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REGULAR ARTICLE

Structural characterization and primary *in vitro* cell culture of locust male germline stem cells and their niche

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Abstract The establishment of *in vitro* culture systems to expand stem cells and to elucidate the niche/stem cell interaction is among the most sought-after culture systems of our time. To further investigate niche/stem cell interactions, we evaluated *in vitro* cultures of isolated intact male germline–niche complexes (i.e., apical complexes), complexes with empty niche spaces, and completely empty niches (i.e., isolated apical cells) from the testes of *Locusta migratoria* and the interaction of these complexes with isolated germline stem cells, spermatogonia (of transit-amplifying stages), cyst progenitor cells, cyst progenitor cell-like cells, cyst cells, and follicle envelope cells. The structural characteristics of these cell types allow the identification of the different cell types in primary cultures, which we studied in detail by light and electron microscopy. In intact testes germline stem cells strongly adhere to their niche (the apical cell), but emigrate from their niche and form filopodia if the apical complex is put into culture with "standard media." The lively movements of the long filopodia of isolated germline stem cells and spermatogonia may be indicative of their search for specific signals to home to their niche. All other incubated cell types (except for follicle envelope cells) expressed rhizopodia and lobopodia. Nevertheless isolated germline stem cells in culture do not migrate to empty niche spaces of nearby apical cells. This could indicate that apical cells lose their germline stem cell attracting ability *in vitro*, although apical cells devoid of germline stem cells either by emigration of germline stem cells or by mechanical removal of germline stem cells are capable of surviving *in vitro* up to 56 days, forming many small lobopodia and performing amoeboid movements. We hypothesize that the breakdown of the apical complex *in vitro* with standard media interrupts the signaling between the germline stem cells and the niche (and conceivably the cyst progenitor cells) which directs the typical behavior of the male regenerative center. Previously we demonstrated the necessity of the apical cell for the survival of the germline stem cell. From these studies we are now able to culture viable isolated germline stem cells and all cells of its niche complex, although DNA synthesis stops after Day 1 in culture. This enables us to examine the effects of supplements to our standard medium on the interaction of the germline stem cell with its niche, the apical cell. The supplements we evaluated included conditioned medium, tissues, organs, and hemolymph of male locusts, insect hormones, mammalian growth factors, Ca^{2+} ion, and a Ca^{2+} ionophore. Although biological effects on the germline stem cell and apical cell could be detected with the additives, none of these supplements restored the *in vivo* behavior of the

Abbreviations: AC, apical cell (a single AC represents the niche of an apical complex); CC, cyst cell; CPC, cyst progenitor cell; GB, gonioblast; GSC, germline stem cell.

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incubated cell types. We conclude that the strong adhesion between germline stem cells and apical cells *in vivo* is actively maintained by peripheral factors that reach the apical complex via hemolymph, since a hemolymph–testis barrier does not exist. The *in vitro* culture model introduced in this study provides a platform to scan for possible regulatory factors that play a key role in a feedback loop that keeps germline stem cell division and sperm disposal in equilibrium.

Introduction

The stem cell niche concept was first proposed more than three decades ago for the human hematopoietic system (Lin, 2002). In the following the stem cell niche concept has also been proposed for several other tissues including the epidermis, the intestinal epithelium, the neural system (Shepherd and Bate, 1990; Taghert et al., 1984; Doe et al., 1985; Doe and Goodman, 1985), and the gonads (Lin, 2002). Insect model systems of the neural stem cell niche have in many respects marked the beginning in developing deeper insights into the niche–stem cell relationship. In this report we focus on the interaction between male germline stem cells (GSCs) and supporting somatic elements, the apical cells (ACs), now known as their niche (Lin, 2002). Early investigators marveled at the intricate morphological interrelations observed in light microscopical preparations of lepidopteron male germinal proliferation centers, the apical complexes of testicular follicles (for review, see Roosen-Runge, 1977). They proposed a regulatory function of ACs by interactions with GSCs, whereas the traditional view of subsequent investigators favored a nutritive role of ACs for the developing spermatogonia (for reviews, see Schmidt and Dorn, 2004; Schmidt et al., 2001). The study of Szöllösi and Marcaillou (1979) on the fine structure of the AC in *Locusta migratoria* sparked new interest on this extraordinary cell and its function. Szöllösi and Marcaillou observed large amounts of smooth endoplasmic reticulum in the AC which prompted them to speculate that the cell may synthesize a lipid, presumably juvenile hormone that regulates spermatogenesis. Smooth endoplasmic reticulum is also prominent in the ACs of the lepidopteran *Euproctis chrysorrhea* (Leclercq-Smekens, 1978). In this case, the authors suspected the production and secretion of a steroid that affects spermatogenesis. None of these speculations have been verified. Recently, the apical complex of *Drosophila melanogaster* has emerged as a successful model for the revelation of signaling interactions between GSCs and their niche, the ACs (lately called *hub cells* in *Drosophila*), and the cyst progenitor cells (CPCs) as well. According to these studies (for reviews, see Li and Xie, 2005; Xie et al., 2005), the niche interacts with GSCs in several ways: it regulates GSC self-renewal and proliferation, it upholds the stemness of GSCs, and it is probably necessary for the survival of GSCs (Zahn et al., 2007). GSCs apparently also have an effect on their niche, which is indicated by the observation that *hub cells* reenter the cell cycle in agametic testes (Gonczy and DiNardo, 1996). Generally, insect ACs do not divide during postembryonic development (Schmidt et al., 2001).

The *Drosophila* model played a crucial role for the establishment of the stem cell–niche theory for an array of tissues ranging from the neural system to the gonads (Lin, 2002). This theory probably applies to all embryonic and postembryonic/adult stem cells of animal and human regenerative tissues (Lin, 2002). A disturbance of the stem cell–niche relationship supposedly causes severe diseases and is therefore the subject of intense research (Reya and Clevers, 2005; Scadden, 2006). In order to maintain tissue homeostasis, it is imperative that death of exhausted or injured differentiated cells is exactly matched by the differentiation of replacing cells generated by stem cells. Stem cell divisions can occur in one of two ways: they can be either symmetric, resulting in two differentiated daughter cells, or asymmetric, resulting in a stem cell (stem cell self-renewal) and a progenitor cell that eventually starts differentiation and replaces a lost tissue cell (Doe, 2008; Morrison and Kimble, 2006; Yamashita et al., 2003, 2007). Signals from the niche are thought to play a decisive role in the decision whether symmetric or asymmetric division takes place and at which rate. A malfunction of the niche could result in either an overproduction of stem cells and/or progenitor cells, leading to tumorigenesis, or a shortage of progenitor cells resulting in tissue disintegration (Holtmann and Dorn, 2009).

Owing to their extremely complex anatomical structures in mammals and humans, identification of stem cell niches has proven to be difficult (Li and Xie, 2005). Consequently, *in vivo* studies to elucidate signals that regulate stem cell activity suffered from this circumstance. Medical application of stem cells for tissue replacement and gene therapy requires a detailed understanding of how the choice between stem cell self-renewal and the onset of differentiation is determined. This knowledge may facilitate the expansion of adult stem cell populations *in vitro* while maintaining essential stem cell characteristics (Jones, 2004). The simplicity of insect GSC/niche arrangement in both sexes and the advanced knowledge of molecular signals from the niche to GSCs make it very tempting to probe this system *in vitro* to potentially gain basic information that can support the understanding of human systems.

Recently, Niki et al. (2006) established a stable cell line of female *Drosophila* GSCs. They used a *bam* (*bag of marbles*) mutant, in which the ovaries show an expansion of GSCs and GSC-like cells (Kai et al., 2005). Since Bam initiates the differentiation of GSCs and cystoblasts, the lack of Bam results in an accumulation of GSC-like cells. GSC maintenance, self-renewal, and division require the growth factor Dpp (decapentaplegic) which is provided by the cap cells (Xie et al., 2005). Cap cells/terminal filament represent the niche of the female GSCs. In the absence of the niche, Dpp had to be substituted in the culture of GSCs/GSC-like cells (Niki et al., 2006). Thus far to our knowledge, there is no

report on the culture of male GSCs of *Drosophila* or any other insect. The testes of the *L. migratoria*, with its components of the testicular regenerative center, were selected for primary *in vitro* culture as a continuation of our previous studies on specific laser ablation of the niche cell in *L. migratoria* testes (Zahn et al., 2007). The GSCs, the apical cell, and the CPCs, including spermatogonia, CCs, and the cells of the follicle wall, have distinct structural characteristics which are maintained in primary culture and allowed us to identify the different cell types *in vitro*. The coincubation of the different components of the regenerative center and studies on partially and completely dissociated apical complexes resulted in unexpected behavior between the different components and may be indicative of the complexity of the regulation of early spermatogenesis.

Results

Cell isolation

Neither the enzymatic nor the treatment with adjudin effectively dissociated the apical follicles. Especially the cells of the follicle wall and the apical complex could not sufficiently be dissociated, whereas CPCs detached rather easily from the surrounding tissue. We therefore separated the cells mechanically with the help of fine tweezers and needles. Of special interest was to isolate the GSCs from their niche, the AC. This proved to be difficult due to a sticky substance that regularly glued the GSCs to the AC. Nevertheless, the separation of some to all GSCs of an apical complex could be achieved. Inevitably, a high percentage of the GSCs and also many ACs were injured by the manipulations. Since one incubation contained about 20 follicular tips (or 20 young follicles, respectively) and therefore 20 apical complexes with about 60 GSCs each, there were potentially 1200 GSCs and 20 ACs in one incubation; each test included 5 to 10 replicas. Thus, despite the high rate of cell injury caused by the preparation technique, which amounted from 40 to 90% (depending on the thoroughness of GSC isolation), a sufficiently high number of vital cells remained for testing. Cell viability was evaluated by the presence of cell movement, podia formation, and morphological evaluation. The last step of tissue dissociation was carried out in the final incubation medium.

Structural characteristics and cell type identification of the follicular apex *in vitro*

The germinal proliferation centers are located in the apical tips of the testicular follicles. They comprise several types of cells which are present in the cultures of dissociated follicular apices. A light and ultramicroscopic analysis proved to be a reliable method for identification during *in vitro* culture. We therefore present a brief structural description of the different cellular components of the intact follicular apex as identified in our cultures. A detailed ultrastructural study of the native AC of *L. migratoria* is given in Szöllösi and Marcaillou (1979).

