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Nature of the Spermatogenic Arrest in *Dazl* −/− Mice

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ABSTRACT

***Dazl* encodes an RNA-binding protein essential for spermatogenesis. Mice that are deficient for *Dazl* are infertile, lacking any formation of spermatozoa, and the only germ cells present are spermatogonia and a few spermatocytes. To gain more insight regarding the timing of the spermatogenic arrest in *Dazl* −/− mice, we studied the spermatogonial cell types present in testis sections and in seminiferous tubular whole mounts. Most of the seminiferous tubular cross-sections contained A spermatogonia as the most advanced cell type, with only very few containing cells up to pachytene spermatocytes. Both 5-bromodeoxy-uridine incorporation and mitotic index indicated that the remaining A spermatogonia were actively proliferating. C-*kit* immunohistochemical studies showed that most of the A spermatogonia were positively stained for the c-Kit protein (~80%). The clonal composition of the A spermatogonia in tubular whole mounts indicated these cells to be A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) spermatogonia. It is concluded that the prime spermatogenic defect in the *Dazl* −/− mice is a failure of the great majority of the A_{al} spermatogonia to differentiate into A_1 spermatogonia. As a result, most seminiferous tubules of *Dazl* −/− mice only contain actively proliferating A_s, A_{pr}, and A_{al} spermatogonia, with cell production being equaled by apoptosis of these cells.**

apoptosis, development, developmental biology, gene regulation, spermatogenesis, testis

INTRODUCTION

Reduced fertility in men has become a concern in recent years. Some cases of oligozoospermia or azoospermia are caused by a genetic defect, namely microdeletions of the long arm of the Y chromosome [1, 2]. One region that has been identified is called the azoospermia factor (AZF) region, which can be divided into three subregions: AZFa, AZFb, and AZFc [3]. Two gene families, *RBM* (RNA-binding motif) and *DAZ* (deleted in azoospermia), both encoding RNA-binding proteins, are present on the AZFb and AZFc region, respectively. Both regions also contain other genes. The *RBM* and *DAZ* transcription is restricted to the male germline [4]. Y-Linked *RBM* homologues are found in all mammalian species, whereas *DAZ* is only found in humans, Old World monkeys, and apes [5]. In all other mammals, it is represented by an autosomal, single-copy gene, *DAZ*-like (*Dazl*). *Dazl* genes have been isolated independently in humans, mice, monkeys, frogs, and flies [6]. In humans, *Dazl* is located on chromosome 3 [7, 8]; in mice, the gene is localized on chromosome 17 [5, 9]. Mice that are deficient for *Dazl* are infertile, lacking any formation of spermatozoa or oocytes. Virtually the only cells that remain in the *Dazl* −/− seminiferous tubules are spermatogonia [10].

In rodents, A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) spermatogonia are at the beginning of the spermatogenic process [11, 12]. The stem cells, the A_s spermatogonia, undergo mitosis and form two new A_s spermatogonia, or the daughter cells stay together, connected through an intercellular bridge, and are called A_{pr} spermatogonia. The A_{pr} spermatogonia divide into chains of 4, 8, 16, and sometimes, 32 A spermatogonia, named A_{al} spermatogonia. These differentiate without mitotic division into type A_1 spermatogonia. Through subsequent mitotic divisions, A_2, A_3, A_4, intermediate, and B spermatogonia are formed, which finally form preleptotene spermatocytes. The A_1 through A_4 spermatogonia are present during epithelial stages VIII until I, whereas the A_s, A_{pr}, and A_{al} spermatogonia are present throughout the epithelial cycle [13, 14].

We studied the spermatogenic arrest in *Dazl* −/− mice in seminiferous tubular whole mounts and in testis sections in which the proliferative activity as well as the nature of the remaining A spermatogonia was determined. The presence of the c-*kit* receptor in these cells, as a marker for differentiation of spermatogonia [15], was also studied.

