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Review

Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood

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Disorders of testicular function may have their origins in fetal or early life as a result of abnormal development or proliferation of Sertoli cells. Failure of Sertoli cells to mature, with consequent inability to express functions capable of supporting spermatogenesis, is a prime example. In a similar way, failure of Sertoli cells to proliferate normally at the appropriate period in life will result in reduced production of spermatozoa in adulthood. This review focuses on the control of proliferation of Sertoli cells and functional maturation, and is motivated by concerns about 'testicular dysgenesis syndrome' in humans, a collection of common disorders (testicular germ-cell cancer, cryptorchidism, hypospadias and low sperm counts) which are hypothesized to have a common origin in fetal life and to reflect abnormal function of Sertoli (and Leydig) cells. The timing of proliferation of Sertoli cells in different species is reviewed, and the factors that govern the conversion of an immature, proliferating Sertoli cell to a mature, non-proliferating cell are discussed. Protein markers of maturity and immaturity of Sertoli cells in various species are reviewed and their usefulness in studies of human testicular pathology are discussed. These markers include anti-Mullerian hormone, aromatase, cytokeratin-18, GATA-1, laminin alpha5, M2A antigen, $p27^{kip1}$, sulphated glycoprotein 2, androgen receptor and Wilms' tumour gene. A scheme is presented for characterization of Sertoli-cell only tubules in the adult testis according to whether or not there is inherent failure of maturation of Sertoli cells or in which the Sertoli cells have matured but there is absence, or acquired loss, of germ cells. Functional 'de-differentiation' of Sertoli cells is considered. It is concluded that there is considerable evidence to indicate that disorders of maturation of Sertoli cells may be a common underlying cause of human male reproductive disorders that manifest at various life stages. This recognition emphasizes the important role that animal models must play to enable identification of the mechanisms via which failure of proliferation and maturation of Sertoli cells can arise, as this failure probably occurs in fetal life.

The Sertoli cell plays a central role in development of a functional testis, and hence in the expression of a male phenotype. Sertoli cells are the first cells to differentiate recognizably in the indifferent fetal gonad, an event which enables seminiferous cord formation, prevention of germ-cell entry into meiosis and differentiation and function of the Leydig cells (Mackay, 2000). The secretions of the Leydig cells (testosterone, insulin-like factor 3) then play vital roles in downstream masculinization events and in descent of the testes into the scrotum (Hutson *et al*., 1997; Sharpe, 2001). The Sertoli cells also ensure regression of the Mullerian ducts via secretion of anti-Mullerian hormone (AMH; Mackay, 2000; Josso *et al*., 2001). When these events have occurred, and some time has passed, the role of the Sertoli cell switches during puberty to the support of spermatogenesis. Without the physical and metabolic support of the Sertoli cells, germ-cell differentiation, meiosis and transformation into spermatozoa would not occur (Sharpe, 1994). Moreover, the number of Sertoli cells will determine the number of germ cells that can be supported through spermatogenesis and hence will numerically determine the extent of sperm production (Orth *et al*., 1988; Sharpe, 1994, 1999), a factor with obvious bearing on fertility.

This brief 'job description' of the Sertoli cell emphasizes its two functionally separate roles, in the process of testis formation or sexual differentiation and in spermatogenesis. These events are separated in time and by function. Thus, the functions of the Sertoli cell expressed in adult life to support spermatogenesis are

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largely absent during fetal life (insofar as is known), and the converse is also true to some extent. The switch from 'fetal' to 'adult' Sertoli cell appears to occur during puberty and is termed 'maturation' or 'differentiation', and is likely to be a multi-step process. The term 'differentiation' applies because the Sertoli cells are terminally differentiating into their adult form, but as this term is also used to describe the morphological differentiation of the Sertoli cells in fetal life, the term 'functional maturation' will be used in this review. Maturation involves loss of proliferative ability, formation of inter-Sertoli cell tight junctions and expression of functions not present in immature Sertoli cells. Though functional maturation of Sertoli cells is temporally separate from Sertoli-cell development and function in fetal life, it is inevitable that what happens to the Sertoli cells during their 'fetal period' (which extends out into neonatal life) will to some extent predetermine their function or behaviour in adulthood. It is the connections between fetal and adult functions of the Sertoli cells and the consequences and manifestation of failure of proliferation or 'functional maturation' of Sertoli cells that are the focus of this review. As this is a broad area, reviews have been cited wherever possible to provide sources of more detailed information.

Testicular dysgenesis syndrome

The motivation for this review is the hypothesis that, in humans, testicular germ-cell cancer, cryptorchidism, hypospadias and some cases of low sperm counts form a syndrome of disorders, testicular dysgenesis syndrome (TDS), with a common origin in fetal life (Skakkebaek *et al*., 2001). These disorders are risk factors for each other and share several pregnancy-related risk factors and, from a cell biology point of view, a strong case can be made for their common origin (Sharpe and Skakkebaek, in press). It is proposed that TDS results from abnormal function of Sertoli and Leydig cells in fetal life with either immediate (testis descent, masculinization) or delayed (testis cancer, low sperm counts) consequences. Analysis of biopsies of the contralateral (tumour-free) testis in patients with testis cancer has revealed a high prevalence of abnormal focal features such as immature Sertoli cells, poorly formed seminiferous tubules and Sertoli cell-only (SCO) tubules (Hoei-Hanson *et al*., in press). Such analyses raise many questions. For example, what should be concluded when an SCO tubule is observed in the adult testis? Does it indicate that the Sertoli cells did not develop normally so that germ cells could not survive? Or is the function of the Sertoli cells normal, but the SCO has arisen because of an inherent problem with the germ cells or because of their postnatal loss (for example, due to infection, orchitis, trauma, irradiation, chemotherapy, heat exposure)? Again, considering the vital role that the number of Sertoli cells plays in determining sperm production or sperm counts in adulthood (see below), are there factors acting in fetal life that may affect proliferation of Sertoli cells at this time or alter the ability of Sertoli cells to proliferate postnatally? These issues are addressed in this review, which focuses on what is known about the factors that can influence the proliferation and functional maturation of Sertoli cells.

Proliferation of Sertoli cells

The number of Sertoli cells in the adult testis determines both testis size and daily sperm production. This relationship occurs because each Sertoli cell has a fixed capacity for the number of germ cells that it can support (Orth *et al*., 1988), though this capacity varies between species (Sharpe, 1994). Only immature Sertoli cells proliferate, so the final number of Sertoli cells is determined before adulthood. At face value, there appear to be fundamental differences between species as to when Sertoli cells proliferate, because in rodents all proliferation occurs in fetal and neonatal life, whereas in rhesus monkeys proliferation occurs predominantly in the peripubertal period (Fig. 1). However, as more data is obtained from more species, a consensus interpretation is now possible (see, for example, Plant and Marshall, 2001). This indicates that Sertoli cells proliferate during two periods of life, in fetal or neonatal life and in the peripubertal period in all species. In some species (for example rhesus monkeys), one period may be far more important than the other (Plant and Marshall, 2001), whereas in most species proliferation of Sertoli cells occurs in both periods, although the fetal or neonatal period is probably the more important (Fig. 1). Confusion has arisen because in species such as the rat, in which the neonatal period overlaps with the peripubertal period, it is difficult to discern the two periods of proliferation of Sertoli cells. In contrast, in humans, these periods are separated by a decade or more and in lower primates by periods of months (Fig. 1). Intermediate between the extremes of rodents and primates are species such as the bull and pig, in which there is a short gap (weeks) between the neonatal and peripubertal periods, but in which two corresponding peaks of proliferation of Sertoli cells can be discerned (De Franca *et al*., 2000).

