

SOX9 and SF1 are involved in cyclic AMP-mediated upregulation of anti-Müllerian gene expression in the testicular prepubertal Sertoli cell line SMAT1

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Lasala C, Schteingart HF, Arouche N, Bedecarrás P, Grinspon RP, Picard JY, Josso N, di Clemente N, Rey RA. SOX9 and SF1 are involved in cyclic AMP-mediated upregulation of anti-Müllerian gene expression in the testicular prepubertal Sertoli cell line SMAT1. *Am J Physiol Endocrinol Metab* 301: E539–E547, 2011. First published June 21, 2011; doi:10.1152/ajpendo.00187.2011.—In Sertoli cells, anti-Müllerian hormone (AMH) expression is upregulated by FSH via cyclic AMP (cAMP), although no classical cAMP response elements exist in the *AMH* promoter. The response to cAMP involves NF- κ B and AP2; however, targeted mutagenesis of their binding sites in the *AMH* promoter do not completely abolish the response. In this work we assessed whether SOX9, SF1, GATA4, and AP1 might represent alternative pathways involved in cAMP-mediated AMH upregulation, using real-time RT-PCR (qPCR), targeted mutagenesis, luciferase assays, and immunocytochemistry in the Sertoli cell line SMAT1. We also explored the signaling cascades potentially involved. In qPCR experiments, *Amh*, *Sox9*, *Sf1*, and *Gata4* mRNA levels increased after SMAT1 cells were incubated with cAMP. Blocking PKA abolished the effect of cAMP on *Sox9*, *Sf1*, and *Gata4* expression, inhibiting PI3K/PKB impaired the effect on *Sf1* and *Gata4*, and reducing MEK1/2 and p38 MAPK activities curtailed *Gata4* increase. SOX9 and SF1 translocated to the nucleus after incubation with cAMP. Mutations of the SOX9 or SF1 sites, but not of GAT4 or AP1 sites, precluded the response of a 3,063-bp *AMH* promoter to cAMP. In conclusion, in the Sertoli cell line SMAT1 cAMP upregulates SOX9, SF1, and GATA4 expression and induces SOX9 and SF1 nuclear translocation mainly through PKA, although other kinases may also participate. SOX9 and SF1 binding to the *AMH* promoter is essential to increase the activity of the *AMH* promoter in response to cAMP.

adenosine 5'-monophosphate; follicle-stimulating hormone; gonadotropin; GATA

IN THE MALE, ANTI-MÜLLERIAN HORMONE (AMH), also known as Müllerian inhibiting substance, is a specific marker of the immature Sertoli cell activity. AMH, secreted by the testis from the time of gonadal differentiation in early fetal life, is responsible for the regression of the Müllerian ducts, the anlagen of the fallopian tubes, the uterus, and the upper vagina (reviewed in Ref. 48). In early fetal life, AMH expression is triggered in Sertoli cells by SOX9, which binds to a specific response element on the *AMH* gene proximal promoter (4, 16, 39). Subsequently, other transcription factors like SF1,

GATA4, WT1, and DAX1 also regulate AMH expression either by direct binding to specific response elements in the *AMH* proximal promoter or by protein-protein interaction (4, 16, 24, 38, 50, 55, 58, 59, 61, 64). These initial steps of fetal testicular AMH expression, conserved from birds to mammalian species (4, 39), are independent of gonadotropins (48).

Although AMH exerts its best-characterized physiological effect in male sex differentiation very early during fetal life (56), Sertoli cells continue to synthesize large amounts of AMH until puberty (2, 26). With the advent of sensitive and highly specific immunoassays for its determination in serum in the 1990s, AMH became a useful circulating marker of Sertoli cell functional activity in diverse physiological and pathological conditions in humans and other mammals (26, 30, 43, 52). For instance, in humans, serum AMH increases progressively in the first months after birth following the neonatal activation of pituitary FSH secretion (1, 8). Accordingly, serum AMH is lower than expected in rodents with an inactivation of the FSH β -subunit gene (2) as well as in patients with congenital hypogonadotropic hypogonadism, a condition characterized by extremely low pituitary FSH secretion (9, 67), and increases in response to FSH administration in both rodents (2) and humans (9, 66). On the other hand, AMH is abnormally high in prepubertal boys with an activating mutation of the G_{α} protein involved in FSH receptor signaling in Sertoli cells (14, 34, 47) and in rams overexpressing the FSH receptor (51). These observations prompted us to study a potential regulation of testicular AMH production by FSH in late fetal life and after birth.

In previous studies (29, 32), we showed that FSH, signaling through its classical pathway involving cyclic AMP (cAMP) and protein kinase A (PKA), induces an increase in AMH expression. Unexpectedly, no classical cAMP response element (CRE) is present on the *AMH* promoter. The objective of this work was to identify alternative pathways not involving a classical CRE that mediate the increase of the *AMH* promoter activity in response to cAMP. Canonical sites for SOX9, SF1, GATA4, and AP1 are present within the proximal *AMH* promoter sequences, and all of these factors are involved in cAMP-mediated regulation of target genes in other cell types (13, 25, 41, 60). Therefore, using quantitative real-time PCR, immunocytochemistry, targeted mutagenesis, and luciferase assays in a previously characterized prepubertal Sertoli cell line named SMAT1 (17, 32), we assessed whether SOX9, SF1, GATA4, and AP1 are involved in cAMP-mediated upregula-

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tion of the *AMH* promoter. Beyond the classic PKA-dependent pathway, additional signaling cascades involving the cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF)-regulated phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB/Akt) pathway (27, 35–37, 49) and the MAPK cascades (15, 35) have been identified in Sertoli cells. Therefore, we also explored the signaling cascades downstream of cAMP potentially involved in the upregulation of the *AMH* promoter.