Follicular apices include one large star-shaped AC that constitutes the niche for the GSCs which are grouped around it (Figs. 1 and 2). The rosette-like configuration is called the apical complex. In the adult, approximately 60 (59 ± 5) GSCs contact the AC surrounded by a corona of CPCs. Gonioblasts and cysts preferentially form laterally from the apical complex (Figs. 1E and 2) and move toward the follicle base during spermiogenesis. A plug of cells is located immediately below the apical complex (Figs. 1B and D and 2). In size, nuclear organization, and fine structure, these cells strongly resemble CPCs; yet, they have no contact with GSCs or GBs. Therefore, we will tentatively name them CPC-like cells. The follicle wall consists of two layers: the very thin perifollicular layer (not discernible in the micrographs shown here) and the inner parietal layer (Figs. 1D and 2).

The apical cell (the niche for GSCs)

Its regular globular nucleus occupies the center of the cell and exhibits finely dispersed chromatin (Fig. 2). The nuclear matrix is electron lucent. Noticeably electron lucent is also the cytoplasm of the AC. It commonly includes one, rarely two, large phagocytotic vacuoles (Figs. 1F and 2) that are most commonly formed by the uptake of degenerated GSCs. The cytoplasm displays a concentric arrangement of organelles around the nucleus (Figs. 2 and 3). Many mitochondria with an electron-dense matrix form a broad ring next to the nucleus which also contains many lysosmal bodies (Fig. 3). These organelles are recognizable as distinct granules in the living cell under the stereomicroscope (cf. Fig. 10F). The periphery of the apical cells shows an abundance of sparsely granulated endoplasmic reticulum (Fig. 3) that is also found in cellular extensions that reach deeply between the basal parts of GSCs (Figs. 3 and 4). Neither mitoses nor BrdU incorporation were observed in ACs.

The GSCs

GSCs show a polar organization along the axis AC/CPCs that affects the shape of the cell and the distribution of cell organelles. GSCs are pear-shaped with the more slender part directed toward the apical cell (Figs. 3 and 4). The nucleus is located in the broader cell part directed toward the CPCs (Fig. 4). It shows patches of condensed chromatin and highly electron-dense nucleoli. The nuclear envelopes present extremely irregular contours. On sections, the deep nuclear constrictions can cause the false impression of bi- or multinucleated cells. Below the nucleus, in the direction to the AC, mitochondria aggregate (Figs. 3 and 4). Their matrix is moderately electron dense. An aggregation of fibrous material (*fibrous body*) is located in this part of the cell, most likely representing nuage (Hardy et al., 1979; Klag and Ostachowska-Gasior, 1997; Mahowald, 1971). Whereas it can be readily identified in living cells (Fig. 9E), it is hardly visible on electron micrographs (Fig. 3). Besides an abundance of free ribosomes, the remaining organelle equipment is inconspicuous.

The mode of GSC division, symmetric versus asymmetric, determines the resulting fate of the daughter cells. For symmetric divisions, the spindle axis runs parallel to the niche, causing both daughter cells to remain in contact with the niche and to develop into stem cells. In asymmetric divisions, the spindle axis runs perpendicular to the niche; one daughter cell losing contact with the niche and entering into differentiation (i.e., becomes a GB), whereas the daughter in contact with the niche remains a stem cell. In the testes of the adult migratory locust, both types of GSC

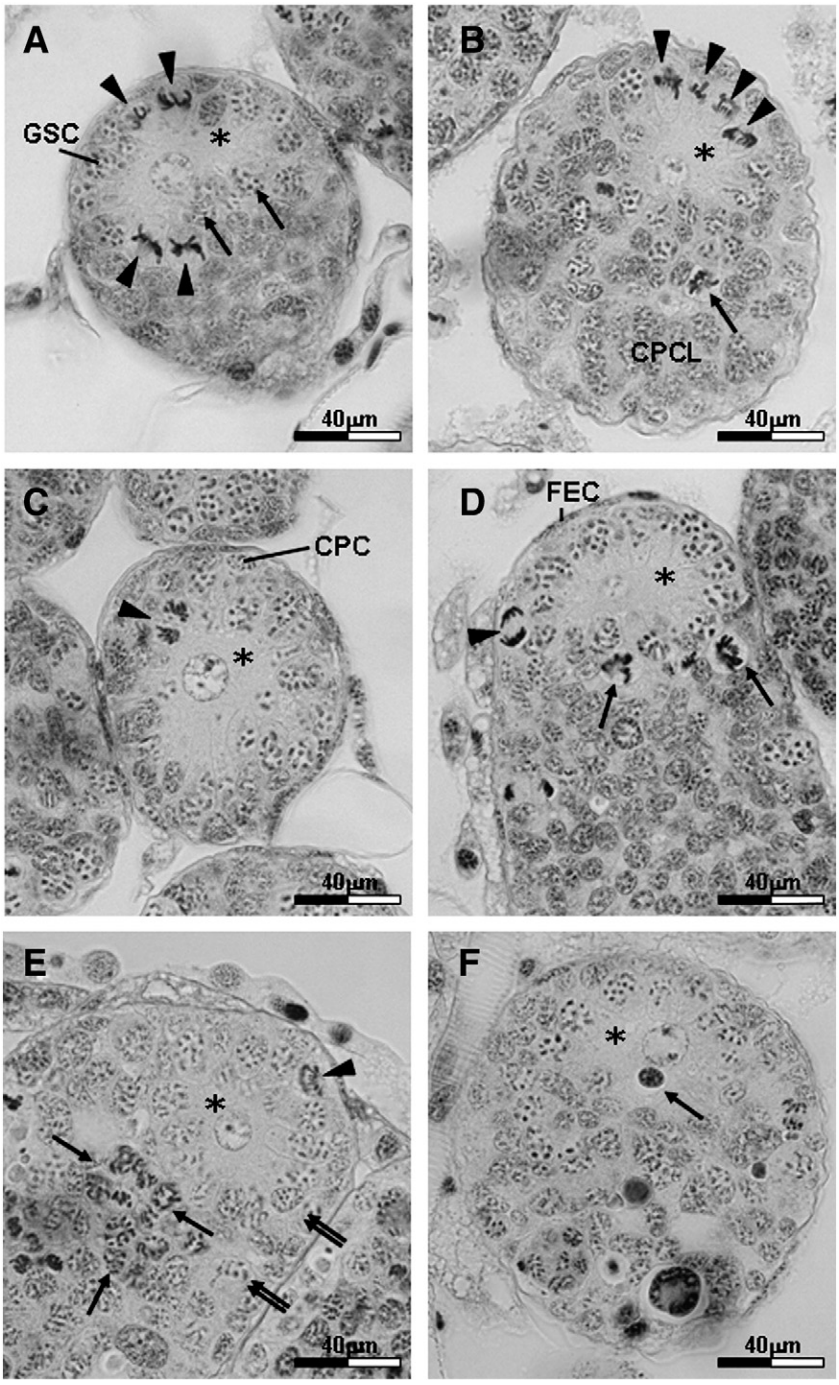

Figure 1 Light micrographs. Images of follicular apices, the regenerative centers of adult male *L. migratoria*. CPC, cyst progenitor cell; CPCL, cyst progenitor cell-like cell; FEC, follicle envelope cell; GSC, germline stem cell; asterisks (*) denote nuclei of apical cells. (A) Tangential section. Two pairs of GSCs performing asymmetric and synchronized divisions are at the metaphase stage (arrowheads). Note that a few GSCs (arrows) are closer to the nucleus of the AC than the others. (B) Tangential section. Whereas four adjacent GSCs are engaged in asymmetric and synchronized divisions (arrowheads), none of the CPCs and only one CPC-like cell (arrow) is in division. (C) Cross section. Anaphase of a GSC completing a symmetric division (arrowhead). (D) Longitudinal section. Division of CPC (arrowhead) and of two GBs (arrows). There are no dividing GSCs. (E) Longitudinal section. Division of one CPC (arrowhead) and of a cluster of CPC-like cells (arrows). Double arrows point to young cysts. There are no dividing GSCs. (F) Tangential section. The AC has phagocytized a GSC (arrow).

division have been observed. Asymmetric divisions are seen in Figs. 1A and B. Note that in Fig. 1, two adjacent GSCs (Fig. 1A) and four neighboring GSCs (Fig. 1B) exhibit synchronized asymmetric mitoses. A symmetric division of a GSC is shown in Fig. 1C. EM studies revealed that GSCs are interconnected by fusomes. In all cases observed, the fusome

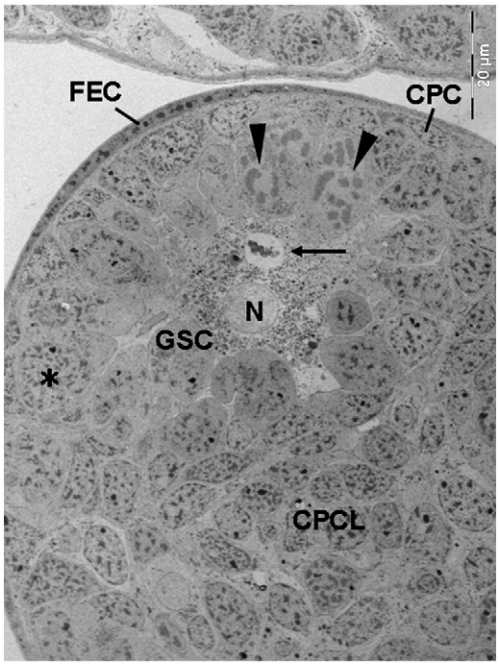

Figure 2 Electron micrograph. Overall view of the follicular apex. Abbreviations as in Figure 1. Note the granular cell organelles around the AC nucleus (N). Arrowheads point to GSCs with condensed chromosomes indicating prophase or late anaphase mitotic stages. Arrow points to a phagosome (probably remnant of a phagocytized GSC). Asterisk (*) indicates a young cyst. (From Zahn et al. (2007) with kind permission of Elsevier.)

indicated a previous symmetric division, since both daughter cells contacted the AC (Figs. 5 and 6), and the spindle axis was oriented perpendicularly to the AC surface. Figure 6 shows remnants of the mitotic spindle.

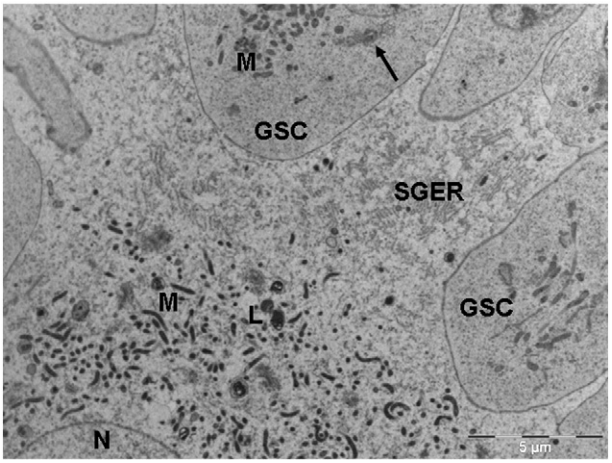

Figure 3 Electron micrograph. Detail of the apical complex revealing that the granular cell organelles around the AC nucleus (N) consist mainly of mitochondria (M) and lysosomes (L), whereas the peripheral parts of the AC that extend between the GSCs mainly include sparsely granulated endoplasmic reticulum (SGER). Arrow points to nuage-like material.

