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High-Resolution Light Microscopic Characterization of Mouse Spermatogonia[1]

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ABSTRACT

Characteristics of spermatogonia were determined in the C57BL/6J strain mouse using high-resolution light microscopy of plastic-embedded tissues and identifying cells during stages of the spermatogenic cycle. The frequency of expecting each spermatogonial cell type was a major factor in identifying and categorizing various cell types. Although numerous characteristics were described, several major differences were noted in spermatogonial cell types. The group comprising A_s, A_{pr}, and A_{al} spermatogonia could be differentiated based primarily on mottling of heterochromatin throughout the nucleus in the absence of heterochromatin lining the nuclear envelope. The A_1 cells displayed finely granular chromatin throughout the nucleus and virtually no flakes of heterochromatin along the nuclear membrane. The A_2 through A_4 spermatogonia contained progressively more heterochromatin rimming the nucleus. Intermediate-type spermatogonia displayed flaky or shallow heterochromatin that completely rimmed the nucleus. Type B spermatogonia showed rounded heterochromatin periodically along the nuclear envelope. Use of gray-scale histograms allowed objective quantification of nuclear characteristics and showed a logical shift in the gray scale to a narrower and darker profile, from four cell types leading to A_1 cells. The ability to differentiate spermatogonial types is a prerequisite to studying the behavior and kinetics of the earliest of the germ cell types in both normal and abnormal spermatogenesis.

mouse, seminiferous tubule, spermatogenesis, spermatogonia, testis

INTRODUCTION

Spermatogonia are the cells within the testis that initiate the process of spermatogenesis. Via mitotic divisions, they greatly increase the number of cells entering meiosis, such that only the remaining two meiotic divisions supplement the cell number that eventually results in the production of millions of sperm daily for a particular species.

Numerous types of spermatogonia exist in the premeiotic lineage of rats and mice. The cells comprising this lineage have recently been reviewed [1]. The A_s (A_{single}) cells may replenish themselves via self-renewal divisions or may undergo differentiation divisions. The stem cells of the testis are termed A_s cells, because they have no intercellular bridge connections with other spermatogonia. Unlike the progeny of stem cells elsewhere, spermatogonial cells remain joined by intercellular bridges [2]. The result of the stem cell division is a pair of spermatogonia or A_{pr} cells. Subsequent divisions lead to chains of 4, 8, and 16 cells (i.e., $A_{al\text{-}4}$, $A_{al\text{-}8}$, and/or $A_{al\text{-}16}$, respectively).

Before the formation of A_1 spermatogonia, the divisions of stem cells and aligned cells occurred at any point in the spermatogenic cycle. In contrast, the transition (i.e., they do not divide) of aligned cells to A_1 cells occurs at a particular time during the cycle, regardless of chain length of the A_{al}. In the rat and mouse, this transition occurs during stages VII and VIII. Once A_1 cells are formed, spermatogonial divisions are synchronized and predictable, occurring at specific stages of the cycle of the seminiferous epithelium. The A_1 cells divide to form A_2 spermatogonia, and these, in turn, divide to form A_3 cells, which, finally, divide to form A_4 cells. The A_4 cells divide to form intermediate-type spermatogonia, and the latter divide to form type B spermatogonia. Type B spermatogonia are the last cells in the spermatogonial lineage. Approximately 9–11 spermatogonial divisions precede meiosis.

The various kinds of type A spermatogonia are notoriously difficult to differentiate. The most mature of the spermatogonial cells, intermediate spermatogonia and type B spermatogonia, are generally known to have nuclear heterochromatin along the nuclear envelope, which distinguishes them from most type A spermatogonia and from each other. To date, the best way to tell them apart is by knowing in which stage of the cycle they are present.

The more primitive spermatogonia, type A spermatogonia, are very difficult to distinguish. Huckins and Oakberg [3] as well as Oakberg [4] have illustrated and described differences in A_1 through A_4 spermatogonia. In addition, in the whole-mount preparations utilized by Clermont and Bustos-Obregon [5], the nuclei of type A_1 through A_4 cells are drawn to indicate that their features are not identical to each other. In all the aforementioned studies, the photographs of actual spermatogonia are low resolution, and the differences are readily apparent only in the accompanying drawings.

For most investigators, knowledge regarding the identifying characteristics of the various spermatogonial cell types would be useful to differentiate the various classes of spermatogonia in both normal and experimental situations. To date, biochemical markers of spermatogonia have not provided unequivocal identification of the various spermatogonial cell types, although some markers, such as c-Kit, will identify a subtype of A_{pr} and A_{al} cells [6, 7]. The present study has utilized the high resolution provided by epoxy embedding and semithin sections to study spermatogonia with light microscopy. Herein, we report substantial progress in identifying and characterizing various spermatogonial cell types.

MATERIALS AND METHODS

The testes of four adult C57BL/6J mice, weighing 27.8 ± 0.52 g (mean ± SEM) and obtained from Jackson Labs (Bar Harbor, ME), were perfused-fixed with according to the fixative method of Sprando [8]. Briefly, each animal was given heparin 15 min before perfusion. Saline was perfused initially to clear the blood from the testis, followed by 5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2–7.4). After a

