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Dynamic Growth of Oocytes of the Medaka, *Oryzias latipes* I. A Relationship between Establishment of the Animal-Vegetal Axis of the Oocyte and Its Surrounding Granulosa Cells

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ABSTRACT—In previtellogenic oocytes of the freshwater teleost, *Oryzias latipes*, the yolk nucleus is a prominent structure in the cytoplasm. The process by which the animal-vegetal (A-V) axis is established in these oocytes was observed by focusing on the yolk nucleus, granulosa cells and attaching filaments using light and electron microscopy. Prior to appearance of the rudiments of attaching filaments a special area was recognized near the yolk nucleus of early stage III oocytes by gathering of granulosa cells with irregular-shaped nuclei. The rudiments of attaching filaments clearly appeared in the area where granulosa cells gathered. The area with a cluster of granulosa cells and the rudiments of attaching filaments corresponded to the position of the yolk nucleus. In late stage III, the future vegetal pole area (VPA) was easily recognizable by the presence of the rudiments of attaching filaments. The present results suggest that the vegetal pole may be primarily determined by the random position of the yolk nucleus and established by reciprocal interactions between the cortical cytoplasm containing the yolk nucleus and its surrounding granulosa cells. It was postulated that the animal pole is determined by differentiation of the micropylar cell from a granulosa cell and by locomotion of granulosa cells around the axis connecting the centers of the germinal vesicle and the yolk nucleus before the initiation of vitellogenesis.

INTRODUCTION

There is generally clear-cut evidence of biochemical and morphological differences between the animal and the vegetal hemispheres of fully grown oocytes in non-mammalian vertebrates. In the freshwater teleost *Oryzias latipes*, the animal-vegetal (A-V) axis is established in the previtellogenic period of oogenesis. The growing oocyte remains in contact with flattened granulosa cells through cytoplasmic projections. The oocyte and granulosa cells are enclosed with a basement membrane. The A-V axis is manifested during formation of the egg envelope (chorion) during which the positions of the micropyle in the animal pole and of attaching filaments in the vegetal pole are determined. The micropyle is a convenient marker for identifying the animal pole. In the oocyte of most teleost species, a micropylar cell with one large cytoplasmic process participates in the formation of the micropyle (cf. Hart, 1990). The micropyle is always formed in the animal pole by a micropylar cell that differentiates from a granulosa cell in previtellogenic medaka follicles (Nakashima and Iwamatsu, 1989). The vegetal pole area (VPA) is established by the relationship between the behavior of the granulosa cells and formation of attaching filaments at the vegetal pole during oogenesis (Iwamatsu, 1992).

In *Spinax* and *Chimaera*, the cells of the follicular epithelium are flat at the animal pole, and columnar at the vegetal pole (Wallace, 1903). We also recently reported that in the medaka follicles there are morphological and physiological differences in granulosa cells that correspond to regional differences along the A-V axis of the oocyte (Iwamatsu *et al.*, 1994). In order to understand the mechanism by which the A-V axis of the oocyte is established, the relationship between establishment of the A-V axis and the differentiation of granulosa cells closely associated with the oocyte was examined in the present study. The results suggest that the oocyte axis is established by reciprocal interactions of the oocyte with granulosa cells during the previtellogenic phase of follicle development.

MATERIALS AND METHODS

Light microscopy

Ovaries of the medaka *Oryzias latipes*, were obtained from the opened body cavities of females after their brains were pithed. The ovaries were fixed for 12 hr at 0-4°C in Bouin's fixative that was diluted 1/3. After dehydration, samples were embedded in paraffin and sectioned 7 μm in thickness. Paraffin sections mounted on glass slides were stained with Delafield's haematoxylin after removal of paraffin. After coverslips were mounted with Canada balsam, the sections were observed with an ordinary light microscope. The stages of oocyte

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development were determined according to the table of oogenesis prepared in a previous study (Iwamatsu *et al.*, 1988).

Fluorescence microscopy

For observation of follicle cell nuclei, dissected ovaries were fixed in 4% glutaraldehyde in 0.1 M Tris-HCl buffer (pH7.4) for 3 hr or more at 4-8°C. Fixed and separated follicles were rinsed once in saline, immersed for 30 min in 10 μg/ml Hoechst 33258 (Sigma, St. Luois, MO) in saline, rinsed once in fresh saline and observed through a fluorescence microscope (× 200, excitation filter G365, emission filter LP420, Olympus).

Transmission electron microscopy

Immediately after ovaries were obtained from mature females, they were prefixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and postfixed for 2 hr in osmium tetroxide at 4°C. These samples were washed in 10 mM phosphate buffer (pH 7.4), dehydrated in ethanol followed by propylene oxide and embedded in Epon 812. Ultrathin sections were observed after staining with lead citrate and uranyl acetate.

RESULTS

Relationship between yolk nucleus and the vegetal pole area

In live follicles less than 100 μm in diameter (stage III), there was no morphological evidence of the A-V axis (see Iwamatsu *et al.*, 1988). Sections of these follicles revealed that the cytoplasm of all the stage III oocytes already had the yolk nucleus which was a round-shaped mass stainable with haematoxylin (Fig. 1). The position of the yolk nucleus in these oocytes was random in relation to the ovarian surface. The yolk nucleus in stage III oocytes mainly consisted of thread-like bodies (granulofibrillar material), smooth endoplasmic reticulum and some mitochondria (Fig. 2). The primitive attaching filaments were first recognized in the vegetal pole area (VPA) as a cluster of verruciform-like rudiments on the surface of the early stage IV oocyte before the chorion began to form, as described in a previous report (Iwamatsu, 1992).