Arguably, the most puzzling observation is why rhesus monkeys, but not humans or marmosets (or Cebus monkeys), exhibit such a low proliferation of Sertoli cells during the neonatal period. Another curious finding is that in normal marmoset monkeys, adult numbers of Sertoli cells are present by the end of the neonatal period, implying that peripubertal proliferation does not occur in this species (Fig. 1). However, it is clear that Sertoli cells can proliferate after the neonatal period in marmoset monkeys. In a study in which proliferation of Sertoli cells was inhibited by neonatal treatment with a GnRH antagonist (to suppress FSH concentrations), a reduction of about 35% in the number of Sertoli cells was evident at the end of the neonatal period.

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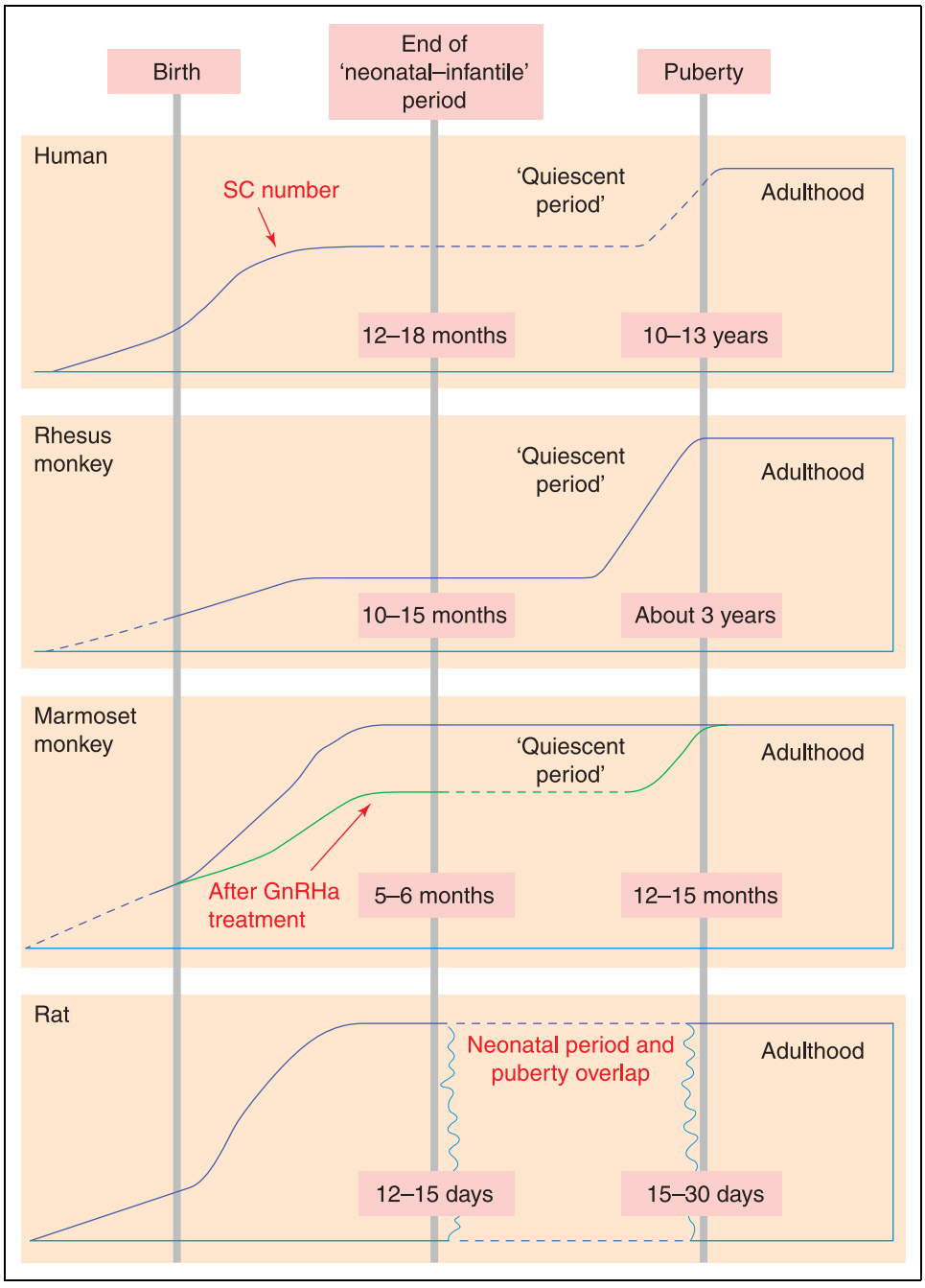

Fig. 1. Schematic diagram illustrating timing of proliferation of Sertoli cells (SC) in humans, rhesus and marmoset monkeys, and rats in relation to birth, the neonatal period and puberty. The dark blue line indicates the change in relative number of Sertoli cells for that species, based on reported data (references below); where there are data gaps, a dashed blue line is shown. Note that in rats, the end of the neonatal period and the onset of puberty overlap so that adult numbers of Sertoli cells are already present by the end of the 'neonatal' period. The green line shown in the panel for the marmoset indicates what happens to the number of Sertoli cells in animals that are treated from birth to the end of the neonatal period with a GnRH antagonist to suppress pituitary gonadotrophin secretion. This diagram is based mainly on data from: Cortes *et al*. (1987); Wang *et al*. (1989); Marshall and Plant (1996); Mann *et al*. (1997) and Sharpe *et al*. (2000).

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Table 1. Effect of various neonatal hormonal manipulations in rats on final number of Sertoli cells, testis mass and blood concentrations of inhibin B

Neonatal treatment	Percentage of appropriate control			Reference(s)
	Number of Sertoli cells	Testis mass	Blood concentration of inhibin B[a]	
GnRH antagonist (suppression of FSH)	45–52%	46%	63–79%	Atanassova *et al*., 1999 Sharpe *et al*., 1999
Treatment with recombinant FSH	149%	124%	nm	Meachem *et al*., 1996
Hemicastration (raises FSH concentrations)	118%	133%	nm	Simorangkir *et al*., 1995
Hypothyroidism	182–257%	127–162%	215%	Hess *et al*., 1993 Simorangkir *et al*., 1995 Sharpe *et al*., 1999
Hemicastration + hypothyroidism	123%	162%	nm	Simorangkir *et al*., 1995
Polychlorinated biphenyls (induces mild hypothyroidism)	132.6–139.4%	114.8–116.5%	nm	Kim, 2001
Hyperthyroidism	50%	52%	nm	van Haaster *et al*., 1993
Diethylstilboestrol (suppresses FSH)	64%	49%	31%	Atanassova *et al*., 1999

[a]Note that FSH concentrations in adulthood usually show the converse change to that indicated for inhibin B.
nm: not measured.

However, when comparably treated males were allowed to grow to adulthood they had restored normal numbers of Sertoli cells (Sharpe *et al*., 2000; Fig. 1); it is presumed that the compensatory proliferation occurred during the peripubertal period.

Hormonal and other factors influencing final number of Sertoli cells

As the number of Sertoli cells determines the number of spermatozoa produced per day, it is important that the correct number of Sertoli cells is generated. The factors that determine the number of Sertoli cells must be partly genetic, although the relevant genes have yet to be identified, apart from the fragile X gene, FMR-1 (Slegtenhorst-Eegdeman *et al*., 1998; Sharpe, 1999).