MATERIALS AND METHODS

Cell culture, transfection, and luciferase assays. SMAT1 cells, an immortalized immature Sertoli cell line (17, 32), were cultured in DMEM (GIBCO Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum, 1× amino acid mix (GIBCO Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO Invitrogen). Cells were plated at 2×10^5 cells/well in six-well plates and transiently transfected with 1 μg of luciferase reporter plasmids, or at 0.5×10^5 cells/well in 24-well plates, and with 0.5 μg of DNA using the LipofectAMINE PLUS Reagent package (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, 1 day after initial plating, DMEM with fetal calf serum was changed for DMEM without serum. Twenty-four hours later, transfections were performed in DMEM without serum. Dibutyl cAMP (dbcAMP; Sigma, St. Louis, MO) was added at 1 mM. Kinase inhibitors H89, LY-294002, SB-203580, and PD-98059 (all from Merck-Calbiochem, Darmstadt, Germany) were used at 10, 25, 20, and 10 μM, respectively. Firefly luciferase activity was determined with the Dual Luciferase Reporter Gene Assay kit (Roche Diagnostics, Indianapolis, IN), using Lumat LB95507 and Junior LB9509 luminometers (Berthold Technologies, Bad Wilbad, Germany), and normalized for renilla luciferase activity.

Plasmids. A 3,078-bp fragment of the 5'-flanking region of the human *AMH* gene (position -3,068/+10 relative to the major transcriptional initiation site) (20) was obtained from pGAMH1 construct (17) by restriction enzyme digestion using *SacI* and *AvrII*. *AvrII* site was rendered blunt using the Klenow fragment of DNA polymerase I, and the resulting construct was subcloned between *SacI* and *BglIII* sites of luciferase vectors pGL2B (Promega, Madison, WI) to obtain -3,068 5'hAMH-luc plasmids, as already described (32). The FSH receptor expression vector pcDNA-FSH-R and its control pcDNA-CAT were kindly provided by Drs. W. Tribley and M. Griswold (Pullman, WA). The use of these vectors has already been validated in SMAT1 cells (32).

Targeted mutagenesis. Plasmids with mutations in binding sites for SOX9, SF1, GATA4, or AP1 of the human *AMH* promoter were generated using the Altered Sites II In Vitro Mutagenesis Systems (Promega, Madison, WI) or the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). One SOX9 binding site was mutated (CTTTGAGA → GGTACCGA) at position -141, two SF1

binding sites were mutated (CAAGG → GGTAC) at positions -92 and -218, three GATA binding sites were mutated (AGATAG → GGTACC) at positions -74, -168, and -408, and one AP1 binding site was mutated (TTGACACATC → TGCGCGCAC) at position -203. Mutagenic oligonucleotide primers (Table 1) were synthesized by Eurogentec (Liège, Belgium). The introduction of a *KpnI* restriction site in SOX9, SF1, and GATA binding elements and of a *BssHII* restriction site in AP1 binding element helped in identifying the mutated plasmids. Sequence changes were verified by direct sequencing.

Quantitative real-time RT-PCR. Levels of gene expression were examined by a reverse transcription real-time quantitative PCR method using a LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) and the TaqMan method. cDNA was prepared from SMAT1 cells submitted to the various experimental conditions, and each cDNA was assayed in duplicate PCRs. Briefly, 2 μg of total RNA was treated with deoxyribonuclease I and reverse transcribed using the Omniscript Reverse Transcription system (Qiagen, Courtaboeuf, France) in a reaction volume of 20 μl as recommended by the manufacturer. Specific reverse and forward oligonucleotide primers for each gene were designed using Probe Finder Version 2.44 for mouse (Roche Applied Science). The sequences of oligonucleotide primers used were the following: mouse *Amh*: forward 5'-GGCTAGGG-GAGACTGGAGAA-3', reverse 5'-AGGTGGAGGCTCTTG-GAACT-3'; mouse *Sox9*: forward 5'-CAGCAAGACTCTGG-GCAAG-3', reverse 5'-TCCACGAAGGGTCTCTTCTC-3'; mouse *Sf1*: forward 5'-AGAATTCTCCTTCCGTTTCAGC-3', reverse 5'-TCACCACACACTGGACACG-3'; mouse *Gata4*: forward 5'-GGAAGACACCCCAATCTCG-3', reverse 5'-CATGGC-CCCACAATTGAC-3'. Real-time PCR was performed in a 96-well plate using the previously synthesized cDNA as template. The PCR contained about 5–10 ng of cDNA, 1× LightCycler 480 Probes Master (Roche Applied Science), and 500 nM of each specific reverse and forward primer. The PCR protocol used an initial denaturing step at 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s, with a transition rate of 20°C/s. Quantification of gene expression is based on a standard curve for each gene, constructed with known amounts of copies of amplification products of SMAT1 cDNAs, which was included in each real-time PCR experiment. Products of mock reverse transcription reactions for each of the SMAT1 RNA samples were included as negative controls to verify the absence of amplification signal attributable to contamination by genomic DNA.

Immunocytochemistry. SMAT1 cells, cultured on four-chamber Lab-Tek Permaxox slides (Nunc, Naperville, IL) at $2-5 \times 10^4$ cells/chamber, were fixed in 4% paraformaldehyde and submitted to immunocytochemistry without antigen retrieval. Primary antibodies were rabbit anti-SOX9 affinity purified polyclonal antibody 0.85 μg/ml (no. AB5535; Chemicon, Temecula, CA), rabbit anti-SF1 ligand-binding domain affinity-purified polyclonal antibody diluted 1:1,000 (no. 18443-5; kindly provided by Drs. N. Stallings and K.

