Characterization of mouse spermatogonia by transmission electron microscopy

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Characteristics of the various type A, intermediate (In) and B spermatogonia were determined in C57BL/6J mice using transmission electron microscopy. Spermatogonia were photographed at all stages of the cycle of the seminiferous epithelium. Over 450 images were taken. Spermatogonia could be classified into A_s, A_{pr}, A_{al}, A₁ cells, A₂ cells, A₃ cells, A₄ cells, intermediate type and type B cells primarily on the basis of nuclear and nucleolar characteristics. The most primitive spermatogonia (A_s, A_{pr}, A_{al}) had mottled chromatin; A₁ cells contained homogeneously finely

granular chromatin throughout the nucleus; A₂, A₃, A₄ and intermediate type spermatogonia had progressively increasing amounts of chromatin encrusting the nuclear envelope; type B spermatogonia had less heterochromatin along the nuclear envelope, although the particles were more dense and rounded than in intermediate type spermatogonia. Mitochondrial size and position of Golgi complexes varied in different types of spermatogonia. These data show that types of spermatogonia can be differentiated such that these characteristics can be used in functional studies.

Introduction

The diploid germ cells that initiate spermatogenesis, the spermatogonia, have been the least studied of all the germ cells. Most studies have examined the kinetics of spermatogonia from stem cells to their division into spermatocytes using whole-mount techniques in which clones of spermatogonia are visible and the cell type can be identified by association with a particular stage of the seminiferous epithelium. The number of cells has also been useful to identify cells by their intercellular bridges and number of cells in a clone (Huckins and Oakberg, 1978; de Rooij and Russell, 2000). It is generally now appreciated that spermatogonia undergo 9–11 divisions before entering meiosis. Studies of spermatogonia have mainly examined their behaviour in whole mounted seminiferous tubules after, for example, irradiation (van Beek *et al.*, 1984), vitamin A depletion (van Pelt *et al.*, 1995) or transplantation (Nagano *et al.*, 1999). There is considerable information about various other types of germ cell, such as spermatocytes and spermatids, but because spermatogonia are thought to 'look similar', most investigators still view the spermatogonia as a morphologically homogeneous population.

In mice and rats, the most primitive cell is an A_s spermatogonium that functions as the stem cell (de Rooij, 2001). It is not connected to other spermatogonia by intercellular bridges and is characterized as a 'single' or 'isolated' cell (A_s). The A_s cells divide, but maintain a telophase connection known as the intercellular bridge. The A_{pr} (paired) cells,

formed from an A_s spermatogonium, divide to form longer chains of interconnected cells called A_{al} (aligned) spermatogonia. The divisions up to the A_{al} stage may occur at any time during the cycle of the seminiferous epithelium. Once the chains of A_{al} cells reach about 16 cells, they transform at a predictable time during the spermatogenic cycle to A₁ cells. A₁ cells divide to form A₂ cells, and A₂ cells divide to form A₃ cells, which divide to form A₄ cells. The A₄ cells divide to form intermediate type spermatogonia, which divide to form type B spermatogonia. All divisions after A₁ occur at predictable stages of the cycle of the seminiferous epithelium (de Rooij, 1998). The chain length of spermatogonia increases progressively with each cell division.

There is evidence that there may be morphological differences in some of the type A spermatogonia visible using a light microscope (Clermont and Bustos-Obregon, 1968; Oakberg, 1971; Huckins and Oakberg, 1978). More recently, Chiarini-Garcia and Russell (2001) used high resolution light microscopy to define the characteristics of many of the types of mouse spermatogonial cell. The ability to distinguish types of spermatogonial cell allows spermatogonia to be studied functionally (Chiarini-Garcia *et al.*, 2001). In the present study, light microscopic observations of spermatogonia have been extended using an electron microscope. Our observations relating to the fine structural differences in the various types of spermatogonia are presented.

