Absence of *mDazl* produces a final block on germ cell development at meiosis

P. T. K. Saunders¹, J. M. A. Turner², M. Ruggiu³, M. Taggart³, P. S. Burgoyne², D. Elliott⁴ and H. J. Cooke³

¹Medical Research Council Human Reproductive Sciences Unit, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK; ²Division of Developmental Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK; ³Medical Research Council Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, UK; and ⁴University of Newcastle, Institute for Human Genetics, International Centre for Life, Central Parkway, Newcastle Upon Tyne, NE1 3BZ, UK

The autosomal gene *DAZL* is a member of a family of genes (*DAZL*, *DAZ*, *BOULE*), all of which contain a consensus RNA binding domain and are expressed in germ cells. Adult male and female mice null for *Dazl* lack gametes. In order to define more precisely the developmental stages in germ cells that require *Dazl* expression, the patterns of germ cell loss in immature male and female wild-type (+/+, WT) and *Dazl* –/– (*Dazl*KO) mice were analysed. In females, loss of germ cells occurred during fetal life and was coincident with progression of cells through meiotic prophase. In males, testes were recovered from WT and *Dazl*KO males obtained before and during the first wave of spermatogenesis (days 2–19). Mitotically active germ cells were present up to and including day 19.

Functional differentiation of spermatogonia associated with detection of c-kit positive cells did not depend upon expression of *Dazl*. RBMY-positive cells (A, intermediate, B spermatogonia, zygotene and preleptotene spermatocytes) were reduced in *Dazl*KO compared with WT testes. Staining of cell squashes from day 19 testes with anti-γ-H2AX and anti-SCP3 antibodies showed that germ cells from *Dazl*KO males were unable to progress beyond the leptotene stage of meiotic prophase I. It was concluded that in the absence of *Dazl*, germ cells can complete mitosis, and embark on functional differentiation but that, in both sexes, progression through meiotic prophase requires this RNA binding protein.

Introduction

The key role of RNA binding proteins in fertility has been highlighted by the identification of two gene families on the human Y chromosome, *RBMY* and *DAZ*, loss of either of which is associated with failure of germ cells to mature during spermatogenesis (for review, see Elliott and Cooke, 1997). *RBMY* (Ma *et al.*, 1993) is conserved on the Y chromosome of mammals and marsupials (Delbridge *et al.*, 1997), whereas *DAZ* (Reijo *et al.*, 1995) is found only on the Y chromosome in man and Old World primates (Cooke *et al.*, 1996; Shan *et al.*, 1996). In contrast, two autosomal homologues of *DAZ* (*DAZL* and *BOULE*) have been identified in numerous species including humans (Saxena *et al.*, 1996; Yen *et al.*, 1996; Xu *et al.*, 2001), mice (Cooke *et al.*, 1996; Xu *et al.*, 2001), *Xenopus* (*XDazl*; Houston and King, 2000), *Caenorhabditis elegans* (Karashima *et al.*, 2000) and *Drosophila* (*Boule*; Eberhart *et al.*, 1996). The *DAZL*/*DAZ* genes evolved after a series of gene duplications from

the ancestral *Boule* gene first identified in the fly (Xu *et al.*, 2001). Although sequence divergence between *Boule* genes in flies and humans is low (Xu *et al.*, 2003), *DAZ* and *DAZL* homologues have diverged rapidly in primates (Bielawski and Yang, 2001). Immunoexpression studies on sections from rodent and human testes using antibodies specific for *DAZ*, *DAZL* or *BOULE* have shown that all are expressed exclusively in the germline lineage although their patterns of expression are distinct (Xu *et al.*, 2001).

The impact of loss of expression of *DAZ*/*DAZL*/*BOULE* family members on germ cell maturation varies among species. For example, in humans, deletion of multiple copies of the *DAZ* gene results in variable germ cell loss (Reijo *et al.*, 1995, 1996) even when *DAZL* expression can be detected (Ruggiu *et al.*, 2000). Targeted disruption of *Dazl* in mice (a species which does not have a Y-encoded *Daz* gene) results in infertility in both males and females (Ruggiu *et al.*, 1997). In testes of adult *Dazl*KO male mice, staining with proliferating cell nuclear antigen (PCNA) antibodies identifies a population of actively proliferating germ cells (Slee *et al.*, 1999) and

Email: p.saunders@ed.ac.uk

analysis indicates that most of these cells are pre-meiotic A type spermatogonia (Slee *et al.*, 1999; Schrans-Stassen *et al.*, 2001). In *C. elegans* and *Drosophila*, loss of *cDazl* or *Boule* respectively is associated with meiotic arrest (Eberhart *et al.*, 1996; Karashima *et al.*, 2000).

