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Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance, a member of the TGF-ß family, is responsible for the regression of Müllerian ducts in the male fetus. In females, AMH is synthesized by granulosa cells of preantral and small antral follicles, and production wanes at later stages of follicle maturation. Using RT-PCR in luteal granulosa cells in primary culture and reporter gene techniques in the KK1 granulosa cell line, we show that FSH and cAMP enhance AMH transcription, and LH has an additive effect. Gonadotropins and cAMP act through protein kinase A and p38 MAPK signaling pathways and involve the GATA binding factor-4 and steroidogenic factor-1 transcription factors, among others. The expression profile of AMH and the dynamics of serum AMH after gonadotropin stimulation have been interpreted as a down-regulating effect of FSH upon AMH production by granulosa cells. The specific effect of gonadotropins upon granulosa cells may be obscured *in vivo* by the effect of FSH upon follicular maturation and by the presence of other hormones and growth factors, acting individually or in concert. **(*Molecular Endocrinology* 25: 645–655, 2011)**

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance, is a member of the TGF-ß family expressed almost exclusively by gonadal somatic cells. Known essentially for its capacity to inhibit the development of Müllerian ducts in male fetuses when secreted by fetal Sertoli cells (reviewed in Ref. 1), AMH has also been detected in granulosa cells, albeit at lower levels (2). AMH expression is initiated in primary follicles, is strongest in preantral and small antral follicles, and wanes subsequently up to ovulation (3–6), becoming nearly undetectable in human follicles larger than 8 mm and disappearing completely in luteal bodies and atretic follicles (7). In large antral follicles, granulosa cells in the cumulus and those close to the antral cavity still express a small variable amount of AMH protein (3, 8–12) and mRNA (5, 6).

Many studies suggest a correlation between the size of the primordial follicle pool and the number of small growing follicles (13), the most active producers of AMH. Indeed, the level of serum AMH is predictive of the age of menopause (14) and of the yield of oocytes after ovarian stimulation (15) and is now considered a reliable marker

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Abbreviations: AMH, Anti-Müllerian hormone; AP2, activation protein-2; hCG, human chorionic gonadotropin; HMG, human menopausal gonadotropin; NFκB, nuclear factor-κB; PCOS, polycystic ovary syndrome; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase; RLU, relative light units; SOX8/9, Sex Determining Region Y gene-related high mobility group box protein 9 or 8.

of ovarian reserve. However, measurement of serum AMH is not an appropriate tool for the study of regulation of AMH production by granulosa cells because its level is modulated not only by the degree of secretion by individual cells but also by the ratio of large *vs.* small follicles, which varies in response to hormonal stimulation. To achieve our purpose, we chose to study isolated human granulosa-luteal cells obtained from women undergoing *in vitro* fertilization treatment. However, the low yield does not allow testing of more than one or two treatments per patient. Furthermore, because these cells resist transfection, sensitive reporter gene techniques cannot be used. Therefore, most of our work was carried out using the KK1 mouse luteinized granulosa cell line (16), which has been previously used successfully to study AMH regulation by growth factors (17). A similar reporter gene approach has been instrumental in unraveling the mechanisms of FSH action upon Sertoli cells (18).

Results

AMH expression is up-regulated by cAMP and FSH in human granulosa-luteal cells and in the mouse KK1 granulosa cell line

We first investigated by real-time RT-PCR the expression of *AMH* by human granulosa-luteal cells obtained after ovarian puncture of women undergoing *in vitro* fertilization treatment. Cells from individual women were studied separately to take into account a variable response to hormonal treatment. Between 6 and 18 $\times$ 10^5 cells were obtained per patient. Because *AMH* expression by primary culture of Sertoli cells is known to decrease with time (19), we first studied the kinetics of *AMH* expression. As shown in Fig. 1A, *AMH* expression fell dramatically after 24 or 72 h of culture in control medium. Addition of FSH or its second messenger cAMP at 24 h increased *AMH* mRNA by 71 and 94%, respectively, at 72 h (Table 1 and Fig. 1B). LH had no effect (Table 1 and Fig. 1B). Similar results were obtained for cAMP in the granulosa cell line KK1 obtained by targeted oncogenesis (16) (Table 1 and Fig. 1C).

AMH transcription is activated by cAMP, FSH, and LH

We then tested the responsiveness to cAMP of a human AMH promoter-luciferase construct (−3068hAMH-luc) composed of 3068 bp of the human *AMH* gene 5′-flanking region upstream of the luciferase gene (18) transfected into KK1 cells. As shown Fig. 2A, cAMP stimulated the transcriptional activity of −3068hAMH-luc, this effect growing more significant after 48 h of treatment. Therefore, in additional experiments, luciferase activity of