Hormones are important, in particular FSH and thyroid hormones, but also growth hormone and various paracrine growth factors (Sharpe, 1994, 1999), and even LH or testosterone may play a role in rhesus monkeys (Ramaswamy *et al*., 2000). FSH increases the rate of proliferation of Sertoli cells. In contrast, thyroid hormones alter the period in which proliferation can occur by regulating maturation of Sertoli cells. How thyroid hormones produce this effect is unclear, but it may involve effects on the size of Sertoli cells (and therefore their density in the epithelium). This interpretation is based primarily on experimental data from rats, and not all aspects may be applicable to other species. The experimental manipulations (in neonatal life) that have been undertaken in rats are shown (Table 1). These illustrate that the neonatal concentration of FSH is important, as its suppression reduces the final number of Sertoli cells by about 40%, whereas experimental increase of FSH by injection or by neonatal hemicastration increases the number of Sertoli cells by 18–49%. Induction of neonatal hypothyroidism has considerably larger effects, increasing the final number of Sertoli cells by 82–157%, whereas induction of neonatal hyperthyroidism reduces the final number of Sertoli cells by about 50%. The last two treatments may induce their effects by delaying (Hess *et al*., 1993; De Franca *et al*., 1995) or advancing (van Haaster *et al*., 1993), respectively, the age at which cessation of proliferation of Sertoli cells (= maturation) occurs. Whether thyroid hormones play a similar role in all species is uncertain, but untreated, juvenile hypothyroidism in humans can be associated with precocious and permanent testicular enlargement (Jannini *et al*., 1995; Sharpe, 1999).

Non-hormonal factors (for example growth factors) are also presumed to play a physiological role in proliferation or function of Sertoli cells, and effects of numerous growth factors have been described *in vitro*, and some *in vivo* (see for example Jégou 1992; Jégou and Sharpe, 1993); space precludes discussion of such effects in this review. Such studies also indicate that cell-contact inhibition (confluency) may play a role in cessation of proliferation of Sertoli cells, as is the case generally for epithelial cells (Schlatt *et al*., 1996).

Variation in number of Sertoli cells

Differences in testis size among species and within strains are explicable by differences in the number of Sertoli cells in animal studies (Sharpe, 1994, 1999; McCoard *et al*., 2001). Testis size and sperm count in the ejaculate always show a clear linear relationship in humans (see, for example, Bujan *et al*., 1989; Lenz *et al*.,

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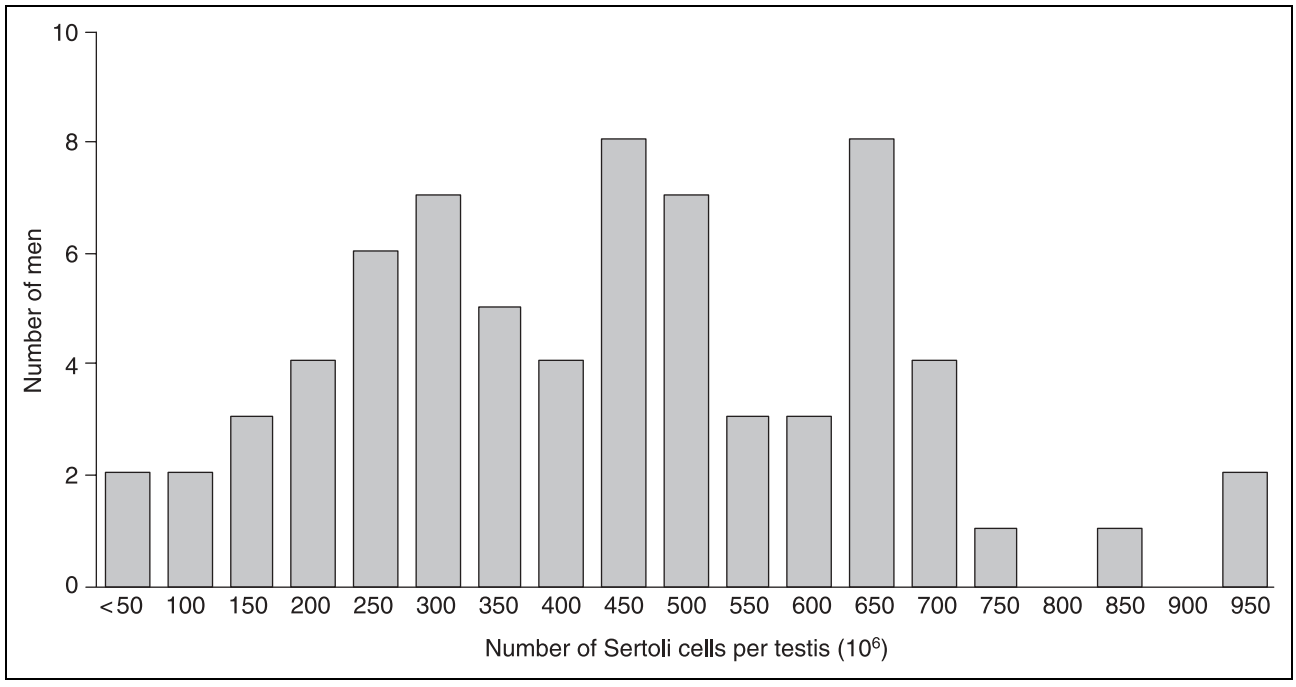

Fig. 2. Variation in number of Sertoli cells in the testes of adult men. The data have been extracted from a study by Johnson *et al*. (1984) of men who died suddenly from a variety of causes.

1993). As also shown in these studies, testicular size and sperm count show wide variation among men, and one interpretation is that variation in the number of Sertoli cells accounts for this (Sharpe, 1999; Sharpe and Franks, 2002). This interpretation is supported by the only study (Johnson *et al*., 1984) that has directly measured the number of Sertoli cells in the adult human testis and equated it to daily sperm production (based on germ-cell counts in the testis). This study showed a linear relationship between the number of Sertoli cells and daily sperm production, but also considerable variation in the number of Sertoli cells among men (Fig. 2). Other than evidence for a modest loss of Sertoli cells with ageing (Johnson *et al*., 1984), this variation was unexplained, but is presumed to result from events in fetal, neonatal or peripubertal life (Sharpe, 1999). Other evidence of high variation in the number of Sertoli cells is the equally high variation in blood concentrations of inhibin B in otherwise normal men (see, for example, Jensen *et al*., 1997). Although secondary absence of germ cells can influence inhibin B concentrations (see below), high variation in blood concentrations of inhibin B has also been reported neonatally in humans (Andersson *et al*., 1998) when Sertoli cells are proliferating.

Storgaard *et al*. (in press) highlight some of these relationships. This study has shown that smoking of >10 cigarettes per day by the mother during pregnancy results in a 30–48% reduction in sperm counts and testis size in adulthood in the exposed male with a corresponding 24% decrease in blood concentrations of inhibin B; other data confirm this work (Sharpe and Franks, 2002). On the basis of the change in inhibin B concentrations, and no evidence for any change in the morphology or motility of the spermatozoa according to exposure, Storgaard *et al*. (in press) concluded tentatively that the effect of maternal smoking probably reflected a reduction in the number of Sertoli cells. However, if the number of Sertoli cells was reduced in fetal life, it is unclear why compensatory adjustment did not take place neonatally or peripubertally, as these are important times for proliferation of Sertoli cells in humans (Fig. 1; Cortes *et al*., 1987). The mechanism via which maternal smoking affects proliferation of Sertoli cells in the fetus remains to be identified.

Recent studies have shown that *in utero* exposure of rats to certain phthalate esters can induce a spectrum of disorders similar to TDS (reviewed in Sharpe, 2001). These include reduced testis mass in adulthood (Mylchreest *et al*., 1998), and neonatal treatment with similar phthalates has been shown to reduce proliferation of Sertoli cells (Dostal *et al*., 1988; Li *et al*., 2000). The present authors' studies indicate that exposure *in utero* to phthalates may permanently arrest some Sertoli cells in an immature, fetal state, incapable of supporting spermatogenesis (Fisher *et al*., 2003). Though the effects of phthalates in rats have involved doses considerably higher than the documented exposure concentrations for humans (Sharpe, 2001), concerns remain that some individuals (mainly women of reproductive age) may have unusually high exposure to phthalates

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(Blount *et al*., 2000). The mechanisms via which phthalates affect development of Sertoli cells remain to be identified.

