Int. J. Exp. Path. (1998), **79,** 67–80

Stem Cell Review

Stem cells in the testis

DIRK G. DE ROOIJ
Department of Cell Biology, Utrecht University, Utrecht, The Netherlands

Received for publication 5 January 1998
Accepted for publication 26 January 1998

Summary. The origin and development of the spermatogenic cell lineage is reviewed, as well as spermatogonial kinetics in adult nonprimate mammals in relation to the cycle of the seminiferous epithelium, the emphasis being on spermatogonial stem cells. A hypothesis is presented for the transition from foetal germ cells, gonocytes, to adult type spermatogonia at the start of spermatogenesis. An overview is given of the present knowledge on the proliferation and differentiation of undifferentiated spermatogonia (spermatogonial stem cells and their direct descendants) and the regulation of these processes. It is concluded that the differentiation of the undifferentiated into differentiating type spermatogonia is a rather vulnerable moment during spermatogenesis and the models for studying this are described. Research into the molecular basis of the regulation of spermatogonial proliferation, differentiation and apoptosis is at its infancy and the first results are reviewed. An exciting new research tool is the spermatogonial stem cell transplantation technique which is described. Finally, reviewing the nature of human germ cell tumours it is concluded that at present there are no animal or *in vitro* models to study these tumours experimentally.

Keywords: testis, spermatogenesis, stem cells, spermatogonia, gonocytes, cell cycle regulation, differentiation

Origin of spermatogonial stem cells

Spermatogonial stem cells originate from the primordial germ cells (PGCs) which in turn derive from epiblast cells (embryonal ectoderm) (Lawson & Pederson 1992). By 7 days post coitum (p.c.) in the mouse embryo, about 100 alkaline phosphatase positive PGCs can be detected in the extraembryonal mesoderm posterior to the definitive primitive streak (Ginsberg *et al.* 1990). Later in development the PGCs migrate from the base of the allantois, along the hindgut to finally reach the genital ridges. The PGCs proliferate during migration and by day 13 of foetal life in the mouse, when these cells have reached the genital ridges, their numbers have increased to about 10 000 in each gonad (Tam & Snow 1981).

Primordial germ cells are single cells that under certain culture conditions can form colonies of cells which morphologically resemble undifferentiated embryonic stem cells (ES cells; Resnick *et al.* 1992). These cells can be maintained on feeder layers for extended periods of time and can give rise to embryoid bodies and to multiple differentiated cell phenotypes in monolayer culture and in tumours in nude mice. Primordial-germ-cell-derived ES

Correspondence: Dirk G. de Rooij, Department of Cell Biology, Utrecht University Medical School, Postbus 80.157, 3508 TD Utrecht, The Netherlands. Fax +31 (0)30 2537419; E-mail: d.g.derooij@med.ruu.nl

cells can also contribute to chimaeras when injected into host blastocysts (Resnick *et al*. 1992). Clearly, PGCs are stem cells, still having the capacity to renew themselves and to differentiate in various directions.

Once arrived in the genital ridges, the PGCs become enclosed by the differentiating Sertoli cells, and seminiferous cords are formed (Figure 1). The germ cells present within the seminiferous cords differ morphologically from PGCs and are called gonocytes (Clermont & Perey 1957; Sapsford 1962; Huckins & Clermont 1968) or various subsequent types of prospermatogonia (Hilscher *et al*. 1974). In rats and mice, after formation the gonocytes proliferate for a few days and then become arrested in the G_0/G_1 phase of the cell cycle (Clermont & Perey 1957; Franchi & Mandl 1964; Huckins & Clermont 1968; Hilscher *et al*. 1974; Vergouwen *et al*. 1991). Shortly after birth the gonocytes resume proliferation to give rise to A spermatogonia (Sapsford 1962; Novi & Saba 1968; Huckins & Clermont 1968; Bellvé *et al*. 1977; Vergouwen *et al*. 1991). This event marks the start of spermatogenesis.

In vitro studies have demonstrated differences between gonocytes and PGCs. For example, gonocytes can only survive in the presence of Sertoli cells (Van Dissel-Emiliani *et al*. 1993), while PGCs can be cocultured with other types of somatic cells (Resnick *et al*. 1992). This suggests that the gonocytes are restricted in their differentiation potential in comparison to the PGCs. Furthermore, in gonocyte mitosis cytokinesis is often not completed and many gonocytes remain interconnected by intercellular bridges (Zamboni & Merchant 1973). As discussed below, in the adult testis the first visible sign of differentiation of the stem cells is the formation of a pair of cells interconnected by an intercellular bridge. These paired spermatogonia are destined to ultimately become spermatozoa. Conceivably, the gonocytes may well be a heterogeneous population of cells of which only some still have stem cell properties, while the rest may be destined to become differentiating cells directly at the start of spermatogenesis after birth.

Spermatogonial multiplication and stem cell renewal in nonprimate mammals

A_s-model. According to the model originally proposed by Huckins (1971a) and Oakberg (1971), a compartment of undifferentiated A spermatogonia exists at the beginning of spermatogenesis. In whole mounts of seminiferous tubules, it becomes apparent that these cells can be subdivided into A_{single} (A_s), A_{paired} (A_{pr}) or $A_{aligned}$ (A_{al}) spermatogonia according to their topographical arrangement on the basement membrane. The A_s spermatogonia are considered to be the stem cells of spermatogenesis. Upon division of the A_s spermatogonia, the daughter cells can either migrate away from each other and become two new stem cells or they can stay together connected by an intercellular bridge and become A_{pr} spermatogonia. In the normal situation about half of the stem cell population will divide to form A_{pr} spermatogonia, while the other half will go through a self-renewing division, thus maintaining stem cell numbers. The A_{pr} spermatogonia divide further to form chains of 4, 8 or 16 A_{al} spermatogonia (Figure 2).

The A_{al} spermatogonia are able to differentiate into A_1 spermatogonia that are the first generation of the differentiating type spermatogonia. These differentiating spermatogonia go through a series of six divisions and *via* A_2, A_3, A_4, Intermediate (In) and B spermatogonia become primary spermatocytes. Especially in tubular whole mounts it can be seen that differentiating spermatogonia

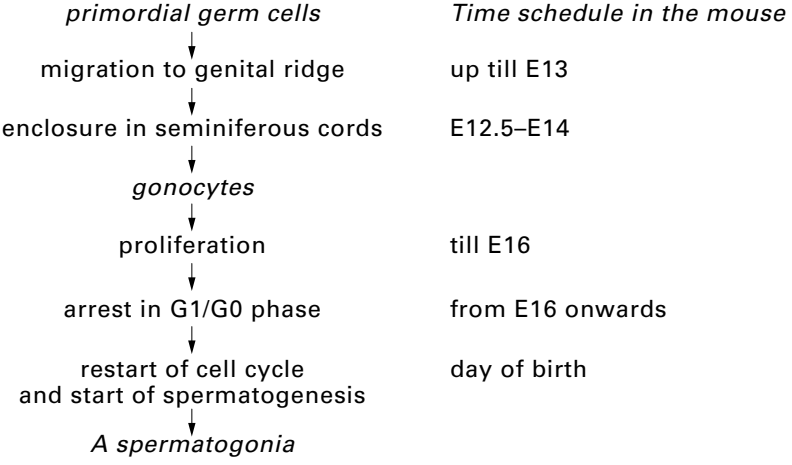

Figure 1. Scheme of the development of the spermatogenic lineage from primordial germ cells to A spermatogonia.

