Differential Regulation of Ovarian Anti-Müllerian Hormone (AMH) by Estradiol through α- and β-Estrogen Receptors

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Background: Anti-Müllerian hormone (AMH) is a member of the TGF-β family, which limits follicle maturation. Recently serum AMH has been recognized as a useful diagnostic and prognostic tool in human reproductive endocrinology.

Objective: The aim of this study was to investigate the regulation of human ovarian *AMH* by estradiol and FSH.

Methods: AMH mRNA were quantified by real time RT-PCR in human granulosa cells (GC). AMH transcription was studied in KK1 GC cotransfected with estrogen receptors (ER)-β or ERα, and normal human AMH promoter-luciferase construct (hAMH-luc) or mutated AMH promoter reporter constructs. Binding sites for estradiol (estrogen response element half-site) and steroidogenic factor 1 were disrupted by targeted mutagenesis. The level of ER in GC was determined by quantitative RT-PCR and Western blotting.

Results: In KK1 cells, estradiol up-regulated and inhibited hAMH-luc in the presence of ERα and ERβ respectively. Disruption of estrogen response element half-site and/or steroidogenic factor 1 binding sites did not modify ERβ-mediated effect of estradiol on hAMH-luc, whereas it affected that conveyed by ERα. The FSH enhancement of hAMH-luc was abolished by estradiol in cells overexpressing ERβ. When both ER were transfected, estradiol inhibited hAMH-luc or had no effect. Estradiol repressed AMH mRNAs in human GC, which express a little more ERα than ERβ mRNA.

Conclusions: Our results show that AMH expression can be differentially regulated by estradiol depending on the ER and suggest that its decrease in GC of growing follicles, which mainly express ERβ, and during controlled ovarian hyperstimulation is due to the effect of estradiol. (***J Clin Endocrinol Metab*** **97: E1649–E1657, 2012)**

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Abbreviations: AMH, Anti-Müllerian hormone; DPN, 2,3-bis(4-hydroxyphenyl)propionitrile; ER, estrogen receptor; ERE, estrogen-response element; $^1/_2$-ERE, an ERE half-site; FCS, fetal calf serum; GC, granulosa cell; hAMH-luc, human AMH promoter-luciferase construct; hGC, human granulosa-luteal cell; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PPT, 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; rhFSH, recombinant human FSH; RPL13a, ribosomal protein L13a; SDHA, succinate dehydrogenase complex subunit A; SF-1, steroidogenic factor 1; WB, Western blotting.

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance, is a member of the transforming growth factor-β family expressed almost exclusively by gonadal somatic cells (1). In males, AMH is expressed by Sertoli cells and is responsible for the regression of Müllerian ducts, the anlagen of uterus, and tubes in females (1). In females, AMH is secreted by granulosa cells (GC) of growing follicles from the beginning of folliculogenesis to menopause (2), and it limits follicle maturation (3). *AMH* expression is initiated in primary follicles, is strongest in preantral and small antral follicles, and decreases up to ovulation, except in cumulus cells (2). Recently serum AMH has been recognized as a useful diagnostic and prognostic tool, as an early indicator of relapse of ovarian GC tumors (4) and a reliable marker of the ovarian follicular status and predictor of the ovarian response to controlled ovarian hyperstimulation (5).

The regulation of ovarian AMH is still poorly documented, and studies on the effect of FSH and estradiol have yielded conflicting results (6–11). The decreased *AMH* expression during late follicular maturation and the fall of serum AMH during controlled ovarian hyperstimulation using exogenous FSH have been interpreted as an inhibitory effect of FSH on AMH secretion by GC without taking into account the possible role of estradiol on *AMH* regulation. However, several reports are consistent with a positive effect of FSH upon ovarian *AMH* expression: gonadotropin suppression results in a drop of AMH serum level in women (12) and injection of a GnRH antagonist decreases *AMH* expression in the marmoset (11), whereas increased AMH production by isolated human follicles (13) and enhanced *AMH* promoter activity in GC cultures have been observed after the addition of FSH (8). The objective of this work was to explore whether estrogens, whose production is stimulated by FSH, might be involved in the regulation *AMH* production. Because the *in vivo* effect of a treatment by estradiol can be obscured by the presence of other hormones or factors acting individually or in concert, we used two complementary models of cultured GC to study the effect of estradiol alone or in combination with FSH: primary cultures of human granulosa-luteal cells (hGC) to study the effect of estradiol on endogenous AMH mRNA, and the KK1 mouse luteinized GC line (14) in which we transfected a human *AMH* reporter gene, to highlight modest effects not detectable by conventional expression studies, and to investigate the mechanism of action of estradiol on the *AMH* promoter.

Estrogens mainly mediate their effects through receptors (ER) that act as ligand-dependent transcription factors (15). In the classic nuclear or genomic pathway of estrogen action, ligand binding to the ER induces a change in receptor conformation, dissociation of the ER-chaperone complex, dimerization, and binding to an estrogen-response element (ERE) or a steroidogenic factor 1 (SF-1) response element (16, 17). Additionally, ligand-bound ER can interact with other transcription factors like activator protein-1 and specificity protein-1 (18), thereby regulating transcription but not through direct DNA binding, or use nongenomic mechanisms of action and rapidly activate protein kinase pathways (19). Two forms of the ER have been identified, ERα (20) and ERβ (21), which have both overlapping and distinct mechanisms of action. Both ER are expressed in the ovary, ERα being predominant in thecal cells and ERβ in GC (22). The human AMH promoter contains an ERE half-site ($^1/_2$-ERE) at -1772 bp (23) and two SF-1 binding sites at -218 bp and -92 bp (24). To investigate the mechanism of action of estradiol on AMH expression, we studied the effects conveyed by each ER and the importance of $^1/_2$-ERE and SF-1 binding sites.