Functional maturation of Sertoli cells

At around the onset of puberty, Sertoli cells undergo a radical change in their morphology and function, heralding the switch from an immature, proliferative state to a mature, non-proliferative state. The nucleus enlarges and becomes tripartite and the nucleolus becomes more prominent (Fig. 3). Adjacent Sertoli cells form tight junctions with each other to create a unique adluminal compartment in which meiotic and post-meiotic steps of spermatogenesis can proceed, as well as allowing formation of a fluid-filled lumen. As a result, the germ cells developing in the adluminal compartment become effectively sealed off from direct access to many nutrients and thus become dependent on the secretion of such factors by the Sertoli cells. Accordingly, the Sertoli cell switches on these functions and they become a distinguishing feature of the 'mature' Sertoli cell (see Jégou 1992; McLaren *et al*., 1993; Sharpe, 1994). The expectation would be that many different functions would be expressed by mature adult Sertoli cells when compared with fetal, proliferating Sertoli cells, but there is a surprising lack of definitive comparative studies.

Whether maturation of Sertoli cells involves a rapid switch from an immature to a mature state or involves a step-wise cascade of changes that may occur over a period of time, is not entirely clear. Though some immature functions of Sertoli cells (for example, aromatase expression) show a clear switch at puberty, others (for example, AMH) do not show such a sharp demarcation (see below). Irrespective of this debate, analysis of the expression of maturational markers of Sertoli cells can be potentially informative, especially in patients in whom disorders of sexual differentiation or testicular development are suspected. Maturational markers that have been studied are discussed below.

Factors affecting maturation of Sertoli cells

Before considering the protein markers of maturation of Sertoli cells, a key question to address is what induces maturation of Sertoli cells. As already mentioned, thyroid hormone (T_3) plays a role in maturation of Sertoli cells in laboratory animals, and though it may play a role in other species, other factors may be involved. For example, in primates with a considerable delay to puberty, it seems likely that FSH, and particularly androgens, may play a role in final maturation of Sertoli cells as patients with complete androgen insensitivity syndrome (AIS) usually exhibit Sertoli cells that show various features of immaturity, such as persistence of AMH expression (Rey *et al*., 1994, 1999; Rajpert-de Meyts *et al*., 1999). An obvious candidate for induction of maturation of Sertoli cells is the influence of newly differentiating germ cells. However, it is evident from both animal models and human patients that absence of germ cells does not result *de facto* in failure of maturation of Sertoli cells, though there may be a delay, such as in the formation of inter-Sertoli cell tight junctions (Means *et al*., 1976). Changes in secretory function of Sertoli cells secondary to the absence of particular types of germ cell can also occur (Jégou and Sharpe, 1993; Sharpe *et al*., 1993; Boujrad *et al*., 1995; Guitton *et al*., 1999; Fig. 3), and others have claimed that absence of germ cells can result in de-differentiation of Sertoli cells, such that functions of immature Sertoli cells are 'switched back on' (see below). However, it is important to recognize that absence of germ cells may also be a reflection of underlying abnormalities in the Sertoli cells, such as failure of their maturation (Fig. 3). The fact that the latter phenotype can occur in focal areas intermixed with other 'types' of seminiferous tubule (see below and Steger *et al*., 1996; Maymon *et al*., 2000) can create obvious problems with interpretation.

Although the evidence from laboratory animals indicates an important role for T_3 in maturation of Sertoli cells, the most likely scenario is that T_3 interacts with androgens, and perhaps FSH, to bring about final maturation of Sertoli cells, and that germ cells then exert modulating effects on these mature Sertoli cells. This implies a step-wise process of maturation. For example, *in vitro* studies in rats have shown that FSH and T_3 both induce androgen receptor (AR) expression in immature Sertoli cells (Arambepola *et al*., 1998a) and suppress AMH expression (Arambepola *et al*., 1998b), and the effects of FSH and T_3 are additive. It is therefore envisaged that, in rats, rising FSH concentrations, which trigger proliferation of Sertoli cells, induce progressively increasing expression of AR, an effect that is upregulated by increasing effects of T_3 which will ultimately lead to functional maturation of the Sertoli cells and consequent loss of AMH expression. Changing sensitivity of Sertoli cells to T_3 during their proliferative phase, due to altered expression of T_3 receptors (Buzzard *et al*., 2000), may be another contributing factor. However, this scenario may differ among species. Thus, in marmosets and humans there is no, or only weak, AR expression in Sertoli cells in the neonatal period despite high FSH concentrations (see, for example, Andersson *et al*., 1998) that are driving proliferation of Sertoli cells (Cortes *et al*., 1987; McKinnell *et al*., 2001). Thereafter, AR expression switches on in marmosets during infancy when FSH or testosterone concentrations are baseline and the Sertoli cells are clearly immature (McKinnell *et al*., 2001; Kelnar *et al*., 2002; see Figs 1 and 4). It is speculated that absence of sufficient T_3, or more likely the absence of T_3 receptors in the Sertoli cell, prevent induction of AR during the neonatal period in marmosets and possibly in humans (Jannini *et al*., 2000).

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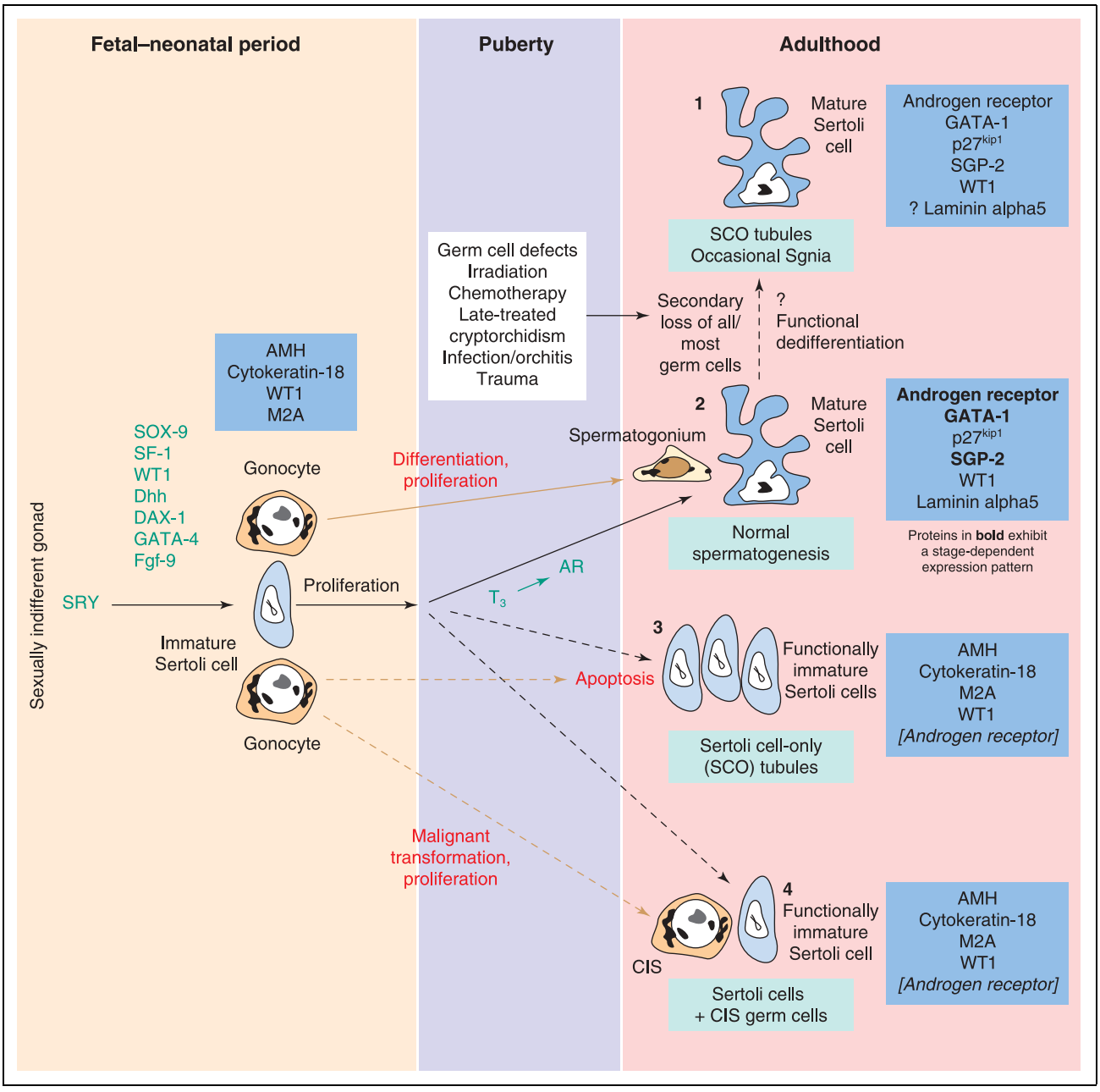

