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DNA and RNA pattern of staining during oogenesis in zebrafish (*Danio rerio*): A confocal microscopy study

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ABSTRACT

Oogenesis involves a sequence of cellular divisions and developmental changes leading to the formation of oocytes, whose role in development is to transfer genomic information to the next generation. During this process, the gene expression pattern changes considerably concomitant with genome remodeling, while genomic information is maintained. The development of the gonad in zebrafish is unique in that it goes through an initial ovarian phase and subsequently into either ovarian or testicular phases. How the germ cells choose to commit to an oogenic fate and enter meiosis or alternatively not to enter meiosis and commit to a spermatogenetic fate remains a key question in development. Lack of suitable markers has hampered the understanding of the principles controlling sex differentiation in zebrafish. The current study was aimed at finding substantive cytochemical markers to identify specific oocyte stages primarily focusing on the DNA and RNA component of cells, using fluorescent dyes: acridine orange and propidium iodide. The pattern of synthesis and appearance of nucleoli was stage specific and may be used to identify stages of oogenesis. A distinguishing and possibly diagnostic feature of the staining pattern observed was the low level of chromatin staining compared to other cellular structures. This may be related to the more diffuse state of chromatin that occurs prior to thickening of chromosomes from the pachytene stage onwards. Although the fluorescent dyes may be useful in determining the localization of nucleic acids in tissue sections, it was not possible to quantify the relative contribution of the DNA and RNA components of specific stages of oocyte growth.

Introduction

The use of zebrafish (*Danio rerio*) has become one of the most widely studied model systems in developmental biology and has led to sophisticated cellular and molecular approaches. Many aspects of zebrafish development have been described including early embryonic patterning (Kimmel, 1993), early development of the nervous system (Kimmel et al., 1994), and aspects of cell fate and lineage determination (Kimmel and Law, 1985; Kimmel and Warga, 1987). Embryonic development and biology of zebrafish have also been described (Streisinger et al., 1981; Warga and Kimmel, 1990; Westerfield, 1993). Other aspects of zebrafish oocyte growth, egg formation including gene expression patterns, have been reported fairly extensively in the literature (Hisaoka and Firlit, 1962; Baumeister, 1973; Takahashi, 1977; Selman et al., 1993; Howley and Ho, 2000; Pelegri, 2003; Lessman, 2009; Okuthe et al., 2009). Although the methods used to classify stages of oocyte growth by the early authors differ from those of Selman et al. (1993), all of the above studies have proved useful in understanding morphological events of oocyte growth in zebrafish.

In general, oocyte growth in zebrafish can be divided into four developmental stages (Selman et al., 1993). The early ovary in zebrafish (Okuthe et al., 2009) 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. In adult ovaries (Selman et al., 1993), stage I oocytes have a large centrally located germinal vesicle (GV) characterized by a smooth and oval outline. At stages II and III, oocytes are characterized by an increase in size due to the accumulation of large amounts of cortical alveoli and yolk respectively, while the GV remains centrally located. At stage IV the oocyte undergoes maturation and is characterized by the migration of the GV to the future animal pole, followed by the dissolution of the GV. After the maturation stage, the oocyte becomes a mature egg. During the entire process, the oocyte structure goes through discrete developmental transitions that include periods of specific gene activity and synthesis of organelles specialized for the egg.

In many multicellular organisms, including fish, the first hours post-fertilization proceed with little or no transcription. As a result, early embryos depend on maternally inherited mRNAs to support all basic cellular functions (Pelegri, 2003) and for the synthesis

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of new proteins (Seydoux, 1996; Heasman, 1997; Nishida et al., 1999). These mRNAs are transcribed during oogenesis and stored in the maternal cytoplasm. In addition to providing essential factors for growth and cell viability, translation of maternal mRNAs also generates products that regulate embryonic polarity and cell fate (Olszańska and Borgul, 1993; Seydoux, 1996; Ding and Lipshitz, 1993). It is therefore, critical for precise regulation of the accumulation of proteins derived from maternal mRNAs, which implies that a large number of ribosomes must accumulate in the germ cell to translate these messages (Wallace and Selman, 1990). This amplification of ribosomal genes allows synthesis of sufficient rRNA to supply the growing oocyte and to maintain the embryo during the first few days of development (Davidson, 1986).

Much of what is known about maternal RNA synthesis and accumulation in vertebrate oocytes and embryos has been derived from studies in *Xenopus* and mouse (Davidson, 1986; Hausen and Riebesell, 1991). In amphibians, it is apparent that expression of amplified genes changes during oogenesis (Hausen and Riebesell, 1991; Spring et al., 1996) and that these changes are accompanied by modifications of the nucleolus. These phenomena appear not unique to amphibian oocytes, but seem also to be common in fish (Bruslé, 1980). RNA regulation is therefore, crucial in the germ line of virtually all organisms and plays a variety of prominent roles.