The present authors were concerned that the phenotype of the adult male *Dazl* knockouts might be consistent with a mitotic defect, whereas loss of germ cells during fetal life in females was consistent with failure of cells to complete meiotic prophase. Therefore, the aims of the present study were to determine whether migration of germ cells to the urogenital ridge is normal, to re-examine the timing of germ cell loss in the female *Dazl*KO and focus on the development of germ cells in male *Dazl*KO mice from birth until day 19 in order to examine their fate as they resume mitotic activity after birth and enter meiosis during the first wave of spermatogenesis. It was confirmed that female germ cells are lost on and before day 17.5 after mating and therefore fail to complete meiotic prophase. In males, germ cells were capable of mitotic activity and expressed markers of differentiation into A and B type spermatogonia (c-kit and RBMY). On day 19, the most advanced germ cells in the testes of *Dazl*KO mice were a few leptotene spermatocytes, but synaptonemal complexes were not formed, demonstrating for the first time that *Dazl* protein is essential for progression of cells to zygotene stage during the first wave of spermatogenesis.

Materials and Methods

Animals and tissue collection

Dazl^{Tm1hgu/m1hgu} mice (Ruggiu *et al.*, 1997) were housed under standard conditions and fed *ad libitum*. Ovaries were removed on days 17.5 and 19.5 after mating, and on days 4 and 12 after birth. Testes were recovered from immature mice on days 2–19 (day 1 is the day of birth) and from adults. Tissues were fixed in Bouin's fixative for 2 h (fetal samples) or 6 h, transferred into 70% ethanol and processed into paraffin wax using standard methods. At the time of fixation, tissue was also taken for genotyping by standard methods.

Generation and breeding of mice was covered by Home Office project licence and was approved by institutional ethics committees.

Antibodies used for immunohistochemistry on tissue sections

A polyclonal antibody directed against murine RBMY (RNA binding motif, Y chromosome; Elliott *et al.*, 1996; Delbridge *et al.*, 1999) was prepared as follows: amino acids 113–232 of mouse RBMY fused with glutathione S-transferase (GST) or thioredoxin were expressed and purified from *Escherichia coli* BL21 cells as described by Elliott *et al.* (2000). A rabbit was immunized with the GST

fusion protein (Diagnostics Scotland, Carlisle), and the resulting antiserum affinity purified using the thioredoxin fusion protein immobilized on a Sulfolink affinity column (Pierce). The purified antiserum recognized a single testis-specific band of the appropriate molecular mass that was preabsorbable with the immunizing protein (data not shown).

PCNA is a protein expressed in cells during early G1 and S phases of the cell cycle that has been used to detect proliferating cells within the mouse testis (Slee *et al.*, 1999). In the present study, it was detected using a monoclonal antibody (PC-10 Waseem and Lane, 1990) supplied by Dako (High Wycombe, Bucks).

C-kit, the receptor for the kit (steel) ligand is not expressed in spermatogonial stem cells in the adult mouse testis but can be detected in differentiating spermatogonia (Aal A1–A4; Schrans-Stassen *et al.*, 1999). C-kit-positive cells were detected with a rabbit polyclonal antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Immunohistochemistry

Sections (5 µm) were cut and mounted on to slides coated with 3-aminopropyl triethoxy-silane (Sigma Chemical Co., Poole, Dorset), dried overnight (50°C) dewaxed, rehydrated in graded ethanols, blocked in methanol–peroxide sections, washed once each (5 min) in distilled water and TBS (0.05 mol Tris–HCl l⁻¹ pH 7.4, 0.85% NaCl) and blocked for 30 min using normal rabbit serum diluted in TBS (1:5, NRS–TBS). Sections were incubated with primary antibody (anti-c-kit at 1/100; anti-RBM at 1 in 50; anti-proliferating cell nuclear antigen (PCNA) 1 in 100) diluted in TBS overnight at 4°C. Sections were washed twice in TBS (5 min each), incubated for 30 min with biotinylated swine anti-rabbit immunoglobulin (c-kit and RBMY) or rabbit anti-mouse immunoglobulin (PCNA) diluted 1:500 in NRS–TBS for 30 min, then washed again in TBS (2 × 5 min). Bound antibodies were detected according to standard methods (Ruggiu *et al.*, 2000). Images were captured using an Olympus Provis microscope (Olympus Optical Co., London) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY).

