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Invited Review

The prepubertal testis: a quiescent or a silently active organ?

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Summary. The development of the testis is characterised by dramatic changes between birth and adulthood. The most conspicuous changes take place during puberty, when the seminiferous tubule diameter increases significantly owing to an important proliferation of germ cells, giving rise to spermatozoa, and to the development of a tubular lumen; in the interstitial tissue characteristic Leydig cells appear and secrete high levels of testosterone. These pubertal changes of the testis can be detected clinically since they result in testicular volume and serum androgen level increments. The prepubertal testis has classically been defined as a quiescent organ. However, since adequate stereological methods for microscopic analysis are available, it has been shown that the male gonad triplicates its volume between birth and the onset of puberty. Sertoli cells and spermatogonia proliferate intensely; this is critical for the development of quantitatively normal adult spermatogenesis. Seminiferous tubule volume increases owing to an increment in tubular length, not diameter. Sertoli cells are also functionally active during childhood: they produce high amounts of anti-Müllerian hormone during the whole prepubertal period, and inhibin B until the age of 2-4 years. Anti-Müllerian hormone and inhibin seem to play a role as modulators of the proliferation and differentiation of Leydig cell precursors.

Key words: Childhood, androgens, Anti-Müllerian hormone, Sertoli cells, inhibin

Introduction

The testes are composed of two morphologically and functionally different compartments: the seminiferous tubules, where the male gamete is produced, and the interstitial tissue, responsible for the secretion of androgens. The seminiferous tubules contain two distinct cell populations: the Sertoli cells are somatic cells which play an essential role in the development of the other population, the germ cells, from which the spermatozoa arise. The interstitial tissue contains a testis-specific component, the Leydig cells which produce androgens, and a non-specific one, mainly represented by connective tissue. The two testicular compartments are functionally interdependent: androgens are essential for Sertoli cell maturation and for spermatogenesis, and Leydig cell development and function are modulated by seminiferous tubule-secreted factors.

The development of the testes, from the time of their differentiation in the foetus until adulthood, is characterised by dramatic morphological and functional changes. These changes will be briefly described hereafter as an introduction to the discussion of one particular period of the postnatal development of the male gonad: the prepubertal period.

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Differentiation and development of the testis

The foetal period

In the human foetus, a thickening of the coelomic epithelium covering the anterior surface of the mesonephros on each side of the body represents the anlagen of the gonads. Although the sex is determined by the chromosomal complement (46,XY or 46,XX) resulting at the time of fertilisation, the male or female gonads cannot be distinguished at the microscopic level before the end of the 7th week; by then, in the male foetus, the expression of the SRY (Sex-determining Region of the Y chromosome) gene results in the differentiation of the gonad along the testicular pathway (Berta et al., 1990; Sinclair et al., 1990). Other autosomal genes are also implicated in gonadal differentiation: WT-1 (Wilm's Tumour 1) (Kreidberg et al., 1993) and SF-1 (Steroidogenic Factor 1) (Luo et al., 1994) are essential for the development of the urogenital ridge, while mutations or deletions of SOX-9 (SRY HMG-box, gene 9) (Foster et al., 1994) or other putative genes located on the short arm of chromosome 9 (Bennett et al., 1993) and on the long arms of chromosomes 7 (Seller et al., 1997) and 10 (Wilkie et al., 1993) result in abnormal testicular differentiation.

The first morphologically identifiable event in testicular differentiation is the development of the Sertoli cells, which aggregate to form the seminiferous cords that will be colonised by the primordial germ cells travelling from the stalk of the allantois. By the end of the 7th week, Sertoli cells begin to synthesise anti-Müllerian hormone (AMH) (Fig. 1), the first Sertoli-cell specific protein expressed by the foetal gonad (Tran et al., 1977; Josso et al., 1993). AMH, also known as Müllerian inhibiting substance (MIS) (Lee and Donahoe, 1993), is responsible for the regression of the Müllerian ducts, which differentiate to form the uterus and Fallopian tubes in the female (Fig. 2). Soon afterwards, Sertoli cells begin to express other specific markers, like SGP-2 or clusterin (Taketo, 1991). The immature aspect of Sertoli cells does not change during the remaining foetal life.

Primordial germ cells, or gonocytes, express a membrane-bound receptor known as c-kit, of vital importance for germ cell migration to the gonad (Orth et al., 1997) and for their adhesion to Sertoli cells (Pesce et al., 1997), which express the c-kit ligand (Rossi et al., 1991; Tajima et al., 1991), known as Steel Factor. Once harboured in the sex cords, where they occupy a central position surrounded by Sertoli cells, the gonocytes proliferate vigorously until late foetal life (Huckins and Clermont, 1968), but they do not enter meiosis.