Acridine orange (AO) is a fluorescent dye that is able to stain differentially double stranded versus single-stranded nucleic acids (Darzynkiewicz, 1994). It gives a red fluorescence when bound to denatured (single-stranded) DNA at 488 nm laser light excitation, while AO associated with double-stranded DNA fluoresces green. AO can also exhibit different spectral characteristics when bound to DNA or RNA thereby allowing for the simultaneous assessment of DNA and RNA content of cells (Darzynkiewicz and Kapuscinski, 1990; Darzynkiewicz, 1994). Its absorption range is 440–480 nm. Propidium iodide (PI) is also a fluorescent molecule that binds to nucleotides. It labels brightly the nuclei of most cells. Because PI binds to the nucleotide pair of guanine and cytosine, it stains not only the nuclear DNAs, but may also label cytoplasmic RNAs. Its peak excitation wavelength is 536 nm, and its emission peak is 620 nm (Krishan, 1975; Zampolla et al., 2008). Tissue sections stained with PI show red fluorescence under blue 568 nm laser excitation. As PI binds to the nucleotide pair of guanine and cytosine, it stains, not only the DNA component of the cell, but may also stain cytoplasmic RNA.

Since a comprehensive study on oocyte development in zebrafish ovary was described by Selman et al. (1993), Othieno (2004) and partially by Hisaoka and Firlit (1962), the aim of the present study was to revisit germ cell differentiation in the zebrafish gonad with the overall purpose of trying to determine substantive cytochemical markers to identify a specific stage (mitotic–meiotic switch) of oocyte growth, primarily focusing on the DNA and RNA component of cells. The switch from mitosis to meiosis is a unique feature of germ cell development and is crucial for animal development consequently any dysregulation in the process may result in clinical problems. The overall pattern of DNA and RNA staining in oocytes was evaluated with two fluorescent dyes: acridine orange and propidium iodide.

Materials and methods

Sampling and tissue preparation

Care and breeding of zebrafish used in the present study were adopted from the study of Westerfield (1993). Original fish stock consisted of an immature, undefined commercial strain, purchased from a local dealer (Rainbow Aquarium, Johannesburg, South Africa). Fish were maintained and raised under standard conditions at ±26 °C on a 12 h:12 h light:dark cycle and embryos were produced by natural spawning. Fish used in this study were selected from hatchery tanks at 9, 12, 21, 36, 42, 45, and 49 days post-fertilization (dpf). Five fish from each age group were sacrificed 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 (0.1 M NaH_2PO_4, 25 ml; 0.1 M Na_2HPO_4, 81 ml; dH_2O, 250 ml; adjusted to pH 7.4 and topped up to 500 ml with dH_2O) for 6 h at room temperature and then washed in phosphate buffer. Fish tissues were then dehydrated through an ethanol series, cleared in methyl benzoate (Sigma–Aldrich, St. Louis, MO, USA) 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 slides coated with 3-amino-propyl-triethoxy saline (Sigma–Aldrich, St. Louis, MO, USA). Experimental protocols for the study were approved by the Animal Ethics Screening Committee (No. 2000/98/1), University of the Witwatersrand.

Staining with acridine orange

Tissue sections were hydrated and then stained with acridine orange (Sigma–Aldrich, St. Louis, MO, USA) made up in distilled water and acetic acid for 30 min, rinsed briefly in 0.5% acetic acid in absolute ethanol and finally rinsed in two additional changes of absolute ethanol. Tissue sections were then rinsed in two changes of xylene and mounted for imaging. Fluorescence-stained sections were examined under a fluorescent microscope equipped with a laser confocal system (Zeiss LSM 410 inverted microscope) comprising a krypton/argon laser, which has lines at 488, 568 and 647 nm excitation. Confocal microscopy was carried out under identical settings for all sections at an excitation of 488/568 nm with green/red excitation filters for dual color image acquisition.

Staining with propidium iodide

Tissue sections were processed as described above. Following dewaxing with xylene, sections were hydrated and stained in propidium iodide (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 1 μg/ml in 50 mM Tris buffer pH 7.4 at 37 °C according to the manufacturer's instructions. This was achieved by placing slides on a flat surface on a water bath and protected from light by covering the container with foil. In order to evaluate the contribution of the RNA and DNA components to the composite staining pattern, RNAse digestion was performed on some tissue sections to eliminate RNA staining. In this case, RNAs were digested by addition of DNA-free RNAse A (Sigma–Aldrich, St. Louis, MO, USA; final concentration 1 mg/ml) in blocking solution 1% bovine serum albumin (BSA)–Tris buffered saline (TBS) for 30 min at room temperature, before staining with propidium iodide. Tissue sections were then rinsed in three changes of TBS for 5 min each and mounted in permafluor (Immunotech, Inc., Marseille, France), for imaging. Imaging was done as described above but by using a laser excitation of 568 nm (green–yellow excitation filter) for single color image acquisition. The intensity of staining with both dyes was scored in arbitrary units from − to +++ (−, no signal; +, weak signal; ++, moderate; +++, intense) with respect to the intensity of change.