MATERIALS AND METHODS

Animals and Tissue Preparation

Knockout animals were generated as described previously [10]. Animals were housed under standard conditions and fed ad libidum. At 9 days and 2–3 mo of age, male animals were killed by cervical dislocation 2 h after i.p. injection of 5-bromodeoxy-uridine (BrdU; 200 mg/kg body weight; Sigma, St. Louis, MO). The testes were removed, and one testis was used for preparing whole mounts of seminiferous tubules according to the method of Clermont and Bustos-Obregon [16] and fixed in Bouin fluid. The other testis was cut in half, after which one half was fixed in methacarn for BrdU labeling and the other half was fixed in Bouin fluid for c-*kit* immunohistochemistry. Animal experiments were carried out under local ethical rules and Home Office license 60/2242.

For histological sections, Bouin-fixed material from adult wild-type and knockout testes was stained with the periodic acid-Schiff reaction and hematoxylin and with hematoxylin-and-eosin for the testes of 9-day-old knockout mice.

Tubular Whole Mounts

Fixed seminiferous tubules were stained with Harris hematoxylin (Polysciences, Warrington, PA) and mounted in toto on microscopic slides. Tubules of six different knockout mice were scored for mitotic clonal sizes; per animal, 50 mitotic clones were studied.

Labeling and Mitotic Index

Fixed testes were embedded in glycol methacrylate Technovit 7100 (Kulzer, Wehrheim, Germany). Five-micrometer sections were cut and mounted on microscopic slides. The BrdU immunohistochemistry was per-

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formed using BrdU-immunogold-silver staining as described by van de Kant and de Rooij [17]. The mitotic index was determined by counting A spermatogonia in metaphase, anaphase, and telophase. At least 200 A spermatogonia in each of six *Dazl* −/− animals were counted to determine the labeling and the mitotic index.

C-kit *immunohistochemistry*

Testes fixed in Bouin fluid were embedded in paraffin (Stemcowax, Adamas Instruments BV, Amerongen, The Netherlands), and c-*kit* immunohistochemical experiments were performed as described previously [15]. Briefly, 5-μm sections were mounted on coated slides and dried overnight. The sections were dewaxed and blocked with 5% (v/v) rabbit serum (Aurion ImmunoGold Reagents & Accessories, Wageningen, The Netherlands) and 5% (v/v) BSA fraction V in PBS and then incubated overnight at room temperature in a moist chamber with c-kit antibody M14 (1:100; sc-1494; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 0.05% (v/v) BSA-c/5% (v/v) rabbit serum/PBS. The next day, slides were washed and incubated with biotinylated rabbit anti-goat (1:100; Aurion) in 0.05% BSA-c/5% rabbit serum/PBS for 1 h at room temperature. Endogenous peroxidase activity was blocked with 0.35% H_2O_2 in PBS for 15 min, after which sections were incubated at room temperature with the avidin-biotin complex (Vector, Burlingame, CA). After washing three times with PBS, sections were washed twice for 10 min in 0.05 M Tris-HCl (pH 7.6), 0.3 M NaCl, and 0.1% Tween. The signal was visualized using 0.5 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride (DAKO, Carpenteria, CA) in 0.05 M Tris-HCl (pH 7.6) and 0.01% H_2O_2. Sections were counterstained with Mayer hematoxylin (Sigma), and slides were mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.). In each of six knockout mice, at least 200 A spermatogonia were scored for c-*kit* staining.

Tubular Diameter

Sections of testes from the BrdU experiments were used to measure the tubular diameter of *Dazl* +/+ and *Dazl* −/− mice. At least 30 round tubules were measured in each of five different animals per genotype.

RESULTS

Histology of the Testes

In testis sections of adult *Dazl* −/− mice, approximately 21% of the seminiferous tubules contained only Sertoli cells, and in 76% of the tubules, A spermatogonia were present, some of which were in apoptosis (Fig. 1A). In approximately 36% of the tubules, clusters of Sertoli cells (Fig. 1B) were present. In some tubules (~3%), spermatocytes were observed (Fig. 2), which underwent apoptosis before or during the pachytene stage (Fig. 1C). No spermatids were found.

In sections of testes from 9-day-old *Dazl* −/− mice, no preleptotene spermatocytes were present (Fig. 1D), whereas in wild-type mice, this cell type was abundant (Fig. 1E). At this age, except for the cell types present, no differences were seen between *Dazl* −/− and +/+ testes.