In the interstitial tissue, Leydig cells differentiate during the 8th week of foetal life. The presence of an LH/hCG receptor capable of binding placental hCG on Leydig cell membrane is essential for further development and multiplication of foetal Leydig cells (Kremer et al., 1995; Laue et al., 1995), which reach peak numbers between weeks 16 and 24; thereafter, Leydig cell number progressively decreases until birth (Codesal et al., 1990). As soon as they differentiate, Leydig cells begin to express steroidogenic enzymes involved in testosterone biosynthesis, as well as other peptides that are secreted and might play a role in paracrine or endocrine regulatory mechanisms (for review, see Pelliniemi et al., 1996). Testosterone secretion is essential for the virilisation of the Wolffian ducts, which differentiate to form the internal male gonaduct, and of the external genitalia.

Fig. 1. Localisation of anti-Müllerian hormone (AMH) expression by in situ hybridisation using a digoxigenin-labelled riboprobe in an 8 week-old male foetus gonad. The slide was slightly counterstained with hematoxylin. Sertoli cells in the seminiferous cords express AMH intensely. x 50

The postnatal period

The aspect of the adult testis is significantly different from that of the gonad in the new-born (Fig. 3). The most notorious changes take place in the seminiferous tubule compartment, where Sertoli cells show morphological maturation (Fig. 4) and spermatogenesis reaches complete development. Spermatogenesis is a complex process, involving mitotic and meiotic divisions and differentiation of germ cells, through which the undifferentiated diploid germ cells give rise to haploid spermatozoa. As a result of the proliferation of germ cells and of the maturation of Sertoli cells, the seminiferous tubules acquire a lumen and dramatically increase their diameter. In the interstitial tissue, Leydig cells differentiate from mesenchymal precursors. The mature male gonad has an intense secreting capacity and produces high quantities of androgens, inhibin, activin, and androgen-binding protein, which have been used as markers of adult testicular function.

Most of the aforementioned changes take place during puberty: in a period of 4 to 6 years, the testicular

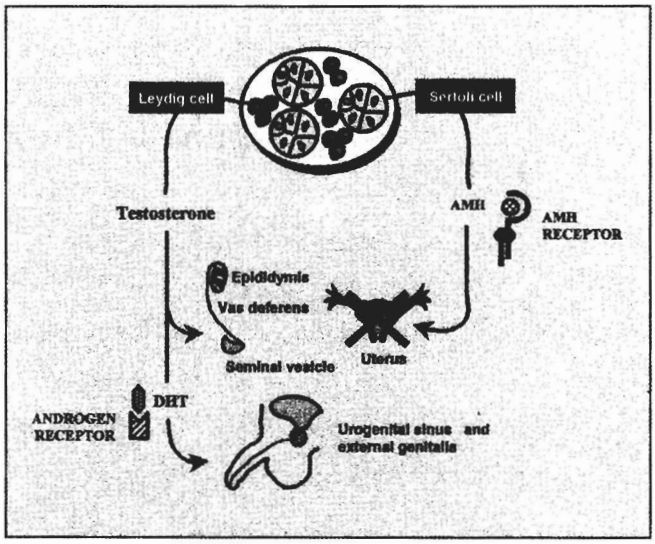

Fig. 2. Hormonal regulation of sex differentiation. Anti-Müllerian hormone, secreted by Sertoli cells and acting through a membrane-bound receptor, inhibits the development of Müllerian ducts, which give rise to the Fallopian tubes, uterus and upper part of the vagina in the female foetus. Testosterone, produced by Leydig cells and acting through the intracellular androgen receptor, induces the development of Wolffian ducts to form the epididymis, vas deferens and seminal vesicle. Transformation of testosterone to dihydrotestosterone (DHT), which also binds to the same androgen receptor, is essential for the virilisation of the urogenital sinus and external genitalia (Reproduced with permission from: Rey R and Picard JY. Embryology and endocrinology of genital development. Baillière's Clinical Endocrinology and Metabolism 1998; 12:17-33).

volume grows from 1-2 cm^3 to 20-25 cm^3, the seminiferous tubule diameter increases from ~50 μm to ~250 μm, and serum testosterone levels that are almost undetectable in the prepubertal boy reach adult levels.

The prepubertal testis

Morphological changes during infancy and childhood

During the first months of postnatal life, testicular function is stimulated by the activated hypothalamic-pituitary axis. Testosterone production by the interstitial tissue of the gonad results in elevated serum androgen levels. Then, the steroidogenic activity of the testis progressively decreases, and the production of testosterone, the most well known marker of testicular function, remains at basal levels until the onset of puberty.