Materials and Methods

Animals, tissue collection and processing

Four adult C57BL/6J mice, 27.80 ± 0.52 g body weight, were obtained from Jackson Labs (Bar Harbor, ME). The

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maintenance of the animals was in full compliance with the standard laboratory animal care protocols approved by Institutional Animal Care and Use Committee (IACUC). Mouse testes were perfusion-fixed according to the technique of Sprando (1990). In brief, each animal was given heparin 15 min before perfusion. Saline was perfused initially to clear the blood from the testis followed by 5% (w/v) glutaraldehyde (pH 7.2–7.4) in 0.05 mol sodium cacodylate buffer l^{-1} . After a glutaraldehyde perfusion of approximately 20 min, the testes were excized, weighed (104 ± 1.36 g; $n = 8$) and cut into 0.5 mm slabs by perpendicular sectioning. The slabs were cut into approximately 2 mm (length $\times$ width) portions and embedded such that cross-sections of seminiferous tubules would be obtained when the samples were cut into sections. After an overnight fixation of the diced slabs, the tissues were washed in three changes of buffer, post-fixed in an osmium:ferrocyanide mixture (Russell and Burguet, 1977), dehydrated, embedded in epoxy resin and cut into sections (1 μm thickness) for light microscopy.

Electron micrograph study

Tissues from four animals used in a previous study were used again in the present study (Chiarini-Garcia and Russell, 2001). Embedded blocks were trimmed to reflect a random collection of stages of the cycle of the seminiferous epithelium. Sections showing silver-gold to gold interference colours were made with a diamond knife and mounted on celloidin-coated slot grids. All type A spermatogonia displaying nuclei less mature than A_3 spermatogonia were photographed in the 12 stages of the cycle of the seminiferous epithelium. As there were numerous A_3 , A_4 , type B and intermediate type spermatogonia within the epithelium, only random images of these cells were photographed. Images displaying grazing sections of the nucleus were not photographed.

The staging criteria used to identify spermatogonia were those of Oakberg (1956), as modified and depicted by Russell *et al.* (1990). In particular, the 'stage range' diagram of Russell *et al.* (1990) was used to determine the beginning and end of each stage. More than 35 micrographs of type A spermatogonia were taken with a Hitachi 500H Electron Microscope (Hitachi Corporation, Tokyo) at each stage. The magnification range of the negatives was $\times 2000$ –4000 original size.

Images were mounted on the wall of a room. Using criteria and the general concepts developed for light microscope study (Chiarini-Garcia and Russell, 2001), spermatogonia were categorized by a single worker into type of cell (A_5 to A_{al} , A_1 , A_2 , A_3 , A_4 , intermediate type and type B) and stage without any prior knowledge of the stage from which the micrograph was taken. After preliminary classification, the stage at which the micrograph was taken was added and the accuracy of the investigator was tested using only cells from stages in which the cell type was known to occur. After providing criteria for a particular type of cell, the spermatogonia were categorized for all stages of the cycle.

Stereological studies

Measurements of subcellular organelles and determination of position of organelles were performed. Transversely sectioned mitochondria that showed spherical profiles were measured from micrographs using the smallest dimension of the cross-section or the smallest diameter in a slightly oblique section, using a scale lupe. All measurements of mitochondria were pooled from a particular type of cell or grouping to yield a mean mitochondrial diameter. Thirty-two to sixty-three diameters were measured for each type of spermatogonia.

The position of the centre of the Golgi complex was also determined from data pooled from all animals for individual types of cell. The centre of the nucleus was considered as the centre of a clock and 12 clock numbers were assigned radially around the nucleus to determine the Golgi position. The position of each Golgi complex was recorded as a clock hour number. As the orientation of the tubule with respect to the rete was not known, horizontal mirror image dimensions of the clock were considered equivalent, for example 3 o'clock and 9 o'clock were equivalent.

A digitizer was used to determine the length of the contact of various types of spermatogonia with the basal lamina. Only cells that showed their entire contact in the micrograph were measured.

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analyses were performed by ANOVA and Student's *t* test for multiple comparison among means. Differences were considered significant at $P < 0.05$.

Results

All but one spermatogonium that was photographed was well fixed. This single poorly fixed spermatogonium had darker than usual cytoplasm and this feature was regarded as an artefact of fixation, as the mitochondria were swollen.

