Development 125, 3323-3328 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV3874

Female mouse germ cells form synchronously dividing cysts

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Accepted 8 June; published on WWW 6 August 1998

SUMMARY

Oocytes from many invertebrates initiate development within distinctive cysts of interconnected cells, which are formed through synchronous divisions of a progenitor cell. Recently, processes underlying cyst formation have been extensively characterized at the molecular level in *Drosophila*. Defects in this process cause sterility in female flies. Early female mouse germ cells are organized as cell clusters as well, but it is uncertain whether these groups are similar to the cysts of invertebrates. We find that mouse germ cells are connected by intercellular bridges in the ovaries of 11.5 to 17.5 days postcoitum embryos; microtubules and organelles have been observed within these bridges. Confocal microscopy shows that cells within mouse clusters divide synchronously and frequently correspond in number to powers of two. Thus, female mouse germ cell clusters exhibit key characteristics of invertebrate germline cysts indicating that the process of germline cyst formation is conserved in the mouse.

Key words: Oogenesis, Cyst, Mouse germ cell

INTRODUCTION

Female germ cells in a wide variety of invertebrates develop in clusters of interconnected cells called cysts that share several distinctive characteristics (Büning, 1994, de Cuevas et al., 1997). Cysts grow progressively by mitosis from individual progenitor cells during a defined developmental period. During cyst growth, cytokinesis of each cell cycle remains incomplete, leaving daughter cells (‘cystocytes’) joined by a network of specialized intercellular bridges called ring canals. In many species, all cystocytes divide synchronously, resulting in cysts containing 2^n cells. Following formation of the cyst, microtubules that pass through the ring canals directionally transport organelles and specific mRNAs into one of the cystocytes. In *Drosophila*, this process underlies the determination of one cystocyte as an oocyte, while the remaining cystocytes become nurse cells. Eventually, the cysts undergo programmed breakdown, each giving rise to one, or in some species more than one, oocyte**.** Specific cellular and molecular processes that accompany cyst formation have been characterized (de Cuevas et al., 1997). The fusome, an unusual vesicle-rich organelle, passes through the ring canals of developing cysts in many species. It arises from a spherical structure known as the spectrosome located within the germline progenitor cell and grows during cystocyte divisions. Spectrin and several related proteins are found in fusomes; mutations in these proteins disrupt cyst formation and abolish fertility (Lin et al., 1994; McKearin and Ohlstein, 1995; de Cuevas et al., 1996). The presence of germline cysts in developing ovaries from diverse organisms raises the question of whether the process of cyst formation has been conserved in vertebrates.

Morphological observations of developing germ cells in several vertebrate species previously identified cell clusters with some properties of cysts during fetal ovary development (Gondos, 1973). Premeiotic germ cells are arranged in small clusters in mice, rabbits, rats, hamsters and humans, and electron microscope studies have shown that intercellular bridges strongly resembling ring canals join early germ cells during this period (Ruby et al., 1969; Spiegelman and Bennett, 1973; Franchi and Mandl, 1962; Gondos et al., 1971; Gondos and Zamboni, 1969; Zamboni and Gondos, 1968; Weakley, 1967; Gondos, 1987). Human, rabbit and hamster germ cells divide synchronously in small groups (Gondos et al., 1971; Gondos and Zamboni, 1969; Zamboni and Gondos, 1968; Weakley, 1967). At the time of these studies, the lack of specific markers and the requirement for serial section reconstruction made a thorough analysis of these stages difficult. Therefore, it has remained uncertain whether early germ cell clusters in vertebrate ovaries are cysts. Recently, Gomperts et al. (1994), using an in vitro assay, suggested that mouse germ cell clusters form by aggregation of individual cells and do not correspond to cysts.

MATERIALS AND METHODS

Mice

Gonads of 10.5-16.5 days postcoitum (d.p.c.) embryos were obtained from CD-1 mice (Charles River Labs). The presence of a vaginal plug the morning after mating was designated 0.5 d.p.c.. All animal experimentation was reviewed and approved by the Institutions’ Animal Care and Use Committee.

