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### Research Article

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## Hormonal and Cellular Regulation of Sertoli Cell Anti-Müllerian Hormone Production in the Postnatal Mouse

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### Abstract

Anti-Müllerian hormone (AMH) is secreted by immature testicular Sertoli cells. Clinical studies have demonstrated a negative correlation between serum AMH and testosterone in puberty but not in the neonatal period. We investigated AMH regulation using mouse models mimicking physiopathological situations observed in humans. In normal mice, intratesticular, not serum, testosterone repressed AMH synthesis, explaining why AMH is downregulated in early puberty when serum testosterone is still low. In neonatal mice, AMH was not inhibited by intratesticular testosterone, due to the lack of expression of the androgen receptor in Sertoli cells. We had shown previously that androgen-insensitive patients exhibit elevated AMH in coincidence with gonadotropin activation. In immature normal and in androgen-insensitive *Tfm* mice, follicle stimulating hormone (FSH) administration resulted in elevation of AMH levels, indicating that AMH secretion is stimulated by FSH in the absence of the negative effect of androgens. The role of meiosis on AMH expression was investigated in *Tfm* and in pubertal *XXSxr<sup>b</sup>* mice, in which germ cells degenerate before meiosis. We show that meiotic entry acts in synergy with androgens to inhibit AMH. We conclude that AMH represents a useful marker of androgen and FSH action within the testis, as well as of the onset of meiosis. (*J. Clin. Invest.* 1997; 100:1335–1343.) Key words: follicle stimulating hormone • meiosis • testosterone • androgen receptor • testicular hormones

### Introduction

Anti-Müllerian hormone (AMH),<sup>1</sup> also known as Müllerian inhibiting substance (MIS) or Müllerian inhibiting factor

(MIF), is a testicular hormone responsible for the regression of Müllerian ducts in male fetuses. This glycoprotein of the TGF- $\beta$  family is produced by Sertoli cells from the time of differentiation of seminiferous tubules in the fetal testis until pubertal maturation (1). AMH is measurable in human serum and has diagnostic applications as a specific marker of immature Sertoli cell number and function (2, 3). In a previous study, we showed that elevation of serum testosterone correlates with a decrease in serum AMH levels during puberty. This inverse relationship is also present in boys with either central or gonadotropin-independent precocious puberty, thus implicating testosterone and not gonadotropins as the major player in AMH downregulation (4). In keeping with this hypothesis, patients with an androgen insensitivity syndrome (AIS) show abnormally elevated serum levels of AMH (5). However, some enigmas remain: while serum AMH is always low in late puberty, i.e., when serum testosterone has reached high levels, AMH concentration is extremely variable in early pubertal boys, and up to 60% of them show low serum AMH together with prepubertal testosterone levels (4). Another unresolved issue is why AMH levels are elevated in spite of the high androgen production by the testis in the fetus and between the second and sixth months of postnatal life (6–9).

Testosterone may not be the only hormone regulating AMH production. In patients with AIS, serum AMH is upregulated only at developmental periods characterized by the activation of the hypothalamic–pituitary–gonadal axis. Cellular factors may also be involved. In a previous clinicopathological study, we showed that AMH expression is present in premeiotic seminiferous tubules, but is no longer detected in neighboring tubules that have entered meiosis (3).

To investigate the hormonal and cellular interactions implicated in AMH regulation, we used mouse models that mimic the different physiological and physiopathological situations observed in humans. *Tfm* mice, a model for AIS, present a mutation in the androgen receptor gene that makes the XY mouse insensitive to androgens (10, 11). To determine the role of meiotic entry on AMH downregulation, we used the *XXSxr<sup>b</sup>* mouse model. Male *XXSxr<sup>b</sup>* mice develop testes with normal

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1. Abbreviations used in this paper: AIS, androgen insensitivity syndrome; AMH, anti-Müllerian hormone; ARE, androgen response element; CHO, Chinese hamster ovary; CRE, cAMP response element; FSH, follicle-stimulating hormone; PMSG, pregnant mare's serum gonadotropin.

androgenic function, but the presence of two X chromosomes leads to germ cell failure before meiotic entry (12–14).

To identify the level at which AMH regulation takes place, we studied AMH RNA levels in the testes by hybridization analysis, protein production in the Sertoli cells by immunohistochemistry, and protein levels in circulation by a specially developed ELISA for mouse AMH.

## Methods

**Animals and treatments.** Normal male mice of the B6/CBA strain were purchased from CERJ (Le Genest St. Isle, France). *Tfm* male mice ( $X^{Tfm}/Y$ ) were obtained by mating female heterozygous carriers of the *Tfm* mutation ( $X^{Tfm}/X^+$ ) from The Jackson Laboratory, Bar Harbor, ME with B6/CBA male mice. Animals were maintained on commercial food and tap water ad libitum and kept at  $22 \pm 2^\circ\text{C}$  under 12-h artificial illumination.

*XXSxr<sup>b</sup>* mice were obtained at the National Institute for Medical Research (Mill Hill, London, UK) by mating *XYsxr<sup>b</sup>* males to normal XX females (15). *XXSxr<sup>b</sup>* males were identified by the absence of PCR amplification of the Y chromosome gene YMT2/B. The myogenin gene was used as positive control for PCR amplification. Normal *XYsxr<sup>b</sup>* males from the same litters were used as controls for the study of AMH expression.

Male mice were killed at various ages between birth and adulthood to provide data on AMH ontogeny in normal and *Tfm* mice. To study the possible effect(s) of gonadotropins and/or testosterone on AMH production in males, one group of *Tfm* mice ( $n = 5$ ) and one group of normal mice ( $n = 10$ ) were injected with 3 U pregnant mare's serum gonadotropin (PMSG) (Chronogest; Intervet, Angers, France) on days 1, 3, and 5 after birth and killed on day 7. *Tfm* ( $n = 6$ ) and normal ( $n = 6$ ) control groups were injected similarly with saline. Further studies were carried out to assess follicle-stimulating hormone (FSH) effect on AMH secretion. Normal male mice were injected every day from postnatal days 1–6 with either 0.5 U recombinant FSH (Gonal F-75; Ares-Serono S.A., Geneva, Switzerland) or saline, and then killed on day 7.