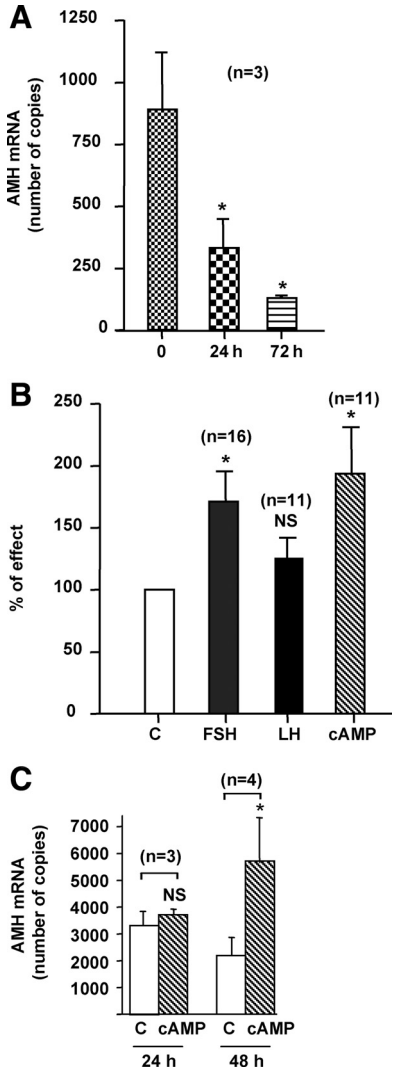

FIG. 1. Expression and regulation of *AMH* mRNAs by gonadotropins in human granulosa-luteal cells and KK1 cells. Panel A, Kinetics of *AMH* expression in granulosa-luteal cells. Cells were cultured in control medium for 24 or 72 h after seeding. RNAs were extracted at these different time points and AMH expression was analyzed by Real Time RT-PCR. Results are expressed in copy numbers normalized by two reference genes, *RPL13a* and *SDHA* and analyzed by the Dunnett test; n represents the number of patients. Panel B, Regulation of *AMH* expression by gonadotropins in human granulosa-luteal cells. Twenty-four hours after seeding, cells were cultured for 48 h in the presence of FSH (1 IU/ml), LH (1 IU/ml), or cAMP (10 μM) before RNA extraction and analysis of *AMH* expression by real-time RT-PCR. Results are expressed as a percentage of effect obtained in cells cultured in control medium (C). The effect of each treatment was compared with cells from the same woman cultured in control medium, using the Wilcoxon paired test; n represents the number of patients. Panel C, Regulation of *AMH* expression by cAMP in KK1 cells. Twenty-four hours after seeding, cells were cultured for 24 and 48 h in control medium (C) or in the presence of cAMP (1 mM) before RNA extraction and analysis of AMH expression by real-time RT-PCR. Results are expressed in copy numbers normalized by *HPRT* gene expression and analyzed using the Wilcoxon paired test; n represents the number of experiments. Data shown correspond to the mean ± SEM. NS, Not significant; *, $P < 0.05$.

AMH constructs was measured after 48 h of treatment. Similar effects were observed when KK1 cells were cultured without serum, ruling out a possible effect of AMH

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

Genes	Nucleotide sequences (5′–3′)		Universal Probe Library probes
	Sense	Antisense	
Human *AMH*	CGCCTGGTGGTCCTACAC	GAACCTCAGCGAGGGTGTT	69
Human *RPL13a*	CTGGACCGTCTCAAGGTGTT	GCCCCAGATAGGCAAACTT	74
Human *SDHA*	GGACCTGGTTGTCTTTGGTC	CCAGCGTTTGGTTTAATTGG	80
Mouse *AMH*	GGCTAGGGGAGACTGGAGAA	AGGTGGAGGCTCTTGGAACT	41
Mouse *SOX9*	CAGCAAGACTCTGGGCAAG	TCCACGAAGGGTCTCTTCTC	66
Mouse *SOX8*	GACCCTAGGCAAGCTGTGG	CTGCACACGGAGCCTCTC	25
Mouse *SF-1*	AGAATTCTCCTTCCGTTCAGC	TCACCACACACTGGACACG	85
Mouse *GATA-4*	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC	13
Mouse *HPRT*	TCAACGGGGGACATAAAAGT	CCAGTGTCAATTATATCTTCAACAATC	22

present in fetal calf serum (Fig. 2B). To compare the effects of cAMP, FSH, and LH, we cotransfected KK1 cells with −3068hAMH-luc and either human *FSH-R* or *LH-R* cDNAs. In control medium, luciferase activity was higher in cells cotransfected with the *FSH-R* or *LH-R* cDNAs alone or in combination than in cells transfected with the backbone vector (pGL2) (Fig. 2C). Then we tested the activation of the *AMH* promoter with concentrations of FSH or LH ranging from 5–1000 mIU/ml. FSH did not have any effect at 5 mIU/ml; it enhanced the AMH reporter by approximately 31% at 25 and 50 mIU/ml and by 60% at 250-1000 mIU/ml (Fig. 2D). The stimulation was statistically different between the two ranges of FSH concentration, showing a dose-dependent effect of FSH. LH activated the AMH reporter by 15–25%, without statistical differences between the concentrations (Fig. 2E).

Additive effect of FSH and LH on the *AMH* promoter

Because stimulation of the *AMH* promoter by either FSH or LH (Fig. 2, D and E) was lower than that obtained with cAMP (Fig. 2A), we cotransfected both receptors (Fig. 2C) together with the −3068hAMH-luc construct, and we treated the cells with a combination of recombinant FSH and LH (1 IU/ml of each) or with human menopausal gonadotropin (HMG) (1 IU/ml), a commercial combination of native FSH and LH. Figure 3A shows that in these conditions, the stimulation of luciferase activity was similar to that obtained with cAMP, indicating an additive effect of FSH and LH combination. Next we tested gonadotropin combinations, with different LH to FSH concentration ratios (0.5, 0.75, 1, 1.5, and 2) and at concentrations similar to those observed in serum at midfollicular phase (10–20 mIU/ml) and at ovulation (50–100 mIU/ml). An additive effect on *AMH* transcription (42–58% of stimulation) was still found with physiological doses of FSH and LH (compared with Fig. 2, D and E), but it was not different between the two ranges of gonadotropin concentrations (Wilcoxon test, $P = 0.07$). Interestingly, activation of the AMH reporter was significantly higher for LH to FSH ratios above 1, corresponding to an abnormal clinical situation often encountered in women with polycystic ovary syndrome (PCOS) (Fig. 3B).

cAMP, FSH, and LH activate the *AMH* promoter through protein kinase A (PKA)/p38-dependent signaling pathways

Intracellular targets of cAMP include PKA, MAPK p38, ERK, and phosphatidylinositol 3-kinase (PI3K) cascades (20) (Fig. 4A). Using inhibitors of each cascade, we investigated the molecular mechanisms involved in the activation of the *AMH* promoter by cAMP, FSH, and LH. KK1 cells were transfected with the −3068hAMH-luc construct alone or with the *FSH-R* or *LH-R* cDNAs and stimulated for 48 h with cAMP, FSH, or LH in the presence or absence of inhibitors of PI3K (LY29402), MAPK (PD98059), p38 MAPK (SB203580), or PKA (H89). H89 reduced the effects of cAMP, FSH, and LH on *AMH* promoter (Fig. 4, B–D). SB203580 also decreased cAMP and LH effects but to a lower extent than H89 (Fig. 4, B and D).

