Lack of Androgen Receptor Expression in Sertoli Cells Accounts for the Absence of Anti-Mullerian Hormone Repression during Early Human Testis Development

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Context: Puberty is associated with increased testicular testosterone (TT) synthesis, which is required to trigger spermatogenesis and to repress anti-Mullerian hormone (AMH) production. However, testicular gonadotropin stimulation during fetal and newborn life neither initiates spermatogenesis nor represses AMH.

Objective: We postulated that a lack of androgen receptor (AR) expression in Sertoli cells (SC) might explain why these processes do not occur during early human development.

Methods and Patients: Using immunohistochemistry and quantitative PCR, we examined the relationship between AR, AMH, and FSH receptor expression in fetal, newborn, and adult human testis. The ability of testosterone to repress AMH secretion was evaluated in male newborns, neonates, and two adults with androgen insensitivity syndrome and also *in vitro* using SMAT1 SC.

Results: FSH receptor was present in SC at all developmental stages. In fetal and newborn testis, AR was expressed in peritubular and Leydig cells but not in SC. This coincided with the absence of spermatogenesis and with strong SC AMH expression. In adult testis, spermatogenesis was associated with AR expression and with a decrease in SC AMH content. Accordingly, AR mRNA expression was lower and AMH mRNA expression higher in fetal testes than in adult testes. In androgen insensitivity syndrome patients, combined gonadotropin stimulation induced an increase in circulating testosterone and AMH, a finding consistent with a failure of TT to repress AMH in the absence of AR signalling. Finally, direct androgen repression of AMH only occurred in AR-expressing SMAT1 cells.

Conclusion: Functional ARs are essential for TT-mediated AMH repression in SC. **(*J Clin Endocrinol Metab* 94: 1818–1825, 2009)**

In the human adult testis, spermatogenesis is under the control of two gonadotropins, namely FSH and LH. FSH acts directly on Sertoli cells (SC), whereas LH induces testosterone (T) production after Leydig cell stimulation. Intratesticular testosterone acts via a paracrine mechanism on androgen receptors (ARs) expressed by target cells situated in the seminiferous tubules. In adulthood, the action of androgens on the seminiferous tubules is essential for full, quantitatively normal spermatogen-

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Abbreviations: AMH, Anti-Mullerian hormone; AR, androgen receptor; CHH, congenital hypogonadotropic hypogonadism; FSHR, FSH receptor; hCG, human chorionic gonadotropin; 3βHSD, 3-β-hydroxysteroid dehydrogenase; MAIS, moderate androgen insensitivity syndrome; rhFSH, recombinant human FSH; SC, Sertoli cell(s); SCARKO, SC-selective AR knockout; T, testosterone.

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esis and fertility. Most evidence suggests that this effect is mediated through an effect on SC, although the precise mechanisms are unclear. Recently, spermatogenesis was found to be arrested in mice specifically lacking AR in their SC (1–3).

Anti-Mullerian hormone (AMH) is produced by SC from fetal life until puberty and is responsible for Mullerian duct regression in male fetuses. AMH and T serum levels correlate negatively during puberty and adulthood, indicating that testicular T is responsible for inhibiting SC AMH production. This was confirmed in adult congenital hypogonadotropic hypogonadism (CHH) patients, whose testicular AMH secretion is inhibited by both human chorionic gonadotropin (hCG) and androgen administration (4, 5).

AMH and T levels are both high during fetal and neonatal life (6, 7), indicating that testicular T is unable to repress AMH during these periods.

One important physiological issue is why the human testis is unable to produce sperm and to repress AMH secretion during fetal and neonatal life, despite stimulation by chorionic and pituitary gonadotropins, respectively. The aim of this study was to test the hypothesis that the absence of spermatogenesis and the AMH repression observed during human fetal and newborn life are related to the absence of AR in SC, despite FSH receptor (FSHR) expression. For this purpose, we compared the expression profiles of AR, FSHR, and AMH proteins and their mRNAs in human fetal, newborn, and adult testes by means of immunohistochemistry and real-time quantitative PCR. In addition, we compared T and AMH levels in umbilical arterial cord blood of male newborns, 20- to 30-d-old neonates, and adults with normal or altered androgen sensitivity to evaluate the ability of T to repress AMH secretion. Our results clearly demonstrate that in humans, AR expression in SC is required to both induce spermatogenesis and repress AMH.

Materials and Methods

Tissue collection and quality

Archival paraffin-embedded human testicular samples were collected at different stages of development. Archival fetal testis samples (n = 46), collected at 14 to 35 wk gestation, were selected from the organ bank of the Pathology Institute of Bari University with local ethics committee approval. Newborn (n = 10) and adult (n = 10) human testis samples were collected between 2 d and 6 months and between 20 and 40 yr of age, respectively. These samples were obtained from the licensed collections of three pathology departments (Bicêtre, Créteil Intercommunal and Necker-Enfants Malades Hospitals, Paris). Snap-frozen testis specimens from nine human fetuses of 16 to 38 wk and from adult men were also obtained from the same licensed tissue collection (French Bioethics law no. 2004-800).