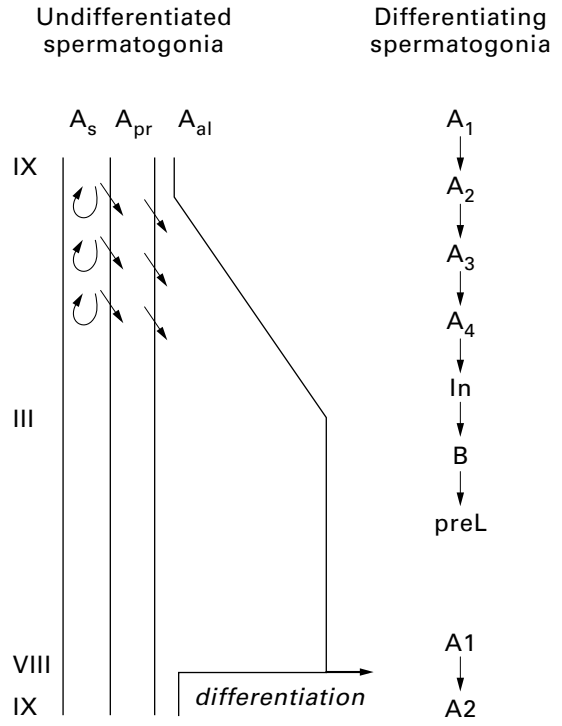

Figure 2. Scheme of spermatogonial multiplication and stem cell renewal in the mouse.

behave in a highly synchronized manner; large areas in which all the differentiating spermatogonia synchronously go through mitosis can be seen. Also the various generations of differentiating spermatogonia will go through S phase or mitosis in particular epithelial stages (see below). The synchrony in the divisions of the differentiating type spermatogonia derives from two causes. First, from the A_{pr} spermatogonia onwards at a spermatogonial division the daughter cells remain connected by intercellular bridges; the comprising cells of such a clone will always synchronously traverse the cell cycle. Second, the areas in which the differentiating type spermatogonia simultaneously go through mitosis are much larger than the size of such a clone. So, there must be an additional mechanism inducing the synchronous behaviour of neighbouring clones. The nature of the latter mechanism is not known yet, but it seems likely that the Sertoli cells are involved since they are known to produce many growth factors (review De Rooij & Van Dissel-Emiliani 1997). In whole mounts of seminiferous tubules, undifferentiated spermatogonia can be distinguished from differentiating type A spermatogonia (A_1-A_4) in those areas in which the latter cells are in late G_2 or M phase, due to the fact that the undifferentiated spermatogonia do not cycle synchronously with the A_1-A_4 spermatogonia. However, there is not enough morphological difference between undifferentiated and differentiating type A spermatogonia to distinguish these cells in all cell cycle phases.

According to the model of Huckins (1971a) and Oakberg (1971), in the adult testis differentiation of the stem cells, the A_s spermatogonia, occurs when following a division the daughter cells remain connected by an intercellular bridge. However, it is not known whether the presence of intercellular bridges among A_{pr} and A_{al} spermatogonia reflects a functional differentiation of the A_s spermatogonia and whether these cells still have stem cell properties when the intercellular bridges become severed, as occurs after irradiation (Van Beek *et al*. 1984). The A_s, A_{pr} and A_{al} spermatogonia can only be distinguished by their topographical arrangement as there are no further morphological differences between these cells. Also, the A_s spermatogonia more or less follow the proliferation pattern of the total population of undifferentiated spermatogonia. Hence, the A_s, A_{pr} and A_{al} spermatogonia and the regulation of the proliferation of these cells, may be rather similar.

The cell cycle properties of the undifferentiated spermatogonia have been studied in the rat (Huckins 1971b) and the Chinese hamster (Lok *et al*. 1983). In both species the duration of the cell cycle of the undifferentiated spermatogonia appeared to be considerably longer than that of the differentiating spermatogonia (Huckins 1971c; Lok & De Rooij 1983a). For example in the Chinese hamster the duration of the cell cycle of the undifferentiated spermatogonia was found to be at least 90 h compared to 60 h for the differentiating type spermatogonia. Furthermore, in both rat and Chinese hamster the second peak of the curve of the labelled mitoses of the A_s spermatogonia was much lower than that of the A_{pr} and A_{al} spermatogonia. This shows that the A_s spermatogonia divide slower than the A_{pr} and A_{al} spermatogonia although the minimum duration of the cell cycle of the undifferentiated spermatogonia is the same. Hence, like in other tissues, in the seminiferous epithelium the stem cells have a lower rate of proliferation than their more differentiated descendants (Lajtha 1979).

A_0 model. The model of Huckins (1971a) and Oakberg (1971) is at variance with that of Clermont & Bustos-Obregon (1968) and Clermont & Hermo (1975). In the latter the A_s and A_{pr} spermatogonia are quiescent in the normal testis and together are called A_0 spermatogonia. The A_0 spermatogonia only come into action after cell loss, for example after irradiation. Furthermore, in this model the A_1, A_2, A_3 and A_4 spermatogonia still retain stem cell properties; the A_4 spermatogonia at their

division rendering both A_1 and In spermatogonia. The A_{al} spermatogonia in this model are considered to be out of phase A_1-A_4 spermatogonia as these cells do not participate in the synchronous waves of division characterizing the A_1-A_4 spermatogonia. The A_0 model is in conflict with several experimental findings. First, the A_s and A_{pr} spermatogonia are not quiescent in the normal testis but have a distinct pattern of proliferative activity during the epithelial cycle (Huckins 1971b; Lok & De Rooij 1983b). Second, the A_{al} spermatogonia have cell cycle properties similar to those of the A_s and A_{pr} spermatogonia and quite different from those of the A_1-A_4 spermatogonia (Huckins 1971b,c; Lok & De Rooij 1983a; Lok *et al.* 1983), explaining the asynchronous behaviour of the A_{al} spermatogonia. Third, when locally the A_1 spermatogonia are specifically removed, leaving the undifferentiated spermatogonia unharmed, then in these areas abnormally high numbers of A_{al} but no In spermatogonia are formed during the ensuing epithelial cycle (De Rooij *et al.* 1985). The latter argues against the idea that the A_{al} spermatogonia are merely out of phase A_1-A_4 spermatogonia, as in that case at least the surplus of (supposed) A4 spermatogonia could have become In spermatogonia. Fourth, there is a considerable difference in radiosensitivity between proliferating A_{al} spermatogonia and A_1-A_4 spermatogonia (Van der Meer *et al.* 1992a,b) also suggesting that A_{al} and A_1-A_4 spermatogonia are different types of cells. Finally, recently Dupressoir & Heidmann (1996) studied the expression of intracisternal A-particle (IAP) retrotransposons in transgenic mice. Transgenic mice containing constructs of the IAP promoter and a lacZ reporter gene, were found to express the lacZ gene specifically in single and paired A spermatogonia and in chains of 4, 8 or 16 A spermatogonia. The topographical arrangement of the latter chains of A spermatogonia was similar to that of the A_{al} spermatogonia and different from that of the A_1-A_4 spermatogonia that are more widely dispersed and have a much larger chain length. Again, this is strong evidence that A_{al} spermatogonia are different from A_1-A_4 spermatogonia.

The transition from gonocytes to spermatogonia

There is still no general agreement about what happens at the start of spermatogenesis. The daughter cells of the gonocytes after birth may be a special type of prespermatogonia that after one or more divisions give rise to adult type spermatogonia (Novi & Saba 1968; Huckins & Clermont 1968; Hilscher *et al.* 1974; Bellvé *et al.* 1977). However, the evidence (only morphological in nature) for the existence of a special kind of prespermatogonia is not conclusive and the gonocytes might also directly give rise to adult type spermatogonia (Figure 3; Kluin & De Rooij 1981).

Van Haaster & De Rooij (1993) studied the appearance of the subsequent types of spermatogenic cells with age. Graphs were made from which the rate of the spermatogenic process could be derived, indicating that the duration of the epithelial cycle in immature animals is much shorter than in the adult. However, these graphs indicating when the various cell types were identified for the first time after the start of spermatogenesis could also be used to extrapolate backwards to the start of spermatogenesis where it should indicate which cell type was formed at the division of the gonocytes. In the Djungarian hamster at day 4 after birth the first labelled gonocytes can be found (Van Haaster & De Rooij 1994), indicating that at that age spermatogenesis starts in this species and at day 5, the first mitoses were seen. From the extrapolation backwards to the

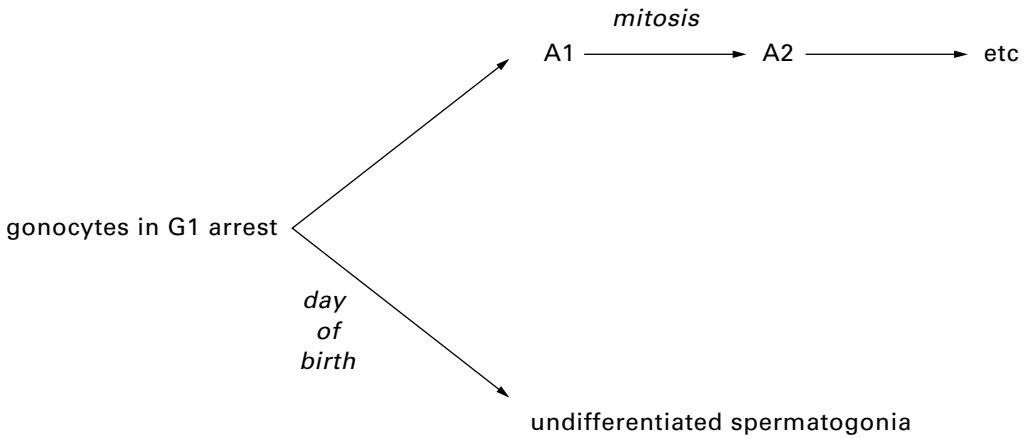

Figure 3. Scheme of the transition from gonocytes to spermatogonia at the start of spermatogenesis.

start of spermatogenesis it can be inferred that at day 5 the epithelium should be in stage IX where in adult spermatogenesis the division of the A_1 into A_2 spermatogonia takes place (Van Haaster 1993). Carrying out the same procedure in the Chinese hamster and the rat rendered the same result (Figure 4).

