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Mitochondria during sea urchin oogenesis

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Summary

Sea urchin represents an ideal model for studies on fertilization and early development, but the achievement of egg competence and mitochondrial behaviour during oogenesis remain to be enlightened. Oocytes of echinoid, such as sea urchin, unlike other echinoderms and other systems, complete meiotic maturation before fertilization. Mitochondria, the powerhouse of eukaryotic cells, contain a multi-copy of the maternally inherited genome, and are involved directly at several levels in the reproductive processes, as their functional status influences the quality of oocytes and contributes to fertilization and embryogenesis. In the present paper, we report our latest data on mitochondrial distribution, content and activity during *Paracentrotus lividus* oogenesis. The analyses were carried out using confocal microscopy, *in vivo* incubating oocytes at different maturation stages with specific probes for mitochondria and mtDNA, and by immunodetection of Hsp56, a well known mitochondrial marker. Results show a parallel rise of mitochondrial mass and activity, and, especially in the larger oocytes, close to germinal vesicle (GV) breakdown, a considerable increase in organelle activity around the GV, undoubtedly for an energetic aim. In the mature eggs, mitochondrial activity decreases, in agreement with their basal metabolism. Further and significant information was achieved by studying the mitochondrial chaperonin Hsp56 and mtDNA. Results show a high increase of both Hsp56 and mtDNA. Taken together these results demonstrate that during oogenesis a parallel rise of different mitochondrial parameters, such as mass, activity, Hsp56 and mtDNA occurs, highlighting important tools in the establishment of developmental competence.

Keywords: Confocal laser scanning microscopy, Hsp56, MitoTracker, mtDNA, PicoGreen

Introduction

Mitochondria are highly dynamic organelles, frequently dividing and fusing, changing size and shape and travelling long distances throughout cell life.

Their number, structure and functions differ in different animal cells and tissues in relation to their energy needs (Yaffe, 1999; Nagata, 2006). As the energy needs of cells can vary during development, differentiation, and in response to physiological or environmental alterations (Pollak and Sutton 1980; Cuezva *et al.*, 1997; Enríquez *et al.*, 1999), the number of organelles can vary throughout complex processes (Goffart & Wiesner 2003; Hood, 2001; Klingenspor *et al.*, 1996).

Oogenesis in most echinoderms is asynchronous so that each ovary lobe may contain hundreds of oocytes at different maturation stages. To begin with, the sea urchin oocyte is about 10 μm; during the vitellogenic phase, it grows becoming 80−100 μm. A mature oocyte appears with its large nucleus, the germinal vesicle (GV), containing a prominent nucleolus (Wessel *et al.*, 2004a). The first indication of meiotic resumption is the migration of GV to a peripheral location at the animal pole. At the end of maturation the GV breaks down (GVBD) (Berg & Wessel, 1997).

Echinoid and Holothurian oocytes achieve meiotic maturation before fertilization, in a different way than other echinoderms or other systems. The eggs can be stored before spawning within the ovaries for several weeks or even months (Wessel *et al.*, 2004b).

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It is well known that mitochondria play, in eukaryotic cells, a primary role in cellular energetic metabolism, homeostasis and death and are directly involved at several levels in the reproductive processes. The quality of oocytes and eggs, the fertilization process, as well as the embryonic development are influenced by the functional status of mitochondria (May-Panloup *et al.*, 2007).

During oogenesis in many animals, including *Drosophila melanogaster*, *Danio rerio*, *Xenopus laevis*, mouse, pig and human, mitochondrial morphology, quantity, subcellular localization, and aggregation change (Schnapp *et al.*, 1997; Barritt *et al.*, 1999; Perez *et al.*, 2000; Sun *et al.*, 2001; Wilding *et al.*, 2001a,b; Barritt *et al.*, 2002; Van Blerkom *et al.*, 2002; Nishi *et al.*, 2003; Chang *et al.*, 2004; Torner *et al.*, 2004; Cao *et al.*, 2007; Dumollard *et al.*, 2007).

A peculiar distribution of mitochondria has been reported in *D. rerio* oocytes at vegetal and animal poles, probably as a result of differences in energy requirement, while active organelles are distributed in all sublocations and undergo fast dynamic movements (Zhang *et al.*, 2008). A comparative study of female gametes of *D. melanogaster* and *D. rerio*, species highly evolutionarily distant, showed suppressed mitochondrial metabolism, probably due to increase in the fidelity of mitochondrial DNA inheritance (de Paula *et al.*, 2013).

A recent study on *Drosophila* and *Xenopus* oocytes revealed an important link between metabolism and oocyte maturation, suggesting the existence of an evolutionarily conserved mechanism. In both species, mitochondria of mature oocytes reach a state of respiratory quiescence by remodelling the electron transport chain, thus accumulating during late oogenesis an extensive amount of glycogen, required for the developmental competence of the oocyte (Sieber *et al.*, 2016).