The fetal samples originated from fetuses that had died *in utero* and had been rapidly delivered and autopsied within 24–36 h postmortem. None of the terminations were performed for fetal abnormalities. Tissue integrity was demonstrated as described in Ref. 8. Neonatal testis samples were obtained from cases of sudden infant death, and adult samples were obtained from men undergoing surgical investigation for obstructive azoospermia. The selected samples had a normal karyotype (46 XY) and normal macroscopic and histological features.

Immunohistochemistry

Briefly, 5-μm-thick tissue sections were deparaffinized and rehydrated in successive baths of toluene and graded alcohol solutions. Slides were then subjected to microwave antigen retrieval for 15 min in pH 6 citrate buffer. For FSHR immunostaining, the sections were preincubated with ready-to-use proteinase K (DakoCytomation, Carpinteria, CA) for 10 min at room temperature before 15 min of microwaving at full power in Tris-EDTA buffer (pH 9). Slides were incubated overnight at 4 C with the following primary antibodies: anti-3-β-hydroxysteroid dehydrogenase (3βHSD) (kind gift from Prof. Van-Luu The, Laval University, Québec, Canada), anti-AMH (9), anti-FSH receptor (FSHR 323) (10), and anti-AR (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:2000, 1:1000, and 1:50 dilution, respectively. Bound Ig were revealed with a streptavidin-biotin-peroxidase-aminoethylcarbazole kit (LSAB+; DakoCytomation).

RT- PCR

Total RNA was isolated from frozen samples with the TRIzol reagent (Invitrogen, Cergy Pontoise, France) as recommended by the manufacturer. RNA integrity was checked on agarose gel before processing for RT-PCR as previously described (8).

Real-time RT-PCR

Specific AMH and AR gene expression was quantified by real-time PCR (for primers, see Supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Total RNA, extracted as described above, was retrotranscribed and amplified on an ABI 7300 Sequence Detector (Applied Biosystems, Foster City, CA) as previously described (8). Ribosomal 18S RNA was used as the internal control for data normalization. Results are expressed as means ± SEM of at least two independent analyses of at least two different reverse-transcribed samples. The relative expression level of each gene is expressed relative to 18S RNA (attomoles of specific gene per femtomole of 18S).

T and AMH measurements in umbilical cords of male newborns, neonates, and adults

Neonatal blood samples were obtained from umbilical cord arteries of 17 full-term eutrophic male newborns immediately after delivery. Written informed consent was obtained from the mothers. The study was conducted in accordance with the Declaration of Helsinki and after approval from the local ethics committee. Blood samples were also obtained between d 20 and 30 of life from 21 normal boys with intrascrotal testes and from 20 normal fertile males aged 17–37 yr being evaluated in the context of female partner infertility. This part of the study was also approved by the local ethics committee, and we only used surplus serum taken for diagnostic purposes. Plasma T was measured with a commercial RIA method with a detection limit of 0.06 ng/ml (0.19 nmol/liter) and intra- and interassay variation coefficients of 5.8 and 8.0%, respectively. Serum AMH levels were measured with an ELISA method (AMH/Mullerian-inhibiting substance ELISA; Immunotech-Beckman, Marseille, France), as previously described (4). The detection limit was 0.7 pmol/liter (0.1 ng/ml), and the intra- and interassay coefficients of variation were 5.3 and 8.7%, respectively, for a serum AMH concentration of 35 pmol/liter and 4.9 and 7.8% for a serum AMH concentration of 1100 pmol/liter.

Clinical investigation of men with moderate androgen insensitivity syndrome (MAIS) or CHH

We analyzed testicular responses to combined human gonadotropins in two azoospermic MAIS patients by comparison with 10 CHH patients receiving gonadotropin combination therapy with hCG (Organon, Puteaux, France) and recombinant human FSH (rhFSH) (Gonal-F; Serono, Aubonne, Switzerland) at respective doses of 1500 and 150 IU, three times a week, for infertility.

MAIS patient 1, who was hemizygous for the M780I mutation of the AR gene, has been described in detail elsewhere (11). MAIS patient 2, a Caucasian boy, was first seen at 19 yr of age with bilateral gynecomastia and impaired virilization but no hypospadias. Testicular volume was 10 ml bilaterally (normal range, 15–30 ml), the penis was less than 4 cm

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long, and his pubic hair was at Tanner stage III. His total plasma T level was 13.4 ng/ml (normal, 3.2–9.7 ng/ml; × 3.467 = nmol/liter), and his LH was 14.8 IU/liter (normal, 4–7 IU/liter). Analysis of two semen samples showed azoospermia. The previously reported A to G transition (12) was found in exon 8 at nucleotide position 2973 causing an unconserved arginine residue to be replaced by a glycine (R871G).