Sequences lying less than 200 bp and more than 2.2 kbp upstream of the *AMH* transcription start site are involved in the response to cAMP

To identify the transcription factors recruited by gonadotropins to stimulate *AMH* transcription, we studied the responsiveness to cAMP of constructs containing various lengths of the *AMH* promoter: 3068, 2202, 423, or 202 bp (Fig. 5A). In control medium, the highest *cis*-activating capacity was obtained with the 2202-bp promoter (Fig. 5B); the 3068-bp construct showed lower activity, suggesting the presence of a repressor element between 2202 and 3068 bp. The *cis*-activating capacity of the 423- and 202-bp constructs was low but at least 10-fold higher than that of the backbone vector. The highest cAMP-dependent stimulation of luciferase activity was obtained with the 3068-bp promoter; the 202-bp and 423-bp promoters showed 50–60% of the 3068-bp pro-

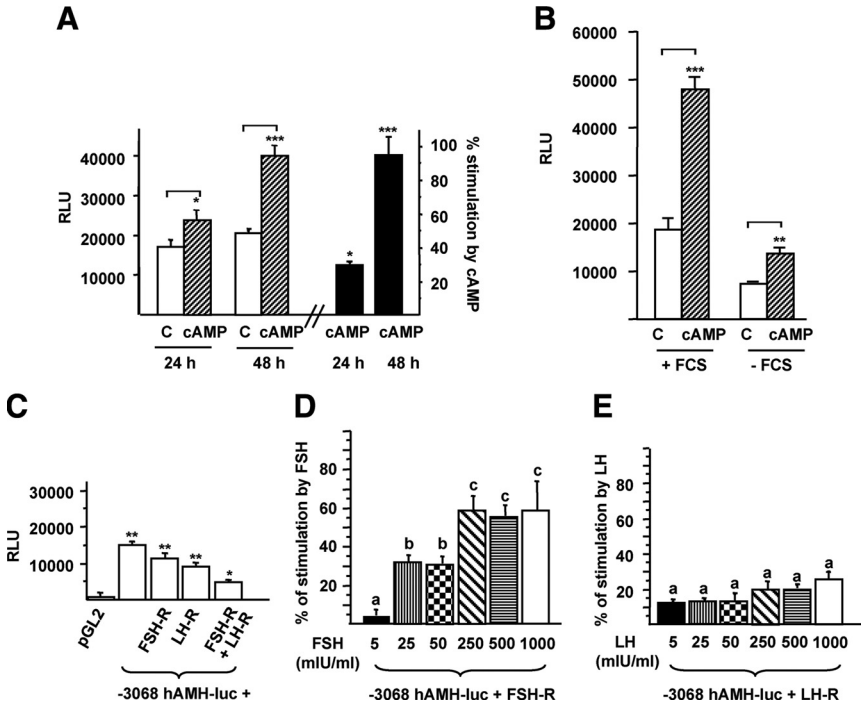

FIG. 2. Stimulation of the AMH promoter by cAMP, FSH, and LH in KK1 cells. Panel A, Stimulation of the −3068hAMH-luc construct by cAMP. Cells were transfected with 1 μg −3068hAMH-luc, and luciferase activity was assessed 24 and 48 h after treatment with cAMP (1 mM) and compared with cells cultured in control medium (C) using a Student's *t* test. Results are expressed in RLU or in percentage of stimulation [(RLU of treated cells − RLU of control cells)/RLU of treated cells) × 100]. Panel B, Effect of fetal calf serum (FCS) on the stimulation of −3068hAMH-luc by cAMP. Cells were transfected with 1 μg −3068hAMH-luc, and luciferase activity was assessed 48 h after treatment with cAMP (1 mM) in presence or absence of FCS and compared with cells cultured in control medium (C) using a Student's *t* test. Results are expressed in RLU. Panel C, *Cis*-activating capacity of −3068hAMH-luc in the presence of *FSH-R* and *LH-R* cDNAs. KK1 cells were cotransfected with 1 μg of the −3068hAMH-luc construct and 1μg of *FSH-R* and/or *LH-R* cDNAs, and luciferase activity was assessed after 48 h of culture in control medium. Results are expressed in RLU. Comparisons of means between different experimental conditions were made by repeated-measures ANOVA, followed by Dunnett *post hoc* test to compare all *vs.* pGL2. Panels D and E, Stimulation of the −3068hAMH-luc construct by FSH or LH. KK1 cells were cotransfected with 1 μg of the −3068hAMH-luc construct and FSH or LH receptor cDNA (1 μg). Luciferase activity was assessed after 48 h of treatment with FSH (panel D) or LH (panel E) (5–1000 mIU/ml). Results are expressed as percentage of stimulation. Responses to the different FSH and LH doses were compared by repeated-measures ANOVA and subsequently by Student-Newman-Keuls tests. *Different superscripts* indicate significant differences between the responses. Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

moter response, whereas the 2202-bp had a 40–50% response (Fig. 5C). Taken together, these results suggest that more than half of cAMP effect on the *AMH* promoter involves sequences lying less than 202 bp of the transcription start site but that full activation requires sequences lying more than 2202 bp upstream.