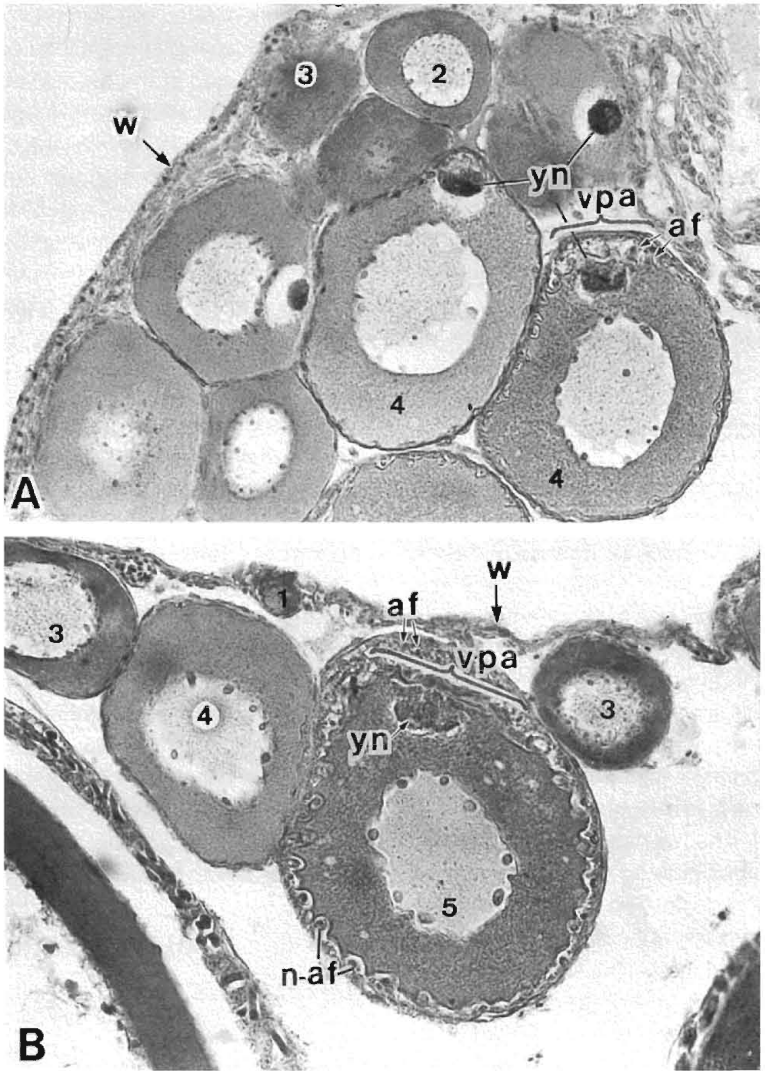

Fig. 1. Micrographs of sections through the ovary of the medaka showing follicles containing oocytes in different stages of oogenesis. The vegetal pole area (vpa) identified by its thick follicular cell layers and attaching filaments (af) in the stage IV oocyte is localized at the yolk nucleus side. In A, previtellogenic oocytes at stage II (2), stage III (3) and stage IV (4) showing the yolk nucleus (yn), positioned randomly to the ovarian surface (w). In B, the stage V oocyte (5) with non-attaching filaments (n-af) contains a large yolk nucleus just dispersing in the region of the vpa. × 260.

In oocytes at this stage the VPA could be recognized by the thickened layer of follicle cells (Fig. 1A, B). In oocytes at stage V the yolk nucleus was more distinctive and was undergoing dispersion near the surface of the oocyte (Fig. 1B). The yolk nucleus which was dispersing around the cytoplasm in this stage oocyte was composed of developed rough endoplasmic reticula, Golgi complexes, various-sized vesicles and prominent mitochondria (Fig. 2). The VPA, which was recognized by the compact distribution of the verruciform-like rudiments of attaching filaments, always corresponded to the