Immunostaining of squashed spermatogenic cells

Squashes of spermatogenic cells from mice killed on day 19 were carried out essentially as described by Page *et al.* (1998). Briefly, tail tips were taken at the time of death to confirm genotype. Testes were dissected from the scrotum, placed individually into small Petri dishes containing PBS, and the tunica albuginea was removed. The tubules were teased apart gently with forceps and then shaken gently to remove extratubular elements. The PBS was removed and replaced with fresh 2% (v/v) formaldehyde (Sigma) in PBS containing

Fig. 1. Ontogeny of germ cell development in wild-type (WT) and DazlKO (KO) mouse ovaries immunostained with proliferating cell nuclear antigen (PCNA). (a,b) Ovaries from fetal day 17.5 after mating. (a) In WT females, intense PCNA immunostaining is present in oogonia (P); most somatic cells are immunonegative. (b) In DazlKO females, in addition to resting oogonia (arrowheads) that appear intact, many immunopositive germ cells have a condensed-distorted nucleus (arrows) and appear depleted when compared with those of wild-type ovaries. (c,d) Day 4 after birth. (c) Follicles have already begun to organize around oocytes (O) in the wild-type ovary and granulosa cells were PCNA-positive, but (d) in the DazlKO females, no follicular structures or normal oocytes are present and the tissue is composed only of disorganized somatic cells.

0.05% (v/v) Triton X-100 (Sigma) at room temperature for 10 min. Small pieces of tubules were placed on ethanol-cleaned microscope slides, teased apart with forceps and squashed by placing a 22 mm $\times$ 50 mm coverslip over the tissue and pressing down briefly. The slides were immersed in liquid nitrogen and stored with coverslips in place at -70°C . Before immunostaining, coverslips were removed and slides were washed three times in PBS. Immunostaining was undertaken as described in detail by Mahadevaiah *et al.* (2001) using antibodies directed against γ -H2AX, histone 1 testis variant (H1t) and synaptonemal complex protein 3 (SCP3); all samples were mounted with Vectastain with 4',6-diamidino-2-phenylindole (DAPI) and viewed using an Olympus IX170 inverted microscope.

Results

Ontogeny of germ cell development in WT and DazlKO female mice

Previous studies have shown that on day 15.5 after mating ovaries from DazlKO and WT mice both contain meiotic germ cells (Ruggiu *et al.*, 1997). Sections demonstrate that within 2 days (day 17.5 after mating)

germ cell loss has occurred and cells with abnormal condensed nuclei are detectable in the DazlKO ovaries (Fig. 1b). On day 4, no germ cells could be detected in the DazlKO females (Fig. 1d) consistent with previous reports that no follicles form in the absence of Dazl expression (McNeilly *et al.*, 2000).

Ontogeny of germ cell development in control and DazlKO male mice

In testes removed on days 2–19, germ cells immunopositive for PCNA were detected in testes from WT^{+/-} (heterozygotes, not shown) and DazlKO mice (Fig. 2). Considerable variation in the numbers of PCNA-positive germ cells was observed from DazlKO animal to DazlKO animal at all ages examined (days 2, 4, 6, 7, 8, 9, 12, 16 and 19). In some but not all testes, fewer germ cells were present in each tubule cross-section of the testes from the DazlKO males as compared with their WT or +/- littermates and this was more marked from day 7 onwards. For example, on day 9 (Fig. 2a,b) in all animals, the WT mice had groups of cells with small round immunopositive nuclei presumed to be preleptotene spermatocytes (arrowheads) and a fluid-filled lumen was detected in some tubules (Fig. 2a), but

Fig. 2. Ontogeny of germ cell development in postnatal wild-type (WT) and *Dazl*KO (KO) mouse testes immunostained with antibodies for proliferating cell nuclear antigen (PCNA) or c-kit. (a,b) The germ cell complement of day 9 testes as revealed by PCNA immunostaining; (a) in WT mice, in addition to spermatogonia, some tubules contain pre-leptotene spermatocytes (arrowheads) and in some tubules a fluid-filled lumen has formed (lu). (b) In *Dazl*KO testes, no lumina have formed and fewer PCNA-positive germ cells are present. (c,d) Day 16 testes. A marked contrast in germ cell complement is evident between (c) WT and (d) *Dazl*KO, with abundant pachytene spermatocytes (P) present in normal males. Testes of *Dazl*KO males contain immunopositive germ cells but apart from those close to the base of the tubule, their nuclei appear abnormal. A central lumen was detected in all WT but in only a few *Dazl*KO (lu) seminiferous tubules. C-kit-positive germ cells (brown immunostaining) were detected in both (e) WT and (f) *Dazl*KO testes on day 8 (arrows).

neither of these was seen in the *Dazl*KO testes, where the centre of the tubule was still occupied by Sertoli cell cytoplasm (Fig. 2b). As maturation of the first wave of spermatogenesis progressed, the seminiferous epithelium of WT mice became populated by germ cells in later stages of development (see Fig. 2c, day 16), whereas those from their *Dazl*KO siblings contained fewer PCNA positive cells and although lumina did form in some tubules (Fig. 2d), germ cells that were not adjacent to the basement membrane appeared abnormal (Fig. 2d).

