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Developmental Biology 281 (2005) 91–101
DEVELOPMENTAL BIOLOGY
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Oogenesis in *Hydra*: Nurse cells transfer cytoplasm directly to the growing oocyte

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Received for publication 22 November 2004, revised 11 February 2005, accepted 15 February 2005
Available online 18 March 2005

Abstract

Oogenesis in *Hydra* occurs in so-called egg patches containing several thousand germ cells. Only one oocyte is formed per egg patch; the remaining germ cells differentiate as nurse cells (Miller et al., Dev. Biol. 224 326–338 (2004)). Whether and how nurse cells contribute cytoplasm to the developing oocyte has been unclear. We have used tissue maceration to characterize the differentiation of oocytes and nurse cells in developing egg patches. We show that nurse cells decrease in size at the same time that developing oocytes increase dramatically in volume. Nurse cells are also tightly attached to oocytes at this stage and confocal images of egg patches stained with the fluorescent membrane dye FM 4–64 clearly show large gaps (10 μm) in the cell membranes separating nurse cells from the developing oocyte. We conclude that nurse cells directly transfer cytoplasm to the developing oocyte. Following this transfer of cytoplasm, nurse cells undergo apoptosis and are phagocytosed by the oocyte. These results demonstrate that basic mechanisms of alimentary oogenesis typical of *Caenorhabditis* and *Drosophila* are already present in the early metazoan *Hydra*.
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Keywords: Oogenesis; *Hydra*; Nurse cells; Cytoplasmic transfer; Apoptosis

Introduction

In metazoan animals, oocytes are derived from germline precursors, which grow to be very large cells. In some cases, sibling germline cells, so-called nurse cells, contribute to this process; alternatively, somatic cells can fulfill this function. For example, in *Drosophila*, individual germ cells divide to form a cluster of precursors one of which will become the oocyte while the others differentiate to nurse cells (de Cuevas et al., 1997). The nurse cells synthesize large amounts of cytoplasm and subsequently transfer the bulk of this cytoplasm through specialized ring canals to the developing oocyte thus contributing to oocyte growth. Following cytoplasm transfer, nurse cells undergo apoptosis and are phagocytosed by follicle cells (Mahajan-Miklos and Cooley, 1994; Nezis et al., 2000).

In the gonad of the nematode *C. elegans*, germline nuclei that fulfill the function of nurse cells develop together with future oocytes within a common syncytium. These nuclei proliferate within a syncytium and contribute to the synthesis of a common pool of cytoplasm. After entering pachytene, about half of these nuclei cellularize, undergo apoptosis and are phagocytosed by sheath cells surrounding the gonad (Gumienny et al., 1999). The cytoplasm, which they contributed to the syncytium, is incorporated into developing oocytes and hence these germline nuclei function as “nurse cells”.

Oogenesis in *Hydra* exhibits some similarities to oogenesis in *Drosophila* and *Caenorhabditis*. A single germline cell within a pool of several thousand sibling germ cells develops into an oocyte while the remaining cells differentiate to nurse cells, undergo apoptosis and are phagocytosed by the oocyte (Honegger et al., 1989; Littlefield, 1991; Miller et al., 2000; Zihler, 1972). The apoptotic program is then arrested (Technau et al., 2003). During cleavage, the apoptotic bodies are distributed to cells of the

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doi:10.1016/j.ydbio.2005.02.015

developing embryo where they remain in the cytoplasm often for several months until hatching. Thereafter, they are degraded and presumably provide nourishment for the hatchling.

Despite the apparent similarity to *Drosophila* and *Caenorhabditis*, the mechanism by which oocytes in *Hydra* grow in size and the contribution of nurse cells to this process are not well understood. By analogy with *Drosophila* and *Caenorhabditis*, it seems likely that nurse cells in *Hydra* contribute to the growth of the oocyte. This could occur by direct transfer of cytoplasm, although no evidence of such transfer has so far been available. The volume of the oocyte is, however, significantly increased by phagocytosis of apoptotic nurse cells at the end of oogenesis. Hence, most authors (Honegger et al., 1989; Miller et al., 2000; Zihler, 1972) have suggested that oocyte growth occurs by phagocytosis of apoptotic nurse cells. A problem with this interpretation is that apoptotic bodies are not degraded until hatching (Gruzova, 1956; Martin et al., 1997), i.e. long after embryogenesis has occurred, and thus their contents cannot contribute to embryogenesis. Moreover, the contents of phagocytosed apoptotic bodies are presumably degraded before being absorbed by embryo cytoplasm. If nurse cells in *Hydra* are to contribute cytoplasm and cytoplasmic organelles such as mitochondria and ribosomes to the developing oocyte, as is the case in *Drosophila* and *Caenorhabditis*, then transfer of cytoplasm must occur before phagocytosis of apoptotic nurse cells.

In view of these questions, we have reinvestigated oogenesis in *Hydra* with the specific goal of understanding the mechanism of oocyte growth. The presence of large numbers of the germ cells in the egg patch (ca. 4000) makes the electron microscopic analysis of oogenesis extremely complicated. We therefore macerated (David, 1973) single egg patches in order to identify and quantitate the cell types at sequential stages of oogenesis. Maceration has also allowed measurements of nuclear DNA content and DNA replication of the different cell types. We have combined the maceration experiments with confocal microscopy of fixed animals in order to analyze oogenesis in situ.

Based on our observations, we propose a scheme for nurse cell and oocyte differentiation in *Hydra* in relation to stages of oogenesis. Our results provide strong evidence that oocyte growth occurs by transfer of cytoplasm from nurse cells to the growing oocyte prior to nurse cell phagocytosis. Confocal images of animals stained with the fluorescent membrane dye FM 4–64 clearly show large gaps (10 μm) in the cell membranes separating nurse cells from the developing oocyte. The increase in oocyte volume is accompanied by a simultaneous decrease in the size of nurse cells. While this process occurs, nurse cells are tightly bound to the developing oocyte. Following this transfer of cytoplasm, nurse cells undergo apoptosis and are phagocytosed by the developing oocyte. Finally, our results indicate that multiple oocytes develop in egg patches and then fuse to form one large oocyte at the end of oogenesis. These observations support the idea that basic mechanisms of alimentary oogenesis typical of *Caenorhabditis* and *Drosophila* were already present in the early metazoan *Hydra*.

Materials and methods

Hydra culture and induction of oogenesis

All analyses were carried out using the AEP female strain of *Hydra* (Martin et al., 1997). Animals were maintained at 18°C in *Hydra* medium containing 0.1 mM KCl, 1 mM NaCl, 0.1 mM $MgSO_4$, 1 mM Tris and 1 mM $CaCl_2$. Stock cultures of AEP were fed with Artemia nauplii two times a week, which kept them in a nonsexual state. To induce gametogenesis, animals were starved for 1 week and thereafter fed again twice a week.

