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Fibroblast Growth Factor May Regulate the Initiation of Oocyte Growth in the Developing Ovary of the Medaka, *Oryzias latipes*

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ABSTRACT—The distribution of fibroblast growth factor (FGF) was investigated in developing and matured ovaries of the medaka, *Oryzias latipes*. In the fry, FGF localized in the cytoplasmic region of all oocytes in the ovary at the pre-vitellogenic stage. Before the initiation of vitellogenesis, it disappeared in the cytoplasmic region and newly appeared around each oocyte, and then it localized around all oocytes in the ovary at the vitellogenic stage. Interestingly, the change in FGF distribution was orderly occurring from the posterior to anterior region of the ovary. In the adult, FGF was detected by immunofluorescence staining around the oocytes. These results suggest that FGF plays a significant role in the initiation of oocyte development through follicle cells, and the expression of FGF is rigidly regulated in the developing ovary of *O. latipes*.

INTRODUCTION

Oocyte development is rigidly regulated by the interaction between germ cells and somatic cells in vertebrates. It is initiated by the effect of gonadotropin from the pituitary gland and regulated by steroid hormone produced in the matured ovary.

In the teleostean ovary, oogenesis involves two sequential steps, the growth of the oocyte and the subsequent maturation, that are influenced by several kinds of hormones, as is true also in other vertebrates. During the growth phase, meiosis proceeds and is arrested at the diplotene stage of the first meiotic division in the oocyte. The egg membrane is formed around the oocyte and the yolk protein accumulates, causing the oocyte to increase in size. Estradiol-17β is produced in the folliclle cells in response to gonadotropin (Nagahama *et al*., 1995), and the former hormone acts as the regulator of these events (Hamazaki *et al*., 1989; Nagahama *et al*., 1995).

In *Oryzias latipes*, germ cell development is initiated earlier in females than males in the developing gonad, and oocytes are seen in the ovary of the fry soon after they hatch out (Onitake, 1972; Satoh and Egami, 1972; Satoh, 1974). Oogenesis continuously occurs from oogonium even in the matured ovary. Sex-reversal can be induced in the fry of the gonad with steroid hormone (Yamamoto, 1958, 1975) as seen in many species of vertebrates (Jimenez *et al*., 1993; Piferrer *et al*., 1994; Ganesh and Raman, 1995). Then, the production of steroid hormone must be rigidly regulated in the developing ovary. It is reported that steroidogenesis occurs in granulosa cells in *O. latipes* (Onitake and Iwamatsu, 1986). However, the mechanism regulating steroidogenesis in the developing ovary is unknown.

Fibroblast growth factors (FGFs) are important regulators of growth and differentiation of the various types of cell in vertebrates. In mammals, it is known that basic FGF supports the proliferation and differentiation of the granulosa cells *in vitro* (Gospodarowicz *et al*., 1977; Gospodarowicz and Bialecki, 1978; Lavranos *et al*., 1994). Thus, it seems likely that basic FGF might be one of the regulators of follicular morphogenesis, acting through granulosa cells (Gospodarowicz, 1990).

In this study, to examine the participation and the mechanism of FGF in controlling oocyte development in the developing ovary of teleost, we investigated the distribution of FGF in the developing ovary of the medaka, *Oryzias latipes*, by immunofluorescence staining.

MATERIALS AND METHODS

Animals

Adult or immature specimens of the medaka, *Oryzias latipes*, were purchased from Morikawa Fish Farm (Yamato-koriyama, Nara, Japan). Immature fish were obtained from the fertilized eggs prepared in our laboratory. They were kept at 26°C in our laboratory before use in experiments.

Histology

The ovaries dissected out from the bodies of the adult and immature fish, which were 7-12 mm in body length, were fixed in Bouin's fixative. Sections of 6 μm thickness were prepared and stained in Delafield's hematoxylin and eosin solution.