C-kit-positive cells were detected in testes from WT and *Dazl*KO mice (Fig. 2e,f) consistent with differen-

tiation of proliferative spermatogonial stem cells into type A spermatogonia (Schrans-Stassen *et al.*, 1999).

RBMV expression was germ cell-specific in all samples (Fig. 3) consistent with the results of studies that used other anti-RBMV antibodies (Mahadevaiah *et al.*, 1998). In adult mice, using the antibody described in this paper, the present authors have consistently detected RBMV staining in the nuclei of spermatogonia (A, B and intermediate) and pre-leptotene and zygotene spermatocytes (data not shown). In testes from WT and *Dazl*KO males recovered from days 2, 4 (Fig. 3a–d) and 6 (data not shown), variable numbers of immunopositive

Fig. 3. Ontogeny of germ cell development detected using immunohistochemistry to the germ cell-specific protein RBMY. Testes from (a,c,e,g) wild-type (WT) and (b,d,f,h) *Dazl*KO (KO) mice were compared and RBMY-positive cells detected in all sections examined. On days 2 (a,b), 4 (c,d) and 7 (e,f), although RBMY-positive cells were variable in most samples from *Dazl*KO males, fewer immunopositive cells were present within the seminiferous epithelium than in age-matched control WT testes. This finding was particularly marked on day 19, when (g) an organized layer of RBMY-positive cells was detected in WT testes, whereas in (h) *Dazl*KO samples, the germ cell complement was disorganized and the number of RBMY-positive cells was much reduced. P: pachytene spermatocytes; arrowheads: spermatogonia in contact with basement membrane; asterisks: RBMY-positive germ cells with abnormal appearance. Scale bar represents 50 μ m.

Fig. 4. Meiotic germ cells from day 19 wild-type (WT) and *Dazl*KO (KO) mouse testes. (a,c,e,g) Blue DAPI staining of all cell nuclei. Red staining is by (b,d) γ -H2AX and (f,h) SCP3. (b,f) WT testes had numerous germ cells that contained a sex body immunopositive for γ -H2AX and axial elements stained by SCP3 (early pachytene spermatocytes: short arrows; late pachytene spermatocytes: long arrows). Germ cells containing a sex body or axial elements were rare in *Dazl*KO samples (data not shown). Germ cells with (d) abundant nuclear staining for γ -H2AX or (h) faint flecks of staining for SCP3 identified as leptotene spermatocytes (arrowheads), were present in both WT and *Dazl*KO testes.

cells were detected with most, but not all, *Dazl*KO testes containing fewer RBMY-positive cells than age-matched controls. Multinucleated cells that had an abnormal appearance (Fig. 3) were detected more frequently in *Dazl*KO than in WT testes on days 2 and 4 (for example,

see Fig. 3b, inset). RBMY-positive cells that were present at the periphery of the seminiferous epithelium and had made contact with the basement membrane of the tubule were detected in the presence or absence of *Dazl* expression (insets to Fig. 3a–c). On days 7 (Fig. 3f), 8, 9 and 16 (data not shown), although the numbers of germ cells detected in the null testes remained highly variable, all samples from *Dazl*KO males contained fewer immunopositive cells than those from WT testes (day 7, Fig. 3e). On day 19, a marked difference in germ cell complement was apparent. Pachytene spermatocytes were present in all tubules in WT testes (Fig. 3g) and an organized layer of RBMY-positive cells was present. In *Dazl*KO samples, the germ cell complement was disorganized and the numbers of RBMY-positive cells much reduced (Fig. 3h).