Antibodies

The EMA mouse monoclonal antibody was raised against Nulli SCC1 mouse embryonal carcinoma cells. It recognizes carbohydrates found on the cell surface of mouse embryonal carcinomal cells and mouse germ cells in 8-13.5 d.p.c. embryos (Hahnel and Eddy, 1986). It was obtained from the Developmental Studies Hybridoma Bank and used at a 1:1 dilution. The GCNA (germ cell nuclear antigen) rat monoclonal antibody was raised against pachytene spermatocytes and recognizes a nuclear antigen in developing male and female germ cells (Enders and May II, 1994). The GCNA antibody was a gift from George Enders and was used at a 1:1 dilution. Anti-α-tubulin antibodies were purchased from Sigma either unconjugated or conjugated to FITC and used at a 1:500 or 1:100 dilution, respectively. FITC, Lissamine-Rhodamine and Cy3-conjugated secondary antibodies were used at a 1:200 or 1:400 dilution. The DNA dyes propidium iodide (Sigma) and Toto-3 (Molecular Probes) were used to detect nuclei.

Immunostaining and fluorescence microscopy

The immunostaining protocol is based on several previously described protocols (de Cuevas et al., 1996; Lin et al., 1994; Karl and Cappel, 1995; Hogan et al., 1994; Patel, 1994). Gonads from CD-1 mouse embryos were dissected in PBS and fixed for 20 minutes at room temperature in 5% Formaldehyde (Ted Pella Inc), washed for 30 minutes in PT (PBS plus 0.1% Triton-X-100), incubated in PT plus 5% BSA for 1 hour at room temperature and then incubated overnight at 4°C in PT plus 5% BSA containing primary antibodies at the appropriate dilution. Gonads were then washed in PT plus 1% BSA for 1.5 hours at room temperature and then incubated for 2 hours at room temperature in PT plus 5% BSA containing the appropriate fluorophore conjugated secondary antibodies. The gonads were washed for 1.5 hours in PT plus 1% BSA, rinsed in PBS and mounted in PBS plus 50% glycerol and 0.1% p-phenylene-diamine (Sigma) for microscopy. Stained gonads were examined by epifluorescence and confocal microscopy using a Leica TCS NT microscope. To visualize nuclei, gonads were incubated before the final wash either in propidium iodide at 5 μg/ml in PT+1% BSA, or in Toto-3 at a 1:2000 dilution for 20 minutes. The gonads were treated with 0.1 mg/ml RNase A for 30 minutes to remove RNA prior to staining.

Analysis of synchronously dividing germ cell clusters

Whole ovaries were double stained with anti-α-tubulin and anti-EMA antibodies. Following staining, each cluster of mitotic germ cells was analyzed by one micron adjacent confocal sections completely spanning each cluster. 5-10 ovaries at each day from 10.5 to 16.5 d.p.c. were examined. More that 50% of each ovary was examined; however, the central portion of each ovary could not be examined completely due to incomplete penetration of the antibodies deep into the tissue. However, no difference between clusters located in inner or outer regions of the ovary was detected.

Electron microscopy

Ovaries were dissected in PBS and processed for transmission electron microscopy as described in Yue and Spradling (1992).

RESULTS

Premeiotic mouse germ cells form clusters

We looked for each of the characteristic properties of germline cysts in mouse ovaries to more definitively address whether cysts are present. Previous studies of this species (see Fig. 1A) demonstrated that germ cells arrive at the gonad by 10.5 d.p.c., increase in numbers, form clusters readily visualized by alkaline phosphatase staining (Fig. 1B), begin to enter meiosis on 14.5 d.p.c., and drastically decrease in number due to degeneration starting at 16.5 d.p.c. (Gomperts et al., 1994; Chiquoine, 1954; Borum, 1961; Tam and Snow, 1981; Ginsburg et al., 1990). Initially, we used confocal microscopy and the specific germ cell markers EMA (Hahnel and Eddy, 1986) and GCNA (Enders and May II, 1994) to directly ask if germ cell clusters originate from single cells and increase progressively in cell number with time prior to meiosis as expected for germline cysts. In 10.5 d.p.c. ovaries, counts of 616 germ cell clusters revealed that 85% were present as single cells, 13% in groups of two, and the remainder in groups of 3 or 4, surrounded by somatic cells (Fig. 1C and data not shown). During the next 7 days of oogenesis, the relative number of single germ cells decreased greatly, while the remainder appeared in clusters (Fig. 1D,E). However, after 10.5 d.p.c., it was difficult to determine the exact number of cells within these clusters because the antibodies failed to clearly distinguish the borders of adjacent clusters.

