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Intercellular bridges between germ cells in the immature golden hamster testis: evidence for clonal and non-clonal mode of proliferation*

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Summary. Intercellular bridges of prespermatogonia and of the first A-spermatogonia in the maturing testes of newborn to 17-day-old golden hamsters have been studied by electron microscopy. Incomplete cytokinesis of dividing M- and T_2-prespermatogonia and A-spermatogonia produces these bridges, which undergo different developmental fates. Bridges of the first A-spermatogonia are stable beyond subsequent mitoses of these cells; this gradually leads to the formation of bridge-connected groups of synchronously developing germ cells. Thus, the clonal mode of male germ cell proliferation is already established in this period of testis maturation. During mitoses, pre-existing bridges reversibly develop structural modifications, i.e. considerable elongation and formation of a bridge-partitioning complex. In contrast, intercellular bridges of prespermatogonia are mostly severed and become lost during subsequent mitoses of the cells involved; this results in separation of the germ cells and represents a mainly non-clonal mode of M- and T_2-prespermatogonial proliferation. Here, too, pre-existing bridges elongate and develop the bridge-partitioning complex during subsequent mitoses of the joined cells, but this is superposed and interrupted by the simultaneous process of disconnection of the bridges.

Key words: Intercellular bridges – Spermatogonia – Prespermatogonia – Mitosis – Testis – Golden hamster, *Mesocricetus auratus* (Rodentia)

Intercellular bridges are a well-known feature of spermatogenesis in animals and in man (Fawcett 1961; Holstein and Roosen-Runge 1981; Huckins 1978; Linares 1983). Originating from incomplete cytokinesis of the dividing germ cells, these bridges provide channels of communication between the members of a germ-cell clone (Braun et al. 1989) and are thought to play an important role in synchronizing germ-cell development (Fawcett et al. 1959; Gondos 1973, 1984). Intercellular bridges between developing germ cells in the immature testis have been studied far less thoroughly, and are mentioned by only a few authors (Franchi and Mandl 1964; Gondos et al. 1973; Hilscher and Schulze 1978; Wartenberg et al. 1971).

Before entering the first meiotic cell divisions and thus the pubertal period of spermatogenesis, the germ cells of the postnatal and prepubertal golden hamster testis successively appear as M-, T_1-, and T_2-prespermatogonia (as defined by Hilscher et al. [1974] for prespermatogenesis in the rat) and as first A-spermatogonia. Of these cell types, M-prespermatogonia (15th day post conceptionem [dpc] – 2nd day post partum [dpp]), T_2-prespermatogonia (8th–11th dpp), and the first A-spermatogonia (from about 13th dpp onwards) represent separate phases of proliferation. The mitoses of these proliferative phases of immature germ-cell development are accompanied by the formation of intercellular bridges between daughter germ cells; this resembles the cell divisions in mature spermatogenesis. However, bridges connecting prespermatogonia frequently do not persist beyond subsequent mitoses of these cells, but become detached and degenerate, and thus do not give rise to the formation of extended germ-cell clones.

The present study provides information about the occurrence, ultrastructural morphology and developmental fate of intercellular bridges between germ cells in the immature testis of the golden hamster.

Materials and methods

The testes of newborn to 17-day-old golden hamsters were obtained and processed in the following way. The anesthetized animals were perfusion-fixed with a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The testes were

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removed, immersed in the above fixative for a further 2 h and postfixed in 1% phosphate-buffered OsO_4 for 4 h. After dehydration in graded alcohols, the samples were embedded in Epon or Durcupan. Series of semithin sections (1 μm) were stained with toluidine blue/pyronine and examined carefully with the light microscope. Selected sections were re-embedded for subsequent ultrathin sectioning (Larramendi 1985); the resulting ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope.

Results

Formation of intercellular bridges

Bridge formation is the result of incomplete cytokinesis at the end of germ-cell mitoses and takes place as follows. In late telophase, cytokinesis stops when the width of the remaining cytoplasmic connection between the two daughter cells measures about 1.5 μm and is occupied by a bundle of interzonal spindle tubules, the midregion of which is surrounded by electron-dense amorphous material (midbody). At the beginning of the following interphase, this amorphous material disperses, and at the same time, a layer of an electron-dense substance lining the bridge plasmalemma becomes visible; this is referred to as the bridge density. After subsequent depolymerization of the remaining microtubules, the bridge provides complete cytoplasmic continuity between the conjoined germ cells.

Intercellular bridges between interphase germ cells

During interphase of bridge-connected germ cells, the bridges are characterized by the following morphological features. They are short (0.5–1 μm), cylindrically formed channels with a diameter of 1.2–1.5 μm (Fig. 1). Their plasmalemma is lined by the bridge density, being approximately 30–40 nm thick. In some cases, bridges between the non-dividing T_1-prespermatogonia of the 3rd–7th dpp are considerably drawn out; this involves parts of the adjacent cell membrane of both the conjoined cells. The bridge cytoplasm is similar to that of the joined germ cells and appears to be freely accessible to cytoplasmic elements, including cell organelles (Fig. 1).

Intercellular bridges between dividing germ cells

During subsequent mitoses of bridge-connected germ cells, the pre-existing intercellular bridges develop a complex pattern of structural alterations, which either result in the detachment and final loss of these bridges or, in other cases, turn out to be completely reversible.

In most cases, bridges between prespermatogonia do not exist beyond the subsequent mitoses of the cells that they connect. Instead, the bridges undergo the following, well-ordered sequence of morphological alterations ending in their final separation and loss:

(1) At the beginning of mitosis, a considerable elongation of the pre-existing bridge occurs, leaving the bridge density at its original length and in an almost central position within the elongated bridge (Fig. 2). Frequently, the bridge is bent at the sites where it originates from the surfaces of the joined cells.

