Cell formation by myxozoan species is not explained by dogma

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Eukaryotes form new cells through the replication of nuclei followed by cytokinesis. A notable exception is reported from the class Myxosporea of the phylum Myxozoa. This assemblage of approximately 2310 species is regarded as either basal bilaterian or cnidarian, depending on the phylogenetic analysis employed. For myxosporeans, cells have long been regarded as forming within other cells by a process referred to as endogenous budding. This would involve a nucleus forming endoplasmic reticulum around it, which transforms into a new plasma membrane, thus enclosing and separating it from the surrounding cell. This remarkable process, unique within the Metazoa, is accepted as occurring within stages found in vertebrate hosts, but has only been inferred from those stages observed within invertebrate hosts. Therefore, I conducted an ultrastructural study to examine how internal cells are formed by a myxosporean parasitizing an annelid. In this case, actinospore parasite stages clearly internalized existing cells; a process with analogies to the acquisition of endosymbiotic algae by cnidarian species. A subsequent examination of the myxozoan literature did not support endogenous budding, indicating that this process, which has been a central tenet of myxozoan developmental biology for over a century, is dogma.

Keywords: Myxosporea; actinospore; development; Myxozoa; endogenous budding; Paramyxida

1. INTRODUCTION

The formation of cells is a fundamental process of biology. Within the Metazoa, new cells form by cytokinesis following either mitotic or meiotic division of the nucleus. Cytokinesis is initiated by the formation of a contractile ring that constricts the cytoplasm between the nuclei, finally cleaving the cell (Glotzer 2001). A notable exception to this process appears within the phylum Myxozoa—a species-rich group composed entirely of obligate parasites, some of which cause disease of substantial ecological and economic importance within the aquatic environment (Kent *et al.* 2001; Sitja-Bobadilla 2009). Here, cells have been reported as forming within other cells by a process referred to as endogenous budding (Lom & Dyková 2006).

Historically, their microscopic size, amoeboid nature and the formation of spores resulted in the Myxozoa being classified within the protistan class Sporozoa, which included other parasitic groups displaying endogenous cell formation (Bütschli 1881). However, the morphological similarity of polar capsules (organelles possessing an extruding filament observed in myxozoan spores) to cnidarian nematocysts was repeatedly commented upon, with some authors promoting placement within the Metazoa (Grassé 1960; Lom 1969).

The majority of molecular phylogenies have confirmed that myxozoans are metazoans (Smothers *et al.* 1994). Several phylogenies have placed them with the Cnidaria, leading to suggestions that they be classified as cnidarians (Siddall *et al.* 1995; Jimenez-Guri *et al.* 2007). If this were adopted then the Myxozoa, with some 2310 described species, would represent a significant addition to cnidarian diversity. However, the placement has proved

controversial with other studies indicating that they are basal bilaterians (Schlegel *et al.* 1996; Zrzavý & Hypša 2003; Evans *et al.* 2008). This ambiguity has led to the suggestion that the Myxozoa may represent a distinct sub-kingdom within the Metazoa between the Cnidaria and Bilateria (Cavalier-Smith 1998).

Reports of endogenous budding arise from the myxozoan class Myxosporea that represent the majority of species; the sister class Malacosporea (three described species) obtain internal cells through engulfment (Morris & Adams 2007, 2008). Where known, myxosporeans have an indirect life cycle incorporating a fish and an annelid host. Within the fish, the parasites may develop into multinucleated plasmodial stages. To form a cell, a nucleus is considered to develop a surrounding mass of endoplasmic reticulum (ER), enclosing it together with some cytoplasm. The ER then transforms into a plasma membrane, resulting in a cell that is internal to the surrounding cell (Lom & Dyková 1992a). The multinucleated plasmodial cell is termed a ‘primary cell’ and the cells contained within this ‘secondary cells’. Where cells are contained within secondary cells, these are termed ‘tertiary cells’; representing a cell, within a cell, within a cell. With the possible exception of the protistan order Paramyxida, this remarkable and somewhat bizarre process (i.e. cell formation not involving an existing plasma membrane) is unique within Eukaryota (Lom *et al.* 1983).