Ten previously untreated men aged 18–31 yr, with idiopathic CHH (n = 5) or Kallmann syndrome (n = 5), were selected for diagnosis and choice of therapy. The diagnostic criteria for CHH were as reported elsewhere (4, 5, 13, 14). None of the patients had a history of cryptorchidism. All had low circulating T and gonadotropin levels. None had previously received gonadotropin or androgen replacement therapy. The patients were offered treatment consisting of FSH combined with hCG to increase testicular size, virilization, and fertility as previously reported (4, 5, 13).

All subjects gave their informed written consent to participate in the study, which was approved by the local ethics committee. Treatment was always well tolerated and compliance, evaluated by measuring circulating FSH and T levels, was excellent (see *Results*). AMH was assayed in blood to evaluate the testicular response. In MAIS and CHH patients, blood samples were drawn before treatment and every month during combined treatment; hormone measurements were performed in a single assay.

Cell culture and transfection

SMAT1 cells were cultured as described in Ref. 15. Cells were seeded at a density of 2×10^5 per well in six-well plates and transiently transfected with 1 μg of human AR expression vector (hAR-pcDNA3) using the Lipofectamine reagent (Invitrogen, Carlsbad, CA). In brief, 1 d after initial plating, DMEM with 10% fetal calf serum was replaced by DMEM with Dextran-coated charcoal treated serum. Transfections were performed 24 h later. Fresh medium containing 10^{-5} M forskolin alone or combined with 10^{-7} M dihydrotestosterone was added to the cells for 24 h. The cells were then rinsed twice with PBS and processed for RNA extraction and real-time PCR as described above.

Western blot analysis and immunoprecipitation assay

Total protein extracts were prepared from SMAT1 cells transiently transfected or not with hAR-pcDNA3. Immunoprecipitation was per-