Materials and Methods

Reagents

Recombinant human FSH (rhFSH) (Gonal-F) was obtained from Serono Pharmaceuticals (Boulogne, France), and 17β-estradiol, 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) and 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) were from Sigma-Aldrich (Saint Quentin Fallavier, France). Monoclonal antibodies against ERα (sc-8002), ERβ (ab16813), and α-tubulin (T5168) were purchased, respectively, from Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, UK),

TABLE 1. Sequence of the primers and probes used for real-time RT-PCR experiments

Genes	Nucleotides sequence written 5′ to 3′ sense	Nucleotides sequence written 5′ to 3′ antisense	UPL probes
Human *AMH*	CGCCTGGTGGTCCTACAC	GAACCTCAGCGAGGGTGTT	69
Human *RPL13a*	CTGGACCGTCTCAAGGTGTT	GCCCCAGATAGGCAAACTT	74
Human *SDHA*	GGACCTGGTTGTCTTTGGTC	CCAGCGTTTGGTTTAATTGG	80
Human *ERα*	TCCTAACTTGCTCTTGGACAGG	GTAGCCAGCAGCATGTCG	22
Human ERβ	GCTCCTGTCCCACGTCAG	AAGCACGTGGGCATTCAG	67
Mouse *HPRT*	TGATAGATCCATTCCTATGACTGTAGA	AAGACATTCTTTCCAGTTAAAGTTGAG	22
Mouse *ERα*	TCCTAACTTGCTCCTGGACAGG	GTAGCCAGCAACATGTCA	22
Mouse *ERβ*	TTCTTTCTCATGTCAGGCACA	CTCGAAGCGTGTGAGCATT	67

and Sigma-Aldrich, and peroxidase-labeled antimouse antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). The human *ERα* isoform 1 and *ERβ* isoform 1 cDNA in pSG5 vector were a generous gift of Dr. Martinez (Unité Mixte de Recherche 6247, Clermont-Ferrand, France).

Targeted mutagenesis

The −3068-bp human AMH promoter reporter construct human AMH promoter-luciferase construct (hAMH-luc), and the same construct with mutations in the two binding sites for SF-1 were obtained as previously described (8, 25). Mutation of the $^1/_2$-ERE (−1772 bp) was generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotide primers 5′-GAT GGT CGC CCT GAG G*GC GGT ACC ACG* AGG AGC CCT CTC TGT C-3′ and 5′-GAC AGA GAG GGC TCC T*CG TGG TAC CGC* CCT CAG GGC GAC CAT C-3′ were synthesized by Eurogentec (Liège, Belgium).

Subjects

Twenty patients, 20–40 yr of age, undergoing *in vitro* fertilization-embryo transfer, were studied prospectively. The inclusion criteria and the ovarian stimulation protocol were the same as previously described (8). An informed consent was obtained from all women, and this investigation received the approval of our internal institutional review board.

Collection and culture of hGC

After oocyte isolation, follicular fluids from each patient were centrifuged separately to isolate hGC as previously described (8), and seeded at 3×10^5 cells/well in six-well plates in DMEM/F-12 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS) (Invitrogen), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Invitrogen). The next day, hGC were treated for 48 h with estradiol (stock solution at 6×10^{-3} M in ethanol, diluted in the culture medium to the desired final concentration), or the same volume of ethanol, in DMEM/F-12 without phenol-red (Invitrogen) and antibiotics (Invitrogen).

KK1 cells culture and transfection assays

The mouse KK1 GC line (14) was cultured in DMEM/F-12 containing 10% FCS and antibiotics. Twenty-four hours after seeding in six-well plates in the same medium except that FCS was charcoal stripped (Invitrogen), subconfluent KK1 cells were transiently transfected with 1 μg/well of the different *AMH* reporter genes with or without 100 ng/well of *ERα*, *ERβ*, *FSH* receptor cDNA, or the same amount of the corresponding vectors, using the Lipofectamine Plus kit (Invitrogen). At the end of the transfection, cells were treated during 48 h with estradiol and/or rhFSH in DMEM/F-12 without phenol-red containing 10% charcoal-stripped FCS and antibiotics.

Western blotting

Lysates from hGC or KK1 cells were prepared as previously described (8). Protein concentration was determined using the BCA assay (Pierce, Rockford, IL). Thirty micrograms were subjected to SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and transferred onto a Protran BA85 nitrocellulose membrane (Whatman, Dassel, Germany). Membranes were incubated with antibodies against ERα (1:1,000), ERβ (1:1,000), or α-tubulin (1:10,000). Then blots were probed with a peroxidase-labeled antimouse at 1:5000. Bands were visualized with the ECL Plus detection reagent (GE Healthcare, Buckinghamshire, UK).

RNA extraction and reverse transcription

Total RNA were extracted from hGC and KK1 cells with the RNA Plus extraction kit (QIAGEN, Valencia, CA). Reverse transcription was performed with the Omniscript reverse transcription kit (QIAGEN) using 1 μg RNA.

Quantitative real-time PCR

Quantification of AMH, ERα, ERβ, ribosomal protein L13a (RPL13a), succinate dehydrogenase complex subunit A (SDHA) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA was performed by real-time PCR using the TaqMan PCR