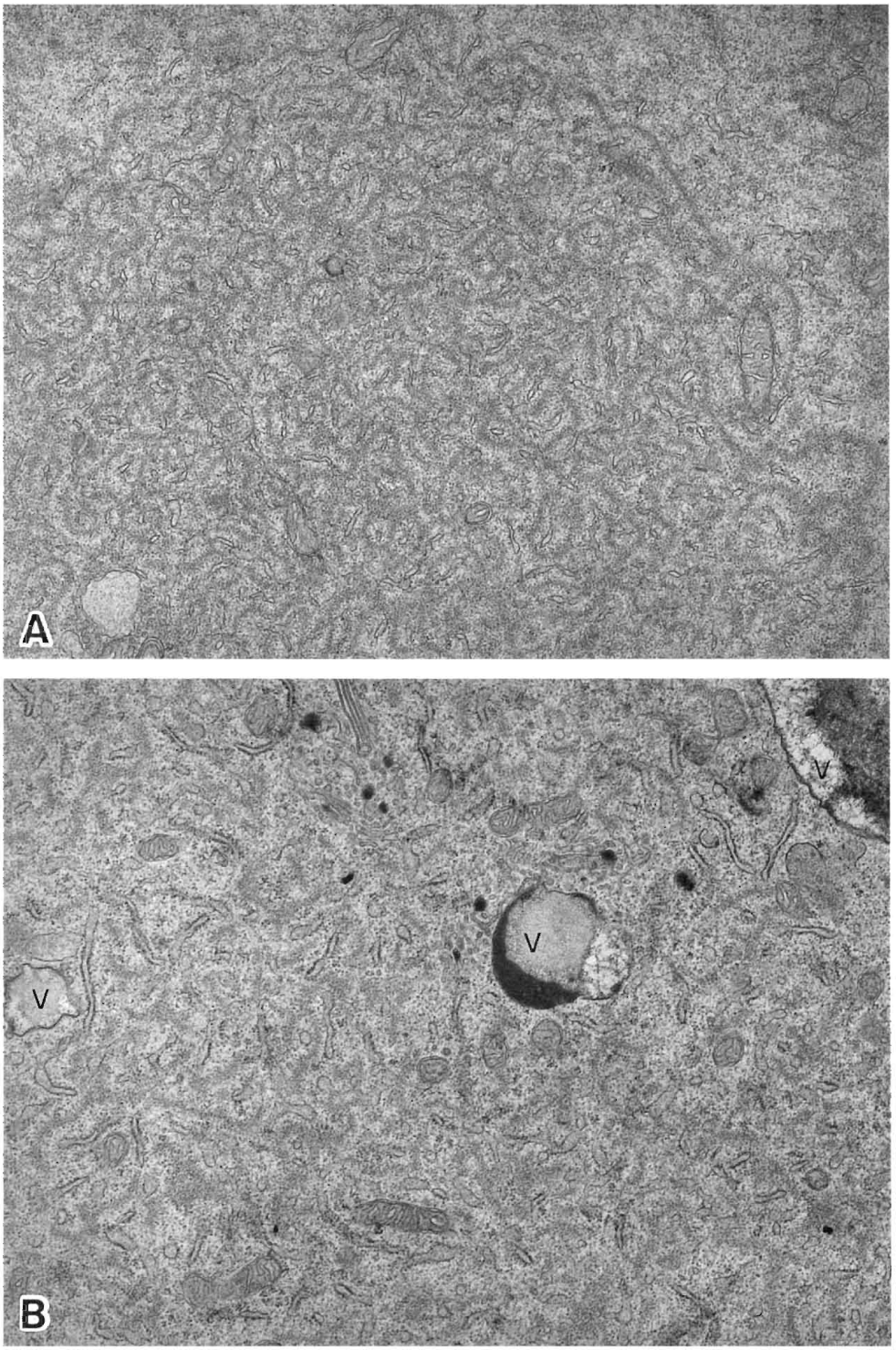

Fig. 2. Electron micrographs of yolk nuclei of previtellogenic medaka oocyes. A, A yolk nucleus in a stage III oocyte. It is composed of aggregates of cytoplasmic organelles such as thread-like structures, smooth endoplasmic reticula and mitochondria. × 11,900. B, A yolk nucleus in a stage IV oocyte showing various sized vesicles (V), Golgi complexes and rough endoplasmic reticula. × 17,000.