(2) At the same time, a number of flattened, parallel cisternae of smooth endoplasmic reticulum appear in the central segment of the bridge, oriented perpendicularly to the longitudinal axis of the bridge (Fig. 2). The space between neighboring cisternae measures approximately 25 nm and is occupied by an electron-dense material that laterally joins the bridge density. This elaborate structural arrangement within the intercellular bridge has been described previously for spermatogenic cells (Nagano 1961; Dym and Fawcett 1971; Weber and Russell 1987) and has been termed the bridge-partitioning complex. At higher magnifications, an intermediate dense midline can be observed within the substance occupying the intercisternal space.

(3) Simultaneously with progression of the mitosis (frequently beginning already during prophase), the bridge gradually becomes pinched off from the conjoined cells, the points of severance being directly adjacent to the sites of the origin of the bridge on the cell surfaces (Figs. 2, 3a).

(4) Concomitantly, the cisternae of the bridge-partitioning complex undergo a disintegration of their well-ordered arrangement. In the course of this disintegration, the cisternae for a short period show an irregular, mainly longitudinally oriented, circular arrangement (Fig. 3b and c), which precedes their final degradation and disappearance.

After its disconnection, the former bridge is usually surrounded by adjacent Sertoli cells (Fig. 4), but occasionally is found to lie within the cytoplasm of a germ cell.

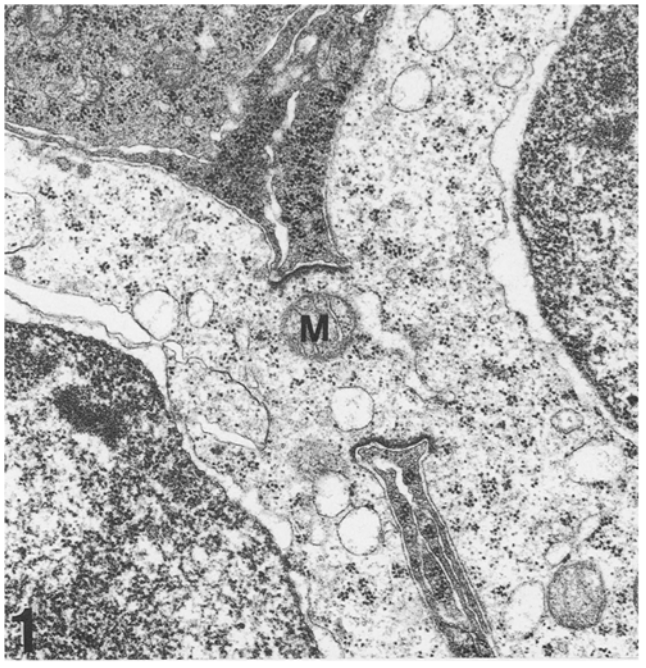
In contrast, intercellular bridges between the first A-spermatogonia regularly prove to be stable beyond subsequent mitoses of the germ cells that they connect. During these mitoses, pre-existing bridges develop similar

Fig. 1. Intercellular bridge of interphase A-spermatogonia (17th day) allowing free cytoplasmic continuity. *M* mitochondrion within bridge lumen. × 21 000

Fig. 2. Pre-existing intercellular bridge of dividing T_2-prespermatogonia (9th day). Alterations include considerable elongation, bridge-partitioning complex in the region of bridge density, and beginning of the process of severance (*arrow*). × 38 500

Fig. 3a–c. Series of three sections showing a pre-existing intercellular bridge of dividing M-prespermatogonia (2nd day). **a** Site of origin from the cell surface with incipient signs of severance (*arrows*). **b, c** Cross sections of the bridge in the region of bridge density, showing the cisternae of the disintegrating bridge-partitioning complex in an irregular, mainly circular arrangement. × 18 000

