pH 7.6, for 2 h at room temperature. After washes in TBS-T, membranes were incubated overnight at 4°C with a rabbit monoclonal antibody against a synthetic peptide derived from a sequence containing the phospho-Ser133 from human CREB (Phospho-CREB (Ser 133), #9198; Cell Signaling Technology), 1:1000 dilution in blocking solution, or a rabbit monoclonal antibody corresponding to residues near the N-terminus of NF- κ B/p65 of human origin (#4764, Cell Signaling Technology), 1:600 dilution, or a rabbit monoclonal antibody against a synthetic peptide derived from a region near the carboxyterminal from human I κ B α (44D4, #4812, Cell Signaling Technology), 1:600 dilution. Proteins were visualized after a chemiluminescence reaction, as described before. The nuclear protein loading control was lamin A, which was detected with a rabbit antibody raised against a synthetic peptide derived from residues 563–664 of Lamin A (Santa Cruz Biotechnology). The cytoplasm loading control was actin, detected with a rabbit antibody raised against an epitope from the aminoterminal region of actin (residues 20–33), which is conserved in all actin isoforms (Sigma, A5060). Lamin A and actin antibodies were used at 1:250 dilution, overnight at 4°C.

Band intensities of phospho-CREB, NF- κ B/p65, I κ B α , Lamin A and Actin (ACTB) from individual experiments were quantified as described above. Results were normalized to

Figure 1 Effect of FSH on Sertoli cell proliferation. (A) [Methyl-³H] thymidine incorporation assay. Cells were incubated in the absence (basal incorporation, control) or in the presence of FSH (100 ng/mL), relaxin (RLN; 50 ng/mL) or in the presence of both hormones (FSH+RLN) for 24 h. Cell-bound radioactivity was determined and the results were expressed in relation to control (mean $\pm$ S.E.M. of three independent experiments performed in triplicate). Statistically significant differences are indicated by different letters (ANOVA followed by Newman–Keuls, $P < 0.05$); ^a indicates difference between control and all other groups; ^b and ^c indicate difference between RLN and FSH groups; ^{b,c} indicates no difference between FSH+RLN group and RLN or FSH groups. (B) Western blotting analysis of the expression of cyclin D1. The graph shows the densitometric analysis of two independent experiments (different cultures). Cells were treated in the absence (control) or in the presence of FSH (100 ng/mL) for 24 h. To obtain the loading control the membranes were stripped and probed with an antibody specific for actin.

the respective Lamin A or ACTB expression and plotted (mean $\pm$ S.E.M.) in relation to control (=1).

Western blotting for detection of cyclin D1

Primary Sertoli cell cultures on culture day 4 were incubated in the absence (control) and presence of 100 ng/mL FSH for 24 h at 35°C. Western blotting assays were performed as described previously (Lucas *et al.* 2010), using a rabbit polyclonal antibody raised against a synthetic peptide derived from residues near the C-terminus of cyclin D1 (#2922, Cell Signaling, 1:500). Actin levels were monitored on the same blot to ensure equal protein loading.

[Methyl-³H] thymidine incorporation assays

Incorporation of [methyl-³H] thymidine into cell DNA was estimated as described previously (Nascimento *et al.* 2012). Briefly, primary Sertoli cell cultures on culture day 4 were initially incubated with 2 μ Ci/mL [methyl-³H] thymidine for 6 h at 35°C. Subsequently, cells were incubated in the

Figure 2 Effects of FSH on AKT and ERK1/2 phosphorylation. Cells were incubated in the absence (control) or in the presence of FSH (100 ng/mL) for different periods. Total cell lysates were resolved on 10% SDS/PAGE, transferred to PVDF membranes, and probed with: (A) antibody specific for phosphorylated AKT (Ser473) (top panel) and antibody that recognizes total AKT (bottom panel); (B) antibody specific for phosphorylated ERK1/2 (p-ERK1/2, top panel) and antibody that recognizes total ERK1/2 proteins (bottom panel). The molecular mass of the proteins are shown at the right. Bars represent the densitometric analysis of the Western blotting. Results were normalized to total AKT or total ERK1/2 expression in each sample and plotted (mean $\pm$ S.E.M.) in relation to controls (=1). The data shown are representative of four independent experiments. Different letters indicate statistically significant differences (ANOVA followed by Newman–Keuls, $P < 0.05$).

absence or presence of 100 ng/mL FSH or 50 ng/mL relaxin or the combination of 100 ng/mL FSH and 50 ng/mL relaxin, for 24 h at 35°C. The reaction was stopped by cooling the cells to 0°C. The medium was aspirated and the cells were rinsed twice with ice-cold PBS and 5% trichloroacetic acid (Sigma). The cells were then solubilized with 0.5 N NaOH, collected with cotton-swabs and transferred to 5 mL OptiPhase HiSafe-3 scintillation liquid (PerkinElmer Life Science Products). Bound radioactivity was determined in a scintillation β counter (LS 6000 IC, Beckman Coulter Inc, Palo Alto, CA, USA). Results were expressed in relation to control, basal levels of [methyl- 3 H] thymidine incorporation (absence of hormones).