Identification of a meiotic defect in *Dazl*KO mice

Squash preparations from day 12 (data not shown) and day 19 testes were stained with antibodies that could differentiate between germ cell stages as they progressed through meiosis to define more precisely the observed block in germ cell development. On day 19, H1t staining was not detectable in the squashes from *Dazl*KO mice (data not shown). This protein is expressed in germ cells from mid-pachytene onwards (Grimes *et al.*, 1992) and therefore these results would be consistent with the absence of these cells. In WT mice on day 19, most of the germ cells stained with antibody directed against γ -H2AX were pachytene spermatocytes with sex body staining (Fig. 4b) and considerably fewer were leptotene spermatocytes (arrowheads). The ratio of pachytene:leptotene spermatocytes was approximately 85:1. However, in their *Dazl*KO littermates (Fig. 4d), the reverse was true with most immunopositive cells showing the abundant nuclear staining characteristic of leptotene spermatocytes (Mahadevaiah *et al.*, 2001) and only 1 in 30 germ cells containing a positive sex body. Consistent with this finding, staining with antibodies directed against SCP3, which is specific for the axial elements formed as precursors of the synaptonemal complex (Dobson *et al.*, 1994), identified cells at leptotene (Fig. 4f,h) in both WT and *Dazl*KO males. However, in the WT males, numerous germ cells containing clear synaptonemal complexes consistent with progression into early and mid-late pachytene were also present, whereas in the *Dazl*KO testes, no cells had commenced synaptonemal complex formation, indicating that they had not progressed beyond leptotene.

Discussion

The importance of the expression of the members of the *DAZ/DAZL/BOULE* gene family in germ cell development has been highlighted by studies in a number

of species from mouse to fly. In *Drosophila*, expression of *Boule* is restricted to testes (Eberhart *et al.*, 1996) and loss of function is associated with male infertility arising from a block in meiotic progression of germ cells at the G2–M transition, although some differentiation of spermatids does occur. In *C. elegans*, loss of *cDazl* has no effect on male fertility but results in meiotic arrest in females (Karashima *et al.*, 2000). In mice, *Dazl* protein is expressed in both male and female germ cells and its expression is essential for the fertility of both sexes (Ruggiu *et al.*, 1997, 2000; Xu *et al.*, 2001). Studies in several species have demonstrated functional conservation among *DAZ/DAZL/BOULE* family members. For example, insertion of human *DAZ* or *DAZL* transgenes into *Dazl*KO mice can increase the numbers of germ cells (Slee *et al.*, 1999; Vogel *et al.*, 2002) but is not able to restore fertility. Meiotic defects in *Boule* mutant *Drosophila* can be rescued by the *Xdazl* gene from frogs (Houston *et al.*, 1998) and by a human *BOULE* transgene (Xu *et al.*, 2003).

In female mice, germ cells enter meiosis on days 12.5–14.5 after mating and meiosis continues throughout fetal development until cells arrest at the diplotene stage of the first meiotic division. In contrast, although the number of male germ cells initially increases as a result of mitotic cell division, proliferation, as measured by incorporation of bromodeoxyuridine, stops on day 15.5 after mating, and is re-initiated on day 2 after birth (Nagano *et al.*, 2000). At around the time male germ cells resume mitosis, they also migrate to make contact with the basement membrane and differentiate to form spermatogonial stem cells (McGuinness and Orth, 1992). The first wave of spermatogenesis occurs synchronously during testis maturation and, in some mouse strains, leptotene spermatocytes at prophase I of meiosis are first detected in the testis at about day 10 (Bellve *et al.*, 1977; Malkov *et al.*, 1998).

In the ovary, mRNA for *Dazl* transcripts has been detected in fetal mice on day 12.5 after mating (Seligman and Page, 1998) and protein has been localized to germ cell cytoplasm on day 17.5 after mating (Ruggiu *et al.*, 1997). Loss of germ cells from the ovary of *Dazl*KO females occurs during fetal life. On day 15 after mating the ovaries of *Dazl*KO females contain early pachytene oögonia (Ruggiu *et al.*, 1997), some of which appear atretic, but, as shown in the present study, by day 17.5 after mating, substantial loss of germ cells has occurred and by day 4 after birth no more germ cells can be detected. These data are consistent with failure of oögonia to progress through meiotic prophase (Dekel, 1996).

The germ cell complements in the testes from WT and *Dazl*KO mice from day 2 to day 19 (day of birth counted as day 1) were examined to establish whether germ cells could develop normally during the first wave of spermatogenesis in the absence of *Dazl* protein. Germ cells that were immunopositive for PCNA, RBM and

c-kit were detected in testicular tissue sections from WT and *Dazl*KO males consistent with *Dazl* protein not being absolutely required for re-initiation of mitotic activity of male germ cells after birth. Previous studies have shown that in the mouse c-kit is not expressed in spermatogonial stem (As) cells (Shinohara *et al.*, 2000) but is detectable in A-type, intermediate and B-type spermatogonia (Schrans-Stassen *et al.*, 1999). These findings indicate that the PCNA-positive cells detected in *Dazl*KO testes include mitotically active spermatogonia other than As cells. Staining with RBMY revealed that the germ cell complement was highly variable among *Dazl*KO animals and that in some the numbers of germ cells in individual tubules appeared to be reduced compared with age-matched controls; these animals were derived from outbred MF1 matings and genetic heterogeneity could contribute to this variability. It is notable that in humans, deletions of the *DAZ* gene(s) result in highly variable testicular defects ranging from complete absence of germ cells (Sertoli cells only), spermatogenic arrest with formation of a few spermatids or severe oligozoospermia (Reijo *et al.*, 1995, 1996).

