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Short communication

Spectrin labeling during oogenesis in zebrafish (*Danio rerio*)

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ABSTRACT

Progression through mitosis and meiosis during early zebrafish ovarian development is accompanied by highly regulated series of transformations in the architecture of oocytes. These cytoskeletal-dependent membrane events may be assumed to be brought about by deployment of proteins. While the cytoskeleton and its associated proteins play a pivotal role in each of these developmental transitions, it remains unclear how specific cytoskeletal proteins participate in regulating diverse processes of oocyte development in zebrafish. Results from this study show that a pool of spectrin accumulates during oogenesis and parallels an increase in volume of oocytes at pre-vitellogenic stages of development. Spectrin labeling is restricted to the surface of oogonia, the cortex of post-pachytene oocytes and later accumulates on the cytoplasm of pre-vitellogenic and vitellogenic oocytes. Results here suggest a correlation between spectrin labeling, increased cytoplasm volume of oocytes, an increase in the number of nucleoli and accumulation of cytoplasmic organelles. Overall, these results suggest that synthesis and storage of spectrin during pre-vitellogenic stages of oogenesis primes the egg with a pre-established pool of membrane–cytoskeletal precursors for use during embryogenesis, and that the presence of spectrin at the oocyte sub-cortex is essential for maintaining oocyte structure.

Introduction

Zebrafish embryos develop first as females, then later during the course of development some females undergo sex reversal and become males (Takahashi, 1977; Othieno, 2004). In general, gonad development in zebrafish can be divided into seven identifiable phases (Othieno, 2004) including juvenile sex inversion phase. The early ovary in zebrafish (Othieno, 2004) consists of oogonia, early meiotic and post-pachytene oocytes, while the adult ovary (Selman et al., 1993) consists of oogonia and oocytes at different stages of development. During its development, the structure of the oocyte goes through discrete developmental transitions that include periods of specific gene activity and synthesis of organelles specialized for the egg.

The major developmental switches exhibited by each oocyte include the transformation from a mitotic stem cell to a meiotically committed egg precursor. This transition is associated with vitellogenesis (Nagahama, 1983, 1986; Selman et al., 1993; Grier, 2002), from a period of slow growth and organelle replication to a period of rapid nutritional incorporation, storage of glycogen and lipid, and accumulation of yolk. It also involves completion of meiotic divisions, which results in the egg's haploid genome and parallels a wholesale change in the mRNA composition and translocation of the cortical granules to the egg cortex (Patiño and Sullivan, 2002). Finally, as a result of fertilization, there are multiple physiological changes in the egg occurring in response to a calcium wave initiated at the point of sperm fusion. These changes include the exocytosis of cortical granules, whose contents merge with the extracellular vitelline layer to form the fertilization envelope (Nagahama, 1983). The increase in size, together with the transformations that occur in the architecture of the germ cells as they undergo the transition from mitosis to growth periods of meiosis, may be assumed to be brought about by the synthesis and deployment of proteins. Alternatively, it can be assumed that the structural components that perform the architectural changes underlying each developmental transition are defined by the cytoskeleton and cytoskeleton associated proteins.

During oogenesis the dynamic assembly properties and structures of the cytoskeleton may affect dramatic cortical changes (Bennet, 1990). Membrane skeletal proteins have been found in many cell types, where they appear to function primarily in organizing discrete, specialized domains of the plasma membrane (Nelson, 1992; Shiel and Caplan, 1995) and also associate with internal organelles (Beck et al., 1994; Beck, 2005; Kloc and Etkin, 1995).

Spectrin is an essential and widely distributed cytoskeletal protein that can bind to actin with specific isoforms found in erythroid and non-erythroid cells. It forms a network in eukaryotes

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(Hayes and Baines, 1992; Winkelman and Forget, 1993) and has been studied extensively in erythrocytes (Marchesi et al., 1970) where it forms a network supporting the plasma membrane and contributing to cell shape (Elgseater et al., 1986). It has been suggested that non-erythroid spectrin has a general, constitutive role, while erythroid spectrin participates in more specialized activities of differentiating cells (Hu et al., 1995). In many cells (Coleman et al., 1989), spectrin plays a role in integrating cell surface and cytoskeletal activities during cellular differentiation including the transport of intracellular vesicles (Aspengren and Wallin, 2004; Watabe et al., 2008). In addition, it has been shown to modulate the cross-linking of microfilaments, microtubules and proteins, as well as potentiating actinomyosin ATPase activity during motile events (Coleman et al., 1989). The timing and localization of spectrin in other organisms, such as *Drosophila*, suggests that it may be involved in germline cell division and differentiation, in other cell types, in protein–protein interactions (Rimm et al., 1995), and in epithelial cells in polarity and differentiation (Hu et al., 1995). Spectrin is also associated with the membranes of organelles such as exocytotic vesicles (Fishkind et al., 1990).