Table 1. Sequences of oligonucleotide primers used for site-directed mutagenesis

Mutation	Sequences	Mutagenesis Protocol
SOX9 -141	ACA GAA AGG GCT GGT ACC GAA GGC CAC TCT G CAG AGT GGC CTT CGG TAC CAG CCC TTT CTG T	B
SF-1 -92	CGG GCA CTG TCC CGC GCG CAC GCG GCA GAG GAG CTC CTC TGC CGC GTG CGC GCG GGA CAG TGC CCG	B
SF1 -218	GGG GAT GGC CCT GGT ACC CGG CAT GTT GAC ACA TC GAT GTG TCA ACA TGC CGG GTA CCA GGG CCA TCC CC	B
GATA -74	AGG TCG CGG CAG AGG GTA CCG GGG TCT GTC CTG C	A
GATA -168	GCT CTA TCA CTG GGG AGG GGG TAC CGC TGC CAG GGA CAG CTG TCC CTG GCA GCG GTA CCC CCT CCC CAG TGA TAG AGC	B
GATA -408	TCC TTG GGG TCT CCG GTA CCC CCA CCA GGG GTG G	A
AP1 -203	CAA GGA CAG CAT GTG CGC GCA CAG GCC CAG CTC T	A

The following mutagenesis kits were used: A, Altered Sites II In Vitro Mutagenesis Systems kit; B, QuikChange II XL Site-Directed Mutagenesis kit.

Parker, University of Texas Southwestern Medical Center, Dallas, TX), rabbit anti-SF1 COOH-terminal domain affinity-purified polyclonal antibody diluted 1:1,000 (no. 4803-2; kindly provided by Drs. N. Stallings and K. Parker), goat anti-GATA4 polyclonal antibody 2 µg/ml (no. sc-1237; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-FOG2 polyclonal antibody 2 µg/ml (no. sc-10755; Santa Cruz Biotechnology). A negative control reaction was performed by replacing the primary antibody with nonimmune rabbit or goat serum. The reaction was revealed using peroxidase Vectastain Universal Elite ABC kit PK-6200 (Vector Laboratories, Burlingame, CA) and DAB Reagent D-3939 (Sigma). The intensity of the immunocytochemical reaction was measured in the nuclei and the cytoplasm of SMAT1 cells submitted to the different experimental conditions using the “mean intensity” tool of the NIS Elements BR Version 3 software for

image analysis (Nikon Instruments, Melville, NY). Results are given in arbitrary units after background subtraction.

Statistical analyses. Data distribution was tested for normality using the D’Agostino-Pearson test. Quantitative results were analyzed using a paired Student’s *t*-test for comparisons between means of two experimental conditions when data distribution passed the normality test or the Mann-Whitney test when distribution was not Gaussian. For comparisons between more than two experimental conditions, we used one-way analysis of variance (ANOVA), when distribution was normal, or the Kruskal-Wallis test, when distribution did not pass the normality test. As posttest, we used Dunnett’s test to compare one experimental condition against all others and Bonferroni’s test to compare between all experimental conditions when data distribution was normal or Dunn’s test when the distribution was not Gaussian. A