Nuclear factor-κB (NFκB), activation protein-2 (AP2), GATA-4, and SF-1 are involved in gonadotropin activation of the *AMH* promoter in KK1 cells

To determine which transcription factors are implicated in gonadotropin activation of the *AMH* promoter, we separately disrupted all the binding sites identified within the 3068 bp of the human *AMH* gene 5′-flanking region (Fig. 5A). Using targeted mutagenesis, −3068hAMH-luc constructs with mutations in binding sites for NFκB (18), AP2 (18), GATA-4, SF-1, SOX9/8, and AP1 were generated (Table 2). In control medium, only the mutation of the Sex Determining Region Y gene-related high mobility group box protein 9 or 8 (SOX9/8)-binding site resulted in a dramatic decrease of the *cis*-activating capacity of the construct (Fig. 6A), precluding a study of its regulation. Disruption of all other binding sites, except AP1, namely NFκB, AP2, GATA-4, and SF-1 resulted in a significant decrease of cAMP stimulation of the −3068hAMH-luc reporter gene (Fig. 6B). As shown in Fig. 6C, only the mutation of GATA-4 or SF-1 sites induced a modest but significant decrease of FSH response. Response to LH was not affected (Fig. 6D), probably because the LH effect on the −3068hAMH-luc reporter gene was too low. Because the importance of upstream binding sites for NFκB and AP2 in the activation of the *AMH* promoter by cAMP had already been shown for Sertoli cells (18), we then focused on the role of transcription factors with binding sites within the first 700 bp of the *AMH* promoter. All these transcription factors are expressed by KK1 cells. After quantification, only *GATA-4*, *SF-1*, and *SOX9* but not *SOX8* mRNAs were slightly but significantly increased after 48 h of treatment with cAMP (Table 1 and Fig. 6E). We also showed that the level of GATA-4 and SF-1 proteins in the nuclear fraction was increased after 48 h of treatment with cAMP (Fig. 6F). Taken together, these results suggest that NFκB, AP2, GATA-4, and SF-1 participate in the stimulation of the *AMH* promoter by gonadotropins in KK1 cells.

Discussion

Despite increasing recognition of the clinical importance of AMH in female reproductive endocrinology, little information is available on its regulation. *AMH* transcription in granulosa cells is up-regulated by bone morpho-

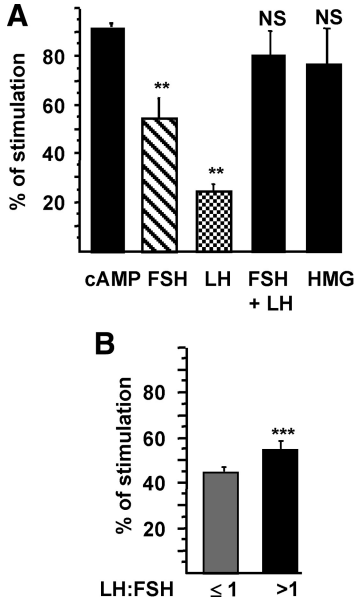

FIG. 3. Additive effect of FSH and LH. KK1 cells were cotransfected with 1 μg of the −3068hAMH-luc construct and cDNAs (1 μg) of the FSH and LH receptors. Luciferase activity was assessed 48 h after stimulation by cAMP (1 mM), FSH (1 IU/ml), LH (1 IU/ml), FSH and LH (1 IU/ml of each), or HMG (1 IU/ml) (A) and combinations of FSH and LH at different ratios (0.5, 0.75, 1. 1.5, 2) in two ranges of concentrations (10–20 and 50–100 mIU/ml) (B). Results are expressed as percentage of stimulation. Comparisons of means between different treatments (A) were made by repeated-measures ANOVA, followed by Dunnett *post hoc* test to compare all *vs.* cAMP. Comparison of means between LH to FSH ratios (B) were made using the nonparametric Mann and Whitney *U* test. Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. NS, Not significant; **, $P <$ 0.01; ***, $P < 0.001$.

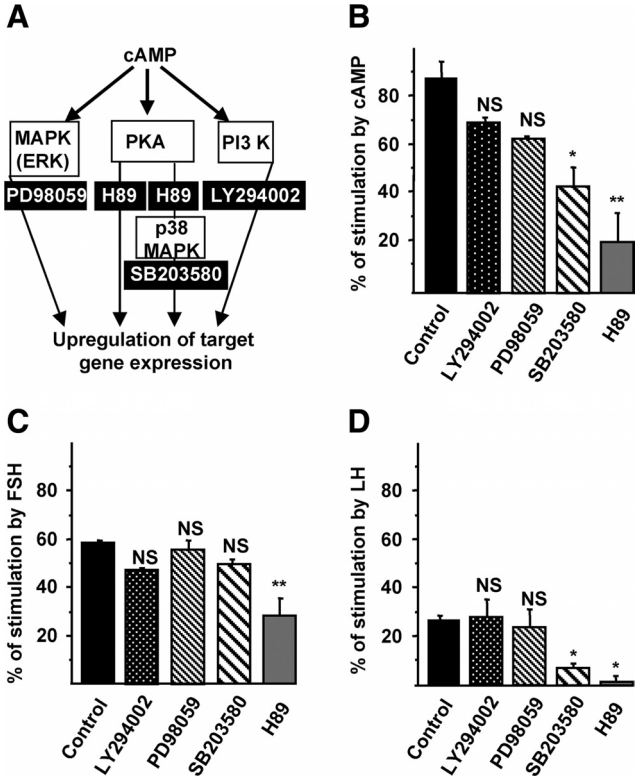

FIG. 4. Signaling pathways used by gonadotropins to activate the *AMH* promoter. A, Diagram of the signaling pathways activated by cAMP. B–D, Signaling pathways used by cAMP, FSH, and LH to stimulate the AMH promoter. KK1 cells were transfected with 1 μg of −3068hAMH-luc construct alone (B) or with the FSH receptor (C) or LH receptor (D) and stimulated during 48 h, with cAMP (1 mM) (B), FSH (1 IU/ml) (C), or LH (1 IU/ml) (D) in the absence or presence of inhibitors of PI3K (LY29402; 25 μM), MAPK (PD98059; 20 μM), p38 MAPK (SB203580; 20 μM), and PKA (H89; 10 μM). Results are expressed as percentage of stimulation and compared with the effect obtained in the same conditions without inhibitor (control). Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. Comparisons of means between different experimental conditions were made by repeated-measures ANOVA, followed by Dunnett *post hoc* test to compare all *vs.* control. NS, Not significant; *, $P < 0.05$; **, $P < 0.01$.

genetic proteins (21, 22) and by coculture with oocytes of preantral or preovulatory follicles (23) and down-regulated by testosterone (24). For estradiol, opposite effects have been reported (see below).