Analysis of the effects of FSH on cell cycle gene expression

Cells were plated on 100 mm dishes and were treated on culture day 4 in the absence (control) or in the presence of FSH (100 ng/mL; 2.8 nM) for 4 h. Total RNA was extracted with the TRIzol reagent (Life Technologies) according to the standard protocol (Chomczynski & Sacchi 1987). Elimination of genomic DNA contamination was performed with the RNeasy mini kit (Qiagen) as described previously (Yasuhara *et al.* 2008). RNA concentration was determined by spectrometry at 260 nm, and the purity of the samples was calculated by A260/A280 ratio. All calculated ratio values were ≥ 1.98 . The first strand of cDNA was synthesized with the RT2 First Strand Kit (Qiagen), following the instructions of the manufacturer.

For gene expression analysis, 1 μ L cDNA was used in the rat cell cycle RT2 Profiler PCR Array (PARN-020Z, Qiagen), which simultaneously evaluates the expression of 84 genes related with cell proliferation and differentiation, using SYBR Green Master Mix (Qiagen). A total of four arrays (a different cDNA per array plate) were performed for each experimental group (control and FSH). The average expression of five genes (P1 ribosomal protein, large, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13A, lactate dehydrogenase A and beta actin) was

Figure 3 Effects of FSH on cyclic AMP production, CREB phosphorylation and NF- κ B expression. (A) Cells were incubated in the absence (control) or in the presence of FSH (100 ng/mL) for 30 min and cAMP production was determined by enzyme immunoassay. Results are expressed as mean $\pm$ S.E.M. of four independent experiments, in triplicate. (B) and (C) Cells were treated or not (control) with FSH (100 ng/mL) for different periods. (B) Expression of phosphorylated CREB was detected in nuclear extracts with an antibody that recognizes phosphorylated CREB at Ser 133 (top panels); (C) Expression of NF- κ B (p65) was determined in nuclear and cytoplasm fractions. To obtain the loading controls the membranes were stripped and probed with an antibody specific for lamin A (nuclear fractions) or actin (cytoplasm fraction). Bars represent the densitometric analysis of the Western blotting. Results were normalized to lamin A or actin expression in each sample and plotted (mean $\pm$ S.E.M.) in relation to controls (=1). The data shown are representative of 3–5 independent experiments. The asterisks indicate statistically significant differences from control (Student's *t*-test, $P < 0.05$).

used as endogenous control. The results were calculated by the $\Delta\Delta CT$ method, using software available at the GeneGlobe Data Analysis Center from Qiagen. The gene expression alteration was calculated by the ratio between $\Delta\Delta CT$ values of FSH and control groups. Only genes with at least twofold increase or decrease in expression were selected for further analysis. Statistical analysis was performed according to Qiagen instructions. P values < 0.05 were accepted as significant.

Validation of the PCR array and analysis of the effects of relaxin and inhibitors of CREB, PKA and Epac on gene expression modulated by FSH

Participation of CREB, PKA and Epac on the FSH-induced effects on gene expression was assessed by pretreating cells in the absence or presence of the PKA inhibitor (H89, 2 μ M, 2 h; Sigma Chemical Co.), the inhibitor of the phospho-CREB–CBP complex, KG-501 (25 μ M, 30 min; Sigma Chemical Co.), or the inhibitor of Epacs (ESI-09; 2 μ M, 30 min, Sigma Chemical Co.). Cells were then stimulated or not (control) with FSH (100 ng/mL) for 4 h. In a separate series of experiments, cells were stimulated or not (control) with FSH (100 ng/mL), in the absence or in the presence of relaxin (50 ng/mL) for 4 h. RNA extraction and cDNA synthesis were performed as described above.

The PCR array results for the genes mostly affected by FSH were validated by quantitative RT-PCR (RT-qPCR), using the SYBR Green system (Applied Biosystems). The primers (Invitrogen) were designed with the Primer3 program (Rozen & Skaletsky 2000), and spanned exon–exon boundaries whenever possible: inhibin alpha (*Inba*), forward 5'-CTTTCCAGCCACAGGTGC-3' and reverse 5'-GTTGGGATGGCCGGAATACA-3'; *Bcl2*, forward 5'-GTAGCCATCCAGGCTGTGT-3' and reverse 5'-GGTAA GTTGAAGGCCCGAA-3'; actin (*Actb*), forward 5'-GTAGC CATCCAGGCTGTGT-3' and reverse 5'-CCCTCATAGATG GGCACAGT-3'. *Actb* was used as an endogenous control and to normalize cDNA amount and PCR efficiency. Primers for all target genes and actin were designed to have approximately the same amplification efficiency (≈ 1), which was calculated as described previously (Cardoso et al. 2010). Controls without cDNA were included in each assay. An aliquot of 1 μ L cDNA was used in the PCR, in a final volume of 25 μ L. Samples were run in triplicates in ABI 7500 Real-Time PCR System (Applied Biosystems), using default conditions of amplification (50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min). To confirm specificity of the amplification, dissociation curves were obtained at the end. The average cycle threshold (CT) was determined with Applied Biosystems software. Data were analyzed by the comparative $\Delta\Delta CT$ method (ABI PRISM User Bulletin #2, Applied Biosystems). A pool of cDNAs from control cultures was used as a calibrator of the experiment. Data are expressed as mean $\pm$ S.E.M. of the $2^{-\Delta\Delta CT}$ from four different cDNAs (four different cultures). At the end of the experiments, samples were run in 2% (w/v) agarose gel electrophoresis to further confirm the absence of nonspecific amplification. The size of the expected products

was compared with a DNA ladder (100 bp ladder, Life Technologies).