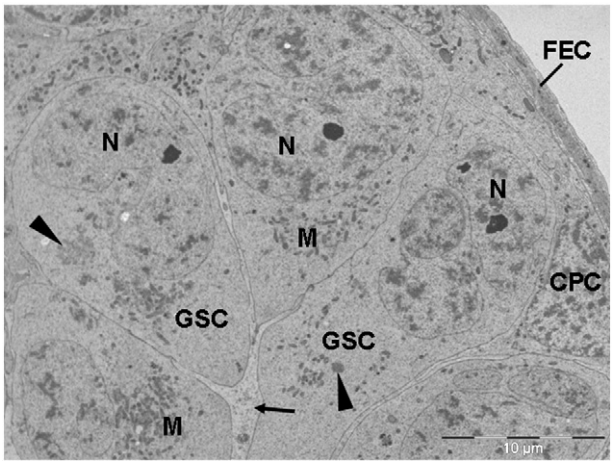

Figure 4 Electron micrograph. Peripheral part of the apical complex with GSCs, CPC, and FEC. Note the structural polarity of GSCs, the extremely irregular profiles of their nuclei (N), and the aggregation of their mitochondria (M) toward the AC (arrow points to extension of AC). Arrowheads point to fibrous structures, most likely representing nuage.

The CPCs and CPC-like cells

The CPCs form a layer around the GSCs (Figs. 1 and 2). Cell extensions reach deeply between the GSCs and often make contacts with extensions of the apical cell (Fig. 7). Nuclear profiles are oval to triangular, do not have indentations and include flakes of condensed chromatin, some of which are attached to the nuclear envelope (Fig. 7). The cytoplasm is scant and not as electron dense as that of GSCs, with divisions occurring sporadically (Figs. 1D and E). The CPC-like cells below the apical complex exhibit the same fine structural characteristics, though contact is lacking between the cells and the GSCs and the AC (Figs. 1B and D and 2). Occasionally, whole clusters of CPC-like cells are observed dividing (Fig. 1E).

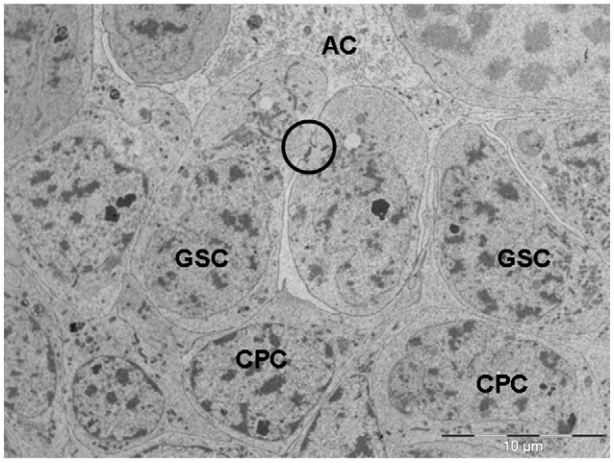

Figure 5 Electron micrograph. Two GSCs bordering the AC are connected by a fusome (encircled). This indicates that these GSCs stem from a symmetric division of their mother cell. CPCs surround the GSCs.

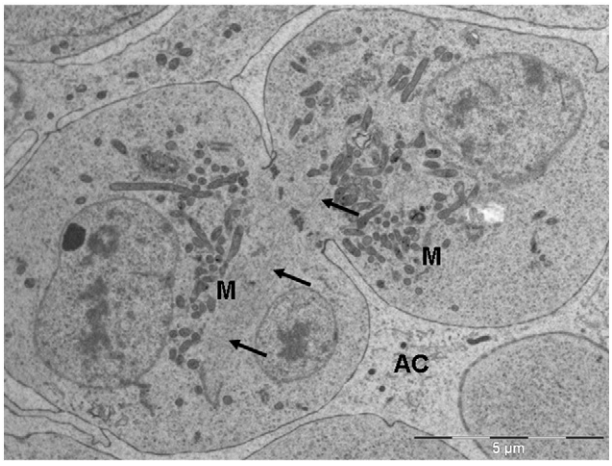

Figure 6 Electron micrograph. Two GSCs connected by a fusome. Remnants of the spindle can be recognized (arrows). The locations of the mitochondrium aggregations (M) are facing each other instead of being directed toward the AC, which indicates that a symmetric division has taken place.

Cysts

Figure 8 shows that in this micrograph 3 CCs enclose the dividing GB. Three CCs per cyst have been observed also in sections of older cysts (not shown). Since serial sections of cysts have not been analyzed, the exact number of CCs per cyst is not known, but is conceivably higher than 3. In addition to CPCs, CPC-like cells may also participate in the formation of a cyst. The nuclei of CCs show the same characteristics as those of CPCs and CPC-like cells (Fig. 8). The cytoplasm, however, forms a thin layer around the spermatogonia.

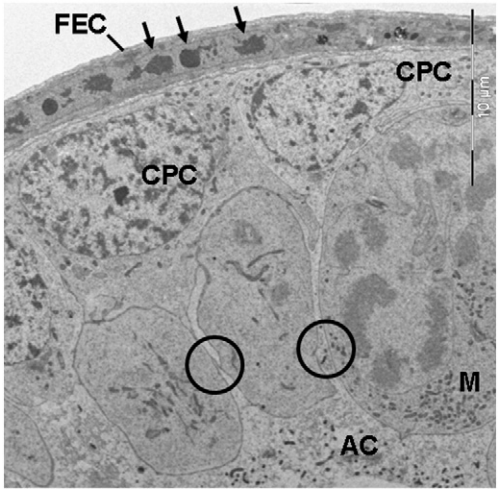

Figure 7 Electron micrograph. Peripheral part of the apical complex with CPCs and follicle envelope. Note the ovoid to rounded profiles of CPC nuclei. Extensions of CPCs reach between the GSCs to the extensions of the AC (encircled). The extremely flattened nucleus of a follicle envelope cell shows many electron-dense nucleoli (arrows). M, mitochondria.

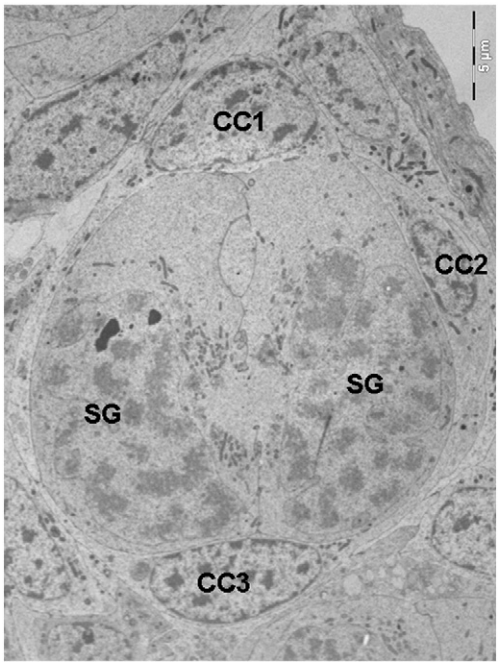

Figure 8 Electron micrograph. Profile of a young cyst that shows two spermatogonia (SG) enclosed by three cyst cells (CC1–CC3). This indicates that cysts are formed by at least three (but probably more) cysts cell—unless their number is varying. Note the irregular nuclear profiles of the spermatogonia.

The parietal cell layer of the follicular envelope

These cells are flat and have a very large diameter (Figs. 1D and 2). Accordingly, their nuclei have a very flat discoid shape. The chromatin is condensed in large clumps, and several nucleoli are found in a nucleus (Fig. 7).

Changes in short-term primary cultures in "standard medium"

Characteristics of newly (up to 2 h) incubated tissue and cells

The preparations included the following tissues: (a) entire young (L1 or L2) follicles, with the young follicles consisting of a fully developed germinal proliferation center without cysts, such as the follicle tips of adults (Fig. 9A); (b) follicle tips (not shown); (c) isolated complete apical complexes, partly with GBs and CPCs (Figs. 9B–E); (d) parts of the apical complex with a various number of GSCs (Figs. 10A–E); (e) cysts of different developmental stages with up to 8 spermatogonia (Figs. 11A–C); and (f) clusters of spermatogonia without enclosing CCs (Figs. 11D–F).

The preparations included the following isolated cells: (a) ACs (Fig. 10F); (b) GSCs (Fig. 10G); (c) GBs/spermatogonia, with GBs and spermatogonia being indistinguishable when isolated (Fig. 10H); (d) CPCs/CPC-like cells/CCs, with CPCs, CPC-like cells, and CCs also being indistinguishable when isolated (Fig. 12A); and (e) cells of the follicle wall (Fig. 13A).

During the first two hours of incubation, tissues and cells maintain their structural characteristics as evidenced in the light and electron microscopic studies. The ACs display the typical concentric arrangement of granular organelles (i.e.,

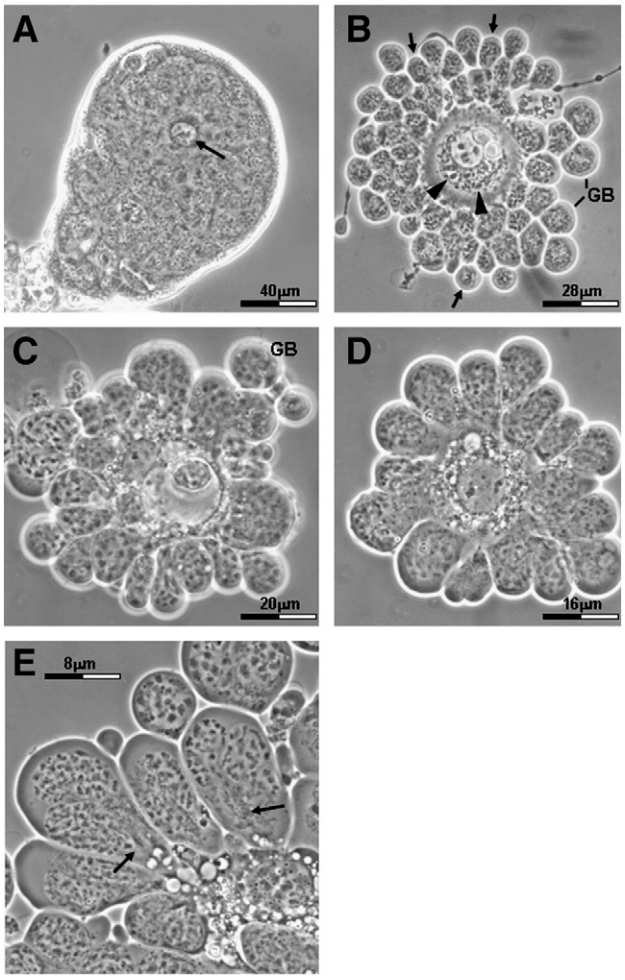

Figure 9 Light micrographs. Newly incubated intact follicular apex (A) and isolated apical complexes (B–E). (A) Intact follicle of a young male (L2) showing the apex with the fully formed apical complex. Arrow points to the AC. (B) Apical complex with adhering GBs and CPCs (arrows). Note the concentric arrangement of cell organelles (arrowheads) around the nucleus in the AC. (C) Apical complexes with few adhering GBs. (D) Apical complex devoid of GBs and CPCs. (E) Part of an isolated apical complex showing the structural polarity of GSCs, their highly irregular nucleus, and fibrous material, probably representing nuage (arrows).