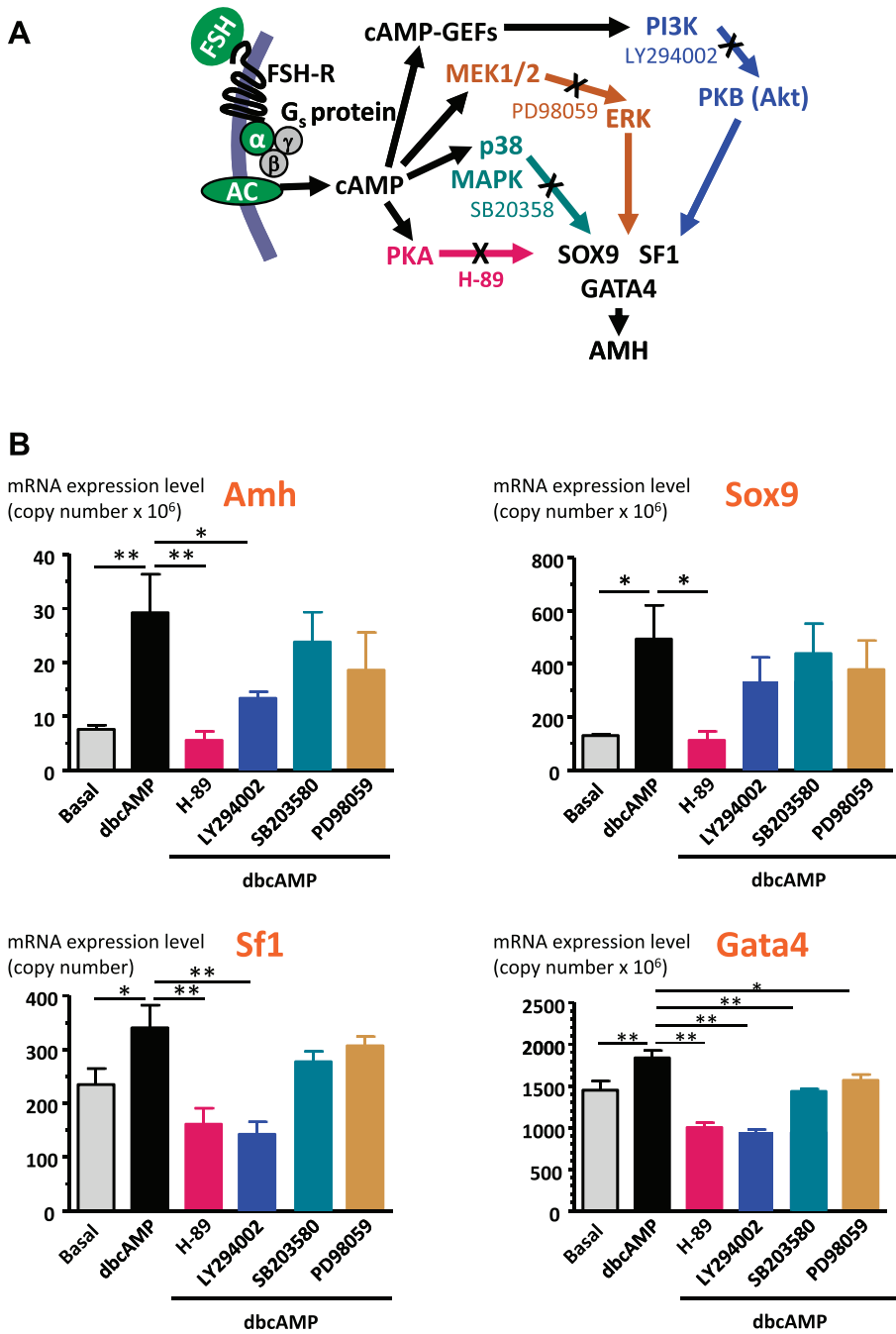


Fig. 1. Intracellular kinases involved in cyclic AMP (cAMP)-dependent increase in mRNA levels of anti-Müllerian hormone (*Amh*) and of its trans-activators *Sox9*, *Sf1*, and *Gata4*. **A:** FSH-mediated upregulation of target genes via cAMP may involve protein kinase A (PKA) or more recently described signaling pathways involving cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), which could be involved in the increase of *Amh* gene expression through the upregulation of *Sox9*, *Sf1*, and/or *Gata4*. Different inhibitors can be used to specifically block each of the various pathways: H-89 to block PKA-dependent pathways, SB-203580 to block p38 MAPK, PD-98059 to block MEK1/2-ERK1/2 pathway, and LY-294002 to block phosphatidylinositol 3-kinase (PI3K)-PKB (Akt) pathway. **B:** SMAT1 cells were incubated for 24 h in basal medium or in medium containing 1 mM dibutyryl cAMP (dbcAMP) alone or with either 10 µM H-89, 25 µM LY-294002, 20 µM SB-203580, or 10 µM PD-98059. Total mRNA was extracted, and levels of *Amh*, *Sox9*, *Sf1*, and *Gata4* were assessed by real-time RT-PCR. Results are expressed in copy numbers normalized by hypoxanthine-guanine phosphoribosyltransferase gene expression and analyzed by ANOVA followed by Dunnett’s test to compare all conditions against dbcAMP. Data shown correspond to the mean ± SD of 3 experiments (samples in duplicate in each experiment). **P* < 0.05, ***P* < 0.01.

difference was considered statistically significant when the P value was <0.05 . All calculations were made using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

RESULTS

cAMP increases Amh gene expression in SMAT1 cells through the PKA pathway. To verify that endogenous AMH expression is responsive to cAMP in SMAT1 cells, we performed quantitative real-time RT-PCR experiments and assessed *Amh* mRNA levels after incubating SMAT1 cells under basal or 1 mM dbcAMP-stimulated conditions. Signaling pathways described as intracellular targets of cAMP include PKA, which can be blocked by H-89, PI3K-mediated PKB (Akt) phosphorylation, which can be blocked by LY-294002, and p38 MAPK and MEK1/2 cascades, which can be blocked by SB-203580 and PD-98059, respectively (57). We used these blocking agents to also assess which signaling pathways were involved (Fig. 1A). As shown in Fig. 1B, *Amh* levels were

increased after incubation with dbcAMP. Coincubation with the PKA inhibitor H-89 resulted in a complete abolition of dbcAMP effect, whereas coincubation with the PI3K/PKB inhibitor LY-294002 provoked a partial inhibition. SB-203580 and PD-98059 had no significant effect. From these results, we conclude that cAMP increases *Amh* expression in SMAT1 cells involving mainly the PKA pathway but also PI3K/PKB. We further hypothesized that this was the result of an increased availability of transcription factors with proven *trans*-activating capacity of the *AMH* promoter, like SOX9, SF1, and GATA4. We tested two hypotheses: 1) dbcAMP upregulates *Sox9*, *Sf1*, and/or *Gata4* expression, and 2) dbcAMP increases SOX9, SF1, and/or GATA4 nuclear translocation.

Sox9, Sf1, and Gata4 expression is upregulated in SMAT1 cells in response to cAMP. To test the first hypothesis, we performed quantitative real-time RT-PCR experiments to assess *Sox9*, *Sf1*, and *Gata4* mRNA levels after incubating SMAT1 cells under basal or 1 mM dbcAMP-stimulated con-