Maceration of isolated egg patches

A method was developed for maceration of individual egg patches excised from tissue. Animals were relaxed in 2% urethane for 2 min and the egg patch region was cut out with the help of a scalpel. The isolated tissue was transferred into a drop of maceration solution (David, 1973), incubated for 5 min and then carefully shaken until the cells had separated to the desired extent. The cells were postfixed by adding a drop of 8% formaldehyde and the cell suspension was then spread on the surface of a gelatinized slide and dried for several hours.

TUNEL assay

TUNEL assays were carried out with the in situ cell death detection kit (Hoffmann La Roche) using fluorescein labeled UTP. Animals were relaxed in 2% urethane for 3 min, and the egg patch region was excised and macerated (see above). The dried slides were washed 2 times in PBS. Permeabilization was carried out on ice (4°C) with 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 5 min. The preparations were again washed 2 times in PBS at room temperature. The area around the sample was dried and 50 μl TUNEL reaction mixture was added. The slides were incubated in a humid chamber for 1 h at 37°C, washed 3 times with PBS and counterstained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and in 1 μM TO-PRO-3 (Molecular Probes) in PBS. Preparations were mounted in Vectashield antifade mounting medium (Vector Laboratories) and sealed with nail polish.

DNA measurements

Nuclear DNA content was determined microfluorometrically using the DNA-specific fluorochrome DAPI. Macerates of isolated egg patches were prepared (see above) and stained with DAPI. Fluorescence measurements were made

on single cells using a Leitz Dialux 20 fluorescence microscope equipped with a photometer (Leitz MPV compact). Nerve cells were used as a standard for diploid (2n) DNA values (David and Gierer, 1974).

Labeling index

To detect cells in the S-phase, 0.5 μl solution of 5 mM 5-bromo-2-deoxyuridine (BrdU; Roche) was injected into the gastric cavity of female polyps at various stages of oogenesis with the help of micro-hematocrit tubes. After 45 min, egg patches were isolated and macerated (see above). Macerated preparations were dried on SuperFrost-Plus slides (Menzel-Gläser, Germany) over night, incubated for 15 min in methanol at room temperature, washed 3 times for 10 min in PBS and permeabilized in 0.5% Triton X-100 in PBS. After a short rinse in PBS, they were incubated in 2 N HCl for 1 h at 37°C. They were then washed 3 times for 10 min in PBS, blocked for 15 min in 1% BSA/0.1% Triton in PBS and incubated over night with anti-BrdU antibody (Roche, 1:10) at 4°C in a humid chamber. The slides were then washed 3 times in PBS for 15 min, incubated with the secondary antibody (Alexa-488 conjugated goat anti-mouse, Molecular Probes, 1:400) for 1 h at 37°C, washed again 3 times for 10 min in PBS and covered with Vectashield mounting medium.

Membrane staining with FM 4–64

Membrane staining was performed on living animals with the membrane specific dye FM 4–64 (excitation 515 nm, emission 640 nm). Nuclei were counterstained with SYTO 16 (excitation 488 nm, emission 518 nm). Both dyes were obtained from Molecular Probes Inc. (Eugene, OR). 1 μl of 500 μM FM 4–64/10 μM SYTO 16 in *Hydra* medium was injected into the gastric cavity of female polyps with the help of micro-hematocrit tubes. The animals were then incubated in 50 μM FM 4–64/10 μM SYTO 16 in *Hydra* medium for 10 to 20 min.

Confocal microscopy

For confocal microscopy, whole-mount *Hydra* were fixed in 2% paraformaldehyde in PBS 1 h at room temperature and stained with 0.5 μg/ml DAPI and 1 μM TO-PRO-3 in PBS.

Living animals were immobilized in a drop of *Hydra* medium containing 4% gelatin and 1% urethane. Confocal optical sections were acquired with a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems, Mannheim). Fluorochromes were visualized with an argon laser (excitation wavelength of 488 nm) and a helium-neon laser (excitation wavelength 633 nm), emission filters 510–540 nm (for SYTO 16) and 640–760 nm (for TO-PRO-3 and FM 4–64). Image resolution was 512 × 512 pixel with a pixel size ranging from 195 to 49 nm depending on the selected zoom factor. The axial distance between optical sections was 280 nm for zoom factor 4 and 1 μm for zoom factor 1. To obtain an improved signal-to-noise ratio, each section was averaged from 4 successive scans. The 8 bit grayscale images were overlayed to an RGB image assigning a false color to each channel and then assembled into tables using Adobe Photoshop 5.5. and ImageJ 1.32j software.

Results

Oogenesis in *Hydra* occurs in discrete patches of germ cells, the so-called egg patch, embedded in the ectoderm of female animals. Each egg patch gives rise to a single oocyte. The time scale and photographs of the different stages of oogenesis are shown in Fig. 1. The stage classification is

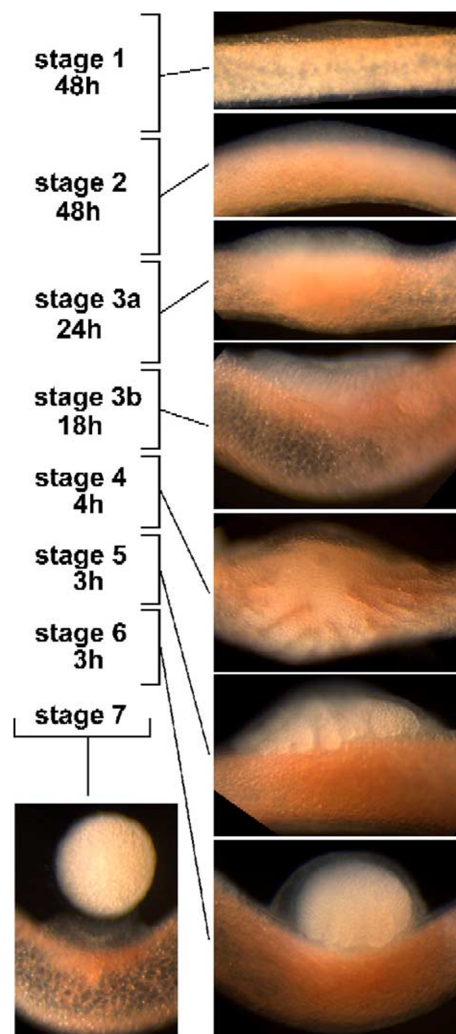

Fig. 1. Stages of oogenesis in *Hydra vulgaris*. The micrographs show morphologically distinct stages and the time scale of oocyte development. Oocyte development begins in a so-called egg patch in the ectoderm of the body column which progressively thickens over about 6 days and then rapidly contracts to form the oocyte. The egg patch contains roughly 4000 germ cells which give rise to one oocyte.

that of Miller et al. (2000). During egg patch development, several different morphological classes of germ cells appear in sequence. Up to Stage 3, it is impossible to predict which of the cells in the egg patch will finally develop into the oocyte and which cells will function as nurse cells. For this reason, we refer to all cells in the egg patch at Stages 1–3 as germ cells. We name the different cell types GCI, GCII, GCIII and GCIV according to their morphology. After a germ cell enters the diplotene phase of meiosis, we refer to it as a developing oocyte.