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Data were analyzed by ANOVA followed by Newman–Keuls for multiple comparisons or Student's t -test for comparisons between two groups.

Figure 4 PCR array analysis of the effects of FSH on expression of cell cycle genes. Cells were stimulated or not (control) with FSH (100 ng/mL) for 4 h. (A) Two-dimensional scatter plots of the PCR array. Each dot represents the average from four independent experiments (different RNAs) for the same gene in control (horizontal axis) and FSH (vertical axis) groups. Genes that were expressed similarly in control and FSH groups appear along the central diagonal ($y = x$); genes differently expressed by the groups appear above or below this line. The two parallel lines mark the limits for twofold difference. The vertical dashed line indicates the minimum limit of gene expression for analysis. (B) Heat map representation of the average effects of FSH on expression of cell cycle genes. The asterisks represent statistically significant difference from control (Student's t -test; $P < 0.05$).

Statistical tests were done with GraphPad Prism 5.01 software. *P* values < 0.05 were accepted as significant.

Results

FSH stimulates proliferation of Sertoli cells from 15-day-old rats

FSH increased by 23% the incorporation of ³H-thymidine (Fig. 1A) and increased by twofold the expression of cyclin D1 in Sertoli cells of 15-day-old rats (Fig. 1B). These results indicate that the hormone stimulates cell proliferation, and corroborate our previous findings (Cardoso *et al.* 2010). Relaxin also increased the incorporation of ³H-thymidine, and the combination of FSH and relaxin did not potentiate the effect of each hormone separately (Fig. 1A), confirming our previous findings. Relaxin did not significantly affect the expression of cyclin D1 in the Western blotting analysis (data not shown).

FSH inhibits phosphorylation of AKT and ERK1/2, and activates cAMP/CREB in Sertoli cells from 15-day-old rats

It has been shown that the proliferative effect of FSH on Sertoli cells from 8-day-old rats depends on activation of PI3K/AKT/mTORC1 pathway (Riera *et al.* 2012). In contrast, we found that FSH reduced basal AKT

phosphorylation in Sertoli cells from 15-day-old rats, which is the final stage of Sertoli cell proliferation (Fig. 2A). The effect of FSH was rapid and lasted for at least 60 min.

Although activation of the ERK1/2 pathway has been shown to mediate FSH-induced proliferation of Sertoli cells from 5-day-old rats (Crépieux *et al.* 2001), FSH induced a rapid and sustained inhibition of ERK1/2 phosphorylation in Sertoli cells from 15-day-old rats (Fig. 2B).

It has been described that FSH does not stimulate or only slightly stimulates the production of cAMP in cultured or freshly isolated Sertoli cells from 5- and 9-day-old rats (Bhattacharya *et al.* 2012). FSH strongly activated cAMP production in Sertoli cells from 15-day-old rats (Fig. 3A).

CREB and NF-κB transcription factors have been implicated on the modulation of gene transcription by FSH in Sertoli cells (Walker *et al.* 1995, Delfino & Walker 1998). The effect of FSH on phosphorylation of Ser133 of CREB was measured in Sertoli cells from 15-day-old rats (Fig. 3B). FSH induced a rapid phosphorylation of CREB, which persisted for at least 30 min.

The p65/p50 heterodimer is the most abundant dimer of NF-κB family present in Sertoli cells (Delfino & Walker 1998). To evaluate the effect of FSH on the activation of NF-κB we measured the expression of p65 (RelA) in the cytoplasm and nucleus of Sertoli

Table 1 Genes significantly affected by FSH in the cell cycle PCR array.

Genes		Function	Fold regulation
Downregulated			
<i>Bcl2</i> (NM_016993)	B-cell CLL/lymphoma 2	Anti-apoptotic	−5.1
<i>Rbl1</i> (NM_001191066.1)	Retinoblastoma-like 1 (p107)	Key regulator of entry into cell division	−2.7
<i>Ppp2r3a</i> (NM_001012202)	Protein phosphatase 2, regulatory subunit B", alpha	G2/M transition of mitotic cell cycle	−2.9
<i>Brca1</i> (NM_012514)	Breast cancer 1	Breast cancer suppressor	−2.4
<i>Mki67</i> (NM_001271366.1)	Antigen identified by monoclonal antibody Ki-67	Marker of cell proliferation	−2.2
<i>Loc367976</i> (XM_346381)	Similar to DNA replication licensing factor MCM3 (DNA polymerase alpha holoenzyme-associated protein P1) (P1-MCM3)	Involved in initiating eukaryotic genome replication	−2.2
<i>Atm</i> (NM_001106821)	Ataxia telangiectasia mutated homolog (human)	DNA damage checkpoint	−2.1
<i>Mcm4</i> (NM_033651)	Minichromosome maintenance complex component 4	DNA replication licensing factor	−2.1
<i>Smc1a</i> (NM_031683)	Structural maintenance of chromosomes 1A	DNA repair, mitotic cell cycle checkpoint	−2.0
Upregulated			
<i>Inba</i> (NM_012590)	Inhibin alpha	Inhibition of FSH secretion, of gonadal stromal cell proliferation; tumor-suppressor activity	6.1
<i>Sfn</i> (XM_232745)	Stratifin	Epithelial marker; involved in the protein kinase C signaling pathway	2.9
<i>Cdkn1a</i> (NM_080782)	Cyclin-dependent kinase inhibitor 1A	Inhibition of the activity of cyclin-CDK complexes; regulator of cell cycle progression at G1	2.4
<i>Cdkn1b</i> (NM_031762)	Cyclin-dependent kinase inhibitor 1B	Inhibition of the activity of cyclin-CDK complexes	2.1