During the whole prepubertal period, i.e. between birth and the onset of puberty, the seminiferous tubules show the same aspect. No lumen is observed and Sertoli cells show immature features: they have scarce cytoplasm, the nuclei are arranged in a palisade-like disposition, have regular outlines and small nucleoli. Germ cells are represented by gonocytes, centrally located, and spermatogonia, attached to the basal membrane. Although meiotic spermatocytes may eventually appear, they soon degenerate: no meiosis progression occurs in the prepubertal testis.

The low of testosterone production and the absence of qualitative histological changes in the testis during chilhood have led to the concept that the prepubertal stage is a quiescent period of testicular development. However, the use of morphometric techniques to study small quantitative changes in testicular histology and the analysis of non-traditional testicular markers have shown that there is an important proliferation of different testicular cell populations and a non-negligible secreting activity of Sertoli cells during infancy and childhood.

Classically, the size of the testes is estimated by palpation and comparison to Prader's orchidometer. Although practical and informative during pubertal development, this way to assess testicular volume clinically is not sensitive enough to detect small changes in the size of the gonads. This certainly explains why the concept exists that testicular volume does not change before the onset of puberty. However, estimating testicular volume by weighing the gonads obtained from autopsies of 50 boys who suffered from sudden death, Müller and Skakkebaek (1983) showed that testicular volume increased from 0.57 cm^3 in the first year of life to 1.50 cm^3 at the age of 10. Although the size of the gonads increases almost threefold during childhood, the change in absolute volume is small, thus clinically undetectable. Similarly, using the Cebus monkey as a model of primate testicular development, we (Rey et al., 1993a) showed that there is a 13-fold increase in testicular volume between birth and the onset of puberty (Fig. 4).

Whereas during pubertal development the increase of testis size is directly dependent on germ cell proliferation, in the prepubertal period testis volume shows a positive correlation with Sertoli cell number. Although germ and interstitial cells also increase in number before puberty, this increment has less incidence in testicular volume. In the Cebus monkey, the total

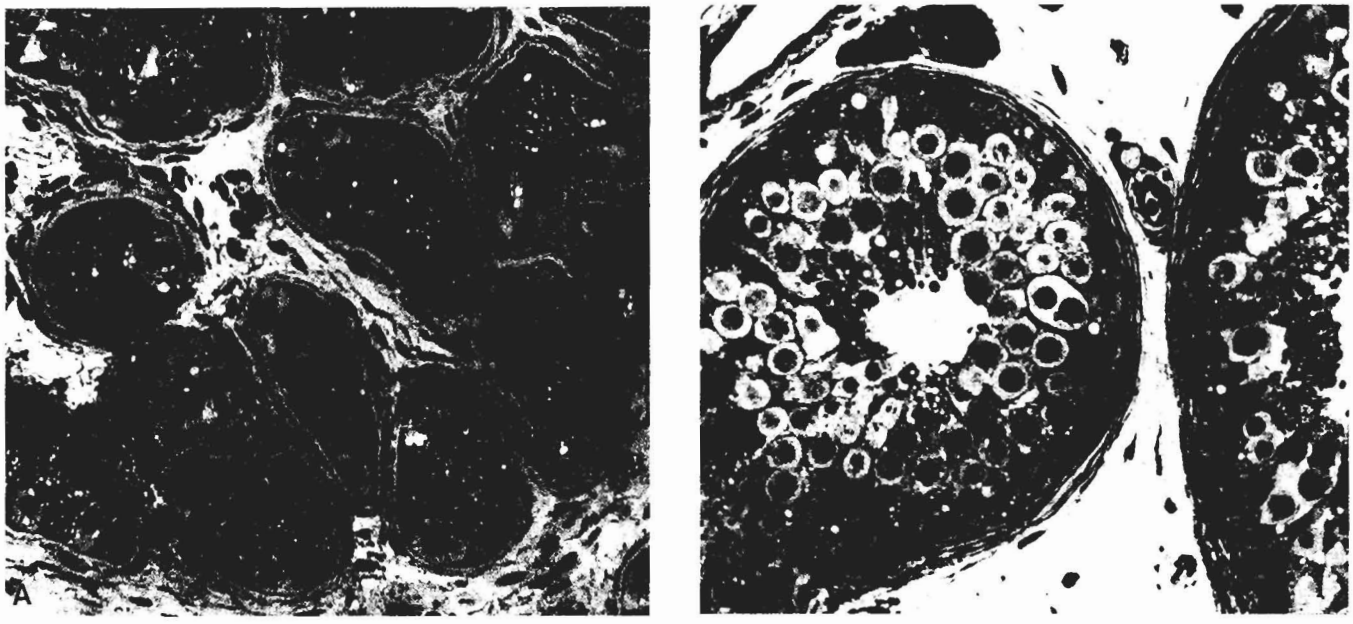

Fig. 3. Histological sections of plastic-embedded normal testicular tissue from an infant **(A)** and an adult **(B)**, stained with hematoxylin and eosin. x 250