Cell composition of the egg patch at different stages of oogenesis

Stage 1 (48 h), the first morphologically detectable indication of oogenesis, is characterized by a transparent swelling of the ectodermal cell layer (Fig. 1). At this stage, three morphological types of germ cells are present in the egg patch (Figs. 2, 3A). GCI cells occur in clusters of 4 and 8 cells which arise by synchronous division of precursors (Littlefield, 1991). The cells in a cluster are held together by cytoplasmic bridges which result from incomplete cytokinesis (Fawcett et al., 1959). GCI cells are 15 μm in diameter and have undifferentiated cytoplasm. GCII cells are larger (20–25 μm) than GCI and contain a single dark granule about 2 μm in diameter in the otherwise homogeneous cytoplasm. The nucleus (10 μm) is larger than GCI nuclei and as a rule contains two nucleoli. GCIII cells are even larger than GCII (40 μm). The cytoplasm is heavily vacuolated and contains numerous granules that have been shown to contain lipids and glycogen (Aizenshtadt, 1975; Honegger et al., 1989).

The morphology of the three germ cell types suggests that they represent progressive stages of differentiation. Experiments described below indicate that GCI are post-mitotic cells, which have initiated oocyte/nurse cell differentiation. They are in G1 phase of the cell cycle. GCI cells turn into GCII cells, which enter a premeiotic S-phase. GCII cells complete DNA replication and turn into GCIII cells, which have 4n DNA content. GCIII cells increase dramatically in size coincident with the synthesis of numerous cytoplasmic vacuoles and granules.

Stage 2 (48 h). At this stage, the egg patch is thicker and no longer transparent and has a milky appearance due to an increased number of highly vacuolated GCIII cells (Figs. 1, 3A). Some clusters of GCIII contain one or two obviously larger cells (100 μm). These are GCIV cells (Fig. 2). The cytoplasm of these cells is similar to the cytoplasm of GCIII but the nucleus is enlarged (20–30 μm) and contains condensed chromosomes typical of early meiotic prophase I. Confocal optical sections of whole mount *Hydra* show that the GCIV cells are mostly located near the mesoglea (Fig. 4A).

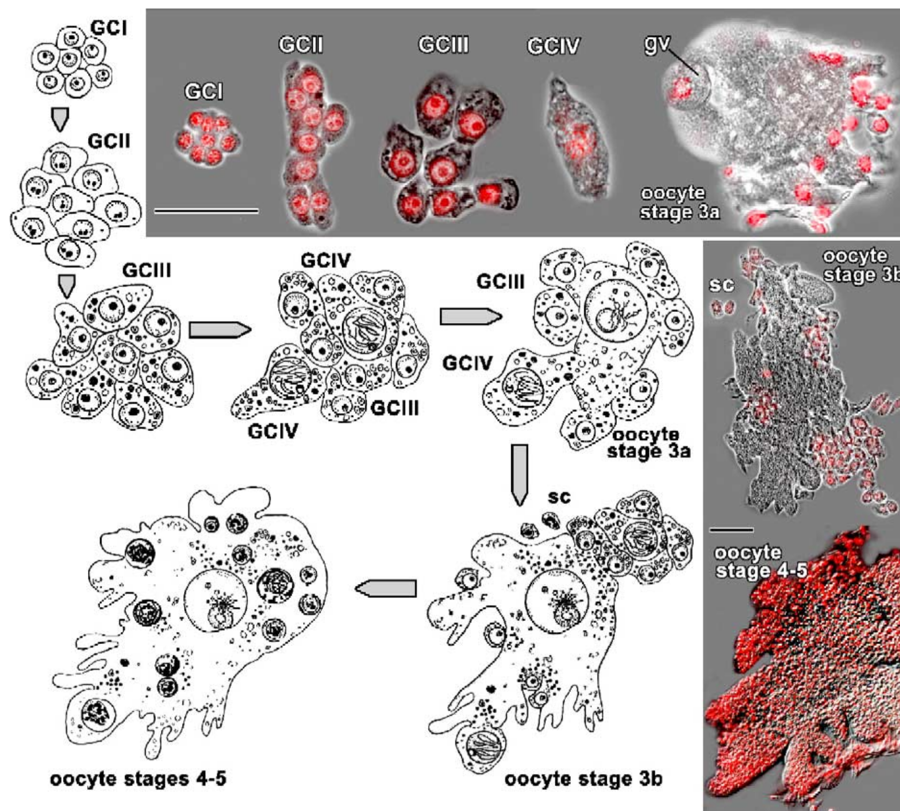

Fig. 2. Germ cell differentiation. The micrographs show morphologically distinct cell types in egg patches which were macerated at different stages of oogenesis. Nuclei are stained with DAPI (shown in red color in the overlay). The schematic drawings document the different stages of germ cell differentiation from GCI to GCII, GCIII, GCIV cells and oocyte (see text). gv—germinal vesicle, sc—small germ cells. Scale bar: 100 μm.

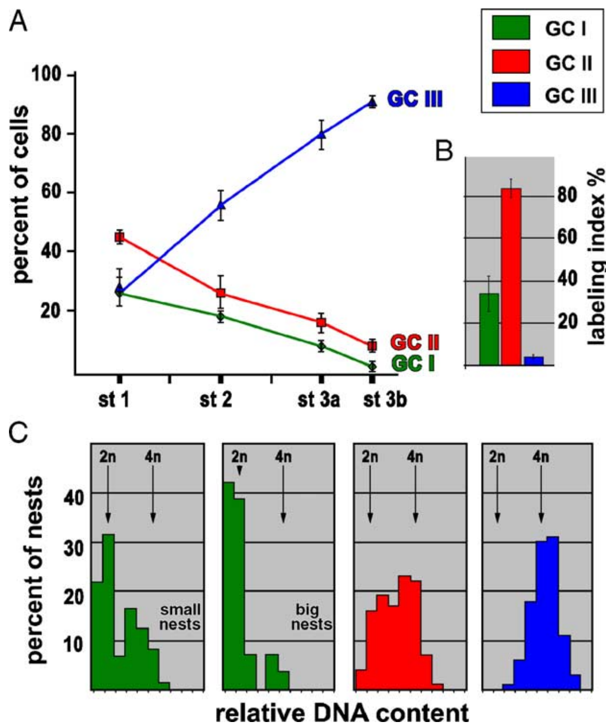

Fig. 3. Cell numbers, labeling index and nuclear DNA content of germ cells in egg patches from Stages 1 to 3b. (A) Single egg patches were macerated and the percentage of each cell type determined. Egg patches contained roughly 4000 germ cells at these stages. (B) Labeling index of GCI, GCII and GCIII cells determined by pulse labeling with BrdU. (C) Nuclear DNA content of GCI, GCII and GCIII cells. The histograms show the percentage of cells with a given DNA content. 2n and 4n DNA contents are shown for reference. GCI (green); GCII (red); GCIII (blue).