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Immunofluorescence staining

At least five individuals were used for each experiment. The methods used were the same as those previously reported (Watanabe *et al*., 1997). In brief, ovaries from the adult or immature fish, 7-12 mm in body length, were fixed in 2% paraformaldehyde in PBS (0.05 M phosphate buffer, pH 7.4) at 4°C for 2 hr, and then they were immersed in 30% sucrose in PBS at 4°C for 1.5 hr. They were embedded in O.C.T. compound (Miles) and frozen in liquid nitrogen. Frozen sections of 8 μm in thickness were serially prepared. They were reacted with a human FGF2-specific monoclonal antibody (described as FGF antibody) (Reilly *et al*., 1989) at room temperature for 30 min, and then with the FITC-labeled antibodies raised in the goat against mouse IgG (diluted 1: 30 in PBS that contained 3% BSA; MBL) at room temperature for 40 min.

In controls, two types of experiments were pursued. One was that 100 ng of bovine FGF2 (Austral Biological) was added to 200 μl of the solution containing the FGF2 specific antibody, and then used for the first immunoreaction. Another was that the chick-specific monoclonal antibody (Watanabe *et al*., 1993) or PBS that did not include any antibody was used for the first immunoreaction instead of the FGF2-specific monoclonal antibody. The sections were observed with a fluorescence microscope (BH-RFK, Olympus).

RESULTS

Distribution of FGF in the developing ovary of *O. latipes*

In the immature fish, 7-8.5 mm in body length, the ovary was localized between the mesonephric tubule and gut in the dorsal-posterior region of the abdominal cavity (Fig. 1a, b). The anterior region of the ovary bound to peritoneum under the air-bladder. Many oocytes in the ovaries were at the pachytene or early diplotene stage of meiotic prophase (Fig. 2a). The size of each oocyte was about 30 μm, which suggested that they were at pre-vitellogenic stage. Follicle cells were seen but not developed. Strong fluorescence was detected in the ovary and muscle by the immunofluorescence staining with the anti-FGF antibodies (Fig. 2b). In the ovary, it was localized in the cytoplasmic region of all oocytes.

In control, auto-fluorescence was seen in the nucleus of somatic cells including follicle cells and muscle (Fig. 2c). It was easily distinguishable from that of FITC by the morphology and the difference in wavelength for excitation. No signal was detected in any oocyte or follicle cell.

In the immature fish of 9.0 mm in body length, the morphological features of the oocytes and follicle cells in the ovary were similar to those in the younger fry. Strong fluorescence was detected in the ovary (Fig. 3a, b). It localized to the cytoplasmic regions of each oocyte in the anterior region of the ovaries (Fig. 3d). Little fluorescence was detected at the sites of follicle cells. This pattern was the same as that seen in the individuals of less than 8.5 mm in body length. However, in the posterior region of the same ovaries, strong fluorescence was seen in the follicle cell layer but not in the oocytes (Fig. 3e). The localization pattern was just inverted compared to that of the anterior region of the same ovary. In control, significant fluorescence was not detected (Fig. 3c).