Fig. 4. Former intercellular bridge, now separated from dividing M-prespermatogonia (1st day) and surrounded by Sertoli cell cytoplasm. *Arrows* indicate remnants of the bridge-partitioning complex. × 40 500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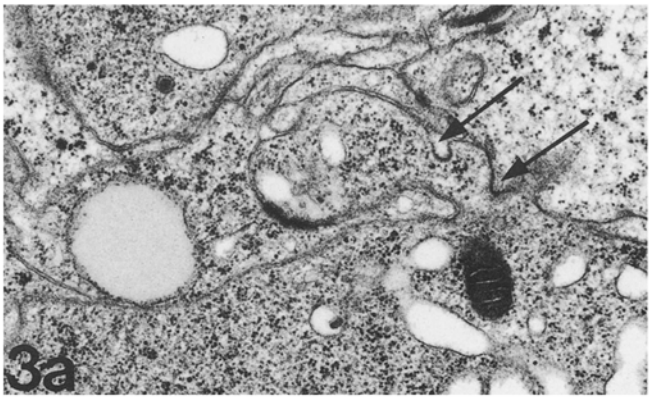
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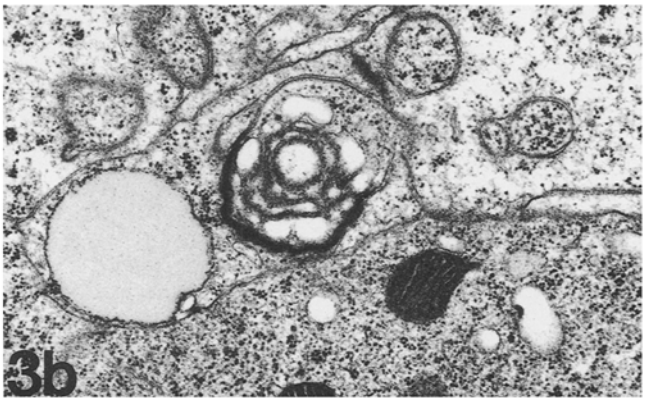
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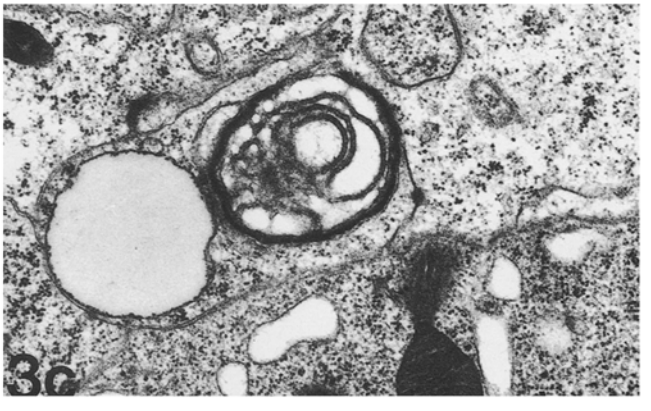
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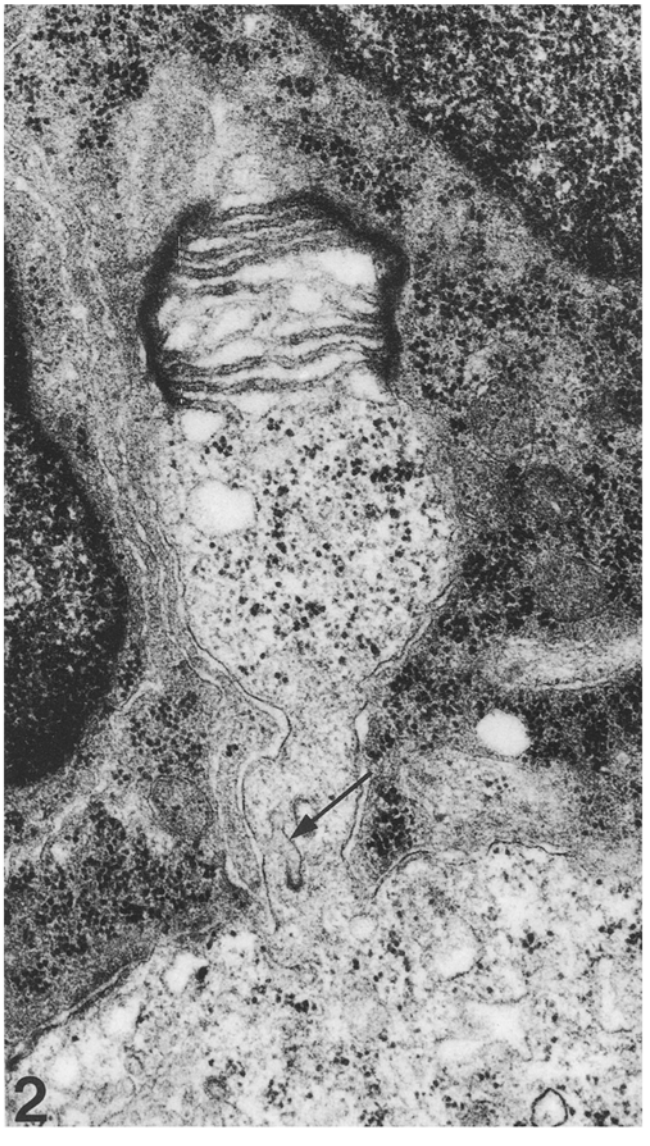
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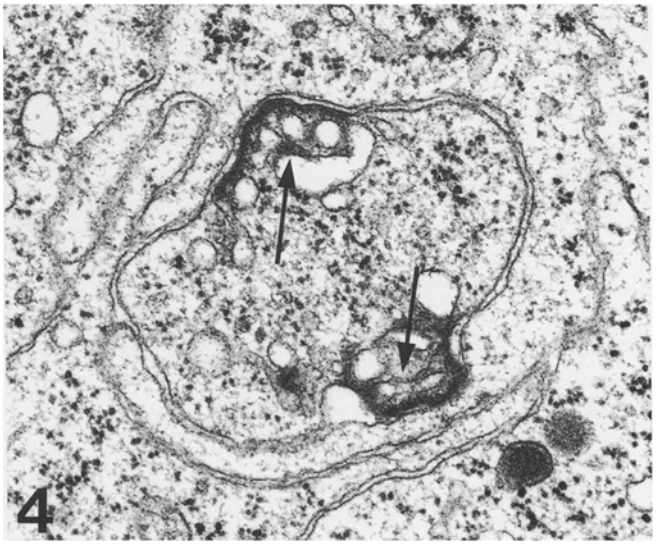
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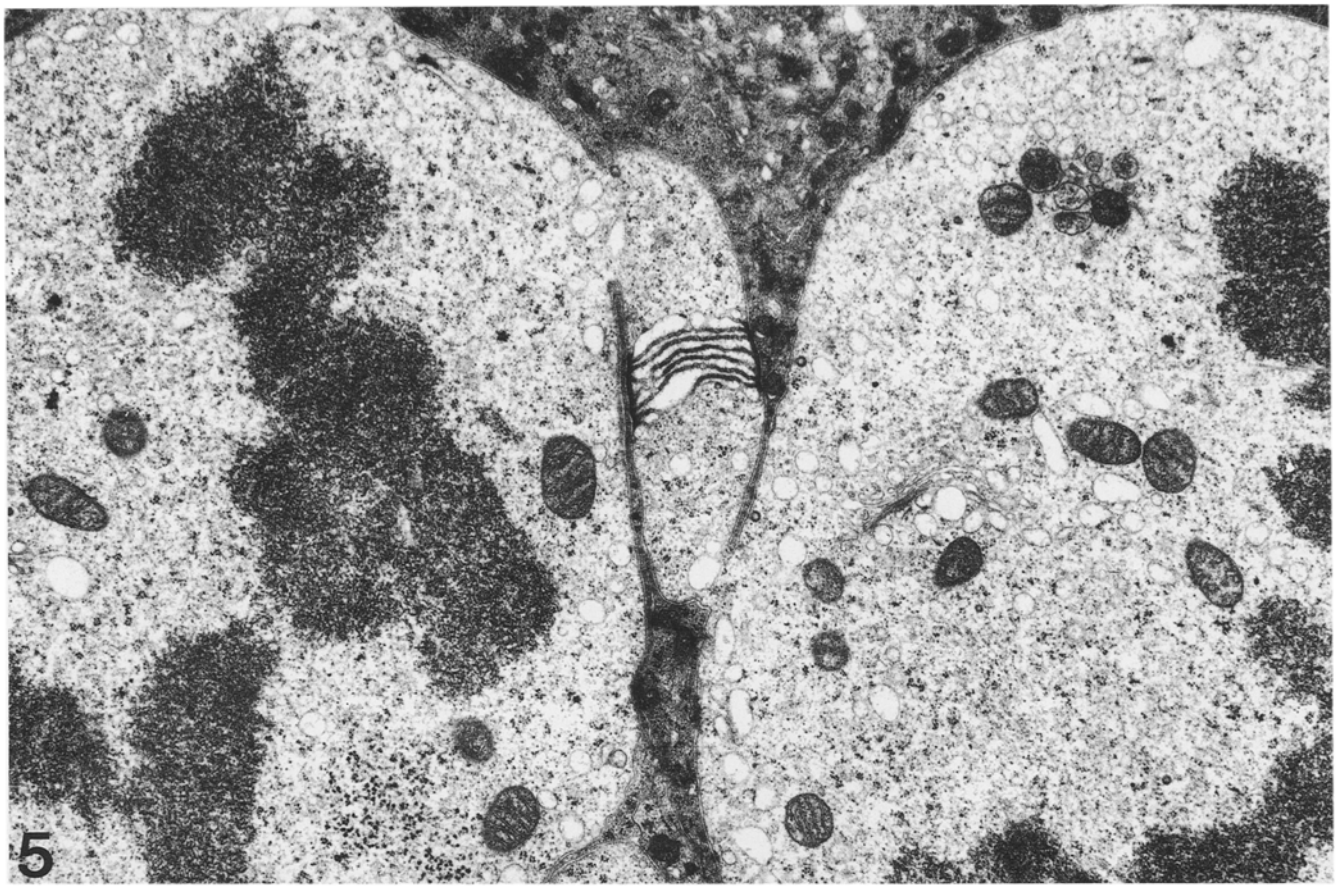