Hence, it can concluded that either gonocytes at their division at the start of spermatogenesis produce A_2 spermatogonia or one would have to accept that in the immature animal generations of differentiating type spermatogonia are skipped, because there is no time for extra generations of spermatogonia. In view of the rather rigid regulation of the spermatogenic process skipping of one or more generations of spermatogonia does not seem a very likely possibility. Moreover, the morphology and the size of the daughter cells of the gonocytes was found to be closely similar to those of A_2 spermatogonia (Kluin & De Rooij 1981). From the gonocytes rendering A_2 spermatogonia at their division one could speculate that these cells differentiate into A_1 spermatogonia upon entering the active cell cycle again.

The spermatogenic process

Spermatogenesis is very strictly regulated, the developmental steps of the spermatogenic cells always taking exactly the same time in a particular species. In addition, mammalian spermatogenesis is a cyclic process. During part of that cycle, the cells at the beginning of spermatogenesis, the undifferentiated spermatogonia, are mostly quiescent and their number is low, only a few A_{al} spermatogonia being present. Then the cells are stimulated and for a certain period of time, active proliferation results in the formation of more and more A_{al} spermatogonia (Figure 5; Lok & De Rooij 1983b). The total number of A_s and A_{pr} spermatogonia does not change much during the epithelial cycle (Lok *et al.* 1982; Van Beek *et al.* 1984; Tegelenbosch & De Rooij 1993). After a period of active proliferation, most of the cells become arrested in G_1 phase of the cell cycle. This period of relative quiescence ends when almost all of the A_{al} spermatogonia formed during the period of proliferation, differentiate into the first generation of the differentiating spermatogonia, the A_1 spermatogonia. The differentiating spermatogonia then go through their six divisions and ultimately become spermatocytes. Subsequently, the spermatocytes go through the lengthy prophase of the first meiotic division, ultimately carry out the meiotic divisions and give rise to spermatids. The spermatids develop into spermatozoa in 16 steps (review Russell *et al.* 1990). The undifferentiated spermatogonia that remain after most of them have differentiated into A_1 spermatogonia start to proliferate again following the division of the A_1 into A_2 spermatogonia to form a new cohort of A_1 spermatogonia for the next epithelial cycle.

The interval of time between the formation of subsequent cohorts of new A_1 spermatogonia is always similar in a particular species and is called the duration of the epithelial cycle. Also the duration of the various steps in the development of the spermatogenic cells is always the same within a particular species. As a consequence the same associations of generations of cells in particular developmental stages are always found together along the tubular wall. Thus, spermatids at a particular step in their transformation into spermatozoa are always found with spermatogonia and spermatocytes of a particular

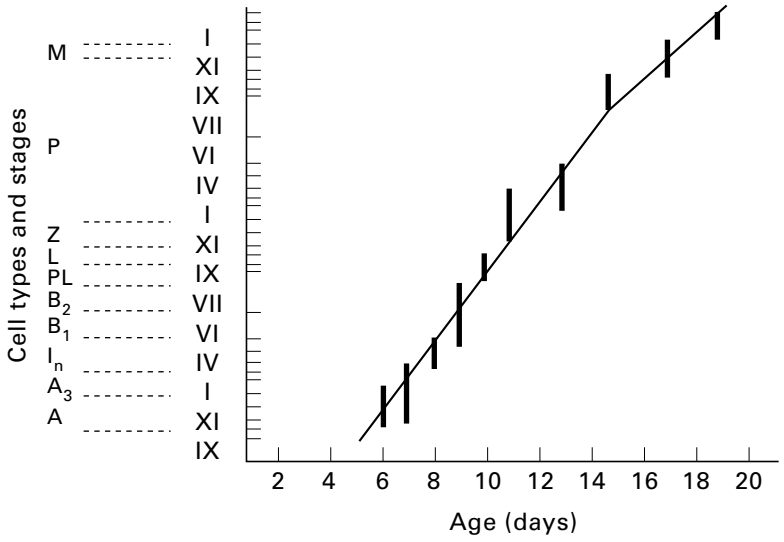

Figure 4. Appearance of the subsequent spermatogenic cell types in the Djungarian hamster during postnatal testicular development (data from Van Haaster 1993 and Van Haaster & De Rooij 1994).

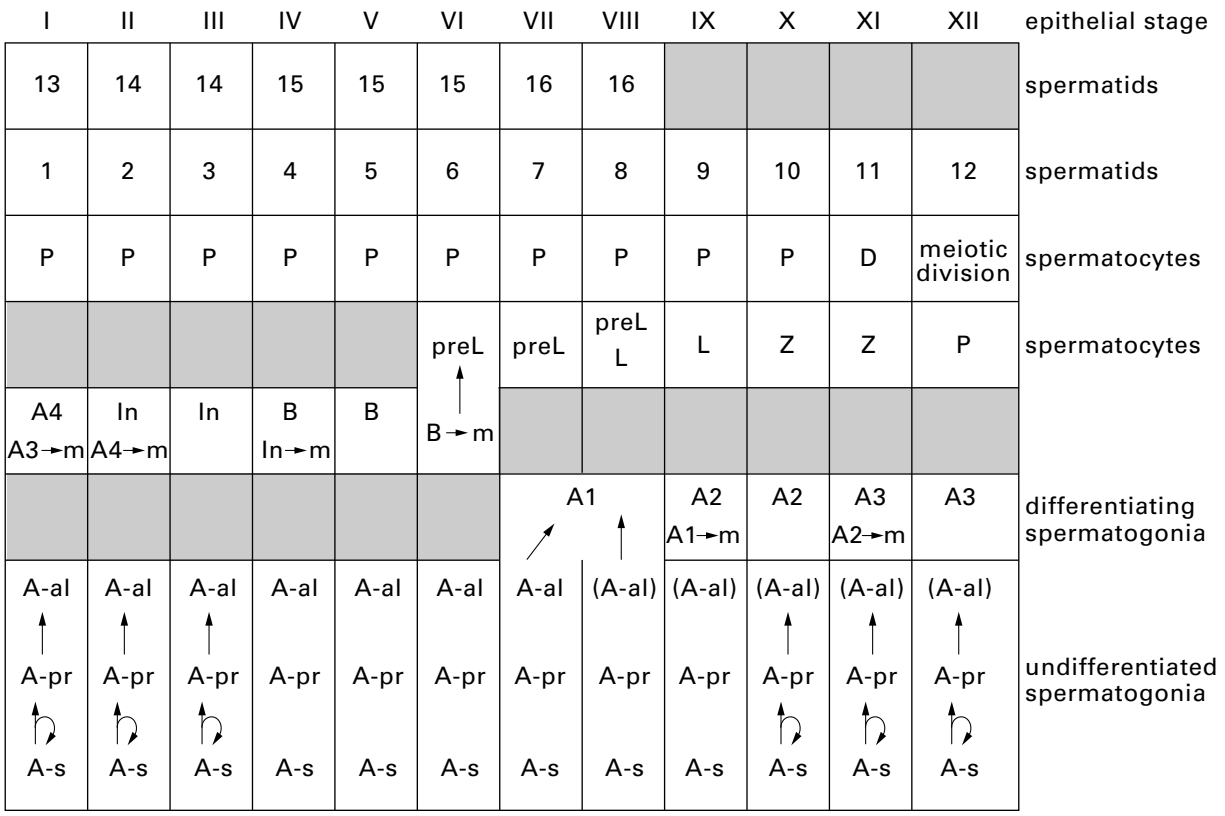

Figure 5. Diagram showing the cellular associations (epithelial stages) during the cycle of the seminiferous epithelium of the mouse. Undifferentiated spermatogonia consist of A_{single} (A_s), A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia that divide during stages X-III as indicated by the arrows between the cells. During stages VIII to XI A_{al} are rare as indicated by the brackets. In the category of differentiating type spermatogonia the moments of division are indicated by an *m*. The spermatocytes go through a G1 and S phase during which these cells are indicated as preL (preleptotene), subsequently these cells traverse the meiotic prophase via leptotene (L), zygotene (Z), pachytene (P) and diplotene (D) phases. The meiotic divisions render spermatids which develop in 16 steps into spermatozoa that are shed into the lumen of the seminiferous tubules. The first 12, well recognizable, steps of spermatid development are used to divide the epithelial cycle into 12 stages (review Russell 1990). The cell types in each column are always found together.

stage in their respective development. This makes it possible to divide the epithelial cycle in stages according to steps in the development of spermatogenic cells. In most species 12 stages are recognized based on the development of the spermatids (reviews by Courot *et al.* 1970; Russell *et al.* 1990). For example, in stage VIII, we find by definition step 8 spermatids, together with step 16 elongated spermatids that are about to leave the seminiferous epithelium, pachytene spermatocytes, preleptotene or early leptotene spermatocytes, A_1 spermatogonia and undifferentiated spermatogonia (Figure 6).

