Mouse Ovarian Germ Cell Cysts Undergo Programmed Breakdown to Form Primordial Follicles

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In many organisms, early germline development takes place within cysts of interconnected cells that form by incomplete cytokinesis and later undergo programmed breakdown. We recently identified similar cell clusters within the fetal mouse ovary, but the fate and functional significance of these germ cell cysts remained unclear. Here, we show that mouse cysts undergo programmed breakdown between 20.5–22.5 dpc, during which approximately 33% of the oocytes survive to form primordial follicles. This process accounts for most of the perinatal reduction in germ cell numbers and germ cell apoptosis reported by previous authors, and suggests that perinatal germ cell loss is a developmentally regulated process that is distinct from the follicular atresia that occurs during adult life. Our observations also suggest a novel function for a transient cyst stage of germ cell development. Prior to breakdown, mitochondria and ER reorganize into perinuclear aggregates, and can be seen within the ring canals joining adjacent germ cells. Cysts may ensure that oocytes destined to form primordial follicles acquire populations of functional mitochondria, through an active process that has been evolutionarily conserved.

Key Words: oogenesis; cysts; germ cells; cell death; mitochondria.

INTRODUCTION

In many mammalian species, it has been reported that one-half to two-thirds of the oocytes die early in development before individual follicles are formed (Baker, 1972). In the human fetus, 7 million oocytes develop initially, but, by birth, only 2 million remain. Germ cell loss also occurs in the mouse (Borum, 1961; Coucouvanis et al., 1993; Ratts et al., 1995), a species in which the biology of oogenesis has been extensively studied (Borum, 1961; Odor and Blandau, 1969a; Peters, 1969; McLaren, 1984; Byskov, 1986). The function of germ cell death is poorly understood but has been viewed as a random process that continues into adulthood, and which can be exacerbated by nutritional deficits or by environmental factors. One proposed function of germ cell death is to eliminate germ cells with chromosomal abnormalities or defective mitochondrial genomes (Baker, 1972; Peters and McNatty, 1980; Coucouvanis, 1994; Krakauer and Mira, 1999; Morita and Tilly, 1999).

The biology of germline development in many nonmammalian species provides another reason for extensive perinatal germ cell loss. The germ cells of these species are arranged in special clusters of cells called germline cysts (Telfer, 1975; Dym and Fawcett, 1971). Cysts arise from progenitor cells that divide synchronously to yield clusters of germ cells that subsequently enter meiosis (for review see de Cuevas et al., 1997). The mitotic divisions are accompanied by incomplete cytokinesis so that the daughter cells remain connected by intercellular bridges called ring canals. Only a fraction of the cells within a completed cyst ultimately survive to form mature oocytes (reviewed in Bünning, 1994). Up to 98% of cyst cells serve as nurse cells, supplying molecules to the oocytes. These nurse cells then undergo programmed breakdown, often at a specific time in germ cell development.

Cell clusters with many of the characteristics of germ cell cysts form in the mouse ovary between 10.5 and 13.5 dpc (days post coitum) (Ruby et al., 1969a; Speigelman and Bennett, 1973; Pepling and Spradling, 1998). This is just prior to the time that germ cells enter meiotic prophase and precedes the time when germ cell losses have been reported (see Fig. 1). By the time mice are a few days old, primordial follicles containing single oocytes are present, indicating that cyst breakdown and perinatal germ cell loss both occur between the time germ cells enter meiotic prophase and form follicles. Thus, a significant amount of germ cell...
apoptosis during this period may be a consequence of the cyst breakdown process. Observations in other vertebrates suggest that similar events may be a common feature of premeiotic germ cell development (Franchi and Mandl, 1962; Gondos et al., 1971; Gondos and Zamboni, 1969; Zamboni and Gondos, 1968; Weakley, 1967; Gondos, 1987).
FIG. 2. Germ cell loss is correlated with cyst breakdown and apoptosis. (A) The number of germ cells per ovary (■) is plotted from 13.5 to 25.5 dpc, revealing the loss of 66% of germ cells at 20.5–22.5 dpc. This corresponds to the time germ line cysts break down, as indicated by the rise in the percentage of single oocytes and primordial follicles (□). (B) The percentages of dying germ cells in representative confocal sections are plotted by using the same time scale as in (A). All apoptotic cells in the ovary were detected by using the TUNEL method (●: "TUNEL+"), while apoptotic germ cells were detected by using an antibody for cleaved PARP in combination with the germ cell-specific antibody, vasa (○: "PARP+"). All data are reported as means. Standard deviation was calculated for each data point (see Table 1). (C–G) Detection of apoptosis in the developing ovary. (C) Example of apoptosis detected in the ovary using the TUNEL method with dying cells labeled in green and germ cells labeled with the germ cell-specific vasa antibody in red. (D) Same section as (C) showing DNA (Toto-3). (E) Examples of dying germ cells detected using an antibody specific for cleaved PARP (green) and the germ cell-specific antibody vasa (yellow). (F) Same section as (E) showing DNA (Toto-3). (G) Dying (arrowhead) and healthy germ cells detected by electron microscopy.
In some species, cysts allow certain germ cells to specialize as nurse cells and provide assistance to others that will develop as oocytes. In the mouse, one possibility is that cysts help provide oocytes with functional mitochondria (Pepling and Spradling, 1998). Mitochondrial DNA is thought to have a higher rate of mutation than the nuclear genome (Brown et al., 1979). Mammals with a heteroplasmic mitochondrial genotype can segregate to a homoplasmic mitochondrial genotype within one generation (Ashley et al., 1989; Hauswirth and Laipis, 1982; Koehler et al., 1991). A genetic bottleneck has been proposed to account for this rapid switch, which is thought to assist in the elimination of less functional mitochondrial genotypes. The bottleneck has been mapped in the mouse to early female germ cell development sometime between the arrival of primordial germ cells at the ovary and the appearance of primordial follicles (Jenuth et al., 1996).