**Tissue and blood samples.** Serum was extracted and stored at  $-20^\circ\text{C}$  until assayed. Testes were weighed and either used for RNA extraction, deep-frozen in liquid nitrogen for testosterone assay, or immersion-fixed in Bouin's fixative overnight, dehydrated in a graded series of ethanol, and finally embedded in paraffin wax.

**RNA extraction and hybridization analysis.** Immediately after dissection, total RNA was extracted from testicular and renal tissue using RNA Plus (Bioprobe Systems, Montreuil-sous-Bois, France) according to the manufacturer's instructions. Concentration of total RNA was estimated by spectrophotometry at 260 nm, considering that 25 U OD corresponds to 1 mg/ml (16, 17). RNA quality was assessed by agarose gel electrophoresis.

For quantitative analysis of AMH expression, RNA dot blots were performed. 10  $\mu\text{g}$  of total RNA from each sample, as estimated by spectrophotometry, was denatured and applied onto a nylon Hybond-N membrane (Amersham International, Les Ulis, France) using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA). The RNA was cross-linked to the membrane by exposure to 0.6  $\text{J}/\text{cm}^2$  UV. Hybridization was performed using a DNA probe spanning the fifth exon of the mouse AMH gene (18), labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by multirandom priming using a Megaprime kit (Amersham International). To assess the exact amount of total RNA blotted in each sample, membranes were stripped and hybridized with a ribosomal oligonucleotide probe labeled with [ $\gamma$ - $^{32}\text{P}$ ]dATP as described (19). After hybridization, blots were exposed to Kodak XAR films (Eastman Kodak Co., Rochester, NY) at  $-80^\circ\text{C}$  with intensifying screens. Quantitative analysis of dot intensity was performed on autoradiographs using Imagenia 3000 software (Biocom, ZI Coutaboeuf, France) and a Biocom 200 image analyzer.

Specificity of probe hybridization was assessed by Northern blot

analysis. Total RNA samples were electrophoresed on 1% agarose/1% formaldehyde gels, blotted onto Hybond-N membranes (Amersham International), and cross-linked. Northern hybridization analysis was performed as described (19), using the same probes as those used in the dot blots.

**Immunohistochemistry.** Testes embedded in paraffin wax were serially sectioned at 5  $\mu\text{m}$  and mounted on slides coated with 3-amino-propyl triethoxy-silane (Sigma Chemical Co., St. Quentin Fallavier, France). Slides were processed using the antigen retrieval technique by microwaves, for AMH detection as described by Rey et al. (3) and for androgen receptor detection as described by Bremner et al. (20).

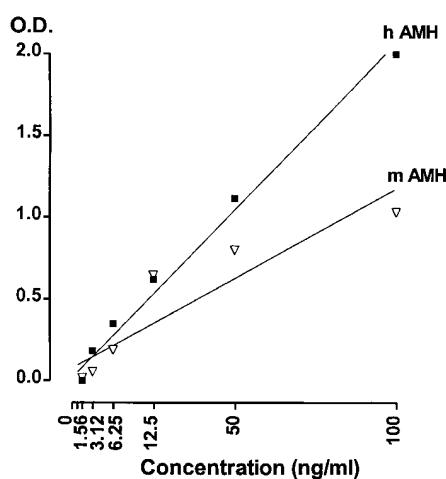
**ELISA for mouse AMH.** The ELISA developed to measure mouse serum AMH is a noncompetitive, solid-phase, double-antibody enzyme immunoassay. The first antibody (Ab L42–43) is a polyclonal antirecombinant human AMH antibody raised in rabbit, immunopurified by chromatography on a bovine AMH-affinity column (21). The second antibody (Ab C1–4) is a polyclonal antirecombinant human AMH antibody raised in guinea pig. Immunoglobulins of the IgG subtype were isolated from the antiserum by affinity chromatography on protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ). The sensitivity and specificity of both antibodies were tested by immunohistochemistry on prepubertal mouse testes and nongonadal tissues. A strong positive signal was observed in the cytoplasm of Sertoli cells of prepubertal mouse, human, rat, and dog testes. No signal was observed in other cell types of prepubertal testes, in adult testes, or in nongonadal tissues, which suggests that both antibodies specifically recognize AMH (data not shown).

For construction of calibration curves, recombinant mouse AMH was produced in permanently transfected Chinese hamster ovary (CHO) cells (22). CHO cells lacking the dihydrofolate reductase gene (23) were transfected by electroporation with expression vector pMDR 902 containing the mouse AMH gene (kindly provided by Dr. R. Behringer, Department of Molecular Genetics, The University of Texas, M.D. Anderson Cancer Center, Houston) under control of the SV40 early promoter and the dihydrofolate reductase gene. Successfully transfected clones were grown in MEM $\alpha$  without ribonucleosides and deoxyribonucleosides (GIBCO BRL, Cergy-Pontoise, France), supplemented with 10% dialyzed FBS. The conditioned medium was clarified by filtration, concentrated 20-fold by ultrafiltration, and semipurified by affinity chromatography using Ab L42–43. The concentration of mouse AMH in the eluate was obtained by comparative densitometric analysis of Western blot bands of recombinant human AMH of known concentration and of recombinant mouse AMH, as revealed by an antibody raised against bovine AMH (21).

Polystyrene plates (Immulon-II; Dynatech Laboratories, Inc., Guyancourt, France) were coated with Ab L42–43 at 10  $\mu\text{g}/\text{ml}$  in  $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ , pH 9.6, 0.1 M at room temperature overnight. Saturation of residual binding sites was carried out by incubating the plates with 1% BSA in PBS (PBS/1% BSA) for 2 h at room temperature. Serum was incubated at four different dilutions (1:4, 1:8, 1:16, and 1:32) in PBS/1% BSA for 1 h. The plates were exposed successively to Ab C1–4 at 5  $\mu\text{g}/\text{ml}$  in PBS/1% BSA and to an alkaline phosphatase-conjugated anti-guinea pig Ig rabbit antibody (Rockland Inc., Gilbertsville, PA) at a 1:1,000 dilution in 1% BSA in Tris-buffered saline. A color reaction was demonstrated with *p*-nitrophenyl phosphate (Sigma Chemical Co.) 1 mg/ml in diethanolamine/HCl 10 mM,  $\text{MgCl}_2$  1 mM, pH 9.8. Absorbance at 405 nm was read in an MRX spectrophotometer (Dynatech Laboratories, Inc.). Data were processed using Biolinx software, version 2.20 (Dynatech Laboratories, Inc.). Calibration curves were constructed using increasing concentrations (0.4–25 ng/ml) of recombinant mouse AMH.