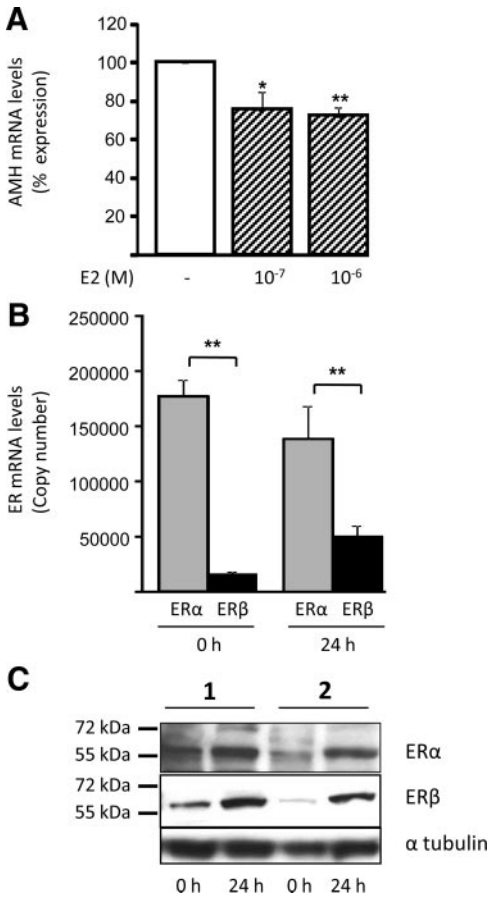

FIG. 1. Estradiol down-regulates AMH mRNA levels in primary cultures of hGC. A, Real-time RT-PCR analysis of *AMH* expression in hGC after 48 h of treatment with estradiol (E2) 10^{-7} M (n = 7) or E2 10^{-6} M (n = 9). Data were normalized to the housekeeping genes *SDHA* and *RPL13a*. Because *AMH* expression was variable among women, results were expressed as percentages ± SEM and compared with the effect obtained in control medium (arbitrarily fixed at 100%). Results were analyzed by the Wilcoxon test for paired comparisons between control and treated hGC. B, Real-time RT-PCR analysis of *ERα* and *ERβ* expression in hGC (n = 5) when they were collected (0 h) and when E2 was added (24 h). Data were normalized to the housekeeping genes *SDHA* and *RPL13a*. Results were expressed in copy number and the levels of *ERα* and *ERβ* mRNAs were compared using a Mann-Whitney *U* test. C, Western blotting analysis of *ERα* and *ERβ* expression in two preparations (1 and 2) of hGC, at collection (0 h) and after 24 h exposure to estradiol (24 h). *, $P < 0.05$; **, $P < 0.01$.

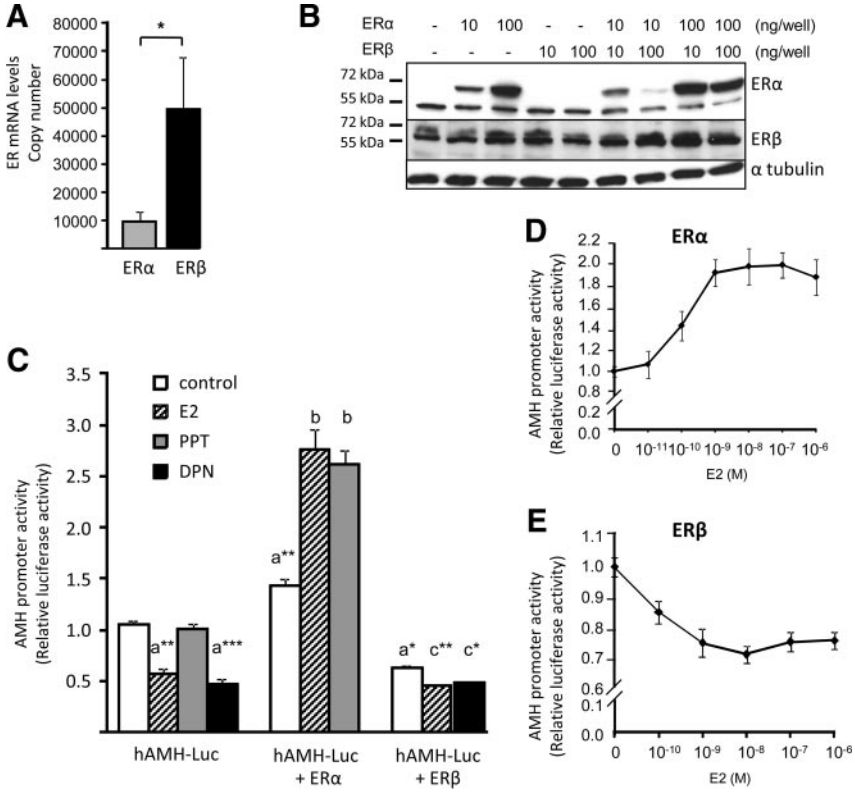

FIG. 2. Differential regulation of *AMH* transcription by estradiol through ERα and ERβ in KK1 GCs. A, Real-time RT-PCR analysis of *ER* expression in KK1 GC. Data were normalized to the housekeeping gene *HPRT*. Results were expressed in copy number and the levels of ERα and ERβ mRNA were compared using a Mann-Whitney *U* test. B, Western blotting analysis of ERα and ERβ in KK1 GC. C, Effect of overexpression of ERα or ERβ or/and ER agonists on hAMH-luc. KK1 cells were transfected with 1 μg/well of hAMH-luc and 100 ng/well of either *ERα* or *ERβ*. Luciferase activity was analyzed after 48 h of culture in control medium or with estradiol (E2) (10^{-6} M), PPT (10 nM), or DPN (10 nM). The relative light units of the first triplicate in control medium for hAMH-luc (hAMH-luc + ERα and hAMH-luc + ERβ) were fixed at one for each experiment, and the other results were normalized to this value (relative luciferase activity). Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. Comparisons of means between different treatments were made by repeated measures ANOVA, followed by Dunnett *post hoc* test to compare all *vs.* controls (hAMH-luc, hAMH-luc + ERα, or hAMH-luc + ERβ in control medium). a, Significantly different from hAMH-luc; b, significantly different from hAMH-luc + ERα; c, significantly different from hAMH-luc + ERβ. D and E, Dose-response curves of estradiol effect on AMH transcription through ERα (D) or ERβ (E). KK1 cells were transfected with 1 μg/well of hAMH-luc and 100 ng/well of either *ERα* (D) or *ERβ* (E) plasmids. Luciferase activity was analyzed after 48 h of culture with increasing concentrations of estradiol (E2). The relative light units of the first triplicate in control medium for hAMH-luc + ERα or hAMH-luc + ERβ were fixed at one for each experiment, and the other results were normalized to this value (relative luciferase activity). Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

method. The primers (Eurogentec) and the Universal Probe Library (UPL) probes (Roche Diagnostics, Indianapolis, IN) used to amplify these genes are indicated Table 1. Real-time PCR was performed with one fifth dilution of the cDNA using the Lightcycler 480 probes master kit (Roche Diagnostics) as previously described (8). To generate external standard curves, different concentrations of the purified and quantified PCR products were amplified. The amplication efficiency was 100% for all genes. For human genes, the effect of treatment or time in culture on *RPL13a* and *SDHA* was expressed as correction factors and copy numbers were divided by the mean of RPL13a and SDHA corrections factors. For mouse genes, each copy number was divided by a correction factor corresponding to the difference between the mean of *HPRT* copy numbers for all samples and the *HPRT* copy number of each sample.