Stage 3a (24 h) is characterized by the appearance of several white dots in the milky region of the thickened ectodermal layer (Fig. 1). Maceration of the egg patch showed that the white dots represent early diplotene oocytes (see below, Fig. 2). The numbers of GCI and GCII are greatly reduced in Stage 3a and GCIII cells now constitute 90% of all cells in the egg patch area (Fig. 3A). Two or three GCIV in the egg patch become obviously bigger than the others (150 μm in diameter) and enter the diplotene stage of meiosis I. The nucleus in these cells enlarges to form a germinal vesicle while the chromosomes condense to form characteristic diplotene bivalent chromosomes. These chromosomes are concentrated in a small region of the germinal vesicle forming a karyosphere (Fig. 7A). We refer to cells at this stage as developing oocytes, although only one such cell per egg patch will complete oocyte differentiation.

Stage 3b (18 h) is relatively short. The egg patch has a star-like appearance when observed from above. The 2–3 oocytes present in the egg patch increase dramatically in size (up to 600 μm in the biggest dimension) and form branched pseudopodia. Confocal optical sections of egg patches at this stage demonstrate that germ cells adjacent to the oocyte have much smaller amounts of cytoplasm than germ cells which are not in contact with the oocyte (data not shown). These cells cannot be separated from the oocyte when the egg patch is macerated indicating tight contact between oocyte and germ cell (see below).

Stage 4 (4 h) is characterized by a shield-like appearance of the egg patch (Fig. 1). One or two large oocytes can be clearly seen in whole mounts. GCIII and GCIV cells, which were in tight contact with the oocyte at Stages 3a–b of oogenesis, now occur as single cells in macerates. These cells are smaller than GCIII cells and have significantly decreased amounts of cytoplasm (Fig. 6B). Chromatin in these cells is condensed forming prominent heterochromatic clumps at the periphery of the nucleus and in the nuclear

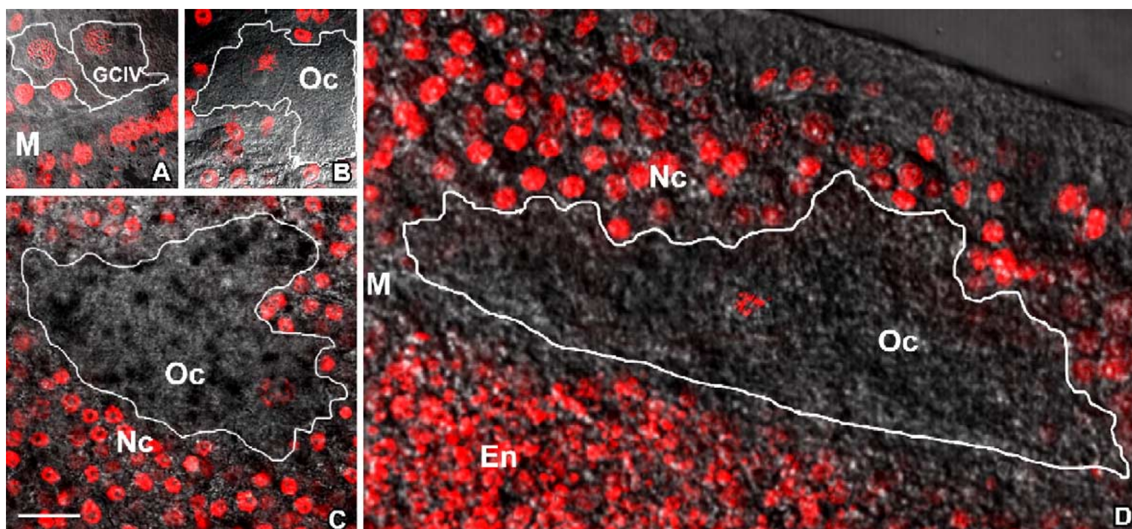

Fig. 4. Confocal images of egg patches at Stages 2, 3a and 3b. Nuclei are stained red with TO-PRO-3. The large areas without stained nuclei or with only one stained nucleus represent growing oocytes. The periphery of growing oocytes is outlined in white. (A) Stage 2 showing two GCIV cells adjacent to the mesoglea. (B) Stage 3a showing an early oocyte with a germinal vesicle. (C) Stage 3b showing growing oocyte (diameter 200 μm) with diplotene chromosomes. (D) Late Stage 3b showing growing oocyte (diameter 600 μm) lying along the mesoglea. The oocyte nucleus contains diplotene chromosomes. Images A–D are at the same magnification for comparison. En—endoderm, M—mesoglea, Nc—nurse cell, Oc—oocyte, Scale bar: 40 μm.

interior. These features are typical of apoptotic cells in *Hydra* (Cikala et al., 1999).

As a result of the rapid increase in oocyte volume, the upper surface of the oocyte(s) comes into contact with the ectodermal surface and the germinal vesicle changes its position from the middle of the oocyte to its upper surface. At this stage, the oocyte(s) start to phagocytize adjacent apoptotic GCIII and GCIV cells. As a result, the ooplasm of the oocytes fills up with numerous phagocytic vacuoles containing apoptotic bodies (Fig. 2).

From **Stage 5** (3 h), only one oocyte is present in the egg patch. The ooplasm of this single oocyte contains a large germinal vesicle (Fig. 7A) and in addition one–two large degenerating nuclei (Fig. 7B). These appear to be the nuclei of oocytes which fused to form the single remaining oocyte (see below).

Stages 6 and 7 (3 h) are short. The oocyte contracts its pseudopodia and becomes round (Fig. 1). The nucleus undergoes two meiotic divisions. At this point, the oocyte breaks through the ectoderm and is ready for fertilization.

Labeling index and nuclear DNA content of germ cells

The changes in the cell composition of the egg patch at different stages of oogenesis as well as the morphology of the different germ cell types suggest that these cells form a differentiation sequence (see Fig. 8). GCI cells give rise to GCII cells which later differentiate into GCIII cells. Some GCIII enter early prophase of meiosis and acquire the appearance of GCIV. In the egg patch, 2–3 GCIV reach the diplotene stage of meiotic prophase and develop as oocytes. Only one of these oocytes, however, completes meiotic division to form the mature oocyte.