**Testosterone measurements.** Serum levels and intratesticular concentrations of testosterone were measured by specific RIA. The testes were homogenized in 1 ml phosphate buffer. The homogenates were then sonicated and transferred to glass tubes. The homogenates as well as the sera were extracted by 10 vol ethyl ether and purified on celite microcolumns as previously described (24). The fractions containing testosterone were measured by RIA as reported (24).

A



B

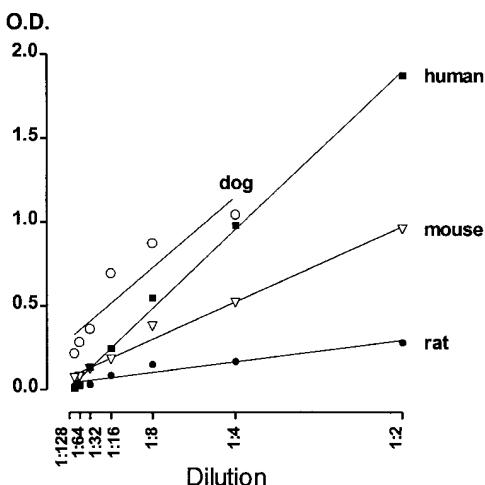


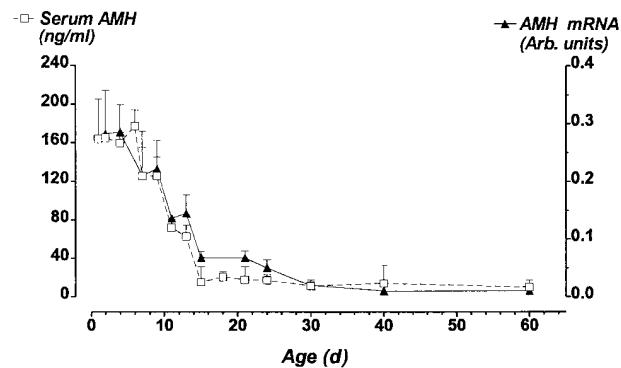
Figure 1. (A) Calibration curves obtained using recombinant human AMH (*h* AMH) and recombinant mouse AMH (*m* AMH). Computed slopes of the curves were  $2.10 \pm 0.11$  for human AMH and  $1.10 \pm 0.21$  for mouse AMH. (B) Curves obtained after assaying AMH in pre-pubertal dog, human, mouse, and rat sera at several dilutions. All slopes were significantly linear and different from zero. O.D., optical density (absorbance at 405 nm).

**Statistics.** For validation of the ELISA technique, slopes, *y* intercepts, linearity of regressions, and regression coefficients were calculated using GraphPad StatMate software (GraphPAD Software for Science, San Diego, CA). Comparison of means between experimental groups was made by Student's *t* test, using the same software.

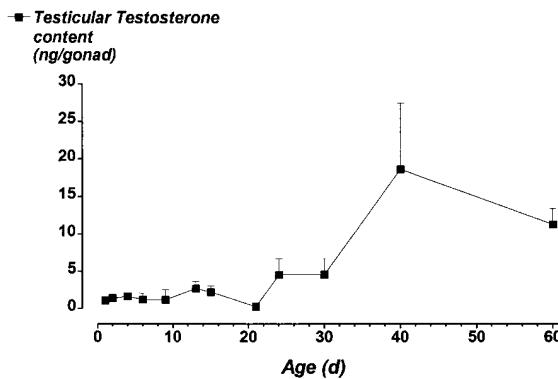
## Results

**Validation of the ELISA technique.** CHO cells permanently transfected with the mouse AMH gene expressed a 2.2-kb mRNA species which was revealed by Northern blot analysis

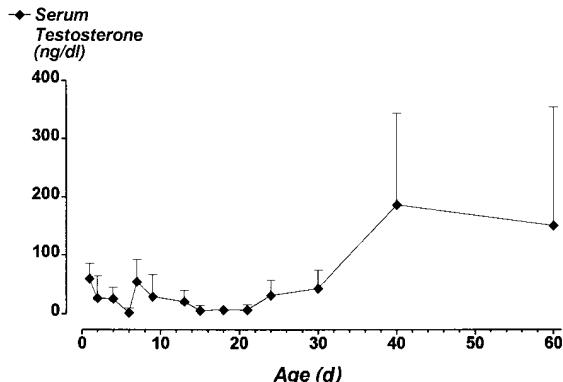
A



B



C



D

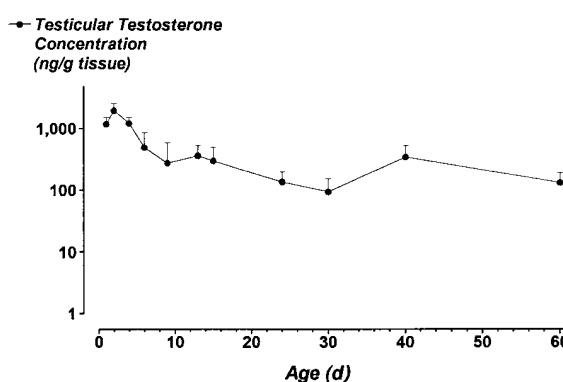


Figure 2. Ontogeny of testicular AMH and testosterone production in normal postnatal male mice. Points and bars, mean  $\pm$  SD of determinations in 5–15 animals per age group.