Tubular Diameter

Because the seminiferous tubules in *Dazl* −/− mice seemed small, the tubular diameter in wild-type and knockout mice was measured. Indeed, the tubular diameter in *Dazl* −/− mice turned out to be half that in wild-type mice, being 261 ± 4 μm (average ± SEM; range, 258–269 μm) and 128 ± 7 μm (range, 118–135 μm) in wild-type and knockout mice, respectively.

Labeling and Mitotic Index

To determine the proliferative activity of the A spermatogonia present in *Dazl* −/− mice, DNA synthesis in these cells was estimated by studying BrdU incorporation. Furthermore, the numbers of mitotic A spermatogonia were counted. We found that 19% ± 5% (range, 11%–25%) of the A spermatogonia were labeled for BrdU, whereas the mitotic index was 26% ± 9% (range, 16%–42%) (Fig. 3).

C-kit *immunohistochemistry*

To gain more insight regarding the characteristics of the A spermatogonia present in *Dazl* −/− mice, the expression of the c-Kit protein was studied. It appeared that 78% ± 5% of the A spermatogonia stained positive for the c-Kit protein (Fig. 4). The Leydig cells stained intensively for c-Kit, whereas no staining was observed in Sertoli cells (Fig. 1F).

Histological Examination of the Whole Mounts

The distribution of the mitotic clonal size of A spermatogonia was studied to further characterize the nature of spermatogonia in the *Dazl* −/− mice. Type A spermatogonia that belong to the same clone go through mitosis simultaneously, because they are connected to each other by intercellular bridges [14]. In this study, more than half the clones (61%) consisted of four or fewer cells (Fig. 5). Approximately 19% of the clones consisted of five or more cells. Although most of the clones consisted of even numbers of cells, 6% of the clones consisted of odd numbers of cells.

DISCUSSION

The present results allow identification of the step at which spermatogenesis becomes arrested in mice deficient for the RNA-binding protein encoded by the *Dazl* gene. In the great majority of the seminiferous tubular cross-sections, A spermatogonia were the only germ cells present. In only 3% did germ cells develop further; ultimately, however, even these cells underwent apoptosis and never developed further than the stage of pachytene spermatocytes. This points to an almost complete arrest at the A spermatogonial level. Further scrutiny of the remaining A spermatogonia in the *Dazl* −/− mice revealed that these cells were actively proliferating and that approximately 95% of the A spermatogonial clones contained 8 or fewer cells and only 3% more than 16 cells. The normal mouse seminiferous epithelium contains single cells (A_s), pairs (A_{pr}), and chains of 4, 8, or 16 A_{al} spermatogonia. The A_{al} spermatogonia differentiate into A_1 spermatogonia, and during each subsequent division, the chains of spermatogonia double in size. Hence, the small clonal sizes found in this study indicate that in the *Dazl* −/− mice, virtually only A_s, A_{pr}, and A_{al} spermatogonia remain, and that despite their vigorous proliferative activity, with a few exceptions, the A_{al} spermatogonia among them are not able to differentiate into A_1 spermatogonia. The few cells that do differentiate do not develop further than the pachytene stage of the meiotic prophase. Despite the proliferative activity of the spermatogonia, no accumulation of germ cells was seen in the *Dazl* −/− mice, with cell production presumably being matched by the observed germ cell apoptosis. Apoptosis of spermatogonia as well as of spermatocytes was seen.

The arrest at the transition of A_{al} to A_1 spermatogonia in *Dazl* −/− mice is another proof that this step in spermatogenesis is subject to a complex regulatory mechanism [18]. The spermatogenic block in *Dazl* −/− mice is comparable to that in *jsd/jsd, Sl17H/Sl17H,* and cryptorchid mice [19]; to that in vitamin A-deficient (VAD) rats and mice [20–22]; and to that in irradiated rats [23]. A similar situation may be present in rats given the Sertoli cell-tox-