Studies in *Xenopus*, (Ryabova et al., 1994) revealed that spectrin is localized diffusely in the ooplasm of pre-vitellogenic oocytes of Stages I to II and forms a network in the ooplasm of Stages II to III oocytes. In fully grown oocytes of Stage IV, spectrin is localized mostly in the cortical and sub-cortical areas, but not in the cytoplasm of mature eggs, and in the nuclei of Stage I to II oocytes. With the onset of vitellogenesis, the nuclei of oocytes of stage IV and germinal vesicle breakdown contained a network of spectrin fibrils (Ryabova et al., 1994).

Given the broad range of cytoskeletal involvement during cellular differentiation, it was of interest to investigate the involvement of spectrin in oocyte and follicle interaction as the oocyte differentiates and grows in zebrafish, with focus on the mitotic-meiotic transformation and beyond. Since several cytoskeletal elements change their distribution during oogenesis in fish, it was of interest to relate the developing cyto-architecture of the oocyte to the stages of oogenesis and to possibly use cytoskeletal changes as a staging tool. Furthermore, an antibody against mammalian alpha and beta spectrin was used to label the protein.

Material and methods

Sampling and tissue preparation

The general methods of zebrafish care and breeding in the present study were adopted from Westerfield (1993). The original stock for this study consisted of immature, undefined commercial strain, purchased from a local dealer (Rainbow Aquarium, Johannesburg). Immature fish of unknown age were purchased and transferred to tanks installed a week in advance. All tanks were kept indoors (Animal Unit of the University of the Witwatersrand). Fish were maintained and raised under standard conditions at ±26 °C on a 12 h light: 12 h dark cycle. Embryos were produced by natural spawning. Fish were thereafter selected from hatchery tanks at 24, 36, 42, 45, and 48 days post-fertilization (dpf). Five fish from each age group were killed by anesthesia with MS222 (4.2 ml tricaine stock solution in 100 ml tank water) as described by Westerfield (1993) and measured for total length. The trunk region of each fish was cut out and fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M-phosphate buffer (pH 7.4) for 6 h at room temperature and then washed in phosphate buffer. Fish tissues were then dehydrated through alcohol series, cleared in methyl benzoate and embedded in Paraplast (Merck, Darmstadt, Germany). Serial sections (5–7 μm thick) of the trunk region of each fish were cut and mounted on 3-amino-propyl-triethoxy saline (Sigma–Aldrich, St. Louis, MO, USA) coated slides. Some embryos were kindly provided by the zebrafish facility of Professor S. Wilson of the Department of Anatomy and Developmental Biology, University College, London, UK. Experimental protocols for the study were approved by the Animal Ethics Screening Committee (No. 2000/98/1), University of the Witwatersrand.

Immunostaining and fluorescence microscopy

After dewaxing with xylene, sections were hydrated, followed by incubation in double strength standard saline citrate (2× SSC) for 40 min at 60 °C. Sections were rinsed in distilled water, washed for 30 min in TBT [Tris buffered saline (TBS) containing Triton-X: (TBS; 50 mM Tris–HCl, pH 7.4, plus 0.1% Triton X-100] incubated for 3 min in TBT–NGS (TBT plus 10% normal goat serum (NGS), (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Sections were then incubated overnight at 4 °C in TBT–NGS containing primary antibody, monoclonal anti-spectrin ((and () mouse (IgG1 isotype), Cat. No. S3396, Sigma–Aldrich, St. Louis, MO, USA) at a dilution of 1:100. Sections were then washed for 30 min in TBT-BSA (TBT plus 2% BSA), incubated for 30 min in TBT–NGS and then for 2 h in a humidity chamber in TBT–NGS containing secondary antibody, FITC-goat anti-mouse IgG (Sigma–Aldrich, St. Louis, MO, USA), at a dilution of 1:200. The stained sections were washed in TBT for 30 min at room temperature and mounted in Permafluor aqueous mounting media (Immunotech, Inc., Marseille, France). Confocal images were obtained using a Zeiss LSM 410 inverted laser scanning microscope at 488 nm wavelengths for FITC. Negative control sections were treated in the same way and incubated with secondary antibody alone. All controls produced undetectable immunosignals when photographed.