Studies of the relationship of FSH with AMH have also yielded conflicting results. Clinical data could be interpreted as supporting the hypothesis that FSH inhibits *AMH* expression, but their value is open to discussion. Obviously, the inverse correlation between low serum AMH and high FSH in infertile women is meaningless, because both are independent markers of decreased ovarian reserve (25, 26). The fall of serum AMH levels during controlled ovarian hyperstimulation with FSH can be at least partly attributed to the acceleration of follicle maturation, leading to the loss of small antral follicles, the main producers of AMH (27, 28). Patients harboring granulosa cell tumors secrete high amounts of AMH that are related to the volume of the tumor, (29–31) whereas endogenous production of FSH is suppressed; however, cancer cells cannot be taken as models, due to disruption of regulatory mechanisms (32).

Granulosa cells express FSH receptors already by the secondary stage of follicle maturation (33) at the time of rising AMH production. The decreased expression of *AMH* during late follicle maturation has been interpreted as supporting a negative effect of FSH upon AMH expression (5). At that stage, local changes in the environment surrounding the follicle may modulate or mask the effect of gonadotropins (34–36). For example, estrogens, whose production is stimulated by FSH, may inhibit AMH production (5, 37), but this is controversial (38). Many other hormones and growth factors emanating from the follicle, oocyte, or theca could be involved too.

The same caveats apply to the outcome of various treatment protocols and may explain some of the conflicting results. On one hand, Baarends *et al.* observed a decrease of *AMH* expression in some, but not all, preantral and small antral follicles in prepubertal rats treated with GnRH antagonist and FSH (5). On the other hand, sev-

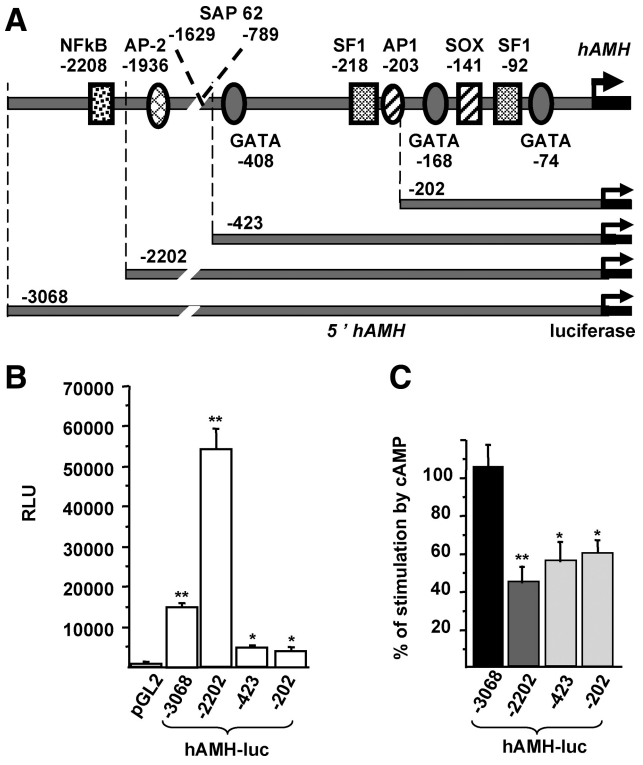

FIG. 5. Determination of cAMP-responsive domains within the *AMH* promoter. A, Schematic representation of the human *AMH* gene-promoter-luciferase constructs. The longest construct contained 3068 bp. Localization and nucleotide positions of binding sites for NFκB, AP2, SF-1, AP1, SOX, and GATA-4 in the human promoter are according to Ref. 18 B, *Cis*-activating capacity of the constructs. KK1 cells were transfected with 1 μg of the previously mentioned constructs, and luciferase activity was analyzed after 48 h culture in control medium. Results are expressed in RLU. Means were compared by repeated-measures ANOVA, followed by Dunnett *post hoc* test to compare all *vs.* pGL2. C, Regulation of the constructs by cAMP. KK1 cells were transfected with 1 μg of each construct, and luciferase activity was assessed 48 h after treatment with cAMP (1 mM). Results are expressed as percentage of stimulation by cAMP compared with untreated cells. Means were compared by repeated-measures ANOVA and subsequently by Dunnett *post hoc* test to compare all *vs.* −3068hAMH-luc. Data shown correspond to the mean ± SEM of at least three experiments, each done in triplicate. *, $P < 0.05$; **, $P < 0.01$.

eral reports are consistent with a positive effect of FSH and/or LH upon *AMH* expression. Injection of a GnRH antagonist decreases *AMH* expression in the marmoset (10). Suppression of gonadotropin secretion results in a drop of AMH serum level in women (39). Xu *et al.* (40) have detected an increased production of AMH by isolated human secondary follicles, cultured in the presence of FSH.

Culture of granulosa cells appears the method of choice for specifically investigating the regulation of ovarian AMH by FSH; however, even with this method, agreement has not been reached. Pellatt and co-workers (9) failed to detect an effect of FSH upon AMH concentration of the culture medium of human granulosa-luteal cells, but the medium also contained testosterone, a potent repressor of AMH production in Sertoli cells (41). Rico *et al.* (22) observed an inhibitory effect of FSH upon the expression of *AMH* by bovine granulosa cells. Voutilainen and Miller (42) detected no effect of FSH but observed a stimulatory effect of cAMP upon *AMH* mRNA of pooled human granulosa cells by Northern blotting. Technical issues and species differences may explain some of the variability. In the present work, we find that FSH and its second messenger cAMP up-regulate *AMH* expression.