Statistical analysis: Student's *t*-test (*P* < 0.05).

Figure 5 Validation of the PCR array and analysis of the effect of inhibition of CREB, PKA or Epac1/2 on FSH-induced expression of inhibin α and *Bcl2*. Cells were pretreated or not (control) with (A) the CREB inhibitor KG 501 (25 μ M, 30 min), (B) the PKA inhibitor H89 (2 μ M, 2 h) or (C) the Epac1/2 inhibitor ESI-09 (2 μ M, 30 min), and stimulated or not (control) with FSH (100 ng/mL) for 4 h. Gene expression was determined by quantitative PCR. Results are expressed as mean $\pm$ S.E.M. of four independent experiments, performed in triplicate. Different letters indicate statistically significant differences (ANOVA followed by Newman–Keuls, $P < 0.05$).

cells from 15-day-old rats (Fig. 3C). FSH significantly inhibited the expression of p65 in the nuclear fraction after 10, 15 and 30 min of treatment.

FSH effects on the expression of cell cycle genes in Sertoli cells from 15-day-old rats

We next investigated the effects of FSH on the expression of cell cycle genes using a PCR array. Whereas FSH rapidly changes cAMP production and CREB, ERK1/2 and AKT phosphorylation, effects of FSH on gene expression are detected later and usually take 4 h to peak, according to our experience. Effects of FSH on cell proliferation require even longer because they involve synthesis of proteins and their action on the cell cycle.

A scatterplot summary of the PCR array expression data including 84 genes related to cell cycle regulation is shown in Fig. 4A, and the heat map representation

Figure 6 Effects of relaxin on cyclic AMP production. (A) Cells were incubated in the absence (control) or in the presence of relaxin (50 ng/mL) for different periods, and cAMP production was determined by enzyme immunoassay. Results are expressed as mean $\pm$ S.E.M. of four independent experiments, in triplicate. The asterisk represents statistically significant differences compared with control (Student's *t*-test; $P < 0.05$). (B) Cells were previously stimulated or not (control) with FSH (100 ng/mL) for 30 min, and afterward relaxin (RLN; 50 ng/mL) was added for different periods. The asterisk represents statistically significant differences compared with control (Student's *t*-test; $P < 0.05$). (C) Cells were treated with FSH (100 ng/mL) and/or RLN (50 ng/mL) for 30 min. Results are expressed as mean $\pm$ S.E.M. of 3–4 independent experiments in triplicate. Different letters indicate statistically significant differences (ANOVA followed by Newman–Keuls, $P < 0.05$).

of the results is shown in Fig. 4B. When the arbitrary difference of at least twofold change was selected, FSH significantly downregulated the expression of 9 genes and upregulated the expression of 4 genes (Fig. 4 and Table 1). The complete list of the FSH effects on cell cycle gene expression is shown in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Although some effects of FSH seemed to be related to cell proliferation (downregulation of *Rb1* and *Ppp2r3a*), most of the effects seemed to be in order to inhibit proliferation or stimulate differentiation (downregulation of *Bcl2*, *Mcm4*, *Loc367976*, *Terf1*, *Smc1a*, *Mki67*, *Atm*, *Brcal* and upregulation of *Inba*, *Cdkn1a*, *Cdkn1b*, *Sfn*).

The most affected genes were B-cell CLL/lymphoma 2 (*Bcl2*; 5.1-fold downregulation), which encodes the anti-apoptotic protein BCL2 and *Inba* (6.1-fold upregulation), which encodes the differentiation marker inhibin alpha. These results were confirmed by quantitative PCR (Fig. 5). The effect of FSH on *Inba* and *Bcl2* expression was blunted by inhibition of the complex CREB–CBP with KG-501 (Fig. 5A), but not by inhibition of PKA with H89 (Fig. 5B). The effects of FSH on *Inba* and *Bcl2* expression were not affected by the Epac inhibitor ESI-09 (Fig. 5C).

Relaxin inhibits basal and FSH-stimulated cAMP production, inhibits CREB and activates NF- κ B in Sertoli cells from 15-day-old rats

We have shown previously, and confirmed presently, that relaxin induces proliferation of Sertoli cells from 15-day-old rats, but does not synergize the effect of FSH (Cardoso *et al.* 2010). We then investigated whether relaxin could modulate the effect of FSH on cAMP production at this time of Sertoli cell development. The time course of relaxin effect on cAMP production is shown in Fig. 6A. Relaxin gradually reduced basal cAMP accumulation. The inhibitory effect, which was almost significant after 15 min ($P=0.058$), was statistically significant after 30 min ($P=0.02$).