Antibodies directed against γ -H2AX, H1t and SCP3 were used to delineate more exactly whether *Dazl*KO germ cells had entered meiotic prophase (Mahadevaiah *et al.*, 2001). The results obtained showed that although nine out of ten spermatocytes in null males expressed both γ -H2AX and SCP3, the staining pattern was consistent with that of leptotene spermatocytes and the few cells that had progressed to zygotene–early pachytene were unhealthy in appearance. Therefore, the conclusion from these findings is that, as in females, *Dazl* is essential for normal progression of male germ cells through meiosis.

The observation that the final block in germ cell development in *Dazl*KO mice is at the leptotene–zygotene stage raises several questions. First, why are germ cell numbers in most animals reduced before leptotene? Possible explanations for this are that *DAZL* has a function in mitosis or a function in commitment to meiosis, the absence of which leads to loss of committed, pre-meiotic cells and the final block observed. The second question is why in adults, most germ cells appear to be blocked from progressing from A_{al} to A1 spermatogonia (although more mature germ cells up to and including pachytene spermatocytes are present in a small number of tubules) (Slee *et al.*, 1999; Schrans-Stassen *et al.*, 2001). A likely explanation for this is that the testicular architecture of the testes of the *Dazl*KO adults is severely disrupted due to the absence of mature germ cells (Ruggiu *et al.*, 1997). There is an intimate relationship between the function of the somatic Sertoli cells within the seminiferous epithelium, the germ cells and the surrounding interstitium (for review, see (Jégou, 1993; Sharpe, 1994; Griswold, 1995). The factors that control differentiation of A_{al} to A1 spermatogonia are

not yet understood but it is notable that increased testosterone inhibits spermatogonial differentiation in *jsd/jsd* mice (Shetty *et al.*, 2001). Androgen receptors are present on Sertoli cells and not on germ cells (Bremner *et al.*, 1994) and therefore the block in spermatogonial maturation observed in the adult *Dazl*KO males may reflect the disturbance in the testicular environment, and particularly in Sertoli cell function, that is associated with the absence of mature germ cells. Failure of A_{al} spermatogonia to differentiate into A1 spermatogonia has been described in several rodent models in which spermatogenesis has been disrupted (for review, see De Rooij, 2001) and this may indicate that the step in germ cell development is particularly vulnerable to disturbances in the testicular environment. The observation that a meiotic defect can manifest itself in the adult mouse testis as a block at an apparently earlier stage of germ cell development has important implications for the interpretation of the histology of adult human biopsies (Busuttill *et al.*, 1983; Mazeyrat *et al.*, 2001).

Finally, why does germ cell loss occur in the *Dazl*KO mice although these mice still contain a functional *Boule* gene? The explanation for this appears to lie in the timing of expression of the *Dazl* and *Boule* proteins. In immature mouse testes, *Dazl* has been immunolocalized to the cytoplasm of spermatogonia, pre-leptotene and zygotene spermatocytes on days 8 and 9 (P. T. K. Saunders, unpublished). In adults, the highest expression is detected in the cytoplasm of pachytene spermatocytes (Ruggiu *et al.*, 1997). In mice, *Boule* is not expressed in spermatogonia and is first detectable in spermatocytes at stage III of the spermatogenic cycle. The highest expression occurs in late pachytene stage spermatocytes and the protein then persists in round spermatids (Xu *et al.*, 2001). Therefore, although sequence homology indicates that functional conservation between mouse *Boule* and *Dazl* is likely (Xu *et al.*, 2001), expression of *Boule* in the mouse testis does not start until cells have entered the pachytene stage of meiotic prophase, after the time when germ cell development has been impaired in the *Dazl*KO males.

In conclusion, in both males and female mice, loss of expression of an intact *Dazl* protein is associated with failure of germ cells to complete meiotic prophase. In molecular terms, the meiotic defect seen in the *Dazl*KO germ cell is downstream of the formation of double strand breaks. In males, germ cells are capable of maintaining mitotic activity and differentiating into A spermatogonia. The apparent reduction in the number of germ cells in testicular tubule cross-sections on days 4–8 indicates that the *DAZL/Dazl* genes have acquired other functions related to germ cell survival before meiotic entry.