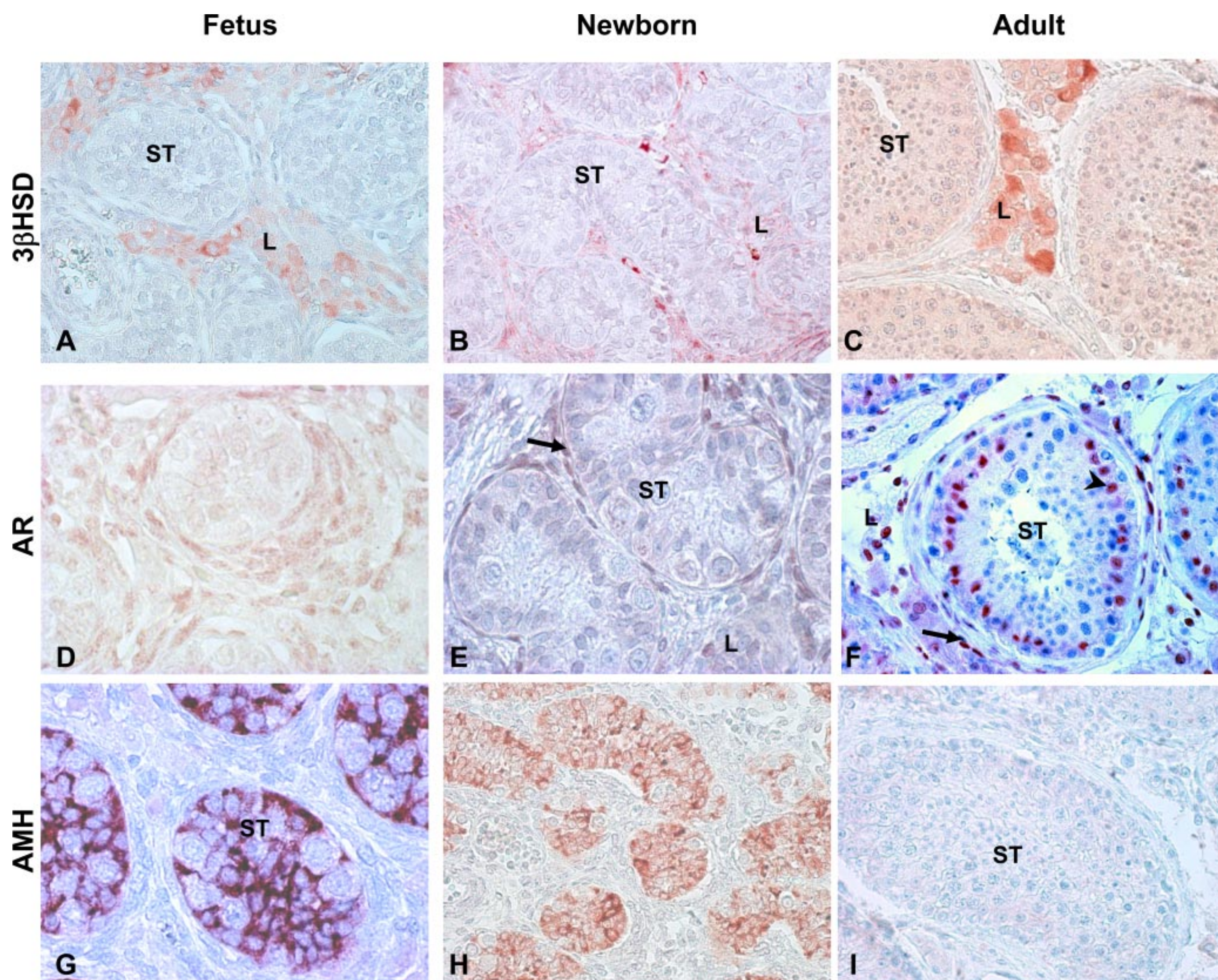

FIG. 1. Immunoexpression of 3βHSD, AR, and AMH in human testis: representative illustrations of immunoreactivity in fetal, neonatal, and adult testis. A–C, Immunoexpression of 3βHSD in fetal (A), neonatal (B), and adult (C) testis. Enzyme expression is localized to the cytoplasm of Leydig cells in fetal, newborn, and adult testis. SC and germ cells are negative. D–F, Immunoexpression of AR in fetal (D), newborn (E), and adult (F) testis. In fetal (D) and newborn testis (E), nuclear AR expression is restricted to Leydig cells and myoid peritubular cells (*arrows*). Both SC and germ cells are negative. In adult testis (F), AR expression is present in the nucleus of Leydig cells, myoid peritubular cells (*arrows*), and also SC (*arrowhead*). G–I, Immunoexpression of AMH in fetal (G), newborn (H), and adult (I) testis. AMH is strongly expressed in SC cytoplasm in fetal (G) and newborn (H) testis. AMH is undetectable in adult human testis. L, Leidig cells; ST, seminiferous tubule. Original magnification, ×20.

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TABLE 1. Summary of AR and AMH immunoexpression in human testis

	Fetal (n = 46)			Newborn (n = 10)			Adult (n = 10)		
	Sertoli	Leydig	Peritubular	Sertoli	Leydig	Peritubular	Sertoli	Leydig	Peritubular
AR	0	2+	2+	0	2+	2+	+	2+	2+
AMH	2+	0	0	2+	0	0	0	0	0

Immunoexpression was classified as: 0, absent or not detectable; +, moderate; and 2+, strong, as described in Ref. 23.

formed by incubating 0.5 mg of total proteins with 1 μg of anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc.) overnight at 4 C. Immunoprecipitates were purified with protein A Sepharose CL-4B beads and submitted to Western blot analysis using the same antibody at 1:200 dilution, followed by incubation with a peroxidase-conjugated goat antirabbit antibody (1:15,000; Vector Laboratories, Burlingame, CA). The blots were visualized with the ECL+ detection kit (GE Healthcare, Velizy, France).

Results

3βHSD expression in human testis

We first examined the immunoexpression pattern of the key enzyme 3βHSD to evaluate the steroidogenic activity of Leydig cells. As expected, the protein was located in the cytoplasm of Leydig cells in all fetal (Fig. 1A), newborn (Fig. 1B) and adult (Fig. 1C) testicular samples.

Immunohistochemical detection of AR and AMH in human testis

To determine the time course of AR and AMH expression during human testicular development, which might account for the relative T resistance of SC, we compared AR and AMH expression in fetal, newborn, and adult testes. In fetal and newborn testes, AR protein was detected in Leydig and myoid peritubular cells, but not in germ cells or SC (Fig. 1, D and E). The absence of AR in SC coincided with very strong AMH expression in the same cells of all fetal and neonatal testis samples (Fig. 1, G and H). In contrast, in all adult human testis samples examined, AR was detected not only in Leydig cells and myoid peritubular cells but also in SC (Fig. 1F), whereas AMH was not expressed (Fig. 1I). Semiquantitative results for AR and AMH expression during human testis development are summarized in Table 1.

AR and AMH mRNA levels in human fetal testis

To examine quantitative changes in AMH and AR expression during testis development, mRNA levels of these genes were determined by real-time quantitative PCR in 10 fetal testes obtained at 16 (n = 2), 20 (n = 1), 21 (n = 1), 22 (n = 1), 24 (n = 2), 25 (n = 2), and 38 (n = 1) wk gestation, by comparison with adult testes (Fig. 2). The relative expression level of AR mRNA in fetal testis was 30% or lower than that in adult testis (Fig. 2A). This difference was probably due to the absence of AR expression in fetal testis SC. We then examined AMH mRNA expression and found that levels were 2- to 30-fold higher in fetal than in adult testes (Fig. 2B). Thus, the AR/AMH mRNA ratio, an index of AR expression in SC, was 151 ± 8 in adult testis, compared with 1–20 in fetal testis, in keeping with the lack of AR immunoexpression in fetal SC.