Figure 7 Effects of relaxin on the activation of CREB and NF- κ B transcription factors. Cells were treated or not (control) with relaxin (50 ng/mL) for different periods. (A) Expression of phosphorylated CREB was detected in nuclear extracts with an antibody that recognizes phosphorylated CREB at Ser 133 (top panels); (B) expression of NF- κ B (p65) was determined in nuclear and cytoplasmic fractions; (C) expression of I κ B- α was determined after stripping the PVDF membranes used to determine expression of NF- κ B (p65) in the cytoplasm. To obtain the loading controls the membranes were stripped and probed with an antibody specific for lamin A (nuclear fractions) or actin (cytoplasmic fraction). Bars represent the densitometric analysis of the Western blotting. Results were normalized to lamin A or actin expression in each sample and plotted (mean $\pm$ S.E.M.) in relation to controls ($=1$). The data shown are representative of 3–5 independent experiments. The asterisks indicate statistical significant differences from control (Student's *t*-test, $P<0.05$).

Relaxin addition for different periods was not able to reduce cAMP production when cells were previously stimulated with FSH for 30 min (Fig. 6B). On the other hand, relaxin significantly reduced, but did not abolish, the stimulation of cAMP production by FSH when both hormones were added together for 30 min (Fig. 6C).

Relaxin significantly inhibited phosphorylation of CREB after 15 min (Fig. 7A). Treatment with relaxin decreased the expression of p65 in the cytoplasm after 10 and 15 min, and increased the nuclear expression of p65 (statistically significant after 15 min) (Fig. 7B). In addition, treatment with relaxin for 10, 15 and 30 min significantly reduced the expression of IKB- α (Fig. 7C).

Effects of relaxin and cAMP/PKA pathway on FSH-mediated inhibition of ERK1/2 and AKT phosphorylation

Confirming our previous findings (Nascimento *et al.* 2012), relaxin rapidly stimulated ERK1/2 and AKT phosphorylation (Fig. 8A and B, respectively). The combined effect of relaxin and FSH was examined after 5 min because we had demonstrated previously that relaxin transiently activated ERK1/2 and AKT phosphorylation with a peak after 5 min (Nascimento *et al.* 2012). When FSH and relaxin were simultaneously added to cells for 5 min, the inhibitory effect of FSH on ERK1/2 phosphorylation was completely blocked (Fig. 8A, graph I), and the inhibitory effect of FSH on AKT phosphorylation was attenuated (Fig. 8B, graph I).

Figure 8 Relaxin and cAMP/PKA pathway modulate the inhibitory effect of FSH on ERK1/2 and AKT phosphorylation. Cells were pretreated or not (control) with PKA inhibitor H89 (2 μ M, 2 h) or phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; 0.5 mM, 30 min) and stimulated or not (control) with FSH (100 ng/mL) and/or relaxin (RLN, 50 ng/mL) for 5 min. Total cell lysates were resolved on 10% SDS/PAGE, transferred to PVDF membranes, and probed with (A) antibody specific for phosphorylated ERK1/2 (p-ERK1/2, top panel) and antibody that recognizes total ERK1/2 proteins (bottom panel); (B) antibody specific for phosphorylated AKT (Ser473) (top panel) and antibody that recognizes total AKT (bottom panel). Bars represent the densitometric analysis of the Western blotting. Results were normalized to total ERK1/2 or total AKT expression in each sample and plotted (mean $\pm$ S.E.M.) in relation to controls (C=1). The data shown are representative of 3–4 independent experiments. Different letters indicate statistically significant differences (ANOVA followed by Newman–Keuls, $P < 0.05$).

Figure 9 The inhibitory effect of FSH on phosphorylation of ERK1/2 and AKT does not involve participation of Epac1/2. Cells were pretreated or not (control) with the Epac1/2 inhibitor ESI-09 (2 μ M, 30 min), and stimulated or not (control) with FSH (100 ng/mL). Total cell lysates were resolved on 10% SDS/PAGE, transferred to PVDF membranes and probed with (A) antibody specific for phosphorylated ERK1/2 (p-ERK1/2, top panel) and antibody that recognizes total ERK1/2 proteins (bottom panel); (B) antibody specific for phosphorylated AKT (Ser473) (top panel) and antibody that recognizes total AKT (bottom panel). Bars represent the densitometric analysis of the Western blotting. Results were normalized to total ERK1/2 or total AKT expression in each sample and plotted (mean $\pm$ S.E.M.) in relation to controls (=1). The data shown are representative of 3–4 independent experiments. Different letters indicate statistically significant differences (ANOVA followed by Newman–Keuls, $P < 0.05$).

Figure 10 Relaxin does not inhibit the effects of FSH on mRNA levels of *Inba* (A) and *Bcl-2* (B). Cells were stimulated or not (control) with FSH (100 ng/mL), relaxin (RLN, 50 ng/mL) or both (FSH+RLN) for 4 h. Gene expression was determined by quantitative PCR. Results are expressed as mean $\pm$ S.E.M. of three independent experiments, performed in triplicate. Different letters indicate statistically significant differences (ANOVA followed by Newman–Keuls, $P < 0.05$).