Reporter assays

Forty-eight hours after the transfection of KK1 cells, firefly luciferase activity was measured as previously described (8) using a Lumat LB95507 luminometer (EG&G Berthold, Thoiry, France). Data shown correspond to the mean ± SEM of at least three experiments each done in triplicate. To take into account all the experiments, the relative light units of the first triplicate obtained for each different DNA mixture in control medium were fixed at one, and the other results were normalized to this value (relative luciferase activity).

Statistics

Control and estradiol conditions were compared using the Wilcoxon paired test for hGC, and the Student's unpaired *t* test, or the nonparametric Mann-Whitney test for KK1 cells. When more than two conditions were tested for the same DNA transfection mixture, comparisons were made by repeated-measures ANOVA followed by Tukey's multiple comparison test or a nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison posttests.

Results

Estradiol down-regulates *AMH* expression in primary cultures of human granulosa cells

Regulation of ovarian AMH by estradiol was analyzed in hGC obtained from women undergoing *in vitro* fertilization. We studied the cells from each woman separately to take into account interindividual variability. As shown in Fig. 1A, AMH mRNA levels decreased significantly after 48 h of culture in the presence of 10^{-7} or 10^{-6} M of estradiol. Because estradiol effects are mediated by ERα and ERβ, we then studied the relative level of these receptors in hGC. Using real-time RT-PCR, we observed that hGC expressed a 3- to 18-fold excess (mean = 9) of ERα mRNA compared with ERβ the day the cells were collected and a 2- to 5-fold excess (mean = 3) of ERα compared with ERβ 24 h after seeding, when estradiol was added to the culture medium (Fig. 1B). Results of Western blotting (WB) analysis of ER expression was in line with RT-PCR results (Fig. 1C).

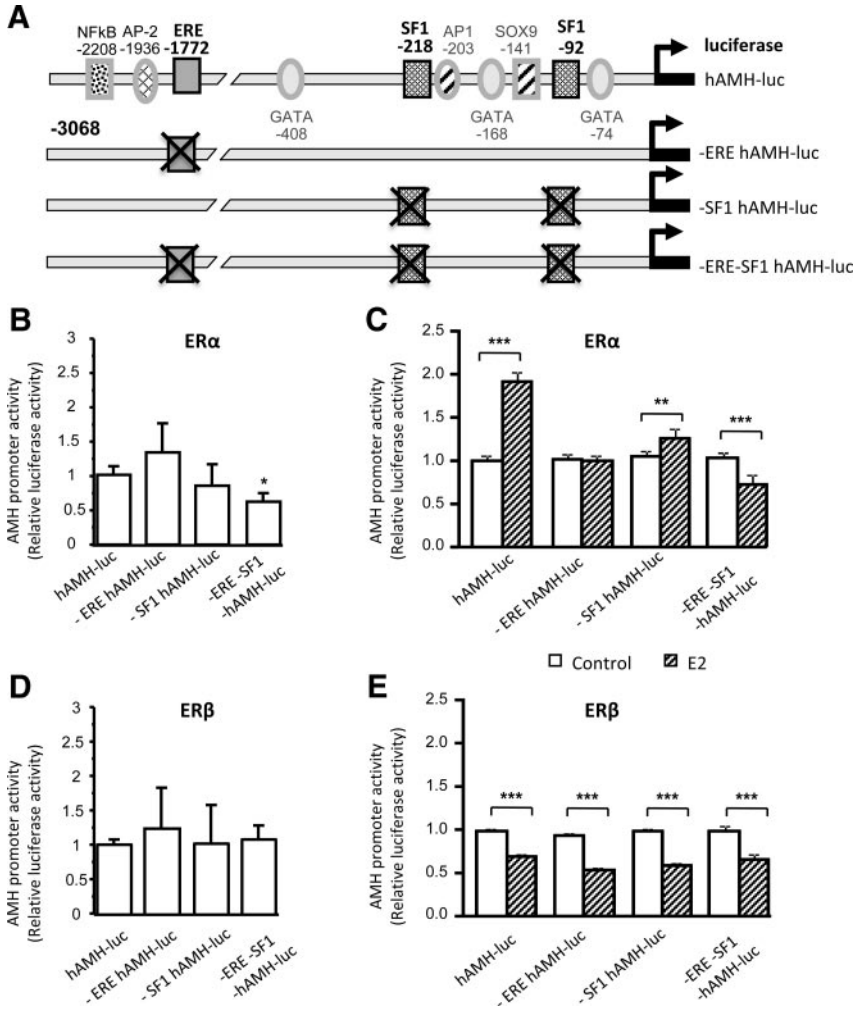

FIG. 3. Differential regulation of AMH reporter constructs by estradiol through ERα and ERβ. A, Schematic representation of the 3068-bp human *AMH* gene promoter-luciferase constructs. Localization and nucleotide positions of binding sites for nuclear factor-κB (NFκB), activating protein 2 (AP2), SF-1, activator protein-1 (AP1), ERE, Sry-type high-mobility-group box transcription factor (SOX), and GATA-4 in the human promoter are according to a published report (25). Targeted mutagenesis (*black rectangles*) was performed on hAMH-luc to disrupt the $^1/_2$-ERE (-ERE hAMH-luc), the SF1 binding sites (-SF1 hAMH-luc), or both the $^1/_2$-ERE and the SF-1 binding sites (-SF1-ERE-hAMH-luc). B to E, KK1 cells were transfected with 1 μg/well of the different AMH promoter reporter constructs and 100 ng/well of either ERα (B and C) or ERβ (D and E) plasmids. Luciferase activity was analyzed after 48 h of culture in control medium or with estradiol (E2) 10^{-6} M. Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. B and D, The relative light units of the first triplicate in control medium for hAMH-luc were fixed at one for each experiment, and the other results were normalized to this value (relative luciferase activity). Comparisons of means between different constructs were made by repeated-measures ANOVA followed by a Tukey's multiple comparison test (B) or a nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison posttests (D). C and E, The relative light units of the first triplicate in control medium for each construct were fixed at one for each experiment, and the other results were normalized to this value (relative luciferase activity). Results were analyzed using unpaired t tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Differential regulation of *AMH* transcription by estradiol through ERα and ERβ