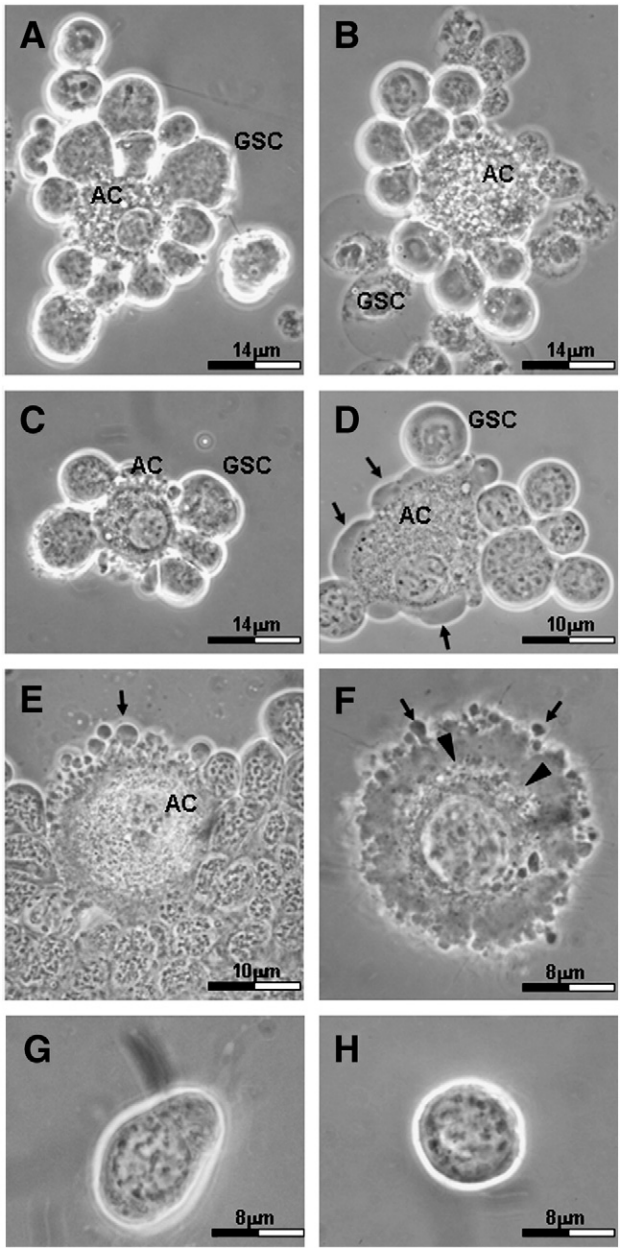

Figure 10 Light micrographs. Newly incubated apical complexes, partly dissociated, with various amounts of empty niche spaces (A–E), completely isolated AC (F), isolated GSC (G), and isolated GB/spermatogonium (H). (A–C) ACs with decreasing numbers of GSCs. (D) Note that the surface of the AC where it is not covered with GSCs has formed broad lobular pseudopodia (arrows). (E) Empty surface of AC with small lobular pseudopodia (arrow). (F) Completely isolated AC. Note the concentric arrangement of the cell organelles around the nucleus (arrowheads) and the formation of many small lobopodia (arrows). (G) Isolated GSC that has maintained its polar organization and pear-shaped form. (H) Germ cell that may represent a rounded off GSC, a GB, or an isolated spermatogonium.

mitochondria and lysosomes) around the nucleus, whereas the peripheral cytoplasm gives a hyaline impression (Figs. 9B and 10F). When isolated, they are the largest cells in culture (~24 μm in diameter), except for the cells of the follicular wall, and form many small lobular bulges (Fig. 10F). After the removal of some GSCs, the exposed surface of the AC also forms lobular projections (Fig. 10E). GSCs attached to the AC show their typical polar organization (Figs. 9C–E). They are pear-shaped and include large nuclei with extremely irregular contours and dark clumps of chromatin. Fibrous, nuage-like material is located toward the AC (Fig. 9E). Mechanically isolated GSCs maintain their pear-shaped outline for only a short period of time (mostly less than 2 h) (Fig. 10G), and then they round up and become indistinguishable from GBs (Fig. 10H). Isolated spermatogonia are somewhat smaller than the GSCs, but their nuclei have the same characteristics as those of GSCs, and it is therefore difficult to differentiate between them. Isolated CPCs, CPC-like cells, and CCs are distinctly smaller than

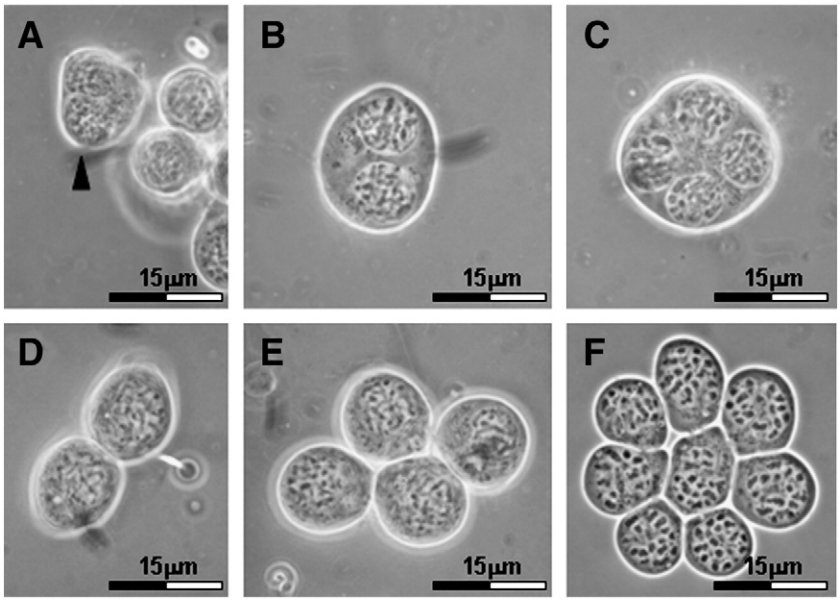

Figure 11 Light micrographs. Newly incubated, young, premeiotic cysts of transit-amplifying stages. (A–C) Intact cysts with CCs, (D–F) cysts whose CCs have been removed. (A) Cyst with GB surrounded by CCs (arrowhead). (B) Cyst with two spermatogonia. (C) Cyst with four spermatogonia. (D) Two, (E) four, and (F) eight spermatogonia without CCs.

GSCs. They have a bipolar or elliptic to round shape and an elliptic to round nucleus (Fig. 12A). Cells of the follicle wall were difficult to separate (which was also not intended on a regular scale). Additionally, these squamous cells have a large diameter (~20 µm) (Fig. 13A).

Changes of isolated cells and tissue during the first five days of incubation

The mechanical dissociation of apical follicle tissues caused some cell injuries. After degeneration of these cells during the first day of incubation, cell death in general occurred at a rate of about 20% until Day 5 in standard medium.

Changes of isolated cells

About 2 h after incubation, GSCs, CPCs, and spermatogonia started to form pseudopodia. Generally, three types of pseudopodia could be distinguished: rhizopodia, lobopodia, and filopodia. A single cell can form several branched rhizopodia of various lengths that show anastomoses (Fig. 16A). Lobopodia are mostly broad and stout (Figs. 16C and 10D). Filopodia can reach the fourfold length of their cell (~40 µm), are thin, and normally do not branch (Figs. 16B and 17I). They originate from a conically shaped base that includes dark granules (Figs. 16B and 17K). Filopodia show vivid tentacle-like movements into all directions. Formation of rhizoform and filiform projections were greatest at Day 1 and ceased after Day 2 of incubation. Thereafter many cells of all types attached to the bottom of the culture dish formed lobopodia and performed slight movements.

GSCs and free spermatogonia (without cyst envelope) expressed either one of the three types of pseudopodia (Figs. 16A–C). Although spermatogonia may be still interconnected by fusomes, they form pseudopodia that

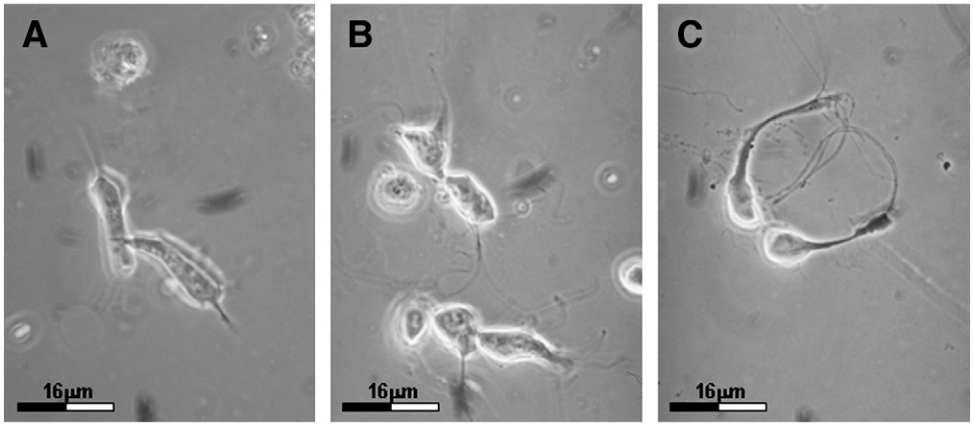

Figure 12 Light micrographs. CPCs/CPC-like cells/CCs. (These three cell types cannot be distinguished after isolation.) (A) Newly incubated cells. Note the formation of rhizopodia. (B, C) Cells 3 days after incubation with long rhizopodia.