After images were printed, the light microscope criteria defined previously were used to make an initial attempt to identify specific types of cell by the other investigator. The initial attempt to identify cells was 85% accurate when compared with the final assignment of type of cell that was made using known staging criteria. Even with knowledge of the stage from which a particular cell was found, the identity of some cells remained in question because cells of different types are found within a particular stage owing to mitotic divisions in that stage. Cells in question were not used in the subsequent morphological and quantitative analyses below, as they were excluded from the initial assessment presented above.

A feature not reported previously for any type A spermatogonia was the presence of multivesicular bodies (Fig. 1a–d). Few internalized vesicles were present in any of the multivesicular bodies. Multivesicular bodies were always located in the juxta-Golgi region. Although multivesicular

Fig. 1. Multivesicular bodies in mouse spermatogonia. (a) Stage I, (b,c) stage XII and (d) stage VI spermatogonia. Spermatogonia show a large vesicular element with sparse, small, vesicles contained within. These were frequently observed near the Golgi apparatus and were thought to be multivesicular bodies (arrows). BL: basal lamina. Scale bars represent 0.5 μm .

bodies were present, lysosomes, expected to be associated with multivesicular bodies, were not detected.

A_s, A_{pr}, A_{al} spermatogonia

The nuclei of this combined group of spermatogonial cells displayed foci of dense heterochromatin flecks (Fig. 2a,b). Moderately dense heterochromatin flecks, measuring no more than $0.23 \pm 0.02 \mu\text{m}$ across, were larger than heterochromatin flecks in any of the other more advanced type A spermatogonia. The nucleus had a mottled appearance with the moderately dense heterochromatin forming the denser regions and virtually no heterochromatin in the lighter regions. No intranuclear vacuoles were noted, as was reported in a recent light microscope study (Chiarini-Garcia and Russell, 2001) and shown previously by electron microscopy (de Rooij, 1973). The nucleolus appears to be reticulated tightly; the reticulation is produced by the folding of the pars fibrosa. Mitochondrial profiles measured $0.57 \pm 0.01 \mu\text{m}$ across (Fig. 3). The Golgi apparatus was located 55.4% of the time in the basal, basolateral and lateral aspects of the cytoplasm, but could be found in any position (Fig. 4).

Fig. 2. *A_s, A_{pr}* and *A_{al}* mouse spermatogonia. (a,b) The nucleus shown contains a mottled appearance with dark speckles of heterochromatin (small arrow), moderately dense heterochromatin measuring $\leq 0.23 \mu\text{m}$ across (large arrow) and rarefied areas (arrowheads). (a) The Golgi apparatus (G) is positioned away from the basal lamina (BL) at the apical aspect of the cell. (b) A portion of a small compact nucleolus (Nu) is visible. (a) Stage II and (b) stage VI. Scale bars represent 1 μm .

Fig. 3. Cross-sectional diameter of mitochondria of various types of mouse spermatogonia. ^{a-d}Different letters indicate significant differences among groups ($P < 0.05$).

A_s, A_{pr}, A_{al} subtype 2 spermatogonia

A small percentage of all spermatogonia were of the *A_s* to *A_{al}* lineage and were morphologically different from most of the cells in the *A_s* to *A_{al}* lineage. The *A_s* to *A_{al}* subtype 2 cells were named for their apparent counterpart in the study of Chiarini-Garcia and Russell (2001). The nucleus of these cells was ovoid and moderately dense chromatin

Fig. 4. Position of the Golgi apparatus of mouse spermatogonia presented as a percentage of the total cells of a particular type. Twelve compartments have been designated radially around the cell from a central position in the nucleus as would be found in a clock. As the orientation of the tubule and the section is not known with respect to the rete, the percentage values for each type of cell are plotted in one-half of the drawing. They correspond to the values of both the left and right sides.

was highly mottled against an electron translucent background (Fig. 5a,b). The mottling was more pronounced in A_{al} subtype2 cells ($0.53 \pm 0.02 \mu\text{m}$) than in any other cells of the A_s to A_{al} lineage ($0.23 \pm 0.02 \mu\text{m}$, $P < 0.05$). Very little heterochromatin lined the nuclear envelope. Small, dense flecks of heterochromatin were present and were similar to those of A_s, A_{pr} and A_{al} cells. The pars fibrosa of the nucleolus was reticulated and the nucleolus was compacted (Fig. 5c). Too few cells were encountered to determine the position of the Golgi apparatus in this subpopulation of cells.