To investigate the mechanism of action of estradiol on *AMH* expression, we used the easily transfectable mouse KK1 GC line. We first studied the ER isoforms content of these cells using real-time RT-PCR (Fig. 2A), and we determined that they expressed a 2- to 7-fold excess (mean = 5) of ERβ mRNA compared with ERα. Only the ERβ protein was detectable by WB (Fig. 2B). We then transfected KK1 cells with a hAMH-luc comprising 3068 bp of the 5′ flanking region of the human *AMH* gene driving the expression of the luciferase gene (25) (Fig. 3A). Both estradiol and DPN, a preferential ERβ modulator, inhibited hAMH-luc (Fig. 2C). In contrast, PPT, an ERα agonist, had no effect. To determine which ER mediated estradiol effects on *AMH* transcription, we then separately overexpressed human ERα or ERβ in KK1 cells and studied the effect of estradiol and ER agonists on hAMH-luc. When ERα was overexpressed, both estradiol and PPT activated hAMH-luc (Fig. 2C), the effect of estradiol being dose dependent with a maximal effect of +96% at 10^{-7} M (Fig. 2D). In contrast, in the presence of ERβ, estradiol and DPN down-regulated hAMH-luc (Fig. 2C), with a maximal inhibition of −29% obtained for estradiol 10^{-8} M (Fig. 2E). Further experiments in KK1 cells were then performed with estradiol 10^{-6} M, which corresponds to the concentration present in the fluid of mature follicles (Figs. 3 and 4).

Transcription factor binding sites involved in ERα- and ERβ-mediated effects of estradiol on *AMH* transcription

The main mechanism of action of estrogens to regulate their target genes involves ER binding to ERE or to SF-1 binding sites, which resemble to ERE (17). To test the implication of these sequences in mediating the effect of estradiol on *AMH* expression, we disrupted those present on hAMH-luc (Fig. 3A) and cotransfected these mutated reporters in KK1 cells with either ERα- or ERβ-expressing plasmids. The *AMH* promoter activity was not affected by the disruption of $^1/_2$-ERE and/or SF-1 binding sites, except in cells overexpressing *ERα*, in which ERE-SF1-hAMH-luc activity was reduced (Fig. 3, B and D). The stimulatory effect of estradiol through ERα was abolished or reduced when the $^1/_2$-ERE or SF-1 binding sites were mutated (Fig. 3C). This was

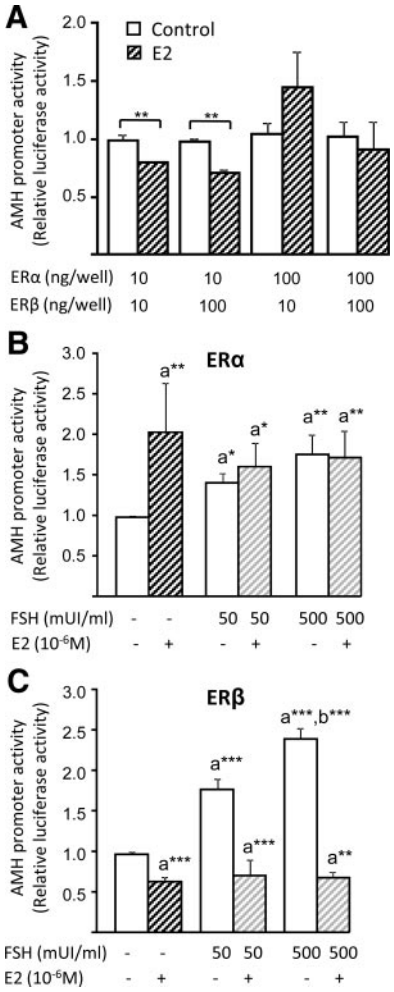

FIG. 4. Effect of estradiol and FSH on AMH transcription in presence of various combinations of ERα and ERβ. A, Effect of estradiol on AMH transcription in the presence of various combinations of ERα and ERβ. Different amounts (10 or 100 ng/well) and ratios (ERα to ERβ: 0.1, 1, or 10) of ERα and ERβ were cotransfected with 1 μg/well of hAMH-luc in KK1 cells, and luciferase activity was analyzed after 48 h of culture in control medium or with estradiol (E2) 10^{-6} M. The relative light units of the first triplicate in control medium for each ratio were fixed at one for each experiment, and the other results were normalized to this value (relative luciferase activity). Comparison of means were made using the nonparametric Mann-Whitney *U* test. B and C, Effect of estradiol and FSH on AMH transcription. KK1 cells were cotransfected with 1 μg/well of hAMH-luc and 100 ng/well of FSH receptor and 100 ng/well of ERα (B) or ERβ (C) plasmids, and luciferase activity was assessed 48 h after treatment with FSH (50 or 500 mIU/ml) with or without E2 10^{-6} M. The relative light units of the first triplicate in control medium were fixed at one for each experiment, and the other results were normalized to this value (relative luciferase activity). Comparisons of means between different treatments were made by a nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison posttests (B) or repeated-measures ANOVA followed by Tukey's multiple comparison test (C). a, Significantly different from control; b, significantly different from FSH 50 mIU/ml. Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

reversed when the $^1/_2$-ERE and SF-1 binding sites were disrupted simultaneously. In contrast, none of these mutations affected ERβ-mediated inhibitory effect of estradiol on the *AMH* promoter (Fig. 3E).