To establish the differentiation pathway in more detail, we determined the labeling index and the nuclear DNA content of the various germ cell types. GCI cells in small nests of 2–4 cells were roughly 50% labeled by a BrdU pulse. By comparison, GCI cells in large nests of 8 were poorly labeled. This suggests that GCI cells in small nests constitute a proliferating population which gives rise to nests of non-proliferating GCI cells following a terminal mitosis. Measurements of nuclear DNA content confirm this idea. GCI cells in small nests have 2n–4n DNA contents typical of proliferating cells in *Hydra* (Fig. 3C). By comparison, GCI cells in large nests have 2n DNA content typical of postmitotic differentiating cells in *Hydra* (David and Gierer, 1974).

In striking contrast to GCI cells in large nests, most GCII cells were labeled after BrdU pulse labeling (Fig. 3B). In agreement with the very high percentage of labeled GCII cells, the DNA content of GCII was typical of cells in S-phase (Fig. 3C). We consider this S-phase to be premeiotic, since at least some GCII cells go on to meiosis (see below). GCIII nuclei were not labeled with BrdU and were characterized by 4n DNA content (Figs. 3B and C). This indicates that GCII cells complete premeiotic S-phase and then acquire the morphology of GCIII cells with 4n DNA content. They increase further in size and acquire typical vacuolated cytoplasm.

Cytoplasm transfer between germ cells and developing oocytes

At Stage 3a, developing oocytes become clearly visible in the egg patch. They are significantly larger than GCIII and GCIV cells. This increase in size is due to a massive (50–100×) increase in cytoplasmic volume which can be clearly seen in confocal sections through egg patches at Stages 3a and 3b (Figs. 4A–D). Maceration of egg patches at these stages also yielded very large oocytes with large amounts of cytoplasm (Fig. 2). The nuclei of such oocytes formed germinal vesicles 50–100 μm in diameter. The chromosomes were in diplotene and condensed indicative of low transcriptional activity. The rapid increase in cytoplasmic volume in the absence of extensive transcriptional activity suggests that the cytoplasm of the growing oocyte is imported from neighboring germ cells. Experiments described below provide evidence for the transfer of cytoplasm between germ cells and the developing oocyte.

In macerations of egg patches at Stages 3a and 3b, we observed that the connections between the growing oocyte and GCIII/GCIV cells became tighter. It was difficult to "clean" the oocyte from neighboring cells even after vigorous shaking in maceration solution (Fig. 2). To investigate whether this tight contact represented adhesion or fusion of nurse cells to developing oocytes, we stained animals with the membrane specific fluorescent dye FM 4–64. Stained animals were scanned in a confocal microscope to locate large developing oocytes (see Figs. 4C–D) and closely associated nurse cells. Fig. 5 shows several examples in which membrane staining was clearly absent over a broad area (10 μm) of contact between nurse cells and the developing oocyte. Where membrane staining was absent, nurse cell and oocyte cytoplasm formed a continuum and the nurse cell nucleus appeared to be in the cytoplasm of the developing oocyte (Figs. 5A–C). Quantitative estimates of the number of nurse cells fusing with oocytes could not be obtained from optical sections, since the duration of the fusion event is not known. Nevertheless, the results suggest that fusion is a frequent event, since many nurse cells in contact with the oocyte at this stage show evidence of fusion. For example, all four nurse cells in Fig. 5C are fused with the developing oocyte.

Cell fusion, documented as the absence of FM 4–64 membrane staining, was restricted to sites of nurse-cell–oocyte contact; at sites of nurse-cell–nurse-cell contact, membrane staining was clearly visible (Fig. 5). Thus, the absence of membrane staining does not seem to be an artefact. This conclusion is also supported by the "reappearance" of membrane staining in neighboring optical sections (compare Figs. 5C with D).

Nurse-cell–oocyte fusion must be a transient event, since individual nurse cell nuclei were never observed in the

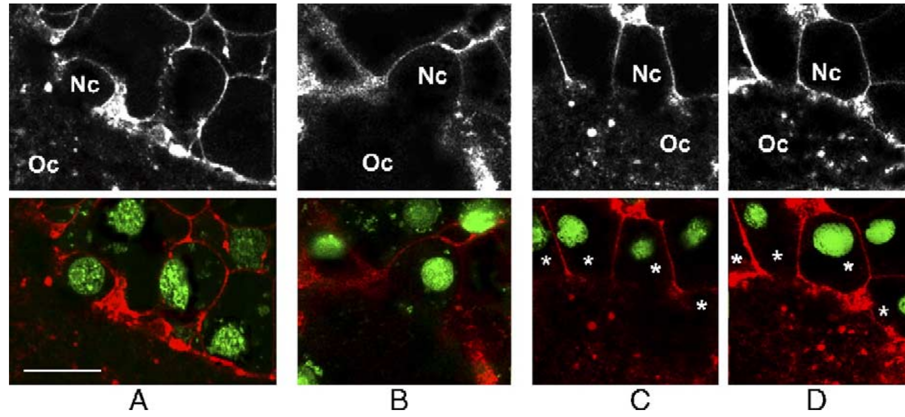

Fig. 5. Membrane staining with FM 4–64 in nurse cells and developing oocytes. Images show the site of contact between nurse cells (Nc) and the developing oocyte (Oc) in three different egg patches (A, B and C–D). Nurse cells are in the upper part, oocyte in the lower part of each image. Membranes are stained with FM 4–64 (red); nuclei are stained with SYTO 16 (green). The images in sections C and D show optical sections of the same cells separated by 3 μm. The nurse cell with an (*) have cytoplasmic connections to the oocyte in section C but not in section D. Scale bar: 20 μm.

cytoplasm of oocytes (see Fig. 4). This suggests that nurse cells recellularize following transfer of cytoplasm to the oocyte. To confirm this conclusion, we examined macerates of Stage 3b for the presence of "small" GCIII and GCIV cells. Such cells were indeed present (Fig. 6B) in significant numbers (530 ± 93 per egg patch, n = 5). These cells had initiated apoptosis as indicated by TUNEL staining (Fig. 6B; see below). We conclude from these results that nurse cells transiently fuse with oocytes, transfer cytoplasm and then recellularize before initiating apoptosis.

The ability of germ cells to fuse was also seen later when the 2–3 oocytes developing in an egg patch fused to form one oocyte. At Stages 3a and 3b, two to three diplotene oocytes were always present in an egg patch. At later stages, however, the egg patch only contained one oocyte due to fusion of the precursor oocytes. Only one nucleus survived in this oocyte (Fig. 7A). The other nuclei degenerated (Fig. 7B). Such degenerating nuclei were not inside vacuoles indicating that they are not derived from phagocytosed germ cells (see below).