A_{al} to A₁ transition spermatogonia

In late stage VII and VIII, numerous forms of spermatogonia were observed that were transitional between the type A_s, A_{pr}, A_{al} spermatogonia and A₁ spermatogonia. There were numerous gradations in morphology in these transitional forms that contained characteristics of both cell types. A single example is shown (Fig. 5d).

A₁ spermatogonia

No heterochromatin lined the nuclear envelope of type A₁ spermatogonia (Fig. 6a,b). The nuclear matrix was uniformly finely granular. Mitochondrial cross-sections were $0.60 \pm 0.01 \mu\text{m}$ in diameter (Fig. 3). The Golgi apparatus lies in the basal lateral region of the cell 95.7% of the time and is observed rarely in the apical portion of the cell (Fig. 4). The nucleolus is compact and the pars fibrosa is slightly

more reticulated than the nucleolus of cells of the A_s, A_{pr}, A_{al} group (Fig. 6a,b).

A₂ spermatogonia

The nuclear matrix of A₂ spermatogonia had denser chromatin than in A₁ cells (Fig. 6c,d). There were more areas in the nucleus that appeared finely mottled than in the precursor A₁ cells. Small, shallow flecks of chromatin lined the nuclear envelope. Generally, < 10% of the nuclear envelope had these chromatin flecks. The nucleolus was no longer compact, but was more spread out throughout the nucleus. The spreading is primarily a result of decompaction of the pars fibrosa. Mitochondrial cross-sectional diameter was the greatest ($0.69 \pm 0.01 \mu\text{m}$) of all the types of spermatogonia (Fig. 3). The Golgi complexes were positioned in the basal, basolateral and lateral aspects of these cells (Fig. 4).

A₃ spermatogonia

A₃ spermatogonia were generally similar to A₂ spermatogonia, but the nucleus of A₃ cells was slightly different in that between 10% and 25% of the nuclear envelope was lined with heterochromatin. The nucleolus was organized loosely and similar to that described for A₂ spermatogonia (Fig. 7a,b). The mitochondrial cross-sectional diameter in A₃ spermatogonia was large ($0.67 \pm 0.02 \mu\text{m}$) and not significantly different from that of A₂ cells, but significantly different from other types of spermatogonia (Fig. 3). The

Fig. 5. (a) The cell on the right is an A_s , A_{pr} , A_{al} subtype 2 spermatogonium, whereas the cell on the left is an A_3 spermatogonium. The former has highly mottled chromatin compared with most of the cells of the A_s , A_{pr} , A_{al} group. Dense flecks of heterochromatin are present throughout the nucleus (small arrow). The moderately dense heterochromatin measures $0.53 \pm 0.02 \mu\text{m}$ across (large arrow) and lies in stark contrast to the rarified nuclear background (arrowheads). The A_3 cell is typical of those described below. (b) A_s , A_{pr} , A_{al} subtype 2 spermatogonium is shown with a small compact nucleolus (enlarged in (c)). (d) A transitional spermatogonium. In stage VII, some cells present as intermediate in morphology between those of the A_s , A_{pr} , and A_{al} group. The heterochromatin of these intermediate cells shows slight mottling and the nucleus or nuclei are moderately compact. A small (in section) multivesicular body is indicated (arrow). (a) Stage I, (b–d) Stage VII. Scale bars represent $1 \mu\text{m}$.

Fig. 6. For legend see facing page.

Golgi apparatus had a similar (basal, basolateral and lateral) distribution to that of A_2 spermatogonia (Fig. 4).

A₄ spermatogonia

The heterochromatin fragments lining the nuclear envelope in A_4 cells were larger than those observed in A_3 cells. Between 25% and 40% of the nuclear envelope was lined with heterochromatin (Fig. 7c,d). The nucleolus was similar to that of A_2 and A_3 nucleoli. The mitochondrial cross-sectional diameter in A_4 spermatogonia was large ($0.64 \pm 0.01 \mu\text{m}$) and not significantly different from A_3 spermatogonia, but significantly different from all other types of type A spermatogonia (Fig. 3). The Golgi apparatus was located primarily in the basal, basolateral and lateral regions of the cell (Fig. 4).

