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Histological Studies on Early Oogenesis in Barfin Flounder (*Verasper moseri*)

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ABSTRACT—There is much information on oogenesis from the resumption of the first meiotic division to oocyte maturation in many vertebrates; however, there have been very few studies on early oogenesis from oogonial proliferation to the initiation of meiosis. In the present study, we investigated the histological changes during early oogenesis in barfin flounder (*Verasper moseri*). In fish with a total length (TL) of 50mm (TL 50mm fish), active oogonial proliferation was observed. In TL 60mm fish, oocytes with synaptonemal complexes were observed. Before the initiation of active oogonial proliferation, somatic cells which surrounded a few oogonial germ cells, started to proliferate to form the oogonial cysts that accompanied oogonial proliferation. In TL 70mm fish, however, the cyst structure of the oocyte was gradually broken by the invagination of somatic cells, and finally the oocyte became a single cell surrounded by follicle cells. Upon comparison of nuclear size, DNA-synthesizing germ cells could be divided into two types: small nuclear cells and large nuclear cells. Based on histological observation, we propose that the small nuclear cells were in the mitotic prophase of oogonia and the large nuclear cells were in the meiotic prophase of oocytes, and that the nuclear size increases upon the initiation of meiosis.

Key words: oogenesis, barfin flounder, oogonial proliferation, meiosis, oocyte

INTRODUCTION

Gametogenesis is an essential process that maintains the base of life. It begins with the differentiation of primordial germ cells (PGCs) to oogonia in females or to spermatogonia in males. These gonial cells develop into eggs or spermatozoa, respectively, through mitotic proliferation and meiosis (Gilbert, 1985).

Oogenesis, or female gametogenesis, in marine teleost species is a complex process that can be divided into four main growth phases: (1) oogonial proliferation by mitotic division; (2) meiotic transformation of oogonia into primary oocytes; (3) oocyte growth; and (4) oocyte maturation (Barr, 1968). In brief, PGCs move into an ovary and then become oogonia. The oogonia proliferate by mitosis. After proliferation, the oogonia undergo meiosis and then develop into primary oocytes.

There is much information on the period during oogenesis from the resumption of the first meiotic division to oocyte maturation in many vertebrates (Wallace, 1985; Wasserman and Smith, 1978; Masui and Clarke, 1979; Kanatani and Nagahama, 1980; Nagahama, 1983; Fostier *et al.*, 1983; Goetz, 1983). However, there have been very few studies on early oogenesis from oogonial proliferation to the development of peri-nucleolus stage oocytes (Yamazaki, 1965; Pisano *et al.*, 1978; Tokarz *et al.*, 1978 a, b; Gonzalez, 1998). One reason for this is that oogenesis up to the prophase of the first meiotic division occurs very quickly in nearly all animal species. In order to investigate early oogenesis in detail, it is important to select an animal species in which oogenesis occurs comparatively slowly.

It is known that female barfin flounders (*Verasper moseri*) is the group-synchronous oocyte development type because they spawn several times during the spawning season (one month a year) (Koya *et al.*, 1994) and their eggs become mature at three years old. This species is very useful for analyzing early oogenesis because oogenesis in this species occurs comparatively slowly. In the present study, we performed histological studies on juvenile barfin flounders in order to investigate early oogenesis.

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MATERIALS AND METHODS

Animals

Juvenile barfin flounders were obtained from the Hokkaido Institute of Mariculture in Shikabe, Japan. Fertilized eggs were obtained from a few breeding males and females in a 1-ton tank. The fertilized eggs were maintained at 8°C, and after they hatched, the temperature was gradually raised to 14°C.

Light and electron microscopic observation

For observation by light and electron microscopy, the gonadic regions and ovaries were excised from juvenile barfin flounders of various total lengths (TLs) (TL 30mm, 40mm, 50mm, 60mm, 70mm, 80mm, 90mm and 100mm±5mm), and fixed in Bouinís solution. They were dehydrated by ethanol and acetone, and embedded in Historesin Plus (Leica, Nussloch, Germany). Five-µm sections were cut and stained with Delafield's hematoxylin and eosin for light microscopic examination. The gonadic regions and ovaries from other juvenile barfin flounders were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.1% cacodylate buffer at pH 7.4 overnight, and then transferred to 10% sucrose in the same buffer for a minimum of 20 min. These samples were then postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hr and embedded in epoxy resin according to standard procedures.