Here, we show that mouse cysts break down during a 2-day window of developmental time and that this process is accompanied by germ cell loss and elevated apoptosis. Two-thirds of all germ cells die, but only a minority of cells within individual cysts undergo apoptosis at any one time. Just prior to cyst breakdown, the number and organization of mitochondria undergo a dramatic change that is preserved in primordial follicles. Changes in the organization of somatic cells indicate that they are likely to participate actively in breaking down cysts into primordial follicles. We propose that cysts play an active role in ensuring that oocytes acquire high-quality mitochondria and that this process corresponds to the mitochondrial bottleneck.

**MATERIALS AND METHODS**

**Mice**

Ovaries of embryos and pups were obtained from CD1 mice (Charles River Labs). The presence of a vaginal plug the morning after mating was designated 0.5 dpc. Birth usually occurs at 19.5 dpc. Times after birth are referred to as dpc for continuity of time scale. All animal experimentation was reviewed and approved by the Institutions' Animal Care and Use Committee.

**Antibodies**

Polyclonal rabbit anti-Vasa antiserum, a gift from Toshiaki Noce, was used at a dilution of 1:500 (Tanaka et al., 2000). Polyclonal rabbit antiserum that specifically recognizes the apoptosis-specific cleaved form of poly(ADP ribose) polymerase (PARP) was obtained from New England Biolabs. The antibodies are directly labeled with Alexa 488 and were used at a dilution of 1:100. Monoclonal mouse anti-cytochrome oxidase subunit I (cox I) antibody was obtained from Molecular Probes and used at a dilution of 1:50. The ApopTag fluorescent TUNEL labeling kit from Intergen Company was used according to the manufacturer's instructions. FITC, Cy3 (Jackson ImmunoResearch Laboratories), Alexa 488, and Alexa 568 (Molecular Probes)-conjugated antibodies were used at a 1:200 or 1:400 dilution. The DNA dyes Toto-3 (Molecular Probes) and propidium iodide (Sigma) were used as previously described (Pepling and Spradling, 1998).

FIG. 3. Breakdown of germ line cysts visualized using anti-Vasa antibodies. Single confocal sections are shown from ovaries at 13.5 (A), 19.5 (B), and 22.5 dpc (C). Note that Vasa staining appears continuous when germ cells are interconnected, while single germ cells stain distinctly, probably due to the interpolation of somatic cell cytoplasm. Scale bar is 15 μm.
TABLE 2
Percent of Different Size Germline Cysts in the Developing Mouse Ovary

<table>
<thead>
<tr>
<th>Age</th>
<th>% Lost(^a)</th>
<th>Cyst size(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>21.5</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>22.5</td>
<td>100</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) Percent of germ cells lost on the corresponding day of development out of total germ cells lost.

\(^b\) Cyst size based on vasa staining.

Immunostaining and Fluorescence Microscopy

For details of the immunostaining protocol, see Pepling and Spradling (1998). To detect both Vasa and cleaved PARP together, the ovaries were stained sequentially first with vasa antibody then the anti-rabbit Alexa 568 secondary antibody and finally with the PARP antibody because both primary antibodies were raised in rabbits.