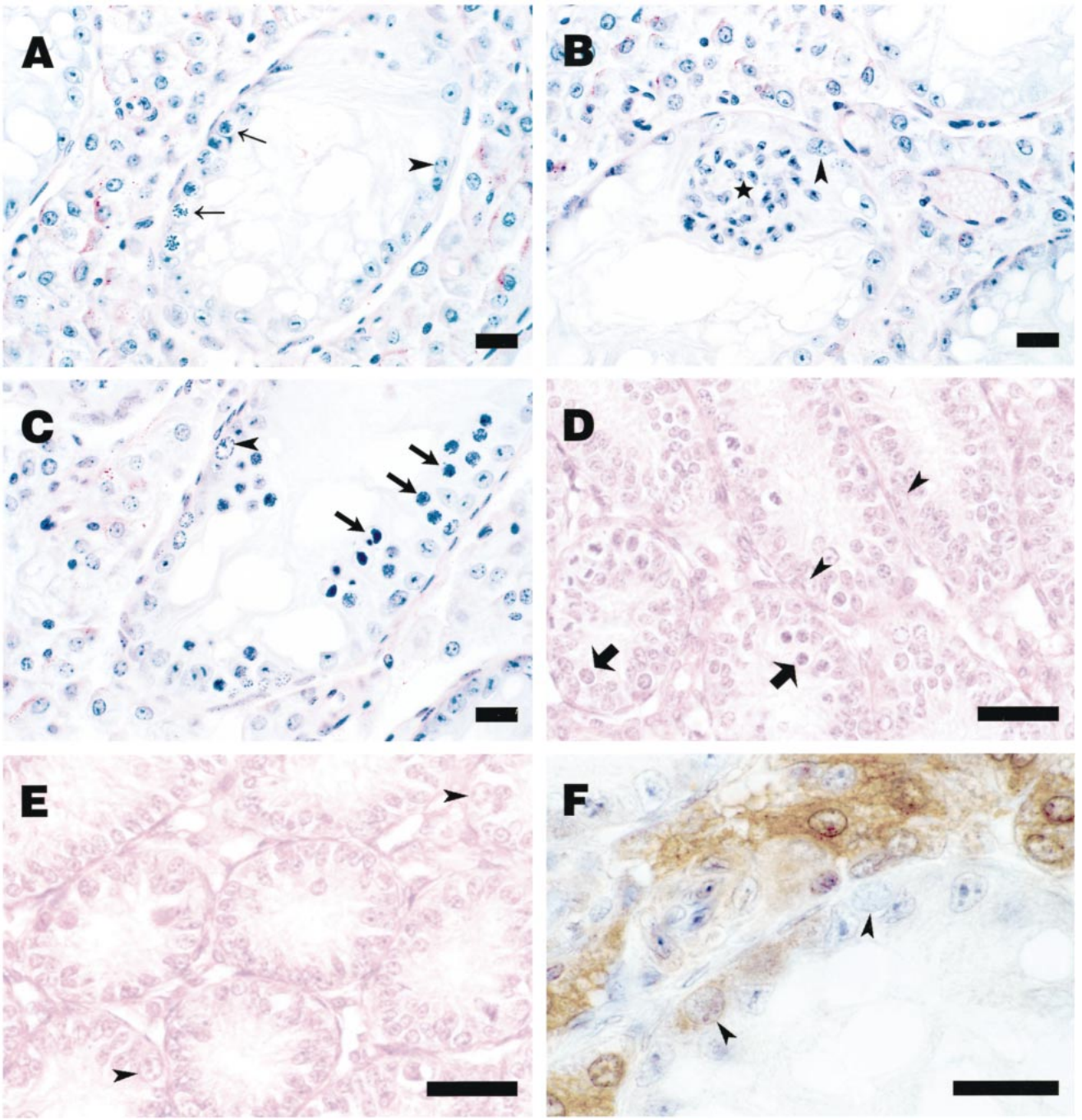

FIG. 1. Histology of the *Dazl* −/− mouse testis. **A**) Seminiferous tubules of *Dazl* −/− mice contain A spermatogonia (arrowhead) and apoptotic spermatogonia (arrows). **B**) A common feature of the testes of *Dazl* −/− mice is the cluster formation of the Sertoli cells (asterisk). **C**) Tubular section showing pachytene spermatocytes (arrows), some of which are in apoptosis, and mitotic spermatogonia (arrowhead). **D**) Testis section of 9-day-old, wild-type mouse. Tubular sections show spermatogonia (arrowheads) and preleptotene spermatocytes (broad arrows). **E**) In testis sections of 9-day-old *Dazl* −/− mice, the seminiferous tubules only show spermatogonia (arrowheads). **F**) Immunohistochemical staining for the c-*kit* receptor in the *Dazl* −/− mouse testis. Both c-*kit*-positive (brown) and negative spermatogonia (arrowheads) can be seen. Bar = 25 μm.