The AMH production of the granulosa-luteal cells obtained after *in vitro* fertilization procedures is extremely low. We therefore switched to a reporter gene approach to highlight modest effects, not detectable by conventional expression studies, and also to investigate the mechanism of action of gonadotropins on the *AMH* promoter. We employed KK1 cells, which have been successfully used to study GATA-4 regulation of *AMH* (17), in which we transfected the human *FSH-R* or/and *LH-R* cDNAs, and a reporter gene constituted by 3068 bp of the human *AMH* gene *5′*-flanking region driving the luciferase gene. We found that both FSH and cAMP stimulate *AMH trans*-activation, the effect of FSH being dose dependent (Fig. 2). A similar reporter gene has been used to demonstrate the up-regulation of *AMH* by FSH in SMAT-1 Sertoli cells (18) because *in vivo*, the strong inhibitory action of testosterone masks the stimulatory effect of FSH upon Sertoli cell secretion of AMH (43, 44).

We also demonstrate, using the reporter gene approach, a significant (25%, $P < 0.01$) enhancement of *AMH* transcription by LH in KK1 cells (Fig. 2). The increase of *AMH* mRNA seen in human granulosa-luteal cells was not significant (25.8%, $P < 0.07$), in keeping with the results of Pellatt *et al.* (9) (28% stimulation, $P = 0.06$). In contrast, Voutilainen and Miller (42) showed an increase of *AMH* mRNAs by human chorionic gonadotropin (hCG) in granulosa-luteal cells. This effect is lower than that obtained with cAMP. In line with these results, we observed a stimulation of the *AMH* reporter gene comparable to that induced by cAMP only when KK1 cells were incubated with both FSH and LH (Fig. 3).

Normally, in large follicles, the LH receptor is highly expressed by granulosa cells close to the basement membrane but not by the ones in the cumulus (45), which express *AMH*; thus, the modest effects of LH reported are not physiological in normal women. However, they could apply to PCOS, the most common cause of infertility, characterized by an overexpression of AMH by granulosa cells (9, 46). Granulosa cells from PCOS follicles are at a prematurely advanced stage of development (47, 48). Furthermore, most PCOS patients present with increased GnRH/LH pulsatility (49, 50) and a LH to FSH

TABLE 2. Sequence of the primers and kits used for targeted mutagenesis

Mutations	Sequences	Targeted mutagenesis kits
GATA −408	TCC TTG GGG TCT CCG GTA CCC CCA CCA GGG GTG G	A
SF-1 −218	GGG GAT GGC CCT GGT ACC CGG CAT GTT GAC ACA TC	B
	GAT GTG TCA ACA TGC CGG GTA CCA GGG CCA TCC CC	
AP1 −203	CAA GGA CAG CAT GTG CGC GCA CAG GCC CAG CTC T	A
GATA −168	GCT CTA TCA CTG GGG AGG GGG TAC CGC TGC CAG GGA CAG	B
	CTG TCC CTG GCA GCG GTA CCC CCT CCC CAG TGA TAG AGC	
SOX9/8 −141	ACA GAA AGG GCT GGT ACC GAA GGC CAC TCT G	B
	CAG AGT GGC CTT CGG TAC CAG CCC TTT CTG T	
SF-1 −92	CGG GCA CTG TCC CGC GCG CAC GCG GCA GAG GAG	B
	CTC CTC TGC CGC GTG CGC GCG GGA CAG TGC CCG	
GATA −74	AGG TCG CGG CAG AGG GTA CCG GGG TCT GTC CTG C	B

A, Altered Sites II *in vitro* mutagenesis systems kit; B, QuikChange II XL site-directed mutagenesis kit.

ratio above 1. Interestingly, when we used a similar combination, gonadotropin stimulation of *AMH* transcription was more effective (Fig. 3). LH stimulation might thus contribute to overexpression of *AMH* by PCOS granulosa cells. Because AMH can activate the expression of FSH and LH ß-subunits (51), it may amplify the syndrome.

Gonadotropins induce cAMP-mediated events predominantly through the PKA-signaling pathway (20). In keeping with this notion, H89, an inhibitor of PKA, prevents FSH, LH, and cAMP stimulation of *AMH* transcription in KK1 granulosa cells (Fig. 4). LH and cAMP effects are also reduced in the presence of SB303580, an inhibitor of p38 MAPK (Fig. 4). However, the negative effect of SB303580 is less potent than that of H89, indicating that PKA is the major pathway involved in *AMH* up-regulation in response to LH. Relatively few gonadotropin/cAMP-responsive genes are regulated through the p38 MAPK pathway in granulosa cells. FSH-stimulated activation of p38 MAPK leads to the phosphorylation of the actin-capping protein heat shock protein-27 (52), triggers *Crtl1* expression (53), and regulates estradiol production (54). LH uses the p38 MAPK signaling pathway to activate epoxide hydroxylase 2 (55) and the transcription factor Runx1 (56).

The best known transcription factor regulated by PKA is the cAMP-response element-binding protein, but its involvement in FSH and LH effects on *AMH* transcription seems unlikely because no cAMP-response element has been identified in the *AMH* promoter. Based upon reporter gene and expression studies, we demonstrate that *AMH* up-regulation by FSH in KK1 granulosa cells mobilizes GATA-4 and SF-1 transcription factors; NFκB, AP2 are involved in the cAMP response. The involvement of NFκB and AP2 on the stimulation of *AMH* transcription by cAMP has also been reported in SMAT-1 Sertoli cells (18). Several *in vitro* and *in vivo* studies have demonstrated the importance of GATA-4, SF-1, and SOX9 transcription factors for *AMH* expression in Sertoli cells and in heterologous systems, SOX9 being absolutely required to trigger *AMH* transcription (57). All directly bind to the *AMH* promoter but also interact with each other and with several cofactors (58, 59). In granulosa cells, Anttonen *et al.* (17) have shown that GATA-4 *trans*-activates the *AMH* promoter and that its cofactor Friend of GATA-2 inhibits this effect. GATA-4 is frequently used by FSH and hCG to regulate their target genes (60), in keeping with our results (Fig. 5 and 6).