Intermediate type spermatogonia

From 40% to 90% of the nuclear margin of intermediate type spermatogonia was occupied by irregularly shaped and low-lying heterochromatin (Fig. 8a,b). The nucleolus was condensed and a pars amorpha and pars fibrosa were clearly visible. The mitochondrial cross-sectional area was markedly and significantly smaller than that of A_4 spermatogonia and all other predecessor cells (Fig. 3). The Golgi apparatus was located primarily in the basal, basolateral and lateral regions, although it was found in all locations (Fig. 4). Mitochondrial cross-sectional diameter ($0.47 \pm 0.01 \mu\text{m}$) was significantly smaller than that of all other predecessor types of spermatogonia.

Type B spermatogonia

In type B spermatogonia, approximately 25% to 45% of the rim of the nucleus was heterochromatin (Fig. 8c,d). The shape of the heterochromatin was less irregular than that of the predecessor cells. Instead, generally rounded and more compacted heterochromatin lined the nuclear envelope. Mitochondrial diameters were the smallest of all the categories of spermatogonia ($0.44 \pm 0.01 \mu\text{m}$) and measured significantly less in cross-sectional diameter compared with that of all other groups except for intermediate type spermatogonia (Fig. 3). The Golgi complexes were distributed almost equally around the nucleus, except they were not present at the basal aspect of the cell (Fig. 4). Golgi complexes were located most frequently in the apicolateral and basolateral aspects of the cell. The pars fibrosa of the nucleolus was more compact than the nucleolus of intermediate type spermatogonia (Fig. 8c,d).

Contact with the basal lamina

The contact of various types of spermatogonia with the basal lamina was measured. The mean $\pm$ SEM (μm) contacts are shown: A_5 to A_{al} : 14.02 ± 2.58 ($n = 4$); A_1 : 14.49 ± 1.78 ($n = 7$); A_2 : 12.59 ± 2.61 ($n = 5$); A_3 : 13.29 ± 1.24 ($n = 17$); A_4 : 11.73 ± 1.42 ($n = 12$); intermediate: 9.31 ± 1.08 ($n = 15$); and B: 10.07 ± 1.00 ($n = 9$). The only significant difference was between A_1 versus B type and intermediate type spermatogonia.

Side-by-side comparison of spermatogonia

In some instances, micrographs of different types of spermatogonia were found side-by-side and viewed in the same micrograph. These images make useful comparisons (Fig. 5a) to show differences in spermatogonia. A yes/no decision tree and a brief description of the various types of spermatogonia are shown (Fig. 9).

Discussion

The present study follows another study using a light microscope in which the various types of spermatogonia were characterized, largely on the basis of nuclear detail (Chiarini-Garcia and Russell, 2001). The results of the present study show that most of the nuclear criteria used for identification of type A spermatogonia with a light microscope also apply at the ultrastructural level.

For example, the relative amount of heterochromatin lining the nucleus is characteristic of specific types of cell; however, the exact percentage of the nuclear envelope with a heterochromatin rim differs in the light and electron microscope studies. The reason for the variance lies in the interpretation of sections of different thicknesses. As the thickness of a section is much greater in light microscope sections than in electron microscope sections, this apparent inconsistency is entirely logical, as structural overlap is greater in thicker sections.

Two characteristics are predominantly responsible for the ability to separate spermatogonia or groups of spermatogonia. Firstly, in the most primitive group of spermatogonia (A_5 , A_{pr} and A_{al}), the mottled appearance of the nucleus is a result of the focal occurrence of moderately dense heterochromatin (although dense heterochromatin flecks are also present) contrasting with regions of no heterochromatin. The mottling slowly goes away in the transition of the primitive group (A_5 , A_{pr} and A_{al}) and is absent in A_1 spermatogonia. Cells that are more mature than A_1 spermatogonia show slight mottling, but never to the extent observed in the primitive group.