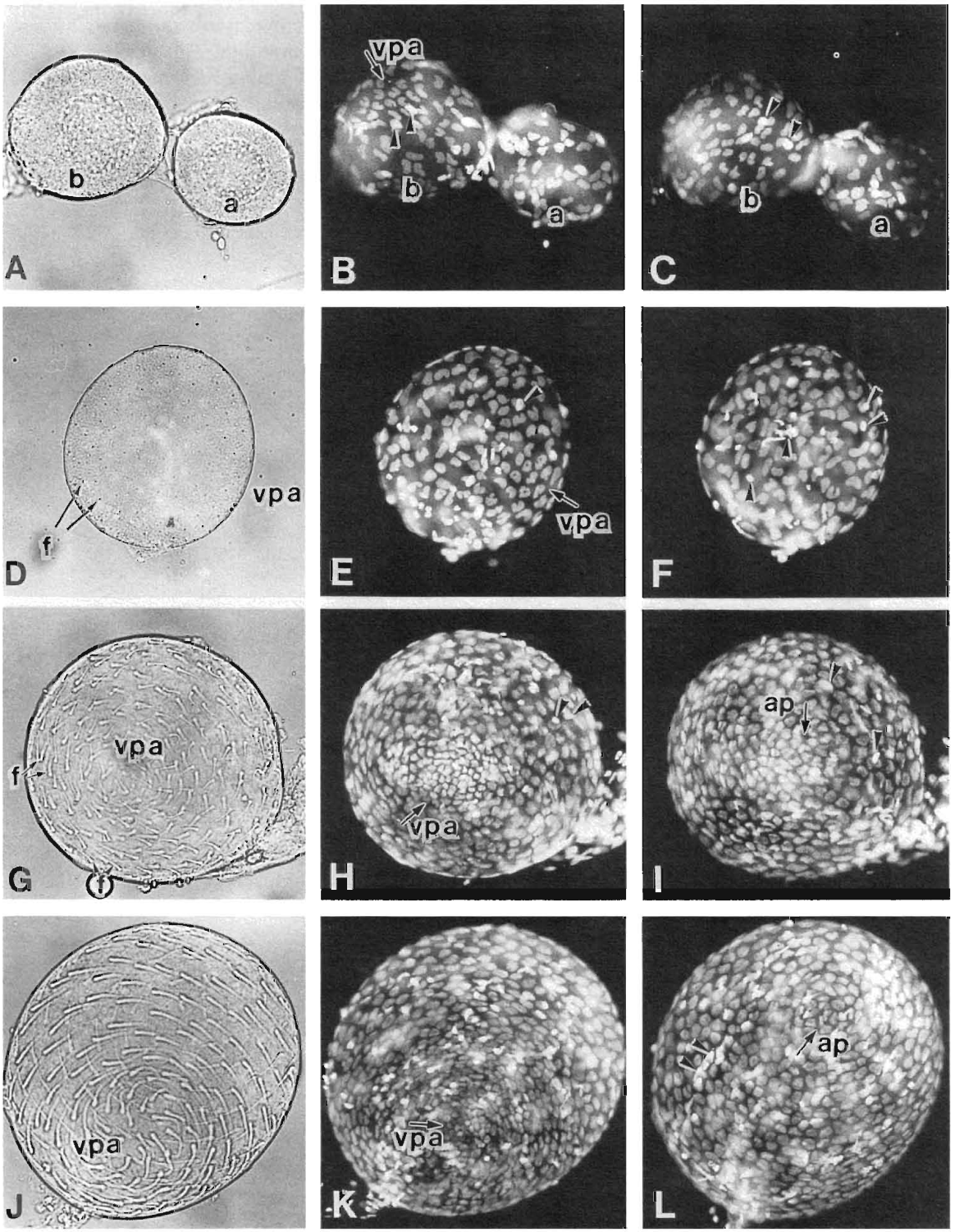

Fig. 3. Distribution of follicle cells in various sized follicles of the medaka. (× 208). The nuclei of the granulosa cells stained with Hoechst dye gathered in the restricted area which is the future site of the vegetal pole area (vpa) in young oocytes (A,D,G and J). The nuclei of thecal cells (arrowheads) are randomly distributed on the granulosa cell layer. One side of the oocyte surface (B,E,H and K) and the opposite side (C,F,I and L) are respectively focused for observing the nuclei of follicle cells. A-C, No filament rudiments are seen on the surface of stage II (a) and stage III (b) oocytes. A cluster of horseshoe-shaped nuclei is seen in the future vpa of the oocyte (b) in B. D-F, Filament rudiments (f in D) can be seen as dots on an oocyte at stage III, and the gathered nuclei (vpa) are observed in E. G-I, The stage IV oocyte shows the unilateral bending of attaching and non-attaching filaments (f) and unilaterally elongated nuclei of granulosa cells. J-L, The stage V oocyte is similar to the stage IV oocyte. In the vegetal pole area (vpa), compactly clustered tall granulosa cells have smaller nuclei than those in the animal pole (ap).

position of the yolk nucleus (Fig. 1A, B). The spiral pattern of attaching and non-attaching filaments on the chorion around the vegetal pole (the position of the yolk nucleus) was observed in oocytes (121-250 μm) at stage IV in the previtellogenic phase (Fig. 3).

Relationship between granulosa cells and attaching filaments

As shown in Fig. 3, flat granulosa cells with an irregular-shaped nucleus were located at random on the surface of stage II oocytes (Fig. 3A-D). A cluster of granulosa cells with horseshoe-shaped nuclei was observed in stage III oocytes before rudiments of attaching filaments appeared. In contrast, a small number of theca cells was distributed randomly on the granulosa cell layer. The rudiments of attaching filaments were formed as verruciform structures near the junctions between granulosa cells on the surface of the eminence of cortical cytoplasm that had no cytoplasmic organelles (Figs. 4 and 5). The number of granulosa cells sharply increased in follicles larger than 130 μm in diameter with the primitive attaching and non-attaching filaments (Fig. 6). The nuclei of the granulosa cells elongated in a unilateral direction perpendicular to the A-V axis of the oocyte. This direction was consistent with the bending direction of the filaments (Fig. 3). Microphotographs of the surface of the follicle revealed that the tall granulosa cells of stage V oocytes contained small nuclei and were distributed more compactly than in other area(Fig. 3H, K). The stalk-like structure by which the oocyte attached to other portion of the ovary originated from the follicular surface without regard to the A-V axis (Fig. 4).

Recognition of the A-V axis by positions of yolk nucleus, attaching and non-attaching filaments, the germinal vesicle and the micropyle

Morphological features of the A-V axis of previtellogenic oocytes included the positions and bending direction of attaching and non-attaching filaments on the chorion, the yolk nucleus, the germinal vesicle and the micropyle. Table 1 summarizes the results of cytoplasmic examinations.

Young transparent oocytes less than 100 μm in diameter had a yolk nucleus in the cytoplasm and a germinal vesicle in the central region. In stage IV oocytes the VPA could be recognized by the compact distribution of primitive attaching filaments. Sections of oocytes revealed that the yolk nucleus was always located in the cytoplasm adjacent to the VPA. Near the end of stage IV of oogenesis, the attaching filaments as well as non-attaching filaments elongated in a unilateral direction around the A-V axis (Figs. 3 and 7). The position of the germinal vesicle began to shift toward the side opposite the VPA, as the yolk mass or its platelets were formed and increased in the VPA during the vitellogenic phase of oogenesis. After this stage, the micropyle was easily found in the thickened animal pole side of the chorion (see Fig. 3 of Iwamatsu *et al.*, 1988).