Results

Staining with acridine orange

Oogonia

Oogonia in larval zebrafish fish are often found in "nests" of two or four corresponding to the second, or third oogonial divisions, or

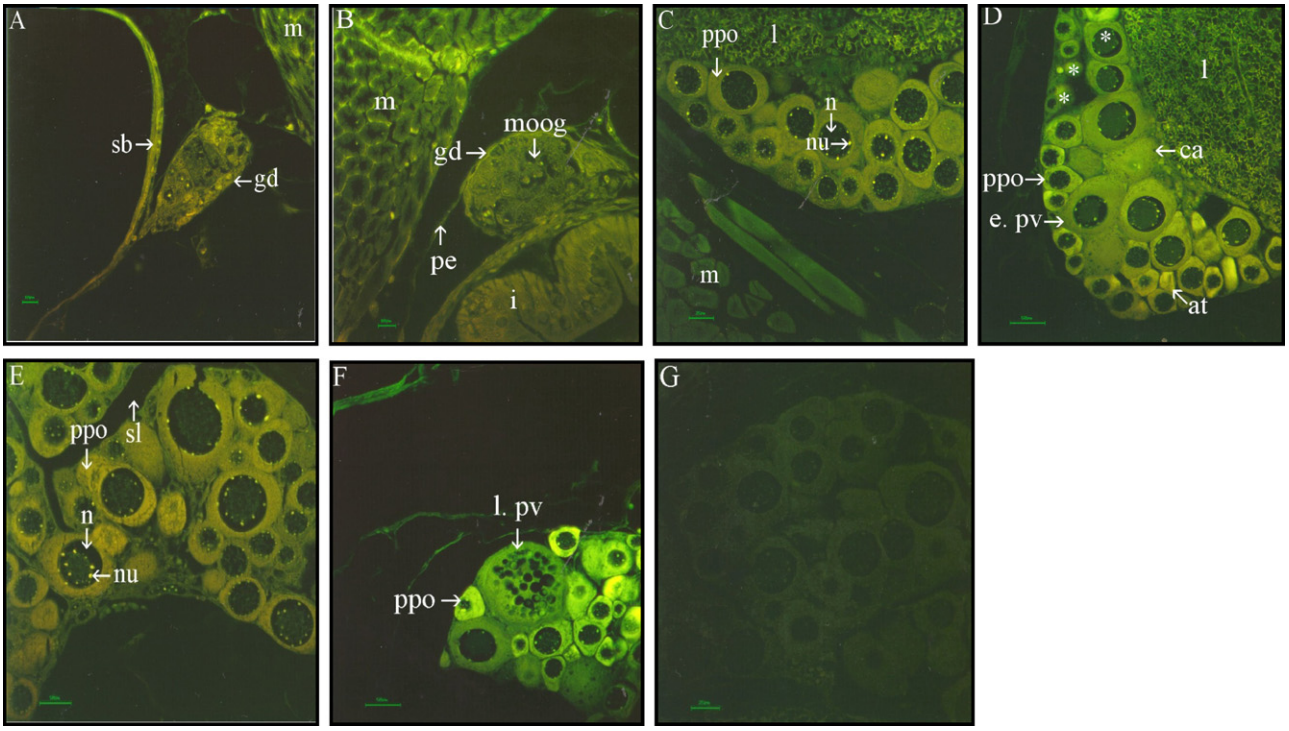

Fig. 1. Confocal images of zebrafish gonad sections stained with acridine orange (AO). (A) AO pattern of staining in oogonia at 21 dpf. Oogonia are round cells, with centrally located nucleoli that appear as yellow/orange dots (scale bar = 10 μm). (B) AO pattern of staining in mitotic oogonia at 25 dpf. Here germ cells stain only faintly for RNA with acridine orange. (C) AO pattern of staining in post-pachytene oocytes (ppo) at 38 dpf. Basophilic cytoplasm and large basophilic nucleoli characterize these oocytes. Larger peripheral nucleoli exhibit a stronger labeling than the smaller ones, which exhibit green fluorescence. The germinal vesicles of these oocytes maintain green fluorescence. (D) AO pattern of staining in early pre-vitellogenic oocytes at 49 dpf. In these oocytes, cytoplasmic staining intensity is shifted to greenish yellow implying a higher proportion of DNA than RNA. Germinal vesicle reveals a green fluorescence (asterisks). Atretic ppo are intensely stained. (E) AO pattern of staining in inverting gonad at 49 dpf. Late ppo are clearly visible with an increased number of basophilic nucleoli (fluorescent yellow dots within the germinal vesicle). Scale bar = . (F) AO pattern of staining in late pre-vitellogenic and in ppo presumed to be undergoing atresia. Post-pachytene oocytes revealed a strong yellow fluorescence. (G) Section of a gonad processed parallel with other sections but omitting the use of acridine orange stain. This was used as a negative control. No fluorescent signals are seen. Scale bars A and B = 10 μm; C and G = 25 μm; D–F = 50 μm. at, atretic oocyte; ca, cortical alveolus; e. pv, early pre-vitellogenic oocyte; gd, gonad; I, intestine; l, liver; l. pv, late pre-vitellogenic oocyte; m, muscle tissue; moog, mitotic oogonia; n, nucleus; nu, nucleolus; pe, peritoneum; ppo, post-pachytene oocyte; sb, wall of swim bladder; sl, slit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