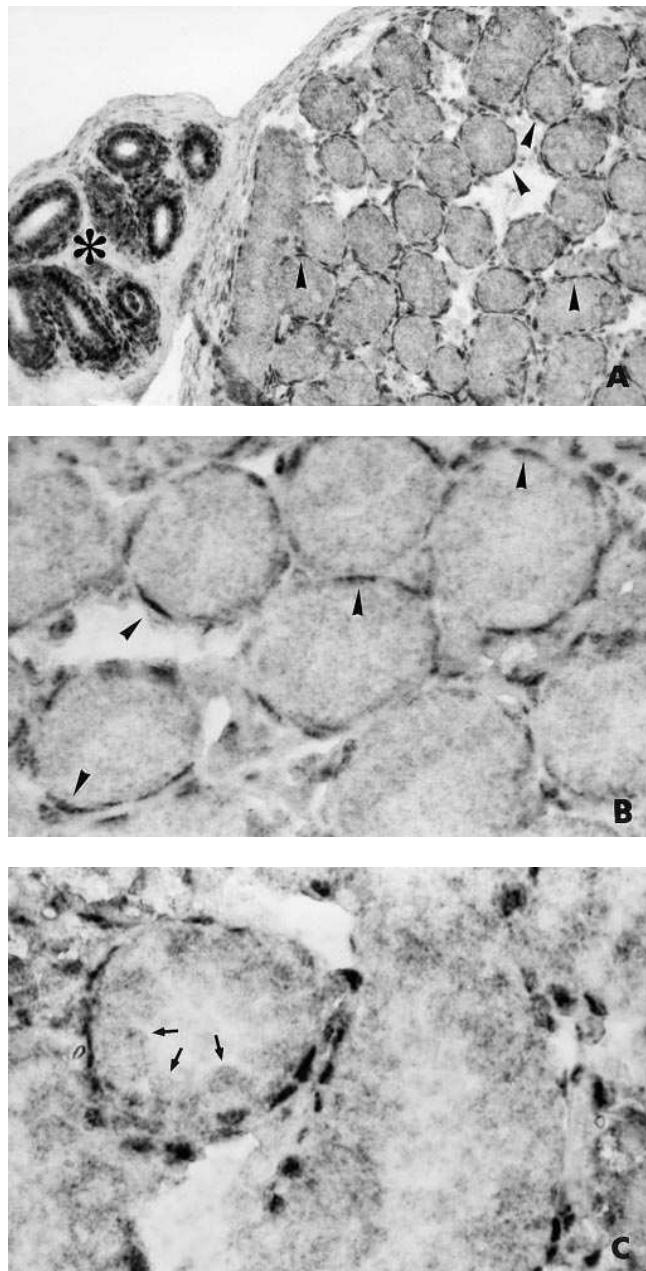
using a specific probe spanning the fifth exon of AMH, as expected (18). Recombinant mouse AMH secreted to the culture medium was detected by Western blotting, using a previously characterized polyclonal anti-bovine AMH antibody (21) and anti-human AMH polyclonal antibodies raised in rabbit (L42-43) and in guinea pig (C1-4) (results not shown). Calibration curves obtained using recombinant mouse and recombinant human AMH are shown in Fig. 1 A. Because both anti-AMH antibodies used in our ELISA were raised against recombinant human AMH, the assay showed a higher sensitivity for the human protein. The lowest detectable concentration of mouse AMH was 1.6 ng/ml. Intra- and interassay variability coefficients were 2.40 and 2.71%, respectively. Our assay also recognized AMH from dog and rat serum (Fig. 1 B). Conversely, purified TGF- $\beta$  was not detected; also, AMH was undetectable in the serum of castrated male mice (results not shown).

**Relationships between AMH, testosterone, and testicular androgen receptor in postnatal life.** In normal male mice, serum AMH was elevated before pubertal onset, i.e., between birth and 6 d of age, dropped significantly from day 7 to day 13, and was at the lowest limit of detection of our assay from day 15 onwards (Fig. 2 A). Testicular AMH mRNA levels showed a similar pattern (Fig. 2 A), which suggests that AMH production by Sertoli cells is regulated mainly at the mRNA level.

To analyze the relationship between androgens and AMH expression in the male mouse, we studied the ontogeny of testosterone secretion from birth to adulthood in males. The total amount of testosterone produced by the testes (Fig. 2 B) and secreted into peripheral blood (Fig. 2 C) remained low until day 30, i.e., 15 d after serum AMH had decreased to its lowest level. However, intratesticular testosterone concentration, i.e., androgen levels to which Sertoli cells are exposed, was maximal at birth, declined slightly until day 20, and remained stable thereafter at the adult range (Fig. 2 D). Intratesticular testosterone never fell to basal values in mice, and were always 5–10 times higher than in the prepubertal human testis (9, 25).

To understand why AMH is not inhibited before day 6, at a time when intratesticular testosterone levels are maximal, we studied testicular androgen receptor expression during postnatal development by immunohistochemistry. We found no positive signal for the androgen receptor in Sertoli cells before the age of 4 d (Fig. 3). At day 7, only a few Sertoli cells showed a faint reaction. Both the number of positive Sertoli cells and signal intensity increased with pubertal development. The androgen receptor was also expressed in myoid peritubular cells at all ages and in Leydig cells, following a similar pattern to that observed in Sertoli cells.

**Effect of gonadotropins on AMH production.** To determine whether gonadotropins play a role in the control of AMH expression, we treated normal and *Tfm* males with PMSG, which contains both FSH and luteinizing hormone activity, from day 1 to day 5 after birth and measured serum AMH on day 7. In normal males, PMSG treatment induced a decrease of serum AMH (Fig. 4 A), probably due to a significant stimulation of androgen synthesis (mean serum testosterone: 83.9 in PMSG-injected vs. 4.7 ng/dl in saline-injected mice). In contrast, in androgen-insensitive *Tfm* males, PMSG increased serum AMH levels (Fig. 4 A). Since the main regulatory gonadotropin for Sertoli cells is FSH, we injected normal male mice with pure recombinant FSH from birth to day 6, which also resulted in an elevation of serum AMH levels (Fig. 4 B).