We noticed a large round structure was labeled with the EMA antibody in a perinuclear position in many 10.5 d.p.c. germ cells (Fig. 1C, arrowheads). The size and location of this structure was

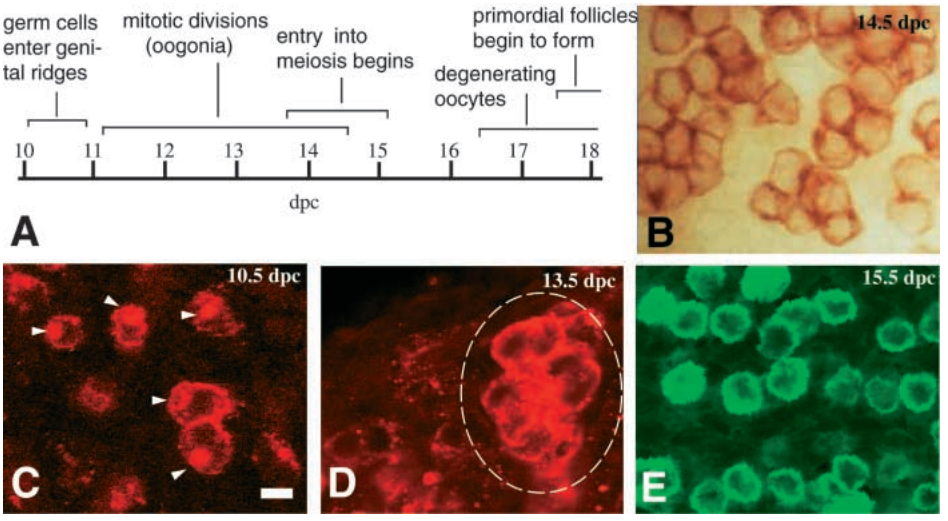

Fig. 1. Time course of female germ cell development. (A) Timeline of germ cell development in the mouse embryo. Mouse gestation is about 20 days. (B) Germ cells detected by alkaline phosphatase activity in a 14.5 d.p.c. mouse ovary (Ginsburg et al., 1990). The germ cells are arranged in clusters. (C) One confocal section showing germ cells detected using the EMA antibody in a 10.5 d.p.c. genital ridge. Several single germ cells and one group of two germ cells surrounded by unlabeled somatic cells are shown. Most of the labeling is observed at the plasma membrane. However, arrowheads indicate a sphere of cytoplasmic staining seen in most cells at this stage. (D) One confocal section showing germ cells detected using the EMA antibody in a 13.5 d.p.c. mouse ovary. One germ cell cluster is outlined. The shape of the clusters tends to be round. (E) One confocal section showing germ cell nuclei detected using the GCNA antibody in a 15.5 d.p.c. mouse ovary. Note that the cyst nuclei at this time tend to be aligned in rows, due to altered associations with somatic cells. Bar, 10 μm.

reminiscent of the spectrosome that is observed in *Drosophila* germ cells prior to cyst formation (Lin and Spradling, 1997). The EMA-rich region is also present in some 11.5-13.5 d.p.c. germ cells. This EMA 'granule' has been observed previously (Hahnel and Eddy, 1986) and may correspond to a cytoplasmic region of intense alkaline phosphatase staining reported in 8.5-11 d.p.c. germ cells that was proposed to be the Golgi (Ginsburg et al., 1990; Jeon and Kennedy, 1973).