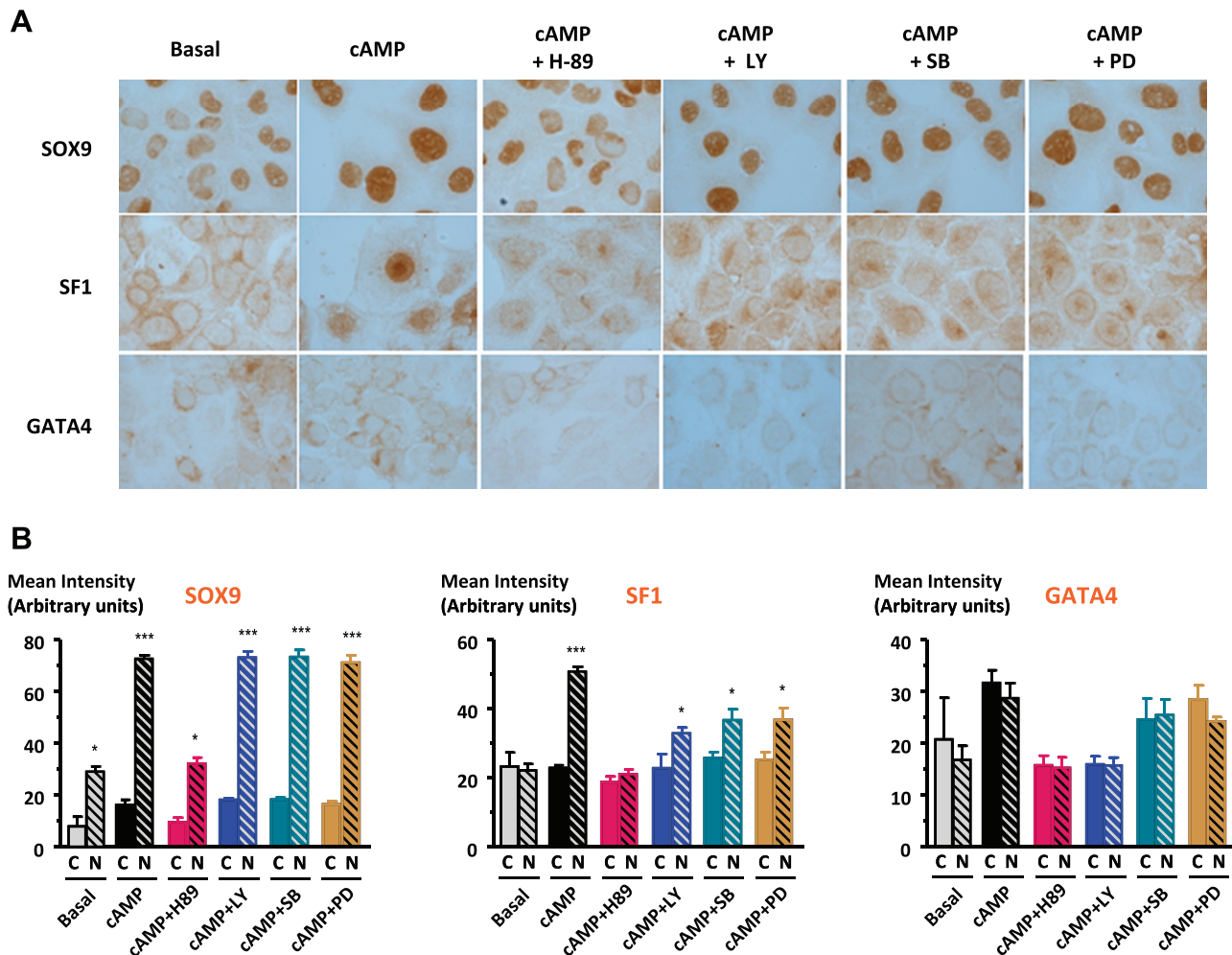


Fig. 2. cAMP-dependent nuclear translocation of *AMH* gene *trans*-activators SOX9, SF1, and GATA4 and the effect of blocking intracellular kinases. SMAT1 cells were incubated for 24 h in basal medium or in medium containing 1 mM dbcAMP alone or with either 10 μ M H-89 (blocks PKA), 25 μ M LY-294002 (LY; blocks PI3K/PKB), 20 μ M SB-203580 (SB; blocks p38 MAPK), or 10 μ M PD-98059 (PD; blocks MEK1/2-ERK1/2). *A*: immunocytochemistry was performed with antibodies against SOX9, SF1, COOH-terminal domain, or GATA4. A negative control reaction was performed by replacing the primary antibody with nonimmune rabbit or goat serum (not shown). The reaction was revealed using horseradish peroxidase and diaminobenzidine, yielding a brownish color reaction. *B*: mean intensity of the immunocytochemical reaction was assessed with the NIS Elements BR Version 3 software for image analysis. Data shown correspond to the mean \pm SD of 10 samples. * $P < 0.05$ and *** $P < 0.001$ for comparisons between the intensity in the nuclei (N) and the cytoplasm (C), as analyzed by ANOVA followed by Bonferroni's test.

ditions in the presence or absence of H-89, LY-294002, SB-203580, or PD-98059. The mRNA levels of *Sox9*, *Sf1*, and *Gata4* were increased in response to dbcAMP (Fig. 1B). *Sox9* increase in response to dbcAMP was abolished by the PKA blocking agent H-89, but not by the other kinase inhibitors. *Sf1* increase was curtailed by H-89 and the PI3K/PKB blocking agent LY-294002, whereas *Gata4* increase was inhibited by all kinase blocking agents tested. We concluded that cAMP increases the availability of the *AMH* gene *trans*-activators SOX9, SF1, and GATA4 in SMAT1 cells. SOX9 upregulation involves exclusively PKA, whereas SF1 upregulation also involves the PI3K/PKB pathway, and GATA4 involves MEK1/2 and p38 MAPK as well.

Nuclear translocation of SOX9 and SF1 is increased in SMAT1 cells in response to cAMP via PKA activation. To test whether dbcAMP regulates nuclear translocation of the transcription factors that mediate *AMH* promoter activation, we performed immunocytochemistry for SOX9, SF1, and GATA4 in SMAT1 cells incubated under basal or 1 mM dbcAMP-stimulated conditions in the presence or absence of H-89, LY-294002, SB-203580, or PD-98059. SOX9 was predominantly nuclear in all experimental conditions; dbcAMP significantly further increased its presence in the cell nuclei, which was impaired by coincubation with H-89 but not with the other kinase inhibitors (Fig. 2). SF1 localization increased in the cell nuclei after incubation with dbcAMP; this effect was abolished by coincubation with H-89 and impaired by LY-294002, SB-203580, and PD-98059. GATA4 was evenly distributed in all conditions. Altogether, these results indicate that cAMP increases nuclear translocation of SOX9 and mainly SF1 involving predominantly the PKA pathway, although PI3K/PKB, MEK1/2, and p38 MAPK might also be involved in SF1 translocation.