**Figure 3.** Immunohistochemical study of the androgen receptor in postnatal mouse testes. (A) Postnatal day 4: a signal is observed only in spindle-shaped peritubular cell nuclei (arrowheads) and in epithelial cell nuclei of the epididymis (\*);  $\times 120$ . (B) Higher magnification of the previous photomicrograph: peritubular cells are positive (arrowheads), but no signal is observed within the seminiferous tubules;  $\times 240$ . (C) Day 7: a faint signal can be detected in a few Sertoli cell nuclei (arrows);  $\times 240$ .

**Relationship between AMH and meiotic entry.** In androgen-insensitive *Tfm* male mice, serum AMH remained stable during pubertal development, in spite of high intratesticular testosterone concentration (day 9:  $2753 \pm 1503$  ng/g; day 21:  $109 \pm 74$  ng/g), but fell after day 25 (Fig. 5). Histological observation of testes at this age showed that most tubules had entered meiosis. To study the temporal correlation between

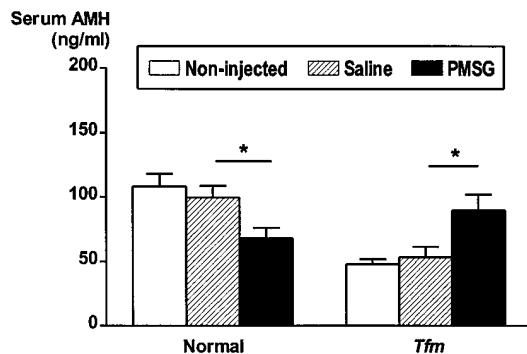
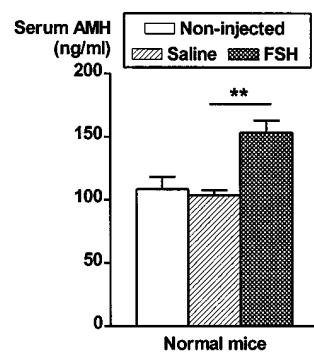
**A****B**

Figure 4. Effect of gonadotropins on testicular AMH secretion. (A) Normal or *Tfm* males were injected with saline or PMSG from birth to postnatal day 5, and serum AMH was assayed at postnatal day 7. (B) Normal males were injected with recombinant FSH from birth to postnatal day 6, and serum AMH was assayed at postnatal day 7. Bars, mean  $\pm$  SD. Differences between means were analyzed by Student's *t* test: \**P* < 0.05; \*\**P* < 0.01.

AMH expression and meiotic development, we performed immunohistochemistry for AMH in normal and *Tfm* mouse testes from birth to adulthood. AMH immunoreactivity was prominent in Sertoli cell cytoplasm of all the seminiferous tubules in normal and *Tfm* prepubertal males (Fig. 6, *A* and *E*). In normal mice by day 9, AMH immunostaining disappeared from tubules containing primary spermatocytes, whereas neighboring premeiotic tubules were still positive (Fig. 6, *B* and *C*). The number of AMH-negative tubules increased with pubertal development as they entered meiosis, and AMH immunostaining was no longer observed in any seminiferous tubule after day 15 (Fig. 6 *D*). In *Tfm* mice, meiotic entry occurred later, around day 25. AMH immunoreactivity remained positive until that age and, as in normal mice, became negative in correlation with meiotic entry of seminiferous tubules (Fig. 6 *F*).

To confirm that meiotic entry is implicated in AMH downregulation, we used the XXSxr<sup>b</sup> mouse model. Before puberty, no differences in AMH mRNA levels, serum AMH, or intratesticular testosterone were observed between XXSxr<sup>b</sup> males and normal XY littermates (Fig. 7). At puberty, although serum AMH was at the lowest limit of detection of our assay in both control and XXSxr<sup>b</sup> mice, AMH mRNA levels were significantly higher in XXSxr<sup>b</sup> mouse testes (Fig. 7). Furthermore, while no positive signal for AMH was seen by immunohistochemistry in control testes in which all seminiferous tubules had reached at least pachytene stage of meiosis, a positive reaction was still observed in Sertoli cell cytoplasm and in the tubular lumen of XXSxr<sup>b</sup> mouse testes, which were devoid of meiotic cells (Fig. 6, *G* and *H*). XXSxr<sup>b</sup> testes showed typical morphological changes of pubertal maturation. A direc-

tional secretion of AMH towards the tubular lumen after the development of tight junctions between Sertoli cells may explain why the maintenance of AMH expression is not reflected in the serum. Altogether, these results show that the failure of germ cells to enter meiosis results in an incomplete inhibition of AMH expression.

## Discussion

The best-documented action of AMH, the regression of the anlagen of the uterus and tubes in the male, takes place very early in fetal life, yet AMH expression persists at high levels until puberty. Whether AMH has biological effects in late fetal life and after birth has been a controversial issue (for a review see reference 1). Recent findings showing that the AMH receptor is expressed within the gonads (19, 26) have given new insight to a possible role for AMH in the postnatal testis. Furthermore, transgenic mice chronically overexpressing AMH show Leydig cell hypoplasia and impaired steroidogenesis (27, 28), while mice in which the genes encoding either AMH (29) or its receptor (30) have been knocked out develop Leydig cell hyperplasia or tumors. Altogether, these observations suggest that AMH might play a role in the control of Leydig cell proliferation and of androgen biosynthesis.