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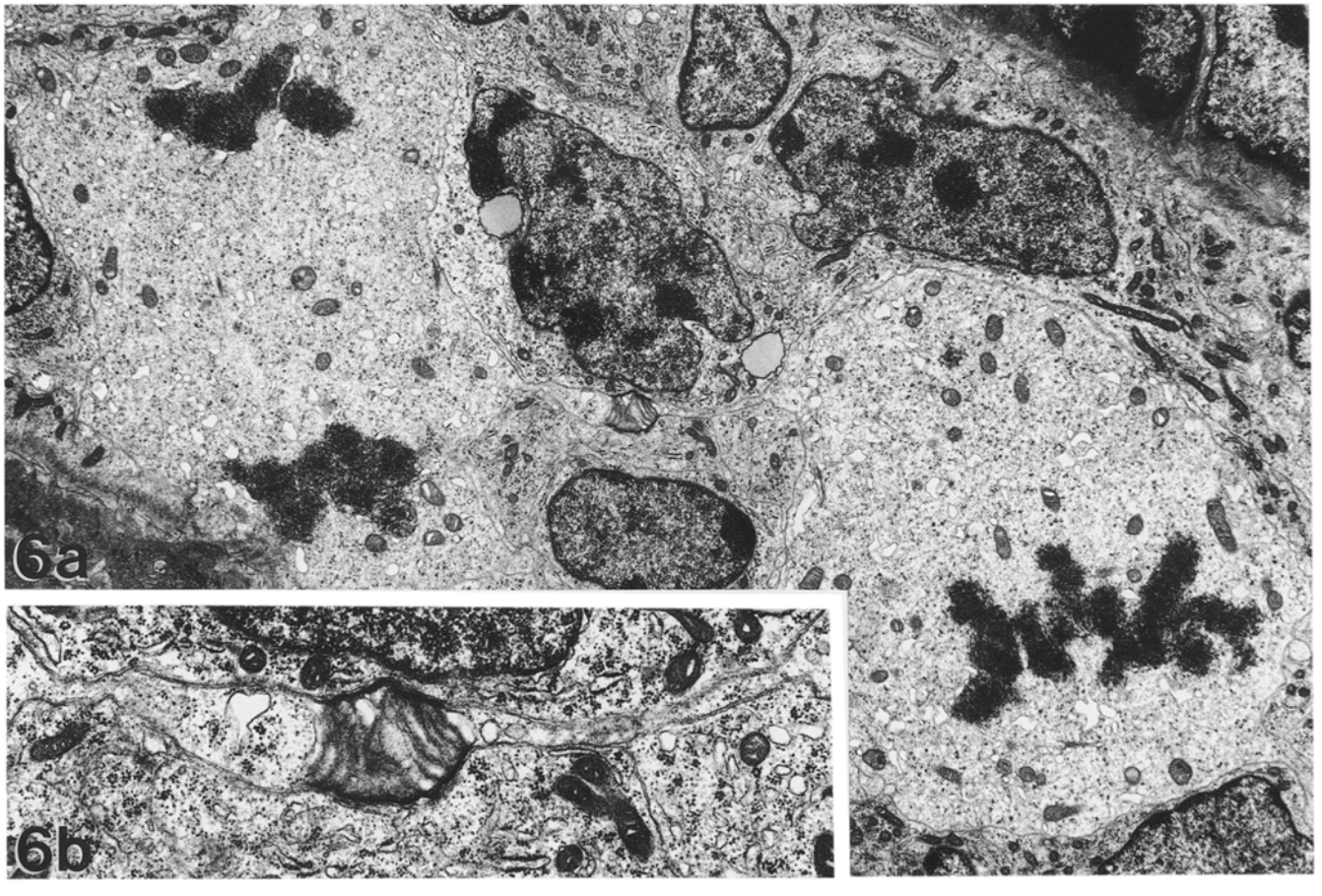