Determination of Germ Cell Number in the Ovary

The number of germ cells in five ovaries was determined for each day of development from 13.5 to 25.5 dpc. First, the volume of each ovary was measured taking into account the geometry of the organ. Then, the number of Vasa-labeled cells in a single representative section was counted. Using the average diameter of a germ cell, the fraction of the ovarian volume represented by the counted section was then calculated. This allowed the number of germ cells in the whole ovary to be computed. At 21.5 dpc and later, the outer cortex and inner cortex were counted separately because the density and developmental age of germ cells are different in these two regions of the ovary (Peters, 1969; Byskov, 1986).

Analysis of Cyst Breakdown

For each day of development, the number of individual germ cells relative to the number of germ cells in cysts was determined by using confocal stacks from five different ovaries. The anti-Vasa antibody was used as a marker for germ cells because it specifically labels the cytoplasm of germ cells and appears continuous when cells are present as cysts. Cyst breakdown begins first in the inner cortex and then expands to the outer cortex. Our analysis focused on the outer cortex of the ovary where the great majority of oocytes reside at this time period.

Electron Microscopy

Appropriately aged ovaries were dissected and processed for transmission electron microscopy as described (Yue and Spradling, 1992). The samples were analyzed with a Phillips Tecnai 12 microscope and images recorded with a GATAN multiscan CCD camera in the Digital micrograph program or by conventional photography. The number of bridges relative to the number of oocytes was determined from sections that were assumed to pass at random within the germ cells.

We calculated the expected frequency of ring canals as follows. Since the section thickness (80 nm) was much smaller than the diameter of the ring canals (1 \(\mu m\)), it could be neglected. In that case, the probability that a section will intersect a single ring of diameter \(d\) on the surface of a spherical cell of diameter \(D\), is just \(d/D\) times the average fraction of the ring canal diameter that is orthogonal to the plane of the section, which is \(2/\pi\). For a cell of diameter 17 \(\mu m\) (see Table 1), this yields a predicted value of 3.7%.

RESULTS

Germ Cell Loss Occurs during a Two-Day Period Prior to Follicle Formation

If developmentally regulated cell death contributes to the germ cell loss observed during early mouse oogenesis, it might be confined to a specific time window. To learn more precisely when germ cells die, the total number of germ cells per ovary was determined in 13.5-25.5 dpc ovaries. Germ cells were specifically labeled with an antibody against mouse Vasa, and the total number of germ cells per ovary was calculated using confocal microscopy. At later time points, we considered the outer and inner cortex of the ovary separately because the inner cortex becomes more developmentally advanced within a few days before birth (see Materials and Methods). At 13.5 dpc, this method showed that CD-1 ovaries contain about 6,000 germ cells per ovary (Fig. 2A; Table 1), which agrees closely with a previous study of the CD1 strain that found 6,575 germ cells per ovary at this time (Pesce and de Felici, 1995). Little change in germ cell number was observed during the next week (up to 20.5 dpc). Over the next 2 days, the number of germ cells decreases sharply until only about 2,000 cells per ovary remain at 22.5 dpc (Fig. 2A; Table 1). Thus, germ cell loss occurs predominantly during a brief period suggesting that the reduction in germ cell number is a regulated developmental process.

The loss of germ cells we observed between 20.5 and 22.5 dpc could result from either random necrosis or apoptosis. Classic morphological as well as more recent molecular studies using internucleosomal cleavage of DNA suggest that germ cell death in the perinatal ovary is due to apoptosis (Borum, 1961; Coucouvanis et al., 1993; Ratts et al., 1995). To see whether a high level of apoptotic cells are present during the time germ cell numbers decline, ovaries