During sea urchin oogenesis, mitochondria are synthesized and accumulated in very high numbers: mitochondrial proliferation and massive amplification of mtDNA take place. Mature eggs indeed contain many thousands of organelles, which are distributed to the daughter cells after fertilization. Maternal mitochondria are able to support development up to the larva stage, without new synthesis of mitochondrial DNA or production of new mitochondria (Matsumoto *et al.*, 1974; Bresch, 1978; Rinaldi *et al.*, 1979a,b). After fertilization, the respiratory rate of sea urchin embryos increases, then decreases, keeping quite constant up to blastula, and slightly increasing after hatching, it reaches the maximum value at the gastrula stage and then this is maintained at a high level up to pluteus (Giudice, 1985; Fujiwara & Yasumasu, 1997).

We have demonstrated previously that during sea urchin embryogenesis the mitochondrial mass does not change, while mitochondrial respiration increases (Morici *et al.*, 2007). Mitochondria consume oxygen at low levels before fertilization, but soon after this step the oxidative activity increases probably because fertilization induces the activation of metabolism in order to guarantee cell duplications, requiring a high energy support supplied by mitochondria (Agnello *et al.*, 2008). A useful mitochondrial marker is represented by the Hsp56 protein, a homologue to Hsp60 chaperonin and located in the organelle matrix (Roccheri *et al.*, 1997). In the *P. lividus* embryos genome, the existence of the gene encoding mitochondrial Hsp60 has been demonstrated constitutively expressed and heat-shock inducible, and some proteins that are able to bind Hsp56 mRNA, possibly regulating its localization, have been detected (Gianguzza *et al.*, 2000; Roccheri *et al.*, 2001; Di Liegro & Rinaldi, 2007; Di Liegro *et al.*, 2008).

The exact situation of mitochondria during oogenesis is to date obscure and current knowledge of these organelles needs further improvement. In the present paper we studied mitochondrial mass, distribution, and oxidative activity during oogenesis of *P. lividus*, by applying CLSM imaging technologies. We incubated oocytes *in vivo* at different growth stages with cell-permeant probes specific for mitochondria, in order to measure mitochondrial membranes and oxidative phosphorylation (OXPHOS) activity. The mtDNA content was studied by incubating *in vivo* with the PicoGreen dsDNA probe. The levels and distribution of constitutive Hsp56 were investigated by immunofluorescence.

Materials and methods

Oocyte collection

P. lividus adults were collected in the west coast of Sicily and oocytes were obtained from the gonads by a slightly modified routine method (Giudice *et al.*, 1972). Ovaries from 10 adult females were excised and gently stirred in Millipore-filtered sea water (MFSW) in order to remove the bulk of mature eggs. Released eggs were removed and the gonads washed three times by suspension in sea water and a further four additional times in Ca- and Mg-MFSW. Gonads were then suspended in 100 ml of Ca- and Mg-free sea water containing 100 mg of pronase (Boehringer Mannheim, cat. no. 165921) and gently stirred by means of a magnetic stirrer spinning at 25°C. After 45 min the ovary suspension was filtered through a mesh filter of 75 μm of diameter and filtered oocytes sedimented at 500 *g* for 30 s and washed twice by re-suspending and centrifuging at the same speed. Sedimented oocytes (about 1.2 ml) were finally suspended in 10 ml of

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MFSW. This suspension contained contemporarily all sizes of the oocytes.

In vivo incubation with mitochondrial fluorescent probes

Next, 200 nM (f.c.) of MTG (FM M-7514) and 100 nM (f.c.) of MTO CM-H2 (TMROS M-7511, Molecular Probes) were added to a suspension of oocytes and eggs. After 60 min of incubation at 18°C in a dark room, in rotation, the samples were washed several times with MFSW and fixed with 0.1% formaldehyde in sea water–Tris−HCl pH 8.0. After incubation with the fluorochromes, an aliquot of embryo suspension was treated for 30 min with 100 µg (f.c.) oligomycin, an inhibitor of ATP synthase (complex V of OXPHOS) and used as the control.

mtDNA staining in living oocytes and eggs was achieved by diluting stock PicoGreen dsDNA solution (Molecular Probes, P7589) at 3 µl/ml directly into cell culture medium, for 1 h at 18°C in a dark room, in rotation. Then the samples were washed several times with MFSW and fixed with 0.1% formaldehyde in sea water−Tris−HCl pH 8.0. Controls were pre-treated with DNase, 10 µg/ml, incubated with PicoGreen dsDNA, and then fixed as previously described.