Female germ cells are connected by intercellular bridges

Cyst cells are connected by ring canals. We next looked for evidence of interconnections between cells within the germ cell clusters. The existence of such junctions was previously reported in 17- and 18-day germ cells (Ruby et al., 1969). We observed many examples of intercellular bridges in 11.5 d.p.c. to 17.5 d.p.c. ovaries using electron microscopy (Fig. 2A). The bridges always connect large round cells with the morphology of germ cells. These bridges are similar in size and appearance to those described in previous studies of mammalian germ cells as well as young *Drosophila* ring canals (Fig. 2B; Ruby et al., 1969; Spiegelman and Bennett, 1973; Franchi and Mandl, 1962; Gondos et al., 1971; Gondos and Zamboni, 1969; Zamboni and Gondos, 1968; Weakley, 1967; Gondos, 1987; Mahowald and Strassheim, 1970; Mahowald, 1971; Koch and Spitzer, 1983; Robinson and Cooley, 1996). Frequently, a thin somatic cell process limited the region of germ cell-germ cell contact to the vicinity of the canal. Some cells contain two bridges (Fig. 2A), suggesting that they undergo more than one round of cell division and bridge formation. Moreover, bundles of microtubules were observed passing through some canals (Fig. 2C), as in invertebrate cysts. Ring canals could no longer be found in 18.5 d.p.c. and 19.5 d.p.c. ovaries following the period of extensive germ cell degeneration. In *Drosophila*, after cyst formation, the ring canals frequently contain mitochondria and vesicles undergoing microtubule-dependent intercellular transport (Mahowald and Strassheim, 1970; Mahowald, 1971; Koch and Spitzer, 1983). We also observed mitochondria in the mouse ring canals at 17.5 d.p.c. (Fig. 2D), consistent with the idea that there is intercellular transport in the mouse bridges as well. Thus, typical intercellular bridges join germ cells during the time that clusters are present.

Small groups of germ cells divide synchronously

We next asked whether the cells of the mouse clusters are mitotically synchronized, like cysts in most studied species. The existence of mitotic synchrony would provide strong evidence that clusters are true cysts, since cell aggregates are expected to remain unsynchronized. Because mitosis occupies a small part of the cell cycle, analyzing only mitotic clusters would allow us to distinguish neighboring clusters and determine cluster size during ovary development. Therefore, mouse ovaries between 10.5 and 16.5 d.p.c. were labeled with an anti-α-tubulin antibody, anti-EMA and, in some cases, DNA-specific dyes, and analyzed by confocal microscopy. We frequently observed adjacent germ cells undergoing synchronous mitosis in 10.5-15.5 d.p.c. ovaries (Figs 3, 4). These cells are germ cells because they were positive for EMA (Fig. 3) or GCNA. Furthermore, the degree of synchrony appeared to be very high. With few exceptions, when one cell was at a particular stage of mitosis such as telophase (Fig. 3B,D) then the other cells in the cluster were at an identical stage of mitosis.