To investigate whether the inhibitory effect of FSH on ERK1/2 and AKT phosphorylation correlated to PKA activation and/or to cyclic AMP levels, we previously treated cells with the PKA inhibitor H89 or with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Figure 8A (graph II) shows that H89 did not significantly affect the inhibitory effect of FSH, but in the presence of H89 the effect of FSH was not significantly different from control. The inhibitory effect of FSH was completely reversed when both relaxin and H89 were used. The increase of cAMP levels induced by IBMX increased the inhibitory effect of FSH and blunted basal phosphorylation of ERK1/2 and the phosphorylation of ERK1/2 induced after simultaneous addition of relaxin and FSH (graph III).

Figure 8B (graph II) shows that pretreatment with H89 attenuated the inhibitory effect of FSH on AKT phosphorylation. Treatment with both relaxin and H89 was not more effective than H89 alone. FSH almost

completely blocked the phosphorylation of AKT, and it was not possible to see an increase of the inhibitory effect of FSH in the presence of IBMX (graph III). Similar to what happened with ERK1/2 activation, treatment with IBMX completely blocked basal phosphorylation of AKT, and the phosphorylation of AKT induced by relaxin in the presence of FSH.

To evaluate the participation of Epacs on the inhibitory effect of FSH on ERK1/2 and AKT phosphorylation, cells were pretreated with the inhibitor of Epac1/2, ESI-09 (Fig. 9A and B). ESI-09 alone significantly inhibited basal phosphorylation of ERK1/2 (Fig. 9A) but not basal phosphorylation of AKT (Fig. 9B). Treatment with ESI-09 did not affect FSH-induced inhibition of ERK1/2 and AKT phosphorylation (Fig. 9A and B).

Relaxin does not inhibit the effects of FSH on gene expression of inhibin alpha and Bcl-2

We next investigated whether the opposite effects of relaxin on signaling pathways affected by FSH would influence the effects of FSH on gene expression. Relaxin did not affect the mRNA levels of *Inba* (Fig. 10A) and *Bcl-2* (Fig. 10B). Relaxin did not affect the FSH-induced stimulation of *Inba* mRNA levels (Fig. 10A) and the FSH-induced inhibition of *Bcl-2* mRNA levels (Fig. 10B).

Discussion

It has been demonstrated that FSH stimulates proliferation of immature Sertoli cells by activation of PI3K/AKT/mTORC1 and MEK/ERK1/2 pathways (Crépieux *et al.* 2001, Riera *et al.* 2012). Mature Sertoli cells remain responsive to FSH but they fail to proliferate. The molecular mechanisms involved in this change are unknown. One possible explanation is that FSH signaling switches from activation of PI3K/AKT/mTORC1 and MEK/ERK1/2 pathways to activation of the cAMP pathway (Bhattacharya *et al.* 2012). To gain insight into the mechanisms involved in this switch, we analyzed the effects of FSH and relaxin on intracellular signaling pathways involved with proliferation and differentiation in Sertoli cells that are close to the transition between these two stages. The results presented herein indicate that both hormones affect the same signaling pathways in opposite directions, and that relaxin may play a role as a paracrine factor that modulates FSH signaling. It seems that the balance between proliferation and differentiation is regulated by the relative contribution of cAMP/PKA/CREB, MEK/ERK1/2 and PI3K/AKT/mTORC1 pathways.

The switch in FSH signaling toward activation of cAMP production occurs before the end of Sertoli cell proliferation. Although the activation of the cAMP pathway seems to direct cells to differentiation, it is not completely incompatible with cell proliferation. FSH stimulates several effects indicative of proliferation in

Sertoli cells of 15-day-old rats, including incorporation of ³H-thymidine, stimulation of cyclin D1 expression and downregulation of *Rb1* and *Ppp2r3a* expression. These proliferative effects are not mediated by the activation of ERK1/2 or PI3K/AKT pathways because FSH inhibits phosphorylation of ERK1/2 and AKT. These proliferative effects are probably mediated by the stimulation of cAMP production and CREB phosphorylation, similar to the proliferative effect of FSH in granulosa cells, which involves cAMP-dependent increase of cyclin D2 (Robker & Richards 1998). It has already been demonstrated by chromatin immunoprecipitation that phosphorylated CREB associates with the promoter of cyclin D1 (Yan *et al.* 2013). It must be noted, however, that cAMP is involved in both proliferation and differentiation in other cell types (Vitali *et al.* 2014, 2015).