TABLE 3
Frequency of Intercellular Bridges

<table>
<thead>
<tr>
<th>Age</th>
<th>Bridges/Oocytes</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.5</td>
<td>2/138</td>
<td>1.4</td>
</tr>
<tr>
<td>17.5</td>
<td>1/137</td>
<td>2.9</td>
</tr>
<tr>
<td>18.5</td>
<td>1/137</td>
<td>2.7</td>
</tr>
<tr>
<td>19.5</td>
<td>6/504</td>
<td>1.2</td>
</tr>
<tr>
<td>20.5</td>
<td>2/501</td>
<td>0.4</td>
</tr>
<tr>
<td>21.5</td>
<td>1/500</td>
<td>0.2</td>
</tr>
</tbody>
</table>
FIG. 4. Distribution of apoptotic cells within germline cysts. Germ cells were identified by staining for Vasa (yellow); dying cells are indicated by staining for cleaved-PARP (green). (A, B) Examples of large cysts (eight or more germ cells) containing one dying germ cell. (C) Bar graph summarizing the number of dying cells in large cysts; n = 51. (D, E) Examples of small cysts (two to four germ cells) with one or two dying cells. (F) Bar graph summarizing the number of dying cells in small cysts; n = 34. Note that some of the dying cells appear to be smaller than the other germ cells (A, D arrowheads). (G) Model of the breakdown of an eight-cell cyst. (Upper Left) Note that gaps represent ring canals. Dying cells (green) cause breakage into smaller cysts, yielding three oocytes (Right) that will form primordial follicles. Scale bar: 5 μm (A, B, D, E).
FIG. 5. Reorganization of mitochondria and ER occurs at 19.5 dpc, just before cyst breakdown. (A) Electron micrograph of a 17.5 dpc oocyte with few mitochondria randomly distributed in the cytoplasm. (B) Electron micrograph of a 19.5 dpc oocyte with an increased number of mitochondria associated in clusters with endoplasmic reticulum (arrowheads). (C, D) Confocal light microscopy of 18.5 dpc oocytes (C) and 19.5 dpc oocytes (D) stained for cytochrome oxidase I, a mitochondrial marker, showing mitochondrial clustering at 19.5 dpc (arrowheads in D). High-magnification electron micrograph of ER–mitochondria associations at 18.5 dpc (E) and 19.5 dpc (F). (G) Electron micrograph showing a mitochondrion and ER-like membranes within a ring canal. Scale bars: 2 μm (A, B); 2.5 μm (C, D); 1 μm (E–G).
were removed from mice 14.5–23.5 dpc, tested for apoptosis using TUNEL-labeling (see Materials and Methods), and the percentage of apoptotic cells plotted (Fig. 2B). To obtain a high level of developmental synchrony, we only studied cells within the outer cortex, where the great majority of germ cells reside. A strong peak in the number of apoptotic cells was observed during 20.5–21.5 dpc (Figs. 2B–2D), the same time that germ cell number declines. Surprisingly, however, none of the TUNEL-positive cells stained with the germ cell marker, Vasa, at any of the times studied. We suspected that Vasa reactivity may be lost in apoptotic germ cells prior to the time they became positive in the TUNEL assay.

To verify that the TUNEL-labeled cells are primarily germ cells, we identified another apoptosis marker, cleaved poly(ADP ribose) polymerase (PARP), that clearly does label VASA-positive cells (see Figs. 2E and 2F). Figure 2B shows that the frequency of cells positive for both PARP and VASA parallels the distribution of TUNEL-positive cells. Moreover, an examination by electron microscopy of ovarian tissue during each day of development between 13.5 and 23.5 dpc revealed that germ cell apoptosis takes place and peaks during 20.5–21.5 dpc, confirming the observations using molecular markers (see Fig. 2G). No other morphological abnormalities were observed, suggesting that the cell death is not due to chromosomal or cell cycle-related abnormalities. The germ cell apoptosis rate is low (~20%) relative to the total percent of germ cell loss (66%). This is most likely because cell death markers are only expressed for a limited period of time and our measurements were separated by 24 h. We conclude that germ cell apoptosis peaks between 20.5 and 22.5 dpc, corresponding to the period when total germ cell number declines sharply.

Germ Cell Loss Is Correlated with Cyst Breakdown

To determine whether the reduction in germ cell number that occurs between 20.5 and 22.5 dpc is related to germ cell cysts, we studied when these cysts break down. Anti-Vasa antibody was critical for these studies because staining with this antibody was found to reveal whether adjacent cells were interconnected. Labeling is continuous when cells are from the same cyst (see Fig. 1B). In contrast, Vasa labeling was interrupted between cells from different cysts, probably because at least a thin layer of somatic cell cytoplasm lies between them. This same effect has been observed in the case of Drosophila ovarian cysts labeled with Vasa (for example, see Xie and Spradling, 1998).

Ovaries from mice 13.5–25.5 dpc were isolated, stained by using stacks of confocal sections (see Materials and Methods). Between 13.5 and 19.5 dpc, germ cells are found in cysts of eight or more cells; single oocytes are rarely observed (Fig. 3A). At 19.5 dpc, large cysts remained but their component cells appear less closely packed (Fig. 3B). However, by 22.5 dpc, most germ cells are single, and begin to form primordial follicles (Fig. 3C). When we plotted the percentage of single oocytes/primordial follicles as a function of time on successive days of development (Fig. 2A), it was clear that cyst breakdown and primordial follicle formation correlated closely with the time of germ cell loss.