Immunofluorescence

Immunofluorescence was performed on whole-mount oocytes and eggs fixed, as previously described by Kiyomoto *et al.* (2007). Subsequently, each sample was incubated for 1 h at room temperature in blocking solution: 0.5% albumin from bovine serum (Sigma-Aldrich, A7906) and 5% heat inactivated goat serum (Sigma-Aldrich, G9023) in PBS-T (phosphate-buffered saline, 0.1% Tween 20) and overnight at 4°C with anti-Hsp60 antibody (Sigma-Aldrich, H 3524), diluted 1:250, in blocking solution. In the negative controls, the primary antibody was omitted. Samples were mounted on glass in 80% glycerol/PBS-T. The observations were performed by CLSM, under a ×60 oil immersion lens.

Microscopic observations

Fifty samples were chosen from 1000s of oocytes and eggs observed, and analysed under CLSM (Olympus FV-300) equipped with argon (488 nm) and helium/neon (543 nm) lasers. Each sample was scanned in layers 3 µm thick, with a PlanApo 60X/1, 40 oil-immersion lenses at 1024 × 1024 pixel resolution. Specimens pre-incubated with MTG absorb laser light at 490 nm wavelength, emitting green fluorescent light at 516 nm; specimens pre-incubated with PicoGreen dsDNA absorb laser light at 485 nm wavelength, emitting green fluorescent light at 530 nm; specimens pre-incubated with MTO absorb laser light at 551 nm emitting red fluorescent light at 576 nm wavelength.

Image acquisition and analysis

The CSLM fluorescence was revealed by photomultiplier tubes at 256 grey levels. As the acquisition software (Olympus Fluoview v.3.3) links a different colour to each channel, green for MTG and PicoGreen dsDNA and red for MTO, we adopted the 8-bit values full range (0–255) with a linear Look up Table (LUT) in order to compare both colours. The images were analysed, measured and processed by the ImageJ software, a public domain Java Image processing programme. The data obtained were elaborated by Microsoft Excel™.

In the negative control images acquisition was performed measuring the intensity of auto-fluorescence. This value became the threshold level for the capture of other samples.

For the specimens incubated with PicoGreen dsDNA, because of the ability of this probe to bind both mtDNA, and nuclear DNA, the analysis of mitochondrial fluorescent signals was performed by subtracting the fluorescence of the nucleus from the total green intensity values for all analysed sections.

Results

Detection of mitochondrial mass and activity

Mass, distribution and OXPHOS activity of mitochondria during *P. lividus* oogenesis, were studied by incubating oocytes *in vivo* with cell-permeant probes specific for mitochondria, MitoTraker green (MTG) and orange (MTO), and analysed by CLSM.

MTG represents a very useful tool for determining the mitochondrial mass and MTO allows the evaluation of mitochondrial oxidative activity (Morici *et al.*, 2007, Agnello *et al.*, 2008). Incubation with both fluorochromes allowed us to simultaneously measure and study the co-localization of both signals.

The oocytes and eggs were grouped into seven classes: 20, 30, 40, 60, 80, 90 µm, and mature egg (90 µm), on the basis of diameter (Fig. 1). Microscopic observations were performed by capturing 2 µm thick layers of specimens, the number of captured sections depending on the different size of each sample. We analysed and processed 20 specimens for each stage, measuring the intensity values of fluorescent signals by densitometry. Results of MTG incubation showed that green fluorescence was more concentrated in some cytoplasmic regions of small oocytes (20/40 µm), and dispersed in the larger oocytes (60/90 µm). Red fluorescence, due to mitochondrial activity, was quite