Effect of estradiol on *AMH* transcription in the presence of various combinations of ERα and ERβ

Because both *ERα* and *ERβ* are expressed physiologically in GC, we then cotransfected different amounts (10 or 100 ng/well) and/or ratios (ERα:ERβ, 1:10, 1:1, 10:1) of the two ER-expressing plasmids with hAMH-luc, and we treated KK1 cells with estradiol for 48 h. The amount of each ER isoform was controlled by WB (Fig. 2B). The intensity of the band corresponding to ERα was proportional to the amount of transfected cDNA. Surprisingly, this was not the case for ERβ. However, cotransfection of *ERβ* reduced in a dose-dependent manner the expression of ERα protein. Estradiol inhibited hAMH-luc when 10 ng of *ERα* were transfected but had no effect in cells transfected with 100 ng of *ERα*, whatever the amount of *ERβ* (Fig. 4A).

Combined effects of estradiol and FSH on *AMH* transcription

Because *in vivo*, FSH stimulates estrogen production by GC, we then tested the combined effect of FSH and estradiol on *AMH* transcription. Because KK1 cells lose their responsiveness to FSH after several passages (8), we cotransfected KK1 cells with hAMH-luc and vectors driving the expression of the human FSH receptor and ER cDNA and cultured the cells during 48 h with rhFSH and/or estradiol. As previously shown (8), a significant stimulation of hAMH-luc was observed when cells were treated by 50 and 500 mIU/ml of FSH (Fig. 4, B and C). Treatment of cells overexpressing *ERα* with both FSH and estradiol did not further increase hAMH-luc activity (Fig. 4B). Interestingly, in the presence of ERβ, estradiol reduced the stimulatory effect of FSH to a level lower than that observed in absence of FSH (Fig. 4C).

Discussion

In this study, we demonstrate that in GC, as reported for other cell types (26, 27), estradiol has opposite effects through ERα and ERβ on *AMH* transcription. In particular, we show that estradiol represses *AMH* expression in GC when ERβ is in excess compared with ERα, the most common situation encountered *in vivo*. We also reconcile data in the literature regarding the effect of FSH on *AMH* expression, showing that the apparent inhibitory effect of FSH is indirect and mediated by estradiol.

Inhibitory effect of estradiol on *AMH* expression through ERβ

Using KK1 cells transfected with a 3068-bp human promoter *AMH* reporter gene, we show that ***AMH*** transcription is inhibited by estradiol and DPN, an ERβ agonist,

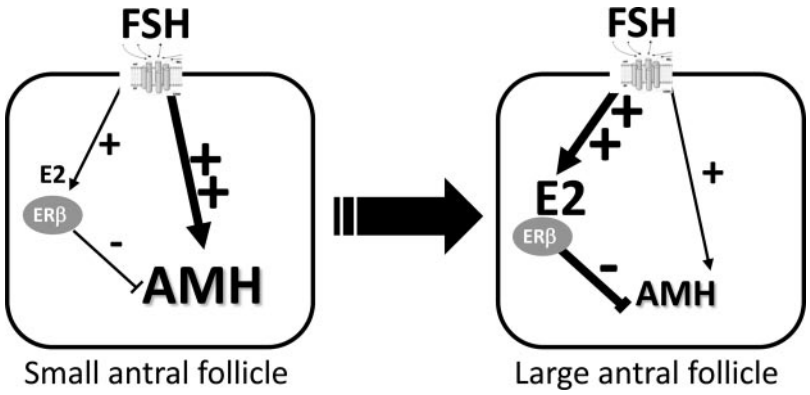

FIG. 5. Schematic diagram of AMH regulation by FSH and estradiol in GC of small and large antral follicles. Until the small antral stage, AMH secretion is stimulated by different factors like FSH. In parallel, estradiol (E2) production also increases under the influence of FSH, but its negative effect on AMH expression through ERβ, which predominates in growing follicles, is overcome by that of stimulatory factors. When estradiol concentration reaches a certain threshold in large antral follicles, it is capable of completely inhibiting AMH expression.

whereas PPT, an ERα agonist, has no effect (Fig. 2C). Because KK1 cells express an excess of ERβ compared with ERα (Fig. 2, A and B), these results demonstrate that the inhibitory effect of estradiol on ***AMH*** **transcription is mediated by ERβ. Overexpression of both ER isoforms in KK1 cells abolished the inhibitory effect of estradiol on** *AMH* transcription only when 100 ng of *ERα* was transfected (Fig. 4A) and a high amount of ERα protein was expressed (Fig. 2B). In keeping with this result, we observed an inhibitory effect of estradiol on AMH mRNA in hGC, which expressed a small excess of ERα mRNA compared with ERβ (Fig. 1, A and B). Because ERβ is predominant in GC of growing follicles (22) and the phenotypes of ER null mice indicate the ERβ but not ERα is critical for mediating the effects of estradiol during follicle maturation (28), our results strongly suggest that estradiol mainly inhibits *AMH* expression by GC during folliculogenesis. Our results are consistent with those of Baarends *et al.* (6), who observed a decrease of *AMH* expression in some, but not all, preantral and small antral follicles in prepubertal rats treated with a GnRH antagonist and estradiol benzoate.

Regulation of *AMH* expression by FSH and estradiol

In vivo, *AMH* expression is high from primary to small antral follicles and then falls at the subsequent stages of follicular development. Our hypothesis (Fig. 5) is that until the antral stage, AMH secretion is stimulated by different factors like bone morphogenetic proteins (29) and FSH (8). In parallel, estradiol production also increases under the influence of these factors, but its negative effect on *AMH* expression through ERβ, which predominates in growing follicles, is overcome by that of stimulatory factors. When estradiol concentration reaches a certain threshold, it is capable of completely inhibiting *AMH* expression. This is supported by our results showing an inhibiting effect of estradiol via ERβ on the *AMH* promoter, which is enhanced in the presence of FSH (Fig. 4C). Consistent with our model, most of the clinical studies report a negative correlation between follicular fluid AMH and estradiol (30) or aromatase expression (31) in small antral follicles but not in larger ones (30). In small follicles that strongly express AMH, its inverse relationship with estradiol concentration could be due to both a negative effect of estradiol on *AMH* expression and a repressive effect of AMH on aromatase (32). In larger follicles, the very low AMH concentration compared with that of estradiol may explain the lack of correlation between the two hormones. No relationship was found between AMH and estradiol concentrations in the serum except during controlled ovarian hyperstimulation cycles with FSH (10), in which a negative correlation was found between the two hormones. This might be due to the fact that, in such conditions, the pool of follicles that account for hormone production is more homogenous than in natural cycles. Thus, the decreased expression of AMH during late follicle maturation and the fall of serum AMH levels during controlled ovarian hyperstimulation with FSH, which both have been interpreted as supporting a negative effect of FSH on *AMH* expression (6, 10), are likely due to the increased levels of estradiol during these processes.