Although FSH affected the expression of genes like *Rb1* and *Ppp2r3a* in order to activate proliferation, most of the cell cycle genes affected by FSH were related to differentiation or inhibition of cell proliferation. FSH strongly activated transcription of the differentiation marker *Inba*, and strongly inhibited the transcription of the anti-apoptotic gene *Bcl2*. Differentiated Sertoli cells from 20-day-old rats express high levels of inhibin alpha. When differentiated Sertoli cells from 20- and 60-day-old rats are forced to proliferate by overexpression of ID proteins (inhibitors of differentiation), the expression of inhibin alpha (gene and protein) is strongly inhibited (Chaudhary *et al.* 2005). Although FSH stimulates proliferation of Sertoli cells from 15-day-old rats, it starts to affect signaling pathways and gene expression to favor differentiation and this effect involves activation of cAMP/ CREB pathway. Considering that the effect of FSH on inhibin A and *Bcl2* gene expression was inhibited by the CREB inhibitor KG-501 but not by the PKA inhibitor H89, some effects of FSH in Sertoli cells may involve PKA-independent mechanisms. For instance, the stimulation of cAMP production by FSH may stimulate calcium influx, with a subsequent activation of calcium-calmodulin kinases, which phosphorylate CREB by a PKA-independent pathway (Gorczyńska & Handelsman 1991, Walker & Cheng 2005).

The effect of FSH on gene expression was greatly dependent on the activation of CREB, but did not involve activation of NF-κB. In fact, FSH inhibited the nuclear expression of NF-κB (p65) at all times analyzed. Activation of NF-κB by FSH in Sertoli cells may be influenced by the culture conditions, because Delfino and Walker (1998) described that FSH increased NF-κB expression in Sertoli cells from 16-day-old rats cultured in Matrigel.

The effect of FSH on cAMP production depends on the stage of Sertoli cell development. FSH stimulated the production of cAMP in cultured or freshly isolated Sertoli cells from 12-, 19- and 20-day-old rats (Meroni *et al.* 2002, Bhattacharya *et al.* 2012, Levallet *et al.* 2013), but had little or no effect on the production of

cAMP in cultured or freshly isolated Sertoli cells from 5- and 9-day-old rats (Crépieux *et al.* 2001, Bhattacharya *et al.* 2012). The reason for this is still under debate. Bhattacharya and coworkers (Bhattacharya *et al.* 2012) suggested that the increase of cAMP production and differentiation of Sertoli cells induced by FSH could be due to increased expression of the FSHR in the plasma membrane and increased FSHR affinity. On the other hand, Musnier and coworkers (Musnier *et al.* 2009) reported no significant differences in FSHR affinity or FSHR density, but a difference in FSH signaling between immature and differentiate Sertoli cells. Lamsam-Casalotti and coworkers (1993) attributed the changes in FSH signaling with development of Sertoli cells of the rat to a gradual increase of expression of G_s between the 7th and the 23rd day of age. In accordance with these data, a recent study from Bhattacharya *et al.* (2015) suggested that the lack of FSH-mediated downregulation of $G_{\alpha i-2}$, the limited expression of $G_{\alpha s}$ and of the G-protein exchange factor Ric8b may underlie limited responsiveness of Sertoli cells to FSH during infancy in primates. In another line of investigation, Levallet and coworkers (Levallet *et al.* 2013) suggested that cell proteoglycans such as syndecan-1 could be involved in the increase of the cAMP response to FSH in the transition between proliferative and differentiate stages of Sertoli cell development.

Activation of ERK1/2 and AKT by FSH also depends on the stage of Sertoli cell development. It has been shown previously that FSH induces activation of ERK1/2 in Sertoli cells from 5-, 10- and 12-day-old rats, which are still proliferating, but not in Sertoli cells from 19- and 20-day-old rats, which are already differentiated (Crépieux *et al.* 2001, McDonald *et al.* 2006, Shupe *et al.* 2011, Levallet *et al.* 2013). Furthermore, FSH increased AKT phosphorylation in Sertoli cells from 8-day-old rats (McDonald *et al.* 2006, Riera *et al.* 2012) but not in Sertoli cells from 19-day-old rats (Dupont *et al.* 2010). The lack of a mitogenic effect of FSH in Sertoli cells from 19-day-old rats was related to activation of PTEN (phosphatase and tensin homolog deleted in chromosome 10), the major regulator of PI3K activity (Dupont *et al.* 2010).

The cAMP/PKA pathway may cause either activation or inhibition of the ERK1/2 pathway, depending on the isoform of the Raf protein kinase expressed in the cell (Luttrell & Luttrell 2003). PKA may phosphorylate the isoform Raf-b, which activates ERK1/2 or the isoform Raf-1, uncoupling it from Ras and inhibiting ERK1/2 pathway (Luttrell & Luttrell 2003). Our results show that the robust activation of cAMP production by FSH in Sertoli cells from 15-day-old rats was related to inhibition of ERK1/2, which corroborates the results from Shupe and coworkers (Shupe *et al.* 2011) and Levallet and coworkers (Levallet *et al.* 2013). In addition, our results do not provide a clear-cut evidence for the involvement of PKA in FSH-induced inhibition

of ERK1/2 phosphorylation, because the effect of FSH in the presence of the PKA inhibitor was not statistically different from control or from the effect of FSH alone.

The stimulation of cAMP production by FSH correlated with the inhibition of AKT, and this effect was PKA-dependent. A similar inhibition of AKT, which involved a cAMP/PKA-dependent activation of Rap-1b, was mediated by TSH in the thyroid follicular cell line PCCL3 (Lou *et al.* 2002).