icant 2,5-hexanedione [24, 25]. In the rat models and in the *jsd/jsd*, cryptorchid, and VAD mice, spermatogenesis starts normally, after which it deteriorates to an A-spermatogonia-only situation. However, sections of Day 9 *Dazl* −/− mouse testes show that already at this age there is no formation of preleptotene spermatocytes. Hence, in *Dazl* knockout mice, differentiation of the A_{al} spermatogonia is already arrested from the start of spermatogenesis onward. This suggests that the RNA-binding protein encoded by the *Dazl* gene is directly involved in this differentiation step. After c-*kit*, *Dazl* is now the second gene known to be involved in the differentiation of A_{al} spermatogonia, as in the *jsd* mutation the actual gene or genes involved are unknown. Furthermore, in the experimental situations discussed, the effect is indirect, via Sertoli cells involving currently unknown genes. A point that makes *Dazl* unique is that the defect clearly is already present during the first wave of spermatogenesis, whereas in *jsd* and *Sl17H/Sl17H* mice, the defect becomes fully visible only after the first wave.

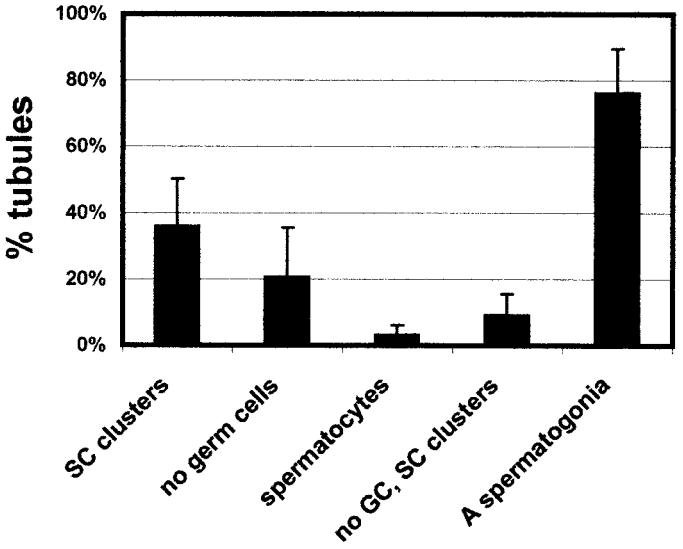

FIG. 2. The cellular contents of the seminiferous tubules in *Dazl* −/− mouse testes. GC, Germ cells (n = 5); SC, Sertoli cell.

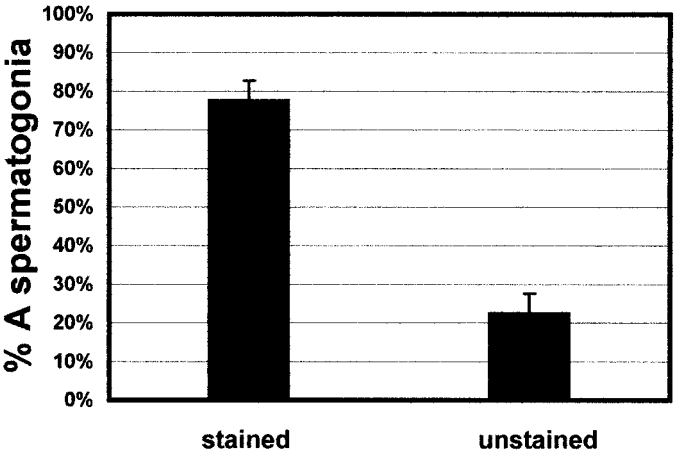

FIG. 4. Percentage of c-Kit-positive and -negative A spermatogonia in *Dazl* −/− mouse testes (n = 6).