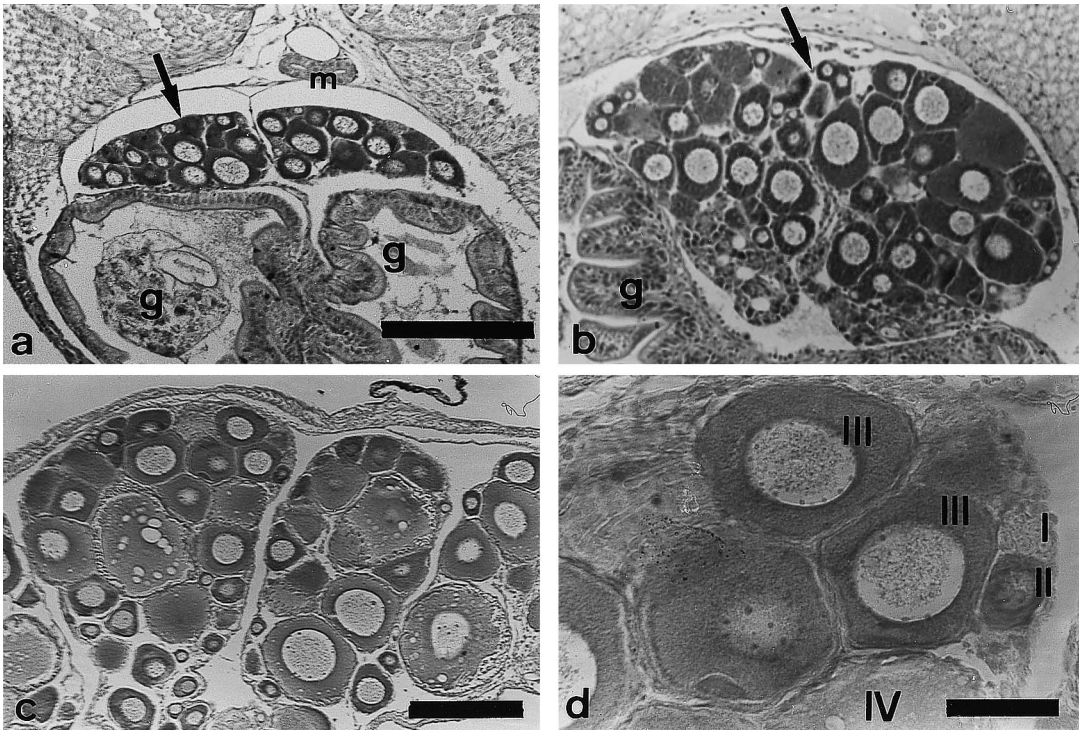

Fig. 1. Sections of the ovary of *O. latipes*. Six μm paraffin sections of the body of the immature fish (**a**, **b**), and the ovary of the adult (**c**, **d**) were cut and stained with hematoxylin-eosin solution. (**a**) A section of the immature fish of 8.5 mm in length. Arrow indicates the ovary. The size of each oocyte was similar. (**b**) A section of the immature fish of 12 mm in length. Arrow indicates the ovary. The number of oocytes was increased and the size of each oocyte varied compared with (**a**). (**c**) Many oocytes at different stages were observed in the ovary. (**d**) High-magnification view of (**c**). I, II, III, and IV indicate the developmental stage of each oocyte (Iwamatsu *et al*., 1988). g, gut; m, mesonephric tubule. Bar: 200 μm (**c**), and 50 μm (**a**, **d**).