To analyze nature of cyst breakdown in more detail, we counted cyst sizes during the critical period between 20.5 and 22.5 dpc (Table 2). At 20.5 dpc, 62% of the cysts are large (8 cells or more) and only 2% appear single. A day later, at 21.5 dpc, only 1% of the cysts remain large. Most are small (2–4 cells), while 40% now contain only one cell. By 22.5 dpc, breakdown has progressed further: 65% of cysts are single, and no large cysts remain. The breakdown of the remaining small cysts continues more slowly. By 26.5 dpc, more than 90% of the germ cells are single. The rapid reduction of cluster size suggests that the cysts first break up into smaller clusters before becoming individual oocytes.

The decline in cluster size as measured by Vasa staining predicts that the frequency of intercellular bridges should decrease to zero over this same time period. To test this, we examined how often intercellular bridges were observed in electron micrographs of oocytes between 14.5 and 21.5 dpc (Table 3). Before cyst breakdown (14.5–18.5 dpc) the frequency of bridges averaged 2.6%. This is lower than the expected value of 7.4%, based on the assumption that mouse germ cells contain an average of two ring canals (see Materials and Methods) and may be due to either the difficulty of detecting ring canals that are not sectioned near their centers or the modification or closure of ring canals. Starting at 19.5 dpc, the frequency of bridges declined drastically and they were virtually undetectable by 21.5 dpc. This result was not simply due to an increase in germ cell diameter (Table 1). This confirms that ovarian germ line cysts break down during 20.5–22.5 dpc, the same interval when germ cell numbers are reduced.

A Subset of Oocytes within Individual Cysts Die

The manner in which germ cell cysts break down is important, ultimately, for understanding their function. For example, if some cells serve as nurse cells, each cyst should contain cells that live and others that die. In contrast, if the purpose of germ cell apoptosis is to eliminate cells with chromosome abnormalities or damaged mitochondrial genomes, then the clonally related cyst cells should usually live or die as a group. To determine the fate of oocytes from individual cysts and distinguish between these two possibilities, ovaries aged 18.5–23.5 dpc were double-stained with antibodies that recognize the germ cell marker Vasa and the apoptosis marker cleaved PARP. Using confocal microscopy, the number of PARP-positive cells within individual cysts was determined (Fig. 4).

It was immediately clear that, regardless of cyst size, only a small fraction of the cells in a cyst was ever positive for PARP. To quantitate the results, we divided the cysts into two groups (large and small) based on their size. In cysts that contained eight or more cells (large cysts), most (66%) had only one dying cell (Figs. 4A–4C). Similarly, in cysts

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with 2–4 cells (small cysts), only one cell was usually (97%) undergoing apoptosis (Figs. 4D–4F). In no case were all the cells of a cyst labeled with PARP. Moreover, dying germ cells that were unconnected to other germ cells accounted for only about 8% of all dying cells during this interval, indicating that most apoptosis occurs while germ cells remain interconnected. At this time, some cyst cells were distinctly smaller than their neighbors, and these often labeled with the PARP antibody (Figs. 4A and 4D, arrows). These results strongly suggest that only a fraction of the cells within a cyst undergo programmed cell death, while others survive to form primordial follicles. Most pathways leading to the sequential death of all cyst cells would generate many more single dying cells than were observed. However, we cannot completely rule out the possibility that all the cells within cyst eventually die, because it was impossible to follow the fate of all the initial cells within a cyst after they become separated into smaller groups. A model of cyst breakdown that is consistent with our results is shown (Fig. 4G).

Mitochondria and Endoplasmic Reticulum (ER)
Reorganize before Cyst Breakdown

If cysts serve a specific developmental function, the structure of their component cells might undergo regulated changes at specific stages prior to cyst breakdown. For example, certain materials might be transferred through the ring canals from cells that are destined to die to those that will survive and form follicles. Early studies of mouse germ cell development describe changes in the organization of mitochondria shortly after birth (Odor and Blandau, 1969; Ruby et al., 1969b; Nogawa et al., 1988). To relate such changes to the process of cyst breakdown, we examined germ cells during this time using electron microscopy. These experiments confirmed that mitochondria undergo significant changes, and revealed that these modifications occur just prior to the time cysts break down (Fig. 5). Until 18.5 dpc, most germ cells contain relatively few mitochondria that appear randomly dispersed within the cytoplasm (Fig. 5A). At this time, a typical cell section contained 26 mitochondria of which 5 were touching another mitochondrion (21%). At 19.5 dpc, mitochondria increase in number and reorganize in a few small aggregates that often curve around portions of the nucleus (Fig. 5B). A typical section contained 77 mitochondria of which 44 were touching another mitochondrion (58%). This change in mitochondrial organization was confirmed by staining mitochondria using anti-cytochrome oxidase I antibody and examining germ cells in the confocal microscope (Figs. 5C and 5D). These experiments also showed that the change in mitochondrial organization begins in a small number of cysts on 18.5 dpc and is essentially complete by 20.5 dpc (data not shown), indicating that the process is very rapid.

