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Follicle cell processes: a shark thing?

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Abstract

Follicle cell processes (FCP) are identified in two species of carcharhinid shark (Selachii) but are absent in the little skate *Leucoraja erinacea* (Batoidea). This suggests that FCPs are either a unique structure that evolved in selachians or were lost by the batoids after their divergence, some 280 MYA. The presence of FCPs in the selachians would be consistent with the evolution of large oocytes in this group of animals.

Keywords

Batoidea; development; oogenesis; Selachii; sharks; skates

The chondrichthyans are a successful group of predatory fishes that exhibit characteristics typically associated with apex predators (*i.e.* long-lived, large size and low fecundity) (Stearns, 1976). To offset the risk associated with low fecundity, they produce large precocial young at birth or hatching. One strategy used by chondrichthyans to produce larger offspring is an increase in egg size, as more nutrients can be stored for the developing embryo. In general, chondrichthyans produce large, yolky eggs which, in some species, can reach extreme proportions. For example, in nurse sharks *Ginglymostoma* spp., gulper sharks *Centrophorus* spp. And the frilled shark *Chlamydoselachus anguineus* Garman 1884, eggs of 10–12 cm in diameter occur (Breder & Rosen, 1966; Wourms, 1977; Tanaka *et al.*, 1990). Producing a single cell of such dimensions raises two prominent questions: how do these egg cells maintain their integrity during ovulation and their transit through the reproductive tract and how are large quantities of metabolites delivered to the developing oocyte?

In regard to cell integrity, one would assume a novel reworking of the oocyte cytoskeleton. Investigations into the cytoskeletal architecture of the developing oocyte revealed a prominent set of structures, although not within the egg. Instead, they were located within the zona pellucida. These structures, termed follicle cell processes (FCP), were first described in 2011 and were hypothesized to be associated with elasmobranchs in general (Davenport *et al.*, 2011). Follicle cell processes are a novel set of tube-like structures that extend from the ovarian follicle cells to the developing oocyte, passing through the zona pellucida. The FCPs potentially play a role in addressing both questions mentioned above. First, the FCPs contain the cytoskeletal protein actin that could play a structural role as in

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microvilli or in the cortical region of cells. They alter their orientation during oogenesis from a radial to a more circumferential pattern, ultimately wrapping around the developing egg, such as bracing bands of a car tyre. These tube-like structures wrapping around the oocyte, embedded within the protective layer of the zona pellucida, would provide a tough but flexible layer around the oocyte during ovulation and its passage through the reproductive tract. Second, actin is used in many systems as a track for myosin-dependent transport. The FCPs allow direct cytoplasmic continuity between the follicle cells and the oocyte, facilitating transport of material to the oocyte. The FCPs are present throughout oogenesis, including vitellogenic stages. To date, these structures have been described in two species of Carcharhiniformes: the Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Richardson 1837), dusky smooth-hound *Mustelus canis* (Mitchill 1815) and one species of Squaliformes, the little gulper shark *Centrophorus uyato* (Rafinesque 1810).

For chondrichthyans that produce egg cells of such significant dimensions, the egg yolk is the only source of nutrition for the developing embryo (lecithotrophy). Thus, the size of the offspring is ultimately restricted by egg size. To produce even larger offspring there is an evolutionary shift to matrotrophy, where the maternal organism continues to supply nutrients to the developing embryo throughout gestation. The chondrichthyans have evolved multiple strategies pertaining to matrotrophy including: surrounding the embryo with a nutrient-rich fluid which embryos imbibe (histotrophy); continued egg production for consumption by the developing embryo (oophagy); interuterine cannibalism (adelphophagy); placental structures. The evolution of nutrient sources during development in chondrichthyans has been reviewed by Wourms & Lombardi (1992). Work presented here is primarily focused on the FCPs and their phylogenetic distribution in chondrichthyan fishes.

Chondrichthyans comprise sharks, skates, rays (Elasmobranchii) and chimaeras (Holocephalii). The Elasmobranchii are subdivided into the sharks (Selachii) and the dorso-ventrally flattened skates and rays (Batoidea). The body cavity in the more cylindrical sharks affords more room for larger eggs than does the reduced body cavity in the flattened skates and rays. As such, skates and rays do not typically produce egg cells or offspring as large as those associated with the Selachii. As FCPs are attributed with evolution of large egg cells, this study is the first step in addressing the phylogenetic distribution of the FCPs to determine whether they are found in all elasmobranchs or instead, confined to the Selachii.