The development of a qualitatively and quantitatively normal Sertoli cell population before puberty is essential for the normal function of the adult testis (31). Although the physiological importance of AMH expression in the postnatal gonad only begins to be understood, AMH has been shown to be a useful clinical marker for studying Sertoli cell function before puberty (2, 7, 32, 33). During human pubertal development, AMH is completely inhibited when serum testosterone is > 200 ng/dl (4), which corresponds to midpuberty (pubertal stage 3, according to the classification by Marshall and Tanner, reference 34). However, AMH is already repressed in a large proportion of boys older than 9 yr of age whose serum testosterone levels and clinical appearance are still prepubertal. Here we show that, in mice also, serum AMH declined at an age when serum testosterone was still low. However, testosterone concentration was already high within the testis, indicating that intratesticular and not serum testosterone is responsible for the inhibition of AMH production by Sertoli cells. This observation might help explain the relationship between testosterone and AMH in early human puberty if we accept that, although prepubertal development is much shorter in rodents

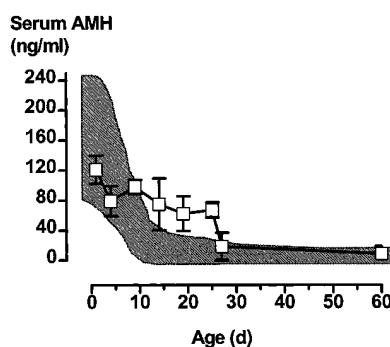
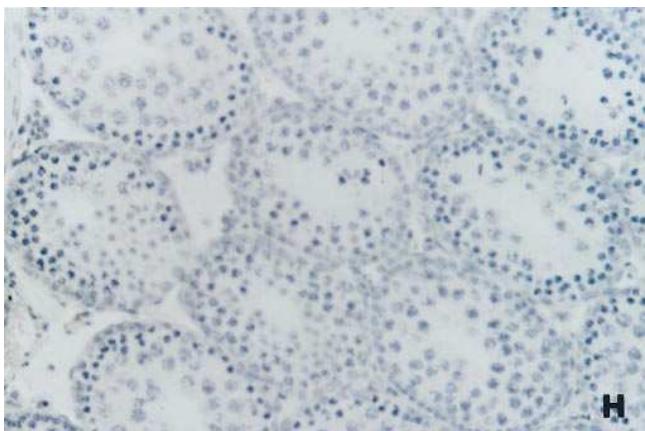
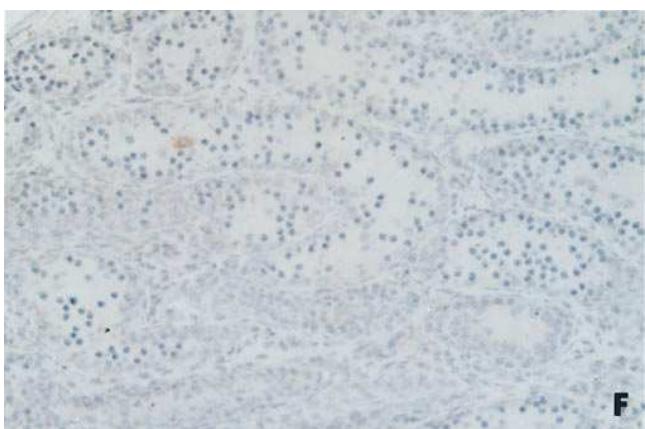
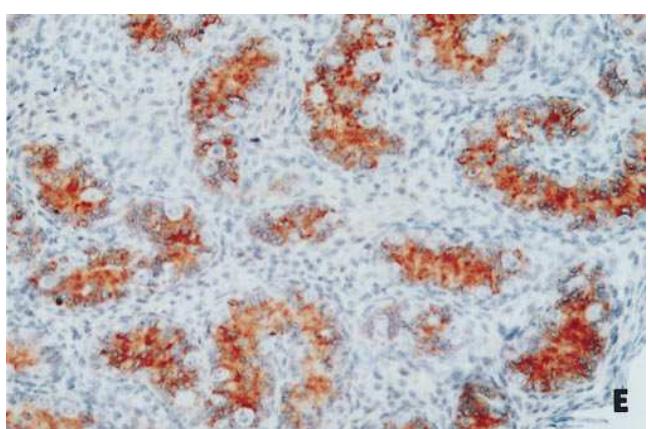
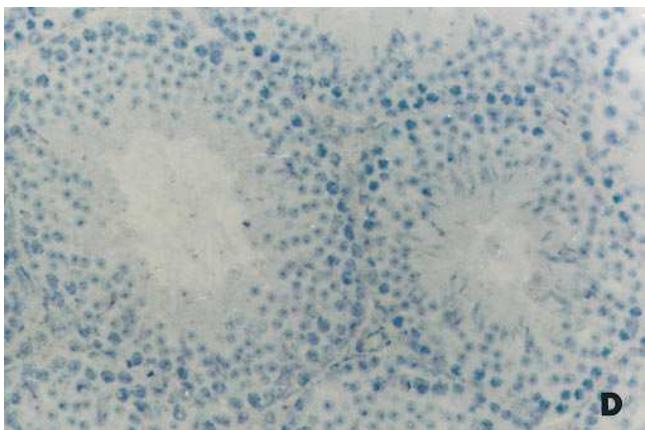
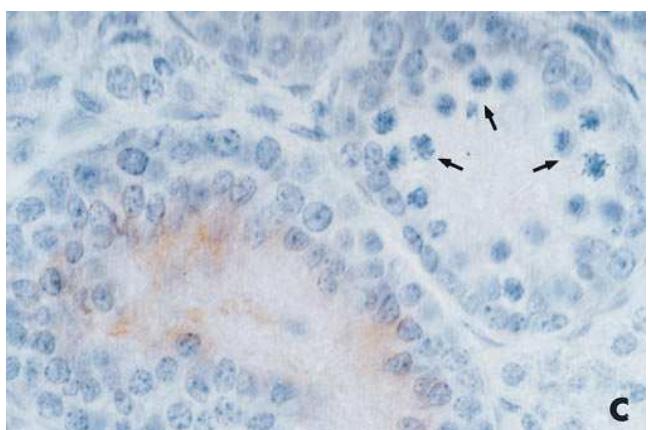
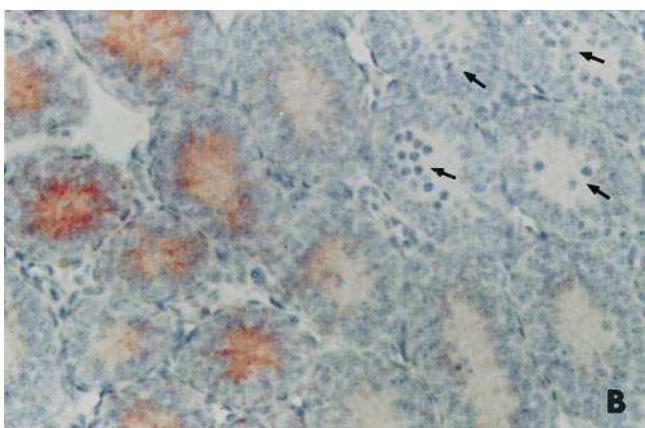
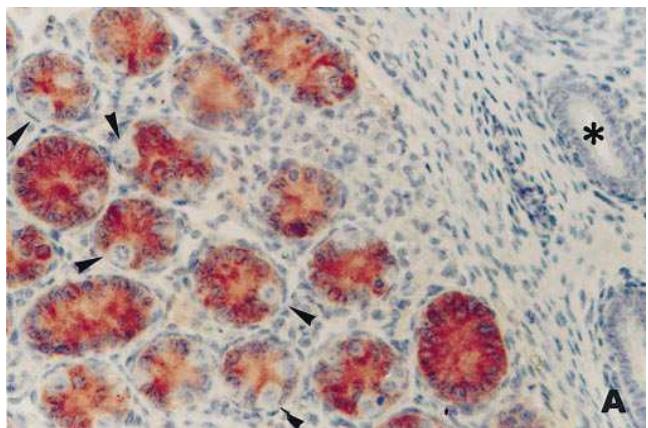
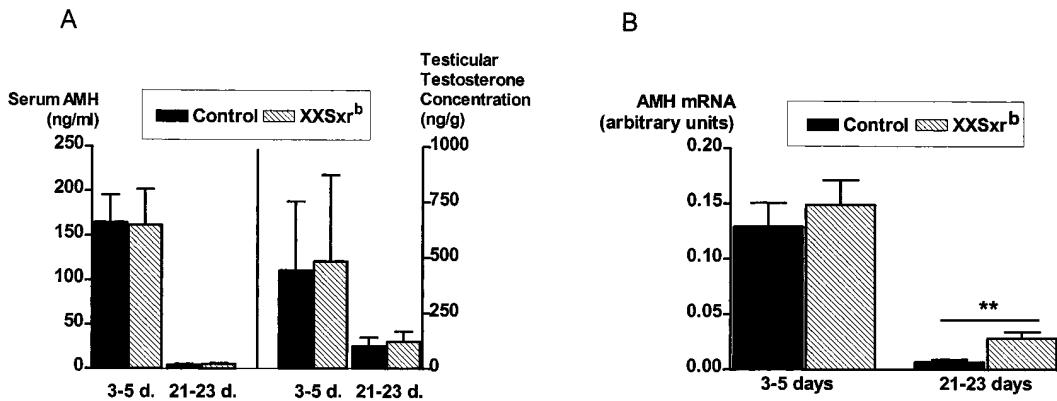