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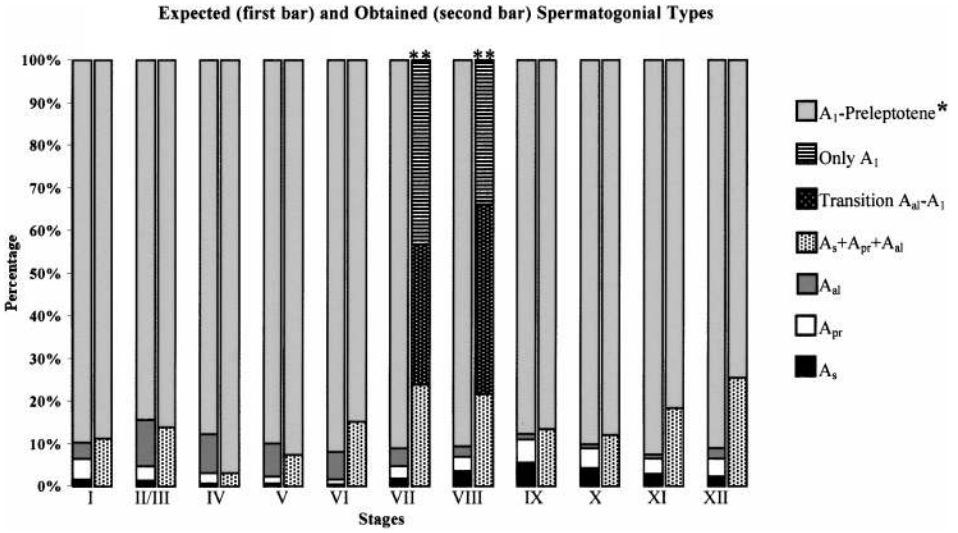


FIG. 1. The expected (first bar) and obtained/photographed (second bar) distributions of various cell types during the cycle of the seminiferous epithelium. *To determine the distribution of the various types of spermatogonia in this category, the reader should refer to Tegelenbosch and de Rooij [12]. **Includes only A_s through A_1 and, thus, is not comparable to the data of de Rooij [14].

glutaraldehyde perfusion of approximately 20 min, the testes were excised, weighed (104 ± 1.36 mg, n = 8), and cut into 0.5-mm slabs by perpendicular sectioning. The slabs were then cut into approximately 2-mm (length × width) portions and embedded such that cross-sections of seminiferous tubules would be obtained on sectioning. After an overnight fixation of diced slabs, the tissues were washed in three changes of buffer, postfixed in an osmium-ferrocyanide mixture [9], dehydrated, embedded in epoxy, and sectioned for light microscopy at approximately 1 μm in thickness.

The plan was to initially study spermatogonia in a single animal. We believed that a thorough examination of a single animal would be more useful, because it would eliminate the variation in fixation that was often obtained using the protocol described above. The single animal utilized (animal 4; body weight, 28.5 g; paired testicular weight, 218 mg) was the one exhibiting the best fixation. After a thorough study of one animal, a more cursory study of the other three animals was undertaken to determine if the characteristics of spermatogonia were similar in the other animals of this group.

All type A spermatogonia were photographed in the 12 stages of the cycle of the seminiferous epithelium of the mouse. The staging criteria used were those of Oakberg [10], as modified and depicted by Russell et al. [11]. In particular, the ''stage range'' was used in the above reference to determine the beginning and end of each stage. More than 200 digital images were taken with a Nikon Optiphot Microscope (Nikon, Inc., Garden City, NJ) of type A spermatogonia in each stage. In addition, numerous images of intermediate and type B spermatogonia were digitized. If a question arose regarding whether digital manipulation of images using the ''curves'' feature of Adobe PhotoShop caused cells to appear different or similar using the criteria measured, we further manipulated the original images to determine if similarities or differences could be obtained solely by manipulation of those images.

Images were adjusted for resolution (600 dpi), sharpness (180%, radius set at 6.0 pixels, threshold set at 0.0), and contrast/gray level (sigmoidal curve) using Photoshop 4.0 (Adobe Systems, Inc., Mountain View, CA). Images of spermatogonia were studied carefully and grouped according to those possessing similar structural characteristics. The proportions of A_s through A_{al} spermatogonia expected at specific stages were determined by examining the data of Tegelenbosch and de Rooij [12]. Matching of the expected percentage of A_s through A_{al} sectioned cells with the actual percentage enumerated in whole mounts provided clues regarding the accuracy of the identification of A_s through A_{al} cells. In identifying cells or groups of cells, consideration was given to the expected transitions of one morphological form to another, such that a logical progression could be obtained from one stage to another.

Gray-scale histograms were made from typical $A_{subtype\ 2}$, $A_{al\ transition}$, A_{al}, and A_1 nuclei using an older version of Adobe PhotoShop (version 3.0). The portion of the nucleus not containing the nucleolus was initially outlined to select the area for the histogram.

RESULTS

Fixation of the testes from the four animals used in the present study ranged from very good to excellent. The animal selected showed some areas of suboptimal fixation, but these areas were largely avoided in the determination of results.

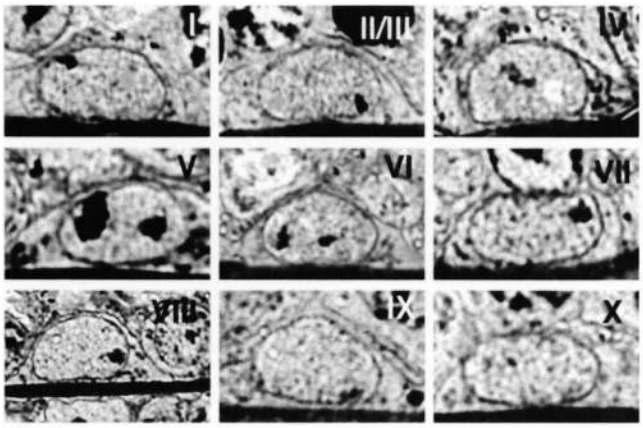
Figure 1 shows the expected and the obtained distributions of A_s through A_{al} spermatogonia as well as the expected distributions of A_1 through A_4 spermatogonia. Note the large difference in expected and obtained distribution in stages VII and VIII. The discrepancy is because we were able to more precisely see the mottling in the transitional cells of the A_s, A_{pr}, and A_{al} population than in the previous study [11] and, thus, classified them as belonging to the A_s to A_{al} group. Because a continuity of cells exists, no clear definition of when a cell is A_{al} or A_1 is available.

Figure 2 illustrates features of the various types of A spermatogonia (various subtypes), intermediate spermatogonia, and B spermatogonia and provides details of their morphology.