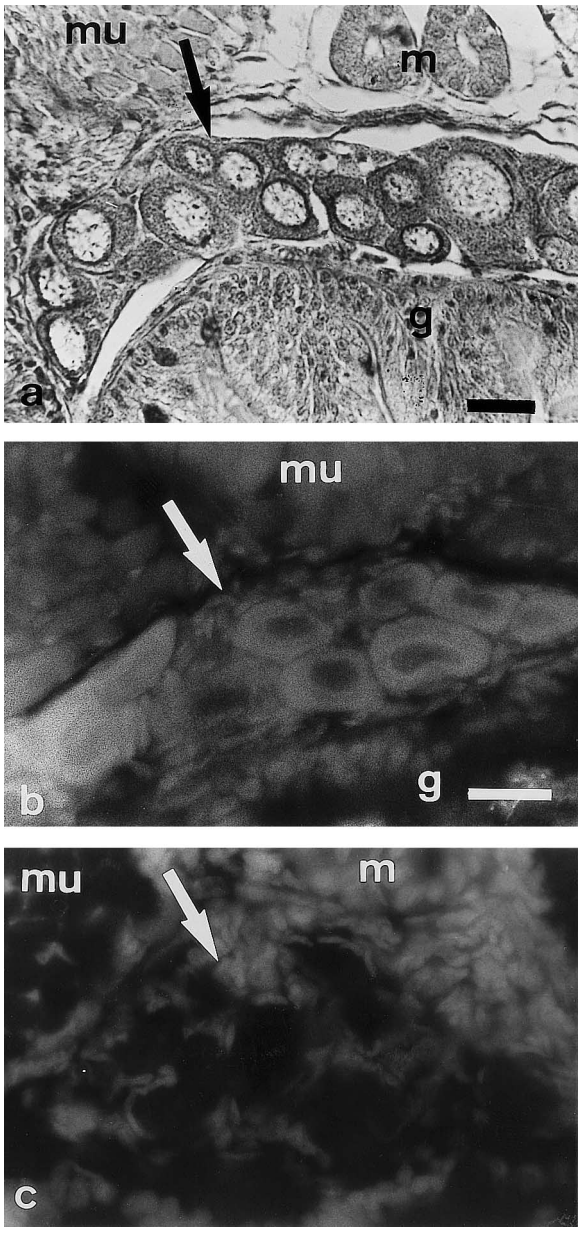

Fig. 2. Immunofluorescence staining of the ovary of fry, 8.5 mm in length, of *O. latipes* with the monoclonal antibody against FGF protein. (**a**) A six μm transverse section was stained with hematoxylin-eosin solution. (**b**) An eight μm frozen section of the fry was stained. The cytoplasmic regions of each oocyte and muscle were strongly stained. (**c**) Control. Bovine FGF2 was added to the solution of FGF-specific antibody and then, used in the first immunoreaction. Autofluorescence was seen in the nucleus of somatic cells. Arrows indicate the ovary. g, gut; m, mesonephric tubule; mu, muscle. Bar: 50 μm.

In the immature fish of more than 10-12 mm in body length, many oocytes were observed in the ovary (Fig. 1b). The size of these oocytes varied (Figs. 1b, 4a), indicating that some had begun to undergo vitellogenesis. Fluorescence was detected not in the oocyte but at the site of follicle cells (Fig. 4b). No difference was seen in the localization pattern of fluorescence. In control, significant fluorescence was not detected (Fig. 4c).

Distribution of FGF in the matured ovary of *O. latipes*

In the ovary of the adult medaka, oocytes at various stages of oogenesis were seen (Fig. 1c, d). Growing oocytes were surrounded by granulosa cells and theca cells. Strong fluorescence was detected around developing oocytes at all stages by the immunofluorescence staining with the anti-FGF antibodies (Fig. 5a). These regions corresponded to the sites of follicle cells. In control, no signal was detected in or around the oocytes (Fig. 5b).

Specificity of the antibodies against FGF

To examine the specificity of the fluorescence staining with the antibodies against human FGF2, the binding of the antibodies to the antigen was blocked with bovine FGF2 in the first immunoreaction. No fluorescence of specific staining was seen in the ovarian sections of the immature (Figs. 2, 4) and the mature fish (Fig. 5b). The results were quite similar as for control.

DISCUSSION

FGFs are regulating factors of the proliferation and/or differentiation of various types of cell in vertebrates (Gospodarowicz, 1990). In this study, we used the immunostaining technique with a monoclonal antibody against human basic FGF for the distribution of FGF in the developing ovary of the medaka. Human FGF2 protein is effective in other species (Dawid *et al*., 1990; Niswander *et al*., 1993). In this study, the first immunoreaction was inhibited by the addition of bovine FGF2 to the solution containing the monoclonal antibody against human FGF2 (Fig. 2b). It has been reported that the antibody can neutralize the effect of basic FGF (Reilly *et al*., 1989). Recently a cDNA fragment of the medaka was isolated with this antibody as a probe in our laboratory. It codes a functional domain of basic and acidic FGF for the binding to the receptor that includes a cystein residue conserved among species (unpublished data). These findings suggest that the antigen stained with anti- FGF antibody in this study was a homologue of FGF in the medaka.

The change in FGF distribution in the developing ovary of the medaka

FGF was localized in the cytoplasmic region of oocytes of the fries, 8.5 mm in body length (Fig. 2). It disappeared in the oocyte cytoplasm of fry, 9 mm in body length, and synchronously appeared in follicle cells (Fig. 3). In teleosts, follicle cells appear at the early stage of development of the ovary (Satoh, 1974; Nakamura and Nagahama, 1985; Nakamura *et al*., 1993). In the fry of less than 9 mm, the oocytes were nearly equal in diameter, as shown in the present study. This indicates that all oocytes were at the pre-vitellogenic stage. And then, some of the oocytes enlarged as the fry grew. These results suggest that FGF acts as a paracrine factor on follicle cells and regulates the initiation of vitellogenesis in the devel-