Another common feature observed in the *Dazl* −/− mice was the presence of Sertoli cell clusters in the seminiferous tubules. Serial section studies will be necessary to establish whether these clusters are shed in the tubule lumen in these mice or still have contact with the tubule wall. If they are indeed shed, this will lead to a progressive shrinkage of the tubules. Sertoli cell abnormalities have also been described in other situations. MacGregor et al. [26] found that transgenic mice carrying an insertional mutation in a gene, named symplastic spermatids, had a defect in spermatogenesis and a vacuolization of Sertoli cells within their cytoplasmic boundaries. Another insertional transgenic mouse mutant, called germ cell deficient, showed approximately 50% seminiferous tubules that were devoid of all germ cells, with Sertoli cells appearing as masses of vacuolated cells. The remaining seminiferous tubules contained normal epithelial stages of spermatogenesis with normal Sertoli cells. The mutation caused a defect during primordial germ cell development, showing no morphological defects in Sertoli cells at that stage [27]. Apparently, morphological alterations of Sertoli cells seem to be common in germ cell-depleted situations [28, 29]. The reason for the formation of the clusters in the *Dazl* −/− mouse testes is not known. It might be possible that, because of the cluster formation, the interaction between germ cells and Sertoli cells is impaired, which could result in a disordered spermatogenesis.

The A spermatogonia that are left behind in the *Dazl* knockout mice are actively dividing, as shown by their high labeling and mitotic index of 20% and 26%, respectively. Studies of irradiated $LBNF_1$ rats, in which spermatogenesis first recovers and then deteriorates until only A_s, A_{pr}, and A_{al} spermatogonia remain, showed a comparable labeling and mitotic index [23]. The mitotic indices of the A spermatogonia in both systems were unexpectedly high, possibly due to a block in mitosis or a long mitotic phase. Shuttlesworth et al. [23] could exclude a block in mitosis as a prelude to apoptosis, because they were unable to find any degenerating TUNEL-positive mitotic cells.

Most of the A spermatogonia in *Dazl* −/− mice are c-*kit*-receptor positive. Although the c-Kit protein is a membrane receptor, the staining was diffusely spread over the cytoplasm. In a previous study [15], we tried three different antibodies, all of which gave cytoplasmic staining. Dym et al. [30], using another antibody, and Sandlow et al. [31] also found cytoplasmic staining. However, three groups have reported membrane staining for c-Kit in combination with cytoplasmic or nuclear staining [32–34]. The

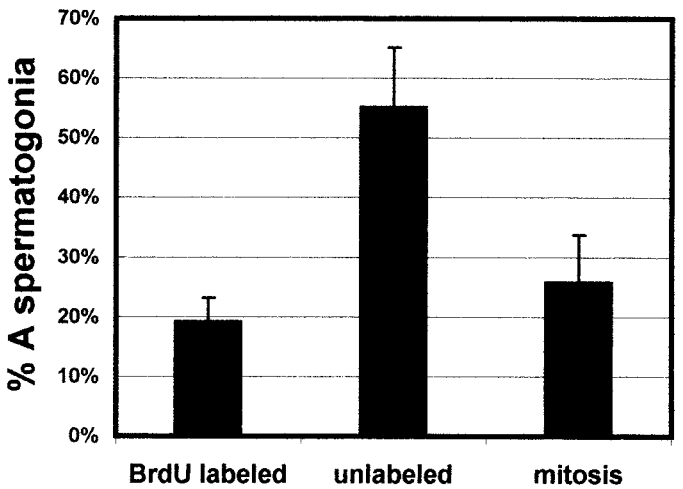

FIG. 3. Percentage of total A spermatogonia labeled or unlabeled for BrdU or in mitosis (n = 6).

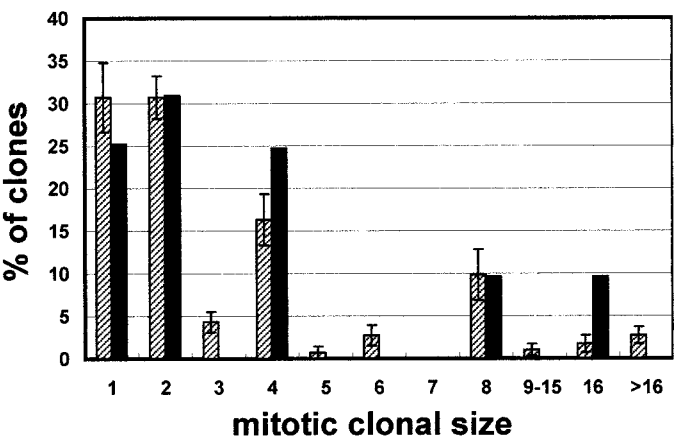

FIG. 5. Mitotic clonal size distribution of A spermatogonia in *Dazl* −/− mouse testes. Black bars indicate the distribution of clonal sizes in stage VI of the cycle of the seminiferous epithelium in C3H/101 F1 hybrid mice [19, 41]. Clonal size is the number of cells composing a clone (n = 6).