A_s, A_{pr}, and A_{al} Spermatogonia

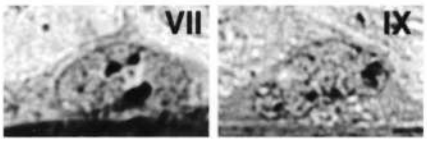
The populations of A_s, A_{pr}, and A_{al} spermatogonia were considered together, although the subtypes described below may represent A_s or A_{pr} or small-chain-length A_{al} (vs. long-chain-length A_{al}). The major population of A_{al} illustrated in Figure 2 showed a nucleus with pronounced, mottled chromatin. Nuclear vacuoles were often present in the plane of the section. Nuclear vacuoles were frequently seen in these cells between stages I through V (~20% of sectioned images) compared with cells present in stages VI through XII (~3% of sectioned images). Although nuclear vacuoles could be seen thereafter, they were rare. The frequency of occurrence in stages I through V suggested that vacuoles may have been present in all cells of this type. The sectioned nucleus contained one or two large nucleoli. Little to no heterochromatin lined the nuclear envelope.

Subtypes of the group described above were noted. In one very rare (1% of all A_s to A_{al} cells) subtype ($A_{al\ subtype\ 1}$), the nuclear chromatin was finely granulated. Generally, the cytoplasm was darker than in other spermatogonia. In a second subtype ($A_{al\ subtype\ 2}$), which was less rare (35% of all A_s through A_{al} cells) than the first subtype ($A_{al\ subtype\ 1}$), a substantially greater mottled appearance of the nucleus was noted than in the major population of A_{al}. Approximately 46% of cells of the second subtype in stages I through V showed vacuoles, whereas only 4% in stages VI through XII showed vacuoles. Micrographs of the first subtype ($A_{al\ subtype\ 1}$) are depicted from stages VII and IX, when the



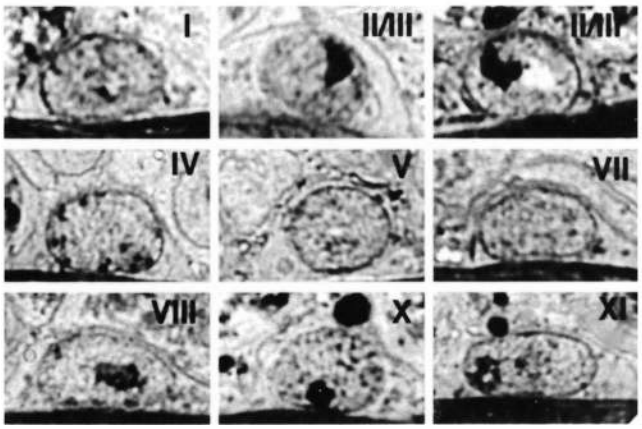
A_s, A_{pr}, A_{al}

Cytoplasmic density - low density
Nuclear shape - oval
Nucleolus - 1 or 2 small, irregular, darkly stained
Heterochromatin - splotchy chromatin of low density throughout the nucleus contrasting with intervening regions of euchromatin (can be described as a mottled nucleus); rare areas of dense heterochromatin along nuclear envelope
Euchromatin - contributes to the mottled appearance.
Nuclear vacuolation - yes, usually 1 in about 20% of sectioned material; present in stages I through V, but less common (3%) in cells in stages VI through XII.
Major distinguishing feature(s) from predecessor cell - see A_{al} subtypes since the latter may be A_{pr} and A_s cells.
Major distinguishing feature(s) from successor cell - The nucleus in A_{al} cells is mottled and not finely granular. The nucleolus is considerably less prominent.



A_{al} subtype 1 (very rare, shown for stage VII - IX)

Cytoplasmic density - sometimes dense
Nuclear shape - oval or somewhat flattened
Nucleolus - 1 or 2 in section
Heterochromatin - splotchy throughout nucleus
Euchromatin - areas between heterochromatin contributing to mottled appearance.
Nuclear vacuolation - occasionally seen in sectioned cells
Major distinguishing feature(s) from A_{al} cells - Since these cells differed from A_s, A_{pr}, A_{al} and A_{al} transition cells (the only cells in stages V-VIII), they were classified as a sub-population of A_{al}. They are possibly affected by fixation.



A_{al} subtype 2

Cytoplasmic density - low density
Nuclear shape - oval
Nucleolus - 1 in section
Heterochromatin - mottled (more so than other A_{pr} cells).
Euchromatin - clear areas between mottled heterochromatin
Nuclear vacuolation - yes, usually 1 in about 46% of sectioned material; present in stages I through V, but less common (4%) in cells in stages VI through XII.
Major distinguishing feature(s) from A_{al} cells -There is an extreme degree of nuclear mottling compared with other A_{al} cells.

FIG. 2. Micrographs showing the characteristics of various cell types and subtypes at specific stages of the cycle of the seminiferous epithelium (indicated in the upper right of each). The characteristics of the cell types are to the right. Original magnification ×1300.

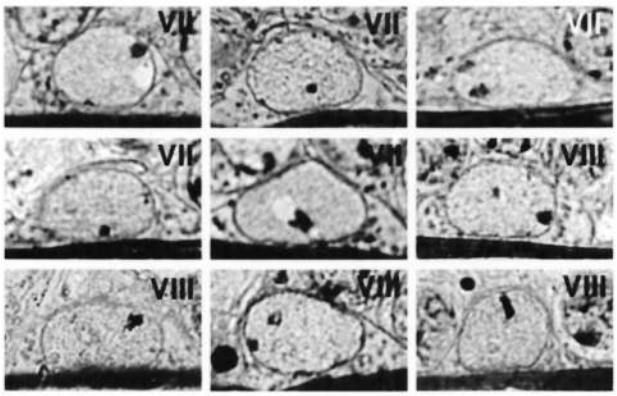
other associated cells (A_1 and A_2) are clearly of contrasting morphologies. The subtypes described displayed a clearly different morphology compared with other cells at these stages.