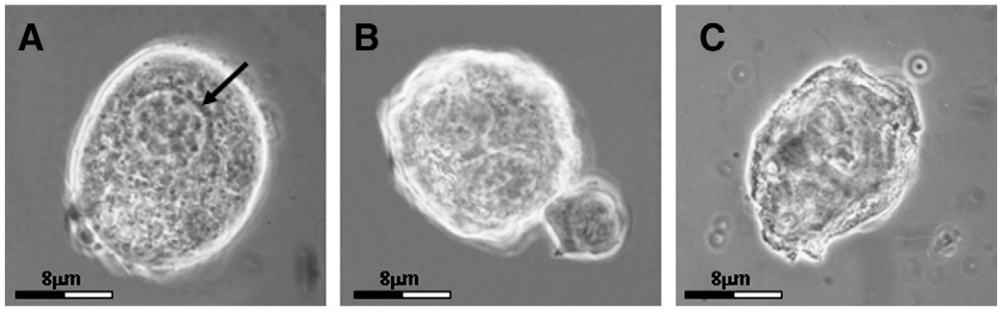

Figure 13 Light micrographs. Isolated cells of the follicular envelope. (A) Newly incubated cell. Arrow points to the nucleus. (B) Cell 3 days after incubation. (C) Cell 10 days after incubation.

are directed toward the periphery of the cell cluster (Figs. 17B–K). CPCs/CPC-like cells/CCs often show a uni- or bipolar shape with short filiform or rhizoform pseudopodia at their pointed ends (Figs. 12A–C). Isolated cyst cells are similarly structured as CPCs. Newly incubated, completely isolated apical cells showed numerous very small cellular protrusions that apparently formed following the retraction of the star-like projections that had extended between the GSCs (Fig. 10F). Sporadically short spiky protrusions could also be observed (Fig. 15C), but never rhizoform and filiform pseudopodia. After Day 1 of incubation, apical cells form large lobopodia (Figs. 15A and B), become attached, and carry out movements.

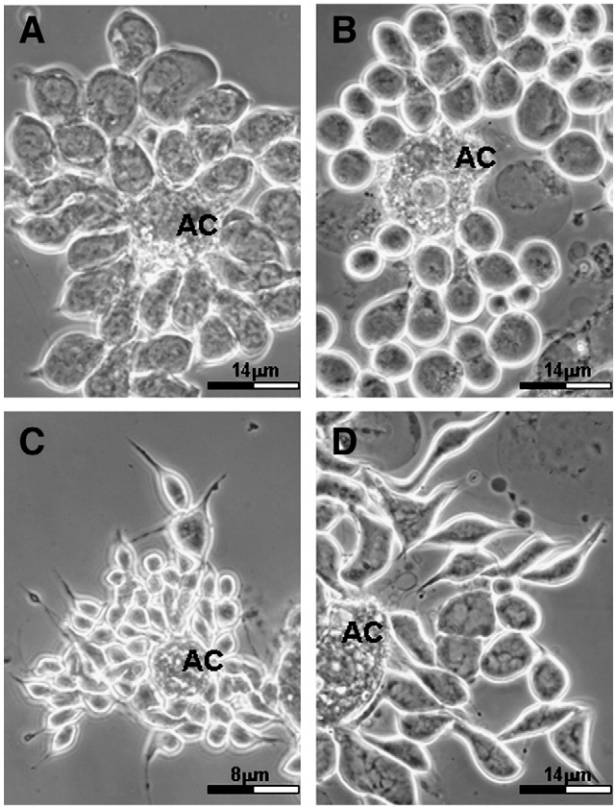

Figure 14 Light micrographs. Apical complexes 24–48 h after incubation. The micrographs show the emigration of the GSCs from the AC, their niche. (A, B) GSCs start to loosen their contact with the AC and to round off. (C, D) GSCs adopt a mostly bipolar shape, form filopodia, and move away from the AC.

Changes of incubated complete and incomplete apical complexes

When complete apical complexes were incubated, GSCs remained attached to the apical cell at least until Day 5, in contrast to GSCs of incomplete apical complexes, where a varying number of GSCs had been peeled off mechanically. In the latter cases, the seemingly intact GSCs dissociated from the AC (between Day 2 and Day 4), but did not degenerate (like injured GSCs). The GSCs seemed to leave the AC (their niche) actively. They loosened their attachment to the AC, adopted a polar or bipolar spindle-shaped form, and moved away from the AC. During this process they formed pseudopodia at their pointy ends (Figs. 14A–D). The dissociation of GSCs from the ACs resulted in an increase of isolated GSCs and isolated ACs. Naked, or almost naked, ACs engaged in amoeboid movements by the extension of lobular pseudopodia (Figs. 15A and B).

Cellular interactions

Isolated GSCs were often present in the immediate neighborhood of ACs that had vacant surface space or that were completely devoid of GSCs. A repopulation of empty spaces on the surface of ACs by isolated GSCs was never observed. Groups of spermatogonia that had lost their CCs during preparation were never ensheathed again, although CPCs/CCs were often located in the vicinity.

Mitotic activity and DNA synthesis

Incubated intact follicles and follicles *in vivo* served as controls with respect to mitotic activity (indicated by the observation of mitotic figures) and DNA synthesis (examined by BrdU labeling) in cultured apical testis tissue. Qualitative estimations revealed that mitotic activity and DNA synthesis occurred in both control groups throughout this examined time interval (Fig. 18A). In dissociated tissues, few mitotic figures were observed until Day 2, and none were observed thereafter. Judged by the morphological characteristics, most dividing cells represented spermatogonia. BrdU labeling revealed that at Day 1 of incubation, DNA synthesis took place in many GSCs, CPCs, and spermatogonia (Figs. 18B and C), but had almost ceased at Day 2 and was completely absent at Day 3 after incubation.

Long-term observations on primary cultures in standard medium

Observations on long-term incubations refer to incubations older than 5 days and up to 80 days in culture when living cells could still be identified. The death rate of cultured cells increased strongly with incubation time. It amounted to 50% at Day 7, 70% at Day 11, and up to 90% at Day 24. Thus, relatively few cells survived Day 10; some, however, lived beyond Day 80.

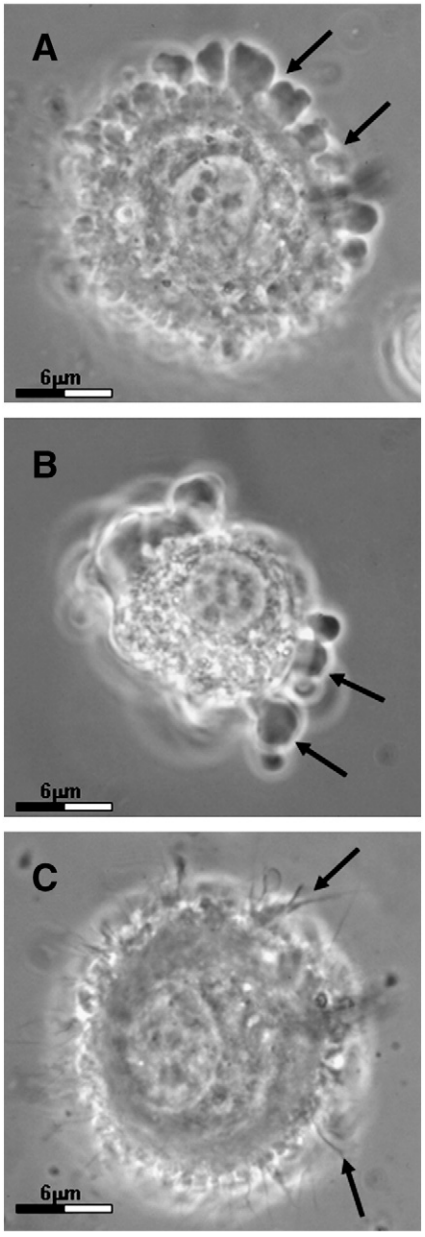

Figure 15 Light micrographs. Isolated ACs (i.e., completely empty niches) after 10 days of incubation. The typical structure of ACs is maintained. (A, B) Directed lobular pseudopodia indicate the direction of the movement of the cell (arrows). (C) The AC shows short rhizopodia (arrows) besides many small lobular pseudopodia.

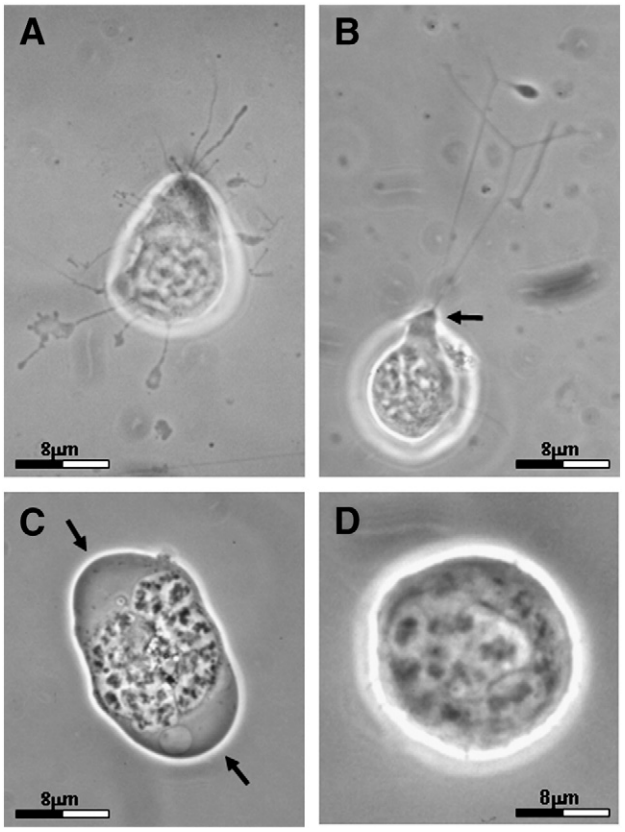

Figure 16 Light micrographs. Germ cells (GSCs/GBs/spermatogonia) 5 days after incubation (A–C) and 10 days after incubation (D). (A) Germ cell with rhizopodia and filopodia. Note the polar structure of the cell. (B) Germ cell with long filipodium that originates on a cone-shaped cellular protrusion (arrow). (C) Germ cell with two broad lobopodia apposing each other (arrows). (D) Germ cell of globular shape with large irregular nucleus that includes dark chromatin in patches and scant cytoplasm.