Regulation of the proliferative activity of the undifferentiated spermatogonia

Thus far no data have become available about the nature of the stimulus that each epithelial cycle induces the reinitiation of the proliferation of the undifferentiated spermatogonia at about stage X. No situations have been described in which the undifferentiated spermatogonia stayed in G1 arrest or in which the reinitiation of the proliferative activity occurred earlier or later.

Fortunately, more is known about how in each epithelial cycle the proliferative activity of the undifferentiated spermatogonia is inhibited in stages II-III. First, it was shown by various groups that the proliferation of the undifferentiated spermatogonia can be partly inhibited by testicular extracts (Clermont & Mauger 1974; Thumann & Bustos-Obregon 1978; Irons & Clermont 1979; De Rooij 1980). The inhibiting factor was found to be tissue- but not species-specific, and was thought to be a chalone but has not yet been purified.

Second, in the Chinese hamster it was found that when the differentiating spermatogonia were specifically

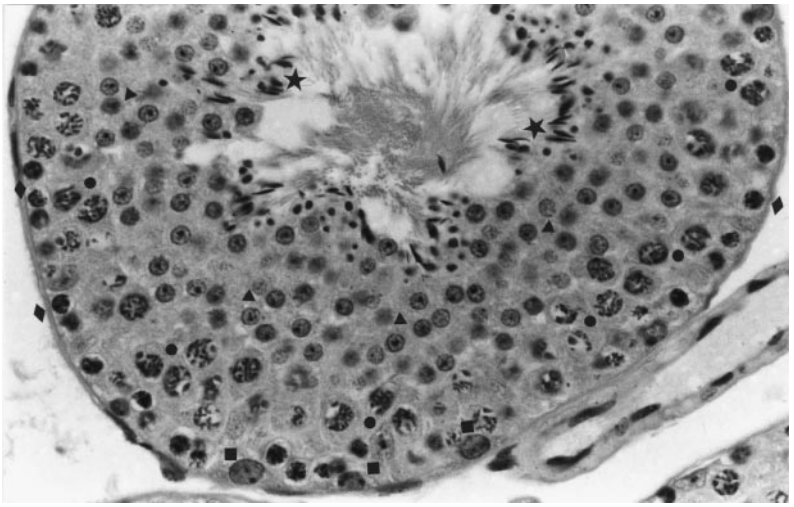

Figure 6. Photograph of a seminiferous tubular cross-section in which the epithelium is in stage VIII of the epithelial cycle (see scheme in Figure 5). In this stage elongated spermatids in step 16 of their development (asterisks) can be found that are about to be shed into the lumen. Furthermore, there are step 8 round spermatids (triangles), pachytene spermatocytes (dots), early leptotene spermatocytes (diamonds) and A spermatogonia (squares). In sections the different types of A spermatogonia cannot be determined in further detail.

removed from the epithelium the inhibition of the proliferative activity of the undifferentiated spermatogonia did not take place (De Rooij *et al*. 1985). It was concluded that from stage III onwards the proliferation of the undifferentiated spermatogonia is inhibited by the In and B spermatogonia present in these stages by way of a negative feedback system. This feedback regulation might work *via* the production of a chalone by the In and B spermatogonia. In this respect it is interesting that the inhibiting activity of the extracts was absent when they were made of testes from which all spermatogonia were removed (De Rooij 1980). Alternatively, the effect of the presence of In and B spermatogonia on the proliferation of undifferentiated spermatogonia may be indirect *via* Sertoli cells.

An important question is whether or not the stem cells are also inhibited in their proliferative activity by this feedback system. In the Chinese hamster the DNA labelling index (LI) of the A_{pr} and A_{al} spermatogonia drops sharply in stage III while the LI of the A_s spermatogonia drops in early stage VII (Lok & De Rooij 1983b). In the rat the LI of the undifferentiated spermatogonia drops in stage II and that of the A_s spermatogonia in stage V (Huckins 1971b). Hence it can be concluded that the A_s spermatogonia are much less sensitive or perhaps completely insensitive to the feedback regulation. If these cells are insensitive we have to assume that the decrease in their proliferative activity later during the epithelial cycle is caused by a lack of stimulation. This would be consistent with the notion that the secretory activity of the Sertoli cells varies during the epithelial cycle (Parvinen 1982).

Stem cell renewal and differentiation

Normal situation. As yet not many data are available about the regulation of the ratio between self-renewal and differentiation of the stem cells. Cell counts in the normal mouse and the Chinese hamster revealed that during the period of active proliferation of the stem cells the number of these cells slowly decreases (Van Beek *et al*. 1984; De Rooij *et al*. 1985; Tegelenbosch & De Rooij 1993). This indicates that in such a situation more than 50% of the daughter cells become A_{pr} spermatogonia as a first step towards differentiation. Furthermore when the period of active proliferation of the undifferentiated spermatogonia is prolonged, the decrease in the number of A_s spermatogonia keeps taking place. Hence there is a relationship between the proliferative activity in the epithelium and the probability of self-renewal of the stem cells.

Attempts have been made to detect a regulatory mechanism that would ensure an even density of stem cells and/or all the undifferentiated spermatogonia by way of changing the ratio between self-renewal and differentiation when necessary (De Rooij & Janssen 1987). No trace of such a regulatory mechanism was found. Areas were found that contained widely different numbers of A_s spermatogonia and/or A_{pr} and A_{al} spermatogonia and consequently produced very many or only few differentiating spermatogonia. It was found that an even distribution of spermatocytes in the epithelium was ensured by a density dependent degeneration of differentiating spermatogonia in such a way that many of these cells degenerated in high density areas and only few or none at all, in low density areas (De Rooij & Lok 1987). Apparently, in the normal epithelium there is no need for a precise regulatory mechanism to keep the density of the stem cells within close limits.

After cell loss. While in the normal situation the overall probability of self renewal of stem cells during the cycle of

the seminiferous epithelium will have to be close to 50%, after stem cell loss this percentage should be higher to enable recovery of stem cell numbers. A first indication that this is indeed happening was obtained from the observation that both after irradiation and after administration of busulfan isolated clones of A_{al} spermatogonia were extremely rare (Van Keulen & De Rooij 1975; Van Beek *et al.* 1986). Isolated clones of A_{al} spermatogonia would arise when a surviving stem cell would directly differentiate into A_{pr} spermatogonia instead of renewing itself.

Clear proof for enhanced self renewal of stem cells after cell loss was obtained by studying the composition of repopulating colonies formed by surviving stem cells, 6–10 days after irradiation (Van Beek *et al.* 1990). Monte Carlo simulations were carried out to understand stem cell behaviour during the first 7 divisions after the inflicted cell loss. It was found that virtually no differentiation of stem cells occurred during the first 5 divisions, and that the percentage of self renewing divisions remained considerably higher than 50% for at least divisions 6 and 7. As yet there are no clues about the nature of the mechanism involved in regulating the ratio between stem cell renewal and differentiation.

Regulation of the differentiation of undifferentiated spermatogonia

Through the years it has become apparent that there exists a step in spermatogonial development which is rather vulnerable to disturbances of the normal situation. More and more situations are being described in which the differentiation of the A_{al} spermatogonia into A_1 spermatogonia seems to have become blocked. These are listed in Table 1.

A first example is the cryptorchid mouse model developed by Nishimune *et al.* (1978). In C57Bl mice that were surgically made cryptorchid, after 2 months only A spermatogonia, presumably undifferentiated spermatogonia, remained that were unable to give rise to more differentiated cells. This was despite the fact that these cells were actively proliferating in the cryptorchid condition (Nishimune & Haneji 1981). The situation, however, remained reversible since after transferring the testes back to the scrotum, normal spermatogenesis was restored.