DISCUSSION

One of the findings in the present study is that the position of the yolk nucleus identified by its electron-dense, thread-like structure (granulofibrillar material) corresponds to the vegetal pole side of the oocyte. This finding confirms the suggestion that in *Xenopus laevis* the area in which the granulofibrillar material disperses becomes the vegetal pole of the oocyte (Takamoto, 1981; Heasman *et al.*, 1984). Similar observations in invertebrates that the axis connecting the center of the nucleus to the yolk nucleus at the vegetal side of the oocyte corresponds to the A-V axis of the egg are cited by Raven (1961). It has also been reported that the subcortical mitochondria in *Lebistes reticulatus* oocytes show a much

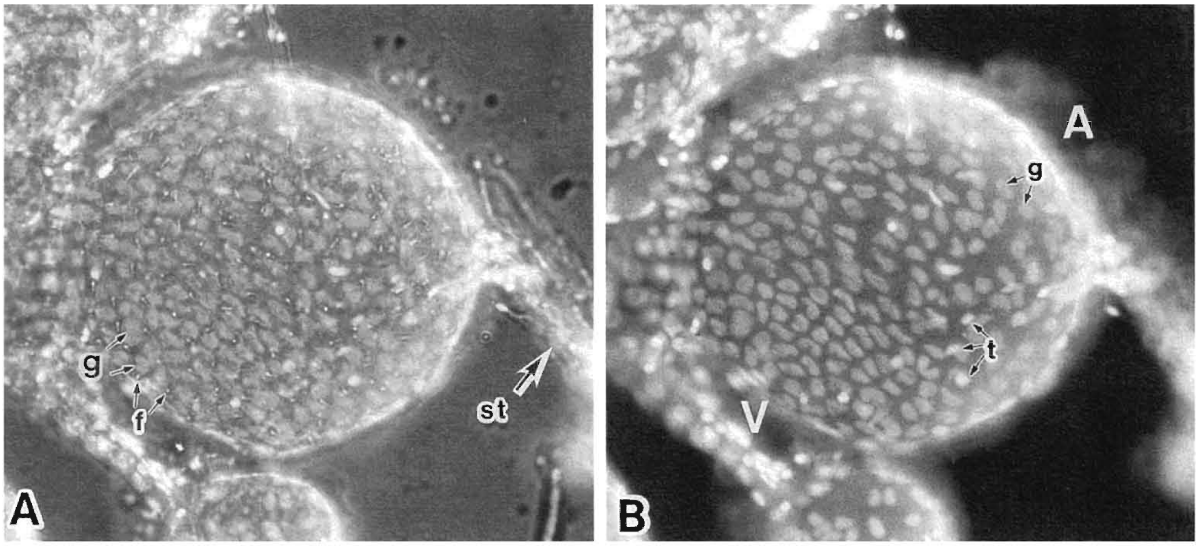

Fig. 4. The relationship between the positions of filaments and granulosa cells. In early stage IV, most differentiating filaments (f) on the oocyte surface are located between the nuclei (g) of granulosa cells (A, ordinary light microscope; B, fluorescence microscope). At the vegetal pole side (V), the nuclei of granulosa cells are compactly distributed. A, the animal pole side; st, the stalk of the follicle; t, thecal cells. × 420.