Ultra-thin sections were stained with uranyl acetate and lead citrate for electron microscopic examination.

Immunohistochemical detection upon injection of 5-bromo-2'-deoxyuridine (BrdU)

BrdU injection was performed as described by Asakawa *et al.* (1991) with some modifications. Intraperitoneal injection of 5-bromo-2'-deoxyuridine (Sigma, St. Louis, MO, USA), 25 µg/g body weight, in two doses every 12 hr was performed on juvenile barfin flounders with a TL of 30mm, 40mm, 50mm, 60mm, 70mm, 80mm, 90mm, 100mm, 110mm, or 120mm±5mm, and they were sacrificed by decapitation 12 hr after the second injection. The gonadic regions and ovaries were excised, fixed in Bouinís solution at 4°C for 24 hr, embedded in paraffin and sectioned at 5 µm. The sections were deparaffinized, stained immunohistochemically with the reagents in the Cell Proliferation Kit (Amersham Pharmacia Biotech, Uppsala, Sweden), and counterstained with Delafield's hematoxylin. The entire staining procedure was performed at 20±1°C. The number of immunolabelled germ cells from three randomly selected sections of each ovary was counted and expressed as a percentage of the total number of germ cells (BrdU index; immunolabelled germ cells/total germ cells X 100). The area of the nuclei of immunolabelled germ cells was measured on photomicrographs of randomly selected sections of each of the three ovaries of TL 50mm, 60mm, 70mm and 90mm±5mm flounders using NIH-image software provided by the National Institutes of Health (Bethesda, MD, USA).

TUNEL (TdT-mediated dUTP nick-end labelling) assay

The TUNEL assay was performed as described by Gavrieli *et al.* (1992) with some modifications. Gonadic regions and ovaries were excised, fixed in Bouinís solution at 4°C for 24 hr, embedded in paraffin and sectioned at 5 µm. The sections were deparaffinized, stained with the reagents in the TACS™ 2 TdT *In Situ* Apoptosis Detection Kit (Trevigen, MD, USA), and counterstained with Delafield's hematoxylin.

Statistical Analysis

Results were expressed as the mean and standard error (SEM). Differences between means were analyzed by one-way ANOVA, and if a significant difference was found ($p<0.05$), the Bonferroni multiple-comparison test was performed. Significance was set at $P<0.05$.

RESULTS

Morphological changes of the ovary during early oogenesis

In the present study, histological observation of early oogenesis in barfin flounders with a TL of 30mm to 100mm was performed. In fish with a TL of 30mm (TL 30mm fish) (Fig. 1A), morphological sex differentiation of the gonad had not yet started and a few PGCs; undifferentiated germ cells were observed in the undifferentiated gonads. In TL 40mm fish (Fig. 1B), the ovarian cavity had begun to form, and morphological sex differentiation of the gonad was completed. Also, cysts of oogonia surrounded by somatic cells; the cells differ from germ cells were observed. Among fish with a TL of 50mm (Fig. 1C) to 70mm (Fig. 1D), the size of the cyst and the number of germ cells per cyst gradually increased as the TL increased. The largest cysts were observed in the ovaries of TL 60mm and TL 70mm fish, and we could not distinguish whether the germ cells were oogonia or oocytes by light microscopic observation. On electron microscopic observation of the germ cells (Fig. 2), many synaptonemal complexes were observed in their nuclei. In the ovaries of fish with a TL of 80mm (Fig. 1E) to 100mm (Fig. 1F), the size of the cyst and the number of germ cells per cyst gradually decreased as the TL increased. Most of the germ cells in the TL 100mm fish were peri-nucleolus stage oocytes, but a few oogonia still existed.