Figure 5. Ontogeny of serum AMH in postnatal male *Tfm* mice. Points and bars, mean  $\pm$  SD of determinations in four to six animals per age group. Shaded area, serum AMH levels (mean  $\pm$  2 SD) in normal male mice.





than in humans, hormonal mechanisms regulating testicular function are basically comparable in both species. One of the earliest landmarks of the onset of human pubertal development of the testis is the abrupt increase of local testosterone concentration occurring at pubertal stage 2 which is not reflected in the elevation of serum testosterone levels until pubertal stage 3 (25, 35, 36). Thus, boys with serum testosterone < 200 ng/dl are probably heterogeneous with regard to their intratesticular testosterone concentration. Those in whom androgen levels are already elevated within the testis will no longer express AMH. Consequently, the decrease in serum AMH is one of the earliest signs of the onset of testicular pubertal maturation and may be a useful biochemical marker for predicting the onset of puberty.

In neonatal mice, AMH is expressed at high levels in spite of the elevated intratesticular testosterone concentration. Since androgens regulate gene expression after binding to a nuclear receptor, we studied the profile of androgen receptor expression in Sertoli cells throughout postnatal development in the normal mouse. In agreement with findings reported by Majdic et al. (37) in the rat testis, we find that the androgen receptor cannot be detected by immunohistochemistry in Sertoli cells before the age of 4 d, which explains why the elevated androgen levels present in the testis are unable to downregulate AMH before that age. AMH is not the only androgen-regulated gene unresponsive to high intratesticular testosterone in fetal and neonatal periods. Morphological maturation of Sertoli cells and establishment of the blood–testis barrier (38, 39), onset and maintenance of spermatogenesis (40), and transcription of most genes expressed in adult Sertoli cells do not take place before puberty (41, 42), in spite of high levels of androgens and gonadotropins in the fetus and the neonate (8). Thus, the lack of androgen repression of AMH in early development reflects a physiological state of transient androgen insensitivity

of the Sertoli cell until the androgen receptor is expressed in adequate levels to allow testosterone action.

In precocious puberty, whether gonadotropin-dependent or -independent, there is clinical evidence that gonadotropins are not implicated in AMH repression (4). To confirm this hypothesis experimentally, we treated prepubertal male mice with PMSG, containing both FSH and luteinizing hormone activity. In normal mice, PMSG induced a decrease in serum AMH, probably due to a significant stimulation of testosterone secretion. However, the possibility that gonadotropins themselves inhibited AMH could not be ruled out. To bypass the negative effect of androgens, we gave the same treatment to male *Tfm* mice, which are insensitive to androgens due to a frameshift mutation in the androgen receptor gene (11). Not only did AMH levels not fall, confirming that gonadotropins do not repress AMH expression in the absence of androgen action, but they rose significantly. In patients with impaired androgen secretion or responsiveness, serum AMH is abnormally high only in the neonatal and pubertal periods, coinciding with an activation of the gonadotropic axis, whereas normal AMH levels are observed during the rest of childhood, when serum gonadotropins are low (5). Here we show that testosterone and gonadotropins exert opposite effects on AMH secretion in mice. The negative effect of androgens seems to be more potent; therefore, the stimulatory effect of gonadotropins may only be observed in the absence of androgen action.