A_{al} to A_1 Transition Spermatogonia

Cells that represent the transition between A_{al} and A_1 showed a distinct morphology and were seen only in stages VII and VIII, in the period when A_{al} cells are known to transition to A_1 cells. The nuclei are larger than previously illustrated A_{al} cells. Chromatin mottling is present, but it is progressively much finer. Nuclear vacuoles are sometimes seen in these cells of stage VII but rarely in those of stage VIII. Heterochromatin is rare along the nuclear envelope. One or two prominent nucleoli are seen.

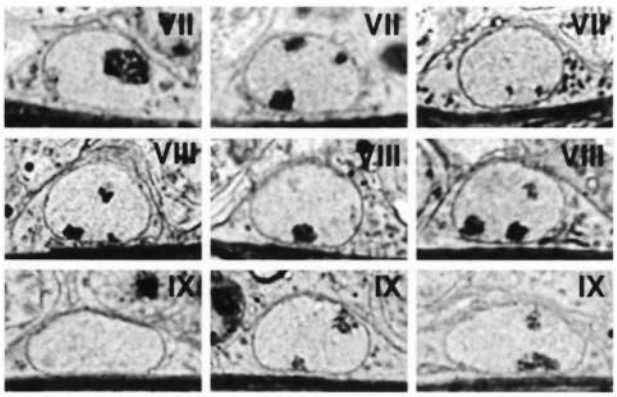
A_1 Spermatogonia

The A_1 cells were first recorded in stage VII. The A_1 spermatogonia possess large nuclei that are oval, yet their



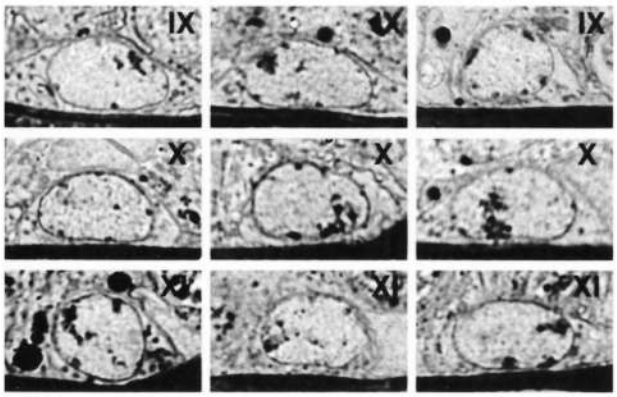
A_{al} to A_1 (transition cell)

Cytoplasmic density - low to moderate
Nuclear shape - oval
Nucleolus - 1 or 2 prominent nucleoli extending from the nuclear envelope centrally
Heterochromatin - low density, finely mottled throughout nucleus
Euchromatin - very small regions between heterochromatin
Nuclear vacuolation - only in stage VII and not in VIII
Major distinguishing feature(s) from predecessor cell - lesser degree of nuclear mottling compared with A_{al}.
Major distinguishing feature(s) from successor cell - greater degree of mottling of the nuclear heterochromain.



A_1

Cytoplasmic density - low to moderate
Nuclear shape - overall more rounded than predecessor cell
Nucleolus - 2-3 prominent, well circumscribed, darkly stained, often reticulated, peripherally located and protruding centrally from nuclear envelope
Heterochromatin - not detected due to almost complet absence of mottling.
Euchromatin - not detected due to almost complet absence of mottling.
Nuclear vacuolation - none
Major distinguishing feature(s) from predecessor cell - texture of nucleus is more finely granular
Major distinguishing feature(s) from successor cell - virtually no heterochromatin along the nuclear envelope



A_2

Cytoplasmic density - low to moderate
Nuclear shape - oval, similar to A_1
Nucleolus - smaller, less compact, more 'stringy'and irregular than A_1
Heterochromatin - flecks of heterochromatin along nuclear envelope occupying less than 10% of the nuclear envelope
Euchromatin - slight indication of fine mottling of the nucleus. Mottling does not approach the degree of that described for A_{al} cells
Nuclear vacuolation - none
Major distinguishing feature(s) from predecessor cell - presence of chromatin flecks along the nuclear envelope; presence of smaller and less compact, but stringy, nucleolus
Major distinguishing feature(s) from successor cell - less heterochromatin lining the nuclear envelope

FIG. 2. Cont'd.

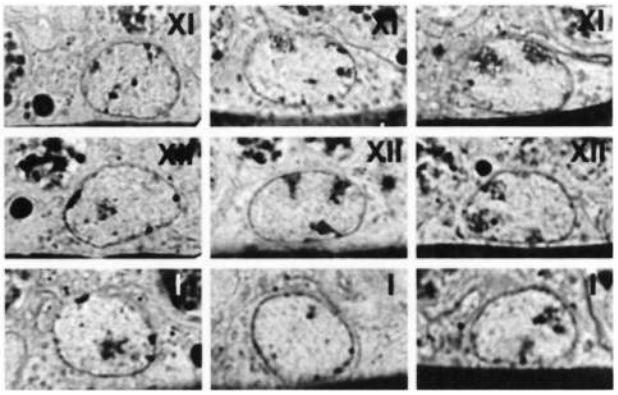
nuclei tend to be slightly more spherical than those of A_s through A_{al} cells. Chromatin is finely granulated and shows pale staining. Chromatin flakes along the nuclear envelope are very rare. From zero to three nucleoli are in the plane of the section. The nucleoli, as seen in sections, appear highly reticulated; nuclei are clearly demarcated from the surrounding nucleoplasm.

A_2 Spermatogonia

Small flakes of heterochromatin rim the nuclear envelope, but less than 10% of the nuclear envelope is rimmed by heterochromatin. Nuclear vacuoles are never seen in this and more advanced cell types. The nucleoli are similar to those of A_1 cells, except they appear more elongated and less well demarcated from the nucleoplasm. Very slight mottling of the nucleus is observed, but not to the degree seen in A_{al} cells.