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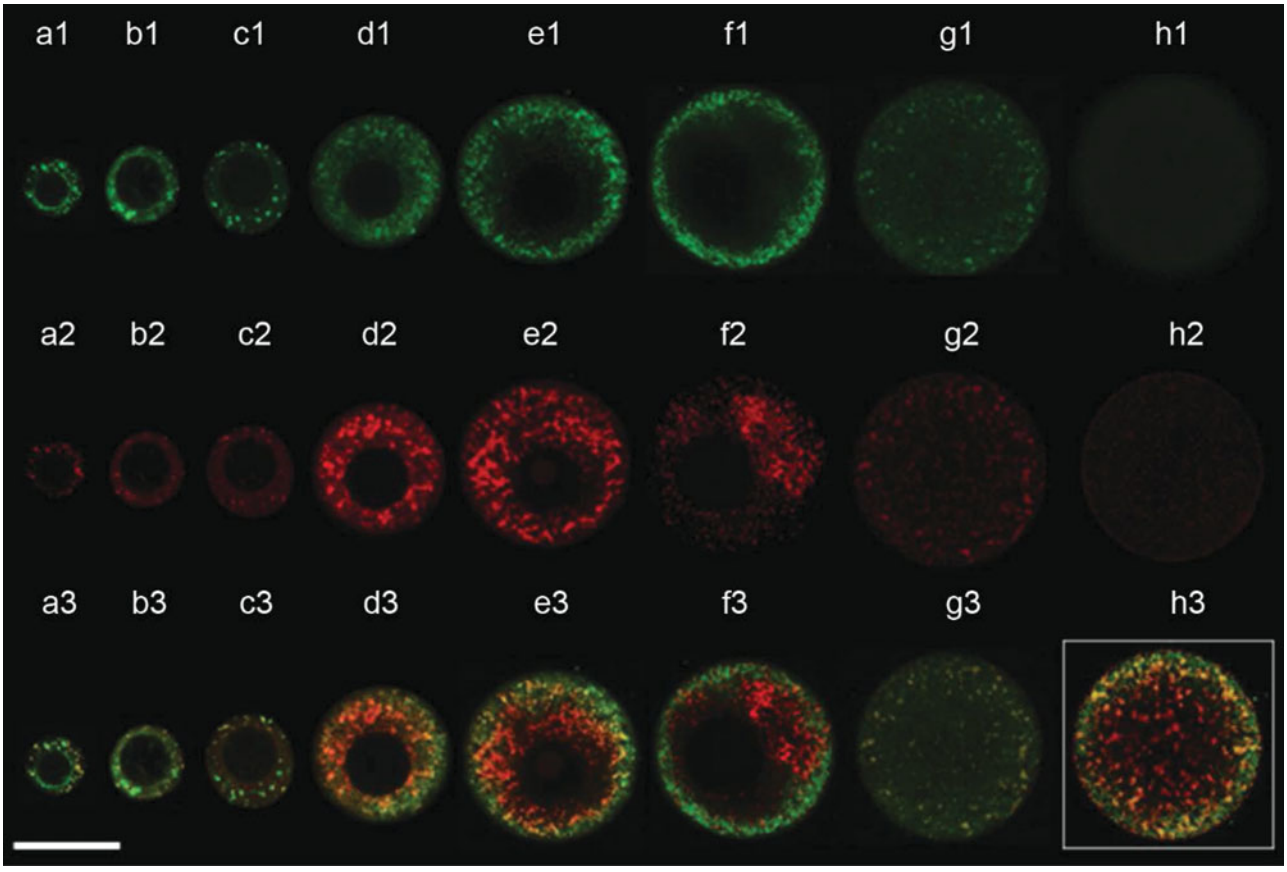

Figure 1 *In vivo* incubation with MTG and MTO: equatorial optical sections of representative oocytes, at different sizes, and mature eggs, captured by CLSM. In green: the mitochondria population stained with MTG. In red: active mitochondria stained with MTO. Bottom: merge of green and red fluorescence. (*a1–a3*): 20 µm oocytes; (*b1–b3*): 30 µm oocytes; (*c1–c3*): 40 µm oocytes; (*d1–d3*): 60 µm oocytes; (*e1–e3*): 80 µm oocytes; (*f1–f3*): 90 µm oocytes; (*g1–g3*): 90 µm mature egg; (*h1, h2*): negative controls after oligomycin incubation; (*h3*): merge of MTG and MTO signals in the zygote, 20 min after fertilization. Bar represents 50 µm.

low in the smaller oocytes, while it was considerably increased in the larger oocytes, where it appeared to be localized mainly around the germinal vesicle, and fluorescence merge tended towards the red. In the mature eggs, red and green fluorescences were quite uniformly distributed and oxygen consumption decreased in comparison with the 90 µm oocyte, probably because basal metabolism, typical of unfertilized eggs, was achieved. As previously demonstrated (Agnello *et al.*, 2008), in the zygote, 20 min after fertilization, the mitochondrial population was more active than in unfertilized eggs and the resulting colour tended to red (Fig. 1*h*3). A treatment with oligomycin, an inhibitor of ATP synthase (complex V of OXPHOS), was used as the control (Fig. 1*h*2, *h*3).

Quantification of MTG and MTO signals

In order to calculate the total mitochondrial mass and activity we integrated the values of the fluorescent signals. Results suggested that green and red signals

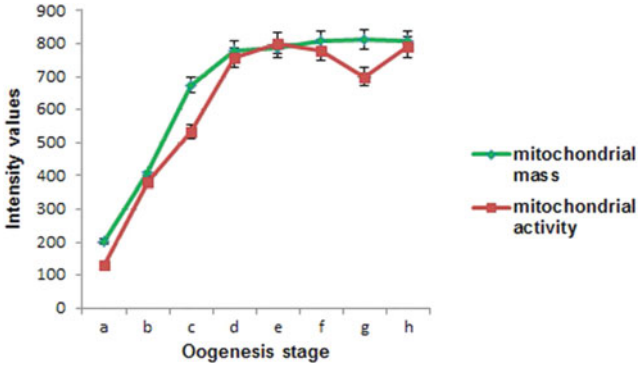

Figure 2 Quantification of fluorescence signals, after incubation of oocytes and eggs with MTG and MTO. In the abscissa (*a*–*h*): 20, 30, 40, 60, 80, 90 µm oocytes, 90 µm mature and fertilized eggs respectively. Ordinate: intensity values, referred to as arbitrary units. Data were reported as a mean of triplicate experiments.

comparably increased during oogenesis, with a little decrease in red fluorescence in the mature egg.