difference between the methods used by the latter groups and those finding cytoplasmic staining is that the latter groups used frozen sections. Apparently, the membrane localization of the c-Kit receptor is better preserved in frozen sections. Previous studies revealed that A_s and A_{pr} spermatogonia do not express c-Kit, and that A_{al} spermatogonia start to express c-kit just before their differentiation into A_1 spermatogonia [15]. In VAD mice, in which only A_s, A_{pr}, and A_{al} spermatogonia are present, approximately 20% of the spermatogonia are A_s and A_{pr} spermatogonia and c-kit negative, whereas 80% are A_{al} spermatogonia that are unable to differentiate into A_1 spermatogonia and c-kit positive [15]. The similarity of the composition of the A spermatogonial populations in VAD and *Dazl* −/− mice as well as the similar percentages of c-kit-positive spermatogonia in both mice suggest that in *Dazl* −/− mice, most A_{al} spermatogonia have stopped developing just before the transition into A_1 spermatogonia and after they acquired the c-Kit receptor. Apparently, the c-Kit and Dazl proteins are directly involved in the differentiation of A_{al} spermatogonia. Because c-Kit protein is expressed in *Dazl* −/− spermatogonia, c-Kit expression probably precedes Dazl protein expression in the cascade of events during A_{al} spermatogonial differentiation. For Leydig cells, c-Kit protein expression in *Dazl* knockout mice was observed, in accordance with results from other groups [35].

In wild-type mice, *Dazl* mRNA is already detectable from embryonic Day 12.5, when only primordial germ cells are present [36]. However, because at the time of birth the testes of wild-type and knockout mice are morphologically similar, and because a clear distinction between wild-type and knockout mice is not visible until Day 9 (unpublished results), it can be concluded that the possible role of *Dazl* during fetal testicular development is redundant. The mRNA expression increases by Day 6, when only spermatogonia are present, until Day 10, at the moment when spermatocytes enter meiosis, and then is sustained at that level during adulthood [9]. The *Dazl* mRNA and protein expression is most abundant in pachytene spermatocytes (at stages I–VIII) but is also present in spermatogonia and preleptotene and zygotene spermatocytes [10, 37]. The pachytene spermatocytes express the Dazl protein in an abundant way, suggesting a role for the protein during this part of the meiotic prophase. Therefore, the lack of this protein in *Dazl* −/− mice might explain why the few cells reaching the prophase of meiosis go into apoptosis before completion of the pachytene stage.

In other species, knockout animals showed different deficiency patterns. In *Drosophila*, loss of the *Dazl* homologue *boule* caused no problems in spermatogonial differentiation, but it was found that this gene is required for the meiotic cell cycle [38]. In *Xenopus*, *Xdazl* was expressed in spermatogonia and spermatocytes as well as in the mitochondrial cloud and vegetal cortex of oocytes. *Xdazl* was able to rescue the meiotic entry of spermatocytes in *boule* flies, although the *Xdazl* transgene flies remained infertile [39]. The maternal *Xdazl* was found to be necessary for the early primordial germ cell differentiation [40]. Likely, the Dazl protein has functions at various steps of spermatogenic development, but the deficiency of this protein does not lead to problems at the same step in all animal phyla or species.

In conclusion, we have shown that the prime spermatogenic defect in *Dazl* −/− mice is a failure of nearly all A_{al} spermatogonia to differentiate into A_1 spermatogonia. Thus, the seminiferous tubules of these knockouts contain predominantly A_s, A_{pr}, and A_{al} spermatogonia that proliferate actively but are unable to develop further. The nature of the spermatogenic defect in *Dazl* −/− mice indicates that the testes of these mice will provide a good source for the purification of actively dividing A_s, A_{pr}, and A_{al} spermatogonia in studying regulation of the proliferation and stem cell renewal of this type of spermatogonia in vitro.

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