Fig. 3. Schematic diagram illustrating normal (and abnormal) maturational development of Sertoli cells from fetal life through puberty to adulthood, and the associated changes in expression of protein markers (blue boxes) of maturity or immaturity. The ability of Sertoli cells to support germ cells is also indicated. Note that only one of the scenarios in adulthood is normal (2), the other three (1,3,4) represent conditions in which only isolated spermatogonia are present or tubules are Sertoli-cell only (SCO; 1), or in which transformed fetal germ cells (carcinoma-*in situ*, CIS) are present (4). Testes exhibiting germ-cell arrest are not illustrated but would fall into categories 1 and 2. The main purpose of the diagram is to distinguish adult phenotypes in which Sertoli cells have failed to mature (3,4) from those in which the Sertoli cells have matured (1,2); the possibility of functional 'de-differentiation' of Sertoli cells, following germ-cell loss is covered by category 1. Factors in green are those that play a role in initial differentiation or final maturation of Sertoli cells. Where factors in boxes are shown in parentheses, this indicates a degree of ambiguity or uncertainty, which is discussed in the text. SGP-2: sulphated glycoprotein 2; WT1: Wilms' tumour gene; AMH: anti-Mullerian hormone; T_3: thyroid hormone; AR: androgen receptor.

AR expression in Sertoli cells in humans is probably a late event in puberty (A. Waring, H. Wallace, C. Kelnar and R. Sharpe, unpublished). The rising concentrations of FSH and testosterone at puberty, coincident with AR expression in Sertoli cells, might enable their final maturation.

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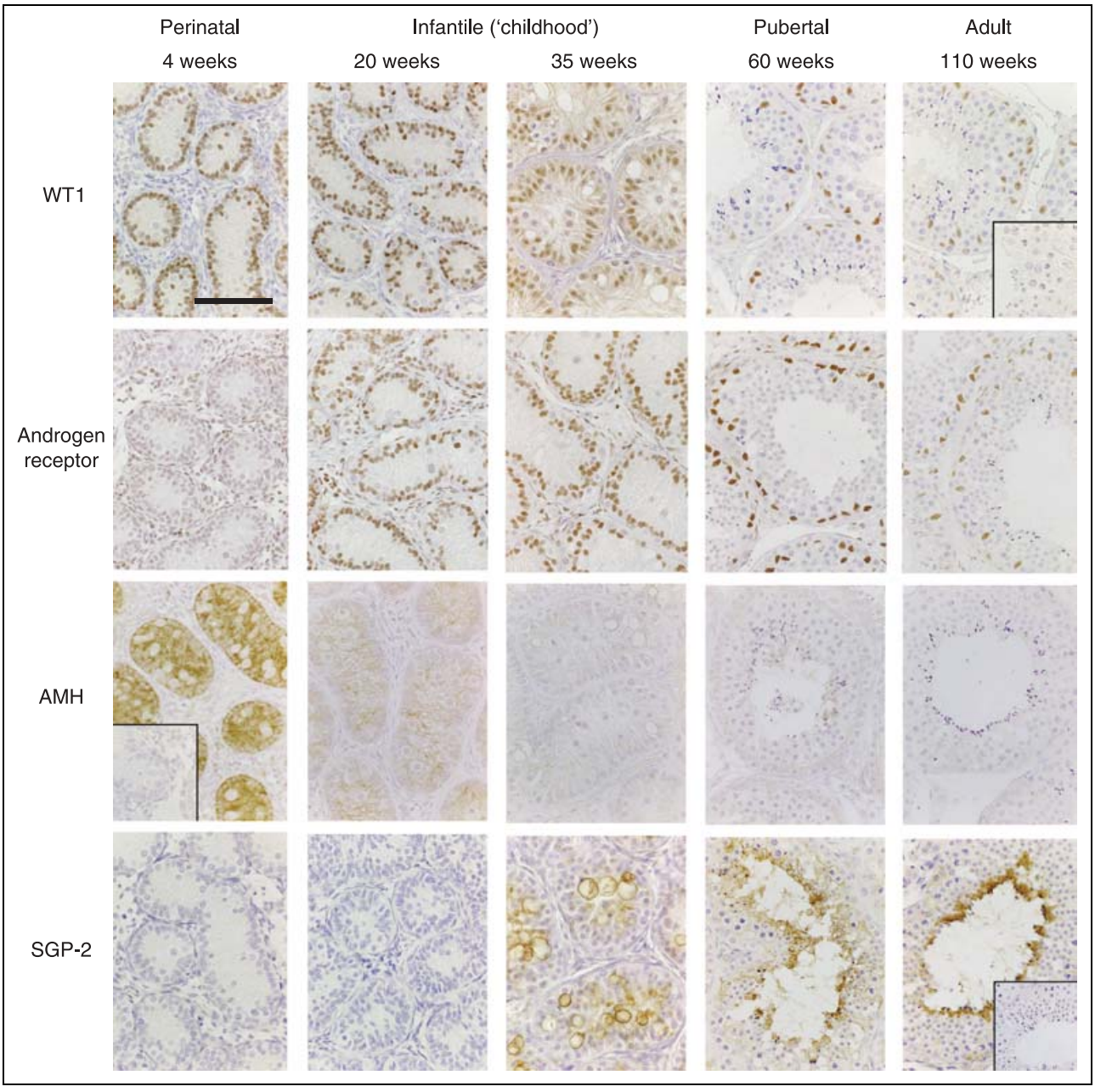

Fig. 4. Changes in the immunoexpression of four protein markers in Sertoli cells from birth to adulthood in marmosets, to illustrate the maturation-dependent patterns of expression. Nuclear immunoexpression of the Wilms' tumour gene (WT1) is evident in Sertoli cells at all ages and shows no obvious maturation-dependent changes. In contrast, nuclear immunoexpression of the androgen receptor is absent in the neonatal period but is apparent from about 20 weeks of age onwards with increased intensity of immunoexpression from 20 to 60 weeks of age. Cytoplasmic immunoexpression of anti-Mullerian hormone (AMH) in Sertoli cells is intense in neonatal life but expression diminishes gradually thereafter and is virtually absent from puberty onwards. In contrast, cytoplasmic immunoexpression of sulphated glycoprotein 2 (SGP-2) is absent until 35 weeks of age and later (note increased intensity of expression at later ages). Insets show negative controls in which the primary antibody was omitted or pre-absorbed by an excess of the immunizing peptide. Scale bar represents 100 μm.

From the information available, it seems most probable that maturation of Sertoli cells is a multi-step process or cascade, and that the precise timing of the different steps may vary according to species. Intrinsic to this explanation is the likelihood that disorders of maturation of Sertoli cells may occur at different steps and that failure to undergo an early step may prevent or interfere with future steps. This might explain observations such as

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the mixed patterns of Sertoli cell AR expression seen in SCO tubules in humans (especially in cryptorchid testes), in which Sertoli cells exhibiting immature nuclear morphology and not expressing AR may be intermixed with Sertoli cells with partly differentiated nuclei that express the AR weakly (Regadera *et al*., 2001).