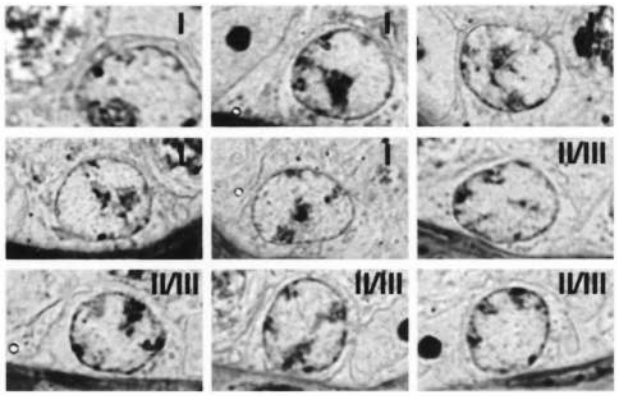
A_3 Spermatogonia

Nuclei of A_3 are more rounded than those of A_2 spermatogonia, although oval forms can be found. Approximately 10–25% of the nuclear envelope is rimmed with heterochromatin. The overall effect of this lining heterochromatin is to make the nuclear envelope appear prominent within the cell compared with A_2 cells. Mottling of the nucleus is very slightly increased compared with that of A_3 spermatogonia. Nucleoli are generally reticulated and not well defined (stained), but they extend deeply into the nucleoplasm.



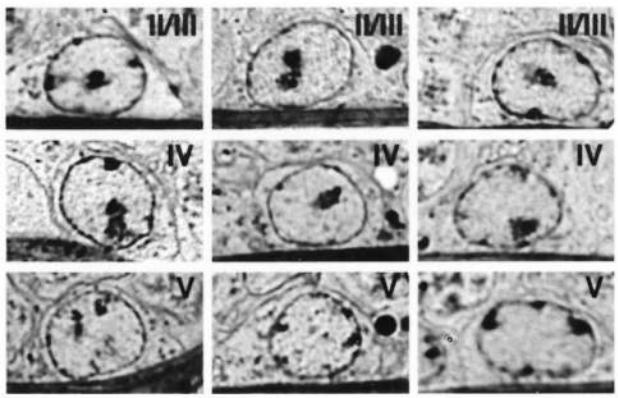
A_3

Cytoplasmic density - low to moderate density
Nuclear shape - oval to round; similar to predecessor
Nucleolus - large, stringy, reticulated and extends deeply into the nucleus; up to 3 nucleoli visualized in section
Heterochromatin - about 25% of the nuclear envelope is lined with heterochromatin
Euchromatin - slight increase in the degree of the mottling of the nucleus
Nuclear vacuolation - none
Major distinguishing feature(s) from predecessor cell - increase in heterochromatin lining the nuclear envelope.
Major distinguishing feature(s) from successor cell - less heterochromatin lining nucleus.



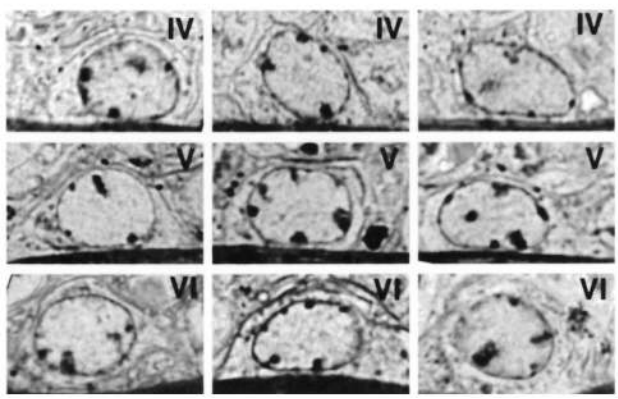
A_4

Cytoplasmic density - low to moderate density
Nuclear shape - oval to round in section; similar to predecessor cell
Nucleolus - Up to 3 nucleoli visualized in section. Nucleoli may 'bridge' across the nucleus.
Heterochromatin - chromatin lines from 30-70% of the sectioned nucleus
Euchromatin - similar to A_3 cells
Nuclear vacuolation - none
Distinguishing feature from predecessor - more heterochromatin lining nucleus.
Distinguishing feature from successor - considerably less heterochromatin riming the nucleus.



Intermediate type

Cytoplasmic density - low to moderate density
Nuclear shape - round to slightly oval
Nucleolus - one or two, compact nucleoli
Heterochromatin - 70% - 100% of the nucleus rimmed by heterochromatin; heterochromatin is shallow in regions and flaky in other regions.
Euchromatin - clear areas are slightly large than A_4 cells
Nuclear vacuolation - none
Major distinguishing feature(s) from predecessor cell - increase in chromatin riming the nucleus.
Major distinguishing feature(s) from successor cell - heterochromatin riming nucleus is both shallow and flaky compared with the compact rounded heterochromatin of type B cells.



Type B

Cytoplasmic density - low to moderate density
Nuclear shape - round to slightly oval in section
Nucleolus - small, compact, and darkly stained; usually only 1 per section
Heterochromatin - rounded heterochromatin clumps located periodically along the nuclear membrane
Euchromatin - mottlely due to contrast between heterochromatin and euchromatin is fine.
Nuclear vacuolation - none
Major distinguishing feature(s) from predecessor cell - balls of dense heterochromatin rim a portion of the nucleus

FIG. 2. Cont'd.

A_4 Spermatogonia

The nuclei of A_4 spermatogonia are the roundest yet in the developmental sequence. These cells closely resemble type A_3 spermatogonia. A slightly larger number of heterochromatin flakes lie on the nuclear envelope, however, and mottling of the nucleus is similar or slightly increased compared with type A_3 spermatogonia. The size of heterochromatin flakes is also increased compared with A_3 spermatogonia. Approximately 30–70% of the nuclear envelope is encrusted with heterochromatin. Only small differences are noted when these cells are compared with A_3 spermatogonia. Numerous cells of each type must be examined to distinguish one from another.