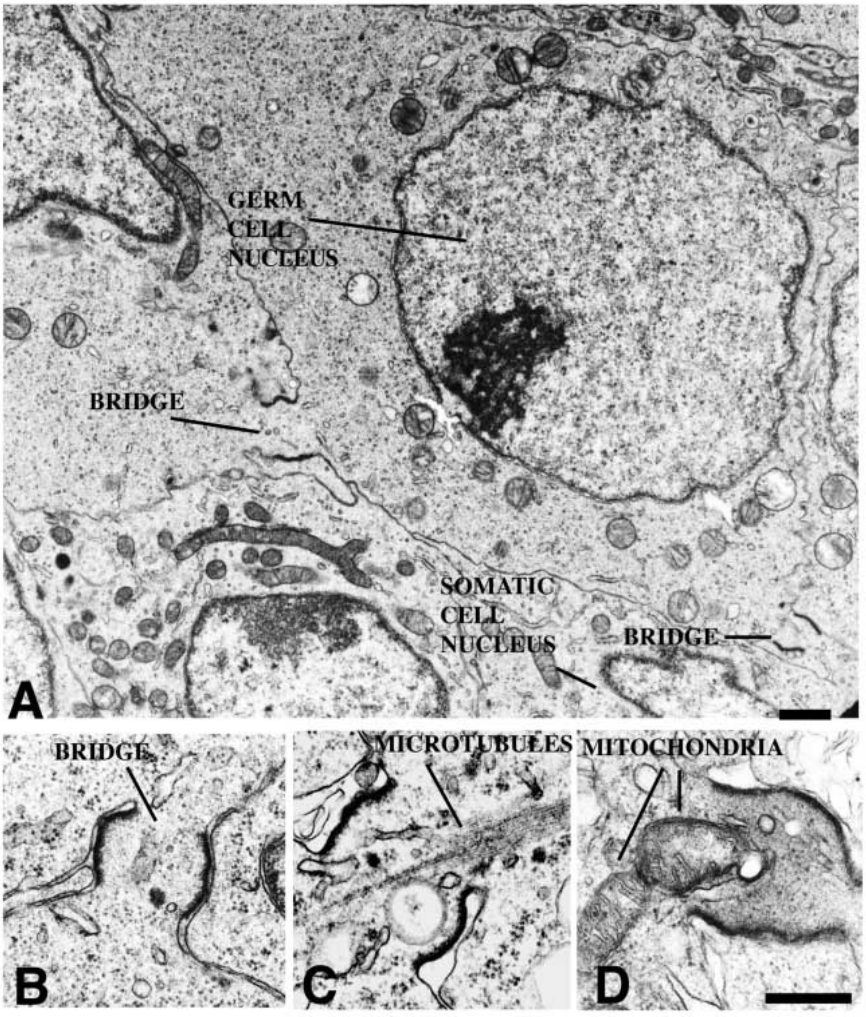

Fig. 2. Germ cells are interconnected by ring canals. (A) 13.5 d.p.c. germ cell from a mouse ovary with two intercellular bridges. The other germ cells that are connected to this germ cell are not visible in this section. Examples of germ cell and somatic cell nuclei are shown. Bar, 0.75 μm. (B) High-power view of a bridge from a 13.5 d.p.c. mouse ovary showing the electron-dense rim of the bridge. (C) High-power view of a bridge from a 12.5 d.p.c. mouse ovary showing microtubules going through the bridge. (D) Germ cell bridge from a 17.5 d.p.c. mouse ovary showing a mitochondrion within the bridge and another nearby. Rings averaged 0.5-1.0 μm in diameter at all stages examined. Bar for B-D, 0.5 μm.

Of key importance, the number of adjacent mitotic cells frequently corresponded to powers of 2 (Fig. 4). Many clusters of two (Fig. 3A,B,D), four (Fig. 3C,E), eight (Fig. 3F,G – six are visible in this section) and 16 cells were observed. In some cases, the EMA aggregate appeared to associate with spindle poles (Fig. 3B). Overall, 74% of the synchronous clusters corresponded to a power of two. However, not all the clusters of mitotic cells fell into this category, especially in older ovaries, even when careful confocal analysis ensured that no cells in a cluster were missed. We observed several clusters in 13.5 d.p.c. and older ovaries where mitotic germ cells were adjacent to other non-dividing germ cells that appeared to be part of the same group (Fig. 3H, arrowheads). This suggests that some of the cells in older clusters become unsynchronized, as has been observed in species such as bees and aphids (Büning, 1994). Nonetheless, the high frequency of clusters that contained 2, 4, 8 or 16 mitotic cells argues strongly that the clusters form by rounds of synchronous divisions with incomplete cytokinesis. Several observations indicate that the clusters of mitotic cells are not due to random coincidence. First, the probability of large clusters containing up to 19 adjacent mitotic cells is negligible in a tissue with an average mitotic index of 1.7%. Second, only mitotic germ cells appear in clusters, while mitotic somatic cells, which divide at a similar rate, are always single. For example, a sample of 2,600 cells (728 germ cells) at 13.5 d.p.c. contained 2 single mitotic germ cells and 6 mitotic clusters, while all 6 somatic cell mitoses were solitary. Finally, if random processes give rise to these mitotic cell groups, then the number of clusters of 3 would be expected to exceed the number with 4, and no preference for 8 or 16 cell clusters should be observed; this is contrary to our evidence.

Further support that these clusters are cysts came from their progressive increase in size from 10.5 d.p.c. until meiosis ensues at 14.5 d.p.c., but not thereafter. Thus, at 10.5 d.p.c. only groups of 2 or 4 cells undergoing mitosis are observed (Fig. 4A). At 11.5-12.5 d.p.c., clusters of 2 or 4 cells still predominate but some larger clusters are also seen. At 13.5 d.p.c. many large clusters are seen, including a significant number with exactly 8 or 16 mitotic cells. Thereafter, the number of mitotic clusters detected per gonad and their size decreased. Thus, synchronous germ cell mitotic divisions decline just as cells are observed to begin entering meiosis. Some of the clusters observed in 14.5 and 15.5 d.p.c. ovaries may represent cysts that started dividing later or whose development is lagging, since the developmental synchrony of mouse germ cells is not absolute. Moreover, some of the smaller clusters of dividing cells seen at 14.5 and 15.5 d.p.c. may be part of larger cysts whose cells are not completely synchronized. Because the largest mitotic clusters contain between 8 and 16 mitotic cells, mouse cysts likely grow no larger than 32 cells.