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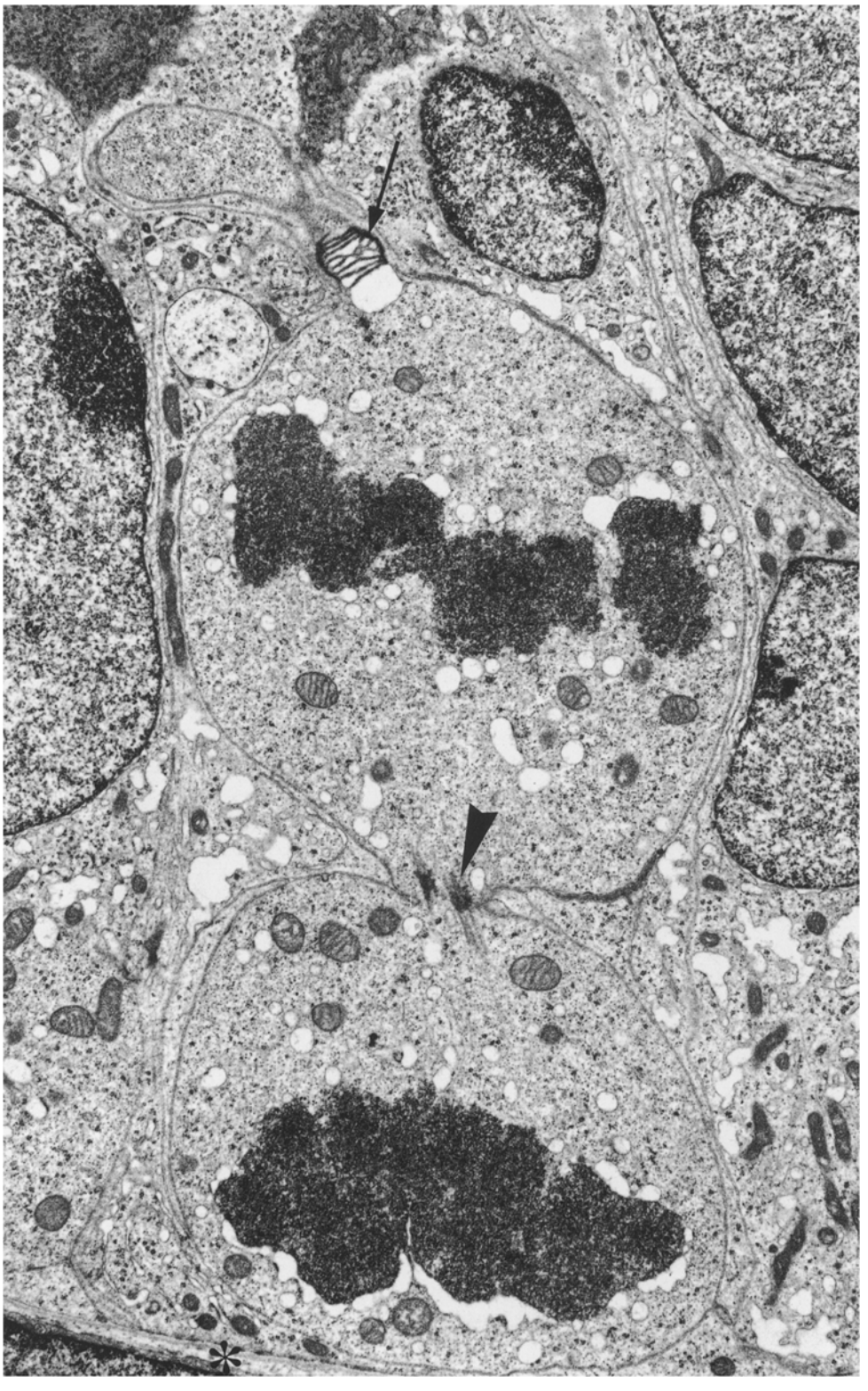
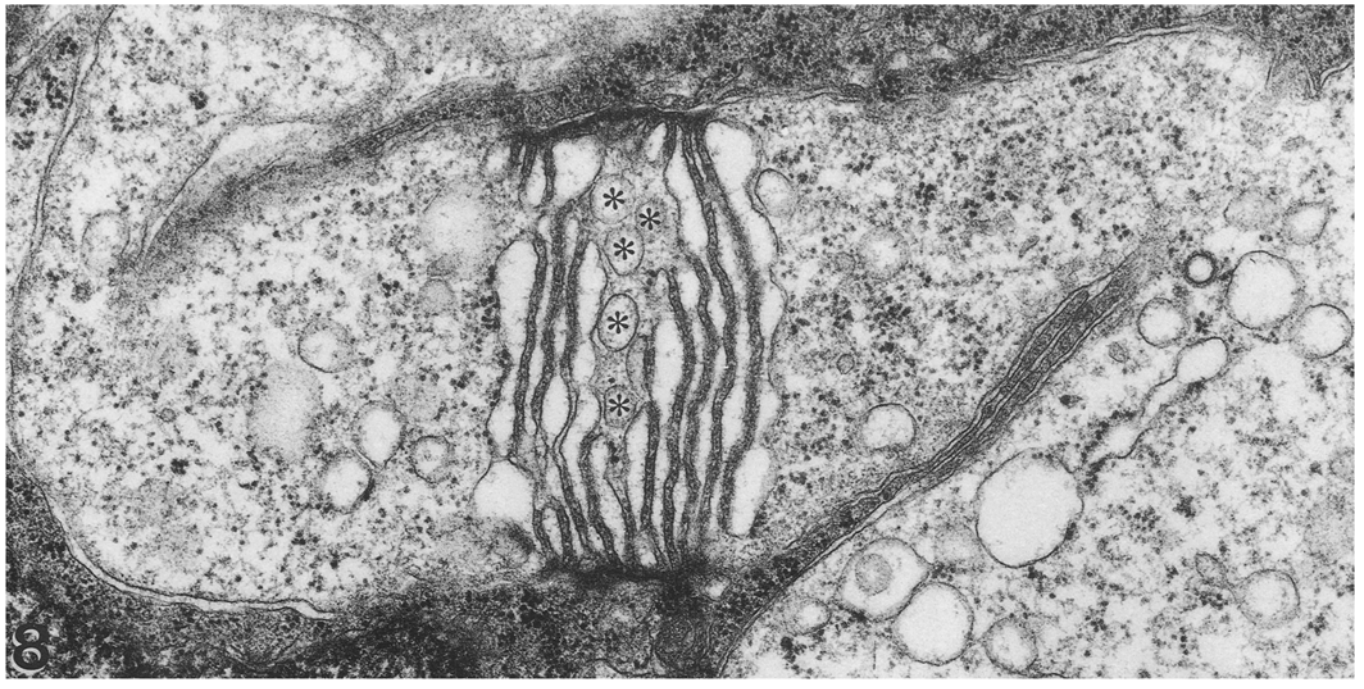
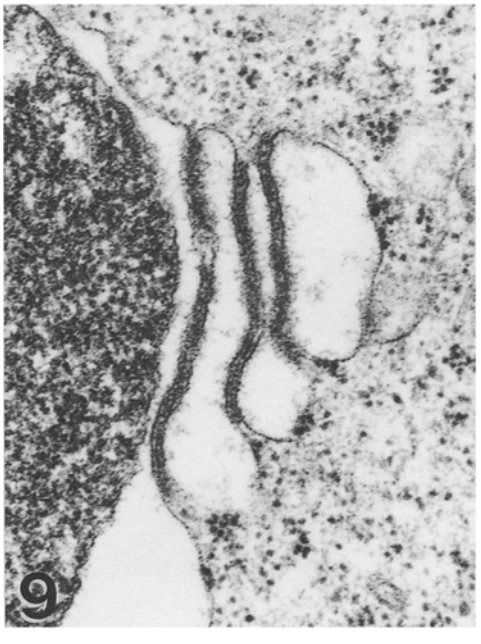