Results

Patterns of spectrin labeling were examined in gonadal sections by immunofluorescence, using a monoclonal anti-spectrin ((and () antibody (Sigma–Aldrich, St. Louis, MO, USA). The staining pattern was widespread as the antibody labeled additional structures other than oocytes. Immunostaining of the gonadal sections produced a honeycomb-like pattern in which individual germ cells were brightly outlined (Fig. 1A). Spectrin labeling was restricted to the surface of oogonia and on the cortex of early post-pachytene oocytes (Fig. 1A), while there was no labeling in the nuclei of early post-pachytene oocytes. During the late post-pachytene/early pre-vitellogenic stages, spectrin labeling was observed in the cortex, nuclei and on the surface of nucleoli (Fig. 1B).

At the advanced stages of the pre-vitellogenic stages of development, spectrin remained dispersed in the cytoplasm of oocytes and on the surface of the nucleoli, while the expression in the nucleus appeared weaker (Fig. 1C). During the vitellogenic stage, spectrin was distributed throughout the cytoplasm, as well as on the surface of cortical alveoli (Fig. 1D). In vitellogenic oocytes, spectrin labeling was pronounced on one pole of the oocyte (Fig. 1C and D). Oocytes in gonads believed to be undergoing sex inversion, with many oocytes in advanced stages of degeneration, displayed a different pattern of spectrin localization, which by contrast, lacked the honeycomb-like pattern of spectrin labeling (Fig. 1E). Negative control sections labeled using FITC-conjugated IgG as secondary antibody following omission of the primary antibodies produced weak or undetectable immunosignals (Fig. 1F).

Discussion

There is very little information in the literature regarding the possible involvement of cytoskeletal proteins in early gonad