This dramatic change in mitochondrial organization was accompanied by coordinated alterations of cytoplasmic membranes that resemble endoplasmic reticulum. Germ cells prior to 18.5 dpc display relatively few ER-like membranes. However, beginning at 18.5 dpc, small pieces of ER-like membranes begin to associate with mitochondria (Fig. 5E). As the mitochondria aggregate, the membrane plates appear to fuse and the mitochondria within the aggregates become separated by sheets of ER-like membrane (Fig. 5F). During this time, mitochondria and ER-like membranes were observed within the intercellular bridges by EM (Fig. 5G), raising the possibility that active intercellular transport of mitochondria and membranes is involved in effecting these changes in cellular architecture.

Somatic Cells Associate Closely with Cysts Prior to and during Breakdown

Classical studies revealed that germ cells interact closely with somatic cells beginning at 14.5 dpc (Odor and Blandau, 1969; Byskov, 1978). Somatic cells clearly intrude between closely associated germ cells as part of the process leading to primordial follicle formation. These observations motivated us to reexamine the role of somatic cells in the processes of cyst development and breakdown. In the electron microscope, we observed that somatic cell processes almost always reside between adjacent cells at the position of the ring canals, whereas, in other places, germ cells directly contact their sisters. This architecture arises very early, as cysts develop, and is clearly visible by 13.5 dpc (Fig. 6A). The somatic cellular processes remain throughout cyst lifetime, although by 18.5 dpc they have become very thin (Figs. 6B and 6C). The intimate association of somatic processes with the ring canals and the movement of somatic cells between germ cells before cyst breakdown strongly suggest a role for somatic cells throughout the processes that break down cysts and build primordial follicles.

DISCUSSION

Cyst Breakdown Is Developmentally Programmed

These studies add to a growing body of both classical and recent evidence that the formation and breakdown of germ-line cysts represent important processes in female mouse germ line development. Although previous work documented the formation of germ line cysts based on the presence of intercellular bridges and cell synchrony (Ruby et al., 1969a; Spiegelman and Bennett, 1973; Pepling and Spradling, 1998), little was known about their fate. We find that cyst breakdown occurs with a high degree of synchrony primarily between 20.5 and 22.5 dpc, just prior to the onset of primordial follicle formation. Thus, cyst breakdown appears to be a developmentally programmed step in the process of oocyte formation. Other mammals also show evidence of germ cell cysts at the corresponding time in development (Franchi and Mandl, 1962; Gondos and Zamboni, 1969; Zamboni and Gondos, 1968), suggesting that cysts represent an evolutionarily conserved stage in both invertebrates and vertebrates (Gondos, 1973; Spradling, 1993; Robinson and Cooley, 1996; Pepling et al., 1999).

Reports of germ cell number in fetal and neonatal mouse
female gonads have varied from 3,500 to 30,000 but these experiments utilized different strains, different developmental times, and different methods (Abe et al., 1996; Jones and Krohn, 1961; Mintz and Russell, 1957; Pesce and deFelici, 1995; Tam and Snow, 1981; Perez et al., 1999). Further studies will be required to confirm the reality of such a large variation in the number of germ cells in different strains. This is the only study in mice in which germ cells from a single strain were followed in detail over the entire interval from meiotic entry until after birth.

Programmed Cyst Breakdown Is Associated with Germ Cell Apoptosis

Previous studies have noted the occurrence of apoptotic germ cells during a wide range of developmental periods both before and after follicle formation (reviewed in Morita and Tilly, 1999). Up to four waves of degeneration prior to follicle formation have been reported depending on the species and the methods used (Baker, 1963; Beaumont and Mandl, 1962; Borum, 1961). Germ cell death at these times may be due to the occasional turnover of entire cysts, the loss of aberrant meiotic cells, or the precocious programmed death of cells within the inner cortex that are more developmentally advanced. However, in these studies, the highest frequency of apoptotic cells was observed at about the time of birth (Baker, 1963; Beaumont and Mandl, 1962; Borum, 1961) as reported here. We also observed a detectable level of germ cell apoptosis in the outer cortex prior to the time of cyst breakdown. However, the frequency of labeled cells was relatively low and their appearance did not correlate with a significant reduction in germ cell numbers. Almost all of the losses in germ cell number took place during the interval of cyst breakdown.