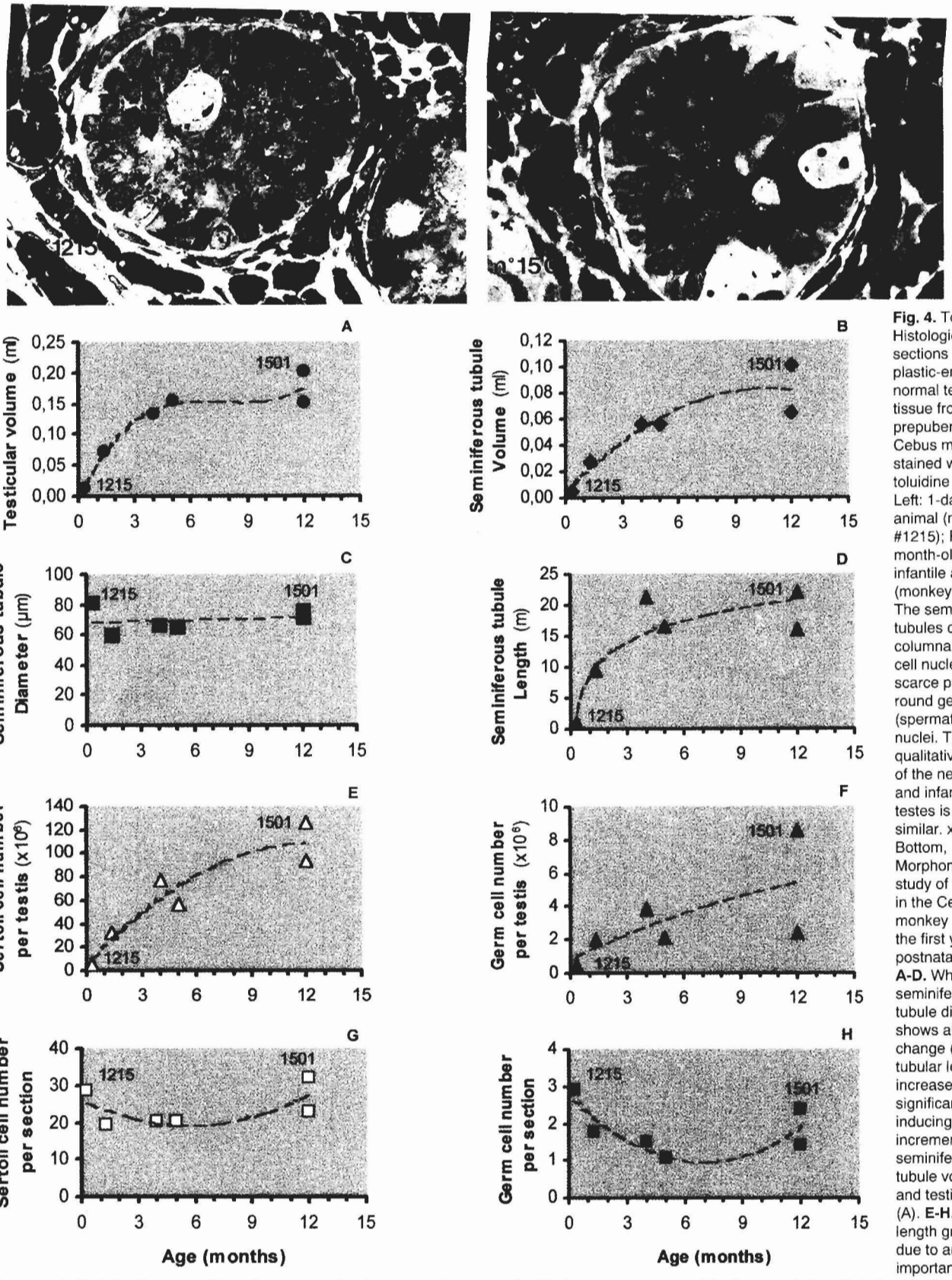

Fig. 4. Top, Histological sections of plastic-embedded normal testicular tissue from prepubertal Cebus monkeys, stained with toluidine blue. Left: 1-day-old animal (monkey #1215); Right: 12-month-old infantile animal (monkey #1501). The seminiferous tubules contain columnar Sertoli cell nuclei and scarce pale, round germ cell (spermatogonia) nuclei. The qualitative aspect of the new-born and infantile testes is quite similar. x 700. Bottom, Morphometric study of the testis in the Cebus monkey during the first year of postnatal life. **A-D.** While seminiferous tubule diameter shows almost no change (C), tubular length (D) increases significantly, inducing an increment in seminiferous tubule volume (B) and testicular size (A). **E-H.** Tubular length growth is due to an important increase in Sertoli cell number (E) and a more moderate increase in germ cells (F). As a consequence of the increase in length, but not in diameter, of the seminiferous tubules, the number of Sertoli (G) and germ cells (H) per tubular cross section does not increase. In all graphs, 1215 and 1501 label the values corresponding to the Cebus monkeys whose histological sections are shown in the Top plate.

number of Sertoli cells per gonad increases more than 10-fold between birth and the onset of puberty (Fig. 4) (Rey et al., 1993a). This is not specific to primates, since in most rodent testes also, Sertoli cells actively proliferate until approximately postnatal day 15-20 (Solari and Fritz, 1978; Kluin et al., 1984; Orth et al., 1988; Vergouwen et al., 1993), when germ cells enter meiosis. The multiplication of Sertoli cells before puberty is critical for the development of quantitatively normal spermatogenesis in the adult, since the number of germ cells in adults depends on the size of the Sertoli cell population (Orth et al., 1988; Kumar et al., 1997). Follicle-stimulating hormone (FSH) alone (Griswold et al., 1977; Almirón and Chemes, 1988; Schlatt et al., 1995; Kumar et al., 1997), or in association with activin (Boitani et al., 1995), is at least in part responsible for Sertoli cell multiplication in the perinatal testis.