Besides the cells of the follicle wall, two cell types could be identified until the end of incubation: the ACs and germ cells. Among the germ cells, it was no longer possible to differentiate among GSCs, GBs, or spermatogonia. Spermatogonia had separated and were no longer connected by fusomes. Germ cells were recognizable by size, having a large irregular nucleus with dark chromatin patches and scant cytoplasm (Fig. 16D). ACs maintained their defining characteristics, i.e., the extraordinary large size, the round, lucent nucleus, and granulation of cytoplasm. The fraction of smaller cells most likely represented CPCs, CPC-like cells, and CCs that had all rounded off.

ACs in long-term culture expressed regularly pseudopodia, were adherent, and showed amoeboid movements. Likewise many of the germ cells formed lobopodia, although more flat in appearance. Such germ cells carried out minor movements and were adherent. The pending death of ACs was indicated by the loss of pseudopodia and rounding off.

Whereas the majority of cells died during the first 10 days of incubation, part of the young germ cells and apical cells survived much longer. Living young germ cells were observed until Day 33 and living apical cells until Day 56. Not definitively identifiable cells were alive until Day 80.

Effects of supplements to the standard medium on primary cultures

The following supplements to the standard medium were tested: conditioned medium of *l(2)mbn* cell cultures; addition of locust hemolymph; coincubation with intact testicular follicles, brain, and fat body; addition of various doses of growth regulators (insulin, EGF, PDGF, and retinoic acid), insect hormones (20-hydroxyecdysone, JH III, and the JH mimic fenoxycarb), and compounds interfering with calcium signaling ($CaCl_2$, EGTA, and the ionophore A 23187). None of these supplements stimulated the division or prolonged the DNA synthesis of the cells in primary cultures. However several supplements had biological effects. Three treatments suppressed the formation of pseudopodia: (1) the addition of native hemolymph; (2) high doses (10^{-5} M) of JH III and fenoxycarb; and (3) a high dose (0.1 μg/μl) of A 23187. The addition of native as well as heat-inactivated hemolymph shortened the survival of incubated cells. Coincubation with intact testicular follicles and/or small portions of fat body did not affect the incubation, whereas the addition of larger parts of fat body or locust brains reduced survival of cell cultures. Low doses of growth regulators had no negative impact on primary cultures, but high doses of insulin shortened cell survival considerably. In cultures with 20% PDGF, a few apical cells survived 84 days of incubation. 20-Hydroxyecdysone, JH III, and fenoxycarb all reduced the survival of incubated cells. About 95% of the cells were dead at Day 17 after treatment with 10^{-7} M 20-hydroxyecdysone. All agents interfering with calcium signaling had a negative effect on cell survival.

Discussion

Insects probably offer the simplest structure of a cellular niche known. In *L. migratoria* the GSC niche consists of a single large cell, the AC, surrounded by about 60 GSCs in the adult. The simplicity, size, and distinct structural characteristics of the cell types of the regenerative center prompted us to study their behavior in primary cultures. Since enzymatic dissociation of the apical complex was not successful, we dissociated the tissue mechanically. Although many cells were injured by this procedure, it had the advantages of preparing ACs (i.e., niches) with varied numbers of remaining GSCs, ranging from the full set to completely naked ACs. This allowed us to address the question of what happens when a niche has space available.

Behavior of complete apical complexes (AC with full set of GSCs) *in vitro*

Isolated apical complexes were almost always devoid of CPCs, indicating that there are no robust cell–cell contacts between CPCs and GSCs. This is supported by the ultrastructural observations which revealed no specialized intercellular junctions between the two cell types (data not shown). GSCs and the AC, in contrast, are extremely firmly attached. Whereas Szöllösi and Marcaillou (1979) did not find intercellular junctions, Zahn et al. (2007) described "close cell–cell contacts" between the GSCs and the AC. Adherens junctions have been demonstrated to exist between GSCs and *hub cells* in *Drosophila* (Yamashita et al., 2003). In *Drosophila* testes, they play a crucial role in GSC anchorage, in the positioning of the spindle, and consequently in the maintenance of stem cell identity (Yamashita et al., 2003, 2007; Song and Xie, 2002). GSC divisions are always asymmetric in adult *Drosophila* testes. In *L. migratoria* the majority of GSC divisions are also asymmetric (Figs. 1A and B), but symmetric divisions do occur (Fig. 1C). Daughter cells derived from symmetric GSC divisions (i.e., both daughter cells contact the AC) are interconnected by fusomes for some period of time (Figs. 5 and 6). Fusomes between GSCs and GBs have not been observed, which may indicate that after asymmetric division the daughter cells are more rapidly separated. In many stem cell niche systems, stem cells show low mitotic activity and retain BrdU labeling for an extended period of time (cf. Schmidt and Dorn, 2004). GSCs of adult locusts, however, are frequently seen in mitosis, and often whole clusters of neighboring GSCs are seen at the same stage of division (Fig. 1B). This corresponds with results obtained from pulsed BrdU administration, where consistently several labeled GSCs per apical complex could be found (Fig. 18). In our studies BrdU incorporation into ACs was not observed.

Isolated apical complexes began changing their structure after incubation (Figs. 14A and B). First, GSCs lose their structural polarity. This parallels the loss of GSC polarity immediately after laser ablation of the apical cell in intact follicles (Zahn et al., 2007). It reveals that the relationship between GSCs and niche has changed. Concomitant with the loss of polarity, the contact between the GSCs and the AC loosens. GSCs often form pseudopodia and finally leave their niche (Figs. 14C and D). The loss of the adhesive force between GSCs and AC *in vitro* suggests that the very strong adhesion in the intact male is actively supported by the "milieu" surrounding the apical complex. It should be noted that the apices of testicular follicles have no effective hemolymph testis barrier (Szöllösi and Marcaillou, 1977). Thus, a complex cocktail of enzymes, nutrients, hormones, growth factors, and other molecules reach and may potentially affect the regenerative center from the periphery. Changes of the milieu, as under culture conditions, obviously cause the breakdown of the adhesive substance. The nature of the adhesive substance, to our knowledge, is not known. In male and female GSCs of *Drosophila*, adhesion to the niche is mediated by DE-cadherin (Song and Xie, 2002; Wang et al., 2006; Song et al., 2002). E-cadherin is also expressed in primordial germ cells of mouse testis and N-cadherin-11 is localized at cell junctions between germ cells and sertoli cells of the rat (Johnson and Boekelheide, 2002; Mackay et al., 1999). Adjudin, shown to disrupt adherens junctions between germ cells and sertoli cells in the rat and human testis (Mruk and Cheng, 2004), could not separate GSCs from the AC in *L. migratoria*. Thus, the nature of the adhesive substance remains to be elucidated.

Niches with empty spaces, completely empty niches, and separated GSCs

It has been demonstrated that experimentally emptied niches of *Drosophila* female GSCs can persist for several weeks, can attract and interact with incoming cells, and

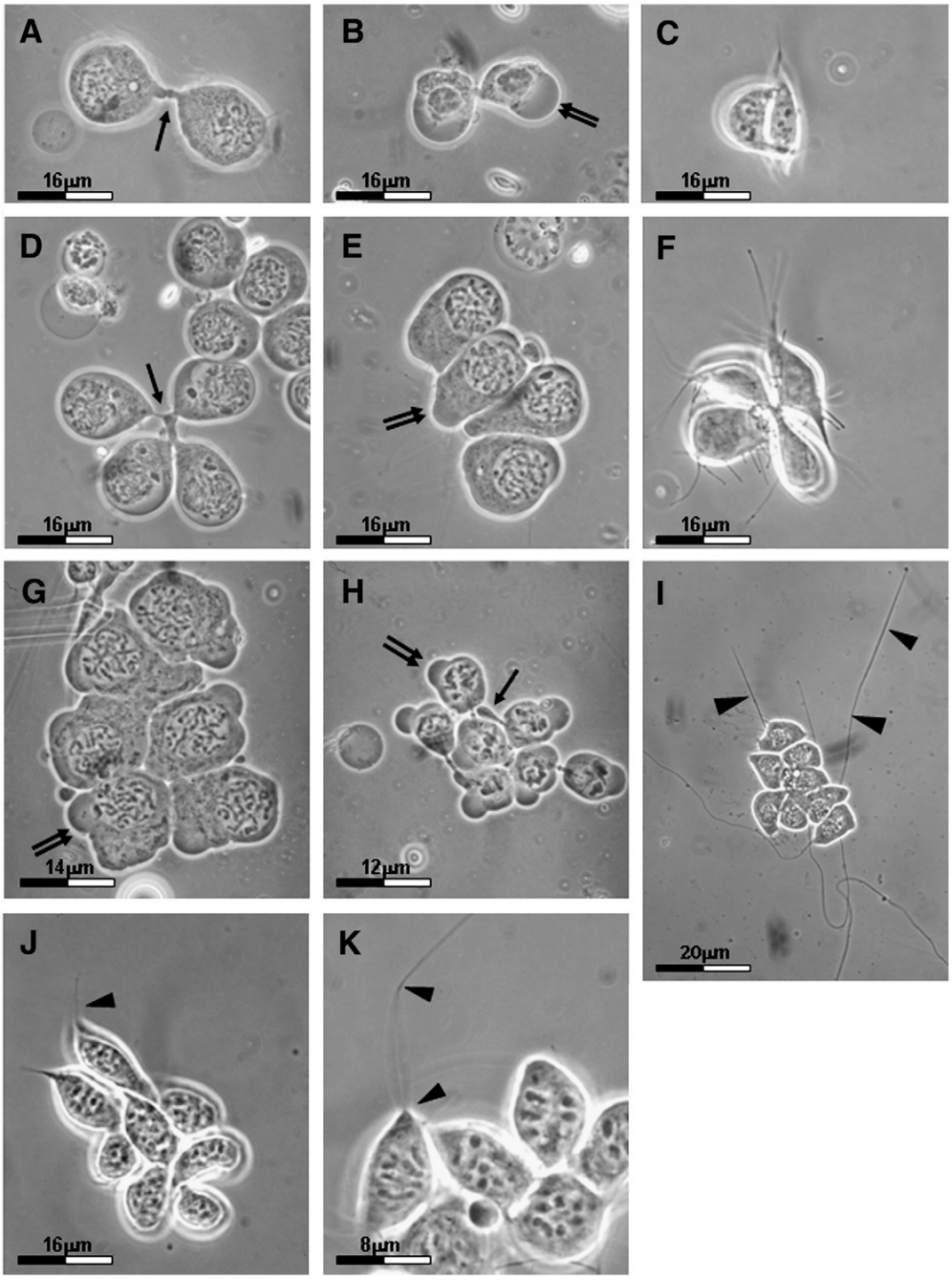

Figure 17 Light micrographs. Spermatogonia isolated from cysts after the first (A–C), second (D–F), and third transitamplifying division (G–K) of GB 5 days after incubation. Note that the spermatogonia maintain their fusomes (arrows) (A, D, H). Spermatogonia may form broad lobopodia (double arrows) (B, E, G, H), rhizopodia (F), or long filopodia (arrowheads) (I–K) which frequently originate from cell cones (K).

support ectopic proliferation (Kai and Spradling, 2003). Both male and female differentiating germ cells can dedifferentiate and revert into functional GSCs (Brawley and Matunis, 2004; Kai and Spradling, 2004). There is even a competition among GSCs for niche occupancy in the *Drosophila* ovary (Jin et al., 2008). Recently Singh et al. described the mutual dependency of GSCs and somatic stem cells and their competitiveness for a shared niche which is regulated by the JAK/STAT pathway (Singh et al., 2010). Thus, it could have been expected that GSCs (and likely spermatogonia of transit-amplifying stages) located in the vicinity of partly or totally emptied ACs would try to contact and repopulate

their niche, since both GSCs and ACs engage in local movements. However, we never observed a repopulation of empty niche spaces by GSCs, nor was there any interaction between ACs and GSCs detectable. On the contrary, GSCs left ACs soon after incubation, as described above (Fig. 14).