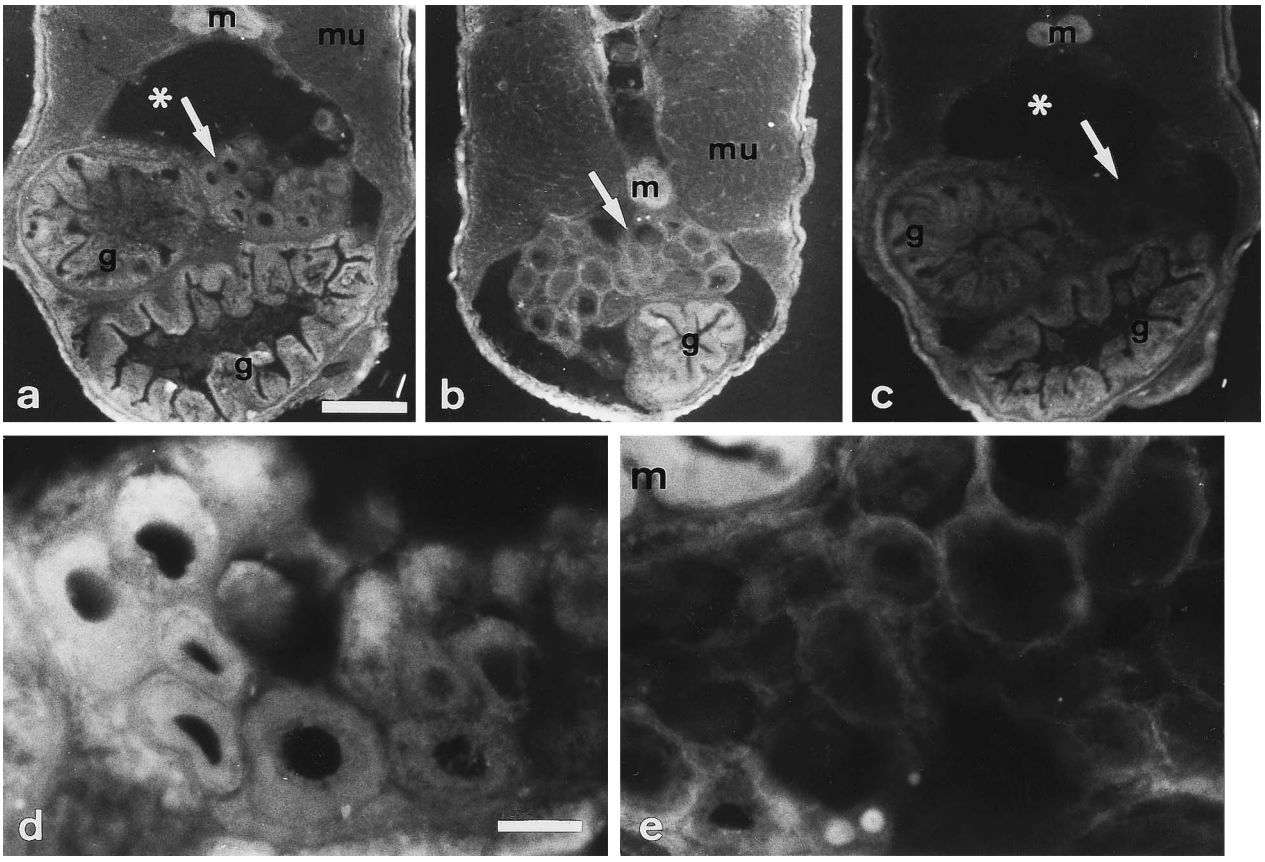

Fig. 3. Sections of the ovary of the fry, 9.0 mm in length, of *O. latipes*. An eight μm frozen section of the immature fish was stained with the monoclonal antibody against FGF. (**a**) The section including the anterior portion of the ovary. An air-bladder (asterisk) and a pair of guts, the features of the anterior region, were sectioned. (**b**) The section including the posterior portion of the same ovary of (**a**). Air-bladder was not seen, and gut was sectioned alone. (**c**) Control. The FGF-specific antibody was not included in the immunoreaction. Auto-fluorescence was seen in mesonephric tubules and gut. No significant fluorescence was seen in the ovary and muscle. (**d**) and (**e**) High magnification view of (**a**) and (**b**). Strong fluorescence was specifically seen in the cytoplasmic region of each oocyte (**d**) and the somatic cells around them (**e**). Arrows indicate the ovary. g, gut; m, mesonephric tubule; mu, muscle. Bar: 200 μm (**a**) and 50 μm (**d**).

oping ovary of the medaka.