A clear example of the proliferation of Sertoli cells during prepuberty is the effect of neonatal hypothyroidism on testicular development in rodents. Induced neonatal hypothyroidism delays pubertal maturation of the testis, and causes prolonged Sertoli cell multiplication (Van Haaster et al., 1992; de França et al., 1995). This may be explained by the fact that triiodothyronine upregulates androgen receptor expression in Sertoli cells (Jannini et al., 1990; Panno et al., 1996) and pubertal Sertoli cell maturation depends on androgen action (Chemes et al., 1979). In adult life, male mice who suffered from neonatal hypothyroidism have increased Sertoli and germ cell numbers - resulting in larger testicular volume - when compared to normal mice (Hess et al., 1993; Jannini et al., 1993).

Germ and peritubular cell populations also increase between birth and the beginning of puberty (Huckins and Clermont, 1968; Kluin et al., 1984; Almirón and Chemes, 1988). The number of spermatogonia increases approximately 30-fold during prepuberty in the mice (Vergouwen et al., 1993) and in the Cebus monkey (Fig. 4) (Rey et al., 1993a). Although this increment is more important in relative terms than that observed in the Sertoli cell population, germ cells represent a small proportion of total testicular cells and their proliferation has a more trivial impact on testicular volume changes.

During infancy and childhood, the diameter of the seminiferous tubules shows almost no change (Fig. 4). How can this be explained when Sertoli and germ cell numbers increase significantly? The enlargement of testicular size before pubertal onset relies mainly on the increase of seminiferous tubule volume, which depends on two parameters: tubular diameter and tubular length. Tubular diameter is easily assessed in histological examination, but tubular length estimation requires adequate stereological analysis. The use of morphometric techniques has shown that the increase of Sertoli, germ and peritubular cell numbers in infantile animals results in an increment of tubular length rather than tubular diameter (Fig. 4) (Rey et al., 1993a; Steger and Wrobel, 1996). This explains why the number of Sertoli and germ cells per tubular cross section remains stable, or even decreases, during prepubertal life (Fig. 4) (Mancini et al., 1960; Rey et al., 1993a).