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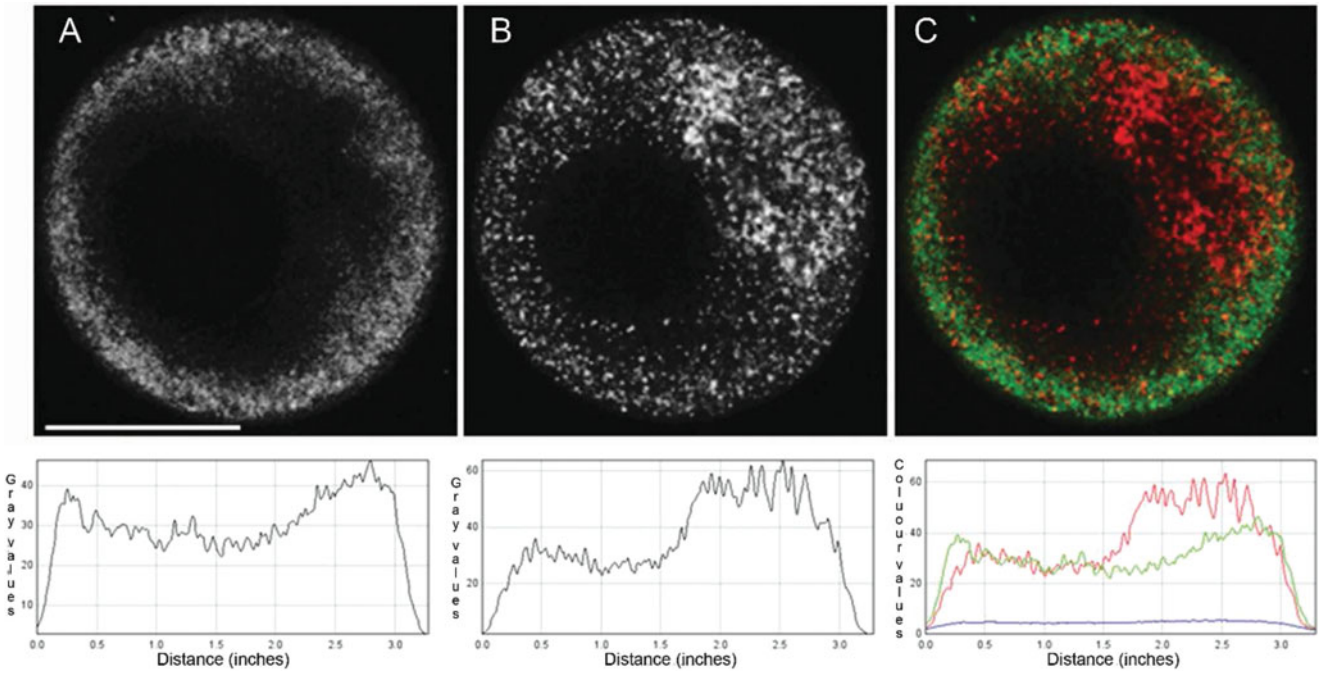

Figure 3 Quantification of fluorescent signals after incubation with MTG and MTO: equatorial optical sections of a representative oocyte captured by CLSM. (*A*, *B*) Green and red signals in grey scale. (*C*) Merge of green and red fluorescence in RGB colours; in the bottom correspondent plot profiles. Abscissa: distance, measured in inches, along the diameter of oocyte; ordinate: intensity values of incorporated fluorescent probes in different areas of an equatorial optical section of the 90 μm oocyte, measured as arbitrary units. Bar represents 50 μm.

After fertilization the mitochondrial mass did not change, while OXHPHOS activity had the similar levels compared with larger oocytes (Fig. 2).

In the larger oocytes (60/90 μm) OXPHOS activity was more pronounced in close proximity to GV region, as shown in Fig. 1. Interestingly this result suggests that at the end of oogenesis, next to meiotic completion, red fluorescence appears to be concentrated more in a specific area near the germinal vesicle, where it reached values almost two times higher than in the other regions (Fig. 3).

Detection and quantification of mtDNA and Hsp56

In order to study the mtDNA *in vivo*, we incubated oocytes and eggs with PicoGreen dsDNA, an ultra-sensitive fluorescent probe which allowed us to detect and quantify even small amounts of DNA, avoiding damage to cellular morphology and/or viability. Results achieved after subtracting the fluorescence values due to nuclear DNA, showed that mtDNA, actively increased in the oocytes from 20 to 80 μm, while in that of 90 μm, as in mature and fertilized eggs, it did not augment, probably because final copy number had been achieved (Fig. 4). As a control, DNase treatments were performed (Fig. 4*i*).