Protein markers of number, function or maturational status of Sertoli cells

Although sperm count may provide a gross indication of function of Sertoli cells, it does not enable easy interpretation of whether there is an underlying deficit in the number, function or maturation of Sertoli cells. Measurement of blood concentrations of inhibin B has shown promise in this regard, as in humans, monkeys and rats it appears to be a specific product of Sertoli cells (reviewed in Anderson and Sharpe, 2000). Various studies have shown that in humans and rats, inhibin B concentrations show their biggest and most rapid increase during the neonatal period (Andersson *et al*., 1998; Sharpe *et al*., 1999), when the number of Sertoli cells is increasing (Fig. 1), whereas in rhesus monkeys there is only a modest increase in inhibin B concentrations neonatally but a very pronounced increase peripubertally (Winters and Plant, 1999), when proliferation of Sertoli cells mainly occurs in this species (Fig. 1). In both rats (Sharpe *et al*., 1999) and rhesus monkeys (Ramaswamy *et al*., 1999), experimental manipulation of the number of Sertoli cells is matched by a corresponding change in concentrations of inhibin B. Blood concentrations of inhibin B are extremely variable in adult men (see, for example, Jensen *et al*., 1997), and though this may reflect variation in the number of Sertoli cells, as described above, it is apparent that other factors are also influential. In particular, the status of spermatogenesis is important, and loss of particular types of germ cell can lead to a marked reduction in blood concentrations of inhibin B (Foppiani *et al*., 1999; Anderson and Sharpe, 2000). Therefore, though a positive relationship between sperm counts and blood inhibin B concentrations in adult men is evident at the population level (see, for example, Jensen *et al*., 1997; Pierik *et al*., 1998), consistent with this reflecting differences in the number of Sertoli cells, it is not possible to interpolate confidently the number of Sertoli cells from the inhibin B concentrations in individual men.

Men with oligozoospermia may undergo testicular biopsy, and this affords the opportunity to investigate directly the functional or maturational status of the Sertoli cells. Expression of certain proteins is clearly associated with immaturity or maturity of the Sertoli cell, and study of the immunoexpression of such markers can potentially aid interpretation of various seminiferous tubule phenotypes that may present. Such studies might indicate whether there has been a fundamental failure of maturation of Sertoli cells (Fig. 3). If maturation of Sertoli cells occurs as a cascade, better understanding of when various protein markers are expressed in relation to acquisition of maturation of Sertoli cells may provide pointers as to when the maturation process failed. At present there is a lack of sufficient detailed understanding to use the available information in this way. Immunoexpression in Sertoli cells of four of the protein markers described below is shown (Fig. 4), as exhibited by marmosets, which show similar developmental phases to humans, but measured in months rather than years (Kelnar *et al*., 2002). This illustrates how different proteins may signal the maturational status of the Sertoli cell. Data for immunoexpression of WT1 and $p27^{Kip1}$ in rats are shown (Fig. 5) for the same purpose.

Wilms' tumour gene (WT1)

The transcription factor WT1 is switched on in Sertoli cells early in fetal life (Mackay, 2000). Thereafter, it continues to be expressed in Sertoli cells, localized primarily to the nucleus, throughout all phases of life (Figs 4 and 5). It provides a stable marker of Sertoli cells, against which other protein markers can be compared.

Anti-Mullerian hormone (AMH)

AMH is one of the first genes to be switched on in Sertoli cells after their differentiation in fetal life (Mackay, 2000; Josso *et al*., 2001), and expression continues while Sertoli cells remain immature (Fig. 4). At puberty, AMH expression in Sertoli cells and its secretion into the bloodstream is severely downregulated, coincident with the appearance of meiotic germ cells and androgen sensitivity of the Sertoli cell, and also with final maturation of the Sertoli cells (Rey *et al*., 1994, 1999; Rajpert-de Meyts *et al*., 1999). Absence of germ cells due to prepubertal testicular irradiation does not prevent downregulation of AMH at puberty (Rajpert-de Meyts *et al*., 1999). Persistence of high AMH expression in Sertoli cells or high concentrations in blood in adulthood may indicate failure of maturation of Sertoli cells (Fig. 3), though it can also reflect deficiencies in androgen action (see below).

Aromatase

This P450 enzyme is expressed in fetal or neonatal Sertoli cells but expression is downregulated during maturation (Palmero *et al*., 1995; Panno *et al*., 1995), such that in the adult rat testis, aromatase is expressed mainly in Leydig cells and in certain germ cells (Carreau *et al*., 1999; Turner *et al*., 2002). Together with other markers of immature Sertoli cells in rats, aromatase expression is downregulated by exposure to T_3 (see, for example, Ulisse *et al*., 1994; Palmero *et al*., 1995; Ando *et al*., 2001). It is uncertain whether downregulation of aromatase, as a feature of maturation of Sertoli cells,

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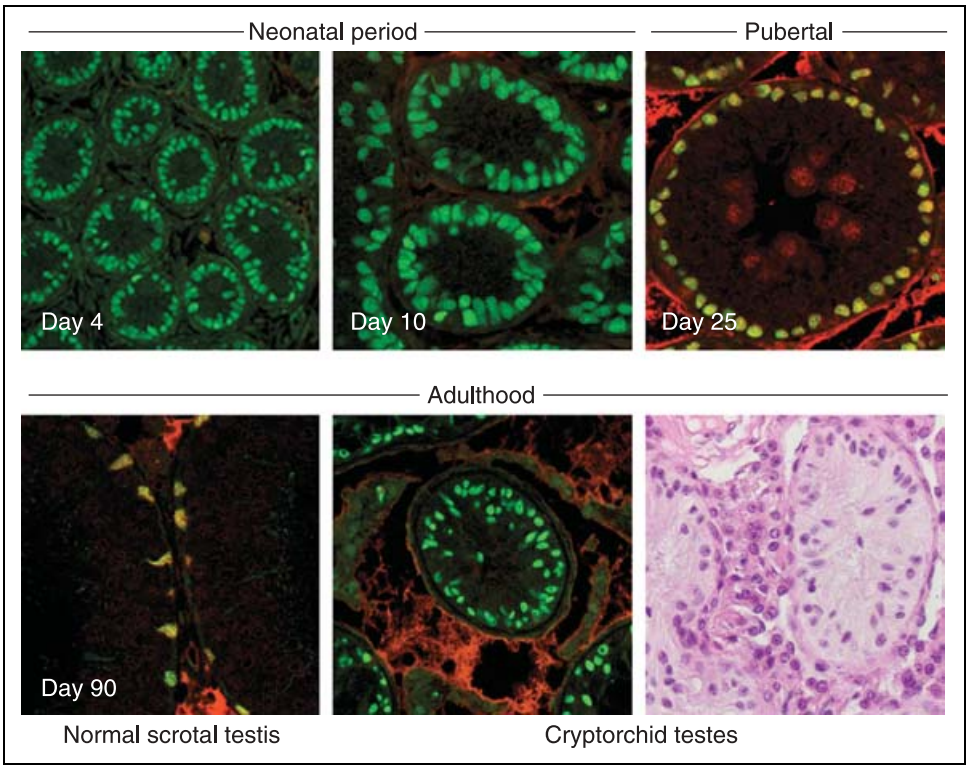

Fig. 5. Maturation-dependent change in Sertoli-cell nuclear protein expression in rats, on the basis of co-immunoexpression of $p27^{kip1}$ by Sertoli cells and the constant Sertoli-cell marker, WT1 (Wilms' tumour gene). Figures shown are confocal images with green nuclear fluorescence indicating nuclear immunoexpression of WT1 alone and yellow nuclear fluorescence indicating nuclear immunoexpression of both WT1 and $p27^{kip1}$. Note that no cells exhibit red-only nuclear fluorescence (that is, immunoexpression of $p27^{kip1}$ but not WT1), though there is some background red fluorescence. Note that co-expression of $p27^{kip1}$ with WT1 is only evident beyond 25 days of age. Also depicted is the lack of $p27^{kip1}$ immunoexpression (indicative of failure of normal maturation) in nuclei of Sertoli cells in a Sertoli cell-only (SCO) tubule from the cryptorchid testis of an adult rat that had been exposed *in utero* to dibutyl phthalate (see Fisher *et al*., 2003). The morphological appearance of such SCO tubules, in which maturation of Sertoli cells has not occurred, is shown in the lower right-hand panel.

is specific to the rat or occurs in most species; this uncertainty stems from the low protein expression of aromatase in the testis (Turner *et al*., 2002). The role of oestrogens produced by immature Sertoli cells is not understood, but it is remarkable that knockout of the genes for either aromatase or for oestrogen receptors α and β can result in the latent appearance of 'Sertoli-like' cells in the ovaries of females (Couse *et al*., 1999; Brit *et al*., 2002). This finding implies a role for oestrogens in maintenance of granulosa-cell or Sertoli-cell differentiation, and maturational loss of aromatase expression in Sertoli cells may be important for this reason.