Recently, Zahn et al. (2007) showed that laser ablation of the AC in intact testicular follicles of *L. migratoria* resulted in the apoptotic death of the affiliated GSCs. Two possibilities for the induction of apoptosis were discussed: first the deprivation by the niche eliciting a pending death program in the GSCs; secondly the release of a signal by the degenerating niche that actively initiates an apoptotic pathway in GSCs. Whereas the authors favored the first possibility, the present study demonstrates that GSCs are able to survive without the AC, at least *in vitro*.

Behavior of ACs, GSCs, spermatogonia, CPCs, CPC-like cells, and CCs in long-term culture in standard medium

Five days after incubation apical complexes had dissolved resulting in naked ACs and isolated GSCs. Also, the spermatogonia had lost their connecting cell bridges, the fusomes. It was therefore no longer possible to distinguish among GSCs, GBs, and spermatogonia, which share the most structural characteristics that were retained until death. In addition, these cells showed an astonishing variety of pseudopodia, as discussed below (Figs. 16 and 17). ACs maintained their typical concentric arrangement of cell organelles until death (Fig. 15). CPCs, CPC-like cells, and CCs all show similar structural characteristics (Fig. 12). The first two days in incubation they express mainly rhizopodia (Fig. 12) and start to round off thereafter.

Proliferation and differentiation of cells

DNA synthesis, indicated by the uptake of BrdU, was never observed in ACs and had ceased at Day 2 of incubation in the other cell types. In *Drosophila* testes, GSCs undergo differentiation and transit amplification on inactivation of signaling from the niche (Brawley and Matunis, 2004; Kiger et al., 2001; Tulina and Matunis, 2001). Furthermore, in the absence of GSCs, *hub cells* start to divide, and CPCs continue to proliferate with cells from the cyst lineage switching to the *hub cell* fate (Gonczy and DiNardo, 1996). In the present *in vitro* study, ACs, germ cells, CPCs, and CPC-like cells were often located at considerable distances from each other. This makes it very unlikely that the signals, which are normally exchanged between neighboring cells, function across the distance. Consequently, effects similar to those observed in GSC-deprived *Drosophila* testes could have been expected, but none of those effects were observed. The arrest of mitoses of all cell types points to the absence of factors that are crucial for cell proliferation and differentiation, in particular for the two stem cell types, the GSCs and CPCs.

Effects of supplements on cultures

Maintenance of tissue homeostasis demands that differentiated cells dying by natural exhaustion or injury are replaced by regenerative cells, the stem cells. To date, circuits that maintain tissue homeostasis have been rudimentarily investigated. Recently we were able to demonstrate in the stick insect that maintenance of midgut epithelium is strongly supported by the stomatogastric nervous system (Holtmann and Dorn, 2009). Interruption of the stomatogastric nervous systems leads to

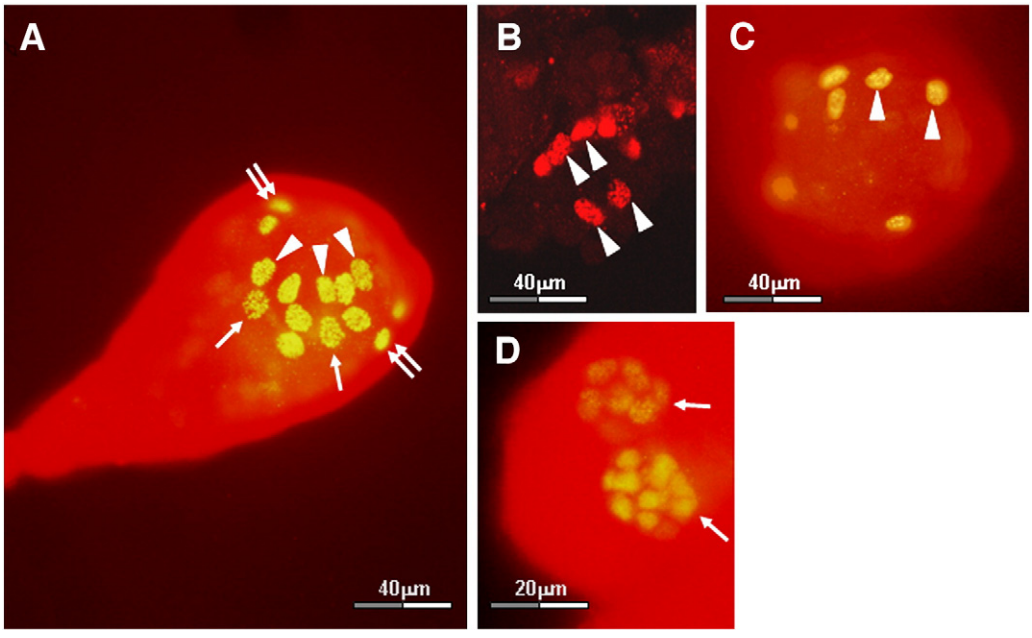

Figure 18 DNA synthesis, indicated by pulsed BrdU labeling, in the regenerative center of incubated testicular tissues. (A) Complete testicular follicle of young (L2) larva after 24 h of incubation. Arrowheads point to GSCs, arrows to labeled GBs, and double arrows to labeled CPCs. (B, C) Dissected apical complexes. Arrowheads point to labeled GSCs. (D) Dissected cysts. Arrows point to two labeled cysts with eight spermatogonia each.

increased apoptotic death of differentiated midgut cells and altered the mitotic rate of intestinal stem cells.

In the case of male reproduction, a reservoir of semen must be maintained by balancing use and production of new sperms. In both cases a feedback mechanism must exist, correlating stem cell mitotic activity with the demand for differentiated cells. The mechanisms orchestrating an appropriate level of sperms in the case of testes are largely unknown in most postembryonic stem cell–niche systems (Lin, 2003). With respect to the insect male regenerative system there is a long-lasting dispute over a role of ecdysteroids. Ecdysteroids have been described to be male sex hormones in general (for review, see (De Loof, 2006)) and/or to perform specific tasks during testis maturation (Gelman et al., 1989; Gillott and Ismail, 1995; Jarvis et al., 1994; Loeb et al., 1982, 2005) or spermatogenesis in particular (Leclercq-Smekens, 1978; Balles et al., 2002). None of these hypotheses have been verified. Likewise, the suggestion of Szöllösi and Marcaillou (1979), that the AC of *L. migratoria* may synthesize juvenile hormone, which was based on the organelle equipment of the AC, remains unconfirmed. However, factors from hemolymph and fat body were shown to have effects on the growth and differentiation of insect midgut stem cells in primary tissue cultures (Hakim et al., 2007). Neural insulin from medial neurosecretory cells in the brain had an influence on ovarian GSC division and cyst growth in *Drosophila* (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). In the present study, we examined the impact of various supplements, including those that proved to have an effect on lepidopteran intestinal stem cells *in vitro* (Hakim et al., 2007; Loeb, 2005; Loeb et al., 2003) and on the different cellular components of the locust regenerative center in the primary culture.

None of the studied supplements promoted the differentiation of GSCs or spermatogonia, nor did they have an effect on the mitotic activity of GSCs, PCPs, spermatogonia, or any other cell type. Several of the supplements reduced the survival of the cells, notably the insect hormones 20-hydroxyecdysone and JH III. This effect concerned all cell types and is therefore not indicative of a special role of the hormones in spermatogenesis. Nevertheless it is conceivable that juvenile hormone or ecdysteroids could act on the regenerative center together with other factors which were not present.

Formation of pseudopodia

All cell types in our culture, except for the testes envelope cells, formed pseudopodia after a few hours to days after incubation. Pseudopodia are generally associated with cell motility and phagocytosis which may also apply to lobopodia and rhizopodia, but the long, agile filopodia, which were only formed by GSCs and spermatogonia (Figs. 16 and 17), may fulfill other functions. It has been shown that cells of the imaginal discs in *Drosophila* that find themselves in the wrong environment or compartment can form cellular extensions reaching into the "appropriate" compartment and thus protect themselves against apoptosis triggered by the foreign environment (Milan et al., 2002; Rorth, 2003). Recent studies also indicate that these structures can be associated with sensory and exploratory functions (Faix and Rottner, 2006). In our primary culture systems, the formation of filopodia of GSCs and spermatogonia may express the search for their appropriate environmental niche, the AC in the case of GSCs, and CCs in the case of spermatogonia. But ACs and CCs apparently lose their specific markers during *in vitro* culture so that the filopodia possessing cells do not migrate or repopulate vacant niches.

An interesting communication via cellular extensions has been identified among cells of the hematopoietic stem cell niche, the PSC (posterior signaling center of the lymph gland), and hemocyte progenitor cells located in the medullary zone of the lymph gland of *Drosophila*. In this system cellular extensions of the PSC deliver hedgehog (Hh) to the medullary zone, keeping the residing precursor cells in an undifferentiated quiescent state (Mandal et al., 2007). The niche composed of the AC in our culture system expressed many small and occasionally broader lobopodia (Fig. 15).

Conclusion

Intriguingly the *in vitro* culture conditions described in this study induced a dissociation of the GSCs from their niche, presumably initiated by the inactivation of the adhesive components between AC and GSCs. Consequently the observed formation of long and motile filopodia by the GSCs may reflect a search of the stem cell to reenter their niche, which most likely has lost its markers. Therefore we postulate that in the intact male, the integrity and proper functioning of the apical complex is supported by factors reaching the complex from the surrounding milieu such as the hemolymph. Since our culture system ensures the survival of the GSC, the system represents an ideal tool for the examination of peripheral factors that are necessary for the maintenance and proper function of the stem cell–niche entity. The mandatory peripheral factors for the circuitry of feedback systems that guarantee tissue homeostasis can be easily examined in this culture system as demonstrated on an exploratory set of additives.