cAMP increases FOG2 expression in SMAT1 cells through the PKA pathway. In granulosa cells, GATA4-dependent *trans*-activation of the *AMH* promoter is inhibited by FOG2 (3). Therefore, we tested whether dbcAMP also regulates *FOG2* expression and nuclear localization. SMAT1 cells were incubated for 24 h in basal medium or in medium containing 1 mM dbcAMP (3 experiments, with samples in duplicate in each

experiment). Total mRNA was extracted, and levels of *Fog2* were assessed by real-time RT-PCR. *Fog2* mRNA expression was significantly increased by dbcAMP (mean \pm SE mRNA copy number $\times 10^6$: basal 56.60 ± 7.85 vs. dbcAMP 300.72 ± 106.71 , $P < 0.05$). To assess the effect of dbcAMP on FOG2 intracellular localization, SMAT1 cells were incubated for 24 h in basal medium or in medium containing 1 mM dbcAMP. Immunocytochemical studies performed with an anti-FOG2 antibody showed that FOG2 protein localization was persistently nuclear (results not shown). From these results, we conclude that the concomitant increase of FOG2 together with GATA4 in response to cAMP may oppose any effect of GATA4 on *AMH* transcription.

SOX9 and SF1 mediate AMH promoter trans-activation in response to cAMP. We next tested whether the canonical sequences in the *AMH* promoter known to be responsive to SOX9, SF1, or GATA4 are essential for *AMH* promoter *trans*-activation in response to cAMP, using luciferase assays. The prepubertal mouse Sertoli cell line SMAT1 was transfected with luciferase constructs containing the 3,063-bp *AMH* promoter, either wild type or with mutations in the sites for SOX9 at position -141, SF1 at positions -92 and -218, or GATA at positions -74, -168, and -408, and incubated in basal medium or medium with 1 mM dbcAMP (Fig. 3A). Since there is an AP1 consensus site at position -203, and AP1 has been shown to be regulated by cAMP in other cells (53), we also mutated this site. As expected, the basal activity of the 3,063-bp *AMH* promoter was lower, although not completely abolished, when mutations of the SOX9 or the two SF1 sites were present in the prepubertal Sertoli cell line SMAT1 (Fig. 3B). Conversely, mutation of the GATA sites did not affect the basal activity of the 3,063-bp *AMH* promoter. The response to dbcAMP observed with the wild-type promoter was abolished by mutations in the SOX9- or SF1-binding sites but not by mutations of the GATA sites. When the AP1 site was mutated, an increase in basal *AMH* promoter activity was observed, and response to dbcAMP was not disturbed. These observations indicate that, like in the fetal Sertoli cell (4, 16, 55), in postnatal Sertoli cells SOX9 and SF1 are also the major regulators of basal *AMH* transcription. AP1 seems to act as an inhibitor of basal *AMH*

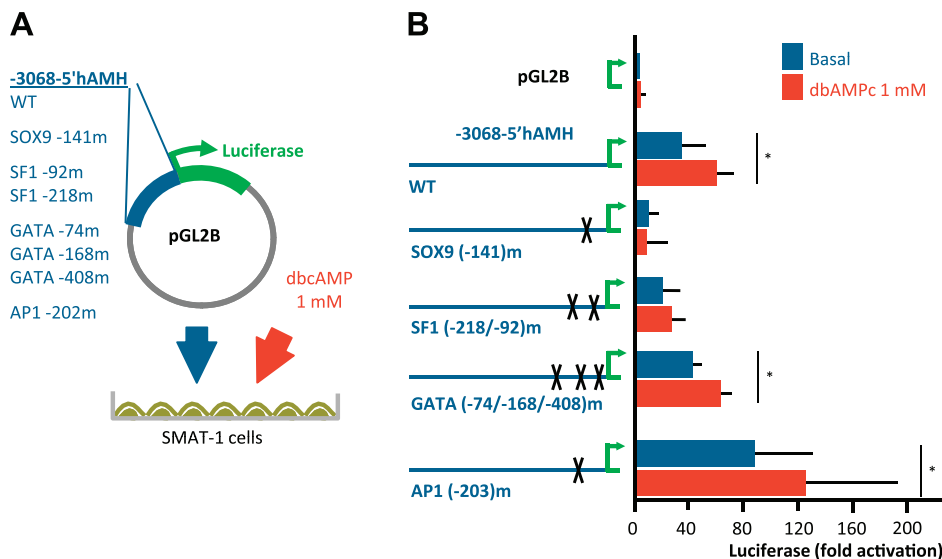


Fig. 3. cAMP enhancement of the *AMH* promoter activity involves SOX9 and SF1 binding sites in the proximal *AMH* promoter. *A*: SMAT1 cells were transfected with 1 μ g of the full-length -3,068-5' *hAMH*-luc construct with wild-type or mutated response elements for SOX9 (1 element at position -141), SF1 (2 elements at -92 and -218), or GATA4 (3 elements at -74, -168, and -408), and luciferase activity was assessed 48 h later. *B*: effect of the incubation with 1 mM dbcAMP during the last 24 h. Results are given as fold induction (mean \pm SE of 4 experiments), considering as 1 the luciferase activity of the pGL2B backbone vector incubated under basal conditions. Results were analyzed using a Student *t*-test to compare basal condition against dbcAMP for each construct. * $P < 0.05$.

promoter activity. Finally, the existence of functional binding sites for SOX9 and SF1, but not for GATA4 or AP1, is essential for AMH upregulation in response to cAMP.