presumably arising from mitotic divisions of primordial germ cells. They were small, round cells with a relatively narrow zone of clear cytoplasm and a single prominent nucleolus (Fig. 1A). The cytoplasm of these germ cells showed weak (yellow) staining intensities with acridine orange. Nucleoli of oogonia were strongly basophilic and indicated a strong affinity for RNA and DNA (brilliant yellow fluorescence). These appear as perfectly circular yellow dots in the middle of the almost unstained region (Fig. 1A). Their basophilic nature appears to be an indication of their high nucleic acid content. Somatic cells as well as the wall of the swim bladder indicated a strong orange fluorescence.

Post-pachytene oocytes

These are formed when a single early meiotic oocyte in a cell nest has undergone growth and become detached from the "nest". Post-pachytene (ppo) oocytes were characterized by an increase in size, caused by the enlargement of the nucleus as well as the cytoplasm. Early post-pachytene oocytes consisted of few large nucleoli aligned peripherally on the nuclear membrane (Fig. 1C), and stained intensely with acridine orange. Late post-pachytene oocytes on the other hand contained multiple nucleoli which, stained moderately with acridine orange. Unlike oogonia, post-pachytene oocytes demonstrated strong cytoplasmic staining intensity for RNA and DNA (yellow-orange fluorescence) with acridine orange. The staining pattern of larger nucleoli was much stronger than other nuclear structures (Fig. 1C and E) indicative of increased nuclear activity.

Early pre-vitellogenic oocytes

Early pre-vitellogenic oocytes (e. pv) were characterized by the presence smaller cortical alveoli situated individually within the cytoplasm or in a thin ring between the germinal vesicle and the newly formed vitelline membrane. These oocytes demonstrated moderate cytoplasmic RNA and DNA, but lower labeling compared to post-pachytene oocytes (Fig. 1D). Nucleoli of these oocytes revealed moderate RNA and DNA staining intensity. Larger nucleoli demonstrated a stronger RNA and DNA (yellow fluorescence) staining intensity, compared with their smaller counterparts which exhibited green–yellow fluorescence, implying lower levels of RNA and moderate levels of DNA.

Late pre-vitellogenic oocytes

During this stage of development, cortical alveoli increased in number throughout the cytoplasm or leaving a small zone of cytoplasm around the nucleus in larger oocytes. These oocytes demonstrated a much lower cytoplasmic staining intensity for RNA and DNA with acridine orange (Fig. 1F). The level of staining appeared to have shifted to green fluorescence rather than the combination of red and green. Late pre-vitellogenic oocytes (l. pv) were