The authors thank J. McNeilly for her assistance with genotyping, C. Heyting for the SCP3 antibody and W. Bonner for the γ -H2AX antibody.

References

- Bellve AR, Cavicchia JC, Millette CA, O'Brien DA, Bhatnagar YM and Dym M (1977) Spermatogenic cells of the prepubertal mouse, isolation and morphological characterization *Journal of Cell Biology* **74** 68–85
- Bielawski JP and Yang Z (2001) Positive and negative selection in the *DAZ* gene family *Molecular Biology and Evolution* **18** 523–529
- Bremner WJ, Millar MR, Sharpe RM and Saunders PTK (1994) Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens *Endocrinology* **135** 1227–1234
- Busuttill A, Orr S and Hargreave T (1983) Histopathology. In *Male Infertility* pp 112–143 Ed. T Hargreave, Springer-Verlag, Berlin
- Cooke HJ, Lee M, Kerr S and Ruggiu M (1996) A murine homologue of the human *DAZ* gene is autosomal and expressed only in male and female gonads *Human Molecular Genetics* **5** 513–516
- De Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells *Reproduction* **121** 347–354
- Dekel N (1996) Protein phosphorylation/dephosphorylation in the meiotic cycle of mammalian oocytes *Reviews in Reproduction* **1** 82–88
- Delbridge ML, Harry JL, Toder R and Graves JM (1997) Only one candidate human spermatogenesis gene is conserved on the marsupial Y chromosome *Nature Genetics* **15** 131–136
- Delbridge ML, Lingenfelter PA, Distechi CM and Graves JA (1999) The candidate spermatogenesis gene RBMY has a homologue on the human X chromosome *Nature Genetics* **22** 223–224
- Dobson M, Pearman RE, Karaiskakis A and Spyropoulos B (1994) Synaptonemal complex proteins: occurrence, epitope mapping and chromosome disjunction *Journal of Cell Science* **107** 2749–2760
- Eberhart CG, Maines JZ and Wasserman SA (1996) Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia [see comments] *Nature* **381** 783–785
- Elliott DJ and Cooke HJ (1997) The molecular genetics of male infertility *Bioessays* **19** 801–809
- Elliott DJ, Ma K, Kerr SM, Thakrar R, Speed R, Chandley AC and Cooke HJ (1996) An RBM homologue maps to the mouse Y chromosome and is expressed in germ cells *Human Molecular Genetics* **5** 869–874
- Elliott DJ, Bourgeois CF, Klink A, Stevenin J and Cooke HJ (2000) A mammalian germ cell-specific RNA binding protein interacts with ubiquitously expressed proteins involved in splice site selection *Proceedings National Academy of Sciences USA* **97** 5717–5722
- Grimes SR, Wolf SA and Koppel DA (1992) Temporal correlation between the appearance of testis-specific DNA-binding proteins and the onset of transcription of the testis-specific histone *H1t* gene *Experimental Cell Research* **201** 216–224
- Griswold MD (1995) Interactions between germ cells and Sertoli cells in the testis *Biology of Reproduction* **52** 211–216
- Houston DW and King ML (2000) A critical role for *Xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. *Development* **127** 447–456
- Houston DW, Zhang J, Maines JZ, Wasserman SA and King ML (1998) A *Xenopus* *DAZ*-like gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophila* *boule* *Development* **125** 171–180
- Jégou B (1993) The Sertoli–germ cell communication network in mammals *International Review of Cytology* **147** 25–96
- Karashima T, Sugimoto A and Yamamoto M (2000) *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not spermatogenesis *Development* **27** 1069–1079
- Ma K, Inglis J, Sharkey A *et al.* (1993) A Y chromosome gene family with RNA binding protein homology: candidates for the azoospermia factor AZF controlling human spermatogenesis *Cell* **75** 1287–1295
- McGuinness MP and Orth JM (1992) Reinitiation of gonocyte mitosis and movement of gonocytes to the basement membrane in testes of newborn rats *in vivo* and *in vitro*. *Anatomical Record* **233** 527–537
- McNeilly JR, Saunders PTK, Taggart M, Cranfield M, Cooke HJ and McNeilly AS (2000) Loss of oocytes in *Dazl* knockout mice results in maintained ovarian steroidogenic function but altered gonadotropin secretion in adult animals *Endocrinology* **141** 4284–4294