Ovarian tissue was collected from two species of carcharhinids over a 3 year period from the Alabama Deep Sea Fishing Rodeo, Dauphin Island, AL, U.S.A. Both carcharhinids studied here are matrotrophic placental species. Samples were obtained from the spinner shark *Carcharhinus brevipinna* (Valenciennes 1839) ($n=6$) and the sandbar shark *Carcharhinus plumbeus* (Nardo 1827) ($n=12$). Ovarian tissue from the little skate *Leucoraja erinacea* (Mitchill 1825) ($n=5$) was kindly donated by B. Lutton. All samples were fixed in 4% buffered formaldehyde. For light microscopy, tissues were embedded in Technovite 7100 (Kulzer; www.heraeus-kulzer.com), sectioned at 1 μm and stained with methylene blue and basic fuchsin. All images were captured with a Nikon DS-Fi1 colour camera mounted to a Nikon Eclipse 50i microscope (www.nikon.com) at $\times 100$ magnification (oil immersion objective). For fluorescent microscopy, tissues were embedded in Tissue-Tek O.C.T. compound (www.sakura-americas.com), cryosectioned at 16–20 μm and stained with Alexa

Fluor 488 labelled phalloidin (green) as a marker for actin and 4',6 diamidino-2-phenylindole dihydrochloride (DAPI) (blue) for distinguishing nuclei. Fluorescence images were acquired using a Nikon A1RSi laser scanning confocal microscope controlled by Nikon Elements software at $\times 40$ magnification (oil immersion objective).

The morphology of the chondrichthyan ovarian follicle follows that of other vertebrates. The centrally located oocyte is surrounded by the acellular zona pellucida, the follicle cell layer, a basal lamina and the theca, respectively. The anomaly is that the zona pellucida reaches extreme widths, in excess of $70\text{ }\mu\text{m}$. In the carcharhinids, the maximum width of the zona pellucida occurs when the oocyte diameter is 2–4mm.

The FCPs can easily be distinguished in both light and confocal micrographs when present (Fig. 1). The zona pellucida reaches its maximum width at an oocyte diameter of *c.* 3mm in *C. brevipinna* and the FCPs appear stretched [Fig. 1(a), (b)]. In *C. plumbeus*, the zona pellucida begins to narrow around 4mm oocyte diameter, which coincides with a more mesh-like arrangement of the FCPs [Fig. 1(c), (d)]. In stark contrast, there are no visible FCPs in the zona pellucida of *L. erinacea* [Fig. 1(e), (f)]. The follicle wall of the two species of carcharhinids is notably different with the follicle cells being a single layer of columnar cells, while in *L. erinacea* they appear cuboidal and multilayered. The zona pellucida is also much narrower in *L. erinacea* than in the carcharhinids.

When looking at general patterns of oogenesis amongst vertebrates, the zona pellucida is consistently narrow throughout oogenesis (Buccione *et al.*, 1990). In vertebrates that typically produce large eggs, the zona pellucida is relatively narrow. For example, in chickens *Gallus gallus* the zona pellucida is *c.* 3–5 μm (Bellairs, 1965) and in the crocodilians around 18 μm (Carmen *et al.*, 2000). Nutrients are supplied to the developing oocyte by diffusion through the narrow zona pellucida, or passed *via* cell junctions between the interdigitating microvilli of the follicle cells and the oocyte (sometimes referred to as the zona radiata; Buccione *et al.*, 1990). What has not been previously documented amongst vertebrates is direct cytoplasmic continuity between the follicle cells and the ovary during vitellogenic stages.

The extreme width of the zona pellucida in chondrichthyan ovarian follicles, which can exceed $70\text{ }\mu\text{m}$ during early oogenesis, is noticeable. The FCPs are uniquely positioned to facilitate the transport of nutrients across this substantial distance, where diffusion alone would be impractical and inefficient. Examples of direct cytoplasmic continuity between follicle cells and developing oocytes are common among invertebrates; for example the intracellular bridges or ring canals of the invertebrate *Drosophila*. These bridges allow for cytoplasmic continuity between the 15 nurse cells and the oocyte. They too contain the cytoskeletal protein actin, which plays a role in transport *via* cytoplasmic streaming or dumping (Gutzeit, 1986; Wheatley *et al.*, 1995; Guild *et al.*, 1997). These intracellular bridges between nurse cells and oocytes of invertebrates are just a few microns in length compared with the much larger dimensions of the FCPs in chondrichthyans. Intracellular bridges have also been described in some vertebrates, including chondrichthyans, but only in pre-vitellogenic stages of egg development (Hamlett *et al.*, 1999). There has been no previous documentation of FCPs or similar structures in any vertebrate during vitellogenic

stages. They might have been missed in previous histological studies of vertebrate oogenesis, as they are not identifiable in paraffin sections stained with haematoxylin and eosin.