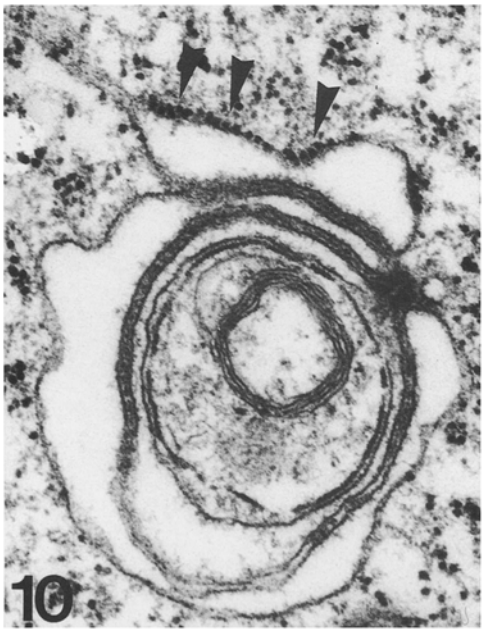
Fig. 7. A-spermatogonial division in late telophase (17th day): pre-existing intercellular bridge with bridge-partitioning complex (*arrow*) and newly forming bridge with arising midbody (*arrowhead*) are in same plane of section. *Asterisk* indicates basal lamina of testicular cord. ×9200

Fig. 5. Pre-existing bridge between dividing A-spermatogonia (17th day). The bridge is elongated and sharply bent at the sites of its origin, and exhibits the bridge-partitioning complex in its midregion. ×13000

Fig. 6. a Considerably elongated pre-existing intercellular bridge between dividing A-spermatogonia in telophase (14th day). ×5800. **b** Same bridge at higher magnification; although elongation is accompanied by a reduced bridge diameter, no severance takes place. Characteristically, this narrowing does not affect the midregion of the bridge, which shows the bridge density and is occupied by the bridge-partitioning complex. ×18000







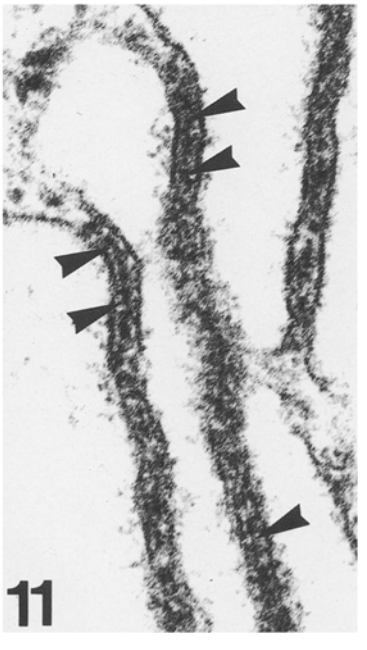


Fig. 8. Pre-existing intercellular bridge of dividing A-spermatogonia (17th day). Bridge-partitioning complex in the midregion of the bridge is composed of several flattened, transversely orientated cisternae, which are separated by a 25-nm-wide space occupied by an electron-dense substance. Two of the cisternae appear to be devolved into single round vesicles (*asterisks*). ×39000