Apoptosis of germ cells following cytoplasm transfer

As described above, macerates of egg patches at Stage 3b contained a new class of "small" GCIII and GCIV cells. Staining with DNA dyes indicated that the nuclei in these cells were apoptotic (Fig. 6B). The chromatin was condensed to small droplets along the nuclear membrane and in

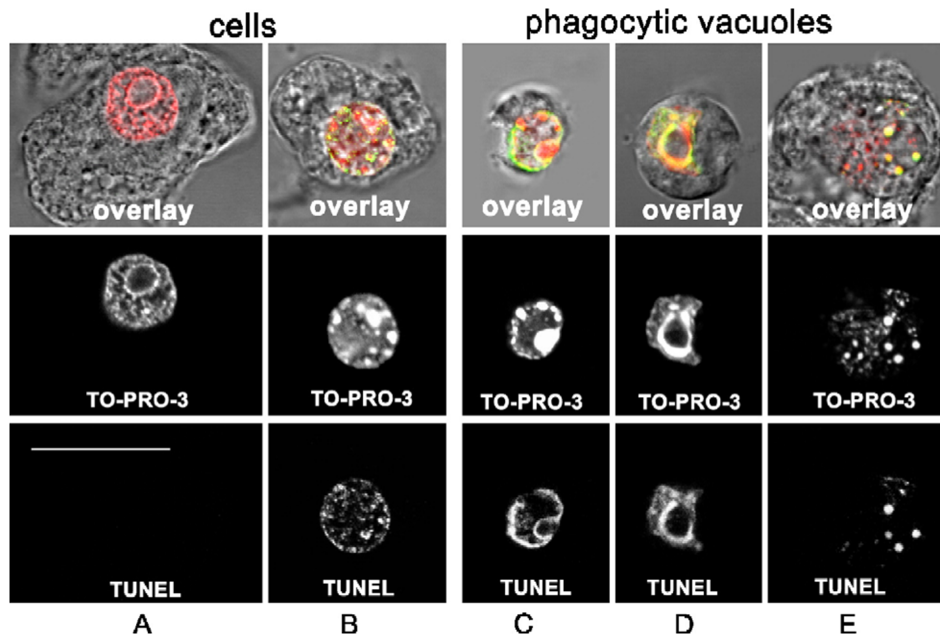

Fig. 6. Nurse cells in the egg patch (A, B) and phagocytosed apoptotic bodies released from macerated oocytes (C–E). DNA is stained red with TOP-RO3, TUNEL staining in green. (A) Large GCIII nurse cell; (B) "Small" GCIII nurse cell with reduced cytoplasmic volume. Nucleus has clumped chromatin typical of apoptosis and is TUNEL positive; (C–E) apoptotic bodies from macerated oocytes differ in size but all have clumped chromatin and are TUNEL positive. Scale bar: 20 μm.

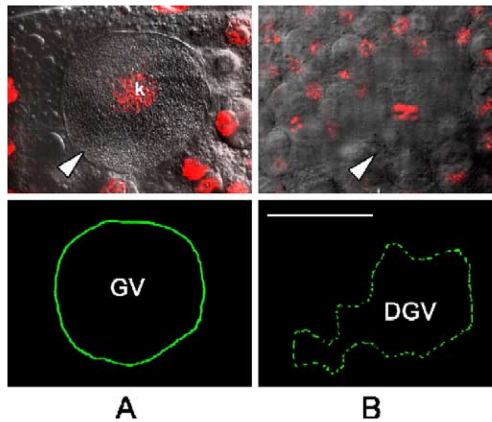

Fig. 7. Intact germinal vesicle and degenerating germinal vesicle in an oocyte at Stage 5. Confocal sections of an egg patch stained with TO-PRO-3 to label DNA. (A) Intact germinal vesicle with intact nuclear membrane (arrowhead) and diplotene chromosomes in a karyosphere. (B) Degenerating germinal vesicle with defective nuclear membrane (arrowhead) and clumped chromosomes. The green line in the lower panels outlines the periphery of the intact and degenerating germinal vesicles. Note the absence of phagocytic membrane. DGV—degenerating germinal vesicle, GV–germinal vesicle, k—karyosphere. Bar: 80 μm.

the interior of the nucleus. To confirm that these cells were apoptotic, we stained the macerates with TUNEL. Small GCIII and GCIV cells present in the macerates were TUNEL positive (Fig. 6B). By comparison, GCIII and GCIV cells with normal amounts of cytoplasm as well as epithelial cells present in the same macerates were TUNEL negative (Fig. 6A and data not shown).

We also squashed mature oocytes in maceration medium to release phagocytosed germ cells and stained them with TUNEL. Three types of apoptotic bodies could be distinguished in the cytoplasm of macerated oocytes (Figs. 6C–E). Medium sized apoptotic bodies (nucleus 10 μm) with loosely organized chromatin were the most common type (Fig. 6D). They appear to represent phagocytosed GCIII cells. Small apoptotic bodies (nucleus 5–7 μm) with highly condensed chromatin represent phagocytosed GCII cells (Fig. 6C). A few very large apoptotic bodies (nucleus 20 μm) with chromatin organized in threads throughout the nuclear area were also found. They represent phagocytosed GCIV cells of which there are about 50 in an egg patch (Fig. 6E). All three types of apoptotic bodies (Figs. 6C–E) had TUNEL positive DNA. Although some of the condensed DNA in the nuclear interior was TUNEL negative, the ring of condensed chromatin visible at the periphery of the nucleus was always TUNEL positive.

Discussion

Cell differentiation scheme

Oocyte precursors in *Hydra* are derived from interstitial stem cells (Bosch and David, 1986, 1987; Littlefield, 1991). Based on observations here, we can now propose a detailed cell differentiation scheme for oogenesis (Figs. 2 and 8). Precursors proliferate throughout the body column, aggregate to form an egg patch (Honegger et al., 1989) and then give rise to a post-mitotic non-cycling population of GCI cells (Figs. 2, 3C). These cells increase their cytoplasmic and nuclear volume, acquire the appearance of GCII cells (Fig. 2) and start premeiotic DNA replication (Figs. 3B, C). After DNA replication, they acquire the features of GCIII cells (Fig. 2) including a nuclear DNA content of 4n (Fig. 3C) and vacuolated cytoplasm filled with lipid droplets, glycogen granules and other inclusions (Aizenshtadt, 1975; Honegger et al., 1989). GCIII cells constitute the predominant cell type in Stages 2 and 3 of oogenesis (Fig. 3A). Aizenshtadt and Marshak (1974) have also suggested that nurse cells in *Hydra* pass through a premeiotic S-phase, since about 90% of germ cell nuclei in the egg patch area were observed to have 4n DNA content.