Stimulatory effect of estradiol on *AMH* expression through ERα

When *ERα* was overexpressed alone in KK1 cells, *AMH* transcription was increased in basal conditions and in the presence of both estradiol and PPT (Fig. 2C). Chen *et al.* (33) also observed an activation of a 273-bp *AMH* promoter reporter in NT2/D1 cells overexpressing *ERα*. Whether the activating effect of estradiol through ERα is physiologically relevant cannot be evaluated with our experimental design. Indeed, the critical ER isoform in mediating the synergistic effect of FSH and estradiol during follicle maturation is ERβ (34). ERα becomes predominant in GC only when they luteinize. One *in vivo* study has shown a positive relationship between estradiol and AMH (7). The influence of other hormones or growth factors locally present might also be involved and could explain the results. Consistent with this hypothesis, *ERα* expression can be induced by the hormonal environment in cul-

tured mice follicles (35). Conversely, *ERβ* is repressed by the LH surge (28), likely explaining why in controlled ovarian hyperstimulation cycles, serum AMH rises between the moment of human chorionic gonadotropin administration and ovarian puncture (36). The estradiol effect through ERβ is inhibited in GC cancers (28), and ERβ expression is reduced in GC of women with a polycystic ovarian syndrome (37), two conditions in which serum AMH is high.

Mechanism of action of estradiol on *AMH* expression

Targeted mutagenesis of the $^1/_2$-ERE or SF-1 binding sites of the human *AMH* promoter respectively prevented or reduced estradiol stimulation of *AMH* transcription via ERα (Fig. 3C), showing that this effect is mediated through classical DNA binding. In contrast, the disruption of $^1/_2$-ERE and/or SF-1 binding sites did not modify ERβ-mediated repression of the *AMH* promoter by estradiol (Fig. 3E), suggesting that ERβ does not directly bind to these sites in the *AMH* promoter and uses other genomic or nongenomic mechanisms. Consistent with this result, the affinity of ERβ for $^1/_2$-ERE or SF-1 sites is low (15). The absence of effect of estradiol via ERβ on the 273-bp *AMH* reporter promoter in NT2/D1 cells (33) probably indicates that ERβ along with a cofactor interacts with upstream promoter sequences, uses a nongenomic pathway or that NT2/D1 cells, which are derived from a human testicular embryonal cell carcinoma, lack the cofactors necessary for mediating the ERβ effect. The reason that transfection of various amounts of *ERβ*-expressing plasmid did not significantly modify the amount of ERβ protein in KK1 cells is unknown (Fig. 2B). Despite this observation, overexpression of ERβ cDNA significantly decreased hAMH-luc activity in control medium (Fig. 2C) and inhibited ERα expression (Fig. 2B), in keeping with other studies reporting an antagonism between ER (38, 39).

In conclusion, our results provide insights into the regulation of *AMH* by estradiol alone or in interaction with FSH, particularly in growing follicles where ERβ is predominant. We demonstrate that estradiol represses *AMH* expression in this situation, and we also provide evidence regarding the effect of FSH on *AMH* expression, showing that the apparent inhibitory effect of FSH is indirect and mediated by estradiol. In pathological conditions such as GC tumors or polycystic ovarian syndrome, the increased secretion of AMH might be partly due to a lack of ERβ-mediated inhibitory effect or an increase of ERα-mediated stimulatory effect of estradiol on *AMH* expression.

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References

1. **Josso N, Picard JY, Rey R, di Clemente N** 2006 Testicular anti-Müllerian hormone : history, genetics, regulation and clinical applications. Pediatr Endocrinol Rev 3:347–358
2. **Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC, Themmen AP** 2004 Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10:77–83
3. **Visser JA, Themmen APN** 2005 Anti-Mullerian hormone and folliculogenesis. Mol Cell Endocrinol 234:81–86
4. **Rey R, Sabourin JC, Venara M, Long WQ, Jaubert F, Zeller WP, Duvillard P, Chemes H, Bidart JM** 2000 Anti-Müllerian hormone is a specific marker of Sertoli and granulosa-cell origin in gonadal tumors. Hum Pathol 31:1202–1208
5. **La Marca A, Sighinolfi G, Radi D, Argento C, Baraldi E, Artenisio AC, Stabile G, Volpe A** 2010 Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). Hum Reprod Update 16:113–130
6. **Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA** 1995 Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951–4962
7. **Ikeda Y, Nagai A, Ikeda MA, Hayashi S** 2002 Increased expression of Müllerian-inhibiting substance correlates with inhibition of follicular growth in the developing ovary of rats treated with E2 benzoate. Endocrinology 143:304–312
8. **Taieb J, Grynberg M, Pierre A, Arouche N, Massart P, Belville C, Hesters L, Frydman R, Catteau-Jonard S, Fanchin R, Picard JY, Josso N, Rey RA, di Clemente N** 2011 FSH and its second messenger cAMP stimulate the transcription of human anti-Mullerian hormone in cultured granulosa cells. Mol Endocrinol 25:645–655
9. **Fanchin R, Schonäuer LM, Righini C, Frydman N, Frydman R, Taieb J** 2003 Serum anti-Müllerian hormone dynamics during controlled ovarian hyperstimulation. Hum Reprod 18:328–332
10. **La Marca A, Malmusi S, Giulini S, Tamaro LF, Orvieto R, Levratti P, Volpe A** 2004 Anti-Mullerian hormone plasma levels in spontaneous menstrual cycle and during treatment with FSH to induce ovulation. Hum Reprod 19:2738–2741
11. **Thomas FH, Telfer EE, Fraser HM** 2007 Expression of anti-Mullerian hormone protein during early follicular development in the primate ovary *in vivo* is influenced by suppression of gonadotropin secretion and inhibition of vascular endothelial growth factor. Endocrinology 148:2273–2281
12. **Arbo E, Vetori DV, Jimenez MF, Freitas FM, Lemos N, Cunha-Filho JS** 2007 Serum anti-Müllerian hormone levels and follicular cohort characteristics after pituitary suppression in the late luteal phase with oral contraceptive pills. Hum Reprod 22:3192–3196
13. **Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK** 2009 *In vitro* grown human ovarian follicles from cancer patients support oocyte growth. Hum Reprod 24: 2531–2540
14. **Kananen K, Markkula M, Rainio E, Su JG, Hsueh AJ, Huhtaniemi**