BrdU incorporation into gonadal cells

To clarify the details of oogonial proliferation, the initiation of the first meiotic division and the process of cyst formation, we observed DNA replication in gonadal cells by BrdU injection into juvenile barfin flounders (Fig. 3). In TL 30mm and 40mm fish, BrdU immunoreaction was rarely observed in the germ cells, although many somatic cells around the germ cell cyst were immunolabelled. In the TL 50mm fish, many germ cells and somatic cells were immunolabelled, and the percentage of immunolabelled germ cells was significantly higher than that in the TL 40mm fish ($P<0.05$). In the TL 60mm fish, a similar level of immunoreactivity was observed as in the TL 30mm and 40mm fish, and the percentage of immunolabelled germ cells was significantly lower than that in the TL 50mm fish ($P < 0.05$). In the TL 70mm fish, many germ cells were immunolabelled and the percentage of immunolabelled germ cells was significantly higher than that in the TL 60mm fish ($P<0.05$), although no immunoreactivity was observed in the somatic cells. In the fish with TL of 80mm to 110mm, BrdU immunoreactivity was observed in only a few germ cells and the percentage of immunolabelled germ cells in the fish with TL of 80mm, 100mm or 110mm was significantly lower than that in the TL 70mm fish ($P<0.05$). In the TL 120mm fish, positive reactivity was not observed in any of the germ cells or somatic cells.

Additionally, we investigated the qualitative differences of the DNA synthesis in the germ cells of TL 50mm to TL

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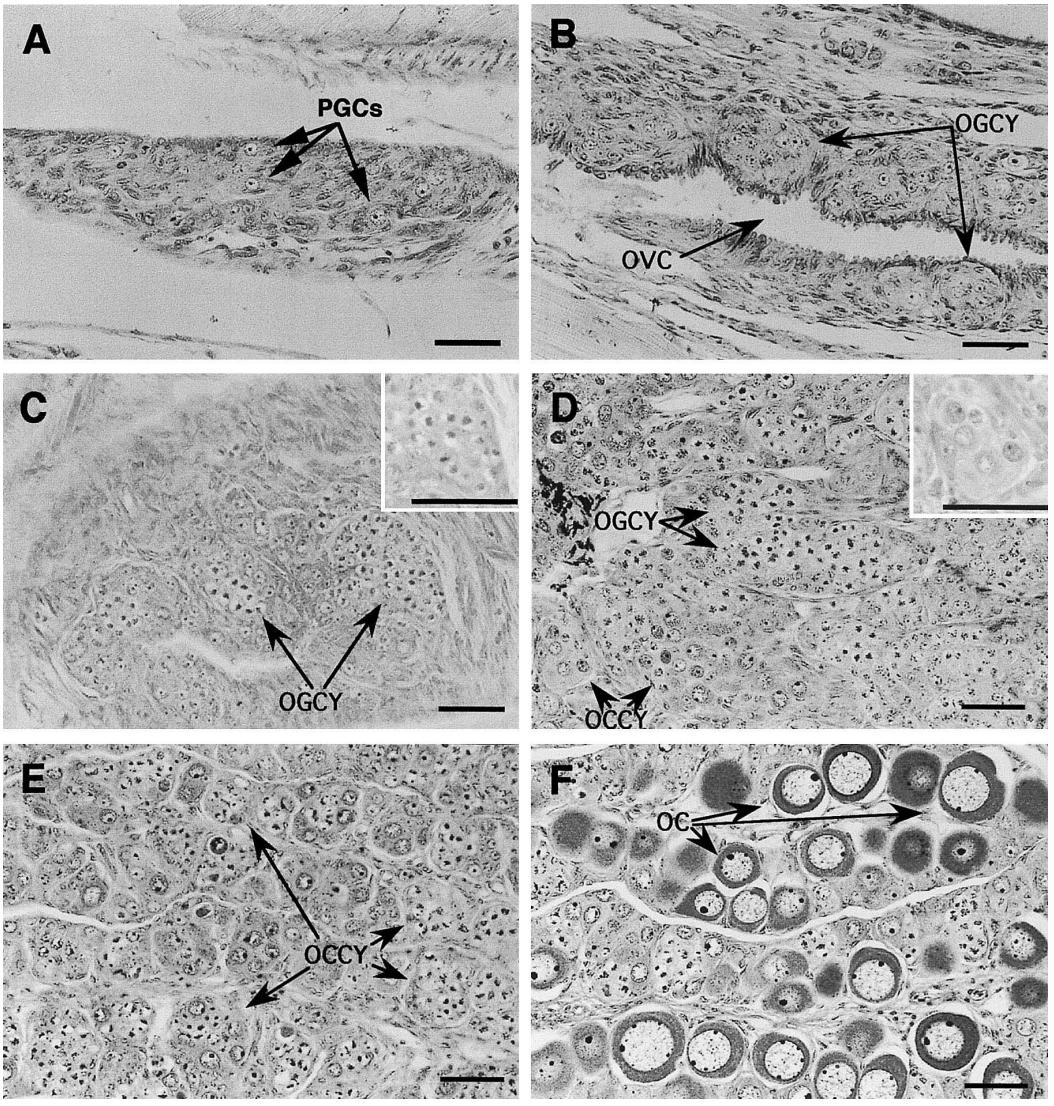