These new findings give further insight into the phylogenetic distribution and potential role of FCPs during oogenesis in chondrichthyans. FCPs may not be a chondrichthyan or even an elasmobranch innovation as previously stated, as it appears to be confined to the Selachii only. As Batoidea produce smaller eggs in general, they do not need such structures. The results of this study support this statement with observations of the general differences in the morphology of the developing follicle between Selachii and Batoidea. During oogenesis in Selachii, the follicle cells become columnar at an early stage and remain as a uniform, single layer of columnar cells in conjunction with a zona pellucida that reaches extreme widths. In contrast, the follicle cells of the batoids are more cuboidal throughout oogenesis and multilayered, while the zona pellucida remains narrow, around 7 μm in width; examples are found in the starry ray *Raja asterias* Delaroche 1809, yellow spotted stingray *Urobatis jamaicensis* (Cuvier 1816), marbled electric ray *Torpedo marmorata* Risso 1810 and the red skate *Zearaja chilensis* (Guichenot 1848) (Andreuccetti *et al.*, 1999; Hamlett *et al.*, 1999; Prisco *et al.*, 2002; Wehitt *et al.*, 2015). This morphology is consistent with the ovarian follicle of *L. erinacea* where no FCPs are present. This alludes to their absence in batoids in general, but this needs to be supported by analysis of additional species in future work.

The split between the Selachii and the Batoidea is thought to have occurred around 280 MYA. The evolution of a flattened body plan reduces the size of the body cavity which correspondingly constrains egg size. Batoids indeed produce smaller eggs and offspring. Conversely, the larger, cylindrical-shaped body form associated with the Selachii affords more room within the body cavity, allowing eggs to become much larger. Two potential scenarios for the absence of FCPs in batoids could be proposed. First, FCPs could have been present in a common ancestor, but were lost as egg size became smaller due to a reduced body cavity. Alternatively, FCPs could be an evolutionary novelty confined to the Selachii as a response to selective pressure for producing larger offspring before matrotrophy. Differentiating between these competing hypotheses could be achieved by looking at patterns of oogenesis in the Holocephalii which split from the Elasmobranchii around 420 MYA (Inoue *et al.*, 2010). If FCPs are present in the Holocephalii and in the Selachii, it would suggest they were lost by the batoids. If they are absent in the Holocephalii and batoids, however, they may well be an innovation for the Selachii. Future studies will aim to fully address the phylogenetic distribution of FCPs throughout the chondrichthyans and the specific transport mechanisms associated with the FCP during oogenesis.

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Fig. 1.

(a, c, e) Light and (b, d, f) confocal micrographs from two species of carcharhinid shark and the skate *Leucoraja erinacea*. (a, b) Section through the developing ovarian follicle wall from *Carcharhinus brevipinna*; oocyte diameter is *c.* 3 mm. (a) The follicle cell processes (FCP) can clearly be seen (▼) embedded in the zona pellucida. (b) The FCP are very prominent (▼). Note the radial orientation of the FCP at this stage. (c, d) A section through the developing ovarian follicle wall from *Carcharhinus plumbeus*; oocyte diameter is *c.* 4mm and at a slightly later stage of development than the *C. brevipinna* above. (c) At this stage, the zona pellucida begins to narrow and the FCP take on a more mesh-like configuration (▼). (d) The more mesh-like pattern of the FCP is highlighted as oogenesis continues. The follicular epithelium appears as a uniform columnar layer in both carcharhinid species. The

theca has separated from the follicle cells during tissue processing in both confocal micrographs. (e, f) Sections through the developing ovarian follicle wall from *Leucoraja erinacea*; oocyte diameter is *c.* 4 mm. (e) No FCP can be seen in the region of the zona pellucida (■). (f) Again, the zona pellucida appears totally void of any FCP as indicated by the complete absence of the green fluorophore (■). The follicular epithelium appears more cuboidal and multilayered, and the width of the zona pellucida is much narrower. (a, c, e) Stained with methylene blue and basic fuchsin, (b, d, f) stained with Alexa Fluor 488 (green) for filamentous actin and DAPI (■) for nuclei. OO, oocyte; FC, follicle cells; T, theca. All scale bars are 20 µm.