IT 1995 Gonadal tumorigenesis in transgenic mice bearing the mouse inhibin α-subunit promoter/Simian virus T-antigen fusion gene: characterization of ovarian tumors and establishment of gonadotropin-responsive granulosa cell lines. Mol Endocrinol 9:616–627

15. **Deroo BJ, Buensuceso AV** 2010 Minireview: estrogen receptor-β: mechanistic insights from recent studies. Mol Endocrinol 24:1703–1714
16. **Klinge CM** 2001 Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res 29:2905–2919
17. **Wilson TE, Fahrner TJ, Milbrandt J** 1993 The orphan receptors NGFI-b and steroidogenic factor-1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 13:5794–5804
18. **Safe S, Kim K, Kim K** 2008 Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J Mol Endocrinol 41:263–275
19. **Micevych P, Kuo J, Christensen A** 2009 Physiology of membrane oestrogen receptor signalling in reproduction. J Neuroendocrinol 21:249–256
20. **Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P** 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320:134–139
21. **Kuiper GG, Gustafsson JA** 1997 The novel estrogen receptor-β subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. FEBS Lett 410:87–90
22. **Sharma SC, Clemens JW, Pisarska MD, Richards JS** 1999 Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. Endocrinology 140:4320–4334
23. **Guerrier D, Boussin L, Mader S, Josso N, Kahn A, Picard JY** 1990 Expression of the gene for anti-Müllerian hormone. J Reprod Fertil 88:695–706
24. **Watanabe K, Clarke TR, Lane AH, Wang X, Donahoe PK** 2000 Endogenous expression of Müllerian inhibiting substance in early postnatal rat Sertoli cells requires multiple steroidogenic factor-1 and GATA-4-binding sites. Proc Natl Acad Sci USA 97:1624–1629
25. **Lukas-Croisier C, Lasala C, Nicaud J, Bedecarrás P, Kumar TR, Dutertre M, Matzuk MM, Picard JY, Josso N, Rey R** 2003 Follicle stimulating hormone increases testicular anti-Müllerian hormone (AMH) production through Sertoli cell proliferation and a non-classical AMP-mediated activation of the AMH gene. Mol Endocrinol 17:550–561
26. **Mitchner NA, Garlick C, Steinmetz RW, Ben-Jonathan N** 1999 Differential regulation and action of estrogen receptors α and β in GH3 cells. Endocrinology 140:2651–2658
27. **Roa J, Vigo E, Castellano JM, Gaytan F, García-Galiano D, Navarro VM, Aguilar E, Dijcks FA, Ederveen AG, Pinilla L, van Noort PI, Tena-Sempere M** 2008 Follicle-stimulating hormone responses to kisspeptin in the female rat at the preovulatory period: modulation by estrogen and progesterone receptors. Endocrinology 149:5783–5790
28. **Drummond AE, Fuller PJ** 2010 The importance of ERβ signalling in the ovary. J Endocrinol 205:15–23
29. **Shi J, Yoshino O, Osuga Y, Koga K, Hirota Y, Hirata T, Yano T, Nishii O, Taketani Y** 2009 Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin β subunits, and anti-Mullerian hormone in human granulosa cells. Fertil Steril 92:1794–1798
30. **Yding Andersen C, Rosendahl M, Byskov AG** 2008 Concentration of anti-Mullerian Hormone and inhibin-B in relation to steroids and age in follicular fluid from small antral human follicles. J Clin Endocrinol Metab 93:2344–2349
31. **Eilso Nielsen M, Rasmussen IA, Fukuda M, Westergaard LG, Yding Andersen C** 2010 Concentrations of anti-Müllerian hormone in fluid from small human antral follicles show a negative correlation with CYP19 mRNA expression in the corresponding granulosa cells. Mol Hum Reprod 16:637–643
32. **di Clemente N, Goxe B, Remy JJ,** *et al.* 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 2:553–558
33. **Chen G, Shinka T, Kinoshita K, Yan HT, Iwamoto T, Nakahori Y** 2003 Role of estrogen receptor α (ER α) in the regulation of the human Müllerian inhibitory substance (MIS) promoter. J Med Invest 50:192–198
34. **Couse JF, Yates MM, Deroo BJ, Korach KS** 2005 Estrogen receptor-β is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. Endocrinology 146:3247–3262
35. **Lenie S, Smitz J** 2008 Estrogen receptor subtypes localization shifts in cultured mouse ovarian follicles. Histochem Cell Biol 129:827–840
36. **Lee JR, Kim SH, Kim SM, Jee BC, Ku SY, Suh CS, Choi YM, Kim JG, Moon SY** 2010 Anti-Mullerian hormone dynamics during controlled ovarian hyperstimulation and optimal timing of measurement for outcome prediction. Hum Reprod 25:2597–2604
37. **Jakimiuk AJ, Weitsman SR, Yen HW, Bogusiewicz M, Magoffin DA** 2002 Estrogen receptor α and β expression in theca and granulosa cells from women with polycystic ovary syndrome. J Clin Endocrinol Metab 87:5532–5538
38. **Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price Jr RH, Pestell RG, Kushner PJ** 2002 Opposing action of estrogen receptors α and β on cyclin D1 gene expression. J Biol Chem 277:24353–24360
39. **Lindberg MK, Movérare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C** 2003 Estrogen receptor (ER)-β reduces ERα-regulated gene transcription, supporting a "ying yang" relationship between ERα and ERβ in mice. Mol Endocrinol 17:203–208