Fig. 1. Light micrographs of a hematoxylin and eosin-stained, 5-µm section of an ovary of barfin flounders. (A) TL 30mm fish; (B) TL 40mm fish; (C) TL 50mm fish with the insert indicating oogonia in a cyst; (D) TL 70mm fish with the insert indicating chromatin-nucleolus stage oocytes in a cyst; (E) TL 80mm fish; (F) TL 100mm fish. PGCs, primordial germ cells; OVC, ovarian cavity; OGCY, oogonial cyst; OCCY, oocyte cyst; OC, peri-nucleolus stage oocyte (bar = 40 µm).

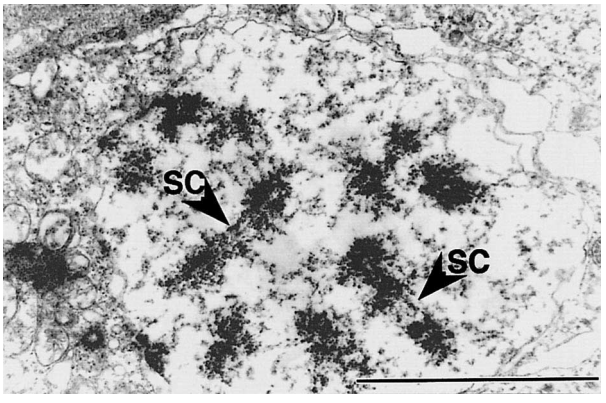

Fig. 2. Electron micrograph of the synaptonemal complex in an oocyte in a TL 70mm fish. SC, synaptonemal complex (bar = 3 µm).

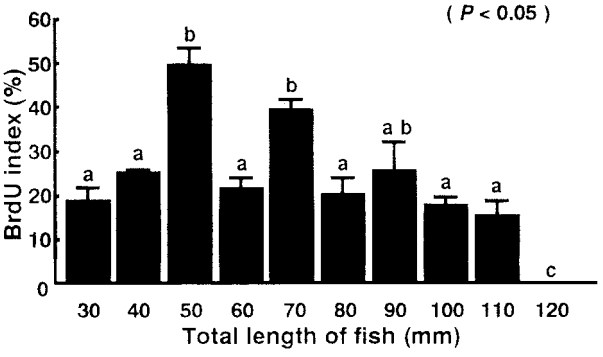

Fig. 3. Percentage of germ cells that were immunolabelled with BrdU (BrdU index) in fish with a TL of 30mm to 120mm. Results are expressed as the mean±SEM. Values with the same lowercase letter are not significantly different ($P > 0.05$). Each bar represents the results from 6 fish.

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90mm fish by comparing the nuclear size of the BrdU-incorporated germ cells. The nuclear size of these BrdU-incorporated germ cells was not uniform (Fig. 4). Many small nuclear germ cells (Fig. 4A) were observed in TL 50mm fish within the oogonial cysts (Fig. 1C), whereas many large nuclear germ cells (Fig. 4B) were observed in TL 70mm fish within the oocyte cysts (Fig. 1D). Next, the nuclear area of each BrdU-incorporated germ cell (GNA) was measured (Fig. 5). In the TL 50mm fish, many small nuclei of germ cells incorporated BrdU and the GNA was concentrated at one peak from 5 to 10 μm^2 (Fig. 5A). In the TL 60mm fish, the percentage of BrdU-incorporated cells with this range of GNAs decreased; the nuclei of some BrdU-incorporated germ cells were larger than those in the TL 50mm fish and the GNA was concentrated at two peaks where one peak was at 5 to 15 μm^2 and the second peak was at 30 to 40 μm^2 (Fig. 5B). The percentage of BrdU-incorporated germ cells with large nuclei increased and their nuclear size gradually increased as the TL of the fish increased. In the TL 90mm fish, the peak of GNAs from 5 to 15 μm^2 disappeared and the GNA was concentrated at one peak from 45 to 50 μm^2 (Fig. 5D).