Fig. 6. (a,b) Type A_1 mouse spermatogonium. The cells shown contain a finely granular chromatin throughout the nucleus without evidence of mottling. The nucleolus is compact and highly reticulated (arrow). (c,d) Type A_2 spermatogonia. Heterochromatin flecks are visible along a small portion ($< 10\%$) of the nuclear envelope (arrowhead). The chromatin shows fine and dense particles, giving a slightly mottled appearance. The nucleoli (arrows) are stringy and several may be seen in section. (d) A multivesicular body is visible near the Golgi at the lower right of the figure (arrow). (a,b) Stage VII, (c) stage IX and (d) stage X. Scale bars represent $1 \mu\text{m}$.

Fig. 7. For legend see facing page.

Fig. 8. (a,b) Intermediate type mouse spermatogonia. The nuclei of intermediate spermatogonia have 40–90% heterochromatin lining the nuclear envelope (arrowheads). The heterochromatin lining the nucleus is generally 'low lying' and the face of the heterochromatin facing the nucleus is highly irregular in shape. The pars fibrosa of the nucleolus (arrow) is loose. (c,d) Type B spermatogonia. Fewer heterochromatin flecks line the nuclear envelope than in predecessor cells, but they are more prominent. The aspect of the heterochromatin facing the centre of the nucleus is generally rounded (arrowheads). Arrows show the slightly more compact nucleoli than type intermediate spermatogonia. (a,b) Stage IV and (c,d) stage VI. Scale bars represent 1 μm .

The dense heterochromatin flecks differ in size in different spermatogonia. In the subpopulation of the more primitive spermatogonia (A_{al} subpopulation 2) cells they are the largest (0.53 μm across) and are larger than the main A_s , A_{pr} and A_{al} population ($\leq 0.23 \mu\text{m}$ across). The presence of heterochromatin flecks and the increased mottling indicate that the A_{al} subpopulation 2 may be a separate population of cells from the larger number of A_s , A_{pr} and A_{al} , and may represent A_s or A_{pr} spermatogonia. Chromatin flecks and

mottling are not present in A_1 cells, but are visible in A_2 and more mature spermatogonia.

The nuclear vacuole described by light microscopy in the A_s , A_{pr} and A_{al} population of cells by Chiarini-Garcia and Russell (2001) was not observed in the present study, although it was observed in the same tissue by light microscopy. The reason for this difference is not known. Even more puzzling is that nuclear vacuoles were described by electron microscopy by de Rooij (1973).

Fig. 7. (a,b) Type A_3 mouse spermatogonia. The amount of heterochromatin (arrowheads) lining the nuclear envelope in the two cells shown is approximately 10–25%. The loosely reticulated nucleoli are indicated (arrows). (c,d) Type A_4 mouse spermatogonia. The heterochromatin flecks of A_4 spermatogonia are considerable larger than those of the predecessor cells. The cells shown have approximately 25–40% of the nuclear envelope decorated with heterochromatin (arrowheads). Nucleoli (arrows) are reticulated loosely, with the pars fibrosa being the most prominent component. (d) Two conjoined A_4 spermatogonia. (a,b) Stage XII, (c) stage II and (d) stage I. Scale bars represent 1 μm .

Fig. 9. A yes/no decision tree allows an investigator to determine the type of spermatogonia based on the criteria provided.

The nucleolus is compacted in the primitive A_s , A_{pr} and A_{al} cells. The nucleolus takes a markedly less compacted form after the division to form A_1 to A_2 cells. Less compacted nucleoli are considered more active in RNA

synthesis (Busch and Smetana, 1970; Schultz and Leblond, 1990). The nucleolar change may be a result of the increased synthetic capacity necessary for the rapid divisions that occur from A_2 onward. The reason for the apparent

movement of the Golgi apparatus is not known, but it may be passive as a result of shape changes in the cell or redistribution of its cytoplasm as a result of pressure from surrounding cells.

The results of the present study also demonstrate that differences in organelles were likely to occur in the progress of spermatogonial division. Mitochondrial cross-sectional area increased, reaching a peak diameter in A_2 and A_3 cells, and then decreased, with the smallest diameter in intermediate and type B cells.