DISCUSSION

Mouse germ cells develop as synchronized cysts

Our experiments reveal that during 10.5-16.5 d.p.c., mouse female germ cells exhibit all the hallmarks of germline cysts. They grow by mitotic division from one progenitor cell, are interconnected by intercellular bridges and divide synchronously, increasing in number by powers of two. Our data also suggest that intercellular transport of vesicles and mitochondria takes place within clusters, possibly along

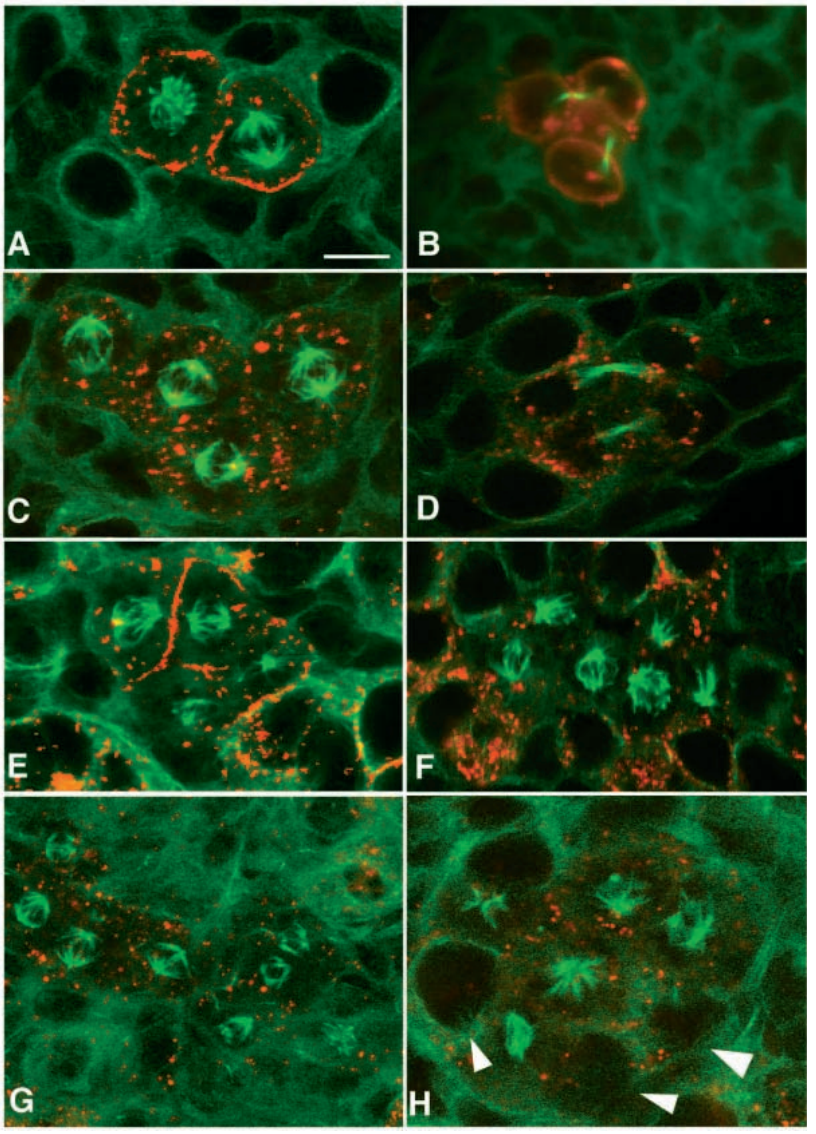

Fig. 3. Many germ cell clusters divide synchronously. Mouse ovaries were stained with EMA (red) and anti-α-tubulin (green) antibodies. A and C-H are from 13.5 d.p.c. embryos and B is from an 11.5 d.p.c. embryo. Scale bar is 10 μm. (A) One confocal section of two germ cells in metaphase. (B) One confocal section of two germ cells in telophase. (C) One confocal section showing four germ cells in metaphase. (D) One confocal section of two germ cells in telophase. (E) A projection of two 1 μm confocal sections showing four germ cells in metaphase. (F) One confocal section showing six germ cells out of a total of eight in metaphase. (G) One confocal section of eight germ cells in metaphase. (H) One confocal section showing a partially dividing cluster. Five of the eight germ cells shown are in metaphase. Three cells in interphase are indicated by arrowheads. A total of nine cells from this cluster were in metaphase.