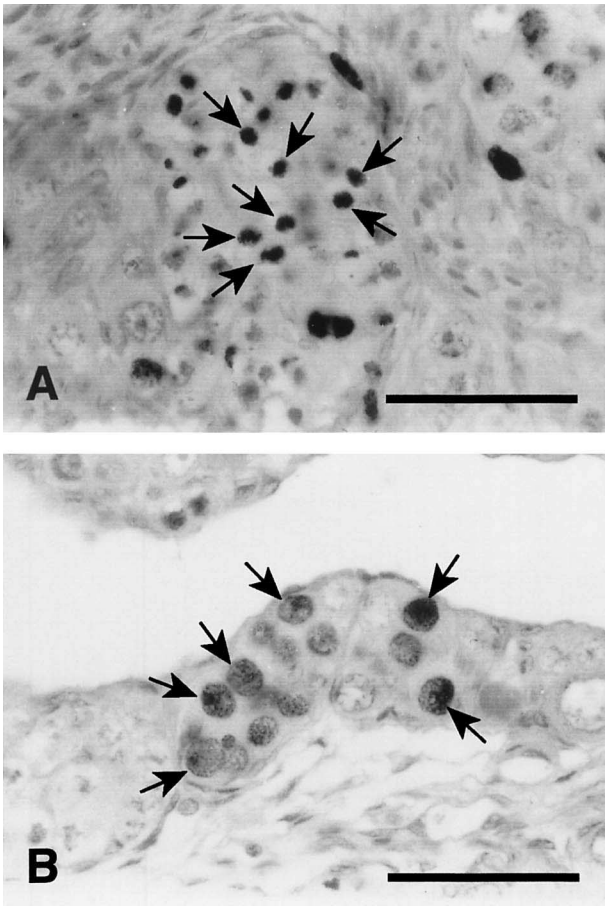

Fig. 4. Light micrographs of BrdU-incorporated nuclei of ovarian germ cells. (A) TL 50mm fish; (B) TL 70mm fish. Arrows indicate BrdU-incorporated germ cells (bar = 40 μm).