DISCUSSION

Serum AMH levels increase in humans and mice in response to FSH (2, 9, 29, 32, 66, 68) or to an abnormally hyperactive $G_s\alpha$ protein (14, 47). This is due to both an increase in the Sertoli cell mass, since FSH induces immature Sertoli cell proliferation (2, 28, 32, 42), and an upregulation of AMH expression in each Sertoli cell in response to FSH signaling through its $G_s\alpha$ -coupled receptor via activation of the classical adenylyl cyclase-cAMP pathway, although no classical CRE is present in the *AMH* promoter (29, 32). In this work, we show that cAMP upregulates SOX9 and SF1 expression and induces their nuclear translocation in the prepubertal Sertoli cell line SMAT1, suggesting that these well-known *AMH* gene *trans*-activators are more available in Sertoli cell nuclei after cAMP levels increase in the cytoplasm. We also show that PKA is the main responsible kinase, whereas the PI3K/PKB, p38 MAPK, and MEK1/2-ERK1/2 pathways may also underlie SF1-increased availability. Finally, results from our luciferase assays indicate that SOX9 and SF1 induction of the *AMH* promoter seems to be critical in mediating cAMP response, since mutations in their response elements completely abolish the effect. Our results also indicate that GATA4 increases its availability in Sertoli cell nuclei in response to cAMP, but its action is not essential for the cAMP mediation of the *AMH* gene promoter activity, as shown by mutations in GATA elements.

To identify alternative pathways not involving a classical CRE that mediate the increase of the *AMH* promoter activity in response to cAMP in Sertoli cells of the prepubertal testis, we used a previously characterized established cell line. Immortalized cell lines are practical tools for transcriptional regulation studies, especially when they are easily transfectable. However, the process of immortalization may result in permanent phenotypic changes that undermine the ability of the experimental model to reflect the physiological condition *in vivo*. We wished to assess the regulation of AMH expression by the FSH receptor second messenger cAMP in the postnatal immature (i.e., prepubertal) Sertoli cell because this is the period of life where FSH is capable of increasing testicular AMH output (48, 66). SMAT1 cells, used as our experimental model, originate from a clonal Sertoli cell line derived from a 6.5-day-old mouse testis. These cells conserve most of the characteristics of the prepubertal Sertoli cell, including the expression of the transcription factors needed for basal AMH production (17). Although SMAT1 cells have lost the expression of the FSH receptor, they keep downstream signaling pathways, and FSH responsiveness is fully recovered when the receptor is transfected (32).

SOX9 and SF1 are transcription factors involved in several steps of gonadal development and function. Both factors are necessary for testicular differentiation from the gonadal ridge in early fetal life (65) and are expressed in Sertoli cells with changing levels through postnatal maturation (18, 23). SOX9 and SF1 are expressed in basal conditions in the SMAT1 cell line used in our experiments. In the newly differentiated fetal Sertoli cell, SOX9 binding to its response element in the proximal *AMH* promoter is essential for the initiation of AMH

expression (4). SF1 binding to its two sites in the proximal *AMH* promoter enhances SOX9-mediated AMH transcription, but its absence does not preclude AMH expression (4, 16). Most of the studies on the relevance of SOX9, SF1, and GATA regulatory sequences on the *AMH* promoter have been performed using less than 500 bp of the *AMH* 5'-sequences (5, 22, 55, 58, 61, 64). Interestingly, the *AMH* proximal promoter sequences involved in the initiation of AMH expression in the early fetal period are not sufficient for the maintenance of AMH expression later in fetal and postnatal life, suggesting that other promoter sequences, likely present far more upstream, are also involved in the regulation of AMH expression in the testis (6). In a previous study, we showed that sequences