The results relating to the contact of the cell with the basal lamina are limited, as the number of cells was small because most spermatogonia contact with the basal lamina extended beyond the limits of the micrograph. The cells most severely restricted by space were the most primitive cells, as they were more flattened on the basal lamina. Despite these limitations, the data show a trend in which contact is greatest in the most primitive cells and least in the more mature spermatogonia. This is a logical result given that the numbers of cells such as A_4 , B and intermediate type spermatogonia are confined to a limited space on the basal lamina. As cell populations expand with the latter divisions, either the contact of germ cells or Sertoli cells will be reduced.

The flow chart (Fig. 9) indicates that it may be possible to identify all the major types of spermatogonia at the ultrastructural level. We suggest a certain degree of caution before reaching this conclusion, for several reasons. Firstly, the criteria presented in the present report are developed only for the specified fixation and embedding conditions. Secondly, only sections that capture the central region of the nucleus can be used. Thirdly, all types of spermatogonia cannot be identified unequivocally. For example, the distinction between A_3 and A_4 spermatogonia is subtle and errors can easily be made. We suggest that at least three cell types of the same kind should be examined before making a determination.

The present study represents the first attempt to differentiate the various types of spermatogonia, using morphological criteria, at the ultrastructural level. Although many differences in various types of spermatogonia were identified, the differences are subtle and their identification requires considerable effort on the part of the investigator. A major research challenge is to identify and characterize the most primitive of the spermatogonia, especially the A_s stem cell. Careful use of the criteria presented above will allow identification of types of cell when performing functional studies of spermatogonia without the necessity of using whole mounts.

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References

- Busch H and Smetana K (1970) *The Nucleus* Academic Press, New York
- Chiarini-Garcia H and Russell LD (2001) High resolution light microscopic characterization of mouse spermatogonia *Biology of Reproduction* **65** 1170–1178
- Chiarini-Garcia H, Hornick JR, Griswold MD and Russell LD (2001) The distribution of Type A spermatogonia in the mouse is not random *Biology of Reproduction* **65** 1179–1185
- Clermont Y and Bustos-Obregon E (1968) Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto" *American Journal of Anatomy* **122** 237–248
- de Rooij DG (1973) Spermatogonial stem cell renewal in the mouse. I. Normal situation *Cell and Tissue Kinetics* **6** 281–287
- de Rooij DG (1998) Stem cells in the testis *International Journal of Experimental Pathology* **79** 67–80
- de Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells *Reproduction* **121** 347–354
- de Rooij DG and Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask *Journal of Andrology* **21** 776–798
- Huckins C and Oakberg EF (1978) Morphological and quantitative analysis of spermatogonia in mouse testis using whole mounted seminiferous tubules. I. The normal testis *Anatomical Record* **192** 519–528
- Nagano M, Avarbock MR and Brinster RL (1999) Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes *Biology of Reproduction* **60** 1429–1436
- Oakberg EF (1956) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal *American Journal of Anatomy* **99** 391–414
- Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse *Anatomical Record* **169** 515–531
- Russell LD and Burguet S (1977) Ultrastructure of Leydig cells as revealed by secondary tissue treatment with a ferrocyanide–osmium mixture *Tissue and Cell* **9** 751–766
- Russell LD, Ettlin RA, Sinha Hikim AP and Clegg ED (1990) *Histological and Histopathological Evaluation of the Testis* Cache River Press, Vienna
- Schultz MC and Leblond CP (1990) Nucleolar structure and synthetic activity during meiotic prophase and spermiogenesis in the rat *American Journal of Anatomy* **189** 1–10
- Sprando RL (1990) Perfusion of the rat testis through the heart using heparin. In *Histological and Histopathological Evaluation of the Testis* pp 277–280 Eds LD Russell, RA Ettlin, AP Sinha Hikim and ED Clegg. Cache River Press, Vienna
- van Beek ME, Davids JA, van de Kant HJ and de Rooij DG (1984) Response to fission neutron irradiation of spermatogonial stem cells in different stages of the cycle of the seminiferous epithelium *Radiation Research* **97** 556–569
- van Pelt AMM, van Dissel-Emiliani FMF, Gaemers IC, van der Burg MJ, Tanke HJ and de Rooij DG (1995) Characteristics of A spermatogonia and pre-leptotene spermatocyte in the vitamin A-deficient rat testis *Biology of Reproduction* **53** 570–578

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