In this study, the change in FGF localization occurred along the anterior-posterior axis in the developing ovary (Fig. 3). It occurred in all oocytes in the ovary before oocytes are not developed. This result suggests that the expression of FGF is regulated independently of oocyte development in the developing ovary. However, the mechanism regulating it is unknown.

In teleosts, vitellogenesis is regulated by estradiol-17β produced by follicle cells (Nagahama *et al*., 1995). FGF may activate steroidogenesis for the initiation of vitellogenesis in the developing ovary of the medaka. It has been reported that sex-reversal could artificially take place in the gonad of the fry of *O. latipes* by the stimulation of steroid hormone (Yamamoto, 1958, 1975). In female, it is induced before the period when steroidogenesis is initiated (Onitake, 1972; Satoh and Egami, 1972; Yamamoto, 1975). The period of loss of the response to sex-reversal corresponds with the period when FGF disappeared in the oocytes. From these points, it is speculated that change in FGF distribution correlates with sex determination of germ cells in the developing gonad. The process of sex determination is not well-known in teleost and FGF may be a useful marker for the analysis of it.

The possible role of FGF in follicle cells of the medaka

In the teleostean ovary, the follicle consists of the oocyte and two layers of follicle cells. During oocyte growth, the oocyte increases in size with the accumulation of yolk protein, and a thick egg membrane is formed between oocyte and follicle cells. It is known that the follicle cells, both granulosa cells and theca cells, are indispensable for the regulation of these events since estradiol-17β is synthesized in them in response to gonadotropin (Nagahama *et al*., 1995). In medaka, it has been reported that steroidogenesis occurs only in granulosa cells (Onitake and Iwamatsu, 1986).

In this study, FGF was found around the oocytes in the ovary of the adult medaka. The distribution of FGF corresponded to the site of follicle cells. In mammal, basic FGF can support the proliferation of granulosa cells in the bovine follicles *in vitro* (Gospodarowicz *et al*., 1977; Lavranos *et al*., 1994). It is also known that basic FGF can affect the differentiation of granulosa cells *in vitro* (Gospodarowicz and Bialecki,