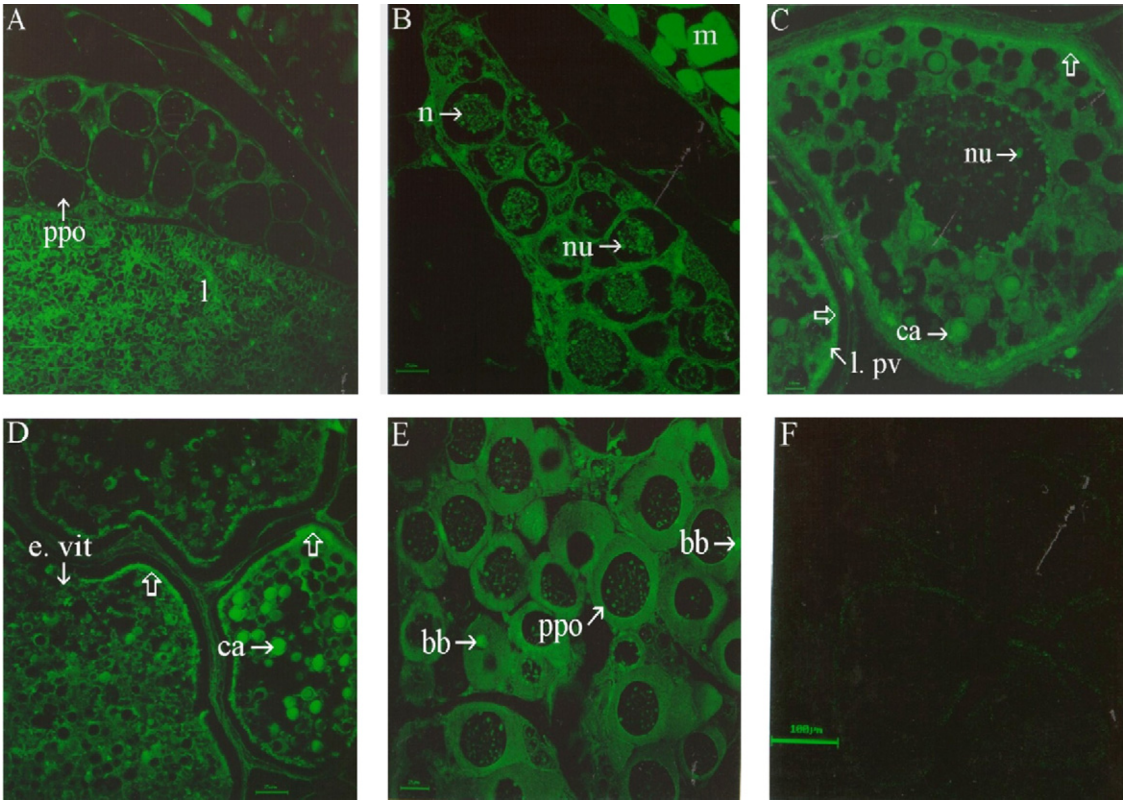

Fig. 1. (A) Spectrin labeling in post-pachytene oocytes at 36 days post fertilization (dpf). Spectrin is restricted to the cortex of oocytes. No labeling is revealed in the nucleus and cytoplasm. Scale bar = 25 μm. (B) Spectrin labeling in late post-pachytene oocytes at 42 dpf. Spectrin labeling is seen at the cortex of oocytes, in the nucleus and on the surface of nucleoli. Scale bar = 25 μm. (C) Spectrin labeling on the cortex of late pre-vitellogenic oocytes (arrows), on the surface of nucleoli and on the surface of the cortical alveoli. The nucleus reveals only faint labeling. Scale bar = 10 μm. (D) Spectrin labeling in a vitellogenic oocyte at 48 dpf. Here labeling is seen all over the cytoplasm and on the surface of cortical alveoli. The intensity of cytoplasmic labeling is stronger on one pole of the oocyte. Scale bar = 25 μm. (E) Spectrin labeling on zebrafish inverting gonad. The loss of the honeycomb-like pattern is evident, however, the Balbiani's body is labeled. Scale bar = 25 μm. (F) Confocal image of zebrafish gonad section labeled with FITC-conjugated anti-mouse IgG. This section was used as a control. Very faint fluorescent signals were observed. Scale bar = 100 μm. *Abbreviations*: ppo, post-pachytene oocyte; l. pv, late pre-vitellogenic oocyte; e. vit, early vitellogenic oocyte; n, nucleus; nu, nucleolus; ca, cortical alveoli; m, muscle tissue; l, liver; bb, Balbiani's body.

development of zebrafish, especially at the time of sex inversion. Identification and detection of instructive and trophic molecules and their stage specific roles in regulating normal development may help elucidate important principles controlling germ cell differentiation.

Results demonstrate spectrin immunoreactivity in early zebrafish gonads. Labeling was mainly coincident with the onset of mass production and storage of organelles during the pre-vitellogenic stage of oocyte growth. The intense labeling of spectrin-like molecule at the oocyte borders, forming a honeycomb-like pattern, suggests that spectrin or spectrin-like molecule is localized in the outermost cortex of these cells. The distribution of spectrin during oogenesis has been reported in other animal species including amphibians (Campanella et al., 1990; Ryabova et al., 1994).

Several studies have also confirmed the expression of isoforms of erythroid membrane proteins in non-erythroid cells (Beck et al., 1994; Kotula et al., 1998). Campanella et al. (1990) showed that spectrin distribution was mainly observed during the vitellogenic stages of development in *Discoglossus pictus*. Unlike *D. pictus*, in the African clawed frog, *Xenopus laevis*, spectrin was already found at the pre-vitellogenic stages of development (Ryabova et al., 1994). Concerning the localization of a spectrin-like molecule to the cortex of pre-vitellogenic oocytes results of the present study are in line with the study by Ryabova et al. (1994), and lead to the suggestion that cytoskeletal structures at the surface of the oocyte, including spectrin, may be necessary for maintaining external tensions required for the transition of oocytes from one developmental stage to another or in cell shaping and adhesion.

Accumulation of cytoskeletal proteins including spectrin on vesicles during oogenesis is thought by some authors to play an important and structural role in the organelles of embryos (Fishkind et al., 1990). Some authors suggest that during oogenesis, spectrin is first assembled at the plasma membrane of oocytes and then recruited onto vesicles during vitellogenesis (Tsukahara and Sugiyama, 1969) and/or during the formation of extracellular matrix-containing granules (Alliegro and McClay, 1988). This, however, would be consistent with models used to describe the assembly of the membrane–skeleton during cellular differentiation in somatic cells where such an organization serves to increase the overall mechanical stability of the organelles (Lazarides and Woods, 1989) and to ensure the appropriate physiological function of organelles. It has also been suggested elsewhere that spectrin labeling on the surface of vesicles serves both in their targeting to the cell surface (Morrow, 1989) and firm anchorage within the actin-rich cortex (Fishkind et al., 1990).