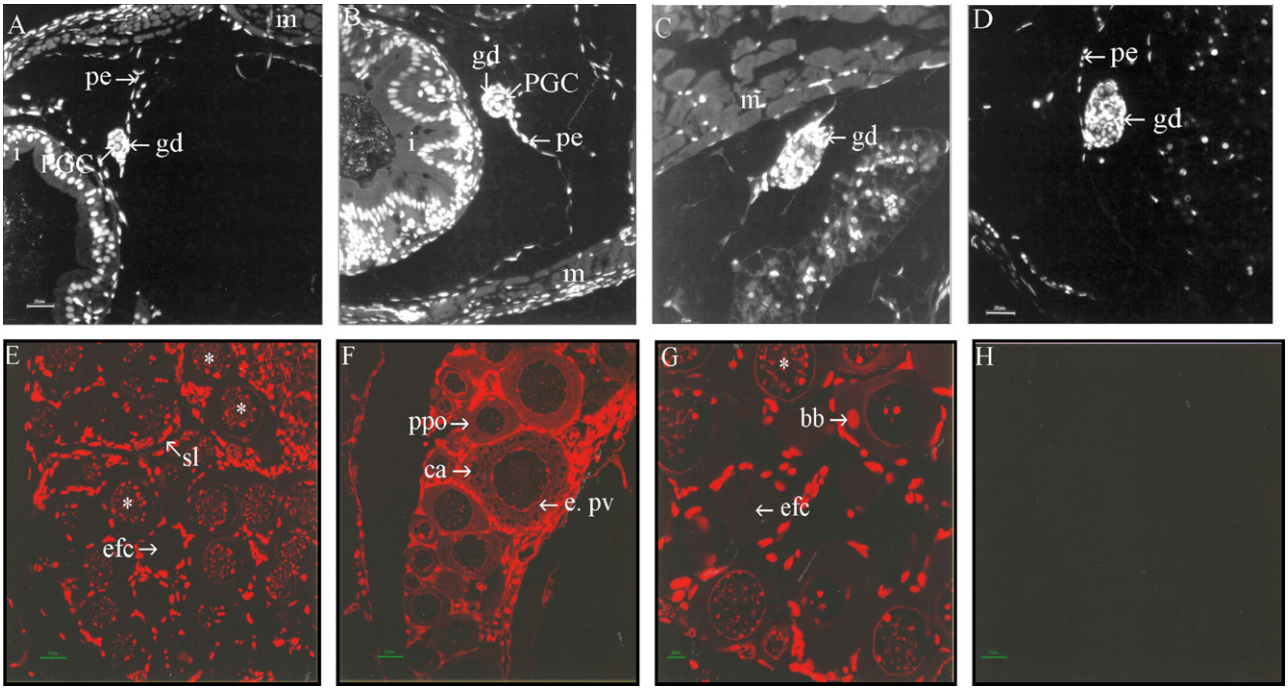

Fig. 2. Confocal images of zebrafish gonadal sections stained with propidium iodide (RNAase A treatment). (A and B) Cross-sections of zebrafish gonads at 8 and 9 dpf respectively. The condensed nuclei of the germ cells are highly fluorescent. These appear as round white bodies in black and white images. Follicle cells are also fluorescent and form a continuous line with the peritoneum. (C and D) Cross-sections of zebrafish gonads at 12 dpf. The number of germ cells has increased. Germ cells are "nested" and reveal very weak signals compared to that of adjacent follicle cells. (E) Confocal image of an ovary at 42 dpf. Note the cytoplasm of post-pachytene oocytes is almost unstained following RNAse treatment. The germinal vesicle of post-pachytene oocytes reveals intense red fluorescence with propidium iodide (asterisks). This applies to the follicle cell that surrounds the oocytes. Empty follicles are also seen following complete resorption of degenerating oocytes possibly by phagocytes. These appear as clear, unstained circular rings surrounded by highly fluorescent follicle cells. (F) Pre-vitellogenic oocyte in a gonad section at 49 dpf. A moderate red cytoplasmic signal is revealed by an oocyte. The germinal vesicle of the oocyte however, exhibits weak red signals. An intense red fluorescence is also seen in the Balbiani's body. This is a large circular body in the cytoplasm close to the nucleus. (H) Gonad section processed parallel with other experimental sections, but omitting propidium iodide stain. This was used as a negative control in all experiments using propidium iodide stain. No fluorescent signals were observed. A–H, scale bars = 25 μm. at, atretic oocyte; bb, Balbiani's body; ca, cortical alveolus; ecf, empty follicle cell; e. pv, early pre-vitellogenic oocytes; gd, gonad; i, intestine; l. pv, late pre-vitellogenic oocyte; m, muscle tissue; pe, peritoneum; ppo, post-pachytene oocyte; PGC, primordial germ cell; sl, slit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the largest germ cells observed here therefore, oocytes in advanced stages of growth beyond this stage are not described.

Staining with propidium iodide (RNAse digestion)

Oogonia

Gonads of larval fish were still very small. Two to four "nested" germ cells were recognized in cross sections enveloped by highly fluorescent somatic cells that formed a continuous layer with the dorsal peritoneal cells (Fig. 2A and B). The condensed nuclei of germ cells exhibited strong fluorescence with propidium iodide indicating a sufficient level of nuclear DNA. These appeared as round white fluorescent bodies surrounded by a narrow clear zone of cytoplasm. Some sections believed to contain oogonia (oog) are also illustrated (Fig. 2C and D). The germ cells were nested and surrounded by fluorescent follicle cells. The germ cell "nests" revealed relatively weak signals compared to surrounding follicle cells.

Post-pachytene oocytes

Post-pachytene oocytes (ppo) demonstrated very low red cytoplasmic fluorescent signals with propidium iodide after RNAase treatment. Nucleoli, on the contrary, revealed intense red signals implying high DNA content. This applied to the follicle cells surrounding the oocytes as well as blood vessels or phagocytes (Fig. 2E). The nuclear envelope exhibited moderate red signals.