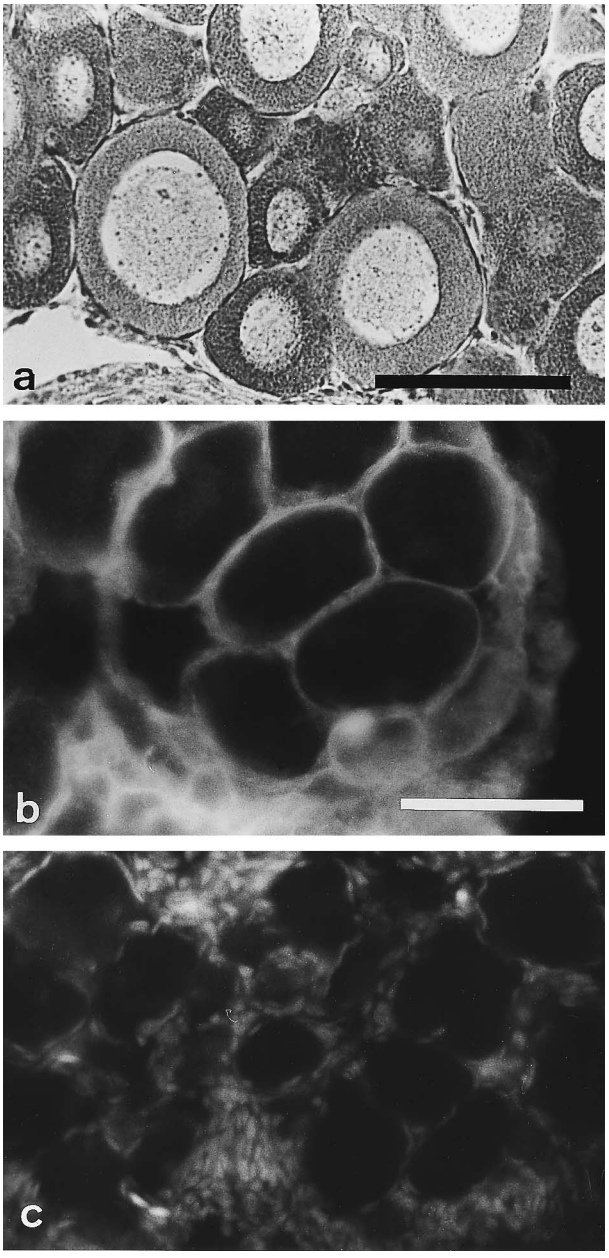

Fig. 4. Immunofluorescence staining of the ovary of fry, 12 mm in length, of *O. latipes* with the monoclonal antibody against FGF protein. (**a**) A six μm section was stained with hematoxylin-eosin solution. (**b**) An eight μm frozen section of the fry was stained. Strong fluorescence was seen around each oocyte. (**c**) Control. Bovine FGF2 was added to the solution of FGF-specific antibody and then, used in the first immunoreaction. Auto-fluorescence was seen in the nucleus of somatic cells. Bar: 50 μm.

1978). Furthermore, very electron-dense lipid granules can be seen in granulosa cells cultured under the influence of FGF (Gospodarowicz *et al*., 1977). These results suggest that FGF acts as a regulator of the proliferation and differentiation of those cells in the medaka.

Steroidogenic cells first appear in the ovaries of fish of 7-9 mm in body length (Kanamori *et al*., 1985). In this study, FGF was first seen at the sites of follicle cells at the same period and continued to localize to the same region (Fig. 4). This suggests that FGF is important for steroidogenesis in follicle cells. The steroidogenic activities of follicle cells increase as oocyte growth proceeds (Nagahama *et al*., 1995). However, the distribution of FGF was not specific for the stages of developing oocytes (Fig. 5). It is reported that TGF-β is produced in the theca cells and stimulates the synthesis of estradiol-17β in the granulosa cells *in vitro* in the bovine (Skinner *et al*., 1987). It seems likely that some other factors are cooperate with FGF to regulate steroidogenesis in follicle cells during oocyte growth.

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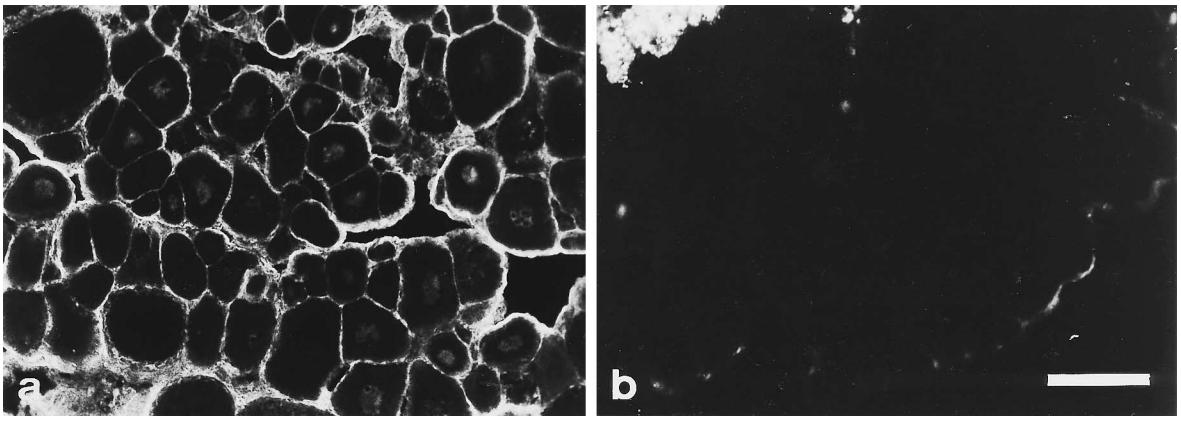

Fig. 5. Immunofluorescence staining of the ovary of mature fish of *O. latipes* with the monoclonal antibody against FGF protein. (**a**) An eight μm frozen section of the ovary of the mature fish was stained. (**b**) Control. The FGF-specific antibody was not included in the immunoreaction. Autofluorescence was seen in haemocytes and the nucleus of follicle cells. Bar: 200 μm.

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