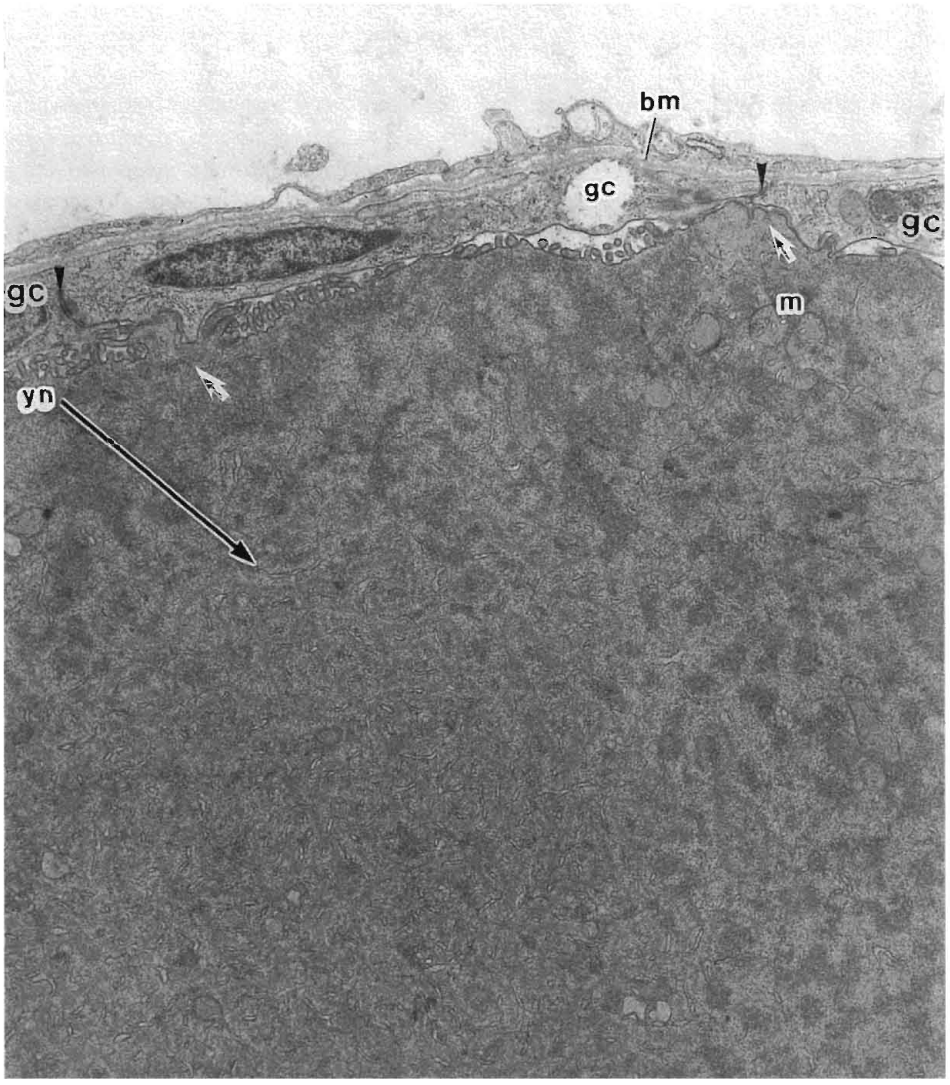

Fig. 5. Electron micrograph of part of a follicle in stage III of oogenesis. The rudiments (small arrows) of attaching filaments are formed on a cytoplasmic eminence lacking cell organelles near the junctions (arrowheads) between granulosa cells (gc) in the vicinity of the yolk nucleus (yn arrowed). bm, basement membrane; m, mitochondria. × 11,350.

Table 1. Recognition of the A-V axis of oocytes by attaching filament, germinal vesicle and micropyle in *Oryzias latipes*

Size (μm) of oocytes	No. of oocytes examined	No. (%) of oocytes showing the A-V axis by			
		YN	AF	GV	MP
60-100	51	51 (100)	0	0	0
101-150	117	104 (88.9)	62 (53.0)	0	0
151-200	80	62 (77.5)	79 (98.8)	0	0
201-300	84	4 (4.8)	84 (100)	0	0
301-400	54	0	54 (100)	2 (3.7)	3 (5.6)
401-500	41	0	41 (100)	14 (34.1)	17 (41.4)
501-600	23	0	23 (100)	23 (100)	21 (91.3)
601-700	24	0	24 (100)	24 (100)	24 (100)
701-800	11	0	11 (100)	11 (100)	11 (100)
801-900	9	0	9 (100)	9 (100)	9 (100)
901-1000	9	0	9 (100)	9 (100)	9 (100)
1001-	6	0	6 (100)	6 (100)	6 (100)