Second, recent data indicate that the c-kit receptor and its ligand Stem Cell Factor (SCF), play a role in the differentiation of A spermatogonia (review Nishimune & Okabe 1993). Koshimizu *et al.* (1991) and Tajima *et al.* (1991) studied the effects of mutations in the dominant white-spotting (W) and Steel (Sl) loci on the differentiation of mouse testicular germ cells using the experimental cryptorchidism and surgical reversal model, described above. Heterozygous mice showed a normal seminiferous epithelium, and when made cryptorchid spermatogenesis in all mice became arrested at the level of A spermatogonia, as in wild type mice. However, upon surgical reversal of the cryptorchidism, spermatogenesis recovered in +/+ mice but not or incompletely in the mice carrying W or Sl mutants, suggesting that these mutations affect the differentiation of the A spermatogonia. These findings indicate that the c-kit receptor/SCF system has a role in A spermatogonial differentiation.

Table 1. Situations in which A spermatogonial differentiation becomes arrested

Abnormality	References
Cryptorchidism	Nishimune & Haneji (1981)
W and Sl mutations	Koshimizu *et al.* (1991)
	Tajima *et al.* (1991)
Vitamin A deficiency	De Rooij *et al.* (1994)
	Van Pelt *et al.* (1995)
jsd mutant mice	Beamer *et al.* (1988)
	Mizunuma *et al.* (1992)
Sertoli cell intoxication with 2,5-hexanedione	Boekelheide (1988)
	Allard *et al.* (1995)
	Allard *et al.* (1996a,b)
Irradiated LBNF1 rats	Kangasniemi *et al.* (1996)
	Meistrich & Kangasniemi (1997)

Third, in vitamin A deficient testes, spermatogenesis deteriorates and ultimately only A spermatogonia remain and in the rat (but not in mice) a few preleptotene spermatocytes also persist (Mitranond *et al.* 1979). Vitamin A replacement or continuous administration of retinoic acid than restore spermatogenesis (Huang & Hembree 1979; Morales & Griswold 1987; Van Pelt & De Rooij 1990, 1991). The A spermatogonia remaining in the vitamin A deficient testis could be characterized as undifferentiated spermatogonia that were apparently unable to differentiate (De Rooij *et al.* 1994; Van Pelt *et al.* 1995).

Fourth, adult male mice homozygous for the mutant gene, juvenile spermatogonial depletion (jsd) are sterile and have small testes (Beamer *et al.* 1988; Mizunuma *et al.* 1992). It was established that in jsd/jsd mice A spermatogonia are unable to differentiate. When seminiferous tubules from cryptorchid mice, containing proliferating A spermatogonia unable to differentiate, were transplanted into the scrotal testes of jsd/jsd mice, these cells differentiated and spermatids were formed. In contrast, in seminiferous tubules from jsd/jsd mice transplanted to testes of W/W mice, which have defective A spermatogonia themselves, no further development took

place. It was concluded that the intertubular environment of jsd/jsd mice is normal and that the defect must be intratubular (Mizunuma *et al*. 1992).

Fifth, another situation in which recovery from damage does not take place because spermatogonial differentiation is impaired, is that in which the Sertoli cell toxicant 2,5-hexanedione is given to rats (Boekelheide 1988). In these rats, spermatogenesis irreversibly deteriorates until only actively dividing A spermatogonia are left (Allard *et al*. 1995). For this model too, it was concluded that the block was at the A spermatogonia (Allard & Boekelheide 1996b). Interestingly, in *in vitro* experiments the proliferation and survival of the remaining A spermatogonia could be stimulated by adding SCF to the cultures (Allard *et al*. 1996a).

Finally, an interesting phenomenon was described in LBNF1 rats (Kangasniemi *et al*. 1996). After doses of 3.5–6 Gy of X-irradiation, there was an initial depletion of the seminiferous epithelium followed by recovery up to 4–6 weeks after irradiation. Surprisingly, thereafter spermatogenesis deteriorated until only actively proliferating undifferentiated spermatogonia remained, apparently unable to differentiate. However, these undifferentiated spermatogonia could be triggered to start normal differentiation again by treatment of the rats with either a gonadotropin releasing hormone agonist or testosterone (Meistrich & Kangasniemi 1997). As yet, hormone receptors have not been conclusively shown to be expressed by spermatogonia, so probably after irradiation Sertoli cell function becomes impaired in these rats and they no longer adequately support spermatogonial differentiation. The transient change in hormone levels may induce normal function again in the Sertoli cells (Table 1).

In conclusion, the differentiation of undifferentiated spermatogonia into A_1 spermatogonia is a crucial phase during the spermatogenic process. Widely different situations such as a higher testicular temperature, vitamin A deficiency, Sertoli cell intoxication and radiation damage can cause a blockage of this differentiation step. As yet there is no clue how the differentiation of the undifferentiated spermatogonia is regulated and how this process becomes arrested in these situations. One possibility could be an impairment of Sertoli cell function which may have happened in all situations described above, except for that in W/W mice which lack the c-kit receptor. However, in this case the supportive action of the Sertoli cells is without effect as the spermatogonia cannot respond to the SCF secreted by the Sertoli cells.

Expression of apoptosis and cell cycle genes in spermatogonia

Recently, initial steps have been made in unraveling the molecular basis of the mechanisms that determine whether cells will progress through the cell cycle, differentiate or go into apoptosis. Many genes appeared to be involved and additional ones are discovered regularly. As yet, only a few of these genes have been studied with respect to their expression in the testis.

One of the genes that has been studied is *p53*, also referred to as the guardian of the genome, which has important functions in cell growth and differentiation (review Levine 1997). Using *in situ* hybridization, during normal mouse spermatogenesis, *p53* mRNA was only detected in early spermatocytes (Schwartz *et al*. 1993). The p53 protein was also found to be expressed in spermatocytes, as determined by using CAT reporter transgenic mice (Almon *et al*. 1993) and immunohistochemistry in the rat (Sjöblom *et al*. 1996). From these results it was concluded that p53 has a role during the meiotic prophase. In these studies no expression of p53 was found in normal spermatogonia, nor after irradiation. In the normal mouse testis, using immunohistochemistry, we also did not see p53 protein expression in spermatogonia. However, after a dose of 4 Gy of X-rays, high levels of p53 staining were observed in spermatogonia (Beumer *et al*. 1998). Because virtually all spermatogonia are doomed to go into apoptosis after a dose of 4 Gy of X-rays (Beumer *et al*. 1997), p53 expression in spermatogonia correlates with apoptosis induction. Moreover, at 10 days after irradiation increased numbers of giant cells were found (Beumer *et al*. 1998). As these giant cells are lethally damaged spermatogonial stem cells that eventually will disappear *via* apoptosis (Van Beek *et al*. 1984), it can be concluded that p53 is involved in stem cell apoptosis.

A well studied group of apoptosis effectors is the Bcl-2 family of proteins which can either induce or protect from apoptosis. Regularly, new proteins are discovered which belong to this family, characterized by the presence of Bcl-2 homology 1 and 2 (BH1 and BH2) domains. These domains allow the Bcl family members to form homodimers or heterodimers with each other. The balance between homo- and heterodimers is crucial for the decision whether or not a cell will enter the apoptotic pathway (Korsmeyer 1995; Kroemer 1997). Bcl-2 is not detected in the mammalian testis (Rodriguez *et al*. 1997; Beumer & De Rooij unpublished observations in the mouse). Nevertheless, Bcl-2 overexpression in spermatogenic cells has dramatic effects on spermatogenesis. During normal spermatogenesis, considerable numbers of spermatogonia go into apoptosis as a consequence of the density regulatory mechanism (see above). When Bcl-2 is overexpressed this density regulation does not take place, a massive accumulation of spermatogonia

occurs and subsequently both spermatogonia and the spermatocytes that are formed, go into apoptosis (Furuchi *et al*. 1996; Rodriguez *et al*. 1997). These results showed that the Bcl-2 family is important in spermatogenesis although the Bcl-2 protein itself does not seem to be normally involved. Probably, another family member, the *Bcl-x* gene is involved which produces at least two splice variants, Bcl-x_S and Bcl-x_L. Whereas Bcl-x_L suppresses apoptosis, Bcl-x_S has opposing effects. In mice overexpressing Bcl-x_L the same phenomena were seen as with overexpression of Bcl-2 (Rodriguez *et al*. 1997).