The present view is that such a mechanism for the positioning of vesicles in the cortex by these proteins, including spectrin, which were first identified in erythrocytes, have isoforms expressed in non-erythroid cells and may also play a role during zebrafish oogenesis when cortical vesicles (cortical granules) move to the oocyte surface during the vitellogenic phase of oocyte growth.

Nucleoli of fish and amphibians are a product of gene amplification that takes place during the lampbrush stage of development (Brown and Dawid, 1968). During this process, a large number of nucleoli accumulate at the nuclear periphery of oocytes from the post-pachytene to the late vitellogenic stages of growth. In the present study the reactivity of nucleoli to anti-spectrin antibody was observed as a weak labeling during the oogonial stages of development, but the intensity gradually increased on the surface of nucleoli with the entry into the pre-vitellogenic stages of oocyte development. Reactivity by nucleoli to anti-spectrin antibody has been demonstrated in the amphibian oocyte (Carotenuto et al., 1997), especially during the early stages of vitellogenesis, and may also be involved in the determination of pigment distribution during *Xenopus* oogenesis (Carotenuto et al., 2000).

As described above, spectrin is a primary component of the erythrocyte cytoskeleton, and is a major actin-binding protein usually associated closely with the cytoplasmic surface of the plasma membrane (Lazarides and Moon, 1984). Cortical actin cytoskeleton has been found in oocytes of other animal species such as starfish (Heil-Chapdelain and Otto, 1996), *Xenopus* (Chang et al., 1999), mouse (Longo, 1987), including late stages of human oocyte maturation (Pickering et al., 1988).

Spectrin tetramers are organized into a meshwork that is fixed to the membrane by the protein ankyrin and is also linked to a transmembrane protein called glycophorin by the protein known as band 4.1. Band 4.1 stabilizes the association of spectrin with actin, as does the protein adducing. This group of four proteins: spectrin, ankyrin, 4.1 and adducing reportedly evolved with the metazoa (Baines, 2010), and has been adapted to a variety of functions at different times during animal evolution (Baines, 2010). Therefore, the spectrin-like protein present in zebrafish oocytes as reported in the current study may be similar and possibly homologous to proteins found in other animal species.

The honeycomb-like structure following spectrin labeling was lacking in some tissue sections examined during this study. This could not be explained from the results obtained but were thought to be associated with the marked distortion of oocytes and the loss of germ cell–germ cell contact at the time of sex inversion. Results suggest that, the reorganization of oocyte membrane structure during sex inversion in juvenile fish interfere with the cytoskeletal architecture of transforming oocytes. The presence of spectrin linking the cytoskeletal elements of the sub-cortex in female gonads appears to be essential for maintaining the rigid structure of the oocyte sub-cortex. Overall, it appears that the components of the membrane skeleton including spectrin play an important role in maintaining structure during the dynamic changes in cell shape that characterize juvenile zebrafish ovarian development.

In conclusion, observations of the present study suggest that the labeling of a protein cross reacting with monoclonal anti-spectrin during zebrafish oogenesis occurs at a time when the oocyte surface undergoes a set of changes associated with the reorganization of the cortical cytoskeleton, at a time of organelle production and marked accumulation of cytoplasmic RNA in female gonads. Based on the results of the present study we believe that the spectrin-like protein starts differentiating between the post-pachytene and pre-vitellogenic stages of oogenesis and parallels an increase in the volume of oocytes at the pre-vitellogenic and vitellogenic stages of development. However, spectrin does not appear to be a sensitive or suitable marker for a specific stage of zebrafish oogenesis.

Results of the present study may not be the first to report the distribution and localization of spectrin during oogenesis in oviparous vertebrates, but as far as we are aware are the first to report spectrin labeling during early gonad development in zebrafish. The unusual labeling pattern in oocytes of gonads believed to be undergoing sex inversion however needs further examination. This study was carried out on Paraplast wax embedded material and it would be important to confirm these findings on fresh cryostat sectioned tissues in the future.

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