In addition, we studied by immunofluorescence the localization of the Hsp56 chaperonin, a well known mitochondrial marker, reacting with an heterologous antibody against Hsp60 that specifically recognizes the corresponding sea urchin protein (Roccheri *et al.*, 2001). The protein, quite uniformly distributed, accumulates over the entire oogenesis, reaching values that remain constant after fertilization, as demonstrated by densitometric analyses (Fig. 5). Negative controls have confirmed the specificity of fluorescent signals.

Discussion

In the present paper we studied mitochondria during *P. lividus* oogenesis, by applying CLSM imaging technologies. For many years CLSM has had wide application in biological sciences, medical fields (Pawley, 1995; Zhang *et al.*, 2000) and marine biological studies, specifically concerning the reproduction and development of marine organisms (Hertzler & Clark, 1992; Summers *et al.*, 1993; Holy, 1999; Buttino *et al.*, 2003; Morici *et al.*, 2007; Agnello *et al.*, 2008). Recently, using this useful tool, the occurrence of autophagic processes during sea urchin oogenesis and early development has been demonstrated (Agnello *et al.*, 2016).

Gametogenesis represents a key step responsible for the success of embryogenesis. Mitochondria

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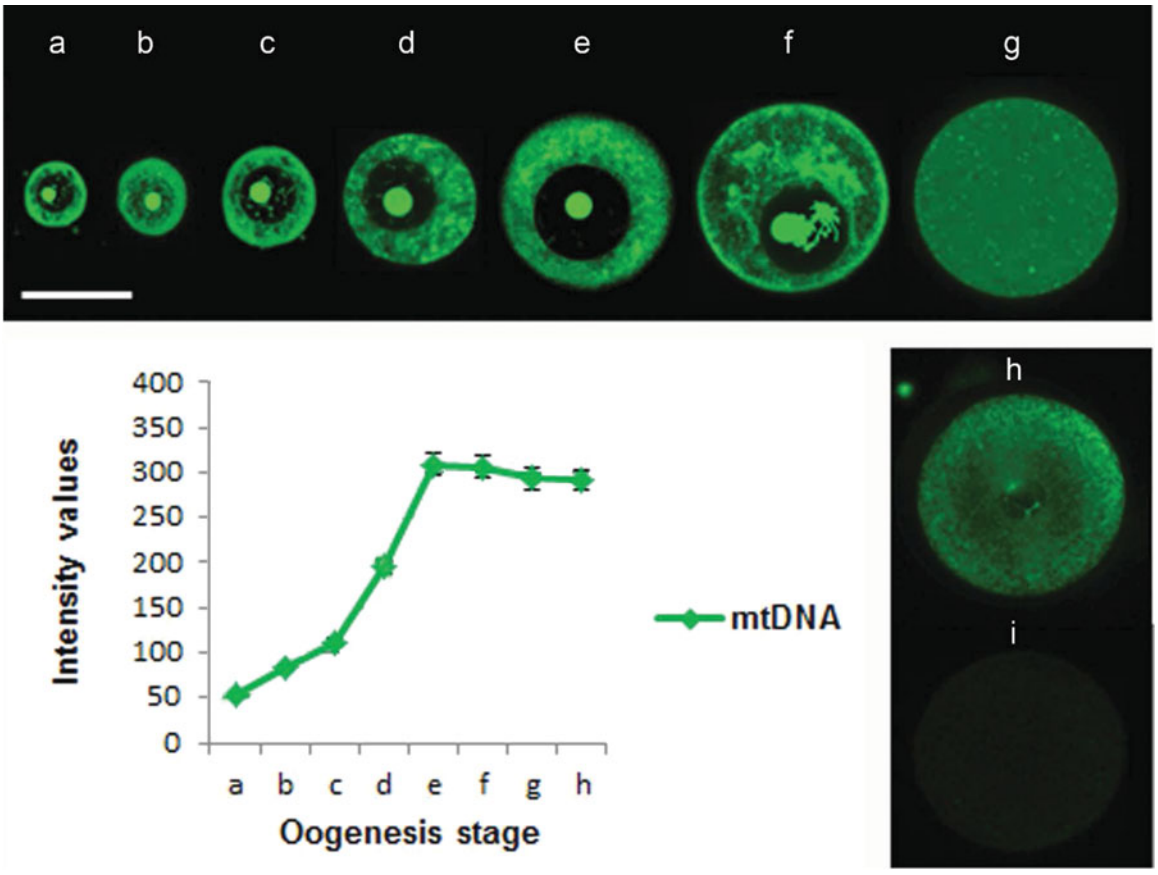