Bax is a 21 kD protein, belongs to the Bcl-2 family and is a potent inducer of apoptosis. While Bax is barely detectable using immunohistochemistry (Krajewski *et al*. 1995), Bax knock out mice were found to be sterile (Knudson *et al*. 1996; Rodriguez *et al*. 1997). The histological features of the testes from Bax knock out mice resembled that of the Bcl-2 and Bcl-x_L transgenic testes, i.e. accumulation of spermatogonia. Although Bax is an apoptosis inducer, apoptosis is massively present in the Bax knock out mouse testis. Hence, spermatogonia and early spermatocytes can also go into apoptosis *via* a pathway not involving the Bax protein. Clearly, the Bcl-2 family has a role in the regulation of apoptosis in germ cells. To date, only a few of the many members of this family have been studied with respect to a possible role in the testis, but Bcl-*x* and Bax seem involved in regulating germ cell numbers and the removal of damaged cells.

Spermatogonial stem cell transplantation

An exciting new development has been the establishment of a method to transplant spermatogonial stem cells from one mouse to another (Brinster *et al*. 1994a,b; Dym 1994). A mixture of testicular cells was obtained from postnatal or foetal mice, and injected into seminiferous tubules of either mice that previously were given a cytostatic agent (busulphan) to destroy their seminiferous epithelium or of mutant W/W mice lacking a functional seminiferous epithelium because of a stem cell defect. The spermatogonial stem cells within the mixture of cells injected were found to be able to move to the basal membrane of the recipient tubules and to repopulate the injected tubules with a seminiferous epithelium that in some tubules was normal while in others some impairment was noted (Russell *et al*. 1996). In addition, some recipient mice were found to become fertile again thanks to spermatozoa derived from donor stem cells. It has also proved possible to transplant rat spermatogonial stem cells into the testes of immunodeficient mice (Clouthier *et al*. 1996). Ultrastructural observations revealed that the rat spermatogonial stem cells initiated repopulating colonies showing a seminiferous epithelium with normal cellular associations (epithelial stages), while being supported by mouse Sertoli cells (Russell & Brinster 1996). This was especially remarkable when one takes into account that the duration of the cycle of the seminiferous epithelium of the rat is about 50% longer than that of the mouse. It further proved possible to cryopreserve spermatogonial stem cells (Avarbock *et al*. 1996). Recently, the method has been extended by transplanting stem cells *via* injection of the rete testis or of the efferent ductules (Ogawa *et al*. 1997).

Although the spermatogonial stem cell transplantation method has not yet been applied experimentally, there are many new possibilities. First, it will become possible to study the effect of a particular treatment or of a particular gene mutation on spermatogenesis and on the somatic cells in the testis (including Sertoli cells) separately. In the normal situation it is often difficult to distinguish between the effects of a treatment or mutation on germ cells or on somatic cells. Second, it provides a new tool to study the role of those genes in spermatogenesis, that upon general over-expression or in a knock-out situation would be lethal to the animals.

Neoplasia

In adult men, seminomas and nonseminomatous germ cell tumours or a combination of both, are the most important testicular tumours. Both tumour types are thought to be derived from a common dysplastic germ cell precursor, the carcinoma *in situ* cells (CIS) (Skakkebaek *et al*. 1987). CIS cells have histological characteristics of gonocytes, are rich in glycogen and express germ cell-specific alkaline phosphatase. Although seminomas and nonseminomatous germ cell tumours are only found in the adult testis, CIS cells can already be seen in the prepuberal testis. This has led to the idea that the origin of the tumour must be sought before puberty and even before birth. Recently, it was found that for a number of immunohistochemical markers CIS cells resemble the gonocytes present in the 9 week-old foetus and much less the primordial germ cells in the 6 weeks old foetus (Jorgensen *et al*. 1995). From this it was concluded that CIS cells most likely derive from gonocytes, and not from the primordial germ cells. Interestingly, CIS cells do not form intercellular bridges (Gondos 1993), indicating that these cells are blocked in the very first visible step of differentiation. An alternative hypothesis for the origin of human germ cell

tumours is that they arise from spermatocytes in the prophase of meiosis (Chaganti *et al*. 1994).

Unfortunately, there are no animal models for seminomas and nonseminomatous germ cell tumours as they occur in the human. Germ cell tumours found in 129/Sv-ter mice and in dogs appeared different from human tumours (Walt *et al*. 1993; Looijenga *et al*. 1994), the seminomas described for the dog resembling spermatocytomas occurring in elderly men. The *in vitro* models developed for human germ cell tumours are not yet suitable for detailed studies on the development of these tumours and to study their progression with time (Berends *et al*. 1991; Olie *et al*. 1994, 1995).

Conclusions and perspectives

The behaviour of spermatogonial stem cells and the other spermatogonial cell types during development and in the adult testis has now been described in considerable detail. Emphasis now will be on studying the regulation of spermatogonial behaviour. While important data have already been obtained on the regulation of the inhibition of the proliferation of the undifferentiated spermatogonia, nothing is known yet about the mechanisms that induce proliferation of these cells. Many experimental situations are known in which an arrest in the differentiation of the undifferentiated spermatogonia into differentiating type spermatogonia occurs; this differentiation step is clearly a rather vulnerable one. Obtaining knowledge on the way the proliferative activity of undifferentiated spermatogonia can be stimulated and on how their differentiation is regulated will be a major goal in future testis research and could well be of clinical importance for treating oligospermic patients.

The first steps are being made in unravelling the molecular mechanisms that regulate spermatogonial behaviour. The P53 and the Bcl-2 families have already been found to be important. However, there are many other regulatory genes the importance of which to the testis remain to be ascertained. In elucidating the role of regulatory genes in spermatogenesis, the recently developed technique of spermatogonial stem cell transplantation will no doubt prove to be invaluable.

References

Allard E.K., Blanchard K.T. & Boekelheide K. (1996) Exogenous stem cell factor (SCF) compensates for altered endogenous SCF expression in 2,5-hexanedione-induced testicular atrophy in rats. *Biol. Reprod.* **55,** 185–193.

Allard E.K. & Boekelheide K. (1996) Fate of germ cells in 2,5-hexanedione-induced testicular injury. II. Atrophy persists due to a reduced stem cell mass and ongoing apoptosis. *Toxicol. Appl. Pharmacol.* **137,** 149–156.

Allard E.K., Hall S.J. & Boekelheide K. (1995) Stem cell kinetics in rat testis after irreversible injury induced by 2,5-hexanedione. *Biol. Reprod.* **53,** 186–192.

Almon E., Goldfinger N., Kapon A., Schwartz D., Levine A.J. & Rotter V. (1993) Testicular tissue-specific expression of the p53 suppressor gene. *Dev. Biol.* **156,** 107–116.

Avarbock M.R., Brinster C.J. & Brinster R.L. (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. *Nature Med.* **2,** 693–696.

Beamer W.G., Cunliffe-Beamer T.L., Shultz K.L., Langley S.H. & Roderick T.H. (1988) Juvenile spermatogonial depletion (jsd): A genetic defect of germ cell proliferation of male mice. *Biol. Reprod.* **38,** 899–908.

Bellvé A.R., Cavicchia J.C., Millette C.F., O'Brien D.A., Bhatnagar Y.M. & Dym M. (1977) Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J. Cell Biol.* **74,** 68–85.

Berends J.C., Schutte S.E., Van Dissel-Emiliani F.M.F., De Rooij D.G., Looijenga L.H.J. & Oosterhuis J.W. (1991) Significant improvement of the survival of seminoma cells *in vitro* by use of a rat Sertoli cell feeder layer and serum-free medium. *J. Natl. Cancer Inst.* **83,** 1400–1403.

Beumer T.L., Roepers-Gajadien H.L., Gademan I.S., Rutgers D.H. & de Rooij D.G. (1997) P21 $^{(Cip1/Waf1)}$ expression in the mouse testis before and after X-irradiation. *Mol. Rep. Dev.* **47,** 240–247.

Beumer T.L., Roepers-Gajadien H.L., van Buul P.P.W., Gil-Gomez G., Gademan I.S., Rutgers D.H. & De Rooij D.G. (1998) The role of the tumor suppressor p53 in spermatogenesis. *Cell Death Differ.* in press.

Boekelheide K. (1988) Rat testis during 2,5-hexanedione intoxication and recovery. I. Dose response and the reversibility of germ cell loss. *Toxicol. Appl. Pharmacol.* **92,** 18–27.

Brinster R.L. & Avarbock M.R. (1994) Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA* **91,** 11303–11307.

Brinster R.L. & Zimmerman J.W. (1994) Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. USA* **91,** 11298–11302.

Chaganti R.S.K., Rodriguez E. & Mathew S. (1994) Origin of adult male mediastinal germ-cell tumours. *Lancet* **343,** 1130–1132.