Intermediate Spermatogonia

The nuclei of intermediate spermatogonia are similar in shape to those of A_4 cells. Shallow heterochromatin lines 70–100% of the nucleus, making the nuclear-cytoplasmic boundary well delineated. The heterochromatin lining of the nuclear envelope is often shallow, and the more prominent heterochromatin is flaky. Mottling of the nucleus is prominent. One or two compact nucleoli can be found per section. It is difficult to differentiate A_4 from intermediate spermatogonia. When several of each type are examined, it becomes clear that the nuclear envelope is almost uniformly darkened by shallow chromatin in intermediate spermatogonia, whereas that of A_4 cells shows areas with little contrast between the nuclear periphery and cytoplasm.

Type B Spermatogonia

The nuclei of type B spermatogonia are more deeply stained and oval, and the euchromatin more homogeneously stained, compared with the predecessor intermediate spermatogonia. Less heterochomatin than seen in intermediate spermatogonia rims the nuclear envelope, with the range of encrustation being 20–100%. Small, densely stained ''balls'' of heterochomatin are attached to the nuclear envelope. The heterochromatin balls and nucleoli are more deeply stained than nuclei of intermediate spermatogonia.

Compared with the single animal studied thoroughly, no differences in spermatogonial characteristics, as described above and depicted in Figure 2, were noted for the three other animals undergoing a more cursory examination.

Side-by-Side Comparison of Spermatogonia

It was useful to view closely spaced spermatogonia of different types to make comparisons. Such comparisons showed differences that could not be attributed to computer adjustment of digital figures. Figure 3 shows a tubule in which two different spermatogonial cell types are seen: an A_3 spermatogonium and an A_{al} spermatogonium in stage XII (Fig. 3A), an A_{al} spermatogonium and an intermediate spermatogonium (Fig. 3B), and an intermediate spermatogonium and an $A_{al\ subtype\ 2}$ spermatogonium (Fig. 3C). A decision tree is also presented to aid in identifying the various spermatogonial types (Fig. 4).

Gray-Scale Histograms of Young Spermatogonia

Figure 5 shows gray-scale histograms of the nuclei of four young cell types ($A_{subtype\ 2}$, A_{al}, A_{al} to $A_{1\ transition}$, and A_1) and the region outlined in the nucleus of these four cell types. The histograms show a logical progression of a broad, gray-scale pattern in the cells thought to be most immature and a narrower band of gray scale in those that are more mature. Thus, the band is widest in $A_{subtype\ 2}$ and narrowest in A_1 cells. In general, a shift to the right (darker profile) was observed for cells as they matured, although the left-right position on the gray scale was somewhat variable, depending the degree of intensity of the staining.

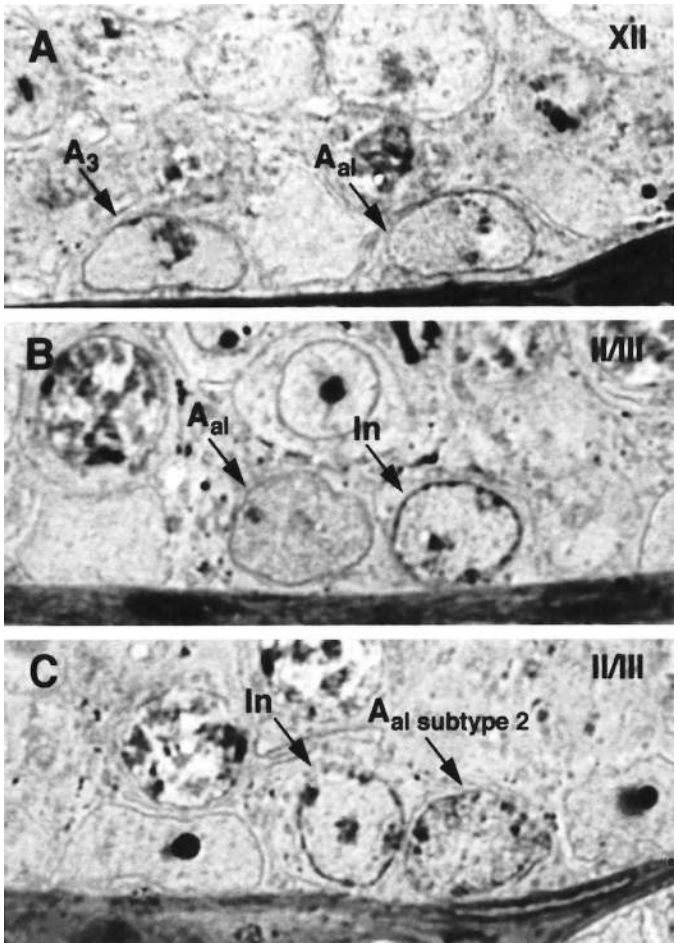


FIG. 3. Comparisons of spermatogonial types adjoining each other within the same seminiferous tubules. **A**) A_3 and A_{al}. **B**) Intermediate and A_{al}. **C**) $A_{al\ subtype\ 2}$ and intermediate. Original magnification ×1600.

DISCUSSION

To our knowledge, this is the first study to describe the morphological features of type A spermatogonia using high-resolution, plastic-embedded tissue. We have shown that differences exist between all spermatogonial types. In some cases, these differences are minor; in others, they are quite marked. The literature suggests that some differences in the various spermatogonial types have been noted previously. For example, the descriptions and drawings of A_1 through A_4 spermatogonia made by Clermont and Bustos-Obregon [5], Huckins and Oakberg [3], and Oakberg [4] suggest that increasing amounts of heterochromatin line the nuclear envelope as cells differentiate from A_1 through A_4. Generally, when whole-mounted seminiferous tubules are utilized, the spermatogonial type is not determined by nuclear features but, rather, by the stage of the cycle in which the spermatogonia are seen as well as by the number of interconnected cells in a clone.