Depending on the cell type, the nucleotide exchange proteins activated by cAMP, Epac1 and Epac2 may, alone or in conjunction with PKA, modulate cell proliferation, and activation of ERK1/2, PI3K/AKT and CREB (Wang *et al.* 2001, Gerlo *et al.* 2005, Vitali *et al.* 2014). Since the Epac1/2 inhibitor ESI-09 failed to reduce the inhibitory effect of FSH on ERK1/2 and AKT phosphorylation, the participation of Epacs was discarded.

As increased cAMP production inhibits AKT and ERK1/2 activation, and since the ability of FSH to stimulate cAMP production increases with cell maturation, it seems reasonable to propose that stronger activation of cAMP production is directly related to Sertoli cell differentiation, as already proposed by other authors (Crépieux *et al.* 2001). The balance between the activation of cAMP production and the inhibition of PI3K/AKT/mTORC1 and MEK/ERK1/2 pathways may therefore determine the end of cell proliferation and the start of cell differentiation.

Our studies also revealed that relaxin, a peptide produced by Sertoli cells, plays not only a direct role on Sertoli cell proliferation (Cardoso *et al.* 2010, Nascimento *et al.* 2012) but may regulate FSH action, and this effect seems particularly important at this transition phase of cell development to ensure the appropriate number

Figure 11 Proposed mechanism for the interactions between FSH and relaxin signaling to modulate the proliferation and differentiation of Sertoli cells from 15-day-old rats. FSH binds to FSHR and stimulates cAMP production, which leads to CREB activation and modulation of gene expression related to cell differentiation. On the other hand, relaxin binds to RXFP1 and inhibits basal and FSH-stimulated production of cAMP, and activates ERK1/2 and PI3K/AKT pathways to stimulate NF-κB-dependent proliferation of Sertoli cells. Activation of cAMP production by FSH inhibits ERK1/2 and AKT phosphorylation, which contributes to reduced cell proliferation.

of Sertoli cells. In general, relaxin affected the same pathways affected by FSH but in opposite direction. In contrast to FSH, relaxin slightly, but significantly, decreased basal cAMP production after 30 min of treatment. This was surprising because the classic mechanism of relaxin action is the activation of cAMP production (Halls 2012). When RXFP1 is overexpressed in HEK293 cells, it couples to multiple G proteins and activates the cAMP production through a mechanism that depends on G_s and G_i. However, it is possible that the lower expression of endogenous RXFP1 allows more selective activation of signaling pathways (Halls 2012). Relaxin also did not increase cAMP production in uterine fibroblasts (Kuznetsova *et al.* 1999) and in vas deferens (Cardoso *et al.* 2010). The effect of relaxin on cAMP production seems therefore cell type dependent. Relaxin did not interfere with the cAMP production induced by prior administration of FSH, which indicates that relaxin did not stimulate cAMP degradation. However, relaxin reduced the effect of FSH when both hormones were added simultaneously for 30 min. These results suggest that relaxin and FSH crosstalk, which occurs after binding of FSH to its receptor and after coupling of the activated FSHR with G_s.

In contrast to testosterone that did not attenuate the inhibition caused by FSH on ERK1/2 phosphorylation in a primary culture of Sertoli cells from 20-day-old rats (Shupe *et al.* 2011), relaxin completely reversed the inhibition of ERK1/2 phosphorylation by FSH when both hormones were added simultaneously. Relaxin seemed a more efficient regulator of FSH effects on ERK1/2 than on AKT pathway.

Different from FSH, relaxin transiently inhibited CREB phosphorylation and increased translocation of NF- κ B (p65) from the cytoplasm to the nucleus. The stimulation of NF- κ B by relaxin seems to involve phosphorylation and subsequent degradation of the repressor protein I κ B- α . Proliferation of Sertoli cells mediated by the estrogen receptor ESR1 also involved NF- κ B, whereas exit of cell cycle and differentiation mediated by the estrogen receptor ESR2 involved CREB (Lucas *et al.* 2014b).

Finally, although relaxin counteracts the effects of FSH on cAMP, ERK1/2 and AKT pathways, relaxin does not inhibit the effects of FSH on expression of at least two genes (inhibin alpha and *Bcl2*). This was expected because the expression of these genes is highly dependent on activation of cAMP–CREB, and FSH still stimulates cAMP production in the presence of relaxin. It seems therefore that relaxin does not inhibit the differentiation process triggered by FSH in Sertoli cells, but further studies are necessary to clarify the mechanisms underlying the role of both hormones in the regulation of Sertoli cell development.

In conclusion, at this stage of Sertoli cell development, FSH and relaxin affect the same signaling pathways in opposite directions (Fig. 11). FSH promotes the coupling of FSHR to G_s, stimulates cAMP production

and CREB phosphorylation, and starts to direct cells to differentiation. Elevation of cAMP production inhibits ERK1/2 and AKT phosphorylation. On the other hand, relaxin binds to RXFP1 and inhibits basal and FSH-stimulated cAMP production, and stimulates cell proliferation through activation of ERK1/2, PI3K/AKT and NF- κ B. Whereas FSH action predominates and seems essential to direct cells to differentiation, relaxin seems to be preferentially related to Sertoli cell proliferation. The higher levels of relaxin in the testis of 15-day-old rats compared with adult animals (Cardoso *et al.* 2010) supports this idea.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0330>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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