Clermont Y. & Bustos-Obregon E. (1968) Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted 'in toto'. *Am. J. Anat.* **122,** 237–248.

Clermont Y. & Hermo L. (1975) Spermatogonial stem cells in the albino rat. *Am. J. Anat.* **142,** 159–176.

Clermont Y. & Mauger A. (1974) Existence of a spermatogonial chalone in the rat testis. *Cell Tissue Kinet.* **7,** 165–172.

Clermont Y. & Perey B. (1957) Quantitative study of the cell population of the seminiferous tubules of immature rats. *Am. J. Anat.* **100,** 241–268.

Clouthier D.E., Avarbock M.R., Maika S.D., Hammer R.E. & Brinster R.L. (1996) Rat spermatogenesis in mouse testis. *Nature* **381,** 418–421.

Courot M., Hochereau-de Reviers M.T. & Ortavant R. (1970) Spermatogenesis. In: *The testis* (Eds. A.D. Johnson, W.R. Gomes N.L. & Vandemark), New York: Academic Press. pp. 339–432.

de Rooij D.G. (1980) Effect of testicular extracts on proliferation

of spermatogonia in the mouse. *Virchows Arch. B. Cell Path.* **33,** 67–75.

DE ROOIJ D.G. & JANSSEN J.M. (1987) The regulation of the density of spermatogonia in the seminiferous epithelium of the Chinese hamster. I. Undifferentiated spermatogonia. *Anat. Rec.* **217,** 124–130.

DE ROOIJ D.G. & LOK D. (1987) The regulation of the density of spermatogonia in the seminiferous epithelium of the Chinese hamster. II. Differentiating spermatogonia. *Anat. Rec.* **217,** 131–136.

DE ROOIJ D.G., LOK D. & WEENK D. (1985) Feedback regulation of the proliferation of the undifferentiated spermatogonia in the Chinese hamster by the differentiating spermatogonia. *Cell Tissue Kinet.* **18,** 71–81.

DE ROOIJ D.G. & VAN DISSEL-EMILIANI F.M.F. (1997) Regulation of proliferation and differentiation of stem cells in the male germ line. In *Stem Cells*. (Ed C.S. Potten). London: Academic Press. pp. 283–313.

DE ROOIJ D.G., VAN PELT A.M.M., DE KANT H.J.G., VAN DER SAAG P.T., PETERS A.H.F.M., HEYTING C. & DE BOER P. (1994) Role of retinoids in spermatogonial proliferation and differentiation and the meiotic prophase. In *Function of somatic cells in the testis*. Ed A. Bartke. New York: Springer Verlag, pp. 345–361.

DUPRESSOIR A. & HEIDMANN T. (1996) Germ line-specific expression of intracisternal A-particle retrotransposons in transgenic mice. *Mol. Cell. Biol.* **16,** 4495–4503.

DYM M. (1994) Commentary: Spermatogonial stem cells of the testis. *Proc. Natl. Acad. Sci. USA* **91,** 11287–11289.

FRANCHI L.L. & MANDL A.M. (1964) The ultrastructure of germ cells in foetal and neonatal male rats. *J. Embryol. Exp. Morph.* **12,** 289–308.

FURUCHI T., MASUKO K., NISHIMUNE Y., OBINATA M. & MATSUI Y. (1996) Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development* **122,** 1703–1709.

GINSBURG M., SNOW M.H.L. & MCLAREN A. (1990) Primordial germ cells in the mouse embryo during gastrulation. *Development* **110,** 521–528.

GONDOS B. (1993) Ultrastructure of developing and malignant germ cells. *Eur. Urol.* **23,** 68–75.

HILSCHER B., HILSCHER W., BÜLTHOFF-OHNOLZ B., KRÄMER U., BIRKE A., PELZER H. & GAUSS G. (1974) Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. *Cell Tissue Res.* **154,** 443–470.

HUANG H.F.S. & HEMBREE W.C. (1979) Spermatogenic response to vitamin A in vitamin A deficient rats. *Biol. Reprod.* **21,** 891–904.

HUCKINS C. (1971a) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat. Rec.* **169,** 533–558.

HUCKINS C. (1971b) The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. *Cell Tissue Kinet.* **4,** 313–334.

HUCKINS C. (1971c) Cell cycle properties of differentiating spermatogonia in adult Sprague-Dawley rats. *Cell Tissue Kinet.* **4,** 139–154.

HUCKINS C. & CLERMONT Y. (1968) Evolution of gonocytes in the rat testis during late embryonic and post – natal life. *Arch. Anat. Hist. Embryol.* **51,** 343–354.

IRONS M.I. & CLERMONT Y. (1979) Spermatogonial chalone (s): Effect on the phases of the cell cycles of type A spermatogonia in the rat. *Cell Tissue Kinet.* **12,** 425–433.

JORGENSEN N., RAJPERT-DE MEYS E., GRAEM N., MULLER J., GIWERCMAN A. & SKAKKEBAEK N.E. (1995) Expression of immunohistochemical markers for testicular carcinoma in situ by normal human fetal germ cells. *Lab. Invest.* **72,** 223–231.

KANGASNIEMI M., HUHTANIEMI I. & MEISTRICH M.L. (1996) Failure of spermatogenesis to recover despite the presence of A spermatogonia in the irradiated LBNF1 rat. *Biol. Reprod.* **54,** 1200–1208.

KLUIN P.M. & DE ROOIJ D.G. (1981) A comparison between the morphology and cell kinetics of gonocytes and adult type differentiated spermatogonia in the mouse. *Int. J. Androl.* **4,** 475–493.

KNUDSON C.M., TUNG K.S.K., TOURTELOTTE W.G., BROWN G.A.J. & KORSMEYER S.J. (1996) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270,** 96–99.

KORSMEYER S.J. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270,** 96–99.

KOSHIMIZU U., SAWADA K., TAJIMA Y., WATANABE D. & NISHIMUNE Y. (1991) White-spotting mutations affect the regenerative differentiation of testicular germ cells: demonstration by experimental cryptorchidism and its surgical reversal. *Biol. Reprod.* **45,** 642–648.

KRAJEWSKI S., BODRUG S., KRAJEWSKA M., SHABAIK A., GASCOYNE R., BEREAN K. & REED J.C. (1995) Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 production suggests a unique role for Mcl-1 in control of programmed cell death *in vivo*. *Am. J. Pathol.* **146,** 1309–1319.

KROEMER G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nature Med.* **3,** 614–620.

LAJTHA L.G. (1979) Stem cell concepts. *Differentiation* **14,** 23–34.

LAWSON K.A. & PEDERSON R.A. (1992) Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. In *CIBA foundation symposium 165 Post implantation development in the mouse*. New York: John Wileys & Sons, pp. 3–26.

LEVINE A.J. (1997) P53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331.

LOK D. & DE ROOIJ D.G. (1983a) Spermatogonial multiplication in the Chinese hamster. I. Cell cycle properties and synchronization of differentiating spermatogonia. *Cell Tissue Kinet.* **16,** 7–18.

LOK D. & DE ROOIJ D.G. (1983b) Spermatogonial multiplication in the Chinese hamster. III. Labelling indices of undifferentiated spermatogonia throughout the cycle of the seminiferous epithelium. *Cell Tissue Kinet.* **16,** 31–40.

LOK D., WEENK D. & DE ROOIJ D.G. (1982) Morphology, proliferation and differentiation of undifferentiated spermatogonia in the Chinese hamster and the ram. *Anat. Rec.* **203,** 83–99.

LOK D., JANSEN M.T. & DE ROOIJ D.G. (1983) Spermatogonial multiplication in the Chinese hamster. II. Cell cycle properties of undifferentiated spermatogonia. *Cell Tissue Kinet.* **16**, 19–29.

LOOIJENGA L.H., OLIE R.A., VAN DER GAAG I., VAN SLUIJS F.J., MATOSKA J., PLOEM-ZAAIJER J. & KNEPFLE C. (1994) Seminomas of the canine testis. Counterpart of spermatocytic seminoma of men? *Lab. Invest.* **71,** 490–496.

MEISTRICH M.L. & KANGASNIEMI M. (1997) Hormone treatment after irradiation stimulates recovery of rat spermatogenesis from surviving spermatogonia. *J. Androl.* **18,** 80–87.

MITRANOND V., SOBHON P., TOSUKHOWONG P. & CHINDADUANGRAT W. (1979) Cytological changes in the testis of vitamin A deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. *Acta Anat.* **103,** 159–168.