FSHR expression in human testis

To determine whether the lack of spermatogenesis in the fetal and newborn testes is related to the lack of FSHR during these periods of life, we studied FSHR expression by immunohistochemistry and RT-PCR. As shown in Fig. 3, A–C, FSHR immunoreactivity was localized to the SC membrane in fetal, newborn, and adult testis. Furthermore, we confirmed that, as in adult testis, FSHR mRNA is also detected in human fetal testis (Fig. 3D). Using FSHR as another SC marker, we confirmed that the AR/FSHR mRNA ratio is higher in adult testis (406 ± 25) than in fetal testis (7.5 ± 1.5), providing additional support for the lack of AR expression in SC during human fetal development.

Inability of combined gonadotropin stimulation to repress AMH in adult patients with an AR signaling defect

We then compared the testicular responses to combined hCG and rhFSH in two adult MAIS patients and 10 CHH patients

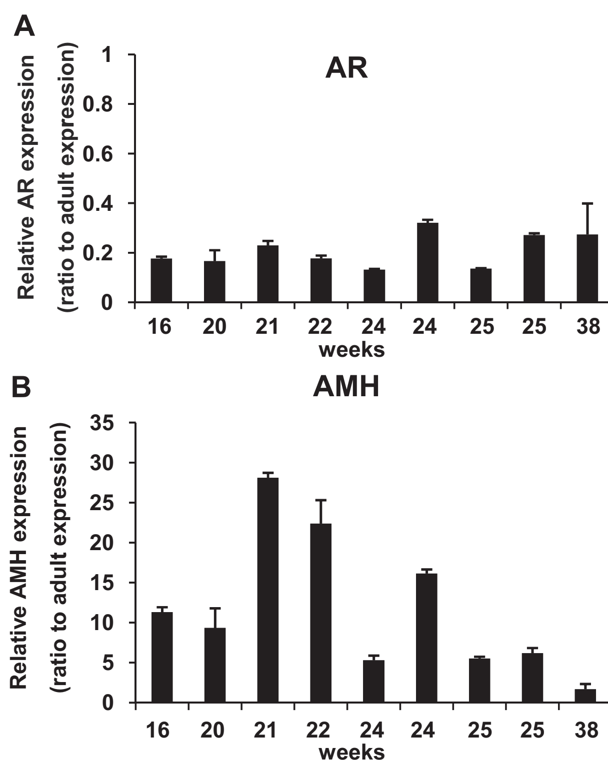

FIG. 2. AMH and AR messenger levels in fetal human testis. Relative expression of AR (A) and AMH (B) mRNA in 10 fetal human testis obtained at 16 (n = 2), 20 (n = 1), 21 (n = 1), 22 (n = 1), 24 (n = 2), 25 (n = 2), and 38 (n = 1) wk gestation. (See Supplemental Table 1 for primers.) Results were normalized to 18S ribosomal RNA. Relative expression in a given sample was calculated as attomoles per femtomole of 18S RNA. Results are expressed as means ± SEM of at least two reverse-transcribed samples run in duplicate and represent the relative expression compared with control adult testes (arbitrarily 1).

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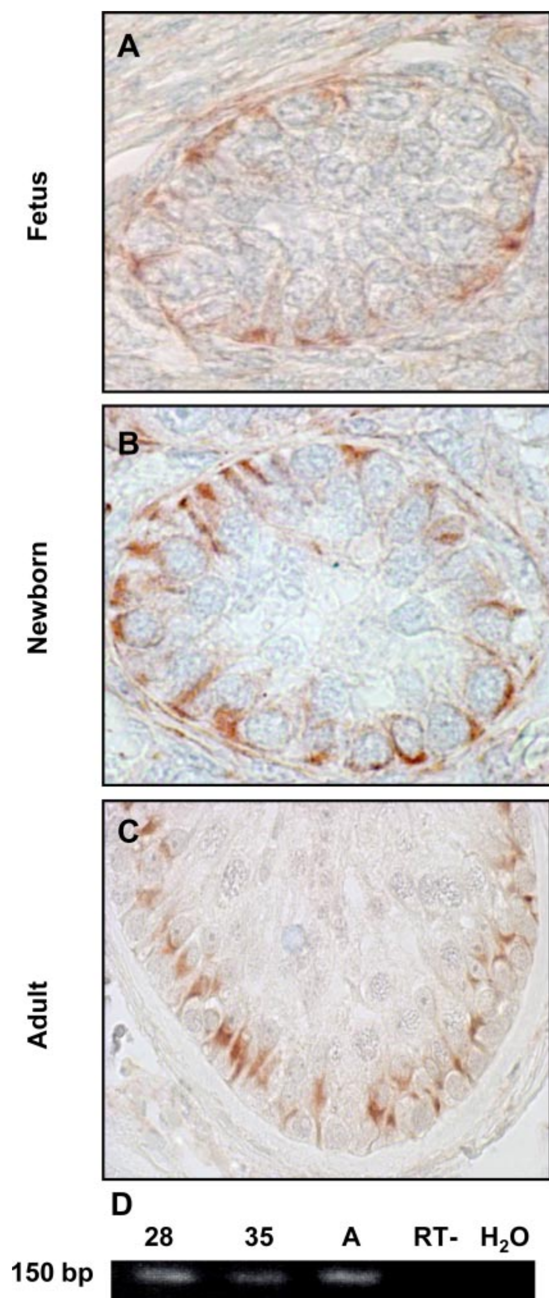