Figure 4 *In situ* detection of fluorescent signals after PicoGreen dsDNA incubation: equatorial optical sections of representative oocytes and eggs, captured by CLSM. (*a*–*h*): 20, 30, 40, 60, 80, 90 µm oocytes, 90 µm mature and fertilized eggs, respectively; i: control after incubation with DNase. Bar represents 50 µm. Graph: quantification of intensity values for mtDNA fluorescence, results obtained by subtracting, through Image J software, the nuclear fluorescence. (*a*–*h*) In the abscissa: same samples showed in the images; ordinate: intensity values, referred to as arbitrary units. Data were reported as a mean of triplicate experiments.

play an essential role throughout oogenesis and embryogenesis, emerging from basic research in model species and clinical studies in human infertility (Van Blerkom, 2011).

During development mitochondria are maternally transmitted with little, if any, paternal contribution, and they originate from a restricted founder population, which is amplified during oogenesis and no further increases in early development (Dumollard *et al*., 2007). Mitochondrial number of mammalian primordial germ cells is amplified during oogenesis (Poulton & Marchington, 2002) and the organelles translocate from a cortical to a perinuclear area just before germinal vesicle breakdown (GVBD). A cloud of mitochondria and ER elements surrounds the spindle during its migration to the cortex, providing energy and spatial information for the spindle displacement (Dalton & Carroll, 2013; Yi *et al*., 2013; Coticchio *et al*., 2015).

Mitochondrial dysfunctions cause a decrease in oocyte quality and interferes with fertility and embryonic development (Babayev and Seli, 2015). The mtDNA copy number of oocyte has been introduced as a useful parameter for prediction of oocyte competence (Pedersen *et al*., 2016).

The localization of mitochondria in the egg during maturation and their segregation to blastomeres in the cleaving embryo are strictly regulated. Gradients of mitochondria distribution in the egg give rise to blastomeres receiving different numbers of organelles, contributing to the viability and definition of embryonic axes. Mitochondrial dysfunction may interfere with development and could cause apoptosis, thus having a dual role, in life and cell death (Dumollard *et al*. 2007).

During frog and zebrafish oogenesis, germ plasm and mitochondria are closely related and mitochondria actively participate to germ plasm formation (Kloc & Etkin 1998; Knaut *et al*. 2000; Kosaka *et al*. 2007; Wilding *et al*. 2001b). In frog, fly, fish, mouse, and other mammals, including humans, the formation of the Balbiani body has been observed, a transient

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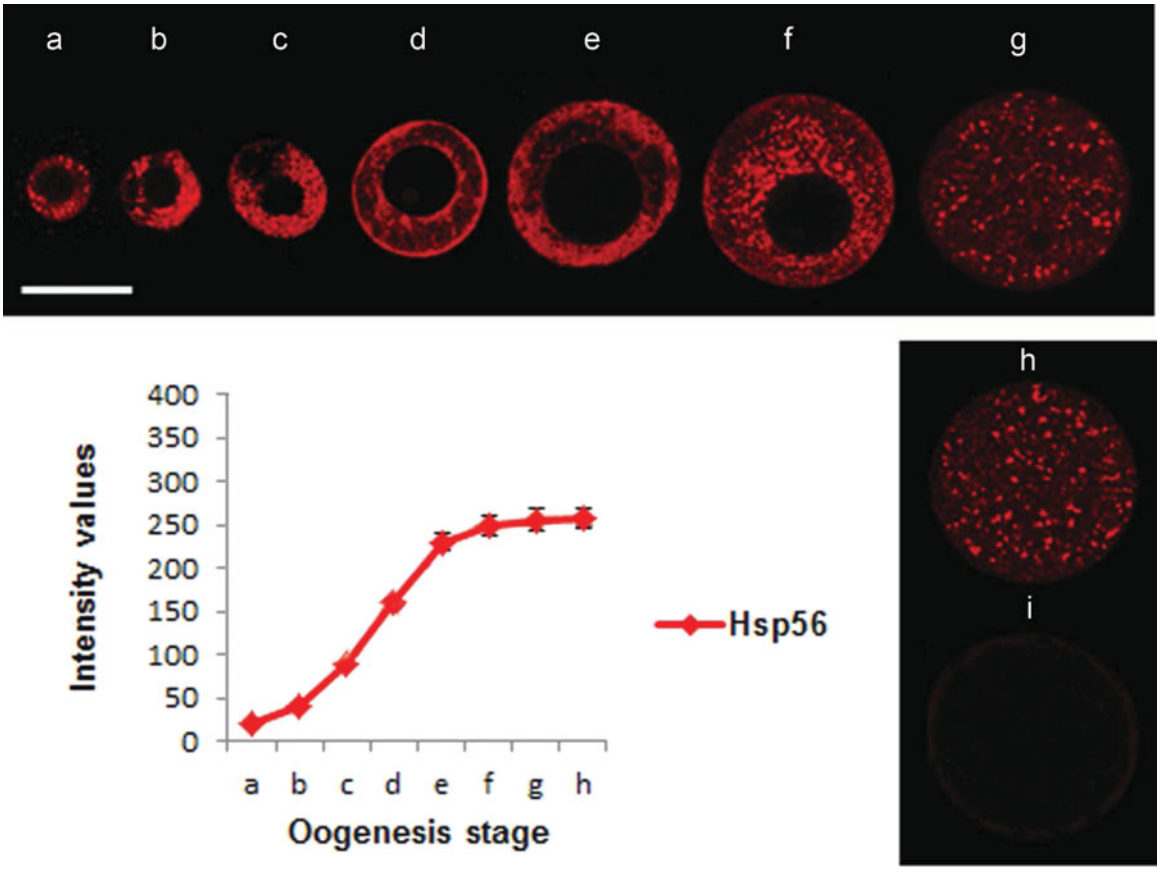