Functional changes in the testis during infancy and childhood

In the human male, after a period of up to 6 months

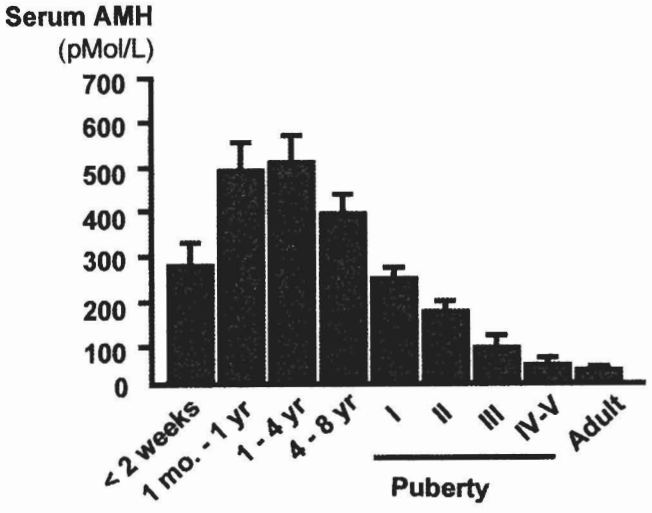

Fig. 5. Serum AMH levels in the normal male after birth, as assayed using the AMH/MIS ELISA kit (Immunotech-Coulter, Marseilles, France). Bars represent mean±SEM. Serum AMH is elevated during prepubertal life and subsequently decreases from pubertal stage I to stages IV-V, when it reaches the typically low values of the adult, downregulated by increasing Leydig cell testosterone production (Reproduced with permission from: Rey R. Endocrine, paracrine and cellular regulation of post-natal anti-Müllerian hormone secretion by Sertoli cells. Trends in Endocrinology and Metabolism 1998; 9:271-276).

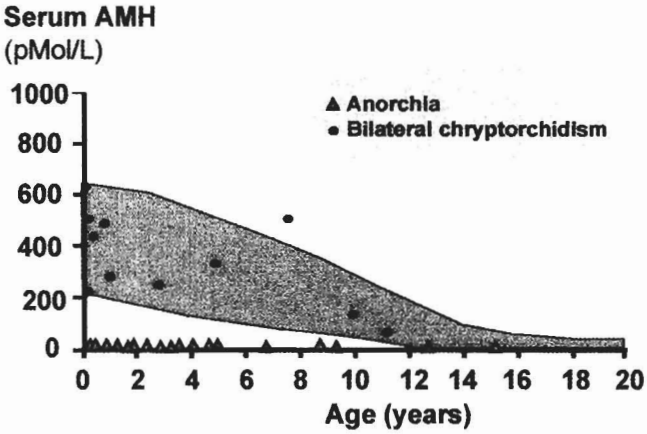

Fig. 6. Serum AMH in boys with non-palpable testes: AMH levels within the normal range indicate the presence of chryptorchid gonads, while undetectable serum AMH is a sign of anorchia (except for the rare cases of patients with a bilateral chryptorchidism owing to a Persistent Müllerian Duct Syndrome due to a mutation in the AMH gene). Notice that serum AMH assay is no longer informative after pubertal development, since it may be undetectable in normal males. The shaded area represents normal serum AMH levels.

after birth in which the testis secretes high levels of androgens as a consequence of an activation of the hypothalamic-pituitary axis, testicular testosterone production by Leydig cells falls to basal levels until puberty. Since most of Sertoli cell proteins are upregulated by androgens, their secretion is also low or undetectable during childhood. However, prepubertal Sertoli cells are not functionally inactive: they express steroidogenic enzymes capable of transforming steroid precursors to testosterone and oestradiol; this activity is regulated by FSH (Welsh and Wiebe, 1976). Many other secreted or intracellular proteins are also produced by prepubertal Sertoli cells, although most of them (e.g., androgen binding protein or ABP) are synthesised in larger quantities after puberty (for review, see Griswold, 1993).

Two gonadal proteins are specifically secreted by Sertoli cells: AMH and inhibin. Although its most well-known action takes place during early foetal life, AMH is produced by Sertoli cells until puberty. AMH is a glycoprotein hormone belonging to the transforming growth factor ß (TGF-ß) superfamily, encoded by a relatively small gene spanning 2.8 kb in the human (Cate et al., 1986; Josso et al., 1993). AMH circulates in blood as a homodimer of 140 kD, which can be detected by ELISA (Lee et al., 1996; Rey, 1998). Testicular AMH production is intense during whole foetal life, infancy and childhood. Except for a transient decrease in serum AMH levels observed from the last weeks of foetal life until the 2nd or 3rd week after birth, high serum AMH levels are detected in prepubertal boys (Fig. 5). Serum AMH is a sensitive, specific and reliable marker of prepubertal Sertoli cell function. Serum AMH assay is used to seek for the presence of ectopic testes in boys with non-palpable gonads (Fig. 6) (Josso, 1995; Lee et al., 1997), but also reflects the number and functional state of Sertoli cells in children with dysgenetic testes (Fig. 7) (Rey et al., 1996). Immunohistochemical localisation of AMH expression in the supporting cells of small gonadoblastomas represents a further proof of the Sertoli cell origin of those cells (Fig. 7). AMH expression in Sertoli cells is activated by the nuclear receptor SF1 (Steroidogenic Factor 1), also known as Ad4BP, present at high levels in the nuclei of Sertoli cells until pubertal maturation (Hatano et al., 1994; Shen