In conclusion, using complementary approaches, we show that FSH and cAMP stimulate *AMH* transcription by granulosa cells. FSH and LH have an additive effect, which may be important in PCOS. Gonadotropins and cAMP act through the PKA and p38 MAPK signaling pathways and the GATA-4 and SF-1 transcription factors among others. These data are in keeping with those already demonstrated for Sertoli cells (reviewed in Ref. 61) and unify the concept of regulatory mechanisms in male and female gonads, despite the sharp differences in the timing of their *AMH* expression.

Materials and Methods

Reagents

Recombinant human FSH (Gonal-F) and LH (Luveris) were obtained from Serono Pharmaceuticals (Boulogne, France) and HMG from Ferring Pharmaceuticals (Gentilly, France). Rabbit polyclonal antibodies against SOX9 (ref AB5535) and SF-1 were purchased, respectively, from Millipore (Temecula, CA) and Upstate Biotechnology (Waltham, MA), goat polyclonal anti-GATA-4 was from Santa Cruz Biotechnology (Santa Cruz, CA); and peroxidase-labeled antigoat and antirabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). KK1 cells and the *LH-R* cDNA construct were a generous gift of Dr. Ilpo Huthaniemi.

Targeted mutagenesis

Most AMH reporter constructs were obtained as previously described (18). Plasmids with mutations in binding sites for GATA-4 (at positions −408, −168, and −74), SF-1 (at position −218 and −92), and SOX9/8 (at position −141) of the human

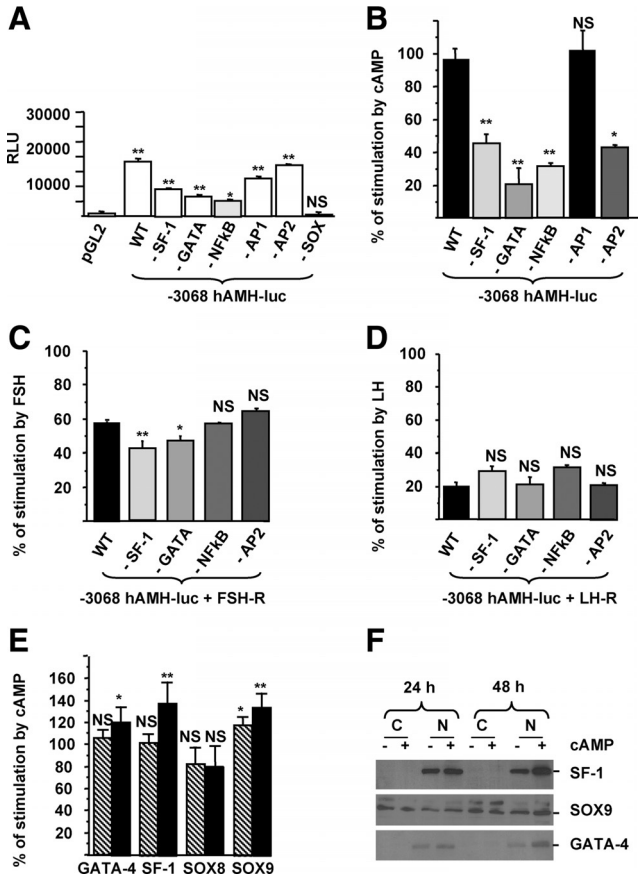

FIG. 6. Identification of the transcription factors involved in cAMP and gonadotropin stimulation of the *AMH* gene. A, *Cis*-activating capacity of the −3068hAMH-luc constructs with mutations in binding sites for NFκB, AP2, GATA-4, SF-1, AP1, and SOX. KK1 cells were transfected with 1 μg of the constructs, and luciferase activity was analyzed after 48 h of culture in control medium. Results are expressed in RLU. B–D, Regulation of the mutant constructs by cAMP (B), FSH (C), and LH (D). KK1 cells were transfected with 1 μg of mutated constructs and 1 μg of *FSH-R* (C) or *LH-R* (D), and luciferase activity was assessed 48 h after treatment with cAMP (1 mM) (B), FSH (1 IU/ml) (C), or LH (1 IU/ml) (D). Results are expressed as percentage of stimulation. Data shown correspond to the mean ± SEM of three experiments, each done in triplicate. Comparisons of means between different experimental conditions were made by repeated-measures ANOVA, followed by Dunnett *post hoc* test to compare all *vs.* control (pGL2 for A and −3068hAMH-luc (WT) for B– D). E, *GATA-4*, *SF-1*, *SOX9*, and *SOX8* mRNAs were analyzed by RT-PCR, and their regulation by cAMP in KK1 cells was quantified by real-time RT-PCR after 24 (*hatched bars*) and 48 (*black bars*) hours of treatment by cAMP (1 mM). Results are expressed as percentage of response compared with untreated cells using the Student's *t* test. Data shown correspond to the mean ± SEM of three experiments, each done in triplicate. F, Nuclear translocation of SF-1, SOX9, and GATA-4 in the presence of cAMP. SF-1, SOX9, and GATA-4 proteins in cytosol and nuclear fractions (50 μg of each) were analyzed by Western blotting after 24 and 48 h of treatment with cAMP. NS, Not significant; *, $P < 0.05$; **, $P < 0.01$.

AMH promoter were generated using the QuikChange II XL site-directed mutagenesis (Stratagene, La Jolla, CA) or Altered Sites II *in vitro* mutagenesis systems (Promega, Charbonnières, France) kits according to the manufacturer's instructions, and mutagenic oligonucleotide primers (Table 2) were synthesized by Eurogentec (Liège, Belgium).