The present study clearly shows the features of spermatogonia in numerous micrographs. It allows for close

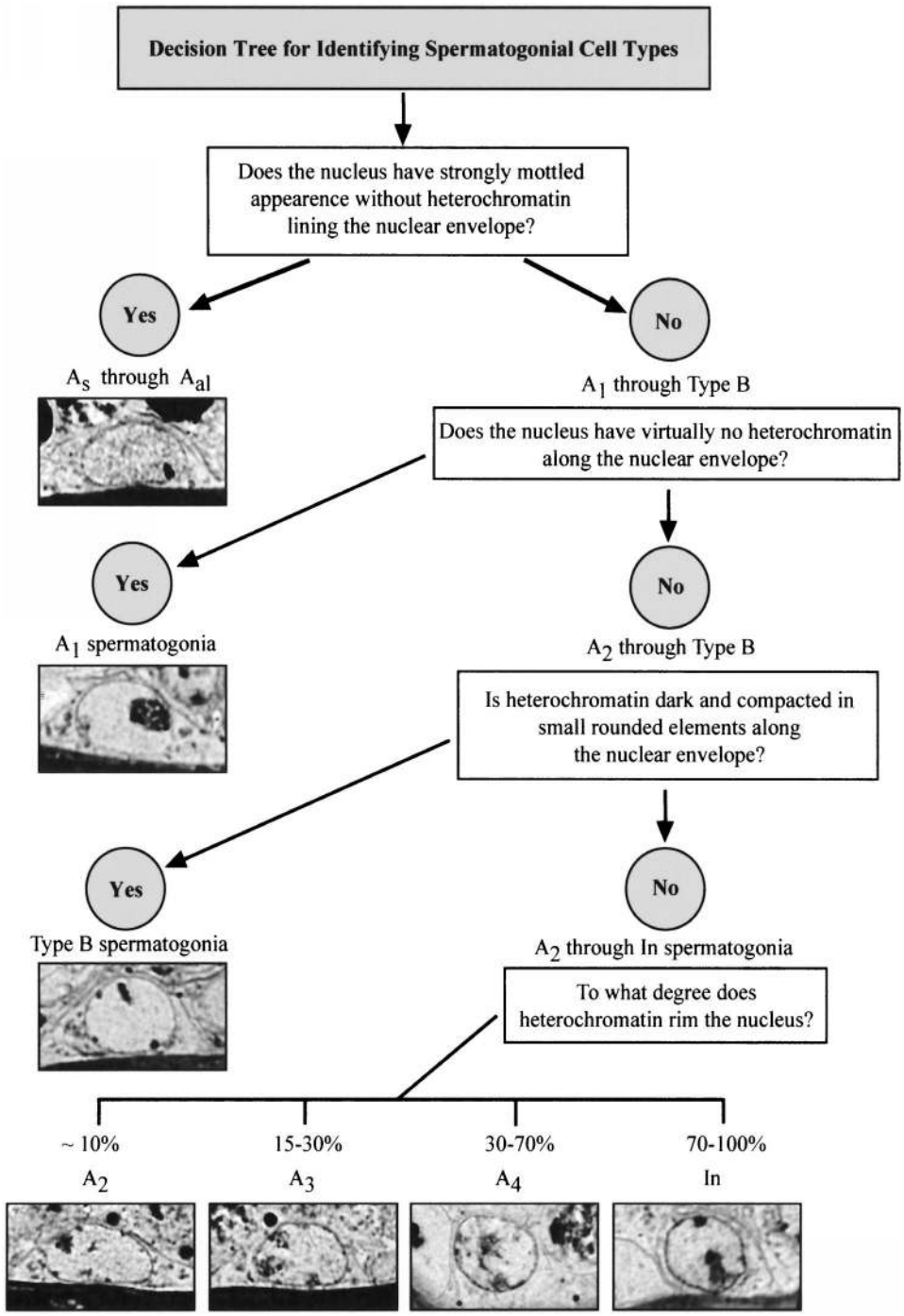


FIG. 4. A yes/no decision tree used to determine the various types of spermatogonial cells. Original magnification ×1300.

comparison of cell types in sections of high resolution. It also allows one to distinguish the population of A_1 through A_4 spermatogonia from the population of less mature A_s through A_{al} spermatogonia without the necessity of using whole mounts and of viewing intercellular bridges. The ability to distinguish the primitive A_s, A_{pr}, and A_{al} cells from the more advanced A_1 through A_4 cells proved useful in a companion study [13].

We believe, however, that some degree of caution should be exercised in distinguishing spermatogonial types based on the morphology presented. Certain spermatogonial cell types are clearly very similar in appearance. For example, A_4 and intermediate spermatogonia appear nearly identical. In addition, sectioned material used in the present study may, or may not, include typical features, such as a nucleolus. The fixation and tissue preparation protocol utilized in the study of spermatogonial cell types emphasizes certain features—features that may not be apparent with material prepared in another manner. We also caution against examining one or two cells; instead, we recommend that a minimum of five well-stained and well-displayed cells of a particular kind be examined before determination of the cell type. For example, an individual who was knowledgeable regarding the criteria for cell identification and with experience in identifying the various spermatogonial cells described in this report made an error in less than 16% of attempts at identifying specific cell types. Cells identified incorrectly in such a manner are never more than one cell distant in the lineage of spermatogonia. Additionally, when we examine very young animals (<10 days of age), many

of the aforementioned cell types are not of similar shape to those in the adult. We believe that the criteria applied herein to the adult should not apply to the young animal, and that these cells should be characterized in a separate study.

What are the essential differences in spermatogonial cell types of the mouse? A brief examination of the cell types is insufficient to differentiate them. We recommend that, when differentiating the various types of spermatogonia is necessary, one exert considerable effort and place micrographs of cells side by side for comparison. Although in *Results* we described the morphology of various types of spermatogonia, the key to cell identification is knowing the essential differences between the cell types. Figure 4 provides a decision tree that can be used to differentiate the various spermatogonial cell types when no staging clues are present.