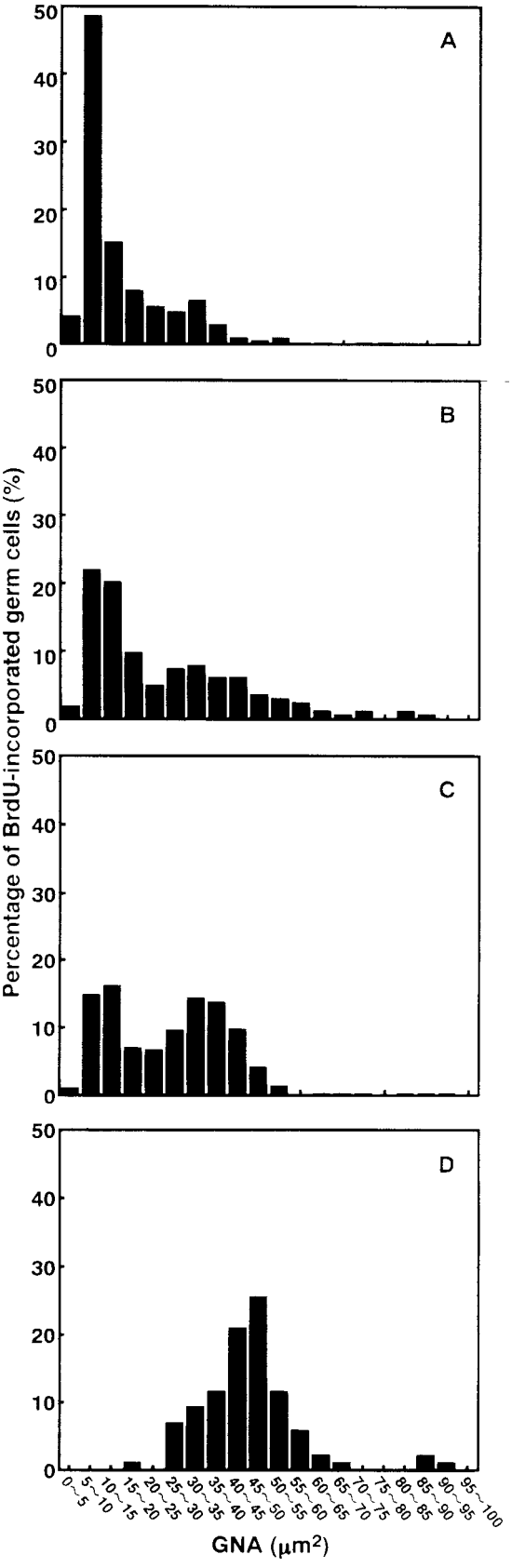

Fig. 5. Frequency distribution of the nuclear area of BrdU-incorporated germ cells (GNA). (A) TL 50mm fish; (B) TL 60mm fish; (C) TL 70mm fish; (D) TL 90mm fish.

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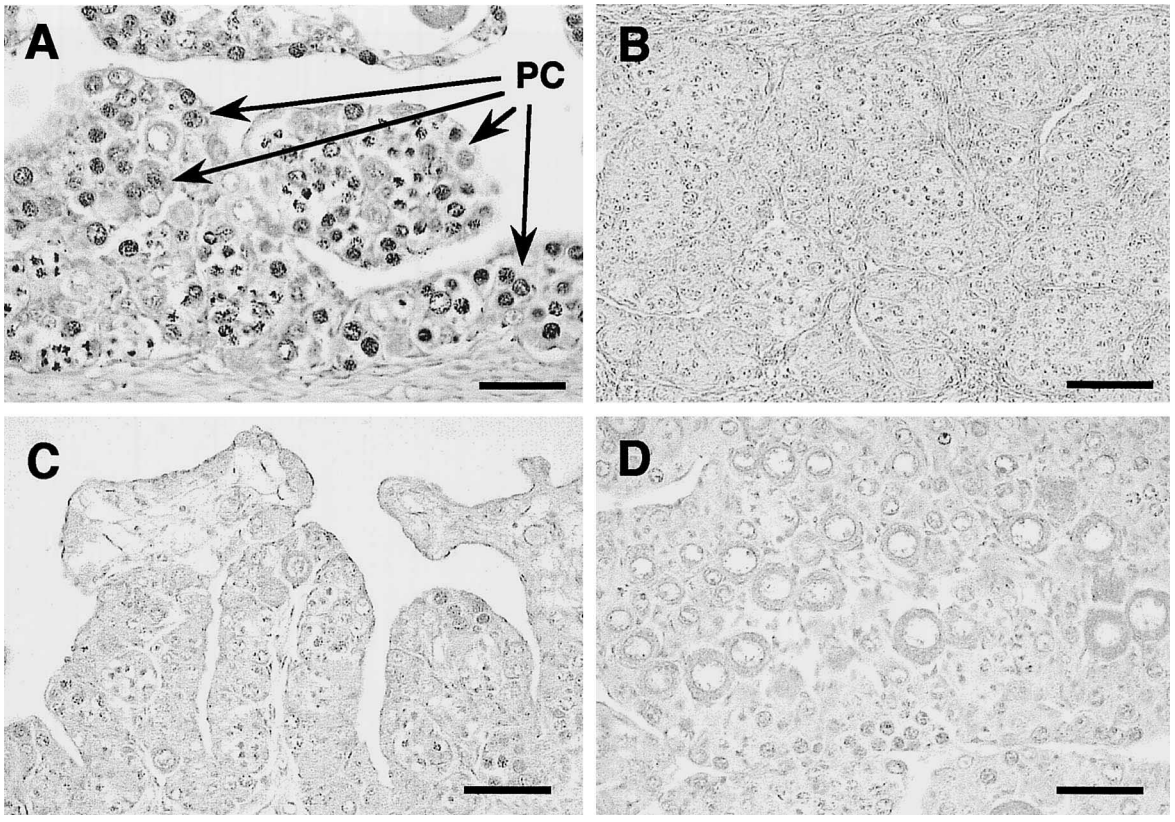

Fig. 6. Light micrographs of ovarian sections of juvenile barfin flounders assayed by TUNEL. (A) positive control; (B) TL 50mm fish; (C) TL 70mm fish; (D) TL 90mm fish. PC, TUNEL-positive cells (bar = 40 μm).