Subjects

Thirty-two infertile *in vitro* fertilization-embryo transfer patients, 20–40 yr of age, were studied prospectively. All of them met the following inclusion criteria: 1) both ovaries present, with no morphological abnormalities, adequately visualized in transvaginal ultrasound scans; 2) menstrual cycle length range between 25 and 35 d; 3) no current or past diseases affecting ovaries or gonadotropin or sex steroid secretion, clearance, or excretion; 4) no clinical signs of hyperandrogenism; and 5) body mass indexes ranging between 18 and 25 kg/m^2. Infertility was due to either tubal or sperm abnormalities. In agreement with the inclusion criteria, no patient suffering from PCOS has been enrolled. An informed consent was obtained from all women, and this investigation received the approval of our internal institutional review board.

Ovarian stimulation

All women received a time-release GnRH agonist (Decapeptyl 3 mg; Organon Pharmaceuticals, Saint-Denis, France) from cycle d 2 onward. About 3 wk after gonadotropin suppression confirmed by the detection of low serum levels of estradiol and gonadotropins, recombinant FSH therapy (Gonal-F; Serono Pharmaceuticals) was initiated at a dosage of 150–225 IU/d. Daily FSH doses and timing of hCG administration were adjusted according to the usual criteria of follicular maturation. Administration of hCG (Gonadotrophine Chorionique 'Endo,' 10,000 IU im; Organon Pharmaceuticals) was performed when at least four follicles were larger than 16 mm in diameter and estradiol levels per mature follicle (>16 mm in diameter) were higher than 200 pg/ml. Oocytes were retrieved 36 h after hCG administration by transvaginal ultrasound-guided aspiration.

Collection of human granulosa-luteal cells

After oocyte isolation, follicular fluids from each patient were centrifuged separately through a one-step density Percoll gradient (vol/vol, PBS/Percoll) at 350 × *g* for 15 min to remove red blood cells. Granulosa-luteal cells were collected at the interface, washed with PBS, and seeded at 3×10^5 cells per well in six-well plates in DMEM/F-12 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Cell culture and transfection assays

KK1 cell line was cultured at 37 C under 5% CO_2 in DMEM/F-12 (Invitrogen) containing 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Twenty-four hours before transfection, KK1 cells were seeded in six-well plates at 3.5×10^5 cells per well in the same medium except that fetal calf serum was charcoal stripped (Invitrogen). Transient transfection was performed with the Lipofectamine Plus kit (Invitrogen).

Western blotting

After 24 or 48 h of treatment in presence of cAMP, KK1 cells were washed and lysed to prepare nuclear and cytosol extracts according to the ProteoExtract subcellular proteome extraction kit (Calbiochem, La Jolla, CA). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins were subjected to SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and transferred onto a Protran BA85 nitrocellulose membrane (Whatman, Dassel,

Germany). Membranes were first incubated with antibodies against SOX9, SF-1, or GATA-4 as described (62). Then blots were probed with a peroxidase-labeled antirabbit or antigoat antibody at 1:5000 (Jackson ImmunoResearch). Bands were visualized with the enhanced chemiluminescence detection reagent (GE Healthcare, Buckinghamshire, UK).

RNA extraction and reverse transcription

Twenty hours after seeding, human granulosa-luteal cells were serum starved for 1 h and cultured for 48 h without serum in the presence of FSH (1 IU/ml), LH (1 IU/ml), or *N*,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (cAMP) (10 μM), and KKI cells were cultured for either 48 or 72 h in the presence of cAMP (1 mM). Total RNAs were extracted with the RNA Plus extraction kit (QIAGEN, Valencia, CA).

Reverse transcription was performed in a total of 20 μl with the Omniscript reverse transcription kit for RT-PCR (QIAGEN) using 1 μg RNA, Omniscript reverse transcriptase, and oligodeoxythymidine primers and random hexamers as recommended by the manufacturer.

Quantitative real-time PCR

Quantification of the content of *AMH*, *RPL13a* (ribosomal protein L13a), *SDHA* (succinate dehydrogenase complex subunit A), *SF-1*, *GATA-4*, *SOX8*, *SOX9*, and *hprt* (hypoxanthine guanine phosphoribosyl transferase) mRNA was performed by real-time PCR using the TaqMan PCR method. The primers and the Universal Probe Library probes (Roche Diagnostics, Indianapolis, IN) used to amplify these genes are indicated in Table 1. Real-time PCR was performed with 1/5 dilution of the cDNAs using the Lightcycler 480 Probes Master kit (Roche Diagnostics, Mannheim, Germany). The PCR protocol used an initial denaturing step at 95 C for 10 min followed by 45 cycles of 95 C for 10 sec, 60 C for 30 sec, and 72 C for 1 sec. To generate external standard curves, different concentrations of the purified and quantified PCR products were amplified. Copy numbers were normalized by two reference genes, *RPL13a* and *SDHA* for human *AMH*, and one, *HPRT*, for mouse *AMH*.

Reporter assays

KK1 cells were transfected with 1 μg of the different *AMH* reporter genes with or without 1 μg of the human *FSH-R* or/and *LH-R* cDNAs. At the end of the transfection, cells were stimulated with cAMP (1 mM) or human recombinant FSH or LH at different concentrations. Forty-eight hours later, cells were washed twice with PBS and lysed for 20 min under rocking in 500 μl passive lysis buffer (Luciferase Assay System; Promega). Twenty microliters were analyzed for firefly luciferase activity according to the manufacturer's instructions using a Lumat LB95507 luminometer (EG&G Berthold, Thoiry, France). Results are expressed in relative light units (RLU) or in percentage of stimulation [(RLU of treated cells − RLU of control cells)/RLU of treated cells) × 100]. Data shown correspond to the mean ± SEM of at least three experiments each done in triplicate.

Statistics

Results were analyzed by the Wilcoxon test for paired comparisons between control and treated cells, Student's *t* test or by nonparametric Mann and Whitney *U* test to compare two means, and repeated-measures ANOVA followed by Dunnett *post hoc* tests to compare all *vs.* control or by Student-Newman-Keuls tests to compare the different conditions to each other.

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