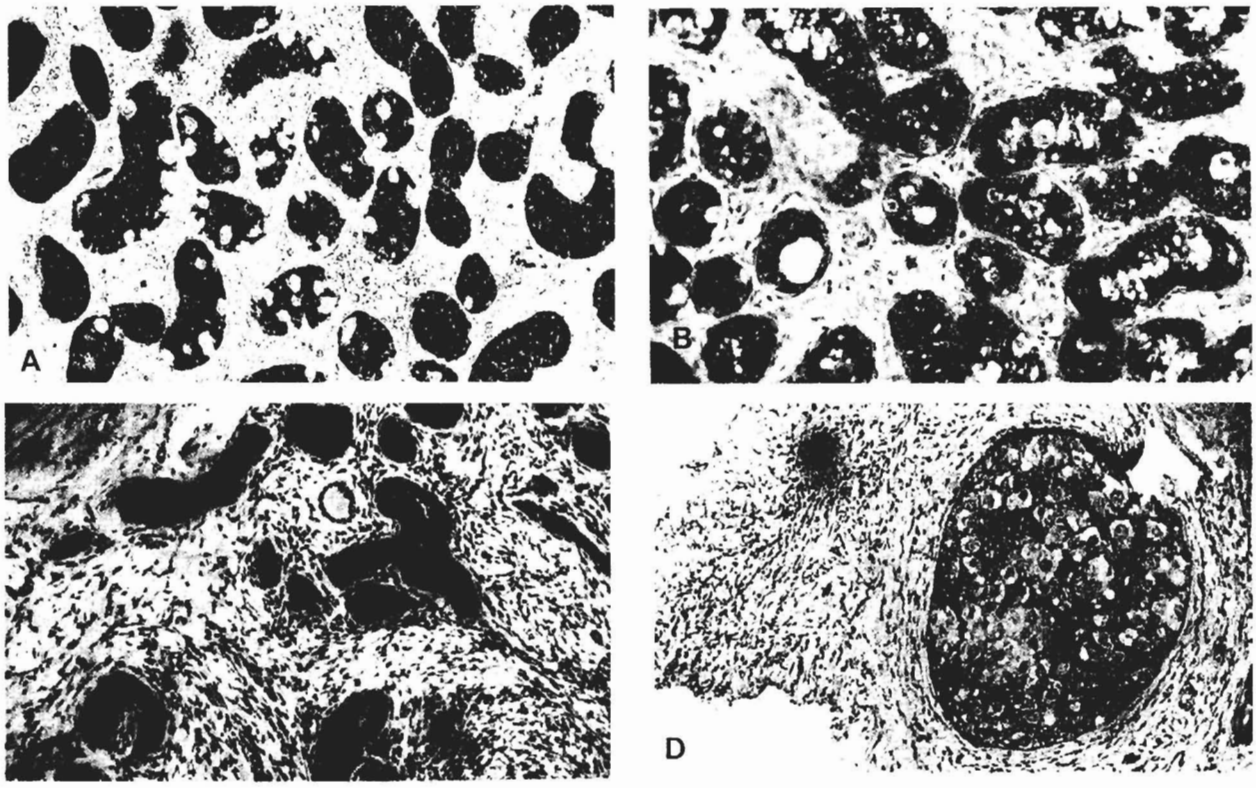

Fig. 7. Localisation of anti-Müllerian hormone (AMH) expression by immunohistochemistry using a polyclonal anti-human recombinant AMH following antigen retrieval by microwaves. **A.** Normal testis from a prepubertal boy: Sertoli cells are positive, while germ cells and interstitial tissue are negative. **B.** Small dysgenetic testis, showing ring tubules with calcareous eosinophilic substance (arrowhead). AMH immunoreactivity is positive in tubules showing dysgenetic characteristics; however due to the decrease in Sertoli cell number, reflected in small testicular volume, serum AMH is low in patients with testicular dysgenesis. **C.** Severely dysgenetic tissue from an ovotestis present in a true hermaphrodite; still, Sertoli cells produce AMH. **D.** Small gonadoblastoma; the small sex-cord derivatives are AMH-positive, confirming their Sertoli cell origin. All sections were slightly counterstained with hematoxylin. x 100. (Modified and reproduced with permission from: Rey R, Al-Attar L, Louis F, Jaubert F, Barbet P, Nihoul-Fékété C, Chaussain JL, Josso N. Testicular dysgenesis does not affect expression of anti-Müllerian hormone by Sertoli cells in pre-meiotic seminiferous tubules. American Journal of Pathology 1996; 148:1689-1698).

et al., 1994). Other Sertoli-cell specific factor(s) are also responsible for the activation of the AMH gene, since no activation of a transgene under AMH promoter control could be obtained by SF1 in HeLa cells (Shen et al., 1994).