Figs. 9, 10. Groups of flattened cisternae arranged in parallel within the cytoplasm of dividing germ cells. The intercisternal space is occupied by an electron-dense substance

Fig. 9. Cisternae are adjacent to the perinuclear cistern of a reconstituting nucleus of an A-spermatogonium in late telophase (17th day). ×48000

Fig. 10. Circular arrangement of cisternae in the cytoplasm of dividing T_2-prespermatogonium (9th day). *Arrowheads* indicate partial studding with ribosomes. ×48000

Fig. 11. High magnification of a detail of four cisternae arranged in parallel, showing the intercisternal space to be bisected by an intermediate dense line (*arrowheads*). ×116000

structural alterations as above, but are not finally severed from the cells.

The bridge shows a considerable elongation (Figs. 5, 6) and frequently a sharp double bend at the sites of its origin from the cell surfaces (Figs. 5, 8). Within the central segment containing the bridge density, flattened cisternae of smooth endoplasmic reticulum build up the bridge-partitioning complex (Figs. 5–8). This complex is stable up to late telophase, only occasionally showing single cisternae devolving into a number of small, mem-

brane-bounded vesicles (Fig. 8). At the start of the following interphase, the bridge is shortened again to its previous length and the bridge-partitioning complex disintegrates; in the course of this process, it transitorily shows a longitudinally oriented circular arrangement of the cisternae. After the bridge-partitioning complex is completely removed, the intercellular bridge again provides unrestricted cytoplasmic continuity between the conjoined germ cells.

Within the cytoplasm of dividing prespermatogonia and A-spermatogonia, small groups of flattened cisternae of endoplasmic reticulum are frequently found arranged in parallel (Figs. 9, 10); this resembles the cisternae of the bridge-partitioning complex existing at the same time. These cisternae are also separated by a space of approximately 25 nm occupied by an electron-dense substance, including the intermediate dense midline (Fig. 11). They either appear in the cell periphery or are directly attached to the perinuclear cistern of a reconstituting nucleus in telophase (Fig. 9). In some cases, these groups of cisternae exhibit a circular arrangement (Fig. 10), or are partially studded with ribosomes (Fig. 10).

Discussion

Intercellular bridges represent an integral feature of germ-cell development and maturation in both invertebrates (Reger and Cooper 1968; King and Akai 1971) and mammals (Fawcett 1961; Gondos 1984; Huckins 1978) including man (Holstein and Roosen-Runge 1981). Throughout spermatogenesis, they are known to be responsible for the building up of extensive germ-cell clones. As these bridges provide channels of cytoplasmic communication between the members of a germ-cell clone, they are thought to have a functional significance in developmental synchronization (Fawcett et al. 1959; Gondos 1973, 1984), regulation of proliferative activities (King and Akai 1971; Gondos 1973) and restriction of motility (Gondos and Hobel 1971) of the conjoined germ cells. Yasuzumi et al. (1988), having studied the ultrastructure of the intercellular bridge membrane, have suggested that these bridges act as a barrier impeding free intercellular membrane flow within a germ-cell clone.

The results of the present study confirm that intercellular bridges are also characteristic of immature germ-cell development. The same mechanism of bridge-formation arising from incomplete cytokinesis, as occurs in spermatogenic cell divisions in the mature testis, is found in dividing prespermatogonia and first A-spermatogonia of the immature golden hamster testis. The cytoplasm of the newly forming bridge is transitionally occupied by a midbody (Mullins and Biesele 1973; Mullins and McIntosh 1982) that gradually disappears, leaving complete cytoplasmic continuity between the daughter germ cells. This channel of communication probably synchronizes the simultaneous entry of conjoined cells into the subsequent mitosis; however, during this mitosis, the bridges between prespermatogonia and those between A-spermatogonia undergo a different fate, revealing two fundamentally dissimilar modes of proliferation.

As a consequence of the stability of their intercellular bridges, the first A-spermatogonia in the immature testis already show a clonal mode of proliferation, leading to the formation of groups of synchronously developing cells. Thus, in this developmental stage of testicular maturation, an important feature of mature spermatogenesis has already been established. This includes the appearance of transitional, completely reversible structural alterations of pre-existing bridges during subsequent mitoses; these are formation of the bridge-partitioning complex and elongation of the bridge.

Bridge-partitioning complexes have been described repeatedly in bridges of dividing spermatogenic cells (Nagano 1961; Dym and Fawcett 1971; Linares 1983; Weber and Russell 1987). They are interpreted as temporary barriers between the joined daughter cells (Nagano 1961), possibly preventing the spread of degeneration throughout a germ-cell clone (Dym and Fawcett 1971). Bridges between dividing somatic cells of the squid embryo develop similar complexes (Cartwright and Arnold 1980, 1981), and for these cells, one type of cell-to-cell communication pathway has been shown to be operative only during interphase (Marthy and Dale 1989). These data substantiate a barrier-function of the bridge-partitioning complex, i.e., it prevents cell-to-cell transport, at least of macromolecules of a molecular weight of more than 9000 (Marthy and Dale 1989). Furthermore, Weber and Russell (1987) have suggested that these complexes stabilize bridges of dividing cells. This stabilization might be necessary to prevent pre-existing bridges from becoming stimulated to complete cleavage (Weber and Russell 1987).

However, another function of the bridge-partitioning complex is conceivable, viz., the prevention of an intercellular flow of cytoplasm. Within a germ-cell clone, which is comparable to a system of communicating, flexible volumina, such a flow of cytoplasmic fluid might possibly become strong enough to disarrange the complex mitotic spindle apparatus. Thus, the bridge-partitioning complex could serve as a protective wall allowing the undisturbed progression of mitotic (and meiotic) karyokinesis.