AF, attaching filament; MP, micropyle; GV, germinal vesicle; YN, yolk nucleus

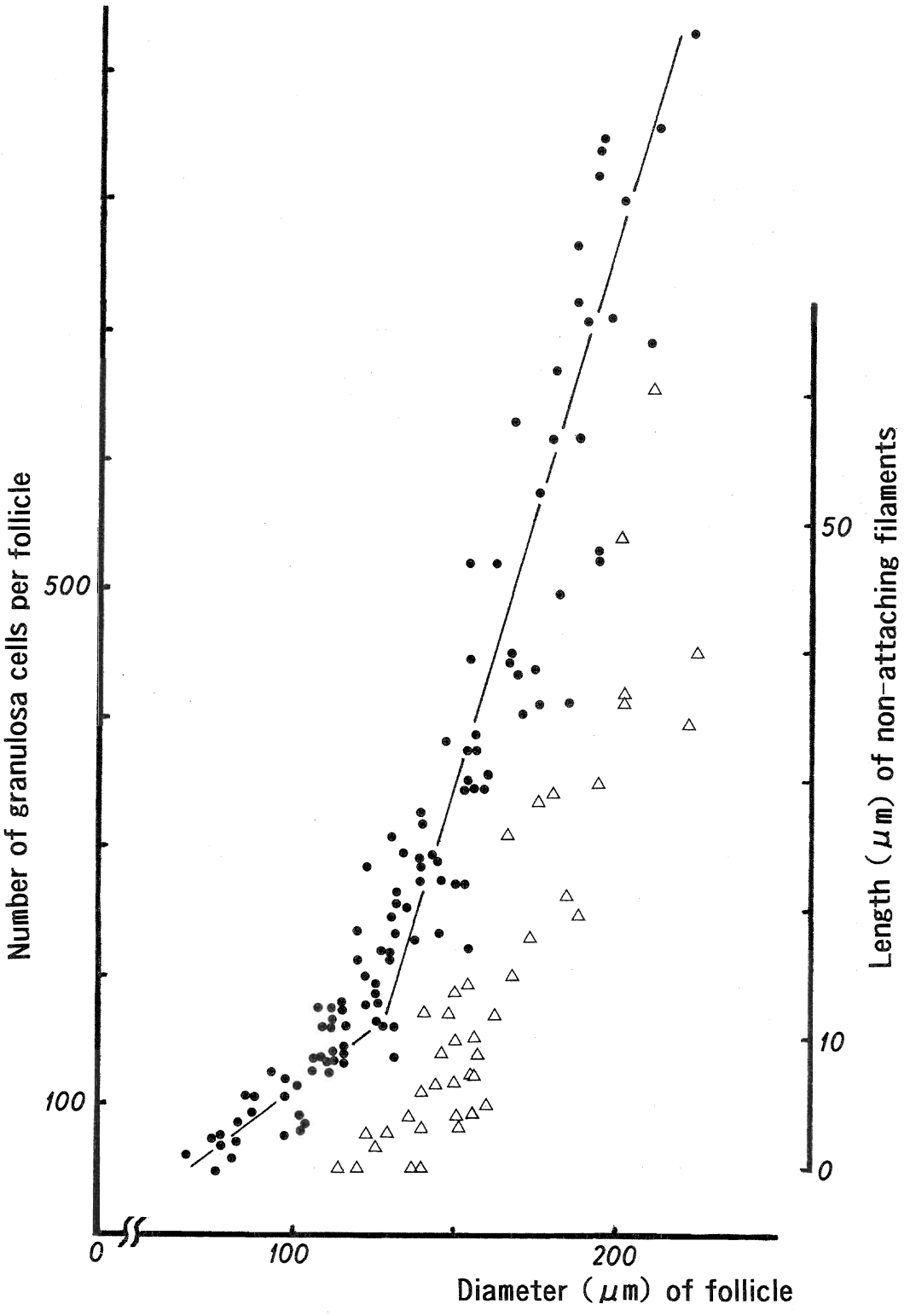

Fig. 6. Relationship between the size of follicles and the number of granulosa cells and the overall length of non-attaching filaments. The number of granulosa cells (dots) increases sharply in follicles more than 130 μm in diameter at stage I or later, whereas the length of the non-attaching filaments (triangles) increases gradually as follicles grow.

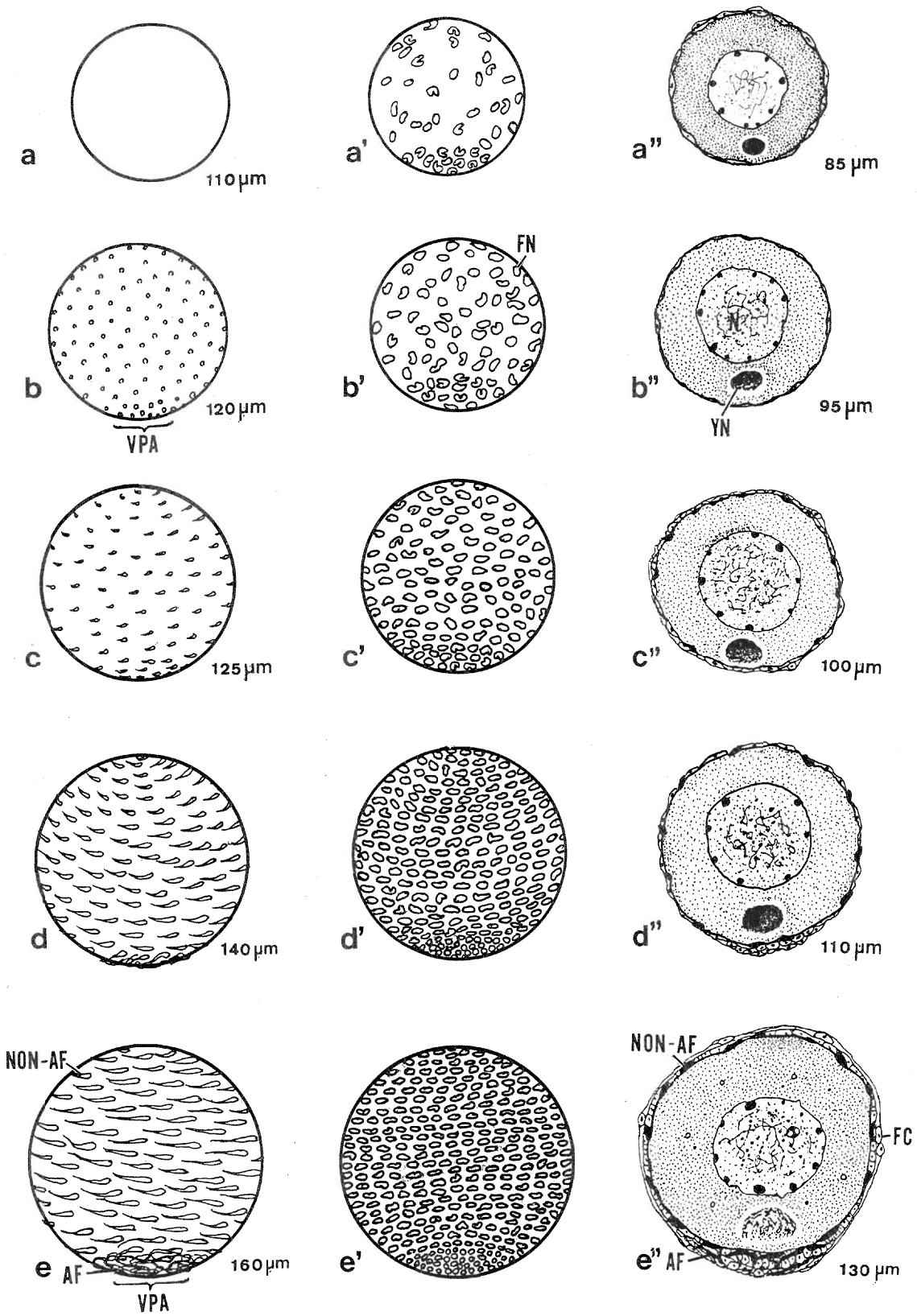

Fig. 7. Diagrams summarizing the observations on the changes in morphology and distribution of the yolk nucleus, the nuclei of granulosa cells and filaments on the oocyte surface. a-e, Differentiation of attaching (AF) and non-attaching (NON-AF) filaments in the stage II (a), III(b,c), IV(d) and V(e). a'-e', Distribution of nuclei of granulosa cells (FN). a"-e", Sectioned oocytes showing the relationship between the position of the yolk nucleus and formation of attaching filaments in the VPA. FC, follicle cell; VPA, vegetal pole area; YN, yolk nucleus.

denser accumulation at one side of the egg axis (Vakaet, 1955). It seems to be common in both invertebrates and vertebrates that the position of the yolk nucleus may determine the future VPA, one end of the egg axis.