Neural cell adhesion molecule (NCAM)

NCAM is expressed by fetal or immature Sertoli cells and is probably important for gonocyte adhesion during their intratubular migration to the basement membrane (Laslett *et al*., 2000; Orth *et al*., 2000). Expression of NCAM is downregulated in the rat during maturation of Sertoli cells (Orth and Jester, 1995; Orth *et al*., 2000) and can be downregulated experimentally by administration of T_3 (Laslett *et al*., 2000), consistent with this interpretation. It is unknown if failure of maturation of Sertoli cells results in persistence of NCAM expression, but if this does occur it might determine whether fetal germ cells and carcinoma *in situ* (CIS) cells could survive without differentiating into spermatogonia (Fig. 3).

Cytokeratin 18

Cytokeratin 18, a marker of intermediate filaments, is expressed in epithelial cells, including Sertoli cells. In humans, cytokeratin 18 is a robust marker of immature Sertoli cells and in adult testes it has been used to

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identify seminiferous tubules in which the Sertoli cells have not matured (Stosiek *et al*., 1990; Steger *et al*., 1996, 1999; Maymon *et al*., 2000), though this has also been indicated as reflecting de-differentiation of mature Sertoli cells (Kliesch *et al*., 1998; Steger *et al*., 1999).

Laminin alpha5

Deposition of a basement membrane is an integral feature of seminiferous cord formation and the component parts may change during maturation and reflect the status of Sertoli cells. One such factor is laminin alpha5, the expression of which is inversely related to that of AMH in the rat (Pelliniemi and Frojdman, 2001). It is unknown whether failure of maturation of Sertoli cells results in absence of laminin alpha5 expression.

GATA-1

GATA-1 is a transcription factor that is first expressed in Sertoli cells in mice as they are maturing, during the first wave of spermatogenesis (Yomogida *et al*., 1994). Initially, GATA-1 is immunoexpressed by all Sertoli cells but, as spermatogenesis expands, its expression becomes stage-dependent, and absence of germ cells in various mutant mice strains results in uniform Sertoli-cell expression of GATA-1 (Yomogida *et al*., 1994). GATA-1 is therefore a protein marker of mature Sertoli cells, but with secondary modulation by later types of germ cell (see also Jégou and Sharpe, 1993). Expression of GATA-1 and AMH are inversely related during testis development and GATA-1 may switch off AMH expression during maturation of Sertoli cells (Beau *et al*., 2000), and may upregulate expression of other Sertoli cell genes (Feng *et al*., 2000). It is not known whether failure of maturation of Sertoli cells results in absence of GATA-1 expression.

p27^{Kip1}

p27^{Kip1}, a cyclin-dependent kinase inhibitor, plays a role in the cell cycle; its expression is associated normally with inhibition of proliferation. Accordingly, immunoexpression of p27^{Kip1}coincides with maturation of Sertoli cells in mice (Beumer *et al*., 1999; Cipriano *et al*., 2001), rats (Fig. 5) and humans (Beumer *et al*., 1999). Failure of maturation of Sertoli cells in rats results in absence of p27^{Kip1} expression, in contrast to the continued expression of WT1 (Fig. 5).

M2A antigen

M2A is an unidentified antigen that is recognized by a monoclonal antibody. M2A is expressed only by immature and not mature Sertoli cells in humans (Baumal *et al*., 1989), and its expression may persist in patients in whom disorders of spermatogenesis are present (Steger *et al*.,1999; Blagosklonova *et al*., 2002).

Androgen receptor (AR)

Nuclear immunoexpression of AR is a feature of mature Sertoli cells, though expression varies according to the stage of the spermatogenic cycle in rats (Bremner *et al*., 1994) and humans (Suarez-Quian *et al*., 1999). However, AR immunoexpression does not coincide exactly with maturation of Sertoli cells in any of the species studied, but first appears before final maturation of Sertoli cells, in which it may play a role. In fetal and early neonatal life, Sertoli cells do not express AR in rats, humans or marmoset monkeys (Fig. 4; Williams *et al*., 2001). In rats, Sertoli cell nuclear AR immunoexpression becomes evident at age 4–8 days (that is, before cessation of proliferation of Sertoli cells; Bremner *et al*., 1994), whereas in the marmoset monkey it occurs at the end of the neonatal period (Fig. 4) when Sertoli cells are still immature (Fig. 4; Kelnar *et al*., 2002), and in humans it probably does not occur until close to the onset of puberty (A. Waring, H. Wallace, C. Kelnar and R. Sharpe, unpublished). Failure of maturation of Sertoli cells in humans, based on nuclear and general morphology, can be associated with absent or weak AR expression (Regadera *et al*., 2001); weak expression might be indicative of maturational failure at a later step than in Sertoli cells that show no expression of AR.

Persistence of immature Sertoli cells in testes with disorders of spermatogenesis

Failure of a Sertoli cell to mature functionally will presumably render it incapable of supporting the survival and development of the various germ cells that appear after puberty. However, immature (fetal) Sertoli cells are clearly capable of supporting fetal germ cells (primordial germ cells, gonocytes) and immature, neonatal or infantile Sertoli cells are capable of supporting spermatogonia. Persistence of immature Sertoli cells in the adult testis would therefore be inconsistent with full spermatogenesis, though in theory such cells might be capable of supporting the survival of normal or transformed (CIS) fetal germ cells or early spermatogonia (Fig. 3). It is also established that the presence of particular meiotic and post-meiotic germ cells has profound effects on the function of mature Sertoli cells (Jégou and Sharpe, 1993; Sharpe *et al*., 1993; Boujrad *et al*., 1995), and their absence (for example due to exposure to irradiation) may lead to secondary changes in Sertoli-cell function such that they bear some functional resemblance to immature Sertoli cells (Fig. 3). When an SCO tubule is observed in the adult testis, how is the mature Sertoli cell that has lost its germ cells distinguished from the 'failure of maturation' type of Sertoli cell? Until fairly recently, the distinction has relied firmly on morphological criteria, such as the persistence of small, spindle-shaped Sertoli-cell nuclei arranged closely in a pallisade in immature Sertoli cells and with

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no evidence of formation of a lumen (see Paniagua *et al*., 1990; Pinart *et al*., 2000; Nistal *et al*., 2002; Fig. 5). These seminiferous tubules are commonly described in cryptorchid testes, but are also reported in scrotal testes of some patients with Klinefelters syndrome (see, for example, Nistal *et al*., 1982), impaired spermatogenesis (see, for example, Regadera *et al*., 2001) or testicular germ-cell cancer (see, for example, Stosiek *et al*., 1990; Kliesch *et al*., 1998).