In addition to fish, endogenous budding may occur during the intra-annelid development of myxosporeans (outlined in figure 1). The mature spores released from these hosts (termed actinospores) are multicellular, composed of a sporoplasm, three valve cells and three capsulogenic cells. The sporoplasm is the infective stage to the fish; the capsulogenic cells contain the polar capsules that anchor the spore to a potential host, while the

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Figure 1. Generalized myxosporean spore development within an oligochaete based on El-Matbouli & Hoffmann (1998). Following a proliferation, binucleate stages transform into pansporocysts, multicellular sac-like stages within which actinospores form. (1) Within the pansporocyst, cell division followed by fusion results in eight 'zygotes' (Z). (2) Each zygote divides twice. Three cells surround the remaining cell to form a sporoblast. (3) The sporoblast initially consists of three sporogonic cells (S) surrounding an internal cell (termed generative cell by El-Matbouli & Hoffmann 1998). (3–4) Transitional stages represented by ?, under debate in this paper; the two alternative interpretations are shown in figure 9 pathways A and B i–iii. (4) The sporoblast transforms into three capsulogenic cells (C) and three valvogenic cells (V). A single sporoplasm is attached to the valvogenic cells. The sporoplasm comprises a multinucleated somatic cell within which germ cells reside. Each germ cell is contained within a tightly fitting vacuole. (5) The sporoplasm enters the cavity formed by the differentiated V and C cells and a mature actinospore is formed. When this is released from the host into water, the V cells expand to form three processes that aid floatation. The shape of the processes defines the actinospore type—in this case an aurantiactinomyxon.

valve cells protect the sporoplasm and aid buoyancy (Lom *et al.* 1997). The sporoplasm is a multinucleated cell that possesses 'germ' cells within it, and endogenous budding is proposed to explain the origin of these cells (Lom & Dyková 1992b; El-Matbouli & Hoffmann 1998). However, detailed ultrastructural studies regarding germ cell formation are lacking.

2. MATERIAL AND METHODS

The material used for this study was obtained from archival material from 2007 and consisted of two *Tubifex tubifex* oligochaetes collected from the same site and infected with the same species of aurantiactinomyxon-type myxosporean. The oligochaetes were collected, fixed and processed into Spurr's resin for electron microscopy as detailed by Morris & Freeman (2010), additional ultrathin sections were cut and stained using uranyl acetate and lead citrate and examined at 120 kV using a Technai Spirit G² electron microscope. A previous study had established that the aurantiactinomyxon had identical developmental characteristics between both oligochaete samples and has thus been considered to represent a single species with no evidence of a coinfection (Morris & Freeman 2010).

Twenty-four grids were examined, each holding a minimum of two ultrathin sections. Myxosporean stages observed were compared with previous reports from the literature to help construct the developmental pathway.

3. RESULTS

Both oligochaetes were heavily infected with the myxosporean, possessing numerous parasites, including binucleate proliferating stages and pansporocysts (the sac-like structure inside of which actinospores form). Development of actinospores within each pansporocyst was roughly synchronous, but asynchronous between pansporocysts. This meant a range of stages were captured in section, allowing for the interpretation of a developmental sequence for the sporoplasm.

Figure 2. Sporoblasts formed when one germ cell (G) is entirely surrounded by at least three sporogonic cells (S). Division of these sporogonic cells presumably occurs during the encirclement of the germ cell. Scale bar, 2.5 µm.

(a) Sporoblast

The initial stage of sporoplasm formation, observed within pansporocysts, was the sporoblast. This structure was composed of a centrally positioned germ cell, enclosed by at least three sporogonic cells, the two cell types separated by a conspicuous fluid-filled space (figure 2).

The sporogonic cells were connected together by dense adherens junctions, whereas filopodia were noted extending from the internal germ cell(s) suggesting a contact with the surrounding cells. Both the germ and sporogonic cells underwent division (figure 3). In sections where sporoblasts possessed few cells, the sporogonic cells appeared positioned roughly equidistant around the germ cell(s), but as cell numbers increased, the sporogonic cells clustered at one pole and appeared differentiated; some cuboidal, whereas others were distended. The maximum number of cells counted in section composing a sporoblast was seven, two germ cells surrounded by five sporogonic cells (figure 4).