Early pre-vitellogenic oocytes

The cytoplasm of early pre-vitellogenic oocytes (e. pv) indicated weak non-specific red signals, but stronger than those observed in the cytoplasm of oocytes in the previous stage. Follicle cells surrounding the oocytes however stained strongly with PI (Fig. 2F and G). Nucleoli of early pre-vitellogenic oocytes revealed moderate red signals. Late pre-vitellogenic oocytes were not observed in gonad sections and are not described.

Sub-sets of juvenile ovaries were sectioned and stained with propidium iodide (PI) without the RNAase digestion. Here, a staining pattern similar to those stained with acridine orange was noticed. The cytoplasm of post-pachytene oocytes indicated intense red fluorescence (Fig. 3A and B). Nucleoli of these oocytes also exhibited strong staining intensity. This applied to oocytes of the late pre-vitellogenic stage (Fig. 3B). However, very weak cytoplasmic staining intensities for RNA and DNA were observed at the peripheral edges of the ovarian wall as well as along the wall of the gonadal lamellae in ovaries believed to be undergoing sex inversion. Such gonads were characterized by the occurrence of slit-like openings in gonads (Fig. 3E and F) and the presence of islets of 'gonial' cells at the peripheral wall of the ovaries. These peripheral 'gonial' cells were characterized by a very narrow clear zone of cytoplasm. These germ cells acquired morphological features similar to oogonia and were most likely spermatogonia. The latter germ cells revealed similar staining patterns seen in oogonia. In gonads presumed to undergoing sex reversal, post-pachytene oocytes in advanced stages of degeneration revealed a different staining pattern. The strength of the propidium iodide

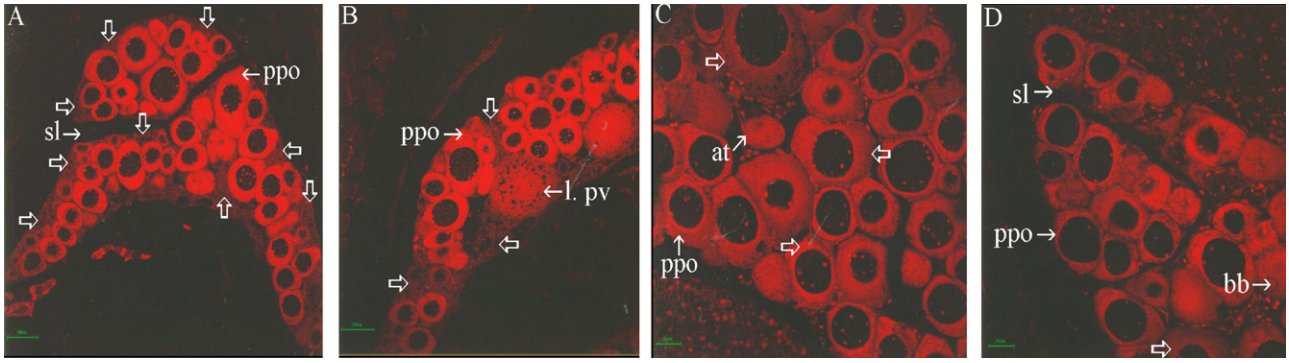

Fig. 3. Confocal images of zebrafish gonadal sections stained with propidium iodide (without RNAase A treatment). (A) Confocal image of a gonad at 46 dpf, showing the completed formation of a slit and the appearance of 'gonial' cells along these slits (arrows). Post-pachytene oocytes dominate the gonad and reveal strong red cytoplasmic signals. The 'gonial' cells seen along the ovarian periphery and along the slits (arrows) revealed very weak red signals. (B) Late pre-vitellogenic oocytes are seen along with post-pachytene oocytes in a gonad at 49 dpf. These show a strong red fluorescence. The cortical alveoli appear as round unstained bodies within the oocyte cytoplasm. (C and D) Confocal images of gonads at 48 dpf showing the weaker cytoplasmic RNA and DNA staining pattern of post-pachytene oocytes (arrows) compared to that seen in (A) and (B). These possibly illustrate phagocytic mechanisms, which appear to occur from the oocyte surface inwards. A and B, scale bar = 50 μm; C and D, scale bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

staining appeared to fade from the oocyte surface towards the nucleus (Fig. 3C and D). Aggregations of cells, believed to be blood cells or phagocytes, were observed close to the degenerating oocytes. A schematic summary of the staining pattern of oocyte stages as observed in the present study is illustrated in Table 1.