FIG. 3. FSHR expression in human testis. A–C, FSHR protein expression in human fetal (A), newborn (B), and adult testis (C). The receptor is expressed on the SC membrane. Germ cells are negative. D, Representative FSHR mRNA expression in human adult and fetal testis at 28 and 35 wk. RNA was extracted and reverse transcribed as described in *Materials and Methods*. (See Supplemental Table 1 for primers.) A, Adult. RT− (omission of the reverse transcriptase) and H_2O are negative controls.

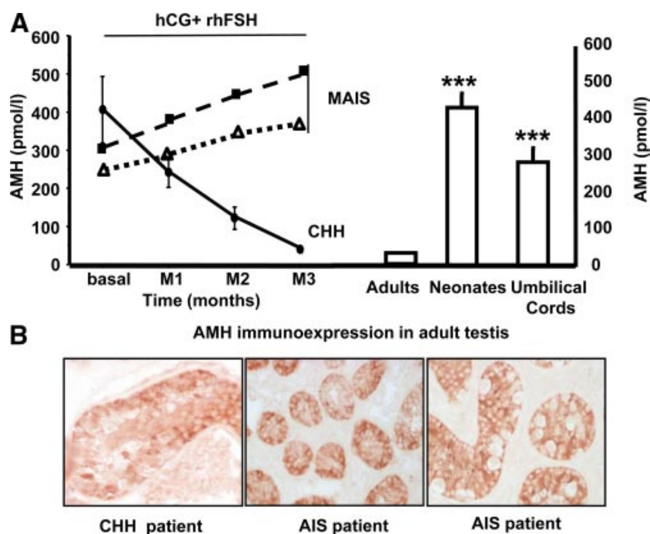

FIG. 4. Relationship between AMH and T levels in normal and androgen-deficient human males. A, *left*, Time course of individual serum AMH levels (pmol/liter) in two adult MAIS patients and mean (±SD) serum AMH in 10 adult CHH patients during combined gonadotropins stimulation (rhFSH and hCG). *Right*, Serum AMH levels (pmol/liter) were measured in adult, neonatal (20 and 30 postnatal days), and umbilical cord arterial bloods. Results are expressed as means ± SEM of at least 19 values. ***, $P < 0.001$ *vs.* adult values, Mann-Whitney test. B, High AMH immunoexpression in testis of an untreated adult patient with CHH and two adults with MAIS.

used as positive controls of preserved AR signaling in postpubertal testes (Fig. 4A, *left panel*). As expected (4, 5) gonadotropin therapy increased the serum levels of FSH (from 0.6 ± 0.3 to 11.2 ± 2.2 IU/liter) and T (from 0.46 ± 0.2 to 5.8 ± 2.2 ng/ml) in CHH patients, leading to a concomitant collapse of AMH levels (from 406 ± 274 to 37 ± 18 pmol/liter; $P < 0.001$) that reached normal adult levels (Fig. 4A, *right panel*). In contrast, in MAIS patients, despite a further increase in their plasma T levels induced by gonadotropin administration (from 11–13.4 to 15–19.4 ng/ml), the serum AMH level also rose, clearly demonstrating that, in these adult patients, testicular T is unable to repress AMH secretion in the absence of functional AR and therefore to prevent the AMH stimulation induced by rhFSH. This response pattern was similar to that observed during the prenatal and postnatal period in which, despite relatively high circulating T levels driven by chorionic and pituitary gonadotropin stimulation, mean AMH levels remain significantly higher than in postpubertal men (6) (Fig. 4A, *right panel*). Interestingly, AMH levels were significantly higher in 20- to 30-d-old boys than in newborns, a finding consistent with our demonstration of FSHR expression in SC and its stimulation by FSH during the early postnatal period (16). In addition, strong AMH immunostaining was observed in SC of both adult patients with androgen insensitivity syndrome and untreated CHH patients (Fig. 4B), contrasting with undetectable AMH expression in adults with normal testicular T and androgen sensitivity (Fig. 1I). Collectively, our results clearly indicate that repression of AMH secretion requires both an appropriate intratesticular T levels and functional AR signaling in SC.