Unlike most Sertoli cell products, AMH expression is inhibited during pubertal development, owing to the action of testosterone and meiotic germ cells (Rey et al., 1993b; Al-Attar et al., 1997). In the absence of these inhibitory effects, FSH is capable of increasing testicular AMH output (Al-Attar et al., 1997). Therefore, during the neonatal and pubertal periods when gonadotropins are elevated, serum AMH is extremely elevated in patients suffering from intersex disorders owing to abnormal testosterone secretion (e.g., Leydig cell aplasia due to LH receptor mutations, steroidogenic enzyme defects) or action (androgen insensitivity syndromes) (Fig. 8). Whether the elevation of serum AMH in these cases is due to increased Sertoli cell numbers or to an upregulation of AMH gene expression in each Sertoli cell remains to be elucidated.

AMH signals through a membrane-bound receptor with serine-threonine kinase activity (di Clemente et al., 1994; Imbeaud et al., 1995). AMH receptor is expressed in Sertoli and Leydig cells, which may suggest an autocrine/paracrine action of AMH in the prepubertal testis. Transgenic mice chronically overexpressing AMH have low serum testosterone owing to Leydig cell hypoplasia (Racine et al., 1998; Rouiller-Fabre et al., 1998), while male mice in which the AMH (Behringer et al., 1990; Lyet et al., 1995) or the AMH-receptor gene (Behringer et al., 1994; Mishina et al., 1996) has been inactivated by recombinant techniques have Leydig cell hyperplasia; a Leydig cell tumour has been reported in one animal. These observations indicate that AMH modulates Leydig cell numbers and function by controlling the differentiation of mesenchymal precursors and the expression of steroidogenic enzymes in the immature testis (Racine et al., 1998).

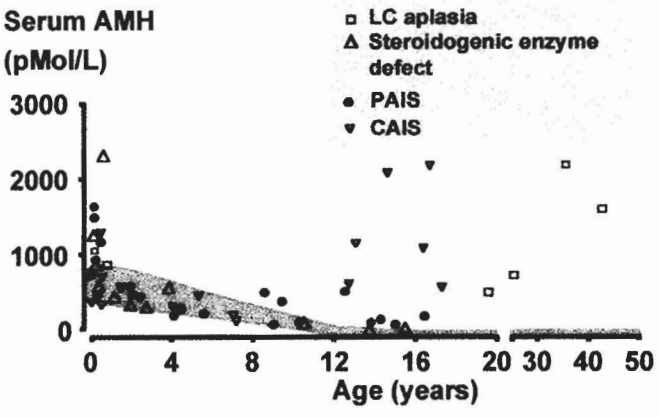

Fig. 8. Serum AMH levels in intersex patients due to abnormal androgen secretion (Leydig cell aplasia and steroidogenic enzyme defects) or action (partial or complete androgen insensitivity syndromes). Owing to the lack of androgen-negative action on AMH expression, serum AMH levels are increased in the periods of activation of the gonadotropin axis, i.e. in the neonatal and pubertal periods.

Inhibin B is another Sertoli-cell specific marker belonging to the TGF- ß superfamily (Burger and Fuller, 1996). Inhibin is a heterodimer composed by one α and one of two ß subunits, ßA (inhibin A) or ßB (inhibin B). While inhibin A is not produced by the testis, inhibin B and free α subunits are synthesised by Sertoli cells. The interpretation of testicular inhibin secretion and of its functional importance remained hampered for years owing to the lack of a reliable assay capable of specifically detecting each molecular form, but the recent development of specific and sensitive immunoassays have shown that the prepubertal testis secretes inhibin B in high levels until the age of 2-4 years (Andersson et al., 1998). This endocrine function of Sertoli cells clearly remains active longer than that of Leydig cells after birth and is never completely abolished during childhood. In boys, sustained serum inhibin B levels may play a role in FSH inactivation during prepubertal life. In rodents also, immature Sertoli cells express high levels of inhibin α mARN (Keinan et al., 1989); inhibin α is a tumour-suppressor gene with gonadal specificity as shown by gene knockout experiences (Matzuk et al., 1992, 1995).

Concluding remarks

Morphometric analysis of the testis by adequate stereological methods and functional assessment of testicular endocrine activity during infancy and childhood clearly show that there is an intense proliferation of Sertoli and germ cells, and that Sertoli cells actively produce AMH and inhibin. These apparently silent activities of the prepubertal testis are of great importance for the control of proliferation and function of other testicular cell populations, and for the establishment of adequate conditions necessary for the development of a quantitatively normal spermatogenesis and a satisfactory reproductive capacity in the adult male.

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