Discussion

DNA and RNA staining with respect to the organization and the state of the chromatin

DNA in eukaryotic cells is organized into nucleosomes, which are further folded into a structure known as chromatin. Chromatin structure plays a critical role in regulating gene expression, DNA replication and other processes. During oogenesis, it is clear that the arrangement and packing of DNA (in the context of chromosomes) shows some remarkable changes. After mitosis, with the entry into meiosis, chromosomes adopt a threadlike structure and then proceed to condense and thicken as cross-over takes place. Chromosomes enter the diplotene phase and lampbrush chromosomes appear with the characteristic loops, which are actively synthesizing RNA.

Table 1
Staining pattern of oocytes stages with nucleic acid binding dyes.[a]

Stages of development	Chromatin	Nucleoli	Cytoplasm
Acridine orange (DNA + RNA)			
oog (oogonia)	–	+++	+
ppo (post-pachytene oocyte)	+++	+++	+++
e. pv (early pre-vitellogenic oocyte)	+++	++	++
l. pv (late pre-vitellogenic oocyte)	N/A	N/A	++
Propidium iodide (DNA + RNA)			
oog (oogonia)	–	+++	+
ppo (post-pachytene oocyte)	+++	+++	+++
l. pv (late pre-vitellogenic oocyte)	N/A	N/A	++
Propidium iodide (after RNAse treatment) – DNA			
oog (oogonia)	–	+++	–
ppo (post-pachytene oocyte)	++	+++	+
e. pv (early pre-vitellogenic oocyte)	++	+++	++

N/A, data not available.
[a] Results are scored in arbitrary units from – to +++.

Staining with acridine orange

Contrary to expectations, distinct red or green fluorescence was not observed in the sections of tissues. Instead, brilliant orange or yellow and yellow–green fluorescence was observed in tissue sections stained with acridine orange, the brilliant orange color resulting probably from the combination of the red and green fluorescence. Weak cytoplasmic signals exhibited by oogonia were indicative of low cytoplasmic RNA and DNA. In contrast, nuclei of oogonia exhibited intense brilliant orange or yellow signals denoting their high nucleic acid content. While the nucleoli exhibited intense signals, the contribution of the DNA and RNA components could not be evaluated from the results.

Strong basophilia seen in the cytoplasm of post-pachytene oocytes is similar to that observed following staining with H&E in other teleost fish (Selman et al., 1986, 1993; Tyler and Sumpter, 1996; Grier, 2000). Basophilia is indicative of intense RNA synthesis that occurs during this stage of development. The appearance of a large number of nucleoli indicates production of an enormous number of ribosomes (Muller, 1997). The peripheral arrangement of nucleoli on the nuclear envelope previously reported in other teleost fish, reptiles and birds may be related to the extrusion of nucleoproteins or ribosomes in to the cytoplasm from these organelles (Wallace and Selman, 1990). Their appearance was probably a manifestation of a previous selective amplification of ribosomal genes. The intense cytoplasmic staining pattern of post-pachytene oocytes may be indicative of the presence of the RNA-containing free ribosomes associated with endoplasmic reticulum. High ribosome content generally characterizes a cell that is actively engaged in protein synthesis. It is probable that these germ cells were at the peak of cytoplasmic RNA deposition.

Chromosomes of both early and late post-pachytene oocytes exhibited green fluorescence with acridine orange, implying presence of nuclear DNA. In general the proportion between yellow and green fluorescence of acridine orange in post-pachytene oocytes appeared to be dependent on the size of individual oocytes. Under identical staining and imaging conditions, the cytoplasm and nucleoli of post-pachytene oocytes exhibited a wide range of staining intensities with green to orange fluorescence. This differential staining pattern could not be explained from results of the present study, but is believed to reflect the different proportions of DNA (green) and RNA (orange) that the nucleoli possess during protein synthesis.

The cytoplasm of pre-vitellogenic oocytes revealed some weak non-specific yellow–green staining implying a mixture of

different proportions of RNA and DNA. It appears that these oocytes had a greater intensity of the green (DNA) in relation to the yellow (RNA) fluorescence. Some greenish areas in the cytoplasm may be detectable at this stage, implying the presence of mitochondrial DNA (mtDNA), evident as nucleic acid fragments present in multiple copies in each mitochondrion. Preliminary data presented in this report are insufficient to establish the exact relationship between DNA packing density and proportion of red and green fluorescence of acridine orange in these oocytes. The localization of mtDNA was not an objective of the present study, but further studies may be necessary to clarify these observations.

While some post-pachytene oocytes exhibited homogenous yellow–green fluorescence or different proportions of yellow/green, others were almost entirely yellow. This, as described above may have been indicative of the different mixing proportions of DNA and RNA in these oocytes or probably due to an apoptotic process. Oocytes with these characteristics were commonly seen in gonads believed to be undergoing sex reversal. Oocytes with strong yellow staining patterns had features of degenerating oocytes, such as deformity in cell shape (Okuthe et al., 2009).