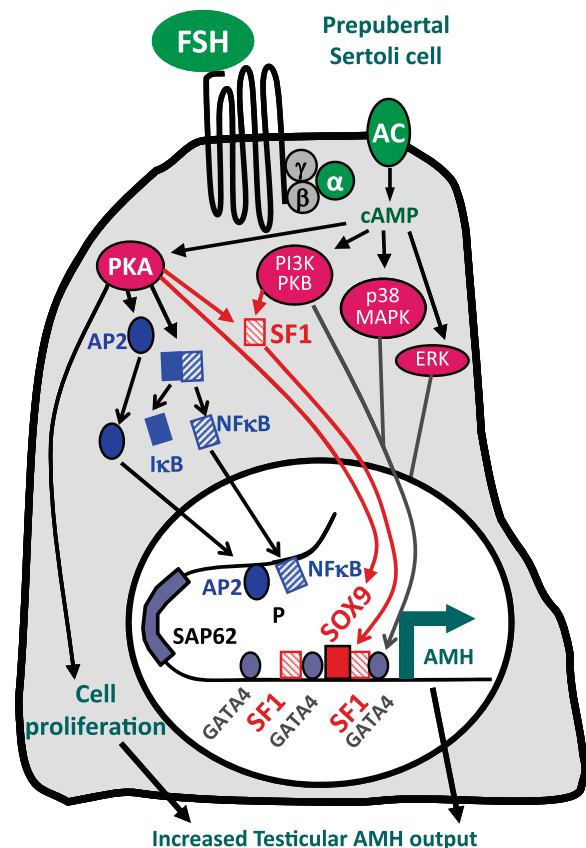


Fig. 4. Schematic model to explain the increase of testicular AMH production following FSH stimulation. In basal conditions, i.e., in early fetal life when the gonadotrope is not yet active and during infancy and childhood, AMH expression depends on the *trans*-activating potential of nuclear factors SOX-9, SF-1, and GATA-4 (4, 16, 19, 55, 58, 64). Upon gonadotrope activation, increasing FSH binds to its G protein-coupled receptor. The α -subunit of the heterotrimeric G_s protein induces adenylyl cyclase (AC) activity, resulting in an increase of cAMP concentration that may activate several kinases like PKA, PI3K-PKB, p38 MAPK, and MEK1/2-ERK1/2. We had shown previously that PKA enhances *AMH* gene expression by inducing AP2 and NF- κ B binding to their response elements in the distal *AMH* promoter (32). Here we show that PKA is also involved in SOX9 and SF1 upregulation as well as SF1 nuclear translocation, resulting in an increased AMH expression by binding to their cognate response elements in the proximal *AMH* promoter. Furthermore, PI3K-PKB, p38 MAPK, and MEK1/2-ERK1/2 may also mediate FSH-dependent AMH expression upregulation by increasing SF1 and GATA4 availability in the Sertoli cell nucleus, yet these pathways do not seem essential. FSH-dependent PKA activity is also known to induce cell proliferation (54). Together, the increase in Sertoli cell number and the enhancement of AMH transcription in each Sertoli cell results in an increase of testicular AMH production and secretion to the circulation.

present between 423 and 3,063 bp upstream of the transcription start site result in a twofold increase of the *AMH* promoter basal activity in the prepubertal Sertoli cells SMAT1 (32). Our present results using more the construct encompassing 3 kb of the *AMH* 5'-sequences confirm the uppermost role described for SOX9 in shorter *AMH* promoters. However, mutation of SOX9 response element did not fully abolish the 3-kb *AMH* promoter activity, indicating that a minor *AMH* transcription can be observed even in the absence of the SOX9 stimulus. This is in line with the observation that AMH is secreted by mammalian granulosa cells of the ovary (45, 62), which do not express SOX9 (21). SF1 and GATA4, which are potent *trans*-activators of the *AMH* gene in a 200-bp *AMH* promoter context (55, 64), play a less relevant role in a longer promoter context, as shown by our present results. Furthermore, persistent, albeit reduced, AMH activity is observed in vivo in mice with a mutated SOX9 binding site in the endogenous *Amh* gene promoter generated using a Cre recombinase/loxP gene targeting strategy (4). On the other hand, this elegant experimental approach clearly proved that, in a physiological context, the SOX9 site present in the proximal *Amh* promoter is the major site responsible for basal promoter activity.

SOX9 expression is high in fetal Sertoli cells, decreases after birth, and increases again during pubertal development (18). This expression pattern observed in vivo is compatible with regulation by FSH, whose circulating levels are high in fetal, neonatal, and pubertal periods of life, with a decline in the infantile phase. Accordingly, in our experimental model, the cognate FSH second messenger cAMP provoked an increase in SOX9 expression and nuclear translocation, thus likely resulting in an increased availability for binding to the *AMH* promoter. Our previous studies had identified a region in the distal *AMH* promoter, with response elements to AP2 and NF- κ B, involved in the upregulation of AMH expression in response to cAMP (32). Directed mutagenesis of AP2 and NF- κ B sites resulted in a decrease, but not a complete abolition, of the response. Those results indicated that other sequences were involved in *AMH* promoter activation by the cAMP pathway. Our present data strongly suggest that SOX9 is the most important factor, with other factors likely playing less potent effects, in the increase of AMH expression in response to FSH via G α protein-cAMP-PKA signaling.

Hormonal regulation of Sertoli cell AMH production is complex (48), and its comprehension may require multiple observational and experimental strategies. The stimulating effect of FSH on testicular AMH production, via its second messenger cAMP, is now clearly established (2, 9, 29, 32, 63, 66, 68). However, during puberty, AMH levels decline in negative correlation with FSH increase (48). The explanation for this unexpected negative association is that during pubertal development there is also an increase in intratesticular testosterone, which behaves as a potent inhibitor of AMH expression (48). The negative effect of testosterone prevails over the positive effect of FSH, resulting in a downregulation of AMH expression. The positive effect of FSH can be observed only in physiological or pathological conditions, when androgen action on Sertoli cells is absent. For instance, in the fetal and early postnatal periods of life, the high intratesticular androgen levels are unable to inhibit AMH expression because Sertoli cells physiologically lack androgen receptor expression (2, 7, 10, 12, 33). Similarly, AMH is not downregulated at pubertal

age in 46,XY patients (31, 40, 44, 46) or mice (2, 11) with androgen insensitivity due to androgen receptor-inactivating mutations or in subjects with impaired androgen synthesis due to steroidogenic enzyme defects (31, 40, 44, 46). Interestingly, in patients with androgen insensitivity or steroidogenic defects, circulating AMH is higher than normal during the early postnatal period and at puberty, in coincidence with high FSH levels (44).

In summary, we have provided further insight into the intracellular mechanisms underlying the upregulation of AMH expression in response to increased cAMP levels in prepubertal Sertoli cells. Schematically, upon binding to its G α -coupled receptor, FSH induces an adenylyl cyclase-dependent increase in cAMP levels, which activate several kinases (Fig. 4). PKA is the main kinase involved in the upregulation of AMH transcription by increasing the nuclear levels of SOX9, SF1, and GATA4, which bind to response elements present in the proximal promoter, as well as of AP2 and NF- κ B, which bind to distal promoter sequences. Although SOX9, SF1, AP2, and NF- κ B pathways are essential for attaining full response to cAMP, GATA4 pathway does not seem to be indispensable. In addition, cAMP promotes prepubertal Sertoli cell proliferation. The increase in Sertoli cell number together with the enhanced AMH expression in each Sertoli cell result in an increased testicular AMH output in response to FSH, which can be observed in physiological conditions in humans and most mammals during the second half of fetal life, early postnatal life, and the onset of puberty as well as in pathological conditions where the FSH pathway is abnormally activated and the androgen pathway is abrogated.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

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