In assigning cell types, we paid attention to the stage of the cycle as well as to a logical progression of features associated with cells. Two notable examples of the latter can be described. First, the nuclei of A_{al} cells showed a marked mottled appearance. As they transitioned into A_1 spermatogonia, the mottling became finer, until the A_1 cells displayed a nucleus that was finely granular. The fine granularity of A_1 spermatogonia was lost after they divided to progressively form A_2, A_3, and A_4 spermatogonia, and a gradual reappearance of mottling then occurred. Mottled cells of this lineage developed their mottling component to the extent that they almost resembled A_{al} spermatogonia, but unlike A_{al} cells, their nuclei were encrusted with heterochromatin. Intermediate spermatogonia contained mottled chromatin, although type B spermatogonia had slightly less. Second, heterochromatin changes along the nuclear envelope were noted and progressed logically. In A_1 cells, virtually no heterochromatin was observed, and the amount of heterochromatin increased with time, such that intermediate spermatogonial nuclei were either completely or almost completely rimmed with heterochromatin. The pattern of nuclear heterochromatin differed in intermediate and type B spermatogonia.

The ability to differentiate a primitive population that we call A_s, A_{pr}, and A_{al} from A_1 through A_4 is yet another argument for the stem cell renewal scheme presented by Huckins and Oakberg versus that proposed by Clermont and Bustos-Obregon (reviewed in [1]). The major difference between the scheme proposed Huckins and Oakberg and that proposed by Clermont and Bustos-Obregon is that the latter theory does recognize the A_{pr} and A_{al} cells as being a separate population from A_1 through A_4 cells. Chain length has, to date, been the major method by which to differentiate these cell populations [1]. Here, we show a clear morphological difference in these cell populations. Not only is morphological difference in the various cell types observed, but also a logical progression of the morphology (as well as histogram patterns) of cells from the A_s to A_{al} group to A_1 and, progressively, from A_1 to A_4 to intermediate and, finally, to type B spermatogonia. If one were to consider this progression according to the Clermont and Bustos-Obregon scheme, it would not be logical for cells with the morphology of A_4 spermatogonia, with extensive heterochromatin, to dedifferentiate to cells like A_1 cells, which contain virtually no heterochromatin.

With attention now focusing on stem cells, we believe that the characterization of spermatogonia will prove increasingly important in distinguishing specific cell type in both normal and abnormal conditions. Markers for spermatogonia will, no doubt, be developed in the future that

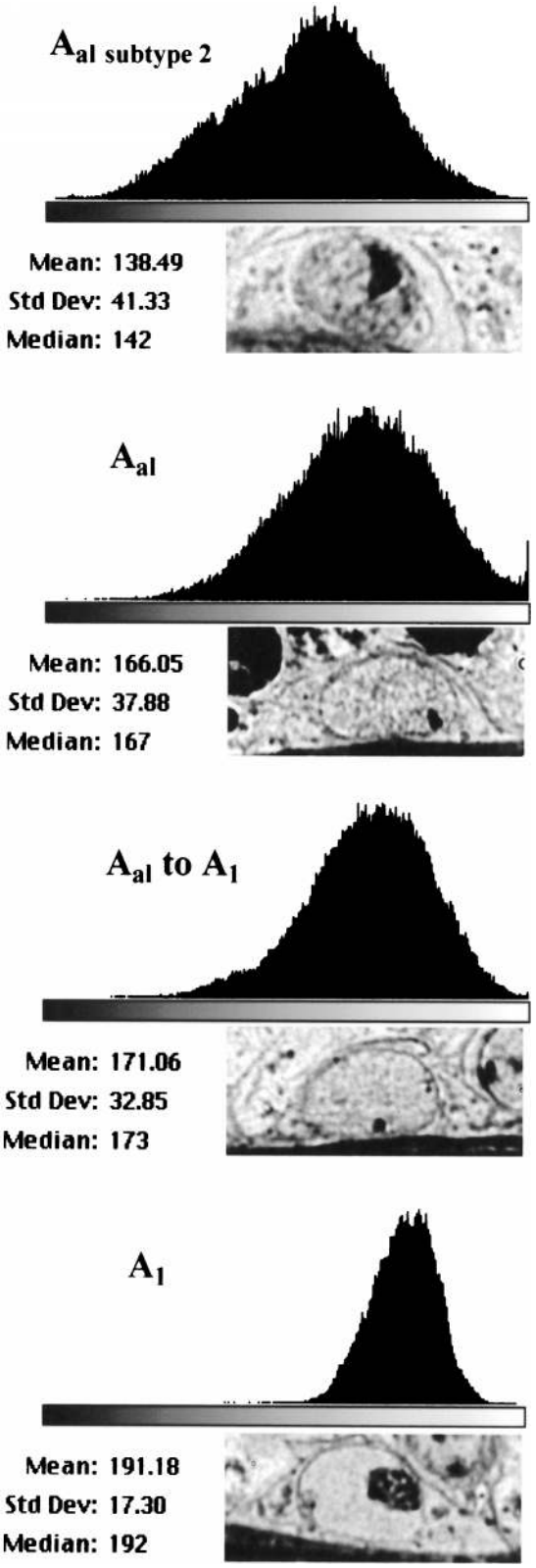


FIG. 5. Gray-scale histograms of the nuclei of types $A_{subtype\ 2}$, A_{al}, A_{al} to $A_{1\ transition}$, and A_1 and the region outlined in the nucleus of these four cell types. Areas with low gray-scale values are to the left of the histogram, and those to the right have the highest values. A shift of the entire profile to the left or the right should not be considered absolute, because the staining of cells from different slides may vary the overall density. However, several gray-scale measurements form similar cells show the trend indicated on the abscissa for the density profiles to be representative of the specific cell types shown. Original magnification ×1400.

will further distinguish spermatogonial cell types. The present study will prove useful to identify the spermatogonial types that display specific markers. We should caution, however, that other means of fixation and embedding that are necessary for histochemical studies will alter the structure that is seen compared with the results of the present study, and that spermatogonial cell type will need to be redefined. This study is valid for mouse tissues of a single strain, fixed in a specific manner, and with tissues sectioned to a specific thickness. It does, however, show the feasibility of distinguishing spermatogonial types based solely on morphology, and a separate study shows the value of doing so. Our laboratory is now extending this study to the ultrastructural level to further characterize the various spermatogonial cell types.

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