Our results strongly suggest that only a fraction of the cells in each cyst undergoes programmed cell death. More than 90% of the dying germ cells are still associated with their sister cyst cells. This finding makes it unlikely that the role of perinatal germ cell death is to eliminate genetically defective oocytes such as those with chromosome abnormalities. If this were the case, then all or none of the cells within a cyst would tend to die because all arise from a common cyst progenitor cell and should be genetically identical. Rather it suggests that differences exist between sister cyst cells, and that they may carry out different functions, like the nurse cells and oocytes of invertebrate cysts.

Although germline cysts form and break down by apoptosis in both vertebrates and invertebrates, the timing of breakdown occurs at different times in oogenesis in different organisms. In mammals, and several lower invertebrates, cysts are present for only a relatively short time early in oocyte development. They break down, as in the mouse, at the time of follicle formation. In contrast, in higher invertebrates such as Drosophila, cysts persist after follicles have formed. Most of the cyst cells differentiate as nurse cells and contribute cytoplasmic components including ribosomes to the future oocyte. The finding that cysts are evolutionarily conserved during early stages in oogenesis, but not in later stages, suggests that there is a separate function for these structures prior to follicle formation. It may have been possible to evolve the role of cysts in rapid ribosome production because this earlier function was already present.

Studies of mice defective in apoptosis may allow the function of developmentally programmed cyst breakdown to be addressed. Young adult mice lacking bcl-2 function, an apoptosis-repressor, contain a reduced number of primordial follicles (Ratts et al., 1995). Loss of the pro-
apoptotic enzyme, caspase 2 leads to an excess of oocytes in the adult (Bergeron et al., 1998). If oocytes that would have normally been programmed to die at the time cysts break down can be shown to survive in this genotype, it might be possible to study whether they are capable of developing and functioning normally as gametes.

**Mechanism of Breakdown**

Our experiments show that mouse cysts break down in a manner that is distinct in several respects from the previously studied breakdown of Drosophila cysts (Foley and Cooley, 1999; Matova et al., 1999). In Drosophila, breakdown occurs synchronously, and is essentially complete within a 30-min period. In contrast, the breakdown of mouse cysts takes place in a stepwise fashion over 1–2 days. Moreover, the final breakdown process extends over several additional days of development in the neonatal mouse. We observed that 10% of the cells still remained within small cysts of two to four cells each at 26.5 dpc. Cysts that resist breakdown at the normal time are likely to explain why 10% of primordial follicles in mice up to 10 weeks of age have been observed to contain two (or rarely more) oocytes (Kent, 1960). Some of these differences may be due to differences in the nature of the interconnections between the cells. All the cells in a Drosophila cyst are joined by similar ring canals that differ only in size; however, the detailed organization of mouse cyst cell interconnections has not yet been elucidated.

Despite these differences, the molecular mechanisms controlling Drosophila cyst breakdown are likely to be relevant to understanding mouse cyst breakdown. Drosophila cysts also break down by apoptosis (Foley and Cooley, 1998) and require a specific caspase (McCaffrey and Steller, 1998). In Drosophila, groups of somatic follicle cells undergo specific movements that are tightly coordinated with cyst breakdown (see Cooley and Theurkauf, 1994). We found that mouse somatic cells are associated with ring canals and appear to play an active role in cyst breakdown. Recent evidence strongly suggests that Drosophila cysts break down in response to local production of the steroid hormone ecdysone (Buszczak et al., 1999; Carney and Bender, 2000). Mouse germ cells within cysts do not progress synchronously through the early stages of meiosis (Peters and McNatty, 1980); perhaps a hormonal signal is responsible for the highly synchronous onset of cyst breakdown that we observed in the outer cortex.