AMH repression by androgens requires functional ARs in SMAT1 cells

To investigate direct androgen regulation of AMH in a SC model, we examined endogenous AMH expression by means of real-time PCR in SMAT1 cells, an immortalized immature SC line that does not express AR (Fig. 5A). Forskolin induced a 2.5-fold increase in AMH expression (Fig. 5B) through a protein kinase A-dependent mechanism, as already reported (15). However, coadministration of dihydrotestosterone did not affect forskolin-induced AMH expression. In contrast, when SMAT1 cells were transiently transfected with a human AR expression vector (Fig. 5, A and C), forskolin-stimulated AMH mRNA levels re-

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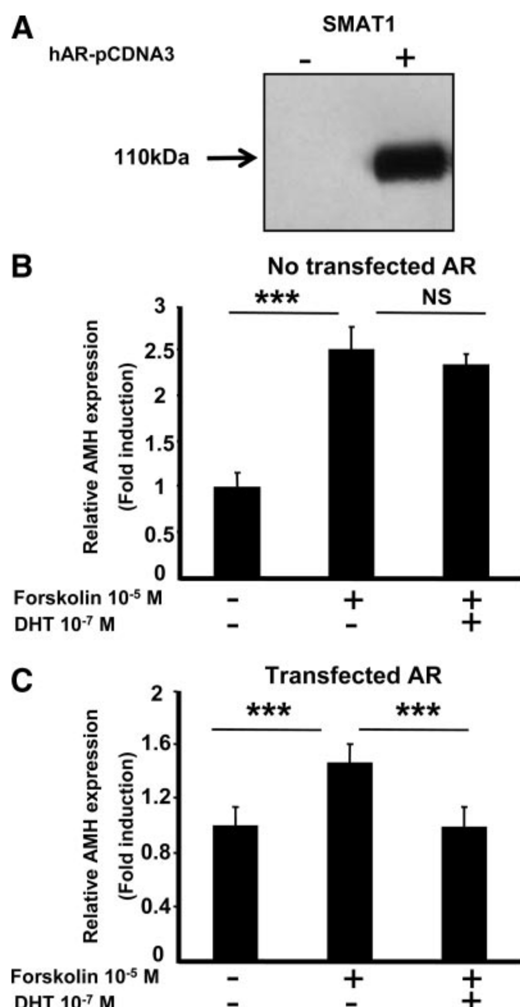

FIG. 5. Androgens repress endogenous AMH expression in SMAT1 SC in the presence of AR. A, AR was expressed in SMAT1 cells transfected with pcDNA3-hAR plasmid. Proteins (0.5 mg) were immunoprecipitated with an anti-AR antibody, followed by Western blotting with the same antibody. AR protein was only expressed in SMAT1 cells transiently transfected with pcDNA3-hAR plasmid. B and C, Relative AMH expression was analyzed by using real-time PCR in control SMAT1 cells (B) and in SMAT1 cells transiently transfected with pcDNA3-hAR plasmid (C). Cells were treated with 10^{-5} M forskolin alone or combined with 10^{-7} M dihydrotestosterone (DHT) for 24 h. Results are expressed as means ± SEM of at least six independent determinations and represent the relative expression compared with basal levels in untreated cells (arbitrarily set at 1). ***, $P < 0.001$ nonparametric Mann-Whitney test. NS, Not significant.

turned to basal values after dihydrotestosterone exposure, providing the first evidence that functional AR are crucial for full AMH repression by androgens in SC.

Discussion

The role of AR in spermatogenesis has been the subject of intense interest for many years. Multiple rodent studies of androgen withdrawal and disruption of AR activity by surgical, chemical, or genetic means have produced convergent results: spermatogenesis rarely occurs in the absence of androgens and functional AR. It has been assumed that the main cellular mediator of this regulatory function is the SC, given its intimate contact with germ cells devoid of AR (17–19). Our findings endorse this assumption. Other rodent studies have revealed the major role played by SC AR in the completion of spermatogenesis (20). Several groups (1–3) recently generated mice with SC-selective AR knockout (SCARKO mice), opening up new possibilities to elucidate the respective contributions to spermatogenesis of AR expression in different testicular cell types. Thus, despite normal testicular descent, all SCARKO mice generated to date display arrested spermatogenesis. These results prove that the initiation and maintenance of spermatogenesis are both crucially dependent on AR activation in SC. Several authors, while studying the profile of AR expression in SC during postnatal development of normal rodents and nonhuman primates (21–24), failed to detect immunochemical AR expression in the immediate postnatal period. This could explain why the elevated T levels present in rodent and marmoset testis are unable to induce morphological maturation of SC or to trigger and maintain spermatogenesis, despite high levels of androgens and gonadotropins in the fetus and neonate.

The main purpose of the present study was to extend to humans this concept of a physiological state of transient androgen insensitivity of SC during fetal and neonatal life. Our results clearly show that, as reported for rodents and marmoset, AR protein is present in Leydig and peritubular cells of fetal and neonatal human testis, but not in SC. This expression pattern is in keeping with the absence of spermatogenesis, as expected (25–27), in our samples. During these two periods of human testicular development, hCG- and LH-induced biosynthesis of T is activated, as indicated by the strong 3βHSD expression in Leydig cells. This lack of AR expression in SC during early testicular development contrasted with the expected (17) strong AR expression we observed in these cells in sperm-producing adult human testis.