MIZUNUMA M., DOHMAE K., TAJIMA Y., KOSHIMIZU U., WATANABE D. & NISHIMUNE Y. (1992) Loss of sperm in juvenile spermatogonial depletion (jsd) mutant mice is ascribed to a defect of intratubular environment to support germ cell differentiation. *J. Cell. Physiol.* **150,** 188–193.

MORALES C. & GRISWOLD M.D. (1987) Retinol-induced stage synchronization in seminiferous tubules of the rat. *Endocrinology* **121,** 432–434.

NISHIMUNE Y., AIZAWA S. & KOMATSU T. (1978) Testicular germ cell differentiation *in vivo*. *Fertil. Steril.* **29,** 95–102.

NISHIMUNE Y. & HANEJI T. (1981) Testicular DNA synthesis *in vivo*: Comparison between unilaterally cryptorchid testis and contralateral intact testis in mouse. *Arch. Androl.* **6,** 61–65.

NISHIMUNE Y. & OKABE M. (1993) Mammalian male gametogenesis: growth, differentiation and maturation of germ cells. *Dev. Growth Differ.* **35,** 479–486.

NOVI A.M. & SABA P. (1968) An electron microscopic study of the development of rat testis in the first 10 postnatal days. *Z. Zellforsch.* **86,** 313–326.

OAKBERG E.F. (1971) Spermatogonial stem-cell renewal in the mouse. *Anat. Rec.* **169,** 515–532.

OGAWA T., ARÉCHAGA J.M., AVARBOCK M.R. & BRINSTER R.L. (1997) Transplantation of testis germinal cells into mouse seminiferous tubules. *Int J. Dev Biol.* **41,** 111–122.

OLIE R.A., LOOIJENGA H.J., DEKKER M.C., DE JONG F.H., DE ROOIJ D.G. & OOSTERHUIS J.W. (1994) Growth of human seminoma cells on STO feeder depends on phenotype, presence of fetal calf serum and added growth factors. *Adv. Biosciences* **91,** 95–106.

OLIE R.A., LOOIJENGA L.H.J., DEKKER M.C., DE JONG F.H., VAN DISSEL-EMILIANI F.M.F., DE ROOIJ D.G., VAN DER HOLT B. & OOSTERHUIS J.W. (1995) Heterogeneity in the *In vitro* survival and proliferation of human seminoma cells. *Br. J. Cancer* **71,** 13–17.

PARVINEN M. (1982) Regulation of the seminiferous epithelium. *Endocrine Rev.* **3,** 404–417.

RESNICK J.L., BIXLER L.S., CHENG L. & DONOVAN P.J. (1992) Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359,** 550–551.

RODRIGUEZ I., ODY C., ARAKI K., GARCIA I. & VASSALLI P. (1997) An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J.* **16,** 2262–2270.

RUSSELL L.D. & BRINSTER R.L. (1996) Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules. *J. Androl.* **17,** 615–627.

RUSSELL L.D., ETTLIN R.A., HIKIM A.P.S. & CLEGG E.D. (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater Fl.

RUSSELL L.D., FRANCA L.R. & BRINSTER R.L. (1996) Ultrastructural observations of spermatogenesis in mice resulting from transplantation of mouse spermatogonia. *J. Androl.* **17,** 603–614.

SAPSFORD C.S. (1962) Changes in the cells of the sex cords and the seminiferous tubules during development of the testis of the rat and mouse. *Austr. J. Zool.* **10,** 178–192.

SCHWARTZ D., GOLDFINGER N. & ROTTER V. (1993) Expression of p53 protein in spermatogenesis is confined to the tetraploid pachytene primary spermatocytes. *Oncogene* **8,** 1487–1494.

SJÖBLOM T. & LÄHDETIE J. (1996) Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p 53 in meiotic recombination and repair. *Oncogene* **12,** 2499–2505.

SKAKKEBAEK N.E., BERTHELSEN J.G., GIWERCMAN A. & MULLER J. (1987) Carcinoma-in-situ of the testis: possible origin from gonocytes and precursor of all types of germ cell tumours except spermatocytoma. *Int. J. Androl.* **10**, 19–28.

TAJIMA Y., SAKAMAKI K., WATANABE D., KOSHIMIZU U., MATSUWAZA T. & NISHIMUNE Y. (1991) Steel-Dickie (Sl^d) mutation affects both maintenance and differentiation of testicular germ cells in mice. *J. Reprod. Fert.* **91,** 441–449.

TAM P. & SNOW M.H.L. (1981) Proliferation and migration of primordial germ cells during compensatory growth in the mouse embryo. *J. Embryol. Exp. Morph.* **64,** 133–147.

TEGELENBOSCH R.A.J. & DE ROOIJ D.G. (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101, F1 hybrid mouse. *Mutat. Res.* **290,** 193–200.

THUMANN A. & BUSTOS-OBREGON E. (1978) An 'in vitro' system for the study of rat spermatogonial proliferative control. *Andrologia* **10,** 22–25.

VAN BEEK M.E.A.B., DAVIDS J.A.G. & DE ROOIJ D.G. (1986) Variation in the sensitivity of the mouse spermatogonial stem cell population to fission neutron irradiation during the cycle of the seminiferous epithelium. *Radiat. Res.* **108,** 282–295.

VAN BEEK M.E.A.B., MEISTRICH M.L. & DE ROOIJ D.G. (1990) Probability of self-renewing divisions of spermatogonial stem cells in colonies formed after fission neutron irradiation. *Cell Tissue Kinet.* **23,** 1–16.

VAN BEEK M.E.A.B., DAVIDS J.A.G., VAN DE KANT H.J.G. & DE ROOIJ D.G. (1984) Response to fission neutron irradiation of spermatogonial stem cells in different stages of the cycle of the seminiferous epithelium. *Radiat. Res.* **97,** 556–569.

VAN DER MEER Y., HUISKAMP R., DAVIDS J.A.G., VAN DER TWEEL I. & DE ROOIJ D.G. (1992a) The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X-irradiation. *Radiat. Res.* **130,** 289–295.

VAN DER MEER Y., HUISKAMP R., DAVIDS J.A.G., VAN DER TWEEL I. & DE ROOIJ D.G. (1992b) The sensitivity for X-rays of spermatogonia committed to differentiate. *Radiat. Res.* **130,** 296–302.

VAN DISSEL-EMILIANI F.M.F., DE BOER-BROUWER M., VAN DER DONK J.A. & DE ROOIJ D.G. (1993) Survival and proliferation of rat gonocytes *in vitro*. *Cell Tissue Res.* **273,** 141–147.

VAN HAASTER L.H. (1993) Regulation of postnatal testicular development in rodents. Thesis, Utrecht.

VAN HAASTER L.H. & DE ROOIJ D.G. (1993) Spermatogenesis is accelerated in the immature Djungarian and Chinese hamster and the rat. *Biol. Reprod.* **49,** 1229–1235.

VAN HAASTER L.H. & DE ROOIJ D.G. (1994) Partial synchronization of spermatogenesis in the immature Djungarian hamster, but not in the Wistar rat. *J. Reprod. Fertil.* **101,** 321–326.

VAN KEULEN C.J.G. & DE ROOIJ D.G. (1975) Spermatogenetic clones developing from repopulating stem cells surviving a high dose of an alkylating agent. I. First 15 days after injury. *Cell Tissue Kinet.* **8,** 543–551.

VAN PELT A.M.M. & DE ROOIJ D.G. (1990) The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. *Biol. Reprod.* **42,** 677–682.

VAN PELT A.M.M. & DE ROOIJ D.G. (1991) Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high, replicate doses support the full development of spermatogenic cells. *Endocrinology* **128,** 697–704.

VAN PELT A.M.M., VAN DISSEL-EMILIANI F.M.F., GAEMERS I.C., VAN DE BURG M., TANKE H.J. & DE ROOIJ D.G. (1995) Characteristics of A spermatogonia and preleptotene spermatocytes in the vitamin A deficient rat testis. *Biol. Reprod* **53,** 568–576.

VERGOUWEN R.P.F.A., JACOBS S.G.P.M., HUISKAMP R., DAVIDS J.A.G. & DE ROOIJ D.G. (1991) Proliferative activity of interstitial cells, Sertoli cells and gonocytes during testicular development in the mouse. *J. Reprod. Fert.* **93,** 233–243.

WALT H., OOSTERHUIS J.W. & STEVENS L.C. (1993) Experimental testicular germ cell tumorigenesis in mouse strains with and without spontaneous tumours differs from development of germ cell tumours of the adult human testis. *Int. J. Androl.* **16,** 267–271.

ZAMBONI L. & MERCHANT H. (1973) The fine morphology of mouse primordial germ cells in extragonadal locations. *Am. J. Anat.* **137,** 299–336.