Sertoli cells express the FSH receptor, and many Sertoli cell secretory products are stimulated by FSH during pubertal development and in adult life (42). Thus, FSH was likely to be the gonadotropin responsible for AMH stimulation in *Tfm* mice treated with PMSG. To test this hypothesis, we injected normal prepubertal mice with pure recombinant FSH and confirmed that AMH production was enhanced. Previous studies on the role of FSH on AMH production had produced diver-

**Figure 7.** Effect of meiotic entry on AMH expression. Serum AMH and intratesticular testosterone levels (A) and AMH mRNA levels (B) in prepubertal (3–5 d-old) and pubertal (21–23 d-old) XXSxr<sup>b</sup> and XY control male mice. Bars, mean  $\pm$  SD. Differences between means were analyzed by Student's *t* test: \*\**P* < 0.01.

**Figure 6.** Immunohistochemical study of AMH in postnatal mouse testes, as revealed by horseradish peroxidase activity on aminoethyl carbazole substrate, which produces the deposit of a red pigment. Slides were counterstained with hematoxylin for identification of Sertoli and germ cell nuclei. A–D, normal mouse testes. (A) Postnatal day 4: a positive signal is observed in Sertoli cell cytoplasm; germ cells (arrowheads), interstitial tissue, and epididymal epithelium (\*) are negative. (B and C) Day 9: seminiferous tubules containing meiotic spermatocytes (arrows) are AMH-negative, while neighboring premeiotic tubules are positive. (D) Adult: all tubules are AMH-negative. E and F, *Tfm* mouse testes. (E) Postnatal day 1: all Sertoli cells are AMH-positive. (F) Day 25: all tubules have entered meiosis and are AMH-negative. G and H, pubertal (23-d-old) Sxr mouse testes. (G) XXSxr<sup>b</sup> testis devoid of meiotic cells show a positive reaction in Sertoli cell cytoplasm and in seminiferous tubule lumen. (H) XY Sxr<sup>b</sup> control testis with normal spermatogenesis is AMH-negative. Magnifications: A, B, and D,  $\times 120$ ; C,  $\times 240$ ; E–H,  $\times 100$ .

gent results: while Kuroda et al. (43) found that FSH given to newborn rats decreased AMH mRNA and immunoreactive protein levels in the testis, Voutilainen and Miller (44) showed that cultured fetal Sertoli cells increased AMH mRNA levels in response to FSH second messenger AMP. Serum AMH levels reflect total AMH production and secretion by the whole Sertoli cell population. The increase of total AMH production after FSH treatment could result from an increase of AMH gene expression or of Sertoli cell number, or both. Since FSH has a proven proliferative effect on Sertoli cells, mainly in the prepubertal testis (42, 45, 46), increase in the number of Sertoli cells secreting AMH is certainly responsible, at least in part, for the elevation in serum AMH levels. Further molecular studies in a Sertoli cell line transfected with a luciferase reporter gene under control of the AMH promoter are currently in progress to determine whether FSH modulates AMH gene expression.

AMH secretion is not downregulated in *Tfm* mice at the beginning of puberty; however, serum AMH is no longer detectable after 1 mo of age, a time when most seminiferous tubules have entered meiosis. Previous reports in mice (18) and humans (3) had shown a correlation between meiotic entry and AMH downregulation. Using mRNA dot blot, immunohistochemistry, and ELISA for AMH, we show here that the decrease in serum AMH levels during pubertal development does not result from an extinction of AMH expression in all seminiferous tubules synchronously, but rather from its selective repression in tubules that enter meiosis.

A possible interpretation of the temporal coincidence of AMH extinction and meiotic entry could be that AMH inhibits meiotic entry; however, this hypothesis is ruled out by the fact that transgenic mice overexpressing AMH throughout life have normal spermatogenesis (27). To determine whether the occurrence of meiosis and AMH downregulation in the same tubule meant a simple coincidence of events regulated by androgens and gonadotropins, or whether meiotic spermatocytes could play a role in AMH inhibition, we used the XXSxr<sup>b</sup> mouse model. We present clear evidence here that meiotic entry does play a direct role, since AMH expression persists in pubertal XXSxr<sup>b</sup> testes, producing adequate androgen levels and expressing the androgen receptor in Sertoli cells, but showing a germinal failure before meiotic entry. Although it is not completely repressed, AMH expression declines during puberty in XXSxr<sup>b</sup> males, indicating that androgens are capable of partially inhibiting AMH expression independent of meiotic entry in mice. These observations are in keeping with the results of a previous study in infertile adult men, in which tubules showing spermatogenesis arrest at the premeiotic level continued to express AMH (47). However, since the fine mechanisms of AMH negative regulation remain unknown, we cannot rule out other interpretations that could possibly explain our findings in both mice and humans.

Steroidogenic factor-1 (SF-1), an orphan nuclear receptor, plays a key role in the induction of AMH expression in Sertoli cells at the time of testicular differentiation (48). However, molecular mechanisms explaining hormonal and cellular regulation of AMH expression in postnatal life still need to be elucidated. Androgens regulate gene expression by binding to a nuclear receptor behaving as a transcription factor. Various androgen response elements (AREs) showing different binding and enhancer activity have been localized in the promoter and in intronic regions of target genes (for reviews see refer-

ences 49 and 50). We have searched for AREs on the AMH gene, from 3,750 bases upstream of the transcription initiation site to the polyadenylation addition site, but no sequences could be found with < 3 mismatches, as compared with 13 previously described ARE sequences (51). FSH action, mediated through cAMP, results in the phosphorylation of transcription factors which bind to cAMP response elements (CREs) present on the promoters of regulated genes (52). The consensus CRE palindrome TGACGTCA (53, 54) is absent from all the AMH genes sequenced so far. The lack of consensus ARE and CRE sequences in the AMH promoter suggests that regulatory mechanisms may involve intermediate or alternate pathways.

In conclusion, using three different mouse models, we have provided experimental evidence that AMH production is stimulated by FSH and inhibited at puberty by a synergistic effect of androgens and meiotic entry. When the androgen receptor expression reaches adequate levels in Sertoli cells, AMH is inhibited as soon as testosterone concentration rises in the testis, before serum androgen levels become elevated, which emphasizes the value of serum AMH as an early clinical marker of the onset of testicular pubertal maturation.

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