Germ cell apoptosis in early oogenesis

To investigate germ cell apoptosis in early oogenesis, we performed the TUNEL assay in fish with a TL of 30mm to 100mm (Fig. 6). In the positive control fish in which the DNA was intentionally fragmented by nuclease treatment of the sections, the TUNEL-positive reaction was observed in the nuclei of many ovarian germ cells. On the other hand, the TUNEL-positive reaction was not observed in any of the cells of the fish with a TL of 3 0mm to 100mm.

DISCUSSION

In the barfin flounder, morphological differentiation of the gonad into either an ovary or testis becomes distinguishable at a TL of 35 mm (Goto *et al.*, 1999), and the oocytes develop into peri-nucleolus stage oocytes at a TL of 133 mm. In the present study, we first investigated the morphological changes in early oogenesis in barfin flounders with a TL of 30mm (before sex differentiation) to 100mm (peri-nucleolus stage oocytes exist) by light and electron microscopy. We found that the gonad had not differentiated in TL 30mm fish, but in TL 40mm fish the ovarian cavity had begun to form and morphological differentiation of the gonad had already started. Among fish with a TL of 30mm to 100mm, the degree of gonadal development and gametogenesis depended on the TL. Since these results corresponded with the report of Goto *et al.* (1999), the gonadal development in the barfin flounders used in the present study was normal and the degree of development of the germ cells was associated with the TL.

In the TL 40mm fish, the proliferated oogonia started to construct a cyst structure surrounded by somatic cells. These somatic cells were directly bound to the germ cells and were separated from other somatic cells by the basement membrane. These somatic cells also actively proliferated by mitosis until the germ cells began to undergo meiosis. We defined these somatic cells as pre-granulosa cells, because they are similar to centrally-located somatic cells (Kanamori, 1985) and will become granulosa cells with the progression of oogenesis. In fish with a TL of 40mm to 70mm, the size of the cyst as well as the number of germ cells per cyst gradually increased as the TL increased. On the other hand, in the fish with a TL of 80mm to 100mm, the cyst size and number of germ cells per cyst gradually decreased as the TL increased. In the Japanese whiting, *Sillago Japonica*, the oogonial cyst is constructed in the same manner as in barfin flounders (unpublished data). In the masu salmon, *Onchorynchus masou,* however, an oogonial cyst structure does not form (Nakamura, 1974). These findings suggest that there are two types of fish; one type forms a germ cell cyst and the other type does not form a germ cell cyst during early oogenesis. In the spawning season, barfin flounder and Japanese whiting hold more eggs than masu salmon; a barfin flounder holds 326,000 to 2,800,000

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eggs (Watanabe and Minami, 2000), a Japanese whiting holds 1,290,000 to 2,800,000 eggs (Asano and Kubo, 1995), and a masu salmon holds 360 to 900 eggs (Atoda, 1974). It seems that oogonial cyst formation is related to the fecundity of the fish species.

In the TL 60mm and 70mm fish, we could not distinguish whether the germ cells were oogonia or oocytes under light microscopic observation; the germ cells in the large cysts were observed to have condensed nuclei. We performed electron microscopic observation to confirm their developmental stage, and observed synaptonemal complexes in all of their nuclei. Since the synaptonemal complex is generally the figure of homologous chromosomal synapsis (Moses, 1968; Roeder, 1990; Wettstein *et al.,* 1984), the ovarian germ cells in TL 60mm and 70mm fish were oocytes at the zygotene or pachytene stage in the prophase of the first meiotic division, and it is suggested that oogonial mitotic division had already switched to meiosis in TL 60mm and 70mm fish.