Figure 3. Cytokinesis of germ cell (G) resulting in the two germ cells of the sporoblast. Sporogonic cells (S) also increase in number through division. In this section, one sporogonic cell has been cut so that its nucleus (Sn) appears in the vacuole occupied by the germ cells. Scale bar, 1 μm .

Figure 4. Sporoblast with differentiating sporogonic cells (S), clustering at one pole, with two internal germ cells (G) contained within a fluid-filled vacuole. Scale bar, 2.5 μm .

Sporoblasts composed of cuboidal cells surmounting distended cells, which surrounded the germ cells, were interpreted to be the next stage of development (figure 5). The cuboidal cells attached to the distended cells by elongated adherens junctions. Owing to the distinctive nature of the cell types and their apparently differing roles during development, the cuboidal cells are now termed valvocapsulogenic (VC) cells and the distended cells, sporoplasmogenic (SP) cells.

(b) Formation of sporoplasm

The simplest sporoplasms observed were two germ cells, contained within a somatic, multinucleated surrounding cell, connected at one pole to three VC cells by adherens junctions (figure 6). The connection to the VC cells was either broad, across all three cells or narrow. For the latter configuration, VC cells formed a rounded structure, attached to the sporoplasm by adherens junctions (figure 7).

The most developed sporoblast noted, prior to the differentiation of the VC cells into valve cells and capsulogenic cells, had three apical VC cells that formed a hollow, rounded structure connected by prominent adherens junctions to the sporoplasm to form an isthmus. The

Figure 5. Sporoblast with VC cells surmounting SP cells that surround the germ cells (G). Arrows indicate adherens junctions between VC and SP cells. Scale bar, 2.5 μm .

Figure 6. Fluid separating SP cells from germ cells (G) removed resulting in sporoplasm (Sp) attached to VC cells. Scale bar, 2 μm .

sporoplasm was multinucleated and contained many germ cells within it. The germ cells separated from the surrounding cell by a double plasma membrane (figure 8). Evidence for any of the processes associated with endogenous budding was not observed to explain the initial occurrence of germ cells within the sporoplasm.

4. DISCUSSION

While some differences can be noted between the myxosporean species observed in this study compared with those of previous ultrastructural studies (notably two cells contained within the early sporoblast as opposed to just one), sporoblasts containing cells in a fluid-filled space and the maturing sporoplasm connected to VC cells by a cytoplasmic isthmus appear consistent across species (El-Matbouli & Hoffmann 1998; Ozer & Wootten 2001). In previous studies, a generative cell (=SP cell) was considered to be the central cell observed in the early sporoblast. The generative cell produced an internal germ cell by endogenous budding resulting in a sporoplasm. The VC cells then separated and the sporoplasm attached to them (figure 9, pathway A). The endogenous budding stage was not demonstrated, only inferred to

Figure 7. Connection between the VC cells and multinucleated sporoplasm (Sp) containing germ cells (G) reduced to form an isthmus (arrow). Scale bar, 2 µm.

explain the resultant structures (Lom & Dyková 1992b; El-Matbouli & Hoffmann 1998; Ozer & Wootton 2001). In examining the development of the aurantiactinomyxon-type myxosporean, endogenous budding does not account for the presence of two cells within the early sporoblast but only one sporoplasm within the actinospore. Instead, it is clear that the sporoplasm forms when the fluid surrounding the two germ cells is removed, collapsing the surrounding SP cells onto them (figure 9, pathway B). Therefore, the previous inferences are not supported. As there is no evidence for endogenous budding within the annelid host, this leads to the question: does endogenous budding occur within the fish host?

The development of myxosporeans in fish has been examined for over a century. Early studies developed the idea of endogenous budding to explain the apparent appearance of internal cells within multinucleated plasmodia, which was commensurate with knowledge of other parasitic protists at the time (Thélohan 1890; Davis 1916; Noble 1944; Shul'man 1966). However, the studies were constrained by the limits of light microscopy and a lack of knowledge concerning myxozoan life cycles. Only one study has previously questioned the existence of endogenous budding, suggesting that the light microscopical evidence supporting it was weak, but this study did not adequately explain the origin of the internal cells (Desser *et al.* 1983). The advent of electron microscopy, and its use to examine myxozoans, resulted in studies that supported endogenous budding, explaining secondary cell formation by *Ceratomyxa shasta* and *M. cerebralis* (Yamamoto & Sanders 1979; El-Matbouli *et al.* 1995), and of tertiary cell formation in parasites putatively identified as *Sphaerospora renicola* (Lom *et al.* 1983). These studies suggested that the formation of an internal cell occurred through the accumulation of 'cytoplasmic membranes' or ER around nuclei. All other studies either have not addressed the issue or presumed that it occurs. In addition, with no alternative hypothesis to explain the origin of internal cells, all review articles, which address myxosporean development, have consistently reiterated the existence of endogenous budding (e.g. El-Matbouli *et al.* 1992;