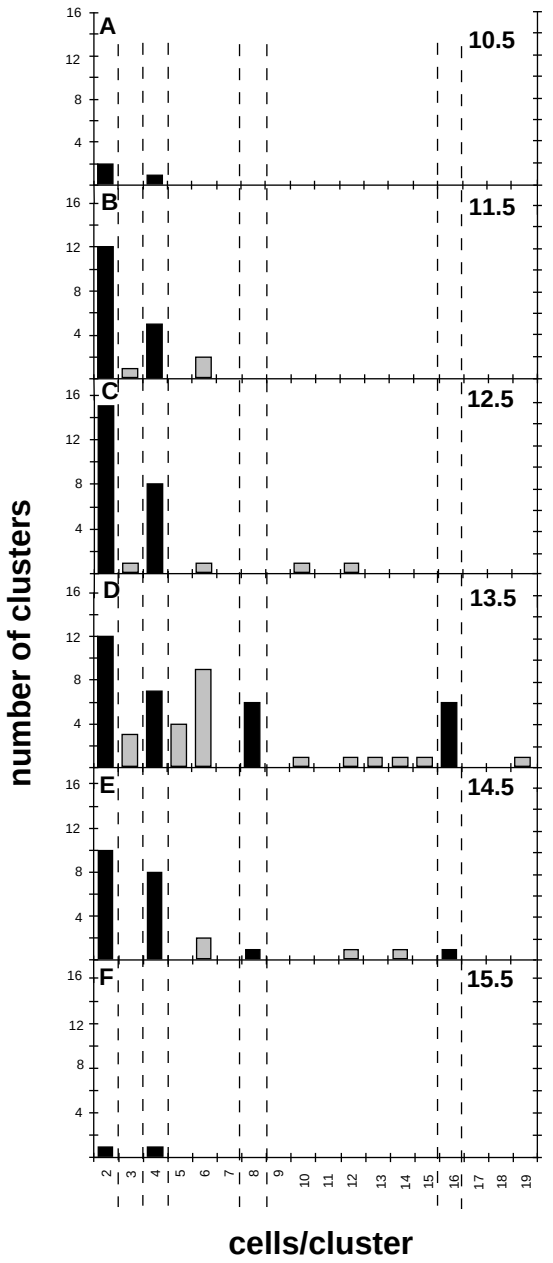

Fig. 4. Synchronously dividing clusters of cells grow in size and frequently correspond to powers of two. Whole ovaries were stained as in Fig. 3, for clusters of 2 or more mitotic cells, and the results plotted: (A) 10.5 d.p.c., 3 mitotic clusters found in 10 ovaries. (B) 11.5 d.p.c., 20 mitotic clusters found in 5 ovaries. (C) 12.5 d.p.c., 27 mitotic clusters found in 5 ovaries. (D) 13.5 d.p.c., 53 mitotic clusters found in 8 ovaries. (E) 14.5 d.p.c., 24 mitotic clusters found in 9 ovaries. (F) 15.5 d.p.c., 2 mitotic clusters found in 6 ovaries. Dark-shaded bars indicate powers of 2.

microtubule bundles that pass through the ring canals. These observations cannot be explained on the assumption that mouse ovarian germ cell clusters are random aggregates. The finding that female germ cells form cysts means they resemble premeiotic male germ cells, which develop as cysts in nearly all organisms including the mouse and other vertebrates (Büning, 1994; Fawcett, 1961). Some of these properties of female germ cell clusters have been reported previously in several vertebrates (Ruby et al., 1969; Spiegelman and Bennett, 1973; Franchi and Mandl, 1962; Gondos et al., 1971; Gondos and Zamboni, 1969; Zamboni and Gondos, 1968; Weakley, 1967; Gondos, 1987). However, this is the first time that a preference for powers of two in cell number has been demonstrated, and the first time that the presence of ring canals and synchronous divisions has been documented throughout the process of cluster formation in a single organism. Consequently, our data strongly indicate that the process of germline cyst formation has been conserved during fetal mouse oogenesis. Moreover, in light of the previous studies, it is likely that cysts form during oogenesis in diverse vertebrates, including humans.