Immunohistochemical evidence for immaturity of Sertoli cells in various pathologies

In human testes with various disorders, some, but not all, SCO tubules express one or more markers of immature Sertoli cells such as AMH, cytokeratin 18 and M2A, and may not express antigens such as AR that are normal features of mature Sertoli cells (Fig. 3). These findings have been interpreted as evidence either for the persistence of immature Sertoli cells (see, for example, Maymon *et al*., 2000, 2002) or, in some instances, as de-differentiation of mature Sertoli cells such that certain immature functions have been re-expressed (see, for example, Kliesch *et al*., 1998; Steger *et al*., 1999; Young *et al*., 1999; Brehm *et al*., 2002). It is difficult to distinguish failure of maturation, which may occur at various steps, from de-differentiation, unless there are obvious differences such as in nuclear morphology. If de-differentiation of Sertoli cells secondary to the loss of germ cells is a real phenomenon, it is clearly not an inevitable consequence of germ-cell loss, as is illustrated by the loss of germ cells from the prepubertal testis of boys who have been treated for cancer. In these otherwise normal boys, pubertal loss of features of immature Sertoli cells, such as expression of AMH, still occurs though perhaps with some delay (Rajpert-de Meyts *et al*., 1999). Irradiation-induced loss of germ cells from the adult testis also fails to alter the differentiation status of Sertoli cells (Giwercman *et al*., 1991). In a similar way, loss of AMH and cytokeratin 18 immunoexpression in Sertoli cells also occurs normally in most (but not all) azoospermic men either with idiopathic absence of germ cells (Rajpert-de Meyts *et al*., 1999), or associated with Y-chromosome microdeletions (Maymon *et al*., 2000; Blagosklonova *et al*., 2002); in oligozoospermic men with germ-cell maturation arrest, similar absence of markers of immature Sertoli cells is evident in most men (Maymon *et al*., 2000, 2002; Blagosklonova *et al*., 2002).

Though the above indicates that AMH expression reflects maturational status of Sertoli cells, this is an over-simplification. Patients with congenital hypogonadotrophic hypogonadism (HH), in whom puberty is consequently delayed, exhibit continued high blood concentrations of AMH, consistent with failure of maturation of Sertoli cells (Young *et al*., 1999). However, patients with acquired HH, in whom normal puberty had earlier occurred, also show a (smaller) increase in blood AMH concentrations, and treatment of these men with hCG or testosterone suppresses AMH concentrations to normal adult concentrations (Young *et al*., 1999). This result implies that AMH reflects status of androgen action on the Sertoli cell, though androgen responsiveness and maturation of Sertoli cells may be coincident, as already discussed. This interpretation is reinforced by studies in patients with complete AIS, in whom the Sertoli cells retain immature morphological and functional features such as expression of AMH (Rajpert-de Meyts *et al*., 1999; Regadera *et al*., 1999; Rey *et al*., 1999). Moreover, in some men with impaired spermatogenesis, especially those who exhibit focal SCO with morphologically immature Sertoli cells, focal expression of AMH and cytokeratin 18 (Steger *et al*., 1996; Rajpert-de Meyts *et al*., 1999; Maymon *et al*., 2000, 2002) and absence of AR expression (Regadera *et al*., 2001) are evident, consistent with failure of maturation of Sertoli cells in these regions. A similar association is frequently seen in men with testicular germ-cell cancer, in particular the association of Sertoli cells with an immature phenotype and the presence of pre-cancerous CIS cells (Fig. 3; Rogatsch *et al*., 1996; Kliesch *et al*., 1998; Brehm *et al*., 2002). However, in such cases it is difficult to distinguish whether continued AMH expression occurs because of the failure of maturation of Sertoli cells or because of failure of expression of androgen receptor, as the latter would result from the former.

In the authors' opinion, the occurrence of immature functional features of Sertoli cells in the testes of adult men with various abnormalities, in particular foci of abnormal seminiferous tubules, are most likely a reflection of failure of maturation rather than re-emergence of an immature phenotype due to de-differentiation of previously mature Sertoli cells. This would explain why such immature Sertoli cells occur most commonly in cryptorchid testes or in testes with germ-cell cancer or CIS, as these conditions originate in fetal life (Skakkebaek *et al*., 2001). In a similar way, persistence of immature Sertoli cells in patients with complete AIS, who are at high risk of CIS and testis cancer, and in whom the failure of maturation has a ready explanation (absence of the AR), is consistent with this view. This interpretation also fits with the hypothesized 'testicular dysgenesis syndrome' in humans, in which fundamental dysfunction of Sertoli cells or Leydig cells is believed to underlie the risk of cryptorchidism, testis cancer and some cases of impaired spermatogenesis (Skakkebaek *et al*., 2001; Sharpe and Skakkebaek, in press). The present findings in rats are entirely consistent with this hypothesis, as they show that treatments that result in focal persistence of immature Sertoli cells are associated with a high incidence of cryptorchidism, spermatogenic impairment, SCO tubules and infertility and abnormal development of fetal germ cells (Fisher *et al*., 2003). Nevertheless, in these situations it is common to find SCO tubules

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in which the Sertoli cells are immature alongside SCO or normal tubules in which the Sertoli cells are functionally mature (that is, not expressing immature Sertoli-cell markers; Fig. 3). In animals, similar foci of apparently immature Sertoli cells (mainly on the basis of morphology) in SCO tubules have been described, usually in cryptorchid testes (see, for example, Pinart *et al*., 2000). These findings support the view that the associated cryptorchidism may be a consequence, rather than the cause, of impaired maturation or development of Sertoli cells (Skakkebaek *et al*., 2001). Another potential implication is that the presence of foci of immature Sertoli cells might be indicative of more subtle, but more widespread, disturbance of maturation or function of Sertoli cells, such that some impairment of spermatogenesis occurs even in seminiferous tubules exhibiting complete spermatogenesis. However, account must be taken of changes in function of Sertoli cells that occur secondary to subnormal androgen action, as may occur with AMH.

Future perspectives

Recognition that immature Sertoli cells may persist from fetal or prepubertal life and are always associated with absence of normal, or even any, spermatogenesis, is a conceptual step forward. It puts the emphasis on early development as the cause of the problem and thus raises the all-important questions of why, how and when the process of maturation of Sertoli cells has gone wrong. How can immature, dysfunctional Sertoli cells persist next to normal mature, fully functional Sertoli cells in the same testis? This seems to indicate that very early events in differentiation of Sertoli cells are at fault, which then render the Sertoli cell non-responsive to the normal cues that drive its step-wise maturation, absence of a functional AR being a case in point. If this dysfunction occurs during fetal life, it may also compromise normal proliferation of the affected cells in postnatal life (Fig. 1), and thus contribute to lowering the sperm count in such individuals. This poses numerous fascinating questions relating to the mechanics of Sertoli-cell and sexual differentiation, but it also has important health implications. The incidence of testicular cancer has doubled approximately every 30 years in Caucasian men in Western countries, such that lifetime risk is now 0.3–0.8%, whereas cryptorchidism remains the commonest congenital malformation in children, affecting 2–4% of boys at birth (Sharpe and Skakkebaek, in press). Cryptorchidism is the most important risk factor for testicular cancer as well as being an important risk factor for low sperm counts (Sharpe and Skakkebaek, in press). If the incidence of testicular cancer is a beacon, signalling that the occurrence of TDS is increasing (Skakkebaek *et al*., 2001), then a better functional understanding of the development and maturation of early Sertoli cells may help to pinpoint how and when this might be affected by our modern lifestyle and environment (Sharpe and Franks, 2002). Studies in dysfunctional human testes that seek to establish the precise point(s) of arrest in differentiation or maturation of Sertoli cells should prove informative in this regard. However, studies in animal models which can shed light on the precise cascade of events in the development of Sertoli cells and can identify how arrest at various points may manifest at birth and in adulthood, will also be essential to link definitively events *in utero* with testicular function in adulthood. In turn, this understanding is likely to explain how transformed fetal germ cells (CIS) can survive into adulthood and give rise to testicular germ-cell cancer, as the persistence of immature Sertoli cells in association with such cells is a common feature, and may perhaps be essential before puberty.

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