The yolk nucleus contains a large amount of RNA (Guraya, 1963; Riehl, 1978). Some investigators have postulated that it is a possible site of vitellogenesis or lipidogenesis (cf. Yamamoto, 1958), since the yolk nucleus disappears shortly before the beginning of yolk formation. Yamamoto (1964) suggested that in medaka oocytes, the yolk nucleus may be the site of production of the RNP particles needed for rapid increase in the cytoplasm, on the basis of observations that it is made from networks of threads consisting of aggregates of small particles resembling RNP particles. However, experiments to support these opinions remain to be performed.

Yolk vesicles and their inclusions appear in the cytoplasm as the yolk nucleus completely disappears. Therefore, the yolk nucleus has long been believed to be the site of production of yolk vesicles (cortical alveoli: Kudo, 1969) or yolk inclusions (Aizenshtadt, 1988). Most ultrastructural studies have shown that yolk bodies do not originate directly within the yolk nucleus, although they begin to arise after the basophilic yolk nucleus substances, mitochondria, Golgi complexes, RNP particles, and other organelles and materials disperse throughout the ooplasm. It has been considered that the gradients of ooplasmic components in the oocyte are originally derived from the yolk nucleus, which contains variable amounts of RNA, proteins, and lipoproteins (Guraya, 1979). However, the exact physiological significance of the yolk nucleus remains to be clarified. In the sea urchin, the ooplasmic gradients seem to relate to the A-V polarity which may depend on qualitative differences in the mRNA found at the two poles of the egg (Rodgers and Gross, 1978). Recently, Mosquera *et al.* (1993) has shown that in addition to Vg 1 mRNA (Melton, 1987) Xcat-2 mRNA is located as a crescent at the vegetal pole of mature *Xenopus* oocytes.

During development of medaka follicles as illustrated in Fig. 7, the first asymmetrical feature in the cytoplasm of oocytes is the position of the yolk nucleus. Secondly, granulosa cells that are randomly distributed around the small oocyte at stage II form a cluster on the restricted oocyte surface area facing the yolk nucleus and then dislocate synchronously in a unilateral direction around the axis connecting the center of the germinal vesicle and the yolk nucleus, judging from the disposition and shape of their nuclei. The positioning of tall granulosa cells is concurrent with the formation of a cluster of primitive attaching filaments in the VPA. The unilateral direction in which the nuclei of granulosa cells elongate coincides with the unilateral bending direction of attaching and non-attaching filaments. The bending may depend on the locomotion of the granulosa cells themselves as they induce oocyte rotation, as inferred from a previous experimental result (Iwamatsu, 1994). The locomotion occurs in small follicles before differentiation of the micropylar cell begins. The position of the animal pole is possibly determined by the oocyte rotation.

Another finding of this study is that some of the granulosa cells begin to proliferate or to gather at a restricted area of the oocyte surface, i.e. the future VPA prior to vitellogenesis. The primitive filaments that are formed on an eminence of the cortical surface of the oocyte appear near the junctions between these granulosa cells. The lesser distance between granulosa cells in this area must cause the compact distribution of the attaching filaments. Thus, the clustered attaching filaments at the VPA may be formed by the reciprocal interaction between the cortical surface of the oocyte and the compactly distributed granulosa cells. The process of formation of the filaments on the chorion still must be verified, although it has been reported that their tubular precursor material appeared to originate in the cisternae of the rough endoplasmic reticulum of ovarian follicle cells (cf. Hart *et al.*, 1984).

The process by which the A-V axis of the medaka oocyte is determined is postulated to be as follows. (1) An intercellular signal (diffusible substance) is emanated from the yolk nucleus to the nearby surrounding granulosa cells via the cortical cytoplasm, (2) the compactly clustered granulosa cells determine the position of the attaching filaments in the VPA, (3) the granulosa cells anchored in the VPA become non-locomotive and tall, while the synchronous dislocation of the remaining granulosa cells in a unilateral direction around the axis connecting the centers of the germinal vesicle and the yolk nucleus induces rotation of the oocyte, (4) the position of the micropylar cell at the animal pole is determined by the oocyte rotation(see a diagram of Iwamatsu, 1994). A diffusible factor(s) may be produced at the side of the oocyte containing yolk nucleus and this factor causes granulosa cells to gather at the position of the attaching filaments on the VPA. The yolk nucleus disappears after the VPA is established in the oocyte during stage V when the micropylar cell is differentiated from a granulosa cell at the animal pole (Nakashima and Iwamatsu, 1989). An intercellular signal from the yolk nucleus that attracts granulosa cells may be detected by further investigations.

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