Previously, Gomperts et al. (1994) showed that migrating primordial germ cells are often connected by long processes that are lost by day 11.5 d.p.c., after arrival in the genital ridges. Because 10.5 d.p.c. germ cells cultured in vitro were observed to aggregate, the authors proposed that germ cell clusters form during 10.5-11.5 d.p.c. by the aggregation of networks of such interconnected germ cells. Our data argue that many germ cell clusters arise by successive rounds of synchronous divisions from single progenitor cells to form cysts of cells interconnected by ring canals. The clusters continue to increase in size after 11.5 d.p.c. Moreover, the finding that mouse germ cell clusters frequently correspond to powers of two is difficult to explain by the aggregation model. However, our experiments cannot exclude that some clusters arise by alternative mechanisms, including aggregation as proposed by Gomperts et al. (1994).

The fusome is associated with synchronous cystocyte divisions in many invertebrate organisms and, in *Drosophila*, it is known to be required for synchronous division and oocyte formation (de Cuevas et al., 1997). We observed a cytoplasmic structure in early germ cells using the EMA antibody that may correspond to the spectrosome, a fusome precursor. This structure was sometimes associated with the poles of mitotic spindles. However, it did not appear to be asymmetrically distributed during cell divisions and failed to persist throughout the entire period of cluster formation. Electron micrographs did not reveal the specialized cytoplasm containing small vesicles that is typical of the *Drosophila* fusome, although an atypical, Golgi-like region was frequently observed. Furthermore, immunofluorescent staining of 13.5 d.p.c. ovaries with antibodies that recognize mouse α-spectrin or mouse fodrin labeled germ cell membrane skeletons, but not a typical fusome in mitotically dividing clusters (data not shown). Mitotic synchrony in mouse clusters was less complete than in species that form cysts of fixed size. These observations suggest that mouse germ cell divisions are synchronized by a process that differs in some respects from *Drosophila*, and that the requirement for a fusome has been altered or eliminated.

Possible roles of germ cell cysts

What might the role of germ cell cysts be in the developing mouse ovary? One possibility is that cysts are required to synchronize the development of small groups of germline cells prior to meiosis. In males of most species, cysts of interconnected germ cells persist throughout spermatogenesis, synchronizing the development of all cyst members. Gene products produced by individual germ cells are shared by intercellular transport throughout the cyst (Braun et al., 1989), and it may be important

that all the cells remain at the appropriate developmental stage to utilize gene products produced by their neighbors. Cysts might be required to facilitate a similar period of gene product sharing among early synchronized female germ cells. Stoneflies are good candidates for such a mechanism because in these organisms cysts of interconnected synchronized cells break down after entry into meiosis and each component cell develops into an oocyte (Gottanka and Büning, 1990).

A second possibility is that cysts make possible an early specialization of germline cells. One or more cyst cells may accumulate materials transported from the others that allow them to develop as oocytes. The transporting cells, known as nurse cells, eventually degenerate. This function for cysts is widespread in insects such as *Drosophila* where centrioles, mitochondria, specific mRNAs and most ribosomes are transported into the oocyte from nurse cells (reviewed in de Cuevas et al., 1997). While cysts from many species give rise to only one oocyte, in organisms such as aphids, 32-cell cysts give rise to 16, 11 or 8 oocytes depending on the particular species (Büning, 1994). If the mouse cysts that we have described have such a function, it would mean that not all premeiotic germ cells are oogonia, but some differentiate as nurse cells (Gondos, 1973). One possible role for such early transport would be to facilitate organelle biogenesis. Only a subset of mouse mitochondria serve as founders for the next generation and the time of their selection has been mapped to early stages of oogenesis (Jenuth et al., 1996). Perhaps a small number of special mitochondria (and possibly other organelles) are transported into the future oocytes and used to found their mitochondrial populations. Although we do not yet understand the purpose of the mouse cysts, conservation of many aspects of this process in mammals suggests that they serve an important function during oogenesis.

We especially thank Susan Dymecki for guidance with mouse work. The GCNA antibody was a gift from George Enders. We thank Mike Sepanski for valuable assistance with electron microscopy. We thank members of the Spradling lab and Chen-Ming Fan for helpful comments on the manuscript. This work was supported by the Howard Hughes Medical Institute. The Developmental Studies Hybridoma Bank is maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD.

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