Figure 8. Germ cells (G) within sporoplasm (Sp) continue to divide as sporoplasm matures. Scale bar, 2 µm.

Garden 1992; Lom & Dyková 1992a, 2006; Molnár 1994; Kent *et al.* 2001; Canning & Okamura 2004; Feist & Longshaw 2006). A similar situation exists for the order Paramyxida of the protistan phylum Cercozoa, the only other group reported to have cell-within-cell development involving internal membranes, comparable to the Myxozoa (Perkins 1976; Ginsburger-Vogel & Desportes 1979; Desportes 1984). Like the Myxozoa, although often cited, any evidence for such development has rarely been documented (Perkins 1976; Desportes 1984).

Studies examining the entire life cycle of *Tetracapsuloides bryosalmonae*, a myxozoan of the class Malacosporea, the sister taxon to Myxosporea, found that all internal cells, secondary and tertiary, initially originated by cells engulfing one another (Morris & Adams 2007, 2008). A reinterpretation of the literature by Morris & Adams (2008) contended that the origin of all myxosporean tertiary cells, including those of *S. renicola*, could be accounted for by engulfment. Therefore, the only myxosporean cell type that results from an endogenous budding would be the secondary cell that occurs within the vertebrate phase of development. As secondary cells have been documented as dividing by mitosis, endogenous budding is not required to explain their proliferation, so proof of its occurrence relies on the origin of the first cell doublet, i.e. a primary cell containing a single secondary cell.

Studies that document the origin of cell doublets are lacking (also noted by Lom & Dyková 1992a). The photomicrograph in Yamamoto & Sanders (1979), reputedly demonstrating endogenous budding within the primary cell of *C. shasta*, appears to show complete cells already formed. The time course study of El-Matbouli *et al.* (1995) examining *M. cerebralis* within the fish host identified the initial presence of cell doublets within an

Figure 9. Two suggested pathways (A and B) for transformation of sporoblast (corresponding to stage 3 in figure 1) to a sporoplasm with internal cells connected to valvogenic cells (corresponding to stage 4 in figure 1). Pathway A uses endogenous budding. (A3i) VC cells divide and separate. (A3ii) Nucleus of SP cell divides, and one nucleus forms ER around it. (A3iii) Internal germ cell forms. Sporoplasm (Sp) consists of one germ cell surrounded by somatic cell. Pathway B uses SP cells. (B3i) Germ and sporogonic (S) cells divide. (B3ii) Sporogonic cells reorganize to form three VC cells and three SP cells. (B3iii) SP cells fuse and fluid surrounding germ cells removed resulting in sporoplasm. VC cells then divide to form three valvogenic cells and three capsulogenic cells B4. Steps for pathway A3i–iii were inferred while steps for pathway B3i–iii have been reported (this study). The number of sporogonic cells in pathway B3i estimated on known number of cells in B4.

enclosing cell during very early infection, but similarly to Yamamoto & Sanders (1979), did not evidence how the doublets formed. Therefore, despite the numerous studies conducted on myxosporean development, none corroborate endogenous budding.

The origin of internal cells (either secondary or tertiary) by myxozoans has only been noted when cells surround other cells. I propose that the internal cells of all myxozoans initially derive from variations of this method, with division explaining any subsequent intracellular proliferation. This trait of internalizing cells, resulting in cell-within-cell complexes, has parallels with the Cnidaria, i.e. the acquisition of algal symbionts by cnidarian cells (Benayahu *et al.* 1992). Therefore, this unusual developmental process, which initially drew comparisons to protistan schizogony, has analogies to the closest relatives of myxozoans. In comparison, endogenous budding that would require a highly specialized process, unique within the animal kingdom but has little to no data supporting its existence, is dogma.

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