Staining with propidium iodide

Oogonia, early and late post-pachytene oocytes exhibited intense (red fluorescence) cytoplasmic labeling similar to that seen in sections stained with acridine orange. This applied both to the nucleoli and the other nuclear structures implying active protein synthesis. Pre-vitellogenic oocytes exhibited moderate cytoplasmic staining with propidium iodide indicative of their involvement in active protein synthesis. Low signals seen in the cytoplasm of degenerating post-pachytene oocytes were thought to reflect some phagocytotic activity. Numerous small fluorescent cells believed to be blood cells or phagocytes were observed in close association with these cells and were believed to be actively involved in phagocytotic activity.

As expected, following RNAase digestion in some tissue sections prior to staining with propidium iodide, DNA stained red in the nuclei post-pachytene oocytes, but not in the cytoplasm indicating that treatment with RNAse eliminated most of the cytoplasmic RNA in these oocytes and that the basophilic characteristics of these oocytes is primarily due to their high RNA content. However, follicle cells and aggregations of blood cells or phagocytes revealed intense staining even after RNAse digestion.

This also applied to the Balbiani's body seen in the cytoplasm of some post-pachytene oocytes. The Balbiani's body, or the mitochondrial cloud, as it is commonly referred to in amphibians (Guraya, 1979; Kloc and Etkin, 1995), is a complex composed of a heterogeneous population of cytoplasmic organelles, including mitochondria, Golgi bodies, smooth endoplasmic reticulum and multivesicular bodies. Such aggregations show variations in their composition and size according to species (Guraya, 1979) and have been described for oocytes of many eutherian mammals, including human (Kress, 1996). The Balbiani's body may be important for trafficking of germ line determinants and other proteins products within the oocyte (Pelegri, 2003). It appears that the red fluorescent staining exhibited by the Balbiani's body following RNAse treatment corresponds exclusively to DNA, although RNA is also known to exist in high concentrations in these structures.

In general, chromosomes of post-pachytene and pre-vitellogenic oocytes stained with acridine orange appeared to exhibit high proportions of green fluorescence. In contrast, in the study of Hisaoka and Firlit (1962) only chromosomes of oogonia and stage I oocytes stained with methyl green and were Feulgen positive. Nuclear DNA could not be demonstrated in pre-vitellogenic oocytes in their study. Their staging scheme is also, different from that of the present study. While slight differences in the yellow/green staining in some oocytes in the present study could be attributed to the ability to bind to acridine orange, the staining pattern of the different stages were clearly detectable in confocal images.

In the study of Baumeister (1973) a high rate of RNA synthesis was observed in fish at prior to the onset of oogenesis. In his study stages of oocyte growth is not mentioned. In a similar study by Hisaoka and Firlit (1962), the greatest amount of cytoplasmic RNA was observed in stage II oocytes, followed by stage III oocytes. Stages IV and V exhibited low levels of cytoplasmic RNA, while stage I oocytes showed the least amount of RNA. In addition, chromosomes of oogonia and stage I oocytes were Feulgen positive, implying a sufficient level of DNA. Chromosomes of stages II to V oocytes were Feulgen negative, probably due to the fact that DNA in these oocytes was too diffuse to be detected by the staining methods used. The staging methods of oocytes used by Hisaoka and Firlit (1962) and Baumeister (1973) however, differ from that of the current study making comparisons difficult.

Conclusions

The technique used in the present study clearly showed that DNA fluoresces a bright green or green (nuclei) and RNA, orange (nucleoli) in tissue sections stained with acridine orange. Sections stained with propidium iodide exhibited red fluorescence in nucleoli, other nuclear structures, in the follicle cells surrounding the oocytes, as well as blood cells or phagocytes. Treatment of tissue sections with RNAse A, prior to staining with propidium iodide, eliminated cytoplasmic staining of RNA from tissue sections. Results also demonstrate that the nucleolus in zebrafish as in most fish and amphibians (Raikova, 1976; Spring et al., 1996; Thiry and Poncin, 2005) undergoes distinct series of transformation during oogenesis. A distinguishing, and possibly diagnostic feature of the staining pattern observed in the current study was the low levels of chromatin staining of with both acridine orange and propidium iodide (RNAase treated) stains as compared to other cellular structures. The reason for these low levels of staining may be related to the more diffuse state of the chromatin that occurs prior to the thickening of the chromosomes from the pachytene stage onwards. The synthesis and appearance of nucleoli was stage specific and may be used as a staging tool for stages of oocyte growth. However, it was not possible to quantify the relative contribution of the DNA and RNA components of specific stages of growth with both dyes. This study was carried out on Paraplast® wax embedded material and it would be interesing to confirm these findings on fresh frozen cryostat sectioned tissues and/or using other cellular and molecular biology protocols in the future.

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