Cysts of germ cells surrounded by somatic cells exist in the testis of teleosts as well as in the ovary of barfin flounders. It was reported that in Japanese eel, Sakhalin taimen, masu salmon, medaka, and zebrafish the spermatogonia in a cyst proliferate synchronously and develop into spermatocytes after a defined number of mitotic divisions (Ando *et al.*, 2000). In BrdU injected fish, we found that DNA syntheses in the germ cells of a cyst were completed at the same time, and that the oogonia divided synchronously and developed into chromatin-nucleolus stage oocyte in a synchronous fashion after several mitotic divisions, during early oogenesis in barfin flounders.

In gametogenesis, DNA synthesis occurs at the time of chromosome replication (S-phase) in the mitotic and meiotic prophases. In the present study, DNA synthesis in germ cells was examined by the incorporation of BrdU that had been injected into fish. High BrdU incorporation in germ cells was observed in the TL 50mm and TL 70mm fish. It was considered that the high BrdU incorporation in the TL 50mm fish was due to mitosis because mitotic metaphase was observed in some of the germ cells of the TL 50mm fish by light microscopy, and that the high BrdU incorporation in the TL 70mm fish was due to meiosis because many oocytes with synaptonemal complexes were observed in the TL 70mm fish. However, high BrdU incorporation probably reflected a mixture of cells in mitotic and meiotic prophases.

In spermatogenesis, primary spermatocytes in the leptotene stages are distinguished from the proliferated late type B spermatogonia by the size of their nuclei; the nuclei of spermatocytes are larger than those of late type B spermatogonia (Miura, 1999). Therefore, it may be possible to distinguish a proliferated oogonium from an oocyte in the early stage by the size of the nucleus. Using this criterion, the BrdU-incorporated germ cells could be divided into two types: the first type of cells had a GNA of 5–15 μm^2 (small nuclear cells), and the second type had a GNA of 30–50 μm^2 (large nuclear cells). Small nuclear cells were mainly seen in TL 50mm fish in which mitotic figures of germ cells were frequently observed, and the percentage of small nuclear cells decreased as oogenesis progressed. On the other hand, large nuclear cells were first observed in TL 60mm fish, and the percentage of these cells increased as the fish grew further. The appearance of large nuclear cells coincided with the appearance of oocytes with a synaptonemal complex in the ovary. These observations indicate that the BrdU-incorporated small nuclear cells are oogonia at the mitotic prophase and that the large nuclear cells are oocytes at the meiotic prophase; the nuclear size increased upon the initiation of meiosis.

This study showed that the size of the germ cell cyst surrounded by pre-granulosa cells changed during early oogenesis as described above. We investigated the formation of the germ cell cyst in detail using BrdU incorporation as the index. In the fish with a TL from 30mm to 60mm, BrdU was incorporated in many pre-granulosa cells. On the other hand, in the fish with a TL of 70mm or longer, BrdU was not incorporated in any pre-granulosa cell. These findings suggest that the germ cell cysts grow in fish between TL 30mm and TL 60mm due to the proliferation of pre-granulosa cells, and that the size of the germ cell cysts decreases after TL 70mm due to the invagination of the pre-granulosa cells, because the pre-granulosa cells did not proliferate and their number was fixed in fish with a TL of 70mm to 100mm.

Cell death is generally classified into two types: programmed cell death and accidental cell death. Apoptosis is programmed cell death characterized by contraction of cell size, nuclear concentration, intense condensation of chromatin and DNA fragmentation (Wyllie *et al.*, 1984). In the later part of early oogenesis in barfin flounder, the number of germ cells in the cyst decreased. It is not known whether this reduction occurs by germ cell apoptosis. Therefore, we investigated germ cell apoptosis by the TUNEL method, and found that apoptosis did not occur in any stage of early oogenesis. In mammals, many oogonia are generally formed but only a few of them are ovulated as oocytes during the life of the animal. In humans (Depol *et al.*, 1998) and mice (Coucouvanis *et al.*, 1993), it has been reported that germ cell apoptosis can occur during oogenesis. On the other hand, the fecundity of fish is much higher than that of other animal species. Germ cell apoptosis in oogenesis has not been reported in fish, which suggests that germ cell apoptosis does not occur in oogonial proliferation, and that all proliferated oogonia become mature oocytes in some fish species including the barfin flounder.

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