The role of FSH in the initiation of human spermatogenesis remains controversial (15, 28–32). To confirm that the lack of FSHR expression is not responsible for the lack of spermatogenic development in the fetal and early postnatal testis, we examined the FSHR expression pattern in SC in the same testicular samples. Immunochemical studies clearly showed FSHR expression in fetal and neonatal SC, as well as in adult SC. These findings, which concord with the FSHR transcript expression we also observed in human fetal testis, are also in line with the presence of FSH binding sites in fetal primate testis (33). It is generally accepted that FSH stimulates SC inhibin B secretion (4, 31, 34). Indeed, the presence of functional FSHR in human neonatal testis is suggested by two recent reports showing an increase in circulating inhibin B in response to the neonatal rise in FSH (16, 35). Collectively, these results rule out the possibility that the absence of spermatogenesis in fetal and neonatal life is due to a lack of FSHR.

AMH, which is responsible for the repression of Mullerian ducts in male fetuses, is measurable in human male umbilical cord serum, as well as in the neonatal period and during infancy (36, 37). The T elevation during puberty correlates with a fall in circulating AMH (6). This negative correlation is also observed in boys with central or gonadotropin-independent early puberty, suggesting that T is the main player in AMH down-regulation.

Our combined T and AMH assays in umbilical cord, neonatal infants, and adult blood provide additional *in vivo* support for

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the lack of functional AR expression in SC during the perinatal period. Indeed, the elevated T levels in fetal and neonatal males, in keeping with previous studies (16), are consistent with an increase in testicular T. However, AMH levels remained elevated during these two developmental stages, in keeping with the absence of AR in SC, as demonstrated here and in another very recent publication (25). Furthermore, circulating AMH levels were markedly increased in neonates compared with cord blood, owing to the reported FSH surge between these two periods (16, 38). This AMH increase is probably related to FSH-induced SC proliferation (which increases the AMH-producing cell population), and also to activation of AMH gene transcription through a pathway mediated by cAMP (15, 21).

The negative correlation between testicular T and AMH levels is not found in neonatal rodents (21), human fetuses (6), or normal or cryptorchid human male newborns (16, 37), indicating that testicular T is unable to repress AMH in the fetal and neonatal periods. In newborn rodents (21, 24) and in adult SCARKO mice (1), the absence of AR expression in SC is also associated with a lack of AMH repression, suggesting that a similar mechanism might exist in human male newborns. Our immunohistochemical findings demonstrating strong AMH expression in SC lacking AR, and the marked fall observed in postpubertal SC (which express AR) support this assumption. These results also argue against a mechanism in which a change in directional testicular AMH secretion (39) causes the fall in serum AMH after puberty.

Patients with androgen insensitivity syndrome or CHH, a pathological condition associated with low testicular T, have abnormally elevated serum AMH levels. As we have previously shown (4, 5), the hCG-driven increase in intratesticular T in the 10 CHH patients studied here was associated with a marked reduction in the serum AMH level. In contrast, AMH levels were not reduced in the two adult MAIS patients who received the same combined gonadotropin treatment; on the contrary, they were markedly enhanced, in keeping with an absence of androgen action owing to the lack of AR signaling in SC. Thus, this AMH response pattern in MAIS is similar to that recently reported in two CHH neonates (35) receiving a similar treatment, indicating that in both cases the failure to repress AMH is related to the absence of functional AR expression in SC.

In accordance with these *in vivo* findings, we obtained the first direct evidence that AMH repression by androgens only occurs when AR is expressed in Sertoli SMAT1 cells. The molecular mechanisms underlying the repressive effect of AR on AMH expression remain to be elucidated. AR might directly repress AMH transcription although no androgen response elements are present in the regulatory regions of the AMH gene. Alternatively, AR might compromise FSHR-mediated AMH stimulation by altering activator protein 2 and nuclear factor κB signaling (15). We are currently investigating the functional interaction between AR and FSHR activation cascade on AMH expression using a SC model.

In conclusion, the absence of AR expression in SC of fetal and neonatal human testis contributes to the lack of germ cell maturation and of AMH repression despite strong testicular T biosynthesis.

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Supplemental table 1

Name	Accession	Amplicon	Sense primer	Antisense primer
18S	AJ844646	70 bp	GTGCATGGCCGTTCTTAGTTG	CATGCCAGAGTCTCGTTCGTT
AR	L29496	150 bp	GTTTTAGACTGCCAGGGAC	TCAGCGGCTCTTTTGAAGAAG
AMH	NM_000479	150 bp	ACATCAGGCCCAGCTCTATCAC	TGTTTGTGCAGGACAGACCC
FSHR	NM_000145	79 bp	TCACAGTCCCCAGGTTCCTTA	TGATGCAATGAGCAGCAGGTA

Table 1: Primer sequences for real time PCR

Abbreviations of the genes, their GENBANK or NCBI accession numbers, and the 5' to 3' nucleotide sequences of their sense and antisense primers