At the end of stage 2 and the beginning of stage 3, about 1% of GCIII cells enter prophase I of meiosis (leptotene to pachytene) thus becoming GCIV cells (Figs. 2 and 8). Two to three GCIV cells develop into diplotene oocytes. These cells increase dramatically in size from 100 μm to 600 μm due to transfer of cytoplasm from adjacent nurse cells (Figs. 4A–D). After this growth phase, the surrounding GCIII and GCIV cells initiate apoptosis and are phagocytosed by the oocyte (Figs. 6C–E). As soon as the growing oocytes (2–3 in the egg patch) come into contact with one another, they fuse into one cell in which only one nucleus (germinal vesicle) survives (Fig. 7). The single remaining oocyte contracts its pseudopodia and breaks though the ectodermal

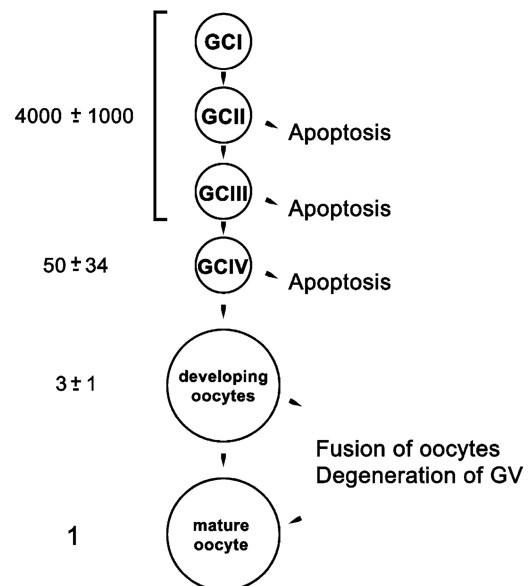

Fig. 8. Summary of nurse cell and oocyte differentiation indicating numbers of germ cells and their cell fate. Egg patches contain about 4000 germ cells which complete a premeiotic S-phase and enter G2 phase. Most of these cells differentiate as GCIII nurse cells; about 50 cells become GCIV cells. Of these, 2–3 cells per egg patch enter diplotene and enlarge to become presumptive oocytes. These oocytes fuse to form one large oocyte in which one germinal vesicle survives. The “excess” germinal vesicles degenerate.

surface (Fig. 1). During this process, the surviving nucleus undergoes two meiotic divisions and the egg is ready for fertilization.

Oocyte number, oocyte fusion and degradation of "excess" oocyte nuclei

Our observation that 2–3 oocytes initially form within an egg patch appears surprising since only one oocyte is finally formed by the egg patch. This result is, however, consistent with previous observations showing that, when egg patches are cut in half, both halves develop an oocyte (Miller et al., 2000). These observations indicate that an egg patch contains more than one potential oocyte even though only one survives in an intact egg patch. A likely explanation for this result is that developing oocytes fuse at the end of differentiation thus forming only one oocyte per egg patch. In such fused oocytes, only one nucleus is expected to survive and undergo meiosis. "Excess" nuclei derived from fusion of 2–3 developing oocytes need to be eliminated. We have indeed observed such degenerating nuclei in the cytoplasm of late stage oocytes (Fig. 7B). As expected from the fusion hypothesis, this nucleus is free in the oocyte cytoplasm and is not surrounded by a phagocytic membrane as are the phagocytosed nurse cells.

Fusion of oocytes is not uncommon in Cnidarian oogenesis. It has been shown for another Hydrozoan *Tubularia croccea* (Boelstreli, 1975) as well as for a representative of Archiannelida *Dinophilus gyrociliatus* (Grün, 1972). Although several authors have proposed a similar scheme for *Hydra* on the basis of electron microscopic investigations (Aizenshtadt, 1978; Zihler, 1972), their data were not sufficient to confirm or reject this possibility (Honegger et al., 1989).

Oocyte growth

After entering the diplotene stage of meiosis, oocytes start to grow. In Stages 3a and 3b, the volume of developing oocytes increases dramatically from 100 to 400 μm in diameter in about 2 days (Figs. 4A–D). It appears unlikely that this 50–100 fold increase in cytoplasmic volume is due to oocyte synthetic activity alone since the transcriptional activity of oocyte nuclei at this stage is not expected to be high. The diplotene bivalents visible at Stage 3a of oogenesis (beginning of the oocyte growth period) are condensed and aggregated in the center of the germinal vesicle forming the karyosphere which is characteristic of transcriptionally silent oocyte nuclei (Gruzova and Parfenov, 1993).

In the absence of extensive oocyte transcriptional activity, cytoplasmic components necessary for early development must be delivered to the oocyte from the other cells. The present results provide strong evidence that oocyte growth in *Hydra* occurs by transfer of cytoplasm from nurse cells to the oocyte. During this process (stages 3a and 3b), nurse cells became tightly bound to oocytes (Figs. 2 and 4) and it was no longer possible to separate them from oocytes during maceration. We interpret this as evidence for a transient fusion of the two cell types and the transfer of cytoplasm from nurse cells to oocyte. Membrane staining with FM 4–64 confirmed this interpretation. Large gaps in the cell membrane were observed at sites of close contact between nurse cells and oocytes (Figs. 5A–D).

The problem of oocyte growth has been recognized by previous investigators but no satisfactory mechanistic explanation has been provided. Honegger et al. (1989) suggested that nurse cells bud off cytoplasmic blebs and that these fuse with oocytes. However, their EM pictures do not show such blebs. Thus, the mechanism of cytoplasm transfer from nurse cells to developing oocytes has been unresolved until the present experiments.

Cell fusion is not common in animal tissue but does occur in specific circumstances, e.g. fusion of sperm and egg during fertilization and fusion of myoblasts with differentiating myotubes. The fusion of myoblasts has been extensively investigated in *Drosophila* (Dworak and Sink, 2002). A number of essential genes have been identified, including several encoding transmembrane proteins expressed in fusion competent myoblasts and myotubes. Although the molecular mechanism of fusion is not yet understood, the process appears to be polarized: myoblasts are donors and myotubes are acceptors. Correspondingly, they express different molecules on their cell membranes. A similar polarity is observed in *Hydra* oogenesis: nurse cells fuse with developing oocytes but not with each other (see Fig. 5).