Figure 5 *In situ* detection of Hsp56 after immunofluorescence with the antibody anti-hsp60: equatorial optical sections of representative oocytes and eggs, captured by CLSM. (*a*–*h*): 20, 30, 40, 60, 80, 90 µm oocytes, 90 µm mature and fertilized eggs, respectively. (*i*): Negative control in which the primary antibody was omitted. Bar represents 50 µm. Graph: quantification of intensity values for Hsp56. (*a*–*h*) In the abscissa: same samples showed in the images; ordinate: intensity values, referred to as arbitrary units. Data were reported as a mean of triplicate experiments.

collection of organelles, inclusions, and molecules (endoplasmic reticulum, mitochondria, Golgi, and proteins), near the nucleus of primary oocytes. The Balbiani body, asymmetrically positioned, segregates germ plasm and vegetal patterning molecules in the oocyte, disappearing prior to stage II of oogenesis (Pepling *et al.*, 2007; Billett & Adam, 1976; Bukovsky *et al.*, 2004; Kloc *et al.*, 2008; de Smedt *et al.*, 2000; Kloc *et al.*, 2004). Recently it has been demonstrated that the oocytes of sea urchins *S. intermedius* have germ plasm-related organelles in the form of germ granules, resembling Balbiani bodies, and perinuclear cloud. Maternal germ plasm, accumulated in late oogenesis at the cell periphery and in the eggs, is associated with cortical cytoskeleton (Yakovlev, 2016).

Maternal mitochondria support *P. lividus* development until the larva stage, without new synthesis of mtDNA or production of new mitochondria (Matsumoto *et al.*, 1974; Bresch, 1978; Rinaldi *et al.*, 1979a,b). Whereas mitochondrial mass does not change, mitochondrial respiration increases (Morici *et al.*, 2007). Mitochondria consume oxygen at low levels before fertilization, but soon after the oxidative activity increases probably because fertilization induces the activation of metabolism in order to guarantee cell duplications, requiring a high energetic support supplied by mitochondria (Agnello *et al.* 2008). An understanding of organelle activity during development is almost completely clear, but mitochondrial behaviour during oogenesis remains poorly understood.

In the present paper we demonstrate a parallel rise of mitochondrial mass and OXPHOS activity during oogenesis, up to 90 µm oocytes, and a decrease in the activity in mature eggs. Interestingly, we detect evident anisotropies of mitochondrial activity: the oxygen consumption appears to be higher around the GV, especially in the larger oocytes, just before the GVBD, probably for energetic aims, and more active mitochondria appear to be concentrated in a specific portion of the cytoplasm.

Recent data have shown that the autophagic process is required for sea urchin oogenesis undoubtedly as a survival mechanism (Agnello *et al.*, 2016). The major

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concentration of vesicular acidic organelles is near the GV, possibly for the energetic need of the following GVBD. Intriguingly, mitochondrial activity is also more pronounced around the GV. The occurrence at the same time in nearby areas of two catabolic mechanisms, OXPHOS activity and autophagy, is interesting because their coexistence could occur to ensure the final steps of meiotic process.

Our results suggest that there is a decrease in mitochondrial activity in the mature eggs; this is not surprising considering the basal metabolism of mature eggs that are characterized by a cell cycle arrested in G0 (Costache et al., 2014). After fertilization OXPHOS activity increases (Morici *et al.*, 2007; Agnello *et al.*, 2008).

Further and significant information was achieved by studying the mitochondrial chaperonin Hsp56 and mtDNA. Results showed a high increase in both Hsp56 and mtDNA, as well as in mitochondrial mass.

Taken together these results demonstrate that during oogenesis a parallel rise of different parameters, such as mass, activity, Hsp56 and mtDNA occurs, highlighting important tools in the establishment of developmental competence.

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