**Germ Line Cysts May Contribute to Balbiani Body Formation**

Developing oocytes in many species transiently display a large aggregate of mitochondria and membranes known as the Balbiani body (reviewed in de Smedt et al., 2000). This large structure is one of the earliest indications of oocyte asymmetry and in Xenopus is associated with germinal granules (Heasman et al., 1984). The mitochondrial aggregates we observed beginning in 19.5 dpc mouse germ cells appeared similar to the mitochondrial “clouds” of Xenopus. The mouse mitochondrial aggregates arise while cells are still interconnected and are retained within follicular oocytes. In the frog oocyte, one cloud eventually grows larger and gives rise to the Balbiani body. Whether any of the mitochondrial aggregates we observed expand during follicular development to produce a full-fledged Balbiani body has not been examined. However, our study provides the first indication that mitochondrial clouds may be linked to cyst function. Developmental studies suggest that mitochondrial clouds in Xenopus also arise initially within 16-cell nests that likely correspond to germline cysts (Al-Mukhtar and Webb, 1971).

The presence of aggregates of mitochondria and membranes in young Xenopus and mouse oocytes suggests a relationship to the Drosophila fusome (for review, see de Cuevas et al., 1997). The fusome contains ER-like membranes, is associated with mitochondria along its length, and requires membrane skeleton proteins such as α-spectrin for its structural integrity. At the time of follicle formation, an aggregate of mitochondria has been observed in association with the remnants of the fusome (Mahowald and Strassheim, 1970). Moreover, in Xenopus, the Balbiani body contains α-spectrin (Kloc et al., 1998). It should be possible to address whether there are functional as well as structural similarities between the fusome and the membrane/mitochondrial aggregates in the mouse by analyzing gene knockouts that disrupt its molecular components.

**The Function of Germline Cysts**

Despite their widespread conservation, the only established function of ovarian germ line cysts is to accelerate oocyte development late in oogenesis in those species where some cyst cells differentiate as nurse cells. A similar specialization of mouse oocyte cells into oocytes and accessory cells may take place early in oogenesis as well. Early nurse cells might produce hormones or signals that act primarily to control the development of their sister oocytes. Alternatively, the two-thirds of the cells that die at the time of cyst breakdown might transfer cellular components into those that survive prior to dying. Our measurements of germ cell diameters (Table 1) suggest that the simplest version of this idea is unlikely to hold. The total volume of germ cells increases 70% from 2.62 × 10³ μm³ at 19.5 dpc to 4.45 × 10³ μm³ at 23.5 dpc. This is not a particularly high rate of volume increase given that a loss of two-thirds of the cells should provide the potential for a rapid increase of 200%. The increase over the previous 4 days is 45% (from 1.80 × 10³ μm³), which is nearly as large. Thus, if anything is transferred between living and dying oocytes, it is likely to be specific cellular components, such as nutrients, mRNAs, or organelles rather than bulk cytoplasm.

Another indication of directional cytoplasmic transfer between cyst cells would be a decrease in cell volume within the donor cell. We observed that some of the cells within 19.5 dpc mouse cysts were smaller than the others.
and smaller than any germ cells earlier in development. However, cells frequently shrink in size following the onset of apoptosis, and it has not yet been possible to distinguish whether the size reductions observed were a harbinger or the result of apoptosis. A similar problem complicates the analysis of differences in the number of mitochondria or other cytoplasmic organelles between individual cysts just prior to the onset of cell death. It may be necessary to observe live cysts using markers for various cellular components in order to directly determine whether materials are passing from one cell to another.

**Role of Cysts in Mitochondrial Inheritance**

In many respects, mitochondria are the most attractive candidate for a cellular component that is transferred intercellularly prior to cyst breakdown. The genetic “bottleneck” for mitochondrial DNA inheritance has been mapped to this period of germ cell development (Jenuth et al., 1996), suggesting that a relatively few mitochondrial genomes give rise to most of the mitochondria of the finished egg. These authors proposed that random segregation of about 200 “segregating units” of mitochondrial DNA would be sufficient to explain the bottleneck they measured. We documented significant changes in the number and subcellular location of mitochondria prior to cyst breakdown. The knowledge that these events are taking place within cysts of interconnected cells raises the possibility that an active process of mitochondrial selection, rather than stochastic inheritance, might take place at this time.

Cyst architecture might greatly increase the efficiency of a mitochondrial genome selection system. Mitochondria with functional and defective genomes would be actively transported into different germ cells. A polarized microtubule network within the cyst, which is known to arise in Drosophila, would facilitate such movement and sorting. The quality of each cell’s mitochondria might then determine whether it survived or entered apoptosis. Indeed, an evolutionarily ancient mechanism in which mitochondria self-selected by programmed suicide during oogenesis might account for the close connection between mitochondria and apoptosis. The selected mitochondria might begin to rapidly proliferate, giving rise to clouds and eventually to the transient appearance of Balbiani bodies. The studies reported here provide a biological basis for testing these ideas in future studies.

**REFERENCES**


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