Apoptosis of nurse cells

Towards the end of Stage 3b, a new type of GCIII/IV cell with less cytoplasm and a TUNEL positive nucleus became abundant in macerates (Fig. 6B). This suggests that, having transferred the main part of their cytoplasm to the oocyte, GCIII/IV cells recellularize and start an apoptotic program. This conclusion agrees well with previous work of Zihler (1972) and Honegger et al. (1989) based on EM observations and of Technau et al. (2003) based on acridine orange staining and TUNEL staining. By contrast, Frobius et al. (2003) have recently suggested that phagocytosed nurse cells actively synthesize macromolecules, since mRNA transcripts of several developmentally relevant genes could be identified by RT-PCR and in situ hybridization. While these results clearly showed that specific mRNAs were present in oocytes and that nuclei isolated from phagocytosed cells expressed low levels of transcription in run-on experiments, active transcription in vivo was not demonstrated. Hence, the balance of evidence at present suggests that phagocytosed cells are apoptotic.

Apoptotic death of nurse cells in the course of oogenesis has been described for many different species including *Drosophila* and *Caenorhabditis*. As with *Hydra*, the

cytoplasm of these cells is transferred to the oocyte before they initiate apoptosis. The cell corpses are then phagocytosed by somatic tissues in *Drosophila* and *Caenorhabditis* (Foley and Cooley, 1998; Gumienny et al., 1999; Nezis et al., 2000). By contrast, in *Hydra*, the apoptotic nurse cells are phagocytosed by the oocyte itself and remain in the cytoplasm up to hatching of the polyp (Martin et al., 1997; Technau et al., 2003; Zihler, 1972). What is the biological significance of engulfing the apoptotic cells by the oocyte? Why are the apoptotic bodies protected from the further digestion until the end of the embryonic development? One possible explanation is nourishment. In *Hydra*, several thousand nurse cells are phagocytosed per oocyte. These cell corpses represent a significant biosynthetic mass which the developing embryo utilizes after hatching. This may constitute a selective advantage.

Comparison of oogenesis in Hydra, Caenorhabditis and Drosophila

Fig. 9 shows a schematic comparison of oogenesis in *Hydra*, *Caenorhabditis* and *Drosophila*. In all three cases, multiple germ cells contribute cytoplasm to the oocyte. In *Drosophila* sibling, germ cells in the ovariole transfer cytoplasm through ring canals to the growing oocyte (for review, see de Cuevas et al., 1997; Mahajan-Miklos and Cooley, 1994). In this case, the biosynthetic product of 15 nurse cell nuclei contributes to the oocyte. In *Caenorhabditis* germline, nuclei proliferate in a syncytium and the gene products of these nuclei contribute to the formation of a common mass of cytoplasm. About half of these nuclei subsequently undergo apoptosis while the remaining nuclei enclose units of the common cytoplasm to form oocytes. In this case, roughly 2 nuclei die for each oocyte formed (Gumienny et al., 1999). In the *Hydra* egg patch, roughly 4000 germline cells contribute to the growing oocyte (Fig. 8). These cells fuse with the oocyte and transfer cytoplasm to it before undergoing apoptosis (Figs. 5, 6).

The difference between *Hydra*, *Caenorhabditis* and *Drosophila* in the number of germ cells contributing to each oocyte parallels the difference in oocyte size. A mature *Hydra* oocyte has a size of approximately 600 μm, whereas a *Drosophila* oocyte is roughly 150 μm and a *Caenorhabditis* oocyte is roughly 50 μm in diameter. These different sizes of oocytes reflect volume ratios of 1700:27:1, which agrees roughly with the different numbers of nurse cells contributing cytoplasm to the oocytes. Thus, alimentary oogenesis represents a strategy to rapidly produce egg cytoplasm by utilizing multiple germline nuclei to synthesize it. The size of the oocyte is proportional to the number of contributing cells.

There are also similarities in the differentiation of nurse cells. In all three species, nurse cells are derived from germline cells which undergo a premeiotic S-phase prior to differentiation. Some of these cells in *Hydra* (GCIV) and two cells in *Drosophila* (de Cuevas et al., 1997) initiate meiosis while all nuclei in *Caenorhabditis* reach meiotic pachytene. At this stage, nurse cells undergo apoptosis and are phagocytosed. In *Caenorhabditis*, this step requires activation of the MAPK pathway. Mutants in MAPK do not exit pachytene and do not undergo apoptosis (Gumienny et al., 1999). Whether a similar signaling

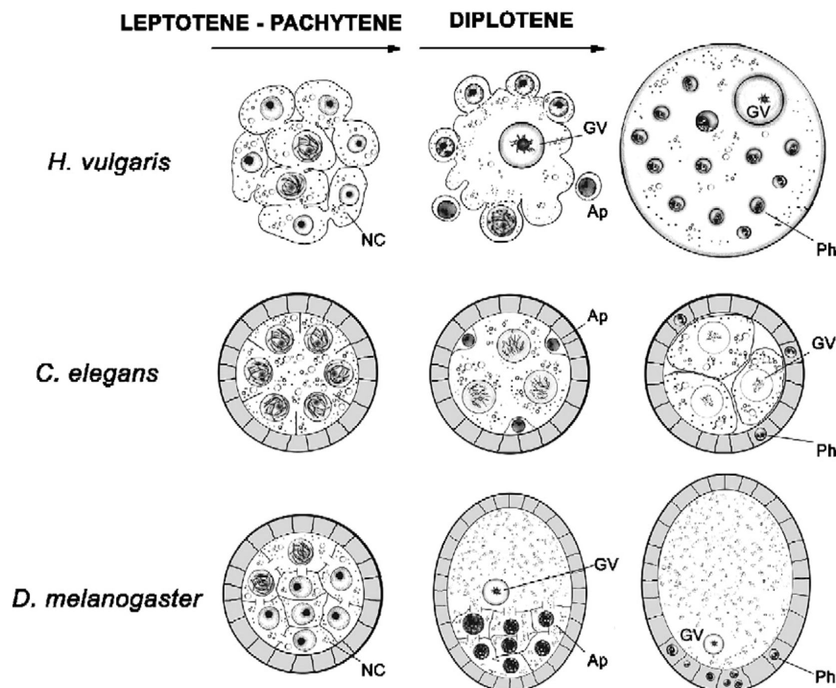

Fig. 9. Schematic comparison of the contribution of nurse cells to oogenesis in *Hydra*, *Caenorhabditis* and *Drosophila*. Ap—apoptotic cell, GV—germinal vesicle, Nc—nurse cell, Ph—phagocytic body. See text for details.

pathway regulates apoptosis in *Hydra* and *Drosophila* is not presently known.

Despite the similarities between these three species in the role of nurse cells, there is one striking difference: the fate of nurse cells. Fig. 9 shows that nurse cells in *Hydra* are phagocytosed by the oocyte itself, whereas in *Caenorhabditis* and *Drosophila* they are phagocytosed by somatic tissue (sheath or follicle cells) surrounding the oocyte (Gumienny et al., 1999; Nezis et al., 2000).

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