- Mahadevaiah SK, Odoriso T, Elliott DJ et al.** (1998) Mouse homologues of the human AZF candidate gene *RBM* are expressed in spermatogonia and spermatids and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities *Human Molecular Genetics* **7** 715–727
- Mahadevaiah SK, Turner JMA, Baudat F et al.** (2001) Recombinational DNA double-strand breaks in mice precede synapsis *Nature Genetics* **27** 271–276
- Malkov M, Fisher Y and Don J** (1998) Developmental schedule of the postnatal rat testis determined by flow cytometry *Biology of Reproduction* **59** 84–92
- Mazeyrat S, Saut N, Grigoriev V et al.** (2001) A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis *Nature Genetics* **29** 49–53
- Nagano R, Tabata S, Nakanishi Y, Ohsako S, Kurohmaru M and Hayashi Y** (2000) Reproliferation and relocation of mouse male germ cells (gonocytes) during spermatogenesis *Anatomical Record* **258** 210–220
- Page J, Suja JA, Santos JL and Rufas JS** (1998) Squash procedure for protein immunolocalisation in meiotic cells *Chromosome Research* **6** 639–642
- Reijo R, Lee TY, Salo P et al.** (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene *Nature Genetics* **10** 383–393
- Reijo R, Alagappan RK, Patrizio P and Page DC** (1996) Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome *Lancet* **347** 1290–1293
- Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J and Cooke HJ** (1997) The mouse *Dazl* gene encodes a cytoplasmic protein essential for gametogenesis *Nature* **389**(6646) 73–77
- Ruggiu M, Saunders PTK and Cooke HJ** (2000) Dynamic subcellular distribution of the DAZL protein is confined to primate male germ cells *Journal of Andrology* **21** 470–477
- Saxena R, Brown LG, Hawkins T et al.** (1996) The DAZ gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned *Nature Genetics* **14**(3) 292–299
- Schrans-Stassen BHGJ, van de Kant HJG, de Rooij DG and van Pelt AMM** (1999) Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia *Endocrinology* **140** 5894–5900
- Schrans-Stassen BHGJ, Saunders PTK, Cooke HJ and de Rooij DG** (2001) Nature of the spermatogenic arrest in *Dazl* $-/-$ mice *Biology of Reproduction* **65** 771–776
- Seligman J and Page DC** (1998) The *Dazh* gene is expressed in male and female embryonic gonads before germ cell sex differentiation *Biochemical Biophysical Research Communications* **245** 878–882
- Shan Z, Hirschmann P, Seebacher T, Edelmann A, Jauch A, Morell J, Urbitsch P and Vogt PH** (1996) A *SPGY* copy homologous to the mouse gene *Dazl* and the *Drosophila* gene *boule* is autosomal and expressed only in the human male gonad *Human Molecular Genetics* **5** 2005–2011
- Sharpe RM** (1994) Regulation of Spermatogenesis. In *The Physiology of Reproduction* 2nd Edn pp 1363–1434 Eds E Knobil and JD Neill/Raven Press, New York
- Shetty G, Wilson G, Huhtaniemi I, Boettger-Tong H and Meistrich ML** (2001) Testosterone inhibits spermatogonial differentiation in juvenile spermatogonial depletion mice *Endocrinology* **142** 2789–2795
- Shinohara T, Orwig KE, Avarbock MR and Brinster RL** (2000) Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells *Proceedings National Academy of Sciences USA* **97** 8346–8351
- Slee R, Grimes B, Speed RM, Taggart M, Maguire SM, Ross A, McGill NI, Saunders PT and Cooke HJ** (1999) A human *DAZ* transgene confers partial rescue of the mouse *Dazl* null phenotype *Proceedings National Academy of Sciences USA* **96** 8040–8045
- Vogel T, Speed RM, Ross A and Cooke HJ** (2002) Partial rescue of the *Dazl* knockout mouse by the human *DAZL* gene *Molecular Human Reproduction* **8** 797–804
- Waseem NH and Lane DP** (1990) Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA): structural comparison and detection of a nucleolar form *Journal of Cell Science* **96** 121–129
- Xu EY, Moore FL and Pera RA** (2001) A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans *Proceedings National Academy of Sciences USA* **98** 7414–7419
- Xu EY, Lee DF, Klebes A, Turek PJ, Kornberg TB and Reijo Pera RA** (2003) Human *BOULE* gene rescues meiotic defects in infertile flies *Human Molecular Genetics* **12** 169–175
- Yen PH, Chai NN and Salido EC** (1996) The human autosomal gene *DAZL*: testis specificity and a candidate for male infertility *Human Molecular Genetics* **5** 2013–2019

Received 2 May 2003.

First decision 30 June 2003.

Revised manuscript received 10 July 2003.

Accepted 28 July 2003.