Materials and methods

Rearing conditions and tissue acquisition

L. migratoria mirgratorioides (R & F) were reared under crowded conditions as reported previously (Zahn et al., 2007). Testes used for tissue culture were harvested from all larval stages (L1 through L5) and young adults, 1 to 8 days old. Testis follicles were isolated under sterile conditions and transferred into any of the media listed below. The further procedure depended on the age of the donors. From larvae older than L2 and adults, the apical tips of the follicles were separated with a microscissor and placed into fresh medium. These follicle tips contained the niche, i.e., the AC, the GSCs, the CPCs, GBs, young cysts, and the part of the follicle wall that covered the follicular apex (Figs. 1 and 2). Follicles of young larvae were nearly spherical and consisted only of the elements found in the follicle tips of advanced stages and did not include cysts. In order to obtain isolated cells or cell clusters of the elements of the follicular apices, both whole follicles of young larvae and follicle tips of older larvae and adults were treated the same way.

Mechanical and enzymatically digestive methods were evaluated to separate cells of young follicles and follicular

tips. The following enzymes and enzyme mixtures were used: (a) trypsin (Invitrogen, Karlsruhe, Germany) 0.01% and 0.05%, respectively, with DNase (Boehringer, Mannheim, Germany) 0.05% in HBSS (Hank's balanced salt solution) (Invitrogen); (b) collagenase type I 0.5% or collagenase type II 0.5% (both from Worthington, St. Katharinen, Germany), in each case with dispase 2% (Invitrogen) dissolved in HBSS. Mechanically we separated the cells with fine tweezers and needles.

Recently the substance adjudin was reported to be able to remove rat germ cells from their niche due to disruption of sertoli-germ cell anchoring provided by adherens junctions (see Mruk and Cheng, 2004 for review). We tested the possibility of a similar function of adjudin (generously supplied by Dr. Cheng) in the locust testes. L2 larvae and young adults were injected with 50 μg adjudin (dissolved in DMSO) per gram life weight. Test animals and controls (injected with DMSO only) were sacrificed 24 h later, and the testicular follicles (in case of L2) or follicle tips (in case of adults) isolated and incubated. Apical complexes (i.e., AC with adhering GSCs) were examined immediately and the following days for the dissociation of GSCs from the AC (i.e., their niche). In addition, isolated follicles of L1 and follicle tips of young adults were incubated in medium containing 500 μg adjudin per milliliter.

Primary culture conditions

Culture devices included petri dishes for cell culture with nunclon surface, 35 mm in outer diameter and 10 mm deep (Nunc, Wiesbaden, Germany), and flat bottom wells, 15 mm in diameter and 1 mm deep, in special glass slides which could be sealed with a greased (Vaseline) coverslip (for details, see Dübendorfer and Eichenberger-Glinz, 1980; Küppers-Munther et al., 2004). The glass slide chambers allowed hanging drop, standing drop, and sandwich cultures. Standing drop cultures in petri dishes were applied routinely, since they allowed an easy exchange of culture medium or the addition of test substances. In long-term incubations, media were changed weekly. The incubation temperature was 26 °C.

First, we established a standard medium that could be used as the base to test and compare effects of putative active compounds affecting the developmental fate of the incubations. In order to select a standard medium we incubated whole testis follicles and vasa efferentia. The following media were examined (see also Zahn et al., 2007): Grace Insect Cell Culture Medium, TC 100 Insect Medium, Schneider's *Drosophila* Medium, and Leibovitz's L-15 Medium for Orthopterans (all media were from Invitrogen, Karlsruhe, Germany). Several supplements were tested: fetal bovine serum (FBS) (Linaris, Wertheim-Bettingen, Germany), active or inactivated at 60 °C, tryptose phosphate broth (Sigma-Aldrich, Taufkirchen, Germany), and lipid concentrate (Invitrogen). The suitability of a medium was judged using the following criteria: (1) occurrence of mitoses in the apical complex of incubated follicles at the same rate as in follicles of intact males of the same age, which was monitored at serial sections of histological preparations (see below); (2) equal cell death rates, as determined by trypan blue staining, between intact and cultured follicles; (3) spontaneous vasa efferentia contractibility after isolation and coincubation with follicles during the incubation period. All tested media fulfilled the requirements. Subsequently, we incubated mechanically dissociated testis tissue in the media listed above. Again, there were no major differences between the media. Therefore we selected as incubation medium of choice Leibovitz's L-15 Medium for Orthopterans with the addition of 6.7 mg/ml tryptose phosphate broth, 0.3% lipid concentrate, 10% active FBS, 10 μg/ml insulin (Sigma-Aldrich), 50 μg/ml gentamicin (Invitrogen), and adjusted to pH and osmolarity of the locust hemolymph, i.e., pH 7.2 and 380 mOsm, termed standard medium in the following text. When media were changed after the first week of incubation, the standard medium contained heat-inactivated FBS instead of active FBS.

Examined additives

The following additives given to the standard medium were examined for their impact on development and differentiation of GSCs, GBs, young cysts, CPCs, and ACs: (1) coincubation with locust tissues, (2) growth regulators, (3) insect hormones and a hormone mimic, and (4) compounds affecting Ca^{2+} signaling.

(1) The locust tissues for coincubation consisted of solid tissue, locust hemolymph, and conditioned media from *Drosophila l(2)mbn* cells cultured for 5 and 10 days (for details on the cell line, see Ress et al., 2000). The solid tissues for coincubation stemmed from the same specimens as the preparations and included whole intact testicular follicles (3 to 5 per incubation), fat body (those parts adhering to the testis), and brain. Hemolymph was supplemented at 2.5 and 25% of the standard medium and was native or heat inactivated and stemmed from males of the same age as the specimens that were sacrificed for the testicular preparations. For media conditioning with *Drosophila l(2)mbn* cells, 33 and 50% of *l(2)mbn* culture medium was added to the standard medium.

(2) We evaluated the growth regulators EGF, PDGF, insulin, and retinoic acid that were recently identified to affect insect stem cells, in particular midgut stem cells (LaFever and Drummond-Barbosa, 2005; Loeb, 2005; Loeb et al., 2003). Insulin, although present in the standard medium, was tested at a 10-fold concentration at 100 μg/ml. EGF (epidermal growth factor, murine) (Sigma) was supplemented at 10 and 100 ng/ml, PDGF (platelet-derived growth factor, porcine) (Sigma) was added at concentrations of 1.3×10^{-8} and 2.6×10^{-8} M and retinoic acid (Sigma) at concentrations of 10^{-6}, 10^{-7}, and 10^{-8} M.

(3) 20-Hydroxyecdysone (Sigma) was added at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M. JH III (juvenile hormone III) (Sigma) and the synthetic JH mimic fenoxycarb (Syngenta, Münchwilen, Switzerland) were each supplemented at concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M. JH III and fenoxycarb are not water soluble. The compounds were therefore dissolved in acetone, and the appropriate amounts of the solution were placed into a culture flask. The solvent was allowed to evaporate. The standard medium was added and shaken for 30 min. Acetone treatment without JH III or fenoxycarb served as control.

(4) For elevation of the extracellular Ca^{2+} concentration, the standard medium was adjusted to 5, 10, 50, 75, and 105 mM $CaCl_2$. For decreasing the extracellular Ca^{2+} concentration, the standard medium contained 10^{-6} to 10^{-9} mM EGTA (ethylene glycol-bis-(2-aminoathylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) (Acros Fisher Scientifific, Schwerte, Germany). The ionophore A23187 (Sigma), that tends to open inward plasma membrane Ca^{2+} channels (e.g., Loeb, 2005), was applied at 0.1 to 0.0001 μg/μl.

Each test comprised 5 to 10 incubations, each incubation containing the preparations of 20 whole testicular follicles (in the case of L1 and L2) or 20 apices of testicular follicles (in the case of L3 through L5 and young adults). Each test incubation was accompanied by control incubations in standard medium that contained testicular preparations from the same developmental stage as the test preparations. If test substances were dissolved in a nonaqueous solvent (e.g., acetone or DMSO), controls were incubated with the solvent.

Determination of cell death and DNA synthesis

Cell death was determined by trypan blue. DNA synthesis was demonstrated by the incorporation of BrdU (5-bromo-2′-deoxyuridine) instead of thymidine during the S-phase of mitotic activity and the visualization of BrdU by a monoclonal primary (mouse anti-BrdU) and a secondary fluorochrome-linked (rhodamine anti-mouse Ig-) antibody. We used the BrdU Immunofluorescence Assay Kit (Roche, Mannheim, Germany). The secondary antibody was from Dianova (Hamburg, Germany). For demonstration of DNA synthesis in testes of intact males, 10 μl of BrdU was injected into larvae (L5) and 20 μl into adults, resulting in a concentration of about 10 μM in the hemolymph. Incorporation was stopped after 30 min by dissection of the testes and fixation in Carnoy's fluid for 30 min at room temperature. For demonstration of DNA synthesis of either whole testes or dissociated follicle apices in culture, BrdU was added to the culture medium, adjusted to a concentration of 10 μM, and stopped after 30 or 60 min. Whole testes were then placed into Carnoy's fluid, whereas cultures with dissociated tissue were transferred into Eppendorf capsules and gently centrifuged, and the pellet was fixed in Carnoy's fluid. Subsequently, all specimens were handled the same way. They were hydrated and rinsed in PBS-TX, pH 7.4, placed in PBS-TX with 2 N HCl for 30 min at room temperature, and rinsed again before incubation with anti-BrdU for 12 h at 4 °C. After rinsing with PBS-TX, specimens were incubated with anti-mouse Ig-rhodamine for 30 min at 37 °C, rinsed again, and embedded with the Anti-Fading Kit Pro Long (Molecular probes, Eugenen, OR). BrdU labeling was analyzed with a confocal laser scanning microscope (Leica TCS 4D, Leica Heidelberg, Germany). Living incubations were photographed with a Zeiss Axioplan (Oberkochen, Germany).

Light and electron microscopy

For light microscopy follicles were fixed in aqueous Bouin's fluid and embedded in methacrylate. Serial sections of 5 μm thickness were stained with azan. For electron microscopy follicles were taken from young adult males and fixed in 2.5% glutaraldehyde in 0.08 M cacodylate buffer (pH 7.4) with 0.1 M sucrose for 1 h, followed by 2% osmium tetroxide in 0.08 M cacodylate buffer (pH 7.4) for 1 h at room temperature. Then the specimens were washed in distilled water, dehydrated, and embedded in araldite. Sections were double-stained in uranyl acetate and lead citrate.

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