The morphological features of the intercisternal space, viz., an even width of 25 nm and the appearance of an intermediate dense line mid-way within the electron-dense substance, resemble the structural organization of the intercellular compartment of epithelial desmosomes. The latter also show an intermediate dense line within the intercellular material (Rayns et al. 1969; Staehelin and Hull 1978); it is thought to represent the linking sites of transmembrane glycoproteins and thus to function as the intercellular desmosomal adhesive (Gorbsky and Steinberg 1981; Garrod 1986). The above morphological similarities suggest that the intercisternal electron-dense material may also exercise a direct adhesive function. Thus, these intercisternal structures are probably crucial components for maintaining mechanical stability of the bridge-partitioning complex as a whole.

The considerable elongation of bridges between dividing A-spermatogonia is different to the occasionally appearing drawing-out of bridges between T_1-prespermatogonia (Miething 1989). The latter cells are passively moved apart by the forces of proliferating supporting cells between them, leading to stretching of the bridges and adjacent parts of the cell membrane. In contrast, bridges of A-spermatogonia show elongation exclusively during mitoses, and this might be important in allowing a limited movability of the joined germ cells relative to one another during the division process.

Intercellular bridges between prespermatogonia, in most cases, are not stable for longer than the duration of the interphase directly following their formation. As these bridges are usually severed from the joined cells and become lost during the subsequent mitosis, M- and T_2-prespermatogonia are characterized by a non-clonal mode of proliferation. Exceptions to this rule are indicated by the occasional appearance of rows of bridge-connected T_1-prespermatogonia (Miething 1989). Presumably, these rows are formed during mitoses in the late M-prespermatogonial phase, directly preceding the non-proliferative T_1-prespermatogonia.

In addition to the comparison of the immature and mature mode of male germ-cell proliferation, a possible analogy with female gametogenesis taking place at the same developmental time can be made. The most conspicuous feature of female gametogenesis at this time is a period of clonal proliferation of oogonia (parallel to non-clonal mitoses of M-prespermatogonia), immediately followed by the entry of primary oocytes into meiotic prophase (parallel to the T_1-prespermatogonial resting phase and a further short non-clonal proliferation of T_2-prespermatogonia) (Wartenberg 1974; Hilscher 1981). Moreover, in male gametogenesis, the onset of meiosis is also preceded by a period of clonal proliferation that, in the golden hamster, begins on about the 13th dpp with the first A-spermatogonial mitoses. In the light of this comparison, the phenomenon of disconnection of the intercellular bridges and the resulting non-clonal mode of prespermatogonial proliferation might possibly be taken to be a prerequisite for allowing a phase of necessary mitotic proliferation without a directly succeeding entry into meiosis; these features may therefore represent one factor responsible for the belated onset of meiosis in male gametogenesis.

The question arises as to why pre-existing bridges of dividing M- and T_2-prespermatogonia develop the bridge-partitioning complex and elongate, although they are fated to become pinched off from the joined cells directly afterwards. It could be a fundamental mechanism in clonal germ-cell proliferation, i.e., operative already in the early developmental stages but without serving a specific function at that time. Phylogenetic conservation of intercellular bridges as a fundamental feature of both invertebrate (Reger and Cooper 1968; King and Akai 1971) and mammalian (Fawcett et al. 1959; Gondos 1984; Holstein and Roosen-Runge 1981) germ-cell development might correspond to ontogenetic stability of this structure, including alterations of pre-existing bridges of dividing cells. Linares (1983) emphasizes this stability and structural self-reliance while supposing intercellular bridges to be organelle in character.

The formation of the bridge-partitioning complex in bridges between dividing prespermatogonia may ensure a more controlled cell-separation process. The functional separation of the cells by the establishment of the bridge-partitioning complex precedes the final structural separation, the latter not being precisely temporally adjusted to the cell division process. Severance does not take place in the midregion of the bridge, but occurs at its two sites of origin on the apposing cells. This observation corroborates the suggestion of Weber and Russell (1987) that the bridge-partitioning complex plays a stabilizing role thereby preventing severance in the central region. Differing from the above-described mode of disconnection of intercellular bridges, the separation of bridges in their midregion is found in spermatids of *Spirostreptus* sp. (Horstmann 1970). Here, the bridge is occupied by a phragmoplast that consists of spindle fibers, a network of endoplasmic reticulum, and granulated osmiophilic material. This structure, however, first divides and retracts from the central zone of the bridge where severance finally occurs.

The configuration of flattened cisternae observed within the cytoplasm of both dividing prespermatogonia and A-spermatogonia might not be of specific functional significance. As they are seldom found in immediate proximity to the pre-existing intercellular bridge, they do not seem to take part directly in the process of development or disintegration of the bridge-partitioning complex. Rather, a mitosis-dependent stimulus causing the formation of the bridge-partitioning complex may be operative throughout the germ-cell cytoplasm.

The results of the present study reveal not only the appearance, but also some of the dynamic structural modifications of intercellular bridges of male germ cells, to have considerable ontogenetic stability. The fundamental mechanisms of bridge formation and of structural alterations of pre-existing bridges are found in incipient spermatogenesis as well as in the preceding proliferative stages of prespermatogenesis. This stability is even more remarkable as it concerns both the stages of clonally proliferating germ cells (A-spermatogonia) and the preceding stages of germ cells, stages that are marked by mainly non-clonal mode of proliferation (M- and T_2-prespermatogonia). The non-clonal mode of proliferation in prespermatogonial germ-cell development is achieved by disconnection of the intercellular bridges during subsequent mitoses, allowing free motility of the germ cells and possibly preventing premature onset of meiosis; thereby, an even distribution of the developing stem-cell population within the testicular cord is assured.

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